Molecular Biology
Lab 6
March 2nd, 2010

DNA replication/ amplification

Use of Polymerase Chain Reaction—PCR to Identify DNA Polymorphisms

Today's lab will introduce several aspects of the DNA amplification technique called Polymerase Chain Reaction or PCR. One can make many copies of a DNA segment *in vitro* using the basic "ingredients" of DNA synthesis and a machine called a Thermal cycler. (Recall, Kornberg was the first to succeed in synthesizing DNA in vitro.)

PCR requires that you know at least some of the linear sequence of the DNA of interest (template). The sequence information is used to design short DNA primers (10-20 nucleotides in length) that are complementary to the ends of the DNA segment of interest. These primers serve the same purpose as the RNA primer used in DNA replication inside cells and allows replication of the DNA by extending the primers (similar to in vivo DNA replication).

Like all replication, PCR also requires DNA molecules to copy. This *template* DNA can be purified from cells (genomic DNA) or be cloned DNA with much more limited sequence (such as the pGLO plasmid). The template chosen, depends on the desired product.

Finally, PCR requires the DNA replication enzyme (DNA polymerase) and ingredients (nucleotides, dNTPs A, T, G, C) and a way to mimic the events of cellular replication. One of the key events of DNA replication is strand separation. In PCR strands are separated by high heat (in the thermalcycler). Primers are not made in the test tube, but are added and allowed to anneal to the ends of the DNA template (by adjusting the temperature). Once primers are annealed the DNA polymerase can synthesize DNA as it does in cells, by linking together dNTPs according to the single stranded template. We use a special, heat-stable, DNA polymerase so that multiple rounds of PCR can be done without adding new enzyme. The enzyme, *taq* polymerase comes from *Thermus aquaticus*, a thermophilic bacterium.

Today's exercise will include preparing the template (molecular biology student genomic DNA from cheek or hair root/sheath cells) and preparing for and carrying out, the PCR amplification. (See attached protocols)

PCR application -- genetic polymorphisms

PCR has many uses in molecular biology. Its sensitivity allows one to amplify virtually any region of DNA of interest regardless of the complexity of the DNA template. Today's exercise will allow us to amplify a very small region of a specific genomic region called **PV92** located on human chromosome 16. The locus is not a gene but serves as an interesting region to study in the population as it differs in a specific way among individuals in the population.

One of the major uses of PCR is in genetic analysis of DNA from various tissue/cell samples to identify individuals. To do such analysis one must analyze a region of DNA that differs among individuals in a population. We call such differences **genetic polymorphisms.** A single nucleotide difference within any gene between you and your lab partner would be an example of a polymorphism. When a nucleotide difference affects a restriction enzyme recognition site, the difference between you and your partner (or any 2 individuals) can be recognized by digesting DNA with the restriction enzyme, noting differences in fragment patterns. This technique does not involve PCR, and is **called Restriction Fragment Length Polymorphism analysis (RFLP analysis)**. (We discussed this previously when learning about restriction enzymes.)

PCR is useful in detecting other types of polymorphisms, including the presence or absence of repetitive DNA. DNA of eukaryotes is full of small (2-6 nucleotides—called microsatellite DNA) or larger (hundreds of nucleotides) repeated sequences. These sequences are often found between genes or within the introns of genes so that they don't affect the proteins encoded by genes. Individuals vary greatly with respect to the number and types of repeats found in specific regions of DNA. PCR can be used to detect the presence or absence (or number of) repeats in a given region DNA by amplifying a region around the repeat(s). If a DNA repeat is present in the amplified region the fragment amplified will be larger than if the DNA repeat sequence is absent.

The PV92 locus has a larger type of repeat sequence called an **Alu** repeat ~300nt in length. Some individuals have one of these Alu repeats, while others do not have an Alu repeat at this locus. Today you will begin to determine your specific **genotype** with respect to this Alu. You genotype will of course depend on the genotype of your parents and the specific chromosome 16 homolog you received from each of them.

