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Improved vectors for selection of transgenic *Caenorhabditis elegans*

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Summary

The generation of transgenic animals is an essential part of research in *Caenorhabditis elegans*. One technique for the generation of these animals is biolistic bombardment involving the use of DNA coated microparticles. To facilitate the identification of transgenic animals within a background of non-transformed animals, the *unc-119* gene is often used as a visible marker as the *unc-119* mutants are small and move poorly and the larger size and smoother movement of rescued animals make them clearly visible. While transgenic animals can be identified from co-bombardment with a transgene of interest and a separate *unc-119* rescue plasmid, placing the *unc-119* in *cis* on the transgene increases confidence that the resulting transgenic animals contain and express both the marker and the transgene. However, placing the *unc-119* marker on the backbone of a plasmid or larger DNA construct, such as a fosmid or BAC, can be technically difficult using standard molecular biology techniques. Here we describe methods to circumvent these limitations and use either homologous recombination or Cre-LoxP mediated recombination in *E. coli* to insert the *unc-119* marker on to a variety of vector backbones.

Keywords

C. elegans; transgenic animals; *unc-119*; microparticle bombardment; recombination; Cre recombinase; plasmid; biotechnology

1. Introduction

In *C. elegans* research, transgenic animals are routinely generated for the purpose of tagging proteins, visualizing gene expression, over-expressing a gene, or otherwise modifying the worm genome (1, 2). As a result, the generation of transgenic animals is an essential aspect of *C. elegans* research, and effective and facile methods to reliably generate animals with stable transgenic arrays or integrated transgenes are necessary for lab productivity. Two commonly employed methods for introducing foreign DNA into the worm genome are microinjection, and biolistic bombardment (2, 3). Both of these techniques require the use of a visible marker to identify animals which carry the transgene. However, these methods differ in the stability, raw number, and proportion of transgenic lines obtained, as well as the ease of carrying out the protocol (1, 2). Biolistic bombardment is the best method to use to obtain multiple lines that are either integrated or extrachromosomal arrays from a single bombardment. This is also an attractive method because it has a much smaller learning curve and success is less dependent on the skill of the operator.

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One challenge to bombardment is that the identification of transgenic worms requires visually screening a large quantity of animals. For this technique, a visible marker that is detectable at lower magnifications is optimal. To date, the best marker for biolistic bombardment is the *unc-119* rescue gene (3). The *unc-119* mutants are smaller, have uncoordinated locomotion, are deficient in their ability to form dauers, and have lower fitness in lab conditions compared to wild type (4). Rescue of the *unc-119* mutation with a transgene produces worms that have normal mobility and size which makes them easy to distinguish from non-transformed animals. One way to use *unc-119* rescue as a selectable marker is to bombard two separate plasmids at the same time – one containing the *unc-119* rescue gene and the other containing the gene of interest. There exists an expansive plasmid collection generated by the lab of Dr. Andrew Fire (available from Addgene, Inc., Cambridge, MA), and a *C. elegans* fosmid library created by the lab of Dr. Donald Moerman (available at Source BioScience, Nottingham, UK) that are ready to be used to create transgenic worms in a co-bombardment fashion. However, a disadvantage to this strategy is that the lack of physical linkage between the selective marker and the transgene of interest can lead to some selected transgenic lines containing the marker but not the gene of interest, and vice versa (5). This is especially a problem when there is no way of easily confirming the presence of the transgene of interest (i.e. by GFP expression, western blot, or PCR genotyping). Physically linking the *unc-119* and the transgene of interest by putting them on the same plasmid ensures that this will not happen. Generating constructs such as these with traditional molecular biological techniques poses challenges such as identification of available restriction sites, which may make this approach not worthwhile.

The following vectors and protocols overcome the obstacles of traditional molecular cloning through the use of homologous or Cre-LoxP recombination, and offer facile methods to produce DNA constructs that improve the selection of transgenic animals (Figure 1). In the first protocol, the *unc-119* rescue gene in the form of either *C. elegans* cDNA or *C. Briggsae* genomic DNA may be inserted into any plasmid with an ampicillin resistance gene by recombination (Figure 1A) (5). Among the plasmids that can be modified by this protocol are the large collection of plasmids generated by the lab of Dr. Andrew Fire, which expedite common tasks such as generating a GFP reporter or expressing a cDNA with a tissue-specific promoter. This collection is available from Addgene Inc. (Cambridge, MA). The second protocol provides a means to modify existing fosmids or BACs containing large portions of genomic DNA or modified genomic DNA, by inserting the *C. elegans* genomic *unc-119* rescue gene via Cre-LoxP mediated recombination (Figure 1B) (6). Using these methods we are able to routinely bombard with vectors from the Fire lab that have been retrofitted to contain the *unc-119* rescue gene, and also to bombard with fosmids containing stretches of modified *C. elegans* genome, including the gene of interest and most of the regulatory elements, and the *unc-119* rescue gene, to obtain transgenic animals (7, 8).

