PCR Investigation

COLLECT EVIDENCE ● ESTABLISH LINKS ● MAKE AN ARGUMENT

Biotechnology Outreach

featuring the
blueGel™ electrophoresis system and mini16 thermal cycler
by miniPCR bio®

_____ _____

student name date

BE A SCIENTIST

- ✓ Listen to and read instructions closely
- ✓ Follow all directions, including safety precautions
- ✓ Wear appropriate safety equipment, including medical gloves
- ✓ Collaborate with your team/group to ensure all work is done efficiently and correctly
- ✓ Take time to think about…
  - …WHAT you're doing.
  - …HOW you're supposed to do it.
  - …WHY you're doing it.

A special "thank you" to the team at miniPCR bio® for their contributions to this project including blueGel™ electrophoresis system and mini16 thermal cycler instructions and diagrams.

A Human DNA Fingerprinting Lab Protocol

Adapted from 1994 Cold Spring Harbor Laboratory DNA Learning Center

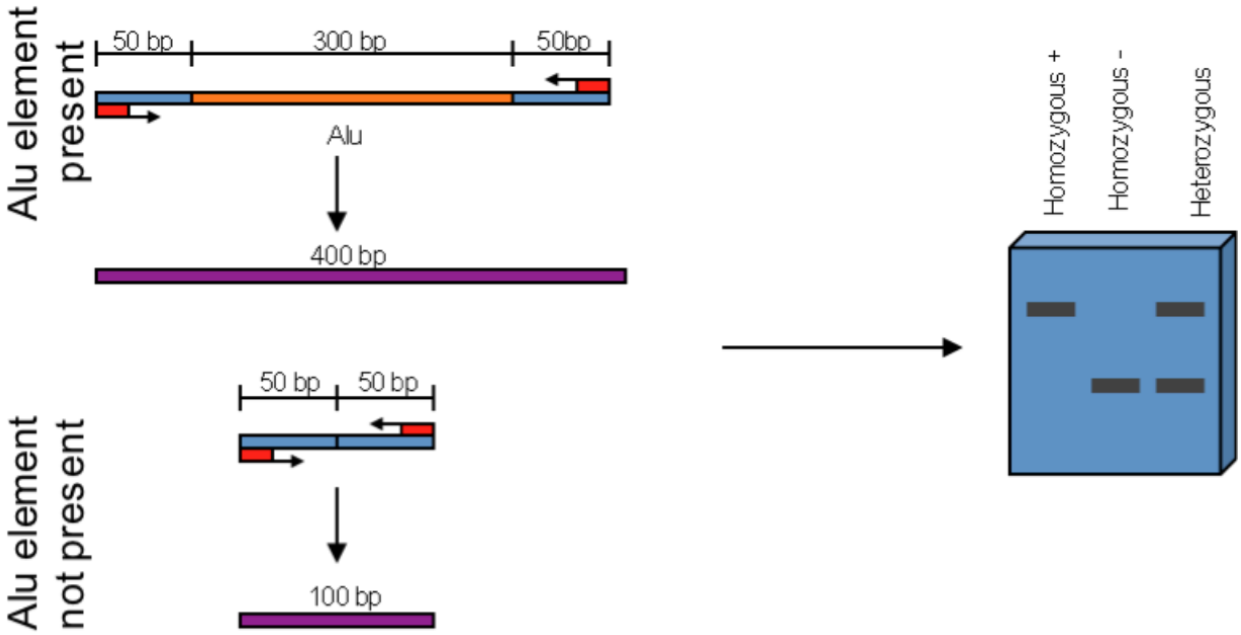
In this exercise, the polymerase chain reaction (PCR) is used to amplify a nucleotide sequence from chromosome 8 to look for an insertion of a short DNA sequence called *Alu* within the tissue plasminogen activator (TPA) gene. Although the DNA from different individuals is more alike than different, there are many regions of the human chromosomes that exhibit a great deal of diversity. Such variable sequences are termed "polymorphic" (meaning many forms) and provide the basis for genetic disease diagnosis, forensic identification, and paternity testing.

