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# 3130xl DNA Sequencing and Submission Protocol

**Researchers are asked to perform cycle sequencing in their laboratories using dideoxy terminator chemistry, and to bring the purified sequencing reaction to the core for electrophoresis and processing, along with a submission sheet and disk.**

**For more in depth information on sequencing, please refer to the BigDye Terminator v3.1 Cycle Sequencing Kit protocol manual available free of charge upon request from the manufacturer. www.appliedbiosystems.com Toll Free: 800-345-5224**

## DNA Prep

Automated sequencing requires **high-quality DNA**. For plasmids, cosmids, PACs and BAC's columns from Qiagen, Promega or Edge Biosystems are recommended. Depending on how much DNA you need either small spin columns or large Maxiprep columns will work.

Sequencing directly from PCR products is also possible if the quality and quantity are sufficiently high. PCR products should be purified with PCR clean up columns such as those from Qiagen or Promega. The Core routinely uses Qiagen QIAquick PCR Purification Kit (250), Cat. # 28106. In addition, we have seen good success with purification using ExoSap-IT from USB Corp.

## Primer Design

The length and sequence of a primer determines its melting temperature and specificity. Most recommendations are for primer lengths of 18-25bp and CG content between 40-60%. There should also be no runs of a single nucleotide greater than 3 within the primer. The primer should also be checked for potential self-annealing or hairpin formation, especially at its 3' end. In addition, possible secondary priming should also be identified, again stressing matches involving the 3' end of the primer.

## DNA Quantity

DNA concentration should be estimated after purification by comparison to standards on an agarose gel or by spectrophotometry.

Plasmids

For standard plasmid preps, 100ng per kb of the total plasmid is a good place to start. In general, 500-750ng of DNA is sufficient for a single sequencing reaction. Cosmids, YACs, PACs and BACs will require more DNA (please inquire).

| Plasmid size in bp | Amount of DNA in ng |
|---|---|
| 1000 | 100 |
| 2000 | 200 |
| 3000 | 300 |
| 4000 | 400 |
| 5000 | 500 |
| 6000 | 600 |
| etc. | |

PCR Products

The quantity of PCR product needed varies with the size of the fragment. It is possible to sequence fragments ranging from 150-12,000bp, although very small (<150bp) and very large (>5kb) products can be challenging.

| PCR size in bp | Amount of DNA in ng |
|---|---|
| 150-250 | 25-40 |
| 250-500 | 40-70 |
| 500-700 | 70-90 |
| 700-900 | 90-110 |
| 900-1200 | 110-140 |
| 1200-2000 | 140-180 |
| 2000-12,000 | 180-250 |

**Sequencing Reaction**

Sequencing reactions should be performed using ABI BigDye Terminator V.3.1 chemistries. ABI now sells all sequencing kits without protocol manuals, but they can be ordered at no cost. Because of the increased sensitivity of the new platform we recommend the user to dilute the terminator reaction kit as indicated in the table below. The manufacturer supplies the dilution buffer needed. **The BCL recommends customers to use a ¼ dilution of the BigDye Terminator Mix when processing samples on the 3130xl for the first time.**

| ABI: | ½ rxn | ¼ rxn | ⅛ rxn |
|---|---|---|---|
| Terminator Chemistry (2.5X) | 4ul | 2ul | 1ul |
| 5X Dilution Buffer | 2ul | 3ul | 3.5ul |
| Primer | 3.2pMoles | 3.2pMoles | 3.2pMoles |
| DNA | | | |
| H2O | | | |
| Rxn total | 20ul | 20ul | 20ul |

Use a thermocycler with rapid ramping times and 0.2ml thin-walled reaction tubes.
For ABI chemistry, program the thermocycler as shown below.

- Initial denaturation of 96°C for 1 min
- 25 cycles of:
  96°C 10sec
  50°C 5sec
  60°C 4min
- Followed by a 4°C hold until ready to purify

After the thermocycler has finished, samples can be stored at -20°C in the dark or taken immediately to Terminator Removal.

For Cosmids, BACs, PACs or difficult templates, alterations to the thermocycling protocol or additives into the reaction such as DMSO, Glycerol or Betaine can be tried. Please inquire with BCL staff or consult BigDye Terminator v3.1 Cycle sequencing kit manual.

**Terminator Removal & Sample prep**
Best sequencing results are obtained when excess dye terminators are removed prior to electrophoresis. Please note for the 3130xl, the unincorporated dye terminators migrate between 60-80 bases and will obscure your data if not completely removed.

1.) Hot SDS Treatment
**BCL recommends this treatment *before* spin column purification.**

- Prepare 2.2% SDS in deionized water. It will be stable at room temperature.
- Add an appropriate amount of the 2.2% SDS solution to each sample to bring the final SDS concentration to 0.2%. (For example: Add 2 ul of 2.2% SDS to each 20 ul sequencing reaction.)
- Seal tubes and mix thoroughly.
- Heat the tubes to 98° C for 5 min. then allow tubes to cool to ambient temperature before proceeding to the next step.

Note: A convenient way to perform this heat and cool cycle is to place the tubes in a thermocycler and set to 98° C for 5 min followed by 25° C for 10 min.

2.) Dye Terminator Removal

- Spin Column Purification, including pre-column SDS treatment. The BCL routinely uses Edge Biosystems Performa DTR Gel Filtration Cartridges, cat. # 42453. The SDS treatment is very effective in removing the unincorporated dye terminators. If you chose column purification, the BCL Core lab highly recommends performing the SDS treatment.

Note: Ethanol/EDTA precipitation is an alternative to spin column purification. This is cheaper than the spin column method, but less effective at removing unincorporated dye terminators.

3.) After terminator removal transfer purified samples to **0.2ml** tubes (strip tubes with **individual caps** may be used). Tubes must be labeled <u>on the side only</u> with the principal investigator or submitter's

initials and a number (Ex. KS1, KS2 etc.). More detailed names can be linked to these numbers on the sample submission sheet. **It is no longer necessary to speedvac samples prior to submission.**

**Sample submission and Turnaround Time**
Samples must be submitted **wet** in 0.2ml tubes (strip tubes with individual caps may also be used), labeled on the side with the principal investigator or submitter's initials and a number (Ex. KS1, KS2 etc). A sample submission sheet much be completed. Samples should be placed in the Core –20 freezer in G25 A/R building by 1:30 PM or in room 214 Rockland Center One by 2:30 PM.

Contact the core as early as possible to let us know you have samples to be run. To have samples run the same day they are submitted it is required that you call the core and let us know before 11:00 AM. Samples should be received in G25 A/R by 1:30 PM or RC1 room 214 Core freezers by 2:30 PM on the day they are to be run (you may also bring your samples directly to the Core anytime before then). If you submit your samples after that time, it is the responsibility of the researcher to bring the samples to the Core lab. Samples will be processed overnight on a first-come first-serve basis and turn around time is typically 24-48 hours.

**Data**
Runs will go overnight and results can be picked up from the mail slots inside the core lab after 10:00 AM the following morning. There are two files generated per sample.

- The first is a chromatogram file. These are available upon request. You can use any of the available free downloads to print and view your chromatograms.
- The second is a text file of the sequence, which will be saved to your shared folder, flash, CD or disk. Text files can be imported into programs such as Macvector as well as PC software.
- In addition, a hard copy printed color chromatogram containing the first 600-625 bps of sequence (the second page can also be printed on request) will be printed by the Core and made available for pickup after 10:00 AM or delivered the your customer mail slot in G25 A/R building by 1:30 PM.