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

Written by Dan Dickinson (daniel.dickinson@austin.utexas.edu) and last updated August 2018.

This protocol was originally published in: Dickinson DJ, Pani AM, Heppert JK, Higgins CD and Goldstein B (2015). Streamlined genome engineering with a self-excising drug selection cassette. ***Genetics*** 200(4): 1035-49. DOI: 10.1534/genetics.115.178335. Changes from the original published protocol appear in blue text.

Before the Experiment

Design and clone plasmids expressing your sgRNA and repair template, using either SapTrap assembly or our Gibson/ccdB-based procedure (see separate protocols).

Injections to Generate Knock-ins

Day 0: Injection

1) Prepare an injection mix containing the following:
   - 50 ng/µL homologous repair template (if your repair template plasmid also contains the sgRNA) or 10-20 ng/µL (otherwise).
   - 50 ng/µL Cas9 plasmid pDD121 (if your repair template plasmid also contains the sgRNA) or unique Cas9-sgRNA construct with your targeting sequence (otherwise).
   - 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)

   Prepare plasmid DNA using Invitrogen's PureLink HQ mini-prep kit (Catalog number K2100-01), which gives high injection efficiencies. Note: The PureLink protocol includes an "optional" wash step just before the ethanol wash (see pages 8 and 11 in the product manual). This step is essential for high injection efficiency and must be included. Prepare the extra wash buffer as follows: Dissolve 38.2 g Guanidinium Chloride (GnHCl) in 30 mL of

water (you may have to heat it to get it to dissolve). Adjust pH to 6.5, then bring volume to 60 mL with water. Finally, add 40 mL of isopropanol to obtain a final concentration of 4 M GnHCl, 40% v/v isopropanol. You can also use Qiagen PB buffer if you don't want to make your own. In either case, wash DNA with 500 µL of this solution immediately after binding to the column, before washing with the ethanol-containing buffer supplied with the kit. Thanks to Dave Matus for making us aware that this extra wash step improves efficiency (we had never omitted it when developing our protocol).

2) Inject the mixture into the gonads of 50-60 young adult worms of strain N2 (or substitute any strain you like).
3) 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 un-injected worms, so that when you do the drug selection it can serve as a negative control.
4) Put the plates at 25°C and let the worms lay eggs without selection for 2-3 days.

Day 2 or 3: Add hygromycin

Prepare and filter sterilize a 5 mg/mL hygromycin solution in water. 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). Swirl gently so that the solution covers the entire surface of the plate, then let it dry. Put the worms back at 25ºC. 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.

Day 6 or 7: Pick initial knock-in worms

1) Examine the plates and identify those that contain Roller (Rol) animals that survived the hygromycin treatment. 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). Do not waste your time picking from plates that have only a few, sick-looking worms.
1) Candidate knock-in animals are L4/adults that 1) survive hygromycin selection; 2) are Rol; and 3) lack the red fluorescent extrachromosomal array markers. 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.

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

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.

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 2 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 2A).

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 2). 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 2B). 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 2C). 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 2C). Use these balanced knock-in worms for subsequent steps.

Day 0: Heat shock

1) 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.
2) 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. After heat shock, return the plates to 20°C or 25°C.

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

Pick wild-type worms to new plates. 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.

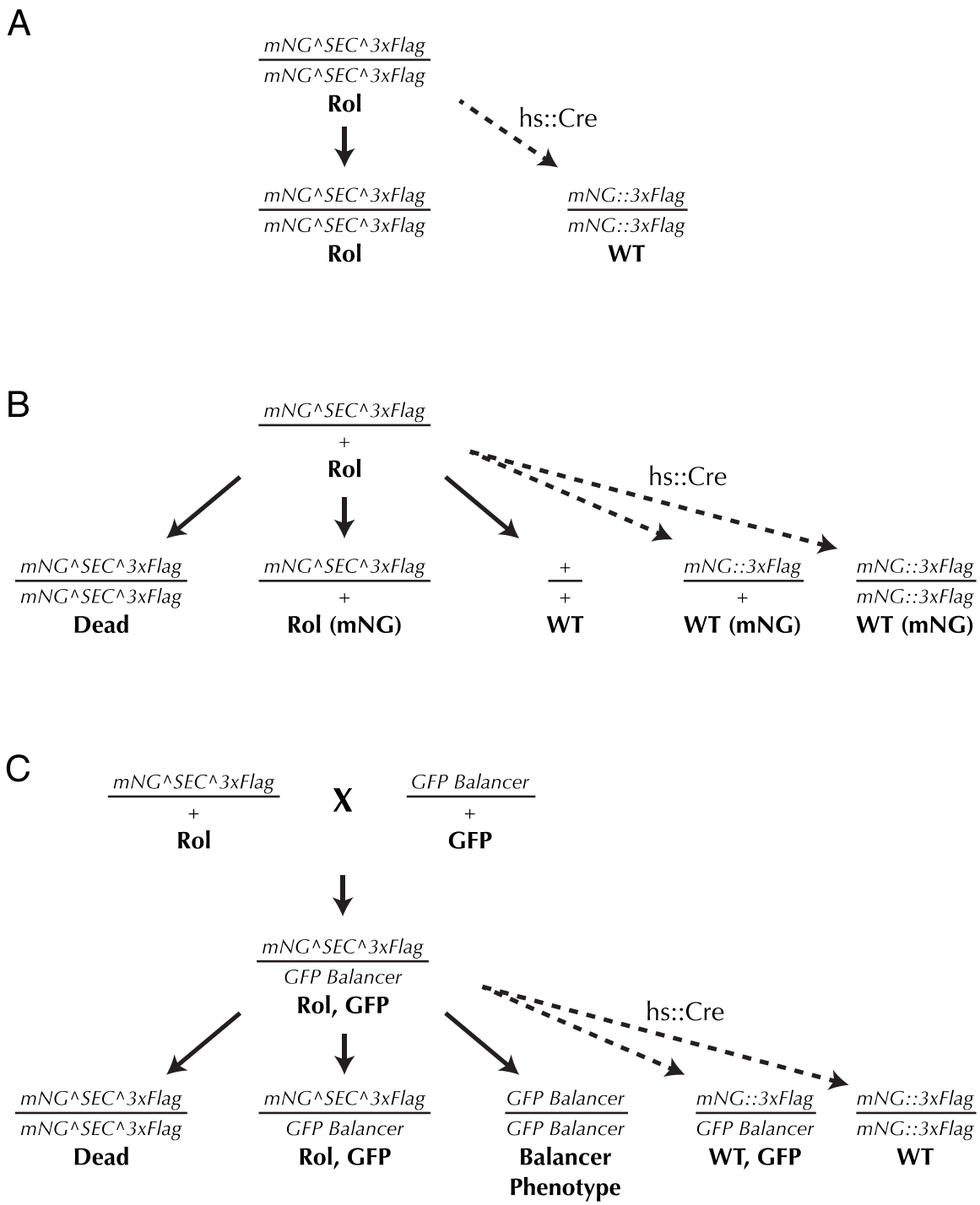

Figure 2: Genetic schemes for marker self-excision. (A) For homozygous viable knock-ins, the situation is simple: after heat shock, any wild-type worms will have lost both copies of SEC. (B) If the knock-in is homozygous lethal, the strain produces 1/4 wild-type progeny at each generation. This makes 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.