

# High-Throughput Screening of Microbial Isolates with Impact on *Caenorhabditis elegans* Health

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## Abstract

With its small size, short lifespan, and easy genetics, *Caenorhabditis elegans* offers a convenient platform to study the impact of microbial isolates on host physiology. It also fluoresces in blue when dying, providing a convenient means of pinpointing death. This property has been exploited to develop high-throughput label-free *C. elegans* survival assays (LFASS). These involve time-lapse fluorescence recording of worm populations set in multiwell plates, from which population median time of death can be derived. The present study adopts the LFASS approach to screen multiple microbial isolates at once for the effects on *C. elegans* susceptibility to severe heat and oxidative stresses. Such microbial screening pipeline, which can notably be used to prescreen probiotics, using severe stress resistance as a proxy for host health is reported here. The protocol describes how to grow both *C. elegans* gut microbiota isolate collections and synchronous worm populations in multiwell arrays before combining them for the assays. The example provided covers the testing of 47 bacterial isolates and one control strain on two worm strains, in two stress assays in parallel. However, the approach pipeline is readily scalable and applicable to the screening of many other modalities. Thus, it provides a versatile setup to rapidly survey a multiparametric landscape of biological and biochemical conditions that impact *C. elegans* health.

## Introduction

The human body harbors an estimated 10-100 trillion live microbial cells (bacteria, archaea fungi), which are primarily found in the gut, skin, and mucosal environments<sup>1</sup>. In a healthy state, these provide benefits to their host, including vitamin production, maturation of the immune system, stimulation of innate and adaptive immune responses

to pathogens, regulation of fat metabolism, modulation of stress responses, and more, with an impact on growth and development, disease onset, and ageing<sup>2,3,4,5</sup>. The gut microbiota also evolves considerably throughout life. The most drastic evolution occurs during infancy and early childhood<sup>6</sup>, but significant changes also occur with age,

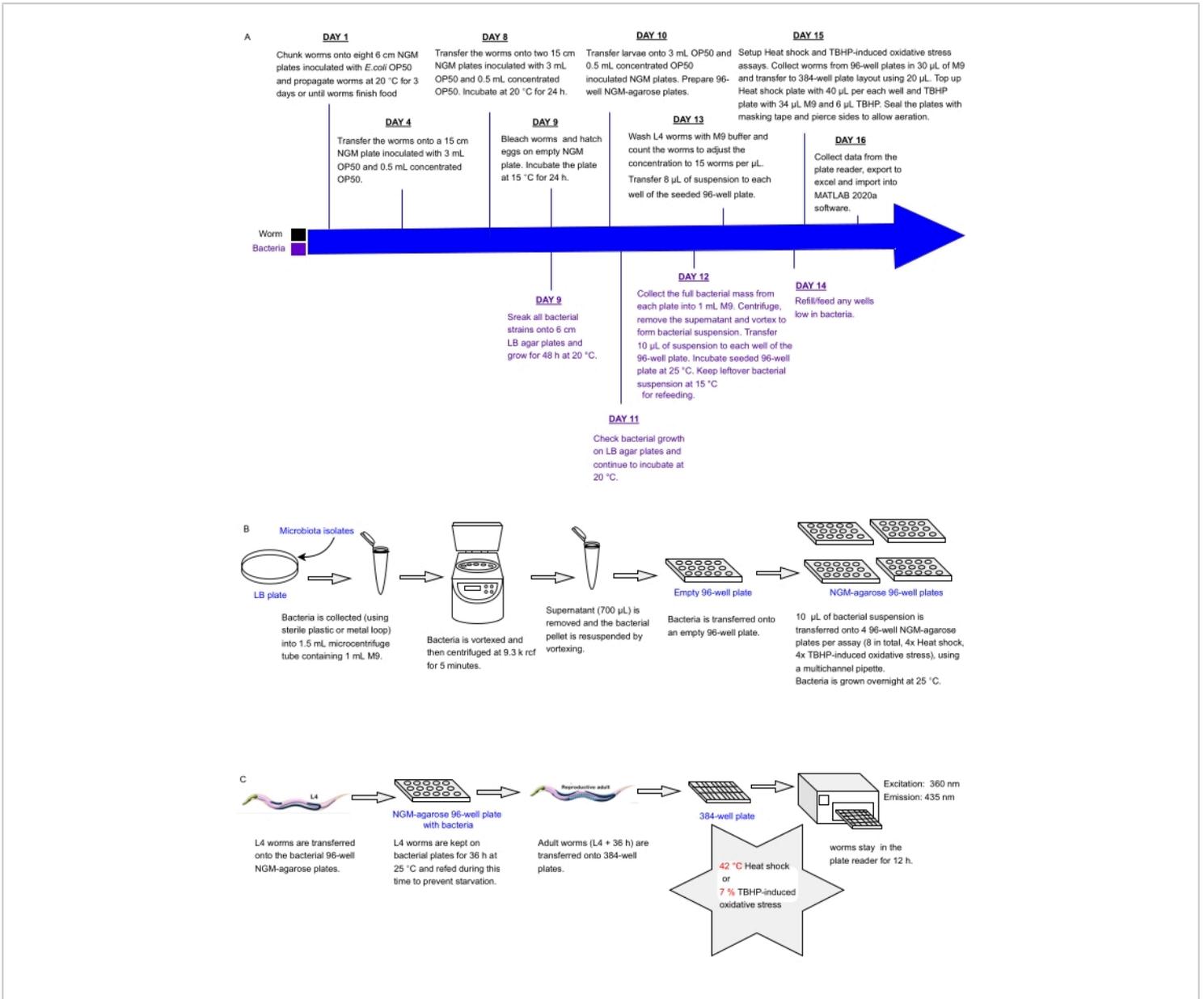
including a decrease in *Bifidobacterium* abundance and an increase in *Clostridium*, *Lactobacillus*, *Enterobacteriaceae*, and *Enterococcus* species<sup>7</sup>. Lifestyle can further alter gut microbial composition leading to dysbiosis (loss of beneficial bacteria, overgrowth of opportunistic bacteria), resulting in various pathologies such as inflammatory bowel disease, diabetes, and obesity<sup>5</sup>, but also contributing to Alzheimer's and Parkinson's diseases<sup>8,9,10,11</sup>.

