Edvo-Kit # 856

Edvo-Kit #856

Environmental Toxicity Responses in *C. elegans*

Experiment Objective:

In this experiment, students will perform a bioassay that tests the impacts of heavy metals on *Caenorhabditis elegans*. Students will also examine RNA editing and genotype – environmental interactions by comparing the reactions of wild-type and mutant strains.

See page 3 for storage instructions.

Version 856.210824

EDVOTEK®

Table of Contents

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

	Component	Storage	Check ✓
A	Wild-type *C. elegans**	Room Temperature	❑
B	ADR-2 Mutant *C. elegans**	Room Temperature	❑
C	*E. coli* OP50 BactoBeads™	4° C Refrigerator	❑
D	S-Buffer	4° C Refrigerator	❑
E	NGM Salts	4° C Refrigerator	❑
F	Sterile Water	4° C Refrigerator	❑
G	Copper Sulfate Solution	4° C Refrigerator	❑
H	Cadmium Chloride Solution	4° C Refrigerator	❑
I	Zinc Sulfate Solution	4° C Refrigerator	❑
•	ReadyPour™ NGM Agar	4° C Refrigerator	❑

Components A&B are live items that cannot be stored. Call 1-800-EDVOTEK to request these two weeks before the experiment's start date. Review page 18 for additional instructions.

Experiment #856 is designed for 10 groups.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Reagents & Supplies *(included with this experiment)*

Store all components below at room temperature.

- Petri Dishes ❑
- Large Transfer Pipets (Sterile) ❑
- Small Transfer Pipets ❑
- 1.5 mL Microcentrifuge Tubes (Snap-top) ❑
- Counting Chambers ❑
- Sterile Loops ❑
- 15 mL Conical Tubes ❑
- 10 mL Pipet ❑

Experiment Requirements *(NOT included with this experiment)*

- Incubator, covered plastic box, or cardboard box (kit box will work as an incubator)
- Microscopes (20-40X magnification)
- Timers
- Pipet pump or bulb
- Adjustable micropipettes and tips (10-100 µL, 100-1000 µL) (optional)
- Marking pens
- Microwave or hot plate
- Distilled or deionized water
- Disposable laboratory gloves
- Bleach solution or laboratory disinfectant
- Water bath (optional)

Background Information

Environmental Toxicology and Bioassays

Environmental toxicology is the study of the effects of harmful chemicals on human health and the environment. This field rocketed into the public consciousness in 1962 with Rachel Carson's book "Silent Spring" which looked at the effects of dichlorodiphenyltrichloroethane (DTT) on humans and wildlife. Today, scientists from a range of backgrounds work to determine where a toxicant will end up in an ecosystem and how long it will remain. Using a series of bioassays – tests that measure the potency of a substance by observing its effects on molecules, cells, tissues or organisms – toxicologists also predict safe exposure limits that will keep the environment and us healthy.

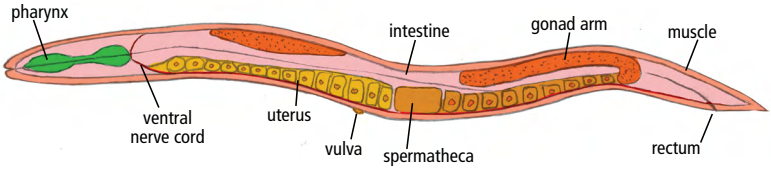


Figure 1: *C. elegans* anatomy

Predictive toxicology focuses on accurately forecasting the interaction between a toxicant and different biological systems and can be generally divided between *in vitro* and *in vivo* studies. *In vitro,* Latin for "in the glass", involves exposing molecules, cells, tissues and certain microorganism to both a standard neutral solution and to a concentration gradient of a toxicant. These targets are then monitored to see whether or when certain adverse effects — called endpoints — occur. For example, common endpoints in cell based *in vitro* studies are membrane damage, nuclear condensation, and apoptosis. *In vitro* tests are easy to carry out in large replicates, less expensive, and allow researchers to focus on specific biological processes.

Alternatively, the potency of a toxicant can be tested by observing how it affects the behavior, growth, reproduction, or lifespan of popular animal models. This is *in vivo* or "within the living" testing. These tests account for the fact that individuals have developed protective mechanisms to reduce the damaging effects of certain toxins, but that these mechanisms mainly manifest at the organism level. For example, when an animal is exposed to a high metal dose, several systems often work together to expel the absorbed molecules, decrease environmental uptake, and activate general stress responses. In addition, *in vivo* studies can highlight emergent health threats that may have been missed during studies that only focus on a certain cell type or a single organ. However, *in vivo* studies are also slow, costly, and raise both legal and moral issues related to animal rights.

Bioassays using *Caenorhabditis elegans* (see-no-rab-DITE-iss el-leh-GANS) offer many of the conveniences of *in vitro* testing with the fuller picture provided by *in vivo* testing. Because *C. elegans* is a popular model organism in other research fields like development, neurology, and genetics there is a wealth of scientific data that can guide and enhance basic toxicology tests. Today, these organisms are increasingly being used to test the safety of potential pollutants.

Why Study *C. elegans*

A model organism is any plant, animal or microorganism that allows us to study fundamental questions in biology that may be hard to study directly in complex organisms like humans. In the 1970s, Dr. Sydney Brenner established the nematode *C. elegans* as a model organism because they have a simple genome, a fast generation time, and are easy and inexpensive to maintain. While characterizing the worm, Brenner, along with Drs. John Sulston and Robert Horvitz, discovered that the developmental fate of every cell in the worm is invariable between animals. They also discovered key genes involved in organ development and programmed cell death. For this work, Brenner, Sulston, and Horvitz were awarded the Nobel Prize in Physiology or Medicine in 2002.

C. elegans have become important to the study of embryogenesis, morphogenesis, development, nerve function, behavior and aging, and genetics. The *C. elegans* genome has been completely sequenced and several thousand genetic mutants are

available for study. This allows scientists to correlate changes at the DNA level with changes in phenotype. Notably, by comparing DNA sequences, it was determined that over 35% of worm genes have human homologs. Many of these genes are important for human health and development.

C. elegans is a free-living, non-parasitic nematode that lives in temperate soil, where it feeds on microbes that are found in decaying organic matter. Adult worms measure approximately one millimeter (mm) in length. The outer cuticle of *C. elegans* is transparent, making it easy to visualize growth and development of internal structures like the pharynx, the intestine, the gonads and the muscles (Figure 1). The worm also has an extensive nervous system – in fact, the nervous system comprises almost 1/3 of the worm's 959 somatic cells! This makes *C. elegans* a valuable model system for neuroscientists (Figure 2).

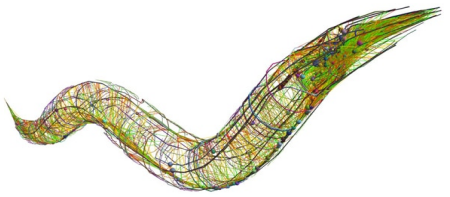


Figure 2: Neural anatomy of *C. elegans*. Source: OpenWorm project. Shared under an MIT License.

Growth and Development of *C. elegans*

There are two naturally occurring sexes in *C. elegans*. The vast majority of worms are self-fertile hermaphrodites, meaning that they produce both the sperm and the eggs used for reproduction. Free-living males represent <1% of the total nematode population. However, free-living males plus a hermaphrodite can produce over 1000 offspring in a generation; in contrast, self-fertilized hermaphrodite worms will produce about 300. Because their sperm will preferentially fertilize a hermaphrodite's eggs and produce more offspring, free-living males are often used to introduce specific genetic mutations into a worm population to be studied.

