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High-throughput assessment of changes in the Caenorhabditis elegans gut microbiome

G3: Genes|Genomes|Genetics

In 1 collection

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1 Works for me dx.doi.org/10.17504/protocols.io.rtzd6p6

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ABSTRACT

The gut microbiome is an important driver of host physiology and development. Altered abundance or membership of this microbe community can influence host health and disease progression, including the determination of host lifespan and healthspan. Here, we describe a robust pipeline to measure the microbiome abundance and composition in the *C. elegans* gut that can be applied to examine the role of the microbiome on host aging processes.

EXTERNAL LINK

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COLLECTIONS

C. elegans related protocols

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PARENT PROTOCOLS

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C. elegans related protocols

GUIDELINES

This protocol starts once you have your nematodes of interest and want to know what type of bacteria, and how many are present in the worms digestive tract.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Levamisole	16595-80-5	Acros Organics

MATERIALS TEXT

Prepare all solutions using MilliQ water and store all reagents at room temperature unless otherwise noted.

1. Bacterial culture and maintenance

1. LB Medium: Add 10 g bacto-tryptone to 900 mL water in 1 L glass autoclavable bottle (e.g., Pyrex), then add 5g bacto-yeast, 5 g NaCl and adjust pH to 7.0 using 5 M NaOH. For LB agar,add 15 g of agar, adjust volumeto 1000 mL and autoclave.
2. Rectangular plates: Stamp out bacterial culture stock in 96 well plate format.
3. Deep well plates: Grow bacterial culture in 96 well plate format (Axygen).

2. *C. elegans* culture and maintenance

1. Nematode Growth Medium (NGM) Agar: Start with 975 mL water in 1 L glass autoclavable bottle (Pyrex, etc) , add 3.0 g NaCl, 2.5 g peptone, 17 g agar and autoclave with a magnetic stir bar. Cool the mixture down to 55°C on a heat plate preheated to 55°C and add the following while stirring it: 0.5 mL of 1 M $CaCl_2$(sterile), 1 mL of 5 mg/mL cholesterol (dissolved in ethanol), 1 mL of 1 M $MgSO_4$(sterile), 25 mL of 1 M potassium phosphate buffer, pH 6.0 (sterile), pour into petri dishes or multiple well plate using sterile technique.
2. M9 buffer: Start with 700 mL water in 1 L glass autoclavable bottle (Pyrex, etc), add6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl , 0.25 g $MgSO_4 \cdot 7H_2O$ or 1 mL of 1 M $MgSO_4$, add water to 1000 mL, and autoclave.
3. Multiwell plates: 12 or 24 well plates with NGM agar for cultivation ofC. elegans.

3. Measurement of microbiome colonization levels

1. M9-wash solution:Prepare 0.01% Triton X-100 solution in M9 buffer form 5% Triton X-100 stock solution in M9 buffer. Sterilize by filtration through 0.22 µm filter.
2. Levamisole solution: Prepare 100 mM stock solution of levamisole in M9 buffer and dilute it to 10 mM to be used as working solution. Sterilize by filtration through 0.22 µm filter. Prepare fresh before use.
3. Egg prep bleach solution: Mix 2 parts of Clorox bleach and 1 part of 5M NaOH. Prepare fresh before use.
4. Wash bleach solution: Prepare a 4% Bleach solution in M9 buffer. Prepare fresh before use.
5. Garnet beads (1.0 mm): for mechanical lysis of worms by bead-beating. Sterilize by autoclaving before use.
6. Phosphate buffer saline(PBS;1X): pH 7.2, 0.22 µm filter sterilized.

4. Measurement of microbiome composition and diversity

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1. PCR primers
2. Silica beads (0.1 mm): for mechanical lysis of bacterial cells by bead-beating prior to DNA isolation. Sterilize by autoclaving before use.
3. 96-well PCR plates: for use in Proteinase K treatments during microbiome DNA extraction and PCR amplifications.
4. PCR master mix
5. Agarose gel electrophoresis supplies
6. Low melt agarose powder
7. TAE buffer (40 mM Tris-acetate, 1 mM EDTA)
8. 1Kb ladder (NEB)
9. Gel loading dye (NEB)
10. PCR purification kit: Removes primers, nucleotides, enzymes and other impurities from PCR product before sequencing.

