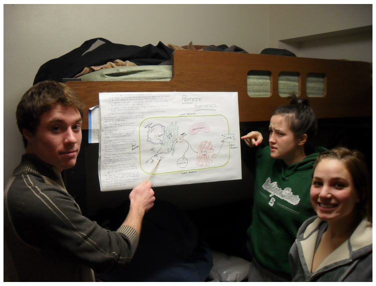
LB-145

Cell, Molecular & Organismal Biology





Lecture & Lab Course Packet

(for students in Douglas Luckie's sections)

This publication is a compendium of work authored by:
Candace Igert, Angela Wright, Cori Fata-Hartley, Michael Haenisch,
Susannah Cooper, Marjia Krha, Joseph Maleszewski, John
Wilterding, Mimi Sayed, and Douglas Luckie

Welcome to LB-145

Welcome to LB 145! The experience you are about to have in the cell & molecular biology course may turn out to be unlike most other lectures/labs that you will have while as an undergraduate. There will be a major emphasis on team effort in this class and your ability to work in a team is crucial to your success in this course and beyond. As a team, you and your partners, will work together to solve biological problems in lecture, discuss scientific ideas, *strategies*, and pursue research projects within the lab to find answers to the unique questions your group will have posed.

Molecular biology is quickly becoming an integral part of science and society. With new discoveries and ongoing discussions of topics such as: cloning, genetically modified foods/organisms, DNA fingerprinting, genomics, bioinformatics, gene patenting (the list goes on) it becomes crucial to understand the concepts of molecular biology not only to have a degree in science, but to be a member of society in general.

Molecular biology is not an easy subject to master. There are complex concepts as well as a great deal of factual information. Nevertheless, the difficulty of this subject adds to its appeal. The staff of LB 145 will work hard to help you glean the information necessary to achieve in this course; however, your hard work is the most essential element to success.

This experience will without a doubt be exciting, frustrating, and almost overwhelming at times -but it is all with purpose. We hope that you will come out of this course with not only a better understanding of molecular biology, but also a better understanding of the scientific method itself.

We look forward to working with you throughout the semester and if you have questions please do not hesitate to contact any one of the TAs, GAs, or the Professor. Good luck and enjoy the semester!

-The 145 Staff

Contents

LB-145: CELL & MOLECULAR BIOLOGY, SPRING

LB-145 = LB-145 (LECTURE) & LB-145L (LAB)

If you strive to think and communicate like a scientist in your work throughout the semester, in the end, you will be a scientist and really know biology (Think like a serious scientist, not like a pretend one).

LECTURER

Douglas B. Luckie, Ph.D., Professor, Lyman Briggs College & Dept. of Physiology
Holmes Hall Office: W-26D
Phone: 353-4606
E-mail: **luckie@msu.edu**

TEXTBOOK

"Integrating Concepts in Biology" by Campbell, Heyer & Paradise, 2024, 1st Edition, Trunity Holdings Inc

COURSE PACKET

LB145 Course Packet [for Dr. Luckie] at Bookstores (includes: syllabus, lab manual, lab notebook, etc)

COURSE WEBSITE **http://ctools.msu.edu/145**

LB-145 = LB-145 (LECTURE) & LB-145L (LAB) OVERVIEW OF CLASSES

There are two overreaching goals in these two connected classes.

1.) To gain a fundamental comprehension of the cellular processes necessary for life and an appreciation why it is important to understand these processes.
2.) To learn how to think like a scientist and be able to adaptively negotiate a question or problem.

During the semester we will study the building blocks of cells, the gross anatomy of the cell, and the structures and organelles that perform the work necessary for cell function. We will also examine several cellular processes at the molecular level, including the *central dogma* of molecular biology: RNA transcription and protein translation. We will examine the bioenergetic processes necessary to sustain life; first photosynthesis, the mechanism by which plant chloroplasts capture light energy to make the carbohydrates that bring life to earth. We then discuss the mitochondria and how they break down carbohydrates to release energy. All topics will be framed within the context of the human physiology. Mastery of these topics will provide you with an understanding of modern molecular and cellular biology.

LECTURE COURSE WORKLOAD

LB-145 is a 5-credit course that consists of two connected classes (lecture 3 credits, laboratory 2 credits) and because it IS two classes it requires twice as many hours of work as one class. Since the lecture is a course worth 3 credits, MSU requires that you work in-class 3 hours each week, and, in addition to the in-class work, for any 3-credit lecture course, MSU expects you to spend 6-9 hours/week outside of class studying and working on assignments. There will be a certain amount of preparation that you and your research group will need to do before each class and reading that you will need to complete (with notes taken) before each lecture. You will be expected to master quite a bit of new material to expand your knowledge of life and science. Come to lecture well-prepared and carefully listen and add additional notes about all the information provided or you will forget things and feel like nobody ever explained it to you.

SCHEDULE

Both the lecture schedule and the lab schedule are found in the subsequent pages. We reserve the right to modify the schedule if necessary. You will be given advance warning if the schedule needs to be changed.

OFFICE HOURS W-26D Holmes Hall, to be posted, and appointments upon request.

ACADEMIC HONESTY

It is your responsibility to know what constitutes cheating. Ignorance is not a defense. ***Turnitin.com*** will allow you and your group members to review written assignments prior to submission. If you are caught cheating you may be assigned a "0" for the assignment, or for the entire course. The policy for academic honesty at LBC can be found at http://lbc.msu.edu/current_students/academics/AcademicPolicies.cfm

GRADING

Your grade in this course (LB145) is based on the total percentage earned in the both the lecture portion and the laboratory portion of the course, each worth half. The course will be graded on a flat scale.

4.0= 90-100%	3.5= 85-89.9%	3.0= 80-84.9%	2.5= 75-79.9%	2.0= 70-74.9%	1.5= 65-69.9%	1.0= 60-64.9%	0.0= <60

A "3.0" score is considered Excellent. It is impressive work, top of the class, and the work was done extremely well but nothing beyond what was expected.
A "3.5" is Most Excellent. Every detail of the work was done extremely well, and they found additional papers and evidence beyond what they were told.
A "4.0" is Outstanding. It has the 3.0, 3.5-level elements + student impresses instructor with how much/well they did the work. They taught Prof something.

Late Policy: Assignments are due in lab/lecture at the beginning of the session indicated (at time of entering room) unless otherwise specified. If an assignment is 1 day late, 1 point will be deducted from the final score. After this 24 hr grace period, the penalty becomes more severe: 20% off for two days late, 30% off for three days late, and so on. After 5 days, you will receive a "0" for the assignment.

Rejected Manuscripts/Reports: Each time a paper is "rejected", because it did not follow the *Instructions to Authors*, 1 point is deducted. This is independent of the Late Policy, both can occur.

Blind Grading: Whenever possible we will score assignments "blind" and ask you not indicate your name but just list your "B-PID" (found on D2L). This helps eliminate bias and makes grading more fair.

***Formal Written Appeal Process**: If you feel that your exam, paper, or quiz was not graded properly you must submit your complaint in writing (on paper, not via email). You must concisely explain why you object to the assigned grade and what elements of your work demonstrate you mastered the material. Please be advised that if you submit a formal grade appeal about one element of an assignment, we always re-grade your *entire* exam, paper or quiz and the score may increase, decrease or stay the same. For group assignments, all authors must sign the written request. How much and how well you provide evidence to support your argument is assessed and students who provide good logical arguments supported well by solid relevant evidence will earn approval (Claim, Evidence, Reasoning; you may cite pages of textbooks or even better published research papers). Avoid emotional arguments that blame others or arguments based on hearsay, e.g. "A TA told me this was correct." If you neither make logical arguments nor provide thoughtful evidence to support them, your appeal will not gain traction or be approved. All discussion concerning score changes must be completed within 7 days from the date the grade was officially posted (on the returned assignment or online). No grade changes will be considered after this time. If illness or other emergency prevents you from completing assignments on time, you must make arrangements with your instructor afterwards.

LB145: Biology Learning Goals

Our **"skills"** learning goals are for you to gain practice and excel in these scientific methods:

1. Design: Apply science process skills, such as: developing hypotheses, making predictions, and designing experiments to test them (e.g. design an experiment to determine whether it's change in temperature or sunlight that causes leaves to turn red in Fall).

2. Analyze: Interpret evidence collected during experiments, looking for patterns and different ways to represent data, and using logical and/or quantitative reasoning to defend or reject hypotheses (claims).

3. Collaborate: Confidently cooperate in teamwork, and practice team building, team communication and leadership. (e.g. use techniques like "that's a good idea, OK, how can we improve it even more?" "Jon, you haven't spoken much, what do you think?")

4. Communicate: Conversation aimed at a variety of audiences important for scientists: (Ben says: "Their data predicts squirrels will hit light speed!" Jen responds: "But they have zero data at that part of the graph.")
    1) *Speaking*: practice speaking and listening to others in large & small groups.
    2) *Reading*: practice careful and critical reading of text, identification of important points & ideas, as well as slow deliberate reading and interpretation of figures and graphs.
    3) *Writing*: practice composition of text, writing manuscripts, building figures and graphs.
    4) *Thinking*: practice identifying data and evaluating author's evidence-based arguments.

5. Reflect: Develop personal learning goals and reflect on your progress throughout the semester. (e.g. regularly consider "OK, what I am supposed to be learning here? Have I mastered that topic? What next?)

Our **"content"** learning goals are for you to understand, describe, and provide examples of how:
(this semester's topics connected to learning goals you studied previously in LB144 are underlined below)

1. Evolution of molecules, organelles, and processes led to the origin of life on the planet earth. (e.g. How is it possible to create carbon-based life on a sterile planet that has none?)

2. Some cells can capture CO2 and transform photonic energy into chemical energy (e.g. ATP) to drive cellular processes and build cellular polymers. (e.g. How does photosynthesis work? How does a chlorophyll pigment molecule capture light energy?)

3. Small organic molecules (nucleotides, amino acids, lipids, carbohydrates) when built into polymers can associate to create cellular surfaces and compartments with which to perform biochemical processes (of life). (e.g. What is a lipid and how is it used to create a cell membrane? When proteins join a membrane does that create intelligence?!)

4. Information in DNA -> becomes (transcribed) information as RNA -> becomes (translated) information in the proteins that determine structure. (e.g. How does a cell make insulin? Transcription make mRNA?)

5. Information in the 3D structure of a molecule determines its function (and influences its evolution). (e.g. the CFTR protein is shaped like a roll of toilet paper in the cell membrane, turns out it's an ion channel)

6. Changes in DNA (mutations that lead to new alleles) -> result in changed RNA that may lead to -> changed protein (structure) that lead to changed function. (e.g. What DNA change leads to sickle cell anemia? or How does a three-base deletion result in the disease cystic fibrosis?)

7. ____
____
____

LB-145: CELL & MOLECULAR BIOLOGY, LECTURE

SCHEDULE:

Date		***Lecture Topic***	***Reading(s) discussed in lecture***
Week 1	M	The course and diseases	*CF paper* in Course Pack pp.267-274
	W	**Evolution of Life**: What is the heritable material?	Textbook's *Foreword*, Ch.1-ICB: 1.2
Week 2	M	EvoLife: What is Evolution? (molecules in life).	Ch.4-ICB: 4.1, ELSI 4.1
	W	EvoLife: Could abiotic molecules form organic?	Ch.4-ICB: 4.2
Week 3	M	EvoLife: Can non-living objects compete, store E?	Ch.4-ICB: 4.3(1st half), 4.4
	W	EvoLife: Were organelles once living bacteria?	Margulis; Photosynthesis (OSB): 8.1
Week 4	M	EvoLife: What chloroplasts do? (photosynthesis)	Photosynthesis (OSB): 8.2
	W	EvoLife: Where is tree mass from? (photosynthesis)	Photosynthesis (OSB): 8.3
Week 5	M	EvoLife: Why is Paraquat used? (photosynthesis)	Ch.11-ICB: 11.1
	W	EvoLife: Brazil affects glaciers? (photosynthesis)	Ch.11-ICB: 11.2
Week 6	M	***EXAM I*:** Evolution of molecules, organelles, photosynthesis	
	W	**Cystic Fibrosis**: How do people get sick?	*CF paper*: Rowe et al. *NEJM* 2005
Week 7	M	CF: How do genetic diseases arise?	Ch.5-ICB: 5.1
	W	CF: What role for prok & eukaryotes? (cells)	Cell Structure (OSB): 4.3
Week 8	M	CF: What happens at endomembranes? (cells)	Cell Structure (OSB): 4.4
	W	CF: Why aren't there giant cells? (cells)	Ch.8-ICB 8.2
Week 9	M	CF: Is passive transport the disease? (membranes)	Plasma Membranes (OSB): 5.2
	W	CF: Is active transport important too? (mems)	Plasma Membranes (OSB): 5.3
Week 10	M	CF: Breathing/ventilation/circulation	Respiratory System (OSB): 39.1
	W	***EXAM II:*** cystic fibrosis, cells, membranes, channels, transporters	
Week 11	M	**Diabetes**: How do people get sick?	*Diabetes paper:* Polonsky *NEJM* 2012
	W	Diabetes: How do you break down food?	Ch.23-ICB: 23.1
Week 12	M	Diabetes: How does DNA shape affect (dogma)?	Ch.1-ICB: 1.4, ELSI-1.1
	W	Diabetes: How to make RNA (central dogma)	Genes and Proteins (OSB): 15.3, 15.5
Week 13	M	Diabetes: How does DNA communicate (dogma)?	Ch.2-ICB: 2.1
	W	Diabetes: How does insulin express itself (dogma)?	Ch.2-ICB: 2.4 (insulin leisure reading)
Week 14	M	Diabetes: How is ATP produced (respiration)?	Ch.10-ICB: 10.4
	W	Diabetes: Homeostasis and regulation (respiration)?	Ch.10-ICB: ELSI-10.1, BME-10.1

***FINAL EXAM*:** comprehensive (finals week, see student.msu.edu for schedule)

THE LECTURE
ASSIGNMENT SCHEDULE

Researchers have found increased structure and active learning increase everyone's ability to learn in introductory biology courses[1]. In addition, every student in our course really does want to slowly carefully read the textbook, learn new information and enjoy mastering topics in biology. Given we believe the textbook we are using is outstanding, we are structuring short readings, with integrating questions in the course, so you more carefully read each section and reflect upon it. A quiz or exercise based on the reading may be given each lecture. These quizzes/exercises are designed to help you assess your own learning before and between exams. They provide you with regular feedback as to how well you are mastering each topic.

ATTENDANCE AND PARTICIPATION: It is essential that you not only come to class but also actively participate in order to construct your own knowledge. While attendance is being "present", participation includes reading and preparing well for class, answering questions verbally, and via clicker questions. Active participation includes the following behaviors:

1. *Bringing forth new ideas, information, or perspectives to academic conversations*
2. *Discussing your readings and reflections with instructors and peers*
3. *Meeting with the instructors to discuss your interests, assignments, or project*
4. *Participating in small group discussions and activities*
5. *Assuming responsibility for personal behavior and learning*

While working on group projects, students should be mindful, all participants should exercise:

- *Respect for themselves, each other*
- *Openness and a positive attitude toward new ideas and other's ideas*
- *Flexibility and tolerance of ambiguity*
- *Good communications amongst themselves.*

EXAMS: There will be two midterm exams and a final exam, each may be comprehensive of all prior material. Midterm exams may be traditional multiple-choice format, or may be essay-style Answers to open-book & take-home exams must also be submitted online to http://turnitin.com/.

Assignments (pts)**:**

Week	**Assignment(s)**	**@Lecture**	**%**
(*all*)	Attendance, Participation, Homework, Quizzes	X	**20**
5	**Exam I**	**X**	**25**
10	**Exam II**	**X**	**25**
15	**Final Exam**	**X**	**30**

Total = 100% of lecture grade

---

[1] Haak, D., J. HilleRisLambers, E. Pitre, and S. Freeman. 2011. Increased structure and active learning reduce the achievement gap in introductory biology. *Science* 332:1213-1216. Freeman, S., D. Haak, and M.P. Wenderoth. 2011. Increased Course Structure Reduces Fail Rates in Biology. *CBE Life Science Education* 10 (2):175-186

LB-145L: CELL & MOLECULAR BIOLOGY, LAB

LAB COORDINATOR

Douglas B. Luckie, Ph.D., Associate Professor, Lyman Briggs College & Dept. Physiology

TEXTBOOK

"Integrating Concepts in Biology" by Campbell, Heyer & Paradise, 2014, 1st Edition, Trunity Holdings Inc

LAB MANUAL

found inside "LB-145 Course Pack," (Luckie) from MSU Library Services via local bookstore

COURSE WEBSITE http://ctools.msu.edu/145

RESEARCH TEAM RATIONALE

Student groups are intended to be research & learning teams. Work with other students to study and discuss biology topics in lecture, as well as share your ideas and research predictions in lab. Teams are better learning environments but also, they are REAL LIFE. While scientists do some things on their own, they more often work in groups to solve problems because a well-functioning team is the most efficient way to work. Working in the same group in both laboratory and lecture will allow you to become more familiar with each other so you will feel comfortable enough to discuss your biology questions. Although it is easier for an instructor to run a class or lab without group work, numerous research studies have shown that working in groups and discussing science with your peers can increase your learning ***considerably*** (although you have to strive to be a "cooperative" group). By pooling your knowledge, members of your group will get "stuck" less often be able to progress far beyond what any individual in the group could do alone.

Week	Laboratory Investigation
1	*Orientation and project introduction*
2	*Group orientation and project planning*
3	Basic lab skills, group's **Proposal Presentation**
4	PCR Lab, group's ***Draft 1 due*****, In-lab LA Interview** (GEA1)
5	PCR Lab (continued)
6	Genome Lab begins
7	Genome Lab (cont.), ***Draft 2 due*** (5 copies), **Prof/GA Interview** (GEA2)
8	Independent Investigations, Ordering, **Written *Peer Review* due**
9	Independent Investigations [PBAs, Notebooks checks and troubleshooting]
10	**Verbal Status Report Presentation due**
11-12	Independent Investigations [PBAs, Notebooks checks]
13	In-lab **Presentations** (formal final talks about your findings) (GEA3)
14	***Final research report (Draft 3) due***

THE LABORATORY

You will need the Laboratory Manual resources provided in the Course Pack. Review the lab guide materials required for each week during the semester. If you know the course pack "inside and out" you will make yourself extremely well prepared (and far less stressed) for all that happens during the week to week events of the course laboratory. Other students will think you are some genius who seems to always know everything, and is always calm, but you will be the only one who reads ahead in the course pack.

This semester, you will design a PCR-based diagnostic test for a known mutation that causes a disease. You will also perform a sociological study where you experience the impact of the disease. Each week, you will examine and practice the methods of a scientist in performing your research. This approach is aimed at mentoring you, so you master the ability to think and work like a serious scientist.

While working on group projects, you should be mindful of other students in your group; therefore, it is important for all participants to exercise:

- Respect for themselves, each other
- Openness and a positive attitude toward new ideas and other's ideas
- Flexibility and tolerance of ambiguity
- Good communications amongst themselves

ASSIGNMENT SCHEDULE

Speaking	*Writing*	*Discussing/Demonstrating*
Proposal Presentation	Group Draft 1 Paper	LA interview
Status Report Presentation	Group Draft 2 Paper	Prof/GA interview
Formal Final Presentation	Group Final Paper	Lab Notebook & PBAs
	Peer Review worksheet	

Week	**Assignment(s) Due**	**Value (%)**
3	*Proposal Presentation (& movie)*	10
4	**Draft 1 Paper**, LA Interview in lab	**10,** practice
7	**Draft 2 Paper (original & 5 copies)**, Interview with Prof	**20,** 10
8	Peer Review worksheet (2 copies, one anonymous)	EC
10	*Status Report Presentation (& movie)*	ReDo option
13	In-lab Formal Final Presentations *(& movie)*	20
14	**Final Paper (Draft 3)**	**30**

Total = 100% of lab grade

MSU & LBC INFORMATION AND POLICIES

WASH HANDS OFTEN

Wash your hands with soap or hand sanitizer.

FEEL SICK? STAY HOME.

Fever, cough, aches, fatigue, nausea? Stay home.

Face Coverings:

Face coverings must be worn by anyone who is not vaccinated versus the SARS-CoV-2 coronavirus (all faculty, staff, students, vendors, and visitors) while participating in MSU-related or MSU-sponsored activities. If you have a medical condition that may prevent you from safely wearing a face covering, you should contact MSU's Resource Center for Persons with Disabilities to begin the accommodation process.

Face coverings should (a) be non-medical grade to maintain supplies for health care use, (b) fit snugly against the side of your face, (c) cover your nose and mouth, (d) be secured with ties or ear loops, and (e) allow for breathing without restriction. Cloth face coverings should only be worn for one day at a time, and they must be properly hand washed or laundered before subsequent use. Face coverings may vary (for example, disposable non-medical face coverings or neck gaiters are acceptable).

Absence due to illness: Students who need to quarantine themselves, have been sick with COVID-19 symptoms, tested positive for COVID-19, or have been potentially exposed to someone with COVID-19 must follow CDC guidance to self-isolate or stay home. Illness or self-isolation will not harm performance or put one at a disadvantage in the class.

Technical Assistance

If you need technical assistance at any time during the course or to report a problem you can:

- Visit the Distance Learning Services Support Site
- Visit the Desire2Learn Help Site (http://help.d2l.msu.edu/)
- Or call Distance Learning Services: (800) 500-1554 or (517) 355-2345

Mental Health Resources

College students often experience issues that may interfere with academic success such as academic stress, If you or a friend is struggling, we strongly encourage you to seek support. Helpful, effective resources are available on campus, and most are free of charge.

- Drop by Counseling & Psychiatric Services (CAPS) main location (3rd floor of Olin Health Center) for a same-day mental health screening.
- Visit https://caps.msu.edu for online health assessments, hours, and additional CAPS services.
- Call CAPS at **(517) 355-8270** any time, day or night.
- 24-Hour MSU Sexual Assault Crisis Line **(517) 372-6666** or visit https://centerforsurvivors.msu.edu/

Resource Persons with Disabilities (RCPD)

- To make an appointment with a specialist, contact: (517) 353-9642
  Or TTY: (517) 355-1293
- Web site for RCPD: http://MYProfile.rcpd.msu.edu

Inform Your Instructor of Any Accommodations Needed

- From the Resource Center for Persons with Disabilities (RCPD): Once your eligibility for an accommodation has been determined, you will be issued a Verified Individual Services Accommodation ("VISA") form. Please present this form to me at the start of the term and/or two weeks prior to the accommodation date (test, project, etc.).

LBC Student Success and Advising Team

LBC advisors work to educate, coach, and support students in our College. For more information about the Student Success and Advising team visit: https://lbc.msu.edu/advising/index1.html

To make a zoom or phone appointment with an advisor visit: https://lbc.msu.edu/advising/advising-appointments.html

To review LBC Academic Policies, including LBC's Academic Grievance Policy, visit: https://lbc.msu.edu/advising/academic-policies.html

Related Policies:
Institutional Data Policy:
https://tech.msu.edu/about/guidelines-policies/msu-institutional-data-policy/
Student Privacy Guidelines and Notification of Rights under FERPA
https://reg.msu.edu/ROInfo/Notices/PrivacyGuidelines.aspx

Commitment to Integrity: Academic Honesty

Article 2.3.3 of the Academic Freedom Report states that "The student shares with the faculty the responsibility for maintaining the integrity of scholarship, grades, and professional standards." In addition, the (insert name of unit offering course) adheres to the policies on academic honesty as specified in General Student Regulations 1.0, Protection of Scholarship and Grades; the all-University Policy on Integrity of Scholarship and Grades; and Ordinance 17.00, Examinations. (See Spartan Life: Student Handbook and Resource Guide and/or the MSU Web site: www.msu.edu.)

Therefore, unless authorized by your instructor, you are expected to complete all course assignments, including homework, lab work, quizzes, tests and exams, without assistance from any source. You are expected to develop original work for this course; therefore, you may not submit course work you completed for another course to satisfy the requirements for this course. Also, you are not authorized to use answers provided by Chegg.com or CourseHero.com or similar "cheat" web sites to complete any course work in this course. Students who violate MSU academic integrity rules may receive a penalty grade, including a failing grade on the assignment or in the course. Contact your instructor if you are unsure about the appropriateness of your course work. (See also the Academic Integrity webpage.)

LINKS TO UNIVERSITY POLICIES

- **Spartan Code of Honor**
- **Academic Integrity**
- **RCPD Disability Accommodations Statement**
- **Mental Health**
- **Tolerance and civility**
- **Religious Observance Policy**
- **Student Athletes**
- **MSU Final Exam Policy**

145 Contract (sign & return)

A syllabus is a form of contract between the instructor and the students. If you, the student, complete tasks with a specific score a predefined grade is awarded. Read the announcements below and the syllabus *in full* before signing and submitting this page.

1. WORKLOAD *As Undersigned student, I am aware the lecture & lab course is worth 5 credits and will require me to work outside of class 10-15 hours each week. Some weeks will require less, and some weeks will require more effort. If I prepare poorly for class, learning will take longer.*

2. TOURISM *I am aware that I will work with a group of students that sit together in lecture, work together as a research team in lab, meet and study together outside of class at night and on weekends. This course is designed for full-time LBC students and if I need to travel off-campus or off-grid frequently, my group members may become very unhappy, and I should discuss this.*

3. MANY READINGS *As the Undersigned student, I am aware that I will have many required reading assignments each week, and unless I read the assigned pages, answer questions, take notes and study them prior to class, it's likely I will become lost in lecture.*

5. VERBAL FINAL *I am aware that the final exam is long, and comprehensive, but an optional "verbal final" is also available. The verbal final is an individual interview with the instructor where I demonstrate my mastery by explaining the biology discussed in the course. The verbal final is pass/fail where passing results in a 100% score for the final exam.*

5. EXAMS *As the Undersigned student, I am aware midterm exams may be purely essay style and provided in advance, and in this case I should work with my group studying the questions and developing excellent answers in the time prior to the test. If I just "cram" my studies and work into 48 hours prior to the exam, it's likely I will get a low score on said midterm.*

6. UNIVERSITY GRADING SCALE *I am aware this course uses a university scale with higher expectations than high school, excellent work is a 3.0, much more is necessary for a 4.0.*

7. GROUP GRADES *I am aware that I, with the help of other students in my research group, will be authoring one research paper (with a number of drafts) and my grade may include both the score of my sections as well as the score for the work as a whole. I realize I will be expected to review the entire project before submission. If this doesn't work well for me, I should discuss it with my group or the prof immediately.*

8. HONOR CODE *In the authoring of assignments, I accept that any piece of work may be submitted to http://turnitin.com for screening. I am aware that if the work authored by me is found to be plagiarized, I will be given a zero for the assignment & perhaps for LB145 course grade.*

I have read the above announcements and syllabus. I understand the expectations are high but I'm up to the challenge. I agree to the tenets of this contract.

Printed Name *Signature* *Date*

145 Contract (sign & return)

A syllabus is a form of contract between the instructor and the students. If you, the student, complete tasks with a specific score a predefined grade is awarded. Read the announcements below and the syllabus *in full* before signing and submitting this page.

1. WORKLOAD *As Undersigned student, I am aware the lecture & lab course is worth 5 credits and will require me to work outside of class 10-15 hours each week. Some weeks will require less, and some weeks will require more effort. If I prepare poorly for class, learning will take longer.*

2. TOURISM *I am aware that I will work with a group of students that sit together in lecture, work together as a research team in lab, meet and study together outside of class at night and on weekends. This course is designed for full-time LBC students and if I need to travel off-campus or off-grid frequently, my group members may become very unhappy, and I should discuss this.*

3. MANY READINGS *As the Undersigned student, I am aware that I will have many required reading assignments each week, and unless I read the assigned pages, answer questions, take notes and study them prior to class, it's likely I will become lost in lecture.*

5. VERBAL FINAL *I am aware that the final exam is long, and comprehensive, but an optional "verbal final" is also available. The verbal final is an individual interview with the instructor where I demonstrate my mastery by explaining the biology discussed in the course. The verbal final is pass/fail where passing results in a 100% score for the final exam.*

5. EXAMS *As the Undersigned student, I am aware midterm exams may be purely essay style and provided in advance, and in this case I should work with my group studying the questions and developing excellent answers in the time prior to the test. If I just "cram" my studies and work into 48 hours prior to the exam, it's likely I will get a low score on said midterm.*

6. UNIVERSITY GRADING SCALE *I am aware this course uses a university scale with higher expectations than high school, excellent work is a 3.0, much more is necessary for a 4.0.*

7. GROUP GRADES *I am aware that I, with the help of other students in my research group, will be authoring one research paper (with a number of drafts) and my grade may include both the score of my sections as well as the score for the work as a whole. I realize I will be expected to review the entire project before submission. If this doesn't work well for me, I should discuss it with my group or the prof immediately.*

8. HONOR CODE *In the authoring of assignments, I accept that any piece of work may be submitted to http://turnitin.com for screening. I am aware that if the work authored by me is found to be plagiarized, I will be given a zero for the assignment & perhaps for LB145 course grade.*

I have read the above announcements and syllabus. I understand the expectations are high but I'm up to the challenge. I agree to the tenets of this contract.

____

Printed Name　　　　*Signature*　　　　*Date*

Owner's Manual

(with lots of ideas and text stolen from great authors, Drs. Alice Dreger and Tanya Noel)

Why is this an "owner's manual" instead of a syllabus?

Most syllabi contain only class schedule information. By contrast, this is more like an "owner's manual" like the sort that comes with a new car. If you read and use this manual, you will understand how this course works, and you will be able to keep the course running smoothly, and do the regular maintenance required to avoid breakdowns. Of course, this course isn't a car. It's more like a bus tour. I believe that a university course is in its essence not a number, and not a topic, but a group of people who share a common goal of learning about some particular thing. In this sense, a course is like a bus tour, a tour to a place which is unfamiliar to most of us. As the teacher, I am the bus driver and chief tour guide. Each member of the course starts off at "home" intellectually and emotionally and comes to the bus station which is the classroom. We agree to "take the tour" together, to get on the bus and travel together for the length of the course even though many of us may never have met before. Together we visit a number of different "places."

So why is this "owner's manual" so long?

I've discovered that the more information I give students, the more comfortable and in control they feel, and the better they learn. This packet contains lots of information. Besides telling you about the mechanics of the course, this packet tells you a lot about my teaching style. I used to provide my students with a separate "statement of teaching philosophy." It now occurs to me it is weird to separate that teaching philosophy from my teaching materials. So now my philosophy is embedded throughout this packet. My teaching style, methods, and philosophy change over time, thanks to students who tell me what works and what doesn't work. I'm counting on you to give me lots of feedback about what is working for you and what is not, and most importantly why. It is very important to me to do a good job for you. In addition to the course learning objectives provided earlier, be aware this course aligns with the following MSU Undergraduate Learning Goals:

Analytical Thinking

A successful student uses ways of knowing from mathematics, natural sciences, social sciences, humanities, and arts to access information and critically analyzes complex material in order to evaluate evidence, construct reasoned arguments, and communicate inferences and conclusions.

- *Acquires, analyzes, and evaluates information from multiple sources.*
- *Synthesizes and applies the information within and across disciplines.*
- *Identifies and applies, as appropriate, quantitative methods for defining and responding to problems.*
- *Identifies the credibility, use and misuse of scientific, humanistic and artistic methods.*

Effective Communication

A successful student uses a variety of media to communicate effectively with diverse audiences.

- *Identifies how contexts affect communication strategies and practices.*
- *Engages in effective communication practices in a variety of situations and with a variety of media.*

Integrated Reasoning

A successful student integrates discipline-based knowledge to make informed decisions that reflect humane social, ethical, and aesthetic values.

- *Critically applies liberal arts knowledge in disciplinary contexts and disciplinary knowledge in liberal arts contexts.*
- *Uses a variety of inquiry strategies incorporating multiple views to make value judgments, solve problems, answer questions, and generate new understandings.*

How does this course work in terms of the day-to-day?

We will meet two times a week for the lecture class and our meetings will consist of discussions of the readings and activities related to the topics we are investigating. Do the readings assigned for the day **before** you come to class and spend enough time thinking about the readings before class. You should come to class ready to summarize the readings and to ask and answer questions about them. Homework and quizzes will often be given on the readings.

Always give yourself plenty of time to do your work, and feel free to contact me whenever you need help or clarification. I like teaching and not only do I feel good when you learn, often when you learn something new, I learn, too.

Generally, we will stick very closely to the attached schedule, however, the point of this class is for you to learn, so if we need to change our scheduled plans to achieve that goal, we will do so. If you feel that you need things to be done somewhat differently in class in order for you to learn better, please let me know and I will work to adjust our schedule or classroom dynamics so that we can maximize learning.

So what's my feeling about teaching?

I love it! And I think it shows – my students have voted me "honorary member of the graduating class of Lyman Briggs" ("teacher of the year") about five times in the last fifteen years, I was given the Teacher-Scholar Award of MSU, and most recently the 2015 MSU Alumni Club of Mid-Michigan Quality in Undergraduate Teaching Award (nominated by MSU faculty and alumni for teaching) and the 2017 Outstanding Faculty Award by the ASMSU Senior Class Council (nominated by MSU graduating seniors for teaching). If you hear that I am tough, I am, but that's because I care about your learning. If I didn't care about your learning, I would have stayed at Stanford University.

I am delighted to have recruited amazing LAs to help you do well in the course. You will find that our LAs share my love of teaching, of biology and dedication to helping you learn. But they are tough too because they want you to learn, lots. They are trained to answer your questions with responses in the form of guiding questions. Why? because it helps you learn and *remember*, and they know your next class (and career) will be far more difficult and demanding than this course, you know this too.

What else besides being in class will be required of you?

Note that this course uses a wider range of assignments than just several exams. This spreads out risk and stress so it's lower level, day to day, and allows you to assess your own learning with lower-stake quizzes to avoid any surprises when facing the bigger exams. Grades are pretty simple, like getting an "A" or "B+" or "C" written at the top of each assignment– and you can always check your grade on the D2L gradebook – but be sure to keep your own spreadsheet and alert me if my gradesheet has an error.

- *Quizzes on readings:* I will frequently give short quizzes on a day's assigned reading at the beginning of the class meeting. These quizzes accomplish two things: (1) reward you for keeping up-to-date on the readings; (2) reward you for spending enough time on the readings to really understand them. If you read carefully, you should have little problem with the quizzes. If you have a lot of trouble with short, fast quizzes, remember there are lots of bonus options in this class you can use as substitutions. If you miss a quiz because you are late or absent, you will receive a "0". These cannot be made up.

A note on grades & FERPA:

To support blind-grading we will often request that you not list your actual name but just provide your PID. Privacy, as required by MSU FERPA regulation, will be maintained by utilizing a code that is NOT your real A-PID, so we'll call it your B-PID. Your B-PID will be listed on D2L in your personal gradebook.

Backstory: In recent years universities have become very afraid of getting in trouble for breaking the law called FERPA (Family Educational Rights and Privacy Act). The law was created back in 1974 to protect the privacy of students and their grades. In response to it all universities created student ID numbers so instead of placing a grade next to a person's name, instructors could place it next to a student number to maintain privacy. Many universities chose to use a student's social security number to also be their student number. When identity theft became a big problem, universities then changed all their student ID numbers from social security to become some number randomly generated in house. In recent years now the student ID number itself has become protected. In fact, while other people are permitted to know your name, and even say it aloud and post it publicly, the student ID number is super protected. Thus instead of using your officially MSU-issued A-PID, in this course MSU requires that we issue a new temporary student ID. We will call these the B-PID, since they are for "who you be" and it’s for blind grading.

Professors can use grades in two ways: they can use grades to "sort" students into "A" students, "B" students, etc.; or they can use grades as learning incentives and rewards. Unfortunately the sorting system generally sorts according to "talents" students either have or don't have before they ever reach a particular classroom, e.g., the talent of being able to memorize and recall a lot of things. I would rather use grades to encourage students to develop their skills, to expand their minds and interests. While students are often only familiar with positive curving (sometime called a mother's curve) a number of university classes use an actual curve that raises or lowers the grading scale with the goals to only permits a few students (like just 10 in a class of 100) to earn a 4.0 and then only a few (perhaps 20) are permitted to have a 3.5 etc. Even if everyone in the class got above a 90% on an exam the grade scale would shift up until only the prescribed number of students got a 4.0 grade. This is a real "curve" and, I will never grade on a curve like this. Our grading scale with stay exactly as stated in the syllabus and each student will get whatever grade she or he has earned by the end of the semester. Nothing would make me happier than if everyone worked hard and learned a lot and got 4.0's. I would feel that we had achieved something great if everyone got a 4.0.

Table 1- University-level grading system: The table below describes the relationships between grades, percent, and performance in the University-level grading system used in our lab and lecture courses. The first column describes the letter/number grade. The second column describes the percentage associated with that grade. The third column describes the performance-level required. Remember, if at any point you feel confused or distressed about your grades, carefully review the syllabus and talk to me.

Letter Grade	***Percentage***	***Performance***
A (4.0)	90 to 100%	*Outstanding Work*- A “4.0” is Outstanding. It literally stands out. It has the characteristics described for 3.0 and 3.5-level elements but in addition, the work by itself impressed with how much & well it was done. The student taught Prof something original.
B+ (3.5)	85 to 89.9%	*Most Excellent Work* - A “3.5” is Most Excellent. Every detail of the work was done extremely well and they found additional papers and evidence beyond what they were told.
B (3.0)	80 to 84.9%	*Excellent Work* - A “3.0” score is considered Excellent. It is impressive work, top of the class, and the work was done extremely well but nothing beyond what was expected.
C+ (2.5)	75 to 79.9%	*Pretty Good Work*- A "2.5" is Pretty Good, the student did the minimum work required and did a pretty good job, this is expected at the university level and near average for the class.
C (2.0)	70 to 74.9%	*Average Work* - A "2.0" is average, the student did the minimum work required.
D+ (1.5)	65 to 69.9%	*Below Average Work* - the student did less than minimum work required.
D (1.0)	60 to 64.9%	*Poor Work*- the student did less than minimum work required and of poor quality.
F (0.0)	0 to 59.9%	*Failing Work*- the student did far less than minimum work required and very poor quality.

Course Structure

This course will use a public website and online tools like Turnitin, CATME, Desire2Learn, and Top Hat. The course website may include online lessons, course materials, and additional resources. Activities may consist of readings, discussion forums, email, journaling, wikis, and other online activities. You will need your MSU NetID to login to the course to access the grades on *D2L* (*http://d2l.msu.edu*).

Definitions, terms, transparency

Admission: I believe caffeine and sugar increase attention and learning but have no empirical data to support this, except for eating donuts, that is documented to work, but just for 15 minutes post-eating. I like the drink called the Cortado (it's coffee, like a tiny latte) but particularly enjoy the moment I pour cane sugar out of the brown paper packet on top of the frothed milk and watch it sink into the drink. When you come to office hours, unless there's a rush, I'll likely offer you an espresso.

Attendance: Student learning is impacted by many things, yet education research has robustly shown it is significantly impacted by these three things: class size, teacher quality and attendance. You are, of course, permitted to skip any class meeting you wish but often a single clicker point is made available to you, to encourage attendance since it correlates with learning. Attendance at the meeting of a class will be defined as being physically present in the room for the full time period of the class meeting. Thus be present, in your seat with you notebook open and pen in hand, at the very beginning when the clock in room strikes the hour and class begins, still there during/throughout the entire duration of the class, as well as at the very end of the official time period (feel free to come and go to visit the restroom, just not off vacationing elsewhere). It's only fair to treat students who arrive late exactly the same as those who depart early. We will often reward students for attendance by using technology to record your presence. If you fail at using your device to click-in for attendance at the beginning middle or end of class, due to whatever reason, be aware we do not micromanage the attendance data (no appeals). Making the choice to schedule another course that has a start or finish time that is proximal or even overlaps with this class is, of course, your choice and entirely acceptable. Yet this will not change the definition of attendance or waive it. University students are adults and literally everything in a course is optional, yet if you want points, in this case for attendance (and more importantly to learn) you have to be there.

Belong: Lyman Briggs College is dedicated to promoting inclusion and fostering diversity. Let's make our classroom comfortable and welcoming for everybody. Let's strive to treat everyone with respect, civility, and empathy and rather than avoid new things to learn from others about different beliefs, practices, and lives. You are all super wonderful smart people and all belong here.

Blind grading: When a computer scores a scantron bubble sheet from a multiple choice exam, it is objective, it doesn't have a pre-conception as to which students are smart, or are nice to it, so it treats everyone the same and just rewards correct answers. Unfortunately, human graders are less objective. LAs, GTAs, and Profs, are all unable to be perfectly objective when they have already had interactions with the person whose work they are grading. While they try hard to be so, education research shows that even knowing what the person's name is will impact the grader and grade (even if they never met the person). Thus imagine if they know the person reasonably well. If they have read prior papers, knew the person's prior grades, or had a number of positive (or negative) conversations with them. Wow, that will cause major problems when trying to be objective while grading, even for the best teacher ever, unless the grader is blind to the identity of the author. Professional journals and grant review panels use single blind or double blind systems to avoid subjective evaluation. We will use this in our class too.

Participation: It turns out participation is different from attendance. It refers to a student who is actively working to perform the work and learn the materials discussed in the course. Students who are active participants do not merely talk during class but also prepare in advance for the class and do work outside of class. For the lecture course, this means carefully completing the readings, taking notes on them (handwritten notes), and preparing for the upcoming class meeting by reviewing notes and highlighting any questions you thought of while preparing for class. To reward this behavior, which enhances learning, often there will be a pop quiz or problem or writing exercise during class which is scored. Also, there are TopHat clicker questions during the lecture, and you earn a point each time you choose a correct answer. You only need to get above 70% of all TopHat points to earn an Outstanding (4.0) grade for lecture participation. For the lab course, this means performing experiments, collecting data for your project each week, and recording it in your official lab notebook (trifecta style). It also means working well with your group, working just as much as your peers. CATME surveys as well as instructors' observations of you and your notebook will be used to evaluate your lab participation. If you prepare well for class (lecture & lab courses), you'll get good grades, and if you don't, you are accountable. This helps increase the number of people who ultimately decide they need to study the material or collect data before class and as a result, also learn more when discussing the material again in class. If you prepare, the class is fun and interesting. If you don't, it can become confusing and frustrating, as it feels like everyone else seems to know all the answers while you don't even understand the questions. The lab participation grade represents half of the final combined Attendance and Participation grade. Here are examples of Outstanding versus 0% participation in the lab course. OUTSTANDING participation would be: you did all CATMEs, got high scores on all, have many full experiments listed in your lab notebook, and many highlighted/read papers on your gene, disease, methods, kept in a notebook. A 0% would be: you did no CATMEs, got low scores on all, have zero experiments listed in your lab notebook, and found/read/contributed no papers to your group.

Random calling in the lecture: How often have you been in a big lecture class that has maybe 8 students who are the only people who ever are called upon to answer the professor's questions in the lecture? The other 100+ students throughout the entire semester will generally never speak aloud during lectures. After a while, you get used to it. Everyone knows that "those students" answer the questions, so we don't have to, cool. Yet deep down you also know, that while it's comfortable to never have to answer a question, it likely reduces your learning, heck some folks fall asleep. My wife tells a story about a small class where the Professor always asked these incredibly difficult questions that nobody ever even understood. Then one day, near the end of the semester, for the very first time, she did the reading before class and during class realized that every single question the instructor asked had always been directly out of the reading. She was embarrassed because she realized the Professor must know nobody does the reading, given no student ever understood the questions he asked, even though they were right out of the first pages of each reading. Because our goal in this class is learning we will use random calling in lectures to help *everyone* increase their learning and gain skills in communication/public speaking. Given that our #1 goal in the course is you to become comfortable and confident at public speaking, this is required to help that happen.

Our no-points grading system: The grading system in the course is based upon the University Grading Scale (described earlier) and grade levels are described by terms e.g. "Pretty Good", "Excellent", "Outstanding" which are equivalent to 2.5, 3.0, 4.0. While some assignments like TopHat, or rubrics for the papers, still use points in evaluation, their final overall grade becomes a grade-level not a precise point total. We actually do not track a point total as the grading system, nor do the instructors track you current grade in either the lecture course or lab course. We just work to make sure the data, the individual grades, are provided and accurate on the D2L grades page. In a University level course students are not treated like children, they have incredible math skills and are expected to do the mathematics needed to regularly calculate and track their own grades. Each assignment's grade often becomes a grade-level and in the end every assignment is just worth a portion (percentage) of the final grade.

Here are descriptions associated with each grade level.

PRETTY GOOD= If a student did mostly what was asked (nearly or at the very minimum required) AND a pretty good job of it, the grade awarded is a "Pretty Good" grade level.
EXCELLENT= If a student did everything that was asked (the very minimum required, but nothing beyond) AND did an excellent job in the work, the grade awarded is an "Excellent" grade level.
MOST EXCELLENT= If a student did everything required AND MORE (did the very minimum in all categories as well as more than the minimum in one or more) AND an excellent job.
OUTSTANDING= If the student did everything that was asked for AND went FAR above and beyond what was asked (more than the minimum in multiple categories) AND did an amazing job!

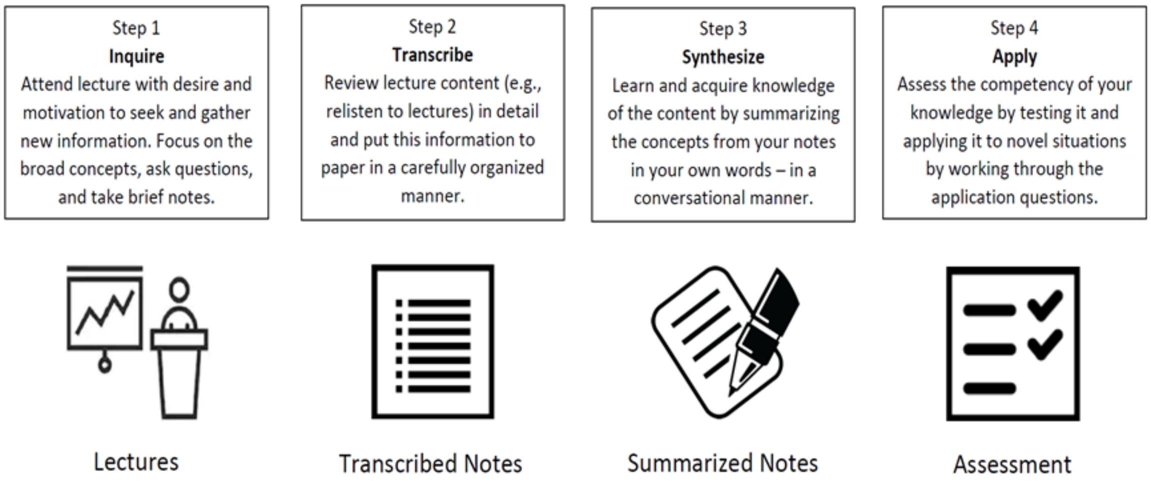
WHEN YOU NEED MORE LEVELS:
If a student did the minimum work required but not pretty good quality, just average quality =AVERAGE
If a student does less than the minimum work required =BELOW AVERAGE
If a student does less than the minimum work required AND of poor quality =POOR
If a student does FAR less than the minimum work required AND of poor quality= FAILING

NOTE: If the listed score for an assignment is not a descriptive "grade level" like those above, but instead just a number, e.g. 100% or 78.2%, which is always the case for exams, that is the final grade. It will not change to become a grade level.

At the end of the semester, in final grades calculations we will convert these grade levels to these numbers and do math to determine each student's final percentage grade:
Outstanding (4.0 level) =95%, **Most Excellent** (3.5) =87.5%, **Excellent** (3.0) =82.5%, **Pretty Good** (2.5) =77.5%, **Average** (2.0) =72.5%, **Below Average** (1.5) =67.5%, **Failing** (1.0 level) =62.5%

Study Skills Tips from Dr. Marty Spranger (plus 18min video)

To gain deep understanding and be able to recall material try these four steps. Here is a link to an 18 minute video where he explains these steps. [Study skills for better learning]



Step 1
Inquire
Attend lecture with desire and motivation to seek and gather new information. Focus on the broad concepts, ask questions, and take brief notes.

Step 2
Transcribe
Review lecture content (e.g., relisten to lectures) in detail and put this information to paper in a carefully organized manner.

Step 3
Synthesize
Learn and acquire knowledge of the content by summarizing the concepts from your notes in your own words – in a conversational manner.

Step 4
Apply
Assess the competency of your knowledge by testing it and applying it to novel situations by working through the application questions.

Exam Information

Exams

What to Expect

In LB 145, we attempt to use various methods of assessment to evaluate your effort and learning during the course. These include homework assignments, quizzes in recitation, writing papers as a group and individually and three exams (2 midterms and a final). While the exams are not the sole source of how your learning is evaluated (and scored for grades) they are a significant part of the course and we would like you to do very well on them. As a result, we have provided some information in the next few pages to help you do well on exams.

The first midterm exam will likely be a multiple-choice exam designed to test basic knowledge and application of course content. The second midterm exam will likely be essay style. In the pages that follow we have provide you with information on the optional verbal final exam.

Your Responsibilities

If there is a take-home exam, you are expected to read the essay questions ahead of the exam and work individually and with your classmates to develop good answers for all the questions provided (you will be required to answer all of them for an exam). Make sure you **really** address the question and topic fully. Make an outline of your answer and make a first draft and second draft to be sure you have a 4.0 level response. Re-read the question frequently to be sure you are still really answering what it asks. Ask Dr. Luckie what the question means or if you got it all before assuming you did. If you don't read these essays and do this work prior to the exam, you shouldn't be surprised if you become overwhelmed and receive a poor grade.

Final Exam

The Verbal Final

What is it?

Throughout the course of LB145, we strive to assess your learning and understanding of the material. This is done using homework assignments, quizzes, PBAs, papers, and several exams. The Verbal Final is a very unique method that we employ which most students may not be familiar with. In this case, it is an optional way to take the final exam and earn a 100% if you pass the exam with some limit on the number of attempts allowed. There is no penalty for not taking it or not passing, you simply must then take the normal final exam.

A Verbal Final is a test of not only your knowledge on the subject matter but also how well you understand the concepts behind it using critical thinking. Material covered is the same as for the final exam but you will answer questions one-on-one verbally and with illustrations you draw. This year, to be able to schedule a verbal final with Dr. Luckie, you must first pass with an LA and have a Verbal Final Receipt filled out and signed. When the last few weeks of class arrives, anyone who has not previously done a verbal final will not be allowed to schedule one with an LA or Dr. Luckie. In the last weeks of class, if you have not previously tried with Dr. Luckie you cannot schedule a time with him. This is in place to provide time for students who have been working hard since VF time slots opened.

Everyone should take advantage of this opportunity. Even if you do not pass with Dr. Luckie, you will have already been studying the material for the final exam as a result of your preparations and will have gained a greater understanding of the material. It is beneficial to everyone who participates and you can read about the evidence that it will increase your learning in LB145 as well as upper-level science courses in the Luckie et al. 2013 research paper from the journal CBE-Life Science Education, proof of paper in the Appendix.

Verbal Final Outline (after passing the VF with a LA/TA you get an appt with Luckie):

Instructor says introductory blurb: "*This is the verbal final for LB-145, it is worth the same amount as the regular final exam. If you pass it you'll receive a 100% for your final exam. Also no matter how many times you take it you always can decide to take the regular written final exam... etc, etc.*"

1. **Draw and Explain Light Reactions**: *"Take your time and draw an illustration of the photosystems and carriers etc important for light reactions. This illustration is so you have something to point at when you explain light reactions to me."*

When you are done explaining light reactions I will ask you questions. First I'll ask about stuff you said that didn't quite make sense to me, then questions like these: What is an absorption spectrum vs action spectrum? Why does a pigment prefer certain colors of light? How are electrons and orbitals involved? Where are we in the cell/leaf? If we had 100 protons in the stroma and 200 in the lumen, how much ATP can we make?

2. **Draw and Explain Calvin Cycle**: "*Take your time and draw an illustration of the Calvin Cycle. This illustration is so you have something to point at when you explain light-independent reactions of photosynthesis to me.*"

When you are done explaining the reactions I will again ask questions. First I'll ask about stuff you said that didn't quite make sense to me or forgot to include [like enzyme names], then questions like these: What does PGA taste like? What are the names of the phases and why? What if we only fixed 1 CO2 molecule, how would that change things?

Transition: OK, let's pretend the glucose you just made in the Calvin Cycle turns into a donut. Preferably a warm Krispy Kreme original glazed donut. Let's eat it.

3. **Draw** {if you'd like} **and Explain Digestion & Absorption**: "*Eat the donut and explain how digestion works in one organ* (I'll pick the organ, you explain function, cells, enzymes, hormones etc) *and then explain absorption at the epithelial villus cell.*"

When you are done explaining digestion or absorption I will again ask questions. First I'll ask about stuff you said that didn't quite make sense or you forgot to include [like enzyme & hormone names] then questions like these: Draw a parietal cell and explain how it makes HCl and the same for the villus cell and how it absorbs glucose. What would happen if the glucose transporter didn't co-transport Na^{2+}, ie it was just a simple channel? How does the glucose molecule get out of the villus cell and into the capillary?

4. **Draw & Explain the Biosynthesis of a protein** (I'll choose which) **and your disease**. *Draw a pancreatic beta cell [or epithelial cell] and explain how it makes/secretes the protein (ie the path DNA -> RNA -> protein -> organelles what they each do and why).*

When you are done I'll ask questions, first about stuff you said that didn't quite make sense to me or forgot to include and then stuff like: What is splicing? How does an hnRNA differ from a mRNA? What is an intron? What is your disease and mutation? What do you predict would happen if we add/subtract some hydrophobic domains/parts to the gene you're studying?

Verbal Final with TA

Student ________________________ Date ___________________

(Start time ____:____ End time ____:____)

☐ The student made a good effort and just needs to study these areas:

- ◊ Light Reactions
- ◊ Calvin Cycle
- ◊ Digestion
- ◊ Absorption
- ◊ Circulation
- ◊ Central Dogma

As well as ____

☐ The student made a great effort and needs to practice in these areas:

- ◊ Light Reactions
- ◊ Calvin Cycle
- ◊ Digestion
- ◊ Absorption
- ◊ Circulation
- ◊ Central Dogma

As well as ____

☐ **The student made an outstanding effort and *passed*. There is no way they will fail when facing Dr. Luckie.**

Teaching Assistant/Learning Assistant NAME ______________________________

Verbal Final with TA

Student ________________________ Date ___________________

(Start time ____:____ End time ____:____)

☐ The student made a good effort and just needs to study these areas:

◊ Light Reactions
◊ Calvin Cycle
◊ Digestion
◊ Absorption
◊ Circulation
◊ Central Dogma

As well as ____

☐ The student made a great effort and needs to practice in these areas:

◊ Light Reactions
◊ Calvin Cycle
◊ Digestion
◊ Absorption
◊ Circulation
◊ Central Dogma

As well as ____

☐ **The student made an outstanding effort and *passed*. There is no way they will fail when facing Dr. Luckie.**

Teaching Assistant/Learning Assistant NAME ______________________________

Verbal Final with TA

Student _________________________ Date ____________________

(Start time ____:____ End time ____:____)

☐ The student made a good effort and just needs to study these areas:

- ◊ Light Reactions
- ◊ Calvin Cycle
- ◊ Digestion
- ◊ Absorption
- ◊ Circulation
- ◊ Central Dogma

As well as ____

☐ The student made a great effort and needs to practice in these areas:

- ◊ Light Reactions
- ◊ Calvin Cycle
- ◊ Digestion
- ◊ Absorption
- ◊ Circulation
- ◊ Central Dogma

As well as ____

☐ **The student made an outstanding effort and *passed*. There is no way they will fail when facing Dr. Luckie.**

Teaching Assistant/Learning Assistant NAME ______________________________

Lecture Handouts

Lecture

What to Expect

LB-145 is fundamentally the examination of what processes go on inside a cell and lead to life. In the first few meetings of the course, we study the gross anatomy of the cell and review the different structures inside eukaryotic and prokaryotic cells. For the remainder of the course, we focus on the eukaryotic cell and move from organelle to organelle deeply examining each and how it works (organelle anatomy & physiology). Specifically, in the first half of the course we'll focus on plant chloroplasts and how they can capture light energy and use it to MAKE carbohydrates (photosynthesis). In the second half of the course, we'll examine mitochondria and what happens inside them to BREAKDOWN carbohydrates and create energy to sustain life (metabolism-respiration), as well as examining the nucleus, ribosomes and endomembrane system where the function of DNA, RNA and proteins is defined (protein synthesis). We will also discuss Physiology to add a larger scale context to what cells are doing.

Class meetings will involve plenty of lectures, but also readings, story- telling, student presentations, discussions, problem-solving, 'biology theatre,' demonstrations and more. You will be a part of a 'study/research group' that should learn together in lecture, lab, and study together outside of class. We will cover an enormous amount of biology but we'll try to make class as active and cooperative as possible. The grading scale for the course will be a flat scale (ie "no curve" 80-84.9%= 3.0, 85-89.9%= 3.5, >90% = 4.0). Thus your grade will not be harmed by other students doing well. In fact, I will have no hesitation giving a 4.0 to every student in the class (this literally is our goal).

There will be in-class quizzes and writing assignments that are due at the end of each class. You will occasionally be given a handout (some which are included in this course packet) yet you will always be expected to take copious notes while reading the textbook and during lecture. There will be two midterm examinations and a comprehensive final examination during finals week.

Your Responsibilities

You are expected to read assignments *ahead* of the class meeting scheduled to address that topic, take detailed notes, and study them. In lecture we will often discuss only particular parts of a chapter or even topics that build upon the reading (not review it). If you don't read prior to the class meeting, you shouldn't be surprised if you become lost during the discussions. If on the other hand you actually read, take notes and study prior to each lecture you will become one of those really smart students, who just seems to know everything in class.

Chapter Checklists for each lecture

I strongly suggest attaching a sheet for the current week to your "work wall" where you can see it at a glance and literally check off items as you complete them.

Before first day of class:

Obtain supplies for course: online textbook, course pack, lecture notebook and TopHat online homework system. *See course website for more details (ctools.msu.edu/145).

1. _______ **Buy** the **LB145 Lecture & Lab Course Packet** (for students in Luckie's sections) at the Collegeville Textbook Store at 321 E. Grand River Ave in East Lansing (ctcmsu.com, 517-922-0013). This Course Pack contains the syllabus, lecture handouts, learning objectives, lab manual, lab notebook, scientific papers and more. A pdf of the Course Packet is also available on the course website for easy 24/7 access.

2. _______ **Buy** our online **Integrating Concepts in Biology (ICB) textbook**. Do not buy an expensive $259 Biology textbook! Please just buy this $39 online textbook. Note: this textbook is custom-assembled just for this class, so be sure to buy Luckie's version of the ICB textbook (http://trunity.org/).

3. _______ **Buy** access to **TopHat online homework** & **clicker system.** Do not spend $100+ for an online homework system like Mastering Chemistry/Biology and then also buy a $50 clicker you might break or lose. Please just buy the $20 TopHat online homework system for the semester, which also permits you to use your phone/tablet/laptop as a clicker. Click the TopHat.com link on course website.

4. _______ **Buy** a traditional paper **Lecture Notebook.** Can be spiral bound or 3-ring bound as long as there is real paper that you'll write on lots (mostly for taking notes when doing the readings prior to lecture). Be sure to write all notes by hand because it greatly increases your learning.[1]

---

[1] **Mueller PA, Oppenheimer DM. 2014. The Pen Is Mightier Than the Keyboard: Advantages of Longhand Over Laptop Note Taking. Psychol. Sci. 25:1159–1168.** (https://www.npr.org/2016/04/17/474525392/attention-students-put-your-laptops-away)

Week 1

(Preparing for the first day of class) **Monday's lecture:**

Budgeting homework time (45 min): Read the 1995 *Review paper* on Cystic Fibrosis by Welsh and Smith in your Course Pack. Take a few handwritten notes in your notebook that focus on defining the normal functions of the CFTR protein and what happens to it that leads to the disease.

(Preparing for the second day of class) **Wednesday's lecture:**

Budgeting homework time (70 min): Ch. 1, section 1.2 is approximately 2600 words in length. At what's considered slow reading speed, 200 words per minute, reading section 1.2 should take 13 minutes. But when done properly, when you pause to review figures, read and think about a few of the Integrating Questions, and take careful notes, if you focus (avoid distraction) it should take you approx. 70 minutes.

1. _______ For the first lecture, read the 1-page **Foreword** written by the very famous Dr. Bruce Alberts, review the Student Resources in **Chapter 0**, and then begin reading **Chapter 1: Heritable Material** of our textbook, Integrating Concepts in Biology (ICB). Read the single Introduction page, and the short section 1.1 of Chapter 1, but you do not need to take notes on any of those pages.

2. ________ Then slowly read the section we will discuss most during lecture, section 1.2 "What is the heritable material?" As you read section 1.2 on your computer or tablet be sure to **take handwritten notes in your lecture notebook** (handwritten notes lead to much greater learning[2]).

3. ________ **Try to answer at least one Integrating Question (IQ) in each set**. As you read the ICB textbook always attempt to answer <u>at least one</u> of the yellow **Integrating Questions** each time you get to a set of them. It will help you test yourself to determine if you got the meaning, or not, while reading the last few paragraphs. Just like taking handwritten notes, this too will greatly increase your learning. If you desire a high grade in the course, try to answer more IQs.
   NOTE: Assume you will be asked a question in lecture which is directly from one of the IQs.

4. ________ **Trifecta**: **Prepare to explain (aloud) <u>Figures 1.2, 1.3</u> and <u>Table 1.1</u> in class**. As you read a section from the ICB textbook always attempt to pause and study **each figure/drawing/table** that is discussed. Some of them are just pictures or drawings and may not require lots of thinking, but others are graphs or tables that contain actual data from research experiments. Spend more time looking at these. In class, during lecture, students will be randomly chosen to explain a Figure or Table aloud (LA will hand you a microphone) so prepare for when your name is called to be sure you are ready. *Some students avoid stress by just writing out in their notebook an explanation of the Purpose, Methods and Findings of each data figure* (we call these three things *the Trifecta*). If it's already written down then you can just read aloud what you wrote, like: "Purpose: Dr. Griffith wanted to determine...., Methods: his group worked with mice and pneumonia bacteria called...., Findings: in the end they found evidence that ...".

5. _________ **Advanced TIP reported from prior student**: "The way the textbook explained this figure did not make sense to me, so I scrolled down to the bottom of the page and clicked on link to the original paper and read about the same figure in that. The way the paper explained it made so much more sense and cleared up what I was confused about. Tell other students about this!"

---

[2] **Mueller PA, Oppenheimer DM. 2014. The Pen Is Mightier Than the Keyboard: Advantages of Longhand Over Laptop Note Taking. Psychol. Sci. 25:1159–1168.** (https://www.npr.org/2016/04/17/474525392/attention-students-put-your-laptops-away)

Week 2

(Preparing for) **Monday's lecture (special TopHat online lecture):**

Budgeting homework time (45 min): Ch. 4, section 4.1 is about 1250 words in length and ELSI 4.1 is 1100 words thus the total is 2350 words. At 200 words per minute, reading section 1.2 & ELSI should take 12 minutes, but when done properly, when you pause to review figures, read and think about a few of the Integrating & Review Questions, and take careful notes, this homework assignment should take you more like 45 minutes (and longer if you are distracted by texts, friends, email etc.).

1. _______ **For the second lecture, read** the introduction page of **Chapter 4: Evolution and Origin of Cells** in the ICB textbook, but you do not need to take notes on that page. Then slowly read section 4.1 "What is Evolution?" and as you read it on your computer be sure to take handwritten notes*. Last, read the section Ethical, Legal, Social Implications (ELSI) 4.1: "Are evolution and religion compatible?" You do not need to take notes on the ELSI reading, just think about it.
2. _______ **Try to answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. Also answer the green Review questions.
3. _______ (Trifecta): **Prepare to explain (aloud) Figures 4.1, 4.2 and ELSI Figure 4.1 in class**. As you read a section from the ICB textbook always attempt to pause and study **each figure/drawing/table** that is discussed. In class, during lecture, you may be randomly chosen to explain these aloud (the LA will hand you a microphone so everyone can hear you in lecture) so prepare well.
4. _______ **Advanced TIP**: scroll down to the bottom of the page and click on the link to an original version of Darwin's Origin of Species, peek at it, and look at some of the other research papers in the Bibliography to get used to, and in a habit of, doing this.

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (70 min): In Ch. 4, the first 2/3's of section 4.2 is 3000 words in length which should take 15 minutes if you just read it. But when done properly, when you pause to review figures, read and think about a few of the Integrating Questions, and take careful notes, this homework assignment should take you more like 70 minutes (and that's if you are not distracted).

1. _______ **For the third lecture of the semester, read Chapter 4's** section 4.2 "Could abiotic molecules form biologically important molecules before life evolved?" and as you read it on your computer be sure to take handwritten notes*. You should focus mostly, and only take detailed notes for, the first 2/3s of the section. You can stop taking notes once you complete the yellow Integrating Questions 5 & 6. Read the remaining section regarding RNA and directed evolution, but no notes needed on this, just be amazed at what is said.
2. _______ **Try to answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. Also try to answer the green Review questions.
3. _______ (Trifecta): **Prepare to explain (aloud) Figures 4.5, 4.6, and 4.8 in class** (Purpose, Methods, Findings)
4. _______ **Advanced**: Click on "Explore More on Abiotic Production of Organic Molecules" to learn a little about research published in 2016. Just take a peek, read the abstract.

Week 3

(Preparing for) **Monday's lecture:**

Budgeting homework time (70 min): In Ch. 4, the first half of section 4.3 is 2000 words in length and section 4.4 is 1500 words, totaling 3500. This should take 17 minutes if you just read it. But when done properly, when you pause to review quite a few figures, read and think about a few of the Integrating Questions, and take careful notes, this homework assignment should take you more like 70 minutes (if you are focused). **Special Allowance**: Your group can divide up the Trifectas for this lecture.

1. _______ **For Monday's lecture, read Chapter 4's** section 4.3(1st half) in the ICB textbook, and then section 4.4. For section 4.3 "Can non-living objects compete and grow?" you only need to carefully read and take notes on items up to and including information related to Figure 4.13. Then stop taking notes and just read the rest to learn about research on vesicles competing with each other. Explore Bio-Math Exploration 4.2 if you find it interesting. Then read and take notes on all of the short reading in section 4.4 "Can non-living objects harvest and store energy?". Be sure to take handwritten notes.

2. _______ **Try to answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _______ (Trifecta): **Prepare to explain (aloud) Figures 4.11, 4.12, 4.13 and 4.17 in class**. *Special Allowance today*: If you wish your group can designate who will be responsible for each figure and thus split up the responsibility and reduce the load (Purpose, Methods, Findings).

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (60 min): In the Chapter **Photosynthesis (OSB)** section 8.1 is 1624 words but has quite a few figures. This should take 8 minutes if you just read it. But when done properly, when you pause to review figures and take good notes, this assignment should take you more like 45 minutes. Give yourself at least 10 more minutes to google and read about Lynn Margulis and take a few notes.

1. _______ **For Wednesday's lecture, first google** "Lynn Margulis" to find, read about, and take notes on, the *endosymbiotic hypothesis*. Then in the chapter **Photosynthesis (OSB),** read section 8.1 "Overview of Photosynthesis" and take handwritten notes.

2. _______ (Tip): **Prepare to explain (aloud) the anatomy of a chloroplast, and Figure 6, in class**.

3. _______ **Advanced**: Take a peek at section 8.2, in particular study Figure 7. Be ready to act out this process in class.

Week 4

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): Photosynthesis (OSB) section 8.2 is 2603 words in length, but has quite a few figures. This should take 13 minutes if you just read it. But when done properly, when you pause to review figures and take careful notes, this assignment should take you more like 60 minutes.

1. _______ **For Monday's lecture,** in the chapter Photosynthesis (OSB) read section 8.2 "The Light-Dependent Reactions of Photosynthesis" and as you read it on your computer be sure to take handwritten notes in your lecture notebook.

2. _______ (Tip): While you are reading focus mostly and take notes regarding **Figures 5, 7, and 8.** We will discuss these in class.

3. _______ **Advanced**: Take a peek at section 8.3, in particular study Figure 1. Take a sneak peek at "Chapter 11: Photosynthesis", section 11.1, study Figures 11.2 and 11.3.

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (60 min): Photosynthesis (OSB) section 8.3 is 1573 words in length, and does not have many figures. This should take 13 minutes if you just read it. But the video is 12 minutes and when done properly, when you pause to review figures and take careful notes, this assignment should take you more like 60 minutes.

1. _______ **For Wednesday's lecture,** read section 8.3 "Using Light Energy to Make Organic Molecules" (1573 words) in the chapter Photosynthesis (OSB) and take handwritten notes.

2. _______ (flipped classroom) Watch the **12min lecture by Mr. Andersen** provided where he gives quickly reviews Photosynthesis. Add to your notes any interesting points he makes that helped you better understand the parts of the cell and what they do.

3. _______ (Tip): While reading, focus mostly and take notes regarding **Figures 1, and 2.** We will discuss these in class.

4. _______ **Advanced**: Take a sneak peek at "Chapter 11: Photosynthesis", section 11.1, in particular study Figures 11.4.

Week 5

(Preparing for) **Monday's lecture:**

Budgeting homework time (70 min): Chapter 11 section 11.1 is long with many figures that require thinking and notetaking. The first 2/3's of it is the most important part and just reading that part, which is 3800 words, should take 21 minutes or longer. The data figures are important while those that are just drawings, like 11.3, 11.6, 11.7, 11.9, are not. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 70 minutes. **Special Allowance**: Your group can divide up the Trifectas for this lecture.

1. _______ **For Monday's lecture,** read **Chapter 11: Photosynthesis**, section 11.1 "Why is paraquat used in America but illegal in Europe?" and as you read it on your computer be sure to take handwritten notes in your lecture notebook. Please read carefully and take good notes for the first 2/3s of the section (which is about 3800 words). You can stop taking notes when you come to the yellow box of Integrating Questions #11-14. Then just read the last 1/3 of the section for deeper thought and better understanding.

2. _______ **Try to answer some Integrating Questions and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _______ (Trifecta): **Prepare to explain (aloud) Figures 11.1B, 11.2A, B, C, 11.4, 11.5, and 11.8A in class**. *Special Allowance today*: If you wish your group can designate who will be responsible for each figure and thus split up the responsibility and reduce the load (Purpose, Methods, Findings).

4. _______ **Advanced**: Take a peek at section 8.3, in particular study Figure 1. Take a sneak peek at "Chapter 11: Photosynthesis", section 11.2.

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (70 min): Chapter 11 section 11.2 is 3593 words in length with several data figures that require thinking and notetaking for the Trifecta. Reading at 200 words per minute would mean the section might take 18 minutes to read. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 70 minutes. It could be shorter if you have been doing homework regularly, ie. training like an athlete, and getting much better at this now that it is week 5.

1. _______ **For Wednesday's lecture,** read **Chapter 11: Photosynthesis**, section 11.2 "How does Brazil's rainforest affect Greenland's glaciers?" (3593 words), and take handwritten notes in your lecture notebook.

2. _______ **Try to answer some Integrating Questions and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _______ (Trifecta): **Prepare to explain (aloud) Figures 11.11** (just pick one graph), **11.12, 11.13, and 11.15** (Purpose, Methods, Findings).

Week 6

Monday lecture:

EXAM I

(Preparing for) Wednesday's lecture:

Budgeting homework time (75 min): This is a professional *Review Paper* that is 4500 words in length from an important medical journal so it will take some time to read, but if you have gained some understanding about CF while working in the lab this semester you should be able to more quickly grasp most of what is discussed. If you struggle with the text, focus on each figure and determine what it is trying to explain. Scientists almost always first focus on the figures.

1. _______ **For Wednesday's lecture,** read Steven Rowe *et al's* review paper "Mechanisms of Disease" on Cystic Fibrosis published in *New England Journal of Medicine* back in 2005. This paper is provided at the back (around page 275) of your Course Pack. You do not need to take notes from all, simply read for your own learning and to help you with your research.

2. _______ Write out in your lecture notebook your explanation for Figures 1, 3, and 5.

3. _______ (Tip): **Prepare to explain (aloud) <u>Figures 1, 3, and 5</u> in class**. While reading, focus mostly on the figures. We will discuss these in class. Be prepared to state aloud what each figure is trying to explain or communicate. Prioritize Figures 1, 3, and 5 as most important to understand and be able to explain in class.

Week 7

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): Chapter 5 section 5.1 is 2090 words in length with several data tables and figures that require thinking and notetaking for the Trifecta. Reading at 200 words per minute would mean the section might take 10 minutes to read. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 60 minutes. It could be shorter if you have been doing homework regularly, ie. training like an athlete, and getting stronger, better, faster at this now that it is week 7.

1. _______ **For Monday's lectu**re, read **Chapter 5: Evolution Applied**, section 5.1: "How do genetic diseases arise?" and as you read it be sure to take handwritten notes.
2. _______ **Try to answer some Integrating Questions and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.
3. _______ (Trifecta): **Prepare to explain (aloud) Figure 5.1, and Tables 5.1, 5.2 and 5.3 in class** (Purpose, Methods, Findings).
4. _______ **Advanced**: Take a peek at several of the published research papers in the Bibliography at the bottom of the page. This may help you understand how to make taq polymerase function more efficiently in your own PCR research project.

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (60 min): The Chapter **Cell Structure (OSB)** section 4.3 is 3060 words in length with a number of art figures (no data figures for trifectas). Reading at 200 words per minute would mean the section might take 15 minutes to read. But the video is 14 minutes and when done properly, when you pause to review figures and take careful notes, this assignment should take you more like 60 minutes.

1. _______ **For Wednesday's lecture,** read section 4.2 "Prokaryotic Cells" and 4.3 "Eukaryotic Cells" in chapter **Cell Structure (OSB)**. For section 4.3 (3060 words) take handwritten notes in your notebook.
2. _______ Compare and contrast the anatomy of a Prokaryote versus Eukaryote. Then also a plant cells versus an animal cell. Which seems most advanced, why?
3. _______ (flipped classroom) Watch the **14min lecture by Mr. Andersen** provided where he gives you a tour of the cell. Add to your notes any interesting points he makes that helped you better understand the parts of the cell and what they do.
4. _______ While reading, focus mostly and take notes regarding **Figures 2 & 3, and 4 & 5,** and note the building block of cell walls/wood in **Figure 9.** We will discuss these in class. Generally, you need to learn the names and functions of each organelle. It's best to create hand-made flash cards with the name on one side and the function on the other. Also add interesting facts, like a drug user would be expected to have more of which organelle? Use these to study prior to class and then prior to the exam too.
5. _______ **Advanced**: Take a sneak peek at section 4.4, in particular study Figure 1.

Week 8

(Preparing for) **Monday's lecture:**

Budgeting homework time (30 min): The Chapter **Cell Structure (OSB)** section 4.4 is 1430 words in length with a number of art figures (no data figures for trifectas). Reading at 200 words per minute would mean the section might take 7 minutes to read. When done properly, when you pause to review figures and take careful notes, this assignment should take you more like 30 minutes.

1. _______ **For Monday's lecture,** read section 4.4 "The Endomembrane System and Proteins" in chapter **Cell Structure (OSB)** (1430 words). Take handwritten notes in your lecture notebook.

2. _______ Compare and contrast the anatomy of a Prokaryote versus Eukaryote. What's similar, what's different. Then also compare a plant cells versus an animal cell. Which one is a eukaryote, why, which cell type do you think seems the most advanced, evolutionarily, why?

3. _______ While reading, focus most on **Figure 1.** We will discuss this process of biosynthesis in class. Generally, you need to learn the names and functions of each organelle in the endomembrane system. In particular what are the functions of that organelle in general as well as in particular during the ***biosynthesis*** of a protein. It's best to create hand-made flash cards with the name on one side and the function on the other. Also add interesting facts, like where does CFTR go and what happens to it during its expression from a gene and conversion into a functioning protein, where does it end up? What about insulin, what happens when it is made, does it do the exact same thing? Use these to study prior to class and then prior to exam too.

4. _______ **Advanced**: Take a sneak peek at section 8.2 for Wednesday.

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (60 min): Chapter 8, section 8.2 (first half) is 2498 words in length with four data tables and figures that require thinking and notetaking for the Trifecta. Reading at 200 words per minute would mean the section might take 13 minutes to read. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 60 minutes.

1. _______ **For Wednesday's lecture**, read **Chapter 8: Cell Structure and Function**, section 8.2: "Why aren't there giant cells?" and as you read it be sure to take handwritten notes on the first half (2498 words). You can stop taking detailed notes when you begin reading the light blue box denoting the second section on "Calculated cell limitations". Just read that short section for deeper understanding.

2. _______ **Try to answer some Integrating Questions and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _______ (Trifecta): **Prepare to explain (aloud) Figures 8.14, 8.15 and 8.16 in class** (Purpose, Methods, Findings)

4. _______ **Advanced**: Take a peek at some of the published research papers in the Bibliography at the bottom of the page with the goal to find the original figure you studied here in the reading and where it is in the paper?

Week 9

(Preparing for) **Monday's lecture:**

Budgeting homework time (70 min): Section 5.2 of **Structure and Function of Plasma Membranes (OSB)** is 3856 words in length with a number of art figures (yet no data figures for trifectas). Reading at 200 words per minute would mean the section might take 20 minutes to read. But the two videos are 6 & 8 minutes each, and when done properly, when you pause to review figures and take careful notes, this assignment should take you more like 70 minutes.

1. _______ **For Monday's lecture,** skim section 5.1 "Components and Structure" in the chapter **Structure and Function of Plasma Membranes (OSB)** then carefully and slowly read section 5.2 "Passive Transport" (3856 words) and for that one please take handwritten notes in your lecture notebook.
2. _______ (flipped classroom) Watch the **6-min lecture by Mr. Andersen** on membrane structure. You do not need to take notes on this.
3. _______ Then review section 5.2 again, now focus and take notes regarding **Figures 1, 2, 3 and 5.** We will discuss these in class.
4. _______ (flipped classroom) Watch the **8-min lecture by Mr. Andersen** provided where he gives you an explanation of the topics: diffusion and osmosis. Add to your notes any interesting points he makes that helped you better understand what they are all about.
5. _______ **Advanced**: Take a sneak peek at the next section 5.3, in particular Figure 1.

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (30 min): Section 5.3 of **Structure and Function of Plasma Membranes (OSB)** is 1532 words in length with four art figures (no data figures for trifectas). Reading at 200 words per minute would mean the section might take 8 minutes to read. If done properly, when you pause to review figures and take careful notes, this assignment should take you more like 30 minutes.

1. _______ **For Wednesday's lecture,** read section 5.3 "Active Transport" in the chapter **Structure and Function of Plasma Membranes (OSB)** (1532 words) and take handwritten notes in your notebook.
2. _______ While reading, be sure you can explain **Figures 1 and 3.** We will discuss these in class.
3. _______ **Advanced**: What is one way to determine whether ion movement is due to passive transport or active transport? Does this hold true for CFTR?

Week 10

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): Read "Systems of Gas Exchange" in the chapter **The Respiratory System (OSB)** with several art figures (no data figures for trifectas). Yet you can focus on the important part, the second half, on "Mammalian Systems" which is only 1400 words. Reading at 200 words per minute would mean the whole section might take 13 minutes to read. If done properly, when you pause to review figures and take careful notes just in the "Mammalian Systems" section, this assignment should take you no more than 30 minutes of reading time. Then watch the 20-minute Khan Academy video to really get a good understanding of pulmonary anatomy & function, and take a few notes. Total estimated time is for homework is 60 minutes.

1. _______ **For Monday's lecture,** read section 39.1: Systems of Gas Exchange, in the chapter **The Respiratory System (OSB)** (2350 words). Take handwritten notes in your lecture notebook on the second half of the reading, starting at the section "Mammalian Systems".

2. _______ (flipped classroom) Watch the really helpful **20-min lecture from the Khan Academy** provided where he gives you an explanation of the topics of ventilation and respiration. Add to your notes any interesting points he makes that helped you better understand what they are all about.

3. _______ **Advanced**: Take a sneak peek at the images and movies in this chapter's section on "Breathing" on the mechanics of breathing particularly related to humans.

Wednesday lecture:

EXAM II

Week 11

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): This is a professional Review paper that is 3700 words in length from an important medical journal so it will take some time to read, but if you have gained some understanding about genetics and diseases while working in the lab this semester you should be able to more quickly grasp most of what is discussed. If you struggle with the text, focus on each figure and determine what it is trying to explain. Scientists almost always first focus on the figures.

1. _______ **For Monday's lecture,** read Kenneth Polonsky's review paper on "The Past 200 Years in Diabetes" published in *New England Journal of Medicine* in 2012. This paper is provided at the back of your Course Pack. You do not need to take notes from all the written text, simply read for your own learning and to help you with your research.

2. _______ While reading, **focus mostly on the figures.** We will discuss these in class. Be prepared to state aloud what each figure is trying to explain or communicate.

3. _______ **Write out in your notebook** your explanation for Figure 2 and how that structure relates to what you learned about the biosynthesis of a protein back in the reading and lecture about *Endomembranes*. Describe how you predict the insulin protein is made DNA -> RNA -> protein and the path it takes via which organelles and what happens to it at each?

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (70 min): Chapter 23, section 23.1 is 3891 words in length with 10 figures and three of which are data tables/figures that will require thinking and notetaking for the Trifecta. On the other hand, these Figures and Tables are not important, Figure 23.4, the second half of Table 23.2, the parietal cell video, and Figure 23.5. When done properly, when you pause to review important figures/tables, try Questions, and take notes, this assignment should take you more like 70 minutes.

1. _______ **For Wednesday's lecture**, read **Chapter 23: Cells in Tissues**, first review the introductory page, and then carefully read section 23.1: "How do you break down and absorb nutrients from the food you eat?" (3891 words). As you read it be sure to take handwritten notes.

2. _______ **Try to answer these Integrating Questions :** #1 about da Vinci, #3 about Prout's research and #7 on Muallem's work and be prepared to share your answers in class.

3. _______ (Trifecta): **Prepare to explain (aloud) Tables 23.1, 23.2(first half) and Figure 23.7 in class** (Purpose, Methods, Findings).

Week 12

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): Read ELSI 1.1 and the first 2/3 of section 1.4 in "Chapter 1: Heritable Material." While this is about 3700 words in both the readings combined, only the 2700 words in section 1.4 need careful reading and notetaking. Also, there are no traditional Trifectas to prepare for, just three simple questions posed below to be ready to answer aloud in class.

1. _______ **For Monday's lecture,** revisit **Chapter 1: Heritable Material** and read section ELSI 1.1: "Who Owns Your DNA?" (975 words), and then read the first 2/3's of section 1.4 "How does DNA's shape affect its function?" Take notes on section 1.4 all the way up until it switches to the new light blue box topic of "DNA Replication" (2700 words). Read on, don't need to take notes.

2. _______ **Try to answer some Integrating Questions** and **Review Questions**.

3. _______ (Tip): **Prepare to explain (aloud) in class:** 1. What's difference between the chemical structure of DNA vs RNA, 2. What's incorrect in Figure 1.10?, 3. What's incorrect in Figure 1.13?

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (50 min): Read two sections in the chapter **Genes and Protein (OSB)**. Section 15.5 is 2500 words in length and requires careful reading and notetaking. There are no traditional Trifectas, just three simple questions and a task posed below to be ready to answer in class.

1. _______ **For Wednesday's lecture**, read chapter **Genes and Proteins (OSB),** first review 15.3: "Eukaryotic Transcription" but don't take notes. Then carefully read section 15.5 "Ribosomes and Protein Synthesis" (2501 words) and as you read it take handwritten notes.

2. _______ (Tip): **Prepare to explain (aloud) in class:** 1. What happens when a gene is going to be expressed?, 2. What happens when a gene is transcribed? 3. Where does folding occur, what if it goes wrong? Also, prepare for this: If **Figure 2** is projected on the screen in class be able to go to the board and explain how translation works, using it.

Week 13

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): Chapter 2, section 2.1 (first half) is 2257 words in length with three data tables and figures that require thinking and notetaking for the Trifecta. Reading at 200 words per minute would mean the section might take 12 minutes to read. Yet figures 2.5 and 2.6 are challenging and require time to think and read about them for the Trifecta. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 60 minutes.

1. _______ **For Monday's lecture,** review the introductory page of **Chapter 2: Central Dogma.** Then carefully read section 2.1: "How does DNA communicate information to the cell?" (2257 words) and take handwritten notes in your lecture notebook.

2. _______ **Try to answer some Integrating Questions** and **Review Questions**.

3. _______ (Trifecta): **Prepare to explain (aloud) Figures 2.3, 2.5 and 2.6 in class** (Purpose, Methods, Findings).

(Preparing for) **Wednesday lecture:**

Budgeting homework time (60 min): Chapter 2, section 2.4 is 2673 words in length with several exercises that require you to use NCBI to look for the insulin gene, find introns, and then the ORF finder to understand the gene further. Reading at 200 words per minute would mean the section might take 12 minutes to read. Yet integrating questions 35-38 are challenging and require time to think and perform what they request of you. Of course, when done properly, when you really try to do the Integrating Questions, and take notes, this assignment will take you more like 60 minutes.

1. _______ **For Wednesday's lecture, read another section of Chapter 2: Central Dogma.** Carefully read section 2.4: "Can cells pick and choose information? (2257 words) and take handwritten notes in your lecture notebook.

2. _______ **Perform the exercises** outlined in **Integrating Questions 35-38** and then answer the remaining IQs and **Review Questions.**

3. _______ **(Tip): Prepare to explain (aloud) in class:** how to use online tools at NCBI and OMIM to find the DNA, RNA and amino acid sequence of any gene, with insulin as an example.

(optional leisure reading for those who are interested and haven't noticed this yet): **Ch.22-ICB: section 22.1,** "How do genetic diseases affect cells and organisms" which has a great section on the genetic disease Sickle Cell Anemia.

Week 14

(Preparing for) **Monday's lecture:**

Budgeting homework time (60 min): Chapter 10, section 10.4 (first half) is 3000 words in length with three data figures that require thinking and notetaking for the Trifecta. Reading at 200 words per minute would mean the section might take 15 minutes to read. Yet all three trifecta figures will require time to think and read about them for class. Thus, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 60 minutes.

1. _______ **For Monday's lecture,** read the introductory page of Chapter 10: Cellular Respiration and read section "10.4: How is ATP produced?" Take notes on section 10.4 all the way up until Figure 10.22 (2900 words). After that please read but do not take notes.

2. _______ **Try to answer some Integrating Questions** and **Review Questions**.

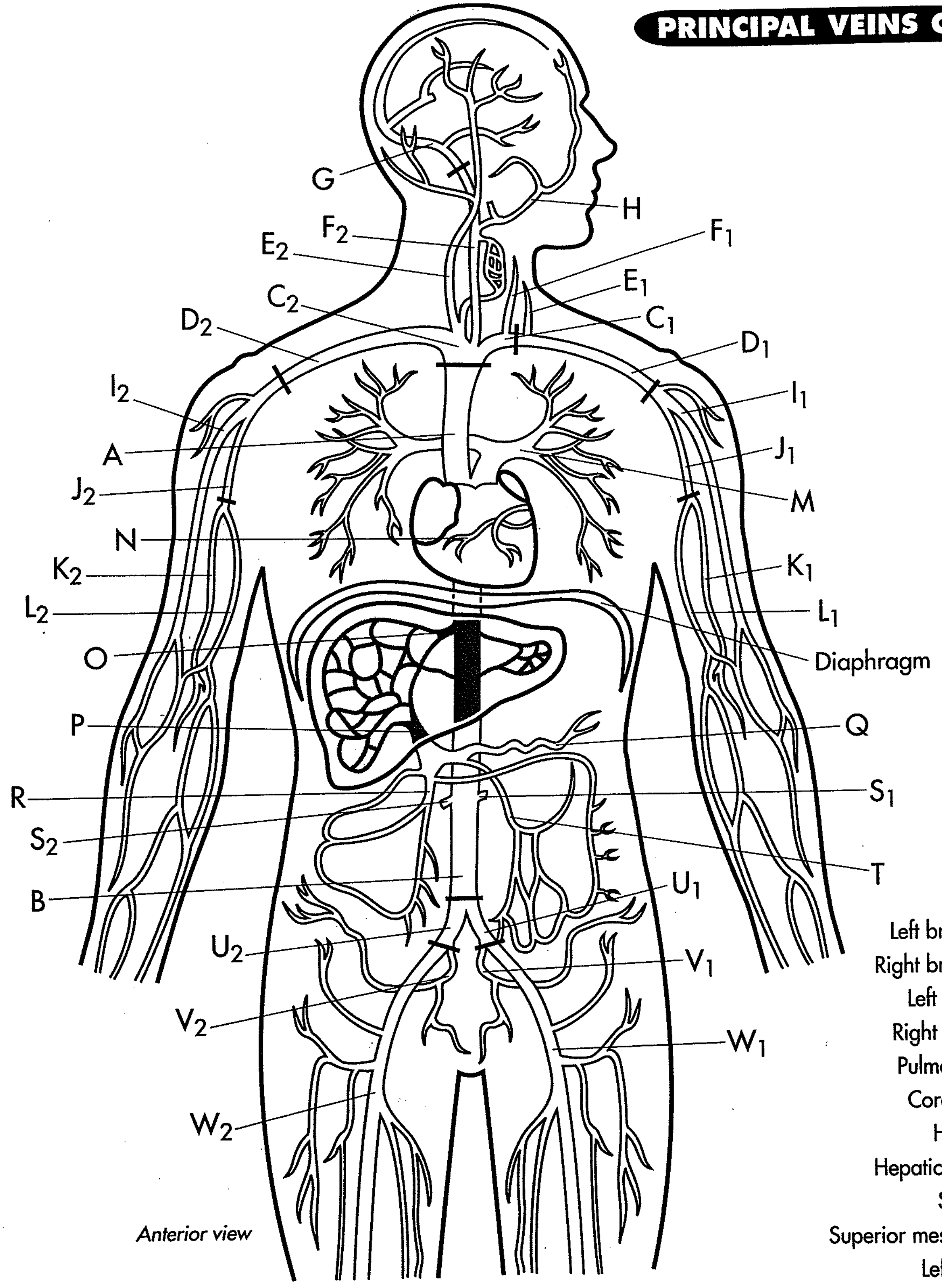
3. _______ (Trifecta): **Prepare to explain (aloud) Figures 10.20, 10.21 and 10.22 in class** (Purpose, Methods, Findings)

(Preparing for) **Wednesday's lecture:**

Budgeting homework time (20 min):

1. _______ **For Wednesday's lecture**, read ELSI 10.1 (1000 words) and BME-10.1 (385 words). No notes necessary.

PRINCIPAL VEINS OF THE BODY



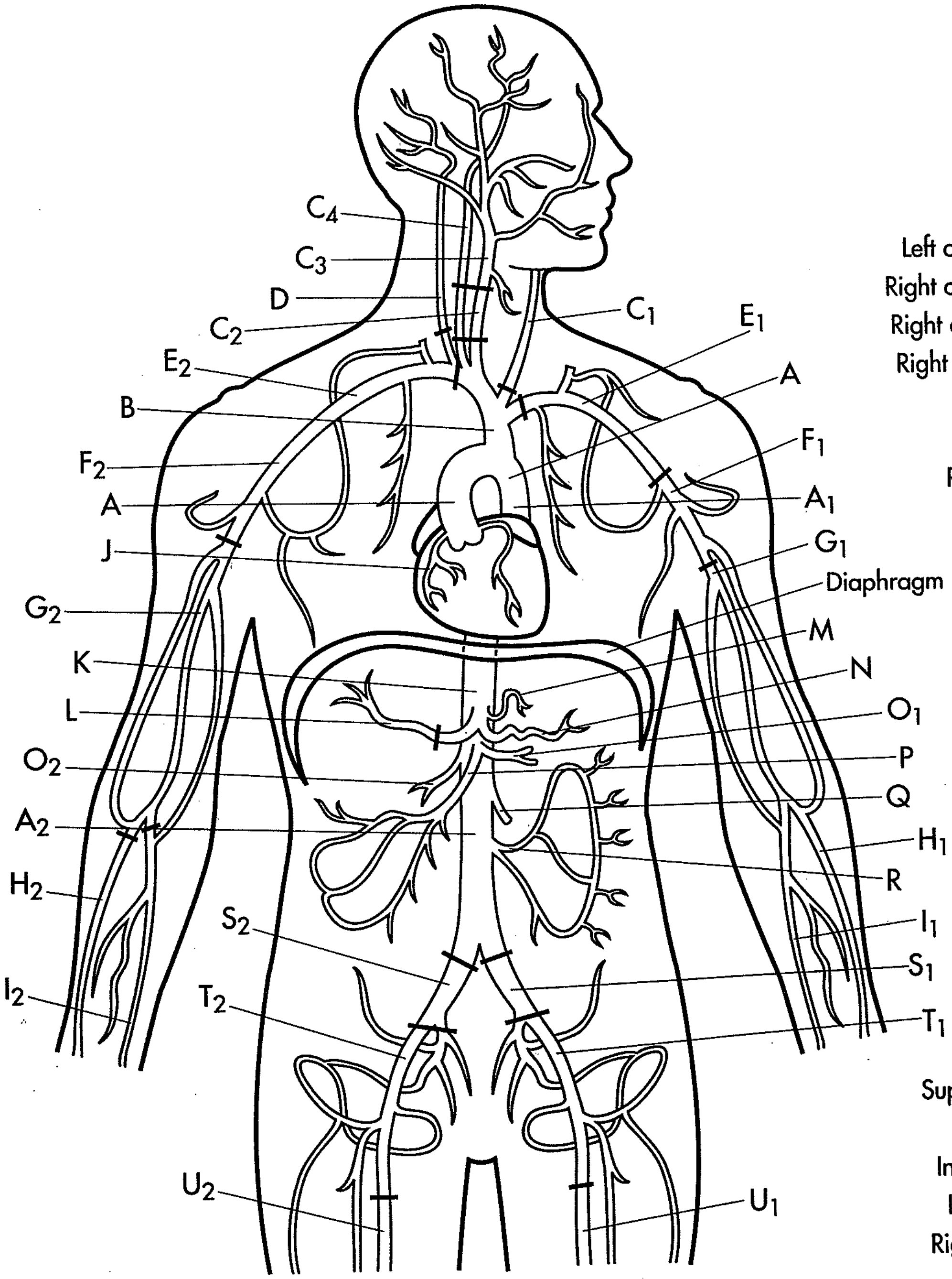
Anterior view

Superior vena cava	A	○
Inferior vena cava	B	○
Left brachiocephalic vein	C_1	○
Right brachiocephalic vein	C_2	○
Left subclavian vein	D_1	○
Right subclavian vein	D_2	○
Left external jugular vein	E_1	○
Right external jugular vein	E_2	○

Left internal jugular vein	F_1	○
Right internal jugular vein	F_2	○
Sigmoid sinus	G	○
Facial vein	H	○
Left cephalic vein	I_1	○
Right cephalic vein	I_2	○
Left axillary vein	J_1	○
Right axillary vein	J_2	○

Left brachial vein	K_1	○
Right brachial vein	K_2	○
Left basilic vein	L_1	○
Right basilic vein	L_2	○
Pulmonary veins	M	○
Coronary sinus	N	○
Hepatic vein	O	○
Hepatic portal vein	P	○
Splenic vein	Q	○
Superior mesentery vein	R	○
Left renal vein	S_1	○
Right renal vein	S_2	○
Left inferior mesenteric vein	T	○
Left common iliac vein	U_1	○
Right common iliac vein	U_2	○
Left internal iliac vein	V_1	○
Right internal iliac vein	V_2	○
Left external iliac vein	W_1	○
Right external iliac vein	W_2	○

PRINCIPAL ARTERIES OF THE BODY



Anterior view

Aorta	A	○
Thoracic aorta	A_1	○
Abdominal aorta	A_2	○
Brachiocephalic trunk	B	○
Left common carotid artery	C_1	○
Right common carotid artery	C_2	○
Right external carotid artery	C_3	○
Right internal carotid artery	C_4	○
Vertebral artery	D	○
Left subclavian artery	E_1	○
Right subclavian artery	E_2	○
Left axillary artery	F_1	○
Right axillary artery	F_2	○
Left brachial artery	G_1	○
Right brachial artery	G_2	○
Left radial artery	H_1	○
Right radial artery	H_2	○
Left ulnar artery	I_1	○
Right ulnar artery	I_2	○
Coronary arteries	J	○
Celiac trunk	K	○
Hepatic artery	L	○
Gastric artery	M	○
Splenic artery	N	○
Left renal artery	O_1	○
Right renal artery	O_2	○
Superior mesenteric artery	P	○
Gonadal artery	Q	○
Inferior mesenteric artery	R	○
Left common iliac artery	S_1	○
Right common iliac artery	S_2	○
Left external iliac artery	T_1	○
Right external iliac artery	T_2	○
Left femoral artery	U_1	○
Right femoral artery	U_2	○

LB-145

Laboratory Guide

Phase I: Designing a PCR Assay
&
Phase 2: Using PCR to detect CF

**Igert, Wright, Fata-Hartley, Haenisch, Cooper,
Krha, Maleszewski, Wilterding, Sayed, Luckie**

Laboratory : Expectations & Effort

Time Commitment- “Don’t be a tourist”

- Remember that the laboratory is an entire class all by itself, and is worth 2 of the 5 credits in LB-145. This means that you should be prepared to spend 6 hours per week in the LB-145 laboratory as well as time outside of the lab with your group each week – so plan ahead.
- The above also means that if you explain to your research team that you are “busy” with social events every evening and intend to go home each weekend, realize you won’t succeed in this course. Do NOT be a TOURIST - make college a priority in your life.

The LB-145 Laboratory is a Research Team Lab

- Your research group will need to meet outside of class at least twice a week to coordinate the projects and the papers you write as a group. It is essential that you read and prepare prior to coming to lab each week, because the experiments require a good amount of organization to complete in a timely manner.
- Please familiarize yourself with the concept behind this lab – it will help you to keep your “eyes on the prize” as the semester progresses.
- Attendance will be taken at the beginning of each lab, so it is important that you arrive ON TIME.
- The key to sucess in the laboratory class is to think and work like a scientist (in fact not like a student) that means do not behave like this is just a class but strive to work as though you are a professional scientist and this is your own research lab and project (which it is).

Research Group Roles in the Laboratory

The Research Teams in LB-145 will usually consist of 4 individuals. You are expected to help out in all tasks but you will have one specialty (your primary job or role in the team). If your team consists of 3 individuals eliminate the "Primary Investigator" and divide those responsibilities among the team members.

Primary Investigator (PI) - *Plan!*

The primary investigator will be responsible for organizing meeting times, overall project planning, as well as implementing troubleshooting techniques throughout the investigations. It is your job to also be sure all members participate in discussions and you record notes. Ask questions when a member hasn't spoken, "What do you think, Jen?". In addition to sharing the final grade for each group paper, the PI is assigned and graded for writing specific sections of the paper as well as editorial duties on all sections in creating all complete papers.

Protocol Expert (PE) - *Protocols!*

This individual is responsible for overseeing the creation of scientific protocols for each week's independent investigation (written experiments and steps you plan to do). It is your job to worry about whether the protocol is appropriate and being followed exactly during the experiment. In addition to sharing the grade for each full draft paper, the PE is assigned and graded for writing sections of the paper as well as editorial duties on all sections in creating all complete papers.

Data Recorder/Documentarian (DRD) - *Notebooks!*

The data recorded is responsible for recording and organizing the results and taking many pictures to document the team's efforts. It is your job to be sure everyone is keeping their notebook up to date and data is being recorded properly. In addition to sharing the grade for each full draft paper, the DRD is assigned and graded for writing sections of the paper as well as editorial duties on all sections in creating all complete papers.

Laboratory Technician (LT) - *Hardware!*

This individual is responsible for learning the many experimental procedures and becoming an expert on how to use the various pieces of equipment. It is your job to read manuals and get the right equipment to work properly. In addition to sharing the grade for each full draft paper, the LT is assigned and graded for writing certain sections of the paper as well as editorial duties on all sections in creating all complete papers.

Working Effectively in Small Groups

As you know, a great deal of the learning that occurs in our class is based upon your interactions with your peers in small groups. But working well in small groups does not happen by magic. There are things that I have to do as an instructor to make sure that you are getting the most out of your experience, such as identifying conflicts between group members that might hinder your work and having TAs listening closely to what you are saying as they stroll around the lab room so that we can help you get the most from your discussions or keep you on track.

You, too, have to work to make your groups all that they can be. Below, are some suggestions of effective small group work. Some of these will also clarify my expectations of you in your groups. Look this over carefully and raise questions in class or to me privately.

Individuals in Groups

There are two things that happen in small groups. Individual students share their thoughts, but those thoughts are also reacted to by one or more students. The group succeeds only insofar as each individual does actually share their thoughts; an individual who is not trying their best will diminish the group's experience. When I evaluate your group participation via GEA forms and TA feedback, I will take into account the quality of your group interactions- how well you did on your own in your group and how well the group did overall. It is up to each individual, and in particular the PI, to encourage teammates to work together to maximize your learning. If someone is not doing the reading or is not participating in lab enough, discuss the problem and see what you can do to help that person. You are all in your groups together!

Group Process

Groups are really processes, steps toward a goal. There are two types of goals toward which you are striving in our class. The first is growth as an individual. Group work should leave you feeling that you understand the material better than you could have had you worked only on your own. The second goal has to do with teamwork. Have you, and the group, learned something about group problem solving through your work together? Do you know how to better communicate with others from different backgrounds and who hold different perspectives? If so, you have achieved the second goal.

In order to make the group process work effectively, please follow these guidelines:

- Everyone should participate in your group.
- Everyone's contributions should be welcomed.
- Everyone should be heard. Listen to and hear one another; ask questions if you do not understand what a group member has said.
- No one should dominate group processes.
- A recorder should take notes for the group during a meeting. If your group meets more than once, the recorder role should be shared equally by all members through time.

Names: ____________________
____
____
____
Section #: ____________________

Team Ground Rules Contract Form

Adapted from Dr. Deborah Allen, Univ. of Delaware and Dr. Rique Campa, MSU

Team Assignment

Purpose: To establish team *norms* in order to promote constructive & productive teamwork.

Directions: To work best groups require that all team members clearly understand their responsibilities to one another. These team ground rules describe the general responsibilities of every member to the team. You can adopt additional ground rules if your group believes they are needed. Your signature on this contract form signifies your commitment to adhere to these rules and expectations.

Some questions to discuss when thinking about these rules:

1) What are your professional goals (i.e., what would you like to do following graduation)? How will working in a team help you achieve some of your professional goals?
2) Besides class time, when are you available to work with your team members (exchange your class and work schedules)?
3) What is the best method(s) and time for your team members to contact you? Share the necessary phone number(s), e-mail addresses, etc... *NOTE:* This contact information is private, so should not be shared with others outside of your team, and should only be used for class-related communication.

All group members agree to:

1. Come to class and team meetings on time.
2. Come to class and team meetings with assignments and other necessary preparations correctly and thoughtfully completed.

Additional ground rules (add as many as you like; see examples on appendix of Smith (2007)):

Example: We will always meet on these two days each week at these times ___________

If a member of the team repeatedly fails to meet these ground rules, other members of the group are expected to take the actions below. When filling in the *"If not resolved"* sections, think about how, when, and who will communicate dissatisfaction to offending team members. Reflect back on what you've learned thus far about your teammates in terms of what previous team experiences they have had, how you have worked together as a group thus far, and how each member tends to feel and deal with conflict.

