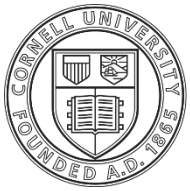
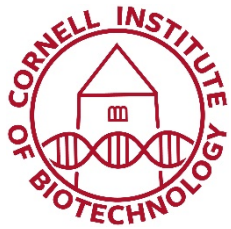


Cornell University



Genomics Facility
147 Biotechnology Building
526 Campus Road
Cornell University
Ithaca, NY 14853-2703

Sanger Sequencing Handbook
READY TO LOAD

Revised May 2021

Contact

Phone: (607) 254-4857

Email: genomics@cornell.edu

Monday -Thursday: 8:00AM-4:30PM

Friday: 8:00AM-4:00PM

This document (“Sanger Sequencing Handbook READY TO LOAD”) explains all the steps you need to take to prepare and submit your samples if **you only need the sequencing itself**.

The information provided assumes you will do the BigDye reaction yourself.

If you want the Genomics Facility to perform both the BigDye reaction and the sequencing service, please consult an alternative document: “Sanger Sequencing Handbook FULL SERVICE”.

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Step 1. Do the BigDye Reaction in your lab

You can choose the reaction the way you prefer. As an indication, we provide a standard protocol in the Appendix 1 at the end of this document.

Step 2. Cleanup your BigDye reaction product

To remove the excess of BigDye, you can use a commercial kit such as the Big Dye Xterminator (Thermo Fisher).

At the Genomics Facility, we perform dye terminator removal by adding 5 µl ddH2O to each 12 µl reaction before loading onto Edge Biosystems 96-well dye terminator removal columns (EdgeBio part number 60122). We then follow the manufacturer's protocol. Reaction products are spun into 96-well plates that fit the 3730xl instruments.

The Edge Biosystems cleanup plates are available to purchase from the Genomics Core.

You can also submit plates for the Genomics Core to perform the cleanup set prior to loading. Please inquire for pricing.

We provide additional methods for BigDye cleanup in the Appendix 2 at the end of this document.

Step 3. Choose the right plates

All Ready-to-load samples must be submitted in plates, no matter how many samples you have.

We require that you use any of the following:

- Applied Biosystems® MicroAmp® Optical 96-Well Reaction Plate (catalog number N8010560)
- Fisher Scientific (catalog number 14230244 and any plate that is compatible with the ABI instruments)
- USA Scientific (catalog number 1402-9200 and catalog number 1402-9300).
- If you have a plate that is not listed above but that you think is compatible, please email us a picture. We will get back to you if they will work with the sequencers or not.
- Note that "fast plates" or "short plates" are not compatible.

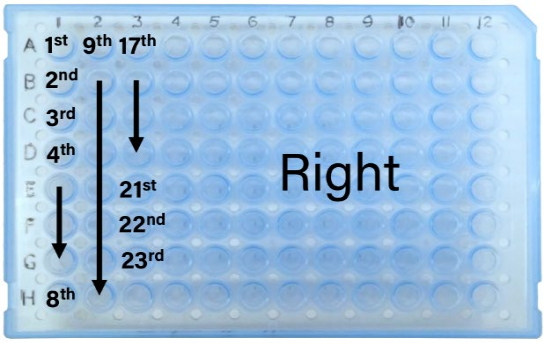
Step 4. Fill up your plates

- Fill **20uL** of your cleaned-up samples *down the columns.*
- If you accidentally skip a well, add it to your order so that your samples are not shifted.
- If you will ship plates by mail: we recommend that you use strip caps and NOT adhesive seal. They can leak and lead to sample contamination.
- If you will personally deliver plates: no need for strip caps. Cover the plate with an adhesive seal.

