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Embryo filtration for preparation of staged populations of *Caenorhabditis elegans* embryos

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We use this protocol and it's working

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Abstract

The protocol describes the use of two different pore size filters purchased from pluriSelect, U.S.A. to separate *Caenorhabditis elegans* embryos from other developmental stages. A large number of embryos with 100% purity are recovered by this technique. The collected embryos can be grown to the desired stages to get synchronized populations for high throughput assays.

Image Attribution

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Materials

1. M9 buffer (Per 1000 mL)
3 g of KH2PO4
6 g of Na2HPO4
5 g of NaCl
Add the three salts to 1000 mL of Milli Q water. Autoclave and store at room temperature for two weeks.

2. Bleach Solution (Per 200 mL)
40 mL NaOCl (Fisher, catalog # SS290-1), add ~ 100 mL Milli Q water
Note: Do not use an opened bottle of NaOCl after one month.
10 mL of freshly made 10 M NaOH (add 4g NaOH pellets to 10 ml of Milli Q water)
Add Milli Q water up to 200 mL
Mix well and store at 4°C until needed. This bleach should be made four days before use.

- 25% bleach solution: (1 mL of the above bleach solution + 3 mL M9 buffer)

3. K medium (Per 500 mL)
51 mM NaCl (5.1 mL of 5 M NaCl)
32 mM KCl (16 mL of 1 M KCl)
3 mM CaCl2 (1.5 mL of 1 M CaCl2)
3 mM MgSO4 (1.5 mL of 1 M MgSO4)
Mix the four salts with increasing water, then fill to 500 mL with Milli Q water. Filter sterilize with Thermo Filter Unit (catalog #566-0020). After filtering, add 1.25 µg/mL unfiltered cholesterol (125 µL of 5 mg/mL cholesterol). Mix well and store at room temperature for up to two weeks. Be sure to check the K medium prior to use for any floating particulates, which is a sign of contamination.

4. 3% Poly ethylene glycol (PEG) (Per 500 mL)
15 g of PEG3350 (Millipore sigma, catalog # P4338)
Add to 500 mL of M9 buffer. Mix well and store at room temperature for 15 days.

CONSUMABLES:

1. 15 mL conicals: FisherBrand Disposable Centrifuge Tube 15mL catalog # 07-200-886
2. 50 mL conicals: FisherBrand Disposable Centrifuge Tube 50mL catalog # 06-443-21
3. pluriStrainer® 40 µm: PluriSelect catalog #: SKU 43-50040
4. pluriStrainer® 20 µm: PluriSelect catalog #: SKU 43-50020
5. Connector Ring: PluriSelect catalog #: SKU 41-50000
6. Funnel: PluriSelect catalog # SKU 42-50000
7. Rubber Policeman: Fisher Scientific catalog # S66001

Safety warnings

Connect the filtration apparatus to a vacuum only for a few seconds (1 - 3 seconds) (Step 19). A prolonged connection to a vacuum might allow the adults that are stuck on the 40 µm filter to pass through and be collected on the 20 µm filter along with embryos.

Strain preparation

1 Using a sterile, autoclaved spatula, chunk the strains from a starved plate onto two fresh OP50-spotted 6 cm plates. This step is generation zero (G_0). Store these 6 cm plates in a 20 °C incubator. Throughout the experiment, make sure that the temperature in all incubators is carefully monitored using the iButton temperature tracking system. Aim to chunk a plate that yields about 50 adult worms, assessed by the number of arrested L1 larvae on the source plate.

2 After 48 hours, pipette 20 µL of bleach solution away from the lawn of a newly seeded 6 cm plate and pick 10 to 20 gravid hermaphrodites into the bleach spot. Swirl gently with the pick to ensure all worms are removed from the pick. Store these 6 cm plates upside down (lid up) in a 20 °C incubator. Keep the incubator consistent throughout the assay.

3 After 24 hours, pick 20 to 30 L1s to three new labeled and seeded 6 cm plates for each strain. If the L1s do not survive the bleach, make a note of it so that larger chunks or fresher source plates can be used for future assays. This pick is generation one (G_1). Store these 6 cm plates in a 20 °C incubator.

