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Most Probable Number Relative Fluorescence Units Microplate Assay

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ABSTRACT

The most probable number (MPN) assay is a culture-dependent method of quantification most commonly used to enumerate microbes from environmental samples 1. The MPN assay determines the density of microbes by serially diluting to extinction a sample in a medium which supports their growth. Using probabilistic principles ²⁻⁴, an MPN value is derived from the consecutive pattern of presence and absence of a measurable signal (e.g., optical density, fluorescence, luminescence), the original sample volume present at each dilution step, and the number of replicates for each dilution series. Application of the MPN assay to a homogenous culture does not result in subjective interpretation of what is being enumerated in a sample. However, tracking a single microbe amongst an heterogenous population requires either complete suppression of non-targets or a mechanism of distinguishing the target within the sample. Here we provide a stepby-step protocol for the MPN assay using a fluorescently tagged soil pathogen and semi-selective media to estimate the concentration of targets from soil samples. To enable high-throughput screening, we perform the MPN assay in microplates ^{5,6}. We refer to this assay as the MPN relative fluorescence units (MPN-RFU) microplate assay.

GUIDELINES

- The estimated cell concentration obtained from an MPN assay is based on the probability theory. There are assumptions that must be met in order for the MPN assay to estimate the true microbial concentration in a sample ^{1,4}. Assumption 1, the inoculum contains random distribution of microbial cells. This means that at each dilution step, samples must be thoroughly mixed before proceeding with further dilutions. Clumps prevent proper dilution of the sample leading to abnormal results with increasing dilutions. Assumption 2, the presence of a single cell within a diluted sample should result in growth.
- When applying the MPN-RFU microplate assay to environmental samples, selective or semi-selective media must be used in order to reduce background noise. Otherwise, this method will result in false-positives. If applying the assay to pure cultures, then there is no need to use selective media.
- In cases where the user is trying to quantify a low-density population from soil samples, consider filtering or centrifuging samples to remove unwanted soil particles. In our setup, we did not perform these additional steps. Therefore, soil particles fully blocked the passage of the excitation light in undiluted samples

(row 1 of microplate). Even though there were soil particulates at 10^{-1} and 10^{-2} dilutions, emission spectra were obtained for these wells.

- In this protocol we are using 96-well microplate, but this assay can be performed with other plate formats as long as the plate reader has different plate configurations.
- A 48-h incubation of a microplate was enough time to allow our tractable system (Ralstonia solanacearum) to grow in the most diluted wells. Depending on the bacterial strain, microplates may need to be incubated for longer times. In such cases, ensure that media levels are carefully replenished in wells showing sign of volume loss due to evaporation. Alternatively, serial dilutions and incubation can be carried out in a deep-well plate, and, at the time of data collection, samples transferred to a plate that meets the specifications of a plate reader.
- We note that although well volume determines the path length and is correlated linearly to the intensity of fluorescence of each well, small differences in volume generally should not impact the determination of a positive sample. Positive samples in our system are, on average, two orders of magnitude (100x) brighter than background, while the threshold for a positive read was nearly twice the average of background RFU. In theory, a volume reduction of 98% would be required to yield a false negative, on average. Path length correction is available in many microplate readers for study systems in which a higher level of sensitivity is required and volume differences may impact the outcome of the analysis.
- An MPN estimate is calculated from the volume of sample used in each dilution step, the number of replicates per dilution, and the number of positive wells for each dilution series ^{1,4,6}.

MATERIALS

Materials for preparing soil microcosms

- 1. Two 8" x 10 34" polypropylene tray and snap-on lid (Bel-Art F1630-0000)
- 2. Ceramic mortar and pestle
- 3. Thermo ScientificTM NalgeneTM large polyproylene sterilizing pan (catalog number 6900-0010PK)
- 4. Thermo ScientificTM ClipTipTM 1250 pipette tip rack (catalog number 94410817)*
- 5. Metal trowel
- 6. Paintbrush
- 7. Rubber bands
- Nylon strainer with ~2-3 mm-diameter holes (Mainstays Scoop & Strainer UPC 08346900775)
- 9. Aluminum foil

- 10. Eppendorf Deepwell Plate 96/2000 uL (catalog number 951033502)
- 11. Greiner Bio-One 96-well, polystyrene, flat-bottom, clear microplate (item 655101) and lid**
- 12. Nitrile gloves***
- 13. Alconox
- 14. 70% Ethanol
- 15. Deionized water
- 16. Autoclave
- 17. Biosafety cabinet
- 18. Sterile 50-mL conical-bottom centrifuge tube (VWR 21008-242)
- 19. Soil capped in a 5-gallon, polyethylene bucket (Lowe's 57640)****
- 20. Meker and/or Bunsen burners
- 21. Compact balance (A&D Company, Limited EJ-120)
 - *Alternatively, one can use a Thermo ScientificTM ClipTip TM 1000 pipette tip rack (catalog number 94420713-HID) instead of item 4.
 - **Alternatively, one can use a new, sterile, polystyrene, flat-bottom, clear microplate with each experiment instead of item 11.
 - ****Prior to filling with soil, a new, 5-gallon, polyethylene bucket can be sterilized with 70% ethanol and/or UV light.

The materials that follow are to be used with the rest of the protocol.

