

A Scalable CURE Using a CRISPR/Cas9 Fluorescent Protein Knock-In Strategy in *Caenorhabditis elegans*[†]

Eric Hastie^{1*}, Ryan Sellers^{1‡}, Bruno Valan^{1‡}, and David R. Sherwood^{1,2}

¹Department of Biology, Duke University, Durham, NC 27708;

²Regeneration Next, Duke University, Durham, NC 27708

INTRODUCTION

Genome editing with CRISPR/Cas9 technology has advanced from the lab bench to clinical application, with the first phase 1 and 2 trials underway to treat β -thalassemia (NCT03655678) and sickle cell disease (NCT03745287). Undergraduate laboratory course introductions to CRISPR/Cas9 commonly feature bacteria or yeast (1, 2) but have also been successfully implemented as course-based undergraduate research experiences (CUREs) featuring zebrafish, at the University of Alabama at Birmingham (3), and *Caenorhabditis elegans*, at Pomona College (using the SapTrap method [4], different than the two-plasmid system presented here [Sara K. Olson, personal communication]).

The nematode *C. elegans* is an excellent model system for undergraduate study: it provides low cost, hermaphroditic sexual reproduction, rapid forward and reverse genetics, ease of imaging, and a broad network of researchers. Readily available fluorescent markers, a two-plasmid CRISPR/Cas9 genome editing system (5), and the opportunity for student-led projects make it ideal for independent, lab-based research experiences.

We demonstrate the ability of undergraduates enrolled in an independent study to use CRISPR/Cas9-mediated homologous recombination to generate knock-in worms using monomeric NeonGreen (mNG) fused to the C terminus of the *C. elegans* zinc metalloprotease 4 (*zmp-4*) gene. Belonging to the matrix metalloproteinase (MMP) family of proteins responsible for extracellular matrix degradation and maintenance, *zmp-4* has orthology and conserved structure with human MMPs (6). Previous knowledge of *zmp-4* is limited to protein expression as a transgenic translational reporter (7). No phenotypes have been reported in *zmp-4*

mutant worms, but as MMPs can facilitate cancer metastasis in humans (8), endogenous tagging of ZMP-4 with mNG and visualization of protein trafficking and localization may allow for better understanding of a role for MMPs in normal development and disease.

In this semester-long exercise, students fulfilled key aspects of an effective CURE: using multiple science practices to conduct student-driven discovery with unknown outcomes (students researched, cloned, and endogenously tagged genes of their choosing for the first time), collaborating with *C. elegans* labs (students spoke to other labs to see what genes might be of interest to them), developing novel tools of broad relevance for the scientific community (strains are distributed by the *Caenorhabditis* Genetics Center), and experiencing potential iterative successes or failures (imaging newly generated strains or rethinking their strategy for genes that are difficult to tag) (9). Here we describe a simplified protocol to generate a CURE that can be scaled from first- to fourth-year undergraduate labs.

PROCEDURE

General overview

We used the *C. elegans* CRISPR/Cas9 genome editing strategy described previously (5), with modifications (10) to streamline the procedure for undergraduate lab success (Fig. 1). Briefly, two plasmids are generated: 1) the Cas9/short-guide plasmid and 2) the self-excising cassette (SEC) repair template plasmid for homologous recombination. The Cas9/short-guide plasmid encodes the Cas9 protein and short-guide (sg) RNA sequence used to direct Cas9 DNA cleavage at the genomic location of interest. The repair template plasmid contains *hygR* (a hygromycin resistance gene) and *sqt-1(d)* (a dominant roller (Rol) phenotype inducing gene) to allow for selection of edited lines. A heat shock inducible Cre recombinase (*hs::Cre*) also is encoded, along with two LoxP sites, to remove the SEC (the *hygR* and *sqt-1* genes) after genome editing. Students conducted every aspect of research and discovery: plasmid building, Cas9 guide selection, worm injection, genome-edited strain selection, and imaging of edited lines. We estimate one to two weeks are needed for generation of the two-plasmid

*Corresponding author. Mailing address: Department of Biology, Duke University, 130 Science Drive, Box 90338, Durham, NC 27708.

E-mail: ehastie@duke.edu.

