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Rapid isolation of wild nematodes by Baermann funnel

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TITLE:
Rapid Isolation of Wild Nematodes by Baermann Funnel

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SUMMARY:
This protocol outlines a method for efficiently extracting live nematodes from natural substrates in the field.

ABSTRACT:
Beyond being robust experimental model organisms, *Caenorhabditis elegans* and its relatives are also real animals that live in nature. Studies of wild nematodes in their natural environments are valuable for understanding many aspects of biology, including the selective regimes in which distinctive genomic and phenotypic characters evolve, the genetic basis for complex trait variation, and the natural genetic diversity fundamental to all animal populations. This manuscript describes a simple and efficient method for extracting nematodes from their natural substrates, including rotting fruits, flowers, fungi, leaf litter, and soil. The Baermann funnel method, a classical nematology technique, selectively isolates active nematodes from their substrates. Because it recovers nearly all active worms from the sample, the Baermann funnel technique allows for the recovery of rare and slow-growing genotypes that co-occur with abundant and fast-growing genotypes, which might be missed in extraction methods that involve multiple generations of reproduction. The technique is also well suited to addressing metagenetic, population-genetic, and ecological questions. It captures the entire population in a sample simultaneously, allowing an unbiased view of the natural distribution of ages, sexes, and genotypes. The protocol allows for deployment at scale in the field, rapidly converting substrates into worm plates, and the authors have validated it through fieldwork on different continents.

INTRODUCTION:
Valuable biological insights are emerging as researchers studying *C. elegans* in the lab expand their focus to *C. elegans* and related rhabditid nematodes in the wild. Studies of wild nematodes

place genes and genomes in their natural context, revealing functions potentially obscured by laboratory conditions[1-5]. These studies generate insights into the prerequisite for evolution itself, genetic variation[6-10]. The natural genetic variation captured by wild samples also provides inroads into the genetic basis of many complex traits[11-13].

When designing studies that require the isolation of natural populations of nematodes, mainly when performing remote fieldwork, practical considerations come to the fore. This protocol aims to cleanly isolate entire populations of active nematodes that can be cultured on OP50 from bait or wild substrates. The method is well suited for extracting free-living rhabditid and diplogasterid nematodes, including *Caenorhabditis*, *Oscheius*, and *Pristionchus*.

There are many techniques for isolating nematodes from their substrates[14-15]. The most basic approach is to place the substrate directly onto a nematode-medium plate, picking animals as they crawl out[8,15]. This method requires large amounts of time and labor if the goal is to isolate all nematodes from a sample. More sophisticated techniques take advantage of the animals' weight, size, mobility, or some combination of these[14]. Each method has its advantages and disadvantages in terms of setup and throughput. They also differ in their sampling biases and may select for certain nematodes if the animals in the sample vary along the axis of the method's separation principle.

The Baermann funnel method was first described in 1917 by Dutch Physician G. K. T. F. Baermann, who invented the device on Java while studying soil-dwelling nematodes, including the parasitic hookworm[16]. The Baermann funnel functions based on the principle of mobility. The substrate is placed in a funnel lined with a cloth or paper filter (a "Kimwipe" is used for this study, referred to as "lint-free wipe" in the current protocol) and sealed shut at the bottom. The funnel is then filled with water, submerging the sample while the filter separates it from the sealed outlet. Active nematodes in the sample release themselves into the water and swim through the filter, eventually settling at the bottom of the funnel. The funnel outlet is opened, and a drop of nematodes is expelled onto a plate (**Figure 1**).

[Place **Figure 1** here]

The Baermann funnel will not work for every kind of nematode (see Discussion section for specific alternatives) and is best suited to those that are active forms in the size range of *Caenorhabditis* or smaller[14]. However, if a study can use the Baermann funnel, there are many advantages. The method is practical in the field, requiring limited setup, hands-on time, and cost. The researcher is left with a clean sample without the obstruction of the substrate on the plate, which makes picking easy. Using a filter also prevents contamination of the plate by insect larvae or mites, which chew up plates or prey on nematodes in the sample. Most importantly, the Baermann funnel efficiently extracts nearly the whole population from the substrate[14], which may be required depending on the study's design. For example, researchers interested in counting wild populations' stage or sex distribution, finding slow-growing or rare genotypes, or sampling nematodes not attracted to OP50 might benefit from this method. This is appropriate for

researchers studying ecological[17], population genetic[18], or metagenetic[19] questions as the sampling scheme takes a snapshot of the population at the sampling time.

The present manuscript describes a complete protocol for isolating populations of nematodes using the Baermann funnel and establishing isofemale lines in the field, using equipment chosen for easy transport. For researchers conducting fieldwork near their labs, many of these steps can be omitted or simplified.

PROTOCOL:

1. Preparation of seeded NGM plates in the field

1.1. Before travel, weigh 23.005 g of Nematode Growth Medium (NGM) (see **Table of Materials**) powder and pre-pack in a sealable plastic bag. Make one bag for each liter of media desired.

NOTE: Pre-packaging before travel bypasses the need for a functional balance in the field.

1.2. Prior to travel, prepare 1 mL of 1M $MgSO_4$, 1 mL of 1M $CaCl_2$, and 25 mL of 1M potassium phosphate buffer for each liter of media desired. To make 1 L of potassium phosphate buffer, dissolve 108.3 g of KH_2PO_4 and 35.6 g of K_2HPO_4 in water, as described in WormBook[20].

1.3. Before travel, make an overnight culture of OP50 (see **Table of Materials**) grown in LB at 37 °C, as described in WormBook[20]. Aliquot the culture into 50 mL conical tubes and wrap the tops with paraffin film to prevent leakage.

1.4. In the field, dissolve the contents of the NGM packet into 973 mL of double-distilled water (ddH_20) or the purest, most sterile water available in a 1 L flask or bottle.

1.5. Place the media bottle or beaker, with a loose cap or aluminum foil cover, in a boiling hot water bath on a hot plate or stove. Stir occasionally until all the powder is dissolved and is clear (this takes ~30 min).

NOTE: If a magnetic hot plate is available, a stir bar is an excellent option to limit the amount of manual stirring.