Reagents

- 1. cycloheximide (Sigma-Aldrich 01810-1G)
- 2. 2,3,5-triphenyltetrazolium chloride (TZC) (Difco 264310)
- 3. bacitracin A (Sigma-Aldrich B0125-50KU)
- 4. penicillin G sodium salt (Sigma-Aldrich P3032-10MU)
- 5. gentamicin sulfate (GoldBio G-400-25)
- 6. N-(3-oxohexanoyl) homoserine lactone (OC6, Sigma-Aldrich K3007)
- 7. 10X phosphate-buffered saline (Fisher Bioreagents FLBP6651)
- 8. N,N-dimethylformamide (Fisher Scientific D119-500)
- 9. agar (Sigma-Aldrich A1296-1KG)
- 10. peptone (Fisher Bioreagents BP1420-500)
- 11. casamino acid (Research Products International C45000-500.0)
- 12. BactoTM tryptone (Gibco 211705)
- 13. D-glucose, anhydrous (Bio Basic 50-99-7)
- 14. sodium chloride (Fisher Scientific S271-1)
- 15. 70% Ethanol (30% v/v sterile de-ionized water, 70% v/v ethyl alcohol, pure, Sigma-Aldrich 459844-4L)
- 16. deionized water

Consumables

1. 500-mL glass flasks

- 2. 250-mL glass flasks
- 3. glass culture tubes
- 4. media reservoirs with lids (VWR 89049-030)
- 5. glass graduated pipettes (5 mL, 10 mL, 25 mL)
- 6. microplate black lids
- 7. silicone mat (Eppendorf 0030127978)
- 8. petri dishes (Fisher Scientific FB0875712)
- 9. deepwell plate 96/2000 µL, white border (Eppendorf 951033502)
- 10. microplate 96/F-PP, white border (Eppendorf 951040005)
- 11. 96-well black microplate (Thermo Scientific 265301)
- 12. sterile syringe filter 0.2 µm PES (VWR 28145-501)
- 13. 10-mL sterile syringes (BD 309604)
- 14. 15-mL sterile polypropylene centrifuge conical tubes (Fisherbrand 05-539-12)
- 15. 50-mL sterile polypropylene centrifuge conical tubes (VWR 21008-242)

Pipettes

- 1. For transferring soil from deepwell plate to row 1 of microplate:
- Pipetman L Multichannel P12x1200L, 100-1200 μL (Gilson FA10040)
- ZAPTM SLIK aerosol pipet tips with wide orifice, 100-1000 µL (Labcon 1148-965-008-9)

2. For serial dilutions:

- Transferpette[®] S-8, 10-100 µL (BrandTechTM BRANDTM 705908)
- 200-μL wide bore low-retention pipette tips, sterile, RNase and DNase free (Teepa Tips TT-200ULWB-C; they fit the Transferpette[®] S-8, 10-100 μL)
- 10-µL FisherbrandTM Sure OneTM Low Retention pipette tips (Fisher Scientific 02-707-009)
- 3. For aliquoting media:
- Impact² 8-channel electronic pipette, 50-1250 μL (Matrix Technologies Corp.)
- 1250-µL sterile Matrix pipet tips (Thermo Scientific 8042)
- 4. For spotting on agar:
- 8-channel Finnpipette[®] F2, 1-10 μL (Thermo Scientific 4662000)
- 10-µL FisherbrandTM Sure OneTM Low Retention pipette tips (Fisher Scientific 02-707-009)

Equipment

- 1. analytical balance (Mettler Toledo AB54-S)
- 2. compact balance (A&D Company, Limited EJ-120)
- 3. incubator, 37°C (Percival I-35 LL)
- 4. low temperature incubator, 30°C (VWR Scientific 2020)
- 5. incubator shaker (Innova[®] 40)
- 6. multi-mode microplate reader for optical density and fluorescence intensity (SpectraMax M3)

- 7. microplate adapter for top read of 96 and 384 well plates (Molecular Devices 0310-4336 Rev B)
- 8. centrifuge (Eppendorf 5810 R 15 amp version)
- 9. swing bucket rotor for centrifuge (Eppendorf A-4-62)
- 10. digital microplate shaker, 120V, 150-1200 rpm (Thermo Scientific 88882005)
- 11. Meker and/or Bunsen burners
- 12. vacuum system for aspiration
- 13. bead bath, 14 Liter, with Lab Armor[®] Beads (Lab Armor[®] 74309-714)
- 14. Isotemp[®] magnetic stirrer (Fisher Scientific 11-100-49s)
- 15. magnetic stir bars coated with PTFE (VWR 58948-138)
- 16. pipet-aid

SAFETY WARNINGS

- •
- It is recommended to wear gloves, closed-toe shoes, goggles, and a mask to avoid abrasion and particulate inhalation of soil. Other personal protective equipment (PPE) may be required based on the biosafety level of the microbes in a particular soil sample and consortia.
- Wear PPE when preparing antibiotic solutions, preparing semiselective media, and while handling microplates with media.
- Handle with care the antibiotic cyclohexamide, as it inhibits protein synthesis in eukaryotic systems.
- It is the responsibility of each user to carefully read the MSDS of each reagent used in this protocol and adhere to their recommendations in the proper handling, storage, and disposal.
 Users can also consult with their institution Environmental Health and Safety department for further assistance.

