

Seamless editing of the *C. elegans* genome using CRISPR/Cas9

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Abstract

This protocol is from:

Alexandre Paix, et al. (2014) [Scalable and Versatile Genome Editing Using Linear DNAs with Microhomology to Cas9 Sites in *Caenorhabditis elegans*](#). *Genetics* 198:1347-1356;doi:10.1534/genetics.114.170423

Please see the [full manuscript](#) or additional details

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Guidelines

Reagents

- QIAprep Spin Miniprep Kit: Qiagen, 27104
- MinElute PCR Purification Kit: Qiagen, 28004
- Phusion High-Fidelity PCR Master Mix with HF Buffer: NEB, M0531L
- GoTaq Green Master Mix: Promega, M7122
- Taq DNA Polymerase: Invitrogen, 10342-046
- Recovery buffer: 5mM HEPES pH 7.2, 3mM CaCl₂, 3mM MgCl₂, 66mM NaCl, 2.4mM KCl, 4% Glucose (w/v)
- 10X M9: 420mM Na₂HPO₄, 220mM KH₂PO₄, 860mM NaCl, 10mM MgSO₄
- Q5 Site-Directed Mutagenesis Kit: NEB, E0554S
- Lysis buffer: 50mM KCl, 10mM Tris pH8.3, 2.5mM MgCl₂, 0.45% NP40, 0.45% Tween20. Before worm lysis, add proteinase K to 0.1 ug/ul.

Design and cloning of the sgRNAs

-Use this website <http://crispr.mit.edu/> to select sgRNAs.

Choose sgRNAs that are 1) as close as possible to the modification site and 2) have few off-target sites. If there are off-target sites, they should have 3 or more mismatches, preferentially near the PAM. If possible, choose more than one sgRNAs near each other. Not every sgRNA works, so it's best to use more than one, until you have one that you know works.

-The website shows sgRNAs with 20 bases, but we only use the last 19 bases (skip the first base).

-Order the following primers:

Forward Q5: (N19)gttttagagctagaaatagcaagt

Reverse Q5: caagacatctcgcaatagg

Forward sequencing (for sequencing sgRNA insertion only): tatgaaatgcctacaccctctc

Design of repair ssODNs

The repair oligo should contain flanking bases at both the 5' and 3' ends for homologous recombination (sequences identical to genomic DNA). Ideally, flanking sequences should terminate with a C or G and contain good sequence diversity at their extremities (no hairpins). 43nt is the shortest flanking sequence we have tested and 100 is the longest. We do not know the optimal homology arm length for ssODNs. Make sure the homology arms extend as close to the sgRNA cut(s) as possible.

The repair oligo should contain mutations that make it resistant to cutting by Cas9/sgRNA. You can mutate the PAM, insert new bases between the sgRNA sequence and the PAM, or mutate the sgRNA sequence near the PAM (we typically create 4 mismatches).

If the sgRNA targets a coding region, be careful to make only silent changes using codons that are used at similar frequency as original codon ([This site](#) for codon usage in *C. elegans* may be useful). If possible, avoid sgRNAs that target non-coding sequences since mutations in these sequences could possibly affect regulatory (splicing, promoters) motifs.

-If possible, engineer a restriction site (RE) in your oligo to facilitate screening. Make sure that the RE site is either directly in your edit, or on the distal side of your edit relative to the cut, to ensure that both are incorporated.

-If you want to insert a premature stop, insert a NheI site between the PAM and sgRNA sequence. Use the TAG codon inside the NheI site. For maximal gene disruption, we recommend also adding a base to create a frameshift after the TAG codon.

-Suggestions for protein tags (capital letters represent bases modified to create a RE site):

V5: ggtaagcctatccctaaccctctcctcggtctAgatAGTAcT (contains XbaI and ScaI sites)

HA: tacccataTgatgtCccGgattacgct (contains NdeI and NciI sites)

TetraCys: tgctgcccaggatgctgc (contains BstNI site)

3xFLAG: gactacaaagaccatgacggtgattataaagatcatgaTatcgaTtacaaggatgacgatgacaag (contains EcoRV and ClaI sites)

Myc: gaacaaaaactGatAtcagaagaggatctg (contains EcoRV site)

OLLAS: tccggattcgccaacGAGCTCggaccacgtctcatgggaaag (contains SacI site)

-Order single stranded oligo from Operon (125nt max, 10nmol, salt free) or from IDT (200nt max, 4nM ultramer, salt free). Reconstitute oligo at 1ug/ul according to the amount provided by the manufacturer.

