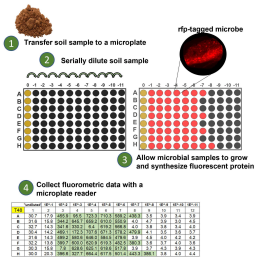
Jul 28, 2023 Version 1

Most Probable Number Fluorescence Microplate Assay V.1



DOI
dx.doi.org/10.17504/protocols.io.q26g7yqk1gwz/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.q26g7yqk1gwz/v1

Protocol Citation: Karla Franco Meléndez, Layla Schuster, Melinda Chue Donahey, Emily Kairalla, Christopher Reisch, Adam R Rivers, Michael Andrew Jansen 2023. Most Probable Number Fluorescence Microplate Assay. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.q26g7yqk1gwz/v1**

Protocol status: Working
We use this protocol and it's working.

Created: June 02, 2023

Last Modified: July 28, 2023

Protocol Integer ID: 82831

Keywords: most probable number assay, MPN, microtiter plates, soil microcosms, soil pathogen, Ralstonia solanacearum, automation, high-throughput screening, relative fluorescence units, MicroMPN, NP301, Genomics and Bioinformatics Research Unit, microplates, plate readers, CRIS 6066-21310-005-00-D

Funders Acknowledgement:
USDA-ARS
Grant ID: 6066-21310-005-00-D
Plant Genetic Resources, Genomics and Genetic Improvement
Grant ID: NP301

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Abstract

Here we provide a step-by-step protocol for running the MPN fluorescence microplate assay with soil samples inoculated with a fluorescently tagged pathogen. We performed the MPN fluorescence assay in 96-well microplates to facilitate automation of fluorescence data collection using a plate reader. Serial dilutions were performed in semi-selective media and in the presence of a homoserine lactone to induce expression of a red fluorescent protein. When the signal is a product of the organism, rather than the medium, it reflects both viability and specificity simultaneously. For brevity, we refer to this method as the MPN-RFU microplate assay.

Guidelines

- The most probable number (MPN) assay is a culture-dependent method of quantification most commonly used to enumerate microbes from environmental samples [1]. In the MPN assay, the density of viable microbes is determined by serially diluting to extinction in a culture medium. An MPN count (MPN/mL or MPN/g) is therefore based on the presence or absence of a measurable signal, such as media turbidity [2] and color change [3,4] .The original MPN assay quantifies a population of microbes based on the properties of the growth medium and is specific to microbes that can metabolize the nutrients in the medium (e.g., anaerobes, methanogens, iron reducers) [3,6,7,8]. For greater specificity, the MPN luminescence variation of the assay relies on microbial strains that produce a detectable signal – such as luminescence [9,10] or fluorescence expression [11-14] – that differentiates the target from the rest of the sample.

- MPN calculations require two fixed quantities: sample volume and number of replicates at each dilution step; calculations of an MPN value are based on probability theory and not on an absolute count [5]. There are assumptions that must be met in order for the MPN assay to estimate the true microbial concentration in a sample [1,5]. 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 even one microbe within the inoculum volume will yield a measurable signal.

- 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.

- MPN calculations require two fixed quantities: sample volume and number of replicates at each dilution step[1,5,16].

Materials

Materials for preparing soil microcosms

1. Two 8” x 10 ¾” polypropylene tray and snap-on lid (Bel-Art F1630-0000)
2. Ceramic mortar and pestle
3. Thermo Scientific™ Nalgene™ large polyproylene sterilizing pan (catalog number 6900-0010PK)
4. Thermo Scientific™ ClipTip™ 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. 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. Bacto™ 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)
- ZAP™ 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 (BrandTech™ BRAND™ 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 Fisherbrand™ Sure One™ Low Retention pipette tips (Fisher Scientific 02-707-009)

3. For aliquoting media:

- Impact^2 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 Fisherbrand™ Sure One™ 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 semi-selective 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

- 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 LuxR/P_{LuxB} promoter-regulator pair regulating an mRFP cassette [17]. 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) [18]. 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.

