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TITLE:

Culture and Assay of Large-Scale Mixed-Stage *Caenorhabditis elegans* Populations

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SUMMARY:

To use *Caenorhabditis elegans* (*C. elegans*) in omics research, a method is needed to generate large populations of worms where a single sample can be measured across platforms for comparative analyses. Here, a method to culture *C. elegans* populations on large-scale culture plates (LSCPs) and to document population growth is presented.

ABSTRACT:

Caenorhabditis elegans (*C. elegans*) has been and remains a valuable model organism to study developmental biology, aging, neurobiology, and genetics. The large body of work on *C. elegans* makes it an ideal candidate to integrate into large-population, whole-animal studies to dissect the complex biological components and their relationships with another organism. In order to use *C. elegans* in collaborative omics research, a method is needed to generate large populations of animals where a single sample can be split and assayed across diverse platforms for comparative analyses.

Here, a method to culture and collect an abundant mixed-stage *C. elegans* population on a large-scale culture plate (LSCP) and subsequent phenotypic data is presented. This pipeline yields sufficient numbers of animals to collect phenotypic and population data, along with any data needed for omics experiments (i.e., genomics, transcriptomics, proteomics, and metabolomics). In addition, the LSCP method requires minimal manipulation to the animals themselves, less user

preparation time, provides tight environmental control, and ensures that handling of each sample is consistent throughout the study for overall reproducibility. Lastly, methods to document population size and population distribution of *C. elegans* life stages in a given LSCP are presented.

INTRODUCTION:

C. elegans is a small free-living nematode that is found throughout the world in a variety of natural habitats[1]. Its relative ease of growth, fast generation time, reproduction system, and transparent body make it a powerful model organism that has been widely studied in developmental biology, aging, neurobiology, and genetics[2,3]. The copious work on *C. elegans* makes it a prime candidate to use in omics studies to comprehensively link phenotypes with complex biological components and their relationships in a given organism.

To use *C. elegans* in collaborative omics research, a method is needed to generate large mixed-stage populations of animals where a single sample can be split and used across diverse platforms and instruments for comparative analyses. Creating a pipeline to generate such a sample requires keen awareness of diet, environment, stress, population structure, and sample handling and collection. Therefore, it is crucial to have standard and reproducible culturing conditions integrated into large-scale pipelines. In *C. elegans* research, two traditional methods are used to culture worms - agar Petri dishes and liquid culture[4].

Historically, when large quantities of *C. elegans* are needed, they are grown in liquid culture[4]. The steps involved in generating a large population of worms in liquid culture require multiple handling steps that often include bleach synchronization to rupture gravid adult cuticles, releasing embryos to achieve the desired population size. However, when bleach synchronization is used, population growth is dependent on starting census size and, therefore, effects subsequent growth and population numbers. In addition, *C. elegans* strains vary in their cuticle sensitivity, exposure time, and stress response to bleach synchronization making it difficult to assay many strains at a time[5-9].

Additionally, worm growth in liquid culture requires a couple of transfer steps as it is often recommended to grow just one generation of worms before harvesting because overcrowding can easily occur if grown for multiple generations and lead to dauer formation despite the presence of food[10]. Dauer formation occurs through small signaling molecules such as ascarosides, often referred to as "dauer pheromones"[11-14], are released into liquid media and effect the growth of the population. Furthermore, growing large worm populations in liquid culture leads to excess bacteria accumulation in the culture, creating difficulties when a clean sample is needed for downstream phenotypic assays. Lastly, when a liquid culture becomes contaminated, it is more difficult to maintain as fungal spores or bacterial cells are easily dispersed throughout the media[15].

The other traditional method of growing *C. elegans* is on agar Petri dishes. Commercially available Petri dishes allow one to easily grow multiple generations of mixed-stage worms without the rapid effects of overcrowding and high dauer formation as seen in liquid cultures. However, a

disadvantage to worm growth on traditional agar Petri dishes are that the largest commercially available Petri dish does not yield large worm populations for an omics study without adding in a bleach synchronization step. In summary, culturing mixed-stage populations of *C. elegans* on agar Petri dishes is more suitable for collecting omics data, but we required a method to generate very large population sizes without liquid culturing.

Here, we present a method to culture and collect large mixed-stage *C. elegans* populations large scale culture plates (LSCP). Collecting samples through this pipeline yields enough sample to gather phenotypic and population data, along with any data needed for omics experiments (i.e., genomics, transcriptomics, proteomics, and metabolomics). In addition, the LSCP method requires minimal manipulation of the animals, less user prep time, provides tight environmental control, and ensures that handling of each sample is consistent throughout the study for overall reproducibility.

PROTOCOL:

1. Sterilize LSCP and equipment

1.1. Prepare glass LSCPs by handwashing, followed by dishwashing, and subsequent autoclaving to ensure glassware is free of contaminants before starting the experiment. Store autoclaved LSCPs in a clean dry location until in use.

NOTE: Ensure LSCPs are dishwasher and autoclave safe. Ensure the LSCP lids are dishwasher safe.

1.2. Prepare LSCP lids by handwashing followed by dishwashing. Store LSCP lids in a clean bin until needed.

1.3. On the day Nematode Growth Media Agarose (NGMA) is prepped, wipe LSCP lids with 10% bleach solution twice, followed by 70% ethanol. Once wiped down with 10% bleach and 70% ethanol, keep LSCP lids in a clean bin in the laminar flow hood where the NGMA will be prepped.

2. Prepare nematode growth media agarose (NGMA)

2.1. Prepare NGMA by combining the following reagents into an autoclaved 2 L Erlenmeyer flask with stir bar on a stir plate: 2.5 g peptone, 3 g NaCl, 7 g agarose, 10 g agar, and 975 mL sterile water[16]. Ensure that the total volume equals 1 L. Tape a foil cap to the flask.

NOTE: The preparation steps for the NGMA as described here will yield enough material for 2.5 LSCPs. The protocol can be tailored to the needed LSCP batch size in a given experiment.

2.2. Autoclave on liquid cycle at 121 °C and 21 p.s.i. for 45 min.

2.3. Turn on the water bath and set to 50 °C. Bring the autoclaved NGMA to the water bath to cool to 50 °C.

2.4. Bring 2 L Erlenmeyer flask of NGMA into the hood or cleaned space and set on a stir plate. Use a thermometer to track NGMA temperature.

2.5. After the NGMA has reached 50 °C, add the following in the order listed with a sterile disposable pipette inside the hood or cleaned space: 25 mL of 1 M KH_2PO_4 (K phosphate buffer), 1 mL of cholesterol (5mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of nystatin (10mg/mL), and 1 mL of streptomycin (100 mg/mL)[16].

2.6. Pour 400 mL of NGMA into a sterile glass LSCP, approximately 1.3 cm deep, allow the LSCP to solidify on flat surface in the hood and place the autoclaved foil lid back on the LSCP.

2.7. Once agar is set, remove the foil, and place a clean air-tight lid onto LSCP and move to 4 °C for storage. Store NGMA in LSCPs at 4 °C until in use and use within 5 days.

3. Generate *E. coli* food for NGMA on LSCP

3.1. To generate a stable food source, generate batches of HT115 (DE3) *E. coli* using a small batch averaging concept consistent with the central limit theorem[17]. Store at -80 °C. When needed pull *E. coli* bacterial stock(s) from -80 °C to thaw[18].

NOTE: In this protocol, *E. coli* bacterial stocks were grown in a bioreactor. At the end of the culture growth, the culture was diluted 1:50, and the measured OD_{600} was 0.4. Thus, the culture had an effective OD_{600} of 20. Bacteria were pelleted, weighed, and resuspended in K-medium at a concentration of 0.5 g/mL (wet weight), transferred into 2 mL aliquots, and frozen[19].

4. Bacterial lawn on NGMA

4.1. Bring NGMA LSCPs out of 4 °C to room temperature (RT) for several hours before spreading the bacterial lawn to allow the entire dish to reach RT.

4.2. Pull out needed *E. coli* bacterial stock(s) from -80 °C to thaw[18].

4.3. Dilute *E. coli* bacterial stock(s) with 2 mL of sterile K-medium to achieve 0.5 g *E. coli* in 4 mL per NGMA LSCP. Carefully pipette 4 mL of *E. coli* in the middle of the NGMA LSCP.

4.4. Use a sterile spreader to spread bacteria into a rectangle leaving approximately 3.8 cm of room around the edges of the NGMA *E. coli* free.

4.5. Leave the NGMA LSCP with *E. coli* in the hood with the fan on for 1 h to ensure the *E. coli* suspension fully dries.

4.6. Once the bacterial lawn is dry, push the lid on tightly and store at 4 °C until used.

5. Chunk worms to reduce stress and age variability across samples

5.1. Streak worms from a frozen worm stock to a newly seeded 6 cm plate[4]. This plate will serve as the "master chunk" plate.

NOTE: Chunking is an optimal method to transfer worms from a homozygous strain[20]. If a strain is heterozygous or needs to be maintained by picking and mating, chunking is not advisable. Chunking frequency may need to be optimized depending on worm genotypes used, temperature chosen for growth, and downstream steps.

5.2. After the master chunk plate is full of healthy gravid adults (approximately 3 days) with plenty of *E. coli* lawn still present, follow standard *C. elegans* chunking guidelines as described in WormBook to produce four total chunk plates[4].

5.3. Store all chunk plates in a controlled temperature (CT) room at 20 °C unless otherwise specified for growth.

NOTE: If users of this protocol do not have access to a CT room as described here it is recommended to use either a small incubator where the temperature can be controlled or a designated room where environmental conditions can be controlled as much as possible. If neither of these alternate options are available, note that variation in sample growth may be greater.

5.4. Once many gravid adults are observed in the 4th chunk plate, move on to Step 6.

6. Spot bleaching gravid adults onto LSCP

NOTE: This bleaching technique is used to eradicate most contaminants and dissolve the cuticle of the hermaphrodites releasing embryos from the adult worm. The bleach solution will soak into the NGMA prior to the embryos hatching.

6.1. Bring LSCPs out to RT for several hours prior to spot bleaching worms.

6.2. Prepare a 7:2:1 ratio of ddH_2O : bleach : 5 M NaOH. Make this alkaline hypochlorite solution fresh just before use.

NOTE: Use the same stock of bleach and NaOH throughout the duration of a given experiment to avoid bleach batch affects. Bleach used in this protocol was 5-10% sodium hypochlorite.

6.3. Light a Bunsen burner and flame a worm pick before proceeding. Scoop fresh *E. coli* onto a sterile pick from the edge of the bacterial lawn on the LSCP.

6.4. Pick a single gravid adult from the 4th chunk plate for spot bleaching.

6.5. Pipette 5 µL of the alkaline hypochlorite solution into one corner of the LSCP away from the *E. coli* lawn.

6.6. Place the picked gravid adult into the 5 µL alkaline hypochlorite solution. Tap the nematode to help disrupt the cuticle and release eggs.

6.7. Repeat steps 6.4 – 6.6 for a total of 4x and place 5 gravid adults evenly around the *E. coli* lawn. Pick all 5 gravid adults from the same 4th chunk plate to ensure nearly genetically isogenic individuals are added to a given sample.

6.8. Place the lid back onto the LSCP.

6.9. Repeat steps for all LSCPs.

7. Worm growth in controlled temperature (CT) room

7.1. Following spot bleaching, place the lid tightly onto the LSCP and place in the CT room set to 20 °C with constant airflow and a 12L:12D photoperiod (12 h light and 12 h darkness).

7.2. Note the time and position where the sample was placed in CT room.

NOTE: Position within the room should always be documented to record any environmental differences samples that could potentially encounter while growing. Once the sample is in the CT room, it should remain in its assigned spot undisturbed. Do not open the lid of the LSCP in the CT Room to decrease the chance of contamination.

