NEB EXTENSION/TERMINATION 35S-dATP SEQUENCING

1. Denature dsDNAonly (use 2.5ug DNA) 4M NaOH 1.0uL 4mM EDTA 1.0uL dH2O 15.5uL DNA 2.5uL 20.0uL
    - Incubate at room temp., 5 min.
    - Ethanol precipitate.
    - Resuspend 6.5uL dH2O
2. Anneal DNA to primer

    Denatured DNA 6.5uL

    -primer 10ug/uL stock 10X Vent buffer 1.5uL

    -dsDNA ~2ug(per reaction) Primer 1.0ul

    -ssDNA~1ug(per reaction)

    -ssDNA: 65 C 5', cool to 42 C ~20' 9.0uL

    Mix.

    Incubate 42 degrees C, 20 minutes.
3. Prepare G A T C tubes; one set for each clone to be sequenced. Add 3uL of each V-1 mix to respectively labelled tubes on ice.
4. Extension reaction:

    Add to annealing reaction:
    - 2.0uL extension mix v (1Xext)
    - 2.0uL 35S-dATP
    - 1.0uL vent (exo-) DNA polymerase (2000u/mL)
    - Mix.
    - Incubae 42 degrees C, 5 minutes.

    Add 3.2uL of extension reaction to each GATC tube, mix. Incubate 72 degrees C, 10 min.

    Spin and quickly add 4 uL of STOP/LOADING solution.
5. Samples may be frozen at -20 degrees C overnight before running on the sequencing gel. Heat samples 2 min. at 72 degrees C just prior to loading. (In other words, the samples as well as the sequencing gel should be hot in order to get good seperation.) Load 2.5 uL of each GATC sample in that order. If you avoid the outermost lanes, you can load 13 loads onto one sequencing gel.

NEB SEQUENCING (VENT POLYMERASE)--ALTERNATE METHOD PREPARING SAMPLES TO BE SEQUENCED

1. Vectors are double stranded and firstly the DNA must be denatured.

   For each tube:

4 M NaOH	1.0 uL
4 mM EDTA	1.0 uL
dH2O	15.5 uL
DNA	2.5 uL
Total = 20 uL	

2. Incubate at room temp. For 5 min.
3. Ethanol precipitate solution to pellet DNA--gets rid of NaOH in solution.

   Using:

   - 4uL of 5 M NH4OAc

   - 2X the volume of EtOH(100%)=48uL EtOH (b/c 20uL solution +4 uL salt (NH4OAc) +24uL) ,

   - then spin for 15'

   - Keep the pellet (hard to see), discard supernatant.
4. Each tube containing pellet was filled with 6.5uL of dH2O to resuspend pellet.
5. Each tube is heated at 95 C for 5' (to denature DNA since NaOH has been removed.
6. After 5' the tubes are placed in an ice bath to "quick-cool" them. (This ensures denatured DNA will not reanneal)
7. Anneal DNA to primer.
   - DNA 6.5uL
   - 10X vent buffer 1.5uL
   - primer(T7 5pmol/uL) 1.0uL
   - Total = 9.0uL
8. Incubate at 42 C for 20'.
9. Extension reaction--add the following to the above;

   Ext. Mix 2.0uL
10. 35S dATP 2.0uL
11. Vent polymerase 1.0uL
12. DNA + primer solution 9.0ul

13. Total=14uL
14. . Incubate at 42 degrees C for 5'.
15. GATC tubes were prepared for each clone -add 3uL of each dNTP solution into its respective GATC tube.
16. Add 2.8uL of extension reaction (14uL) for each clone to its respective GATCset of tubes. The tubes were then mixed by flicking with finger or vortex, and were then incubated at 72 degrees C for 10'. (The reaction proceeds very quicly ~ 30bp/sec)
17. The solution were spun and 4uL of STOP/LOADING solution was added to each solution. The tubes can then be frozen.
18. . The samples were heated before loading at 72 degrees C for 2 min (or 7 min if previously frozen) , so that they are the same temp. as the gel.
19. The combs were inserted into the warming gels.
20. 2.0uL of each clones GATC tubes (whle still in the heat blacK) were quickly added to the wells.