Dideoxynucleotide Sequencing

Protocol courtesy of Jeff Habig and Kristen Ostrow of the Loeb Lab

Preparation of ddNTP/dNTP Mixes for Vent Exo- Sequencing

Concentrations in μM, each solution prepared in 1x ThermoPol Buffer

	Adenine Mix	**Cytosine Mix**	**Guanine Mix**	**Thymine Mix**
ddATP	900	-	-	-
ddCTP	-	480	-	-
ddGTP	-	-	400	-
ddTTP	-	-	-	720
dATP	15	30	30	30
dCTP	100	19	100	100
dGTP	100	100	19	100
dTTP	100	100	100	17

Preparation of Sequencing Dye

1ml deionized formamide
20ul 0.5M EDTA pH 8.0
10mg Xylene Cyanol FF ("ΞΨ")
10mg Bromophenol Blue ("BΦB")

Sequencing Protocol:

1. To pre-labeled tubes, add 3ul of one of the ddNTP/dNTP mixes A, C, G, or T.
2. Prepare a Master Mix of all components besides the NTP's as follows:
   - 2.5ul 20ng/ul template DNA
   - 1.5ul 10x ThermoPol Buffer
   - 10ul 0.1 pmol/ul ^{32}P-end labeled primer
   - 1ul Vent Exo- (2 U/ul)
3. Add 3.2ul of the Master Mix to each of the ddNTP/dNTP containing tubes A, C, G, and T. Mix gently.
4. Overlay the reaction with 15ul mineral oil due to the small reaction volume (e.g. 6.2ul).
5. Run a PCR Reaction as follows:
   - 10-20x [96^{o}C, 1min; Tm primer – 5^{o}C, 1min; 72^{o}C 1min]
   - Hold 4^{o}C
6. Separate sample from the mineral oil. Add 2-4ul of Sequencing Dye to each sample. Can store the sample at –20^{o}C until ready to run the gel.
7. Just before the sample is ready to be run on the gel, bring to RT, then heat at 80^{o}C for 15min to evaporate the aqueous fraction of the sample, leaving the volume added as the Sequencing Dye (e.g. 2-4ul) and heat denaturing the DNA strands.

Preparation of Denaturing 6% PAGE

1. Combine the following in a 150ml beaker:
   23g Urea
   20ml dH_2O
   5ml 10x TBE
2. Stir until urea has dissolved completely. This reaction is endothermic, but only stir, do not heat.
3. Prepare the glass plates
   a. Wash the plates, spacers and the comb with RODI-RDD (Reverse Osmosis De-Ionized Water with Regular Dish Detergent). Do not use sponges, nor paper towels, but rather rub the plates with your hand when wearing latex gloves.
   b. Rinse the plates, spacers and the comb with dH_2O. Change gloves and wash with water again to remove all traces of detergent.
   c. Rinse the plates, spacers and the comb with 70% EtOH, allow to air dry.
   d. Place the dry spacers on the dry long plate, 'bunny ears' up. Place the dry short plate on top of the spacers. Bring the plates/spacers to a vertical position and make sure that the bottom edge of the plates and the spacers are flush, as well as both sides.
   e. Attach two binder clips to one side of the plates. Apply tape to the non-clipped side, smoothing it. Remove clips and tape the other side. Apply two clips to both sides. Tape the bottom of the plates twice. Finally, add the signature "Dan Loeb" pieces of tape on the sides of the plates at the bottom corners to minimize leaking.
4. Add 450ul 10% APS, 7.5ml 40% 19:1 acrylamide, 63ul TEMED to the PAGE solution. Swirl contents of beaker and draw up most of the PAGE into the 60cc syringe without a needle.
   a. Pouring Trick #1: Lift the top end of the glass plates with one hand and place the tip of the syringe at the top edge of the long plate. Use the outside portion of the 'screw-on' region of the syringe normally used to attach the needle as a guide by pushing it against the top side of the long plate.
   b. Pouring Trick #2: Once you begin to push PAGE from the syringe, CONTINUE to do this without stopping nor slowing down too much in order to keep a steady flow and prevent air bubbles from forming.
   c. Pouring Trick #3: If you need to stop the flow of PAGE for any duration (i.e. to raise the plates to vertical to remove large bubbles), tilt the plates to one side such that the PAGE reaches the top corner of the gel and resume pouring without stopping until it is full.
   d. Pouring Trick #4: Insert the comb so that the top of the tooth lines up with the top edge of the shorter plate (to avoid setting the wells too deep) and layer more PAGE on top of the comb and on the sides of the comb to replace PAGE when it drips out of the bottom
5. Allow the gel to dry in a horizontal position completely (15-30min). Then cut away the bottom portion of the tape on the sides and remove the tape below this point and on the bottom of the plates.

6. Assemble the gel electrophoresis apparatus (adjustable height with 3 white pillar supports works great) and clip in the plates against the gasket. Clip on an aluminum plate to the front of the plates to radiate heat and prevent the plates from breaking. IMPORTANT NOTE: do not allow the aluminum plate to touch the bottom well of buffer.
7. Fill the top buffer well with 1x TBE, add enough 1x TBE in the bottom buffer well to cover the bottom edge of the plates.
8. Use a disposable pipette tip to scrape PAGE away from the front of the comb to ease comb removal. Pull up evenly on the comb to avoid disturbing the gel wells.
9. Use a 3cc syringe and 18-gauge needle to wash each well with 1x TBE from the top buffer well in order to flush out liquid PAGE and/or urea. This will improve resolution and help to prevent the smiley face effect.
10. Pre-run the gel at 2000V, 50mA, 50W at RT for at least 1 hour to both equilibrate the gel temperature throughout and remove free radicals that have formed during the polymerization of the gel.
11. Load sample and electrophoresis at the same conditions until the BΦB has migrated through the gel (~1.5-2hrs).
12. Remove aluminum plate and remove glass plates from the gel apparatus. Remove the gel spacers and with a thin metal instrument (e.g. forceps), carefully separate the two plates leaving the gel on one of them.
    OPTIONAL STEP: Soak the gel while it is still adhered to one of the plates in 10% glacial acetic acid to remove the urea from the gel for 10-15min.
13. Place a large enough piece of Whatman paper to cover the entire gel on top of the gel. Invert the gel so that the glass plate is now on top of the gel. Slowly peel the Whatman Paper/Gel away from the glass plate. Cover the gel in Saran Wrap. Trim massive excess Whatman paper and Saran Wrap.
14. Dry the gel in a gel dryer at 80C for 1 hr.
15. Trim excess Whatman paper and Saran Wrap to just outside the bounds of the gel.
16. Expose the dried gel to a storage phosphor screen or to autoradiography film 2hrs-o/n.