

CRISPR-Cas9-Guided Genome Engineering in *C. elegans*

UNIT 31.7

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The CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated) system is successfully being used for efficient and targeted genome editing in various organisms, including the nematode *C. elegans*. Recent studies have developed various CRISPR-Cas9 approaches to enhance genome engineering via two major DNA double-strand break repair pathways: non-homologous 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, as well as injection methods required for delivering Cas9, sgRNAs, and repair template DNA into the *C. elegans* germline. © 2016 by John Wiley & Sons, Inc.

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INTRODUCTION

Overview of the CRISPR-Cas9 System and Function

The CRISPR-Cas system is primarily a 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 (clustered regularly interspaced short palindromic repeats), as well as genes encoding CRISPR-associated (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 et al., 2010; Horvath and Barrangou, 2010; Mali et al., 2013). In brief, this system consists of two non-coding RNAs, crRNA and trRNA, which are transcribed from the CRISPR locus. The crRNA, or CRISPR-targeting RNA, consists of a 20-nucleotide 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 which is later cleaved by RNase III to form a crRNA-trRNA hybrid, thereby directing the Cas9 RGN to make a double-stranded break (DSB) at the target site as long as the target is directly 5' to a so-called protospacer adjacent motif (PAM) with the sequence NGG (Deltcheva et al., 2011). The DSB is within ~3 bases of the target site's PAM. The CRISPR locus itself is not cleaved by the RGN because it does not contain any NGG sequences (Fig. 31.7.1).

The *S. pyogenes* CRISPR-Cas9 system has been utilized for genetic engineering because the *S. pyogenes* crRNA and trRNA are functional when fused as a single RNA molecule



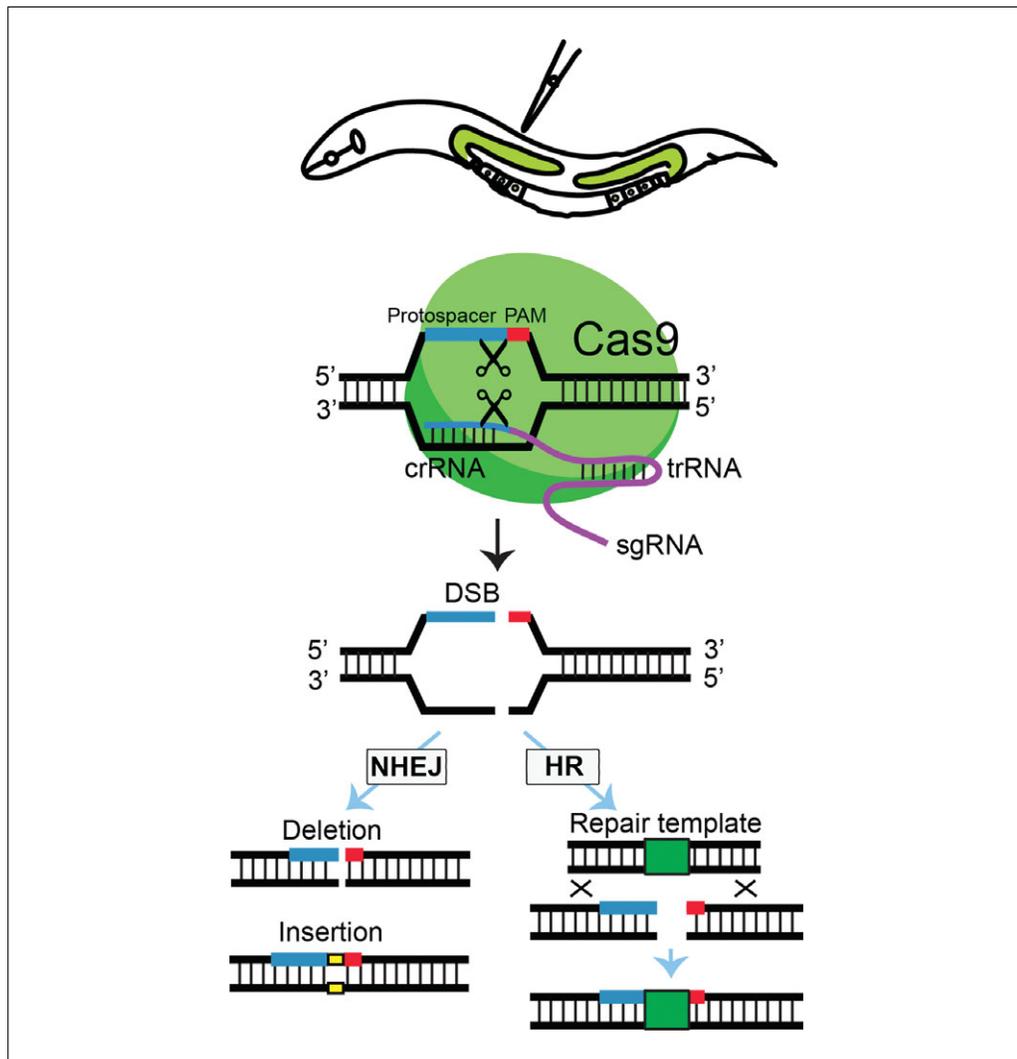


Figure 31.7.1 Schematic representation of the CRISPR-Cas9 genome editing approach in *C. elegans*. Young adult hermaphrodites are injected with the CRISPR-Cas9-containing DNA mixture. A DSB generated by Cas9 is repaired via error-prone NHEJ or error-free HR. The yellow box represents small nucleotide insertion and the green box represents insertion of the GFP tag.

