# Bezanilla Lab

# Moss Methods Manual

April 2008

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# Contents

# Tissue Propagation

Moss tissue should be propagated weekly. For routine culturing of moss, the following unusual items are needed:

Cellophanes
Tissue homogenizer

These items can be acquired from the following sources:

Cellophanes:

Gel drying cellophanes from Research Products International, catalog #1080

It is ideal to have the cellophanes cut in circles the appropriate size for standard disposable Petri dishes. It is possible to acquire a "hole" punch that will cut out circles of the correct size from the following arts and crafts site: http://www.1stopsquare.com/gigapunc.html Order the Paper Punch Craft, Marvy Uchida Giga - Circle 3".

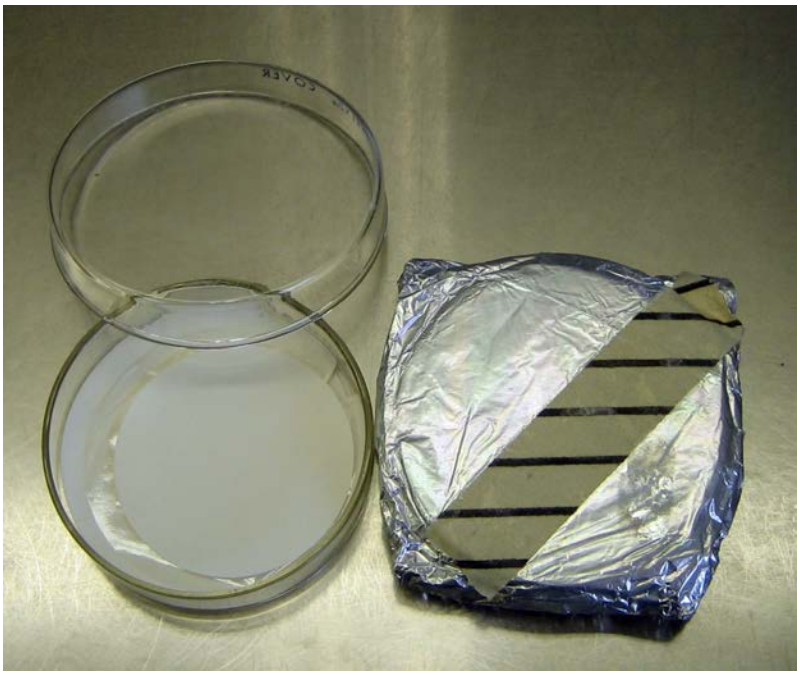
Tissue Homogenizer:

Powergen 125 homogenizer – Fisher Scientific 14-261-02
Adaptor for disposable generators – Fisher Scientific 14-261-35
Disposable generators 110 mm in length – Fisher Scientific 14-261-26

These generators can be placed in glass culture tubes, covered with aluminum foil and autoclaved. After use they can be washed in water, air-dried and re-autoclaved. Typically these generators can be re-autoclaved at least 20 times.

**Preparing Cellophanes**

The cellophane comes on 11" rolls, which can be cut into 8-1/2 X 11" sheets with a paper cutter. Then stack 3 layers of cellophane alternating with 3 layers of copy paper, staple the stacks in the corners, and cut out the circles. The punch works best with copy paper on top of the stack—you can cut 8 circles from an 8.5 X 11” stack. The stacks of circles (paper and cellophane) can be put directly in glass Petri dishes that are then wrapped in aluminum foil and autoclaved on a liquid cycle. The paper keeps the cellophane from sticking together during autoclaving.



**Weekly Tissue Propagation**

All manipulations should be carried out with sterilized items and in a laminar flow hood.

Prepare as many $PpNH_4$ plates as you will need by layering a cellophane onto each plate.

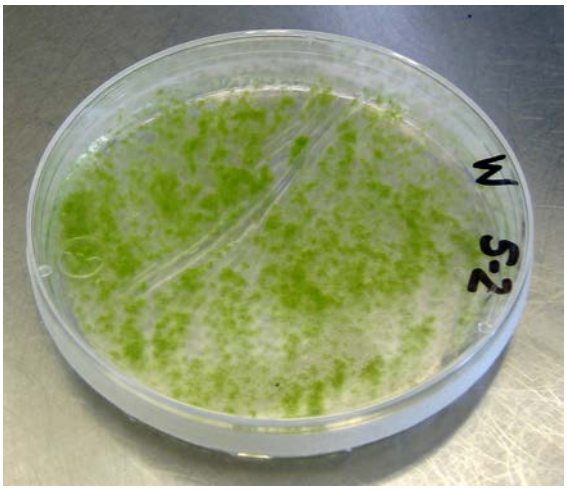
Using a metal spatula gently scrape moss tissue off the 1-week old plate. Put in 4 mls of $H_20$ in a 15 ml conical tube. Use the tissue homogenizer to grind the tissue until all large chunks have been disrupted and the water is uniformly green. With the Powergen 125 model, it usually takes around 10-30 seconds with the maximum power setting.

Plate 0.5 mls per plate. Gently swirl the plate to evenly distribute the tissue.

To check for contamination, a small amount of homogenized tissue can be plated on a LB plate with no antibiotics and stored at room temperature for 1 week

For routine culturing, plates dry easily unless they are well sealed with micropore tape.

Growth conditions:
We use Percival chambers that are set at 25°C and have light for 16 hours and dark for 8 hours.



## Longer-Term Propagation and Generation of Gametophores

Protonemal tissue from routine culturing, or tissue from plants of any age can be propagated by moving small pieces of tissue with forceps and placing in spots directly onto $PpNH_4$ plates (with no cellophanes) – this can be considered spot inoculums. Gametophores should appear within 3-4 weeks.

Plants that have grown up from spot inoculums can be expanded by putting a plant ~2 cm in diameter into 2 mls of $H_20$. Grind with tissue homogenizer and plate 0.5 mls per plate onto a cellophane-covered $PpNH_4$ plate.

# Cleaning a line of contamination

In order to prevent the selection of antibiotic microbes, it is recommended that this treatment be used only if there is no other source of healthy tissue.

**Antibacterial stocks (250mg/ml - 1000X)**

- **Cefotaxime**
- **Timentin**
- **Vancomycin**

**Antifungal – Fungigone (Plant Media) comes as 100X Stock**

1. Add Antibacterials to $H_2O$ at proper dilution before adding Fungigone. (Fungigone will form an insoluble precipitate with antibacterials if combined before adding to water).

2. Add contaminated tissue and grind using tissue homogenizer.

3. Let the ground tissue sit in the antimicrobials for at least two hours.

4. Plate onto $PpNH_4$ and let grow for 7-10 days.

5. Take healthy green tissue, homogenize it and plate on a new $PpNH_4$ plate. Check for contamination by plating a small amount on an LB plate with no antibiotics. Store plate at room temperature for 1 week.

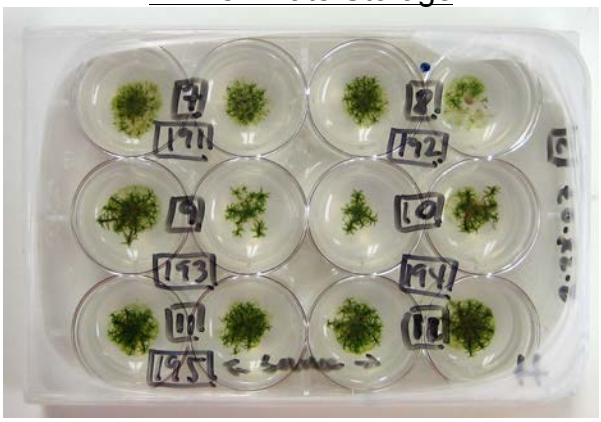
# Tissue Storage

Fill 12-well plates or individual screw cap tubes with $PpNH_4$ agar. Put single spot inoculums into each well. Allow these plants to grow robustly under normal growth conditions. When the plants are at least 1-2 cm in diameter remove and put in storage:

> Percival Chamber set to 4°C, no light for 22 hours and 6°C, light for 20 hours

Plants can be stored for at least 2 years (at least for 12-well plates) under these conditions. To recover from long-term storage, a portion of the plant should be picked and placed on a fresh plate. If the plant has been in storage for < 6 months, the whole plant can be ground in water and plated onto cellophane-covered plates.

12-well Plate Storage



Individual Tube Storage

Potential advantages – takes up less space, and minimizes contamination.

