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Prerequisites
Basics of worm maintenance.

Concepts
These are big worms. Only grow them on 10 cm plates.

They will starve by the third generation (F_2). When the second generation (F_1) reaches adulthood, wash them off the plates, and move them onto at least 3 times the amount of plates you started with.

Larvae do not like to settle in 50 mL conical tubes.

Bacteria in your sample will eat up reads, that is, DNA from bacteria will be sequenced in place of the nematode. We separate bacteria from nematodes by letting the nematodes settle in 15 mL conical tubes. Bacteria in the supernatant will be discarded.

We discard plates with mites, mold, or bacterial growth. These typically make for unhealthy worms. We also discard plates where the worms have burrowed into the agar. Loose pieces of agar and agarose in your sample can interfere with downstream reactions.

They don't like M9. I substitute with sterile water or PBS throughout.

Notes
This protocol is based on development at 25 ˚C.

Seeding bacteria
Xenorhabdus griffinae, HGB2587
Xenorhabdus griffinae, HGB2587, concentrated

Plates
10 cm NGM plates with 1.0 % agar and 0.7 % agarose (herein 10 cm NGM plates) seeded with *X. griffiniae*.

Reagents and equipment
Eppendorf tubes, 1.5 mL
Conical tubes, 15 and 50 mL

Centrifuges compatible with 1.5 mL, 15 mL and 50 mL tubes
Sterile water
PBS, 1X
Funnel, washed with Sparkleen and 10% bleach before and after using
CellMicroSieve™ nylon mesh filters, 30 and 50 µm, washed with Sparkleen and 10% bleach before and after using
Shaker
Calbiochem Protease Inhibitor Cocktail Set I (539131), 100X in water
PMSF, 100 mM in isopropanol
Liquid nitrogen or dry ice and ethanol

For fixed collections, you will also need…
Formaldehyde, 37% (w/v)
Glycine, 2.5 M
Nutator

Time
7-8 days

Yield
200 µL of packed early stage larvae from 3 10 cm plates

Protocol
1. Transfer 10 infective juveniles (F_0) to n_1 10 cm NGM plates seeded with HGB2587. **Tip:** 3 plates will give you a good yield. **Tip:** get infective juveniles from starved plates (see Appendix A).

2. Place plates at 25 ˚C for 6 days or until most of the F_1 worms reach young adulthood. Feed as necessary with **concentrated food**.

3. Add 8 mL of **sterile water** to each plate, and place them on a shaker for 3 minutes.

4. Move the worms to a 50 mL conical tube with a strippetor.

5. Let the worms settle, remove the supernatant, and resuspend in **sterile water**.

6. Repeat step 5 two times for a total of three washes. **Expected:** 500-600 µl of settled adults.

7. Resuspend the worms in **sterile water**, and transfer them to at least $3n_1$ 10 cm NGM plates seeded with HGB2587. **Tip:** I resuspend them in about 1800 µL water.

8. Let the plates dry on the bench top. **Tip:** the adults will settle at the bottom of the pipette tip. Drop the worms in the center of the plate, and swirl or tilt the plate before they dry to spread out the worms.

9. Place plates at 25 ˚C for 2 days or until the F_2 are mostly early stage looking larvae. Feed as necessary with **concentrated food**.

10. Add 8 mL of **sterile water** to each plate, and place plates on a shaker for 3 minutes.

11. Move the worms onto a 50 µm nylon mesh filter in a funnel over a 50 mL conical tube. Only larvae will pass through the filter.

12. Pass the filtrate through a 30 µm filter. Only early stage larvae will pass through the filter. **Note:** if you skip step 11, the adults will clog the filter.

13. Transfer the filtrate to 15 mL conical tubes.

14. Let the larvae settle for 10 minutes, discard the supernatant (i.e., bacteria), and resuspend in **sterile water**.

15. Let the larvae settle for 10 minutes and discard the supernatant.

16. Combine tubes with **sterile water**. Transfer 20 µL of resuspended worms onto a seeded 10 cm NGM plate and place at 25 ˚C. You will image from this plate later. Pipette 20 µL onto a depression slide and check before proceeding. You should not have adults, embryos, or many late stage larvae (L4).

17. Spin for 1 minute at 2000 x g in a swinging bucket rotor. Discard supernatant.

18. Follow the protocol in Ercan_worm_collection_vs3 for collecting crosslinked or native larvae with the following edit: replace **1X M9** with **1X PBS** (or see Appendix B).

19. Choose the biggest worms from the plate you set aside in step 16, and image their germline with the protocol in Ercan_staging_by_germline_proliferation_v1.