[referred to as a single guide RNA (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 N₂₀-NGG by engineering an sgRNA molecule in which the first 20 nucleotides correspond to a 20-nucleotide target sequence directly 5' of an NGG (PAM) sequence.

Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR)

DNA double-strand breaks (DSBs) induced by the Cas9 RGN at the target site can be repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) (Fig. 31.7.1). In the absence of a repair template, DSBs introduced by CRISPR-Cas9 are repaired by NHEJ, which results in small insertions and/or deletions (InDels) at the targeted site (Fig. 31.7.1). In the generation of InDels, nucleotides are randomly inserted and/or deleted, and this can result in the early termination of a protein either due to sequence alteration or a 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.

Table 31.7.1 Types of CRISPR-Cas9 Methods Developed in *C. elegans*

Selection marker	References
Marker-free	Single-CRISPR (Friedland et al., 2013; Tzur et al., 2013; Paix et al., 2014) Co-CRISPR (Arribere et al., 2014; Kim et al., 2014; Ward, 2015)
Marker-dependent	<i>unc-119(+)</i> (Dickinson et al., 2013) Drug resistance (Dickinson et al., 2013, 2015; Norris et al., 2015a, 2015b)

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 to the target site, which can be used for DSB repair (Fig. 31.7.1).

Various approaches have been developed by several laboratories to engineer the nematode genome, and they can be divided into two major categories based on their dependency on a phenotypic marker that probes/marks the edited genome sequence (Table 31.7.1). Here, we describe a simple and reproducible marker-free protocol using *S. pyogenes* Cas9 in *C. elegans* to create heritable genome modifications via either the NHEJ or HR pathways. The overall protocol, which is broken down into four separate basic protocols, involves (1) generating the sgRNA (Basic Protocol 1), (2) generating the repair template DNA if homologous recombination is going to be employed to specifically modify a particular gene (Basic Protocol 2), (3) introducing the *cas9* gene, sgRNA, and repair DNA templates into the *C. elegans* animals on separate plasmids (Basic Protocol 3), and (4) screening for transgenic worms carrying the CRISPR-Cas9-mediated gene editing event(s) (Basic Protocol 4). Other published methods utilize a single plasmid expressing both the *cas9* gene and the sgRNA (Dickinson et al., 2013).

GUIDE RNA PREPARATION

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

Materials

sgRNA_top: 5'-ATTGCAAATCTAAATGTTT N19/N20
GTTTTAGAGCTAGAAATAGC-3' (synthesized by DNA synthesis service)

sgRNA_bottom: 5'-GCTATTTCTAGCTCTAAAAC N19/N20 reverse complement
AAACATTTAGATTTGCAAT-3' (synthesized by DNA synthesis service)

pHKMC1 empty sgRNA (Addgene, cat. no. 67720)
Plasmid miniprep kit (GeneJet, cat. no. K0502 or Qiagen, cat. no. 27104)
Nuclease-free water (Qiagen, cat. no. 129114, or equivalent)
*Bam*HI (NEB R0136S)
*Not*I (NEB R0189S)
Gel DNA Extraction Kit (Zymoclean, cat. no. D4001)
2× Gibson assembly Master Mix (NEB, cat. no. E2611S)
Chemically competent *E. coli* cells (NEB, cat. no. C2987I, or equivalent)
LB plates containing 100 μg/ml ampicillin
M13F: 5'-GTAAAACGACGGCCAGT-3'
M13R: 5'-AACAGCTATGACCATG-3'

PCR thermal cycler (BioRad T100 or equivalent)
Heat block (VWR Scientific standard heat block, or equivalent)

BASIC PROTOCOL 1

Genome Editing

31.7.3

Sterile pipet tips or toothpicks for picking colonies
PCR thermal cycler (BioRad T100 or equivalent)
Sequence analysis software (e.g., NCBI BLAST, UCSC Genome Browser, BLAT, LaserGene)

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000), transformation of *E. coli* (*UNIT 1.8*; Seidman et al., 1997), and DNA sequencing (Chapter 7)

Identify a sgRNA targeting sequence

1. To construct an appropriate sgRNA (single guide RNA), first identify a targeting sequence within ~70 bp of the intended genomic target site in the form 5'-N19/20-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 of generating off-target mutations, use software such as NCBI BLAST or search engines such as <http://crispr.mit.edu/> 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.

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 Gibson assembly.

2. Order both top (forward) and bottom (reverse complement) oligos containing 19 to 20 nucleotides of homology (N19/N20) to the target sequence from a DNA synthesis service (Fig. 31.7.2A). When N1 ≠ G, add one additional 'G' in front of N1 to ensure expression from the U6 promoter.

*The following are the generic sgRNA sequences (in capital letters) as well as an example of the sgRNA sequence design (in lowercase) adopted for C-terminal tagging of the *C. elegans ztf-8* gene with GFP (Kim and Colaiacovo, 2015b).*

Generic sgRNA sequences:

sgRNA_Top: 5'-ATTGCAAATCTAAATGTTT N19/N20 GTTTTAGAGCTAGAAATAGC-3'

sgRNA_Bottom: 5'-GCTATTTCTAGCTCTAAAAC N19/N20 reverse complement AAACATTTAGATTTGCAAT-3'

sgRNA adopted for C-terminal GFP tagging (Kim and Colaiacovo, 2015b)

ztf-8_Top: 5'-ATTGCAAATCTAAATGTTT *gagatgatcgaggctctcga* GTTTTAGAGCTAG AAATAGC-3'

ztf-8_Bottom: 5'-GCTATTTCTAGCTCTAAAAC *tcgagagcctcgatcatctc* AAACATTTAGA TTTGCAAT-3'

A recent study showed that the presence of GG at positions N19 and 20 on the target sequence enhances the efficiency of recombination (Farboud and Meyer, 2015).

