DNA Testing Laboratory

Record of Chain of Custody of Biological Samples for Paternity Testing using PCR and Southern blot preparation followed by gel electrophoresis analysis.

This is to certify that prior to obtaining samples, proper identification was verified, samples were collected from the persons named below and samples were handled without tampering while in packaging and transit. **Please complete all information. Check that spelling is correct on all forms and labels.**

Name of person collecting the specimen: Tiffany Scholle

Signature of person collecting the specimen: [signature]

Name of person DNA Sample 1 was collected from:

Last: Andrews **First:** Kevin

Type and amount of sample: buccal/2 swabs **Race:** African-American

Name of person DNA Sample 2 was collected from:

Last: Jones **First:** Tommy

Type and amount of sample: buccal/2 swabs **Race:** African American

Name of person DNA Sample 3 was collected from:

Last: Doe **First:** John (# 34)

Type and amount of sample: buccal/2 swabs **Race:** African-American

Name of person DNA Sample 4 **was collected from:**

Last: Smith **First:** Elijah

Type and amount of sample: buccal / 2 swabs **Race:** African-American

Testing Requested: X Paternity ____ Maternity ____ Full Sibs ____ Half Sibs ____ Twin

____ Immigration X Forensics ____ Other/Type: ____

Sample received by ____ Time ____ Date ____ Location ____

Sample received by ____ Time ____ Date ____ Location ____

Sample received by ____ Time ____ Date ____ Location ____

******************************* Laboratory Use Only *******************************

Condition of Samples: Satisfactory ____ Unsatisfactory/Reason ____

Evidence of Tampering: Yes ____ No ____

Documents Agree with Sample Labeling: Yes____ No____ Tech ____ Date ____

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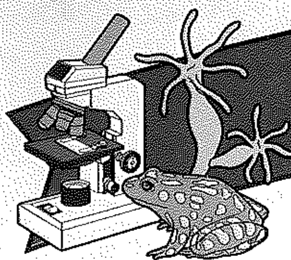

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DNA Paternity Testing

Student Laboratory Kit

Introduction

The question of whether or not a child is biologically related to a probable father has been around for many years. Prior to the 1920s, paternity questions were resolved by observing the child's phenotype—does the child physically resemble the alleged father? This method was not accurate since genetic recombination that occurs during the formation of gametes, results in a unique set of genes and typically, a blend of parental features. Recently, paternity testing has become a very reliable, high tech procedure with the development of DNA testing for relatedness.

Biological Concepts

- Blood typing
- Gel electrophoresis
- Polymerase Chain Reaction (PCR)
- Paternity testing
- Restriction Fragment Length Polymorphism (RFLP)
- Southern Blot
- Variable Number Tandem Repeats (VNTR)

Background

The first laboratory testing for paternity involved *blood typing*. Proteins found on the surface of red blood cells determine whether someone has blood type A, B, AB, or O. Two proteins, called A and B, are coded on chromosome 9. Blood typing was only able to exclude someone as a father if the child and father had different dominant blood types. For example, a father with a type B blood and a mother with a type O blood could not have a child with the type A or AB blood. If the child was found to have the type B blood, the tested father could not be excluded from paternity along with any other type B male. As is often the case in biology, it was later discovered that inheritance of blood type is not as simple as it was first thought. Inheritance of A, B and AB blood types are confounded by the presence of a third protein, type H, which when inherited in the homozygous recessive form (hh) can block the production of the A and B proteins. This leads to a type O phenotype even if the parents both have type A or type B blood. As a consequence of this "Bombay phenotype," blood typing is no longer considered an accurate method for determining paternity.

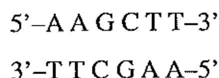
In the 1940s, laboratories began testing for *Rh factors* as part of blood typing. The Rh blood group system includes more than 40 antigens. The most commonly known Rh types are Rh+ and Rh–. These relate to the presence or absence of only the type D antigen. Homozygous DD and heterozygous Dd are Rh+, while homozygous recessive dd is Rh–. In reality, Rh is a lot more complicated than a simple Rh+ or Rh–. Rh inheritance actually involves three different pairs of genes at three different loci on chromosome 1. Like ABO blood typing, Rh typing is only able to exclude possible relationships, not prove biological paternity or relatedness.