2. Materials

2.1 Retrofitting Ampicillin Resistant Plasmids by Inserting the *unc-119* Marker

2.1.1 Strains

1. DH5 α (*fhuA2* Δ *argF-lacZ*)U169 *phoA* *glnV44* Φ 80 Δ *lacZ*)M15 *gyrA96* *recA1* *relA1* *endA1* *thi-1* *hsdR17*) *E. coli* bacteria transformed with the pKD78 plasmid (9). (see Note 1).

¹The pKD78 plasmid encodes the lambda red recombination machinery control of the arabinose inducible *araB* promoter (9). The pKD78 plasmid is chloramphenicol resistant and uses the temperature-sensitive repA101 replicon so it must be grown at 30°C. The plasmid is available from the Coli Genetic Stock Center (CGSC) (<http://cgsc.biology.yale.edu/index.php>) in the BW25141 bacterial strain. BW25141 is not compatible with this technique as it is *pir*⁺ and will allow the growth of the *punc-119* plasmids. The pKD78 plasmid can be isolated via mini-prep and retransformed into DH5 α for subsequent use.

2. DH5 α E. coli bacteria (Invitrogen Corp., Carlsbad, CA).

2.1.2 Plasmids

1. *punc-119c* plasmid containing the R6K replication origin, kanamycin resistance, and *unc-119* cDNA (Addgene Inc., Cambridge, MA) (5). (see Note 2 and ³).
2. *punc-119cR* plasmid which is similar to *punc-119c* except that the mCherry gene is fused to the C-terminus of the *unc-119* cDNA (Addgene Inc., Cambridge, MA) (5). (see Note 2 and ³).
3. *punc-119cbr* which is similar to *punc-119c* except that the *C. briggsae unc-119* gDNA is used (Addgene Inc., Cambridge, MA). (see Note 2 and ³).
4. Fire lab vectors or other plasmids containing the ampicillin resistance gene on the plasmid backbone.

2.1.3 Equipment

1. Electroporator, such as the Eppendorf 2510 electroporator (Eppendorf AG, Hamburg, Germany), with 0.1 cm gap cuvettes.
2. Microbial incubator.
3. Shaking incubator.
4. Refrigerated centrifuge with rotors
5. Microcentrifuge
6. Pipettors
7. 1.5 mL microfuge tubes
8. 14 mL round bottom snap cap culture tubes
9. 750 mL centrifuge bottles
10. Apparatus for agarose gel electrophoresis
11. Spectrophotometer
12. -80°C freezer
13. 4°C refrigerator
14. Dry ice
15. 2 L Erlenmeyer flask

2.1.4 Reagents and Media

1. 34 mg/mL chloramphenicol stock solution (1000X): Dissolve 0.34 g. chloramphenicol powder in 10 mL absolute ethanol. Store at -20°C.

²All of these plasmids use the R6K replication origin which is only able to replicate in bacteria which express the *pir* gene, which is uncommon among routine lab bacterial strains. These are supplied in the EC100D *pir-116* (F^- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80d *lacZ* Δ M15 Δ *lacX74* *recA1* *endA1* *araD139* Δ (*ara*, *leu*)7697 *galU* *galK* λ^- *rpsL* *nupG* *pir-116*(DHFR bacteria (Epicentre Biotechnologies, Madison, WI) which express *pir* at high level and permit replication of the plasmid at medium copy number.

³We have successfully obtained transgenic animals using either of three forms of the *unc-119* rescue gene (*C. elegans* cDNA, cDNA fused to mCherry, or *C. briggsae* gDNA). However, the *C. elegans* cDNA seems to require a higher copy number to produce the rescue phenotype, therefore, if a lower copy number of the transgene is desired, use of the *C. briggsae* genomic DNA is recommended. The mCherry is particularly useful as it is visible using a fluorescent dissecting microscope and permits the transgene to be identified if it is crossed out of strains with the *unc-119* mutation (5).