We will amplify the polymorphic (di-morphic) region of Chromosome 16 using PCR and then determine our genotype by using electrophoresis to analyze the amplified DNA.

See attached protocol.

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Protocol

Template preparation (see attached sheets):

Today we will collect either cheek cells or hair follicle cells as a source of our own DNA to analyze. The protocols for collection are similar but not identical. Make sure you follow the correct protocol according to your choice of sample. The treatment of the cheek cells or hair root cells will release the DNA to be used as the template in PCR reactions. We would expect members of the lab group to have different genotypes for the PV92 locus. Be sure not to contaminate each other's samples!

**If you choose cheek cells for your sample, you will collect them in a separate tube, then transfer them to the matrix solution.

***If you choose to use a hair sample, make sure the hair you pluck does not break off, but has the root attached. You should see a tiny white blob of cells attached to the hair if you do it correctly. Make sure the hair fragments include this blob and watch carefully to see the hairs in the matrix solution throughout the procedure.

PCR amplification (see attached sheets)

You will combine 20 μl of your prepared DNA template solution with 20 μl of PCR "master mix". Recall DNA amplification requires:

Template DNA
dNTPs
oligonucleotide primers from the region of interest
DNA polymerase (*Taq* polymerase) + necessary ions (mg++, pH, temp)

I will prepare the Master Mix, which will include all of the above, just before you need it.

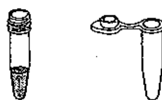
**** I will ask 2 groups to prepare control samples for PCR. (Using templates from +/+, +/-, ajd -/- genotypes, with respect to the Alu repeat.)

Analysis of PV92 locus—Next lab, we will analyze the PV92 amplification products using gel electrophoresis. I will ask for volunteers to prepare 2-3 gels for the class.

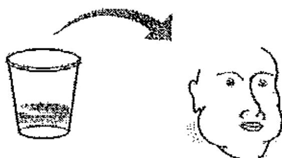
Quick Guide

Lesson 1 Cheek Cell DNA Template Preparation

1. Label one 1.5 ml micro test tube with your initials. Label one screwcap tube containing 200 µl of InstaGene matrix with your initials.

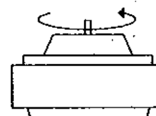


2. Obtain a cup containing saline solution from your instructor. Pour the saline into your mouth and rinse vigorously for 30 seconds. Expel the saline back into the cup.



3. Transfer 1 ml of your saline rinse into the micro test tube (NOT the screwcap tube) with your initials. If a P-1000 micropipet is not available, **carefully** pour ~1 ml of your saline rinse into your micro test tube (use the graduations on the side of the micro test tube to estimate 1 ml).

4. Spin your tube in a balanced centrifuge at full speed for 2 minutes. When the centrifuge has completely stopped, remove your tube. You should see a match-head sized pellet of whitish cells at the bottom of the tube. If you don't see a pellet of this size, decant the saline, refill your tube with more of your oral rinse, and repeat the spin.



Centrifuge

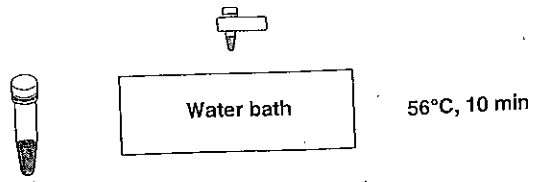
5. After pelleting your cells, pour off the saline. Being careful not to lose your pellet, blot your tube briefly on a paper towel or tissue. It's OK for a small amount of saline (< 50 µl, about the same size as your pellet) to remain in the bottom of the tube.

