Standard Operating Procedure @ ICMAB-CSIC

PROTOCOL	***C. elegans* maintenance and handling**
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Index

1. Purpose

This procedure describes the materials and protocols used for maintaining and storing *Caenorhabditis elegans (C. elegans).*

2. Background

Caenorhabditis elegans (C. elegans) is a 1 mm long free-living nematode postulated as an animal model in 1965 by Sydney Brenner. The ability to grow hundreds of animals on a single Petri dish feeding on bacteria, transparency, rapid life cycle (3 days), short lifespan (2-3 weeks), and facile and inexpensive growth in the lab position the worm as an ideal model. The advances in genetics and molecular biology allowed the identification of all 959 cells of *C. elegans* and its complete genome, demonstrating a high conservation of biological mechanisms between the worm and vertebrates. C. elegans has some limitations as an experimental model since it lacks some specific tissues (i.e., bones), organs (i.e., eyes and ears), and systems (i.e., the circulatory system). However, since its establishment as a model organism, the impact of *C. elegans* has extended from biology to other fields, including chemistry, materials science, and medicine.

C. elegans were obtained from the *Caenorhabditis Genetics Center (CGC)* at the University of Minnesota. Upon arrival, *C. elegans* were expanded in a fresh, clean agar plate with *Escherichia coli* OP50 food. After two generations, cryovials with the *C. elegans* generations were frozen. A working bank of *C. elegans* is maintained at the -80°C freezer at the CSIC-ICMAB premises.

C. elegans´ life cycle is described in the worm atlas book (Figure 1), and the maintenance and handling are considered.

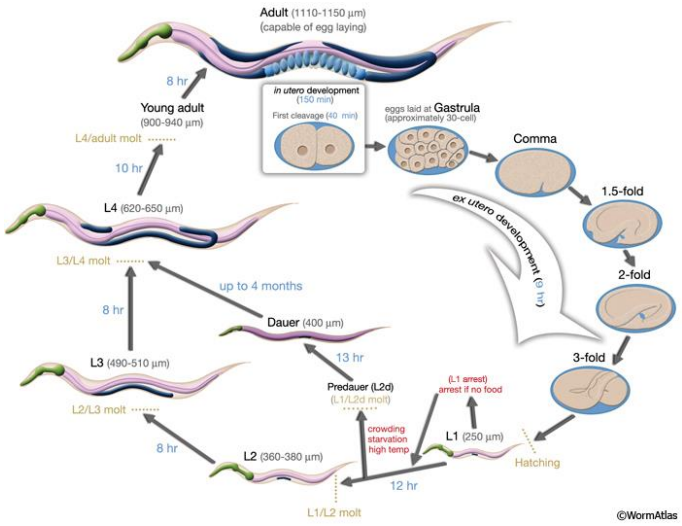

Figure 1 . *C. elegans*'s life cycle. Extracted from Altun, Z.F. and Hall, D.H. 2009. Introduction. In *WormAtlas*

Typically, *C. elegans* is cultivated utilizing *E. coli* strain OP50 as its primary food source and maintained on Nematode Growth Medium (NGM) agar poured into Petri plates. Various sizes of petri plates are available for different purposes, ranging from 35 mm for mattings or when using expensive drugs to 60 mm for general strain maintenance and 100 mm for growing larger quantities of worms. NGM agar can be poured into petri plates using a peristaltic pump, with a constant amount dispensed into each plate, reducing the need for microscope refocusing when switching between plates. Drugs can be added to the NGM solution just before pouring if desired.

3. Procedure

The reagents and materials used are sterile, and all the procedures are performed under a laminar flow cabinet.

C. elegans stocks are best maintained between 16°C and 25°C, typically at 20°C. They can be kept for months between transfers as worms enter the dauer form. *C. elegans* and bacteria stocks can also be stored frozen in liquid nitrogen or at –80°C with good viability. The maintenance of *C. elegans* involves the following steps: i) Preparation of Nematode Growth Media (NGM); ii) Preparation of the bacterial food source; iii) Transference of *C. elegans*.

