Generating Cas9-mediated fluorescent protein knock-ins with a self-excising selection cassette (SEC)

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Abstract

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Guidelines

Please see the author's website for updates and additional details.

Before the Experiment

Choose the Cas9 target site
1) Identify a 100-200 bp region in which the Cas9 target site should be located. We generally use a 200 bp window centered on the start codon (for N-terminal tags) or stop codon (for Cterminal tags.

2) Submit this genomic sequence to the Zhang lab's CRISPR design tool at http://crispr.mit.edu. Make sure you have selected *C. elegans* as the genome for checking specificity.

3) The design tool returns a list of potential targeting sequences, ranked in order of predicted specificity. We always try to choose target sites with a specificity score >95, and in most cases find a site that scores 98 or 99 (100 indicates perfect specificity). If there are several candidate sites with high specificity, choose the site that is closest to the desired insertion site (start or stop codon). The best case scenario is to have the insertion site within the guide sequence and within 10 bp of the PAM (NGG motif), so that insertion of mNG^SEC^3xFlag will disrupt the target site (Figure P1A). If this is not possible, then choose a Cas9 target sequence that is within the coding region of your gene. For the targeting sequence you choose, copy and paste the list of potential off-target sites into a Word document or Excel sheet and save this list for future reference.

Add the target sequence to the Cas9–sgRNA construct

1) The design tool returns target sites of the form 5'N20-NGG-3', where N is any base. You need to insert the N20 sequence into the Cas9–sgRNA construct (pDD162, Addgene #47549). We use NEB's

Q5 Site-Directed Mutagenesis Kit to do this. Use forward primer 5'-N20GTTTTAGAGCTAGAAATAGCAAGT-3', where N20 is your 20 bp targeting sequence from the design tool, and reverse primer 5'-CAAGACATCTCGCAATAGG-3'.

2) IMPORTANT: Do not include the PAM (NGG motif) in your primers for the Cas9-sgRNA construct. The NGG motif must be present in the target DNA, but it is not part of the sgRNA.

3) We use sequencing primer 5'-GGTGTGAAATACCGCACAGA-3' to verify correct insertion of the targeting sequence.

<u>Design primers to add homology arms to an FP–SEC vector</u>

Figure 1C shows our strategy for cloning homology arms into FP–SEC vectors. Homology arms are generated by PCR and inserted in place of the ccdB negative selection markers, which are flanked by restriction sites. We chose these particular restriction sites so that no residual sequence is left behind after addition of the homology arms. ccdB negative selection makes this cloning strategy exceptionally robust and efficient: in a pilot experiment, we generated 8 repair templates in parallel in a single afternoon. 7/8 of these reactions yielded >80% correct clones; the remaining reaction also yielded correct clones, albeit at a lower frequency.

You need to design four primers: two for each homology arm. These primers will amplify the homology arms and add sequence overlaps for Gibson assembly to the ends of each arm. If FP::SEC insertion will not disrupt the Cas9 target site, your primers will also need to introduce silent mutations to prevent Cas9 from cutting the repair template.

You have a choice of two possible pairs of restriction enzymes to digest the FP–SEC vector: AvrII+SpeI or ClaI+SpeI. If AvrII and SpeI are used, the repair template will include a flexible linker between the 5' homology arm and FP (this is useful for generating C-terminal tags). If ClaI and SpeI are used, the 5' homology arm will be fused directly to the FP, with no added sequence (this is useful for N-terminal tags, or when no flexible linker is desired). Figure P1 shows sample primer designs for each situation.

Detailed primer design instructions:

1) First, decide whether additional mutations are needed to prevent Cas9 from cutting the repair template. We make additional mutations whenever the insertion site is not within the 10 bp of the target sequence closest to the PAM. Additional mutations are made using synonymous codons so that the amino acid sequence is not altered (See Figure P1B for an example). If possible, the simplest and most effective approach is to mutate the PAM (NGG motif), since this motif is absolutely required for cleavage of a substrate by Cas9. If a PAM mutation is not feasible, introduce as many mutations as possible (at least 5-6) in the target sequence.

