Mike's Moss Transformation Protocol

(Compiled from various sources, mostly Cove protocol) *Updated 3/25/2019*

I. Preparation

A. 8.5% D-Mannitol Solution (10 ml/transformation + 55 ml/protoplast strain)
B. 10.28% D-Mannitol Solution (9 ml/protoplast strain)
C. 1% MES pH 5.6 (autoclave) (1 ml/protoplast strain)
D. 1M $MgCl_2$ (autoclave) (150 µl/protoplast strain)
E. 1M $Ca(NO_3)_2$ (autoclave) (500 µl/~20 transformations)
F. 1M Tris•HCl pH 8 (autoclave) (50 µl/~20 transformations)
G. Autoclave 2.0 g PEG-6000 in a 15 ml tube (1 tube/~20 transformations)
H. PRML medium BCD + 2.5 mM DAT, 8% D-mannitol, 0.08% Agar + 10 mM $CaCl_2$ (@50°C)
I. PRMB plates BCD + 2.5 mM DAT, 6% D-mannitol, 0.8% Agar, 10 mM $CaCl_2$ (@50°C)
J. 5-day-old moss chloronema grown on BCD+DAT + 0.6 mM $CaCl_2$ (± glucose)
K. 10 to 30 µg of each plasmid DNA in ≤30 µl total. For transient/CRISPR transformations, use supercoiled plasmid; for non-CRISPR-facilitated stable transformations, linearize the plasmids, then extract, ethanol precipitate, dry, and resuspend with ≤30 µl sterile TE.
L. BCD+DAT Medium (800 ml): 1.6 ml 500 mM $MgSO_4$; 1.6 ml 616 mM KH_2PO_4/303 mM K_2HPO_4 pH 6.5; 8 ml 1 M KNO_3; 800 µl Trace element solution; 10 mg $FeSO_4•6H_2O$; 6.4 g Agar; 0.74 g diammonium tartrate; 800 µl 1 M $CaCl_2$ (add @50°C)

II. Day 1

Before starting, set water baths to 45°C and 65°C, start DNA precipitation.

A. Prepare 2% Driselase Solution in 8.5% D-Mannitol (0.2g/10ml) 15 minutes at RT, Centrifuge 2500 *g*, Filter sterilize supernatant.
B. Collect protonema to a sterile 50 ml tube, pipet away excess liquid, and add ~15 ml 8.5% D-Mannitol per plate of tissue.
C_1. Add 1/3 volumes of 2% Driselase Solution (0.5% final). Incubate 30 to 60 at RT with gentle shaking (<2 hours). While waiting, complete step C_2.
C_2. Start PEGT 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.
D. Filter digested tissue through 70 µ mesh basket. (or 1st through 100µ basket then 40/70µ)
E. Pellet at 100-200 *g* for 4 minutes with no braking (1000 RPM in CL2 centrifuge).
F. Resuspend in 10 ml 8.5% D-Mannitol. Re-pellet and resuspend two additional times.
G. Count protoplasts and prepare enough fresh MMM Medium
Total # protoplasts = [# protoplasts in 1 mm^2 (5×5) grid] x [# ml protoplasts] × 10^4
MMM = 8.85 ml 10.28% D-Mannitol, 150 µl 1M $MgCl_2$, 1 ml 1% MES pH 5.6.
H. Pellet as before and resuspend in MMM at $2×10^6$ cells/ml. (= hemocytometer count ÷ 20)
I. Add ≤30 µl plasmid DNA to 15 ml conical tubes
J. Add 300 µl protoplasts
K. Add 300 µl PEGT (swirl to mix and incubate for ~5 minutes)
L. 45°C for 5 minutes
M. 25°C for 10 minutes
N. Gently add 10 ml 8.5% D-Mannitol and mix by swirling
O. Incubate for 1 hour (from 30 minutes to multiple hours is okay.) While waiting, label PRMB plates and place cellophanes on surfaces.
P. Pellet cells as before
Q. Resuspend in 2 ml PRML.
S. Dispense 500 µl per PRMB+cellophane plate.
T. Put in growth room for 4-5 days

III. **Day 5 or 6**: Transfer cellophane to Selective BCD(±DAT) plates (grow 10-17 days)
20 µg/ml G418, 15 µg/ml Hygromycin, 50 µg/ml Zeocin, 75-100 µg/ml Gentamicin

IV. **Day ~13**: Transfer cellophane to non-selective BCDAT plates (grow 7-14 days)

V. **Day ~21**: Pick a few cells at 2-3 edges to selective media (grow 10+ days)

VI. **Day ~35**: Check insertion site PCR (5' native/foreign, 3' native/foreign, empty site)