

Drug selection in worms

The puromycin selection was developed at the Lehner lab:

"Rapid selection of transgenic *C. elegans* using antibiotic resistance." Semple JI, Garcia-Verdugo R, Lehner B. Nature Methods 7(9):725-7, (Sep, 2010).

The G418 selection was developed in the Dupuy lab:

"An antibiotic selection marker for nematode transgenesis." Giordano-Santini R, Milstein S, Svrzikapa N, Tu D, Johnsen R, Baillie D, Vidal M, Dupuy D. Nat Methods. 2010 Sep;7(9):721-3.

Both labs have adapted their vectors to contain both resistance markers but they have different cloning sites, and have worked on different applications:

The Lehner lab vectors contain the 3-way Gateway R4R3 site, and we have developed the dual antibiotic operon vector for bombardment.

The Dupuy lab have the Gateway R4R1 site and have developed new vectors for mosSCI (single copy integration). Please contact them for further details.

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1. Applications

- Select large populations of extrachromosomal transgenic worms (the cheaper chemical equivalent to a worm sorter).
- Create stable transgenes by bombardment in any genetic background
- Positive selection in *C. elegans*, *C. briggsae*, *C. remanei*, *C. brenneri*.
- Maintain extrachromosomal lines on selective agar plates without picking
- Rapid selection for single copy insertions (Dupuy lab)

2. Which drug?

G418 is considerably cheaper than puromycin (if you find a cheap source of puromycin then let us know!), so for applications requiring selection on plates, G418 is a better choice. However in our hands puromycin works really well in liquid culture where small volumes can be used, and we see a better killing efficiency with puromycin in very high density cultures. For bombardment we use a mixture of both drugs.

3. Vectors for drug selection in worms

The basic *Prpl-28::PuroR* puromycin resistance cassette is the same in all the vectors and works well in *C. elegans*, *C. briggsae*, *C. remanei* and *C. brenneri*.

The reason we have made multiple vectors after the originally published pBCN21 and pBCN22 is because of various modifications made to the backbone:

- a) The minimal *myo-2* promoter in pBCN22 does not work in some non-*elegans* species so we have cloned the full length promoter (1 kb). We have tested this version in *C. elegans*, *C. briggsae*, *C. remanei* and *C. brenneri* and it works in all of them.
- b) Previously the *Pmyo-2::mCherry* reporter construct used the *unc-54* 3'UTR. This 3'UTR is frequently used as a generic UTR to add to the gene of interest. To reduce the likelihood of any intra-vector recombination we changed the 3'UTR of the reporter gene to the *myo-2* 3'UTR
- c) In pBCN22 we had an intronless version of mCherry. We switched this for mCherry derived from the Jorgensen lab pCFJ104 vector because introns seem to improve expression in non-*elegans* species such as *C. briggsae*. We have also created a version of the dual selection vector with GFP. If *myo-2* fluorescence is not compatible with your application, the fluorescent reporter genes can be easily cut out with a single *Apa I* digest and replaced with a reporter gene of your choice.
- d) The old vectors were derived from pCG150, which requires transformation into DB3.1 *ccdB* resistant cells. These cells are no longer commercially available. The "ccdB Survival" cells now provided by Invitrogen are for some reason is not compatible with the pCG150 Gateway cassette (we see one coding and some non-coding variation in the sequence). To remedy this inconvenience, we 'cut and pasted' the region containing the *ccdB* cassette from pDONR221 into our vectors so they are now compatible with the commercially available *ccdB* Survival cells.
- e) The old bombardment vectors only had one unique restriction site that was conveniently positioned for linearising the vectors prior to bombardment (*Spe I* site). Depending on the gene of interest inserted into the vector this may not be usable. Therefore we modified the vector to contain several more unique sites (*Spe I*, *Bgl II*, *Xcm I*, *Avr II* and *Sac I*).

We are sorry if the multiple versions of the vectors cause any confusion. Below we explain the features of each vector from the final 'collection'. In Appendix A we include information about some of the older vectors as these have been distributed over the last year by ourselves and Addgene and having the information 'out there' might help avoid any confusion. The only reasons we can think of to choose vectors from Appendix A over the new ones are:

- You want to try zeocin selection (pBCN25) - we haven't really tested this, but if someone wants to they can.

- You want the mosSCI pCFJ150 vector with puromycin resistance. Note that the unc-119 gene is still in the backbone of this vector, we just added the PuroR. You might be better off contacting the Dupuy lab for this application.
- For some reason you really want an mCherry reporter gene that is 500 bp shorter (pBCN22) (but remember this may not work in non-elegans species).

A note about Gateway vectors: All vectors that contain the Gateway cassette (Invitrogen) should be transformed into *E. coli* cells which are *ccdB* resistant. For the old vectors this had to be DB3.1 cells which are no longer commercially available, but the new batch of vectors (pBCN39-pBCN45) is also compatible with 'ccdB Survival' cells from Invitrogen. The plasmid should be grown with both ampicillin (ampicillin resistance on backbone) AND chloramphenicol (resistance in the Gateway cassette) to avoid loss of the Gateway cassette by recombination. Once the Gateway cassette has been replaced with your construct of interest, it is transformed into *ccdB* sensitive cells (most strains, e.g Top10 or DH5alpha) and should be selected with ampicillin only as it no longer has the chloramphenicol resistance gene. See the Invitrogen manuals for further information about the Gateway system.

