Experiment 4 and 5
Human Genetics / Detection of the ALU Insertion

I. Introduction

The previous experiment dealt with bacterial genetics, this exercise will give you the opportunity to study human genetics. The techniques that you will be using are the same as those used in genetic testing and the forensic analysis of evidence. You will be using the polymerase chain reaction (PCR) to amplify a short region of your chromosome eight to look for an insertion sequence called Alu. As you know, the DNA from individuals is more alike than different (there is only a 2% difference between human DNA and mouse DNA). However there are many regions of human chromosomes which exhibit great degrees of diversity; these sequences are referred to as polymorphic. It is these polymorphic regions that afford the basis for genetic testing and forensic identification.

The Alu family of insertions sequences is found throughout primate genomes. The Alu sequence that you will be looking for in this exercise is called TPA-25 and it is found within an intron of the tissue plasminogen activator gene. The TPA-25 insertion, which is phenotypically neutral (what does this mean?) is present in some people but not in others. Therefore, PCR technology can be used to screen individuals for the presence or the absence of the TPA-25 insertion.

The PCR reaction allows for the amplification of a piece of DNA of unknown sequence if the flanking sequences are known. In the case of the TPA-25 insertion, oligonucleotide primers have been designed to amplify a 400 bp fragment when TPA-25 is present and a 100 bp fragment when it is absent. Since you receive one chromosome of each pair from each parent there are three possible genotypes depending upon your inheritance: homozygous for the presence of TPA-25 (400 bp fragment only), homozygous for the absence of TPA-25 (100 bp fragment only), or heterozygous (400 bp and 100 bp fragments). These genotypes are distinguished by performing electrophoresis in an agarose gel of the PCR products.

You will use DNA that has been isolated from cells obtained by a saline mouthwash as a template. The cells are collected by centrifugation and resuspended in a solution containing the resin "Chelex". The resin binds any metal ions that would inhibit the PCR reaction. Next the cells are lysed by boiling and the cellular debris is removed by centrifugation. A sample of the remaining DNA is mixed with Taq polymerase, oligonucleotide primers, the four deoxynucleotides, and magnesium. (What is the function of the magnesium?) The reaction mixture is then put on a thermal cycler where the following steps occur: 1) denaturing of the template DNA, 2) annealing of the primers to the template, and 3) extension of a complementary strand of DNA. This process is then repeated over and over to amplify the target DNA. The following primers are used to bracket the TPA-25 locus: the "upstream primer,"
'5-GTAAGAGTTCCGTAACAGGACAGCT-3' and the "downstream primer," '5-CCCCACCCTAGGAGAACTTCTCTTT-3'.

As previously mentioned, once you have completed the amplification, the PCR products will be compared by electrophoresis on an agarose gel. It is extremely important for you to have good pipetting technique for your experiment to succeed.

**Polymerase Chain Reaction**

PCR is actually a "simple" method for amplifying nucleic acids. It is similar to the natural DNA replication process in that the number of DNA molecules generated by the PCR reaction doubles after each "cycle" (see diagram)

In the PCR technique, the typical "cycle" consists of 3 steps

1) Denaturing the double stranded template DNA by heating it to a high temperature (94°C), thereby producing two single strands of DNA.

2) Annealing of the "target-specific" primers to the two separated DNA strands by cooling the reaction mixture to a lower temperature (58°C for our PCR)

3) Extending the annealed primers (adding the proper nucleotides) with "Taq DNA Polymerase" enzyme by warming the reaction mixture to an intermediate temperature (72°C).

The product of the reaction (if the primers have "extended" properly) will be double stranded DNA molecules whose end will be determined by the set of primers used. One cycle has been completed. By repeating the cycle many times- heating to separate the double stranded DNA (94°C) - annealing the primers again (58°C) - and, in the presence of nucleotides, extending the primers (72°C) we can "amplify" the piece of DNA many fold. After this amplification process, we can run the DNA on an agarose gel, and by staining with ethidium bromide, visualize this DNA. When you are starting out with very small amounts of DNA (as we did with our rinsing cells out of the mouth with saline) or in analyzing DNA from crime scenes (where you may have very small amounts of blood or fluids to analyze) the PCR technique allows visualization of DNA segments that would otherwise be impossible to analyze.
II. Procedure

A. To Make Template DNA

1) Use a permanent marker to label your name on a 50 ml orange top tube containing 10 ml of a sterile 0.9% saline solution.

2) Pour all of the saline solution into your mouth, and vigorously swish for 10 seconds. Expel the saline mouthwash back into the 50 ml test tube.

3) Make sure that the cap is secured to the 50 ml centrifuge tube and load your tube into the rotor of the centrifuge. All tubes must be balanced in a centrifuge; therefore, load tubes opposite each other in the rotor. Your T.F. will assist you.
Centrifuge at 1000 Xg for 10 minutes to pellet the cells on bottom of the culture tube.

4) Carefully decant the supernatant into the sink; do not disturb the cell pellet. Place the tube containing the cell pellet on ice.

5) Using a pipettor add 500 µl of 10% Chelex to the cell pellet. Be sure to resuspend the Chelex beads first. Resuspend your cells with the Chelex beads by pipetting up and down several times. There should be no visible cell clumps.

