A simplified, robust, and streamlined procedure for the production of C. elegans transgenes via recombineering

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Stage I. Pre- *gal* K recombineering

1. Oligo design and ordering. We order 100 nM scale - gel purified oligos from IDT.

galK Recombineering

1. Design *galK* primers with 50 bp homology to an area flanking the desired site to be modified.

2. The 3' end of these primers bind to the *galK* cassette which is present in pMOD4 *galK*-G and pMOD4 *galK*-GT.

   Forward :

   5'-------50 bp homology -------CCTGTTGACAATTAATCATCGGCA-3'

   Reverse:

   5'-------50 bp homology compl. strand ------- TCAGCACTGTCCTGCTCCT-3'

galK-G Recombineering

1. Design pMOD4*galK*-G primers with 50 bp homology to an area flanking the desired site to be modified. Be sure to keep the fusion protein in frame. The ATG can be discarded if desired.

2. The 3' end of these primers bind to the *galK*-G cassette. (The first and last codons of GFP are underlined to demonstrate the reading frame).

   Forward :

   5'-------50 bp homology ------- ATGGATTACAAGGACGATGACGATAAGATGAG -3'

   Reverse:

   5'------- 50 bp homology compl. strand ------- CAAAGCTTGTGGGCTTTTGTATAG-3'

galK-GT C-term TAP Recombineering

1. Design pMOD4 *galK*-GT primers with 50 bp homology to an area flanking the desired site to be modified. Be sure to keep the fusion protein in frame.
2. The 3' end of these primers bind to the *galK*-GT cassette. (The first and last codons of TAP are underlined to demonstrate the reading frame).

Forward :

5'-------50 bp homology ------ ATGGAAAAGAGAAGATGGAAAAAG - -3'

Reverse:

5'------- 50 bp homology compl. strand ------- GGTTGACTTCCCCGC -3'

galK-GT N-term TAP Recombineering

1. Design pMOD4 *galK*-GT primers with 50 bp homology to an area flanking the desired site to be modified. Be sure to keep the fusion protein in frame.

2. The 3' end of these primers bind to the *galK*- GT cassette. (The first and last codons of TAP are underlined to demonstrate the reading frame).

Forward :

5'-------50 bp homology ------ ATGGCAGGCCTTGCGC - -3'

Reverse:

5'-------50 bp homology compl. strand -------AAGTGCCCCGGAGGATGAGATTTTCT -3'

2 . Order the above oligos and as well as a set of flanking oligos for PCR in step #3 and later as well as sequencing. They are standard PCR oligos which should bind 50-100 bp upstream and downstream of insertion site.

3. Ordering of fosmid clone of Gene of interest (G.O.I) from GeneService using Wormbase as a guide. When selecting clones, we choose ones that have the GOI in the center of the sequence. Ones that exclude neighboring genes might be preferable but are often hard to find.

4. Culture of fosmid clone of Gene of interest (G.O.I). Be sure to freeze an aliquot for future use as a standard glycerol stock. Verify that the G.O.I. is present via PCR using the flanking oligos.

5. Preparation of electrocompetent SW106 cells. SW106 must be grown at 30-32ºC.

1. Grow a 5 ml overnight culture of SW106 cells from a single SW106 colony in a 14 mL BD Falcon tube with LB broth at 32ºC.

2. Inoculate 1 mL into 100 mL of LB in a 2 L flask

3. Grow them to an OD_{600} 0.6–0.8. **Do NOT heat-shock.**

4. Pellet and wash twice in 50 mL ice-cold 10% glycerol.

5. Aspirate all but ~500 µl of each supernatant

6. Resuspend the pellets by gentle vortexing.

7. Store 100 µl aliquots at -80°C until use.

5. Mini-preparation of fosmid DNA. We have been using the Epicentre fosmid prep kit mini-prep and 1.5 mL of overnight culture. We follow the alternate protocol described in the instructions which involves adding the Riboshredder mix at an earlier step.

6. Transform the fosmid DNA into electrocompetent SW106 cells.

1. Electroporate ~50 ng fosmid DNA of gene of interest (G.O.I) by using an Eppendorf 2510 electroporator at 1350 volts in 0.1 cm gap cuvettes. Other electroporators should be fine as well.

