Efficient Genome Editing in *Caenorhabditis elegans* with a Toolkit of Dual-Marker Selection Cassettes

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ABSTRACT Use of the CRISPR/Cas9 RNA-guided endonuclease complex has recently enabled the generation of double-strand breaks virtually anywhere in the *C. elegans* genome. Here, we present an improved strategy that makes all steps in the genome editing process more efficient. We have created a toolkit of template-mediated repair cassettes that contain an antibiotic resistance gene to select for worms carrying the repair template and a fluorescent visual marker that facilitates identification of *bona fide* recombinant animals. Homozygous animals can be identified as early as 4–5 days post-injection, and minimal genotyping by PCR is required. We demonstrate that our toolkit of dual-marker vectors can generate targeted disruptions, deletions, and endogenous tagging with fluorescent proteins and epitopes. This strategy should be useful for a wide variety of additional applications and will provide researchers with increased flexibility when designing genome editing experiments.

KEYWORDS *C. elegans*; CRISPR; Cas9; genome editing

CLUSTERED, regularly interspaced, short, palindromic repeat (CRISPR) RNAs and the *Streptococcus pyogenes* CRISPR-associated endonuclease, Cas9, have been used to generate custom mutations, indels, and transgene insertions in a wide variety of organisms (Doudna and Charpentier 2014), including *Caenorhabditis elegans* (Chen *et al.* 2013; Chiu *et al.* 2013; Cho *et al.* 2013; Dickinson *et al.* 2013; Friedland *et al.* 2013; Katic and Grosshans 2013; Lo *et al.* 2013; Tzur *et al.* 2013; Waaijers *et al.* 2013; Arribere *et al.* 2014; Kim *et al.* 2014; Paix *et al.* 2014; Zhao *et al.* 2014; Katic *et al.* 2015; Farboud and Meyer 2015). This revolutionary technology has enabled researchers to target cells in the *C. elegans* germline by using Cas9 complexed with single-guide RNAs (sgRNAs) to produce double-strand breaks at desired locations in the genome. Since the initial discovery repurposing this ribonucleoprotein complex for targeted genome editing, several other applications have emerged (Sternberg and Doudna 2015), expanding the ability of biologists to make specific perturbations in cells and organisms. As a testament to the robustness of CRISPR/Cas9-mediated genome editing in *C. elegans*, several methodologies initially generated mutants and transgenic animals by injection with different combinations of Cas9 and sgRNA encoded by DNA on plasmids (Chen *et al.* 2013; Dickinson *et al.* 2013; Friedland *et al.* 2013; Tzur *et al.* 2013; Waaijers *et al.* 2013), through injection of *in vitro*-transcribed sgRNA and RNA encoding Cas9 (Chiu *et al.* 2013; Katic and Grosshans 2013; Lo *et al.* 2013), and even by direct injection of Cas9 protein preassembled with sgRNA (Cho *et al.* 2013).

Since the initial implementation of these approaches, several studies have explored improving the efficiency of CRISPR/Cas9-mediated editing in *C. elegans*. It has been of major interest to reduce the amount of brute force screening by PCR genotyping and sequencing of mutations. Along this line, it has been demonstrated that some injected P_0 mothers can give rise to "jackpot broods" with higher frequencies of desired genome editing events (Paix *et al.* 2014). Thus, measures taken to identify these jackpot broods can significantly reduce the number of animals required to screen by PCR. Moreover, prescreening animals for editing at a locus that gives a visible phenotype when mutated (co-CRISPR or co-conversion) enriches for successful editing of the desired locus (Arribere *et al.* 2014; Kim *et al.* 2014; Ward 2015). These results indicate that cells accommodating editing

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at one locus exist in an ideal state (proper CRISPR/Cas9 transgene expression and assembly in the nucleus, and competency for repair of double-strand breaks) for editing at many loci. Another significant advance is the finding that sgRNAs that contain 20 nucleotide protospacer sequences of the form $N_{18}GG$ dramatically enhance the probability of generating targeted double-strand breaks (Farboud and Meyer 2015). It is currently speculated that the NGG sequence at protospacer positions 18,19, and 20 acts to mimic a PAM sequence and possibly increases the residence time of Cas9 in the vicinity of where a double-strand break is desired (Farboud and Meyer 2015).

In addition to methods requiring PCR-based identification of animals carrying genome modifications, a number of applications have employed selection schemes involving *unc-119* or antibiotic resistance genes in traditional transgenesis experiments (Giordano-Santini *et al.* 2010; Semple *et al.* 2010, 2012; Radman *et al.* 2013), transposon-based applications (Frokjaer-Jensen *et al.* 2008, 2010, 2012, 2014), and CRISPR/Cas9 editing (Chen *et al.* 2013; Dickinson *et al.* 2013; Kim *et al.* 2014). Selection-based approaches are powerful because they eliminate the need for laborious screening. However, one caveat of most selection-based approaches in *C. elegans* is that some form of negative selection is required to select against animals carrying the positive selection marker in the form of extra-chromosomal arrays (Mello *et al.* 1991), increasing the amount of time required to identify transgenic animals of interest.

Inspired by these selection-based approaches, we sought to develop an improved method for genome editing using a selection scheme and our original published CRISPR reagents. Here, we present a very effective strategy and vector toolkit that will simplify cloning of repair templates and will be complementary to recently developed methods improving CRISPR/Cas9 genome editing in *C. elegans* (Arribere *et al.* 2014; Dickinson *et al.* 2015; Farboud and Meyer 2015; Paix *et al.* 2014; Ward 2015). Our approach enables rapid identification of recombinant animals without the need of special genetic backgrounds through positive selection with a neomycin resistance transgene and circumvents the need for strong negative selection of extrachromosomal array carrying animals by tracking a co-integrated pharyngeal GFP-expressing transgene. We successfully applied our suite of vectors to create gene disruptions and larger deletions, as well as to endogenously tag genes with fluorescent protein transgenes and epitopes. We believe this new approach and set of reagents will provide *C. elegans* researchers with increased flexibility when designing genome editing experiments.

Materials and Methods

Strains and maintenance

The Bristol N2 strain was used for all experiments. Animals were grown at room temperature on nematode growth medium (NGM) plates seeded with *Escherichea coli* strain OP50 as previously described (Brenner 1974), unless otherwise noted.

sgRNA cloning

New sgRNA vectors were constructed in one of two streamlined methods. First, using our original p*U6*::*klp-12* or p*U6*::*unc-119* sgRNA vectors (Friedland *et al.* 2013) as a template, PCR was performed around the plasmid using a forward primer incorporating the new targeting sequence (Supporting Information, Table S1 for all primers used in this study), and the universal reverse primer sg uni R. The amplified linear plasmids were then incubated with T4 polynucleotide kinase (NEB) in T4 DNA ligase buffer (NEB) at 37° for 20 min, followed by addition of T4 DNA ligase (NEB) and incubation at room temperature for 1 hr. An aliquot of the ligation reaction was used for transformation into NEB 5-alpha cells (NEB) as recommended by the manufacturer. In the second method, for *lsd-1* and *him-18* sgRNA cloning, we designed ~60-nt oligonucleotides containing the sgRNA target sequence flanked by ~20-nt homology both up and downstream of the targeting sequence. Forward and reverse oligonucleotides were hybridized to generate dsDNA and ligated to *Bam*HI and *Not*I digested empty sgRNA plasmid (addgene.org/67720) using Gibson Assembly, and transformation was performed as described above.