# Generating Protoplasts

**Items Needed in Advance:**

2% Driselase (Stored at -20°C, thaw at room temperature for use)
8.5% Mannitol (stored at room temperature)
Top agar PRMT or Plating Media (stored at room temperature)

PRMB plates covered with cellophanes are needed to plate transformations.
For most transient transformations, use 1 plate per transformation and plate in liquid plating media. Seal the plate with micropore tape.
For stable transformations, use 2 plates per transformation and plate in top agar (PRMT), seal with micropore tape.

Filters for separating protoplasts from undigested tissue:

Home-made:

Purchase ~100 µm metal mesh. Purchase plastic funnels (~7 cm diameter, ~12 cm in length). Heat the plastic to near melting in a bunsen burner and place directly onto metal mesh. Once the plastic has cooled, cut off the excess mesh. This process is messy but by melting the plastic onto the wire mesh it will withstand autoclaving indefinitely. These funnels can then be placed in an Erlenmeyer flasks and covered with aluminum foil for autoclaving. These filters work very well, can be used indefinitely, and are very economical.

Alternative commercial source:

70 µm Cell Strainer – BD Falcon 352350
These are excellent and fit into the top of a 50 ml conical tube.

**Making protoplasts:**

1. Use 2-4 plates of 5-7 day old tissue. (For smaller amounts of tissue see below.)
2. Put 15 ml of Mannitol in a Petri dish.
3. Add tissue to the Mannitol.
4. Add 5 ml of 2% Driselase.
5. Incubate with shaking for 1 hr – 1 hr 15min.
6. Filter through 100 µm mesh.
7. Spin at 250g for 5 min.
8. Wash with 10 ml of Mannitol.
9. Spin at 250g for 7 min.
10. Wash with 10 ml of Mannitol.
11. Spin at 250g for 7 min.
12. Wash with 10 ml of Mannitol

Count Protoplasts:

Take ~10 µl of the protoplast solution and count in a hemocytometer.

**To make smaller numbers of protoplasts, we suggest the following:**

For 1 plate of 5-7 day old tissue:

Use 5 ml of Mannitol in a 6 cm Petri dish.
Digest with 1.5 ml of 2% Driselase.

For 1/3-1/4 plate of 5-7 day old tissue:

Use 1.5 Ml of Mannitol in a 3.5 cm Petri dish.
Digest with 0.5 ml of 2% Driselase.

# Transformation – PEG Mediated in Protoplasts

**PEG-mediated Transformation**

**Items Needed in Advance**

Protoplasts – Concentration required for Transformation:
$2X10^6$ protoplasts/ml
PEG – for highest efficiency transformation make fresh (can store at -20°C, bring up to room temp for use)
3M (stored at 4°C, use at room temp)
Beaker of water
45°C Water Bath

While preparing the protoplasts, aliquot desired DNAs into 15 ml conical tubes. Normal transformation takes 15-30 µg of highly pure maxi-prep DNA.

**Transformation Procedure:**

1. Spin protoplasts at 250g for 7 min.
2. Resuspend protoplasts at $2X10^6$ protoplasts/ml in 3M Solution.
3. IMMEDIATELY PROCEED AFTER 3M BUFFER.
4. Add 300 µl of protoplasts to 15 µg of DNA.
5. Shake well.
6. Add 300 µl of PEG.
7. Shake well.
8. Incubate at room temperature for 10 minutes.
9. Heat shock at 45°C for 3 min.
10. Put in a beaker of room temperature $H_2O$ for 10 min.

***** If using PRMT for plating, at this point melt the PRMT in the microwave. Add 1 ml of 500 mM $CaCl_2$ to each 50 mls of PRMT, shake well, and place PRMT to cool in 45°-55°C water bath. *****

11. Add Mannitol to 5 mls.
12. Incubate at room temperature for 30 min.
13. Spin transformations at 250g for 7 min.

***** If using liquid plating media, be sure to add 1 ml 500 mM $CaCl_2$ per 50 mls of media before plating. *****

14. Resuspend in 0.5 ml liquid plating media (for transients) or 2 ml of PRMT (for stables).
15. Plate 0.5 mls/plate (for transients) or 1 ml/plate (for stables) onto PRMB plates.

# Transformation – Particle Bombardment in Tissue

Items needed:

DNA
Petri dishes with filter paper to hold the macro-carriers.
100% EtOH
70 % EtOH
Petri dish containing 100% Isopropanol to soak the rupture discs.

Gold aliquot (see preparation instructions)

2.5 M $CaCl_2$ (store at -20°C)

0.1 M Spermidine (single-use aliquots are stored at -20°C at a concentration of 1M, be sure to dilute to 0.1 M befreo use)

Preparing the Gold

1. Weigh out 60 mg of 1 μm gold particles
2. Add 1 ml 70% Ethanol
3. Vortex 20 seconds
4. Let stand 5 minutes
5. Spin 1 minute
6. Remove supernatant
7. Add 1 ml dH2O
8. Vortex 10 seconds
9. Let stand 1 minute
10. Spin 1 minute
11. Remove supernatant
12. Repeat steps 7-11 three times
13. Add 1ml 50% glycerol and store at 4°C

Preparing the DNA

1. Vortex 50 µl aliquot of gold for at least 5 minutes.
2. While vortexing the tungsten, prepare tubes with your DNA. Put 2.5 µg of maxiprep, or 20 µl of miniprep into each tube and label with the name of the plasmid.
3. While vortexing, add 25 µl of tungsten OR gold to the DNA.
4. While vortexing, add 25 µl 2.5 M CaCl2.
5. While vortexing, add 10 µl 0.1 M Spermidine. (Spermidine are single use aliquots. Discard after done preparing all DNAs.)
6. Vortex for an additional 1 minute and let sit for 1 minute.
7. Spin at 4000 RPM for 30 seconds.
8. Remove the supernatant carefully and discard.
9. Add 70 µl 70% EtOH. Vortex for 10 sec and sonicate for 10 sec. Make sure the pellet is completely dispersed.
10. Spin at 4000 RPM for 10 seconds.
11. Remove the supernatant carefully and discard.
12. Add 70 µl 100% EtOH. Vortex for 10 sec and sonicate for 10 sec. Make sure the pellet is completely dispersed.
13. Spin at 4000 RPM for 10 seconds.
14. Remove the supernatant carefully and discard.
15. Add 24 µl 100 % EtOH. Vortex for 10 sec and sonicate for 10 sec. Make sure the pellet is completely dispersed.
16. From a completely dispersed sample, take 6 µl of the sample and place on macro-carrier (macro carriers should be wet in 100% Ethanol and dried on filter paper).
17. Let the gold/DNA dry before proceeding to bombardment

Bombardment conditions:

Turn the register on the helium to 1300.
Turn on PDS-1000.
Turn on vacuum.
Rupture discs of 900 psi (1100 psi also work) (soaked in Isopropanol)

For each shot, try to reach a vacuum of 24 in Hg. Place gold carrying macro-carrier 2 levels below the rupture disc and place the tissue two levels below the gold.

# DNA Isolation

Simple DNA prep for PCR genotyping:

1. Freeze a small amount of tissue in an eppendorf tube in liquid $N_2$.
2. Grind frozen tissue in eppendorf tube with chilled small blue pestle.
3. Add 400 µl Shorty Extraction Buffer, continue to grind until most of the large chunks of tissue have been eliminated. Vortex lysate thoroughly.
4. Spin 5 minutes at high speed in micro-centrifuge.
5. Transfer 300 µl of supernatant to a fresh eppendorf containing 300 µl isopropanol.
6. Mix by inversion and spin 10 minutes at top speed in micro-fuge.
7. Pour off liquid and dry pellet by letting it sit upside down on a paper towel. You should not expect to see a pellet, but there is DNA there. Worry only if you want to (worrying is unnecessary).
8. Once the tube is dry, add 400 µl TE (10 mM Tris pH 8, 1 mM EDTA) and resuspend by gently pipetting up and down. For full elution of the invisible DNA pellet, it is best to let the DNA elute overnight at 4°C.
9. Use 0.5-2 µl of prep per PCR reaction.

| Shorty Extraction Buffer | Stock Solutions | 500 ml |
|---|---|---|
| 0.2 M Tris-HCl, pH 9.0 | 1 M Tris-HCl | 100 ml |
| 0.4 M LiCl | 2 M LiCl | 100 ml |
| 25 mM EDTA | 0.5 M EDTA | 25 ml |
| 1% SDS | 10% SDS | 50 ml |
| $H_2O$ | | 225 ml |