7.3. Take LSCP to a microscope, outside of the CT room, to observe the population growth and density.

NOTE: Each *C. elegans* strain and sample will vary in its growth, so monitor samples closely. While it is recommended to not disturb the growth of the LSCP while in the CT Room, LSCPs were transported out of the CT Room and lids were opened every 2 days to monitor sample growth. Taking the sealed lids off the LSCPs every 2 days also allows for O_2 to flow into the LSCP.

7.4. Before harvesting ensure that the LSCP has become full of a large population of worms. Use the following criteria to decide if the LSCP is ready to be collected.

7.4.1. Ensure that the LSCP is full of gravid adult worms.

7.4.2. Ensure that the plate contains a large population size (i.e., worms cover the entire surface of the agar).

7.4.3. Ensure that the plate does not have many eggs on the surface of the agar (i.e., the maximum number of worms should have hatched).

7.4.4. Ensure that the plate has minimal to no *E. coli* left, indicating that the worms would starve and generate dauer larvae if left on the plate for an additional two days.

NOTE: Although most LSCPs are ready to harvest between 10 to 20 days, depending on strain and sample, check each LSCP frequently upon establishing this protocol to determine normal harvest times.

7.5. Clean gloves and area with 70% ethanol between handling LSCPs to avoid cross contamination between strains.

8. Harvesting the LSCP sample

8.1. Turn on and allow the centrifuge to cool to 4 °C prior to harvesting samples.

8.2. Prepare three 50 mL conical tubes with 50 mL of M9 solution per LSCP to be harvested.

8.3. Label one 15 mL conical tube per LSCP.

NOTE: All centrifugation steps are performed in the 15 mL conical tube, because worms tend to pellet well in these tubes.

8.4. Pour 50 mL of M9 solution (from one 50 mL conical tube in Step 8.2) onto the LSCP surface and swirl around to ensure that M9 covers the entire NGMA surface.

8.5. While M9 sits on the LSCP surface, prime a sterile serological pipette with M9.

NOTE: By priming the sterile serological pipette with M9 this ensures that less worms stick to the inside of the plastic pipette, preventing sample loss.

8.6. Tilt the LSCP so M9 and the worm population gather in one corner of the LSCP.

NOTE: The mixture of M9 solution and worms from the LSCP will be referred to as the “worm suspension” in downstream steps.

8.7. Using a primed serological pipette with an automatic pipettor, pipette worm suspension and place into the original 50 mL conical tube. Once 50 mL of worm suspension is collected, place the conical tube on a rocker to disrupt bacteria clumps and debris.

8.8. Repeat steps 8.4 - 8.7 collecting 150 mL of worm suspension per LSCP.

8.9. Transfer 15 mL of worm suspension, from one of the three 50 mL conical tubes, by pouring to a labeled 15 mL conical tube set aside in step 8.3. Centrifuge the 15 mL conical tube at 884 *x g* for 1 min at 4 °C. The majority of worms will pellet at the bottom of the tube.

8.10. Aspirate off the supernatant ensuring to not disturb the worm pellet.

8.11. Continue adding approximately 13 mL of worm suspension to the same 15 mL conical tube repeating steps 8.10 and 8.11 until all 150 mL of worm suspension are consumed. Invert the tube and disturb the pellet between centrifugations to wash and aspirate off as much bacteria and debris as possible.

NOTE: At this step, the contents from all three 50 mL conical tubes are condensed in a single 15 mL tube.

8.12. Add 10 mL of clean M9 to the 15 mL conical tube and agitate the worm pellet by inverting. Centrifuge the 15 mL conical tube at 884 *x g* for 1 min at 4 °C. Aspirate off the supernatant ensuring to not disturb the worm pellet. Repeat twice.

NOTE: If there is a great amount of debris or bacteria in sample, repeat step 8.12 until the sample is clean.

8.13. Once the sample is clean, add ddH_2O to the worm pellet for a total of 10 mL of ddH_2O and worms. Agitate the worm pellet by inverting. Move quickly into step 9.1, as worms must remain in ddH_2O for 5 min or less to avoid osmotic stress.

NOTE: Suspending worm pellets in ddH_2O is the preferred solvent for downstream omics steps. Worms can be suspended in other solvents or buffers if they are compatible with a given experimental workflow.

9. Estimate population size

NOTE: Move through Steps 9.1 – 9.7 quickly. The mixture of ddH_2O and worms from step 8.13 are referred to as the "worm sample" in subsequent steps.

9.1. Prior to pipetting the worm sample, prime pipette tip to be used with M9 to avoid worms sticking to the inside of the plastic pipette preventing sample loss and reducing count variation.

9.2. Take a 100 µL aliquot of worm sample and dilute it into 900 µL of M9. Mix well and make a serial dilution (1:10, 1:100, 1:1000). Repeat this step twice to achieve a total of three sets of aliquot replicates.

NOTE: Pipetting worms can cause high variability in sample population counts. Ensure that the worm sample is homogenous prior to pipetting the desired aliquot.

9.3. Set the 15 mL conical tube on a rocker to continue moving culture while aliquots are counted.

9.4. Ensure the worm sample is well mixed and homogenous. Pipette 5 µL from the 1:10 worm sample, dispense it onto a microscopy slide, and count the number of worms. If this number is <~50, then also count the 1:100 and 1:1000 dilutions. If it is more than 50, move to the next serial dilution.

NOTE: If too many worms cannot be accurately counted, use the next serial dilution for counting instead.

9.5. Count each aliquot replicate of each dilution 3x. At the end of counting, for most cultures, 9 total counts will be documented (i.e., 3 total counts for each aliquot replicate).

9.6. Average the dilution counts to determine the estimated population size of the worm sample. These dilution counts will determine the volume of worm sample needed to create desired aliquot size for omics steps.

NOTE: In this experiment, aliquots of approximately 200,000 mixed-stage worms were generated. In addition, one aliquot of approximately 50,000 mixed-stage worms was set aside for sorting in a large particle flow cytometer (described in Step 10).

9.7. Once the worm sample has been split into appropriate aliquots, flash freeze in liquid nitrogen and store the sample at -80 °C.

NOTE: Do not freeze the aliquot intended for large particle flow cytometry.

10. (Optional) Prepping sample for large particle flow cytometry

NOTE: Steps 10, 11, and 12 are the authors' preferred method to record sample growth (i.e., population size and population distribution of *C. elegans* life stages) and determine success of a culture. Users of this protocol can substitute optional Steps 10, 11, and 12 with their own metrics of growth success. Steps 10, 11, and 12 are described here for two reasons; First, so users who have equipment used in Steps 10, 11, and 12 can replicate these steps and secondly, to show validation of this growth method. Step 9 above provides a good estimation of total number of worms to determine aliquot sizes, and step 10 is a more quantitative metric to estimate the number and population distribution of worms in a given sample.

10.1. Bring the aliquot of approximately 50,000 mixed-stage worms (set aside in Step 9.6) up to 10 mL total volume in M9 solution.

10.2. Make a solution composed of 1 mg/mL of *E. coli* and a 1:50 dilution of 0.5 µM red fluorescent microspheres[19].

10.3. Add 200 µL of this solution to the 10 mL of mixed-stage worms in M9 and incubate while rocking for 20 min.

10.4. After 20 min, centrifuge the 15 mL conical tube at 884 *x g* for 1 min at 4 °C.

10.5. Aspirate off the supernatant ensuring to not disturb the worm pellet.

10.6. Wash the worm pellet twice with M9 solution to eliminate excess bacteria and red fluorescent microspheres.

10.7. Add 5 mL of M9 to the worm pellet and ensure that the pellet looks clean. If the pellet is clean, add 5 mL of M9 with 50 mM sodium azide to both straighten and kill the worms for accurate counting and sizing[21].

10.8. Document time and date when sodium azide is added to sample.

10.9. Set the sample aside on rocker until needed for large particle flow cytometry.

NOTE: Sodium azide is known to affect nematode physiology (i.e., body length, metabolism, and thermotolerance). Therefore, it is critical to note the time worms are exposed to sodium azide as many of these physiological affects happen within a matter of minutes[22]. Due to the known physiological effects of sodium azide on worms, this treatment will affect downstream image quality and should be considered.

11. (Optional) Documenting population distribution and prepping 384 well plate for imaging

NOTE: Step 11 uses a large particle flow cytometer (LPFC). Basic knowledge of a LPFC is assumed in this protocol. Other methods can be substituted to document the growth and population distribution of samples. Steps documented here are for users who plan to use a LPFC in their pipeline[23].

11.1. Turn on, clean and prime the LPFC, and allow laser(s) to warm for 1 h prior to sorting samples.

11.2. After the laser has warmed, open the "**Histogram**" profile and scale to a Time of Flight (TOF) of 2050.

11.3. Add a bar region to the "**Histogram**" spanning a TOF range of 100. The first bar region covers a TOF of 50-150.

11.4. Continue to create twenty bar regions each spanning a TOF range of 100. These bar regions will span the entire TOF range from 50 – 2050. See **Supplementary Table 1** for the exact gated regions to use across the TOF distribution.

11.5. Save this Histogram set up as an "**Experiment**" to use in future LPFC runs.

11.6. Select calibrated 384 well plate or calibrate instrument to a 384 well plate to dispense objects into.

11.7. Once in the calibrated 384 well plate template, set template to dispense 20 gated objects into four wells (four technical replicates of each gated region) for each of the 20 bar regions created during Steps 11.3-4. See **Supplementary Table 2** for an example layout of how to dispense worms into the 384 well plate.

11.8. Transfer sample from step 10.9 into a 50 mL conical tube and add additional M9 solution to achieve approximately 40 mL total volume.

11.9. Start automatically sorting the sample per the parameters set in step 11.7 while continuously stirring the sample to prevent settling and simultaneously dispensing objects from the sample into the calibrated 384 well plate.

NOTE: Ensure the flow rate of the LPFC is operating between 15-20 objects per second and specify no doubles to be sorted.

11.10. Once entire sample has been sorted and the maximum number of gated regions have been dispensed into the 384 well plate, take the sample off LPFC and clean instrument.

NOTE: When larger TOF regions are reached, it may become challenging to continue to fill the 384 well plate due to low event counts in that TOF region. Fill as many of the gated regions as possible to get the best idea of where *C. elegans* life-stages fall within the LPFC distribution prior to running out of sample.

11.11. Place a sealed film on top of the 384 well plate until imaged.

NOTE: Image plate as quickly as possible after sorting since samples are treated with sodium azide[22]. Red fluorescent microspheres can be seen in the collected LPFC data files (i.e. PH Red data in output text file) based on the level of red fluorescence emitted in each sorted object to help identify which objects are alive worms, dead worms, dauers, or junk[24].

12. (Optional) Imaging 384-well plate

NOTE: Step 12 uses a plate-reading micro confocal microscope. Basic knowledge of a micro confocal microscope is assumed in this protocol. Other methods can be substituted to document the growth and population distribution of samples.

12.1. Using a plate-reading micro confocal microscope with a 20x lens.

12.2. Open the "**Objective and Camera**" tab and set to "**10x Plan ApoLambda**" mode.

12.3. Open the "**Camera Binning**" tab and set to "**2**".

12.4. Open the "**Sites to Visit on Plate**" tab and set to "**4**" sites per well and "**overlap sites 10%**" to later stitch together images.

12.5. Open "**Wavelength**" tab and set to "**Brightfield 1**".

12.6. Open "**Illumination**" tab and set to "**Transmitted light, bright sample**".

12.7. Place the 384 well plate in the microscope and set the "**Z Stack**" to "**Calculate Offset**" and find the proper focal plane for the samples in the 384-well plate.

