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Lethal mutation co-conversion using *pha-1(ts)* repair and NHEJ inactivation by *cku-80* RNAi

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Strain maintenance

- Get GE24 *pha-1(e2123)* III from CGC, make glycerol stocks.
- Maintain at 15ºC on NGM-lite plates seeded with HB101 (available from CGC). If making a lot of knock-ins, I chunk to a fresh 10 cm plate every day, so that I have correctly staged animals at any given time. I inject either young adults or day 1 adults with a nice row of eggs.
- One additional advantage of *cku-80* RNAi (besides increased knock-in efficiency) is that it is easier to pick worms off of HT115 bacteria compared to HB101.

cku-80 RNAi

- Streak out *cku-80* RNAi strain on LB+Amp+Tet. Also streak out a positive control that produces a visible phenotype (ie. *nhr-25)*.
- Pick single colony into 50 mls of LB+Amp, grow overnight at 37ºC, shaking at 225 rpm.
- Pellet culture at 4000 rpm, 15 minute, remove supernatant and resuspend pellet in 5 mls of LB+Amp. I use this stock for up to 2 weeks.
- Seed 6 cm RNAi plates with 50 µl of the concentrated *cku-80* and positive control RNAi bacteria; leave overnight on the bench to induce. I prefer to seed the plates freshly and put worms on the next day.
- Pick four adult *pha-1(ts)* worms onto the seeded RNAi plates. Incubate at 15ºC. It typically takes 4-8 days to get adults in the F1 generation that one can inject. Confirm RNAi efficacy with the positive control.
- If one expects to be injecting frequently, plate *pha-1(ts)* on *cku-80* RNAi daily during the work week.

Construct and oligo design

- Identify the desired insertion or knock-in site in the genomic DNA.
- Take 50 bp of flanking sequence 5' and 3' to the insertion site and identify possible CRISPR cut sites (PAMs). I typically use Feng Zhang's MIT site, though there are others out there. http://crispr.mit.edu
- Pick high scoring PAMs with no off-target sites in genes, as close as possible to the insertion site. My data suggests that DSBs closer to an insertion site may be more effective, though differences in PAMs may play a role as well. I typically choose two PAMs per desired modification. **Save the output as a webarchive so that you have a list of the sgRNAs, their scores, and potential offtarget sites.**
- If using PCR-derived sgRNAs, order oligos with the sgRNA target sequence in place of "N_{20}" in 5'-cctcctattgcgagatgtcttg(N_{20})gtttaagagctatgctgg-3'. This keeps the size at 60 bp, which is the cheapest synthesis scale at IDT, the oligo supplier that I use. **Note: do not include the "NGG" PAM sequence in the oligo.**
- For oligo-templated knock-ins, use 35-80 basepairs of flanking sequence. For epitope tags, it helps to include a flexible spacer sequence encoding a restriction

site (ie. a *Bam*HI site encodes glycine-serine). This inclusion facilitates knock-in screening by restriction digestion. One can have the oligos PAGE purified, which leads to higher knock-in efficiency, or avoid this cost and inject more animals and screen more F1s. **Inactivate PAMs in repair template.** If possible, silently mutate one of the guanines in the "NGG" sequence. If this is not possible (ie. some codons do not allow the possibility of silent mutation of these guanines), make 5-6 silent mutations in the sgRNA target sequence.

- Design oligos for amplifying the insertion site (typically aiming for a 600 bp product); I use Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to design these oligos.
- Also design knock-in specific screening oligos. The oligo sits at an insertion junction with the two most 3' bases binding to the insert and the remaining sequence binding to genomic sequence.
- In theory, it should be possible to enrich for similar modifications as described by Paix *et al.* (2014)(ie. oligo-templated insertion of stop codons or deletions, knock-in of PCR-derived GFP cassettes), but I have not formally tested these templates yet.

Generation of P*U6::sgRNA* templates by PCR fusion

- Amplify P*U6* from pJW1310 using oligos 1787 and 1788 (attgtgttcgttgagtgaccc and caagacatctcgcaataggagg, respectively). I use this PCR product repeatedly for fusion reactions.
- Amplify new *sgRNA* template using a target specific 60mer and oligo 1790 (aaaaataggcgtatcacgagg); use pJW1311 as a template for PCR
- Use 0.5 µl of each PCR product in a 100 µl PCR (I use Phusion for PCR fusion ☺) with the following parameters: The: i) 98ºC denaturation; ii) 35 cycles of 98ºC-10 sec, 61ºC-30 sec, 72ºC-20 sec; iii) 72ºC 1 min final extension.
- Clean and concentrate PCR product (I like Zymo kit) and elute in 15 µl of nuclease-free water. Nanodrop. It's now ready for injection
- If you need more P*U6::sgRNA* or if the product is dirty, perform a nested PCR on the fusion PCR with oligos 1793 and 1794 (aacgtcgtgactgggaaaacc and ggtgtgaaataccgcacagatgc, respectively).

