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PEG-mediated moss protoplast transformation V.1

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1 Works for me dx.doi.org/10.17504/protocols.io.bghcjt2w

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BEFORE STARTING

Prepare all solutions and material.
Prepare 15µg DNA in max 30µL of dH_2O for each transformation.

Day 1

1 Turn on waterbath at 45°C and flame forceps in laminar flowhood.

2 Prepare 1% driselase solution in 8% mannitol. To do so weight **100mg driselase** in the cap of a 15ml falcon tube. In flow hood add **10mL 8% mannitol**. Mix by shaking, wrap tube in foil and leave for 15 minutes at room temperature.

3 Meanwhile, defrost MaMg (150µL needed/transformation) and PEG (300µL needed/transformation) in waterbath at 45°C.

4 Centrifuge 1% driselase solution for **2 minutes at 2000rpm**.

5 Filter sterilize the supernatant with 0.45µM filter and 10mL syringe, and transfer to sterile 50mL falcon tube.

6 Scrape protonemal tissue (4-5 days old max!) off from approx. **5-6 plates** with a spatula and transfer it into driselase/mannitol solution. Leave it at room temperature with occasional gentle stirring until filamentous tissue becomes invisible. It takes approx. **1hr**. Do not digest longer than 1hr30.

7 Meanwhile, take MaMg and PEG microtubes out. For each transformation (usually 4 per experiment) prepare one 2mL sterile microtube and one 10mL round-bottom tube. Aliquot 150µL MaMg per microtube and 300µL PEG per round-bottom tube. Centrifuge round-bottom tube with PEG in order to sink it to the bottom for 1 minute at 2000rpm.

Every step must be carried out very gently from now on

8 Place the protoplast suspension onto a 100µm filter carefully, collect the protoplasts in a sterile petri dish and transfer in 50mL-falcon tube. Wrap foil around the tube. If using Pipetboy, set it to the slowest speed. Let the protoplast solution run very gently along the tube.

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9 Spin down at **700rpm for 4minutes** with acceleration set at 2 and **brake set at 0 (swing rotor centrifuge)**.

10 Remove the supernatant, leaving a very small volume of the supernatant. Resuspend the protoplasts initially with the remainder of supernatant to loosen the pellet, then top up with 10mL 8% mannitol.

11 Repeat steps 9 and 10 twice

12 Before the third centrifugation set aside a small aliquot (100µL) of the protoplast suspension and count the density while running the centrifuge. To do so use Neubauer counting chamber. Pipette 10µL of protoplast suspension with cut-yellow tip onto Neubauer counting chamber and count cells in 5 big squares.

The general formula is: Concentration (cells/mL) = (number of cells x 10,000) / (number of squares)

Then for 10mL of suspension the number of protoplasts is: **((number of cells x 10,000) / 5) x 10**

For more information about Neubauer counting chamber check the protocol in annex.

13 Resuspend protoplasts in 8% mannitol at 2x the required density for transformation: **3.2 million (M) cells/mL**. The optimum density for transformation is 1.6M cells/mL. To measure the approx. volume of protoplast, use 1mL pipette to pipette the protoplast suspension and check the volume. Add 8% mannitol to get the final volume you need to have a density of 2 M cells/mL.

14 Set volume of each pipette: P1000 on 350µL, P200 on 150µL (cut tip) and P20 on DNA volume (eg. 10µL).

15 Pipette DNA in 2mL microtube with MaMg.

16 Pipette 150µL of protoplast suspension into 2mL microtube.

17 Using blue tips, pipette the 2xMaMg+DNA+protoplast suspension, mix it gently by pipetting up and down twice and transfer the mix to 10mL round-bottom tubes with PEG. Let the mixture run along the tube very gently and mix by gently tapping the tube 10 times.

18 Repeat steps 15-17 for each transformation.

19 Heat-shock the protoplast mixture by placing the tubes in the waterbath at **45°C for 5 min exactly (NOT LONGER)**.

20 Take the tubes out from the waterbath and cool them in water at room temperature for a further 10min.

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21 Switch off the waterbath (and turn off the light if too bright in the room).

