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High-Throughput Cultivation and Dilution-to-Extinction Protocol for Field-Grown Crops

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We use this protocol and it's working

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Abstract

This protocol describes a high-throughput cultivation method for isolating and identifying bacteria from field-grown crop plants using dilution-to-extinction, two-step library preparation, and amplicon sequencing. The approach enables the systematic recovery of diverse bacterial strains from root-associated microbiomes while minimizing competition from fast-growing species. The protocol integrates automated liquid handling, culture-based microbial isolation, and high-throughput sequencing to streamline bacterial cultivation and identification. The expected results include the isolation of a diverse set of cultivable root-associated bacteria, comprehensive taxonomic identification using 16S rRNA amplicon sequencing, and the generation of a curated culture collection for downstream applications, including functional screening and synthetic community design. This method improves efficiency, reproducibility, and scalability in microbial cultivation studies.

Guidelines

Troubleshooting Guide

- **Drying of the Liquid Medium in Border Wells (Steps 7, 15)**

If the liquid medium in the outer wells of the 96-well plates dries out during incubation, the most likely reason is incomplete sealing with Parafilm. To prevent this, tightly seal the edges of the plates before incubation. This ensures that evaporation is minimized and that bacterial cultures remain hydrated throughout the experiment.

- **No Bacterial Growth in Wells After Incubation (Steps 8, 8.1)**

If no bacterial growth is observed after incubation, this may be due to over-dilution of the bacterial suspension. To resolve this, reduce the dilution factor or increase the starting inoculum concentration to ensure that enough viable bacteria are present in the culture medium.

- **Excessive Growth in All Wells (Step 8.1)**

If growth appears in all wells, including high-dilution wells, the bacterial concentration in the dilution series may be too high. To optimize microbial diversity selection, prepare a more diluted suspension before plating.

- **Cross-Contamination Between Wells (Step 9)**

If bacterial growth is detected in negative control wells or unexpected microbes are found across plates, cross-contamination may have occurred during pipetting. To avoid this, use slow and controlled pipetting, and centrifuge the plate before pipetting to minimize splashing.

- **DNA Degradation After Extraction (Step 12)**

Overheating during alkaline lysis can degrade DNA, leading to poor PCR amplification. Ensure that the PCR plates are tightly sealed to prevent evaporation and that the samples are incubated at 95°C for no longer than 30 minutes.

- **No Visible PCR Product on Gel Electrophoresis (Steps 16, 17)**

If no PCR bands are visible on the gel, the DNA extract may contain PCR inhibitors from the root microbiome. Try diluting the DNA template 1:10 or purify the DNA using a cleanup kit before reattempting PCR.

- **Unexpected Bands or Smearing in Gel Electrophoresis (Steps 17, 36)**

If gel electrophoresis results show smearing or unexpected band sizes, non-specific amplification may have occurred due to incorrect annealing temperature. Adjust the PCR conditions by increasing the annealing temperature or modifying the primer concentration to improve specificity.

- **Negative Control Shows Amplification (Steps 24, 28)**

If negative control wells show amplification during PCR, contamination in the PCR reagents is the most likely cause. Always perform PCR reactions using fresh reagents, nuclease-free water, and work under a laminar flow hood. Setting up reactions in a dedicated PCR workspace can further reduce contamination risks.

- **Low-Quality Sequencing Reads (Step 50)**

If sequencing results show low-quality reads, this could be due to poor primer specificity or degraded PCR products. To improve sequencing quality, use high-fidelity polymerase, verify the primer design, and ensure that the PCR products are of good quality before sequencing.

- **Unequal Sequencing Depth Across Plates (Step 53)**

Substantial differences in sequencing depth among plates often result from uneven PCR product concentrations. To prevent this issue, always double-check and normalize PCR product concentrations before pooling the samples for sequencing.

- **No Visible Growth After 7 Days (Step 64)**

If bacterial growth is not detected after 7 days of incubation, it could be due to low inoculum size or slow-growing bacteria. Try inoculating with a larger bacterial colony or pre-growing bacteria on solid agar media before transferring them to liquid cultures.

- **Low Recovery Rate After Freezing (Step 67)**

If bacterial recovery rates after freezing are low, this may be due to the sensitivity of natural isolates to the freeze-thaw process. To improve recovery, increase the bacterial concentration in glycerol stocks and avoid repeated freeze-thaw cycles.

- **Loss of Viability in Glycerol Stocks (Step 68)**

If bacteria fail to grow after thawing, the most common reason is insufficient mixing of glycerol and bacterial suspension before freezing. Ensure that the glycerol and bacterial cultures are thoroughly mixed before storing at -80°C to maintain cell viability.

Limitations of the High-Throughput Cultivation Method

1. **Restricted to Bacteria:** This cultivation method has only been tested for bacteria and is not optimized for the growth of fungi, oomycetes, or archaea. Additional modifications would be required to accommodate non-bacterial microbes.

2. **Limited to Liquid Medium Adapted Microbes:**

The protocol is optimized for bacteria that grow in liquid media. Many microbes, particularly those requiring solid surfaces for biofilm formation or colony development, may not grow efficiently under these conditions.