Dual-marker repair vector suite synthesis

A series of "empty" vectors designed for insertion of our dual-marker cassette were synthesized by Genscript. These vectors contained loxP sites flanking convenient restriction sites *Sna*BI and *Sal*I. The loxP sites were designed to reside in an empty vector (loxP_genscript) or in a synthetic strongly spliced intron sequence within fluorescent protein (GFP_loxP_genscript, mCherry_loxP_genscript, GFP::3xHA_loxP_genscript) and/or 3xHA (3xHA_loxP_genscript) coding sequences. (See Table S2 for relevant information on all vectors in our toolkit and their features.)

We separately amplified and stitched together P*myo-2*, and *GFP*::*unc-54* 3′*UTR* fragments from PDD04 (obtained from Addgene, kindly deposited by the Dupuy lab) (Giordano-Santini *et al.* 2010) and cloned the final stitched product into the *Spe*I and *Bgl*II restriction sites in the pCFJ910 neoR miniMos vector (obtained from Addgene, kindly deposited by the Jorgensen lab) (Frokjaer-Jensen *et al.* 2014). This created a miniMos vector containing our dual-marker cassette (P*myo-2*::*GFP*::*unc-54 3′*UTR *and* P*rps-27*::*neoR*::*unc-54 3′UTR* transgenes). The dual-marker cassette was then excised from the miniMos vector by digestion with *Sna*BI and *Sal*I and inserted by Gibson Assembly into each of the loxP_genscript series of vectors above that were made linear by PCR amplification. This strategy created the vectors loxP_myo_neoR (disruption/deletion), GFP_loxP_myo_neoR (GFP tag), mCherry_loxP_myo_neoR (mCherry tag), GFP::3xHA_loxP_myo_neoR (GFP::3xHA tag), and 3xHA_loxP_myo_neoR (3xHA tag) (Table S2).

Cloning of homology arms into repair vectors

To clone homology arms into the disruption vector, the loxP_myo_neoR vector was digested with *Sac*II and *Not*I

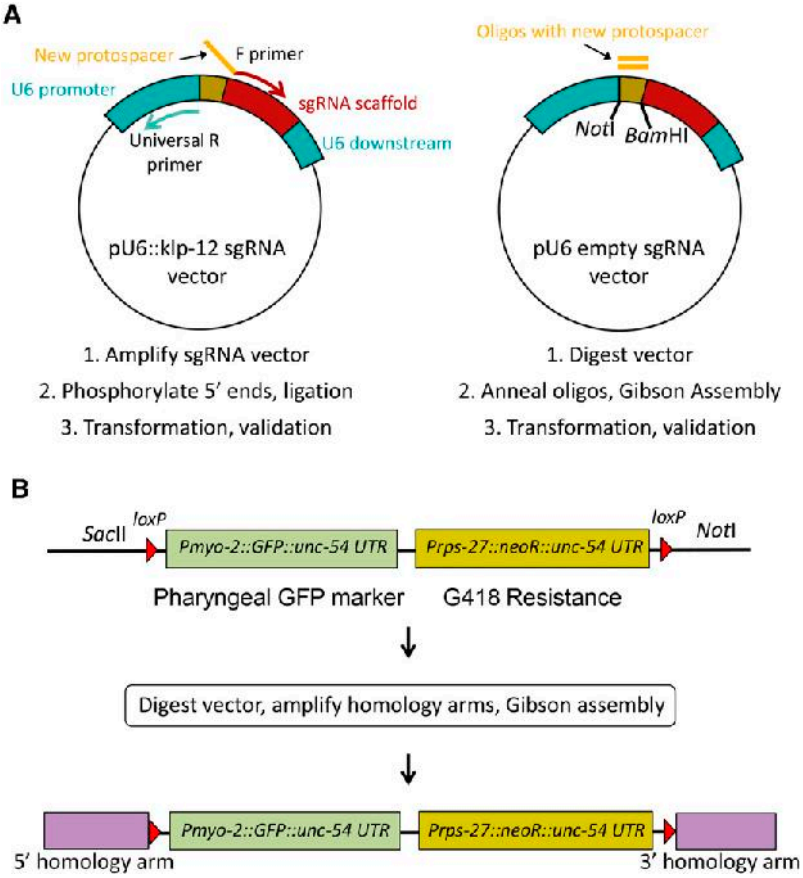

Figure 1 Improved strategies for creation of sgRNA expression vectors and customized repair templates with a dual-marker selection cassette. (A, left) New sgRNA vectors are easily generated from an existing sgRNA plasmid using a universal reverse primer (green curved arrow) and a protospacer-specific forward primer (yellow and red curved arrow) to PCR amplify the entire plasmid, followed by 5′ phosphorylation, ligation, and transformation. (A, right) Alternative sgRNA vector cloning strategy where an empty protospacer sgRNA vector backbone is digested with *Not*I and *Bam*HI restriction enzymes, followed by insertion by Gibson assembly of annealed oligonucleotides carrying a new protospacer. (B) Custom repair templates are generated by digesting a dual-marker template vector with two convenient restriction enzymes, PCR-amplifying appropriate homology arms (purple boxes), and performing a Gibson assembly followed by transformation. Red triangles, green box, and yellow box represent loxP sites, a pharyngeal GFP marker transgene, and a neoR transgene, respectively.

enzymes (Thermo Scientific). The four tagging repair vectors were digested with *Spe*I and *Not*I. These digestion reactions were then purified on a geneJET PCR purification column (Thermo Scientific) as recommended by the manufacturer. Upstream and downstream homology arms (ranging from 600 to 1800 bp) were either amplified by PCR using N2 genomic DNA as a template or synthesized as gBlocks (IDT) (Table S1). PCR products were gel purified using the geneJET gel extraction kit (Thermo Scientific), and corresponding upstream and downstream homology arms were combined with digested vector fragments and stitched together by Gibson Assembly.

DNA preparation and micro-injection

Our original *Peft-3*::*Cas9_SV40_NLS*::*tbb-2 3′UTR* vector was purified using a Qiagen midiprep kit. pCFJ90 and pCFJ104 (P*myo-2* and P*myo-3*::*mCherry* vectors) were purified using the Invitrogen purelink HQ miniprep kit. sgRNA and repair template vectors were purified with the geneJET plasmid miniprep kit (Thermo Scientific), followed by further cleanup and concentration using a DNA clean and contentrator-5 kit (Zymo).

The injection mix was prepared (final concentrations of plasmids were 50 ng/μl Cas9, 100 ng/μl sgRNA, 50 ng/μl repair template, 2.5 ng/μl pCFJ90, 5 ng/μl pCFJ104), and injected into young adult N2 animals as previously described (Kadandale *et al.* 2009).

