FINAL SCRIPT: APPROVED FOR FILMING

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Project Page Link: https://review.jove.com/account/file-uploader?src=21024308

Title: CRISPR-Cas9-Based Mutagenesis in the Entomopathogenic Nematode *Steinernema hermaphroditum* and the Maintenance of Mutant Lines

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

   If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

   Zeiss Axio Observer 3 for microinjection

   Leica Inveta 3 for nematode manipulation

   SCOPE: 2.3.1-2.3.2, 3.4.3, 3.6.1-3.6.2, 3.7.1-3.7.2, 3.8.2-3.4.4, 3.10.1-3.10.3,3.12.1-3.12.2
   Videographer: Please capture the above shots with a SCOPE KIT

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, in the same building**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length
Number of Steps: 21
Number of Shots: 44

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Sally Ireri:** We are working on the genetics of nematode-bacteria symbiosis which has agricultural applications.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What technologies are currently used to advance research in your field?~~

1.2. **Mengyi Cao:** Genetic tools in *Xenorhabdus* are well developed, but EPN genetics remain limited, relying mostly on RNAi and EMS screens.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What significant findings have you established in your field?~~

1.3. **Mengyi Cao:** My lab expanded my postdoctoral work on EPN genetics, and CRISPR is now established in three *Steinernema* species.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What advantage does your protocol offer compared to other techniques?~~

1.4. **Sally Ireri:** In this protocol we establish an efficient technique for creating stable heritable mutants using CRISPR-Cas9 gene editing.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What new scientific questions have your results paved the way for?~~

1.5. **Sally Ireri:** With stable CRISPR-Cas9 gene editing we can dissect the molecular mechanisms of the *Steinernema-Xenorhabdus* symbiosis.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Mengyi Cao, Principal Investigator, Carnegie Institution for Science**: (authors will present their testimonial statements live).

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Mengyi Cao, Principal Investigator, Carnegie Institution for Science**: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Nematode Preparation and CRISPR Target Selection for Microinjection in *Steinernema hermaphroditum*

Demonstrator: Sally Ireri

2.1. To begin, obtain nematode agar plates seeded with *Xenorhabdus griffiniae* (*ZEE-noh-rab-dus grih-FIN-ee-ay)* and *Comamonas aquaticus (KOH-muh-MOH-nas uh-KWAH-ti-kus)* **[1]**.

2.1.1. WIDE: Talent looking at seeded NGM plates.

2.2. Pick *Steinernema hermaphroditum (STY-ner-NEE-muh her-MAFF-roh-DYE-tum)* nematodes onto the prepared NGM plates containing *Xenorhabdus griffiniae* **[1].** Incubate them at 25 to 28 degrees Celsius for optimal growth **[1]**.

2.2.1. Talent picking out labeled nematode plates seeded with *X. griffiniae*.

2.2.2. Talent placing them in an incubator.

2.3. The night before microinjection, pick J3-stage hermaphrodites onto fresh NGM agar plates seeded with *Xenorhabdus griffiniae* **[1]**. On the day of microinjection, pick young adult hermaphrodites for injection **[2]**.

Videographer: Please capture all shots labeled SCOPE using a SCOPE KIT

2.3.1. SCOPE: Talent transferring J3 nematodes onto fresh NGM plates seeded with *X. griffiniae*.

2.3.2. SCOPE: Talent selecting young adult nematodes.

2.4. Extract the target gene sequence from the *Steinernema hermaphroditum* reference genome using BLASTP *(Blast-P)* **[1-TXT]**.

2.4.1. SCREEN: SCREEN_2.4.1.mp4 01:38-02:02 **TXT: Accession ID: PRJNA982879**

2.5. Select an early coding exon, preferably the first exon **[1]** and input that sequence into CRISPRScan *(Crisper-Scan)* using default settings to generate candidate crRNAs *(crisper-R-N-Ase)* **[2]**. Blast the top candidate crRNAs against the *Steinernema hermaphroditum* genome to ensure there are no off-target matches **[3]**.

2.5.1. SCREEN: SCREEN_2.5.1.mp4 00:23-00:28

2.5.2. SCREEN: SCREEN_2.5.2.mp4 00:08-00:26

2.5.3. SCREEN: SCREEN_2.5.3.mp4. 00:04-00:25, 00:36-00:40

2.6. Store the selected crRNAs and universal trans-activating CRISPR RNAs at 100 micromolar each in a duplex buffer at minus 80 degree Celsius **[1]**. Melt 2 percent agarose in water using a microwave **[2]**.