BEFORE START INSTRUCTIONS

- In this protocol we do not cover how to engineer a bacterium to express fluorescence. We expect the user to have their strains already transformed and characterized for the best expression conditions.
- As an example of the application of this protocol, we are using the plant pathogen *Ralstonia solanacearum* strain Rs5 transformed with an inducible red fluorescent protein carried in the vector "pFLxR5" available through Addgene (plasmid ID 149482). The vector pFLxR5 has the antibiotic marker gentamicin and a <u>LuxR/P_{LuxB}</u> promoter-regulator pair regulating an mRFP cassette ⁷. Expression of RFP was induced in *Ralstonia* by N-(3-oxohexanoyl) homoserine lactone (OC6) added to media at a working concentration of 10.2 μM. To prevent loss of plasmid throughout incubation in broth and agar, gentamicin at a concentration of 10 mg/L (gent-10) was always included in the media.
- Transformation of *Ralstonia* was done as described by Perrier et al. (2018) ⁸. To ensure plasmid retention, cultures of *Ralstonia* were grown in the presence of gentamicin at a concentration of 10 mg/L. No selective pressure was applied to soil samples. We profiled the stability of the plasmid in *Ralstonia* for a duration of three days and did not observe any biological loss of vector.
- This protocol was tested with clay soil with native microflora. Therefore, we are using semi-selective media optimized for *Ralstonia* and our expression needs. Users will need to optimize media for their own conditions.
- This protocol describes in detail how to apply the MPN-RFU microplate assay to enumerate concentrations of *Ralstonia solanacearum* in soil samples. For other bacterial systems and soil types, users will have to optimize and adapt each step to their own conditions.
- To calculate an MPN value from RFU data and microplate formats, users can use our command line package "MicroMPN". A separate protocols.io was created to guide users through the process of running MicroMPN.

Preparing soil microcosms

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1 In this section, we cover how to assemble high-throughput soil microcosms. Play the video below to overview how to load a 96-deepwell plate with soil in less than 2 minutes.



In our system, we used clay soil which was retrieved in 2021 from the University of Florida, North Florida Research and Education Center (Quincy, Florida, USA). Soil was collected from four sections of a plot (30.551302, -84.599687) at a depth of 15 cm and combined as one. Soil was stored in air-tight, polyethylene containers at 4°C for 1 year.

In our system, we dried soil at room temperature in a sterile tray for 6 days. Finalizing day 6 of drying, we loaded 2-mL deepwell plates, which we refer to as soil microcosms. Plates were covered with a sterile lid and left on a bench at room temperature. Early morning of the proceeding day, we inoculated soil wells with rfp-tagged *Ralstonia*. Inoculated systems were

incubated in the dark at $30 \,^{\circ}\text{C}$ (± 2°C) and $30 \,^{\circ}\text{C}$ (± 5%) relative humidity, for a total of 72 h.

- 2 The following items were used to assemble our soil microcosms. Some of these items are autoclavable, while others need to be cleaned and air dried in a biosafety cabinet.
 - 1. Two 8" x 10 34" polypropylene trays and their snap-on lids (Bel-Art F1630-0000)
 - 2. Ceramic mortar and pestle
 - 3. Thermo ScientificTM NalgeneTM large polyproylene sterilizing pan (catalog number 6900-0010PK)
 - 4. Thermo ScientificTM ClipTip TM 1250 pipette tip rack (catalog number 94410817)*
 - 5. Metal trowel
 - 6. Paintbrush
 - 7. Rubber bands
 - 8. Nylon strainer with ~2-3 mm-diameter holes (Mainstays Scoop & Strainer UPC 08346900775)
 - 9. Eppendorf Deepwell Plate 96/2000 mL (catalog number 951033502)
 - 10. ~1 m of aluminum foil
 - 11. Greiner Bio-One 96-well, polystyrene, flat-bottom, clear microplate (item 655101) and lid**
 - 12. Nitrile gloves***

*Alternatively, one can use a Thermo Scientific TM Clip Tip TM 1000 pipette tip rack (catalog number 94420713-HID) instead of item 4.

	See steps below for specific sterilization and cleaning protocols.
3	Gather items 1-9 and follow the sub-steps below to clean and sterilize them.
3.1	Wash with Alconox.
3.2	Rinse with hot tap water.
3.3	Rinse with deionized water.
3.4	Dry with brown paper towels.
3.5	Wrap each item individually with foil.
3.6	Sterilize with an autoclave cycle of 30 minutes at 121°C and a dry cycle of 30 minutes.
4	Follow the sub-steps below to clean, sanitize, and air dry the Greiner Bio-One 96-well, polystyrene, flat-bottom, clear microplate (item 655101) and its lid.

^{**}Alternatively, one can use a new, sterile, polystyrene, flat-bottom, clear microplate with each experiment instead of item 11.

- **4.1** Wash with Alconox.
- **4.2** Rinse with hot tap water.
- **4.3** Rinse with deionized water.
- **4.4** Spray with 70% ethanol.
- 4.5 Air dry in a biosafety cabinet, with the inner sides of the plate and lid facing down. To allow for air flow underneath, prop one against the other in an A-shape. If there are many to dry, stack them in a circle like fallen dominos.

