SOP: ABI 377 Automated Sequencing

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AREA OF EXCELLENCE M E R I T

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Part I: Cycle sequencing using the Rdhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, PE)

1. Add the following components to a 0.2 ml PCR tube:

DNA template	8 μl (200-500 ng)
Primer (2 μM)	4 μl
Reaction mix	8 μl
	Total: 20 μl

Note: To get reasonably strong signals for plasmid DNA, double or triple the amount of template (i.e. about 1 μg).

Resuspend and place on ice.

2. Input the following cycle profile to a PCR machine:

1 cycle:
96°C 1 min

25 cycles:
96°C 20 sec
50°C 7 sec
60°C 4 min

Start the program and place all reaction tubes to the PCR block when it reaches 96°C.

Note: Annealing temperature can be adjusted in regard to the length of the primer used. For example, to perform direct sequencing of purified PCR product using a 20-23 mer gene-specific primer, the annealing temperature can be raised to 55-60°C for enhancing specificity.

3. When the program has finished, place the tubes immediately on ice. Transfer all of the reaction (20 μl) to a 1.5 ml microcentrifuge tube. Add 2 μl NaOAc (3M, pH 4.6) and 50 μl absolute ethanol.

4. Leave on ice (or -20°C) for at least 15 min.

5. Centrifuge at 14,000 rpm for 15 min at 4°C.

6. Carefully remove the supernatant and add 100 μl chilled (-20°C) 70% ethanol.

7. Centrifuge at 14,000 rpm for 5 min at 4°C.

8. Carefully remove the supernatant and air-dry the DNA pellet for 5-10 min.

9. Thoroughly resuspend the DNA pellet in 2 μl loading dye [5 vol. deionized formamide + 1 vol. 25 mM EDTA (pH 8.0) with blue dextran (50 mg/ml)].

10. Denature the samples at 100°C for 2 min. Quench on ice immediately before loading.

11. Samples can be also stored at -20°C.

Part II: The Automated DNA Sequencer

A. Gel mix preparation

1. Add the following reagents to a 50ml centrifuge tube:

Urea	18 g
40% acrylamide*	6.25 ml
10X TBE	5 ml
Milli-Q H_2O	make to 50 ml

We use RapidGel-XL-40% Concentrate from USB.

2. Allow the gel mix to fall to room temp.

3. Add 0.05 g of APS to a 1.5 ml tube and add 500μL Milli-Q H_2O to dissolve. Note: APS must be freshly prepared.

10X TBE Preparation

Tris Base	108 g
Boric Acid	55 g
$Na_2EDTA2H_2O$	7.44 g
Milli-Q H_2O	make to 1 L

Filter through a 0.45 μm filter. Note: Do not use if precipitate forms.

B. Gel pouring

1. Put the cassette on two metal bars. Use kimwipe to clean the glass plates with methanol. Put the rear plate on the cassette and put the spacers on the margin of the plates before cover it with the front plate (always use upper side to face the gel).

2. Adjust the glass plates to make sure they are parallel to each other and lock on the fixing clamps except those pairs at the top and bottom of the cassette. Slide the back support at the upper end of the cassette.

3. Attach the bottom fixture to the lower end of the plates, turn the side knobs to bring the rubber gasket into contact with the bottom of the plates and fix it with the clamps.

4. Filter the gel mix with a 0.45 μm filter into a clean beaker. Add 250 μl of APS and 35 μl of TEMED into the gel. Mix thoroughly.

5. Use another syringe to draw in all the gel mix and screw it into the hole of the clamp (make sure no air bubbles are in the syringe).

6. Slowly inject the gel mix into the space between the plates and tab the glass plates by a hand to prevent the formation of bubbles until the gel is poured out of the upper end of the plates.

7. Insert the flat edge of the comb into the gel. Put on the top fixture and hold it with the clamps. Wait for about 2 hours for it to set.

8. Remove the syringe and the fixture, wash them with tap water and rinse with Milli-Q H_2O.

C. Gel running

1. Remove the holder and comb, clean the well (created by the comb) with Milli-Q H_2O and make sure there is no gel residue at the wells. (which may hinder sample loading). Insert the comb into the well until the teeth are a bit inside the gel.

