Biology 324 **Moyer/Leaf**
Winter 2000 **Lab #6 – Cloning & Sequencing**

Now that you have successfully isolated gDNA, you will being a series of molecular biological manipulations that will result in the cloning and sequencing of a specific gene of interest. We will be targeting the SSU rRNA gene as much is know about these genes in general and because they are extremely good "biomarkers" used in determining the phylogenetic placement of all known biological taxa.

Our strategy will be this, first to PCR SSU rDNA genes from our gDNA samples. Next to clone these PCR products into the pTA vector using the AdvanTAge PCR cloning kit from Clontech (see user manual for detail and protocol). Then to screen potential or putative positive clones with α-complimentation. Next, to confirm the positive clone by PCR amplification using primers specific to the plasmid vector. Then to grow cells and isolate purified plasmid containing your insert using the Qiaprep Spin plasmid Kit (see handout for protocol and details). Then, finally to PCR amplify your SSU rDNA gene of interest using flanking vector primers to be used as the template for cycle sequencing. You will also prepare a cryopreserved sample of your *E. coli* strain at the same time you do your plasmid preparation.

This approach will require you to construct three separate PCR reactions, each of which will use a different form of template DNA. In the first you will have to quantify your gDNA using the spectrophotometer and get an estimate for both purity and yield. Second, you will use a toothpick to place a small amount of cells directly from a single isolated colony into your PCR cocktail. Third you will add a given volume of your purified plasmid preparation to the PCR to get the final double stranded template to be used for your asymmetric sequencing PCR. Make sure to modify your master mixes accordingly!

Initial gDNA PCR amplification reaction mixtures are as follows: 5 µl of 10X PCR reaction buffer, 10 µg of acetylated BSA, 200 µM dNTPs, 1.0 µM forward and reverse primers, 2.5 mM $MgCl_2$, and 5.0 Units of *Taq* DNA polymerase in a total volume of 50 µl. Reactions are mixed with everything except the *Taq* polymerase, incubated for 2 min at 95°C, and then placed on ice (true-hot-start). The *Taq* polymerase is then added and followed by 30 cycles of denaturation, annealing and extension at 94°C for 1 min, 55°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 10 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.

The colony screening PCR reaction mixtures are as follows: 2 µl of 10X PCR reaction buffer, 4 µg of acetylated BSA, 200 µM dNTPs, 0.5 µM forward and reverse primers, 2.5 mM $MgCl_2$, and 2.0 Units of *Taq* DNA polymerase in a total volume of 20 µl. Template is added using toothpick transfer of single isolate colony method, smaller is better! Reactions are incubated for 1 min at 95°C (pseudo-hot-start) followed by 30 cycles of

denaturation, annealing and extension at 94ºC for 1 min, 55ºC for 1.5 min, and 72ºC for 3 min, respectively. This gets followed by an additional extension at 72ºC for 7 min, and a 4ºC hold. A 10 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.

Sequencing template preparation PCR mixtures are as follows: 5 µl of 10X PCR reaction buffer, 10 µg of acetylated BSA, 200 µM dNTPs, 0.5 µM forward and reverse primers, 2.5 mM $MgCl_2$, and 5.0 Units of *Taq* DNA polymerase in a total volume of 50 µl. Plasmid template is added by using 2 µl of plasmid prep. Reactions are incubated for 1 min at 95ºC (pseudo-hot-start) followed by 30 cycles of denaturation, annealing and extension at 94ºC for 1 min, 55ºC for 1.5 min, and 72ºC for 3 min, respectively. This gets followed by an additional extension at 72ºC for 7 min, and a 4ºC hold. A 10 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.

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

Primers: 50 or 20 µM (aka pmol/µl!)

50X BSA: 10 µg/µl

***Taq* polymerase:** 5 units/µl

[Mg^{++}]: 25 mM