2) Each homology arm should be 500-700 bp long. The positions of the two primers most proximal to the FP^SEC^3xFlag module (i.e., the reverse primer for the 5' homology arm and the forward primer for the 3' homology arm) are fixed by the need to insert FP^SEC^3xFlag at a specific location. The positions of the distal primers are more flexible. We design the proximal primers first based on our desired insertion site, and then use Primer-BLAST to pick the best possible distal primers.

3) Decide with FP you want to insert, and consult Table P1 for the sequence that needs to be added to the end of each primer to allow Gibson assembly.

4) Ideally, the primer length should be less than 60 bp, because longer primers are much more expensive and fail more often. If you find you need a longer primer because your Cas9 target site is far away from the insertion site, it might be more cost effective to purchase a synthetic DNA fragment (we like IDT's gBlocks) containing the homology arm instead of using PCR.

5) Before ordering primers, double check that the mNG::3xFlag will be in frame with your gene of interest.

Injections to Generate Knock-ins
Day 9 or 10: Look for homozygous plates

Look for plates where 100% of L4s and adults are Rollers. These are homozygous knock-in animals. They can be maintained indefinitely, outcrossed if desired, or mated to another genetic background. The strong Rol phenotype makes it very easy to follow the knock-in in crosses (but note that Rol males mate poorly). You can also take L1s from these plates and proceed directly to heat shock to remove the selectable markers.

It is straightforward to generate lethal mutations with our strategy, because knock-in alleles can be isolated and maintained as heterozygotes. You will know that your knock-in is lethal if you see only heterozygous plates (i.e., plates with ~1/4 wild-type worms and 1/4 dead embryos). You should expect your initial knock-in to be lethal if you are making an N-terminal tag on an essential gene, because the initial knock-in is a transcriptional null mutation.

Note: It is impossible to tell whether two strains that originated from the same injection plate derive from independent insertion events or 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.

Selectable marker removal

Figure P2 shows the overall scheme for selectable marker removal. In most cases, the initial knock-in is homozygous viable and marker removal is extremely simple (Figure P2A).

If the initial knock-in is lethal, marker removal is slightly more complicated because heterozygous knock-in animals segregate wild-type animals at each generation, which makes it impossible to identify animals that have excised the marker based on wild-type phenotype alone (Figure P2). In this situation, there are two choices. If your knock-in strain is visibly fluorescent, you can simply heat shock heterozygotes and identify animals that have excised the marker based on a wild-type phenotype plus visible fluorescence (Figure P2B). If fluorescence in your knock-in strain is too dim to see by eye, you need to mate in a GFP-marked balancer chromosome first (Figure P2C). Mate males carrying an appropriate GFP-marked balancer to Rol knock-in hermaphrodites. Pick GFP-positive, Rol animals from the F1 progeny. These animals should now no longer segregate wild-type progeny in the absence of heat shock (Figure P2C). Use these balanced knock-in worms for subsequent steps.

