The PTPN22 Gene and its’ Role in Familial Autoimmune Disorders

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In association with the American Association of immunologists – John H Wallace High School Teacher
Summer Research Program

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TEACHER GUIDE

The following are indicators from the Applied Genetics Biology Unit in the Montgomery County Public Schools, MCPS Science Curriculum Framework.

**Essential Indicators:**
- HS3.3.2.6 *use a pedigree to interpret patterns of inheritance within a family.*
- HS3.3.4.1 *identify the beneficial or harmful effects of mutations on an individual, society and/or environment.*
- HS1.2.7 *use relationships discovered in the lab to explain phenomena observed outside the laboratory.*
- HS1.4.2 *analyze data to make predictions, decisions, or draw conclusions.*

**Important Indicators:**
- HS1.3.1 *develop and demonstrate skills in using lab and field equipment to perform investigative techniques.*

**Supplemental Indicators:**
- HS3.3.2.1 *identify phenotypes as the expression of inherited characteristics.*

**I. Overview**

The rationale for this paradigm is for the fundamental understanding of the immune system via autoimmune disease incorporating common laboratory techniques used in most research labs today. The labs incorporated are PCR, restriction digest and gel electrophoresis. This model may be used to introduce PCR or used as a culminating activity within an immune system chapter in advanced placement biology, anatomy and physiology, or molecular biology class. Each module in this unit requires the results for the subsequent lab. Therefore, it is best if students have been introduced to the lab practices of restriction digest and gel electrophoresis prior to this experience.

Students will learn the mechanisms of the immune system focusing primarily on the acquired immune system involving B and T lymphocytes and how allelic forms of proteins involved in T cell interactions manifest in autoimmune disorders. Students are introduced to the relatively common variant of PTPN22, 620W, which codes for protein tyrosine phosphatase and confers susceptibility in four different autoimmune disorders.

Students will construct a family pedigree that is suspected to have multiple members with assorted autoimmune disorders. Students will PCR DNA derived from individuals with a predisposition to various forms of autoimmune disease. They will be looking for the variant PTPN22, R620W allele. The amplified DNA will next be cut using the restriction enzyme EagI and run on a gel to separate potential DNA fragments. Analysis of the gel fragments will determine the presence of the PTPN22 allele.
The technical skills acquired in this unit involve mastery of the micropipette and sterility protocols. Students will gain an understanding of the role of restriction enzymes and how they function as well as with how gel electrophoresis separates DNA molecules present in a mixture and the relationship between fragment size and migration rate in a gel. Laboratory safety is an unremitting practice throughout this unit.

The significance of this unit is to demonstrate the diagnostic tools of PCR, restriction analysis, and gel electrophoresis. Throughout this multi-lab process students will gain an understanding that each lab is required for facilitation of the subsequent protocol. Then, through these results, students will recognize the role of DNA alterations and the outcomes of there expression in disease.

II. Science Background

Background Knowledge of the Immune System and Autoimmune Disease

The body protects itself from invading foreign organism’s (e.g., bacteria, viruses, and parasites) through immune system recognition of molecules called antigens. This system has two components known as the innate and acquired system. The innate system is non-specific and includes physical barriers such as the skin and mucus membranes. The acquired or specific system involves the actions of either B or T lymphocytes. These B and T cells enable the body to remember antigens and to distinguish self from nonself (foreign). Lymphocytes circulate in the bloodstream and lymphatic system and move into tissues as needed.

B cells work chiefly by secreting substances called antibodies into the body's fluids. Antibodies associate with antigens and microbes in the body. Each B cell is programmed to make one specific antibody. For example, one B cell will make an antibody that blocks a virus that causes the common cold, this is one function of antibodies but they have many more. Antibodies belong to a family of large molecules known as immunoglobulins. When a B cell encounters its triggering antigen, it gives rise to many large cells known as plasma cells. Every plasma cell is essentially a factory for producing an antibody. Each plasma cell comes from a given B cell manufacturing millions of identical antibody molecules.

Unlike B cells, T cells do not recognize free-floating antigens. In most cases, T cells only recognize an antigen if it is carried on the surface of a cell by one of the body's own MHC, or major histocompatibility complex, molecules. MHC molecules are proteins recognized by T cells when distinguishing between self and nonself. A self MHC molecule provides a recognizable scaffolding to present a foreign antigen to the T cell. Normally, the immune system can distinguish between “self” and “nonself” and only attacks those tissues that it recognizes as “nonself.”

