CRISPR-Cas9-Guided Genome Engineering in *Caenorhabditis elegans*

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The CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated protein) system is being used successfully for efficient and targeted genome editing in various organisms, including the nematode *Caenorhabditis elegans*. Recent studies have developed a variety of CRISPR-Cas9 approaches to enhance genome engineering via two major DNA double-strand break repair pathways: nonhomologous end joining and homologous recombination. Here, we describe a protocol for Cas9-mediated *C. elegans* genome editing together with single guide RNA (sgRNA) and repair template cloning (canonical marker-free and cassette selection methods), as well as injection methods required for delivering Cas9, sgRNAs, and repair template DNA into the germline. © 2019 by John Wiley & Sons, Inc.

Basic Protocol 1: Guide RNA preparation
Alternate Protocol 1: sgRNA cloning using fusion PCR
Basic Protocol 2: Preparation of a repair template for homologous recombination
Alternate Protocol 2: Preparation of repair template donors for the cassette selection method
Basic Protocol 3: Injecting animals
Basic Protocol 4: Screening transgenic worms with marker-free method
Alternate Protocol 3: Screening transgenic worms with cassette selection method

Keywords: *C. elegans* • Cas9 • CRISPR • CRISPR-Cas • genome editing • genome engineering

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INTRODUCTION

Overview of the CRISPR-Cas9 System and Function

The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) system is a primarily nuclease-based defense mechanism utilized by bacteria against bacteriophages to avoid viral attacks (Barrangou et al., 2007). Key features of all major CRISPR-Cas systems are the presence of an array of direct repeats, referred to as CRISPR, as well as genes encoding Cas proteins, including an RNA-guided site-specific nuclease (RGN). CRISPR loci are composed of palindromic repeats with spacer regions originating from viral or plasmid DNA and are accompanied by adjacent Cas genes, including a gene that encodes the RGN. The so-called type II CRISPR system from *Streptococcus pyogenes* is the best-studied system for genome editing (Garneau

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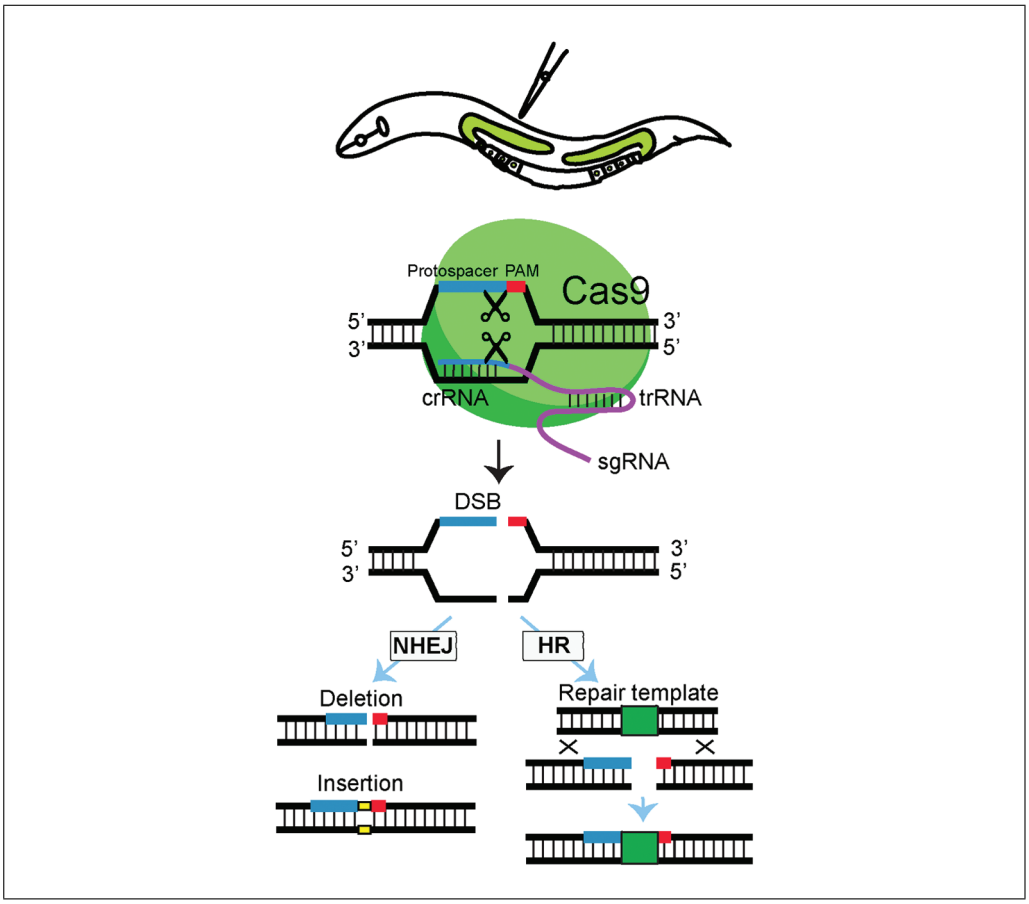

Figure 1 Schematic representation of the CRISPR-Cas9 genome editing approach in *C. elegans*. Young adult hermaphrodites are injected with the CRISPR-Cas9-containing DNA mixture. A DSB generated by Cas9 is repaired via error-prone NHEJ or error-free HR. Yellow box represents small nucleotide insertion and green box represents insertion of GFP tag.

et al., 2010; Horvath & Barrangou, 2010; Mali et al., 2013). In brief, this system consists of two noncoding RNAs, the crRNA and trRNA, which are transcribed from the CRISPR locus. The crRNA or CRISPR targeting RNA consists of a 20-nucleotide (nt) sequence from the spacer region of the CRISPR locus and corresponds to a viral DNA signature. The trRNA, or *trans*-activating RNA, is complementary to a pre-crRNA, thus forming an RNA duplex that is later cleaved by RNase III to form a crRNA-trRNA hybrid, thereby directing the Cas9 RGN to create a double-stranded break (DSB) at the target site as long as the target is directly 5′ of a so-called protospacer adjacent motif (PAM) with the sequence NGG (Deltcheva et al., 2011). The DSB is within ~3 bases from the target site's PAM. The CRISPR locus itself is not cleaved by the RGN because it does not contain any NGG sequences (Fig. 1).

The *S. pyogenes* CRISPR-Cas9 system has been used for genetic engineering because the *S. pyogenes* crRNA and trRNA are functional when fused as a single RNA molecule (referred to as a single guide RNA, or sgRNA) and because the *S. pyogenes* RGN is a single-subunit protein. This system can thus be used to introduce a DSB in vivo at the locus N_{20}-NGG by engineering an sgRNA molecule in which the first 20 nt correspond to a 20-nt target sequence directly 5′ of an NGG (PAM) sequence.

Non-Homologous End Joining and Homologous Recombination

DNA DSBs induced by the Cas9 RGN at the target site can be repaired by either nonhomologous end joining (NHEJ) or homologous recombination (HR; Fig. 1). In the absence of a repair template, DSBs introduced by CRISPR-Cas9 are repaired by NHEJ,

Table 1 Types of CRISPR-Cas9 Methods Developed in *C. elegans*

Selection marker	References
Marker-free	Single-CRISPR (Friedland et al., 2013; Paix et al., 2014; Tzur et al., 2013) Co-CRISPR (Arribere et al., 2014; Kim et al., 2014; Ward, 2015)
Marker-dependent	*unc-119*$^{+}$ (Dickinson et al., 2013) Drug resistance (Dickinson et al., 2015, 2013; Norris et al., 2015)

which often results in small insertions and/or deletions (InDels) at the targeted site (Fig. 1). The random insertion and/or deletion that occurs in the generation of InDels can result in the early termination of a protein due either to sequence alteration or to frame shift when the targeted site is located in an open reading frame. Importantly, when aiming for gene disruption, targeting of the N-terminus of a gene is preferred. However, the presence of potential cryptic start codons has to be evaluated to confirm the loss of gene function.

Unlike error-prone NHEJ-driven InDel events, HR is error free and can be utilized with the CRISPR-Cas9 system for the insertion of tags and/or to generate precise point mutations in a specific gene. This requires introducing a repair template carrying homology both upstream and downstream of the target site that is used for DSB repair (Fig. 1).

Various approaches have been developed by different laboratories to engineer the *Caenorhabditis elegans* genome, which can be divided into two major categories based on their dependency on a phenotypic marker that probes or marks the edited genome sequence (Table 1). Here, we describe reproducible marker-free and marker (selection cassette)-based methods using *S. pyogenes* Cas9 in *C. elegans* to create heritable genome modifications via either the NHEJ or HR pathway. The overall strategy, which is broken down into four separate basic protocols with some alternative options, involves (1) generating the sgRNA (Basic Protocol 1 or Alternate Protocol 1); (2) generating the repair template DNA, if HR is going to be employed to specifically modify a particular gene (Basic Protocol 2 or Alternate Protocol 2); (3) introducing the *cas9* gene, sgRNA, and repair DNA templates into *C. elegans* worms on separate plasmids (Basic Protocol 3); and (4) screening for transgenic worms carrying the CRISP-Cas9-mediated gene-editing event (Basic Protocol 4 or Alternate Protocol 3) (Fig. 2). This strategy can generate targeted deletions or disruptions, point mutations, and endogenous tagging

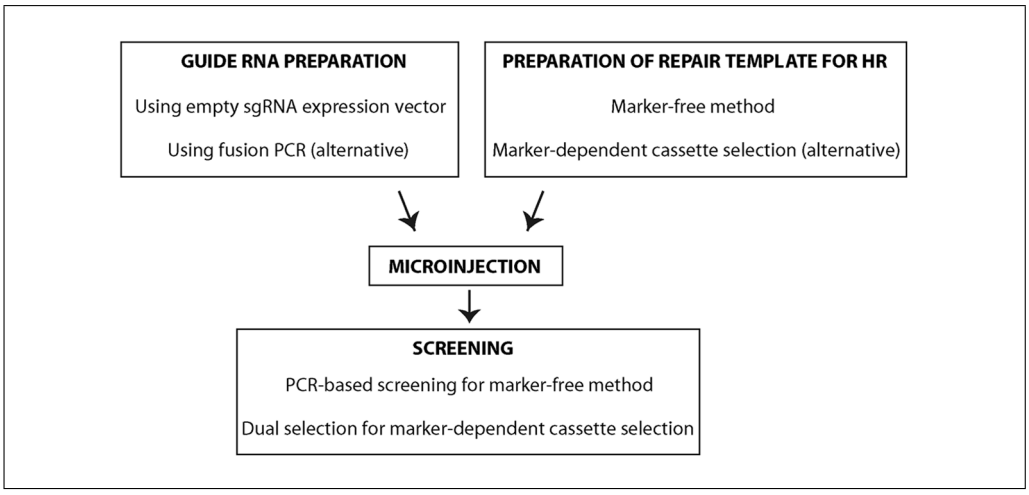

Figure 2 Outline of the process of building genome engineered *C. elegans* in this unit. Note that we present two alternative protocols for guide RNA and repair template. Each requires its own screening strategy.

through canonical marker-free and marker-based (cassette selection) methods. Advantages afforded by each method, whose assessment can aid in deciding what method to follow, are presented in the Critical Parameters.

