

## File S1

### Detailed protocol for CRISPR/Cas9 mediated genome editing with dual marker cassettes

#### A) Preparing sgRNA vectors

We've modified the strategy to create new sgRNA vectors starting with the *kfp-12* vector as a template. The strategy from the original papers works just fine as well, but these modifications reduce the amount of work involved. We thank Arneet Saltzman for suggesting this protocol.

In the new strategy we amplify around the plasmid by PCR, incorporating the new targeting sequence in a forward PCR primer, and a universal reverse primer. Following PCR amplification of the vector, the free 5' ends are phosphorylated and then ligated in sequential steps with no need to clean up the PCR product. An aliquot of the ligation reaction then goes directly into a transformation reaction. Minipreps are performed on a few clones and validated directly by sequencing. Usually nearly every clone we sequence has the new target sequence incorporated.

#### New primer considerations

##### sgRNA-specific F primer:

5'-G(*N*<sub>20</sub>)GTTTTAGAGCTAGAAATAGCAAG-3'\*

Where *N*<sub>20</sub> is the new 20mer targeting sequence to introduce

\*Notes:

*It has been found that a terminal GG at positions *N*<sub>19</sub> and *N*<sub>20</sub> can dramatically increase the efficiency of targeting (FARBOUD and MEYER 2015).*

*The G in front of the *N*<sub>20</sub> is only required if the *N*<sub>1</sub> nucleotide is not a G. This G is only present to ensure that the transcription start site from the U6 promoter begins with a G. If nucleotide *N*<sub>1</sub> is a G, then the primer should be of the form:*

5'-(*N*<sub>20</sub>)GTTTTAGAGCTAGAAATAGCAAG-3'

##### sgRNA universal R primer:

5'-AAACATTTAGATTTGCAATTCAATTA-3'

Protocol:

1. Prepare 50 uL Phusion reaction on ice according to manufacturer's instructions:

MasterMix for 1x (50uL)

ddH <sub>2</sub> O	32.5
5xHF Buffer	10
10mM dNTPs	1
sgRNA universal R primer(10uM)	2.5
<i>kfp-12</i> sgRNA vector (20pg/uL)	1
<u>Phusion polymerase</u>	<u>0.5</u>
	47.5 uL

Individual items:

<u>sgRNA specific F primer(10uM)</u>	<u>2.5</u>
	50 uL

2. PCR settings

1. 98° 30s
2. 98° 30s
3. 56° 10-30s
4. 72° 1min45s
5. go to step 2, 9 times
6. 98° 30s
7. 61° 30s
8. 72° 1min45s
9. go to step 6, 14 times
10. 72° 5min
11. 4° hold ∞

3. Run 5 uL of PCR reaction out on agarose gel. Should see a clean band. If not you can still move forward with protocol or consider increasing cycle number to 30 or 35, although this could risk increasing chance of point mutations.
4. Purify reaction using any PCR purification column, and elute with 26 uL elution buffer with 2 min incubation on column at room temperature.
5. To purified DNA, add 3 uL 10x T4 DNA Ligase\* buffer (NEB), and add 0.5 uL T4 PNK (NEB).

\*Note:

*10x T4 ligase buffer is identical to 10x PNK buffer except it already has the ATP in it (1mM at 1x) (needed by both enzymes but normally not added to PNK buffer for flexibility in downstream applications).*

6. Pipette or flick and quick spin to mix. Incubate 37°C for 20 min.
7. Remove 10 uL for “No ligase” control.
8. To rest of DNA (~19uL), add 1 uL T4 DNA ligase (NEB). Pipette or flick and quick spin to mix.
9. Incubate room temperature 1 hr .
10. Transform competent *E. coli* (e.g. DH5α or equivalent) with 1uL of reaction and control (No Ligase).
11. Should see many colonies on reaction plates and very few colonies on control plates. Pick 1-3 single colonies to miniprep and validate insertion of new protospacer directly by sequencing (can use M13 F primer that is available at most companies).

