

 Protocol

Preparation and Immunolabeling of *Caenorhabditis elegans*

Sarah Crittenden and Judith Kimble

Adapted from *Basic Methods in Microscopy* (eds. Spector et al.). CSHL Press, Cold Spring Harbor, NY, USA, 2006.

INTRODUCTION

The use of antibodies to visualize the distribution and subcellular localization of gene products powerfully complements genetic and molecular analysis of gene function in *Caenorhabditis elegans*. The challenge to immunolabeling *C. elegans* is finding the fixation and permeabilization methods that effectively make antigens accessible without destroying the tissue morphology or the antigen. Embryos are surrounded by a chitinous eggshell and larvae and adults are surrounded by a collagenous cuticle, each of which must be permeabilized to allow penetration of antibodies. In addition, antigens and antibodies are sensitive to different fixing and permeabilizing conditions. This protocol describes two methods for tissue fixation. The whole-mount freeze-cracking method is a good starting point as it is easy and works well with most antibodies and with embryos, larvae, and adults. In the tissue extrusion method, gonads and intestines, which are extruded from the carcass, are well fixed and permeabilized. Tissues remaining in the carcass are not usually stained well. The protocol concludes with an antibody incubation procedure in which fixed worms are incubated overnight with primary antibody, subsequently exposed to secondary antibody, and mounted for viewing.

RELATED INFORMATION

The whole-mount freeze-cracking method is based on [Strome and Wood \(1982\)](#), [Albertson \(1984\)](#), and [Bowerman et al. \(1993\)](#). The tissue extrusion method is based on [Crittenden et al. \(1994\)](#). [Figure 1](#) shows gonads fixed using the tissue extrusion method. An alternative protocol for antibody-based detection can be found in [Antibody Addition and Detection for Staining *Caenorhabditis elegans*](#) ([Harlow and Lane 2006a](#)). A protocol that describes [Media for Mounting Fixed Cells on Microscope Slides](#) ([Fischer et al. 2008](#)) is also available.

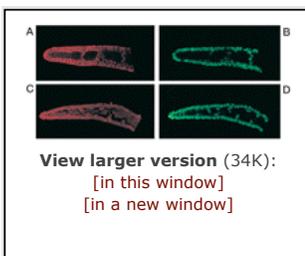


Figure 1 . Gonads from wild-type *C. elegans* hermaphrodites were fixed according to the tissue extrusion method. Primary antibodies were rat anti-GLP-1 ([Crittenden et al. 1994](#)) and mouse anti-DNA (Chemicon, MAB030). Secondary antibodies were Cy3-conjugated donkey anti-rat and FITC-conjugated donkey anti-mouse (Jackson ImmunoResearch). Images were obtained on a Bio-Rad MRC 1024 laser scanning confocal microscope. (A,B) Well-fixed gonad. The transmembrane receptor, GLP-1, is membrane-associated in a crisp honeycomb pattern. DNA staining is crisp, and discrete structures within the nucleus are visible. (C,D) Poorly fixed gonad. GLP-1 is punctate and diffuse. In addition, there is a faint red haze in the nuclei. DNA staining is bright, but nuclear morphology is poor; the nuclei are fuzzy, and discrete structures are not discernible. The poor morphology was due to a contaminant in the primary antibody solution. (Photo provided by S. Crittenden, University of Wisconsin.)

MATERIALS

Reagents

 Acetone (for whole-mount freeze-cracking method)

 Precool on dry ice (~10 min) in a Coplin jar.

Antibodies, primary and secondary

For the secondary antibody, we use Jackson ImmunoResearch's purified IgG coupled to Cy3, Cy5, FITC (fluorescein isothiocyanate), or rhodamine.

C. elegans of desired stage (more than 10 adults or more than 40 larvae for whole-mount freeze-cracking method; 5-10 adult hermaphrodites for tissue extrusion method)

 DAPI (4',6-diamidino-2-phenylindole dihydrochloride) (optional for antibody incubation procedure; see Step 20)

 DMF (*N,N*-dimethylformamide) (optional for whole-mount freeze-cracking method; see Step 7)

 Precool on dry ice (~10 min) in a Coplin jar.

 Formaldehyde (5%)

Dilute to 1% for use in Step 12.

 Levamisole (0.25 mM) (for tissue extrusion method)

 M9 buffer for *C. elegans* (for fixation procedures)

 Methanol (for whole-mount freeze-cracking method)

 Precool on dry ice (~10 min) in a Coplin jar.