Construction of PCR donor templates for GFP insertion

Optional Nested PCR step: The nested PCR step is included to amplify your PCR further and to eliminate any long primers remaining from the first PCR. This step may be omitted if your PCR is already >500ng/ul and if you used relatively short primers (<60 bases) that are efficiently removed by the minelute PCR purification kit. Perform three nested PCR on this purification using 22-25nt Fw/Rev primers complementary to the 5'/3' ends of the template (45s elongation step, 30 cycles, 3*50ul reaction, 60°C annealing). Check the PCR products on agarose gel.

Optional Dpn1 digestion step: This step may not be necessary but is included to eliminate any remaining GFP plasmid template that could form an extrachromosomal array and give you a false GFP+. Add to each 50ul PCR reactions 30ul of H₂O, 10ul of Dpn1 (200 units, NEB), 10ul of 10X cutsmart buffer, and incubate at 37°C for 12h and next at 80°C for 20min.

PCR Screening

We recommend testing each gene-specific PCR assay before starting the injections.

We recommend the following Taq polymerases:

If the PCR product will be processed enzymatically (restriction enzyme): Invitrogen recombinant taq (follow the manufacturer recommendation for a 50ul reaction, do 30 cycles, 1min or more by kb). Most of restriction enzymes work with the Invitrogen recombinant Taq buffer.

If the PCR product does not need processing (only looking for a size shift or a positive PCR using GFP internal primer):
Promega Go Taq 2x Master mix.

For PCR products >1.5kb: NEB Phusion 2X Master mix, 30s by kb, 30 cycles. Note that, although regular Taq could amplify larger fragment, the full length DNA will compete with the edited one, and for amplification of both fragments in the same mixture, the Phusion Taq is a better choice. Most of the restriction enzymes work with the Phusion buffer.

-PCR strategies (Figure 1B):

For large insertions, use a primer specific to the insert ("internal primer"): This is best for detecting a large insert (such as GFP) in a large pool of F1s (8). Use your gene-specific forward external primer and an internal reverse primer (for GFP use the GFP reverse primer: cattaacatcaccatctaattc). Do the PCR using GoTaq, 50ul reaction, 30 cycles, 45s-1min elongation. Note

that the GFP reverse primer works well with annealing temperatures from 55 to 60 °C using a GoTaq.

For small insertions and modifications, design forward and reverse external primers around the edited site (but OUTSIDE the sequences in the repair template). Ideally, the PCR product should be about 500 bp centered around the edited site. Test your primers on N2 lysis with the appropriate Taq, using a gradient from 55 to 65 °C for regular Taq or 60 to 72 °C for Phusion Taq.

PCR size shift: use a 2.5% gel for insertion/deletion >18bp. For deletion > to 200bp, 1.25% agarose gel is the preferred.

A large number of F1s can be pooled together when looking for a deletion, but we generally pool 8 F1s (makes it easier to recover edits among F2/3s).

Restriction Enzyme (RE) site insertion: This is the preferred method for a small modification/insertion. 2 F1s can be pooled. Use 5ul of the PCR product and add RE/buffer/H₂O to 10ul. Do the reaction overnight. Most of the REs work with this approach (we prefer NheI because it works very well at 37 °C, is available in master mix package from NEB, and contains a putative STOP codon). Run the digestion product on 2.5% gel, short run.

-Controls:



When screening F1s, we make a reaction master mix (PCR reagents and primers) that is added to each of the F1 lysis samples. We also prepare several N2 worm lysis samples with master mix only (negative control) and a few with master mix + a positive control (see below). Be sure to add this positive control PCR after all the other tubes have been closed to avoid contamination.

Positive control for RE digestion: use a PCR fragment containing the selected restriction sites. This control could be amplified from a plasmid using the same polymerase than the one used for the screen. Use 5ul of the unpurified PCR.

