EDVOTEK®

THE **BIOTECHNOLOGY**
EDUCATION COMPANY®

Edvo-Kit #
851

Edvo-Kit #851

The Effect of Alcohol on *Caenorhabditis elegans*

Experiment Objective:

This experiment explores how model organisms are used to study complex diseases, like addiction, where susceptibility is both genetic and environmental. Students will compare the acute alcohol tolerance of wild-type and NPR-1 deficient *Caenorhabditis elegans* to observe how genetic changes translate into behavioral differences.

See page 3 for storage instructions.

E-Z elegans™

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*	4° C Refrigerator	❑
B	NPR-1 Mutant *C. elegans*	4° C Refrigerator	❑
C	*E. coli* OP50 BactoBeads™	4° C Refrigerator	❑
D	S-Buffer	4° C Refrigerator	❑
E	Rehydration Buffer	4° C Refrigerator	❑
F	Sterile Water	4° C Refrigerator	❑
G	Alcohol Solution	4° C Refrigerator	❑
•	ReadyPour™ NGM Agar	4° C Refrigerator	❑
•	NGM Salts	4° C Refrigerator	❑

Experiment #851 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.

Component	Check ✓
• 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
- 100-1000 µl Adjustable volume micropipets (optional)
- Marking pens
- Microwave or hot plate
- Distilled or deionized water
- Disposable laboratory gloves
- Bleach solution or laboratory disinfectant

Background Information

Alcohol is a popular recreational drug. However, all types of drug use become problematic when individuals abuse the drug or become dependent. The societal and individual cost of alcohol and drug misuse, alcoholism, and addiction are considerable and growing. A major challenge in addressing these public health issues is that there is insufficient understanding about their underlying biological causes, the best treatments, and prevention options. Studying simple model organisms, like *Caenorhabditis elegans* (see-no-rab-DITE-iss el-leh-GANS), provides essential information that helps us understand and treat addition.

WHY STUDY *C. elegans*?

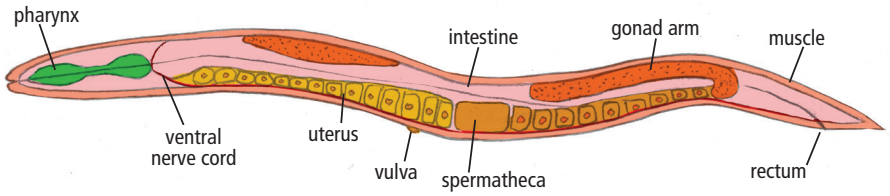


Figure 1: *C. elegans* anatomy

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 1970's, Dr. Sydney Brenner established the nematode *C. elegans* as a model organism. *C. elegans* have a simple genome, a fast generation time, and are easy and inexpensive to maintain. While characterizing this species, Brenner, along with Drs. John Sulston and Robert Horvitz, discovered that the developmental fate of every cell in the nematode 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.

These model organisms have become important to the study of embryogenesis, morphogenesis, development, nerve function, behavior and aging, and how specific genes regulate these processes. 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 their DNA sequences, it was determined that over 35% of *C. elegans* genes have human homologs. Many of these genes are important for human health and development.

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

REPRODUCTION AND DEVELOPMENT IN *C. elegans*

There are two naturally occurring sexes in *C. elegans*. The vast majority of nematodes are self-fertile hermaphrodites, meaning that they produce both the sperm and the eggs used for reproduction. However, free-living males represent <1% of the total population. Free-living males plus a hermaphrodite can produce over 1000 offspring in a generation; in contrast, self-fertilized hermaphrodite individuals 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 population to be studied.

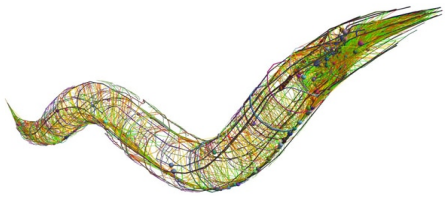


Figure 2: Neural anatomy of *C. elegans*. Source: OpenWorm project. Shared under an MIT License.

C. elegans develop from embryo to adult in four days, allowing for rapid studies in the laboratory (Figure 3). The nematodes are grown on agar plates or in liquid culture and they feed on *E. coli*. After being laid, the embryo will develop for approximately 14 hours before hatching. Juveniles 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 nematodes ar roductively 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.

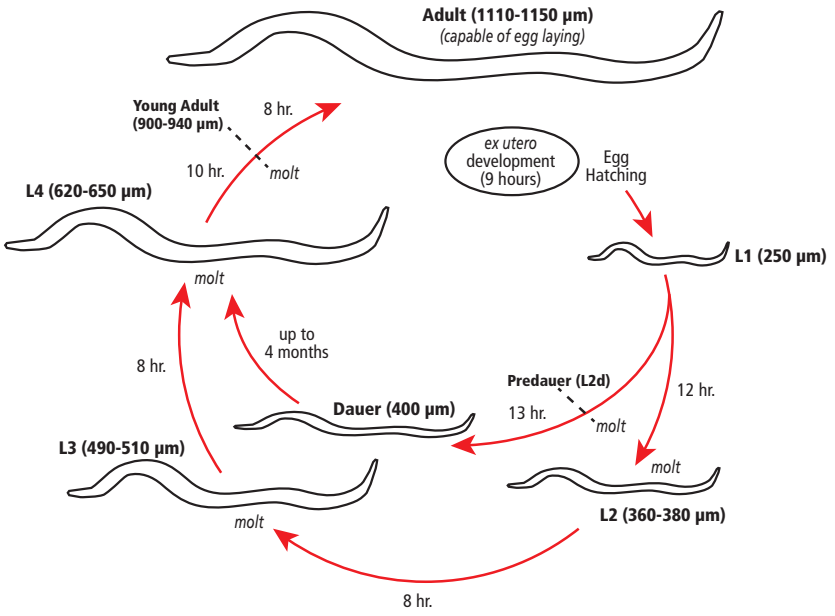


Figure 3: Life Cycle of *C. elegans*

USING *C. elegans* TO UNDERSTAND BEHAVIORS AND DISEASES

Scientists use *C. elegans*' genome and neural system to study how genetics influences certain behaviors and mental health conditions. One approach is to study nematodes where a gene of interest has been modified or silenced in order to discover the biological function of that gene. Such studies also need a control – animals without the mutation. These nematodes are referred to as "wild-types". In this experiment, you will compare the behavior of wild-type nematodes to nematodes with non-functioning *npr*-1 genes.

