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High-throughput Larval Development Assay (HTLDA)

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We use this protocol and it's working

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Abstract

The high-throughput larval development assay (HTLDA) is used to prepare selfing *Caenorhabditis* strains and quantitatively measure the development of animals using the Molecular Devices ImageXpress Nano micropscope. Synchronized L1 larval stage animals are grown for 48 hours and then measured for animal length as a proxy for development. The HTLDA can be scaled to measure the development of up to 96 unique strains at a time, which provides an efficient method to obtain quantitative measurements of selfing *Caenorhabditis* animals.

Materials

Equipment:

- Eppendorf 5810R
- Plastic shoe box (IRIS USA CNL clear Latching Box, 6 Qt, 18 Count; or similar box)

Consumables:

- 12-channel reservoirs (USA Scientific 12-well V-Bottom Reservoir, Cat #: 3824*3412)
- Gas permeable sealing film (Fisher, Cat #: 14-222-043)
- Kanamycin (US Biological, Cat #: K0010)
- Sodium azide
- Bleach solution (Per 200 mL):

Bleach solution recipe (per 200 mL):

1) Make 10 mL of freshly made 10 M sodium hydroxide (NaOH) (add 4 g NaOH pellets to 10 mL of ddH2O).
1a) Add 4 g NaOH pellets to an autoclaved glass media storage bottle.
1b) Measure 10 mL of ddH2O in a graduated cylinder and add the 10 mL of ddH2O to the 4 g NaOH pellets.
2) In a graduated cylinder, measure out 40 mL NaOCl (Fisher, cat #SS290-1). Do not use store-bought bleach.
3) Add ~ 100 mL ddH2O to the graduated cylinder with NaOCl.
4) Pour the 140 mL of NaOCl + ddH2O solution to the glass media storage bottle.
5) Add ~ 50 mL ddH2O to the graduate cylinder to rinse out the remaining NaOCl and add to the glass media storage bottle.
6) Mix well and store at 4ºC until needed.

Note: This bleach should be made four days before use.

K medium solution recipe (per 500 mL):

1) Combine the following into a graduated cylinder:

- 51 mM NaCl (5.1 mL of 5 M NaCl)
- 32 mM KCl (16 mL of 1 M KCl)
- 3 mM $CaCl_2$ (1.5 mL of 1 M CaCl2)
- 3 mM $MgSO_4$ (1.5 mL of 1 M MgSO4)

2) Mix the four salts with increasing water, then fill to 500 mL of ddH_2O.
3) Filter sterilize with Thermo Filter Unit (Cat #566-0020).
4) Add 1.25 µg/mL unfiltered cholesterol (125 µL of 5 mg/mL cholesterol) to the K medium solution after filtering.
5) Mix, label, and store for up to two weeks.

Note: Be sure to check the K medium before use for any floating particulate, which is a sign of contamination. If K medium is contaminated, make new K medium.

Sodium azide solution:

Add 6.5 g sodium azide in 2 L 1x M9

Section 1: Growing Strains

3d

1

Note

General notes on amplifying strains:

- Ensure that your source NGMA (nematode growth medium agarose) starved plate is no more than one month old before chunking. If your NGMA starved plate is older than one month, generate a newly starved source plate to use in the assay.
- All source NGMA plates should be stored at 15 °C.
- For picking each generation, use 6 cm plates from the same batch of NGMA and OP50 to account for variation in NGMA and *E. coli* effects on the assay.
- Ensure 6 cm NGMA plates are put at room temperature the night before picking to ensure that you will not be picking to cold plates that have condensation.
- Organize 6 cm NGMA plates containing the same strain into the same cardboard storage box when possible, stacking no higher than five high. Whenever possible, organize groups of strains into cardboard boxes such that similar strain names (*e.g.*, JU3291 vs. JU2391) are not in the same box to avoid potential strain confusion. Also, mark plates with similar strain names so that researchers will more easily notice the differences between the strain names.
- It is NECESSARY to monitor your strains throughout the whole HTPA process. If strains are growing slowly, you may leave the worms at 21.5 °C (or higher) the day/night before you pick them. Animals (*Caenorhabditis elegans* and *Caenorhabditis briggsae*) are reared under standard conditions, including generation zero of the HTLDA. Start monitoring at the first generation to make subsequent picking as easy as possible. If animals are growing faster or slower, they can be moved to a lower or a higher temperature, respectively, for a generation or two. Be careful of temperature-sensitive strains or mutants, because they are most affected by shifting temperatures.
- Monitor the temperature in all incubators to ensure that the incubators match the temperature in the HTLDA protocol. iButtons are good for monitoring.