6) Transfer 500 µl of the resuspended Chelex/cell solution to a sterile 1.5 ml microfuge tube labeled with your name. Using a hot 10-gauge needle make a hole in the cap of the tube.

7) Incubate the tube with your sample on the hot block for 10 minutes at 100°C.

8) Remove your tube from the hot block and cool on ice for about 1 minute.

9) Place the sample tube in a balanced microfuge rotor and spin for 30 seconds to pellet the Chelex beads.

10) Transfer 500 µl of the supernatant to a clean, sterile 1.5 ml tube labeled with your name and place it on ice. Avoid transferring any of the Chelex resin. Dispose of the tube with the Chelex in the biohazardous waste bins.

B. Set up the PCR Reaction

1) Label a 0.2 ml. tube (already containing the “Ready to use PCR beads”). These beads contain everything necessary for the PCR reaction, except the primers and the template DNA. They contain the nucleotides, buffers, and taq DNA polymerase enzyme. All you have to add is sterile distilled water, the primers and your DNA (the template). These tubes are designed to hold a total of 25 µl of the reaction mixture.

2) Add 20.5 µl of sterile distilled water; 2.5 µl of your template DNA, and 1.0 µl of each primer.

3) Cap the tube lightly, mix and place in the PCR thermocycler programmed as follows:
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94ºC</td>
<td>1 minute</td>
<td>30</td>
</tr>
<tr>
<td>58ºC</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>72ºC</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>4ºC</td>
<td>HOLD</td>
<td></td>
</tr>
</tbody>
</table>

4) Remove the tube and freeze at -20º for analysis during the next lab.

**NOTE:** Because PCR is such a sensitive technique it is essential that sterile tips be used for each reactant to avoid the amplification of any contaminating DNA. Touch the solutions with the only with the end of the pipette tip itself. Wear gloves and be sure to change tips for each solution.

C. Preparation of 2% Agarose Gel

**Wear gloves when preparing and running gels.**

1) Prepare a 2% agarose gel by weighing out the appropriate amount of agarose to be dissolved in 50 ml of TBE buffer. Put the agarose and the 50 ml of 1x TBE buffer into a 125 ml Erlenmeyer flask. Weigh the flask.

2) Put the flask in the microwave to dissolve the agarose. Be careful the flask will be hot. Once the agarose has dissolved reweigh the flask and compensate for any weight loss by adding water. (Why would you do this?)

3) Pour the agarose into the gel casting tray. Your T.F. will demonstrate how this is put together.

4) While the gel is hardening, prepare your DNA. Using a pipettor with a tip on it pierce the wax on top of your amplified DNA and remove 20 µl to a clean tube. Add 5 µl of sample buffer to this tube. Find your unamplified DNA tube that should be at -20ºC. Remove 20 µl of unamplified DNA to a clean tube and add 5
µl of sample buffer. This will be a control. Each student should have an amplified DNA sample and an unamplified control DNA sample to run on the gel. Each gel has 8 lanes; we will be able to run 3 student samples per gel. It is necessary to run a 100 bp ladder in one of the other lanes. Obtain a tube of 100bp ladder from the freezer; this will be loaded directly on the gel. (Why do you run the 100 bp ladder?)

5) Once your gel is set remove the comb and place the gel in the running box. Cover the gel with TBE buffer. Load your DNA samples in the lanes; keep track of what DNA is in which lanes.

6) When all of the samples are loaded on the gel place the lid on the gel box being careful to connect the position and negative terminals correctly. (Toward which terminal will your DNA run? Have your T.F. check your gel). Connect the wires to the power supply and run the electrophoresis at constant current and 100 volts for 45 minutes to 1 hour.

7) Once the electrophoresis is complete turn off the power supply, disconnect the wires from the power supply and remove the lid from the gel box. Remove the gel from the tray and place it in a staining dish. Cover the gel with the ethidium bromide solution. **WEAR GLOVES!** Ethidium bromide is a mutagen.

8) Stain your gel for 10 minutes in the ethidium bromide. Pour the ethidium bromide back into the original bottle and wash your gel with distilled water for 10 minutes.

9) Before examining your gel on the UV transilluminator put on your safety glasses. Place your gel on the UV transilluminator and examine your results. Before removing your gel photograph it. Your T.F. will help with this.

**III. Discussion**

1) Describe and interpret your results in your notes. Include in your interpretation what each band is. Do you see primer dimers? What are they? What do the other bands tell you about your genetic composition?

2) Using the posted data for the class determine the genotype distribution for the class. That is how many students are (+/+), (+/-), and (-/-).

3) If your gel has problems and the interpretation was difficult describe any sources of error.
4) After this experiment you should understand PCR and gel electrophoresis.
Experiment 5
Appendix

Materials

* 10% Chelex
* 0.9% sodium chloride (sterile)
* oligonucleotide primers
* Taq polymerase
* 10x PCR buffer
* 25 mM MgCl₂
* dNTP’s
* sterile distilled water
* loading dye
* agarose
* 1x TBE buffer
* ethidium bromide