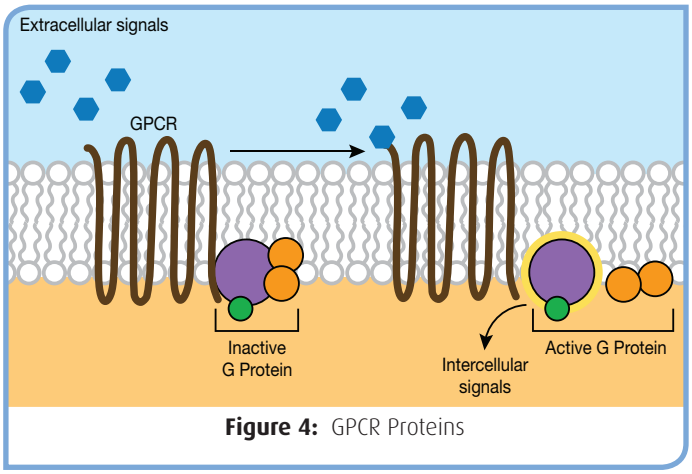


Figure 4: GPCR Proteins

The *npr*-1 gene encodes a G-protein coupled receptor (GPCR) protein. GPCRs are a huge and diverse group of cell membrane receptor proteins that detect molecules outside of a cell (Figure 4). In turn, G proteins act as molecular switches within a cell. When an extracellular ligand binds to the GPCR it causes a conformation change inside the cell that can increase or decrease the production of hundreds of internal messenger molecules. GPCR proteins have played a huge part in the development of new medical treatments – close to one half of currently available drugs are thought to target them!

The NPR-1 protein is a receptor for neuropeptides, protein-like molecules produced by other cells, and is found in several types of *C. elegans* neurons. Nematodes with high *npr*-1 gene expression show solitary feeding habits, heat tolerance, and a low sensitivity to oxygen levels. In contrast, nematodes with low *npr*-1 gene expression show social feeding, thermal avoidance, and aerotaxis (movement toward certain oxygen levels). This has led scientists to hypothesize that the receptor may slow or inhibit certain sensory pathways. Scientists have also observed a link between alcohol tolerance and *npr*-1 activity. While the *npr*-1 gene is not found in humans, it is homologous to the NPY receptor family which has been linked to pain sensation, circadian rhythms, food consumption, anxiety, and drug tolerance.

Tolerance refers to a decrease in response to an environmental condition over time. Scientists studying addiction and alcoholism are interested in drug tolerance - the progressive decrease in the effect of a substance on an individual over time. This can be due to metabolic, physiological, or behavioral changes that occur at the cellular, tissue, organ, and organ system levels. Tolerance can occur as a result of repeated (chronic) use but also during a single exposure (acute). Acute tolerance is why individuals often report feeling less impaired when their blood drug levels are decreasing than when their blood drug concentrations were identical but increasing earlier during a single using event (Figure 5.)

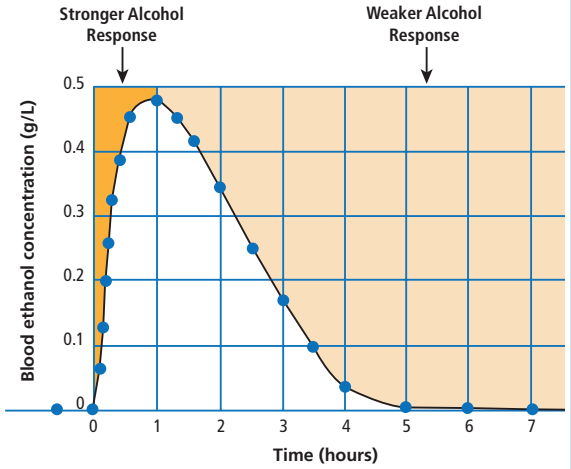


Figure 5: Time Changes in Blood Alcohol Concentration.

Tolerance can influence drug use behavior. For example, chronic users often experience a reduced reaction to a drug and require larger doses in order to experience the desired effect. This can lead to serious medical conditions or overdose. Likewise, individuals who show an initial acute tolerance to a substance may be more likely to abuse it. One possible reason for this is that many of a drug's pleasurable effects are experienced in the beginning of consumption while adverse effects like impaired judgment, dizziness, nausea and headaches occur later.

It is likely that both chronic and acute tolerance have a genetic component. For example, rats bred to prefer alcohol also build alcohol tolerance more quickly than wild-type rats. Similarly, when scientists compared the (grown) children of alcoholic and nonalcoholic parents, the children of alcoholic parents were affected more strongly by alcohol early in a drinking session but then experienced fewer cognitive impairments later on.

Alcohol exposure effects *C. elegans* behavior in numerous ways (Table 1). Decreased mobility - as measured by the number of thrashes per minute - is one of the most common and easily tracked changes. A thrash occurs when the anterior end of the nematode bends towards the posterior end to form a "U" shape. When first introduced to a high ethanol environment *C. elegans* will slow or cease thrashing. However, over time the thrash rate will increase to normal again – even when blood and tissue concentrations of ethanol are kept constant. In such cases this increase is likely due to physiological and behavioral changes that are a part of acute ethanol tolerance.

Cell signaling involving NPR-1 likely depresses the development of acute tolerance. *C. elegans* with null or comprised npr-1 genes often quickly develop tolerance to alcohol. In contrast, *C. elegans* with normal or higher *npr*-1 expression exhibit a much slower recovery. In this experiment, you will observe how genetic mutations translate into behavioral differences by quantifying acute tolerance in wild-type individuals and individuals with an *npr*-1 loss of function mutation.

