

## **Isolation and transformation of protoplasts from Arabidopsis thaliana leaves and suspension cultures for transient expression assays.**

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This protocol works well for a number of *Arabidopsis* ecotypes that we have tried. It is the long version of our protocol described briefly in our first two published descriptions of transient expression from promoters recognized by RNA polymerases I and II in protoplasts derived from suspension cultures or leaves. The suspension cultures must be vigilantly maintained at low density to obtain good protoplasts, therefore we recommend the leaf protocol. Our published papers describing the optimization of expression of a pol II reporter gene and rRNA are:

- 1) Doelling and Pikaard. 1993. Transient expression in *Arabidopsis thaliana* protoplasts derived from rapidly established cell suspension cultures. *Plant Cell Reports* 12: 241-244.
- 2) Doelling, Gaudino and Pikaard. 1993. Functional analysis of *Arabidopsis thaliana* rRNA gene and spacer promoters in vivo and by transient expression. *Proc. Natl. Acad. Sci. USA* 90: 7528-7532.

### Protocol for making protoplasts from *Arabidopsis* Leaves (solution recipes are in the back).

Sterilize *Arabidopsis* seeds in a 1.5 ml microcentrifuge tube: add desired amount of seeds to eppendorf tube. Fill tube with 95% EtOH and incubate at room temperature 2 - 3 minutes. Remove EtOH and add 700 ul ddH<sub>2</sub>O (distilled deionized water) and 700 ul chlorox bleach (undiluted). Mix every few minutes for 20 - 30 minutes. In laminar flow hood, remove chlorox solution and rinse seeds 5 times with 1 ml sterile ddH<sub>2</sub>O.

Grow plants in deep petri dishes (100 x 25mm) on GM (germination medium). Spread sterilized seeds onto GM. I use a P20 pipetman set at 10 ul with yellow tips that have been cut to increase the size of the opening. I then plant the seeds individually in rows with about 100 seeds per plate, distributed evenly. Other methods will likely be quicker but the seeds will be less evenly distributed. Wrap plates with parafilm and introduce air holes with hypodermic needle. Incubate the plates in growth chamber (16 hour, 24°C days and 8 hour, 21°C nights) for 14-15 days at which time the plantlets are ready to harvest. At about 2<sup>1</sup>/<sub>2</sub> weeks, the plants will begin to bolt and flower and are past their prime. Older plants don't have much more leaf tissue, they are harder to work with and the resulting protoplasts are more fragile-we don't use them.

Isolation of protoplasts: In hood, open petri dishes, pull plants from GM with flamed forceps and place them in the petri dish lid. Chop plants with flamed scissors and immediately transfer plant material to deep petri dish containing 15 ml 0.5M mannitol (combine plants grown on two or three GM plates into one petri dish of mannitol). Plasmolyze in the mannitol solution for approximately 1 hour. Replace mannitol solution with 15 ml cell wall digestion solution (filter sterilized through a 0.45 um syringe filter) :

|                                | <u>per 15 ml</u>                          |
|--------------------------------|---|
| 0.4M mannitol                  | 1.1 g                                     |
| 0.33% Cellulase "onozuka" R-10 | 50 mg                                     |
| 0.17% Macerozyme R-10          | 25 mg                                     |
| 3mM MES, pH 5.7                | 3 ml 5x MES solution:                     |
| 7mM CaCl <sub>2</sub>          | 15mM MES, 35mM CaCl <sub>2</sub> , pH 5.7 |

Wrap plates with parafilm, cover with foil and place on orbital shaker in growth chamber. Shake gently (45 - 50 rpm on our shaker). Allow digestion to continue overnight. Expect a yield of approximately  $1 \times 10^7$  protoplasts/GM plate (100 seedlings).

#### From suspension culture

Grow Arabidopsis suspension cultures in 2x Gam +1 +0.05. Maintain cultures by transferring callus material to new flasks or by exchanging growth medium once a week. It is helpful to also exchange medium 2-3 days before isolating protoplasts from suspension cultures. It is best to keep the cultures fairly thin.

Flame mouth of suspension culture flask and pour contents into deep petri dish. Remove the growth medium and replace with 15 ml enzyme solution (filter sterilized):

|                               | <u>per 15 ml</u>                          |
|-------------------------------|---|
| 0.4M mannitol                 | 1.1 g                                     |
| 1.0% Cellulase "onozuka" R-10 | 150 mg                                    |
| 0.25% Macerozyme R-10         | 40 mg                                     |
| 0.5% Driselase                | 75 mg                                     |
| 3mM MES, pH 5.7               | 3 ml 5x MES solution:                     |
| 7mM CaCl <sub>2</sub>         | 15mM MES, 35mM CaCl <sub>2</sub> , pH 5.7 |

Wrap plates with parafilm, cover with foil and place on shaker in growth chamber (45 - 50 rpm). Allow digestion to continue overnight.