2

Using a sterile, autoclaved spatula, chunk from a starved NGMA (nematode growth medium agarose) "**source**" **plate** onto two fresh OP50-spotted 6 cm NGMA plates. Store these 6 cm NGMA "**chunk**" **plates** in a 20 °C incubator.

Note

Ensure your starved NGMA **source plate** is no more than one month old before chunking. Aim to chunk a plate that yields about 50 adult worms, assessed by the number of arrested larvae on the **source plate**.

3 After 48:00:00, pipette 15 µL of bleach solution (*see Materials for recipe*) away from the *E. coli* lawn of a new 6 cm NGMA plate. Pick ten to twenty gravid hermaphrodites from the **chunk plate** into the bleach spot. Swirl gently with the pick to ensure all animals are removed from the pick. Store these 6 cm "**spot bleach**" **plates** upside down (lid up) in a 20 °C incubator. 2d

4 After 24:00:00, pick 20-30 L1s to a new labeled 6 cm plate for each strain. If the L1s did 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. Store these 6 cm "**generation one (G1) L1**" NGMA plates in a 20 °C incubator. 1d

Note

Keep your **spot bleach plates** along with your **G1 L1 plates** in case you need to go back to them in Step 5.

5 After 48:00:00, pick five late-stage L4 hermaphrodites to labeled 6 cm NGMA plates for each strain. These animals are distinguished by a distinct white half-moon shaped patch near the midsection of the animal, containing what looks like a classic Christmas tree where the vulva is developing. These plates are "**G1 L4"** plates. Pick at least three 6 cm **G1 L4 plates** per strain. Grow this generation of animals at 20 °C.

Note

Animals might have grown into young adults by this time, in which case, pick the youngest adult animals you can find. If you find males on the **G1 L1 plates**, avoid picking young adults from these plates. Check the **G1 L1 plates** one and two days after picking to ensure that they will be properly staged for the generation two pick.

Note

Depending on how many **generation two** (**G2**) **L4 plates** are needed in Step 6, one might need to modify the number of **G1 L4 plates** that are picked. Here are two examples of how one can determine how many **G1 L4 plates** to pick:

Example 1: If one needs to pick five NGMA plates per strain for their **G2 L4** pick, pick at least three **G1 L4** plates. Even though it may be possible to obtain five **G2 L4** plates worth of animals from one **G1 L4** plate, picking three **G1 L4** plates here safeguards against your single plate becoming contaminated or containing males at the next step, ending your assay.

Example 2: If one needs to pick 36 plates per strain for their **G2 L4** pick, pick at least five **G1 L4** plates in this step.

6 Three days later in the morning, pick five late-stage L4 hermaphrodites (look for the developing vulva in the crescent) of each strain to each plate for this generation. This pick is the **G2 L4** pick (AKA the “big pick”). **Try to pick as fast as possible.** You do not want one strain to be four hours older than the last strain. Do not pick males and avoid picking L4 hermaphrodites from plates with males if other plates have enough hermaphrodites. Grow the **G2 L4** pick at 21.5 °C . Check the 6 cm **G2 L4** plates for L2 and L3 animals after two days and L4 and young adults after three days.

Note

If one predicts that the **G2 L4** pick will take more than four hours, split up the picking between multiple people to speed up the picking process.

Based on calculations from previous experiments, each **G2 L4** 6 cm plate provides approximately 1,000 embryos for the average *C. elegans* strain (using hypochlorite preparation). Based on your experimental requirements, calculate the number of plates you need to pick.