- The MPN-RFU microplate assay is not restricted to soil samples. It can be adapted and optimized for other sample types. We recommend users who are interested in learning how to set up and run the assay to go directly to sections "Setting up a 96-well microplate for serial dilutions", steps 31-33, "Recording relative fluorescence units (RFU) before starting incubation (T0)", "Replenishing media at 24 h", and "Recording RFU measurements at T48".

- **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 [20].**

Preparing soil microcosms

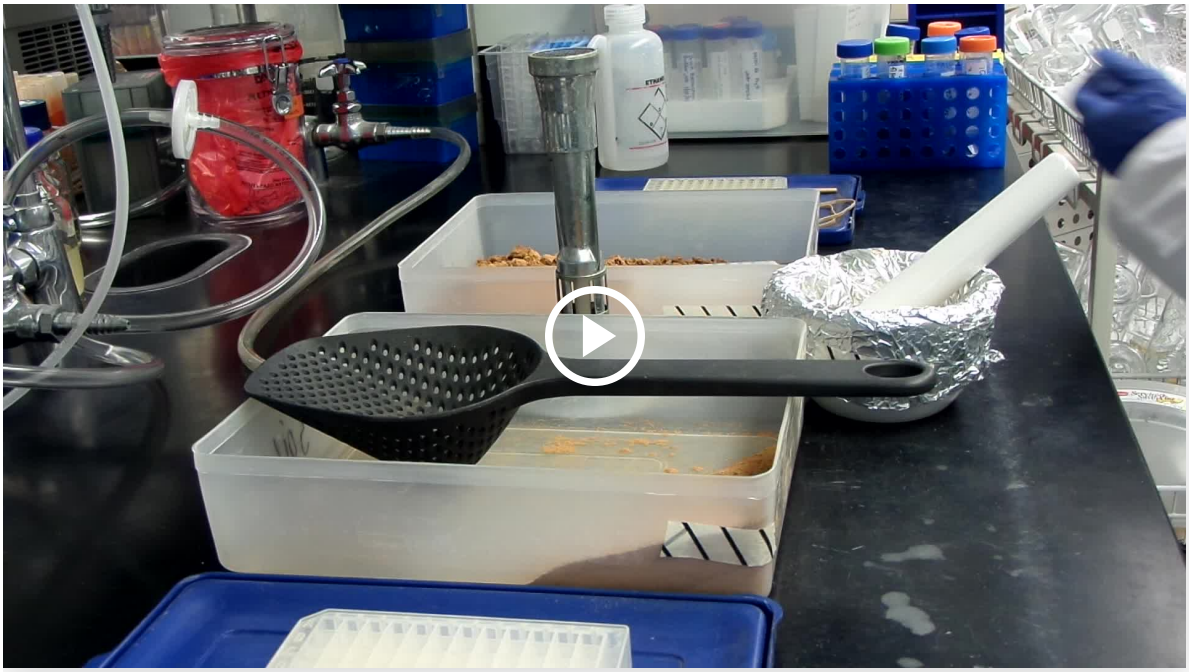
6d

1 3d

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 about 4 minutes.

Note: The MPN-RFU microplate assay is not restricted to soil samples. It can be adapted and optimized for other sample types. We recommend users who are interested in learning how to set up and run the assay go directly to the following sections:

- "Setting up a 96-well microplate for serial dilutions",
- steps 31-33,
- "Recording relative fluorescence units (RFU) before starting incubation (T0)",
- "Replenishing media at 24 h", and
- "Recording RFU measurements at T48".



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.

Before use, we dried soil at room temperature in a sterile tray for 6 days. At the end of day 6 of drying we loaded 2-mL deepwell plates, the individual wells of which we refer to as soil microcosms. Plates were covered with a sterile lid and left on a bench at room temperature. In the early morning of the following day, we inoculated soil wells with rfp-tagged *Ralstonia*.

