Mike's Moss Transformation Protocol

(Compiled from various sources, mostly Cove Lab protocol) *Updated Aug 7, 2024*

I. Preparation (* = prep each time)

A.* 10 to 30 µg plasmid DNA in ≤30 µl.
I use Zymo midipreps for plasmids; it yields 200 μl 1.0±0.4 μg/μl plasmid. For oligo-mediated edits, use 10 μl of 25 μM double-stranded oligos after annealing with PCR machine. For transient/CRISPR transformations, use supercoiled plasmid.

B.* 7-day-old moss chloronema grown on BCDAT (1 plate/7 transformations, at best)
The moss should be 4-7 days old (from last chop) and not too dense. The more chloronemata, the better. If the starting material had a lot of gametophores, you might need to go through two rounds of chopping.

C.* Autoclave 2.0 g PEG-6000 in a 15 mL tube (1 tube/~20 transformations)
Add to 15 ml tube. Autoclave with cap loose covered with foil.

D.* 8.5% D-Mannitol Solution (~15 mL/transformation + 50 ml/protoplast strain)

E. 10.3% D-Mannitol Solution (9 ml/protoplast strain)

F. 1% MES pH 5.6 (autoclave) (frozen aliquots in freezer) (1 ml/protoplast strain)

G. 1 M $MgCl_2$ (autoclave) (frozen aliquots in freezer) (150 µl /protoplast strain)

H. 1 M Tris•HCl pH 8 (autoclave) (frozen aliquots in freezer) (50 µl/~20 transformations)

I. 1 M $Ca(NO_3)_2$ (autoclave) (500 µl/~20 transformations)

J.* PRML medium=BCD + 2.5 mM DAT, 8% D-mannitol, 0.08% Agar, 10 mM $CaCl_2$ (@50°C)
or PRMT medium=BCD + 2.5 mM DAT, 8% D-mannitol, 0.5% Agar, 10 mM $CaCl_2$ (@50°C)
or 1.5% alginate+ 8.5% 8.5% D-mannitol plus 8.5% D-mannitol + 10 mM $CaCl_2$

K.* PRMB plates BCD + 2.5 mM DAT, 6% D-mannitol, 0.8% Agar, 10 mM $CaCl_2$ (@50°C)
(need 3 or 4 plates per transformation plus 1-2 plates per starting strain.)
The more transformants you expect, the more plates you need. I generally split single-plasmid transformations to four plates and split two/three-plasmid transformations to three plates.

L.* BCDAT selection plates (not needed until Day 5-8)

II. Day 1 *Before starting, set water baths to 45°C and 65°C and turn on burner.*

A. Prepare 20 mg/ml Driselase + 3 mg/ml Cellulase R-10 Solution in 8.5% D-Mannitol. Leave 15 minutes at RT, Centrifuge 2500 *g* for 10 min. Filter sterilize (0.22µ) supernatant.
Take driselase out of freezer and warm it up before opening so that water doesn't condense inside the bottle. I make 5 ml if I only have a few transformations and 10 ml if I need more. Adding Cellulase R-10 seems to improve digestion. I leave them to dissolve in the hood with the light off, inverting the tube occasionally. I usually filter-sterilize the driselase/cellulase directly to the moss+mannitol tube in step C to save a tube.

B. Collect protonema to a sterile 50 ml tube and add ~5 ml 8.5% D-Mannitol per plate of tissue using cell scraper and/or forceps.
Choosing how many plates of moss to harvest is tricky. If all goes well, you should get enough for at least 7 transformations per plate. I tend to start with more than enough if available, but this can be wasteful. I add 3 ml 8.5% Mannitol per ml of driselase solution being prepared.

C_1. Add 1/3 volume of 20 mg/ml Driselase Solution (5 mg/ml final). Incubate 30 to 60 at RT with occasional gentle shaking (<2 hours). While waiting, complete steps C_2.
I set the tube in a clean tray on the shaker under the centrifuge set at the slowest speed. Protoplasts are fragile. If you don't see clumps breaking up within 30 minutes, there may be a problem.