Recipes

Bacterial culture

Dark LB
Add 10 g NaCl, 10 g tryptone, and 5 g yeast extract to a 1 L bottle wrapped in aluminum foil with a magnetic stir bar. Add 950 mL milliQ water and dissolve. Transfer to a graduated cylinder and add water to 1000 mL. Move back to the bottle, remove the foil, and autoclave for 45 min at 121.5 ˚C. Let the bottle cool down in a dark place. Wrap it in aluminum foil and store in a dark place.

Kanamycin (50 mg/mL)
Add 10 mL milliQ water to 0.5 g kanamycin sulfate in a 15 mL conical tube. Dissolve on a nutator. Filter sterilize (0.22 micron) and make 1 mL aliquots. Store at -20 ˚C.

LB pyruvate (0.1%) plates with kanamycin
Add 5 g agar, 2.5 g NaCl, 2.5 g tryptone, 1.25 g yeast extract, 0.25 g sodium pyruvate, and 250 mL milliQ water to a 1 L Erlenmeyer flask with a magnetic stir bar. Autoclave for 45 min at 121.5 ˚C. Let the flask cool to 55 ˚C on a magnetic heat block while stirring. Add 250 µL kanamycin (50 mg/mL) and mix. Pour into about 10 10 cm petri dishes. Let plates solidify overnight on the benchtop. Store them upside down at 4 ˚C for up to a month. The sodium pyruvate will quench any photoproducts. You do not need to store the plates in the dark until they are seeded.

***Xenorhabdus griffinae* for seeding**
X. griffinae is sensitive to photoproducts produced in LB. Therefore, use dark LB unless supplemented with sodium pyruvate. *X. griffinae* also does not survive for long at 4 ˚C. Instead, store at room temperature for up to two weeks. We used the strain HGB2587, which we received from Mengyi Cao. It is resistant to kanamycin, streptomycin, and chloramphenicol. It constitutively expresses attTn7-TurboRFP, which makes the colonies and cultures pink. See

Xu, J. & Hurlbert R. E. 1990. Toxicity of irradiated media for Xenrhabdus spp. *Appl Environ Microbiol* 56:815-818.

St Thomas NM, Myers TG, Alani OS, Goodrich-Blair H, Heppert JK. 2024. Green and red fluorescent strains of Xenorhabdus griffiniae HGB2511, the bacterial symbiont of the nematode Steinernema hermaphroditum (India). *MicroPubl Biol.*

1. Place an LB pyruvate (0.1%) plate with kanamycin at 30 ˚C for at least 40 minutes.
2. Remove a glycerol stock from -80 ˚C, and streak (tons of protocols online for this. I really like this one) the prewarmed plate.
3. Incubate the plate upside down at 30 ˚C overnight.
4. Store at room temperature for up to two weeks. The colonies may appear colorless at first, but will turn pink/red with time.
5. In a 125 mL sterile Erlemeyer flask, inoculate 20 mL dark LB and 20 µL kanamycin (50 mg/mL) with one colony. Wrap the flask in aluminum foil.
6. Shake at 200 rpm at 30 ˚C overnight in the dark.

Seeds about 15-20 plates. I like to make six patches. I use a strippetor to drop a couple drops onto the plate for each patch, and let it dry overnight in the dark. More than a couple drops per patch will take forever to dry.

Concentrated *X. griffinae*
1. In a 125 mL sterile Erlemeyer flask, inoculate 20 mL dark LB and 20 µL kanamycin (50 mg/mL) with one colony.
2. Shake in the dark at 200 rpm at 30 ˚C overnight. **The next morning, the culture should be pink.**
3. The next morning, prepare Terrific Broth (16 g tryptone, 32 g yeast extract, 10 mL sterile glycerol (50%), 1200 mL MilliQ H_2O) in a 2 L Erlenmeyer flask with a magnetic stir bar, dissolve, and divide it amongst four baffled 1 L Erlenmeyer flasks.
4. Autoclave for 45 minutes at 121.5 ˚C.
5. Let the broth cool down in a dark place like a cabinet.
6. Add 33 mL sterile 10X phosphate buffer (23.1 g KH_2PO_4, 125.4 g K_2HPO_4, 1000 mL H_2O) and 330 µL kanamycin (50 mg/mL) to each flask and mix.
7. Inoculate each flask with 5 mL of the starter culture.
8. Shake in the dark at 200 rpm at 30 ˚C overnight, and preferably for 24 hours. **The broth will turn from a dark to light brown.**
9. Transfer the culture to 2 1 L sterile centrifuge bottles.
10 Spin at 6000 rpm with a fixed angle rotor at 4 ˚C for 20 min.
11. Remove the supernatant and weigh each pellet.
12. Prepare a 5% glycerol solution in M9.
13. Add the glycerol solution to each pellet in a 1:1 g to mL ratio.
14. Place bottles in a shaking incubator set to 200 rpm and 30 ˚C to dissolve the pellet. You can wrap the bottom of the bottles in paper towels to make them fit in the adapters.
15. Make 5 mL aliquots and store at 80 ˚C.