3.1. Equipments

- *C.elegans* incubator (Memmert ICP110)
- Laminar flow cabinet (ESCO Laminar Flow Cabinet AVC-3D1)
- Stereoscope microscope (Nikon SMZ800N)
- Table centrifugue (J.P. Selecta. S.A. 7002239)
- -80°C Ultrafreezer (Telstar Boreas)
- Vortex (Ibx instrumentos V05 series)3
- Pipet controller (BrandTech™ accu-jet™ Fisher scientific)
- Platform agitator

3.2. Materials

- Eppendorf® tubes
- Falcon tubes
- Autoclavable glass flasks with screw lid
- Metallic spatulas
- Petri dishes (90 mm)
- Petri dishes (35 mm)
- Spreaders
- Inoculation loops
- Platinum wire
- Ethanol Burner
- Glass pasteur pipettes
- Serological pipettes 10 ml
- Serological pipettes 25 ml

3.3. Reagents

- NaCl (S9888, Merck)
- Agar (01916-500G, Merck)
- Peptone (211677 Thermofisher)
- $CaCl_2$ dihydrate (223506 Merck)
- KH_2PO_4 (P0662-500G, Merck)

- K_2HPO_4 (795496-500G, Merck)
- $Na_2HPO_4 \cdot 12\ H_2O$ (Emsure Supelco 1.06579.0500)
- Cholesterol (C8667-5G, Merck)
- Ethanol (99%, 493546-1L, Merck)
- LB broth (12780052, Thermo Fisher)
- Sodium hypochlorite (219250025, Thermo Fisher)
- PFA (158127, Merck)
- NaOH (S5881-500G, Merck)
- $MgSO_4$ (M7506 Merck)
- NGM plates. 30 ml per plate of NGM (3 g/l NaCl, 1 ml/l $MgSO_4$ 1M, 1 ml/l cholesterol 5 mg/ml, 17 g/l agar, 2.5 g/l peptone, 1 ml/l $CaCl_2$ 1M, 25 ml/l K_3PO_4 buffer, MilliQ® water)
- M9 buffer (5g/l NaCl, 1 ml/l $MgSO_4$ 1M, 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, MilliQ® water)
- K_3PO_4 buffer (108.3 g/l KH_2PO_4, 35.6 g/l K_2HPO_4, MilliQ® water)
- Glycerol (15514011 Thermo Fisher)

3.3.1. NGM agar plates preparation

To prepare NGM plates, mix 3 g NaCl, 17 g agar, and 2.5 g peptone. Add 975 ml H_2O. Autoclave for 20 min at 121°C. Wait until it colds down to 55°C. Add 1 ml 1 M $CaCl_2$, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$, and 25 ml 1 M K_3PO_4 buffer. Swirl to mix well. Using sterile procedures, dispense 30 ml NGM solution into petri plates using a pipet controller.
They are stored at room temperature in a dry ambient for two weeks.

3.3.2. OP50 solution preparation

From a single *E. coli* OP50 colony, inoculate LB broth and allow cultures to grow overnight at 37°C. Afterward, centrifuge to precipitate bacteria and replace the supernatant with M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 l).
E. coli OP50 is inactivated with PFA. This step is essential to avoid the interference of bacteria metabolism with the experiment's final result. *E. coli* OP50 is the usual food source for worms.

1) Inoculate LB culture with one colony of *E. coli* OP50 and incubate overnight (17h) at 30°C.
2) Mix vigorously with 3 ml of PFA 8% for each 45 ml of culture.
3) Incubate for another 20 min more at 30°C.
4) Centrifuge at 14500 rpm for 10 min. Discard the supernatant.
5) Resuspend the pellet in the same volume of PBS buffer.
6) Repeat steps 4 and 5 three times more.
7) Concentrate it 100 times (OP50-100x).
8) Spread 3 drops of 3 µl in an NGM plate. Culture at 30°C to know if it is completely activated.
9) Store it at 4°C until needed.
10) Spread 80 µl 100x OP50 onto 35 mm NGM plates. Store plates in the fridge until they are needed.

3.3.3. OP50 lawn petri dishes preparation

Dispense approximately 0.5 ml of *E. coli* OP50 liquid culture onto 60 mm NGM plates or 0.25 ml onto 35 mm NGM plates using a pipette. *E. coli* OP50 is an uracil auxotroph with restricted growth on NGM plates. Maintaining a limited bacterial lawn is preferable to facilitate easier observation and enhance worm mating.