### Harvesting protoplasts

Separate protoplasts from undigested leaf or callus tissue by filtering the digestion mixture through wire mesh sieves ( purchased from Small Parts Inc., P.O. Box 4650, Miami Lakes, FL 33014-0650. Tel: (305) 557-8222. Two sequential filtering steps, first using CX-60 (230um) and then CX-400 (38um), are probably sufficient for protoplasts being isolated from leaf tissue. I usually go through a 3 step filtration procedure when working with suspension culture material since the sieves clog quickly. The sieves are used in the order CX-60, CX-150 (104um) and finally CX-400. I usually filter into small petri dishes or the lid of the petri dish used for digestion. Transfer protoplasts into a 15 ml conical tube and spin 10 minutes at 50xg. Remove enzyme solution (supernatant) and wash protoplasts with a mixture of 6 ml 0.5M mannitol and 3 ml 0.2M CaCl<sub>2</sub>. Spin for 5 - 10 minutes at 50xg to remove supernatant. Repeat the wash before resuspending protoplasts in 0.5M mannitol solution for counting.

### Counting protoplasts

Use a hemacytometer to determine the concentration of protoplasts in the mannitol solution. Place cover slip over counting grid and introduce resuspended protoplasts (make sure they are well mixed since they tend to aggregate) into slot until liquid covers the entire grid area. Count the number of protoplasts in one of the 9 large squares by viewing with microscope at 100x magnification (10x eyepiece, 10x lens). Multiply this number by 10<sup>4</sup> to get # of protoplasts per ml. It is best to repeat this for several squares to make sure your count is accurate. Multiply the number of protoplasts per ml by the number of ml of protoplast solution to get the total number of protoplasts.

### Transformation of protoplasts

The following is the procedure we used for a CaMV 35S promoter-luciferase reporter gene construct, pWB216 (obtained from Dr. Wayne Barnes at the Washington University Medical Center) or an RNA polymerase I promoter construct ( in which case we need to measure transcripts directly and need to scale up ten-fold).

Resuspend protoplasts in MaMg solution at 1 x 10<sup>7</sup>/ml. Perform transformations in 15 or 50 ml disposable screw cap tubes. Place tube on ice and add in order, mixing after each addition:

#### pWB216 Transfection

130 ul MaMg  
 10 ul CT (calf thymus)DNA (10 ug/ul)  
 +/- 10 ug pWB216  
 150 ul protoplasts (2 x 10<sup>6</sup>), hold on ice 10 minutes.  
 300 ul 40% PEG/CMS to give 20% [PEG] final

#### pol I promoter transfection

1.6 ml MaMg  
 200 ul CT DNA  
 +/- 200 ug plasmid  
 3.0 ml protoplasts (3.2 x 10<sup>7</sup>)  
 5.0 ml 40% PEG/CMS

Incubate on ice 30 minutes. Now, dilute the PEG solution stepwise with W<sub>5</sub>, waiting a few minutes between additions. Mix carefully to avoid protoplast breakage. I generally dilute no more than two fold at a time (+ 600 ul, 1 ml, 1 ml, 2 ml, 2 ml for small transfection reaction, e.g. pWB216; or +5 ml, +10 ml, +10 ml, +20 ml for scaled-up transfection) and dilute about ten fold (and at least 5 fold) in total. Centrifuge 10 minutes at the lowest speed possible to sediment the protoplasts effectively (26 x g in our hands). Remove PEG solution and wash protoplasts by resuspension in 2 ml 0.5M mannitol + 0.5 ml W<sub>5</sub> (or 10 ml 0.5M mannitol + 2.5 ml W<sub>5</sub> for scaled-up reaction). Centrifuge as before and again remove supernatant. Resuspend the protoplasts in 3 ml (or 15 ml) 2x Gam +1 +0.05 containing 0.4M mannitol. Pour the contents of each tube into a separate small petri dish (60 x 15 mm) (or measure 3 ml into each of 5 small petri dishes for scaled-up reaction) and tilt dish until the medium covers the entire bottom surface. Place dishes in container and wrap with foil. Place container in growth chamber 20 - 24 hours to allow expression of gene(s) of interest (see our Plant Cell Reports paper for how we determined best time of expression-this could vary).

#### Protein determination

|                        |                           |
|------------------------|---------------------------|
| In eppendorf tube add: | 800 ul ddH <sub>2</sub> O |
|                        | 50 ul protein extract     |
|                        | 200 ul Biorad concentrate |

Use acetylated BSA as a protein standard. I usually use 0, 1, 2, 4, 6 and 9 ug tubes prepared as above except that the protein extract is replaced by 50 ul protoplast lysis buffer and the appropriate amount of BSA. Measure A<sub>595</sub> of each sample and graph the values obtained from BSA measurements. Approximate the concentration of protein in each sample based on graph.

