

## Sample Preparation for DNA Sequencing

### General

We aim for a turnaround time of 24 hours or less if you deliver the samples to the core facility or pickup location before 11am. Much of the process is automated. The speed at which we can cycle, load, and run samples depend on how the samples are presented to us. We need to work within constraints of the computer systems and of the liquid handling system. File names and sample size are the most important. If we have to work with individual samples that do not conform, it takes longer for everyone to get their data and the per sample price goes up.

### Sample names

The Molecular Biology and Proteomics Core has switched to a new sample [database management system](#). This system makes it easier to submit samples while giving the user automated data delivery, direct access to old data, sample billing information, etc. An important feature of this system is that it will assign a sample number to each of the samples you submit. For the system to work correctly you will need use the assigned numbers on the sample tubes you submit. See these [instructions](#) for details.

## Delivering Template and Primer to the Core Facility

### Template and Primer

If you prefer to have the Core Facility do your chemistry, please prepare your samples in the following manner: mix template and primer in a single tube in the proportion indicated in the table below. Samples should be delivered in 1.5 ml eppendorf tubes. Final sample volumes should be 20 microliters. If they are lower, add Milli-Q water to bring them to volume. The liquid handling equipment requires at least a 20 ul sample. If the sample is less than 20ul your data may be compromised. The Core Facility performs all cycling and cleanup operations and generally delivers the data via email the following morning.

Type of Template	Amount
Double stranded template	200-500
Single stranded template	50-100 ng
PCR Product 100-200 bp	1-3 ng
PCR Product 200-500 bp	3-10 ng
PCR Product 500-1000 bp	5-20 ng
PCR Product 1000-2000 bp	10-40 ng
PCR Product 2000 bp	40-100 ng
cosmid, BAC	0.5-1.0 ugm
Primer	3.2pMole
dH2O	q.s. 20ul

## Preparing the sample yourself

### Protocol for Cycle Sequencing

The Molecular Biology Core Facility distributes ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits v3.0 with AmpliTaq DNA polymerase, FS at cost. The kits are available in various sizes to accommodate large and small projects. The MBCore tests each lot before it is distributed. We advise reading the manual for the kit before preparing your template and primers. You can download this manual in PDF format from the Applied Biosystems web site. An abstracted version, containing only the essential information necessary to process your sample, appears below. Please refer to the manual or inquire at the Core for information regarding the preparation of a template for sequencing. High quality template and a good primer are essential for obtaining good results. Quantitation of the template is of primary importance. Taking an OD of your sample is not the best way to estimate the amount of DNA in the sample. We recommend running the template on an agarose gel with quantitative DNA standards. This will produce the best estimate of the amount of DNA in the preparation. For a successful sequencing reaction, the molar ratios must be within the windows specified below for the various types of template.

### Mixing Reagents

We currently recommend "half" reactions, using half the 8 microliters of BigDye reaction mix recommended by the manufacturer. The addition of 5X buffer allows for a 20 microliter total reaction volume which is the smallest reaction the average thermal cycler will handle. If you have a thermal cycler capable of 10 ul reaction volumes, eliminate the buffer and q.s. to 10 ul. IF YOU DO 10ul REACTIONS, YOU MUST ADD 10 ul OF MILLI-Q WATER TO YOUR REACTIONS BEFORE PURIFICATION.

Please pay particular attention to the section on purifying extension products below. For each reaction, mix the following reagents in a labeled tube of the appropriate size:

Reagent	Amount	Concentration	Volume
BigDye Terminator Mix			4ul
Double stranded template	200-500 ng		Not to exceed 10.8ul
Single stranded template	50-100 ng		Not to exceed 10.8ul
PCR Product 100-200 bp	1-3 ng		Not to exceed 10.8ul
PCR Product 200-500 bp	3-10 ng		Not to exceed 10.8ul
PCR Product 500-1000 bp	5-20 ng		Not to exceed 10.8ul
PCR Product 1000-2000 bp	10-40 ng		Not to exceed 10.8ul
PCR Product 2000 bp	40-100 ng		Not to exceed 10.8ul
cosmid, BAC	0.5-1.0 ugm		Not to exceed 10.8ul
Primer	1pmole/ul		3.2ul
5x Buffer*			2ul
dH2O			q.s.
Total Volume			20ul

\*The Core facility supplies the 5X buffer at no cost.

We recommend making a "master mix" containing enough BigDye and 5x buffer in the above proportions then adding 6 ul to each reaction containing template DNA, Primer and Milli-Q water in the amounts specified above.

### Thermal Cycler Conditions

This protocol was developed for use with PE/ABI thermal cyclers. If you have a different make of thermal cycler you may need to modify these conditions. Regardless of the thermal cycler that you use, we advise heating the reactions to 96 degrees C for 5 minutes before cycling. If using the DNA Thermal Cycler (TCI)

or the DNA Thermal Cycler Model 480, overlay the reaction mixture with one drop of light mineral oil (approximately 40 µl).

### **Cycle Sequencing on the GeneAmp PCR Systems 9700, 9600 and 2400**

1.) Place the tubes in the thermal cycler, begin thermal cycling as follows:

- Rapid thermal ramp to 96 degrees C
- 96 degrees C for 10 seconds
- Rapid thermal ramp to 50 degrees C
- 50 degrees C for 5 seconds
- Rapid thermal ramp to 60 degrees C
- 60 degrees C for 4 minutes

2.) Repeat for 25 cycles.

3.) Rapid thermal ramp to 4 degrees C and hold.

4.) Purify extension products.

Note: If condensation is observed on the walls of the tubes at the end of the reaction, it is recommended that the reaction mixture be centrifuged prior to removal of the unincorporated dye terminators.

### **Cycle Sequencing on the DNA Thermal Cycler (TCI) and the DNA Thermal Cycler Model 480**

1.) Place the tubes in a thermal cycler, and begin thermal cycling as follows:

- Rapid thermal ramp to 96 degrees C
- 96 degrees C for 30 seconds
- Rapid thermal ramp to 50 degrees C
- 50 degrees C for 15 seconds
- Rapid thermal ramp to 60 degrees C
- 60 degrees C for 4 minutes

2.) Repeat for 25 cycles.

3.) Rapid thermal ramp to 4 degrees C and hold.

4.) Purify extension products.

### **Purifying extension products**

The purification of the extension products is very important. If the unincorporated dye terminators are not thoroughly removed from the reaction, your sequence will be rendered unreadable due to the overwhelming fluorescence of the aggregated dye terminators. There are several acceptable methods for removing the unincorporated dye terminators. The cheapest way is ethanol precipitation. However, we have found this to be subject to a great deal of variability and do not recommend it. We recommend spin column purification of extension products. Two products are available for this procedure. The Remsen stock room carries Edge BioSystems columns. These columns are relatively inexpensive and give good results on the average sample. However, in our hands, they give less than a quantitative yield of extension products and we have observed some batch-to-batch variation. A more quantitative product is available from Princeton Separations. These columns give a better yield of extension products. However, they are not as convenient to use and are more expensive. Whichever column you choose, be sure to follow the package directions carefully, paying particular attention to calculating the G force in the centrifugation step. There are other methods available, such as magnetic bead technology and a membrane filtration method. However, they are designed for high throughput 96-well plate technology and would be of little use for small numbers of samples. If you intend to do full 96 well-plate sequencing, please visit the Core and allow us to assist you in setting it up. There is a discount for a full plate of completed samples but there are specific guidelines.