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# Sperm and Oocyte Isolation Methods for Biochemical and Proteomic Analysis

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## Summary

The *Caenorhabditis elegans* gonad is a simple model to investigate molecular mechanisms that regulate fundamental cell and developmental processes. The strength of the model is that *C. elegans* is amenable to genetic manipulation. The complete genome sequence, advances in mass spectrometry, and RNA-mediated interference (RNAi) are now providing a technical infrastructure that complements biochemical and proteomic approaches. This chapter describes simple methods for sperm and oocyte isolation and gonad liberation that can be performed routinely in the lab, without expensive equipment. These methods are ideal for biochemical and proteomic applications, including those aiming to identify proteins based on affinity or biological activity. Germline mRNA expression profiles, RNAi feeding clones, and Gateway®-engineered ORFeome vectors are available to help validate experimental results.


**Key Words:** Oocyte; sperm; biochemistry; proteomics; mass spectrometry; gonad; reproduction; germ line.

## 1. Introduction

*Caenorhabditis elegans*' success as a model organism is primarily owing to the powerful genetic methods with which to study gene function. Biochemical approaches have been impeded by the large amount of starting material required for protein analysis. In the postgenomics era, a collision between two unrelated fields, mass spectrometry and bioinformatics, has changed the rules of protein identification ***(1)***. In organisms with sequenced genomes, as little as 10 ng of protein can be identified from one- and two-dimensional polyacrylamide gels ***(2)***. Computer algorithms make accurate predictions from isolated protein spots using mass spectra and genome sequence data. Already, *C. elegans* researchers have exploited this technology in biochemical purifications and more general proteomic applications ***(3–5)***.

From: *Methods in Molecular Biology, vol. 351:* C. elegans*: Methods and Applications*
Edited by: K. Strange © Humana Press Inc., Totowa, NJ

The gonad has several advantages that facilitate proteomic analyses. Germ cells are by far the most abundant cell type in adults and their proteins can be metabolically labeled for quantitative analysis ***(5)***. Temperature-sensitive mutants are available that allow comparison of protein content in gonads undergoing spermatogenesis or oogenesis to those lacking germ cells. Transgenes encoding epitope or affinity-tagged proteins can be expressed specifically in the hermaphrodite germline ***(6)***, making techniques such as tandem immunoaffinity purification possible. After candidates are identified by mass spectrometry, methods are necessary to validate the results. In this context, genome-wide surveys of genes transcribed during spermatogenesis and oogenesis are available for cross-referencing ***(7,8)***. RNA-mediated interference (RNAi) clone libraries facilitate rapid testing of candidate gene function by the feeding method ***(9)***, which creates effective knockdowns in oocytes, although not sperm. Finally, an ORFeome library engineered for use with the Gateway® recombination system expedites cloning into yeast two-hybrid vectors to test for direct interactions ***(10,11)***.

This chapter describes sperm and oocyte isolation procedures designed for routine practice in the lab. The procedures are biased toward applications requiring less than 2 mL of gametes, such as those aiming to identify proteins from polyacrylamide gels. For biochemical applications that require more material, we provide references for scaling up the procedures. Isolated gametes can be used to purify multiprotein complexes, signaling proteins, or even organelles. Also included is a simple procedure to liberate gonads *en masse* from the tough cuticle, making them readily accessible to extraction buffers.

