

## Sample Preparation

The DNA template and primer should be mixed together ahead of time according to the recipe listed below. We do not sequence samples that are not premixed.

### **Premixed Sequencing Reactions: (Template + Primer + water)**

#### Template

dsPlasmid (3-6 kB) – 0.25 ug.

dsPlasmid (6-10 kB) – 0.25-0.50ug.

dsPlasmid (10-20 kB) – 0.50-1.0ug.

dsPlasmid (>20 kB) - Contact us first.

ssDNA - Use 50% of the dsDNA amount.

PCR fragment – 5-10ng per 100bp ( ex. 400 bp = 20-40 ng).

#### Primer

~30ng (4.8pmoles primer) (18-24mer)

Water to a total volume of 12ul.

The amount of DNA template used in a sequencing reaction can affect the quality of the data. Too much template makes the data top heavy with strong peaks at the beginning that fade rapidly. Too little template or primer reduces the signal strength and results in faint or blank samples.

The two most important parameters for successful DNA sequencing are high-quality template and primer.

### **Template Recommendations:**

DNA suitable for restriction enzyme analysis and manual DNA sequencing may not work well for cycle sequencing. Residual salts, organic chemicals or incomplete removal of cellular components such as RNA, proteins, polysaccharides, and chromosomal DNA will all affect the template quality. Applied Biosystems recommends that all DNA templates be purified from (endA-) E. coli strains using either ABI Prism Qiagen miniprep kits according to the manufacturer's instructions. Applied Biosystems also states that CsCl banded DNA works well if all the CsCl and EtBr have been completely removed via dialysis. In addition, many other commercially available purification kits prepare plasmid DNA suitable for cycle sequencing. The A260/A280 ratio should be 1.8-1.9; lower ratios usually indicate contamination by protein or organic chemicals. Templates should be dissolved in H<sub>2</sub>O or 10mM Tris pH 8.0 (1xTE also works). Higher concentrations of EDTA or other impurities will inhibit the PCR reaction and result in little or no readable sequence. The DNA concentration of the template is very important and should be accurately determined by Abs. 260nm. Many laboratories also run aliquots of their templates on agarose gels prior to sequencing. This is often very helpful in detecting contaminating DNAs or RNAs and getting accurate quantitation. PCR product templates should contain only one band or be gel purified. They must also be completely free of other oligonucleotides prior to DNA sequencing. PCR products can be purified using Qiagen Qiaquick Gel Extraction kit, Promega PCR clean-up kits, Centricon-100, Microcon 50 spin columns, or equivalent.

### **Primer Recommendations**

High-quality primers, 18-24 bases long, 40-60% GC, with a 3' G or C and a  $T_m \sim 55^\circ\text{C}$  are recommended. Avoid primers with runs of 4 or more identical nucleotides (especially G's), palindromes because they can form secondary structures, and primers that can hybridize to form primer-dimers. If your primer has a GC content below 50%, lengthen it until the  $T_m > 52^\circ\text{C}$ . The following formula can be used to estimate the melting temperature:

$$T_m = [(\# \text{ A} + \text{ T}) \times 2^\circ\text{C}] + [(\# \text{ G} + \text{ C}) \times 4^\circ\text{C}].$$

Almost all oligonucleotides obtained from commercial sources (including IDT oligonucleotides purchased through the Duke University DNA Analysis Facility) can be used without further purification. As with the DNA template, an accurate DNA concentration of the primer is required. Applied Biosystems recommends using 4.8 pmole primer per reaction; that translates into 28.2ng, 31.2ng, and 37.5ng for average 18, 20 and 24mers respectively.

The DNA Sequencing Facility offers a limited number of tested sequencing primers for sale. Download the Sequencing Primers for sale file for more information.