MOSS TRANSFORMATION

This protocol takes about six weeks from start to finish. Essentially, protoplasts are transformed using PEG and colonies are initially regenerated on non-selective medium. Stable and non-stable transformants are subsequently selected on antibiotics and then transferred back onto non-selective medium. At this stage, non-stable transformants lose the transforming DNA. A final stage of growth on selection medium, allows stable transformants to be identified.

Stock solutions

Stock solution B:

$MgSO_4.7H_2O$ (magnesium sulphate 7-hydrate)	2.5 g
(or 1.2 g of anhydrous $MgSO_4$)	
dH_2O	to 100 ml

Store at 4°C.

Stock solution C:

KH_2PO_4 (potassium phosphate)	2.5 g
dH_2O	to 50 ml

Adjust pH to 6.5 with minimal volume of 4 M KOH, then make up to 100 ml with additional dH_2O. Store at 4°C.

Stock solution D:

KNO_3 (potassium nitrate)	10.1 g
$FeSO_4.7H_2O$ (iron sulphate 7-hydrate)	0.125 g
dH_2O	to 100 ml

Store at 4°C.

1x trace element solution:

H_3BO_3 (boric acid)	614 mg
$AlK(SO_4)_2.12H_2O$ (aluminium potassium sulphate 12-hydrate)	55 mg
$CuSO_4.5H_2O$ (cupric sulphate 5-hydrate)	55 mg
KBr (potassium bromide)	28 mg
LiCl (lithium chloride)	28 mg
$MnCl_2.4H_2O$ (manganese chloride 4-hydrate)	389 mg
$CoCl_2.6H_2O$ (cobalt chloride)	55 mg
$ZnSO_4.7H_2O$ (zinc sulphate 7-hydrate)	55 mg
KI (potassium iodide)	28 mg
$SnCl_2.2H_2O$	28 mg
dH2O	to 1L

Store at 4°C.

The following solutions should be autoclaved and stored at RT or 4°C as indicated below:.

1M $MgCl_2$. Store at RT.
1% MES pH5.6. pH with drops of 0.1MKOH, store at 4°C.
1M $Ca(NO_3)_2$. Store at 4°C.
50% glucose. Store at 4°C.
500mM $CaCl_2$. Store at RT.
500mM ammonium tartrate. Store at 4°C.

Keep sterile – only open in flow hood and do not use for other experiments.

Solutions to prepare the day before you start (Autoclave all)

- 910mg mannitol dissolved in 8.85ml dH_2O
- ~100ml 8% mannitol
- 10ml 8% mannitol
- liquid BCD + mannitol - 300µl solution B
  300µl solution C
  300µl solution D
  30µl 1x trace elements
  2.4g mannitol
  dH_2O to 29.1 ml
- 2g PEG 6000 (in a flat bottomed autoclavable vial)
- 140µM nylon cloth-fitted glass funnel, wrapped in foil
  (I use a cut out falcon tube cap and a tube cut off at ~2 cm from the top. Put a piece of nylon on the top of the tube and tighten the cap. Secure the cap upside down on a glass funnel with bluetack. Wrap the whole thing with foil and autoclave it in 100ml plastic beaker.)

Note: There is no real need to prepare solutions above fresh apart from liquid BCD which precipitates if you leave it longer than o/n. Mannitol solution will last but use unopened bottles each time. Please note that the volumes above are for 6 tubes of transformation. If you are planning to do more than 6 tubes you need more 8% mannitol and liquid BCD + mannitol.

Other things you need on day 1

- 3-5 5ml or 10ml sterile syringes
- 3-5 0.2µm syringe filters
- a pack of sterile 14ml round bottomed falcon tubes
- 45°C waterbath
- DNA, cut, precipitated and washed with 70% ethanol. Concentrated to > 330ng/µl

- 1-2 plates of healthy moss filamentous tissue. It is very important to have healthy moss. Ideally take a 2 week old plate regenerated from a good stock culture and make 3-4 plates fresh plates from it. Culture for 7 days and then sub-culture onto 2-3 fresh and grow for a further 5 days, Use this for transformation. (Growth conditions - 25°C, continuous light). If you are transforming stable knockout mutants, it's best to grow tissues without selection.