This realization has critically contributed to refining the concept of the gut-brain axis (GBA), where interactions between gut physiology (now including the microbes within it) and the nervous system are considered the main regulator of animal metabolism and physiological functions<sup>12</sup>. However, the precise role of microbiota in gut-brain signaling and the associated mechanisms of action are far from being fully understood<sup>13</sup>. With gut microbiota being a key determinant of healthy aging, how bacteria modulate the aging process has become a subject of intense research and controversy<sup>6,14,15</sup>.

With the demonstration that the roundworm *Caenorhabditis elegans* hosts a bonafide gut microbiota dominated—as in other species—by *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*<sup>16,17,18,19,20</sup>, its rapid rise as an

experimental platform to study host-gut commensal interactions<sup>21,22,23,24,25,26</sup> has significantly expanded our investigative arsenal<sup>26,27,28,29</sup>. In particular, high-throughput experimental approaches available for *C. elegans* to study gene-diet, gene-drug, gene-pathogen, etc. interactions, can be adapted to rapidly explore how bacterial isolates and cocktails impact *C. elegans* health and aging.

The present protocol describes an experimental pipeline to screen at once arrays of bacterial isolates or mixtures set in multiwell plates for effects on *C. elegans* stress resistance as a proxy for health, which can be used to identify probiotics. It details how to grow large worm populations and handle bacterial arrays in 96- and 384-well plate formats before processing worms for automated stress resistance analysis using a fluorescence plate reader (**Figure 1**). The approach is based on label-free automated survival assays (LFASS)<sup>30</sup> that exploit the phenomenon of death fluorescence<sup>31</sup>, whereby dying worms produce a burst of blue fluorescence that can be used to pinpoint the time of death. Blue fluorescence is emitted by glucosyl esters of anthranilic acid stored in *C. elegans* gut granules (a type of lysosome-related organelle), which burst when a necrotic cascade is triggered in the worm gut upon death<sup>31</sup>.



**Figure 1: Experimental workflow for high-throughput screening of bacterial isolates with impact on *C. elegans* resistance to stress. (A) Timeline for worm and bacterial maintenance and assay setup. (B) 96-well bacterial plate array setup and handling. (C) 384-well worm plate setup. [Please click here to view a larger version of this figure.](#)**

## Protocol

The two *C. elegans* strains used in parallel for the present study were Bristol N2 wild type and HT1890: *daf-16(mgDf50)*, which grow at similar rates. However, the protocol can be replicated with any combination of two strains that

have similar growth rates. Note that, when testing other strains in parallel (for instance, wild type and slow-growing *daf-2* mutants), differing growth rates must be considered, and accordingly, the protocol needs to be adjusted. The timescales and quantities of worms and bacteria in the following protocol are optimized for parallel testing of 48

bacterial isolates on two worm strains in two LFASS assays in tetraplicates. Adjustments will be needed if more conditions are to be tested in parallel. *Escherichia coli* OP50 bacteria strain was obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota. The 48 bacterial isolates were obtained from the Schulenburg lab and maintained on LB agar.

### 1. *C. elegans* culturing on OP50 (Days 1 - 8)

**NOTE:** The current approach aims to grow *C. elegans* hermaphrodites on a solid medium at all stages and avoids unnecessary dietary changes (i.e., using alternate faster-growing *E. coli* strains such as NA22 or richer growth media such as egg plates) to remain as close as possible to the standard growth conditions<sup>32,33</sup> that are still widely used. Worm growth temperature (here set at 15 °C) depends on the *C. elegans* strain(s) used and may need adjusting (for instance, to avoid or trigger the expression of a temperature-sensitive phenotype or biomarker). For information on worm husbandry, please see reference<sup>33</sup>.

1. Prepare eight 6 cm diameter NGM plates (10 mL of nematode growth media agar, NGM, **Supplementary File 1**)<sup>32,33</sup> per worm strain and let them dry for 1 day at room temperature.
2. Prepare a saturated liquid culture of *E. coli* OP50 bacteria by seeding a single bacterial clone from a freshly grown Lysogeny Broth agar (LB agar, **Supplementary File 1**) plate in 25 mL of OP50 medium (**Supplementary File 1**) in a 50 mL conical tube. Grow the culture overnight at 37 °C in a shaker incubator.
3. Inoculate the eight 6 cm NGM plates per strain with 100 µL of saturated liquid culture of *E. coli* OP50 per plate and keep the plates at 20 °C for 2 days before use.

4. Using a scalpel, cut and transfer a square agar chunk of 0.5 cm with worms from a recently starved NGM plate onto each of the eight inoculated 6 cm NGM plates and incubate these plates at 20 °C for 3 days (or until the worms finish the food).
5. Prepare five 15 cm NGM plates per worm strain (30 mL of NGM medium per plate) and inoculate with 3 mL of OP50. Let the plates dry before incubating at 37 °C overnight. Keep the plates at 20 °C until use in later steps.
6. Using a P-1000 pipette, add up to 3 mL of sterile M9 buffer (**Supplementary File 1**) to the 6 cm NGM plates (Step 1.1.) to resuspend the worms, and collect the worm solution from all eight plates per strain in a single 15 mL conical tube.
  1. Centrifuge at 142 x *g* for 2 min at 4 °C. Carefully remove the supernatant using a P-5000 pipette or a water pump equipped with a sterile Pasteur pipette or tip. Add 10 mL of sterile M9 buffer to wash the worm pellet. Repeat 2x.
  2. Remove the supernatant (as much as possible) and transfer the worms onto a 15 cm OP50 inoculated NGM plate (Step 1.5.) using a pipette. Add 0.5 mL of concentrated OP50 culture.
  3. To make concentrated OP50, inoculate each of four 1 L bottles of LB with 2 mL of OP50 starter culture (prepared in Step 1.2.), and grow in a shaking incubator for 6 h at 37 °C and 160 x *g*. Pellet the bacteria at 3057 x *g* and 20 °C for 15 min. Discard the supernatant, resuspend the bacterial pellets with 6 mL of OP50 medium, and collect in a sterile 50 mL conical tube.

