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

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Abstract

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 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 and injection methods required for delivering Cas9, sgRNAs and repair template DNA into the *C. elegans* germline.

Keywords

CRISPR; Cas9; genome editing; genome engineering; *C. elegans*; CRISPR-Cas

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 a RNA duplex which is later cleaved by RNase III to form a crRNA-trRNA hybrid, thereby directing the Cas9 RGN to

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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 from the target site's PAM. The CRISPR locus itself is not cleaved by the RGN because it does not contain any NGG sequences. (Figure 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 (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 a 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) (Figure 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 (Figure 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.

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 that can be used for DSB repair (Figure 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 which probes/marks the edited genome sequence (Table 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 4 separate basic protocols, involves 1) generating the sgRNA, 2) generating the repair template DNA if homologous recombination is going to be employed to specifically modify a particular gene, 3) introducing the *cas9* gene, sgRNA, and repair DNA templates into *C. elegans* animals on separate plasmids, and 4) screening for transgenic worms carrying the CRISPR-Cas9-mediated gene editing event(s). Other published methods utilize a single plasmid expressing both the *cas9* gene and the sgRNA (Dickinson et al., 2013).

Outline (for review purposes only)

GUIDE RNA PREPARATION: Identifying a sgRNA targeting sequence and preparing a repair template

sgRNA cloning using an empty sgRNA expression vector

sgRNA cloning using fusion PCR (alternative method)

PREPARATION OF REPAIR TEMPLATE FOR HR: Designing the repair template DNA

INJECTING ANIMALS

SCREENING FOR TRANSGENIC WORMS

BASIC PROTOCOL 1: GUIDE RNA PREPARATION

Materials

*Bam*HI (NEB R0136S)

*Not*I (NEB R0189S)

*Eco*RI (NEB R0101S)

*Hind*III (NEB R0104S)

Nuclease-free water (Qiagen 129114 or equivalent)

T4 DNA ligase (NEB M0202S)

Sterile pipet tips or toothpicks for picking colonies

Gibson assembly Master Mix (NEB E2611S)

pHKMC1 - Empty sgRNA (Addgene #67720)

Chemically competent *E. coli* cells (NEB C2987I or equivalent)

High Fidelity Phusion DNA polymerase (NEB M0530S or equivalent)

Gel DNA Extraction Kit (Zymoclean D4001)

Plasmid Miniprep Kit (GeneJet K0502 or Qiagen 27104)

Plasmid Midiprep Kit (Qiagen 12143)

Heat Block (VWR Scientific Standard Heat Block or equivalent)

PCR thermo cycler (BioRad T100 or equivalent)

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

sgRNA_Bottom: 5'-GCTATTTCTAGCTCTAAAAC N19/N20 Reverse Complement
AAACATTTAGATTTGCAAT-3'

M13F: 5'-GTAAAACGACGGCCAGT-3'

M13R: 5'-AACAGCTATGACCATG-3'

P1: 5'-CGGGAATTCCTCCAAGAACTCGTACAAAAATGCTCT-3'

P2: 5'-(N19/20-RC) + AAACATTTAGATTTGCAATTCAATTATATAG-3' (where N19/20-RC is the reverse complementary sequence of the N19/20 target sequence used in primer P3).

P3: 5'-(N19/20) + GTTTTAGAGCTAGAAATAGCAAGTTA-3' (where N19/20 represents the sgRNA target sequence)

P4: 5'-CGGAAGCTTCACAGCCGACTATGTTTGGCGT-3'

Identifying 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 other 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.

1. Order both top (forward) and bottom (reverse complement) oligos containing 19-20 nucleotides of homology (N19/N20) to the target sequence from a DNA synthesis service (Figure 2A). When N1 = G, add one additional 'G' in front of N1 to ensure expression from the U6 promoter. Following are the generic sgRNA sequences (in CAPs) as well as an example of the sgRNA sequence design (lower case) 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
GTTT TAGAGCTAGAAATAGC-3'

sgRNA_Bottom: 5'-GCTATTTCTAGCTCTAAAAC N19/N20 Reverse Complement
AAACATTTAGATTTGCAAT-3'

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

ztf-8_Top: 5'-ATTGCAAATCTAAATGTTT gagatgatcgaggctctcga
GTTTTAGAGCTAGAAATAGC-3'

ztf-8_Bottom: 5'-GCTATTTCTAGCTCTAAAAC tcgagagcctcgatcatctc
AAACATTTAGATTTGCAAT-3'

NOTE: 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).