Puromycin selection:

pBCN42 - a modified version of the published pBCN21 vector. Contains *ccdB* cassette flanked by AttR4 and AttR3 sites for 3-way Gateway recombination (e.g. promoter + ORF + 3pUTR insertions). Puromycin resistance is conferred by a *Prpl-28::PuroR::let-858* 3'UTR cassette. This cassette contains the promoter of the ribosomal protein large subunit 28 gene upstream of the puromycin resistance ORF in which two artificial introns were inserted, followed by the *let-858* gene 3' flanking sequence.

We recommend the use of a visual marker in conjunction with the puromycin selection in order to easily keep track of how the selection is working. This vector contains no such marker and can be used when the gene of interest itself gives a visual phenotype or fluorescence, or can be co-injected with another plasmid with a visual marker.

pBCN43 - same as pBCN42, but a visual reporter gene was inserted in a unique *Apal* site. The visual reporter is composed of mCherry with introns from the Jorgensen lab pCFJ104 vector, flanked by the *myo-2* promoter (1 kb of 5' flanking sequence of the *myo-2* CDS), and the *myo-2* 3'UTR (340 bp of 3' flanking sequence of the *myo-2* CDS). This reporter gene works well for expression in various species of *Caenorhabditis* (tested in: *C. elegans*, *C. briggsae*, *C. remanei* & *C. brenneri*).

G418 selection:

pBCN44 - Same as pBCN42 but contains the neomycin resistance gene for G418 selection instead of the puromycin resistance gene. NeoR was amplified from the pDestDD04Neo Dupuy lab vector, and PCR-stitched to the same *Prpl-28* promoter and *let-858* 3'UTR sequences that flank our PuroR gene.

pBCN45 - Same as pBCN43 but contains the neomycin resistance gene for G418 selection instead of the puromycin resistance gene (as in pBCN44).

Dual selection Puromycin-Neomycin operon vector for bombardment:

last updated: 24th Oct, 2011

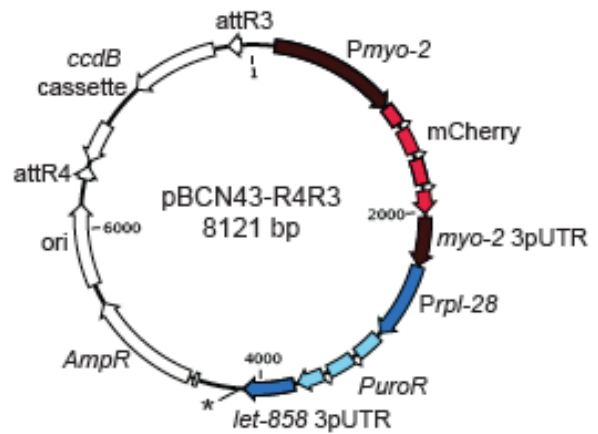
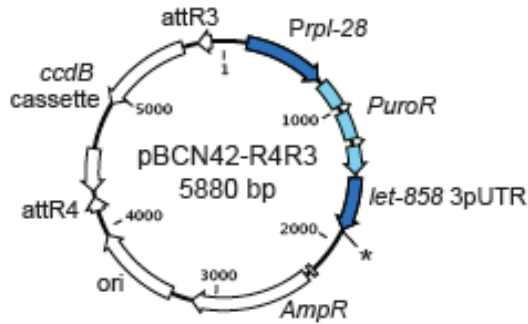
pBCN39 - Dual resistance operon vector. Same as pBCN42 or pBCN44, but the puromycin and neomycin genes are expressed as a single operon. The whole operon is under the control of the *rpl-28* promoter and the two ORFs are joined by the outtron upstream of the *rpl-16* gene. This vector is useful for selection at high dilution ratios such as those needed after bombardment (where few transformed worms are mixed with hundreds of thousands of wild type worms). The joint action of the two drugs is more effective than each drug on its own. This operon confers resistance to both drugs at once.

Even with this vector, selection is never 100%, so we strongly recommend the use of a visual marker in conjunction with the drug selection in order to pick transgenic worms at the end of the procedure. This vector contains no such marker and can be used when the gene of interest itself gives a visual phenotype or fluorescence.