## **B) Preparing homology arm repair vectors**

### 1. Digest the vectors\* and alkaline phosphatase treatment.

- 1a. The Pmyo-2::GFP neoR loxP disruption/deletion vector is cut sequentially with SacII (Thermo) followed by cleanup with a PCR purification kit, followed by the NotI fastdigest enzyme (Thermo). SacII has much better activity in its own buffer. We usually digest a big batch of this vector so that it's good for many Gibson assembly reactions.

ddH2O	34 – x uL
10x SacII Buffer	4uL
SacII enzyme	2uL
<u>Pmyo-2::GFP neoR loxP</u>	<u>x uL (2ug)</u>
	40 uL

Incubate at 37°C for 4 hours, run through PCR purification column and elute in 34 uL elution buffer or water, then set up second digest:

10x FastDigest Buffer	4uL
FastDigest NotI enzyme	2uL

<u>Pmyo-2::GFP neoR loxP</u>	34 uL
	40 uL

Incubate at 37°C for 2 hours and 30 minutes, then add 1 uL of FastAP (Thermo - alkaline phosphatase), incubate at 37°C for another 30 minutes, and run sample through PCR purification column. Nanodrop DNA to assess yield.

1b. The GFP, RFP, 3xHA, and GFP::3xHA Pmyo-2::GFP neoR loxP tagging vectors have been modified such that they can be cut in one step with FastDigest SpeI and FastDigest NotI enzymes (Thermo).

ddH <sub>2</sub> O	32 - x uL
10x FastDigest Buffer	4uL
FastDigest SpeI enzyme	2uL
FastDigest NotI enzyme	2uL
<u>Tagging vector</u>	x uL (2ug)
	40 uL

Incubate at 37°C for 2 hours and 30 minutes, then add 1 uL of FastAP (Thermo - alkaline phosphatase), incubate at 37°C for another 30 minutes, and run sample through PCR purification column. Nanodrop DNA to assess yield.

\*NOTE:

*Vectors prepared this way will liberate the Pmyo-2::GFP neoR selection cassette which is then left together with the vector in the same tube. These digested vector and cassette fragments are then assembled with the homology arms in one 4 piece Gibson Assembly reaction. While this does generally work well, it's less efficient in our hands than sequentially cloning in each homology arm by Gibson Assembly. As an alternative approach you can cut with the individual enzymes and insert the homology arms one at a time, which works very efficiently but has the drawback that you need multiple steps to generate the vector. In this latter approach, you need to make sure the order of inserting the homology arms is compatible with the enzymes being used to cut the vector (i.e SacII/NotI or SpeI/NotI).*

## C) Preparing Homology arms

### 1. Designing primers

We are cloning everything by Gibson Assembly, so primers\* are designed to have 25 nucleotides of overlap at their ends to the relevant regions of the digested vectors:

Pmyo-2::GFP neoR loxP (disruption/deletion vector) primers

Upstream F: AACGACGGCCAGTGAATTCCCGCGG(N<sub>x</sub>)  
 Upstream R: TTATAGGCCCGCTGATGCGCCGCGG(N<sub>x-RC</sub>)  
 Downstream F: ATAGTTGCAGGACCACTGCGGCCGC(N<sub>x</sub>)

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC(N<sub>x-RC</sub>)

Where N<sub>x</sub> and N<sub>x-RC</sub> are homology arm specific sense and reverse complementary sequences, respectively.

GFP-Pmyo-2::GFP neoR loxP (GFP tagging vector) primers

Upstream F: AACGACGGCCAGTGAATTCAGT(N<sub>x</sub>)

Upstream R: GTTCTTCTCCTTTACTCATACTAGT(N<sub>x-RC</sub>)

Downstream F: TGGATGAACTATACAAAGCGGCCGC(N<sub>x</sub>)

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC(N<sub>x-RC</sub>)

RFP-Pmyo-2::GFP neoR loxP (RFP tagging vector) primers

Upstream F: AACGACGGCCAGTGAATTCAGT(N<sub>x</sub>)

Upstream R: CTCACCCCTTTGAGACCATACTAGT(N<sub>x-RC</sub>)