Alcohol Effects in *C.elegans*	Alcohol Effects in Humans
Decline in activity/immobility	Reduced inhibitions
Reduced feeding and egg laying	Slurred speech
Frequent spontaneous reversals	Motor impairment
Fewer C-shaped postures during swimming	Confusion
Impaired navigation ability (withdrawal)	Memory problems
Increased social feeing (withdrawal)	Concentration problems
	Coma
	Breathing problems
	Death

TABLE 1: Effects of Alcohol

Experiment Overview

EXPERIMENT OBJECTIVE:

This experiment explores how model organisms are used to study complex diseases, like addiction, where susceptibility is both genetic and environmental. Students will compare the acute alcohol tolerance of wild-type and NPR-1 deficient *Caenorhabditis elegans* to observe how genetic changes translate into behavioral differences.

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:
90 minutes.

PRELAB QUESTIONS

1. What are some effects of alcohol that you expect to be able to observe in both strains?
2. Predict how wild-type individuals might react to alcohol initially. What about mutant individuals?
3. Predict how wild-type individuals might react to alcohol after prolonged exposure. What about mutant individuals?

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, pipets, 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:

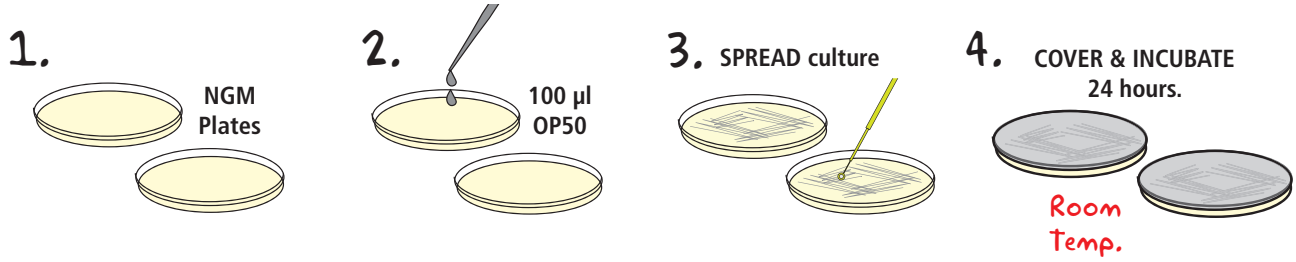
      - Autoclave at 121° C for 20 minutes.
        Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into 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 incubate two petri plates with *E. coli* strain OP50 bacteria. The plates will be incubated for 24 hours to prepare a bacterial lawn for *C. elegans* cultivation in Module II.

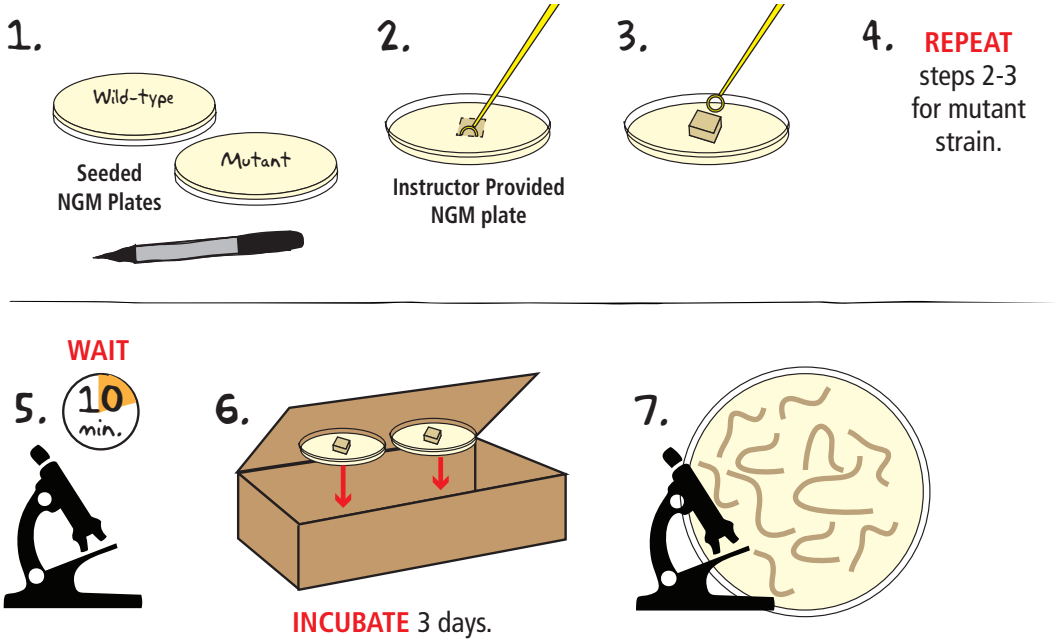


1. **OBTAIN** two Nematode Growth Medium (NGM) plates, the OP50 culture, a small transfer pipet, and a sterile inoculating loop from your instructor. **LABEL** the bottom of both plates with your group number or names.
2. While maintaining sterile technique, **ADD** two drops (100 µl) of OP50 culture 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: Be careful to avoid gouging or scratching the agar surface as this can affect visibility as well as worm movement.

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

In this module, you will transfer wild-type and NPR-1 mutant *C. elegans* strains 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 one 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 3 days.

