Efficient genome editing in *C. elegans* with a toolkit of dual marker selection cassettes

Adam D. Norris¹⁺, Hyun-Min Kim²⁺, Mónica P. Colaiácovo²⁺, and John A. Calarco¹⁺

¹FAS Center for Systems Biology, Harvard University, Cambridge, MA, 02138

²Department of Genetics, Harvard Medical School, Boston, MA, 02115

⁺These authors contributed equally to this work

#Address correspondence to:

John Calarco
jcalarco@fas.harvard.edu

Mónica Colaiácovo
mcolaiacovo@genetics.med.harvard.edu
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.
Introduction

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* (Arribere et al. 2014; Chen et al. 2013; Chiu et al. 2013; Cho et al. 2013; Dickinson et al. 2013; Farboud and Meyer 2015; Friedland et al. 2013; Katic and Grosshans 2013; Katic et al. 2015; Kim et al. 2014; Lo et al. 2013; Paix et al. 2014; Tzur et al. 2013; Waaijers et al. 2013; Zhao et al. 2014). 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 pre-assembled 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 *P₀* 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, pre-screening 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 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 N18GG 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; Radman et al. 2013; Semple et al. 2012; Semple et al. 2010), transposon-based applications (Frokjaer-Jensen et al. 2012; Frokjaer-Jensen et al. 2010; Frokjaer-Jensen et al. 2008; Frokjaer-Jensen et al. 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 extra-chromosomal 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 E. 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 pU6::klp-12 or pU6::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 (See Supplementary Table 1 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°C for 20 minutes, followed by addition of T4 DNA ligase (NEB) and incubation at room temperature for one hour. 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 ~20nt homology both up and downstream of the targeting sequence. Forward and reverse oligonucleotides were hybridized to generate dsDNA and ligated to BamHI and NotI 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 SnaBI and SalI. 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 Supplementary Table 2 for relevant information on all vectors in our toolkit and their features).

We separately amplified and stitched together Pmyo-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 SpeI and BglII 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 (Pmyo-2::GFP::unc-54 3’UTR and Prps-27::neoR::unc-54 3’UTR transgenes). The dual marker cassette was then excised from the miniMos vector by digestion with SnaBI and SalI, 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) (Supplementary Table 2).

**Cloning of homology arms into repair vectors**

To clone homology arms into the disruption vector, the loxP_myo_neoR vector was digested with SacI and NotI enzymes (Thermo Scientific). The four tagging repair vectors were digested with SpeI and NotI. 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 600bp-1800bp) were either amplified by PCR using N2 genomic DNA as a template, or synthesized as gBlocks (IDT) (Supplementary Table 1). 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 microinjection**

Our original Peft-3::Cas9_SV40_NLS::tbb-2 3’UTR vector was purified using a Qiagen midiprep kit. pCFJ90 and pCFJ104 (Pmyo-2 and Pmyo-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 concentrator-5 kit (Zymo).

The injection mix was prepared (final concentrations of plasmids were 50 ng/uL Cas9, 100 ng/uL sgRNA, 50 ng/uL repair template, 2.5 ng/uL pCFJ90, 5 ng/uL 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°C for 24 hours. 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°C for an additional 4-11 days. During this time window or until starvation of the plate, six to eight F2-F3 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/uL and pCFJ90 (2.5 ng/uL final concentration) as described previously (DICKINSON et al. 2013). Injected animals were allowed to recover for 1 hour, and then grown at 25°C for 2-3 days. 25-30 F1 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.

**Fluorescence Microscopy**

Transgenic extra-chromosomal and recombinant animals were screened using a Zeiss AxioZoom v16 microscope. Images of integrants and extra-chromosomal 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 °C: 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).
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 panel). 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 panel). 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 non-complementary guanine nucleotide upstream of the protospacer to ensure efficient 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; KIM et al. 2014; TZUR et al. 2013). 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 in order 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 extra-chromosomal arrays (MELLO et al. 1991). Using our dual marker cassette,
such extra-chromosomal 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 extra-chromosomal 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 extra-chromosomal 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°C for one day, followed by selection with G418. During the selection phase, we screened plates daily to look at fluorescence patterns. Intriguingly, as early as four days post injection, around the time when F2 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 extra-chromosomal 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 P0 animals, we obtained three independent insertion lines (15% of P0 animals). We transferred four to six single F2 animals from each independent insertion line to new standard growth plates without antibiotics and followed the inheritance pattern of pharyngeal GFP expression. F3 animals
inherited GFP fluorescence in expected Mendelian ratios, consistent with a recombination event that had occurred in the germline of the P₀ or F₁ generation. Based on F₃ inheritance patterns we often confirmed that some F₂ 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 P₀ mothers.

