Supplemental Materials: Lab Documentation for

A Metabolic Murder Mystery: A Case-Based Experiment for the Undergraduate Biochemistry Laboratory

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A. The Case Study

A Metabolic Murder Mystery

On July 7, 1989, Mrs. Patricia Stevens brought her three-month old son, Ryan to the Emergency Room of Cardinal Glennon Children’s Hospital in St. Louis, MO. Ryan had been ill for the past three days, had been vomiting extensively and was developing breathing difficulties. The attending physician, a toxicologist, thought that Ryan’s symptoms were characteristic of ethylene glycol poisoning. Ethylene glycol is the main ingredient in antifreeze. It is highly toxic if consumed. Samples of Ryan’s blood and urine were collected and analyzed for the presence of ethylene glycol by both the hospital’s laboratory and a commercial laboratory. Both laboratories reported the presence of a large amount of ethylene glycol in Ryan’s blood as well as in the formula that his mother had been feeding him. These findings seemed to confirm the physician’s diagnosis.

Ryan recovered, was removed from his parents care and was placed in a foster home. His parents were allowed to see him only under supervised conditions. On one such visit, Mrs. Stevens was accidentally allowed to feed Ryan a bottle without supervision. Shortly after that, Ryan died. Mrs. Stevens was arrested and charged with first degree murder.

While in jail, Mrs. Stevens found that she was pregnant and gave birth to a second son, David. David was placed in foster care but began to show the same symptoms that Ryan had displayed. David was diagnosed as having a rare genetic disorder, methylmalonic aciduria (MMA). Victims of MMA show symptoms nearly identical with those characteristic of ethylene glycol poisoning.

Although Mrs. Stevens could not have possibly poisoned David, the State decided to proceed with her trial. The presiding judge ruled that David’s illness was irrelevant to the case and could not be introduced as evidence. Mrs. Stevens was convicted of murder in the first degree and was sentenced to life in prison without the possibility of parole.

Two academic scientists who had been following Patricia Stevens’ trial in the media thought that there was a possibility that she might have been wrongly convicted. They managed to obtain samples of Ryan’s blood. Their analysis of the blood sample failed to reveal the presence of any ethylene glycol. When they attempted to obtain a sample of the formula that Ryan had been drinking immediately prior to his death, they were told that it had “disappeared”.

The scientists were convinced that the laboratory results were in error and were “unacceptable”. The labs had apparently detected something unusual in Ryan’s blood (methylmalonic acid) and had assumed that it was ethylene glycol with no additional proof.

The academic scientists presented their findings to the prosecutor, George McElroy. Mr. McElroy reviewed all of their data and extensively questioned the scientists as well as the hospital and commercial lab personnel involved in the analysis of Ryan’s blood. After reviewing all of the information he had gathered, he concluded that the work done by the hospital and commercial laboratories was in error and was “no longer believable”. All charges against Mrs. Stevens were subsequently dropped and she was released with an apology in September 1991.
B. Notes and Procedures for Instructors

An excellent resource for learning about PCR techniques has been published by Cold Spring Harbor Laboratory Press (Dieffenbach, C. D., G., (Ed.) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2003.)

Preparation of PCR Samples: Students are provided with two tubes containing 10 ng of DNA—one that contains the native MUT gene (positive control) and one unknown sample that may or may not have a defective MUT gene. (The concentration of the plasmid DNA can be determined by the absorbance at 260 nm. An optical density = 1 corresponds to 50 µg/mL of double stranded (plasmid) DNA.) They are also supplied with 10X PCR Buffer (100 mM Tris-HCl, pH 9, 500 mM KCl, 1% Triton X-100), 250 mM MgCl₂, and 5 mM dNTP mix (each dNTP is 5 mM). Based on a pre-laboratory discussion, the students should know the correct concentration ranges for MgCl₂ (3-5 mM) and dNTPs (0.25-0.4 mM) and assemble their PCR reactions. Once all students have prepared their samples, they are supplied with the Taq DNA polymerase; therefore, all PCR reactions can be started simultaneously.

