# Measuring Embryonic Viability and Brood Size in *Caenorhabditis elegans*

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## Abstract

*Caenorhabditis elegans* is an excellent model organism for the study of meiosis, fertilization, and embryonic development. *C. elegans* exist as self-fertilizing hermaphrodites, which produce large broods of progeny-when males are present, they can produce even larger broods of cross progeny. Errors in meiosis, fertilization, and embryogenesis can be rapidly assessed as phenotypes of sterility, reduced fertility, or embryonic lethality. This article describes a method to determine embryonic viability and brood size in *C. elegans*. We demonstrate how to set up this assay by picking a worm onto an individual Modified Youngren's, Only Bacto-peptone (MYOB) plate, establish the appropriate timeframe to count viable progeny and non-viable embryos, and explain how to accurately count live worm specimens. This technique can be used to determine viability in self-fertilizing hermaphrodites as well as cross-fertilization by mating pairs. These relatively simple experiments are easily adoptable for new researchers, such as undergraduate students and first-year graduate students.

## Introduction

Sexual reproduction in eukaryotic organisms requires the production of functional gametes that merge to form an embryo through the process of fertilization. Maternal and paternal gametes, ovum (eggs), and sperm are created through the specialized cell division and differentiation processes of meiosis and gametogenesis[1]. Meiosis starts with a single diploid cell and ends with the formation of daughter cells that contain half the number of chromosomes of the original parental cell. From the reduction of ploidy to the shuffling of genetic material *via* independent assortment and crossover recombination, meiosis serves multiple important functions[1]. Errors within meiosis can result in aneuploidy, in which there are too many or too few chromosomes within a gamete. Incidences of aneuploidy have tremendous impacts on human health, as chromosomal imbalances are a major cause of miscarriages and developmental disorders such as Down syndrome and Edwards syndrome[2].

Fertilization is the process by which the maternal and paternal gametes fuse to generate a new organism[3]. Gamete-gamete recognition is facilitated by proteins on the gamete surface[3].

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Errors with gamete compatibility lead to infertility as sperm and egg fusion is unable to proceed. The fusion of sperm with an oocyte triggers a host of events that lead to the proper formation of an active embryo that can begin the developmental journey from a single-cell embryo to a fully functional multicellular organism *via* mitotic divisions[4]. Throughout embryogenesis, molecular events that regulate development must be tightly regulated and precisely timed to enable proper growth of the organism[5]. Proper cellular differentiation during early development is crucial as the organism transitions from a pluripotent embryo to a fully-fledged organism. Due to the complexities of these events, disruptions can lead to developmental defects that result in embryonic lethality.

*Caenorhabditis elegans* is an excellent model organism to study meiosis, fertilization, and embryonic development. *C. elegans* is a transparent nematode that has two sexes, males and hermaphrodites. *C. elegans* hermaphrodites, which are capable of self-fertilization, are the predominant sex[6,7]. The hermaphrodite gonad first produces sperm during the fourth larval (L4) stage, which are stored in the spermatheca. At the L4 to adulthood transition, the germ line switches to producing oocytes, which are then fertilized *via* the stored sperm. Males, which arise in hermaphrodites at a rate of less than 0.2%, only produce sperm and can mate with hermaphrodites. Upon cross-fertilization, male sperm outcompete hermaphrodite sperm in the fertilization of oocytes[8]. This allows for the relatively easy maintenance of homozygous mutants through self-fertilizing stocks and for genetic manipulations through genetic crosses. The two sexes allow for studies exploring differences between meiosis in male and female germ lines. Further, due to the transparent nature of *C. elegans* and its eggs, the processes of meiosis, gametogenesis, fertilization, and embryogenesis can be studied in live, intact animals using fluorescence imaging techniques.

When analyzing new mutations in genes that may play a role in meiosis, fertilization, and/or embryonic development in *C. elegans*, a crucial first step is determining embryonic viability and brood size since errors in these processes often lead to a failure or reduction in the production of viable progeny. This paper describes a protocol for assessing fertility, embryonic viability, and brood size from either self-fertilizing hermaphrodites or crosses between hermaphrodites and males. While this classic assay has been used in many *C. elegans* studies, we provide a standardized protocol for setup and accurate quantification. In this protocol, individual worms or male/hermaphrodite pairs are isolated to allow mating and progeny production. Progeny production and viability are observed over a series of days to determine the number of viable progeny and non-viable embryos. At the conclusion of the experiment, individual broods are analyzed to calculate the embryonic viability percentage and total brood size.

