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Slim Cycler Student Lab: Who killed Carol?

Introduction:
This curriculum is developed by Aas Incorporated with the goal of providing students with genetic tools they can use to solve real world problems that involves DNA profiling. This Slim Cycler lab will allow students to conduct state-of-the-art molecular biology experiments and understand how these techniques are performed in the real world forensic science labs.

How can DNA evidence solve crimes?
DNA profiling is the use of molecular genetics methods to determine the exact genotype of a DNA sample to distinguish one human being from another. This powerful tool is now routinely used around the world for investigations of crime scene, missing persons, mass disasters, human rights violations, paternity testing and allows early diagnosis of heritable diseases. Crime scene often leaves traces of biological evidence (such as blood, semen, sweat, hair, saliva, bones, skin cells) from which DNA can be extracted. If the DNA of an individual matches the DNA samples found in the crime scene, that individual is incriminated whereas if his/her DNA does not match with those found in the crime scene, that individual is excluded from the crime investigation. In addition, DNA profiling can help inform individuals if they inherited a genetic disease so they are guided as to what the next necessary steps are in alleviating the phenotype of their genetic condition. Moreover, any dispute over ambiguities on who the father is of a child in question will be solved by comparing the DNA of all likely sex partners, the DNA of the mother and the child DNA. DNA profiling provides a powerful and definitive evidence that can help solve previously unresolved cases.

What kinds of human DNA sequences are used in crime scene investigations?
The human genome is made up of 3 billion base pairs of DNA but only about 5% are composed of **genes or exons** which are genomic sequences that code for a functional protein. The rest of the genome is made up of non-coding sequences called **introns** which are sequences of DNA that do not code for any protein and has no clear function so they are often referred to as 'junk DNA'. Non-coding sequences contain many repeat sequences that vary in sizes and are classified according to the length of the core repeat units, the number of contiguous repeat units and the overall length of the repeat region. These repeats vary among individuals hence they are called **polymorphic** (many forms) sequences. DNA sequences with short repeat units composed of 2-9 base pairs are called **short tandem repeats** or **STRs** which are found to inhabit the area surrounding the chromosomal centromere. Each individual inherits one copy of the STR allele from each parent which may or many not have the same repeat size. Because the number of repeats vary widely from person to person, every person possess a distinct STR signature that is unique for each individual. This makes STR technology a powerful and effective tool in human identification especially in forensic DNA profiling.

STRs have become popular markers for DNA profiling because they are easily amplified by polymerase chain reaction (PCR). For human identification purposes, it is important to have the DNA markers exhibit the highest possible variation in order to discriminate between samples. It

is often difficult to obtain PCR amplification products from forensic samples because either the DNA in those samples are degraded or mixed, such as DNA samples obtained from a sexual assault case.

The smaller size of STRs make them better candidates for use in forensic applications because STRs are easily amplified with low mutation rates hence they work exceptionally well with degraded or mixed DNA samples. At present, there are 13 internationally recognized STR loci that are routinely used in STR typing test in the FBI laboratory. These also include the core genetic loci for CODIS (Combined DNA Index System). They include CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S818, D8S1179, D13S539, D18S51, D21S11, and AMEL.

Case Overview:

A group of five male students, Sam, Ken, Pete, John and Brian attended a party at their Lisa's house one Friday night. Lisa, the party host, invited many of her friends from school aside from her five close male friends. When these five fellows arrived, the house was already crowded and there was a lot of drinking, dancing, loud music and laughing from everywhere. The party was on a roll when suddenly, a loud gunshot noise emanating from one of the bedrooms reverberated throughout the house which stymied everyone. This was followed by two more loud gunshots and then silence. The music was suddenly stopped and everyone was hushed and one could almost hear a needle drop. In the far end of the house where the sound of the gunshots came from, they heard muffled voices and footsteps rushing towards the backdoor but they could not see who they were as they were indistinguishable from a distance. Then they heard the backdoor closing and footsteps of people running away. Everyone was still for a minute but then a burst of panic ensued and everyone scrambled as fast as they could to their cars and swiftly drove away from the scene of the crime. When the police came, they saw Lisa crying with her hands filled with blood sitting beside her best friend Carol who lay there motionless with a gunshot wound in her chest. The police looked at traces of evidence and they saw three pairs of footsteps, some blood smear on the edge of the backdoor, some blood drippings on the grass, and there was a bloody shoe left. Carol was declared dead a moment later and the police opened a murder investigation to determine WHO MURDERED CAROL.