C. elegans develop from embryo to adult in four days, allowing for rapid studies in the laboratory (Figure 3). The worms are grown on agar plates or in liquid culture and they feed on *E. coli*. After being laid, the worm embryo will develop for approximately 14 hours before hatching. Juvenile worms progress through four larval stages (L1-L4) over the next two days, increasing in size with each stage. After the fourth larval molt (L4), the worms are reproductively mature, meaning that they can be used for further genetic studies. Adults will live for 2-3 weeks, over which time they gradually age and lose vigor.

Bioassays with Multiple *C. elegans* Strains

Another advantage of *C. elegans* is the availability of over 3000 strains whose behavior and genetic make-up has been researched and documented. In biology, a strain is a genetic variant or subtype that falls below the taxonomical level of species. By creating multiple strains, scientists can identify the function of different genes, observe how different genotypes respond to environmental stimuli, or modify a phenotype for a particular task. *C. elegans*' short generation time, male fecundity, and susceptibility to multiple DNA alteration techniques have allowed scientists to create thousands of different mutational strains in this species. In environmental toxicology, different strains are used to better understand how an individual's genetics and its environment interact to determine the biological consequences of toxic exposure. In addition, mutant strains that are more susceptible to toxicants enable toxicologists to run faster and more sensitive bioassays.

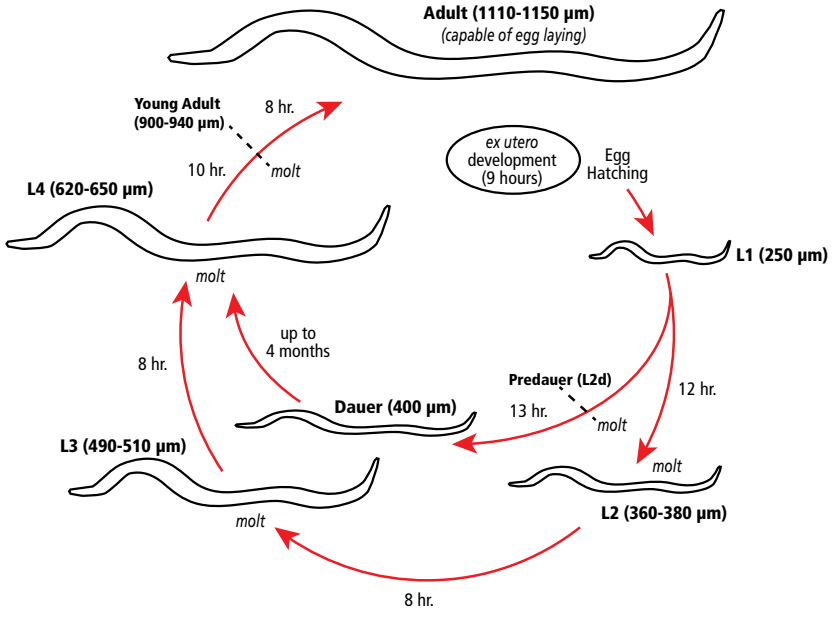


Figure 3: Life Cycle of *C. elegans*

In this experiment you will be using a strain with a loss of function mutation in the *adr-2* gene that makes individuals more vulnerable to environmental pollution. The *adr-2* gene codes for an RNA editing enzyme called ADAR - short for Adenosine Deaminases Acting on RNA. This enzyme converts the nucleoside Adenosine (A) into the nucleoside Inosine (I). Such editing allows multiple proteins to be derived from a single gene region by creating new splice sites, stop points, and different amino-acid codes (Figure 4). Mutant *adr-2* individuals show no ADAR activity and have lower protein diversity. Expressions studies in wild-type (non mutant) individuals have indicated that *adr-2* is particularly active in neurons where added protein diversity is needed to construct the many receptors, ion channels, and adhesion molecules necessary for strong intercellular signaling.

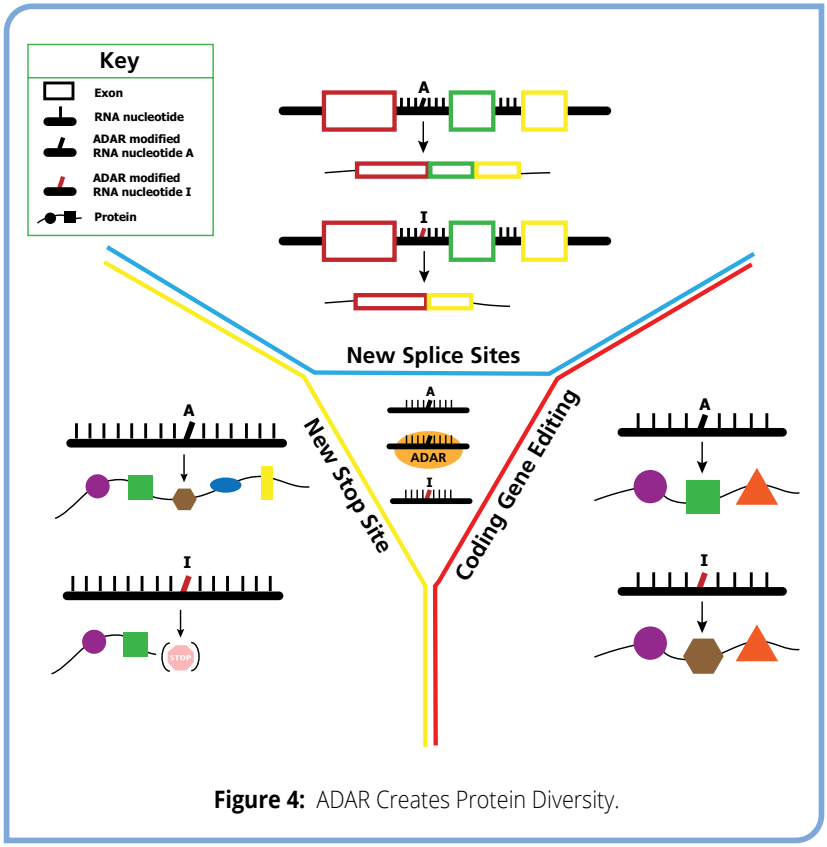


Figure 4: ADAR Creates Protein Diversity.

ADAR proteins are a highly conserved group of enzymes that are also found in most animals. In humans, mutations in the ADAR gene are associated with Aicardi-Goutieres syndrome – an early onset childhood disease characterized by microcephaly, seizures, glaucoma, skin lesions, and general abnormal neurology. More generally, abnormal RNA editing in humans has been linked to depression, schizophrenia, and epilepsy. In *C. elegans* loss of function mutations in the ADAR genes are non-lethal but do result in several defects including difficulty interpreting external signals like smells and trouble maintaining homeostasis in the presence of heavy metals.

Heavy Metal Pollution and Human Health

Heavy metals are a loosely defined group of high-density chemical elements (Figure 5). While naturally occurring, many of these elements have become concentrated in certain areas as a result of mining, industrial waste, agricultural runoff, and use in products like paint and treated wood. Heavy metals also tend to bioaccumulate in living organism because they are often stored in fat tissue rather than metabolized. Some heavy metals such as iron, zinc, copper, cobalt, and manganese play crucial biological roles in processes like oxygen delivery, free radical removal, and cell signaling. However, these trace nutrients can become toxic at higher doses. Other heavy metals such as mercury and lead have no known biological benefits but can cause long-lasting and irreversible biological damage.