## 2. Materials

1. *fog-2(q71)*, *fem-1(hc17)*, *glp-4(bn2)*, or *fer-1(hc1)* mutants. These strains can be obtained from the *Caenorhabditis* Genetics Center (http://biosci.umn.edu/CGC/CGChomepage.htm).
2. LB liquid medium (2–6 L): add 10 g Bacto tryptone, 5.0 g Bacto yeast extract, and 5.0 g NaCl to 1.0 L $H_2O$, and autoclave (*see* **Note 1**).
3. Alkaline hypochlorite solution (100 mL): prepare fresh by adding 5.0 mL 5 *N* NaOH to 71 mL $H_2O$ and 24 mL bleach.
4. S-medium (1 L): add 5.9 g NaCl to 933 mL distilled deionized (dd) $H_2O$. Add 50 mL 1 *M* $KH_2PO_4$ (pH 6.0) and autoclave. When cool, add 3.0 mL 1 *M* $CaCl_2$, 3.0 mL 1 *M* $MgSO_4$, 10 mL trace metal solution (0.364 g $FeSO_4{\cdot}7H_2O$, 0.93 g $Na_2EDTA$, 0.098 g $MnCl_2{\cdot}4H_2O$, 0.144 g $ZnSO_4{\cdot}7H_2O$, 0.012 g $CuSO_4{\cdot}5H_2O$ in 500 mL $ddH_2O$), and 1.0 mL cholesterol (5 mg/mL). Store at 4°C and warm to room temperature before use.
5. M9 buffer (1 L): add 3.0 g $KH_2PO_4$, 6.0 g $Na_2HPO_4$, and 5.0 g NaCl to $ddH_2O$, adjust to 999 mL, and autoclave. When cool, add 1.0 mL sterile 1 *M* $MgSO_4$.
6. Egg salts buffer (1 L): add 6.9 g NaCl and 3.58 g KCl to 800 mL $ddH_2O$. Add 5 mL 1 *M* HEPES, pH 7.4, 2.0 mL 1 *M* $MgCl_2$, and 2.0 mL 1 *M* $CaCl_2$. Bring to 1.0 L with $ddH_2O$, adjust pH if necessary, and autoclave.

7. X-large Petri dishes (150 mm diameter × 15 mm height).
8. Nitex nylon nets (10, 20, 35, and 45 µm). Lab Paks® containing 12-in. squares are available at Sefar (www.sefar.us).
9. 5-in. Plastic embroidery hoops.
10. 6-in. Benchtop vise (25,000 psi or higher casting) mounted to a 2- × 10- × 40-in. wood slab or lab bench (*see* **Note 2**).
11. 6–8 in. Square Plexiglas plates (two).
12. 6–8 in. Square 0.5 in. thick hard wood spacers (two).
13. Razor blades.
14. Nutator® single-speed orbital mixer or general platform rocker.
15. 15- and 50-mL polypropylene conical centrifuge tubes.
16. Glass Pasteur pipets with bulbs.
17. Automatic pipetor with 10-mL disposable pipets.
18. Liquid nitrogen and Dewar flask for quick freezing.

## 3. Methods

### *3.1. Sperm Isolation*

This procedure is modified from that of Klass and Hirsh ***(12)***. It relies on nylon nets with precise pore sizes for filtration, and pressure to liberate sperm from males. We use *fog-2(q71)* feminizing mutations to generate populations consisting of an equal ratio of males and females ***(13)***. Males are separated from females using a 35-µm net. Sperm preps are often greater than 90% pure with a single pass of filtration, and most yields are between 100 and 200 µL sperm. Because the isolation steps can be accomplished in 1 d, multiple procedures can be performed in 1 wk if *fog-2(q71)* cultures are staged. As much as 2 mL of sperm can be isolated in 1 mo.

1. Grow *fog-2(q71)* animals on 4 X-large nematode growth medium (NGM) plates, ensuring that the animals are well fed (*see* **Note 1**).
2. When gravid adults are abundant, collect the worms by pipetting 10 mL of M9 buffer onto a plate. Tilt the plate back and forth, then dump worms and buffer onto the next plate. Continue until all worms are floating in a single plate. Transfer worms with a glass Pasteur pipet to a 15-mL conical polypropylene centrifuge tube. To collect the residual worms from the plates, repeat this process with 10 mL fresh M9 buffer. Centrifuge both tubes in a clinical centrifuge for 2 min at 600*g*. Remove M9 buffer and combine worms into a single polypropylene tube. Centrifuge again and remove as much buffer as possible.
3. Add 5 mL freshly made alkaline hypochlorite solution to worms. Cap and vortex for 3–4 min, then centrifuge at 800*g* for 45 s (*see* **Note 3**). Hatched worms are dissolved, but eggs survive. Quickly wash the eggs with 5 mL M9 buffer, repeat four more times, then resuspend them in S-medium. Incubate with rocking for approx 20 h at 25°C. The following day, estimate the number of hatched L1 larva per µL S-medium.
4. Plate L1 larva onto 40–60 X-large NGM plates at a density of 6000–10,000 per plate. Grow for approx 60 h at 25°C until the plates contain males and gravid females (*see* **Note 4**). Add *Escherichia coli* concentrate as necessary (*see* **Note 1**).