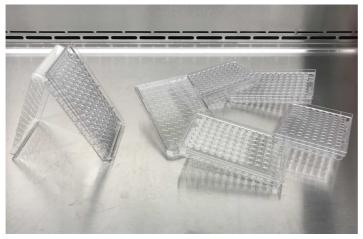


Fig. 1. How to stack microplates and lids, facing downward, in an A or circular, fallendominos configuration to allow air flow for drying in a biosafety cabinet.

5 After all items previously listed in this section have dried (usually overnight), follow the sub-steps

below to dry soil. Soil will be dried in a sterile polypropylene container.

Wear PPE when performing the following sub-steps.

- **5.1** Wipe down benches and hard work surfaces with 70% ethanol.
- **5.2** Place a label on the lidded polypropylene tray. On this label, write the starting date and time of soil drying.
- 5.3 Using a sterile trowel, proceed to mix the soil in the storage bucket. Once mixed, transfer soil to the pre-labeled polypropylene tray. Usually, one to two scoops are sufficient to load several deepwell plates. On average, each well receives 0.4 grams of soil. For example, 0.4 g x 96 wells = 38.4 grams of soil needed per plate. This should give users an idea of how many grams of soil they will need depending on their experimental design.

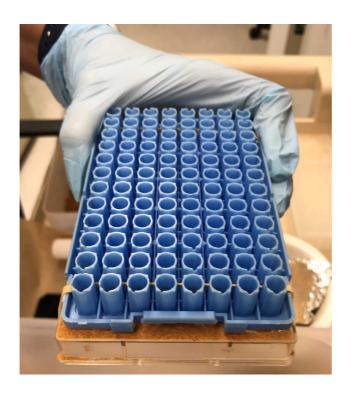
It is good practice to record how much soil was used for each experiment. This can serve as reference for future scaling of experiments. Without the lid, tare and weigh soil in tray using a regular balance.

- Before drying the soil, cover the tray with brown paper towels and affix with tape. The paper towels provide a breathable membrane that will allow water to evaporate. Place the covered tray where it is not under lights and not humid. Allow the soil to dry at Room temperature, for 6 days or 144:00:00.
- Once the soil has dried for 6 days, proceed with loading it into a 96-deepwell plate by following the sub-steps below.
- **6.1** Start by removing the paper towels covering the tray, then proceed to weigh the tray and record changes in soil mass. Users will note a drop in weight. Drier soil makes it easier to load deepwell plates using the technique shown in the video.
- **6.2** By the flame, use a sterile 50-mL conical-bottom centrifuge tube to transfer soil into a sterile, aluminum foil-lined mortar.

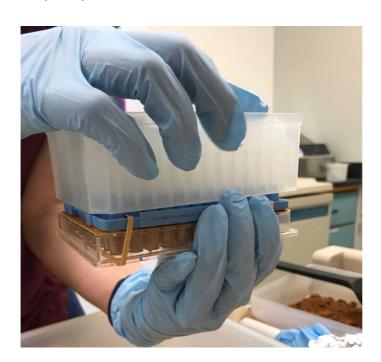
- **6.3** Pulverize the dried soil with the pestle.
- **6.4** Sift the pulverized soil with the sterile strainer into a **new sterile polypropylene tray** (not shown in the video).
- **6.5** Place large particles remaining in the strainer back with the rest of the un-processed soil.
- **6.6** Repeat sub-steps 6.2 6.5 until enough soil has been pulverized.
- **6.7** Use the conical tube to transfer the strained, pulverized soil into the Greiner microplate. The microplate wells will function as measuring cups.



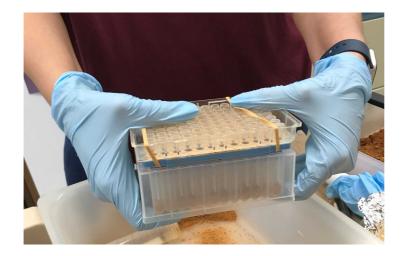
- **6.8** Use a spatula and gloved hand to level the soil flush with the top of the microplate.
- 6.9 Overlay the sterile pipette tip rack on top of the filled microplate. The rack will function as a funnel. Align the rack holes with the microplate holes to ensure the soil will be able to funnel through later. Use two rubber bands to hold the assembly together.



6.10 Now, overlay the tip rack with a sterile deepwell plate. Be careful to avoid lateral movement. The bottom portion of the assembly must maintain alignment in order to funnel soil into the deepwell plate.



6.11 Invert the entire assembly to load the deepwell plate with soil. Pat the microplate if clumps of soil do not release. Then remove the assembly of rubber bands, tip rack, and microplate.



6.12 Brush away excess soil from the skirt of the deepwell plate with the paintbrush.



- 6.13 As required by experiments executed on the same day, repeat the above sub-steps to load additional deepwell plates with dried soil.
- On the outside of a deepwell plate, label the date and experiment name. If you have multiple plates, write a description to distinguish between plate treatments. As a precaution, label both sides of the plates with the same information.