Selection and screening of integrants and excision of the cassette

Injected animals were transferred to new plates and grown at 25° for 24 hr. After this period, G418 (Sigma Aldrich) was added directly to the plates at an estimated final concentration of 1.25–1.5 mg/ml. Animals were incubated at 25° for an additional 4–11 days. During this time window or until starvation of the plate, six to eight F_2–F_3 animals growing on G418, and possessing uniform pharyngeal GFP expression as well as absence of mCherry expression, were singled to new NGM plates. Animals were followed in subsequent generations to assess inheritance of the pharyngeal GFP signal and homozygotes were identified.

Once obtained, homozygous animals were then injected with an injection mix containing a plasmid encoding germline expressed Cre recombinase (pDD104, obtained from Addgene, kindly deposited by the Goldstein lab) at a final concentration of 50 ng/μl and pCFJ90 (2.5 ng/μl final concentration) as described previously (Dickinson *et al.* 2013). Injected animals were allowed to recover for 1 hr and then grown at 25° for 2–3 days. A total of 25–30 F_1 animals expressing mCherry in the pharynx were isolated and placed on new plates (four animals per plate), and their progeny were screened for loss of both GFP and mCherry pharyngeal expression. These latter animals had both copies of the selection cassette excised.

Table 1 Summary of genome editing experiments

Strain	sgRNA targeting sequence	Homology arm lengths in bp (5′/3′)	Distance (bp) of cut from site from exogenous DNA insertion	No. of P0s successfully injected	No. of insertions	Insertion frequency per P0 (%)	Modification
klp-12 disruption	GATCCACAAGTTACAATTGG	1514/1517	1	20	3	15	Disruption
mec-8 deletion	AAGAGTGGAGCACGCAGAGG	1514/1536	72	6	1	16	Deletion
his-72::*GFP*	AAAAGTGGTGATGAGATCGG	1574/650	112	20	2	10	C-term. Tag
his-72::*GFP*	AATTCAATTCAAAACTGCGG	1574/650	269	20	5	25	C-term. Tag
his-72::*mCherry*	AATTCAATTCAAAACTGCGG	1574/650	269	20	1	5	C-term. Tag
his-72::*3xHA*	AATTCAATTCAAAACTGCGG	1574/650	269	15	3	20	C-term. Tag
3xHA::*lsd-1*	ACATGGCTTCTGGGACTCGG	803/851	16	20	4	20	N-term. Tag
GFP::*3xHA*::*lsd-1*	ACATGGCTTCTGGGACTCGG	801/851	13	20	2	10	N-term. Tag
him-18::*3xHA*	AAGACGGGAAGAGCACGTGG	1027/967	22	20	4	20	C-term. Tag

Table includes information on protospacer sequences used for sgRNA construction, length of 5′ and 3′ homology arms in repair template vectors, distance of double-strand break sites from nonhomologous sequence inserted during editing, number of P_0 adults successfully injected, number of independent insertions obtained, and efficiency of editing reported as a percentage of a P_0 animals injected.

Fluorescence microscopy

Transgenic extrachromosomal and recombinant animals were screened using a Zeiss AxioZoom v16 microscope. Images of integrants and extrachromosomal array-containing animals, as well as HIS-72::GFP and HIS-72::mCherry-tagged animals, were imaged with a Zeiss Axioskop 2 inverted epifluorescence microscope. Acquired images were further processed in ImageJ.

Immunofluorescence

Whole-mount preparations of dissected gonads, fixation, and immunostaining procedures were carried out as described in Colaiacovo *et al.* (2003). Primary antibodies were used at the following dilutions with incubation at 4°: mouse α-HA (1:240, Cell Signaling) and mouse α-GFP (1:700, Life Science). The secondary antibody used was: Cy5 anti-mouse (Jackson Immunochemicals) at 1:400 for LSD-1 experiments and Cy5 anti-mouse at 1:300 for HIM-18 experiments.

Immunofluorescence images were collected at 0.2-μm intervals with an IX-70 microscope (Olympus) and a CoolSNAP HQ CCD camera (Roper Scientific) controlled by the DeltaVision system (Applied Precision). Images were subjected to deconvolution by using the SoftWoRx 3.3.6 software (Applied Precision).

Data availability

Strains are available upon request. Table S2 contains a list of available plasmids and strains that will be distributed by the Calarco Lab. The empty cloning sgRNA vector is available for distribution from Addgene (addgene.org/67720). Up to date versions of protocols and new toolkit plasmids will be maintained on our CRISPR resources page (https://sites.google.com/site/calarcola.boratory/crispr_resources).

Results

Streamlined cloning of original sgRNA vectors and considerations for sgRNA design

In the course of incorporating new protospacer sequences into our original pU6::klp-12 and pU6::unc-119 sgRNA vectors (Friedland *et al.* 2013), we found that the construction of these plasmids could be greatly simplified and developed two alternative approaches. In the first approach, analogous to site-directed mutagenesis protocols, we used a universal reverse primer and a protospacer sequence-specific forward primer to amplify the entire plasmid sequence by PCR. This amplification step incorporated the new protospacer of interest and was followed by sequential enzymatic steps phosphorylating the free 5′ ends of the PCR product and ligation of the newly amplified vector in a single reaction tube (Figure 1A, left). For each new sgRNA vector to be synthesized, only a single primer containing the new 20 nucleotide protospacer sequence needs to be designed. Moreover, the new method no longer requires any restriction enzyme-based cloning or PCR stitching, greatly simplifying the construction process.

In the second approach, we designed an empty protospacer sgRNA expression vector that contains two convenient restriction enzyme cleavage sites located between the U6 promoter and sgRNA scaffold sequences (Figure 1A, right). For each new sgRNA vector to be constructed, a pair of oligonucleotides are synthesized, annealed, and then inserted into the digested vector by Gibson assembly. Both new procedures require less than half a day of hands on time and are very efficient, such that one or two transformed clones can be selected and directly verified by sequencing.