Step 1: If not resolved, what will your team do? How? When?:

Step 2: Meet as a team with your lab instructor. If not resolved, what will your team do? How? When?:

Step 3: The quit or fire clause: If the steps above have been completed without resolving the problem, any team member may quit the team. Alternatively, if all other team members are in agreement, the offending team member may be fired from the team. In either case, the individual no longer working as part of a team is required to complete the remaining class activities and assignments individually.

The LB145 teaching team reserves the right to make final decisions to resolve difficulties that arise within a team. Before this becomes necessary, the team should try to find a fair and equitable solution to the problem.

Group Name:______________

Member's Names (printed), Signatures, and date:

1.____________________________

2.____________________________

3.____________________________

4.____________________________

CF & PCR Research Studies

CF-PCR Project: Getting Started

***Assignment*: Develop a PCR-based DNA diagnostic assay for a mutant form of the gene that causes a cystic fibrosis.**

What's your research question?

☐ I want to design a PCR test for the R117H mutation in CFTR that causes a mild form of cystic fibrosis.

☐ I want to design a multiple site PCR assay for the top ten mutations that cause 99% of all cases of cystic fibrosis.

☐ I want to design PCR primers that will allow me to find if the CFTR gene has an intron junction mutation that causes alternative splicing.

☐ I am designing a PCR assay for the nonsense mutation(W1282X) that causes CF in the Ashkenazi Jews.

☐ Dude, I want to do something *way* cooler ....

Review what previous students have done in their PCR research projects on the course website.

LAB 0.1: INTRODUCTION TO THE LB-145 RESEARCH STREAM

Project/Experimental Objectives:

- Form research teams. Assign group roles, exchange contact information and schedule open lab times.
- Become familiar with the concepts of this semester's research theme.
- Schedule times to meet outside of lab to plan for next week's proposal presentation and in-lab interview.
- Learn how to use a micropipette.

Learning Objectives:

- Learn who the people in your research team are.
- What is cystic fibrosis (CF)?
- What is a polymerase chain reaction (PCR)?
- How can PCR be used to test for CF?
- How can you use this lab manual to help you design a PCR-based test for CF?

Lab 0.1 is intended to familiarize you with how the LB-145 research lab works. The goal is to outline the entire 14-week research stream in a single session while still giving you time to work with your groups and get started on your homework for next week. In other words, be ready to hit the ground running. First, you will need to form your research teams. You will be working together extensively both inside and outside of lab this semester, so take a moment to get to know each other, exchange emails and contact information, and discuss your schedules to find out what times you will all be able to come to open labs and meet with your group at night/weekends outside of lab. You'll be getting a brief introduction to the research we would like you to do this semester, and then be given time to start planning your projects. Next week in lab, you will be presenting your research proposal for the whole semester and convincing your TAs your group deserves 4.0s. To successfully impress your TAs, it is expected that your group will read all labs, primers and meet outside of the lab several times this weekend to plan your project and prepare a presentation.

Read primers 1, 2 and 3 on the metric system, keeping a notebook and using a pipette.

1. Collect 4, 1.8mL eppendorf (microfuge) tubes from the container in the drawer at your lab bench and label them 1 through 4.
2. Record the mass of each tube in you laboratory notebook.
3. Use various pipetmen to add the following volumes of water to the tubes.

Tube	**1**	**2**	**3**	**4**
Volume of H20	**500 ul**	**200 ul**	**20 ul**	**740 ul**

4. Record the mass of each tube in your notebook.
5. Remove 250uL from tubes 1 & 4 and record their masses in your lab notebook.
6. Use any resource (internet, textbook, etc) to find the density of water. Based on this information, what mass should each tube have? Compare these numbers to those you measured. How closely do they match? Record your findings and conclusions in your lab notebook.

“CF & PCR” Research Project *Scientific Method* Learning Objectives

Science Skill	**Your Learning Objectives (can you do this?)**
System Identification	• Identify and define experimental system.
Question Asking	• Ask thoughtful and probing questions about an experimental system.
Hypothesis Formation	• Identify and assess a hypothesis. • Develop hypotheses based on previous observations. • Distinguish hypotheses from predictions.
Experimental Design	• Evaluate an experimental design. • Identify variables and controls. • Develop an experiment to address a given hypothesis with appropriate variables and controls. • Make predictions based on hypothesis.
Data Analysis and Interpretation	• Evaluate data from a given experiment by determining the results of control and experimental treatments and relating the results to the experimental question or hypothesis. • Formulate conclusions based on data. • Create a model to explain the data.

LAB 0.2: INTRODUCTION TO THE LB-145 RESEARCH LAB

Experimental Objectives:

1.) Make a dilution series.
2.) Measure the absorbance of the dilution series.
3.) Make a Beer's Law plot (aka: graph or standard curve) of the data
4.) Keep a notebook that would allow a 144 student to repeat the experiment.

Learning Objectives:

1.) Learn to use standard equipment including spectrophotometers, micropipetters, pipette aids and graphing programs.
2.) Learn how to keep a laboratory notebook.
3.) Understand the technique and mathematics associated with a dilution series.
4.) Be able to pipette solutions accurately.
5.) Be able to create a graph using Excel.

Lab 0.2 is intended to familiarize you with basic lab practices, skills, techniques and equipment. Good scientists are always **careful** and **conscientious** (look it up). You should make every attempt to limit human error as a variable in your experiments. Many mistakes can be avoided if you take your time and pay careful attention to the task at hand. **Citing "human error" is NEVER an acceptable explanation for unexpected results**. Good record keeping is also a critical component of scientific research if you are to have result to interpret. This lab is intended to introduce the sound practices that should be used throughout this semester as well as in any laboratory setting you may encounter during your education and career.

Top 10 Laboratory Survival Rules/Tips

Safety- First.

1.) Imagine you're a responsible adult and act accordingly.
2.) If something is wrong (ie: a common reagent is low, an alarm is going off, or a piece of equipment is broken), report it to a TA.
3.) If you were never expressly told to do something, ask a TA before you do.

Courtesy- You're not the only one in here.

4.) Do not change the location of any common equipment, supplies, or reagents; and put things back promptly when you are finished with them.
5.) Clean up your own mess promptly.

Integrity- Or lose credibility as a scientist.

6.) Assume nothing. If you do not have a good answer, be honest and try to find one.
7.) Honesty is the best policy. Don't cover-up your mistakes- that can only make them worse. Trust your data- NEVER modify it to fit your expectations. Unexpected results are often the most interesting results.

Perseverance- If you want something done…

8.) If at first you don't succeed, modify your protocol and try again.
9.) If you don't know what something means, look it up. This is the information age.
10.) Stop working when you are finished, not when time is up.

Read Primers 1-6. Be sure to understand how to use the equipment and do the calculations.

Dilution Procedure: This experiment is your first experiment in LB-145. Record all information in your lab notebook. This procedure requires distilled H_2O and bromophenol blue solution.

1.) Obtain 8 clean, dry test tubes. Label the tubes 1 through 8.

2.) Using the pipette filler, draw up 1 ml distilled H_2O. Dispense the 1 ml in a single tube.

3.) Repeat for all 8 tubes so that each tube contains 1 ml. *Each team member should fill 2 tubes.*

4.) Using a micropipetter, draw up 1 ml of blue solution (Bromophenol blue). Dispense the blue solution into tube #1.

5.) Vortex the tube gently. Be careful not to allow the liquid to splash out of the tube.

6.) Change the pipette tip. This is very important during a dilution series. Draw up 1ml of the solution in tube #1, dispense it into tube #2, and gently votex tube #2 as before.

7.) Change the pipette tip. This is very important during a dilution series. Draw up 1ml of the solution in tube #2 dispense it into tube #3, and gently votex tube #3 as before.

8.) Continue in the same manner for every tube. *Each team member should perform 2 dilutions.* The last tube should contain 2 ml while tubes 1 through 7 should have 1 ml.

9.) Transfer 1 ml of solvent (in this case, distilled H_2O) to a spectrophotometer cuvette. This will serve as a blank. Check to make sure 1mL is a large enough volume for the cuvettes you are using.

10.) Transfer 1 ml of solution from tubes 1 through 8 to 8 cuvettes. Using the protocol on the next page, read the absorbance of each solution at a wavelength of 450nm. Record the data IN YOUR NOTEBOOK.

Tube	Absorbance at 450nm	Concentration
Blank		NA
1		
2		
3		
4		
5		
6		
7		
8		

Math Minute: Assume the blue solution contains 10mg/ml bromophenol blue. Determine the concentration of crystal violet in each tube. Record in the table and your notebook.

Vernier Spectrometer protocol:

If you use the BioRad spectrophotometers, consult the manual to find how to use it for different experimental protocols

1. Plug the spectrophotometer into the computer using the USB cable.

2. Open the LoggerPro software by clicking the icon on the toolbar.

3. Select "Calibrate Spectrometer" from the experiment menu and wait for the lamp to warm up.

4. Place a cuvette of "blank" solvent into the cuvette slot and click "Finish Calibration."

5. Place a sample in the cuvette slot and click "start collection" in the experiment menu. This will display a graph as well as a chart of absorbance v. wavelength.

6. Click "stop collection" in the experiment menu.

7. Click "store latest run" in the experiment menu.

8. Swap samples and repeat step 5-8. As you store more samples, the data will be saved in the table on the left hand side of you screen. You can use the re-sizing tabs in the corners of this box to be able to see all of the data at once. The data can be exported to excel by copying and pasting.

Graph all of your data in excel. Be sure to add a trendline and show the equation and R^2 of this line. Print this graph and paste / tape it into your lab notebook.

LAB 1: POLYMERASE CHAIN REACTION (PCR)

Note: You will be analyzing your PCR results by Gel Electrophoresis. Please review gel electrophoresis in your textbook (Ch. 20).

Adapted from the original author: John Urbance, Ph.D. by Doug Luckie, Ph.D., and Mike Haenisch

Learning Objectives:

1. Learn how to design a PCR reaction cocktail and what each ingredient does.
2. To learn enough about PCR to design your own primers to target a known mutation.
3. Learn how to calculate annealing temperatures for primers given only the DNA sequence.
4. To learn to troubleshoot and optimize PCR reactions to help you in your research.
5. To develop a PCR reaction to be used as a positive control during your research.

Experimental Objectives:

1. To learn how to set up and run PCR reactions and the role of the reaction ingredients.
2. Learn how to mix a PCR reaction cocktail with correct concs diluted from stock solutions.
3. Learn how to mix and make an agarose gel for electrophoresis.
4. To further gain expertise in working with DNA laboratory equipment (e.g. micropipettors, thermocycler, agarose gels).

Introduction

Have you ever wondered how forensic scientists get enough DNA from a single drop of dried blood or from a single hair to conduct investigations? Or how, out of the millions of base pairs that make up an organism's genome, scientists isolate a particular gene, or set of genes, for analysis? They can accomplish these things (and many others) by *"amplifying"* the targeted region of the genome using a technique called the *Polymerase Chain Reaction (PCR).* In PCR we essentially replicate the desired target region of the DNA in a test tube using the same enzyme that the cell uses for DNA replication (DNA polymerase). These days, there are very few genetic analyses that don't include a PCR step somewhere in the process. It is arguably the most important technique in the molecular biologist's repertoire (important enough to win its inventor, Kary Mullis, the Nobel Prize) and has become a ubiquitous and powerful tool in diagnostics, forensics and research biology. PCR is an method of synthesizing ("amplifying") large quantities of a targeted region of DNA *in vitro*. The DNA is synthesized the same way that cells do it—using a DNA polymerase (the enzyme that cells use to replicate their DNA). Once amplified, PCR products can simply be visualized by agarose gel electrophoresis or can be further analyzed by subsequent enzymatic digestion for DNA fingerprinting, by cloning or by DNA sequencing.

PCR works by using a thermostable DNA polymerase (Taq polymerase) and short DNA fragments, called **'primers'**, to direct the synthesis of *a* specifically-targeted region of the genomic DNA. The synthesis reaction is repeated numerous times called **'cycles'**. The products of previous synthesis cycles serve as template for the next cycle. This results in an exponential amplification of the targeted region of DNA—every cycle will double the copy number of the target region.
This repeated cycling is made possible by the use of Taq polymerase, a thermostable (heat-tolerant) DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*, originally isolated from a hot springs in Yellowstone National Park (ambient temperature 80ºC!). Because it comes from a heat-adapted bacterium, *Taq* polymerase can withstand the repeated, high-temperature DNA denaturation steps (94ºC) that are part of the PCR procedure.

The Primers

All DNA polymerases require a short segment of double-stranded nucleic acid (a primer) to initiate DNA synthesis. During DNA replication, cells use short stretches of complementary RNA—synthesized by enzymes called 'primases'—to initiate polymerization. In the laboratory, short, complementary DNA primers are also used in PCR to initiate DNA synthesis and to designate the specific target region to be amplified. The primers (also called oligonucleotides—meaning small number of nucleotides) are easily synthesized and can be designed to be complementary to any known DNA sequence. They can range in size from 10 to 100 nucleotides in length, but typically they range from 15 to 30 bases for PCR. It is the amplification primers that determine target specificity (i.e. which segment of the template DNA will get amplified) of the PCR reaction.

The Cycles

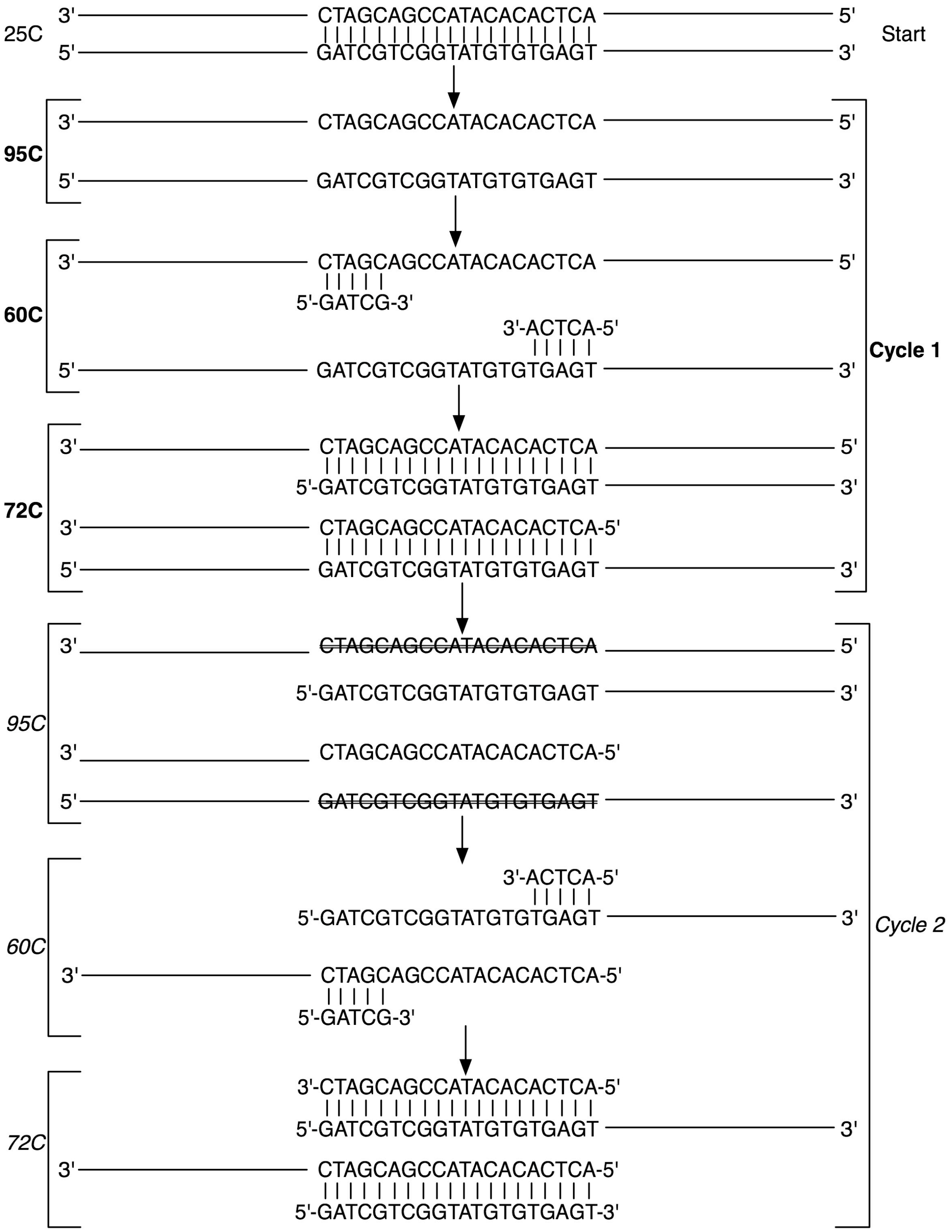
Denaturation (HOT). During the denaturation step, the reaction cocktail is exposed to high temperature, usually 95C. This high temperature will denature the DNA-- meaning the two complementary strands of the DNA molecule unravel, exposing the nucleotide bases. The high temperature of the denaturing step has the added advantage of denaturing proteins and disrupting cells so you don't have to always start with purified DNA as your amplification template, you can often amplify DNA directly from cell lysates—or even whole cells.

Primer Annealing (COOL). During the second step of each cycle, the temperature is lowered to an annealing temperature, allow annealing of the primers to their complementary targets on the DNA template (one for each DNA strand). These are designed to flank the desired target region of your DNA template and serve as the starting points for DNA synthesis by the *Taq* polymerase. Each pair of primers will have a particular annealing temperature determined by the length of the primers and their nucleotide content. Using an annealing temperature that is too low can result in non- specific amplification (amplifying the wrong region of the DNA). Using an annealing temperature that is too high can result in no amplification at all.

Extension (JUST RIGHT). The reaction cocktail is now brought to the optimum reaction temperature for *Taq* polymerase (68 to 72C). During this step, the *Taq* will bind to each DNA strand and "extend" from the priming sites (synthesize a complementary strand of the targeted DNA).

Notice that these three steps are accomplished simply by varying the incubation temperature of the reaction tubes. Typically, PCR reactions are run for 30 to 35 cycles, which are performed by a specialized machine called a thermocycler designed to rapidly heat and cool the reaction tubes to the desired temperatures. *For this exercise,* you will first perform your PCR reaction *manually* using water baths to control the temperatures (FUN!).

<u>What happens in each step of the thermal cycle.</u>



Methods

PCR Materials

You should have or collect the following materials at your bench.

- 0.2 ml PCR tubes and a tube rack
- micropipettors and tips
- *E. coli* bacterial colony, or, Lambda virus genomic DNA @ 20 ng/ml
- nuclease-free H_2O
- 10X PCR buffer (stock is 10X needs to become 1X in PCR reaction tube)
- deoxynucleotides (dCTP, dATP, dTTP & dGTP) (stock is 10 mM, final should be 200uM)
- forward primers (stock conc is often 10 pmole/ul or 100uM, final should be 2uM)
- reverse primers (same as forward)
- *Taq* polymerase @ 5 U/ul (need only have 1 U=unit of activity in a PCR reaction)

Part 1: Making your agarose gel

Follow the protocol for pouring agarose gels described in your textbook and by your TA or LA. While your gel is solidifying you can perform your manual PCR reactions.

Part 2: Plan your Manual PCR

Your goal is for your group to work together to make a plan to perform PCR without using a thermal cycler. We recommend you use 50 ul reaction volumes (i.e. each tube will ultimately contain 50 ul of reaction cocktail). You'll use primers that you choose and assign different group members to different PCR steps or cycles, Joe= 95 degrees. Set up 3-temperature cycles just like the PCR machines will do. While preparing you need to figure the optimum temperature for *Taq* and the best annealing temperature for your primers. Water heated in beakers on hot plates can serve as your baths.

Work with your group members to create your own protocol for this experiment. Write it all up in your notebook before coming to lab. Plan to mix up 0.8% agarose solution of agarose powder with 1X TAE buffer, heat it and pour a gel. Make a table that lists all the ingredients and amounts you need to add to your PCR tube. Then plan what temperatures and how long you'll hold the tube (with tongs) in each temperature etc. You can choose to test the lambda virus genome for the "Rz gene" or the *E. coli* genome looking for "16S rDNA genes" by using the appropriate primers [more information below]. *REPEAT: Write a plan in your laboratory notebook before lab. *Your LA will check each notebook as you enter lab, admission to the lab room will require your preparation in advance.*

Table 1: ? What primers, times & temperatures will you use _____ _____ _____ ?

Single reaction cocktail ingredients	**Manual PCR**
? ul H_2O	
? ul 10X PCR buffer	
? ul 10 mM dNTPs	
? ul 100 uM forward primer	
? ul 100 uM reverse primer	
1 ul DNA Template	
1 ul *Taq* polymerase	

Stuff you need to know to do this lab

What's in the reaction cocktail?

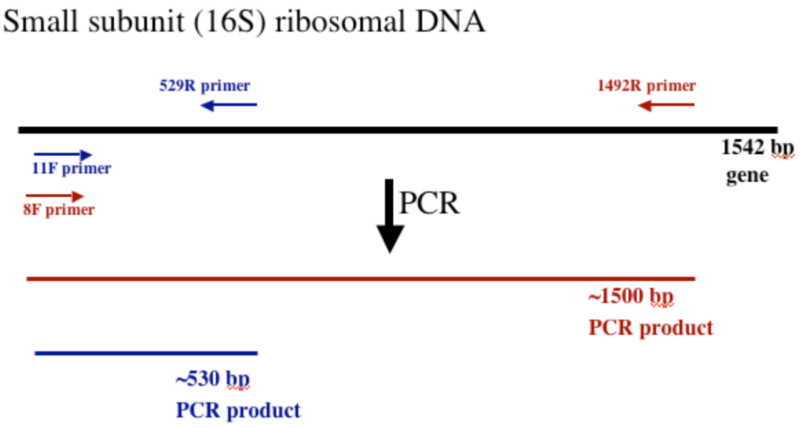
You must create a reaction cocktail in your tube that provides everything the enzyme (in this case *Taq* polymerase) needs to function as it would in the cell during DNA Replication. Below is an inventory of what is required.

- Taq Buffer: Each cellular enzyme has a specific salt concentration and pH (and temperature) required for its optimum performance. At 1X your PCR reaction buffer provides the proper salt (KCl) concentration and pH (~8.5) for *Taq* polymerase.
- dNTPs: (i.e. nucleotide bases) DNA is a polymer of these four nucleotides [A,T,G,C]. They are the building blocks polymerase uses to synthesize new DNA.
- Oligonucleotide primers: Their roles in DNA replication and in PCR were described above. PCR usually requires two primers, one targeted to each DNA strand.
- DNA Template: In the cell, DNA polymerases use denatured, genomic DNA as a template upon which to synthesize complementary DNA strands.
- *Taq* polymerase: You can't carry out an enzymatic reaction without the enzyme.

What's primers are available?

E. coli ribosome 16S gene primers

These are two sets of primers that amplify different segments of the small subunit rRNA gene (16S rDNA) of *E. coli*. Figure 1 illustrates the 1542 bp 16S rDNA gene, the target sites of the two primer pairs and the resulting sizes of the PCR products.



Primer	*E. coli* positions	5' -> 3' sequence
8F	8-27	AGA GTT TGA TCC TGG CTC AG
11F	11-27	GTT TGA TCC TGG CTC AG
1512R (1492R)	1492-1512	ACG GTT ACC TTG TTA CGA CTT
529R	515-529	CGC GGC TGC TGG CAC

Lambda virus Rz gene primers

The primer pair below will target the "Rz gene" of bacteriophage lambda, a virus that infects *E. coli*. The Rz gene encodes for a protein associated with lysis of the *E. coli* cell wall. These primers will amplify a fragment of the Rz gene ~ 500 base pairs long.

The primers are:

lRz1F primer:
5'-GATGTATGAGCAGAGTCACCGCGAT-3'
l- Rz 1R primer:
5'-GAGGGTGAAATAATCCCGTTCAG-3'

How do I get template DNA?

1. To get *lambda* genomic DNA for amplification ask the LA for a sample.

2. To get *E. coli* genomic DNA for amplification just add some living E. coli to your PCR reaction tube. They will lyse (explode) during the PCR and their genome will serve as the template DNA.

> ❐ Specifically, use an inoculating loop to add a tiny (I MEAN TINY) amount of colony directly to the PCR cocktail and just run the PCR. This has a very good success rate with gram negatives and somewhat less-so with gram positives. Too much colony frequently poisons the reaction. A variation on this is to make a cell suspension (again, very dilute) in 20 ul of DNA-grade water and use different volumes (1-3 ul) of this as your template.

Troubleshooting Your Research

Figure out how to make experiments work better for your research (see Troubleshooting Primer)

Date: Name:

Experiment Performed:

Goal of the Experiment:

Did the experiment work? If not, what went wrong? Describe the specific details of the problem (colors, volumes, incubations, etc.).

If you were to repeat this experiment, what would you do to address any problems? How would you improve this experiment in the future? Be specific.

LAB 2: (DESIGN PROTOCOL) PURIFICATION OF GENOMIC DNA

Note: You find protocols supplied by professional researchers for use of Chelex

Learning Objectives:

1. To better understand the nature of science today: finding existing protocols for commonly used techniques provided by fellow scientists.
2. To be able to follow and optimize existing protocol and make it work for you.

Experimental Objectives:

1. Isolate enough DNA from cells to support/be used in your PCR research experiments.

Introduction

During your LB-145L PCR research project this semester you'll need genomic DNA from cells to serve as template. This is the lab when you get that DNA. NOTE: This is NOT a cookbook lab designed solely to teach you how to do something, it is also your opportunity to actually get enough genomic DNA from living cells for your research for the whole semester.

This laboratory will serve as your opportunity to practice working with modern DNA protocols developed by others and optimize them for yourself. You must both determine how to mix up an appropriate stock solution of Chelex-100 chelating resin, as well as develop a protocol to use that solution to extract DNA from human cheek cells from saliva or buccal swabbing (which also requires a protocol for collecting cells). The genome, literally human chromosomes, will serve as an excellent “template” for your PCR research. Protect the DNA you isolate, keep it cold so enzymes don’t degrade it. This is your very precious material for the semester.

Methods

We currently plan to provide you with the Chelex resin from the company Sigma Inc. Check with the LA to confirm which vendor and what reagents are provided (in case there are more). Prior to coming to lab, visit the vendor’s website and search the internet for existing protocols provided by other scientists for using Chelex to do genome purification from saliva with cheek, or buccal, cells. Work with your group to review the protocol(s), choose one, and then distill it down to the basic steps you’ll have to perform that seem the most appropriate to your project. Write up the protocol in your laboratory notebook.

Your LA will check each person’s notebook as you enter lab, admission to the lab room will require your preparation in advance.

Troubleshooting Your Research

Figure out how to make experiments work better for your research (see Troubleshooting Primer)

Date: Name:

Experiment Performed:

Goal of the Experiment:

Did the experiment work? If not, what went wrong? Describe the specific details of the problem (colors, volumes, incubations, etc.).

If you were to repeat this experiment, what would you do to address any problems? How would you improve this experiment in the future? Be specific.

30 DAYS

LAB 30: “30 Days” Designing Investigations into Health & Privilege

Authors: Dr. Douglas Luckie, Ph.D. and Marija Krha, M.S.

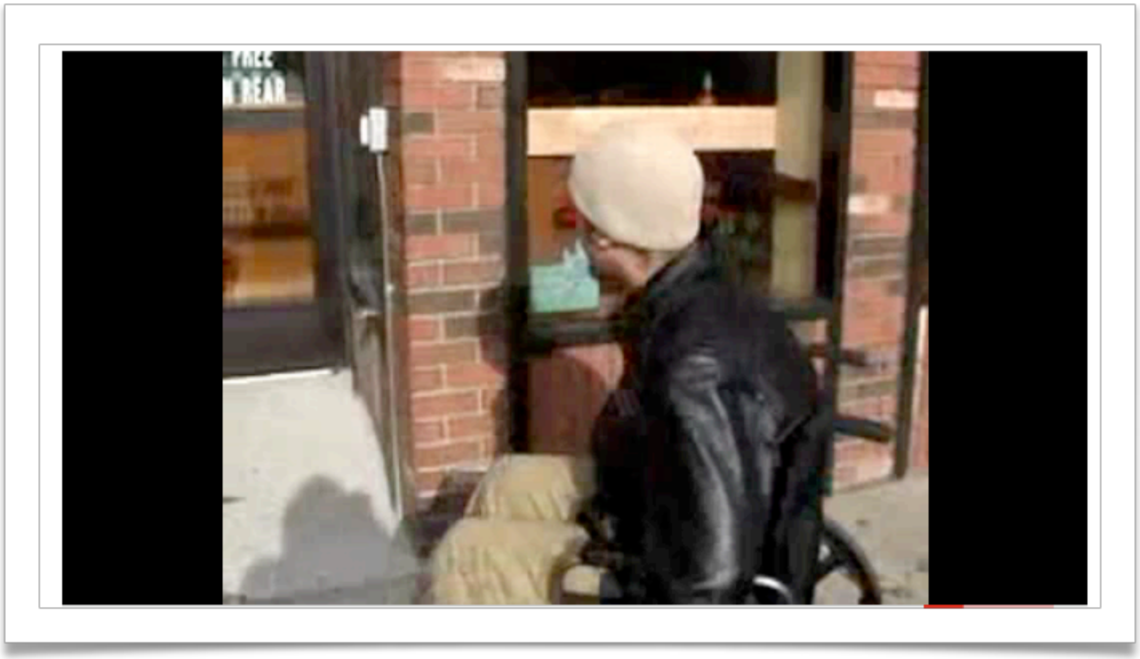
(Optional)

Learning Objectives:

1. To better reflect upon your own learning & connect it to other disciplines.
2. To gain a greater focus and ability to write/speak about health & disease

Experimental Objectives:

1. To build skills designing experiments & experiences with real-world impacts.



[TOPIC TO BE ANNOUNCED]

General Primers

(in this case the word *primer* means an introduction)

Primer 1: The Metric System

You will find it very hard to exist in lab this semester without knowing and being able to use the metric system. The short story is that a base unit (meters, grams, calories, joules, etc) is given a prefix to indicate the scale of the unit (kilo = 1000 base units; mili = 1 / 1000 base units). You should memorize at least the units in bold since they are the most likely to be used this semester and you should also be able to convert from one unit to another (ie: know how to convert 500mg into 0.5g).

List of metric prefixes, symbols, and their multipliers.			
Prefix	Symbol	Numerical multiplier	exponential
yotta	Y	1,000,000,000,000,000,000,000,000	10^{24}
zetta	Z	1,000,000,000,000,000,000,000	10^{21}
exa	E	1,000,000,000,000,000,000	10^{18}
peta	P	1,000,000,000,000,000	10^{15}
tera	T	1,000,000,000,000	10^{12}
giga	**G**	**1,000,000,000**	**10^{9}**
mega	**M**	**1,000,000**	**10^{6}**
kilo	**K**	**1,000**	**10^{3}**
hecto	**H**	**100**	**10^{2}**
deca	**da**	**10**	**10^{1}**
NA		**1**	**10^{0}**
deci	**d**	**0.1**	**10^{-1}**
centi	**c**	**0.01**	**10^{-2}**
milli	**m**	**0.001**	**10^{-3}**
micro	**µ**	**0.000001**	**10^{-6}**
nano	**n**	**0.000000001**	**10^{-9}**
pico	p	0.000000000001	10^{-12}
femto	f	0.000000000000001	10^{-15}
atto	a	0.000000000000000001	10^{-18}
zepto	z	0.000000000000000000001	10^{-21}
yocto	y	0.000000000000000000000001	10^{-24}

Primer 2: Keeping a Laboratory Notebook

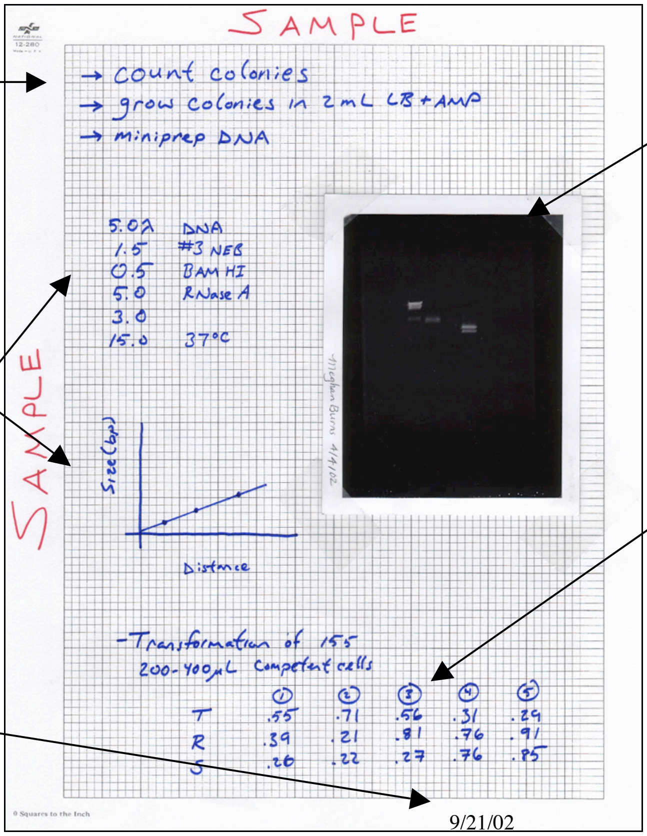
When scientists leave the lab (either to go to lunch or to go to work in another lab), their notebooks stay behind as a testament to what they did there. Other researchers in the lab may wish to know how someone had done a previous experiment, or what the results were from a different trial. Thus the notebook should be organized in such a way as to be intelligible to someone proficient in the field without any input from the author.

You are required to keep a notebook for LB 145. There is graph paper provided for you in the back of this notebook which will be checked to assess your notebook score (so use it as your notebook). BEFORE you leave lab each week, **You MUST get your Notebook stamped and initialed by a TA/LA.** This will be checked & graded throughout the semester and will be a means of taking attendance. Below is shown a sample page from a lab notebook. Although there is one data recorder for the group ALL students will be responsible for transferring the data to their notebooks before leaving lab. Each days entry in your notebook should include the date, the purpose of the experiment, the techniques used, and ORGANIZED data. Your time in lab will be used most effectively if you prepare as many of these elements beforehand as possible. For example, outline what your next experiment is and why you are doing it, write in the protocol or a clear reference to it (be sure to leave room for modifications), and prepare a section to enter data in (what will it look like? Do you need a graph, a table, or something else?). When a protocol is used frequently with only slight modifications, many scientists will type a copy of the protocol in a word-processor and leave blanks in which to fill in important variables (ie: make a protocol for PCR, but leave blanks for things like polymerase concentrations, primer types, etc).

Making a "To Do" list is very useful to do before coming into lab.

Jotting down both numerical as well as pictorial data is extremely useful to you and your TAs when examining your data.

ALWAYS date your work



→ count colonies
→ grow colonies in 2 mL LB + AMP
→ miniprep DNA

5.0λ	DNA
1.5	#3 NEB
0.5	BAM HI
5.0	RNase A
3.0	
15.0	37°C

-Transformation of 155
200-400μL Competent cells

	①	②	③	④	⑤
T	.55	.71	.56	.31	.29
R	.39	.21	.81	.76	.91
S	.26	.22	.27	.76	.85

9/21/02

Notice that some material may need to be taped into your lab notebook – this is totally acceptable.

Keeping a record of all data gathered during an experiment at time intervals is absolutely a MUST.

Primer 3: How to Pipette

Below is a picture of 2 different micropipetters. Notice that**although the windows show the same numbers, these micropipettes will not deliver equal volumes** The amount of volume depends on what TYPE of pipette you are using. The one on the left is a 200µL pipette (set at 200), while the one on the right is a 20µL pipette (set at 20). Look at the pipettes at your bench and see if you can tell the difference.

Micropipette Procedure

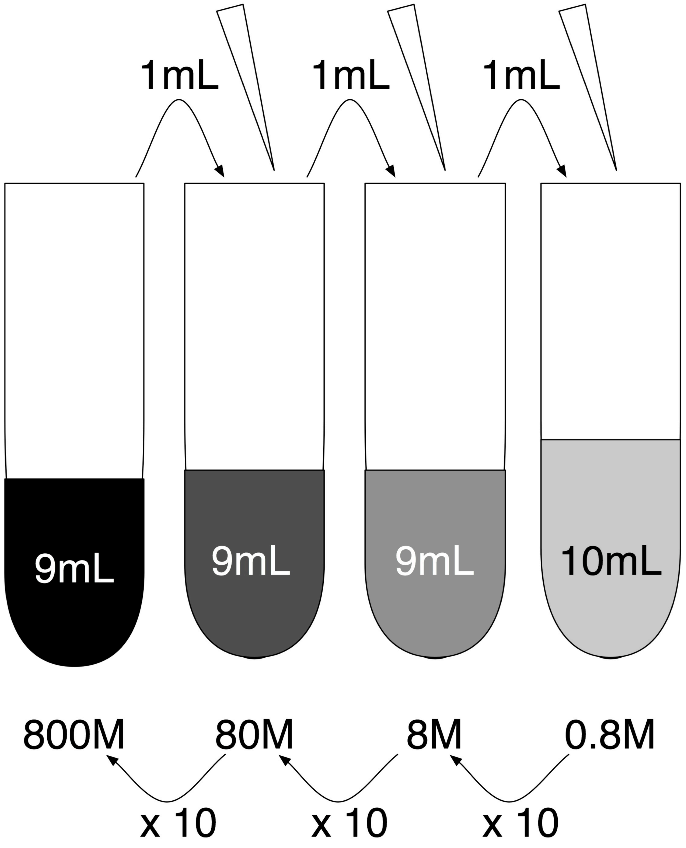
1.) Identify the right pipette for the volume you would like to transfer. The range that any pipette can transfer should be written on it somewhere. Find out where and select an instrument that can accommodate your desired volume. Avoid using a pipette to transfer a volume that is near it's lower limit, the will reduce precision.
2.) Set the pipette to the desired volume by turning the black knobs located between the plunger and the window display. This can be done by twisting the plunger too, but this practice should be avoided since plungers are easy to bend.
3.) Put a disposable tip on the micropipetter. Be sure to use the correct tip size.
4.) Push the plunger down a few times – notice that there are two natural stops to the plunger. If you push lightly, it will stop once and then you can push a bit harder and it will move down to the second stop. KNOW THE DIFFERENCE BETWEEN THE STOPS.
5.) To draw up your desired volume, push the plunger down to the FIRST STOP before putting the tip into the solution. Insert the tip into the fluid you are withdrawing from and SLOWLY ease back on the plunger and watch to ensure you do not draw air bubbles up with your fluid. DO NOT SUDDENLY LET GO.
6.) To dispense your volume, carefully put the pipette tip against the wall of the receiving vessel. SLOWLY push down to the FIRST STOP. You will notice a small volume remaining in the tip. You will now push down to the SECOND STOP to expel fluid.

Pipette Aid Procedure

1.) Obtain an individually wrapped, sterile pipette and pull apart the wrapper near the end with a cotton plug. Leave the majority of the pipette wrapped.
2.) Place the cotton-plugged end in the pipette aid and pull off the plastic cover.
3.) Draw up the liquid by depressing the upper button on the pipette aid. DO NOT allow liquid to enter the cotton plug. If this occurs, inform an LA.
4.) Expel the liquid by depressing the lower button on the pipette aid.
DO NOT allow liquid to enter the cotton plug or inside the pipette aid.

Primer 4: Dilution Series

Making a dilution is a common task performed in both research and clinical laboratories. Many assays (tests) can only detect compounds of interest in a given concentration range. You'll most frequently simply dilute a stock solution from something like "10X" (e.g. 10mM) to "1X" (1mM), and you need to get used to that. Yet when you need to dilute a solution significantly (perhaps 10million X) such that you would need to put a drop of it into a swimming pool to lower it's concentration enough, you can use a dilution series instead of finding a pool. Dilution series can also be used to make a panel of concentrations to test when the starting concentration is too high to measure, or if multiple trials are to be done with different concentrations. Using the formula $C_1 x V_1 = C_2 x V_2$, the concentration of any substance in any sample of a dilution series can be calculated. By using the same volumes for each subsequent dilution, a very large dilution can occur. This also makes the math required for your calculations much easier. While the actual volumes can change as dictated by an experiment, the overall scheme of a dilution series is generally the same. A schematic of a dilution series is shown in the diagram below.

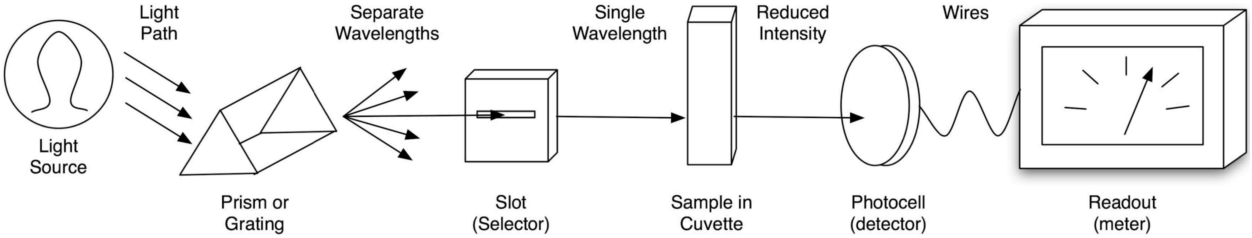


Primer 5: Spectrophotometry, Beer's Law and Standard Curves

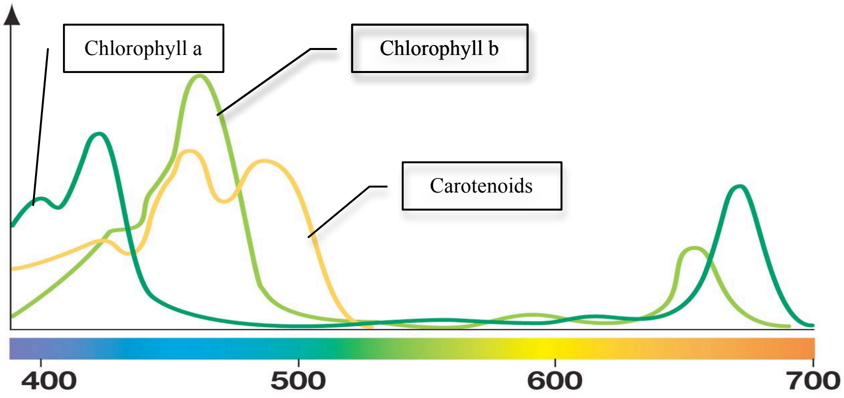
Mike Haenisch

Spectrophotometry is one of the most fundamental and most widely used techniques in modern biology labs. You will use spectrophotometers this semester to quantify DNA from cell extracts and evaluate its purity. This section will first discuss how the absorbance of light is measured at specific wavelengths, then how this can be used to identify compounds based on which wavelengths of light they absorb most strongly, and finally how we can use this information to quantify compounds in biological and chemical samples.

As shown below, broad-spectrum light (many wavelengths) is emitted from a bulb and the wavelengths are separated into different directions by a grating or prism. By rotating the prism, it is possible to angle a different wavelength of light towards the selection slot. Only the desired wavelength passes through the slot and eventually to the sample in the cuvette. Light that is not absorbed by the sample passes through to a photocell which generates an electric current when struck by light. The higher the intensity of the light that strikes the photocell, the stronger the electrical current that is generated (just like solar power). The strength of the current is converted into a display that can be recorded.



Every compound absorbs specific wavelengths of light. The wavelengths absorbed depend on the types of chemical bonds in a compound. Measuring the absorbance of any compound over a range of wavelengths generates a type of fingerprint for that compound called an absorption spectrum. Below are the absorption spectra of several photosynthetic pigments (Taken from Campbell). You can see that these pigments absorb red and blue light most strongly.



Primer 6: Graphing in Excel

Mike Haenisch

1.) Input your data into excel.

2.) Use your cursor to highlight the data and then open the "chart wizard" either by clicking the button on the toolbar, or by clicking chart in the insert menu. The "Chart Wizard" will guide you through most of the rest of the process.

3.) To make a standard curve, select "XY (scatter)" from the chart type options and click "Next".

4.) The third window of the wizard allows you to give the chart a title and label the axes. Be sure to use units on your axes. This would also be a good time to check that the independent variable (the one that we have control over) is on the x-axis. If it isn't, then switch the position of your data columns right to left (ie: conc on the left, or ABS on the left).

5.) The fourth and final window gives you the option to create your graph as an object in the spreadsheet or as a new sheet. If you make the graph as a new sheet, it will show up as a new tab at the bottom of your screen. You can move between sheets and graphs by clicking on these tabs.

6.) A trendline can be added by right clicking on a datapoint and selecting "add trendline" from the drop-down menu. You can also select your data and select "add trendline" from the chart menu. This will open the trendline wizard. The style of trendline you use will depend on the type of data you have, so choose carefully. Use the options tab to further format your graph. You should always show the equation and R^2 of your trendline, but the intercept may not always be set to a constant.

7.) Generally, double-clicking on the various areas of the graph will allow you to change its features (colors, styles, scales, gridlines and much more). Figures don't need to be fancy, but they need to be presentable.

Primer 7: Analysis of Gel Electrophoresis

David Maison

If you're very lucky, then sometime this semester you will have a band in your gel from something other than your DNA ladder (hopefully from your PCR reactions). Having a band is good evidence that your PCR reactions worked, but it's not conclusive. Based on how you designed your primers, you should know how large of a PCR product should be in the gel. This primer will explain how to use the molecular weight marker (DNA ladder) to determine the size of a band in your gel.

Semi-Log Plots

Sometimes the only ways to see whether bands have changed, are unknown, or are the size you are looking for, is to use a visual analysis. In gel electrophoresis, the creation, and use, of a semi-log plot is a frequent and simple method for doing this.

A semi-logarithmic plot is used if one range of numbers is spread out over numerous orders of magnitude. It is important to note that applying the logarithmic scale is essential to standardize the graph. This creates a linear scale and allows determination of unknown bands.

Gel Electrophoresis and Plotting

When plotting a semi-log plot from your gel electrophoresis product, the y-axis corresponds to the base pair equivalent size of the molecule and is expressed in a logarithmic scale. This is because the bands can often range in size from 10^1 to 10^5 (10^4 for CFTR). The y-axis is partitioned into cycles, which are separated from one another by a factor of 10. The x-axis, migration distance when pertaining to DNA in gel, is expressed in a linear, non-logarithmic scale, often in equivalent centimeter intervals.

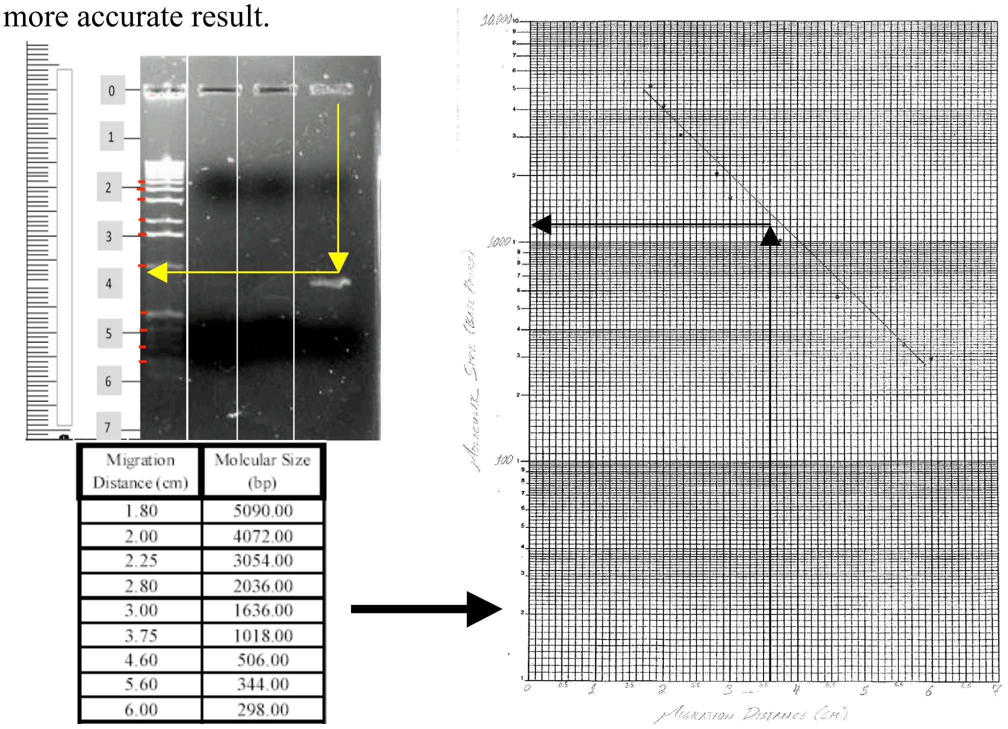
The standard curve (the line that is formed by plotting these two variables from your molecular weight standard) is what you will be using to determine the band size, of your unknowns. Since all of the bands in the molecular weight standard are of a known size, their migration pattern can be used to determine the size of unknown bands. The distance traveled by the DNA running in gel electrophoresis is not only proportional to the size of the DNA, but also the time the gel was allowed to run. As a consequence to this, the same DNA run on different gels will not be directly comparable.

Methods to Use:

A rough estimate of size can be made by comparing your band to the ladder on the gel (light arrows), but you should always make a standard curve to accurately analyze your results.

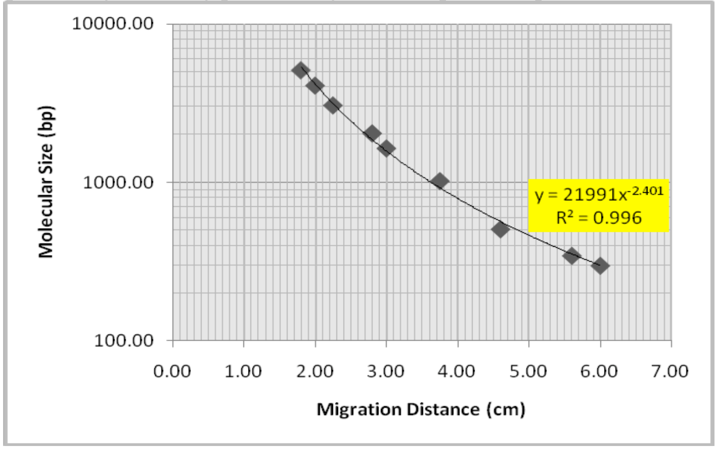
STANDARDIZED CURVE ON PAPER:

1) Measure the distance from the well to each band in the ladder.
2) Plot this distance (x-axis) verses band size (y-axis) on the semi-log paper in the back of your notebook and draw the best-fit straight line through the points. This is your standard curve.
3) Measure the distance your unknown band migrated and find that distance on your standard curve. Follow the gridlines up until you intercept the best-fit line, then follow the grid over to find the size of your unknown.
4) Enter this same data in excel and use the trendline function to solve for the equation of the best-fit line. You can use this equation to solve for your unknown band size will give you a more accurate result.



Migration Distance (cm)	Molcular Size (bp)
1.80	5090.00
2.00	4072.00
2.25	3054.00
2.80	2036.00
3.00	1636.00
3.75	1018.00
4.60	506.00
5.60	344.00
6.00	298.00

(Figure 1 – Sequence for producing semi-log plot from gel electrophoresis product)



(Figure 2 – semi-log plot generated from Microsoft Excel)

Primer 8: Introduction to the Troubleshooting Process

So you've researched your topic, planned an experiment, prepped all the necessary material, sat at the bench for 4.5 straight hours following every step of the protocol to the letter, and after all of that… the data you collected defies the laws of physics. Don't feel bad. You'd be surprised how many times a day researchers break the "laws of physics." We've all come to expect it, but since you're new to this, we're going to assume that you might be wondering what to do next.

The fact of the matter is that much of the actual scientific process is not spent analyzing data, but rather is spent trying to find ways to make experiments *work* in a way that provides any sort of useful data at all, and doing tests to make sure that the data that is being generated is a reliable representation of reality. Confirming that your data represents reality is why we have controls, but to get stuff to work or any useful data, we need to "troubleshoot" our experiments. This is the creative side of laboratory science that does use logic and the scientific method, but there isn't always a well defined process that will inevitably lead you to a successful experiment. *On the other hand, there are some things you can do that should help.*

1. Try to identify all the things in your experiment that *did* work. This is not just meant to make you feel better, it's important to identify which aspects of your experiment should NOT be changed.
2. Next identify *where* you noticed things beginning to not work well in your experiment. The problem must somewhere in-between "did work" & "didn't".
3. Sit down and look at the protocol in-between what did and did not work. At each step of the protocol, ask yourself, what could go wrong at this step? How can you tell if this is what actually went wrong? Generate a list of possible problems, ways to tell if those are the actual problems, and ways to fix the problems. What you should end up with is a list of experiments that you can do to try to isolate and fix the problem. Take that list to your TA and see if they have any advice on how best to proceed.

For example, lets say you did PCR on the 16S ribosome gene of *E. coli* that was supposed to generate a 500 base pair long fragment but you didn't have anything that looked like a DNA band on the 500bp region of your DNA agarose gel.

1. *What worked*: If you knew where the 500bp region of your gel was, that means you had a MW ladder that worked, therefore, your DNA gel was made and run properly.
2. *Where is problem*: The PCR product did not show up. The problem was before this.

3. *Possible problem(s)*	*Solution(s)*
PCR ingredients wrong concentrations.	Review your protocols and notes, review the Lab Guide and internet for proper concs, show your findings to your LA.
One of the PCR ingredients could be bad.	Check if other groups in your section used the same material as you, and their reaction worked on a different gene.
Your primers could be bad.	Check to see if anyone had success with them. If designed by you evaluate sequences again, you may need to redesign.
You may not have had enough clean template.	"Dirty" DNA doesn't always amplify. Check your DNA sample's quality and try to improve it with an extraction.
There may be a problem in your PCR cycle.	Change the variables (time and temperature) to increase the probability of annealing and amplification.

Primer 9: Find Me a Project!

Helpful Guide by Zach Gaudette

1. Log onto http://www.genome.gov/10001204 or utilize Google searches to learn about the causes, symptoms, and significance of multiple genetic diseases.

2. Discuss which diseases interest you and why. Select ~2 diseases that you would like to research further. For those diseases, determine which genes are affected. Record the genes' common symbols/nomenclature for future use. *For example, the disease Cystic Fibrosis is caused by mutations to the "Cystic Fibrosis Transmembrane Conductance Regulator" gene – more often abbreviated as the CFTR gene.*

3. Log onto http://omim.org and enter a gene of interest into the search bar.

4. The first search result will often be the name of your gene, preceded by a red asterisk or other symbol. Click on it.

5. The resulting page contains a great wealth of information on your gene of interest and should be bookmarked immediately. You should explore this entire page yourself but, for now, scroll down to the bolded subheading "ALLELIC VARIANTS."

6. Each of the entries in this section, often labeled with numbers like ".0001," describes the clinical and scientific significance of a specific mutation of your gene. Unless you have a much cooler project in mind, your work this semester will likely be based around detection of specific mutations like these. Read about multiple mutations. Ensure that their presence leads to (or is a marker of) your disease of interest.

7. Select a few mutations and research them further. You can do so by clicking on links to past research papers. *Example:* Yang et al. (2003) Clicking on these links will take you down the page to the bibliography section, where the paper's full information is listed. The bibliography entry is often followed by links to PubMed or other full-text versions of the paper.

8. Determine which mutation(s) you would like to base your semester's work on. Return to the subheading (mentioned is step 6) of that/those mutation(s). There is often a blue link below the mutation's heading with a format similar to this example: [dbSNP:rs5030858]. Don't worry if such a link is not present. This often means that your mutation affects more than just a few nucleotides of DNA. Such large mutations often make for an especially interesting experimental design and project but, for now, we will assume that a link is available. This means that the mutation affects a small number of nucleotides, often just one. Click on the link.

9. This link will take you to a site containing more cataloged information about your mutation. Scroll down to the bolded subheading "Explore this variation." It should contain multiple buttons that you'd be wise to explore on your own but, for now, click on the button titled "Flanking sequence."

10. The resulting page should look similar, but notice that the "Explore this variation" subheading has been replaced with the title "Flanking Sequence." Below this subheading will now be a list of letters. This page is presenting you will the DNA nucleotide sequence directly adjacent to your mutation of interest. This is great information to construct your PCR primers from – bookmark this page.

11. To avoid confusion, look to the left of this page and click on the blue button with a cogwheel symbol labeled "Configure this page."

12. From the three selection bars present on the resulting pop-up screen, select:
    1) Length of reference flanking sequence to display: <u>1000bp</u>
    2) Sequence selection: <u>Upstream and downstream sequences</u>
    3) Show variations in flanking sequence: <u>No</u>
    **Once finished, click the checkmark in the upper-right corner to return to the sequence screen

13. Now the nucleotide sequence flanking your mutation of interest (the red underlined letter) has been cleared of all other genetic variations possible in the area, and spans for 1000 base pairs to each side. Highlight this string of letters and transfer them to a blank Microsoft Word document. Delete the resulting spaces, but not the letters! Save this document and you will have a workable piece of genomic sequence to develop PCR primers from later.

14. Rejoice!

Primer 10: Examples of how to write an email to a researcher

Here are some examples of the style and wording you should go for when emailing a scientist to seek a sample of genomic DNA with your particular mutation.

Your goal is to establish an authentic one-on-one conversation between two peer researchers. Be a scientist not a student. Be sure to send the email from your msu.edu email account. They will look at that first. It establishes credibility.

Dear Dr. X,

I just read your article in The Canadian Journal of Neurological Sciences on dystrophin mutations and seek your advice. I'm currently designing a customized PCR assay to detect the deletion of exon 47 on the dystrophin gene and I'm curious if there is anyone in the field you might recommend I contact to obtain a small sample of genomic DNA with the mutation to serve as a control for testing my assay.

Any help you can send my way would be greatly appreciated.

Sincerely,

Kim Vi
MSU Diagnostics Lab
Michigan State University
vikim@msu.edu

Dear Dr. Fang,

I'm studying the SMN1 gene and just read your 2012 article in Biomedcentral Medical Genetics on SMN1 gene mutations in SMA patients of Chinese descent. I'm currently designing a diagnostic assay for the Arg288Met mutation in SMN1 using PCR. I'm contacting you for advice. Do you know of anyone in the field who I might contact to obtain a small sample of genomic sequence to serve as a positive control in my work?

Any help you could send my way would be greatly appreciated.

Best Regards,

Rajvinder Singh
Research Laboratory of Natural Science
Michigan State University

singhr10@msu.edu

Dear Douglas Lawrence,

I just read your PNAS 2008 paper on CARD15 mutations and I am curious if you can give me some advice. My research lab at Michigan State University is looking at the CARD15 mutation XYJ15, which is very similar to those in your studies. I was curious to know if you might have or know where I could seek a positive cell or DNA sample for XYJ15 mutation of CARD15 in order to further our research. Your time and consideration is greatly appreciated.

Alex Pierce

Dear Dr. Kanavak,

I just read your 2003 paper in Clinical Chemistry detailing the genotyping of the HbS mutation. I'm currently working on a related project, building a PCR-based diagnostic assay to detect HbS. I am contacting you for advice. Might you know someone who may be able to supply me with a small sample of HbS genomic DNA to serve as a control in my tests? Any information you could send my way would be greatly appreciated.

Best Regards,

Sarah Newman
Lymann Briggs College
Michigan State University
newmans7@msu.edu

Primer 11: How to read a research paper like a (busy) scientist

By Candace R. Igert

At some point during the semester, you are going to need to read a research paper. If you are striving to do well in this course, you will likely be reading a lot of research papers or journals. Just like everything else in science, there is an effective method to make this less painful and time-consuming. Below is a set of steps an experienced scientist will take when reading a journal or even deciding if it is relevant to what they are looking for. The scientist's goal is getting important information fast. They do not read from beginning to end, but skip around.

Step 1: **A scientist will read the title.** This may sound simple and kind of silly to have it as a step, but the title of a paper can tell you what species/cells were used, what technique was used, what was found, and/or what the research aims were. Sometimes, by this information alone, you can decide if a paper is useful or relevant.

Step 2: **A scientist will then look at the authors and whom they are affiliated with.** This step can help to give credibility to the article or to give you a better idea of what the paper is about. If after reading X amount of journals on CF, you may know that Dr. Smith is an expert on only one aspect of CF or perhaps Dr. Smith works at Harvard, his lends credibility to his/her work. Scientists read slowly and carefully when they determine if the paper is important and/or done by outstanding people.

Step 3: **A scientist will now read critically read the abstract.** At this point, a scientist would read the abstract and glean as much information from it as possible such as the research question, the hypothesis, the predictions, the methods used, the outcome, and how it is interpreted (in other words, what does all of it mean?). All of these aspects are not always apparent or present in every abstract as each journal has different requirements in layout. A great abstract will often share the most important findings and data.

Step 4: **A scientist will read the Introduction if not familiar with the topic.** If the subject that this journal focuses on is not one that you are familiar with, reading the introduction is a way to quickly get yourself up to speed. Perhaps you are familiar with the topic then read the introduction until you catch yourself shaking your head in confirmation or going "Yea, I know this."

Step 5: **A scientist will then go on to look at the figures and tables.** Using the figure and table legends, which are usually pretty extensive and descriptive, a scientist will try to discern what is shown in each figure including what it means in terms of the research aims and interpret data presented in tables. If you come upon something you do not understand by just reading the legend, proceed to Step 5.

Step 6: **A scientist will then read the text to clarify.** When a scientist finds that s/he does not understand a figure or table and needs further explanation, s/he will locate where that figure is referenced in the text and read that portion for clarification. S/he will first just scan the Results section to find where that figure or table is cited e.g. "(Figure 3)".

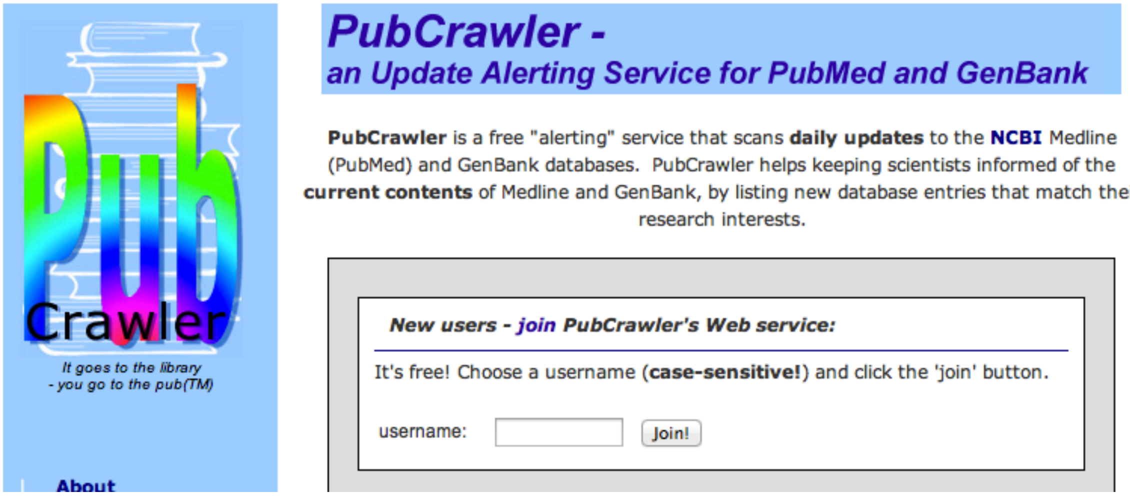
Step 7: **<u>When relevant, a scientist will finally read the discussion.</u>** After reading the title, looking at the authors and their affiliates, critically reading the abstract, and understanding the figures, you may wonder what they concluded from all of that, what they think went wrong/can be fixed in the future, or where they plan to take their research in the future. If this is the case then read the discussion/conclusion.

What information you gain or want from a paper will vary depending on your research needs at that point in time. If you are looking for a primer sequence, then you may skip Step 4 and read the Methods sections to see if their sequence is listed. As you learn how to read papers, you will also learn how to effectively customize the process depending on the occasion.

** Disclaimer: If asked to read a research paper for a class, it is probably best not to only employ this method.

Primer 12: How to use Pubcrawler to find research papers

By: Candace R. Igert



PubCrawler -
an Update Alerting Service for PubMed and GenBank

PubCrawler is a free "alerting" service that scans **daily updates** to the **NCBI** Medline (PubMed) and GenBank databases. PubCrawler helps keeping scientists informed of the **current contents** of Medline and GenBank, by listing new database entries that match their research interests.

New users - join PubCrawler's Web service:

It's free! Choose a username (**case-sensitive!**) and click the 'join' button.

Pubcrawler is a free service that scans NCBI Medline (PubMed) and GenBank publication databases for updates on new published papers on a daily basis. A user can select keywords related to his/her interests and it will search for all new related papers each morning and send an alert via email when a scan comes back with found papers. To get started, go to **http://pubcrawler.gen.tcd.ie** on an Internet browser.

Then **choose a username** and click "**Join!**" You will then be taken to the New User Registration Page where you will be required to **fill in information** such as a password selected by you, and an email address for the alerts to be sent to. Next click "**Register!**"

It will then congratulate on your registration. Below that box is another one titled "**New PubMed query**". This is where you can create a "query" or refined search for articles based on search terms. Each query can be given an alias or name to allow you to have multiple different searches. **Type in a term(s)**, **select a search field** (which is where you want the term to be searched for), and if you would like to add another term, select a connector.

To start make a query for CF papers using keywords: "cystic fibrosis" or "CFTR." Once you group decides on a mutation to focus on, create a daily query for papers related to it.If you want to have more search terms than the allotted slots, towards the bottom of the page, you can change the number of terms you wish to search for. When you have finished with the terms, fields, connectors, and have the number of terms you want searched for, click "**Finish!**"

Happy Paper Searching!

Primer Design

(in this case the word *primer* means "oligonucleotide")

Introduction to “Primer Design” for PCR

Jayme Olsen

Oligonucleotides, also referred to as primers, are short single strands of nucleic acids that are synthesized from either DNA or RNA in order to bind to a complementary strand. Primers have a target area where they bind and act as the starting point for polymerase to extend from, and thus determine what segment of DNA gets amplified. DNA consists of a double stranded helix. One strand of the DNA is named the “sense” strand and the other strand is the “anti-sense” strand. These two DNA strands are complements of each other. During PCR, the denaturing step will break the hydrogen bonds, separating the two strands. This allows the primers to anneal to the target region on the DNA during the annealing step. One primer is designed to anneal to the sense strand and the other primer needs to bind to the anti-sense strand.

When designing primers for PCR it is necessary to take into consideration things like: how many primers are needed, the length of the primer, the 5’ and 3’end, the mutation location in primer, the primer melting/annealing temperature, the G-C content, “primer dimmer” and the distance between the forward and reverse primers.

How Many Primers?

When ordering oligonucleotides for your particular CFTR mutation 3 or 4 primers should be used. Since experiments often fail you cannot design a good PCR diagnostic test where *failing* (a negative result) is considered a dependable diagnosis. You don’t want to tell the parents with a baby who might have CF: “We didn’t get a band on the gel so she maybe doesn’t have CF, or we just screwed up the gel.” Your goal is to design an assay that can diagnose either: (i) if the mutation *is* present by seeing a band on the gel (ie getting a positive result) or (ii) if the normal DNA sequence is present you can see a different band on the gel. If you attempt to make only 3 primers: The wild-type primer could anneal to the anti-sense strand if the mutation is not present on the DNA. The mutant primer could be identical to the wild-type primer, annealing to the anti-sense strand, but with the mutation sequence that will allow it to only anneal if the mutation is present in the DNA. The reverse primer could then be the same for both the wild-type and mutant primer. It will anneal downstream in the opposite direction on the sense strand. With three primers the bands are the same size on the gel, if you use 4 primers you can also design the experiment so two bands of different lengths/sizes show up on the gel.

Length

The length of the primers need to between 15 and 30 base pairs so that they are long enough for adequate specificity and short enough for them to anneal to the DNA template.

The 5’ and 3’end

The primers need to be designed so that the 3’ end of the forward primer will extend toward the reverse primer. The 3’ end of the reverse primer need to also extend toward the forward primer. The 3’ ends of the forward and reverse primers should be facing each other from opposite DNA strands. This will facilitate the continued replication of the desired strand of DNA. If, for instance, the 3’ ends do not elongate in opposite directions (i.e., toward each other) replication will not work and a PCR product will not be obtained.

Mutation Location

The best way to distinguish the genotype is to put the mutation on the 3' end of the primer. Placing the mutation closer to the 5' end of the primer may allow for hairpins to occur, where the primer skips over the mutant base pair and will re-anneal around it.

Primer Melting Temperature (pretty much the same as the annealing temperature)

The Primer Melting Temperature (Tm) is important for the annealing phase of PCR. Preferred temperatures should be between 50°C and 65°C. The forward and reverse primer melting temperatures should be no more than 2° different. To calculate the Tm see the next page on Calculating Annealing Temperatures.

G-C Content

The G-C content of the primer sequence should be relatively high as it has a direct relationship with the Tm. There should be a base composition of G-C of about 50%-60%. The 3' end of the primer should finish with at least one G or C to promote efficiency in annealing due to the stronger bonding.

Distance between the Forward and Reverse

The forward primer and the reverse primer should be between 300 and 2,000 base pairs apart. This distance determines how big the band will be in your gel. Larger bands are easier to see. If they are too close, the amplified region the product will be too small and run off the gel and if they are too big, the product will not make it out of the well. Refer to Ch. 20 in your book.

Beware of "Primer Dimer"

Primer Dimer is an artifact of PCR where primers bind to each or to themselves other instead of the template DNA and thus act as their own template to make a small PCR product and appear faintly on an electrophoresis gel. To avoid "primer dimers", be sure there are not many complementary areas in the base sequence of your forward and reverse primers where the primer strands would be able to bind to each other instead of the gene.

Things to Avoid

- To avoid non-specific binding, design the primers with high annealing temperatures.
- To make sure the primers designed will only bind to the target area submit the sequence to the BLAST website.
- The $MgCl_2$ and pH conditions can also be adjusted for improved amplified product.
- Watch out for runs of singles bases of G's, C's, A's, and T's when developing primers because they can allow mis-priming.
- Keep in mind that the more nucleotide bases that the primer is made up of, the more expensive they are. The shorter the primers are, the less specificity they have in PCR.

Introduction to Calculating PCR annealing temperatures of oligonucleotide primers

The most crucial factors that need to be optimized in a PCR reaction are the magnesium concentration, enzyme concentration, DNA concentration and annealing temperature of the primer. The G+C content of the primers should generally be 40-60% and care should be taken to avoid sequences that produce internal secondary structures as well as “primer dimer” where primers bind to each other. The annealing temperature for a PCR cycle is generally 3-5 degrees Celsius below the melting temperature (Tm) of the primer. There are several formulas for calculating melting temperatures. In all cases these calculations will give you a good starting point for determining appropriate annealing temperatures for PCR primers. The exact optimum annealing temperature must be determined empirically, however. There are numerous websites that help with primer design and annealing temperature calculations, search for them. Here's Promega’s website at http://www.promega.com/BioMath.

Basic Melting Temperature Calculations

1) The simplest "rule of thumb" formula is as follows:

Tm=4°C x (#G’s + C’s in the primer) + 2°C x (# A’s + T’s).

2) This formula is valid for oligos of less than 14 bases and assumes that the reaction is carried out in 50mM monovalent cations. For longer primers the formula is modified.

Tm= 64.9°C + 41°C x (number of G’s and C’s in the primer -16.4)/N

Where N is the length of the primer. For example, Promega’s T7 promoter primer (TAATACGACTCACTATAGGG) is a 20-mer that has 5 T’s, 7 A’s, 4 C’s, and 4 G’s. Thus, its melting temperature would be:

64.9°C + 41°C x (8-16.4)/20= 47.7° C

3) A third formula calculates the Tm with salt concentrations taken into consideration:

Tm = 81.5 +16.6 • (log10[Na+]) + 0.41 • (%G+C) – 675/n

Where [Na+] is the molar salt concentration ; [K+] = [Na+] and n = number of bases in the oligonucleotide primer.

Other useful formulae are:

- Nanogram of primer = picomole of primer x 0.325 x # bases
- MicroMolar concentration of primer = picomoles of primer/ volume (µL) in which the primer is dissolved.

ADVANCED APPROACH: Introduction to The Yaku-Bonczyk Primer Design Method

Vincent Cracolici

The What?

The Yaku-Bonczyk method is an advanced protocol by which primers can be designed in order to increase PCR stringency as well as decrease the chance of false positives or negatives seen in a gel. In 2008, Yaku et al presented this design method; it was then further investigated by LB 145 student Sarah Bonczyk in spring 2009. By slightly altering the "classic" 3' single base pair difference between wild-type and mutant primers, a research team can drastically increase primer discrimination against nonspecific binding. Similar results were shown by Wittwer et al (1993).

How does it work?

The standard method of primer design for a genetic mutation, like one on CFTR, typically involves two forward primers which are identical save for the base pair nearest the 3' end: one primer is complementary to wild-type DNA and the other to mutant DNA. However, the single base-pair mismatch between these two primers is often not enough to ensure that the wild-type primer will not anneal to and extend mutant DNA, and vice versa.

The Yaku-Bonczyk method differs from the standard because the primers are designed to better discriminate against non-complementary DNA by always incorporating an intentional mismatch into the primer. The Yaku-Bonczyk method involves the most 3' base pair of each forward primer again being complementary to the mutant/wild-type DNA it is seeking, the second base pairs in are designed to always anneal to either type of DNA, and the third base pairs in are designed as an intentional mismatch that will never anneal to either type of DNA (see illustration).

If a primer and its complementary DNA strand anneal to each other, the single mismatch three base pairs in from the 3' end is not enough to prevent extension and will result in only a small hairpin in the sequence. Additionally, if a primer and a non-target DNA strand anneal to each other, the complementary match at the second base pair from the 3' end of the primer is not strong enough to pull the two strands together and is unlikely to allow for extension. Therefore, nonspecific binding is decreased.

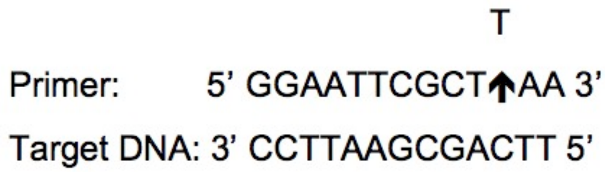
Points to Ponder…

-The intentional mismatch that exists on all the primers three base pairs in from the 3' end provides an excellent opportunity to boost your primer's G/C content.
-Should this intentional mismatch still be included in the calculations of annealing temperatures?
-Remember to consider purine/pyrimidine interactions with themselves and each other in designing the intentional mismatch.

The Yaku-Bonczyk Method

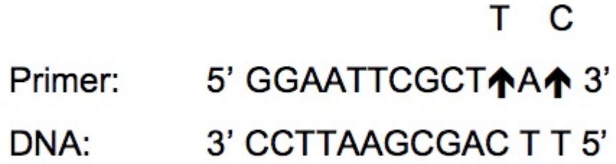
Primer: 5’ AACGTGGTC**XYZ** **3’**

X: Should be designed to **NEVER** anneal to mutant **OR** wild-type DNA
Y: Should be designed to **ALWAYS** anneal to mutant **AND** wild-type DNA
Z: Should be site specific: anneal to **EITHER** mutant **OR** wild-type DNA



```
                                    T
Primer:      5’ GGAATTCGCT↑AA 3’
Target DNA: 3’ CCTTAAGCGACTT 5’
```

A primer designed with the Yaku-Bonczyk method will anneal to and extend target DNA despite the intentional mismatch. The force of the single repulsion will not hinder the primer as a whole.



```
                                    T   C
Primer:      5’ GGAATTCGCT↑A↑ 3’
DNA:         3’ CCTTAAGCGAC T T 5’
```

A primer designed with the Yaku-Bonczyk method will not anneal to nor extend non-target DNA as a result of the two mismatches. The attraction at the second base pair in on the primer is not enough to allow for extension at the 3’ end.

ADVANCED APPROACH: Mutation Construction through Site-Directed Mutagenesis

Mitchell Wood

Introduction:

PCR is a powerful tool in molecular biology, specifically for genotypic identification of a given sample of DNA. Some novel mutations in the CFTR gene have only been noticed in a few patients; therefore the number of DNA samples with that genotype is limited. Genetic tests that are generated should cover all mutations known in the CFTR gene, including these novel ones. But to test for these rare mutations positive controls must be found, or generated, to experiment upon before the test is given to a patient. As an alternative to contacting researchers across the globe for positive control samples, a relatively simple alternative is to use PCR to replicate DNA with this rare mutation. This process is called Site-Directed Mutagenesis, which in principle uses imperfect stringency in primer annealing to direct a mutation into the replicated DNA.

Methods:

The length of the primer with the forced mutation is the foremost limitation of the replicated DNA. When the primer anneals and is replicated with the intentional mismatch, the resulting PCR product will begin with the 5' end of the primer. Therefore, the length of the primer used in the allele specific positive control test can not exceed the length of the site-directed mutagenesis primer. However, the length of the allele-specific primer must not be too short (under ~18 base pairs) otherwise it is more probable for non-specific binding on non target DNA. To minimize the complications that come with a lengthy primer, the forced mutation can be placed as close to the 3' end of the oligo as possible in order to leave the remaining length to fit the allele specific primer. Refer to Yaku et al. (2008) for ideas and clarification.

1. Design allele specific primers.
2. To design mutagenic primers, add several nucleotides to the 3' end of your allele specific primers (the exact number should be determined by Yaku et al (2008)).
3. Predict the sequence of the PCR product and confirm that it is the one you want.

5'-TAC ACG CCC AAG TAC GGT TCC ACA-3' →Primer with mutation
3'-CCG TCG ATG TGC GGG TTC ATG CCA AAG TGT CTG-5' →DNA Template

Replication with above primer will yield a new DNA template with the forced mutation, but the DNA segment will only be the length between the forward and reverse primers from above. Therefore the direction of the replication in the next ASPCR will have to be closely watched.

5'-TAC ACG CCC AAG TAC GGT TG-3'→ASPCR Primer using Yaku Method.
3'-ATG TGC GGG TTC ATG CCA ACG TGT CTG-5' → Complement to primer with forced mutation.

ORDERING: When do we order our DNA primers?

Please prepare to bring all your DNA primer sequences to lab at the start of Week 4.

We'd like to help check over the DNA sequences each group has designed and plans to order for their PCR primers during Week 4 in lab (***before** you order them). For both sets of primers, see #1 & 2 below, bring the DNA sequences you created and be prepared to draw in chalk on your benchtop both the genome/gene sequence (ie. template) and where the primers will bind to appropriate complementary sequences during the annealing phase of PCR.

1. Show us your set of two "control" PCR primers: These are DNA primer sequences that you have obtained from a published research paper where the authors designed primers to diagnose the same mutation as the one your group chose on the CFTR gene. Bring the original paper in hard copy and bring the full DNA sequence of the CFTR gene and DNA sequence of the primers that target your mutation (bring on your computers). Your group is expected to order these two primers first after getting feedback from TAs/Luckie. Then you will attempt to repeat the Methods provided by the authors of the paper to make their primers/their assay work in your hands. Scientists do this quite frequently.
2. Show us your set of "designed" primers: Please also bring your set of designed primers that your group has custom-designed to be used to diagnose your specific mutation. These are primers your group created on your own and are not published anywhere.

NOTE: Once your group successfully gets your control primers to work in your experiments (above in #1), and presents evidence in your gel photos and graphs (see Primer 7) to support it, then your group may order your set of designed PCR primers and turn your focus to completing the final experiments necessary to create you own custom diagnostic assay.

We also ask that all primers be shipped to one address (see below). That way we can track for sure and know if the primers have arrived or not.

All oligonucleotides/primers should be shipped to this address:

Fernando Rosado (for Student Name-LB145)
Lyman Briggs College-MSU
919 E. Shaw Lane, E35 Holmes Hall
East Lansing, MI 48825-1107

Writing Information

Instructions to Authors

Follow these instructions or your paper will be returned to you, and incur late penalties.

One of the learning objectives of your research project in the course is to develop your scientific writing skills. In science, writing is the most important means of communicating research findings. Major scientific findings are rarely kept secret. Instead, scientists share their ideas and results with other scientists, encouraging critical review and alternate interpretations from colleagues and the entire scientific community. In most cases, scientists report the results of their research activities in scientific journals in a standard written format. In this course, you will practice writing using this same standard scientific format and style.

4.0 TIP: Write like a scientist. Write your papers so that anyone who reads your manuscript could not tell it was from a student, but assumes it must have been from a scientist in a lab at MSU.

A scientific paper includes the following: a **TITLE** (statement of the question or problem), an **ABSTRACT** (short summary), an **INTRODUCTION** (background and significance of the problem), a **METHODS** section (report of exactly what you did), a **RESULTS** section (presentation of data), a **DISCUSSION** section (interpretation and discussion of your results), and **REFERENCES** (books and periodicals used). Data is also represented by **FIGURES** and **TABLES**.

Throughout the laboratory, you will practice scientific research and writing. Your papers will be reviewed by the course professor, TAs, and your peers in order to point out your areas of weakness and make suggestions for future improvements. By the time you have completed the course, you will have submitted the equivalent of two full scientific papers. If you are not certain about the level of independence and what constitutes plagiarism in this program, ask your instructor to clarify the class policy. ***Plagiarism will not be taken lightly and will be evaluated by instructors and software at turnitin.com. See syllabus for more info.***

Predictions: Science is not about explanation, but in fact it is about the ability to predict. All scientists must have models or hypotheses that can be used to then make predictions of what will occur. Thus prediction is a very important part of writing you papers. As a result in your early DRAFT1 and DRAFT2 in many cases your predictions may be all the data you have on a certain experiment and thus they should be well supported by papers from the literature. Use future tense when discussing Predictions. In general whether it's in the Abstract, Introduction, Results or Discussion, whenever you discuss something that you predict it should be in the format of:

"We predict..[what].. because..[rationale].. (citation of paper)."

**Note: A hypothesis is different than a prediction. The hypothesis is the model that explains how you believe things are working (e.g. we hypothesize electricity sparks gas in the cylinders of a car engine) while the prediction is what you think should happen during the experiment (e.g. if hydrogen gas is injected into the cylinder of a functioning engine we predict there should be a significant explosion).

Overview of Manuscript Sequence & Format
(This is what professional journals expect)

1. Page one is the Title Page (≤100 characters in title)
2. Page two will have ONLY the Abstract (≤250 words)
3. Page three will start with the Introduction. The Methods, Results, and Discussion sections can follow without starting a new page for each one (although you may start a new page if you are near the bottom of the current page). Clearly label each section with the section headings (ex. Introduction) and who authored it (Written by: Jill Sanders, Revised by: Bob Roberts).
4. The **Introduction** will provide the reader with the background information necessary to understand the rest of the paper.
5. **Methods** section will list materials used (Bought Vitamin C with Rose hips from the General Nutrition Store (GNC) 324 E. Grand River East Lansing MI) how stock solutions were prepared and explain exactly what you did in your research. After reading the Methods section, an incoming student should be able to repeat your work. Reference the original protocol.
6. The **Results** section follows Methods. This section will clearly and succinctly state what you observed upon performing each experiment.
7. The **Discussion** section follows Results. In this section you will discuss the significance of results and how your results relate with research performed by others.
8. The **References** section follows the Discussion. This is a list of the references cited within the paper.
9. Start a new page with the **Figures** section after the References. Figures will be sequentially numbered in the order that they were cited in the Results section (figures are most always cited ONLY in the Results section, not in Methods, not in Discussion). One figure per page with extensive figure legend paragraph ONLY at the bottom of the figure. The first sentence of a figure legend is its title. Follow the title with sentences explaining the figure as if someone did not have the Results section or in fact any other part of the paper available as a reference.
10. The **Tables** section will follow the Figures section. Tables get a title ONLY on the top with some explanation. Tables will be sequentially numbered in the order that they were cited in the Results section (Tables are most always cited ONLY in the Results). One table per page.
11. Figures and tables MUST be created on a computer unless otherwise instructed.
12. After the Tables section, a single white page will follow entitled, **Appendix**. Then append any laboratory notebook pages that indicate signed data for all members (photocopies from your notebook) and a photocopy of the first page of any articles cited and referenced in report.
13. Double space or 1.5 space typeface is required. Preferred font size is 12 point.

***Once returned, rejected papers (like late papers) lose one point in the first 24 hrs grace period but then the penalty becomes more severe: 10% off for 2 days late, 20% off for 3 days, and so on. After 5 days, you will receieve a "0". Unlike late papers, a rejected paper also loses 1 pt per rejection.**

A more detailed description of each section of a scientific paper follows also, review the published papers provided in the course packet for examples. As you write your paper, clearly label each section (except the title page), placing the title of the section on a separate line, centered, bold, but not underlined (like shown below).

Title Page and Title

The title page is the first page of the paper and includes the title of the paper, your name, the course title, your lab time, your lab instructors' names, the due date for the paper, and your groups' website address. The title should be as short as possible and as long as necessary to communicate to the reader the question being answered in the paper. Consider the following titles for a paper that describes the molecular mechanism of an antiviral drug.

1. "Inhibition of Mengovirus Replication by Dipyridamole"
2. "Antiviral Action of Dipyridamole"
3. "A Study Examining the Inhibitory Effects of the Drug Dipyridamole on Mengovirus Replication"

Title 1 is short and communicates the question being investigated. It conveys the mechanism of action (inhibition of replication), the name of the virus being inhibited (Mengovirus), and the name of the drug doing the inhibiting (Dipyridamole). Title 2 is short but too vague for the reader to know the subject matter of the paper. Title 3 is too long. The words "A Study Examining" are superfluous, and "Drug" and is redundant.

Place the title about 7 cm from the top of the title page. Place "by" and your name(s) in the center of the page, and place the course title, lab time, lab instructors' names, due date, and your groups' website address, each on a separate centered line, at the bottom of the page. Leave about 5 cm of white space below this information.

Abstract

The abstract is placed at the beginning of the second page of the paper, after the title page. The abstract summarizes the question being investigated in the paper, the methods used in the experiment, the results, and the conclusions drawn. The reader should be able to determine the major topics in the paper without reading the entire paper. As mentioned previously, predictions are an essential element of science and thus should appear in the Abstract of DRAFT1 and DRAFT2 and in the format: ***"We predict..[what].. because..[rationale].. (citation of paper)."***

Introduction

Start the introduction on page three. The introduction should generally be short, only 4-5 paragraphs in length and focus are background information of the following types:

1. Describe the question and hypothesis being investigated and background on the importance of the topic.
2. Review the background information that will allow the reader to understand the purpose and topics of the paper. There is usually a paragraph on the specimens studied, also one that provides evidence to support the hypothesis posed. A hypothesis is an educated guess; the Introduction should provide the "education." Include only information that directly prepares the reader to understand the question investigated. Most of this information should come

from outside sources, such as scientific journals or books dealing with the topic you are investigating.*

3. In a paragraph state background information on the methods chosen to investigate the hypothesis. Explain how these methods will address the question and describe the predicted outcomes. Why were they chosen?
4. In the last paragraph briefly state a hint of the results and conclusions of the investigations (or predictions). This generally comes only at the very end of the Introduction.

*All sources of information must be referenced and included in the References section of the paper, but the introduction must be in your own words. ***No "quotations" are permitted in any part of the paper.*** Refer to the references when appropriate. As you describe your investigation, include only the question and hypothesis that you actually investigated. It is a good idea to write down each item (question, hypothesis, supporting evidence, prediction) before you begin to write your introduction.

Write the introduction in past tense when referring to elements of your experimental investigation that are completed. When relating the background information, use present tense when referring to another investigator's published work. Use future tense when discussing Predictions.

Methods

The Methods section describes your experiment in such a way that it may be repeated exactly. Make the Methods professional just like in published papers, but target as your audience a student in LB-144. The majority of the information in this section comes from the Procedures or Protocols section of the Laboratory Guide and in your paper, this information should not be a list of steps. Write the Methods section in a paragraph format in past tense. Be sure to include levels of treatment, numbers of replications, and control of treatments. If you are working with living organisms, include the species and the sex of the research organism. Do not include failed attempts unless other investigators may wish to try the technique used. Do not try to justify your procedures in this section of the report.

If you describe an experiment from the lab guide, unless instructed otherwise, you may simply refer to the procedures listed in the guide (and page numbers). Under those circumstances, your Methods section should point out changes in procedure that are not indicated in the Lab Manual. When writing a full Methods section (with no reference to the Lab Guide), write these procedures concisely, but in paragraph form. The difficulty comes as you decide the level of detail to include in your paragraphs. You must determine which details are essential for the investigator to repeat the experiment. For example, if in your experiment you incubated potato pieces in different concentrations of sucrose solution, it would not be necessary to explain that the pieces were incubated in plastic cups labeled with a wax marking pencil. In this case, the molarity of the sucrose solutions, the size of the potato pieces and how they were obtained, and the amount of incubation solution are important items to include

Results

The Results section consists of two components: (1) one or more paragraphs that describe the results of each experiment/test and include the actual data with observations, specific numbers [and units] and math, (2) reference to figures (graphs, diagrams, pictures), and reference to tables.

In referencing figures, remember to number figures and tables consecutively in the order that they are mentioned in this section. Refer to figures and tables within the paragraph as you describe your results, using the word Figure or Table in parentheses, followed by its number, for example, "(Figure 1)." Avoid citing a figure with a full sentence or statement such as, "please see figure 1 for graphed data points" or even just "please see table 2." DO NOT place each figure or table at the end of each paragraph in which it is cited. Place figures and tables after the References section. If you have performed a statistical analysis of your data, such as chi-squared, include this data in the Results section. Explain what calculations you did and the result and direct the reader to the Methods section for more details. Then show an example of the results and refer the reader to a table with all the data.

The most common error a student makes is only making general comments and not including actual data (ie the results) in the Results. Results should include detailed observations (what did you see: odor, texture, etc.) and specific findings (what did you record: 15 grams, 20 degrees C, 20% increase, 32,000 cells etc.). Report your data as accurately as possible in the order that they happened. It is important to have separate paragraphs and topic sentences that introduce the results of each test but do not spend much time discussing the meaning of your findings, save that for the Discussion.

For "***Predicted Results***" - which will be the only focus of Draft 1 and will be included in subsequent drafts - these are what you predict or expect will happen, and these predictions must be based upon the primary literature you have gathered throughout your research on your topic/thesis. When including predictions follow this format: ***"We predict..[what].. because..[rationale].. (citation of a published paper or source)."***

Discussion

The Discussion section is where you will analyze and interpret the results of your experiments. The Discussion should show a possible relationship between observed facts—those observed by you and those observed by others and reported in published research papers. Write as clearly and succinctly as possible. A good Discussion will include the following:

1. Summarize the Introduction and restate the question and hypothesis being addressed.
2. Briefly summarize the results of the experiments. Do not include details regarding methods.
3. Interpret the results. Explain how the results answer the questions posed. State whether your results support or refute your hypothesis. Do not use the word "prove" in your conclusions. Your results will support, verify, or confirm your hypothesis. They also may negate, refute, or contradict your hypothesis. The word prove is not appropriate in scientific writing.
4. Discuss how your results and interpretations relate with previously published research. This will require you to cite outside references. Some may come from the Introduction, while you will also find new references that specifically relate to your findings. You can speculate and propose theoretical implications of your work.

5. Describe weaknesses in experimental design or technical difficulties that arose during the research. Explain how these problems specifically affected the outcome of the research. Any human errors (spills, etc) discussed should have been then corrected by certain steps.
6. Discuss experiments that would be performed if the research were to be continued. Explain how those experiments would contribute to answering the questions addressed by the research.

Figures

All figures should be computer generated. The format of the figure will depend on the type of data collected. Your figures will include mostly photographs and graphs. The photos and graphs must be done in a professional manner and include computer generated labels when appropriate, and always with only one figure per page. Under each photo or graph, there must be a legend paragraph. The legend paragraph will include the Figure number, a title sentence, and a description of what was done in the experiment and shown in different labeled parts of the figure. A reader must be able to understand the general concept of the experiment performed without reading the Methods section. In fact if a student from LB-144 picks up a single piece of paper with one of your figures on it, they should be able to explain to you what that experiment is about from the legend.

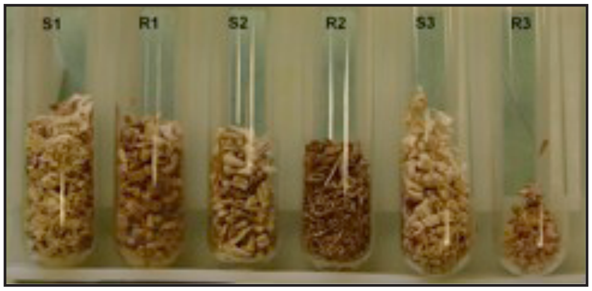


Figure 1. Preparation of corn root and stalk samples for sugar analysis. Samples were cut into units no greater than 4 mm3 using a surgical scalpel. Three stalk samples of 70 grams each and three root samples of 80 grams each were prepared. Root 1 (R1), Root 2 (R2), Root 3 (R3), Stalk 1 (S1), Stalk 2 (S2), and Stalk 3 (S3).

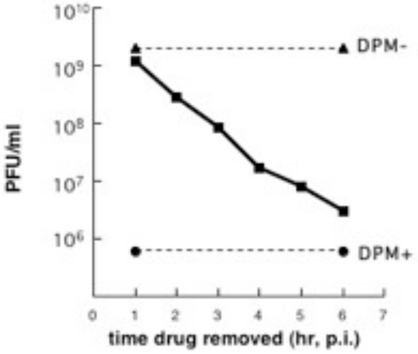


Figure 2. DPM and Virus Yield. HeLa cell monolayers were infected with Mengovirus at a multiplicity of 50 pfu/cell. DPM+ samples had 80 μM DPM (in ethanol) added to the media at the time of infection. DPM- samples were dosed with an equivalent volume of ethanol. Medium from DPM+ cultures was exchanged with drug-free medium at the indicated times. Virus was harvested at 8 hrs PI and the titer determined by plaque assay.

Tables

While Figures are often used in papers (graphs, photographs, gel images) tables are rare. Tables should only be used when all the data being presented cannot be reported in a simple and comprehensible manner in the Results section. The title appears at the top of the table; there is no legend. A footnote may be necessary to clarify an important point in the table.

Table 2. Mengovirus plaque phenotypes in the presence of DPM.

Concentration, µM DPM	Plaque Reduction (%)[a]	Relative Plaque Size[b]
80	100	N/A
60	98	minute
40	93	+
20	68	++
10	25	++
0	0	++++

[a] Values represent the average of two experiments each done in triplicate.

[b] Plaques in the absence of DPM averaged about 2mm in diameter.

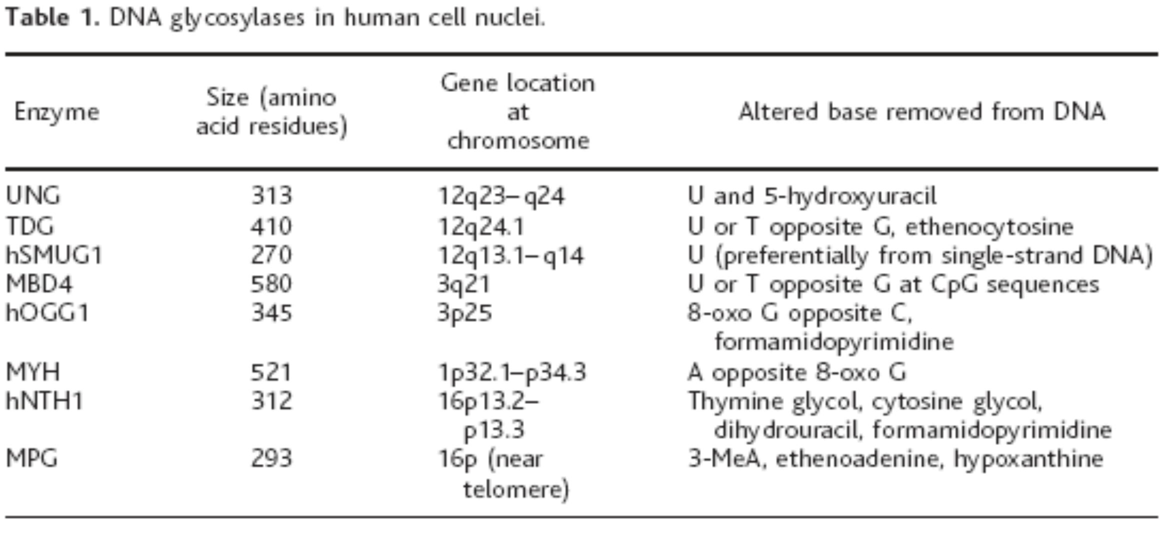


Table 1. DNA glycosylases in human cell nuclei.

Enzyme	Size (amino acid residues)	Gene location at chromosome	Altered base removed from DNA
UNG	313	12q23–q24	U and 5-hydroxyuracil
TDG	410	12q24.1	U or T opposite G, ethenocytosine
hSMUG1	270	12q13.1–q14	U (preferentially from single-strand DNA)
MBD4	580	3q21	U or T opposite G at CpG sequences
hOGG1	345	3p25	8-oxo G opposite C, formamidopyrimidine
MYH	521	1p32.1–p34.3	A opposite 8-oxo G
hNTH1	312	16p13.2–p13.3	Thymine glycol, cytosine glycol, dihydrouracil, formamidopyrimidine
MPG	293	16p (near telomere)	3-MeA, ethenoadenine, hypoxanthine

Reference Citation Formatting

A References section lists only those references cited in the paper. You will cite all the references you used when you wrote your paper. In the text of the paper, cite the references using the author's name and publication year. If there are two authors you must state both of their names if there are greater than two authors, state the first authors name followed by et al.

For example: We predict that the 95% alcohol solution will kill all of the NIH-3T3 cells in our culture flask because Smith performed a similar experiment in his paper with another cell type (Smith et al, 1998). In previous research the p58 protein was associated with increased cell growth in C127 cells (Johnson, 2001; Benenson and Kortemeyer, 2003; Haenisch et al, 2006).

Types of Literature:

The vast collection of scientific literature can be generally divided into three categories based on how 'close' they are to the original experiments and descriptions of scientific phenomena. **1) Primary literature**: The bulk of scientific journal articles are primary, meaning that they report the findings of specific experiments or descriptive studies. **2) Secondary literature**: From time-to-time investigators write review articles or books that summarize what is and is not known about a particular topic. Rather than conducting new experiments, these authors rely heavily on the primary literature, therefore these review articles and books are considered a part of the secondary literature. **3) Tertiary literature**: More general texts that summarize what has been reported in review articles comprise the tertiary literature.

Most new research relies heavily on previous work reported in primary literature. However, review articles can be extremely helpful in understanding how your research project fits into the larger scope of scientific investigation, and can be used as a source to locate primary literature references for the topic of interest.

Note that websites were not included in the above description of scientific literature sources. This is because they are not refereed — that is, just about anyone can publish something on the web without some impartial reader reviewing it beforehand. Web pages are often wonderful sources of information, but they can just as often be replete with bad information. At this point, it is very difficult to determine the reliability of web sources and, in general, they should generally only be used as a starting point about a particular topic. ***Thus websites are only allowed as citations in DRAFT1 manuscripts.***

Examples of Proper Citation Formatting for the listings in your Reference section:

Journal articles:

Single Author:

Belsky, A. J. 1986. Does herbivory benefit plants? A review of the evidence. American Naturalist 127: 870–892.

Two Authors:

Brown, J. H. and D. W. Davidson. 1977. Competition between seed-eating rodents and ants in desert ecosystems. Science 196: 880–882.

Multiple Authors:

Free, C. A., J. R. Beddington, and J. H. Lawton. 1977. On the inadequacy of simple models of mutual interference for parasitism and predation. Journal of Animal Ecology 46: 543–554.

If the source was published in an online journal do not cite the URL, treat it the same as printed:

Thomas, J. A., M. G. Telfer, D. B. Roy, C. D. Preston, J. J. D. Greenwood, J. Asher, R. Fox, R. T. Clarke, and J. H. Lawton. 2004. Comparative losses of British butterflies, birds, and plants and the global extinction crisis. Science 303: 1879-1881

Books:

Chapter within a book:

Goldberg, D. E. 1990. Components of resource competition in plant communities. Pp. 27-50 in J. B. Grace and D. Tilman, eds., Perspectives on Plant Competition. Academic Press, San Diego.

An entire book:

Hynes, H. B. N. 1970. The Ecology of Running Waters. University of Toronto Press, Toronto.

Theses:

Watson, D. 1987. Aspects of the population ecology of Senecio vulgaris L. Ph.D. thesis, University of Liverpool.

The textbook:

Campbell NA. and Reese JB. 2007. Biology – 8th ed., Chapter 13 "Mendel and the Gene". Benjamin Cummings, CA.

The Lab Manual:

Igert, et al. 2013. LB145 Course Pack. MSU Printing Services, East Fee Hall. Michigan State University, East Lansing, MI

A Web Site: [only allowed in DRAFT1 manuscripts and follows a text citation (Author(s), Year published).]

Anonymous. 2002. Wisconsin Fast Plants Web Site. http://www.fastplants.org /Introduction/ Introduction.htm, last accessed 7/10/02

References (used in the creation of this appendix):

McMillian, V.E. 2001. Writing Papers in the Biological Sciences, 3rd ed. St. Martin's Press, Inc., New York.

Appendix

Note everything you place in your appendix will be discarded during grading.

An Appendix section includes only materials that are not actually required in the paper and could be thrown away with no effect to the paper. Often special extra information is included here. In LB-145 you are expected to place any laboratory notebook pages that indicated signed data for all members (photocopies from your notebook) and a photocopy of the first page of any articles cited and referenced in the report.

Reminders

Before writing your paper, refer to the following hints to make your paper stronger:

1. Write clearly in short, logical, but not choppy sentences.
2. Use past tense in the Abstract, Methods, and Results sections when discussing things that have been completed. Also use past tense in the Introduction and Discussion sections when referring to your experiment. Use future tense when making predictions about future experiments.
3. Write in grammatically correct English, but use METRIC UNITS.
4. When referring to the scientific name of an organism, the genus and species should be italicized, the first letter of the genus is capitalized, but the species is in lower case; for example *Drosophila melanogaster*.

Supplemental Requirements: Final Research Paper

Here are special requirements for your final paper in addition to those listed in the *Instructions to Authors*.

TIP: Please re-read the "Instructions to Authors" (I to A) and carefully review the requirements of the research project to raise your score.

These are requirements specific to the final draft of your paper: (Title Page, Methods and sections not mentioned are unaffected).

- **Introduction** section is limited to 3 pages (ie. no more than 3 pages long).
- **Abstract** section is expanded to a maximum of 350 words (ie. no longer 250 words).
- **Results** section should be no longer than 2 pages. Should include only actual findings/outcomes of experiments. No predicted results should remain here.
- **Discussion** section must be limited to no more than 4 pages. It can include original predictions and ultimate findings.
- **Future Directions** should be a separate section, 1 page in length, with it's own title.
- **Figures and Tables** are limited to no more than 8 in total (figures and tables combined, e.g. 6 figures and 2 tables; or 1 figure and 7 tables etc). A single figure may have multiple images or graphs but they must be clearly viewable, labeled and well-documented in the one legend at the bottom. No *predicted* figures or tables.
- **Appendix** must include photocopies of pages from your laboratory notebook during the weeks since submission of DRAFT2 (can be from just one member of your group with best notebook). The scientific notebook should look like a personal journal that shows the date, purpose, and methods of each day you visited lab as well as any gel photos, recorded data etc that serve as evidence of all your work (see Course Packet for more info on keeping a Notebook).

