Sequencing

I. Denature DNA - for double-stranded rxns.

To each tube add:

2 µl DNA (~1.5 µg/µl)	• 5 min. @ 37° (not more)
2 µl 2M NaOH + trace EDTA	
6 µl dH_2O	

Then add:	• mix EtOH + NaOAc, then add together
60 µl EtOH (~~100~~ 95%)	• vortex immediately + put in dry ice / EtOH bath for 10'
10 µl 1M NaOAc	• spin cold 15'; take off snv
	• 70% EtOH rinse
	• speed-vac dry

II. Annealing primer.

To each tube containing dry pellet, add:

2 µl "Rx buffer"	• add H_2O + buffer first
2 µl primer	• immediately add primer
6 µl H_2O	• 30' @ 37°
	• 10' (up to 4-5 h.) RT
	• if using SS DNA, put instead in 65° bath + cool to RT (~30-45 min.); same ingredients

III. Reactions

Prepare plate:

2.5 µl termination mixes to each well (ddA, C, G, T) - for dGTP or dITP.

Cover + set aside — otherwise will evaporate!

Then add:	• mix DTT, H_2O, label mix (dGTP or dITP), + ^{35}S dATP; put on ice
1.0 µl DTT	• add enzyme separately, to cold dilution buffer, then to rest of stock sol'n. Use ASAP!
1.4 µl H_2O	
0.4 µl label mix	
0.7 µl dATP (^{35}S)	• add 5.5 µl stock to each tube from above, leave in tip
0.25 µl enzyme	
1.75 µl enz. dilution buffer	• add 3.5 µl from each tube into each well on plate

Sequencing, cont.

...
- cover + spin down to mix contents
- 37° for 3-5′
- add stop sol'n, 5.5 μl to each well
- cover + freeze
- before loading, heat at least 2-3′.
- total time from adding stock sol'n (5.5 μl) + putting in 37° bath should be 2-5′.

IV. Gel

5% acrylamide - 1 L. (for long runs)		7% (short + long)	6% (... a compromise ...)
47.5 g	acrylamide	66.5 g	57 g
2.5 g	bis	3.5 g	3 g
0.5 x	TBE	0.5 x	0.5 x
498 g (480 g?)	urea (= 8.3 M)	498 g	498 g

immed. before pouring:

30 μl TEMED
500 μl 10% APS

Polymerize ≥ 1 h.

To pre-run: use 0.5 x TBE (or other buffer); make sure air is out of lower + upper areas between plates. Run at 35-50 mAmps; 1000 to 2000 + V. Should be warm to the touch; pre-run 45 min - 1 h., or until warm + over ~ 1500 V.

Run; short runs ~ 1 h 45 m; long runs ~ 3 h 30 min. Before running, force urea out of top loading area. After loading, rm. bubbles. Take down + fix in 10% EtOH / 10% Glacial acetic acid. Dry, + put on film.