12.8. Run the 384 well plate on the micro confocal microscope collecting four images per well.

12.9. Montage the four images together to create one image per well.

REPRESENTATIVE RESULTS:

Growth of *C. elegans* using the LSCP method yields an average of approximately 2.4 million mixed-stage worms per sample over 12.2 days. Growth of *C. elegans* using the LSCP method enables users to generate large mixed-stage populations of *C. elegans* with little handling and manipulation of the animals, which is ideal for large-scale omics studies **(Figure 1)**. Once a LSCP has become full of adult worms, reached a large population size, and has minimal bacteria left, users can harvest and estimate the population size. This point can also serve as a quality control by evaluating whether the population is sufficient to use in an omics pipeline (**Figure 2**). Population dynamics are dependent on the strain itself, behavior of the strain (i.e., burrowing strains tended to have lower worm recovery), and growth success (i.e., contamination). The LSCP method was tested on 15 strains of *C. elegans* containing a mixture of *Caenorhabditis* Genetics Center (CGC) mutants and *Caenorhabditis elegans* Natural Diversity Resource (CeNDR) wild strains[25], strain genotypes are described in **Supplementary Table 3**.

The LSCP method yielded population sizes from approximately 94,500 to 9,290,000. The mean population size within the reference strain, PD1074, and across strains was approximately 2.4 million worms **(Figure 3)**. No significant differences were found in estimated population sizes between *C. elegans* strains over the course of an average of 12.2 LSCP growth days **(Figure 4)**. PD1074 LSCPs took between 10 – 14 days to grow to a full mixed-stage population. The mean growth time across PD1074 was 10 days. The slowest growing strain grew for a maximum of 20 days, and the fastest growing strain grew for a minimum of 10 days (**Figure 4**).

Therefore, using this LSCP method, users can easily integrate new strains of interest into a study with little knowledge of developmental timing and background expertise. Note that strains and phenotypes that have to be maintained by picking, have fecundity defects, are heterozygous, or have growth defects may not work well in this pipeline.

Large particle flow cytometry and imaging of samples allows users to document population distribution. A wide variety of platforms can be used to measure successful population growth.

For reproducible omics measurements, it is important to grow consistent cultures. The metrics of culture reproducibility are number of worms and a consistent size distribution for a given strain. We show the sample distribution for the reference strain, PD1074 – a variant of the original N2 Bristol strain, using the LPFC[23,26] and micro confocal microscope images as proxies for growth success. As worms were measured from the L1 stage through gravid adult on the LPFC distribution **(Figure 5),** subsequent imaging **(Figure 6)**, and the variation in the population distribution across samples **(Figure 7),** we can see that this pipeline generated a mixed-stage population of *C. elegans*.

To take a closer look at the population distribution of our mixed-stage samples, we looked at the distribution of 35 PD1074 LSCPs by looking at the percent of worms that fall within each region across the entire Time of Flight (TOF) (i.e., body length) distribution **(Figure 7A, B)**.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the LSCP worm growth pipeline. (**A**) Once received in the lab, all strains were prepared and frozen for long-term storage at -80 °C[2]. (**B**) A "master chunk" plate was prepared from a frozen worm stock and stored at 15 °C to be used for no longer than one month. (**C**) Each sample went through four successive chunking steps to reduce generational stress prior to growing on the LSCP. (**D**) 5 individual gravid adults were picked from the "chunk 4" 6 cm plate in Step (D) and spot bleached on five given areas of the LSCP. (**E**) The LSCP was placed in a Controlled Temperature room and grown at 20 °C until the LSCP was full of adult worms, reached a large population size, and had minimal bacteria left. (**F**) The worm population was harvested and collected for downstream steps. (**G**) Aliquots were created from the LSCP and were flash frozen for downstream desired applications.

Figure 2: Overview of LSCP harvesting and estimating population size. (**A**) 50 mL of M9 were used to wash worms off the NGMA surface. Worm suspension was pipetted into a 50 mL conical tube. Step (A) was repeated twice. (**B**) 15 mL of worm suspension was poured into a new 15 mL conical tube. Worms were pelleted by centrifuging. M9 + debris were aspirated off without disturbing worm pellet. Step (B) was repeated until all 150 mL of worm suspension were collected. (**C**) The worm pellet was washed and centrifuged three times with M9 to eliminate remaining debris. Once the sample was clean, the worm pellet was resuspended in 10 mL of ddH_2O. (**D**) A serial dilution of the sample was created to estimate worm population size. The dilution factor(s) that allowed worms to be counted accurately were used. The dilution factor(s) used changed depending on the population size of the LSCP. (**E**) Once the dilution factor(s) were chosen, all worms from all three aliquot replicates of that dilution were pipetted onto a clean slide and worms were counted under a dissecting microscope. (**F**) Sample was split into appropriate-sized aliquots.

Figure 3: LSCP method generated on an average a population of 2.4 million mixed-stage worms. The LSCP yields population sizes in the smallest population growths at around 94,500 and at the biggest population growths at around 9,290,000. The mean population size across all strains was 2.4 million worms. Bars underneath *C. elegans* strain names indicate whether a strain is a CGC

mutant or CeNDR natural isolate. LSCP sample size is displayed for each strain. Comparisons for all pairs using Tukey's HSD Test were performed. No significant differences were observed between estimated population sizes across *C. elegans* strains ($F(14,108) = 0.7$, $p = 0.77$). Colored bars indicate standard color displays for respective *C. elegans* strain representation.

Figure 4: LSCP method generated large mixed-stage populations of worms in 10 – 20 days. A given *C. elegans* LSCP grew until the sample was full of adult worms, reached a large population size, and had minimal bacterial lawn left. LSCPs took between 10 – 20 days to grow to a full mixed-stage population, depending on the strain. The mean growth time across the strains was 12.2 days. LSCP sample size is displayed for each strain. Each error bar was constructed using 1 standard deviation from the mean. Levels not connected by same letter are significantly different. Comparisons for all pairs using Tukey's HSD Test. A significant difference was found in the amount of growth time on LSCP needed across *C. elegans* strains ($F(14,108) = 8.8$, $p < 0.0001$*). Colored bars indicate standard color displays for respective *C. elegans* strain representation.

Figure 5: Mixed population and growth measurement of the wild-type reference strain, PD1074. A representative LPFC distribution of one LSCP growth of the wild-type reference strain, a variant of the original N2 Bristol strain, (PD1074) documents the size distribution and event counts of a mixed-stage population. The x-axis displays the length (Time of Flight, TOF) of the worms sorted. The y-axis displays the optical density (optical extinction, EXT) of the worms sorted. Each data point is a worm that was documented in the sample. Each TOF region that was used for image analysis is displayed in a different color. Twenty TOF regions were created (R2 – R21) ranging from a TOF of 50 to 2050. Details on each TOF region can be found in Supplementary Table 1.

Figure 6: Images of worms sorted from TOF regions ranging from R2 – R12 show the PD1074 LPFC distribution. In region R2, L1 worms can be identified and in region R9 predominately gravid adults are identified, spanning the two developmental larval extremes giving us approximate regions within the flow cytometer distribution of where stages are expected in the distribution. Scale bar represents 1 mm. Representative images were taken from the LPFC distribution displayed in Figure 5, and the colored boxes correspond to regions from Figure 5.

Figure 7: Population distribution across time of flight (TOF) regions in the wild-type reference strain, PD1074. Distribution of worms across the entire TOF region showing in the regions where worms were found. Each PD1074 LSCP is represented as an individual color. (**A**) The x-axis shows the twenty TOF regions (R2 – R21) observed and counted for the LSCP, displaying the entire size distribution. The y-axis shows the percent of worms from a given LSCP that had a body size that fell into a given TOF region. (**B**) As a smaller fraction of the worm population falls between the R7-R21 regions, the log of the percent of worms that fell within each region was taken to display the population distribution. The x-axis displays the R7-R21 TOF regions. The y-axis displays the log of the percent of worms from a given LSCP that had a body size that fell into a given TOF region.

Supplementary Figure 1: Mean daily temperature (°C) of growth conditions under which the LSCP was grown and handled. Reported temperatures of the Controlled Temperature (CT) room were documented and collected throughout the six-month span of sample growth and collection. The average daily temperature is reported here. No significant differences were observed between the temperature in which the LCSP grew during the duration of the project ($F(5,24) = 2.59$, $p = 0.0524$). The entire temperature difference spanned no greater than 0.003 °C throughout the six-month duration of sample growth and generation.

Supplementary Table 1: TOF gated regions used to sort worms into 384-well plates for imaging. Binned regions were created to span a TOF of 100 across the entire TOF distribution from 50 – 2050. Gated regions can be changed and optimized to suit your needs. Each TOF region that was used for image analysis is displayed in a different color.

Supplementary Table 2: 384-well plate template of TOF regions and replicate layout. Every sample was sorted into a 384-well plate for imaging. Four replicates were created for each region selected for sorting. Gated regions can be changed and optimized to suit your needs. See Supplementary Table 1 for specific gated regions created and used in this protocol. Each TOF region that was used for image analysis is displayed in a different color.

Supplementary Table 3: *C. elegans* strains used in this protocol contain a mixture of CGC and CeNDR strains. The strain, genotype, strain source, and details are described in this table.

DISCUSSION:

A variety of vessels can be used as a LSCP. In this protocol, a standard glass baking dish was used. The LSPCs in use had outer dimensions of 35.56 x 20.32 cm, inner dimensions of 27.94 x 17.78 cm, and approximately 4.45 cm deep and came with a fitted lid. Thus, the amount of bacteria used here has been optimized for a LSCP with the above dimensions to yield a large population of mixed-stage worms. Bacterial volume and concentration can be adjusted to fit the experimental needs.

Contamination by mold, fungi, or other bacterial sources can occur at any step in the LSCP method, so handle samples with care. Prior to starting any step in the protocol, ensure that the working space is cleaned with 70% ethanol and 10% bleach. If available, treat used areas with UV light for 30 min and turn on a HEPA air-filter 30 min prior to starting each step.

By growing the LSCP in a controlled setting (i.e., in a CT room set at 20 °C), the user can more easily track the growth of the sample and document potential contamination. If the surface of the LSCP becomes contaminated, either cut out the contamination when possible and let the sample continue to grow or discard the sample if the contamination is not possible to control. It is imperative to address contamination quickly to reduce unwanted growth and to ensure it is not outcompeting worms for resources.

This method is meant for those who want to grow large-scale mixed-population cultures of *C. elegans*. Although it may be possible to grow synchronized populations of worms on the LSCP as

done on commercially available Petri dishes and in liquid culture, the authors have not tested this option. Additionally, if users wish to grow more than approximately 2.4 million worms on average in a given sample, a different method is recommended[4]. Growth success is dependent on the strain being processed in the pipeline. The authors were able to successfully grow populations of approximately 2.4 million worms in at least five biological replicates of 15 *C. elegans* strains, indicating that the method is robust.

Prior to starting the experiment note that the age and health of a given worm can influence fecundity and subsequent population growth time. Ensure that worms are maintained in healthy conditions with minimal stress prior to being used in this pipeline. It is assumed that stock samples have been created, frozen, and kept at -80 °C to reduce genetic drift over time.

Depending on the needs of a given experiment, the number of starting gravid adults on a LSCP can be changed. Altering the number of starting gravid adults on the LSCP will change the growth rate and thus time to harvest. Five gravid adults are used to seed each LSCP for the following reasons: (1) A simple, fast, and efficient way to seed many *C. elegans* strains onto LSCPs at one time was needed and (2) to reduce the age differences amongst the gravid adults picked that could lead to the growth heterogeneity.

This method allows the user to harvest large populations of worms with all life cycle stages present. With current methods available, collecting large-scale samples of *C. elegans* requires bleach synchronization to obtain the number of worms desired for downstream work. Given this approach, one can now grow as many worms as previously possible in fermenters or large-scale liquid cultures without the difficulties associated with bleach synchronizing and multiple handling steps. Our protocol allows one to target strains of interest efficiently, use minimal handling time in growing the sample itself, and isolate stages of worms or the population as needed in downstream pipelines.