Although MHC molecules are required for T-cell responses against foreign invaders, they also pose a difficulty during organ transplantations. Basically every cell in the body is covered with MHC class I proteins, but each person has a different set of these proteins on his or her cells. If a T cell recognizes a nonself MHC molecule on another cell, it will destroy the cell. Therefore, doctors must match organ recipients with donors who have the closest MHC makeup. Otherwise
the recipient's T cells will likely attack the transplanted organ, leading to a rejection. This is also the reason blood typing is so important. Mixing incompatible blood can lead to clumping and agglutination.

Sometimes the immune system's recognition apparatus breaks down, and the body begins to manufacture T cells and antibodies directed against its own cells and organs. Misguided T cells and autoantibodies, as they are known, contribute to many diseases. For instance, T cells that attack pancreas cells contribute to diabetes, while an autoantibody known as rheumatoid factor is common in people with rheumatoid arthritis. People with systemic lupus erythematosus (SLE) have antibodies to many types of their own cells and cell components.

**Commonality among Autoimmunity Diseases**

Collectively, autoimmune diseases are estimated to affect at least 5-8% of the population. The clustering of multiple autoimmune disorders in families and evidence for overlapping disease susceptibility loci between different autoimmune diseases suggest that certain alleles contribute to multiple autoimmune disorders within families. This includes type I diabetes, rheumatoid arthritis, Greaves' disease, and systemic lupus erythematosus (SLE). It is known that a single-nucleotide polymorphism at nucleotide 1850 (C1850T) in codon 620 (R620W), in the intracellular tyrosine phosphatase (PTPN22) confers risk of the previously mentioned autoimmune disorders. Clustering of these autoimmune diseases within families' maybe explained genetically and environmentally. Common genes and shared environmental conditions contribute to the expression of the genes involved. Environmentally, some research suggests exposure to cigarette smoke and Epstein-Barr virus infection increase the risk of multiple autoimmune diseases.

The humoral abnormalities, or anomalies associated with antibody production, in the PTPN22 contribute to autoantibody production which is a prominent feature in all human autoimmune diseases associated with PTPN22. This gene is located on chromosome 1p13 which is important in the negative control of T lymphocyte activation. Ultimately, this variant may cause T-cell receptor-associated kinases to induce T-cell activation in an uncontrolled manner and this may increase the overall reactivity of the immune system and predispose an individual to autoimmune disease.

The premise of this lab is entirely based on the missense C1850T polymorphism. This region of the human DNA on chromosome 1 can be targeted through the process of PCR. The targeted PCR region is 355 bp in length. Due to the nucleotide substitution at base 1850, this sequence is unique to the restriction enzyme Eag1. Therefore, individuals with the variant will produce two bands from the restriction enzyme and this will appear on a 2% gel. The fragments produced by the restriction digest of Eag1 are 202 bp and 153 bp in length. Individuals lacking the variant will appear as a single band (355) on the gel.
III. Student Outcomes

The focus of this unit is to study a single mechanism of autoimmunity as a function of genetics. Students will learn the effects of a point mutation in the PTPN22 gene and explore its familial existence. Students will combine previously used lab protocols as a diagnostic tool to identify the existence of the mutated PTPN22 allele. It allows students to see and experience the translation of laboratory practices into real life outcomes.

IV. Learning Objectives

- Students will learn one mechanism of autoimmunity through the PTPN22 gene.
- Students will construct a family pedigree based on a written family history.
- Students will perform a PCR using DNA of two individuals possible containing a mutated for of a gene.
- Students will perform a restriction digest and gel electrophoresis to determine the existence of allele type.
- Students will consider and evaluate possible options when facing genetic decisions.

V. Time Requirements

Total time allotments for this entire module are approximately ten hours. This unit would require ten, forty-five minute class periods. Two days of lecture are needed for introducing the PTPN22 gene and the mechanism to which its pathway is altered. Constructing the family pedigree is one class period. The PCR uses a class to mix the reagents but three hours are used in the machine outside of the class. Day five is used to perform the restriction digest, day six to make a gel and day seven to load and run the gel. The teacher will be responsible for staining and taking a picture of the gel. Day eight and nine are used to analyze and complete the lab write up. Day ten is for the quiz.