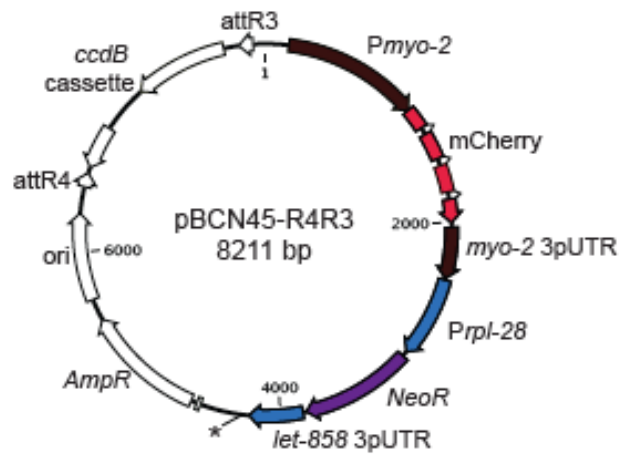
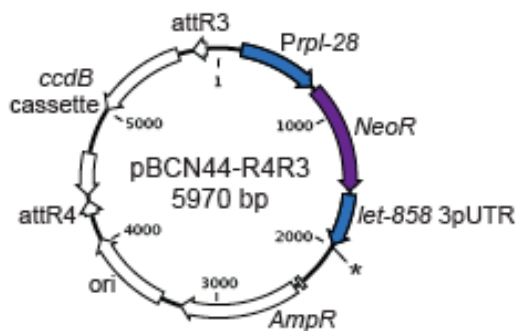
pBCN40 - Same as pBCN39, but with the *Pmyo-2::mCherry* fluorescent reporter gene from pBCN43 also present in the backbone.

pBCN41 - Same as pBCN40, but the mCherry ORF has been replaced with GFP containing synthetic introns amplified from the pPD129.57 plasmid from the Fire lab.

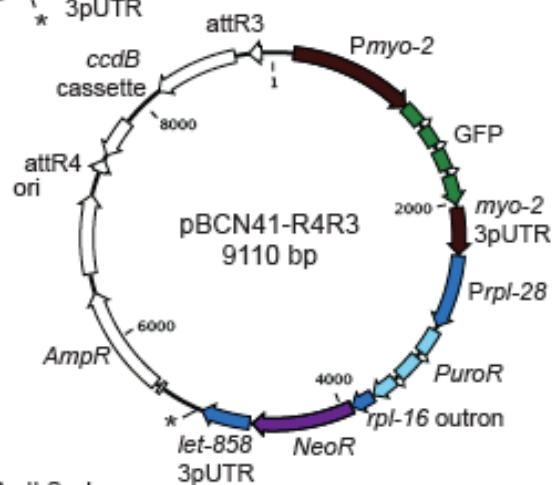
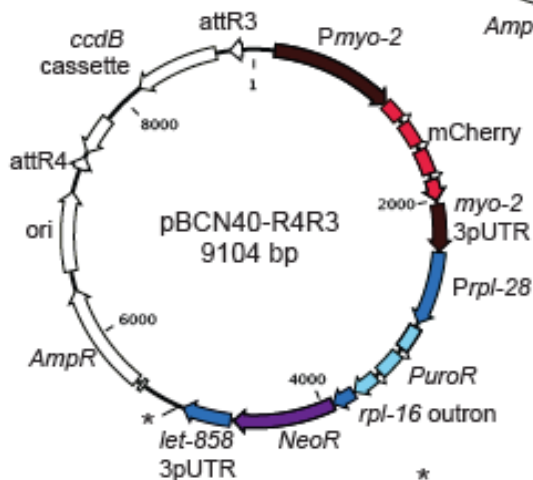
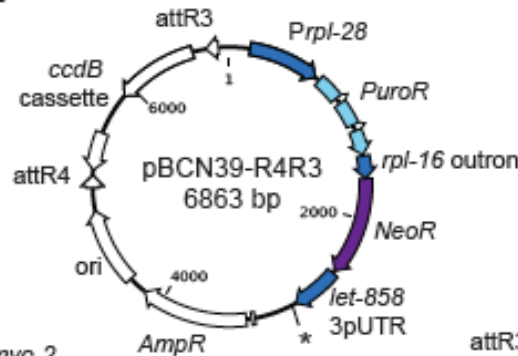
Puromycin selection