Inoculated systems were incubated in the dark at 30 °C (± 2°C) and 80 % (± 5%) relative humidity, for a total of 3 days or 72:00:00 .

In the remainder of this section, we describe step-by-step how to assemble high-throughput soil microcosms.

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 ¾” polypropylene trays and their snap-on lids (Bel-Art F1630-0000)
2. Ceramic mortar and pestle
3. Thermo Scientific™ Nalgene™ large polyproylene sterilizing pan (catalog number 6900-0010PK)
4. Thermo Scientific™ ClipTip ™ 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™ ClipTip ™ 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.*

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.

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.

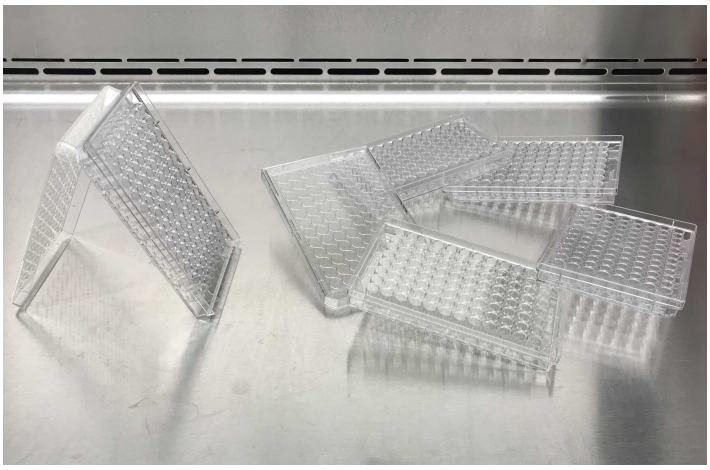


Fig. 1. How to stack microplates and lids, facing downward, in an A or circular, fallen-dominos configuration to allow air flow for drying in a biosafety cabinet.

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, 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 much 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.

5.4 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. 6d

6 Once the soil has dried for 6 days, load it into a 96-deepwell plate by following the sub-steps below.

6.1 Start by removing the paper towels covering the tray, then 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**.

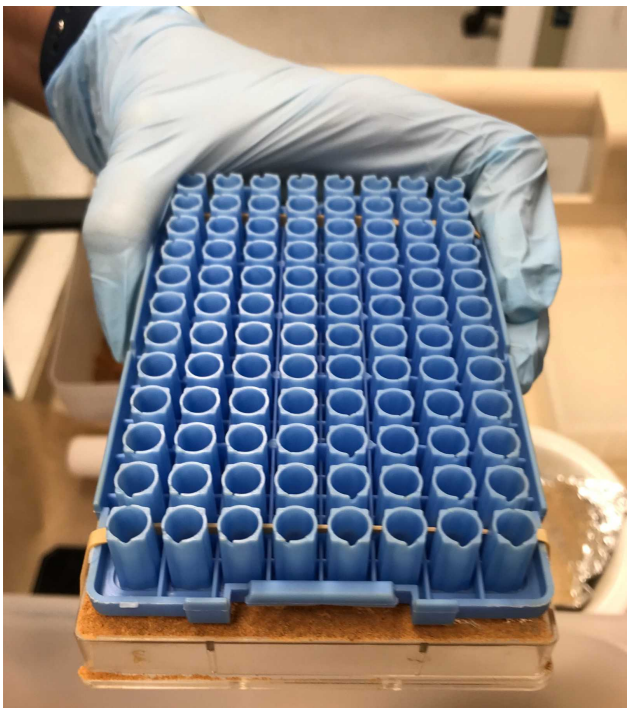
6.5 Place large particles remaining in the strainer back with the rest of the un-processed soil.