**NOTE:** Bacteria can be stored at 4 °C for up to 1 week.

7. Grow each worm strain on a 15 cm diameter NGM plate for 3-4 days at 15 °C by re-feeding the worms with 0.5 mL of concentrated OP50 daily.

1. Once the worms have nearly finished the food, collect and wash in M9 buffer (Step 1.6.1.), transfer each worm strain culture to two 15 cm NGM plates (Step 1.5.), and propagate worms at 20 °C until ~95% of the population are gravid adults (it will take about 24 h for wild type Bristol N2).

**NOTE:** Gravid adults are characterized by the presence of eggs within the worm, and the ideal plate should also have an abundance of unhatched eggs laid on the plate without too many larvae<sup>33</sup>.

## 2. Maintenance of gut microbiota isolate collections (Day 9)

1. Streak the 48 bacterial isolates on individual 6 cm LB agar plates and grow for 48 h at 20 °C.

**NOTE:** Bacteria can be grown at 25 °C for 24-36 h if needed sooner, but the longer 20 °C growth allows for spotting potential contaminants.

2. Synchronize a large number of *C. elegans*.

1. Bleach gravid adult worms by following the standard egg preparation method<sup>33</sup> and transfer the eggs onto two unseeded 15 cm NGM plates for 24 h at 15 °C to allow for all the L1 larvae to hatch and grow synchronously in the subsequent steps.

**CAUTION:** Be careful while handling bleach solutions.

## 3. Growing large *C. elegans* cultures (Day 10)

1. Once hatched, collect the L1 larvae (from Step 2.2.1.) in 3-4 mL of M9 in a clean conical 15 mL tube. Pipette

four 10 µL drops of worm solution onto a slide or a plate and count the number of worms in each drop under a stereomicroscope at 16x magnification. Determine the worm concentration of the solution by averaging the number of larvae from all the drops of worm solution. Multiply this value by the volume left and estimate the total worm count for each strain.

**NOTE:** 46,000-50,000 L1 larvae per strain are required at this stage to later fill a 384-well plate or two half-plates.

1. For each strain, transfer all the L1 larvae onto two 15 cm NGM plates (23,000-25,000 L1 per plate) previously inoculated with 3 mL of OP50 (Step 1.5.) and re-seeded with 0.5 mL of concentrated OP50.

2. Incubate at 15 °C, topping up with 0.5 mL of concentrated OP50 daily as needed until the worms reach the L4 stage.

**NOTE:** The L4 stage is characterized by a slightly darker intestine and a half-disk or crescent-shaped white patch where the vulva will eventually form<sup>32, 33</sup>.

3. Prepare 96-well NGM-agarose plates following the steps below.

1. Prepare eight 96-well NGM-agarose plates by filling each well with 125 µL of NGM-agarose (four plates per assay).

**NOTE:** It is recommended to plate some extra plates if some get contaminated in the subsequent steps. Two plate readers will be required to run assays in parallel, but they may also be run successively starting with the heat stress assay as it can be run for as little as 6 h. For these plates, the <4% ash agar is substituted with agarose (see **Table of Materials**), enabling slower and more even drying across the NGM plugs and reducing worm burrowing for better recovery.

2. Ensure that the wells are filled evenly and bubble-free. Use a heat block set at 70 °C (with slow heat transfer through the plastic of the multiwell plate, the NGM-agarose may only heat up to about 55-60 °C) to keep the mixture from solidifying during the process. To remove bubbles within wells, use a sterile flame-heated needle.
  3. Allow the 96-well plates to set at room temperature in a sterile environment before inverting (lid down to prevent condensation) and store at 4 °C in a clean box until needed.
  4. On Day 11, check on the worms from Step 3.2., ensuring no contaminations have appeared and the worms are still replete.
  5. On Day 12, check on the worms from Step 3.1., ensuring no contaminations have appeared and the worms are still replete. Also, check the worms' developmental stage.
- NOTE:** The sex/strain and the developmental stage of the worm, such as L4 or L4 + 24 h worms used, are dependent on the treatments the worms are subjected to. Here, wild-type hermaphrodites were exposed to bacterial isolates from L4 for 36 h.

#### 4. Preparing gut microbiota isolate collections for re-feeding worms

1. Monitor bacterial growth on the LB agar plates from Step 2.1. and continue to incubate at 20 °C.
- NOTE:** While it is not ideal, in case some clones do not grow or reveal contaminations, bacteria may be re-streaked from clean stocks onto 6 cm LB plates and grown at 25-28 °C for 24 h to be ready for the experiment.
2. Define a 96-well array layout for the bacterial collection being tested, facilitating systematic plate seeding and

data analysis in the subsequent steps (**Supplementary Table 1**).

3. Collect the bacterial mass from each 6 cm bacterial plate (Step 4.1.), and transfer it to a labeled 1.5 mL microcentrifuge tube containing 1 mL of M9 buffer. Perform this by using either a single-use 2 mm diameter sterile plastic loop or a 5 mm diameter metal loop. Sterilize the metal loop between bacterial strains by dipping in 100% ethanol, flaming, and cooling down for 5 s.
4. Vortex the microcentrifuge tubes until the bacterial pellets are fully resuspended (depending on the bacterial strain, this may take ~1-10 s).
5. Spin down at 9,300 x g for 5 min at room temperature, remove 700 µL of supernatant, and resuspend the bacterial pellet by vortexing.
6. Transfer 200 µL of each bacterial suspension into a single well of an empty sterile 96-well plate according to the layout set out in Step 4.2.
7. From this plate, inoculate eight 96-well NGM-agarose plates (prepared in Step 3.3.) with 10 µL of bacterial solution using a multichannel pipette and incubate with the lid on at 25 °C for 24 h. Do not seal the plates to allow for plate drying and bacterial aerobic growth and to avoid excess condensation.
8. Seal the 96-well suspension plate prepared in Step 4.6. with clean adhesive sealing film (see **Table of Materials**), and store at 15 °C for up to 5 days. This will be used for worm re-feeding as needed.

