

Polymerase Chain Reaction (PCR) Protocol

First PCR of Nested Protocol

In today's lab we will begin to amplify a partial sequence of the mitochondrial cytochrome C oxidase subunit I (CO I) gene from the freshwater mussel *Elliptio complanata*. The procedure we will use to amplify the CO I sequence is a nested PCR protocol. A nested protocol uses two separate rounds of PCR. PCR protocols allow us to synthesize DNA in a test tube. The key to this synthesis is a DNA polymerase that is stable at high temperatures, such as 94° C. The DNA polymerase we will use in our PCR protocol is from a eubacterium called *Thermus aquaticus*. *Thermus aquaticus* is a species that was originally isolated from a hot spring in Yellowstone National Park in 1969. The DNA polymerase from this bacterium is designated by the first letter of the generic name (T) and the first two letters of the specific name (aq) or Taq DNA polymerase. The form of the enzyme we are using has been modified through genetic engineering. The DNA polymerase needs to be stable at high temperatures because we will repeatedly heat our reaction mix to separate the two strands of the DNA molecules.

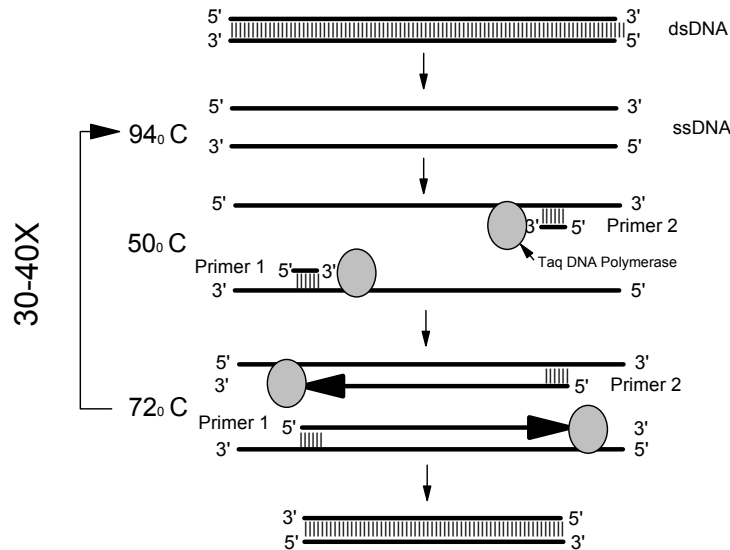
Taq DNA polymerase is similar to all other DNA polymerases in that it requires a preexisting piece of DNA as a starting point for synthesis. The requirement of a starting point by the DNA polymerase actually makes the PCR protocol a powerful technique. We will be able to control which portions of the DNA molecules that are replicated by adding specific starting points for DNA synthesis. These starting points are short single stranded pieces of DNA called primers that contain between 15 and 30 bases. To synthesize both strands of a DNA molecule we will need to add two primers to the reaction mix, one for each strand. These primers locate and form hydrogen bonds with (anneal to) their complimentary sequences. In order for the hydrogen bonds to form between the primers and their complimentary regions we must lower the temperature after we separate the DNA strands. How much we lower the temperature will set the specificity of the PCR protocol. The higher the annealing temperature the more specific the PCR product. However, as we will see there is a balance between making the wrong PCR product (too low an annealing temperature) and making no PCR product at all (too high an annealing temperature). An indication of the annealing temperature is the melting temperature (T_m) of the primer. We will use the T_m when the reaction conditions contain 1M Na⁺ to estimate the melting temperature of our primers (our reactions do not contain this much salt, but this T_m is a better indicator of the melting temperature of our primers than the 50 mM T_m). Usually as a starting point for PCR protocols, you use an annealing temperature 10° C lower than the T_m .

After the primers have had a chance to anneal, we will raise the temperature to 72° C. At 72° C, the Taq DNA polymerase adds about 1000 bases per minute to an existing DNA strand. What other conditions are important for the DNA polymerase to work optimally? Look at the buffer composition to see if you can figure out what is important. Does the Taq DNA polymerase work only at 72° C? What is the consequence of the enzyme working at a temperature at or lower than the annealing temperature? To prevent the enzyme from working below the annealing temperature while the reaction mix is being constructed or before the tube in the PCR machine reaches the annealing temperature, the folks at Life Technologies have attached an antibody to the Taq DNA polymerase. The enzyme is inactive while the antibody is attached to it. At the start of the PCR protocol we will heat the tubes at 94° C for 2 minutes to remove the antibody from the enzyme. Preventing the enzyme from working until the reaction mix is heated to a temperature above the annealing temperature is called a **hot start**.

Our PCR protocol will therefore consist of a series of steps in which we raise or lower the temperature of the reaction mix (see below). We will heat the tubes to 94° C to denature the DNA,

lower the temperature to the annealing point so that primers can find their complimentary sequences, and raise the temperature to 72° C to rapidly lengthen the DNA strands. We will repeat these three steps 30 to 40 times. Through these cycles we will create millions to billions of copies of the DNA sequence we want to amplify (Fig. 1).

Figure 1 A General PCR Cycle



Protocol

I. Target Dilution - Concentration of Target should be no more than 1 µg/µl

II. Primer Dilution and Preparation of Primer Mix

We need to make a working solution for each primer at a concentration of 10 µM. Use the information in the table below to determine how many µl of primer stock solution and autoclaved ddH₂O you will use to produce 25 µl of a Primer Mix in which each primer has a final concentration of 10 µM..

Primer Mix - Prepare in a 0.5 ml microcentrifuge tube.

| Primer | Conc. μM | Volume Primer (μl) | Final Conc. μM |
|-------------------------------|---------------------|---------------------------------|---------------------------|
| CO I P1 | | | 10 |
| CO I P2 | | | 10 |
| Autoclaved ddH ₂ O | | | |
| Total Volume | | | |

II. Reagent Cocktail - Build Cocktail in - 0.5 ml microcentrifuge tube

| Reagent | μl | Multiplication Factor | μl | [Final] in RXN Mix Below |
|--|---------------|-----------------------|---------------|----------------------------|
| 10X PCR Buffer | 2.50 | x _____ | | |
| 50 mM MgCl ₂ | 0.75 | x _____ | | |
| 10 mM dNTP Mix | 0.50 | x _____ | | |
| 10 μM Primer Mix | 1.00 | x _____ | | |
| autoclaved ddH ₂ O | | x _____ | | |
| Platinum Taq DNA Polymerase (5u/ μl) | 0.25 | x _____ | | Total number of units/tube |
| Total Volume | | x _____ | | |

- Vortex briefly and centrifuge briefly - Remember to use an adapter so that small tubes are not damaged during the spin

III. Reaction Mix - Build Reaction Mix in Thin-walled PCR Tubes - 0.2 ml

| Component | μl |
|-------------------------------|---------------|
| Reagent Cocktail | |
| DNA | |
| autoclaved ddH ₂ O | |
| Total | 25 |

- Vortex briefly and centrifuge briefly - Remember to use an adapter so that small tubes are not damaged during the spin

- Place tube in PCR Machine.

IV. PCR Machine Program for first round of the nested PCR Protocol

| Step Number | Temperature °C | Time(min:sec) |
|-------------|----------------|---------------|
| 1 | 94 | 1:30 |
| 2 | 94 | 0:30 |
| 3 | 50 | 0:30 |
| 4 | 72 | 1:00 |
| 5 | Repeat 2-4 | 40X |
| 6 | 72 | 10:00 |
| 7 | 4 | Hold |