2. 10% arabinose stock solution: Dissolve 1 g. arabinose in 10 mL miliQ water. Filter sterilize. Store at -20°C .
3. 30 mg/mL kanamycin stock solution (1000X): Dissolve 0.3 g kanamycin in 10 mL milliQ water. Filter sterilize. Store at -20°C .
4. LB broth: Dissolve 10 g. tryptone, 5 g. NaCl, and 5 g. yeast extract in 1 L. miliQ water. Autoclave for 20 minutes. (*see* Note 4).
5. LB agar: Dissolve 10 g. tryptone, 5 g. NaCl, 5 g. yeast extract, and 15 g. agar in 1 L. miliQ water. Autoclave for 20 minutes. (*see* Note 5).
6. LB broth with 34 $\mu\text{g}/\text{mL}$ chloramphenicol: Using sterile technique, add 5 μL chloramphenicol stock solution to 5 mL of LB broth which has been autoclaved and allowed to cool to room temperature.
7. LB agar with 34 $\mu\text{g}/\text{mL}$ chloramphenicol: Using sterile technique, add 1 mL chloramphenicol stock solution to 1 L. LB agar which has been autoclaved and allowed to cool in a water bath to 60°C . Mix by swirling and pour agar into 10 cm. Petri dishes. Allow plates to cool and harden at room temperature before use.
8. LB broth with 30 $\mu\text{g}/\text{mL}$ kanamycin: Using sterile technique, add 500 μL kanamycin stock solution to 500 mL of LB broth which has been autoclaved and allowed to cool to room temperature.
9. LB agar with 30 $\mu\text{g}/\text{mL}$ kanamycin: Using sterile technique, add 1 mL kanamycin stock solution to 1 L. LB agar which has been autoclaved and allowed to cool in a water bath to 60°C . Mix by swirling and pour agar into 10 cm. Petri dishes. Allow plates to cool and harden at room temperature before use.
10. SOB with 34 $\mu\text{g}/\text{mL}$ chloramphenicol and 0.015% Arabinose: Dissolve 10 g. tryptone, 2.5 g. yeast extract, 0.25 g. NaCl, 1.4 g. MgSO_4 , and 0.092 g. KCl in 500 mL miliQ water in a 2 L. flask, autoclave, and let the media cool to RT. Using sterile technique, add 500 μL chloramphenicol stock solution and 750 μL arabinose stock solution. (*see* Note 6).
11. 10% glycerol solution: Measure 10mL glycerol, and add water to a volume of 1 L. Autoclave, and chill on ice or in a cold room to 4°C .
12. BamHI, ScaI, and XhoI restriction endonucleases and buffers 9. Zymo DNA Clean & Concentrator kit (Zymo Research Corp., Irvine, CA)
13. Qiagen QIAprep@Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) or similar mini-prep reagents.
14. 0.8% agarose gel

2.2 Inserting *unc-119* into Fosmids by Cre-LoxP Recombination

2.2.1 Strains

1. SW106 (*mcrA* Δ (*mrr-hsdRMS-mcrBC*) Δ *lacX74* *deoR* *endA1* *araD139* Δ (*ara*, *leu*) 7697 *rpsL* *recA1* *nupG* ϕ 80*dlacZ* Δ *M15* [λ c1857 (*cro*-*bioA* Δ *Tet*)] (*cro*-*bioA* Δ *araC*-*P*_{BAD} *Cre* Δ *galK*) strain (10). (*see* Note 7).

⁴LB broth mixes can be also purchased from many suppliers. We use LB mix from EMD4 Biosciences (San Diego, CA). LB broth containing chloramphenicol or kanamycin can be made by adding these antibiotics from the described stock solutions as a 1:1000 dilution.

⁵LB agar mixes can be also purchased from many suppliers. LB agar plates containing chloramphenicol or kanamycin can be made by cooling the molten agar to 55°C and then adding these antibiotics from the described stock solutions as a 1:1000 dilution.

⁶SOB media mix can also be purchased. We use SOB mix from EMD4 Biosciences (San Diego, CA).

