ECKER LAB PROTOCOL

Nested PCR and Sequencing of T-DNA Junctions in Arabidopsis

Plant Growth Conditions

The germination and DNA extraction of individual lines are done in 96-deep-well plates (Doc Frugal #33-196 or Beckman 267006 with corresponding lids). For the plate lid, punch holes with a needle in every well except those on the edge. A metal spoon was created to scoop about 30 seeds from an independent T2 plant line. Seeds were plated starting at position A1 and moving down the plate, then across (A1 to H1, then A2 to H2, etc.). Seed plates and lids are first gas-sterilized using bleach-HCl (25 ml bleach and 1 ml HCl) in a 5-liter hermetic chamber placed inside a fume hood. Keep the seed plates and lids in the gas for 30 minutes, then take them out and leave them (plates open) in a sterile hood overnight. Pour twenty milliliters of 0.5x MS salt solution (Gibco #11117-074) containing diluted Daconil2787 (Ortho) [3 ml of 100 µl daconil/50 ml H_2O stock per 20 ml media] and 60 µl 10 mM Gibberellic Acid (Sigma #G-7645) into a sterile petri dish. Add 60 µl of this solution to each well of the seed plate. Spin the plate(s) to 1500x g then stop. Keep the plates at 4°C for three days. Remove the plates from the cold and transfer them to a clean growth chamber set for constant light and a temperature of 25°C. Allow seedlings to grow about 6-8 days, then transfer the plates to the dark for another 3 days.

I. Genomic DNA Prep

Fill the lid of a 96-well plate (CLP #2407 or Beckman #267002) with a layer of 2.5mm zirconia/silica beads (Biospec Products #11079125z). Remove extra beads by tilting the lid to 45 degrees. The wells should contain 1-3 beads each. Put the plate with the germinated seedlings in an inverted position on top of the lid, making sure that the holes of the plate and lid are aligned. Invert the plate and lid so the beads fall into the wells. The extraction protocol uses the Wizard 96 well magnetic bead system for plants (Promega #FF3761).

1) For 1 96-well plate mix 15 ml Lysis buffer A with 75 µl RNAse A (4mg/ml). RNA from the plant tissue may compete with DNA for the magnetic beads.
2) Add 150 µl of Lysis Buffer A to each well of the 96 well plate.
3) Seal the plates tightly with two layers of aluminum foil tape.
4) Grind the seedlings 2.5 minutes in a Harbil 5G-HD Mixer (Fluid Management)
5) Centrifuge plates 10 minutes at 2700x g and 4°C
6) Carefully transfer 125µl of lysate to a new "U" bottom 96-well microtiter plate, making sure not to disturb the pellet.
7) Premix 5280 µl of Lysis Buffer B with 820 µl of resuspended Magnesil particles.
8) Add 60µl of Lysis B/Magnesil mix to the 125 µL of cell lysate.
9) Mix the plate to allow the DNA to bind to the Magnesil. This can be done using an eight-channel pipette for each well, or cover the plate very carefully with clear adhesive cover (ScotchPad from 3M), vortex and centrifuge (quick spin). Place the plate on the Magnabot and remove the supernatant.
10) Remove the plate from the Magnabot and add 100 µl of Wash Solution to each of the DNA/Magnesil wells. Mix the beads in the wash either by pipetting a few times per well, or carefully covering the plates with the adhesive cover and vortex then centrifuge. Place the plate

on the Magnabot. Invert plate over sink and place paper towel under the plate to soak up remaining wash solution. Repeat wash again using 100 µl of Wash Solution. If the wash still contains green color perform a third wash. After wash is complete, let the DNA/Magnesil dry at 37°C until the beads are slightly moist and alchol has evaporated. Resuspend in 40 µl of 10 mM Tris, pH 8.3 for 15 minutes to overnight.

11) Place the plate on the Magnabot and transfer the DNA solution to a new plate. Run 1 µl from row C and F (columns 3 to 10) on a gel to check quality.

II. Genomic Digest-Adaptor Ligation

Adaptor Primers:

LongAd: 5' GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GC 3'
ShortHind: 5' P-AGC TGC AGC CCG-NH2 3'*
ShortEco: 5' P-AAT TGC AGC CCG-NH2 3'*
ShortAse: 5' P-TAG CAG CCC G-NH2 3'*

If the above Ase adaptor doesn't work well, use the following primers to make the adaptor:

LongAdv.2: 5' GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GTG C 3'
ShortAsev.2: 5' P-TAG CAC AGC CCG-NH2 3'*