4 After 48 hours, pick five late-stage L4 hermaphrodites to five labeled 6 cm plates from each strain. These animals are distinguished by a distinct clear half-moon-shaped patch near the midsection of the worm, containing what looks like a classic Christmas tree where the vulva is developing. This pick is still G_1. Note: Worms might have grown into young adults by this time. In which case, pick the youngest adult worms that you can find. If you find males on source plates, avoid picking young adults from these plates. Check the plates one and two days after picking to make sure that they will be properly staged for the generation two pick (G_2). Grow this generation of animals at 20 °C. Pick at least five 6 cm plates per strain for backup. Note: Check these plates every day. If you feel they might not be ready to reach the G2 adult stage by the fourth day, transfer them to 21.5 °C for faster growth. Four days later, you will have a plate full of G_2 adults that are ready for bleaching.

Bleaching of adults to collect embryos for filtration

5 Wash the G_2 worms off plates with M9 buffer into a labeled 15 mL conical tube. You can pour the M9 buffer onto one plate of Strain A, transfer the liquid to another plate of Strain A, etc. Wash all plates from a single strain into one labeled 15 mL conical tube. We typically pour 2 mL of M9 buffer onto one plate and transfer the same solution to

four other plates before pouring it into the conical. Repeat for all strains into separate 15 mL conical tubes.

6 Spin down the worms at 254 g (Eppendorf 5810R, 1,100 rpm) for one minute using the table-top clinical centrifuge. Aspirate off the M9 buffer, being careful to not suck up any worms.

7 Add 7 mL of bleaching solution to each 15 mL conical for pellet sizes of 100 µL and up. If pellet sizes are smaller, adjust the volume of the bleaching solution accordingly. We use 4 mL of the bleach solution for all smaller pellets. The freshness of the bleach solution matters. An ineffective bleaching solution (kept at 4 °C longer than one month or kept at Room temperature for longer than eight hours) will require more time to bleach and will not dissolve the worms uniformly. Shake the tubes manually and vigorously by holding the tubes by the cap to avoid the transfer of warmth from the hands to the solution. After four minutes of shaking, use a Differential Interference Contrast (DIC) microscope to check if the adult worms are dissolved every 30 seconds until complete. Be very careful not to over-bleach the worms. Once you see that the carcasses are nearly dissolved, move to the next step. If only embryos are left, then you have gone too long. If you find a strain that is lagging, shake this strain more vigorously to catch it up to the other strains.

8 Once you find that only a few adults remain, move quickly to spin down the embryos at 254 g (Eppendorf 5810R, 1,100 rpm) for 30 seconds. It is best to have the reagents next to the waste/pour-off receptacle and prepared for the next few steps. Some table-top centrifuges do not have an option of 30 seconds, so you must stop the centrifuge manually after 30 seconds. If you feel you have under-bleached your worms, you may let them spin down for a full minute.

9 Immediately decant off the bleach into the waste/pour-off receptacle carefully and quickly without disturbing the worm pellet.

10 Pour 10 mL of M9 buffer to each 15 mL conical tube. Spin down the embryos at 254 g (Eppendorf 5810R, 1,100 rpm) for 30 seconds. Decant off the M9 buffer into the sink/waste container carefully and quickly without disturbing the worm pellet.

11 Repeat step 6 twice with M9 buffer.

12 After the final wash, resuspend the embryos in 2 mL of M9 buffer (called embryo suspension). The embryos collected are generation three (G_3).

13 Determine the titer of embryos by counting the number of embryos in 3 µL in five replicates. Average the counts to get the mean number of embryos in 3 µL.

14 Calculate the volume of the embryo suspension required for 450 G_3 embryos. For example, if the average number of embryos in 3 µL is 60, add 22.5 µL of the embryo suspension to get 450 embryos on the desired number of 6 cm OP50 bacteria plates.

Note: The number of 6 cm OP50 bacteria plates made with 450 G_3 embryos will depend on the number of G_4 embryos required from filtration to set up the assay.
Note: We have optimized the number of G_3 embryos to be plated to 450 based on the *C. elegans* reference strain N2. The number may need to be adjusted based on the brood size of the strain to get the maximum G_4 embryos from one 6 cm plate after filtration.