A recent study has found that protospacers of the form $N_{18}GG$ result in dramatic enhancement in generating double-strand breaks (Farboud and Meyer 2015) without additional modifications to our original sgRNA expression vectors. As such, all sgRNAs designed in the present study conformed to this protospacer configuration. One additional requirement of our sgRNA expression vector, which uses a U6 promoter, is that transcription is most efficient when the +1 nucleotide is a purine. In principle, this caveat could further restrict availability of suitable protospacer sequences for genome editing. However, in instances when the first nucleotide of the protospacer is not a purine, the addition of a noncomplementary guanine nucleotide upstream of the protospacer to ensure efficient

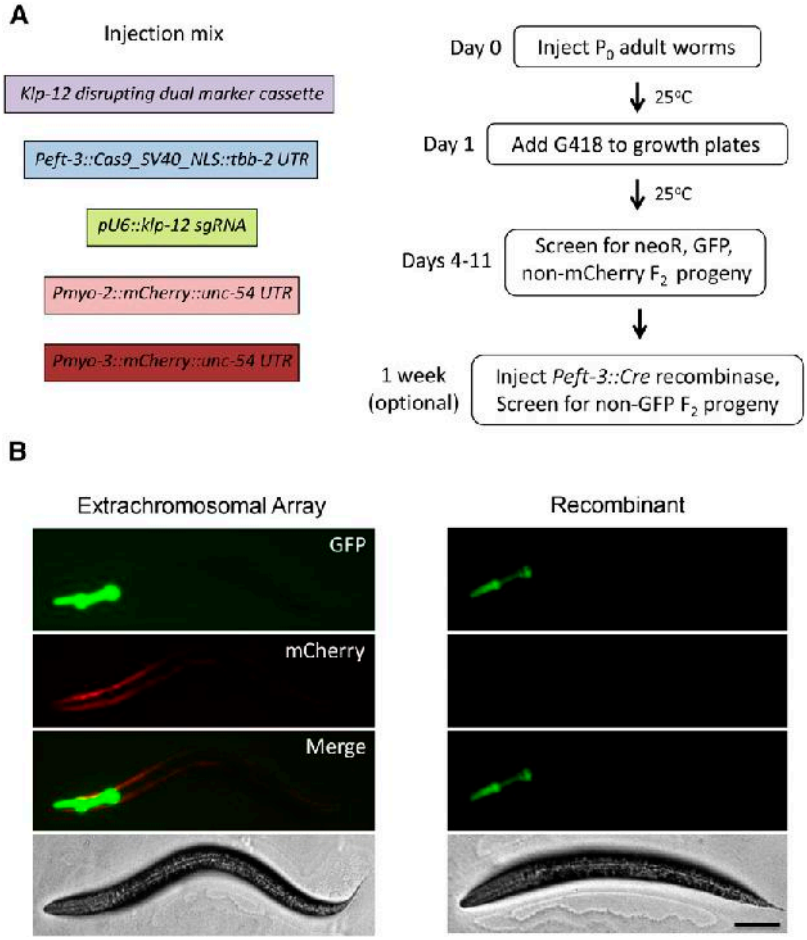

Figure 2 Strategy for generating and identifying successful recombinants. (A) Injection mix for disruption of the *klp-12* locus. Adult animals are injected with a cocktail of five plasmids (left), including Cas9 and sgRNA expression vectors, two co-injection markers, and a *klp-12* disrupting dual-marker cassette repair template. The 24-hr post-injection animals are treated with G418 (right) and allowed to grow for 4–11 days, at which point plates are screened for recombinants. If excision of the dual-marker cassette is required, an additional injection of *Cre* recombinase is then performed. (B) Visual strategy for identifying successful recombinants. Extra-chromosomal array-bearing animals (left) exhibit bright, mosaic GFP expression in the pharynx and also express one or both mCherry co-injection markers. Recombinant animals (right) exhibit dim, uniform GFP expression in the pharynx and loss of mCherry expression, demonstrating integration of the selection cassette and loss of the extrachromosomal array.

transcription does not seem to negatively influence activity of the expressed sgRNA (Farboud and Meyer 2015). Our results, discussed below, are consistent with these observations (Table 1). Taken together, our new cloning strategies and improved protospacer choice will allow researchers to construct highly effective sgRNA vectors in a more streamlined manner.

A dual-marker repair template facilitates rapid identification of recombinants without the need for strong negative selection

We and others initially demonstrated that double-strand breaks generated by Cas9/sgRNA ribonucleoprotein complexes can be repaired from a plasmid harboring a DNA template containing homology on both sides flanking the lesion (Chen *et al.* 2013; Dickinson *et al.* 2013; Tzur *et al.* 2013; Kim *et al.* 2014). In several instances, homology-directed repair from plasmid templates is efficient enough that PCR screening can be used to identify recombinant animals without additional selection schemes. However, more often than not, many animals have to be screened by PCR to find rare recombinants, making the process laborious and not scalable for generating larger numbers of transgenic animals. To circumvent this problem, we sought to develop a selection protocol that allowed for rapid identification of recombinant animals, enabled near seamless editing of loci, and provided a means to screen for excision of the selection cassette without requiring a special genetic background.

To fulfill all of these criteria, we created a dual-marker cassette containing both a reporter expressing green fluorescent protein (GFP) in the pharynx (*Pmyo-2*::*GFP*::*unc-54 3′UTR*) and a neomycin resistance transgene (*Prps-27*::*neoR*::*unc-54 3′UTR)*. These two marker transgenes were flanked with loxP sites and two restriction enzyme sites for convenient insertion of homology arms of interest (Figure 1B).

In *C. elegans* transgenesis experiments, animals can stably maintain plasmid DNA in extrachromosomal arrays (Mello *et al.* 1991). Using our dual-marker cassette, such extrachromosomal array harboring animals will also carry the *Prps-27*::*neoR*::*unc-54 3′UTR* and therefore will be resistant to G418. To circumvent this challenge, several groups have developed effective selection schemes to negatively select for animals carrying extrachromosomal arrays, including the use of the heat shock-inducible toxin PEEL-1 (Frokjaer-Jensen *et al.* 2012; Frokjaer-Jensen *et al.* 2014) or the use of ivermectin in a mutant genetic background (Shirayama *et al.* 2012). Although our selection scheme can also make use of these counter-selectable markers, we first wanted to test whether we could distinguish between extrachromosomal array carrying animals and recombinant animals by taking advantage of the *Pmyo-2*::*GFP*::*unc-54 3′UTR* transgene in our cassette.

As a proof of principle, we attempted to disrupt the *klp-12* gene by inserting our dual-marker cassette. We injected a DNA cocktail that included our original *Peft-3*::*Cas9_SV40_NLS*::*tbb-2 3′UTR* vector, a *klp-12*-targeting sgRNA vector, our dual-marker cassette with *klp-12* homologous sequences, and co-injection marker plasmids expressing mCherry in the pharynx and body wall muscles (*Pmyo-2*::*mCherry*::*unc-54 3′UTR* and *Pmyo-3*::*mCherry*::*unc-54 3′UTR*, respectively) (Figure 2A). Injected animals were allowed to recover and lay eggs at 25° for 1 day, followed by selection with G418. During the selection phase, we screened plates daily to look at fluorescence patterns. Intriguingly, as early as 4 days post-injection, around the time when F_2 progeny begin to emerge, we identified animals that were surviving G418 selection, had no mCherry expression, but did express GFP in all pharyngeal muscle cells in a uniform manner (Figure 2B). This population of animals was easily distinguishable from animals carrying extrachromosomal arrays, which displayed mosaic expression of both GFP and mCherry markers (Figure 2B). Screening for pharyngeal GFP marker expression also had the added benefit of distinguishing putative recombinant animals from wild-type animals that simply escape G418 selection, which has been demonstrated to occur at low frequency (Semple *et al.* 2012). From 20 injected P_0 animals, we obtained three independent insertion lines (15% of P_0 animals). We transferred four to six single F_2 animals from each independent insertion line to new standard growth plates without antibiotics and followed the inheritance pattern of pharyngeal GFP expression. F_3 animals inherited GFP fluorescence in expected Mendelian ratios, consistent with a recombination event that had occurred in the germline of the P_0 or F_1 generation. Based on F_3 inheritance patterns we often confirmed that some F_2 animals in the population were already homozygous for the dual-marker cassette, both in this experiment and others described below. These results suggest that repair events inserting our dual-marker cassette likely occur most frequently in the germline of injected P_0 mothers.