A LPFC was utilized as a tool to document the population distribution and size in a given LSCP. The LPFC used is a continuous flow system that analyzes, sorts, and dispenses worms based on their size (TOF) and optical density. As a given worm passes through the flow cell, the axial light-loss detector captures the amount of signal light blocked by a 488 nm-solid state laser for the length of time it takes a worm to pass through, giving the user the TOF and optical density of the worm. Fluorescence collection optics and detectors can also be utilized to maximize fluorescence sensitivity and collection on each sample. LPFC collection parameters will vary based on instrument. Users can employ a variety of platforms to capture worm size and are not limited to using this protocol if a LPFC is not available.

The authors are using samples grown in the method described here to identify unknown metabolites in various strains of *C. elegans* via Liquid Chromatography – Mass Spectrometry, NMR spectroscopy, and RNA sequencing. The authors plan to continue to use this method for growth of samples in this pipeline with a variety of *C. elegans* strains as new strains of interest can be easily processed using this pipeline.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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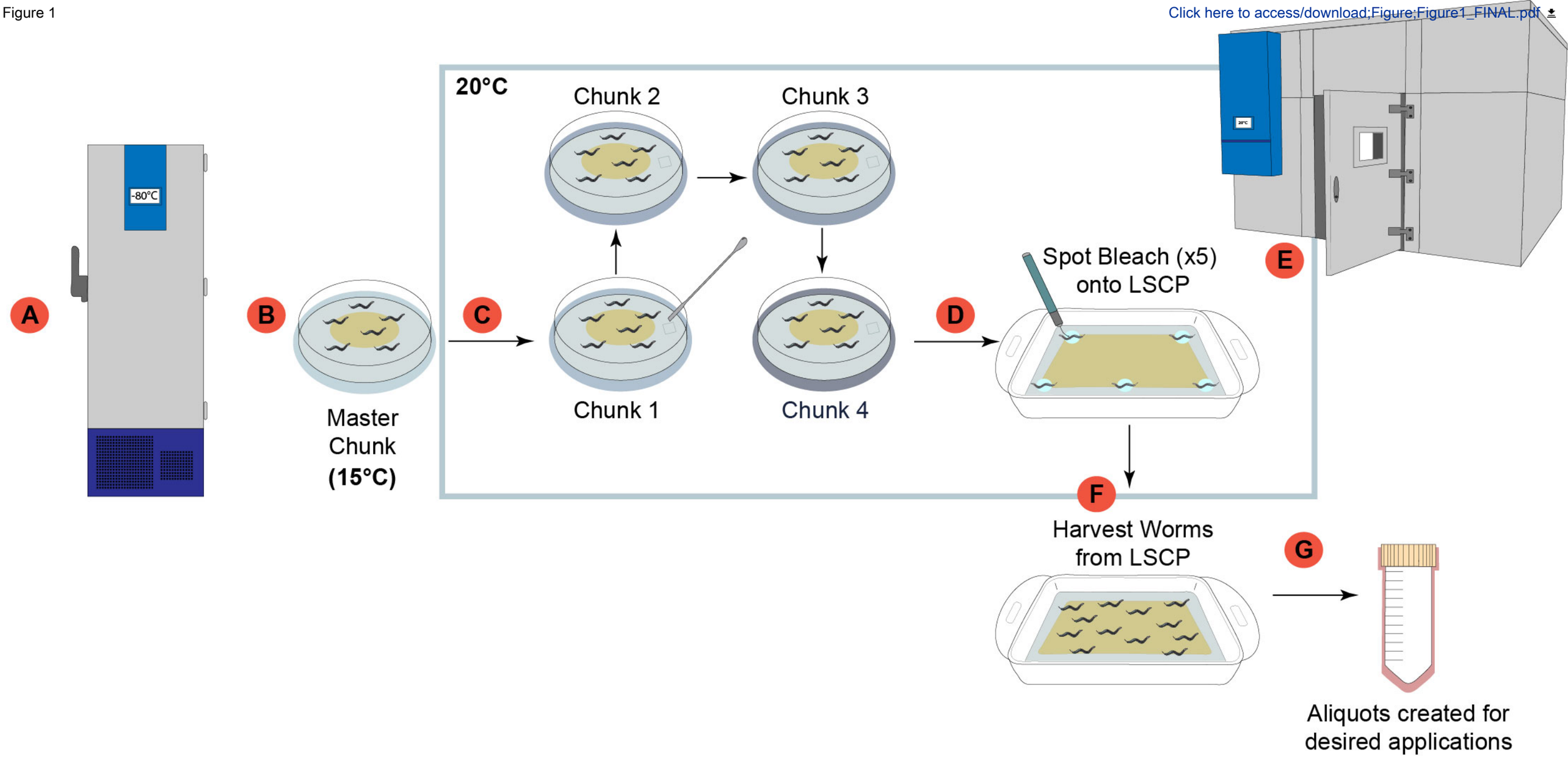

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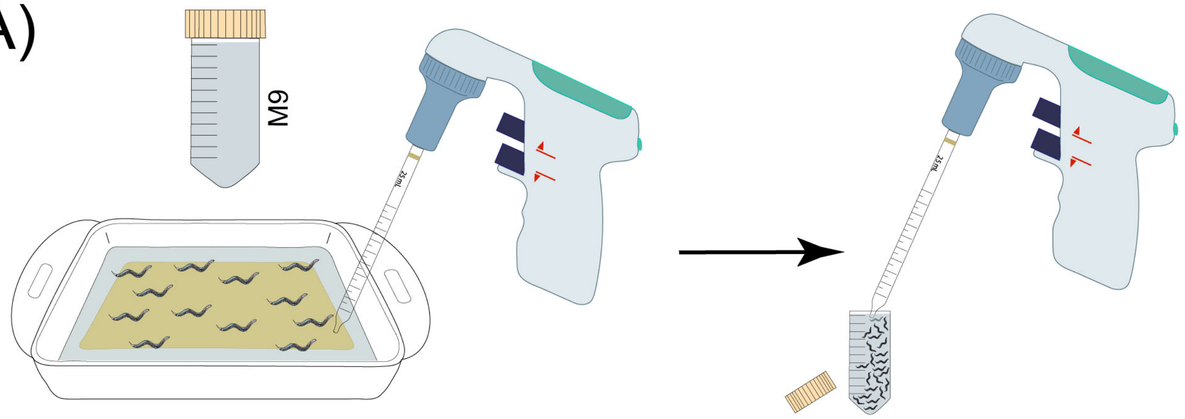
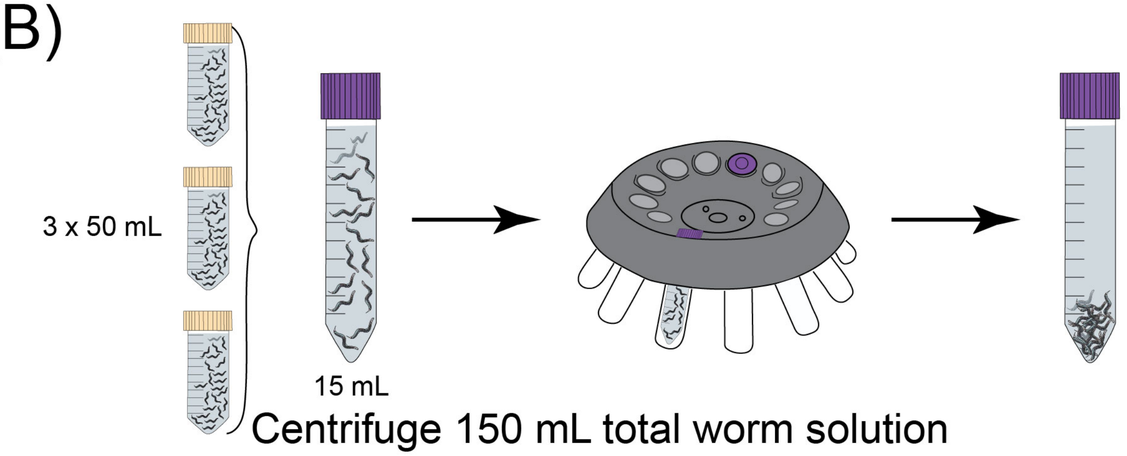
(A)



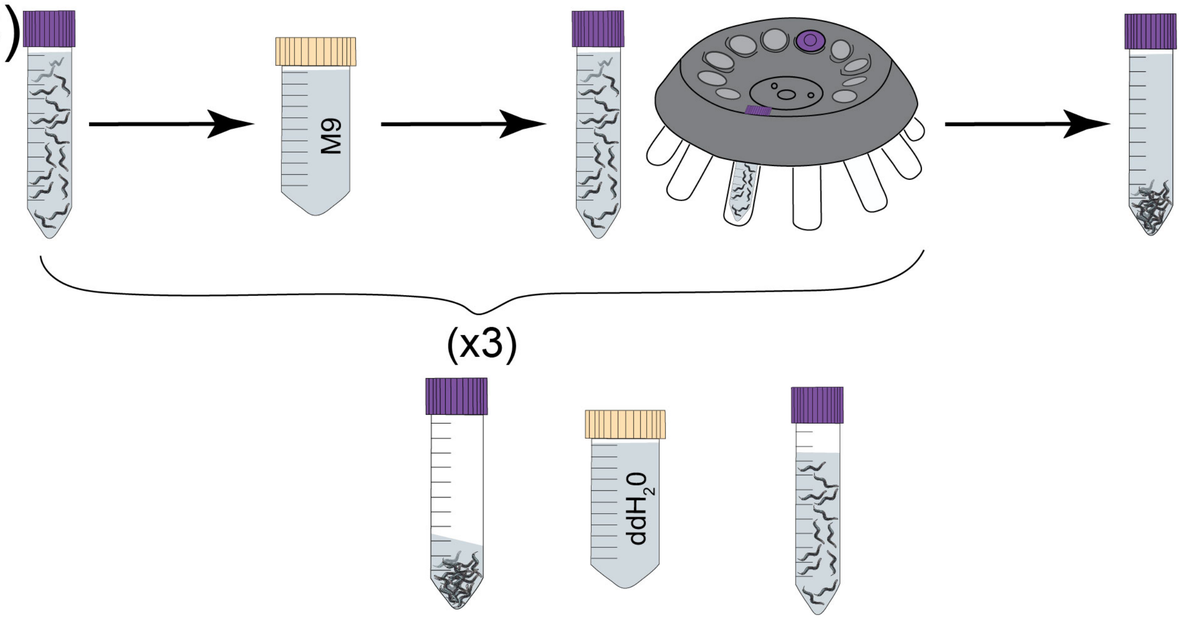
Collect 50 mL worm solution from LSCP (Repeat x2).

(B)



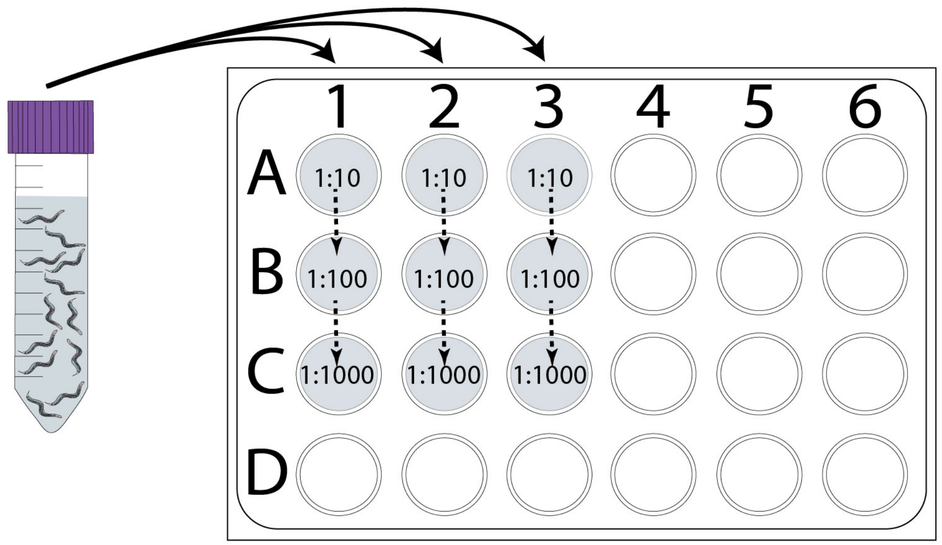
Centrifuge 150 mL total worm solution into one 15 mL conical tube.