GEEKING OUT

The *Alu* family of short interspersed repeated DNA elements are distributed throughout primate genomes. Over the past 65 million years, the *Alu* sequence has amplified via an RNA-mediated transposition process to a copy number of about 500,000, comprising an estimated 5% of the human genome. *Alu* sequences are thought to be derived from the 7SL RNA gene which encodes the RNA component of the signal recognition particle that functions in protein synthesis. *Alu* elements are approximately 300-bp in length and derive their name from a single recognition site for the endonuclease *Alu*I located near the middle of the *Alu* sequence.

An estimated 500-2,000 *Alu* elements are mostly restricted to the human genome. A few of these have inserted recently, within the last one million years, and are not fixed in the human species. One such *Alu* element, called TPA-25, is found within an intron of the tissue plasminogen activator gene. This insertion is dimorphic, meaning that it is present in some individuals and not in others. PCR can be used to screen individuals for the presence (or absence) of the TPA-25 insertion.

In this exercise, oligonucleotide primers, flanking the insertion site, are used to amplify a 400-bp fragment when TPA-25 is present and a 100-bp fragment when it is absent. Each of the three possible genotypes - homozygous for the presence of TPA-25 (400-bp fragment only), homozygous for the absence of TPA-25 (100-bp fragment only), and heterozygous (both 400-bp and 100-bp fragments) – and can be distinguished following electrophoresis in agarose gels.



The source of template DNA is a sample of several thousand cells obtained from the inner cheek wall. The cells are collected and suspended in an extraction buffer supplied by the miniPCR bio™ company. The cells are lysed by heating and centrifuged to remove cell debris. A sample of the supernatant containing genomic DNA is mixed with a PCR-to-Go™ PCR bead which contains Taq polymerase, oligonucleotides, and buffers. Temperature cycling is used to denature the target DNA, anneal the primers, and extend a complementary DNA strand.

GEEKING OUT

The "upstream" primer, 5'- GTA AGA GTT CCG TAA CAG GAC AGC T -3', brackets one side of the TPA locus, while the downstream primer, 5' CCC CAC CCT AGG AGA ACT TCT CTT T -3', brackets the other side. The size of the amplification products(s) depends on the presence or absence of the *Alu* insertion at the TPA-25 locus on each copy of chromosome 8.

In order to compare the genotypes from a number of different individuals, aliquots of the amplified sample and those of other experimenters are loaded into wells of an agarose gel, along with the DNA size markers. Following electrophoresis, amplification products appear as distinct bands in the gel; the distance moved from the well is solely determined by the presence or absence of TPA-25 insertion. One or two bands are visible in each lane, indicating that an individual is either homozygous or heterozygous for the *Alu* insertion. (See image.)

PCR Lab Activity

TPA-25 Alu Sequence

General equipment:

- 2-20µl micropipettors
- pipette tips (non-sterile is okay)
- microcentrifuge
- medical gloves in various sizes
- disposal containers for pipette tips, toothpicks, etc.

PCR section:

- miniPCR® mini16 thermal cycler
- 1 PCR tube with 50µl X-Tract™ Buffer per student
- 1 Ready-to-Go™ PCR bead tube per student
- 1 tube with 10µl of TPA-25 Alu Forward (Up) primer (provided by ISU) per group of 5 students
- 1 tube with 10µl of TPA-25 Alu Reverse (Low) primer (provided by ISU) per group of 5 students
- sterile water
- sterile flat toothpicks

Gel Analysis:

- miniPCR® blueGel™ electrophoresis system
- 1 SeeGreen™ tablet per gel
- approx. 30 ml of 1X TBE buffer per gel
- 16µl 100bp ladder
- loading/migration dye

Part I: Cell collection and prep using the miniPCR X-Tract™ buffer

Step 1. Gently scrape the inside of your cheek about 5 times with a flat-end toothpick, collecting cells at the tip. Rub the toothpick gently…it shouldn't hurt!

Step 2. Dip the toothpick into the 200µl tube containing X-Tract™ buffer and swirl gently to release the cells. Cap the tube tightly.

Step 3. Everyone should be assigned an individual number. Label the upper side of the 200µl PCR tube with your number.

Step 4. Incubate the tube at 95° C for 10 minutes. You may do this using a hot water bath or thermal cycler. (If using a mini16 miniPCR® thermal cycler, refer to Part 3 for instructions and use the "Add HEAT BLOCK" program to set up your incubation. Page 10 demonstrates how to open/close the miniPCR® thermal cycler.)