## Protocol

NOTE: See the **Table of Materials** for details about all materials used in this protocol.

### 1. Preparing experimental plates

1. Prepare 35 mm diameter Petri dishes with Modified Youngren's, Only Bacto-peptone (MYOB) media. To make MYOB plates, add 20 g of Bacto agar, 2 g of NaCl, 0.55 g of Trizma-HCl, 0.24 g of Trizma-OH, 3.1 g of Bacto peptone, and 1.6 mL of cholesterol to 1 L of dd$H_2O$ and autoclave for 40 min at 121 °C. Let it cool to 55 °C and using sterile technique, pour 4 mL of the molten media into each 35 mm Petri dish.

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NOTE: A liter of MYOB makes ~250 plates, which can be stored at 4 °C for up to 6 months. We recommend a minimum of 10 individuals per replicate with three replicate sets for each strain.

2. Using sterile technique, seed the plates with a small spot (approximately 50 µL) of OP50 bacteria in the middle of the plate.

   NOTE: Small lawns are especially important for assessing cross-fertilization as the smaller spot leads to increased encounters between males and hermaphrodites, thus increasing the chance of mating.

## 2. Embryonic viability assays (hermaphrodite self-fertilization)

1. Day 1
   1. Label the back of each plate. Be sure to keep track of each plate and their respective worms throughout the experiment.

      NOTE: Preferred labeling technique-Day 1: worm 1, Day 1: worm 2, … etc.
   2. Transfer an individual L4 stage hermaphrodite onto each plate. Ensure no embryos or other worms are transferred onto the plate. Allow the worms to develop into adults and lay self-progeny for 24 h at the standard culturing temperature of 20 °C. Score these plates on day 3.

      NOTE: The temperature of experiments can be altered in the case of temperature-sensitive mutations. For temperature sensitive mutations, embryonic viability assays should be performed at both the permissive (15-16 °C) and nonpermissive temperatures (24-26 °C).
2. Day 2
   1. Label a set of new plates-Day 2: worm 1, Day 2: worm 2, … etc.
   2. Transfer day 1 individual worms onto the new plates.

      NOTE: These worms should have reached adulthood, and wild-type worms should have already started laying embryos.
   3. Allow the worms to lay embryos for 24 h at 20 °C or other appropriate temperature. Score these plates on day 4.
3. Day 3
   1. Label a set of new plates. Day 3: worm 1, Day 3: worm 2, … etc.
   2. Transfer day 2 individual worms onto new plates. Allow them to lay progeny for 24 h at 20 °C or other appropriate temperature. Score these plates on day 5.

      NOTE: In cases of lower temperatures, hatching times will be increased. Adjust accordingly.
   3. Draw a grid pattern on a 35 mm lid using a fine marker. Place the gridded lid under the test plate for counting to keep track of worms previously counted (**Figure 1A-B**).
      1. Using a differential cell counter, score the day 1 plates for the presence or absence of progeny. Count the live larvae and unhatched embryos.

         NOTE: At this point, a sufficient time has lapsed such that any viable embryos should have hatched. Any unhatched embryos are presumed dead.
      2. Within an individual square, count the unhatched embryos and live larvae that are entirely within the square (**Figure 1C-F**).

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3. For worms that are on the square borders, count based on the location of the worm head. Count worms with heads touching the top and left edges of the square (do not count those touching the bottom or right edges; **Figure 1D**). Do not count unfertilized oocytes for this assay (**Figure 1F**).

4. Record the number of live larvae and unhatched embryos in a laboratory notebook.

4. Day 4

1. Score the day 2 plates by counting the live larvae and unhatched embryos, as described in step 2.3.3.1. Record the number of live larvae and unhatched embryos in a laboratory notebook.