Alternative DNA prep using a bullet blender

1. Prepare microcentrifuge tubes with beads to ~200uL marker. They do not need to be sterilized. Add 200 uL H20 to the beads.
2. Add plant tissue in the flow hood using sterile forceps. Be sure to label the side of the tube not the top (labels on the top will be removed by the bead beater).
3. Grind for 3 min at level 10, then add 200uL 2X Shorty buffer. Invert to mix.
4. Remove all liquid to a new tube and spin 5 min @ max speed.
5. Pour supernatant into a new tube and add 350 uL Isopropanol. Invert to mix.
6. Leave on ice 10 min, then spin 10 min @ max speed.
7. Remove supernatant and air-dry pellet.
8. Resuspend in 400 uL TE.

| 2X Shorty Buffer | Stock Solutions | Making 50 mL |
|---|---|---|
| 0.4 M Tris, pH 9.0 | 1 M Tris | 20 mL |
| 0.8 M LiCl | 5 M LiCl | 8 mL |
| 50 mM EDTA | 0.5 M EDTA | 5 mL |
| 2% SDS | 10% SDS | 10 mL |
| $H_2O$ | | 7 mL |

# Protein Isolation

## Making Protein Extracts from Tissue:

1. Harvest 1-2 plates of evenly grown protonemal filaments. Pat dry in a paper towel. Store in aluminum foil. Directly freeze in liquid $N_2$ or put at –80°C for future processing.

2. Take tissue from liquid $N_2$ and put in a pre-chilled mortar and pestle. Grind tissue evenly to a fine powder. Put powder into chilled eppendorf tubes or falcon tubes, depending on the quantity of the powder.

3. For protein extracts, put ~100-200 µl of powder in an eppendorf tube. Add 300 µl grinding buffer + more protease inhibitors. Vortex well.

4. Spin for 10 minutes at maximum speed in centrifuge at 4°C.

5. Remove supernatant to a clean tube and put on ice. There should be a largish green pellet. The supernatant will be yellowy and thick if the concentration of protein is high. Measure the concentration of the supernatant with a Bradford assay. A good extract will be ~1-10 mg/ml.

**Grinding Buffer**

| Final Concentration | Stock Solution | To make 50 ml |
|---|---|---|
| 100 mM NaPhosphate pH 7 | 1 M | 5 ml |
| 10 mM DTT | 1 M | 500 µl |
| 20 µg/ml Leupeptin | 10mg/ml | 100 µl |
| 20% Glycerol | 100% | 10 ml |

Methanol Precipitation may be required if your protein is less than 30 kDa.

**Methanol Precipitation**

1. Calculate the volume of sample needed for desired amount of protein to be loaded on gel.
2. Bring volume up to total of 400 µl with grinding buffer or water.
3. Add 100 µl Chloroform. Add 400 µl Methanol. Vortex well.
4. Spin 5 minutes at max speed in microfuge at room temperature.
5. Carefully remove as much of the upper phase as possible while leaving the interphase intact. There should be a visible white protein pellet on the interphase.
6. Add 400 µl Methanol. Gently tap tube to ensure that the Methanol and chloroform are well mixed.
7. Spin 5 minutes at maximum speed in microfuge at room temperature.
8. Remove the entire supernatant. Careful to not disturb the pellet. Air dry.
9. Add 1-2.5X SDS sample buffer to pellet. Boil 10 minutes. Mix well with pipet before loading into gel.

**Making Protein Extracts from Protoplasts:**

1. Harvest protoplasts by spinning 15 ml conical tubes in centrifuge. Pour out the mannnitol. Resuspend the pellet in the little bit of Mannitol that is still present. Transfer to an eppendorf tube. Spin at max speed for one minute. Take off supernatant. Proceed to next step or store pellets at –80°C.

2. Resuspend pellet in 400 µl grinding buffer (see above). Vortex to resuspend well.

3. Put in liquid $N_2$. Take out of liquid $N_2$, uncap tube, put in 37°C water bath. Let extract thaw completely. Vortex thoroughly, at least 30 seconds.

4. Repeat step #2 two more times. After final vortex, place on ice until all samples are done.

5. Spin at maximum speed for 10 minutes in the cold room. Move supernatant to a fresh tube. Proceed to methanol precipitation for western blot analysis.

**Methanol Precipitation**

1. Add 100 µl Chloroform. Add 400 µl Methanol. Vortex well.
2. Spin 5 minutes at max speed in microfuge at room temperature.
3. Carefully remove as much of the upper phase as possible while leaving the interphase intact. There should be a visible white protein pellet on the interphase.
4. Add 400 µl Methanol. Gently tap tube to ensure that the Methanol and chloroform are well mixed.
5. Spin 5 minutes at maximum speed in microfuge at room temperature.
6. Remove the entire supernatant. Careful to not disturb the pellet. Air dry, or brief speed vac, or place in 42°C heat block for <5 minutes.
7. Add 50 µl 1X SDS sample buffer to pellet. Boil 10 minutes. Mix well with pipet before loading into gel. (Loading 10 µl per lane may be sufficient for most westerns.)

# GUS Assays

## Histochemical GUS Staining Protocol

Make GUS staining buffer following the recipe below:

| Final Concentration | Concentration of Stock Solution | Volume required for 10 ml |
|---|---|---|
| 100 mM $NaPO_4$ pH 7.0 | 1 M | 1 ml |
| 10 mM EDTA pH 8.0 | 0.4 M | 0.25 ml |
| 0.5 mM $K_4Fe(CN)_6$ | 50 mM | 0.1 ml |
| 0.5 mM $K_3Fe(CN)_6$ | 50 mM | 0.1 ml |
| 0.1 % Triton X-100 | 10% | 0.1 ml |

X-Gluc stock is 20mg/ml in DMSO
Store X-Gluc and the K-salts at –20°C.

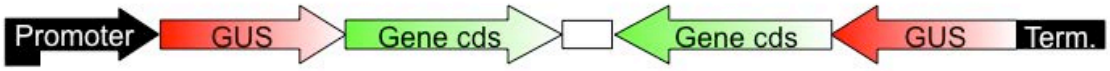
Add 1 µl of X-Gluc per ml of GUS staining buffer for staining.

1. Put tissue in staining buffer. (This works very well in multi-well plates – minimizes volumes needed.)
2. Add appropriate amount of X-Gluc to moss and staining solution.
3. Incubate for at 37°C. (1hr to overnight, could be less, especially for NLS-4) Incubation time will vary with your particular transgenic plant. This needs to be determined empirically.
4. Wash tissue with 70% ETOH.
5. Store in water.

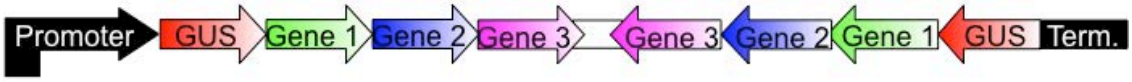
# RNA Interference

## Strategies for silencing a multi-gene family

Use highly conserved coding sequence in construct:




String together specific sequences for each gene in the construct:




For first strategy, choose at least a 400 bp region of cDNA that is > 85 % identical at the nucleotide level. For the second strategy, any region of cDNA can be used. At least 200 bp of each gene should be used. Using untranslated regions is best for performing complementation experiments.

**For first strategy: protocol for cloning cDNA fragment into Gateway RNAi vector**

Identify region of cDNA that will be amplified. Sometimes it is possible to identify a highly conserved region from a single exon. In this case amplification of the region can be performed using genomic DNA. It is not recommended to have introns in the construct. So if the region does contain introns, then amplification should be performed on cDNA.

