PEG-mediated DNA transformation into protoplasts of *Physcomitrella patens*

Stable transformation using protoplasts and PEG
Foreign DNA is introduced into protoplasts prepared from propagated protonemata. The isolation efficiency of protoplasts depends on the age (days from subculture) of the protonemata. We obtained the best results using protonemata grown for 4 to 6 days. When older protonemata were used it was difficult to digest the cell walls using the enzyme driselase. For optimum results, vigorous, light green protonemata should be used.

Transformation schedule
We routinely transformed protonemata according to the schedule below.

1. (Day 1)
Subculture the propagated protonemata at 25˚C for approximately 4-6 days.

2. (Day 5 at the earliest)
DNA transfer takes 2 days. After the transfer of DNA, incubate for 5 days.

3. (Day 12)
Transfer cellophane with top agar containing regenerating protoplasts onto a selection plate supplemented with adequate antibiotics. Cultivate for 5 days on the selection medium.

4. (Day 17)
Transfer cellophane with moss colonies onto a non-selection plate (no antibiotics). Cultivate for 5-7 days on the selection medium.

5. (Day 24)
Transfer cellophane with moss colonies onto a selection plate supplemented with adequate antibiotics. Cultivate for 5-7 days on the selection medium.

6. (Day 31)
Transplant a selection of the peripheral section of the colonies (100-200 lines) onto a non-selection plate.
Cultivate for 7-10 days on the non-selection medium.

7. (Day 41)
Transplant a selection of the peripheral section of the colonies onto a selection plate
Cultivate for 7-10 days on the selection medium.

8. (Day 51)
Transplant colonies surviving on the selection medium as stable transformants onto non-selection medium.

1. Propagation of protonemata

1) Growth medium
Stock medium
All stock media are stored at 4˚C. Stock D should be used within 2-3 months before iron precipitates.

Stock B (x 100) Autoclave

$MgSO_4$ $7H_2O$	2.5 g (0.1 mM)

Fill up to 100 ml with H_2O

Stock C (x 100) Autoclave

KH_2PO_4	2.5 g (1.84 mM)

Adjust to pH6.5 with 4M KOH

Fill up to 100 ml with H_2O

Stock D (x 100) Do NOT autoclave

KNO_3	10.1 g (1 M)
$FeSO_4$ $7H_2O$	125 mg (4.5 mM)

Fill up to 100 ml with H_2O

Alternative TES (x 1000) Autoclave

$CuSO_4$ $5H_2O$	5.5 mg (0.22 mM)
H3BO3	61.4 mg (10 mM)
$CoCl_2$ $6H_2O$	5.5 mg (0.23 mM)
Na_2MoO_4 $2H_2O$	2.5 mg (0.1 mM)
$ZnSO_4$ $7H_2O$	5.5 mg (0.19 mM)
$MnCl_2$ $4H_2O$	38.9 mg (2 mM)
KI	2.8 mg (0.17 mM)

Fill up to 100 ml with H_2O

500mM Ammonium Tartrate (x 100) Autoclave
Ammonium tartrate 9.21 g
Fill up to 100 ml with H_2O

100mM $CaCl_2$ (x 100) Autoclave
$CaCl_2$ $2H_2O$ 1.47 g
Fill up to 100 ml with H_2O

[Medium used for protonemata culture]

BCDAT medium	**1000 ml**
Stock B	10 ml
Stock C	10 ml
Stock D	10 ml
Alternative TES	1 ml
500mM Ammounim tartrate	10 ml (final 5 mM)
50mM $CaCl_2$ $2H_2O$	10 ml (final 1 mM)
Agar (INA agar; TC-6)	8 g (final 0.8%)

Fill up to 1000 ml with H_2O

After autoclave, pour into 50 dishes of 9 cm and solidify for 30 min. on a clean bench. Store at room temperature.

2) sub-culture
The 4-6 days culture on a 9 cm-dish is harvested with dental forceps, suspended in 8-10 ml of sterile water per cultured dish and blended using a homogenizer. 2 ml of suspension is inoculated in a new dish overla with cellophane. After 4-6 days of incubation at 25˚C under continuous white light (40 μmol/m^2/s), growing protonemal tissue, consisting mainly of chloronemata, is obtained on the dish. The dish may be sealed with medical surgical tape to prevent contamination.