Step 5. Spin the tube at high speed (~5000rpm) in a microcentrifuge for 1 minute to create a pellet of cell debris at the bottom of the PCR tube.

Step 6. Place the tube on ice until ready to move on to Part 2.

Part 2: DNA prep

Step 7. Each member of your group will receive a 200µl PCR tube containing a PCR bead and enough sterile water and primers to complete the reaction matrix in Step 8.

Step 8. Using the same number from Step 3, label the upper side of the 200µl PCR bead tube.

Step 9. Use a 2-20µl pipet to add the following reagents to the PCR tube according to the matrix below. **Keep the primers on ice until ready to use.**

Sterile water	16.0µl
Upper primer (5pmol/µl)	2.0µl
Lower primer (5pmol/µl)	2.0µl
Cheek cell solution from Part 1	5.0µl
Total volume	***25.0µl***

Step 10. Gently flick the PCR tube to mix the contents. Place the tubes in the microcentrifuge and spin at ~5000rpm for 30 seconds.

MAKING SENSE

PCR Reaction Components

DNA + nucleotides + Taq polymerase + buffer + primers

DNA: from your cheek cells

***Nucleotides:** also called 'bases', these are adenine (A), thymine (T), cytosine (C), and guanine (G). They serve as the building blocks for the new copies of DNA made during the PCR reaction.

***Taq polymerase:** this enzyme adds new DNA nucleotides to the end of the primer sequences. We use this enzyme because it is stable at the high temperatures needed to denature DNA sequences.

***Buffers:** helps maintain proper pH levels in the reaction mixture

Primers: these short, single-stranded DNA segments are used to amplify targeted fragments of DNA. They always come in sets, with a forward and reverse direction.

**the PCR beads contain nucleotides, Taq polymerase, and buffers*

Part 3: Thermalcycling with the miniPCR® mini16 thermal cycler

Note: if you already have the miniPCR app and have connected to your thermal cycler, you can skip to Step 16.

DOWNLOAD

Step 11. Download the miniPCR® app for iOS or Android. Downloadable versions are also available for Windows 10 and Mac at www.minipcr.com/downloads.

Step 12. Install and open the app by tapping the miniPCR® icon.

CONNECT

Step 13. Turn on the mini16 using the on/off switch on the back of the unit. A flashing blue LED on the front of the mini16 indicates that it is ready to connect to your device.

Step 14. Click the Devices tab (Win/Mac) or the miniPCR® icon (mobile, top center of the screen). mini16 units within Bluetooth range will be listed. In the app, click on the Bluetooth symbol of the mini16 unit that matches the number on the white sticker above the power switch.

Step 15. Successful pairing is indicated by green text "Connected" and by the blue LED staying always on. You are ready to use your miniPCR® thermal cycler.

MAKING SENSE

Polymerase Chain Reaction: PCR is a type of in vitro DNA replication. PCR is unique from other methods of in vitro DNA replication in that two primers are used. The primers are complementary to opposite strands, so that both strands can be synthesized at the same time. In addition, an important feature of PCR is that the length of the product sequence is determined by the position of the two primers (the target region is bracketed by the two primer sequences). Thus, the copies made are of a defined length and from a specific location on the template. Because DNA synthesis occurs by extension from the primer (the primer is included in the newly synthesized strand), the new DNA strands include binding sites for the primers to anneal, and thus the new strands function as templates for new synthesis.

Denaturation: The first step is denaturation of the double-stranded DNA template so that it becomes single stranded. This is done by heating the DNA to a high temperature (~ 94°C) so that the hydrogen bonds between the complementary bases of the DNA are broken.

Annealing: In the second step, the annealing step, the temperature is lowered so that the primers can bind to the complementary sequences on the single-stranded template by forming hydrogen bonds between the bases. The primers are typically about 18-25 bases in length and chosen so that they flank the region of DNA to be amplified.

Extension: The third step is the extension step, in which the temperature is raised to 72°C to optimize the process of incorporating complimentary nucleotides into the growing DNA chain. The Taq polymerase enzyme contained on the PCR beads facilitates these reactions.

PROGRAM

Step 16. Open the miniPCR® App in your device and remain on the "Library" tab.

Step 17. Click on the  button on the top right corner.

Step 18. Select "Flex" from the protocol type menu.