5. Day 5

1. Score the day 3 plates by counting the live larvae and unhatched embryos, as described in step 2.3.3.1. Record the number of live larvae and unhatched embryos in a laboratory notebook. Analyze the data obtained using the method described in the data analysis.

NOTE: Some genetic mutants may have either a delayed or expanded reproductive cycle. Monitor individual strains for the continued laying of embryos beyond the day 3 plates. If embryo production has not ceased by day 4, continue to transfer adults to new plates.

### 3. Embryonic viability assays (male/hermaphrodite cross-fertilization)

1. Day 1

1. Label the back of each plate. Be sure to keep track of each plate and their respective worms throughout the experiment.

2. Transfer an individual L4 hermaphrodite worm onto each plate. Ensure no embryos or other worms are transferred onto the plate.

NOTE: Feminized strains, such as *fog-2* loss-of-function mutants, which do not produce sperm, can be used in place of hermaphrodites.

3. Transfer a single L4 male worm onto each labeled plate containing a L4 hermaphrodite. Ensure no embryos or other worms are transferred onto the plate.

NOTE: Strains such as *plg-1* variant males can be used to identify whether mating has occurred, as these males deposit a copulatory plug on the hermaphrodite vulva after mating.

4. Allow the worms to mate and lay progeny for 24 h at 20 °C, or another appropriate temperature. Score these plates for live larvae versus unhatched embryos on day 3.

2. Day 2

1. Label a set of new plates and transfer the worms, as in day 2 above. In this case, make sure to transfer both the hermaphrodite and male onto new plates. Ensure that the hermaphrodite has reached adulthood.

2. Allow the hermaphrodites to lay progeny for 24 h at 20 °C, or another appropriate temperature. Score these plates for live larvae versus unhatched embryos on day 4.

3. Day 3

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1. Label a set of new plates and transfer the worms, as in day 3 above. Make sure to transfer both the hermaphrodite and male onto new plates.
2. Allow to lay progeny for 24 h at 20 °C, or another appropriate temperature. Score these plates for live versus unhatched embryos on day 5.
3. Using a differential cell counter, count the live larvae and unhatched embryos from the day 1 plates, as described in step 2.3.3.1.

   NOTE: Do not discard the plates, as they are required for day 4.
4. Record the number of live larvae and unhatched embryos in a laboratory notebook.

4. Day 4
   1. Count the live progeny and unhatched embryos from the day 2 plates, as described in step 2.3.3.1. Record the number of live larvae and unhatched embryos in a laboratory notebook.
   2. Check the day 1 plates for males. If mating has occurred, the expected genetic ratio of hermaphrodites to males should be 50:50. If day 1 plates do not contain any males, mating between the male and hermaphrodite did not occur. Discard this mating pair and record this observation in the laboratory notebook.

5. Day 5
   1. Count the live larvae and unhatched embryos from the day 3 plates, as described in step 2.3.3.1. Record the number of live larvae and unhatched embryos in a laboratory notebook. Analyze the data obtained using the methods described in the data analysis.

## 4. Data analysis

1. Calculate the embryonic viability percentage of a given biological replicate of a strain using equation (**1**).

   NOTE: The numbers of live progeny and unhatched embryos are obtained by totaling the daily count across the experimental period.

$$\textit{Embryonic viability} = \left[\frac{\#\ \textit{live progeny}}{\#\ \textit{live progeny} + \#\ \textit{unhatched embryo}}\right] \times 100 \qquad (\mathbf{1})$$

2. Calculate the brood size per worm of a given strain by summing the number of progeny (unhatched embryos and larvae) produced by the parent hermaphrodite. Calculate the average brood size using equation (**2**).

$$\textit{Average Brood size} = \frac{\Sigma(\#\ \textit{live progeny} + \#\ \textit{unhatched embryo})}{\#\ \textit{parent hermaphrodites}} \qquad (\mathbf{2})$$

3. Report the embryonic viability percentage and average brood size with the average and standard deviation between biological replicates. Perform Student's *t*-test comparing the control and experimental data.

