PCR amplification detecting 1278insTATC mutation

and analysis of Tay-Sachs handicaps

Original By: A42172323

First Revision By: A41892397

Second Revision By: A42772376

*By:* A42772376, A42001872,

A41892397, and A42172323

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Professor Douglas B. Luckie

Vincent Cracolici and Katie Oleski

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**Abstract**

**Original by: A42172323**

**First revision by: A41892397**

**Final revision by: A42772376**

The mutation 1278insTATC in the Hexosaminidase alpha polypeptide (HEXA) gene on chromosome 15 is the major cause of infantile Tay-Sachs disease (TSD) and can be visualized with PCR and electrophoresis (Myerowitz and Costigan, 1988). Thus, we hypothesized the primers designed to amplify a 1019- or 1016-bp sequence in PCR, could detect the presence of the insertion. Testing purified wild-type DNA from S9 epithelial cells has not yet been successful in detecting the sequence without the insertion. Varying annealing temperatures and magnesium concentrations, only obtained non-specific binding, faint bands, and primer dimers. In a sociological experiment, researchers experienced handicaps associated with TSD. The Global Assessment of Functioning (GAF) scale, which rates social, psychological, and occupational functioning allowed for comparison of degree of debilitation caused by the handicap. We hypothesized immobility would be most debilitating because of the greater need of assistance from others (Rintala et al., 1998). An ANOVA test revealed significance between the scores of immobility and muteness.

**Introduction**

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Tay-Sachs disease is inherited in an autosomal recessive manner and is most prevalent in people of Eastern European Ashkenazi Jewish descent. The researched mutation 1278insTATC accounts for 70% of the Tay-Sachs mutations in the Ashkenazi Jewish carrier population. This mutation is a four base pair insertion on chromosome 15, exon 11, of the alpha chain of the HEXA gene (Myerowitz and Costigan, 1988). This frameshift mutation causes premature termination of the polypeptide (Campbell and Reece, 2007).

The site of the mutation on the HEXA gene codes for the α-chain polypeptide of the lysosomal enzyme β-hexosaminidase A which, when defective or absent, is the underlying cause of Tay-Sachs (Shore and Myerowitz, 1990). The enzyme, β-hexosaminidase A, is composed of an alpha-subunit and a beta-subunit, both of which are required for proper lipid digestion. β-hexosaminidase A works as part of a complex that breaks down the lipid GM2 ganglioside through hydrolysis. If no functioning alpha subunit β-hexosaminidase A can form a hydrolyzing complex with the GM2 activator protein, GM2 gangliosides cannot be broken down (Yamanaka et al., 1994). The GM2 gangliosides build up to toxic levels in the lysosome and eventually kill nerve cells. The buildup of the GM2 gangliosides is a gradual process, which explains the lack of symptoms in those homozygous recessive and less than three months old.

Symptoms of infantile TSD begin at three to six months of age when the child seems more irritable, has decreased eye contact and is easily startled. This progresses to seizures and noticeable debilitations, including the inability to grasp for objects, roll over, or crawl. Symptoms eventually reach blindness, deafness, and loss of motor skills, as well as abnormal body tone and feeding difficulties. Regression is gradual until the child becomes virtually unresponsive to their environment with death typically occurring between 2-5 years of age (Filho and Shapiro, 2004).

In the United States approximately one out of every thirty people of Jewish decent are carriers for the TSD mutation. Heterozygotes, however, produce a sufficient amount of β-hexosaminidase A to be asymptomatic. In the Ashkenazi Jewish population, 1 out of 3,600 people are homozygous recessive for the mutation (Triggs-Raine, 1990). The carrier frequency in the Ashkenazi Jew population is ten times higher than in the general population (Shore and Myerowitz, 1990). It was concluded that the high concentration of the 1278insTATC in the Ashkenazi Jewish population spread was a result of a founder effect in a population growing quickly from a bottleneck (Frisch et al., 2004).

Although there is currently no cure for TSD, DNA testing has been done to screen for heterozygotes in the Ashkenazi Jewish population since 1983, and has reduced the birth rate of children born with TSD by more than 90% in the population (Triggs-Raine, 1990). Prenatal testing for Tay-Sachs can be performed around the 11th week of pregnancy by means of chronic villi sampling or with amniocentesis around the 16th week. The polymerase chain reaction (PCR) assay was designed to provide these prenatal tests with a reliable method of testing for the presence of the 4bp insertion causing TSD.