2. Perform a miniprep of an empty sgRNA vector (3482bp, Addgene #67720) which contains the U6 Promoter and sgRNA scaffold sequence as illustrated in Figure 2A. Digest 1-2 µg of empty sgRNA vector with *Bam*HI and *No*I. Extract the 3470 bp band from an agarose gel using the Gel DNA recovery kit. This will be sufficient for ~10 reactions.
3. To generate double stranded DNA, mix equal amounts of the “Top” and “Bottom” strand of sgRNA targeting oligonucleotides (e.g. 5µl 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.

4. Alternatively, place the tube containing the Top and Bottom oligos in a heat block at ~90°C for ~3 minutes. 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 of annealed oligonucleotides + ~100 ng *Bam*HI and *No*I digested empty sgRNA + ½ of total volume of Gibson assembly mix.
6. Use 1-2 µl for bacterial transformation and select ampicillin resistant colonies the following day.
7. Pick and inoculate ~10 ampicillin-resistant colonies from plate containing bacteria, purify plasmids, and screen for insertion of sgRNA by restriction digest analysis.
8. Verify sgRNA insertion by Sanger sequencing using the primers M13R and M13F.

NOTE: For colony PCR screening, M13F and M13R primers will amplify ~890bp from the empty vector and this PCR product can be used for sequencing and/or be further analyzed with a *Bam*HI or *No*I 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 a 530bp and a 360 bp products. Although *Bam*HI and *No*I-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 *Bam*HI and *No*I.

ALTENATE 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).

1. Design primers to amplify upstream (P1 + P2; PCR-up) and downstream (P3 + P4; PCR-down) of the empty sgRNA vector (Figure 2B). Primers P2 and P3 contain the sgRNA target sequence N19/20 (19-20nt) (Figure 2B). When N1 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 3 nucleotides at the 5' end of P1 and P4 are not complementary to the empty sgRNA vector, but the remaining sequences (33nt and 28nt for P1 and P4) are complementary to the empty sgRNA.

P1: CGGGAATTCTCTCCAAGAACTCGTACAAAAATGCTCT

P2: N19/20-RC + AAACATTTAGATTTGCAATTCAATTATATAG
(where N19/20-RC is the reverse complementary sequence of the N19/20 target sequence used in primer P3).

P3: N19/20 + GTTTTAGAGCTAGAAATAGCAAGTTA (where N19/20 represents the sgRNA target sequence)

P4: CGGAAGCTTCACAGCCGACTATGTTTGGCGT

2. Amplify upstream (P1 + P2; PCR-up) and downstream (P3 + P4; PCR-down) of sgRNA expression vector using empty sgRNA as a template (Figure 2B):
 - 10 µL 5x HF buffer
 - 1 µL 10 mM dNTP mix
 - 1.25 µL 10 uM each of P1 and P2 for PCR-up, P3 and P4 for PCR-down
 - 2 µL 50 ng/uL Empty sgRNA vector
 - 0.5 µL Phusion polymerase
 - 34 µL ddH₂O (Total 50 µl)
3. Run reactions in a thermal cycler as described in manufacturer's instructions (NEB Phusion High-Fidelity DNA Polymerase).
4. Gel purify PCR amplicons by agarose gel electrophoresis (1% agarose gel) and by using a gel DNA extraction kit.
5. Amplify a stitched sgRNA amplicon by using amplicons from PCR-up and -down as templates (PCR-whole).
 - 10 µL 5x HF buffer
 - 1 µL 10 mM dNTP mix
 - 1.25 µL 10 uM each of P1 and P4

1 μ L amplicon each from PCR-up and PCR-down (5-10 ng)

0.5 μ L Phusion polymerase

35 μ L ddH₂O (Total 50 μ l)

6. Run reactions in a thermal cycler as described in manufacturer's instructions (NEB Phusion High-Fidelity DNA Polymerase).
7. Clean up PCR product from PCR-whole reaction using a PCR purification kit.
8. Digest empty sgRNA vector and purified PCR amplicon with *Eco*RI and *Hind*III restriction enzymes. Gel purify digested vector backbone and PCR amplicon using agarose gel electrophoresis (1% agarose gel).
9. Ligate the digested amplicon with the digested vector. A control ligation with “vector only” will help assess enrichment of transformed bacteria carrying amplicon insertions in the vector. Control ligation can be set up by dropping out stitched amplicon from the reaction.

50ng digested vector

50 ng digested stitched PCR products

2 μ L 10x T4 DNA ligase buffer

2 μ L T4 DNA ligase

Add ddH₂O to make a total 20 μ l

10. Incubate ligation reactions at room temperature for 1 hour. Transform 5 μ L of each ligation reaction into 45 μ L DH5alpha competent cells according to manufacturer's recommendations, and then spread bacteria onto LB + ampicillin plates. 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 using primers P1 and P4.