## Representative Results

We performed embryonic viability and brood sizing assays on N2 (wild type) and on two strains harboring mutations in genes involved in meiosis, *him-5(e1490)* and *spo-11(ok79)*. As both *him-5* and *spo-11* play a role in meiotic crossover formation, mutations in these two genes result in the formation of aneuploid gametes. This embryonic viability assay for N2 yielded a viability percentage of 98.9%, while both *him-5(e1490)* and *spo-11(ok79)* showed a reduction in progeny viability with a percentage of 74.9% and 0.8%, respectively (**Figure 2A**; $p < 0.0005$). These results are consistent with previously published results[7,9]. The average brood size of N2, *him-5(e1490),* and *spo-11(ok79)* were

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determined to be 217, 105, and 219, respectively (**Figure 2B**). Consistent with previous publications, *him-5(e1490)* has a significantly reduced brood size compared to wild type, whereas *spo-11(ok79)* does not[7,9].

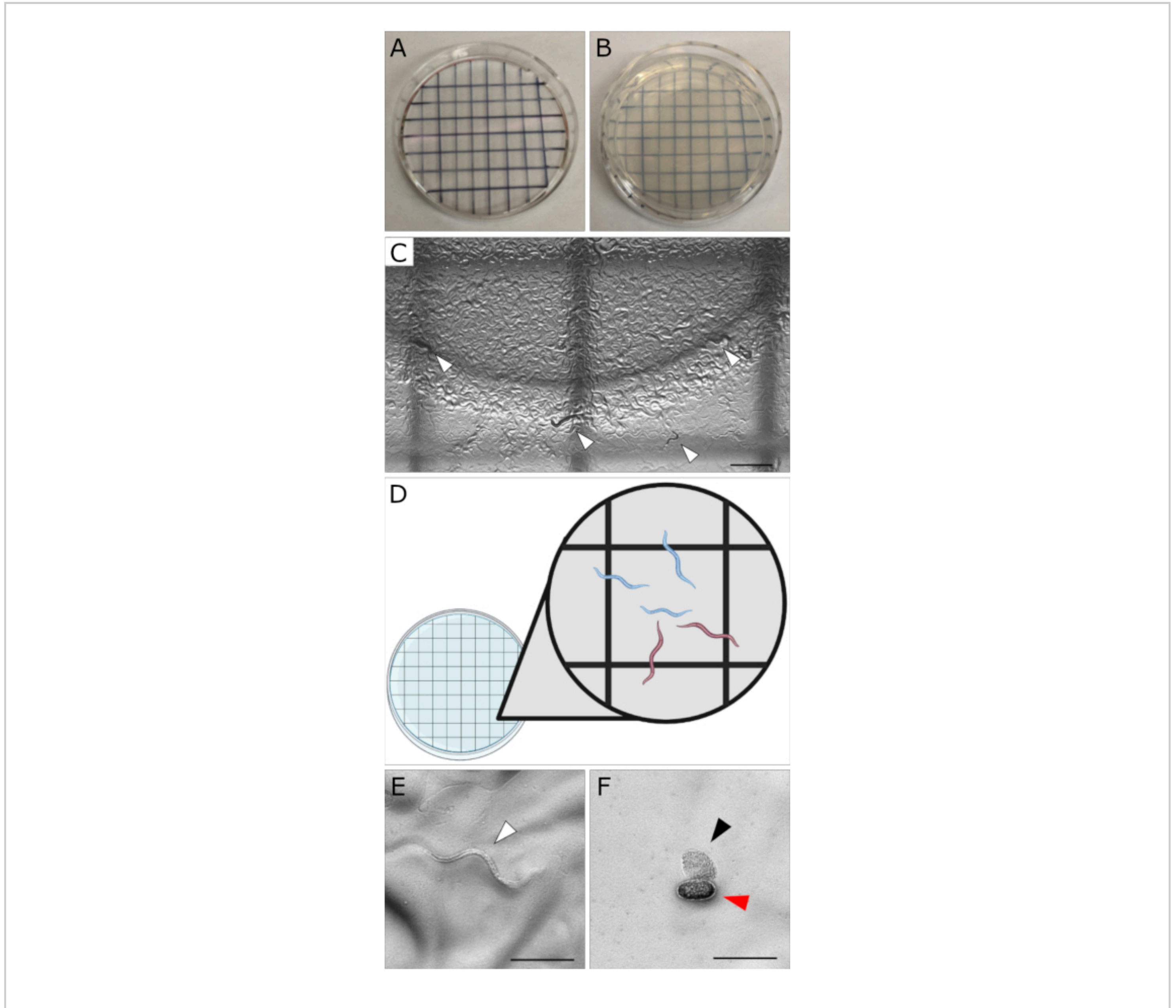


**Figure 1: Demonstration of counting setup and embryo morphology on plates.** (**A**) Image of a lid with a cross-hatched grid pattern drawn using a fine sharpie. (**B**) A 35 mm MYOB plate with the patterned lid underneath for counting. (**C**) Image demonstrating a low magnification (10x) field of view showing multiple boxes of the grid. White arrowheads point out several larvae within this field of view. Counting should be done at a higher magnification (only one square in field) to observe both larvae and embryos. Scale bar = 1,000 µm. (**D**) Cartoon of the lid with the grid pattern placed under a 35 mm plate. The inset denotes the proper technique to determine which larvae are counted in a given square. Count from top to bottom, left to right.

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Count any worms that are entirely within the square. Worms touching the boundary should be counted based on the position of the worm head, not the tail. Count worms facing the current grid or grids that have been previously counted (i.e., top and left edges). Do not count worms with heads touching the bottom or right edges. From the inset, blue worms will be counted while red worms will not. (**E**) Representative image of healthy N2 L1 hatched larvae (white arrowhead). (**F**) Representative image of an unfertilized oocyte (black arrowhead) and an unhatched embryo (red arrowhead). Unfertilized oocytes should not be counted for this assay. Scale bars (**E,F**) = (100 µm). Note: Images in **C**, **E**, and **F** were taken with a Zeiss AxioZoom microscope, with a camera attached for the purpose of demonstrating the appearance of larvae, embryos, and oocytes on worm plates. For embryonic viability assays, counts are done while observing the plates using a stereomicroscope (no camera). Please click here to view a larger version of this figure.