VI. Advanced Preparation

PTPN22 primers: down stream 5’TTCGCAGACATCATAACC 3’
  up stream 5’CTGTTGCCGCAACCTGT 3’

Primers can be ordered at Invitrogen at http://tools.invitrogen.com/content.cfm?pageid=9716.
Restriction enzyme Eagl and both plasmids (pMal-pIII and pMal-p5X) were ordered from New England biolabs http://www.neb.com/nebecomm/products/categories.asp.
The PCR tubes with beads, agarose, and the ethidium bromide are ordered through Edvotek.
VII. Materials and Equipment

Reagents
DNA samples from Barbara’s children:
A. Timmy
B. Lindsey
Restriction enzyme EagI
PTPN22 primers
Agarose
1X TBE buffer
PCR tubes with beads
Loading Dye
Ethidium bromide

Supplies
0.5 – 10 µl micropipette with tips
10 - 100 µl micropipette with tips
Balance
Microwave
Water bath 37°C
PCR machine
Gel electrophoresis box
Power supply
Glass jar for agarose
Tissues
Hot glove
1.5 mL tubes

VIII. Students Prior Knowledge

This unit is designed to accompany an immunology chapter in a secondary biology class such as AP or molecular biology. Within these courses, students will have completed the immunology unit in which both the innate and adaptive systems are covered. The focus of this module is on autoimmunity within the immune system, particularly how the mutated form of PTPN22 can lead to autoimmune disorders. Therefore, a solid understanding of T lymphocytes and their communication is essential.

Technical skills required are relatively common in today’s advanced science courses such as gel electrophoresis and restriction analysis. Skills involved with these protocols are sterility techniques, micropipetting proficiency and general knowledge on the use of relevant equipment. Students may not be required to program the PCR machine.

IX. What is Expected from Students

Students are expected to complete a laboratory report consisting of purpose, materials, procedure, data, analysis, and answers to questions which go in the conclusion section. A picture or drawing of the gel is required under the Data section. Analysis will be an interpretation of the gel. Students will have to identify the DNA banding patterns of each child as homozygous or heterozygous. Conclusion questions will be answered in the Conclusion section of the lab write-up.
X. Anticipated Results

The DNA banding patterns of the two individual children include Lindsey who is homozygous for the normal allele will have a single 355 base pair fragment. Timmy, who is heterozygous, will have a banding pattern with 3 bands at 355, 202, and 153 base pairs.

Students should at least get the DNA of the plasmid appearing in the gel near the edge of the well. If no bands appear other than the 7000 bp plasmid then the primers did not anneal to their target. If Timmy’s results show only one band at 355 bp then this indicates the restriction digest did not work.

XI. Classroom Discussion

It is imperative that the students understand the possible DNA band patterns. It would be best to go through every possibility with the class before doing the gel.

Wells 1, 3, and 6 all show the 355 base pair band, homozygous for the normal allele. Wells 4 and 5, show individuals who are heterozygous for the normal allele (355 bp) and the mutated allele (202 bp and 153 bp).

XII. Assessment

Students are responsible for background questions on the PTPN22 gene, a laboratory report including analysis and conclusion questions, as well as a nine-point quiz shown below.
PTPN22 QUIZ

1. Which one of the following is an example of an antigen?
   a. virus       b. bacteria       c. parasite       d. all of these

2. The PTPN22 gene is located on which chromosome?
   a. 1        b. 2       c. 11       d. 12

3. PTPN22 is found in the protein _____.
   a. thymine phosphatase
   b. tyrosine photosynthesis
   c. thalidomide phosphate
   d. tyrosine phosphatase

4. Which of the following is not an autoimmune disease?
   a. Type 1 Diabetes   b. Thyroid Cancer   c. Graeves’ Disease   d. Lupus

5. If an individual has the mutated form of the PTPN22 gene, how will the DNA appear on a gel?
   a. One single band at base pair 355.
   b. One single band at 153 base pairs.
   c. One single band at 202 base pairs.
   d. Two bands at 153 and 202 base pairs.