Neomycin selection



Dual selection PuroR-NeoR operon vectors for bombardment



* SpeI-BglII-XcmI-AvrII-SacI

4. Bacterial expression plasmids.

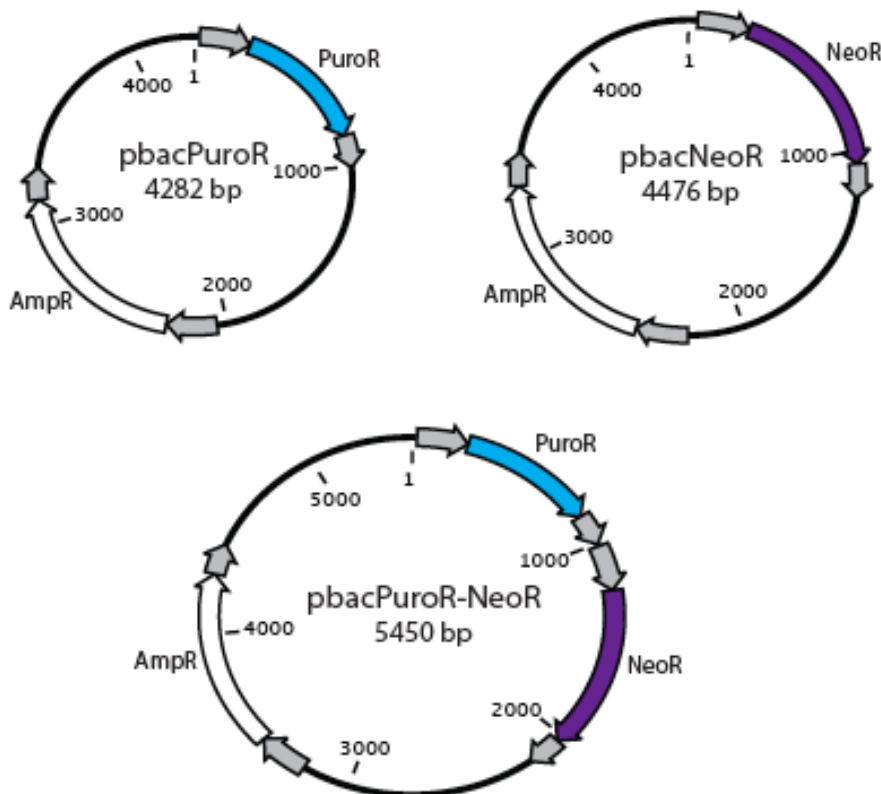
When puromycin or neomycin are used for selection on agar plates the OP50 bacterial lawn grows very poorly. Therefore we created plasmids and bacterial strains expressing the PuroR and NeoR genes under bacterial promoters. We used these bacteria as a food source on selective plates instead of OP50. They form a thinner lawn on selective plates than we normally get with OP50, but it is enough to support worm growth.

We transformed *DH5alpha* bacteria with these vectors. We used this strain to avoid recombination within the plasmid (especially since the sequences around *AmpR* and *PuroR* are the same). We use these bacteria as a food source on selective plates instead of OP50. They form a thinner lawn on selective plates than we normally get with OP50, but it is enough to support worm growth.

pbacPuroR - Upstream and downstream flanking sequences of the ampicillin resistance gene were fused with an intronless version of the puromycin resistance gene by PCR and inserted into the *Hind III* and *NheI* sites of pPD49.78 vector from the Fire kit. The vector still contains ampicillin resistance for bacterial selection.

pbacNeoR - Upstream and downstream flanking sequences of the ampicillin resistance gene were fused with the neomycin resistance gene by PCR and inserted into the *Sall* and *KpnI* sites of pPD49.78 vector from the Fire kit. The vector still contains ampicillin resistance for bacterial selection.

pbacPuroR-NeoR - The bacterial neomycin resistance cassette from pbacNeoR was inserted into the the *Sall* and *KpnI* sites of pbacPuroR vector to give a dual resistance vector. The vector still also contains ampicillin resistance for bacterial selection.



5. Protocols

We use puromycin from Sigma (P7255) and prepare a stock of 100 mg/ml in sterile water. We store the puromycin at -20°C in small aliquots to avoid too many rounds of freeze-thawing. When cold, the puromycin stock forms a white precipitate, but this dissolves at room temperature.

5.1 Puromycin selection of extrachromosomal arrays

The gene of interest can be inserted into the Gateway 3-way recombination site of the puromycin resistance plasmid or co-injected with this plasmid.

We recommend the use of a visual marker, such as the *Pmyo-2::mCherry* pharyngeal marker present on pBCN43, in order to easily evaluate the selection. If your gene of interest gives a visual phenotype, the pBCN42 plasmid can be used instead.

We normally use 1-5 ng/μl of the selection plasmid out of a total 100 ng/μl in the injection mix. (Excess amounts of the vector carrying the *Pmyo-2::mCherry* marker may be toxic).

5.1.1 Selection of large populations of transgenic worms from extrachromosomal lines in liquid

Some experiments require pure, large populations of transgenic worms. Puromycin selection in liquid medium is an easy method to achieve this without the need for expensive equipment such as a worm sorter, or the labour intensive integration of extrachromosomal arrays.

- Worms can be selected at high density (10-15 worms/μl). For example, 50,000 worms can be selected in only 5 ml NGM medium, thus requiring much less puromycin than that needed for selective agar plates.
- Worms can be selected as synchronised L1s in the absence of food, which means that they can then be placed back on non-selective plates and allowed to develop to the developmental stage of interest, or placed under the experimental conditions of interest without the need to continue puromycin selection.
- Selection can also be carried out with other developmental stages in liquid medium with a food source, however, as with the agar plates, these mixed stage cultures will also contain non-transgenic larvae. Nevertheless, these larvae die before they reach adulthood in selective medium.