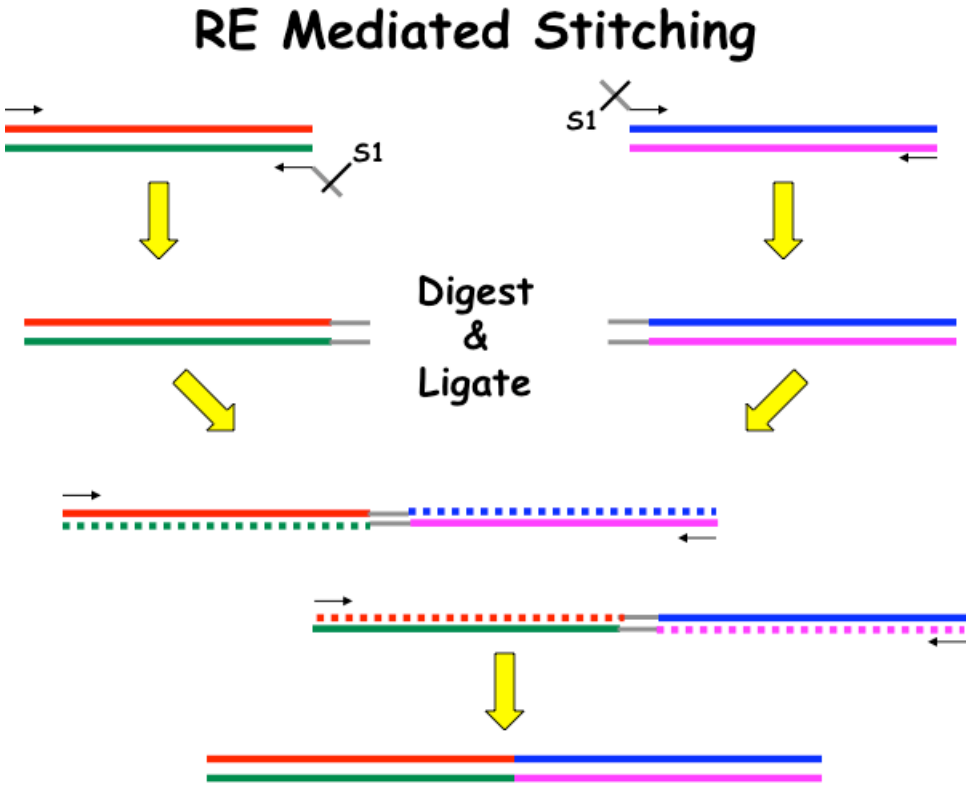
1. Clone PCR product into pENTR vector. pENTR/D-TOPO Kit catalog number: K2400-20
   Be sure to add CACC sequence to the forward primer. The reverse primer does not need anything special.
   It is important to use a blunt end producing polymerase, such as Phusion (can be purchased from NEB).

2. The pENT reaction is efficient (see Appendix III). Usually pick 4 colonies from the resulting ligation and isolate DNA. You can test for your insert by digesting the DNA with Not I/ Asc I. This should result in a vector only band of 2600 bp and a fragment the size of your amplified PCR product (as long as there are no Not I or Asc I sites in your amplified region). Sequence the resulting pENT clone using M13Forward and M13Reverse primers.

3. Perform LR clonase reaction (see Appendix III) with pENT clone and moss destination vector (pUGGi).
   Do the clonase reaction 1 hour or overnight. Make sure to transform DH5α or Top10 cells.

   Control moss vectors for moss transformation:
   pUGi – ubi promoter driving inverted repeats of GUS

**For second strategy: protocol for stitching cDNA fragments together**




1. Design primers to the fragments.

   Fragment 1:
   Forward primer: CACCfragment sequence
   Reverse primer: fragment sequenceRESTSITE (S1 in figure)

Fragment 2:
Forward primer: CACCRESTSITE (S1 in figure) fragment sequence
Reverse primer: reverse sequence

2. PCR fragments off appropriate template (genomic DNA if cDNA region of interest is in an exon, otherwise cDNA).

3. Gel Purify the fragments (we use the Qiaquick gel extraction kit from Qiagen). Elute with 50 µl of elution buffer.

   *Note: If you want to clone the individual fragments in pENT, then you will need to measure the concentration and proceed with a pENT ligation (described above) with a fraction of this PCR product.*

4. Digest the purified fragments with the appropriate restriction enzyme. Use a 50 µl reaction volume and use 40 µl of the purified fragment.

5. Use the PCR purification procedure (from the Qiagen kit) to remove the restriction enzyme after digestion.

6. Measure the concentration of your fragments. Set up the following 20µl ligation:
   15 ng fragment 1 + 15 ng fragment 2
   use 1 µl of ligase (we use T4 DNA ligase from NEB)
   use the rapid ligation buffer (2X) (we use the rapid ligation buffer from NEB)

7. PCR off the ligation mixture using the forward primer of fragment 1 and the reverse primer of fragment 2. Set up three PCR reactions. Use 1 µl of ligation mix as template, 1 µl of the 1:10 dilution of the ligation mixture, and 1 µl of the 1:50 dilution.

8. Gel purify the PCR reaction. Repeat this process for as many fragments as necessary. Once the product containing all the desired gene fragments is constructed, it can be cloned into pENT as described above.

**For transformation into moss:**
Make a maxiprep of the RNAi construct and the control (pUGi, or pZUGi). Transform 30 µg of DNA. Plate onto a single plate. On day 4, move to antibiotic selection. On day 7, use a fluorescent stereo microscope to identify and silenced plants. Take pictures of at least 25 silenced plants per transformation. It is best to do these experiments blinded to the observer.

## Isolating RNA from RNAi plants

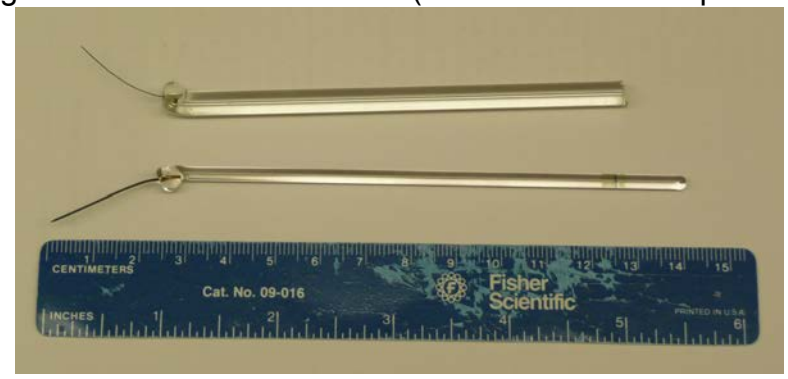
***Plant Isolation***

- Transform RNAi construct into NLS4 line. The number of plates you need will depend on the size of your silenced plants and the efficiency of your transformation.

    - If similar in size to control RNAi plants, 3-5 plates are more than sufficient.

    - Smaller plants will require a greater number of plates, the smallest plants will require 12-15 plates.

- Using a fluorescence stereo microscope identify and pick individual silenced plants using a fine needle or a thin wire (see below for example of needles).




- Transfer plants into an eppendorf tube with ~200µl sterile $H_2O$. Submerge the tip of the needle/wire in the water and then shake to get the plant off.

- Allow plants to settle to the bottom of the tube and then remove as much excess water as you can without losing plants. One way to do this is to use a twisted kimwipe to wick out the water from the tube. ***Be very careful to not touch the plants because they will stick to the kimwipe and you will lose them.***

- Immediately freeze in liquid $N_2$ or store at -80°C.

- Please note that you will have a **very** small amount of tissue so it is critical to ensure that your micropestles will actually reach to the bottom of the eppendorf tube. Otherwise, the tissue will not be thoroughly ground and the RNA concentration may be too low to work with.