Yield can vary depending on how long they are in culture. You can expect 20-50 mL.

I have kept them at -80 ˚C for about a year without problems. Perhaps they can last longer. I have not checked. If they're alive, the drop should become thicker and pink(er).

To thaw, leave in the dark at room temperature, and store for up to 4 days. Perhaps they can last longer. I always use up a 5 mL aliquot within 4 days. You can test them by checking the viscosity. With time, the bacteria will die, and the solution will become more viscous. You can also test as described above.

To feed, pipette up to 400 µL onto an NGM plate with worms (I drip it onto the plate so that the plate looks like it has spots. You can also tap the sides of the plate to spread the drops around). Let the droplets dry on the benchtop and place the plates back in the incubator. Repeat as necessary.

Worm culture

NGM plates with 1.0 % agar and 0.7 % agarose
1. In a 2 L flask, add 980 ml milliQ water, 3 g NaCl, 10 g agar, 7 g agarose, and 2.5 g peptone
2. Add a stir bar inside the flask.
3. Cover the mouth of the flask with aluminum foil, and autoclave for 45 minutes at 121.5 ˚C.
4. Let the flask cool while stirring on a hot plate set to 55 ˚C for 30-40 minutes or until it's about bath temperature.
5. While stirring, and next to flame, add – IN THIS ORDER – 1 ml 1 M CaCl2, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$, and 25 ml 1 M KPO_4 buffer .
6. Using sterile technique, dispense the NGM solution into 10 cm petri dishes.
7. Leave the plates to dry at room temperature for one day.
8. Seed with *Escherichia coli*, OP-50 (using a 5 mL pipette tip and Stripettor, add 1 drop onto a 6 cm plate, or 4 drops onto a 10 cm plate).
9. Dry overnight by leaving the plates at room temperature.
10. You can store unseeded or seeded plates at 4 ˚C to make them last longer.

Sterile water
Filter milliQ water through a 0.22 micron vacuum filter, and autoclave for 45 minutes at 121.5 ˚C. Store at room temperature.

PBS, 1X
I buy this at 10X and dilute with sterile water because I don't trust our pH meter. It's also cheap. Store at room temperature.

Other

Protease inhibitors, 100X
Dissolve Calbiochem protease inhibitor cocktail set I (catalog number 539131) as per the manufacturers instructions to make a 100X solution. Store at -20 ˚C and add fresh.