(C)



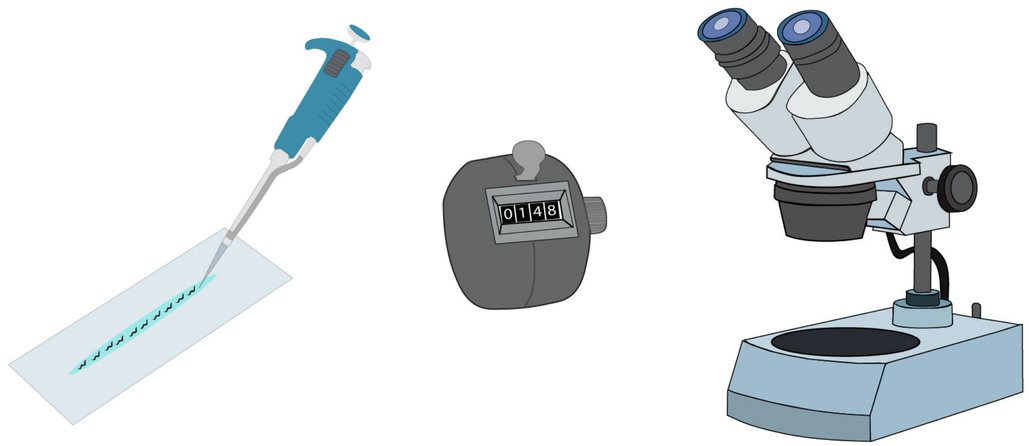
Wash sample with M9 (x3).
Resuspend worm pellet in 10 mL ddH_20.

(D)



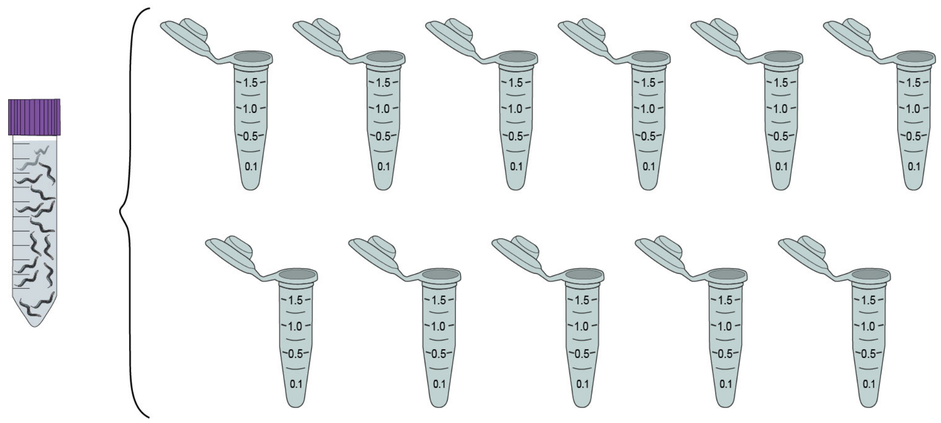
Prepare 3 sets of serial dilutions to estimate sample population counts.

(E)



Count aliquot of worms to estimate population size.

(F)



Split into desired aliquots.

Figure 2

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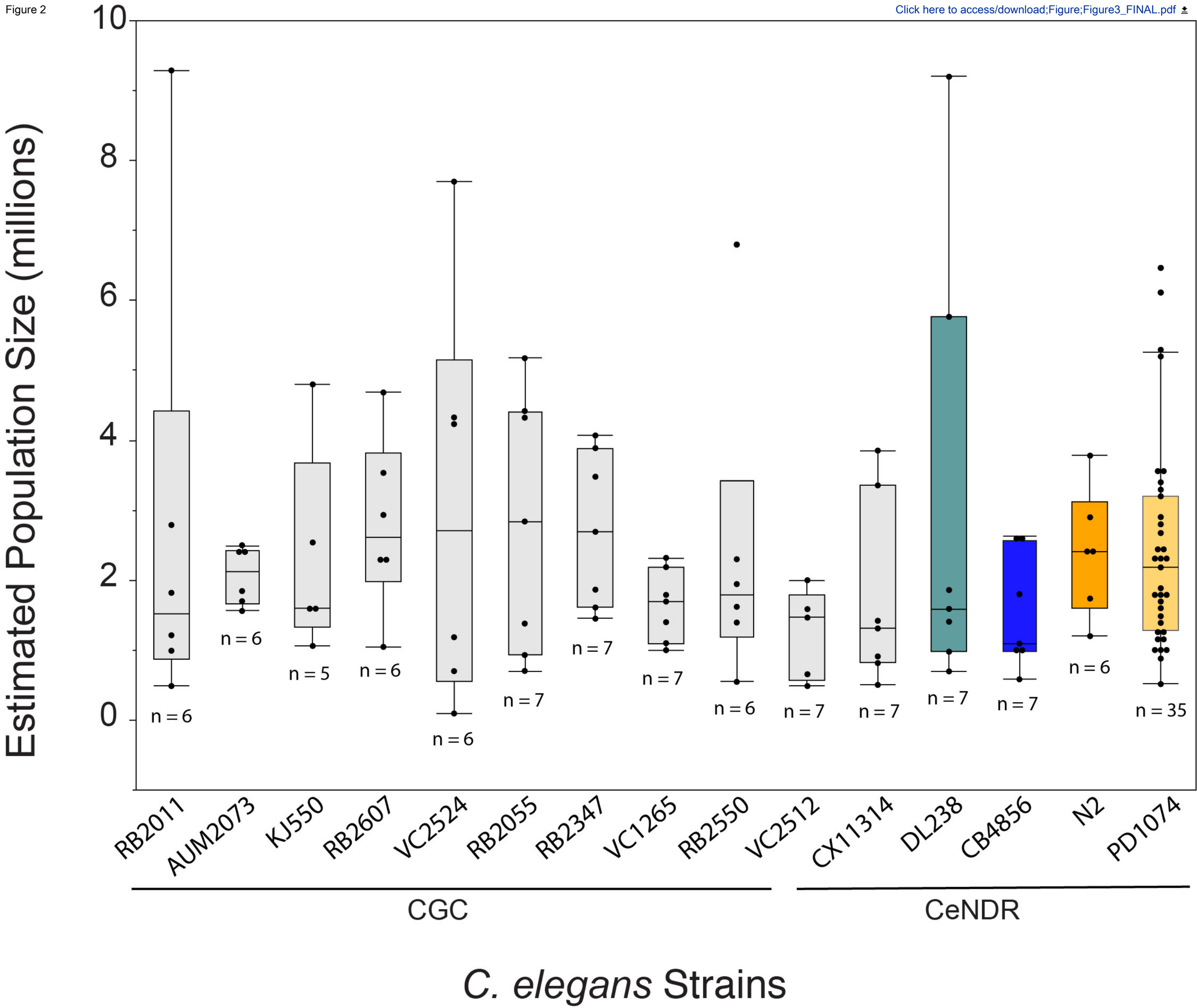

Figure 4

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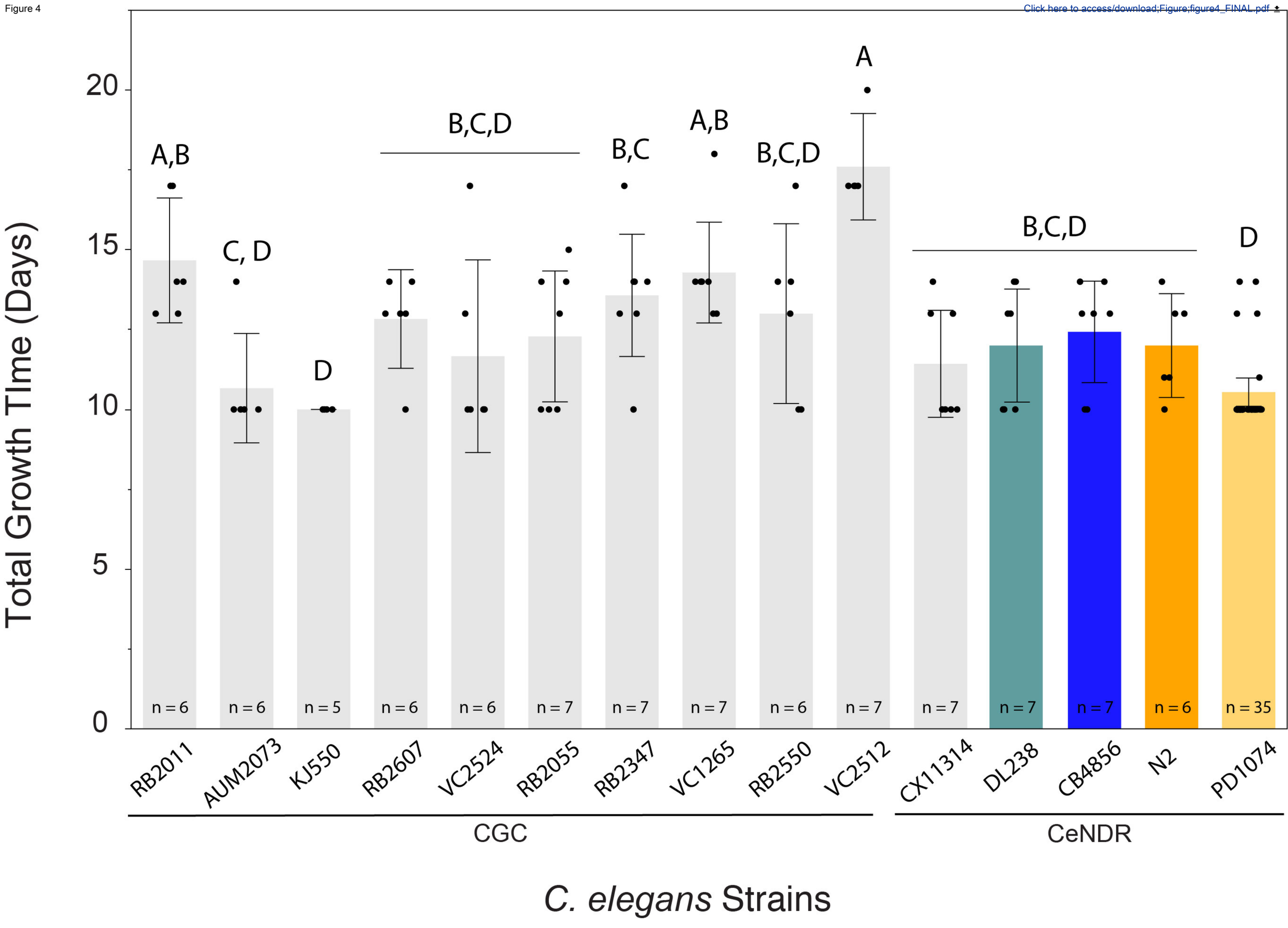