***RNA Isolation***

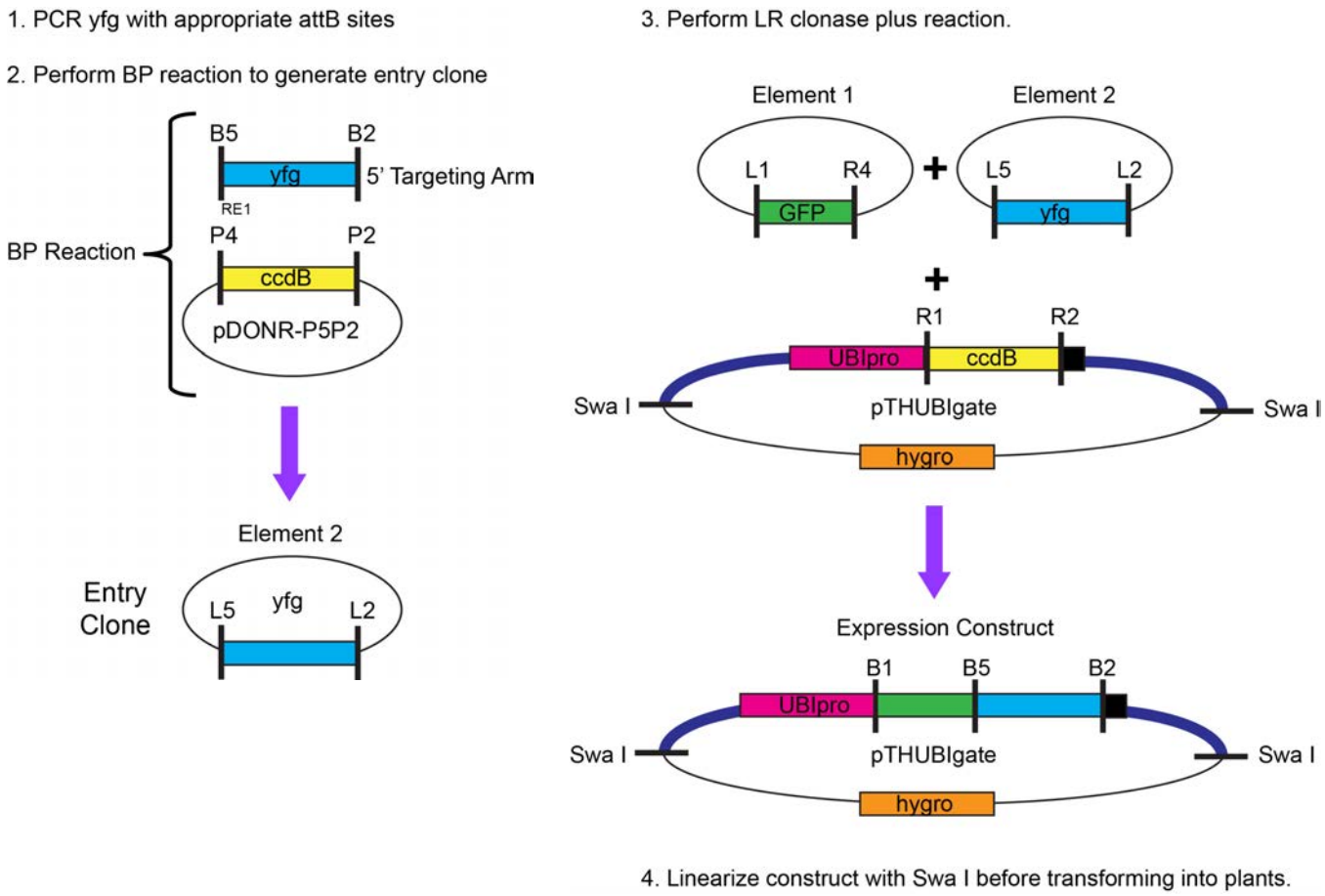
- We use the RNeasy plant mini kit (Qiagen) and protocol for RNA isolation from moss. To grind the tissue, remove tissue from liquid N2 and grind with micropestle that has been equilibrated in liquid N2. Grind as carefully as possible, add buffer with micropestle still in tube. Carefully keep grinding. Then remove the micropestle from the tube and vortex vigorously.

If isolating from ground tissue, harvest ~1/4 - 1/2 plate, remove excess water from tissue and immediately freeze in liquid $N_2$ or store at -80°C.

# 2-Fragment Recombination for N- and C- terminal tagging

We have generated a 2-fragment recombination system to tag your gene of interest. mEGFP, 3XmEGFP (3 tandem copies of mEGFP), mCherry, and 3XmCherry have been cloned into pDONR-P1P5r for N-terminal tagging and pDONR-P5P2 for C-terminal tagging.

As an example (see below), to tag your favorite gene (yfg) on the N-terminus with mEGFP, you would need to clone yfg into the pDONR-P5P2 donor vector. This is done by amplifying yfg with primers that incorporate attB5 and attB2 sites on the 5' and 3' ends, respectively. Then you perform a BP reaction to clone the amplified product into pDONR-P5P2. Once you have obtained this clone, you would perform a 2-fragment recombination reaction with mEGFP-P1P5r, yfg-P5P2, and your favorite destination vector, such as pTHUBI-gate. The resulting clone will be mEGFP-yfg in the pTHUBI vector. There is a small linker between mEGFP and yfg; it is an attb5 site. Following the instructions in the multi-site Gateway manual, it is pretty straightforward to design the primers for yfg so that it will result in frame with the mEGFP after the 2-fragment recombination reaction.



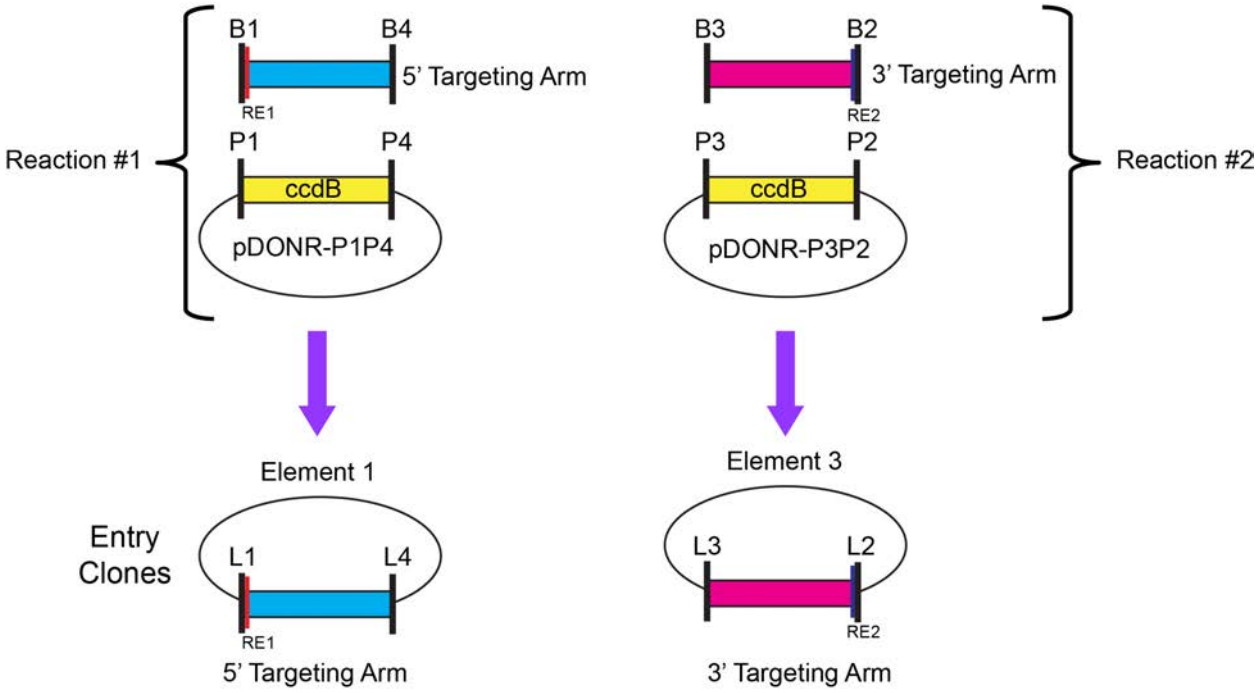
# 3-Fragment Recombination for Making Knock Outs

**Design a 5' targeting arm (element 1).** Design a 5' targeting arm. This should contain ~1 KB upstream of your gene. It is useful to include the start codon in the 5' targeting arm. The forward primer will have the attB1 site, followed by a restriction site and ~20 bp genomic sequence. In choosing a restriction enzyme, it is ideal to choose one that has a half site present in the genomic sequence. Also the site(s) should not be in the amplified genomic regions (5' or 3'), or the antibiotic resistance cassette (element 2). The reverse primer will contain the attB4 site, followed by ~20 bp genomic sequence. PCR amplify off of genomic DNA and perform a BP reaction to clone into pDONR-P1P4 vector. Frame does not matter.

**Design a 3' targeting arm construct (element 3).** This should contain ~1 KB downstream of your gene. It is useful to include the stop codon of your gene in the 3' targeting arm. The forward primer will have the attB3 site followed by genomic sequence and the reverse primer will contain the attB2 site, followed by a restriction site, followed by ~20bp of genomic sequence. In choosing a restriction enzyme, your should use the same considerations described above. PCR amplify and clone into pDONR-P3P2 vector. Frame does not matter.