Importantly, the *Pmyo-2*::*GFP*::*unc-54 3′UTR* transgene also serves as an excellent marker for removal of the dual-marker cassette. After identification of animals carrying insertions of the cassette, we excised the cassette by injecting a germline-expressed Cre recombinase and a co-injection marker expressing pharyngeal mCherry and screened the F_2 progeny of these injected animals for absence of pharyngeal GFP expression. We found this step to be very efficient as previously described (Dickinson *et al.* 2013), requiring the injection of only 5–10 animals and selection of 25–30 mCherry-expressing F_1 animals to obtain several independent lines that excised the cassette.

To confirm that both the initial insertion of the dual-marker cassette and its excision occurred precisely, we sequence verified the relevant recombined boundaries at the *klp-12* locus in a subset of lines (data not shown). Insertion of the cassette precisely occurred in the expected location, and its removal also occurred as expected. Taken together, our revised editing procedure can yield homozygous animals carrying disruptions in as early as 1 week with minimal PCR screening required. Once homozygous animals are obtained containing insertions, excision of the cassette can be achieved in 1 more week with minimal hands on time (Figure 2A).

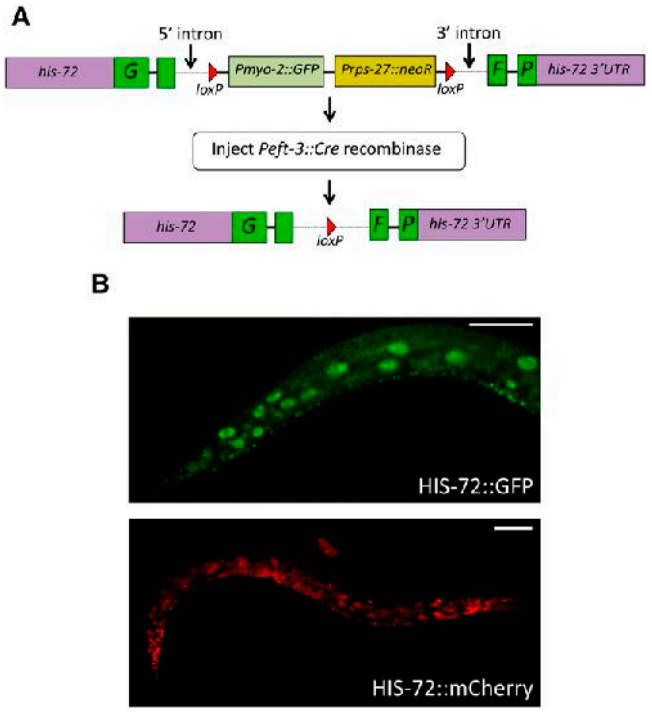

Figure 3 Dual-marker cassettes facilitate tagging of genes with fluorescent protein transgenes or other epitopes. (A) Schematic of a dual-marker cassette *his-72*::*GFP* tagging vector. The selection cassette is housed within a constitutively spliced synthetic intron (dotted lines denote 5′ and 3′splice sites), such that after *Cre*-mediated excision of the cassette, the remaining loxP site will be spliced out of the mature GFP mRNA open reading frame, creating near seamless editing. (B) Fluorescent micrographs of endogenously tagged HIS-72::GFP (top) and HIS-72::mCherry (bottom). Scale bars, 100 μm.

A suite of dual-marker repair templates for tagging and deleting genes

Given the success of our *klp-12* disruption experiment, we constructed a set of vectors to facilitate cloning of new repair templates for genes of interest while providing flexibility and diversity of available epitope and fluorescent protein tags (GFP, mCherry, 3xHA, and GFP::3xHA tags). The full complement of available vectors in our toolkit is listed in Table S2. As described above, our approach relies on the use of Cre recombinase to excise the inserted dual-marker cassette once a recombinant animal is identified and propagated. However, after Cre-mediated recombination and removal of the cassette, a single loxP site remains behind in the genome. For endogenous gene-tagging applications, we thought it would be best to have the smallest footprint possible after editing, but still wanted to benefit from our selection scheme. Therefore, we placed the cassette within a synthetic constitutively spliced intron in a similar location that was previously