***Note**: the 5' Phosphate and 3' C7 amino mod. These primers are HPLC purified.

When primers are obtained from Operon they arrive lyophilized. A data sheet is included. On the data sheet is the number of pmol of primer. Divide this number by 200 pmol/µl (this will be the final concentration) to determine the volume of water (or 10 mM Tris, pH 8.3) to add to the stock tube.
Here is an example:

Seq Name	Seq 5' to 3'	OD	pmol	Len	MW	ug	Tm	Scale	Purif.
ShortHind		6.25	56838.19	12	3920.53	222.84	53.98	0.2 umol	HPLC
ShortEco		6.32	55140.65	12	3919.56	216.13	47.15	0.2 umol	HPLC
ShortAse		5.31	57119.94	10	3302.14	188.62	43.6	0.2 umol	HPLC
LongAd		47.19	105815.9	47	14506.37	1535	80.43	1.0 umol	HPLC

ShortHind: 56838.19 pmol / 200 pmol/µl = 284 µl of H_2O to stock tube
ShortEco : 55140.65 pmol / 200 pmol/µl = 275 µl of H_2O to stock tube
ShortAse : 57119.94 pmol / 200 pmol/µl = 285.6 µl of H_2O to stock tube
LongAd : 105815.9 pmol / 200 pmol/µl = 529 µl of H_2O to stock tube

To make the adaptor, add to a small eppendorf tube:

Hind Adaptor
82.5 µl NanoPure H_2O
10 µl 10mM Tris, pH 8.3
3.5 µl 200 pmol LongAd primer
4.0 µl 200 pmol ShortHind primer

Eco Adaptor
81.5 µl NanoPure H_2O
10 µl 10mM Tris, pH 8.3
3.5 µl 200 pmol LongAd primer
5.0 µl 200 pmol ShortEco primer

Ase Adaptor
84.5 µl NanoPure H_2O
10 µl 10mM Tris, pH 8.3

Ase Adaptor v.2
85 µl NanoPure H_2O
10 µl 10mM Tris, pH 8.3

3.5 µl 200 pmol LongAd primer	3.5 µl 200 pmol LongAd primer
2.0 µl 200 pmol ShortAse primer	1.5 µl 200 pmol ShortAse primer

Vortex and place tube on a wet heat block 96°C for 2 minutes, then turn power off to heat block and remove the block from the heat mantle. Let cool to room temperature.

Note: This method sometimes fails to work when a new stock is tested. This may be a result of incorrect DNA concentration, impure primers, etc. Always have some good primer stock stored so that new stocks can be compared to the primer standard. Primers can be compared via spec. and/ or gel electrophoresis. The volume of primer stocks used to make the adaptor may be modified as required.

The *Hind*/*Eco* digestion and ligation steps are performed at the same time.

For one 96-well PCR plate prepare the following:

550 µl MilliQ H_2O or similar PCR quality water
100 µl NEB Ligase Buffer
25 µl Hind Adaptor
25 µl Eco Adaptor
10 µl NEB *HindIII* (2 U/rxn)
10 µl NEB *EcoRI* (2 U/rxn)
5 µl NEB T4 DNA Ligase (20 U/rxn)
5 µl 10 mM ATP

Add 7 µl of mix to each well. Add ~100 ng of Genomic DNA to each reaction (2-3 microliters of the Magnesil DNA prep). Incubate overnight at room temperature.

The *AseI* digest and ligation steps are performed separately.

Digest for one 96-well plate:

250 µl MilliQ H_2O or similar PCR quality water
60 µl Ligase buffer
20 µl *Ase I* (2 U/rxn)

Add 3.5 µl Digest mix to well
Add 2.5 µl DNA
Digest 4 hours at 37°C

Ligation for one 96-well digest:

275 µl MilliQ H_2O or similar PCR quality water
40 µl Ligase buffer
70 µl *AseI* Adaptor
12 µl 10 mM ATP
17 µl T4 DNA Ligase

Add 4 µl Ligation mix to well

Incubate 20°C overnight

III. PCR

PCR1 Primers:
T-DNA Left Border a1 (Lba1): 5' TGG TTC ACG TAG TGG GCC ATC G 3'

Adaptor Primer (AP1): 5' GTA ATA CGA CTC ACT ATA GGG C 3'

PCR2 Primers:
T-DNA Left Border b1 (LBb1): 5' GCG TGG ACC GCT TGC TGC AAC T 3'

Adaptor Primer (AP2-C): 5' TGG TCG ACG GCC CGG GCT GC 3'

PCR1:
This protocol uses TaKaRa ExTaq and dNTPs.
For one 96-well PCR plate prepare **1 tube** of the following:

1650 µl MilliQ H_2O or similar PCR quality water
200 µl 10x ExTaq PCR Buffer
80 µl 10 mM dNTPs
3 µl 200 pmol LBa1 primer (4 µl of 6/12/03 stock)
3 µl 200 pmol AP1 primer (4 µl of 6/12/03 stock)
6 µl ExTaq

Pipette 20 µl of mix into each well of a 96-well PCR plate. Add 1 µl of ligated *Hind*/*Eco* DNA (1.5 µl ligated Ase DNA) to each reaction. Cover with rubber mat and immediately load on MJ paused at the initial 96 °C step.

