Moss Methods Sheet

Transformation of *Physcomitrella patens* and *Ceratodon purpureus* using direct DNA uptake by isolated protoplasts.- David Cove

(last update 9th August 2004)

Except where stated otherwise, all procedures may be carried out at room temperature, providing this is between 20° and 25°. Otherwise use a waterbath.

You will need to prepare the following in advance

Tissue	Protoplasts are best isolated from young protonemal tissue obtained from cellophane-overlay plates. It is very difficult to obtain protoplasts from gametophyte tissue. The Leeds lab. use tissue cultured on BCD + ammonium (using cellophane overlays) for 6 days at 25° in continuous light. This gives about 500 mg fresh weight of tissue per plate. 5-6 day old tissue is optimal, and usually yields 2 - 4 $\times 10^6$ protoplast per plate of tissue. Older tissue may leave clumps of undigested tissue, which can act like glue and stick protoplasts together. One plate of tissue usually yields sufficient protoplasts to carry out 3 transformations.
Sterile filters	One per strain to be transformed. These can be made of stainless steel or nylon with a mesh size of about 100 µm × 100 µm.
Driselase stock sol'n.	5 ml of stock solution is enough to digest four plates of tissue
PEG	See recipes - 2 g is enough for about 15 transformations

1 M $MgCl_2$ sol'n.

1% MES pH 5.6 sol'n

1% $Ca(NO_3)_2$ sol'n

1M Tris buffer pH 8.0

8% mannitol

CaPW

4 plates PRMB + cellophanes per transformation

PRMT

Protoplast isolation and regeneration

Based on Grimsley et al. 1977 with modifications

Prepare 0.5% Driselase, MMM and PEG solutions. Melt PRMT and place in 45° water bath.

Then proceed as follows:

Note: all centrifugations of protoplast suspensions are carried out at 100 to 200 × g for 4 min, with no braking.

1 Add tissue at the rate of about 100 mg (fresh wt.) per ml of sterile Driselase solution, *i.e.* we use 5 ml 0.5% Driselase solution per plate of tissue.

2 Incubate for 30 to 60 min, with occasional gentle shaking. The time taken to protoplast some strains may be longer, but longer exposure to Driselase (upto 2 h) does not appear to affect protoplast viability greatly, but it vibility will fall off as incubation times increase.

3. To isolate protoplasts from undigested tissue, filter sterilely through mesh with a pore size of approx. 100 µm × 100 µm.

4. Sediment protoplasts by centrifugation.

5. Remove supernatant

6. Resuspend protoplast pellet in sterile 8% (w/v) D-mannitol (use about the same volume as the volume of enzyme solution used for digestion).

7. Repeat steps 4 and 5

8. Resuspend protoplast pellet in CaPW, again using about the same volume as the volume of enzyme solution used for digestion. Estimate protoplast density using a haemocytometer.

This should yield about 5×10^{6} to 10^{7} viable protoplasts per g fresh wt. of tissue (or using Leeds growth regime, about 2 to 4×10^{6} per plate of tissue). If you wish you can check for protoplast viability at this stage, or you can carry out a “no DNA” control treatment.

Transformation

Based on Schaefer et al., 1991 with modifications

1. For each transformation, you should aim to pipette a volume of protoplast suspension from step 8 above, that contains 5×10^5 protoplasts into tubes. These tubes will need to be able to contain at least 12.5 ml of liquid.

2. Sediment protoplasts by centrifugation.

3. Resuspend each pellet in 300 µl of D-mannitol/$MgCl_2$/MES solution.

5. Dispense 10 to 30 µg of DNA (volume no more than 30 µl, but otherwise not critical) into the protplast suspension.

6. Follow the following time schedule (times in minutes). Ten transformations can be carried out in parallel, at 30 second intervals.

0	Add 300 µl PEGT solution.
5	Heat for 5 min at 45°.
10	Return to room temperature
20	Add 600 µl CaPW, mix gently
25	Add 1 ml CaPW, mix gently
30	Add 2 ml CaPW, mix gently
35	Add 3 ml CaPW, mix gently
40	Add 4 ml CaPW, mix gently
45	Sediment protoplasts by centrifugation (100 to 200 × g for 4 min)

7. **Either:** go directly to 11;
or resuspend in 5 ml PRML

note: In my hands, the 24 h incubation in darkness makes no difference, and I prefer to omit it.

8. Incubate for 24 h at 25° in darkness.

9. Sediment protoplasts by centrifugation.

10. Remove supernatant

11. Resuspend protoplast pellet in 1 ml of sterile 8% (w/v) D-mannitol.

13. Add 7 ml of molten PRMT medium (kept molten at 45°).

14. Dispense onto four 90 mm plates of PRMB overlayed with cellophane at rate of 2 ml per plate.

15. Incubate in light for 5 days at 25°

16. Transfer protoplasts on top layer to selective medium if appropriate.

Recipes

8% D-mannitol solution

D-mannitol	80 g
distilled H20	1 l

Sterilise by autoclaving.

Driselase solution

There is some variation between batches but a final concentration of 0.5 % (w/v) is usually satisfactory. Test each new batch to check this. Our latest batch needs 0.1%. It is convenient to store Driselase as a 2% (w/v) solution at –20°, and dilute on the day you wish to obtain protoplasts. You will need 5 ml of dilute Driselase per plate of tissue.

2% Driselase

1. Dissolve Driselase in 8% D-mannitol solution.
2. Gently mix (do not shake vigorously).
3. Leave to stand at room temperature for 15 min.
4. Centrifuge at 2500 × g for 5 min.
5. Remove the clear supernatant
6. Filter sterilise.
7. Dispense into convenient aliquots and freeze.

Calcium protoplast wash (CaPW)

D-mannitol	80 g
$CaCl_2.2H_20$	7.35 g
distilled H_2O	1 l

Sterilise by autoclaving.

D-mannitol/$MgCl_2$/MES (MMM) solution

On day of use, mix:

D-mannitol	910 mg
1M $MgCl_2$ solution	150 µl
1% MES pH5.6 solution	1 ml
distilled water	8.85 ml

Filter sterilise.

1M $MgCl_2$

$MgCl_2.6H_20$	203.3 g
distilled water	1 l

1% MES pH 5.6

Use 1% (w/v) 2-[N-morpholino]ethanesulphonic acid) in distilled water.
Adjust to pH 5.6 with 0.1M KOH.
Sterilise by autoclaving. Store at 4^oC.

PEG solution for transformation (PEGT)

PEG 6000 2 g

Autoclave in a glass Universal bottle or equivalent vessel.

On day of transformation, melt PEG (this can be done in a water bath at 37° or above, or in a microwave.) Add 5 ml D-mannitol/$Ca(NO_3)_2$ solution and mix well. Leave at room temperature for about 2 h before use.

D-mannitol/$Ca(NO_3)_2$ solution

Make up fresh, on day of use

8% (w/v) D-mannitol solution	9 ml
1M $Ca(NO_3)_2$ solution	1 ml
1M Tris buffer, pH 8.0	100 µl

Filter sterilise

1M $Ca(NO_3)_2$

$Ca(NO_3)_2.4\ H_2O$	236.1 g
distilled water	1 l

Sterilise by autoclaving. Store at 4^oC.

References

Grimsley,N.H., N.W.Ashton & D.J.Cove. 1977. The production of somatic hybrids by protoplast fusion in the moss, *Physcomitrella patens*. Molec Gen Genet 154, 97-100.

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