Preparing cultures of rfp-tagged Ralstonia

- A loaded deepwell plate with soil is used immediately the day after. **Users must time drying soil** with the steps described in this new section.
 - In our experiments we used the bacterium *Ralstonia solanacearum* strain Rs5 to inoculate

soil. We made *R. solanacearum* tractable by transforming the bacterium with the plasmid pFLxR5 (Addgene plasmid ID 149482). This plasmid encodes for a red fluorescent protein, whose expression in controlled by the LuxR/P_{LuxB} promoter-regulator pair. The transcriptional regulator LuxR binds to inducer N-(3-oxohexanoyl) homoserine lactone (OC6), which then recruits the RNA polymerase to the promoter LuxB to initialize transcription of mRFP. Additionally, the plasmid encodes for a gentamicin resistance cassette. To prevent loss of plasmid throughout incubation in broth and agar, the antibiotic gentamicin was always included in the media.

Sreak *Ralstonia* from a glycerol stock on casamino acid-peptone-glucose (CPG)⁹ agar supplemented with 10 mg/L of gentamicin. Perform this step under aseptic conditions.

Preform the following sub-steps a few days prior to streaking.

Prepare a stock of gentamicin with a concentration of 20 mg/mL (20 g/L). Resuspend powder in autoclave de-ionized water and filter sterilize (0.22 μ m). Keep stock at 4 °C.

8.2 CPG agar recipe:

A	В
Component	Per Liter
Peptone	10 g
Casamino Acids	1 g
Glucose (dextrose)	5 g
Yeast Extract	1 g
Agar	16 g
De-ionized water	up to 1 L

Depending on the volume needed, users should scale the number of grams from each component of the agar recipe.

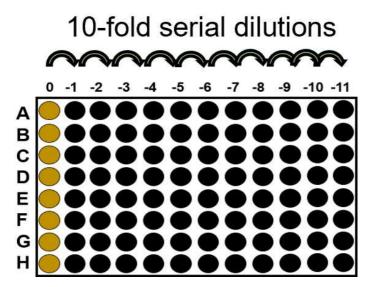
Include a stir bar with the agar. Autoclave at 121°C for 30 minutes, cool to about 55°C before adding gentamicin. For 1 L of agar, add 500 μ L of gentamicin from the stock prepared earlier, for a final concentration of 10 mg/L. Allow agar plates to solidify before storing at $\frac{1}{2}$ 4 °C.

9 Incubate streak plate at \$\mathbb{g}\$ 30 °C for at least 3 days.

- From a streak plate, prepare an overnight culture. Pick three independent colonies and inoculate 6 mL of CPG broth supplemented with 10 mg/L of gentamicin.
 - *Depending on the number of soil wells to be inoculated, prepare sufficient volume of bacterial culture. In our experiments, soil wells were inoculated with 150 μ L. Therefore, 6 mL should be enough volume for inoculating approximately 30 35 wells.
 - * The number of soil wells to inoculate with bacteria will be determined by how the user choses to distribute soil replicates per a 96-well microplate. Remember, samples must be diluted to extinction in order to estimate the original microbial load in soil. That being said, there are different ways in which a user could structure sample distribution across a microplate.
 - *Whether a microplate needs to shake or not will also affect how many wells of soil can be processed at a time. In our experimental design, to speed up bacterial growth and promote synthesis of fluorescent protein, we maintained microplates under shaking conditions. For this step we used a digital microplate shaker which fits 4 plates at a time.
 - * We used 10-fold serial dilutions, but users can use other dilution series.

For example:

• In our case, from a single soil well we aliquoted 8 replicates of soil to column 1 of a microplate and performed eleven 10-fold serial dilutions. This means that for every single well of soil, an MPN value was calculated from a single microplate.



- In the above layout, users might process 2 soil samples per plate, for 4 replicates per sample. Replicates for sample 1 could occupy rows A D, while the rest of the rows are used for sample 2. Alternatively, the plate could be split in half and include 4 samples each with 4 replicates.
- Instead of performing dilutions horizontally, users can perform dilutions going in the vertical direction. For example, a user could include 4 soil samples, each with 3 replicates.

Alternatively, 3 soil samples, each with 4 replicates.

Preform the following sub-steps prior to preparing an overnight culture.

10.1 CPG broth recipe:

A	В
Component	Per Liter
Peptone	10 g
Casamino Acids	1 g
Glucose (dextrose)	5 g
Yeast Extract	1 g
De-ionized water	up to 1 L

Depending on the volume needed, users should scale the number of grams from each component of the media recipe.

Include a stir bar in the broth. Autoclave at 121°C for 30 minutes, cool to about 55°C before adding gentamicin. For 1 L of agar, add 500 μ L of gentamicin from the stock prepared earlier, for a final concentration of 10 mg/L. Allow agar plates to solidify before storing at $\frac{1}{2}$ 4 °C.

Shake overnight culture in an incubator shaker for 16 h at (5 250 rpm, 30°C)

Inoculating soil microcosms with rfp-tagged Ralstonia

- In this next section, users will inoculate the pre-loaded with soil deepwell plate with the overnight of *Ralstonia* prepared in the above section. Before soil inoculation we remove the antibiotic gentamicin by centrifuging and resuspending the overnight culture in sodium chloride.
- Transfer overnight culture to a 50 mL polypropylene centrifuge conical tube. Pellet sample 4000 rpm, 23°C for 30 min.
- 14 Decant supernatant aseptically and without disturbing the pellet.