Downstream F: TGGATGAAATTGTATAAGGCGGCCGC(N<sub>x</sub>)

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC(N<sub>x-RC</sub>)

3xHA-Pmyo-2::GFP neoR loxP (3xHA tagging vector) primers

Upstream F: AACGACGGCCAGTGAATTCAGT(N<sub>x</sub>)

Upstream R: CTGGAACGTCGTATGGGTAAGT(N<sub>x-RC</sub>)

Downstream F: ACGTCCCAGATTACGCTGCGGCCGC(N<sub>x</sub>)

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC(N<sub>x-RC</sub>)

GFP-3xHA-Pmyo-2::GFP neoR loxP (GFP-3xHA tagging vector) primers

Upstream F: AACGACGGCCAGTGAATTCAGT(N<sub>x</sub>)

Upstream R: GTTCTTCTCCTTTACTCATACTAGT(N<sub>x-RC</sub>)

Downstream F: ACGTCCCAGATTACGCTGCGGCCGC(N<sub>x</sub>)

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC(N<sub>x-RC</sub>)

\*Notes:

1) When designing homology arms, need to consider if you will need to mutate the PAM in order to avoid having the repair plasmid DNA also be a target for cleavage. For the Pmyo-2::GFP neoR loxP deletion vector, we usually design our homology arms to be outside the region of cleavage, so this is typically not an issue. But for the endogenous tagging vectors, you will need to pay attention to this.

2) The primers above for the tagging vectors are designed for C-terminal fusions. The GFP, RFP, and GFP-3xHA ORFs have start codons built into the vector, but they do not have stop codons (see plasmid maps for reference). You will need to pay attention to make sure your final edited locus will have appropriate start and stop codons where you want them and if you are making an N or C terminal fusion protein strain.

*The 3xHA ORF has no start or stop codons, so you will need to also design your homology arms accordingly depending on if you want to make an N or C terminal tagged fusion protein strain.*

## 2. PCR amplification of homology arms

There are several options for amplifying homology arms. We have had success with amplifying directly out of genomic DNA or plasmid/fosmid DNA. Alternatively, you could also design gBlocks in situations where amplicons are difficult to obtain. We usually gel purify the PCR products if they are not very clean (i.e multiple bands on gel).

## **D) Gibson Assembly of Homology Arms with vector and selection cassette**

### 1. Set up assembly reaction and control in PCR tubes

assembly reaction:

ddH <sub>2</sub> O	10 - x - y - z uL
Cut vector + selection cassette	x uL (100ng)*
Upstream homology arm	y uL (100ng)
Downstream homology arm	z uL (100ng)
<u>2x Master Assembly mix</u>	<u>10uL</u>
	20 uL

control reaction:

ddH <sub>2</sub> O	20 - x uL
<u>Cut vector + selection cassette</u>	<u>x uL (100ng)</u>
	20 uL

2. Incubate at 50°C for 1 hour in thermal cycler and transform into DH5α or equivalent competent cells.

3. Screen and validate clones that have both homology arms and selection cassette inserted.

\*Notes:

*1) These amounts for the assembly reaction are just rough estimates. You can scale down accordingly if you don't have as much material. Protocol suggests two-fold molar excess of inserts, but it has worked well in our hands using the amounts above.*

*2) We have recently switched over to the NEB HiFi assembly kit since NEB claims it's more efficient for multi-piece assembly with smaller amounts of material, and it's the same price as the Gibson mix.*

## E) Microinjection of vectors and drug selection/screening

### 1. Set up injection mix:

<u>Plasmid</u>	<u>Final concentration (ng/uL)</u>
*Peft-3::Cas9_SV40-NLS::tbb-2_UTR	50
pU6::sgRNA vector	100
Repair template	50
*pCFJ90 (Pmyo-2::cherry)	2.5
*pCFJ104 (Pmyo-3::cherry)	5

#### \*Notes

*We recommend making high quality DNA preps of the vectors with asterisks (midiprep for Cas9 vector because a lot of it is used in each injection and Invitrogen Purelink HQ for pCFJ90 and pCFJ104).*