Typical concentrations of the components required for PCR amplification are:
- MgCl₂: 3-5 mM
- dNTPs: 0.25-0.4 mM
- Primers: 0.5-1.0 µM each
- Template: 1-10 ng per 50 µL reaction
- Taq DNA Polymerase: 0.5-1 unit per 50 µL reaction
- 1X PCR Buffer (supplied with the enzyme if purchased from a commercial source or can be homemade, for example 10 mM Tris-HCl, pH 9, 50 mM KCl, and 0.1% Triton X-100)

Thus, an example of the volumes and concentrations of reagents added to a PCR reaction is:
- 5 µL of 10 X PCR Buffer
- 3.3 µL of 5 mM dNTP Mix
- 0.8 µL of 250 mM MgCl₂
- 1 µL of 100 µM Primer A
- 1 µL of 100 µM Primer B
- 1 µL of the DNA template for the native MUT gene
- 36.9 µL of H₂O
- 1 µL of 1 unit/ µL Taq DNA polymerase

We use the following cycle conditions for our PCR reactions:
1.) An initial denaturation step: 95 °C for 1 min
2.) 25 cycles of: 95 °C for 20 s; 45 °C for 20 s, 72 °C for 45 s
A final incubation at 72 °C for 4 min can be completed if desired.

Depending upon the amount of the PCR reaction that is loaded on the gel, 15-25 cycles are sufficient for visualization on an agarose gel stained with ethidium bromide (*CAUTION: Known carcinogen!*) or SYBR Safe DNA Stain (Invitrogen).

Agarose Gel Electrophoresis: During PCR amplification, students should pour agarose gels; however, they can also be poured by the instructor. The percentage of the gel depends on the size of the PCR products. We typically use 1.5% (w/v) agarose gels with 1X TBE Buffer (89 mM Tris, pH 8.3, 89 mM Boric Acid, and 2 mM EDTA) since they run more quickly. Completely dissolve 0.75 g of agarose in 50 mL of 1X TBE buffer using a microwave or a Bunsen burner. If pre-staining of gels is desired, add 2-3 µL of 10 mg/mL ethidium bromide (toxic!) or 5 µL of 10,000X SYBR Safe DNA Stain per 50 mL of
agarose solution. Alternatively, the gels can be stained post-electrophoresis (see below). Pour the agarose solution into the gel caster and insert the comb. Students should use caution when pouring agarose gels since the solution is hot.

After the PCR amplification has finished, the PCR samples are prepared for loading onto an agarose gel. The simplest way is to add 0.5 volumes of 50% glycerol (v/v) containing a trace of dye (0.1% (w/v)) such as Orange G, Bromophenol Blue, or Xylene Cyanol. For example, a standard PCR reaction is completed in 50 µL. In this case, 25 µL of 50% (v/v) glycerol (or 0.5 \times 50 \mu L) would be added. We prefer Orange G since it does not co-migrate with most DNA fragments. The dye aids in the loading process and serves as a visual indicator of the maximum distance a DNA fragment has migrated. (Orange G migrates approximately as a dNTP.)

Load about 10 µL of each sample in a different well. It is helpful to also load a 100 base pair ladder on the agarose gel as a marker. Separation of DNA fragments is achieved by electrophoresis at 100-150 V for 20 min. Remember, electrophoresis can be stopped at any time, the gel stained (if the stain has not been added directly to the gel prior to electrophoresis) and/or imaged, and then run longer if separation is not sufficient.

If the agarose gels were prepared without ethidium bromide or SYBR Safe DNA Stain, they are then stained using SYBR Safe DNA Stain and the manufacturer’s protocol. The green bands can be visualized by using a standard transilluminator or hand-held lamp.

**Instructor Notes**

Note that deoxyribonucleotides and Taq DNA polymerase should be kept on ice.

To save reagents, smaller PCR reactions (10 µL) can be completed although this requires a great deal of accuracy when students pipet.

