C. elegans injection

Alt-R™ CRISPR-Cas9 System ribonucleoprotein delivery

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The methods presented here are provided by customers who have used the Alt-R CRISPR-Cas9 System. This can serve as a starting point for using the Alt-R CRISPR-Cas9 System in similar model organisms but may not be fully optimized for your gene or application. IDT does not guarantee these methods, and application specialists at IDT can only provide general guidance with limited troubleshooting and support.

Screening

The Dernburg lab usually enriches for edited F1 progeny using 1 of 2 published methods. One method involves co-injection of 2 plasmids carrying red fluorescent transgenes (pCFJ90 [*Pmyo-2::mCherry*] and pCFJ104 [*Pmyo-3::mCherry*]), and then picking and screening red fluorescent F1 progeny for correctly edited genes. The second method is based on the *dpy-10* co-CRISPR strategy described by the Fire lab [1].

Both methods are quite effective. It is somewhat easier to screen for *dpy-10* mutations (by identifying Roller or Dumpy progeny), but the high mutation efficiencies can make it difficult to find many Rollers (*dpy-10* heterozygotes), and these mutations may need to be separated from the desired edits by crossing. The advantage of co-injecting red fluorescent transgenes is that they are not integrated into the genome. Many F1 progeny that carry the desired edits are already homozygous for the insertions, and when they are not, they often have indels at the second locus, indicating that Cas9 cleavage is extremely robust. When the Dernburg lab performs *dpy-10* co-CRISPR with injected protein, they obtain "jackpot" broods in which virtually all F1 produced during a time window after injection show a Dumpy or Roller phenotype, further indicating that a good injection often leads to extremely high cutting/editing efficiency.

Using Cas9 protein with Alt-R CRISPR tracrRNA + crRNA as an RNP complex, the Dernburg lab has seen editing efficiencies ranging from 25–77% of red fluorescent or Roller F1 progeny carrying (small) insertions, which are often homozygous, using several different target genes/target sequences. By comparison, plasmid-based delivery of Cas9 and sgRNA typically gave the desired insertion in only ~1% of red fluorescent or Roller F1 progeny. Larger insertions (e.g., fluorescent protein fusions) have been somewhat less efficient and more variable, but still occur at frequencies high enough to make positive selection (e.g., selection for hygromycin resistance) unnecessary.

For simple gene disruptions, one can inject Cas9 + gRNA without a repair template and screen for indels, but it is often useful to include a template that will insert a small sequence, e.g., with a rare restriction site, which can greatly facilitate screening and (later) genotyping for strain construction or maintenance.

Methods

1. Prepare injection mixture

Note: Assemble the Cas9:crRNA:tracrRNA, ribonucleoprotein (RNP) complex following the IDT Alt-R CRISPR RNP protocol, but use higher concentrations of both protein and RNA oligos as described here.

a. Anneal Alt-R CRISPR crRNA and tracrRNA (200 µM each) in IDT Nuclease-Free Duplex Buffer: 5 min at 95°C, followed by 5 min at room temperature (RT).

For the *dpy-10* co-CRISPR strategy [1], try a ratio of 1:12 [*dpy-10*:target gene] crRNAs. (The *dpy-10* crRNA target sequence used by the Fire lab is GCUACCAUAGGCACCACGAG [1].)

b. Mix 27 µM crRNA:tracrRNA duplex (from step 1a) with 27 µM Cas9-NLS protein and incubate for 5 min at RT.

c. Assemble the injection mix with the following final concentrations:

- 17.5 µM Cas9 protein
- 17.5 µM crRNA:tracrRNA duplex(es)
- 6 µM ssDNA repair template(s) or 0.16–2 µM dsDNA repair template(s), (higher concentrations preferred)

If using *dpy-10* co-CRISPR, also include:

- 0.5 µM ssDNA repair template for *dpy-10*

If using red fluorescent transgene co-injection, also include:

- 2.5 ng/µL pCFJ90 injection marker plasmid (toxic, if too highly expressed)
- 5 ng/µL pCFJ104 injection marker plasmid

2. Inject 10–15 young adult hermaphrodites (or more) with sufficient injection mix to see visible "puffing" of the gonad, and recover onto individual plates.

3. After an appropriate number of days post-injection*, pick and transfer fluorescent F1 progeny (if fluorescent markers were co-injected) or Rollers† from jackpot broods (for *dpy-10* co-CRISPR) to individual plates. Allow them to produce self-progeny, then lyse and screen by PCR—typically using one primer within the insertion and one in a flanking sequence.

* Injected P0s may be incubated at 25°C to obtain transgenic F1 progeny more quickly. In this case, pick red fluorescent F1 progeny after 2 days. Rollers from *dpy-10* co-CRISPR develop more slowly than WT animals, so you may need to wait an additional day to pick them: ~3 days after injection at 25°C or 4 days at 20°C.

† Roller F1 progeny are usually heterozygous for *dpy-10* mutations. You can also pick the Dumpys, but they are likely homozygous *dpy-10* mutants, so you may need to cross this out later, depending on your goals.

Templates for homology directed repair

Single-stranded repair templates

The Dernburg lab currently uses single-stranded DNA (ssDNA) templates to insert both small and large tags, with flanking homologous ends ranging from ~35–500 bases. Although their evidence was preliminary at the time this protocol was written, in several direct comparisons, they observed higher insertion frequencies with long ssDNA templates than the corresponding dsDNA (plasmid or PCR product). Using Cas9 RNPs + ssDNA templates, they also see fewer aberrant events (e.g., complex indels) than with plasmid delivery of Cas9.

They have found that either forward or reverse single stranded templates work better than the corresponding dsDNA template. This is consistent with recent work in mammalian tissue culture cells [2]. Single-stranded templates can be prepared from the corresponding double-stranded gBlocks® Gene Fragments, PCR products, or plasmids by a variety of methods, including: 1) primer extension/asymmetric PCR, 2) PCR using one phosphorylated oligonucleotide followed by Lambda exonuclease digestion of the 5′-phosphorylated strand, or 3) *in vitro* transcription of one strand followed by reverse transcription.

Geraldine Seydoux's lab has also recently published work showing that overlapping short ssDNA templates can successfully direct repair of Cas9-induced breaks in *C. elegans* [3].

Double-stranded repair templates

For insertion templates that are too large to be synthesized as an Ultramer® oligo (i.e., longer than 200 bases), the Dernburg lab used gBlocks® Gene Fragments or constructed a plasmid template.

References

1. Arribere JA, Bell RT, et al. (2014) Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. Genetics, 198(3):837–846.
2. Richardson CD, Ray GJ, et al. (2016) Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotech, 34(3):339–344.
3. Paix A, Schmidt H, et al. (2016) Cas9-assisted recombineering in *C. elegans*: Genome editing using *in vivo* assembly of linear DNAs. Nucleic Acids Res, 44(15):e128.

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