3. Perform a miniprep of an empty sgRNA vector (3482 bp, Addgene cat. no. 67720) that contains the U6 promoter and sgRNA scaffold sequence as illustrated in Figure 31.7.2A. Digest 1 to 2 μg of empty sgRNA vector with *Bam*HI and *Not*I. Perform agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000). Extract the 3470-bp band from the gel using the Gel DNA Extraction Kit.

This will be sufficient for ~10 reactions.

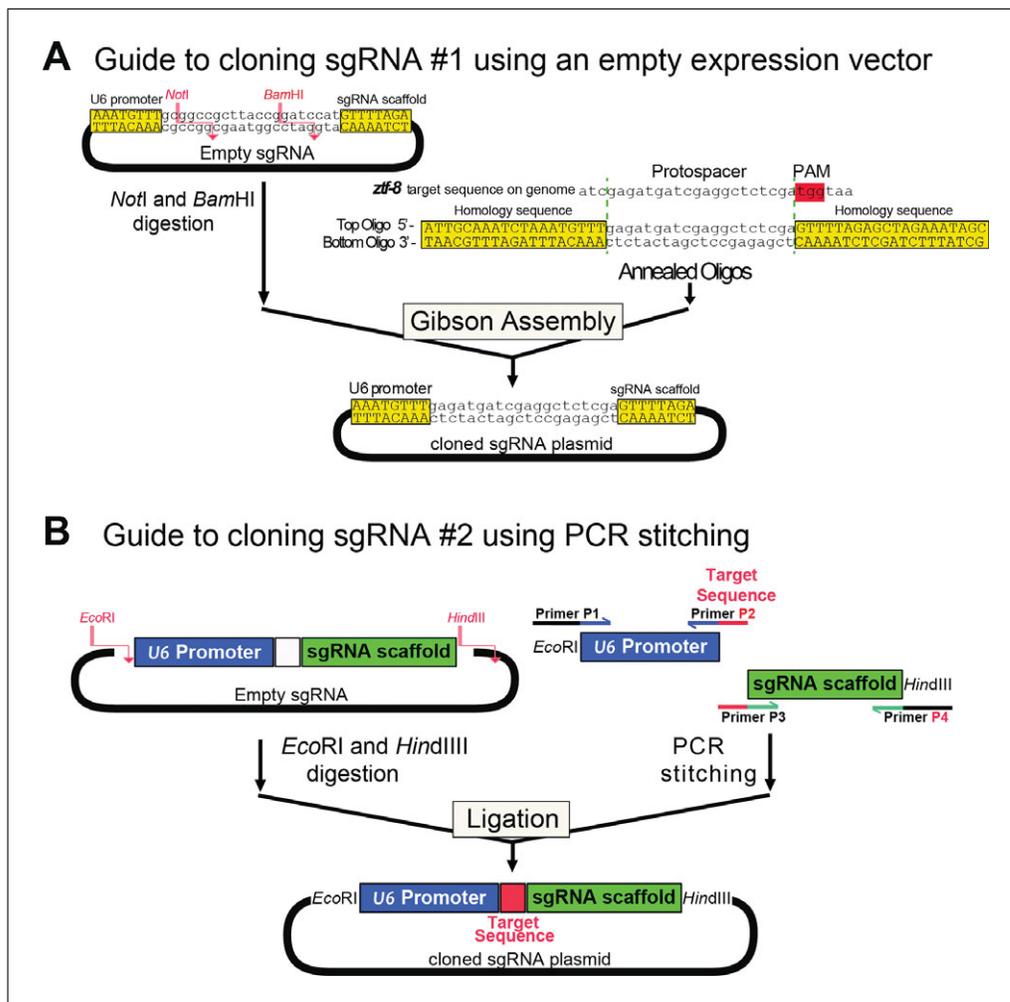


Figure 31.7.2 Schematic representation of sgRNA cloning by two alternative methods. **(A)** sgRNA cloning using an empty sgRNA expression vector. The sgRNA comprising a pair of annealed oligos specifically targeting *ztf-8* is cloned into the *NotI*- and *Bam*HI-digested empty sgRNA vector (left) using Gibson assembly. **(B)** sgRNA cloning using fusion PCR. To generate an sgRNA containing the *Eco*RI-*Hind*III fragment, two amplicons are stitched by PCR (right). Both the fused PCR fragment and the empty sgRNA vector are digested with *Eco*RI and *Hind*III and ligated to create the sgRNA expression vector.

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

Heat to 95°C for ~2 min

Slowly ramp down to 25°C over ~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.

- Set up the Gibson assembly reaction:

5 μ l of annealed oligonucleotides (from step 3)

~100 ng *Bam*HI- and *Not*I-digested empty sgRNA (from step 2)

2 \times Gibson assembly mix to one-half the total volume.

Incubate the reaction in a thermal cycler at 50°C for ~60 min.