NOTE: 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).

NOTE: 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 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 (Figure 3A). Here we present an example of C-terminal tagging with GFP (Figure 3B). Using DNA from Bristol N2 worms as template, PCR amplify both upstream and downstream homology arms (aim to amplify between 500 to 1500bp of homology sequence flanking each side of the target site). Consider designing PCR primers with ~20-30nt 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 *SaI* side of pUC19 (gray line). GFP is PCR amplified 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

KpnI (NEB R0142S)

SaI (NEB R0138S)

pUC19 (NEB N3041S)

pPV477 (Addgene plasmid #42930)

37°C water bath incubator

42°C water bath incubator for bacterial transformation

Spectrophotometer for measuring DNA concentration

PCR thermo cycler (Biorad T100 or equivalent)

LB agar plate containing 100 µg/ml ampicillin (UNIT 1.1)

LB liquid medium containing 100 µg/ml ampicillin (UNIT 1.1)

Gibson assembly Master Mix (NEB E2611S)

High Fidelity Phusion DNA polymerase (NEB M0530S or equivalent)

Plasmid Miniprep Kit (GeneJet K0502 or Qiagen 27104)

Plasmid Midiprep Kit (Qiagen 12143)

Nuclease-free water (Qiagen 129114 or equivalent)

Gel DNA Extraction Kit (Zymoclean D4001)

Q5® Site-Directed Mutagenesis Kit (NEB E0554S)

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

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

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)

GFP-F: 5'-ATGAGTAAAGGAGAAGAACT-3'

GFP-R: 5'-TTTGTATAGTTCGTCCATGC-3'

M13F: 5'-GTAAAACGACGGCCAGT-3'

M13R: 5'-AACAGCTATGACCATG-3'

Repair template cloning

1. PCR amplify the upstream sequence of a gene of interest (500-1500bp) (Fig. 3B).

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

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

2. PCR amplify the downstream sequence of a gene of interest (500-1500bp) (Fig. 3B).

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 the 867bp GFP fragment from plasmid pPV477 (Addgene #42930).

GFP-F: 5'-ATGAGTAAAGGAGAAGAACT-3'

GFP-R: 5'-TTTGTATAGTTCGTCCATGC-3'

4. Digest pUC19 (or pUC18) DNA with *KpnI* and *SaI* (Figure 3B, top left). Run digest on an agarose gel to confirm plasmid linearization at 2686 bp. Since the result of a successful double digest (2669 + 17bp) 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 5-10 µg of the digested vector on a gel and using the Gel DNA extraction kit extract the 2669 bp size band resulting from the *KpnI* and *SaI* 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 *SaI* digested vector + 3 PCR fragments (Upstream, GFP, and Downstream) as described in the manufacturer's instructions (Figure 3B). After the Gibson assembly reaction, use 1-2 µl for bacterial transformation and select ampicillin resistant colonies.
6. Inoculate ~10 colonies for plasmid minipreps and analyze them by Sanger sequencing using the M13F and M13R primers.

NOTE: 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 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.

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

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

BASIC PROTOCOL 3. INJECTING ANIMALS

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

Materials

Cas9 Expression Plasmid (Addgene plasmid #46168).

pCFJ90 - *Pmyo-2::mCherry::unc-54utr* (Addgene plasmid #19327)

pCFJ104 - *Pmyo-3::mCherry::unc-54* (Addgene plasmid #19328)

pMA122 - *peel-1* negative selection (Addgene plasmid #34873, Optional)

Microinjection apparatus

Microwave

24 × 40mm Glass Coverslips (VWR 470145-746 or equivalent)

Petri plates (6 cm) containing nematode growth medium seeded with OP50 (NGM plates).

25 °C incubator (PRECISION 815 or equivalent)

M9 buffer

Recovery solution: M9 with 4% glucose.

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

E. coli OP50 for seeding nematode growth medium plate (<http://www.cgc.cbs.umn.edu/>)

Agarose pads for microinjection

1. Prepare agarose pads consisting of ~ 2 % agarose in distilled water melted in a microwave. Line up three 22 × 40 mm size cover slips and place a dime sized drop of melted agarose onto each cover slip 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 cover slips with the agarose pads to air dry overnight.

Preparing good quality DNA

2. Good quality DNA is required for efficient CRISPR-Cas9 genome editing. Use a Qiagen midiprep kit or equivalent for plasmid extraction.