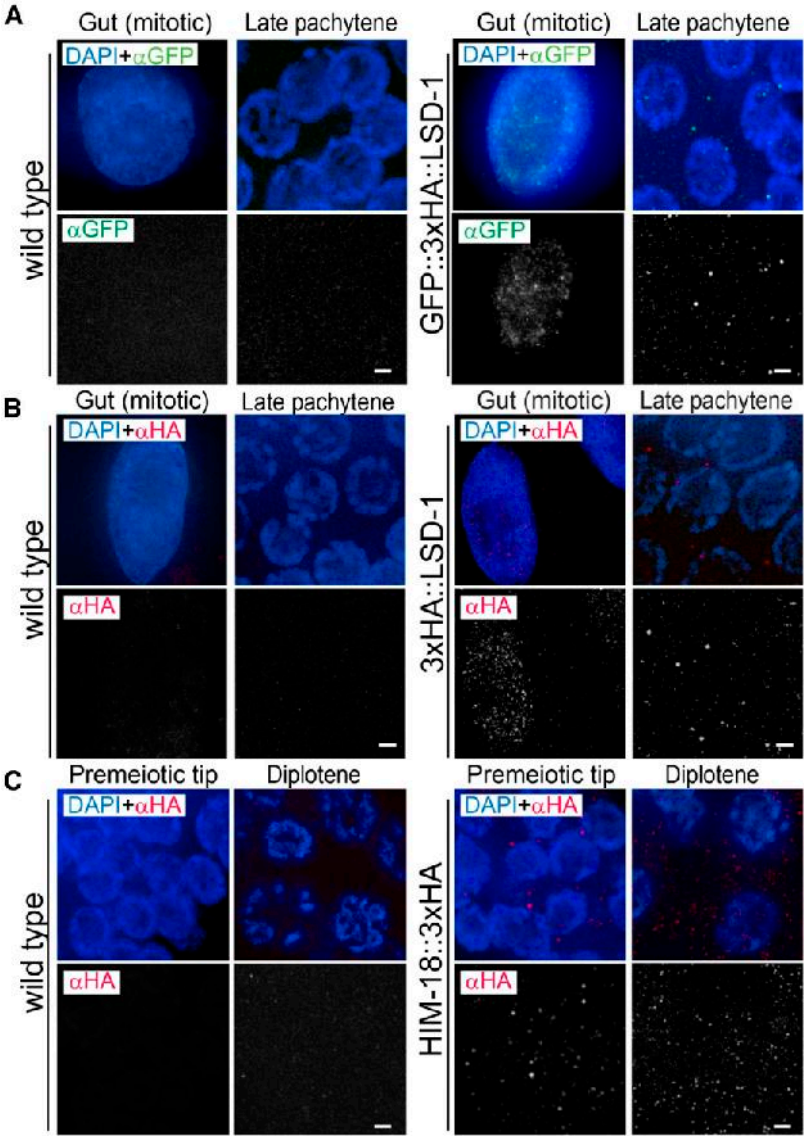

Figure 4 Dual-marker cassette generated epitope tagged LSD-1 and HIM-18 strains. (A and B) Expression of GFP::3xHA::LSD-1 and 3xHA::LSD-1 detected with anti-GFP (A) and anti-HA (B) antibodies, respectively. LSD-1 localization is detected as foci in both somatic (gut) and germline (late pachytene) nuclei in both CRISPR/Cas9 engineered lines. (C) Expression of HIM-18::3xHA detected with anti-HA antibody. HIM-18 localization is detected as foci in the germline as shown here for premeiotic tip and diplotene nuclei. Scale bars, 2 μm.

described in a fosmid recombineering approach (Tursun *et al.* 2009) (Figure 3A). In this configuration, after removal of the dual-marker cassette the remaining loxP site will ultimately be spliced out of the mature messenger RNA encoding the transgenic fusion protein (Figure 3A).

To test our dual-marker tagging repair templates, we targeted the histone H3.3 gene *his-72*, the histone demethylase gene *lsd-1*, and *him-18*, a gene involved in the repair of double-strand breaks in the germline. We carried out our transgenesis protocol to generate *his-72*::*GFP* and *his-72*::*mCherry* transgenic animals as described above and established lines carrying our dual-marker cassette (Table 1). After insertion of the cassette, only pharyngeal GFP fluorescence expression was observed. However, after excision of the dual-marker cassette with Cre recombinase, nuclear-localized GFP and mCherry could be detected in many different cell and tissue types in each of the corresponding strains (Figure 3B), consistent with previously reported expression patterns for this gene (Ooi *et al.* 2006).

Similarly, we generated *GFP*::*3xHA*::*lsd-1*, *3xHA*::*lsd-1*, and *him-18*::*3xHA*-tagged lines, and examined dissected gonads from 24-hr post-L4 young adult worms by immunofluorescence (Figure 4). We observed LSD-1 signal in both somatic (gut) as well as meiotic germline nuclei for both tagged lines, consistent with the predicted roles for LSD-1 as a histone H3 lysine 4 demethylase and as a transcriptional corepressor (Figure 4, A and B). Importantly, the localization observed for LSD-1 is specific since the same immunofluorescence signal was not observed in the control wild-type worms. Similar somatic and germline localization was observed with an anti-LSD-1 antibody (S. Beese-Sims and M.P. Colaiacovo, unpublished results). We also observed specific signal for HIM-18 in the germline. HIM-18 signal was observed in mitotic nuclei at the distal tip region of the germline (premeiotic tip), with a reduction in signal upon entrance into meiosis followed by increased nuclear signal once again from late pachytene through the end of diakinesis, consistent with the previously described immunolocalization

for HIM-18 (Saito *et al.* 2009) (Figure 4C and data not shown).

We next sought to test whether our dual-marker cassette could facilitate the construction of strains containing larger deletions. As a proof of principle we targeted the RNA binding protein gene *mec-8*, originally characterized among other mutants with mechanosensory defects (Chalfie and Sulston 1981). We inserted homology arms flanking the *mec-8* gene into our dual-marker cassette vector such that most of the *mec-8* locus (~2 kb) would be deleted after homology-directed repair (Figure 5A). Using a single sgRNA and our standard injection protocol, we identified animals heritably carrying our cassette (Table 1). We recovered homozygote mutant animals and genotyped them by PCR and sequencing at the relevant boundaries of the lesion (Figure S1), confirming that deletion and replacement of the *mec-8* locus had occurred as expected. We further assayed homozygous mutants for defects in responding to gentle touch (Chalfie and Sulston 1981). Consistent with previous observations, we confirmed that our CRISPR/Cas9 generated *mec-8* deletion strain had defects in mechanosensation (Figure 5B). These results indicate that our dual-marker cassette is also effective at generating larger deletion alleles.

Consistent with our experiments targeting the *klp-12* locus, we recovered recombinant animals with insertions from between 5 and 25% of the P_0 animals injected in all of our experiments (Table 1). These results suggest that our dual-marker-based approach and vector toolkit will be generally efficient for a variety of editing requirements across most genes.

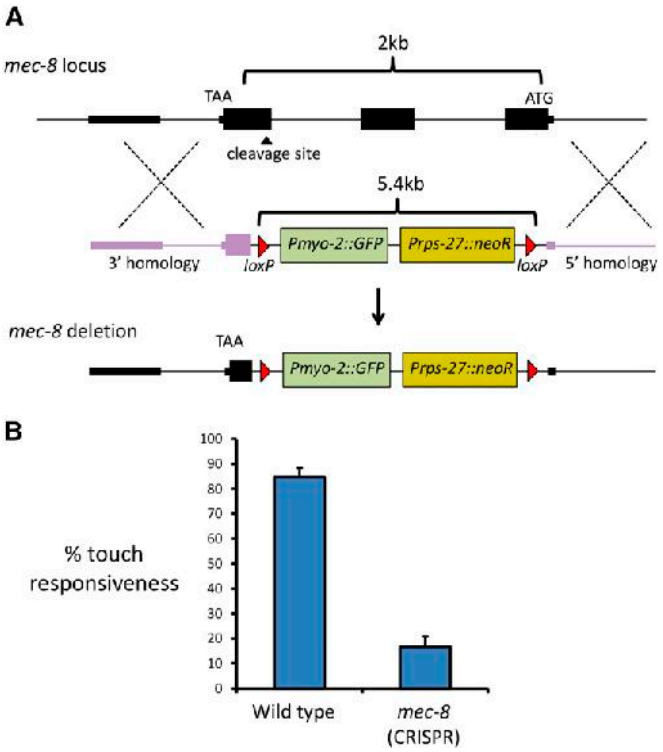

Figure 5 Dual-marker cassettes facilitate large deletions. (A) Homology arms were designed at the 5′ and 3′ ends of the *mec-8* locus such that the repair template replaced most of its coding sequence, creating a ~2-kb deletion and simultaneous 5.4-kb insertion of the dual-marker cassette. (B) Animals deleted for *mec-8* have severe defects in touch sensitivity. Worms were touched 10 times each with an eyelash and scored for the percentage of time they responded by initiating locomotion. Error bars represent standard error of the mean.

