CRISPR for C. elegans with dpy-10 coCRISPR and pre-loaded Cas9

Introduction

This method follows the strategy developed by the Sedoux lab (Arribere et al. 2014) for coCRISPR and the protocol from the Dernburg lab on the IDT website for using Cas9 protein. The strategy is to introduce a mutation into the *dpy-10* locus along with editing the target gene. Edits in *dpy-10* indicate that Cas9 is active. This allele, *dpy-10(cn64),* causes a dominant Rol phenotype. Deletion of *dpy-10* leads to Dpy animals. It has been shown that there is no correlation between CRISPR at different loci. Therefore, non-Rol animals can be picked and will have the same chance to be edited at the target locus as those that are edited for *dpy-10*.

We have recently reduced the amount of dpy-10 to very little or none. Picking a few F1 has been enough to get deletions, small insertions and even GFP insertions.

Guide RNAs and Cas9 can be purchased from IDT or other companies

IDT now sells crRNAs that need to be combined with tracerRNA, or sgRNAs that can be used as is.

There is also an NEB kit with which one can generate sgRNAs from oligos (10 reactions are ~$200).

For design of guide RNA:
Use Benchling tool!
http://chopchop.cbu.uib.no/
http://crispr.mit.edu
IDT website.
The rating for the guides differs between these sites. What is a top hit for ChopChop results in a low-efficiency warning at IDT.

Repair template can also be purchased if it is short enough or if the lab has $$ to burn. Longer repair templates can be made by PCR.
See entry "making repair templates"

Materials

For injection mix

- Duplex buffer from IDT
- Gene-specific crRNA from IDT at 200µM in duplex buffer (resuspend 2nmol in 10µl duplex buffer). Alternatively, buy sgRNAs, which are more expensive but seem to have higher efficiency.
- dpy10 crRNA at 200µM in duplex buffer (ordered from IDT using this sequence GCTACCATAGGCACCACGAG)
- tracerRNA from IDT at 200µM in duplex buffer
- Cas9 from IDT, which has a concentration of 62µM (610 pmol in 10µl) or 10µg/µl (10µl cost around $200). Keep in aliquots at -70ºC!
- Alternatively GeneScript also offers CRISPR reagents: https://www.genscript.com/protein/Z03385_100-GenCrispr_Cas9_C_NLS_Nuclease.html. Their nuclease is at a lower concentration. This nuclease comes with a Cas9 working buffer that, however, does not contain potassium.
- dpy10 repair template (SEA-107 from Sarah Albritton in the Ercan lab) at 1µM concentration in water (Sequence from Arribiere et al.: cacttgaacttcaatacggcaagatgagaatgactggaaaccgtaccgcATgCggtgcctatggtagcggagcttcacatggcttcagaccaacagcct).

- gene-specific repair template single stranded 20µM in water short:
  - Order ssDNA from a company
  - Ordering repair templates: **IDT** offers regular oligos up to 60 nt long. For longer pieces, one can order "ultramers", which go up to 200 nt. **Sigma** offers standard oligos up to 120 nt long and long oligos up to 180 bases but these are much more expensive than the ultramers.
  - IDT ships the longer oligos at a 2 nmol concentration. We make a 100µM stock in TE buffer (add 20 µl) and then dilute 1:5 with water.
- Long gene-specific repair template single stranded or double stranded
  - See separate Benchling entry for more information
  - To make Use 400ng dsDNA template if it is ~1kb long, 2µl of one primer @ 25µM in a 50µl reaction with high fidelity PCR enzyme (e.g. Perfect Taq Plus). PCR protocol without annealing step, 72ºC extension for 1 minute and 40 cycles. Run on gel, there should be two bands one at the size of the dsDNA product and one that is smaller; but this depends on the sequence. Purify with column purification kit. Because ssDNA does not bind as well to the matrix, it is advised to set up two 50µl reactions and purify them on the same column. Elute with the smallest possible volume. Concentration measurement with Nanodrop is also not accurate, but using the Nanodrop read as a guide has worked in the past.
  - Larger repair templates can also be ordered from IDT as so-called Megamer. For the mip-2 repair template (inserting GFP), we received 12 pmol (= 3µg). I diluted this in 6µl TE buffer to get a 2µM concentration and used 1µl for the injection mix (0.2µM concentration). IDT does not recommend to dissolve this DNA in such a small volume, but it worked.
  - For double stranded repair templates, melt at 95 ºC for 5 minutes and slam on ice.
- Worms
  - The worms should be young adults, ideally without any or just very few embryos in their uteri. Dauers that were kept at 20ºC for 36-48 hours are also good. Worms should not have been starved at any time in their life (unless they are dauers).
- Seeded 3.5 cm plates
  - It is best if the patch of OP50 does not touch the side of the plate, worms sometimes crawl on the wall of the plate and get lost.
- For screening:
  - Proteinase K (0.2mg/ml in sterile water, but we use a solution from Takara that is stable at 4ºC, we use it at a 1:10 dilution in lysis buffer)**NOTE: i made a new lysis buffer +proteinase K solution. we have a Qiagen Proteinase K (20mg/mL) which i diluted to 2mg/mL. i then added this solution to the lysis buffer (1ul of 2mg/mL proteinase K per 100 ul of lysis buffer).**
  - Lysis buffer (see below)
  - Primers to detect the edit
  - PCR enzyme and buffer (we are using Perfect Taq Plus)
  - Materials to run an agarose gel for 48 samples
- Lysis buffer recipe
  - 50 mM KCl (2.5 ml of 2 M per 100 ml buffer)
  - 10 mM Tris-HCl, pH 8.3 (500 µl of 2M stock per 100 ml buffer)
  - 2.5 mM MgCl2 (125 µl of 2 M per 100 ml buffer)
  - 0.45% Nonidet P-40 (450 µl per 100 ml buffer)
  - 0.45% Tween-20 (or Tween-80) (450 µl per 100 ml buffer)
  - 0.1% Gelatin (0.10 g per 100 ml buffer)

› (96 ml sterile deionized water)

› Mix and autoclave.