2.6.1. Talent placing labeled microfuge tubes into a –80 °C freezer.

2.6.2. Talent placing a flask of agarose solution into a microwave.

2.7. Transfer 50 microliters of the melted agarose onto a cover glass **[1]**, immediately cover it with a second cover glass **[2]**. After a 20-minute drying time, remove the top cover glass **[3]** then label the front side of the agarose pad **[4].**

2.7.1. Talent pipetting molten agarose onto a cover glass.

2.7.2. Talent placing the second cover glass on top.

2.7.3. Shot of the top cover glass being removed.

2.7.4. Talent labeling the front side of the agarose pad.

2.8. Store the agarose pads at room temperature for long-term use for months to years **[1]**.

2.8.1. Talent stacking labeled agarose pads in a dry box.

2.9. To prepare the needles for injection, pull quartz capillaries on a laser filament puller **[1]**, and store the needles in a pipette storage box **[2]**.

2.9.1. Shot of quartz capillaries being pulled on a laser filament puller.

2.9.2. Talent loading the pulled needles into storage.

3. CRISPR-Cas9 Microinjection and Post-Injection Recovery in *Steinernema hermaphroditum*

3.1. Prepare fresh guide RNA duplexes by mixing the selected crRNAs and universal trans-activating CRISPR RNAs in a 1 to 1 ratio **[1]**. Incubate the tube at 94 degrees Celsius for 2 minutes, then cool to room temperature to anneal duplexes **[2]**.

3.1.1. Talent micropipetting crRNAs and tracrRNA into a tube.
AND
TEXT ON PLAIN BACKGROUND:
crRNA #1 (100 µM): 1.5 µL

crRNA #2 (100 µM): 1.5 µL

tracrRNA (100 µM): 3.0 µL
Video Editor: Please play both shots side by side in a split screen

3.1.2. Talent placing the tube in a PCR cycler and inputting the settings.

3.2. Assemble the injection mix by combining Cas9 *(Kass-nine)*, potassium chloride and the gRNA duplexes and incubate **[1]**.

3.2.1. Talent pipetting of each component into the tube.

AND
TEXT ON PLAIN BACKGROUND:
Cas9 (10 mg/mL): 2 µL

1M KCl: 0.58 µL

gRNA duplexes :2.7 µL

Incubation: RT, 5 min

Video Editor: Please play both shots side by side in a split screen

3.3. Flick the PCR tube three times to mix **[1]**, then spin briefly for 10 to 15 seconds in a mini centrifuge **[2].** After the final mixing, spin for up to 1 minute to collect contents **[3]**.

3.3.1. Talent flicking the tube.

3.3.2. Talent placing the tube in a mini centrifuge.

3.3.3. Shot of centrifuged tube with pellet.

3.4. Next, use a microloader tip to load 1 to 2 microliters of injection mix into the needle **[1]**. Mount the needle onto the holder **[2]**, break open the tip by dragging across double-sided tape under halocarbon oil **[3]**, and test the size of the opening using the **CLEAN** function of the microinjector **[4]**.

Videographer: Please capture all shots labeled SCOPE using a SCOPE KIT

3.4.1. Talent attaching a microloader tip and loading the mix into the needle.

3.4.2. Talent assembling the needle on the holder.

3.4.3. SCOPE: Shot of the tip being dragged across a double-sided tape under halocarbon oil.

3.4.4. Talent pressing the **CLEAN** option on the instrument screen.

3.5. Place a small droplet of halocarbon oil onto the 2 percent agarose pad **[1]**.

3.5.1. Talent pipetting oil onto an agarose pad.

3.6. Using a dissecting microscope and a soft platinum wire, pick up a young adult hermaphrodite **[1].** Wash it in M9 buffer to remove the bacteria **[2].**

3.6.1. SCOPE: Shot of the worm being picked up with a platinum wire.

3.6.2. SCOPE: Shot of the worm being placed in M9 buffer.

3.7. Transfer the nematode onto an agarose pad and place it a droplet of halocarbon oil **[1].** Use the soft platinum wire to gently brush the nematode until immobilized **[2]**.

3.7.1. SCOPE: Talent placing worm into oil on agarose pad.

3.7.2. SCOPE: Show gentle brushing to immobilize the worm.

3.8. Mount the pad on the microinjection stage **[1].** Locate one gonadal arm and penetrate

it with the needle **[2]**. Use the **CLEAN** function of the injector to deliver the injection mix at 99 pounds per square inch **[3]** until gonadal flow is visible, confirming reagent access to the syncytium **[4]**. If accessible, reposition the stage and inject the second gonadal arm similarly **[5]**.

NOTE: VO and shot numbers edited to accommodate the added shot

3.8.1. Talent placing the pad on the microinjector stage. *Added shot: A gonadal arm is being located and penetrated with a needle*

3.8.2. SCREEN: Show the injector interface and execution of **CLEAN** and injection pressure.

3.8.3. SCOPE: Show the worm's gonad under microscope with flow of liquid.

3.8.4. SCOPE: Show repositioning and injecting second arm.

3.9. Immediately after injection, resuspend the injected animal in 1 to 2 microliters of M9 buffer on the agarose pad **[1]**.

3.9.1. Shot of M9 buffer being pipetted onto the pad and mixing with the worm.

3.10. Use a thin platinum wire to pick the injected worm off the pad **[1]**, then wash off oil with M9 *(M-Nine)* buffer **[2]**, and recover it onto the recovery NGM plate seeded with *C. aquaticus (C-Ackwa-T-cuss)* **[3]**.