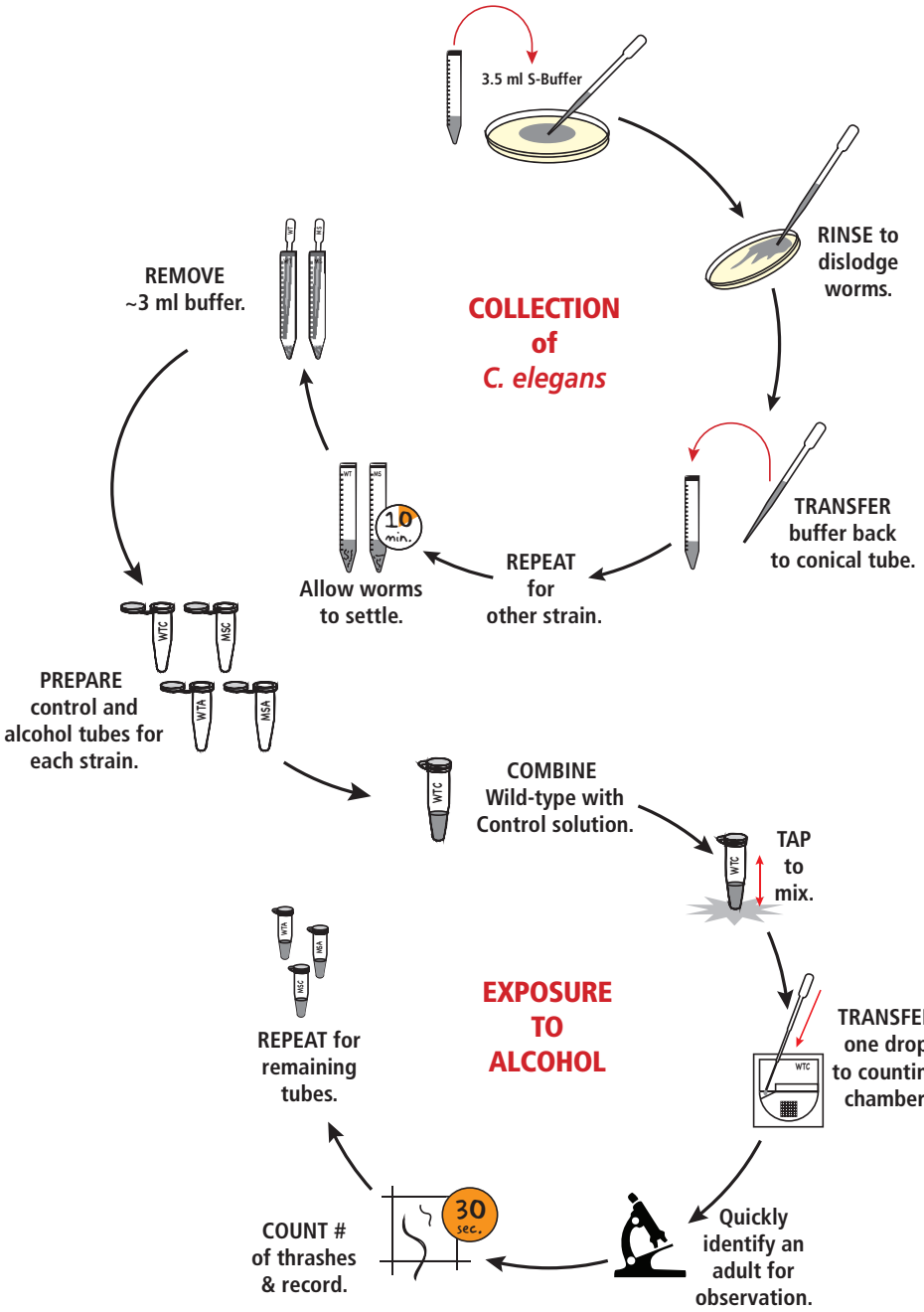
OPTIONAL STOPPING POINT:
Plates may be stored for up to a week but need extra OP50 to avoid drying and to feed the growing population. See Appendix A.

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 expose both strains to two environmental conditions (an alcohol solution and a control water solution) and monitor thrash rates at the beginning, middle and end of a 45 minute exposure period. Different tests can be carried out over multiple lab periods or staggered to fit into one single 90 minute lab.

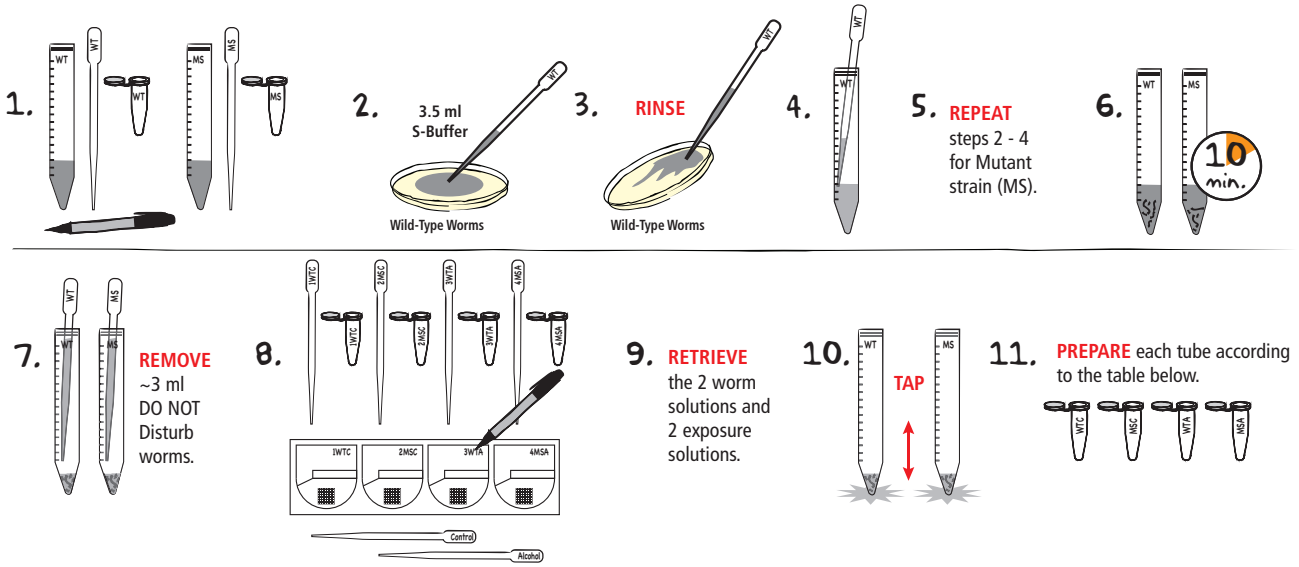


Harvesting
Time Requirements:
25 minutes

Initial Set-Up
Time Requirements:
5 minutes

Thrash Counts plus Additional Set-Up
Time Requirements:
60 minutes.