[Material]

- Solid medium (BCDAT) – 9 cm-petri dish
- Sterile water
- Cellophane (autoclave) – Preparation of cellophane is as follows:

1) Place cellophane in a glass petri dish and add 5 mM EDTA solution (pH8.0). Autoclave.
2) Wash with MilliQ water several times.
3) Add MilliQ water to the dish. Autoclave.

- Pipette man P-1000
- Dental forceps
- Homogenizer Ultra turrax T-8 (IKA)
- Ultra turrax T-8 generator shaft S8N-8G (autoclave)
- glass tube (30-40 ml)(autoclave)

[Procedure]

1. Overlay the solid medium with cellophane.
2. Add 8-10 ml of sterile water (per cultured dish) in a glass tube.
3. Recover propagated protonemata from one cultured dish (4-to-6-days old) with dental forceps and add to the glass tube.
4. Blend for about 10 sec. with Ultra turrax at max speed. Used generator shaft must be washed as soon as possible to prevent the adhesion of moss cells inside.
5. Inoculate 2 ml of suspension into a new 9 cm-petri dish.
6. Incubate at 25˚C under continuous white light.

2.1 Transformation (on the First day)

[Materials]

- 500 ml beaker x 1 (for discarding waste solution)
- 10 ml disposable pipet x 3 (sterile)
- 35 ml centrifuge glass tube x 2 (autoclave)

- Funnel with a 80 μm nylon mesh (autoclave)
- Forceps x 1
- Cellophane (cut into circles) (autoclave)
- Yellow tips for P200 and blue tips for P1000 (autoclave)
- 0.22 μm syringe-driven filter unit and 10 ml syringe x 2
- 0.45 μm syringe-driven filter unit and 50 ml syringe x 1
- Neubauer hemacytometer (Becton Dickinson no. 424011)
- Water bath x 2
- Centrifuge with swing roter
- 50 ml conical tube (Nunc 373687, etc) (sterile) x1
- 15 ml conical tube (Nunc 366060, etc) (sterile) x2
- 15 ml conical polystyrene tube (Falcon 2057) (sterile) x number of sample
- 6-cm Petri dish (Falcon 351007)(sterile) x number of sample
- Surgical tape
- Parafilm

[Solution]

- 500 ml 8% (w/v) mannitol solution (autoclave)
- 2 g of PEG6000 and a small stir bar in a 50 ml medium bottle (autoclave)
- 1% (w/v) MES (pH5.6)

- 100 ml protoplast liquid medium (autoclave)

Stock A	1 ml
Stock B	1 ml
Stock C	0.1 ml
5 g/lAmmonium tartrate	1 ml (final 50 mg/l)
Mannitol	6.6 g (final 6.6%)
Glucose	0.5 g

Fill up to 100 ml with H_2O

Divide into 4 flasks (25 ml in each flask) and autoclave

- 500 ml 8% (w/v) Mannitol (Sterile)
- 1 M $Ca(NO_3)_2$ Solution
- 1 M $MgCl_2$ Solution
- Driselase (We use driselase C-20, kindly provided by Kyowa Hakko Co., Ltd.)
- 30 µl of linearized plasmid DNA (1µg/µl) digested adequate restriction enzyme (Purify by phenol/chloroform after digesion)

[procedure]

1. Add 1 ml of 1 M $Ca(NO_3)_2$ and 100 µl of 1M Tris-HCl (pH8.0) into 9 ml of 8% (w/v) mannitol solution and mix. Filter the solution with a 0.22 µm filter.

2. Preparation of PEG/T solution
   Add 5 ml of the filtered solution in step 1 to autoclaved bottle containing warm PEG. Dissolve the PEG completely. This solution is called PEG/T.

3. Preparation of MMM solution
   Mix 910 mg of mannitol, 0.15 ml of $MgCl_2$, 1 ml of 1% MES (pH5.6) and 8.85 ml of H_2O and filter the solution with a 0.22 µl filter.

4. Add 0.5 g of driselase in a 50 ml conical tube and then add 25 ml of 8% mannitol solution. Vortex for more than 30 min.