6. Use 1 to 2 μ l for bacterial transformation of chemically competent *E. coli* (UNIT 1.8; Seidman et al., 1997). Spread the transformants onto LB plates containing 100 μ g/ml ampicillin, and incubate overnight.
7. The following day, pick and inoculate \sim 10 ampicillin-resistant colonies from plate containing bacteria, purify plasmids, and screen for insertion of sgRNA by restriction digest analysis.

For colony PCR screening, M13F and M13R primers will amplify \sim 890 bp from the empty vector; and this PCR product can be used for sequencing and/or be further analyzed with a BamHI or NotI digestion. The plasmid containing the sgRNA insert can be distinguished from either the undigested or self-ligated vector lacking an insert via digestion with either of these two restriction enzymes, since an undigested or self-ligated vector will produce both 530-bp and 360-bp products. 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 (see Chapter 7) using the primers M13R and M13F.

ALTERNATE PROTOCOL

sgRNA CLONING USING FUSION PCR (ALTERNATIVE METHOD)

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)

P1: 5'-CGGGAATTCCTCCAAGAAGCTCGTACAAAAATGCTCT-3' (synthesized by DNA synthesis service)

P2: 5'-(N19/20-RC) + AACATTTAGATTTGCAATTCAATTATATAG-3' (where N19/20-RC is the reverse complementary sequence of the N19/20 target sequence used in primer P3; synthesized by DNA synthesis service)

P3: 5'-(N19/20) + GTTTGTAGAGCTAGAAATAGCAAGTTA-3' (where N19/20 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 \times HF buffer

10 mM dNTP mix (10 mM each dNTP)

PCR purification kit

*Eco*RI (NEB, cat. no. R0101S)

*Hind*III (NEB, cat. no. R0104S)

T4 DNA ligase (NEB, cat. no. M0202S) and 10 \times buffer

Additional reagents and equipment for PCR (UNIT 15.1; Kramer and Coen, 2000) and agarose gel electrophoresis (UNIT 2.5A; Voytas, 2000)

1. Design primers to amplify upstream (P1 + P2; PCR-up) and downstream (P3 + P4; PCR-down) of the empty sgRNA vector (Fig. 31.7.2B). Primers P2 and P3 contain the sgRNA target sequence N19/20 (19 to 20 nt) (Fig. 31.7.2B). When N1 \neq G, add one additional 'G' in front of N1 to ensure expression from the U6 promoter. P1 and P4 contain *Eco*RI and *Hind*III restriction sites, which will be used for ligation to the vector plasmid. Note that three nucleotides at the 5' end of P1 and P4 are not complementary to the empty sgRNA vector, but the remaining sequences (33 nt and 28 nt for P1 and P4) are complementary to the empty sgRNA.

- Amplify (by PCR; *UNIT 15.1*; Kramer and Coen, 2000) upstream (P1 + P2; PCR-up) and downstream (P3 + P4; PCR-down) of sgRNA expression vector using empty sgRNA as a template (Fig. 31.7.2B). Use the following reaction mix:

10 μ l 5 \times HF buffer
1 μ l 10 mM dNTP mix
1.25 μ l 10 μ M each of P1 and P2 for PCR-up, P3 and P4 for PCR-down
2 μ l 50 ng/ μ l empty sgRNA vector
0.5 μ l Phusion polymerase
34 μ l H₂O (Total 50 μ l).

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

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

- Gel purify PCR amplicons by agarose gel electrophoresis (1% agarose gel; *UNIT 2.5A*; Voytas, 2000) and by using a gel DNA extraction kit.

- Amplify (by PCR; *UNIT 15.1*; Kramer and Coen, 2000) a stitched sgRNA amplicon by using amplicons from PCR-up and -down as templates (PCR-whole). Use the following reaction mix:

10 μ l 5 \times HF buffer
1 μ l 10 mM dNTP mix
1.25 μ l 10 μ M each of P1 and P4
1 μ l amplicon each from PCR-up and PCR-down (5 to 10 ng)
0.5 μ l Phusion polymerase
35 μ l H₂O (total 50 μ l).

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

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

- Clean up PCR product using a PCR purification kit.

- Digest empty sgRNA vector and purified PCR amplicon with *EcoRI* and *HindIII* restriction enzymes. Gel purify digested vector backbone and PCR amplicon by agarose gel electrophoresis (1% agarose gel; *UNIT 2.5A*; Voytas, 2000).

- Ligate the digested amplicon with the digested vector. Use the following reaction mix:

50 ng digested vector
50 ng digested stitched PCR products
2 μ l 10 \times T4 DNA ligase buffer
2 μ l T4 DNA ligase
Add H₂O to make a total 20 μ l.

A control ligation with “vector only” will help assess enrichment of transformed bacteria carrying amplicon insertions in the vector. A control ligation can be set up by dropping the stitched amplicon from the reaction.

10. Incubate ligation reactions at room temperature for 1 hr. Transform 5 μ l of each ligation reaction into 45 μ l competent cells according to manufacturer's recommendations, and then spread bacteria onto LB plates containing 100 μ g/ml ampicillin. Incubate at 37°C overnight.
11. Pick and grow up bacterial colonies from plate containing bacterial transformants, purify plasmids, and screen for insertion of the sgRNA cassette by standard restriction digest analysis.
12. Further verify correct sgRNA insert by sequencing the vector by Sanger sequencing (see Chapter 7) using primers P1 and P4.

Instead of the empty vector, another sgRNA containing vector can be used for the above described steps 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 used in the amplification cycles of the "PCR-whole" reaction.