Solutions to make fresh on day 2

PRMT (Protoplast Regeneration Media Top layer)

200µl solution B
200µl solution C
200µl solution D
200µl 500mM ammonium tartrate
20µl 1x trace elements
1.6g mannitol
0.1g agar (moss agar)
dH_2O to make up to 19.6 ml
Autoclave
Keep at 50°C after autoclaving and add 400µl 500mM $CaCl_2$ before use as indicated in the protocol.

PRMB

5 ml solution B
5 ml solution C
5 ml solution D
5 ml 500mM ammonium tartrate
0.5ml 1x trace elements
30g mannitol
4g agar
dH_2O to make up to 485 ml
Autoclave
Keep at 50°C after autoclaving, and add 10 ml sterile 500mM $CaCl_2$ & 5ml sterile 50% glucose before use as indicated in the protocol.

Solutions to make on day 7 & day 35

Selection plates (BCD + ammonium tartrate + appropriate antibiotics)

5ml solution B
5ml solution C
5ml solution D
5ml 500mM ammonium tartrate
4g agar
dH_2O to 500ml

Autoclave and then add 1ml sterile 500mM $CaCl_2$.
Add 1ml 25mg/ml G418 kanamycin (final conc; 50μg/ml) and/or 200μl 50mg/ml Hygromycin B (final conc; 20μg/ml).

As you need to keep these plates for 2 weeks, prepare thick plates. Thin plates can dry out. Aim to pour ~16 plates from 500ml media.

Solutions to make on day 21

Non – selective plates

5ml	solution B
5ml	solution C
5ml	solution D
5ml	500mM ammonium tartrate
4g	agar
dH_2O to 500ml	

Autoclave and then add 1ml sterile 500mM $CaCl_2$.

Day 1

1. Clean the flow hood and sterilise Gilsons, tip boxes and racks with ethanol.

2. Make mannitol/$Ca(NO_3)_2$ solution & filter-sterilise
   9ml 8% maniitol
   1ml 1M $Ca(NO_3)_2$
   50µl 2M Tris pH8.0

3. Melt PEG in the vial in microwave at ~300W for 1-2 min

4. Add 5ml of mannitol/$Ca(NO_3)_2$ solution to melted PEG. Swirl until it is thoroughly mixed and leave it at RT for 2-3hrs*

5. Make MMM solution & filter sterilise
   8.85ml 910mg mannitol
   150µl 1M MgCl2
   1ml 1% MES pH5.6

6. Dissolve 60mg Driselase*[2] in 6ml 8% mannitol in a 14ml tube by incubating at RT for 15-20 min. Gently invert to mix at intervals. Spin at 3.3k for 3 min.

7. Pour the supernatant into a Petri dish and filter sterilise it. Collect the filtrate in a 14 ml falcon tube.

8. Add 1.5-2 plates*[3] of moss tissue to the enzyme solution.

9. Digest the tissue for 35-40 min at RT. Invert very gently to mix at 5-10 min intervals.

10. Place the autoclaved 140µM nylon-fitted funnel in the 14ml tube and pour the digest onto the nylon

11. Leave for further 5-10 min incubation

12. Place the cap on the tube and spin at 120 x g for 3min. Remove the supernatant without disturbing the cells.

13. Wash the cells by resuspending them in 6ml 8% mannitol and then spin at 120 x g for 3 min.

14. Repeat the wash.

15. Resuspend cells in 6-10 ml*[4] 8% mannitol

16. Estimate the protopast yield using a haemocytometer (Take 5 readings of the number of cells in 25x16 small squares*[5], which is 1/10,000 ml. Multiply the mean value of the readings by 10,000 x volume (ml) of the cell suspension to get the total no. of cells.)