15 After incubating the 450 G_3 embryo plates at 21.5 °C for three days, the plates will be ready to use for the filtration protocol.

Filtration

16 Set up the filtration apparatus as shown (Figure 1). A connector ring is attached on top of a clean and labeled 50 mL conical tube. Above the connector ring, a filter stack consisting of a 20 µm filter (green) at the bottom and two 40 µm filters (blue) at the top are added. A funnel is attached to the 40 µm filter to pour the solution through the filtration apparatus.

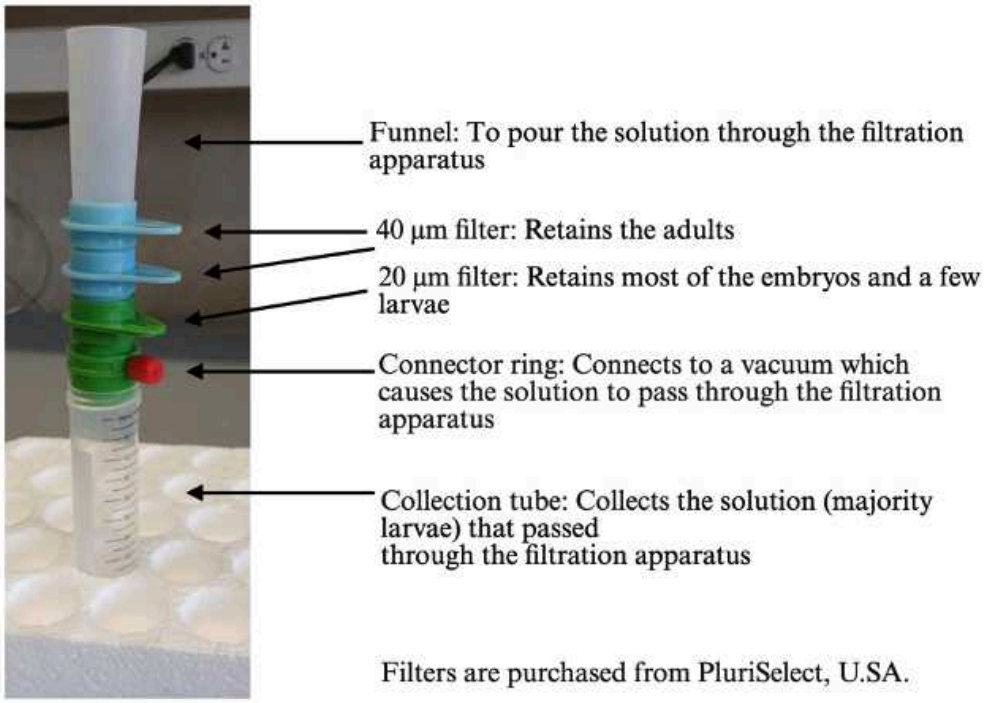

Figure 1. Set up of the filtration apparatus.

17 Add 2 mL M9 buffer to each plate. Swirl and discard the liquid. Repeat the process one more time. This step is to get rid of the G_3 adults and G_4 larvae that are not stuck to the OP50 bacteria. You will NOT be keeping these worms. However, the G_3 adults can be used to set up another assay by bleaching and starting at step 5.

18 Add 2 mL of the M9 buffer to each plate. Take a rubber policeman (Figure 2) and gently scrub the plate to get the G_4 embryos in the M9 buffer.

Figure 2. Rubber policeman is used to scrape the embryos from the OP50 bacteria plate.

19 Collect the G_4 embryo solution in a 15 mL tube (Figure 3A). Repeat the wash step one more time. This solution (G_4 embryo solution) will be used for filtering through the filtration apparatus (Figure 3B). Mix the embryo solution by inverting the 15 mL tube four to five times to ensure that the embryos are in solution and not settled at the bottom of the tube. Pour the embryo solution through the funnel of the filtration apparatus (Figure 3C). Connect the connector ring to the vacuum. Start the vacuum to pass the embryo solution through the filtration apparatus. Make sure that the solution is collected in the 50 mL conical tube. The vacuum should only be applied for 1 - 3 seconds, which is enough time for the embryo solution to pass through the filter (Figure 3D).