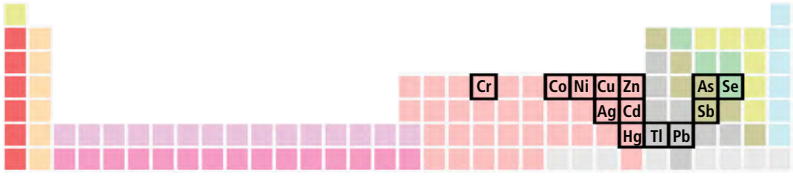


Figure 5: Examples of Toxic Heavy Metals.

Exposures to heavy metals are a common and dangerous environmental health issue. The fast metabolic rates and limited self-repair abilities of nerve cells, as well as the large number of chemical messengers used in interneuron communication, make the nervous system particularly vulnerable to these chemicals. This is especially true for the still-developing nervous

systems of children and infants. Regardless of age, damage to the nervous system can affect everything from learning to motor coordination to the regulation of internal systems like circulation and digestion (Table 1). While some of these effects can be observed immediately after exposure, others take months or even years to manifest. Because it is often difficult to detect early symptoms of exposure and because damages to the nervous system can be permanent, health strategies related to heavy metal neurotoxicity focus on prevention through the early identification and removal.

C.elegans have a similar nervous system to humans and are susceptible to most heavy metals. Mercury, copper, lead, chromium, nickel, cadmium, aluminum, cobalt, zinc, and manganese have all been observed to have a negative effect on the wild-type strain. While toxicology studies have traditionally focused on lethal doses or lethal concentrations (LC), advances in chemical detection technologies have led to a growing interest in the effects of smaller, and often more environmentally relevant concentrations, as well as the synergistic effects of two or more chemicals.

In this experiment, you will be investigating the effects of low-level heavy metal exposure on both wild-type and *adr-2* mutant *C. elegans*. Each strain will be exposed to a potentially damaging chemical for four minutes. (Copper Sulfate, Cadmium Chloride, and Zinc Sulfate are provided but you or your teacher may bring in additional solutions to test.) You will observe and count the thrash rate of a single worm at the beginning, middle, and end of this time period. In addition, you will carry out controls where both strains are exposed to a non-harmful buffer solution for four minutes and again observe and calculate the start, middle, and end thrash rates. Results can be graphed and shared with other lab groups.

TABLE 1: Symptoms of Heavy Metal Neurotoxicity

- Headache
- Loss of Vision
- Imbalance
- Loss of Circulation
- Flu-like symptoms
- Tingling, Weakness or Paralysis of the Limbs
- Loss of memory and cognitive function
- Uncontrollable obsessive and/or compulsive behavior
- Sexual dysfunction
- Depression
- Chronic Fatigue
- Attention Deficit Disorder
- Predisposition to Alzheimer's Disease, Multiple Sclerosis, Parkinson's Disease

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment students will perform a bioassay that tests the impacts of heavy metals on *Caenorhabditis elegans*. Students will also examine RNA editing and genotype – environmental interactions by comparing the reactions of wild-type and mutant strains.

BEFORE YOU START THE EXPERIMENT

1. Read all instructions before starting the experiment.
2. Answer the prelab questions (below).
3. Write a hypothesis that reflects the experiment and predicts experimental outcomes.

Module I:
Preparation of *C. elegans* Food Source

Time Requirements:
10 minutes plus
1 day incubation.

Module II:
Cultivation of *C. elegans*

Time Requirements:
15 minutes plus
3+ day incubation.

Module III:
Collection of *C. elegans* and Exposure to Alcohol

Time Requirements:
55-90 minutes.

PRELAB QUESTIONS

1. What are possible adverse effects or end points that *C. elegans* may display after exposure to an unsafe concentration of a heavy metal?

2. Apart from genotype what other factors could affect an individual's reaction to different heavy metal solutions? How could you control for these during a bioassay experiment?

LABORATORY NOTEBOOKS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:
- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:
- Record (draw) your observations, or photograph the results.

After the Experiment:
- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Laboratory Safety

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!


Wear gloves and safety goggles

1. Wear gloves and goggles while working in the laboratory.

2. Exercise caution when working in the laboratory – you will be using reagents that can be dangerous if used incorrectly.

3. Neither the *E. coli* nor the *C. elegans* used in this experiment are considered pathogenic. Regardless, it is good practice to follow simple safety guidelines in handling and disposing of materials contaminated with living organisms.

   A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.

   B. All materials, including petri plates, pipettes, transfer pipets, loops and tubes, that come in contact with living organisms should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:

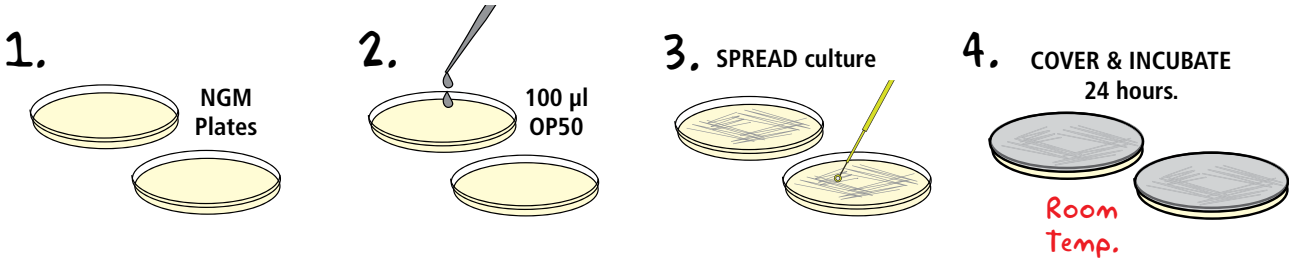
      - Autoclave at 121° C for 20 minutes.
        Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.

      - Soak in 10% bleach solution.
        Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves, goggles, and a lab coat when working with bleach.

   C. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.

Module I: Preparation of *C. elegans* Food Source ("Seeding" the Plates)

In this module, you will seed two petri plates with a favorite *C. elegans'* food (*E. coli* OP50 strain bacteria).



1. **OBTAIN** two Nematode Growth Medium (NGM) plates, the OP50 culture, a small transfer pipet, and a sterile inoculating loop from your instructor. **LABEL** the bottom of both plates with your group number or names.

