

# DUKE UNIVERSITY DNA ANALYSIS FACILITY

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## Fluorescent DNA Sequencing Instructions

**Introduction:** We use the Applied Biosystems Dye Terminator Cycle Sequencing system with AmpliTaq DNA Polymerase combined with ABI 3730 PRISM DNA Sequencing instruments in this facility. Currently, we are using Big Dye terminator v1.1 sequencing chemistry. With dye terminator labeling, each of the four dideoxy terminators (ddNTPs) is tagged with a different fluorescent dye. This system has the advantages of 1) any unlabeled primer can be used, 2) all four sequencing reactions are performed in one tube and run in one lane on the gel, 3) false stops caused by anything other than incorporation of a ddNTP are not fluorescently labeled and thus not detected, and 4) sequencing difficulties caused by DNA conformation and base content are minimized by performing the PCR reaction at 60°C. In this system the two most important parameters for successful sequencing are high-quality template and primer.

**Template:** DNA suitable for restriction enzyme analysis and manual DNA sequencing may not work well for cycle sequencing. ABI recommends that all DNA templates be purified from (endA-) E. coli strains using either ABI Prism or Qiagen miniprep kits according to the manufacturer's instructions. ABI also states that CsCl banded DNA works well if all the CsCl and EtBr have been completely removed via dialysis. In addition, Promega Wizard Plus purification kits and Clontech NucleoBond kits prepare plasmid DNA suitable for cycle sequencing. 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 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.

**Primers:** High-quality primers, 18-24 bases long and ~50% GC, with high specificity and a T<sub>m</sub> = 55-60°C are recommended. Almost all oligonucleotides obtained from commercial sources can be used without further purification. As with the DNA template, an accurate DNA concentration of the primer is required. Perkin Elmer recommends 4.8 pmole primer per reaction be used; that translates into 28.2ng, 31.2ng, and 37.5ng for average 18, 20 and 24mers respectively

**DNA Sequence:** We use the ABI PRISM DNA Sequencing Analysis software program to analyze the DNA sequencing gel and read the bases. The overall quality is, of course, very dependent on DNA quality and signal strength. A typical run generates about 800 bases with an accuracy of 99% for the first 500 bases. As you read past 500 bases the accuracy decreases. Readable sequence begins about 50 bases from the primer. High quality samples often generate accurate sequence to 650 bases and beyond.

**Cost** (Please contact the DNA Analysis Facility for current pricing)

- "Peer-reviewed" Duke Cancer Center members are eligible for a discount.
- Volume discounts (per sample) are available for:

-\$1.00 for ≥ 48 samples	-\$2.00 for ≥ 96 samples
-\$3.00 for ≥ 500 samples	-\$4.00 for ≥ 2500 samples

Contact us for details.

- Additional charges (per sample):
  - + \$2.00 manual data handling
  - + \$2.00 special reaction fee (Genomic samples - to cover addition reagent charges)

Please provide a current Duke grant code in the Duke accounting format (ex. 0010 3031234 WBSE) or PO#. A billing record is provided with each customer's sequencing data. In order to keep costs as low as possible, this is the only record that will be provided. Each customer should give this information to their laboratory business manager.

**Data Output:** The Finch LIMS system is an online data ordering and retrieval system. Email notifications are sent once your data is completed. <http://lims.duhs.duke.edu:8080/Finch/Core/loginForm> You are welcome to check the status of your order at any time. You can do so by logging in with your user name and password. Instructions on how to view and download your results are available on our website.

In order to view chromatographs you need a sequence analysis program capable of reading the file. Our current favorite freeware viewing program is FinchTV from Geospiza for Linux, Mac OSX, Windows, and Solaris (downloadable from <http://www.geospiza.com/finchtv/index.htm>). ABI provides a freeware program named ABI Prism EditView DNA Sequence Viewer for older Apple computers that allows you to view, edit, and print the electropherogram. EditView can be downloaded from Applied Biosystems or obtained from the Sequencing Facility. There is also a viewing program for PC computers called Chromas that can be downloaded from [www.technelysium.com.au/chromas.html](http://www.technelysium.com.au/chromas.html). In addition, a number of commercial sequence analysis programs (ex. Sequencher) are able to view and edit electropherogram files.

**Disclaimer:** The DNA Analysis Facility is a non-profit core service established to meet the DNA sequencing needs of the Duke University scientific community. The charge per reaction covers the expense of labor, reagents and instrument usage. We will be performing positive controls for the cycle sequencing reactions and electrophoresis of the samples on every sequencing run. Therefore, laboratories will be charged for every reaction performed regardless of whether or not it succeeds so long as our controls work. We recommend that laboratories inexperienced in cycle sequencing start with only a few reactions until they are confident their samples are working.

We will be happy to help you troubleshoot your reactions if your samples are not working.

The DNA Sequencing Facility is able to sequence samples for **research purposes only**.

**Turn Around Time:** The DNA Analysis Facility operates on a first-come, first-served basis. At this time, members of Duke University receive priority over non-Duke customers. We sequence samples every business day. Samples need to be dropped off no later than 4PM the day before they are processed. Turn around time is 2 business days unless there is a backlog or technical problems. It is not necessary to call ahead of time to reserve spaces but you can call to determine if there is a backlog.

**Dropping Off Samples:** Users should drop off their samples in 135 Jones. Instructions for dropping off samples are posted in 135 Jones. The room will be open most days Mon.-Fri. 9AM-4PM. A completed Sequencing Request Form is required for all samples prior to sequencing. Blank Request forms are available in 135 Jones or can be downloaded from our web site. All tubes must be labeled with your initials or some other unique label. Tubes should be placed in the freezer.

#### **Reaction Recipe:**

The DNA templates and primers should be mixed together ahead of time. We do not sequence samples that are not premixed.

Premixed reactions (each rxn):

0.25-0.5 ug plasmid Using too much template will result in less than optimal results!  
(If plasmid > 12kb, contact us first)

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

+ 30 ng primer (18-24mer) Use 2x (60ng) if your primer is larger.

water to a total volume of 12 ul

Big Dye™ ver 1.1 is currently the default DNA sequencing chemistry used.

We are interested in your questions and comments. Please feel free to contact us at any time.