## 5. LFASS heat shock and oxidative assay setup (Days 13 - 14)

1. Looking at the plates from Step 3.5., assess the worms' developmental stage. Once >90% of worms have reached L4<sup>34</sup>, collect the worms in up to 10 mL of sterile M9 solution in 15 mL conical tubes.
2. Wash the worms extensively (at least 4x) by spinning down at 142 x g for 2 min at 4 °C, removing the supernatant, and adding 10 mL of fresh sterile M9 between each wash to get rid of OP50 bacteria. Resuspend the worm pellet in 10 mL of M9.
3. Transfer 50 µL of worm solution into a low surface binding tube (see **Table of Materials**) containing 950 µL of M9. After gently mixing the tube contents to avoid worm sedimentation, quickly use a wetted low-bind pipette tip to transfer 3-4 separate 10 µL drops onto a glass slide or an NGM plate, and count worm numbers under a stereomicroscope (see **Table of Materials**) at 16x magnification. Average the counts from the 3-4 drops and determine the number of worms per microliter in the worm solution (see Step 3.1.).
4. Adjust the worm concentration in the 10 mL tube to reach ~120 worms in 8 µL. If the solution prepared in Step 5.2. is not concentrated enough, spin the worms down and remove M9 accordingly to reach 120 worms per 8 µL.
5. Transfer 8 µL of worm solution (~120 worms) into each of the wells of the eight 96-well NGM-agarose plates from Step 4.7., using a multichannel pipet or a repeat pipet. Ensure to use low retention tips to limit worm loss. It might also be necessary to cut the tip ends to allow for large adult worms to limit mechanical stress on adult worms.

**NOTE:** The assay requires a minimum of 30 live healthy worms to work reliably but works best with about 100 worms per well.

6. Incubate the worm and bacterium-seeded 96-well NGM-agarose plates at 25 °C for 36 h.
7. Check the plates between 12-24 h, ensuring the worms remain replete throughout. If re-feeding is required, resuspend the bacteria within the 96-well bacterial array plate stored at 15 °C in Step 4.8., and add up to 10 µL of the corresponding bacterial solution to the 96-well NGM-agarose plates where worms are at risk of starvation before the end of the 36 h incubation period (starved worms will produce vastly differing results, so this is very important).

**NOTE:** The following steps need to be conducted on Day 15. Prior to starting the assay, it may be necessary to optimize the reading height. The optimal reading will be achieved 20-50 µm above the bottom of the well. This will be dependent on the model of the plate reader. Some offer the possibility of a Z-scan, while others allow manual height input. Set the optimal height at the level where the highest blue fluorescence (365 nm/430 nm) signal is detected. Some plate-readers may operate at a fixed height optimized for adherent cell assays and might not be ideal for LFASS assays.

8. After 36 h, dispense 30 µL of M9 into each well of the 96-well plate.

**NOTE:** For thermal stress assays, the plate reader needs to have reached the required temperature to perform the assay and may need to be turned on ahead of time. The current protocol uses 42 °C to maximize killing speed, but the approach applies to other temperatures above 30 °C.

9. Transfer worms (about 20  $\mu$ L) to the 384-well plate according to set layouts, using low-retention tips (consider cutting off the end of the tips to allow large worms to reduce mechanical stress for adult worms).

**NOTE:** For the present study, two different plate reader settings are used for the two assays described here (thermal stress and oxidative stress), and thus samples intended for these two assays must not be plated in the same 384-well plate.

10. Ensure the plate readers are set up properly (**Table 1**).

11. Top up the 384-well plates with more M9, aiming for a final volume of 60  $\mu$ L per well. For thermal stress assay, add 40  $\mu$ L of M9, and for *t*-BHP-induced oxidative stress, add 34  $\mu$ L of M9 in 6  $\mu$ L of *t*-BHP (see **Table of Materials**).

1. Start the assay within 2 min of adding *t*-BHP (ideally, all worms must be exposed to *t*-BHP simultaneously, the assay time resolution being 2 min). If not possible, use a timer to estimate the time spent pipetting *t*-BHP before the start of the assay to allow for later adjustment of the median time of death.

12. Close the plates with their transparent lid. Seal the edges of the 384-well plates with masking tape (taping over the plate and lid), ensuring that the tape does not go over the lid or under the plate. Slit the tape between lid and plate at intervals using a scalpel to allow air exchange while minimizing evaporation during the assay.

13. Insert the plate into the plate reader (see **Table of Materials**) and start the run. Aim to excite at 365 nm and detect emission at 435 nm every 2 min for 6-12 h (**Table 1**).

**NOTE:** Typically, 6 h is enough for 42 °C heat stress assays and 8 h for 7% *t*-BHP oxidative stress assays.

## 6. Plate-reader data handling

1. Save the raw fluorescence data from the plate-reader as comma- or tab-separated .txt, .csv, or .xls /.xlsx formats, and then convert to xls /.xlsx format. Depending on the data format, reorganize them to match the excel sheet layout needed for LFASS analysis. Follow the detailed instructions provided in reference<sup>30</sup>.

**NOTE:** While data can be analyzed manually, normalizing each time series and looking for the time when death fluorescence reaches the half maximum, automated analysis can be carried out in Matlab running the LFASS routine<sup>30</sup>.

2. Download and install Matlab (version 2014a or above) and the LFASS software package from <https://github.com/ABA80/LFASS>. Follow the guidelines and annotations provided within it.

**NOTE:** **Figure 1C** gives a brief description of the approach. Matlab is required to run the LFASS routine. Alternatively, the Matlab code may be translated into Oracle, except for the fitting function, which is proprietary. New smoothing and sigmoid functions can be rewritten to enable use in a fully open-source platform.