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



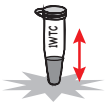
1. **LABEL** one 15 ml tube of S-Buffer, a large transfer pipet, and a snap-top tube with "WT" (Wild-Type) and the other 15 ml tube, large transfer pipet, and snap-top tube with "MS" (Mutant Strain).
2. Using the WT transfer pipet, **TRANSFER** 3.5 ml of S-Buffer from the WT conical tube to the Petri 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 transfer pipet and then expel the buffer near the top so that it 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 pipet.
5. **REPEAT** steps 2-4 for mutant *C. elegans* 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** Appendixes B and C for additional thrash count guidance.
7. Using the appropriately labeled 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 µl and 500 µl of buffer with worms should remain at the bottom of both tubes.
8. **LABEL** 4 tubes and 4 small transfer pipets with "1 WTC", "2 MSC", "3 WTA", "4 MSA". Also label the outer edge of four counting chambers with these numbers or acronyms. Finally, label two small transfer pipets "Control" and "Alcohol".
9. **RETRIEVE** the two *C. elegans* strains from step 7 and the alcohol and control (water) solutions from your teacher.
10. **MIX** the *C. elegans* solutions by tapping the tubes 3-4 times so that the worms are again evenly suspended.
11. **PREPARE** each tube according to Table 2. Begin by **COMBINING** 50 µl of Wild-Type *C. elegans* and 450 µl of control (water) solution in the "1 WTC" tube.

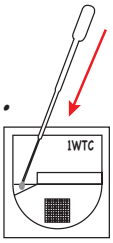
NOTE: We strongly recommend staggering the preparation, mixing, and counting of tubes 2, 3, and 4 by three minutes. This will help your group accurately count the number of thrashes. A timetable with a 3-minute delay between each count is provided in Appendix C.

	1(WTC)	2(MSC)	3(WTA)	4(MSA)
***C. elegans* Suspension**	50 µl (1drop) Wild-Type	50 µl (1drop) Mutant Strain	50 µl (1drop) Wild-Type	50 µl (1drop) Mutant Strain
Exposure Solution	450 µl (9 drops) Control (water)	450 µl (9 drops) Control (water)	450 µl (9 drops) Alcohol	450 µl (9 drops) Alcohol

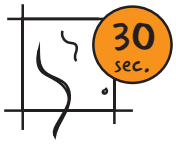
TABLE 2: Preparing control and experimental solutions.

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

12. TAP 

13. 

14. 

15. COUNT 

Record in Table 3.

16. REPEAT steps 10-15 for remaining tubes.

17. CONTINUE with additional thrash counts. Record in Table 3.

12. **MIX** the 1 WTC tube by tapping the bottom 3-4 times or until worms are uniformly resuspended throughout the solution.
13. With the WTC small pipet, **TRANSFER** 1 drop (~10 µl) of the WTC mixture to the opening of the corresponding chamber.

NOTE: If correctly placed the solutions 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 openings are facing upwards.

14. Quickly **PLACE** the chamber under a microscope and **IDENTIFY** an adult *C. elegans* (900-1200 µm long) for observation.
15. **COUNT** the number of thrashes that occur over a 30 seconds time period. **RECORD** your count in Table 3.
16. **REPEAT** steps 10 through 15 for the remaining tubes according to the timetable in Appendix C or your own timetable.
17. **CONTINUE** with second (15 minute), third (30 minute), and fourth (45 minute) thrash counts for each conditions. **RECORD** results.

Table 3

Wild-Type *C. elegans*

	Thrash Count in 30 seconds in Control Solution	Thrash Count in 30 seconds in Alcohol Solution	% Decrease in Mobility
Time 1 (0 min.)			
Time 2 (15 min.)			
Time 3 (30 min.)			
Time 4 (45 min.)			

Mutant Strain *C. elegans*

	Thrash Count in 30 seconds in Control Solution	Thrash Count in 30 seconds in Alcohol Solution	% Decrease in Mobility
Time 1 (0 min.)			
Time 2 (15 min.)			
Time 3 (30 min.)			
Time 4 (45 min.)			

18. **CALCULATE** the difference in Mobility using the equation:

$$\frac{\text{(Thrash Count in Control Solution – Thrash Count in Alcohol Solution)}}{\text{Thrash Count in Control Solution}} \times 100$$

19. (Optional) **GRAPH** the change in mobility over time in wild-type and mutant strains.

Study Questions

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

1. What is a model organism?
2. What are some advantages to using *C. elegans* as a model system?
3. What does GPCR stand for? Where in the cell are these proteins found and what do they do?
4. Vulnerability to alcoholism and addiction is a complex trait - multiple environmental and inherited factors determine the likelihood that someone will become an alcoholic or addict. Research and then describe a gene or environmental factor that may play a role in this disease.

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. Because of long incubation times we recommend setting the experiment up over 2 weeks and using the weekend to cultivate nematodes between Module II and III. We recommend prelab preparations begin at least 7 days before the Module I lab activity.

Preparation For:	What to do:	When:	Time Required:
E-Z elegans™ Preparation	Prepare NGM Agar Plates	7 days before Module I	45 min.
	Prepare OP50 culture	6 days before Module I	15 min. (plus 20+ min. incubation)
	Prepare a microscope with 20-40X magnification	Before E-Z elegans™ rehydration	Varies
	Rehydrate E-Z elegans™ and Seed NGM Plates	6 days before Module I	30 min. (plus 24-48 hour incubation)
	Monitor E-Z elegans™ growth	5 days before Module I	15 min. (plus 3-5 day incubation)
Module I	Prepare classroom 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 a 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 a 2 days before the class period of Module III	15 min.

*Prelab and experimental times are based on room temperature incubation. However, seeded OP50 plates can be prepared using overnight incubation at 37° C. In addition, an incubator can be used to slow down the life cycle of *C. elegans* - see Appendix A for additional details.

Suggested Implementation Schedule

This schedule is an example. Incubation times may be longer or shorter depending on growth conditions and extended stopping points can be introduced at various stages.

