

## **DNA Cycle Sequencing Protocol**

### **For use with BIG DYE Terminators + Amplitaq FS**

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This protocol is for BIG DYE chemistry. These terminators work well for vector/based clones. PCR amplified product may not be as effective. This chemistry gives more even peaks, and longer unambiguous bases. You can obtain the kits from the UWBC ABI Freezer.

- 1.) For cycle sequencing template quality and concentration is extremely important!
- 2.) For plasmid DNA the Qiagen mini-kits work well, (for larger amounts of template the midi/maxi columns will also work well). Follow the protocol according to manufacturers directions, with one exception, resuspend DNA in H<sub>2</sub>O.
- 3.) For PCR amplified product, the DNA must be free of MgCl +2 ions, so post-PCR clean-up is recommended. The Qiagen QiaQuick PCR purification columns work well. Again follow manufactures directions, but use water as resuspension agent.
- 4.) Quantify DNA on spec to determine exact concentration. You may wish to double check  
this on a mini-gel with appropriate standards.

Note... Before beginning to sequencing one should aliquot out the sequencing pre-mix into pre-defined volumes (enough for 4 rxns + error). The pre-mix is light sensitive and must be kept frozen. Set-up rxns as this 1 clone x 2 directions x 2 reps = 4 rxns. Use one tube of premix per clone and dispose of tube after use!

- 5.) Set-up the PCR reaction in the laminar flow hood, to prevent contamination of reactions. Use the strip tubes and caps (This eliminates the use of Mineral oil overlay). Add the following in the exact order, on ICE. (Turn on thermocycler now, in order to warm-up)
  - 1.) X ul of H<sub>2</sub>O (not to exceed 10ul)
  - 2.) X ul of DNA (250-500ng for ds Plasmid) or (20-50ng of PCR product)
  - 3.) X ul of Primer (3.0-5.0pmol) for pUC primers I used 3.5ul of primer
  - 4.) 4ul of Sequencing 2.5X Buffer
  - 5.) 4ul of BIG DYE pre-mix  
Ex of reactions that worked for Jason  
7.0ul of H<sub>2</sub>O  
2ul of DNA (400ng of Plasmid which had been linearized) (more DNA = better reads)  
3.0ul of Primer (5.5 pmol)  
8ul of Pre-mix + Buffer (if using BIG-DYE use 4ul of pre-mix and 4ul of 2.5X Buffer)
  - 6.) Mix well with Pipetting, be careful not to incorporate bubbles.
- 6.) Seal with strip caps, be sure to obtain good seal otherwise reaction will evaporate in thermocycler
- 7.) Place reaction plate in thermocycler seal lid and begin cycling.

To obtain good unambiguous sequences I use the following method

96 C HOLD for 3 minutes (to denature)

25 cycles of

96 C for 20 sec

50 C for 15 sec (17 bp primer)

60 C for 5 minutes (yes, this is long, but it improves peaks beyond 400 bp)

4 C HOLD for infinity (I would take it out 5 minutes after it reaches 4C).

I prefer to run the reaction in a room without the lights on (don't ask why... just know that it works)

- 8.) Before reaction is complete set up 1.5ml microfuge tubes with 50ul of 95% EtOH and 2.2ul of 3M NaOAc (pH 4.6), label appropriately, mix and place at -20 for 15 minutes
- 9.) Remove caps from tubes, avoid jerking the reaction plate.
- 10.) Place entire 20ul reaction in bottom of 1.5ul tubes containing the EtOH (at this point you should see precipitate). Using same tip remove 20ul from the surface of the EtOH, and place back into reaction tube, mix and recover, dispense back into 1.5ul tube, and mix via pipetting.
- 11.) Place tubes at room temperature for 25 minutes (now go and turn on condenser and heater for speedvac in Spooner's lab (room 278) then place in microcentrifuge (with tabs all up in same direction) and spin at 14,000rpm for 20minutes
- 12.) Carefully remove tubes from microfuge as not to disrupt the pellet. Remove EtOH with a pipette (set to 85ul). Slowly remove liquid, a small pellet should be visible. Gently tap on lab bench and place upside down with cap open. Proceed with remaining tubes in same manner.
- 13.) Add 250ul of 70% EtOH to microfuge tubes containing pellet. Slowly dribble down side of tube opposite of pellet. Let sit on bench at room temperature for 5 minutes.
- 14.) Spin at 14,000rpm for 15 minutes
- 15.) Remove tubes, even more carefully than the first time. Using a P200 set to 150ul remove EtOH with two motions. Again a very tiny clear pellet should be stuck to the side of the tube. Aspirate all possible liquid. With caps open gently tap on lab bench. Remove any remaining EtOH from side of tube with sterilized Q-tips (this eliminates many 'Dye blobs' from data set.) Allow to dry briefly on lab bench.
- 16.) Carry tubes down to speedvac. Place tubes into rotor, close lid. Turn off the heater, turn on the rotor, turn on the vacuum, allow pump to begin, then close stopcock. Start timing the spin. Let go no longer than 3-4 minutes.
- 17.) Open stopcock and allow vacuum to break before shutting off pump. Once pump is off, turn off rotor and open lid. Carefully examine tubes... No liquid should be visible and a

very small dot of clear stuff maybe present. Close tube lid, and hold up to light. If tube is dry, you are done :)

- 18.) If it is Monday→Friday 10a.m. to 4p.m. walk (be sure not to expose them to sunlight) your samples over to biotech center, second floor north hallway room 2305. Place your samples in the freezer. Fill out appropriate form. (Biotech P.O.# 5533560) Samples should be done and on UWBC server within 3 working days. I would recommend not taking samples over any later than Thursday morning. They will sit in their freezer over the weekend.
- 19.) If it is the weekend, place tubes in -20 until Monday morning.
- 20.) Make sure tubes are CLEARLY labeled, with VERY SIMPLE letters and numbers. I write on both the top and sides of tubes. They load the tubes according to PI and sample I.D. So I label the tops of the tubes with a 3 or 4 number (4 is MAX) system

Clone ID (first initial)

Primer ID (F for forward, R for reverse)

Rep ID (A or B = 1<sup>st</sup> or 2<sup>nd</sup> respectively)

Another Identifier if any of the above three are the same letter (ex. AF1 AF2X) that way you can ID them easily.

You will also rename these when you get the computer files back so it would be useful to write this in your lab manual so you have a record of it.

- 21.) To retrieve sequences log onto the UWBC server via the Appletalk Network
  - 1.) Start up the Power Mac
  - 2.) Under the apple menu item select apple talk, choose Ethernet close window and save settings.
  - 3.) Open the chooser under the apple menu, click on Appleshare, and select the zone 'biotech'
  - 4.) Click on the 'the new UWBC DNA server' and click ok.
  - 5.) Click Ok again, and log in as  
User: Havey  
Pass: havey
  - 6.) The icon for the UWBC server will appear on the desktop. Click on it to open the directory. Select users, and open the folder named Havey.  
You can create your own directory under the P.I.'s folder. Open that folder and copy your sequences to the desktop.
  - 7.) The files come named as you named them on the form/tubes. Rename the files so they are clearly understandable.
  - 8.) Move these files to your folder on the Mac, it is recommended that you create a new folder for each date you run sequences.
  - 9.) Open the files with Sequencer 3.0