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Maintenance of *C. elegans* in axenic growth cultures and generation of large numbers for biochemical studies.

The ability to grow *C. elegans* in a defined growth medium adds a new dimension to *C. elegans* research which can be exploited to dissect genetic and molecular determinants in nutritional and toxicological studies. Furthermore, large quantities of worms can be grown axenically in small manageable volumes for biochemical analyses including enzyme assays and chromatography.

This unit presents growth conditions for liquid medium. The protocol is a modification of the original CeHR medium developed by Eric Clegg's group (http://www.usacehr.org/cehr_medium.htm). There are two alternate protocols: The first one is with reduced metals for studies with metalloproteins and gene regulation, and the second protocol eliminates all cations by metal chelation followed by addition of known quantities of metal ions.

NOTE: All reagents and equipment coming into contact with worms must be sterile and proper sterile techniques within a laminar flow hood must be used.

BASIC PROTOCOL 1

MAINTENANCE AND CULTURE OF WORMS in mCeHR-1:

This protocol describes how to culture and maintain *C. elegans* in liquid axenic medium. Worm strains can be obtained from the *Caenorhabditis* Genetics Center (<http://www.cbs.umn.edu/CGC/>). Cultures are initiated and/or developmentally synchronized using bleach and maintained by subculturing. Aliquots of these cultures can be frozen at -80°C or in liquid nitrogen for long-term storage.

Materials

Laminar flow hood

mCeHR growth medium (see recipe)

C. elegans wild-type Bristol N2 strain [CGC DR subclone of CB original (Tc1 pattern I)]

25 or 75 cm² flasks (Nunc #136196 or 178891)

20°C refrigerated incubator equipped with platform shaker or rocker (Labline Orbit Shaker or Hofer Rocker).

Beckman CS-6R centrifuge with GH3.7 horizontal rotor (or equivalent rotor)

Screw cap cryostat freezing vials

15 ml / 50 ml sterile conical tubes

Phase contrast inverted microscope with 10X objective

Stereoscope with bottom illumination

Begin culture of C. elegans

1. Grow N2 worms on ten 60 mm NGM agar plates spotted with OP-50 *E. coli* strain (obtained from CGC) until there are large number of gravid worms with very little bacteria on the plates (freshly or nearly starved).
2. Rinse the plates with M9 buffer and transfer the worms to a 50 ml conical tube. If there are numerous embryos remaining on the agar they can be loosened and collected by swirling and tapping the plates. Alternatively, a gloved finger or 'rubber policeman' can be used to quickly liberate embryos from the agar surface.
3. Let the tube stand for a few minutes (5-10 mins) and slowly remove the buffer from the top. This step will remove a significant amount of bacteria because the worms are denser and tend to sink faster to the bottom of the tube compared to *E. coli*.
4. Resuspend the worm pellet in M9 buffer and repeat step 3 twice.
5. Resuspend the worms in 0.1N NaCl in a volume appropriate for bleaching (see Embryo Preparation protocol below)
6. After bleaching, use a Pasteur pipette to transfer the embryos into a 25 cm² flask containing mCeHR growth medium supplemented with 100 µg/ml of tetracycline; use sterile techniques.

To ensure that the worm cultures are established and to prevent any bacterial carryover during the initial period, the growth medium can also be supplemented with a triple antibiotic cocktail of tetracycline (100 µg/ml), streptomycin (250 µg/ml) and nalidixic acid (250 µg/ml). We use this cocktail very sparingly.

7. Incubate at 20°C in a refrigerated incubator on a rocker platform set at ~70 RPM.
The growth rate of worms in the first round will be slow (typically about 7-10 days) as they become established for growth in liquid medium, but successive generations will be closer to 4 days. Some mutant strains (e.g. rol) will take longer to grow (5-6 days).
8. Let worms grow to an appreciable density such that bleaching gravid worms will yield sufficient number of larvae to start a subculture.

Embryo preparation / Bleaching

All work must be carried out using sterile techniques in a laminar flow hood.