6. Resuspend the pellet by vortexing or flicking the tube so that no clumps of cells remain.

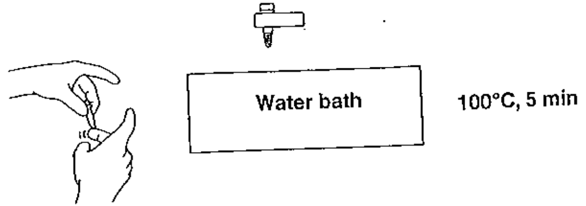
7. Using a 2–20 µl adjustable-volume micropipet set to 20 µl, transfer all of your resuspended cells to the screwcap tube containing InstaGene.

8. Screw the cap tightly on the tube. Shake or vortex to mix the tube contents.

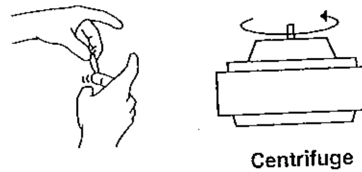
9. When all members of your team have collected their samples, place the tubes in the foam micro test-tube holder, and incubate at 56°C for 10 minutes in a water bath. At the halfway point (5 minutes), shake or vortex the tubes gently, then place back in the 56°C water bath for the remaining 5 minutes.



10. Remove the tubes, shake or vortex, and place the tubes in a boiling water bath (100°C). Incubate at 100°C for 5 minutes.



11. Remove the tubes from the boiling water bath and shake or vortex the contents to resuspend. Pellet the matrix by spinning at 6,000 x g for 5 minutes (or 2,000 x g for 10 minutes) in a centrifuge.



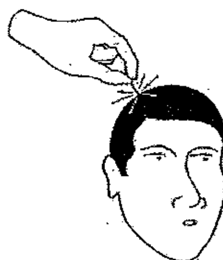
12. Store your screwcap tube in the refrigerator until the next laboratory period (or proceed to step 2 of Lesson 2).

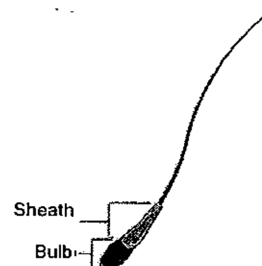
Quick Guide

Lesson 1 Hair Follicle DNA Template Preparation

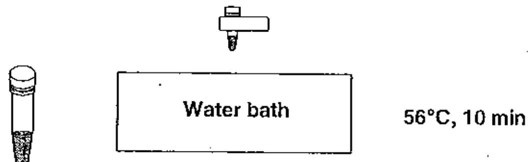
1. Label 1 screwcap tube containing 200 µl of InstaGene matrix plus protease with your initials.

2. Collect 2 hairs from yourself. Select hairs that have either a noticeable sheath (a coating of epithelial cells near the base of the hair), or a good sized root (the bulb-shaped base of the hair). Trim the hair, leaving the last ~2 cm of the base of the hair. Place the trimmed hairs into the screwcap tube with your initials.

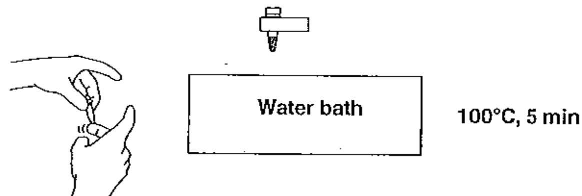




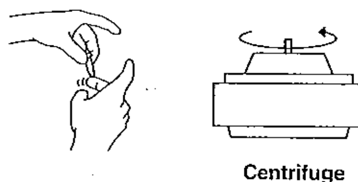
3. Place your tube in the foam micro test-tube holder and incubate at 56°C for 10 minutes in a water bath. At the halfway point (5 minutes), shake or vortex gently, then place it back in the 56°C water bath for the remaining 5 minutes.



4. Remove the tubes, gently shake or vortex, and place them in a boiling water bath (100°C). Incubate at 100°C for 5 minutes.



5. Remove the tubes from the boiling water bath and shake or vortex the contents to resuspend. Pellet the matrix by spinning at 6,000 x g for 5 minutes (or 2,000 x g for 10 minutes) in a centrifuge.