For example:

An assay with eight strains and twelve conditions for each strain with three replicate bleaches and four technical replicates per condition, would require:

1) 12 conditions x 4 technical replicates x 3 bleaches/assays x 30 embryos = 4,320 embryos
2) 4,320 embryos / 1,000 embryos / 6 cm plate = Five 6 cm **G2 L4** plates (rounded up)

Therefore, for this example experiment, one should pick five 6 cm **G2 L4 plates** per strain to meet the experimental requirements.

In subsequent experiments, if you find that you are producing a vast excess or dearth of embryos compared to your experimental requirements for your strain(s) of interest, you should adjust the number of picked plates accordingly. The above calculations provide an approximate starting point.

7 In the afternoon of the **G2 L4** pick (*i.e.*, four days before bleaching (~84-90 hours, the same day as your generation **G2 L4** pick)), make fresh bleach solution for your assay (*see recipe in Materials section*). Store bleach solution in the dark at 4 °C until needed.

8 In the afternoon of the **G2 L4** pick, make 500 mL batches of fresh K medium for your assay (*see recipe in Materials section*).

At any point in the assay, if you notice your K medium has gone bad (any solute crashing out and appearing as white flakes or granules floating), make a new batch.

Section 2: Preparation of synchronized animals

9 Four days after picking the **G2 L4** plates, check the strains to ensure your worms are staged properly for the bleach. Plates are staged properly for the bleach when the population on the plate comprises mostly gravid adults, and adults have begun to lay embryos throughout the plate and have begun migrating away from what remains of the bacterial lawn.

10 Take your bleach solution out to room temperature (keep it in the dark) 30-45 minutes before you start washing your 6 cm plates, allowing the bleach to warm up a little bit helps more effectively bleach worms.

Note

Because of the variation between bleaches, it is highly recommended to perform multiple (three) bleaches per day. Make sure to pick enough 6 cm plates in the **G2 L4 pick** and make sure your independent bleaches are performed independently (not at the same time). If possible, bleach all strains for a bleach-assay together. If it is not possible, bleach the same set of strains together for each bleach-assay.

Follow instructions for conical bleaching in Steps 11 - 22.

11 Wash worms off plates into a labeled 15 mL conical tube. Pour M9 onto one 6 cm plate of Strain A, transfer the liquid to a second 6 cm plate of Strain A, transfer the liquid to a third 6 cm plate of Strain A, and finally transfer the liquid to a fourth 6 cm plate of Strain A. Wash off a maximum of four plates from one strain at a time.

Wash the same 6 cm plates of Strain A a second time, pouring M9 onto the last 6 cm plate and transferring M9 to each plate in the opposite direction as was done the first time.

Pour all M9 media plus worms into one labelled 15 mL conical tube per strain.

Note

It is easy to pour from the final 6 cm plate into the 15 mL conical with a little bit of practice. We typically pour ~ 2 mL of M9 onto one plate and transfer the same solution to four other 6 cm plates before pouring it into the conical. Repeat for all strains.

12 Spin down worms in 15 mL conical tubes at 254 g (Eppendorf 5810R, 1100 rpm) for 1 minute using a table-top clinical centrifuge.

13 Aspirate off the M9, being careful to not suck up any of your worms.

Once all strains are pelleted in a 15 mL conical tube and all M9 is aspirated off, proceed to the next step.

14 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 volume of bleaching solution accordingly. We use 4 mL of bleach for all smaller pellets.

Note

The freshness of the bleach solution matters. Ineffective bleaching solution (*e.g.*, kept at 4 °C longer than one month or kept at room temperature for longer than eight hours) will require more time to bleach worms and will not dissolve the worms uniformly.

14.1 Shake the tubes manually and vigorously. If multiple people are bleaching, try to ensure that each individual is shaking in approximately the same way to ensure greater consistency between strains bleached by multiple individuals.

After three minutes of shaking, check to see 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 there are only embryos 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.

Note

We have found it useful to shake to the beat of a song; use a playlist with songs that have the same beats per minute (bpm). We used 130 bpm. Shake by holding the tubes by the cap to avoid the transfer of warmth from the hands to the solution. You can bleach multiple strains at the same time, but depending on experience, you may want to start with only doing 4-5 strains at once. The most one should bleach at at time is 10 strains.

