

# Melting dsDNA donor molecules greatly improves precision genome editing in *C. elegans*

## Cas9 Based Genome Editing

### I. Materials:

1. *S. pyogenes* Cas9 3NLS (10  $\mu\text{g}/\mu\text{l}$ , IDT)
2. tracrRNA (IDT)
3. crRNA (2 nmol or 10 nmol, IDT)
4. ssODN 4 nmol Ultramer (standard desalting, IDT)
5. PRF4::*rol-6*(*su1006*) plasmid (high quality Midi or Maxiprep)
6. SPRI paramagnetic beads (AMPure XP, Beckman Coulter)

### Re-suspension (Stock Solutions):

1. Aliquot 0.5  $\mu\text{l}$  (5  $\mu\text{g}$  or 30 pmol) of Cas9 protein and store at  $-80^{\circ}\text{C}$  (avoid freeze/thaw cycles)
2. tracrRNA – 0.4  $\mu\text{g}/\mu\text{l}$  (18  $\mu\text{M}$ ) in IDT nuclease free duplex buffer, store at  $-20^{\circ}\text{C}$  (aliquots at  $-80^{\circ}\text{C}$ )
3. crRNA – 0.4  $\mu\text{g}/\mu\text{l}$  (34  $\mu\text{M}$ ) in TE PH 7.5 (IDT), store at  $-20^{\circ}\text{C}$  (aliquots at  $-80^{\circ}\text{C}$ )
4. ssDNA oligo donor – 1  $\mu\text{g}/\mu\text{l}$  in ddH<sub>2</sub>O, store at  $-20^{\circ}\text{C}$
5. PRF4::*rol-6* (*su1006*): 500 ng/ $\mu\text{l}$ , store at  $-20^{\circ}\text{C}$

### II. Injection mixture preparation:

Add components of the injection mixture to the tube containing Cas9 in the following sequence:

1. Cas9 – 0.5  $\mu\text{l}$  of 10  $\mu\text{g}/\mu\text{l}$  stock (30 pmol)
2. Add tracrRNA – 5  $\mu\text{l}$  of 0.4  $\mu\text{g}/\mu\text{l}$  stock (90 pmol)
3. Add crRNA – 2.8  $\mu\text{l}$  of 0.4  $\mu\text{g}/\mu\text{l}$  stock (95 pmol) (if two guides are needed add 1.4  $\mu\text{l}$  of each)
4. Pipette the mixture gently several times and incubate @ $37^{\circ}\text{C}$  for 15 minutes. In our experience adding any double stranded DNA before RNP complex formation reduces HDR efficiency.
5. Add ssODN donor – 2.2  $\mu\text{l}$  of 1  $\mu\text{g}/\mu\text{l}$  stock (see note (3) below) (or)  
Add melted dsDNA – 500 ng (final concentration: 25 ng/ $\mu\text{l}$  for ~1kb donors or 45 fmol/ $\mu\text{l}$ )
6. Add PRF4::*rol-6* (*su1006*) plasmid – 1.6  $\mu\text{l}$  of 500 ng/ $\mu\text{l}$  stock
7. Add nuclease free water to bring the final volume to 20  $\mu\text{l}$  and pipette gently several times.
8. To avoid needle clogging, centrifuge the mixture @14000rpm for 2 min, transfer about 17  $\mu\text{l}$  of the mixture to a fresh tube and keep the tube on ice; proceed to loading the needles.

### Notes:

1. All the above steps in section II can be performed at room temperature
2. Aggregation is not an issue under these Cas9 concentrations.
3. Although we haven't explored the optimal dose range for ssODNs, given the efficiencies obtained with dsDNA at 25 ng/ $\mu\text{l}$ , much lower doses of ssODN could be used.
4. Final injection mixture can be stored at  $4^{\circ}\text{C}$  and re-used for several months (up to 6 months) without compromising efficiency; we have not yet tested mixes that are older than 6 months.

