DNA Sequencing Analysis using Microtiter Trays

Experimental considerations

- This protocol has been modified to fit reactions into 96 well plates; this makes the method easier, faster and cheaper.
- Although single-stranded DNA gives the cleanest sequence, we typically use double stranded DNA with good results (the DNA must be denatured, hoever, by boiling in an alkali solution, see below).
- Several commercially available sequencing kits are available and we typically use "Sequenase version 2.0 DNA Sequencing Kit" (United States Biochemical). Sequenase is a modified bacteriophage T7 DNA polymerase (Tabor and Richardson 1987, PNAS 84:4767) that has high processivity and elongation rate with no exonuclease activity.
- For internal sequencing, specific primers must be designed; we routinely design 17-mer oligonucleotides whose 5' end is 50-75 base pairs from the sequence of interest.

Protocol

I. DNA denaturation

- Bring DNA volume up to 20 ul with H_20 [DNA generated by either the boiling miniprep method (10ul), or $CsCl_2$ purified maxiprep DNA (2-10ug) give the best results].
- Add 2 ul 2N NaOH, tap with finger to mix.
- Incubate for 5 minutes at 90°C
- Spin down briefly
- Add: 3 ul 3M Sodium Acetate, pH 5.0
  3 ul primer (10ng/ul, or 6 pmoles)
  7 ul H_2O
- Mix by tapping with finger.
- Add 80 ul Absolute Ethanol.
- Mix by vortexing.
- Spin at high speed in microfuge for 20 minutes.
- Take off (or aspirate) the EtOH solution.
- Wash with 500 ul 70% EtOH (stored at –20°C) [i.e. add 70% EtOH, vortex, spin 5 minutes (high speed in microfuge) and take off EtOH].
- Dry in speedy vac and suspend DNA in 8 ul H_20.

II. Annealing reaction

- Add 2 ul 5x Sequenase buffer (provided with sequencing kit. However, for recipe, see "Solutions"), tap with finger to mix.
- Incubate for 5 minutes at 68°C.
- Transfer tubes to 37°C for 20 minutes.
- Spin tubes briefly.
- Leave samples at room temperature until next step.

III. Sequencing reactions

- Place 2.5 ul "termination mix" (i.e. ddA, ddC, ddG or ddT) per well in separate wells of a 96 well microtiter tray and set tray aside momentarily.
- In a fresh eppy tube, set up "Reaction Mix":

Reagent	x4 (ul)	x6 (ul)	x10 (ul)	x12 (ul)	x16 (ul)
0.1 M DTT	3.7	5.6	9.3	11.2	15.0
Labelling mix	2.3	3.4	5.6	6.8	9.0
^{35}S-dATP	2.3	3.4	5.6	6.8	9.0
Sequenase	1.5	2.2	3.6	4.4	6.0
H_20	14.0	21.0	35.0	42.0	55.0

Note: We never add Sequenase diluent as mentioned in vendor's instructions.

- Add 5.5 ul Reaction Mix to each annealed reaction tube containing the DNA templates and mix by tapping gently with finger.
- Allow reaction to proceed for 5 minutes at room temperature (Elongation reaction), however, while this incubation is proceeding, aliquot 3.0 ul of the reaction (i.e. DNA plus Reaction Mix) to the side of each of the wells (in the microtiter tray) containing ddA, ddC, ddG, and ddT (note: Do not place more than 3.0 ul, otherwise the drop will slip to the bottom of the well and prematurely mix with termination reaction).
- After the 5 minute extension reaction, start termination reaction by spinning microtiter plate briefly (i.e. a few seconds).
- Incubate tray at 37^{o}C for 5 minutes to allow the termination reaction to proceed (we typically place the plate in a water bath, carefully].
- After 5 minutes, aliquot 4.0 ul of "Stop Solution" to the side of each well.
- Spin down briefly (Stop reaction).
- Heat samples at 68^{o}C for 10 minutes.
- Load 3.0 ul onto a Sequencing gel.

Solutions

- Sequenase 2.0 Kit (USB; Kit = cat #70770; Sequenase Enzyme only = cat #70775Y)

- 10mCi/ml α^{35}S-dATP (500 to 1200 Ci/mmol)

5 x Sequenase Buffer
200 mM Tris-HCl, pH 7.5
100 mM $MgCl_2$
250 mM NaCl
25 mM DTT

Stop solution
95% (v/v) deionized formamide (Fluka)
10 mM EDTA
0.1% (w/v) Xylene Cyanol
0.1% (w/v) Bromophenol Blue

- Store indefinitely at –20^{0}C

Sequencing gel (SequaGel kit [National Diagnostic, Inc – cat #EC-833])

50 ml SequaGel	6%	8%
Concentrate	12 ml	16 ml
Diluent	33 ml	29 ml
Buffer	5 ml	5 ml
Total	50ml	50ml