1.6. Remove the media from the water bath and cool to ~58 °C with occasional shaking or with a stir bar. Once the media is cooled to 58° C, use serological or standard pipettes to add 25 mL of 1M potassium phosphate buffer, 1 mL of 1M $MgSO_4$, and 1 mL of 1M $CaCl_2$, mixing well between each step.

1.7. In the most sterile environment available, pipette or pour the media into plates of the desired size and allow to cool overnight so that it gets solidified. Pour one 60 mm plate (~10 mL)

for each substrate sample. Pour one 35 mm plate (~3.5 mL) for each isofemale line; the number of small plates needed is difficult to predict in advance.

1.8. Pipette 50 µL of OP50 culture onto each plate and allow it to dry and grow overnight before use.

2. Collection of the nematode substrates

2.1. Identify a bacteria-rich substrate in the field. Some examples include rotting fruit, flowers, fungi, and stems of herbaceous plants. Soil and leaf litter are also suitable, though they rarely contain *Caenorhabditis*.

2.2. With a gloved hand, place a sample of this substrate (1-15 cm^3) into a sealable plastic bag (see **Table of Materials**) labeled with a unique sample ID (**Figure 1A**).

2.3. Record the sample ID, latitude, longitude, date, description of the substrate, and any other local environmental measurements relevant to the experiment, including ambient and substrate temperature, time of collection, condition of substrate, presence of substrate-associated macroinvertebrates, and so on. A smartphone app is available to streamline this process[21].

3. Preparation of an array of Baermann funnels

3.1. For each funnel, use scissors to cut a segment of rubber tubing (see **Table of Materials**) ~3 cm long.

3.2. Fit the tubing segment over the end of a plastic funnel (see **Table of Materials**). This may take some effort as the fit is very tight.

3.3. Slide a tubing clamp over the rubber tubing and clamp it shut.

3.4. To make a funnel holder, use a scalpel to cut circular holes of 35 mm diameter in the bottom of a cardboard fly-vial tray (see **Table of Materials**) that has not been folded together from its flat shipping orientation. A standard tray can accommodate 12 of these holes in a 3 x 4 array.

3.5. Invert the cardboard, fold down the sides once (not twice, as one would to make a fly-vial tray), and tape the sides together to elevate the inverted cardboard tray (**Figure 2**).

3.6. Place funnels in the holes, first making sure that the tubing clamps are in the closed position.

[Place **Figure 2** here]

4. Transfering of samples into the funnels

4.1. Pour water (as sterile as available) into each funnel, filling it about 3 cm below the rim. If air bubbles are trapped in the tubing, tap the funnel to release them.

4.2. With gloved hands, place a lint-free wipe or specifically a Kimwipe (folded in half to make a square) over the funnel, and press down on the center so that it is submerged in the water.

4.3. Manually break large solid pieces of natural substrates (fruit, flower, soil, leaf litter, etc.) into smaller fragments to minimize the distance worms must travel to fall out of the substrate.

NOTE: Leaf litter and awkwardly shaped samples can be preprocessed in a food processor or blender.

4.4. Gently place a sample of the natural substrate (1-15 cm^3) onto the tissue/lint-free wipe in a funnel without puncturing the tissue and without the sample protruding above the rim.

4.5. Label the funnel, or the cardboard next to the funnel, with the sample ID corresponding to field collection notes.

4.6. Fold the corners of the tissue/lint-free wipe over the sample (**Figure 1B**). Be careful to keep the sample contained within the tissue/lint-free wipe so that no soil or debris can pass to the bottom of the funnel.

NOTE: This step is to prevent the corners from draping over the edge of the funnel, where they would wick the water from the funnel over the sides.

4.7. With hands, a spatula, or a pipette tip, pick out any active insects, millipedes, or other animals that may travel from funnel to funnel, cross-contaminating samples. Wrap the tissue/lint-free wipe entirely around the sample or lay a second tissue across the top of the sample can help prevent cross-contamination.

4.8. Add more water to each funnel so that the entire sample is submerged (**Figure 3**).

[Place **Figure 3** here]

5. Extraction of nematodes from the funnels

5.1. Wait for ~12 h or overnight. During this time, active worms will wriggle out of the substrate, through the tissue/lint-free wipe and down to the bottom of the clamped funnel.

NOTE: Waiting much longer than 12 h risks worm mortality due to hypoxia or pathogenic infection, which may also be a risk at shorter durations for samples that are particularly crowded with worms and bacteria.

5.2. Write the sample ID of a funnel on the bottom of a 60 mm NGM worm plate seeded with a spot of OP50 *E. coli* bacteria. Remove the lid from the plate.

5.3. Remove the funnel containing that sample from the funnel stand. Using one hand to hold the funnel upright above the open worm plate, use the other hand to release pressure on the tubing clamp, allowing one drop of water to fall from the tubing onto the worm plate (**Figure 1C**). As soon as water drops from the funnel, quickly clamp it shut again to prevent flooding the NGM plate.

NOTE: To select worms attracted to OP50, including *Caenorhabditis* species, release the drop away from the bacterial lawn. When the water soaks into the plate or evaporates, *Caenorhabditis* nematodes will crawl into the bacterial lawn.

5.4. Clean up: Throw out the contents of the funnels. Wash the funnels with hot water for subsequent reuse.

6. Establishing the cultures

6.1. Observe the isolated nematodes under the stereomicroscope at a magnification of 5x-50x. The plates should include nematodes and small oligochaete annelids, tardigrades, rotifers, and small crustaceans at much lower frequencies (**Figure 4**).

NOTE: If the funnel has been set up correctly, no mites, insects, or visible non-living material will have made it through the funnel.

[Place **Figure 4** here]

6.2. To establish isohermaphrodite or isofemale lines, use a worm pick to transfer each L4 hermaphrodite or mated adult female (recognizable by their larger body size and lack of the distinct male tail[22]) to a separate 35 mm NGM plate seeded with OP50 (**Figure 1D**). Use a lighter to sterilize the worm pick before and after transferring worms.