C_2. Start PEG/T preparation: add 4.45 ml 8.5% D-mannitol 500 µl $Ca(NO_3)_2$ 50 µl Tris pH 8, to a sterile PEG tube. Dissolve at 65° then allow to cool completely before use.
I combine the ingredients and put in the 65°C bath, eat lunch, then return and vortex the PEG/T. It should be mostly-but-not-completely melted/dissolved. Return to 65°C for ~10 more minutes and vortex until completely dissolved. Chill—it needs to be at room temperature by step L.

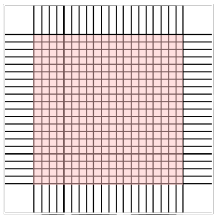
D. Filter digested tissue through 70 µ strainer. (or 1st through 100 µ strainer then 40 µ)
I usually start the filtering the protoplasts after 1 h. I generally filter through a 70 μ strainer, but it sometimes clogs, taking a lot more time to filter. Filtering first with a 100 μ strainer, then though a 40 μ strainer seems to clog less and results in less debris in the prep (but wastes more plastic). Use heat-sterilized forceps to place the strainer(s) into new 50 ml Corning tubes, and add digested cells using a sterile transfer pipet.

E. Pellet at 100-200 *g* for 4 minutes with no braking (1000 RPM in CL2 centrifuge = 167*g*).
I use the CL2 centrifuge at 1000 RPM. CJ used the Eppendorf at 250*g*.

F. Pour off supernatant in one motion. Resuspend in 10 ml 8.5% D-Mannitol.
Immediately after it stops, take tube out and pour into a waste beaker in one motion then recap immediately to limit contamination. If you hesitate, the pellet will be partially resuspended.

G. Repeat centrifugation and resuspension. CJ did a 3rd wash.

H. Count protoplasts and prepare enough fresh MMM Medium
Volume (ml) MMM needed = average hemocytometer count ÷ 17
The hemocytometer and counter are in a box above the Eppendorf centrifuge. Swirl tube to get protoplasts fully suspended, sterilely add ~20 µl to both halves of the hemocytometer, then cover with coverslip. Under the microscope, locate one of the central 1 mm × 1 mm grids and count the intact (spherical) protoplasts. Repeat with the other 1 mm × 1 mm grid. *Math: the coverslip sits 100 µm over the grid, so the volume (1 mm × 1 mm × 0.1 mm) is 0.1 µl. Multiply the average of the two counts by $10^4 \times 10$ to get the total number of protoplasts in the 10 ml. Divide that number by 1.7×10^6 to calculate the number of ml of MMM needed to get 1.7×10^6 protoplasts/ml.*



I. Pellet as before and resuspend in calculated volume of MMM.
10 ml MMM = 8.85 ml 10.3% D-Mannitol, 150 µl 1 M $MgCl_2$, 1 ml 1% MES pH 5.6.

J. Add ≤30 µl plasmid DNA to 15 mL conical tubes, quick spin to get to tip
I pipet the DNA(s), protoplasts, and PEG to successively higher gradation marks on the tubes so that I don't have to change tips between samples. I use polystyrene tubes (Falcon 352095) instead of standard polypropylene tubes because cells may stick to them less.

K. Add 300 µl protoplasts

L. Add 300 µl PEG/T and mix gently
After adding the PEG/T to each sample, I gently swirl and tip the tube nearly horizontal to mix in droplets stuck to side of tube

M. 45°C for 5 minutes
Use smaller metal tube rack. While waiting, add ~2" water to an ice bucket and add ice until 20°C

N. 20°C for 5-10 minutes
After ~5 minutes, I dry the tubes with paper towel and put back in a dry rack in the hood.

O. Slowly add 10 ml 8.5% D-Mannitol and mix by swirling.
I gently invert them until the cells in the tip become unstuck. Leave tubes horizontal rather than vertical.

