Advanced Cell Biology Labs 6 and 7.
Polymerase Chain Reaction (PCR)

March 5 and 19, 2012

Part 1. PCR preparation and running

Background

Shortly following the discovery of DNA structure, important experiments showed DNA is replicated in a semiconservative manner, in which each strand serves as a template for synthesis of a new strand, (key experiments by Messelson and Stahl). The sequence of the template strand determines the sequence (complementary) of the new strand. Arthur Komberg added to the understanding of DNA replication with his identification of the enzymes that catalyze the DNA polymerization reaction. These enzymes, DNA polymerases, first isolated from *E. coli*, perform 5' to 3' DNA synthesis.

Many details of DNA replication were worked out in the last 50 years. We know that a host of proteins work together at a replication fork to unwind DNA and maintain a single stranded template. We know that DNA polymerases must have a short existing stretch of nucleic acid with a free 3' OH group on which to add new nucleotides (nucleoside triphosphates, dNTPs). In cells, this primer is a short RNA molecule.

Much of the work in DNA replication was based on in vitro experiments, using a purified DNA template and showing DNA replication could occur in *E. coli* or in vitro, in the presence of the essential elements used in the cell. (DNA polymerase, primers, dNTPs). In 1988, Kary Mullis published his invention of a DNA replication method that is arguably one of the most important advances in molecular biology. The technique, called Polymerase Chain Reaction (PCR) is a super sensitive method for replicating any region of DNA using a fast, simple method of consecutive rounds of DNA synthesis.

Mullis realized DNA synthesis can occur with a minimum of components if one can mimic the action of some proteins in other ways (like manipulating temperature). PCR can be performed with as few as 4 ingredients:

1. Template DNA (to copy or amplify)
2. dNTPs (dATP, dTTP, dCTP, dGTP)
3. short DNA/RNA sequences to prime synthesis of the DNA template (primers)
4. DNA polymerase enzyme

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 in between the primers.

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 (small plasmid).

Finally, PCR requires the DNA replication enzyme (DNA polymerase) and ingredients (nucleotides, dNTPs: A, T, G and 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. 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.

The essential components for DNA synthesis are combined in one tube and placed in a thermal cycler. The tubes are subjected to repeated cycles of heating (to denature the template to its single strands) and cooling (to allow complementary binding of primers to the region to be amplified). The specific region amplified in PCR depends entirely upon the primers used. A third temperature may be used according to the active temp for the polymerase (see **Fig. 1**).

Typically 30-40 rounds of repeated cycles are used to create enough copies of the DNA to be visualized (e.g. by DNA electrophoresis). The number of copies of the DNA of interest is dependent on the number of cycles of PCR done. For each starting copy there will be 2n copies produced where n is the number of cycles.

The exponential amplification of DNA allows many applications, including detection of rare DNA sequences. PCR is used to detect viral or bacterial DNA in humans and animals. It is used to produce probes for genetic analysis. Most procedures that match a suspect's DNA profile to the crime scene utilize PCR.

Assignment

The template is a genomic DNA of Eurasian spruces obtained on the previous lab; we will need to amplify a variable fragment located inside circular chloroplast DNA, between two genes encoding tRNAs: *trn*T(UGU) and *trn*L(UAA) (see **Fig. 2**). The region size should be about 600 base pairs.

Today we will set up PCR on a single template, ans use one primer set: so-called primers a and b.

Materials

1. 2 thin walled PCR tubes
2. Template DNA
3. Forward primer a, 10 µM
4. Reverse primer b, 10 µM
5. 10× PCR buffer
6. dNTPs (2.5 mM each)
7. *Taq* polymerase (1 unit per 1 µl)
8. H_20

Procedure

1. Label your PCR tubes according to your DNA extraction ID. **Keep tube on ice** for the duration of the procedure steps.
2. Pipette 15.5 µL H_20 into each PCR tube.
3. Pipette 2.5 µL 10× PCR buffer into each PCR tube.
4. Pipette 1 µL of DNA template into each PCR tube.
5. Pipette 2 µL dNTPs into each PCR tube.
6. Pipette 2 µL forward primer into each PCR tube.
7. Pipette 2 µL of reverse primer into each PCR tube.
8. Pipette 1 µL of *taq* polymerase into each tube.
9. Set a pipette to 20 µL and gently mix by pipetting the entire mixture. Try not to introduce bubbles. If bubbles exist, short centrifugation will remove them.
10. Cap the tubes tightly and place in thermal cycler (wait for instructor's assistance!)