6.6 Repeat sub-steps 6.2 - 6.5 until enough soil has been pulverized.

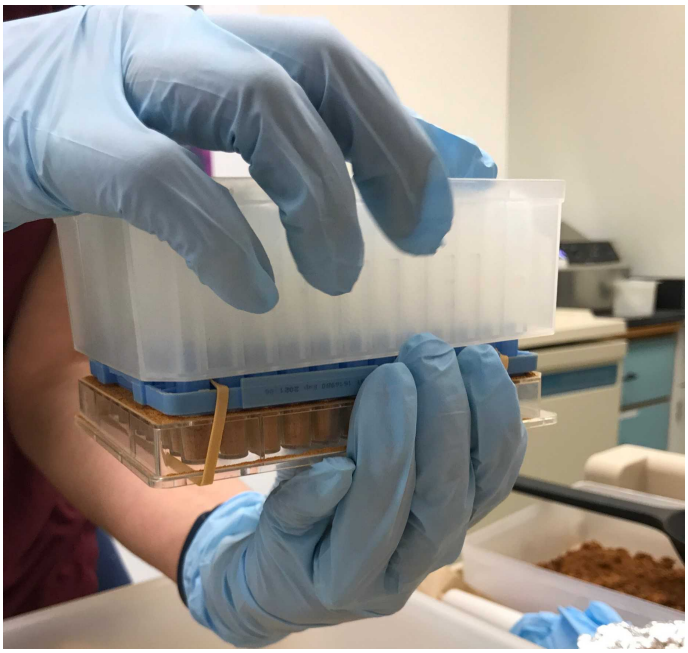
6.7 Use the conical tube to transfer the strained, pulverized soil into the Greiner microplate. The microplate wells will function as measuring cups.

6.8 Use a spatula and gloved hand to level the soil flush with the top of the microplate.

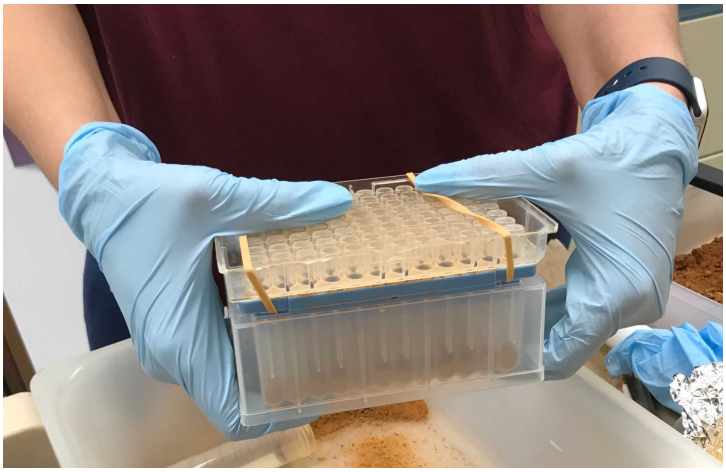
6.9 Overlay the sterile pipette tip rack on top of the filled microplate. The rack will function as a funnel. Align the rack holes with the microplate wells to ensure the transfer of soil from the microplate to a deepwell plate. Use two rubber bands to hold the assembly together.



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, carefully remove the tip rack and the empty 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.

6.14 On the outside of a deepwell plate, label the date and experiment name. If there are 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*

7 A loaded deepwell plate with soil is used the next day. **Users must time the drying of 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. The antibiotic gentamicin was always included in the media (broth and agar), to prevent loss of plasmid throughout incubation.

8 Streak *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.

Below we describe the preparation of CPG agar. ***Perform these sub-steps a few days prior to streaking.***

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

8.2 CPG agar recipe:

	A	B
	Component	Per 1.0 Liter
	Peptone	10 g
	Casamino Acids	1 g
	Glucose (dextrose)	5 g
	Yeast Extract	1 g
	Agar	16 g
	De-ionized water	fill to 1 L

Depending on the volume needed, users should scale the mass of each component of the 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 µL of gentamicin from the stock prepared earlier, for a final concentration of 10 mg/L. Allow agar plates to solidify before storing at 4 °C .