5. Float animals off the plates with M9 buffer as in **step 2**. Transfer them to a 50-mL polypropylene tube or several 15-mL tubes. Centrifuge at 600*g* for 2 min. Combine worms to a single 15-mL tube and wash twice with M9 buffer to remove bacteria.
6. Gravid females have a diameter greater than 40 µm and cannot swim through a 35-µm net; males are thinner and can freely pass through. Place a 35-µm net in an embroidery hoop. Next, place the hoop in an X-large Petri dish so that the net faces the bottom. Place two small spacers, such as stacks of glass slides, underneath the perimeter of the hoop so that it is raised approx 0.5 cm off the dish bottom. The hoop will form a barrier around the perimeter of the net. Using a glass pipet, transfer worms to the net. Add M9 buffer to the worms until the buffer level is approx 0.5 cm above the net. Sift animals back and forth every few minutes. After 15–20 min, use a stereo microscope to examine male accumulation in the plate. Wait until most males have passed through the net. Collect the males and spin at 600*g* for 2 min. Remove the supernatant, leaving one additional volume M9 buffer above the volume of males.
7. Carefully applied pressure to males will trigger release of sperm. Place concentrated males on a Plexiglas plate. Make sure that the worms are positioned so they are near the contact site of the vise arms, where the pressure is highest. Sandwich males between the two Plexiglas plates, and then sandwich the Plexiglas plates between the wood spacers. Place the plates in the vise so the worms are directly between the arms, and apply pressure. To determine whether sperm are being liberated, remove the Plexiglas plates from the vise and examine males using a stereo microscope. Sperm appear as small dots that refract light (*see* **Note 2**).
8. When most males have released their sperm, separate the plates. Quickly add M9 buffer to the carcasses to prevent sperm dehydration. Wash off sperm and carcasses into an X-large Petri dish. Use a razor blade if necessary to sweep sperm off the plates. Repeat **steps 7** and **8** until all males have been used.
9. Sperm have a diameter of approx 5 µm and can freely pass through a 10-µm net. Place a 10-µm net in an embroidery hoop and place the hoop in an X-large dish with the net face down, as in **step 6**. Using a Pasteur pipet, transfer sperm and carcasses to the net so there is an even distribution. Slowly add M9 buffer and wash. Once the buffer level reaches 0.5 cm above the net, shake the net back and forth, and up and down (*see* **Note 5**). Sperm will accumulate in the bottom of the dish.
10. Transfer sperm to a 15-mL polypropylene tube(s) and centrifuge at 800*g* for 10 min. Remove all but 1 mL of M9 buffer and the sperm pellet. Resuspend the pellet and take a 2-µL aliquot to check for purity under a stereo or high power microscope. If the purity is not satisfactory, add 10 mL fresh M9 and centrifuge at 600*g* for approx 5 min, until about 95% of sperm are in the pellet. Remove the supernatent, which contains less dense debris, and repeat. Alternatively, perform a second filtration step with the 10-µm net.
11. When sperm purity is satisfactory, spin the sperm again and transfer them to an Eppendorf tube (*see* **Note 6**).

12. Combine the preps and spin sperm in a microcentrifuge at high speed. Remove all M9 buffer from the sperm pellet, and freeze it quickly in liquid nitrogen. Store at –80°C.

### 3.2. Oocyte Isolation

In addition to sperm, oocytes can be isolated using nylon nets ***(14,15)***. One of two temperature-sensitive mutant strains is used as starting material. *fer-1(hc1)* hermaphrodites produce defective sperm at 25°C ***(16)***. The mutant sperm still stimulate oocyte meiotic maturation and ovulation, so large numbers of unfertilized oocytes can be isolated. However, most of these oocytes are endomitotic and exhibit features typical of necrotic cells. *fem-1(hc17)* hermaphrodites produce no sperm whatsoever at 25°C ***(17)***, and many isolated oocytes have not been ovulated. The disadvantage with this strain is that significantly fewer oocytes are isolated. In most cases, the yield using *fer-1(hc1)* is between 400 and 800 µL of oocytes.