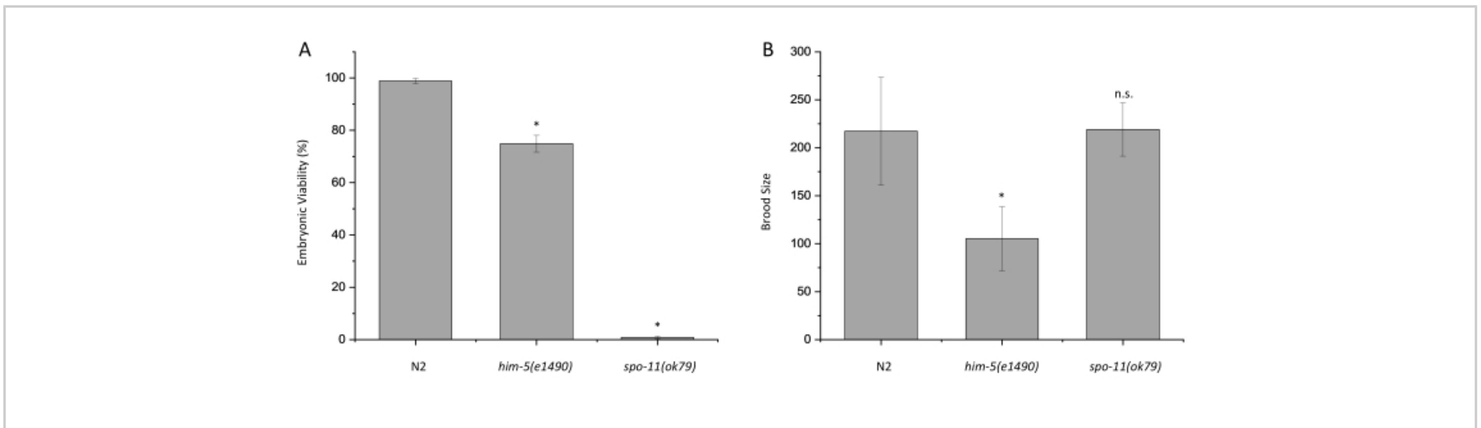


**Figure 2: Representative graphs depicting the results from embryonic viability assays and brood sizes.** (**A**) Embryonic viability percentage of N2, *him-5(e1490),* and *spo-11(ok79)* at 20 °C. (**B**) Brood sizes of N2, *him-5(e1490),* and *spo-11(ok79)* at 20 °C. The progeny of at least 28 hermaphrodites were scored for each strain. The total number of unhatched embryos plus larvae scored were N2 = 6302, *him-5(e1490)* = 2,945, and *spo-11(ok79)* = 7,230. Error bars indicate the standard deviation across three separate individual replicates. Statistics calculated using Student's *t*-test, n.s. = not significant, *$p < 0.0005$. Please click here to view a larger version of this figure.

## Discussion

Propagation of sexual reproducing species requires the formation of haploid gametes (i.e., eggs and sperm) through meiosis, which are then unified at fertilization, restoring the diploid chromosome number and initiating embryonic development. Errors in any of these processes can lead to infertility, embryonic lethality, and/or birth defects. *C. elegans* is a powerful model system to study sexual reproduction. The effects of gene mutations or gene expression knockdown (e.g., RNA interference) can be assessed relatively quickly and easily using the embryonic viability and brood sizing assays described above. We have used these methods for the initial characterization of genes involved in meiotic chromosome segregation and fertilization/egg activation[10,11,12]. An observed reduction in embryonic viability or brood size indicates a disruption in meiosis, gametogenesis, fertilization, or embryogenesis.

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As embryonic viability and brood sizing are assessed relatively easily through counting progeny and a simple mathematical calculation, these are optimal introductory experiments for research novices either in the laboratory or the classroom. The ease of *C. elegans* husbandry and economic advantages make them particularly suited to experimental biology classes. The students gain valuable research experience through *C. elegans* husbandry, learn to use dissecting microscopes, and can ask biological questions in a developmental system that can be answered in a relatively short amount of time (approximately 5 days with the protocol described in this paper).

The timing of progeny counts is very important for embryonic viability assays. At 20 °C, embryogenesis takes approximately 16 h, and reproductively mature adults begin laying eggs approximately 60 h after hatching as L1 larvae. As the life cycle is rapid, it is important to count progeny within the appropriate window, allowing sufficient time for embryos to hatch but before the progeny themselves begin to lay eggs. It is also important to note that growth periods vary depending on temperature. Growth is approximately 2.1 times faster at 24-25 °C than 15-16 °C, and approximately 1.3 times faster at 20 °C than 15-16 °C[13]. In this protocol, we recommend that counts occur 48 h after the adults are placed on a fresh plate. This timeframe ensures that all embryos with wild-type development have sufficient time to hatch (>16 h), but do not age to the point of reproductive capability. Assays performed at temperatures lower than 20 °C may need to be extended (transfer animals for 4 days) for embryos to hatch and for hatched progeny to reach larval stages that are easier to observe among the bacteria on the MYOB plates (L3-L4 stages).

A limitation to embryonic viability assays and brood sizing is that the specific developmental process that is perturbed is not readily apparent. However, these initial assays can be followed up with cytological techniques to determine which process is affected. For example, dissection of the adult worms to release the gonad followed by 4',6-diamidino-2-phenylindole (DAPI) staining and careful analysis of DNA morphology within the germline can reveal whether meiotic processes are disrupted. In addition, DAPI staining of embryos can reveal at which stage embryogenesis arrests.

In conclusion, we have described a protocol for assaying the number of embryos produced (brood) and the percentage of embryos that are viable for various *C. elegans* mutants. This assay can be used for both self-fertilizing hermaphrodites and for male/hermaphrodite crosses. With the short life cycle of *C. elegans,* this protocol can be completed in less than 1 week. Embryonic viability assays and brood sizes can be used as first analyses of genes involved in meiosis, fertilization, or embryonic development, and are appropriate protocols for more advanced researchers and research novices (undergraduate and first-year graduate students) alike.

## Disclosures

The authors have no conflicts of interest to declare.

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