Positive control for GFP screen: we recommend making a synthetic template containing both your gene-specific external forward primer and the GFP internal reverse primer. Using 0.1ul of the repair GFP template, do a PCR using GoTaq, the GFP internal reverse primer, and a new forward primer containing the sequence of your forward external primer fused to 25-28nt complementary to the 5' end of your repair GFP template. Do the PCR at 60 °C annealing, 50ul reaction, 30 cycles, 45s elongation. Purify the PCR product and use it as a positive control (100pg).

Another control that can be used when screening for GFP are lysed N2s that receives the PCR master mix as well as the external reverse primer (positive control).

Materials

- QIAprep Spin Miniprep Kit 27104 by [Qiagen](#)
MinElute PCR Purification Kit 28004 by [Qiagen](#)
 Phusion High-Fidelity PCR Master Mix with HF Buffer - 500 rxns (50 ul vol) [M0531L](#) by [New England Biolabs](#)
GoTaq Green Master Mix [M7122](#) by [Promega](#)
Taq DNA Polymerase [10342-046](#) by [Thermo Scientific](#)
 Q5 Site-Directed Mutagenesis Kit - 10 rxns [E0554S](#) by [New England Biolabs](#)

Protocol

Design and cloning of the sgRNAs

Step 1.

Design the sgRNAs and order the primers (see the guidelines).

Design and cloning of the sgRNAs

Step 2.

Clone the sgRNAs in pDD162 using Q5 mutagenesis kit (NEB).



REAGENTS



Q5 Site-Directed Mutagenesis Kit - 10 rxns [E0554S](#) by [New England Biolabs](#)



NOTES

Alexandre Paix 01 Oct 2015

pDD162 is the Cas9/sgRNA plasmid from Dickinson et al., 2013, available at [Addgene](#).

Design and cloning of the sgRNAs

Step 3.

Mix together the following:



PROTOCOL

. [Seamless editing of sgRNAs cloning mixture](#)

CONTACT: [Alexandre Paix](#)

Step 3.1.

Q5 2X master mix (12.5ul)

Step 3.2.

Forward primers 10uM (1.25ul)

Step 3.3.

Reverse primers 10uM (1.25ul)

Step 3.4.

pDD162 from a 1.5ml bacterial culture miniprep (0.5ul)

Step 3.5.

H2O (9.5ul)

Design and cloning of the sgRNAs

Step 4.

Prepare a negative control mix without the Forward primer.

Design and cloning of the sgRNAs

Step 5.

Do the PCR as follows:

30s at 98°C

10s/98°C + 20s/60°C + 4.30min/72°C for 25 cycles

2min at 72°C

10°C forever

Design and cloning of the sgRNAs

Step 6.

Digest away pDD162 for 5min at RT with:

1ul of Q5 PCR

5ul of KLD 2X buffer

1ul of KLD 10X enzyme

3ul of H2O

 DURATION

00:05:00

Design and cloning of the sgRNAs

Step 7.

Add 5ul of the digested reaction to 50ul of kit-provided competent cells

Design and cloning of the sgRNAs

Step 8.

Heat shock at 42°C for 30s

 DURATION

00:00:30

Design and cloning of the sgRNAs

Step 9.

Add 950ul of SOC medium and shake for 1h at 37°C.

 DURATION

01:00:00

Design and cloning of the sgRNAs

Step 10.

Plate 25ul on Carb plate.

Design and cloning of the sgRNAs

Step 11.

Centrifuge the remaining 975ul for 3min at 5K and also plate the pellet.

 DURATION

00:03:00

Design and cloning of the sgRNAs

Step 12.

One half (or more) of the colonies will have the correct insertion. Pick 6 colonies to grow each in 2ml

of bacterial culture.



REAGENTS

QIAprep Spin Miniprep Kit 27104 by [Qiagen](#)

NOTES

Alexandre Paix 01 Oct 2015

Keep several good clones. It's best to mix at least two clones for injection to avoid potential clones with mutations in Cas9.

Design and cloning of the sgRNAs

Step 13.