Three primers were designed to test for the presence of the 1278insTATC mutation in human DNA. Previous studies have detected the insertion using PCR and gel electrophoresis (Shore and Myerowitz, 1990). Therefore, it was hypothesized that with the designed primers and experimentally determined annealing temperatures, PCR and gel electrophoresis could be used to accurately identify the 1278insTATC mutation in the HEXA gene. DNA was purified using the QIAGEN Genome Capture. PCR was then used to amplify a targeted region of the genome by making copies of a desired sequence that included the site of the mutation on exon 11. These PCR products were then run through gel electrophoresis, which induces a voltage causing the products in the wells of the gel to move through the gel at a rate determined by their molecular mass (Wright et al., 2010). A UV transilluminator was then used to visualize the products for interpretation. Based on the primer design, it was predicted that using forward and wild-type reverse primers would produce a 1019bp band using forward and wild-type reverse primers with wild-type DNA and no band using mutant DNA. A 1016bp band was also predicted using forward and mutant reverse primers with mutant DNA and no band using wild-type DNA.

In addition to diagnosing the 1278insTATC mutation, the sociological effects of TSD were examined. Each researcher attempted to experience a handicap common in TSD, which included blindness, deafness, immobility, and inability to speak. Psychological effects on the handicapped person, on the caretaker, and observations of reactions of those around us to the handicap were observed and compared for later use with the Global Assessment of Functioning (GAF) scale. In previous research examining the relationship between severity of chronic pain and subjective well-being, patients’ self-assessed health was worse with a greater overall pain (Rintala et al., 1998). Applying these results, it was hypothesized immobility would be most debilitating to overall functioning as it requires the greatest amount of assistance from others. Using the GAF scale, which rates social, psychological, and occupational functioning on a scale of 0 to 100, the apparent degree of debilitation was assessed by outside sources by comparing the qualitative data collected and formulating a score for each handicap.

**Methods**

**Written by A42772376**

**First revision by A42001872**

**Final revision by A41892397**

 The experiment consisted of two parts- one biological, one sociological. The biological portion included purification of genomic human DNA, PCR amplification of a 1019- or 1016-bp segment of this DNA using primers designed to detect the 1278insTATC mutation, and analysis of the PCR products using gel electrophoresis. The sociological portion involved researchers experiencing handicaps first hand, and analyzing how these handicaps affected their social, psychological, and occupational functioning as rated by the Global Assessment of Functioning scale.

*DNA Purification*

 Qiagen Inc’s “Generation Capture Column Kit” protocol was used to purify DNA from cultured cells **(**QIAGEN, 2007). The cells used were S9 cells, which are bronchial epithelial cells from a cystic fibrosis patient with the ΔF508 mutation on chromosome seven. The successfully purified DNA was then quantified using the Gen5 1.09 program by BioTek® Instruments, Inc. The quantification through this program gave the following values: Absorbance ratio 1.315 260/280, and the DNA concentration 0.008 mg/ml.

*Primer Design*

Three primers were then designed for use in PCR to amplify a segment of this purified DNA that included the location of the 1278insTATC insertion in the HEXA gene.

Forward primer: 5’-3’ TTTAATGGTCGGAGCACTCT

Reverse wild-type primer: 5’-3’ CAGTCAGGGCCATAGGATAT

Reverse mutant primer: 5’-3’ TCAGGGCCATAGGATAGATATAC

The annealing temperatures were estimated at 53.6°C for the forward primer, 53.4°C for the reverse wild-type primer, and 52.7°C for the reverse mutant primer. When the forward primer was paired with the reverse wild-type primer, a 1019-basepair segment was amplified. When the forward primer was paired with the reverse mutant primer, a 1016-basepair segment was amplified. The primers were designed using the Primer Design section of the LB145 Coursepack (Wright et al., 2010). Primers were designed using the HEXA sequence obtained from the National Center for Biotechnology Institute GenBank. Primers were purchased from Integrated DNA Technologies.

*Polymerase Chain Reaction*

 These primers were then used in polymerase chain reaction to amplify the segment of the purified DNA. The cocktail used in each PCR trial contained: 40μL H2O, 5μL Promega Thermophilic DNA Poly. 10x Buffer, 1μL 10mM Promega dNTP mix, 1μL of each of the appropriate forward and reverse 100μM primers, 1 μL purified DNA, 1μL *Taq* polymerase (Wright et al., 2010). The samples were then run in a Perkins-Elmer GeneAmp 2400 Thermal Cycler to amplify exon region 11 of the HEXA gene.