6.3. Use paraffin film to wrap plates thoroughly for travel.

REPRESENTATIVE RESULTS:

This protocol was used to isolate nematodes from fruit, flowers, fungi, soil, and stems on Barro Colorado Island, Panamá, at the Smithsonian Tropical Research Institute field station in August of 2018. Of 131 substrates processed by a single investigator over four days, 130 substrates (99.2%) yielded nematodes. Forty-four of the substrates (33.6%) yielded *Caenorhabditis* nematodes (**Figure 5**). Subsequent analysis of cultures established from these forty-four substrates, by PCR and mating tests[23], revealed the presence of six different *Caenorhabditis* species—*C. becei*, *C. tropicalis*, *C. briggsae*, *C. sp. 24*, *C. sp. 57*, and *C. panamensis*.

This protocol was used again to isolate nematodes from various substrates in the Chernobyl Exclusion Zone, Ukraine, over four days in August of 2019. Live worms were recovered from 62 out of 63 soil samples, 1 out of 17 invertebrate samples, 31 out of 75 fruit samples, 1 out of 12 bait samples (see Discussion section), and no worms were recovered from mushroom, river reed, or wolf feces samples (one sample collected of each). Subsequent sequencing of 18S ribosomal DNA[15] identified these nematodes as *Oscheius*, *Panagrolaimus*, *Acrobeloides*, *Mesorhabditis*, *Panagrellus*, *Pristionchus*, and *Pelodera*, but no *Caenorhabditis* were identified (**Figure 5**).

[Place **Figure 5** here]

FIGURE LEGENDS:

Figure 1: Summary of the Baermann funnel technique. (**A**) Collection of a bacteria-rich sample from a site of interest. (**B**) Submerging the sample in a Baermann funnel and waiting for worms to wriggle out and sink. (**C**) Releasing a single drop from the funnel. (**D**) Moving single hermaphrodites or mated females to separate plates. Illustration created by Ramin Rahni.

Figure 2: Repurposed cardboard fly vial trays, folded and cut to support 12 Baermann funnels each.

Figure 3: Assembled Baermann funnels. Each sample is wrapped in tissue/lint-free wipe and submerged under water in the funnel, which is clamped shut. Over a period of ~12 h, the nematodes will migrate through the tissue and to the bottom of the funnel.

Figure 4: Contents of the first droplet released from a Baermann funnel onto an NGM plate.

Figure 5: Success rates of two collection trips. Panama in 2018 (left) and Ukraine in 2019 (right).

DISCUSSION:

The central principle of this method is that nematodes will pass through the tissue submerged in water, while their substrate and larger invertebrate contaminants will not. The critical steps of the protocol are (1) collecting an appropriate substrate, (2) submerging the substrate, wrapped in a filtering material, in water, (3) collecting worms that have passed through the filter and sunk to the bottom of the water, and (4) isolating individual worms to create isofemale or isohermaphrodite lines. All other parts of the method are amenable to modification as necessary according to the resources available, the nature of the substrates, or the fieldwork goals. Some modifications worth considering are as follows.

Baiting the worms by planting fruit

If there is not an ample supply of bacteria-rich rotting material to be found in the field site, one may wish to bring a sample, such as a piece of apple or tomato, to leave to rot. Pin the bait down well with a few stakes so that larger animals do not remove them and so it can easily be found later for collection. Avoid direct sunlight, where the bait might dry before it rots.

Constructing funnel apparatus out of whatever materials are available

Any structure with holes large enough to accommodate the tubing clamp and stable enough to support a top-heavy funnel will work. For a single funnel, a drinking glass is a suitable holder. Any kind of tissue—facial tissue, toilet paper, or paper towels—can be used.

Putting less material in funnels, or adjusting the wait time, to prevent hypoxia or infection

If the sample has a very high concentration of worms or bacteria, nematodes may begin dying from hypoxia or infection before the 12 h incubation is complete. If this is a concern, the researcher may check funnels earlier or prepare an additional funnel with a very small subsample of the highly populated substrate.

Adjusting the NGM plate preparation to experimental needs and constraints

NGM plates can be prepared with whatever media and food source is appropriate to the experiment. The field protocol described above is designed to minimize baggage weight. Depending on baggage limitations and the fieldwork timing, bringing already-poured NGM plates—either prepared in the lab or purchased commercially—may be preferable rather than pouring plates in the field.

Performing the funnel isolations in the laboratory

The protocol describes carrying out the complete procedure under field conditions to capture the nematode population as it occurs in nature. For some research goals, it may be sufficient to isolate nematodes later, after traveling back to the lab with samples in sealed bags. Even then, the Baermann funnel method provides a cleaner and complete sample of the surviving nematodes than other isolation methods. However, samples in sealed bags may experience selection during travel, as they are exposed to potential extremes of temperature and hypoxia. This can be minimized by performing isolations as soon as possible after sample collection.

A common alternative method to the Baermann funnel involves placing the substrate directly onto an NGM plate and waiting for worms to crawl out, which is either highly labor-intensive or results in the incomplete collection of the population. It also yields plates contaminated with mites and insect larvae. The Baermann funnel method is a low-cost, low-tech, low-labor strategy for quickly separating the entire population of active worms from their substrate.

The Baermann funnel method is not universally applicable for collecting all types of wild nematodes. Some plant-dwelling nematodes take much longer than 12 h to emerge from their substrate and will be absent from a droplet released too early, while some insect parasites will crawl to the top of the funnel rather than the bottom, also evading collection[14]. Alternatives to the Baermann funnel either require more specialized equipment or more labor to recover the worms. However, they may still be preferred if the caveats above are a problem for the experiment. Alternative options, reviewed by van Bezooijen[14], include the funnel spray method, which provides a constant mist of water to funnels, adding oxygen and allowing the overflow of bacteria in suspension. This allows for a more extended extraction period of nematodes from plants. The blender centrifugal flotation method recovers slow-moving, inactive, or upward-crawling nematodes by separating them by their specific gravity, the Oostenbrink elutriator