Approximately three groups of students can share a single gel containing 10 wells.

Approximately 2-3 µL of 10 mg/mL ethidium bromide or 5 µL of 10,000X SYBR Safe DNA Stain is sufficient to stain a 50 mL agarose gel. The ethidium bromide can be added after the agarose has been dissolved but before pouring (Alternate: this procedure is the fastest. It also allows bands to be visualized throughout electrophoresis if desired.) or the gel can be stained post electrophoresis by submerging the gel in 100 mL of 1X TBE and 5 µL of 10 mg/mL ethidium bromide or 10 µL of 10,000X SYBR Safe DNA Stain.

To stain post-electrophoresis with SYBR Safe, follow the instructions provided by the manufacturer.

If the instructor prefers that the teams use their own primers, primer design should be completed one week prior to the laboratory period.

**Class Management**

The instructor should provide an overview of PCR amplification before giving the students the sequences of the native and mutated MUT gene. An animation containing introductory information about PCR by Cold Spring Harbor Laboratory’s Dolan DNA Learning Center can be found at
Prior to beginning the laboratory experiments, the instructor should show the students how to program the thermocycler. This is most effectively completed in groups of 2-4 students. Students are encouraged to select their own PCR cycling conditions. If a gradient thermocycler is available, multiple annealing temperatures can be accommodated. Otherwise, the class should be asked to determine a set of parameters that will likely result in successful PCR amplification for all groups. Since the instructor approves all primer selections and PCR cycling conditions, this is possible.

The students work in instructor assigned two-person teams to PCR amplify two samples and analyze them by agarose gel electrophoresis: a PCR of the native (known) MUT gene and their assigned unknown. The report that they write should include accurately labeled copies of the agarose gel stained with ethidium bromide or SYBR Safe DNA Stain and a clear interpretation of the results.

Required Chemicals and Equipment

**Required Chemicals:**
- Agarose [9012-36-6]
- Boric Acid [10043-35-3]
- 2'-Deoxyadenosine 5’-triphosphate (dATP) [1927-31-7]
- 2'-Deoxycytidine 5’-triphosphate (dCTP) [102783-51-7]
- 2’-Deoxyguanosine 5’-triphosphate (dGTP) [93919-41-6]
- 2’-Deoxycytidine 5’-triphosphate (dTTP) [18423-43-3]
- Ethylenediamine tetraacetic acid (EDTA) [6381-92-6]
- Glycerol [56-81-5]
- Magnesium chloride [7791-18-6]
- Orange G [1936-15-8]
- Potassium chloride [7447-40-7]
- Taq DNA polymerase [9012-90-2]
- Tris(hydroxymethyl)aminomethane (Tris) [77-86-1]
- Triton X-100 [9002-93-1]

- Primers No CAS Number
- 100 bp DNA Ladder No CAS Number

Detection of DNA in agarose gels can be completed with ethidium bromide (toxic!) or SYBR Safe DNA Stain:
- Ethidium bromide [1239-45-8]
- SYBR Safe DNA Stain No CAS Number

**Required Equipment:**
- PCR Tubes
- Pipets
- Pipet Tips
- Thermocycler (or three water baths/heat blocks set to 95 °C, 72 °C, and 50 °C)
- Agarose gel caster and comb
- Agarose gel apparatus
• Power Supply
• UV Transilluminator or hand-held lamp (254 nm)
• Orbital shaker (optional)