Mounting medium (for antibody incubation procedure; see [Media for Mounting Fixed Cells on Microscope Slides](#) [[Fischer et al. 2008](#)])

Rehydration series (for whole-mount freeze-cracking method), choices as follows:

 Methanol (90%, 70%, 50%, followed by 1X TBS) ([Lin et al. 1995](#))

Ethanol (2 min each in 95%, 70%, 50%, and 30%, followed by 1X TBS) ([Miller and Shakes 1995](#))

 Acetone series (*Goldstein and Hird 1996*)

 Subbing solution, freshly prepared

 TBSB (Tris-buffered saline [TBS] containing 0.5% BSA)

  TBSBTx (TBSB with 0.1% Triton X-100) (for tissue extrusion method)

 Worm acetone powder (adapted from **Preparing Acetone Powders** [Harlow and Lane 2006b]; optional for antibody incubation procedure; see Step 18)

Equipment

Chamber, humidified

Make a humidified chamber from a plastic Petri dish (or other container) with a wet paper towel taped to the lid.

Coplin jars

Coverslips, 18- x 18-mm #1

Laboratory tissues (e.g., Kimwipes)

 Metal plates precooled on dry ice (~10 min) (for whole-mount freeze-cracking method)

We use plates 1/4 in. thick. Use the plates to make a cold, flat surface for the animals to sit on.

Micropipettor with tips

Microscope slides

Nail polish (for antibody incubation procedure)

Razor blade (for whole-mount freeze-cracking method)

Syringe needle, 25-gauge (for fixation procedures)

METHOD

Fixation of Animals and Tissue

For whole-mount freeze-cracking method, follow Steps 1-9 and then proceed to Step 16 for antibody incubation. For tissue extrusion method, follow Steps 10-15 and then proceed to Step 16 for antibody incubation.

Whole-Mount Freeze-Cracking Method

1. Prepare subbed slides by soaking clean slides in subbing solution in a Coplin jar for 5 min to 1 h. Air-dry the slides and store at room temperature.

Subbed slides can be used for weeks. Several batches of slides can be subbed in the same subbing solution. When slides become less sticky, it is time to make a new solution.

2. Place *C. elegans* of desired stage into 6 μ L of M9 buffer on a subbed slide. Cut the animals open with a 25-gauge syringe needle if early embryos or extruded germ lines or intestines are to be stained.

Use more than 10 adults or more than 40 larvae; some animals or tissues will be lost from the slide during the staining procedure.

3. (Optional) Add 2 μ L of 5% formaldehyde.

Concentrations between 1% and 5% of formaldehyde are commonly used (e.g., Bowerman et al. 1993; Evans et al. 1994; Lin et al. 1995); adjust concentration for ideal staining.

Formaldehyde fixation improves morphology. However, it can be omitted if it interferes with antibody binding.

4. Set an 18- x 18-mm #1 coverslip carefully on top of the animals. Use a needle to apply gentle pressure several times over each animal or region of the slide. The animals will flatten; usually a few burst. This procedure aids in opening the eggshell or cuticle. Alternatively, use a tissue to wick excess liquid from under the coverslip until the worms flatten.

5. If formaldehyde was added, let the sample incubate 30 sec to 30 min at room temperature in a humidified chamber (e.g., Bowerman et al. 1993; Evans et al. 1994; Lin et al. 1995); adjust time for ideal staining.

6. Put the slide on a metal plate on top of dry ice for at least 10 min.

7. Pop the coverslip off with a razor blade and immerse the slide immediately in a Coplin jar containing 100% cold methanol for 5 min, followed by 100% cold acetone for 5 min.

For some antigens, it is better to omit the acetone incubation.

For some antigens, incubation in cold DMF works better than methanol or acetone (Lin et al. 1998).

8. Air-dry the slide for 5 min. This enhances the adhesion of the animals to the slide. Rehydrate the sample through a rehydration series of increasingly aqueous solutions (see Materials).