BASIC PROTOCOL 2

PREPARATION OF REPAIR TEMPLATE FOR HR

Designing the Repair Template DNA

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), since that determines what primer sequences you must use (Fig. 31.7.3A). Here, we present an example of C-terminal tagging with GFP (Fig. 31.7.3B). Using DNA from Bristol N2 worms as template, PCR amplify both upstream and downstream homology arms (aim to amplify between 500 to 1500 bp of homology sequence flanking each side of the target site). Consider designing PCR primers with ~20 to 30 nt of overlapping sequence for cloning via Gibson assembly. Specifically, the "upstream" PCR product will contain overlapping sequences for the *KpnI* 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 contain overlap with the 3' end of GFP (green line) and the *SalI* side of pUC19 (gray line). GFP is PCR amplified because sufficient homology is provided by the upstream 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

- UP-F:** 5'-ACGGCCAGTGAATTCGAGCTCGGTA + ~N18-24-3' (N18-24 from upstream of a gene of interest)
- UP-R:** 5'-GTGAAAAGTTCTTCTCCTTACTCAT + ~N18-24 (RC)-3' (N18-24 from upstream of a gene of interest) (reverse complement)
- DN-F:** 5'-TGGCATGGACGAACACTATACAAA + ~N18-24 -3' (N18-24 from stop codon of a gene of interest)
- DN-R:** 5'-ACGCCAAGCTTGCATGCCTGCAGG + ~N18-24 (RC)-3' (N18-24 from downstream of a gene of interest) (reverse complement)
- GFP-F:** 5'-ATGAGTAAAGGAGAAGAAGACT-3'
- GFP-R:** 5'-TTTGTATAGTTCGTCCATGC-3'
- 10 mM dNTP mix (10 mM each dNTP)
- High Fidelity Phusion DNA polymerase (NEB M0530S or equivalent) and 5 \times HF buffer
- pPV477 (Addgene, plasmid no. 42930)
- pUC19 (NEB, cat. no. N3041S)
- Nuclease-free water (Qiagen 129114 or equivalent)

*Kpn*I (NEB, cat. no. R0142S)
*Sal*I (NEB, cat. no. R0138S)
 Gel DNA Extraction Kit (ZymoClean, cat. no. D4001)
 Gibson assembly Master Mix (NEB, cat. no. E2611S)
 Plasmid Miniprep Kit (GeneJet, cat. no. K0502 or Qiagen, cat. no. 27104)
 LB agar plates containing 100 μg/ml ampicillin (*UNIT 1.1*; Elbing and Brent, 2002)
 LB liquid medium containing 100 μg/ml ampicillin (*UNIT 1.1*; Elbing and Brent, 2002)
M13F: 5'-GTAAAACGACGGCCAGT-3'
M13R: 5'-AACAGCTATGACCATG-3'
 PCR thermal cycler (BioRad T100 or equivalent)

Additional reagents and equipment for PCR (*UNIT 15.1*; Kramer and Coen, 2000), agarose gel electrophoresis (*UNIT 2.5A*; Voytas, 2000), and sequencing (Chapter 7)

Repair template cloning

1. PCR amplify (*UNIT 15.1*; Kramer and Coen, 2000) the upstream sequence of a gene of interest (500 to 1500 bp) (Fig. 31.7.3B).

UP-F: 5'-ACGGCCAGTGAATTTCGAGCTCGGTA + ~N18-24 –3'. N18-24 from upstream of a gene of interest.