More accurate relatedness tests were developed in the 1970s when *human leukocyte antigen* (HLA) typing came into prominence. HLA is the general name of a group of genes found on chromosome 6 in humans. These genes code for the cell-surface, antigen-presenting proteins. The proteins encoded by HLAs are the proteins on the outer part of body cells that the immune system uses to differentiate self and non-self cells. Blood samples are tested for these HLA proteins, since these proteins are found in most cells of the body including white blood cells. HLA testing can eliminate 80% of the male population from being the possible father, and in some cases it is possible to say with 90% probability that someone is the father, depending on the father's HLA type. However, HLA testing is limited in that it cannot differentiate between related alleged fathers.

Beginning in the 1980s, *Restriction Fragment Length Polymorphism* (RFLP) has been used to determine relatedness. RFLP analysis of DNA samples provided by the mother, child, and alleged father can produce a probability of paternity of 99.99% or greater. RFLP is based on the variation in the number of repeating DNA base-pair sequences that are interspersed between genes

on the chromosomes. These repeating DNA base-pair sequences are called *Variable Number Tandem Repeats* or VNTR. These VNTR sequences vary in number from person to person with the general small number or large number of repeats being inherited from the parents.

In order to test a person's VNTR profile, long strands of DNA are extracted from cells collected from the mother, child, and alleged father. The strands of DNA are cut into fragments using special enzymes called restriction enzymes. The enzymes which break DNA molecules at internal positions are called *restriction endonucleases*. Enzymes that degrade DNA by digesting the molecule from the ends of the DNA strand are termed *exonucleases*. There are several different restriction enzymes available to molecular biologists. Each restriction enzyme recognizes a specific nucleotide sequence. The enzyme "scans" the length of the DNA molecule and then digests it (breaks it apart) at or near a particular recognition sequence. The specific sequence may be five to sixteen base pairs long. For example, the HindIII endonuclease has the following six-base-pair recognition sequence:



It breaks the DNA at the locations indicated by the dotted line and produces jagged ends which molecular biologists call sticky ends. Other endonucleases cut the DNA cleanly at one specific base-pair and produce blunt ends.

Since the average human DNA sequence contains more than 3 billion base-pairs, there may be as many as 750,000 fragments of DNA after a single restriction enzyme completes the digestion of a single cell's DNA.

The size of each fragment in the DNA profile of a child depends on the DNA inherited from the biological mother and the biological father. Each person's DNA is composed of 23 chromosomes inherited from the mother's egg cell and 23 chromosomes inherited from the father's sperm cell. Consequently, half of the child's fragments should match DNA fragments from the mother, and the rest should match DNA fragments from the father. If too many fragments do not match the DNA of an alleged father then that person may be excluded.

RFLP testing is conclusive but it requires large amounts of sample and a longer analysis time than the newest type of genetic testing. Developed in the 1990s, the *Polymerase Chain Reaction* (PCR) can be performed on just a few cells collected from almost any part of the body. Even old, degraded cells can be analyzed via PCR making it the best choice for posthumous (after death) testing. The PCR technique creates billions of copies of a small segment of DNA in a process called amplification. Sixteen different DNA segments are copied simultaneously in a typical paternity test. The sixteen segments or loci selected by scientists for PCR testing have a higher degree of variation in humans. Once the amplification of DNA is complete, the sample is analyzed in the same manner as RFLP samples.

The general procedure for DNA analysis is as follows. The RFLP fragments generated by the restriction enzyme are loaded into wells made in an agarose gel. Agarose is a refined form of agar. The agarose gel is positioned between two electrodes with the wells toward the cathode (negative electrode). When a voltage is applied to the electrondes, the negatively charged DNA fragments move toward the anode (positive electrode). The electrophoresis chamber is filled with a buffer solution, bathing the gel in a solution that shields the system from changes in pH. The gel acts like a molecular sieve, creating a maze for the fragments to move through on their way toward the anode. Smaller fragments move faster through the holes or pores in the gel, while larger fragments move slower because of their size.

The DNA fragments are white to colorless and appear invisible in the gel. Molecular biologists add colored tracking dyes so they can visualize the sample moving through the gel. Typically, two dyes are added—one that migrates at a rate similar to the smaller DNA fragments and one that migrates at a rate similar to the largest DNA fragments. Once the first dye migrates to within 1 cm of the end of the gel, the power is shut off to the electrophoresis chamber. All DNA fragments stop migrating because the electromotive force stops.