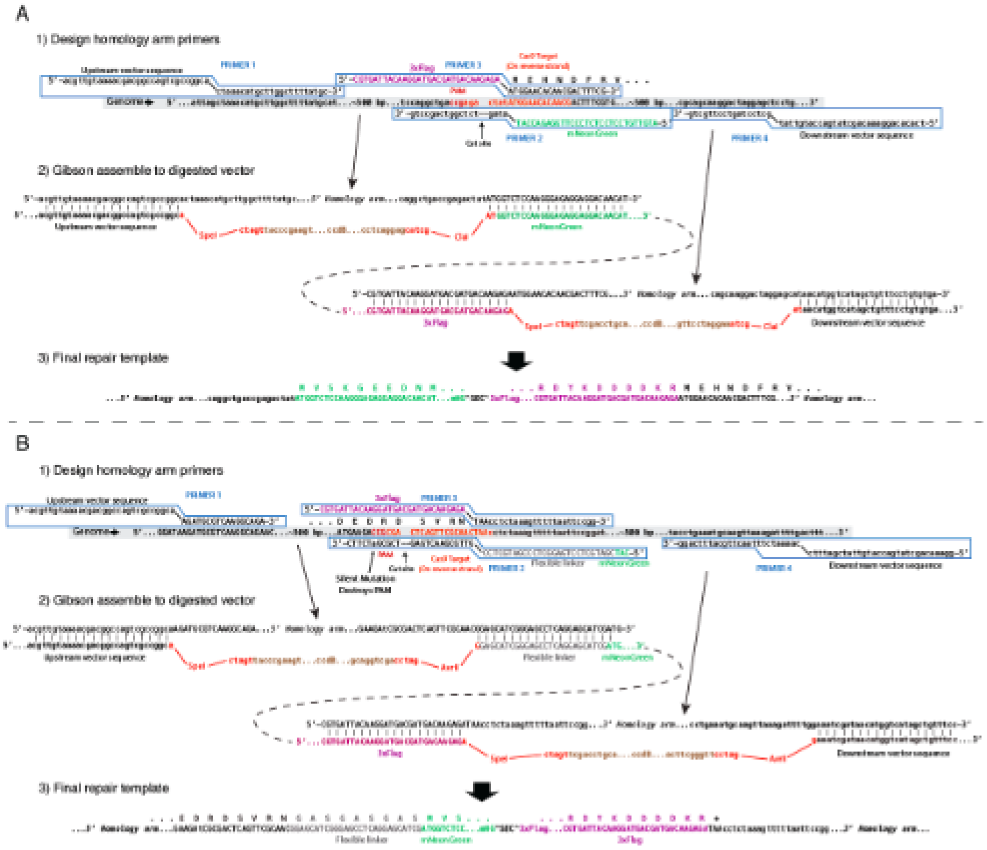

Figure P1: Primer design examples. (A) Primers for an N-terminal tag. In this example, mNG^SEC^3xFlag insertion disrupts the Cas9 target site, so no mutations are required. (B) Primers for a C-terminal tag, including the optional flexible linker that is built into the FP–SEC vectors. In this example, a silent PAM mutation is introduced to block Cas9 cleavage.

N-terminal mNeonGreen::3xFlag		
	Digest vector pDD268 with ClaI and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATGTTGTCCTCCTCTCCCTTGGAGACCAT-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-tcacacaggaaacagctatgaccatgttat-(Homology arm sequence)-3'
C-terminal mNeonGreen::3xFlag (with flexible linker)		
	Digest vector pDD268 with AvrII and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATCGATGCTCCTGAGGCTCCCGATGCTCC-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-ggaaacagctatgaccatgttatcgatttc-(Homology arm sequence)-3'
N-terminal GFP::3xFlag		
	Digest vector pDD282 with ClaI and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-TCCAGTGAACAATTCTTCTCCTTTACTCAT-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-tcacacaggaaacagctatgaccatgttat-(Homology arm sequence)-3'
C-terminal GFP::3xFlag (with flexible linker)		
	Digest vector pDD282 with AvrII and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATCGATGCTCCTGAGGCTCCCGATGCTCC-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-ggaaacagctatgaccatgttatcgatttc-(Homology arm sequence)-3'
N-terminal YPET::3xFlag		
	Digest vector pDD283 with ClaI and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-TCCTGTAAATAACTCTTCTCCTTTTGACAT-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-tcacacaggaaacagctatgaccatgttat-(Homology arm sequence)-3'
C-terminal YPET::3xFlag (with flexible linker)		
	Digest vector pDD283 with AvrII and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATCGATGCTCCTGAGGCTCCCGATGCTCC-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-ggaaacagctatgaccatgttatcgatttc-(Homology arm sequence)-3'
N-terminal TagRFP-T::3xFlag		
	Digest vector pDD284 with ClaI and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CTTGATGAGCTCCTCTCCCTTGGAGACCAT-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-tcacacaggaaacagctatgaccatgttat-(Homology arm sequence)-3'
C-terminal TagRFP-T::3xFlag (with flexible linker)		
	Digest vector pDD284 with AvrII and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATCGATGCTCCTGAGGCTCCCGATGCTCC-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-ggaaacagctatgaccatgttatcgatttc-(Homology arm sequence)-3'
N-terminal mKate2::3xFlag		
	Digest vector pDD285 with ClaI and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATGTTTTCTTTAATGAGCTCGGAGACCAT-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-tcacacaggaaacagctatgaccatgttat-(Homology arm sequence)-3'
C-terminal mKate2::3xFlag (with flexible linker)		
	Digest vector pDD285 with AvrII and SpeI	
	5' arm forward primer:	5'-acgttgtaaaacgacggccagtcgccggca-(Homology arm sequence)-3'
	5' arm reverse primer:	5'-CATCGATGCTCCTGAGGCTCCCGATGCTCC-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm forward primer:	5'-CGTGATTACAAGGATGACGATGACAAGAGA-(Cas9 target mutations)-(Homology arm sequence)-3'
	3' arm reverse primer:	5'-ggaaacagctatgaccatgttatcgatttc-(Homology arm sequence)-3'