Thermal cycler settings:

1. 95°C 5 min (complete denaturing) Then 35 cycles of:
   (a) 94°C 1 min
   (b) 50°C 1 min
   (c) 72°C 2 min
2. 72°C 7 min
3. 4–10°C unlimited time (stable storage of DNA)

On next lab, we will monitor the outcome of our PCR experiment using DNA gel electrophoresis.

Part 2. Analysis of PCR: agarose gel electrophoresis

Background

Today we will determine our success in amplifying the regions of the MLL gene by PCR. You should have PCR samples in which you used 2 different primer pairs to analyze.

A standard method for analyzing the size and amount of DNA produced in a PCR reaction is agarose gel electrophoresis. Agarose is a product of seaweed that can be melted and then solidified, forming a gel/matrix for separation of DNA. The gel is submerged in a buffer which allows an electrical potential to be applied. DNA is loaded into wells made in the gel and an electrical current is applied. DNA is highly negatively charged and will migrate through the pores of the gel toward the positive pole. DNA fragments of different sizes will migrate different distances in a given period of time (see below). DNA is typically visualized by staining the whole gel with a dye that is taken up by DNA. One common dye is called Ethidium Bromide (there also are SYBR dyes which are generally weaker but safer). This fluorescent dye binds between the paired bases of the nucleotides and is detectable in UV light. (illustrated by small circles below)

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log10 of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the log10 of either their molecular weights or number of base pairs, a roughly straight line will appear.

We will do this following the electrophoresis.

Materials

1. **PCR samples**
2. **An electrophoresis chamber and power supply**
3. **Gel casting trays**, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
4. **Sample combs**, around which **molten agarose** is poured to form sample wells in the gel.
5. **Electrophoresis buffer**, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
6. **Loading buffer**, which contains something dense (e.g. glycerol) to allow the sample to sink into the sample wells, and one or more tracking dyes, which migrate in the gel and allow visual monitoring of how far the electrophoresis has proceeded.
7. **Ethidium bromide**, a fluorescent dye used for staining nucleic acids. *NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical—wear gloves while handling.*
8. **Transilluminator** (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light!

Procedure

A. Prepare 1% agarose gel following steps below. USE EYE PROTECTION and OVEN MITTS!

1. Prepare casting tray for gel. Use the masking tape to dam the ends.
2. Place a comb in the casting tray.
3. Weigh agarose for 100 ml gel (1% w/v agarose)
4. Measure 100 ml electrophoresis buffer (1× TAE—mixture of Tris base, acetic acid and EDTA) in flask (125 ml or larger)
5. Add powdered agarose to electrophoresis buffer, swirl gently and plug with paper towel.
6. Heat in microwave till boiling starts—watch carefully.
7. Remove from microwave and GENTLY swirl
8. Return to microwave and bring just to boil
9. Remove from microwave and GENTLY swirl

10. Check for undissolved crystals—if none stop here. If not dissolved repeat heating.
11. Let cool 5 minutes
12. Put on gloves (latex)
13. Add 1 μL Ethidium bromide to the warm agarose. Ethidium Bromide is a strong mutagen—take care when using the concentrated solution and wear gloves when handling the gel from this point on!
14. Pour the gel slowly into the casting tray and allow it to solidify.
15. Place your gel into a plastic box, cover it with electrophoresis buffer. OR wrap the gel as instructed.

B. Prepare DNA samples and molecular weight standard sample (DNA ladders)

1. Label a small microfuge tube "*1 kb DNA ladder*"
2. Label a second small microfuge tube "*100 bp DNA ladder*"
3. Add 23 μL H_20 to each of the DNA ladder tubes
4. Add 2 μL of each ladder DNA to the appropriate tube.
5. Add 3 μL of loading dye to each DNA ladder tube
6. Add 3 μL of loading dye to each of the 2 PCR sample tube.