Prior to the lab

Store E-Z elegans™ in the fridge as soon as they arrive. Unopened vials can be stored for two weeks.

Day 1
- Prepare NGM Agar plates.

Day 2
- Prepare an OP50 culture, warm the rehydration buffer to room temperature, and seed four NGM plates.
- Incubate seeded plates at room temperature to allow a bacterial lawn to grow.

Day 3 or 4
- Rehydrate E-Z elegans™.

Day 5 - 10
- Incubate and monitor the four plates. Plates with >50 *C. elegans* are ready for Module II.

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

Day 6
- Prepare classroom OP50 culture and warm NGM plates to room temperature. Label and distribute student supplies.
- Students seed NGM plates (10 minutes). Incubate for 24 hours.

Module II: Cultivation of *C. elegans*

Day 7
- Confirm that at least one wild–type and one mutant E-Z elegans™ plate is ready. Set up microscopes and distribute student supplies.
- Students transfer *C. elegans* to their seeded plates (15 minutes).

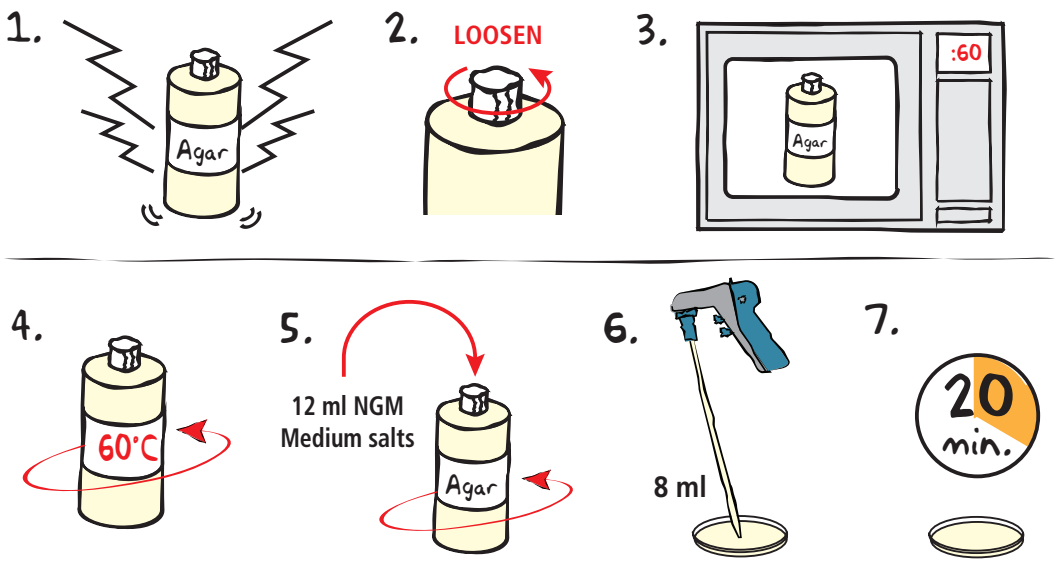
Day 8 – 10
- Incubate and monitor student plates. Plates with >50 *C. elegans* are ready for Module III.

Module III

Day 11
- Aliquot solutions and distribute student supplies.
- Set up microscopes.
- Students expose both strains to alcohol and a control solution and observe thrash rates (90 minutes).*

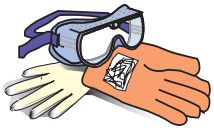
*Students can test four experimental conditions (wild-type in alcohol, wild-type in control solution, NPR-1 mutants in alcohol, and NPR-1 mutants in control solution). Each test involves four 30 seconds thrash counts over 45 minutes. In order to fit the preparation and execution of these four time tests into a typical lab period we suggest staggering the experiments and following the timetable in Appendix C. However, such continuous counting requires organization and familiarity with thrash counts. An alternative is to assign each group a single experimental condition and then share results as a class.

Pre-Lab Preparations



POURING NGM PLATES

NOTE: One bottle of ReadyPour™ NGM Agar should make 25 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. **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**.
7. **WAIT** at least twenty minutes for the agar to solidify. For optimal results, leave plates at room temperature overnight.
8. **KEEP** four plates at room temperature, to rehydrate the E-Z elegans™, and **STORE** the remaining plates in the refrigerator (4° C). To prevent drying, keep plates inverted and in a sealable plastic bag.

Pre-Lab Preparations

PREPARING REHYDRATION BUFFER AND OP50 CULTURE FOR E-Z ELEGANS™

1. **INCUBATE** Rehydration Buffer (Component E) overnight at room temperature. This solution should be between 15-25° C when adding to *C. elegans* vials.
2. **TRANSFER** 1 ml of Sterile Water (Component F) to a 1.5 ml snap-top tube. **LABEL** it as "OP50".
3. **REMOVE** one OP50 BactoBead™ (Component C) from the vial using a sterile loop and **ADD** it to the OP50 tube.
4. **MIX** and **INCUBATE** the tube at room temperature for 20 minutes.

SEEDING NGM PLATES

1. **ADD** 100 µl of the OP50 culture to each of the four NGM plates using a pipet tip or transfer pipet.
2. Using a sterile loop, **SPREAD** the liquid over the entire surface of each plate.
3. **INCUBATE** the plates at room temperature for 24-48 hours.
4. **SAVE** the remaining OP50 culture in the fridge. This additional OP50 can be used if *C. elegans* petri plates need to be stored for additional days.

REHYDRATION OF E-Z ELEGANS™

E-Z elegans™ takes advantage of C. elegans' ability to arrest development and go into an alternative larval state when rapidly dehydrated. These dauer larvae are stable in the refrigerator for up to two weeks. To prepare the E-Z elegans™, you will gently rehydrate and then incubate for one week. Due to the stress of drying and rehydrating, we expect a mixture of live and dead nematodes for several days, but this extended incubation time allows the dauers to return to normal development and lay eggs. The student experiments will be carried out on this second generation of worms.

NOTE: The C. elegans are fragile during rehydration and resuspension. Be very gentle during steps 2 through 6 particularly when pipetting.