Miniprep (Qiagen kit, include the PB wash, elute in 50ul of H₂O) and send to sequencing using the forward sequencing primer in the guidelines.



REAGENTS

QIAprep Spin Miniprep Kit 27104 by [Qiagen](#)

NOTES

Alexandre Paix 01 Oct 2015

Keep several good clones. It's best to mix at least two clones for injection to avoid potential clones with mutations in Cas9.

Design of repair ssODNs

Step 14.

Design and order the repair ssODNs as described in the guidelines.

Design of repair ssODNs

Step 15.

Reconstitute oligo at 1ug/ul according to the amount provided by the manufacturer.

Construction of PCR donor templates for GFP insertion

Step 16.

Amplify the GFP plasmid [pCM1.53](#) (available at Addgene) with primers containing the desired flanking regions (30-60 bp), mutations in the sgRNA site(s) and GFP sequence.

NOTES

Alexandre Paix 30 Sep 2015

Be sure that you place GFP in frame with your ORF and that you introduce mutations to prevent recutting (as described in section for design of repair ssODNs).

Alexandre Paix 01 Oct 2015

Fw: (flanking region/sgRNA site mutations) agtaaaggagaagaacttttcactggagttg

Rev: (flanking region/sgRNA site mutations) tttgtatagttcgtccatgccatgtgtaatccc

Alexandre Paix 01 Oct 2015

FYI: In one experiment where we inserted GFP right at the cut site, we obtained the highest frequencies using 33/33 bp arms. 15/19 bp arms did not work, and longer arms did not increase edit frequency (Figure 4, Paix et al.).

Construction of PCR donor templates for GFP insertion

Step 17.

PCRs are performed using Phusion taq 2X Master Mix (NEB), 45s elongation step, 30 cycles, 50ul reaction. Annealing step is done using a gradient from 60°C to 72°C.

Construction of PCR donor templates for GFP insertion

Step 18.

Run PCR reactions on agarose gel to confirm the amplification

Construction of PCR donor templates for GFP insertion

Step 19.

Pool positive PCRs (typically three reactions) and purify using a minelute PCR purification kit (Qiagen, elution with 10ul of H2O).

REAGENTS

MinElute PCR Purification Kit 28004 by [Qiagen](#)

NOTES

Alexandre Paix 01 Oct 2015

See the guidelines for the "Optional Nested PCR step" and the "Optional Dpn1 digestion step"

Construction of PCR donor templates for GFP insertion

Step 20.

Pool the reactions and purify them using one minelute PCR purification column and measure the concentration. The DNA concentration should be >500ng/ul (at this concentration, the amount of PCR oligo remaining in the mixture will be low enough to avoid any toxicity).

NOTES

Alexandre Paix 30 Sep 2015

Oligos can significantly reduce brood size (Mello et al, 1991) and make it difficult to obtain enough F1 worms for screening.

Preparation of the injection mix

Step 21.

We use pRF4 roller plasmid at 120ng/ul, but you can use any marker that you find convenient.

NOTES

Alexandre Paix 30 Sep 2015

The marker is included to identify successfully injected mothers. Another option to identify broods likely to give edits is to use a Co-CRISPR method (e.g. Arribere et al., 2014).

Preparation of the injection mix

Step 22.

Miniprep from 3ml of bacterial culture, as for the Cas9/sgRNA plasmid. Do not let cultures grow for more than 16 hours.

DURATION

16:00:00

Preparation of the injection mix

Step 23.

Mix in a small 0.5ml eppendorf tube (Concentrations indicated are final concentrations in injection mix):

pRF4 (120ng/ul)

Repair template (30ng/ul for a 125nt ssODN, 50ng/ul for a PCR template)

Cas9/sgRNA clones (50ng/ul)

Add H2O to 15ul

NOTES

Alexandre Paix 01 Oct 2015

When using multiple oligos, we still keep each around 30ng/ul but BEWARE too much oligo can significantly reduce brood size. We use the same ssODN concentration as recommended in Zhao et al., 2014.

Alexandre Paix 01 Oct 2015

When using multiple sgRNAs, we still keep each individual Cas9/sgRNA plasmid around 50ng/ul. We use the same concentration of Cas9/sgRNA as recommended in Dickinson et al., 2013.