2. Put the upper buffer chamber on the plates and lock it with clamps.

4. Clean the inside of the laser beam safety stop and the scanning region with kimwipe and methanol. Lock the beam stop.

5. Put the lower buffer chamber in the sequencer and put the gel in with knobs on. Connect both cables to the sequencer.

6. Turn on the sequencer and restart the computer (always turn the sequencer on first).

7. Open *ABI 377 Data Collection Software* on the Desktop, choose *New* at the File on menu bar, click *Sequence Sample* to open the sample sheet. Fill in the sample names, choose dRhodamine or BigDye for DyeSet and BD and dRhodamine Matrix for Matrix (choose appropriately otherwise the results will be completely different). Save and close the sample sheet.

8. Click *New* again at File menu. Click *Sequence Run* to open the run window. Choose:

48E-18	for Run Module
E-18	for Plate Check Module
Sharp comb 377-18 well sharks tooth	for Comb
Big Dye and dRodamine matrix	for Gel Matrix
10 hours	for Collect Time
48cm	for Well-to-Read Distance
Date and time you filled the sheet	for Sample Sheet

9. Click *Plate Check* to check the cleanliness of the plates. Make sure there are 4 colored flat lines appear on the scan window (if there are peaks at the scanning region (middle of the gel), click *Pause*, *Cancel*, remove the gel out and clean that region again).

10. Click *Freeze Update* at the top left corner of the scan window. Click *Pause*, *Cancel* to stop the Plate Check (always click *Pause*, then *Cancel* to stop an action).

11. Fill the upper chamber and lower chamber (buffer must cover the plates) with 1XTBE.

12. Click *Pre-Run*. Click *Window* Menu and choose *Status* to open the status window. Make sure the 4 green lights are on and wait until the temperature is 51°C (it takes about 25 min).

13. Click *Pause*. Use a syringe to wash away the urea in the wells. Load the first set of denatured samples in alternate lanes and click *Resume* to run for about 3 min. Click *Pause* and load the second set of samples, click *Resume* to pre-run for another 3 min. Click *Pause* and *Cancel* to stop the pre-run. Wait for 10s (remember not to click *Run* immediately).

13. Click *Run*. Open the *Gel Image* from Window Menu. Ensure the machine is scanning the gel (black image will appear at the top of the window and running downward). If not, cancel and run it again.

14. Choose *Finder* at the right top corner of the Desktop. Click *Sleep* at Window from the menu bar (the screen went blank). Collect data the following day (10 hr).

15. Sign the Log Book.

D. Data collection

1. Turn on the screen by pressing the button at the top right corner of the keyboard.

2. Essentially the computer will automatically extract the data if the signals are strong enough.

3. Otherwise, choose *Adjust Gel Contrast* from the Gel menu. Drag down the top 4 triangles to a desire level (the lower position, the greater contrast). Click *Apply*.

4. Choose *Mark All Lanes For Extraction* from Gel Menu. Move the diamonds to their corresponding lanes. Track the lanes according to the signal intensity by using the 'scissors' (or select the diamonds and choose *Mark Lane For Extraction* if want to track certain lanes; White Diamond: Selected Lane, Blue Diamond: Unselected Lane).

5. Choose *Extract Lanes* from Gel menu. Click *OK* to retrieve the data.

6. The sample data will be extracted in the Sample Manager. Click on the sample name to see and edit the results (in form of electropherogram or text).

7. All data will be saved in the Run Folder specified with date in Run Alias on desktop (the most recent data will be found at the bottom).

8. Save the data by dragging the files into the disk (only the text files can be opened in PC).

E. Cleaning of glass plates

1. Turn the sequencer off. Remove the gel out. Insert spectular between the glass plates at the bottom left corner and twist it slowly to separate them. Remove the gel by covering it with towel paper.

2. Clean the plates and accessories with tap water (Don't use any detergent) and then rinse with Milli-Q H_2O.