Here is the protocol we follow to get pure large populations of transgenic worms without needing to make selective plates:

- a) Pick 8-10 adult transgenic worms onto 90mm non-selective NGM plates and allow the population to expand for 4-5 days at 20°C.
- b) Bleach the worms. Wash the embryos 3 times in M9 and allow the embryos to hatch overnight in M9.
- c) Count the number of L1s hatched in 5-15μl aliquots of the overnight culture and calculate the total number of worms.
- d) Collect the worms by centrifugation and resuspend them at a final concentration of 10 worms/μl in an appropriate volume of liquid NGM supplemented with 0.1% Triton and 0.5 mg/ml

puromycin. Triton enhances the effect of puromycin, and at this concentration does not show any toxicity on its own.

e) Grow for 4 days at 20 °C with gentle shaking (<100 rpm) and good aeration (no more than 5 ml culture in a 15 ml tube, and place tubes horizontally in shaker. Small conical flasks would probably work better than closed tubes, but we have not tried this.)

f) After 4 days count the number of viable L1 larvae in 3-5µl aliquots of the culture and plate on non-selective NGM plates seeded with OP50. (A density of 1,500 worms on a 90 mm plate normally lets the population reach adulthood before the food runs out).

5.1.2 Easy maintenance of strains with extrachromosomal arrays on selective plates

As this protocol requires larger volumes of media, we would recommend the use of G418 selection, as it is cheaper. However, it is possible to use puromycin for plate selection as well. Standard NGM agar plates are supplemented with 0.5 mg/ml puromycin just before pouring (when the agar is cool). We also add 100 µg/ml ampicillin to help prevent bacterial infections. Note that when performing selection on agar plates we do not use Triton as this is detrimental to the formation of a bacterial lawn.

Plates are allowed to dry for 24 h and then spotted with bacteria containing the pbacPuroR as a food source. Allow to dry overnight and store at 4°C till required.

Place a few worms carrying the extrachromosomal array on the plate and chunk to a new plate when the food runs out.

The plates normally contain a mixture of transgenic and non-transgenic larvae, but all the adults are transgenic and the transgene is easily maintained by simply chunking without the need to pick worms.

5.2 Puromycin selection in *C. briggsae* and other *Caenorhabditis* species

Puromycin selection works well in *C. briggsae*, but requires twice the amount of puromycin (1 mg/ml). We use the same amount of Triton (0.1%).

For successful transformation of *C. briggsae* we use complex arrays containing at least 90 ng/µl of AF16 genomic DNA (N2 genomic should do the trick too), and the rest of the mix made up to 100 ng /µl with any other plasmids you wish to transform not exceeding 4 ng/µl of any one plasmid. We linearise all the plasmids and also cut the genomic DNA with restriction enzymes. If co-injecting several plasmids, remember to always have an excess of the other plasmids over the selection marker plasmid.

When we injected *C. remanei* and *C. brenneri* we also prepared a mix with genomic DNA as described above, and we got transgenic lines on our first try. *C. remanei* (PB4641) is more sensitive to G418, and efficient killing is achieved with 0.5-1 mg/ml of the drug. *C. brenneri* is more sensitive to puromycin and is efficiently killed with 0.5 mg/ml of the drug.

5.3 Puromycin selection following bombardment

Puromycin selection is powerful enough to select 'rare' transgenic worms in a large population of transgenic worms. However, direct application of puromycin selection to the current bombardment protocol is not really possible: At a concentration of 10 worms/µl, starting with 100,000 worms each

with about 100 progeny, you would need the culture volumes of 1l (+1g puromycin) for each selection experiment which would be too expensive. If you were to use higher worm concentrations (>50 worms/ μ l), selection is not as efficient and you start to get problems of hypoxia .

To resolve these issues we have made two key modifications to the standard protocol:

- a) We perform selection only on the F1 progeny, removing the adults from the culture
- b) We use a dual selection vector containing both puromycin and neomycin resistance. Although both drugs target the ribosome, they target different stages of translation and the combination of the drugs seems more effective than each one on its own.

We have managed to obtain integrated lines by bombardment of *C. elegans*, *C. briggsae* and *C. remanei*. We have also tried *C. brenneri* several times without success. Our problems were probably in growing up the worms, since the selection itself does work when we inject the plasmids. The main problem was that *C. brenneri* and *C. remanei*, and many of the more 'wildtype' strains of *elegans* and *briggsae* dig into the egg plates and we could not recover them. We then tried growing in liquid, which gave us partial success, but with the male-female species (*C. brenneri* and *C. remanei*) the worms did not seem to mate well in liquid so we did not get fertilisation and so no progeny. At the recent worm meeting people with more experience with these species, kindly pointed out that the solution is actually quite simple: use plates with more agar (2.5%)! We tried it out and found that 2.5% agar works fine for *C. remanei* (PB4641), whereas *C. brenneri* requires an even higher percentage of agar (3% or 3.5%). *C. brenneri* worms tend to chew up the agar a bit, releasing fragments which one accidentally collects when washing the plates. We have started using a sucrose gradient to remove these fragments before bleaching, otherwise they seem to store up some bleach and then kill the embryos left overnight to hatch in M9.