BASIC PROTOCOL 1

GUIDE RNA PREPARATION

Plasmids containing sgRNAs are necessary for Cas9-mediated genome editing. This basic protocol outlines the steps required to prepare these plasmids for microinjection in two alternative ways (Basic Protocol 1 and Alternative Protocol 1).

Materials

Oligonucleotides:
- sgRNA Top: 5′-ATTGCAAATCTAAATGTTT-$N_{19/20}$-GTTTTAGAGCTAG AAATAGC-3′ (where $N_{19/20}$ represents a 19- or 20-nt sequence corresponding to the target sequence of interest)
- sgRNA Bottom: 5′-GCTATTTCTAGCTCTAAAAC-$N_{19/20}$-RC-AAACATT TAGATTTGCAAT-3′ (where $N_{19/20}$-RC represents the reverse-complementary sequence of the $N_{19/20}$ target sequence)
- M13F: 5′-GTAAAACGACGGCCAGT-3′
- M13R: 5′-AACAGCTATGACCATG-3′

pHKMC1 (3482 bp, encoding empty sgRNA for cloning; Addgene #67720)
*Bam*HI (NEB R0136S or equivalent)
*Not*I (NEB R0189S or equivalent)
Plasmid Miniprep Kit (GeneJet K0502, Qiagen 27104, or equivalent)
Plasmid Midiprep Kit (Qiagen 12143 or equivalent)
Gel DNA Recovery Kit (Zymoclean D4001)
Gibson Assembly Master Mix (NEB E2611S)
Chemically competent *Escherichia coli* cells (DH5α, NEB C2987I, or equivalent)
LB agar plates containing 100 µg/ml ampicillin (Elbing & Brent, 2002)
Nuclease-free water (Qiagen 129114, Zymo Research W1001-10, or equivalent)

Heat block (VWR Scientific Standard Heat Block or equivalent)
PCR thermal cycler (Bio-Rad T100 or equivalent)
Sterile pipet tips or toothpicks for picking colonies

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000), bacterial transformation of chemically competent *E. coli* (Seidman, Struhl, Sheen, & Jessen, 1997), and Sanger sequencing (Slatko et al., 2011)

Identifying an sgRNA targeting sequence

1. To construct an appropriate sgRNA, first identify a targeting sequence within ∼70 bp of the intended genomic target site in the form 5′-$N_{19/20}$-NGG-3′ using nucleotide sequence analysis software or a text editor program such as Notepad.

   The 3′-NGG is the PAM sequence that is necessary for Cas9 binding to the target sequence but is not included in the sgRNA expression vector.

   To minimize the potential for generating off-target mutations, use software such as NCBI BLAST to find possible off-target sites. Once the targeting sgRNA sequence is identified, generate an sgRNA expression vector as described below. We present two alternative reliable methods that we have implemented successfully.

   The presence of GG at positions N_{19} and N_{20} on the target sequence enhances the efficiency of recombination (Farboud & Meyer, 2015).

   Instead of NGG, an NGA PAM can be used with the VQR Cas9 variant, achieving the same efficiency as with wild-type Cas9 (Bell, Fu, & Fire, 2016).

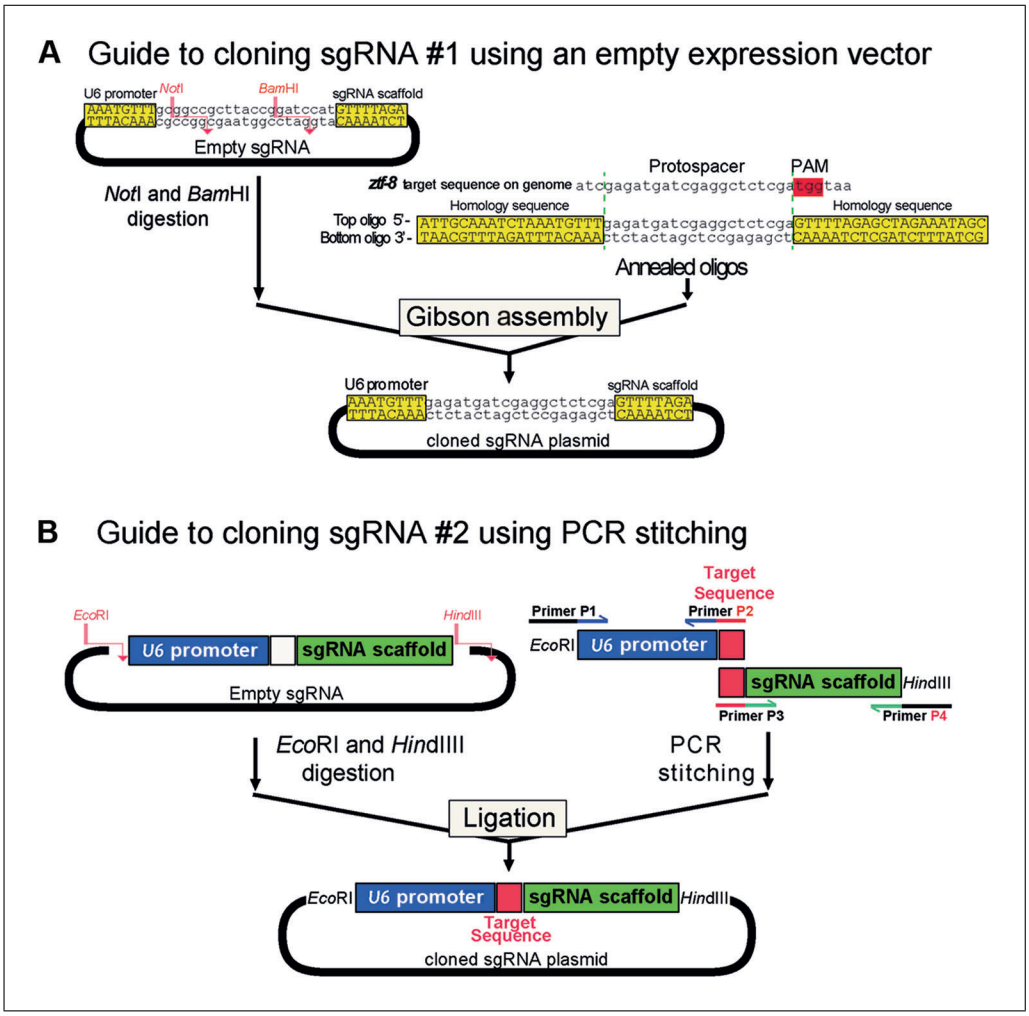

Figure 3 Schematic representation of sgRNA cloning by two alternative methods. (**A**) sgRNA cloning using an empty sgRNA expression vector. The sgRNA comprising a pair of annealed oligos specifically targeting *ztf-8* is cloned into the *Not*I- and *Bam*HI-digested empty sgRNA vector (left) using Gibson assembly. (**B**) sgRNA cloning using fusion PCR. To generate an sgRNA containing the *Eco*RI-*Hin*dIII fragment, two amplicons are stitched together by PCR (right). Both the fused PCR fragment and the empty sgRNA vector are digested with *Eco*RI and *Hin*dIII and ligated to create the sgRNA expression vector.

sgRNA cloning using an empty sgRNA expression vector

This section describes how to engineer an sgRNA in an empty sgRNA expression vector using restriction digestion and either Gibson or HiFi DNA assembly.

2. Order both top (forward) and bottom (reverse complement) oligos containing 19 or 20 nt of homology ($N_{19/20}$) to the target sequence from a DNA synthesis service (Fig. 3A). When $N_1 \neq G$, add one additional G in front of N_1 to ensure expression from the U6 promoter.

   Following are the generic sgRNA sequences (in CAPITAL LETTERS), presented in the context of an example with the specific sgRNA sequence design (lower case) adopted for C-terminal tagging of the C. elegans ztf-8 gene with GFP (Kim & Colaiacovo, 2015b).

   Generic sgRNA sequences:

   sgRNA_Top: 5′-ATTGCAAATCTAAATGTTT-$N_{19/20}$-GTTTTAGAGCTAG AAATAGC-3′

sgRNA_Bottom: 5′-GCTATTTCTAGCTCTAAAAC-$N_{19/20}$-RC-AAACATTT AGATTTGCAAT-3′

sgRNA adopted for C-terminal GFP tagging of the *C. elegans ztf-8* gene (as described in Kim & Colaiacovo, 2015b)

*ztf-8*_Top: 5′-ATTGCAAATCTAAATGTTT-gagatgatcgaggctctcga-GTTTT AGAGCTAGAAATAGC-3′

*ztf-8*_Bottom: 5′-GCTATTTCTAGCTCTAAAAC-tcgagagcctcgatcatctc-AA ACATTTAGATTTGCAAT-3′

3. Perform a miniprep of the empty sgRNA vector (Addgene #67720), which contains the U6 promoter and sgRNA scaffold sequence as illustrated in Figure 3A. Digest 1-2 µg empty sgRNA vector with *Bam*HI and *Not*I and separate on an agarose gel. Extract the 3470-bp band from the gel using the Gel DNA Recovery Kit.

   This will be sufficient for ~10 reactions.

4. To generate double-stranded DNA, mix equal amounts of the Top and Bottom strand of sgRNA targeting oligonucleotides (e.g., 5 µl of 200 pM each) in distilled water and anneal them using a thermal cycler.

   Heat to 95°C for ~2 min.
   Slowly ramp cool to 25°C over a period of ~40 min.

   Alternatively, place the tube containing the Top and Bottom oligos in a heat block at ~90°C for ~3 min. Remove the block from the heat block apparatus and allow it to cool at room temperature for ~40 min until it reaches ~30°C.

5. Set up the Gibson assembly reaction:

   5 µl annealed oligonucleotides
   ~100 ng *Bam*HI- and *Not*I-digested empty sgRNA
   1 vol Gibson mix (i.e., volume of mix equal to the entire volume of oligos and digested sgRNA)

6. Use 1-2 µl of reaction for bacterial transformation of chemically competent *E. coli* (Seidman et al., 1997). Spread the transformants onto LB plates containing 100 µg/ml ampicillin and incubate overnight.

7. The following day, pick and inoculate ~10 ampicillin-resistant colonies, purify plasmid DNA using a plasmid miniprep kit, and screen for insertion of sgRNA by restriction digest analysis.

   For colony PCR screening, M13F and M13R primers will amplify ~890 bp from the empty vector, and this PCR product can be used for sequencing and/or be further analyzed through a BamHI or NotI digestion. The plasmid containing the sgRNA insert can be distinguished from either undigested vector or self-ligated vector lacking an insert via digestion with either of these two restriction enzymes, as an undigested or self-ligated vector will produce both a 530-bp and a 360-bp product. Although BamHI- and NotI-cleaved ends are not compatible with each other, Gibson assembly has 5′-to-3′ resection activity and this may enhance the self-ligation of empty vectors by removing the staggered incompatible ends produced by BamHI and NotI.

8. Verify sgRNA insertion by Sanger sequencing using the primers M13R and M13F.

ALTERNATE PROTOCOL 1

sgRNA CLONING USING FUSION PCR

This section describes how to clone a sgRNA targeting sequence using fusion PCR (adapted from Friedland et al., 2013).

