**­Supplemental File S1**

**Detailed Protocol to generate putative null mutants by inserting the universal STOP-IN cassette
 (Wang H., *et al.*, G3, 2018)**

Step (**A**) Select a guide sequence and order crRNA and tracrRNA

1. Search for guide sequences that are followed by the PAM site (5’-NGG-3’) for the target gene, using the website established by the Moerman lab (<http://genome.sfu.ca/crispr/search.html>) (Au *et al.* 2018).
2. Select a guide sequence, such that the Cas9 cut site is within the coding regions of the target gene. Other considerations for the selection of the guide sequence include:
* Shared by all isoforms of the gene
* Close to the start codon ATG of the target gene
* Folding energy between -2.0 to 0
1. Order the crRNA corresponding to the selected guide sequence from the IDT website (https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system). Under the section “Use your own designs”, click “Order IN TUBES” to go to the webpage with the order form (<https://www.idtdna.com/site/order/oligoentry/index/crispr>), select the default scale (Alt-R® CRISPR-Cas9 crRNA, 2 nmol), and enter the 20 DNA bases immediately 5’ to the PAM site **(do not include the PAM site)** in the order form.
2. Order the universal 67mer tracrRNA. Under the section “CRISPR-Cas9 tracrRNA” in the IDT website (<https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system>), select the amount of Alt-R® CRISPR-Cas9 tracrRNA (Typically, we order 20 or 100 nmol).
3. Dissolve crRNA and tracrRNA with accompanying nuclease-free water to make 100 µM stock solutions. Aliquot the tracrRNA soution into small volumes. Store stock solutions at -80 °C.

Step (**B**) Design single stranded DNA repair oligos with two short homology arms

1. Copy the genomic sequence of the target gene (the coding strand from the start codon to stop codon) from WormBase and paste it into ApE (<http://biologylabs.utah.edu/jorgensen/wayned/ape/>) or your preferred program to manipulate DNA sequences.
2. Select the whole sequence in ApE, and label all exons of the target gene as a cDNA feature.
3. Label the guide sequence selected in (**A**) as a separate feature.
4. Insert the STOP-IN cassette (sequence below) at the Cas9 cut site (-3 nt upstream of the PAM site) in the coding strand (see the blue triangle below).

**The STOP-IN cassette sequence (43 nucleotides):** GGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAAGCTAGC

(Exogenous Cas9 target site with PAM site underlined, stop codons, NheI site)

1. Design the single stranded repair oligo by selecting ~35 nt flanking the STOP-in cassette for a total of ~35 + 43 + ~35 = ~113 nucleotides as described below. We always use repair oligo on the PAM strand (the strand where NGG is located).

**Note:** It is recommended that the first and last nucleotide of the repair oligo should be guanine (G) or cytosine (C) (Paix *et al.* 2014).

1. Order the DNA repair oligo from IDT (<https://www.idtdna.com/site/order/oligoentry/index/ultra>) and select the default scale (4nmole UltramerTM DNA Oligo).
2. Dissolve the DNA repair oligo in nuclease-free water to make 100 µM stock solution and store at ‑20 °C or -80 °C.

Guidelines for DNA repair oligos:

1) If the **PAM** site is on the **coding strand** of the gene of interest:

NNNNNNNNNNNNNNNTCGATCTGCTAAACGTCTTG**TGG**NNNNNNNNNNNNNN

Then the repair oligo to order is the following sequence(~35 bp for both homology arms):

(N18)TCGATCTGCTAAACGTCGGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAAGCTAGCTTG**TGG**(N29)

2) If the **PAM** site is on the **non-coding strand** of the gene of interest:

NNNNNNNNNNNNNNN**CCA**CGATCTGCTAAACGTCTTGTNNNNNNNNNNNNNN

Then the repair oligo to order is the **REVERSE COMPLEMENT** of the following sequence:

(N29)**CCA**CGAGGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAAGCTAGCTCTGCTAAACGTCTTGT(N18)

**Note**: TCGATCTGCTAAACGTCTTG**TGG** and **CCA**CGATCTGCTAAACGTCTTGT are example Cas9 target sequences with PAM sites underlined. The blue triangles indicate Cas9 cleavage sites.

Step (**C**) Make Cas9 ribonucleoprotein injection mix

We used *dpy-10* or *unc-58* as a co-CRISPR marker (Arribere *et al.* 2014). Protocols from the Seydoux lab (Paix *et al.* 2017), the Dernburg lab (UC Berkeley), and the IDT website were used as references. The volumes of the following mixtures can be scaled up, if needed.

1. Make the guide RNA duplex, which consists of crRNA and tracrRNA at a 1:1 ratio.

# crRNA: crRNA:

 *dpy-10* crRNA (100 µM) 0.5 µL **or** *unc-58* crRNA (100 µM) 1.0 µL

 your gene crRNA (100 µM) 2.0 µL your gene crRNA (100 µM) 1.5 µL

 tracrRNA (100 µM) 2.5 µL tracrRNA (100 µM) 2.5 µL

Total volume: 5.0 µL Total volume: 5.0 µL

1. Incubate at 94 °C for 2 min in a PCR machine and then let it cool down to room temperature on the bench.
2. Assemble injection mix as described below. Mix Cas9 protein and guide RNA duplex first and incubate for 5 min at room temperature. Then add repair oligos and mix well.