⇒ **30 Days projects (not required) may be included as an element of your paper.**

⇒ **Two copies of the final Research Manuscript are required at time of submission**. **One** will have an attached appendix (appendix not bound). Spiral-bound final papers are preferred, yet TAs will still reject poorly prepared papers even when bound thus be sure the paper is complete before binding. **Grading** criteria will be the same as indicated in the rubric unless altered by this document. Utilize the grading sheets to determine if your paper has all the expected elements as well as will receive an excellent score.

⇒ **Website**: Please do not create a website of your own design or by using a web-based company to whip up a website from their templates (don't use weebly.com or wix.com or wordpress.com etc), instead just convert an abbreviated version of your group's final manuscript into a single page html document that will function as a single page website (with an added link to your 30 Days movie). Review details at end of grading rubric precisely describing the website, as well as the information in the course pack on how to use netfiles.msu.edu to move files around and place your website online in your MSU web folder.

Samples: Student Paper

Genotypic Identification of CF Patients with the R553X Mutation using IB3 and S9 Cell Lines and Allele Specific PCR

By: Sarah Bonczyk, Nathan Johns, Elizabeth LeMieux, and Mitch Wood

LB 145 Cell and Molecular Biology
Tuesday 7 PM
Ashley Coulter and Jason Mashni
4/24/2009

http://teamhouselb145.tripod.com/

(Title page written by: Nathan Johns
Revised by: Mitch Wood,
Finalized by: Liz LeMieux)

Abstract

Written by: Nathan Johns
Revised by: Mitch Wood
Finalized by: Liz LeMieux

The R553X mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) accounts for 0.7% of all cystic fibrosis cases (Hull *et al,* 1993). The mutation involves a single base pair substitution in the 553rd amino acid from CGA (Arginine) to TGA, a stop codon, leading to a truncated protein (Bal *et al,* 1991). Allele specific polymerase chain reaction (ASPCR) was used to determine whether samples of DNA are wild-type, heterozygous, or homozygous for the R553X mutation in the CFTR gene. DNA was extracted from epithelial bronchial cells of known CF patients (Qiagen Inc, 2007). We hypothesized that by controlling annealing temperatures and salt concentrations in the PCR reaction, a single base pair mismatch can be used to determine the presence of the R553X mutation, based on previous PCR diagnostic testing (Chavanas *et al,* 1996). The resulting amplified DNA was then analyzed using agarose gel electrophoresis to determine the genotype of the DNA. We were able to determine whether samples were heterozygous, homozygous wild-type, or homozygous for the mutation by the presence or absence of bands 1,056 base pairs long and based on which forward primer used (Wu *et al*, 1989). Research surveys were distributed and analyzed in order to examine different public opinion on genetic testing between students of various studies at Michigan State University. Results showed no significant difference in opinions on genetic testing among various residential colleges. These tests are significant in helping doctors diagnose cystic fibrosis patients for specific mutations faster and more accurately than previous testing.

Introduction

Written by: Nathan Johns
Revised by: Mitch Wood
Finalized by: Liz LeMieux

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The role of the CFTR protein is to serve as a chloride ion channel in epithelial cells (Rowe *et al*, 2008). Epithelial cells with a CFTR mutation, most often in the lungs, pancreas, and intestines, secrete large amounts of mucus, which builds up and creates complications in the affected tissues (Welsh and Smith, 1995). Mucus buildup in the respiratory tract often leads to pulmonary infection, the most common cause of death in CF patients (Golshahi *et al,* 2008). Current treatments attempt to remove this mucus in the lungs and avoid affection, often by using percussive therapy and antibiotics (Welsh and Smith, 1995).

Over one thousand mutations in the CFTR gene are identified to cause CF, with the ΔF508 mutation, a deletion of three base pairs at position 508, being the most common, and accounting for approximately 70% of all cases (Teem *et al*, 1993). The R553X mutation is the sixth most common, accounts for 0.7% of cases, being most prevalent in German communities (Hull *et al*, 1993). R553X is a nonsense mutation caused by a C to T substitution at the 553rd amino acid. This changes what would normally be arginine to a stop codon (Hull *et al,* 1993). A nonsense mutation is a mutation that causes the DNA sequence to result in a premature stop codon, or a nonsense codon in the mRNA which results in a truncated, incomplete and nonfunctioning protein. In the case of R553X, research has shown that the premature stop codon often results in exon skipping in RNA translation (Aznarez *et al*, 2007). The loss of the exon causes an unstable mRNA of the truncated protein and therefore does not undergo the process of translation (Aznarez *et al*, 2007). The R553X mutation is a class I mutation because of the unstable mRNA synthesized in the nucleus caused by nonsense alleles. (Gambardella *et al.* 2006)

Polymerase chain reaction (PCR) is a technique used to amplify a desired section of DNA (Saiki *et al*, 1988). In PCR, DNA is heated during a denaturing step in order to break the hydrogen bonds between nucleotide bases to separate complimentary 5' and 3' strands. Once

Methods

Written by: Liz LeMieux
Revised by: Sarah Bonczyk
Finalized by: Mitch Wood

Primers

Before the PCR tests were run, primers for the PCR tests were designed using the Cystic Fibrosis Mutation Database. Forward and reverse allele-specific primers that worked with both the wild type and with the mutation were needed. FPrimer1 is a forward primer ending in Guanine, complementary to the wild type amino acid where the mutation should be present. The second of the forward primers, Fprimer2, seeks the mutant type base sequence at the mutation site, base pair number 1789, which results in the primer ending in Adenine. The reverse primer, Rprimer, was designed to bind to the DNA strand 1,022 base pairs past the mutation site between base pairs 2828 and 2811 and was used in both tests. Fprimer1 is 16 base pairs long with the sequence of: GACTCACCTCCAGTT**G** and should properly bind to the wild type gene sequence of **C**AACTGGAGGTGAGTC. Fprimer2, the mutant seeking primer, is also 16 base pairs long with the sequence of: GACTCACCTCCAGTT**A**; the only difference from the previous forward primer is the last base pair, which should properly bind to the mutant R553X gene sequence of **T**AACTGGAGGTGAGTC. The reverse primer that will be used for both tests is 18 base pairs long with the sequence of: CATGAGAGAAGAGAC, which should bind to the gene sequence of TCTGTCTCTTCTCTCATG, which is the same in both mutant and wild type genes. All of the primers are written in 5’ to 3’. These primers were ordered from the biological laboratory company Integrated DNA Technologies (IDT).

After the primers were designed, the annealing temperatures at which the primers would bind were determined by first calculating the theoretical melting temperatures of each primer using the following formula:

$$T_m = 64.9° \text{ C} + 41° \text{ C} \times (\text{number of G's and C's in the primer} - 16.4)/N$$

where N is the length of the primer (Wright *et al,* 2009). The calculated theoretical melting temperatures were: Fprimer1- 45.94°C, Fprimer2 – 43.36°C, and Rprimer – 45.77°C. Based on these calculations, an annealing temperature of 42°C was used in the PCR tests.

DNA Purification

DNA Purification was used to collect DNA from cultured cells of both mutant type and wild type samples to be used in PCR. Two different sources of Human DNA came from Human bronchial epithelial cells from a CF patient without the R553X mutation (IB3 stock cells) while another set of stock cells was found to obtain the R553X mutation, therefore were used as the mutant type test (S9 cells). Each of these stock cells were used to contrast the effectiveness of the allele specific primers. (Gambardella *et al*, 2006) Before beginning purification, all reaction vessels were kept on ice until used and frozen cultured cells were allowed to thaw in a water bath set to 37°C. Then, 200ul of the sample (either human bronchial epithelial cells without the mutation or cells from a CF patient with the mutation, depending on the PCR test being done) was added to a capture column and was incubated at room temperature for 30 minutes. Afterwards, 400ul of DNA Purification Solution 1 was added to the sample. The resulting mixture was incubated for 1 minute at room temperature. After incubation, the mixture was centrifuged for ten seconds at 2,000-12,000 times the force of gravity (xg). The capture column was then transferred into a new waste collection tube. Another 400ul of DNA Purification Solution 1 was added to the mixture and the solution was incubated at room temperature for 1 minute. The solution was then centrifuged again for ten seconds at 2,000-12,000 x g. Then, 200ul of DNA Elution Solution 2 was added and the mixture was centrifuged for ten seconds at 2,000-12,000 x g. The capture column was transferred to a clear DNA collection cube and 100ul of DNA Elution Solution 2 was added. The solution was incubated for ten minutes at 99°C and then centrifuged for 20 seconds at 2,000-12,000 x g. (Quigen Inc. 2007)

PCR

To analyze DNA obtained from the purification process, two different Polymerase Chain Reaction (PCR) tests were designed. Test #1used Fprimer1 and the Rprimer. Test #2 used Fprimer2 and the Rprimer. For each of the tests, the combination of 2.0ul of the target DNA template obtained through DNA purification, 5.0ul 10X PCR buffer, 0.2ul Taq polymerase, 2.0ul forward primer (100 uM), 2.0ul reverse primer (100 uM), 1.0ul 10mM deoxynucleotide building blocks (dNTP) of DNA and 40.4ul water was added to a test tube on ice. The combination was mixed and spun down in a centrifuge. Once the sample was placed in the Labnet thermocycler, the cocktail went through five steps: initial denaturation, denaturation, primer annealing, extension and final extension. In the initial denaturation stage the temperature was raised to 94°

Predicted Results

Authored by: Kevin Werner

In this experiment for the template used in PCR, genomic DNA will be extracted and purified from human cells. Cultured cells from Crohn's Disease patients with the R702W-CARD15 mutation, without the mutation, and heterozygous for the mutation will be obtained from CRISI Inc (Sacramento, CA). For our diagnostic assay, PCR will be used to amplify a DNA segment at a locus containing the R702W mutation on the CARD15 gene that causes a form of Crohn's Disease using a specialized approach for detecting SNPs developed by Hidenobu Yaku (Yaku et al, 2008). The resulting PCR products will be analyzed using agarose gel electrophoresis in either a traditional TBE or fast LB buffer system.

Primer Design

We predict PCR of the genomic DNA will amplify the anticipated products from the R702W locus on the CARD15 gene on chromosome 16 (Figure 1). This will occur because of carefully selected primer design, reactant concentrations, and PCR temperatures and times (Saiki et al, 1998). Thus primers R (annealing at 20,866 base pairs to 20,887 base pairs on the CARD15 gene) and CDF (annealing at 19,866 base pairs to 19,881 pairs on the CARD 15 gene) will amplify a 1021 base pair product, and primers R and WTF (annealing at 19,866 base pairs to 19,881 base pairs on the CARD15 gene) will amplify a 1021 base pair product (Wright, et al. 2010) (Figure 2). Also, the forward primers CDF (forward primer designed to anneal to mutant type template) or WTF (forward primer designed to anneal to wild type template) will not anneal when their bases are not completly complimentary to the DNA template bases at the respective annealing loci of the primers, preventing amplification from occurring (Schochetman et al, 1988). The forward primers are known as discriminating primers because they are designed to be completely complimentary to either the mutant type or wild type genotype, but not both (Wittwer et al, 1993). Hence it is also predicted that heterozygous DNA will allow for some amplification with both of the discriminating primers. It then follows that amplification of DNA template from the CDF discriminating primer supports the presence of the R702W mutation, which could cause a maladaptive pro-inflammatory response in Paneth cells along with other genetic and environmental factors (Figure 3) (Lala et al, 2003).

We did a preliminary PCR amplification of the 1542 base pair long 16S rDNA locus of *Escherichia coli* to use as a control throughout our experiment (Haffar et al, 2010). The bands we produced during gel electrophoresis exhibited non-specific binding that we were able to reduce by raising the annealing temperature (Figure 4) (Livak et al, 2010). Also, the validity our concentrations of forward primer, reverse primer, dNTPs, buffer, and taq polymerase will supported by these experiments, thus supporting the validity of our Crohn’s experiment.

Genomic Purification

In our preliminary research and assay design we found that DNA is extracted from samples in four steps: cell lysis, membrane disruption with a detergent, protein removal, and precipitation (Qiagen 2010) (Figure 5). We predict DNA yield of purified samples will be in the range of 3-8 μg, because this is the normal theoretical yield of understanding of issues like genetic testing significantly more than any other surveyed group (Figure 6). Using the 1-10 scale for all questions pertaining to genetic testing, all surveyed groups on average responded above the median answer of 5, from which it can be interpreted that Michigan State University students tend to be supportive and knowledgeable about genetics (Table 2).

Discussion

Written by: Mitchell Wood
Revised by: Nathan Johns
Finalized by: Sarah Bonczyk

Experiment Summary

Cystic fibrosis, the most common autosomal recessive disease in Caucasians (Aznarez *et al*, 2007), has dramatic effects on multiple organs, including the lungs, pancreas, intestines, and liver (Welsh and Smith, 1995), due to mutations of the CFTR gene on chromosome seven, causing defects in sodium and chloride transport in epithelial cells (Aznarez *et al*, 2007). The R553X mutation is a specific variation of cystic fibrosis, involving a single base pair substitution at the 1789th base pair in the 553rd amino acid, from cytosine to thymine (Hull *et al*, 1993). The subsequent change from the amino acid arginine to a premature stop codon causes early truncation of the CFTR protein, thus altering the folding sequence (Gambardella *et al.* 2006). Although PCR has been proven effective for diagnosing genetic disorders such as cystic fibrosis (O'Leary *et al*, 1997), the question we are addressing is whether or not a PCR test can be designed to identify this specific mutation. We hypothesized that allele specific primers and a single base pair mismatch could be used to develop an accurate diagnostic test for patients with the R553X mutation using experimentally determined optimal conditions of PCR in terms of annealing temperature, primer concentration, and salt concentration.

In addition to primer design, we bridged the gap between laboratory experiments and the sociology behind diagnosing genetic diseases by surveying student opinion on genetic screening and the effects of genetic diseases on the human race in the long run. In recent years modern medicine has extended the life expectancy of people with cystic fibrosis allowing those affected to live to childbearing age (Ratjen 2008), meaning CF genes are more likely to be passed down to future generations. Samples were taken from Lyman Briggs, James Madison, the College of

Natural Science, and general university students. It was hypothesized that Lyman Briggs students would be more supportive of genetic testing due in part to their background in required integrated studies and their greater understanding of how the frequency of a genetic disease could impact a gene pool (Singer *et al*, 2008).

Original Predictions

By amplifying DNA from IB3 human bronchial epithelial cells from a CF patient and S9 epithelial cells from a leukemia patient via allele specific PCR, the length of the amplified DNA was interpreted through gel electrophoresis to show the presence or absence of the R553X mutation. Two different forward primers, Fprimer1 and Fprimer2, were designed to discriminate between the wild-type and mutant CFTR genes through allele specificity based on a single base pair mismatch on the 3' end. The mismatch was positioned on the 3' end of the primers to more effectively reduce the amplified product by decreasing DNA polymerase and dNTP binding efficiency (Yaku *et al*, 2008). Successful annealing of the primers and the subsequent extension phase was hypothesized to result in a band of 1,056 base pairs, thus indicating a positive test. The lack of a band was hypothesized to indicate a disruption in the extension phase due to the single base pair mismatch (Chavanas *et al*, 1996). A homozygous wild-type genotype was expected to show a band of 1,056 base pairs when using Fprimer1 and show no band when using Fprimer2. In contrast, a homozygous mutant genotype was expected to show a band of 1,056 base pairs when using Fprimer2 and show no band when using Fprimer1. Lastly for heterozygous genotypes, faint bands 1,056 base pairs long were expected to appear in both tests, using either Fprimer1 or Fprimer2 due to the replication of both genotypes during PCR, causing neither set of forward primers to completely discriminate against the specific mutation site (Chavanas *et al*, 1996).

Results and Ultimate Findings

In order to determine optimal PCR conditions, multiple experimental trials were run with adjustments in DNA concentration and primer concentration. In addition, multiple experiments were run to establish the optimal annealing temperature, which can directly affect the annealing rates of designed primers (Elnifro *et al*, 2000) and magnesium chloride ($MgCl_2$) concentration to alter magnesium ion concentrations, which directly affect DNA polymerase activity in PCR (Ignatov *et al*, 2002). The optimal annealing temperature was determined to be 46ºC based off of the calculated primer melting temperatures (see Methods section) and which annealing

References

Written by: Mitchell Wood
Revised by: Nathan Johns
Finalized by: Sarah Bonczyk

Aznarez, I., J. Zielenski, J.M. Rommens, B.J. Biencome, and L.C. Tsui. 2007. Exon skipping through the creation of a putative exonic splicing silencer as a consequence of the cystic fibrosis mutation R553X. *Journal of Medical Genetics*. 44(5): 341-346.

Bal J., M. Stuhrmann, M. Schloesser, J. Schmidtke, and J. Reiss. 1991. A cystic fibrosis patient homozygous for the nonsense mutation R553X. *Journal of Medical Genetics*. 28(10):715-717.

Chavanas, S., L. Pulkkinen, Y. Gache, F. Smith, W.H. McLean, J. Uitto, J. Ortonne, and G. Meneguzzi. 1996. A Homozygous Nonsense Mutation in the PLEC1 Gene in Patients With Epidermolysis Bullosa Simplex with Muscular Dystrophy. *The American Society for Clinical Investigation, Inc*. 98(10): 2196-2200.

Elnifro, E.M., A.M. Ashshi, R.J. Cooper, P.E Klapper. 2000. Multiplex PCR: Optimization and Application in Diagnostic Virology. *Clinical Microbiology Reviews.* 13(4):559-570.

Gambardella, S., M. Biancolella, M.R. D'Apice, F. Amati, F. Sangiuolo, A. Farcomeni, G. Chillemi, S. Bueno, A. Desideri, and G. Novelli. 2006. Gene expression profile study in CFTR mutated bronchial cell lines. *Clinical and Experimental Medicine*. 6(4):157-165.

Golshahi, L., K. Seed, J. Dennis, W. Finlay. 2008. *Journal of Aerosol Medicine and Pulmonary Drug Delivery.* 21(4):351-359.

Hull, J., S. Shackelton, and A. Harris. 1993. Short Communication: The Stop Mutation R553X in the CFTR Gene Results in Exon Skipping. *Genomics.* 19:362-364.

Ignatov, K.B., A.I. Miroshnikov, V.M. Kramarov. 2002. A New Approach to Enhanced PCR Specificity. *Russian Journal of Bioorganic Chemistry.* 29(4):368-371.

Jacobsen, P.B., H.B. Vadimarsdottir, K.L. Brown, K. Offit, 1997. Decision-Making About Gentic Testing Among Women at Familial Risk for Breast Cancer. *Journal of Biobehavioral Medicine.* 59(5): 459-466.

Lynch, H.T, P. Watson, T.G. Shaw, J.F. Lynch, A.E. Harty, B.A. Franklin, C.R. Kapler, S.T. Tinley, B. Liu. 1998. Clinical impact of molecular genetic diagnosis, genetic counseling, and management of hereditary cancer. *Cancer.* 86(11): 2449-2456.

Mones, L., P. Kulhanek, J. Florian, I. Simon, M. Fuxreiter. 2007. Probing the Two-Metal Ion Mechanism in the Restriction Endonuclease BamHI. *Biochemistry.* 46:14514-14523.

Predicted Figures:

Authored by: Kevin Werner

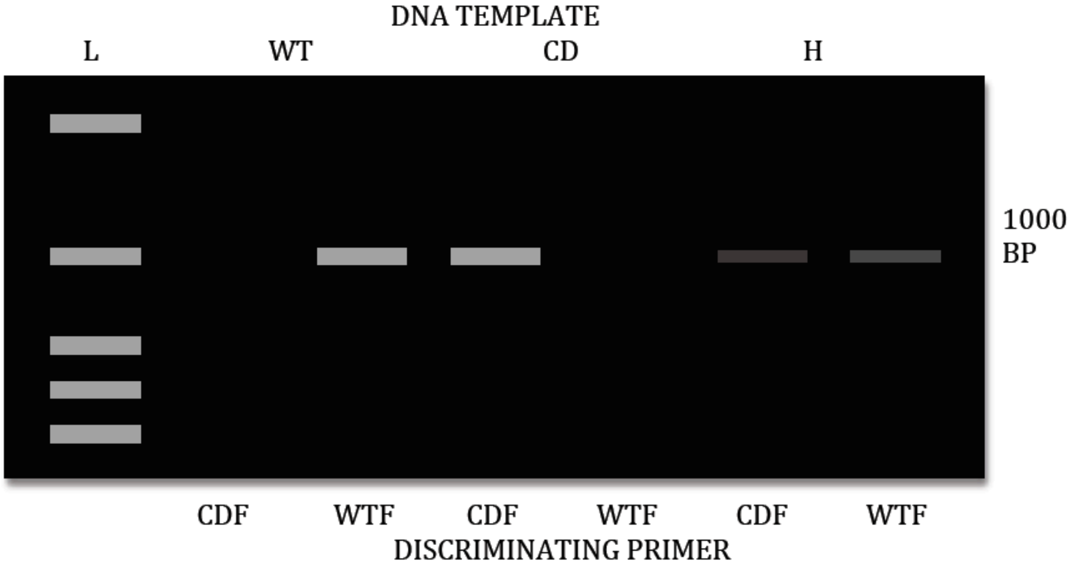


Figure 1 – Predicted results of from amplified products using gel electrophoresis. The non-discriminating oligonucleotide reverse primer, R, is 2% 3'-GTGGCTGCAGGGTTACAACTA-5'. The discriminating oligonucleotide forward primer CDF is 2% 5'-CGGGACGAGGCCGCG-3'. The discriminating oligonucleotide forward primer WTF is 2% 5'-GCGGGACGAGACCGCG-3'. The predicted binding site is on chromosome 16 on the CARD 15 gene from position 19,866 b.p. to 19,881 b.p. for the forward primer CDF, from position 19,866 b.p. to 19,881 b.p. for the forward primer WTF, and from position 20,866 b.p. to 20,887 b.p. for the reverse primer R with respect to the CARD 15 gene. It is predicted there will be a yield of 1.7 billion copies of the target DNA, along with a negligible amount of much larger DNA fragments (a by-product of PCR), and the original DNA template. The denaturing temperature will be set at 95°C, the annealing temperature at 55°C, and the extension temperature at 72°C. An initial denaturing time of two minutes will be used, then the times will be 30 seconds denaturing, 45 seconds annealing, one minute extending, and a final extension of 7 minutes for 30 cycles. The agarose gel contains 10% agarose. All bands are predicted to appear at 1021 base pairs beside the ladder. Bromphenol blue dye will be added to the gel to indicate when the gel electrophoresis is complete. L is the molecular marker or ladder, and the DNA templates WT, CD, and H are 2% DNA purified products from a wild type, mutant, and heterozygous genotype, respectively.

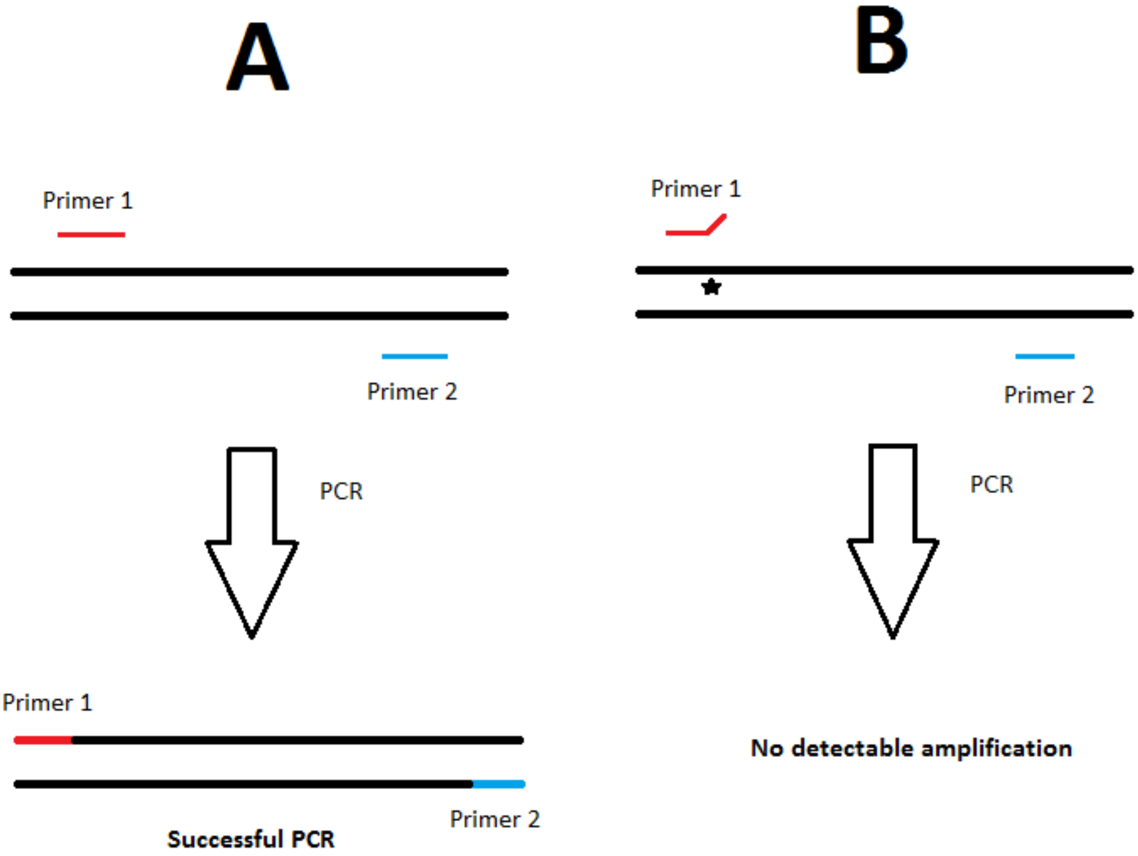


Figure 8. Representation of allele specific PCR. Column A represents wild-type DNA that was extracted using the "generation capture column kit". Column B represents mutant DNA. Primer 1 represents the forward wild-type primer and primer 2 represents the reverse primer. When primer 1 is used with wild-type DNA as seen in column A, PCR completes successfully. This is because the oligonucleotide bases match up with the DNA template and primers. However, when we use primer 1 with mutant DNA as shown in column B, the result is that there is no detectable amplification of DNA.

Figures

Written by: Sarah Bonczyk
Revised by: Liz LeMieux
Finalized by: Nathan Johns

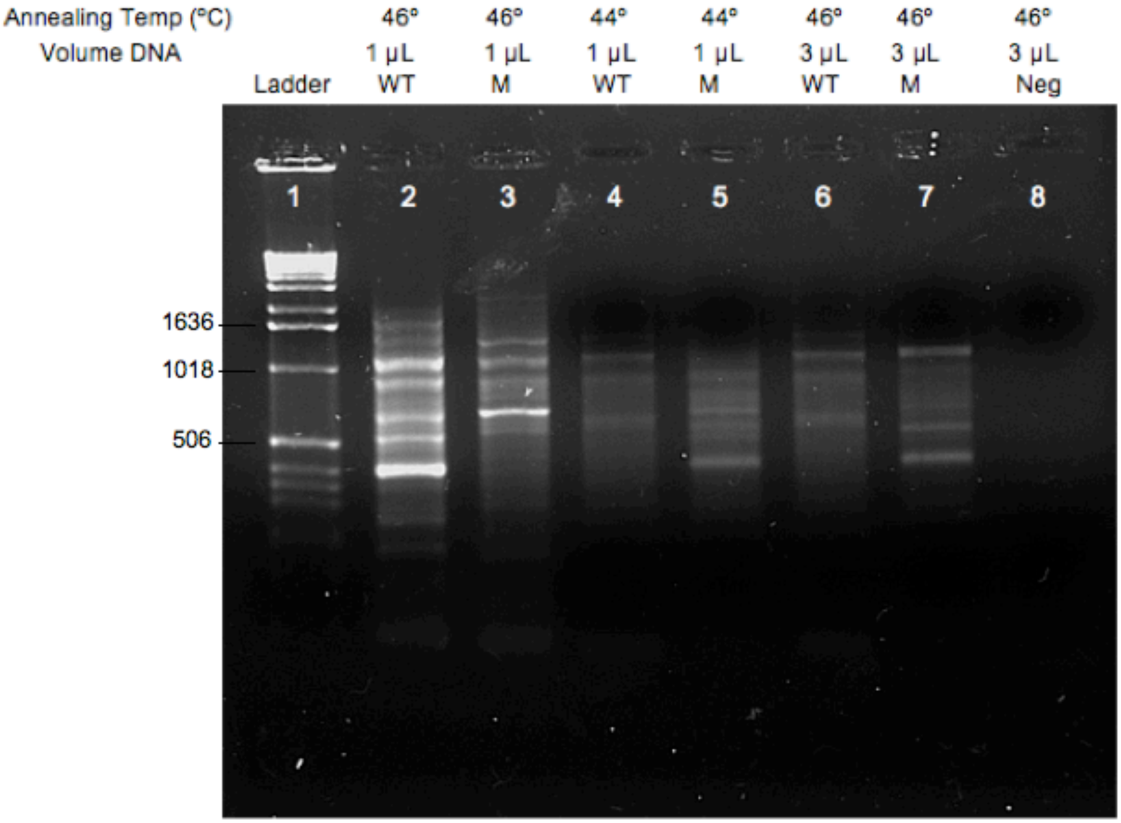


Figure 1: Amplification of DNA segment containing R553X mutation site while varying annealing temperatures and initial S9 DNA volumes. Thermocycling conditions included a 5-minute denaturation at 94 ºC with 30 cycles of 30 s at 94 ºC, 30 s at 46 or 44 ºC, and 60 s at 72 ºC, with a final elongation phase at 72 ºC for 7 minutes. All lanes show non-specific binding. Lanes 2,3,4,6, and 7 show bands near 1018. The targeted region of DNA is 1056 base pairs in length. Lanes using an annealing temperature of 46 ºC and lower initial DNA volumes of 1µL show higher intensity bands than other lanes. From this test it was determined that lower initial concentrations of DNA and an annealing temperature of 46 ºC are optimal for our designed primers.

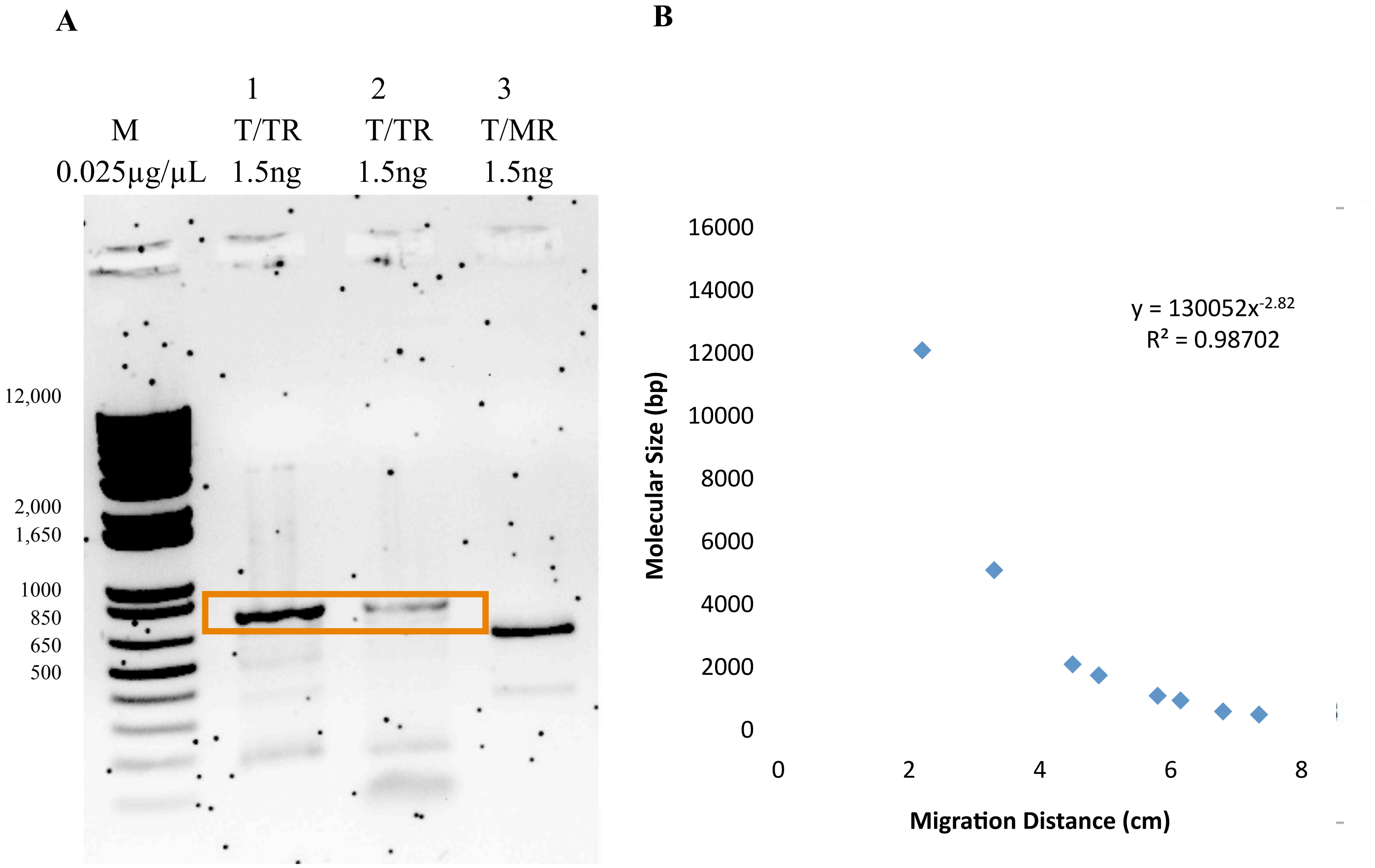


Figure 2. PCR amplification of the DMD gene from human DNA at an annealing temperature of 48ºC. A. After PCR amplification, gel electrophoresis was conducted in a 0.8% TBE gel run at 115V for 30 minutes to detect amplified regions of DNA. M is the molecular marker (1.25ng) 1-Kb Plus Ladder. Lanes 1 and 2 correspond to wild-type DNA samples tested with the wild-type forward/reverse primer set (T/TR). 10µL (1.5ng) of wild-type DNA was added to each of these lanes, and the expected amplification of a 765bp long fragment was observed. Lane 3 corresponds to a wild-type DNA sample amplified with the mutant forward/reverse primer set (T/MR). 10µL (1.5ng) of DNA was added to this lane and a PCR product of 589bp long was observed. For each one of the lanes, non-specific binding was also seen but the expected band was quite distinct.

B. Semi-log plot for 1.25ng of 1-Kb Plus ladder. Each point of the graph depicts the specific distance traveled by each band of the 1-Kb Plus ladder with respect to its well. An R^2 value of 0.98702 ($p<0.05$) was obtained for the logarithmic trend line of the plotted traveled distances. The equation obtained from the trend line was used to calculate the traveled distances of the bands from lanes 1, 2, and 3 to get more accurate band size values. Values of 757.83 ± 8.75bp, 775.33 ± 8.75bp and 589.03 ± 5.21bp were obtained for lanes 1, 2, and 3 respectively.

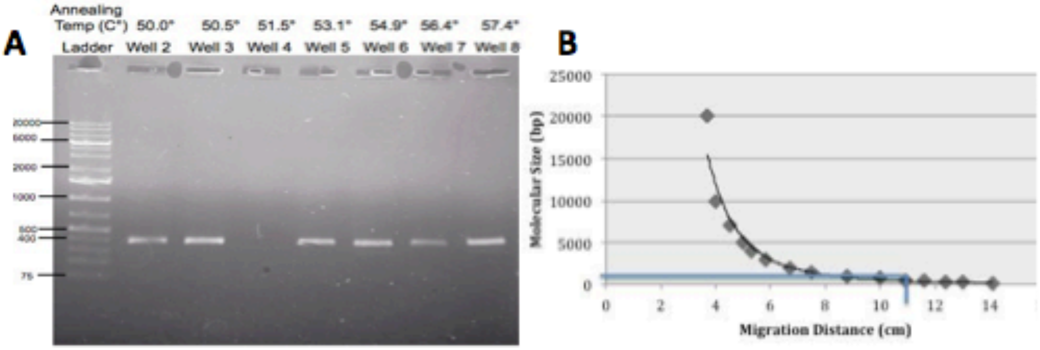


Figure 3: Amplification of lambda RZ gene by PCR and analysis by gel electrophoresis. **(A)** A target DNA sequence of about 400 bp was amplified using PCR in which two primers were used: Rz1R and Rz1F. The PCR cocktails contained 38 µL of nuclease free water, 7 µL of 10X PCR buffer, 1 µL of Taq polymerase, 1µL of dNTPs, 1µL of lambda DNA template, and 1µL of each primer. The cocktails were run in the thermocycler at 95° C for an initial 3 minutes and then cycled between 30 seconds at 95° for the denaturing phase, 30 seconds at the annealing temperature for the annealing phase, and 1 minute at 72° for the elongation phase. The annealing temperatures were set on a gradient from 50° to 58° and 25 cycles were completed. Annealing temperatures are noted above the wells. A 0.8% agarose gel made using TBE (Tris/Borate/EDTA) buffer, agarose, and GloGreen was run at 135V in which 7µL of DNA and 3 µL of loading dye was pipetted into wells 2-8 and 5µL of Fermentas' 1kb Plus ladder was pipetted into well 1. The gel was then observed under an ultraviolet light in order to see the bands created by the DNA product. **(B)** Migration distance vs. molecular size of 1 kb Plus DNA ladder used to analyze PCR products from Rz gene. A target sequence of lambda DNA from the Rz gene was amplified using PCR. An 0.8% agarose gel was made using TBE (Tris/Borate/EDTA) buffer, agarose, and GloGreen. 5 uL of 1 kb Plus ladder was used and analyzed using a semi-log plot shown above in which the x-values represent the distance in centimeters that the bands of the ladder migrated away from the well and the y-values represent the size of the molecules in base pairs. A trend line was added in order to obtain an equation for further analysis. This equation was used to calculate the base pair length of the lambda DNA PCR product in which the distance migrated from the wells was inputted for the x-value of the equation, yielding a y-value representing the base pair length of the product. For the bands produced by the lambda DNA (Figure 5), the base pair length produced was calculated to be 400 bp for all 6 wells yielding a band. The R2 value given in the figure represents the fit of the trend line; 1 represents a perfect fit.

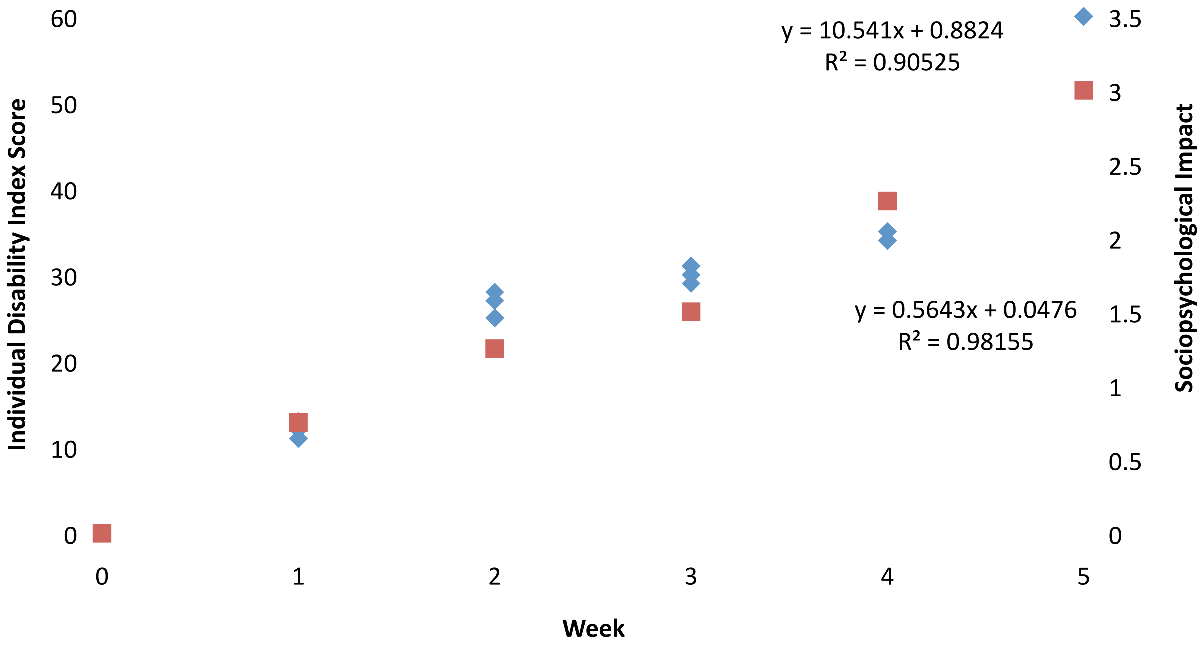


Figure 5. Correlation between the degenerative nature of Duchenne muscular dystrophy and the deteriorating socio-psychological state associated with the disease. Duchenne muscular dystrophy's symptoms were progressively added to each one of the researchers' lifestyles every week for a five-week period. At the end of each week, each researcher completed the Disability Index of Stanford University's Health Assessment Questionnaire (HAQ) in order to gauge the severity of disability that the researchers were living with. The HAQ gives scores between 0 and 60, with zero pertaining to no disability at all and sixty symbolizing complete disability and immobility. In addition to the HAQ, each researcher completed a survey that measured, on a scale of 0 to 3, the socio-psychological impact on the researcher as each symptom was added. A linear regression for the individual Disability Index scores is shown in blue while the linear regression for the Socio-psychological impact test scores is shown in red. An R^2 value of 0.90525 ($p<0.05$) was obtained for the Disability Index score and an R^2 value of 0.98155 ($p<0.05$) was obtained for the socio-psychological impact test.

Table 1. Chi-square test on adverse reactions to CF symptoms in public locations.

Location	Total People Observed	Expected Frequency*	People Observed with Reaction	People Expected with Reaction	$(O-E)^2/E$
Elevator	40	.717	27	28.68	.0984
Cafeteria	25	.717	17	17.93	.0482
Study Lounge	15	.717	9	10.75	.284
Restaurant	10	.717	6	7.17	.191
Total	**90**	**.717**	**59**	**64.53**	X^2=.6216
				n=4	df=3
					p value associated with X^2=.90

*Values representative of the results of question one of Figure 5.

Grading Rubrics

(for) Research Papers

Guide to Paper Grading Rubrics

During LB-145L, your team will write a first draft, a second draft, and a final draft. The rubrics on the following pages will describe the sections and information that should be included in each paper as well as how things will be graded. This series of drafts is designed so that your group will slowly build a final paper while receiving constructive feedback along the way. While individuals in the group are assigned different tasks for each paper, it is essential that every member of the group also read the entire manuscript. *See page 80 "Research Group Roles" to review roles for a group of 3.

DRAFT1: This is where you will first attempt to articulate what you plan to do. Once you think of a research question/project you are interested in doing, you will need to do some preliminary literature research in order to find references that will support your hypothesis. At this point you will complete the first draft of the full research paper. The First Draft should contain all section of the full paper and all parts of your full research investigation (could include 30 Days too).

Roles: PI writes Title, Abstract and Introduction;
PE authors the Methods section;
DRD does Predicted Results, Tables and Figures;
LT authors Discussion and References.

DRAFT2: Use the feedback received from the first draft to get the Methods and completed experiments and information into final quality shape. The primer and genome pre should be near final quality. It is best to approach this draft as if it were the final draft for those sections.

Roles: PI revises Discussion, Refs ;
PE updates Results, Figures & Tables;
DRD does Methods section;
LT revises Title, Abstract, Introduction.

Final Draft & Website: It is essential that you address all the comments from previous drafts in the final paper. You should also apply these comments to the new material that is written for the final draft. If you do both of these things, you have a very good chance of getting a high grade for the final paper.

Roles: PI finalizes Results, Figures & Tables;
PE finishes Title & Abstract, Introduction,
DRD revises Discussion and Refs;
LT authors the final version of Methods section, everyone helps author web & "Future Directions".

****It is imperative that you read the grading rubrics and "Instructions to Authors" carefully. Failure to follow the rubrics and "Instructions to Authors" may result in point losses.****

Group Inquiry Research Paper DRAFT 1 (30 points)

Due at the start of Lab (week 4)

REJECT and HAND BACK IMMEDIATELY if…

__ The individual authorship of sections is unclear. Lacking who did which (use B-PIDs for blind grading)
__ "Group responsibility" signature page is not completed with tasks done and B-PID listed
__ Submitted manuscript format doesn't not follow "Instructions to Authors"
__ Manuscript does not have first page of Turnitin originality report (with each author's handwritten B-PID)

ORIGINALITY/CREATIVITY/PROFESSIONALISM: (WORTH 2 POINTS *EC*)

Appendix: (WORTH 2 POINTS) Do they have copies of the first page of all articles?

Title: (WORTH 3 POINTS)

__ Paper gets 2 pts for having a title that says something about **methods** and their **specimen**.
__ Paper gets 1 points if their title predicts anything about their **findings**.

Abstract: (WORTH 5 POINTS)

__ Paper gets 3 points for having an abstract that explains well what they **will do and why.**
Including: ___purpose ___hypothesis ___experimental design
__ 2 points if abstract ***predicts*** anything about their expected **findings/results** (with support from citations).

Introduction: (WORTH 5 POINTS)

__ Paper gets 4 points for having an introduction that tells you some **background** (several paragraphs) about what they plan to do and (one paragraph) what they **expect**/predict to see.
__ Paper gets the final 1 point if their introduction talks about **anyone else's research** on this topic or other reference information from a book or paper etc.
__ Paper gets 1 BONUS point for having an Introduction that makes you **excited** and want to read on.

Methods: (WORTH 5 POINTS)

__ Paper gets 5 points for having a detailed methods section that clearly tells what they will do in a few pages. If you were an LB-144 student and ONLY had the Methods section, **could you repeat** the work?
Including: ___source of materials ___procedures and data analysis ___narrative & in past tense

"Predicted" Results and Figures: (WORTH 5 POINTS)

__ Paper gets 2 point if "Predicted" Results section discusses expected/predicted data that will be collected. Read the Results section and look at each Figure (no tables allowed). Is cited properly, does it make sense?
Including: ___outcomes of experiments predicted with support ("We predict ... because... citation/paper")
__ Paper gets 1 point for each "prototype" figure that seems highly appropriate for their investigation.
Including: ___do Figures address research question ___ have a professional appearance ___have long legends with title and extensive description (so a 144 student could explain it with only that page).

Discussion: (WORTH 10 POINTS)

Try to develop a hypothesis that might be tested in your research. Present **why** you have predicted the results you did? The Discussion should be based on **evidence** found in your citations: books, magazines, literature.

__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point; *requires citations*
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing objections; *requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used. *requires citations*
__ 10. Indicates original PREDICTIONS and ultimate findings

“Group Responsibility” Signature Page (DRAFT 1)

Attach this sheet to the front of any written work that is to be turned in for a grade.

I, the undersigned, have read, edited and approved of the full manuscript my group is now submitting for grading. I can explain any part of it to Dr. Luckie and I am willing and excited to discuss the research on a moment’s notice. In addition to the above, I am verifying that as a member of the Lyman Briggs community, I have held myself and my peers to the highest measures of honesty and integrity. My group has neither given nor received any unauthorized assistance in completing this work and we submitted our manuscript to http://turnitin.com/ for screening.

Group Name _____________________________ Date __________

Primary Investigator: Name ________________________ Signature _________________________

I developed/revised these sections of the paper: ____

Since the Proposal, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Protocol Expert: Name ________________________ Signature _________________________

I developed/revised these sections of the paper: ____

Since the Proposal, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Data Recorder: Name ________________________ Signature _________________________

I developed/revised these sections of the paper: ____

Since the Proposal, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Laboratory Technician: Name ________________________ Signature _________________________

I developed/revised these sections of the paper: ____

Since the Proposal, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Group Inquiry Research Paper DRAFT 2 (40 points)

Due at the start of Lab (week 7)

****REJECT and HAND BACK IMMEDIATELY if…***

__ Don't have 5 copies (one original with appendix and 4 photocopies without appendix)
__ "Group responsibility" signature page is not completed
__ The individual responsible for each section is unclear (ie "revised by Jim Smith").
__ Submitted manuscript format doesn't follow "Instructions to Authors"
__ Submitted manuscript has not yet been uploaded to http://turnitin.com/ (have receipt?)

ORIGINALITY/CREATIVITY/PROFESSIONALISM: (WORTH UP TO 5 POINTS **EC)**

__ Paper gets 1-5 pts for having experimental design that is very creative and unique -or- extremely professional ie if they take previous research, cite it well, and extend it in an impressive scientific style.

APPENDIX

__ Do they have copies of signed data *and* the 1st page of articles they've cited in References?

Title: (WORTH 3 POINTS)

__ Paper gets 2 pts for having a title that says something about **methods** and their **specimen**.
__ Paper gets 1 points if their title predicts anything about their **findings**.

Abstract: (WORTH 3 POINTS)

__ Paper gets 3 points for having an abstract that explains well what they **will or did do and why.**
Including: ___purpose ___hypothesis ___exp design ___outcomes/data ___significance in science
__ Paper gets 2 points if the abstract discusses *both* actual *and* predicted **findings/results**.
__ Subtract points if authors make claims or assertions without any evidence to support those predictions.

Introduction: (WORTH 7 POINTS)

__ Paper gets 2 points for having an introduction that tells you some **background** (several paragraphs) about what they plan to do.

Paper gets 0.5 point for each of these present in the Introduction:
__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point; *requires citations*
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing objections; *requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used; *requires citations*
__ 10. Indicates original PREDICTIONS and some hint of findings

Methods: (WORTH 5 POINTS) The *Methods* section must appear complete for ALL experiments and ready for publication.

__ Are there all the protocols necessary to repeat their experiments exactly for their completed research?
__ Are there all the recipes and protocols clarified for their research during the upcoming weeks?
__ If an LB-144 student ONLY had the Methods section and a course pack, **could they repeat** the work?
Including: ___source of materials ___procedures ___data analysis ___narrative & in past tense

Results: (WORTH 4 POINTS)

__ Paper gets 4 points if Results section discusses actual findings alongside the expected/predicted data that was collected. Read the Results section and look at each cited Figure or Tables, is it cited properly?

Including: ___outcomes of experiments stated? ___has actual #'s from data? ___cite figures/tables properly?

__ Subtract points if authors make claims or assertions without any evidence to support those predictions.

Figures/Tables: (WORTH 5 POINTS)

__ Paper gets 1 point for each actual (not predicted) figure that seems highly appropriate and professional for investigation. At week 6, should have completed/actual 30 Days research figures and those from Genomic Prep.

Including: ___do F/T address research question? ___ have a professional appearance? ___have long legends with title and extensive description (so a 144 student could explain it with only that page)?

__ If they have NO photographs of research results from any experiment, subtract points (no evidence).

__ Paper gets up to 2 **BONUS** points if Figures completely ROCK!

Discussion: (WORTH 10 POINTS)

Try to develop a hypothesis that might be tested in your research. Present **why** you believe you have found those results or predicted the results you did? The Discussion should be based on **evidence** found in your experiments and citations: books, magazines, literature.

__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point; *requires citations*
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing objections; *requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used; *requires citations*
__ 10. Indicates original PREDICTIONS and ultimate findings

Completed experiments: (3 POINTS) Paper must have *final draft* profession quality for completed work from previous weeks. At week 7 some research should be mostly complete, genomic prep also complete.

__ Does the Introduction, Results and Discussion sections have a professional complete quality for all completed experiments in the research?

__ Do the Figures (photographs, graphs, drawings etc) and Tables for all completed research appear complete and ready for publication?

"Group Responsibility" Signature Page (DRAFT 2)

Attach this and the rubric sheet to the front of any written work that is to be turned in for a grade.

I, the undersigned, have read, edited and approved of the full manuscript my group is now submitting for grading. I can explain any part of it to Dr. Luckie and I am willing and excited to discuss the research on a moment's notice. In addition to the above, I am verifying that as a member of the Lyman Briggs community, I have held myself and my peers to the highest measures of honesty and integrity. My group has neither given nor received any unauthorized assistance in completing this work and we submitted our manuscript to http://turnitin.com/ for screening.

Group Name ____________________________ Date __________

Primary Investigator: Name _______________________ Signature ________________________

I was responsible for these sections of DRAFT2: ____

Since DRAFT1, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Protocol Expert: Name _______________________ Signature ________________________

I was responsible for these sections of DRAFT2: ____

Since DRAFT1, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Data Recorder: Name _______________________ Signature ________________________

I was responsible for these sections of DRAFT2: ____

Since DRAFT1, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Laboratory Technician: Name _______________________ Signature ________________________

I was responsible for these sections of DRAFT2: ____

Since DRAFT1, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Group Research Paper FINAL DRAFT (100 points)

Due at the start of Lab (week 14)

REJECT and HAND BACK IMMEDIATELY if…
__ The individual authorship of sections is unclear.
__ "Group responsibility" signature page is not completed
__ Submitted manuscript format doesn't not follow "Instructions to Authors"
__ Submitted manuscript has not yet been uploaded to http://turnitin.com/ (have receipt?)

CONTENT:

Title: (WORTH 6 POINTS)
__ Paper gets up to 4 pts for having a title that says something about **methods** and their **specimen**.
__ Paper gets up to 2 points if their title explains anything about their **findings**.

Abstract: (WORTH 8 POINTS)
__ Paper gets up to 6 points for having an abstract that explains what they "**did**" and "**found**."
Including: ___purpose ___hypothesis ___exp design ___outcomes/data ___significance in science
__ Paper gets up to 2 points if their abstract sounds like **scientists** wrote it (not obvious that its by students).

Introduction: (WORTH 15 POINTS)
__ Paper gets 3 points for having an introduction that tells you some background (several paragraphs) about what they plan to do and (one/final paragraph) what they expect/predict to see.
__ Paper gets up to 4 points (2 bonus are possible) if their Introduction sounds like **scientists** wrote it (not obvious that its by students) and makes you interested and want to read on.

Paper gets 1 point for each of these in the Introduction:
__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point; *requires citations*
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing objections; *requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used, *requires citations*
__ 10. Indicates original PREDICTIONS and some hint of findings

Methods: (WORTH 10 POINTS)
__ Paper gets up to 10 points for having a methods section that tells what they did and allows a student in LB-144 to easily replicate their experiments (*all* in past tense).
Including: ___source of materials ___procedures ___data analysis ___narrative & in past tense

Results: (WORTH 5 POINTS)
__ Paper gets up to 5 points if Results section discusses data that was collected in a very professional fashion. Read the Results section and look at each Figure or Table to be sure it is cited properly. Does it make sense? Do you now know what they tested and what they found? No predictions, just findings.
Including: ___outcomes of experiments stated ___actual #'s from data ___cite figures/tables properly

Figures/Tables: (WORTH 10 POINTS)

__ Paper gets up to 10 points if Figures and Tables (F/T) are very professional and really DO document their evidence. If they have NO photographs of actual results of experiments subtract 5 points (no evidence).

Including: ___do F/T address research question ___ have a professional appearance ___have long legends with title and extensive description ____No PREDICTED FIGURES allowed in Final paper

__ Paper gets up to 2 BONUS points if Figures completely ROCK!

Discussion: (WORTH 25 POINTS)

__ Paper gets up to 2 points if they develop an excellent hypothesis that they evaluate.

__ Paper gets up to 3 points (for various topics and DIY) if they present again a brief summary of their intro, methods and results and then proceed to **interpret** these (what's it mean?)

Also 2 points for each of these.

__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);*requires citations*
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point;
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing reasonable objections;*requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used;*requires citations*
__ 10. Indicates original PREDICTIONS and ultimate findings

Discussion - "Future Directions" subsection: (WORTH 6 POINTS)

__ Paper gets up to 2 points for each assay that didn't work completely where they troubleshoot it well and propose detailed plans to resolve the problem and get useful results in future research experiments. What didn't work well, why is it likely it failed, how would you re-design your experiment(s) or whole research project if you had six weeks longer to work and it was very worthwhile for your grade.

HOW GOOD (QUALITY & QUANTITY) WAS THE RESEARCH : (WORTH 10 POINTS)

Did very *few* trials/experiments 0 1 2 3 4 5 6 7 8 9 10 Did *many* complex trials exps

"The quantity and quality of their research experiment: If they made a sincere effort and did many trials and controls and were rigorous in their research design and execution they will be generously rewarded. If on the other hand, their group did very few experiments, trials, controls and in general it's clear their effort was minimal, their score will be minimal."

View their graphs and check if they have multiple trials indicated and include statistics with data.

DO THEY HAVE A WEBPAGE? YES / NO (WORTH 5 POINTS)

*WEBPAGE: In addition, we request you create a website that contains some information from your final paper for publication on the web. The info requested is: Title, Authors, Abstract, 1 Figure or Table (the best one that shows the most important finding), and your full Discussion section. List the URL for us to view it on the coverpage of your paper. This webpage will be placed in a public website to "publish" your results. Students in the future (as you did) will read about your work and extend your published research.

"Group Responsibility" Signature Page (Final DRAFT)

Attach this sheet to the front of any written work that is to be turned in for a grade.

I, the undersigned, have read, edited and approved of the full manuscript my group is now submitting for grading. I can explain any part of it to Dr. Luckie and I am willing and excited to discuss the research on a moment's notice. In addition to the above, I am verifying that as a member of the Lyman Briggs community, I have held myself and my peers to the highest measures of honesty and integrity. My group has neither given nor received any unauthorized assistance in completing this work (ie. We used no data, images, text etc from other groups or from published resources in print, internet or other domains).

Group Name ____________________________ Date _________

Primary Investigator: Name _______________________ Signature ________________________

I revised these sections of the paper: ____

Since DRAFT 2, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Protocol Expert: Name _______________________ Signature ________________________

I revised these sections of the paper: ____

Since DRAFT 2, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Data Recorder: Name _______________________ Signature ________________________

I revised these sections of the paper: ____

Since DRAFT 2, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Laboratory Technician: Name _______________________ Signature ________________________

I revised these sections of the paper: ____

Since DRAFT 2, I performed these duties inside and outside of lab towards the completion of this part of our group research project (e.g. assays, meetings, growing/buying plants, etc)

____

Characteristics of a 4.0 Final Manuscript

1. Reads like a scientist wrote it. The author is clearly well-read on the topics they studied, understands their goals and methodologies and communicates that well in a concise, smart, composition.
2. References section contains scientific research papers, recent books and news articles.
3. Figures looks professional with clear and detailed legends.
4. Introduction and Discussion sections have numerous citations and they are used regularly every few sentences. All sections are clearly broken up into sub sections, with sub titles, that focus on each step of the project (such as: Quantitation of DNA Found, DNA purification, PCR analysis).
5. Each step in the research project is addressed individually and thoroughly with context "why and how it was done" as well as evidence "photographs," data sets, mathematical analysis.
6. Error analysis is scientific and thoughtful; citing difficulties found by other researchers in publications, limitations or possibilities that explain problems in data collection.

Characteristics of a 0.0 Final Manuscript

1. Reads like a high school student wrote it. The author is clearly has not read about the topics they studied (even material presented in the course pack), and does NOT understand their project or communicate well in a composition.
2. References section contains anonymous websites and citations are not complete.
3. Figures looks incomplete with short confusing legends.
4. Introduction and Discussion sections have very few citations and all sections are just long paragraphs with no topic sentences and no sub-headings.
5. Each step in the research project is NOT addressed individually and the author seems only focused on quickly being done.
6. Error analysis is trivial where all the human error items listed could easily have been fixed by using controls or doing replications (or even coming to lab more than once a week and paying attention).

(for) Presentations

Talks/Posters: Presenting research in a 10-minute talk

Research Presentation requirements and expectations.

In LB145 you will have multiple opportunities to practice informal and formal research presentations. Review this information for guidance. Each member of your group may present your research at a Research Symposium. If so, you might choose to present a poster or a 10-minute talk. In either case you would give a talk explaining your research project and findings in your lab experiments. Here are places to get help.

FOR ORAL PRESENTATIONS: If you are doing an oral presentation, please keep in mind that during a Symposium talks might be only 10 minutes per presentation, with 5 minutes between presentations. The 5 minute space between presentations is there to allow you time to field questions from the audience and to allow the next presenter time to set up or arrange any equipment or material he/she needs.

A few years ago Dr. James Zacks prepared a web page with links to some helpful sites that offer advice on how to prepare for giving an oral presentation.

http://www.msu.edu/user/zacks/OnGivingATalk.htm

FOR POSTER PRESENTATIONS: If you are making a poster, please keep in mind that the display surface that events will provide you to hang your poster is often 36" tall x 48" wide (check this to be sure). You can make your poster presentation any size that will fit into that area (feel free to measure and test your rough poster). Your poster may need to be able to be hung with thumb tacks, tape or Velcro tape.

Here's a helpful website to get tips about making a scientific poster:

http://www.kumc.edu/SAH/OTEd/jradel/Poster_Presentations/PstrStart.html

Group ______________________________

Rubric for scoring Proposal Presentations (LB145)

Each individual competed the Proposal Worksheet very well [attach all four of this group]

_____ [P/F/0-_pt] BACKGROUND INFO ON **DISEASE** ________________: Student name ________

We read papers _______, ________, and _____ and found that [insert disease here] has symptoms like _____________, a lifespan of X years, and the disease is treated with these drugs and strategies. [insert gene here] normal wild-type genomic DNA sequence is XXX bases in length and the protein is YYY amino acids in length.

_____ [P/F/0-__p t] OUR **MUTATION**: ________________: Student name ________

We searched Google and NCBI Database website that is in the Laboratory Information section of the LB145 website. The ______________ mutation of _______ causes the disease because___________ . The acronym of the mutation is ___________ and that acronym means ___________. The acronym refers just to the amino acids that changed. The DNA base(s) that were mutated to lead to a codon change and amino change were…..

_____ [P/F/0-__ pt] **LITERATURE** RESEARCH: ________________: Student name ________

We used Google Scholar and PubMed to search research papers and reviews on [insert disease here] and this particular mutation. So far we have found X research papers and Y books that discuss this mutation in ... The first paper is entitled _____ and it demonstrates _______. The second paper is a review ….. We found two research papers where the authors designed PCR based diagnostic assays and can now identify primer DNA sequences that should work in PCR.

_____ [P/F/0-___] (**30 Days -OPTIONAL EXPERIMENT-** ________________: Student name ________

To further connect our biological research with other fields of study, we plan to generate a project that tests whether a our own historical/philosophical/sociological understanding of genetic diseases and evolution …..

_____ [P/F/0-__p t] OUR **PRIMERS-PCR**: ________________: Student name ________

We read the "Primer Design" section of the Lab Guide and reviewed the full genomic sequence of We plan to design our own primers this way, primer (#1) that will only bind at this location in the DNA sequence if the mutation IS present. It will look something like this. Here is ……

Once we know the base sequence of the primer we can calculate it's expected annealing temperature….

_____ [P/F/0-__p t] **CONTROLS**: ________________: Student name ________

As a "PCR positive experimental control" we will also create a primer #3 that binds well to the normal gene sequence (non-mutant) at the primer 1 location. We also plan to design….

Verbal Status Report Grading Rubric (Talk is 10 minutes in length, individually graded)

Assignment Explanation: Each person is responsible for only ONE figure in both the talk & mini-paper. IMPORTANT the figure on your slide should look identical to the same figure in the paper, except on the slide you should have almost zero figure legend (ie nearly none of the text). Slides should contain both the most relevant actual data figures to represent what is done [or in progress] and predicted figures to represent precisely what still must be completed. If a gel image has some unusual smears, bands, etc, did the student troubleshoot what they might be and how controls/control experiments could be used to help better clarify their identity? Possible Topics: Primers, Genome Prep, E.coli, 30Days.

Student Names: Group Name:

Figure 1: Student ______________________ Topic ___________________

Evaluate Slide (visual) ____ Score _____________

Evaluate Explanation (verbal) ___________________________________ Score _____________

Figure 2: Student ______________________ Topic ___________________

Evaluate Slide (visual) ____ Score _____________

Evaluate Explanation (verbal) ___________________________________ Score _____________

Figure 3: Student ______________________ Topic ___________________

Evaluate Slide (visual) ____ Score _____________

Evaluate Explanation (verbal) ___________________________________ Score _____________

Figure 4: Student ______________________ Topic ___________________

Evaluate Slide (visual) ____ Score _____________

Evaluate Explanation (verbal) ___________________________________ Score _____________

Figure 5: Student ______________________ Topic ___________________

Evaluate Slide (visual) ____ Score _____________

Evaluate Explanation (verbal) ___________________________________ Score _____________

Grading Scale:

Pretty Good: Average work, respectable, middle of the class.

Excellent. It is impressive work, top of the class, and the work was done extremely well but nothing beyond what is expected.

Most Excellent. Every detail of the work was done extremely well and they found additional papers and evidence beyond what they were told.

Outstanding. It has the 3.0, 3.5-level elements + student impresses instructor with how much/well they did the work. They taught Prof something.

Progress Report

Group Name: __________________ Lab Section: __________________

Fill out with Group
Progress Fill in: **<u>Complete</u>** **<u>Incomplete</u>** **<u>Haven't Started</u>**

Paper	**Section**	**Progress**	**If not complete, Who is working on it?**
	Title		
	Abstract		
	Introduction		
	Methods		
	Results		
	Discussion		
	References		
	Figures and Tables		

Lab	**Phase 1**	**Progress**	**If not complete, Who is working on it?**
	E. coli/λ gel		
	+ Analysis		
	Genome Prep gel		
	+ Analysis		
	Published Primers		
	+ Analysis		
	Phase 2	**Progress**	**If not complete, Who is working on it?**
	Customized Primers		
	+ Analysis		
	Mt DNA?		
	Phase 3	**Progress**	**If not complete, Who is working on it?**
	30-day Experiment		
	+Analysis		
	Movie		
	Website		

Final Talk: 15-minutes (Introduction, 8 figures, future directions and film).

Group Name is ________________ Lab Section Name is ______________

Introduction slide(s): Shared responsibility, Score (out of 5 pts) _______________

Does it have: ___good purpose ___good hypothesis ___experimental design ___significance in science

Primary Investigator is responsible for presenting and explaining two figure/table slides

Student's Name _______________ **Score** (out of 10 pts) _______________

Figure/Table 1: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

Figure/Table 2: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

___ did the student carefully review each figure, its methods, findings and meaning/interpretations

___ did they discuss how controls/control experiments were used to clarify findings

Laboratory Technician is responsible for presenting and explaining two figure/table slides

Student's Name _______________ **Score** (out of 10 pts) _______________

Figure/Table 3: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

Figure/Table 4: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

___ did the student carefully review each figure, its methods, findings and meaning/interpretations

___ did they discuss how controls/control experiments were used to clarify findings

Data Recorder is responsible for presenting and explaining two figure/table slides

Student's Name _______________ **Score** (out of 10 pts) _______________

Figure/Table 5: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

Figure/Table 6: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

___ did the student carefully review each figure, its methods, findings and meaning/interpretations

___ did they discuss how controls/control experiments were used to clarify findings

Protocol Expert is responsible for presenting and explaining two figure/table slides

Student's Name _______________ **Score** (out of 10 pts) _______________

Figure/Table 7: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

Figure/Table 8: ___did its appearance and its presentation address research question ___ have a professional nature

__ deserve **Bonus** points for being super-spectacular?

___ did the student carefully review each figure, its methods, findings and meaning/interpretations

___ did they discuss how controls/control experiments were used to clarify findings

Conclusion slides: Shared responsibility, Score (out of 5 pts)

Appendix

Handouts

THE ELEMENTS OF A GOOD HYPOTHESIS

The American Heritage Dictionary defines hypothesis as *"a tentative explanation for an observation, phenomenon, or scientific problem that can be tested by further investigation"*. A hypothesis is a possible answer to a question, from which predictions can be made and tested. There can be multiple hypotheses used to answer a single question and for each hypothesis, multiple predictions can usually be made.

The foundation for high quality, biological research is a good hypothesis. A good hypothesis is more than just an educated guess.

THE HYPOTHESIS SCORE CARD…

A good hypothesis must:

1.) explain how or why: provide a mechanism

2.) be compatible with and based upon the existing body of evidence.

3.) link an effect to a variable.

4.) state the expected effect.

5.) be testable.

6.) have at least two outcomes.

7.) have the potential to be refuted.

Hypotheses can be scored based on these elements. When considering a hypothesis, give one point for each of the elements. An *accomplished hypothesis* will have a score of 7. An *incomplete or developing hypothesis* will have a score of 5-6. A score below 5 is an *attempted hypothesis* or *not a hypothesis*. You should use this scoring procedure when developing your own hypotheses or when evaluating hypotheses of others.

Proposal Worksheet (optional aid) Name(s) ____________________

____

Use readings on cystic fibrosis and PCR in your textbook, guide as well as additional sources to complete the following worksheet.

1. Summarize the overview of your proposed PCR research project. What mutation are you studying and why? Write down a proposed schedule/timeline for your researh project.

2. **PCR** is the process of using DNA Replication in a tube to create a genomic ID. On which pages of the Lab Guide do you find instructions on how to properly do such a task? Which methods on PCR and genome isolation will you use in your project, **when**?

3. To do PCR which **materials** will you need, and **why**? How will you design/choose your particular primers?

4. What resources beyond the *Laboratory Guide* will be helpful in completing your research project (list ALL relevant items you need to search for: papers, supplies, experts)? Costs $?

Group Interview (due Week 4)

During the lab period, your group will participate in an interview with an LA/TA. The purpose of the interview is to determine if you have a well prepared research plan. Below is a list of sample questions your group should be prepared to answer during the interview. Each member of the group will be asked questions about your plan, all members must be prepared.

Student Names:

Group Name:

Lab Assignment:

Interviewer:

What is the overall purpose of Labs **1, 2, and** 30? and each protocol? What types of protocols will you be performed during these labs? What new ones? (points)

***The interview may stop here if the group is clearly not prepared.**

What is the hypothesis or question being addressed by the research? (points)

What does the group predict the outcome will be and why? (points)

Are the experiments well planned? Do they address the hypothesis or research question in an appropriate manner? (points)

What types of controls will be used in the experiments? (points)

Interview questions

1. What was the official lab assignment, what are you doing this semester?
2. Pretend I am your mom/dad. Explain to me your research in lay-terms without diluting your purpose/finding.
3. What makes your project same vs. different from previous publications?
4. What research paper(s) is the most influential for your project (show me)?
5. Provide brief explanations about the significance and/or importance of background literature you have used?
6. Pull out a hard copy of one of the research papers you've read for your research project and explain to me this {title/figure/abstract/methods}.
7. Pull out a hard copy of the {Wittwer-PCR/Rowe-CF/Welsh-CF/Polonsky-Diabetes/Tokarz-Diabetes} paper & explain {title/figure/abstract/methods}.
8. What is the difference between a hypothesis and a prediction? Examples?
9. Define, provide examples, what is a positive control vs. negative control?
10. What are your hypotheses for your research project? What are the associated predictions for each hypothesis?
11. Identify relevance & purpose of the Lab 30 (30 Days) studies?
12. How do you plan to analyze your data?
13. Can you provide information on the CFTR gene (Give specifics: located? base pairs? function?)
14. How does CFTR gene correlate with pathologies seen in cystic fibrosis?
15. Imagine you are in an interview for your dream job, or for your dream med/vet/grad school, in 3 sentences, explain the research project you led this semester and impress me so much you can feel confident "you got it".

TURN IN TWO COPIES (One with your name, one without) **PID** ________________________

PEER REVIEW of DRAFT 2
Due at the start of your assigned Lab in week 8

TITLE OF PAPER-> ____

____

'GROUP NAME' OF AUTHORS ->____

Your peer review will be graded on both the quantity and quality of comments you write on this worksheet. This worksheet should take approximately one hour to complete.

Evaluate the paper's TITLE: (1pt)

A. How many characters does the paper have in its title?

B. What are the names of the specimens they are investigating?

C. Based only on the words in the paper's title, explain in 3 sentences what their research project is doing (methods) studying (specimen) and has found.

Evaluate the paper's ABSTRACT: (1pt)

A. How many words does the paper have in its abstract? What is the word limit?

B. In three sentences, state what the authors **did** and what they **found**.

C. In your own words, explain what hypotheses these scientists are testing in their research? Does their data support it or refute it. Why? Or Why Not?

Evaluate the paper's INTRODUCTION: (2pts)

A. Are you interested in learning more about this topic after reading the Introduction section?

B. Does the introduction discuss anyone else's research on this topic or other reference information from a book or paper etc? How many different citations are used?

C. What is their hypothesis, what question(s) are they investigating?

D. Identify the *topic* and *purpose* of the first 4 paragraphs in the *Introduction*. What topic of material are they discussing in each paragraph and why is it useful to the research project they plan to explain?

Paragraph 1**: Topic?**

Purpose?

Paragraph 2**: Topic?**

Purpose?

Paragraph 3**: Topic?**

Purpose?

Paragraph 4**: Topic?**

Purpose?

Evaluate the paper’s FIGURES/TABLES: (2pt)

A. Do all the figures and tables make sense and appear professional and are actually USED (ie cited) in the *Results* or other sections of the paper?

Look at the first 4 figures separately and decide, if you were a student in LB-144 and ONLY read this figure and it’s legend and NO other part of the manuscript, could you explain it to your mom? If not indicate why and list suggestions for improvement.

Evaluate Figure 1: If you ONLY read this figure and it’s legend “Can you explain it to Mom”?
Suggestions for improvement:

Evaluate Figure 2: If you ONLY read this figure and it’s legend “Can you explain it to Mom”?
Suggestions for improvement:

Evaluate Figure 3: If you ONLY read this figure and it’s legend “Can you explain it to Mom”?
Suggestions for improvement:

Evaluate Figure 4: If you ONLY read this figure and it’s legend “Can you explain it to Mom”?
Suggestions for improvement:

Evaluate the paper's DISCUSSION: (4pts)

Answer these questions using the paper's *Discussion* section as your ONLY source of information.

__ 1. What is the paper's TOPIC;

__ 2. What is the paper's clear, and preferably original, specific POINT (also known as a THESIS);

__ 3. What is the paper's adequate SUPPORT (REASONING and EVIDENCE) for that point;

__ 4. How the paper's discussion is ORGANIZED so that the audience can follow the points and examples;

__ 5. Two examples of where the paper employs CLEAR, PRECISE LANGUAGE;

__ 6. Two examples of where the paper is factually ACCURATE and also FAIR, including recognizing reasonable objections;

__ 7. Two examples of where the paper is presented in a way that is ENGAGING to the audience;

__ 8. Evidence that the paper is of a REASONABLE SIZE for the circumstances (not too short or too long);

__ 9. One example of where the paper clearly CREDITS OTHERS when their ideas and words are used.

__ 10. Two examples of where the paper indicates original PREDICTIONS and ultimate findings

LB-145 Interview Receipt

Student Names:________________________________

Group Name:___________

Lab Assignment:__________________

INTERVIEW of Student Preparation and Plan

☐ This group has successfully completed their interview and my proceed and begin their independent research.

LB-145 Prof/GA Interview

Student Names:____

Group Name:_______________________ Lab Assignment:___________________

INTERVIEW of Student Preparation and Plan (10 points)

Ask each member general questions about the laboratories and protocols in the Course Packet (write down responses). Then ask someone: What tests are done in each of your labs, how/what is your Do-It-Yourself-Lab? (creative, original assay) (points) etc

---you may stop your interview here if they're clearly not yet prepared---

What do they propose as their long-term research question(s)?

____

Ask question to determine these items:

Group clearly understands the questions their research is asking and explained what they think will happen (hypothesis-predictions) (points).

Group has good general plans for their research during the three independent research weeks (what are you doing during week 1, 2, 3?) (points)

Group identified appropriate 'control' experiments (and replications) they have planned to show the reagents and assays functioned properly (points).

Group clearly understands the different methods they plan to use each week ie "PCR", "Primer design and other Assays (Genome Prep, Gels)", "Do-It-Yourself methods" (points).

NAME __________________ DATE _______

“Figure Focus” Analysis Sheet

Use this worksheet to dissect important parts of a figure presented in a research paper

Figure Number**:**

INSTRUCTIONS: Answer each of the following questions as concisely as possible.

1) “Official” **title** for this figure or table (from the legend/caption) is:

2) My (simplified, decoded, in regular language) title for this figure or table is:

3) The *specific **hypothesis** being tested,* or *specific question asked* in the experiment here is:

4) *First*, review the important parts of the figure that show what the experimenters did and the information you wrote above. *Then*, answer the following for each figure or table:

a) The representations of their **data** (graphs, pictures etc):

If we compare these part(s)__________ and __________, of the figure to __________ and

___________, we learn about____

If we compare panel(s)__________ and __________, or columns __________ and

___________, we learn about____

If we compare panel(s)__________ and __________, or columns __________ and

___________, we learn about____

b) The ***controls*** in this experiment are:

They are represented where (in which part of the chart or graph, or what figure panels?)

5) Overall, what we learn from this figure is:

NAME ______________________________ DATE _______________

"Figure Focus" Analysis Sheet

Use this worksheet to dissect important parts of a figure presented in a research paper

Figure Number:

INSTRUCTIONS: Answer each of the following questions as concisely as possible.

1) "Official" title for this figure or table (from the legend/caption) is:

2) My (simplified, decoded, in regular language) title for this figure or table is:

3) The *specific* ***hypothesis*** *being tested* or *specific question asked* in the experiment here is:

4) *First*, review the important parts of the figure that show what experimenters did and the information you wrote above. *Then*, answer the following for each figure or table:

a) The representations of their data (graphs, pictures, etc.):

If we compare these part(s) ______________ and _____________, of the figure to ____________ and

____________, we learn about ____

If we compare these panels ______________ and _____________, or columns ____________ and

____________, we learn about ____

If we compare these panels ______________ and _____________, or columns ____________ and

____________, we learn about ____

b) The ***controls*** in this experiment are:

They are represented where (in which part of the chart or graph, or what figure panels?)

5) Overall, what we learn from this figure is*:*

NAME ______________________________ DATE _______________

“Figure Focus” Analysis Sheet

Use this worksheet to dissect important parts of a figure presented in a research paper

Figure Number:

INSTRUCTIONS: Answer each of the following questions as concisely as possible.

1) “Official” title for this figure or table (from the legend/caption) is:

2) My (simplified, decoded, in regular language) title for this figure or table is:

3) The *specific* ***hypothesis*** *being tested* or *specific question asked* in the experiment here is:

4) *First*, review the important parts of the figure that show what experimenters did and the information you wrote above. *Then*, answer the following for each figure or table:

a) The representations of their data (graphs, pictures, etc.):

If we compare these part(s) ______________ and _____________, of the figure to ____________ and

____________, we learn about ____

If we compare these panels ______________ and _____________, or columns ____________ and

____________, we learn about ____

If we compare these panels ______________ and _____________, or columns ____________ and

____________, we learn about ____

b) The ***controls*** in this experiment are:

They are represented where (in which part of the chart or graph, or what figure panels?)

5) Overall, what we learn from this figure is*:*

NAME ______________________________ DATE _______________

"Figure Focus" Analysis Sheet

Use this worksheet to dissect important parts of a figure presented in a research paper

Figure Number:

INSTRUCTIONS: Answer each of the following questions as concisely as possible.

1) "Official" title for this figure or table (from the legend/caption) is:

2) My (simplified, decoded, in regular language) title for this figure or table is:

3) The *specific* ***hypothesis*** *being tested* or *specific question asked* in the experiment here is:

4) *First*, review the important parts of the figure that show what experimenters did and the information you wrote above. *Then*, answer the following for each figure or table:

   a) The representations of their data (graphs, pictures, etc.):

If we compare these part(s) ______________ and _____________, of the figure to ____________ and

____________, we learn about ____

If we compare these panels ______________ and _____________, or columns ____________ and

____________, we learn about ____

If we compare these panels ______________ and _____________, or columns ____________ and

____________, we learn about ____

   b) The ***controls*** in this experiment are:

They are represented where (in which part of the chart or graph, or what figure panels?)

5) Overall, what we learn from this figure is*:*

Performance Based Assessment Sheets

Name ______________________________ Date ____________

Performance-Based Assessment

Topic: PCR Cycle Profile

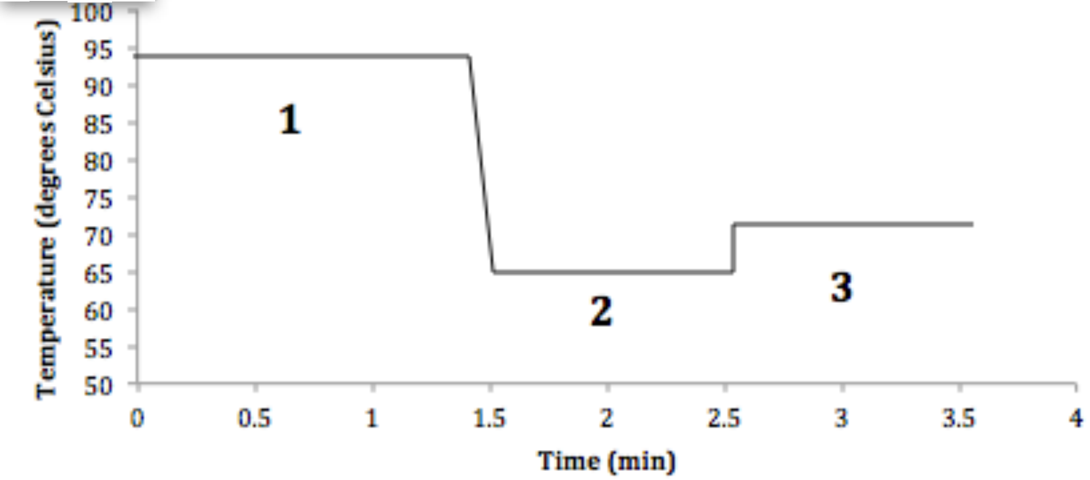


Fig 1. Typical PCR

Give the name of and explain, in one or two sentences (max), what is happening in the tube at each step.

1. Step 1 (2 pts)

2. Step 2 (2 pts)

3. Step 3 (2 pts)

Name ________________________________ Date _____________

Performance-Based Assessment

Topic: Primer Role in PCR

Briefly explain what role(s) the primers play in the PCR reaction.

Name ________________________________ Date _____________

Performance-Based Assessment

Topic: PCR Temperatures

Why can an enzymatic PCR reaction still function given the extremely high temperatures needed to carry out the process?

Name ______________________________ Date ____________

Performance-Based Assessment

Topic: PCR Ingredients

PCR synthesizes DNA strands using *taq*, a DNA polymerase. What ingredient of the PCR reaction cocktail is, in fact, the substrate for *taq* enzyme?

A. Reaction Buffer

B. The primers

C. Deoxynucleotides

D. Magnesium

E. *Taq* polymerase doesn’t need a substrate

Name ____________________________

Mutation Translation Exercise

1. Using the **base pairing rules** (Note: RNA U binds to A) and the **codon table**, determine the mRNA and protein sequences produced by the CFTR gene (below).

CFTR gene 5’ - ATGTACAACCGAGGGTAAAAAA - 3’ sense strand

mRNA 5’ - ____________________________________- 3’

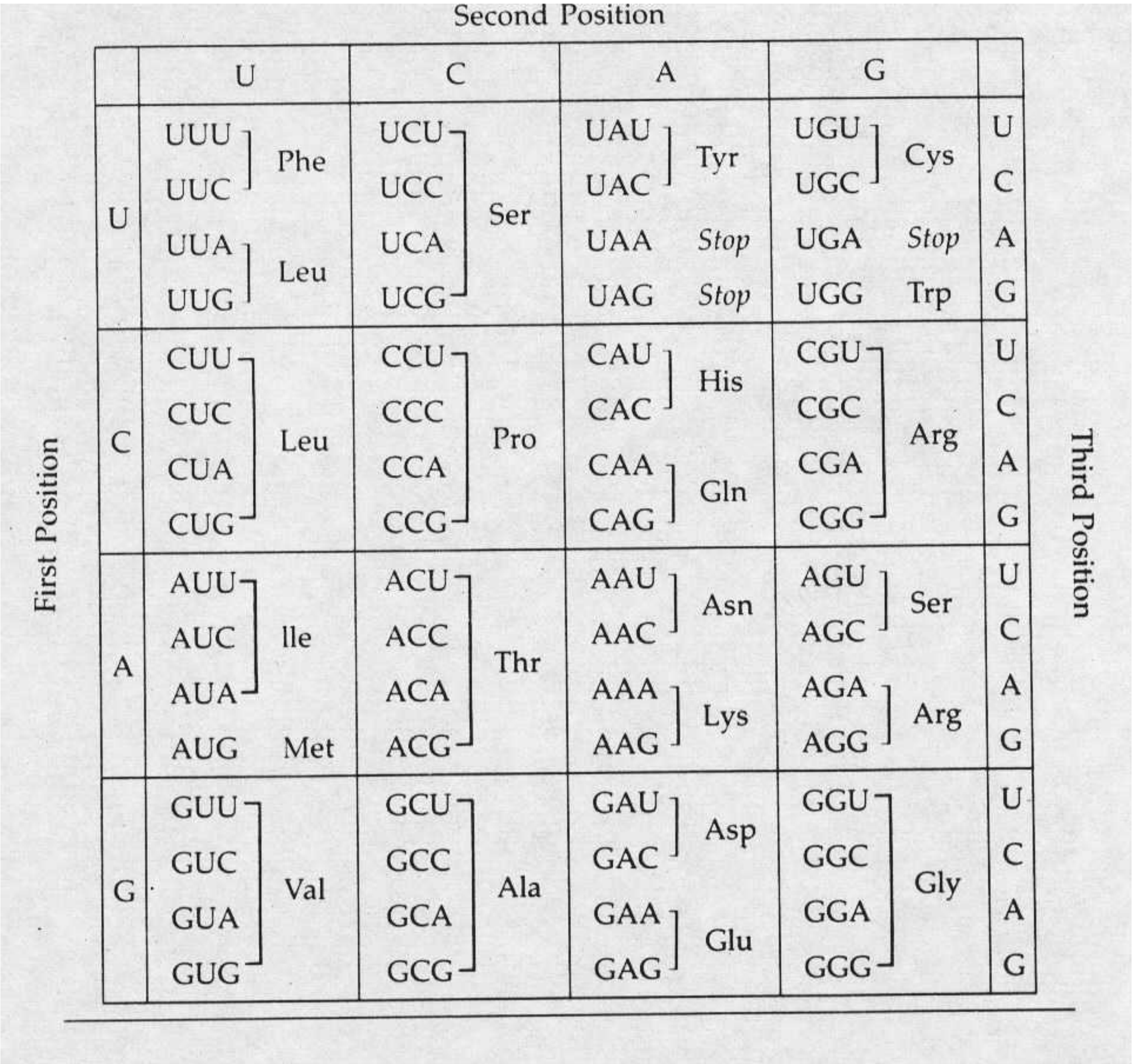
protein N-term.- ____________________________________ C- terminus

2. Exposure to cigarette smoke (a known mutagen) **deletes base #6** (cytosine) on the gene above. Now determine the resulting mRNA and protein sequences of the new mutant gene.

CFTR gene 5’ - ATGTA~~C~~AACCGAGGGTAAAAAA - 3’ sense strand

mRNA 5’ - ____________________________________- 3’

protein N-term.- ____________________________________ C- terminus



	Second Position								
First Position	U		C		A		G		Third Position
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA	Leu	UCA		UAA	*Stop*	UGA	*Stop*	A
	UUG		UCG		UAG	*Stop*	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

Product Literature Sheets

Invitrogen™
life technologies

1 Kb Plus DNA Ladder

Cat. No. 10787-018 **Size: 250 µg**
Conc.: 1 µg/µl **Store at -20°C.**

Description:

The 1 Kb Plus DNA Ladder is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000 bp to 12,000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band contains approximately 8% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ^{32}P input; (ii) Labeling the 5′ ends with T4 polynucleotide kinase; (iii) Filling in the 3′ recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:

10 mM Tris-HCl (pH 7.5)
1 mM EDTA
50 mM NaCl

Recommended Procedure:

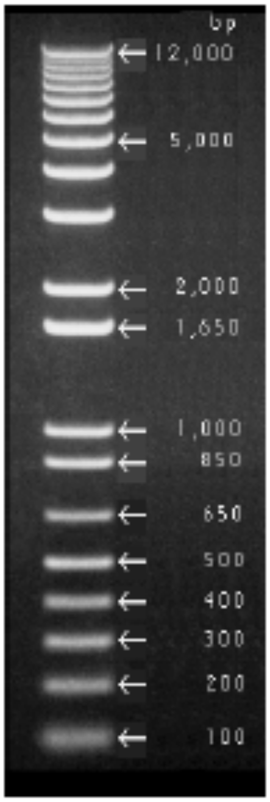
Invitrogen recommends the use of 10X BlueJuice™ Gel Loading Buffer (Cat. No. 10816-015) at a concentration of 2X for electrophoresis of DNA standards on agarose gels. Alternately, the DNA standard can be diluted such that the final concentration of NaCl is 20 mM. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that all bands in the ladder are distinguishable and are of approximate equal intensity by ethidium bromide staining.

Doc. Rev.: 021802

For technical questions about this product, call the Invitrogen Tech-Line℠ U.S.A. 800 955 6288



1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

$$\begin{array}{l} 5'\text{-GATCC}\text{———}\text{G} \\ \qquad\;\;\text{G}\text{———}\text{CCTAG-}5' \\ \qquad\quad\underbrace{\hphantom{———}}_{(996\text{ bp})_n} \end{array}$$

Notes:

During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

Cat. No. 10787-018

Invitrogen
life technologies

100 bp DNA Ladder

Cat. No. 15628-019 **Size: 50 µg**
Concentration: 1 µg/µl **Store at -20°C.**

Description:

The 100 bp DNA Ladder consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp. The 600 bp band is approximately 2 to 3 times brighter than the other ladder bands to provide internal orientation. This ladder is not designed for quantitation.

Storage Buffer:

10 mM Tris-HC1 (pH 7.5)
1 mM EDTA

Recommended Procedure:

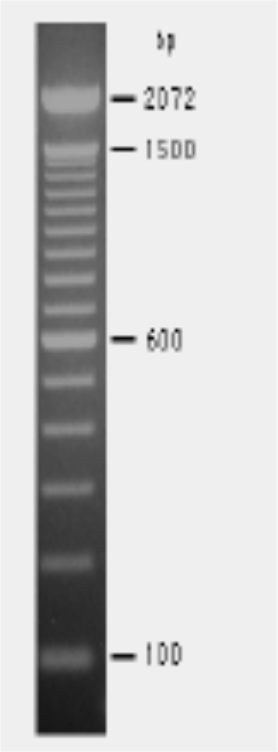
A final concentration of 20 mM NaCl is recommended for gel electrophoresis. Apply approximately 0.1 µg of ladder per mm lane width. **Do not heat** before loading.

Quality Control:

Agarose gel analysis shows that the bands between 100 to 1500 bp are distinguishable. The 600 bp band must be more intense than any other band except the band at 2072 bp.

Doc. Rev.: 102501

For technical questions about this product, call the Invitrogen Tech-Line℠ U.S.A. 800 955 6288



100 bp DNA Ladder
0.5 µg/lane
2% agarose gel stained with ethidium bromide.

Note:

During 2% agarose gel electrophoresis with tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates near the 100-bp fragment. The 100-bp band migrates behind the bromophenol blue marker on 6% polyacrylamide gels with tris-borate (pH 8.0) as the running buffer.

Part of the 600-bp band may migrate anomalously slowly in polyacrylamide gels (1,2,3). This band may appear as an extra band near or on top of the 700-bp band.

References:

1. Hsieh, C., et al. (1991) *Mol. Gen. Genet. 225*, 25.
2. Stellwagen, N.C. (1983) *Biochemistry 22*, 6186.
3. Jordan, H. and Hartley, J. (1997) *Focus® 19*, 9.

invitrogen™
by life technologies™

10 mM dNTP Mix, PCR Grade

Cat. nos.	**Size**	**Store at –30°C to –10°C**
18427-013	100 µL	
18427-088	1 mL	

Pub. Part no. 18427.pps MAN0001398 **Rev. Date** 7 Sep 2011

Description

10 mM dNTP (2'-deoxynucleoside 5'-triphosphate) Mix consists of all four nucleotides (dATP, dCTP, dGTP, dTTP), each at a concentration of 10 mM, in a solution of 0.6 mM Tris-HCl (pH 7.5). The 10 mM dNTP Mix is suitable for use in polymerase chain reaction (PCR), sequencing, fill-in, nick translation, cDNA synthesis, and TdT-tailing reactions.

Volumes and Concentrations

20 µL Final Reaction Volume

Final dNTP Conc.	dNTP Volume	Rxns per Kit	
		100 µL Kit	1 mL Kit
0.2 mM	0.4 µL	250	2,500
0.5 mM	1 µL	100	1,000
1.0 mM	2 µL	50	500
1.5 mM	3 µL	33	333

25 µL Final Reaction Volume

Final dNTP Conc.	dNTP Volume	Rxns per Kit	
		100 µL Kit	1 mL Kit
0.2 mM	0.5 µL	200	2,000
0.5 mM	1.25 µL	80	800
1.0 mM	2.5 µL	40	400
1.5 mM	3.75 µL	26	266

Intended Use: For research use only.
Not intended for any animal or human therapeutic or diagnostic use.

50 µL Final Reaction Volume

Final dNTP Conc.	dNTP Volume	Rxns per Kit	
		100 µL Kit	1 mL Kit
0.2 mM	1 µL	100	1,000
0.5 mM	2.5 µL	40	400
1.0 mM	5 µL	20	200
1.5 mM	7.5 µL	13	133

100 µL Final Reaction Volume

Final dNTP Conc.	dNTP Volume	Rxns per Kit	
		100 µL Kit	1 mL Kit
0.2 mM	2 µL	50	500
0.5 mM	5 µL	20	200
1.0 mM	10 µL	10	100
1.5 mM	15 µL	6	66

Additional Products

Product	Volume	Catalog no.
2.5 mM dNTP Mix	1 mL	R725-01
100 mM dNTP Set	4 × 250 µL	10297-018
100 mM dNTP Set	4 × 2.5 mL	10297-117

Reference

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) *Science 239*, 487.

Certificate of Analysis

The Certificate of Analysis (CofA) is available at www.invitrogen.com/cofa, and it is searchable by product lot number, which is printed on each box.

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com
www.lifetechnologies.com

invitrogen
by life technologies

Taq DNA Polymerase, recombinant

Cat. nos.	**Size**
10342-053	100 units
10342-020	500 units
10342-046	1,500 units
10342-178	5,000 units

Conc. 5 U/µL
Store at –30°C to –10°C

Pub. Part no. 10342.pps MAN0000814 **Rev. Date** 18 November 2011

Description

Taq DNA Polymerase is purified from *E. coli.* expressing a cloned *Thermus aquaticus* DNA polymerase gene. This enzyme has a 5' → 3' DNA polymerase and a 5' → 3' exonuclease activity but lacks a 3' → 5' exonuclease activity. The enzyme consists of a single polypeptide with a molecular weight of approximately 94 kDa. *Taq* DNA polymerase is heat-stable and synthesizes DNA at elevated temperatures from single-stranded templates in the presence of a primer.

Contents

Component	Kit Size			
	100 U	**500 U**	**1,500 U**	**5,000 U**
Taq DNA Polymerase	20 µL	100 µL	300 µL	1000 µL
10X PCR Buffer, Minus Mg^{++}	1.25 mL	2.5 mL	7.5 mL	20 mL
50 mM Magnesium Chloride	1 mL	1 mL	3 mL	10 mL

Storage Buffer

20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol, stabilizers.

Product Use: For research use only.
Not intended for any animal or human therapeutic or diagnostic use.

10X PCR Buffer

200 mM Tris-HCl (pH 8.4), 500 mM KCl.

The supplied PCR Buffer is a 10X concentrate and should be diluted for use.

Unit Definition

One unit incorporates 10 nmol of deoxyribonucleotide into DNA in 30 minutes at 74°C.

Basic PCR Protocol

The following basic protocol serves as a general guideline and a starting point for any PCR amplification. Optimal reaction conditions (incubation times and temperatures, concentration of *Taq* DNA Polymerase, primers, $MgCl_2$, and template DNA) vary and need to be optimized.

Critical parameters and troubleshooting information are documented in Innis et al., 1988. Assemble PCR reactions in a DNA-free environment. We recommend the use of clean dedicated automatic pipettors and aerosol resistant barrier tips. **Always** keep the control DNA and other templates to be amplified isolated from the other components.

1. Add the following components to a sterile 0.5-ml microcentrifuge tube sitting on ice:

Components	**Volume**	**Final Concentration**
10X PCR buffer minus Mg^{++}	10 µL	1X
10 mM dNTP mixture	2 µL	0.2 mM each
50 mM $MgCl_2$	3 µL	1.5 mM
Primer mix (10 µM each)	5 µL	0.5 µM each
Template DNA	1–20 µL	n/a
Taq DNA Polymerase (5 U/µL)	0.2–0.5 µL	1.0–2.5 units
Autoclaved distilled water	to 100 µL	n/a

   We recommend preparing a master mix for multiple reactions, to minimize reagent loss and enable accurate pipetting.

2. Mix contents of tube and overlay with 50 µL of mineral or silicone oil.
3. Cap tubes and centrifuge briefly to collect the contents to the bottom.

Continued on the next page

4. Incubate tubes in a thermal cycler at 94°C for 3 minutes to completely denature the template.
5. Perform 25–35 cycles of PCR amplification as follows:

Denature	94°C for 45 seconds
Anneal	55°C for 30 seconds
Extend	72°C for 1 min 30 seconds

6. Incubate for an additional 10 minutes at 72°C and maintain the reaction at 4°C. The samples can be stored at –20°C until use.
7. Analyze the amplification products by agarose gel electrophoresis and visualize by ethidium bromide staining. Use appropriate molecular weight standards.

"Hot Start" Protocol

In the "hot-start" method, the addition of *Taq* DNA Polymerase is withheld until the reaction temperature is at 80°C, to ensure high specificity of the products being synthesized.

1. Add all components as in the Basic PCR Protocol, except for the *Taq* DNA Polymerase.
2. Mix contents of tube and overlay with 50 µL of mineral or silicone oil.
3. Cap tubes and centrifuge briefly to collect the contents to the bottom.
4. Incubate tubes in a thermal cycler at 94°C for 3 minutes to completely denature the template.
5. After denaturation at 94°C, maintain the reaction at 80°C.
6. Add 0.2–0.5 µL of *Taq* DNA Polymerase (1.0–2.5 U) to each reaction. Be certain to add the enzyme beneath the layer of oil.
7. Continue with 25–35 cycles of denaturation, annealing and extension as in the Basic PCR Protocol.

Reference

Innis, M.A., Myambo, K.B., Gelfand, D.H. and Brow, M.A.D. (1988) *Proc. Natl. Acad. Sci. USA 85*, 9436.

Product Qualification and SDS

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/sds**.

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com
www.lifetechnologies.com

Research Publications

Cystic Fibrosis

Steven M. Rowe is an assistant professor of medicine, pediatric pulmonology, and physiology and biophysics at the University of Alabama at Birmingham.

J. P. Clancy is a professor of pediatric pulmonary medicine at Cincinnati Children's Hospital Center and the University of Cincinnati.

Eric J. Sorscher is a professor of medicine and a professor of physiology and biophysics at the University of Alabama at Birmingham.

MEDICINE

A BREATH OF FRESH AIR

Fundamental understanding of basic biology has set the stage for new treatments for cystic fibrosis

By Steven M. Rowe, J. P. Clancy and Eric J. Sorscher

Illustration by John Hendrix

IN 1989 WHEN SCIENTISTS DISCOVERED THE DEFECTIVE GENE THAT CAUSES CYSTIC FIBROSIS, a serious hereditary disorder that primarily strikes children of European descent, it seemed as though a long-hoped-for cure might soon follow. After all, tests in many laboratories showed that providing normal copies of the gene should enable patients to make healthy copies of the protein specified by the gene. If successful, that feat would go a long way toward restoring health in the tens of thousands of people around the world who suffered from cystic fibrosis and typically died in their late 20s. (Half of all patients now live to their late 30s or beyond.) The question was whether researchers would be able to reliably insert the correct gene into the proper tissues in patients' bodies to rid them of the illness forever.

That task proved harder than anyone had believed. Although scientists successfully engineered viruses to ferry copies of the correct gene into patients' cells, the viruses did not do the job well. By the late 1990s additional unexpected complications made it increasingly obvious that another approach to addressing the fundamental problem in cystic fibrosis would need to be found.

Meanwhile cell biologists and their colleagues undertook the long, challenging task of determining exactly what the normal protein looked like, how it functioned and how defects led to the symptoms of cystic fibrosis. These efforts included understanding the protein's three-dimensional shape in increasingly fine detail as well as the various ways the abnormal protein failed in its cellular duties. Instead of creating normal proteins by replacing the broken gene with an effective one—as was gene therapy's goal—this group of researchers focused on a different objective: finding a drug that allowed the deficient protein to work better. A fruitful search might give people with cystic fibrosis many additional years of a healthier life.

Today it looks as though the gradual but steady approach is paying off. Several new compounds are in the final stages of being tested for use in the treatment of cystic fibrosis—and one of them looks particularly promising for certain patients. If successful, it would be the first medication that targets the underlying cause of the disease, as opposed to dealing with symptoms. But that is not all. Preliminary studies indicate that these potential new treatments may also work against other, more common conditions, such as bronchitis, chronic sinusitis and pancreatitis, among others.

A PROBLEM WITH SALT

THE STORY OF HOW these drugs were identified begins with a dogged search to understand the basic biology of cystic fibrosis. The disease has long been known to result generally from a failure in the ability of certain body tissues to transport salt (sodium chloride) across the membranes that envelop cells. The cells in these tissues extrude the chloride part of the salt to help maintain the right balance between their fluid-filled interior and the watery exterior environment. As the chloride ions accumulate on the outside of the cell, water molecules follow suit, diffusing across the membrane to the outside. When the cell is finished constructing these tiny chloride channels, it inserts them into the membrane, where each protein forms a passageway that spans the cell border.

The gene that in 1989 was found to cause cystic fibrosis codes for one of these proteins, known as the cystic fibrosis transmembrane conductance regulator, or CFTR. The normal version of this molecule is made of a precise sequence of ap-

IN BRIEF

Cystic fibrosis is a serious hereditary disorder that fills certain organs in the body with a sticky mucus that interferes with the ability to digest food or breathe.

When researchers discovered the gene that causes cystic fibrosis in 1989, a long-hoped-for cure—in the form of gene therapy—seemed possible.