1. This kit contains four vials of *C. elegans*, two wild-type and two mutant strain. Before rehydrating the E-Z elegans™, **LABEL** the bottom of the four seeded NGM plates with either "wild-type"(2 plates) or "mutant"(2 plates).
2. Carefully open the vials of E-Z elegans™ and **ADD** 250 µl of Rehydration Buffer (Component E) to each tube.
3. **INCUBATE** the E-Z elegans™ for 1 hour at room temperature.
4. **RESUSPEND** the wild-type nematodes by gently pipetting the solution up and down 3-4 times in both vials using a large sterile transfer pipet.
5. **REPEAT** this process for the mutant strain nematodes using a new large sterile transfer pipet.
6. **TRANSFER** each vial of rehydrated nematodes to an appropriately labeled NGM plates. Each vial should contain ~250 µl of solution which is enough to inoculate a single plate.
7. **OBSERVE** the 4 plates under a microscope. You should see many nematodes spread evenly around the plate – it is not necessary to see any movement at this point!
8. **COVER** and **INCUBATE** the plates at room temperature for 3 days.

NOTE: Not all worms will survive the rehydration process. However, you should observe movement from many living C. elegans within 3-6 days of recovery in at least one wild-type plate and at least one mutant strain plate.

continued

Pre-Lab Preparations

REHYDRATION OF E-Z ELEGANS™, CONTINUED

9. **OBSERVE** the worms under a microscope after 72 hours for mobility. Often the first signs of mobility will be worm trails (Figure 6). Refer to Appendix A "Troubleshooting" if you do not observe a moving worm or a trail.
10. Continue to **INCUBATE** the worms at room temperate for an additional 3 days. This will allow a second (and non dauer) generation of worms to grow.
11. **OBSERVE** the worms under a microscope daily. Plates are ready when they contain approximately 50 live worms and will be used in Module II.

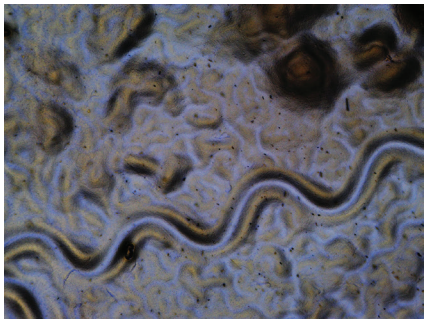


Figure 6: Worm trails.

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** a classroom OP50 culture by combining 3 ml of Sterile Water (Component F) with three OP50 Bacto-Beads™ (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. **REMOVE** NGM plates 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 pipet 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. **CONFIRM** that at least one wild-type and one mutant strain E-Z elegans™ plates has approximately 50 moving worms.
3. **PREPARE** incubation oven or box(es) for student's plates.
4. **DISTRIBUTE** two sterile loops to each group.

PREPARE AND ALIQUOT REAGENTS FOR MODULE III

1. **ALIQUOT** 3.5 ml of S-Buffer (Component D) to twenty 15 ml conical tubes. These tubes will also be used to hold collected *C. elegans* so remind students not to dispose of them once the S-Buffer is used.
2. **ALIQUOT** 1.2 ml of 10% Alcohol (Component G) into 10 snap-top tubes labeled "Alcohol Solution".

CAUTION: Alcohol is a highly flammable liquid. Handle carefully. Commercial alcohol preparations contain other chemicals to make the solution non-potable. Do not take internally.

3. **ALIQUOT** 1.2 ml of Sterile Water (Component F) into 10 snap-top tubes labeled "Control solution".
4. **DISTRIBUTE** two S-Buffer tubes, Alcohol and Control solution tubes, 2 large transfer pipets, 6 small pipets, 4 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 questions are:

- How does the starting concentration of alcohol affect acute tolerance development?
- Do individuals at different life stages show different degrees of acute tolerance?
- Are mutant nematodes more successful at finding food in a low ethanol environment than wild-type nematodes?
- How does exposure to alcohol alter both strains' population growth?
- Do worms show similar responses to other over the counter drugs like dimenhydrinate (motion sickness) and acetaminophen (pain relief)?

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 7)*

1. **What are some effects of alcohol that you expect to be able to observe in both strains?**

   The main effect that will be observed in this experiment is a decrease in mobility as measured by lower thrash counts in intoxicated nematodes. Additional effects that may be observed are less pronounced "c" shapes and fewer directional changes when an intoxicated individual does thrash. In longer experiments it would also be possible to observe fewer eggs, smaller nematodes, and more clumping in plates where the population was exposed to alcohol.

2. **Predict how wild-type individuals might react to alcohol initially. What about mutant individuals?**

   Initially both strains should be equally affected by alcohol and show one or more of the behaviors described in question 1.

3. **Predict how wild-type individuals might react to alcohol after prolonged exposure to alcohol. What about mutant individuals?**

   After prolonged exposure, mobility should begin to increase in mutant strain individuals because they have a higher acute tolerance. An increase in thrash count may also be observed in wild-type individuals but this should occur later in the experiment.

Expected Results

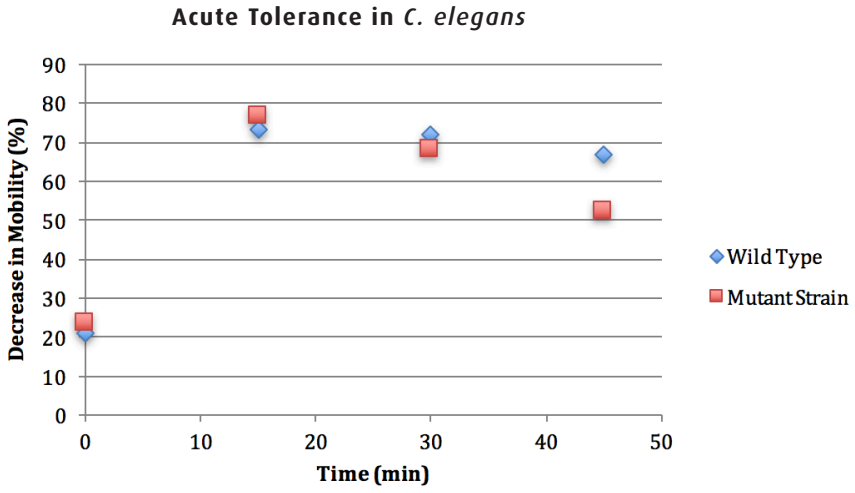
These are representative results from an in house experiment. Your results will vary.