3. Between LFASS analyses, move the data and results to a new location as the LFASS analysis will process all files in the data folder and overwrite files in the Results folder.

## 7. Data inspection

1. Open the excel file and label the rows according to the well position on the 384-well plate. **Supplementary File 2** shows an example of the excel file of the raw fluorescence data generated for the heat shock assay.

Use the well position on the 384-well plate to label the worm and bacterial strains.

2. Ahead of Matlab analysis, visually inspect the data in excel, plotting fluorescence intensity over time for a representative well. Depending on the plate-reader used, data may be noisy but should display a clear peak. In particular:

1. Determine a fluorescence value below which a peak would not be significantly different from noise (setting such a threshold in LFASS will speed up analysis by excluding empty wells).
2. Note the earliest time point when fluorescence fluctuations dampen prior to rising (animals may thrash vigorously for up to 30 min, leading to fast fluctuating blue fluorescence readings).

**NOTE:** The peak fitting may be improved by excluding these early timepoints from the curve fitting window.

3. Note the time points between which minimal and maximal fluorescence values are expected to fall (look at several wells to identify these ranges) as they will be used for curve fitting.
4. Check if the amplitudes of the fluorescence peaks vary significantly between the wells, normalize the data prior to further analysis using the following formula:

$$\text{Normalized fluorescence well}_n(t) = \frac{(\text{Fluorescence well}_n[t] - \text{minimum fluorescence well [Dt]})}{(\text{maximum fluorescence well [Dt]} - \text{minimum fluorescence well [Dt]})}$$

where "n" is the current well number, "t" is the timepoint, and "Dt" is the series of time points for the assay.

## 8. LFASS data processing

**NOTE:** Details are provided at <https://github.com/ABA80/LFASS> and in the supplementary materials of Reference<sup>30</sup>.

1. Create two subfolders within the LFASS folder, one for the data to be analyzed and one for results, for example, "my data" and "results".
2. Copy the assay excel data file into the LFASS subfolder "my data" after data inspection.
3. Launch MATLAB, navigate to the **LFASS** folder, type, and run **fitfolder** in the command window (**Supplementary File 3**). Then follow the on-screen instructions.
4. After typing in "**fitfolder**", the system asks for the name of the folder in which the excel file is located, for example, '**my data**'. Type in the name of your data folder (in this example, "my data").
5. Follow the on-screen instructions, providing the various parameters requested.
  1. Enter "**2**" for the time interval between successive measurements in the current protocol (specifying this allows for the results to be expressed in minutes instead of timepoint units).

**NOTE:** The time interval can be modified to perform fluorescence measurements more or less frequently (to decrease or increase time resolution) and also depending on the plate reader capabilities (i.e., the time interval may need to be increased for plate readers that cannot perform fast enough measurements). Ensure always to match the experimental time interval with the LFASS routine.

2. Assign the upper tolerance threshold by typing "0.95" (this can be changed as needed to improve fit) and the lower tolerance threshold by typing "0.05" (this can be changed as needed to improve fit) to constrain the sigmoid fit.

**NOTE:** Other time parameters are based on user notes from the data inspection (Step 7.2.).

6. Choose whether to display or not fitted and smoothed curves by typing "y" for YES or "n" for NO when prompted. To visually inspect the converging fits, select the former.

**NOTE:** The latter is useful to visualize all smoothed data but is usually not selected because it generates too many pop-up graphs. Following this, Matlab will execute the LFASS routine, which may take 1-10 min if multiple excel files are being processed in one go. Pop-up windows with curves will appear according to the selection in Step 8.6. **Supplementary File 4A** shows an example of a fitted curve.

7. Choose whether to (1) analyze curves identified as noise or (2) refit poorly fitted curves with an [y/n] option. Type y to approve and n to reject.

**NOTE:** Approving refitting is recommended, especially if there are many poorly fitted or unfitted curves. This will allow the user to provide tailored curve fitting parameters for each curve as they appear on the screen and only ask for earlier and later boundaries for the sigmoid fit. It can be attempted as many times as required.

8. Once the data is analyzed, close Matlab and open the **LFASS** folder.
9. Click on the LFASS subfolder **My results**, as the result files are saved automatically in the Results folder as .txt.

**NOTE:** Matlab generates three .txt files: "Batch-fitted.txt", "Batch and noise-fitted.txt", and "Refitted.txt". The former two are saved as a precaution in case of a computer crash or user error during the refitting. The file containing the most accurate complete analysis is "Refitted.txt".

10. Open the file **Refitted.txt** with Microsoft Excel and save as .xls for further processing. **Supplementary File 4B** shows an example of such a result file.

**NOTE:** For each well (organized in rows), three values are provided in the columns that give estimates of the median time of death of the worm population: "Raw": reports the time intersecting at the half-maximum of the experimental data peak; "Batch-fitted": reports the time to intersect at the half-maximum of the batch-fitted curve; "Refitted": reports the time to intersect at the half-maximum of the refitted curve.

11. Save the file in .xls format as a copy in a safe location. Failing to do this will run the risk of the files being overwritten during the next run of the LFASS routine.