9. Pipette nematode suspension into sterile conical tubes and centrifuge at 800 x g for 5 mins at 4°C in a GH-3.7 rotor.
10. Aspirate the supernatant using a Pasteur pipette and resuspend the worm pellet in 10 ml of 0.1 M NaCl by gently pipetting. Incubate nematodes on ice for 5 min.
11. Aspirate supernatant using a Pasteur pipette, including worms that have not settled to the bottom of the tube. Resuspend the pellet in 0.1 M NaCl in a volume that is a multiple of three. For example, 1.5 ml, 3 ml, 6ml, 9 ml, etc.

Individual worms and not clumps should be visible in the liquid suspension by eye when the worm suspension is swirled in the conical tube.

12. Add 5N NaOH and 5% bleach to the worm suspension in a 1:2:6 ratio.

For example: If the worms were resuspended in 6 ml of 0.1 M NaCl, then add 3 ml of NaOH/Bleach mix (2 ml 5% bleach : 1 ml 5N NaOH).

13. Mix by vortexing (number 10 setting on a Vortex Genie-2). Continually monitor the worms under a phase-contrast inverted microscope with periodic vortexing. The

bleaching is continued until most embryos are released from the gravid adult. It should take less than 10 min for the worms to burst open.

Worms should be constantly monitored under a phase contrast microscope with a 10X objective.

14. Let the worms remain in bleach for an additional 30-40 sec to further dissolve the worms and immediately centrifuge them at 800 x g for 45 sec at 4°C. Aspirate the supernatant with a Pasteur pipette.
15. Add sterile water to the embryo pellet to a volume of 5-10 ml depending on the pellet size and vortex for 5 sec. Centrifuge to pellet the eggs at 800 x g for 45 sec at 4°C and aspirate the supernatant using a Pasteur pipette. Repeat this step once more.
16. Add 10 ml M9 buffer to the embryos and transfer them to a sterile 25 cm² flask. Incubate the flask overnight at 20°C on a platform shaker at 70 RPM. The embryos will hatch in the M9 buffer as synchronized starved L1 larvae. Use the larvae within 2 days.

L1 larvae stored in M9 buffer for longer time periods tend to have a greater lag time and get asynchronized when re-introduced to mCeHR medium. Changes to fresh M9 buffer every third day may help alleviate this problem.

Maintain and subculture of C. elegans cultures

17. Transfer L1 larvae in M9 buffer to 50 ml conical tubes and centrifuge the worms at 800 x g for 45 sec at 4°C. Resuspend the larvae in mCeHR medium and transfer them to a 25 cm² flask. Incubate flask at 20°C on a shaker platform at 70 RPM.

Antibiotics are no longer needed if proper sterile techniques are used. With two successive rounds of bleaching, contaminating bacterial carryovers are usually eliminated. A density of 3000 worms/ml/cm² ensures that adequate nutrients are available to the worms. Growth rates should be carefully recorded at this point (L1 to gravid adults) and are easy to monitor in a synchronized population.

Freeze worms

18. Pellet mixed population of worms (asynchronous) and resuspend them in S buffer. Aliquot 0.5 ml of worms into a 2 ml freezing vial and add equal volume of Freezing Solution. Vortex briefly. Freeze samples at -80°C.

Worms freeze well when frozen in concentrated number of ~1 million worms in 1 ml per vial. Although a pure synchronized L1 population can be frozen if available, it is convenient to freeze a mixed culture. Because early larval stages tend to survive better when thawed, a mixed culture ensures that there are sufficient quantities of young larvae.

19. To thaw worms place them directly in a 37°C water bath till the ice has mostly melted (< 2 mins). Gently pipette worms with a Pasteur pipette into mCeHR medium.

Set-up for a growth curve

20. Pellet synchronized L1 larvae by centrifugation at 800 x g for 5 min. Resuspend the pellet in ~ 2 ml of M9 buffer. The volume of buffer can be increased if the resuspended larvae look dense.