15 Once you find that only a few adults remain, move quickly to spin down the embryos at 254 g (Eppendorf 5810R, 1100 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.

Note

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.

16 Immediately decant off the bleach into the waste/pour-off receptacle carefully and quickly without disturbing the worm pellet. **It is critical to move as fast as possible from Step 16 to Step 21.**

17 Pour 10 mL of M9 from a 50 mL conical to each 15 mL conical tube.

18 Invert three times as you walk back to the clinical centrifuge.

19 Spin down the embryos at 254 g (Eppendorf 5810R, 1100 rpm) for 30 seconds.

20 Decant off the M9 into the sink/waste container carefully and quickly without disturbing the worm embryo pellet.

21 Repeat steps 16-20 twice with M9.

22 Pour 10 mL of K medium from a 50 mL conical to each 15 mL conical tube.

23 Invert three times as you walk back to the clinical centrifuge.

24 Spin down the embryos at 254 g (Eppendorf 5810R, 1100 rpm) for 30 seconds.

25 Decant off the K medium into the sink/waste container carefully and quickly without disturbing the worm embryo pellet.

26 After the final wash, resuspend the embryos in 3 mL of K medium. Adjust the volume of K medium to the number of plates you bleached. For 15 plates per bleach, we find 3 mL is a good volume. For fewer plates, 2 mL might be more appropriate.

27 Determine the titer of the embryos by counting the number of embryos in five replicates of 3 µL .

Pulse vortex, by starting and stopping vortexing every few seconds, each 15 mL conical of embryos for at least ten seconds to ensure embryos are homogenously dispersed in the K medium.

Note

If embryos are **too numerous** to count, you may dilute the embryo suspension further to ensure accurate counts.

If embryos are **too dilute**, spin down the embryos for 1 minute at 254g (Eppendorf 5810R, 1100 rpm) and carefully pipette off some of the K medium and repeat the embryo counts.

28 Adjust the resuspension liquid (K medium) to obtain the desired concentration (*e.g.*, 0.6 embryo per µl).

29 Pulse vortex, by starting and stopping vortexing every few seconds, each 15 mL conical of 0.6/µL embryos for at least ten seconds before pouring into a sterile reservoir.

30 Using a multi-channel pipette, pipette immediately and ensure proper mixing before pipetting into assay plates. For mixing, take 50 µL from the bottom of the trough and dispense it higher up on the side of the reservoir, repeat on the other side for a total of ten times (five per side).

Pipette 50 µL of embryo solution to distribute approximately 30 animals into each well of the tissue-culture treated 96-well flat-bottom plate (USA Scientific 96 well Cellstar Clear TC PLT*FLAT Cat #: 5665-5180 OR RPI 96 Well Assay Plate, Sterile, Flat Bottom with Lid, Individually Wrapped, 100 per Case, Cat #: 141380).

Note

Example: With 12-channel reservoirs, you can prepare a row of the 96-well assay plate in each reservoir.

31 Seal all 96-well plates with a gas-permeable sealing film (Fisher Cat #: 14-222-043).

Try to avoid wrinkles in the film and use a finger nail to seal edges of the plate and wells.

31.1 Place all 96-well plates in a humidity chamber.

To make a humidity chamber, place damp paper towels in a clean (bleached and rinsed) plastic box (IRIS USA CNL clear Latching Box, 6 Qt, 18 Count; or similar box).

31.2 After filling the humidity chamber with plates, close the chamber using the lid, and seal the chamber using parafilm.

> Note
>
> These chambers hold 18 plates, so scale up the number of boxes according to your experimental set up.

32 Shake overnight at 170 rpm at 20 °C (Excella E24R shaker or the INFORS HT Multitron shaker) in a freshly made humidity chamber.

Section 4: Feeding strains

33 To keep your 96-well plates clean and debris-free, conduct the following steps:

A) Approximately 30 minutes prior to feeding strains, place an air filter on the bench where you plan to feed strains. Keep this air filter on during the feeding process.

B) Place on a face mask and a lab coat.

