Puromycin Selection

"Rapid selection of transgenic C. elegans using antibiotic resistance." Semple JI, Garcia-Verdugo R, Lehner B. Nature Methods 7(9):693-5, (Sep, 2010)**.**

Contents:

1. Applications:

- Select large populations of extrachromosomal transgenic worms (the cheap chemical equivalent to a worm sorter).
- Maintain extrachromosomal lines on selective agar plates without picking
- Positive selection in both *C. elegans* and *C. briggsae.*
- Single copy insertions (Dupuy lab)
- Rapid selection after bombardment (in development)

2. Drug selection vectors for worms

The basic P*rpl-28*::*PuroR* puromycin resistance cassette is the same in all the vectors and works well in both *C. elegans* and *C. briggsae*.
The reason we currently have multiple puromycin vectors is because the minimal *myo-2* promoter in pBCN22 does not work in *C. briggsae* and we are attempting to get a visual marker 'version' that works well in different species. Hopefully, pBCN28 will fulfill these requirements, but we still have not got round to testing it. With *C. elegans* there is no problem and the visual marker in any of these vectors works.

A note about Gateway vectors*:* All vectors that contain the Gateway cassette (Invitrogen) should be transformed into DB3.1 *E. coli* which are *ccdB* resistant (the *ccdB* resistant bacteria sold by Invitrogen do not seem to work so well). 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 (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 without a fluorescent marker (tested in C. elegans & C. briggsae):

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 P*rpl-28*::*PuroR*::*unc-54* 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 *unc-54* 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.

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

pBCN22 - same as pBCN21, but an additional visual marker was inserted in a unique ApaI 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 minP*myo-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 (C. briggsae & C. elegans):

pBCN23 - 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 and we recommend the use of 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 P*myo-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 fluoresence *in vivo* in *C. elegans*, *C. briggsae*, *C. remanei* and *C. brenneri*. We have yet to test the puromycin selection in *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 P*rpl-28*::*PuroR*::*unc-54* 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

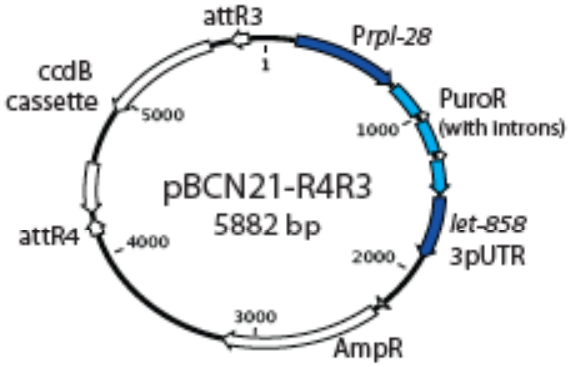
method as suggested by the Dupuy lab, thus removing the need to cross various Mos insertions strains into an unc-119 background.