Synopsis:

Students will help solve Carol's murder case by assisting the forensic investigators in analyzing numerous DNA samples from the scene of the crime using molecular biology techniques such as polymerase chain reaction (PCR) and gel electrophoresis. This exercise will familiarize students with molecular biology techniques used in real-world applications such as DNA profiling in a crime scene investigation.

Required techniques: PCR, agarose gel electrophoresis, DNA visualization
Time required: 90 minutes
Reagents needed: PCR reagents and gel electrophoresis reagents

PCR Reagents:

1. Choose one Mastermix of your choice:
   WizPure™ PCR 2X Master for standard PCR
   WizPure™ HS-PCR 2X Master for Hot Start PCR
   WizPure™ Pfu 2X Master – for high fidelity PCR using *Pfu* Polymerase
   WizPure™ FX-PCR 2X Master – for high Fidelity PCR using *Pfx* Polymerase

   ****NOTE:** All PCR Mastermix reagents (provided by A'as Inc.) already contain DNA polymerase, dNTPs and the appropriate reaction buffer. All you have to do is add the primers and sample template DNA.

2. Forward and reverse primers (2 uM final concentration)
3. DNA sample (10 pg – 250 ng)

Gel Electrophoresis Reagents

1. 1X TAE Buffer
2. Ethidium bromide
3. Bromphenol blue
4. 100 bp DNA ladder

Equipment needed: T-heater Slim PCR cycler (from A'as Inc.), gel electrophoresis apparatus, DNA transilluminator

Suggested skill level: Familiarity with DNA and its properties, DNA amplifications and basic micropipetting techniques

Reaction setup: This experiment can be done either at room temperature or in ice. Make sure to mix and centrifuge the PCR components before use. All Mastermix solutions provided by A'as are at a 2X concentration and are ready to use. Dilute this to 1x for the PCR reaction.

Procedure:

1. Obtain sterile 200 µl (0.2 ml) microfuge tubes and label them with your sample name.
   Tube 1 – blood sample from grass
   Tube 2- blood sample from shoe
   Tube 3- blood sample from backdoor
   Tube 4 –blood sample from the bedroom floor
   Tube 5 – DNA swab sample from the bathroom doorknob
   Tube 6 – hair sample from pillow
   Tube 7 –semen sample from bed cover

2. Prepare the reaction mastermix. Sample volumes and concentrations for a single reaction are listed in the table below. Use these amounts to prepare your mastermix for multiple reactions:

Component	**20 µl**	**50 µl**	**Final Concentration**
Nuclease-free water	4 (variable)	10 (variable)	
2X Master Mix	10	25	1X
forward primer	2	5	2 µM
reverse primer	2	5	2 µM
Sample template DNA	2 (variable	5 (variable)	10pg – 250 ng
TOTAL	**20**	**50**	

Table 1. Components of the PCR reaction mix.

****NOTE:** All PCR Mastermix reagents (provided by A'as Inc.) already contain DNA polymerase, dNTPs and the appropriate reaction buffer. All you have to do is add the primers and sample template DNA.

****NOTE:** When you make your reaction mastermix, remember to add 1-2 reactions in addition to the number of samples you have to make room for pipetting discrepancies and account for the surface tension properties of water (water molecules tend to stick to the pipet tips).