1. PCR 5' and 3' targeting arms

2. Perform BP reactions to generate entry clones

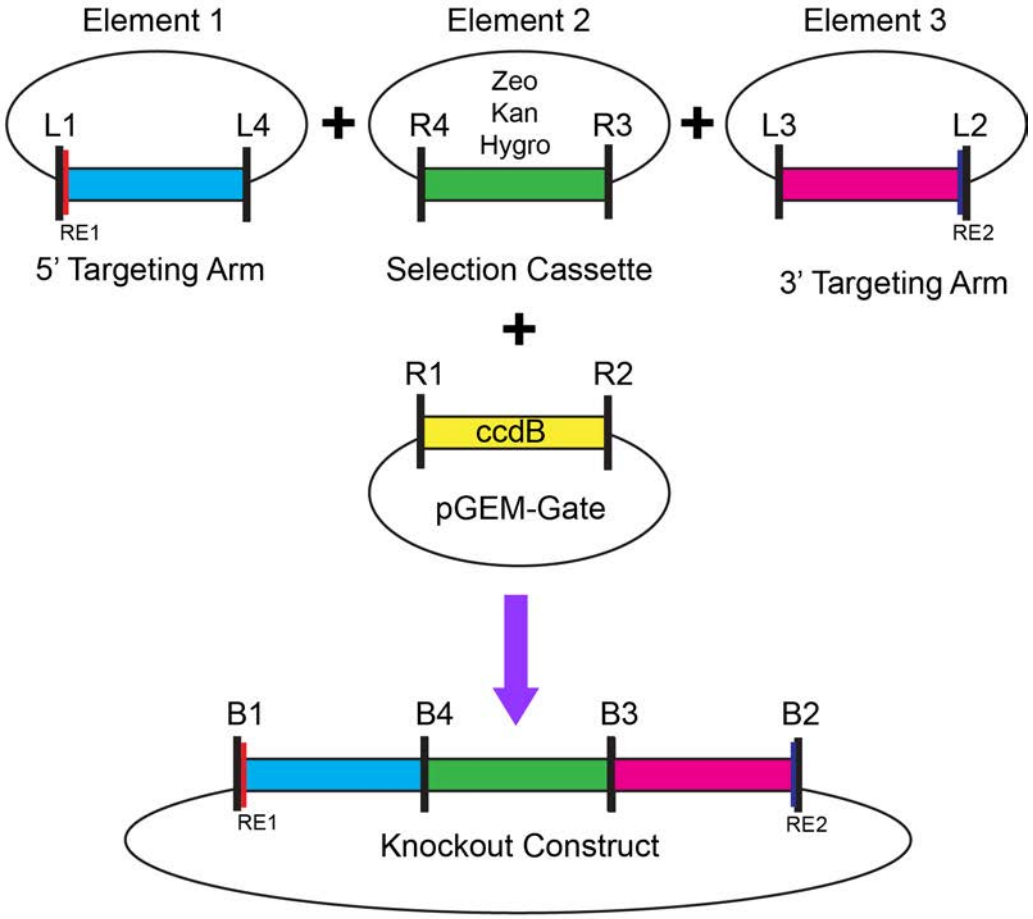



Useful Restriction Enzyme Sites: Swa I and Pme I (do not cut in element 2).

**Selection cassette (element 2).** Noster-Lox-35S::Hygromycin(or Zeo or Kan)-Lox is already cloned into the pDONR-P4rP3r vector with a BP reaction. The names of these plasmids are **Hygro-R4R3, Zeo-R4R3, and Kan-R4R3.**

When the three entry clones are made, perform LR reaction with LR clonase II plus into destination vector (pGEM-gateway). Make a maxiprep of the knock out construct to have enough DNA for several transformations. Finally, linearize knock out construct before transforming using designed restriction sites.

3. Perform LR clonase plus reaction.




4. Linearize construct with restriction enzymes (RE1 and RE2)

# 4-Fragment Recombination for Making Knock Ins

## For tagging the C-terminus of a gene:

### Design a 5' targeting arm that will replace the end of your gene of interest (Element 1).

Design primers to amplify ~1KB of sequence upstream of the stop codon.

-Forward primer begins with the attB1 sequence, followed by a restriction enzyme site that does not appear anywhere else in the construct. In choosing a restriction enzyme, it is ideal to choose one that has a half site present in the genomic sequence. Also the site(s) should not be in the amplified genomic regions (5' or 3'), the tagging cassette (element 2), or the antibiotic resistance cassette (element 3). In this example, we will use the restriction enzyme SwaI (a blunt cutter sequence ATTT/AAAT). Locate a genomic AAAT sequence ~1KB upstream of the stop codon followed by ~20bp. Incorporation of the SwaI site is used to linearize the final construct before transformation. It is critical that when the construct is digested with SwaI before transformation, no sequence is deleted and the frame is not shifted.

-Reverse primer contains the attB5r sequence, followed by ~20 bp of the genomic sequence that precedes the stop codon. Do not include the stop codon in the primer. PCR amplify off of genomic DNA, do BP clonase reaction to clone into pDONR P1-P5r vector.

Example:
Green = attB site, Blue = half of the SwaI site, (bold and underlined is the full SwaI site) Red = genomic sequence

Forward Primer- GGGGACAAGTTTGTACAAAAAAGCAGGCT
**ATTTAAAT**NNNNNNNNNNNNNNNNNNN

Reverse Primer:
GGGGACAACTTTTGTATACAAAGTTGTNNNNNNNNNNNNNNNNNNN

### Design 3' targeting arm that will replace the region downstream of your gene of interest (Element 4).

Design primers to amplify ~1KB downstream of the gene within ~200bp-500bp downstream of stop codon. Make sure the size of your 5' arm is very close to the size of your 3' arm. In the case of the 3' arm, staying in frame does not matter, but make sure a gene downstream will not be disrupted. The forward primer starts with the attB3 sequence, followed by ~20bp from the genomic sequence. The reverse primer contains the attB2 as well as the same restriction site used in the 5' targeting arm and a restriction enzyme half site (in this example a Swa I site) followed by ~20bp from the genomic sequence. PCR amplify off of genomic DNA and clone into pDONR P3-P2 vector.

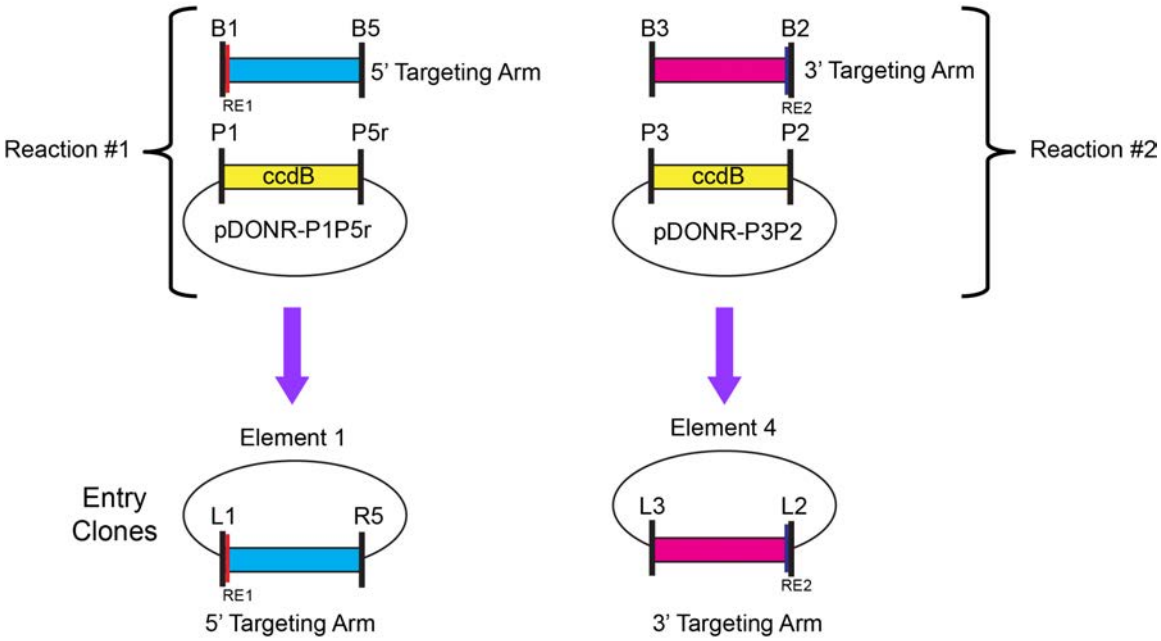
Example:
Green = attB site, Blue = half of the SwaI site, (bold and underlined is the full SwaI site) Red = genomic sequence