2. EPI300 (*F⁻mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻rpsL nupG trfA tonA*) strain. (see Note 8).

2.2.2 Plasmids, Fosmids, and Oligos

1. Fosmids containing *C. elegans* genomic DNA in the pCC1FOS vector. (see Note 9 and 10).
2. pLoxP *unc-119* plasmid contains the *unc-119* gene, ampicillin resistance gene, and an R6K origin of replication (Addgene Inc., Cambridge, MA) (6). (see Note 2).

3. Oligos for colony PCR

unc-119 F :CAAATCCGTGACCTCGACAC

unc-119 R :CACAGTTGTTTCTCGAATTTGG

2.2.3 Equipment

1. Electroporator such as the Eppendorf 2510 electroporator (Eppendorf AG, Hamburg, Germany), with 0.1 cm gap cuvettes.
2. Microbial incubator.
3. Shaking incubator.
4. Refrigerated centrifuge with rotors
5. Microcentrifuge
6. Pipettors
7. 1.5 mL microfuge tubes
8. 14 mL round bottom snap cap culture tubes
9. 250 mL centrifuge bottles
10. PCR machine
11. Apparatus for agarose gel electrophoresis
12. Spectrophotometer
13. -80°C freezer
14. 4°C refrigerator
15. Dry ice
16. 2 L and 250 mL Erlenmeyer flask

⁷The SW106 strain contains an arabinose inducible Cre transgene in addition to the heat inducible lambda red recombination machinery (10). To avoid inducing the recombination proteins, SW106 needs to be grown at 32°C or below. SW106 is available from the NIH (<http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx>).

⁸The EPI300 strain lacks the Cre recombinase which prevents the *unc-119* marker from being lost via the reverse Cre-LoxP recombination event. EPI300 also permits induction of certain fosmids and BACs to high copy number to facilitate DNA purification. This strain is available from Epicentre Biotechnologies (Madison, WI).

⁹Genomic DNA library fosmids in this vector have a loxP site on the vector backbone which is used in the recombination reaction. The pCC1FOS vector can also be amplified in the EPI300 bacteria strain. Fosmids from the library generated by the lab of Dr. Donald Moerman are available from Source BioScience, Nottingham, UK. Other fosmid or BAC clones can also be used as long as they have a LoxP site and are not ampicillin resistant.

¹⁰Prior to insertion of the *unc-119* marker, the fosmids can be modified by recombineering in the SW106 bacterial strain to add a TAP, GFP, or other tags or to introduce mutations following protocols described by our lab and others (6, 12–14).

2.2.4 Reagents and Media

1. 12.5 mg/mL chloramphenicol stock solution (1000X): Dissolve 0.125 g chloramphenicol in 10 mL absolute ethanol. Store at -20°C .
2. LB broth: Dissolve 10 g. tryptone, 5 g. NaCl, and 5 g. yeast extract in 1 L. miliQ water. Autoclave for 20 minutes. (*see* Note 4).
3. LB agar: Dissolve 10 g. tryptone, 5 g. NaCl, 5 g. yeast extract, and 15 g. agar in 1 L. miliQ water. Autoclave for 20 minutes. (*see* Note 5).
4. 50 mg/mL ampicillin stock solution (1000X): Dissolve 0.5 g ampicillin in 10 mL miliQ water. Filter sterilize. Store at -20°C .
5. LB broth with 12.5 $\mu\text{g/mL}$ chloramphenicol: Using sterile technique, add 500 μL chloramphenicol stock solution to 500 mL of LB broth which has been autoclaved and allowed to cool to room temperature.
6. LB agar with 12.5 $\mu\text{g/mL}$ chloramphenicol: Using sterile technique, add 1 mL chloramphenicol stock solution to 1 L. LB agar which has been autoclaved and allowed to cool in a water bath to 60°C . Mix by swirling and pour agar into 10 cm. Petri dishes. Allow plates to cool and harden at room temperature before use.
7. LB broth with 50 $\mu\text{g/mL}$ ampicillin and 12.5 $\mu\text{g/mL}$ chloramphenicol: Using sterile technique, add 500 μL chloramphenicol stock solution and 500 μL ampicillin stock solution to 500 mL of LB broth which has been autoclaved and allowed to cool to room temperature.
8. LB agar with 50 $\mu\text{g/mL}$ ampicillin and 12.5 $\mu\text{g/mL}$ chloramphenicol: Using sterile technique, add 1 mL chloramphenicol stock solution and 1 mL ampicillin stock solution to 1 L. LB agar which has been autoclaved and allowed to cool in a water bath to 60°C . Mix by swirling and pour agar into 10 cm. Petri dishes. Allow plates to cool and harden at room temperature before use.
9. Two sterile 2 L flasks and one sterile 250 mL flask.
10. 10% glycerol solution: Measure 10mL glycerol, and add water to a volume of 1 L. Autoclave, and chill on ice or in a cold room to 4°C .
11. 10% arabinose stock solution: Dissolve 1 g. arabinose in 10 mL miliQ water. Filter sterilize. Store at -20°C .
12. LB + 0.1 % arabinose (10mL): Add 100 μL 10% arabinose stock solution to 10 mL sterile LB.
13. CopyControl™ Fosmid Autoinduction Solution (Epicentre Biotechnologies, Madison, WI).
14. FosmidMAX fosmid DNA purification kit (Epicentre Biotechnologies, Madison, WI).
15. GoTaq DNA polymerase master mix (Promega Corp., Madison, WI).
16. 0.8% agarose gel