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[‡]R.S. and B.V. contributed equally to this article.

system, with another three to four weeks to inject the plasmids and obtain an edited strain.

Generation of the Cas9/short-guide plasmid

Students conducted a literature search on *zmp-4* to determine a genomic location for knock-in. Next, the genomic DNA sequence was obtained from Wormbase, and crispor.tefor.net was used to identify the short guide location within the genomic region of interest. With the genome editing protocol provided (Appendix 1), students designed primers to amplify the 200-bp short-guide PCR product to be inserted into the digested Cas9/short-guide plasmid with Gibson assembly (Fig. 1A). The Gibson assembly mix was transformed into DH5 α bacteria and incubated overnight. Colony PCR, diagnostic digest (NdeI, XbaI), and sequencing were used to verify correct 200-bp insertion.

Generation of the repair template plasmid

To generate the SEC repair template plasmid, students were given a plasmid containing ~3 kb of genomic DNA (gDNA) centered on the C terminal *zmp-4* insertion site for mNG (~3 kb plasmid was made ahead by the instructor as gDNA amplification was a bottleneck for student progress). With the genome-editing protocol provided (Appendix 1), students designed primers to amplify the ~700-bp 5' and 3' homology arm PCR products to be inserted into the digested SEC repair template plasmid with Gibson assembly (Fig. 1B). The Gibson assembly mix was transformed into

DH5 α bacteria and incubated overnight. Colony PCR, diagnostic digest (SphI, EcoRV), and plasmid sequencing were used to confirm accurate homology arm insertion.

Injection of worms and edited strain selection

Students were trained on the injection microscope and became proficient after two uses. Injection of 20 worms with a mix of the Cas9/short-guide plasmid (50 ng/ μ L) and SEC repair template plasmid (100 ng/ μ L) could be completed in less than two hours and was sufficient to obtain an edited line. Individual injected worms were placed on an OP50 plate and allowed to self-fertilize and produce progeny at room temperature for four days, then treated with 500 μ L hygromycin B (20 mg/mL). During the next two weeks, students examined plates for the Rol phenotype and hygromycin B resistant worms. Rol allows for identification of successful injection while hygromycin B resistance indicates a successful homologous recombination (Fig. 2A). From plates that contained all Rol worms, a mixed-age population of ~10 worms was picked to a new plate and incubated at 34°C for four hours to induce heat shock excision of the SEC. Wild type (non-Rol) worms were singled to new plates and maintained for genotyping (described in Wormbook, Section: Worm Methods, Reverse Genetics) (Fig. 2B) and imaging (Fig. 3).

Imaging of genome-edited worms

Worms were imaged using a Zeiss Axiolmager confocal microscope with a 40 \times Plan-APOCHROMAT objective and