Forward primer-
GGGGACAACTTTGTATAATAAAGTTGCTNNNNNNNNNNNNNNNNNNNN

Reverse primer-
GGGGACCACTTTGTACAAGAAAGCTGGGTA**ATTTAAAT**NNNNNNNNNNNNNNNNNNNN

1. PCR 5' and 3' targeting arms

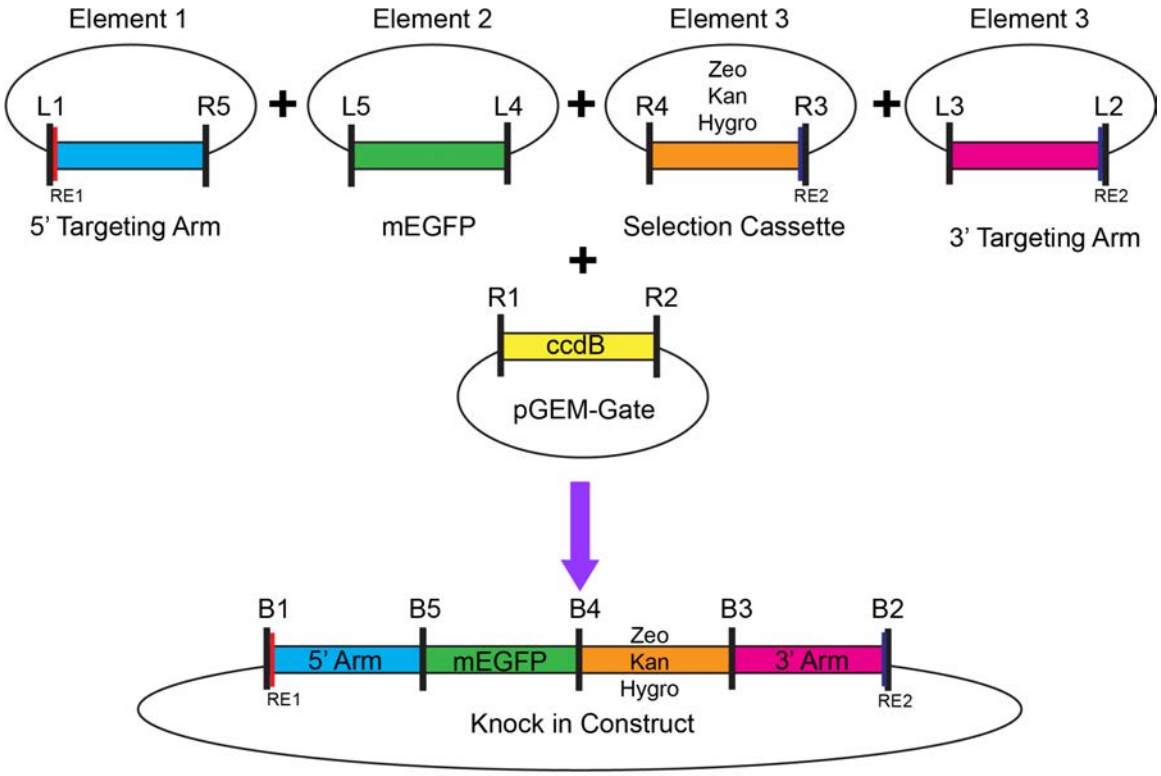
2. Perform BP reactions to generate entry clones



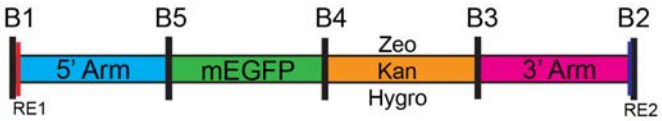

**Fluorescent protein tag (Element 2).**
In this example, GFP-Stop was cloned into the P5-P4 vector using a BP reaction. The name of the plasmid is **mEGFP-L5L4**.

**Selection cassette (Element 3).** In this example, Noster-Lox-35S::Hygromycin-Lox was cloned into the P4r-P3r vector with a BP reaction. The name of the plasmid is **Nos-Lox-Hygro-Lox-L4L3.**

3. Perform LR clonase plus reaction.




4. Linearize construct with restriction enzymes (RE1 and RE2)




**With all four pieces, perform 4-way gateway reaction with LR clonase II plus to put your construct into a destination vector (pGEM-gateway).**

**6. Linearize the knockin construct before transforming using designed restriction sites.**

## AFTER TRANSFORMATION:

1. In all cases, the protocol for after transformation is the same. Transfer plants to antibiotic after 4 days. One week later, transfer to $PpNH_4$. One week later, transfer to antibiotic resistance. One week later, transfer to $PpNH_4$. ~One week later, pick individual plants to antibiotic resistance.

2. Allow plants to grow large enough so they can be genotyped for the presence of the insertion.

3. You are ready to analyze!

## Linearizing Constructs for transformation to generate stable lines:

### Restriction digestions for stable transformations

- **60 μg DNA** (Good for two transformations)
- **30 μl appropriate restriction enzyme buffer**
- **H2O up to 295ml**
- **5 μl Restriction enzyme(s)**

- Mix well and incubate at appropriate temp for 2-4 hours, or overnight.

- Check 1 μl of digest on gel to ensure that the digest has gone to completion.

### Ethanol precipitate digest

- Add 30 μl of 3M Na-Acetate pH5.2
- Add 800 μl of 100% Ethanol
- Incubate at -20°C for at least 30 minutes
- Centrifuge 5 minutes at top speed
- Decant supernatant
- Add 500 μl of 70% Ethanol, spin 2 minutes at top speed
- Decant supernatant in a sterile laminar flow hood
- Air-dry pellet
- Resuspend pellet in 50 μl of sterile TE
- Incubate at 37°C for 10 minutes and mix to resuspend pellet
- Let stand at room temp for 10 minutes and resuspend until pellet is dissolved
- This is enough for two transformations, plate each transformation with top agar in 2-4 plates per transformation

### Alternative: PCR Amplification of constructs

The outermost primers used to make the targeting arms can be used to amplify the entire construct (alternatively primers lacking the attb sites), but to get enough DNA it is necessary to perform ~10 50μl PCR reactions, and then ethanol precipitate

# Appendix I – Moss Growth Media Recipes

## Micro Elements 1000X (used for $PpNH_4$ and $PpNO_3$):

Measure out the following chemicals with fine balance.

| | |
|---|---|
| $H_3BO_3$ (Amresco 0588-500G) | 614 mg |
| $CuSO_4 \cdot 5H_2O$ (Sigma C-6283) | 55 mg |
| $MnCl_2 \cdot 4H_2O$ (Sigma M-3643) | 389 mg |
| $CoCl_2 \cdot 6H_2O$ (Sigma C-3169) | 55 mg |
| $ZnSO_4 \cdot 7H_2O$ (Sigma Z-0501) | 55 mg |
| KI (Sigma P-4286) | 28 mg |
| $Na_2MoO_4 \cdot 2H_2O$ (Sigma S-6646) | 25 mg |

Add to 1 liter of $H_2O$. Aliquot 200 ml into 250 ml bottles. Autoclave and store at 4°C.

## Stock Solutions for $PpNH_4$:

| | |
|---|---|
| 127 g/L $MgSO_4 \cdot 7H_2O$ (Sigma 230391-500G) | 500X |
| 125 g/L $KH_2PO_4$ | 500X |
| 400 g/L $CaNO_3 \cdot 4H_2O$ (Sigma 237124-500G) | 500X |
| 250 g/L Di-ammonium tartrate (Sigma A4767-500G) | 500X |

Autoclave and store at 4°C.

**PpNH$_4$:**
**Recipe makes 4 liters of media.**

Fill Beaker with distilled $H_2O$ (~ 3.5 L).

| | |
|---|---|
| 8 ml | $MgSO_4 \cdot 7H_2O$ (500X) |
| 8 ml | $KH_2PO_4$ (500X) |
| 8 ml | $CaNO_3 \cdot 4H_2O$ (500X) |
| 50 mg | $FeSO_4 \cdot 7H_2O$ (Amresco 0387-500G) MUST WEIGH THIS OUT with fine balance |
| 4 ml | Micro Elements (1000X) |
| 8 ml | Di-ammonium tartrate (500X) |

Bring volume up to 4.0 L.