## **Cas12a (Cpf1) Based Genome Editing**

### **I. Materials:**

1. A.s. Cas12a Ultra (10  $\mu\text{g}/\mu\text{l}$ , IDT)
2. Cpf1-crRNA 21 bases long (2 nmol or 10 nmol, IDT)
3. ssODN 4 nmol Ultramer
4. PRF4::*rol-6*(*su1006*) plasmid (high quality Midi or Maxiprep)
7. SPRI paramagnetic beads (AMPure XP, Beckman Coulter)

### **Re-suspension (Stock Solutions):**

1. Aliquot 0.5  $\mu\text{l}$  (5  $\mu\text{g}$  or 32 pmol) of Cas12a protein and store at  $-80^{\circ}\text{C}$  (avoid freeze/thaw cycles)
2. Cas12a-crRNA – 40  $\mu\text{M}$  in TE PH 7.5 (IDT), store at  $-20^{\circ}\text{C}$  (aliquots at  $-80^{\circ}\text{C}$ )
3. ssDNA oligo donor – 1  $\mu\text{g}/\mu\text{l}$  in ddH<sub>2</sub>O, store at  $-20^{\circ}\text{C}$
4. PRF4::*rol-6* (*su1006*): 500 ng/ $\mu\text{l}$ , store at  $-20^{\circ}\text{C}$

### **II. Injection mixture preparation:**

Add components of the injection mixture to the tube containing Cas9 in the following sequence:

1. Cas12a – 0.5  $\mu\text{l}$  of 10  $\mu\text{g}/\mu\text{l}$  stock (32 pmol)
2. Add cas12a-crRNA – 2.5  $\mu\text{l}$  of 40  $\mu\text{M}$  stock (100pmol)
3. Add TE PH 7.5 – 3.0  $\mu\text{l}$
4. Pipette the mixture gently several times and incubate @ $37^{\circ}\text{C}$  for 15 minutes
5. Add ssODN donor – 2.2  $\mu\text{l}$  of 1  $\mu\text{g}/\mu\text{l}$  stock (see note (3) below) (or)  
Add melted dsDNA – 500 ng (final concentration: 25 ng/ $\mu\text{l}$  for ~1kb donors or 45 fmol/ $\mu\text{l}$ )
6. Add PRF4::*rol-6* (*su1006*) plasmid – 1.6  $\mu\text{l}$  of 500 ng/ $\mu\text{l}$  stock
7. Add nuclease free water to bring the final volume to 20  $\mu\text{l}$  and pipette gently several times.
8. To avoid needle clogging, centrifuge the mixture @14000rpm for 2 min, transfer about 17  $\mu\text{l}$  of the mixture to a fresh tube and keep the tube on ice; proceed to loading the needles.

#### *Notes:*

1. *All the above steps in section II can be performed at room temperature*
2. *TE is added in step 3 for easier pipetting; by further diluting the crRNA stock this step can be omitted.*
3. *Although we haven't explored the optimal dose range for ssODNs, given the efficiencies obtained with dsDNA at 25 ng/ $\mu\text{l}$ , much lower doses of ssODN could be used.*

### III. Donor Design and Generation

#### ssODN donors:

To generate ssODN donor, add 35 bases of 5' homology sequence in front of the tag (or mutations) and 35 bases of the 3' homology sequence at the end. 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 or if silent mutations are introduced between the guide cleavage site and the desired insertion site, length of homology sequence should be 35bp from the last mutation.

#### dsDNA donors:

Generate dsDNA donors by PCR either by using unmodified oligos or 5' SP9-modified oligos.