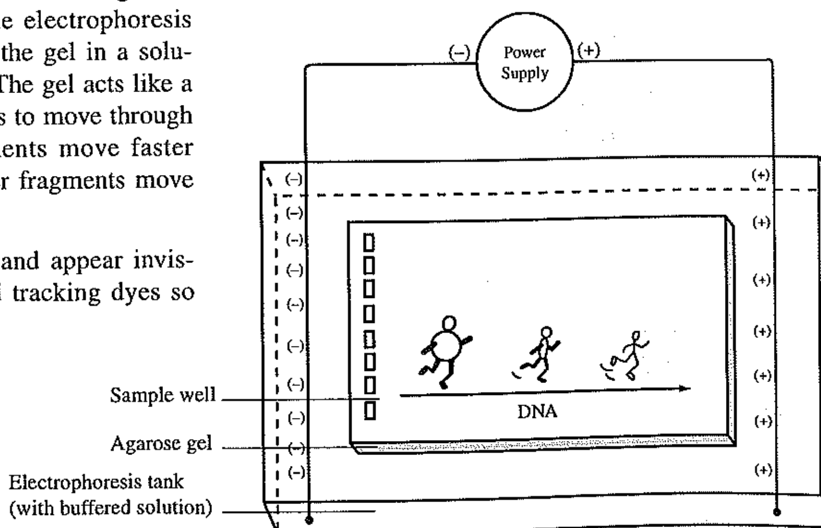


Figure 1.

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When electrophoresis is finished, a *Southern Blot* analysis is performed on the sample. In a Southern Blot, the gel is removed from the chamber and soaked for 45 minutes in a basic (pH) solution that denatures the DNA. *Denaturing* means that the double-stranded DNA separates into single strands, which makes them easier to analyze. The single-stranded DNA fragments remaining on the gel are then transferred to an inert nylon membrane in a process known as blotting. By simple capillary action, the DNA fragments are transferred to the membrane in exactly the same pattern and location as they existed in the gel. The final step of the process involves immersing the membrane in a solution containing small radioactive DNA sequences called "probes." These specially made probes are designed to attach only to complementary sequences on the DNA fragments.

After overnight incubation with the radioactive probes, the membrane is washed again to remove any unbound probes and then placed on top of a piece of X-ray film. When developed, black spots, called bands, appear on the film wherever a radioactive probe was joined to its complementary DNA sequence. These X-ray pictures of DNA fragments are known as *autoradiographs* or *autorads*. In this experiment, a colored dye that can be viewed with white light is used rather than radioactive probes and X-ray film. The banding pattern is unique since the DNA sample is unique to each individual or organism, except identical twins. The banding pattern will be compared to that obtained from a known standard of human DNA, plus samples of the mother, the child ,and likely fathers. The more bands from the child's sample that match bands from the possible father's sample the more likely he is to be the father of the child.

A second sample may be run using a different restriction enzyme. A different banding pattern will be revealed for the same DNA samples because the DNA sequence is cleaved at a different base-pair location. The probability that two individuals will have identical banding patterns for two different DNA cuts is greater than the current human population (with the exception of identical twins who share identical genotypes). The theoretical risk of a coincidental match is estimated to be 1 in 100 billion.

Experiment Overview

Paternity testing is just one type of relatedness that can be tested. Tests can determine if a person is related to a mother, father, sister, brother, or grandparent. In this lab, the relationship between descendents of one man will be determined.

The purpose of this activity is to demonstrate the separation technique known as gel electrophoresis.

Materials

- Agarose gel
- Beaker, 600 mL, 2
- DNA samples:
  - DNA Sample 1
  - DNA Sample 2
  - DNA Sample 3
  - DNA Sample 4
- Electrophoresis chamber with power or battery supply
- Light box or other light source (optional)
- Marker
- Methylene blue electrophoresis staining solution, 50 mL
- Paper towels
- Paper, white
- Pipets, disposable, needle-tip, 8
- Resealable bag
- Ruler, metric
- Staining tray
- TAE electrophoresis buffer, 200 mL
- Water, tap, 100 mL

Safety Precautions

Electrical Hazard: Treat these units like any other electrical source—very carefully! Be sure all connecting wires, terminals and work surfaces are dry before using the electrophoresis units. Do not open the lid of the unit while the power is on. Use heat protective gloves and eye protection when handling hot liquids. Methylene blue will stain skin and clothing. Wear chemical splash goggles, chemical-resistant gloves, and a chemical-resistant apron. Wash hands thoroughly with soap and water before leaving the laboratory.