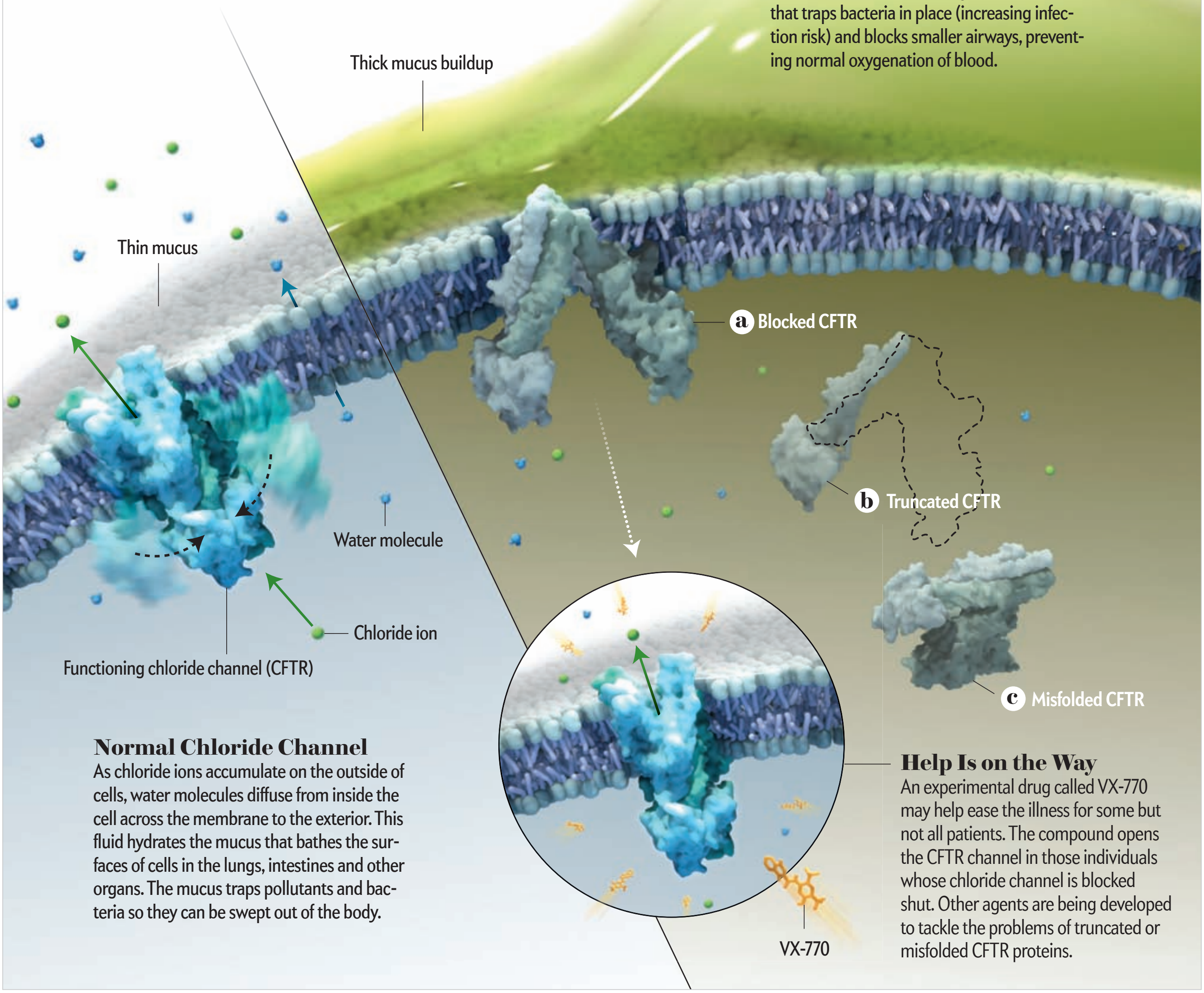
There were setbacks. But a new and different approach is now poised to deliver the first medications that address the fundamental causes of the disorder.

The Food and Drug Administration will probably begin to consider whether to approve a drug based on this latest research this year.

ANATOMY OF GENE DEFECT

Pathways to Trouble

To survive, cells constantly adjust their internal environment, such as by actively moving chloride ions out of the cell through a membrane-spanning protein called the CFTR channel. Mutations in the gene coding for this channel can lead to cystic fibrosis.



Abnormal Chloride Channel

Mutations in the cystic fibrosis gene cause illness in several ways, such as by leading to production of CFTR channels that are permanently blocked (*a*), truncated (*b*) or misfolded (*c*). The result: an abnormally thick mucus that traps bacteria in place (increasing infection risk) and blocks smaller airways, preventing normal oxygenation of blood.

Normal Chloride Channel

As chloride ions accumulate on the outside of cells, water molecules diffuse from inside the cell across the membrane to the exterior. This fluid hydrates the mucus that bathes the surfaces of cells in the lungs, intestines and other organs. The mucus traps pollutants and bacteria so they can be swept out of the body.

Help Is on the Way

An experimental drug called VX-770 may help ease the illness for some but not all patients. The compound opens the CFTR channel in those individuals whose chloride channel is blocked shut. Other agents are being developed to tackle the problems of truncated or misfolded CFTR proteins.

proximately 1,500 amino acids folded intricately and gracefully into a series of three-dimensional loops and sheets that spiral or plunge to form a number of different subsections. The flow of water molecules elicited by the movement of chloride ions through the channel helps to move the mucus that coats the surfaces of the body's airways, as well as the many ducts found in the intestines, pancreas and liver. The CFTR channel can also transport certain other ions, such as bicarbonate.

Here is where the trouble starts. Mutation of the gene results in the body lacking a proper CFTR channel. As a result, people with cystic fibrosis produce a sticky mucus that is so thick it interferes with many physiological processes. In the lungs, the gel-like mucus hampers the diffusion of oxygen into the air sacs and makes the simple act of breathing, as one of our young patients described it, like "trying to breathe with someone's hands over your face." Furthermore, the viscous buildup becomes an ideal breeding ground for serious infections by harmful bacteria, often *Pseudomonas aeruginosa*. In the pancreas, the thick and immobile secretions prevent the passage of digestive enzymes through various ducts into the intestines, interfering with proper digestion and, as a result, frequently causing people with cystic fibrosis to be underweight

Illustration by AXS Biomedical Animation Studio

or undernourished. Meanwhile bile becomes trapped in the liver, so fats are not properly processed, and blockages in the intestines lead to constipation and sometimes even a life-threatening shutdown of the entire gastrointestinal tract.

Before the advent of antibiotics to treat recurrent lung infections and the discovery of better nutritional therapy, most children with cystic fibrosis died in infancy. Over the past few decades advances in medical and supportive care have substantially prolonged the lives of individuals with the disease. Some of the treatments can seem rough to the uninitiated: parents or others are taught how to vibrate or pound their children's chest to help move the thick secretions in the lungs and dislodge any mucus plugs. Several drugs have been developed that open the airways, suppress infection or help thin the airway secretions. Supplemental vitamins and enzymes aid the digestive process. It is as a result of these and other measures that half of all cystic fibrosis patients now live to 37 years of age or older. None of these treatments, however, addresses the underlying cause: the insufficient flow of chloride and other ions out of cells.

THREE PATHWAYS

THE FIRST STEP in finding a drug that might restore at least some function to a deficient chloride channel was to better understand on a microscopic level the precise details of what goes wrong. Geneticists have tested DNA samples from cystic fibrosis patients around the world and have so far discovered more than 1,600 different mutations in the CFTR gene that lead to serious illness. The deleterious effects on the resulting CFTR protein can be divided into several groups. In three of the best-studied categories, the channel never gets put in place in the cell membrane, or a truncated channel is synthesized (and the directions for making it are rapidly degraded), or a channel of normal length is made but is unable to open or transport chloride or other ions. A single drug developed to repair one of these problems might not be of much help for the other two. Therefore, it is likely that to help the entire population of cystic fibrosis patients, different drugs will need to be developed—each based on the genetic defect responsible for an individual's condition.

A completely missing chloride channel at the cell surface stems from the most common genetic mutation, which results in the deletion of just one of the channel's 1,500 amino acid building blocks. Because the missing amino acid is phenylalanine (designated "F" in protein parlance) and is the 508th amino acid in the chain, the mutation is referred to as F508del.

The F508del mutation causes disease in a fashion that was at first surprising. Despite the mutation, the cell is able to build a chloride channel, amino acid by amino acid. The final product is equipped to transport chloride ions to a limited extent. But the cell's own molecular quality-control apparatus prevents it from doing so. The cell has several hundred helper proteins and enzymes that ferry the nascent CFTR molecules around the cell, inspect the ways they are being folded and help to insert CFTRs in the cell membrane. Even seemingly minor defects in folding—such as the F508 omission—can be rapidly recognized, leading the cell to quickly destroy the mutant. As a result, the somewhat functional chloride channel never even makes it to the cell membrane.

Because F508del is the most common cause of cystic fibrosis, numerous facilities around the world (including our own) are trying to locate the precise cellular checkpoints at which the F508del CFTR molecule fails to "make the grade" and is routed to the recycling bin. The goal is to aid in the discovery of compounds that will ease cystic fibrosis by helping the protein fold correctly and avoiding its destruction without interfering with the ability of the cells to recognize and eliminate other aberrant amino acid chains.

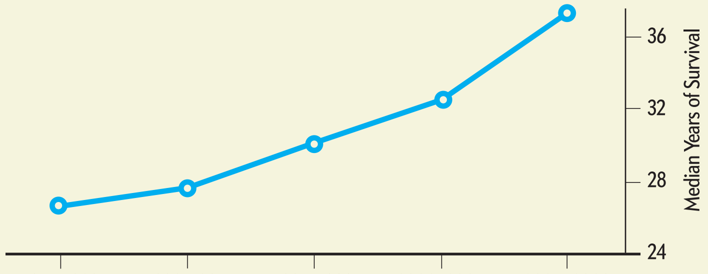
Figuring out how to adjust the cell's quality-control systems could offer benefits beyond treating cystic fibrosis. A number of chronic diseases—such as defects in cholesterol metabolism and some lung disorders (among them, alpha-1-antitrypsin deficiency)—occur because of protein misfolding. In at least certain diseases, evidence is growing that the true culprit is not the altered function of the protein per se but rather the propensity of the quality-control mechanism to degrade the abnormal molecule or deposit it in a tangled clump. It is conceivable that a number of slightly misfolded, mutant proteins would retain significant function if they were spared and allowed to do their intended jobs. Therapies that tackle the quality-control mechanism might therefore provide valuable insight into the biology and treatment of a wide range of diseases.

Mutations that result in abnormally foreshortened CFTR channels account for about 10 percent of cystic fibrosis cases worldwide. One

STEP BY STEP

Steady Progress

People with cystic fibrosis are already living longer thanks to new drugs and new approaches to delivering care. But agents that address the underlying biological cause would also help tremendously.



1994 A drug called dornase alpha improves pulmonary function by breaking down thick mucus in the lungs

1997 Routine spraying of the antibiotic tobramycin into the lungs helps to control infections

2002 The Cystic Fibrosis Foundation launches a quality-improvement program that standardizes treatment of U.S. patients

2009 Mandatory screening of newborns across the U.S. for cystic fibrosis allows for earlier detection and treatment

SOURCE: CYSTIC FIBROSIS FOUNDATION PATIENT REGISTRY: ANNUAL DATA REPORT 2009

Graphic by Jen Christiansen

such genetic defect, dubbed W1282X, underlies about 40 percent of cystic fibrosis in Israelis. The protein ends up being truncated because the gene contains misguided instructions telling the protein-synthesizing machinery to stop attaching amino acids to the growing protein molecule at position 1282 in the chain, where the amino acid tryptophan (signified by "W") would normally reside. Such genetic instructions are called nonsense codons and are crucial to the proper manufacture of proteins—provided they occur in the right place. In this case, however, the protein-manufacturing process comes to a premature halt. In addition, the intermediate instructions (known as messenger RNA) that guide the production process are also recognized as abnormal and destroyed so that even if the stop signal could somehow be skipped over, not enough of the now functional protein would be made. Therefore, drug treatments for W1282X may need to attack two problems and not just one.

The last set of mutations we consider here disables the channel's ability to open and accounts for about 5 percent of cystic fibrosis cases worldwide. In effect, these mutations cause the doorway through the membrane to be stuck in the closed configuration, which leaves the channel less able to transport chloride ions to the extracellular environment. One of the mutations that act in this way (called G551D) causes particularly severe symptoms. Although it is generally true that each group of genetic defects may require its own specifically targeted treatment, researchers have shown that compounds designed to prop open a mutant CFTR gate might aid patients who do not have this mutation. For example, take the case of a drug that enabled a small amount of the F508del CFTR to travel to its proper location in the cell membrane. A second drug that braced the channel door open would allow this somewhat sluggish version of the channel to pass more chloride out of the cell.

PROMISING DRUG CANDIDATES

THE NEXT STEP in the long process of drug development was to search for compounds able to alleviate the effects of specific mutations in the CFTR gene. It made sense to start with F508del because of its high prevalence in patients with cystic fibrosis and because the resulting protein retains some residual functionality; if researchers could help F508del CFTR evade premature degradation, the protein could arguably provide partial activity and improve lung function without further prodding.

Knowledge of the ways the F508del CFTR protein misfolds and of how the cell's quality-control machinery detects that folding defect is far from complete. It is, however, reasonably straightforward to determine whether a particular compound can alleviate the effects of the folding error in human cells. By loading fluorescent molecules into a cell, investigators can measure small changes in the concentrations of chloride or other ions moving across the cell membrane. When ions traverse the membrane after exposure to a potentially therapeutic drug, researchers can infer that the impaired CFTR channel has regained some function. If the ions do not make it across, the search continues for a more active compound. By automating and computerizing the drug-screening process, millions of compounds can be analyzed in a relatively short time.

Biotechnology company Vertex Pharmaceuticals identified one compound, known as VX-809, that had encouraging preliminary results but that did not significantly improve lung function in test subjects. Another company, PTC Therapeutics, is directing clinical trials of a drug called ataluren that addresses the less common, CFTR-truncation mutations. This agent causes the protein-making machinery to read through some of the misplaced "stop" instructions, thereby allowing the chloride channel protein to avoid being foreshortened. This agent is also being tested for other hereditary disorders that involve aberrant stop codes, such as Hurler syndrome and Duchenne muscular dystrophy.

Adjusting the cell's quality-control systems could offer benefits beyond treating cystic fibrosis, such as fixing defects in cholesterol metabolism and other disorders.

Scientists have had the best results counteracting the G551D mutation. After testing 228,000 different potential compounds against cells harboring chloride channels that did not open easily, a group of researchers at Vertex discovered a compound that selectively activates the energy switch of the CFTR channel, boosting function to 50 percent of normal levels. The laboratory results were good, and the compound, named VX-770, has now undergone extensive testing. In these trials, the ability to breathe improved substantially in patients with the G551D mutation within weeks, and the benefits were sustained for the length of a one-year study. Just as important, treated individuals were hospitalized less often and gained an average of seven to eight pounds. One of us (Rowe) was the first clinician in the U.S. to administer VX-770 in a patient. Sometime later this year Vertex plans to petition the Food and Drug Administration for the right to bring the drug to market. Further testing to use VX-770 in combination with other agents is also showing some promise in clinical trials. (The University of Alabama at Birmingham is one of the sites for clinical trials of drugs developed by PTC Therapeutics and Vertex; Rowe and Clancy have advised both companies about study design.)

These clinical results are groundbreaking, and the discovery of several drug candidates that take aim at the root cause of cystic fibrosis validates decades of research into its basic biology and funding of such work by the National Institutes of Health and the Cystic Fibrosis Foundation. Although more clinical trials are under way to establish whether the available compounds will be safe and effective in the long term for a larger group of patients, optimism is growing that one day we will finally be able to treat the underlying causes of this difficult disease.

MORE TO EXPLORE

Cystic Fibrosis. Steven M. Rowe, Stacey Miller and Eric J. Sorscher in *New England Journal of Medicine*, Vol. 352, pages 1992–2001; May 12, 2005. **www.nejm.org/doi/full/10.1056/NEJMra043184**

Managing Cystic Fibrosis: Strategies That Increase Life Expectancy and Improve Quality of Life. Malena Cohen-Cymberknoh, David Shoseyov and Eitan Kerem in *American Journal of Respiratory and Critical Care Medicine*, Vol. 183, No. 11, pages 1463–1471; June 2011.

PubMed Health on cystic fibrosis: **www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001167**

SCIENTIFIC AMERICAN ONLINE
Young people with cystic fibrosis share their lives, philosophies and daily routines on video at **ScientificAmerican.com/aug2011/cf-video**

Cystic Fibrosis

The genetic defects underlying this lethal disease have now been shown to eliminate or hobble a critical channel through which a constituent of salt enters and leaves cells

by Michael J. Welsh and Alan E. Smith

Woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die. This adage, from northern European folklore, is an early reference to the common genetic disease recognized today as cystic fibrosis. As the saying implies, the disorder once routinely killed children in infancy and is often identifiable by excessive salt in sweat. A salty brow is one of the more benign manifestations. The inherited genetic abnormality can also destroy the lungs and cause serious impairment of the pancreas, intestines and liver. Advances in therapy over the past few decades have brightened the outlook for afflicted children, enabling more than half of them to survive into their late twenties or beyond. But none of the approved treatments can yet correct the biochemical abnormality at the root of the condition, and none can remove the specter of an early death.

Hoping to do better, investigators began trying in the early 1980s to identify the specific genetic derangement that gives rise to cystic fibrosis. After almost a decade of struggle, they isolated the affected gene and pinpointed the mutation that most often leads to the disease. At the time, they could only guess at the gene's normal function—that is, at the role played by the protein produced from the healthy DNA. Since then, in an exciting series of discoveries, researchers have learned that the protein serves as a channel through which chloride, one component of salt, enters and leaves cells. They also have explained how damage to the gene blocks chloride transport, and they are exploring how the loss of chloride movement brings on the overt signs of cystic fibrosis. As was hoped, such findings are suggesting new ideas for therapy, some of which may one day cure the disorder.

The molecular advances that have led to this promising moment in medical history could not have been achieved without the pioneering efforts of physicians, many of whom gleaned their initial understanding of cystic fibrosis at the bedside. Indeed, for decades, clinical research yielded more information about the nature of the disease than did biochemical investigation.

One of the first major contributions came in 1938 from Dorothy H. Andersen of Columbia University. After performing autopsies on infants and children and reviewing the youngsters' case histories, Andersen provided the first comprehensive description of the symptoms of cystic fibrosis and of the changes produced in organs. Those changes, she noted, almost always included destruction of the pancreas (even in infants) and, often, infection of and damage to the lung airways. Andersen also gave the disease its name, calling it "cystic fibrosis of the pancreas," on the basis of microscopic features she observed in pancreatic tissue.

By the late 1940s physicians had further realized that ductal systems and other passageways in the organs affected by cystic fibrosis generally become clogged with unusually thick secretions. In the pancreas, for instance, ducts that deliver digestive enzymes to the intestines almost always become occluded, impairing the body's ability to break down food and extract nutrients from it.

In the lung it is the bronchial tubes and bronchioles that become obstructed. Those passages are usually bathed by a thin layer of mucus that traps inhaled particles and carries them to the throat for removal. But in patients with cystic fibrosis, the mucus is excessively thick and resistant to removal. This change by itself can narrow air passages and impair breathing. Moreover, when bacteria remain in the air passages, they can establish infections readily. These infections, which tend to recur, harm lung tissue by recruiting immune cells that secrete injurious chemicals and enzymes. As time goes by, chronic infection progressively destroys the bronchial passages and, together with the plugging of airways, ultimately leads to respiratory failure.

By 1946 studies of patients had also revealed something about the genetics of cystic fibrosis. After examining the pattern of disease inheritance in families, researchers deduced that cystic fibrosis was a recessive condition, probably caused by mutation of a single gene. If an infant inherited a damaged copy of the gene from both parents and therefore made no normal molecules of the protein specified by the gene, the child became ill; however, receipt of one good copy and one damaged copy did not produce disease.

Cystic fibrosis is now known to be among the most common genetic diseases and to strike mostly whites. About 5 percent of white Americans are asymptomatic carriers, harboring a single mutant version of the gene in their cells. One child in approximately 2,500 of European descent carries two defective copies and has the disease. In the U.S. such numbers translate into about 1,000 new cases a year and a total of some 30,000 people who live with the disorder today.

ABRAHAM MENASHE

Help from a Heat Wave

Roughly seven years after the inheritance pattern was delineated, New York City baked in a heat wave. Hospitals saw a disproportionate number of children with cystic fibrosis, who apparently became dehydrated more readily than other youngsters. Paul di Sant'Agnese and his colleagues at Columbia University then found that boys and girls with cystic fibrosis lose an excessive amount of salt in sweat. The reason for the increased saltiness would not be discerned for many years, but the observation had great clinical value. It resulted in development of a test that remains the cornerstone of diagnosis: measurement of the chloride content in perspiration.

Over the years, such clinical work has led to earlier, more accurate diagnosis and better treatments. For example, pancreatic failure is rarely life-threaten-

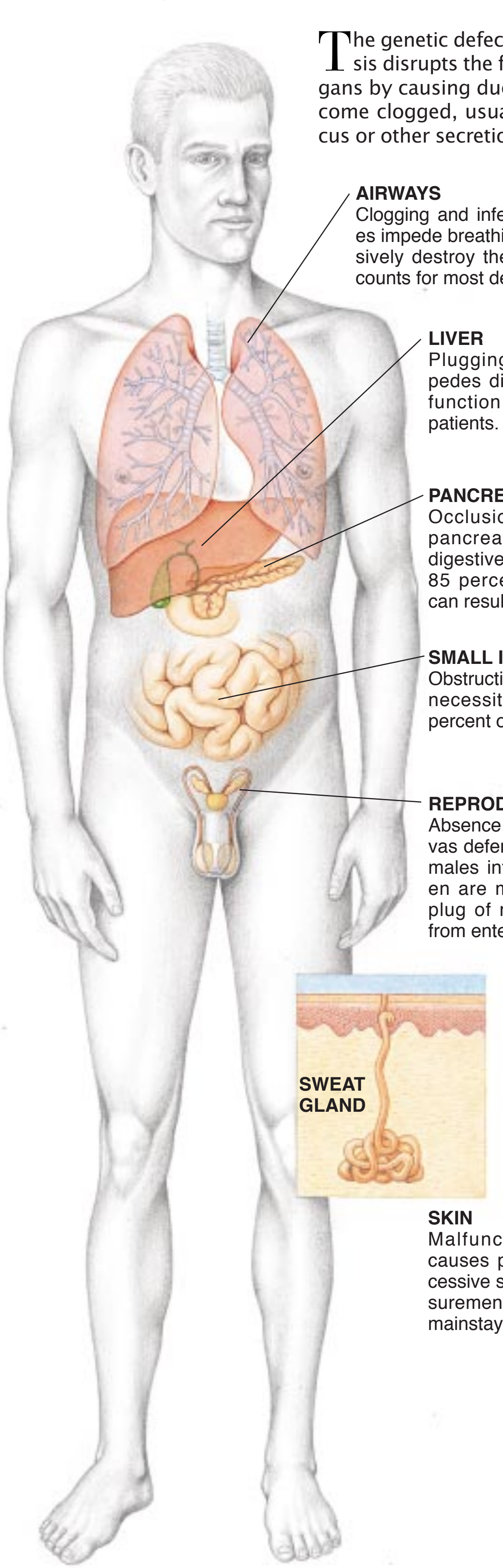
GENTLE POUNDING ON THE CHEST, or chest percussion, has long been a standard treatment for cystic fibrosis. The procedure aims to clear mucus from clogged airways in the lungs. Investigators hope that growing understanding of the molecular basis of the disease will lead to drug therapies that prevent airway obstruction in the first place. The child here is being tapped by her mother. The white unit on her arm delivers intravenous antibiotics to combat infection of the lung.

ing today because patients can replace their missing digestive enzymes with capsules taken when they eat. Now that the digestive problems can generally be controlled, the lung impairment accounts for more than 90 percent of the disability and death in patients with cystic fibrosis. Treatment options for the lung disease have expanded as well. Current therapy does include old standbys called postural drainage and chest percussion. Patients lie so that their head is tilted downward; someone then pounds gently and rapidly on their back or chest—as if hitting the bottom of a ketchup bottle—to try to clear mucus from the airways. But patients also benefit from a range of antibiotics that help to control the repeated infections (although usually without eliminating them). And about two years ago another treatment became available: inhalation of a drug called DNase. This compound aims to break up mucus by digesting long, sticky strands of DNA released from dying cells.

Research into the biochemical underpinnings of cystic fibrosis progressed more slowly than did the clinical work, but the pace intensified in the first half of the 1980s. During that time, scientists realized that malfunction of epithelial tissue was at fault in every organ impaired by cystic fibrosis. (An epithelium is a sheet of cells that forms a barrier between different compartments of the body; such sheets, which often secrete mucus, line the intestines and many ducts.) In particular, two avenues of investigation revealed that the epithelia of patients with cystic fibrosis were relatively impermeable to chloride. This discovery implied that some chloride-transporting channel in epithelial tissue was malfunctioning.

Organs Affected by Cystic Fibrosis

The genetic defect underlying cystic fibrosis disrupts the functioning of several organs by causing ducts or other tubes to become clogged, usually by thick, sticky mucus or other secretions.



AIRWAYS
Clogging and infection of bronchial passages impede breathing. The infections progressively destroy the lungs. Lung disease accounts for most deaths from cystic fibrosis.

LIVER
Plugging of small bile ducts impedes digestion and disrupts liver function in perhaps 5 percent of patients.

PANCREAS
Occlusion of ducts prevents the pancreas from delivering critical digestive enzymes to the bowel in 85 percent of patients. Diabetes can result as well.

SMALL INTESTINE
Obstruction of the gut by thick stool necessitates surgery in about 10 percent of newborns.

REPRODUCTIVE TRACT
Absence of fine ducts, such as the vas deferens, renders 95 percent of males infertile. Occasionally, women are made infertile by a dense plug of mucus that blocks sperm from entering the uterus.

SKIN
Malfunctioning of sweat glands causes perspiration to contain excessive salt (sodium chloride). Measurement of chloride in sweat is a mainstay of diagnosis.

ROBERTO OSTI

In one set of those investigations, Paul M. Quinton of the University of California at Riverside found that the epithelia lining the ducts of sweat glands failed to take up chloride efficiently from the cavity, or lumen, of the glands. This finding finally explained why people with cystic fibrosis have unusually salty sweat. Sweat is normally produced at the base of sweat glands; it then flows to the skin surface through a narrow duct. Initially the sweat is a solution rich in sodium and chloride ions—that is, the constituents of salt. But as the fluid traverses the duct, the ions escape into the epithelium, leaving the water behind. Thus, the sweat that emerges to cool the skin surface is only slightly salty. In patients with cystic fibrosis, in contrast, the inability of epithelial tissue to absorb chloride and the consequent impairment of sodium absorption from the duct lumen cause sweat to retain excess sodium and chloride and to become abnormally salty.

In the other line of study, Michael R. Knowles and Richard C. Boucher of the University of North Carolina at Chapel Hill examined the lungs. They found that chloride movement from epithelial tissue into the airway lumen was diminished and that sodium uptake by the epithelium was enhanced. Reduced chloride transport has now been demonstrated as well in the epithelia of the pancreatic ducts in mice and of the intestines in patients.

Finally, the Gene Is Found

As these studies of chloride transport were progressing, many scientists were engaged in an intense race to find the gene responsible for cystic fibrosis. That effort culminated in 1989, when a large group of collaborators, led by Lap-Chee Tsui and John R. Riordan of the Hospital for Sick Children in Toronto and by Francis S. Collins, then at the University of Michigan, announced it had isolated the gene. Aware that the protein product of the gene probably influenced the movement of chloride directly or indirectly, they named the protein the cystic fibrosis transmembrane conductance regulator (CFTR). While searching for the gene, the team also identified an abnormality in the DNA that appeared to account for about 70 percent of cystic fibrosis cases. That aberration, often denoted as the ΔF508 mutation, consists of the deletion of three nucleotides (DNA building blocks) from the gene. That loss causes the protein product of the gene to lack a single amino acid: phenylalanine at position 508.

The report was extraordinarily exciting for everyone concerned with cystic fibrosis; it promised to open new vistas of understanding and new options for therapy. Nevertheless, investigators desired additional evidence that the correct gene had been isolated. Strong support could be obtained by inserting a healthy version into cells from a patient with cystic fibrosis and thereby correcting the chloride transport defect. Frustratingly, workers had difficulty constructing even a streamlined version of the gene. By the summer of 1990, however, our colleague Richard J. Gregory of Genzyme Corporation had solved the problem.

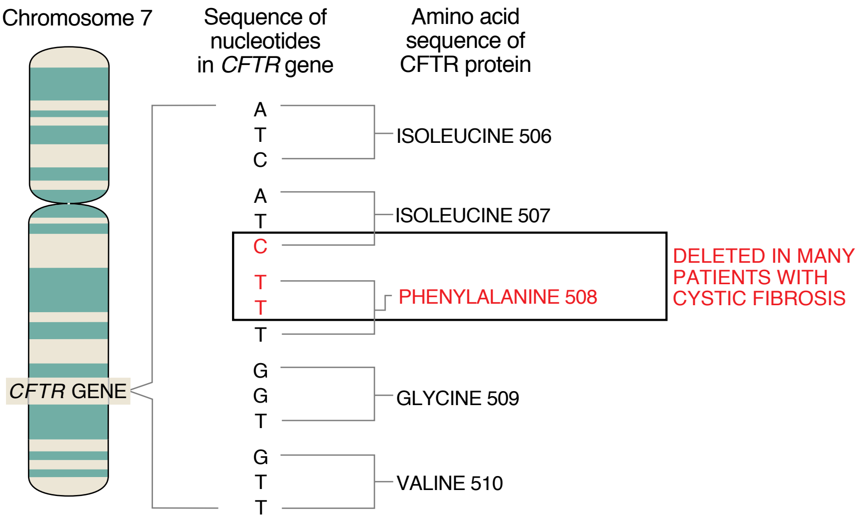
The two of us and our co-workers lost no time inserting the gene into epithelial cells isolated from the airways of patients with cystic fibrosis. Next we exposed the cells to cyclic AMP, a molecule that normally stimulates chloride transport in airway epithelium but has no effect on tissue from patients with cystic fibrosis. We were thrilled to see that cyclic AMP now caused chloride to stream out of the treated cells; the gene had apparently made the cells normal. We were not alone in our delight. Collins and a number of his colleagues had obtained similar findings using different methods in pancreatic epithelial cells.

The successes with cultured cells suggested that delivery of healthy *CFTR* genes to patients might correct their underlying biochemical abnormality—a tantalizing possibility. But we also knew, as will be seen, that there were many obstacles to attaining that goal. Meanwhile another obvious problem loomed over the field: resolving exactly how the CFTR protein influenced chloride movement.

What Does This Protein Do?

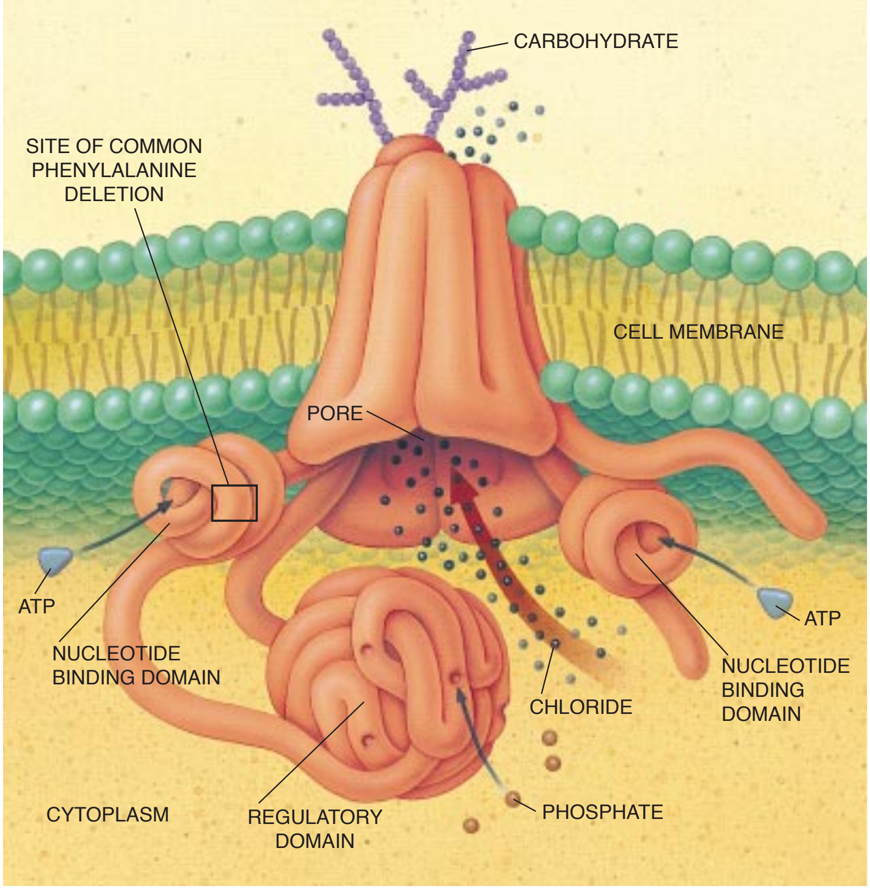
The linear sequence of amino acids in the protein, which was easily deduced once the gene was isolated, offered some immediate clues to the protein's normal behavior. Notably, the sequence was much like that found in a family of proteins called traffic ATPases or ABC transporters (because they carry what is known as an *A*TP *b*inding *c*assette). The similarity implied that the CFTR protein might also resemble the family in its behavior and in its folded, three-dimensional structure.

The traffic ATPase family includes a number of proteins used by bacteria to pump nutrients across their cell membrane; it also includes the drug-resistance protein that unfortunately ejects chemotherapeutic drugs from cancer cells [see "Multidrug Resistance in Cancer," by Norbert Kartner and Victor Ling; SCIENTIFIC AMERICAN, March 1989].



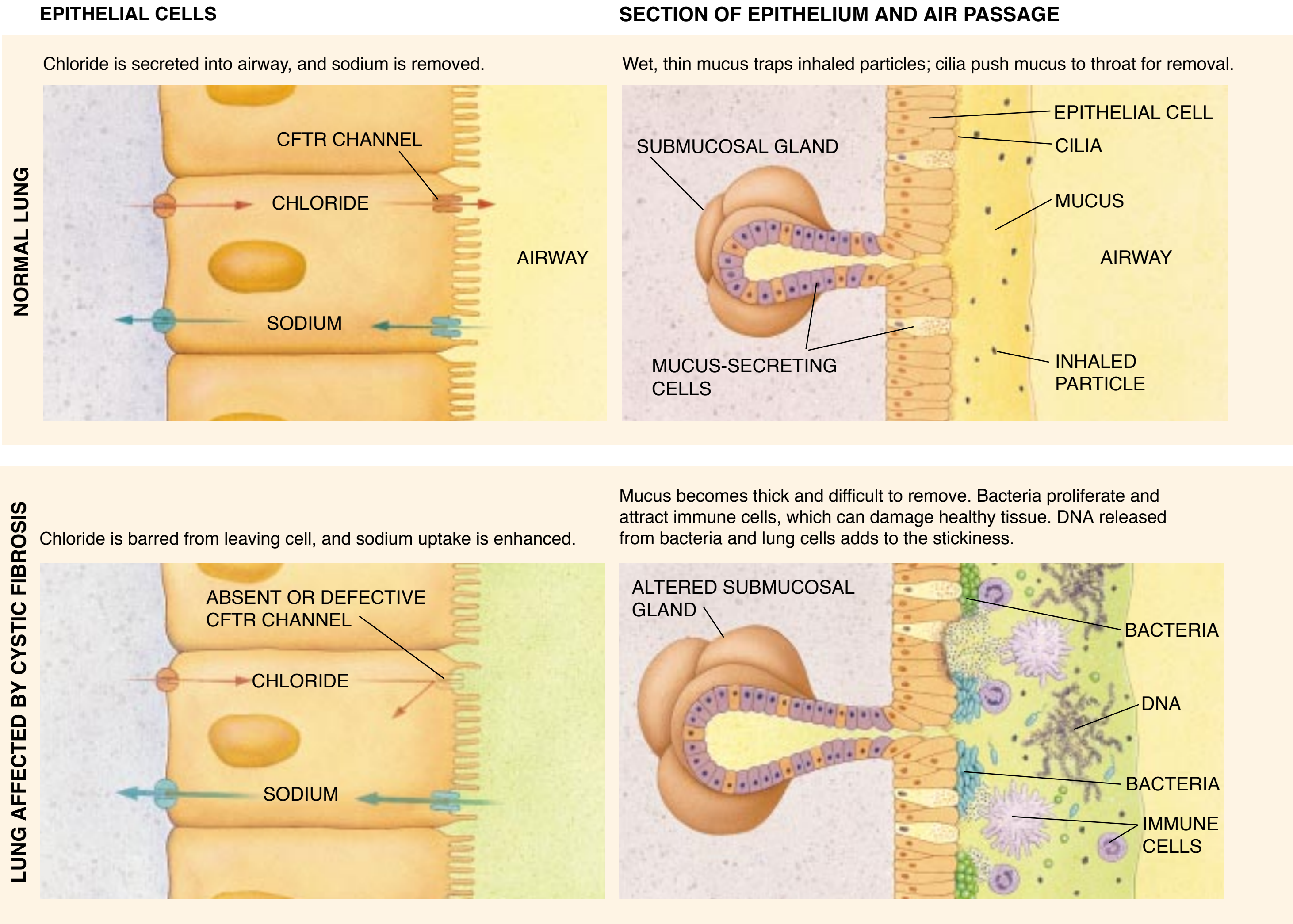
CAREY BALLARD

CYSTIC FIBROSIS GENE resides on chromosome 7 (*left*) and normally gives rise to a protein called the cystic fibrosis transmembrane conductance regulator (CFTR). The defect that most often leads to the disease is the deletion of three nucleotides from the gene (*red letters in center column*); this alteration, known as the ΔF508 mutation, results in the loss of one amino acid—phenylalanine at position 508—in the CFTR protein (*right*). Phenylalanine is lost because the protein-making machinery of the cell now sees ATT (an alternative way to encode isoleucine) at the gene region coding for the protein's 507th amino acid, followed by the GGT sequence for the glycine that normally follows phenylalanine.



ROBERTO OSTI

INTACT CFTR PROTEIN forms a chloride-permeable channel in the outer membrane of many cells. The precise structure has yet to be determined, but movement of chloride through the pore is known to be regulated by three cytoplasmic domains of the protein. Passage is allowed only when the two nucleotide binding domains dock with and cleave adenosine triphosphate (ATP) and when the regulatory domain becomes studded with phosphate groups.


EPITHELIAL CELLS

SECTION OF EPITHELIUM AND AIR PASSAGE

NORMAL LUNG

Chloride is secreted into airway, and sodium is removed.

Wet, thin mucus traps inhaled particles; cilia push mucus to throat for removal.

LUNG AFFECTED BY CYSTIC FIBROSIS

Chloride is barred from leaving cell, and sodium uptake is enhanced.

Mucus becomes thick and difficult to remove. Bacteria proliferate and attract immune cells, which can damage healthy tissue. DNA released from bacteria and lung cells adds to the stickiness.

MOLECULAR BASIS OF LUNG DISEASE in patients who have cystic fibrosis is complex. In healthy individuals (*top row*), the main epithelial cells lining the airways (*left panel*) display at least two types of channels at the surface facing the air passage. One—the CFTR channel (*red*)—releases chloride into the passage; the other (*blue*) takes up sodium. This arrangement somehow enables mucus made by other cells to remain wet, thin and easy to remove from the airways (*center panel*), and so the airways remain open (*right panel*). In patients with cystic fibrosis (*bottom row*), absence or malfunction of the CFTR channel prevents chloride movement (*left panel*) and indirectly causes cells to take up extra sodi-

When folded, these ATPases generally have four main structural parts, or domains: two that span the membrane (each of which contains several transmembrane segments) and two that dwell in the cytoplasm. The last two units, known as nucleotide binding domains, take up and cleave ATP (the nucleotide adenosine triphosphate) to obtain the energy required for pumping. The CFTR molecule was predicted to take essentially the same shape and, as will be seen, to have an added component residing in the cytoplasm.

Based on the activities of the ATPases, some researchers favored the hypothesis that CFTR was an ATP-driven pump that actively transferred some substance into or out of epithelial cells; the transported substance then induced chloride transport across the cell membrane through a separate channel. They posited this complex scheme because no known ion channels (such as would be needed to move chloride more directly) resembled the predicted folded structure of CFTR.

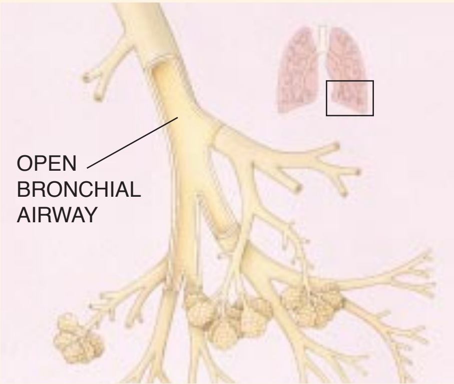
A second hypothesis proposed that CFTR itself attached to chloride channels and influenced their activity. And a third hypothesis held that CFTR might serve directly as a chloride channel even though its structure was unusual for any ion channel recognized at the time. In this scenario, the two membrane-spanning domains would form the pore through which chloride ions passed across the membrane.

As the work advanced, the data confirmed the third idea: CFTR formed a chloride channel on its own. We found that transfer of a gene for CFTR into chloride-impermeable cells conferred the ability to move that ion. If the gene was first altered in ways that affected parts of the CFTR protein thought to help chloride move through the channel, the channel's affinity for chloride decreased; this effect was shown by our colleague Matthew P. Anderson of the University of Iowa. Any last doubts were dispelled when Riordan and his colleagues inserted highly purified CFTR proteins into artificial cell membranes (lipid bilayers) containing no other channellike proteins. Addition of the protein allowed the ions to travel across the membrane.

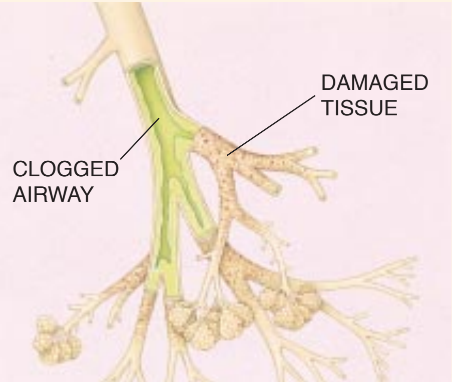
Subsequent investigations clarified the function of the "extra" CFTR component not found in traffic ATPases. On the basis of certain short sequences within that component, the mysterious

BRONCHIAL TUBES AND BRONCHIOLES

Airways stay clear for breathing.



Airways become plugged and begin to deteriorate.



ROBERTO OSTI

um (*thick blue arrow*). Then the mucus becomes thicker and more resistant to removal (*center panel*), and bacteria trapped there flourish. Together these changes plug the airways and lead to their destruction (*right panel*).

segment was deduced to be a regulatory domain—R—whose activity in the cytoplasm was controlled by the addition and removal of phosphate groups. Various experiments, including those by our colleagues Seng H. Cheng of Genzyme and Devra P. Rich of the University of Iowa, showed that when the R domain lacks phosphate groups, chloride ions cannot flow into the channel pore. But when chemical changes in a cell (specifically, rising levels of cyclic AMP) cause enzymes to dot the domain with phosphate, the addition promotes chloride movement through the pore.

It is helpful, though overly simplistic, to imagine that when the regulatory domain is not phosphorylated, it behaves like a gate blocking the cytoplasmic opening of the membrane pore. Addition of the phosphates somehow displaces the domain (opens the gate), allowing chloride ions to pass into the pore. Other analyses have demonstrated that the nucleotide binding domains influence the activity of the channel as well. For ions to go through the pore, those domains must bind to and probably cleave ATP.

How the Mutations Make Mischief

Knowing that the CFTR protein forms a chloride channel and having some idea of how the molecule functions leaves an important question still to be answered: Exactly how do mutations in the *CFTR* gene lead to loss of chloride transport? The effect of the most common DNA mutation—the deletion that leads to omission of phenylalanine 508 from the CFTR protein—has been the most extensively studied.

This deletion engenders what is known as an intracellular trafficking defect. Many proteins, among them the normal CFTR molecule, are processed after they are synthesized. They gain some sugar groups in a cellular compartment called the endoplasmic reticulum, after which they take up more sugar in the Golgi apparatus before being dispatched to the cell membrane. The mutant protein, in contrast, fails to leave the endoplasmic reticulum. Its travel is halted presumably because the quality-control system in the endoplasmic reticulum discerns that the protein is folded improperly. Proteins that are identified as defective are marked for degradation rather than being allowed to undergo further processing.

Although the phenylalanine 508 mutation is the most common one, hundreds of others have now been identified in people with cystic fibrosis. As is true of the 508 mutation, many of these changes block the protein from making its way to the cell membrane. Some prevent the CFTR protein from being made at all, and still others allow the protein to be produced and inserted into the cell membrane but bar the CFTR molecule from operating properly. In the last instance, the mutations may forestall chloride movement by disrupting the function of a nucleotide binding domain or by introducing a flaw into the lining of the ion-transporting pore.

In general, people whose cells carry two copies of the gene bearing the phenylalanine 508 mutation tend to have severe disease, probably because little if any of the mutated protein escapes from the endoplasmic reticulum. In people whose genes permit at least some CFTR to reach the cell membrane and to transport chloride to an extent,

Testing Dilemmas

Now that many genetic mutations leading to cystic fibrosis have been pinpointed, prospective parents can easily find out whether they are likely to be carriers of the disease—that is, whether their cells silently harbor a defective copy of the *CFTR* gene. Couples can also learn whether an already developing fetus has inherited two altered copies of the gene (one from each parent) and will thus be afflicted with cystic fibrosis.

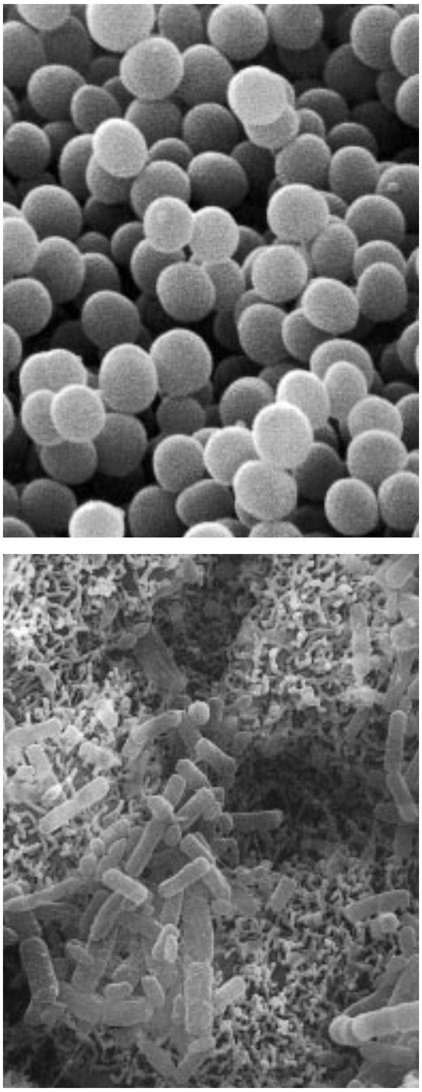
The difficulty for many people is deciding how to proceed once they receive their test results. The trouble arises in part because the laboratories that perform the genetic analyses do not detect every mutation in the *CFTR* gene. Consequently, a reassuring negative finding may not fully rule out the possibility that someone is a carrier or is affected with cystic fibrosis. (A favorable prenatal test result will be conclusive, however, if the fetus is shown to lack the specific *CFTR* mutants known to be carried by the parents.) Moreover, it is not yet possible to predict the extent of symptoms in a person who inherits two *CFTR* mutants; even if the inherited genes are usually associated with highly severe or less severe disease, such associations do not necessarily hold true in every individual.

Some couples may be tempted to think that research will progress fast enough to protect children born today from the life-threatening lung damage characteristic of cystic fibrosis. Yet medical investigations often hit unexpected obstacles and suffer setbacks before they achieve their ultimate goals. Hence, although it is probable that treatment will become more effective—perhaps markedly so—in the coming years, no one can foretell exactly when cystic fibrosis will become significantly easier to manage. Prospective parents need to understand, therefore, that a child born with cystic fibrosis today will still have to cope with the disease and may not be spared a premature death.

Such uncertainties render decision making extremely challenging. This is an exciting time in cystic fibrosis research, but it is also a trying one for couples caught in the gap between current technology and anticipated advances that have not yet become a reality.—*M.J.W. and A.E.S.*

the residual activity can make for somewhat less severe symptoms. These patterns do not always hold, however, and so making predictions in individual cases remains problematic. Indeed, two patients with exactly the same mutations in both copies of their *CFTR* gene can differ significantly in the extent of organ damage they suffer. This divergence arises because other genetic and environmental factors that remain poorly understood can probably influence the course of the disease.

It is humbling to note that burgeoning understanding of the genetic defects has not yet fully explained how disordered chloride transport in the lung epithelium alters sodium transport and how those changes result in the accumulation of mucus in the bronchial passages. It has also been discovered that submucosal glands—mucus producers that lie below the surface epithelium—produce a large amount of the CFTR protein. What role do these glands play in the disease? Scientists are further puzzled by the fact that the airways of patients with cystic fibrosis are predisposed to infection by some bacteria more than by others. For instance, infections by *Pseudomonas aeruginosa* and *Staphylococcus aureus* are particularly common. An understanding of why certain organisms thrive is only now beginning to emerge.



TONY BRAIN *Science Photo Library*

DAVID M. PHILLIPS *Population Council, Science Source*

BACTERIA that often cause severe infections in the lungs of patients with cystic fibrosis include *Staphylococcus aureus* (*top*) and *Pseudomonas aeruginosa* (*bottom*). Once the infections are established, they almost invariably recur.

Investigators wonder as well whether the CFTR protein has functions beyond its role as a chloride channel. Among the possibilities being considered is that CFTR may help regulate chloride channels distinct from CFTR. Researchers have also posited that the molecule may indirectly alter the mix of sugars on the epithelial surface in ways that favor colonization by certain bacteria.

Strategies for Treatment

In spite of the unanswered questions, the knowledge gained since 1989 has already suggested several avenues for attacking cystic fibrosis. One is to compensate for the loss of the CFTR chloride channel by increasing the activity of a different class of chloride channel. For instance, channels controlled by calcium ions are known to exist in the lumen-facing surface of epithelial cells. Those molecules usually fail to counteract the loss of the CFTR channel, but perhaps their chloride conductance can be increased artificially. This possibility is being tested in patients.

One day doctors also might deliver purified CFTR proteins to the cells that need them. Studies of cells in culture have shown that the protein molecules can correct chloride flow in cells carrying a mutant *CFTR* gene. In theory, another tactic would be to administer drugs able to escort mutant CFTR molecules from the endoplasmic reticulum through the Golgi apparatus and into the cell membrane. This idea seems worth pursuing because ΔF508 mutant CFTR proteins that become stuck in the endoplasmic reticulum usually function fairly well when experimentally inserted into the outer membrane of cells. At present, however, we know of no drugs that can correct the intracellular trafficking abnormality. A different approach, not yet tested, would be to use drugs to increase the activity of any mutant CFTR channels that do find their way into the cell membrane.

The treatment option attracting the most attention, however, is gene therapy, which aims to deliver a normal copy of the *CFTR* gene to the cells that need it. If all goes well, the DNA inserted into target cells should direct synthesis of the normal CFTR protein and reverse the primary biochemical abnormality at the root of cystic fibrosis. Introduction of the gene is a favored approach because it should replace all functions of the CFTR protein, including any that have not yet been recognized.

The best-studied method of gene therapy exploits the ability of viruses to enter cells, bringing their DNA with them. We and others have paid special attention to adenoviruses as gene carriers, or vectors, because those microbes are naturally able to infect human airways but will usually produce relatively innocuous disease, such as the common cold. The adenoviruses are altered in two ways: certain viral genes are removed to prevent the virus from reproducing in cells and causing symptoms. And the excised DNA is replaced with a normal *CFTR* gene. Our group, as well as those of Ronald G. Crystal, then at the National Heart, Lung and Blood Institute, and James M. Wilson, then at the University of Michigan, has demonstrated that such vectors can deliver the *CFTR* gene to cultured epithelial cells and to airway cells in animals. What is more, the cells use the DNA to synthesize CFTR molecules that function as healthy chloride channels.

On the basis of such experiments, several research groups have begun attempting to deliver the *CFTR* gene to patients via genetically engineered adenovirus vectors. The aim of these early experiments is primarily to assess safety. Even so, we and others have also tested the ability of a *CFTR*-bearing adenovirus to correct chloride transport in the nasal epithelium of patients. We chose the nasal epithelium because it is similar to that of the bronchial passages but is easier to reach.

Our first test was encouraging. For experimental purposes, we applied the altered virus directly to a small patch of epithelium in the nose. The treatment partially corrected chloride transport for a time. Since then, however, a similar study by us has been less successful, and one by another group showed no increase in chloride flow. These findings indicate that adenoviral vectors need to be improved substantially before they can serve as gene-delivery agents in therapy.

Even if ways are found to increase the efficiency of gene delivery by the viruses, another challenge would remain. Most cells in epithelial tissue are replaced every few months. Therefore, gene therapy would probably have to

Some Strategies for Treating Lung Abnormalities

The lung disease characteristic of cystic fibrosis can be attacked at many levels. Potential strategies range from reversing the genetic defect at the root of the pulmonary problems to replacing a failed lung with a healthy one.

ABNORMALITY	APPROACH	STATUS
Mutation in *CFTR* gene	Provide normal gene through gene therapy; provide normal CFTR protein to cells	Gene therapy is being tested in preliminary clinical trials; methods for protein delivery are inefficient
Defective delivery of CFTR protein to outer cell membrane	Supply drugs able to escort protein to cell membrane of epithelial cells	No candidate “escorts” have been identified
Defective movement of chloride ions through CFTR channels in cell membrane	Deliver drugs that increase activity of other classes of chloride channel in epithelial cells	Such drugs are being tested in preliminary clinical trials
Clogging of air passages by viscous mucus	Pound back and chest to help clear secretions; administer DNase and other drugs to liquefy secretions	Chest percussion is standard therapy; DNase is now in wide use, and similar drugs are being tested in animals
Development of recurrent infections that can damage lungs	Deliver antibiotics to destroy bacteria or provide antibodies (special molecules of immune system) to remove microbes	Antibiotics are in wide use; antibodies are being tested in preliminary clinical trials
Tissue damage caused by immune response to bacteria	Administer drugs that reduce harmful effects of immune response	Steroidal anti-inflammatory drugs are sometimes used; nonsteroidal anti-inflammatory agents (mainly ibuprofen) are being tested
Destruction of lung	Transplant healthy lung	Transplantation is sometimes an option

be administered a few times a year—at least until the rare, long-lived cells that give rise to the replacement cells can be induced to take up a normal *CFTR* gene permanently. Aside from inconvenience and expense, the need for multiple treatments is a concern because people respond to adenoviruses by mounting an immune response that ultimately eliminates the microbes and prevents repeated infection. For gene therapy to be successful, investigators will have to find ways to “hide” the adenoviruses from the immune system or to create viral or other vectors that do not elicit an immune response.

One appealing alternative to relying on viruses would be to coat the therapeutic gene with fatty molecules that are not recognized by the immune system but that nonetheless enable the DNA to enter cells. Recent studies conducted on human patients by Eric Alton and his co-workers at the Royal Brompton Hospital in London suggest this approach can restore chloride permeability to airway epithelium, although this group, like ours, has so far studied only nasal tissue. Moreover, delivery of genes by nonviral systems needs to be made more efficient.

Scientists have much to learn before they understand exactly how loss of the CFTR protein leads to the manifestations of cystic fibrosis. And a host of technical challenges must be eliminated before any therapy will routinely compensate for that loss. Nevertheless, progress is being made on many fronts. It is difficult not to be optimistic that the ongoing work will produce improved therapies within the next several years.

The Authors

MICHAEL J. WELSH and ALAN E. SMITH have collaborated for several years. Welsh, a Howard Hughes Medical Institute investigator, is professor of medicine and of physiology and biophysics at the University of Iowa College of Medicine. He earned his M.D. at Iowa in 1974 and held fellowships at the University of California, San Francisco, and the University of Texas Medical School at Houston before returning to Iowa in 1981. Smith is senior vice president of research at Genzyme Corporation in Framingham, Mass. He has also been head of the biochemistry division of the National Institute for Medical Research in Mill Hill, England, and scientific director and vice president of Integrated Genetics, also in Framingham.

Further Reading

CYSTIC FIBROSIS: MOLECULAR BIOLOGY AND THERAPEUTIC IMPLICATIONS. Francis S. Collins in *Science,* Vol. 256, pages 774–779; May 8, 1992.

CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR: A CHLORIDE CHANNEL WITH NOVEL REGULATION. M. J. Welsh, M. P. Anderson, D. P. Rich, H. A. Berger, G. M. Denning, L. S. Ostedgaard, D. N. Sheppard, S. H. Cheng, R. J. Gregory and A. E. Smith in *Neuron,* Vol. 8, No. 5, pages 821–829; May 1992.

THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR. J. R. Riordan in *Annual Review of Physiology,* Vol. 55, pages 609–630; 1993.

MOLECULAR MECHANISMS OF CFTR CHLORIDE CHANNEL DYSFUNCTION IN CYSTIC FIBROSIS. M. J. Welsh and A. E. Smith in *Cell,* Vol. 73, No. 7, pages 1251–1254; July 2, 1993.

CYSTIC FIBROSIS. M. J. Welsh, L. C. Tsui, T. F. Boat and A. L. Beaudet in *Metabolic and Molecular Basis of Inherited Disease.* Edited by C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle. McGraw-Hill, 1994.

The NEW ENGLAND JOURNAL of MEDICINE

REVIEW ARTICLE

MECHANISMS OF DISEASE

Cystic Fibrosis

Steven M. Rowe, M.D., Stacey Miller, B.S., and Eric J. Sorscher, M.D.

From the Gregory Fleming James Cystic Fibrosis Research Center (S.M.R., S.M., E.J.S.), and the Departments of Medicine (S.M.R., E.J.S.), Pediatrics (S.M.R.), Genetics (E.J.S.), and Physiology and Biophysics (E.J.S.), University of Alabama at Birmingham, Birmingham. Address reprint requests to Dr. Sorscher at the Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, 1530 3rd Ave. S., MCLM 796, Birmingham, AL 35294, or at sorscher@uab.edu.

Dr. Rowe and Ms. Miller contributed equally to this article.

N Engl J Med 2005;352:1992-2001.

Advances in the care of patients with cystic fibrosis have improved survival, and as a result, patients with the disease now often live beyond the third decade.[1] In addition, developments in the understanding of the genetics and molecular mechanisms of cystic fibrosis have led to new targets for treatment and an increasingly hopeful outlook. This review summarizes the mechanisms underlying the disease, the sequelae stemming from the absence of a functioning cystic fibrosis transmembrane conductance regulator (CFTR), and the therapeutic strategies devised to correct these abnormalities. Progress in the supportive care of patients with the disease has been reviewed elsewhere.[2-4]

HISTORICAL BACKGROUND

In 1949, Lowe et al. postulated that cystic fibrosis must be caused by a defect in a single gene (and therefore a single protein) on the basis of the autosomal recessive pattern of inheritance of the disease.[5] The characterization of the molecular mechanism, therefore, included early attempts to identify the causative protein. High levels of salt in the sweat of patients with cystic fibrosis suggested an abnormality in fluid and electrolyte transport in the sweat gland, and Quinton established that sweat ducts in such patients are impermeable to chloride.[6] Studies of nasal epithelium and subsequent membrane patch-clamp analysis of epithelial cells from the airways of patients with cystic fibrosis provided conclusive evidence of a defect in chloride permeability of plasma membranes in the lung.[7-10] These findings, which were confirmed by several laboratories worldwide, led to the hypothesis that a defective chloride channel situated in the apical membranes of the lung surface or glandular epithelium accounts for respiratory failure and that this abnormality could explain the other clinical manifestations of cystic fibrosis.

Soon after the discovery of abnormal chloride transport in cystic fibrosis, Collins, Riordan, Tsui, and colleagues identified the gene that is responsible for the disease with the use of linkage-based techniques, independently of any prior knowledge of the structure of the cystic fibrosis protein.[11-13] With a measure of circumspection, they named the gene product CFTR, since the predicted protein sequence did not resemble other ion channels. This name posed a problem for those who thought that a chloride channel was primarily responsible for the clinical manifestations of cystic fibrosis.

STRUCTURE AND FUNCTION OF CFTR

The hypothesized structure of CFTR placed it squarely in the ATP-binding cassette (ABC), or traffic ATPase, gene family. As is characteristic of this gene family, CFTR protein contains 2 ATP-hydrolysis domains (also termed nucleotide-binding domains) and 12 membrane-spanning alpha helixes (Fig. 1). ABC proteins were known to function as mediators of organic solute transport and included, for example, the genes that encode multidrug resistance (e.g., *MDRs*, or P-glycoprotein genes), a gene that encodes chloroquine resistance in *Plasmodium falciparum*, and a number of prokaryotic and eukaryotic small nutrient and molecular transporters.[14] Because there was no reason to suspect that the cystic fibrosis chloride channel should require ATP hydrolysis, and since the ABC protein family was not characteristic of other ion transporters, the possibility was left open that CFTR might indirectly regulate cellular chloride permeability by other means, rather than act as a chloride channel itself.

These findings caused a measure of scientific drama that was ultimately resolved in two ways. First, Welsh and colleagues showed that the expression of *CFTR* in cells that lacked chloride channels led to the appearance of a new chloride permeability pathway.[15] In a formal sense, these studies still did not distinguish between the role of CFTR as a chloride channel and its role as a regulator of anion conductance that was unmasked by the expression of *CFTR* in cells lacking chloride channels. The subsequent observations that point mutations in the *CFTR* gene caused subtle alterations in ion-channel selectivity, single-channel conductance, channel gating, and other properties of CFTR supported a direct role of the CFTR protein as a chloride channel. Bear et al. made a second major contribution when they synthesized CFTR in cell-free systems, reconstituted the polytopic membrane protein in lipid bilayers,[16] and definitively established that purified CFTR could act (at least in part) as an ion channel regulated by cyclic AMP (cAMP). These experiments thus reconciled a scientific debate concerning the relationship between CFTR and epithelial ion transport.

Numerous laboratories have now established that CFTR conducts chloride across the cell membrane and is regulated by protein kinase A (PKA) in a cAMP-dependent fashion. The PKA sites within the protein serve as targets for phosphorylation. ATP hydrolysis is mediated by nucleotide-binding domains within the full-length ion channel. A number of other cellular functions have been ascribed to CFTR: it down-regulates transepithelial sodium transport, in particular the epithelial sodium channel[7,17]; it also regulates calcium-activated chloride channels and potassium channels and may serve important functions in exocytosis and the formation of molecular complexes in the plasma membrane.

In the apical plasma membrane, CFTR is part of a multiprotein assembly. The final three amino acids (threonine, arginine, and leucine) anchor the protein to PDZ-type receptors (PDZ domains occur in intracellular signaling proteins and other proteins associated with the plasma membrane), in close proximity to a number of membrane receptors, ion channels, and the cytoskeleton.[18] Thus, it appears that the role of CFTR in epithelial cells may extend well beyond chloride permeability. In human cystic fibrosis, and in mice with targeted deletions of the *Cftr* gene,[19] the absence of CFTR influences the expression of several other gene products, including proteins important in inflammatory responses, maturational processing, ion transport, and cell signaling. These other proteins are potential modifiers of the cystic fibrosis phenotype and may help explain the substantial differences in clinical severity among patients with the same mutations in *CFTR*.

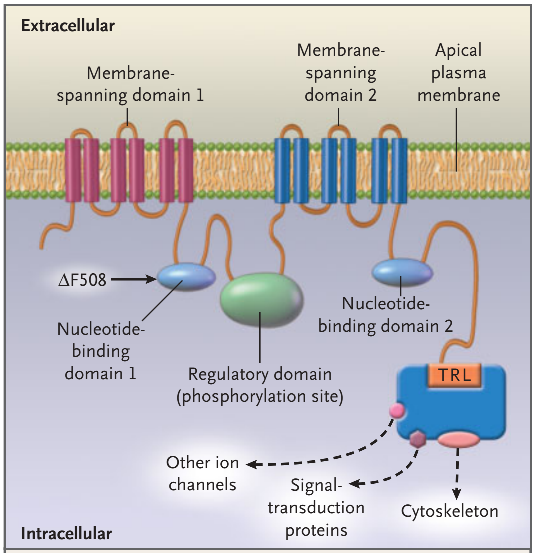
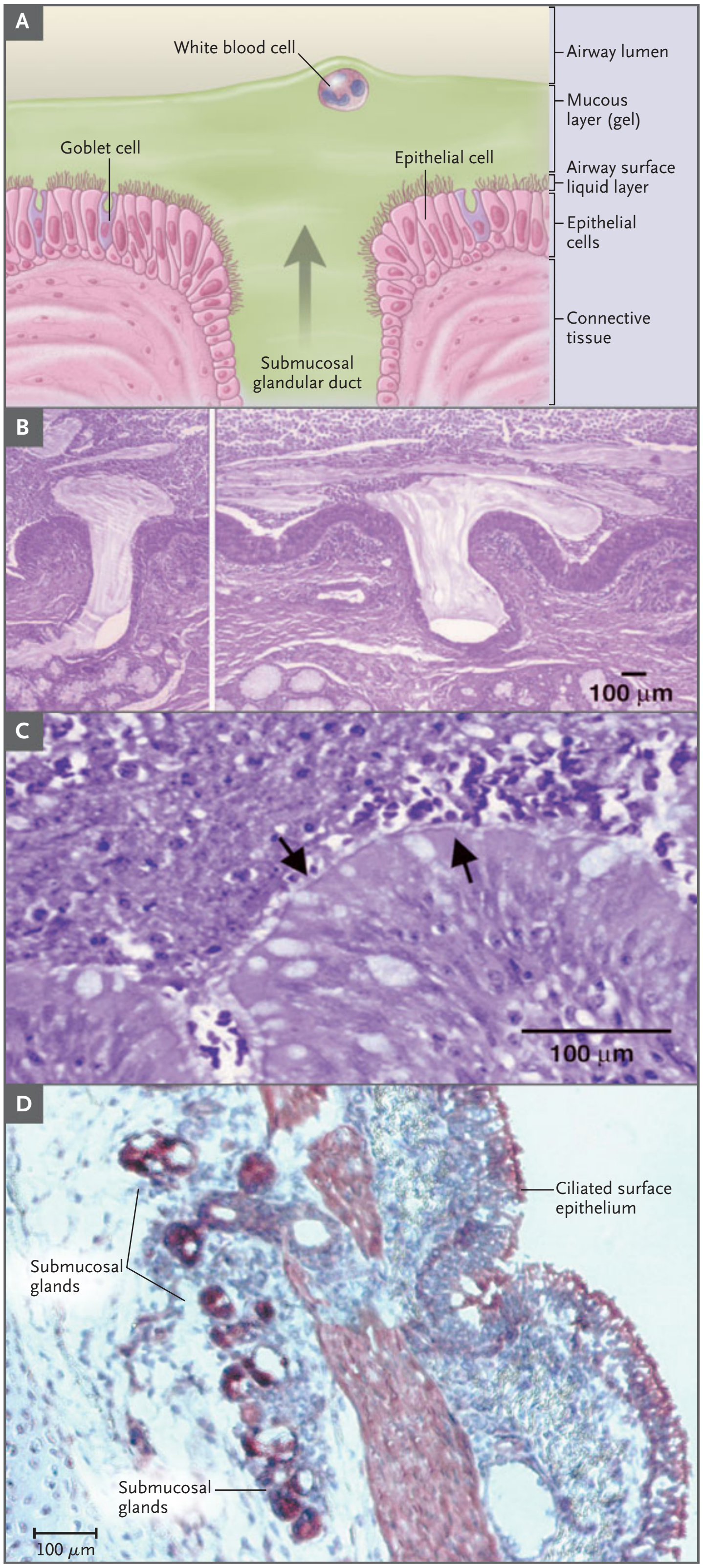


Figure 1. Hypothesized Structure of CFTR.

The protein contains 1480 amino acids and a number of discrete globular and transmembrane domains. Activation of CFTR relies on phosphorylation, particularly through protein kinase A but probably involving other kinases as well. Channel activity is governed by the two nucleotide-binding domains, which regulate channel gating. The carboxyl terminal (consisting of threonine, arginine, and leucine [TRL]) of CFTR is anchored through a PDZ-type–binding interaction with the cytoskeleton and is kept in close approximation (dashed arrows) to a number of important proteins. These associated proteins influence CFTR functions, including conductance, regulation of other channels, signal transduction, and localization at the apical plasma membrane. Each membrane-spanning domain contains six membrane-spanning alpha helixes, portions of which form a chloride-conductance pore. The regulatory domain is a site of protein kinase A phosphorylation. The common ΔF508 mutation occurs on the surface of nucleotide-binding domain 1.



PATHOBIOLOGIC FEATURES OF ORGAN SYSTEM DISEASE

Mucosal obstruction of exocrine glands is the chief contributor to morbidity and mortality in patients with cystic fibrosis (Fig. 2). In the human lung, thick, tenacious secretions obstruct the distal airways and submucosal glands, which express CFTR.[20] Ductular dilatation of these glands (associated with blockage by mucus) and the plastering of airway surfaces by thick, viscous, neutrophil-dominated mucopurulent debris are among the pathological hallmarks of the disease. Glandular hyperplasia in submucosal regions is prominent and surrounded by peribronchiolar inflammation and scar tissue. Submucosal gland ducts are inconspicuous in the lungs of patients without cystic fibrosis, whereas luminal dilatation by mucus is one of the earliest discernible changes in the lungs of newborns and children with cystic fibrosis. Pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Staphylococcus aureus*, and *Haemophilus influenzae* become well established within firmly fixed airway secretions in patients with cystic fibrosis and are not effectively eradicated. *P. aeruginosa*, for example, specifically adapts to the pulmonary microenvironment in patients with cystic fibrosis through the formation of macrocolonies (or biofilms) and the production of a capsular polysaccharide (an alginate product) that inhibits penetration by anti-

Figure 2. Extrusion of Mucus Secretion onto the Epithelial Surface of Airways in Cystic Fibrosis.

Panel A shows a schematic of the surface epithelium and supporting glandular structure of the human airway. In Panel B, the submucosal glands of a patient with cystic fibrosis are filled with mucus, and mucopurulent debris overlies the airway surfaces, essentially burying the epithelium. Panel C is a higher-magnification view of a mucus plug tightly adhering to the airway surface, with arrows indicating the interface between infected and inflamed secretions and the underlying epithelium to which the secretions adhere. (Both Panels B and C were stained with hematoxylin and eosin, with the colors modified to highlight structures.) Infected secretions obstruct airways and, over time, dramatically disrupt the normal architecture of the lung. In Panel D, CFTR is expressed in surface epithelium and serous cells at the base of submucosal glands in a porcine lung sample, as shown by the dark staining, signifying binding by CFTR antibodies to epithelial structures (aminoethylcarbazole detection of horseradish peroxidase with hematoxylin counterstain).

microbial agents and confers the mucoid phenotype.[21,22]

Pulmonary inflammation is another major cause of the decline in respiratory function in patients with cystic fibrosis and may precede the onset of chronic infection. Elevated levels of interleukin-8, interleukin-6, tumor necrosis factor α, and leukotriene B_4, along with reduced levels of antiinflammatory cytokines and proteases, have been found in the airways of patients with cystic fibrosis. Toll-like receptors, which recognize a variety of inflammatory mediators (including neutrophil elastase, bacterial lipopolysaccharide, and other microbial products), mediate inflammatory effects in part by activating the transcription factor nuclear factor-κB, which governs a molecular pathway that induces the production of inflammatory proteins and cytokines.[23-26] Recently, an elevated ratio of arachidonic acid to docosahexaenoic acid was found in mucosal scrapings from patients with cystic fibrosis, as compared with scrapings from normal persons and from patients with inflammatory bowel disease; thus, the altered ratio cannot be explained by systemic inflammation alone.[27] These and other inflammatory mediators such as mannose-binding protein and alpha$_1$-antitrypsin influence the progression of lung disease.[27-32]

The absence of normal CFTR activity in patients with cystic fibrosis also engenders obstruction in other organs. Mucinous impaction and thick concretions within pancreatic ducts lead to chronic fibrosis, fatty replacement of the gland, or both and to formes frustes of the disease in a large subgroup of patients with a previous diagnosis of idiopathic or alcoholic pancreatitis.[33,34] Nearly 10 percent of patients with the disease are born with intestinal obstruction (meconium ileus), a fatal condition if left untreated. Men with cystic fibrosis are frequently infertile because of glandular obstruction of the vas deferens in utero, which causes involution of the wolffian duct, vas deferens, and associated structures. *CFTR* mutations can also cause infertility in otherwise normal men as a result of the cystic fibrosis variant, called congenital bilateral absence of the vas deferens.[35] Similar obstruction of bile canaliculi frequently causes hepatic damage and, in some patients, overt cirrhosis.

MECHANISM UNDERLYING THE SWEAT-GLAND ABNORMALITY

There is widespread agreement that defects in ion transport, salt homeostasis, or both are intimately linked to organ damage in cystic fibrosis. The precise molecular basis for this connection, however, is unknown. Conversely, sweat glands in patients with cystic fibrosis, which usually do not become obstructed or show major pathologic abnormalities, have pronounced abnormalities in sodium chloride homeostasis that are well understood. In human sweat glands, primary secretion elaborated in the glandular coil is modified as it traverses the sweat duct, before emerging on the surface of the skin. Under normal conditions, sodium (followed by chloride counter-ion) is avidly reabsorbed from the ductular lumen, primarily through apical sodium channels and CFTR (Fig. 3). In patients with cystic fibrosis, the absence of functioning CFTR restricts reabsorption of chloride, thereby limiting the amount of salt that can be reclaimed. Because there is no other pathway for effective chloride reabsorption in the duct, sodium is also poorly absorbed, and sweat emerging on the skin surface contains a high level of salt. By the same token, in cystic fibrosis, the transepithelial potential difference across the sweat duct (the lumen-negative transepithelial voltage) is two to three times the normal value. The increased lumen-negative surface charge is caused by an inability to reabsorb chloride despite the continued existence of pathways for sodium uptake.

BIOELECTRIC MEASUREMENTS IN THE LUNG

Abnormalities of salt and fluid metabolism have also been examined in vivo in human and murine lungs. Knowles et al. popularized a method for measuring the transepithelial potential difference across nasal and lower airways in human subjects with the use of mucosal superperfusion.[36] This in vivo bioelectric measurement reveals virtually pathognomonic defects and can be used as a diagnostic test for cystic fibrosis. For example, under basal conditions, the transepithelial potential difference due to sodium uptake is two to three times as great (lumen negative) in patients with cystic fibrosis as in persons without the disease.

A traditional way of explaining airway mucosal obstruction (the low-volume model) argues persuasively that pulmonary surface epithelium in cystic fibrosis behaves in a fashion essentially opposite that of the sweat duct (Fig. 4). In this model, the absence of CFTR leads to overactivity of sodium absorption through the epithelial sodium channel (Fig. 4D). The mucosal surface has a more negative charge because overall chloride permeability is less than that of sodium. However, because Cl^- permeability (through non-CFTR Cl^- uptake pathways) is thought to be available in the lung, the result is a relative increase in the absorption of sodium, chloride, and fluid. This increase causes the dehydration of airway surfaces and defective mucociliary transport. Evidence in favor of the low-volume model derives from compelling in vitro experiments and a mouse model characterized by excessive functioning of the epithelial sodium channel with a lung phenotype similar to that in human cystic fibrosis.[37]

An alternative hypothesis (the high-salt model) contends that the airway epithelial surface in patients with cystic fibrosis behaves similarly to sweat ducts, in that CFTR is the major pathway for counter-ion absorption (Fig. 4C). The model was advanced in part to help unify bioelectric findings in the disease. When CFTR is absent, chloride cannot be reabsorbed — a situation that again resembles that of sweat-gland epithelium in cystic fibrosis. Consequently, the transepithelial potential difference becomes hyperpolarized: the inability of Cl^- ions to follow Na^+ results in a more negatively charged mucosal surface. One interpretation of this hypothesis is that an elevated level of sodium chloride in the airway-surface liquid would inactivate endogenous antimicrobial peptides and thereby predispose patients with the disease to bacterial infections with pathogens such as *P. aeruginosa*.[38]

When normal airway mucosa is superperfused with a solution designed to open CFTR or induce luminal chloride secretion, additional changes in the potential difference occur. These are compatible with chloride secretion (i.e., an increase in the lumen-negative potential difference) in normal persons but not in those with cystic fibrosis. Therefore, regardless of which model of sodium chloride reabsorption (low-volume or high-salt) better accounts for certain aspects of pulmonary insufficiency in cystic fibrosis, there is a strong consensus that in patients with the disease, the airways lack the normal ability to secrete chloride through CFTR.

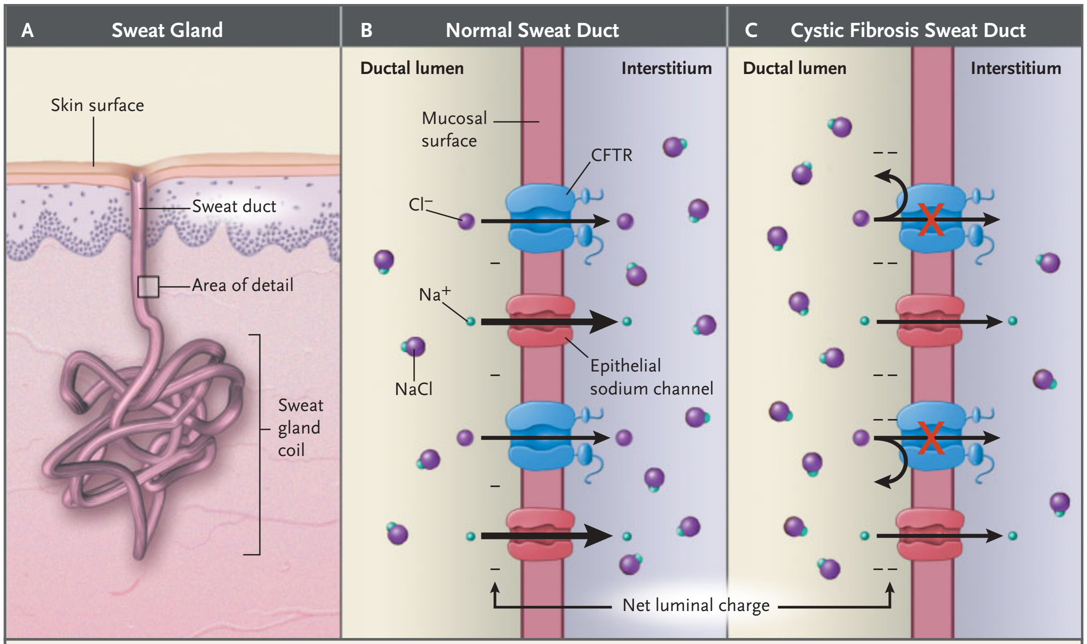


Figure 3. Mechanism Underlying Elevated Sodium Chloride Levels in the Sweat of Patients with Cystic Fibrosis.

Sweat ducts (Panel A) in patients with cystic fibrosis differ from those in people without the disease in the ability to reabsorb chloride before the emergence of sweat on the surface of the skin. A major pathway for Cl^- absorption is through CFTR, situated within luminal plasma membranes of cells lining the duct (i.e., on the apical, or mucosal, cell surface) (Panel B). Diminished chloride reabsorption in the setting of continued sodium uptake leads to an elevated transepithelial potential difference across the wall of the sweat duct, and the lumen becomes more negatively charged because of a failure to reabsorb chloride (Panel C). The result is that total sodium chloride flux is markedly decreased, leading to increased salt content. The thickness of the arrows corresponds to the degree of movement of ions.

Compounds such as adenosine, genistein, and phosphodiesterase inhibitors augment residual chloride secretion by mutant CFTR channels and form the basis of clinical experiments to restore function in patients with cystic fibrosis. A unified model of organ-system pathophysiology emanates naturally from a universally acknowledged defect in chloride secretion in the disease.

GLANDULAR SECRETION AND SMALL-AIRWAY PHYSIOLOGY

Abnormalities in sodium chloride absorption or antimicrobial peptide function that are invoked to explain specific pulmonary defects in cystic fibrosis are unlikely to contribute to disease-related damage in other organs. In the pancreas, there is no evidence of either sodium hyperabsorption or overactivity of the epithelial sodium channel, and abnormalities of antimicrobial peptides are not believed to contribute to exocrine pancreatic insufficiency in cystic fibrosis. In the intestine, neither immune alterations nor increased susceptibility to clinically apparent infection has been implicated. In tissues in which sodium transport has been examined (e.g., in samples of rectal mucosa and proximal ileum from patients with cystic fibrosis and in the obstructed intestine in the mouse model of the disease[39]), the activity of sodium-uptake pathways is variable and in some cases is decreased. By contrast, virtually every tissue that is affected by the disease is defective in chloride and fluid secretion. It is generally believed that in pancreatic ducts, the failure of the release of anions (including those of chloride and bicarbonate) due to CFTR dysfunction and deficient fluid secretion impairs flushing

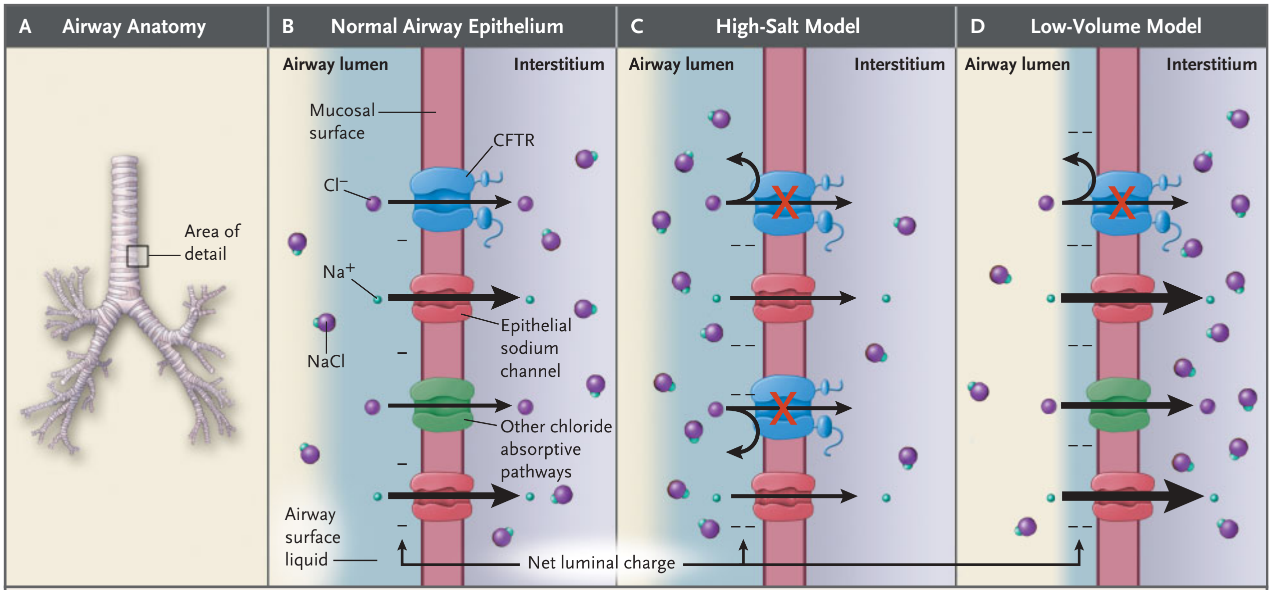


Figure 4. Models Explaining the Transepithelial Potential Difference across the Airway Epithelium in Cystic Fibrosis.

Under normal conditions, sodium chloride is absorbed from the airways (Panel A). The first step of this process uses sodium and chloride absorptive pathways present in the luminal (apical) membranes of airway-surface epithelial cells, designated as the mucosal surface (Panel B). In a bioelectric assay (a measurement of the transepithelial potential difference), the lumen is negative in part because of the relative impermeability of chloride as compared with sodium. The relative contribution of CFTR and other non-CFTR Cl^- permeability pathways is not known. The transepithelial potential difference is markedly hyperpolarized (i.e., the lumen is much more negatively charged) in cystic fibrosis. Two models have been proposed to explain this difference. In the high-salt model (Panel C), the situation resembles that of the sweat duct, in which the absence of CFTR leads to the inability to reabsorb chloride ion from airway-surface liquid. Because of the continued activity of sodium ion reabsorption, which is dependent on epithelial sodium channels, the airway surface negativity is increased (lumen-negative). According to this model, although a large charge separation is observed (with positively charged sodium ions moving across the airway wall and negatively charged chloride ions remaining behind), the net sodium chloride reabsorption decreases because of the inability to reabsorb chloride counter-ions. In the low-volume model (Panel D), both sodium and chloride are hyperabsorbed. The airways of patients with cystic fibrosis are slightly less permeable to chloride ions than they are to sodium ions, a process that leads to an increased transepithelial potential difference. This model predicts a depletion in the volume of airway-surface liquid (shown in blue). The thickness of the arrows corresponds to the degree of movement of ions.

of the exocrine glands, causing an accumulation of mucus, obstruction, and consequent end-organ damage. A pronounced abnormality in chloride secretion by intestinal tissues has been well documented in cystic fibrosis and provides a compelling explanation for end-organ dysfunction. Similar arguments can be made regarding the hyperviscous secretions in the liver, vas deferens, and other glands in cystic fibrosis.

New tools have emerged for testing the hypothesis that defective chloride secretion in airways is also a fundamental cause of pulmonary failure in cystic fibrosis. The finding of dilated glandular structures (which occurs very early in cystic fibrosis–associated lung disease) implicates abnormal clearance of glandular mucus from the ducts (a process reminiscent of the situation in the pancreas, liver, and intestine in cystic fibrosis). The levels of CFTR in airway serous glandular cells are among the highest of any cell type in the body (Fig. 2D).[20] In the small airways, where surface epithelial cells contribute to the production of mucus, an analogous defect in chloride and fluid secretion could result in decreased surface liquid in airways and similar mucosal obstruction.

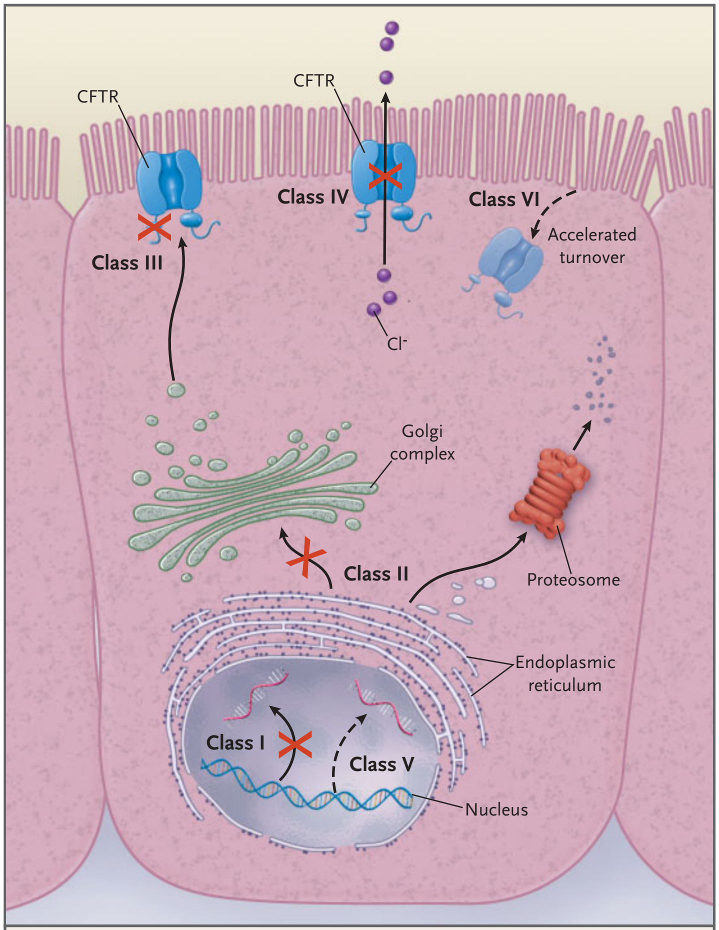


Figure 5. Categories of *CFTR* Mutations.

Classes of defects in the *CFTR* gene include the absence of synthesis (class I); defective protein maturation and premature degradation (class II); disordered regulation, such as diminished ATP binding and hydrolysis (class III); defective chloride conductance or channel gating (class IV); a reduced number of CFTR transcripts due to a promoter or splicing abnormality (class V); and accelerated turnover from the cell surface (class VI).[46]

These considerations have led to the idea that lung disease in cystic fibrosis is primarily attributable to a failure of CFTR-dependent flushing of mucous secretion from the glands, small airways, or both. Joo et al. have directly measured glandular secretions in lung tissues in cystic fibrosis.[40,41] Their studies suggest fundamental abnormalities in the mobilization and clearance of mucus from submucosal glands (a process that is also crucial in the distal small airways). Similar conclusions have been reached by Verkman and colleagues.[42,43] Attempts to activate CFTR-independent chloride conductance with secretagogues such as uridine 5′-triphosphate or related compounds (which may restore or enhance mucociliary clearance) have yielded encouraging results in clinical trials.[44,45] Models based on defects in glandular and airway secretion as primary contributors to respiratory decline therefore continue to represent a vital and unifying means for understanding pulmonary destruction due to defects in CFTR.

MUTATIONS IN *CFTR* AND THEIR CONSEQUENCES

The *CFTR* gene encompasses approximately 180,000 base pairs on the long arm of chromosome 7. The protein contains 1480 amino acids (Fig. 1). More than 1000 disease-associated mutations have been described in the coding sequence, messenger RNA splice signals, and other regions. These mutations can be classified on the basis of the mechanism by which they are believed to cause disease (Fig. 5). The most common mutation, which is termed ΔF508 and is present in approximately 70 percent of defective *CFTR* alleles and in 90 percent of patients with cystic fibrosis in the United States, is categorized as a class II defect. CFTR with the ΔF508 mutation lacks a phenylalanine (F) residue at position 508. The defective protein retains substantial chloride-channel function in cell-free lipid membranes. When synthesized by the normal cellular machinery, however, the protein is rapidly recognized as misfolded and is degraded shortly after synthesis, before it can reach its crucial site of action at the cell surface. Like ΔF508, several other clinically important mutations — such as N1303K, G85E, and G91R — lead to misfolded CFTR protein that is prematurely degraded.

About 5 to 10 percent of *CFTR* mutations are due to premature truncation or nonsense alleles (designated by "X," such as G542X, a class I mutation). As a result of a genetic founder effect, prematurely truncated CFTR is particularly prevalent among persons of Ashkenazi Jewish descent. Other *CFTR* mutations encode properly processed, full-length CFTR protein that lacks normal ion-channel activity. For example, the G551D mutation (class III) is believed to possess little or no chloride-channel function in vivo because of abnormal function of a nucleotide-binding domain, resulting in disordered regulation. The A455E mutation (class IV) exhibits only partial CFTR ion-channel activity, a feature that probably explains a less severe pulmonary phenotype.[47] Other mutation classes include reduced numbers of CFTR transcripts (class V) and defective CFTR stability at the cell surface (class VI).[48-50]

INTERVENTIONS TAILORED TO SPECIFIC CFTR DEFECTS

Insight into the cellular consequences of defective CFTR suggests a role for tailored therapies, a predominant theme in clinical research on cystic fibrosis. For example, robotic drug screening of more than a million random compounds has led to the discovery of compounds that correct the ΔF508 abnormality by restoring the mutant protein to its normal position at the cell surface (thereby partially restoring chloride-channel function).[51,52] The recently elucidated crystal structure of nucleotide-binding domain 1 localizes the crucial phenylalanine 508 residue to a loop on the external surface of the domain. Because of this structure, drug-screening laboratories can test the specificity of ΔF508-correcting compounds by co-crystallization with the CFTR protein.[53] Curcumin, a nontoxic compound and the major constituent of the spice tumeric, has been shown to correct ΔF508 processing in a number of in vitro model systems and prolong life in mice that are homozygous for the ΔF508 mutation.[54,55] New compounds that correct this fundamental processing abnormality in CFTR should undergo clinical testing in the near future.

A number of agents have also been shown to suppress premature stop codons in *CFTR* (class I) mutations, including the surprising finding that ribosomally active drugs such as gentamicin may be capable of correcting premature stop codons in human subjects.[56,57] This same concept has been applied to other diseases caused by premature stop codons, including Duchenne's muscular dystrophy, Hurler's syndrome, and disorders resulting from mutations in the p53 tumor-suppressor gene.[58-60] High-throughput screening programs specifically designed to identify drugs that activate residual CFTR activity (class III and IV mutations) have also been successful.[61-64]

SUMMARY

Important advances have improved our understanding of the role of the CFTR protein in the progression of suppurative pulmonary failure in cystic fibrosis. These discoveries are ushering in a new era of translational research that incorporates specific therapeutic targets and new cellular pathways. Progress in research on cystic fibrosis will continue to rely on an improved understanding of CFTR function and its relationship to mucociliary clearance, airway secretion, and other ion channels. Clinical advances directed at the correction of CFTR function predict an optimistic future for patients with cystic fibrosis and their families.

Dr. Sorscher is coinventor of a method of making and using human papillomavirus vectors for transduction of host cells and a coinventor of a method of activating chloride secretion. Patents on these methods are unlicensed and held by the University of Alabama.

We are indebted to Drs. J.P. Clancy, James Hagood, Jeff Wine, Jonathan Widdecome, Paul Quinton, Michael Welsh, and Garry Cutting for their useful discussion and suggestions; to Drs. Zsuzsa Bebok and David Kelly for their scientific contributions to the figures; to Mikelle Foster, Jane Schell, and David Fischer for assistance in the preparation of the manuscript; and to Chris Valarie, Anne Marie Seibel, and Hughes Evans for initiating some of the analysis described in the article.

REFERENCES

1. Cystic Fibrosis Foundation National Patient Registry Annual Data Report 2003. Bethesda, Md.: Cystic Fibrosis Foundation, 2004.
2. Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med 2003;168:918-51.
3. Ratjen F, Doring G. Cystic fibrosis. Lancet 2003;361:681-9.
4. Yankaskas JR, Marshall BC, Sufian B, Simon RH, Rodman D. Cystic fibrosis adult

care: consensus conference report. Chest 2004;125:Suppl:1S-39S.

5. Lowe CU, May CD, Reed SC. Fibrosis of the pancreas in infants and children: a statistical study of clinical and hereditary features. Am J Dis Child 1949;78:349-74.

6. Quinton PM. Missing Cl conductance in cystic fibrosis. Am J Physiol 1986;251:C649-C652.

7. Knowles M, Gatzy J, Boucher R. Relative ion permeability of normal and cystic fibrosis nasal epithelium. J Clin Invest 1983;71:1410-7.

8. Welsh MJ, Liedtke CM. Chloride and potassium channels in cystic fibrosis airway epithelia. Nature 1986;322:467-70.

9. Welsh MJ. An apical-membrane chloride channel in human tracheal epithelium. Science 1986;232:1648-50.

10. Schoumacher RA, Shoemaker RL, Halm DR, Tallant EA, Wallace RW, Frizzell RA. Phosphorylation fails to activate chloride channels from cystic fibrosis airway cells. Nature 1987;330:752-4.

11. Riordan JR, Rommens JM, Kerem B, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989;245:1066-73. [Erratum, Science 1989;245:1437.]

12. Kerem B, Rommens JM, Buchanan JA, et al. Identification of the cystic fibrosis gene: genetic analysis. Science 1989;245:1073-80.

13. Rommens JM, Iannuzzi MC, Kerem B, et al. Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 1989;245:1059-65.

14. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002;71:537-92.

15. Rich DP, Anderson MP, Gregory RJ, et al. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. Nature 1990;347:358-63.

16. Bear CE, Li CH, Kartner N, et al. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell 1992;68:809-18.

17. Kunzelmann K, Schreiber R, Nitschke R, Mall M. Control of epithelial Na+ conductance by the cystic fibrosis transmembrane conductance regulator. Pflugers Arch 2000;440:193-201.

18. Short DB, Trotter KW, Reczek D, et al. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J Biol Chem 1998;273:19797-801.

19. Xu Y, Clark JC, Aronow BJ, et al. Transcriptional adaptation to cystic fibrosis transmembrane conductance regulator deficiency. J Biol Chem 2003;278:7674-82.

20. Engelhardt JF, Yankaskas JR, Ernst SA, et al. Submucosal glands are the predominant site of CFTR expression in the human bronchus. Nat Genet 1992;2:240-8.

21. Wyckoff TJ, Thomas B, Hassett DJ, Wozniak DJ. Static growth of mucoid Pseudomonas aeruginosa selects for non-mucoid variants that have acquired flagellum-dependent motility. Microbiology 2002;148:3423-30.

22. Whiteley M, Bangera MG, Bumgarner RE, et al. Gene expression in Pseudomonas aeruginosa biofilms. Nature 2001;413:860-4.

23. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. J Pediatr 2002;141:811-7.

24. Konstan MW, Davis PB. Pharmacological approaches for the discovery and development of new anti-inflammatory agents for the treatment of cystic fibrosis. Adv Drug Deliv Rev 2002;54:1409-23.

25. Koehler DR, Downey GP, Sweezey NB, Tanswell AK, Hu J. Lung inflammation as a therapeutic target in cystic fibrosis. Am J Respir Cell Mol Biol 2004;31:377-81.

26. Muir A, Soong G, Sokol S, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol 2004;30:777-83.

27. Freedman SD, Blanco PG, Zaman MM, et al. Association of cystic fibrosis with abnormalities in fatty acid metabolism. N Engl J Med 2004;350:560-9.

28. Garred P, Pressler T, Madsen HO, et al. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J Clin Invest 1999;104:431-7.

29. Puchelle E, De Bentzmann S, Hubeau C, Jacquot J, Gaillard D. Mechanisms involved in cystic fibrosis airway inflammation. Pediatr Pulmonol 2001;Suppl 23:143-5.

30. Sagel SD, Accurso FJ. Monitoring inflammation in CF: cytokines. Clin Rev Allergy Immunol 2002;23:41-57.

31. Hilliard JB, Konstan MW, Davis PB. Inflammatory mediators in CF patients. Methods Mol Med 2002;70:409-31.

32. Frangolias DD, Ruan J, Wilcox PJ, et al. Alpha 1-antitrypsin deficiency alleles in cystic fibrosis lung disease. Am J Respir Cell Mol Biol 2003;29:390-6.

33. Cohn JA, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. N Engl J Med 1998;339:653-8.

34. Sharer N, Schwarz M, Malone G, et al. Mutations of the cystic fibrosis gene in patients with chronic pancreatitis. N Engl J Med 1998;339:645-52.

35. Chillón M, Casals T, Mercier B, et al. Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. N Engl J Med 1995;332:1475-80.

36. Knowles M, Gatzy J, Boucher R. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. N Engl J Med 1981;305:1489-95.

37. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. Nat Med 2004;10:487-93.

38. Smith JJ, Travis SM, Greenberg EP, Welsh MJ. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell 1996;85:229-36.

39. Grubb BR. Ion transport across the normal and CF neonatal murine intestine. Am J Physiol 1999;277:G167-G174.

40. Joo NS, Irokawa T, Wu JV, Robbins RC, Whyte RI, Wine JJ. Absent secretion to vasoactive intestinal peptide in cystic fibrosis airway glands. J Biol Chem 2002;277:50710-5.

41. Joo NS, Saenz Y, Krouse ME, Wine JJ. Mucus secretion from single submucosal glands of pig: stimulation by carbachol and vasoactive intestinal peptide. J Biol Chem 2002;277:28167-75.

42. Jayaraman S, Joo NS, Reitz B, Wine JJ, Verkman AS. Submucosal gland secretions in airways from cystic fibrosis patients have normal [Na(+)] and pH but elevated viscosity. Proc Natl Acad Sci U S A 2001;98:8119-23.

43. Verkman AS, Song Y, Thiagarajah JR. Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. Am J Physiol Cell Physiol 2003;284:C2-C15.

44. Olivier KN, Bennett WD, Hohneker KW, et al. Acute safety and effects on mucociliary clearance of aerosolized uridine 5′-triphosphate +/– amiloride in normal human adults. Am J Respir Crit Care Med 1996;154:217-23.

45. Deterding RR, La Vange L, Mathews D, et al. Safety and efficacy of INS37217 inhalation solution, a novel P2Y2 receptor agonist, in patients with mild cystic fibrosis: results of a phase 2 multi-center study. Pediatr Pulmonol Suppl 2004;38:249. abstract.

46. Zeitlin PL. Pharmacologic restoration of delta F508 CFTR-mediated chloride current. Kidney Int 2000;57:832-7.

47. Gan KH, Veeze HJ, van den Ouweland AM, et al. A cystic fibrosis mutation associated with mild lung disease. N Engl J Med 1995;333:95-9.

48. Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 1993;73:1251-4.

49. Zielenski J, Tsui LC. Cystic fibrosis: genotypic and phenotypic variations. Annu Rev Genet 1995;29:777-807.

50. Haardt M, Benharouga M, Lechardeur D, Kartner N, Lukacs GL. C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis: a novel class of mutation. J Biol Chem 1999;274:21873-7.

51. Verkman AS, Pedemonte N, Du K, et al. Small-molecule activators (potentiators and correctors) of DF508-CFTR identified by high-throughput screening. Pediatr Pulmonol Suppl 2003;36:290. abstract.

52. Verkman AS. Drug discovery in academia. Am J Physiol Cell Physiol 2004;286:C465-C474.

53. Lewis HA, Buchanan SG, Burley SK, et al. Structure of nucleotide-binding domain 1

of the cystic fibrosis transmembrane conductance regulator. EMBO J 2004;23:282-93.
54. Zeitlin P. Can curcumin cure cystic fibrosis? N Engl J Med 2004;351:606-8.
55. Egan ME, Pearson M, Weiner SA, et al. Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. Science 2004;304:600-2.
56. Clancy JP, Bebok Z, Ruiz F, et al. Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. Am J Respir Crit Care Med 2001;163:1683-92.
57. Wilschanski M, Yahav Y, Yaacov Y, et al. Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and *CFTR* stop mutations. N Engl J Med 2003;349:1433-41.
58. Keeling KM, Brooks DA, Hopwood JJ, Li P, Thompson JN, Bedwell DM. Gentamicin-mediated suppression of Hurler syndrome stop mutations restores a low level of alpha-L-iduronidase activity and reduces lysosomal glycosaminoglycan accumulation. Hum Mol Genet 2001;10:291-9.
59. Wagner KR, Hamed S, Hadley DW, et al. Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. Ann Neurol 2001;49:706-11.
60. Keeling KM, Bedwell DM. Clinically relevant aminoglycosides can suppress disease-associated premature stop mutations in the IDUA and P53 cDNAs in a mammalian translation system. J Mol Med 2002;80:367-76.
61. Galietta LV, Jayaraman S, Verkman AS. Cell-based assay for high-throughput quantitative screening of CFTR chloride transport agonists. Am J Physiol Cell Physiol 2001;281:C1734-42.
62. Galietta LJ, Springsteel MF, Eda M, et al. Novel CFTR chloride channel activators identified by screening of combinatorial libraries based on flavone and benzoquinolizinium lead compounds. J Biol Chem 2001;276:19723-8.
63. Ma T, Vetrivel L, Yang H, et al. High-affinity activators of cystic fibrosis transmembrane conductance regulator (CFTR) chloride conductance identified by high-throughput screening. J Biol Chem 2002;277:37235-41.
64. Gill S, Gill R, Lee SS, et al. Flux assays in high throughput screening of ion channels in drug discovery. Assay Drug Dev Technol 2003;1:709-17.