9. Gently drop 200 μ L of TBSB onto the animals and incubate at room temperature for 30 min.

Tissue Extrusion Method

10. Put five to 10 adult hermaphrodites into 5 μ L of 0.25 mM levamisole on a subbed slide (prepared as in Step 1).
11. Using a 25-gauge syringe needle, cut off the heads or tails of the animals, allowing the gonads and intestines to extrude from the animals. *Levamisole causes the animals to contract, which results in their germ lines and intestines being extruded efficiently.*
12. Gently drop 100 μ L of 1% formaldehyde onto the cut animals. Incubate at room temperature for 10 min in a humidified chamber.
13. Remove the formaldehyde and add 50 μ L of TBSBTx at room temperature for 5 min.
14. Remove the TBSBTx and wash two times with 200 μ L of TBSB.
15. Incubate samples in 200 μ L of TBSB at room temperature for \sim 30 min.

Antibody Incubation Procedure

16. Incubate fixed worms with primary antibodies overnight at 4°C or for several hours at room temperature in a humidified chamber. Use 30-50 μ L of antibody solution per slide.
17. Wash by gently covering the worms with 200 μ L of TBSB. Wash three times for 15 min each at room temperature. Alternatively, immerse the slides in a Coplin jar if the worms are well attached to the slide.
Worms generally do not stick well to slides, so start with plenty of animals and be gentle when doing washes.
18. (Optional) To reduce nonspecific background, treat secondary antibody with worm acetone powder as follows before use in Step 19:
 - i. Add \sim 1 mg of worm acetone powder/200 μ L of secondary antibody solution.
 - ii. Incubate the secondary antibody/worm acetone powder mix at 4°C for 15 min to 1 h.
 - iii. Centrifuge at 10,000 rpm for 5 min in a microcentrifuge to pellet the worm acetone powder. Use the supernatant as the secondary antibody solution.
19. Dilute secondary antibodies to the recommended concentration in TBSB. Use 100 μ L per slide and incubate at room temperature for 1-2 h.
20. Wash worms as in Step 17. If desired, add the DNA stain, DAPI, to 0.5 μ g/mL in the final wash.
21. After removing the last wash, wick off excess moisture from the slide by touching the edges with a tissue. Add 8 μ L of mounting medium (see **Media for Mounting Fixed Cells on Microscope Slides** [Fischer et al. 2008]), put an 18- x 18-mm #1 coverslip over the worms, and seal with nail polish.

TROUBLESHOOTING

Problem: There is high background staining.

[Step 21]

Solution: Consider the following:

1. Determine whether high background is due to the primary or secondary antibody. Try diluting the antibodies further, affinity-purify the primary antibody, and preabsorb the primary antibody with worm or bacterial acetone powder or with fixed worms. Use null mutants so that the specific antibody will not be depleted.
2. Background problems can also be due to intestine autofluorescence on the DAPI and fluorescein channels, causing a dim nuclear stain.

Problem: The nuclei and cytoplasm are hazy.

[Step 21]

Solution: Do not let the worms dry after they have been fixed and rehydrated; this tends to give a nonspecific haze to the nuclei and cytoplasm.

Problem: Morphology is poor.

[Step 21]

Solution: Consider the following:

1. Try to fix the worms more quickly.
2. Contaminated solutions can cause poor morphology. DAPI-stained DNA should look well defined and crisp; if it does not, be suspicious (e.g., Fig. 1 C,D)

Problem: Staining is poor.

[Step 21]

Solution: Use different fixatives or make small changes in the concentration of fixative or time of fixation. These changes can make a big difference in the quality of staining.

DISCUSSION

C. elegans larvae and adults can either be picked from plates or washed off with M9 buffer. Young embryos (one to 50 cells) are easily obtained by cutting open gravid hermaphrodites. Older embryos can be obtained by adding M9 buffer to a plate, washing off the adults and larvae, then scraping the remaining embryos off with a Pasteur pipette into additional M9 buffer. The worms or embryos are then pelleted by centrifuging for 1-2 min at 1000 rpm in a microcentrifuge. To remove *Escherichia coli*, more M9 buffer is added, and the worms are pelleted again. Then, 6-8 μ L of concentrated worms can be dropped onto a slide. When staining larvae, it helps to stage the animals so that they are similar in size. This way, the amount of pressure can be adjusted for the size of the worms being fixed. For example, if there are lots of large adults (or larvae), it is difficult to permeabilize L1s without completely squashing the adults. It is increasingly more difficult to effectively permeabilize the worms as they get older. For tissues that can be extruded from the cuticle, such as germ lines and intestines, the morphology is generally better using a non-freeze-crack method such as the tissue extrusion method.