21. Vortex/shake the tube vigorously and immediately pipet 100 μ l of worms into a microfuge tube. Add equal volume of 10 mM sodium azide (NaN_3) to immobilize/anesthetize the worms. Mix and incubate for ~5-10 min.
22. Vortex and spread 40-50 μ l of the solution in an S-shape on a glass slide. Usually this volume should contain between 50 to 150 worms. Dilute concentrated worms with 5 mM sodium azide. Counting less than <20 worms usually leads to errors.
23. Calculate the number of worms/ml in the original 2 ml solution and dilute to ~ 1 worm/ μ l. Seed ~50 worms/ cm^2 / 0.5 ml. For example, a single well (2 cm^2) of a 24-well plate should contain ~100 worms in 1 ml growth medium.

Perform a heme dose-response

24. Prepare a fresh solution of 10 mM hemin stock in 0.3M NH_4OH , pH 8.0. For lower final concentrations of hemin make additional stocks of 1 mM and 0.1 mM.
25. Add 800 μ l of growth medium into individual wells of a 24-well plate. Aliquot 100 μ l of 0.3M NH_4OH , pH 8.0 containing hemin to a desired final concentration. The total volume of 0.3M NH_4OH , pH 8.0 in each well should be the same to ensure consistency.
26. Finally, 100 μ l of M9 buffer containing ~100 L1 larvae is added to the culture medium. Mix tube well prior to aliquoting the worms because they tend to sink to the bottom of the tube. The final volume of the growth medium plus worms in each 2 cm^2 well is 1ml.
27. Check the plate under the microscope to ensure that all the wells have equal number of larvae. Incubate plates at 20°C on a rocker platform at 70 RPM. Cover the plates with an aluminum foil because hemin is light sensitive.

The day the worms are added to the culture medium is day 0. Worms are thereafter monitored each day for growth and are usually counted on days 3, 6, and 9. If 12-well (4 cm^2) or 6-well (10 cm^2) plates are used, then the number of worms and volume of the growth medium is proportionately changed to accommodate the difference in surface area.

Count worms

28. Swirl the plate gently so that all worms are in the center of the well and not in the corners. Pipette the entire contents (~1ml) of each well into a microfuge tube. Occasionally, the volumes will be slightly lower due to evaporation in the incubator. If this is the case, then bring the volume to 1ml with sterile M9 buffer.
29. Vortex vigorously and pipette 100 μ l into another microfuge tube. Add 100 μ l 10mM NaN_3 and incubate for 5-10 min. Count worms in either 20 or 40 μ l as described previously.

At low and high hemin concentrations worms are usually dead or severely growth retarded. If this is the case, count the entire contents of that well by centrifugation at 800g for 5 min and resuspending the pellet in 50 μ l of 5 mM NaN_3 .

REAGENTS AND SOLUTIONS

Use sterile deionized, distilled water in all recipes and protocol steps. For common stock solutions, see Appendix XX; for suppliers, see Appendix XX. Make small volumes of stocks A, B and C so they are used quickly and are not stored for extended periods.

A. Mineral mix, 1L

MgCl ₂ .6H ₂ O	4.1 g
Sodium Citrate	2.9 g
Potassium Citrate. H ₂ O	4.9 g
CuCl ₂ .2H ₂ O	0.07 g
MnCl ₂ .4H ₂ O	0.2 g
ZnCl ₂	0.1 g
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	0.6 g
CaCl ₂ .2H ₂ O (always add last)	0.2 g

B. Vitamins and Growth Factor mix, 100 ml

(i) To 60 ml of water add:

N-Acetyl-a-D-glucosamine	0.15 g
DL-Alanine	0.15 g
Nicotinamide	0.075 g
D-Pantethine	0.0375 g
DL-Pantothenic acid, hemi calcium salt	0.075 g
Pteroylglutamic Acid (Folic Acid)	0.075 g
Pyridoxamine.2HCl	0.0375 g
Pyridoxine.HCl	0.075 g
Flavin mononucleotide, sodium salt	0.075 g
Thiamine hydrochloride	0.075 g