Below is the protocol we have developed:

Preparation for bombardment:

- Grow up a large population of N2 worms for bombardment, by any standard method. We use egg plates: Chunk N2s onto 1-3 egg plates. When the worms have grown, bleach them and allow to hatch overnight in M9. The next day, plate 20,000 L1 worms per egg plate (3-5 plates) and grow for 3 days at 20°C to obtain young adults. Five egg plates usually give 1 ml of worms (~100,000 worms). We get successful bombardments with only ~0.5 ml worms from three egg plates, but more worms means a greater chance of success. *C. briggsae* are thinner than *C. elegans* and develop slower, so we always use 5 egg plates and also let them develop for 3.5-4 days before bombardment. For strains and species that tend to dig into the agar (such as *C. remanei* and *C. brenneri*) we suggest the use of plates with 2.5% agar at all stages, although we have not tested this yet.

Bombardment (day 0):

- Bombardment with the BioRad PDS 1000/He system using the hepta adaptor and DNA-coated gold beads is carried out according to the standard protocol (Berezikov *et al.* 2004, NAR 32:e40). We use between 0.5-1 ml of worms per bombardment. We tried using lower amounts of worms to improve the selection, but we generally find that the closer we are to 1 ml of worms the more successful the bombardment. Most likely due to the probability of a gold particle hitting a worm on a given surface area.

- We use the pBCN39, pBCN40 or pBCN41 dual selection vectors for bombardment. It is important to have a visual reporter - either your gene of interest, or in the backbone.
- Although not properly tested, we find bombardments work best when we use linearised vector. pBCN39-41 can be linearised with SpeI, BglII, XcmI, AvrII or SacI, as long as they don't affect your gene of interest. We precipitate the digestion reaction onto the gold beads directly, without any purification. (7 µg vector digested in 70 µl reaction volume. We precipitate according to a protocol from the Hope lab: digested vector + 70 µl of gold beads (60mg/ml stock), followed by 300 µl 2.5 M CaCl₂ and 112 µl 0.1 M spermidine with 1 min of vortexing at each stage.... Washed with 70% ethanol and 100% ethanol)
- After bombardment, leave the worms to recover for 10 min to 1h at 20°C. If you bombarded 1 ml of worms, add 2 ml M9 to the bombarded plate to wash off the worms and transfer ~0.5 ml to each of four 90 mm NGM plates seeded with OP50 bacteria.
- Try to recover even more worms from the bombarded plate with a further 1 ml M9 and transfer ~0.25 ml to each of the new 90 mm plates. Also keep the original bombardment plate, as there are usually some worms still left on it.
- Incubate all five plates at 20°C for 24h. The small amount of food allows the worms to lay eggs, but newly hatched L1s arrest due to starvation.

Harvesting L1s and selection (day 1):

- Wash off the worms from the 4x 90 mm plates by adding ~4 ml M9 to the original bombardment plate and transfer the worms+liquid sequentially from one plate to the next and eventually to a 15 ml conical tube. Repeat twice more, combining the washes in the same 15 ml conical tube.
- Allow the adult worms to separate out by gravity for 10 min at room temperature.
- Transfer the top ~10-12 ml to a new 15 ml falcon, taking care not to disturb the 'pellet' of adults at the bottom of the tube.
- Centrifuge this 'supernatant' at 1,800 rpm for 2 min in order to recover the L1s, and remove the excess M9.
- Add another ~12 ml M9 to the original tube containing adults, invert several times to resuspend the worm 'pellet' and repeat the sedimentation of adults by gravity for 10 min at room temperature.
- Transfer the top ~10-12 ml supernatant and combine with the first L1 fraction.
- Centrifuge the L1s at 1,800 rpm for 2 min and remove the excess M9 (we normally get a 100-300 µl 'pellet' of L1s at this stage).
- The adults can be re-plated onto 4x90 mm plates for a further 24 h to recover another batch of L1s tomorrow, or they can be discarded at this stage (we normally don't bother with a second batch of selection).
- Prepare 10-15 ml liquid NGM medium supplemented with 0.1% Triton, 1 mg/ml puromycin and 0.5 mg/ml G418.
- Resuspend the L1s in this medium and divide into two 50 ml conical tubes (5-7.5 ml culture in each, to allow adequate aeration). Using small 25-50 ml conical flasks (Erlenmeyer) will probably work better, but we have not tried this (aeration will be better but evaporation might be a problem).

- Place conical tube horizontally in a 20°C shaking incubator and incubate for 4 days with very gentle shaking (no more than 100 rpm, because the Triton in the medium could make them sensitive to violent shaking).

Plating selected worms (day 5):

- Pellet the worm cultures by transferring the contents of each 50 ml conical tube to a 15 ml conical tube and then wash out the empty 50 ml tube with M9 and combine it with the rest of the culture.
- Centrifuge for 2 min at 1,800 rpm. Remove the supernatant, taking care not to disturb the worm pellet. Transfer the worms from each 15 ml tube to a single 60 mm NGM plate containing seeded with bacteria. It is possible to use G418 selective plates (0.3-0.5 mg/ml) at this stage, with a lawn of resistant bacteria (transformed with pBacNeoR) to improve selection, but we don't bother.
- Incubate the plate at 20°C for 3 days to allow viable transformed worms to develop to adults).