Another commonly used procedure for whole mounts is the reduction/oxidation method of [Finney and Ruvkun \(1990\)](#), which is described in detail in [Miller and Shakes \(1995\)](#).

The tissue extrusion method works well for at least some membrane proteins ([Crittenden et al. 1994](#); [Henderson et al. 1994](#)), but not for the cytoskeletal proteins actin and tubulin. For cytoskeleton, try fixing first in 100% methanol at room temperature for 5 min followed by 1% formaldehyde at room temperature for 25 min ([Crittenden et al. 1994](#)). Other protocols have been used for fixing extruded tissues ([Strome 1986](#); [Francis et al. 1995](#)) and/or the cytoskeleton ([Strome 1986](#); [Waddle et al. 1994](#); [Francis et al. 1995](#)).

The following controls should be considered when using the antibody incubation method described here:

- Use an antibody that is known to work to test for morphological preservation, permeability, and fixation. Some useful control antibodies are anti-DNA monoclonal mAb 030 (Millipore MAB030), anti-actin clone C4 (Millipore) ([Strome 1986](#); [Evans et al. 1994](#)), and anti- β -tubulin (e.g., Promega) ([Crittenden et al. 1994](#); [Evans et al. 1994](#); [Waddle et al. 1994](#)).
- Stain a null mutant and look for loss of staining.
- Compete for staining with proteins that contain the antigen used to raise the antibodies.
- If a null mutant is not available, it is possible to abolish the antigen in embryos from animals that have been injected with antisense RNA ([Guo and Kemphues 1995](#)).

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Caution

Acetone

Acetone causes eye and skin irritation and is irritating to mucous membranes and upper respiratory tract. Do not breathe the vapors. It is also extremely flammable. Wear appropriate gloves and safety glasses.



Caution

DAPI (4',6-Diamidino-2'-phenylindole dihydrochloride)

DAPI (4',6-diamidino-2'-phenylindole dihydrochloride) is a possible carcinogen. It may be harmful by inhalation, ingestion, or skin absorption. It may also cause irritation. Avoid breathing the dust and vapors. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

Caution

DMF (*N,N*-Dimethylformamide, dimethylformamide, $\text{HCON}[\text{CH}_3]_2$)

DMF (*N,N*-dimethylformamide, dimethylformamide, $\text{HCON}[\text{CH}_3]_2$) is a possible carcinogen and is irritating to the eyes, skin, and mucous membranes. It can exert its toxic effects through inhalation, ingestion, or skin absorption. Chronic inhalation can cause liver and kidney damage. Wear appropriate gloves and safety glasses. Use in a chemical fume hood.

Caution

Dry ice (Carbon dioxide; CO_2)

CO_2 (carbon dioxide; dry ice) in all forms may be fatal by inhalation, ingestion, or skin absorption. In high concentrations, it can paralyze the respiratory center and cause suffocation. Use only in well-ventilated areas. In the form of dry ice, contact with carbon dioxide can also cause frostbite. Do not place large quantities of dry ice in enclosed areas such as cold rooms. Wear appropriate gloves and safety goggles.

Caution

Methanol (MeOH , H_3COH)

Methanol (MeOH , H_3COH) is poisonous and can cause blindness. It may be harmful by inhalation, ingestion, or skin absorption. Adequate ventilation is necessary to limit exposure to vapors. Avoid inhaling these vapors. Wear appropriate gloves and goggles. Use only in a chemical fume hood.

Caution

Triton X-100

Triton X-100 causes severe eye irritation and burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles.

Information Panel

Media for Mounting Fixed Cells on Microscope Slides

Andrew H. Fischer, Kenneth A. Jacobson, Jack Rose, and Rolf Zeller

Adapted from "Preparation of Cells and Tissues for Fluorescence Microscopy," Chapter 4, in *Basic Methods in Microscopy* (eds. Spector and Goldman). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2006.

INTRODUCTION

After a specimen is labeled, coverslips containing cells or tissues are mounted onto microscope slides, or slides containing sections are overlaid with a coverslip. A number of recipes for commonly used mounting media are presented in this article, each with particular recommendations.