Discussion

Since the initial demonstrations that the CRISPR/Cas9 system could be used for genome editing in *C. elegans*, several strategies have been used effectively, and significant improvements have been made to facilitate the generation and identification of edited animals. Here, we present another effective strategy and vector toolkit that will be complementary to these recently developed methods. Our approach enables rapid identification of animals carrying insertions at desired loci through the use of a selectable marker conferring resistance to G418 and further discrimination of these animals from extrachromosomal array carrying animals through a co-integrated pharyngeal GFP expressing transgene. The extra fluorescent marker provides a reliable means to independently verify and track the heritability of an insertion in the genome, and as such our approach does not rely on the use of negative selection markers such as the PEEL-1 toxin. This strategy will likely prove useful and increase the speed of obtaining and following recombinant transgenics in genome editing approaches using transposons in *C. elegans* such as MosSCI and miniMos (Frokjaer-Jensen *et al.* 2012, 2014). Moreover, we believe that this new approach will provide researchers with increased flexibility when designing a genome editing experiment. Below we compare and contrast our current approach with recently developed methods.

The main advantage of our approach is that minimal PCR screening is required to identify *bona fide* recombinant animals, because insertions at the desired locus are directly selected for by the presence of both an antibiotic resistance marker and a fluorescent reporter gene. This technique contrasts with recent effective protocols making use of coconversion markers that give visible phenotypes or direct screening of jackpot broods (Arribere *et al.* 2014; Kim *et al.* 2014; Paix *et al.* 2014; Ward 2015), where more initial PCR screening is required because not all animals that are edited at the first locus are also edited at the desired locus.

In our present approach, several applications will require the removal of the dual-marker cassette through a second step where Cre recombinase is injected and animals are screened for loss of pharyngeal GFP expression. In our experience, this step is quite robust and simpler than previous versions relying on reversion of an *unc-119* mutant phenotype (Dickinson *et al.* 2013). This extra step is not required in the recently described coconversion approaches, which instead require an extra step to segregate the coconversion marker from the allele of interest or the use of special mutant backgrounds

for coconversion. Encouragingly, it was recently demonstrated that introducing a heat shock-inducible Cre recombinase transgene within a selection cassette can facilitate excision of that cassette without the requirement of an extra injection step (Dickinson *et al.* 2015). Our dual-marker cassette contains a number of unique cloning sites that will enable this heat-inducible Cre recombinase transgene to be inserted in future designs, along with additional useful markers and transgenes developed by other labs to suit the diverse needs of the community. We have also included a series of empty tagging vectors in our plasmid toolkit (Table S2), allowing researchers to introduce selectable markers tailored to their needs, within the context of a synthetic intron that will splice out residual loxP sites. Ultimately, the implementation of any strategy will now cater to the preferences of the experimenter, where under certain conditions it may be preferable to screen by coconversion and PCR and in other circumstances having a selection scheme would be more ideal.

Several studies have indicated that in addition to using large homology arms in the context of plasmid repair templates, single-strand DNA oligonucleotides or double-strand PCR products with much smaller homology arms can be used to introduce small modifications, deletions, and medium-sized (~1-kb) insertions (Lo *et al.* 2013; Arribere *et al.* 2014; Paix *et al.* 2014; Zhao *et al.* 2014; Farboud and Meyer 2015; Katic *et al.* 2015). However, it appears that one drawback when using smaller homology arms is that the location of the double-strand break needs to be very close to the desired site of editing. The use of selectable cassettes with longer flanking homology arms appears to allow for insertion of larger sequences during the repair process and more flexibility in the distance between the site of cleavage by Cas9 and the location of modification. Indeed, in our current study, one sgRNA protospacer we selected generated double-strand breaks as far as 269 nucleotides away from the nonhomologous sequences we introduced into the genome (Table 1). This added flexibility will be particularly useful in situations when there is limited availability of appropriate protospacers very close to the desired site of modification.

Another advantage of our dual-marker cassette, when designed to disrupt or delete a gene, is that it provides a simple way to maintain heterozygote animals with recessive alleles that cause lethality or significant fitness defects, reducing the explicit requirement for balancers. For example, heterozygotes can simply be maintained on growth media containing G418 or identified under the fluorescent microscope by pharyngeal GFP expression.

Finally, the method presented here is similar in concept to a recently described selection scheme making use of two transgenes: one conferring hygromycin resistance and another creating a dominant roller phenotype (Dickinson *et al.* 2015). It is encouraging that we have observed similar results through coupling a neomycin resistance transgene with pharyngeal GFP expression. It will be interesting to test whether introducing new combinations of drug-resistance transgenes and easy to score markers into such selection schemes will open the door to more complex genome editing experiments making use of multiple repair templates.

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Efficient Genome Editing in *Caenorhabditis elegans* with a Toolkit of Dual-Marker Selection Cassettes

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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 *klp-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_{20})GTTTTAGAGCTAGAAATAGCAAG-3'*
Where N_{20} is the new 20mer targeting sequence to introduce

*Notes:
It has been found that a terminal GG at positions N_{19} and N_{20} can dramatically increase the efficiency of targeting (FARBOUD and MEYER 2015).

The G in front of the N_{20} is only required if the N_1 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_1 is a G, then the primer should be of the form:

5'-(N_{20})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)	
ddH2O	32.5
5xHF Buffer	10
10mM dNTPs	1
sgRNA universal R primer(10uM)	2.5
klp-12 sgRNA vector (20pg/uL)	1
Phusion polymerase	0.5
	47.5 uL
Individual items:	
sgRNA specific F primer(10uM)	2.5
	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
Pmyo-2::GFP neoR loxP	x uL (2ug)
	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

Pmyo-2::GFP neoR loxP	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).

ddH2O	32 – x uL
10x FastDigest Buffer	4uL
FastDigest SpeI enzyme	2uL
FastDigest NotI enzyme	2uL
Tagging vector	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_x)
Upstream R: TTATAGGCCGCCTGATGCGCCGCGG($N_{x\text{-}RC}$)
Downstream F: ATAGTTGCAGGACCACTGCGGCCGC(N_x)

Downstream R: ATGATTACGCCAAGCTTGCGGCCGC($N_{x\text{-}RC}$)

Where N_x and $N_{x\text{-}RC}$ are homology arm specific sense and reverse complementary sequences, respectively.