17. Spin the protoplasts down at 140 x g for 3 min.

18. While cells are spinning, decide how many tubes you are going to use. It depends on how much cell suspension you will have at step 20. You need 300μl suspension for one tube of transformation. Don't forget to include one tube for –DNA control. Add 10ug DNA to each tube, and add the same volume of sterile dH_2O to the control tube. Tap the tubes to bring the liquid down to the bottom.

19. Once protoplasts are pelleted, remove the supernatant.

20. Add MMM solution to get a cell density of 1.2-1.62x10^6 /ml (normally make it to 1.5x10^6 /ml). Resuspend cells by swirling the tube or by pipetting up and down very gently*[6].

21. Add 300μl protoplast prep to each tube of DNA, slowly pipetting the cells down the side of the tube. Swirl to mix with DNA.

22. Add 300μl PEG solution in drops*[7]. Swirl to mix gently at each addition.

23. Heat-shock cells at 45°C for 5 min, then incubate them at RT for 5 min.

24. Add 300μl 8% mannitol to each tube, 5 times at 4-6 min intervals. Swirl or tilt the tubes slightly to move suspension around to mix gently after each addition.

25. Add 1ml 8% mannitol to each tube, 5 times at 4-6 min intervals. Mix cells by tilting tubes after each addition.

26. Add 600μl 500mM $CaCl_2$ and 300ul 50% glucose to the liquind BCD + mannitol. Filter sterilise $CaCl_2$ and Glucose solution if they have not been sterilised.

27. Spin cells at 140 x g for 4 min and remove the supernatant.

28. Resuspend each pellet with 5ml liquid BCD+mannitol+$CaCl_2$+glucose

29. Wrap tubes them in foil to keep them dark and leave them in 25°C o/n.

Day 2

1. Prepare PRMB and PRMT and autoclave the media, forceps and cellophane discs. After autoclaving, keep PRMT at 50°C.

2. Add $CaCl_2$ and glucose to PRMB and pour it in 90mm plates (3-4 plates for each tube of transformation). Place cellophane discs on top.

3. Spin cells down 120 x g for 4 min.

4. While cells are spinning, add 400µl sterile 500mM $CaCl_2$ into 20ml PRMT. Mix by swirling not shaking. Shaking seems to encourage phosphates to precipitate faster than otherwise. Store at 37°C.

5. Remove the supernatant and gently resuspend each pellet in 0.5 ml 8% mannitol if plating out on 3 plates, or in 0.8 ml if using 4 plates. Use mannitol from 10ml 8% mannitol that is autoclaved separately.

6. Add 2.5ml PRMT to each cell suspension if plating out on 3 plates, or 3.2ml PRMT if using 4 plates. Quickly but gently mix by pippetting up and down and plate 1ml on each PRMB plate. You can do up to 4 tubes at a time, or 5 if you are quick, before PRMT starts to solidify.

7. For –DNA control, make 1/10 dilutions with PRMT i.e. 1/10 volume of the total cell suspension added to 3 ml or 4ml PRMT, and plate out at 1 ml per plate. From these plates you can count numbers of regenerants, which will give you a measure of regeneration efficiency.

8. Incubate plates at 25°C in continuous light for 5 days.

Day 7

1. Prepare selection plates and sterile forceps*[8]

2. Using 2 pairs of forceps, transfer cellophane discs from PRM plates to the selection plates.

3. Seal the plates with micropore tape and incubate at 25°C in continuous light

Day 14-17 (only necessary if colonies are growing too close together)

It is very important to keep individual colonies separate. Therefore, if some of the colonies are growing too close together, you need to sub culture them.

1. Prepare BCD + ammonium tartrate plates (non-selective) and place sterile cellophane discs on top.

2. Using a sterile scalpel, pick individual colonies and put them in grids on the new non-selective plates. Flame the scalpel after handling each colony to avoid cross-contamination. (Remember to cool the blade as you can burn the moss). Leave more than 1 cm between colonies.