Table P1: Primers for insertion of homology arms into FP::SEC vectors.

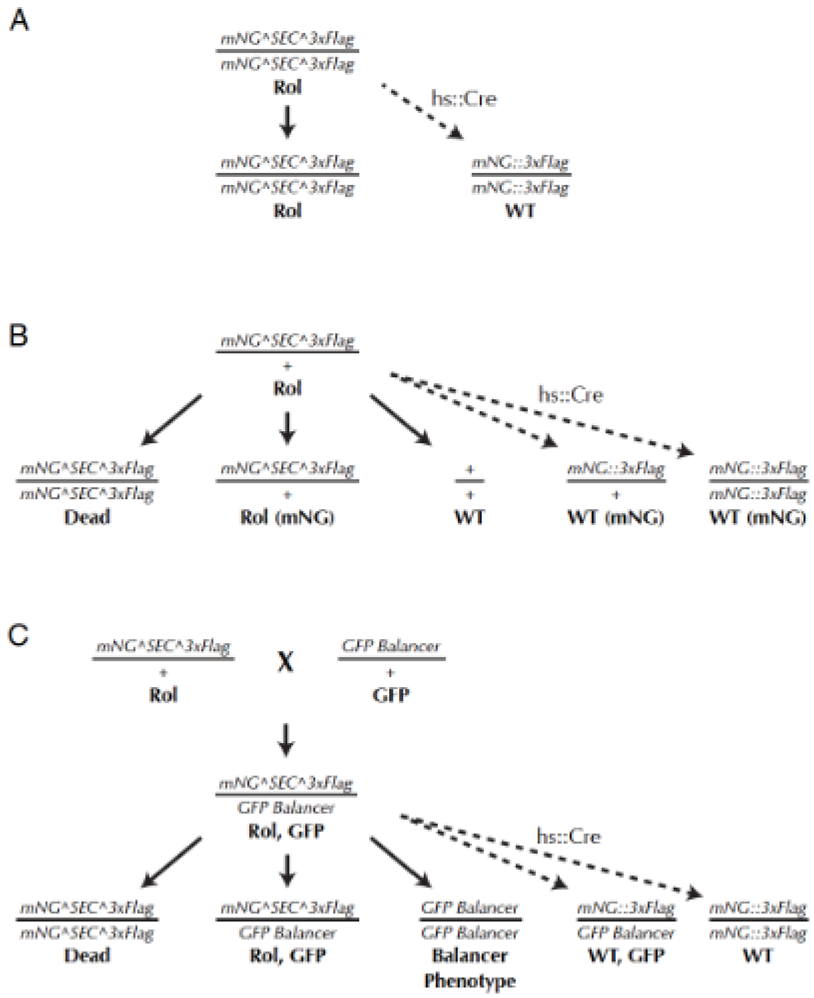

Figure P2: Genetic schemes for marker self-excision. (A) For homozygous viable knock-ins, the situation is simple: after heat shock, any wild-type worms will have lost both copies of SEC. (B) If the knock-in is homozygous lethal, the strain produces 1/4 wild-type progeny at each generation. This makes it impossible to unambiguously identify worms that have lost SEC based on wild-type phenotype alone, although knock-ins may be identifiable if they show visible fluorescence. (C) A simple, 1-step cross to introduce a GFP balancer chromosome results in a strain that does not segregate any wild-type progeny. Heat shock-induced marker excision in this background generates wild-type animals that can be easily and unambiguously identified.

Protocol

Preparation

Step 1.
See the Guidelines for:
1. Choosing the Cas9 targets site
2. Adding the target sequence to the Cas9–sgRNA construct
3. Designing primers to add homology arms to an FP–SEC vector

Add homology arms to the repair template: Preparing the vector

Step 2.
Grow bacteria carrying the FP–SEC vector and miniprep the plasmid DNA. Note that, prior to replacing the ccdB elements with homology arms, FP–SEC vectors must be grown in cells that are resistant to ccdB (ccdB Survival cells).

Add homology arms to the repair template: Preparing the vector

Step 3.
Digest an entire miniprep of FP–SEC vector overnight at 37°C (consult Table P1 for which construct and enzymes to use).

DURATION
18:00:00

Add homology arms to the repair template: Preparing the vector

Step 4.
Purify the digested vector using a PCR cleanup spin column to remove the enzymes. Process the entire digested vector as one sample; do not attempt to gel purify individual bands.

Add homology arms to the repair template: Preparing the vector

Step 5.
The digested, purified vector may be stored at 4°C for at least a few months and reused to construct multiple repair templates.