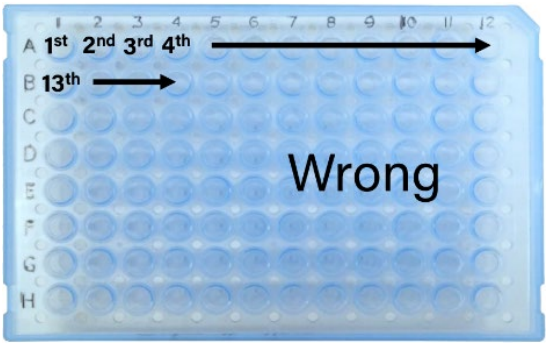
Example:

Your first sample goes in A1, your second sample in B1, your third samples in C1 and so one. When you are done with the first column, you will fill the next column. Sample 9 will be in A2, sample 10 in B2, sample 11 in C2 etc. If you have 23 samples, your 23rd sample is in G3.

You can put up to 96 samples per plate. If you have less than 96 samples, fill the empty well with 20uL of water.



Example with 23 samples



Step 5. Place your order online

All requests for sequencing must be submitted online. You can access our online ordering system by clicking “Submit samples” on our website.

We cannot process your samples if your order is not in our online ordering system.

To upload your sample names in the online ordering system, you can either upload a file or type all the sample names manually. If you choose to type in the sample names, go directly to step 2.

1. Create a text file with all the sample names to upload

- Using Microsoft Excel:

  Write your sample names with one name per line starting with well A1 and proceeding down the columns (A1, B1, C1, D1...) of the plate.

  Name1
  Name2
  Name3
  …
  Name23

  If you have empty wells, leave the cell blank. You do not need to enter any name for empty wells. Save as a Text (Tab-delimited) file.

- Using a text editor:

  Write your sample names with one name per line starting with well A1 and proceeding down the columns (A1, B1, C1, D1...). If you have empty wells, leave the line blank. You do not need to enter any name for empty wells. Save as a plain text file.

2. Log in to your account

3. Scroll down to the bottom of the page and select the plate you are submitting, 96 or 384 well, under **"Ready-To-Load Sanger Sequencing (BigDye reactions performed by customer)"**.

4. Enter billing information

5. Upload the text file you prepared with your sample names. Don't forget to click "Upload File". You should see the sample names auto-populate the online form.

6. **Enter a plate name** at the bottom of the form (letters, numbers, hyphens, and underscores only).

7. Click "Proceed to verification" and submit

8. Write the order number you will receive on the long side of the plate.

External customers will need a Purchase Order for payment. Please send us a copy of the PO at brc_payment@cornell.edu. We cannot start processing your samples until we receive a copy of the PO. Email is preferred over a paper copy; it will expedite the process.

Step 6. Send your samples or bring them to our facility

There are different methods to send you samples to sequencing.

- **In person.** You can come and drop your samples off in room 147 in Biotechnology Building. Our work hours are written on the first page of this document.
- **By FedEx or UPS**. Avoid USPS as packages are not delivered directly to the lab. Our mailing address is written on the first page of this document. Please note that there is no delivery on weekends or holidays. Also, note that we have received numerous broken plates, so please cushion and seal your plates before mailing.
- **Fed Ex drop box**. This option is available for customers from the Weill Medical College and Memorial Sloan Kettering Cancer Center in NYC. The Office of Sponsored Programs manages the drop box, so please direct all questions regarding the drop box to them.

Whether you send or bring your samples, don't forget to write your name, contact number, lab location and **order number (that you obtained through the online system)** on the outside of the plate and package.

If you have any question, our contact information is on the first page of this document.

Step 7. Receive and analyze your results

Our usual turnaround time is 1-2 business days.

You can track the progress of your samples from our ordering online system

When your results are ready, we will send you an email pointing you to our secure website where your electropherograms and text files can be downloaded.

Always look at the electropherogram, not just the text file. The sequencing software calls the strongest signal (highest peak) at any location. However, if the noise level is high, weaker signals may not be distinguishable from the background noise, resulting in questionable calls. Be sure that each peak is clearly stronger than any background at that site.

We also strongly recommend that you load the AB1 file into a program, and not rely uniquely on the picture provided by the facility.

To this end, Thermo Fisher Scientific offers a cloud-based data storage and data analysis software.

The Bioinformatics Facility can also provide software and support for primary sequence analysis.

Step 8. Store your data

Data will be available for 30 days. After this, your results will no longer be available on your account. If you want to access your files, contact us with your order number.