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

Upstream F: AACGACGGCCAGTGAATTCACTAGT(N_x)
Upstream R: GTTCTTCTCCTTTACTCATACTAGT($N_{x\text{-}RC}$)
Downstream F: TGGATGAACTATACAAAGCGGCCGC(N_x)
Downstream R: ATGATTACGCCAAGCTTGCGGCCGC($N_{x\text{-}RC}$)

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

Upstream F: AACGACGGCCAGTGAATTCACTAGT(N_x)
Upstream R: CTTCACCCTTTGAGACCATACTAGT($N_{x\text{-}RC}$)
Downstream F: TGGATGAATTGTATAAGGCGGCCGC(N_x)
Downstream R: ATGATTACGCCAAGCTTGCGGCCGC($N_{x\text{-}RC}$)

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

Upstream F: AACGACGGCCAGTGAATTCACTAGT(N_x)
Upstream R: CTGGAACGTCGTATGGGTAACTAGT($N_{x\text{-}RC}$)
Downstream F: ACGTCCCAGATTACGCTGCGGCCGC(N_x)
Downstream R: ATGATTACGCCAAGCTTGCGGCCGC($N_{x\text{-}RC}$)

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

Upstream F: AACGACGGCCAGTGAATTCACTAGT(N_x)
Upstream R: GTTCTTCTCCTTTACTCATACTAGT($N_{x\text{-}RC}$)
Downstream F: ACGTCCCAGATTACGCTGCGGCCGC(N_x)
Downstream R: ATGATTACGCCAAGCTTGCGGCCGC($N_{x\text{-}RC}$)

*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:

ddH2O	10 – x – y – z uL
Cut vector + selection cassette	x uL (100ng)*
Upstream homology arm	y uL (100ng)
Downstream homology arm	z uL (100ng)
2x Master Assembly mix	10uL
	20 uL

control reaction:

ddH2O	20 – x uL
Cut vector + selection cassette	x uL (100ng)
	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:

Plasmid	Final concentration (ng/uL)
*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_0 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_0s surviving and throwing ~25-30 Ex F1 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_1 progeny across all injected animals. It's recommended to inject more animals if injections do not generally yield large amounts of extra-chromosomal F_1 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

Plasmid	Final concentration (ng/uL)
*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_1 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_1 animals, screen plates for F_2 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.

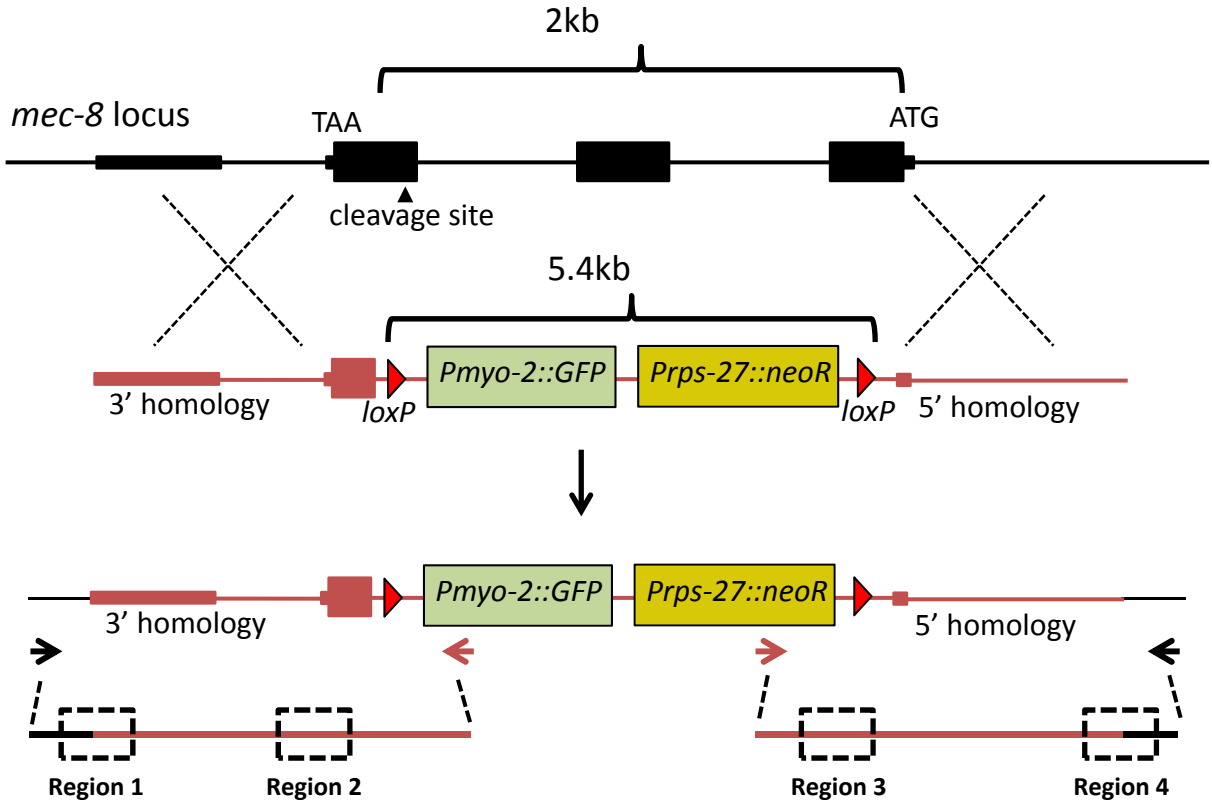
6. Propagate animals for appropriate downstream applications.

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.

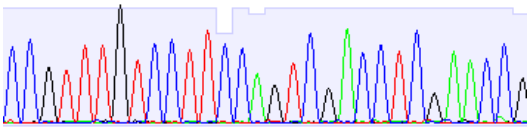


Region 1

EXPECTED: CGGTTCGAGGTCGACTGGAAGGACAAACGG

VERIFIED: C C G T T T G T C C T T C C A G T C G A C C T C G A A C C G

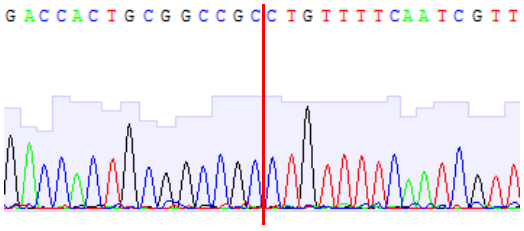
(reverse-complement sequence)



Region 3

GACCACTGCGGCCGCCTGTTTTCAATCGTT

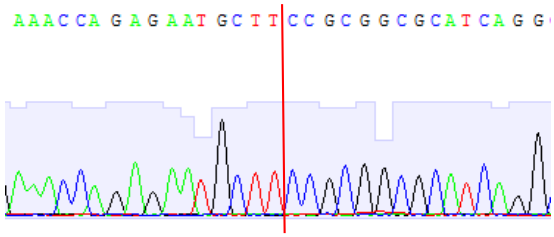
G A C C A C T G C G G C C G C C T G T T T T C A A T C G T T



Region 2

EXPECTED: AAACCAGAGAATGCTTCCGCGGCGCATCAGG

VERIFIED: A A A C C A G A G A A T G C T T C C G C G G C G C A T C A G G



Region 4

TTTGGCAGTCAGAAACTAGAAGCTTTTTTG

T T T G G C A G T C A G A A A C T A G A A G C T T T T T T G

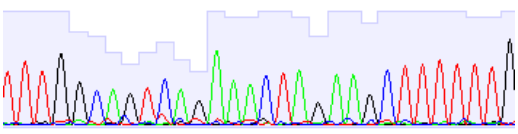


Figure S1 Schematic of *mec-8* locus deletion strain generation and validation by PCR and sequencing. Upper panels display genomic *mec-8* locus (in black), repair template with 5' and 3' homology arms flanking loxP sites and dual marker cassette (homology arms labeled as red lines), and the resulting 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

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

Table S2 List of available and recommended reagents in toolkit