Add homology arms to the repair template: Preparing the homology arms

Step 6.
Generate two PCR products (the homology arms) using genomic DNA as the template and the primers you designed above.

Add homology arms to the repair template: Preparing the homology arms

Step 7.
Mix the two PCR products and purify them together on a single PCR cleanup spin column.

Add homology arms to the repair template: Preparing the homology arms

Step 8.
Mix **1 µL** of vector, **4 µL** of homology arms and **5 µL** of isothemal assembly enzyme mix (we use NEBuilder HiFi DNA assembly mix from NEB).

REAGENTS
NEBuilder HiFi DNA Assembly Master Mix - 10 rxns E2621S by New England Biolabs

DURATION
01:00:00

Add homology arms to the repair template: Preparing the homology arms

Step 9.
Incubate 1h @ 50°C or as directed by the enzyme manufacturer.

DURATION
01:00:00

Add homology arms to the repair template: Preparing the homology arms

Step 10.

Transform **2 µL** of the reaction to suitable competent cells.

Add homology arms to the repair template: Preparing the homology arms

Step 11.

Isolate DNA from 3-6 clones and sequence with M13 Forward and Reverse primers to verify correct insertion of the homology arms.

NOTES

Tracey DePellegrin 02 Oct 2015

This cloning procedure is efficient enough that screening clones prior to sequencing is not necessary.

Injections to Generate Knock-ins- Day 0: Injection

Step 12.

Prepare an injection mix containing the following:

10 ng/µL homologous repair template	
50 ng/µL Cas9-sgRNA construct with your targeting sequence	
Fluorescent co-injection markers (to label extrachromosomal arrays):	
	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)

Injections to Generate Knock-ins- Day 0: Injection

Step 13.

Prepare plasmid DNA using Invitrogen's PureLink mini-prep kit, which gives high injection efficiencies.

Injections to Generate Knock-ins- Day 0: Injection

Step 14.

Inject the mixture into the gonads of 50-60 young adult worms of strain N2 (or substitute any strain you like).

Injections to Generate Knock-ins- Day 0: Injection

Step 15.