**Suggested Vendors:**
- dNTP Mix: New England BioLabs (catalog #: N0447S); Promega (catalog #: U1330); Sigma-Aldrich (catalog#: DNTP10)
- Taq DNA Polymerase: New England BioLabs (catalog #: M0273S); Promega (catalog #: M3001); Sigma-Aldrich (catalog#: D1806)
- Custom Primers: Integrated DNA Technologies (IDT)
- M13 Primers: [IDT](http://www.idtdna.com/catalog/ReadyMadePrimers/Page1.aspx); New England Biolabs (catalog #'s: S1201S and S1211S)
- 100 bp DNA Ladder: New England BioLabs (catalog #: N3231S); Promega (catalog#: G2101); Sigma-Aldrich (catalog #: P9364)
- SYBR Safe DNA Stain: Invitrogen (catalog # for 1X solution: S33100; catalog # for 10,00X solution: S33102)
- Thermocycler: BioRad (catalog #: PTC-1148C); Thermo Scientific* Hybaid PCR Sprint Thermal Cycler (catalog #: HBSP05110)
- Agarose gel apparatus: BioRad (catalog #: 170-4468EDU); Thermo Scientific Owl B2 EasyCast Mini Gel System
- PCR Tubes: Thermo Fisher (catalog #: PCR05C); VWR (catalog #: 22234-052)

**Safety and Disposal**

Safety glasses and gloves should be worn at all times. Waste materials should be placed in properly labeled aqueous or organic waste containers. Ethidium bromide is a known carcinogen. Agarose gels containing ethidium bromide should be disposed of as hazardous waste. Liquids containing ethidium bromide can be disposed of as hazardous waste or decontaminated according to one of the methods reported in Molecular Cloning: A Laboratory Manual (J. Sambrook, E. F. Fritsch, and T. Maniatis; Cold Spring Harbor Laboratory Press, 1989; ISBN: 0-87969-309-6).

UV transilluminators and hand-held UV lamps emit UV radiation that is damaging to skin and eyes. A cabinet and/or face shield should be used when visualizing bands in gels stained with ethidium bromide or SYBR Safe DNA stain.

Gel electrophoresis apparatus should never be touched while gels are running (attached to a power supply) due to the risk of electrocution.
C. Additional Details for Cloning MUT Genes Used in Biochemistry Experiments

Construction of plasmids: Plasmids are available gratis from Canisius College or from Addgene (http://www.addgene.org/pgvec1) for a nominal fee.

Plasmids were purchased from GenScript Corporation. The following constructs were inserted into pUC57 using the EcoRV restriction site by GenScript. Exons are shown in bold; intron 7 is in italics; intron 8 is underlined.

Human MUT Exon 7 – Intron 7 – Exon 8
ctcatataag aatggaagaa atggccccag ctgtctctga gggataacag ttgaagaatg
tgctgccga acaagagtcc agaatatag tgaattc tcatatatgc aatccagtcc
tggtgaga taacatgtat agtaaactaa acaaaaataa gttctagag tggattctgc gattcatag tagaactatg
taaagatat taatttaag taatttaag taatattctga cttaaattatatga gttattc tggattc
tggttctga ggttaaataa attattctgc gatctaatctg
tctagatctacatag taatattctga cttaaattatatga gttattc tggattc
tggttctga ggttaaataa attattctgc gatctaatctg
tctagatctacatag taatattctga cttaaattatatga gttattc tggattc
tggttctga ggttaaataa attattctgc gatctaatctg

tctctgctt ctctttcttt ccttgacttt ccagatacg

gttctga ggttaaatg
ttcttttttttt tttttttttaag tattttctgt gaactgtgaa ctgggctctg gctaagcttg
tttatctctc tcaatgtaaa
ttgcccctcttgac
tttttccata g

tctctgctt ctctttcttt ccttgacttt ccagatacg

If all students use the same primer set, M13 primers are recommended. These are common, inexpensive primers that can be purchased from many companies.

Good resources to learn about techniques in molecular biology and cloning are:


D. Instructions for Biochemistry Students:

Testing For A Genetic Mutation That Causes Methylmalonic Acidemia (MMA)

A) A Genetic Mutation That Causes MMA

Propionyl-Coenzyme A (propionyl-CoA) is generated from a variety of cellular processes including the oxidation of fatty acids with odd numbers of carbons and the oxidation of various amino acids. The normal biochemical pathway for the conversion of propionyl-CoA into succinyl-CoA (an intermediate in the citric acid cycle) is shown in Figure 1. Three enzymes are required, propionyl-CoA carboxylase, methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase (MUT). Defects in any of these enzymes are deleterious to cellular function.