Procedure

Prepare loaded Cas9

1. mix at equimolar concentrations

   0.5µl gene-specific crRNA @200µM
   0,5µl tracerRNA @ 200µM (if two cut sites, use 1µl)

2. mix at equimolar concentrations

   0.5µl dpy-10 crRNA
   0.5µl tracerRNA
   .
   because both RNAs are at 200µM, this will result in a 100µM concentration of the duplex.

3. heat both at 95ºC for 5 minutes and allow to cool to RT

4. dilute dpy-10 RNA complex with 5µl duplex buffer

5. Add 0.6µl of this mix to the target gene RNA complex (if using, otherwise, add water)

6. mix 0.5µl RNA mix and 0.5µl CAS9 enzyme.

   It is difficult to pipet such small volumes of a protein in glycerol. Control under the stereoscope that the pipet tip is properly filled. The concentrations should be equimolar.

7. incubate at room temperature for 5 minutes

Make injection mix

8. mix

   1µl RNP mix from above
   1µl SEA-107 at 1µM concentration
   1µl repair template for gene of interest at 20µM
   2µl 1x TE buffer
   5µl water

9. Centrifuge at maximum speed for 1 minute

10. Load 2 needles, optional: use Eppendorf microloader pipet tips.

11. **Note:** the injection mix can be kept at -20ºC for several month without reduced activity.

Injections

12. Inject both gonads. Keep only worms that were injected into both gonads to avoid screening offspring that had no opportunity to be edited. This is best done on Thursday. I now inject fewer than 10 worms.

13. After worms have recovered, move surviving hermaphrodites to individual plates. Keep at room temperature or 20ºC (F_0).

Screening for simple edits

14. Simple edits are: Deletions with two cuts and a repair template. Insertions of small things like restriction sites or edits to modify DNA sequences (e.g. TF binding sites), even insertion of fluorescent proteins if the PAM site is very near the insertion site.

15. Pick 8-16 F1 hermaphrodites as L4 on individual plates. PCR screen or simply look for mutation in the next generation (in case of deletions that cause a phenotype).

Screening for difficult edits

16. Once F1 generation is at L4 stage and if using dpy-10 co CRISPR, check that there are RoI and Dpy worms on the plates. Discard plates that have no RoI or Dpy.

17. Pick more than one hermaphrodite (up to 5) onto new plates. WT and RoI worms can be picked, but Dpy worms should be avoided. These are homozygous for the *dpy-10* mutation, which would have to be outcrossed, and they have a very low brood size. In the beginning, we picked iup to 48 plates with 5 hermaphrodites each, but this is probably not necessary any longer. If picking more than one hermaphrodite, match age (L4 is fine but all worms have to be L4). Keep over night at 25ºC.

18. On the next day, check that there are embryos on the plates and then pick all mothers from one plate into the same PCR tube with 10µl lysis buffer supplemented with proteinase K. Make sure to label tubes and plates carefully. Put plates at 15C.

19. Freeze-thaw in liquid N_2 5 times, then perform lysis in PCR machine with this protocol: 60ºC for 90 minutes, 95ºC for 15 minutes, cool down.

20. Use 1µl lysate for 10µl PCR reactions with primers that detect the edit.

    > For small insertions like 3x FLAG and for small deletions, flanking primers work if the product is run on a 1.5% gel. The insert can be seen as a larger band.
    > For large insertions, one not always heterozygous alleles since the small product (no insertion) is amplified preferentially. It can thus be better to use a primer set that binds inside and outside of the insert. Here, an edit yields a band, no edits do not yield bands. Unfortunately, it is not possible to test these PCR primers prior to the experiment.
    > Very small edits, like insertions or deletions of a few nucleotides can be detected as heteroduplexes. (Karin will add the details for this method)

21. Add loading dye (1µl is fine) to PCR reaction and load 5µl onto a 1% or 1.5% agarose gel.

22. Identify the plates for which PCR indicates an edit, discard all other plates.

If individual hermaphrodites were put on the plates and no homozygous edit was found, continue with a heterozygous plate as usual: pick 12-16 F2 to self.
If 5 F1 hermaphrodites were put on the plate, Pick 20 F2 L4 hermaphrodites from each positive plate onto individual plates and let them mature and lay eggs. This gives a >90% chance to pick an edited worm. The next day, pick mother into 10µl lysis buffer and perform lysis and PCR as before. Put plates at 16ºC. A positive band identifies a mother that was edited. If there is no positive band, more worms can be picked from the original plates and the PCR can be repeated.

23. Keep all plates from positive mothers. Depending on the edit, these worms can be screened for a phenotype (GFP, genetic phenotype). Otherwise, pick 12 L4 hermaphrodites from positive plates onto new plates and repeat PCR. If all of them show the positive band, the mother was homozygous, otherwise, repeat this step until homozygous line is identified.

24. It is advised to outcross the edited line, although off-target effects are not as much of a problem in *C. elegans* as in mammals.

    We are actually not doing this right now. A paper on him-5 CRISPR also did not outcross (reference in him-5 CRISPR entry).