5. Set water baths at 45˚C and 20˚C.

6. Centrifuge the driselase solution at 4000 rpm for 5 min.

7. Transfer the supernatant to a 50 ml syringe with a 0.45 μm filter unit and filter it into a 35 ml centrifuge glass tube.

8. Put propagated protonema into the driselase solution and incubate at 25˚C for 30 min. Mix very gently every 5 min.

9. Filter the protoplast cells of protonemata through an 80 μm nylon mesh.

10. Centrifuge freshly isolated protoplasts at 1000 rpm (180 x g) for 2 min. and discard supernatant. Suspend protoplasts gently and completely in remaining driserase solution and then gently add 35 ml of 8% (w/v) mannitol. Repeat this washing procedure twice.

11. Count the finally suspended protoplasts with hemacytometor, and re-suspend at 1.6 x 10^6 protoplasts/ml in the MMM solution.
MMM (ml) = number of protoplasts per square (large nine squares) x 10^4 (cell/ml) x 35 (ml) / (1.6 x 10^6)

12. Add 30 μl of plasmid DNA (1μg/μl) into a 15 ml polystyrene tube (Falcon 2057). Add 300 μl of protoplast suspension and 300 μl of PEG solution to the tube and tap gently.

13. Incubate the transformation mixture at 45˚C for 5 min. and then at 20˚C for 10 min.

14. Dilute the transformation mixture 5 times by adding 300 μl of protoplast liquid medium every 3 min. and then dilute 5 times with 1 ml of protoplast liquid medium at 3 min. intervals.

15. Pour the diluted protoplast solution into a 6 cm-dish and incubate at 25˚C overnight in darkness.

[Key points]

- Suspend protoplasts in the MMM solution by pipetting gently if protoplasts aggregate on the bottom of the centrifuge tube.
- Use a 15 ml falcon polystyrene tube (2057) for transfer of plasmid.
- Keep the temperature of the water bath at 20˚C after heat-shock.
- Use of plasmid DNA purified by CsCl gradient increases transformation efficiency.

2.2 Transformation (on the second day)

[Materials]

- 10 ml disposable pipet (sterile) x number of sample
- Forceps x 1 (autoclave)
- Cellophane (autoclave)
- 15 ml conical tube (Nunc 366060) x number of sample
- Centrifuge with swing rotor
- Surgical tape
- parafilm

[Solution]

- PRM/T (200 ml) Autoclave

Stock B	2 ml
Stock C	2 ml
Stock D	2 ml
Alternative TES	0.2 ml
500mM Ammonium tartrate	2 ml (final 5 mM)
Mannitol	16 g (final 8%)
$CaCl_2$ $2H_2O$	0.29 g (10 mM)
Agar (INA agar; TC-6)	1.6 g

Fill up to 200 ml with H_2O

After melting agar divide it between 10 flasks, 20 ml in each flask, and autoclave.

- PRM/B (1000 ml) Autoclave

Stock B	10 ml
Stock C	10 ml
Stock D	10 ml
Alternative TES	1 ml
500 mM Ammonium tartrate	10 ml (final 5 mM)
Mannitol	60 g (final 6%)
$CaCl_2$ $2H_2O$	1.47 g (final 10 mM)
Agar (INA agar; TC-6)	8 g (final 0.8%)

Fill up to 1000 ml with H_2O

After autoclave, pour into 50 dishes of 9 cm each and solidify for 30 min on a clean bench. Store at room temperature.

[Procedure]

1. Overlay cellophane on a 9 cm-dish containing PRM/B medium.

2. Remove the protoplast solution into a 15 ml conical tube (Nunc) and centrifuge at 1000 rpm for 2min.

3. Re-suspend the protoplasts in 8 ml of PRM/T medium pre-warmed at 48˚C by pipetting.

4. Pour 2 ml of protoplast suspension on a 9 cm-dish containing PRM/B medium overlaid with cellophane.

5. Incubate the plate at 25˚C for 5 days under continuous daylight with a light flux of 40 µmol/m^2/s.

3. Transfer to selection medium

[Materials]

- Forceps x 2
- Surgical tape

[Solution]

- Selection medium (BCDAT supplemented with adequate antibiotics)

Stock B	10 ml
Stock C	10 ml
Stock D	10 ml
Alternative TES	1 ml
500mM Ammonium tartrate	10 ml (final 5 mM)
100mM $CaCl_2$ $2H_2O$	10 ml (final 1 mM)
Agar (INA agar; TC-6)	8 g (final 0.8%)

Fill up to 1000 ml with H_2O

After autoclave, allow to cool at ~60˚C and add adequate antibiotics into the medium. Store at 4˚C.