2. While maintaining sterile technique, **ADD** two drops (100 µL) of OP50 culture to each plate.

3. Using the loop, **SPREAD** the culture over the entire surface of the NGM plates. **COVER.**

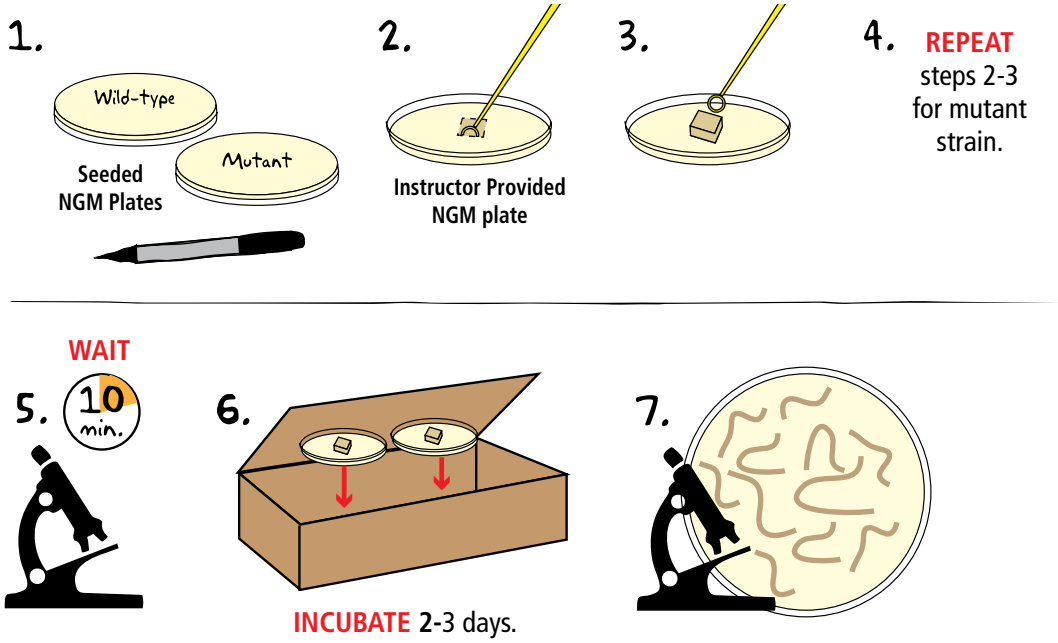
4. **INCUBATE** the plates inverted and at room temperature for 24 hours.
NOTE: Seeded OP50 plates can also be prepared overnight (~12 hours) by incubating at 37 °C.

HINTS for Step 3:

- Avoid gouging or scratching the agar surface as this can affect visibility as well as worm movement.
- Widely spreading the bacteria creates a larger lawn for the worms but stop just before the plate's edge. This discourages the worms from crawling up the plate's sides and drying out.

Module II: Cultivation of *C. elegans* ("Chunking" the Plates)

In this module, you will be "chunking" your *C. elegans* strains. This means that you will transfer wild-type and ADR-2 mutant *C. elegans* to the plates you prepared in Module I. Over the next few days, both strains will establish new populations in their individual plates. Once these populations have expanded to at least 50 individuals you will continue to Module III.



1. **LABEL** the bottom of the seeded NGM plates (from Module I) with "Wild-type" or "Mutant".
2. Your instructor will have NGM plates containing wild-type or mutant *C. elegans*. Using a sterile loop, **CUT OUT** a small portion of the NGM plate containing the wild-type strain (~1 cm square). Make sure to completely cut the agar by pushing the loop all the way to the bottom plate.
3. **REMOVE** the "chunk" from the plate. **PLACE** the chunk, worm side down, in the center of the Module I Wild-type plate.
4. With a new sterile loop, **REPEAT** step 2 and 3 for the mutant strain.
5. After 5-10 minutes, use a microscope to **CONFIRM** the presence of *C. elegans* on the "chunked" plates.
   NOTE: Occasionally, a "chunk" is transferred nematode side up. Worms in this position will eventually migrate to the plate. If the "chunk" is incorrectly positioned, keep the plate but wait 1 hour before confirming the presence of C. elegans and continuing on to Step 6.
6. **COVER** and **PLACE** the plates into a cardboard box. **INCUBATE** at room temperature for 2-3 days.

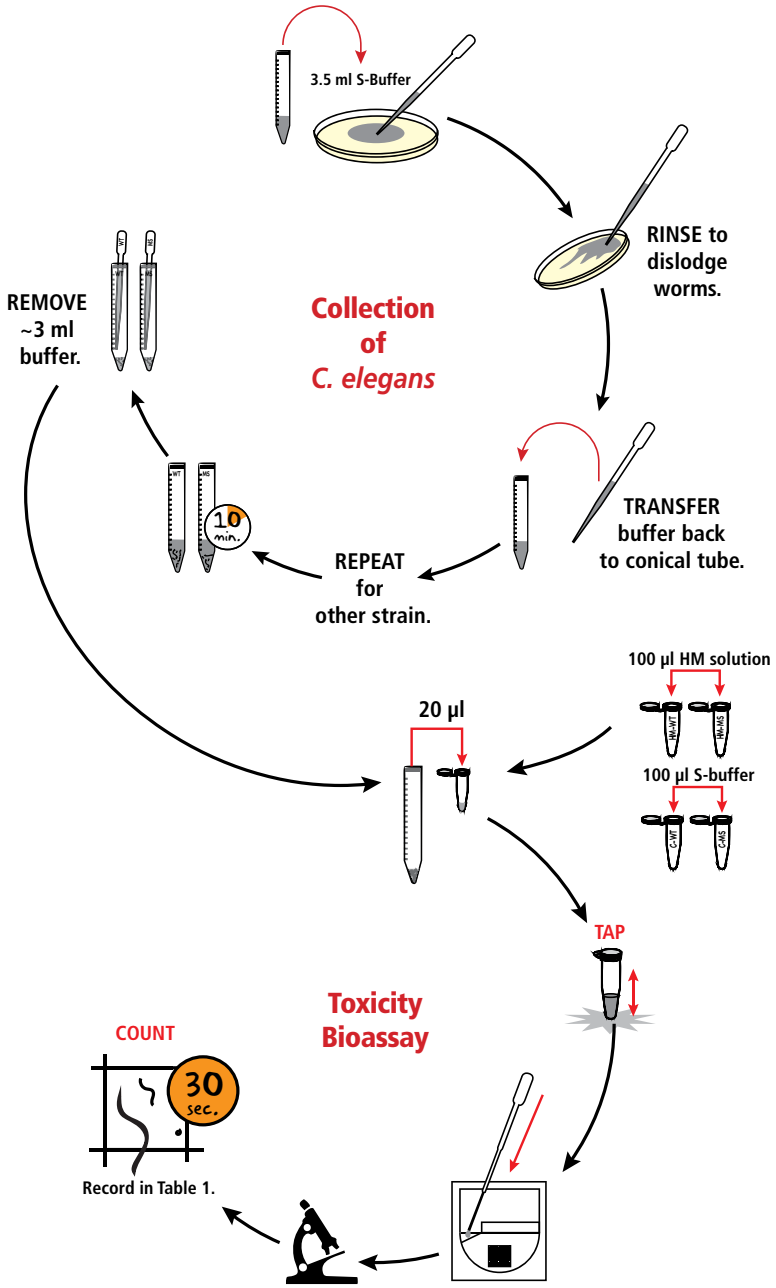
OPTIONAL STOPPING POINT:
Plates may be stored for up to a week but need extra OP50 to avoid drying and to feed the growing population. See Appendix A.

7. **CHECK** growth of *C. elegans* under a microscope. If the plate contains 50 or more worms, proceed with Module III. If the plate contains fewer than 50 worms, continue incubating at room temperature.

HINT for Step 7:
To quickly confirm worm numbers, divide the plate into quarters. If you see 12 or more in the first quarter, the plate is ready.

Module III Overview

In this module, you will first harvest your *C. elegans* populations. You will then test the effects of copper, zinc or cadmium on both wild-type and *adr-2* mutant individuals by monitoring thrash rates over a 4-minute exposure period. The bioassay sequence we recommend is: (1) test the wild-type strain in a heavy metal solution, (2) test the mutant strain in the same heavy metal solution, (3) test the wild-type strain in S-buffer, and (4) test the mutant strain in S-buffer. If time permits, you may test additional heavy metal solutions.