![Diagram of biochemical pathway](image)

Figure 1

One disease caused by a defect in the conversion of propionyl-CoA into succinyl-CoA is methylmalonic academia (MMA). One major cause of MMA is a genetic defect in the MUT gene that is responsible for the conversion of L-methylmalonyl-CoA into succinyl-CoA. In affected individuals, impaired MUT activity leads to build up of propionyl-CoA and other organic acids. One mutation identified is the deletion of a coding region (170 bp) within the MUT gene. In this laboratory experiment, you will determine if a patient has MMA caused by a deletion in MUT using Polymerase Chain Reaction (PCR) and agarose gel electrophoresis.

B) Introduction to Polymerase Chain Reaction (PCR) & Agarose Gel Electrophoresis

**Polymerase Chain Reaction (PCR):**

Polymerase Chain Reaction, or PCR, is a powerful method used to amplify small amounts of DNA (typically nanograms) in order to obtain usable quantities for downstream analyses (µgrams). Remember, DNA is a polymer of deoxyribonucleotides (dNTPs, or dATP, dGTP, dCTP, and dTTP) that is synthesized by a family of enzymes called DNA polymerases in a process called replication. In order for replication to begin, DNA polymerase binds to a single stranded piece of DNA to which a short RNA
segment, or primer, has bound. This primer is required for DNA polymerase to start copying the remaining segment of DNA. (Think of priming a lawn mower with gas before it is started, for example.) Since DNA is ordinarily double stranded and both strands must be copied, two primers are required that each bind a different strand. Therefore PCR requires: a DNA template that is to be copied many times, or “amplified”; two primers; dNTPs (the starting material to make the DNA product); and DNA polymerase. DNA polymerases also require Mg²⁺ to be active. Therefore, MgCl₂ is also added to the PCR reaction.

Typical concentrations of the components required for PCR amplification are:
- MgCl₂: 3-5 mM
- dNTPs: 0.25-0.4 mM
- Primers: 0.5-1.0 µM each
- Template: 1-10 ng per 50 µL reaction
- Taq DNA Polymerase: 0.5-1 unit per 50 µL reaction
- 1X PCR Buffer (supplied with the enzyme if purchased from a commercial source or can be homemade, for example 10 mM Tris-HCl, pH 9, 50 mM KCl, and 0.1% Triton X-100)

Typically, there are three steps to PCR that are repeated 25-30 times: denaturation, annealing, and extension. These three steps collectively are known as one cycle. The denaturation step is typically conducted at 95 °C. In this step, the DNA is separated into two strands, or denatured. In the second, or annealing, step, the temperature is decreased (usually to 50-60 °C). The lower temperature allows the primers to bind, or anneal, to the denatured DNA strands. The extension step is conducted at 70-75 °C. This is the optimal temperature for Thermus aquaticus (Taq) DNA polymerase, which extends the short primers into exact copies of the DNA template strand. T. aquaticus is a thermophile that lives in hot springs. DNA polymerases from other organisms are denatured at temperatures used in PCR. (The creator of PCR, Kary Mullis, won a Nobel Prize in Chemistry in 1993; visit http://nobelprize.org/nobel_prizes/chemistry/ for more information). Figure 2 shows this process schematically.

Therefore a typical program for PCR amplification is:

1.) An initial denaturation step: 95 °C for 1 min
2.) 25 cycles of: 95 °C for 20 s; 50 °C for 30 s, 72 °C for 1 min
3.) A final incubation at 72 °C for 4 min can be completed if desired.