(ii) Prepare each of these chemicals in ~5 ml 1N KOH

p-Aminobenzoic Acid	0.075 g
d-Biotin	0.0375 g
Cyanocobalamine (B ₁₂)	0.0375 g
Folinic Acid, calcium salt	0.0375 g
Nicotinic Acid	0.075 g
Pyridoxal 5'-phosphate	0.0375 g

(iii) (±)-a-Lipoic Acid, oxidized form
in 1 ml Ethanol 0.0375 g

Mix solutions (i), (ii), and (iii) and bring final volume to 100 ml. Store in dark at 4°C or freeze aliquots at -20°C.

C. Nucleic acid mix, 100 ml

To 60 ml of water add:

Adenosine 5' monophosphate, sodium salt	1.74 g
Cytidine 5' phosphate	1.84 g
Guanosine 2' and 3' monophosphate	1.82 g
(or Guanosine 5' phosphate)	2.04 g
Uridine 5' phosphate, disodium salt	1.84 g
Thymine (add last-will dissolve)	0.63 g

Bring stock solution to 100 ml and store in the dark at 4°C or freeze aliquots at -20°C.

D. Other stock solutions:

Prepare each of the following solutions separately in water unless noted otherwise:

KH ₂ PO ₄	450 mM
Choline di-acid citrate	2 mM
i-Inositol	2.4 mM
d-Glucose	1.46 M
HEPES, Na salt	1 M
Cholesterol	5 mg/ml in Ethanol
Lactalbumin hydrolysate (GIBCO #18080-036)	170 mg/ml

Hemin:

2 mM hemin chloride in 0.1 N NaOH. Adjust pH to 8.0 with conc. HCl.

If the pH falls below 6.0 (acidic), hemin will precipitate.

Higher concentrations of hemin and other metal substituted porphyrins can be made in 0.3M NH₄OH.

Milk:

High temperature ultra-pasteurized (UHT) skim milk can be obtained in a grocery store (eg: Horizon's Organic UHT skim milk). Using sterile techniques open the container of milk and streak on Brain Heart Infusion [DIFCO #237500] agar plates. Incubate plates at 37°C and 30°C to test for growth of any microbes. Transfer milk to 50 ml sterile conical tubes and freeze at -80°C.

We found that 10 % milk is usually sufficient with no discernable differences in worm growth compared to mCeHR-1 medium supplemented with 20 % milk.

M9 buffer, 1L

6.0 g Na₂HPO₄ (42 mM)

3.0 g KH₂PO₄ (22 mM)

5.0 g NaCl (86 mM)

0.25 g MgSO₄.7H₂O (1 mM)

Sterilize by autoclaving and store at 4°C.

NaOH/Bleach solution

5N NaOH

5 % Chlorox Bleach

Use one part NaOH to 2 parts bleach.

Commercial bleach is ~6% and should be diluted fresh to 5% in water. Use NaOH/Bleach mix immediately.

Assembly of CeHR Medium, 1L

Make media fresh in a laminar flow hood with sterile techniques. The pH without milk is 6.0-6.5.

Filter the following volumes of stock solutions in the order described with a 1 L 0.2 μ m filter unit.

10 ml Choline

10 ml Vitamin mix

10 ml i-Inositol

10 ml Hemin

250 ml Water

Apply filter

20 ml Nucleic acid mix

100 ml Mineral mix

20 ml lactalbumin

20 ml Essential Amino Acid Mix (GIBCO #11130-051)

10 ml Non-essential Amino Acid Mix (GIBCO #11140-050)

20 ml KH_2PO_4

50 ml d-Glucose

10 ml HEPES

250 ml Water

1 ml cholesterol

Apply filter

Can be stored at 4°C. Add 20% (v/v) UHT skim milk just prior to use.

Note: Milk cannot be filtered through a filter unit.