1. Grow *fer-1(hc1)* or *fem-1(hc17)* animals on four X-large NGM plates at 15°C, ensuring that animals are well fed (*see* **Note 1**). When the plate is full of gravid adults, perform alkaline hypochlorite treatment to generate a synchronized L1 stage population, as in **Subheading 3.1.**, **steps 1–3**. Remember to incubate eggs overnight at the restrictive temperature, 25°C (*see* **Note 3**).
2. Plate L1 larva onto 40–60 X-large NGM plates at a density of 6000–10,000 per plate. Grow at 25°C until adult worms are full of oocytes, about 65–75 h after plating. Add *E. coli* concentrate as necessary (*see* **Note 1**).
3. Collect worms in egg salts buffer by floating them off the plates, as in **Subheading 3.1.**, **step 2**. Transfer them to a 50-mL polypropylene tube or several 15-mL tubes. Centrifuge at 600*g* for 2 min to pellet the worms. Combine tubes, if necessary, and wash twice with egg salts buffer to remove bacteria (*see* **Note 7**).
4. Transfer animals to a 60- × 15-mm Petri dish. Fill the dish so it is about half full with densely packed worms. When females are cut in egg salts buffer, their gonads tend to extrude into the surrounding medium. Continued cutting releases oocytes from the reproductive tract. With a clean razor blade, cut worms for 5 min using rapid up and down motion. Check under a stereo microscope for extruded gonads and free oocytes. Continue until nearly all worms have been cut. Massive numbers of oocytes should be observed in the dish (*see* **Note 8**).
5. Most oocytes are between 25 and 40 µm in diameter, and will pass freely through a 45-µm net. Place an embroidery hoop containing a 45-µm net in an X-large Petri dish, so that the net faces the bottom of the dish, as in **Subheading 3.1.**, **step 6**. Using a glass pipet, transfer the oocytes and carcasses to the net so they are evenly distributed. Add egg salts buffer to the top of the net and wash. Once the buffer level reaches 0.5 cm above the net, sift the carcasses back and forth, and up and down. Check using a stereo microscope to determine whether oocytes are passing through the net.

6. Transfer the oocytes from the bottom of the dish to two 15-mL polypropylene tubes and spin at 600*g* for 4 min. Remove all but 4-mL buffer from each tube and resuspend the oocytes. Transfer the suspension to a new X-large Petri dish containing a 20-µm net, and wash as in the previous step. Smaller contaminating particles will pass through the net, but oocytes will not. Collect oocytes into a new Petri dish by washing the net with egg salts buffer. Transfer oocytes to two 15-mL disposable conical tubes, and spin at 600*g* for 5 min to pellet the oocytes. Remove the buffer leaving one additional volume of buffer above the volume of oocytes.
7. Gently resuspend the oocytes, take a 2-µL aliquot, and examine the purity using a stereo or high power microscope. Generally, a single pass of filtration yields approx 60–80% pure oocytes. Excess debris can be removed from oocytes by low speed centrifugation or repeated filtration. If more worms remain from **step 3**, repeat the procedure. Combine the preps, and spin to pellet oocytes. Remove the supernatant and freeze oocytes quickly in liquid nitrogen. Store at –80°C.

### 3.3. Gonad Liberation

For some applications, isolated gametes are not required. Complete extraction from whole worms is relatively straightforward, but it results in a complex mixture of total protein. Liberating gonads into the surrounding medium makes them readily accessible to extraction buffers, including those that are likely to maintain protein complexes. With the exception of intestinal and gonadal cells, other cell types tend to remain in the body column, where the tough cuticle helps protect them from extraction. Temperature-sensitive mutant strains can be used to evaluate germ line specificity. For example, to test whether a given protein is expressed in the germ line, *fem-1(hc17)* mutants, whose germ line is feminized ***(17)***, are compared with *glp-4(bn2)* mutants, which lack germ cells ***(18)***. To test whether a protein is expressed in germ cells committed to the oocyte fate, *fem-1(hc17)* mutants are compared with *fem-3(q20)* mutants, whose germ line is masculinized ***(19)***. These strains have the additional advantage that they do not produce progeny at the restrictive temperature, leaving a homogeneous population of adults for extraction.