6. The PTPN22 mutation is a result of a base pair substitution at base pair 1850, this involves a substitution of the nucleotide _____ to _____.
   a. thymine to cytosine
   b. cytosine to thymine
   c. adenine to thymine
   d. thymine to adenine

7. Which cells work chiefly by secreting antibodies into the body’s fluid?
   a. T cells        b. B cells        c. platelets        d. Langerhorn cells

8. Which cell works by using specialized antibody-like receptors that see fragments of antigens on the surfaces of infected or cancerous cells?
   a. T cells        b. B cells        c. platelets        d. Langerhorn cells

9. Which component of the immune system uses physical barriers such as skin and mucus membranes?
   a. acquired system
   b. innate system
   c. autoimmune system
   d. specific system

Answers: 1. d, 2. a, 3. d, 4. b, 5. d, 6. b, 7. b, 8. a, 9. b
XIII. References


I. Rationale

Our DNA came from our parents, which they received from their parents. The good, the bad and the ugly can exist in our DNA, but if it exists in our DNA, it also exists or existed in our parents DNA and all our next of kin. When things go awry in our DNA, it can have terrible consequences. The genes in our DNA code for approximately 30,000 proteins, but if there is just a single nucleotide base substitution, then a protein’s function can be rendered ineffective. Proteins that function in the immune system are explored in this unit and their allelic forms. In order to develop an inheritance pattern for the PTPN22 gene, a pedigree created from a written family history of a lupus patient will illustrate the prevalence for familial existence. The patient then wants to determine if her own children carry the gene and requests DNA testing for both kids. As a laboratory technician, you will be using the protocols of PCR, restriction digest and gel electrophoresis.

The PCR procedure is designed to bracket the region on chromosome 1 where the PTPN22 exists. This is a fragment of 355 bp. Once the fragment is isolated from the entire genome, it can be determined whether or not the individual contains a mutated form of the PTPN22 gene. Within this 355 bp fragment exists the point mutation. It is this single nucleotide difference which leads to the predisposition to various autoimmune disorders. To verify the existence of this base pair substitution, a restriction analysis is performed. The restriction enzyme Eag1 has a restriction site for the sequence where the point mutation resides. If this mutation is present, the restriction enzyme will produce two fragments from the PCR product of 202 bp and 153 bp. Subsequently, this restriction site does not exist in the normal form of the allele and the 355 bp fragment remains intact.

Once the digest is complete, these products are run on a 4% gel using gel electrophoresis for the purpose of visualizing the DNA fragments. There are three possible genetic combinations that individuals could retain within their DNA. These possible conditions are homozygous for the normal or wild type allele, which produces a single 355 bp fragment. This condition is shown in the gel in Figure 1, lane 2. A second possibility is an individual who is homozygous for the mutated form that produces two fragments one at 202 bp and a second fragment at 153 bp, in Figure 1 this is shown in lane 3. A third option is for the person to be heterozygous. This situation indicates the person has one chromosome which has the normal form of PTPN22 and the other chromosome contains the mutated allele, which would produce three bands; one at 355 bp, 202 bp and 153 bp, this is illustrated in lane 4. The 355 bp fragment is faint but does exist.

Introduction

The body protects itself from invading foreign substances called antigens, such as bacteria, viruses, and parasites through its immune system. This system has two components known as the innate and acquired system. The innate system is non-specific and includes physical barriers such as the skin and mucus membranes. The acquired or specific system involves the actions of lymphocytes. Lymphocytes may be T cells or B cells. These cells enable the body to remember
antigens and to distinguish self from nonself (foreign). Lymphocytes circulate in the bloodstream and lymphatic system and move into tissues as needed.

B cells work chiefly by secreting substances called antibodies into the body's fluids. Antibodies ambush antigens circulating the bloodstream. Each B cell is programmed to make one specific antibody. For example, one B cell will make an antibody that blocks a virus that causes the common cold, while another produces an antibody that attacks a bacterium that causes pneumonia. Whenever antigen and antibody interlock, the antibody marks the antigen for destruction. Antibodies belong to a family of large molecules known as immunoglobulins. When a B cell encounters its triggering antigen, it gives rise to many large cells known as plasma cells. Every plasma cell is essentially a factory for producing an antibody. Each plasma cell comes from a given B cell manufacturing millions of identical antibody molecules and pours them into the bloodstream.

Unlike B cells, T cells do not recognize free-floating antigens. Rather, their surfaces contain specialized antibody-like receptors that see fragments of antigens on the surfaces of infected or cancerous cells. T cells contribute to immune defenses in two major ways: some direct and regulate immune responses; others directly attack infected or cancerous cells. In most cases, T cells only recognize an antigen if it is carried on the surface of a cell by one of the body's own MHC, or major histocompatibility complex, molecules. MHC molecules are proteins recognized by T cells when distinguishing between self and nonself. A self MHC molecule provides a recognizable scaffolding to present a foreign antigen to the T cell. Normally, the immune system can distinguish between “self” and “nonself” and only attacks those tissues that it recognizes as “nonself.”