Step 19. Enter a protocol name; for example, "ISU PCR".

Step 20. Click on "Add step" and choose "PCR". Configure Step 1 using the following parameters:

Denature	94°C	300 seconds
Annealing	54°C	15 seconds
Extension	72°C	60 seconds
Number of cycles: 1		

Step 21. Click on "Add step" and choose "PCR". Configure Step 2 using the following parameters:

Denature	94°C	15 seconds
Annealing	54°C	15 seconds
Extension	72°C	60 seconds
Number of cycles: 33		

Step 22. Click on "Add step" and choose "PCR". Configure Step 3 using the following parameters:

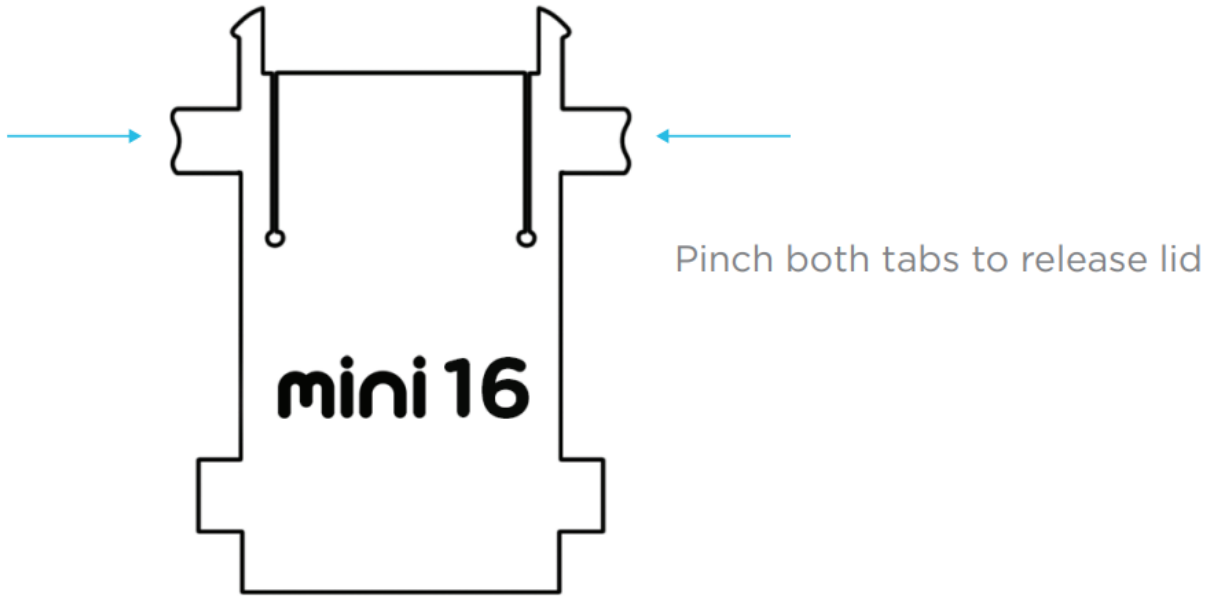
Denature	94°C	300 seconds
Annealing	54°C	15 seconds
Extension	72°C	600 seconds
Number of cycles: 1		

Step 23. Click on "Add Step" and choose "Heat Block." This step will act as our infinite hold and can be increased, decreased, or omitted as needed. Configure Step 4 using the following parameters:

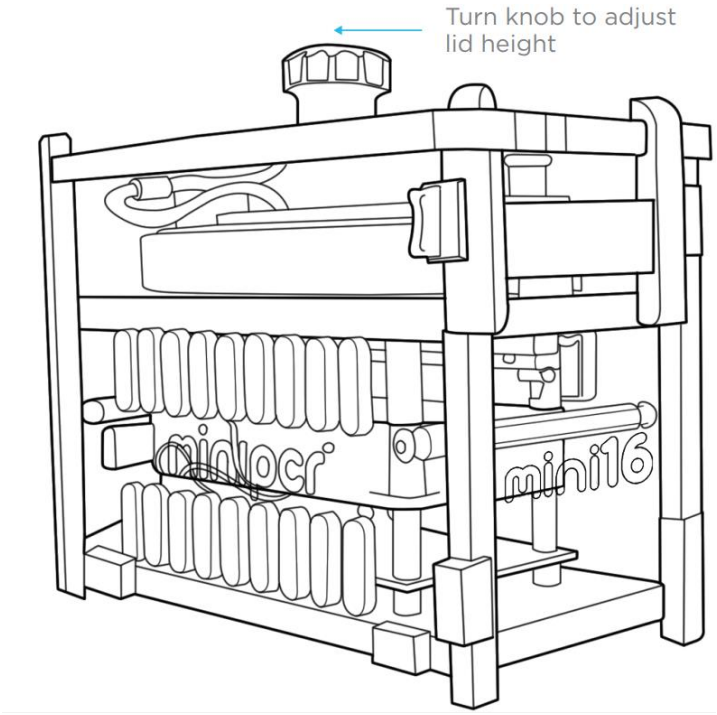
Temperature	25°C
Time	120min

START THE RUN

Step 23. Open the mini16 thermal cycler by pinching the side tabs on the front plate of the machine. Use your thumb and index fingers to pinch, and lift the lid with your other hand. Load the PCR tubes from Step 10 into the metal block.



Step 24. Fully untwist (counterclockwise) the adjustment knob and press the lid down until the lid clicks shut. Tighten the adjustment knob (clockwise) until you feel **light** resistance from the tube caps. Be careful not to over-tighten.



Step 25. Move the power switch on the back of the mini16 to the ON position.

Step 26. Connect your miniPCR® thermal cycler via Bluetooth.

Step 27. Select the desired protocol from your Library. Click the "Run" button. The run will automatically begin, signaled by the greed LED turning on.

- It is not necessary to maintain a USB or Bluetooth connection during a run. The miniPCR® will keep running the protocol while the power switch remains in the ON position.
- Toggling the power switch OFF and ON will restart the last protocol programmed.
- A flashing yellow LED indicates an error; reinitialize the device or contact support.
- Only ONE Bluetooth device may be connected with the mini16 thermal at a time.

Tip: The end of a run will be signaled by the green, yellow and red LEDs staying ON.

MONITORING THE RUN

Step 28. Click on the "Monitor" tab to see the real-time run status. You can pause or stop the reaction at any time from this screen.

Step 29. When all four mini16 LEDs are on (solid), the protocol is complete. Remove the PCR tubes and place on ice.

Part 4: Gel analysis with the miniPCR® blueGel™ electrophoresis system

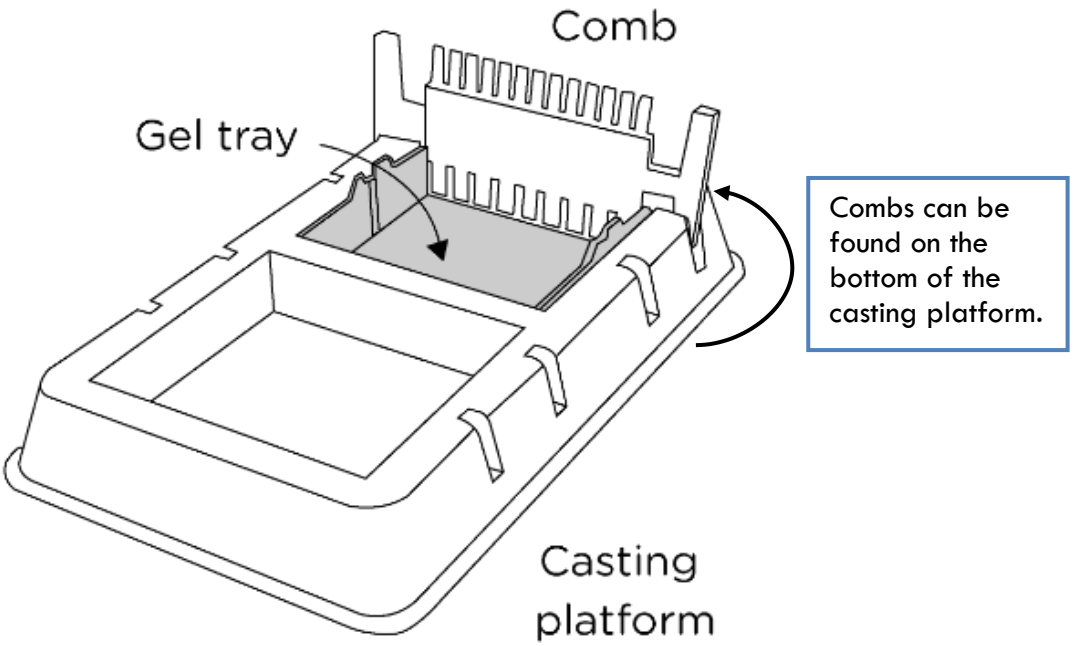
Step 30. Place the gel tray inside the casting platform and add combs from the bottom side of the tray (we will use the wider, 9-well comb). Place on a level surface to ensure uniform gel thickness. Determine the percentage gel to make; we recommend a 2.0% gel for this lab.