JOURNAL EDITORIAL FELLOW

The *Journal's* editorial office invites applications for a one-year research fellowship beginning in July 2006 from individuals at any stage of training. The editorial fellow will work on *Journal* projects and will participate in the day-to-day editorial activities of the *Journal* but is expected in addition to have his or her own independent projects. Please send curriculum vitae and research interests to the Editor-in-Chief, 10 Shattuck St., Boston, MA 02115 (fax, 617-739-9864), by September 30, 2005.

Polymerase Chain Reaction

CLIN. CHEM. 39/5, 804–809 (1993)

Rapid Cycle Allele-Specific Amplification: Studies with the Cystic Fibrosis ΔF_{508} Locus

Carl T. Wittwer,[1,5] Bruce C. Marshall,[2] Gudrun H. Reed,[3] and Joshua L. Cherry[4]

Rapid cycle DNA amplification is a polymerase chain reaction technique with improved product specificity and cycle times of 20–60 s, allowing complete 30-cycle reactions in 10–30 min. The presence or absence of the ΔF_{508} deletion and wild-type allele was determined in 104 cystic fibrosis patients by rapid cycle DNA amplification. In separate allele-specific assays, sequences on both sides of the ΔF_{508} locus were amplified with the 3′ end of a discriminating primer at the ΔF_{508} locus, with either a 3-bp or a 1-bp mismatch. With rapid cycling (35-s cycles), single-base discrimination was achieved over a broad range of annealing temperatures (50 °C or lower); with conventional cycling and "hot starts" (160-s cycles), only annealing temperatures of 61–62 °C sufficiently discriminated between alleles. With rapid cycling, genotype could still be assessed with annealing temperatures as low as 25 °C. We conclude that faster temperature cycling can improve the results of allele-specific amplification.

Indexing Terms: *polymerase chain reaction · heritable disorders · DNA probes · screening*

Systematic study of the times required for in vitro DNA amplification reveals interesting facts (*1*). Thirty cycles can routinely be completed in 15 min or less. Sample denaturation and annealing times of "0"–1 s not only are sufficient, but appear to improve the specificity of reaction. Limitations on the speed of DNA amplification arise not from kinetic requirements of the underlying reactions, but rather from the instrumentation usually used for temperature cycling. The fastest system reported makes use of capillary tubes as containers and air as the heat-transfer medium (*1–3*). Specificity of reaction is particularly important in allele-specific amplification. Single-base mismatches at the 3′ end of one primer may or may not be amplified, depending on the type of mismatch and reaction conditions (*4*). The potential advantages of rapid temperature cycling for allele-specific amplification in clinical diagnostics have not previously been tested.

Cystic fibrosis is the most common autosomal recessive disease in Caucasians, with a carrier rate of ~1:25 (*5*). A 3-bp deletion within the cystic fibrosis transmembrane conductance regulator gene produces a phenylalanine deletion at residue 508 (ΔF_{508}) and occurs in 70–75% of cystic fibrosis chromosomes (*6*). In anticipation of mass screening, much attention has been directed toward simple, reliable, and inexpensive assays for this mutation. Most methods are based on the polymerase chain reaction. Alleles are detected by sequencing (*7*), allele-specific oligonucleotides (*8*), restriction enzymes (*9*), size-fractionation on polyacrylamide gels (*10*), or allele-specific amplification (*11*). Allele-specific amplification with direct visualization on agarose gels with ethidium bromide requires the least effort and postamplification processing.

We have applied rapid cycle DNA amplification to the allele-specific amplification of ΔF_{508} in 104 patients with cystic fibrosis. For validation, two independent allele-specific amplifications were developed, each for both ΔF_{508} and wild-type alleles. The differential amplifications were based on either a 3-bp mismatch or a single base pair mismatch. Temperature cycle conditions for discriminating the single-base mismatch were rigorously determined for both rapid cycling and conventional temperature cycling.

Materials and Methods

Patients with cystic fibrosis were recruited from the Intermountain Cystic Fibrosis Center at the University of Utah. After informed consent, acid/citrate/dextrose-anticoagulated peripheral blood was obtained for purification of genomic DNA. Leukocyte nuclei were digested in 10 g/L sodium dodecyl sulfate and 100–500 mg/L proteinase K at 60 °C for 1 h, extracted with phenol/chloroform, and ethanol-precipitated (*12*). DNA was resuspended in 10 mmol/L Tris–0.1 mmol/L EDTA by boiling.

DNA amplification reactions were performed with 10 mmol/L Tris, pH 8.3 (at 25 °C), 3 mmol/L $MgCl_2$, 50 mmol/L KCl, 500 mg/L bovine serum albumin, 10 g/L Ficoll 400, 1 mmol/L tartrazine, and 0.5 μmol of each primer, 200 μmol of each dNTP, 50 ng of human genomic DNA, and 0.4 U of Taq polymerase per 10 μL. One unit (U) of polymerase activity was the amount of enzyme required to incorporate 10 nmol of [^{3}H]dTTP in 30 min at 80 °C, as defined by the manufacturer (Promega, Madison, WI). *Mse* I was from New England Biolabs (Beverly, MA).

Oligonucleotide primers were synthesized from the phosphoramidites (Gene Assembler Plus; Pharmacia-LKB, Piscataway, NJ). Two amplification primer sets were synthesized, one on each side of the ΔF_{508} mutation locus. Primer 1 (GACTTCACTTCTAATGATGA) was located at the 5′ end of exon 10 of the cystic fibrosis gene (*6*). The 3′ end of primer 2 (primer 2cf: TCATCATAGGAAACACCAAT, or primer 2wt: TCATCATAGGAAACACCAAA) annealed at the ΔF_{508} mutation locus.

Departments of [1] Pathology, [2] Medicine, and [4] Human Genetics, University of Utah Medical School, Salt Lake City, UT 84132.

[3] Associated Regional and University Pathologists, Inc., 500 Chipeta Way, Salt Lake City, UT 84108.

[5] Author for correspondence.

Received September 17, 1992; accepted December 3, 1992.

Primers 2cf and 2wt differed from each other only at the 3′ base. The other amplification set utilized primer 3 (CTCTTCTAGTTGGCATGCTT) located at the 3′ end of exon 10 and either primer 4cf (CCATTAAAGAAAATATCATTGG) or primer 4wt (CCATTAAAGAAAATATCATCTT). Primers 4cf and 4wt differed from each other by three bases at the 3′ end.

Temperature cycling equipment included a conventional heat block instrument (DNA Thermal Cycler; Perkin-Elmer Cetus, Norwalk, CT), a second-generation heat block instrument (System 9600; Perkin-Elmer Cetus), and a hot-air cycler (Idaho Technology, Idaho Falls, ID). A custom hot-air cycler was also used for some experiments (*1*); the temperature/time profiles of the commercial and custom air cyclers were similar. When the conventional heat block instrument was used, all reaction components except the enzyme were mixed (90 µL) and heated to 80 °C. A 10-fold-concentrated enzyme solution at 80 °C was rapidly added and mixed for a "hot start" (*13*). The reaction mixture was then overlaid with 60 µL of mineral oil at 80 °C. Cycling was performed in "STEP cycle" mode for 35 cycles with denaturation at 94 °C for 35 s, annealing for 35 s at various temperatures, and elongation at 72 °C for 45 s. These times are the minimal denaturation and annealing times necessary for the sample to reach temperature in this system (*1*). The temperature of a 20-µL sample in a MicroAmp™ tube was monitored in the second-generation heat block instrument. Although annealing times of <10 s are "not recommended," we used a 1-s annealing time to minimize the time spent at the annealing temperature. When the air-cycling instrument was used, 10-µL samples were placed in 0.5 mm (i.d.) glass capillary tubes (Idaho Technology). The tubes were used as supplied by the manufacturer without siliconization, and hot starts were not attempted. Thirty-five cycles were performed with denaturation at 94 °C for "0" s, annealing for "0" s at 45 °C unless otherwise specified, and elongation at 72 °C for 10 s. For annealing temperatures of ≤40 °C, the cycler was placed in a cold room at 2 °C.

Amplification products (10 µL) were fractionated by electrophoresis in 1.5% agarose gels unless otherwise stated and made visible by ethidium bromide staining and transillumination with ultraviolet light. The size markers either were a *Hae* III digest of $\phi \times 174$ RF or were produced by cleavage of pUCBM21 DNA with *Hpa* I and *Dra* I + *Hin*dIII (DNA molecular weight marker VIII; Boehringer Mannheim, Indianapolis, IN). The sample temperature in all systems was monitored with a 0.2 mm (o.d.) thermocouple with a 0.005-s time constant (IT-23; Sensortek, Clifton, NJ), placed in the center of a mock sample.

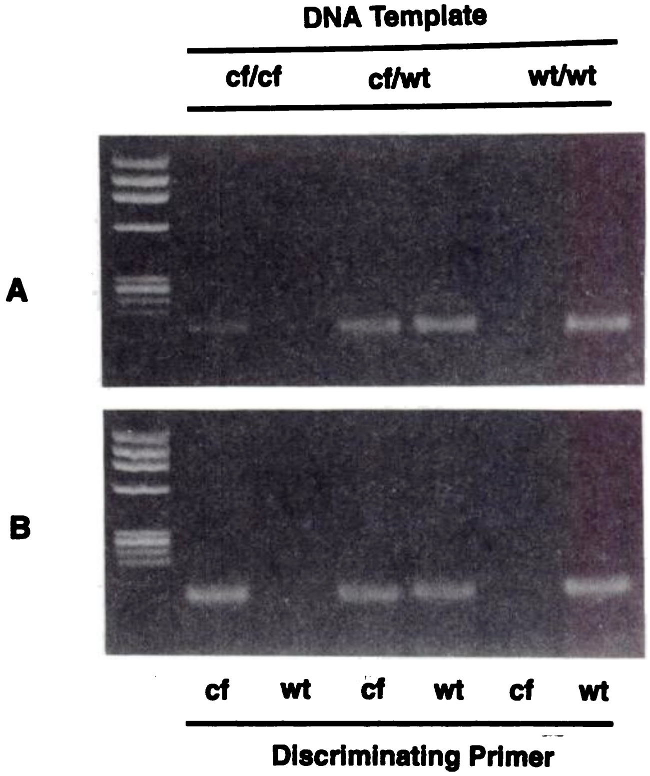


Fig. 1. Allele-specific amplification of ΔF_{508} (cf) and wild-type (wt) sequences utilizing a single-base mismatch (*A*) and a 3-bp mismatch (*B*)

(*A*) Primer 1 was combined with either primer 2cf or primer 2wt and amplification performed as described. (*B*) Primer 3 was combined with either primer 4cf or primer 4wt. Genomic DNA was either homozygous ΔF_{508} (cf/cf), heterozygous ΔF_{508} (cf/wt), or homozygous wild-type (wt/wt). *Leftmost sample:* molecular size marker (*Hae* III digest of $\phi \times 174$ RF)

Results

The expected products were amplified from the cystic fibrosis locus in genomic DNA with rapid cycling (Figure 1). Primer pairs 1 and 2cf/2wt amplify a 147/150-bp product and primer pairs 3 and 4cf/4wt amplify a 80/83-bp product. The identity of the amplification product was confirmed by restriction enzyme digestion; e.g., digestion of the primer 1/primer 2wt product with *Mse* I gave bands at ~58, 54, and 34 bp (data not shown). The amplifications were allele specific; when there was a mismatch at the 3′ end of the discriminating primer, no observable amplification occurred.

The validity of genotype determinations in 104 cystic fibrosis patients was tested by the concordance of the two independent allele-specific amplifications. In the initial testing, performed without knowledge of the results of the other test, 103/104 of the genotypes concurred. The exception was a case that appeared heterozygous with primers 1 and 2cf/2wt, but homozygous ΔF_{508} with primers 3 and 4cf/4wt. When a sample of the original DNA stock was rediluted, the discrepancy could not be repeated and the patient tested homozygous for ΔF_{508}. The parents were both heterozygous for ΔF_{508}. Sixty of the 104 patients were homozygous ΔF_{508} (57.7%), 35 were heterozygous (33.6%), and 9 (8.7%) were homozygous wild type. The frequency of the ΔF_{508} allele was 0.75 in our cystic fibrosis population.

The allele-specific amplification dependent on a single base change (primer 1, primer 2cf/2wt) was optimized for both rapid and conventional temperature cycling. Figure 2 shows amplification products obtained with conventional temperature cycling and hot starts at

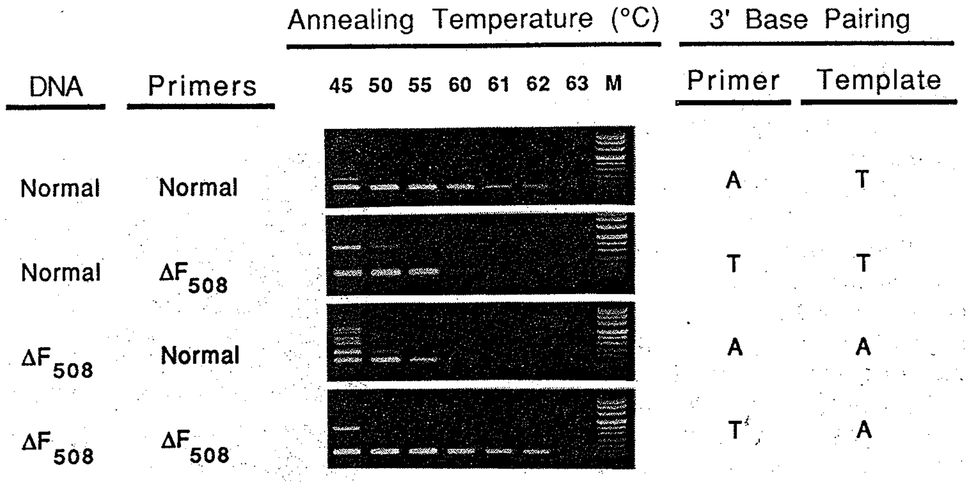


Fig. 2. Single-base discrimination by DNA amplification in a conventional heat block instrument

Amplification reactions with various annealing temperatures were performed with all four combinations of homozygous normal or homozygous ΔF_{508} genomic DNA and normal or ΔF_{508} primers. Cycle times varied from 160 s with 62 °C annealing to 255 s with 45 °C annealing. Hot starts (*13*) were used with all samples and 35 cycles were performed. Primers were perfectly matched to the template in the samples shown in the *top* and *bottom* gel photographs. Amplification of the expected 150-bp (*top*) or 147-bp (*bottom*) fragment occurred with annealing temperatures ≤62 °C. Because some amplification occurred with the T-T 3′ mismatch at 60 °C, only a narrow range (61–62 °C) was acceptable for mismatch discrimination. Figs. 2–4: *M*, molecular weight marker VIII from Boehringer Mannheim

various annealing temperatures. Adequate discrimination occurred with annealing temperatures of 61 and 62 °C, but not at 60 or 63 °C. Single-base discrimination was lost when the annealing temperature was lowered to 60 °C or below. In addition, multiple products of various sizes were amplified with annealing temperatures of 50 °C or below. Inclusion of Ficoll and the electrophoresis indicator dye, tartrazine, in the amplification mixture did not affect yield or specificity (data not shown).

Figure 3 shows amplification products obtained with rapid cycling at various annealing temperatures. No product was observed with an annealing temperature of 55 °C, a temperature at which very efficient amplification occurred with either matched or mismatched primers on a conventional cycler. Single-base discrimination was achieved with rapid cycling at annealing temperatures of 50 °C or lower. Some undesired amplification occurred with annealing temperatures of 35 °C or lower. However, most of this undesired amplification resulted in products that were not the size expected for the cystic fibrosis locus, even when the annealing temperature was 25 °C (Figure 4).

Figure 5 shows the profiles of sample temperatures near an annealing temperature of 55 °C for three different instruments. All systems were empirically adjusted for the fastest possible approach to, and the least possible duration at, 55 °C. The sample size used in each instrument was that generally recommended by the manufacturer and varied for each instrument. Samples in the conventional instrument spend about 12 s between 55 and 56 °C. The second-generation instrument is capable of faster temperature transitions, especially during cooling, but samples still spend about 8 s between 55 and 56 °C. Samples in the rapid cycling instrument are at 55 to 56 °C for just under 1 s.

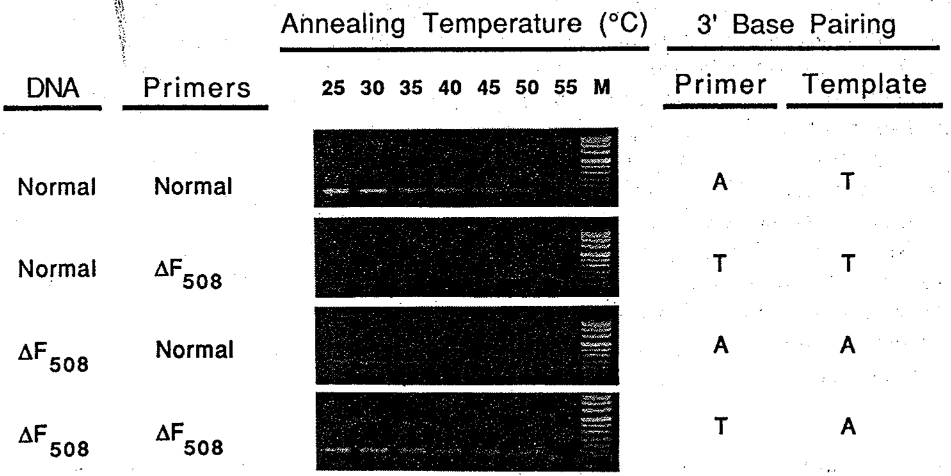


Fig. 3. Single-base discrimination by DNA amplification in a rapid air cycler

Primers and template DNA as described in Fig. 2. Cycle times varied from 30 s at 55 °C annealing to 60 s at 25 °C annealing because of longer transition times associated with lower annealing temperatures. Thirty-five cycles were performed. The optimal annealing temperature for single-base discrimination by rapid cycling differs from that for conventional cycling. With perfectly matched primers, amplification occurred at 50 °C but not at 55 °C. Single-base discrimination was possible with annealing temperatures of ≤50 °C. See Fig. 4 for greater detail

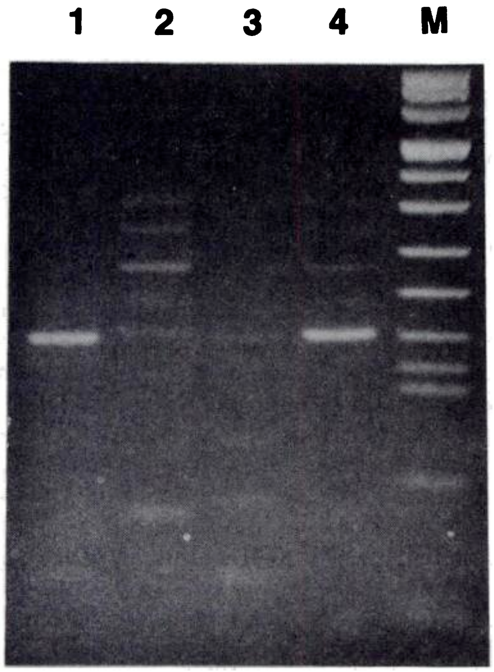


Fig. 4. Allele-specific amplification with 25 °C annealing and rapid cycling

Amplification products were electrophoresed in a 4% agarose gel. *Lane 1*, ΔF_{508} DNA amplified with ΔF_{508} primers (*bottom* gel in Figs. 2 and 3). *Lane 2*, ΔF_{508} DNA amplified with normal primers (A-A mismatch). *Lane 3*, wild-type DNA amplified with ΔF_{508} primers (T-T 3′ mismatch). *Lane 4*, normal DNA amplified with normal primers (*top* gel in Figs. 2 and 3). Although some undesired amplification is evident in *lanes 2 and 3*, most of the products are of different sizes than expected for amplification at the ΔF_{508} locus

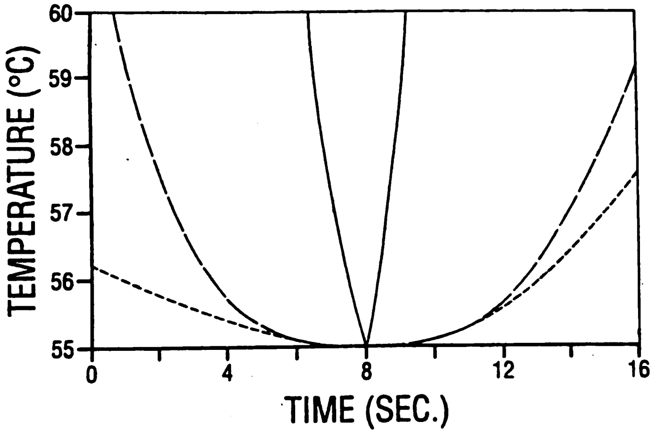


Fig. 5. Temperature/time profiles near an annealing temperature of 55 °C for three different temperature cyclers

Cycling variables on each instrument were empirically adjusted to minimize the time that the sample was at 55 °C. (- - -) 100-µL sample on a standard heat block instrument; (— —) 20-µL sample on a second-generation heat block instrument; (—) 10-µL sample in a commercial rapid air cycler. The rapid air cycler produces annealing segment "spikes," in contrast to the annealing temperature "plateaus" of the other instruments

Discussion

The polymerase chain reaction is an extremely popular technique. Although not widely recognized, increasing the speed of temperature cycling and (or) decreasing the annealing time can significantly improve specificity (*1*). With conventional temperature cycling, 30 cycles of DNA amplification often require ~4 h, although 2-h amplifications are possible if adjustments to minimize sample denaturation and annealing times are made. Second-generation instruments can complete 30 cycles in about 1 h (*7*). "Rapid cycle" DNA amplification, as used here, refers to completing 30 cycles in 10–30 min. Details on the construction of rapid cycling instruments and corresponding sample temperature/time profiles have been published elsewhere (*1–3*).

Specificity of amplification is particularly important in allele-specific amplification, where detection of an allele may be dependent on fine sequence discrimination, even a single base change. The ΔF_{508} mutation of the cystic fibrosis gene (*6*) can be detected by allele-specific amplification (*11, 12*). Several different primer pairs have been devised for discrimination of this mutation from the wild-type allele. Most often, sequences between the ΔF_{508} locus and the 3′ end of exon 10 are amplified, and a 3-bp mismatch between the discriminating primers and template is utilized for specificity. The primers used here for 3-bp discrimination (3 and 4cf/4wt) are similar, but not identical, to those previously reported (*11*).

When sequences between the 5′ end of exon 10 and ΔF_{508} are amplified, the bases that can be used to discriminate between ΔF_{508} and wild-type alleles are limited because of the sequence similarity between the ΔF_{508} bases (CTT) and the immediately adjacent 5′ sequence (CAT). When a single-base mismatch at the 3′ end of the discriminating primers is used (primers 1 and 2cf/2wt), specificity depends on either an A-A mismatch or a T-T mismatch (Figures 2 and 3). A prior attempt to discriminate ΔF_{508} from the wild-type sequence, based on this single-base match/mismatch with use of similar primers, was unsuccessful (*14*). Under "standard" polymerase chain reaction conditions and with model human immunodeficiency virus templates, a 3′ mismatch of T-T did not show discrimination, whereas an A-A mismatch reduced amplification 20-fold (*4*). In another study, 69 single-base mismatches for allele-specific amplifications included 1 A-A mismatch and 1 T-T mismatch that were reported refractory to amplification (*15*). Although details are not given, these results were apparently achieved by alteration of primer oligonucleotide and magnesium concentrations. Single-base discrimination from A-A and T-T mismatches have only rarely been reported and seemed good tests for the putative specificity advantages of rapid cycling.

The redundancy of using two allele-specific amplifications for ΔF_{508} can be used to detect infrequent errors. With rapid temperature cycling for ΔF_{508} genotype determination, 1 of 104 paired analyses was initially discordant. Repeat analysis of the discrepancy showed that the error occurred with the primer 1–2cf/2wt amplification, dependent on single-base discrimination. Use of two separate allele-specific amplifications can prevent rare technical errors, albeit at twice the work. Genotyping errors have also been reported with the polymerase chain reaction because of unexpected polymorphisms at primer binding sites (*16*); these would also be detected in a redundant system.

A total of ~3 h was required for the DNA purification from blood, amplification, and electrophoresis described here. The DNA purification was the most time-consuming step (2 h). Although the genotyping system described here is rapid and inexpensive, it is not ideal for population screening. Current capillary-based systems are awkward when large numbers of samples are manipulated. Automated systems that detect not only ΔF_{508} but also multiple cystic fibrosis mutations (for a 90% detection rate) are desirable for population screening (*17*).

Nevertheless, an understanding of the factors contributing to "nonspecific" amplification is important not only for ΔF_{508} testing, but also for any allele-specific amplification and for polymerase chain reaction techniques in general. Single-base discrimination with conventional cycling and hot starts (*13, 18*) was possible only in a narrow annealing range of 61–62 °C (Figure 2). At 60 °C, the T-T mismatch failed to prevent amplification. At 55 °C, both the T-T and A-A mismatches were extended with good efficiency, leading to complete failure of the test for genotype.

With rapid cycling, optimal annealing temperatures for single-base discrimination were about 15 °C lower than for conventional cycling (Figure 3). Whereas conventional cycling required a precise annealing temperature of 61–62 °C, a wide range of annealing temperatures at $\leq$50 °C was acceptable with rapid cycling. Although the claimed precision of most instruments is better than 1 °C, temperature variations greater than this have been reported (*19*). A wide range of acceptable annealing temperatures increases tolerance for calibration errors, minor compositional differences between samples, and sample-to-sample temperature variations. Surprisingly, any annealing temperature between 25 °C to 50 °C could be used to genotype with rapid cycling. Even with annealing at 25 °C, the major undesired amplification products from rapid cycling were of a different size from those expected for the cystic fibrosis locus (Figure 4). In contrast, single-base specificity was entirely lost at an annealing temperature of 55 °C with conventional cycling, even with hot starts (Figure 2).

It is interesting that the optimal annealing temperatures for conventional and rapid cycling are so disparate. The shape of the sample temperature/time curve near the annealing temperature is very different for rapid and conventional cycling (Figure 5). Both the temperature transition rates and the minimum amount of time that can be spent at the annealing temperature vary greatly. Sample annealing times of <1 s can be achieved with rapid cycling; the minimal annealing times in conventional cycling are 10 times longer, even with second-generation instrumentation. The short annealing times and (or) the rapid temperature transitions must be important for the single-base specificity of rapid cycling.

Nonspecific amplification in the polymerase chain reaction usually refers to either template-independent amplification (primer dimers or oligomers) or amplification of template regions other than the desired target. Amplification of such nontarget sequences generally leads to products of sizes different from the desired sequence. Hot starts have been used to minimize this kind of undesired amplification and allow single-copy detection after amplification with ethidium bromide staining (*18*). Nevertheless, hot starts will not prevent undesired amplification if the annealing temperature during cycling is too low (Figure 2).

With allele-specific amplification, an additional kind of nonspecific amplification can occur. A mismatched primer may anneal at the expected sequence but extend through the mismatch, giving a product of the same size as a perfectly matched primer. This kind of undesired amplification means failure of the method as a test for genotype. Rapid cycling strongly disfavors extension of a mismatched primer annealed at the expected site and should be useful in the design and implementation of other allele-specific amplifications.

A detailed study of the required annealing times for different annealing temperatures in DNA amplification and their effect on product yield and specificity has not been performed. A rigorous analysis based on annealing kinetics and polymerase extension rates is also conceivable. However, such studies are limited by current instrumentation. Conventional temperature cyclers have long transition times, whereas current rapid cycling instruments lack full control of temperature transition rates.

We gratefully acknowledge Idaho Technology for providing a commercial hot-air rapid cycler and Associated Regional University Pathologists, Inc., for financial assistance. The University of Utah has applied for a patent on rapid air cycling instrumentation. This technology has been licensed to Idaho Technology, in which C.T.W. holds equity interest.

References

1. Wittwer CT, Garling DJ. Rapid cycle DNA amplification: time and temperature optimization. BioTechniques 1991;10:76–83.
2. Wittwer CT, Fillmore GC, Hillyard DR. Automated polymerase chain reaction in capillary tubes with hot air. Nucleic Acids Res 1989;17:4353–7.
3. Wittwer CT, Fillmore GC, Garling DJ. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. Anal Biochem 1990;186:328–31.
4. Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky JJ. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucl Acids Res 1990;18:999–1005.
5. Collins FS. Cystic fibrosis: molecular biology and therapeutic implications. Science 1992;256:774–9.
6. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989;245:1066–73.
7. Jalanko A, Kere J, Savilahti E, Schwartz M, Syvänen A-C, Ranki M, Söderlund H. Screening for defined cystic fibrosis mutations by solid-phase minisequencing. Clin Chem 1992;38:39–43.
8. Kerem BS, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, et al. Identification of the cystic fibrosis gene: genetic analysis. Science 1989;245:1073–80.
9. Friedman KJ, Highsmith WE, Silverman LM. Detecting multiple cystic fibrosis mutations by polymerase chain reaction-mediated site-directed mutagenesis. Clin Chem 1991;37:753–5.
10. Taylor GR, Noble JS, Hall JL, Quirke P, Stewart AD, Mueller RF. Rapid screening for ΔF_{508} deletion in cystic fibrosis [Letter]. Lancet 1989;ii:1345.
11. Ballabio A, Gibbs RA, Caskey CT. PCR test for cystic fibrosis deletion [Letter]. Nature 1990;343:220.
12. Thomas SM, Moreno RF, Tilzer LL. DNA extraction with

organic solvents in gel barrier tubes. Nucl Acids Res 1989;13:5411.
13. Mullis KB. The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. PCR Methods Appl 1991;1:1–4.
14. Wagner M, Schloesser M, Reiss J. Direct gene diagnosis of cystic fibrosis by allele-specific polymerase chain reactions. Mol Biol Med 1990;7:359–64.
15. Sommer SS, Groszbach AR, Bottema CDK. PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. BioTechniques 1992;12:82–7.
16. Fujimura FK, Northrup H, Beaudet AL, O'Brien WE. Genotyping errors with the polymerase chain reaction [Letter]. N Engl J Med 1990;322:61.
17. Silverman LM, Highsmith WE. Automation and the single-gene disorder: cystic fibrosis as a model [Editorial]. Clin Chem 1992;38:7–8.
18. Chow Q, Russell M, Birch DE, Raymond J, Bloch W. Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. Nucl Acids Res 1992;20:1717–23.
19. Linz U. Thermocycler temperature variation invalidates PCR results. BioTechniques 1990;8:286–93.

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2757–2760, April 1989
Genetics

Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia

(genetic diseases/base-pair mismatch/DNA polymerase/oligodeoxyribonucleotide/polymerase chain reaction)

DAN Y. WU*, LUIS UGOZZOLI†, BIJAY K. PAL‡, AND R. BRUCE WALLACE*

*Department of Molecular Biochemistry, Beckman Research Institute of the City of Hope, Duarte, CA 91010; †Laboratorio di Immunogenetica, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy; and ‡Department of Biological Sciences, California State Polytechnic University, Pomona, CA 91768

Communicated by Eugene Roberts, December 27, 1988 (received for review December 12, 1988)

ABSTRACT **A rapid nonradioactive approach to the diagnosis of sickle cell anemia is described based on an allele-specific polymerase chain reaction (ASPCR). This method allows direct detection of the normal or the sickle cell β-globin allele in genomic DNA without additional steps of probe hybridization, ligation, or restriction enzyme cleavage. Two allele-specific oligonucleotide primers, one specific for the sickle cell allele and one specific for the normal allele, together with another primer complementary to both alleles were used in the polymerase chain reaction with genomic DNA templates. The allele-specific primers differed from each other in their terminal 3′ nucleotide. Under the proper annealing temperature and polymerase chain reaction conditions, these primers only directed amplification on their complementary allele. In a single blind study of DNA samples from 12 individuals, this method correctly and unambiguously allowed for the determination of the genotypes with no false negatives or positives. If ASPCR is able to discriminate all allelic variation (both transition and transversion mutations), this method has the potential to be a powerful approach for genetic disease diagnosis, carrier screening, HLA typing, human gene mapping, forensics, and paternity testing.**

Sickle cell anemia is the prototype of a genetic disease caused by a single base-pair mutation, an A → T transversion in the sequence encoding codon 6 of the human β-globin gene. In homozygous sickle cell anemia, the substitution of a single amino acid (Glu → Val) in the β-globin subunit of hemoglobin results in a reduced solubility of the deoxyhemoglobin molecule and erythrocytes assume irregular shapes. The sickled erythrocytes become trapped in the microcirculation and cause damage to multiple organs.

Kan and Dozy (1) were the first to describe the diagnosis of sickle cell anemia in the DNA of affected individuals based on the linkage of the sickle cell allele to an *Hpa* I restriction fragment length polymorphism. Later, it was shown that the mutation itself affected the cleavage site of both *Dde* I and *Mst* II and could be detected directly by restriction enzyme cleavage (2, 3). Conner *et al.* (4) described a more general approach to the direct detection of single nucleotide variation by the use of allele-specific oligonucleotide hybridization. In this method, a short synthetic oligonucleotide probe specific for one allele only hybridizes to that allele and not to others under appropriate conditions.

All of the above approaches are technically challenging, require a reasonably large amount of DNA, and are not very rapid. The polymerase chain reaction (PCR) developed by Saiki *et al.* (5) provided a method to rapidly amplify small amounts of a particular target DNA. The amplified DNA could then be readily analyzed for the presence of DNA sequence variation (e.g., the sickle cell mutation) by allele-specific oligonucleotide hybridization (6), restriction enzyme cleavage (5, 7), ligation of oligonucleotide pairs (8, 9), or ligation amplification (10). PCR increased the speed of analysis and reduced the amount of DNA required for it but did not change the method of analysis of DNA sequence variation. In this paper, we investigated whether PCR could be done in an allele-specific manner such that the presence or absence of an amplified fragment provides direct determination of genotype.

PCR utilizes two oligonucleotide primers that hybridize to opposing strands of DNA at positions spanning a sequence of interest. A DNA polymerase [either the Klenow fragment of *Escherichia coli* DNA polymerase I (5) or *Thermus aquaticus* DNA polymerase (11)] is used for sequential rounds of template-dependent synthesis of the DNA sequence. Prior to the initiation of each new round, the DNA is denatured and fresh enzyme is added in the case of the *E. coli* enzyme. In this manner, exponential amplification of the target sequences is achieved. We reasoned that if the 3′ nucleotide of one of the primers formed a mismatched base pair with the template due to the existence of single nucleotide variation, amplification would take place with reduced efficiency. Specific primers would then direct amplification only from their homologous allele. After multiple rounds of amplification, the formation of an amplified fragment would indicate the presence of the allele in the initial DNA.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer by the phosphoramidite method. They were purified by electrophoresis on a urea/polyacrylamide gel followed by high-performance liquid chromatography as described (12).

Source and Isolation of Human DNA. All genomic DNA samples with the exception of the β-thalassemia DNA were isolated from the peripheral blood of appropriate individuals. The β-globin genotype of these individuals was previously determined by hybridization with allele-specific oligonucleotide probes (4) as well as by hemoglobin electrophoresis. Thalassemia major DNA was obtained from an Epstein–Barr virus-transformed lymphocyte cell line obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). Thalassemia DNA was isolated from the cultured cells. All DNA preparations were performed according to a modified Triton X-100 procedure followed by proteinase K and RNase A treatment (13). The average yield of genomic DNA was ≈25 μg per ml of blood.

PCR. Hβ14A (5′-CACCTGACTCCTGA) and BGP2 (5′-AATAGACCAATAGGCAGAG) at a concentration of 0.12 μM were used as the primer set for the amplification of the

---

Abbreviations: PCR, polymerase chain reaction; ASPCR, allele-specific PCR.

normal β-globin gene (*a* primer set). Similarly, 0.12 μM Hβ14S (5′-CACCTGACTCCTGT) and 0.12 μM BGP2 were used as the primer set for the amplification of the sickle cell gene (*s* primer set). Both primer sets directed the amplification of a 203-base-pair (bp) β-globin allele-specific fragment. As an internal positive control, all reaction mixtures contained an additional primer set for the human growth hormone gene comprised of 0.2 μM GHPCR1 (5′- TTCCCAACCATTCCCTTA) and 0.2 μM GHPCR2 (5′-GGATTTCTGTTGTGTTTC) (*hGH* primer set). GHPCR1 and GHPCR2 direct the amplification of a 422-bp fragment of the human growth hormone gene. All reactions were performed in a vol of 50 μl containing 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, template DNA (0.5 μg/ml), and 0.1 mM each dATP, dCTP, dGTP, and TTP. Reactions were carried out for 25 cycles at an annealing temperature of 55°C for 2 min, a polymerization temperature of 72°C for 3 min, and a heat-denaturation temperature of 94°C for 1 min on a Perkin-Elmer Cetus DNA thermal cycler. At the end of the 25 rounds, the samples were held at 4°C in the thermal cycler until removed for analysis.

Analysis of the PCR Products. An aliquot (15 μl) from each of the completed PCR reactions was mixed with 5 μl of 5× Ficoll loading buffer (1× = 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol/3% Ficoll) and subjected to electrophoresis in a 1.5% agarose gel. Electrophoresis was performed in 89 mM Tris·HCl/89 mM borate/2 mM EDTA buffer for 2 hr at 120 V. At the completion of electrophoresis, the gel was stained in ethidium bromide (1.0 μg/ml) for 15 min, destained in water for 10 min, and photographed by ultraviolet trans-illumination.

RESULTS

Experimental Design. The scheme describing allele-specific PCR (ASPCR) is shown in Fig. 1. Primer P1 is designed such that it is complementary to allele 1 but the 3′-terminal nucleotide forms a single base-pair mismatch with the DNA sequence of allele 2 (Fig. 1*B*, *). Under appropriate annealing temperature and PCR conditions, there is normal amplification of the P1–P3 fragment with DNA templates containing allele 1 (homo- or heterozygous), while there is little or no amplification from DNA templates containing allele 2. In a similar way, a primer (P2) could be designed that would allow the specific PCR amplification of allele 2 but not allele 1 DNA.

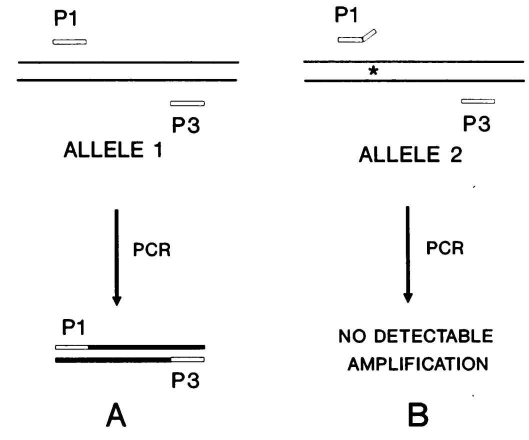


FIG. 1. Schematic representation of the ASPCR. P1 and P3, synthetic oligonucleotide primers that anneal to opposing strands of a single copy gene. P1 anneals to the region of a gene in the region of a DNA sequence variation such that its terminal 3′ nucleotide base pairs with the polymorphic nucleotide of the template. P1 is completely complementary to allele 1 (*A*) but forms a single base-pair mismatch with allele 2 at the 3′-terminal position due to one or more nucleotide differences relative to allele 1 (*B*).

We designed two 14-nucleotide-long allele-specific primers, Hβ14S and Hβ14A, complementary to the 5′ end of the sickle cell and normal β-globin genes, respectively. The oligonucleotide primers differ from each other by a single nucleotide at the 3′ end, Hβ14S having a 3′ T and Hβ14A having a 3′ A corresponding to the base pair affected by the sickle cell mutation. The oligonucleotide primer BGP2 (7) complementary to the opposite strand 3′ of the allele-specific primers was used as the second primer for PCR. The amplification product with these primer pairs was 203 bp. Also included in each reaction was a second pair of primers that directed the amplification of a 422-bp fragment of the human growth hormone gene. These primers were included as an internal positive control.

Discrimination Between the Normal and Sickle Cell Alleles. Genomic DNA was isolated from peripheral blood leukocytes of individuals of known β-globin genotypes (β^A/β^A, β^A/β^S, β^S/β^S). In addition, DNA was isolated from an Epstein–Barr virus-transformed cell line containing a homozygous deletion of the β-globin gene (β^{th}/β^{th}). DNA was subjected to 25 rounds of PCR using either the sickle cell-specific primer set (Hβ14S and BGP2) or the normal gene-specific primer set (Hβ14A and BGP2) using an annealing temperature of 55°C. The results are shown in Fig. 2*A*. It can be seen that a 203-bp fragment is observed using the sickle cell-specific primer set only with the β^A/β^S and β^S/β^S genomic DNA templates and not with the β^A/β^A genomic DNA templates. Conversely, the normal gene-specific primer set only gave rise to an amplification product with β^A/β^S and β^A/β^A genomic DNA templates. As expected, the thalassemia DNA did not give rise to a β-globin gene amplification product with either primer set. The internal growth hormone gene control gave rise to a 422-bp fragment in all samples, demonstrating that in no case was the absence of a globin-specific band due to a failure of the PCR.

In a single blind study, the DNA from 12 individuals with different β-globin genotypes was analyzed with the two primer sets. The results are shown in Fig. 2*B*. Individuals 1, 2, 3, and 5 are predicted to be β^A/β^A; individuals 6, 9, 10, and 11 are predicted to be β^S/β^S; and individuals 4, 7, 8, and 12 are predicted to be β^A/β^S. In each case, the genotype was correctly and unambiguously predicted from the pattern of fragment amplification (see legend to Fig. 2 for clinically diagnosed genotype).

DISCUSSION

The results presented above indicate the potential usefulness of ASPCR for sickle cell diagnosis. The method is rapid and the result is obtained without the use of radioactivity, since all that is required is to visualize the band on a gel with ethidium bromide staining. It should be possible to further improve the technique by elimination of the gel separation step. One strategy for this is shown in Fig. 3. As proposed recently by Yamane *et al.* (15), the two primers for the PCR could be labeled differently, one with biotin and one with a fluorescent group such as fluorescein or tetramethyl rhodamine. The product of the PCR could be captured on streptavidin-agarose and the presence of the amplified sequence could be detected with the fluorescence. In this case, if one allele-specific primer were labeled with one fluorescent group and the other were labeled with a different one, then the ASPCR could be done simultaneously.

In this study, we have used PCR primers that form either an A·A or a T·T mismatch. It is not clear that other mismatches will give equally effective discrimination. Since G·T mismatches are more stable than other mismatches (16), G·T should probably be avoided when designing primers.

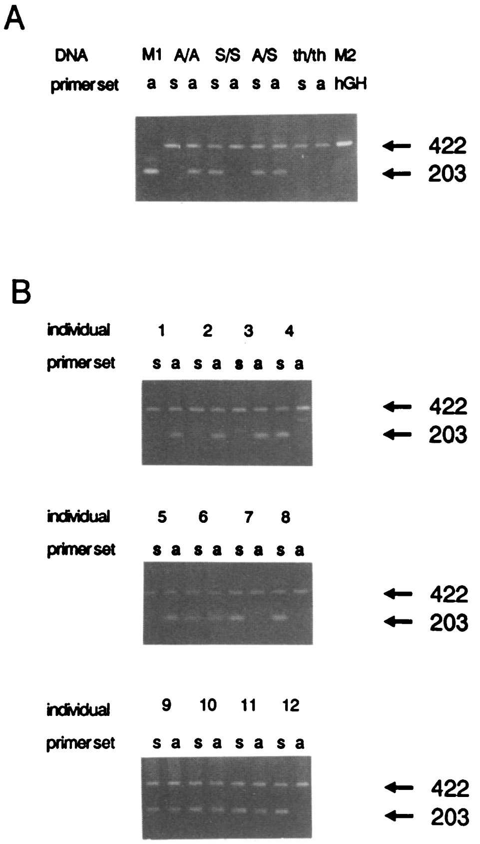


FIG. 2. (*A*) Identification of the normal (β^A) and the sickle cell (β^S) alleles by ASPCR. Normal (β^A/β^A), homozygous sickle cell (β^S/β^S), heterozygous sickle cell (β^A/β^S), and homozygous β-thalassemia (β^{th}/β^{th}) DNA samples (0.5 μg each) served as template using either the normal (*a* primer set) or the sickle cell (*s* primer set) for the ASPCRs. As an internal positive control, all reaction mixtures contained an additional primer set for the human growth hormone gene (*hGH* primer set) that directed the amplification of a 422-bp fragment of the human growth hormone gene. After amplification, 15 μl from each reaction mixture was subjected to electrophoresis in a 1.5% agarose gel for 2 hr at 120 V. Ethidium bromide staining of the agarose gel was used to detect PCR amplified fragments. Positive β-globin ASPCR can be identified by the presence of a 203-bp fragment using either the *a* or the *s* primer set reaction. As a marker for the globin-specific fragment, 0.3 μg of plasmid pHβ^A containing the normal human globin gene (β^A) was amplified with the *a* primer set alone (M1). As a marker for the growth hormone-specific fragment, 0.1 μg of plasmid pXGH5 containing a 3.8-kilobase fragment of the human growth hormone gene (14) was amplified with the growth hormone primer set (hGH) alone (M2). (*B*) A single blind trial using ASPCR to diagnose the β-globin genotype of genomic DNA samples. Genomic DNA samples from 12 individuals (4 each of normal, homozygous, and heterozygous sickle cell individuals) were randomly assigned numbers 1–12 by the hematology laboratory and blinded to the investigators. ASPCR was performed using both the normal (*a*) and the sickle cell-specific (*s*) primer sets as described above. Genotypes were identified as homozygous normal (β^A/β^A) if the single 203-bp fragment appears exclusively in the *a* primer set reaction, as homozygous sickle cell (β^S/β^S) if the 203-bp fragment appears only in the *s* primer set, or as heterozygous sickle cell trait (β^A/β^S) if the fragment appears in both reactions. The genotypes of these DNA samples were previously determined by hemoglobin electrophoresis (results not shown). The genotypes of the 12 individuals are as follows: 1, 2, 3, and 5, β^A/β^A; 6, 9, 10, and 11, β^A/β^S; 4, 7, 8, and 12, β^S/β^S.

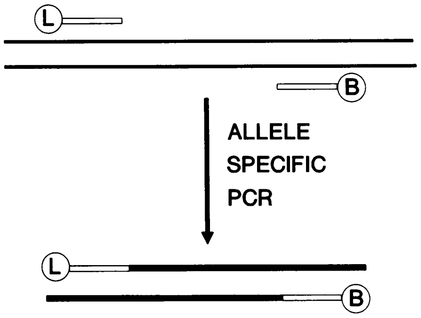


FIG. 3. Schematic representation of a dual labeling system suitable for the detection of the ASPCR products. One of the oligonucleotide primers is labeled at the 5′ end with a fluorescent group such as fluorescein or tetramethyl rhodamine (L) and the other primer is labeled with biotin (B). The ASPCR amplification product would therefore have the 5′ end labeled on both strands. The biotin is suitable for capturing the amplified fragment on a streptavidin-agarose column, while the fluorescent group is suitable for measuring the amount of fragment produced.

This can be done by designing the primer so that it is complementary to the strand with which it forms an A·C mismatch. It may be possible to use a competition approach, as we have previously used to improve the discrimination provided by oligonucleotide hybridization probes (17). In this case, a competitive primer could be designed that was not able to prime, for example, by including in it a 3′ dideoxynucleotide or a 3′ ribonucleotide that has been oxidized. A mixture of a labeled allele-specific primer complementary to allele 1 plus an unlabeled priming-defective primer complementary to allele 2 should then allow the specific amplification of allele 1.

The ability of an oligonucleotide to prime on a DNA template is governed by two kinetic variables: the rate at which the annealed primer dissociates from the template before initiating polymerization (r_{off}) and the rate at which the DNA polymerase extends the primer (r_{pol}). Efficient priming in PCR should take place whenever $r_{pol} > r_{off}$, the addition of the first few nucleotides to the primer then greatly stabilizing the oligonucleotide–template complex and allowing continued extension of the primer. For a given primer r_{pol} is an intrinsic property of the polymerase. Studies with *E. coli* DNA polymerase I have suggested that this polymerase may be able to discriminate between primers that either do or do not form a mismatch with the template at the 3′-terminal nucleotide (18). In this case, r_{pol} for the mismatched primer was slower than r_{pol} for the perfectly matched primer. For the present study, we designed the allele-specific primers such that the allele-specific nucleotide in the template was complementary to the 3′-terminal nucleotide of the primer. In this way, the 3′ nucleotide of the primer specific for one allele would form a mismatch with the other allele. This design allows one to take advantage of the difference between r_{pol} of the perfectly matched and mismatched primers as well as to optimize primer concentration, priming temperature, primer length, and primer sequence, all of which will affect the difference in the r_{off} for the two allele-specific primers.

We reasoned that a set of conditions should exist such that $r_{pol} > r_{off}$ for the perfectly matched primer, while $r_{pol} < r_{off}$ for the mismatched primer. The results shown here clearly demonstrate this to be true. In our study, the allele-specific primers were 14 nucleotides long. We found (data not shown) that discrimination between the β^A and β^S alleles was not possible at low annealing temperatures (e.g., 44°C and 50°C). Presumably the short length of the oligonucleotides as well as the high annealing temperature combined to provide the discrimination.

Taq polymerase is well suited for using ASPCR for the discrimination of two alleles that differ by a single nucleotide because it lacks a 3′ → 5′ exonuclease activity (19). Such an activity would correct the mismatched base pair in the mismatched primer–template complex and then permit efficient priming with the one-nucleotide-shorter primer. Since the specificity of the ASPCR is determined in the initial several cycles of PCR, the fact that the primer remains uncorrected enhances the discrimination of the reaction. PCR is an exponential reaction; the yield of product is very dependent on the efficiency of each round (5). Only very minor changes in the efficiency of each round of amplification have profound effects on the overall yield after many rounds. For example, if the efficiency of the reaction with the perfectly matched primer is 90% and with the mismatched primer is 60%, there would be 73-fold more product produced in the reaction with perfectly matched primer than with the mismatched primer.

The ASPCR should find application in the fields of genetic diagnosis, carrier screening, HLA typing, and any other nucleic acid-based diagnostic in which the precise DNA sequence of the priming site is diagnostic for the target. In the case of HLA typing, recent advances have used PCR amplification followed by allele-specific oligonucleotide hybridization for the determination of *DR*, *DQ*, and *DP* alleles (6, 20–22). It should be possible to use ASPCR for the direct analysis of HLA types.

We have recently proposed a process for the simultaneous determination of multiple polymorphic loci based on the concept of producing locus-specific amplification products each with a unique length (23). In such a system, since ASPCR would produce allele-specific products, the simultaneous analysis of the genotype of the target DNA at multiple loci should be possible.

This work was supported by Grant DCB-8515365 from the National Science Foundation (R.B.W.). D.Y.W. is a M.D./Ph.D. candidate at Loma Linda University. R.B.W. is a member of the Cancer Center of the City of Hope (NIH CA33572). L.U. is a fellow of AIRC (Associazione Italiana per la Ricerca sul Cancro).

1. Kan, Y. W. & Dozy, A. M. (1978) *Lancet* **ii**, 910–912.
2. Geever, R. F., Wilson, L. B., Nallaseth, F. S., Milner, P. F., Bittner, M. & Wilson, J. T. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5081–5085.
3. Chang, J. C. & Kan, Y. W. (1982) *N. Engl. J. Med.* **307**, 30–32.
4. Conner, B. J., Reyes, A. A., Morin, C., Itakura, K., Teplitz, R. L. & Wallace, R. B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 278–282.
5. Saiki, R. K., Scharf, S., Falcona, F., Mullis, K., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230**, 1350–1354.
6. Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1986) *Nature (London)* **324**, 163–166.
7. Chehab, F. F., Doherty, M., Cai, S., Kan, Y. W., Cooper, S. & Rubin, E. M. (1987) *Nature (London)* **329**, 293–294.
8. Landegren, U., Kaiser, R., Sanders, J. & Hood, L. (1988) *Science* **241**, 1077–1080.
9. Wu, D. Y. & Wallace, R. B. (1989) *Gene*, in press.
10. Wu, D. Y. & Wallace, R. B. (1989) *Genomics*, in press.
11. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
12. Dembek, P., Miyoshi, K. & Itakura, K. (1981) *J. Am. Chem. Soc.* **103**, 706–708.
13. Bell, G. I., Karam, J. H. & Rutter, W. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5759–5763.
14. Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. & Moore, D. D. (1986) *Mol. Cell Biol.* **6**, 3173–3179.
15. Yamane, A., Nakagami, S., Kawasoe, T. & Miyoshi, K. (1988) *Nucleic Acids Res.* **20**, 91.
16. Kidd, V. J., Wallace, R. B., Itakura, K. & Woo, S. L. C. (1983) *Nature (London)* **304**, 230–234.
17. Nozari, G., Rahbar, S. & Wallace, R. B. (1986) *Gene* **43**, 23–28.
18. Atkinson, M. R., Deutscher, M. P., Kornberg, A., Russel, A. F. & Moffet, J. G. (1969) *Biochemistry* **8**, 4897–4904.
19. Tindall, K. R. & Kunkel, T. A. (1988) *Biochemistry* **27**, 6008–6013.
20. Morel, P. A., Dorman, J. S., Todd, J. A., McDevitt, H. O. & Trucco, M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8111–8115.
21. Angelini, G., Bugawan, T., Delfino, L., Erlich, H. & Ferrara, G. B. (1988) *Hum. Immunol.* **23**, 77.
22. Scharf, S., Saiki, R. & Erlich, H. (1988) *Hum. Immunol.* **23**, 143.
23. Skolnick, M. H. & Wallace, R. B. (1988) *Genomics* **2**, 273–279.

Diabetes

200 NEJM ANNIVERSARY ARTICLE

The Past 200 Years in Diabetes

Kenneth S. Polonsky, M.D.

From the Department of Medicine, University of Chicago, Chicago. Address reprint requests to Dr. Polonsky at the Department of Medicine, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, or at polonsky@bsd.uchicago.edu.

N Engl J Med 2012;367:1332-40.
DOI: 10.1056/NEJMra1110560

DIABETES WAS FIRST RECOGNIZED AROUND 1500 B.C.E. BY THE ANCIENT Egyptians, who considered it a rare condition in which a person urinated excessively and lost weight. The term diabetes mellitus, reflecting the fact that the urine of those affected had a sweet taste, was first used by the Greek physician Aretaeus, who lived from about 80 to 138 C.E. It was not until 1776, however, that Matthew Dobson actually measured the concentration of glucose in the urine of such patients and found it to be increased.[1]

Diabetes was a recognized clinical entity when the *New England Journal of Medicine and Surgery* was founded in 1812. Its prevalence at the time was not documented, and essentially nothing was known about the mechanisms responsible for the disease. No effective treatment was available, and diabetes was uniformly fatal within weeks to months after its diagnosis owing to insulin deficiency. In the intervening 200 years, major fundamental advances have been made in our understanding of the underlying causes of diabetes and the approach to its prevention and treatment (see timeline, available with the full text of this article at NEJM.org). Although diabetes is still associated with a reduced life expectancy, the outlook for patients with this disease has improved dramatically, and patients usually lead active and productive lives for many decades after the diagnosis has been made. Many effective therapies are available for treating hyperglycemia and its complications. The study of diabetes and related aspects of glucose metabolism has been such fertile ground for scientific inquiry that 10 scientists have received the Nobel Prize for diabetes-related investigations since 1923 (Table 1). Thus, as a result of the efforts of the past 200 years, there is much good news to report regarding diabetes.

A timeline is available at NEJM.org

Ironically, although scientific advances have led to effective strategies for preventing diabetes, the pathway to cure has remained elusive. In fact, if one views diabetes from a public health and overall societal standpoint, little progress has been made toward conquering the disease during the past 200 years, and we are arguably worse off now than we were in 1812. Two centuries ago, severe insulin deficiency dominated the clinical presentation of diabetes. Although it is possible that some people had milder forms of hyperglycemia at that time, they largely escaped clinical detection. In 2012, the commonly encountered spectrum of diabetes is quite different. Although severe insulin deficiency still occurs, it now accounts for only about 10% of cases overall and can be readily treated with insulin. The vast majority of patients with diabetes are overweight and have a combination of insulin resistance and impaired insulin secretion. The prevalence of this form of diabetes has been increasing dramatically, particularly in the past three to four decades, resulting in a worldwide epidemic that has made diabetes one of the most common and most serious medical conditions humankind has had to face.

Table 1. Nobel Prizes for Diabetes-Related Research.

Year	Category	Recipient	Contribution
1923	Medicine	F.G. Banting and J.J.R. Macleod	Discovery of insulin
1947	Medicine	C.F. Cori and G.T. Cori	Discovery of the course of the catalytic conversion of glycogen
1947	Medicine	B.A. Houssay	Discovery of the role of hormones released by the anterior pituitary lobe in the metabolism of sugar
1958	Chemistry	F. Sanger	Work on the structure of proteins, especially insulin
1971	Medicine	E.W. Sutherland	Discoveries concerning the mechanisms of action of hormones
1977	Medicine	R. Yalow	Development of radioimmunoassays for peptide hormones
1992	Medicine	E.H. Fischer and E.G. Krebs	Discoveries concerning reversible protein phosphorylation as a biologic regulatory mechanism

THE SCIENTIFIC BASIS OF CURRENT TREATMENT APPROACHES

STUDIES OF GLUCOSE METABOLISM

In the past 200 years, we have made dramatic advances in our understanding of the regulation of normal glucose metabolism. Beginning in the mid-19th century, Claude Bernard showed that blood glucose levels are regulated not just by the absorption of dietary carbohydrate but also by the liver, which plays a central role in producing glucose from nonglucose precursors.[2] Other investigators built on this discovery to identify the enzymes responsible for the synthesis and breakdown of glycogen,[3] the role of anterior pituitary hormones in glucose metabolism and the onset of diabetes,[4] the role of reversible protein phosphorylation by a protein kinase,[5] and the discovery of cyclic AMP and its role in hormonal action, particularly that of epinephrine and glucagon, both of which elevate the blood glucose concentration and contribute to diabetic hyperglycemia.[6]

THE ROLE OF THE PANCREAS AND THE DISCOVERY OF INSULIN

In 1889, Joseph von Mering and Oskar Minkowski found that removing the pancreas from dogs resulted in fatal diabetes, providing the first clue that the pancreas plays a key role in regulating glucose concentrations.[7,8] In 1910, Edward Albert Sharpey-Schafer hypothesized that diabetes was due to the deficiency of a single chemical produced by the pancreas; he called this chemical insulin, from the Latin word *insula*, meaning island and referring to the pancreatic islet cells of Langerhans. In 1921, Frederick Banting and Charles Best actually discovered insulin when they reversed diabetes that had been induced in dogs with an extract from the pancreatic islet cells of healthy dogs.[9,10] Together with James Collip and John Macleod, they purified the hormone insulin from bovine pancreases and were the first to use it to treat a patient with diabetes. The production of insulin and its therapeutic use quickly spread around the world. This series of events may be the most dramatic example of the rapid translation of a discovery in basic science into a benefit for patients. Once insulin injections became available, young people with insulin deficiency who had previously faced almost certain, painful death within weeks to months were able to survive for prolonged periods of time. Figure 1 shows a patient before and after she was treated successfully with insulin in 1922.[11]

INSULIN CHEMISTRY, BIOLOGY, AND PHYSIOLOGY

The dramatic discovery of insulin and the rapid demonstration that it is essential for human health stimulated intense interest in its chemistry and biology. A number of landmark discoveries resulted, some of which reached beyond diabetes research. For example, Frederick Sanger was awarded the Nobel Prize in Chemistry for developing methods to sequence the amino acids of proteins, and he used insulin as an example of his approaches.[12] Insulin was the first hormone for which the three-dimensional crystal structure was determined (by Dorothy Hodgkin, who had previously received the Nobel Prize in Chemistry for determining the structure of vitamin B_{12}). Donald Steiner's demonstration in 1967 that the two-polypeptide insulin molecule is derived from a

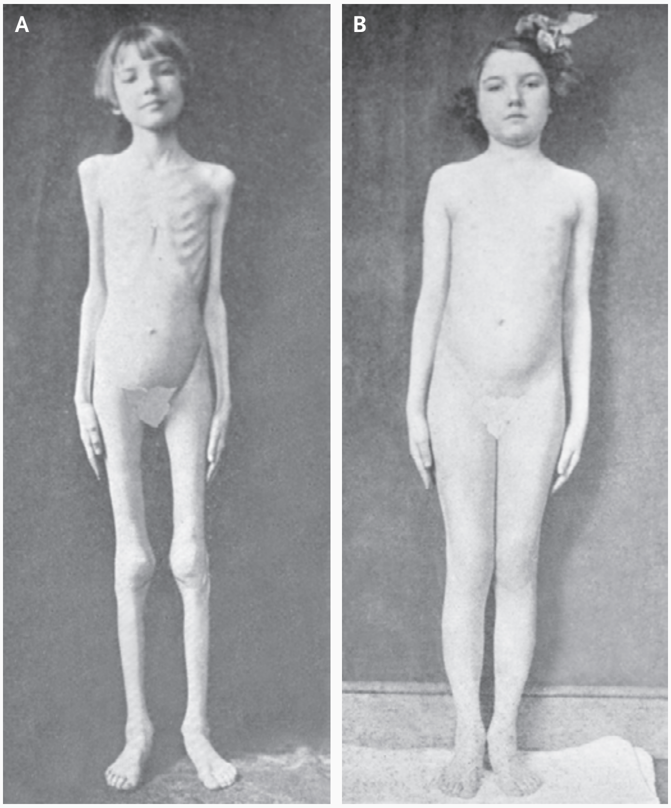


Figure 1. **Effects of Insulin Therapy.**
These photographs from 1922, in a case described by Geyelin,[11] show a young girl with insulin-deficient diabetes before treatment with insulin (Panel A) and after treatment (Panel B).

single-chain precursor proinsulin[13] was important not only for our understanding of the biochemistry of insulin but also because it applies to other peptide hormones that are transcribed as single-chain precursors. Insulin was the first hormone to be cloned[14] and then produced for therapeutic use by means of recombinant DNA technology, which provided an unlimited supply of this important molecule and laid the foundation for the biotechnology industry. Figure 2 shows the structure of insulin.

The development of the radioimmunoassay for insulin by Rosalyn Yalow and Solomon Berson in 1959 permitted the quantitative measurement of pancreatic beta-cell function in animals and humans and established the radioimmunoassay as a powerful tool for measuring proteins, metabolites, and other chemicals present in very low concentrations.[15] Much of our current understanding of diabetes has resulted from the ability to measure serum insulin levels.

PATHOGENESIS OF DIABETES

INSULIN RESISTANCE AND INSULIN DEFICIENCY

Over the past two centuries, we have learned that diabetes is a complex, heterogeneous disorder. Type 1 diabetes occurs predominantly in young people and is due to selective autoimmune destruction of the pancreatic beta cell, leading to insulin deficiency. Type 2 diabetes is much more common, and the vast majority of people with this disorder are overweight. The increase in body weight in the general population, a result of high-fat, high-calorie diets and a sedentary lifestyle, is the most important factor associated with the increased prevalence of type 2 diabetes. Older adults are most likely to have type 2 diabetes, although the age at onset has been falling in recent years, and type 2 diabetes is now common among teenagers and young adults.

Harold Himsworth first proposed in 1936 that many patients with diabetes have insulin resistance rather than insulin deficiency.[16] We now know that insulin resistance is essential in the pathogenesis of type 2 diabetes and that the disease results from both insulin resistance and impaired beta-cell function.[17] A clinical phenotype widely called the metabolic syndrome, which includes insulin resistance, upper-body obesity, hypertension, hypertriglyceridemia, and low levels of high-density lipoprotein cholesterol,[18] identifies persons at high risk for glucose intolerance and diabetes. Such persons are also at high risk for cardiovascular disease and should be targeted for preventive strategies.

GENETIC FACTORS

Genetic factors play an important role in the development of diabetes. Type 1 and type 2 diabetes are polygenic disorders, and multiple genes and environmental factors contribute to the development of the disease. A few forms of diabetes (e.g., maturity-onset diabetes of the young and neonatal diabetes) are single-gene disorders that affect the pancreatic beta cell[19,20] but account for only 1 to 2% of cases. In type 1 diabetes, alleles at the human leukocyte antigen locus on the short arm of chromosome 6 appear to explain up to 50% of the cases of familial clustering.[21,22] In contrast, a predominant genetic susceptibility lo-

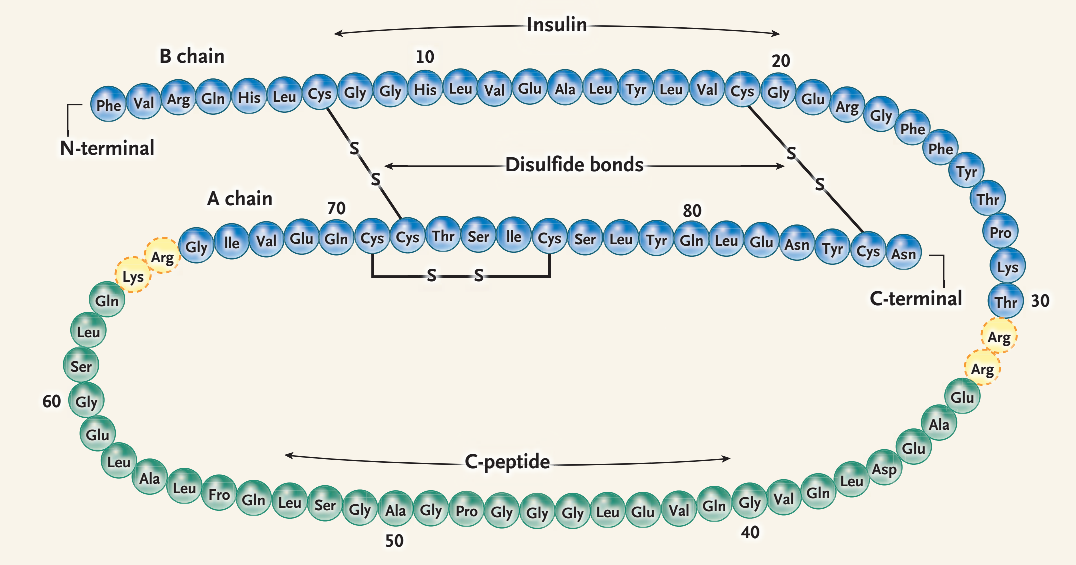


Figure 2. The Structure of Human Proinsulin.

Proinsulin is converted to insulin by proteolytic converting enzymes that remove the connecting peptide (C-peptide) and the lysine-arginine (Lys-Arg) and arginine-arginine (Arg-Arg) sequences of dibasic amino acids, leaving the mature insulin molecule, which consists of A and B chains connected by disulfide bonds.

cus for type 2 diabetes has not been found. Genetic studies have identified over 40 genetic variants that increase the risk of type 2 diabetes, but in the aggregate these variants account for only about 10% of the heritability of the disorder.[23,24] Individually, persons with these variants have an increased risk of diabetes of 10 to 15%, as compared with persons without the variants. The multiplicity of genes that contribute to the risk of type 2 diabetes makes it difficult to determine this risk precisely or to develop selective preventive or therapeutic strategies based on the genetic profile.

PREVENTION AND TREATMENT OF DIABETES

The approach to the prevention and treatment of diabetes has been transformed since the discovery of insulin, which led to the rapid development of a widely available and lifesaving new treatment and initiated a series of advances that have fundamentally enhanced the daily lives of patients with diabetes and dramatically extended their life expectancy. Many advances have resulted from important clinical trials that were reported in the *Journal* and elsewhere.[25-49] Some highlights of these studies include the use of biosynthetic human insulin, which has virtually eliminated local reactions at the injection site; insulin syringes and needles that are small and convenient to use and have reduced the pain of injections; home glucose monitoring,[25] which together with measurements of glycated hemoglobin,[26] allows therapy to be altered on the basis of accurate assessments of glucose control; and insulin pumps[27] driven by computer algorithms[28] that adjust insulin doses on the basis of the continuous measurement of glucose levels to achieve glucose concentrations within the physiologic range (Fig. 3). Preventive strategies and treatments for diabetic complications have undergone impressive improvements. The beneficial effects of angiotensin-receptor blockade, angiotensin-converting–enzyme inhibition, and protein restriction in preventing diabetic nephropathy have been shown.[29-34] Advances in kidney transplantation have extended the lives of patients with advanced diabetic kidney disease, and laser photocoagulation has preserved the vision of millions of patients with diabetic retinopathy.[35] Advances in islet-cell and pancreas transplantation have also been impressive.[36,37] Recent evidence exemplified by the results of two randomized, controlled clinical trials reported this past spring in the *Journal* suggests that bariatric surgery to induce weight loss in patients with

Detection devices

Detection of diabetes has progressed from use of the saccharometer in the 1800s to measure urine density (a proxy for urinary glucose content) to instruments that monitor blood glucose levels at home.

Insulin syringes

Insulin syringes were initially glass and were used on multiple occasions, with needles that were also reused. Insulin pens, which became available in the 1990s, allow patients to vary the injected dose and to administer insulin discreetly.

Insulin preparations

The first highly refined form of insulin was extracted from porcine or bovine pancreas. Recombinant human insulin is now readily available.

Insulin pumps

The first insulin pumps, such as the Mill Hill infuser (near right), were invented in 1976 and weighed more than 0.5 kg. Current pumps are much smaller and more portable. Pumps that simultaneously infuse insulin and monitor glucose, allowing instantaneous feedback, are currently under investigation.

Figure 3. Milestones in Diabetes Diagnosis and Management.

Photographs of the saccharometer and the early insulin preparation are from the Science Museum collection at the Science & Society Picture Library.

type 2 diabetes is much more effective than either standard or intensive medical therapy alone in lowering glucose levels and even in achieving disease remission.[38,39] Advances in technology have thus profoundly improved our ability to monitor diabetic control (from urine testing to home glucose meters to continuous glucose monitoring) and to treat this disease and its complications (laser therapy for diabetic retinopathy, kidney transplantation for diabetic renal disease, and bariatric surgery to induce disease remission).

Diabetes care has been at the forefront of efforts to develop team-based approaches to patient care that involve physicians, nurses, nutritionists, social workers, podiatrists, and others and in developing models of care delivery for chronic illness. Using such an approach, the Diabetes Prevention Program showed that physical activity and weight loss can reduce the risk of diabetes in predisposed persons by 58%.[40] Major effects are also seen after treatment with metformin[40] or pioglitazone.[41] The Diabetes Control and Complications Trial showed that improved glucose control reduces microvascular complications in type 1 diabetes,[42] and the United Kingdom Prospective Diabetes Study showed the same for type 2 diabetes.[43] Intensive insulin therapy to prevent hyperglycemia improves outcomes in critically ill patients.[44,45]

The effect of diabetes treatment on cardiovascular outcomes and mortality is a critical issue. The Steno-2 Study showed that a multifactorial intervention aimed at improving control of glucose levels, lipid levels, and blood pressure led to a 50% reduction in cardiovascular mortality among patients with type 2 diabetes.[46,47] Among patients with type 1 diabetes, improved glucose control leads to a reduction in macrovascular disease, an effect that becomes apparent only many years after the improvement has been achieved.[48] The recent Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial showed that aggressive glycemic control of type 2 diabetes reduced the risk of nonfatal myocardial infarction but increased overall mortality.[49] The reasons for these differences between studies are not clear, but in type 2 diabetes, multiple factors increase the predisposition to cardiovascular disease. Indeed, treatment of hyperlipidemia and hypertension appears to be more effective in reducing cardiovascular events than does treatment to lower glucose levels. As a result of these and other findings, the treatments available for patients with diabetes have improved dramatically, particularly over the past 30 to 40 years.

PREVALENCE OF DIABETES — A WORLDWIDE EPIDEMIC

Unfortunately, the improvement in outcomes for individual patients with diabetes has not resulted in similar improvements from the public health perspective. The worldwide prevalence of diabetes has continued to increase dramatically. The difficulty in applying the principles of diabetes care from the individual patient to the population reflects the unique challenges of implementing research findings and effecting behavioral change. Figure 4 shows the number and percentage of persons in the U.S. population with diagnosed diabetes between 1980 and 2010 (http://www.cdc.gov/diabetes/statistics/prevalence_national.htm). During this period, the number of diagnosed cases of diabetes increased from 5.6 million to 20.9 million, representing 2.5% and 6.9% of the population, respectively. Nearly 27% of persons over 65 years of age have diabetes. If current trends continue, 1 in 3 U.S. adults could have diabetes by 2050. The American Diabetes Association estimated that the cost of diagnosed diabetes in the United States was $174 billion in 2007,[50] and efforts to prevent and treat diabetes threaten to overwhelm health systems throughout the world.

FUTURE CHALLENGES

Given the surge in the prevalence of diabetes, timely prevention of this disease at the population level is essential. Opportunities abound for the implementation of preventive public policies. Rigorous scientific methods will be needed to evaluate the effects of policy and legislative initiatives to eliminate trans fat from the diet; require restaurants to provide the caloric content of items on their menus; reduce the availability of high-calorie, high-fat foods in school cafeterias; and impose a tax on sugar-sweetened beverages. Lifestyle modification will undoubtedly play a key role in the ultimate solution to the problem of diabetes, but the necessary modifications have not been easy to implement, and more definitive solutions will depend on the ability of basic science to point prevention and treatment in new directions. Advances in basic immunology — in particular, the transformation of primitive stem cells into pancreatic beta cells — offer promise for the preven-

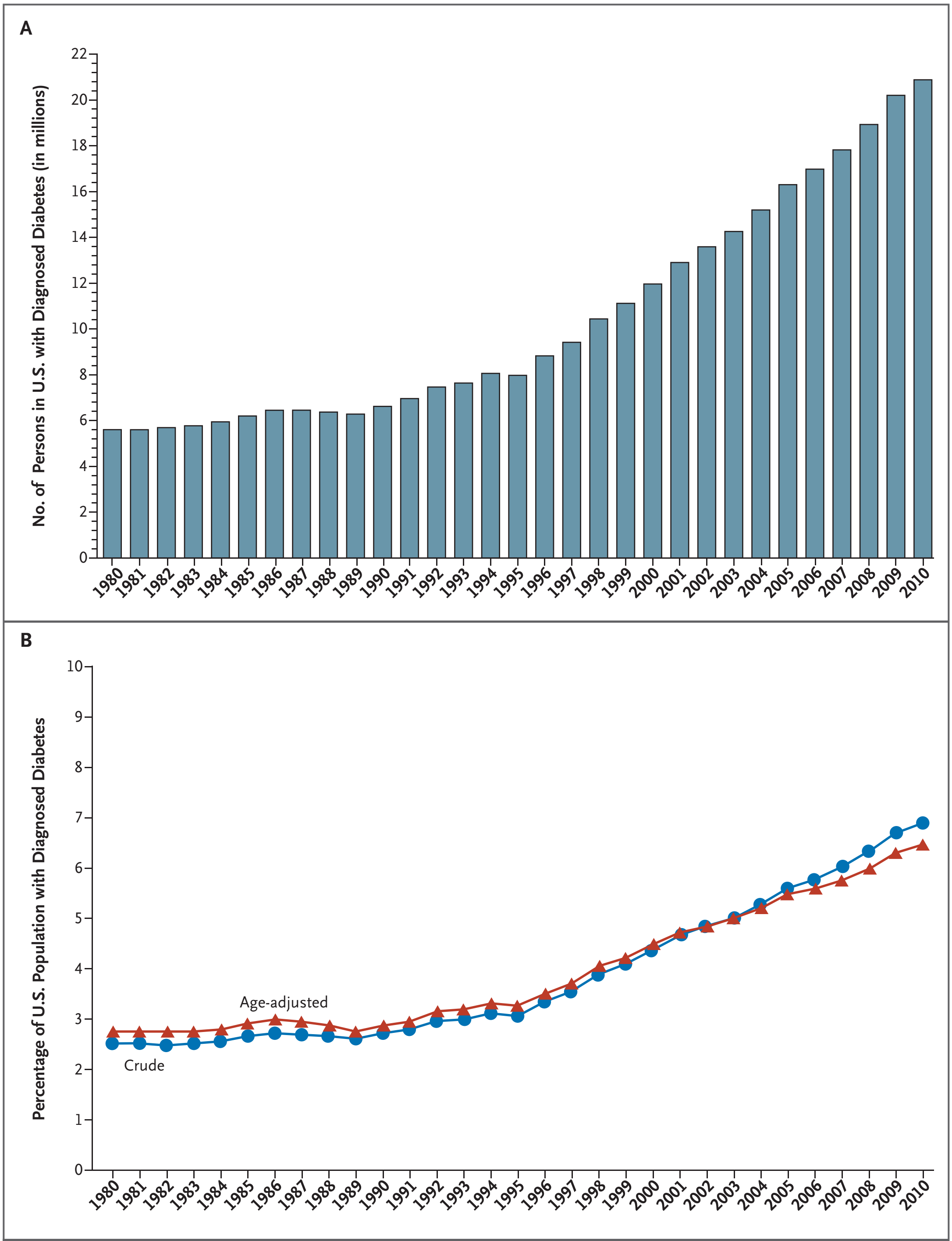


Figure 4. Number of Persons and Percentages of the Population with Diagnosed Diabetes in the United States, 1980–2010.

Panel A shows the number of cases of diagnosed diabetes between 1980 and 2010 among U.S. adults 18 to 79 years of age. During this period the number increased from 5.6 million to 20.9 million. Panel B shows the crude and age-adjusted percentages of the U.S. population with diagnosed diabetes for this same period of time. Data are from the Centers for Disease Control and Prevention (http://cdc.gov/diabetes/statistics/prevalence_national.html).

tion and treatment of autoimmunity in patients with type 1 diabetes. Advances in the identification of diabetes-susceptibility genes should clarify the relative role of insulin resistance and beta-cell dysfunction and identify molecular pathways and new drug targets, leading to more effective approaches to the prevention and treatment of type 2 diabetes. Although the challenges are still substantial, if we build on past accomplishments, there is every reason for optimism that another breakthrough as dramatic as the discovery of insulin will occur in the foreseeable future, with a similarly dramatic impact.

Disclosure forms provided by the author are available with the full text of this article at NEJM.org.

REFERENCES

1. Dobson M. Nature of the urine in diabetes. Medical Observations and Enquiries 1776;5:218-30.
2. Robin ED. Claude Bernard: pioneer of regulatory biology. JAMA 1979;242:1283-4.
3. Cori CF, Cori GT. Carbohydrate metabolism. Annu Rev Biochem 1946;15:193-218.
4. Houssay BA, Smyth FS, Foglia VG, Houssay AB. Comparative diabetogenic action of the hypophysis from various animals. J Exp Med 1942;75:93-106.
5. Fischer EH. Phosphorylase and the origin of reversible protein phosphorylation. Biol Chem 2010;391:131-7.
6. Sutherland EW. Studies on the mechanism of hormone action. Science 1972; 177:401-8.
7. von Mering J, Minkowski O. Diabetes mellitus nach Pankreasextirpation. Arch Exp Pathol Pharmacol 1890;26:371-87.
8. Brogard JM, Vetter T, Bickle JF. Discovery of pancreatic diabetes in Strasbourg. Diabete Metab 1992;18:104-14. (In French.)
9. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Encore: pancreatic extracts in the treatment of diabetes mellitus: preliminary report, 1922. CMAJ 1991;145:1281-6.
10. Bliss M. The discovery of insulin. Chicago: University of Chicago Press, 2007.
11. Geyelin HR, Harrop G, Murray MF, Corwin E. The use of insulin in juvenile diabetes. J Metabolic Res 1922;2:767-92.
12. Sanger F. The free amino groups of insulin. Biochem J 1945;39:507-15.
13. Steiner DF, Oyer PC. The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. Proc Natl Acad Sci U S A 1967;57:473-80.
14. Ullrich A, Shine J, Pictet R, Tischer E, Rutter WJ, Goodman HM. Rat insulin genes: construction of plasmids containing coding sequences. Science 1977;196: 1313-9.
15. Yalow RS, Berson SA. Assay of plasma insulin in human subjects by immunological methods. Nature 1959;184:Suppl 21: 1648-9.
16. Himsworth HP. Diabetes mellitus: its differentiation into insulin-sensitive and insulin-insensitive types. Lancet 1936;1: 127-30.
17. Cavaghan MK, Ehrmann DA, Polonsky KS. Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. J Clin Invest 2000;106:329-33.
18. Reaven GM. Why Syndrome X? From Harold Himsworth to the insulin resistance syndrome. Cell Metab 2005;1:9-14.
19. Fajans SS, Bell GI, Polonsky KS. Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. N Engl J Med 2001;345:971-80.
20. Støy J, Steiner DF, Park SY, Ye H, Philipson LH, Bell GI. Clinical and molecular genetics of neonatal diabetes due to mutations in the insulin gene. Rev Endocr Metab Disord 2010;11:205-15. [Erratum, Rev Endocr Metab Disord 2012;13:79-81.]
21. Nerup J, Platz P, Andersen OO, et al. HL-A antigens and diabetes mellitus. Lancet 1974;2:864-6.
22. Ounissi-Benkalha H, Polychronakos C. The molecular genetics of type 1 diabetes: new genes and emerging mechanisms. Trends Mol Med 2008;14:268-75.
23. Stolerman ES, Florez JC. Genomics of type 2 diabetes mellitus: implications for the clinician. Nat Rev Endocrinol 2009;5: 429-36.
24. Ahlqvist E, Ahluwalia TS, Groop L. Genetics of type 2 diabetes. Clin Chem 2011;57:241-54.
25. The Juvenile Diabetes Research Foundation Continuous Glucose Monitoring Study Group. Continuous glucose monitoring and intensive treatment of type 1 diabetes. N Engl J Med 2008;359:1464-76.
26. Nathan DM, Singer DE, Hurxthal K, Goodson JD. The clinical information value of the glycosylated hemoglobin assay. N Engl J Med 1984;310:341-6.
27. Tamborlane WV, Sherman RS, Genel M, Felig P. Reduction to normal of plasma glucose in juvenile diabetes by subcutaneous administration of insulin with a portable infusion pump. N Engl J Med 1979; 300:573-8.
28. Bergenstal RM, Tamborlane WV, Ahmann A, et al. Effectiveness of sensor-augmented insulin-pump therapy in type 1 diabetes. N Engl J Med 2010;363:311-20. [Erratum, N Engl J Med 2010;363:1092.]
29. Barnett AH, Bain SC, Bouter P, et al. Angiotensin-receptor blockade versus converting-enzyme inhibition in type 2 diabetes and nephropathy. N Engl J Med 2004;351:1952-61. [Erratum, N Engl J Med 2005;352:1731.]
30. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting–enzyme inhibition on diabetic nephropathy. N Engl J Med 1993;329:1456-62. [Erratum, N Engl J Med 1993;330:152.]
31. Brenner BM, Cooper ME, de Zeeuw D, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med 2001;345:861-9.
32. Lewis EJ, Hunsicker LG, Clarke WR, et al. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. N Engl J Med 2001;345:851-60.
33. Parving H-H, Lehnert H, Bröchner-Mortensen J, Gomis R, Andersen S, Arner P. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. N Engl J Med 2001; 345:870-8.
34. Klahr S, Levey AS, Beck GJ, et al. The effects of dietary protein restriction and blood-pressure control on the progression of chronic renal disease. N Engl J Med 1994;330:877-84.
35. Frank RN. Diabetic retinopathy. N Engl J Med 2004;350:48-58.
36. Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355:1318-30.
37. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M. Reversal of lesions of diabetic nephropathy after pancreas transplantation. N Engl J Med 1998;339:69-75.
38. Mingrone G, Panunzi S, De Gaetano A, et al. Bariatric surgery versus conventional medical therapy for type 2 diabetes. N Engl J Med 2012;366:1577-85.
39. Schauer PR, Kashyap SR, Wolski K, et al. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. N Engl J Med 2012;366:1567-76.
40. Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 2002;346:393-403.
41. DeFronzo RA, Tripathy D, Schwenke DC, et al. Pioglitazone for diabetes prevention in impaired glucose tolerance.

N Engl J Med 2011;364:1104-15. [Erratum, N Engl J Med 2011;365:189, 869.]
42. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977-86.
43. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet 1998; 352:854-65.
44. Van den Berghe G, Wouters P, Weekers F, et al. Intensive insulin therapy in critically ill patients. N Engl J Med 2001; 345:1359-67.
45. Van den Berghe G, Wilmer A, Hermans G, et al. Intensive insulin therapy in the medical ICU. N Engl J Med 2006;354: 449-61.
46. Gaede P, Vedel P, Larsen N, Jensen GVH, Parving H-H, Pedersen O. Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. N Engl J Med 2003;348:383-93.
47. Gaede P, Lund-Anderson H, Parving H-H, Pedersen O. Effect of a multifactorial intervention on mortality in type 2 diabetes. N Engl J Med 2008;358:580-91.
48. Nathan DM, Cleary PA, Backlund JY, et al. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. N Engl J Med 2005;353: 2643-53.
49. The ACCORD Study Group. Long-term effects of intensive glucose lowering on cardiovascular outcomes. N Engl J Med 2011;364:818-28.
50. American Diabetes Association. Economic costs of diabetes in the US in 2007. Diabetes Care 2008;31:596-615.

JCB Journal of Cell Biology

REVIEW

The cell biology of systemic insulin function

Victoria L. Tokarz[1,2]*, Patrick E. MacDonald[3]*, and Amira Klip[1,2,4]

Insulin is the paramount anabolic hormone, promoting carbon energy deposition in the body. Its synthesis, quality control, delivery, and action are exquisitely regulated by highly orchestrated intracellular mechanisms in different organs or "stations" of its bodily journey. In this *Beyond the Cell* review, we focus on these five stages of the journey of insulin through the body and the captivating cell biology that underlies the interaction of insulin with each organ. We first analyze insulin's biosynthesis in and export from the β-cells of the pancreas. Next, we focus on its first pass and partial clearance in the liver with its temporality and periodicity linked to secretion. Continuing the journey, we briefly describe insulin's action on the blood vasculature and its still-debated mechanisms of exit from the capillary beds. Once in the parenchymal interstitium of muscle and adipose tissue, insulin promotes glucose uptake into myofibers and adipocytes, and we elaborate on the intricate signaling and vesicle traffic mechanisms that underlie this fundamental function. Finally, we touch upon the renal degradation of insulin to end its action. Cellular discernment of insulin's availability and action should prove critical to understanding its pivotal physiological functions and how their failure leads to diabetes.

Introduction

Preceded by valiant efforts in Berlin, Strasbourg, Baltimore, and Bucharest, insulin was discovered in Toronto in 1921 by Fredrick Banting and Charles Best, with auspicious advice and support from John Macleod, and its purification was made possible by James Collip. The story of its discovery is legendary and was awarded the Nobel Prize in Physiology or Medicine in 1923 (Karamitsos, 2011), but the journey of this hormone in the body has not been "romanced" as much. Insulin is the paramount anabolic hormone (promoting dietary carbon source deposition), and its synthesis, quality control, delivery, and action are exquisitely regulated in different organs or "stations" of its bodily journey. These functions are enacted by highly orchestrated intracellular mechanisms, starting with production in the β-cells of the pancreas, on to its partial clearance by the liver hepatocytes, followed by its delivery and action on the vascular endothelium and its functions at level of the brain, muscle fibers, and adipocytes (major action sites), and ending with insulin degradation in the kidney. As such, the journey of insulin in the body is a superb example of integrated cellular physiology.

In this *Beyond the Cell* review, we focus on five stages of the journey of insulin through the body and the captivating cell biology that underlies its connections with each organ. We analyze insulin's biosynthesis in and release from β-cells of the pancreas, its first pass and partial clearance in the liver, its action on the blood vasculature and exit from the capillary beds, its action in the central nervous system in brief, followed by its stimulation of muscle and adipose cell glucose uptake, and its degradation in the kidney to finalize its action (Fig. 1).

By necessity, many aspects of the metabolic actions of insulin are not reviewed here; rather, we present the most current picture of each phenomenon, highlighting up-to-date concepts and spatial-temporal coordinates. By applying a cell biology lens to the five fundamental stages in insulin's journey in the body, we hope to render an integrated view of insulin "within and beyond the cell." Of major relevance, though not individually discussed here, defects in each station of the hormone's journey in the body have been correlated and often causally related to insulin resistance, hypertension, and type 2 diabetes (Taniguchi et al., 2006; Hoehn et al., 2008; Odegaard and Chawla, 2013; Boucher et al., 2014; DeFronzo et al., 2015; Samuel and Shulman, 2016; Haeusler et al., 2018; also see other important highlights in the text box).

Biosynthesis and export of insulin in pancreatic β-cells

Insulin synthesis, processing, and packaging in pancreatic β-cells

Humans have a single insulin gene, *INS* (rodents have two, *ins1* and *ins2*), located on chromosome 11, the transcription of which is controlled largely by upstream enhancer elements that bind key transcription factors that include IDX1 (PDX1), MafA, and NeuroD1 along with numerous coregulators (Artner and Stein, 2008). In the insulin-producing pancreatic β-cells, these are

---

[1]Cell Biology Program, The Hospital for Sick Children, Toronto, Ontario, Canada; [2]Department of Physiology, University of Toronto, Toronto, Ontario, Canada; [3]Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada; [4]Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada.

*V.L. Tokarz and P.E. MacDonald contributed equally to this paper; Correspondence to Amira Klip: amira@sickkids.ca.

Rockefeller University Press
J. Cell Biol. 2018

https://doi.org/10.1083/jcb.201802095

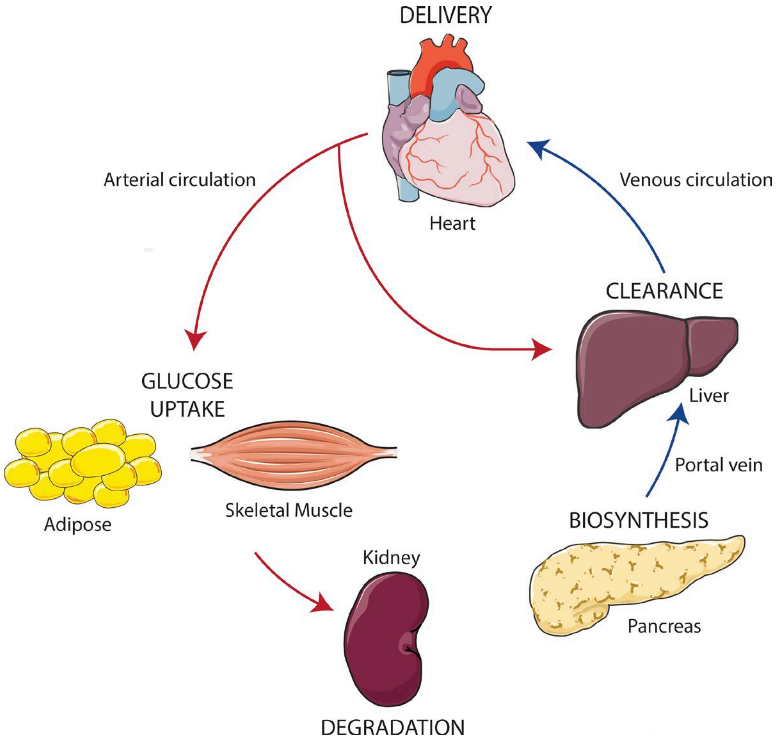


Figure 1. **Journey of insulin in the body.** Insulin is transcribed and expressed in the β-cells of the pancreas, from whence it is exported through the portal circulation to the liver. During this first pass, over 50% of insulin is cleared by the hepatocytes in the liver. The remaining insulin exits the liver via the hepatic vein, where it follows the venous circulation to the heart. Insulin is distributed to the rest of the body through the arterial circulation. Along the arterial tree, insulin promotes vasodilation. Arterially delivered insulin exerts its metabolic actions in the liver and is further cleared (second pass). Insulin exits the circulation at the level of the microvasculature, reaching muscle and fat cells, where it stimulates GLUT4 translocation and glucose uptake. Remaining circulating insulin is delivered to and finally degraded by the kidney. This review analyzes the cellular processes at each stage of this journey. This figure was created using Servier Medical Art (available at https://smart.servier.com/).

required for insulin gene expression and contribute to the regulation of *INS* transcription in response to glucose and autocrine insulin signaling (Andrali et al., 2008). Given the role of these enhancer elements, transcription factors, and their coregulators in controlling the expression of insulin and many additional components of the β-cell secretory pathway, such as glucose transporter 2 (GLUT2) and the insulin processing enzyme PC1/3, they are key defining contributors to the establishment and maintenance of β-cell identity (Gao et al., 2014).

Insulin is translated initially as a preproinsulin (Fig. 2 A), which is then processed to proinsulin in the RER upon cleavage of its signal sequence by a signal peptidase. In the RER, proinsulin is folded and stabilized in its 3D proinsulin configuration, linking the semihelical A domain and helical B domain via the formation of three disulfide bonds. After transit to the Golgi apparatus, the properly folded proinsulin is sorted into still-immature secretory granules where it is processed via the prohormone convertases PC1/3 and PC2, which cleave the C-peptide. Subsequently, carboxypeptidase E removes C-terminal basic amino acids from the resulting peptide chains, yielding mature insulin consisting of A- and B-peptide chains linked by disulfide bonds (Hutton, 1994).

Transit of immature secretory granules through the TGN, and their subsequent budding and maturation, is controlled by a host of regulatory proteins, including newly identified vesicle-sorting by proteins such as SORCS1 (Kebede et al., 2014) and HID-1 (Du et al., 2016). Insulin biosynthesis in this manner is generally rapid (less than ~2 h) and efficient, with only 1–2% of the protein remaining as proinsulin within mature secretory granules where insulin couples with Zn^{2+} and exists as a hexameric crystal with the cation. Transport of the insulin hexamer into the secretory granules is thought to be mediated by ZnT8 or related zinc transporters (Lemaire et al., 2009).

Insulin granule pools and their intracellular traffic

Most insulin granules (perhaps 75–95% of an estimated 10,000) are stored within the β-cell cytoplasm at some distance away from the cell membrane (Rorsman and Renström, 2003). The remainder move to the cell periphery along microtubule networks in an AMPK- and kinesin1-dependent manner (McDonald et al., 2009). To reach the plasma membrane, however, granules must cross a cortical actin network that acts as a physical barrier to insulin secretion (Li et al., 1994). Actin reorganization is

Selected examples of mechanistic defects in the five stages of the journey of insulin, associated with insulin resistance and type 2 diabetes

- Defective insulin exocytosis from diabetic β-cells (Ferdaoussi and MacDonald, 2017; Gandasi et al., 2017) and impaired pulsatile secretion of insulin in diabetic individuals (Lang et al., 1981; Hollingdal et al., 2000; Laedtke et al., 2000)
- Reduced hepatic insulin clearance (Jung et al., 2018) and CEACAM1 expression (Lee, 2011) in obesity
- Impaired vasoactive effects of insulin during insulin resistance, including capillary recruitment (de Jongh et al., 2004; Clerk et al., 2006; Keske et al., 2009); reduced insulin delivery to muscle in obesity and diabetes (Broussard et al., 2016)
- Diminished GLUT4 translocation to the muscle membrane in diabetic rodents and humans (Klip et al., 1990; Zierath et al., 1996; Garvey et al., 1998; Hoehn et al., 2008; Czech, 2017) and lowered expression of Rac1 (Sylow et al., 2013) as well as a number of proteins of the GLUT4 vesicle fusion machinery (Aslamy and Thurmond, 2017); the underlying defects include alterations in the maintenance of the storage compartment (Foley et al., 2011; Samuel and Shulman, 2012) and in the insulin-derived signals that trigger GLUT4 vesicle release from storage and interaction with the plasma membrane
- Compromised glomerular function in obesity (Kanasaki et al., 2013) that may alter insulin bioavailability; sodium retention and down-regulation of the natriuretic peptide system in insulin resistance (Spoto et al., 2016) that may herald hypertension

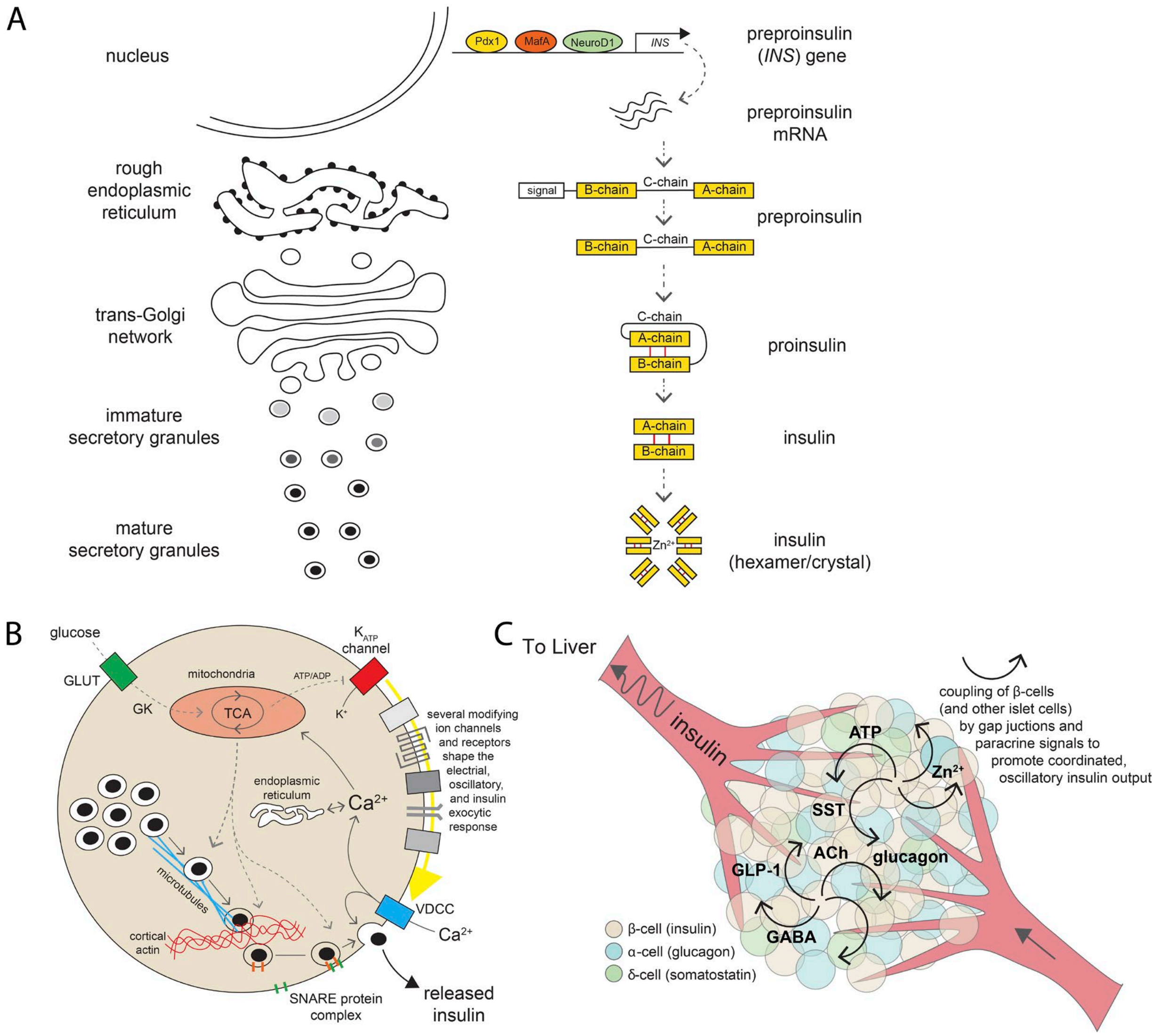


Figure 2. **Insulin biosynthesis and secretion. (A)** Insulin maturation along the granule secretory pathway. Preproinsulin mRNA is transcribed from the *INS* gene and translated to preproinsulin peptide. As this transits through the RER and TGN, the prepropeptide is processed to its mature form and ultimately stored as hexameric insulin/Zn^{2+} crystals within mature secretory granules. **(B)** Glucose sensing and metabolic signals leading to insulin granule secretion. The release of insulin via exocytosis of secretory granules from pancreatic β-cells is controlled by a series of metabolic and electrical signals arising as a result of glucose entry through GLUTs, phosphorylation by GK, and entry into the TCA cycle. The closure of ATP-dependent K^+ (K_{ATP}) channels triggers electrical events that culminate in Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCCs), which triggers exocytosis mediated by SNARE complex proteins. The overall secretory response is modulated by numerous receptors, channels, intracellular Ca^{2+} stores, metabolic signals, and cytoskeletal elements. **(C)** Islet communication for coordinated pulsatile insulin secretion. Within an islet, β-cells communicate with each other and with glucagon-producing α-cells and somatostatin (SST)–producing δ-cells to coordinate their activity. Many putative intraislet messengers have been implicated, including ATP, Zn^{2+}, γ-aminobutyric acid (GABA), glucagon-like peptide-1 (GLP-1), acetylcholine (ACh), and others. These, along with electrical coupling via gap junctions, are likely important for the physiological coordination of pulsatile insulin secretion.

therefore an important component of the early journey of insulin before it can exit the β-cell. The process is coordinated by the action of several small G-proteins and their activating nucleotide exchange factors. This includes the glucose- and Cdc42-dependent activation of Rac1, which, when released from an inhibitory RhoGDI and in its GTP-bound form, promotes cortical actin remodeling, perhaps via an interaction with gelsolin (Kalwat and Thurmond, 2013). Finally, secretory granules must dock at the plasma membrane and be chemically "primed" in response to an intracellular Ca^{2+} signal (Fig. 2 B).

The coordinated interaction of exocytic machinery proteins in association with Ca^{2+} channels (Gandasi et al., 2017) ensures assembly of an insulin granule–exocytic site complex that is "ready to go" when needed. These events likely underlie the well-described biphasic nature of glucose-evoked insulin secretion seen in vitro: a rapid first phase resulting from fusion and secretion by already "docked and primed" secretory granules that in human lasts up to 10 min, and a subsequent second-phase secretion that is associated with actin reorganization thought to allow granule recruitment to the plasma membrane (Wang and Thurmond, 2009).

The orderly arrival, priming, docking, and fusion of granules is exquisitely coordinated in response to physiological inputs initiated by glucose and decoded by the β-cell, as described next.

Sensing glucose: Metabolism-controlled electrical signals and Ca^{2+} activity

Glucose is the paramount metabolic signal eliciting insulin secretion, and a consensus model reveals a relay of chemical to electrical on to mechanical signals (Fig. 2 B). In brief, glucose enters through the cell membrane glucose transporters GLUT2 in rodents and GLUT1 in humans (McCulloch et al., 2011). Glucose is rapidly phosphorylated by glucokinase (GK) to generate glucose-6-phosphate, which, through glycolysis, feeds the mitochondrial TCA cycle. GK, an isoform of hexokinase, effectively generates downstream signaling metabolites (i.e., ATP and pyruvate) within a range of glucose concentrations that matches the normal physiological range for plasma glucose homeostasis (Meglasson et al., 1983). For this reason, the tandem GLUT1/2 and GK is often referred to as a glucose-sensor controlling blood sugar levels. Mutations that alter the glucose-dependent activity of GK effectively adjust the set point for whole-body glucose homeostasis (Gloyn et al., 2003).