C) Clean your bench and all used supplies with 10% bleach and 70% ethanol.

34 The morning after bleaching, check one or two 96-well plates that were aliquoted the previous
day. Visually inspect the plates for successful hatching of L1 larvae under a stereoscope.

If there was successful hatching (judged by nicely swimming L1s), continue with protocol. Also, check for leftover chunks/contamination from the bleach and crystals; you want your wells to be as clean and contamination-free as possible. Crystals often arise if M9 is not washed out by K medium.

35 Make food from frozen HB101 OD100 previously prepped.

If you are running many 96-well plates, start making food early in the morning.

Note

We recommend using the Iterative Batch Averaging Method (IBAT) to make your OD30 HB101 (Step 35.1) to feed the animals in the HTLDA. IBAT produces a sustainable food source that can be used across HTLDAs.

For example, if 10 mL of HB101 OD100 food is needed to feed animals in 96-well plates, take one mL from ten unique HB101 food preps, allowing you to average food prep effects across ten batches.

IBAT reference: **https://pubmed.ncbi.nlm.nih.gov/34156835/**

35.1 Dilute freshly thawed OD100 HB101 to a concentration of OD30 with K medium. This dilution should be made up in an autoclaved Pyrex media storage bottle.

35.2 Add kanamycin (US Biological, Cat #: K0010) to the OD30 food mix. The final kanamycin concentration in the well should be 50 µM (final concentration in the food dilution, prior to plating, will be 150 µM).

That is 18.75 µL of 80 mM stock per 10 mL of solution.

36 If you are testing the effects of drugs or toxicants on worms, prepare drug dilutions and add to OD30 HB101 food with kanamycin.

36.1 If you are testing the effects of drugs or toxicants on worms, ensure you also prepare the appropriate control conditions for those drugs or toxicants. In our experience, we use either fresh milli-Q water or DMSO.

37 Start with one or two 96-well plates. Make sure to resuspend the HB101 food before you add it to
the 96-well plates by swirling (if a large volume of OD30 HB101 is made in an autoclaved Pyrex
media storage bottle) or vortexing (if using smaller batches of OD30 HB101 made up in a conical tube or otherwise vortexable container).

38 Steps 34 - 36 will describe how to feed your 96-well plates of animals to measure animal development in a quantitative fashion.

Your goal is to feed one 96-well plate on average every 3-4 minutes. Use a timer to make sure you do not forget. Ensure that the HB101 food is mixed into each well. Every well

should look cloudy.

Note

It is easiest to see when you look at the wells from above and on a black background.

38.1 Mix enough HB101 and drug (drug is optional) (at the appropriate concentration) for three replicate 96-well plates. Pour this mixture into a single channel trough (USA Scientific: ChannelMate 100 mL Reservoir, Cat #: 1306-1010).

Using a micropipette, add 25 µL of food (+ drug) to every well that has worms in it, for each of the replicate plates.

39 Seal with a gas permeable sealing film (Axygen BF-400 gas permeable sealing film: Fisher Cat #: 14-222-043), label the plate top with the plate number and any condition information, and record the time that you added the food solution.

40 Put the 96-well plates back in the humidity chamber once they are fed. When done for the day, make sure your paper towels are still damp and parafilm the chamber shut. Return the humidity chamber to the incubator and ensure the shaking restarts.

41 Shake for 48 hours at 180 rpm at 20 °C (Excella E24R shaker or the INFORS HT Multitron shaker).

Section 5: Scoring animals

42 **EXACTLY** 48-hours after a 96-well plate was fed and drugged, add 167 µL of 50 mM sodium azide (*see Materials for recipe*) in M9 buffer to each well twice 10 minutes before scoring.

42.1 Use a single-channel trough to hold the sodium azide solution and dispense the azide using either an 8- or 12-channel pipette. For each step, dispense at opposing angles to each well.

Note

Opposing angles help alleviate heterogeneous swirling of well contents and uneven illumination of images. Also, incubating in sodium azide for too long (> 15-20 min) might cause uneven illumination as the bacteria can clump.

43 Follow your lab's protocol to image each 96-well plate on the ImageXpress.