3. Methods

3.1 Retrofitting Ampicillin Resistant Plasmids by Inserting the *unc-119* Marker

In this protocol, plasmid based vectors carrying an ampicillin resistance gene can be modified to carry the *unc-119* marker on the vector backbone via homologous

recombination in *E. coli* (Figure 1A) (5). This procedure involves addition of an *unc-119* and kanamycin resistance gene cassette which is flanked by sequences homologous to the ends of the ampicillin resistance gene, and is then followed by destruction of the unmodified parental plasmid to obtain a pure population of modified plasmid. The destruction of the unmodified plasmid is necessary as most plasmids exist as multiple copies in *E. coli* so only a minority of the plasmids are modified during the recombination step (11). The final plasmid contains a kanamycin resistance gene and *unc-119* marker in place of the ampicillin resistance gene and is now kanamycin resistant.

3.1.1 Induction of competent pKD78 transformed DH5α cells for electroporation

1. Streak out pKD78 transformed DH5α on LB + 34 µg/mL chloramphenicol agar plates. Grow colonies at 30° C overnight in an incubator.
2. Inoculate 5 mL of LB + 34 µg/mL chloramphenicol with a colony of pKD78 transformed DH5α. Grow overnight at 30° C in a shaking incubator at 250 rpm.
3. Save 1 mL of the SOB + 34 µg/mL chloramphenicol and 0.015% arabinose to use as a blank for the spectrophotometer to determine the optical density of the bacteria.
4. Inoculate 500 µL of the pKD78 transformed DH5α overnight culture in the SOB + chloramphenicol + arabinose, and grow in a 30° C shaking incubator, until the bacteria reach an optical density of between 0.6 and 0.8. This will take between 2 and 4 hours.
5. Immediately spin down the bacteria in a 750 mL centrifuge bottle at 6000×g for 15 min. at 4°C, and wash once with 500mL 10% ice cold glycerol. (see Note 11).
6. Wash twice with 50 mL 10% ice cold glycerol, leaving approximately 5 mL of glycerol solution after the final wash. Keep the bacteria on ice in between washes. Resuspend the pellet in the remaining glycerol, make 100 µL aliquots in 1.5 mL microfuge tubes, and freeze on dry ice.
7. Store aliquots at –80° C. Long term storage for weeks to months in a –80° C freezer does not seem to reduce the efficiency of these cells.

3.1.2 Preparation *unc-119* – kanamycin resistance cassette

1. Cut 1 µg. *punc-119c*, *punc-119cR*, or *punc-119cbr* with BamHI for 1 hour at 37°C. If this protocol will be carried out to modify multiple plasmids, a larger quantity of plasmid DNA may be digested at this step, and the fragment stored at 4°C for later use.
2. Desalt and purify the digest with the Zymo DNA Clean & Concentrator kit and elute in 10 µL of water. Gel purification of this fragment does not improve the results.

3.1.3 Homologous recombination of *unc-119* cassette with target vector

1. Thaw an aliquot of the induced pKD78 transformed DH5α cells on ice.
2. Mix 100 ng. of the *unc-119* – kanamycin resistance cassette with 50 ng. of the ampicillin resistant recipient plasmid in a 1.5 mL microfuge tube.

