SCHNABLE LAB
Plant Genomics

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ISOLATION OF PLASMIDS FOR HTP SEQUENCING

(Last Revised: April, 2007)

This Protocol is used by the Schnable Laboratory (Iowa State University) based to a large extent on one obtained from Rod Wing's group at Clemson University. Please contact Dr. Patrick Schnable (schnable@iastate.edu) regarding questions or corrections.

DAY 1: Spread library

1. Spread 1 ul (about 500 colonies) of phagemid cDNA library on dyt plate containing appropriate antibiotics for selection

- **Recipe for dyt Plates**

To prepare 1 L

Bacto-trypton	16 g
Yeast extract	10 g
NaCl	5 g
Agar	15 g

Add dH_2O up to 1000 ml.
Autoclave 25 minutes at 121PSI. Cool to ~45ºC before adding appropriate antibiotic(s). Pour 150 x 15mm plates (Fisher cat# 08-75-7-14). Each liter should make ~40 plates.

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DAY 2: Prepare Glycerol Cultures

1. Use Q-Fill to fill each well of a 96-well plate (Nunc MicroWell plates with lids, Fisher 12-565-65) with 180 ul of Wu broth plus appropriate antibiotic. Use 80 ml for four 96-well plates. Note: it is important to autoclave the tubes, bottles, and the manifold needed for Q-Fill prior to use.
2. Pick single colonies from the plates spread previous day with phagemid cDNA library into individual wells of the Wu broth plate.
3. Grow for 24 hours in a 37ºC non-shaking incubator with lid on.

- **Recipe for Wu Broth**

To prepare 1 L

K_2HPO_4	6.27 g
KH_2PO_4	1.8 g
NaCitrate	0.5 g
$(NH_4)_2SO_4$	0.9 g
Tryptone	10.0 g
yeast extract	5.0 g
NaCl	10.0 g
Glycerol (BRL ultrapure)	44.0 ml

Adjust pH to 7.2 using 5M KOH
Add dH_2O to 1 L
Autoclave 25 minutes at 121PSI.
After cooling to RT (room temperature), add 200 ul of 2M $MgSO_4$

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DAY 3: Stamp from Glycerol Culture to Culture Block

1. Use Q-Fill* to fill each well of a 96-well culture block (Dot scientific, cat# R-6530; or Qiagen, cat# 19579) with 1.2 ml of Magnificent Broth (see recipe below) plus appropriate antibiotic(s).
2. Flame the 96-pin stamper (V&P Scientific, cat# VP408A) with 95% alcohol
3. Cool stamper in autoclaved dH_2O
4. Stamp the source glycerol plate, and dip into the culture block. Be sure to maintain the correct orientation.
5. Seal culture block with AirPore tape (Qiagen, cat# 19571) and grow at 37ºC shaking at 325 rpm for 16 hrs.
6. After stamping the culture block, seal the source plate with aluminum tape (Fisher, cat# 07-200-683) and store at –80ºC.

- **Recipe for Magnificent Broth**

To prepare 1 L

Magnificent Broth powder 50 g (MacConnell Research, cat# MR2001)

Add dH_2O to 1000 ml
Autoclave 25 minutes at 121PSI.