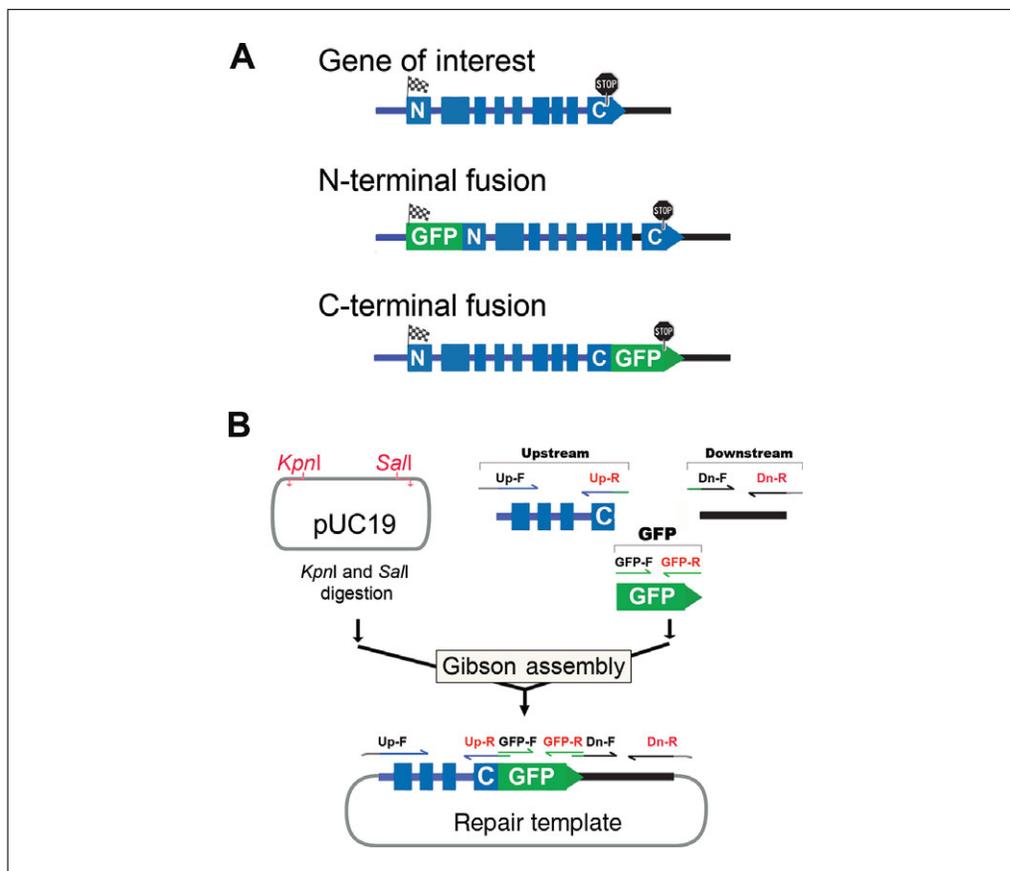


Figure 31.7.3 Schematic representation of the construction of the repair template. **(A)** Diagrams illustrate the N- and C-terminal GFP tagging of a gene of interest. Note the position of GFP as well as the start and stop codons of the protein. The flag represents the start codon. **(B)** Schematic representation of the 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).

UP-R: 5'-GTGAAAAGTTCTTCTCCTTTACTCAT + ~N18-24 (RC)-3'.
N18-24 from upstream of a gene of interest (reverse complement).

Prepare the following PCR mix:

Template DNA (<250ng)
2 μ l of 10mM dNTP mix
2 μ l each of 10 μ M forward and reverse primer
10 μ l 5 \times HF buffer
0.5 μ l Phusion polymerase
Distilled deionized H₂O up to total 50 μ l

Use the following PCR thermal cycling conditions:

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

2. PCR amplify (*UNIT 15.1*; Kramer and Coen, 2000) the downstream sequence of a gene of interest (500 to 1500 bp) (Fig. 31.7.3B) using the reaction mixture and PCR cycling conditions described in step 1.

DN-F: 5'-TGGCATGGACGAACTATACAAA + ~N18-24 -3'. N18-24 from stop codon of a gene of interest.

DN-R: 5'-ACGCCAAGCTTGCATGCCTGCAGG + ~N18-24 (RC)-3'. N18-24 from downstream of a gene of interest (reverse complement).

3. PCR amplify (*UNIT 15.1*; Kramer and Coen, 2000) the 867-bp GFP fragment from plasmid pPV477 using the reaction mixture and PCR cycling conditions described in step 1.

GFP-F: 5'-ATGAGTAAAGGAGAAGAAGACT-3'

GFP-R: 5'-TTTGTATAGTTCGTCCATGC-3'

4. Digest pUC19 (or pUC18) DNA with *KpnI* and *SalI* (Fig. 31.7.3B, top left). Run digest on an agarose gel (*UNIT 2.5A*; Voytas, 2000) to confirm plasmid linearization at 2686 bp. Since the result of a successful double digest (2669 + 17 bp) is barely distinguishable from the outcome of a single restriction enzyme digest due to the subtle change in size, make sure both enzymes are working properly. Load 5 to 10 μ g of the digested vector on a gel and, using the Gel DNA Extraction Kit, extract the 2669-bp band resulting from the *KpnI* and *SalI* digestion of the vector from the gel.

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

5. Perform Gibson assembly with the *KpnI*- and *SalI*-digested vector plus three PCR fragments (Upstream, GFP, and Downstream) as described in the manufacturer's instructions (Fig. 31.7.3B). After the Gibson assembly reaction, use 1 to 2 μ l for bacterial transformation, spread bacteria onto LB plates containing 100 μ g/ml ampicillin, and select ampicillin-resistant colonies the following day.
6. Inoculate ~10 colonies for plasmid minipreps into LB liquid medium containing 100 μ g/ml ampicillin to expand them. Analyze by Sanger sequencing (Chapter 7) using the M13F and M13R primers.

Aim to introduce a silent mutation at the PAM site of the repair template to avoid it 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, use a site-directed mutagenesis kit (*Q5 site-directed mutagenesis kit*; NEB, cat. no. E0554S) if you are working with a pre-existing repair template (donor vector). 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.

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.

INJECTING ANIMALS

At 20 to 24 hr post L4 stage, young adult *C. elegans* are injected with the CRISPR, Cas9, and, if appropriate, the repair template DNA plasmid.

Materials

General purpose agarose (Bioexpress, cat. no. E-3119-500BX)

Plasmids (good-quality DNA is required for efficient CRISPR-Cas9 genome editing; use a Qiagen midprep kit or equivalent for plasmid extraction):

Cas9 expression plasmid (Addgene, plasmid no. 46168)

pCFJ90 - *Pmyo-2::mCherry::unc-54utr* (Addgene, plasmid no. 19327)

pCFJ104 - *Pmyo-3::mCherry::unc-54* (Addgene, plasmid no. 19328)

pMA122 - *peel-1* negative selection (Addgene, plasmid no. 34873; optional)

N2 *C. elegans* wild-type worms for injection (<http://www.cgc.cbs.umn.edu/>)

Recovery solution (M9 buffer with 4% glucose)

Halocarbon oil (Sigma, cat. no. H8898)

E. coli OP50 for seeding nematode growth medium plate

(<http://www.cgc.cbs.umn.edu/>)

Nematode growth medium (NGM; Stiernagle, 2006; http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html) in 6-cm Petri plates

Microwave oven

24 × 40 mm glass coverslips (VWR, cat. no. 470145-746, or equivalent)

22 × 22 mm coverslips (VWR, cat. no. 48366-227)

Microloaders (Eppendorf, cat. no. 930001007)

Sutter P-97 needle puller or equivalent

Glass microscope slides

Microinjection apparatus

25°C incubator (PRECISION 815 or equivalent)

Prepare agarose pads for microinjection

1. Prepare agarose pads consisting of ~2% agarose in distilled water melted in a microwave oven. Line up three 22 × 40-mm size coverslips, place a dime- or ~1.8-cm-sized drop of melted agarose onto each coverslip, and quickly place a glass slide on top of the drops to flatten the agarose. Wait for 1 min, remove the glass slide, and allow coverslips with the agarose pads to air dry overnight.