*For the repair template and sgRNA vectors however, high quality DNA preps are good but can be replaced by standard minipreps with an extra purification step. When we use sgRNA vectors prepped by our standard GeneJET minipreps (Thermo Scientific), we see a lot of sterility in the injected P<sub>0</sub> mothers. Midipreps can be a bit time consuming and wasteful, especially if the sgRNA and repair template vectors are only used once. We have recently taken our GeneJET miniprep prepared sgRNA and repair template vectors and passed them through a Zymo clean and concentrator-5 kit and elute with water, and this effectively eliminated the sterility effect and gave really good transgenesis results. We would recommend this extra step as a convenient method for cleaning up and concentrating the sgRNA vectors from standard miniprep kits. We are not sure if other standard miniprep kits give similar toxicity/sterility, but in our hands this extra cleanup step makes a huge difference from no transgenics to many.*

2. Inject animals. We typically will aim for 15-20 animals if injections usually lead to most P<sub>0</sub>s surviving and throwing ~25-30 Ex F<sub>1</sub> progeny each by 24 hours post injections (we typically inject both gonad arms of most animals). In general, we aim for seeing several hundred transgenic extra-chromosomal F<sub>1</sub> progeny across all injected animals. It's recommended to inject more animals if injections do not generally yield large amounts of extra-chromosomal F<sub>1</sub> progeny. Alternatively, some extra practice at injections with one of our positive control injection mixes will be helpful for future injection success.

3. After injection, let animals recover for a half hour to one hour at room temp. Then move 3-4 adults per plate and place in 25°C incubator.

4. Day after injection and recovery, add 500 uL\*\* of 25 mg/mL G418 (Sigma) to each plate.

**\*\*Note**

*A recent protocol using G418 selection states that for best selection conditions, you should weigh your agar plates (FROKJAER-JENSEN et al. 2014). A standard 6 cm plate contains about 8g of agar, and that is what this 500 uL volume is designed for. If your plates weigh dramatically different, you can scale the amount of drug added.*

5. Swirl drug over surface of plate to ensure complete coating. Transfer plates back to 25°C incubator with the lids removed to allow the drug solution to soak in. Check after a half hour and an hour. When plates are completely dry, return lids and continue incubation at 25°C for the next week to ten days.

6. About three to five days after adding drugs\*, should primarily see transgenic animals on plates (Ex and possible integrants)\*\*. Real CRISPR integrant animals should be distinguishable from Ex animals because they will usually have dim GFP pharyngeal expression\*\*\* that are completely fluorescent and uniform, and they will also lack the mCherry co-injection marker expression. Extra-chromosomal array animals will have mosaic and very bright GFP expression in the pharynx, and will have one or both of the cherry expressing markers.

**\*Note**

*The earliest we have seen integrants start to appear is four days post-injection. This is usually most obvious by day five and six, but we have also seen later arising transgenics in days 7-11. We recommend checking for insertions up until plates starve out.*

**\*\*Note**

*In our hands, the G418 treatment does not lead to perfect selection, but rather serves as an enrichment scheme. We do notice non-transgenic wild type escapers present in our populations. Screening for pharyngeal GFP expression is thus critical for success in the procedure.*

**\*\*\*Note**

*Occasionally we also observe bona fide integrants that have much brighter GFP fluorescence. These animals are still easy to spot, because they lack mCherry fluorescence markers and have uniformly bright and complete pharyngeal GFP expression.*

7. Single 6-8 candidate integrants to isolate homozygous or heterozygous animals. If marker is truly heritable, you will see 75% of progeny (heterozygous) or 100% of the progeny (homozygous) with pharyngeal GFP expression.

## **F) Excision of the dual marker cassette with Cre recombinase**

Homozygous animals\* can be used to excise the selection cassette by microinjecting *Peft-3::Cre* recombinase (DICKINSON et al. 2013). With our dual marker cassette, you

can screen for loss of GFP expression in the pharynx of F2 animals to isolate homozygous animals that have excised the selection cassette.