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DAY 4: Plasmid Isolation

1. Pellet the cells from culture blocks at 4000 rpm (Jouan C412 centrifuge, cat#11175335) at room temperature (RT) for 10 min. Discard the liquid, place plates upside down on a paper towel to drain for 3-5 min.
2. Add 800 ul RNAse A (Roche/Boehringer Mannheim, cat# 109 169) to 40 ml of Solution I. This will be enough for processing four 96-well plates. Add 100 ul of Solution I plus RNAse A to each well of the culture block using 8-channel pipettor (Impact 1250 ul multichannel pipetman, Matrix , cat# 6004), vortex using the Fisher Votexer Genie 2. (Fisher Scientific, cat# 12-812) at a speed of 5-6 until all pellets are resuspended, and incubate at RT for 5 min.
3. Prepare Solution II from stock solutions. Add 100 ul fresh Solution II to each well of the culture block using Impact 8-channel pipettor, briefly vortex at speed of 1-2, incubate at RT for 10 min, vortex briefly.
4. Add 100 ul Solution III to each well using Impact 8-channel pipettor, vortex at speed of 1-2, incubate at RT for 15 min with occasional vortexing.
5. Meanwhile, place a MultiScreen NA filter plate (Millipore , cat#MANANLY50) on top of a MultiScreen Centrifuge Alignment Frame (Millipore cat# MACF09604).
   Both of these should be placed on top of a 96-well assay block (Fisher, cat# 07-200-724). Be sure to align the filter plate and assay block in the correct orientation. Tape all four sides of the three components to avoid cross contamination.
6. Use Impact 8-channel pipettor to transfer all solution from the culture block to the 96-well MultiScreen NA filter plate. Pay attention to the orientation of the two plates. This is a time-consuming step. Use of larger tips helps; use Matrix (cat# 8051) 1250 ul tips. Centrifuge at 4000 rpm at 4ºC for 10 min. After centrifuging, if some wells still contain liquid, transfer them individually to the correct receiving well. Discard the filter plate. An additional centrifuge step (4000 rpm at RT for 5 min) before proceeding to next step will reduce the amount of debris that can block the filter, but normally this is not necessary.
7. Use Impact 8-channel pipettor to add 200 ul of isopropanol to each well. Tape the plate with Qiagen Tape Pads, (cat# 19570) and invert the plates 5-10 times. Allow plasmid DNA to precipitate at RT for 15 min.
8. Pellet DNA at 4000 rpm in the Sorval RC5B centrifuge for 15 min; discard the liquid, place the plates upside down on paper towel to drain for 2 min.
9. Wash DNA pellets with 400 ul 70% ethanol and centrifuge at 4000 rpm (Jouan C412 centrifuge, cat # 11175335) for 10 min; discard liquid; place the plates upside down on paper towels for 2 min. Meanwhile, place at least two-layers of paper towels on the centrifuge carrier. Then invert the plate on the paper towel in the carrier and centrifuge at a max speed of 400 rpm for 30 sec in Jouan.
10. Dry the plate(s) in the sterile hood by placing them on their sides at a 45o angle to the airflow for 10 min. This will prevent loss of the DNA pellets. The pellets will usually be clear at this stage. Because ethanol negatively affects the sequencing reactions, it is very

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important that all the ethanol be allowed to evaporate prior to proceeding to the next step.

11. Use 8-channel Pipet-Lite (Rainin, cat# L8-200) to add 30 ul dH_2O to each well to hydrate the DNA. Briefly vortex at speed of 5-6 and centrifuge in Jouan at 3500 rpm for 30 sec. Load 1~2 ul of DNA solution into an 0.8-1% agarose gel and electrophoresis to check the DNA concentration. Transfer DNA from blocks to Nunc plates (Fisher cat# 12-565-65) for long-term storage.

Recipes

Solution I (500 ml) [50 mM glucose, 10 mM EDTA (pH 8.0) and 25 mM Tris-HCl (pH 8.0)]

Glucose	4.5 g
EDTA (pH 8.0)	10 ml of a 0.5 M stock
Tris-HCl (pH 8.0)	25 ml of a 1 M stock

Add dH_2O to 500 ml
Autoclave 25 minutes at 121PSI.

Solution II (0.2 N NaOH, 1% SDS)
Equal volume of 0.4 N NaOH and 2% SDS
Prepare fresh before each use

Solution III (500 ml) (3 M KOAc with pH 4.8)
To 300 ml ddH_2O, add

KOAc	147.2 g
Acetic acid, glacial	57.5 ml

After salt dissolved, adjust volume to 500 ml with ddH_2O

RNAse A 10mg/ml (20 ml)
To 19.8 ml ddH_2O, add

RNAse A(Roche/Boehringer Mannheim, cat# 109 169)	200 mg
Tris-HCl (pH 7.5, 1 M)	200 ul

NaCl (5 M) 60 ul
Boil for 5 min