ABI Prism 310 Cycle Sequencing Protocol – Economy Version

Leslie Vosshall 8/24/2001

Revised Richard Benton/Thomas Paschke 10/10/2003

This protocol uses much less Big-Dye Ready Reaction Mix, both by halving the volume of the PCR reaction (from 20 ul to 10 ul), and reducing the quantity of Big-Dye (from 40% of volume to 10% volume i.e. 1 ul in a 10 ul reaction). To maintain correct buffer composition, you have to add 3 ul of 'Sequencing Buffer' (200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$), to make up for the reduction in volume of Big-Dye Mix used. Aliquots of this buffer are stored in 1.5 ml Eppendorf Tubes in the same box as the Big-Dye.

In our hands, this new mix gives very good quality sequence for plasmid DNA prepared using the Qiagen Miniprep kit. To sequence PCR products, it may be necessary to increase the volume of Big-Dye, but see Richard for advice.

SEQUENCING REACTION

1. Remove Big Dye Ready Reaction Mix from Freezer and place in wet ice bucket. Thaw on ice! Do not thaw at room temperature! Once thawed, flick tub to mix contents well. Place back on ice.

2. Mix together in a 0.2 ml PCR tube (strip or individual tube) using barrier tips and wearing gloves:

DNA 0.5 ug (typically ~1 ul of a miniprep DNA sample)
Primer 15 ng (use 1.5 ul of 10 ng/ul stock)
dH_2O to 6 ul final volume

3. To this, add 1 ul of thawed Big Dye Ready Reaction Mix, and 3 ul of Sequencing Buffer (200mM Tris-HCl pH 9.0, 5 mM $MgCl_2$).

RETURN BIG DYE TO FREEZER AS SOON AS YOU FINISH USING IT; THE REAGENT COSTS $675!

4. Mix well by flicking tube.

5. Run PCR reaction:

1] 96°C 10 seconds
2] 50°C 5 seconds

3] 60°C 4 minutes
4] Return to 1, 24 times
5] 4°C 10 minutes
6] End
(Samples can be frozen at this point for several days)

COLUMN PURIFICATION

1. For each reaction, prepare one MicroSpin G50 Column. Invert column and vortex to mix resin.

2. Snap off the bottom of column, loosen lid 1/4 turn, and place column in a microcentrifuge tube.

3. Centrifuge 3200 rpm for 1 minute. Discard lid of column.

4. Discard flowthrough and return column to same tube. Apply 500 ul ultrapure water to column.

5. Centrifuge 3200 rpm for 1 minute.

6. Discard flowthrough and return column to same tube.

7. Centrifuge 3200 rpm for 1 minute.

8. Transfer column to a clean microcentrifuge tube, labeled with the name of the sample.

9. Carefully pipet the entire PCR reaction (20 ul) into the center of the column.

10. Centrifuge 3200 rpm for 1 minutes.

11. Purified DNA will elute into tube. Discard the column.

12. Place all samples into the SpeedVac to dry samples. Make sure that all residual liquid has dried, but do not over dry sample (20-30 minutes should suffice). A single drop of aqueous liquid will prevent your reaction from running properly in the capillary gel!
(Samples can be frozen at this point for several days)

SAMPLE LOADING PREPARATION

1. Ensure that samples are completely dry! Sequence will not work if there is any trace of liquid in sample!

2. Add 20 ul of TSR (Template Suppression Reagent) to sample. Mix thoroughly on a vortex mixer.

3. Denature for 2 minutes at 95^{o}C.

4. Chill samples on ice. Vortex to mix. Spin at 13,000 rpm for 30 seconds to collect volume at bottom of tube.

5. Transfer entire volume of sample to sample tube. Make sure you do not introduce any air bubbles.

6. Cap sample tube with grey rubber gasket and make sure it is evenly sealed. Place sample on ice or in the refrigerator until you load it into the ABI Prism 310 Instrument. Load into machine within several hours of this step.

TO RUN SAMPLES IN INSTRUMENT

1. Activate the ABIPrism 310 Collection program, if it is not already running. Check status of machine. Go to ABI Prism Collection program and view the STATUS window. It should say "IDLE." If it says "RUNNING," this indicates that the machine is in use. You can estimate the remaining run time of current samples: each sample takes 40 minutes.

2. If machine is "IDLE":
Create a SAMPLE SHEET. Choose NEW from the FILE menu. Click:
Sequence Sample Sheet 48 Tube
In the Sample Name column, type sample name according to this convention:
name of plasmid/name of oligo
Do not give your samples vague names like 1, 2, 3, 4, etc…!
Don't change DYE SET/PRIMER or MATRIX columns.
When finished, close the window. You will be prompted to save the Sample Sheet. Click Save (the computer will give the Sample Sheet a name, according to that day's date and time).

3. Create an INJECTION SHEET. Choose NEW from the File menu. Click:
Sequence Injection List 48 Tube
Click the arrow in the Sample Sheet field and choose the Sample Sheet you just created. The Injection List is automatically filled in. Make sure that the run time is set to 36 minutes. If it is set to 120 minutes, you have to change the settings from Extended Run to Quick Run (see Leslie).

4. Open the door of the instrument, push the tray button, and insert your samples. Start in the far right corner and move forward, according to the order:
A1, A3, A4, A7, A9…
3
B2, B4, B6, B8, B10…
Etc.

5. Make sure the buffer levels are OK. Reservoir at left of capillary block is filled with 1X POP6 buffer (dilute 10X buffer **with EDTA** from ABI with distilled water). Remove reservoir by twisting gently and pulling down, then rinse with distilled water, wipe with Kim Wipe and fill with 1X POP6 buffer to the fill line – not above!).
At the front of the sample tray are three other buffer vials:
The left one is filled with 1X POP6 buffer. Glass vial with white plastic stopper and grey rubber gasket. Fill to fill line.
The middle one is filled with ultrapure water. Glass vial with white plastic stopper and grey rubber gasket. Fill to fill line.
The right one is filled with ultrapure water. Microcentrifuge tube with lid removed. Fill nearly to top with ultrapure water.
Push the Tray button again to return capillary and tray to normal running position.

6. Close the door of the instrument and hit the RUN button. Machine will warm laser for 30 minutes, then samples will begin to be processed at a rate of 40 minutes per sample.

7. At end of sequencing run, STATUS window will indicate “IDLE.” Insert a 100Mb ZIP disk into the ZIP drive and copy the appropriate RUN folder to the ZIP disk by dragging. Your run folder will be in the “RUNS” folder on the hard drive. It will be named according to the date/time of the start of the run. Drag the icon for the ZIP disk into the trash to eject disk (don’t simply push the eject button).

8. Transfer data to our Macintosh and view ABI electropherogram files with Factura or .seq files with SimpleText. If you need to assemble multiple overlapping sequences from the same plasmid, use Autoassembler.