22 Dilute the PEG-protoplast mixture with 8% mannitol over the next 30min-1hr in 6-7 steps. In each dilution step the protoplasts must be thoroughly, but very gently mixed with diluents by tilting the tubes.

23 Add 300µL 8% mannitol and wait 7min.

24 Add 600µL 8% mannitol and wait 7min. Repeat once.

25 Add 1mL 8% mannitol and wait 7min. Repeat once.

26 Add 8% mannitol to fill up the tube and gently mix by tilting the tube.

27 Leave the diluted protoplasts on the bench for one hour to let them sink at the bottom of the tubes.

28 Meanwhile, add sterile $CaCL_2$ to PRM-L and PRM-B (to reach 10mM $CaCL_2$ final concentration). To do so add **2mL $CaCl_2$ per 100mL PRM**.

29 Prepare 4 PRM-B plates for each transformation and store at RT until Day 2.

30 Spin down protoplasts at 700rpm for 4minutes with acceleration set at 2 and **brake set at 0**.

31 Remove the supernatant, wet 5cm Petri-dish with 5mL PRM-L and add those 5mL PRM-L in each tube. Mix it gently in the tube by pipetting once up and down and pour the suspension to the 5cm Petri-dish.

32 Seal the Petri-dishes with **Parafilm** and place them in black cardboard box. Incubate them at 23°C in the dark overnight.

Day 2

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33 Next morning turn on waterbath set at 45°C.

34 Add CaCl2 to sterile PRM-T to be 10mM at the final concentration. To do so add **2mL $CaCl_2$ per 100mLPRM-T**.

35 Dispense 10.5mL of PRM-T in 50mL Falcon tubes for each transformation. Cool them down in waterbath set at 45°C for 1h.

36 Take the Petri-dishes out from the box and pour the protoplast suspension in round-bottom tubes.

37 Let the protoplasts sink down for 30min.

38 Meanwhile, put cellophane disks on PRM-B Petri-dishes.

39 Spin down protoplasts at 700rpm for 4minutes with acceleration set at 2 and brake set at 0.

40 Remove the supernatant from the protoplast tube. Add PRM-T, mix by pipetting up and down one time and pour onto PRM-B plates overlaid with cellophane. Pour 2.5mL on each plate, i.e. 4 plates for each transformation.

41 Incubate the plates at 23°C under 16hr light condition. Most of the live protoplasts should start dividing several days after embedding.

42 After 5-7 days transfer the cellophane disks with protoplasts on BCDAT medium with appropriate antibiotic.

43 14 days after transformation transfer them on BCDAT without antibiotics. Let grow on medium without BCDAT medium for 7 days minimum.21 days after transformation isolate individual colonies on BCDAT medium with appropriate antibiotic.

44 21 days after transformation isolate individual colonies on BCDAT medium with appropriate antibiotic.

Solutions to prepare before start

45 **PRM L. Liquid protoplast regeneration medium**
Stock B..... 1mL
Stock C..... 1mL

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Stock D..... 1mL
Stock AT..... 1mL
Stock TE (Trace Elements)..... 100µL
D-Mannitol (Sigma)..... 6g (6%)
dH_2O..... 100 mL

Autoclave
Add CaCl2 to 10mM at the final concentration just before use..... 1mL

PRM B. Protoplast regeneration medium bottom layer
Stock B..... 4mL
Stock C..... 4mL
Stock D..... 4mL
Stock AT..... 4mL
Stock TE..... 400µL
D-Mannitol (Sigma)..... 24g (6%)
Agar..... 2.2g (0.55%)
dH_2O..... QSP 400 mL

Autoclave
Add CaCl2 to 10mM at the final concentration just before use..... 8mL

PRM T. Protoplast regeneration medium top layer
Stock B..... 1mL
Stock C..... 1mL
Stock D..... 1mL
Stock AT..... 1mL
Stock TE..... 100µL
D-Mannitol (Sigma)..... 12g (6%)
Agar..... 0.4g (0.4%)
dH_2O..... QSP 100mL