 Each PCR trial consisted of 5-minutes at 95°C (initial denaturation), followed by 30 PCR cycles of 30 seconds at 95°C (denaturation), 30 seconds at various temperatures between 45°C and 53.3°C (annealing), and 30 seconds at 72°C (extension) with a final extension for 8-minutes at 72°C. Annealing temperatures tested were: 45°C, 45.5°C, 46°C, 46.5°C, 47°C, 48°C, 49°C, 50°C, and 53.3°C.

 For additional trials, 5μL magnesium-free buffer was used in place of 5μL Promega Thermophilic DNA Poly. 10x Buffer and 1, 2, or 3 μL MgCl2 was added.

*Positive Control Trial*

 PCR was also conducted to amplify a segment of Lambda virus DNA to serve as a control. The cocktail was mixed using Promega Thermophilic DNA Poly. 10x Buffer as described above. The segment was amplified using the following primers:

Forward primer 5’-3’ GATGTATGAGCAGAGTCACCGCGAT

Reverse primer 5’-3’ GAGGGTGAAATAATCCCGTTCAG.

Annealing was achieved at 55°C and a 497bp band resulted.

*Gel electrophoresis*

 Agarose gel electrophoresis was conducted to analyze the results of amplification. The PCR products were run at 200V for 25 minutes in a 1% agarose gel prepared from 40mL 1x lithium bromide and .4grams agarose powder (Sambrook et al., 1989). The gel also contained 2 μL ethidium bromide to allow for DNA fragments to fluoresce under UV light. The Invitrogen 1KB ladder was placed in lane 1 to serve as a molecular weight marker. Results were then viewed using the UV transilluminator and the Kodak Image Capture software.

*Sociological Experiment*

 Researchers also conducted a sociological experiment to experience the effects of handicaps associated with Tay-Sachs disease on social, psychological, and occupational functioning. Each researcher experienced a handicap on four different days for a period of two hours in various environments, such as the cafeteria, the laboratory, and the classroom setting. Wearing glasses that blocked all vision simulated blindness; immobility was experienced by having to ride in a wheel chair and not move arms or legs; wearing earplugs simulated deafness; muteness was experienced by simply not speaking. After each trial, the researcher recorded feelings and thoughts regarding social, psychological, and occupational effects and functioning. Using these quotes, outside raters then rated these conditions using the Global Assessment of Functioning scale (American Psychiatric Association, 2000). The scores between raters were averaged, and the lowest average score indicated the most debilitating handicap. A four-sample ANOVA was run to test the significance between the GAF scores for each handicap (Cheruvelil, 2010).

**Results**

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*Genome extraction and DNA Qualification*

Quantification using the Gen5 1.09 program gave the following values: Absorbance ratio 1.315 OD260/280 and the DNA concentration 0.008 mg/ml.

*Polymerase Chain Reactions and Gel Electrophoresis*

In the experiment the following variables were manipulated to optimize the PCR and gel electrophoresis results: annealing temperature, buffer type (buffer with standard magnesium concentration or magnesium-free buffer), and magnesium chloride concentrations.

Seven annealing temperatures were tested in search of a temperature that allowed for optimal binding of primers. The following temperatures were used to attempt annealing in separate PCR trials: 45°C, 45.5°C, 46°C, 46.5°C, 47°C, 48°C, 49°C, 50°C, and 53.3°C. All were found to be ineffective in optimizing primer binding to allow for amplification of the intended segment of DNA. The non-specific binding shown in lanes 3 and 4 indicates that the primers annealed to segments of DNA at 45°C that they were not intended to anneal to (Figure 1A). The streaking in lanes 5 and 6 indicates the presence of DNA material.

 Magnesium concentrations in PCR cocktails were varied by adding 1μl, 2μl, or 3μl. Results of electrophoresis show that this did not aid in achieving accurate primer binding and DNA amplification (Figure 1B). At 45°C, the primers bound together when magnesium was added and when standard magnesium concentration was used. At 45.5°C, no binding occurred (Figure 1B).

*Sociological Experiment*

Through experiencing select handicaps associated with Tay-Sachs disease, researchers found that even a single symptom was extremely debilitating and had a profound effect on social, psychological, and occupational functioning. In the interviews with the participants of the sociological experiment, the individuals noted that the when experiencing the handicaps, they felt significantly impaired (Table 1). Common debilitations included feelings of depression and loneliness, increased dependence on others, and decreased ability to perform necessary daily and occupational tasks.

Degree of debilitation was quantified using the Global Assessment of Functioning (GAF) scale. Average GAF scores ranged from 40.5 to 62, with immobility rating the lowest and muteness rating the highest. An ANOVA test was performed to determine statistical significance between the GAF scores for each handicap. It was found that there was only a statistically significant difference between muteness and immobilility (p= 0.025070).