3. To prepare your primers, you will need to dilute your stock solution so you can add appropriate amounts of the primers into your PCR mix.

   - Oftentimes, the stock solution is very concentrated that the amount you need is too small to be dispensed by a micropipette so you need to make a diluted working solution so you can easily pipet the primers you need for the experiment.

- Let's say you have 100 μM stock solution of each primer and you want to make 100 μls of 10 μM working stock solution of each primer to use for the experiment. First, we need to calculate how much of the stock we need to dilute.

- We will use the classic formula of $C_1 \times V_1 = C_2 \times V_2$ for dilution where:

  C_1 = 100 μM (Concentration of the stock solution)
  V_1 = Unknown (Volume of the stock solution needed to make the new solution)
  C_2 = 10 μM (Concentration of the working solution you want to make)
  V_2 = 100 μl (Total volume of the working solution you want to make)

  To calculate the amount of stock solution you need to make 100 μls of 10 μM working stock solution :

  $C_1 \times V_1 = C_2 \times V_2$;

  Plug in the values:
  $(100\ \mu M)(V_1) = (10\ \mu M)(100\ \mu l)$

  So to to get V_1, we will rearrange the formula:

  $V_1 = (10\ \mu M)(100\ \mu l) / (100\ \mu M)$

  V_1 = 10 μl of the stock solution.

- This is the amount of stock solution needed to make 10 μM of working primer solution. You can then add 10 μl of your stock primer solution to 99 μls of sterile distilled water for a total of 100 μls.

- After you made the diluted working solution, add 2 μls of that solution in a 20-μl PCR reaction mixture to obtain a final primer concentration of 2 μM.

4. In this experiment, you have 7 samples (20 μls/reaction) to amplify using PCR. To make the reaction mastermix, multiply each of the components in the table by 8 (*7 samples plus an extra reaction to cover pipetting discrepancies*) so you will have:

Component	**20 μl**	**x 8 = ___μl**
Nuclease-free water	4	32
2X Master Mix	10	80
Forward primer	2	16
Reverse primer	2	16

Sample DNA	2	16
TOTAL	**20**	**160**

Table 2. Reaction Mastermix of 7 samples (20 μl reaction).

****NOTE:** The amount of water and DNA sample are variable. Depending on the concentration of your DNA sample, add the appropriate amount of your DNA sample first then adjust to the final volume using nuclease-free water.

****NOTE:** The use of highly purified DNA sample greatly enhances the quality of your PCR products.

5. Pipet out 18 uls of the master mix solution into each of the labeled tubes.

6. Add the sample DNA into each of their corresponding tubes.

7. After you have dispensed all the components into each tube, pipet up and down gently to mix. Close the lids and centrifuge. Now your samples are ready for PCR.

8. Transfer the tubes from room temperature/ice to the pre-heated Slim PCR Cycler.

   Thermocycling conditions for routine PCR are illustrated in Table 3 below:

Initial denaturation	98°C	30 seconds
25-35 cycles Denaturation Annealing Extension	 98°C 45-72°C 68-72°C	 5 – 10 seconds 10 – 30 seconds 30 seconds – 1 minute per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

Table 3. Thermocycling conditions for routine PCR.

****NOTE:** For samples with high GC content, you may want to increase the denaturation and annealing times because it takes more time and energy to break GC bonds.

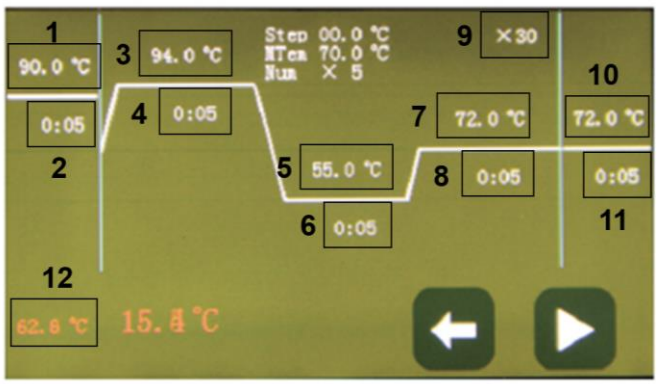
9. For this experiment, you will use the following PCR conditions:

   Initial denaturation 98°C for 30 seconds

Denaturation	98°C for 10 seconds
Annealing	58°C for 30 seconds
Extension	68°C for 30 seconds
Number of Cycles	25 cycles
Final extension	68°C for 5 minutes
Heated lid	ON

10. Turn on the Slim cycler PCR machine.

11. Enter the settings indicated in step #9. To change a setting, first tap it on the touch screen and then adjust its value by turning the rotary knob clockwise (to increase) or counterclockwise (to decrease).



1. Set initial denaturation temperature to 98°C
2. Set initial denaturation time to 30 s
3. Set denaturation temperature to 98°C
4. Set denaturation time to 10 s
5. Set annealing temperature to 58°C
6. Set annealing time to 30 s
7. Set extension temperature to 68°C
8. Set extension time to 30 s
9. Set number of cycles to 25
10. Set final extension temperature to 68°C
11. Set final extension time to 5 min
12. Set heater lid to ON by tapping it to white

12. Press START to run your PCR program.

Start button

13. After your PCR program is done, turn the PCR machine off. And store the tubes at -20^{o}C. Remember that DNA is not stable when stored in this buffer and may denature over time.

Gel Electrophoresis

14. While the PCR is running, prepare 1% agarose gel by adding 0.3g of agarose to 29.7 mls of TAE buffer in an Erlenmeyer flask and cover it loosely.

15. Put the solution in the microwave oven to dissolve the agarose but make sure that the flask is covered loosely to avoid evaporation. You need to watch the solution as it boils. Make sure to stop the microwave in time as the solution boils to prevent the liquid from spilling.

16. After the agarose has dissolved, let the solution cool down and add the ethidium bromide and mix by swirling.

17. Pour the solution into the gel box with the combs in place and allow it solidify. Make sure that no bubbles are trapped in the agarose gel as this will interfere with the migration of DNA.

    ** **NOTE:** *If you prepared the gel the day before the experiment, make sure to soak your gel in TAE buffer and cover the container tightly to avoid drying. In this case, you can just get your gel from the refrigerator and carefully lay it down the gel box before pouring the TAE buffer.*

18. After the gel has solidified, gently remove the comb by adding a little bit of TAE buffer to loosen it. Once the combs are removed, add the rest of the 1x TAE Buffer (with 1x ethidium bromide) and fill the gel reservoirs just enough to cover the gel and the wells.

19. Make sure that you position the gel so that your samples run from the anode to the cathode. Add 1x of the electrophoresis tracking dye bromphenol blue to each of your samples so you can see the bands as they migrate down the gel.

20. Load the samples into each well carefully with a micropipette. Make sure that the tip of the micropipet is on the mouth of the well before you release your samples.

21. Add the 100 bp ladder in one of the lanes so you have a reference for the molecular weights of the DNA bands in your samples.

22. Close the gel apparatus and turn on the power supply. Make sure that the electrode terminals fit snugly in place.

23. Run the gel at 100-130 Volts for 15 to 25 minutes. After you turn on the apparatus, notice the bubbles forming on the wires connected to the electrodes at the bottom of the gel reservoirs. This means that your gel electrophoresis is running.

24. Once the tracking dye has reached about 85 % of the length of the gel, turn off the power supply.

25. Carefully remove the gel from the gel box and visualize DNA bands by putting the gel on a UV illuminator. Normally, we cannot see DNA but the ethidium bromide you added earlier in your gel and in TAE buffer is a fluorescent dye that intercalates tightly with the nitrogenous bases of DNA, which makes DNA visible under UV illumination.

26. If possible, take a picture of your gel and put it in your notebook.