Gel %	Per 1 SeeGreen™ Tab	Yield (no. of gels)
1.0%	40mL distilled water	2 blueGel gels
1.5%	27mL distilled water	2 blueGel gels
2.0%	20mL distilled water	1 blueGel gel

Step 31. Soak one SeeGreen™ all-in-one agarose tab in distilled water according to the table above. Use a container at least three times larger than the desired gel volume.

Step 32. Swirl about 3 minutes until the Tab is fully broken down.

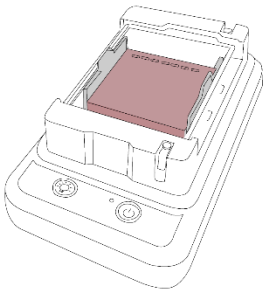
Step 33. Wearing heat-resistant gloves, heat the solution until it is clear and all particles are dissolved. To do this, microwave on high for 15 seconds, remove and swirl the flask, and then heat for another 15 seconds. Allow to cool to 60-70°C (or when the bottom of the flask is comfortable to touch). DO NOT add any DNA stain.

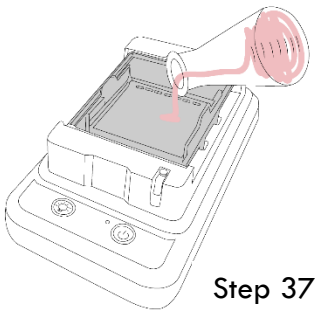


Step 34. Pour the solution into the gel tray to a depth of approximately 8mm; if using a 2.0% gel, this will be the whole solution. 2-3mm of the comb should be below the level of the solution. Allow the gel to completely set before moving on (approximately 20 minutes).

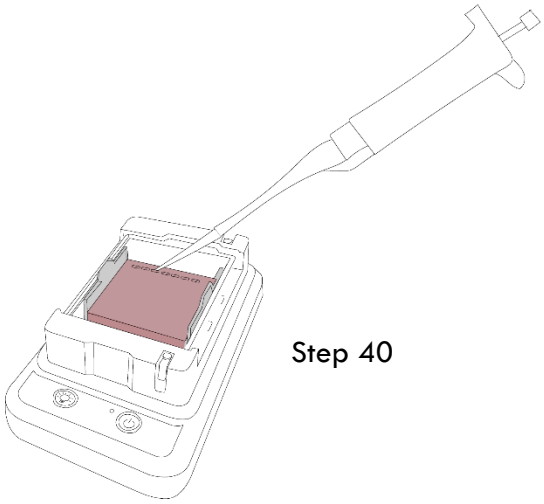
Step 35. Slowly remove the combs from the gel. Remove the gel tray from the casting platform. If a small amount of gel has formed underneath the gel tray, wipe it off and discard it. Note: you may store gels in a cool, dark place for up to 5 days. Keep the gel moist in a resealable zip bag with paper towel saturated with water.

Step 36



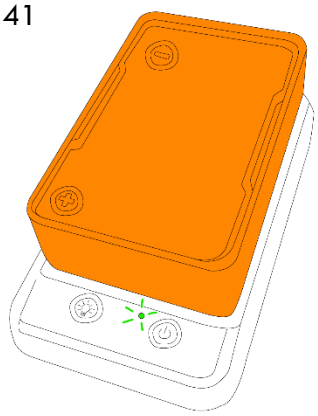


Step 37



Step 40

Step 41



Step 36. Place the gel tray containing a gel in the buffer chamber and place the buffer chamber inside the blueGel™ base. The wells should be closest to the (-) end.