9 Incubate streak plate at 30 °C for at least 3 days.

10 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. Prepare overnight cultures on the same day as the deepwell plate loading.

**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 µL. 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. Samples must be diluted to extinction in order to estimate the original microbial load in soil; however, there are different ways in which a user could structure sample distribution across a microplate.*

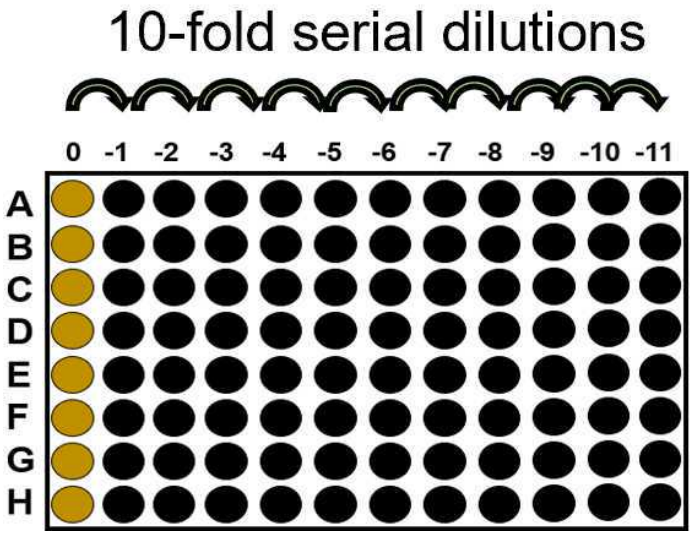
**Whether a microplate needs to shake or not will also affect how many wells of soil can be processed at a time. In our experimental design, to speed up bacterial growth and promote*

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.

Below we describe the preparation of CPG broth. ***Perform these sub-steps prior to preparing an overnight culture.***

10.1 CPG broth recipe:

	A	B
	Component	Per 1.0 Liter
	Peptone	10 g

	A	B
	Casamino Acids	1 g
	Glucose (dextrose)	5 g
	Yeast Extract	1 g
	De-ionized water	up to 1 L

Users should scale the media recipe depending on the final volume needed.

Include a stir bar in the broth. Autoclave at 121ºC for 30 minutes. Cool to about 55ºC before adding gentamicin. For 1 L of agar, add 500 µL of gentamicin from the stock prepared earlier, for a final concentration of 10 mg/L. Allow agar plates to solidify before storing in the dark at 4 °C.

11 Shake overnight culture in an incubator shaker for 16 h at 250 rpm, 30°C.

Inoculating soil microcosms with rfp-tagged *Ralstonia*

12 In this next section, users will inoculate the soil-loaded deepwell plate with the overnight culture of *Ralstonia* prepared in the above section. Before soil inoculation we remove the antibiotic gentamicin by centrifuging and resuspending the overnight culture in a dilute sodium chloride solution.

13 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.

15 Resuspend the pellet in 6 mL of sterile 0.5% NaCl prepared in de-ionized water. On average, under these conditions we recuperate ~ 1 x 10^9 CFU/mL of *Ralstonia.*

16 Once pellet is resuspended, decant supernatant into a sterile reservoir (VWR 89049-030).

17 Using a p200 or p1000 multichannel, add 150 µL of mixed culture to each well of soil. Try to place the tips as near as possible to the soil, but without touching the soil, to ensure all of the volume is added to the soil.