- Resuspend the pellet in 6 mL of sterile 0.5% NaCl prepared in de-ionized water. On average, under these conditions we recuperate $\sim 1 \times 10^9$ CFU/mL of *Ralstonia*.
- Once pellet is resuspended, decant supernatant into a sterile reservoir (VWR 89049-030).
- Using a p200 or p1000 multichannel, add 150 μ L of mixed culture to each well of soil. Try to place the tips as close as possible to soil to ensure all of the volume is added to soil.
- 18 Change tips between additions to prevent cross-contamination of pure culture.

Incubating of soil microcosms

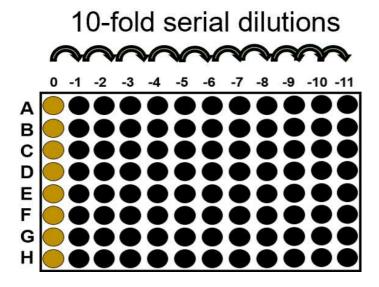
- Once soil samples have been inoculated with *Ralstonia*, proceed to incubate deepwell plate at
 - **₽** 30 °C
- **80** %

relative humidity, and no light. In our system, incubation lasted for 72 h.

Setting up a 96 well microplate for serial dilutions

While soil microcosms are being incubated, start media and supplement preparations for serial dilutions.

In our system of study, a single microplate was used per soil well. Each microplate has 8 replicates with 11 dilutions. If the volume of media per microwell equals 90 μ L, then on average 1 microplate requires \sim 10 mL of media.



21 Follow the sub-steps below to prepare the semi-selective media used in serial dilutions. CPG broth is supplemented with several antibiotics, whose masses were derived from the modified SMSA media recipe ¹⁰. As recommended by the SMSA media recipe, we also include 2, 3, 5-triphenyl tetrazolium chloride, which is a redox indicator of cellular respiration. Additionally, we add gentamicin to the media to ensure retention of vector pFLxR5.

Perform the following sub-steps in advance of setting up microplates.

Prepare stocks of media supplements and store at 4 °C. Some of the supplements are light sensitive.

Equipment:

pipet-aid

Resuspension solutions needed:

- a) autoclaved de-ionized water
- b) 70% ethanol made with autoclaved de-ionized water

Consumables:

0.22 μm syringe filters
sterile syringes
15 or 50 mL polypropylene conical tubes
glass graduated pipettes (5 mL, 10 mL, 25 mL)

Antibiotic stock concentrations:

1% solution (w/v) of Cyclohexamide - Resuspend powder in 70% ethanol.

1% solution (w/v) of Bacitracin - Resuspend powder in autoclave de-ionized water and filter sterilize.

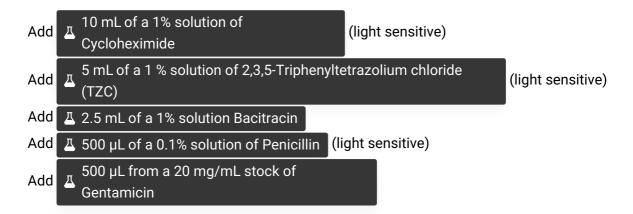
0.1% solution (w/v) of Penicillin - Resuspend powder in autoclave de-ionized water and filter sterilize.

20 mg/mL of Gentamicin - Resuspend powder in autoclave de-ionized water and filter sterilize.

Redox indicator:

1% solution (w/v) of Triphenyl Tetrazolium Chloride - Resuspend powder in autoclave deionized water and filter sterilize.

- 21.2 Prepare CPG broth.
- 21.3 Wait until broth cools down to about 55°C before adding supplements. The following volumes are meant for 1 L of CPG media. Stir to mix supplements properly.



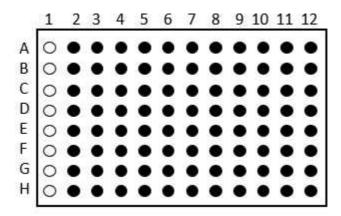
- If media won't be used immediately, store at immediately the next day.
- Remember to warm up media to room temperature before use.
- Using these volumes as reference, users can tailor how much of each component to add if they are not preparing 1 L of broth.
- To induce expression of RFP, add inducer OC6 at a final concentration of 10.2 μM. Typically, we add inducer to an aliquot of semi-selective media, not to the full 1 L of media. For example, to 100 mL of semi-selective broth add 100 μL of OC6. Mix properly.

*Only add inducer OC6 the day of the experiment.

- Make a stock of OC6 at a concentration of 10.2 mM. Resuspend powder with the solvent dimethylformamide. The inducer is light sensitive. Once resuspended, store stock at \$\circ\$ -20 °C.
- Once OC6 is added to semi-selective media, proceed to aliquot 90 µL of broth per microwell.

^{*} To avoid reagent degradation, we would prepare new stocks every two weeks.

- For ease of media addition, we carried this step using a sterile reservoir and an electronic multichannel. Make sure to always mix media in the reservoir before proceeding to add to wells.
- In our system, each microwell has 90 μL of broth for 10-fold serial dilutions.
- Perform this step under aseptic conditions.
- Cover plates with black lids to prevent contamination and reduce light exposure of inducer.



Do not add media to wells A1- H1.