Figure 5

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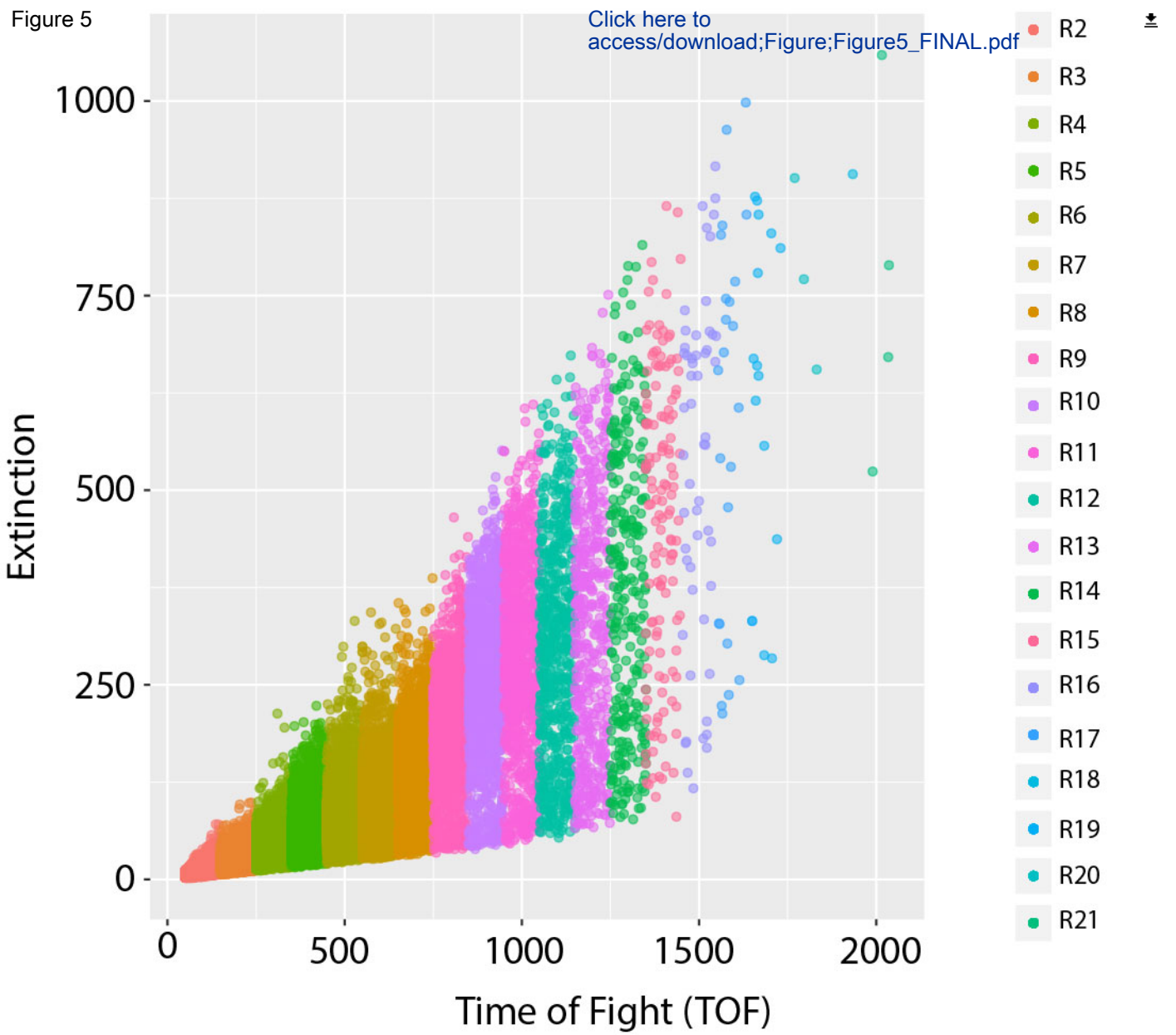

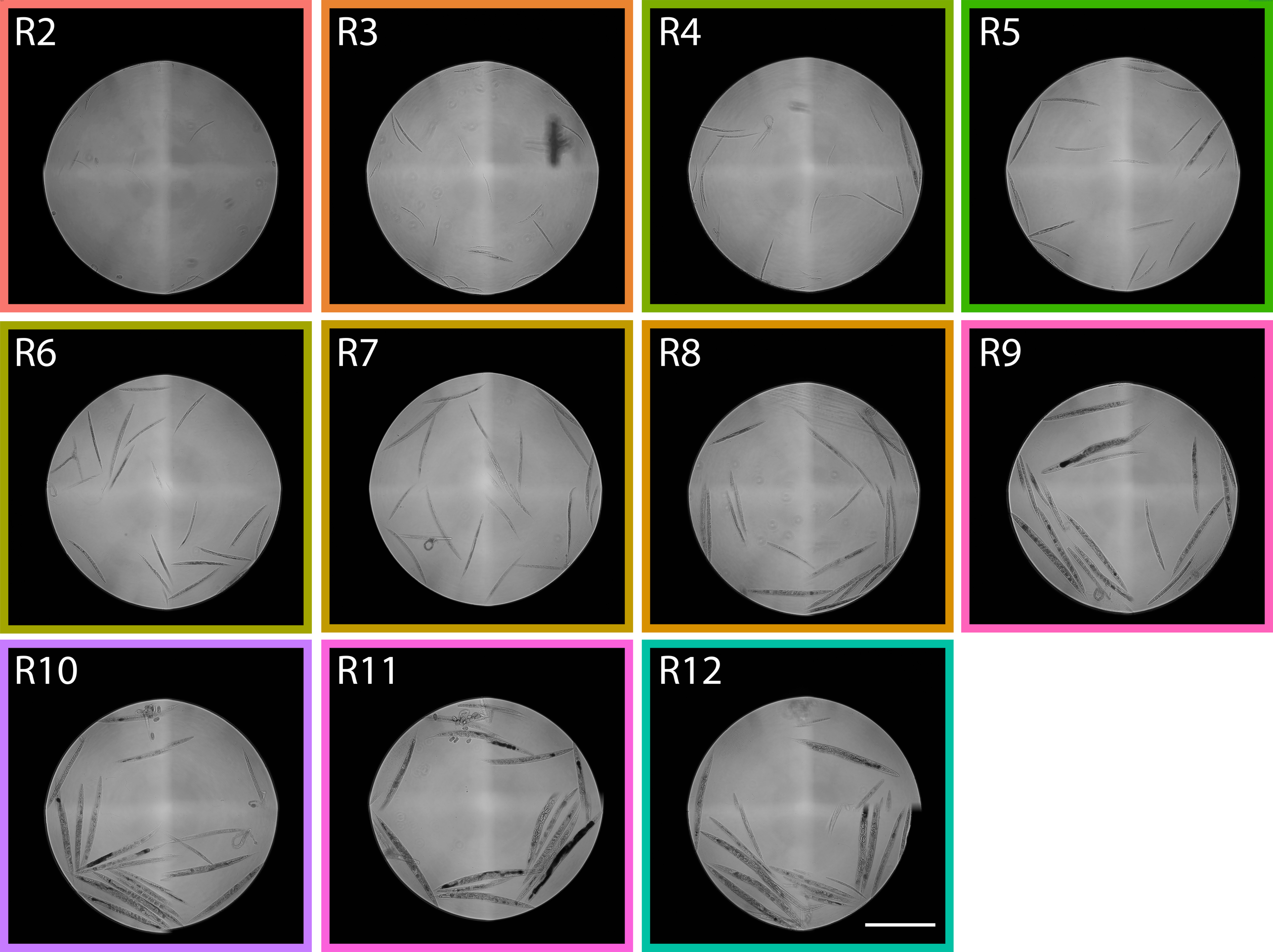
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Figure 7

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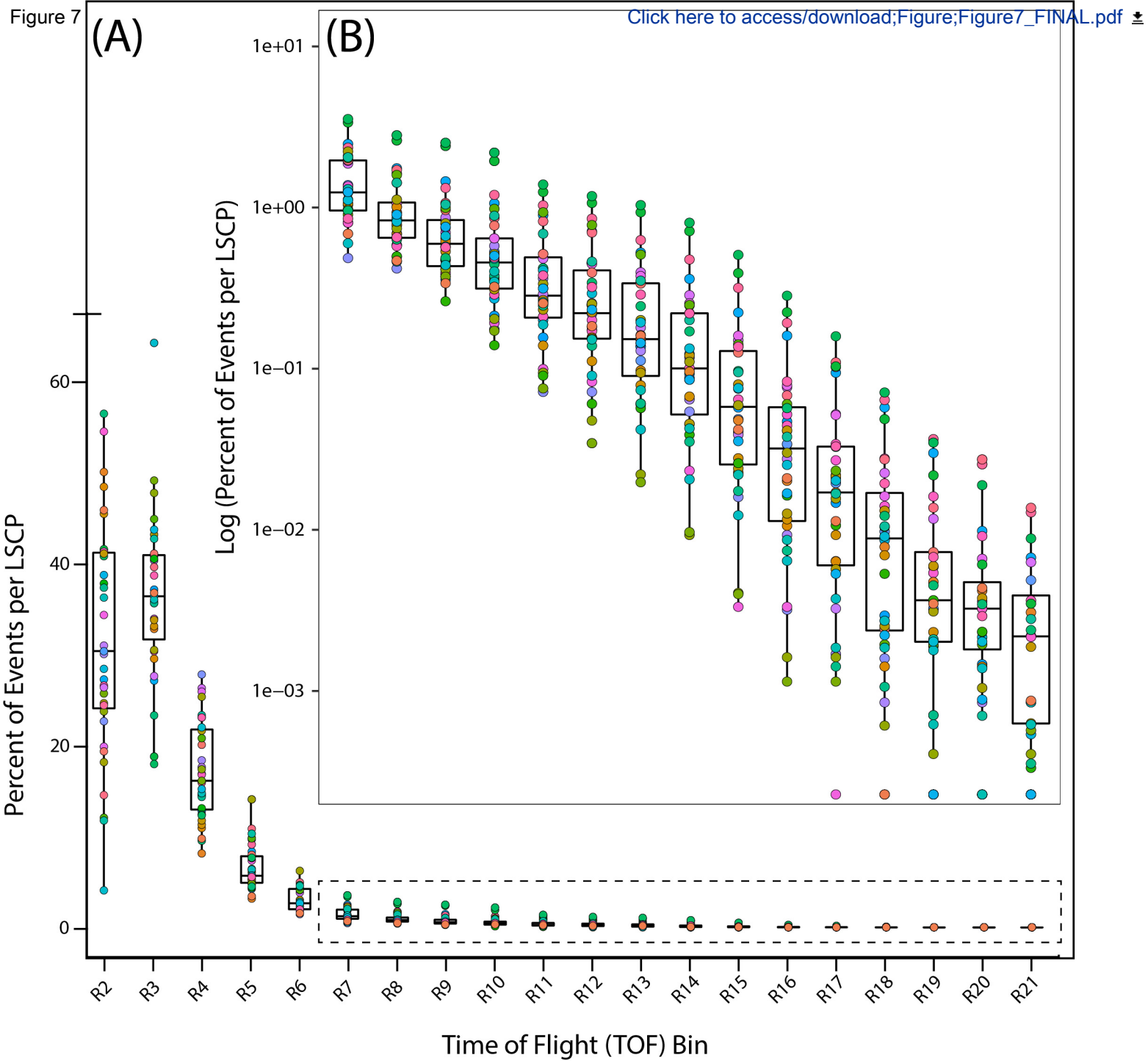

