

## Sequencing with the ABI Prism 377

### I. Sequencing PCR Products:

Perform PCR reaction to amplify region-of-interest. Pass 10  $\mu\text{l}$  of products over Sephacryl-300 columns (prepared in Millipore plates) to remove primers and salts. Place sample onto center of column – otherwise it may slip through cracks at edge of column and primers won't be removed. Avoid adding mineral oil to column, as this will clog it. (Mineral oil on the tip of the pipette can be wiped off on a KimWipe.) Run 2  $\mu\text{l}$  on agarose gel to estimate [DNA]. Add 50-100 ng of DNA template (which can typically be obtained from 2-4  $\mu\text{l}$  of a good PCR reaction) to the Big Dye Sequencing reaction described below:

### II. Sequencing plasmid DNA:

Add 0.5-1  $\mu\text{g}$  plasmid DNA to the Big Dye Sequencing reaction described below:

### III. Preparation of Dye-labeled Samples:

Add DNA template to the following reaction:

#### 1x Big Dye Sequencing Reaction:

1  $\mu\text{l}$  Big Dye Reagent  
0.5  $\mu\text{l}$  20  $\mu\text{M}$  primer  
0.5  $\mu\text{l}$  DMSO (optional – for CG-rich sequences)  
1.5  $\mu\text{l}$  5x Buffer (400 mM Tris HCl or Tris Acetate, pH9 and  
10 mM  $\text{MgCl}_2$ )  
2-4  $\mu\text{l}$  PCR template (50-100 ng)  
 $\mu\text{l}$   $\text{dH}_2\text{O}$

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final volume = 10  $\mu\text{l}$

Run samples on Tetrad using the cycling program BDSeq (in Standard folder. This is one of the recommended cycle sequencing

programs from Abi.)

#### **IV. Purification of Dye-labeled Samples:**

Remove unincorporated nucleotides by passing over a Sephadex G50 column. (Again, avoid getting mineral oil on column...) Optional: add 1  $\mu$ l loading dye to samples prior to passing over Sephadex G50 column. This may allow you to better distinguish between your sample and the mineral oil. Evaporate samples by heating on PCR machine at a constant 80 °C, with lid left open. Sample should be dry in about 20 minutes.

#### **V. Pouring Gel on Otter:**

Wash glass plates with Micro60 liquid detergent and scrubber. Rinse with hot water and dry with squeegee. Plates are oriented as follows: outsides have writing, and notched edges belong at base of gel. Place larger plate on end of otter and lay spacers along edges. Spacers will remain in position with a few drops of water added to their underside. Add smaller glass plate to other end of otter, such that the two plates overlap by about an inch.

Prepare the gel matrix:

30 mls Urea/Sequagel/TBE Mix (recipe follows in section XI)  
21  $\mu$ l TEMED  
150  $\mu$ l 10% APS

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final volume = 30 mls

Mix and pour into region of overlap between two glass plates. As this region fills with gel matrix, slowly begin to slide the upper glass plate over the lower one, while continuing to pour the gel. If bubbles develop at far end of glass plate, wait a moment before proceeding. After plates are flush and filled with gel matrix, insert the flat side of a comb (either 36, 48, or 64 wells) until it is flush with the plates. (Be careful not to introduce bubbles into the region around the comb.) Fasten clips around the edges of the plate. Finally, add two clamps to the top end of the plates, being careful not to damage the comb in the process. The gel should polymerize in approximately 20 minutes.

## **VI. Creating Files on the ABI Prism 377:**

Restart computer before each run (to avoid bugs which cause it to crash during the run). Also restart ABI 377. Open *ABI Prism 377XL Collection* software. Open a new file, of type "Sequence Run". Set the appropriate parameters in the menu (3.5 hours is standard run time, and enter # of samples). Now either enter a new "Sequence Sample" program, or use a pre-existing template (such as "Lahn Lab"). This feature assigns a numerical value or a title to each sample.

## **VII. Assembly of Gel:**

Remove comb from gel, and rinse gel in sink using hot water. Be careful to remove all stray gel pieces from region where comb will be replaced (run your nail along this region, while rinsing with running water). If there are still stray gel pieces in the region where the comb will be re-inserted, fish them out with the teeth of the comb. Dry plates with squeegee. If there are still non-water-soluble smears on the plates, try removing them with 70% EtOH. Replace comb, this time with teeth facing into gel matrix. The teeth should only slightly penetrate the matrix by about 0.5-1mm (so that flat top edge of gel is maintained).

Place gel in frame, and fasten in place with knobs. Now do a quick test with the laser to see if your plates are sufficiently clean before proceeding: Mount gel on Abi377 and fasten in place. Hit "Plate Check" on the menu. If the resulting colored lines are relatively flat, gel is clean enough to proceed. If there are large peaks of color, dismount gel and clean the plates again.

Mount upper buffer chamber, which should hang from the top of the gel plates, and be fastened in place with the knobs. Lower buffer chamber should also be in position. Fasten all knobs. Add 0.5X TBE to upper buffer chamber. If there are no leaks, then add buffer to lower chamber, too. Loosen middle knobs and add jacket. Connect the two hoses which circulate water through the jacket. Also connect the three power cords (to the jacket and to the gel).

## **VIII. Pre-running Abi377:**

Use a syringe to expunge urea from the sample wells (urea is dense and if present, it will prevent your sample from entering wells). Hit "Pre-run" on menu, and then open "windows" and check "status". Gel should be pre-run for 45 minutes, or until temperature of gel reaches at least 40-45 °C.

### **IX. Running Abi377:**

Add 1 µl loading dye to each sample. (Loading dye is 5:1 formamide:TE containing Dextran Blue.) Centrifuge briefly, then heat samples to 94 °C for 1 minute (use PCR machine) to reconstitute and denature them. Cancel "pre-run" on menu and use syringe to expel urea from sample wells. Using a 2.5 µl pipette, draw up 0.8 µl sample (for the 64-well comb), and carefully add to well. Try to avoid any spillover into neighboring lanes. Optional: to eliminate bleeding into empty lanes at edges of gel, fill empty lanes with loading dye. Now "Run" gel. Samples can be visualized while they run by pulling up the "Gel Image Window".

### **X. Cleanup:**

If you are around when your sequencing run ends, clean up by removing the gel and the buffer from the machine. If you are not there when your run finishes, clean the plates immediately upon your return, so that they are available for subsequent users.

### **XI. Reagents:**

Urea/Sequagel/TBE Mix:

200g urea  
88.9 ml Sequagel concentrate (25% acrylamide; 19:1  
acrylamide:bis)  
ml dH<sub>2</sub>O

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Final volume = 450 mls

Add 5 g Amberlite resin and stir for 30 mins. Filter, then add 50 mls 10 X TBE. Store at 4 °C in aluminum foil.