Additional Materials *(also see Basic Protocol 1)*

Oligonucleotides:
P1: 5′-CGGGAATTCCTCCAAGAACTCGTACAAAAATGCTCT-3′
P2: 5′-($N_{19/20}$-RC)+AAACATTTAGATTTGCAATTCAATTATATAG-3′ (where $N_{19/20}$-RC is the reverse complementary sequence of the $N_{19/20}$ target sequence used in primer P3; synthesized by DNA synthesis service)
P3: 5′-($N_{19/20}$)+GTTTTAGAGCTAGAAATAGCAAGTTA-3′ (where $N_{19/20}$ represents the sgRNA target sequence; synthesized by DNA synthesis service)
P4: 5′-CGGAAGCTTCACAGCCGACTATGTTTGGCGT-3′ (synthesized by DNA synthesis service)
High Fidelity Phusion DNA polymerase (NEB M0530S), or equivalent, and 5× HF buffer
10 mM 4dNTP mix (10 mM each dNTP)
PCR purification kit (Thermo Fisher GeneJET K0701 or equivalent)
*Eco*RI (NEB, cat. no. R0101S, or equivalent)
*Hin*dIII (NEB, cat. no. R0104S, or equivalent)
T4 DNA ligase (NEB, cat. no. M0202S, or equivalent) and 10× buffer

Additional reagents and equipment for PCR (Kramer and Coen, 2001) and agarose gel electrophoresis (Voytas, 2000)

1. Design primers to amplify upstream (P1 + P2; PCR-up) and downstream (P3 + P4; PCR-down) of the empty sgRNA vector (Fig. 3B).

   Primers P2 and P3 contain the sgRNA target sequence $N_{19/20}$ (19-20 nt) (Fig. 3B). When $N_1 \neq G$, add one additional "G" in front of N_1 to ensure expression from the U6 promoter. P1 and P4 contain EcoRI and HindIII restriction sites, which will be used for ligation to the vector plasmid. Note that 3 nt at the 5′ end of P1 and P4 are not complementary to the empty sgRNA vector, but the remaining sequences (33 and 28 nt for P1 and P4, respectively) are complementary to the empty sgRNA.

2. To amplify either upstream (P1 + P2; PCR-up) or downstream (P3 + P4; PCR-down) of sgRNA expression vector using empty sgRNA as a template (Fig. 3B), mix:

   10 µl 5× HF buffer
   1 µl 10 mM 4dNTP mix
   1.25 µl 10 µM each either P1 and P2 (PCR-up) or P3 and P4 (PCR-down)
   2 µl 50 ng/µl empty sgRNA vector
   0.5 µl Phusion polymerase
   34 µl distilled, deionized H_2O (ddH_2O; to 50 µl final).

3. Run reactions in a thermal cycler using the following PCR thermal cycling program:

1 cycle:	30 s 98°C (initial denaturation)
30 cycles:	5-10 s 98°C (denaturation)
	10-30 s 45°C-72°C (annealing)
	15-30 s/kb 72°C (extension)
1 cycle:	5-10 min 72°C (final extension).

4. Gel purify PCR amplicons by agarose gel electrophoresis (1% agarose gel) followed by the use of a Gel DNA Recovery Kit.

5. To amplify a stitched sgRNA amplicon by using amplicons from PCR-up and PCR-down as templates (PCR-whole), mix:

   10 µl 5× HF buffer
   1 µl 10 mM 4dNTP mix
   1.25 µl 10 µM each P1 and P4

1 µl (5-10 ng) each amplicons from PCR-up and PCR-down
0.5 µl Phusion polymerase
35 µl ddH_2O (to 50 µl final).

6. Run reactions in a thermal cycler using the following PCR thermal cycling program:

1 cycle:	30 s 98°C (initial denaturation)
30 cycles:	5-10 s 98°C (denaturation)
	10-30 s 45°C-72°C (annealing)
	15-30 s/kb 72°C (extension)
1 cycle:	5-10 min 72°C (final extension).

7. Clean up PCR product using a PCR purification kit.

8. Digest empty sgRNA vector and purified PCR amplicon with *Eco*RI and *Hin*dIII restriction enzymes. Gel purify digested vector backbone and PCR amplicon by agarose gel electrophoresis (using a 1% agarose gel) and a Gel DNA Recovery Kit.

9. To ligate the digested amplicon with the digested vector, mix:

50 ng digested vector
50 ng digested stitched PCR products
2 µl 10× T4 DNA ligase buffer
2 µl T4 DNA ligase
ddH_2O to 20 µl final.

A control "vector-only" ligation will help assess enrichment of transformed bacteria carrying amplicon insertions in the vector. A control ligation can be set up by omitting the stitched amplicon from the reaction.

10. Incubate ligation reactions 1 hr at room temperature. Transform 5 µl of each ligation reaction into 45 µl *E. coli* DH5α competent cells according to manufacturer's recommendations, and then spread bacteria onto LB + ampicillin plates. Incubate at 37°C overnight.

11. Pick and grow ampicillin-resistant colonies from the plate containing bacterial transformants, purify plasmids, and screen for insertion of the sgRNA cassette by restriction digest analysis.

For PCR screening, M13F and M13R primers (or P1 and P4) will amplify ~890 bp from the empty vector, and this PCR product can be used for sequencing and/or be further analyzed through a BamHI or NotI digestion as described in step 7 of Basic Protocol 1.

12. Further verify that correct sgRNA insert is present by sequencing the vector by Sanger sequencing using primers P1 and P4.

Instead of the empty vector, another sgRNA containing vector can be used for the steps above, such as pU6::klp-12_sgRNA or pU6::unc-119_sgRNA as described in Friedland et al. (2013).

Although gel purification is not generally necessary for PCR stitching, the original PCR template used in the PCR-up and PCR-down reactions contains an empty sgRNA vector. Gel purification helps ensure that the original template is not utilized in the amplification cycles in the "PCR-whole" reaction.

BASIC PROTOCOL 2

PREPARATION OF A REPAIR TEMPLATE FOR HOMOLOGOUS RECOMBINATION

We present two independent protocols for donor template preparation: the canonical marker-free and marker-based cassette selection methods. Since the latter requires both drug and fluorescent selections followed by removal of a cassette, the donor templates

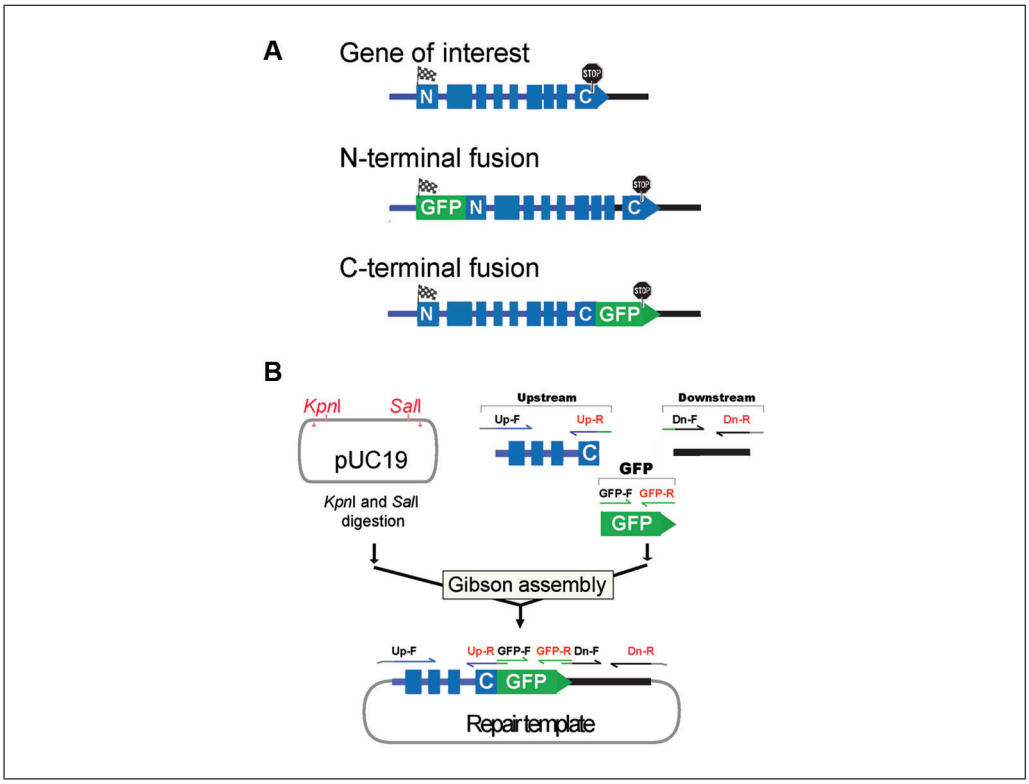

Figure 4 Schematic representation of the construction of the repair template. (**A**) Diagrams illustrate the N- and C-terminal GFP tagging of a gene of interest. Note the position of GFP as well as the protein start and stop codons. The checkered flag represents the start codon. (**B**) Schematic representation of repair template cloning for C-terminal GFP tagging. Three PCR amplicons are assembled into a *Kpn*I- and *Sal*I-digested pUC19 vector to create a repair template (donor plasmid).

for the two protocols are not compatible with each other, and each method requires its own screening procedure (Fig. 2), which are presented here and Alternate Protocol 2, respectively. While it takes more to complete the screening process with the marker-based cassette selection method (see Time Considerations), this method is considered less laborious due to its drug and fluorescence screening capabilities. However, because it uses multiple cassette vectors, this system is tailored for endogenous tagging and deletions but not for generating point mutations. In contrast, the marker-free method is universal and customizable and does not require specific cassette vectors. However, it often entails laborious PCR screening. Therefore, these two methods present both pros and cons that need to be considered when deciding which approach to use.

In designing a repair template DNA for use in the canonical marker-free method, first decide whether you are aiming for an N- or C-terminal fusion of your protein of interest before designing the oligonucleotides for the repair template (donor vector) because that determines what primer sequences you must use (Fig. 4A). Here we present an example of C-terminal tagging with GFP (Fig. 4B). Using DNA from Bristol N2 worms as template, PCR amplify both upstream and downstream homology arms (aim to amplify between 500 and 1500 bp of homology sequence flanking each side of the target site). Design PCR primers with ~20-30 nt of overlapping sequence for cloning via Gibson assembly. Specifically, for Gibson assembly, the "upstream" PCR product will contain overlapping sequences for the *Kpn*I side of pUC19 (gray line of Up-F primer) as well as for the 5′ end of GFP (green line). The "downstream" PCR product will overlap with the 3′ end of GFP (green line) and the *Sal*I side of pUC19 (gray line). The GFP fragment can be PCR

amplified from a GFP-containing plasmid such as pPV477 because sufficient homology is provided by the up- and downstream overhangs. The same strategy can be applied to generate similar fusion tags using, for example, mCherry, HIS, or Flag, by simply replacing the GFP fragment.