18 Change tips between additions to prevent cross-contamination of pure culture.

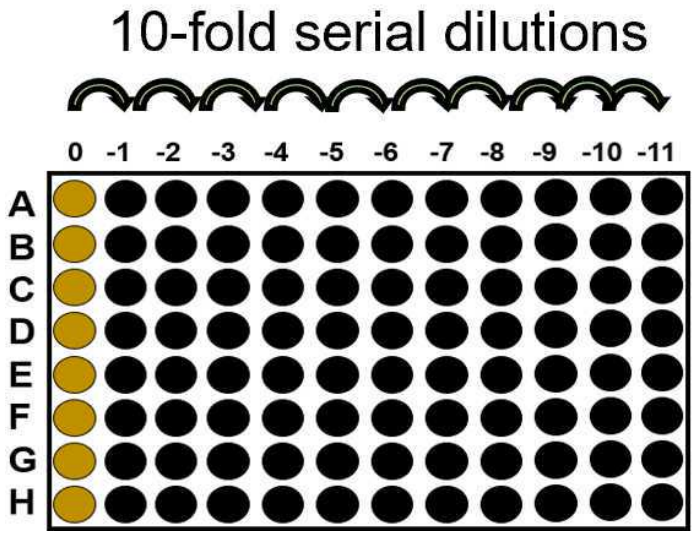
Incubating of soil microcosms

19 Once soil samples have been inoculated with *Ralstonia*, 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

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

In our study system, a single microplate was used per well of soil. Each microplate has 8 replicates with 11 dilutions, across a total of 88 microwells. If 90 µL of media is added to each of these microwells, then (on average) 1 microplate requires ~ 7.92 mL of media. We prepared an excess (~ 10 mL) of media per plate, and used the extra volume to replenish media lost to evaporation at 24 hrs.



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 [19]. 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.

Below we describe the preparation of semi-selective media. ***Perform these sub-steps prior to setting up microplates.***

21.1 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 de-ionized water and filter sterilize.

**To avoid reagent degradation, new stocks were prepared every two weeks.*

21.2 Prepare CPG broth.

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

Add 10 mL of a 1% solution of Cycloheximide (light sensitive)
Add 5 mL of a 1 % solution of 2,3,5-Triphenyltetrazolium chloride (TZC) (light sensitive)
Add 2.5 mL of a 1% solution Bacitracin

Add 500 µL of a 0.1% solution of Penicillin (light sensitive)

Add 500 µL from a 20 mg/mL stock of Gentamicin

- If media won't be used immediately, store at 4 °C. In our system, we used the media 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.

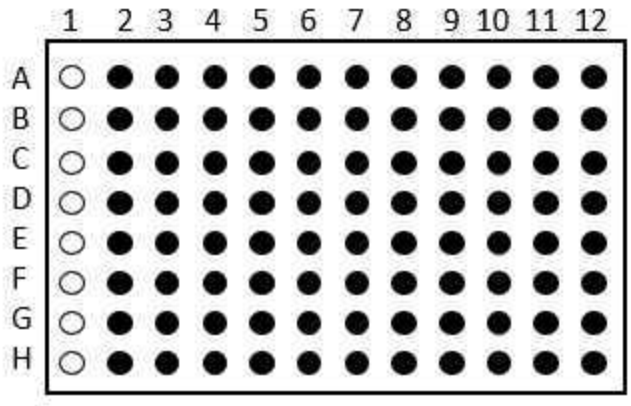
22 To induce expression of RFP, add inducer OC6 at a final concentration of 10.2 µM. Typically, we add inducer to an aliquot of semi-selective media, not to the full 1 L of media. For example, to 100 mL of semi-selective broth add 100 µL of OC6. Mix properly.

**Only add inducer OC6 the day of the experiment.*

22.1 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 -20 °C.

23 Once OC6 is added to semi-selective media, proceed to aliquot 90 µL of broth per microwell.

- 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 adding 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.