Processing soils samples via the MPN-RFU microplate assay

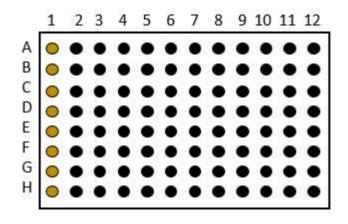
- 24 At the 72 h mark of incubation, remove deepwell plate with *Ralstonia*-inoculated soil microcosms from the incubator.
- Add sterile de-ionized water to a reservoir. Then proceed to add 1 mL of water to every inoculated well. For ease of water addition, we carried this step using an electronic multichannel. If by mistake the tips touch the walls of deepwell plate, then proceed to change tips to avoid cross-contamination.
- Seal deepwell plate with a silicone mat. It is imperative to seal-off wells to prevent crosscontamination during soil resuspension. At the end, we use a roller to finish the sealing process.

^{*} Black lids were re-used between experiments. To clean lids, place lids in a 10% bleach bath for at least 1 h. Then transfer lids to warm tap water and remove bleach residue with a brush. Proceed to rinse lids in clean de-ionized water. Spray lids with 70% ethanol prior to use. Allow ethanol to evaporate in a biosafety cabinet.



Example of silicone mat used to cover an Eppendorf deepwell plate.

- Resuspend the soil samples by manually shaking for 2 min. If 2 min is not enough time to fully resuspend all of the wells in a plate, then continue shaking. Typically, wells that are not mixing display a static soil pellet at the bottom of a deepwell plate. Resuspension of these harder wells can also be done by pipetting up and down.
- Tap the silicone membrane to detach soil slurry from its surface.
- Turn on vacuum system and attach a sterile wide bore tip.
- Carefully proceed to remove silicone mat one column at a time (columns 1 12). At each column, aspirate liquid residue from the silicone mat before moving on to the next set of samples.
 - * We re-use silicone mats. To clean a mat, incubate mat in 10% bleach for no more than a day. Then rinse off all bleach residue with de-ionized water. Finally, place mats in 70% ethanol, at least for a full day. Then decant ethanol and let silicone mats dry in a semi-closed container.
- Using a p1000 multichannel with wide bore tips, mix soil slurries in the deepwell plate, and transfer 100 μ L to column 1 of a microplate. With a plate lid cover columns 2 12 to prevent contamination.



Soil resuspensions added to column 1 of microplate.

- 32 Using p100 with wide bore tips, mix content from column 1 and transfers 10 μL to column 2.
- Dispose of tips. Continue 10-fold serial dilutions using regular p100 tips. Change tips in between dilution steps.

Recording relative fluorescence units (RFU) before starting...

- Once soil sample is serially diluted, quickly take RFU measurements. These are used to estimate an RFU cutoff value for downstream MPN calculations.
- Place microplate on top of SpectraMax plate adapter. Fluorescence readings were made from the top of each well. Our protocol also included a 5 second shaking step prior to taking fluorescence readings. Excitation wavelength was 571 nm, and emissions collected at 620 nm.

36

	"undiluted"	1E^-1	1E^-2	1E^-3	1E^-4	1E^-5	1E^-6	1E^-7	1E^-8	1E^-9	1E^-10	1E^-11
T0	1	2	3	4	5	6	7	8	9	10	11	12
Α	17.461	10.275	4.558	3.777	3.809	4.111	3.106	3.472	2.815	3.378	3.342	4.23
В	18.705	11.294	4.145	3.023	3.753	4.085	3.226	3.34	3.756	3.437	3.588	3.71
С	18.984	10.807	3.986	3.881	3.511	3.631	3.153	3.685	3.349	3.572	3.331	4.245
D	16.891	11.225	3.95	4.205	3.644	3.899	3.416	3.609	3.051	3.158	3.204	4.599
E	17.691	10.101	3.764	3.44	3.991	3.495	3.677	3.324	3.302	2.852	3.52	4.294
F	17.713	9.766	4.399	3.009	3.427	3.581	3.733	3.707	2.964	3.176	3.509	4.581
G	19.354	11.517	4.06	3.371	3.402	3.784	2.722	3.593	3.583	3.836	3.256	4.195
Н	17.668	10.239	3.902	3.508	3.309	3.226	3.542	3.468	3.035	3.4	3.665	4.924

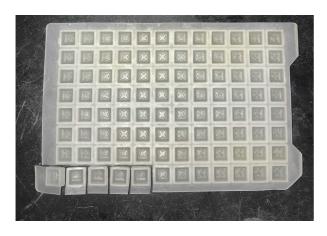
Example of T0 RFU readings.

- Using RFU data from T0, calculate RFU threshold above which wells will be considered positive for the presence of *Ralstonia*. Calculate an average (avg) RFU value for columns 3 12. Then calculate the standard deviation (sd) for the same set of wells. RFU threshold = avg + (5 * sd).
- Cover plate with a clean lid and proceed to incubate microplate at 30 °C under shaking conditions (900 rpm).
 - * Although the lids should be already clean, as a last measure of sterility, wipe lids with 70% ethanol. Allow ethanol to evaporate before use.