5. Equipment

1. Multiple channel pipette: 12 channel pipettes (1000ul and 200ul) are commonly used in the assay to dispense liquid reagentsand transfer volumes between plates.
2. Plate reader: for assessment of optical density (OD) in 96 well clear flat bottom plates.
3. PCR thermocycler: for use in Proteinase K treatment during microbiome DNA extraction and generation of 16S rRNA gene amplicon libraries.
4. Mixer mill: for use in lysis of animals and microbial cells by bead-beating (Retsch MM400).
5. Microplate aspiration manifold: for use in rapid removal of supernatants during wash steps (VP1171A V&P scientific).

EQUIPMENT

NAME	CATALOG #	VENDOR
V&P scientific	VP1171A	

BEFORE STARTING

Before starting the Colonization assay you should have the following:

1. An egg prep of the desired worm strains
2. Seeded NGM plate(s) with desired bacterial strain(s)
3. Drop ~30 L1 worms on seeded plates and incubate at 20°C. Allow the worm to grow until desired age

High-throughput Colonization Assay

1 Wash worms from bacterial lawns with M9 buffer + [M]**0.01 % volume** Triton X to a sterilized 2 ml 96-well deep plate.

- Use **1000 µl** M9+ Triton X to wash worms from a 12-well plate
- Use **600 µl** M9+ Triton X to wash worms from a 24-well plate

M9 Medium
by Adrien Assie,
Baylor College of Medicine
PREVIEW RUN

1.1 Start with **700 mL water**

1.2 **6 g** of Na2HPO4 ([M]**42 Milimolar (mM)**)

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1.3 **3 g** of KH2PO4 (**22 Milimolar (mM)**)

1.4 **5 g** of NaCl (**86 Milimolar (mM)**)

1.5 **0.25 g** of MgSO4•7H2O

OR

1 mL **1 Molarity (M)** MgSO4

1.6 Adjust water to **1000 mL**

1.7 Autoclave

2 Bring each well to **1.8 mL** total with M9 + **0.01 % volume** Triton X using a multichannel pipette

3 Centrifuge deep well plate at **00:01:00 at 300g** to pellet down worms, then remove liquid by using an aspirating manifold. Alternatively worms can be allowed to settle in the deep well plate by gravity without centrifugation to separate adults from progeny.

V&P scientific VP1171A
Aspirating manifold, allow liquid aspiration from 96 well plates

4 Repeat steps 2-3 **5 times** with M9 to further remove bacteria in liquid.

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5 Bring the volume in each well down to **100 µl** using the aspirating manifold.

6 Add **100 µl** of **10 Milimolar (mM)** levamisole in 1X M9 + 0.01% Triton X to each well and allow the worms to paralyze for **00:05:00** . Confirm paralysis via microscopy before proceeding to the next step.

Levamisole
by Acros Organics
Catalog #: 16595-80-5

7 Add **200 µl** of 4% bleach solution (2:1 Bleach:5M NaOH) in M9 to each well for **00:02:00**

This will further eliminate bacteria in liquid and attached on worm cuticle.

8 Repeat step 2-3 2 times with 1X M9 + **0.01 % volume** Triton X to remove bleach and levamisole.

Optional

9 Save **100 µl** of supernatant from the last wash to use as a control to assess the background bacterial residual before host lysis.

High-throughput Colonization Assay

10 Bring liquid volume in each well to **1 mL** 1X M9 + **0.01 % volume** Triton X, mix and transfer **100 µl** of the mixture to a flat bottom 96 well plate (Costar 3370, Corning).

11 Take bright field images for each well for later analysis of worm population size, life stages, and individual length.

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12 Aspirate remaining liquid volume down to **300 µl** and add 1.0 mm sterilized garnet beads (Biospect) to each well (enough to cover well bottom).

13 Seal the plate with a sterile plastic mat and lyse worms in Mixer Mill (Restch) or TissueLyser at 25 Hz speed for 5 min.
00:05:00 Bead beating

14 Mix by pipetting and transfer **20 µl** of cell lysate to a 96-well plate prepared with **180 µl** 1x PBS per well (1:10 dilution) and mix

15 Transfer **20 µl** of the dilution from step 14 to **180 µl** LB in 96 well flat bottom for liquid OD growth assay.

Calculate CFU based on liquid growth curve

16 Fill a flat-bottom 96-well plate with **180 µl** of sterile filtered PBS. Pipette **20 µl** of the microbiome mixture used in the assay into four replicate wells in column 1 of the plate (e.g., A1, B1, C1, and D1). For controls, pipet **20 µl** of LB in the remaining wells of column 1.