Freezing Solution

S Buffer + 30% Glycerol (v/v)

S Buffer, 1L

129 ml 0.05 M K_2HPO_4

871 ml 0.05 M KH_2PO_4

5.85 g NaCl

pH 6.0.

Sterilize by autoclaving.

COMMENTARY

Background Information

Although large quantities of *C. elegans* can be grown in liquid culture using concentrated *E. coli* for food (Lewis and Fleming, 1995 and Stiernagle 2005.), there are several disadvantages to using this method for nutritional and toxicology studies. The foremost is the use of another complex organism as a food source which would confound results because nutrients could be assimilated and modified by *E. coli*, prior to ingestion by worms. Other issues are related to biochemical assays, such as ensuring the enzyme activity measured or the protein purified is not from *E. coli*.

To circumvent these problems, defined growth medium (CbMM) for worms was developed more than 30 years ago (Vanfleteren, 1978). More recently a chemically defined derivative of CbMM, *C. elegans* Maintenance Medium (CeMM), was developed that would allow for axenic growth of *C. elegans* in liquid. However, worms grown in CeMM medium take 2 to 3 times longer to develop compared to worms grown on standard NGM agar plates, and these worms appear to be nutrient starved (Szewczyk et al 2003). Thus, it becomes imperative that experimental results derived from animals grown in liquid medium are comparable to results obtained from animals grown on NGM plates.

In our attempt to control nutrient levels and closely mimic the growth rate of *C. elegans* on NGM plates, we re-formulated the original CeHR medium that was developed for toxicological studies at the U.S. Army Center for Environmental Health Research (E. D. Clegg, personal communication). In the past three years we have systemically tested different components in the growth medium and fine-tuned the concentrations of individual chemicals to achieve maximal growth rates for *C. elegans* that is comparable to NGM plates (Rao et al, 2005). The mCeHR-1 medium has now been used to culture other nematode species including *Panagrellus redivivus*, *Oscheius myriophila*, and *C. remanei* which are male female species, unlike the hermaphroditic *C. elegans*.

Critical Parameters and Troubleshooting

Alteration of hemin concentrations results in changes in worm growth rate. Although 20 μM hemin is optimal, changes in hemin levels can dramatically alter worm growth. To circumvent this issue, worms can be grown in the presence of 50 μM hemin without any obvious changes. However, we have determined that several heme-responsive genes are significantly down-regulated at this heme concentration, and users should be aware of this caveat.

The pH of the growth medium dictates whether its components will precipitate (especially hemin) during worm culture. The use of HEPES sodium salt is important in maintaining the pH. Increasing the concentration of HEPES from 10 mM to 30mM will permit greater buffering capacity. Nucleic acid stock solutions tend to precipitate at room temperature. However, omission of this precipitate does not hinder worm growth as individual components are already in excess.

Although NaOH/bleach treatment will yield a large number of synchronized worms (>90%), it is difficult to achieve complete synchronization. The presence of large number of dead embryos, the day after bleaching, usually is indicative that the bleaching procedure was sub-optimal. Bleaching is a key step for obtaining and maintaining large numbers of progeny, and for preventing contamination.

If worms are grown for prolonged lengths of time in the same growth medium, dauer worms appear in the culture. This alternate life stage can be problematic as dauer larvae are more resistant to bleach than their non-dauer counterparts, making them difficult to eliminate from the culture once they take hold. The growth medium should be changed every 5 days to avoid dauer formation.

Anticipated results

Worms grown in the mCeHR growth medium at 20°C are robust and healthy. They should be moving/swimming most of the time, and at the adult gravid stage contain 6-8 healthy embryos. If the worms are not developing and have been static for more than 3 days, this is usually a sign of sub-optimal media formulations.

Time Considerations

Assembling mCeHR medium from pre-existing stock solutions takes < 1 h. Making *C. elegans* axenic in CeHR medium will take 8-10 days. However, it is important to monitor worm growth and development daily. Once axenic worms have been established, it should take no more than 4 days for wild-type N2 worms to become gravid adults.

Literature Cited

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