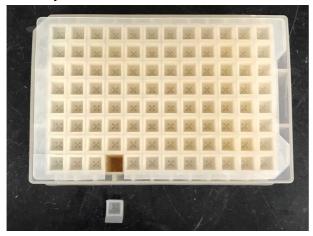
Determining the average grams of soil per well

38 Soil wells from the deepwell plate that were not used for inoculation are used to estimate the average amount of soil in each well. The averaged gram of soil per plate will be used to normalize the estimated MPN value to obtain an MPN/g.

For this part, cut off a portion of a silicone mat.



38.1 Proceed to seal off the wells with water and use the smaller pieces of silicone for wells that have dry soil.



Once everything is covered, proceed to weigh the soil from the selected wells. Place a piece of paper on the balance and tare it. Then, invert the deepwell plate and decant the soil onto the paper. We usually weigh between 5 and 8 wells of soil per experiment.

Replenishing media at 24 h

A 48-h incubation of a microplate was enough time to allow *Ralstonia solanacearum* to grow in the most diluted wells. However, to ensure that enough media remained in wells to continue the incubation process, we had to replenish the evaporated media with fresh broth.

*We note that although well volume determines the path length and is correlated linearly to the intensity of fluorescence of each well, small differences in volume generally should not impact the determination of a positive sample. Positive samples in our system are - on average - two orders of magnitude (100x) brighter than background, while the threshold for a positive read was nearly twice the average of background RFU. In theory, a volume reduction of 98% would be required to yield a false negative, on average. Path length correction is available in many microplate readers for study systems where a higher level of sensitivity is required, and volume differences may impact the outcome of the analysis.

Carefully stop the plate shaker and remove the lid. Take RFU measurements using the SpectraMax plate adapter. Although we did not use this RFU data to calculate an MPN value, it enabled us to confirm that the experiment was working and track which wells required further incubation.

	"undiluted"	1E^-1	1E^-2	1E^-3	1E^-4	1E^-5	1E^-6	1E^-7	1E^-8	1E^-9	1E^-10	1E^-11
T24	1	2	3	4	5	6	7	8	9	10	11	12
A	33.488	17.448	480.362	69.978	17.903	3.436	2.909	3.54	3.389	3.02	2.757	3.552
В	32.836	17.142	389.501	182.095	13.868	3.279	3.285	3.314	3.624	2.577	2.977	3.197
С	33.299	16.215	394.848	148.058	2.902	3.483	3.501	3.333	3.297	3.365	3.25	2.925
D	32.496	16.473	502.66	132.068	26.078	3.761	3.296	3.568	3.266	3.08	2.926	3.308
E	31.657	20.171	504.098	189.407	26.814	3.481	2.715	2.686	3.241	2.801	3.132	2.888
F	32.855	17.546	411.637	189.292	46.379	3.605	2.673	2.86	2.789	3.033	2.726	3.369
G	32.539	22.514	6.823	244.67	67.396	4.165	3.015	3.168	3.204	3.072	2.895	2.976
н	33.386	25.938	408.426	182.204	41.241	3.351	2.91	3.162	2.739	3.099	3.215	3.167

Example of T24 RFU readings. Highlighted in green are wells positive for rfp-tagged Ralstonia.

- Replenish the wells with more semi-selective media lacking inducer OC6. In our system, we would add ~ 30 40 μ L per well. This additional volume should not cause wells to overflow. Otherwise, this will lead to cross-contamination.
- **41.1** Replenish wells with dilutions 10^-2 to 10^-11 using the leftover media prepared earlier.
- **41.2** Transfer media to a sterile reservoir.
- **41.3** Replenish wells with media starting from the most diluted samples. We recommend changing tips in between dilution steps to avoid cross-contamination.
- 42 Cover the microplate with a clean lid and continue incubation for the remaining 24 h.

Recording RFU measurements at T48

43 Carefully stop the plate shaker and remove the lid. Take RFU measurements using the SpectraMax plate adapter.

	"undiluted"	1E^-1	1E^-2	1E^-3	1E^-4	1E^-5	1E^-6	1E^-7	1E^-8	1E^-9	1E^-10	1E^-11
T48	1	2	3	4	5	6	7	8	9	10	11	12
Α	30.731	17.946	455.882	95.484	723.326	710.278	589.153	438.318	3.51	3.889	3.435	3.949
В	31.577	15.791	344.181	645.707	659.22	609.989	550.889	3.987	4.657	3.928	3.034	4.466
С	32.668	14.326	341.61	330.182	6.429	619.234	566.758	4.032	3.786	3.83	3.444	4.009
D	30.411	14.158	469.088	172.275	707.629	671.296	578.231	479.924	4.13	3.539	3.642	3.685
E	31.595	14.33	499.213	580.556	645.975	584.489	479.636	3.918	4.489	3.962	4.204	4.169
F	32.206	13.835	399.741	599.977	620.921	619.279	482.488	380.272	3.624	3.662	3.956	3.55
G	30.276	15.835	7.797	628.614	625.079	618.613	517.805	3.878	3.735	4.255	3.89	4.266
Н	30.048	20.28	396.572	327.656	664.395	617.465	501.418	443.293	386.115	3.755	4.01	4.374

Example of T48 RFU readings. Highlighted in green are wells positive for rfp-tagged *Ralstonia*. Since the MPN assay is based on the principle of dilution to extinction, columns 1 and 2 are also considered positive.

Remember that this data corresponds to a single well of soil.

44 Export RFU data for MPN calculations.