Cas9 protein (10 µg/µL) 2.0 µL

1 M KCl solution**\*** 0.58 µL

Guide RNA duplex from step 13 in (**C**) 2.7 µL

repair oligo for the target gene (10 µM) 0.3 µL

*dpy-10* repair oligo (5 uM) **or** *unc-58* repair oligo (10 µM)**\*\*** 0.3 µL

Total volume: 5.88 µL

 **\*Note**: In the first few trials of our CRISPR experiments, we observed light white precipitation when we mixed Cas9 protein with annealed tracrRNA and crRNAs, and successfully obtained knock-in alleles. KCl solution has been reported to improve the solubility of Cas9 protein (Paix *et al.* 2017; Prior *et al.* 2017), thus we added 0.58 µl of 1 M KCl solution to make the final concentration of KCl in the injection mixture to 150 µM, the same concentration as the buffer for Cas9 protein (20 mM Hepes pH7.5, 150 mM KCl, 10% Glycerol, 1 mM DTT).

 **\*\*Note**: Please note the differences in concentration

1. Briefly spin down the injection mix, put it on ice and inject right away (the mixture is effective at least within four hours in our hands).

Step (**D**) Microinjection and picking F1 animals with co-conversion markers

1. Inject the Cas9 ribonucleoprotein mix prepared in (**C**) into the gonads of adult *C. elegans,* using a standard microinjection protocol (Mello and Fire 1995). Typically, we inject 10-20 P0 animals for each mixture, transfer each P0 onto a new NGM plate, and culture them at room temperature.

1. After 3-4 days, pick 10-20 F1 animals with the co-conversion marker and single each animal into a separate plate.
2. After 3-4 days, genotype those plates that contain F2 animals with the co-conversion marker.

Step (**E**) Genotyping to identify successful knock-in alleles

We only genotype F2 progeny of F1 co-conversion animals to ensure that we obtain heritable genome editing. We use two pairs of primers (one outer primer pair and one outer + inner primer pair, as described in Figure 1F) for each candidate plate to ensure that we isolate F1 animals that are heterozygous for the desired knock-in alleles. The outer primer pair is selected, such that the PCR products of wild type and the knock-in allele (43 bp difference) can be easily separately by agarose gel electrophoresis. Typically, the wild type PCR products with outer primers are less than 500 bp.

1. Worm lysis procedure
2. Add 50 ul Proteinase K (10mg/ml; Invitrogen #25530-015) to 200 µL worm lysis buffer\*.
3. Pipette 10 µL per each PCR tube.
4. Pick 5-10 F2 worms into lysis buffer in PCR tube.
5. Quickly spin down the PCR tubes (recommended!!).
6. Put the PCR tubes at -80 °C for 15 min.
7. Incubate the PCR tubes in a PCR machine at 65°C for 50 min, then at 95°C for 15 min.
8. Quickly spin down and keep at -20 °C.
9. Use 2 µL lysate as the templates for a 15 µL PCR genotype reaction.

\*worm lysis buffer: 50 mM KCl, 10 mM Tris pH 8.0, 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin.

1. PCR protocol

Make master mix by taking following recipe and multiplying by the number of samples + extra (10-15%). Then aliquot 13 uL of the master mixture into each PCR tube and then add 2 ul of worm lysate.

1) PCR mix for 1 reaction

|  |  |
| --- | --- |
| **Content** | **Volume** |
| 10x PCR reaction buffer (Roche) | 1.5 µL |
| 10 µM forward primer | 0.3 µL |
| 10 µM reverse primer | 0.3 µL |
| 10 mM dNTP (NEB, #N0447L) | 0.3 uL |
| Taq DNA polymerase (Roche, #11596594001) | 0.1 µL |
| ddH2O | 10.5 µL |
| Total master mix | 13 µL |
| Add worm lysate as template DNA | 2 µL |
| Final PCR reaction volume | 15 µL |

2) PCR condition

|  |  |  |
| --- | --- | --- |
| **Step** | **Temp** | **Time** |
| Initial Denaturation | 94 °C | 2 min |
| Cycles (30-35) | 94 °C 58 °C (variable\*)72 °C | 30 sec30 sec30 sec (variable ; 1 kb/min) |
| Final Extension | 72 °C | 10 min |
| Hold | 5 °C |  |

\* Temp for extension depends on Tm of the primers. You can use IDT, OligoCalc, etc.

1. Run the PCR products on 2.0-2.5% agarose gel to identify F1 clones that were heterozygous for the positive knock-in alleles.
2. Single out a few F2 progeny without the co-conversion marker from the positive F1 plates and genotype the F3 progeny using the outer primer pair to identify F2 animals that are homozygous for the desired knock-in alleles.
3. Sequence the PCR product (with outer primer pair) of the F2 homozygotes to confirm the successful insertion of the universal STOP-IN cassette, using the outer primer that is further away from the Cas9 cut site.

**References**

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