# Protocol: Double Stranded DNA Sequencing

## I. Preparing Template;

1. Plasmid DNA = 10 μL (3-5 ug)
   (2M) NaOH = 2 μL
   Total vol = 12 μL
2. Incubate at 37 C for 20 min
3. Add 10 μL of (3M) NaAc (pH5.2) + 78 μL of TE
4. Extract with 100 μL of phenol/chloroform mix (pH 7.0. BRL)
5. Extract with 100 μL of chloroform
6. Add 300 ul of ice-cold 100% ethanol. Precipiate at –80 C for 10 min.
7. Spin in microfuge for 30min at 4 C
8. Wash with 100 ul of 70% ethanol. Dry pellet. Resuspend in 6.0 μl of water.

## II. Annealing Reaction:

1. Add 2.0 μl of Sequenase reaction buffer and 10 picomoles (1 μl) of primer to above.
2. Incubate at 70 C for 2 min. Cool slowly to room temp. Place on ice.

## III. Labeling Reaction:

1. Dilute the 5X Labeling mix with water (example: 4 ul of LM + 16 ul of water). Dilute Sequenase enzyme 1:8 with Enzyme Dilution Buffer (example 2.5 l of enzyme + 17.5 ul of enzyme dilution buffer). Leave both on ice. Use immediately.
2. Extension reaction:

| | |
|---|---|
| Template+Primer mix | = 9.0 μl |
| 100 mM DTT | = 1.0 μl |
| Diluted Labeling mix | = 2.0 μl |
| [α-32P] dTTP | = 1.0 μl |
| Diluted Sequenase | = 2.0 μl |
| Total vol | = 15.0 μl |

3. Mix and incubate at room temp for 5 min.

## IV. Termination Reaction:

1. Label four tubes G, A, T, C. Add 2.5 μl of ddGTP, ddATP, ddTTP and ddCTP termination mix to the four tubes respectively. Incubate at 37 C for 2 min.
2. Add to each tube 3.5 μl of the Labeling Reaction mix.
3. Incubate at 37 C for 5 min.
4. Add 4 μl of 2X Sequencing Loading Buffer (2XSLB) to stop the reaction.
5. Store on ice until ready to load on 6% sequencing gel.

6. Heat samples at 90 C/2min. Place on ice. Load immediately 1.5 µl/ lane.

**NOTES:**

To sequence close to the primer dilute the 5X Labeling mix to 0.25X (20-fold) and add 2.0 µl to the Labeling Reaction. Also add 1 µL of supplied Mn Buffer to the Labeling reaction.