1. Order unmodified (or 5' SP9 modified) oligos with standard desalting (IDT); 35nt as homology arms and 20nt complementary to insert (eg: GFP). SP9 modifications are available at 100nmol scale from IDT.
2. Perform PCR with an insert-containing plasmid as the template for amplification; use High-Fidelity polymerase.
3. Run a few microliters of PCR on agarose gel to check if a single bright band is obtained. If non-specific amplification is observed, set up a temperature gradient and find the optimal temperature.
4. PCR clean-up: use one of the following three options depending on your experimental conditions.
  - a. Purify the PCRs using spin-columns and elute DNA in 20  $\mu$ l of nuclease free water. Generally, column purification is sufficient, and you may proceed to step 5. However, some primer pairs produce long (~80bp) primer dimers that may contain the entire homology arms. Spin-columns may not be able to remove dimers of this length completely. We found that these short "dimer donors" are preferentially used as templates over full-length donors with the desired insert (such as GFP). *Note:* Dimers may or may not be visible on the agarose gel.
  - b. If dimer formation is a concern, use 0.6x SPRI beads (AMPure XP) to perform the clean-up instead of spin-columns. For example: add 60  $\mu$ l of beads to 100 $\mu$ l of PCR, wash with 70% ethanol twice, elute in nuclease free water (refer to the bead manufacturer's protocol for further details).
  - c. If primer dimers are clearly visible on the gel, then it is best to gel-extract the DNA. However, gel extracted DNA can be toxic, presumably due to the presence of guanidine hydrochloride (component of binding buffer) in the final elute. To reduce salt contamination, incubate the column with wash buffer for 10 min before centrifugation; repeat washes 2-3 times. Strong absorbance at 230nm on Nanodrop suggests GuHCl contamination. For best results, gel-extracted DNA should be further purified with 1x to 1.5x AMPure XP beads (strongly recommended).
5. After purification, dilute a portion of dsDNA PCR donor to 100 ng/ $\mu$ l and transfer about 5.5  $\mu$ l to a PCR strip tube and proceed to the heating step.
6. Heat to 95 °C and cool to 4 °C using thermal cycler (95 °C-2:00 min; 85 °C-10 sec, 75 °C-10 sec, 65 °C-10 sec, 55 °C-1:00 min, 45 °C-30 sec, 35 °C-10 sec, 25 °C- 10 sec, 4 °C-hold. Ramp down at 1 °C/sec at every step).
7. Add melted donor DNA to rest of the injection mixture only after pre-incubating RNP complexes.

*Note: we store purified donors at -20 °C and melt them right before adding to the injection mix. We have not explored storage and re-use of melted donors.*

#### IV. Micro-injection and Screening:

1. Inject 5 to 10 young adults and transfer them onto individual plates. If both arms of the hermaphrodite gonad are injected, a good injection should yield 20 to 40 F1 Rollers.
2. After about 72 hours post injection, score for number of F1 Rollers and choose 2 plates with the highest number of Rollers.

*Note:* We generally culture the injected animals at room temperature (~22°C-23°C).

3. a. For indels: choose 2 P0 plates that segregate the highest number of F1 Rollers; pick 12-24 F1 Rollers from these 2 plates and place them onto separate plates.  
b. For ssODN-based editing: choose 2 P0 plates that segregate the highest number of F1 Rollers; pick about 24 F1 Rollers from these 2 plates and place them onto separate plates.  
c. For dsDNA-based editing: Choose 2 plates that segregate the highest number of F1 Rollers and from these 2 plates, pick ~24 non-Rollers that are younger than Rollers and place them onto separate plates. Younger animals among the Roller cohorts can also be picked. For inexperienced injectors, we recommend using 5' end-modified dsDNA donors and picking F1 Rollers.
4. To avoid false positives due to mosaicism in F1 animals, pick several F2s from each plate, perform pooled lysis and genotype. Genotyping primers should lie outside of the homology arms to avoid amplification from transiently retained donor molecules.
5. Alternatively, correct insertions of fluorescent tags can be screened under a fluorescence dissecting scope or by using high magnification fluorescence microscope. For high magnification screening, mount several F2 animals onto 2% agarose pads and immobilize with levamisole.