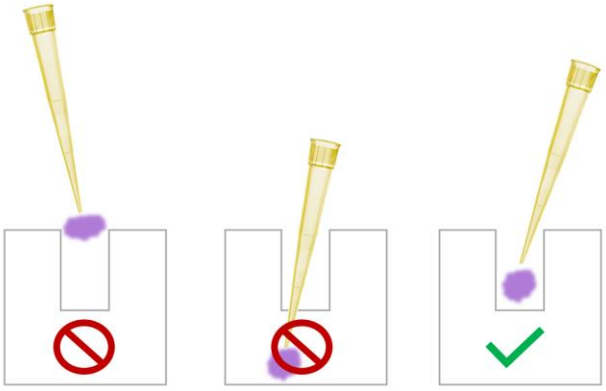
Step 37. Add approximately 30ml of 1X TBE buffer to the buffer chamber. The buffer should just cover the agarose gel. CAUTION: Do not overfill the gel chamber as it may overflow when the cover is placed over the gel.

Step 38. Remove air bubbles (if any) trapped between the gel and the gel tray, or between the gel tray and the buffer chamber.

Step 39. Into your PCR tube, pipette 4µl of loading/migration dye. Also obtain a tube containing 16µl of 100bp ladder. Add 4µl of loading/migration dye to the ladder, as well.

Step 40. To rinse the pipette tip and mix the DNA (PCR products or ladder) and the dye, fill and unload the pipettor with the sample three times. The dye is used to monitor the migration of the DNA during electrophoresis. Spin or use pipette tip to pull all liquids to the bottom of the tube.

Step 41. Load both the PCR products and the ladder into separate wells using a pipettor. 9-well combs hold up to 20µl and 13-well combs hold up to 10µl. Be careful not to puncture the gel with the micropipette tip. Use the diagram below as a guide. Record the contents of each well on **page 15.**



Step 42. Place the orange cover on the blueGel™ base. The cover contains the electrodes and will only fit in one direction, with the (+) electrode positioned to attract the negatively charged DNA.

Step 43. Press the power button to start the run. The green LED indicator located next to the power button should light up. Small bubbles will form near the electrodes. Run for approximately 35-50 minutes.

SAFETY NOTE

For safety, the blueGel™ system's power will not turn on if:

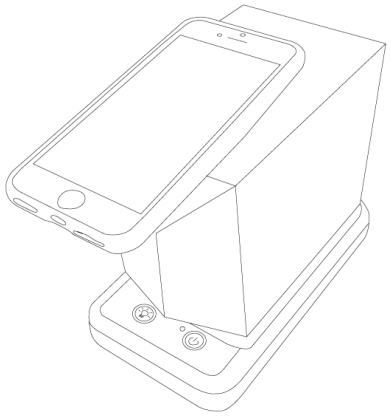
- The cover is not correctly placed on the base, and electrodes are not making contact
- There is no buffer in the buffer chamber
- Using the incorrect buffer (too diluted or too concentrated)

Step 44. At any time during the run press the lightbulb button to visualize the DNA. The orange cover filters the excess blue light allowing easier visualization of the fluorescence emitted by DNA. To protect the bulb, only turn the light on while you are viewing the gel.

TECH TIP

To document the run, turn on the blue light and take a picture with a smartphone, tablet or other camera device.

