

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 μ l) add:
 - 1 μ l DTT, 0.1M
 - 2 μ l Dilute labeling mix
 - 0.5 μ l [35 S] dATP
 - 2 μ l Diluted Sequenase
 Mix and incubate at room temperature 2-5 minutes.
7. Termination Reaction:
Transfer 3.5 μ l 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 μ l 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 μ l 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. ³⁵S 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.