3. Tape all the plates and incubate them at 25°C in continuous light for two weeks. Proceed directly to Day 35 step.

Day 21

Colonies growing on the selectivion plates include both stable and unstable transformants. The following step selects stable transformants from this population.

1. Prepare BCD + ammonium tartrate plates (non-selective) and sterile forceps.

2. Transfer the cellophane discs from selective plates to non-selective plates. You may still need to pick some colonies at this stage or at a later stage if they are growing too close to each other.

3. Tape the plates and incubate at 25°C in continuous light for 2 weeks.

Day 35

1. Prepare selection plates, sterile forceps and sterile metal spatulas.

2. Transfer the colonies from non-selective plates to fresh selective plates. This time as you pick up the disc with two pairs of forceps, turn it upside down so that the aerial part of the moss is now facing down into the agar plate. Push the moss into the agar by smoothing the discs with a sterile spatula from the top. Be careful not to push moss colonies into each other. As the moss is transferred from the disc to the plate, remove the disc using a pair of forceps. Some colonies may stick to the disc as you try to remove it. If this happens, push the colony back into the agar with the spatula.

3. Tape the plates and incubate them at 25°C in continuous light.

Unstable transformants should have lost the construct during the 2 wks growth on non-selective plates and should now die on selective plates. Those that do grow on selective plates are the stable transformants. The difference between survivors and non-survivors should become clear by 7 days after the transfer.

Screening stable transformants for targeted integration by PCR.

1. Using a sterile scalpel, take 1/4-1/2 of each stable colony and place into separate Eppendorf tubes. Flame the blade each time to avoid cross-contamination. Keep the plates sterile and put them back in the incubator to allow colonies to grow back.

2. Squeeze dry the colonies and snap-freeze tubes in liquid N_2

3. Extract DNA following the moss CTAB-DNA extraction protocol.

4. Set up PCR using 1ul each of the DNA prep.

Characterization of insertion events by Southern blot analysis

When you have identified stable transformants with targeted insertions, grow them on for southern analysis. From the southern analysis you can assess the number of construct repeats at the target locus and determine whether there have been any additional integrations of the construct into non-target sites.

1. Use the remainder of the transformed colony to make 2 x 90mm culture plates and 2-3 spot culture stocks. Grow the culture plates at 25°C in continuous light for 10-14 days.

2. Make 3 fresh culture plates from the two plates and grow for 7 days as above.

3. Harvest 250-350 mg of squeeze-dried tissue and snap-freeze in liquid N_2.

4. Extract DNA using the moss CTAB-DNA extraction protocol.

5. Cut DNA with an enzyme that cuts the construct only once or twice.

Mutant analysis

It is best to analyse the phenotypes on the growth media without selection.

NOTES

* It normally takes 2hrs to get to step 22 from here,

*[2] Driselase seems to vary in efficiency from batch to batch. If 1% solution does not digest tissue well, try 1.5%-1.8%.

*[3] It depends on how much tissue you have. You don't want to add too little or too much. Use 1 and 1/2 plates if moss covers 70-80% of the surface. If moss has grown less, use 2 plates.

*[4] It depends on how much tissue you used and how well it has been digested. Judge by colour or the size of the pellet.

*[5] Smallest square should represent 1/400mm^{2}x0.1mm volume.

*[6] Cells are not happy in MMM solution so you should swiftly proceed to the next steps without a break until step 24 where you start diluting the mix.

*[7] PEG solution is thick and harsh mixing will burst cells or reduce regeneration rates. The changes need to be gradual. You dilute the mix slowly at steps 24 and 25 for the same reason.

*[8] Forceps have been a main source of contamination at this stage. Make sure they are sterile, and dip in ethanol after handling several discs if necessary (Do not flame them as it will damage the tips, which makes it difficult to pick the cellophane discs up). Remember that you need to keep each disc contamination-free for another 4 weeks.