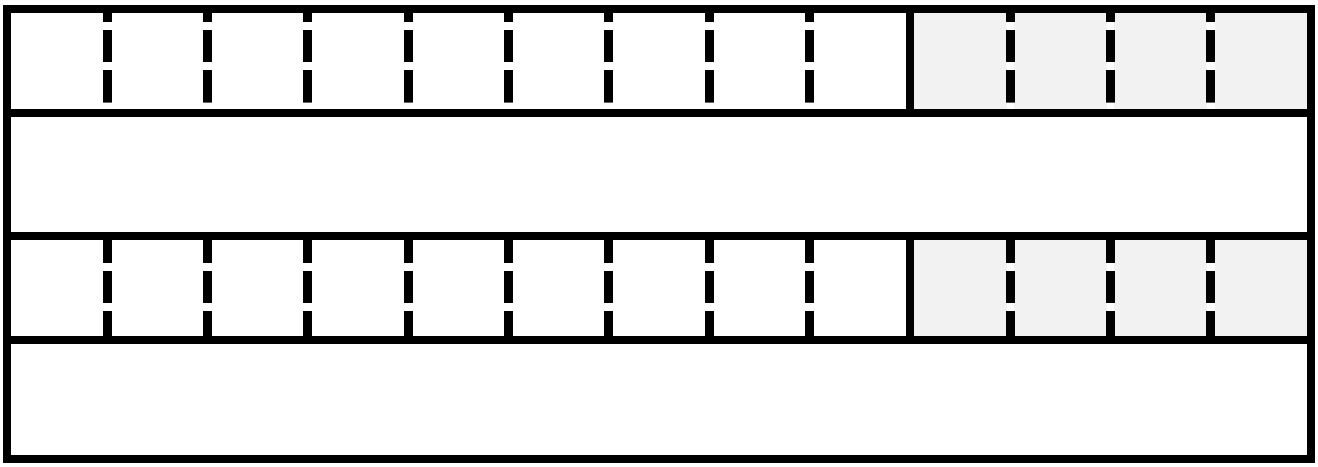
Tip: If DNA is not easily visible, dim or turn off ambient light. To document gels in bright ambient light, use the supplied Fold-a View™ photo documentation hood. Pop up the Fold-a-View™ following the instructions on its side and place it on the blueGel™ orange cover, sliding it down until it fits snugly around the cover's edges. Place your camera on top, and align the camera lens with the circular opening on the Fold-a-View™.



If needed, softly wipe condensation off the inside of the orange cover with the supplied lens cleaning cloth to improve visibility.

LABEL YOUR SAMPLES

Use the diagram below to label the contents of each well on your gel. Space for 2 rows have been provided – large combs create 9 wells, small combs create 13 wells each.



Step 45. Determine your TPA-25 status using the DNA ladder diagram provided. Compare answers with your partner and with the rest of the class.

MAKING SENSE

DNA Ladders

One of the elements of a quality scientific experiment is a good control to measure against. In this experiment (and gel electrophoresis, in general), we use a DNA ladder to ensure that the resulting bands are of the size we expect them to be. The image below shows what the DNA ladder should look like on your gel and gives the length of the DNA strand in base pairs, or bp. Using the ladder, you should be able to determine if your DNA contains a 400bp and/or 100bp band, and thus if you are heterozygous or homozygous for TPA-25.

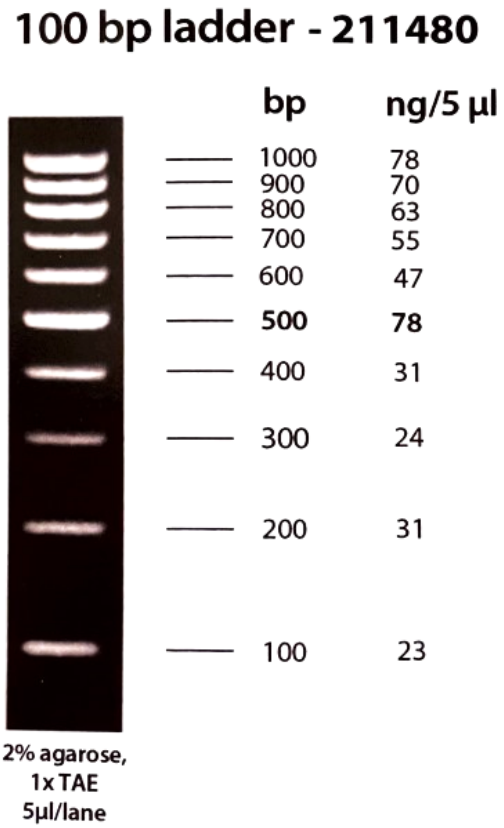


Image from Carolina Biological Supply.

INVESTIGATION QUESTIONS:

1) Why is it important to change pipette tips after each use?

2) What are some things that could cause a PCR to fail?

3) Why are there upper (forward) and lower (reverse) primers?

4) Some PCR labs include the use of a negative control. This is a mixture of all PCR reaction components except for the targeted DNA template. Why would this be a useful addition to our investigation?

5) How is this investigation similar to or different from the PCR diagnostic test for COVID-19? You may need to do some research!

INVESTIGATION NOTES

INVESTIGATION NOTES

Biotechnology Outreach

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