A range of temperatures was given for the annealing step. There are various ways to estimate the proper annealing temperature. One simple method is based on the number of GC and AT pairs that the primer and DNA template form (Rychlik, W. and Rhoads, R.E. (1989) Nucleic Acids Res. 17, 8543):

\[
\text{Annealing Temperature} = 4 \times (\# \text{ of GC pairs formed}) + 2 \times (\# \text{ of AT pairs formed})
\]

A more rigorous way is to use the nearest neighbor model. The nearest neighbor model takes into account that the stability of a given base pair is dependent on the base pairs immediately to its 5’ and 3’, or its nearest neighbors. Websites are available that calculate annealing temperatures based on this model:

- OligoCalc: [http://www.basic.northwestern.edu/biotools/oligocalc.html](http://www.basic.northwestern.edu/biotools/oligocalc.html)

**Agarose Gel Electrophoresis:**

Nucleic acids can be separated by their mass/charge ratios using a technique called gel electrophoresis. For separating mixtures containing nucleic acids that are very different in size (at least 50 base pairs), agarose gel electrophoresis is commonly used.

Recall that nucleic acids are negatively charged due to their phosphodiester backbones. Therefore, a gel or matrix is prepared with the appropriately sized pores (afforded by the percentage of agarose with which the gel is prepared) for the size of the nucleic acids. After loading the sample in separate wells (rectangular holes created by adding a comb before the gel solidifies), an electrical current is applied, with the gel oriented such that the positively charged pole is farthest from the wells. The negatively charged nucleic acid is then attracted to the positively charged pole and moves through the gel based on its mass/charge ratio. Larger nucleic acid fragments travel shorter distances than smaller ones. Since the mutated gene has an approximately 170 base pair deletion, it should move farther through the gel than the native gene. See Figure 3 for a schematic of agarose gel electrophoresis.

![Figure 3](image-url)
C) Objectives

Your objectives in this case-based experiment are:

1) To design PCR primers that can be used to distinguish between the wild type gene that encodes for MUT and the mutated gene that contains an exon deletion.

2) To design cycling conditions to successfully PCR amplify both forms of the MUT gene (wild type and mutated).

3) Use PCR and subsequent analysis of the PCR product(s) by agarose gel electrophoresis to determine whether the patient has a genetic mutation that causes MMA.

D) Experimental Procedure

Part 1. Design primers to distinguish between the native MUT gene and MUT gene with a deletion.

Below are the sequences of the native and mutated MUT gene. A “_” indicates where the deletion occurs.

Native MUT Gene Sequence:
ctcattaatg aaattgaaga atgggcaag atgcgcacct aagaatcag ttgatcctat gctctgttag ggacgtccttc aatgtcgtct gcttcatatg atctatgt gctgtcaaac ggttaaatg gttgctatt tgaataactg tagatagt ataatatat

MUT Gene Sequence with Exon Deletion:
ctcattaatg aaattgaaga atgggcaag atgcgcacct aagaatcag ttgatcctat gctctgttag ggacgtccttc aatgtcgtct gcttcatatg atctatgt gctgtcaaac ggttaaatg gttgctatt tgaataactg tagatagt ataatatat

Answer the following questions:

1. What are the sequences of your primers?

2. Estimate the annealing temperature for PCR using the equation on page 9.
3. What is the expected size (in bp) of the PCR product for the native MUT gene?

4. What is the expected size (in bp) of the PCR product for the mutated gene?

Part 2. PCR amplification of the native MUT gene and the unknown sample: Using the supplied reagents prepare two PCR samples.

1. PCR amplification of the native MUT gene. Add the following to a PCR tube:

   5 µL of 10 X PCR Buffer*
   3.3 µL of 5 mM dNTP Mix
   0.8 µL of 250 mM MgCl\textsubscript{2}
   1 µL of 100 µM Primer A
   1 µL of 100 µM Primer B
   1 µL of the DNA template for the native MUT gene
   36.9 µL of H\textsubscript{2}O
   1 µL of 1 unit/ µL Taq DNA polymerase

2. PCR amplification of your unknown. Add the following to a PCR tube:

   5 µL of 10 X PCR Buffer*
   3.3 µL of 5 mM dNTP Mix
   0.8 µL of 250 mM MgCl\textsubscript{2}
   1 µL of 100 µM Primer A
   1 µL of 100 µM Primer B
   1 µL of the DNA isolated from the patient
   36.9 µL of H\textsubscript{2}O
   1 µL of 1 unit/ µL Taq DNA polymerase

Place your samples in the thermocycler.