Materials

Oligonucleotides:
UP-F: 5′-ACGGCCAGTGAATTCGAGCTCGGTA+~$N_{18\text{-}24}$-3′ (where $N_{18\text{-}24}$ refers to 18-24 nt upstream from a gene of interest)
UP-R: 5′-GTGAAAAGTTCTTCTCCTTTACTCAT+~$N_{18\text{-}24}$(RC)-3′ (where $N_{18\text{-}24}$ refers to 18-24 nt upstream from a gene of interest; the reverse complement)
DN-F: 5′-TGGCATGGACGAACTATACAAA+~$N_{18\text{-}24}$-3′ (where $N_{18\text{-}24}$ refers to 18-24 nt from the stop codon of a gene of interest)
DN-R: 5′-ACGCCAAGCTTGCATGCCTGCAGG+~$N_{18\text{-}24}$(RC)-3′ (where $N_{18\text{-}24}$ refers to 18-24 nt downstream from a gene of interest; the reverse complement)
GFP-F: 5′-ATGAGTAAAGGAGAAGAACT-3′
GFP-R: 5′-TTTGTATAGTTCGTCCATGC-3′
M13F: 5′-GTAAAACGACGGCCAGT-3′
M13R: 5′-AACAGCTATGACCATG-3′
10 mM 4dNTP mix (10 mM each dNTP)
High Fidelity Phusion DNA polymerase (NEB M0530S or equivalent)
pUC19 (NEB N3041S)
pPV477 (Addgene plasmid #42930)
*Kpn*I (NEB R0142S)
*Sal*I (NEB R0138S)
Gel DNA Extraction Kit (Zymoclean D4001)
Gibson Assembly Master Mix (NEB E2611S)
LB agar plate containing 100 µg/ml ampicillin (Elbing & Brent, 2002)
LB liquid medium containing 100 µg/ml ampicillin (Elbing & Brent, 2002)
Plasmid Miniprep Kit (GeneJet K0502 or Qiagen 27104)
Plasmid Midiprep Kit (Qiagen 12143)

PCR thermal cycler (Bio-Rad T100 or equivalent)
Water bath incubators, 37°C and 42°C

Additional reagents and equipment for PCR amplification (Kramer and Coen, 2001)

1. PCR amplify (see Kramer and Coen, 2001) the upstream sequence of a gene of interest (500-1500 bp; Fig. 4B) using the following forward and reverse primers:

   UP-F: 5′-ACGGCCAGTGAATTCGAGCTCGGTA+~$N_{18\text{-}24}$-3′
   UP-R: 5′-GTGAAAAGTTCTTCTCCTTTACTCAT+~$N_{18\text{-}24}$ (RC)-3′,

   First, prepare the following PCR mix:

   Template DNA (<250 ng)
   2 µl mM 4dNTP mix
   2 µl each 10 µM forward and reverse primers
   10 µl 5× HF buffer
   0.5 µl Phusion polymerase
   ddH_2O to 50 µl final.

2. Run reactions in a thermal cycler using the following PCR thermal cycling conditions:

1 cycle:	30 s 98°C (initial denaturation)
30 cycles:	5-10 s 98°C (denaturation)
	10-30 s 45°C-72°C (annealing)
	15-30 s/kb 72°C (extension)
1 cycle:	5-10 min 72°C (final extension).

3. PCR amplify the downstream sequence of a gene of interest (500-1500 bp, Fig. 4B) using the reaction mixture and PCR cycling conditions described in steps 1 and 2 and the following forward and reverse primers:

   DN-F: 5′-TGGCATGGACGAACTATACAAA+~N_{18-24}-3′
   DN-R: 5′-ACGCCAAGCTTGCATGCCTGCAGG+~N_{18-24} (RC)-3′.

4. PCR amplify the 867-bp GFP fragment from plasmid pPV477 (Addgene #42930) using the reaction mixture and PCR cycling conditions described in steps 1 and 2 and the following forward and reverse primers:

   GFP-F: 5′-ATGAGTAAAGGAGAAGAACT-3′
   GFP-R: 5′-TTTGTATAGTTCGTCCATGC-3′.

5. Digest pUC19 (or pUC18) DNA with *Kpn*I and *Sal*I (Fig. 4B, top left). Run digest on an agarose gel to confirm plasmid linearization at 2686 bp. Because the result of a successful double digest (2669 + 17 bp) is barely distinguishable from the outcome of a single restriction enzyme digest because of the subtlety of the change in size, make sure that both enzymes are working properly by comparing the size of undigested to digested plasmid with either restriction enzyme. Separate 5-10 µg of the digested vector on a gel and then use the Gel DNA Extraction Kit extract the 2669-bp band resulting from the *Kpn*I and *Sal*I digestion of the vector from the gel.

   Other DNA cloning vectors can be used instead of pUC vectors.

6. Perform Gibson assembly with the *Kpn*I- and *Sal*I-digested vector plus three PCR fragments (upstream, GFP, and downstream) as described in the manufacturer's instructions (Fig. 4B). After the Gibson assembly reaction, use 1-2 µl for bacterial transformation, spread bacteria onto LB plates containing 100 µg/ml ampicillin, and select ampicillin-resistant colonies the following day.

7. Inoculate ~10 ampicillin-resistant colonies for plasmid minipreps and analyze them by Sanger sequencing using the M13F and M13R primers.

   Aim to introduce a silent mutation at the PAM site of the repair template to prevent it from being cut by Cas9 when it is subsequently injected into the nematodes. This can be achieved by incorporating mutations on primer tails when designing the repair template. Alternatively, if you are working with a pre-existing repair template (donor vector), use a site-directed mutagenesis kit. There are also companies that will synthesize a whole DNA fragment, although this may be a costly option. If it is not feasible to introduce a silent mutation at the PAM site, introduce multiple silent mutations in the sequence corresponding to the sgRNA sequence. See the example in Figure 5A.

   Any fusion protein, such as HA, Flag, or GFP, must be in frame and contain a start (AUG) and/or a stop (UGA, UAG, or UAA) codon.

   The repair template is required only for gene editing using HR, not for NHEJ.

   A single-stranded oligonucleotide or PCR product can be used as a repair template, which avoids the hassle of the cloning procedure (Paix et al., 2014; Zhao, Zhang, Ke, Yue, & Xue, 2014). Insertion of a GFP fragment with ~60-nt homology arms has been

reported. Small insertions, such as insertions of 3×Flag, Myc, His, and HA tags, can be achieved with oligonucleotides with ~60-nt homology arms. Therefore, these can be an alternative option, especially when cloning is not feasible due to technical issues such as difficulties with Gibson assembly stemming from the presence of repetitive sequences, although it remains to be determined whether the vector-free method is as efficient as the vector-based method.

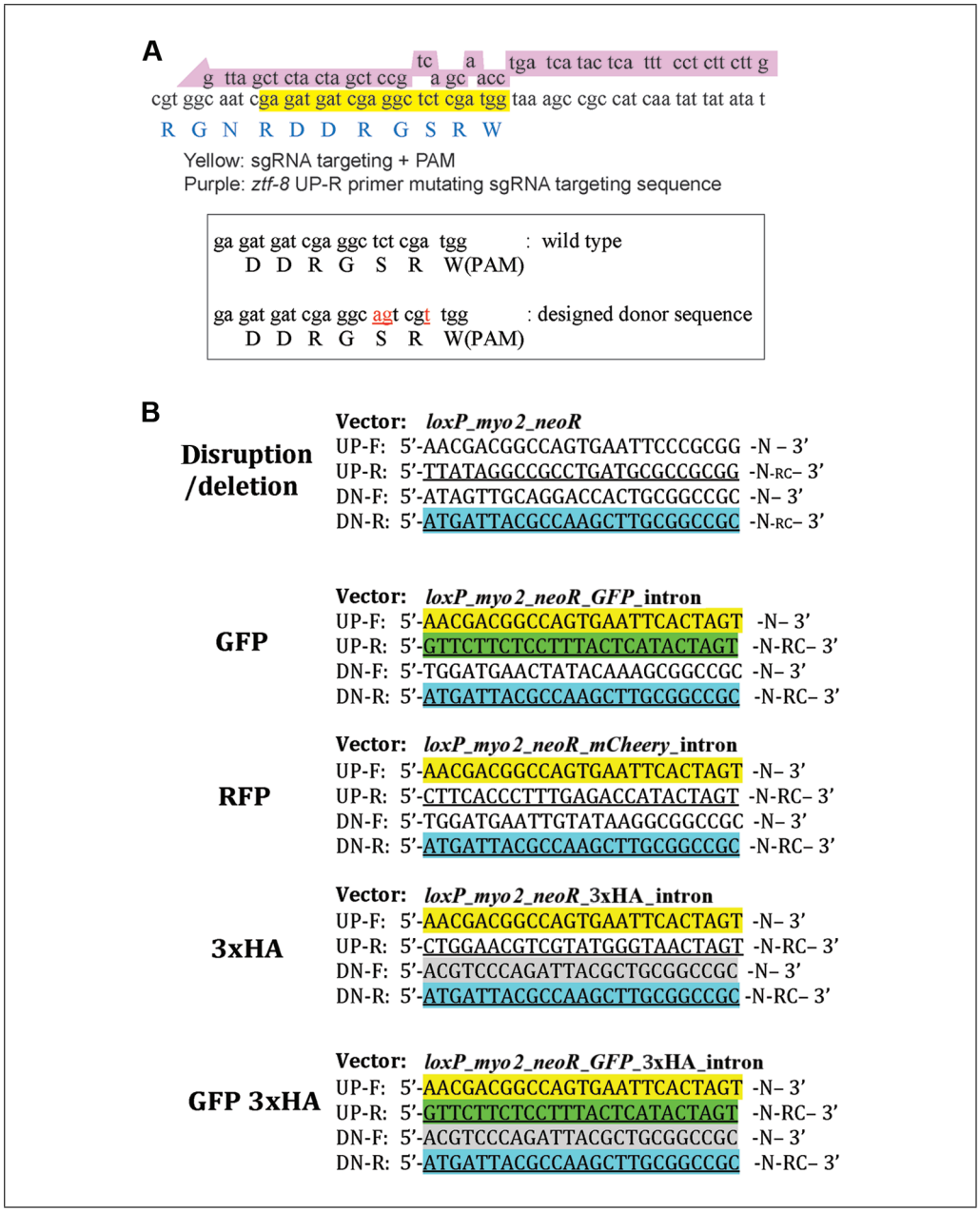

Figure 5 Preparation of repair template. (**A**) Introduction of silent mutations in S (serine) and R (arginine) codons with the primer *ztf-8* UP-R to prevent restriction enzyme cutting of the repair template. The original wild-type and designed repair template sequences are compared. (**B**) Primer sequences for amplifying homology arms for the cassette selection method. Identical sequences are highlighted with the same colors. For example, all constructs share the downstream R primer (cyan color) in common. The reverse complement (RC) is underlined. N, ~20-bp sense complementary sequence to your gene of interest for a PCR reaction. N-RC, ~20-bp reverse-complementary sequence to your gene of interest for a PCR reaction (underlined).