2. Recover bacteria in 1 mL LB /SOC for 1 hour at 32ºC.

3. Plate aliquots on LB plate with chloramphenicol (12.5 µg/mL) and incubate at 32ºC O/N.

7. (Optional) PCR verification of the presence of G.O.I. We grow a 5 mL O/N culture and add 0.5 µL of culture to a standard PCR reaction with the flanking oligos. We usually use the GoTaq mastermix from Promega. We increase the initial 95ºC incubation to 5 minutes to lyse the bacteria prior to PCR.

Stage II. *gal* K recombineering

1. PCR amplification of pMOD4 galK-G or pMOD4 galK-GT cassettes using the primers designed above. We use either Phusion from NEB or Pfu Ultra from Stratagene. Use 1-2 ng template. The parameters are: 98°C 15 sec, 50°C 20 sec., 72°C 30 sec.., for 30 cycles using Phusion. We usually set-up a 50 µL reaction volume and then run 2 lanes on a gel.

2. Gel purify the resulting band. We use either the Zymo Research mini kit or use a Qiagen kit from very strong bands. Quantify the yield by gel or Nanodrop spectrophotometer. This PCR product is then ready for step 13.

3. Inoculate an overnight culture of SW106 cells containing the fosmid DNA in 5 ml LB + chloramphenicol (12.5 µg/µL). Grow at 32ºC.

4. Set shaking water bath to 42ºC to warm-up with a sterile 250 ml flask in the holder. (Using a shaking water bath is critical for getting high efficiency).

5. Add 1 ml of O/N culture to 100 ml of LB and chloramphenicol in a 2 L flask. Grow to an O.D. 0.6-0.8 (going a little over is OK). This usually takes 3-4 hours.

6. Transfer 50 ml of SW106 cells to the 250 ml flask and heat-shock at 42°C for exactly 20 min. in a shaking waterbath at 100 r.p.m. Leave the remaining bacteria at 32°C as the uninduced control.

7. After 20 min., the two samples, induced and uninduced, are briefly cooled on ice.

8. Transfer the samples to two sterile centrifuge tubes and pellet at ~5000x*g* for 5-10 minutes.

9. Pour off all of the supernatant and resuspend the pellet in 1 ml ice-cold 10% glycerol by gentle vortexing (i.e. setting 3-4)..

10. When resuspended, add another 49 ml ice-cold 10% glycerol and pellet the samples again.

11. Repeat step 8-10 twice.

12. Remove all supernatant by inverting the tubes, and resuspend the pellet in the remaining liquid (approximately 500 µL each). Aliquot into 100 µL samples, freeze on dry ice, and store at -80ºC. These are good for weeks to months. (We usually stop here and perform the electroporation the following day).

13. Transform150 ng of PCR product into the electrocompetent SW106 cells. We use 100 µL cells with an Eppendorf 2510 electroporator set at 1350 volts in 0.1 cm gap cuvettes.

14. After electroporation of the PCR product, the bacteria are recovered in 1 ml LB (in a 14 ml Falcon tube) for 4.5 hours in a 32°C shaking incubator.

15. After the recovery period the bacteria are washed twice in 1xM9 salts to remove any rich medium (see below for recipe) as follows: 1 ml culture is pelleted in an eppendorf tube at 13,200 RPM for 15 sec. and the supernatant removed with a pipette. The pellet is resuspended in 1 ml 1xM9 salts, and pelleted again.

M9 medium (1 liter)
6 g Na_2HPO_4
3 g KH_2PO_4
1 g NH_4Cl

0.5 g NaCl
AUTOCLAVE

16. After the second wash, the supernatant is removed and the pellet is resuspended in 1 mL 1xM9 salts before plating serial dilutions in 1xM9 (100 ul, 100 ul of a 1:10 dilution, and 100 ul 1:100) onto MOPS minimal media (available from Teknova #M2106 but do not add the included glucose) containing 0.2% galactose in addition to leucine (45 mg/L), biotin (1 mg/L), chloramphenicol (12.5 µg/mL), and add 15 grams agar/L. We autoclave the water and agar together and then add separately autoclaved galactose (20%). The other ingredients are added from filter sterilized stocks when the agar is cooled to 60ºC. See http://recombineering.ncifcrf.gov/ for help with these stocks. We often resuspend the uninduced bacteria in 0.5 mL as the Copeland lab has reported a higher death rate due to electroporation in this control.

