

Moss Methods Sheet

Somatic hybridisation of *Physcomitrella patens* and *Ceratodon purpureus* by (PEG)-induced fusion of 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

<u>Tissue.</u>	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 5 - 8 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 for each hybridisation is usually sufficient.
<u>Sterile filters.</u>	One per strain to be used. These can be made of stainless steel or nylon with a mesh size of about 100 μm \times 100 μm .
<u>PEG.</u>	See recipes.
<u>Protoplast fusion sol'n</u>	See recipes.
<u>Media, etc</u>	5 plates of appropriate PRMB selection medium per hybridisation, overlaid with cellophane, plus, if you wish to check for survival of the component strains, a further 2 plates of each of the two appropriate PRMB selection media, overlaid with cellophane, You will also need Driselase stock solution, 8% mannitol, CaPW and PRMT

Protoplast isolation and regeneration

Based on Grimsley et al. 1977 with modifications

Prepare 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 \times 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 viability 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 μ m \times 100 μ m.
- 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 \times 10⁶ to 10⁷ viable protoplasts per g fresh wt. of tissue (or using Leeds growth regime, about 2 to 4 \times 10⁶ 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.

Somatic hybridisation (based on Grimsley *et al.*, 1977 with modifications)

This method can be performed sterilely and requires no sophisticated apparatus. Although the efficiency is low, hybrids are readily obtained by using complementary auxotrophic mutants or transgenic antibiotic-resistances as selective markers in the strains being hybridised (it is now routine to obtain hybrids using strains that are hygromycin or G418 resistant, selecting hybrids on medium containing both antibiotics).

1. Following step 7, resuspend protoplast in CaPW at rate of 2.5 ml per plate of tissue used.
2. Approximately equal quantities of protoplasts of the two strains to be hybridised should be mixed. Protoplast density can be judged by eye. If the protoplast suspensions are approximately the same density, add 2.5 ml each of the two strains to be hybridised. If preferred, density can be estimated using a haemocytometer. However, if one component strain protoplasts poorly, the yield of hybrids involving that strain can be maximised by mixing with an excess of the other strain.
3. Sediment the mixed protoplasts.
4. Resuspend pellet in 250 µl CaPW.
5. Now follow the following time schedule (times in minutes). Multiple hybridisations can be carried out in parallel. It should be possible to do these at 30 second intervals.

0	Add 750 µl protoplast fusion solution, mix gently
40	Add 1.5 ml CaPW, mix gently
50	Add 10 ml CaPW, mix gently
60	Add 10 ml CaPW, mix gently
70	Sediment protoplasts by centrifugation (100 to 200 g for 4 min)
6. Resuspend pellet in 1 ml CaPW
7. If you wish to estimate the survival of the individual component strains, add 50 µl of suspension to 8 ml molten PRMT, and plate 2 ml onto each of four plates of PRMB overlaid with cellophane.
8. Add the remainder of the protoplast suspension to 9 ml of PRMT and plate 2 ml onto each of 5 plates of PRMB overlaid with cellophane.
9. After 5 - 6 days (when protoplasts can be seen to have regenerated, but growth is still not great), transfer the protoplasts on cellophane from 7 onto the appropriate BCD media to select for one or other of the component strains, and from 8 to the appropriate BCD selective medium to select for hybrids. Selection of hybrids using vitamin auxotrophies usually takes about 3 weeks: using transgenic antibiotic resistances, hybrids can usually be identified after only 7 days.

Ceratodon protoplasts regenerate quite well on PRMB, and so the survival treatments can be simplified somewhat, by plating protoplasts directly onto appropriately-supplemented PRMB plates. However, these are best overlaid with cellophane to aid spreading PRMT, and so only the subsequent transfer to BCD is eliminated. *Ceratodon* hybrids are more readily identified if transfer to selective BCD is carried out.

Recipes.**8% D-mannitol solution**

D-mannitol	80 g
distilled H ₂ O	1 l

Sterilise by autoclaving.

Driselase solution

There is some variation between batches but a final concentration of 0.5 – 1.0 % (w/v) is usually satisfactory. It is convenient to store Driselase as a 2% (w/v) solution at –20°, and dilute to the required concentration on the day you wish to obtain protoplasts. You will need 5 ml of 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 \times 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 ₂ O	7.35 g
distilled H ₂ O	1 l

Sterilise by autoclaving.

Protoplast fusion solution

dissolve:

CaCl ₂ .6H ₂ O	1.09 g	(CaCl ₂ .2H ₂ O	735 mg)
in 100ml distilled water			

add this to:

polyethyleneglycol (PEG) M.Wt. 6000 50 g

which has been melted in a microwave oven or water bath.

Mix

Dispense into appropriate aliquots (750 μ l needed for each fusion).

Sterilise by autoclaving.

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.
- Knight, C.D., D.J. Cove, A.C. Cuming & R.S. Quatrano. 2002. Moss gene technology. In "Molecular Plant Biology Volume Two" eds: P.M. Gilmartin & C. Bowler, pp285 – 301, Oxford University Press, 2002