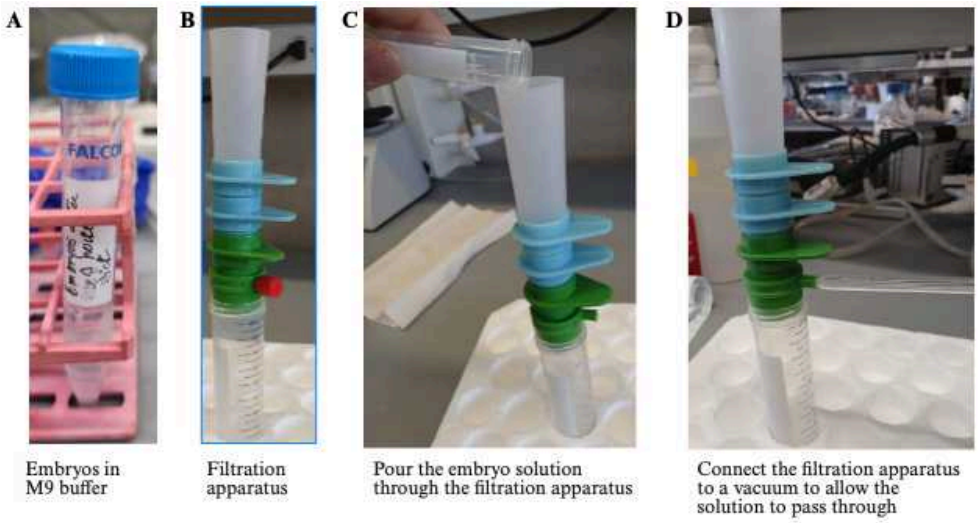

Figure 3. Steps of filtration. (A) G_4 embryos collected in M9 buffer using a rubber policeman. (B) Filtration apparatus. (C). Pouring the embryo solution through the filtration apparatus. (D). Connecting the connector ring to a vacuum to allow the embryo solution to pass through the filtration apparatus.

20 Remove the funnel and the two 40 µm filters (blue color).

21 To collect the embryos retained on the 20 µm filter, reverse the direction of the 20 µm filter (green) and place it on top of a clean and labeled 50 mL conical tube. Make sure the top of the filter is facing the inside of the 50 mL tube (Figure 4).

Figure 4. Reversing the direction of the 20 µm filter to harvest the G_4 embryos that are collected on the 20 µm filter.

22 Using a serological pipette, add 4 mL of 25% bleach solution to the reversed 20 µm filter.

23 Once all the solution has passed through the filter, start the timer for 30 seconds. Keep swirling the tube during this incubation time. Ideally, only the G4 embryos should be recovered from the 20 µm filter. However, the purity of the embryos is approximately 94 - 98% (based on data collected from at least 20 independent batches of filtrations). The lack of 100% purity is because a few larvae are stuck to the 20 µm filter and are collected with the embryos. Adding 25% bleach solution will kill the larvae, which is important because we do not want any larval contaminations to have a synchronous population for downstream assays.

24 After 30 seconds, add 6 mL of 3% polyethylene glycol (PEG) in M9 buffer (w/v) on top of the filter to make up the volume to 10 mL. This step will allow for any embryos that are still stuck on the 20 µm filter to be washed and collected in the 50 mL collection tube. Transfer the contents to a clean and labeled 15 mL conical tube and centrifuge at 3,197 g (Eppendorf 5810R, 3,900 rpm) for one minute to pellet the embryos.

25 Discard the supernatant and add 10 mL of 3% PEG in M9 buffer. Centrifuge at 3,197 g (Eppendorf 5810R, 3,900 rpm) for one minute. Repeat the step once more.

26 Discard the supernatant and add 3 mL of K medium. This one single wash will not remove all PEG from the reaction. However, we have observed no effects on some drug response assays. The user should note this potential issue.

27 Determine the titer of embryos by counting the number of embryos in 3 µL in five replicates. Average the counts to get the mean number of embryos in 3 µL. The collected embryos can be used to set up the required assays.

Acknowledgements

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