PREPARATION OF REPAIR TEMPLATE DONORS FOR THE CASSETTE SELECTION METHOD

ALTERNATE PROTOCOL 2

This section describes an alternative to using the marker-free method. The main advantages of the cassette selection method are that (1) it provides platforms for tagging reporters (GFP, RFP, 3× HA, GFP + 3× HA) and deletion/disruption backbone (Fig. 5B); and (2) it involves the use of dual selection (drug + fluorescent) cassettes for easier screening of transgenic worms. This section describes how to prepare repair template donors with a cassette selection toolkit as described in Norris, Kim, Colaiacovo, & Calarco (2015). With this method, it is important to screen the transgenic worms at a specific time point after injection as described in Time Considerations.

Materials

Construct vector—choose one of the below:
- P*myo-2::GFP neoR loxP* (disruption/deletion; Calarco lab, *http://www.calarcolab.ca/*)
- *GFP*-P*myo-2::GFP neoR loxP* (GFP tag; Calarco lab, *http://www.calarcolab.ca/*)
- *RFP*-P*myo-2::GFP neoR loxP* (RFP tag; Calarco lab, *http://www.calarcolab.ca/*)
- 3×HA-P*myo-2::GFP neoR loxP* (3×HA tag; Calarco lab, *http://www.calarcolab.ca/*)
- *GFP*-3×HA-P*myo-2::GFP neoR loxP* (GFP 3×HA tag; Calarco lab, *http://www.calarcolab.ca/*)

*Not*I (NEB HF R3189S)
*Sac*II (NEB R0157S)
*Spe*I (NEB HF R3133S)
Thermo-alkaline phosphatase (Fermentas FastAP EF0651)
Oligonucleotides:
- UP-F primer: 5′-AACGACGGCCAGTGAATTCACTAGTcgtaaggttacatattaattc-3′
- UP-R primer: 5′-GTTCTTCTCCTTTACTCATACTAGTccaACGACT gcctcgatcatctcgattg-3′
- DN-F primer: 5′-TGGATGAACTATACAAAGCGGCCGCtaaagccgccatcaatatta-3′
- DN-R primer: 5′-ATGATTACGCCAAGCTTGCGGCCGCcggtctcgatacgacaaat-3′
- M13F: 5′-GTAAAACGACGGCCAGT-3′
- M13R: 5′-AACAGCTATGACCATG-3′

PCR purification kit (Thermo Fisher GeneJET K0701 or equivalent)
Phusion polymerase (NEB E0553S) or PrimeStar GXL polymerase (Takara R050B)
Gibson Assembly Master Mix (NEB E2611S)
Chemically competent *E. coli* cells (NEB C2987I or equivalent)
Spectrophotometer for measuring DNA concentration

Vector and alkaline phosphatase treatment

1. Based on the design of your donor template, choose the desired template vector among the five types of vectors and set up the appropriate restriction enzyme digestion of the vector as suggested in Table 2.
2. Incubate 4-6 hr at 37°C to ensure complete digestion.

   A longer digestion period is preferred to avoid incomplete digestion that can result in false positive ampicillin-resistant clones.

Table 2 Setting Up Restriction Digestion Reactions

	Deletion/disruption template donor			Four types of tagging template donors	
1	*loxP_myo2_neoR* (deletion/disruption)	*x* µl (~2 µg)	1	*loxP_myo2_neoR_GFP*_intron (GFP) *loxP_myo2_neoR*_mCherry_intron (mCherry) *loxP_myo2_neoR*_3×HA_intron (3×HA) *loxP_myo2_neoR_GFP_*3×HA_intron (*GFP*::3×HA)	*x* µl (~2 µg)
2	CutSmart buffer (10×)	4 µl	2	CutSmart buffer (10×)	4 µl
3	*Sac*II	2 µl	3	*Spe*I HF	2 µl
4	*Not*I HF	2 µl	4	*Not*I HF	2 µl
5	ddH_2O	32 – *x* µl	5	ddH_2O	32-× µl
	Total volume	40 µl		Total volume	40 µl

For deletion/disruption, set up a reaction according to the left column; for tagging with GFP, mCherry, 3×HA, or GFP::3×HA, set up a reaction based on the right column.

Table 3 Expected Sizes with Restriction Enzyme Digestion

Types	Deletion or tagging vectors	*Not*I (bp)	*Sac*II (bp)	*Spe*I (bp)	Double digest (bp)
Deletion or disruption	*loxP_myo2_neoR*	2828	2828	N/A	2651, 177
GFP	*loxP_myo2_neoR_GFP*_intron	3708	N/A	3708	2648, 1060
mCherry	*loxP_myo2_neoR_mCherry*_intron	3702	N/A	3702	2648, 1054
3×HA	*loxP_myo2_neoR*_3×HA_intron	2982	N/A	2982	2648, 334
GFP::3×HA	*loxP_myo2_neoR_GFP_*3×HA_intron	3798	N/A	3798	2648, 1150

Single-restriction-enzyme digestion linearizes plasmid in all cases.

3. To verify that the plasmid has been fully and correctly digested, run ~4 µl of the digestion mix (200 ng) on an ~0.8% agarose gel.

   Note the changes in the sizes of the bands for the single and double digestions (Table 3). For example, either a single 2828-bp band or both 2651-bp and 177-bp bands are expected from single and double digestions of loxP_myo2_neoR, respectively. If incorrect sizes are detected after digestion, you may try single and double digestions to verify the restriction enzyme or vector. Also, make sure you do not have either partially digested or undigested plasmids present, as they can grow on LB-ampicillin plates.

4. Add 1 µl FastAP thermo-alkaline phosphatase and incubate 30 min at 37°C.

5. Run samples from step 4 through PCR purification using PCR purification kit. Elute DNA in ~30 µl.

6. Measure the amount of DNA with a spectrophotometer such as a Nanodrop.

   NotI HF, SpeI HF, and SacII are all compatible with the CutSmart buffer, which makes digestion convenient because they all work in the same reaction buffer.

   2 µg digested DNA is usually enough for ~15 reactions. It is convenient to keep the prepared/digested plasmids at −20°C for a future reaction.

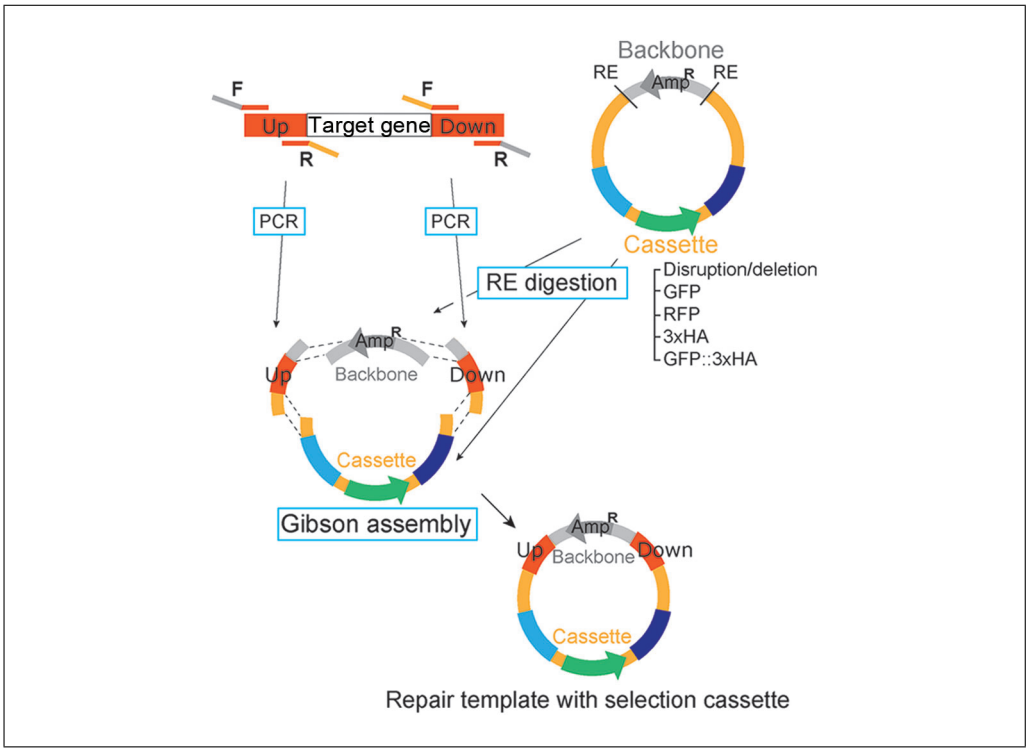

Figure 6 Schematic representation of the construction of the repair template with a selection cassette toolkit. Diagram illustrates the PCR amplification of sequences up- and downstream of the target gene (top left). Vector containing backbone and selection cassette (top right) is digested with restriction enzyme (RE) and stitched together with up- and downstream homology arms through a four-fragment Gibson assembly reaction (bottom). Note that five types of vectors (disruption/deletion, GFP, RFP, 3×HA, and GFP::3×HA) are available for generating disruption/deletion or tagging template. The cassette in the tagging vectors contains selection markers (GFP + neomycin) and DNA sequences for tagging, whereas the cassette in the disruption/deletion vector contains only selection markers. Color code denotes homology-containing DNA sequences.

Restriction enzyme digestion will release the selection cassette and backbone for all five vectors (Fig. 6). Cassette and backbone stay together in the same tube after digestion. Along with two homology arms, a total of four pieces (cassette + backbone + two homology arms) will be stitched together by the Gibson assembly reaction.

Preparation of homology arms: Designing primers for homology arm amplification

7. Find your primer sequences in Figure 5B. For example, for GFP tagging, you need homology sequences for a *loxP_myo2_neoR_GFP*_intron vector.

8. Design primers that bridge your target gene and cassette/backbone.

   As an example, for a *ZTF-8::GFP* tagging donor: the DN-F and DN-R primers will amplify 1011 bp downstream and the UP-F and UP-R primers will amplify 1083 bp upstream from the ZTF-8 gene. Sequences shown in lower case anneal to the *ztf-8* gene.

   UP-F primer: AACGACGGCCAGTGAATTCACTAGTcgtaaggttacatattaattc
   UP-R primer: GTTCTTCTCCTTTACTCATACTAGTccaACGACTgcctc gatcatctcgattg
   DN-F primer: TGGATGAACTATACAAAGCGGCCGCtaaagccgccatcaatatta
   DN-R primer: ATGATTACGCCAAGCTTGCGGCCGCcggtctcgatacgacaaat

   For tagging donors, mutate the PAM sequence on your donor to avoid having your template targeted by the sgRNA. You may want to consider introducing a silencing

mutation for an exon region when designing primers. However, for deletion donors, the sgRNA targeting sequence can be omitted in the donor template; therefore, this is not an issue in general.

The sgRNA can target both genomic and donor sequences when the donor sequence contains the target sequence. For disruption or deletion donors, target sequences can be omitted in the donor and this is not a problem. However, for tagging donors, you may need to mutate the targeting sequence of the donor template. As an example, we designed a silent mutation in the sgRNA target region of the donor DNA, which will be introduced by PCR with a partially complementary primer (UP-R). In general, mutating the PAM (NGG) is convenient; however, in the example, TGG (PAM) is the only codon that encodes W (tryptophan). Therefore, we introduced silent mutations in the S (serine) and R (arginine) codons with the primer ztf-8 UP-R (Fig. 5A).

Tags contain start codon but no stop codon, except for the 3×HA tag, which also lacks a start codon. When designing C-terminal tagging, make sure you have a stop codon after a tag. You can utilize the endogenous stop codon. Similarly, for N-terminal tagging, the start codon must be present in the final version.