**\*Note**

*If working with essential genes and Cre-mediated excision is required, it is recommended to cross insertion animals into an appropriate balancer strain to propagate heterozygotes and facilitate differentiating animals with alleles that have truly excised the dual marker cassette versus animals with the wild type allele.*

1. Set up injection mix

<u>Plasmid</u>	<u>Final concentration (ng/uL)</u>
*Peft-3::Cre (pDD104)	50
*pCFJ90 (Pmyo-2::cherry)	2.5

**\*Note**

*We prepare these vectors using the Invitrogen purelink HQ kit*

2. Inject animals. We typically inject 5-10 animals.

3. After injection, let animals recover for a half hour to one hour at room temp. Then move 3-4 adults per plate and place in 25°C incubator.

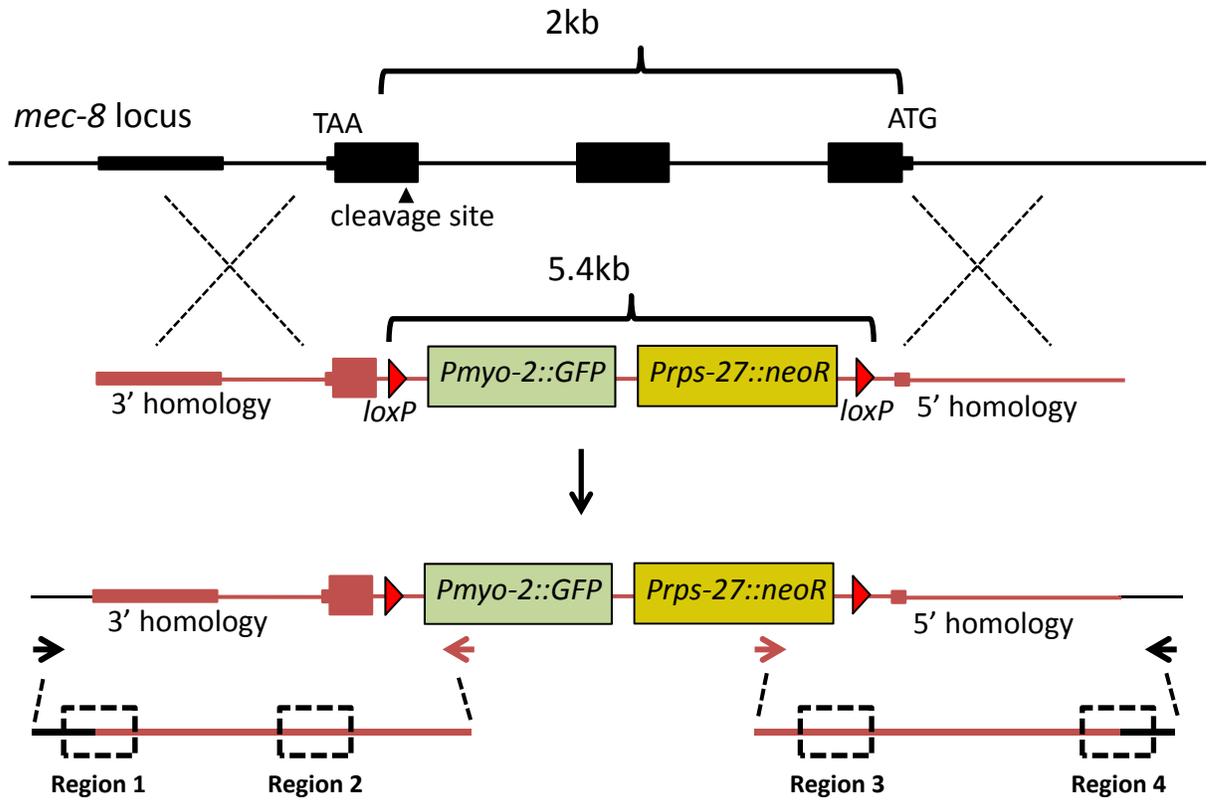
4. Two days after injection, pick 25-30 F<sub>1</sub> progeny that are expressing the Pmyo-2::mCherry marker. Transfer 4-5 worms per plate and return for growth at 25°C.

