DIDEOXY SEQUENCING OF DOUBLE STRANDED DNA

Alkali denaturation of double stranded DNA:

1. Dilute 2-5 µg DNA with dH_2O to a final volume of 18 µl. For mini prep DNA use 18 µl DNA solution. Add 2 µl denaturing solution. Incubate 5 minutes at room temperature.
   Denaturing solution: 2 N NaOH, 2 mM EDTA

2. Place on ice. Add:
   7 µl dH_2O
   7 µl NaOAc
   75 µl absolute ETOH
   Mix and precipitate in dry ice for 10 minutes.

3. Microcentrifuge for 10 minutes at 13,000 RPM, 4˚C.

4. Remove supernatant. Wash pellet with 1 ml 75% ETOH.

5. Microcentrifuge for 5 minutes at 13,000 RPM, 4˚C.

6. Decant supernatant. Speed-Vac dry. Resuspend DNA in 6 µl dH_2O.

Sequencing Reactions:

(Using United States Biochemical DNA Sequencing Kit with sequenase version 2.0 T7 DNA polymerase.)

1. Annealing mixture:
   6 µl denatured DNA from step 6 above
   2 µl sequencing buffer
   2 µl primer (1.0 pmoles) *(Note: 1 µg of a universal 19-mer primer is approximately equal to 160 pmoles)*
   Anneal by heating for 10 minutes at 65˚C in a heat block. Cool slowly to <35˚C by removing the entire heat block from the heater and placing it at room temperature.

2. Microcentrifuge 3 seconds to spin down condensate. Chill on ice for use in step 6.

3. While cooling, label and fill microtitre plate wells with 2.5 µl of each Termination Mixture. (Use 4 wells for each reaction; each well for 30 seconds immediately before termination reaction.

4. Dilute Labeling Mix 1:5 in dH_2O.

5. Dilute enough Sequenase Version 2.0 for all templates in ice cold Enzyme Dilution buffer 1:8

6. Labeling Reaction:
To annealed DNA mixture (10 µl) add:
1 µl DTT, 0.1M
2 µl Dilute labeling mix
0. 5µl [^{35}S] dATP
2 µl Diluted Sequenase
Mix and incubate at room temperature 2-5 minutes.

7. Termination Reaction:
Transfer 3.5 µl of labeling reaction to each prewarmed termination well (G, A, T and C), mix, and continue incubation of the termination reactions at 37-44˚C for 5 minutes. (44˚C decreases G-C compressions.)

8. Stop the reactions by adding 4 µl stop solution. Place on ice or chill in PCR machine at 4˚C until ready to load on gel.

9. Heat samples to 75˚C for 2 minutes immediately before loading. Place on ice.

Electrophoresis:

1. Immediately after heating load 4 µl onto a 6 to 8%, (depending on desired sequence range) pre-run, urea/acrylamide denaturing gel.

2. Run one comb gels at 35W and two comb rigs at 70W. Gel should not run hotter than 60˚C.

3. After approximately 2.5 hours (the bromophenol blue should have just come off the gel) load again (in new wells) to achieve greater sequence. Run 2.5 hours more for a total of 5 hours. Increase or decrease time according to which part of sequence you wish to read.

Fixation:

1. Pry apart gel plates using a metal spatula.

2. Gently place the glass plate which has the gel stuck to it directly down onto a double thickness of Whatman paper which is soaking in a 5% acetic acid, 5% methanol bath so that the gel sticks to the paper. Remove glass plate.

3. Fix 10 minutes.

4. Remove paper with attached gel and dry 45 minutes at 80˚C.

5. Expose at room temperature, without a layer of plastic wrap.

Note:

1. ^{35}S sequencing reactions can be stored at 4˚C for approximately 1 week without a significant increase in background.

References:

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proceedings of the National Academy of Sciences of the United States* **74** (12): 5463-7.

Tabor, S. and Richardson, C. C. (1987) *Proceedings of the National Academy of Sciences of the United States* **84** (14): 4767-71.

United States Biochemical Protocol for DNA sequencing with Sequenase Version 2.0 T7 DNA polymerase.