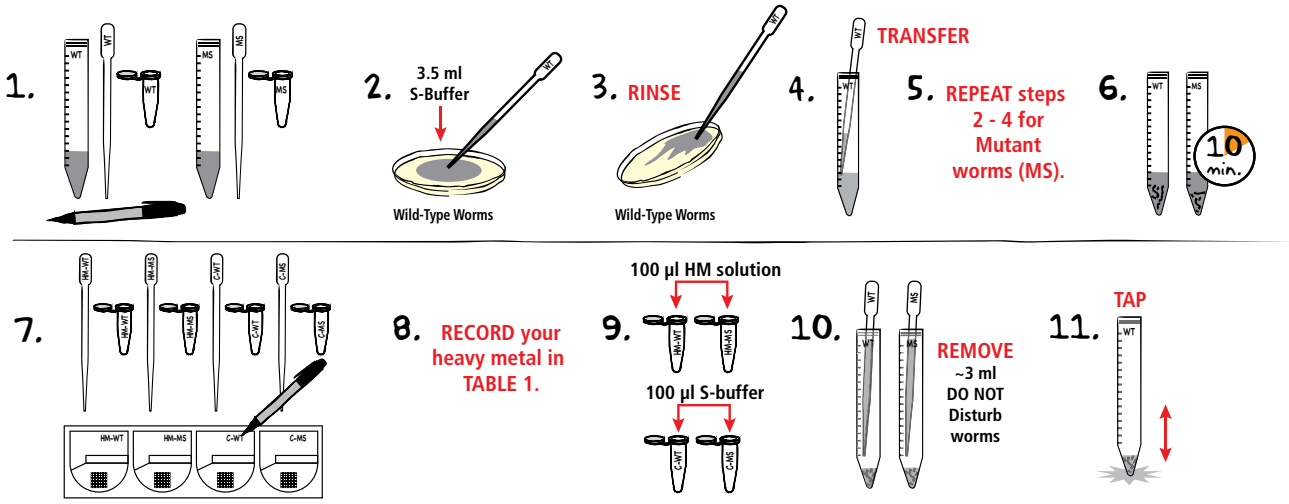
Harvesting
Time Requirements: 25 minutes

Heavy Metal & Control Set Ups & Thrash Counts
Time Requirements: 20 minutes

Additional Heavy Metal Set Ups and Thrash Counts (optional)
Time Requirements: 20 minutes

Graphing (optional)
Time Requirements: 25 minutes

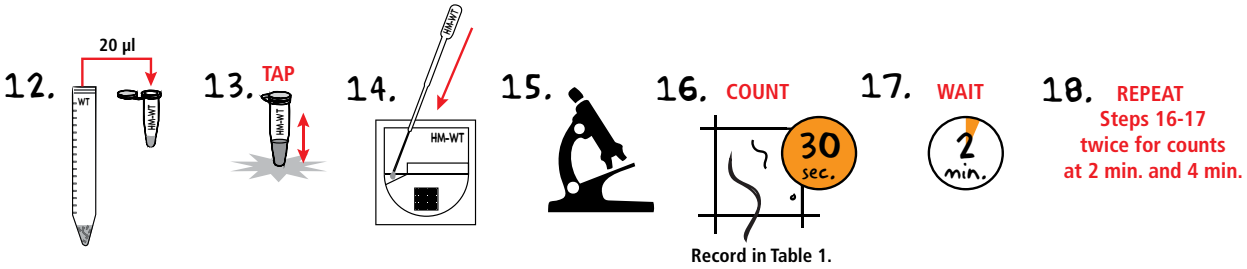
Module III: Collection of *C. elegans* and Toxicity Bioassay



1. **LABEL** one 15 mL tube of S-buffer, large transfer pipet, and snap top tube with "WT" (Wild-Type) and the other 15ml tube of S-buffer, large transfer pipet, and snap top tube with "MS" (Mutant Strain).
2. Using the WT large transfer pipet, **TRANSFER** 3.5 mL of S-buffer from the WT conical tube to the Petri dish containing the wild-type C. elegans.
3. **DISLODGE** worms by rinsing the dish several times. **RINSE** the dish by either (a) swirling the plate or (b) holding the plate at a slight angle and allowing the buffer to collect near the bottom. Next suck up the buffer using the WT transfer pipet and then expel the buffer near the top so that the buffer runs down the plate.
4. Once most worms are suspended in the buffer, **TRANSFER** the worms and buffer back to the WT 15 mL conical tube using the WT transfer pipets.
5. **REPEAT** steps 2-4 for mutant worms using the MS labeled items.
6. Keep the conical tubes still and upright to **ALLOW** the worms to settle to the bottom of the tubes (~10 minutes). While you wait, **REVIEW** Appendix B and complete Steps 7 through 9.
7. **LABEL** four 1.5 mL snap-top test tubes, counting chambers, and small transfer pipets with "HM-WT", "HM-MS", "C-WT", and "C-MS".
8. **RECORD** which heavy metal you plan to test in your lab book or in Table 1 (page 14).
9. **ADD** 100 µL of your heavy metal solution to both "HM" tubes and 100 µL of the S-buffer to both "C" tubes.
10. Using the large transfer pipets, slowly **REMOVE** ~3 mL of the cleared S-buffer supernatant without disturbing the worms that have settled to the bottom of the tubes. Between 300 and 500 µL of buffer with worms should remain at the bottom of both tube.
11. **MIX** the WT tube by tapping the bottom 3-4 times or until worms are uniformly resuspended throughout.

continued

Module III: Collection of *C. elegans* and Toxicity Bioassay, continued



12. Immediately, **ADD** 20 µl of the wild-type worms to the "HM-WT" tube.
13. **MIX** by tapping this tube 3-4 times.
14. Using the HM-WT small pipet, **TRANSFER** 1 drop of the mixture from the "HM-WT" tube to the opening of the corresponding chamber.

NOTE: If correctly placed, the solution will rapidly move into the chamber by capillary action. If the solution does not move into the chamber, check that the chamber is orientated so that the triangular opening is facing upwards.

15. Quickly **PLACE** the chamber under a microscope and **IDENTIFY** an adult worm (900-1200 µm long) for observation.
16. **COUNT** the number of thrashes that occur over a 30-second time period. **RECORD** your count in Table 1.
17. **WAIT** 2 minutes.
18. **REPEAT** steps 16 and 17 twice so that you have additional counts at 2 and 4 minutes.
19. After the final count, **RECORD** any additional notes about the final appearance of the worms including movement, body shape, body curvature, and any clumping. Also record any general observations about the effect of each heavy metal on the worms and how these changed over time.
20. To collect data for the effects of your chosen heavy metal on the mutant strain, **REPEAT** steps 11 through 19 using the mutant strain and your HM-MS labeled items. **RECORD** your results in Table 1.

continued

TABLE 1: Heavy Metal_____

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

Module III: Collection of *C. elegans* and Toxicity Bioassay, continued

21. To collect data for the effects of the control solution on the wild-type strain, **REPEAT** steps 11 through 19 using the wild-type strain and your C-WT labeled items. **RECORD** your results in Table 2.
22. To collect data for the effects of the control solution on the mutant strain REPEAT steps 11 through 19 using the mutant strain and your C-MS labeled items. **RECORD** your results in Table 2.

TABLE 2: Control Solution

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

23. (Optional) If class time permits, test the other two provided heavy metals. Four test tubes, counting chambers, and small transfer pipets are provided. **LABEL** theses items, **RECORD** the selected heavy metals, and **REPEAT** steps 11 through 19 with these two new solutions on both strains. **RECORD** the results in Tables 3 and 4. Note that the control does not need to be repeated.

