SCHNABLE LAB
Plant Genomics

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

HTP SEQUENCING OF PLASMID DNAs

(Last Revised: April, 2007)

This Protocol is used by the Schnable Laboratory at Iowa State University. Please contact Dr. Patrick Schnable (*schnable@iastate.edu*) regarding questions or corrections.

1. Standard Sequencing Reaction (10 ul):

	1 rxn
BigDye v2 mix (Applied Biosystems, cat# 4314414)	2 ul
5X sequencing reaction buffer	1 ul
DNA template (total 0.5 ug/rxn)	X ul
Sequence primer (final concentration, 0.32 uM)	1 ul of **3.2 uM soln**
dH_2O	Y ul
Total	10 ul

The value of X depends on DNA concentration. Use enough volume of DNA template (X ul) to provide 0.5 ug; Y is calculated as Y=6-X.

To make enough master mix to set up a 96-well plate of sequencing reactions:

BigDye v2 mix (Applied Biosystems, cat# 4314414)	200 ul
5X sequencing reaction buffer (recipe below)	100 ul
Sequence primer	64 ul of **5 uM** stock soln
dH_2O	236 ul
Total	600 ul

Note: this example is based on using 4 ul of DNA in the standard sequencing reaction; for other concentrations of DNA calculate master mix recipe by multiplying volumes from the standard sequencing reaction by 100 reactions.

SCHNABLE LAB
Plant Genomics

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

Use either single-channel pipettor (Matrix, cat# 1002) or multiple-channel pipettor (Rainin, cat# L12-20) to aliquot 6 ul of the master mix into each well of a sequencing plate (Fisher, cat# 21-402-130). Use a Rainin multiple-channel pipettor to add the DNA from the DNA source plate to the sequencing plate.

Note: Keep BigDye on ice and minimize its exposure to light.

5x sequencing reaction buffer:

$MgCl2\text{-}6H_2O$	0.203 g
Tris	4.85 g

*Add to 80 ml of dH_2O, adjust pH to 9.0 with 10N NaOH and bring up volume to 100 ml with dH_2O

2. **PCR program:**
   Step 1 96C, 0:30
   Step 2 50C, 0:15
   Step 3 60C, 4:00
   Step 4 go to step 1, 25 cycles
   Step 5 4C, forever,
   Step 6 END

3. **Sequence Reaction Clean Up:**

Preparation of Multiscreen Filter Plates containing Sephadex G-50:

1) Add dry Sephadex G-50 (Sigma, cat# G-50-150) to Millipore 45 ul column loader (Millipore , cat# MACL 09645).
2) Remove excess resin from the top of the column loader with the supplied scraper.
3) Place multiscreen HV plate (Millipore, cat# MAHV N45) upside-down on top of the column loader and invert both multiscreen HV plate and column loader. Tap on top of the column loader to release the resin.
4) Using a Impact 8-channel pipettor (Matrix , cat# 6004), add 300 ul of ddH_2O to each well. Let stand at room temperature for 3 hours during which time the resin will swell. You may set up your sequencing reactions during this period.
5) Once the resin has swollen in the MultiScreen plates, these plates can be sealed with Saran Wrap and stored at 4C for several weeks in a sealed plastic container containing a damp towel to assure that they are kept moist.
6) To prepare for use, place the G50-containing Multiscreen filter plate on a blue MultiScreen Centrifuge Alignment Frame (Millipore cat# MACF09604) which is itself

2035C Roy J. Carver Co-Laboratory
Plant Sciences Institute
Iowa State University
Ames, Iowa 50011-3650
515 294-7209
FAX 515 294-5256

placed on top of a 96-well assay block (Fisher, cat# 07-200-724). Stabilize the three-component 'sandwich' with lab tape.

7) Centrifuge the sandwich for 5 min at 2500 rpm in a Sorvall RC5B or Jouan C412 at room temperature. This packs the columns and removes excess water.
8) Add 150 ul dH_2O to each well in the G50-containing Multiscreen filter plate and spin for an additional 5 min at 2500 rpm using the Sovall or Jouan at room temperature. Disassemble the sandwich and discard the water that collects in the assay block.

Purification of Sequencing Reactions using Filter Plates

1) Add 10 ul dH_2O to the 10 ul of sequencing reaction volume present in each well of the sequencing plate. (To prevent damage to the capillary tubes of the 3700 sequencer, add 20 ul of water to any empty wells in the sequencing plate, i.e., those that do not contain sequencing reactions).
2) Place the G50-containing Multiscreen filter plate on top of an ABI (Applied Biosystems cat# N801-0560) or a Biodot (Dot Scientific, cat#951-PCR) collection plate. Insert the collection plate into a compatible PCR plate rack (e.g., Fisher, cat# 05-541-55). Tape the sides of the two components together so they do not fly apart during centrifugation. (We have been told that the Multiscreen filter plates can be reused following appropriate washing, but we have not tested this.)
3) Transfer all 20 ul from each well of the sequencing plate into the center of each well of the G50-containing Multiscreen filter plate. Spin at 2500 rpm for 5 min at room temperature. Make sure there is at least 20 ul in each well of the ABI collection plate.
4) Spin again for 5 min if the filtered volume is less than 20 ul.
5) Disassemble the two plates.
6) Seal the ABI collection plate with aluminum sealing tape (Fisher, cat# 07-200-683) and submit to the DNA sequence facility.
7) In parallel, fill in the on-line request form at http://www.dna.iastate.edu/3700.html