Autoclave
Add CaCl2 to 10mM at the final concentration just before use..... 2mL

Mannitol 8%
D-Mannitol (Sigma)..... 32g
dH_2O..... QSP 400mL

Autoclave

Driselase from Basidiomycetes >10% (Sigma D9515)..... 100mg in 10ml 8% mannitol
note: solution may be prepared, aliquoted and stored @ -20°C before start *or* prepared fresh (Step 2)

PEG stock solution..... 300µL/transformation
MaMg stock solution.....150µL/transformation

Stocks solutions

46 **2xMaMg stock solution**
$MgCl_2, 6H_2O$..... 6.1g
D-Mannitol (Sigma)..... 8g
MES..... 0.2g
Dissolve all the ingredients in 90 mL dH_2O
Adjust the pH to 5.6 with 1M KOH
dH_2O..... 100 mL
Filter sterilize with 0.45µM filters and aliquot in 2mL microtubes. Store at -20°C.

PEG stock solution
$Ca(NO_3)_2, 4H_2O$..... 1.18g
HEPES..... 0.238g

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D-Mannitol (Sigma)..... 3.64g
PEG6000 (Sigma)..... 4g

Dissolve all but PEG in dH_2O in the order of the above expect the PEG6000. Make sure the chemical is completely dissolved before adding the next one. Bring up the volume to 30mL. Measure pH with pH paper and adjust pH with 1M KOH to 7.5 (pH between 7-8 is acceptable). Add PEG and incubate the solution in 37°C waterbath to dissolve it. Shake occasionally. Top up to 50 mL with dH_2O. Filter sterilize with 0.45µM filters and 10mL syringe and aliquot 1mL in 1.5mL microtubes. Store at -20°C.

Stock solution B

$MgSO_2, 7H_2O$..... 25g
dH_2O..... to 1L

Stock solution C

KH_2PO_4..... 25g
dH_2O..... to 800mL

Adjust pH to 6.5 with minimal volume of 4 M KOH. Then make up to 1L with additional dH_2O.

Stock solution D

KNO_3..... 101g
$FeSO_4, 7H_2O$..... 1.25g
dH_2O..... to 1L

Stock solution AT

$C_4H_{12}N_2O_6$..... 92g
dH_2O..... to 1L

Stock solution trace element (from Harrison Lab)

H_3BO_3..... 614mg
$AlK(SO_4)_2, 12H_2O$..... 55mg
$CuSO_4, 5H_2O$..... 55mg
KBr..... 28mg
LiCl..... 28mg
$MnCL_2, 4H_2O$..... 389mg
$CoCl_2, 6H_2O$..... 55mg
$ZnSO_4, 7H_2O$..... 55mg
KI..... 28mg
$SnCl_2, 2H_2O$..... 28mg
dH_2O..... to 1L

or alternative trace element (from NIBB PHYSCObase)

H_3BO_3..... 614mg
$CuSO_4, 5H_2O$..... 55mg
$MnCL_2, 4H_2O$..... 389mg
$CoCl_2, 6H_2O$..... 55mg
$ZnSO_4, 7H_2O$..... 55mg
KI..... 28mg
$NaMoO_4, 2H_2O$..... 25mg
dH_2O..... to 1L

Material to prepare before start

47 1 box of blue tips (autoclaved)
1 box of yellow tips (autoclaved)

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1 box of wide-bored yellow tips (cut yellow tips) (autoclaved)
2mL microtubes in a magenta tub (autoclaved)
2x 100µM stainless steel mesh in small magenta tub (autoclaved)
2x 250mL flask with wide neck (autoclaved)
Cellophane discs EDTA-treated (autoclaved)

Forceps
Rubber pipettors
Electric pipettors
Plastic pipettes
50mL Falcon tubes
15mL Falcon tubes
10mL round bottom plastic tubes
Tube racks for 50mL, 15mL and 2mL tubes or microtubes
Petri dishes 9cm x 5.5cm
Spatulas
Parafilm
Pens

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