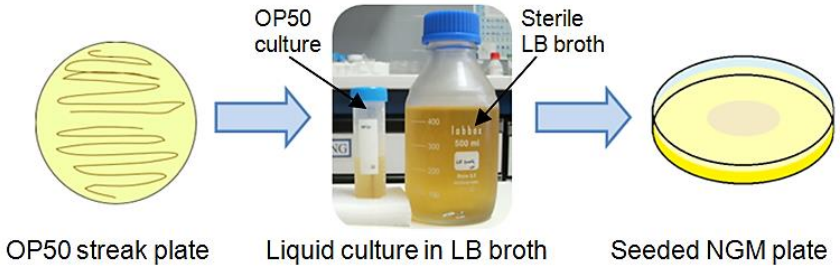

Figure 2 Preparation of the bacterial food source of *C. elegans.*

3.3.4. Worms transference

Worms are transferred from old plates to fresh plates by chunking. 'Chunking' involves moving a chunk of agar from an old plate to a fresh plate using a sterilized spatula. In the new plate, worms crawl out of the chunk and spread onto the bacterial lawn. Chunking is useful for transferring worms when food is scarce and starvation is not desired. The old plates used for chunking must not be contaminated. Otherwise, contaminants will be transferred to the new plate within the chunk (i.e. spores of yeast, bacterial cells).

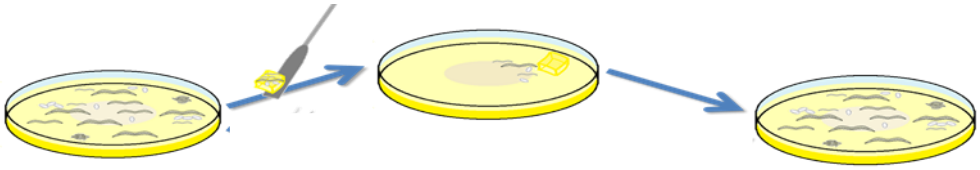

Figure 3. Scheme of the transference of worms. The procedure shows transference by chunking.

3.4. Worms synchronization

This protocol is useful for synchronizing worms and removing contamination. It is done in the laminar flow cabinet.

1. Chunk the worms strain you want two days before the bleaching and culture at 20°.
2. On the bleaching day, check that most of the worms in the gravid worm stage and eggs are on the plate. In addition, check that there is still food on the plates. If not, the worms have suffered from starvation and could grow badly in the next steps.
3. Spread 3 ml M9 buffer onto NGM plates with worms.
4. Using a Pasteur pipette, take the liquid and return it to the plate to remove worms from the NGM plate.
5. Transfer worms to 15 ml falcon tubes.
6. Centrifuge worms at 6000 rpm for 2 min.
7. Remove the supernatant until 5 ml of the tube.
8. Repeat steps d) and e) three times. Adjust the final volume to 5 ml.

9. Prepare the bleaching solution: 2 ml 5M NaOH, 13 ml sodium hypochlorite, 35 ml distilled water.
10. Add 5 ml of bleaching solution.
11. Vortex tubes at 1 min max speed. In this process, the bleaching solution dissolves the worm's tissues.
12. Centrifuge tubes 6000 rpm for 2 min.
13. Remove supernatant until 1 ml of liquid is left on the tube.
14. Add 9 ml of M9
15. Repeat steps 12-14 three times. Finally, worms were resuspended in 5 ml of M9.

3.5. Freezing procedure

Worms can be maintained in the incubator and the freezer. In the incubator, they can survive three months in the same plate, but mutations can appear and change the strain's phenotype. Therefore, preparing a frozen stock when you receive a new strain in the lab is highly recommended. Some protocols suggest freezing the L1-L2 synchronized population, but we have seen that a mixed population can also survive.

1. Prepare two 60 mm-petri dishes with 500 µl *E. coli* OP50 with an optical density at 600 nm (OD600)=1 and put them at 30°C overnight.
2. Do a chunk from a plate with the worms you want to freeze. Put the plate at 20°C (or another temperature where your worms grow) and wait until it is overcrowded with worms (it depends on the growth rate; usually, two days is enough).
3. Recollect worms from plates with M9 as described previously for synchronization.
4. Clean them with M9 to remove bacteria from the solution (at least three times). Centrifuge them at 4400 rpm for one min.
5. Once they are cleaned, worms are resuspended in 2 ml M9. This tube is left in the platform agitator overnight to starve worms.
6. The following day, add an equal volume of glycerol of 30% (Diluted with M9 buffer). Mix the final solution well.
7. Separate the final solution in cryovials (1ml each).
8. Add 4 drops of *E. coli* OP50 with OD600=1.
9. Place them in the freezer at -80°C.
10. Thaw one of the cryovials after one week to see the viability.

3.6. Thawing procedure

1. Remove a vial from the freezer and let it thaw at room temperature.
2. Pour the contents onto one large NGM plate with 500 µl of *E. coli* OP50 OD600=1.
3. Wait until the liquid has dried and incubate the plate overnight at 20°C.
4. The next day, you should check if the worms are alive. If it is, the freezing protocol has worked.
5. Transfer by chunking to a new, fresh plate. Pass worms throw two consecutive generations to avoid any interference from the freezing step.