17. Incubate 3 days at 32°C in an incubator. (Be patient as the true positives grow slowly).

18. Streak a few colonies onto MacConkey + 1% galactose + chloramphenicol indicator plates. We buy the MacConkey powder from BD (#281810). All of the colonies appearing after the last step should be Gal+, but in order to get rid of any Gal- contaminants (hitch-hikers), it is important to obtain single, bright red colonies before proceeding to the second step. Gal- colonies will be white/colorless and the Gal+ bacteria will be bright red/pink due to a pH change resulting from fermented galactose after an overnight incubation at 32°C.

19. Pick a single, bright red (Gal+) colony and inoculate a 5 ml LB + chloramphenicol overnight culture. Incubate at 32°C.

20. Repeat steps 3 -12 above to obtain electrocompetent SW106 cells ready for the second round of recombination.

21. PCR amplify the tag fragments from pMOD4 GFP, pBS1761 (N-term TAP), or pBS1479 (C-term TAP) using the same oligos used in the first round or using shorter GFP or TAP-specific oligos (the inner sequences in Step 1). If you are making multiple constructs, it is particularly useful to use the shorter oligos as the same PCR product can be used for all of the constructs. The PCR products are gel purified and ~100 ng are electroporated into induced and uninduced competent SW106 cells as above.

22. Recover in 1 ml LB in a 14 ml snap-cap tube and incubate in a 32°C shaker for 4.5 hours.

23. As in step 15-16, pellet 1 ml culture and wash twice in 1xM9 salts, and resuspend in 1 ml 1xM9 salts after the second wash before plating serial
dilutions (100 ul, 100 ul of a 1:10 dilution, 100 ul 1:100, and 100 ul 1:1000). Again we usually suspend the uninduced sample in 0.5 mL of 1xM9 salts. Plate bacteria on MOPS minimal media plates containing 0.2% 2-deoxy-galactose (DOG) and 0.2% glycerol in

addition to leucine (45 mg/L), biotin (1 mg/L), chloramphenicol (12.5 µg/mL), and ~15 grams agar/L . We buy 5 grams of DOG from Sigma, suspend it in 25 mL of water, filter-sterilize, and the freeze the 20% stock at -20ºC.

24. Incubate at 32°C for three days.

25. 4 colonies are used to make 5 mL overnight cultures in LB with chloramphenicol (12.5 µg/mL) and these are used for colony PCR as above to confirm that the cassette was inserted. We use both shorter GFP/TAP specific oligos and the flanking oligos to demonstrate the right insert and right site. GFP is ~850 b.p. and TAP is ~600 b.p. whereas *galK* is 1.4-1.5 k.b.

26. DNA sequencing using flanking oligos is also used to verify correct modification of the fosmid.

Stage III. Addition of *unc-119* gene

1. Preparation of competent SW106 bacteria carrying the modified fosmid as above in step 5 of Stage I. Do NOT induce at 42ºC.

2. Electroporate with 50 ng. pLoxP *unc-119* from a mini-prep using the same settings as above.

3. Recover bacteria in LB containing 0.1% arabinose for 1 hour.

4. Plate an aliquots on LB plates containing ampicillin (50µg/mL) and chloramphenicol (12.5 µg/mL) which selects for integration of pLoxP *unc-119* into the fosmid.

5. PCR verification of the presence of the *unc-119* gene

unc-119 F	CAAATCCGTGACCTCGACAC
unc-119 R	CACAGTTGTTTCTCGAATTTGG

Stage IV. Maxi Preparation and Bombardment

1. Grow 5 mL overnight culture of an amp and chloramphenicol colony at 32ºC. Use Epicentre fosmid kit mini-prep to isolate fosmid for use in EPI300 bacteria (ava lable competent from Epicentre). Electroporate ~50 ng into the EPI300 bacteria. These allow the fosmid to be amplified for purification and makes this process much easier. Recover bacteria in LB for 1 hour at 37ºC. Plate aliquots on LB + ampicillin (50 µg/mL) and chloramphenicol (12.5 µg/mL).

2. Grow and induce the EPI300 bacteria containing the modified fosmid. Epicentre now sells an autoinduction media which we have not tried, but a collaborator got a good yield from a 50 ml culture. Be sure to add chloramphenicol and ampicillin.

3. We use 10 ug. of fosmid DNA to bombard DP38 using the protocol in this paper from the Plasterk lab. Berezikov, E., C.I. Bargmann, and R.H. Plasterk. 2004. Homologous gene targeting in Caenorhabditis elegans by biolistic transformation. Nucleic Acids Res. 32:e40.