¹¹Careful washing with the 10% glycerol is important to remove salts from the LB which could produce arcing during the electroporation. To facilitate resuspension of the pellet we use gentle vortexing on setting 3–4.

3. Mix the DNA mixture with the competent cells, and immediately add this to a 0.1 cm gap electroporation cuvette. Electroporate the bacteria at 1350 V.
4. Immediately add 1 mL LB broth to the bacteria, and transfer to a 14 mL snap-cap bacterial culture tube.
5. Place the tube in a shaking 37° C incubator, and allow the cells to recover for 2 hours. (*see* Note 12).
6. Plate 100 µL of bacteria on one LB + 30µg/mL kanamycin agar plate, and the rest of the culture on three additional plates.
7. Incubate the plates at 37° C overnight. 50–500 colonies are expected following this step.
8. Inoculate four individual colonies in 5 mL LB + 30µg/mL kanamycin in 14 mL snap-cap bacterial culture tubes. Grow overnight in a shaking incubator at 37° C.

3.1.4 Confirming the presence of the *unc-119* cassette

1. Purify the plasmid DNA from the overnight culture with a QIAprep®Spin Miniprep Kit..
2. Digest 1/10 of the mini-prep DNA with XhoI for 1 hour and run the digested DNA on a 0.8% agarose gel. (*see* Note 13 and ¹⁴).

3.1.5 Destruction of the parent plasmid by ScaI digestion followed by retransformation

1. Digest 1/10 of the mini-prep DNA with ScaI (or another enzyme from Table 1). (*see* Note 15).
2. Desalt and purify the digest using the Zymo DNA Clean & Concentrator kit. Elute the DNA in 10 µL of water.
3. Chemically transform DH5α with the digested DNA, and plate aliquots of the bacteria on LB + 30µg/mL kanamycin. Incubate at 37° C overnight.
4. Select individual colonies to inoculate two 5 mL cultures in LB + 30µg/mL kanamycin. Incubate at 37° C overnight in a shaking incubator.
5. Mini-prep and test digest the DNA using XhoI. Look for clones which only have the recombinant plasmid.

¹²Incubation at 37° C inhibits growth of the pKD78 plasmid which will be subsequently lost from the DH5α bacteria. All subsequent growth steps can be carried out at 37°C. The prolonged outgrowth is needed as the recombination event usually only affects a single strand of the plasmid so the plasmid needs to replicate to give a double-stranded recombinant plasmid.

¹³All of the *unc-119* – kanamycin resistance cassettes contain two XhoI sites so the insertion of the cassette into the target plasmid can be verified by restriction digest which yields a diagnostic band from the cassette and produces a change in the banding pattern compared to the parent plasmid. For *punc-119c* cassette the diagnostic band is 2329 b.p. in size; for *punc-119cR* it is 3171 b.p., and for *punc-119cbr* it is 2306 b.p. The XhoI digest allows plasmids lacking a map to be successfully retrofitted by looking for the diagnostic band and a change compared to the parent plasmid digested in parallel.

¹⁴The digest usually gives a gel pattern that corresponds to a combination of the parent ampicillin resistant plasmid mixed with the new recombinant plasmid. This occurs because the parent plasmids are present as multiple copies within the bacteria so most colonies consist of a mix of modified and unmodified plasmids (11). This is in contrast to BACs and fosmids which are present at only one copy per cell and are either modified or unmodified. We have found that enzymatic digestion of the mini-prep DNA followed by retransformation is the most effective means to obtain bacteria containing only the modified plasmid.

¹⁵For the ScaI digestion to be successful, the parent plasmid must contain a ScaI site and the recombinant plasmid must NOT contain a ScaI site. ScaI sites are present in almost all ampicillin resistant genes and appear to be uncommon in vectors and worm DNA. If a ScaI site is present elsewhere in the vector, an alternate enzyme from Table 1 can be selected for use. Alternatively, the parent plasmid may be removed by diluting the plasmid prep and re-transforming, however, this technique has been significantly less successful in our lab.

6. Generate a glycerol stock and use the bacterial culture for a midi or maxi-prep to obtain sufficient DNA for bombardment.