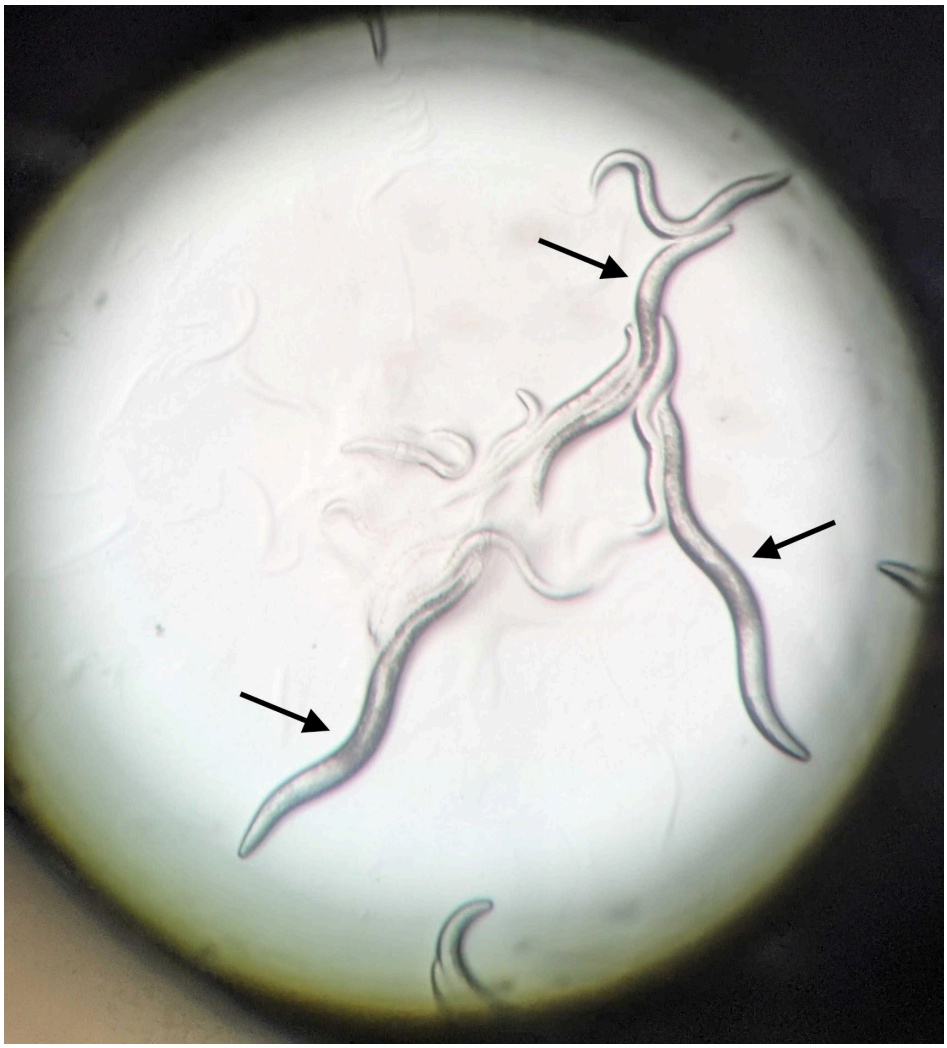
Product	Cat. No.	Mol. Wt.	1X Concentration	Target Protease
AEBSF, Hydrochloride	101500	239.5	500 µM	Serine Proteases
Aprotinin, Bovine Lung, Crystalline	616370	6512	150 nM	Serine Proteases and Esterases
E-64 Protease Inhibitor	324890	357.4	1 µM	Cysteine Proteases
EDTA, Disodium	-	372.2	0.5 mM	Metalloproteases
Leupeptin, Hemisulfate	108975	475.6	1 µM	Cysteine Proteases and Trypsin-like Proteases

The above table taken from the manufacturer shows five protease inhibitors in this cocktail.

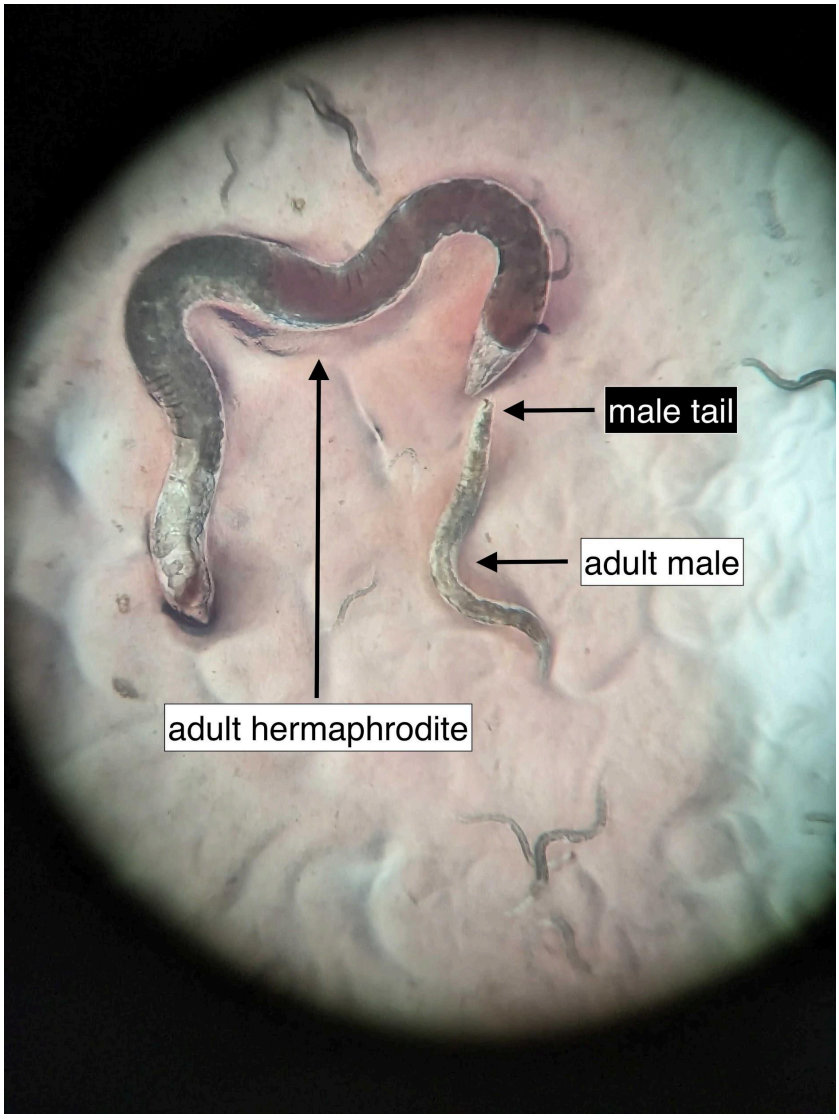
PMSF, 100 mM
Add 348 mg of PMSF and 20 mL of Isopropanol to a conical tube. Nutate to dissolve. Make 1.5 mL aliquots and store at -20 °C. Add to a final concentration of 1 mM as a protease inhibitor. PMSF is inactivated in aqueous solutions. The rate of inactivation increases with increasing pH and is faster at 25°C than at 4°C. The half-life of a 20 mM aqueous solution of PMSF is about 35 min at pH 8.0. Therefore, always add it fresh!