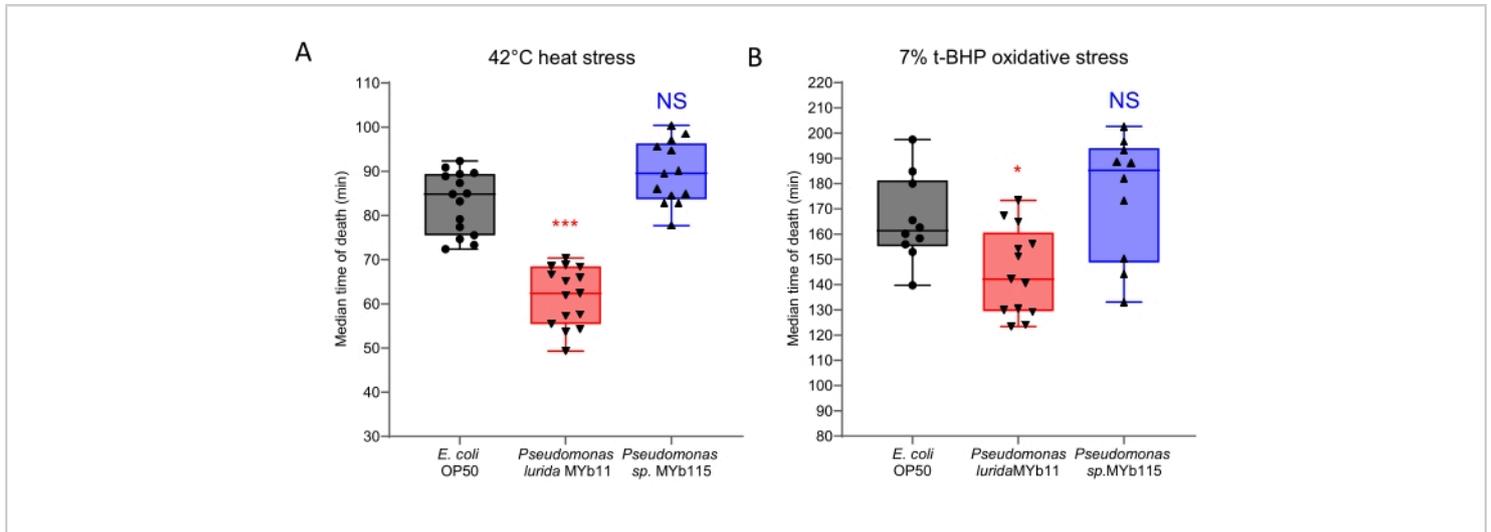
**NOTE:** The results can then be further processed for graphing or statistical analysis.

## Representative Results

LFASS assays provide robust, high-throughput, and rapid screening of multiple test conditions at once, such as screening numerous genetic and microbiota parameters that contribute to stress resistance and aging. It only takes 2-3 weeks for the experiment to acquire an extensive dataset of multiple test conditions. L4 + 36 h adult wild-type worm populations were exposed to 42 °C thermal stress and 7% t-BHP-induced oxidative stress after a 36 h culture on 48 gut microbial isolates for 36 h. The assay was performed four times, with each condition replicated four times in each assay. Across all the conditions tested, the median time of

death varied between 40-130 min for the thermal stress assay and between 90-240 min for the *t*-BHP induced oxidative stress assay. Early adulthood thermal stress assays usually display more consistent results and less inter-day and intra-day variability than oxidative stress assays and are better predictors of subsequent longevity<sup>30</sup>. The difference in the

median time of death of worms fed on different microbial diets conclusively exemplifies how gut commensals impact host stress resistance. **Figure 2** shows typical results from 16 biological replicates for Bristol N2 wild-type worms on two gut microbiota isolates of interest and the standard laboratory strain *E. coli* OP50.



**Figure 2: Representative results from paired heat/oxidative stress resistance assays from worms grown on two bacterial isolates and a control strain. (A)** Heat stress assay. Wild-type worms were fed for 36 h on either MYb11 (*Pseudomonas lurida*) or MYb115 (*Pseudomonas* sp.) bacterial isolates or OP50 control bacteria. MYb11 led to a significantly earlier median time of death ( $p < 0.001$ ). **(B)** Oxidative stress assay. Wild-type worms were fed for 36 h on either MYb11 or MYb115 bacterial isolates or OP50 control bacteria. MYb11 led to a significantly earlier median time of death ( $p < 0.05$ ). 120-150 worms were analyzed in four sets of samples, and each experiment was performed in quadruplicate. Wells with poor quality/poorly fitted data led to missing values. Box plot representations indicate single well values, minimum, median, and maximum values. \*Indicates statistical significance at  $p < 0.05$  and \*\*\* $p < 0.001$ , with one-way ANOVA. [Please click here to view a larger version of this figure.](#)

<b>A</b>		
<b>Parameter</b>	<b>Setting</b>	<b>Comment</b>
Temperature	25 °C	
Fluorescence excitation	365 nm	10 nm bandwidth
Fluorescence emission	430 nm	20 nm bandwidth
Number of flashes	8 flashes, 1ms settle time	
Gain	100 to 130	To be adjusted depending on sample. Aim for 2-5 k read value at start of assay
Lag time	1 $\mu$ s	
Integration time	30 $\mu$ s	
Read time interval	Every 2 min	Depending on stress severity, duration and time interval may need adjusting (8 h is enough at 7% t-BHP)
Duration	8-12h	
Read direction	From below	
<b>B</b>		
<b>Parameter</b>	<b>Setting</b>	<b>Comment</b>
Temperature	42 °C	
Fluorescence excitation	365 nm	10 nm bandwidth
Fluorescence emission	430 nm	20 nm bandwidth
Number of flashes	8 flashes, 1ms settle time	
Gain	100 to 130	To be adjusted depending on sample. Aim for 2-5 k read value at start of assay
Lag time	1 $\mu$ s	
Integration time	30 $\mu$ s	
Read time interval	Every 2 min	Depending on stress severity, duration and time interval may need adjusting (6 h is enough at 42 °C)
Duration	6-12h	

Read direction	From below		
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**Table 1: Oxidative and heat stress assay settings.** Examples of settings used for oxidative (A) and heat (B) stress assays on the fluorescence plate readers used in this study.

