Riverbend North, Room 161
110 Riverbend Road
Athens, GA 30602

The University of Georgia
Georgia Genomics Facility

Telephone (706) 542-6409
Fax (706) 542-6414
http://dna.uga.edu

Single tube sequencing

a) Gather Samples and Paperwork

i) Paperwork will be processed by the front office, issued a work order number, and then placed in the appropriate inbox. Check this inbox often, as samples are submitted throughout the day.

ii) Each reaction should correspond to a unique barcode listed in the work order. Check that each sample and primer listed in the work order is present. If you notice that there is an extra/missing sample, notify the front desk. Some samples/primers may have previously been processed, and therefore may be stored at GGF in its associated short-term archive location. Any missing samples/primers should be communicated to the front desk and/or to the researcher.

b) Sample Quantitation

i) The DNA concentration must be measured for each sample. The Nanodrop provides an estimate of how much DNA is in the sample based on its spectral qualities. The quantitative value allows us to determine what volume of the sample to use for each BigDye reaction.

ii) Both plasmids and PCRs must be measured. The sample information must include the size of the PCR or plasmid template. The size of the insert is not necessary for quantitation (but may be useful during sequence analysis), it is

the total size if the plasmid of PCR that is used for calculation. Use the following formula to determine DNA volume to use:

(a) $\frac{\text{total size of template (bp)}}{100\text{ bp}} \times 5\text{ng} = \text{amount (ng) of DNA to use}$

(b) Divide this answer by the actual DNA concentration (from Nanodrop) to determine what volume to use in the BigDye reaction.

For example:

5000bp plasmid; concentration measured at 210.5ng/uL

$\frac{5000\text{bp}}{100\text{bp}} \times 5\text{ng} = 250\text{ng}$ $\qquad \frac{250\text{ng}}{210.5\text{ng/uL}} = 1.18765$ or 1.2uL sample/ RXN

This formula is based on sample parameters that ask for 5ng of DNA per 100 bases of template. Again, the entire template is used in this formula, not the insert itself.

c) Creating a gel and binding samples

i) Log in to the database, and create a new gel. Always use next consecutive number (Gel Reference) after previous gel.

ii) Choose **650** for **Maximum Sequence Length**

iii) Enter **1** for **Number of Standards**

iv) Organize paperwork according to Work Order#, as reaction will appear on the gel file in this order. Make sure that all concentrations (**Q)** and volumes to be used (**T**) have been recorded into sample information.

v) On the menu to the left, select **Express Lane.** This will prompt a box on the right to appear; the box is named **Scan O' Lane.** For each reaction, type the sample concentration value (*sans* units), press enter, and then scan the barcode for that reaction. Press enter after each scan.

vi) When all entries have been scanned, click the option for **No Number Bind.** The reactions are now "attached" to the gel, but are not yet bound in numerical order. Go through each reaction and verify that each reaction is in fact on the gel. Once this is confirmed, click **Auto Number** to assign a numbered lane to each reaction.

vii) Next, type in each volume amount in the empty box in the **Qty Template** column. Do not press enter until all values have been entered. The pGEM standard quantity will vary, and depends on the concentration (usually b/t 0.5 and 0.8uL). Once all volumes have been entered, click **Update Recalculate.**

viii) Select **Print Gel Table.** Once all pages have printed, assign well positions to lanes on the gel table (start with A1 thru H1, A2-H2, etc.).

d) Building the gel

i) Pipette the each sample to its designated well based on the calculations from above, and add appropriate primer. Generally, primer concentrations between 10 and 30uM work best; use 1 uL of primer at this concentration for each reaction.

ii) Turn on thermal cycler and select BigDye program; do this several minutes before to allow thermal cycler to warm to temperature.

iii) Dry down plate using the evaporator centrifuge. With the Eppendorf repeat pipetter, add 10uL BigDye mastermix to each well.