3.2 Inserting *unc-119* into Fosmids by Cre-LoxP Recombination

In this protocol, fosmid or BAC based vectors carrying a LoxP site on the vector backbone can be modified to carry the *unc-119* marker via Cre-LoxP recombination in *E. coli* (Figure 1B) (6). This procedure involves addition of a replication incompetent plasmid carrying the *unc-119* gene and the ampicillin resistance gene along with a LoxP site. The SW106 bacterial strain has a transgene which expresses Cre recombinase using the inducible arabinose promoter (10). A brief treatment with arabinose produces sufficient Cre to allow recombination between the LoxP sites on the vector and the fosmid/BAC backbone, and selection on LB containing ampicillin is then used to select for the recombinant plasmid.

3.2.1 Purify fosmid DNA containing the gene of interest

1. Streak out bacteria containing the fosmid of interest from a glycerol stock on a LB + 12.5 µg /mL chloramphenicol agar plate and incubate overnight at 37° C to obtain colonies.
2. Inoculate 5 mL LB + 12.5 µg /mL chloramphenicol with a single colony and grow overnight in a 37° C shaking incubator.
3. Purify the fosmid DNA from the overnight culture using the FosmidMAX DNA purification kit following the alternative protocol for purification of 1.5 mL of bacterial culture. (*see* Note 16)

3.2.2 Generate Electrocompetent SW106 Bacteria

1. Streak SW106 on a LB agar plate and incubate overnight at 32°C to obtain colonies.
2. Inoculate 5 mL LB with a single colony and grow overnight in a 32° C shaking incubator.
3. Transfer 200 mL of sterile LB broth to a 2 L Erlenmeyer flask. Save 1 mL from the flask to use as a blank for measuring optical density.
4. Add 200 µL of the overnight culture to the LB in the 2 L Erlenmeyer flask and grow at 32° C to an OD₆₀₀ of between 0.6 and 0.8. This should take ~4 hours.
5. Transfer the bacteria to a 250 mL centrifuge bottle, and pellet the bacteria at 6000 X g for 15 minutes at 4°C. Wash once with 200mL 10% ice cold glycerol. (*see* Note 11).
6. Wash twice with 50 mL 10% ice cold glycerol, leaving approximately 2 mL of glycerol solution after the final wash. Keep the bacteria on ice in between washes. Resuspend the pellet in the remaining glycerol, make 100 µL aliquots in 1.5 mL microfuge tubes, and freeze on dry ice.
7. Transfer aliquots to a –80°C freezer for storage. The bacteria can be used for electroporation for >1 year.

¹⁶The FosmidMAX DNA prep kit has two protocols for the small scale preparation of fosmids. The standard protocol adds the RiboShredder RNase blend at step 18. This makes quantifying the yield using a spectrophotometer more difficult because the nucleotides from the digested RNA are still present. We instead use the alternate protocol which adds the Riboshredder mix at step 12 which allows the nucleotides to be removed by the ethanol precipitation in a later step.

3.2.3 Transform SW106 Bacteria with the Fosmid

1. Thaw competent SW106 bacteria on ice.
2. Mix 100ng of purified fosmid DNA from the steps above with the bacteria, and electroporate at 1350 volts in a 0.1 cm gap cuvette.
3. Recover in 500 μ L LB in a 32° C shaking incubator for 1 hour.
4. Plate 100 μ L and 200 μ L on LB + 12.5 μ g/mL chloramphenicol plates, and incubate overnight at 32° C.

3.2.4 Preparation of Electrocompetent fosmid transformed SW106 cells

1. Inoculate 5 mL of LB + 12.5 μ g/mL chloramphenicol with a single colony from the above transformation. Grow overnight in a 32° C shaking incubator.
2. Transfer 100 mL of sterile LB broth with 12.5 μ g/mL chloramphenicol to a 2 L Erlenmeyer flask. Save 1 mL from the flask to use as a blank for measuring optical density.
3. Add 100 μ L of the overnight culture to the LB in the 2 L Erlenmeyer flask and grow at 32° C to an OD₆₀₀ of between 0.6 and 0.8.
4. Transfer the bacteria to a 250 mL centrifuge bottle, and pellet the bacteria at 6000 X g for 15 minutes at 4°C. Wash once with 100mL 10% ice cold glycerol. (*see* Note 11).
5. Wash twice with 50 mL 10% ice cold glycerol. Keep the bacteria on ice in between washes. Resuspend the pellet in the remaining glycerol, make 100 μ L aliquots in 1.5 mL microfuge tubes, and freeze on dry ice.
6. Transfer aliquots to a –80°C freezer for storage. The bacteria can be used for electroporation for at least several months.