Injections

- I make a 20 µl of an injection mix consisting of [50 ng/µl of pJW1285 (CRISPR/Cas9 plasmid targeting *pha-1*)+50 ng/µl *pha-1(ts)* repair oligo (unpurified 80mer works well)+50 ng/µl of knock-in repair template+25 ng/µl of each PCR-generated P*U6::sgRNA* template]. Bring up to 20 µl with nuclease-free water. For a new target site, I try screening at least two sgRNAs. If multiplexing, just add 50 ng/µl of the additional repair template and 25 ng/µl of PCR derived P*U6::sgRNA* template
- I filter the injection mixture through a SpinX microfuge tube filter (CoStar) and keep at 4ºC
- For each injection mixture, I inject 24-48 animals (more if you get a lot of sterile or dead animals). *pha-1(ts)* animals can be a bit sickly, so it takes time to get your efficiency up. Best to inject more animals, as one begins to use *pha-1(ts)* co-

selection. After animals recover from the injection, single P0 animals onto plates. I like to use 24-well plates seeded with OP50. Shift plates to 25ºC. On days 3 and/or 4, check the plates. Record the number of sterile and viable P0s. Look for rescued F1 progeny. They will be obvious. Aside from the injected P0, they should be the only animals on the plate that develop past L1 or L2. In my hands, lots of rescued F1s is either really really good or really really bad ☺ For my *2xFLAG::smo-1* knock-in (Ward, 2014) I had 14 rescues, or which 11 were correct. In an experiment with a failed sgRNA, I've had up to 46 rescues with no knock-in.

- Single these rescued F1s to new OP50 seeded plates. Again, I like 24-well plates. Best practice is to keep track of which P0 the F1s came from, so that if multiple knock-ins are recovered, one can choose independent knock-ins (ie. from different P0s). Incubate 2-3 days at 25ºC to allow progeny develop. Remove the parental F1 and perform single-worm PCR on it. I like using 30 µl PCRs using Phusion polymerase (NEB), Phusion HF buffer, and 3 µl of single worm lysate. Five µl of this PCR can be use in a restriction digest to identify knock-ins. The remaining PCR can be cleaned and sequence to validate hits from the restriction digestion assay.
- Growth at 25ºC will allow survival of *pha-1(ts)* rescued heterozygotes. Genotype *pha-1* locus by single worm PCR using oligos caatttggcagccattcatgtg and tcgcgcactactgaatcagagtc

Frequently asked questions (FAQs)

1) Have you tried using NHEJ mutants?

In the direct selection experiments I performed, using a *lig-4* null, I did seem to yield better efficiency. However, it was always hard to tell if it was significant when screening hundreds of F1s and getting a single knock-in! One consideration that could be either beneficial or a hindrance is that the NHEJ mutations are all near *pha-1*, to varying extents: *cku-70* is 0.49 cM from *pha-1*, *cku-80* is 10.11 cM, and *lig-4* is 3.84 cM. I had tried to knock-in a stop codon into *cku-70* exon 1; such a strain could be maintained with a GFP marked hT2 balancer. Furthermore, due to tight linkage, one could easily outcross both *pha-1* and the *cku-70* mutation. Unfortunately, the sgRNAs I tested were inactive. I also didn't have any success with two *lig-4* sgRNAs. One could cross in existing deletion alleles to the *pha-1* strain; I didn't do this as these NHEJ mutant strains needed outcrossing, and then a recombination event was requited to get the *pha-1* linked mutation. Unfortunately, I did not have time for these experiments with the revision timeline for the manuscript. Still, I think that it's an outstanding question as to whether the NHEJ mutants could lead to further increases in efficiency, though potentially at a cost of background mutations.

2) What about other NHEJ RNAis?

Reviewing the literature, *lig-4* and *cku-80* RNAi both produced phenotypes; I did not find reports of robust phenotypes for *cku*-70. In a single repetition of the *klp-12 Mfe*I site deletion assay and a single test of a GFP frameshift reporter (NHEJ restores GFP

expression, similar to that described by Waaijers et al, 2013), *cku-80* RNAi produced phenotypes, whereas *lig-4* RNAi was inactive. As these were single experiments, I did not report them in the Genetics manuscript, but my feeling was that only *cku-80* RNAi was worth pursuing.

3) Have you tried linear dsDNA templates, à la Paix *et al.* (2014)?
Regarding dsDNA repair templates generated by PCR, I don't see any reason why it shouldn't work with *pha-1(ts)* co-conversion, but I have not yet tried using these templates. I'm now trying to do some of those experiments, knocking GFP constructs into the same locus which ttTI5605 is inserted. This sgRNA has been validated (Dickinson *et al.* 2013), and I will post updates as the experiments progress. Again, this is an important question, as we still know very little about the mechanism of homologous recombination using ssDNA vs dsDNA templates.