1. Grow *fem-1(hc17), fem-3(q20)*, or *glp-4(bn2)* mutants on four X-large NGM plates at 15°C, ensuring that animals are well fed (*see* **Note 1**). When gravid hermaphrodites are abundant, perform alkaline hypochlorite treatment, as described in **Subheading 3.1.**, **steps 1–3**. Incubate eggs overnight at 25°C (*see* **Note 3**).
2. Plate L1 larva onto 40–60 X-large NGM plates at a density of 6000–10,000 per plate. Grow at 25°C for approx 60 h, adding concentrated *E. coli* as necessary (*see* **Note 1**).
3. Collect worms as described in **Subheading 3.1.**, **step 2**, except using egg buffer instead of M9 buffer. Transfer them to a single 50-mL polypropylene tube or several 15-mL polypropylene tubes, and centrifuge at 600*g* for 2 min. Transfer worms to a single 15-mL tube, and wash twice with egg salts buffer to remove

bacteria. Using the procedure described in **Subheading 3.2.**, **step 4**, liberate gonads with a razor blade, and monitor progress using a stereo microscope. Continue until nearly all worms have been dissected and gonads are exposed in the medium. Using a glass pipet, transfer the gonads and carcasses to a 15-mL polypropylene tube, and centrifuge in a clinical centrifuge at 800*g* for 5 min. Remove the supernatant, and freeze the worms quickly in liquid nitrogen. Store at –80°C.

## 4. Notes

1. Food is required for gamete proliferation and growth. If worms consume all bacteria on the plates, additional food must be added. Grow 2.0 L OP50 or NA22 bacteria in LB medium overnight at 37°C until the culture is dense. Centrifuge for 20 min at 1500*g* to pellet the bacteria. Pour off the supernatant and resuspend bacteria in 40 mL M9 buffer. Store the *E. coli* concentrate at 4°C. Add food to worms as needed, and allow plates to dry. As an alternative, use chicken eggs to grow a rich food source ***(12,15)***.
2. We mounted a 42-lb vise onto a 2- × 10- × 40-in. piece of lumber, creating a portable worm smasher that can be placed next to a stereo microscope. Alternatively, the vise can be mounted to a lab bench. Worms should be oriented on the Plexiglas plate so they receive maximum pressure. A good tug on the vise handle is required to trigger sperm liberation (these guys are tough). Higher worm densities require greater pressure. The wood spacers help protect the Plexiglas plates from being damaged, but they are not necessary. More pressure can be generated without them. For preps containing more than a million males, a laboratory press is recommended ***(12)***.
3. To ensure that eggs remain viable, but carcasses are dissolved, vortex worms in alkaline hypochlorite solution for 3 min, then spin in a clinical centrifuge. Worm carcasses are dark brown, whereas eggs are much lighter. If greater than 20% of the pellet is dark brown, remove the old alkaline hypochlorite solution and add 5 mL fresh solution. Vortex for 30 s to 2 min, depending on the fraction of carcasses that remain, then centrifuge again and wash the eggs with M9 buffer. If the alkaline hypochlorite treatment is too long or the eggs are not washed thoroughly, many will not hatch.
4. When growing worms on X-large NGM plates, avoid overcrowded conditions. Crowding can trigger dauer formation and loss of synchrony in growth. Good separation of males from females requires that the population consist almost entirely of adults. The optimal time for separation is when nearly all females are gravid, yet few progeny have hatched on the plates. Larvae can be problematic because they pass through 35-µm nets with the males. If your culture has too many larvae, they can be separated from adults using a 20-µm net.
5. Worm carcasses get trapped in the net and prevent sperm and oocyte flow. If the net gets too crowded, switch to a clean net to achieve maximum yields.
6. Sperm isolated from males have not been activated, and are not motile. Activation can be stimulated in vitro using Pronase or triethanolamine in sperm medium ***(20)***.

7. If small larvae are found in the *fer-1(hc1)* cultures, separate them from adults using a 20-µm net. *See* **Subheading 3.1., step 6** for the separation technique.
8. Cutting worms with a razor blade releases oocytes from the proximal gonad and uterus. Alternative methods using chemicals or sonication release oocytes from the uterus ***(14,15)***. Although these methods have the advantage that less contaminating debris is generated, the liberated oocytes are endomitotic and exhibit features typical of necrotic cells. Others have used a small blender to release oocytes.

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