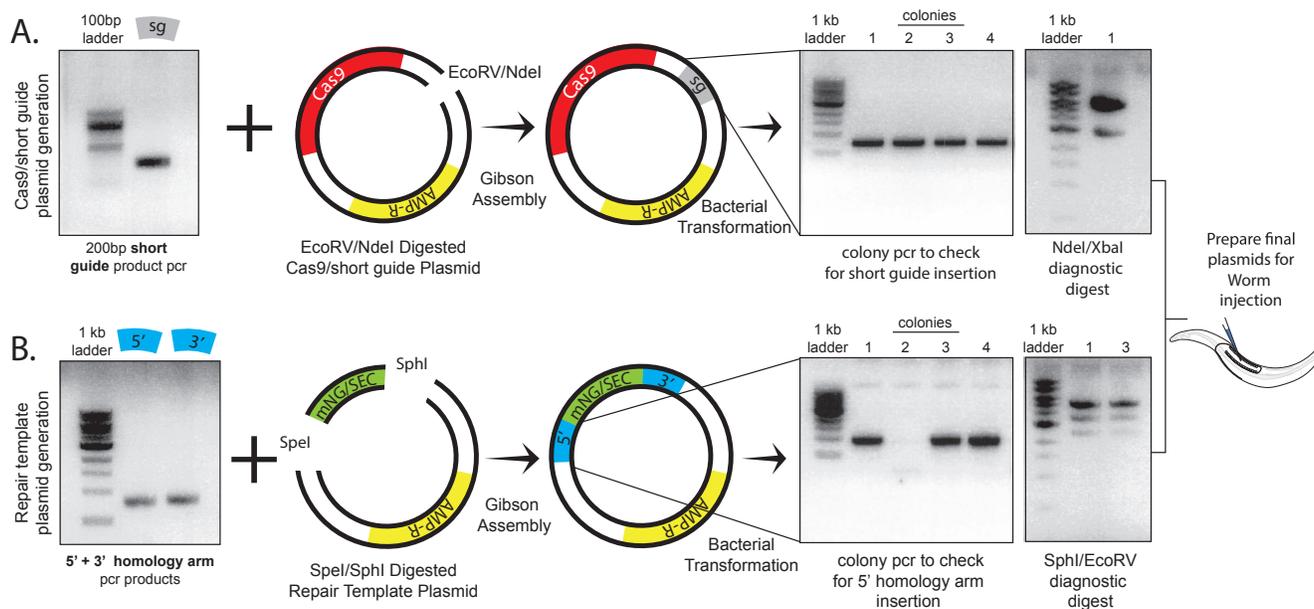


FIGURE 1. A. Cas9/short-guide plasmid generation. Guide primers (Appendix 2) were used to generate a 200-bp PCR product for Gibson assembly with the digested Cas9/short-guide plasmid. Colony PCR was conducted to check insertion, and plasmids were digested and sequenced to confirm Cas9/short-guide plasmid generation. B. Repair template plasmid generation. Homology arm primers were used to generate 5' and 3' arm PCR products for Gibson assembly with the digested repair-template plasmid. Colony PCR was conducted to check insertion of the 5' arm, and plasmids were digested and sequenced to confirm repair-template plasmid generation.

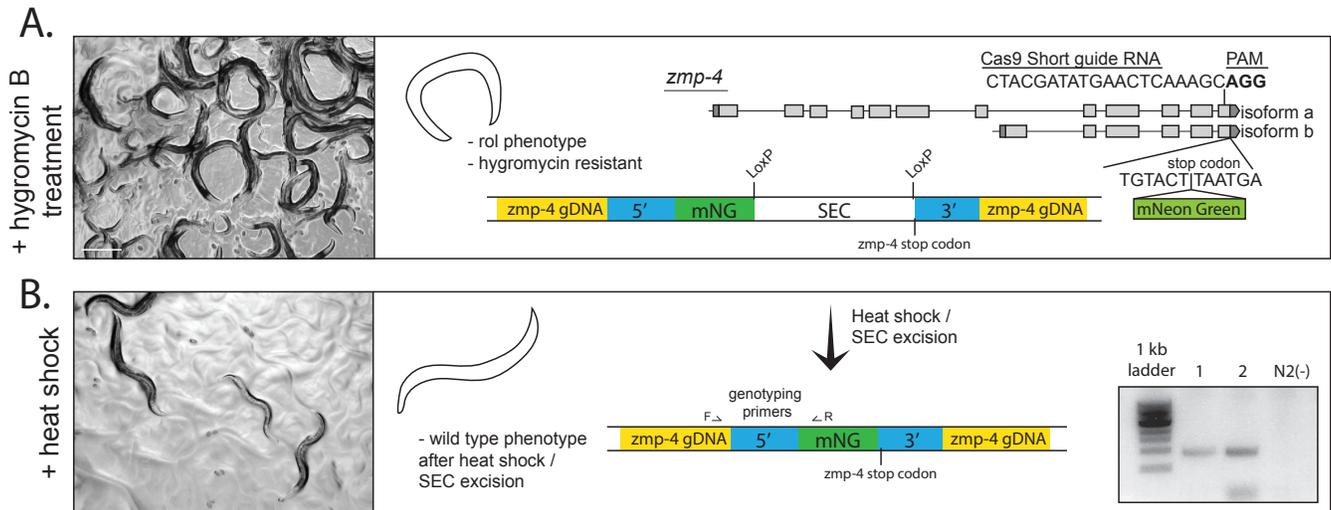


FIGURE 2. A. Hygromycin B selection. Four days following plasmid injection, worms were treated with hygromycin B to select for genome-edited worms. B. SEC excision. Worms from plates containing hygromycin B resistant and Rol phenotype worms were heat-shock treated to excise the selection cassette. After one generation, wild-type worms (all hermaphrodites) were singled to establish potential genome edited lines. Genotyping confirmed mNG insertion at the correct genomic location. Scale bars, 500 μ m.