TABLE 3: Heavy Metal_____

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

TABLE 4: Heavy Metal_____

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

24. (Optional) Graph the change in thrash rate over time for each condition you tested (solution and strain combination) with time on the x-axis and thrash rate on the y-axis.
25. (Optional) Share your group's findings with another lab group or with the class.

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. How are *in vivo* and *in vitro* bioassay similar? How are they different?
2. What is a model organism?
3. What are some advantages to using *C. elegans* as a model system?
4. What is RNA editing and how does it help in optimizing nervous system function?
5. In 1976, the EPA introduced the Toxic Substances Control Act as preventative medicine legislation. This legislation put the EPA in charge of demonstrating that chemical is a risk to human or environmental health. In 1998, the EU proposed REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals), which requires manufacturers to test and ensure that chemicals do not pose a risk. Which of these mirror image approaches do you think is more effective? Why?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation For:	What to do:	When:	Time Required:
IMPORTANT: Arrange for live item delivery of C. elegans approximately 2 weeks before Module I.			
MODULE I	Prepare NGM Agar Plates	Up to a week before the class period of Module I	45 min.
	Prepare OP50 culture	Up to a week before the class period of Module I	15 min.
	Distribute plastics and reagents	The day of the class period of Module I	10 min.
MODULE II	Prepare microscopes with 20-40X magnification	Before Module II	Varies
	Distribute plastics and reagents	Up to 2 days before the class period of Module II	10 min.
MODULE III	Prepare microscopes with 20-40X magnification	Before Module III	Varies
	Aliquot and distribute reagents	Up to 2 days before the class period of Module III	15 min.

Suggested Implementation Schedule

Two Weeks Before Module I: Call Edvotek to request that live items be delivered. (This is also a great time to run through your experiment's time line with one of our scientists!)

Before Module I: Inspect and monitor *C. elegans* plates. Prepare NGM plates and OP50 culture.

Day 1: Students "seed" plates (Module I).

Day 2: Students "chunk" *C. elegans* (Module II).

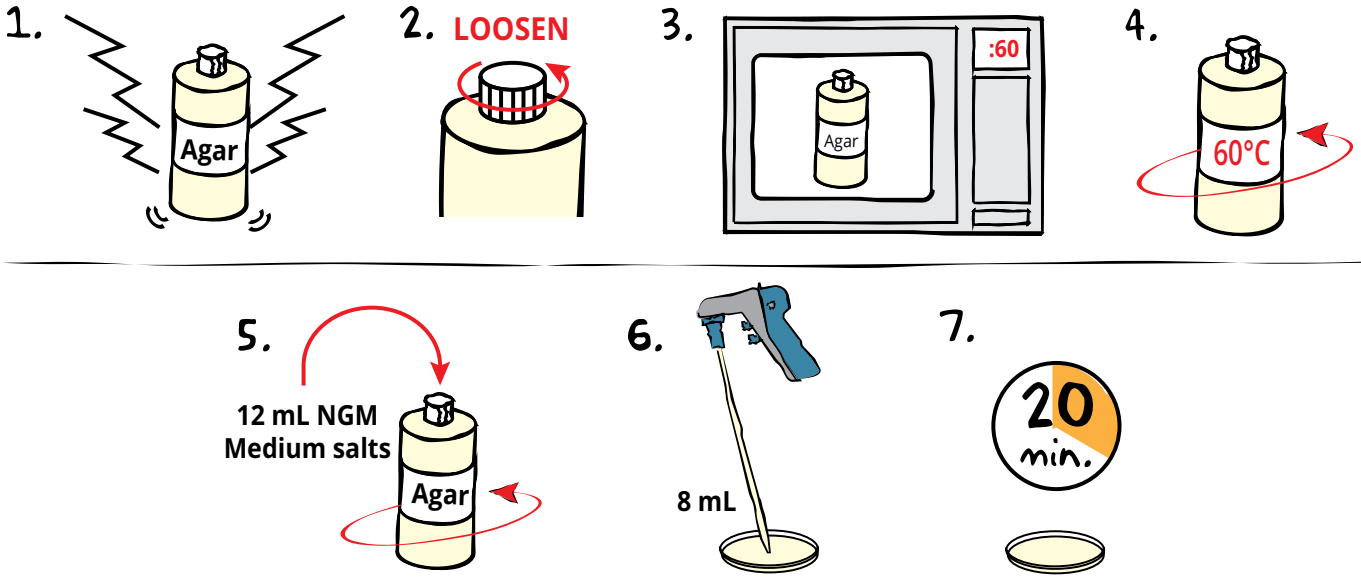
Days 3/4/5*: Students check plates and count *C. elegans* (Module II).

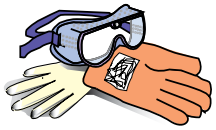
Day 4/5*: Students expose both strains to a heavy metal solution(s) and a control solution and observe thrash rates (Module III).

**This experiment works best when each plate has a population of ~50 C. elegans. This usually takes 2-3 days following the chunking (Module II). If this does not work with your class schedule you can store chunked plates for up to a week but will need to add extra OP50 to feed the growing populations and to delay plate drying. Alternatively, Module I and II can be incorporated into the prelab. In this case, students will only need a single class period for this experiment.*

Pre-Lab Preparations

POURING NGM PLATES





Wear Hot Gloves and Goggles during all heating steps.

1. **BREAK** solid ReadyPour™ medium into small chunks by vigorously squeezing and shaking the plastic bottle.
2. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ medium bottle. This allows the steam to vent during heating. *CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.*
3. **MICROWAVE** the ReadyPour™ medium on high for 60 seconds to melt the agar. Carefully **REMOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the solution should be free of small particles).
4. **COOL** the ReadyPour™ to 60 °C with careful swirling to promote even dissipation of heat.
5. **ADD** 12 mL of NGM Medium Salts (Component E). **RECAP** the bottle and **SWIRL** to mix the reagents. ONLY ADD REAGENTS TO COOLED MEDIUM.
6. Using a fresh 10 mL pipet, **POUR** 8 mL of the medium into 24 small petri plates. **COVER**. If you have additional petri plates around the lab consider pouring extra plate. These can be used to practice thrash counts before the experiment (see Appendix B) or for additional projects (see Openings for inquiries). One bottle of ReadyPour™ NGM Agar can make ~28 plates.
7. **WAIT** at least twenty minutes for the agar to solidify. For optimal results, leave plates at room temperature overnight.
8. If you are using plates within 2 days, **STORE** them at room temperature. Otherwise, **STORE** plates in the refrigerator (4 °C) inverted and in a sealable plastic bag in order to prevent drying out.

Pre-Lab Preparations

TAKING CARE OF *C. elegans* LIVE CULTURES

1. **CALL** and **ORDER** components A and B (Wild-type and Mutant *C. elegans*). Strains are sent on NGM petri plates with the worms still feeding on *E. coli* OP50. Plates are sealed with parafilm and with strain information attached to each plate. **KEEP** plates sealed for as long as possible as this minimizes the dual risks of contamination and drying. It takes 2-5 days for a *C. elegans* request to be processed, filled, and delivered.
2. Upon arrival **STORE** plates at room temperature. *C. elegans* can be maintained at wide range of temperatures (16 °C to 25 °C). However, temperature effects growth rate and food consumption - populations at 25 °C grow twice as fast than those at 16 °C! In this experiment, time estimates and feeding instructions assume a ~20 °C environment.
3. Before Module II, **INSPECT** both plates under a microscope to confirm that there are at least 50 *C. elegans* on each plate. **KEEP** plates covered as much as possible to reduce contamination risks.