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Procedure

Part A. Loading a Gel

1. Assemble the electrophoresis unit according to the teacher's instructions.
2. Place the electrophoresis unit in a horizontal position on top of a piece of white paper on a level table or countertop. Do not move the unit after loading the samples.
3. Gently slide a gel from a resealable bag into the casting tray with the wells toward the cathode (–) end of the gel tray.
4. Carefully position the gel and tray into the electrophoresis chamber. *Caution:* Be careful not to break or crack the gel. If the gel is damaged it should not be used as the breaks and cracks will affect the results.
5. Pour enough electrophoresis buffer into the unit to submerge the entire gel surface to a depth of 2–5 mm. If the gel begins to float away, reposition it on the tray.
6. By convention, DNA gels are read from left to right, with the wells located at the top of the gel. With the gel lined up in the electrophoresis chamber and the wells to the left, load the contents of DNA Sample 1 into the well closest to you. Consequently, when the gel is turned so that the wells are at the top, "1" will be in the upper left corner. If there will be empty wells in the gel, leave the outside (end) wells empty, since they are most likely to give aberrant results.
7. Place the DNA Banding Worksheet on the counter in the same orientation as the electrophoresis unit. The small rectangles on the paper correspond to the wells in the gel (see Figure 1).
8. Shake the microcentrifuge tubes containing the DNA samples and lightly tap the bottom of each tube on the tabletop to return the contents to the bottom of the tube.
9. Withdraw 10 μL of DNA Sample 1 from microcentrifuge tube by filling only the needle tip of a clean pipet. *Note:* Fill the tip by squeezing the pipet just above the tip, not the bulb. Be careful not to draw the sample further up the pipet (see Figure 2).
10. Dispense the sample into the first well by holding the pipet tip just inside the well. The sample will sink to the bottom of the well. *Caution:* Do not puncture the bottom or sides of the well. Do not draw liquid back into the pipet after dispensing the sample (see Figure 3).

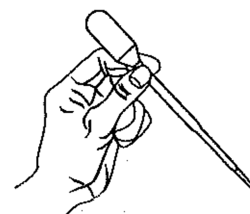

Figure 2.

11. Record the sample name on the DNA Banding Worksheet in the appropriate well box.
12. Using a fresh pipet, withdraw 10 μL of DNA Sample 2 and load it into well 2, adjacent to DNA Sample 1.
13. Record the sample name on the DNA Banding Worksheet in the appropriate well box.
14. Repeat steps 12 and 13 for the remaining DNA samples. Use a clean pipet for each sample. Load each sample into the adjacent well. Each student group will load four wells.

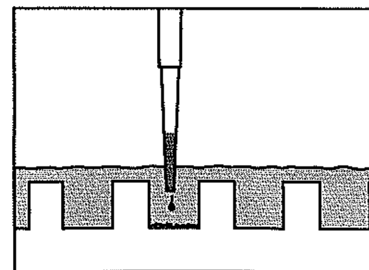

Figure 3.

Part B. Running a Gel

1. Place the lid on the electrophoresis chamber and connect the unit to the power supply according to your teacher's instructions.
2. Run the gel as directed by your teacher. *Note:* Bubbles should form along the electrodes in the chamber while the sample is running. The bubbles are the result of the electrolytic decomposition of water—hydrogen at the cathode and oxygen at the anode.
3. Turn off the apparatus to stop the gel when the first tracking dye is 1 cm from the positive end of the gel. (This may take 30 minutes to 2 hours. The time necessary to run a gel depends on the type of electrophoresis apparatus and the applied voltage.)
4. When the power is off, remove the cover and carefully remove the gel tray from the chamber. Place the gel tray on a piece of paper towel. *Note:* Be careful not to break or crack the gel.

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Part C. Staining the DNA

For best results, stain the gel immediately, and then place in a refrigerator overnight with water to destain.