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 mL Sterile Serological Pipettes	VWR	89130-898	
10 ul pipette tips	VWR	89079-438	
100 ul pipette tips	VWR	89079-442	
1000 mL Graduated Cylinder	VWR	10124-380	
1000 ul pipette tips	VWR	89079-488	
15 mL conical tubes	VWR	89039-668	
190 Proof Ethanol	VWR	89125-166	
2 L Wide Neck Erlenmeyer Flask	VWR	75804-654	
50 mL conical tubes	VWR	75874-294	
Agar	Sigma	05040-100G	
Agarose	Sigma	A9539-500G	
BVC Control G Fluid Aspiration System	Vacuubrand		
Calcium Chloride	Sigma	449709-10G	
Cholesterol	Sigma	C3045-25G	
Clorox Bleach	VWR	89414-502	
Conviron Control Temperature Room	Conviron		https://www.conviron.com/environmental-rooms
Corning Low Volume 384 Well Black with Clear Flat Bottom Polystyrene TC-Treated Microplate	VWR	89089-866	
Fisher Scientific Accuspin 3R	Fisher		
Flat-Bottom 24-Well Plate	VWR	29443-952	
Honeywell True HEPA Purifier 465 sq ft.	Home Depot	204390560	
HT115 *E. coli* (DE3)	CGC	HT115(DE3)	https://cgc.umn.edu/strain/HT115(DE3)
Kimwipes	VWR	470224-038	
Large Scale Culture Plate (LSCP)	Pyrex	1090948	Pyrex 2-quart Glass Baking Dish with Red Lid
Magnesium Sulfate	Sigma	C86677-25G	
$MgSO_4$	VWR	97062-998	

Microscope Plain Slides	VWR	16004-422	
Millipore Filter	Millipore	1.11727.2500	
Molecular Devices ImageXpress	Molecular Devices	Model Number:IXMConfocal	https://www.moleculardevices.com/products/cellular-imagin
Nystatin (10mg/mL)	Sigma	N6261-25MU	
Peptone	Sigma	P7750-100G	
Petri Dishes (6 cm)	VWR	25384-092	
Pipette Controller	VWR	613-4180	
Potassium Chloride	Fisher	P217-3	
Potassium Phosphate Monobasic	VWR	0781-500G	
Potasssium Hydroxide	Fisher	P250-500	
Red Fluroscent Microspheres	Polysciences	19507-5	
Sodium Chloride	Sigma	746398-500G	
Sodium Hydroxide	Fisher	111357	
Sodium Phosphate Dibasic Anhydrous	Fisher	BP332-500	
Standard Gilson Pipette Set	Gilson	FA10002M, FA10004M, FA10006M	
Streptomycin (100mg/mL)	Sigma	S6501-25G	
Union Biometrica COPAS BioSorter	Union Biometrica		https://www.unionbio.com/biosorter/ , authors used: Flow P

ıg-systems/high-content-imaging/imagexpress-micro-confocal#gref , Authors used MetaXpress Software Version 6.5.4.532

ilot software version 1.6.1.3.

Rebuttal Letter
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The University of Georgia

Complex Carbohydrate Research Center

April 30, 2020

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Bajaj,

We are grateful for the very helpful suggestions by both reviewers and editors. The pages attached to this cover letter contain our responses, and the resubmitted manuscript is greatly improved as a result of the changes.

We also appreciate the extra time you gave us to make these changes. It has been a challenging time with our lab shutdown due to COVID-19.

Sincerely,

Arthur S. Edison
Professor and GRA Eminent Scholar
Departments of Genetics and Biochemistry
Institute of Bioinformatics and Complex Carbohydrate Research Center
University of Georgia

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Rebuttal Document | Method to Culture and Assay Large-Scale Mixed-Stage *C. elegans* Populations for Omics Studies

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Overall formatting has been addressed and document has been checked for copy errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Document formatting fits these guidelines.

3. Please ensure the Summary clearly describe the protocol and its applications in complete sentences between 10-50 words

Summary fits the word count and has been modified to encompass the LSCP growth method and subsequent methods to document population growth.

4. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

Abstract fits the word limit and has been modified to include all the goals of this protocol.

5. Please define all abbreviations during the first-time use.

All abbreviations are defined upon first use in the manuscript.

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For example: Union Biometrica BioSorter, Flow Pilot software version 1.6.1.3, Molecular Devices ImageXpress Micro Confocal Model Number IXMConfocal, Molecular Devices, etc.

All commercial language has been emitted from the manuscript and only documented in the Table of Materials and Reagents.

7. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed from the protocol section of the manuscript.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

The protocol section has been rewritten in imperative tense.

9. The Protocol should contain only action items that direct the reader to do something.

The protocol has been revised to involve only action items for the reader unless in a note section.

10. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

The protocol has been revised to not include more than 3 action sentences per step.

11. Please do not have have non-numbered sentences in the protocol. Please either have numbered action steps or notes.

All sentences are numbered or a note in the protocol.

12. Please ensure you answer the “how” question, i.e., how is the step performed?

13. 3. Please make numbered action steps for this heading

All action steps have been numbered.

14. 12.2: Please make complete sentences

Fixed incomplete sentence in Step 12.2.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Protocol is ~ 9 pages long.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Protocol and highlighted sections fit within these guidelines.

17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

We have covered items a-e in the discussion section.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate the journal titles.

We have used the Journal of Visualized Experiments referencing guidelines for formatted references.

19. Please sort the materials table in alphabetical order.

Table of materials has been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method presented in this manuscript described an efficient and reproducible protocol to prepare large mixed-stage C. elegans populations on NGM agar plates for omics studies. I only have a few questions regarding the procedure:

Major Concerns:

1. Condensation (and consequently pooling on the plate) could be a concern when culture the plates with lids for such a long period (10 to 20 days). The authors also suggest not opening the lids in the CT room during the worm growth period to reduce the chance of contamination (line 336-337). Did the authors observe condensation as an issue? Did they apply the technique described earlier (line 170-173) to remove liquid in a laminar flow hood during the worm growth period?

Addressed concern in protocol section 7, pasted below for reference:

NOTE: While checking population growth on the LSCP, ensure condensation is not accumulating. Significant condensation accumulation was not observed in the worm growth process reported here, however if condensation accumulation occurs take a clean delicate task wiper and remove the condensation to avoid pooling on the agar.

2. While C. elegans has broad oxygen tolerance, blocking air exchange by tightly sealing the lid for 10 to 20 days may create conditions not favorable for certain studies (e.g. genes that are sensitive to O2 or CO2 levels). The authors may consider alternative sealing methods (e.g. gas permeable film).

Addressed concern in protocol section 7, pasted below for reference:

NOTE: While it is recommended to not disturb the growth of the LSCP while in the CT Room, LSCPs were transported out of the CT Room and lids were opened every 2 days to monitor sample growth. Taking the sealed lids off the LSCPs every 2 days also allows for O_2 to flow into the LSCP.

3. The current harvesting procedure is not very efficient, especially for large number of LSCPs. Did the authors estimate how much loss would be caused by directly pelleting worms in 50 mL conical tubes (leave 5 mL in each tube) and then transfer to the 15 mL tube for subsequent washing?

Addressed concern in protocol section 8, pasted below for reference:

8.10 Transfer 15 mL of worm suspension, from one of the three 50 mL conical tubes, by pouring to the labelled 15 mL conical tube set aside in Step 8.4. Centrifuge the 15 mL conical tube at 884 x G for 1 minute at 4°C. The majority of worms will pellet at the bottom of the tube.

8.12 Continue adding approximately 13 mL of worm suspension to the same 15 mL conical tube repeating steps 8.10 and 8.11 until all 150 mL of worm suspension are consumed. Invert the tube and disturb the pellet between centrifugations to wash and aspirate off as much bacteria and debris as possible.

NOTE: At this step, the contents from all three 50 mL conical tubes are condensed in a single 15 mL tube.

4. Which bacteria strain was used in this manuscript? Different food source (bacteria strains) may have significant impact on worm population size or total growth time. According to "Table of Materials", I am guessing the authors used HT115(DE3) for their study?

Bacterial strain used is described in protocol section 3 and in the "Table of Materials" (note pasted here):

NOTE: In order to generate a stable food source, batches of HT115 (DE3) *E. coli* have been generated using a small batch averaging concept consistent with the central limit theorem[17].

5. Can the authors estimate the variations of worm distribution using this method? Was there a significant difference amongst R2 to R21 between experiments?

We added Figure 6 to visually display the variation between TOF bins across 35 PD1074 samples to show the reader what one could expect.

6. To avoid the toxic effect of sodium azide, did the authors try levamisole or tricaine for this study?

No, the authors did not try levamisole or tricaine for this study. The authors had previously used an optimized protocol described in more detail in Nika et al. reference. Included Nika et al. reference in the sodium azide description in protocol section 10 to help readers dig more into that process.

Minor Concerns:

1. The authors should use metric system for the units, e.g. 28 cm instead of 11".

Replaced all "inch" descriptions with "cm" throughout the text.

2. Line 652: "…can be found in Supplementary Table 3." should be "…can be found in Supplementary Table 2."

Corrected the text - "Supplementary Table 3" with "Supplementary Table 2".

Reviewer #2:

Manuscript Summary:

The authors present the novel idea of pouring a large NGM plate in a pyrex dish. Thus, they are able to generate experimental sample of large numbers of worms while without the variability in growth conditions and/or the physiological stress induced by current liquid or multi-plate cultivation methodologies. I'm not sure how much this method differs from just growing experimental batches on multiple 10 cm plates. Their plate has 486 square cm while a 10 cm plate has 79 cm. This suggests the same population size can be maintained on 6 10 cm plates. Still it is nice to have all the worms for a batch grown under the exact same conditions.

Major Concerns:

1. I'm not sure how the analysis of all the mutant lines furthers the main story of their new method. The authors should address why they are using these mutant lines or consider wrapping them all up into one set. It would also be good to have more detail on what they look for in the plate before they harvest.

By adding in additional mutant lines outside of N2/PD1074 readers can see that this workflow works across strains that have different growth rates/defects and not just a wild-type strain.

Described what we look for in more detail when harvesting in protocol section 7:

7.4 Once the LSCP has become full of a large population of worms, it is ready to be harvested. The following criteria are used to decide if a LSCP is ready to be collected (7.5 – 7.8):

7.5 The LSCP is (1) full of gravid adult worms,

7.6 (2) reached a large population size (*i.e.* worms cover the entire surface of the agar),

7.7 (3) does not have many eggs on the surface of the agar (*i.e.* the maximum amount of worms should have hatched), and

7.8 (4) has minimal to no *E. coli* left indicating the worms would starve and generate dauer larvae if left on the plate for an additional two days. If the LSCP meets all four of the criteria described in 7.5-7.8, the sample is ready to be harvested.

2. I'm not sure how the extensive flow cytometry with the main innovation of the paper, the LSCP method. A mixed population should be stereotypical, so I am not sure how measuring all the sizes give you any more information about the experiment.

While flow cytometry is an optional part of the method and is not innovative itself, it is our method of choice to show we can produce a large mixed-stage population as shown in Figures 5 and 6.

3. It would be interesting to determine the number of age synchronized animals that can be cultivated on the large plates. The omic experiments they describe could be done more sensitively on age synchronized populations. Using mixed populations could smear out the signal since the worm physiology is strongly dependent on their developmental state.

The authors intended for and want a mixed-stage population and growing synchronized animals on the LSCPs was not a part of this workflow as described throughout the text.

Minor Concerns:

1. Seems odd to make protocol for 2.5 plates.

As stated in the protocol section 2, the NGMA material described makes enough material for 2.5 LSCP. The protocol can be tailored to the user's needs.

NOTE: The preparation steps for the NGMA as described here will yield enough material for 2.5 LSCPs. The protocol can be tailored to the needed LSCP batch size in a given experiment.

2. The E. Coli was grown to OD = 20. Standard OD600 readings are not accurate above 1.00. Do they make a 20-fold dilution and then quantify it? Could you let the E. coli grow on the plate for 24 hours to generate a larger amount of food for worm growth?