Transfer the injected worms to new seeded plates (three animals per plate works well in our hands). Use regular NGM plates (no drug) at this stage. Also make a control plate with uninjected worms, so that when you do the drug selection it can serve as a negative control.

Injections to Generate Knock-ins- Day 0: Injection

Step 16.

Put the plates at 25°C and let the worms lay eggs without selection for 2-3 days.

Injections to Generate Knock-ins- Day 2 or 3: Add hygromycin

Step 17.

Prepare and filter sterilize a 5 mg/mL hygromycin solution in water.

Injections to Generate Knock-ins- Day 2 or 3: Add hygromycin

Step 18.

For 6 cm plates poured with 10 mL agar plates, pipet 500 µL of drug onto the surface of each plate of worms, for a final concentration of 250 µg/mL (if using different size plates, adjust the volume accordingly).

Injections to Generate Knock-ins- Day 2 or 3: Add hygromycin

Step 19.

Swirl gently so that the solution covers the entire surface of the plate, then let it dry. Put the worms back at 25ºC.

NOTES

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Note: In our hands, it does not make any difference whether we add the drug on the second or third day after injection, but the drug must be added no later than the third day in order to kill untransformed F1 progeny before they reproduce and overcrowd the plates.

Injections to Generate Knock-ins- Day 6 or 7: Pick initial knock-in worms

Step 20.

Examine the plates and identify those that contain Roller (Rol) animals that survived the hygromycin treatment.

NOTES

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Do not waste your time picking from plates that have only a few, sick-looking worms.

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Knock-in plates should be obvious: there should be lots of animals, they should look totally healthy, and L3 and older worms should be Rol (the Rol phenotype is not expressed in L1 or L2 larvae).

Injections to Generate Knock-ins- Day 6 or 7: Pick initial knock-in worms

Step 21.

Candidate knock-in animals are L4/adults that 1) survive hygromycin selection; 2) are Rol; and 3) lack the red fluorescent extrachromosomal array markers.

NOTES

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Note that we occasionally see a plate with many wild-type worms that survived selection, but do not pick these – they typically carry extrachromosomal arrays or rearrangements. Also note that, in our experience, rare non-fluorescent animals on plates with lots of mCherry(+) animals (i.e., lots of array animals) are usually false positives.

Injections to Generate Knock-ins- Day 6 or 7: Pick initial knock-in worms

Step 22.

Single 5-10 candidate knock-in adults to new plates without hygromycin.

NOTES

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If you do not see any candidate knock-ins at this stage, or if you have fewer lines than you'd like, wait 3 days and then examine the plates again. We sometimes find knock-ins 9-10 days after injection (when the F3 are young adults) that were missed during the first round of screening.

Injections to Generate Knock-ins- Day 9 or 10: Look for homozygous plates

Step 23.

Look for plates where 100% of L4s and adults are Rollers. (See the **Guidelines** for details.)

Selectable marker removal- Day 0: Heat shock

Step 24.

Pick 6-8 L1/L2 larvae to each of three new plates. It is possible to perform marker excision using older animals, but using young larvae results in the highest efficiency because the germ cells have not yet begun to divide.

NOTES

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See the **Guidelines** for additional information on selectable marker removal

Selectable marker removal- Day 0: Heat shock

Step 25.

Heat shock the plates at 34°C for 4 hours (or at 32°C for 4-5 hours) in an air incubator to activate expression of hs::Cre.

DURATION

04:00:00

Selectable marker removal- Day 0: Heat shock

Step 26.

Return the plates to 20°C or 25°C.

Selectable marker removal- Day 5-7: Pick knock-in animals that have lost the marker

Step 27.

Pick wild-type worms to new plates.

NOTES

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The animals you will pick will be the F1 progeny of the L1/L2 larvae that you heat shocked in the previous step. Be careful not to pick these animals too early, since the Rol phenotype conferred by sqt-1(d) does not appear until L3. To be safe, we only pick L4 and adult animals at this step.