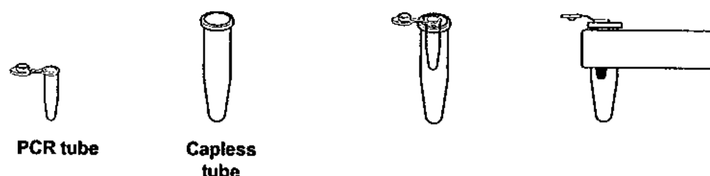


6. Store your screwcap tube in the refrigerator until the next laboratory period (or proceed to step 2 of Lesson 2).

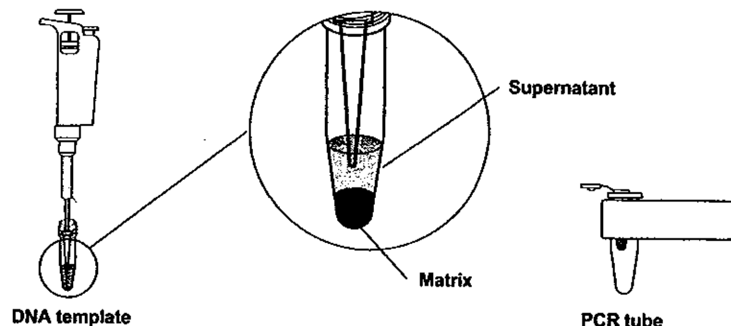
Lesson 2 PCR Amplification (Lab Protocol)

1. Obtain your screwcap tube that contains your genomic DNA template from the refrigerator. Centrifuge your tubes for 2 minutes at 6,000 x g or for 5 minutes at 2,000 x g in a centrifuge.

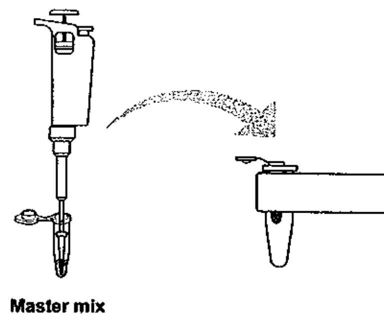
2. Each member of the team should obtain a PCR tube and capless micro test tube. Label each PCR tube on the side of the tube with your initials and place the PCR tube into the capless micro test tube as shown. Place the PCR tube in the foam micro test tube holder.



3. Transfer 20 µl of your DNA template from the supernatant in your screwcap tube into the bottom of the PCR tube. **Do not transfer any of the matrix beads into the PCR reaction because they will inhibit the PCR reaction.**



4. Locate the tube of yellow PCR master mix (labeled "Master") in your ice bucket. Transfer 20 µl of the master mix into your PCR tube. Mix by pipetting up and down 2–3 times. Cap the PCR tube tightly and keep it on ice until instructed to proceed to the next step. Avoid bubbles, especially in the bottom of the tubes.



Step 4 b. Obtain DNA from Control tubes:

1. Pipette 20 μl of the +/+ template into an appropriately labeled tube.
2. Pipette 20 μl of the +/- template into an appropriately labeled tube.
3. Pipette 20 μl of the -/- template into an appropriately labeled tube.
4. Pipette 20 μl of water into an appropriately labeled tube.

5. Pipette 20 ul of the Master Mix into each of the control tubes and into the negative control water tube. **USE A NEW PIPETTE TIP EACH TIME.**

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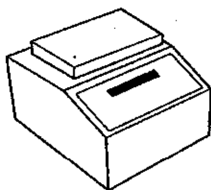
5. Remove your PCR tube from the capless micro test tube and place the tube in the Gene Cycler or MyCycler thermal cycler.



6. When all of the PCR samples are in the thermal cycler, the teacher will begin the PCR reaction. The reaction will undergo 40 cycles of amplification, which will take approximately 3 hours.

7. If your teacher instructs you to do so, you will now pour your agarose gels (the gels may have been prepared ahead of time by the teacher).