Robust genome editing with short single-stranded and long, partially single-stranded DNA donors in C. elegans

Materials:

1. *S. pyogenes* Cas9 3NLS (10 μg/μl, IDT)
2. tracrRNA (IDT# 1072532)
3. crRNA (2nmol or 10nmol, IDT)
4. ssDNA donor (standard desalting; 4 nmol Ultramer, IDT)
5. PRF4::*rol-6(su1006)* plasmid

Re-suspension:

- Aliquot 0.5 μl (5μg) of Cas9 protein and store at -80°C to avoid freeze/thaw cycles. Use 1 aliquot per injection and add all the other reagents sequentially to the Cas9 tube.
- tracrRNA – 0.4 μg/μl in IDT duplex buffer, store at -20°C
- crRNA – 0.4 μg/μl in IDTE P.H 7.5, store at -20°C
- ssDNA oligo donor – 1 μg/μl in ddH2O, store at -20°C
- PRF4::*rol-6 (su1006):* 500 ng/μl

Donor Design and Generation

ssODN donors:

For generating short inserts (<130bp)

To generate a ssODN donor, add 35 bases of 5′ homology sequence in front of the tag and 35 bases of the 3′ homology sequence at the end. Remember to mutate the PAM site or the guide binding sequence if it is not already disrupted by the insert. If the guide binding sequence is mutated, length of homology sequence should be 35bp from the last mutation (Paix *et al.* 2015).

For generating point mutations:

Pick 35bp homology upstream and 35bp homology downstream of your guide cut site, which should ideally be within 20bp of the desired mutation site. Introduce the desired mutations in the donor and the PAM/ guide binding sequence.

For large deletions with 2 guides:

Pick 35bp homology upstream of the left-guide cut site and 35bp homology downstream of the right-guide cut site and put them together. Everything in between will be removed. Deletions up to 1kb can be easily achieved through this strategy. In principle, this should work for larger deletions as well.

dsDNA asymmetric-hybrid donors:

1. Order 140bp oligos from IDT as Ultramers; 120bp as homology arms and 20bp complementary to GFP (or any other desired insert). Also, order standard oligos just complementary to your insert (no homology arms).
2. Generate two PCR products as shown below in figure; one with 120bp homology arms and the other without any homology arms (only insert sequence) using an insert containing plasmid as the template for PCR; perform 4 to 8 50 μl reactions for each product.

3. Run 5 μl on an agarose gel to check if a single bright band at ~1050bp (gfp +120bp+120bp) is obtained (in some cases the template plasmid band might be detected. It can be ignored as it does not interfere with HDR.). If non-specific amplification is observed, set up a temperature gradient and find the optimal temperature. *(OPTIMIZATION NOTE: we find that amplification of challenging templates with long ultramer primers is aided by increasing the template plasmid concentration and decreasing primer concentrations.)*
4. Combine all the PCR reactions of each product and column purify (we use Qiagen minElute kit), elute in 10-20 μl of water depending on brightness of the band, aiming to get >300ng/ul concentration.
5. Mix 1:1 of the purified PCRs (2μg:2μg for 20μl injection mix), heat to 95ºC and cool to 4ºC to re-anneal (95ºC-2:00 min; 85ºC-10 sec, 75ºC-10 sec, 65ºC-10 sec, 55ºC-1:00 min, 45º-30 sec, 35º-10 sec, 25º- 10 sec, 4º-forever.)
6. Add this donor cocktail to the rest of the injection mixture ONLY after pre-incubating Cas9, crRNA and tracrRNA (see below).

(STORAGE NOTE: we store individual purified donors at -20C and pre-assemble the cocktail directly before injection. We have not explored storage and re-use of pre-assembled cocktails)

Preparing injection mixtures:

Add components of the injection mixture in the following sequence:

1. Cas9- 0.5 μl of 10 μg/μl stock
2. Add tracrRNA – 5μl of 0.4 μg/μl stock
3. Add crRNA – 2.8μl of 0.4 μg/μl stock (if you are using two guides add 1.4 μl of each)
4. Incubate this mixture @37°C for 10 minutes before adding any DNA. Adding any double stranded DNA before RNP complex formation reduces HDR efficiency dramatically.
5. Add ssODN donor – 2.2 μl of 1μg/μl stock or dsDNA donor cocktail – 200 ng/μl (total 4 μg) in the final injection mixture
6. Add PRF4::*rol-6 (su1006)* plasmid – 1.6 μl of 500 ng/μl stock
7. If needed, add nuclease free water to bring the volume to 20μl
8. To avoid needle clogging, centrifuge the mixture @14000rpm for 2 min, transfer about 17 μl of the mixture to a fresh tube and proceed to loading the needles.

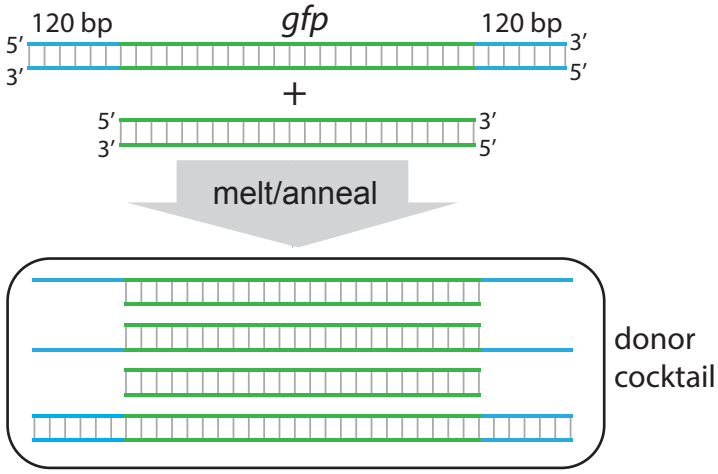
Note: some protocols provide additional KCl in the injection mixture to reduce Cas9 aggregation. Under the reduced Cas9 concentration conditions we have not found aggregation to be an issue and find that KCl from IDT's buffer added to tracrRNA is sufficient.

Micro-injection and screening:

1. Inject 10 to 20 animals and transfer them onto individual plates. After about 3 days, score for F1 rollers and place each roller onto a separate NGM plate.
2. In general, injections with ssODNs yield more number of F1 rollers per injected animal compared to the injections with dsDNA.
    a. For ssODN-based editing: Pick 2 plates that segregate the most number of F1 rollers and from these 2 plates, pick about 24 F1 rollers and place them onto separate plates.
    b. For dsDNA-based editing: Pick at least 24 F1 rollers from several plates and place them onto to separate plates.
3. To avoid false positives due to mosaicism in F1 animals, pick several F2s from each plate, perform lysis and genotyping. Genotyping primers should lie outside the homology arms to

avoid false positives from transiently retained donor molecules. In some circumstances large inserts do not readily amplify in heterozygotes (because the small wild type band amplifies preferentially) In those situations it might be necessary to employ one primer inside the insert for each junction.

4. Alternatively, if the expression levels are detectable, insertions of fluorescent tags can be screened under a microscope either by using high magnification fluorescence microscope (mount several F2 animals onto 2% agarose pads) or by using a fluorescence dissecting scope.



Reference:

Paix, A., A. Folkmann, D. Rasoloson and G. Seydoux, 2015 High Efficiency, Homology-Directed Genome Editing in Caenorhabditis elegans Using CRISPR-Cas9 Ribonucleoprotein Complexes. Genetics 201**:** 47-54.