Although MHC molecules are required for T-cell responses against foreign invaders, they also pose a difficulty during organ transplantations. Basically, every cell in the body is covered with MHC proteins, but each person has a different set of these proteins on his or her cells. If a T cell recognizes a nonself MHC molecule on another cell, it will destroy the cell. Therefore, doctors must match organ recipients with donors who have the closest MHC makeup. Otherwise the recipient's T cells will likely attack the transplanted organ, leading to a rejection. This is also the reason blood typing is so important. Mixing incompatible blood can lead to clumping and agglutination.

Sometimes the immune system's recognition apparatus breaks down, and the body begins to manufacture T cells and antibodies directed against its own cells and organs. Misguided T cells and autoantibodies, as they are known, contribute to many diseases. For instance, T cells that attack pancreas cells contribute to diabetes, while an autoantibody known as rheumatoid factor is common in people with rheumatoid arthritis. People with systemic lupus erythematosus (SLE) have antibodies to many types of their own cells and cell components.

**Commonality among Autoimmunity Diseases**

Collectively, autoimmune diseases are estimated to affect at least 5-8% of the population. The clustering of multiple autoimmune disorders in families and evidence for overlapping disease susceptibility loci between different autoimmune diseases suggest that certain alleles contribute
to multiple autoimmune disorders within families. This includes type 1 diabetes, rheumatoid arthritis, Greaves' disease, and systemic lupus erythematosus (SLE). It is known that a single-nucleotide polymorphism at nucleotide 1850 (C1850T) in codon 620 (R620W), in the intracellular tyrosine phosphatase (PTPN22) confers risk of the previously mentioned autoimmune disorders. Clustering of these autoimmune diseases within families’ maybe explained genetically and environmentally. Common genes and shared environmental conditions contribute to the expression of the genes involved. Environmentally, some research suggests exposure to cigarette smoke and Epstein-Barr virus infection increase the risk of multiple autoimmune diseases.

The humoral abnormalities, or anomalies associated with antibody production, in the PTPN22 contribute to autoantibody production which is a prominent feature in all human autoimmune diseases associated with PTPN22. This gene is located on chromosome 1p13 which is important in the negative control of T lymphocyte activation. Ultimately, this variant may cause T-cell receptor-associated kinases to induce T-cell activation in an uncontrolled manner and this may increase the overall reactivity of the immune system and predispose an individual to autoimmune disease.

The premise of this lab is entirely based on the missense C1850T polymorphism. This region of the human DNA on chromosome 1 can be targeted through the process of PCR. The targeted PCR region is 355 bp in length. Due to the nucleotide substitution at base 1850, this sequence is unique to the restriction enzyme Eag1. Therefore, individuals with the variant will produce two bands from the restriction enzyme and this will appear on a 2% gel. The fragments produced by the restriction digest of Eag1 are 202 bp and 153 bp in length. Individuals lacking the variant will appear as a single band (355) on the gel.

Background questions for PTPN22

1. What is an antigen?
2. What is a lymphocyte and what part of the immune system does it belong to?
3. How does a B cell work, be sure to include the mechanism as well as other cells produced?
4. What are the two ways that T cells contribute to immune defenses?
5. How does a T cell recognize an antigen?
6. What does MHC stand for?
7. What are autoantibodies and list two diseases which result from their activity?
8. Where is the PTPN22 gene located, list the specific base number?
9. What type of mutation occurs in the PTPN22?
10. What diseases result from this mutation?
11. What is another factor involved in getting an autoimmune disorder?
12. The T cell will only attack when prompted but when the mutation occurs describe how this gene causes the T cell to go awry.
II. Materials

Reagents
DNA samples from Barbara’s children:  
   C. Timmy  
   D. Lindsey
Restriction enzyme Eag1  
PTPN22 primers  
Agarose  
1X TBE buffer  
PCR tubes with beads  
Loading Dye  
Ethidium bromide

Supplies
0.5 – 10 µl micropipette with tips  
10 - 100 µl micropipette with tips  
Balance  
Microwave  
Water bath 37°C  
PCR machine  
Gel electrophoresis box  
Power supply  
Glass jar for agarose  
Tissues  
Hot glove  
1.5 mL tubes

III. Procedure

Construct a Family Pedigree

When constructing a pedigree use the required symbols and their representations:

- A circle represents a female.
- A square represents a male.
- A shaded circle or square refers to a person having some form of an autoimmune disorder.
- A circle or square (either shaded or open) with a diagonal slash through it represents a person who is deceased.
- In the presence of high incidents of autoimmune disorders within the family pedigree indicates a genetic mutation in the PTPN22 gene and a predisposition to these disorders.
- A line between a circle and a square indicate a marriage line.
- A vertical line indicates the couple has children.
The clustering of multiple autoimmune disorders in families and the evidence for overlapping disease susceptibility loci between different autoimmune diseases suggest that certain alleles contribute to multiple autoimmune disorders. In this investigation, it will be determined if familial clustering of various autoimmune disorders exist in the Wynne family. The first step is to establish a family pedigree for patient Barbara Wynne. Barbara, age 42, was recently diagnosed with SLE (systemic lupus erythematosus) after a year of evaluations and testing. She had complained of joint pain and swelling along with muscle pain. She often had fever with no known cause and red rashes that appeared on her face after being in the sun. Through genetic testing, it was discovered she had the PTPN22 mutation. She is African American. Lupus is more common among African American, Asian, Hispanic, and Native American women. As part of her medical work-up, a specialist had inquired about her family history of autoimmune disorders. The geneticists informed her that other autoimmune diseases including: Type 1 diabetes, rheumatoid arthritis, Graves' disease, Addison's disease and Alopecia Areata. In talking with her husband, Jeff, it appears his family members are free from any autoimmune diseases. Barbara is well aware of family members' autoimmune disorders and recalled the following information about her family to her doctor. With this information, chart Barbara's pedigree.

- Her maternal grandmother, Helen, suffered from rashes on her face and chronic joint pain. She was never diagnosed with lupus, but she had many of the same symptoms as Barbara. Barbara and her mother now consider the grandmother to have had lupus. She died at age 65.
- Cheryl (age 66), Barbara’s mother, was never diagnosed with an autoimmune disease.
- Barbara’s maternal grandfather, Thomas, was free of any autoimmune disorders and is 87 years old.
- Cheryl's sister, Tammy (age 64), suffers from a hyper thyroid known as Graeves disease.
- Cheryl’s brother, Greg (age 60) suffered from Alopecia Areata (hair loss) at a very early age. This is also an autoimmune disorder.
- Her maternal cousin, Timmy (son of Tammy), was diagnosed with type 1 diabetes at age 15.
- Her cousin (Timmy's sister), Adrienne, is in good health.
- Timmy's other two sister's Denise and Kathy are in good health.
- Kathy's only child, Cindy has rheumatoid arthritis at age 25.
- Barbara's sister, Melissa (age 38), is in good health.
- Melissa's son Vince was diagnosed with Alopecia at 18.
- Melissa's other son, Jason (age 16), and daughter, Amy (age 13), are free of autoimmune diseases.

Barbara has two children:

Timmy (age 14)
Lindsey (age 12)

Both children show no signs of autoimmunity at this time. Barbara has asked for her children to have their DNA tested for the PTPN22 mutation.
When constructing Barbara’s pedigree chart be sure to shade in the individuals with the disorder and name the disorder below the person. This may take several drafts until you complete the chart so be patient.

Detection of the PTPN22 Mutation in a Family with High Susceptibility to Autoimmune Disorders

Objective: Students will gain an understanding of the PTPN22 gene and its association in familial autoimmune disorders.

Procedure:

**PCR Protocol**

1. Get two 0.5 ml PCR tubes and label them T (Timmy) and L (Lindsey). Also, identify each tube with your group name.
2. Add 2 µl of child’s DNA to the appropriate initialed tube.
3. Add 18 µl of the master mix to each tube. The master mix includes taq polymerase, dNTP’s, primers, and water.
4. Pulse all four tubes in a microfuge at 8000 rpm’s to collect liquid in the bottom of the tube.
5. Add 20 µl of mineral oil to the top of the DNA solution as an insulator if your PCR machine does not have a heated lid.
6. Place in PCR machine and run the following protocol:

<table>
<thead>
<tr>
<th>Incubate</th>
<th>Cycles</th>
<th>94°C</th>
<th>57°C</th>
<th>72°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>94°C</td>
<td>57°C</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>3 minutes</td>
<td>for 30 seconds</td>
<td>for 30 seconds</td>
<td>for 30 seconds</td>
<td></td>
</tr>
</tbody>
</table>