Clarified how OD was calculated in the protocol section 3:

NOTE: In this protocol, *E. coli* bacterial stocks were grown in a bioreactor. At the end of the culture growth, the culture was diluted 1:50, and the measured OD_{600} was 0.4. Thus, the culture had an effective OD_{600} of 20. Bacteria were pelleted, weighed, and resuspended in K-medium at a concentration of 0.5 g/mL (wet weight), transferred into 2 mL aliquots, and frozen.

3. The adding of the worms to the plate seems critical to the rate of growth on LSCP.

Yes, adding worms to the plate is critical for the rate of growth. Noted in the protocol section 6:

NOTE: Depending on the needs of a given experiment, the number of starting gravid adults on a LSCP can be changed. Altering the number of starting gravid adults on the LSCP will change growth rate and thus time to harvest.

The reason 5 gravid adults were used in this experiment is described in the protocol section 6:

NOTE: Five gravid adults are used to seed each LSCP for the following reasons: (a) A simple, fast, and efficient way to seed many *C. elegans* strains onto LSCPs at one time was needed and (b) to reduce the age differences amongst the gravid adults picked that could lead to growth heterogeneity.

4. Line 311 - Should all chunk plates should be isogenic? Do you mean physiologically isogenic?

No – it is meant to say "nearly genetically isogenic" as described in the text as described in the protocol section 6:

6.9 Repeat steps 6.6 – 6.8 four times for a total of five gravid adults placed evenly around the *E. coli* lawn. Pick all five gravid adults from the same 4th chunk plate to ensure nearly genetically isogenic individuals are added to a given sample.

5. Avoid tautology like in line 327 " Due to the long nature of growing a LSCP, it is imperative to create a clean environment to reduce contamination on the LSCP"

We deleted the redundancy here.

6. In 8.13 - why do you add dH2O here? Are you worried about osmotic shock to the animals? It could take some time to count and then aliquot animals. After 5 min in dH2O the adult worms will have become "sticks". This is acute stress and thus could likely influence the physiological state of the animals and thus bias subsequent omics data.

Addressed why we use water in the protocol section 8 NOTE: Suspending worm pellets in ddH_20 is the preferred solvent for downstream omics steps. Worms can be suspended in other solvents if they are compatible with a given experimental workflow.

7. I am not sure how the FACS sorting followed by imaging is relevant to the LSCP methods. It seems like it belongs in a different methods article.

We agree that the sorting and imaging is not necessary to use the LSCP method however it is how we validated the success of this method and feel it is worthwhile to share. We have tried to make it clearer that these are optional steps in the protocol at the start of section 10.

NOTE: Steps 10, 11, and 12 are the authors' preferred method to record sample growth (i.e. population size and population distribution of *C. elegans* life stages) and determine success of a culture. Users of this protocol can substitute optional Steps 10, 11, and 12 with their own metrics of growth success. Steps 10, 11, and 12 are described here for two reasons; First, so users who have equipment used in Steps 10, 11, and 12 can replicate these steps and secondly, to show validation of this growth method.

8. The protocol calls for a temperature-controlled room and even put an illustration of one in Fig 1. Most facilities do not have these. The authors should address how researchers can use their technique without TC room access.

Acknowledged that not all users may not have access to a CT room in the protocol section 7.

NOTE: If users of this protocol do not have access to a CT room as described here it is recommended to use either (A) a small incubator where the temperature can be controlled or (B) a designated room where environmental conditions can be controlled as much as possible. If neither of these alternate options are available, note that variation in sample growth may be greater.

9. More justification for starting with 5 individually bleached animals should be given. Why this way? Why not start with 100s or 1000s of animals?

The reason 5 gravid adults were used in this experiment is described in the protocol section 6.

NOTE: Five gravid adults are used to seed each LSCP for the following reasons: (a) A simple, fast, and efficient way to seed many *C. elegans* strains onto LSCPs at one time was needed and (b) to reduce the age differences amongst the gravid adults picked that could lead to growth heterogeneity.

10. The working in Fig 2B is confusing. Why quantify with a 1:10 dilution series. Is it to find one with a reasonable number of animals to count?

I think the reviewer is referring to Figure 2D not 2B. Comments below are about 2D.

In the protocol section 9 we have described how to choose the correct dilution type for your LSCP.

11. Worms can be lost on pipette tips and plastic tubes. They should address how this could influence their population estimations.
In the protocol

We have addressed this in the protocol section 9.

9.1 Prior to pipetting the worm sample, prime pipette tip being used with M9 to avoid worms sticking to the inside of the plastic pipette preventing sample loss and reducing count variation.

12. I found Fig 3 confusing. Why such a large variation between reps of the same genotype?

We've addressed this in the representative results section on Figure 3.

The LSCP method yielded population sizes from approximately 94,500 to 9,290,000. The mean population size across strains was approximately 2.4 million worms (Figure 3). No significant differences were found in estimated population sizes between *C. elegans* strains over the course of an average of 12.2 LSCP growth days.

We did not find significant variation across the strains. The variation within a strain follows what would be expected given the variance in growth.

13. The authors should put the genotype descriptions rather than Strain IDs Some explanation for why those mutant lines chosen for this data would be helpful.

We added in an additional table that shares genotype description in Supplementary Table 3 and mentioned this in the representative results section:

The LSCP method was tested on 15 strains of *C. elegans* containing a mixture of *Caenorhabditis* Genetics Center (CGC) mutants and *Caenorhabditis elegans* Natural Diversity Resource (CeNDR) wild strains[26], strain genotypes are described in Supplementary Table 3.

14. * Do you think the steps listed in the procedure would lead to the described outcome? Unclear if they would because it's not stated clearly when plates should be harvested.

Answered this above in another comment, addressing here as well from the protocol in section 7:

7.4 Once the LSCP has become full of a large population of worms, it is ready to be harvested. The following criteria are used to decide if a LSCP is ready to be collected (7.5 – 7.8):

7.5 The LSCP is (1) full of gravid adult worms,

7.6 (2) reached a large population size (*i.e.* worms cover the entire surface of the agar),

7.7 (3) does not have many eggs on the surface of the agar (*i.e.* the maximum amount of worms should have hatched), and

7.8 (4) has minimal to no *E. coli* left indicating the worms would starve and generate dauer larvae if left on the plate for an additional two days. If the LSCP meets all four of the criteria described in 7.5-7.8, the sample is ready to be harvested.

NOTE: Although most LSCPs are ready to harvest between 10 to 20 days, depending on strain and sample (see Figure 4), check each LSCP frequently upon establishing this protocol to determine normal harvest times.

15. * Are the steps listed in the procedure clearly explained?
* Generally, the sentence construction makes the text difficult to follow as well as some minor grammar issues throughout. It'd be helpful to rewrite a good portion of the text in first person active voice.

Text has been reconstructed to be written in the first person active voice as much as possible.

Text has been rewritten to address grammatical issues.

16. * Are any important steps missing from the procedure?
There were not many details about when it's fine to harvest the plates except "Grew until the sample was full of adult worms, reached a large population size, and had minimal bacterial left." Maybe more details could be incorporated into the text with specific steps added?

Addressed this above in major comment 1 and minor comment 14.

17. Are appropriate controls suggested?
* They had wild type N2 metrics as a positive control.

While we do have N2 in our list of strains grown, we used PD1074, a variant of the N2 Bristol strain, that was used as our control strain in this study. We made this clearer throughout the text.

18. Are all the critical steps highlighted?
* I feel it'd be useful for some LSCP details prior to step 6 to be highlighted as well. Maybe the flow cytometry section could be dropped to make room?

We'd be happy to have more of the LSCP process highlighted however we have already met our quota of what we can highlight based on the JoVE journal guidelines.

19. Is there any additional information that would be useful to include?
* Photos of plates would be nice. Also, some photos showing what the mixed population looks like when it is ready to harvest could be useful.

This would be a good item to film so users can see different stages of the LSCPs.

20. Are the anticipated results reasonable, and if so, are they useful to readers?
* Why such a large range in population size? Could the worms with the lower populations (e.g. 94,500) just be grown longer to generate more worms? Is that because all the strains are harvested at the same time and so their developmental rates affect the final number? I'm not sure how the analysis of all the mutant lines furthers the main story of their new method. It would be good to have more detail on what they look for in the plate before they harvest. This would give more clarity to the total growth time figure as well.

We mention in the discussion section that leaving worms on the plate for a longer period of time will not lead to more worms:

Additionally, if users wish to grow more than approximately 2.4 million worms on average in a given sample, a different method is recommended as leaving worms on LSCPs for a longer growth time will lead to more dauer formation and suppression of growth.

21. Are any important references missing and are the included references useful?
* References look good!

A few additional references were added based on addressing reviewer comments.

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary_Table1_FINAL.xlsx

Gated Region	TOF Distribution (.2us)
R2	50 - 150
R3	150 -250
R4	250 - 350
R5	350 - 450
R6	450 - 550
R7	550 - 650
R8	650- 750
R9	750 - 850
R10	850 - 950
R11	950 - 1050
R12	1050- 1150
R13	1150 - 1250
R14	1250 - 1350
R15	1350- 1450
R16	1450 - 1550
R17	1550 - 1650
R18	1650 - 1750
R19	1750 - 1850
R20	1850 - 1950
R21	1950 - 2050

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary_Table2_FINAL.xlsx

	1	2	3	4	5	6
A	R2	R2	R2	R2	R3	R3
B	R8	R8	R8	R8	R9	R9
C	R14	R14	R14	R14	R15	R15
D	R20	R20	R20	R20	R21	R21
E						
F						
G						
H						
I						
J						
K						
L						
M						
N						
O						
P						

7	8	9	10	11	12	13
R3	R3	R4	R4	R4	R4	R5
R9	R9	R10	R10	R10	R10	R11
R15	R15	R16	R16	R16	R16	R17
R21	R21					

14	15	16	17	18	19	20
R5	R5	R5	R6	R6	R6	R6
R11	R11	R11	R12	R12	R12	R12
R17	R17	R17	R18	R18	R18	R18

21	22	23	24
R7	R7	R7	R7
R13	R13	R13	R13
R19	R19	R19	R19

Supplemental File (Figures, Permissions, etc.)
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary_Table3_FINAL.xlsx

Strain	**Geneotype**	**Source**
N2	WT	CeNDR
RB2011	*ugt-62* (ok2663)	CGC
AUM2073	*unc-119*	CGC
KJ550	*aco-1*	CGC
DL238	WT	CeNDR
RB2607	ugt-49	CGC
VC2524	*gpd-2*	CGC
CX11314	WT	CeNDR
RB2055	*ugt-1* (ok2718)	CGC
RB2347	*idh-2*	CGC
VC1265	*pyk-1*	CGC
RB2550	*ugt-23* (ok3541)	CGC

CB4856	WT	CeNDR
VC2512	ugt-60	CGC
PD1074	WT	CeNDR

About
WT
Homozygous. Gene knockout *ugt-62*
GSK-3 promotes S-phase entry and progress in germline stem clls to maintain tissue output
aco-1 encodes an aconitase that is homologous to mammalian iron regulatory protein-1 (IRP1); *aco-1* activity is required for normal brood sizes and, under iron stress conditions, for normal lifespan and L4-to-adult growth rates
WT
Homozygous. Gene knockout *ugt-49*
gpd-2 encodes one of four glyceraldehyde-3-phosphate dehydrogenases (GAPDHs)
WT
Homozygous. Gene knockout *ugt-1*
idh-2 encodes a predicted mitochondrial isocitrate dehydrogenase; by homology, IDH-2 is predicted to catalyze the formation of alpha-ketoglutarate from isocitrate as part of the citric acid cycle
pyk-1 encodes for one of two pyruvate kinases. Essential for embryonic devleopment
Homozygous. Gene knockout *ugt-23*

WT
Homozygous. Gene knockout *ugt-60*
WT

Supplemental File (Figures, Permissions, etc.)
Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplementaryFigure1_FINAL.pdf