(1) Recipe for BigDye mastermix:

73.5uL BigDye
98uL DMSO
147uL Seq. buffer
661.5uL dH2O

(2) Recipe for BigDye mastermix with dGTP:

55.1uL BigDye
18.4uL dGTP
98uL DMSO
147uL Seq. buffer
661.5uL dH2O

iv) Add translucent sealing mat, spin down, and load onto thermal cycler.

(1) Thermal Cycling Parameters for BigDye RXN:

(a) Lid temp: 104°C

(b) 96°C, 10s

(c) 50°C, 5s

(d) 60°C, 4min

(e) Hold at 4°C

e) Clean-up of PCR

i) After BigDye RXN has completed, remove plate from thermal cycler and spin plate to collect any evaporation.

ii) Build a **Sephadex** plate for clean-up:

(1) Obtain Millipore multiscreen filter plates (96-well). Pour Sephadex on plate loader and fill all holes. Replace any excess Sephadex back into container. Turn filter plate upside-down onto loader plate, turn over, and tap.

(2) Add 300uL dH20 to each well. Place a v-bottom 96-well plate underneath to catch water. Let stand 15 minutes.

(3) Spin plate with balance for 5min at 910G with program 40. Empty catch-plate.

(4) Add 175uL dH20 to each well. Spin again; program 40 (910G; 5min; 4°C. Empty catch plate. Sephadex is now ready to filter sample.

(5) Obtain a semi-skirted sequencing plate, label, and add 10uL dH20 to each well. Insert plate into plate-block, and cover with the hydrated Sephadex plate, taping the sides to ensure alignment.

(6) Transfer 10uL of PCR product to each corresponding well of the Sephadex plate. Spin for 5 min with program 40. Samples are now filtered through to the sequencing plate. Add barcode with gel name to the plate and load onto 3730 for sequencing.

f) Managing the data

i) To create a sample sheet: Log into the database and retrieve the gel. Right-click on **Get ABI377File,** and save it to the desktop. Open the file in WordPad, delete all spaces between sample names. Delete all characters after the primer name. **Replace** all characters with underscore, including spaces. Copy-all, and paste into the template named: **3730GenericSeq_IBL (**found in folder labeled GGF Templates; also on Aerie server in Working-2). Enter the gel name for **Container name** and **Plate ID**. Save this as a txt. Tab-delimited file to the H: drive in order to access it from 3730 computer. Import this sample sheet into the 3730 for analysis.

ii) Once data are generated through 3730 analysis, they are uploaded the **Data** folder on the desktop (E: drive). Copy this folder and paste into H:Working folder. Go to computer with Sequencing Analysis software. Copy folder from H:Working to C:Cheeto. Copy the name only of the gel file, and paste into Cheeto program. This program changes the name of the reaction to the number associated with that well. Copy the newly generated folder from Cheeto and place in H:Working. Delete all other files in H:Working.

iii) Open SequencingAnalysis. Add samples (from H:Working), and select analyze. Open each file and inspect its results.

(1) Raw data should appear uniform and neat, forming a slightly descending plateau shape.

(2) Peaks should be uniform and clear. This is also indicated by the uniformity and consistency of the vertical blue lines above (these denote quality of peaks).

iv) Keep notes on the gel table, passing acceptable sequence and noting what improvements or changes must be made to each sample to improve its sequence on the rerun.

v) When finished viewing the analysis, login to the database. Open the gel and mark the failed reactions. Mark either **fail**, or **rerun,** then select Pass **gel.**

vi) Make sure that only folder in H:Working is the current gel (converted in Cheeto). Go to the Mac computer, select the folder, insert one space after its name, press enter, and then run the **Daily Routine** program. This will bind the data files to the database so that the owner of each reaction will now have access to their data.

vii) Go back to PC and click on **Contacts List.** Scroll down and select **Send mail to marked addresses.** Do the same for the **Failure Contacts List.** Now emails have been sent to each customer with links to their data in the database.