BACKGROUND

Fading and/or bleaching of a labeled specimen can be a major problem while making observations using fluorescence microscopy. Anti-fade reagents in the mounting medium can decrease the rate of fading, allowing longer observation times. Many factors influence the fluorescence intensity and bleaching of fluorophores, including the intensity of the excitatory light, the pH, the embedding medium, and the presence of other substances that may quench fluorescence. In general, a mounting medium is made up in a glycerol base with a buffer and anti-fade reagent. For long-term preservation of specimens, a polyvinyl alcohol mounting medium is better than a glycerol-based medium. Generally, fluorescently labeled slides store well in the cold; slides mounted with paraphenylenediamine and kept at -70°C in the dark look as good as new even after 6 mo of storage.

The rate of fading of fluorescent signals can be retarded by the addition, to mounting media, of certain free-radical scavengers, such as paraphenylenediamine (Johnson and Nogueira Araujo 1981) or *n*-propyl gallate (Giloh and Sedat 1982). A mounting medium with a pH at or above 8.0-8.5 is reported to increase the initial intensity of fluorescein isothiocyanate (FITC) fluorescence and to reduce its fading.

MOUNTING MEDIA FOR FIXED CELLS

Paraphenylenediamine Mounting Medium

This recipe was modified from Johnson and Nogueira Araujo (1981). Purity of the compounds is crucial to producing an effective mounting medium.

MATERIALS

Reagents

 Carbonate-bicarbonate buffer (5X)

Glycerol (Polysciences)

  *p*-phenylenediamine solution (20 mg/mL)

Equipment

Aluminum foil

pH paper

Pipette (10-mL graduated serological)

Scintillation vial (20-mL glass)

Stir plate and stir bar

METHOD

1. Using a 10-mL graduated serological pipette, place 8.5 mL of glycerol in a 20-mL glass scintillation vial containing a stir bar.
2. Wrap the vial in aluminum foil and add 500 μ L of *p*-phenylenediamine (20 mg/mL)
p-phenylenediamine is UV-sensitive; wrapping the vial in aluminum foil extends the shelf life of the medium.
3. Stir the solution until all of the *p*-phenylenediamine has dissolved. The final color of the medium should be dark yellow to light orange. (Stirring at room temperature for 1 h should be sufficient.)
4. Add carbonate-bicarbonate buffer to the medium to reach a final pH of 8.0. Check pH with pH paper.
A pH of 7 will result in fading of fluorescence.
5. Store, wrapped in aluminum foil, in a -20°C freezer.
Triton X-100 quenches the oxidizing anti-fade agents, so do not use it in final washes before mounting.

p-phenylenediamine oxidizes readily in air when exposed to light, yielding a fluorescent product that binds to nuclei. If the anti-fade solution seems to be increasing the background or staining nuclei (particularly noticeable with fluorescein filter sets), use purer *p*-phenylenediamine. The oxidation products are dark brown, whereas pure *p*-phenylenediamine is nearly white. *p*-phenylenediamine is only slightly soluble in cold H_2O , whereas the oxidation products are readily soluble. Washing the *p*-phenylenediamine powder with about five times its weight of cold H_2O will remove most of the fluorescent material.

Glycerol Anti-fade Mounting Medium

This recipe was adapted from [Shuman et al. \(1989\)](#).

MATERIALS

Reagents

 1,4-diazobicyclo-[2,2,2]-octane (DABCO)

Glycerol

 NaOH (pellets)

 *p*-phenylenediamine (Sigma-Aldrich)

 *n*-propyl gallate

Equipment

Aluminum foil

Stir plate and stir bar

METHOD

1. Dissolve in 100 mL of glycerol:
 - 5 g *n*-propyl gallate
 - 0.25 g DABCO
 - 0.25 g *p*-phenylenediamine
2. Bring the pH above neutral by adding several pellets of NaOH.
3. Stir thoroughly (>1 d).
4. Store in aliquots at -20°C wrapped in aluminum foil.

When using this medium, note the following:

- The effectiveness of glycerol-based antifade solution is greatly diminished by small amounts of H_2O . To minimize residual H_2O in the sample, it is best to first drain off all the buffer before mounting, cover the specimen with this solution, let it sit for 15 min or so, then drain it all off and mount in fresh anti-fade solution.

- Small amounts of residual Triton X-100 (used for permeabilization) and perhaps other detergents convert this anti-fade solution into a very powerful quenching agent. If the fluorescence disappears when you apply this solution, remove the coverslip from the slide by flooding with buffer and place it cell side up in a Petri dish. Rinse the sample well with excess buffer to remove the detergent, then remount the coverslip with fresh mounting medium.
- Some lectins that bind to surface carbohydrates tend to dissociate in 100% glycerol. In that case, the anti-fade agents can be dissolved in polyvinylpyrrolidone.
- See the note about *p*-phenylenediamine at the end of the Paraphenylenediamine Mounting Medium recipe.