17 Using a multichannel pipette, serially dilute the microbiome mixture (1:10) in columns 2-12 to create a dilution series for all wells of the plate. Change tips between each dilution/column.

18 From the serial dilution plate, plate **10 µl** of each dilution onto a rectangular LB plate using a multichannel pipette. Incubate at optimal growth temperature overnight. Count colonies for each of the wells where possible.

19 Prepare a growth plate by filling each well of a flat bottom 96-well plate with **180 µl** of LB. Transfer 20 µL from the serial dilution plate to the corresponding well of the growth plate. Incubate at the optimal temperature with intermittent shaking and monitor OD_{600} regularly (e.g., every 15-30 min) until growth is observed in all wells.

20 Use a plotting software (Microsoft Excel, R or otherwise), to visualize growth curves by plotting OD_{600} reading vs time for each well on a single chart. Verify that the growth patterns are consistent between replicates and growth curves are ordered by the dilution factor.

21 Identify the range of OD_{600} values within the linear portion of the growth curves. Use the linear range identified and the following equation to calculate the optical density Threshold:

$$Threshold = \frac{(OD_{600max} - OD_{600min})}{2} + OD_{600min}$$

22 Using the only data points where the OD_{600} readings for each well fall between the linear portion, calculate the Slope and Intercept for each well

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23 To calculate the time at which each well reached the threshold (Y-axis of calibration curve), use the following equation for each well:

$$Time = \frac{(Threshold - Intercept)}{Slope}$$

24 Plot CFU values collected in **Step 19** vs.Time of Threshold crossing for each well, and calculate the R squared value and exponential trendline equation. Use the obtained equation to calculate CFU from the respective threshold time of each sample.

25 Determine colonization levels on a per animal basis for each sample using the following formula:

$$CFU/animal = \frac{(CFU * Dilution factor)}{number of animals}$$

DNA extraction and 16S rRNA PCR

26 Centrifuge the deep-well plate at 4000g for 10 mins to pellet worms and bacteria, then bring the remaining liquid volume down to **190 µl**

00:10:00 Centrifugation at 4000g

27 Freeze the lysate in -80 °C freezer overnight.DNA extraction and 16S rRNA PCR

-80 °C Freezing

28 Thaw the plate and add 0.1 mm sterile zirconia/silica beads (BioSpec products) to each well (enough to cover well bottom) and bead-beat in Mixer Mill (Restch) or TissueLyser for 5 mins to further lyse bacterial cells.

00:05:00 Shaking/Lysis

29 Add **10 µl** of **20 mg/ml** proteinase K to each well.

30 In a ThermoCycler run a program as follow:

01:00:00 step at **60 °C**

00:15:00 step at **95 °C** to desactivate the proteinase K

keep on hold at **4 °C**

31 Centrifuge the plate **00:10:00 at 4000g** to pellet down cellular fractions.

Transfer **100 µl** of supernatant to a clean 96 well PCR plate.

- If using the plate again soon, place at **4 °C** , otherwise, store at **-80 °C**
- Use filter tips for this step, and do not allow the area above the 'groove' of the tips to enter the plate

32 In a 5 mL microcentrifuge tube, mix:

- **1.25 mL** of 2x Taq Master Mix (1 tube)

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- **100 µl** of 515F primer
- **1 mL** H_2O

33 Add **23 µl** of newly mixed master mix into each well of a new 96 well PCR plate

34 Add **1 µl** of reverse primer 806R to each well of the 96 well PCR plate.

35 Add **1 µl** of the supernatant from the PCR plate prepared in step 23 as DNA template for the PCR.

The remaining supernatant can be frozen at **-80 °C** for storage.

36 Place the PCR plate in the ThermoCycler and run the following program

Temperature	Time, 96-well	Repeat
94 °C	3 min	
94 °C	45 s	x35
50 °C	60 s	x35
72 °C	90 s	x35
72 °C	10 min	
4 °C	hold	

37 Verify PCR amplification results with an agarose gel. Prepare a 1.5% agarose gel. Mix 2 µL of loading dye with 4 µL of PCR product per sample and load 5 µL in the gel along with 1 kb ladder.

38 Purify PCR product using commercial PCR purification kit.

39 Quantify the intensity of PCR product band from each library (gel or fluorometry), normalize PCR product amount by volume and pool libraries together with an equivalent amount of PCR product into a single tube.

40 Submit samples for multiplex sequencing along with sequencing primer

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