PREPARE AND ALIQUOT REAGENTS FOR MODULE I

NOTE: The OP50 culture can be prepared ahead of time and stored in the refrigerator for several days.

1. **PREPARE** 0P50 culture by combining 3 mL of Sterile Water (Component F) with three OP50 BactoBeads™ (Component C) in a 15 mL conical tube.
2. **MIX** and **INCUBATE** at room temperature for 10 minutes.
3. **LABEL** ten snap-top tubes "OP50 culture" and aliquot 250 µL of the classroom OP50 culture to each group.
4. If NGM plates were refrigerated, **REMOVE** from the refrigerator and let sit for at least 30 minutes at room temperature.
5. **DISTRIBUTE** an OP50 tube, two NGM plates, a sterile loops, and a small transfer pipet or fixed volume pipette to each group.

PREPARE AND ALIQUOT REAGENTS FOR MODULE II

1. **PROCURE** and set up microscopes. We suggest top illumination or bright field microscopes with 20-40X magnification. (These microscopes will also be required for Module III.)
2. **INSPECT** wild-type and mutant strain plates.
3. **PREPARE** incubation oven or box(es) for student's plates.
4. **DISTRIBUTE** two sterile loops to each group.

ADJUSTABLE PIPET ALTERNATIVES

Students in this experiment will need to aliquot solutions in the following volumes: 10 µL, 30 µL, 40 µL, 100 µL, 3 mL, and 3.5 mL. Adjustable pipettes can provide easy transfer and are by far the most accurate option. However, absolute precision is not required for this bioassay. An alternative is for students to use small and large transfer pipets (Figure 6). Students can use the highlighted volumes to estimate an approximate height for their experimental volumes. Have students mark these heights with a permanent pen. Similarity between sample measurements is still very important.

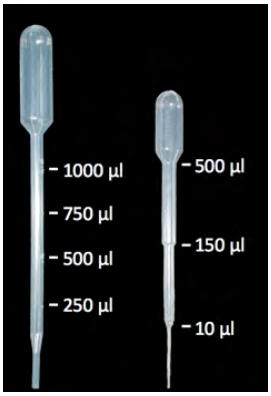


Figure 6: Transfer pipets.

Pre-Lab Preparations

PREPARE AND ALIQUOT REAGENTS FOR MODULE III

1. **DISPENSE** 3.5 mL of S-buffer (Component D) to two 15 mL conical tubes. These tubes will also be used to hold collected worms so remind students not to dispose of them once the S-buffer is used.

2. **LABEL** 10 tubes "Copper" (Component G) and dispense 220 µL Copper Sulfate Solution in each. Repeat for Cadmium Chloride Solution (Component H) and Zinc Sulfate Solution (Component I).

Caution: Wear gloves when handling all three heavy metal stocks. Cadmium in particular is a known carcinogen and chronic toxin. The solution provided is diluted to well below permissible exposure levels.

3. **DISPENSE** 220 µL of S-Buffer into 10 snap top tubes labeled "Control".

4. **DISTRIBUTE** two S-buffer tubes, three heavy metal solution tubes, one control solution tube, 2 large transfer pipets, 8 small pipets, 8 snap top tubes and a counting chamber to each group.

Openings For Inquiry

This experiment is set up for each student group to have similar experimental conditions. An alternate approach is for each student group to design and perform their own investigation. The material and information provided here can be used as a starting block for such open-ended experiment. Examples of possible experiments are:

- Make it local! Have student research regional pollutants and then test these. Alternatively, bring in samples from nearby water sources to test their environmental health.
- Metals in the environment almost always exists in complex mixtures. Test multiple toxins simultaneously to see if these mixtures have a synergetic effect.
- In humans, children are particularly sensitive to neurotoxin exposure. Have groups focus on different *C. elegans* life stages to see if there is a similar relationship between age and susceptibility.
- Have groups propose and test different endpoints. For example, students could test changes in chemotaxis ability using a nose touch experiment or a decrease in reproduction rate by monitoring egg production in separate populations.
- Combine a *C. elegans* bioassay with a water treatment experiment! Collect samples from spiked water before, during, and after treatment (soil filtration, plant/microbial bioremediation, charcoal absorption, chamber etc.) then measure the efficiency of the treatment using this kit. For example, use the provided copper to pollute 500 ml of water and then test the ability of hydroponically grown mustard seeds to remove copper from the water over a two week period by monitoring the mutant strain's response.

A full inquiry lab may require additional time for students to formulate their question, redesign the experiment, and interpret the results. Additional material may also be needed. If you have any questions, please contact us!

Answers to Prelab Questions *(from page 8)*

1. **What are possible adverse effects or endpoints that *C. elegans* may display after exposure to an unsafe concentration of heavy metal?**

   One possible adverse effect is death. Sublethal endpoints include shorter lifespan, slow growth, delayed development, fewer eggs, slow movement, delay response times, limited chemotaxis, reduced enzyme activity, cell apoptosis, or changed genetic expression.

2. **Apart from genotype what other factors could affect an individual's reaction to different heavy metal solutions?**

   This should get students thinking about variables that need to be controlled - such as the size and age of worms, the concentration of the metal, the exposure times, if the worms were stressed before the experiment, as well as the chemical makeup of the NGM media/OP50 culture/S-buffer.

Expected Results and Analysis

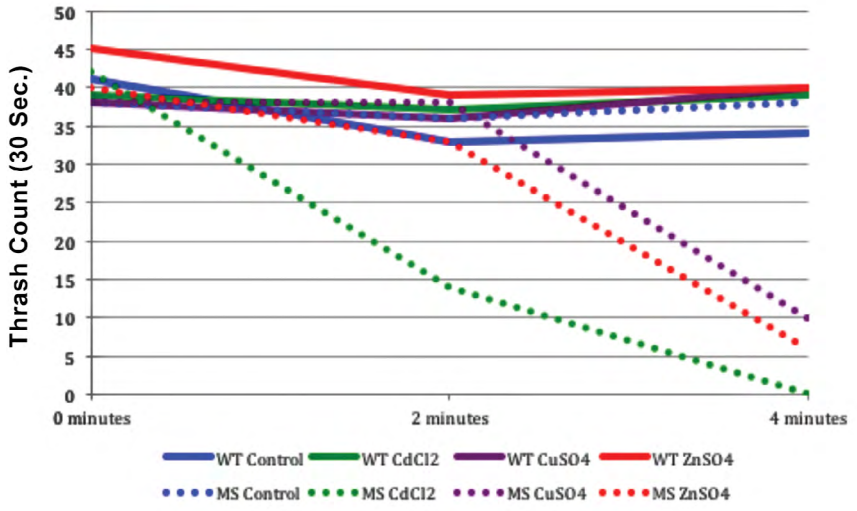
Copper, zinc, and cadmium should have a minimal effect on wild-type worms. In contrast, thrash rates in the mutant strain should decrease in the presence of these heavy metals. Zinc and copper can cause significantly slowing in the mutant strain worms while cadmium can cause complete mobility loss within the time frame of this experiments. Below are representative results from in house testing. Exact values will vary.