3.10.1. SCOPE: Show picking the worm.

3.10.2. SCOPE: Show washing off oil.

3.10.3. SCOPE: Show transferring onto recovery plate.

3.11. Incubate the recovery plate at 25 degrees Celsius overnight **[1]**.

3.11.1. Talent placing the plate placed into an incubator.

3.12. The next day, isolate each injected P0 *(p-Zero)* nematode onto individual NGM plates seeded with *Xenorhabdus griffiniae* **[1-TXT].** ~~and allow them to produce F1 progeny overnight for phenotyping and genotyping **[2].**~~

3.12.1. SCOPE: Show picking individual P0 worms onto fresh plates. **TXT: Allow them to produce F1 progeny overnight for phenotyping and genotyping**

~~3.12.2. SCOPE: Show eggs or bagging of F1 progeny.~~ AUTHOR'S NOTE:~~-~~The microscope shot was not clear enough to show eggs or bagging worms.

Results

4. Results

4.1. Two CRISPR RNAs were designed targeting the *Steinernema hermaphroditum* homolog of *unc-22 (Unk-Twenty-Two)* at previously published Pam *(Pam)* sites **[1].** Jerk analysis of nematodes exposed to nicotine showed distinct motion differences between unc-22 mutants and wild-type nematodes **[1]**.

Added shot: 4.1.0: SCOPE shot of distinct twitching or jerking motion seen in the unc-22 mutants compared to the non-twitching WT (wild-type).

4.1.1. LAB MEDIA: Figure 2A

4.1.2. LAB MEDIA: Figure 2B. *Video editor: emphasize the blue "unc" trace*

4.2. Genotyping of twitching progeny by PCR showed multiple faint bands at the target site for both Pam 3 and Pam 5 **[1]**.

4.2.1. LAB MEDIA: Figure 2C, D. *Video editor: Highlight lane 5 in the gel labeled "Pam 3" and "Pam 5"*

4.3. Sequencing confirmed insertion and deletion events at the targeted unc-22 site **[1]**.

4.3.1. LAB MEDIA: Figure 2E.

Pronunciation Guide:

 CRISPR
Pronunciation link: https://www.merriam-webster.com/dictionary/CRISPR
IPA: /ˈkrɪs.pɚ/
Phonetic Spelling: KRIS·per
 Cas9
Pronunciation link: https://www.howtopronounce.com/cas9
IPA: /kæs ˈnaɪn/
Phonetic Spelling: kas·NYNE
 Mutagenesis
Pronunciation link: https://www.merriam-webster.com/dictionary/mutagenesis
IPA: /ˌmjuː.ɾ̯əˈdʒɛn.ə.sɪs/
Phonetic Spelling: myoo·tuh·JEN·uh·sis
 Entomopathogenic
Pronunciation link: https://www.merriam-webster.com/dictionary/entomopathogenic
IPA: /ˌɛn.toʊˌmɑː.pəˈθɛdʒ.ə.nɪk/
Phonetic Spelling: en·toh·mah·puh·THEDJ·uh·nik
 Nematode
Pronunciation link: https://www.merriam-webster.com/dictionary/nematode
IPA: /ˈnɛməˌtoʊd/
Phonetic Spelling: NEM·uh·tohd
 Steinernema
Pronunciation link: https://www.howtopronounce.com/steinernema
IPA: /ˌstaɪ.nɚˈniː.mə/
Phonetic Spelling: sty·ner·NEE·muh
 Hermaphroditum
Pronunciation link: https://www.howtopronounce.com/hermaphroditum
IPA: /hɝːˌmæf.rəˈdaɪ.təm/
Phonetic Spelling: her·MAF·ruh·DYE·tum
 Xenorhabdus
Pronunciation link: https://www.howtopronounce.com/xenorhabdus
IPA: /ˌzɛn.oʊˈræb.dəs/
Phonetic Spelling: ZEN·oh·RAB·dus
 Comamonas
Pronunciation link: https://www.howtopronounce.com/comamonas
IPA: /ˌkɑː.məˈmoʊ.nəs/
Phonetic Spelling: kah·muh·MOH·nuhs
 Microinjection
Pronunciation link: https://www.merriam-webster.com/dictionary/microinjection
IPA: /ˌmaɪ.kroʊ.ɪnˈdʒɛk.ʃən/
Phonetic Spelling: my·kroh·in·JEK·shuhn

 Agarose
Pronunciation link: https://www.merriam-webster.com/dictionary/agarose
IPA: /ˈæg.əˌroʊs/
Phonetic Spelling: AG·uh·rohs
 Capillaries
Pronunciation link: https://www.merriam-webster.com/dictionary/capillary
IPA: /ˈkæp.əˌlɛr.i/
Phonetic Spelling: KAP·uh·lair·ee