Antibiotics used for selection

1) **Genetecin** (G418)
Nacalai (cat.no. 16513-84) 50 mg/mL solution
Use at the final concentration of 20 µg/l

2) **HygroGold**
Invivogen (cat.no. ant-hg-1) 100 mg/ml solution
Use at the final concentration of 30 µg/l in the medium.

3) **Zeocin**
Invivogen (cat.no. ant-zn-1) 100 mg/ml solution
Use at the final concentration of 50 µg/l in the medium.
Zeocin is light sensitive. Store zeocin solution and plates or medium containing zeocin in the dark.

4) **BlasticidinS**
Invivogen (cat.no. ant-bl-1) 100 mg/ml solution
Use at the final concentration of 75 µg/l in the medium.

[Procedure]

1. Transfer the cellophane with moss colonies to BCDAT medium supplemented with adequate antibiotics using 2 pairs of forceps.
2. Incubate at 25˚C for 5 days under continuous light.

4. Transfer to antibiotics-free medium (non-selection medium)

[Procedure]

1. Transfer the cellophane with moss colonies to BCDAT medium

2. Incubate at 25˚C for 7 days under continuous light.

5. Transfer to selection medium

[Procedure]

Repeat step 3.

6. Transfer to non-selection medium

[Materials]

- Forceps (sharp) x 5-7
- BCDAT plate
- Surgical tape

[Procedure]

1. Transfer each colony under antibiotic selection to an antibiotic-free BCDAT medium.
2. Culture at 25˚C for 10 days under continuous light.

7. Transfer to selection medium

[Procedure]

1. Transfer the peripheral cells of each colony grown under antibiotic selection to an antibiotic-free BCDAT medium. Do not transfer a whole colony or gametophores.

2. Culture at 25˚C for 7-10 days under continuous light.

8. Transfer to non-selection medium

[Procedure]

After incubation for 10 days, select transformants that were able to survive on selection medium as stable transformants.

9. Screening for homologous recombinants by PCR

[Materials]

- Forceps (sharp) x1
- Sterile water
- 1.5 ml tube x number of samples
- PCR tube or PCR plate
- Clear chip for P2 pipette man
- liquid N_2
- Thermal cycler (BioRad iCycler etc)
- PCR enzyme (BlendTaq (TOYOBO), ExTaq (TAKARA bio) etc.)
- 10x PCR buffer
- 25mM dNTPs
- 10 μM each primer set (for WT and recombinant)
- agarose

- electrophoresis aparatus

[Procedure]

1. Transfer two picks ofcells from the peripheral region of each colony to a 1.5 ml tube containing sterile water on a clean bench. Do not transfer a whole colony or gametophores.

2. Transfer each colony to two separate PCR tubes for detection of genotypes (WT and recombinant) in transformants.

3. Remove the remaining water in the PCR tube using P2 tip

4. After removing water, immediately add 5 µl of 10x PCR buffer.

5. Crack moss cells by P2 tip

6. Incubate PCR tubes in thermal cycler at 45˚C for 30 min and then 68˚C for 15min.

7. Freeze PCR tubes by soaking in liquid N_2 and thaw by hand warming. Repeat at least 5 times.

8. Add PCR mixture except PCR buffer.

9. Perform PCR by following cycle and mixture;
   Cycle; 1 cycle of 94˚C for 2min and 55 cycles of 94˚C for 30sec, primer Tm − 5˚C for 1min and 72˚C for 1min/kbp.
   PCR mixture; 1x PCR buffer, 0.25mM dNTPs, 0.1 µM primer pair, 0.75U Blend Taq (or ExTaq)

[Key points]

- If fresh and vigorous moss colonies are not used, no PCR fragments will be amplified.
- The moss cell freeze-thaw cycle will increase PCR efficiency. Do not skip this step.