Microinjection

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

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

3. Prepare the injection mixture:

- 50 to 200 ng/ μ l of Cas9 expression vector
- 50 to 200 ng/ μ l of sgRNA expression vector (see Basic Protocol 1)
- 50 ng/ μ l of repair template vector (for HR; see Basic Protocol 2)
- 2.5 ng/ μ l of pCFJ90 injection marker for mCherry expression in pharynx muscle
- 5 ng/ μ l of pCFJ104 injection marker for mCherry expression in body-wall muscle
- 10 ng/ μ l of pMA122 negative-selection plasmid (optional).

To avoid clogging of the microinjection needle, centrifuge the injection mixture in a tabletop centrifuge for 10 min at $\sim 18,000 \times g$, room temperature. Use injection mixture from the top of the tube to load your needle.

4. Inject worms:

- a. Pick 60 to 100 L4 worms a day before microinjection (it takes ~ 37 hr from eggs to L4 at 20°C).
- b. To load the microinjection needle, use an Eppendorf microloader tip and load 1 to 2 μ l of injection mix to the 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 \times 22-mm and 22 \times 40-mm 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, and, under the stereo microscope place 1 to 10 worms on the pad.
- f. Transfer worms on the 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 (by 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 the worms on the agarose pad have been injected. It is recommended to start with 1 to 2 worms the first time. After injection, transfer the agarose pad back to the stereo microscope and add 2 to 3 μ l of recovery buffer to the injected worms. With a worm pick, gently rescue the injected worms and place 2 to 4 rescued worms onto an OP50-seeded NGM plate.

5. Rehydrate injected worms in recovery buffer and place on NGM plates seeded with OP50 (~ 3 per plate). Incubate animals at 25°C for ~ 3 days until the screening step.

pMA122 is a negative selection marker to eliminate array-carrying worms. This requires an additional 2 hr heat-shock at 34°C (Frokjaer-Jensen et al., 2012).

SCREENING FOR TRANSGENIC WORMS

You can start screening for mCherry-expressing F1 worms ~3 days after injection. Note that the pCFJ90 marker is expressed in the pharynx muscle and that the pCFJ104 marker is expressed in the body-wall muscle. Some *C. elegans* mutants may grow more slowly than wild-type N2 animals, and may exhibit a developmental delay. Therefore, if injecting such mutants, the screening period needs to be extended because they grow slower.

Materials

Injected worms for analysis (Basic Protocol 3)
E. coli OP50-seeded nematode growth medium (NGM) plates
(<http://www.cgc.cbs.umn.edu/>)
Worm lysis buffer (see recipe)
10 mM dNTP 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
PCR thermal cycler (BioRad T100 or equivalent)

Additional reagents and equipment for PCR (*UNIT 15.1*; Kramer and Coen, 2000) and DNA sequencing (Chapter 7)

Single mCherry-expressing F1 worms

1. Pick mCherry-expressing F1 worms using a fluorescent stereomicroscope and place one mCherry⁺ worm on each OP50-seeded NGM plate.

In general, you can expect 80 to 300 mCherry⁺ worms when 60 to 100 worms are injected.

Genotyping potential candidates

Restriction fragment length polymorphisms 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 five nucleotides can be detected by PCR using 15% polyacrylamide gels without the need for 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, primers located outside of the repair template sequence region are recommended. Alternatively, if the region is too large for PCR amplification, it is possible to amplify the junction of the repair template and flanking sequence.

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 F1 worms at 25°C for 1 to 2 days, then sacrifice for single-worm PCR genotyping, as described below.

Protocol adapted from Williams et al. (1992) and He (2011).

Worm lysis

3. Transfer a single worm directly from a plate to 5 μ l of lysis buffer in a PCR tube.
4. Microcentrifuge capped PCR tubes briefly to bring the 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 inactivate protease K at 95°C for 15 min. Store the worm lysate at -80°C if needed.

Single worm PCR

7. Prepare the following PCR mix:

1 to 2 μ l of worm lysate
2 μ l 10 mM dNTP mix
2 μ l 10 μ M each of forward and reverse primer located outside of the repair template
10 μ l 5 \times HF buffer
0.5 μ l Phusion polymerase
Add H₂O to 50 μ l.

8. Run reactions in a thermal cycler as described in the manufacturer's instructions for NEB Phusion High-Fidelity DNA Polymerase.
9. Once F1 screening is completed, re-genotype the F1 progeny (F2s and F3s) from the potential candidates to eliminate possible false-positives and non-heritable mitotic mutants (Arribere et al., 2014; Farboud and Meyer, 2015). Using a fluorescent microscope, make sure that the F2 and/or F3 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 immunofluorescent staining, western/northern blot, or qRT-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 in Kim and Colaiacovo (2015b).