Glycine, 2.5 M
Add 93.8 g glycine to 500 mL water, dissolve and autoclave for 45 min at 121.5 ˚C. Store at room temperature.

Appendix A. Identifying males and infective juveniles



Black arrows point to infective juveniles, which are easily found on starved plates. Notice the long white head, and the white going down the length of its inner body.



Adult males are so much smaller than adult hermaphrodites. You can find them wrapping themselves around the hermaphrodites when mating. Note the male tail . It does not have the distinct hook or fan like structure of *C. elegans*, but is pretty distinct. It has almost like its own kind of hook (zoom in to see). I don't really have too much issue with males dominating the plates.

Appendix B. Step 18 (adapted from Ercan_worm_collection_vs3)

If collecting uncrosslinked larvae for western, IP, ATAC-seq, etc., then follow these steps.

1. Wash larval pellet with 2-3 mLs of **1X PBS**, and spin for 1 minute at 2000 x g (worms need to be resuspended for a proper wash. Pipette up and down to invert the tube to resuspend).

2. Check that the worms pelleted. Discard supernatant with a pipette.

3. Transfer 100-200 µL larval pellet with 1 mL **1X PBS** to a 1.5 mL tube.

4. Add **protease inhibitors** to 1X and **PMSF** to 1 mM and mix (can add to PBS in step 3 instead as long as it is added fresh because PMSF has a short half life in aqueous solutions). **Note:** You can only freeze/thaw a pellet once. For large pellets, e.g., 200 µL or more, split it between multiple tubes to get more samples out of one collection.

5. Spin for 1 minute at 6000 x g[#]. Check that the worms pelleted[#]. Discard the entire supernatant with a pipette.

6. Snap freeze in **liquid nitrogen** or **dry ice/ethanol**. For liquid nitrogen, dip the tube into a dewar until it stops bubbling. For the dry ice ethanol bath, add a layer of dry ice to an ice bucket, and pour enough ethanol into the bucket to submerge the pellet. Dip your tube(s) in the ethanol until frozen.

7. Store at -80 ˚C, and enter information on Ercan_Lab_WormCollections_Extracts .

If collecting formaldehyde crosslinked larvae, follow these steps.

1. To the larval pellet, add **1X PBS** to 9.5 mL

2. Add **formaldehyde** to a 2% final concentration (560 µL of 37% formaldehyde to 10 mL).

3. Nutate at room temperature for 30 minutes.

4. Quench formaldehyde by adding **glycine** to 125 mM (550 µL of 2.5 M glycine to 10 mL), and nutate for 5 minutes.

5. Spin at 2000 x g for 1 minute. Check the pellet and discard supernatant with a pipette.

6. Wash with 10 mL **1X PBS**. Mix well.

7. Spin at 2000 g for 1 minute. Check the pellet and discard supernatant with a pipette.

8. Wash with 2-3 mL **1X PBS**. Mix well.

9. Spin at 2000 x g for 1 minute. Check the pellet and discard supernatant with a pipette.

10. Transfer pellet to a 1.5 mL tube with 1 mL **1X PBS**.

11. Add **protease inhibitors** to 1X and **PMSF** to 1 mM and mix.

12. Spin for 1 minute at 6000 x g[#]. Check that all larvae are pelleted and discard supernatant.

13. Snap freeze in **liquid nitrogen** or **dry/ice ethanol** (see above).

14. Store at -80 ˚C and enter information on Ercan_Lab_WormCollections_Extracts.

[#] If you notice worms on the walls/in the supernatant of the 1.5 ml microfuge tube, spin for 5 minutes at 6000 x g. Lower speed for longer is better to prevent hard brake, which shoots up the worms.