**Supplementary File 1: Media, buffer, and culture recipes.** Composition of different media, buffers, and cultures used in the present study. [Please click here to download this File.](#)

**Supplementary File 2: Raw fluorescence data from the plate reader after being exported and converted to excel .xls format.** (A) The excel file shows the raw fluorescence data for a heat shock assay. Raw fluorescence data is shown at 2 min time interval. (B) The column with "well position" on the 384-well plate is highlighted, which can be used to label the worm and bacterial strains. (C) The file is labeled with worm and bacterial strains. [Please click here to download this File.](#)

**Supplementary File 3: LFASS analysis using Matlab.** (A) After opening the LFASS folder in Matlab, a command window pops up. (B) "fitfolder" is typed in the command window to start the program, and the folder's name with the file to be analyzed is indicated. (C) Once the program finds the data excel file for analysis, the user must follow on-screen instructions, providing the various parameters requested. [Please click here to download this File.](#)

**Supplemental File 4: Analyzed LFASS data using Matlab.** (A) An example of a curve fitted through Matlab analysis, where the black line indicates the time point corresponding to the earlier time boundary and the blue line the later time boundary for the curve fitting. The fitted sigmoid curve is

displayed in red. (B) The results file generated by Matlab is opened in .xls format. The values are highlighted and displayed in number format. The second column provides the time when the value on the raw data curve first exceeds 50% of the maximum value. The third column provides the time when the fitted curve's value equals 50% of the maximum value, as determined during the batch-fitting analysis. The fourth column provides the time at which the value on the fitted curve equals 50% of the maximum value after batch analysis and final refitting where necessary. The fourth column should, thus, provide the most reliable result. [Please click here to download this File.](#)

**Supplementary Table 1: Example experimental plate layouts.** (A) 96-well plate layout for testing 47 different worm gut microbiota bacterial isolates on two separate worm strains, with live OP50 being used as a control. Four copies of this plate are required for each assay being run. (B) 384-well layout for heat or oxidative stress assays, corresponding to the previous 96-well plate assay in (A). This setup allows tetraplicates to be performed for each condition, with each worm strain having its own OP50 control. [Please click here to download this Table.](#)

## Discussion

*C. elegans* offers many advantages for rapidly screening multiple experimental parameters at once, owing to its small size, transparency, fast development, short lifespan, inexpensiveness, and ease of handling. Its considerably

simpler genome, body plan, nervous system, gut, and microbiome, yet complex and similar enough to humans, make it a powerful preclinical model, where mechanistic insight can be gained while testing for bioactive efficacy or toxicity. As interest is growing in developing microbial interventions for diseases<sup>8,9,34,35</sup>, taking advantage of such preclinical models to rapidly investigate probiotics is an attractive option with the largely untapped and near-unlimited therapeutic potential of microbes<sup>34</sup>. The protocol described here is suitable to study the impact of environmental bacteria on *C. elegans* health but can be readily adapted for other purposes.

### Versatility

The different "modules" that form this pipeline can be adapted to match experiment- or lab-specific requirements. For instance, bacterial isolates can be substituted for bacterial mixtures, bacterial mutants, or RNAi-producing bacteria, and the setup can be used to screen for bacterial genes involved in host-microbe interactions, screen for host genes involved in stress resistance, or study the impact of individual microbes as part of defined bacterial communities. This method describes the screening of several bacterial isolates in a single worm's genetic background. However, the assay is scalable, and multiple host and bacterial genotypes can be studied at once. This can be further combined with differential drug treatments and/or dietary regimens to gain insight into host gene-microbe gene-diet interactions.

The protocol is yet to be attempted on other nematode species, but it should also be directly applicable to *C. remanei* and *C. briggsae*, and it may apply to L3 stage parasitic nematode species that share morphological, size, and death fluorescence features with *C. elegans*<sup>30</sup>. Finally, other stressors (juglone, paraquat, arsenate, other

temperatures) can be used within the same pipeline to provide complementary or confirmatory information.

### Potential challenges

The time investment up to day 12 to grow worms and bacteria before the assays is significant, but due to the throughput of the approach, it is far outweighed by the breadth of the collected data. Nevertheless, care in planning such assays is essential to avoid wasting time by repeating the preparation steps because materials are suboptimal by the time the assays can start. Main early issues include sample contamination (worms or bacteria), poorly synchronized worm populations, and running out of materials (worm populations eat through their food faster than expected and re-feed or transfer animals more often than planned).

### Avoiding contaminations

Due to the scale and duration of the protocol, one major potential issue is contamination by fungi and bacteria, whose occurrence would certainly confound results. The ideal setup is, thus, an air-conditioned and air-filtered dedicated lab space without direct airflow above the experimental bench, using sterile consumables. However, observing regular cleaning procedures (decontaminating benches before and after work, cleaning plate boxes, and using sterile consumables) and working by a flame in a contained space (avoid hallways, spaces near open doors, and windows) are usually sufficient to maintain a clean environment. If mild contaminations occur prior to egg preparation in Step 2.2.1., the bleaching procedure will take care of them, and the protocol can progress as planned. Conversely, contaminations that affect the growing worm population prior to transferring onto test bacterial plates will signify the end of that attempt, and the protocol will need to be restarted from Step 1. Later contaminations will tend to affect

single bacterial strains or test conditions. Depending on their extent, it may be worth going on or restarting the protocol.

### Worm synchronization

As resistance to thermal and oxidative challenges changes when worms develop and grow, test conditions that lead to different developmental stages by the time LFASS assays are carried out cannot be readily compared. It is essential to yield well-synchronized populations for endpoint assays. As the assays are performed on worms collected in bulk at L4 or L4 + 48 h stages, in the absence of a worm sorter or other sorting procedure to collect perfectly staged worms, the worm populations must be very well synchronized at L1. It should be ensured that hatchlings are kept off food long enough for all live eggs to hatch before growing the test cohort (>15 h at 20 °C and >18 h at 15 °C). Genotype may affect the spread in developmental timing, which is also affected by temperature. Growing worm populations at 15 °C give better control over this while limiting the impact of temperature-sensitive mutations during the worm population expansion phase (notably when working with insulin signaling mutants). The main reason to study the effects of microbes from L4 or L4 + 48 h is to bypass the developmental impact of a differing diet and study the impact of live gut bacteria rather than their impact on diet. Worms grown in an earlier larval stage on different bacterial isolates or mixtures will inevitably lead to uneven developmental rates and a loss of synchrony.