A $5 internal and an $8 external fee per order is associated with data retrieval, so please remember to download and save your results as soon as they are ready.

NOTE ON DNA STORAGE:

All samples will be discarded two weeks after they are processed.

Appendix 1: BigDye Reaction

The following is the master mix and protocol that the Genomics Core at Cornell University follows for Sanger sequencing premixed template and primer sample.

Mix for each sample in 96 or 384 well PCR plate:

Reagent	**Quantity**
One primer (forward or reverse)	6.25 pmole[1]
PCR product	determined by product length[2]
BigDye (**version 3.1**)	1 uL
5X sequencing buffer	2.5 µL
5M betaine (or DMSO)	0.5 µL
ddH2O	as needed
Final volume	**12uL**

The Sequencing Handbook FULL SERVICE contains additional calculations and recommendations for large PCR products and large constructs, as well as additional information on purity considerations.

Standard ABI thermocycling program

96°C 4 min
Followed by 30 cycles of:
96°C 10 sec
50°C 5 sec
60°C 3 min
4°C hold

[1] If your primer is at 3.2uM (i.e. 3.2 umol/L or 3.2 pmol/uL), you need to use 2.03 uL (rounded to 2 uL) for the reaction.

[2] The amount of template (PCR product) required for sequencing reaction approximately:
ng needed = (length of PCR product in bp)/5
Example: For a 250 bp product, the amount needed is 250/5 = 50 ng ÷ 4 = 12.5 ng per reaction.

Appendix 2: BigDye Cleanup

Post-sequencing reaction dye terminator removal, or “clean-ups", can be done by different methods: gel filtration columns, alcohol precipitation, or magnetic bead cleanup.

Ethanol precipitation are the cheapest but runs the highest risk of carryover gunk that will foul the sequencer's capillaries and/or obliterate peaks early in the sequence due to dye blobs.

A. Gel filtration or desalting columns

Method 1: Edge Biosystems 96-well dye terminator removal column

This is the techniques that the Genomics Facility uses, as explained in “Cleanup your BigDye reaction product” on page 4 of this document.

Method 2: User-prepared sephadex plates

Protocol from Martha Hamblin, Cornell University.

Reagents needed: Sephadex G-50 fine (e.g. Amersham Pharmacia 17-0042-01 100 gm), filter plate (Corning #3511).

Mix 6.5 gm Sephadex with 100 ml water and let swell for several hours at room temperature. Remove excess water (about 20 ml) so you have a medium-thick slurry and store at 4 degrees (for long-term storage, autoclave).

Prepare filter plates by swirling Sephadex solution to resuspend, then pipet 0.3 ml into each well of a 96-well filter plate placed atop a collection plate (a wide-bore pipet tip works best to pipet the slurry). It takes a little practice to get the Sephadex slurry to the right consistency so that it flows nicely into the wells but is not too dilute. Spin the plate (swinging-bucket rotor) for 2 minutes at 2000 rpm to remove excess water. An additional spin may be needed if the tips of the columns are touching the eluate in the collection plate. Replace the collection plate with a clean ABI sequencing plate. It is important to proceed quickly before the Sephadex dries out. Apply sequencing reactions carefully to the center of the Sephadex plugs and spin again for 2 minutes. You should recover most of the volume you applied in the collection plate. The ABI sequencing plate can be loaded directly onto the sequencer.

The filter plates can be rinsed thoroughly with distilled water to remove all Sephadex and air dried for re-use. The Corning plates can be used at least 5 times.

B. Ethanol/Isopropanol Precipitation

Protocol from Willie Swanson, University of Washington

1. Add 26 ul 95% ethanol to each well of sequencing plate
2. Mix and put at -20C for 15 minutes
3. Centrifuge plate for 30 minutes at 3,000 G
4. 4. Remove supernatant by inverting plate
5. Centrifuge INVERTED on top of paper towel for 1 minute at 700 G (yes, plate is upside down)
6. Add 125 ul of 70% ethanol
7. Centrifuge 10 minutes at 3,000 G
8. Remove supernatant by inverting plate
9. Centrifuge inverted plate for 1 minute at 700 G
10. Resuspend in 10-20 ul to load