equipped with a Yokogawa CSU-10 spinning disc confocal controlled by Micromanager software. Images were processed using ImageJ. The students discovered ZMP-4::mNG localization matched the reported transgene in the body wall muscle and head (7) but showed additional localization in the basement membrane of the intestine, vulval/uterine muscles, coelomocytes, and the germ cell membranes. Diffuse mNG expression may indicate previously unreported cleavage of ZMP-4 (Fig. 3).

Safety issues

All work here is BSL1. Students should complete training required to work in a molecular biology lab with recombinant DNA and *C. elegans*.

CONCLUSION

The use of *C. elegans* and this molecular biology approach provide an inexpensive method to offer a scalable CURE for first-year through fourth-year undergraduate students. Importantly, of the approximately 50 genes that we have endogenously tagged in our lab, this approach has been successful in 48 cases, indicating it is robust.

We envision a lab with a designated time for lecture and discussion combined with open hours where molecular biology can occur at the students' pace (with deadlines and expected outcomes in place for progress and assessment). Additionally, strains generated in one class can be used for discovery in future classes. These strains can also be sent to the Caenorhabditis Genetics Center for distribution, giving students a sense of ownership and contribution to the scientific community (11). For institutions without the

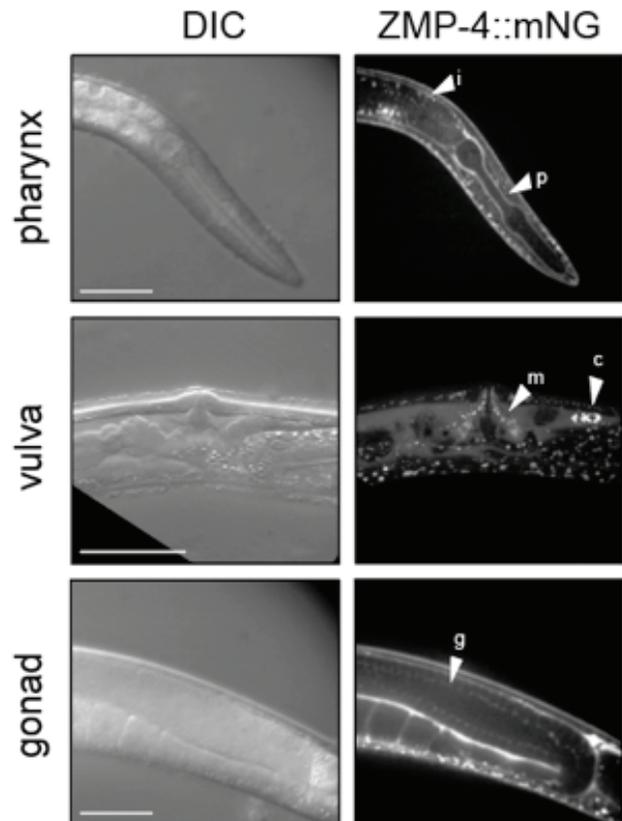


FIGURE 3. Imaging of genome-edited worms. Differential interference contrast (DIC) and fluorescence microscopy of ZMP-4::mNG. Expression of the tagged ZMP-4::mNG appears in the basement membrane around the pharynx (p, feeding organ) and intestine (i), the vulval/uterine muscles (m, facilitate egg laying) and coelomocytes (c, mesodermal cells that endocytose body cavity fluid), and the germ cell membranes (g) (white arrows). Scale bars, 50 μ m.

injection or imaging capability, this lab is perfectly suited to collaboration. Students can build the constructs, send them to be injected, obtain and screen the injected worms, and work with other labs for imaging. In summary, this CURE provides a satisfying research experience in that success is likely, and it involves cutting-edge CRISPR/Cas9 technology with transgenics to connect students with the most advanced therapies in society today.

SUPPLEMENTAL MATERIALS

Appendix 1: Genome editing protocol / troubleshooting
Appendix 2: Primer ordering table

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