PCR amplification of homology arms

9. Set up a PCR to amplify homology arms from genomic DNA prepared from a single-worm PCR (Basic Protocol 4) with primers designed from the previous section.

   A plasmid or fosmid harboring C. elegans genomic DNA can be an alternative template DNA for PCR. In general, 500-1000 bp of homology arm length is preferred.

10. Confirm that the PCR product contains a specific band. If not, perform a gel extraction to obtain a specific band.

    We suggest using a high-fidelity polymerase such as Phusion polymerase (NEB E0553S) or PrimeStar GXL polymerase (Takara R050B).

Setting up Gibson assembly reaction using up- and downstream homology arms with a vector backbone and a selection cassette

11. Set up assembly reaction and control in PCR tubes according to Table 4.

12. Incubate at 50°C for 1 hr in a PCR thermal cycler and transform into competent cells such as DH5α cells (NEB C2987I).

13. After transformation, screen the ampicillin-resistant clones that have both homology arms and selection cassette. Amplify homology arms from the bacterial colony PCR (or plasmid miniprep) using primers UP-F and UP-R and/or DN-F and DN-R. You are expecting clones that contain both up and down homology arms.

14. Validate candidate clones by Sanger DNA sequencing using M13F for upstream and M13R primer for downstream verification.

Table 4 Setting Up a Gibson Assembly Reaction

	Deletion template donor		Negative control	Positive control
1	Digested vector (backbone + cassette)	*x* µl (~100 ng)	*x* µl	10 µl (provided by NEB)
2	Upstream homology arm	*y* µl (~100 ng)	0 µl	0 µl
3	Downstream homology arm	*z* µl (~100 ng)	0 µl	0 µl
4	2× master assembly mix	10 µl	10 µl	10 µl
5	ddH_2O	$10 - x - y - z$ µl	$10 - x$ µl	0 µl
	Total volume	20 µl	20 µl	20 µl

A negative control is useful to validate the absence of self-ligation or incomplete digestion of the vector. On the other hand, a positive control is useful to ensure the quality of reagents provided by the vendor.

NEB HiFi works more efficiently than Gibson assembly (at the same cost) in our hands.

INJECTING ANIMALS

BASIC PROTOCOL 3

At 20-24 hr post L4 stage, young adults are injected with the CRISPR, Cas9, and if appropriate, repair-template DNA plasmids.

Materials

General-purpose agarose (Bioexpress, cat. no. E-3119-500BX, or equivalent)
N2 wild-type *C. elegans* worms for injection (*http://www.cgc.cbs.umn.edu/*)
Plasmids (good-quality DNA is required for efficient CRISPR-Cas9 genome editing; use a Qiagen Midiprep Kit or equivalent for plasmid extraction)
- Cas9 expression plasmid: P*eft-3::Cas9_SV40*_NLS::*tbb-2*_3′UTR (Addgene plasmid #46168)
- Repair template DNA vector for HR *OR* repair template containing selection cassette
- sgRNA expression vector *OR* p*U6*::sgRNA vector
- pCFJ90: P*myo-2::mCherry::unc-54*_3′UTR (Addgene plasmid #19327; for mCherry expression in pharynx muscle)
- pCFJ104: P*myo-3::mCherry::unc-54*_3′UTR (Addgene plasmid #19328; for mCherry expression in body wall muscle)
- pMA122 (Addgene plasmid #34873; for *peel-1* negative selection), optional)

Halocarbon oil 700 (Sigma H8898)
M9 buffer (see recipe)
Recovery solution: M9 with 4% glucose (can be stored 2–3 months at 4°C)
6-cm petri plates containing nematode growth medium (NGM)
E. coli OP50 for seeding NGM plates (*http://www.cgc.cbs.umn.edu/;* Stiernagle, 2006)

25°C incubator (Precision 815 or equivalent)
Microinjection apparatus
Microloader (Eppendorf 930001007)
Sutter P-97 needle puller or equivalent
24 × 40–mm and 22 × 22–mm glass coverslips (VWR 470145-746 and 48366-227 or equivalent)
Worm pick

Prepare agarose pads for microinjection

1. Prepare ~2% agarose in distilled water, melting it in a microwave. Line up three 22 × 40–mm coverslips, and then place a dime-sized (~18-mm-diameter) drop of melted agarose onto each coverslip and quickly place a glass slide on top of the drops to flatten the agarose. Wait 1 min, remove the glass slide, and allow coverslips with agarose pads to air dry overnight.

Microinjection

2. Pick L4 worms and incubate them at 20°C-25°C for ~24 hr. Plan to inject between 60 and 100 24-hr post-L4 worms for each target, although because the recombination rate relies on the quality of the injection, the number of worms that will need to be injected is difficult to predict.

   L4-stage worms can be obtained either by hand picking L4 worms or from a synchronized population from eggs obtained by bleaching of gravid hermaphrodites as described in Kim & Colaiacovo (2015a).

3a. *For marker-free method*: Prepare the injection mixture using the following concentrations and spin it down using a tabletop centrifuge for 4 min at ~18,000 × *g*, room temperature.

50-200 ng/µl Cas9 expression vector
50-200 ng/µl sgRNA expression vector
50 ng/µl repair template vector
2.5 ng/µl pCFJ90 injection marker
5 ng/µl pCFJ104 injection marker
10 ng/µl pMA122 negative selection plasmid (optional).

Centrifuging the injection mixture helps avoid clogging of the microinjection needle. Use injection mixture from the top of the tube to load your needle.

pMA122 (optional) is a negative selection marker to eliminate array-carrying worms.

3b. *For cassette selection method*: We suggest using similar components for injection when following the cassette selection method (Norris et al., 2015). Spin down the mixture using a tabletop centrifuge for 4 min at 18,000 × *g*, room temperature. For example:

50 ng/µl Cas9 expression vector
100 ng/µl sgRNA expression vector
50 ng/µl repair template containing selection cassette
2.5 ng/µl pCFJ90 (P*myo-2::mCherry*)
5 ng/µl pCFJ104 (P*myo-3::mCherry*).

Centrifuging the injection mixture helps avoid clogging of the microinjection needle. Use injection mixture from the top of the tube to load your needle.

4. Inject worms:

a. One day before microinjection: Pick 60-100 L4 worms (it takes 37 hr for worms to develop from eggs to L4 at 20°C).
b. On the day of microinjection: To load the microinjection needle, use an Eppendorf microloader tip and load 1-2 µl injection mix into a needle pulled with a Sutter P-97 needle puller or equivalent.
c. Place the needle into the needle holder of the microinjection module, and adjust needle alignment so that the needle is visible through the objective lenses.
d. To break the tip of the needle, prepare a sandwich with coverslips of two different sizes (22 × 22–mm and 22 × 40–mm coverslips or similar). Place the smaller coverslip on top of the larger one, and add a drop of halocarbon oil along the area where the two coverslips meet. Transfer the sandwiched coverslips onto the stage of the microinjection microscope. Gently move the needle to the edge of the smaller coverslip where the halocarbon oil was added and tap the edge with the needle tip. Make sure the tip of the needle has been successfully opened by pumping the injection mix through it.
e. Add a drop of halocarbon oil onto the agarose pad. Under the stereomicroscope, place 1-10 worms on the pad.
f. Transfer worms on agarose pad onto the microinjection microscope for injection.
g. Looking through the objectives, find the proper focal plane in which you can see the worm's gonad, and align the needle at a 30° angle parallel to the gonad. Lower the needle, and carefully move either the stage or the needle (using the micromanipulator) to inject the DNA mixture into the gonad. When the DNA mixture is injected, the gonad will briefly swell. Repeat this step until all worms on the agarose pad have been injected. Start with one or two worms the first time is recommended.

h. After injection, transfer the agarose pad back to the stereomicroscope and add 2-3 µl of recovery buffer to the injected worms. With a worm pick, gently rescue the injected worms and place two to four rescued worms onto an OP50-seeded NGM plate.

5. Rehydrate injected worms in M9 or recovery buffer and place on NGM plates (Stiernagle, 2006) seeded with OP50 (~3 per plate). Let worms recover at room temperature for 1 hr. Incubate worms at 25°C until screening (Basic Protocol 4).

If using pMA122 to eliminate array-carrying worms (see step 3a), this requires an additional 2 hr heat shock at 34°C (Frokjaer-Jensen, Davis, Ailion, & Jorgensen, 2012).

For the cassette selection method, aim to inject 30 or more animals to obtain hundreds of transgenic mCherry-glowing F_1 progeny. Add the G418 solution to the worms 1 day after the injection as described in Alternate Protocol 3.

SCREENING TRANSGENIC WORMS WITH MARKER-FREE METHOD

BASIC PROTOCOL 4

This section describes screening for the marker-free method. You can start screening for mCherry-expressing F_1 worms ~3 days after injection. Note that the pCFJ90 marker is expressed in the pharynx muscle and the pCFJ104 marker in the body wall muscle. Some *C. elegans* mutants may grow slower than wild-type N2 animals and may exhibit a developmental delay. Therefore, if injecting such mutants, the screening period may need to be extended.

Materials

Injected worms for analysis (Basic Protocol 3)
6-cm petri plates containing nematode growth medium (NGM)
E. coli OP50 for seeding NGM plates (*http://www.cgc.cbs.umn.edu/;* Stiernagle, 2006)
Worm lysis buffer (see recipe)
10 mM 4dNTP mix (10 mM each dNTP)
Primers
High Fidelity Phusion DNA polymerase (NEB M0530S or equivalent) and 5× HF buffer
N2 *C. elegans* wild-type worms for injection (*http://www.cgc.cbs.umn.edu/*)

Fluorescence stereomicroscope
PCR tubes
Heat block (VWR Scientific Standard Heat Block or equivalent)
25°C incubator
Deep freezer (Thermo ULT2580 or equivalent)
PCR thermal cycler (Bio-Rad T100 or equivalent)

Additional reagents and equipment for PCR (Kramer and Coen, 2001) and DNA sequencing (see Shendure et al., 2011)

Isolation of single mCherry-expressing F_1 worms

1. Pick mCherry-expressing F_1 worms using a fluorescence stereomicroscope and "single" them onto OP50-seeded NGM plates.

In general, you can expect to obtain 80-300 mCherry$^+$ worms after injecting 60-100 worms.

Genotyping of potential candidates

Restriction-fragment-length polymorphisms (RFLPs) are useful when screening for changes in only a few nucleotides such as those in InDel or point mutations driven

by HR. InDels or point mutations at the site normally recognized by a restriction enzyme prevent digestion at that position by the restriction enzyme, and this can be useful for screening purposes. Alternatively, when a restriction enzyme site is not available, worms carrying fluorescent markers can be used for PCR analysis followed by Sanger sequencing. Although this can be a laborious process, it is still achievable using 96-well plates and can be further shortened by pooling DNA samples for PCR, as shown in Paix et al. (2014). Alternatively, changes as small as 5 nt can be detected by PCR using 15% polyacrylamide gels without the need of additional restriction enzyme digestion or Sanger sequencing (Kim et al., 2014).

