GENERAL NOTES ON RECOMBINANT DNA
Barrick Lab Manual

This set of notes provides some general guidelines and philosophy for working with recombinant DNA (PCR, DNA preps, restriction analysis of plasmids and cloning vectors, mutagenesis, ligation, transformation, and sequencing). In addition to these notes, which are not going to be comprehensive, you should read a recombinant DNA manual to get up to speed in various methods. Some suggestions, in addition to the numerous protocols in the three volumes of Maniatis et al., are

1.) Carson, S. & Robertson, D. (2005) Manipulation and Expression of Recombinant DNA. Acadmeic Press.

2.) Metzenberg, S. (2007) Working with DNA. Taylor & Francis. (I have this one in the lab).

First, some philosophy…

There is not a single experiment we have done in the lab that has not begun with recombinant DNA methods. Typically, a project involves initial cloning of a gene into an expression vector, often involving PCR. But even when we obtain a gene of interest already cloned into an expression vector, we still need to take the DNA and transform it into the right bacteria, and often it is a good idea to check to make sure the DNA is what was advertised. However, this initial work *never* gets the glory in any paper we publish or any talk we give outside the lab. It is necessary, but it is tempting to regard it as uninteresting and to approach it as routine and automatic.

This is a mistake. Even if you have subcloned a hundred constructs, each time you do it you should regard it as science. You should think about each cloning experiment hard, be careful, precise, systematic, and keep your eyes open for any sign of trouble (is the band at the right size?). Most importantly, you should always do as many controls at each step as you can. Appropriate controls will be mentioned as they are encountered in specific protocols, but examples are “no insert” reaction in ligations, using a separate plasmid standard in every transformation, doing a no primer (or no pfu turbo) standard in a quickchange, and running uncut (but incubated) DNA when you do a restriction digest.

Equally important, you must carefully document each step of the cloning process in your notebook. Design constructs you plan to build using computer files, integrating outputs from DNA strider (or another program), indicating primer design and placement of restriction sites. Print these out and put them in your notebook. Write up protocols in advance, each time you do them (even minipreps), including the samples are being analyzed and why they are being analyzed. Then write about the results, taping gel pictures into your notebook, identifying your size markers, and interpreting your results (e.g. “I was expecting a 1.3 kb band when I double-digested, and a 6 kb vector. The bands I see are at about 1.2-1.4 kb and 6-7 kb, so it looks like I have my insert in clone seven”).

If you are careful in your recombinant DNA work, paying stringent attention to the details, you will get your constructs expediently. When things don't work, you will become aware at the earliest possible time (e.g. PCR analysis rather than when you get your sequences back and they are the wrong thing), and when things don't work, your controls will suggest what the problem was (and how to fix it).

Below are some general comments that apply to DNA work:

1. DNA is generally stored in TE buffer. TE is described in Maniatis. It is 10 mM Tris•HCl, pH 8.0, 1 mM EDTA, pH 8.0. You can make it from 1 M and 0.5 M stock solutions, respectively, but the pH of the stocks (and the diluted DNA) must be 8.0. TE should be regarded as a preservative for DNA. The buffer (Tris) keeps the pH from becoming extremely alkaline (which hydrolyzes DNA) and the EDTA chelates metals (especially Mg^{2+}), which knick DNA.

The following are exceptions to TE storage of DNA in water:

A. Sequencing samples. EDTA appears to inhibit the sequencing reaction, so when you are preparing DNA that will be used for sequencing, resuspend it in water.

B. Electroporation. If you are going to be using your DNA to transform E. coli by electroporation (either after ethanol precipitating a ligation or moving a plasmid from one strain to another), even the low salt concentration of TE buffer is enough to give dielectric breakdown (an arc, a flash of light, and a lot of dead *E. coli*). Here again, dissolve this DNA in water (or if it is a plasmid, dilute from TE resuspension in water—it only takes a tiny fraction of an intact miniprep to get colonies.

2. Unless it is dried (i.e. lyophilized), DNA is always kept on ice, in an ice bucket, unless it is being used in a reaction at a specific temperature (rx digest, ligation) or it has been loaded into a gel. This goes for minipreps, PCR products, ligations, etc. Ice should be used when preparing samples for various reactions, such as restriction digests. Nearly all chemistry goes faster at elevated temperatures, and this includes degradation of DNA by residual nucleases or metals.

3. When you run gels, ALWAYS RUN A SIZE STANDARD. If we are out, don't run your gel. If you can't compare your DNA to a standard, there is not much point in running a gel. When you do run a gel with size standards, ALWAYS compare the size of your samples to the standards and confirm that it makes sense. A lot of time has been wasted on samples that were clearly the wrong thing, but the discrepancy went unnoticed. The great thing about DNA electrophoresis is that it does not lie. If the band is in the wrong place, the band is wrong.

4. Buffers (restriction, ligase, PCR, etc) must be kept cold. Melt the buffer you need, make sure it is throroughly mixed and dissolved, and immediately put it in your ice bucket once you have melted it. Ligase buffer contains an energy source (ATP or

NADPH, depending on enzyme), which will be damaged by being warm for too long. The same goes for dNTPs used in PCR and other polymerase reactions.

5. Enzymes must always be kept at minus 20°C! When you need an enzyme, go to the freezer with your micropipettor already dialed to the needed volume with a clean box of tips, remove the enzyme from its freezer box, immediately withdraw what you need, and put the enzyme back BEFORE you even pipette into your reaction mix. When doing multiple reactions of the same type (e.g. 12 *Nde* I digests), make a cocktail of large enough volume for all the reactions with buffer and dH_2O, then add the enzyme to that cocktail. That way, you only go into the enzyme tube *once*. Plus, it saves pipetting steps, which is good both for your sanity and to avoid making a mistake.

6. Do not open a new tube of enzyme when another one is open. Finish what is there first. These things are not cheap. Moreover, when we get to the point where we have eight tubes of DNA ligase, for example, they all get old, and they all stop working. By working from one tube, we ensure that the tube is fresh. When you do open another tube, label it with the opening date.

7. If you do encounter a bad reagent (enzyme, buffer, size standard…), THROW IT AWAY AND ORDER A NEW ONE. Don’t let others in the lab suffer the same fate that you suffered.