Wild-type *C. elegans*

	Thrash Count in 30 seconds in Control Solution	Thrash Count in 30 seconds in Alcohol Solution	Decrease in Mobility
Time 1	57	45	21.05%
Time 2	45	12	73.33%
Time 3	43	12	72.09%
Time 4	39	13	66.67%

Mutant Strain *C. elegans*

	Thrash Count in 30 seconds in Control Solution	Thrash Count in 30 seconds in Alcohol Solution	Decrease in Mobility
Time 1	55	42	23.64%
Time 2	47	11	76.60%
Time 3	44	14	68.18%
Time 4	40	19	52.50%



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

Appendices

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

Appendix A
EDVOTEK® Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
E-Z elegans™ not visible in vials.	E-Z elegans™ are small and translucent so difficult to see.	A faint ring may be seen around the circumference of the vial. Even if no ring is seen continue with rehydration steps.
Nematodes on E-Z elegans™ plates do not move when observed through a microscope.	Nematodes are still dormant.	The majority of E-Z elegans™ are shipped in a dauer phase and will take several days to recover. Keep plate covered to prevent drying. If there are no signs of movement after 3 days of rehydration contact Edvotek customer service for assistance.
	Nematodes are dead.	While many nematodes will not survive the rehydration process even a few nematodes can quickly repopulate a plate. If there are any moving nematodes or any nematode trails allow the plate to incubate for additional days. If there are no signs of movement check the alternative E-Z elegans™ plate or contact Edvotek customer service for assistance.
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 OP50 solution to plate.
	Nematodes are dead.	Examine initial E-Z elegans™ 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.
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 new OP50 liquid culture.
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 rehydrating E-Z elegans™ or 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.

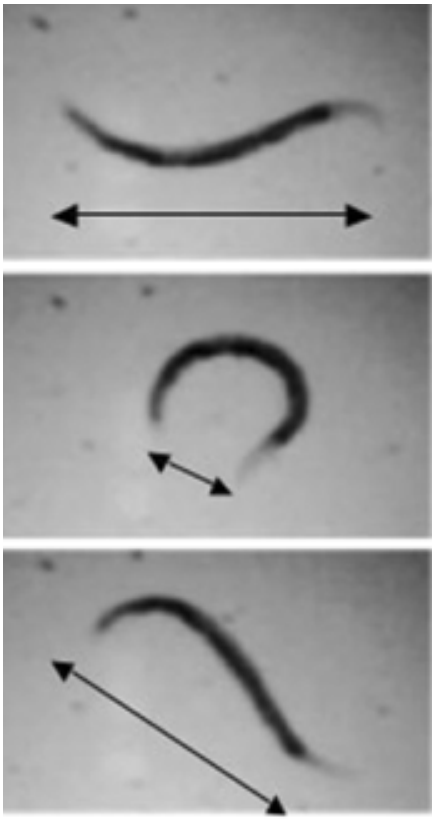


Figure 7

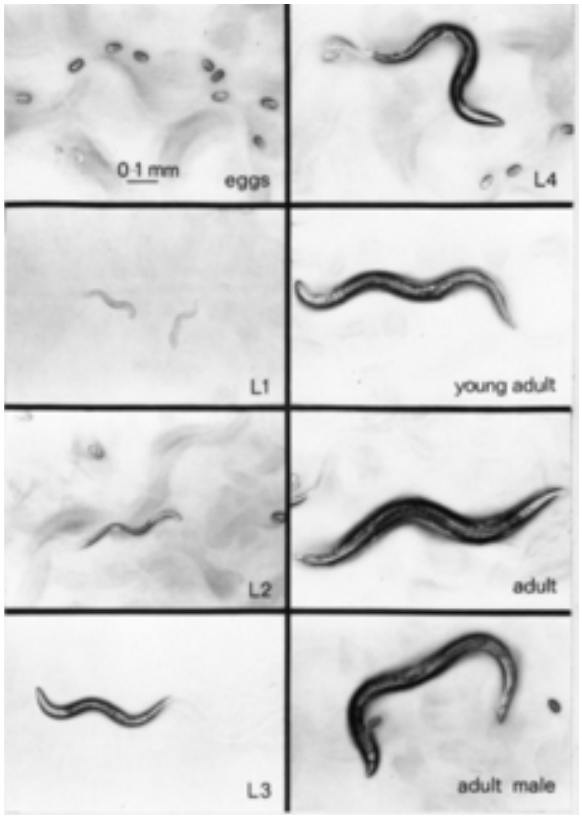


Figure 8

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).

Appendix C

Experimental Timetable for Staggered Counts

Alcohol Exposure Experiment	
Time (min)	**Action**
0	First 30 second Thrash Count Tube 1
1	
2	
3	First 30 second Thrash Count Tube 2
4	
5	
6	First 30 second Thrash Count Tube 3
7	
8	
9	First 30 second Thrash Count Tube 4
10	
11	
12	
13	
14	
15	Second 30 second Thrash Count Tube 1
16	
17	
18	Second 30 second Thrash Count Tube 2
19	
20	
21	Second 30 second Thrash Count Tube 3
22	
23	
24	Second 30 second Thrash Count Tube 4
25	
26	
27	
28	
29	
30	Third 30 second Thrash Count Tube 1
31	
32	
33	Third 30 second Thrash Count Tube 2
34	
35	
36	Third 30 second Thrash Count Tube 3
37	
38	
39	Third 30 second Thrash Count Tube 4
40	
41	
42	
43	
44	
45	Fourth 30 second Thrash Count Tube 1
46	
47	
48	Fourth 30 second Thrash Count Tube 2
49	
50	
51	Fourth 30 second Thrash Count Tube 3
52	
53	
54	Fourth 30 second Thrash Count Tube 4