3.2.5 Addition of the *unc-119* marker by Cre-LoxP recombination

1. Thaw an aliquot of the competent fosmid transformed SW106 bacteria.
2. Mix 50 ng. pLoxP *unc-119* DNA with the SW106 bacteria. (*see* Note 17).
3. Electroporate in a 0.1 cm gap cuvette at 1350 volts.
4. Add 500 μ L LB + 0.1% arabinose to the cuvette, and put the bacteria in a 32°C shaking incubator for a 1 hour recovery.
5. Plate 50 μ L and 150 μ L aliquots of the bacteria on LB + 50 μ g/mL ampicillin and 12.5 μ g/mL chloramphenicol agar plates. Incubate overnight at 32°C to obtain colonies.
6. Select 2 – 4 individual colonies to inoculate 5 mL of LB + 50 μ g/mL ampicillin and 12.5 μ g/mL chloramphenicol in 14 mL snap-cap culture tubes. Grow in a 32°C shaking incubator overnight. (*see* Note 18).

¹⁷The pLoxP *unc-119* DNA can be prepared by mini-prep of the EC100D *pir-116* bacteria carrying the plasmid. If this protocol will be carried out more frequently, larger amounts of DNA can be obtained from a single maxi-prep, and the DNA can be stored at 4°C for later use.

¹⁸We no longer routinely check the modified fosmid for the presence of the *unc-119* gene. The success rate of the protocol is very high in our lab, so we often move directly to the steps in section 3.2.7 using an ampicillin-resistant colony.

3.2.6 Detecting the presence of *unc-119* by colony PCR

1. Set up a PCR reaction using GoTaq DNA polymerase master mix including:
 - 0.5 µL bacteria from overnight culture
 - 1 µL 10 µM *unc-119* F oligo
 - 1 µL 10 µM *unc-119* R oligo
 - 7.5 µL water
 - 10 µL GoTaq master mix
2. Perform PCR with an initial incubation to 5 minutes at 95°C to lyse the bacteria; 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72° for 5 minutes.
3. Run the PCR products on a 0.8% agarose gel. A single 248 b.p. band indicates the presence of the *unc-119* insert in the fosmid.

3.2.7 Transfer of the *unc-119* containing fosmid to EPI300 bacteria

1. Isolate fosmid DNA from 1.5 mL of an *unc-119* positive culture using the FosmidMAX DNA purification kit, as before.
2. Electroporate the electrocompetent EPI300 bacteria in 0.1 cm gap cuvettes at 1350 volts. Recover in 1 mL LB for 1 hour with shaking at 37°C.
3. Plate 100 µL and 300 µL on LB + 50 µg/mL ampicillin and 12.5 µg/mL chloramphenicol agar plates. Incubate at 37°C overnight.
4. Pick a single colony and grow overnight in 5 mL LB broth + 50 µg/mL ampicillin and 12.5 µg/mL chloramphenicol in a 14 mL snap-cap culture tube. Use this culture to make a glycerol stock and seed a 40 mL culture of LB + 50 µg/mL ampicillin and 12.5 µg/mL chloramphenicol in a 250 mL flask for fosmid purification.
5. Induce the bacterial culture with the CopyControl induction solution to amplify the fosmid according to the manufacturer's instructions, and purify the fosmid using the FosmidMAX DNA purification kit. The purified DNA is usually sufficient for several bombardments.

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Figure 1.

Schematic overview of vector modifications. (A) For plasmid vectors with an ampicillin resistance gene, the *unc-119* marker can be added via homologous recombination between the ampicillin resistance gene and a kanamycin resistance – *unc-119* cassette which is flanked by regions of homology to the ampicillin resistance gene. This results in the cassette replacing the ampicillin resistance gene. (B) For vectors such as fosmids containing a *LoxP* site, Cre-*LoxP* recombination is used to insert a replication-defective plasmid containing the *unc-119* marker and the ampicillin resistance gene on to the fosmid backbone.

Table 1

Enzymes which cut within the ampicillin resistance gene and the number of sites present in the *unc-119* – kanamycin cassettes

Enzyme	<i>unc119c</i>	<i>unc119cR</i>	<i>unc119cbr</i>
Scal	0	0	0
FspI	0	0	0
AhdI	1	1	0
BcgI	1	1	0
BmrI	0	0	0