Microinjection

3. Pick L4 worms and incubate them at 20-25 °C for ~24 hrs. 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.

NOTE: 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 and Colaiacovo, 2015a).

4. Prepare the injection mixture using the following concentrations and spin it down using a tabletop centrifuge at ~18,000 RCF for 4 min.

50-200 ng/uL of Cas9 expression vector

50-200 ng/uL of sgRNA expression vector

50 ng/uL of repair template vector (for HR)

2.5 ng/uL of the pCFJ90 injection marker for mCherry expression in pharynx muscle

5 ng/uL of the pCFJ104 injection marker for mCherry expression in body wall muscle

10 ng/uL of the pMA122 negative selection plasmid (Optional)

5. Inject 60 - 100 worms for each target as described in (Kadandale et al., 2009).
6. Rehydrate injected worms in M9 or recovery buffer and place on NGM plates (Stiernagle, 2006) seeded with OP50 (~3 per plate). Incubate animals at 25°C for ~3 days until the screening step.

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

BASIC PROTOCOL 4. 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 slower 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

Worm Lysis Buffer

Petri plates (6 cm) containing nematode growth medium agar seeded with OP50

25°C incubator

Deep Freezer (Thermo ULT2580 or equivalent)

PCR thermo cycler (Biorad T100 or equivalent)

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

E. coli OP50 for seeding nematode growth medium plate (<http://www.cgc.cbs.umn.edu/>)

Single mCherry expressing F1 worms

1. Pick mCherry expressing F1 worms using a fluorescent stereo microscope and “single” them to OP50 seeded NGM plates. 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 prevents 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 further shortened by pooling DNA samples for PCR, as shown in (Paix et al., 2014). Alternatively, changes as small as 5 nucleotides 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, primers located outside of the repair template sequence region are 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 F1 worms at 25 °C for 1 - 2 days and are sacrificed for single worm PCR genotyping as described below. Protocol adapted from (He, 2011; Williams et al., 1992).

Worm lysis

3. Transfer a single worm directly from a plate to 5 µl of 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 h, then inactivate protease K at 95°C for 15 min. Store the worm lysate at -80°C if needed.

Single worm PCR—1 - 2 µl of worm lysate

2 µL 10 mM dNTP mix

2 µL 10 µM Primers (Forward + Reverse)

5 µL 5x HF buffer

0.25 µL Phusion polymerase

Add ddH₂O to 50 µl

7. Run reactions in a thermal cycler as described in the manufacturer's instructions (NEB Phusion High-Fidelity DNA Polymerase).

Once F1 screening is completed, it is advisable to re-genotype the F2s and/or 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 scope make sure that the F2 and/or F3 have lost the mCherry extrachromosomal signal. pMA122 can also be used for negatively select ing arrays with an additional 2 hour heat-shock at 34°C (Frokjaer-Jensen et al., 2012).

8. Order Sanger sequencing analysis to confirm mutations.
9. 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).

10. It is recommended to outcross the derived transgenic/edited lines 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).

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.

Plasmids

Cas9 Expression Plasmid (Addgene plasmid #46168)

pHKMC1 - Empty sgRNA Cloning Plasmid (Addgene plasmid #67720)

pCFJ90 - *Pmyo-2::mCherry::unc-54utr* (Addgene plasmid #19327)

pCFJ104 - *Pmyo-3::mCherry::unc-54* (Addgene plasmid #19328)

pMA122 - *peel-1* negative selection (Addgene plasmid #34873, Optional)

pUC19 (NEB N3041S)

pPV477 (Addgene plasmid #42930)

Worms

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

Reagents

LB agar plate containing 100 µg/ml ampicillin (UNIT 1.1)

LB liquid medium containing 100 µg/ml ampicillin (UNIT 1.1)

Plasmid Miniprep Kit (GeneJet K0502 or Qiagen 27104)

Plasmid Midiprep Kit (Qiagen 12143)

Nuclease-free water (Qiagen 129114, Zymo research W1001-10 or equivalent)

Gel DNA Extraction Kit (Zymoclean D4001)

Chemically Competent *E. coli* cells (NEB C2987I or equivalent)

High Fidelity Phusion DNA polymerase (NEB M0530S or equivalent)

T4 DNA ligase (NEB M0202S or equivalent)

*Bam*HI (NEB R0136S)

*Not*I (NEB R0189S)

*Eco*RI (NEB R0101S)

*Hind*III (NEB R0104S)

*Kpn*I (NEB R0142S)

*Sal*I (NEB R0138S)

Sterile pipet tips or toothpicks for picking colonies

Halocarbon oil 700 (Sigma H8898)

M9 buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 ml 1 M MgSO₄, H₂O to 1 liter. Sterilize by autoclaving.)