#### RNA isolation

After 20 - 24 hour incubation, transfer protoplasts to 15 ml tube and centrifuge at 26 x g to separate protoplasts from supernatant. Estimate the volume of protoplasts in each tube. This equals 1 volume. For our polymerase I experiments this is about 1 ml. Add:

|  |         |
|--|---------|
| 1 volume solution D and vortex immediately                               | ~1 ml   |
| 1/5 volume 3M NaOAc pH 4.0 and mix well                                  | ~0.2 ml |
| 1 volume acid phenol and mix well  | ~1 ml   |
| 1/5 volume CHCl <sub>3</sub> :isoamyl alcohol 24:1 and vortex vigorously | ~0.2 ml |

Hold on ice 20 minutes before centrifuging 10 minutes at 2,500 rpm. Transfer aqueous phase to two eppendorf tubes (750 ul per tube). Add an equal volume isopropanol, mix well and place in freezer 1 hour - overnight. Centrifuge 15 minutes at full speed in microfuge (about 12,000 x g) to

pellet RNA. Resuspend RNA in 300 ul DEPC-treated ddH<sub>2</sub>O, centrifuge 5 minutes to remove any insoluble material and transfer supernatant to new tube. Add 300 ul 4M LiCl and hold on ice 3 hours. Spin 15 minutes at full speed in microfuge, decant supernatant and drain on towel. Resuspend RNA pellet in 200 ul DEPC-treated ddH<sub>2</sub>O. Dilute 5 ul into 1 ml H<sub>2</sub>O to read OD<sub>260</sub> and OD<sub>280</sub> in order to estimate quantity and purity of RNA. I store the RNA as a precipitate (add NaOAc to 0.25M and 2.5-3 volumes of absolute ethanol) in the -20°C freezer. A note of caution: Be careful to not dry EtOH precipitated RNA pellets too long since they can become very difficult if not impossible to resuspend.

The isolated RNA is now ready to be analyzed by either S1 nuclease protection or primer extension.

Solution RecipesGM (for growth of Arabidopsis plants) per L

0.43% MS salts + Gamborg vitamins (Sigma) 4.3 g

2.0% sucrose 20 g

pH to 5.7 with KOH

0.8% agar (weigh separately for each bottle) 8.0 g

autoclave 20 minutes with no more than 400 ml per bottle

2x Gam +1 +0.05 (for growth of Arabidopsis suspension cultures)per L

0.62% Gamborg salts 6.2 g

0.2% Gamborg vitamins 2.0 ml

2.0% sucrose 20 g

0.06% MES 0.6 g

pH to 5.7 with KOH

distribute into 125 or 250 ml bottles (to lesson the risk of contaminating cultures) and autoclave 20 minutes. When media is cool, add:

1 mg/L 2,4D (Sigma) 200 ug/200ul

0.05 mg/L kinetin (Sigma) 10 ug/200ul

MaMg per 100 ml15 mM MgCl<sub>2</sub> 1.5 ml 1M MgCl<sub>2</sub>

0.1% MES 0.1 g

0.4 M mannitol 7.3 g

pH to 5.6 with KOH

autoclave 20 minutes in 125 ml bottles

PEG CMSper 100 ml

40% PEG 8,000

40 g

0.4 M mannitol

7.3 g

0.1 M Ca(NO<sub>3</sub>)<sub>2</sub>

2.4 g

adjust to pH 5.7 if necessary and filter sterilize by using vacuum unit (solution is very viscous) and store at -20°C

W<sub>5</sub>per L

154 mM NaCl

9.0 g

125 mM CaCl<sub>2</sub>

18.4 g

5 mM KCl

0.37 g

5 mM glucose

0.9 g

0.03% MES

0.3 g

pH to 5.8 with KOH

autoclave 20 minutes in 125 or 250 ml bottles

Protoplast lysis bufferper 15 ml

100 mM Na phosphate pH 7.2

1.5 ml 1 M Na phosphate pH 7.2

5 mM DTT

75 ul 1 M DTT

2X Luciferase assay buffer (+/- rATP)per 15 ml

60 mM Tris pH 8.0

900 ul 1 M Tris pH 8.0

20 mM MgCl<sub>2</sub>300 ul 1 M MgCl<sub>2</sub>

20 mM DTT

300 ul 1 M DTT

2 mM EDTA

60 ul 0.5 M EDTA pH 8.0

if + rATP, 2 mM rATP

300 ul 100 mM rATP

Solution Dper 50 ml

4 M guanidinium thiocyanate

23.7 g

25 mM Na citrate pH 7.0

1.25 ml 1 M Na citrate pH 7.0

0.5% Sarkosyl

2.5 ml 10% Sarkosyl

0.05M DTT

2.5 ml 1 M DTT

add DTT just before use (I usually only add DTT to the volume I need.)