applies an undercurrent to separate settling sediment from suspended nematodes, and Cobb's Method uses a series of sieves to isolate nematodes by their size, shape, and sedimentation rate[14]. To collect rhabditids, though, the Baermann funnel effectively produces clean samples quickly and with minimal effort.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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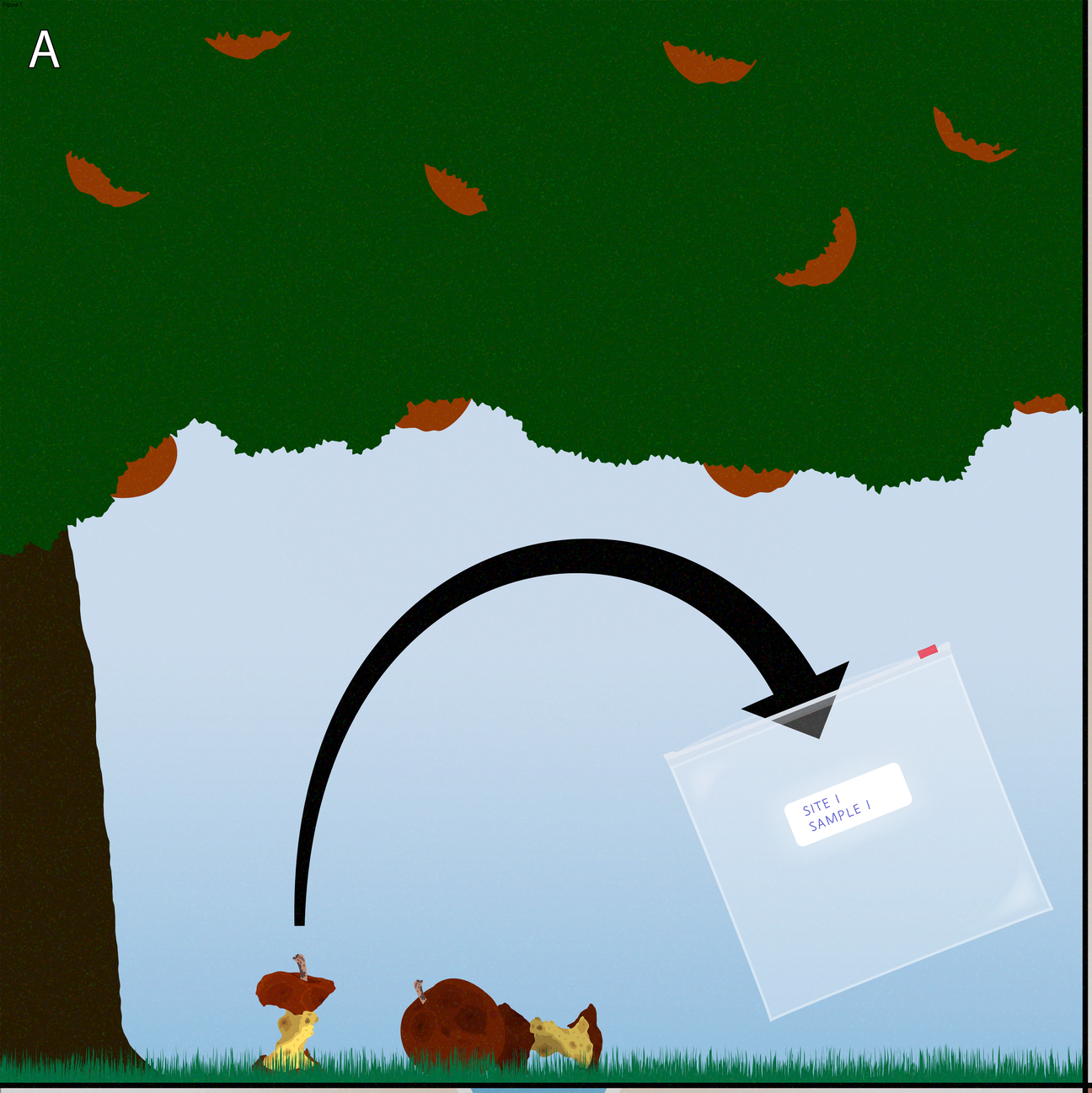