Recovery solution: M9 with 4% glucose.

24 × 40mm Glass Coverslips (VWR 470145-746 or equivalent)

E. coli OP50 for seeding NGM plate (<http://www.cgc.cbs.umn.edu/>)

Petri plates (6 cm) containing nematode growth medium (NGM, (Stiernagle, 2006)) seeded with OP50

Worm Lysis Buffer: 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl₂, 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 with final concentration 60 µg/ml.

Equipment

25 °C and 37°C incubators

42°C water bath incubator for bacterial transformation

Microinjection apparatus

Spectrophotometer for measuring DNA concentration

Sequence analysis software (e.g. NCBI BLAST, UCSC Genome Browser BLAT, LaserGene)

PCR thermo cycler (Biorad T100 or equivalent)

Heat Block (VWR Scientific Standard Heat Block or equivalent)

Deep Freezer (Thermo ULT2580 or equivalent)

Primers

For sequencing and screening purpose—M13F: 5'-GTAAAACGACGGCCAGT-3'

M13R: 5'-AACAGCTATGACCATG-3'

For sgRNA expression vector—sgRNA_Top: 5'-ATTGCAAATCTAAATGTTT + N19/20 + GTTTTAGAGCTAGAAATAGC-3'

sgRNA_Bottom: 5'-GCTATTTCTAGCTCTAAAAC + N19/20 Reverse Complement + AACATTTAGATTTGCAAT-3'

P1: 5'-CGGGAATTCCTCCAAGAACTCGTACAAAAATGCTCT-3'

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).

P3: 5'-N19/20 + GTTTTAGAGCTAGAAATAGCAAGTTA-3' (where N19/20 represents the sgRNA target sequence)

P4: 5'-CGGAAGCTTCACAGCCGACTATGTTTGGCGT-3'

For Repair Template Vector—UP-F: 5'-acggccagtgaattcgagctcggt + ~N18-3'. N18 from Upstream of a gene of interest

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

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

DN-R: 5'-acgccaagcttgcatgcctgcagg + ~N18(RC)-3'. N18 from Downstream of a gene of interest (Reverse complement)

GFP-F: 5'-ATGAGTAAAGGAGAAGAACT-3'

GFP-R: 5'-TTTGTATAGTTCGTCCATGC-3'

COMMENTARY

Background Information

Engineering precise modification 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 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; Kim and Colaiacovo, 2015b; 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 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 protocol presented in this Unit is straight-forward 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.

Trouble shooting

Table 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 - 16.7% genome targeting efficiency for HR with injection of 7-13 worms and 24-72 mCherry expressing F1 worms (Tzur et al., 2013). A 0.5 - 80.3% gene disruption frequency was reported for InDels from four different targeting loci (Friedland et al., 2013).

Time Considerations

See Table 3 and Figure 4 for a description of the time required for each step of the protocols described in this unit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance statement (required)

Since the CRISPR-Cas system was identified as part of the bacterial immune system that confers resistance to foreign DNA such as from bacteriophages (Barrangou et al., 2007), CRISPR-Cas has been at the forefront due to its versatile genome editing capability. This new technology is poised to tremendously advance the life sciences due to its unprecedented flexibility, proficiency, and accuracy. Among a number of Cas enzymes identified thus far, the best known is Cas9 from *Streptococcus pyogenes*, which can induce precise DNA breaks at targeted genomic locations in various species from bacteria to humans. This feature of inducing precise breaks in the genome can be co-opted for genome editing such as for repairing a faulty gene or creating a knockout allele.

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.

A Guide to cloning sgRNA #1 using an empty expression vector

B Guide to cloning sgRNA #2 using PCR stitching

Figure 2.

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

Figure 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. Flag represents start codon. **B.** Schematic representation of the repair template cloning for C-terminal GFP tagging. Three PCR amplicons are assembled into a *KpnI* and *SalI* digested pUC19 vector to create a repair template (donor plasmid).

Figure 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 2 for details.

Table 1Types 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	<i>unc-119(+)</i> (Dickinson et al., 2013) Drug resistance (Dickinson et al., 2015; Dickinson et al., 2013; Norris et al., 2015a; Norris et al., 2015b)

Table 2

Trouble shooting 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 Either too low or too high Cas9 and/or sgRNA concentrations When injecting mutant worms which exhibit a developmental delay	Use plasmid midiprep kit Adjust Cas9 and/or sgRNA concentration 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 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