***n*-Propyl Gallate Anti-fade Medium**

This recipe was adapted from [Giloh and Sedat \(1982\)](#).

1. Prepare 2% (w/v) *n*-propyl gallate in glycerol.
2. Adjust the pH to 8.0.

Polyvinyl Alcohol Mounting Medium

Semipermanent mounts can be made using Celvol mounting medium. This medium is not autofluorescent, is viscous, and hardens slowly. Preparations mounted in this way can be stored in the dark at either 4°C or -20°C with little loss of image quality. The original recipe was prepared with Gelvatol (Monsanto), which is no longer available. Celvol 205 (Celanese Chemicals) has been substituted for the Gelvatol. Prepare the medium as follows:

1. Prepare the Celvol solution by dissolving 0.35 g of Celvol 205 in 3 mL of H₂O (or 1X PBS) and 1.5 mL of glycerol.
2. Heat the solution with stirring in a boiling water bath until the Celvol is completely dissolved, and add antifade agents (see above media) as desired.
The mounting medium can be stored at 4°C for long periods (months) with no loss of properties; contamination with mold usually determines the usable shelf life.

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Recipe

Formaldehyde (5%)

-  NaOH (4 N)
-  Paraformaldehyde
-  Phosphate-buffered saline (PBS) (10X)

METHOD

1. Add 0.25 g of paraformaldehyde to 4.2 mL of H₂O.
2. Add 2 μL of 4 N NaOH.
3. Heat at 65°C until dissolved.
4. Add 0.5 mL of 10X PBS; bring volume to 5 mL with H₂O.
5. Filter and store at 4°C for no more than 1 wk.

For *C. elegans* fixation, concentrations of between 1% and 5% formaldehyde are commonly used; adjust concentration for ideal staining.

Recipe

Levamisole (0.25 mM)

-  Levamisole (Sigma)
-  M9 buffer for *C. elegans*

To make 5 mL of 0.25 M levamisole, dissolve 0.3 g of levamisole in 5 mL of M9 buffer. Aliquot and store at -20°C.

For 0.25 mM levamisole, add 1 μL of 0.25 M levamisole to 1 mL of M9 buffer. Store at room temperature.

Recipe

M9 buffer for *C. elegans*

3.0 g KH_2PO_4

6.0 g Na_2HPO_4

0.5 g NaCl

 1.0 g NH_4Cl

Bring to 1 L with H_2O .

Recipe

Subbing solution

Chrome alum

Gelatin

Poly-L-lysine (m.w. >300,000; Sigma)

 Sodium azide

METHOD

1. Bring 200 mL of H_2O to 60°C.
 2. Add 0.4 g of gelatin and cool to 40°C.
 3. Add 0.04 g of chrome alum.
 4. Add sodium azide to 1 mM.
 5. Add poly-L-lysine to 1 mg/mL.
 6. Put the subbing solution in a Coplin jar and store it at 4°C.
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Recipe

TBSB

1 mL of 5% BSA (bovine serum albumin)

9 mL of 1X TBS (Tris-buffered saline)

This recipe makes enough solution for one or two staining experiments.

Recipe

Worm acetone powder

MATERIALS

Reagents

 Acetone, prechilled to -20°C

C. elegans

 M9 buffer for *C. elegans*

Equipment

Centrifuge

Dounce homogenizer

Filter paper

Ice

Mortar and pestle

Microcentrifuge tube

METHOD

1. Homogenize worms in Dounce homogenizer; use ~1 g of worms/mL of M9 buffer.
2. Set on ice for 5 min.
3. Add 4 mL of -20°C acetone/mL of worm suspension. Mix vigorously.
4. Set on ice for 30 min with occasional vigorous mixing.
5. Centrifuge at 10,000g for 10 min.
6. Resuspend pellet with fresh -20°C acetone.
7. Mix vigorously on ice for 10 min.
8. Centrifuge at 10,000g for 10 min.
9. Spread pellet on clean filter paper and allow to dry at room temperature.
10. When dry, break up chunks in a mortar and pestle.
11. Transfer powder to a microcentrifuge tube and store at 4°C.

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