**Discussion**

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Tay-Sachs disease in humans is the result of a mutation in the HEXA gene on chromosome 15. The most common mutation in Ashkenazi Jews, the population in which this disease is most prevalent, is 1278insTATC in exon 11 (Triggs-Raine, 1990). This insertion causes dysfunctional beta-hexosaminidase A enzymes to be produced, thus preventing it from breaking down GM2 gangliosides. GM2 gangliosides build up to toxic levels in the brain and spinal cord, resulting in the destruction of neurons and the death of the affected individual. (Yamanaka et al., 1994). The diagnosis of Tay Sachs disease rests in genetic testing. It was anticipated that a better genetic test could be made so that people are better informed about their chances of having a child born with Tay-Sachs. This could in the long run preventing more children from being born with TSD (Park et al., 2010). Prior studies with Tay-Sachs using PCR and gel electrophoresis have yielded positive results in detecting the 1278insTATC mutation (Shore and Myerowitz, 1990). Therefore, it was hypothesized that with the designed primers and experimentally determined annealing temperatures, PCR and gel electrophoresis could be used to accurately identify the 1278insTATC mutation in the HEXA gene. If the optimal conditions are achieved thus that the primers anneal at the intended position on the DNA sequence, then the wild-type or mutant primer would anneal, depending on the presence of the mutation.

Researchers conducted a psychological experiment measuring the effects the handicaps associated with Tay-Sachs disease would have on affected patients and their caretakers. Test participants experienced blindness, loss of hearing, muteness, and immobility while their caretakers assisted the participants through their daily functions. Researchers hypothesized that immobility would be the most debilitating handicap associated with Tay-Sachs Disease because it would require the greatest need of assistance from others. Researchers also predicted that these handicaps would hinder the patient’s and the caretaker’s casual relationships and daily functioning.

Preliminary research done using Lambda virus provided the basis of understanding of the specificity of PCR and gel electrophoresis. Bands appeared in the gel at the expected length of 497 basepairs. This indicates that the PCR cocktail was mixed correctly, the ingredients were working properly, and the gel was running properly.

The annealing temperatures were estimated at 53.6°C for the forward primer, 53.4°C for the reverse wild-type primer, and 52.7°C for the reverse mutant primer. Numerous annealing temperatures between 45°C and 53.3°C were tested in PCR to attempt to achieve amplification of the correct segment of the HEXA gene. Annealing temperatures that were too low resulted in non-specific binding and annealing temperatures too high did not allow primers to anneal (Wu et al., 1989). Researchers have not yet been successful in determining the optimum annealing temperature that allows for intended binding and amplification. The occurrence of non-specific binding at 45°C and no binding at higher temperatures suggests that primers were not designed to allow for annealing at the intended location.

The same PCR and gel electrophoresis experimental design could be used to detect genetic mutations of other diseases. Genetic testing in less privileged areas of the world can lead to a decreased number of cases of fatal diseases, resulting in a better quality of life (Triggs-Raine, 1990).

 In the sociological experiment, researchers observed mixed results in the surrounding environments as they conducted their experiments. Most onlookers showed mixed body language, from shock to carelessness. Friends of the researchers showed concern because they could not effectively interact with their disabled friends. Other friends laughed as they watched the test participants run through their daily routines. This suggests that the level of social interaction and response with the test subject is dependent on relationship status (Mattila et al., 2010). Additionally, humans view others differently based on previous interactions, and exhibit behavior toward strangers based on their own personal experiences (Gracia et al., 2005). This is supported by differing environmental interactions noted during the experiment.

 The GAF Scale was used to measure the most debilitating handicap associated with Tay-Sachs disease**.** A One-Way ANOVA of 4 Independent Samples statistical test was performed to determine the significance of the GAF Scale ratings. The GAF Scale ratings between muteness and immobility were found to be statistically significant, with immobility being the most debilitating. However, no one single conclusive test can 100% accurately measure the most debilitating handicap associated with Tay-Sachs disease. This is because the GAF scale is subjective and humans will rate handicaps differently based on past experiences (Gracia et al., 2005). Experiencing the handicaps of a certain disease for a portion of time does not accurately depict the hardships endured by the patient and their caretaker. While researchers could increase the duration that they experience a handicap, this would not fully compare to an individual living with the handicap. To ultimately gain the true understanding of the hardships faced by caretakers and patients with a disease without actually possessing the disease, one must personally care for an affected individual(Beach et al., 2000). This understanding will be even deeper if the person is in close relation with the affected individual(Oliver 1996).

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