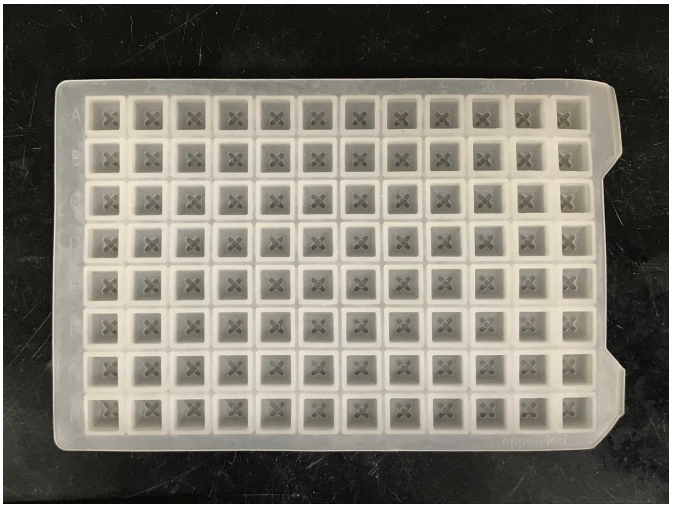
** Black lids were re-used between experiments. To clean lids, place in a 10% bleach bath for at least 1 h. Then transfer lids to warm tap water and remove bleach residue with a brush. Proceed to rinse lids in clean de-ionized water. Spray lids with 70% ethanol prior to use. Allow ethanol to evaporate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

Processing soils samples via the MPN-RFU microplate assay

24 At the 72 h mark of incubation, remove the deepwell plate with *Ralstonia*-inoculated soil microcosms from the incubator.

25 Add sterile de-ionized water to a reservoir. Then add 1 mL of water to every inoculated well. For ease of water addition, we carried out this step using an electronic multichannel. If the tips accidentally contact the walls of deepwell plate, then change tips to avoid cross-contamination.

26 Seal the deepwell plate with a silicone mat. It is imperative to seal-off wells to prevent cross-contamination during soil resuspension. At the end, we used a roller to finish the sealing process.



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

27 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.

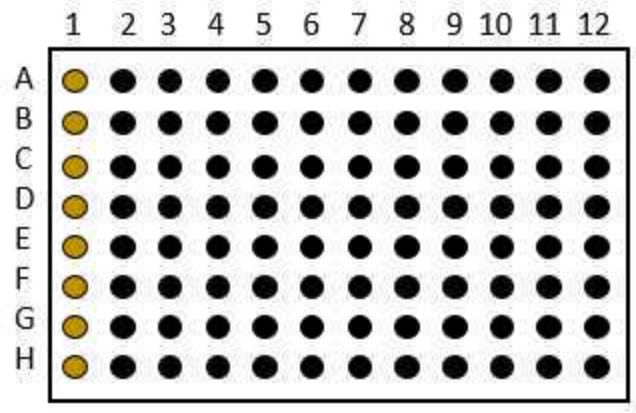
28 Tap the silicone membrane to detach soil slurry from its surface.

29 Turn on the vacuum system and attach a sterile wide-bore tip.

30 Carefully remove the silicone mat, proceeding 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-used silicone mats. To clean a mat, incubate n 10% bleach for no more than a day. Rinse off all bleach residue with de-ionized water. Finally, place mats in 70% ethanol for at least a full day. Then decant ethanol and let the silicone mats dry in a semi-closed container.*

31 Using a p1000 multichannel with wide-bore tips, mix the soil slurries in the deepwell plate, and transfer 100 µL 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 a p100 with wide bore tips, mix the contents of column 1 and transfer 10 µL to column 2.

33 Dispose of tips. Continue 10-fold serial dilutions using regular p100 tips. Change tips between dilution steps.

Recording relative fluorescence units (RFU) before starting incubation (T0)

34 Once the soil sample is serially diluted, quickly take RFU measurements. These are used to estimate an RFU cutoff value for downstream MPN calculations.

35 Place the microplate on top of the 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. The excitation wavelength was 571 nm, with 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
A	17.461	10.275	4.558	3.777	3.809	4.111	3.106	3.472	2.815	3.378	3.342	4.23
B	18.705	11.294	4.145	3.023	3.753	4.085	3.226	3.34	3.756	3.437	3.588	3.71
C	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
H	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.