P. Incubate 1 hour (30 minutes to a few hours is okay). Meanwhile, label PRMB plates then add cellophanes.
Label them with at least the transformation number. I usually add a short description and the date.

Q. While waiting, melt the PRMT if you are using it instead of PRML, then add $CaCl_2$.

R. Pellet as before. I do 4 at a time.

S_1. If using PRMT (top agar), gently resuspend the pellets with 500 µl 8.5% D-Mannitol and get the plates ready for the first transformation. Add [# plates – 0.5] ml molten PRMT to the tube and immediately suck it back into the pipet and drip 1ml across each plate.
I usually drip ~8 drops to different areas of each plate and then spread around with tip before it solidifies.

S_2. If using alginate, gently resuspend the pellets with 1.5 or 2 ml of 10 mM $CaCl_2$ + 8.5% D-Mannitol (depending on whether you're plating to 3 or 4 plates). Add an equal volume of 1.5% alginate + 8.5% D-Mannitol to the cells then pipet 1 ml per plate.
The calcium causes the alginate to gel—add more $CaCl_2$ if you want it to gel faster/firmer, add less if it gels too quickly. With Sigma's A1112, 5 mM $CaCl_2$ gels well enough, but 3.75 mM did not. (Pierre-François Perroud uses for the alginate they use.) After moving to BCDAT plates (1 mM $CaCl_2$), the alginate seems to liquify some making cellophane transfers tricky. Don't use Fisher's 17777-2500; it gelled way too quickly with even 2.5 mM $CaCl_2$ and the gel disk shrunk ~60%.

S_3. If using PRML, resuspend with 2 ml (4 plates) or 1.5 ml (3 plates) and dispense 500 µl per plate.
PRML is faster and easier than PRMT but lowers the regeneration rate. I use it when I'm not worried about getting enough transformants. I use a 5 ml pipet to add 1.5 or 2 ml per transformation then use the same pipet to resuspend and pipet 500 µl to each plate. I use a transfer pipet or P1000 to pipet the others.

T. *Optional* I also recommend plating ~100 µl of the untransformed protoplast strain to a PRMB plate using the same method. This control is useful if you see poor regeneration or if the selection doesn't seem to have worked.

U. Tape and put in growth chamber

III. **Day 5-8**: Transfer cellophane to BCDAT selection plates: *25 µg/ml G418, 15 µg/ml Hygromycin, 50 µg/ml Zeocin, 75-100 µg/ml Gentamicin, 125-150 µg/ml kanamycin.*

I always had moved cellophanes to selection after 4 days on PRMB, but Pierre-François' lab says you get more transformants if you wait 7 days. This increased transformation rate may be true, but I also saw an increased rate of plasmids meant to be maintained transiently that were stably integrated somewhere in the genome. For the no-DNA control plate, I cut the cellophane with a heat-sterilized scalpel and move strips to a plain BCDAT plate and to plates with each antibiotic. This has proven to be a helpful control a few times.

CRISPR deletions and CRISPR+oligo-mediated editing:

As soon as resistant plants are distinguishable (~week on selection), pick 24-96 to non-selective BCDAT. After growing for 7-14 days, pick a few cells from edge of each protonema to a new plate (reduces mosaicism). After growing for 7-14 more days, transfer a few cells to a new plate and use the rest for DNA preps for genotyping. PCR across the deletion/edit site. Confirm absence of the Cas9 plasmid by PCR or antibiotic resistance.

Targeted gene replacement with integrated selection cassette (with or without CRISPR facilitation):

After 7 days on selective media, transfer either the whole cellophanes or many transformants to non-selective media. After growing for 7-14 days, pick a few cells from edge of each protonema to a new selection plate (for the inserted resistance cassette, not the Cas9 plasmid's) and grow for 10+ days. Transfer a few cells to a new plate and use the rest for DNA preps for genotyping. PCR across both sides of the insertion site and confirm the absence of the deleted sequence and of Cas9.