Pyruvate generated from glycolysis enters the mitochondria via mitochondrial pyruvate carriers (Patterson et al., 2014), where the TCA cycle–dependent generation of NADH promotes the export of H^+ from the mitochondrial matrix by the electron transport chain, and then generation of ATP from ADP by ATP synthase, which itself appears dependent on mitochondrial Ca^{2+} uptake (Tarasov et al., 2013). Subsequent increases in the cytosolic ATP/ADP ratio control cell membrane potential by inhibiting ATP-sensitive K^+ (K_{ATP}) channels, eliciting a membrane depolarization that is modulated by a number of additional ion channels (Fig. 2 B). This represents the conversion of chemical to electrical signaling. When the membrane potential depolarizes sufficiently (approximately −50 mV), the activation of voltage-dependent Na^+ and Ca^{2+} channels cause repetitive action potential spiking and a rise in intracellular Ca^{2+} (Rorsman et al., 2012). Ca^{2+} thus becomes the "currency" that triggers granule fusion with the plasma membrane. The increase in cytosolic Ca^{2+} is rapidly reversed by very active Ca^{2+} pumps such as the ER sarco-ER Ca^{2+}-ATPase (SERCA), and the use of ATP in the entire process might feed back to activate AMPK and promote insulin granule migration toward the cell periphery. Additional important feedback between Ca^{2+} and intracellular signals should be noted. For example, feedback from oscillatory Ca^{2+} signals controls mitochondrial ATP generation (Tarasov et al., 2012), and recent work shows that Ca^{2+} oscillations and the ER protein TMEM24 interact at ER–plasma membrane contact sites to maintain phosphatidylinositol levels required for β-cell signaling and insulin secretion (Lees et al., 2017).

Importantly, cells across the entire islet, and islets across the entire pancreas, coordinate their Ca^{2+} signals to effect insulin secretion that occurs as rhythmic oscillations. Although pancreatic β-cells are electrically excitable in response to glucose, they do not work in isolation: they talk to each other. The electrical and Ca^{2+}-responses of β-cells within an islet are synchronized (Zarkovic and Henquin, 2004) and perhaps even coordinated by pacemaker (or "hub") β-cells within the islet (Johnston et al., 2016). Gap junction coupling between β-cells via connexin36 plays a critical role, the loss of which results in dysregulation of insulin secretion (Ravier et al., 2005). Paracrine and autocrine signaling among β-cells also likely contributes to the coordination and amplification of electrical and Ca^{2+} responses. Transmitter molecules secreted by β-cells themselves, such as ATP (Gylfe et al., 2012), among others, likely modulate the excitatory activity of nearby β-cells, thus controlling islet Ca^{2+} and insulin secretory responses (Fig. 2 C).

Thus, communication between cells within an islet likely contributes to the well-described phenomenon of insulin secretory oscillations, which occur in healthy humans with a periodicity of 5–10 min (Satin et al., 2015). The electrical activity and intracellular Ca^{2+} responses in β-cells within rodent and human islets also oscillates, ranging from tens of seconds to ∼5 min (Dean and Matthews, 1970; Henquin et al., 1982). Further information is provided in Fig. 2 B and in recent modeling that integrates metabolic, electrical, and Ca^{2+} feedback to produce these oscillations (Bertram et al., 2018). Importantly, this translates into oscillations of insulin secretion from isolated islets, again with a periodicity of 1–5 min (Bergsten et al., 1994).

On top of this, the translation of this single-islet oscillatory activity into a pulsatile release of insulin from the whole pancreas in vivo requires coordination among many individual islets (perhaps a million within a human pancreas). It is not entirely clear how islets within a pancreas communicate in order to synchronize their oscillations. Strong recent evidence suggests a key role for an intrapancreatic neural network, which could coordinate activity among disparate islet populations. This "neuroinsular network" was most recently demonstrated by elegant 3D imaging techniques in rodent (Tang et al., 2018a) and human (Tang et al., 2018b) pancreata.

The glucose-dependent increase in cytosolic ATP/ADP, closure of K_{ATP} channels, and initiation of electrical activity to increase Ca^{2+} and trigger insulin exocytosis has been a useful consensus model for regulated insulin secretion for more than 35 yr. However, this model oversimplifies the physiological regulation of insulin secretion. It has been long recognized that additional signals from gut-derived hormones, autonomic inputs, glucose metabolism itself, and paracrine signals from neighboring α- and δ-cells impinge on this model to exert important control on insulin secretion (Fig. 2 C). Many of these signals "amplify" the secretory response, either by modulating the electrical/Ca^{2+} responses of β-cells or by controlling the efficacy of Ca^{2+}-triggered insulin exocytosis. For example, the gut-derived hormones glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide together mediate the "incretin" effect whereby nutrient sensing in the gut signals to islets to augment the insulin secretory response to glucose (Drucker et al., 2017). These hormones act on classical G-protein–coupled receptors via $G\alpha_s$-activation of adenylate cyclase to increase cAMP, which causes PKA-dependent phosphorylation of the exocytic machinery and PKA-independent effects mediated by Epac2A to promote the release of Ca^{2+} from intracellular stores (Kolic and MacDonald, 2015). Recently, Epac2A was also shown to regulate insulin granule priming (Alenkvist et al., 2017).

Although a glucose-dependent rise in the intracellular ATP/ADP ratio is critical for eliciting β-cell electrical and Ca^{2+} responses, other mitochondria-derived signals are also important determinants of the secretory response to that Ca^{2+} rise. Hence, glucose not only controls the Ca^{2+} signals that trigger insulin secretion but also generates signals that improve the efficacy of Ca^{2+} on the secretory process, likely by acting on the Ca^{2+} sensitivity of diverse components in the pathway (Henquin, 2000). This is likely behind the glucose-dependent improvement in insulin granule docking-priming, which is also promoted by additional metabolism-derived signals such as glutamate, the fatty acid metabolite monoacylglycerol, and NADPH (Ferdaoussi and MacDonald, 2017). Together, these inputs interact with various elements of the downstream signaling machinery to effectively amplify secretory responses to a Ca^{2+} signal. The metabolic signals controlling electrical activity and exocytic function ultimately determine the timing and magnitude of insulin secretion. This concerted mechanism accounts for the first phase insulin that takes place with 30 min (during a glucose tolerance test in humans). A second phase lasting up to 120 min ensues that may involve new insulin synthesis.

Insulin granule exocytosis

Insulin granules in apposition to the plasma membrane dock with the membrane through the coordinated interaction and recruitment of exocytic SNARE proteins that include SNAP-25, VAMP-8, and syntaxins 1A and 3 (Gaisano, 2017). Loss of key SNARE proteins results in impaired insulin secretion (Liang et al., 2017). The formation and fidelity of the SNARE complex mediating granule docking is regulated by a number of proteins such as Munc18 and syntaxin isoforms (Gandasi and Barg, 2014; Zhu et al., 2015). Assembly of the exocytic site in β-cells includes the association of insulin granules with L-type Ca^{2+} channels (Gandasi et al., 2017), which ensures efficient delivery of Ca^{2+} to the secretory vesicle Ca^{2+} sensor, synaptotagmin VII. Collectively, these mechanisms trigger the fusion of the insulin granule bilayer with the plasma membrane, with subsequent release of insulin.

Insulin release occurs directly into the interstitial space of the pancreas, which is surrounded by a fenestrated endothelial vasculature. In this way, released insulin readily finds its way into the portal circulation to be delivered directly to the liver for "first pass."

Insulin clearance by the liver: Its receptor-mediated endocytosis and degradation

Pulsatile delivery of insulin through the portal vein to the liver

The liver is the first organ that insulin encounters along its journey. Accordingly, the liver is uniquely exposed to higher concentrations of insulin than other insulin-responsive tissues such as muscle and fat. The portal vein delivers insulin from the pancreas to the liver in discrete pulses that occur every ~5 min (Song et al., 2000), where the amplitude of these insulin pulses is 0.5–1 nmol/liter in the fasted state and rises to ~5 nmol/liter after a meal (Pørksen et al., 1995; Song et al., 2000). Pulsatile insulin delivery to the liver is an important physiological signal that regulates both hepatic insulin action (Matveyenko et al., 2012) and insulin clearance (Meier et al., 2005), although the cellular underpinnings of how hepatocytes sense pulsatility are unknown.

The liver acts as a gatekeeper that regulates the amount of insulin reaching peripheral tissues through a process called insulin clearance, which was first observed in dogs (Stevenson et al., 1985). The concentration of insulin arriving to the liver by the portal vein can be up to 10-fold higher than the concentration in the peripheral circulation (Horwitz et al., 1975), and the maintenance of this portal-systemic gradient is mediated by substantial insulin degradation by the liver. In humans, simultaneous measurements of portal vein and peripheral vein insulin concentrations during constant glucose infusion revealed that upwards of 50% and possibly even 80% of insulin arriving to the liver by the portal vein is degraded during first-pass hepatic clearance (Meier et al., 2005), and ~25% of the circulating insulin is degraded upon its second pass through the liver, so that the circulating concentration of insulin is one third that in the portal circulation (Stevenson et al., 1985). This degradation is coupled to pulsatile delivery, such that the liver preferentially clears insulin that arrives in pulses (Meier et al., 2005). Although it seems counterintuitive that so much insulin would be disposed of, degradation appears to be the default mechanism that is however modulated by demand to achieve the insulin concentration required at the periphery. Indeed, adaptive decreases in the rate of hepatic insulin clearance (Ader et al., 2014; Jung et al., 2018) have been observed during insulin resistance and act to compensate for decreased insulin sensitivity (Jung et al., 2018). Moreover, although insulin internalization is not required for many of the metabolic actions of insulin in the liver, the internalized insulin receptor (IR) continues to signal at least within early endosomes (Bevan et al., 1995). Endosomal signaling may have a differential impact from that emanating exclusively from the cell surface, akin to the differential location-based signaling of the EGF receptor (Bergeron et al., 2016).

The portal circulation delivers insulin into the capillaries of the sinusoids, which are not supported by a basement membrane and their endothelial cells contain fenestrations (Wisse, 1970; Braet et al., 1995), together permitting the exchange of contents between the blood and the surrounding liver cells. The unique structure of the hepatic sinusoids allows insulin to easily diffuse out of the circulation and into the perisinusoidal space, where it comes into contact with hepatocytes (Fig. 3 A).

Hepatocytes are the major site of insulin clearance. Early electron microscopy studies revealed that IRs bind ^{125}I-insulin on microvilli (interdigitations) of the hepatocyte membrane (Carpentier et al., 1985). After binding, ^{125}I-insulin–IR complexes move to the base of the microvilli, where they associate with clathrin-coated pits (Pilch et al., 1983) and internalize by clathrin-mediated endocytosis (Fehlmann et al., 1982). Although still unknown for hepatocytes, IR autophosphorylation is required for insulin uptake by CHO cells (Carpentier et al., 1992). Consistent with earlier studies, liver-specific IR knockout mice provided direct evidence that receptor-mediated degradation regulates systemic insulin levels and that impairments in this process lead to severe hyperinsulinemia that, in turn, contributes to whole-body insulin resistance (Michael et al., 2000). Of note, mice lacking in the liver the IR

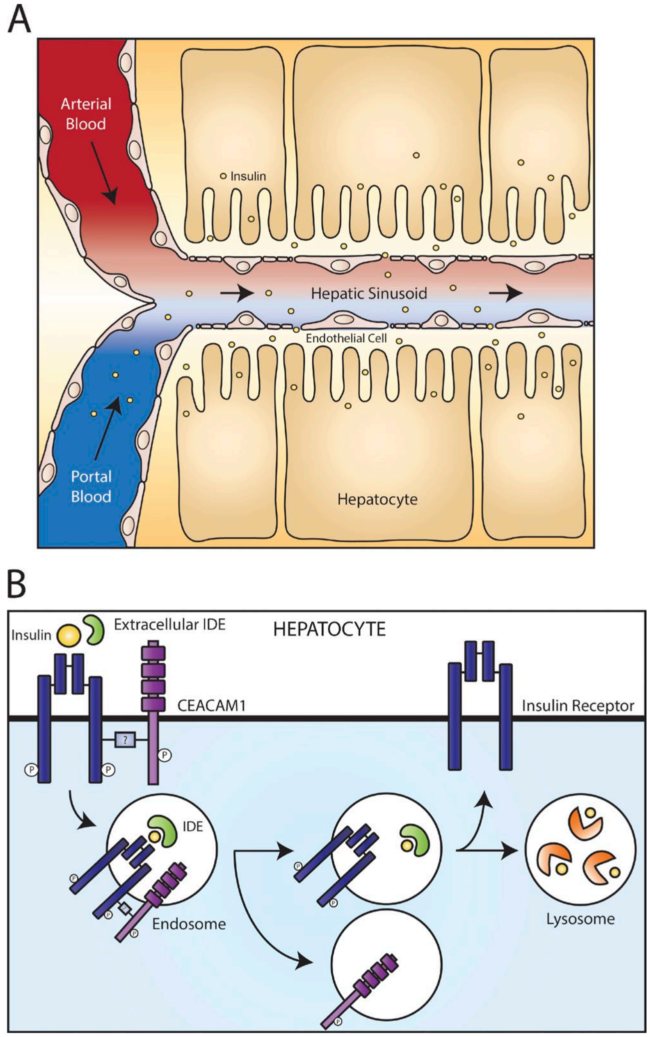


Figure 3. **Insulin clearance in the liver. (A)** Insulin is delivered to the hepatic sinusoid, where it freely accesses the liver hepatocytes through the fenestrated sinusoidal endothelium. **(B)** Proposed mechanism for insulin degradation in hepatocytes. Insulin binds to the IR and forms a complex with CEACAM1. Prior to internalization, extracellular IDE begins to degrade receptor-bound insulin. After internalization, endosomal IDE degrades receptor-bound insulin and, once the endosome acidifies and the complex dissociates, also frees insulin. Any remaining insulin or insulin fragments progress toward lysosomes for their complete proteolytic degradation.

substrates 1 and 2 (IRS1,2; adaptor proteins that can bind to the IR to initiate signal transduction) have less severe hyperinsulinemia (Dong et al., 2008) than mice lacking the IR (Michael et al., 2000; Cohen et al., 2007), suggesting that canonical insulin signaling via IRS1,2 may not participate in insulin clearance. However, there is no direct mechanistic proof of this or of the involvement of other classical insulin signals.

After insulin binds to its receptor on the hepatocyte surface, endocytosis of the receptor–ligand complex causes a concomitant loss of surface IR (Goodner et al., 1988), which is followed by rapid recycling and reinsertion of intact, unbound IRs in the plasma membrane (Goodner et al., 1988). These findings are concordant with the physiological intervals of pulsatile delivery, suggesting that hepatocyte IR internalization and reinsertion into the membrane is adaptively entrained to insulin delivery (Meier et al., 2005). In contrast to IR recycling, the fate of internalized insulin differs, as we will describe.

CEACAM1 and insulin-degrading enzyme (IDE): Hepatic molecules driving insulin clearance

Although hepatocytes are not exclusive in their ability to internalize insulin, they highly express the transmembrane glycoprotein CEACAM1 (carcinoembryonic antigen–related cell adhesion molecule 1), which mediates rapid and effective IR-mediated insulin endocytosis (Najjar, 2002). Mechanistically, CEACAM1 is phosphorylated by the IR, enhancing the formation of an insulin–IR–CEACAM1 complex (Najjar et al., 1995; Fig. 3 B). It is hypothesized that a so-far-unidentified adaptor protein targets the tripartite complex to the AP2 adaptor complex for clathrin-mediated endocytosis (Najjar, 2002). Tests in nonhepatic cells, on the other hand, show that the IR target protein SHC binds dynamin (a GTPase required for the scission of endocytic vesicles), and this complex contributes to IR internalization (Baron et al., 1998). It is tempting to hypothesize that, in hepatocytes, SHC might be the protein linking the IR to CEACAM1 and thus brings the complex to dynamin-rich regions prone for endocytosis. Consistent with the crucial role of CEACAM1 in hepatic insulin clearance, impairments in insulin-stimulated hepatic CEACAM1 phosphorylation or whole-body depletion of hepatic CEACAM1 cause severe hyperinsulinemia and, consequently, insulin resistance and hyperglycemia (Poy et al., 2002; Russo et al., 2017). Notably, hepatocytes from these mice have impaired insulin-dependent IR endocytosis, which can be rescued by liver-specific reexpression of CEACAM1 (Poy et al., 2002; Russo et al., 2017). Hence, the level of CEACAM1 at the plasma membrane and its phosphorylation may impart physiological fine-tuning regulation to the process of insulin clearance.

The majority of insulin that binds to hepatic IRs is degraded (Duckworth, 1988). The degradation process begins on the membrane immediately after insulin binding, where some insulin is reported to be partially degraded by extracellular IDE before internalization (Yokono et al., 1982). After internalization, additional IDE is thought to be targeted to endosomal membranes through its interaction with phosphatidylinositol phosphates (Song et al., 2017), where it begins to degrade receptor-bound insulin in endosomes (Yonezawa et al., 1988) before acidification occurs (Hamel et al., 1991). As endosomes acidify, any remaining insulin or partially degraded insulin that escaped complete degradation by IDE dissociates from the IR (Murphy et al., 1984; Fig. 3 B). Ultimately, these degradation products and any remaining intact insulin are delivered to lysosomes for complete proteolysis (Duckworth, 1988), although lysosomal degradation of insulin is thought to play a minor role in insulin clearance (Duckworth et al., 1981).

Although the in vitro data strongly indicate that IDE is essential for hepatocyte insulin degradation, the role of IDE in hepatic insulin clearance in vivo is controversial, as some studies report that loss of IDE results in hyperinsulinemia (Farris et al., 2003; Abdul-Hay et al., 2011), whereas others observed no changes in systemic circulating insulin in the absence of the enzyme (Steneberg et al., 2013).

Insulin that is not degraded in the liver exits through the hepatic vein, reaching the heart, which pumps insulin into the arterial circulation to be delivered to its target tissues (e.g., skeletal muscle, liver, adipose tissue, and the brain). It is important to note that insulin returns to the liver, this time via the hepatic artery, which pours again into the hepatic sinusoid, where the hormone is subject to a second round of insulin degradation (second pass) within hepatocytes.

Beyond first- and second-pass insulin clearance, the hepatocytes are essential metabolic responders to insulin, where one of the major actions of the hormone is to suppress gluconeogenesis and glycogenolysis (Lin and Accili, 2011). This ensures that a portion of dietary glucose is effectively stored in the liver and is only released to the rest of the body upon cessation of insulin action (between meals) or upon metabolic demand enacted by other "counterregulatory" hormones (Samuel and Shulman, 2018). This is a vast area of study that is however not further discussed here.

Insulin interaction with the vasculature

Hemodynamic insulin action on arteries and arterioles

The peripheral actions of insulin begin inside the vessels of the systemic circulation, where the hormone exerts its hemodynamic effects on endothelial cells to promote blood flow and ensure its delivery to peripheral tissues (Barrett et al., 2009). Endothelial cells line each blood vessel and constitute a crucial interface between the circulation and the tissue parenchyma. In large blood vessels such as the aorta and large arteries, insulin acts on the IR of endothelial cells, causing phosphorylation of the major endothelial IR substrate, IRS2. This leads to activation of class I phosphatidylinositol 3-kinase (PI3K), which signals downstream to the serine and threonine kinase Akt/PKB. In turn, Akt activates endothelial NO synthase to catalyze the conversion of L-arginine to NO (Palmer et al., 1988; Zeng et al., 2000). NO is a potent vasodilator that rapidly diffuses to the vessels' outer layer of smooth muscle cells, where it activates intracellular guanylate cyclase to increase cyclic guanosine monophosphate production (Arnold et al., 1977). Cyclic guanosine monophosphate–dependent reductions in intracellular Ca^{2+} concentration (Carvajal et al., 2000) prevent phosphorylation of myosin light chain required for cytoskeletal cross-bridge formation and contraction (Lee et al., 1997; Mizuno et al., 2008), thereby resulting in vessel relaxation (Fig. 4 A).

As a consequence of endothelial NO production, insulin stimulates dilation of arteries and arterioles (Steinberg et al., 1994; Vincent et al., 2002). Within minutes, vasodilation of precapillary arterioles irrigates previously collapsed capillaries with blood carrying insulin (capillary recruitment), thereby promoting insulin delivery to the tissue (Vincent et al., 2002). With continued insulin circulation (~30 min), the hormone induces relaxation of larger, upstream resistance vessels to further promote limb blood flow (Baron et al., 1996). Insulin action in target tissues is temporally linked to these vascular effects (Barrett et al., 2009); in particular, the full stimulation of skeletal muscle glucose uptake in vivo is contingent on prior NO-mediated vasodilation (Vincent et al., 2003; Bradley et al., 2013).

Insulin transit across the microvascular endothelium

Once insulin arrives at the capillaries of skeletal muscle and adipose tissue, it must exit the circulation to reach the parenchymal cells (muscle fibers and adipocytes). Unlike the fenestrated capillaries of the liver, the capillary endothelium in skeletal and adipose tissues is continuous, functioning as a stringent barrier between the circulation and the interstitial space. Each capillary is constituted by a single layer of endothelial cells, supported by interendothelial junctions that selectively restrict the passage of contents between the blood and the tissue. Bergman et al. first reported a delay in muscle insulin action relative to the rise in insulin in the circulation (Kolka and Bergman, 2012), and several studies have documented that the capillary endothelium is a barrier to insulin delivery to muscle in vivo, maintaining a disequilibrium between circulating and interstitial insulin levels (Jansson et al., 1993; Herkner et al., 2003).

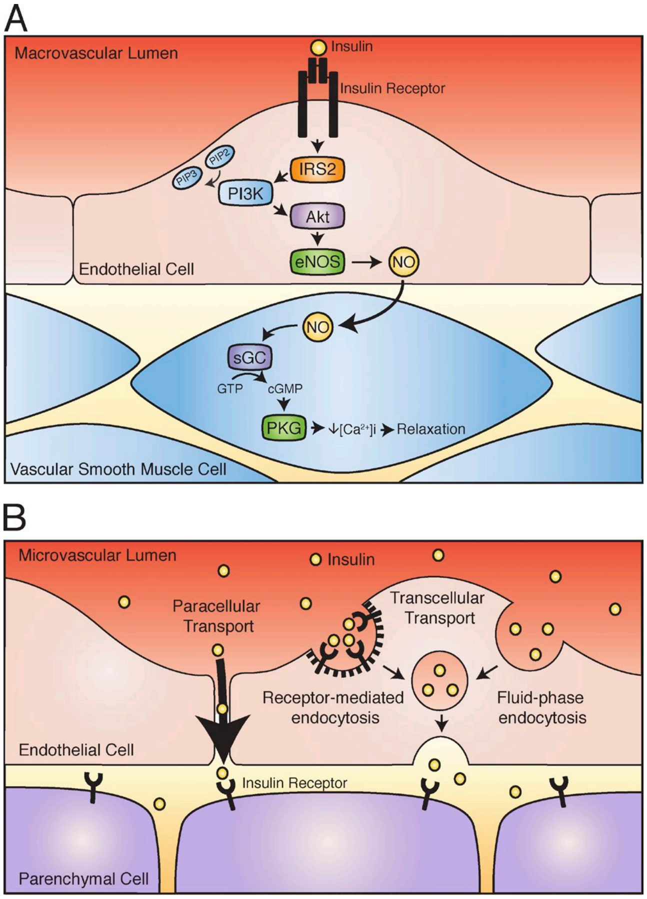


Figure 4. **Insulin interactions with the vasculature. (A)** Endothelial insulin signaling leading to vasodilation in the macrovasculature. The endothelial cell IR engages its major substrate in these cells, IRS2, leading downstream to activation of Akt. Akt phosphorylates endothelial NO synthase (eNOS), which catalyzes the production of NO from L-arginine. NO freely diffuses to the underlying vascular smooth muscle layer, where it leads to cyclic guanosine monophosphate production to induce vasorelaxation. **(B)** Possible routes for insulin exit across microvascular endothelial cells toward the interstitial space in muscle and fat tissue. Insulin may cross the microvascular capillary endothelium either paracellularly (between adjacent endothelial cells) or transcellularly (through individual endothelial cells). For the transcellular route, both receptor-mediated and fluid-phase mechanisms of transport have been proposed.

Insulin may cross the tight capillary endothelia by two potential routes: transcellular (through individual cells) or paracellular (between neighboring endothelial cells; Fig. 4 B). Although there is evidence that expression of the IR or vascular insulin signaling is required for overall insulin egress from the circulation toward tissues (Kubota et al., 2011; Majumdar et al., 2012; Meijer et al., 2016; Konishi et al., 2017), opposing studies challenge a receptor-mediated (Vicent et al., 2003; Duncan et al., 2008; Williams et al., 2018) or saturable process (Steil et al., 1996). To this day, the exact route (intracellular vs. paracellular) and supporting mechanisms remain a matter of debate (Lee and Klip, 2016). The impasse in discerning this route lies in the limitation to differentiate in vivo between a distinctly local role of the endothelial IR in the cellular transport of insulin across the endothelium from its complementary role in capillary recruitment. Recent studies that have bypassed the hemodynamic concerns have also yielded opposite results. Thus, even with chemically induced vasodilation, a new endothelial cell-specific IR knockout mouse model (Konishi et al., 2017) shows defective insulin delivery and action. In contrast, sophisticated imaging of the muscle distribution of somewhat high doses of fluorescent insulin injected into the circulation was best fitted to a model of distribution that does not obey saturation kinetics, suggesting that the IR may not be a major conduit under these conditions (Williams et al., 2018).

Controversy about the mechanism of insulin transit across the microvasculature also arises upon scrutiny in vitro, as cell culture studies have rendered inconsistent results regarding the precise role of the endothelial IR in the uptake of fluorescently conjugated insulin, potentially dependent on their niche origin: microvascular (Azizi et al., 2015) or macrovascular (Wang et al., 2008). Moreover, imaging the internalized insulin, needed to establish the hormone's intracellular route, has required the use of supraphysiological doses of insulin to achieve detectable levels (Wang et al., 2008; Azizi et al., 2015), confounding the identification of the physiological mechanism. On the other hand, the uptake of physiological levels of ^{125}I-insulin into microvascular endothelial cells has uniformly revealed participation of the IR (Jialal et al., 1984; Gray et al., 2017; Jaldin-Fincati et al., 2018). This includes the transfer of insulin across cells of the blood–brain barrier (Jialal et al., 1984; Gray et al., 2017), an important conduit for the now-recognized neuronal actions of the hormone evinced by the neuron-specific IR gene depletion (Brüning et al., 2000). How internalized insulin is spared from degradation in endothelial cells, as opposed to its fate in hepatocytes (described in Insulin clearance by the liver: Its receptor-mediated endocytosis and degradation), remains unsolved. Potentially, this may involve routing of insulin into sorting tubules akin to those recently described for transferrin receptor-mediated transcytosis through blood–brain endothelia (Villaseñor et al., 2017).

Central insulin action: Brief focus on the brain

Emerging from the circulation, insulin begins its multifaceted action on central and peripheral tissues. As outlined above, insulin crosses the blood–brain barrier through a receptor-mediated process (Woods et al., 2003). Thought to be insulin unresponsive in the past, the central nervous system is well recognized to be exquisitely responsive to the incoming hormone (Porte et al., 2005). The concentration of insulin in the cerebrospinal fluid is one third that in the circulation, but it nonetheless fluctuates according to the latter and acts on IR on neurons and glial cells. Notable among the evoked central functions is the regulation of appetite and energy expenditure (Filippi et al., 2013; García-Cáceres et al., 2016). Insulin regulates appetite by reducing expression of neuropeptide Y and Agouti-related peptide (orexigenic) and, conversely, elevating expression of pro-opiomelanocortin (anorexigenic; Schwartz et al., 2000). Insulin also exerts trophic and developmental actions on neurons and glial cells, and new evidence suggests it modulates cognition, memory, and mood (Lee et al., 2016). Conversely, central defects in insulin action are emerging as a potential contributor to the development of Alzheimer's disease (Griffith et al., 2018), possibly as a result of abnormal phosphorylation of tau protein (Kleinridders et al., 2014). Insulin acting centrally also evokes efferent inputs into peripheral tissue metabolism (Ferris and Kahn, 2016), contributing to the suppression of gluconeogenesis in the liver and the counterregulatory response to hypoglycemia (Diggs-Andrews et al., 2010). Acting centrally on IR, insulin contributes to thermoregulation by activating heat-liberating mechanisms in brown adipose tissue (Kleinridders et al., 2014).

The cellular mechanisms underlying each of these complex, integrated responses are still to be elucidated, especially in so far as identification of the specific intra- and intercellular neuronal responses that are likely to be carefully decoded through spatial, temporal, and amplitude parameters. Although rich information is being gathered through electrophysiological approaches (van der Heide et al., 2005; Könner et al., 2007; Korol et al., 2018), there is a rich opportunity to explore additional mechanisms through the advent of real-time intravital imaging of the central nervous system (Forli et al., 2018).

Insulin in action: Stimulation of glucose uptake in muscle and fat cells

The actions of insulin on the parenchyma of peripheral tissues are diverse, and paramount among them is the regulation of glucose metabolism. The major function of insulin in muscle and adipose tissues is to increase their uptake of carbon sources and store them for the energetic needs of tissue. With glucose transport into these tissues being rate limiting for its storage (as glycogen and triglycerides, respectively), it is no surprise that insulin regulates glucose uptake. This is brought about by an exquisite series of signals that cooperate in bringing glucose transporters (GLUT4 isoform) to the cell surface. This process is generically known as GLUT4 translocation, and 30 years of research has revealed regulation at a number of stages in this intracellular process (Bryant and Gould, 2011; Kandror and Pilch, 2011; Stöckli et al., 2011; Bogan, 2012; Leto and Saltiel, 2012; Klip et al., 2014; Jaldin-Fincati et al., 2017).

GLUT4 translocation takes places within minutes of insulin binding to its receptors at the surface of myocytes and adipocytes and does not involve internalization of the hormone. Major aspects of GLUT4 translocation are illustrated in Fig. 5.

The unique GLUT4 compartment

The molecular signature of GLUT4 allows it to be diverted away from the continuously recycling pathway (a ubiquitous intracellular route that constantly removes and returns membrane proteins by internalization toward endosomes and reexternalization)

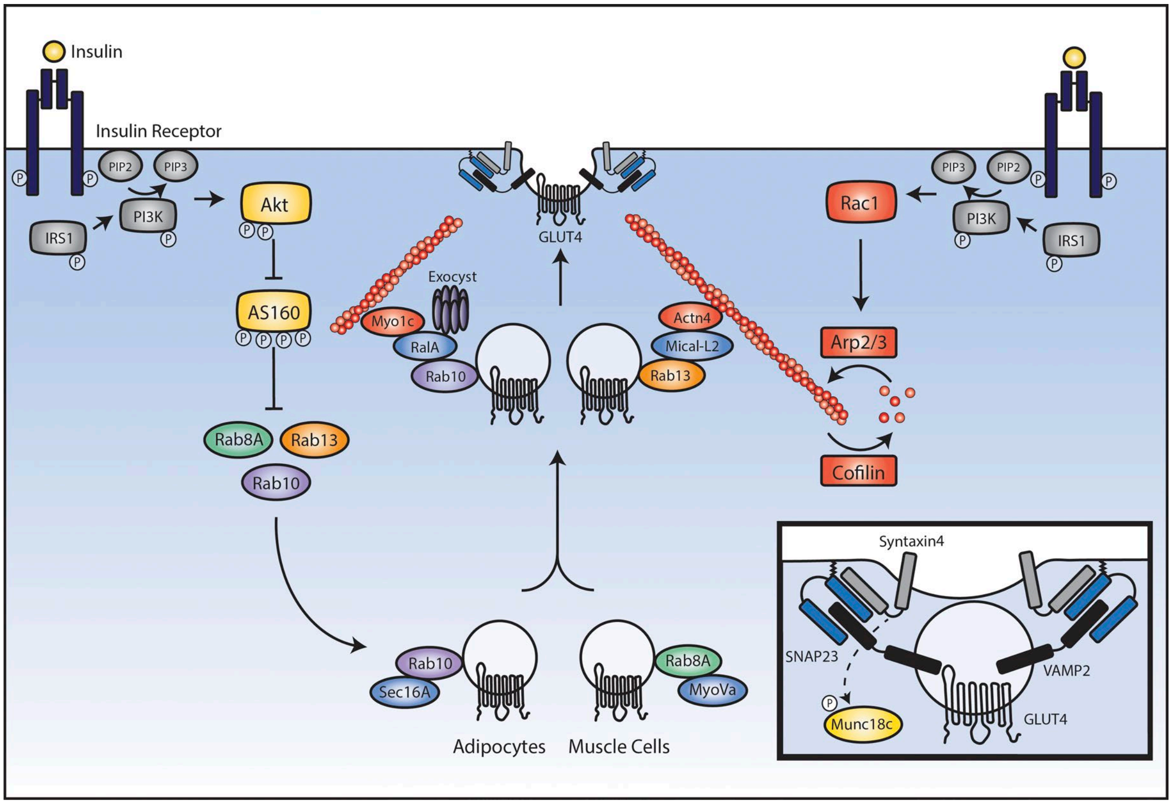


Figure 5. **Insulin signaling in muscle and adipose cells leading to recruitment of GLUT4 to the plasma membrane.** Insulin binds to its receptor on the surface of muscle or fat cells and activates the canonical insulin-signaling cascade to PI3K and Akt. Downstream of Akt, phosphorylation of AS160 allows for the full activation of Rab8A and Rab13 (in muscle cells) and Rab10 (in adipocytes). In the perinuclear region, Rab8A engages with its effector, MyoVa, and Rab10 with its effector, Sec16A, to promote outward vesicle traffic. Near the plasma membrane, Rab13 engages with MICAL-L2 and Actinin-4, whereas Rab10 engages with RalA, Myo1c, and Exocyst components. Simultaneously, downstream of PI3K, insulin leads to activation of Rac1 that promotes a dynamic cycle of cortical actin remodeling. Together, these actions tether GLUT4 vesicles to the actin cytoskeleton near the plasma membrane. Inset: Docked GLUT4 vesicle ready to fuse with the plasma membrane. Immobilized GLUT4 vesicles fuse with the membrane through formation of a SNARE complex between vesicular VAMP2 and syntaxin4 and SNAP23 on the plasma membrane.

to constitute a functionally defined "organelle" called GLUT4 storage vesicles. Several elements contribute to the genesis and maintenance of this storage compartment, including sortilin (Huang et al., 2013), the Rab GTPase-activating protein (GAP) AS160/TBC1D4, syntaxin 6/16 (Bryant and Gould, 2011; Klip et al., 2014), the cleavable tether protein TUG (Belman et al., 2015), and, in human muscle, clathrin heavy chain 22 (Vassilopoulos et al., 2009). The storage compartment is in dynamic communication with recycling endosomes (Coster et al., 2004; Karylowski et al., 2004; Kandror and Pilch, 2011). This dynamic sorting generates a steady state whereby the majority (~90–95%) of GLUT4 resides intracellularly at any point in time in both muscle and adipose cells. This effective removal from the plasma membrane at any point in time is rather unique for GLUT4, as is its intracellular sorting to a compartment that is only slowly or ineffectively available for recycling.

Insulin signals quickly mobilize GLUT4-containing vesicles of ~70 nm in diameter from perinuclear/cytosolic depots toward the cell periphery. These emanate directly from the storage compartment; however, insulin also appears to redirect vesicles from this compartment toward the general recycling endosomes, from whence they reach the cell periphery in the form of somewhat larger vesicles (Xu et al., 2011). A current model proposes that the initial gain in surface GLUT4 emanates from the storage compartment, whereas maintenance of the steady state involves the endosomal route (Bryant and Gould, 2011; Kandror and Pilch, 2011; Stöckli et al., 2011; Bogan, 2012; Leto and Saltiel, 2012; Klip et al., 2014; Jaldin-Fincati et al., 2017). However, another prevailing model proposes that the majority of vesicles furnishing the cell membrane with GLUT4 contain the fusogenic protein VAMP2, which segregates away with the storage compartment and is largely absent from recycling endosomes (Randhawa et al., 2000, 2004; Török et al., 2004). Once at the cell periphery, insulin signals further promote vesicle fusion with the plasma membrane. Within minutes, this concerted action brings about a new steady state with double the number of GLUT4 units at the plasma membrane in muscle cells. Although this gain represents only ~20% of the total GLUT4, given the large mass of muscle in vivo, this gain sustains the vastly preferential deposition of diet-ingested glucose into skeletal muscles. In adipocytes, the insulin-dependent gain in surface-exposed GLUT4 ranges from twofold (human) to 10-fold or higher (rodents) and is typically calculated that this gain involves 30–50% of the total GLUT4 complement in these cells. In both muscle and fat cells, the new steady state lasts for as long as insulin is present.

The GLUT4 polypeptide has a very long lifetime (∼40 h); hence, its continuous removal from the membrane allows for multiple rounds of endocytosis, sorting, and translocation. It is understood that insulin promotes GLUT4 exit from retention in the storage compartment (Xu and Kandror, 2002; Coster et al., 2004; Martin et al., 2006; Bogan et al., 2012) and also regulates GLUT4 vesicle tethering, docking, and fusion with the plasma membrane through mechanisms that borrow principles from those of synaptic vesicle and insulin granule fusion.

Insulin signals involved in GLUT4 translocation

The connection between IR-derived signals (Klip et al., 2014) and the elements that mobilize GLUT4 vesicles and enact their fusion with the membrane is beginning to unravel. Insulin activates the IR tyrosine kinase activity toward autophosphorylation by inducing structural rearrangement of the transmembrane domains to bring them into close proximity with each other (Gutmann et al., 2018), and the consequent activation of the IR tyrosine kinase toward phosphorylation of its major substrates IRS1,2 (Copps and White, 2012). Phosphorylation sites on IRS1,2 constitute entropic information to attract class I PI3K, which rapidly generates membrane domains enriched in PI(3,4,5)P_3 (PIP_3) within minutes (Ruderman et al., 1990). Two major consequences of the PIP_3 burst relevant for GLUT4 translocation are activation of the kinases Akt1, 2 (Brozinick and Birnbaum, 1998; Wang et al., 1999) and of the Rho-family GTPase Rac1 (Chiu et al., 2011).

For the first signal, PIP_3 attracts the PH domain of Akt, which makes the protein available for phosphorylation by two kinases, PDK-1, and mTORC2. Activated Akt1,2 migrates to the cytosol and intracellular membranes (Zheng and Cartee, 2016), where it phosphorylates AS160, a substrate of 160 kD more appropriately named TBC1D4 (Sano et al., 2003; Lansey et al., 2012). The TBC domain of AS160/TBC1D4 defines its GAP activity toward Rab family small GTPases. Phosphorylation of AS160/TBC1D4 inhibits its GAP activity; hence, insulin signaling leads to inactivation of an inhibitor of Rab GTPases. This realization constituted the first involvement of elements capable of specifically regulating vesicle traffic in the pathway, as Rab GTPases regulate vesicle fission, destination, and fusion. AS160/TBC1D4 targets a cluster of Rabs, particularly the phylogenetically related Rabs 8A, 10, and 13. In addition, these three GTPases are stabilized by the holdase chaperone RABIF/MSS4 (Gulbranson et al., 2017). As a result of AS160/TBC1D4 inactivation, these Rab GTPases prevail in their active, GTP-loaded state; hence, their regulation is largely via inhibition of their GAP (whereas their currently incompletely identified guanine nucleotide exchange factors (GEFs) might be constitutively active, as in the case of the Sec10, the GEF for Rab10 (Sano et al., 2011; Fig. 5).

In parallel to activation of this "Akt cascade," the burst in plasma membrane–associated PIP_3 leads to activation of GEFs (and possibly inhibition of GAPs) for Rac1 (Takenaka et al., 2014, 2016). The resulting Rac1 activation leads to a dynamic remodeling of cortical actin filaments via cycles of actin filament branching enacted by Arp2/3 and actin severing enacted by cofilin, which is best mapped in muscle cells (Chiu et al., 2010) and tissue (Sylow et al., 2013; Fig. 5).

From signals to effectors: Mechanical elements of GLUT4 vesicle translocation

Rab GTPases lie at the crux of signal transmission to mechanical transduction, as several Rab GTPase effectors collude with actin filaments (whether those remodeling at the cell cortex and/or other filamentous configurations) to de facto mobilize GLUT4 to the plasma membrane. In adipocytes, Rab10 is the preferred GTPase in GLUT4 translocation (Sano et al., 2008), whereas in muscle cells, it is Rab8A and Rab13 (Ishikura et al., 2007; Sun et al., 2010, 2014, 2016). Although Rab10, Rab8A, and Rab13 have been studied the most, other Rab family GTPases contribute to the overall GLUT4 traffic, such as Rab28, which is also a substrate of AS160/TBC1D4; Rab14, involved in intracellular GLUT4 sorting; Rabs 4 and 11, involved in constitutive GLUT4 cycling; and Rab5, involved in early GLUT4 endocytosis (Jaldin-Fincati et al., 2017).

In adipocytes, Rab10 promotes GLUT4 mobilization from the perinuclear region toward the plasma membrane (Sano et al., 2007; Bruno et al., 2016), specifically by interacting with Sec16A (Sano et al., 2007; Bruno et al., 2016). In addition, a function for Rab10 at the cell periphery was also proposed (Chen and Lippincott-Schwartz, 2013), as will be discussed.

In muscle cells, the perinuclear Rab8A engages its effector Myosin Va thereby promoting GLUT4 exit from the storage compartment (Sun et al., 2014). This processive molecular motor allows migration of GLUT4 vesicles along actin filaments toward the cell periphery. Rab13 is more peripherally located, and its effector is the cortically located protein MICAL-L2, which in turn binds the cortical cytoskeleton protein α-actinin4. In response to insulin, these three proteins can be visualized near the cell surface along with GLUT4 and cortical actin (Sun et al., 2016). In this way, Rab8A and Rab13 ensure GLUT4 vesicle mobilization toward the periphery and tethering to cytoskeletal elements in this region, respectively.

In addition to the Rab13–MICAL-L2–α-actinin4 connection, GLUT4 vesicles tether to actin filaments via Myosin 1c (Bose et al., 2002; Boguslavsky et al., 2012). This restricts GLUT4 mobility beneath the membrane, a phenomenon nicely documented through total internal reflection fluorescence microscopy of muscle and adipose cells (Bai et al., 2007; Xiong et al., 2010; Boguslavsky et al., 2012; Lizunov et al., 2012). GLUT4 vesicle retention near the membrane also involves the exocyst subunit Exo70 (Lizunov et al., 2012). Tethering may be regulated by insulin, as stimulation leads to phosphorylation of Exo84 (Uhm et al., 2017). In addition, active Rab10 binds to Exoc6/6b (Sano et al., 2015), and the Rab10 effector RalA and its GEF, Rlf, interact with exocyst components (Karunanithi et al., 2014).

The GLUT4 vesicle fusion machinery

GLUT4 vesicles immobilized at the cell periphery rapidly fuse with the membrane. This is brought about through formation of a SNARE complex between VAMP2 on the vesicles and syntaxin4 and SNAP23 on the plasma membrane (Cheatham et al., 1996; Foster and Klip, 2000; Thurmond and Pessin, 2001). The formation of the SNARE complex is regulated by a fine balance of a number of proteins such as Munc18c, Synip, and Doc2b, which receive input emanating from Akt and the phosphatase

PTP-1B (Yamada et al., 2005; Fukuda et al., 2009; Bakke et al., 2013; Garrido-Sanchez et al., 2013; Fig. 5).

The kinetics, stoichiometry, and upstream regulation of the fusion step still need to be fully investigated. Intriguingly, there are studies of additional participation of Ca^{2+}-regulated proteins such as Doc2b, Tctex1d2, and E-Syt1 (Lalioti et al., 2009; Friedrich et al., 2010; Shimoda et al., 2015), and insulin-dependent Ca^{2+}-spikes have been recorded in muscle cells (Contreras-Ferrat et al., 2014), suggesting that the ion may impart some fine-tuning to the fidelity and timeliness of GLUT4 vesicle fusion. Lastly, and importantly, the fusion event requires insulin-induced actin polymerization, evincing the contribution of the actin cytoskeleton at different steps in the process of GLUT4 translocation (Lopez et al., 2009).

The end: Insulin degradation in the kidney

Insulin is no longer detectable in the circulation 30 min after its release from the pancreas, and its half-life once in the circulation is ~6 min (Robbins et al., 1985; Marino, 2009). In addition to its clearance by the liver (50% in first and another 25% in second pass), the hormone is also slowly internalized by most cells, including myoblasts and adipocytes, where it is routed to the lysosome for degradation. This is a mechanism to end insulin action, but it accounts for the destruction of only a fraction of the circulating insulin. The brunt of the degradation of the circulating hormone remaining after second pass through the liver occurs when it reaches the kidney. Here, its fate is threesome. Upon filtration at the level of the glomeruli, insulin enters the luminal space and reaches the proximal tubule, from whence it is rapidly reabsorbed by the renal epithelial cells. This reabsorption involves saturable binding to low-affinity, high-capacity sites at the brush border membrane, which are demonstrated to be not the IR (Meezan et al., 1988; Sato et al., 1991; Nielsen, 1993, 1994) but possibly scavenger receptors such as megalin (member of the low-density lipoprotein receptor family; Christensen et al., 1998; Kolman et al., 2009) and cubilin, proteins that recover a number of proteins by endocytosis. Insulin thus internalized enters the retroendocytic pathway, where it dissociates from its binding sites to proceed to lysosomes for degradation.

Second, about an equal amount of insulin also enters renal tubular cells from the contraluminal side facing the renal peritubular capillaries, especially in the convoluted tubule (Rabkin et al., 1984). Here, IRs on the epithelial cells bind insulin and transport it intracellularly for degradation (Nielsen et al., 1987). In addition, these IR are important sites sensing the hormone to stimulate important functions such as reabsorption of sodium, phosphate, and glucose (Rabkin et al., 1984; Hale and Coward, 2013). It has been proposed that these two renal mechanisms of insulin internalization are responsible for clearing up to 6–8 U insulin per day (Palmer and Henrich, 2017) amounting to up to 25% of the insulin secreted by the pancreas, or ~50% of the circulating insulin, although this might be an overestimation. Nonetheless, renal insulin clearance may explain the curious fact that type 1 diabetic patients with onset renal failure can end up reducing their requirement for injected insulin (Rubenstein and Spitz, 1968; Rabkin et al., 1984).

Third, though most of the internalized insulin is degraded by the above pathways, a small fraction is reabsorbed back to the renal circulation through retroendocytosis (Dahl et al., 1989). Notably, alterations in insulin renal clearance prolong the permanence of insulin in the blood (Dahl et al., 1989), evincing the importance of this process to insulin's half-life in the circulation.

Concluding remarks

We have analyzed the fundamental physiological journey of insulin in the body by alternating a bird's-eye view of the integrative phenomenon with close-ups into the key cellular processes of the hormone's secretion, partial clearance in the liver, distribution to the circulation and exit to target tissues, its action to promote glucose uptake in muscle and fat, and ultimately its degradation in the kidney. In spite of the depth of knowledge available to us on each of these cellular stages in the journey, there are many mechanistic and integrated aspects that remain unknown. However, the current knowledge already allows us to understand how each stage is in communication with the other. The temporal periodicity of insulin secretion out of the pancreas is sensed by the hepatocytes, which synchronously clear a portion of the secreted insulin; insulin action on the macrovasculature allows recruitment of the microcirculation for full enactment of insulin delivery to tissues; and insulin action in the liver, muscle, and fat cells results in a lowering of blood glucose, thus terminating the prime stimulus for insulin secretion. In pace with insulin action, the kidney engages in its subsequent degradation, putting an end to the hormone's action with just the right time delay to ensure optimal metabolic homeostasis.

Acknowledgments

We thank profusely the input received for the analysis presented herein from Drs. Sonia M. Najjar, Alan D. Cherrington, and Philip J. Bilan.

Work in P.E. MacDonald's laboratory on insulin secretion is funded by the Canadian Institutes of Health Research (foundation grant FRN 148451). Work in A. Klip's laboratory on insulin delivery and action is funded by the Canadian Institutes of Health Research (foundation grant FRN: FND-143203). A. Klip is the recipient of the Tier I Canada Research Chair "Cell Biology of Insulin Action." V. Tokarz was supported by the University of Toronto (Ontario Graduate Scholarship) and The Hospital for Sick Children (Restracomp Scholarship).

The authors declare no competing financial interests.

A. Klip conceived the subject and format of this article; V.L. Tokarz, P.E. MacDonald, and A. Klip analyzed the literature, discussed the material, wrote the article, and conceived the figures. V.L. Tokarz produced the majority of the figures.

Submitted: 15 February 2018
Revised: 21 March 2018
Accepted: 23 March 2018

References

Abdul-Hay, S.O., D. Kang, M. McBride, L. Li, J. Zhao, and M.A. Leissring. 2011. Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance. *PLoS One.* 6:e20818. https://doi.org/10.1371/journal.pone.0020818

Ader, M., D. Stefanovski, S.P. Kim, J.M. Richey, V. Ionut, K.J. Catalano, K. Hucking, M. Ellmerer, G. Van Citters, I.R. Hsu, et al. 2014. Hepatic insulin clearance is the primary determinant of insulin sensitivity in the normal dog. *Obesity (Silver Spring).* 22:1238–1245. https://doi.org/10.1002/oby.20625

Alenkvist, I., N.R. Gandasi, S. Barg, and A. Tengholm. 2017. Recruitment of Epac2A to Insulin Granule Docking Sites Regulates Priming for Exocytosis. *Diabetes.* 66:2610–2622. https://doi.org/10.2337/db17-0050

Andrali, S.S., M.L. Sampley, N.L. Vanderford, and S. Ozcan. 2008. Glucose regulation of insulin gene expression in pancreatic beta-cells. *Biochem. J.* 415:1–10. https://doi.org/10.1042/BJ20081029

Arnold, W.P., C.K. Mittal, S. Katsuki, and F. Murad. 1977. Nitric oxide activates guanylate cyclase and increases guanosine 3′:5′-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. USA.* 74:3203–3207. https://doi.org/10.1073/pnas.74.8.3203

Artner, I., and R. Stein. 2008. Transcriptional regulation of insulin gene expression. *In* Pancreatic Beta Cell in Health and Disease. Springer, Tokyo. 13–30.

Aslamy, A., and D.C. Thurmond. 2017. Exocytosis proteins as novel targets for diabetes prevention and/or remediation? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 312:R739–R752. https://doi.org/10.1152/ajpregu.00002.2017

Azizi, P.M., R.E. Zyla, S. Guan, C. Wang, J. Liu, S.-S. Bolz, B. Heit, A. Klip, and W.L. Lee. 2015. Clathrin-dependent entry and vesicle-mediated exocytosis define insulin transcytosis across microvascular endothelial cells. *Mol. Biol. Cell.* 26:740–750. https://doi.org/10.1091/mbc.E14-08-1307

Bai, L., Y. Wang, J. Fan, Y. Chen, W. Ji, A. Qu, P. Xu, D.E. James, and T. Xu. 2007. Dissecting multiple steps of GLUT4 trafficking and identifying the sites of insulin action. *Cell Metab.* 5:47–57. https://doi.org/10.1016/j.cmet.2006.11.013

Bakke, J., A. Bettaieb, N. Nagata, K. Matsuo, and F.G. Haj. 2013. Regulation of the SNARE-interacting protein Munc18c tyrosine phosphorylation in adipocytes by protein-tyrosine phosphatase 1B. *Cell Commun. Signal.* 11:57. https://doi.org/10.1186/1478-811X-11-57

Baron, A.D., G. Brechtel-Hook, A. Johnson, J. Cronin, R. Leaming, and H.O. Steinberg. 1996. Effect of perfusion rate on the time course of insulin-mediated skeletal muscle glucose uptake. *Am. J. Physiol.* 271:E1067–E1072.

Baron, V., F. Alengrin, and E. Van Obberghen. 1998. Dynamin associates with Src-Homology Collagen (Shc) and becomes tyrosine phosphorylated in response to insulin. *Endocrinology.* 139:3034–3037. https://doi.org/10.1210/endo.139.6.6131

Barrett, E.J., E.M. Eggleston, A.C. Inyard, H. Wang, G. Li, W. Chai, and Z. Liu. 2009. The vascular actions of insulin control its delivery to muscle and regulate the rate-limiting step in skeletal muscle insulin action. *Diabetologia.* 52:752–764. https://doi.org/10.1007/s00125-009-1313-z

Belman, J.P., R.R. Bian, E.N. Habtemichael, D.T. Li, M.J. Jurczak, A. Alcázar-Román, L.J. McNally, G.I. Shulman, and J.S. Bogan. 2015. Acetylation of TUG protein promotes the accumulation of GLUT4 glucose transporters in an insulin-responsive intracellular compartment. *J. Biol. Chem.* 290:4447–4463. https://doi.org/10.1074/jbc.M114.603977

Bergeron, J.J.M., G.M. Di Guglielmo, S. Dahan, M. Dominguez, and B.I. Posner. 2016. Spatial and Temporal Regulation of Receptor Tyrosine Kinase Activation and Intracellular Signal Transduction. *Annu. Rev. Biochem.* 85:573–597. https://doi.org/10.1146/annurev-biochem-060815-014659

Bergsten, P., E. Grapengiesser, E. Gylfe, A. Tengholm, and B. Hellman. 1994. Synchronous oscillations of cytoplasmic Ca2+ and insulin release in glucose-stimulated pancreatic islets. *J. Biol. Chem.* 269:8749–8753.

Bertram, R., L.S. Satin, and A.S. Sherman. 2018. Closing in on the Mechanisms of Pulsatile Insulin Secretion. *Diabetes.* 67:351–359. https://doi.org/10.2337/dbi17-0004

Bevan, A.P., J.W. Burgess, P.G. Drake, A. Shaver, J.J. Bergeron, and B.I. Posner. 1995. Selective activation of the rat hepatic endosomal insulin receptor kinase. Role for the endosome in insulin signaling. *J. Biol. Chem.* 270:10784–10791. https://doi.org/10.1074/jbc.270.18.10784

Bogan, J.S. 2012. Regulation of glucose transporter translocation in health and diabetes. *Annu. Rev. Biochem.* 81:507–532. https://doi.org/10.1146/annurev-biochem-060109-094246

Bogan, J.S., B.R. Rubin, C. Yu, M.G. Löffler, C.M. Orme, J.P. Belman, L.J. McNally, M. Hao, and J.A. Cresswell. 2012. Endoproteolytic cleavage of TUG protein regulates GLUT4 glucose transporter translocation. *J. Biol. Chem.* 287:23932–23947. https://doi.org/10.1074/jbc.M112.339457

Boguslavsky, S., T. Chiu, K.P. Foley, C. Osorio-Fuentealba, C.N. Antonescu, K.U. Bayer, P.J. Bilan, and A. Klip. 2012. Myo1c binding to submembrane actin mediates insulin-induced tethering of GLUT4 vesicles. *Mol. Biol. Cell.* 23:4065–4078. https://doi.org/10.1091/mbc.E12-04-0263

Bose, A., A. Guilherme, S.I. Robida, S.M.C. Nicoloro, Q.L. Zhou, Z.Y. Jiang, D.P. Pomerleau, and M.P. Czech. 2002. Glucose transporter recycling in response to insulin is facilitated by myosin Myo1c. *Nature.* 420:821–824. https://doi.org/10.1038/nature01246

Boucher, J., A. Kleinridders, and C.R. Kahn. 2014. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb. Perspect. Biol.* 6:a009191. https://doi.org/10.1101/cshperspect.a009191

Bradley, E.A., S.M. Richards, M.A. Keske, and S. Rattigan. 2013. Local NOS inhibition impairs vascular and metabolic actions of insulin in rat hindleg muscle in vivo. *Am. J. Physiol. Endocrinol. Metab.* 305:E745–E750. https://doi.org/10.1152/ajpendo.00289.2013

Braet, F., R. De Zanger, M. Baekeland, E. Crabbé, P. Van Der Smissen, and E. Wisse. 1995. Structure and dynamics of the fenestrae-associated cytoskeleton of rat liver sinusoidal endothelial cells. *Hepatology.* 21:180–189.

Broussard, J.L., A.V.B. Castro, M. Iyer, R.L. Paszkiewicz, I.A. Bediako, L.S. Szczepaniak, E.W. Szczepaniak, R.N. Bergman, and C.M. Kolka. 2016. Insulin access to skeletal muscle is impaired during the early stages of diet-induced obesity. *Obesity (Silver Spring).* 24:1922–1928. https://doi.org/10.1002/oby.21562

Brozinick, J.T. Jr., and M.J. Birnbaum. 1998. Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle. *J. Biol. Chem.* 273:14679–14682. https://doi.org/10.1074/jbc.273.24.14679

Brüning, J.C., D. Gautam, D.J. Burks, J. Gillette, M. Schubert, P.C. Orban, R. Klein, W. Krone, D. Müller-Wieland, and C.R. Kahn. 2000. Role of brain insulin receptor in control of body weight and reproduction. *Science.* 289:2122–2125. https://doi.org/10.1126/science.289.5487.2122

Bruno, J., A. Brumfield, N. Chaudhary, D. Iaea, and T.E. McGraw. 2016. SEC16A is a RAB10 effector required for insulin-stimulated GLUT4 trafficking in adipocytes. *J. Cell Biol.* 214:61–76. https://doi.org/10.1083/jcb.201509052

Bryant, N.J., and G.W. Gould. 2011. SNARE proteins underpin insulin-regulated GLUT4 traffic. *Traffic.* 12:657–664. https://doi.org/10.1111/j.1600-0854.2011.01163.x

Carpentier, J.L., M. Fehlmann, E. Van Obberghen, P. Gorden, and L. Orci. 1985. Redistribution of 125I-insulin on the surface of rat hepatocytes as a function of dissociation time. *Diabetes.* 34:1002–1007. https://doi.org/10.2337/diabetes.34.10.1002

Carpentier, J.L., J.P. Paccaud, P. Gorden, W.J. Rutter, and L. Orci. 1992. Insulin-induced surface redistribution regulates internalization of the insulin receptor and requires its autophosphorylation. *Proc. Natl. Acad. Sci. USA.* 89:162–166. https://doi.org/10.1073/pnas.89.1.162

Carvajal, J.A., A.M. Germain, J.P. Huidobro-Toro, and C.P. Weiner. 2000. Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J. Cell. Physiol.* 184:409–420. https://doi.org/10.1002/1097-4652(200009)184:3%3C409::AID-JCP16%3E3.0.CO;2-K

Cheatham, B., A. Volchuk, C.R. Kahn, L. Wang, C.J. Rhodes, and A. Klip. 1996. Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins. *Proc. Natl. Acad. Sci. USA.* 93:15169–15173. https://doi.org/10.1073/pnas.93.26.15169

Chen, Y., and J. Lippincott-Schwartz. 2013. Insulin triggers surface-directed trafficking of sequestered GLUT4 storage vesicles marked by Rab10. *Small GTPases.* 4:193–197. https://doi.org/10.4161/sgtp.26471

Chiu, T.T., N. Patel, A.E. Shaw, J.R. Bamburg, and A. Klip. 2010. Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells. *Mol. Biol. Cell.* 21:3529–3539. https://doi.org/10.1091/mbc.E10-04-0316

Chiu, T.T., T.E. Jensen, L. Sylow, E.A. Richter, and A. Klip. 2011. Rac1 signalling towards GLUT4/glucose uptake in skeletal muscle. *Cell. Signal.* 23:1546–1554. https://doi.org/10.1016/j.cellsig.2011.05.022

Christensen, E.I., H. Birn, P. Verroust, and S.K. Moestrup. 1998. Membrane receptors for endocytosis in the renal proximal tubule. *Int. Rev. Cytol.* 180:237–284. https://doi.org/10.1016/S0074-7696(08)61772-6

Clerk, L.H., M.A. Vincent, L.A. Jahn, Z. Liu, J.R. Lindner, and E.J. Barrett. 2006. Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. *Diabetes.* 55:1436–1442. https://doi.org/10.2337/db05-1373

Cohen, S.E., E. Kokkotou, S.B. Biddinger, T. Kondo, R. Gebhardt, J. Kratzsch, C.S. Mantzoros, and C.R. Kahn. 2007. High circulating leptin receptors with normal leptin sensitivity in liver-specific insulin receptor knock-out (LIRKO) mice. *J. Biol. Chem.* 282:23672–23678. https://doi.org/10.1074/jbc.M704053200

Contreras-Ferrat, A., S. Lavandero, E. Jaimovich, and A. Klip. 2014. Calcium signaling in insulin action on striated muscle. *Cell Calcium.* 56:390–396. https://doi.org/10.1016/j.ceca.2014.08.012

Copps, K.D., and M.F. White. 2012. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia.* 55:2565–2582. https://doi.org/10.1007/s00125-012-2644-8

Coster, A.C.F., R. Govers, and D.E. James. 2004. Insulin stimulates the entry of GLUT4 into the endosomal recycling pathway by a quantal mechanism. *Traffic.* 5:763–771. https://doi.org/10.1111/j.1600-0854.2004.00218.x

Czech, M.P. 2017. Insulin action and resistance in obesity and type 2 diabetes. *Nat. Med.* 23:804–814. https://doi.org/10.1038/nm.4350

Dahl, D.C., T. Tsao, W.C. Duckworth, M.J. Mahoney, and R. Rabkin. 1989. Retroendocytosis of insulin in a cultured kidney epithelial cell line. *Am. J. Physiol.* 257:C190–C196. https://doi.org/10.1152/ajpcell.1989.257.2.C190

Dean, P.M., and E.K. Matthews. 1970. Glucose-induced electrical activity in pancreatic islet cells. *J. Physiol.* 210:255–264. https://doi.org/10.1113/jphysiol.1970.sp009207

DeFronzo, R.A., E. Ferrannini, L. Groop, R.R. Henry, W.H. Herman, J.J. Holst, F.B. Hu, C.R. Kahn, I. Raz, G.I. Shulman, et al. 2015. Type 2 diabetes mellitus. *Nat. Rev. Dis. Primers.* 1:15019. https://doi.org/10.1038/nrdp.2015.19

de Jongh, R.T., E.H. Serné, R.G. IJzerman, G. de Vries, and C.D.A. Stehouwer. 2004. Impaired microvascular function in obesity: implications for obesity-associated microangiopathy, hypertension, and insulin resistance. *Circulation.* 109:2529–2535. https://doi.org/10.1161/01.CIR.0000129772.26647.6F

Diggs-Andrews, K.A., X. Zhang, Z. Song, D. Daphna-Iken, V.H. Routh, and S.J. Fisher. 2010. Brain insulin action regulates hypothalamic glucose sensing and the counterregulatory response to hypoglycemia. *Diabetes.* 59:2271–2280. https://doi.org/10.2337/db10-0401

Dong, X.C., K.D. Copps, S. Guo, Y. Li, R. Kollipara, R.A. DePinho, and M.F. White. 2008. Inactivation of hepatic Foxo1 by insulin signaling is required for adaptive nutrient homeostasis and endocrine growth regulation. *Cell Metab.* 8:65–76. https://doi.org/10.1016/j.cmet.2008.06.006

Drucker, D.J., J.F. Habener, and J.J. Holst. 2017. Discovery, characterization, and clinical development of the glucagon-like peptides. *J. Clin. Invest.* 127:4217–4227. https://doi.org/10.1172/JCI97233

Du, W., M. Zhou, W. Zhao, D. Cheng, L. Wang, J. Lu, E. Song, W. Feng, Y. Xue, P. Xu, and T. Xu. 2016. HID-1 is required for homotypic fusion of immature secretory granules during maturation. *eLife.* 5:241. https://doi.org/10.7554/eLife.18134

Duckworth, W.C. 1988. Insulin degradation: mechanisms, products, and significance. *Endocr. Rev.* 9:319–345. https://doi.org/10.1210/edrv-9-3-319

Duckworth, W.C., K.R. Runyan, R.K. Wright, P.A. Halban, and S.S. Solomon. 1981. Insulin degradation by hepatocytes in primary culture. *Endocrinology.* 108:1142–1147. https://doi.org/10.1210/endo-108-4-1142

Duncan, E.R., P.A. Crossey, S. Walker, N. Anilkumar, L. Poston, G. Douglas, V.A. Ezzat, S.B. Wheatcroft, A.M. Shah, M.T. Kearney, and M.I. Kearney. 2008. Effect of endothelium-specific insulin resistance on endothelial function in vivo. *Diabetes.* 57:3307–3314. https://doi.org/10.2337/db07-1111

Farris, W., S. Mansourian, Y. Chang, L. Lindsley, E.A. Eckman, M.P. Frosch, C.B. Eckman, R.E. Tanzi, D.J. Selkoe, and S. Guenette. 2003. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc. Natl. Acad. Sci. USA.* 100:4162–4167. https://doi.org/10.1073/pnas.0230450100

Fehlmann, M., J.L. Carpentier, A. Le Cam, P. Thamm, D. Saunders, D. Brandenburg, L. Orci, and P. Freychet. 1982. Biochemical and morphological evidence that the insulin receptor is internalized with insulin in hepatocytes. *J. Cell Biol.* 93:82–87. https://doi.org/10.1083/jcb.93.1.82

Ferdaoussi, M., and P.E. MacDonald. 2017. Toward Connecting Metabolism to the Exocytotic Site. *Trends Cell Biol.* 27:163–171. https://doi.org/10.1016/j.tcb.2016.10.003

Ferris, H.A., and C.R. Kahn. 2016. Unraveling the Paradox of Selective Insulin Resistance in the Liver: the Brain-Liver Connection. *Diabetes.* 65:1481–1483. https://doi.org/10.2337/dbi16-0010

Filippi, B.M., M.A. Abraham, J.T.Y. Yue, and T.K.T. Lam. 2013. Insulin and glucagon signaling in the central nervous system. *Rev. Endocr. Metab. Disord.* 14:365–375. https://doi.org/10.1007/s11154-013-9258-4

Foley, K., S. Boguslavsky, and A. Klip. 2011. Endocytosis, recycling, and regulated exocytosis of glucose transporter 4. *Biochemistry.* 50:3048–3061. https://doi.org/10.1021/bi2000356

Forli, A., D. Vecchia, N. Binini, F. Succol, S. Bovetti, C. Moretti, F. Nespoli, M. Mahn, C.A. Baker, M.M. Bolton, et al. 2018. Two-Photon Bidirectional Control and Imaging of Neuronal Excitability with High Spatial Resolution In Vivo. *Cell Reports.* 22:3087–3098. https://doi.org/10.1016/j.celrep.2018.02.063

Foster, L.J., and A. Klip. 2000. Mechanism and regulation of GLUT-4 vesicle fusion in muscle and fat cells. *Am. J. Physiol. Cell Physiol.* 279:C877–C890. https://doi.org/10.1152/ajpcell.2000.279.4.C877

Friedrich, R., A. Yeheskel, and U. Ashery. 2010. DOC2B, C2 domains, and calcium: A tale of intricate interactions. *Mol. Neurobiol.* 41:42–51. https://doi.org/10.1007/s12035-009-8094-8

Fukuda, N., M. Emoto, Y. Nakamori, A. Taguchi, S. Miyamoto, S. Uraki, Y. Oka, and Y. Tanizawa. 2009. DOC2B: a novel syntaxin-4 binding protein mediating insulin-regulated GLUT4 vesicle fusion in adipocytes. *Diabetes.* 58:377–384. https://doi.org/10.2337/db08-0303

Gaisano, H.Y. 2017. Recent new insights into the role of SNARE and associated proteins in insulin granule exocytosis. *Diabetes Obes. Metab.* 19(Suppl 1):115–123. https://doi.org/10.1111/dom.13001

Gandasi, N.R., and S. Barg. 2014. Contact-induced clustering of syntaxin and munc18 docks secretory granules at the exocytosis site. *Nat. Commun.* 5:3914. https://doi.org/10.1038/ncomms4914

Gandasi, N.R., P. Yin, M. Riz, M.V. Chibalina, G. Cortese, P.-E. Lund, V. Matveev, P. Rorsman, A. Sherman, M.G. Pedersen, and S. Barg. 2017. Ca2+ channel clustering with insulin-containing granules is disturbed in type 2 diabetes. *J. Clin. Invest.* 127:2353–2364. https://doi.org/10.1172/JCI88491

Gao, T., B. McKenna, C. Li, M. Reichert, J. Nguyen, T. Singh, C. Yang, A. Pannikar, N. Doliba, T. Zhang, et al. 2014. Pdx1 maintains β cell identity and function by repressing an α cell program. *Cell Metab.* 19:259–271. https://doi.org/10.1016/j.cmet.2013.12.002

García-Cáceres, C., C. Quarta, L. Varela, Y. Gao, T. Gruber, B. Legutko, M. Jastroch, P. Johansson, J. Ninkovic, C.-X. Yi, et al. 2016. Astrocytic Insulin Signaling Couples Brain Glucose Uptake with Nutrient Availability. *Cell.* 166:867–880. https://doi.org/10.1016/j.cell.2016.07.028

Garrido-Sanchez, L., X. Escote, L. Coin-Aragüez, J.C. Fernandez-Garcia, R. El Bekay, J. Vendrell, E. Garcia-Fuentes, and F.J. Tinahones. 2013. Munc18c in adipose tissue is downregulated in obesity and is associated with insulin. *PLoS One.* 8:e63937. https://doi.org/10.1371/journal.pone.0063937

Garvey, W.T., L. Maianu, J.H. Zhu, G. Brechtel-Hook, P. Wallace, and A.D. Baron. 1998. Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J. Clin. Invest.* 101:2377–2386. https://doi.org/10.1172/JCI1557

Gloyn, A.L., K. Noordam, M.A.A.P. Willemsen, S. Ellard, W.W.K. Lam, I.W. Campbell, P. Midgley, C. Shiota, C. Buettger, M.A. Magnuson, et al. 2003. Insights into the biochemical and genetic basis of glucokinase activation from naturally occurring hypoglycemia mutations. *Diabetes.* 52:2433–2440. https://doi.org/10.2337/diabetes.52.9.2433

Goodner, C.J., I.R. Sweet, and H.C. Harrison Jr. 1988. Rapid reduction and return of surface insulin receptors after exposure to brief pulses of insulin in perifused rat hepatocytes. *Diabetes.* 37:1316–1323. https://doi.org/10.2337/diab.37.10.1316

Gray, S.M., K.W. Aylor, and E.J. Barrett. 2017. Unravelling the regulation of insulin transport across the brain endothelial cell. *Diabetologia.* 60:1512–1521. https://doi.org/10.1007/s00125-017-4285-4

Griffith, C.M., T. Eid, G.M. Rose, and P.R. Patrylo. 2018. Evidence for altered insulin receptor signaling in Alzheimer's disease. *Neuropharmacology.*:S0028-3908(18)30008-X. https://doi.org/10.1016/j.neuropharm.2018.01.008

Gulbranson, D.R., E.M. Davis, B.A. Demmitt, Y. Ouyang, Y. Ye, H. Yu, and J. Shen. 2017. RABIF/MSS4 is a Rab-stabilizing holdase chaperone required for GLUT4 exocytosis. *Proc. Natl. Acad. Sci. USA.* 114:E8224–E8233. https://doi.org/10.1073/pnas.1712176114

Gutmann, T., K.H. Kim, M. Grzybek, T. Walz, and Ü. Coskun. 2018. Visualization of ligand-induced transmembrane signaling in the full-length human insulin receptor. *J. Cell Biol.*:jcb.201711047.

Gylfe, E., E. Grapengiesser, H. Dansk, and B. Hellman. 2012. The neurotransmitter ATP triggers Ca2+ responses promoting coordination of pancreatic islet oscillations. *Pancreas.* 41:258–263. https://doi.org/10.1097/MPA.0b013e3182240586

Haeusler, R.A., T.E. McGraw, and D. Accili. 2018. Biochemical and cellular properties of insulin receptor signalling. *Nat. Rev. Mol. Cell Biol.* 19:31–44. https://doi.org/10.1038/nrm.2017.89

Hale, L.J., and R.J.M. Coward. 2013. Insulin signalling to the kidney in health and disease. *Clin. Sci. (Lond.).* 124:351–370. https://doi.org/10.1042/CS20120378

Hamel, F.G., M.J. Mahoney, and W.C. Duckworth. 1991. Degradation of intraendosomal insulin by insulin-degrading enzyme without acidification. *Diabetes.* 40:436–443. https://doi.org/10.2337/diab.40.4.436

Henquin, J.C. 2000. Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes.* 49:1751–1760. https://doi.org/10.2337/diabetes.49.11.1751

Henquin, J.C., H.P. Meissner, and W. Schmeer. 1982. Cyclic variations of glucose-induced electrical activity in pancreatic B cells. *Pflugers Arch.* 393:322–327. https://doi.org/10.1007/BF00581418

Herkner, H., N. Klein, C. Joukhadar, E. Lackner, H. Langenberger, M. Frossard, C. Bieglmayer, O. Wagner, M. Roden, and M. Müller. 2003. Transcapillary insulin transfer in human skeletal muscle. *Eur. J. Clin. Invest.* 33:141–146. https://doi.org/10.1046/j.1365-2362.2003.01106.x

Hoehn, K.L., C. Hohnen-Behrens, A. Cederberg, L.E. Wu, N. Turner, T. Yuasa, Y. Ebina, and D.E. James. 2008. IRS1-independent defects define major nodes of insulin resistance. *Cell Metab.* 7:421–433. https://doi.org/10.1016/j.cmet.2008.04.005

Hollingdal, M., C.B. Juhl, S.M. Pincus, J. Sturis, J.D. Veldhuis, K.S. Polonsky, N. Pørksen, and O. Schmitz. 2000. Failure of physiological plasma glucose excursions to entrain high-frequency pulsatile insulin secretion in type 2 diabetes. *Diabetes.* 49:1334–1340. https://doi.org/10.2337/diabetes.49.8.1334

Horwitz, D.L., J.I. Starr, M.E. Mako, W.G. Blackard, and A.H. Rubenstein. 1975. Proinsulin, insulin, and C-peptide concentrations in human portal and peripheral blood. *J. Clin. Invest.* 55:1278–1283. https://doi.org/10.1172/JCI108047

Huang, G., D. Buckler-Pena, T. Nauta, M. Singh, A. Asmar, J. Shi, J.Y. Kim, and K.V. Kandror. 2013. Insulin responsiveness of glucose transporter 4 in 3T3-L1 cells depends on the presence of sortilin. *Mol. Biol. Cell.* 24:3115–3122. https://doi.org/10.1091/mbc.E12-10-0765

Hutton, J.C. 1994. Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases. *Diabetologia.* 37(Suppl 2):S48–S56.

Ishikura, S., P.J. Bilan, and A. Klip. 2007. Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochem. Biophys. Res. Commun.* 353:1074–1079. https://doi.org/10.1016/j.bbrc.2006.12.140

Jaldin-Fincati, J.R., M. Pavarotti, S. Frendo-Cumbo, P.J. Bilan, and A. Klip. 2017. Update on GLUT4 Vesicle Traffic: A Cornerstone of Insulin Action. *Trends Endocrinol. Metab.* 28:597–611. https://doi.org/10.1016/j.tem.2017.05.002

Jaldin-Fincati, J.R., R.V.S. Pereira, P.J. Bilan, and A. Klip. 2018. Insulin uptake and action in microvascular endothelial cells of lymphatic and blood origin. *Am. J. Physiol. Endocrinol. Metab.*:ajpendo.00008.2018. https://doi.org/10.1152/ajpendo.00008.2018

Jansson, P.-A.E., J.P. Fowelin, H.P. von Schenck, U.P. Smith, and P.N. Lönnroth. 1993. Measurement by microdialysis of the insulin concentration in subcutaneous interstitial fluid. Importance of the endothelial barrier for insulin. *Diabetes.* 42:1469–1473. https://doi.org/10.2337/diab.42.10.1469

Jialal, I., G.L. King, S. Buchwald, C.R. Kahn, and M. Crettaz. 1984. Processing of insulin by bovine endothelial cells in culture. Internalization without degradation. *Diabetes.* 33:794–800. https://doi.org/10.2337/diab.33.8.794

Johnston, N.R., R.K. Mitchell, E. Haythorne, M.P. Pessoa, F. Semplici, J. Ferrer, L. Piemonti, P. Marchetti, M. Bugliani, D. Bosco, et al. 2016. Beta Cell Hubs Dictate Pancreatic Islet Responses to Glucose. *Cell Metab.* 24:389–401. https://doi.org/10.1016/j.cmet.2016.06.020

Jung, S.-H., C.-H. Jung, G.M. Reaven, and S.H. Kim. 2018. Adapting to insulin resistance in obesity: role of insulin secretion and clearance. *Diabetologia.* 61:681–687.

Kalwat, M.A., and D.C. Thurmond. 2013. Signaling mechanisms of glucose-induced F-actin remodeling in pancreatic islet β cells. *Exp. Mol. Med.* 45:e37. https://doi.org/10.1038/emm.2013.73

Kanasaki, K., M. Kitada, M. Kanasaki, and D. Koya. 2013. The biological consequence of obesity on the kidney. *Nephrol. Dial. Transplant.* 28(Suppl 4):iv1–iv7. https://doi.org/10.1093/ndt/gft098

Kandror, K.V., and P.F. Pilch. 2011. The sugar is sIRVed: sorting Glut4 and its fellow travelers. *Traffic.* 12:665–671. https://doi.org/10.1111/j.1600-0854.2011.01175.x

Karamitsos, D.T. 2011. The story of insulin discovery. *Diabetes Res. Clin. Pract.* 93(Suppl 1):S2–S8. https://doi.org/10.1016/S0168-8227(11)70007-9

Karunanithi, S., T. Xiong, M. Uhm, D. Leto, J. Sun, X.-W. Chen, and A.R. Saltiel. 2014. A Rab10:RalA G protein cascade regulates insulin-stimulated glucose uptake in adipocytes. *Mol. Biol. Cell.* 25:3059–3069. https://doi.org/10.1091/mbc.E14-06-1060

Karylowski, O., A. Zeigerer, A. Cohen, and T.E. McGraw. 2004. GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. *Mol. Biol. Cell.* 15:870–882. https://doi.org/10.1091/mbc.E03-07-0517

Kebede, M.A., A.T. Oler, T. Gregg, A.J. Balloon, A. Johnson, K. Mitok, M. Rabaglia, K. Schueler, D. Stapleton, C. Thorstenson, et al. 2014. SORCS1 is necessary for normal insulin secretory granule biogenesis in metabolically stressed β cells. *J. Clin. Invest.* 124:4240–4256. https://doi.org/10.1172/JCI74072

Keske, M.A., L.H. Clerk, W.J. Price, L.A. Jahn, and E.J. Barrett. 2009. Obesity blunts microvascular recruitment in human forearm muscle after a mixed meal. *Diabetes Care.* 32:1672–1677. https://doi.org/10.2337/dc09-0206

Kleinridders, A., H.A. Ferris, W. Cai, and C.R. Kahn. 2014. Insulin action in brain regulates systemic metabolism and brain function. *Diabetes.* 63:2232–2243. https://doi.org/10.2337/db14-0568

Klip, A., T. Ramlal, P.J. Bilan, G.D. Cartee, E.A. Gulve, and J.O. Holloszy. 1990. Recruitment of GLUT-4 glucose transporters by insulin in diabetic rat skeletal muscle. *Biochem. Biophys. Res. Commun.* 172:728–736. https://doi.org/10.1016/0006-291X(90)90735-6

Klip, A., Y. Sun, T.T. Chiu, and K.P. Foley. 2014. Signal transduction meets vesicle traffic: the software and hardware of GLUT4 translocation. *Am. J. Physiol. Cell Physiol.* 306:C879–C886. https://doi.org/10.1152/ajpcell.00069.2014

Kolic, J., and P.E. MacDonald. 2015. cAMP-independent effects of GLP-1 on β cells. *J. Clin. Invest.* 125:4327–4330. https://doi.org/10.1172/JCI85004

Kolka, C.M., and R.N. Bergman. 2012. The barrier within: endothelial transport of hormones. *Physiology (Bethesda).* 27:237–247.

Kolman, P., A. Pica, N. Carvou, A. Boyde, S. Cockcroft, A. Loesch, A. Pizzey, M. Simeoni, G. Capasso, and R.J. Unwin. 2009. Insulin uptake across the luminal membrane of the rat proximal tubule in vivo and in vitro. *Am. J. Physiol. Renal Physiol.* 296:F1227–F1237. https://doi.org/10.1152/ajprenal.90351.2008

Konishi, M., M. Sakaguchi, S.M. Lockhart, W. Cai, M.E. Li, E.P. Homan, C. Rask-Madsen, and C.R. Kahn. 2017. Endothelial insulin receptors differentially control insulin signaling kinetics in peripheral tissues and brain of mice. *Proc. Natl. Acad. Sci. USA.* 114:E8478–E8487. https://doi.org/10.1073/pnas.1710625114

Könner, A.C., R. Janoschek, L. Plum, S.D. Jordan, E. Rother, X. Ma, C. Xu, P. Enriori, B. Hampel, G.S. Barsh, et al. 2007. Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. *Cell Metab.* 5:438–449. https://doi.org/10.1016/j.cmet.2007.05.004

Korol, S.V., A. Tafreshiha, A.K. Bhandage, B. Birnir, and Z. Jin. 2018. Insulin enhances GABA$_A$receptor-mediated inhibitory currents in rat central amygdala neurons. *Neurosci. Lett.* 671:76–81. https://doi.org/10.1016/j.neulet.2018.02.022

Kubota, T., N. Kubota, H. Kumagai, S. Yamaguchi, H. Kozono, T. Takahashi, M. Inoue, S. Itoh, I. Takamoto, T. Sasako, et al. 2011. Impaired insulin signaling in endothelial cells reduces insulin-induced glucose uptake by skeletal muscle. *Cell Metab.* 13:294–307. https://doi.org/10.1016/j.cmet.2011.01.018

Laedtke, T., L. Kjems, N. Pørksen, O. Schmitz, J. Veldhuis, P.C. Kao, and P.C. Butler. 2000. Overnight inhibition of insulin secretion restores pulsatility and proinsulin/insulin ratio in type 2 diabetes. *Am. J. Physiol. Endocrinol. Metab.* 279:E520–E528. https://doi.org/10.1152/ajpendo.2000.279.3.E520

Lalioti, V., G. Muruais, A. Dinarina, J. van Damme, J. Vandekerckhove, and I.V. Sandoval. 2009. The atypical kinase Cdk5 is activated by insulin, regulates the association between GLUT4 and E-Syt1, and modulates glucose transport in 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA.* 106:4249–4253. https://doi.org/10.1073/pnas.0900218106

Lang, D.A., D.R. Matthews, M. Burnett, and R.C. Turner. 1981. Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man. *Diabetes.* 30:435–439. https://doi.org/10.2337/diab.30.5.435

Lansey, M.N., N.N. Walker, S.R. Hargett, J.R. Stevens, and S.R. Keller. 2012. Deletion of Rab GAP AS160 modifies glucose uptake and GLUT4 translocation in primary skeletal muscles and adipocytes and impairs glucose homeostasis. *Am. J. Physiol. Endocrinol. Metab.* 303:E1273–E1286. https://doi.org/10.1152/ajpendo.00316.2012

Lee, W. 2011. The CEACAM1 expression is decreased in the liver of severely obese patients with or without diabetes. *Diagn. Pathol.* 6:40. https://doi.org/10.1186/1746-1596-6-40

Lee, W.L., and A. Klip. 2016. Endothelial Transcytosis of Insulin: Does It Contribute to Insulin Resistance? *Physiology (Bethesda).* 31:336–345.

Lee, M.R., L. Li, and T. Kitazawa. 1997. Cyclic GMP causes Ca2+ desensitization in vascular smooth muscle by activating the myosin light chain phosphatase. *J. Biol. Chem.* 272:5063–5068. https://doi.org/10.1074/jbc.272.8.5063

Lee, S.-H., J.M. Zabolotny, H. Huang, H. Lee, and Y.-B. Kim. 2016. Insulin in the nervous system and the mind: Functions in metabolism, memory, and mood. *Mol. Metab.* 5:589–601. https://doi.org/10.1016/j.molmet.2016.06.011

Lees, J.A., M. Messa, E.W. Sun, H. Wheeler, F. Torta, M.R. Wenk, P. De Camilli, and K.M. Reinisch. 2017. Lipid transport by TMEM24 at ER-plasma membrane contacts regulates pulsatile insulin secretion. *Science.* 355:6171.

Lemaire, K., M.A. Ravier, A. Schraenen, J.W.M. Creemers, R. Van de Plas, M. Granvik, L. Van Lommel, E. Waelkens, F. Chimienti, G.A. Rutter, et al. 2009. Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. *Proc. Natl. Acad. Sci. USA.* 106:14872–14877. https://doi.org/10.1073/pnas.0906587106

Leto, D., and A.R. Saltiel. 2012. Regulation of glucose transport by insulin: traffic control of GLUT4. *Nat. Rev. Mol. Cell Biol.* 13:383–396. https://doi.org/10.1038/nrm3351

Li, G., E. Rungger-Brändle, I. Just, J.C. Jonas, K. Aktories, and C.B. Wollheim. 1994. Effect of disruption of actin filaments by Clostridium botulinum C2 toxin on insulin secretion in HIT-T15 cells and pancreatic islets. *Mol. Biol. Cell.* 5:1199–1213. https://doi.org/10.1091/mbc.5.11.1199

Liang, T., T. Qin, L. Xie, S. Dolai, D. Zhu, K.J. Prentice, M. Wheeler, Y. Kang, L. Osborne, and H.Y. Gaisano. 2017. New Roles of Syntaxin-1A in Insulin Granule Exocytosis and Replenishment. *J. Biol. Chem.* 292:2203–2216. https://doi.org/10.1074/jbc.M116.769885

Lin, H.V., and D. Accili. 2011. Hormonal regulation of hepatic glucose production in health and disease. *Cell Metab.* 14:9–19. https://doi.org/10.1016/j.cmet.2011.06.003

Lizunov, V.A., K.G. Stenkula, I. Lisinski, O. Gavrilova, D.R. Yver, A. Chadt, H. Al-Hasani, J. Zimmerberg, and S.W. Cushman. 2012. Insulin stimulates fusion, but not tethering, of GLUT4 vesicles in skeletal muscle of HA-GLUT4-GFP transgenic mice. *Am. J. Physiol. Endocrinol. Metab.* 302:E950–E960. https://doi.org/10.1152/ajpendo.00466.2011

Lopez, J.A., J.G. Burchfield, D.H. Blair, K. Mele, Y. Ng, P. Vallotton, D.E. James, and W.E. Hughes. 2009. Identification of a distal GLUT4 trafficking event controlled by actin polymerization. *Mol. Biol. Cell.* 20:3918–3929. https://doi.org/10.1091/mbc.E09-03-0187

Majumdar, S., A.J. Genders, A.C. Inyard, V. Frison, and E.J. Barrett. 2012. Insulin entry into muscle involves a saturable process in the vascular endothelium. *Diabetologia.* 55:450–456. https://doi.org/10.1007/s00125-011-2343-x

Marino, M.2009. Diabetes drugs: Insulin. https://www.diabetesselfmanagement.com/blog/diabetes-drugs-insulin/ (accessed March 15, 2018).

Martin, O.J., A. Lee, and T.E. McGraw. 2006. GLUT4 distribution between the plasma membrane and the intracellular compartments is maintained by an insulin-modulated bipartite dynamic mechanism. *J. Biol. Chem.* 281:484–490. https://doi.org/10.1074/jbc.M505944200

Matveyenko, A.V., D. Liuwantara, T. Gurlo, D. Kirakossian, C. Dalla Man, C. Cobelli, M.F. White, K.D. Copps, E. Volpi, S. Fujita, and P.C. Butler. 2012. Pulsatile portal vein insulin delivery enhances hepatic insulin action and signaling. *Diabetes.* 61:2269–2279. https://doi.org/10.2337/db11-1462

McCulloch, L.J., M. van de Bunt, M. Braun, K.N. Frayn, A. Clark, and A.L. Gloyn. 2011. GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: implications for understanding genetic association signals at this locus. *Mol. Genet. Metab.* 104:648–653. https://doi.org/10.1016/j.ymgme.2011.08.026

McDonald, A., S. Fogarty, I. Leclerc, E.V. Hill, D.G. Hardie, and G.A. Rutter. 2009. Control of insulin granule dynamics by AMPK dependent KLC1 phosphorylation. *Islets.* 1:198–209. https://doi.org/10.4161/isl.1.3.9608

Meezan, E., D.J. Pillion, and A. Elgavish. 1988. Binding and degradation of 125I-insulin by isolated rat renal brush border membranes: evidence for low affinity, high capacity insulin recognition sites. *J. Membr. Biol.* 105:113–129. https://doi.org/10.1007/BF02009165

Meglasson, M.D., P.T. Burch, D.K. Berner, H. Najafi, A.P. Vogin, and F.M. Matschinsky. 1983. Chromatographic resolution and kinetic characterization of glucokinase from islets of Langerhans. *Proc. Natl. Acad. Sci. USA.* 80:85–89. https://doi.org/10.1073/pnas.80.1.85

Meier, J.J., J.D. Veldhuis, and P.C. Butler. 2005. Pulsatile insulin secretion dictates systemic insulin delivery by regulating hepatic insulin extraction in humans. *Diabetes.* 54:1649–1656. https://doi.org/10.2337/diabetes.54.6.1649

Meijer, R.I., S.M. Gray, K.W. Aylor, and E.J. Barrett. 2016. Pathways for insulin access to the brain: the role of the microvascular endothelial cell. *Am. J. Physiol. Heart Circ. Physiol.* 311:H1132–H1138. https://doi.org/10.1152/ajpheart.00081.2016

Michael, M.D., R.N. Kulkarni, C. Postic, S.F. Previs, G.I. Shulman, M.A. Magnuson, and C.R. Kahn. 2000. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol. Cell.* 6:87–97. https://doi.org/10.1016/S1097-2765(05)00015-8

Mizuno, Y., E. Isotani, J. Huang, H. Ding, J.T. Stull, and K.E. Kamm. 2008. Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo. *Am. J. Physiol. Cell Physiol.* 295:C358–C364. https://doi.org/10.1152/ajpcell.90645.2007

Murphy, R.F., S. Powers, and C.R. Cantor. 1984. Endosome pH measured in single cells by dual fluorescence flow cytometry: rapid acidification of insulin to pH 6. *J. Cell Biol.* 98:1757–1762. https://doi.org/10.1083/jcb.98.5.1757

Najjar, S.M. 2002. Regulation of insulin action by CEACAM1. *Trends Endocrinol. Metab.* 13:240–245. https://doi.org/10.1016/S1043-2760(02)00608-2

Najjar, S.M., N. Philippe, Y. Suzuki, G.A. Ignacio, P. Formisano, D. Accili, and S.I. Taylor. 1995. Insulin-stimulated phosphorylation of recombinant pp120/HA4, an endogenous substrate of the insulin receptor tyrosine kinase. *Biochemistry.* 34:9341–9349. https://doi.org/10.1021/bi00029a009

Nielsen, S. 1993. Sorting and recycling efficiency of apical insulin binding sites during endocytosis in proximal tubule cells. *Am. J. Physiol.* 264:C810–C822. https://doi.org/10.1152/ajpcell.1993.264.4.C810

Nielsen, S. 1994. Endocytosis in renal proximal tubules. Experimental electron microscopical studies of protein absorption and membrane traffic in isolated, in vitro perfused proximal tubules. *Dan. Med. Bull.* 41:243–263.

Nielsen, S., J.T. Nielsen, and E.I. Christensen. 1987. Luminal and basolateral uptake of insulin in isolated, perfused, proximal tubules. *Am. J. Physiol.* 253:F857–F867.

Odegaard, J.I., and A. Chawla. 2013. Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis. *Science.* 339:172–177. https://doi.org/10.1126/science.1230721

Palmer, B.F., and W.L. Henrich. 2017. Carbohydrate and insulin metabolism in chronic kidney disease. https://www.uptodate.com/contents/carbohydrate-and-insulin-metabolism-in-chronic-kidney-disease (accessed March 15, 2018).

Palmer, R.M.J., D.S. Ashton, and S. Moncada. 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* 333:664–666. https://doi.org/10.1038/333664a0

Patterson, J.N., K. Cousteils, J.W. Lou, J.E. Manning Fox, P.E. MacDonald, and J.W. Joseph. 2014. Mitochondrial metabolism of pyruvate is essential for regulating glucose-stimulated insulin secretion. *J. Biol. Chem.* 289:13335–13346. https://doi.org/10.1074/jbc.M113.521666

Pilch, P.F., M.A. Shia, R.J. Benson, and R.E. Fine. 1983. Coated vesicles participate in the receptor-mediated endocytosis of insulin. *J. Cell Biol.* 96:133–138. https://doi.org/10.1083/jcb.96.1.133

Pørksen, N., S. Munn, J. Steers, S. Vore, J. Veldhuis, and P. Butler. 1995. Pulsatile insulin secretion accounts for 70% of total insulin secretion during fasting. *Am. J. Physiol.* 269:E478–E488.

Porte, D. Jr., D.G. Baskin, and M.W. Schwartz. 2005. Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. *Diabetes.* 54:1264–1276. https://doi.org/10.2337/diabetes.54.5.1264

Poy, M.N., Y. Yang, K. Rezaei, M.A. Fernström, A.D. Lee, Y. Kido, S.K. Erickson, and S.M. Najjar. 2002. CEACAM1 regulates insulin clearance in liver. *Nat. Genet.* 30:270–276. https://doi.org/10.1038/ng840

Rabkin, R., M.P. Ryan, and W.C. Duckworth. 1984. The renal metabolism of insulin. *Diabetologia.* 27:351–357. https://doi.org/10.1007/BF00304849

Randhawa, V.K., P.J. Bilan, Z.A. Khayat, N. Daneman, Z. Liu, T. Ramlal, A. Volchuk, X.R. Peng, T. Coppola, R. Regazzi, et al. 2000. VAMP2, but not VAMP3/cellubrevin, mediates insulin-dependent incorporation of GLUT4 into the plasma membrane of L6 myoblasts. *Mol. Biol. Cell.* 11:2403–2417. https://doi.org/10.1091/mbc.11.7.2403

Randhawa, V.K., F.S.L. Thong, D.Y. Lim, D. Li, R.R. Garg, R. Rudge, T. Galli, A. Rudich, and A. Klip. 2004. Insulin and hypertonicity recruit GLUT4 to the plasma membrane of muscle cells by using N-ethylmaleimide-sensitive factor-dependent SNARE mechanisms but different v-SNAREs: role of TI-VAMP. *Mol. Biol. Cell.* 15:5565–5573. https://doi.org/10.1091/mbc.E04-03-0266

Ravier, M.A., M. Güldenagel, A. Charollais, A. Gjinovci, D. Caille, G. Söhl, C.B. Wollheim, K. Willecke, J.-C. Henquin, and P. Meda. 2005. Loss of connexin36 channels alters beta-cell coupling, islet synchronization of glucose-induced Ca2+ and insulin oscillations, and basal insulin release. *Diabetes.* 54:1798–1807. https://doi.org/10.2337/diabetes.54.6.1798

Robbins, D.C., S.E. Shoelson, H.S. Tager, P.M. Mead, and D.H. Gaynor. 1985. Products of therapeutic insulins in the blood of insulin-dependent (type I) diabetic patients. *Diabetes.* 34:510–519. https://doi.org/10.2337/diab.34.5.510

Rorsman, P., and E. Renström. 2003. Insulin granule dynamics in pancreatic beta cells. *Diabetologia.* 46:1029–1045. https://doi.org/10.1007/s00125-003-1153-1

Rorsman, P., M. Braun, and Q. Zhang. 2012. Regulation of calcium in pancreatic α- and β-cells in health and disease. *Cell Calcium.* 51:300–308. https://doi.org/10.1016/j.ceca.2011.11.006

Rubenstein, A.H., and I. Spitz. 1968. Role of the kidney in insulin metabolism and excretion. *Diabetes.* 17:161–169. https://doi.org/10.2337/diab.17.3.161

Ruderman, N.B., R. Kapeller, M.F. White, and L.C. Cantley. 1990. Activation of phosphatidylinositol 3-kinase by insulin. *Proc. Natl. Acad. Sci. USA.* 87:1411–1415. https://doi.org/10.1073/pnas.87.4.1411

Russo, L., H.T. Muturi, H.E. Ghadieh, S.S. Ghanem, T.A. Bowman, H.L. Noh, S. Dagdeviren, G.Y. Dogbey, J.K. Kim, G. Heinrich, and S.M. Najjar. 2017. Liver-specific reconstitution of CEACAM1 reverses the metabolic abnormalities caused by its global deletion in male mice. *Diabetologia.* 60:2463–2474. https://doi.org/10.1007/s00125-017-4432-y

Samuel, V.T., and G.I. Shulman. 2012. Mechanisms for insulin resistance: common threads and missing links. *Cell.* 148:852–871. https://doi.org/10.1016/j.cell.2012.02.017

Samuel, V.T., and G.I. Shulman. 2016. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *J. Clin. Invest.* 126:12–22. https://doi.org/10.1172/JCI77812

Samuel, V.T., and G.I. Shulman. 2018. Nonalcoholic Fatty Liver Disease as a Nexus of Metabolic and Hepatic Diseases. *Cell Metab.* 27:22–41. https://doi.org/10.1016/j.cmet.2017.08.002

Sano, H., S. Kane, E. Sano, C.P. Mîinea, J.M. Asara, W.S. Lane, C.W. Garner, and G.E. Lienhard. 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J. Biol. Chem.* 278:14599–14602. https://doi.org/10.1074/jbc.C300063200

Sano, H., L. Eguez, M.N. Teruel, M. Fukuda, T.D. Chuang, J.A. Chavez, G.E. Lienhard, and T.E. McGraw. 2007. Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metab.* 5:293–303. https://doi.org/10.1016/j.cmet.2007.03.001

Sano, H., W.G. Roach, G.R. Peck, M. Fukuda, and G.E. Lienhard. 2008. Rab10 in insulin-stimulated GLUT4 translocation. *Biochem. J.* 411:89–95. https://doi.org/10.1042/BJ20071318

Sano, H., G.R. Peck, A.N. Kettenbach, S.A. Gerber, and G.E. Lienhard. 2011. Insulin-stimulated GLUT4 protein translocation in adipocytes requires the Rab10 guanine nucleotide exchange factor Dennd4C. *J. Biol. Chem.* 286:16541–16545. https://doi.org/10.1074/jbc.C111.228908

Sano, H., G.R. Peck, S. Blachon, and G.E. Lienhard. 2015. A potential link between insulin signaling and GLUT4 translocation: Association of Rab10-GTP with the exocyst subunit Exoc6/6b. *Biochem. Biophys. Res. Commun.* 465:601–605. https://doi.org/10.1016/j.bbrc.2015.08.069

Satin, L.S., P.C. Butler, J. Ha, and A.S. Sherman. 2015. Pulsatile insulin secretion, impaired glucose tolerance and type 2 diabetes. *Mol. Aspects Med.* 42:61–77. https://doi.org/10.1016/j.mam.2015.01.003

Sato, H., K. Yoshioka, T. Terasaki, and A. Tsuji. 1991. Receptor-mediated endocytosis of A14-125I-insulin by the nonfiltering perfused rat kidney. *Biochim. Biophys. Acta.* 1073:442–450. https://doi.org/10.1016/0304-4165(91)90213-Z

Schwartz, M.W., S.C. Woods, D. Porte Jr., R.J. Seeley, and D.G. Baskin. 2000. Central nervous system control of food intake. *Nature.* 404:661–671. https://doi.org/10.1038/35007534

Shimoda, Y., S. Okada, E. Yamada, J.E. Pessin, and M. Yamada. 2015. Tctex1d2 Is a Negative Regulator of GLUT4 Translocation and Glucose Uptake. *Endocrinology.* 156:3548–3558. https://doi.org/10.1210/en.2015-1120

Song, E.S., H. Jang, H.-F. Guo, M.A. Juliano, L. Juliano, A.J. Morris, E. Galperin, D.W. Rodgers, and L.B. Hersh. 2017. Inositol phosphates and phosphoinositides activate insulin-degrading enzyme, while phosphoinositides also mediate binding to endosomes. *Proc. Natl. Acad. Sci. USA.* 114:E2826–E2835. https://doi.org/10.1073/pnas.1613447114

Song, S.H., S.S. McIntyre, H. Shah, J.D. Veldhuis, P.C. Hayes, and P.C. Butler. 2000. Direct measurement of pulsatile insulin secretion from the portal vein in human subjects. *J. Clin. Endocrinol. Metab.* 85:4491–4499.