Cycle on an MJ-200 Thermal Cycler with the following conditions:

1) 96°C for 0:20
2) 96°C for 0:15
3) 72°C for 2:20
4) Goto 2, 10 times
5) 96°C for 0:20
6) 67°C for 2:20
7) Goto 5, 25 times
8) 4 °C for ever

Dilute the *Hind*/*Eco* PCR with 80 µl of MilliQ H_2O. Go to Pcr 2.
Load 6.5 µl of *Ase* pcr product on gel to visualize products. Go to Exonuclease step.

PCR2:
This protocol uses Eppendorf HotMaster Taq.
For one 96-well PCR plate prepare **2 tubes** of the following:

1650 µl MilliQ H_2O or similar PCR quality water
200 µl 10x HotMaster PCR Buffer
80 µl 10 mM dNTPs
3 µl 200 pmol LBb1 primer (3 ul of 11/19/02 stock)
3 µl 200 pmol AP2-C primer (4 ul of 11/01/02 stock)
5 µl HotMaster Taq

Add 20 µl of PCR2 mix to each well of a 96-well PCR plate. Transfer 1.5 µl of diluted PCR1 product to each well. Cover with rubber mat and immediately load on MJ paused at the initial 96 °C step.

Cycle on an MJ-200 Thermal Cycler with the following conditions:

1) 96°C for 1:00
2) 94°C for 0:20
3) 72°C for 2:20
4) Goto 2, 5 times
5) 94°C for 0:20
6) 67°C for 0:20
7) 72°C for 2:10
8) Goto 5, 23 times
9) 72°C for 3:00
10) 4°C hold

Examine 3.5 µl of PCR2 product on a 1.0% agarose gel.

IV. PCR Cleanup

Exonuclease I (NEB # M0293S) is used to degrade the primers.
For one 96-well PCR plate prepare the following:

275 µl MilliQ H_2O or similar PCR quality water
30 µl 10x Sigma PCR Buffer
5 µl Exo I (10 U/µl)

Add 3 µl of mix to each well of the plate. Add 20 µl of *AseI* Pcr product or 10 µl of *Hind*/*Eco* PCR2 product to each well. Incubate at 37°C for 1.5 hours, then 80°C for 20 minutes. Store at 4°C.
If the exo rxn is performed in a 384-well plate for the *AseI* products, an additional concentration step is required prior to precipitation. Place the plate in the speed-vac and put on high heat one hour. The liquid should be completely gone from the wells. Add 10

µl water to each reaction and let the DNA resuspend at least one half hour (overnight may be better) before precipitating.

Store at 4°C until ready to sequence.

V. Cycle Sequencing

Precipitate 10-14 µl PCR product:
Add 20 µl of EtOH/NaOAc mix (105 ml 95% EtOH, 7.5 ml 3M NaOAc pH 4.7, 2.5 ml H2O). Cover and mix thoroughly. Incubate at –80°C for 15 minutes. Spin at 3000x g for 25 minutes. Remove supernatant by inverting plate on paper towel and gently flicking the plate. Wash pellets with 25 µl 70% EtOH. Spin 10 minutes at 2700x g. Dry the DNA by inverting the plates on a paper towel and pulse the centrifuge to 20x g.
This protocol uses ABI Big Dye version 3.1 (#4336921)

5x Sequencing buffer is composed of 400 mM Tris-HCl pH 9.0 and 10 mM $MgCl_2$.

For one 96-well PCR plate prepare the following:

700 µl MilliQ H_2O or similar PCR quality water
200 µl 5x Sequencing Buffer
70 µl Big Dye v. 3
1 µl 200 pmol LBb1 primer (5 µl 40 pmol LBb1 primer) *

* primer concentration can be increased or decreased as needed.

Add 10 µl of mix to each well on the plate.

Cycle on Gene Amp 9700 (Applied Biosystems) with heated lid using the following program:

1) 96°C for 5:00
2) 96°C for 0:25
3) 63°C for 2:30
4) Goto 2, 47 times
5) 4°C hold

Precipitate the sequenced products using the EtOH/NaOAc protocol mentioned previously. To the dry sequence products add 10 µl of water. Load onto an ABI 3700 DNA sequencer.

REFERENCE

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