Injection and Selection Protocols for Cas9-triggered homologous recombination

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Cas9-triggered Homologous Recombination with unc-119 selection

Our selection protocol is based on, and is highly similar to, the MosSCI protocol developed by Christian Frøkjær-Jensen and colleagues. We are greatly indebted to Dr. Frøkjær-Jensen for developing such an effective procedure and for sharing advice and reagents.

Before the Experiment

- Obtain worms of an appropriate strain for injection. Although we used strain DP38 (*unc-119(ed3)* III) for initial experiments, we have subsequently switched to the outcrossed derivative HT1593. HT1593 has a slightly weaker phenotype than DP38 (HT1593 animals are less Dpy and more active), but this has not affected our ability to recognize plates with rescued animals or isolate recombinants. Therefore we recommend that others use HT1593 for *unc-119* selection experiments. In principle, any strain compatible with your selectable marker can be substituted. Our selection procedure is also optimized for *unc-119* selection, and the use of other selectable markers may require subtle changes. If using *unc-119* selection, note that *unc-119* worms are healthier and easier to inject when grown on HB101 bacteria at 15°C.
- The night before injection, pick ~80 L4 animals to a fresh plate and allow them to mature into adults at 15°C overnight.

Day 0: Injection

- Prepare an injection mix containing the following plasmids:
  - 10 ng/µL homologous repair template
  - 50 ng/µL Cas9-sgRNA construct with your targeting sequence
  - 10 ng/µL pMA122 (heat-shock driven PEEL-1 negative selection; Addgene #34873)
  - 10 ng/µL pGH8 (*Prab-3::mCherry* neuronal co-injection marker; Addgene #19359)

- 5 ng/µL pCFJ104 (*Pmyo-3::mCherry* body wall muscle co-injection marker; Addgene #19328)
- 2.5 ng/µL pCFJ90 (*Pmyo-2::mCherry* pharyngeal co-injection marker; Addgene #19327)

DNA should be prepared using Invitrogen's PuroLink mini-prep kit, which gives the highest injection efficiencies. We do not linearize plasmids prior to injection.

- Inject the mixture into the gonads of the young adult worms.
- Transfer the injected worms to new seeded plates (three animals per plate works well in our hands).
- Put the plates at 25°C until the food bacteria are consumed and the Unc worms make piles (these are visible to the naked eye as big clumps of worms on the plate, where the edge of the bacterial lawn used to be).

Day 7–9: Heat Shock

- Examine the plates and identify those that contain *unc-119* rescued (i.e. normally moving) animals. In our experience, plates with rescues are obvious because they contain many crawling worms. You should not have to spend a lot of time searching to spot rescued worms. The number of plates with rescued animals will vary depending on your injection experience, but is usually greater than half in our hands.
- Heat shock rescued plates at 34°C for 4 hours in an air incubator. This activates expression of the PEEL-1 toxin, which kills animals that carry extrachromosomal arrays. After heat shock, return the plates to 25°C overnight to allow the array-carrying animals to die.

Day 8–10: Pick Candidate knock-in animals

- The day after heat shock, examine the plates for normally moving animals that survived heat shock. Such plates are candidates for a knock-in event. Again, these plates are usually obvious in our hands – intensive searching for survivors should not be necessary. Efficiency can vary, but we routinely recover between two and six independent candidate knock-ins lines from each set of injections.
- Using a fluorescence dissecting scope, verify that the worms that survived heat shock also lack expression of the red fluorescent co-injection markers. From each candidate knock-in plate, single 5–10 non-Unc animals lacking co-injection marker fluorescence to new plates (some of these worms will be sterile after the heat shock, so don't try to get away with picking fewer).

- After these worms produce progeny, look at the plates and identify those that did not segregate Unc progeny. These are likely to be homozygous knock-in strains. Failure to identify any homozygotes may indicate that your genomic modification is homozygous lethal, or it may be a sign that you don't have a true knock-in. Use PCR to distinguish between these two possibilities. If you are making a GFP knock-in, you can also look for fluorescence at this stage to establish whether you have an integrant. Note, it is impossible to tell whether two strains that originated from the same injection plate are independent insertion events or just progeny from a single insertion event. Therefore, although we single 5-10 worms from each plate in the previous step, we keep only one line from each plate.

PCR verification

- Design primers that anneal inside and outside the homologous repair.
- Isolate genomic DNA from the strain to be characterized, using detergent and proteinase K digestion followed by phenol-chloroform extraction (for a detailed protocol, see http://thalamus.wustl.edu/nonetlab/ResourcesF/worm%20genomic%20DNA.pdf). A crowded 5 cm plate yields more than enough DNA. Also isolate DNA from your parental strain as a control.
- Set up PCR reactions with primer pairs flanking both insertion junctions and spanning the entire modified locus. We have found that LongAmp Taq DNA polymerase from NEB gives particularly robust amplification from genomic DNA.
- Note: the primer pairs outside the homologous repair template (flanking the insertion site) are particularly useful since you should see a band shift for a knock-in, and the disappearance of the wild-type band indicates that your strain is homozygous.
- If desired, sequence the PCR products obtained in this step to verify a mutant allele.

Removal of the selectable marker with Cre recombinase

These instructions assume you are using *unc-119* selection, but the same basic approach should be applicable to other selectable markers.

- The night before injection, pick 25–30 L4 knock-in worms to a fresh plate and allow them to mature into young adults overnight.
- Prepare an injection mix containing the following:
  - 50 ng/µL pDD104 (*Peft-3::Cre::tbb-2 3'UTR*; Addgene #47551)
  - 2.5 ng/µL pCFJ90 (*Pmyo-2::mCherry* pharyngeal co-injection marker) or any other fluorescent marker.
- Inject the mixture into the gonads of young adult worms. Place the injected worms at 25°C, three worms per plate.
- Two days after injection, single 10–20 F1 progeny that express the fluorescent marker to new plates. These animals are progeny resulting from successful injections and can segregate Uncs in the next generation. Do not put more than one animal per plate at this stage – more animals will cause the plates to be overcrowded and make screening difficult.
- When the F2 progeny have reached adulthood, pick Unc animals to new plates to establish lines. Timing is important here: at 25°C, *unc-119* animals are slightly egg laying-defective (Egl), and therefore are easiest to spot when they are old enough to have accumulated some eggs. However, it is important not to wait too long, as screening becomes more difficult once the plates become crowded.
- Note: because our Cre expression construct also carries *unc-119(+)*, only animals that have 1) Excised both genomic copies of the *unc-119(+)* cassette and 2) Lost the extrachromosomal array generated by injecting the Cre expression construct will have an Unc phenotype. Loss of the array can be verified by loss of the fluorescent co-injection marker.
- If desired, confirm removal of the selectable marker by PCR or sequencing. At this stage, the Unc animals resulting from this procedure can be used in another round of homologous recombination to produce more complicated modifications, or outcrossed to remove the *unc-119* mutation.