Spoto, B., A. Pisano, and C. Zoccali. 2016. Insulin resistance in chronic kidney disease: a systematic review. *Am. J. Physiol. Renal Physiol.* 311:F1087–F1108. https://doi.org/10.1152/ajprenal.00340.2016

Steil, G.M., M. Ader, D.M. Moore, K. Rebrin, and R.N. Bergman. 1996. Transendothelial insulin transport is not saturable in vivo. No evidence for a receptor-mediated process. *J. Clin. Invest.* 97:1497–1503. https://doi.org/10.1172/JCI118572

Steinberg, H.O., G. Brechtel, A. Johnson, N. Fineberg, and A.D. Baron. 1994. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Invest.* 94:1172–1179. https://doi.org/10.1172/JCI117433

Steneberg, P., L. Bernardo, S. Edfalk, L. Lundberg, F. Backlund, C.-G. Ostenson, and H. Edlund. 2013. The type 2 diabetes-associated gene ide is required for insulin secretion and suppression of α-synuclein levels in β-cells. *Diabetes.* 62:2004–2014. https://doi.org/10.2337/db12-1045

Stevenson, R.W., A.D. Cherrington, and K.E. Steiner. 1985. The relationship between plasma concentration and disappearance rate of immunoreactive insulin in the conscious dog. *Horm. Metab. Res.* 17:551–553. https://doi.org/10.1055/s-2007-1013604

Stöckli, J., D.J. Fazakerley, and D.E. James. 2011. GLUT4 exocytosis. *J. Cell Sci.* 124:4147–4159. https://doi.org/10.1242/jcs.097063

Sun, Y., P.J. Bilan, Z. Liu, and A. Klip. 2010. Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. *Proc. Natl. Acad. Sci. USA.* 107:19909–19914. https://doi.org/10.1073/pnas.1009523107

Sun, Y., T.T. Chiu, K.P. Foley, P.J. Bilan, and A. Klip. 2014. Myosin Va mediates Rab8A-regulated GLUT4 vesicle exocytosis in insulin-stimulated muscle cells. *Mol. Biol. Cell.* 25:1159–1170. https://doi.org/10.1091/mbc.E13-08-0493

Sun, Y., J. Jaldin-Fincati, Z. Liu, P.J. Bilan, and A. Klip. 2016. A complex of Rab13 with MICAL-L2 and α-actinin-4 is essential for insulin-dependent GLUT4 exocytosis. *Mol. Biol. Cell.* 27:75–89. https://doi.org/10.1091/mbc.E15-05-0319

Sylow, L., T.E. Jensen, M. Kleinert, K. Højlund, B. Kiens, J. Wojtaszewski, C. Prats, P. Schjerling, and E.A. Richter. 2013. Rac1 signaling is required for insulin-stimulated glucose uptake and is dysregulated in insulin-resistant murine and human skeletal muscle. *Diabetes.* 62:1865–1875. https://doi.org/10.2337/db12-1148

Takenaka, N., N. Yasuda, Y. Nihata, T. Hosooka, T. Noguchi, A. Aiba, and T. Satoh. 2014. Role of the guanine nucleotide exchange factor in Akt2-mediated plasma membrane translocation of GLUT4 in insulin-stimulated skeletal muscle. *Cell. Signal.* 26:2460–2469. https://doi.org/10.1016/j.cellsig.2014.07.002

Takenaka, N., Y. Nihata, and T. Satoh. 2016. Rac1 Activation Caused by Membrane Translocation of a Guanine Nucleotide Exchange Factor in Akt2-Mediated Insulin Signaling in Mouse Skeletal Muscle. *PLoS One.* 11:e0155292. https://doi.org/10.1371/journal.pone.0155292

Tang, S.-C., L. Baeyens, C.-N. Shen, S.-J. Peng, H.-J. Chien, D.W. Scheel, C.E. Chamberlain, and M.S. German. 2018b. Human pancreatic neuro-insular network in health and fatty infiltration. *Diabetologia.* 61:168–181. https://doi.org/10.1007/s00125-017-4409-x

Tang, S.-C., C.-N. Shen, P.-Y. Lin, S.-J. Peng, H.-J. Chien, Y.-H. Chou, C.E. Chamberlain, and P.J. Pasricha. 2018a. Pancreatic neuro-insular network in young mice revealed by 3D panoramic histology. *Diabetologia.* 61:158–167. https://doi.org/10.1007/s00125-017-4408-y

Taniguchi, C.M., B. Emanuelli, and C.R. Kahn. 2006. Critical nodes in signalling pathways: insights into insulin action. *Nat. Rev. Mol. Cell Biol.* 7:85–96. https://doi.org/10.1038/nrm1837

Tarasov, A.I., E.J. Griffiths, and G.A. Rutter. 2012. Regulation of ATP production by mitochondrial Ca(2+). *Cell Calcium.* 52:28–35. https://doi.org/10.1016/j.ceca.2012.03.003

Tarasov, A.I., F. Semplici, D. Li, R. Rizzuto, M.A. Ravier, P. Gilon, and G.A. Rutter. 2013. Frequency-dependent mitochondrial Ca(2+) accumulation regulates ATP synthesis in pancreatic β cells. *Pflugers Arch.* 465:543–554. https://doi.org/10.1007/s00424-012-1177-9

Thurmond, D.C., and J.E. Pessin. 2001. Molecular machinery involved in the insulin-regulated fusion of GLUT4-containing vesicles with the plasma membrane (review). *Mol. Membr. Biol.* 18:237–245. https://doi.org/10.1080/09687680110082400

Török, D., N. Patel, L. Jebailey, F.S. Thong, V.K. Randhawa, A. Klip, and A. Rudich. 2004. Insulin but not PDGF relies on actin remodeling and on VAMP2 for GLUT4 translocation in myoblasts. *J. Cell Sci.* 117:5447–5455. https://doi.org/10.1242/jcs.01421

Uhm, M., M. Bazuine, P. Zhao, S.-H. Chiang, T. Xiong, S. Karunanithi, L. Chang, and A.R. Saltiel. 2017. Phosphorylation of the exocyst protein Exo84 by TBK1 promotes insulin-stimulated GLUT4 trafficking. *Sci. Signal.* 10:5085.

van der Heide, L.P., A. Kamal, A. Artola, W.H. Gispen, and G.M.J. Ramakers. 2005. Insulin modulates hippocampal activity-dependent synaptic plasticity in a N-methyl-d-aspartate receptor and phosphatidyl-inositol-3-kinase-dependent manner. *J. Neurochem.* 94:1158–1166. https://doi.org/10.1111/j.1471-4159.2005.03269.x

Vassilopoulos, S., C. Esk, S. Hoshino, B.H. Funke, C.-Y. Chen, A.M. Plocik, W.E. Wright, R. Kucherlapati, and F.M. Brodsky. 2009. A role for the CHC22 clathrin heavy-chain isoform in human glucose metabolism. *Science.* 324:1192–1196. https://doi.org/10.1126/science.1171529

Vicent, D., J. Ilany, T. Kondo, K. Naruse, S.J. Fisher, Y.Y. Kisanuki, S. Bursell, M. Yanagisawa, G.L. King, and C.R. Kahn. 2003. The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *J. Clin. Invest.* 111:1373–1380. https://doi.org/10.1172/JCI15211

Villaseñor, R., M. Schilling, J. Sundaresan, Y. Lutz, and L. Collin. 2017. Sorting Tubules Regulate Blood-Brain Barrier Transcytosis. *Cell Reports.* 21:3256–3270. https://doi.org/10.1016/j.celrep.2017.11.055

Vincent, M.A., D. Dawson, A.D.H. Clark, J.R. Lindner, S. Rattigan, M.G. Clark, and E.J. Barrett. 2002. Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. *Diabetes.* 51:42–48. https://doi.org/10.2337/diabetes.51.1.42

Vincent, M.A., E.J. Barrett, J.R. Lindner, M.G. Clark, and S. Rattigan. 2003. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am. J. Physiol. Endocrinol. Metab.* 285:E123–E129. https://doi.org/10.1152/ajpendo.00021.2003

Wang, Z., and D.C. Thurmond. 2009. Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J. Cell Sci.* 122:893–903. https://doi.org/10.1242/jcs.034355

Wang, H., A.X. Wang, Z. Liu, and E.J. Barrett. 2008. Insulin signaling stimulates insulin transport by bovine aortic endothelial cells. *Diabetes.* 57:540–547. https://doi.org/10.2337/db07-0967

Wang, Q., R. Somwar, P.J. Bilan, Z. Liu, J. Jin, J.R. Woodgett, and A. Klip. 1999. Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* 19:4008–4018. https://doi.org/10.1128/MCB.19.6.4008

Williams, I.M., F.A. Valenzuela, S.D. Kahl, D. Ramkrishna, A.R. Mezo, J.D. Young, K.S. Wells, and D.H. Wasserman. 2018. Insulin exits skeletal muscle capillaries by fluid-phase transport. *J. Clin. Invest.* 128:699–714. https://doi.org/10.1172/JCI94053

Wisse, E. 1970. An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. *J. Ultrastruct. Res.* 31:125–150. https://doi.org/10.1016/S0022-5320(70)90150-4

Woods, S.C., R.J. Seeley, D.G. Baskin, and M.W. Schwartz. 2003. Insulin and the blood-brain barrier. *Curr. Pharm. Des.* 9:795–800. https://doi.org/10.2174/1381612033455323

Xiong, W., I. Jordens, E. Gonzalez, and T.E. McGraw. 2010. GLUT4 is sorted to vesicles whose accumulation beneath and insertion into the plasma membrane are differentially regulated by insulin and selectively affected by insulin resistance. *Mol. Biol. Cell.* 21:1375–1386. https://doi.org/10.1091/mbc.E09-08-0751

Xu, Z., and K.V. Kandror. 2002. Translocation of small preformed vesicles is responsible for the insulin activation of glucose transport in adipose cells. Evidence from the in vitro reconstitution assay. *J. Biol. Chem.* 277:47972–47975. https://doi.org/10.1074/jbc.C200486200

Xu, Y., B.R. Rubin, C.M. Orme, A. Karpikov, C. Yu, J.S. Bogan, and D.K. Toomre. 2011. Dual-mode of insulin action controls GLUT4 vesicle exocytosis. *J. Cell Biol.* 193:643–653. https://doi.org/10.1083/jcb.201008135

Yamada, E., S. Okada, T. Saito, K. Ohshima, M. Sato, T. Tsuchiya, Y. Uehara, H. Shimizu, and M. Mori. 2005. Akt2 phosphorylates Synip to regulate docking and fusion of GLUT4-containing vesicles. *J. Cell Biol.* 168:921–928. https://doi.org/10.1083/jcb.200408182

Yokono, K., R.A. Roth, and S. Baba. 1982. Identification of insulin-degrading enzyme on the surface of cultured human lymphocytes, rat hepatoma cells, and primary cultures of rat hepatocytes. *Endocrinology.* 111:1102–1108. https://doi.org/10.1210/endo-111-4-1102

Yonezawa, K., K. Yokono, K. Shii, J. Hari, S. Yaso, K. Amano, T. Sakamoto, Y. Kawase, H. Akiyama, M. Nagata, et al. 1988. Insulin-degrading enzyme is capable of degrading receptor-bound insulin. *Biochem. Biophys. Res. Commun.* 150:605–614. https://doi.org/10.1016/0006-291X(88)90436-6

Zarkovic, M., and J.-C. Henquin. 2004. Synchronization and entrainment of cytoplasmic Ca2+ oscillations in cell clusters prepared from single or multiple mouse pancreatic islets. *Am. J. Physiol. Endocrinol. Metab.* 287:E340–E347. https://doi.org/10.1152/ajpendo.00069.2004

Zeng, G., F.H. Nystrom, L.V. Ravichandran, L.N. Cong, M. Kirby, H. Mostowski, and M.J. Quon. 2000. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation.* 101:1539–1545. https://doi.org/10.1161/01.CIR.101.13.1539

Zheng, X., and G.D. Cartee. 2016. Insulin-induced Effects on the Subcellular Localization of AKT1, AKT2 and AS160 in Rat Skeletal Muscle. *Sci. Rep.* 6:39230. https://doi.org/10.1038/srep39230

Zhu, D., L. Xie, N. Karimian, T. Liang, Y. Kang, Y.-C. Huang, and H.Y. Gaisano. 2015. Munc18c mediates exocytosis of pre-docked and newcomer insulin granules underlying biphasic glucose stimulated insulin secretion in human pancreatic beta-cells. *Mol. Metab.* 4:418–426. https://doi.org/10.1016/j.molmet.2015.02.004

Zierath, J.R., L. He, A. Gumà, E. Odegoard Wahlström, A. Klip, and H. Wallberg-Henriksson. 1996. Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NID DM. *Diabetologia.* 39:1180–1189. https://doi.org/10.1007/BF02658504

Education Research

(on LB145)

Adv Physiol Educ 36: 325–335, 2012;
doi:10.1152/advan.00017.2012.

Less teaching, more learning: 10-yr study supports increasing student learning through less coverage and more inquiry

Douglas B. Luckie,[1,2] Jacob R. Aubry,[2] Benjamin J. Marengo,[2] Aaron M. Rivkin,[2] Lindsey A. Foos,[2] and Joseph J. Maleszewski[3]

[1]*Lyman Briggs College, Michigan State University, East Lansing, Michigan;* [2]*Department of Physiology, Michigan State University, East Lansing, Michigan; and* [3]*Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota*

Submitted 3 February 2012; accepted in final form 27 August 2012

Luckie DB, Aubry JR, Marengo BJ, Rivkin AM, Foos LA, Maleszewski JJ. Less teaching, more learning: 10-yr study supports increasing student learning through less coverage and more inquiry. *Adv Physiol Educ* 36: 325–335, 2012; doi:10.1152/advan.00017.2012.—In this study, we compared gains in student content learning over a 10-yr period in which the introductory biology laboratory curriculum was changed in two ways: an increase of inquiry and a reduction of content. Three laboratory formats were tested: traditional 1-wk-long cookbook laboratories, two 7-wk-long inquiry laboratories, and one 14-wk-long inquiry laboratory. As the level of inquiry increased, student learning gains on content exams trended upward even while traditional content coverage taught decreased. In a quantitative assessment of content knowledge, students who participated in the 14-wk-long inquiry laboratory format outscored their peers in both 7- and 1-wk-long lab formats on Medical College Admissions Test exam questions (scores of 64.73%, 61.97%, and 53.48%, respectively, $P < 0.01$). In a qualitative study of student opinions, surveys conducted at the end of semesters where traditional 1-wk laboratories ($n = 167$ students) were used had low response rates and predominately negative opinions (only 20% of responses were positive), whereas those who participated in 7-wk ($n = 543$) or 14-wk ($n = 308$) inquiry laboratories had high response rates and 71% and 96% positive reviews, respectively. In an assessment of traditional content coverage in courses, three indexes were averaged to calculate traditional forms of coverage and showed a decrease by 44% over the study period. We believe that the quantitative and qualitative data support greater student-driven inquiry in the classroom laboratory, which leads to deeper learning in fewer topic areas (less teaching) and can reap gains in scientific thinking and fundamental understanding applicable to a broader range of topic areas (more learning) in introductory biology.

laboratory; inquiry; cooperative; undergraduate; research

EACH YEAR, the field of biology grows with new developments in knowledge and skills that require increased mastery of topics by our students. While we, the faculty, are concerned that the increased number of topics taught in lecture and laboratory courses might not lead to increased learning, we hope that if we speak clearly and energetically enough, perhaps it will. We often consider our best laboratories to be those with detailed protocols, which have been refined over the years to produce experiments that work in the hands of students, yet these are also "cookbook" in nature (10, 18). Unfortunately, this evolution to increased content coverage and more structured experiments for majors in the course lecture and laboratory does not produce learning gains to match our aspirations (11, 18, 21). Nor does this inspire creativity, flexibility, and inquisitiveness in our students or help them develop deeper critical and integrative thinking skills (7, 8, 22, 23, 27).

In the late 1990s, our department's approach to teaching introductory biology laboratory and lecture courses was predominantly that of a traditional format, with many weekly cookbook laboratories strung together, each focused on a different biological topic. Just as lecture topics jumped from one chapter to the next, so did topics in the laboratory. For example, the week that the topic of photosynthesis was covered in lecture, we would also have photosynthesis "experiments" in the laboratory. In the past, this approach was considered the most efficient for increasing student gains because it enabled teachers to reinforce material presented in lecture. However, student feedback and research data have suggested that these traditional laboratories provide little gain in student learning (18, 23).

In the late 1990s, our faculty members revisited the learning goals of our curriculum and came to an agreement that in the laboratory portion of a course we wanted our students to learn *1*) more about the topic studied, *2*) the techniques used, and *3*) the process of research. Past and current evidence have suggested that the majority of our students learned little of the above when performing cookbook laboratories (7, 11, 27). Upon review of our assessments as evaluated by Bloom's taxonomy, we also found that our laboratory assignments did not require higher-level or critical thought and thus needed revision (19). The literature suggests that our experience is not unique. When reviewing traditional undergraduate biology courses, Momsen et al. (21) found that of 9,713 assessments as evaluated by Bloom's taxonomy, 93% leveled 1 or 2 (knowledge and comprehension) and <1% were a 4 or above on Bloom.

Our review of the education literature and consultations with experts as well as negative student comments on course evaluations catalyzed a formal curricular reform and research effort. We redesigned introductory biology courses to increase inquiry as well as instituted standardized assessments to collect data regarding student opinions and academic performance (17, 19, 26, 28). While our previous publication in 2004 simply compared traditional structured/cookbook laboratories to "teams and streams" inquiry laboratories, in this report we extended those studies to compare gains in student performance in three different laboratory formats: traditional 1-wk-long confirmatory laboratories, two 7-wk-long inquiry laboratories, and one 14-wk-long inquiry laboratory. A full decade of data now supports that the learning gains found in 2004 were sustained and trend upward as emphasis in the laboratories

---

Address for reprint requests and other correspondence: D. B. Luckie, Dept. of Physiology, Michigan State Univ., 2100 Biomedical and Physical Sciences Bldg., East Lansing, MI 48824-3320 (e-mail: luckie@msu.edu).

shifted from traditional content coverage to inquiry-driven laboratory formats.

METHODS

In the last decade, the laboratory format we used to teach introductory biology to undergraduate physiology majors has changed dramatically. It evolved from 3-h-long structured cookbook laboratories where students were expected to work individually to multiweek-long inquiry laboratories where research is done by students in groups in a format we defined as "teams and streams" laboratories. In these teams and streams group inquiry laboratories, student teams pose a scientific question, propose an experimental design, and perform multiweek investigations and, along the way, present their research via posters, interviews, papers, and talks. What we refer to as the "two-stream" format uses two separate 7-wk-long inquiry experiments each focusing on a different biological topic (e.g., *stream I*: DNA fingerprinting with PCR and gels, and *stream II*: cellular responses to the environment with cell culture and drugs). In the "one-stream" laboratory, student research teams spend the full 14 wk on a single research topic (e.g., cell and molecular biology stream: develop a PCR-based diagnostic assay to detect a mutation known to cause a genetic disorder/disease).

Population and sample. Lyman Briggs College of Science is an undergraduate science program established at Michigan State University (MSU) in 1967. It is a residential college modeled after those at Oxford University that creates a learning community focused on educating undergraduates in a liberal science curriculum. Overall, its goal is to establish a solid foundation in the sciences as well as a significant liberal education in the history, philosophy, and sociology of science.

The Introduction to Cell and Molecular Biology course is a five-credit freshmen-level course. It is the second in a core two-semester introductory biology sequence for science majors (and the last taken before a two-semester physiology sequence). It is taught in sections of ~100 students with accompanying recitation and laboratory sections led by the professor or teaching assistants. Students attend two lectures and one recitation (50 min each) and two laboratory sections (3 h each) per week.

Chronology of curricular reform. While in the late 1990s we made changes to the lecture and recitation portions of the course, from the year 2000 forward we focused on the laboratory and started revising our traditional cookbook laboratory sequence. Early efforts just altered the sequence of cookbook laboratories. We rearranged three cookbook laboratories in a natural multiweek sequence (or stream), specifically *1*) DNA transformation of bacteria, *2*) miniprep purification of plasmid DNA, and *3*) restriction analysis and gel electrophoresis, where the same "unknown" plasmid DNA sample was tested by each pair of students over several weeks in an effort to determine the identity of the plasmid (28). This format, as described in detail in our report in 2002, was still dominated by traditional cookbook laboratories and low-level assessments but was the initial small change that sparked the larger reform to follow. It is the traditional format shown in Table 1 and in our data set is represented by two semesters, spring 2000 and fall 2001.

In 2001, we more dramatically renovated the laboratory curriculum by purposefully introducing two elements: authentic inquiry and formal group work. The details of all the changes done to instruction and assessments in this phase is described in our 2004 publication (19) and the corresponding website (http://surf.to/teamstreams/). The two-stream model used during spring 2001–spring 2007 contained two 7-wk-long laboratory sequences each semester. The first stream typically consisted of macromolecule, enzyme, and photosynthesis experiments, and the second stream dealt with techniques of molecular biology (Table 1). In this new format, the majority of time was focused on mentoring independent inquiry research by student teams of four students. Each of the three experimental topics (e.g., macromolecules, enzymes, and photosynthesis) was initiated with 1 wk for a cookbook laboratory designed to help students to learn techniques followed by 1 wk for independent investigations, where students then applied those techniques to the study of the their chosen research

Table 1. *Curricular organization and timeline: cookbook versus inquiry laboratories in the tradition, two-stream, and one-stream laboratory formats*

Week	Traditional Curriculum	Two-Stream Curriculum	One-Stream Curriculum
1	No laboratory	Introduction to Teams and Streams	Introduction to Teams and Streams and Project Goals
2	Lab 1: Cell Structure Microscope Analysis*	Lab 1: Carbohydrate Chemistry and Lipid Tests*	Introduction to Basic Lab Skills; Students Propose Projects
3	Lab 2: Plant Tissue Culture: Start Hormones Study*	Lab 1 Inquiry: Apply Lab 1 Tests to Your Question/Investigation	Lab 1: PCR of the *Esherichia coli* Genome*
4	Lab 3: Carbohydrate Chemistry and Lipid Tests*	Lab 2: Photosynthesis I: the Light Reactions*	Lab 1 (cont.): Repeat Attempts Until Successful
5	Lab 4: Photosynthesis I: the Light Reactions*	Lab 2 Inquiry: Apply Lab 2 Tests to Your Question/Investigation	Lab 2: Genome Preparation From Cells*
6	Lab 5: Photosynthesis II: the Dark Reactions*	Lab 3: Enzymes (and Proteins): Kinetics Study*	Lab 2 (cont.): Repeat Attempts Until Successful
7	Lab 6: Enzymes (and Substrates): Kinetics Study*	Lab 3 Inquiry: Apply Lab 3 Tests to Your Question/Investigation	Lab 3: Design an Ethical/Historical/Social Experiment
8	Enzymes (cont.) and Finish Study*	Debriefing Stream I and Introduction to Stream II	Independent Investigations
9	Lab 7: Bacterial Transformation*	Lab 1: Bacterial Transformation*	Independent Investigations
10	Bacterial Transformation (cont.)	Bacterial Transformation (cont.)	Independent Investigations
11	Lab 8: DNA Purification*	Lab 2: DNA Purification*	Independent Investigations
12	DNA Purification (cont.)	DNA Purification (cont.)	Independent Investigations
13	Lab 9: Restriction of DNA*	Lab 3: Restriction of DNA*	Investigations and In-Lab Final Practice Presentations
14	Restriction of DNA (cont.)	Restriction of DNA (cont.)	Final Paper and In-Lecture Final Formal Presentations
15	No laboratory	Wrap up final experiments	Briggs Symposium (present research to public)

*Cookbook laboratories.

question. Later, these revisions were applied throughout the biology curriculum in Lyman Briggs College of MSU. The methods used by two faculty members who converted cookbook laboratories to teams and streams inquiry laboratories for another course, Introduction to Organismal Biology, have been described in a recent publication (26).

During semesters from fall 2007 to spring 2011, we implemented a new one-stream inquiry format, allowing student groups to focus on one project for the full 14 wk of a semester. It was designed to naturally allow more time for student teams to repeat and revise experiments and gain experience at troubleshooting. In this one-stream format, only two of the laboratory weeks remained cookbook and were used to teach the students laboratory protocols and techniques such as PCR, gel electrophoresis, and genome purification. An example of this one-stream semester-long laboratory sequence is described in Table 1. After the two introductory cookbook laboratories used to teach techniques, students were given more freedom and were able to choose their experiment's direction with careful monitoring. This one-stream format also allowed students more time to read scientific publications related to their research topic and apply their findings to their own research. For example, students who pursued PCR projects had more time to read research papers on PCR and then design and redesign their experiments to include better controls, replications, and adjustments to variables (reagent concentrations, temperature, etc). Instructors also had more opportunities to challenge students to spend time troubleshooting and problem solving, e.g., careful analysis of their gel results with peers in their research group. Teaching assistants were trained to respond to student questions with answers in the form of guiding questions and always direct the individual student to return and confer with their group to seek answers, rather than literally telling students what to correct.

Revisions in assessment. Changes in the way that the students were taught in the reformed laboratory course (in both two-stream and one-stream formats) were accompanied by changes in the way that student learning was assessed. New assessments, such as interviews, concept mapping, and peer reviews, were introduced in an attempt to evaluate student learning at higher cognitive levels than those previously used. Quizzes and exams were modified to include more short-answer responses rather than just multiple-choice responses. Evaluation of a student's laboratory research results as well as their content understanding had always been dominated by writing, yet how we implemented writing assignments changed from requiring each student to author many short laboratory reports to expecting a group to generate many drafts of a single research manuscript. These changes were described in detail in our 2004 publication (19) and the corresponding website (http://surf.to/teamstreams).

Collection of data on student performance and opinion. Two sources were used as primary data sets for analysis of student opinion and performance during the experiment. Content exams with questions derived verbatim from Medical College Admissions Test (MCAT) practice tests were used as a standardized posttest each semester. This served as a comparative measure of student learning. Student feedback via extended written responses on end-of-semester course evaluation forms was used to evaluate student opinions and assess affective or qualitative elements in response to different laboratory formats.

Quantitative data. We developed and administered a small, standardized exam named the medical assessment test (MAT) as a posttest during the final week of all semesters. Our MAT exam was built from a subset of unaltered MCAT practice test questions developed, validated, and purchased from the Association of American Medical Colleges. This MCAT-style exam was a 40-question multiple-choice test composed of relevant and rich passage-style questions (31). The MAT exam instrument consisted of questions from five general categories: cell structure and function, oncogenes/cancer, respiration, microbiology, and DNA structure and function. Student performance on the MAT exam was tracked and compared with the laboratory format used each semester. The MAT instrument has been used since the year 2000 and can be found online (http://surf.to/teamstreams/).

To attempt to calibrate variation that would naturally occur semester to semester due to shifts in student cohort academic ability, student ACT science scores were used to normalize MAT data. The average ACT score for all students in each course was used to calibrate MAT comparisons between semesters. Normalized MAT scores were calculated for fall 2000 to fall 2011 semesters to generate the data shown in Fig. 1. Scores were normalized by setting the highest course average ACT score to 100% and converting the remaining course average ACT scores to appropriate relative percentages. These percentages were then used to determine the appropriate multipliers needed to adjust each semester's MAT score proportionally to normalize all results.

In addition, since every MAT exam question was carefully coded for topic, we were able to compare what percentage of questions on the exam were covered in any formal way during any part of the course each semester. While the MAT exam itself remained unchanged, the percentage of questions covered by traditional teaching methods from course to course decreased greatly during the 10-yr study.

Qualitative data. The course evaluation form used in this study was the Student Instruction Rating Survey (SIRS) form commonly used at MSU. Rather than interviewing all students in the study, we focused our attention on the comments section on each student's SIRS form. The back of each form is entirely for free response feedback. To interpret the student feedback on these forms, each individual's written opinions were examined and coded depending on the nature of the comments. Individual quotes that were noticeably frequent or typical among students in the class each semester are shown in Table 2. In addition, we studied the frequency of positive versus negative feedback on the topics of the course laboratory (laboratory) or course lecture (class) as well as for each of coded category (groups, laboratory skills, mental skills, and inquiry) from student surveys over the semesters studied (see Tables S3 and S4 in the Supplemental Material).[1]

Analysis of content coverage. To evaluate the changes in traditional coverage that occurred over the 10-yr period, past syllabi were analyzed regarding topics covered in the laboratories and lectures from spring 2000 to fall 2011. We quantitated content coverage and compared that with performance scores over the decade. We quantitated topics covered in laboratories by counting the number of weeks committed purely to cookbook laboratories (Table 1, laboratories marked by asterisks). To quantitate "pages covered," the pages that were required readings of the textbook for the course were counted for each semester. Finally, and perhaps most interestingly, while the MAT exam was originally created based on what was covered in the course back in the late 1990s, in 2011 we no longer have formal instruction on a number of the topics still tested on the MAT. We therefore determined the number of exam questions that were on topics covered in class lecture for each semester. As a result, we could document "MAT coverage," i.e., the percentage of the MAT exam that the instructor covered each semester, and compare that over time. We then could also calculate student performance on MAT questions that were or were not addressed in the course.

Data analysis. Significant differences between group means of measured variables were determined using general linear model ANOVAs and *t*-tests. Post hoc comparisons were done using Tukey's honestly significant difference test. For all statistical tests, differences were considered statistically significant at P values of ≤ 0.05. Data are presented as arithmetic means ± SE.

Institutional human subjects review. With the approval of the Institutional Review Board of MSU (nos. X00-475 and 10-543),

---

[1] Supplemental Material for this article is available at the *Advances in Physiology Education* website.

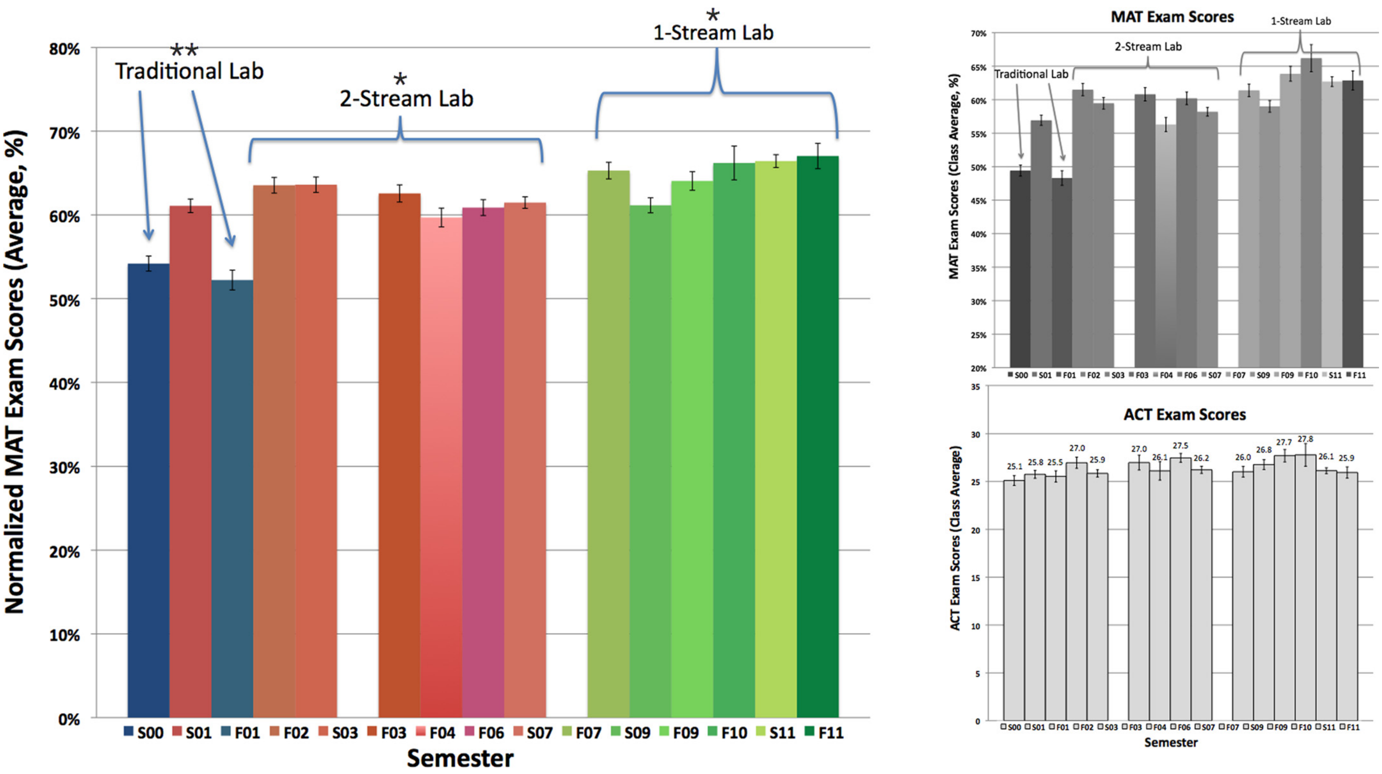


Fig. 1. Normalized medical assessment test (MAT) student score results from 15 semesters. Moving chronologically *left* to *right* is a comparison of the normalized class average score on MAT exams from semesters from 2000 to the present. Course instructors varied in the semesters shown, and these results are from semesters in which the MAT instrument was used as a summative evaluation in the last week of classes. The "teams and streams" (TS) inquiry laboratories were first introduced in spring (S)2001, and in fall (F)2001, the instructor then reverted back to traditional cookbook laboratories. TS laboratories were used again in fall 2002 and continued to be used until the present. Blue indicates a traditional cookbook laboratory, red indicates a two-stream version of the TS laboratories format used until fall 2007, and green indicates a one-stream version of the TS lab format used ever since. *Top inset*: for comparison, MAT scores in all semesters are shown before normalization. *Bottom inset*: class average scores on the ACT for the semesters studied are represented and were used to normalize MAT scores. Error bars are SEs. Student scores from both TS laboratories formats were found to be statistically significant from students who participated in the traditional cookbook laboratory format (**$P < 0.0001$ by ANOVA). Scores of students who participated in the two-stream format were also significantly different from those in the one-stream laboratory format (*$P < 0.01$ by ANOVA).

student data were collected from the 1,018 students who completed our Introduction to Cell and Molecular Biology course at MSU from 2000 to 2011. All students were enrolled at MSU; the research program was described to all participants, and participant consent was obtained.

RESULTS

In 2004, we reported results from 4 yr of data collection that simply compared two curriculums: traditional cookbook laboratories versus a form of inquiry laboratory we termed "teams and streams." We have now revisited the original data set and added all the years since that publication to create an even more robust longitudinal study.

Curricular change. Before 2000, we implemented only traditional cookbook approaches in our weekly course laboratories. A reform of both curriculum and assessments generated the nontraditional laboratory formats tested, two versions of teams and streams inquiry laboratories. The reformed inquiry formats implemented in the laboratory raised the time committed for inquiry over the semester from what was 20% in 2000 to >60% today (Table 1).

Quantitative results. We administered a standardized content exam, the MAT, during the final week of semesters from the years of 2000 to 2011. Our MAT exam was built from MCAT practice test questions developed, validated, and purchased from the Association of American Medical Colleges. The normalized MAT scores from each semester indicated that students who participated in the inquiry formats of the course laboratory made significant gains in learning. One-stream inquiry laboratory semester score averages (64.73%) were found to be significantly higher than two-stream inquiry laboratory scores (61.97%, $P < 0.01$), and students in both scored significantly higher than those in the traditional course laboratory (53.48%, $P < 0.0001$; Fig. 1). Raw MAT scores for all semesters were normalized for variations in each cohort's prior academic performance using ACT scores (Fig. 1, *insets*).

In addition, when the student performance data were pooled into three bins based on the laboratory format used, a stepwise movement of the distribution became apparent (Fig. 2). The mode for performance of students who participated in the full semester-long one-stream laboratory was 70%, whereas the two-stream laboratory format had a mode of 65% and the cookbook laboratory format mode was much lower, at 45%. Not surprisingly, higher levels of content mastery, for example, scores of 75% and 80%, were more frequent among students who participated in one-stream laboratories than the other two formats (Fig. 2).

We examined student performance on each subtopic area evaluated on the MAT instrument. The data showed a positive

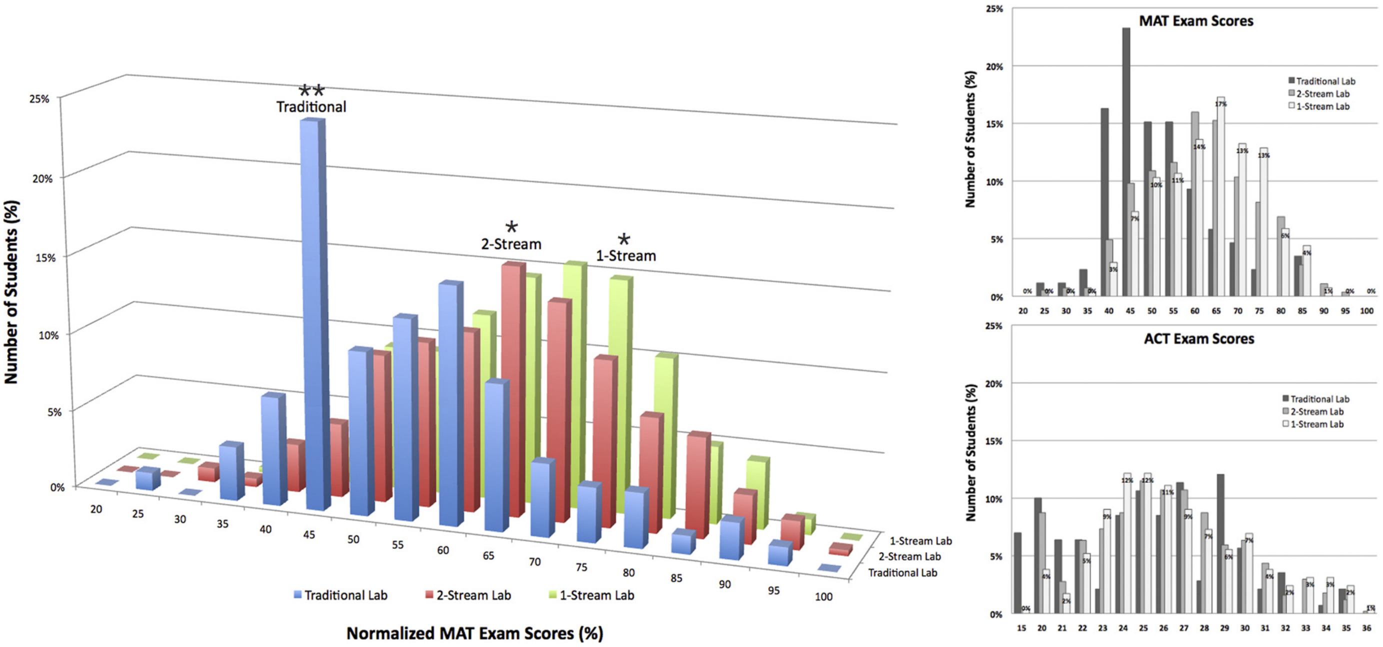


Fig. 2. Students who took courses that offered inquiry laboratories scored higher on the MAT exam than their peers with traditional cookbook laboratory experiences, and students who participated in the recent one-stream version of inquiry laboratories did best. Normalized MAT performance distribution compared students who participated in two semesters that offered traditional cookbook laboratories ($n = 86$) versus two-stream inquiry laboratories between 2001 and 2006 ($n = 551$) and one-stream inquiry laboratories ($n = 306$) between 2007 and 2011. Student scores are represented by sorting MAT scores into bins ranging from 20.01% to 25.0% (labeled 25), 25.01% to 30.0% (labeled 30), and so on. No students scored below 20.01% or above 95.0%. Students in courses with traditional laboratories are represented by blue. Students in courses with a two-stream version of the inquiry laboratory are represented by red. Students in courses with a one-stream inquiry laboratory are represented by green. *Top inset*: for comparison, the distribution of MAT scores in all semesters is shown before normalization. *Bottom inset*: ACT performance distribution comparing students in the three treatment groups. ACT scores for the semesters studied were used to normalize all students' MAT scores. Student scores from both TS laboratory formats were found to be statistically significant from students who participated in the traditional cookbook laboratory format (**$P < 0.0001$ by ANOVA). Scores of students who participated in the two-stream format were also significantly different from those in the one-stream format (*$P < 0.01$ by ANOVA).

trend for normalized scores in all five subtopic categories on the MAT (Fig. 3). Slopes for the associated linear trend lines for MAT subtopics of cell structure and function, cancer, respiration, microbiology, and DNA were +.014, +.0103, +.0037, +.0039, and +.0039, respectively, and were not significantly different from each other. The 95% confidence intervals shown in Fig. 3 demonstrate that students who participated in inquiry laboratories performed far better than their peers on topics of cell structure and function, cancer, and, in some semesters, respiration.

We also looked for trends in the data that might literally support what in 2004 we put forward as our philosophy of "less teaching, more learning." Student normalized performance on the MCAT-based content exam increased from a 54% average

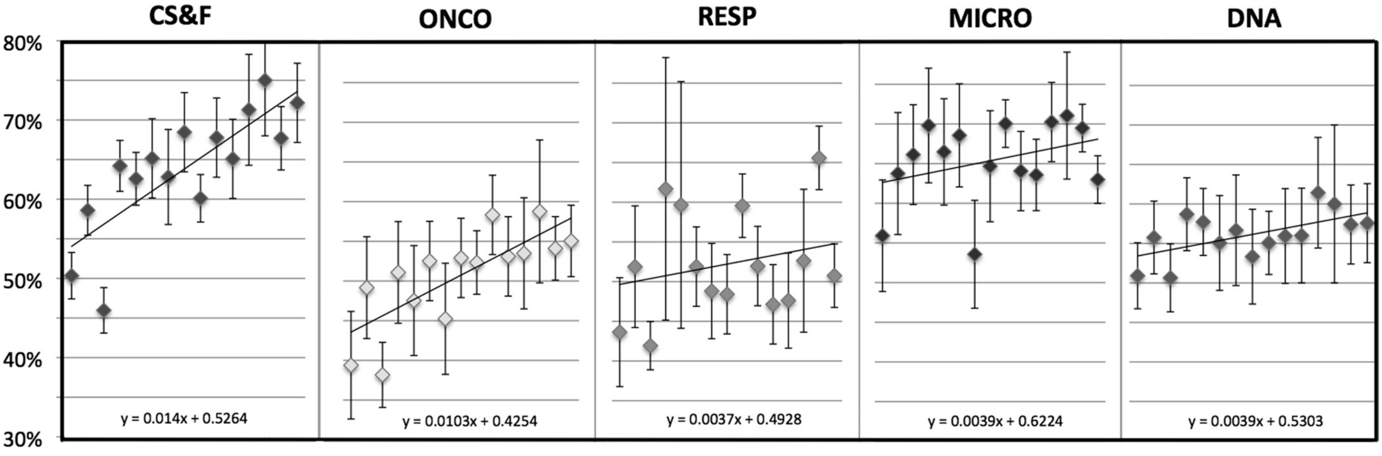


Fig. 3. Student performance on separate topics of the MAT exam. Semester averages and associated trend lines on MAT subtopics are from 15 semesters from 2000 to 2011. The y-axis of the average score for each category (in %) is the same scale for all graphs. The x-axis is time (in semesters; not shown). The 40 questions on the MAT exam were divided into 5 categories based on the topic tested: cell structure and function (CS&F; $n = 16$ questions), oncogenes and cancer (ONCO; $n = 6$ questions), respiration (RESP; $n = 4$ questions), microbiology (MICRO; $n = 4$ questions), and DNA structure and function (DNA; $n = 10$ questions). Error bars represent 95% confidence intervals for each point. A linear trendline was plotted for each set of points, and the resulting equation is shown.

in 2000 to 67% in 2011, yet at the same time several traditional indicators of content coverage have clearly decreased. We quantitated content coverage and compared that graphically with the performance scores over the decade (Fig. 4). Until 2000, the classroom laboratory was composed of 100% traditional cookbook laboratory experiments, but since that time the amount of traditional cookbook laboratory content has continually decreased to the present, relative, 33%. Additionally, the number of pages covered in the textbook for lecture also decreased over the semesters from what was set to be 100% coverage in 2000 (281 pages) to 73.63% coverage in 2011 (241 pages). Finally, and perhaps most interestingly, while the MAT exam was originally created in the late 1990s based on what was covered in the course, by 2011 we no longer had formal instruction on a number of the topics still tested on the MAT. In a careful examination of each test question, we found that the number of exam questions that were on topics covered in class decreased over the semesters, from 87.5% (35 questions covered) to 60% (24 questions covered). While this varies somewhat from instructor to instructor, we rarely cover the topics of viruses, oncogenes, or some organic chemistry and microbiology that we once did. Overall, it seems that there has been a great decrease (44% when averaged over three indexes) in traditional coverage (Fig. 4).

Since we gave the same test to every cohort, the data suggest that students are doing as well (or better) on an exam for which they receive less traditional preparation (Fig. 5). Surprisingly, student performance even rose on questions in topic areas not covered in any traditional way in the course (Fig. 5, *inset*). Therefore, while coverage decreased by as much as 44%, the normalized MAT scores increased by 13%, suggesting that less teaching, or perhaps more accurately less time dedicated to instructor talking, can lead to more learning.

Qualitative survey results. To assess student feedback about the classroom and laboratory, MSU SIRS forms were examined during all semesters studied from spring 2000 to spring 2011. Throughout these semesters, the classroom lecture and recitation have remained reasonably fixed in format, whereas laboratory experiences have changed significantly from traditional laboratories to those implementing inquiry approaches, and so have the responses on the SIRS reports.

Student feedback and comments were sorted/coded using a number of categories. Examples of frequent student comments in categories of laboratory skills (writing), laboratory skills (technical), mental skills, affective, and groups are shown in Table 2. Broadly, feedback from the one- or two-stream laboratories suggested that the students often felt that the workload was higher than that of traditional laboratories; however, students also commented on the great knowledge gains they believed they had made from the inquiry laboratory experience.

In addition to the comments describing the students' experience in the class, we also studied the frequency of positive comments and negative comments related to the general topic areas of "lecture" and "laboratory" and the coded categories described above (Fig. 6). This was done on a semester basis and sorted according to whether the students experienced a semester of traditional cookbook laboratories, a two-stream inquiry laboratory, or a one-stream inquiry laboratory (see Table S3 in the Supplemental Material). Very few comments were given for the traditional laboratory format (<1% response rate), and only 20% of students gave positive responses (n =

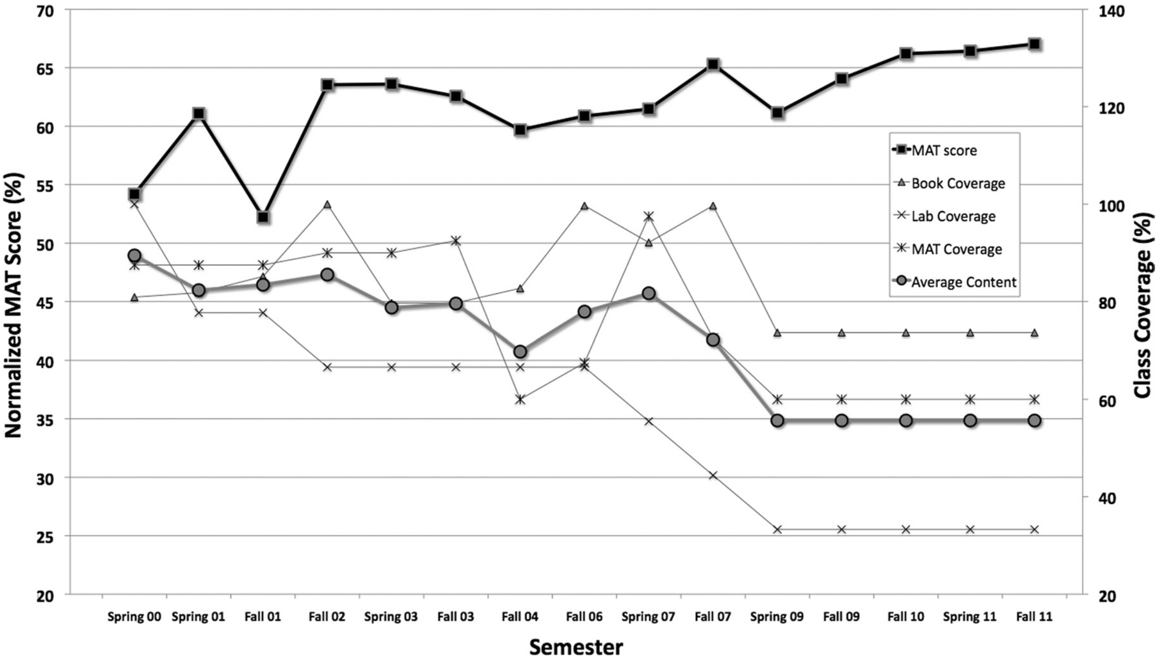


Fig. 4. Comparison of student performance on the MAT (exam scores) with the amount of material "covered" in course. The black line (with squares) shows the change in normalized MAT exam scores over the study period (2000–2011) with the y-axis on the *left*. The y-axis on the *right* is used for the percentage of coverage. The thin gray line (with triangles) depicts the change in the required reading of pages covered in the course textbook; the highest number was set to be 100%. The thin gray line (with gray ×) represents the change in laboratory coverage over time. The thin gray line (with gray *) corresponds to the percentage of MAT questions that were on topics covered in the course. The broad gray line (with circles) represents the content average of laboratory, textbook, and MAT coverage by semester. While the amount of material covered decreased, student performance did not follow; in fact, an upward trend in MAT scores occurred.

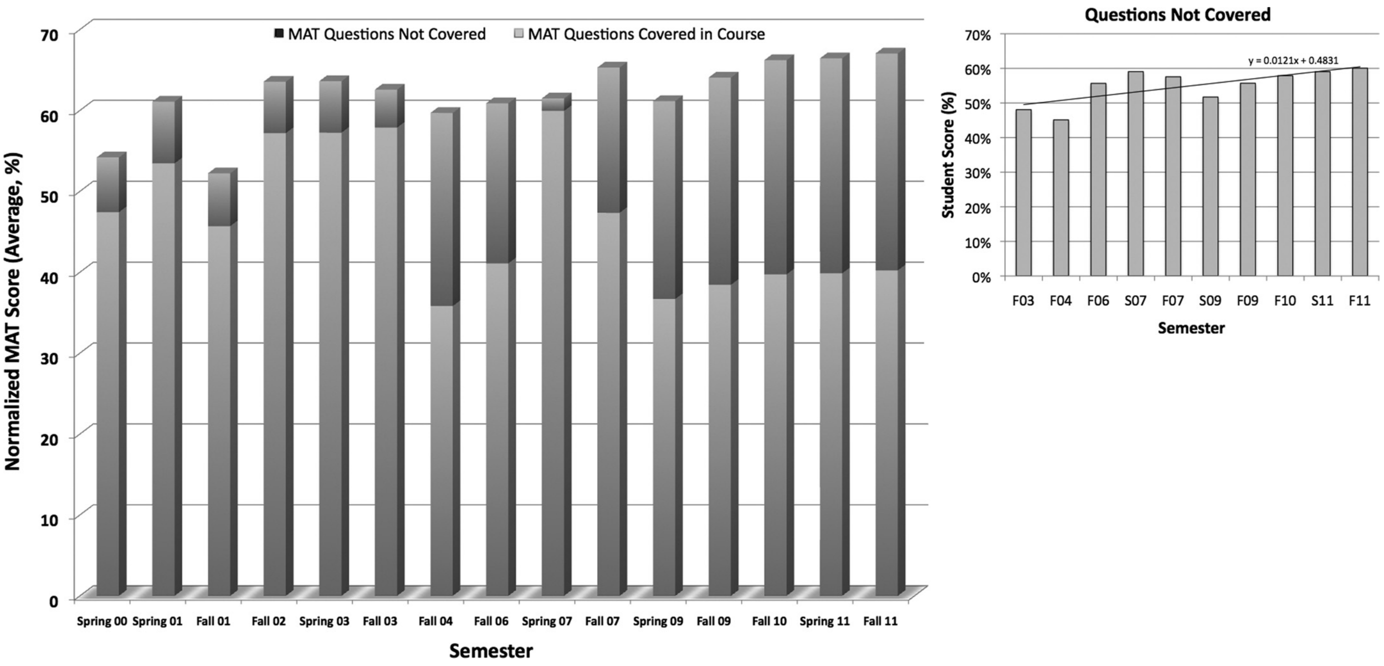


Fig. 5. MAT exam scores trended upward each semester, yet fewer questions on the exam were "covered" in class. Each semester's normalized MAT exam score is represented with the proportion of the exam questions that were covered (*bottom*, light gray) by explicit instruction in the course that semester. Over the years, the number of questions on the MAT exam not covered (*top*; dark gray) has grown. *Inset*: scores achieved by students on MAT questions not covered in recent semesters also trended upward.

70 comments; Fig. 6). In comparison, we found that feedback from all semesters using the two-stream inquiry approach was more plentiful (50% response rate) and averaged 71% positive responses ($n = 473$). Additionally, once the one-stream approach to laboratory was implemented, the response rate increased further, and the approval rating rose to 96% in the course evaluation reports ($n = 304$, 86% response rate). Student feedback regarding lecture or "class" remained generally

Table 2. *Examples of evidence collected from student response forms from fall 2003 to spring 2011*

Category	Learning Outcomes Reported	Semester
Laboratory skills (writing)	• "Also working in a group on the papers helped me to see areas in my writing that could use improvement."	Fall 2003
	• "The papers, as much of a pain as they were–helped me master the material because they forcer me to think a step further than basic lab protocols."	Fall 2003
	• "I feel that my scientific writing has improved due to the many manuscripts and drafts we had to write for lab. I walk out of here feeling accomplished and like I learned something."	Fall 2011
Laboratory skills (technical)	• "I actually learned so much about my disease, primer design, gel electrophoresis and troubleshooting. My confidence in performing these tasks has really gone up."	Spring 2011
	• "Since we were not doing cookbook labs every week it gave me more time to develop and really learn the skills that will be valuable in for any future lab position."	Fall 2009
	• "Because of the lab I now feel extremely confident conducting an experiment, using nearly all lab tools, and being able to analyze my results in a way that will allow me to continue with the experiment and build on it."	Fall 2010
Mental skills	• "I have learned more from this lab than any other lab because of the freedom of experimental design."	Spring 2007
	• "Feel confident I could leave this lab and lab work and do the things I did here somewhere else."	Fall 2010
	• "I know I made great gains in my precision and attention to detail as a scientist in the laboratory."	Spring 2011
	• "[Stream labs] Increased learning a lot–forced me to know details and use conceptual thinking/troubleshooting."	Fall 2006
Affective responses (positive)	• "I really enjoyed this class. My favorite class yet!"	Fall 2006
	• "Its (sic) cool to do your own research and come up with your own topics and experiments."	Fall 2007
	• "[Stream labs] Make it so much interesting to learn, and I learned so much!"	Spring 2009
	• "The lab not only increased my confidence in my knowledge of biology and lab techniques, it also made me feel like a scientist."	Fall 2010
	• "This lab was not your typical cookbook lab, which was a breath of fresh air."	Spring 2011
Groups	• "The groups are great! Since I was already part of a group in class, it was easy to find a study group or to find someone to ask questions."	Fall 2003
	• "I like having the group experience far more than just the lab, but also in the class and outside of the class."	Fall 2007
	• "Working in groups made a huge difference in my understanding of the material."	Fall 2010

Categories were arranged based on gains that reported by the students. Categories such as laboratory skills, mental skills, affective responses (positive or negative), and groups are addressed further in Tables S3 and S4 (available in the Supplemental Material).

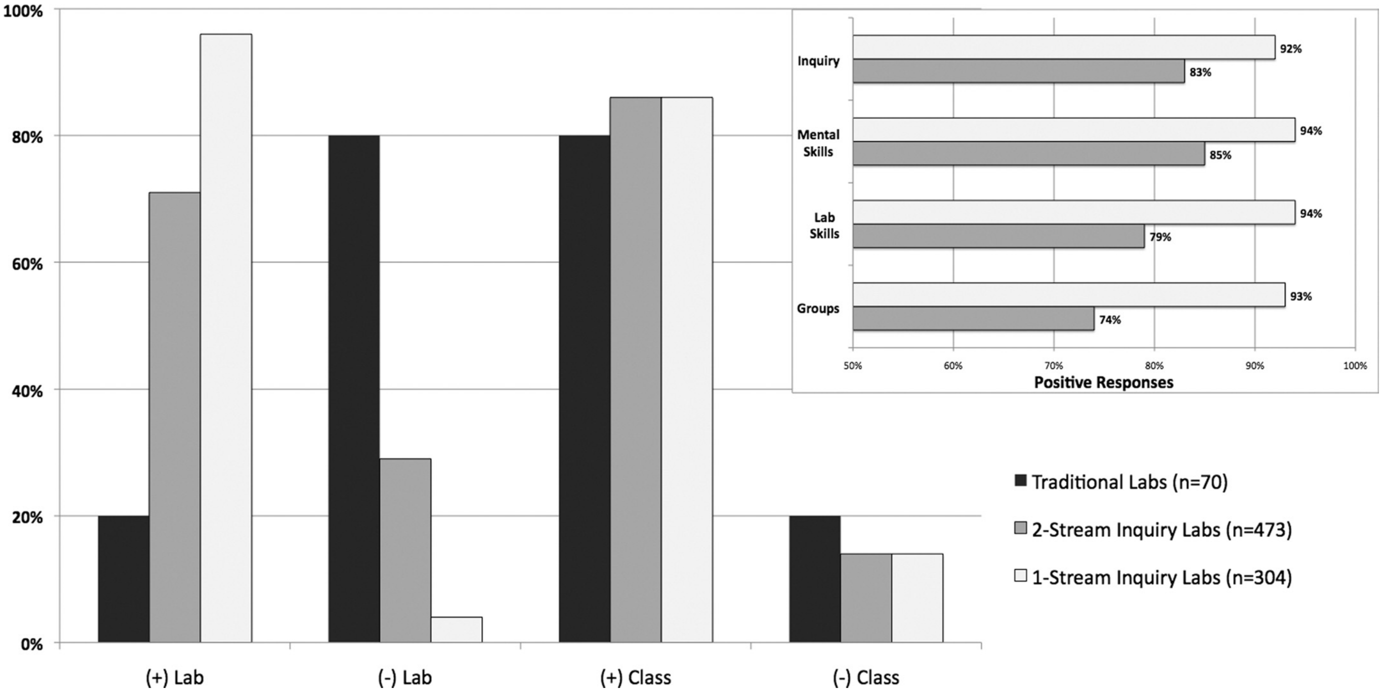


Fig. 6. Student feedback about their laboratory and class experiences from course evaluation forms. Data from student feedback surveys (Michigan State University Student Instruction Rating Survey and Student Assessment of their Learning Gains forms) show the frequency of positive (+) versus negative (−) feedback on the topic of the "lab" or "class" over the semesters studied using the traditional structured laboratory versus those semesters using two versions of the TS inquiry laboratories. Feedback on each topic area was coded and summed (traditional laboratories: $n = 70$ comments, two-stream inquiry laboratories: $n = 473$, and one-stream inquiry laboratories: $n = 304$). *Inset*: feedback on several subtopics associated with the laboratory over semesters using two different formats of the TS inquiry laboratories. Feedback was coded and summed for topics related to working in "groups" ($n = 97$), gain of "lab skills" (physical, $n = 194$), gain of "mental skills" ($n = 307$), and use of "inquiry" ($n = 195$). The numbers of student comments (n) do not equal the numbers of students enrolled in each course.

positive and unchanged throughout the time period studied (80%, 84%, and 86%, respectively; Fig. 6). We also compared student feedback for semesters that used two-stream laboratories versus those that used a one-stream format (see Table S4, in the Supplemental Materials). One-stream laboratories had 92% positive feedback for inquiry, 94% for mental skills, 94% for laboratory skills, and 93% for group work compared with 83%, 85%, 79%, and 74%, respectively, for the two-stream approach (Fig. 6, *inset*).

DISCUSSION

In previous years, we reported increased learning in student cohorts as we transitioned from traditional cookbook laboratory sequences to multiweek group inquiry investigations in our freshmen-level introductory biology classroom (19, 28). In recent years, we have implemented a full semester-long research experience in the hope that it would deepen the inquiry and raise the learning gains with more time spent on scientific writing, oral presentations, analysis of laboratory results, and troubleshooting during the same project. In this study, we compared student opinions and academic performance over a 10-yr period in which the science course laboratory curriculum changed dramatically. The three formats compared were as follows: traditional weeklong cookbook laboratories, two 7-wk-long inquiry laboratories, and one 14-wk-long inquiry laboratory. As expectations of the level of inquiry were raised in the classroom and far more time was dedicated to each project, a decade of data supports that learning gains on content exams trended upward even while the amount of traditional content coverage taught moved downward.

Students make significant learning gains when participating in inquiry laboratories. To gauge students' learning of content, through the decade study period we examined student performance on a 40-question MAT exam built with MCAT test questions. We found that students engaged in the one-stream inquiry laboratory scored the highest on the end-of-semester MAT exam. These students significantly outperformed those in the two-stream inquiry laboratories and those who participated in traditional cookbook-style laboratories (MAT raw scores: one-stream format, 62.6%; two-stream format, 59.3%; and traditional format, 48.9%). When we used students' prior performance on the ACT exam to normalize the MAT scores from each semester, the statistical significance of the increasing trend seen with raw performance scores was maintained (MAT normalized scores: 64.73%, 61.97%, and 53.48%, respectively). The data support our belief that students in the inquiry laboratories had the greatest gains in the understanding of biology compared with those in cookbook experiences and that the shift to longer full-semester inquiry may lead to the greatest learning.

Another aspect of this study involved documenting changes in the traditional content provided during the course. From 2000 to 2011, the amount of overall class coverage declined by ~44%, whereas the averages on MAT exams increased by 13% over the same period. Even when we evaluated test questions on topics not covered in any traditional way in the course, student performance still increased. We believe that these data may help lessen a concern of our colleagues that they should at least "touch on" certain topics. Our data suggest that a more efficient use of time is mastering fewer topics deeply while fostering the development of critical thinking skills that enable the student to apply known information (with greater confidence) to new topics. We believe that these skills of critical thinking and transference are necessary for becoming a productive scientist as well as being useful in making informed life decisions.

Students appreciate the change to inquiry experiences. Student opinion was a motivator of change for our faculty. In the late 1990s, students' opinions about their laboratory experience were less than enthusiastic. Students asked biology faculty advisors whether they should leave the Physiology major because "I don't think I could do that stuff, like in intro bio lab, for my career." Comments on course evaluations were frequently just "labs are boring and time consuming." During interviews, students told us that it wasn't how they imagined science would be. Upon reflection, we agreed that "real science" was very different than the way we were teaching it. This led to the decade of changes that were evaluated and presented in this study. Looking backward in time, through the analysis of course evaluation forms, noticeable differences were observed between students who participated in each format of laboratory curricula. When examining the positive comments versus negative comments related to the laboratory, only 20% of students left positive comments regarding the early traditional cookbook laboratories. However, as the format of the laboratory changed, the number of positive responses increased to 71% of all responses for the two-stream format and 96% of all responses for the one-stream format of laboratories. The qualitative data showed positive changes in both the response rate on the topic of the laboratory as well as student opinions toward traditional versus active inquiry forms of pedagogy. The student responses strongly support that the students believed the changes in the laboratory curriculum were beneficial to their learning.

Challenges for instructors when changing to inquiry laboratories. Often faculty members find it hard to step out of their comfort zone to teach an inquiry laboratory and may find this especially difficult since they were not taught this way (25). However, this change in the curriculum has proven to be beneficial in a variety of classrooms and results in better test scores and increased learning in the long term (4, 5, 30). We found that changing the classroom laboratory from cookbook to inquiry was uncomfortable at first but ultimately liberating to faculty instructors. At each step in the process, our faculty members discovered the classroom laboratory became a more familiar environment, just like working with students in their own research laboratory. Once we became accustomed to running inquiry laboratories, we found the day-to-day operation of the laboratory to be much easier and in fact less expensive than the traditional "different laboratory each week" paradigm. The incredible effort associated just with preparing supplies and training teaching assistants each week for the next cookbook laboratory became a distant memory. Graduate students who serve as teaching assistants also spend a great amount of time working in their own, real laboratory, and although they too resist change, this is the type of change they can more quickly appreciate. Perhaps surprisingly, a recent study (9) has even documented that graduate students who teach in inquiry laboratories tend to master the critical thinking and practice of experimental design more quickly during their graduate studies.

How physiology majors respond to inquiry laboratories. Our students are a population dominated by Premed and Physiology majors, many with differing views of which teaching approach stimulates their learning most. A number of studies (3, 5, 14, 15, 18, 29, 30) have found that students find an inquiry-based research experience more beneficial than traditional cookbook laboratories. Furthermore, students tend to believe that their knowledge is not tested by cookbook laboratories (12). Student opinions certainly played a role in stimulating our faculty to switch to a creative, active, inquiry-based classroom laboratory, and we hoped this premed motivation might also help students engage with and value their performance on our standardized MAT posttest exam, even though it had no impact on their course grade. Given that exams like the MCAT include a number of higher-level thinking problems (31), superficial content coverage may not help students prepare well. If students have more time to focus on one topic in a laboratory and on the process of doing science, greater scientific literacy can be gained. Some studies (13, 15) have found that students experiencing inquiry laboratories become better able to integrate their knowledge and move seamlessly between published research and topics discussed in class, resulting in higher learning.

Less teaching, more learning. We define our use of the term "less teaching" as moving the burden of active effort from the teacher to the student. Given that active and collaborative construction of knowledge "works" (20) and represents a student-centered classroom, having instructors do all the work does not make sense. A number of studies (16, 24) have suggested that depth instead of breadth of coverage in introductory science classes correlates well with later success.

The research literature suggests our findings apply not only to introductory biology courses but also to upper-level classes. In many physiology classes, there is likely too much coverage to allow deep learning, and, as a result, many students reject meaningful learning and resort to using rote memorization to succeed. However, evidence presented in the literature suggests if less content was covered and students experienced an active learning environment, they could achieve greater gains in knowledge (6, 7, 30).

Conclusions. One of the goals of attempting the 14-wk-long one-stream inquiry laboratory was to give students more freedom to develop their own ideas and opportunities to troubleshoot and experience the process of improving their experimental design. The time that students devote to designing (and redesigning) their experiments can be extraordinarily beneficial and has been linked to a greater understanding and improved test scores (2). This greater freedom, combined with the challenges of an ill-structured problem, brings both "pain and gain" that rarely occurs in traditional cookbook laboratories. This challenging environment can ultimately help students make greater gains in learning and in the mastery of scientific laboratory skills (1, 23). Overall, our findings align well with the literature that suggests that by participating in a laboratory curriculum that is enriched for problem solving and authentic inquiry, students have a greater chance to gain more interest in biological research, increase learning, improve critical thinking and analysis, and become better suited for future endeavors (27).

Over the years, faculty instructors in our department who made the change from teaching cookbook laboratories reported that using multiweek inquiry laboratories, especially the one-stream format, "felt more natural, like work done in my own lab." In our own basic science research, we take great care and time exploring our research topic deeply; we spend months/years performing experiments, reading literature, and thinking about our research. When students experience a similar opportunity in the classroom, the education research literature supports our own intuition, specifically that more time on task to achieve deeper understanding in fact "works." Students gain deeper more meaningful understanding of topics, techniques, and the process of doing science and can respond to probing questions like "OK, now design for me an experiment to test that idea." Pursuing longer inquiry experiences with more time yields more formal and informal opportunities for students to talk and write about science, which, not surprisingly, allows students to also makes gains in communication skills like scientific writing and public speaking (10).

While the curricular revisions reported in this study are similar to other reports in the literature, many scientific educators have yet to be convinced to change their teaching to be a more active, student-driven laboratory. The majority of undergraduate laboratory experiences in the United States remain predominately the traditional cookbook style. We hope with this publication and the support of others completing similar research with both qualitative and quantitative studies, more of our peers in science will begin to examine these findings with interest and make progress moving deliberately to challenge students with active, engaging teaching methods and, in particular, using more inquiry in the classroom laboratory.

ACKNOWLEDGMENTS

The authors thank Dr. John Wilterding, Dr. Jim Zablotny, Dr. Jim Smith, Dr. Chuck Elzinga, Dr. Sarah Loznak, Dr. Marija Krha-Massey, Dr. Diane Ebert-May, Dr. Merle Heideman, Dr. Joyce Parker, Dr. John Merrill, Dr. Janet Batzli, Dr. Karl Smith, Dr. Seth Hootman, Dr. Tom Adams, Dr. Mimi Sayed, and Dr. Duncan Sibley for helpful discussions about teaching and learning and assistance during this study. The authors also thank the many teaching assistants who promoted and implemented these renovations in curriculum and made a fantastic environment for learning in the classroom laboratories.

GRANTS

This work was supported by grants from the Cystic Fibrosis Foundation and Pennsylvania Cystic Fibrosis, Incorporated (to D. B. Luckie), for the physiology cystic fibrosis research program and by grants (to D. B. Luckie) from Michigan State University and the National Science Foundation for the education research program.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.B.L. and J.J.M. conception and design of research; D.B.L. and J.J.M. performed experiments; D.B.L., J.R.A., B.J.M., A.M.R., L.A.F., and J.J.M. analyzed data; D.B.L., J.R.A., B.J.M., A.M.R., L.A.F., and J.J.M. interpreted results of experiments; D.B.L., J.R.A., B.J.M., A.M.R., and L.A.F. prepared figures; D.B.L., J.R.A., B.J.M., A.M.R., and L.A.F. drafted manuscript; D.B.L., J.R.A., B.J.M., A.M.R., L.A.F., and J.J.M. edited and revised manuscript; D.B.L., J.R.A., B.J.M., A.M.R., L.A.F., and J.J.M. approved final version of manuscript.

REFERENCES

1. **Anjur SS.** Student-centered physiology in high schools. *Adv Physiol Educ* 35: 161–167, 2011.
2. **Aronson BD, Silveira LA.** From genes to proteins to behavior: a laboratory project that enhances student understanding in cell and molecular biology. *CBE Life Sci Educ* 8: 291–308, 2009.
3. **Casem ML.** Student perspectives on curricular change: lessons from an undergraduate lower-division biology core. *CBE Life Sci Educ* 5: 65–75, 2006.
4. **Casotti G, Rieser-Danner L, Knabb MT.** Successful implementation of inquiry-based physiology laboratories in undergraduate major and nonmajor courses. *Adv Physiol Educ* 32: 286–296, 2008.
5. **Derting TL, Ebert-May D.** Learner-centered inquiry in undergraduate biology: positive relationships with long-term student achievement. *CBE Life Sci Educ* 9: 462–472, 2010.
6. **DiCarlo SE.** Cell biology should be taught as science is practised. *Nat Rev Mol Cell Biol* 7: 290–296, 2006.
7. **DiCarlo SE.** Too much content, not enough thinking, and too little fun! *Adv Physiol Educ* 33: 257–64, 2009.
8. **Donovan MS, Bransford JD, Pellegrino JW.** *How People Learn: Bridging Research and Practice*. Washington, DC: National Academy, 1999.
9. **Feldon DF, Peugh J, Timmerman BE, Maher MA, Hurst M, Strickland D, Gilmore JA, Stiegelmeyer C.** Graduate students' teaching experiences improve their methodological research skills. *Science* 333: 1037–1039, 2011.
10. **FitzPatrick KA, Campisi J.** A multiyear approach to student-driven investigations in exercise physiology. *Adv Physiol Educ* 33: 349–355, 2009.
11. **Handelsman J, Ebert-May D, Beichner R, Bruns P, Chang A, DeHaan R, Gentile J, Lauffer S, Stewart J, Tilghman SM, Wood WB.** Education. Scientific teaching. *Science* 304: 521–522, 2004.
12. **Henige K.** Undergraduate student attitudes and perceptions toward low- and high-level inquiry exercise physiology teaching laboratory experiences. *Adv Physiol Educ* 35: 197–205, 2011.
13. **Hoskins SG, Stevens LM.** Learning our LIMITS: less is more in teaching science. *Adv Physiol Educ* 33: 17–20, 2009.
14. **Howard DR, Miskowski JA.** Using a module-based laboratory to incorporate inquiry into a large cell biology course. *Cell Biol Educ* 4: 249–260, 2005.
15. **Jensen JL, Lawson A.** Effects of collaborative group composition and inquiry instruction on reasoning gains and achievement in undergraduate biology. *CBE Life Sci Educ* 10: 64–73, 2011.
16. **Knight JK, Wood WB.** Teaching more by lecturing less. *Cell Biol Educ* 4: 298–310, 2005.
17. **Krontiris-Litowitz J.** Articulating scientific reasoning improves student learning in an undergraduate anatomy and physiology course. *CBE Life Sci Educ* 8: 309–315, 2009.
18. **Lord T, Orkwiszewski T.** Moving from didactic to inquiry-based instruction in a science laboratory. *Am Biol Teacher* 68: 342–345, 2006.
19. **Luckie DB, Maleszewski JJ, Loznak SD, Krha M.** Infusion of collaborative inquiry throughout a biology curriculum increases student learning: a four-year study of "Teams and Streams". *Adv Physiol Educ* 28: 199–209, 2004.
20. **Luckie D, Harrison SH, Ebert-May D.** Model-based reasoning: using visual tools to reveal student learning. *Adv Physiol Educ* 35: 59–67, 2011.
21. **Momsen JL, Long TM, Wyse SA, Ebert-May D.** Just the facts? Introductory undergraduate biology courses focus on low-level cognitive skills. *CBE Life Sci Educ* 9: 435–440, 2010.
22. **Morgan E.** *BIO2010: Transforming Undergraduate Education for Future Research Biologists* (online). http://www.nap.edu/openbook.php?isbn=0309085357 [21 Oct. 2011].
23. **Myers MJ, Burgess AB.** Inquiry-based laboratory course improves students' ability to design experiments and interpret data. *Adv Physiol Educ* 27: 26–33, 2003.
24. **Schwartz MS, Sadler PM, Sonnert G, Tai RH.** Depth versus breadth: how content coverage in high school science courses relates to later success in college science coursework. *Sci Educ* 93: 798–826, 2009.

25. **Silverthorn DU, Thorn PM, Svinicki MD.** It's difficult to change the way we teach: lessons from the Integrative Themes in Physiology curriculum module project. *Adv Physiol Educ* 30: 204–214, 2006.
26. **Smith J, Cheruvelil K.** Using inquiry and tree-thinking to "March through the animal phyla": teaching introductory comparative biology in an evolutionary context. *Evol Educ Outreach* 2: 429–444, 2009.
27. **Treacy DJ, Sankaran SM, Gordon-Messer S, Saly D, Miller R, Isaac SR, Kosinski-Collins MS.** Implementation of a project-based molecular biology laboratory emphasizing protein structure-function relationships in a large introductory biology laboratory course. *CBE Life Sci Educ* 10: 18–24, 2011.
28. **Wilterding JH, Luckie DB.** "Stream"-lined education: increasing student-initiated learning with an investigative DNA "stream". *J Coll Sci Teach* 31: 303–307, 2002.
29. **Wood WB.** Inquiry-based undergraduate teaching in the life sciences at large research universities: a perspective on the Boyer Commission Report. *Cell Biol Educ* 2: 112–116, 2003.
30. **Wright RL, Klymkowsky MW.** Points of view: content versus process: is this a fair choice?: undergraduate biology courses for nonscientists: toward a lived curriculum. *Cell Biol Educ* 4: 189–196, 2005.
31. **Zheng AY, Lawhorn JK, Lumley T, Freeman Assessment S.** Application of Bloom's taxonomy debunks the "MCAT myth". *Science* 319: 414–415, 2008.

CBE—Life Sciences Education
Vol. 12, 1–15, Fall 2013

Article

Verbal Final Exam in Introductory Biology Yields Gains in Student Content Knowledge and Longitudinal Performance

Douglas B. Luckie,* Aaron M. Rivkin,* Jacob R. Aubry,* Benjamin J. Marengo,* Leah R. Creech,† and Ryan D. Sweeder†

Q1 *Department of Physiology, Michigan State University, East Lansing, MI 48825; †STEM Learning Laboratory, Lyman Briggs College, Department of Physiology, Michigan State University, East Lansing, MI 48824-3320

Submitted April 25, 2012; Revised May 7, 2013; Accepted May 8, 2013
Monitoring Editor: Michelle Kathleen Smith

Q2 We studied gains in student learning over eight semesters in which an introductory biology course curriculum was changed to include optional verbal final exams (VFs). Students could opt to demonstrate their mastery of course material via structured oral exams with the professor. In a quantitative assessment of cell biology content knowledge, students who passed the VF outscored their peers on the medical assessment test (MAT), an exam built with 40 Medical College Admissions Test (MCAT) questions (66.4% [$n = 160$] and 62% [$n = 285$], respectively; $p < 0.001$);. The higher-achieving students performed better on MCAT questions in all topic categories tested; the greatest gain occurred on the topic of cellular respiration. Because the VF focused on a conceptually parallel topic, photosynthesis, there may have been authentic knowledge transfer. In longitudinal tracking studies, passing the VF also correlated with higher performance in a range of upper-level science courses, with greatest significance in physiology, biochemistry, and organic chemistry. Participation had a wide range but not equal representation in academic standing, gender, and ethnicity. Yet students nearly unanimously (92%) valued the option. Our findings suggest oral exams at the introductory level may allow instructors to assess and aid students striving to achieve higher-level learning.

INTRODUCTION

The spoken word is a unique device. Our words can affect others' behavior in dramatic ways and can profoundly affect our own thoughts and learning. Whether one reviews the classic work of Piaget and Vygotsky or other literature of cognitive development (Inhelder and Piaget, 1958; Vygotsky, 1962; Bruner, 1973), learning (Posner *et al.*, 1982; Guest and Murphy, 2000; Krontiris-Litowitz, 2009), memory (Baddeley and Hitch, 1974), or metacognition (Schunk, 1986; Pintrich, 1988; Labuhn *et al.*, 2010), the data support that the action of speaking thoughts aloud strongly impacts one's own understanding and mastery. In fact, for many years, the literature has even suggested that a student who speaks aloud his or her answer to a question will have higher retention and comprehension than the student who silently thinks the same answer (Gagne and Smith, 1962; Carmean and Wier, 1967; Bruner, 1973). Overt verbalization is a powerful tool for learning and increases self-evaluative judgment and cognitive commitment (Wilder and Harvey, 1971; Schunk, 1986; Labuhn *et al.*, 2010).

The literature suggests that exams and other high-stakes assessments tend to drive student learning in the classroom (Tobias, 1992; Tobias and Raphael, 1997). The data are persuasive, and upon reflection, most experienced instructors would concede this is very likely to be true. No matter how much time and energy a professor puts into challenging students to think critically, no matter how much we implore them to think like a scientist, if the exams they face do not test higher-level thinking and problem solving, students are not likely to master the material (Traub and MacRury, 1990; Momsen *et al.*, 2010). Students perceive that the information on our high-stakes assessments is what we truly value (Entwistle and Entwistle, 1992), and the exam format itself can change how students study and approach problem solving.

DOI: 10.1187/cbe.12-04-0050
Address correspondence to: Douglas Luckie (luckie@msu.edu).

As instructors, our exams and other assessments certainly also impact us. We genuinely want our students to learn things that Benjamin Bloom (1956) would have considered higher level, but we struggle with how to assess it. Consider this learning goal: to think like a scientist and be able to adaptively negotiate a question or problem. It is an appropriate learning goal, but if we attempt to align it with our traditional assessments (quizzes, exams, homework, etc.), we rarely find robust sources of evidence that demonstrate mastery (Momsen *et al.*, 2010). In mentoring our graduate students, we tend to teach with a questioning style much like that of Socrates (Stumpf, 1983). We interact one-on-one and ask questions to evaluate as well as to determine how to best guide our students. In this setting, we orally assess whether our students are mastering or achieving "how to think like a scientist" (Paul and Elder, 2006).

A number of years ago, a new assessment was introduced in our biology classroom, a structured clinical interview or oral exam with Socratic questioning called the "verbal final" (VF). It was used to assess higher-level thinking, while at the same time allowing verbalization to better impact learning. We observed students' ability to think critically about topics taught and adaptively negotiate a posed question or problem as addressed in the literature (Schubert *et al.*, 1999; Thorburn and Collins, 2006). The assessment was predicted to allow students to also participate in a metacognitive process as they grappled with the topic and how to explain it to others (Baddeley and Hitch, 1974). The buy-in from students created a novel culture of learning outside the classroom driven by this high-stakes assessment (DiCarlo, 2009) and appears to have a lasting impact on student learning.

METHODS

Population and Context

Lyman Briggs College is an undergraduate science program established at Michigan State University (MSU) in 1967 (Sweeder *et al.*, 2012). It is a residential college modeled after those at Oxford University in the United Kingdom and has a focus of educating undergraduates in a liberal science curriculum defined as a "solid foundation in the sciences and a significant liberal education in the history, philosophy, and sociology of science." Introduction to Cell and Molecular Biology (Bio II) is a five-credit, freshmen-level course. It is the second in a core two-semester introductory biology sequence for science majors. It has an enrollment of ∼100 students and each student attends two lectures and one recitation (50 min each) and two laboratory sections (3 h each) per week.

With the approval of MSU Institutional Review Board (IRB X00-475 and 10-543), student data were collected from 445 students who completed our Introduction to Cell and Molecular Biology course at MSU; the data were from cohorts in the eight semesters studied between the years 2002 and 2011. All students were enrolled at MSU, and participant consent was obtained while students were enrolled in the course. The registrar provided ACT science and composite scores and demographic data for 407 of the 445 students who participated. With the approval of MSU-IRB (IRB 07-446), student data were also collected for longitudinal tracking and comparison purposes from all 21,528 students who completed select upper-level science courses at MSU from 1997 to 2010.

The VF process is structured to incentivize students to participate with high reward and to decrease anxiety by having no penalty. The 1-h exam is graded on a pass/not-pass basis. Students who pass the VF earn a 100% for the final exam score in the course. Students may retake the VF a number of times. If they do not pass, they take the written final exam. In addition to the method listed below (and in greater detail in the Supplemental Material), we have made additional resources available, including audio and video recordings of students taking the VF, on the public VF website (www.msu.edu/∼luckie/verbalfinals).

VF Protocol

During the exam process, instructors keep notes of questions asked and student errors that occur (Figure 1). They also make an effort to frequently use the phrase "What do you predict is likely?" to communicate being open to imperfect answers as long as students provide reasonable support for their predictions. If students are performing at a very high level, then assessment of even more advanced concepts, such as experimental design, can be attempted ; for example, "Okay, great prediction, now design an experiment to test that prediction, what could you try?"

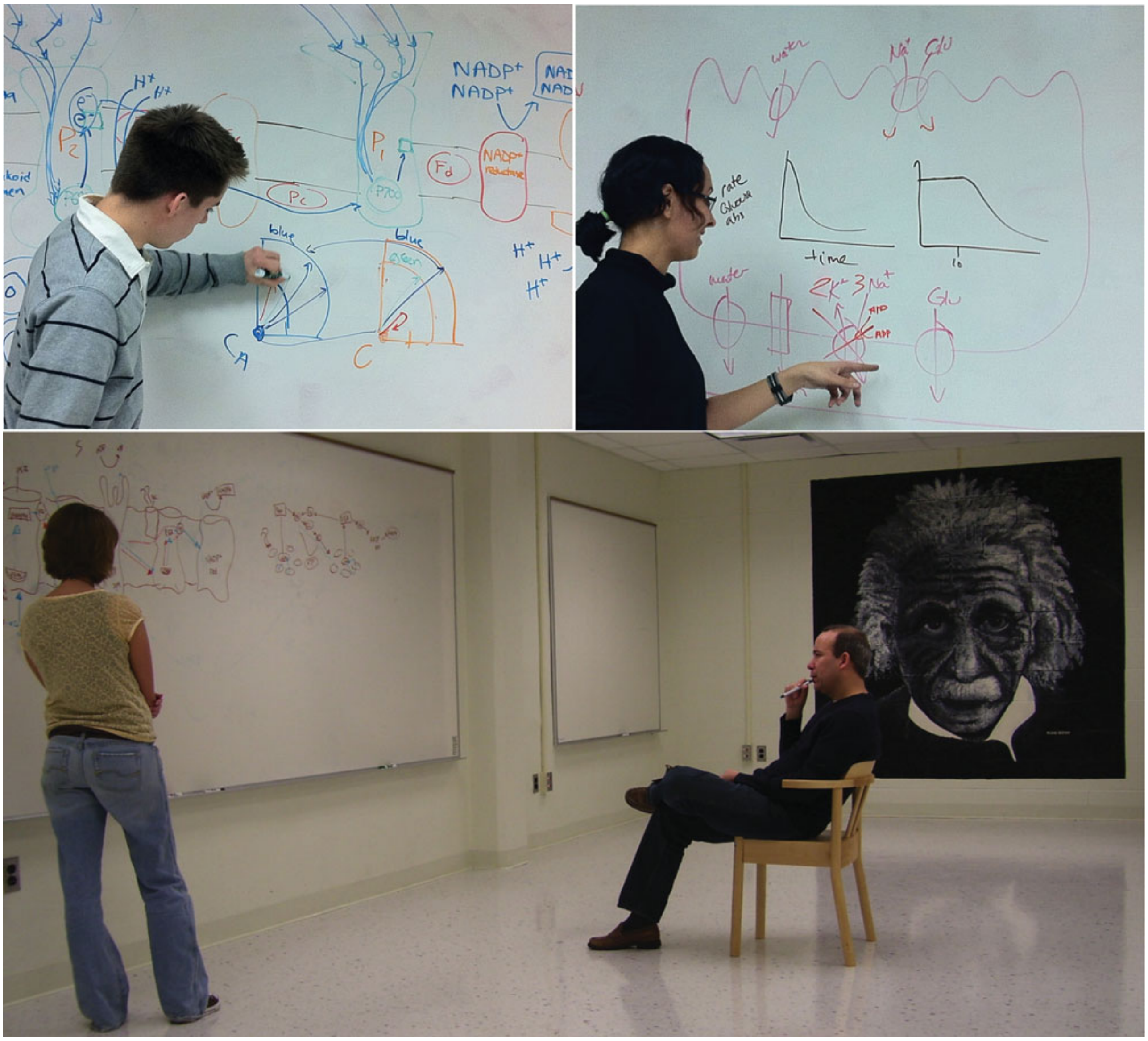
At the beginning of the exam, the instructor reads an introductory statement aloud to outline what is expected and give the guidelines for the process. After this, several topics are discussed in sequence. Below are the topics discussed in the VF used for this study (photosynthesis, digestion/absorption, central dogma/biosynthesis).

Phase 1. Explanation of Process of Photosynthesis

Light Reactions. The first phase of the exam is designed to require the student to draw and explain the light reactions of photosynthesis. At the start of the official exam time period, the student is told to draw an illustration of the photosystems and carriers important for light reactions. This illustration serves as an aid as the student explains the process. After the student is done, the instructor asks probing questions; first about whatever the student said that might not have been completely clear or accurate, then to determine the depth of student understanding. Example questions are: What is an absorption spectrum? How do the pigments absorb light in a photosystem? If there were 100 protons in the stroma and 200 in the thylakoid lumen how much ATP do you predict would be made? What do you predict would happen if we put a hole in the membrane?

When the student successfully completes phase 1, he or she is told of any strikes (significant errors) he or she may have accrued. If there are fewer than three strikes and time remains in the hour, the student may proceed.

Calvin Cycle. The student is instructed to draw and explain the Calvin cycle, including names of enzymatic and structural compounds. When the student is done explaining the process, the instructor again asks questions to clarify and check for errors and omissions, and then follows this up with probing



Q6 **Figure 1.** The event of the VF gives the student one-on-one time with the professor. They both follow a structured exam protocol outlined in advance. The topics discussed during the VF are framed in one, albeit simplistic, story with three parts: how photosynthesis converts light into sugar/food, how humans digest and circulate food, and how a pancreatic cell makes insulin in response to the presence of sugar. The student creates an illustration and explains the way he or she believes the biology works. Once the student completes the explanation about each topic, the professor then asks questions for clarification and follows up with probing questions to test the student's confidence and depth of knowledge. (Permissions for publication of photos were obtained.)

questions: What are the names of phases and why? What is reduction? What would happen if we only fixed one CO_2 molecule? What does G3P taste like, and why does that makes sense from an evolutionary perspective?

If the student successfully completes the discussion of the Calvin cycle, he or she is then told of any additional strikes he or she may have accrued. As was the case before, if there are fewer than three strikes and time remains in the hour, the student may proceed. As a transition, we state: "Okay, let's pretend the glucose you just made in the Calvin cycle turns into a donut. Let's discuss its digestion and absorption."

Phase 2. Explanation of the Process of Digestion and Absorption

Digestion and Absorption of Macromolecules. In the second phase, the student is asked to explain digestion and absorption. We require students to know a sample set of organs and their functions (oral cavity, stomach, small intestine, liver, gall bladder, and pancreas, as well as a set of events; organ function; relevant cells; relevant enzymes; hormones; processes), but for efficiency sake, we often ask them to explain how digestion works in organ X, or alternatively, to explain how

Q7 food group X is digested and absorbed. Randomizing which organ or macromolecule they are asked to discuss requires students to prepare for all questions, but less time is required for the actual exam. When the student has completed his or her explanation of digestion the instructor may ask questions for clarification or to check for errors and omissions, but does not probe the organ level of this topic deeply. Instead, the instructor asks the student to then focus on cell biology. Our goal is to more quickly get to the topics of membrane transporters, gradients, and enzymes in the domain of cell biology, using the parietal cell and/or the villus epithelial cell as models in context.

Parietal or Villus Cell Biology. In this section of the digestion phase, we probe students' understanding of how a cell works and ask students to make predictions about how membrane transporters and chemical reactions may respond to perturbations. For example, we will ask a student to draw a stomach's parietal cell and explain how it makes HCl or to draw the small intestine's villus epithelial cell and explain how it absorbs glucose. We evaluate the explanation and probe for understanding with questions such as: What would happen to this process in the parietal cell if a drug inhibited the enzyme carbonic anhydrase? Indicate which reaction rates would change by placing a check mark next to its arrow in your drawing, how might that change the pH in the stomach lumen or the bloodstream? Similar transporter questions can be used for the villus epithelial cell; for example: What would happen if a drug inhibited the Na/K pump? How would this affect other transporters? How would this affect the membrane potential? How would this affect the rate of glucose absorption/uptake? We vary and alter these questions for different students by, for example, first asking what might happen if the membrane potential is changed.

As an affective transition to the last phase of the test, we request that the student tell us some detail of the path an absorbed macromolecule (e.g., glucose) must take to travel from a capillary at the basolateral side of the villus cell to a capillary beside a beta cell in the pancreas. The student is expected to name some basic vessels and the path (valves/chambers) through the heart. We often request they do this without an illustration, if possible. When the student is done, we ask questions only if errors or omissions occurred; otherwise, we talk about the last steps, in which glucose exits the capillary and enters the beta cells. We probe for understanding of diffusion via questions such as: How does glucose exit the capillary as well as into the pancreatic cell? Does it need a carrier or channel or something? Why does it move?, What drives it? Why do you predict this would occur?

Phase 3. Central Dogma—Biosynthesis of Insulin

Biosynthesis of Proteins. The last phase of the VF requires the student to explain central dogma using the biosynthesis of a particular protein, usually insulin, as we discuss Q8 insulin biosynthesis frequently in class. Students are required to draw a eukaryotic cell and explain the processes of transcription, translation, and biosynthesis in terms of steps that occur at each organelle. When the student has completed his or her explanation of insulin biosynthesis and secretion, we ask follow-up questions for clarity and to check for errors and omissions, and then probe with queries such as: What if there's a mutation? What do you predict would happen if we add some hydrophobic domains to the gene?

Biosynthesis of Other Proteins. After the student shows an acceptable understanding of how insulin is created, we ask what path would be taken in the biosynthesis of an apical channel protein (e.g., CFTR), or a cytoplasmic protein (e.g., Q9 tubulin). We ask the student to draw an large transport vesicle and indicate where insulin or CFTR might be found. We also often request that a student create an illustration to show how a transport vesicle behaves when it reaches the cell membrane.

Timing and Constraints of VF

We have refined the process to avoid wasting time when student performance indicates that a student was unable to prepare adequately. We start promptly and end promptly. If the student has not completed the exam by the end of 60 min, we just stop and require the student to set up another attempt. If it quickly becomes clear during the exam that a student does not yet know basic information, we just end the exam and ask the student to make another appointment. To communicate these rigorous guidelines regarding time and other elements, we provide handouts at the start of the semester, and the instructor reads aloud an introductory statement the first time a student takes the exam. To increase student preparation and performance, during the eight-semester period discussed in this study, the exam process was considered a public one, with classroom doors kept open and interested colleagues permitted to observe if granted prior permission by student.

Collecting Data on Student Performance at End of Semester

We administered a small, standardized exam, the Medical Assessment Test (MAT) as a posttest during the final week (week 15) of all semesters. The MAT exam was built from Medical College Admissions Test (MCAT) test questions developed, validated, and purchased from the Association of American Medical Colleges (Luckie *et al.*, 2004). The MAT exam is a 40-question, multiple-choice test composed of relevant passage–style questions. MCAT passage questions have been studied by others and are deemed to assess higher-level content knowledge than typical multiple-choice exams (Zheng *et al.*, 2008). The MAT exam instrument consisted of questions from five general topic categories: cell structure and function, oncogenes/cancer, cellular respiration, microbiology, and DNA structure and function. Performance of each individual student on the exam as a whole and on questions related to each category was examined, and cohorts were compared. The MAT instrument used in this study is provided online (see VF website). In addition to the MAT instrument, the course written final exam was not changed and was therefore standard across semesters and served as an additional assessment of content knowledge.

Raw MAT scores for all semesters were normalized for variations in each cohort's prior academic performance using ACT scores. In our previous tracking research (Creech and Sweeder, 2012), we determined incoming university grade point average (GPA) tends to be the best predictor of how well a student will do in a university course. Yet the first-semester university GPA, much like high school GPA, is less predictive. Given that our freshmen recently took the ACT, a carefully administered, valid, and reliable exam, we felt the ACT was the best available metric to use for normalization of data for this particular freshmen biology course.

Student ACT science scores ranged from 15–36 and ACT composite scores from 17 to 35. MAT scores were normalized by determining the mean ACT score for each experimental and control group and calculating the numerical multiplier required to elevate the lower score to parity. This multiplier was then used to adjust respective MAT scores proportionally to normalize all results. VF participation and pass rates were calculated for cohorts by using academic standing, gender, and ethnicity (four students did not report ethnicity). Academic standing quartiles were generated by sorting students first for ACT composite score and secondarily for ACT science score. The resulting sorted list was then divided into equal quartiles. The lowest quartile contained students with composite scores below 25. The cutoff for the second-lowest quartile was a composite score of 27 and science score of <26. The next quartile ranged from students with a composite score of 27 and a science score of 26 or higher to a composite of 29 and a science score of 28 or lower. The highest quartile consisted of composite scores of 29 and a science score of 29 or higher and all higher composite scores.

Student's *t* test, analysis of variance (ANOVA), and *z* test of proportions were used to perform statistical comparisons of MAT data. The *t* test was used in comparisons between the experimental cohort (students who passed the VF) and the matched peer control cohort (students who did not pass) as well as comparisons between pooled data sets of experimental and control groups. ANOVA was also used. The figure legends indicate trial numbers and test applied. Error bars on figures were generated by calculating the SEM, unless otherwise indicated.

Longitudinal Analysis of Student Performance in Upper-Level Courses

Tracking analysis of student performance in their upper-level science courses was performed. Grades earned by the cohort of all students who passed the VF in Introduction to Cell and Molecular Biology (LB 145, Bio II) were compared with grades earned by other MSU students who enrolled in upper-level science courses. The courses used were Physiology I (PSL 431) and II (PSL 432), Biochemistry I (BMB 461) and II (BMB 462), Advanced Introductory Microbiology (MMG 301), Genetics (ZOL 341), Organic Chemistry I (CEM 251) and II (CEM 252), and Advanced Organic Chemistry I (CEM 351) and II (CEM 352). The data set consisted of grades in upper-level science courses, introductory courses (biology, chemistry, and organic chemistry), cumulative GPA by semester, and student classification information (gender, ethnicity, major, honors college, Lyman Briggs College). Data were analyzed using SPSS 20 (IBM, Chicago, IL).

We examined student performance in multiple fashions. We initially compared students who passed the VF with all MSU students between 1997 and 2010; when the data were visualized, we saw higher performance of students who passed the VF in a wide range of upper-level science courses. However, during analysis, we realized that 1) student incoming ACT scores increased over the time and 2) students in the Lyman Briggs College major tend to have better incoming GPAs. When aligning the time period to 2002–2010 and examining peer students who were Lyman Briggs majors, we still saw higher grades but smaller to nonexistent significance. Given this approach suffers from the fact that there are multiple instructors teaching in Lyman Briggs courses, our final analysis looked exclusively at those peer-matched students who had enrolled in the lower-division LB 145 (Bio II) course over the same time period and with the same instructor who offered the VF exam option. Table 1 lists courses studied in the analysis and side-by-side comparison of final GPA of the experimental and control cohorts in each course. In addition, Figure 5 (later in this article) compares ACT quartile versus earned course grade average in physiology and biochemistry courses. Quartiles were established using ACT scores, as described above. The trial numbers (*n*) as indicated are lower in the analysis, because not all students in the study had taken their upper-level science courses nor do all students enroll in all the courses we studied. An expansive Supplemental Table S1 lists all courses studied in the analysis and side-by-side comparison of final GPA of the experimental and control cohorts in each quartile in each course.

Table 1. Tracking performance in upper-level courses of students who passed VF vs. controls[a]

Course	Cohort	Average grade (SEM)	*n*
ZOL341	VF	3.26 (0.12)	38
	Control	2.80 (0.10)	98
MMG301	VF	2.69 (0.18)	29
	Control	2.69 (0.12)	59
PSL431	VF	2.72 (0.21)	32
	Control	2.42 (0.12)	85
PSL432	VF	2.78 (0.21)	30
	Control	2.47 (0.13)	78
CEM251	VF	3.44 (0.10)	65
	Control	2.88 (0.07)	180
CEM252	VF	3.39 (0.12)	60
	Control	2.79 (0.08)	168
CEM351	VF	3.70 (0.13)	15
	Control	3.18 (0.14)	31
CEM352	VF	3.44 (0.19)	17
	Control	3.26 (0.19)	27
BMB401	VF	3.15 (0.33)	13
	Control	2.23 (0.17)	37
BMB461	VF	3.11 (0.10)	47
	Control	2.51 (0.09)	104
BMB462	VF	2.91 (0.12)	40
	Control	2.49 (0.09)	85

[a] Average grade in course of students who passed the VF compared with average grade of peer controls from the same classes (*n* = number of students in sample). CEM, chemistry; BMB, biochemistry; PSL, physiology; MMG, microbiology; ZOL, zoology.

Additional methods of data analysis included linear modeling (LM) and graphical analysis. LM was also used to create a model for estimating students' upper-level science performance (Spicer, 2005; Rauschenberger and Sweeder, 2010). LM used the raw data and was executed stepwise, excluding cases listwise. Graphical representations of the data were created through SPSS 20 to visualize categorical differences in the data. Average grades in specific courses for students who did and did not take the VF are compared for students clustered based on incoming GPA and using scatter plots (Supplemental Figure S1). Students' GPAs were clustered to the nearest

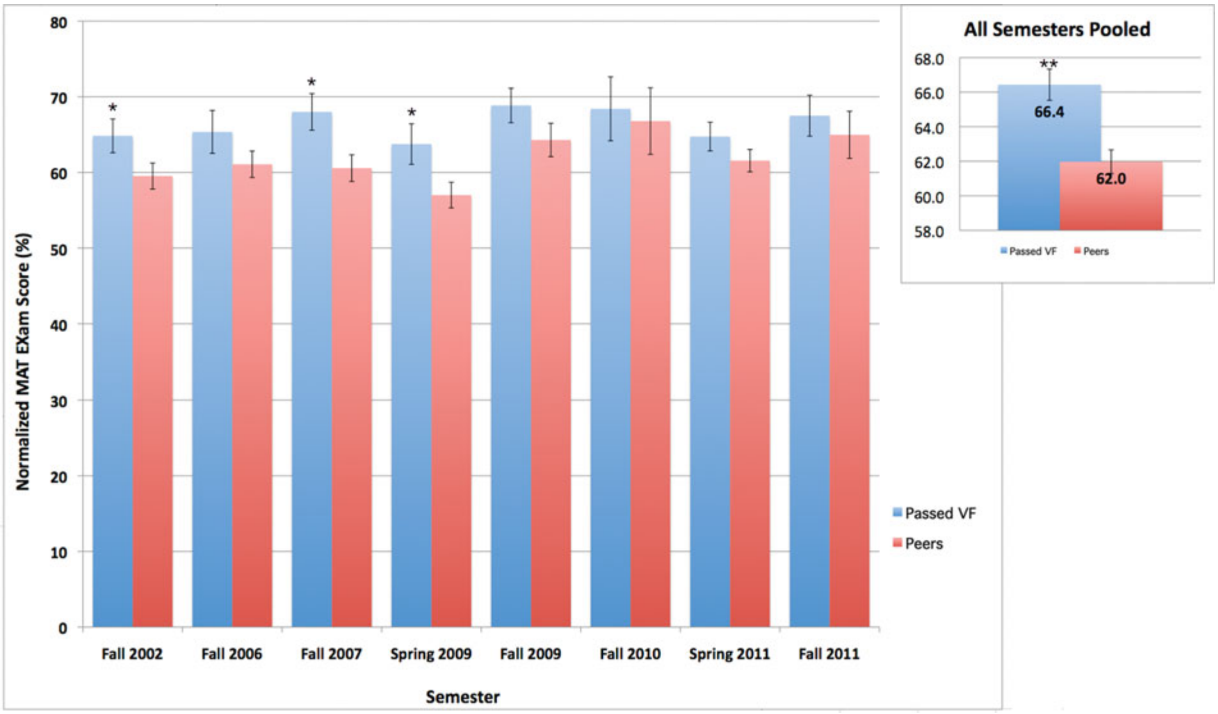


Figure 2. Performance of students who passed the VF vs. peers on a standardized MAT content exam. The blue bar corresponds to the collective normalized average on the MAT of students who passed the VF each semester. Likewise, the red bar illustrates normalized MAT scores of peers who did not pass the VF. Fall 2002 ($n = 77$ students, 31 passed:46 not), Fall 2006 ($n = 63$, 21:42), Fall 2007 ($n = 64$, 20:44), Spring 2009 ($n = 70$, 16:54), Fall 2009 ($n = 42$, 13:29), Fall 2010 ($n = 19$, 11:8), Spring 2011 ($n = 77$, 29:42), and Fall 2011 ($n = 33$, 19:14). The inset compares average MAT scores of all semesters pooled for those who passed the VF ($n = 160$) and those who did not ($n = 285$). *, $p < 0.05$; **, $p < 0.001$; t test compared students who passed the VF with cohorts who did not for each course/semester and for pooled data in inset.

quarter grade in the plots, and the error bars represent the SEM.

Collecting Student Opinion Data via Student Self-Report on Course Evaluation Forms

End-of-semester course evaluation surveys were used to evaluate student opinion and assess affective or qualitative elements in response to the optional VF. We evaluated the course using the Student Assessment of Learning Gains (SALG; Seymour *et al.* 2000). Beginning in 2009, a new prompt, "Do you think the VF option helps student learning?," was added to the course evaluation form administered at the end of each semester in Bio II. Response choices were: 1—no help, 2—a little help, 3—moderate help, 4—much help, 5—great help, 6—not applicable. In addition, in Fall 2010 and 2011, the prompt "Do you think the VF option helps student learning, if so why?" also solicited student feedback via extended written responses. We reviewed the Likert-scale responses to questions posed on the topic of the VF and the written comments prompted by extended-response questions. While only sample feedback is presented in this report, comprehensive student feedback from five semesters (Spring 2009: $n = 72$; Fall 2009: $n = 43$; Fall 2010: $n = 20$; Spring 2011: $n = 77$; and Fall 2011: $n = 34$) is also provided online (see VF website). All student comments are hyperlinked to their full SALG feedback form online.

RESULTS

Students Who Passed the VF Scored Higher on MCAT Questions

During the VF, students demonstrated their mastery of course material via a structured oral exam with the professor (Figure 1). Students who passed the VF performed better than their peers on MCAT questions included on an end-of-semester standardized MAT instrument (Figure 2; MAT scores were normalized with ACT performance). The average normalized MAT score for all students who participated and passed the VF was 66.4% ($n = 160$), while the mean of peers who did not pass the VF was 62% ($n = 285$; $p < 0.001$; Figure 2, inset). In addition, when the student performance data were replotted in a frequency distribution, a rightward shift was apparent (Figure 3). The mode for performance of students who passed the VF was 72.5%, while those who did not pass had a mode of 65%. Higher levels of content mastery, for example, MAT scores of 80–90%, were more frequent among students who passed the VF.