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

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. Use molecular biology-grade nuclease-free water for PCR, ligation, and Gibson assembly. See APPENDIX 2 for common stock solutions.

M9 buffer

3 g KH₂PO₄
6 g Na₂HPO₄
5 g NaCl
1 ml 1 M MgSO₄
H₂O to 1 liter
Sterilize by autoclaving
Store at room temperature

Worm lysis buffer

50 mM KCl
10 mM Tris·Cl, pH 8.3 (APPENDIX 2)
2.5 mM MgCl₂
0.45% Nonidet P-40
0.045% Tween 20
0.01% (w/v) gelatin
Autoclave
Store up to 6 months at 4°C
Immediately before use, add proteinase K to the lysis buffer for a final concentration of 60 µg/ml

COMMENTARY

Background Information

Engineering of 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.

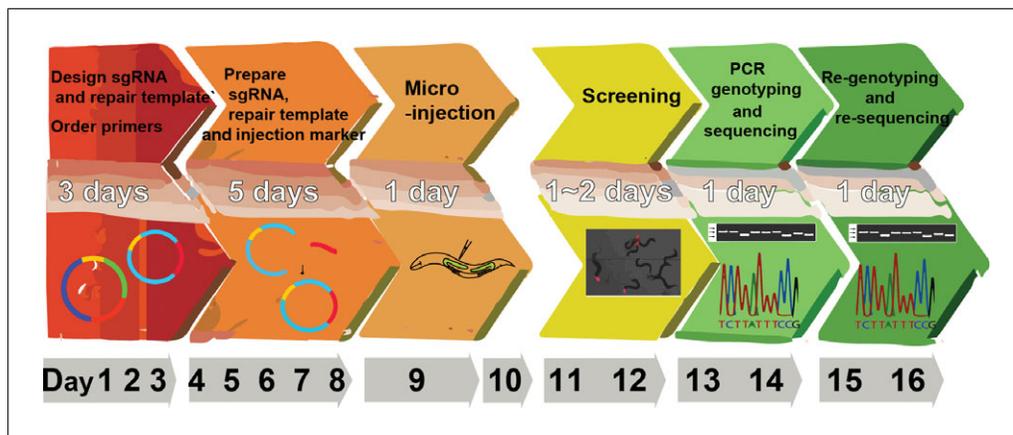
Previous studies using the *S. pyogenes* type II CRISPR system, which requires the Cas9

Table 31.7.2 Troubleshooting Common Problems Associated with CRISPR-Cas9 Genome Editing

Problem	Potential causes	Solutions
Low yield of mCherry expressing F1 worms	Purity of DNA mixture is low	Use plasmid midiprep kit
	Either too low or too high Cas9 and/or sgRNA concentrations	Adjust Cas9 and/or sgRNA concentration
	When injecting mutant worms which exhibit a developmental delay	Screening period needs to be extended as worms grow slower
High embryonic lethality among F1 worms	Cas9 and/or sgRNA causing toxicity in the worms	Reduce the concentration of Cas9 and/or sgRNA
High larval lethality among F1 worms	Cas9 and/or sgRNA causing toxicity in the worms	Reduce the concentration of Cas9 and/or sgRNA
Low genome targeting efficiency	gRNA targeting efficiency varies significantly	Design and test multiple sgRNAs

Table 31.7.3 Workflow and Time Considerations for CRISPR-Cas9 Protocols

Step	From-To	Duration	Procedure	Stopping point
1	Day 1-3	3 days	Design sgRNA, repair template (HR) and order oligonucleotides	Yes
2	Day 4-8	5 days	Prepare sgRNA, repair template (HR) and 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 (mCherry ⁺) expressing F1 worms	No
5	Day 13-14	1 day	PCR genotyping of F1 worms	No
6	Day 15-16	1 day	Re-genotyping and/or re-sequencing F2 worms.	Yes

**Figure 31.7.4** Workflow and timeline for CRISPR-Cas9-guided genome editing in *C. elegans*. The entire procedure takes 16 days to complete assuming that each step is not delayed. See Table 31.7.2 for details.

nuclease, a targeting crRNA, and an additional trans-activating trRNA, have shown that a fusion of the targeting and trans-activating RNAs to form a single guide RNA (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 a simple and reproducible marker-free protocol using the *S. pyogenes* Cas9 in *C. elegans* to create heritable genome modifications via the NHEJ or HR pathways.

Critical Parameters

The protocols described in this unit are based on marker-free strategies used in several previous studies in our lab (Friedland et al., 2013; Tzur et al., 2013; Kim and Colaiacovo, 2015b). Due to the absence of selective markers, these protocols rely on relatively time-consuming screening procedures to iden-

tify animals with the desired modification using PCR techniques compared to marker-dependent Unc, Rol, or drug-selection protocols (Dickinson et al., 2013; Arribere et al., 2014; Kim et al., 2014; Dickinson et al., 2015). However, in contrast, the protocol presented in this unit is straightforward with respect to the design of repair templates, and it progresses very quickly from microinjection to screening since it requires only one round of injection to obtain the final product of genome editing.

Troubleshooting

Table 31.7.2 describes common problems encountered with the protocols described in this unit, together with accompanying solutions.

Anticipated 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 and 24 to

72 mCherry-expressing F1 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).

Time Considerations

See Table 31.7.3 and Figure 31.7.4 for a description of the time required for each step of the protocols described in this unit.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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