Regular PCR combined with agarose gel analysis allows for effective screening of transgenic lines when the insertion or deletion is >10 bp in size. To avoid amplifying from the repair template DNA in the case of HR, using primers located outside the repair template sequence region is recommended. Alternatively, if the region is too large to PCR amplify, it is possible to amplify the junction of the repair template and flanking sequence.

The screening for transgenic worms can be further facilitated if, as a result of the genomic editing, they now exhibit phenotypes that can be easily identified, such as GFP or mCherry expression in a specific tissue or a Dpy or Unc phenotype on plates.

2. Incubate singled mCherry-expressing F_1 worms at 25°C for 1-2 days and sacrifice for single-worm PCR genotyping as described below.

   Protocol adapted from He (2011) and Williams, Schrank, Huynh, Shownkeen, & Waterston (1992).

Worm lysis

3. Transfer a single worm directly from a plate to 5 µl lysis buffer in a PCR tube.
4. Spin capped PCR tubes briefly to bring worm down to the bottom of the tube.
5. Freeze at −80°C for 10 min or longer (up to a week).
6. Heat sample at 60°C for 1 hr, then at 95°C for 15 min to inactivate protease K. Store the worm lysate at −80°C if needed.

Single-worm PCR

7. Prepare the following PCR mix:

   1-2 µl worm lysate
   2 µl 10 mM 4dNTP mix
   2 µl 10 µM each forward and reverse primer located outside the repair template
   10 µl 5× HF buffer
   0.5 µl Phusion polymerase
   ddH_2O to 50 µl final.

8. Run reactions in a thermal cycler as described in the manufacturer's instructions for NEB Phusion High-Fidelity DNA Polymerase.
9. Once F_1 screening is completed, it is advisable to re-genotype the progeny (F_2 or F_3 worms) from the potential candidates to eliminate possible false positives and nonheritable mitotic mutants (Arribere et al., 2014; Farboud & Meyer, 2015). Using a fluorescence scope, confirm that the F_2 and/or F_3 worms have lost the mCherry extrachromosomal signal. pMA122 can also be used for negatively selecting arrays with an additional 2 hr heat-shock at 34°C (Frokjaer-Jensen et al., 2012).

10. Order Sanger sequencing analysis (see Chapter 7) to confirm mutations.

11. To verify an anticipated genome alteration as well as relevant protein expression, further analysis can be performed, such as immunofluorescence staining, western or northern blotting, or quantitative real-time PCR. A tagged protein's localization can be assessed by comparing it to the localization observed using protein-specific antibodies if these are available, as described by Kim & Colaiacovo (2015b).

12. It is recommended that the derived transgenic/edited lines be outcrossed several times to eliminate potential off-target mutations, although there are yet no reports of such events resulting from CRISPR-Cas9 genomic editing in *C. elegans* (Dickinson et al., 2013; Friedland et al., 2013).

SCREENING TRANSGENIC WORMS WITH CASSETTE SELECTION METHOD

ALTERNATE PROTOCOL 3

This section describes how to identify transgenic worms through a cassette selection method after microinjection.

Materials

Transgenic worms (Basic Protocol 3)
25 mg/ml G418 (Sigma G418-RO)
pDD104: P*eft-3::Cre* (Addgene plasmid #47551)
pCFJ90: P*myo-2::mCherry::unc-54*_3′UTR (Addgene plasmid #19327)
6-cm petri plates containing nematode growth medium (NGM)
E. coli OP50 for seeding NGM plates (*http://www.cgc.cbs.umn.edu/;* Stiernagle, 2006)

Fluorescence stereomicroscope
25°C incubators
Microinjection apparatus

1 day after worm injection (in Basic Protocol 3)

1. Add 500 µl 25 mg/ml G418 to each plate of worms. Swirl G418 solution over the surface of each plate to cover the entire plate.

2. Open the lid of each plate and incubate until G148 is soaked in, which may take 30-90 min. Once the agar is dry, return lids to plates and continue incubation at 25°C for the next week to 10 days.

4-6 days after injection

3. Screen for CRISPR-integrant animals, which usually have dim GFP pharyngeal expression, are completely uniformly fluorescent, and lack the mCherry co-injection marker expression. In contrast, animals bearing extrachromosomal arrays will have mosaic, very bright GFP expression in the pharynx and will have one or both of the mCherry-expressing markers. Single six to eight candidate worms on NGM plates seeded with OP50. You should expect three-quarters of progeny for heterozygous or 100% of the progeny for homozygous with pharyngeal GFP expression.

   (1) Some transgenic worms appear late such as days 7-11 after injection, although most appear at 4-6 days. (2) G418 treatment will not lead to perfect selection but kills the majority of non-transgenic worms. (3) In most cases, bright GFP$^+$ worms are not integrants. However, in rare cases, you may get bright GFP$^+$ integrants that still lack the mCherry markers and exhibit uniformly bright pharyngeal GFP.

4. Homozygous animals containing the selection cassette can be treated to excise the cassette by microinjecting Cre recombinase (Dickinson et al., 2013). You can screen

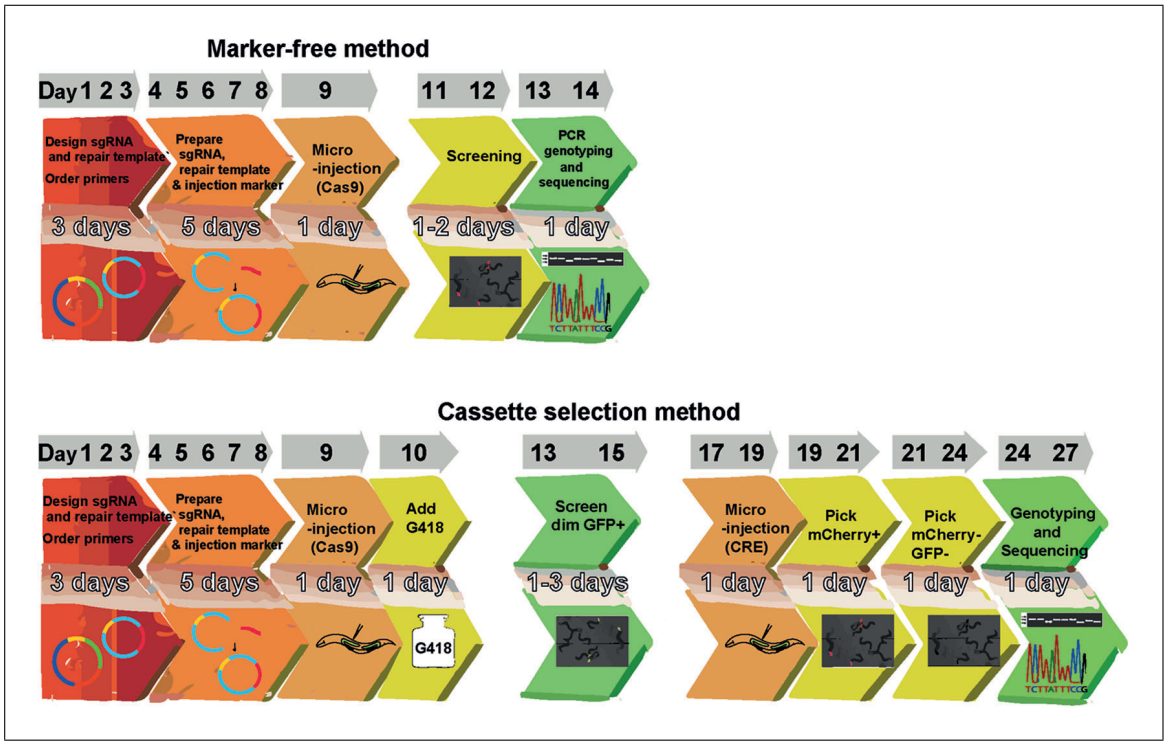

Figure 7 Workflow and timeline for CRISPR-Cas9-guided genome editing in *C. elegans*. The expected timelines (14 days and 27 days for completion for the marker-free and cassette selection methods, respectively) assume that each step is not delayed. See Table 2 for details.

Table 5 Troubleshooting Common Problems Associated With CRISPR-Cas9 Genome Editing

Problem	Potential causes	Solutions
Low yield of mCherry-expressing F_1 worms	Purity of DNA mixture was low	Use Plasmid Midiprep Kit
	Cas9 and/or sgRNA concentrations were either too low or too high	Adjust Cas9 and/or sgRNA concentration
	Occurs when injecting mutant worms which exhibit a developmental delay	Screening period needs to be extended as worms grow slower
High embryonic lethality among F_1 worms	Cas9 and/or sgRNA caused toxicity to the worms	Reduce the concentration of Cas9 and/or sgRNA
High larval lethality among F_1 worms	Cas9 and/or sgRNA caused toxicity to the worms	Reduce the concentration of Cas9 and/or sgRNA
Low genome-targeting efficiency	gRNA targeting efficiency varies significantly	Design and test multiple sgRNAs

for loss of GFP expression in the pharynxes of F_2 animals to isolate homozygous animals that have lost the selection cassette.

If you are working with essential genes and Cre-mediated excision is required, it is recommended that animals bearing insertions be crossed 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. If you are deleting an essential gene, you may want to balance your worms before Cre injection because the homozygous deletion can be lethal.

Table 6 Workflow and Time Considerations for CRISPR-Cas9 Protocols Corresponding to Figure 7

Step	Days	Duration	Actions	Stopping point?
Marker-free method				
1	Day 1-3	3 days	Design sgRNA, repair template (HR); order oligonucleotides.	Yes
2	Day 4-8	5 days	Prepare sgRNA, repair template (HR), injection markers.	Yes
3	Day 9	1 day	Microinject CRISPR-Cas9 DNA mix into young adult worms.	No
4	Day 11-13	1-2 days	Screen the injection marker-expressing ($mCherry^+$) F_1 worms.	No
5	Day 13-14	1 day	Perform PCR genotyping of F_1 worms.	No
6	Day 15-16	1 day	Re-genotype and/or re-sequence F_2 worms.	Yes
Cassette selection method (alternative method)				
1	Day 1-3	3 days	Design sgRNA, repair template (HR); order oligonucleotides.	Yes
2	Day 4-8	5 days	Prepare sgRNA, repair template (HR), injection markers.	Yes
3	Day 9	1 day	Microinject CRISPR-Cas9 DNA mix into young adult worms.	No
4	Day 10	1 day	Add G418 to each plate. Swirl G418 solution.	No
5	Day 13-15	1-3 days	Screen for dim pharyngeal GFP expression (GFP^+, $mCherry^-$).	No
6	Day 17-19	1 day	Microinject CRE recombinase.	No
7	Day 19-21	1 day	Pick $mCherry^+$ worms.	No
8	Day 21-24	1 day	Screen for $mCherry^-$, pharyngeal GFP^- worms.	No

On the day of Cre recombinase injection

5. Set up injection mix:

   50 ng/µl pDD104
   2.5 ng/µl pCFJ90.

6. Inject ~10-15 animals.

7. After injection, place three or four injected worms each on separate 6-cm NGM plates. Let them recover 1 hr at room temperature, and then incubate at 25°C.

2 days after Cre injection

8. Pick 25-30 F_1 progeny displaying an $mCherry^+$ phenotype. Transfer four or five worms per plate and return for incubation at 25°C.