1. Slide the gel off the tray and into the staining tray. *Note:* Do not stain the gel tray.
2. *Gently* pour 40 mL of the methylene blue electrophoresis staining solution into the staining tray.
3. Allow the gel to stain for 5–10 minutes.
4. Pour off the stain into a glass beaker. The stain may be reused. Be careful not to damage the gel.
5. To destain the gel, gently pour room temperature tap water into the staining container. *Note:* Do not exceed 37 °C—warmer water may soften the gel.
6. Occasionally agitate the water for 10 minutes.
7. Pour off the water into a waste beaker.
8. Repeat steps 5–7 until the DNA bands are distinctly visible.
9. If the bands are too faint to be observed, repeat steps 2–8.

Part D. Storing the Gel

Stained gels may be stored in a laboratory refrigerator for several weeks.

1. Label a resealable bag with the group name and the date.
2. Place the stained gel into the resealable bag.
3. Add 2 mL electrophoresis buffer and 3 drops of methylene blue electrophoresis staining solution to bag.
4. Place into a refrigerator as directed by your teacher.

Disposal

Consult your instructor for appropriate disposal procedures.

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Name: ____________________

Paternity Testing Worksheet

Questions

1. Explain the limitations of one type of paternity testing.

2. Why are colored tracking dyes used when running the fragments through the gel?

3. When analyzing the DNA banding pattern, where would you expect to find the smallest fragments produced by the restriction enzyme?

4. What type of test should be used to determine paternity if the likely father is deceased?

5. Summarize the steps involved in analyzing a set of DNA samples for relatedness testing.

6. List three errors that could affect the outcome of any gel electrophoresis procedure.

7. Evaluate the resulting banding patterns of the DNA samples. In your opinion, is anyone a descendent of Leonardo? Justify your opinion.

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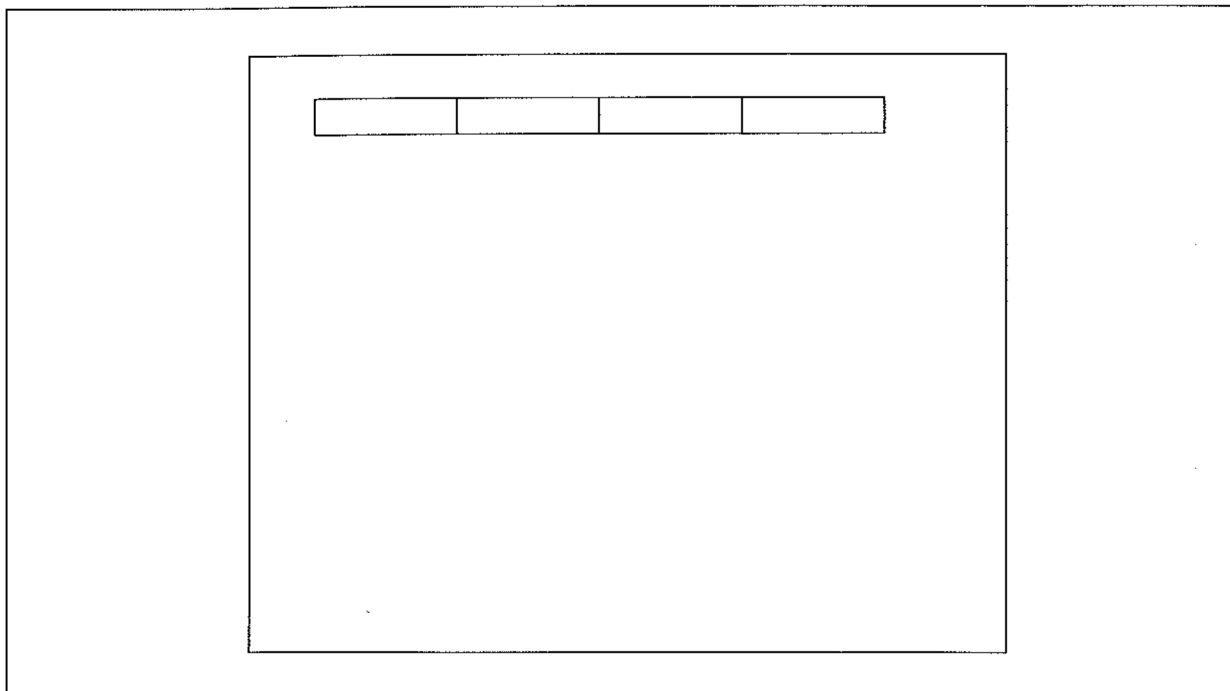
Name: ______________________________

DNA Banding Worksheet

(−) cathode (−)



(+) anode (+)

Analysis

1. Using a metric ruler, measure the migration distance in millimeters for each band and sketch the observed DNA banding patterns for each sample on the DNA Banding Worksheet.
2. Complete the Data Table.