Picking transgenic worms (day 8):

- Visually screen the 2x60mm plates obtained from the bombardment to look for viable transgenic worms. And pick worms into individual wells of 12 well NGM plates (non-selective) seeded with OP50 bacteria.
- Because we are picking the F1 generation every single picked worm, if it transmits the transgene, can be considered an independent line.
- Please note that not all worms that survive this selection procedure are transgenic, which is why it is important to include a second visual marker such as the Pmyo-2::mCherry or GFP in our pBCN40 or pBCN41 vector (this visual marker is flanked by Apal sites and can be easily replaced by a marker of your choice if this one is not convenient for you).
- The visual marker is helpful in distinguishing worms that are more likely to transmit the transgene to their progeny. We generally find that worms with very patchy expression patterns of the Pmyo-2::mCherry in the pharynx are less likely to yield a stable line and are therefore not worth picking. The more uniform the expression the more likely it is to be a transmitting or integrated line. Note that low copy number integrants will have much weaker expression than most of the transgenic worms on the plate, so keep an eye open for dimmer but uniform expression of the mCherry marker.
- We normally re-screen the original 60mm plates 2-3 days later to make sure we have not missed any transgenic worms. Any lines obtained at this stage cannot be counted as independent lines as they could include F2 progeny of the same F1 (unless they com from different 60mm plates).
- As a rough guide we generally get 200-500 surviving worms per plate, of which ~30 are transgenic, a few (~10) are transmitting lines, and ~1 integrated line. About 50% of our bombardments generate an integrated line.

Identifying stable lines (day 11):

- Check the wells of the 12 well plates for transmitting lines. If transmission is high, and it looks like it might be an integrated line (~75% transmission), re-pick 6-12 worms into individual wells of 12 well plates seeded with OP50 to identify homozygotes.

5.4 Single copy integration with puromycin selection.

A single copy of the puromycin resistance gene is sufficient to confer resistance to the drug. The pBCN27 plasmid is adapted from the MosSCI pCFJ150 plasmid (Frokjaer-Jensen, *et al.* Single-copy insertion of transgenes in *Caenorhabditis elegans*. (2008) Nat Genet 40, 1375-1383.), and contains the puromycin selection gene in addition to unc-119. We have not actually tried this ourselves, but it might be possible to reduce the length of the MosSCI protocol by performing puromycin selection directly

after heat shock. Furthermore, drug selection can be used to target any other Mos insertion strain without the need to back cross it into the *unc-119*^{-/-} background. The Dupuy lab have developed neomycin and puromycin vectors that target a couple of different Mos insertion sites.

This ability to target many Mos insertions in different worm strains without having to introduce the *unc-119* background could be particularly important in the new Mos-Deletion protocol (Frokjaer-Jensen *et al.* Targeted gene deletions in *C. elegans* using transposon excision. (2010) Nat Methods 7, 451-453.).

5.5 G418 selection

The NeoR selection was developed by the Dupuy lab (Giordano *et al.* Nat. Methods, Aug. 2010). We simply cloned their NeoR ORF into our backbone because we are using a different Gateway destination vector (ours is the three-way gateway R4R3 site). When we tested sensitivity to G418 we found *C. elegans* was killed efficiently by 0.5 mg/ml G418 in liquid (the next lowest concentration we tried was 0.25 mg/ml, which gave more false positives, but we didn't try anything in between). In solid agar plates, the Dupuy lab use 0.3 mg/ml, which seems to be enough to maintain a transgenic population. We have not actually tried out the pBCN24 vector in vivo, but the *rpl-28* promoter works well for PuroR and the NeoR cassette successfully in the operon bombardment vectors, so we have every reason to believe it should work fine.

6. Media recipes

Liquid NGM

For 1 litre:

3g NaCl

2.5g Peptone

Make up to 1l with water and autoclave.

Keep at 4°C (probably fine at room temp) until use.

When you are about to set up the culture, take the required volume of incomplete NGM and for every 1ml supplement with:

1 µl cholesterol solution (5mg/ml stock in ethanol)

1 µl of 1M calcium chloride (CaCl₂)

1 µl of 1M magnesium sulphate (MgSO₄)

25 µl of 1M pH 6 potassium phosphate buffer (see below)

0.8 µl Fungizone

1 µl of 100 mg/ml Ampicillin

For selection also add the appropriate amount of triton and antibiotic, e.g. for *C. elegans*:

10 µl of 10% Triton (v/v in water) - mix well.

5 µl of 100 mg/ml puromycin (Sigma P7255, dissolve in H₂O)

1µl of 500 mg/ml G418 (A1720-1G from Sigma, or G4185 from ForMedium, dissolve in H₂O)

Potassium phosphate buffer:

For 1 litre:

136.1 g KH₂PO₄ + 17.9 g KOH.

Add water to 750 ml, adjust pH to 6.0 with KOH and make volume up to 1l. Autoclave.

