

## DIDEOXY SEQUENCING USING $^{35}\text{S}$

### **A. Pouring of gel**

When using  $^{35}\text{S}$  for sequencing, the gel must be dried down. The following procedure involves binding of the sequencing gel to one plate, a technique which allows the gel to be dried directly onto the plate. This increases resolution and also avoids the necessity of transferring the gel to filter paper. The glass plate must fit into an x-ray film cassette.

1. Treat large plate with a solution of 6 ml ethanol, 180  $\mu\text{l}$  10% acetic acid 30  $\mu\text{l}$   $\gamma$ -methacryloxypropyl-trimethoxy silane; apply to plate thoroughly, let stand 5 minutes, remove excess reagent with tissues.
2. Treat small plate with 3 ml of 99% dichloromethyl silane; apply to plate thoroughly, let stand 5 minutes, remove excess reagent with tissues.
3. Assemble gel apparatus and place at a  $15^\circ$  angle to horizontal.
4. Gel solutions can be stored at  $-20^\circ\text{C}$  in 55 ml aliquots and thawed just before use. For five 6% acrylamide/8 M urea gels: 16.5 g acrylamide, 0.825 g bis-acrylamide, 137.5 g urea (Schwarz Mann), 3.3 g Tris base, 1.65 g boric acid, 5.5 ml 100 mM EDTA pH 8.3,  $\text{dH}_2\text{O}$  to 275 ml; dissolve completely, filter through Whatman no. 1 filter, divide into 55 ml aliquots and store at  $-20^\circ\text{C}$ .
5. Thaw 55 ml gel, de-gas under vacuum; add 200  $\mu\text{l}$  of freshly prepared 10% ammonium persulfate and 50  $\mu\text{l}$  TEMED; mix well and pour into 60 cc syringe with stopcock.
6. Fill plate sandwich by continuous flow from syringe barrel, tap interface between gel and air to avoid bubble formation.
7. Place gel onto horizontal, insert comb with 1 cm of teeth into gel and clamp.
8. Allow to polymerize for at least one hour.
9. Remove comb, rinse wells with  $\text{dH}_2\text{O}$  and 1x Tris-borate-EDTA buffer. Pre-run gel for  $>1$  hour at 1500 volts (30-50mA).
10. Gel is run in 1x Tris-borate-EDTA buffer (4 P: 48 g Tris base, 24.8 g boric acid, 80 ml 100 mM EDTA pH 8.3,  $\text{dH}_2\text{O}$  to 4 liter); preheat buffer to  $60^\circ\text{C}$  for upper chamber of "Poker face" apparatus.

### **B. Annealing reaction.**

1. Use 4 ng of primer for each DNA sample; calculate amount of template needed to achieve 1:1 template:primer molar ratio. For example, a clone of 8000 bp total length would require approximately 1.9  $\mu\text{g}$  of template with 4 ng of primer to achieve a 1:1 molar ratio.
2. Add  $\text{H}_2\text{O}$  to DNA to 7.5  $\mu\text{l}$ ; add 1.5  $\mu\text{l}$  annealing buffer (100 mM Tris-Cl pH 8, 50 mM  $\text{MgCl}_2$ ) and 2  $\mu\text{l}$  of 2 ng/ $\mu\text{l}$  solution of primer. Mix well.
3. Place at 75VC in metal block in  $\text{dH}_2\text{O}$  bath; turn off and allow to slowly come to room temperature (2 hours).

### C. Dideoxy synthesis

1. Make ddN + dN mixes as follows:

A': 7  $\mu$ l 0.72 mM dCTP, 7  $\mu$ l 0.72 mM dGTP, 7  $\mu$ l 0.72 mM dTTP, 2.0  $\mu$ l 2 mM ddATP, 68.25  $\mu$ l dH<sub>2</sub>O.

C': 1  $\mu$ l 0.72 mM dCTP, 10  $\mu$ l 0.72 mM dGTP, 10  $\mu$ l 0.72 mM dTTP, 3.5  $\mu$ l 2 mM ddCTP, 65  $\mu$ l dH<sub>2</sub>O.

G': 10  $\mu$ l 0.72 mM dCTP, 1  $\mu$ l 0.72 mM dGTP, 10  $\mu$ l 0.72 mM dTTP, 4.5  $\mu$ l 2 mM ddGTP, 65  $\mu$ l dH<sub>2</sub>O.

T': 10  $\mu$ l 0.72 mM dCTP, 1  $\mu$ l 0.72 mM dGTP, 1  $\mu$ l 0.72 mM dTTP, 11.25  $\mu$ l 2 mM ddTTP, 57.75  $\mu$ l dH<sub>2</sub>O.

Note: These dideoxy nucleotide concentrations were determined empirically and may vary for each set of stock solutions and each DNA sequence, to achieve the optimum balance between chain elongation and chain termination.

2. For each clone, label four 0.5 microfuge tubes: A, C, G, T.
3. Add 1.25  $\mu$ l annealing mix into each of the four tubes.
4. Prepare four reaction mixes, one for A, one for C, etc, in sufficient quantity for the number of clones sequenced. 1x: 1.625  $\mu$ l ddN + dN(A', C', G' or T), 0.125  $\mu$ l 0.2 M DTT, 0.475  $\mu$ l a[<sup>35</sup>S] dATP (New England Nuclear, 500 Ci/mmol), 0.25  $\mu$ l sequencing buffer (100 mM Tris-Cl pH 7.5), 0.1  $\mu$ l Klenow fragment.
5. Add 0.25  $\mu$ l of reaction mix, A, C, G, or T to each reaction tube of the corresponding nucleotide. Spin down to start reaction.
6. Incubate at room temperature for 20 minutes.
7. Add 0.6  $\mu$ l of chase solution (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 2 mM dTTP) to each and incubate at room temperature 15-20 minutes.
8. Stop reactions by adding 3  $\mu$ l stop solution to each (10 mls: 9 ml deionized formamide, 10 mM EDTA pH 8.3, 0.1% bromophenol blue, 0.1% xylene cyanol).
9. If two gel loadings are desired, aliquot portions into microfuge tubes and place at -20°C until used.
10. Heat samples to 95°-100°C for 3 minutes, quickly chill on ice and load quickly onto pre-run gel.
11. Run gel at 1500 volts (30-40mA) for two hours (to read from base 20 to 225) or 6-8 hours (to read from base 200 to 350).
12. After run, quickly disassemble gel and soak gel attached to glass plate in large pan with 5% acetic acid/5% methanol for 15 minutes with gentle agitation.
13. Run gel under H<sub>2</sub>O for 5 minutes, drain; dry gel onto plate with hair dryers.
14. Place on film at -70°C without intensifying screen.

### References:

- Sanger, F., S. Nicklen and A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467.
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