Data Table

DNA Sample 1		DNA Sample 2		DNA Sample 3		DNA Sample 4	
DNA Fragment No.	Migration Distance (mm)	DNA Fragment No.	Migration Distance (mm)	DNA Fragment No.	Migration Distance (mm)	DNA Fragment No.	Migration Distance (mm)

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Preparation of 0.8% Agarose Gel

Materials Needed, Casting Agarose Gel

Agarose, 3 g
Balance, 0.01-g readability
Casting trays with well combs
Cotton, non-absorbent or foam plug
Erlenmeyer flasks, borosilicate, 250-mL, 6
Marker or wax pencil
Microwave, hot water bath or stirring hot plate
Stirring rod
TAE electrophoresis buffer, 360 mL
Weighing dishes, small or weighing paper

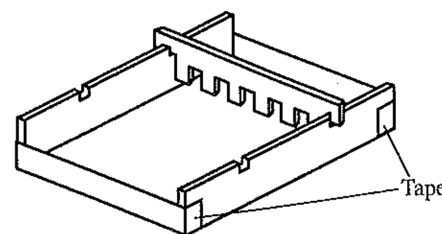
Safety Precautions

Wear chemical splash goggles and heat protective gloves when handling hot liquids. Be careful not to superheat the solution because it will NOT boil until stirred whereupon it will boil over. Wash hands thoroughly with soap and water before leaving the laboratory. Please consult current Material Safety Data Sheets for additional safety, handling and disposal information.

Preparation of one 0.8% agarose minigel

1. Stir 0.48 g of agarose into 60 mL of diluted (1X) electrophoresis buffer in an Erlenmeyer flask. Stopper with a non-absorbent cotton or foam plug.
2. Mark the height of the solution on the Erlenmeyer flask.
3. Dissolve agarose by heating in a microwave, hot water bath, or on a hot plate. *Caution:* Be careful not to superheat the solution because it will NOT boil until you disturb or disrupt it, whereupon it may spontaneously boil out.
   a. Microwave: 30–40 seconds, stir, repeat.
   b. Hot water bath: do not boil the water
   c. Hot plate: do not boil or scorch the agarose solution
4. Heat until the solution is clear and the agarose appears to be fully dissolved.
5. Stir frequently and do not allow solution to exceed 65 °C.
6. Use heat-protective gloves to remove the flask.
7. Check the level of the solution. Add TAE buffer, if needed, to bring the solution volume back to 60 mL.
8. To prevent damage to the casting trays, allow the agarose to cool to 55°C before pouring.

Prepare the casting trays while waiting for the agarose solution to cool.



1. Attach the rubber dams to the ends of the casting tray or use tape to create the end walls.
2. Place the well-forming comb in the grooves at one end of the gel box.
3. Ensure the casting tray is on a level surface.
4. Slowly pour the melted agarose into the assembled casting tray, being careful not to create bubbles in the gel. Use a stirring rod or pipet tip to push any bubbles to the edge of the casting tray. Only add enough agarose to equal the height of the indentations in the well-forming comb—do not fill the tray to the top.
5. Thoroughly rinse out the Erlenmeyer flask immediately.
6. Allow the gel to sit undisturbed for at least 20 minutes until the gel is firm to the touch. The set gel will appear opaque and somewhat white. 60 minutes is optimal.
7. Once the gel is thoroughly set, carefully remove the well-forming comb by rocking it gently from side to side and then pulling it upward. Remove the end dams and carefully slip the gel out of the form.
8. Slide each gel into a separate resealable bag, add 5 mL of buffer, and refrigerate. *Note:* A solidified gel can be stored under buffer in a laboratory refrigerator for up to two weeks.

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