Heavy Metal - COPPER		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	39	38
2 min.	40	38
4 min.	38	10

Control Solution		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	45	38
2 min.	43	36
4 min.	41	38

Heavy Metal - ZINC		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	44	40
2 min.	39	33
4 min.	37	6

Heavy Metal - CADMIUM		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	39	42
2 min.	37	14
4 min.	39	0



Please refer to the kit insert for the Answers to Study Questions

Appendices

A EDVOTEK® Troubleshooting Guide

B Counting Thrashes

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Appendix A
EDVOTEK® Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
OP50 growth is not visible on a plate after incubation.	An OP50 lawn on agar can be hard to see.	Examine under the plate under the microscope OR use a sterile loop to gently scrape the top of the agar for growth OR smell plate for bacterial odor. If no growth is still observed re-seed the plate with OP50 liquid culture.
While transferring the nematodes to a new plate the agar was flipped in the wrong orientation.	It is difficult to transfer the small piece of agar.	Nematodes will leave the chunk and move towards their food regardless of orientation, but allow extra time for migration.
No nematodes are visible on chunked plates when observed through a microscope.	Nematodes haven't migrated from the chunk onto plate.	Wait for an additional 5-10 minutes and observe under the microscope again.
	Chunk did not contain enough live nematodes.	Make new chunk of agar from the source plate and repeat the transfer process.
Nematodes on chunked plates do not move when observed through a microscope.	Nematodes have become dormant.	Add 200 µl of additional 0P50 solution to plate.
	Nematodes are dead.	Examine initial plates or a classmate's plates for living nematodes and re-chunk.
Nematode population is growing slowly.	Inhospitable conditions are slowing nematode development.	This is most likely a food supply issue. Add 200 µL of additional OP50 culture. Also check that classroom temperatures are between 20 °C and 25 °C.
The plates containing the nematodes show a white/pink growth, white threads, or brown/green/ yellow spots.	The plates are contaminated with bacteria, yeast, or mold.	Most contamination will not harm the nematodes but can make observation more difficult. If necessary, prepare a new NGM plate and transfer a chunk from a clean region of the contaminated plate.

Appendix A
EDVOTEK® Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
NGM agar is too thin and does not cover the full plate.	Incorrect volume was poured into the plates.	It is important to have the correct thickness of agar in the plates. Be sure to pour the amount recommended by the protocol.
	The plates are dry.	Keep plates sealed and inside of a box or bag. If they are in a high temperature environment cover the edges with parafilm or saran wrap. Dry plates can be partially rehydrated with 200 µL of OP50 solution.
More OP50 liquid culture is needed.	Experiment involves extra steps that require OP50.	Large (>10 mL) OP50 solution can be created from a single BactoBead™ or from a small amount of old solution. Incubate the new solution at room temperature overnight, ideally on a shaking incubator. Alternatively, order new OP50 Bactobeads.
Cannot see nematodes in the counting chamber when placed under the microscope.	Too few nematodes in the experimental solution.	Check the 15 mL collection tube. If nematodes are visible thoroughly re-mix this solution and then re-make the experimental solutions. If nematodes are not visible they may not have settled properly or may have been accidently aspirated with the buffer. In this case use a classmate's collection.
	Nematodes are out of focus.	Use a lower magnification to locate a specimen. Next adjust the contrast, intensity of illumination, and focus. Once you can clearly see and track the specimen move to a higher focus.
Unable to count thrashes.	Unfamiliarity with *C. elegans* observation.	Practice following *C. elegans* and counting thrashes in one of the empty counting chambers. If counting continues to be challenging focus on one experimental conditions and share results as a class.
	Nematodes are dead.	Repeat test conditions with new nematodes and observe their behavior under the microscope immediately.
Need to delay the experiment after chunking student plates.	Life happens.	Nematode populations can be maintained for a week or more. Feed these populations with 200 µL of OP50 liquid culture every 3-4 days. If a longer delay is expected prepare additional NGM plates and chunk the nematodes onto a new plate every two weeks. Alternatively incubate plates at 16 °C for up to a month with weekly feeding.

Appendix B
Counting Thrashes

In a liquid environment, *C. elegans* display a rhythmic flexing motion centered on their midpoint that helps them move and navigate. A single thrash is defined as a complete movement though the midpoint and back (Figure 7).

For each count period, focus on a single worm. This may require moving the chamber on the microscope platform. The long times between counts make it hard to follow a single individual throughout the entire experiment. To minimize variability, between counts select similarly aged and sized worms (Figure 8). We suggest focusing on larger worms (right column) because the nervous system of *C. elegans* is not fully developed until after the L4 stage.

If time permits, practice counting thrashes under the microscope before beginning Module III. There are extra counting chambers and extra solution for this purpose.

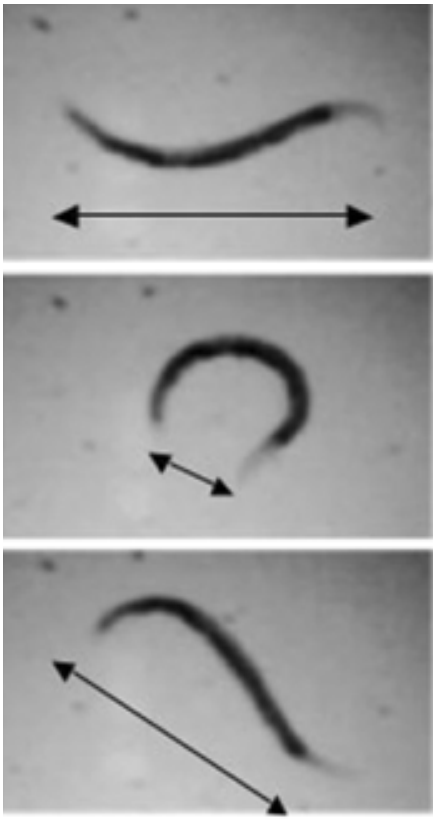


Figure 7

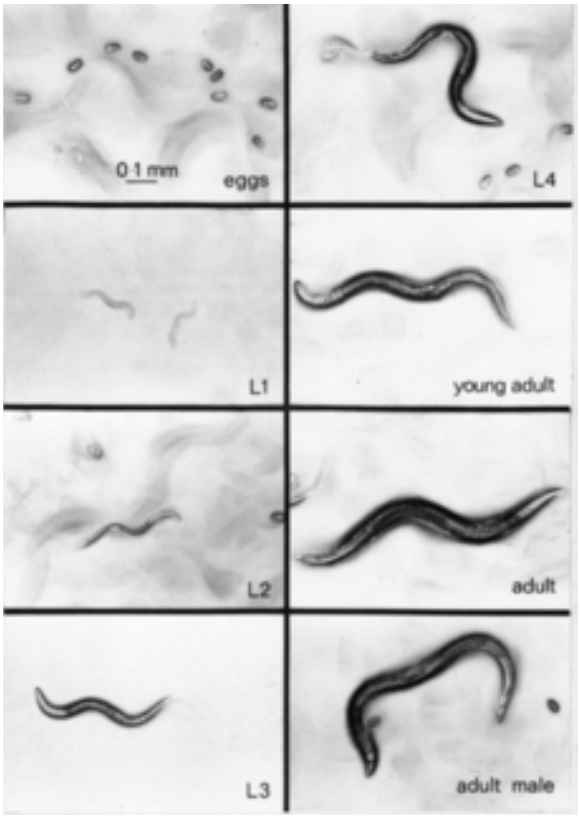


Figure 8

Image Sources
Hart, Anne C., ed. Behavior (July 3, 2006), WormBook, ed. The C. Elegans Research Community, WormBook, doi/10.1895/wormbook.1.87.1, http://www.wormbook.org.

Wood, W.B. in The Nematode *Caenorhabditis elegans* (ed. Wood, W.B.) 1-16 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY,1988).