Biology 324 **Moyer/Leaf**
Winter 2000 **Lab #4 – The Polymerase Chain Reaction**

This laboratory is designed to provide you with the basics for generating a standard PCR from a plasmid DNA template. You will need to calculate the volumes needed to acquire your "master mix" prior to adding the template DNA. The success of a PCR depends largely upon the stringency of primers binding to their target template DNA during the hybridization phase. This stringency is impacted by two major factors, (1) the temperature of annealing, and (2) the concentration of free Mg^{++} ions. We will focus on latter for this exercise, as [Mg^{++}] directly affects the performance of *Taq* polymerase. Reaction components, including template DNA, chelating agents, dNTPs, and proteins all affect the amount of free Mg^{++}. *Taq* polymerase is inactive in the absence of Mg^{++}, and with an excess the polymerase has a greatly reduced fidelity that may increase the level of nonspecific amplification.

Another major factor involved with a successful PCR is quality and quantity of the template DNA (target dose). Reagents commonly used to purify nucleic acids (salts, guanidine, proteases, organic solvents, SDS) are potential inhibitors of *Taq* polymerase. A final EtOH ppt of the template DNA usually will eliminate most of these inhibitory agents. The amount of template required is dependent upon the complexity of the template DNA. For example, in a 4kb plasmid containing a 1kb insert, 25% of the input DNA is the target of interest. Conversely, a 1 kb gene in the human genome (3.3×10^9 bp) represents approximately 0.00003% of the input DNA. Therefore, approximately 10^6 more template DNA would be required in this case to maintain the same target dose (i.e., number of target copies). As a rule of thumb, start with around $>10^4$ to 10^6 copies to obtain a signal in 25-30 cycles, but keep the final [DNA] of the reaction at $\geq$10ng/µl.

Basic PCR reaction mixtures are as follows: 5 µl of 10X PCR reaction buffer, 5 µg of acetylated BSA, 200 µM dNTPs, 0.5 µM primer #1, 0.5 µM primer #2, 1.5 to 2.5 mM $MgCl_2$(usually), and 2.5 Units of *Taq* DNA polymerase in a total volume of 50 µl. Reactions are incubated for 1 min at 95°C (pseudo-hot-start) followed by 30 cycles of denaturation, annealing and extension at 94°C for 1 min, 50°C for 1.5 min, and 72°C for 3 min, respectively. This gets followed by an additional extension at 72°C for 7 min, and a 4°C hold. A 15 µl aliquant of each amplification is assayed for size and purity by electrophoresis on a 1% agarose mini-gel containing 0.4% (w/v) ethidium bromide.

Construct a **master mix** with everything except the template DNA and Mg^{++} and aliquot into 11 tubes (calculate volumes based on n = 12 to account for pipetting carryover & error). The first array of 5 reactions will vary in Mg^{++} with the final concentrations at 0.0, 1.0, 2.0, 3.0, 5.0 mM $MgCl_2$, holding the template amount constant at 10 ng. The second array of 5 reactions will vary the plasmid (template DNA) amounts at 10 ng, 1.0 ng, 100 pg, 10 pg, and 1.0 pg, holding the [Mg^{++}] constant at 2.0 mM. Final construct a negative control with no template and 2.0 mM $MgCl_2$. Remember to account for residual volumes in MM!

PCR Stock Solutions:

10X PCR buffer:
500 mM KCl
100 mM Tris-HCl (pH 9.0 @ 25°C)
1.0% Triton X-100 (nonionic detergent)

dNTPs: 2.5 mM each

High quality water: q.s. to 50 µl

[Plasmid]: 1 or 10 ng/µl

Primers: 50 or 20 µM (aka pmol/µl!)

100X BSA: 10 µg/µl

***Taq* polymerase:** 5 units/µl

[Mg^{++}]: 25 mM

PCR Worksheet:

Template Name	HQ H_2O	10X PCR Buffer	dNTPs	Primer #1	Primer #2	BSA	*Taq* Pol	Mg^{++}	Template DNA
Control									n/a

Volumes needed for Master Mix Cocktail:

H.Q. H_2O =
10X PCR Buffer =
dNTPs =
Primer #1 =
Primer #2 =
BSA =
Taq Pol =