Add 7 g of agar (Sigma A9799-1KG) per liter of media.

Autoclave.

## $PpNO_3$ – Minimal Medium

Stock Phosphate Buffer for $PpNO_3$
Dissolve 25 g of $KH_2PO_4$ (Sigma P0662-500G) in 100 ml $H_2O$ and titrate to pH 7 with 4 M KOH.
This is a 1000X stock. Autoclave and store at 4°C.

Macro elements:

| | |
|---|---|
| $CaNO_3 \cdot 4H_2O$ (Sigma 237124-500G) | 0.8g/L |
| $MgSO_4 \cdot 7H_20$ (Sigma 230391-500G) | 0.254g/L |
| $FeSO_4 \cdot 7H_2O$ (Amresco 0387-500G) | 0.0125g/L |

MUST WEIGH THIS OUT!!

1ml/L of Micro element stock solution
1ml/L of Phosphate buffer

Add 7 g of agar (Sigma A9799-1KG) per liter of media.

## Protoplast Regeneration Media (PRMB)

Need 0.5 M $CaCl_2 \cdot 2H_2O$. This can be made in advance, autoclaved and stored at room temperature.

**Recipe makes 4 liters of media.**

Fill Beaker with 3.8 liters distilled $H_2O$.

| | |
|---|---|
| 8 ml | $MgSO_4 \bullet 7H_2O$ (500X) |
| 8 ml | $KH_2PO_4$ (500X) |
| 8 ml | $CaNO_3 \bullet 4H_2O$ (500X) |
| 50 mg | $FeSO_4 \bullet 7H_2O$ (Amresco 0387-500G) MUST WEIGH THIS OUT with fine balance |
| 4 ml | Trace Elements (1000X) |
| 8 ml | Di-ammonium tartrate (500X) |
| 240 g | Mannitol (Sigma M1902-1KG) |

Check pH is ~5.5, pH paper is ok.

Add 8 g of agar (Sigma A9799-1KG) per Liter of media.

Autoclave.

Let stand 25 minutes.

**Add 20 ml of 500 mM $CaCl_2 \bullet 2H_2O$ to each liter bottle. Shake very well.**

# Appendix II – Moss Transformation Reagents

## Stock Solutions Required for Transformation Buffers:

1M $Ca(NO_3)_2 \cdot 4H_2O$
Dissolve 236.1 g into 1 liter of $H_2O$.

1M $MgCl_2 \cdot 6H_2O$ (Amresco 0288-1KG)
Dissolve 203.3 g into 1 liter of $H_2O$.

1% MES (Sigma M-2933) pH 5.6
1 g in 100 ml of $H_2O$. Bring to correct pH with 0.1 M KOH.

1M Tris (Amresco 0497 10 kg) pH 8.0

Autoclave all solutions and store at 4°C.

8.5% Mannitol (Sigma M1902-1KG)
85 g in 1 liter
Autoclave. Store at room temperature.

## Driselase for Protoplasting:

Makes 200 ml:
Dissolve 4 g Driselase (Sigma D9515-25G) into 200 ml 8.5% Mannitol.
Gently stir for 30 minutes at room temperature.
Keep at 4°C for 30 minutes.
Stir 5 minutes at room temperature.
Spin at 2,500g for 10 minutes in 50 ml Falcon Tubes.
Filter sterilize with 0.22 µm filter.
Aliquot 10 ml into 15 ml Falcon Tubes.
Store at –20°C.

3M Solution for Transformation:

Makes 50 ml:

| | |
|---|---|
| 4.55 g | Mannitol |
| 750 µl | 1M $MgCl_2 \cdot 6H_2O$ |
| 5 ml | 1% MES pH 5.6 |

Add $H_2O$ to 50 ml.
Vortex well.
Filter sterilize with 0.22 µm filter.
Store at 4°C.

Recipe can be scaled up to 200 ml easily for use with larger filtration unit.

PEG Solution for Transformation:

1. Prepare Man/$Ca(NO_3)_2$ Solution.

| | |
|---|---|
| 9 ml | 8.5% Mannitol |
| 1 ml | 1M $Ca(NO_3)_2 \cdot 4H_2O$ |
| 100 µl | 1M Tris pH 8.0 |

   Vortex well. Filter sterilize with 0.22 µm filter.

2. Prepare PEG
   Weigh out 4 g of PEG 8000 (Sigma P-2139) into 50 ml Falcon Tube.
   Melt in microwave, watching carefully.

In sterile hood add 1 and 2 together. Vortex well. Make sure all is well dissolved. Solution can be used after 2 hours or can be stored at –20°C for long-term storage.

Liquid Plating Media:

$PpNH_4$ + 8.5% Mannitol.
Filter Sterilize.

**Before plating – Make sure to add Calcium.**
**Add 1 ml of 500 mM $CaCl_2 \cdot 2H_2O$ per 50 ml of media.**

Top Agar (PRMT):

Make 1 liter of $PpNH_4$ in 800 ml of $H_2O$.
Add 60 g of Mannitol
Bring up to 1 liter with $H_2O$.
Measure 300 mg of Agar for each of 20 100 ml bottles.
Add 50 ml of $PpNH_4$+Mannitol to the bottles.
Autoclave. Let solidify. Store at room temperature.

**Before plating – Make sure to add Calcium.**
**Add 1 ml of 500 mM $CaCl_2 \cdot 2H_2O$ per 50 ml of Top Agar.**

# Appendix III – Cloning Reactions

**pENT-D Topo ½ Reaction**

0.5 µl vector
0.5 µl salt solution (from kit)
2 µl $H_2O$ plus fragment:
    Use 0.5-2.5 ng of a 1kb PCR product
    or 2.5-5 ng of a 2 kb PCR product
    NOTE: the PCR product should be blunt. Using a polymerase such as Phusion will generate a blunt PCR product

Incubate for 5-30 minutes at room temperature.

Transform into competent cell:
Add 50 µl of cells to reaction above
Incubate on ice 20-25 minutes
Heat shock 1 min at 42°C
Put tube back on ice and recover for 2 min
Add 500 µl of LB
Recover with shaking at 37°C for 20-40 minutes
Spin down
Remove most of sup
Resuspend cells gently in 50-100 µl
Plate all onto LB-KAN

## BP Reaction

### 1/4 Reaction

3.75-37.5 ng attB PCR product
0.25 µl pDONR vector (supercoiled, 150 ng/µl)
up to 2 µl with 1X TE Buffer, pH 8.0
Add 0.5 µl BP clonase II enzyme mix

Mix well
Incubate at 25°C for 1 hr
Add 0.25 µl Proteinase K
Incubate at 37°C for 10 min

Transform all into 50 µl competent cells
Add 500 µl LB for recovery.
Spin down and plate all.

### 1/8 Reaction

1.8-18ng attB PCR product
0.13 µl pDONR vector (supercoiled, 150 ng/µl)
up to 1 µl with 1X TE Buffer, pH 8.0
Add 0.25 µl BP clonase II enzyme mix

Mix well
Incubate at 25°C for 1 hr
Add 0.13 µl Proteinase K
Incubate at 37°C for 10 minutes

Transform all into 20 µl competent cells
Add 150 µl LB for recovery
Spin down and plate all.

## LR Reaction

### 1/4 Reaction

12.5-37.5 ng (0.25-1.75 µl) entry clone
0.25 µl Destination vector (150 ng/µl)
up to 2 µl with 1X TE Buffer, pH 8.0

Thaw LR clonase II enzyme mix on ice for 2 min, vortex (2 sec)
Add 0.5 µl clonase
Return clonase to -80°C freezer

Incubate at 25°C for 1-16 hr
Add 0.25 µl Proteinase K
Incubate at 37°C for 10 min

Transform all into 50 µl competent cells
Add 500 µl LB for recovery.
Spin down and plate all.

### 1/8 Reaction

6.3-18.7 ng entry clone
0.13 µl Destination vector (150 ng/µl)
up to 1 µl with 1X TE Buffer, pH 8.0

Thaw LR clonase II enzyme mix on ice for 2 min, vortex (2 sec)
Add 0.25 µl clonase
Return clonase to -80°C freezer

Incubate at 25°C for 1-16 hr
Add 0.13 µl Proteinase K
Incubate at 37°C for 10 min

Transform all into 25 µl competent cells
Add 150 µl LB for recovery.
Spin down and plate all.