Preparation of the injection mix

Step 24.

Centrifuge at 13K for 15min on tabletop centrifuge.

🕒 **DURATION**

00:15:00

Preparation of the injection mix

Step 25.

Load injection needles with the injection mix.

📝 **NOTES**

Alexandre Paix 30 Sep 2015

Be careful not to touch bottom of tube with loading needle – to avoid loading precipitates that will clog your injection needle. Loading the needle under a stereomicroscope can help.

Preparation of worms for injection

Step 26.

Bleach a large plate of worms

Preparation of worms for injection

Step 27.

WashA: wash with M9.

Preparation of worms for injection

Step 28.

WashB: wash with M9.

Preparation of worms for injection

Step 29.

Plate embryos (less than 2000) on NA22 large plate. (NOT completely covered with NA22 bacteria)

Preparation of worms for injection

Step 30.

Incubate multiple plates at different temperatures to ensure to have at least one with young adults (few embryos /one embryo row) on the day of injection.

Preparation of worms for injection

Step 31.

Pick hermaphrodites with a sharp pick from areas of the plate where there are no bacteria and place on injection pad.

📝 **NOTES**

Alexandre Paix 01 Oct 2015

As a baseline: for our lab N2 strain, bleached embryos reached the correct stage for injection after 55 hours at 25°C, or at 15°C for the first night followed by 2 days at 25°C, or at 20°C for 2 days followed by one night at 25°C.

Injections

Step 32.

Inject 30-40 worms.

🔗 NOTES

Alexandre Paix 30 Sep 2015

See [Worm book protocol](#)

Worm recovery after injection

Step 33.

Adding M9 #1: add **5ul** of M9 (Start adding M9 every 5-10 minutes, about 1h after the worms have been put in recovery buffer)

🕒 DURATION

00:05:00

Worm recovery after injection

Step 34.

Adding M9 #2: add **5ul** of M9

🕒 DURATION

00:05:00

Worm recovery after injection

Step 35.

Adding M9 #3: add **5ul** of M9

🕒 DURATION

00:05:00

Worm recovery after injection

Step 36.

Adding M9 #4: add **10ul** of M9

Worm recovery after injection

Step 37.

Adding M9 #5: add **10ul** of M9

🕒 DURATION

00:05:00

Worm recovery after injection

Step 38.

Adding M9 #6: add **15ul** of M9

🕒 DURATION

00:05:00

Worm recovery after injection

Step 39.

Adding M9 #7: add **15ul** of M9

🕒 DURATION

00:05:00

Worm recovery after injection

Step 40.

Adding M9 #8: add **15ul** of M9

🕒 DURATION

00:05:00

Worm recovery after injection

Step 41.

Adding M9 #9: add **20ul** of M9

DURATION

00:05:00

Worm recovery after injection

Step 42.

Adding M9 #10: add **20ul** of M9

DURATION

00:05:00

Worm recovery after injection

Step 43.

Put a drop of 20ul of M9 on a new OP50 plate, outside the bacteria layer.

Worm recovery after injection

Step 44.

With a pick, transfer 5 to 10 injected worms from the recovery buffer to the M9 drop and push them away from the M9 drop towards the food.

Worm recovery after injection

Step 45.

Repeat until all the worms are transferred. Even if the worms look inert at this or the next step, they are worth transferring as they may yield edited progeny.

Worm recovery after injection

Step 46.

Leave the injected worms on OP50 plates at room temperature for 5h and then transfer each worm (P0) to a new OP50 plate (1 P0 per plate).

DURATION

05:00:00

NOTES

Alexandre Paix 01 Oct 2015

We recommend using fresh OP50 plates with thin bacterial lawns – easier to identify roller F1s.

P0 and F1 handling

Step 47.

Allow the P0s to lay eggs at 20°C for 1 or 2 days.

P0 and F1 handling

Step 48.

Transfer the P0s to fresh OP50 plates between the first and second day.

NOTES

Alexandre Paix 30 Sep 2015

Most of the Roller F1s are found on the first-day plates. Edits obtained using ssODNs are found on both days. Most edits made using PCR templates are found on the second day plate (see Table S4). Edits are found in both Roller and non-Roller worms but are only found among broods that contain Rollers.