7. Remove tube from the PCR machine and store in freezer until ready to perform the restriction digest.

**Restriction Digest**

7. Label two 1.5 ml tubes T and L. Also, identify each tube with your group name.
1. If you used oil to PCR the reaction mixture, you must first separate the DNA from the oil. Use the following directions for this DNA isolation.
   - Isolate the PCR DNA from the oil in the tube.
   - Set yellow micropipette to 19 µl and place tip through the oil and into the DNA.
   - Slowly extract ONLY the DNA.
   - Place contents of tip on a piece of paraffin wax paper.
   - The center of the drop will be the DNA.
   - Get a fresh tip and set to 17.5 µl.
   - Carefully extract the DNA from the top of the drop trying not to get any oil.
   - Place the 17.5 µl of DNA into the appropriate tubes labeled T and L.

STOP POINT
2. If you did not use oil in your PCR reaction then add 17.5 µl of the PCR reaction mixture directly to the appropriate tube. Add T to T and L to L.
3. Add 24 µl of dH2O to each tube.
4. Add 3 µl of 10x buffer to each tube.
5. Add 1.5 µl of the restriction enzyme, Eag1 to both tubes.
6. Place both tubes in a 37°C water bath for 2 hours.
7. Take tubes out of the water bath and place in freezer until ready to load.

**STOP POINT**

**Prepare a 4.0% Gel**
1. Measure out 4 grams of agarose on a balance and pour into a glass jar.
2. Measure 100 ml of 1x TBE buffer in a graduated cylinder and pour into the jar containing the agarose.
3. Gently swirl to mix. Place a tissue in the opening of the jar and microwave at 30 second increments. Alternating swirling and microwaving. Continue this process until the solution boils. Immediately stop the microwave once boiling begins.
4. Remove the hot jar from the microwave.
5. While the agarose solution cools on counter.
6. Tape ends of gel tray and caulk with cooling solution where the tape and gel tray meet. This will prevent the gel solution from melting through the tape and oozing on to the counter.
7. Place comb into slots of gel tray and pour the gel solution into the tray when it is comfortable to the touch.
8. Do not disturb the tray until the gel solidifies. It will appear a cloudy white color when it has hardened.
9. Once the gel is solid, gently remove the comb and tape. Place the entire tray and gel into the gel electrophoresis chamber.
10. Fill the gel box with 1x TBE buffer, covering the entire surface of the gel.

**STOP POINT**

**Loading the Gel**
1. Using the DNA from the restriction digest, add 3 µl of loading dye to both reaction tubes.
2. Record the order and well to which you loaded your DNA.
3. Load 30 µl of each reaction tube into a separate well in gel. Use a fresh tip for each reaction.
4. Close the top of electrophoresis box and connect electrical leads to a power supply, anode to anode (red-red) and cathode to cathode (black-black).
5. Turn power supply on, and set to 100 volts. Check the electrophoresis box for air bubbles coming off the cathode side of the chamber. If current is not detected, check connections again.
6. Electrophorese for 40-60 minutes. Stop electrophoresis before bromophenol blue band runs off the end of the gel.
7. Turn off power supply, disconnect leads from the inputs, and remove the top of electrophoresis box.
8. Carefully remove casting tray from electrophoresis box, and slide gel into disposable weigh boat or other shallow tray.
Stain Gel with Ethidium Bromide and View

1. Flood gel with ethidium bromide solution (1 µg/ml), and allow to stain for a minimum of 30 minutes.
2. Following staining, decant ethidium bromide solution into a storage container.
3. View under ultraviolet transilluminator or other UV source.
4. Take time for responsible clean up.
   a. Wipe down camera, transilluminator, and staining area.
   b. Wash hands before leaving lab.

Photograph Gel if possible.

IV. Data Collection

Data will be in the form of a picture or drawing of the gel containing the DNA bands of a 100 base pair ladder and both Timmy and Lindsey’s DNA.

V. Discussion/Analysis

The analysis must include a description of the quantity of bands, the approximate base pair number for each band and the implication of each fragment. This description would include if the person is homozygous for the normal or mutated allele or heterozygous. This understanding is revisited in the conclusion questions.

The conclusion questions are:

1. What is the difference between Timmy and Lindsey’s DNA?
2. What do these differences indicate?
3. Can a physician proceed with a diagnosis on this molecular biology data?
4. What factors should Barbara consider with the outcome of the PTPN22 gene and her children?