4-5 days after Cre injection

9. Screen F_2 progeny for worms that have completely lost both pharyngeal GFP and mCherry expression. These animals should now be homozygous for the excised dual marker cassette. Pick and single candidates for further applications.

7-8 days after Cre injection

10. Use GFP^- $mCherry^-$ worms for genotyping and/or Sanger DNA sequencing.

REAGENTS AND SOLUTIONS

NOTE: Use deionized, distilled water in all recipes and protocol steps. Use molecular biology-grade nuclease-free water for PCR, ligation, and Gibson assembly. See Moore (1996) for common stock solutions.

M9 buffer

3 g KH_2PO_4
6 g Na_2HPO_4
5 g NaCl
1 ml 1 M $MgSO_4$
H_2O to 1 L.
Sterilize by autoclaving.

Worm lysis buffer

50 mM KCl
10 mM Tris·Cl, pH 8.3
2.5 mM $MgCl_2$
0.45% Nonidet P-40
0.045% Tween-20
0.01% (w/v) gelatin

Autoclave and store up to 6 months at 4°C. Right before use, add proteinase K to the lysis buffer to 60 µg/ml final.

COMMENTARY

Background Information

Engineering precise modifications of endogenous genomes has long been desired, and different technologies including zinc-finger nucleases and transcription-activator-like effector nucleases have been developed for this purpose in the past. Recently, the type II CRISPR-Cas9 system has been shown to be the most proficient and adaptable system to create desired genome modifications.

Previous studies using the *S. pyogenes* type II CRISPR system, which requires the Cas9 nuclease, a targeting crRNA, and an additional *trans*-activating trRNA, have shown that a fusion of the targeting and *trans*-activating RNAs to form an sgRNA is sufficient to direct Cas9-mediated target cleavage (Jinek et al., 2012). This strategy has been used in *C. elegans* (Friedland et al., 2013; Tzur et al., 2013) and provides a convenient approach for generating mutants via a marker-free strategy. In this protocol, we describe both marker-free and marker-based (drug and fluorescent selection) protocols using the *S. pyogenes* Cas9 in *C. elegans* to create heritable genome modifications via the NHEJ or HR pathways.

Critical Parameters

The protocols for marker-free strategies described in this article stem from several previous studies in our lab (Friedland et al., 2013; Kim & Colaiacovo, 2015b; Kim, Beese-Sims, & Colaiacovo, 2018; Tzur et al., 2013). Due to the absence of selective markers, these protocols rely on relatively time-consuming screening procedures to identify animals with the desired modification using PCR techniques as compared to marker-dependent Unc, Rol, or drug-selection protocols (Arribere et al., 2014; Dickinson et al., 2015; Dickinson et al., 2013; Kim et al., 2014). However, in contrast, the procedure presented in this article is straightforward with respect to the design of repair templates, and it progresses very quickly from microinjection to screening because it requires only one round of injection to obtain the final product of genome editing. In contrast, the marker-based cassette selection method facilitates the step of screening for transgenic animals due to the presence of both drug and fluorescent selection markers. However, it is a relatively longer method than the marker-free

approach (Fig. 7). Cassette selection can be a useful alternative because it provides various vectors for endogenous gene tagging.

Troubleshooting

Table 5 describes common problems encountered with the protocols described in this article together with accompanying solutions.

Understanding Results

The efficiency of genome editing varies for different targeting sites. We observed 1.3% to 16.7% genome targeting efficiency for HR, with injection of 7 to 13 worms producing 24 to 72 mCherry-expressing F_1 worms (Tzur et al., 2013). A 0.5% to 80.3% gene-disruption frequency was reported for InDels from four different targeting loci (Friedland et al., 2013). Frequencies of 5% to 25% were reported for disruptions, deletions, or insertions at six different loci using the cassette selection method (Norris et al., 2015).

Time Considerations

See Table 6 and Figure 7 for a description of the time required for each step of the protocols described in this article.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Literature Cited

Arribere, J. A., Bell, R. T., Fu, B. X., Artiles, K. L., Hartman, P. S., & Fire, A. Z. (2014). Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. *Genetics*, *198*(3), 837–846. doi: 10.1534/genetics.114.169730.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., ... Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, *315*(5819), 1709–1712. doi: 10.1126/science.1138140.

Bell, R. T., Fu, B. X., & Fire, A. Z. (2016). Cas9 variants expand the target repertoire in *Caenorhabditis elegans*. *Genetics*, *202*(2), 381–388. doi: 10.1534/genetics.115.185041.

Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., ... Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, *471*(7340), 602–607. doi: 10.1038/nature09886.

Dickinson, D. J., Pani, A. M., Heppert, J. K., Higgins, C. D., & Goldstein, B. (2015). Streamlined genome engineering with a self-excising drug selection cassette. *Genetics*, *200*(4), 1035–1049. doi: 10.1534/genetics.115.178335.

Dickinson, D. J., Ward, J. D., Reiner, D. J., & Goldstein, B. (2013). Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nature Methods*, *10*(10), 1028–1034. doi: 10.1038/nmeth.2641.

Elbing, K., & Brent, R. (2002). Media preparation and bacteriological tools. *Current Protocols in Molecular Biology*, *59*(1), 1.1.1–1.1.7. doi: 10.1002/0471142727.mb0101s59.

Farboud, B., & Meyer, B. J. (2015). Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. *Genetics*, *199*(4), 959–971. doi: 10.1534/genetics.115.175166.

Friedland, A. E., Tzur, Y. B., Esvelt, K. M., Colaiacovo, M. P., Church, G. M., & Calarco, J. A. (2013). Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nature Methods*, *10*(8), 741–743. doi: 10.1038/nmeth.2532.

Frokjaer-Jensen, C., Davis, M. W., Ailion, M., & Jorgensen, E. M. (2012). Improved Mos1-mediated transgenesis in *C. elegans*. *Nature Methods*, *9*(2), 117–118. doi: 10.1038/nmeth.1865.

Garneau, J. E., Dupuis, M. E., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., ... Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, *468*(7320), 67–71. doi: 10.1038/nature09523.

He, F. (2011). Single worm PCR. *Bio-Protocol, Bio101*, *E60*. Retrieved from *Bio-Protocol*, *http://www.bio-protocol.org/e60*. doi: 10.21769/BioProtoc.60.

Horvath, P., & Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. *Science*, *327*(5962), 167–170. doi: 10.1126/science.1179555.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816–821. doi: 10.1126/science.1225829.

Kim, H., Ishidate, T., Ghanta, K. S., Seth, M., Conte, D., Jr., Shirayama, M., & Mello, C. C. (2014). A co-CRISPR strategy for efficient genome editing in *Caenorhabditis elegans*. *Genetics*, *197*(4), 1069–1080. doi: 10.1534/genetics.114.166389.

Kim, H. M., Beese-Sims, S. E., & Colaiacovo, M. P. (2018). Fanconi anemia FANCM/FNCM-1 and FANCD2/FCD-2 are required for maintaining histone methylation levels and interact with the histone demethylase LSD1/SPR-5 in *Caenorhabditis elegans*. *Genetics*, *209*(2), 409–423. doi: 10.1534/genetics.118.300823.

Kim, H. M., & Colaiacovo, M. P. (2015a). DNA damage sensitivity assays in *Caenorhabditis elegans*. *Bio-Protocol*, *5*(11), e1487.

Kim, H. M., & Colaiacovo, M. P. (2015b). New insights into the post-translational regulation of DNA damage response and double-strand break repair in *Caenorhabditis elegans*. *Genetics*, *200*(2), 495–504. doi: 10.1534/genetics.115.175661.

Kramer, M. F., & Coen, D. M. (2001). Enzymatic amplification of DNA by PCR: Standard procedures and optimization. *Current Protocols in Molecular Biology*, 15.1.1–15.1.14. doi: 10.1002/0471142727.mb1501s56.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., . . . Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*, *339*(6121), 823–826. doi: 10.1126/science.1232033.

Moore, D. D. (1996). Commonly used reagents and equipment. *Current Protocols in Molecular Biology*, *35*(1), A.2.1–A.2.8. doi: 10.1002/0471142727.mba02s35.

Norris, A. D., Kim, H. M., Colaiacovo, M. P., & Calarco, J. A. (2015). Efficient genome editing in *Caenorhabditis elegans* with a toolkit of dual-marker selection cassettes. *Genetics*, *201*(2), 449–458. doi: 10.1534/genetics.115.180679.

Paix, A., Wang, Y., Smith, H. E., Lee, C. Y., Calidas, D., Lu, T., . . . Seydoux, G. (2014). Scalable and versatile genome editing using linear DNAs with microhomology to Cas9 Sites in *Caenorhabditis elegans*. *Genetics*, *198*(4), 1347–1356. doi: 10.1534/genetics.114.170423.

Seidman, C., Struhl, K., Sheen, J., & Jessen, T. (1997). Introduction of plasmid DNA into cells. *Current Protocols in Molecular Biology*, *36*(1), 1.8.1–1.8.10. doi: 10.1002/0471142727.mb0108s37.

Shendure, J. A., Porreca, G. J., Church, G. M., Gardner, A. F., Hendrickson, C. L., Kieleczawa, J., & Slatko, B. E. (2011). Overview of DNA sequencing strategies. *Current Protocols in Molecular Biology*, *96*(1), 7.1.1–7.1.23. doi: 10.1002/0471142727.mb0701s96.

Slatko, B. E., Kieleczawa, J., Ju, J., Gardner, A. F., Hendrickson, C. L., & Ausubel, F. M. (2011). "First generation" automated DNA sequencing technology. *Current Protocols in Molecular Biology*, *96*(1), 7.2.1–7.2.28. doi: 10.1002/0471142727.mb0702s96.

Stiernagle, T. (2006). Maintenance of *C. elegans*. Retrieved from *WormBook* (Ed. The C. *elegans* Research Community). http://wormbook.org/chapters/www_strainmaintain/strainmaintain.html.

Tzur, Y. B., Friedland, A. E., Nadarajan, S., Church, G. M., Calarco, J. A., & Colaiacovo, M. P. (2013). Heritable custom genomic modifications in *Caenorhabditis elegans* via a CRISPR-Cas9 system. *Genetics*, *195*(3), 1181–1185. doi: 10.1534/genetics.113.156075.

Voytas, D. (2000). Agarose gel electrophoresis. *Current Protocols in Molecular Biology*, *51*(1), 2.5A.1–2.5A.9. doi: 10.1002/0471142727.mb0205as51.

Ward, J. D. (2015). Rapid and precise engineering of the *Caenorhabditis elegans* genome with lethal mutation co-conversion and inactivation of NHEJ repair. *Genetics*, *199*(2), 363–377. doi: 10.1534/genetics.114.172361.

Williams, B. D., Schrank, B., Huynh, C., Shownkeen, R., & Waterston, R. H. (1992). A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics*, *131*(3), 609–624.

Zhao, P., Zhang, Z., Ke, H., Yue, Y., & Xue, D. (2014). Oligonucleotide-based targeted gene editing in *C. elegans* via the CRISPR/Cas9 system. *Cell Research*, *24*(2), 247–250. doi: 10.1038/cr.2014.9.