Egg plates:

We prepare egg yolk plates as described in Berezikov *et al.* 2004, NAR 32:e40.

7. Appendix A - Previous versions of the vectors

The puromycin selection is the same in these vectors as in the new ones. So if you have the old vectors and they are working for you then just keep using them. The newer versions (see above) have got modifications to the fluorescent reporter gene to improve its expression in non-*elegans* species, and modifications to the backbone to make them compatible with commercially available ccdB Survival *E. coli*, and easier to linearise for bombardment.

Puromycin selection without a fluorescent marker:

pBCN21 - a modified version of the pCG150 vector from the Seydoux kit. Contains ccdB cassette flanked by AttR4 and AttR3 sites for 3-way Gateway recombination (e.g. promoter + ORF + 3pUTR insertions). The *unc-119* cassette present in the backbone of the original vector was removed and replaced with a *Prpl-28::PuroR::let-858* 3'UTR cassette. This cassette contains the promoter of the ribosomal protein large subunit 28 gene upstream of the puromycin resistance ORF in which two artificial introns were inserted followed by the *let-858* 3' flanking sequence.

We recommend the use of a visual marker in conjunction with the puromycin selection in order to easily keep track of how the selection is working. This vector contains no such marker and can be used when the gene of interest itself gives a visual phenotype or fluorescence, or can be co-injected with another plasmid with a visual marker.

Puromycin selection with fluorescent marker (C. elegans only):

pBCN22 - same as pBCN21, but an additional visual marker was inserted in a unique Apal site. The visual marker is composed of mCherry preceded by the minimal *myo-2* promoter and 8 copies of the C183 pharyngeal enhancer (Thatcher JD, *et al.* (1999). *Development* 126, 97-107). This reporter gene works well for expression in *C. elegans* while minimising the size of the backbone, to avoid difficulties when cloning large genes of interest into the Gateway site. However, this minPmyo-2::mCherry reporter gene does not work in *C. briggsae* and we recommend the use of pBCN28 for species other than *C. elegans*.

Puromycin selection with fluorescent marker (various Caenorhabditis species):

pBCN23 - (now replaced by pBCN28) Same as pBCN22 but the minimal *myo-2* promoter and C183 enhancers have been replaced by the full length *C. elegans myo-2* promoter. This vector works in *C. briggsae*, however expression is a bit patchy so we created pBCN28, which contains mCherry+introns (we get the impression that *C. briggsae* is much more fussy about needing introns for good expression).

pBCN28 - Same as pBCN21 but a new *Pmyo-2::mCherry::unc-54* 3'UTR cassette was created, containing mCherry + introns from the Jorgensen pCFJ104 vector, for better expression in species other than *C. elegans*. (This vector replaces pBCN23. We have confirmed good Pmyo-2::mCherry fluorescence *in vivo* in *C. elegans*, *C. briggsae*, *C. remanei* and *C. brenneri*).

MosSCI vector with puromycin selection:

pBCN27 - MosSCI puromycin resistance vector. This vector is a modification of the Jorgensen pCFJ150 MosSCI vector that targets the ttTi5605 Mos insertion site. The *Prpl-28::PuroR::let-858* 3'UTR cassette was inserted into the backbone of the vector (*XhoI* & *SpeI* sites) without removing

the *cb-unc-119* rescue gene. We have not tried to select for MosSCI directly by puromycin, but we have shown that the resistance works well even in single copy and therefore we believe that rapid drug selection can be used directly for this method as suggested by the Dupuy lab, thus removing the need to cross various Mos insertions strains into an *unc-119* background.

pBCN27-Pmyo-2::GFP::unc-54 - same as pBCN27, but with the myo-2 promoter, GFP ORF and unc-54 3'UTR fragments inserted into the 3 way Gateway site. (Can be used as a positive control to test for direct puromycin selection for single copy integration).

Neomycin and Zeocin selection plasmids:

pBCN24 - Same as pBCN21 but with the neomycin resistance gene for G418 selection instead of the puromycin resistance gene.

pBCN25 - Same as pBCN21 but with the Zeocin resistance gene for selection with zeocin/bleomycin/phleomycin. (We have not tested this vector but it is available in case anybody wants to try a third antibiotic resistance gene).

Puromycin-Neomycin operon selection vector for bombardment:

pBCN26 - Dual resistance operon vector. Same as pBCN23, but the puromycin and neomycin genes are expressed as a single operon. The whole operon is under the control of the *rpl-28* promoter and the two ORFs are joined by the outtron upstream of the *rpl-16* gene. This vector is useful for selection at high dilution ratios such as those needed after bombardment (where few transformed worms are mixed with hundreds of thousands of wild type worms). The joint action of the two drugs is more effective than each drug on its own. This operon confers resistance to both drugs at once.

pBCN30 - Same as pBCN26, but with the Pmyo-2::mCherry construct from pBCN28 that contains introns and expresses well in *C. briggsae*, *C. brenneri* and *C. remanei*.

Old versions of the vectors