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).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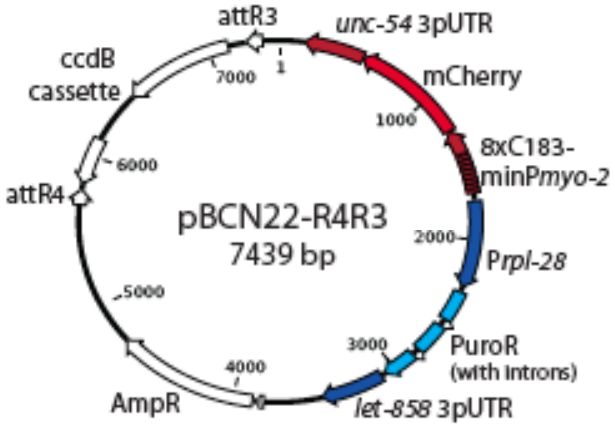
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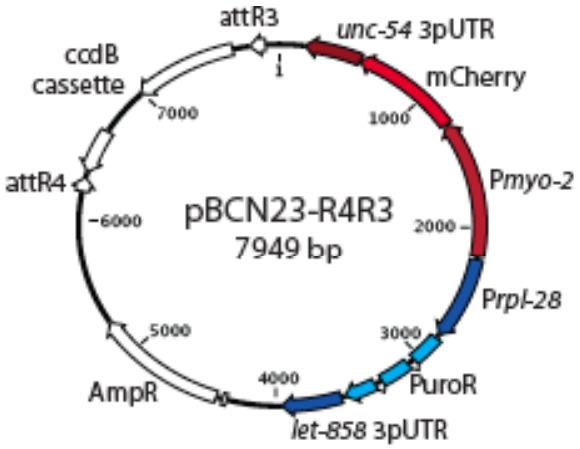
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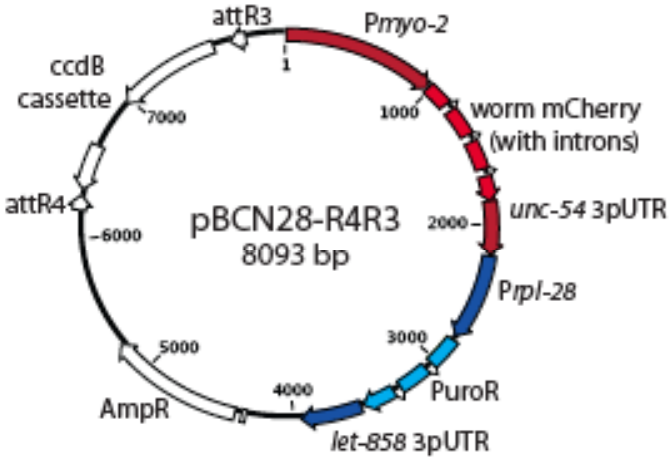
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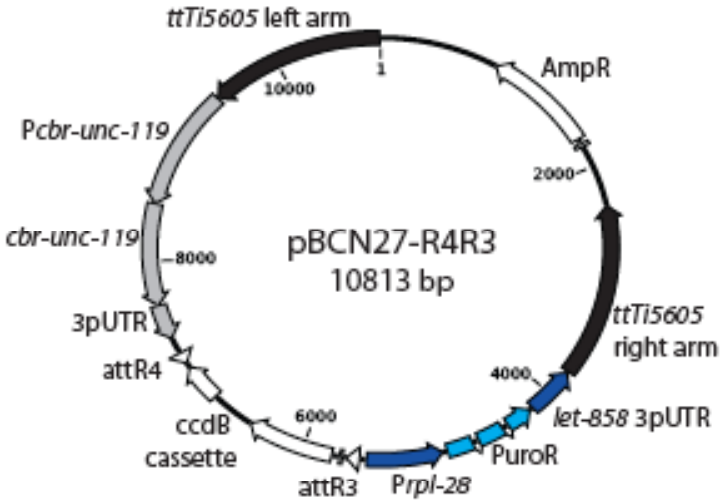
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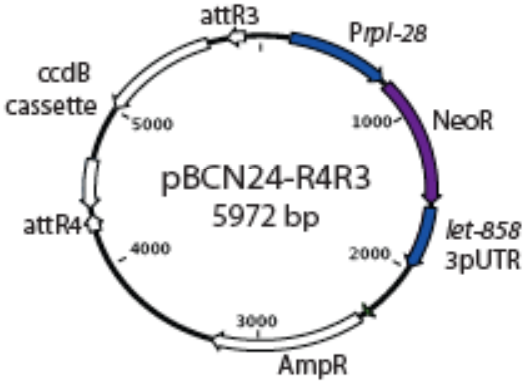
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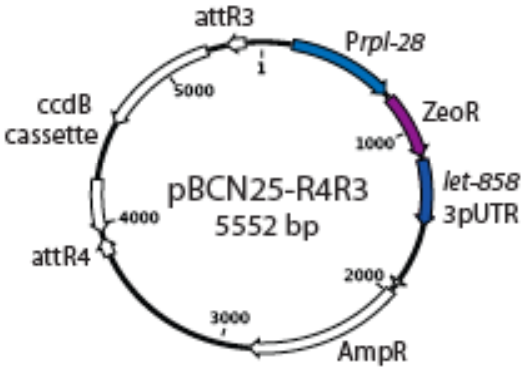
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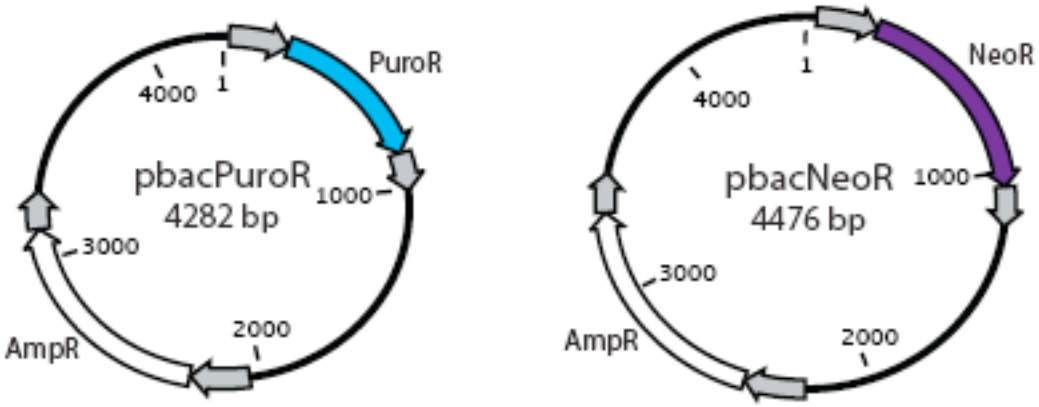
3. Bacterial expression plasmid

When puromycin is used for selection on agar plates the OP50 bacterial lawn grows very poorly. Therefore we created plasmids expressing the *PuroR* and *NeoR* genes under bacterial promoters.

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 SalI and KpnI sites of pPD49.78 vector from the Fire kit. The vector still contains ampicillin resistance for bacterial selection.



4. 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.

4.1 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 P*myo-2::mCherry* pharyngeal marker present on pBCN22 and pBCN28) in order to easily evaluate the selection. If the gene of interest gives a visual phenotype, the pBCN21 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).

4.1.1 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 by liquid selection:

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 over night 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 and good aeration.

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).

4.1.2 Easy maintenance of strains with extrachromosomal arrays on selective plates

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 pbacPuro 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.

4.2 Puromycin selection of C. briggsae

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.

4.3 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 practical: At a concentration of 10 worms/μl the culture volumes would be too big to be cost efficient, and at much higher worm concentrations selection is not as efficient. We are currently developing a protocol that overcomes these problems and will try to make it available as soon as possible.

4.4 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.).

4.5 G418 selection

The NeoR selection was developed by the Dupuy lab (Giordano *et al.* Aug. 2010 Nat. Methods). We simply cloned his construct 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 we have used the NeoR cassette successfully in another context, so we have every reason to believe it should work fine.

5. Media recipes

Liquid NGM
For 1 litre:
3g NaCl
2.5g Peptone
Make up to 1l with water and autoclave.
Keep at 4C (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 ($CaCl_2$)
1 μl of 1M magnesium sulphate ($MgSO_4$)
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 H_2O)

Potassium phosphate buffer:
For 1 litre:
136.1 g KH_2PO_4 + 17.9 g KOH.
Add water to 750 ml, adjust pH to 6.0 with KOH and make volume up to 1l. Autoclave.