

Sequencing and Sephadex Protocols

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Sequencing with BigDye Terminator (v.3)

ExoSap Cleanup (optional, can do no cleanup sometimes, or GeneClean, or isopropanol precipitation)

1. This gets rid of unincorporated dNTP's and primers
2. Add:
 - a. 7.5uL PCR product (or enough for 2 sequencing reactions; 200-400ng)
 - b. 1.5uL ExoSap (made with 5uL ExoI and 15uL SAP)
3. Vortex/spin quickly
4. Run with cleaning cycle on thermal cycler ("ExoSap", =37°C 15min, 80°C 15min, 4°C hold)

Quantify

1. Estimate amount of DNA in PCR by running 5ul on an agarose gel with a size marker of known concentration
2. Take upstairs to PhosphoImager (Typhoon)
 - a. Put gel on lower left of glass plate with wells to the left
 - b. Log in
 - c. Start 'typhoon' software
 - d. Check that the 'Acquisition mode' = Fluorescence.
 - i. Optional: change PMT to 400 if you expect really bright bands on your gel
 - e. Highlight squares on the grid that match where your gel is on the machine
 - f. Hit 'scan' and type in a name with your gel file, saving it into the Cruzan folder
 - g. If there are any red spots on your gel, re-scan it at PMT400. The red spots are where the image is saturated, and you won't get a good quantification.
 - h. Open ImageQuant
 - i. Open the gel picture that the Typhoon just made
 - j. Select the box tool on the L.H. side of the screen
 - i. Draw boxes around each band
 - ii. Just draw one box, then copy and paste it to the other places you want quantified – the size should be exactly the same
 - iii. Include a box for your size standard and one for a background correction
 - k. Background correction
 - i. Go to 'analysis' and 'background correction'
 - ii. Select all 'objects' in pop-up screen (click&drag)
 - iii. Select Method='Object Average' and select the background box
 - iv. Select 'set'
 - l. Analyze
 - i. Go to 'analysis' and 'volume report'
 - ii. Select all rectangles, select 'report'
 - iii. Double-click on any cell to export the table to excel
 - iv. Save as excel sheet (the only column of interest is 'volume')
 - m. Email to yourself or save to disc

Sequencing Reaction

Here's the sequencing recipe (slightly modified from Susan Masta):

- 1uL BigDye
- 1.5uL 5x Sequencing Buffer (400mM Tris pH 9.0, 10mM MgCl₂)
- 1uL M13Forward *or* Reverse (2.5uM)
- DNA (0.05xpbp = approximate ng needed)
- ddH₂O to 15uL

You're welcome to make a master mix of this for all your sequencing reactions – just remember that the BigDye tends to settle out quickly, so make sure you vortex vortex vortex. Also, make sure the BigDye is aliquotted into small amounts so it doesn't go through multiple freeze-thaw cycles.

Run the reaction on the sequencing cycle ("Seqnce"):

1. 98°C 3min
2. 98°C 15sec
3. 50°C 10sec
4. 72°C 4min
5. goto 2, 30X
6. 4°C hold

Cleaning Sequencing Reactions with Sephadex (slightly modified from Lisa Karst)

Sephadex slurry consists of 10g G-50 Sephadex + 150mL ddH₂O, hydrated in fridge overnight

1. Make sure PCR product is at least 20uL (add water to make up the volume)
2. Place Centri-Sep columns into collection tubes (i.e. 1.5mL microcentrifuge tubes)
3. Pipet 650uL of the sephadex into a Centri-Sep column.
4. Place column and collection tube in centrifuge, making sure orientation is marked on the column (black dot facing up)
5. Spin in centrifuge for 2min at 750xg (to show 'g' or rcf on our centrifuge, push both up and down speed arrows at once)
6. Place the column in a new, labeled microcentrifuge tube
7. Add your sample to the *center* of the sephadex column, being careful not to touch the column
8. Spin for 2min at 750xg
9. Dry in Speed Vac if available – otherwise, ethanol ppt:
 - a. Add 3x volume 95% EtOH
 - b. Vortex and incubate 15-30min in -20°C freezer
 - c. Spin for 10min at max speed
 - d. Pipette off EtOH
 - e. Let dry *completely* (can be stored in -20 at this point if desired)
10. Clean out columns and save for next time