DNA Sequencing With Sequenase

**Alkaline Denaturation of Plasmid dsDNA**
1. Begin with 1-3 µg of plasmid DNA in 1-10 µl of TE. Optimal template is 0.5 pmol (e.g., 1 µg of 3 kb plasmid). Adjust to final volume of 30 µl with ddH₂O. Set up in 0.5 ml tube.

2. Add 10 µl of 1 M NaOH. Vortex. Incubate at 25°C for 5 min. For plasmids > 5 kb, incubate at 37°C.

3. Add 60 µl of 4 M NH₄OAc, pH 5. Vortex. Do not use pH 7 NH₄OAc.

4. Add 250 µl of 100% EtOH. Vortex. Incubate at -70°C for 15 minutes (or more).

5. Spin at top speed for 30 minutes at 4°C. Remove supernatant carefully.

6. Add 150 µl of 70% EtOH. Vortex. Spin at top speed for 5 minutes at 4°C.

7. Dry pellet in Speed-Vac for 2-5 minutes. Cover tubes with parafilm with holes.

**Annealing**
1. Resuspend the DNA in 7 µl of ddH₂O.

2. Add 2 µl of 5X Sequencing Reaction Buffer.

3. Add 1 µl (1.0-2.0 pmol) of primer. Vortex.

4. Heat to 65°C for 2 minutes, slowly cool to ~25°C over ~30 minutes. Spin briefly. Place on ice. Can be stored at 0-4°C overnight.

**Labeling Reaction**
1. Combine on ice: 10 µl of Template DNA
   - 1 µl of 0.1 M DTT
   - 2 µl of diluted Labeling Mix: 1.6 µl ddH₂O
   - 0.4 µl Labeling Mix
   - 0.5 µl of 10 µCi/µl [³⁵S]dATP
   - 2 µl of diluted Sequenase 2.0: 1.75 µl Enzyme Dilution Buffer
   - 0.25 µl Sequenase

   For multiple samples, prepare a “Master Mix” (MM) consisting of all components except DNA. Do this on ice. Add 5.5 µl of the MM to each of the templates. To read close to the primer, add 0.5-1.0 µl Mn++ buffer to the labeling reaction (do only short run on gel).

2. Label at 20°C for 2-5 minutes. Incubate at 10-15°C to read close to primer.

**Termination**
1. For each Labeling Reaction, add 2.5 µl of ddATP, ddCTP, ddGTP, or ddTTP to wells in a microwell culture dish. Order of wells is GATC. Keep dish covered on ice.

2. Prewarm the microwell dish to 42°C. Use an inverted dry block and a wet paper wick.

3. Add 3.5 µl of labeling mix to each well. Mix by pipetting. Incubate at 42°C for 2-5 minutes.

4. Add 4 µl of stop solution to each well. Mix by pipetting. Aliquot stop solution in separate tube.

5. Place microwell dish on ice, and/or store at -20°C.