*10X PCR Buffer contains 100 mM Tris-HCl, pH 9, 500 mM KCl, 1% Triton X-100. Alternatively, 10X or 5X PCR Buffer is supplied with Taq DNA polymerase when purchased from a commercial source.

“10X” means that the concentration of all components in the buffer is 10 times more than the concentrations required for the assay. Likewise, “5X” means that the concentrations of all components in the buffer is 5 times more than the concentrations required for the assay. In the case above, the concentrations required for PCR, or 1X, are 10 mM Tris-HCl, pH 9, 50 mM KCl, and 0.1% Triton X-100.
Part 3. Preparation of an agarose gel: While your samples are in the thermocycler, prepare a 1.5% agarose gel by adding 0.75 g of agarose to 50 mL of 1X TBE Buffer in a 250 mL beaker. Microwave the solution until all of the agarose is dissolved. *CAUTION! The solution is hot!* Pour the solution into a gel caster and place a comb at one end.

Part 4. Preparation of samples for agarose gel electrophoresis: You cannot directly pipet your samples into the wells of the agarose gel that you prepared in Part 3. The density of your sample is the same as the running buffer used during electrophoresis. In order to increase the density of your solution (so it sinks in the wells), add glycerol to a final concentration of at least 10% (v/v). Glycerol has been prepared for your use that contains an orange dye (orange G).

1. Add 12.5 µL of 50% glycerol + 0.1% orange dye to each of your samples

Below is a sample calculation to determine how much glycerol should be added to a PCR sample for analysis by gel electrophoresis. It uses the simple formula $M_1V_1 = M_2V_2$ that you learned in general chemistry.

$$M_1V_1 = M_2V_2$$

$$(50 \mu\text{L PCR reaction} + X \mu\text{L 50% glycerol (v/v)}) \times (10\% \text{ glycerol (v/v)}) = (X \mu\text{L 50% glycerol (v/v)}) \times (50\% \text{ glycerol (v/v)})$$

$$500 + 10X = 50X$$

$$X = 12.5 \mu\text{L}$$

2. Prepare a molecular weight marker to determine the size of your PCR products. Mix the following in a microcentrifuge tube:

- 1 µL of 1 µg/µL 100 bp DNA ladder
- 1 µL of 50% glycerol (v/v) + 0.1% orange dye (w/v)
- 4 µL of H$_2$O

Molecular weight ladders are usually generated by digesting larger pieces of DNA with restriction enzymes. Here’s an example of a 100 bp ladder that was separated by agarose gel electrophoresis (http://www.neb.com/nebecomm/products/productN3231.asp).
**Part 5. Agarose Gel Electrophoresis:** Carefully pipet your samples into a well of the agarose gel you prepared in Part 3. (See Figure 3.) Once you are finished loading your gel, replace the lid on the gel apparatus and set the power supply to 100 V. *CAUTION! Do not touch the electrophoresis apparatus when the power supply is on. Risk of electrocution!* After the gel has run for ~20 min, stain your gel with SYBR Safe DNA Stain. Place you gel in a plastic container. Pour enough SYBR Safe solution into the container to completely cover the gel. Incubate the gel for 30 min at room temperature on an orbital shaker. Your instructor will help you image your gel.

Analyze your results:

1. What is the approximate size (in bp) of the PCR product of the native gene? (Use the 100 bp ladder (Figure 4) as an estimate.)

2. Compare the expected size of the PCR product of the native gene reported in Part 1 to the answer in question #1.

3. What is the approximate size (in bp) of the PCR product of your unknown? (Use the 100 bp ladder (Figure 4) as an estimate.)

4. Based on the size of the PCR product of the unknown (in comparison to size of the PCR product of the native MUT gene), does the patient have MMA?