Mike's Moss Transformation Protocol

(Compiled from various sources, mostly Cove protocol) *Updated 2/11/2018*

I. Preparation

A. 8.5% D-Mannitol Solution (8+ mL/transformation + ~30 mL/protoplast strain)
B. 10.28% D-Mannitol Solution (10 mL/protoplast strain)
C. CaPW (8.5% D-Mannitol + 10 mM $CaCl_2$; autoclave) (10 mL/protoplast strain)
D. 1% MES pH 5.6 (autoclave) (1 mL/protoplast strain)
E. 1M $MgCl_2$ (autoclave) (<1 mL/protoplast strain)
F. 1M $Ca(NO_3)_2$ (autoclave) (<1 mL/protoplast strain)
G. 1M Tris•HCl pH 8 (autoclave) (50 µL/~20 transformations)
H. Autoclave 2.0 g PEG-6000 in a 15 mL tube (1 tube/~20 transformations)
I. PRMT medium BCD + 2.5 mM DAT, 8% D-mannitol, 0.4% Agar + 10 mM $CaCl_2$ (@50°C)
J. PRMB plates BCD + 2.5 mM DAT, 6% D-mannitol, 0.8% Agar, 10 mM $CaCl_2$ (@50°C)
K. 5-day-old moss chloronema grown on BCD+DAT + 0.6 mM $CaCl_2$ (± glucose)
L. 10 to 30 µg linearized plasmid DNA in <30 µL (Digest, extract, ethanol precipitate, dry)
M. BCD+DAT Medium (700 ml): 700 µl 500 mM $MgSO_4$; 700 µl 616 mM KH_2PO_4/303 mM K_2HPO_4 pH 6.5; 7 ml 1 M KNO_3; 0.7 ml Trace element solution; 8.75 mg $FeSO_4•6H_2O$; 5.6 g Agar; 0.644 g diammonium tartrate; 1 mM $CaCl_2$ (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 occasional gentle shaking (<2 hours). While waiting, complete steps C_2 to C_5.
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.
C_3. Pellet the plasmid DNA, rinse with 70% ethanol, aspirate, dry, and resuspend to 1µg/µl
C_4. Melt PRMT and add 10mM $CaCl_2$
C_5. Label and place cellophanes on plates
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 CaPW. Repeat once but use 8.5% D-Mannitol.
G. Count protoplasts and prepare enough fresh MMM Medium
Total # protoplasts = [# protoplasts in 1 mm^2 (5x5) grid] x [# mL protoplasts] x 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 $1.67x10^6$ cells/mL
I. Add <30 µL linearized plasmid DNA to 15 mL conical tubes (or round-bottom tubes)
J. Add 300 µL protoplasts
K. Add 300 µL PEGT (5 minutes)
L. 45°C for 5 minutes
M. 25°C for 10 minutes
N. Add 300 µL 8.5% D-Mannitol and swirl, **repeat 4 additional times** at ≥1 intervals.
O. Add 1 mL 8.5% D-Mannitol and swirl, **repeat 4 additional times** at ≥3 intervals.
P. Pellet as before (if round-bottom tubes were used, transfer to conical tubes.)
Q. Resuspend in 1 mL 8.5% D-mannitol.
R. Add 5 mL molten PRMT (45°C).
S. Dispense 2 mL per PRMB+cellophane plates.
T. Put in growth room

III. Day 5 or 6

Day 5 or 6: Transfer cellophane to Selective BCD(±DAT) plates (grow 10-17 days)
20-50 µg/ml G418, 15-30 µg/ml Hygromycin, 50 µg/ml Zeocin (replace weekly?)*, 100-150 µg/ml Gentamicin*

IV. Day ~20

Day ~20: Move (or pick resistant protonemata) to non-selective media (grow 7-14 days)

V. Day ~34

Day ~34: Pick a few cells at 2-3 edges to selective media (grow 10+ days)

VI. Day ~48

Day ~48: Check insertion site PCR (5' native/foreign, 3' native/foreign, empty site)