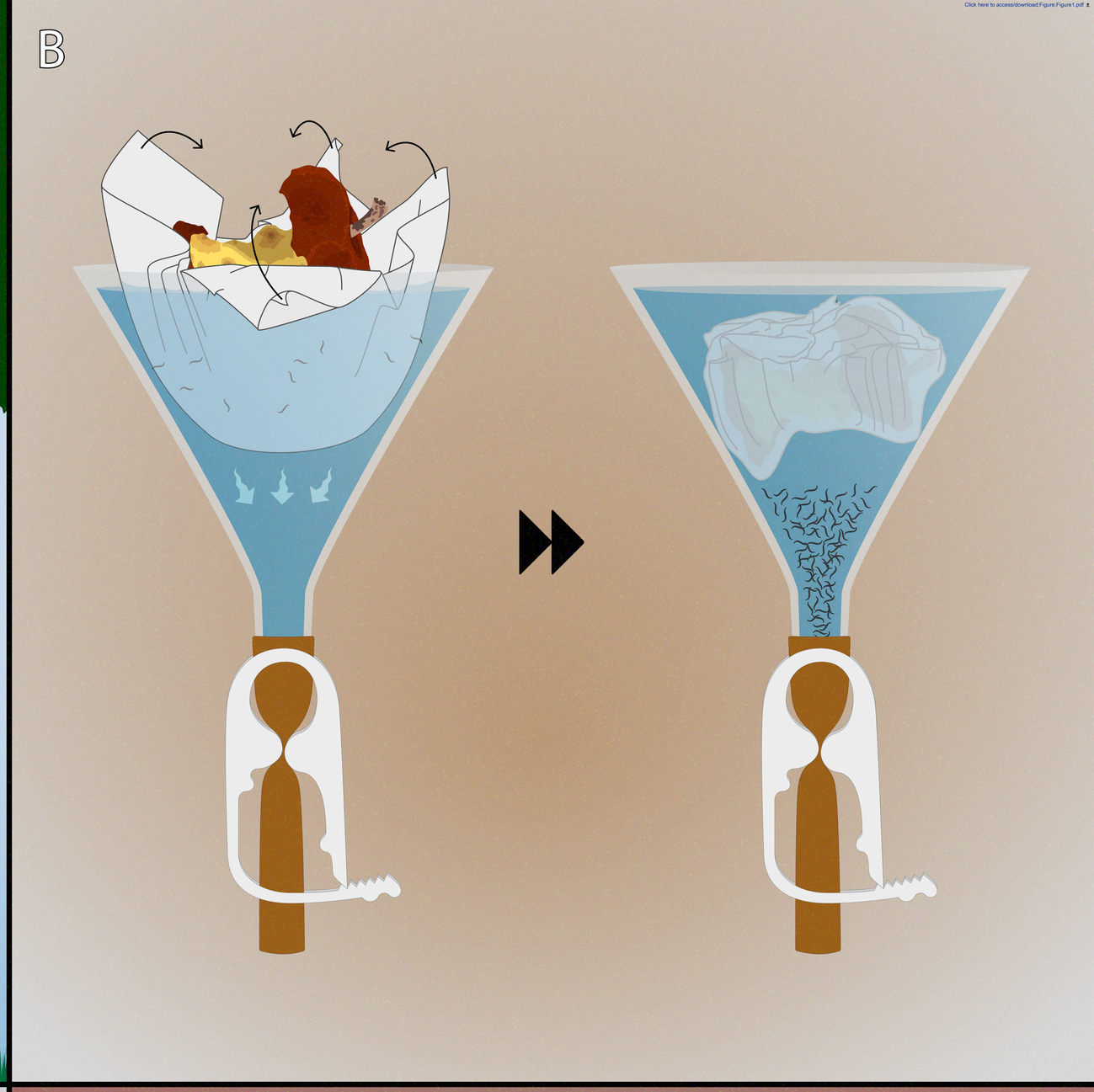

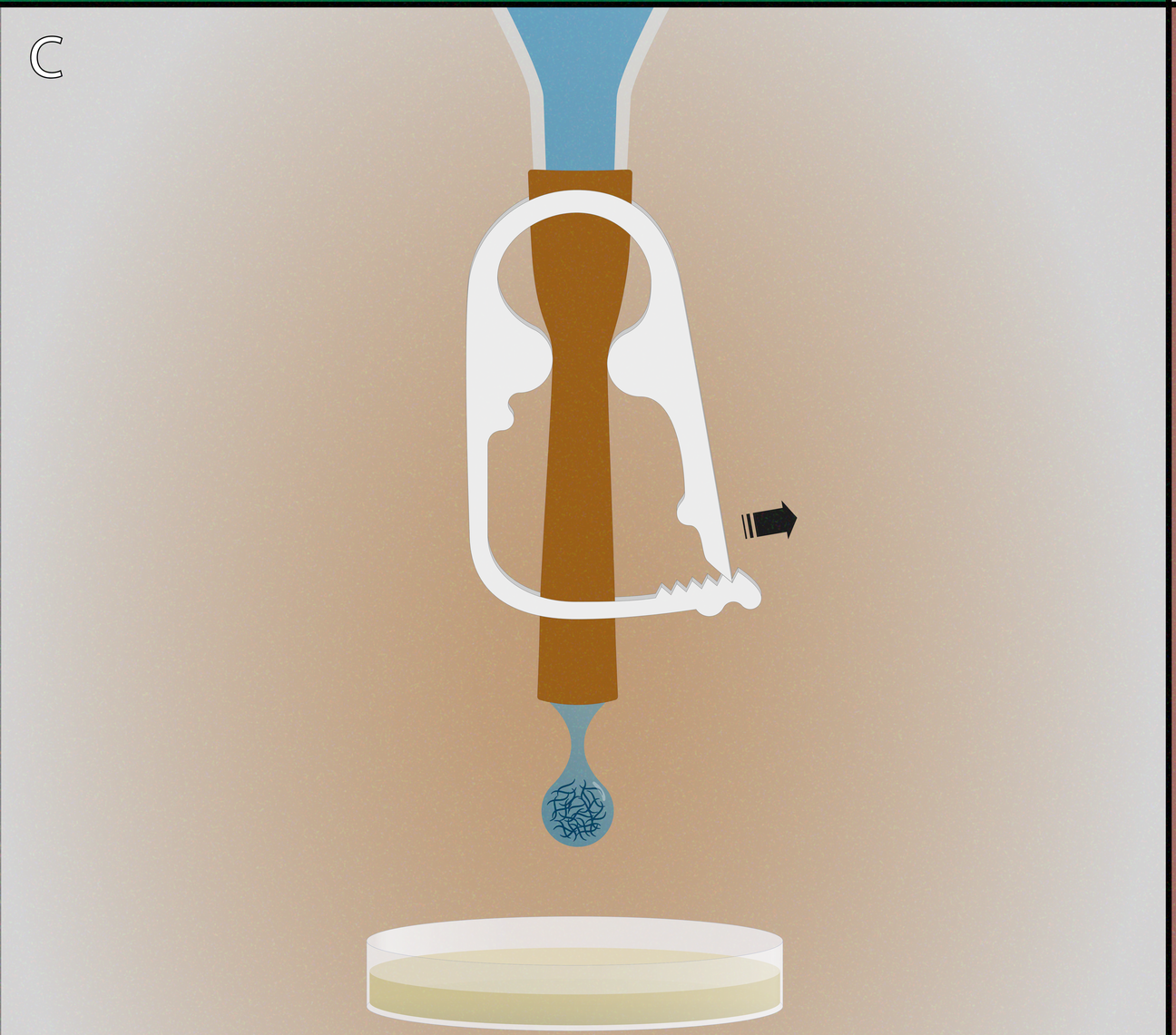

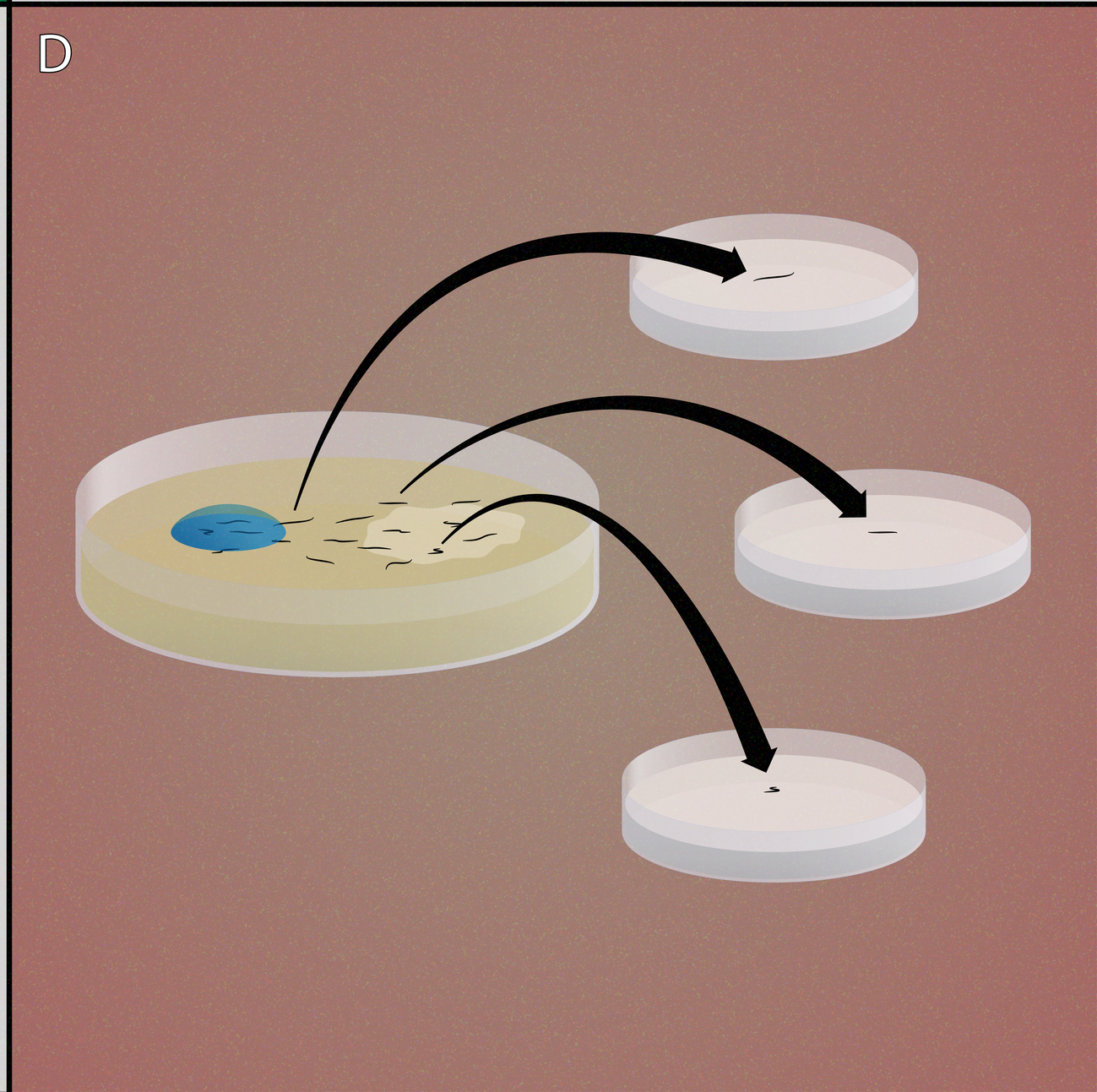

Figure 2

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Figure 3

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Figure 4

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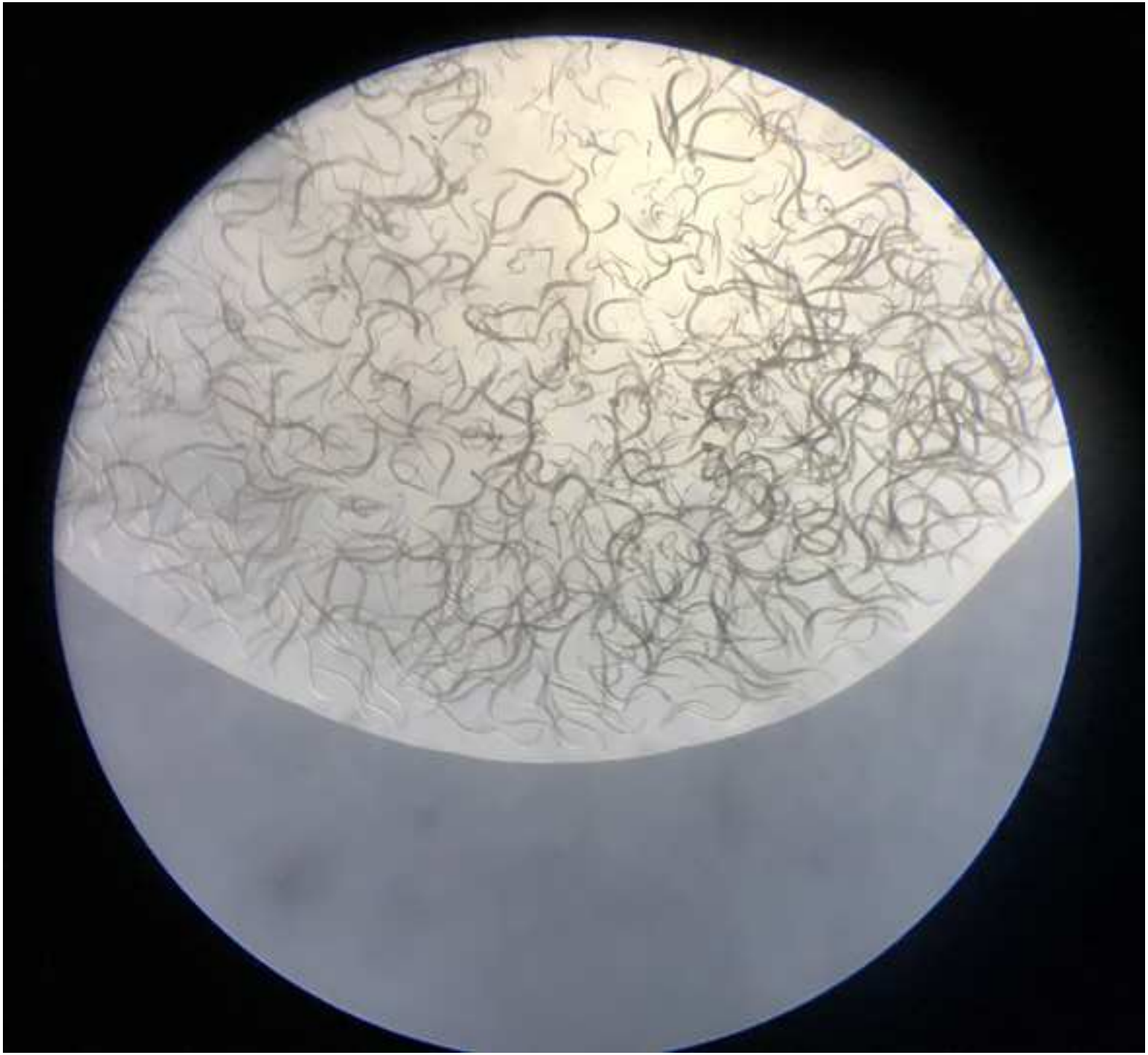
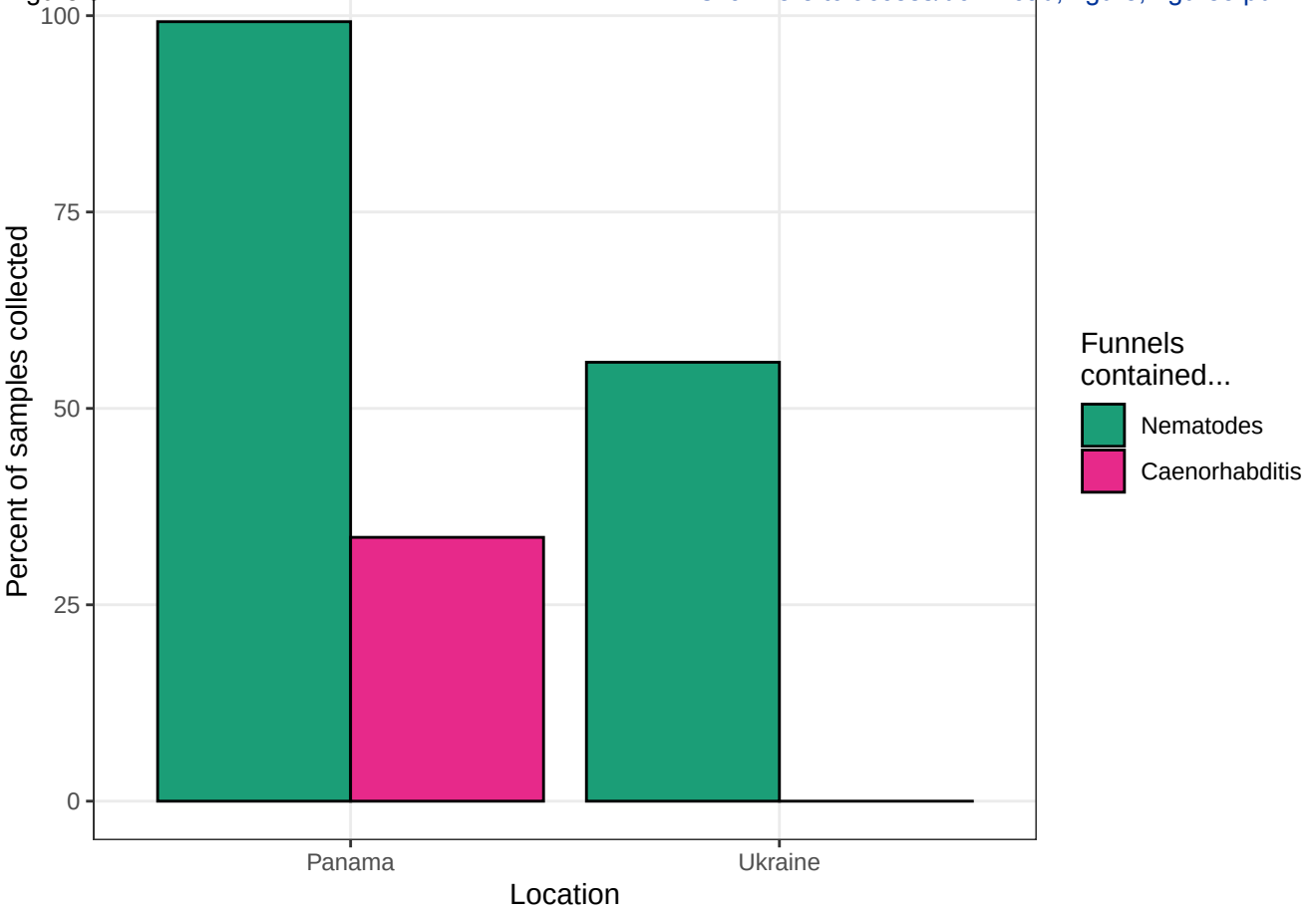


Figure 5



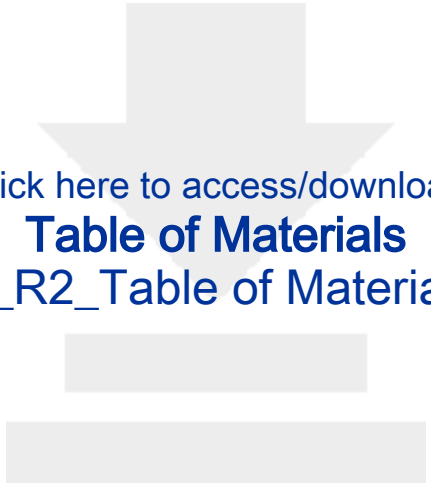


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Table of Materials

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Dear Colleagues,

We appreciate your thoughtful feedback and the comments from yourself and from the reviewers. After considering each comment we have modified our manuscript as described below.

We thank all reviewers and the editor for their generous consideration, and we feel that addressing these comments has strengthened our manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript again, and to the best of our knowledge no errors remain.

2. Please provide suitable citations for lines 54-55, 71-72, 79-83.

We have added appropriate citations for the requested lines.

3. Use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, °C, etc. Use h, min, s, for hour, minute, second.

We have scoured the manuscript for unabbreviated units, and have changed some instances of “hours” to “h”. No other unabbreviated units remain, to our knowledge.

4. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We reviewed the protocol for steps that were unnecessarily long and either simplified them or split them up. Most notably we split step 4.3 into three discrete steps.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have replaced any mention of “we” with “the authors” or “the manuscript”, and any mention of “you” with “the researcher” or “the experiment”. We believe that this has made our manuscript clearer, more consistent, and easier to read.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We carefully reviewed the protocol and added explicit detail about how to do each step. Most steps that lacked this kind of detail were steps in which the strategy is simple and analog (break the substrate up—with your hands, transfer the sample—with your hands, etc). We have added these specifications which should make the protocol easier to follow.

Step 6.1: Were any specific microscope settings used to visualize the microorganisms? If yes, please provide the microscope settings along with the magnification used.

We added a specific range of magnifications that should be suitable for visualizing nematodes of all ages on a stereomicroscope.

Step 6.2: How were the L4 hermaphrodites or mated adult females identified? Please describe in brief.

We have added a line to this step referencing the body size and visible anatomical structures distinguishing males from female and hermaphrodites. We included a reference as well, which will provide more than enough detail for anyone not already familiar with distinguishing the sexes by eye.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted in yellow the sections that we think will be most informative for JoVE readers who are unfamiliar with this method. The only section we did not include is preparing the worm petri dishes with Nematode Growth Media, which could be considered prep work and should be most familiar to nematode biologists.

8. Please also include in the Discussion the following in detail with citations:

a) Critical steps within the protocol

We have added text at the very beginning of the Discussion to clearly delineate the critical steps of the protocol.

b) The significance with respect to existing methods

We have added text to the Discussion that compares our protocol not only to higher-tech alternatives, but also to the lower tech and less efficient methods that are more commonly used.

Reviewer #1:

Manuscript Summary: None Major Concerns: None Minor Concerns: None

We appreciate the reviewer's support for our manuscript and confidence in our execution.

Reviewer #2:

Line 18. The authors must use correct terms, for example "wild nematodes" modify it for free live nematodes, in nematodology this last term is correct.

The reviewer brings up an interesting point which we spent a lot of time thinking about before our submission. "Free live nematodes", as the reviewer recommends, specifically refers to nematodes that are free living, meaning not parasitic. We do not want to restrict ourselves to this term, because many parasitic worms have life stages where they may be recovered in a funnel in the manner described in our protocol. Additionally, the substrate one may wish to collect worms from could be a chopped-up invertebrate, in which case this method is perfectly suitable to collecting parasitic worms as well. We felt, after some debate, that "wild nematodes" is in fact the most accurate term for this occasion.

Line 23. Specify what type of fungi, endophytes nematophages, ovicides, endozoics, etc.

It is not clear to us exactly what the reviewer is referring to, or where mention of such organisms and agents should fit into our manuscript. We have not made any mention of endophytes, nematophages, ovicides, or endozoics in this manuscript, nor do we know how these organisms and agents would be relevant to this protocol, so we are not sure which part of the manuscript the reviewer would like us to add these specific details to. Perhaps the reviewer and editor could add some more specifics to this comment to help us understand the recommendation a little better. If so, we would be happy to give it another look to try to clarify the issue.

Line. What are the advantages and disadvantages of the classic Baerman funnel method with this protocol? Add cost-benefit information.

We have added text to the Discussion that compares this method to both the higher-tech and more thorough protocols (which may be preferred if the researcher wishes to collect specific types of worms with unique collecting challenges), as well as the lower tech, more common protocols.

line 165. Specify clearly and in detail that it is a "kimwipe", since it is confusing.

We are not sure how to make that particular line the reviewer cited clearer, since the third word and object of the sentence is already "Kimwipe", but we have added additional clarification earlier in the protocol that a cleaning tissue, traditionally a kimwipe, is used. Hopefully this elaboration earlier in the manuscript, particularly in the introduction, will help with clarity.

Line 39. Improve the writing of the entire document, following the scientific method.

We have further revised the text with an eye on language and structure. Hopefully this has improved the readability of the document, particularly the parts following the description of the method.

Line 49. The authors must add the species or species of the nematodes described; likewise, improve the writing.

We have added additional details about the nematodes collected from each of the field trips cited, including the full list of 6 *Caenorhabditis* species collected from Panama, as well as a more comprehensive list of the variety of nematodes found in Ukraine. We have made additional edits to that section to improve the clarity of the writing.

What was your experimental design? Statistical analysis?

We have added additional detail about our methods of worm collection and identification to the "representative results" section. There were no statistical analyses involved in the collection part of these projects.

Reviewer #3:

This publication offers a kind of sturdy and economically possible Baermann funnel set-up, which is a traditional approach for recovering a range of nematode species and is described in detail in the literature. Using a methodology described by the authors, it is possible to extract a significant number of wild nematodes from the field without contaminating the sample.

We are glad that the reviewer appreciated the value of this approach, and we appreciate the suggestions to improve clarity.

I think the authors must line the funnel cone with a piece of sieve or muslin cloth.

Our method does describe lining the funnel with a tissue, traditionally a Kimwipe, which serves the purpose that the reviewer's suggested sieve or muslin. We have elaborated on the first mention of the Kimwipe and its function, and added additional focus on this in the introduction. Hopefully this will improve clarity around this aspect of the protocol.

In a field setting, nematodes would coexist with a large number of saprophytic bacteria and fungi, which might result in a shortage of oxygen for the worms to thrive and an increased danger of being prayed by entomopathogenic fungi. The writers must devise strategies for resolving this problem in their protocol.

We appreciate this concern. We had previously included commentary on how to avoid worms dying from hypoxia, but have expanded it to include pathogenic bacteria and fungi as well. We now mention the issue in step 5.1 as well as in the discussion.

Additionally, what is the length of the funnel they have retained? The authors assert that they are not targeting any specific nematode but rather all types of wild worms. All worms may not come down same day. So how long one must wait in the field if they use author's protocol?

In the protocol we describe here we've specified a 12-hour incubation in the funnel, which the reviewer is correct to point out will not be sufficient for all types of worms. We mention this concern in the third paragraph of the discussion (starting "The Baermann funnel method is not universally applicable for collecting all types of wild nematodes") and devote the rest of the paragraph to describing alternative methods that may be more suitable to other types of nematodes. We have also added a parenthetical to the Introduction to foreshadow this issue.