C. Loading and running the gel

1. Design a plan for samples in your gel.
2. Set a micro pipettor to 20 μL.
3. Carefully load 20 μL of each sample into wells of the gel (wait for demonstration!)
4. Place the lid on the gel, noting the orientation of the electrodes. Run the gel at 100 Volts for 30 minutes.
5. View on UV transilluminator, noting presence or absence of DNA in your lanes, and differences in migration of DNA.
6. Document and discuss your results. Using the DNA ladder, estimate the sizes of the DNA fragments you amplified.
7. Plot the migration of the DNA ladder fragments and compare migration of your PCR-amplified fragments.

Figures

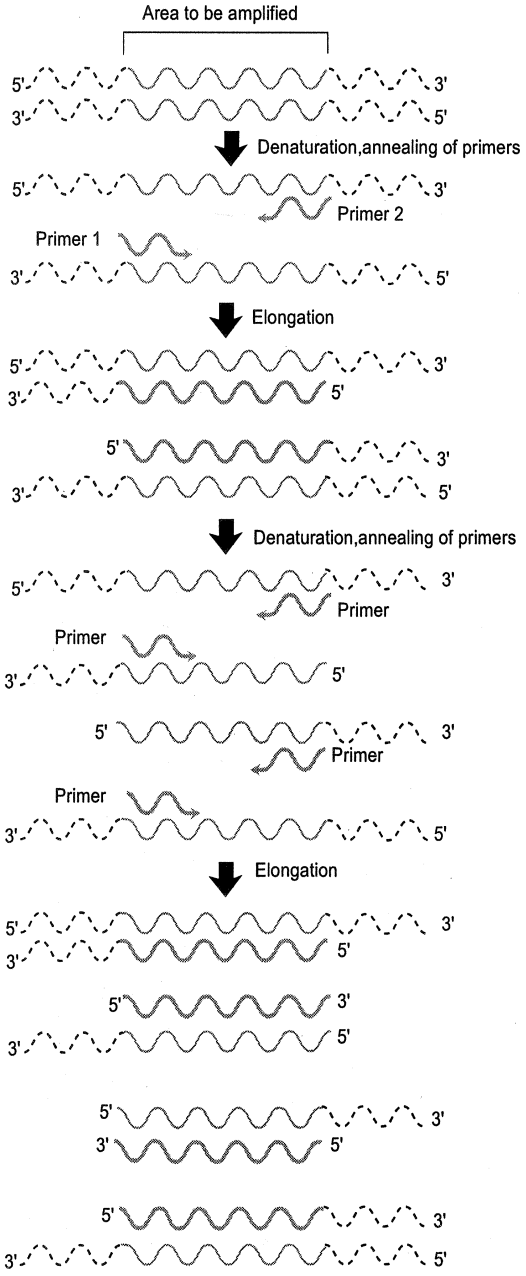

Fig. 1. PCR.

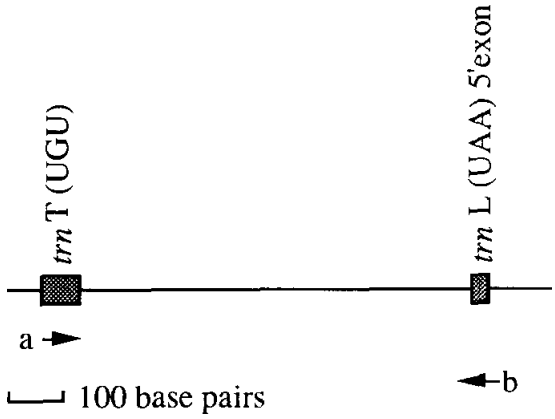

Fig. 2. Chloroplast *trn*T(UGU) — *trn*L(UAA) intergeneric spacer and primers.

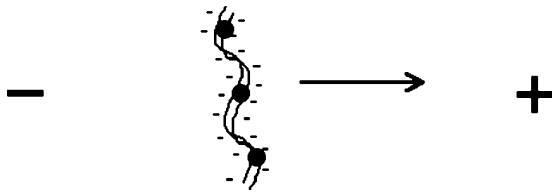

Fig. 3. DNA electrophoresis.