ThermoSequenase DNA Cycle Sequencing Protocol

Adapted from BV's Cookbook by T.-C. He, 10/24/2000

This protocol describes the use of the Amersham DyeTerminator/ThermoSequenase Kit for Cycle sequencing PCR products, plasmids, or BACs. See http://www.apbiotech.com/technical/documentation/dna_sequencing/XY0430003.pdf for more information.

A. Prepare templates:

A-1. Plasmid DNA minipreps:

Plasmid DNA purified 2ml overnight coli culture using alkaline lysis procedure can be routinely used in cycle sequencing reactions. If the final volume of a miniprep is 50-70ul, it usually requires 4ul per primer reaction (or 1ul per A/C/G/T reaction).

A-2. BAC clones:

If BAC DNA is used, it usually requires approximately 0.5-1.0ug per sequencing reaction.

A-3. PCR products:

If you are sequencing a PCR product, you may want to remove excess amplification primers from the PCR reaction. Purify the PCR product by either of the methods listed below:

I) $NaClO_4$ Precipitation:

1). Bring volume of PCR reaction to 600 ul in TE or ddH_2O.
2). Perform PC8 extraction.
3). Transfer supernatant to a new 1.7ml tube, add:

2 ul	glycogen (or 3ul seeDNA)
200 ul	2M $NaClO_4$
400 ul	2-propanol

4). Microfuge 10', RT.
5). Decant supernatant, wash twice with 70% EtOH.
6. Air dry the pellet, and resuspend DNA pellet in appropriate volume of ddH_2O to allow convenient delivery of about 0.5-5ug PCR product per sequencing reaction (usually about 20ul total volume).

II) Gel Purification:

1). Run PCR product on agarose gel of appropriate concentration (0.7-2.0%).
2). Cut out appropriate band.
3). Place gel slice into upper reservoir of Spin-X tube (Costar), or regular spin-column.
4). Microfuge 15', RT.
5). Bring volume of liquid collected in lower reservoir of Spin-X tube to 400ul.
6). Add:

2 ul	3ul see DNA
40 ul	7.5M NaOAc
800 ul	100% EtOH

7). Microfuge 5', RT.
8). Decant supernatant, wash twice with 70% EtOH.
9). Air dry and resuspend DNA pellet in appropriate volume of TE or ddH_2O to allow convenient delivery of about 0.5ug PCR product per sequencing reaction (usually about 20ul total volume).

B. Cycle Sequencing Reactions

1. Set up Reaction Mixture:

2ul	10 X Reaction Buffer
4ul	plasmid DNA, minipreps
	[or ~2 ul PCR product (50-500 ng)]
	[or for BAC DNA, use ~10% of a Nucleobond Midiprep]
1ul	Primer (20-40 ng/ul; or 0.5-2.5 pmol)
12ul	Water (or 20 ul minus the volume of all other components)
1ul	ThermoSequenase (USB, 4U/ul) (in some cases, 2ul can be used)

2. Make 4 master termination mixtures (G, A, T, C) containing 2N ul dGTP termination mix and 0.5N ul of the appropriate [α-33P]ddNTP, where N = the total number of sequencing reactions to be run. (Alternatively, use dITP termination mix instead to dGTP termination mix; dITP is recommended for resolving compressions, while dGTP supposedly yields more even band intensities).

3. Aliquot 2.5ul of each master termination mixture (G, A, T, C) to separate wells of a 96-well PCR plate.

4. To each well, add 4.5 ul of Reaction Mixture. Overlay with 10-20ul mineral oil.

6. Cycle reactions:

dGTP	**Reactions**	**dITP Reactions**	
Stage 1	$94^0C \times 2'$ ×1 cycle	Stage 1	$94^0C \times 2'$ ×1 cycle
Stage 2	$92^0C \times 20''$	Stage 2	$92^0C \times 20''$
	$55^0C \times 30'' \times 25$ cycles		47^0C (orTm-8)× 30'' × 25cycles
	$72^0C \times 1'$		$60^0C \times 4'$

25 cycles has been successful with fairly robust reaction products. More cycles may increase background. Note: **T_m= 4(G+C)+2(A+T).**

7. Add 4 ul **Stop Solution** to each reaction. Check for blue dye in the aqueous layer.

8. Heat to 70°C for 5'. Load 3-4 ul, avoiding oil layer.

NOTE: DMSO can enhance sequencing with high GC templates. The protocol is the same except that the following Reaction Mixture is substituted for that in step 1 above:

2 ul	10 X Reaction Buffer
4 ul	Miniprep plasmid DNA
1 ul	Primer (20-40ng/ul; 0.5-2.5 pmol)
11.5 ul	ddH_2O
0.5 ul	DMSO
1 ul	ThermoSequenase (USB, 4U/ul)