P0 and F1 handling

Step 49.

Let the F1s grow at 20°C. When all the F1s have reached the young adult stage (4 days at 20°C), check for rollers.

NOTES

Alexandre Paix 01 Oct 2015

Ignore P0s that don't give any rollers at all.

Alexandre Paix 01 Oct 2015

Expect around 20-50% of P0s to give rollers, but there may be less – in our hands this has varied greatly from experiment to experiment. Recheck plates on subsequent days if you don't see rollers on initial examination. Rollers grow more slowly than non-rollers, may not be obvious, and may be very few.

P0 and F1 handling

Step 50.

GFP fusions: if you know what you are looking for, it is possible to screen directly for GFP expression in the F1 (or F2) animals.

🔗 NOTES

Alexandre Paix 01 Oct 2015

Keep in mind that, if your sgRNAs are working, 20-30% of broods will have a very high number of edits (20-60%) on the second day ("jackpot broods"). To identify jackpot broods, we recommend screening ~20 F1s from the second day plate for all the broods that segregate rollers. Once you have identified GFP+ broods, clone F1s from these to isolate homozygous edits. You can use the PCR screening to confirm the edit and to sequence it.

P0 and F1 handling

Step 51.

PCR screening: Transfer the F1 rollers and their non-roller F1 siblings to new plates (2 to 8 F1s per plate).

🔗 NOTES

Alexandre Paix 30 Sep 2015

We typically try to screen all the F1s (roller and non roller) from P0s with roller progeny, but that may not be necessary depending on the efficiency of your sgRNA.

P0 and F1 handling

Step 52.

PCR Screening: Let the F1 lay eggs for 24h at 20°C.

🕒 DURATION

24:00:00

P0 and F1 handling

Step 53.

Lyse the F1s for PCR: In each 10 uL tube of lysis buffer, put 2 to 8 F1s.

🔗 NOTES

Alexandre Paix 01 Oct 2015

Use 5ul of lysis for PCR (50ul final volume) (2ul in 25ul final volume PCR could also be used, however some multichannel pipettes are not accurate for volume under 5ul).

Alexandre Paix 01 Oct 2015

Up to 8 F1s may be added to each tube for lysis, if and only if you are screening for a large deletion (50% or less of the full length PCR fragment) or for a positive PCR (using internal primer for GFP insertion).

PCR Screening

Step 54.

We recommend testing each gene-specific PCR assay before starting the injections. See the guidelines for PCR Screening details.

🔗 NOTES

Alexandre Paix 30 Sep 2015

We recommend testing each gene-specific PCR assay before starting the injections.

F2/3 handling

Step 55.

Clone the F2/3s from positive F1 plates. It is useful to let the worms crawl on a no-bacteria plate before picking to avoid accidental transfer of siblings.

If 2 F1s were pooled per plate, clone **16 F2s**.

If 8 F1s were pooled per plate, chunk the starved plate if necessary and clone **24 to 32 F2/F3s**.

F2/3 handling

Step 56.

Lyse and PCR F2/3s using the same methods as for the F1s. **EXCEPT:** When looking for homozygous GFP worms, use primers that flank the GFP fusion.

F2/3 handling

Step 57.

Use the PCR product for sequencing: Clean 25ul of the PCR reaction using Qiagen Minelute kit, elute with 10ul of H₂O. Use 7ul for this elution as a template and use a primer inside the PCR product for sequencing.



REAGENTS

MinElute PCR Purification Kit 28004 by [Qiagen](#)



NOTES

Alexandre Paix 30 Sep 2015

If the sequence contains mismatches, use the remaining worm lysis samples to perform a second PCR with Phusion Taq (Phusion Taq has lower error rate than other Taqs).

F2/3 handling

Step 58.

Once a homozygous F2/3 plate is identified, it is recommended to clone 4 worms again to new plates and to verify their genotype to ensure that the line is truly homozygous.

F2/3 handling

Step 59.

Freeze the worms. We recommend freezing at least two independent lines (derived from different P0s if possible or different F1s) for each type of edit.