5. Two-three days after picking F<sub>1</sub> animals, screen plates for F<sub>2</sub> progeny that have completely lost pharyngeal GFP expression and have also lost mCherry expression. These animals should now be homozygous for the excised dual marker cassette.

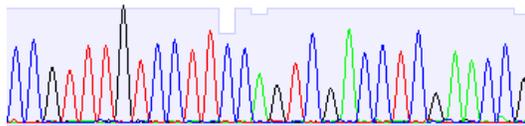
6. Propagate animals for appropriate downstream applications.

## References

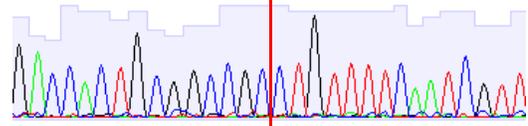
- DICKINSON, D. J., J. D. WARD, D. J. REINER and B. GOLDSTEIN, 2013 Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nat Methods* **10**: 1028-1034.
- FARBOUD, B., and B. J. MEYER, 2015 Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. *Genetics* **199**: 959-971.
- FROKJAER-JENSEN, C., M. W. DAVIS, M. SAROV, J. TAYLOR, S. FLIBOTTE *et al.*, 2014 Random and targeted transgene insertion in *Caenorhabditis elegans* using a modified Mos1 transposon. *Nat Methods* **11**: 529-534.



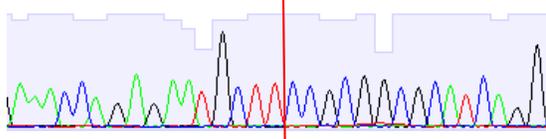
**Region 1**  
**EXPECTED:** CGGTTGAGGTTCGACTGGAAGGACAAACGG  
**VERIFIED:** CCGTTTGTCCCTCCAGTCGACCTCGAACCG  
 (reverse-complement sequence)



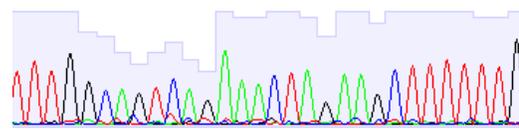
**Region 3**  
**EXPECTED:** GACCACTGCGGCCGCTGTTTTCAATCGTT  
**VERIFIED:** GACCACTGCGGCCGCTGTTTTCAATCGTT



**Region 2**  
**EXPECTED:** AAACCAGAGAATGCTTCCGCGGCATCAGG  
**VERIFIED:** AAACCAGAGAATGCTTCCGCGGCATCAGG



**Region 4**  
**EXPECTED:** TTTGGCAGTCAGAACTAGAAGCTTTTTTG  
**VERIFIED:** TTTGGCAGTCAGAACTAGAAGCTTTTTTG



**Figure S1** Schematic of *mec-8* locus deletion strain generation and validation by PCR and sequencing. Upper panels display genomic *mec-8* locus (in black), repair template with 5' and 3' homology arms flanking loxP sites and dual marker cassette (homology arms labeled as red lines), and the resulting recombined locus where most of the *mec-8* locus is replaced with the dual marker cassette. PCR primers (red and black arrows) were designed to selectively amplify recombinant-specific products on the 5' and 3' sides of the breakpoints. These PCR products (displayed below the arrows as red and black lines) then underwent Sanger sequencing to confirm expected editing. The boxed regions highlight relevant boundaries between vector-encoded homology arms and genomic regions outside of homology arms (Regions 1 and 4), or boundaries between dual marker cassette and deletion sites (Regions 2 and 3). Sequencing traces below demonstrate expected base calls if editing and repair occurred precisely as planned. Red vertical lines in Region 2 and 3 traces indicate deletion breakpoints.

**Tables S1-S2**

Available for download as Excel files at [www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.180679/-/DC1](http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.180679/-/DC1)

**Table S1** List of primers and synthetic DNA fragments used in this study

**Table S2** List of available and recommended reagents in toolkit