Students Who Passed the VF Scored Higher on Every Topic Area and Made Greatest Gains on Cellular Respiration Q10

When performance on the MAT exam was examined, those who passed the VF did significantly better than peers on every topic (DNA, cell biology, respiration, cancer, and microbiology; Figure 4). The greatest gain in performance on the MAT

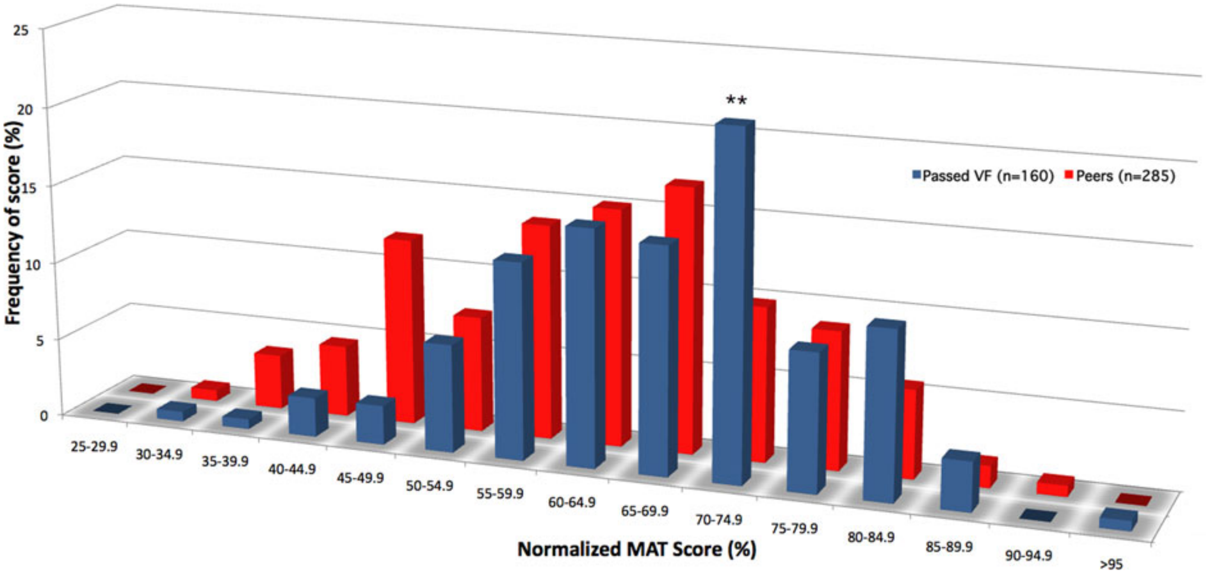


Figure 3. Students who passed the VF scored higher on the MAT exam than peers who did not. Normalized MAT performance distribution (score in percentage vs. frequency in percentage) is shown comparing cohort of students who passed the VF ($n = 160$) with students who did not pass ($n = 285$). No students scored below a 30%, while one student scored a 95%. Students who passed the VF are represented in blue. Students who did not pass the VF are represented in red. **, $p < 0.001$; t test compared pooled data of two cohorts.

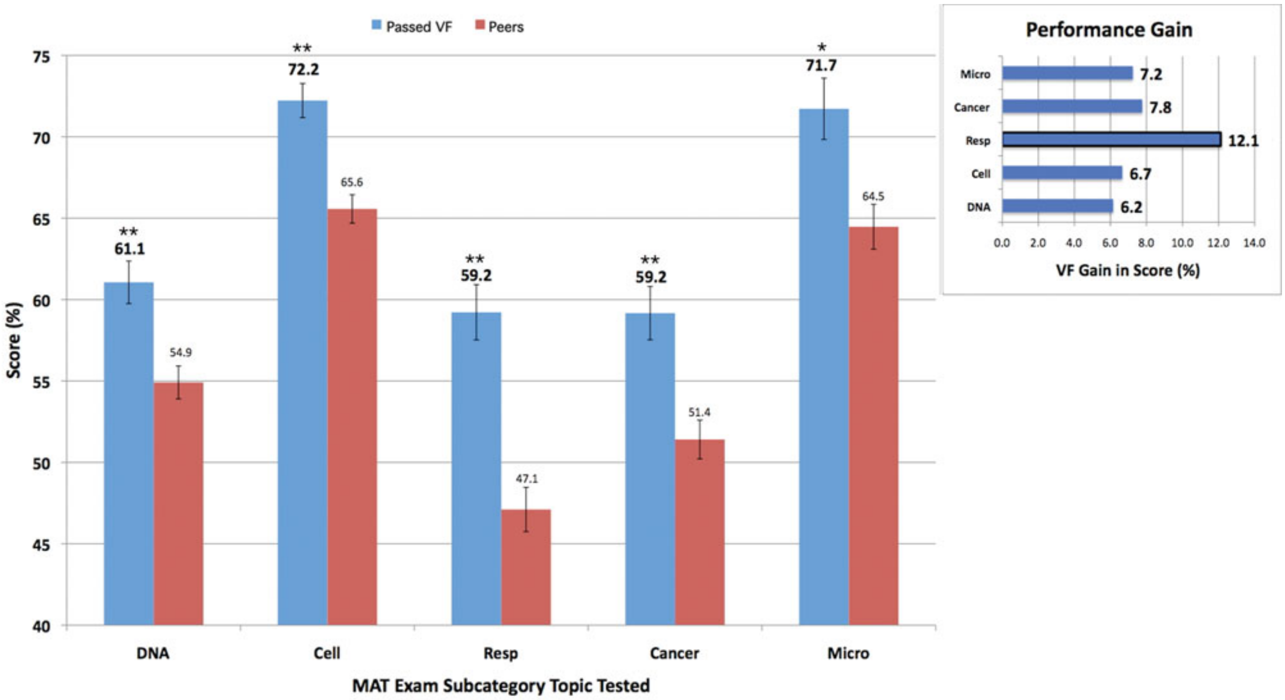


Figure 4. Performance of students on each topic area tested by the MAT exam. The bars set to the left for each topic (DNA, Cell, Resp, etc.) correspond to the collective average score on that topic of all students who passed the VF ($n = 160$). The bar to the right in each pair represents the average score for that topic of all students who did not pass the VF ($n = 285$). Moving from left to right along the x-axis labels, the unabbreviated categories are: DNA structure and function, cell structure and function, cellular respiration, cancer, and microbiology topics, respectively. Error bars were generated using SEM. The inset illustrates the gain in learning of content knowledge obtained for each topic area tested by subtracting the lower score from the upper. t test: *, $p < 0.05$; **, $p < 0.001$).

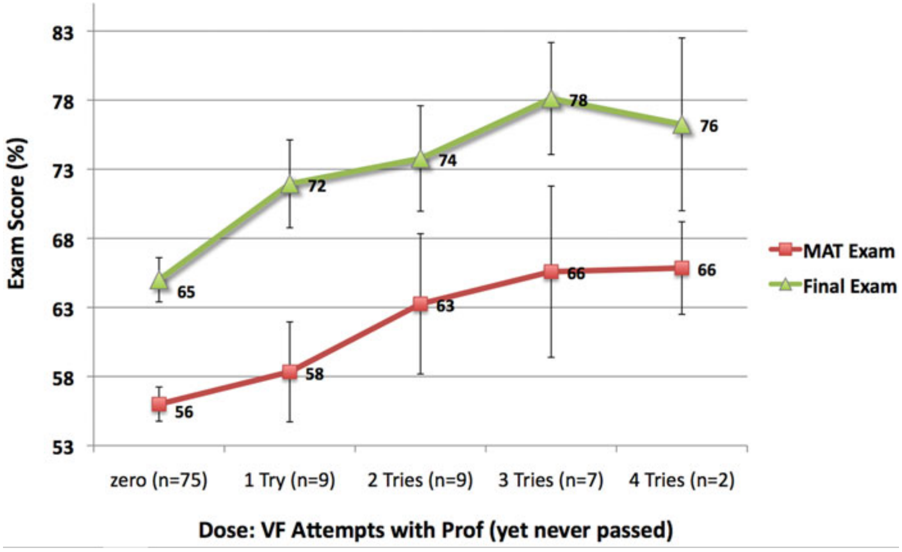


Figure 5. Just attempting the VF may increase student learning. Students who made a greater number of attempts at the VF (but did not pass) may benefit and increase their learning. Students who did not pass the VF were required to take the traditional written final exam of the course. Thus, for this cohort, both the MAT and final exam scores were used to assess their content knowledge. The number of attempts made by students is compared with their exam performance. MAT scores are represented by the red line with square symbols; the green line with triangle symbols indicates final exam scores. SEM is indicated, and differences are not statistically significant.

was on questions related to the topic of cellular respiration (Figure 4, inset). The VF focuses upon the topics of DNA, cell Q11 biology, photosynthesis, and digestion. While cellular respiration is not discussed on the VF, the conceptually similar topic of photosynthesis is given the greatest amount of time and has the most in-depth treatment in the VF. This gain may be evidence of transfer of knowledge of photosynthesis to aid in solving problems on respiration.

Just Attempting the VF Once May Increase Student Learning

In only three semesters were records retained that indicated number of attempts for students who did not pass the VF. When examining these data, we see correlating increases in average performance on the MAT with number of VF attempts. While the trial numbers are quite low, the trend in the data supports a hypothesis that just participating in the process may lead to increased learning (Figure 5). Because these students participated but did not ultimately pass the VF, they were required to take the written final exam. So, for this cohort, the standardized final exam was a second assessment to compare with and complement the MAT. The performance of students had a near identical trend. With one exception, performance on both the written final exam and MAT instrument increased, suggesting a dosage effect is possible as students make more attempts to pass the VF. If one views each attempt as a formative rather than summative assessment, then the process would be expected to improve student understanding.

Students Who Passed the VF Performed Better in Upper-Level Science Courses

We tracked student performance in later years, when they took their upper-level science courses. Several rounds of analQ12 yses were performed. First, grades earned in upper-level science courses by the cohort of all students who passed the VF ($n = 160$) were compared with those earned in the same courses by all other students from 1997–2010 ($n = 21{,}528$). This approach showed greater performance by the VF "passers" in all courses; however, the increase in incoming ACT scores over the time period may have conflated these results. Additional studies were done and final analysis was performed comparing two tightly matched cohorts: all students who passed the VF in the same course (Bio II) and other students who had taken Bio II the same semesters with the same instructor. This method again showed higher grades earned by VF passers than "nonpassers" (Table 1). This analysis, while promising, has the potential to be biasing the VF cohort toward students of higher academic potential, as the upper quartiles of students pass at higher rates. An expansive supplemental table lists courses studied in the analysis and side-by-side comparison of final GPA of the experimental and control cohorts in each quartile in each course (Table S1). Figure 6 illustrates a comparison quartile-by-quartile performance on course grade in biochemistry and physiology courses. Although many quartiles suffer from low n values, we note consistently higher grades earned by the VF passers across the board and significance in physiology and biochemistry courses.

Higher performance in later classes does not demonstrate causation, as it is possible that the results may reflect intrinsic differences in motivation, study skills, and so on. We used LM as a means to dissect this possibility and modeled student performance in the upper-level courses while normalizing for incoming university GPA and gender. In this LM approach, we found the VF to only show statistical significance for organic chemistry, CEM 351 (Figure S1). However, the low number of students in this path ($n = 41$) suggests caution in interpreting these results. On the other hand, passing the VF results in a higher incoming GPA, thus masking its impact somewhat. Using pre–biology course predictors to estimate upper-level science grades resulted in weak models that explained only a small portion of the variance.

Participation Balance from a Perspective of Academic Ability, Gender, and Ethnicity

Overall, of the 445 students in the study, 160 (36%) passed the VF. In an attempt to tease apart participation from pass

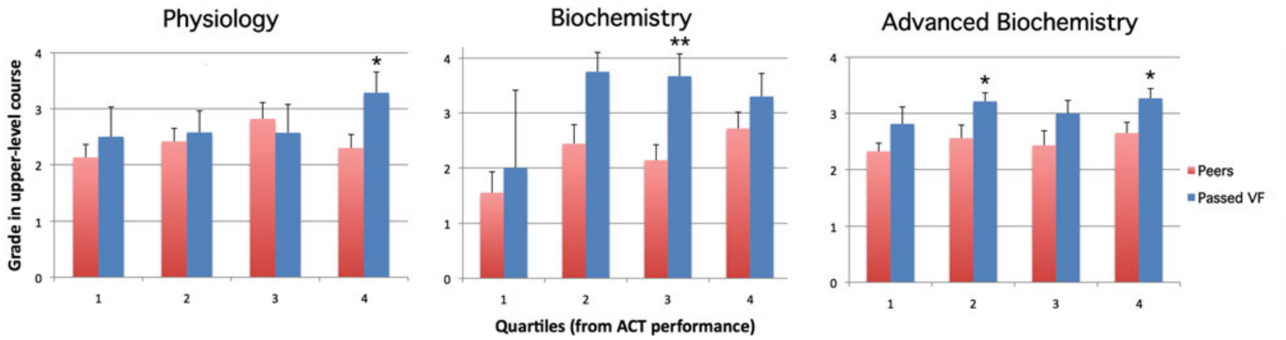


Figure 6. A comparison of students who passed the VF vs. peers in upper-level science courses. Performance differences in upper-level science courses comparing students who passed VF with matched peer controls who were in the same major over the time period and took the LB145 course with same instructor. Average course GPA (trial numbers [n], pairs left to right) in PSL 431 (Physiology I: $n = 27/5$, 18/13, 14/7, 15/7), BMB 401 (Basic Biochemistry: $n = 9/3$, 9/2, 7/3, 9/5), and BMB 461 (Advanced Biochemistry I: $n = 31/8$, 17/14, 21/12, 20/13) for VF passers and nonpassers by ACT quartiles. ANOVA: **, $p < 0.01$; *, $p < 0.05$.

rates, we also evaluated three semesters in which records were retained that indicated students who participated in the VF but never passed. Of the 174 students in those courses, 70 (40%) passed the VF, 29 (17%) participated but did not pass, and 75 (43%) did not participate.

To evaluate participation balance from a perspective of academic ability, we stratified students in quartiles by ACT scores. We found the percentage of students who passed the VF was higher in the upper half of distribution, yet a good representation of students from all academic ability levels passed (Figure 7, top). In the three semesters with participation information, participation in each quartile ranged from 19.6 to 30.8%. Albeit from just a sample of the larger data set, these data indicate participation in the VF occurred from students in all levels (Figure 7, top, bottom graph). Students from all quartiles chose to participate, and the pass rates for the top three quartiles were statistically equivalent, not skewed to the top quartile. The full range of students, not just the academically elite, availed themselves of this optional assessment.

When evaluating gender, we found a reasonable balance. While there were no statistical differences overall between the rate of male and female students attempting or passing the VF (Figure 7, bottom), it was interesting that there was a difference between the rate of male and female students attempting the VF in the third quartile (although the rate of passing within this quartile was no different).

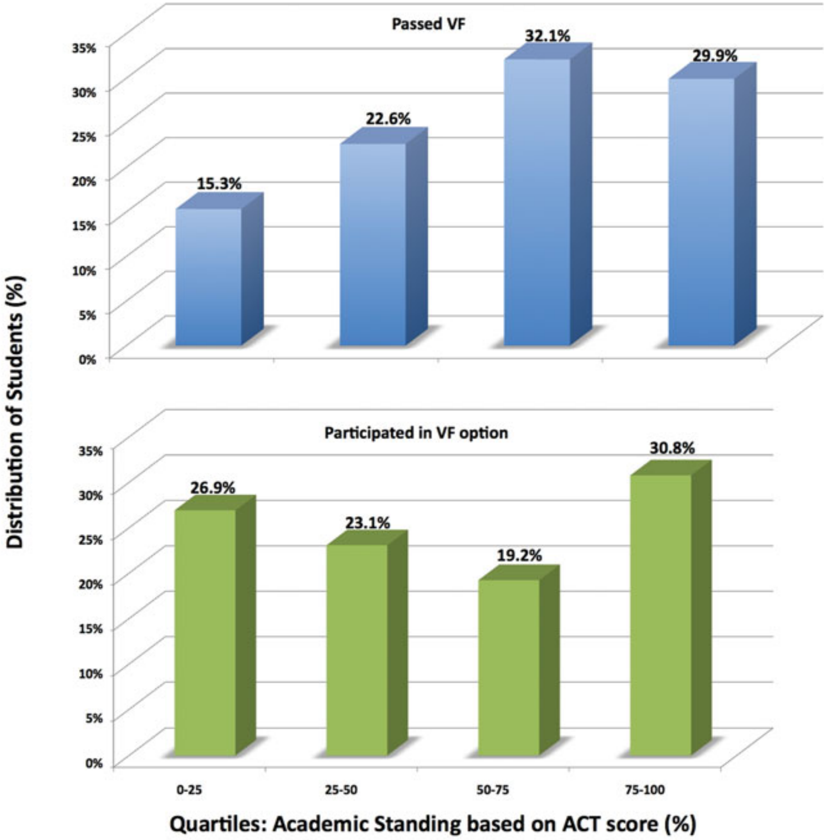
Participation balance from ethnic groups was also evaluated. We note that students who self-identified as African American and Asian had pass rates of 10 and 18% respectively, which while not statistically significant, trended below average. This finding gives pause. The pass rate of another ethnicity group, students who self-identified as “two or more races” was 60%, the highest of all cohorts. Given the error introduced by diametrical performance in overlapping categories and small sample size, we do not yet have enough data to make any statistically significant conclusions regarding potential cultural bias but have made this a focus of future study.

Student Feedback Suggests That the VF Is Valued and Increases Study

When a challenging intervention is added to a curriculum, the student response can be an important determinant of its success. The student response has been nearly unanimous in its support for the VF. Starting in 2009, we added new prompts about the VF on the course evaluation forms. Student feedback in response to the question “Do you think the VF option helps student learning?” was unanimously positive (“great or much help”: 92%; “moderate or little help”: 8%; “no help”: 0%; $n = 243$). We are analyzing student quotations as being supportive of predictions from the educational literature or alternative explanations for their learning gains. Representative exemplar comments are provided in Table S2 from both VF participants and abstainers; comprehensive student feedback is available online (Table S2; see VF website). Students’ informal feedback suggested that they recognized the VF had value, because it increased the amount of time they spent studying, changed the way they studied, and required them to verbalize their thinking. Further study is needed to determine the extent to which these variables can explain the gains students made.

DISCUSSION

Over the past decade, the biology group in our residential college has adopted a number of best practices for teaching, among them inquiry laboratories, active and cooperative learning, and a more diverse set of assessments (Luckie *et al.*, 2004, 2011, 2012; Smith and Cheruvelil, 2009, Fata-Hartley, 2011). During this process, we also had opportunities to interview students and became more keenly aware of how little they were actually learning. Even when we spoke to our “best” students, those who did extremely well on traditional exams, we often found shocking gaps in their knowledge on the most fundamental topics. These realizations were a catalyst for change. They were also somewhat liberating, because they freed instructors from a belief that coverage equaled learning (DiCarlo, 2009; Schwartz *et al.*, 2009; Knight and Wood, 2005; Morgan, 2003; Wright and Klymkowsky, 2005). We decided to cover fewer topics but to dwell in each longer and have discussions that went deeper. We also identified the fundamental ideas or concepts that we were really teaching, or more accurately, that we really wanted the students to learn, underneath the conventional notion that students needed to know certain chapters or topics (Boyer, 1998; Ausubel, 2000).



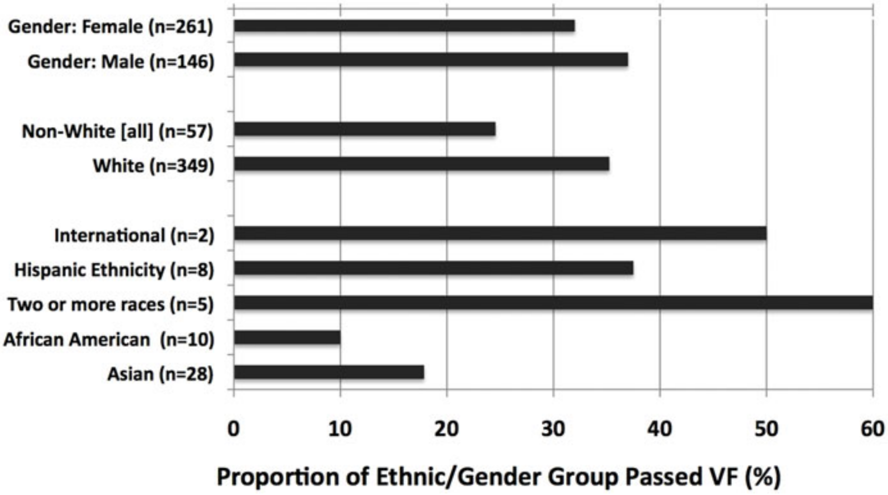


Figure 7. Participation occurs from a broad range of students with a variety of academic and cultural backgrounds. Top, the ACT was used to divide the student population of each semester into academic performance–based academic standing quartiles. All students who passed the VF were counted for each quartile ($n = 160$, z test of proportions at the $p < 0.05$ level). (a) Pass rates for quartile 1 and 2 are equivalent; (b) quartiles 2, 3, and 4 are equivalent). Distribution of students who participated but did not pass is also represented. Bottom, the self-reported ethnicity and gender of students who passed the VF was also examined. Total number of students in study from each group is represented by n, percentage of that group who passed the VF is plotted (four students did not report ethnicity). Q14

As a result, we reorganized our lecture sequence so we would be able to cover in the first half of the semester all the ideas critical for students to master in the course. We then could revisit those same concepts a second time by covering a different set of chapters or topics in the second half of the semester. Our goal was to give each student a "double dose" of the important ideas of biology. For example, for a learning objective such as "membranes work as barriers, and cells use transporters to move ions and create gradients," either the topic of photosynthesis or respiration can serve to frame the context in which we teach this core principle. A major concept in one topic, such as the role of the ATP synthase in photosynthesis, is revisited in cellular respiration, in which it has the same name and performs the same function. We hoped that reframing and revisiting the same idea or example in different contexts would help model for the students how an expert views the systems and perhaps would lead to a greater opportunity for Q15 knowledge transfer, which is further deepened by the VF experience.

This reorganization of topics taught in our introductory biology course enabled this experiment with the VF to occur. Because we discussed all of the important concepts during the first half of the course, we could administer a VF (repeatedly) to all interested students throughout the entire second half of the semester. The topics discussed during the VF were framed in one story with three parts: how photosynthesis coverts CO_2 into sugar/food, how humans digest and circulate sugar/food, and how a pancreatic cell makes insulin in Q16 response to the presence of sugar. The emphasis for greatest depth was focused on the beginning and end of the story, photosynthesis and central dogma, while the middle part was used as an affective bridge and opportunity to examine examples of cell biology in context.

Student Content Knowledge Increased

One goal in this research was to assess student learning and generate data that would be of the relevance and rigor most convincing to scientific colleagues. One important motivator for many of our students is to do well on the MCAT or Graduate Record Exam. They have a great desire to do well on placement exams and profess a desire to learn material in depth and to retain that knowledge beyond the semester. We have also found our science colleagues value student performance on those same exams, in particular the MCAT, far more than any other test we might find or create. As a result, Q17 in order to gauge student learning of content, throughout the study period, we examined student performance on a 40-question MAT exam built with MCAT test questions (Luckie *et al.*, 2004). Given that Zheng *et al.* (2008) demonstrated the MCAT assesses more than just lower-level knowledge and that it is a recognized exam for which performance is valued by our students and faculty peers, use of validated reliable passage questions derived from it seemed appropriate.

We found that students who engaged in the process and passed the VF scored the highest on end-of-semester MAT exams. These students significantly outperformed their peers who never participated in the process and those who made attempts but never passed the exam (passed VF: 66%; made attempt: 58–63%; no attempt: 57% score). Those who passed the VF appear to have more content knowledge. We also saw correlating increases in average performance on the MAT with number of VF attempts. With one exception, performance on both the course final exam and MAT instrument trend upward, suggesting a dosage effect is possible as students make more attempts to pass the VF. These data tend to support that the Socratic-styled VF is a useful assessment and learning aid.

Interestingly, students made significant gains in all topic areas tested, and the greatest gain in performance on the MAT was on questions related to the topic of cellular respiration. This topic is discussed in lecture but not during the VF. While Q18 cellular respiration is not discussed on the VF, the conceptually similar topic of photosynthesis is given the greatest amount of time and has the most in-depth treatment in the VF. We believe that this finding may be evidence of knowledge transfer, a goal in all teaching but a rather difficult task for students (McKeough *et al.*, 1995; Perkins, 1999; Schonborn and Bogeholz, 2009). Instructors always hope that, if a student masters the fundamentals and learns some examples, he or she can use that understanding to make predictions and solve problems on related but different topics or tasks. In literature reporting testing of this idea, knowledge transfer is rarely noted to occur (McKeough *et al.*, 1995; Schonborn and Bogeholz, 2009). Students who passed the VF performed better on a broad range of topics, which suggests that they were better able to grasp the fundamentals and apply their knowledge more efficiently to novel situations; this is an indicator of higher-level learning.

We predicted that students who participated in the VF option might make gains in their understanding of biology through the experience, which is a form of cognitive apprenticeship (Brown *et al.*, 1989; Collins *et al.*, 1989). The VF clearly helped them in the course itself, but the idea that the experience may help students integrate and transfer knowledge, combined with long-term effects found by Derting and others, might predict greater success in upper-level classes (Derting and Ebert-May, 2010). We therefore did a longitudinal tracking study to follow how the students did in other science courses. Several rounds of analyses were performed that showed greater performance by the VF passers in a number of courses. An expansive supplemental table lists courses studied in the analysis and side-by-side comparison of final GPA of the experimental and control cohorts in each quartile in each course (Table S1). Passing the VF correlated with higher performance in later classes.

Students Reported Gains But Not All Participate

Evidence collected indicates that students support the VF as a valued option. Student comments also present opinions that parallel those from learning theory in the literature. Students believe preparation for the VF increases study time, engagement, revisiting concepts in different contexts, and making connections between ideas from different topics. This particular intervention is not controversial and has a positive effect on the affective elements of the classroom.

Analysis revealed that, while the percentage of students who passed the VF is higher in the upper half of distribution, a good representation of students from all academic standing levels passed. In addition, a good balance of students from all quartiles chose to participate in this alternative path. Regarding gender, there is evidence of near balance. The majority of students who passed the VF over the study period were female (61%), and while slightly lower, this percentage was not statistically different from their proportion of enrolled students (64%). When evaluating ethnic background, there is more concern. African-American and Asian students had below-average participation rates, and while the high pass rate of students who self-identified themselves as "two or more races" muddles the data, it seems likely stereotype Q19 threat is impacting participation of students from minority groups.

The existence of stereotype threat, speech impairments, test anxiety, and other barriers to success is a strong argument to Q20 keep the VF an optional path with no negative consequences (Steele, 1995, 2002; Spangler, 1997; Spencer *et al.*, 1999) but more needs to be done. We purposefully designed the process to lower test anxiety and give each student the best chance to engage his or her higher-level thinking without concern that failure would be catastrophic for his or her grade. Rather than making an oral assessment mandatory in an attempt to increase learning for more students, we recognize its limitations, and our focus is to make it an event wherein demonstration of mastery only leads to rewards. New studies are in the works to test potential stereotype threat effects on participation. Using a private rather than public setting (e.g., closing classroom doors) may alter the participation and performance for female or African-American students. We also would like to understand how the presence of a female or nonmajority instructor administering the VF might improve participation.

Time on Task May Be the Mechanism

Overt verbalization and previously discussed theoretical mechanisms likely contribute to the learning gains seen. Yet an additional mechanism leading to this improvement may simply be time on task (Peters, 2004; Guillaume and Khachikian, 2011), and student self-report feedback supports this rationale. If students spend more time on any topic or task, it is likely they will gain more mastery in it. Interestingly, some education psychology research suggests that when students believe they are pursuing knowledge mastery expertise as a goal, rather than just high performance on an assessment, they will spend far more time, persist longer, and use more **Q21** elaborate study strategies to succeed (Nolen and Haladyna, 1990). This certainly could be true for the VF. The amount of time students spend preparing for the VF appears to be great. Student practice at available classroom whiteboards is evidenced by illustrations of photosynthesis found throughout the residential college each morning. Students report that they form study groups to practice together, ask students who have just passed the exam to be visiting experts in their study groups, and even recruit help from upper-level students who passed the VF in previous semesters. Teaching assistants also arrange practice VF appointments with interested students. Hence, we believe that time on task is a critical factor in student success.

Given that our goal is increased student learning, we view increasing the motivation of the students as a positive change. We are presenting the students with a different assessment approach that challenges and encourages them to spend more time preparing than they would otherwise. This activity is very public and creates an engaged culture focused on academics and learning that benefits all parties involved (DiCarlo, 2009). Logistical constraints in allowing students to retake exams make it difficult with most tests (Henderson and Dancy, 2007), but the VF is conducive to this philosophy.

Limited Faculty Time Leads to Solutions

While our colleagues may agree it is debatable whether traditional final exams are good methods to evaluate learning (John Harvard's Journal, 2010) and alternative assessments may be worth considering, it is difficult for faculty members to make changes in their teaching practices due to many factors, including limited time (Silverthorn *et al.*, 2006; **Q22** Henderson and Dancy, 2007; Ebert-May *et al.*, 2011). In the interests of efficiency, instructors implementing the VF have tested a number of different models that, when combined, may offer solutions. During the first few weeks of implementing the VF, the instructor administers exams on only one day of the week. Limitations are also set during the final weeks; for example, no new students can start the process after a certain deadline, and no more VF appointments are permitted after a certain final date (several days prior the written final exam). These strategies can limit the time the faculty member's time commitment to 5–10 h per week.

Additional time-saving approaches have included one recently used by R.D.S. in his chemistry course, in which each student only gets one chance to take the VF with the professor (Sweeder and Jeffery, 2012). Also, one published approach focuses on the learning benefits of verbal exams administered to students as a group (Boe, 1996; Guest and Murphy, 2000). Either of these alternative approaches alone or in combination could dramatically decrease the time commitment from a faculty member. Alternatively, one could test whether many of the benefits of the VF could still be retained in a nonverbal format. One expert suggested that the kinds of questions asked during the oral exam are quite sophisticated, but they could be asked on a written exam that gave students the opportunity to revise their work continuously until their work was correct. For some faculty members, the written exam idea may well be more desirable and efficient in terms of faculty time.

One very promising and effective approach has been the use of teaching assistants. In recent semesters, we required that students pass with a teaching assistant prior to getting an appointment with the professor. Our teaching assistants, called learning assistants (LAs), are undergraduates who participated in the VF process when students. Because they were outstanding students who demonstrated great interest in teaching others the biology topics they were passionate about, they were selected to be LAs. We have found LAs can be trained to be rigorous in their administration of the VF and can carry the lion's share of the load. They become just as invested in the process as when they were students (and the success of their students is a point of pride). This assistance by LAs enables faculty time to be much more protected, because well-prepared students can pass with the faculty on the first attempt. In addition to helping protect limited faculty time, this approach may provide two other benefits.

The participation by undergraduate teaching assistants in the VF process may increase participation from reluctant (and minority) students and may yield learning gains for the undergraduate LA students themselves. Our LAs are far more approachable and diverse than the typical professor. More than half are female, and a significant number are of nonmajority ethnicity. They can serve as a less threatening starting point for reluctant students and as learning coaches in the shared goal that students master the material and pass the test. Our LAs have also shared with us that participating greatly solidifies their own understanding of the material and connects it to other courses. Here are some of their reflections regarding their own learning:

> "I learned a lot from the students. They do so much extra studying which causes them to learn much more than what I will ask them . . . they tell me things that I did not know."

> "I believe that the verbal final exam experience has greatly increased my learning as an LA who administered the exams. Students will inevitably discover, and bring to the LA's attention, information that neither

> person knew before. Every year, I become a better verbal final exam proctor simply because my students are teaching me biology."

> "I believe administering the VF broadened my understanding of the material. Questioning and evaluation presents another facet to the learning process; additionally, discourse with students often challenge[s] our understanding and require[s] constant de- and reconstruction."

> "Giving verbal finals has in some ways increased my learning. I think the biggest thing is just going over the material that I learned in 145 again, but also seeing how I've learned more about certain topics in other classes like PSL and BMB. Students ask a lot of questions and sometimes make you think about/look up things as well."

> "I certainly had information re-introduced that I had forgotten since last taking LB 145. Everything came back quickly after some reviewing (probably because it was so ingrained the first time), but going over the verbal final material once more solidify [sic] certain concepts once again. Just after reviewing them for LB 145, these systems were discussed in my comparative anatomy course."

Limitations of the Study

As one considers the implications of this work, it is important to recognize some of the limitations. The course itself was offered to students who were in a science-themed residential college program, so all of the enrolled students were science majors. Although there is no reason to suspect that nonmajors would be any less capable of succeeding on a VF, the participation rate in a general biology class may be anticipated to decrease, as many students may not be as interested in gaining a deeper level of understanding of biology if they do not see an apparent benefit to their long-term career plans.

While ACT normalization helps equalize for motivation and background knowledge in the data presented in Figures 2–4, the higher level of performance of students in upper-level courses in Figure 6 should be interpreted cautiously. The statistically higher grades earned by the VF passers in all quartiles indicate only a correlation, not a causation. Taking the VF may more accurately represent a high level of motivation, dedication, or other characteristic that also leads to success in the upper-level science courses. Except in one case, CEM351, the more powerful statistical approach of using linear modeling did not show the VF as being a crucial indicator of success; however, it relied on the students' college GPA entering each course. This GPA would already reflect learning gains and concomitant inflation as a result of passing the VF (through the elevated biology grade earned and other classes it may have impacted); thus some evidence of impact would have been removed.

CONCLUSIONS

An optional VF has provided an alternate venue for assessing student understanding in our introductory biology course. As one might predict, in a quantitative assessment of cell biology content knowledge, students who passed the VF outscored their peers on related MCAT exam questions. The higher-achieving students performed better on MCAT exam questions from all topic categories tested. Success on the VF also correlated with enhanced success in multiple, subsequent, upper-level science courses.

While we provide detailed *Methods* to enable other instructors to understand the process we used in our course, these will not be very helpful to faculty members who do not teach a similar course. Perhaps more helpful are just the principles that the VF offers. We believe some important elements are these: 1) having students verbalize processes as they illustrate them; 2) allowing students to first explain a process and, when ready, to accept questions; 3) focusing on just a few topics that contain the most important fundamental concepts; 4) enabling students to understand they can repeat attempts and ultimately take the written final exam no matter whether they pass or not; and 5) using a transparent protocol with a clear rationale for when something is incorrect and giving opportunities to revise.

The evidence evaluated in this study supports that Socratic-styled oral exams can allow instructors to assess and aid students striving to achieve higher-level learning. The intervention is a significant one, but student feedback demonstrates it has no negative effect on the affective atmosphere of the classroom. In fact, the opposite is true; students who participated in the option reported it increased their study and learning, and those who chose to not participate remained supportive of the option.

While the affective (happiness) element of students is often carefully tracked in teaching, another rarely tracked element important to the success of student learning is the affective state of the instructor. **Q23** First and foremost, if you employ a verbal exam ask your students questions about the topics taught in the class, you will get a much better idea of what they learned and their level of mastery. But perhaps equally important, you will also have enjoyable experiences listening to your students thoughtfully and passionately explain to you the processes taught in your course. You will get opportunities to correct, clarify, and raise each student's understanding of the material. And you will find great satisfaction when students show increased mastery about the topics so important to their understanding of the discipline.

We hope this publication and other qualitative and quantitative studies (Dressel, 1991; Bairan and Farnsworth, 1997; **Q24** Schubert *et al.*, 1999; Mills *et al.*, 2000; Ehrlich, 2007; Elfes, 2007) help provide evidence and inspiration to our peers in science on the benefits of moving deliberately to challenge students with active, engaging teaching methods and, in particular, to use more higher-level assessments in the classroom.

ACKNOWLEDGMENTS

We thank Amanda (Gnau) Seguin for the creative idea that enabled the study presented in this article. We thank Drs. Joseph Maleszewski, John Wilterding, James Smith, Cori Fata-Hartley, Diane Ebert-May, Janet Batzli, John Merrill, Merle Heideman, Karl Smith, Mimi Sayed, Sarah Loznak, and Duncan Sibley for helpful discussions about teaching and learning and assistance during this study. We also thank the many LAs who promoted and supported these interventions. Our cystic fibrosis research laboratory is supported by Cystic Fibrosis Foundation and Pennsylvania Cystic Fibrosis grants for our physiology cystic fibrosis research program; our STEM Learning laboratory is supported by Lilly and Quality grants from MSU and

Course Curriculum and Laboratory Improvement and Transforming Undergraduate Education in Science grants from the National Science Foundation for our education research program.

REFERENCES

Baddeley AD, Hitch G (1974). Working memory. In: The Psychology of Learning and Motivation: Advances in Research and Theory, ed. GH Bower, New York: Academic, *8*, 47–89.

Bairan A, Farnsworth B (1997). Oral exams: an alternative evaluation method. Nurse Educ *22*, 6–7.

Bloom BS (ed.) (1956). Taxonomy of Educational Objectives: Book 1, Cognitive Domain, New York: Longman.

Q25 Boe BL 1996. Diversity in assessment procedures. Paper presented at the 48th American Association of Colleges for Teacher Education Conference, held 21–24 February 1996, in Chicago, IL.

Brown JS, Collins A, Duguid P (1989). Situated cognition and the culture of learning. Educ Res *18*, 32–42.

Bruner JS (1973). The Relevance of Education, New York: Norton.

Carmean SL, Wier MW (1967). Effects of verbalization on discrimination learning and retention. J Verbal Learn Verbal Behav *6*, 545–550.

Collins A, Brown JS, Newman SE (1989). Cognitive apprenticeship: teaching the crafts of reading, writing, and mathematics. In: Knowing, Learning, and Instruction: Essays in Honor of Robert Glaser, ed. LB Resnick, Hillsdale, NJ: Lawrence Erlbaum, 453–494.

Creech LR, Sweeder RD (2012). Gender performance differences in life science courses. CBE Life Sci Educ *11*, 396–391.

Derting TL, Ebert-May D (2010). Learner-centered inquiry in undergraduate biology: positive relationships with long-term student achievement. CBE Life Sci Educ *9*, 462–472.

DiCarlo SE (2009). Too much content, not enough thinking, and too little fun! Adv Physiol Educ *33*, 257–264.

Dressel JH 1991. The formal oral group exam: challenges and possibilities—the oral exam and critical thinking. Paper presented at the 81st Annual Meeting of the National Council of Teachers of English, held 22–27 November 1991, in Seattle, WA. **Q26**

Ebert-May D, Derting TL, Hodder J, Momsen JL, Long TM, Jardeleza SE (2011). What we say is not what we do: effective evaluation of faculty professional development programs. BioScience *61*, 550–558.

Ehrlich R (2007). Giving bonus points based on oral exams. Am J Phys *75*, 374.

Elfes C (2007). Oral exam tests your decision-making skills. Practitioner *251*, 44–45.

Entwistle A, Entwistle N (1992). Experiences of understanding in revising for degree examinations. Learn Instruct *2*, 1–22.

Fata-Hartley C (2011). Resisting rote: the importance of active learning for all course learning objectives. J Coll Sci Teach *40*, 36–39.

Gagne RM, Smith EC (1962). A study of effects of verbalization on problem solving. J Exp Psychol *63*, 12–18.

Guest K, Murphy D (2000). In support of memory retention: a cooperative oral final exam. Education *121*, 350.

Guillaume DW, Khachikian CS (2011). The effect of time-on-task on student grades and grade expectations. Assess Eval High Educ *36*, 251–261.

John Harvard's Journal (2010). Bye-bye, blue books? Harvard Magazine *Jul–Aug*, 64–65. http://harvardmagazine.com/2010/07/bye-bye-blue-books. **Q27**

Henderson C, Dancy MH (2007). Barriers to the use of research-based instructional strategies: the influence of both individual and situational characteristics. Phys Rev ST Phys Educ Res *3*, 020102.

Inhelder B, Piaget J (1958). The Growth of Logical Thinking from Childhood to Adolescence, New York: Basic.

Krontiris-Litowitz J (2009). Articulating scientific reasoning improves student learning in an undergraduate anatomy and physiology course. CBE Life Sci Educ *8*, 309–315.

Labuhn AS, Zimmerman BJ, Hasselhorn M (2010). Enhancing students' self-regulation and mathematics performance: the influence of feedback and self-evaluative standards. Metacogn Learn *5*, 173–194.

Luckie D, Harrison SH, Ebert-May D (2011). Model-based reasoning: using visual tools to reveal student learning. Adv Physiol Educ *35*, 59–67.

Luckie DB, Aubry JR, Marengo BJ, Rivkin AM, Foos LA, Maleszewski JJ (2012). Less teaching, more learning: 10-yr study supports increasing student learning through less coverage and more inquiry. Adv Physiol Educ *36*, 325–35.

Luckie DB, Maleszewski JJ, Loznak SD, Krha M (2004). Infusion of collaborative inquiry throughout a biology curriculum increases student learning: a four-year study of "Teams and Streams." Adv Physiol Educ *28*, 199–209.

Q28 McKeough A, Lupart JL, Marini A (1995). Teaching for Transfer: Fostering Generalization in Learning, New York: Psychology Press.

Mills PA, Sweeny WV, DeMeo S, Marino R, Clarkson S (2000). Using poster sessions as an alternative to written examination—the poster exam. J Chem Educ *77*, 1158–1161.

Momsen JL, Long TM, Wyse SA, Ebert-May D (2010). Just the facts? Introductory undergraduate biology courses focus on low-level cognitive skills. CBE Life Sci Educ *9*, 435–40.

Paul R, Elder L (2006). The Art of Socratic Questioning, Dillon Beach, CA: Foundation for Critical Thinking.

Q29 Perkins D (1999). The many faces of constructivism. Educ Leadership *57*, 6–11.

Peters E (2004). Maximize student time on task. Science Scope *28*, 38–39.

Pintrich PR (1998). A process-oriented view of student motivation and cognition. In: Improving Teaching and Learning through Research: New Directions for Institutional Research, ed. J Stakr and L Mets, San Francisco: Jossey Bass, 57, 65–79.

Posner GJ, Strike KA, Hewson PW, Gertzog WA (1982). Accommodation of a scientific conception: toward a theory of conceptual change. Sci Educ *66*, 211–227.

Rauschenberger MM, Sweeder RD (2010). Gender performance differences in biochemistry. Biochem Mol Bio Ed *38*, 380–384.

Schonborn KJ, Bogeholz S (2009). Knowledge transfer in biology and translation across external representations: experts' views and challenges for learning. Int J Sci Math Educ *7*, 931–955.

Schubert A, Tetzlaff JE, Tan M, Ryckman JV, Mascha E (1999). Consistency, inter-rater reliability, and validity of 441 consecutive mock oral examinations in anesthesiology: implications for use as a tool for assessment of residents. Anesthesiology *91*, 288–298.

Schunk DH (1986). Verbalization and children's self-regulated learning. Contemp Educ Psychol *11*, 347–369.

Q30 Seymour E, Wiese D, Hunter A, Daffinrud SM (2000). Creating a better mousetrap: on-line student assessment of their learning gains. Paper presented at the National Meeting of the American Chemical Society, in San Francisco, CA.

Silverthorn DU, Thorn PM, Svinicki MD (2006). It's difficult to change the way we teach: lessons from the Integrative Themes in Physiology curriculum module project. Adv Physiol Educ *30*, 204–214.

Smith J, Cheruvelil K (2009). Using inquiry and tree-thinking to "March through the animal phyla": teaching introductory comparative biology in an evolutionary context. Evol Educ Outreach *2*, 429–444.

Spangler G (1997). Psychological and physiological responses during an exam and their relation to personality characteristics. Psychoneuroendocrinology *22*, 423–441.

Spencer S, Steele CM, Quinn D (1999). Stereotype threat and women's math performance. J Exp Soc Psych *35*, 4–28.

Spicer J (2005). Making Sense of Multivariate Data Analysis, Thousand Oaks, CA: Sage.

Q31 Steele CM, Aronson J (1995). Stereotype threat and the intellectual test performance of African Americans. J Pers Soc Psychol *69*, 797–811.

Q32 Steele CM, Spencer SJ, Aronson J (2002). Contending with group image: the psychology of stereotype and social identity threat. Adv Exp Soc Psych *34*, 379–440.

Stumpf SE (1983). Philosophy: History and Problems, New York: McGraw-Hill.

Q33 Sweeder RD, Jeffery KA (2012). A comprehensive general chemistry demonstration. J Chem Educ *90*, 96–98.

Sweeder RD, Jeffery KA, McCright AM (2012). Lyman Briggs College: an innovative living-learning community for STEM education. Qual Approach High Educ *3*, 7–14.

Thorburn M, Collins D (2006). Accuracy and authenticity of oral and written assessments in high-stakes school examinations. Curriculum J *17*, 3–25.

Tobias S (1992). Revitalizing Undergraduate Science: Why Some Things Work and Most Don't, Tucson, AZ: Research Corporation.

Tobias S, Raphael J (1997). The Hidden Curriculum—Faculty-Made Tests in Science, Part 1: Lower-Division Courses, New York: Plenum.

Traub RE, MacRury K (1990). Multiple-choice vs. free response in the testing of scholastic achievement. In: Test und tends 8: jahrbuch der pädagogischen diagnostik, ed. K Ingenkamp and RS Jager, Weinheim, Germany: Beltz Verlag, 128–159.

Vygotsky LS (1962). Thought and Language, Cambridge, MA: MIT Press.

Wilder L, Harvey DJ (1971). Overt and covert verbalization in problem solving. Commun Monogr *38*, 171–176.

Zheng AY, Lawhorn JK, Lumley T, Freeman S (2008). Assessment. Application of Bloom's taxonomy debunks the "MCAT myth." Science *319*, 414–415.

HIGHLIGHT:

The authors studied gains in student learning when curriculum was changed to include an optional verbal final exam (VF). Students who passed the VF outscored peers on MCAT questions (66.4% [n=160] and 62% [n=285], respectively; $p < 0.001$), and passing the VF also correlated with higher performance in a range of upper-level science courses.

Queries

Q1: AQ: Should second affiliation read: "STEM Learning Laboratory, Lyman Briggs College, and Department of Physiology"? Also, are affiliations correct as set?

Q2: AQ: This article has been lightly edited for grammar, style, and usage. Please compare it with your original document and make changes on these pages. Please limit your corrections to substantive changes that affect meaning. If no change is required in response to a question, please write "OK as set" in the margin. Copy Editor

Q3: AQ: Is "predicated" meant here? Please check sentence.

Q4: AQ: Okay to add?

Q5: AQ: Okay?

Q6: AQ: Okay to add?

Q7: AQ: Okay as edited?

Q8: AQ: Okay edits here?

Q9: AQ: Please define.

Q10: AQ: Okay as edited?

Q11: AQ: Okay as edited?

Q12: AQ: Okay to add?

Q13: AQ: Please add references to list for these citations and Boyer (1998) and Ausubel (2000) below.

Q14: AQ: No (a) and (b) seen on top part of figure. Please check.

Q15: AQ: Okay?

Q16: AQ: Okay to add?

Q17: AQ: Not clear whether this phrase applies to gauging student learning or examining student performance.

Q18: AQ: Okay as edited? Please note this repeats text from earlier in the article.

Q19: AQ: Change to "muddies"?

Q20: AQ: Please add references to list for Steele citations. Or are Steele and Aronson and Steele *et al*. meant here?

Q21: AQ: Please add reference to list for this citation.

Q22: AQ: Please check edits here.

Q23: AQ: Okay to add?

Q24: AQ: Okay as cited?

Q25: AQ: Okay to place here? Or "48th Annual"?

Q26: AQ: Okay?

Q27: AQ: Give recent access date.

Q28: AQ: Okay as edited?

Q29: AQ: Okay pages as added?

Q30: AQ: Please supply dates for meeting.

Q31: AQ: Please cite in text.

Q32: AQ: Please cite in text.

Q33: AQ: Okay as updated?

SAFETY IN THE LABORATORY, AN INFORMED CONSENT STATEMENT FOR STUDENTS IN LYMAN BRIGGS COLLEGE CLASSES

Regulations and guidelines, however well conceived, are not sufficient to achieve safe laboratory practice. It is the skill, knowledge and basic common sense of the individual laboratory worker that is crucial to a safe laboratory situation. To this end, I assume the following responsibilities:

1. To make myself aware, to the best of my ability, of all safety instructions supplied by the instructor in charge of my laboratory section and to read all safety instructions issued to me. To be familiar with the Right-to Know information, Material Safety Data Sheets, and Disposal of Hazardous Waste Documents. If new hazards come to my attention, I will communicate these to the instructor in charge of my laboratory, to the person in overall charge of the laboratory instruction program, or to the unit safety committee.
2. To comply fully with all established safety regulations and practices. This applies especially, but is not restricted, to hazards connected with biological materials, chemicals, radioactive materials, electrical devices, mechanical projectiles, devices involving heat or flames, glassware and other breakable materials, and laser and other bright light sources.
3. To consult the laboratory or course instructor for advice in circumstances where safe practice is in doubt.
4. To limit laboratory work strictly to projects and procedures that are scheduled, described and authorized by the course syllabus, laboratory manual or instruction sheets, by the person in charge of my laboratory, or by my instructor.
5. To warn visitors to the laboratory of existing hazards, and when necessary to inform them of School and University safety rules and regulations. Unoccupied laboratories must be locked.

I have read and understand the above responsibilities and agree to observe them in my work in this laboratory. I recognize that I will be working with potentially hazardous materials and/or equipment and risk is involved if safety regulations are not adhered to.

____________________	____________________
Student Signature	Date
____________________	____________________
Student Number	Course/Sect & Semester/Year

Michigan State University

Release of Information Authorization form

Student Name ____ **Student Number** _____________________

I hereby authorize Michigan State University to release the following educational record information:

____
____
____

to (*provide name and address of person/agency to receive information)*: _____________________________________
____
____

for the purpose of:____
____
____

I understand that I have the right not to consent to the release of my education records and I have the right to receive a copy of such records upon request.

Time limit *(consult with the department/office to determine the most appropriate option)*:

__________ I understand this consent is in effect this one instance; once this request is fulfilled, the consent will be null and void.

__________ I understand this consent shall remain in effect until revoked by me, in writing, and delivered to Michigan State University. However, any revocation shall not affect disclosures previously made by Michigan State University prior to the receipt of any such written revocation.

____ ______________________________
Student's Signature Date

Information released to a third party pursuant to this authorization is subject to the confidentiality provisions provided under the Family Educational Rights and Privacy Act (FERPA) and may not be made available to any other party without the written consent of the student.

By George Edward Hyde, Esquire

Draconian Contract

- **Purpose**

  To establish contractually binding ground rules for a team if the previously existing contract generated by the group is unclear and inadequately detailed.

- **Professional Agreement**

  As a member of this Research Team, I agree to do my part of the project—based on my designated Team Role (Pg.21). In addition to doing my part, I agree to putting in an equal work effort in order to pull my weight for the team. Also, I agree to bring an outstanding work ethic to the team—so that we may achieve a 4.0 level grade in the course. Therefore, I agree to take on all of the course work that a 4.0 level grade entails. Finally, I agree to treat my teammates with the upmost respect—by uphold all terms of this binding contract.

- **Clause I -Established Meeting and Communication Expectations:** Ground rules that will set up a known time for students to meet—thus allowing them to guarantee that additional out of lab hours will be put it for this course. In addition, to set up a known communication plan that includes the means of communication that will be utilized (with the preference included). Finally, establishing time and place to meet prior to the beginning of lab.

  A. Team Meeting
     1. **As a team we will meet bi-weekly for laboratory.** The location of the meeting will be the East Lounge of East Holmes Hall.
        I. Sunday – 9am to 10am
           a. Prepare for the upcoming weeks lab.
           b. Discuss the assigned pre-readings.
           c. Formulate a plan for use of in lab hours.
              i. See Appendix for Plan Worksheet
           d. Observe Proposed Animal Communication Behavior
        II. Friday – 6pm to 7pm
           a. Discuss what was presented in lab that week.
              i. Lecture topic notes
              ii. Assignments
           b. Gather data from Observations
              i. Work on Manuscript and Documentary
                 - Divide and Conquer using Team Roles (Pg.21)
           c. Formulate a plan for the next meeting
              i. See Appendix for Meeting Plan Worksheet
     2. **All members MUST attend the Team Meetings.**
        I. In the case where a member will be unable to attend, they must contact all members 24 hours in advance.
           a. Maximum Excused Absences = 2 meetings

   II. If a member does not contact the team 24 hours in advance, or misses more than 2 meetings—**Go To Clause II.**

B. Team Communication
  1. As a team we will communicate through the following means in order…
     I. Cell Phone Group Message
        a. ALL members will respond to a given message within an HOUR of message being delivered.
           i. If a member does not follow the ground rules—**Go To Clause II.**
     II. Team Email—using strictly @msu.edu
        a. ALL members will respond to a given message within an HOUR of message being delivered.
           i. If a member does not follow the ground rules—**Go To Clause II.**

C. Pre Lab Meetings
  1. ALL members will show up to each scheduled lab class meeting
     I. Prior to lab beginning, ALL members will meet 10 minutes before regular class, and 15 minutes before class when important assignments are due (i.e. Manuscripts)
        a. Location = Directly Outside of C4 Holmes Hall

❖ **Clause II -Meeting and Communication Breach of Contract Incidents:** Ground rules to establish a known protocol concerning how the team will deal with a breach of contract. A breach of contract, by definition, is time when a binding agreement has not been honored by one or more parties involved in the agreement. When breach of contract has occurred, a statement must be filed with the LB144 teaching assistant (TA) that states how the contract was breached and who has not honored the agreement.

A. Not Attending Meetings
  1. As stated in Clause IA Paragraph 2 Subset I—Any member who is not able to attend a scheduled mandatory meeting must contact his/her team 24 hours in advance. Also, any member is allowed 2, and ONLY 2, excused absences from the mandatory meetings. While Clause IC Paragraph 1 states the team will meet prior to lab.
     I. If any member fails to uphold this portion of the agreement a breach of contract has occurred.

        a. First Incident Claim
           i. If any member fails to contact the team 24 hours in advance of missing a mandatory team meeting or has missed a total of 3 team meetings or has missed a pre lab meeting, the team must declare this breach of contract BOTH in the CATME feedback surveys as well as in writing. The CATME feedback surveys will occur regularly throughout the

semester. A hard copy of written notice must be given directly to the offender(s) and a hard copy also provided to the TA in lab section. The hard copy allows the LB144 Teaching Team to date and file the initial claim for record.

  - ➢ The form for breach of contract written claims can be found in the Appendix.
    - ▪ A new clam is not permitted for 2 weeks.

b. Second Incident Claim

i. If any member fails to contact the team 24 hours in advance of missing a mandatory team meeting (for the Second Time) or has missed a total of 4 team meetings or has missed a pre lab meeting, the team must declare this breach of contract BOTH in the CATME feedback surveys as well as in writing. The CATME feedback surveys will occur regularly throughout the semester. While the writing must be given directly to the offender(s) and a hard copy provided to the TA in class. The hard copy allows the LB144 Teaching Team to date and file the claim for record.

  - ➢ The form for breach of contract written claims can be found in the Appendix.

ii. In addition, the team and TA must arrange a date for a Face-to-Face Encounter—in which ALL team members MUST attend.

  - ➢ Failure to attend the Face-to-Face encounter will result in immediate dismissal of the member(s) from the team—**Go To Clause IV.**
    - ▪ A new claim is not permitted for 2 weeks.

c. Third (and Final) Incident Claim

i. If any member fails to contact the team 24 hours in advance of missing a mandatory team meeting (for the Third Time) or has missed a total of 5 team meetings or has missed a pre lab, the team must declare this breach of contract BOTH in the CATME feedback surveys as well as in writing. The CATME feedback surveys will occur regularly throughout the semester. While the writing must be given directly to the offender(s) and a hard copy provided to the TA in class. The hard copy allows the LB144 Teaching Team to date and file the claim for record.

  - ➢ The form for breach of contract written claims can be found in the Appendix.

- ii. In addition, a hard copy of the claim must be provided to the professor (Dr. Luckie). The professor hard copy must comprehensively detail all past events, which led to all prior claims, and the current event.
    - ➢ The *professor form* for breach of contract written claim can be found in the Appendix.
- iii. In addition, the team must contact the professor to setup a mandatory Face-to-Face encounter—in which ALL members must attend.
    - ➢ Failure to attend the Face-to-Face encounter will result in immediate dismissal of the member(s) from the team—**Go To Clause IV.**
    - ➢ If the professor agrees with the claims…
        - ▪ Divorce Clause proceedings begin immediately and can only be completed 2 weeks after the professor meeting.
    - ➢ If the professor does not agree with the claims…
        - ▪ The team will remain with all members in good standing. Unless the team decides to initiate the Divorce clause themselves—which at that point can only be completed 4 weeks after the professor meeting.

B. Not Following Communication Norms

1. As stated in Clause IB—any member of the must communicate in a timely and respectable fashion. The time period for all response must be within an hour of the original message.
    - I. If any member fails to uphold this portion of the agreement a breach of contract has occurred.

a. First Incident Claim

- i. If any member fails to respond to a group message within an hour, the team must declare the breach of contract to the LB144 TA. In order for the team to successfully submit a claim they must provide a hard copy to the offender(s) and provide a hard copy to the TA, along with a screenshot proving a lack of communication, during class. The hard copy allows the LB144 Teaching Team to date and file the initial claim for record.
    - ➢ The form for breach of contract can be found in the Appendix.
        - ▪ A new claim is not permitted for 2 weeks.

b. Second Incident Claim

- i. If any member fails to respond to a group message within an hour, the team must declare the breach of contract to the LB144 TA. In order for the team to successfully submit a claim they must provide a hard copy to the offender(s) and provide a hard copy to the TA, along with a screenshot proving a lack of communication, during class. The hard copy allows the LB144 Teaching Team to date and file the claim for record.
    - ➢ The form for breach of contract can be found in the Appendix.
- ii. In addition, the team and TA must arrange a date for a Face-to-Face encounter—in which ALL members must attend.
    - ➢ Failure to attend the Face-to-Face Encounter will result in immediate dismissal of the member(s) from the team—**Go To Clause IV.**
        - ▪ A new clam is not permitted for 2 weeks

c. Third (and Final) Incident Claim

- i. If any member fails to respond to a group message within an hour, the team must declare the breach of contract to the LB144 TA. In order for the team to successfully submit a claim they must provide a hard copy to the offenders(s) and provide a hard copy to the TA, along with a screenshot proving a lack of communication, during class. The hard copy allows the Lb144 Teaching Team to date and file the claim for record.
    - ➢ The form for breach of contract can be found in the Appendix.
- ii. In addition, a hard copy of the claim must be provided to the professor (Dr. Luckie). The professor hard copy must comprehensively detail all past events, which led to all prior claims, and include all screenshots provided to the TA.
    - ➢ The *professor form* for breach of contract can be found in the Appendix.
- iii. In addition, the team must contact the professor to setup a mandatory Face-to-Face encounter—in which ALL members must attend.
    - ➢ Failure to attend the Face-to-Face encounter will result in immediate dismissal of the

member(s) from the team—**Go To Clause IV.**

- If the professor agrees with the claims…
  - Divorce Clause proceedings begin immediately and can only be completed 2 weeks after the professor meeting.
- If the professor does not agree with the claims…
  - The team will remain with ALL members in good standing—in which the professor drafts a new communication clause. Unless the team decides to initiate the Divorce clause themselves—which can only be completed 4 weeks after the professor meeting.

- **Clause III- Break of Contract Grievances related to *Performance Expectations*:** Ground rules to establish a known protocol concerning how the team will deal with grievances. A grievance, by definition, is a feeling of being wronged (or treated unfairly)—in which a statement is made stating <u>HOW</u> one was wronged and <u>WHY</u> one is not satisfied with an occurrence. Thus, in LB144 a grievance can occur from the following situations listed in this clause.

A. **Performance Expectations**

1. **As stated by the Professional Agreement section of this contract—ALL members who sign this document agreed to** …
   - I. Do their part based on Team Roles (Pgs. CoursePack)
   - II. Put forth Equal Work Effort.
   - III. Work Ethic equivalent to achieve a 4.0-level grade.
     - a. Completing all work needed for a 4.0-level grade.
   - IV. Respect all terms of the contract.

2. **Failure to uphold any of the four previously stated Professional Agreements may lead to grievances within the team.**

   - **I. First Incident Claim**
     - a. If an individual feels any of the Professional Agreements are not being fulfilled, they must declare it in the CATME feedback survey, provide a written hard copy for the TA, and have an interventional meeting (5 minutes maximum) in lab. The hard copy allows the LB144 Teaching Team to date and file the initial grievance for record.
       - i. A new grievance is not permitted for 2 weeks.
         - The grievance claim form can be found in the Appendix.

II. **Second Incident Claim**

   a. If an individual feels any of the Professional Agreements are not being fulfilled, they must declare it in the CATME feedback survey, provide a written hard copy for the TA, and have an interventional meeting (5 minutes maximum) in lab. The hard copy allows the LB144 Teaching Team to date and file the initial grievance for record.
      i. A new grievance is not permitted for 2 weeks.
         - ➢ The grievance claim form can be found in the Appendix.

   b. In addition, the team and TA must arrange a date for a Face-to-Face Encounter—in which ALL members must attend.
      i. Failure to attend the Face-to-Face Encounter will result in immediate dismal of the member(s) from the team—**Go To Clause IV.**
         - ➢ A new grievance is not permitted for 2 weeks.

III. **Third (and Final) Incident Claim**

   a. If an individual feels any of the Professional Agreements are not being fulfilled, they must declare it in the CATME feedback survey, provide a written hard copy for the TA, and have an interventional meeting (5 minutes maximum) in lab. The hard copy allows the LB144 Teaching Team to date and file the initial grievance for record.
      i. The grievance claim form can be found in the Appendix

   b. In addition, a hard copy of the claim must be provided to the professor (Dr. Luckie). The professor hard copy must comprehensively detail all past events, which lead to prior grievances, and the current event.
      i. The professor grievance claim form can be found in the Appendix.

   c. In addition, the team and professor must arrange a date for a Face-to-Face Encounter—in which ALL members must attend.
      i. Failure to attend the Face-to-Face Encounter will result in immediate dismal of the member(s) from the team—**Go To Clause IV.**
      ii. If the Professor agrees with the claim…

        - ➢ Divorce Clause begins immediately and can only be completed 2 weeks after the professor meeting.
    - iii. If the Professor does not agree with the claim…
        - ➢ The team will remain with ALL members in good standings. Unless the team decides to initiate the Divorce Clause themselves—which can only be completed 4 weeks after the professor meeting.

- ❖ **Clause IV: *Prenuptial* agreement basis for "Divorce" proceedings.** In any case where a team feels that the environment is no longer suitable—and a member is "fired" or decides to leave. The intellectual property of the group must be distributed, which is outlined in this clause.

    A. **QUITTING** In the case where a Team Member decides to willingly separate from their group…
    1. ALL Intellectual Property is maintained within the Team.
        - I. Deciding to separate from your team is equivalent to deciding to separate from a company. As is the case with a corporation, all products you created are retained by the company you departed. You have no rights to those products. All you the intellectual property—including your own work is retained by the remained group members. You therefore must start the lab over from zero as a group of 1 person. You cannot plagiarize your own prior writing, use your prior citations, methods, experimental design etc.
            - a. In short, you must start an entirely NEW and DIFFERENT research project.

    B. **FIRING** In the case where a Team Member is fired from the Team…
    1. ALL Intellectual Property is maintained with the Member of the Team who was fired.
        - I. Deciding to fire a member is equivalent to the team as a whole deciding to separate from the individual and all of their intellectual property. The party who was fired in this case gains the rights of the corporation when all the other group members quit. The remaining group members must, therefore, start from the lab over from Square 1 and cannot use/plagiarize their own prior writing, prior citations, methods, experimental design etc

    C. **MISC/OTHER** In any case of Divorce, one party maintains all intellectual property and the other party loses all intellectual property—having to start over. Depending on the week in which the divorce is finalized, all graded assignments prior to the divorce will remain. However, the party that is required to start over will have lost the opportunity to have the feedback from these assignments for their NEW research project.

1. Example
    - I. A team member decides to separate from the team after the Proposal and Half-Draft of the Research manuscript. As stated previously, this team member will be giving up all rights to all intellectual property of the team. In addition, all grades for these assignments will be maintained after the divorce. However, since the individual member must start a new and entirely different research project they have lost the opportunity to receive feedback at either the Proposal and Half-Draft stage of authorship of manuscript. Therefore, the first official graded assignment under this new research project will be the Draft1 of the manuscript.

D. **ONLY SOLO** In the case of any form of divorce—any party who either separates from the group, or is fired, MUST finish the semester as an INDIVIDUAL. Forming a new group or joining a pre-existing group is NOT permitted.

Contract Appendix

Sunday Team Meeting Plan Worksheet

Name(s): ____

Section: ___________________ Date: ________________________

Course-Pack Readings (Pages): ___________________

Reading Summary:

____

____

____

____

Observations:

____

____

____

____

Detailed Plan for Use of In-Lab Hours:

____

____

____

____

____

____

TA Signature: X________________________________

Friday Team Meeting Plan Worksheet

Name(s): ____

Section: ________________ Date: ____________________

Homework:

____

____

Describe Lecture Topic:

____

____

____

____

Manuscript & Documentary—What was Worked On and By Who?

____

____

____

____

Next Meeting Agenda:

____

____

____

____

TA Signature: X___________________________

TA Breach of Contract Form

Names: ____

Offender(s): ____

Claim: _____ First _____ Second _____ Third

Reason: _____Team Meeting _____ Communication _____ Pre-Lab

Date Occurred: ____________________

Description:

____

____

____

____

____

____

Section: ________ TA: _____________________________________

Student Signature: X ____

TA Signature: X ____

Date Issued: _______________________

Next Claim Issue Date (2 weeks): ________________________

Professor Breach of Contract Form

Names: ____

Offender(s): ____

Date Occurred: ____________________ Section: __________

Description:

____

____

____

____

____

____

____

____

____

____

____

____

____

Student Signature: X ____

Date Issued: _______________________

Next Claim Issue Date (2 weeks): ________________________

TA Grievance Form

Names: ____

Offender(s): ____

Claim: _____ First _____ Second _____ Third

Date Occurred: ___________________

Description:

____

____

____

____

____

____

Section: ________ TA: ___________________________________

Student Signature: X ____

TA Signature: X ____

Date Issued: ______________________

Next Claim Issue Date (2 weeks): _______________________

Professor Grievance Form

Names: ____

Offender(s): ____

Date Occurred: ____________________ Section: __________

Description:

____

____

____

____

____

____

____

____

____

____

____

____

____

Student Signature: X ____

Date Issued: _______________________

Next Claim Issue Date (2 weeks): ________________________

Divorce Form

Name: ____

Divorce Type: (Please Check One)

_____ I, ______________________, willingly have decided to separate from my group.

_____ As a team, we have decided to fire ___________________________________ .

Reason:

____

____

____

____

____

____

Date Issued: ____________________ Effective Date: _________________

Signature of ALL Team Members:

X ____

X ____

X ____

X ____

Signature of TA:

X ____

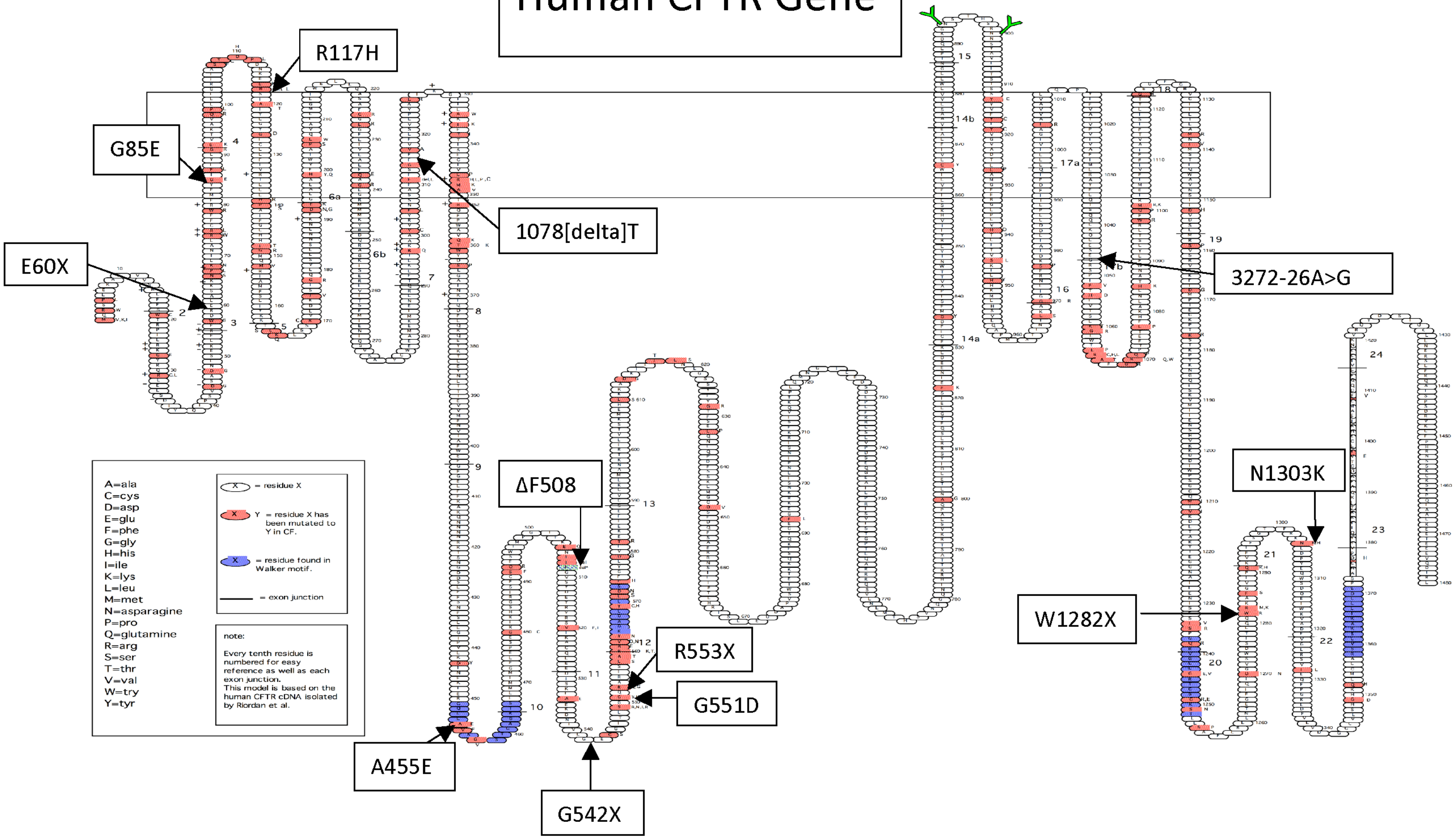
Signature of Professor:

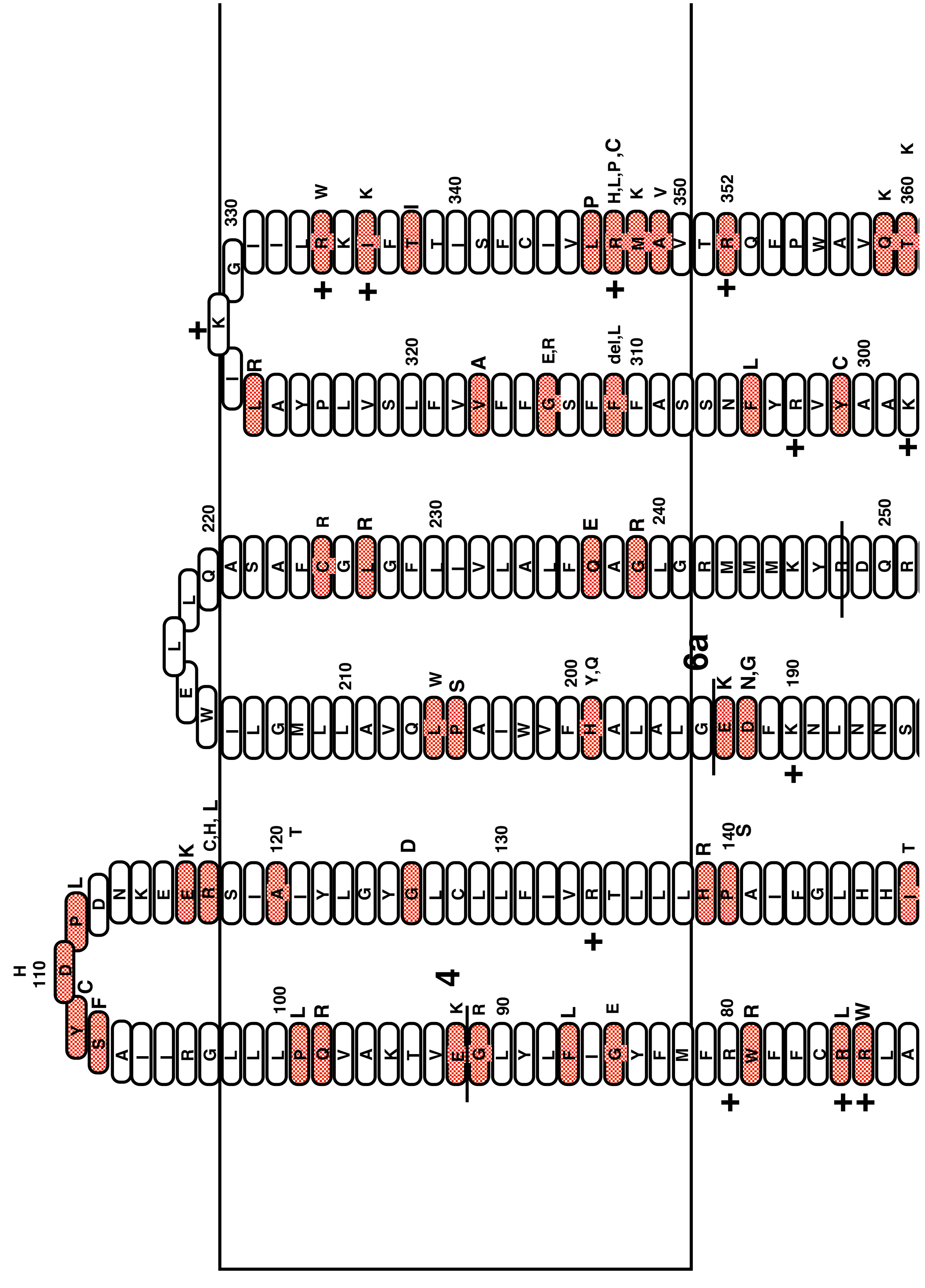
X ____

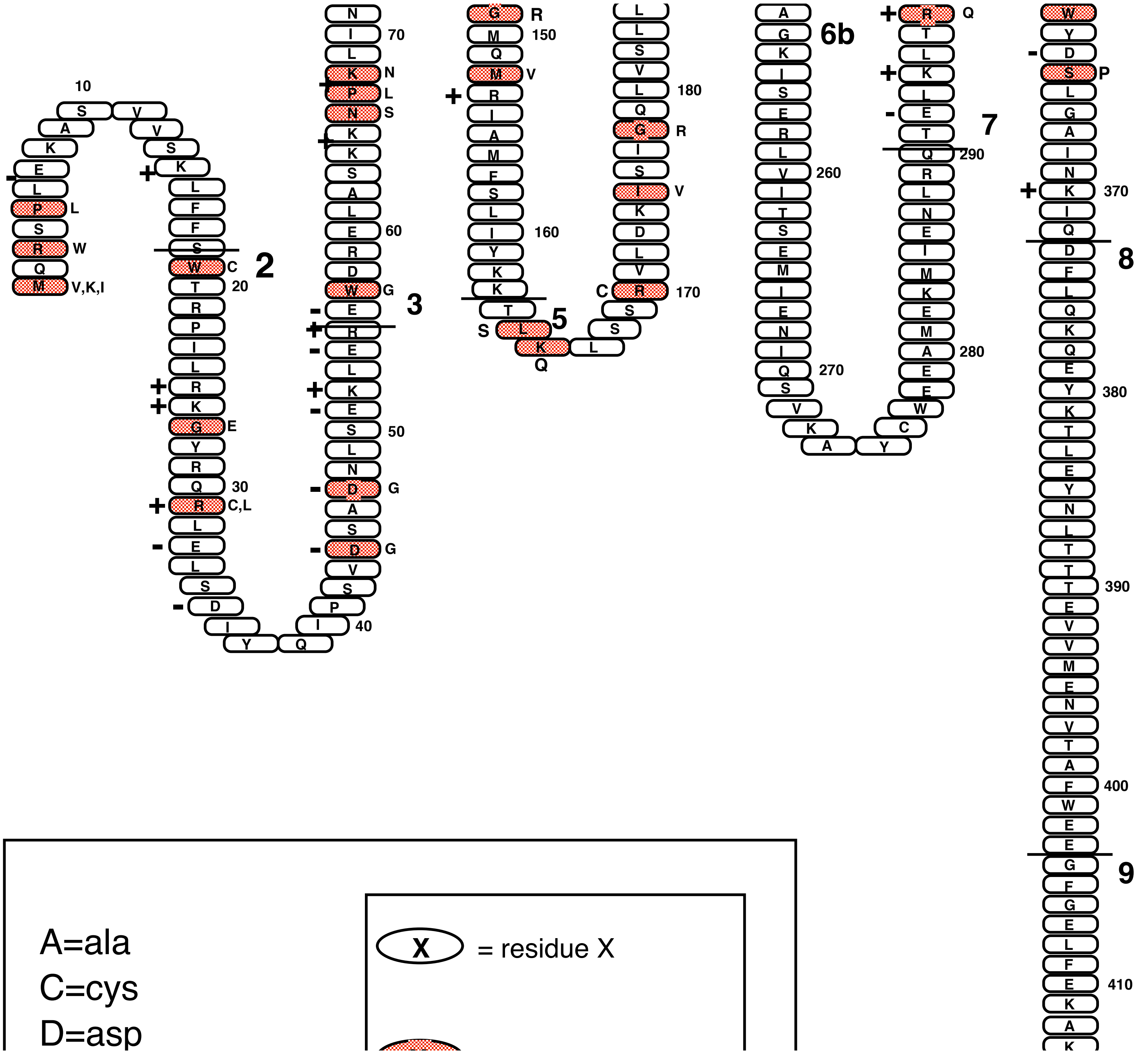
CFTR Model Poster

(cut out and piece together a poster)

Human CFTR Gene




4
6a
80
90
100
110
120
130
140
190
200
210
220
230
240
250
300
310
320
330
340
350
352
360


10
20
30
40
50
60
70
150
160
170
180
260
270
280
290
370
380
390
400
410
2
3
5
6b
7
8
9
X
= residue X
A=ala
C=cys
D=asp

E=glu
F=phe
G=gly
H=his
I=ile
K=lys
L=leu
M=met
N=asparagine
P=pro
Q=glutamine
R=arg
S=ser
T=thr
V=val
W=try
Y=tyr

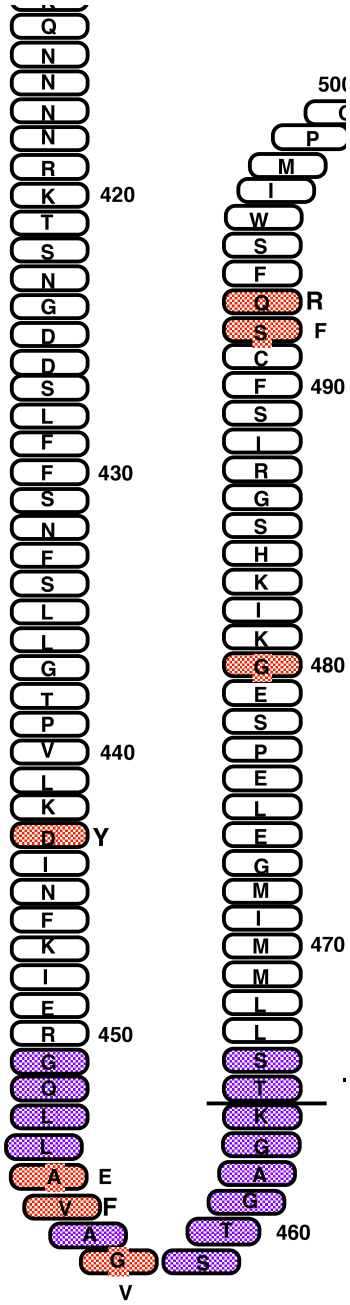
X **Y** = residue X has been mutated to Y in CF.

X = residue found in Walker motif.

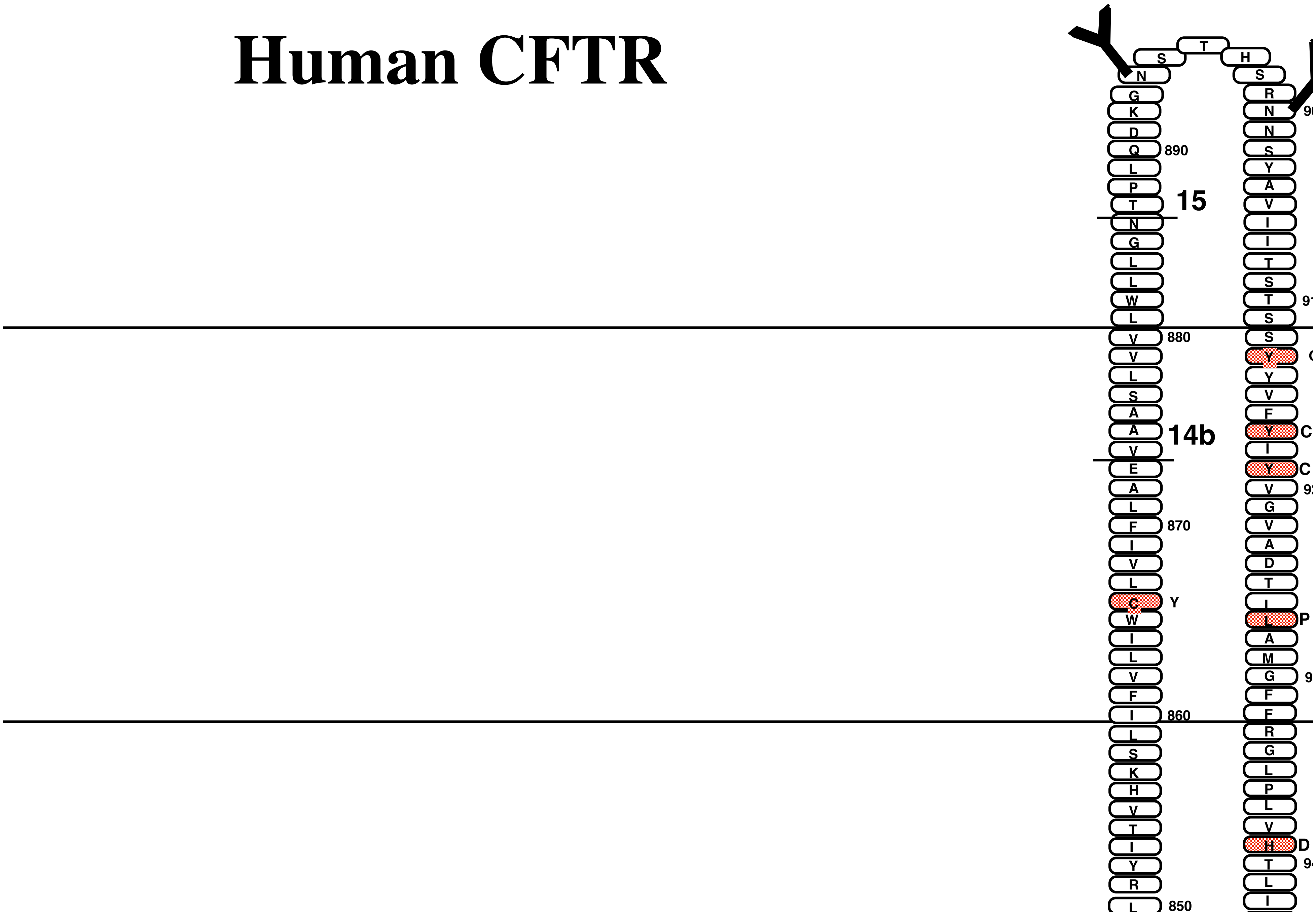
— = exon junction

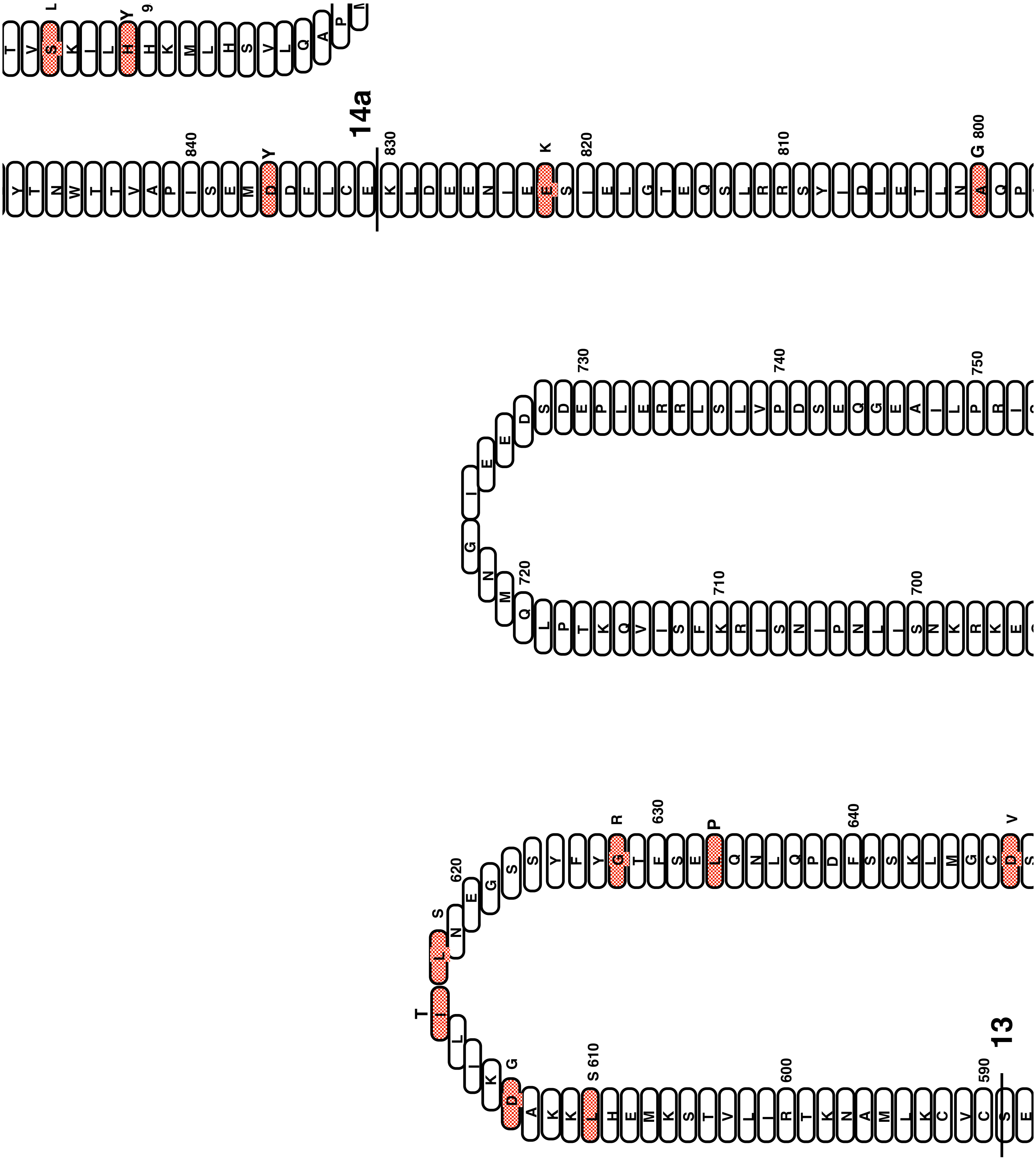
note:

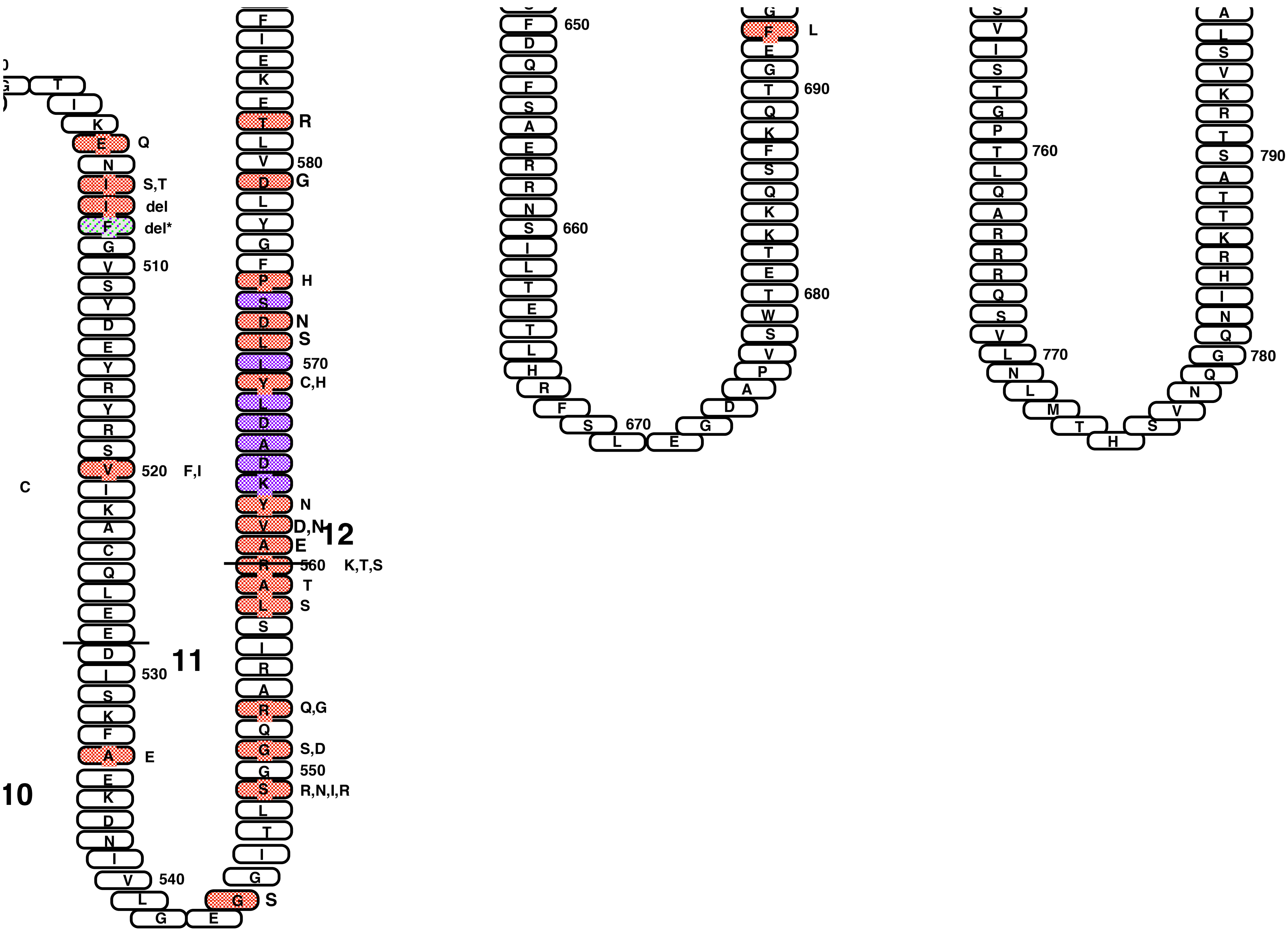
Every tenth residue is numbered for easy reference as well as each exon junction.
This model is based on the human CFTR cDNA isolated by Riordan et al.



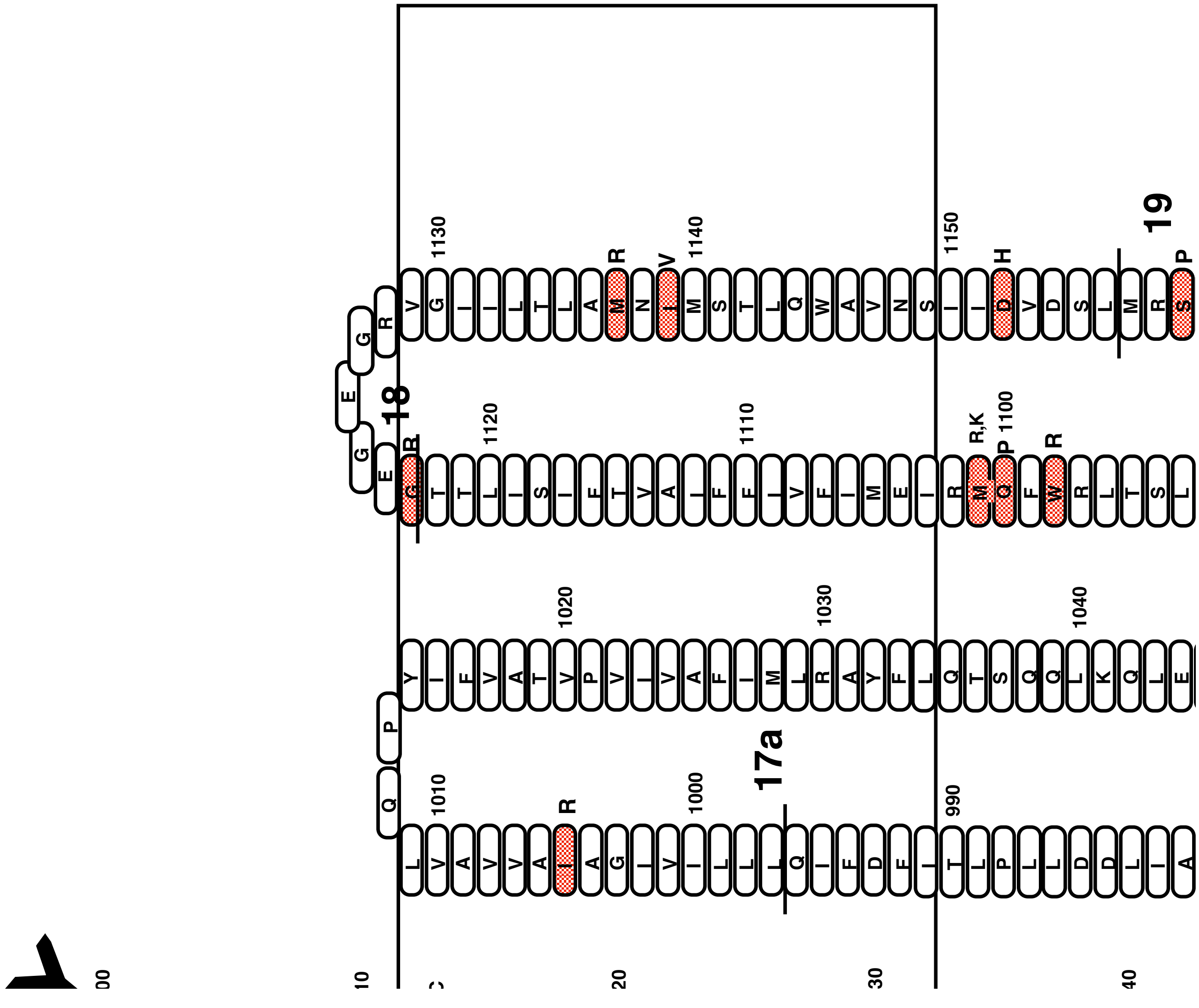
Human CFTR

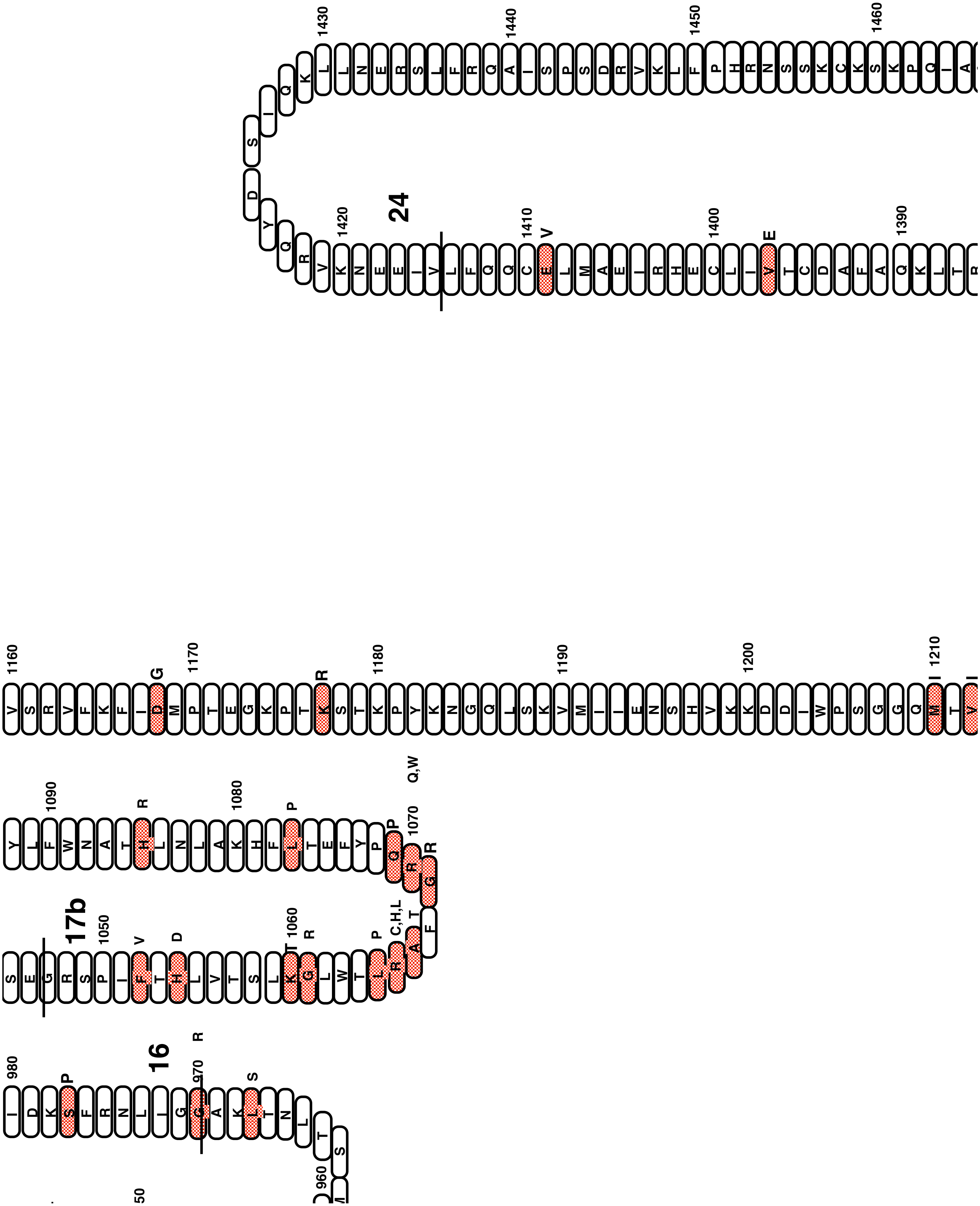


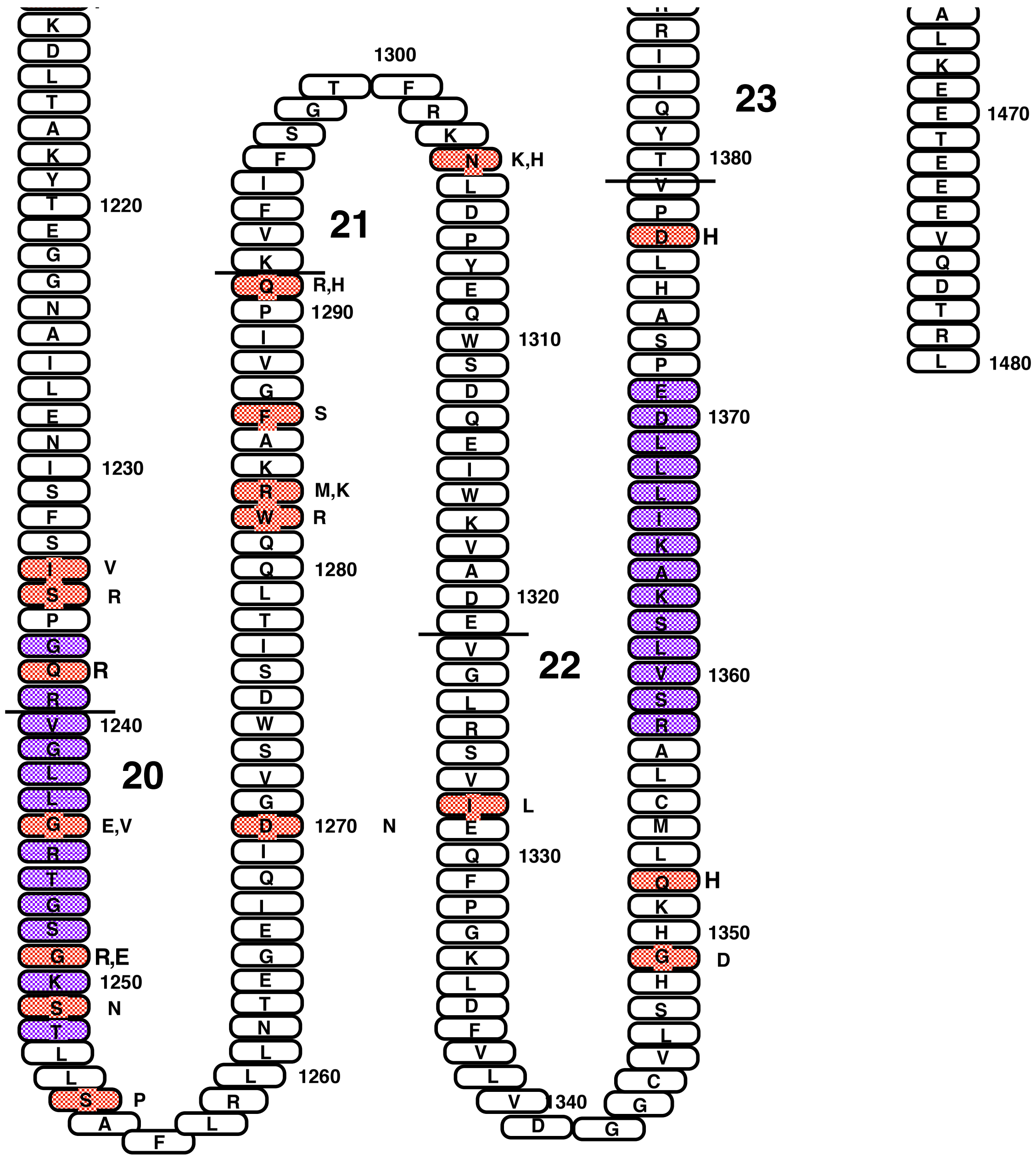

14a
13
590
600
610
620
630
640
700
710
720
730
740
750
800
810
820
830
840
9



10
11
12
C
510
520
530
540
550
560
570
580
650
660
670
680
690
760
770
780
790
Q
S,T
del
del*
F,I
E
S
R,N,I,R
S,D
Q,G
S
T
K,T,S
E
D,N
N
C,H
S
N
H
G
R
L


17a
18
19
990
1000
1010
1020
1030
1040
1100
1110
1120
1130
1140
1150
R
R
R,K
P
R
V
H
P
00
10
20
30
40


16
17b
24
50
960
970
980
1050
1060
1070
1080
1090
1160
1170
1180
1190
1200
1210
1390
1400
1410
1420
1430
1440
1450
1460



20
21
22
23
1220
1230
1240
1250
1260
1270
1280
1290
1300
1310
1320
1330
1340
1350
1360
1370
1380
1470
1480
V
R
R
E,V
R,E
N
P
N
M,K
R
S
R,H
K,H
L
D
H
H

Student Laboratory Notebook