### Consequences of relying on blue fluorescence

Being able to pinpoint death in LFASS assays without the need for individual worm tracking or additional reagents such as Sytox green<sup>36</sup>, but instead exploiting an endogenous signal in a fluorescence range that is unlikely to overlap with traditional worm biomarkers, is a clear benefit. However, it remains critical to ensure that worm death fluorescence

can be consistently detected across many test conditions. While the assay does not rely on fluorescence quantification but on its dynamics, sufficient fluorescence still needs to be emitted by the worms to estimate the median time of death (TOD) accurately. As the assay does not allow for resolution of single worm death, the blue fluorescence bursts emitted by dying individuals need to have sufficient temporal overlap for fluorescence accumulation to give out a single population fluorescence peak. Only then can median TOD be accurately inferred. This means worms unable to produce sufficient levels of blue fluorescence cannot be used in this protocol, which notably includes some mutants of the kynurenine pathway such as *tdo-2* and *kynu-1*<sup>30,31</sup>, as well as starved animals. Hence, it is important to ensure worms are kept in replete conditions throughout. This can notably be an issue when performing HT115-driven RNAi screens as HT115 lawns on NGM medium supplemented with IPTG and antibiotics tend to be thinner than OP50 lawns. Monitoring worm food supply during the days leading to the assays is, thus, critical.

Another consequence of the dynamics of death fluorescence is that the quicker the assay, the better the sensitivity, as worms from the same population will die more synchronously and produce a more defined population death fluorescence peak. Thus, the longer the assay, the larger the population size needed to generate good death fluorescence peaks. Mutants with low metabolisms, such as *eat-2* and *daf-2*<sup>30</sup>, will also give out less fluorescence and require higher numbers per well. While 10-15 wild-type worms per well are sufficient for a 42 °C thermal stress assay that kills all of them within 4 h, nearly double that amount is required for *daf-2(e1370)* worms in the same assay, and >100 wild-type worms are required for an assay that would kill worms over a 24h period.

In the context of environmental bacterial isolates, differing microbial metabolism can also differentially impact the kynurenine pathway that produces the blue fluorescing compounds. Hence, some bacterial isolates lead to lower peak death fluorescence, while others lead to higher peaks than OP50. As a result, one needs to aim for enough worms per well to accommodate all eventualities.

Lastly, correctly optimizing plate-reading parameters is essential. This protocol covers the setting of fluorescence excitation and emission parameters and the depth at which readings should ideally be performed. With a possibly wide range of death fluorescence yields across multiple conditions in a single assay, it is important to ensure that weak signals are detected but that detectors are never saturated. Instruments with detectors that boast high dynamic ranges will, thus, perform better.

### Limitations of the method

The method described here is robust and flexible and can be readily adapted to various conditions. For convenience, key restrictions and limitations are mentioned along the protocol where they come into play. Briefly, the reliance on death fluorescence as an endogenous marker of death is both a strength and a limitation of these assays.

Under a limited number of specific dietary conditions (low tryptophan diets or starvation) and in mutant conditions affecting tryptophan metabolism (i.e., *tdo-2*, *kynu-1*, some *daf-2* alleles), the death fluorescence (DF) may be strongly attenuated, to the extent that the peak fluorescence is difficult to detect and TOD estimates are less reliable.

Conversely, some microbes may produce blue fluorescing compounds whose excitation and emission spectra overlap with DF signals (i.e., *Enterococcus faecalis*). While their

dynamics differ from the DF and can often be uncoupled, they may confuse the analysis.

Next, the assays rely on the cumulation of signals from dying individual worms, but DF is quenched over time. Hence, if worms die too far apart, individual fluorescence peaks will fail to produce a population peak, preventing the measurement of a population median TOD. The slower the assay kills, the higher the worm number required in each well<sup>30</sup>. Hence, 10-15 adult worms per well are sufficient for a 42 °C heat stress assay that kills half the worms within 2 h, but over 70 worms per well are necessary for a bacterial fast-killing assay that kills half the worms in 12 h.

Lastly, users need access to a plate reader to perform such assays that can perform time-lapse fluorescence measurements at the required wavelengths. Excitation and emission wavelengths may also vary slightly between systems and vary between nematode species, as mentioned<sup>30</sup>.

### Comparison and complementarity with other *C. elegans* assays

Over the past 10-15 years, many assays have been developed to assess *C. elegans* health in multiple conditions at once, employing a diversity of strategies and offering different levels of data depth and breadth<sup>30,36,37,38,39,40,41,42,43,44,45,46</sup>. Several powerful assays have notably been developed to automatically monitor many worms at once on solid media across their lifespan, using camera arrays and scanners with multi-worm tracking algorithms and/or microfabricated individual worm arrays<sup>37,38,39,40,41,43</sup>. In the context of host-microbe interactions, similar approaches to the protocol described above but not relying on DF have also enabled genome-wide RNAi screening of *C. elegans* genes

involved in bacterial infections<sup>46,47,48</sup>. However, these high-throughput assays rely on handling worms in liquid throughout, which is not desirable when dealing with multiple bacterial types at once due to the increased risks of cross-contamination and because liquid culture affects host and microbial metabolisms. Hence, in traditional infection, stress, healthspan, and longevity assays, *C. elegans* are maintained and aged on bacterial lawns grown on solid media. As is also the case in this protocol, results from screens performed with this pipeline can be more readily related to and predict the outcome of downstream confirmatory experiments with screen hits.

The advantages of the LFASS approach itself have been well-covered in Reference<sup>30</sup>. Compared to more elaborate continuous imaging setups that could be repurposed for similar studies, LFASS is cheaper (provided median TOD is the only readout needed), and its simplicity means it does not require much technical ability or specialized equipment. LFASS is mainly about breadth over depth. LFASS data do not provide information other than the median time of death of a worm population and an indirect readout of a kynurenine pathway output. Conversely, with lower but still decent throughput, complex traits can be measured from other assays<sup>37,38,39,40,41,43</sup> over the whole lifespan of worms, making them excellent downstream approaches to study hits from an LFASS screen.

## Disclosures

The authors have nothing to disclose.

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