Importantly, the \textit{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 F₂ progeny of these injected animals for absence of pharyngeal GFP expression. We found this step to be very efficient as previously described (DICKINSON \textit{et al.} 2013), requiring the injection of only 5-10 animals and selection of 25-30 mCherry-expressing F₁ 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 \textit{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 one week with minimal PCR screening required. Once homozygous animals are obtained containing insertions, excision of the cassette can be achieved in one more week with minimal hands on time (Figure 2A).

\textit{A suite of dual marker repair templates for tagging and deleting genes}

Given the success of our \textit{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 Supplementary Table 2. 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 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 hour 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 4A and 4B). 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 (Beese-Sims and 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 kilobases) 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 (Supplementary Figure 1), 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% to 25% of the P0 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.

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; Frokjaer-Jensen et al. 2014). Moreover, we believe 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 co-conversion 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 co-conversion approaches, which instead require an extra step to segregate the
co-conversion marker from the allele of interest or the use of special mutant backgrounds for co-conversion. 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 (Supplementary Table 2), 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 co-conversion 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 stranded DNA oligonucleotides or double stranded PCR products with much smaller homology arms can be used to introduce small modifications, deletions and medium sized (~1kb) insertions (ARRIBERE et al. 2014; FARBOUD and MEYER 2015; KATIC et al. 2015; LO et al. 2013; PAIX et al. 2014; ZHAO et al. 2014). 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 non-homologous 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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References


Figure Legends

**Figure 1:** Improved strategies for creation of sgRNA expression vectors and customized repair templates with a dual marker selection cassette. *(A, left panel)* New sgRNA vectors are easily generated from an existing sgRNA plasmid using a universal reverse primer (blue curved arrow) and a protospacer-specific forward primer (gold and red curved arrow) to PCR amplify the entire plasmid, followed by 5’ phosphorylation, ligation and transformation. *(A, right panel)* 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.

**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 panel), including Cas9 and sgRNA expression vectors, two co-injection markers, and a *klp-12* disrupting dual marker cassette repair template. 24 hours post-injection animals are treated with G418 (right panel) 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 panel) exhibit bright, mosaic GFP expression in the pharynx, and also express one or both mCherry co-injection markers. Recombinant animals (right panel) exhibit dim, uniform GFP expression in the pharynx and loss of mCherry expression, demonstrating integration of the selection cassette and loss of the extra-chromosomal array.
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 (upper panel) and HIS-72::mCherry (lower panel). Scale bars represent 100 µm.

Figure 4: Dual marker cassette generated epitope tagged LSD-1 and HIM-18 strains. (A, 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 represent 2µm.

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 ten 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.

Table 1: Summary of genome editing experiments. 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 non-homologous sequence inserted during editing, number of P0 adults successfully injected, number of independent insertions obtained, and efficiency of editing reported as a percentage of a P0 animals injected.
1. Amplify sgRNA vector
2. Phosphorylate 5’ ends, ligation
3. Transformation, validation

1. Digest vector
2. Anneal oligos, Gibson Assembly
3. Transformation, validation

**Norris, Kim et al. Figure 1**

**A**

- **U6 promoter scaffold**
- **sgRNA scaffold**
- **New protospacer**
- **F primer**
- **Universal R primer**
- **U6 downstream**

**pU6::klp-12 sgRNA vector**

**Oligos with new protospacer**

**pU6 empty sgRNA vector**

**B**

- **SacII**
- **loxP**
- **Pmyo-2::GFP::unc-54 UTR**
- **Prps-27::neoR::unc-54 UTR**
- **G418 Resistance**

**Pharyngeal GFP marker**

**Digest vector, amplify homology arms, Gibson assembly**

**5’ homology arm**

**Pmyo-2::GFP::unc-54 UTR**

**3’ homology arm**
Inject P₀ adult worms

Add G418 to growth plates

Screen for neoR, GFP, non-mCherry F₂ progeny

Days 4-11

1 week (optional) Inject Peft-3::Cre recombinase, Screen for non-GFP F₂ progeny

Klp-12 disrupting dual marker cassette

Peft-3::Cas9_SV40_NLS::tbb-2 UTR

pU6::klp-12 sgRNA

Pmyo-2::mCherry::unc-54 UTR

Pmyo-3::mCherry::unc-54 UTR

Norris, Kim et al. Figure 2

A

Injection mix

Day 0

Day 1

Days 4-11

25°C

25°C

Screen for neoR, GFP, non-mCherry F₂ progeny

B

Extrachromosomal Array

GFP

mCherry

Merge

Recombinant

Merge
Inject Peft-3::Cre recombinase

A

B

Norris, Kim et al. Figure 3
Norris, Kim et al. Figure 4
A mec-8 locus

2kb

TAA

ATG

cleavage site

5.4kb

3' homology

5' homology

mec-8 deletion

TAA

Pmyo-2::GFP

Prps-27::neoR

B

% touch responsiveness

Wild type

mec-8 (CRISPR)