36.1 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).

** These columns were chosen because the values of column 3 were not different from subsequent columns, indicating that sample dilution was sufficient to reach the limit of sensitivity for the instrument. Thus, variation between wells in these columns represents the level of background noise for the instrument on these samples. The threshold for a positive sample is then set to 5-sigma above this noise level.*

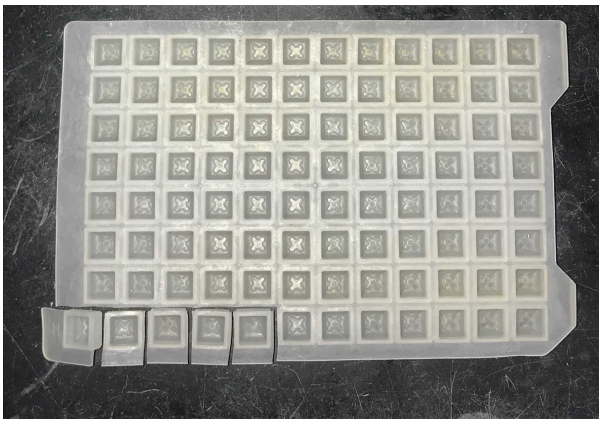
37 Cover the plate with a clean lid and incubate the microplate at 30 °C under shaking conditions (900 rpm).

** Although the lids should already be clean, as a final measure of sterility, wipe lids with 70% ethanol. Allow ethanol to evaporate before use.*

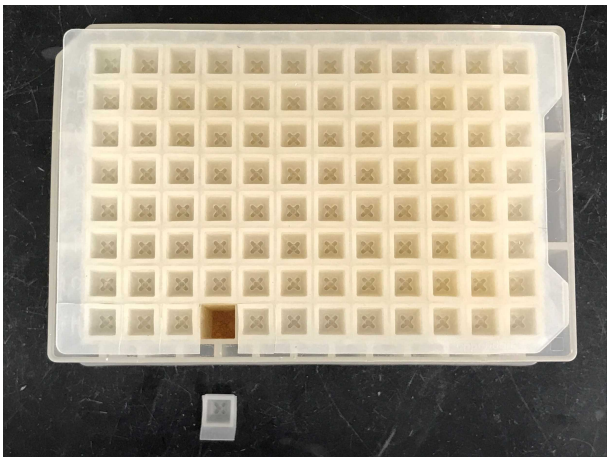
Determining the average grams of soil per well

38 Soil wells from the deepwell plate that were not used for inoculation are instead used to estimate the average amount of soil in each well. The average mass 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 Use the uncut portion of the silicone mat to seal off the wells containing liquid and use the smaller pieces of silicone for wells that have dry soil.



38.2 Once all wells are covered, weigh the soil from the selected unsused 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 weighed between 5 and 8 wells of soil per experiment.

Replenishing media at 24 h

39 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.

40 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
B	32.836	17.142	389.501	182.095	13.868	3.279	3.285	3.314	3.624	2.577	2.977	3.197
C	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
H	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*.

41 Replenish the wells with more semi-selective media lacking inducer OC6. In our system, we would add ~ 30 - 40 µL per well. This additional volume should not cause wells to overflow. Otherwise, this will lead to cross-contamination. In the following 3 sub-steps, we describe the procedure to replenish media in partially evaporated wells.

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
A	30.731	17.946	455.882	95.484	723.326	710.278	589.153	438.318	3.51	3.889	3.435	3.949
B	31.577	15.791	344.181	645.707	659.22	609.989	550.889	3.987	4.657	3.928	3.034	4.466
C	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
H	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.

This microplate data in this example yields an MPN estimate for a single well of soil.

44 Export RFU data for MPN calculations.

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