3. **Aerobic Bias**

The protocol is designed for aerobic bacteria and has not been tested under anaerobic conditions. Obligate anaerobes and microaerophiles may not survive or proliferate unless additional steps, such as anaerobic chambers or gas-purging techniques, are incorporated.

4. **Underrepresentation of Slow-Growing and Fastidious Bacteria**

- Some slow-growing or nutrient-demanding microbes may be outcompeted by fast-growing taxa, leading to an underrepresentation of rare or ecologically important bacteria.
- Additional strategies such as longer incubation times or specialized growth media may be required to recover these microbes.



5. Potential for Loss of Interdependent Microbes

The dilution-to-extinction approach isolates bacteria individually, which may disrupt microbial interactions that are essential for the survival of some species. Syntrophic or symbiotic microbes that rely on co-cultivation may not grow independently.

6. Selective Bias Introduced by TSB Medium

- The use of tryptic soy broth (TSB) as the main culture medium may favor specific groups of bacteria while excluding oligotrophic or extremophilic microbes that require alternative nutrient sources.
- The protocol may need modifications to accommodate nutrient-poor or highly selective media for specific microbial communities.

7. Genome Representation May Be Biased

Due to cultivation biases, the cultured isolates may not accurately represent the full microbial diversity present in the original root microbiome.

8. Not Tested for Extreme Environmental Microbes

This protocol has not been tested on extremophiles, such as halophiles, acidophiles, or thermophiles, which require highly specific growth conditions (e.g., high salt, low pH, or elevated temperatures).

Materials

Biologic materials

Freshly collected roots from corn (*Zea mays*) from 2023 and peas (*Pisum sativum*) from 2022 growing seasons.

Reagents

- Tryptic soy broth (TSB) (Bacto™ Tryptic Soy Broth cat # DF0370173)
- Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich, cat. no. M2670-100G)
- Sodium chloride (NaCl, VWR cat. no. X190-1kg)
- Sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, Sigma-Aldrich cat. no. 10028-24-7)
- Sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, Sigma-Aldrich, cat. no. 71504-250G)
- Sodium hydroxide (NaOH, Sigma-Aldrich, cat. no. S8045-500G)
- $\text{Na}_2\text{-EDTA} \cdot 2\text{H}_2\text{O}$ (JT Baker cat. no.4040-01)
- Tris-HCl (IBI, cat. no. IB70162)
- Glycerol (Thermo Scientific, cat. no. A16205.0F)
- Agar (Thermo Scientific Agar powder cat. no. AAA107520E)
- Agarose (Apex cat. no. 20-102GP)
- Ethanol (Fisher cat. no. BP2818-500)
- Nuclease-free water (Qiagen, cat. no. 129114)
- PCR primers (IDT)
- KAPA Hotstart polymerase (Roche Diagnostics cat. no. KK2602)
- 100-bp Plus DNA Ladder (New England Biolabs, cat. no. N3231S)
- Midori Green Advance DNA Stain (Bulldog Bio, cat. no. MG04)
- Mag-Bind® TotalPure NGS magnetic beads for clean-up PCR products for NGS (Omega Bio-tek, SKU no. M1378-01)
- Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Fisher Scientific cat. no. Q32851) including Quant-iT PicoGreen dsDNA reagent, 20× TE and Lambda DNA Standard.
- Tris (Vetec, cat. no. V900483-500G)
- Acetic acid (Rhawn, cat. no. R049946-500ML)
- Loading buffer (Takara, cat. no. 9156)
- DNeasy Plant Pro Kit (QIAGEN, cat. no. 69204)

Equipment

- Forceps (VWR, cat. no. 232-0085 and 232-0106)
- Scissor (VWR, cat. no. 233-1149)
- Scalpel (Excel Disposable #10 Sterile Scalpels, Fisher Scientific cat. no. 14-840-000)
- Filter papers (Tisch Scientific, cat. no. **5201-935**)
- Falcon tubes, polypropylene, 50 ml (BD Falcon, cat. no. 352070)
- 1 L glass bottles
- Plastic grinding rods (Fisherbrand Pellet Pestles, Fisher Scientific 12-141-363)
- White reservoirs (Southern Labware cat. no. P8050-5)
- Standard microcentrifuge tubes (1.5 and 5 ml; Eppendorf, cat. no. 0030125.150, 30119401)
- 96-well cell culture plate (Amazon, NEST, cat. no. 701001)



- 96-well PCR plate (Hard-Shell, BIORAD, cat. no. HSS9601)
- 96-well EIA/PIA plate (Costar, cat. no. 3590)
- 2.0-ml cryogenic vial (NEST, cat. no. 607001) or Microbank (Pro Lab, cat. no. PL.170/M)
- Multi-channel pipettes (Rainin cat. no. 17013803, 17013805, 17014496)
- Single channel pipettes (Rainin cat. no. 17014393, 17014388, 17014384 and 17014382)
- Pipette tips (nuclease-free, sterile filter tips; 20, 200 and 1,000 µl; Rainin cat. no. 17014961, 30389239, and 30389213)
- Parafilm (VWR, cat. no. 291-1213))
- Plate seals (VWR cat. no. 732-3212)
- Class II biological safety cabinet (Nu-Aire model NU425-400 series 30)
- Vortex mixer (VWR cat. no. 10027-194)
- Electronic balance (Mettler Toledo, cat. no. AL104)
- Incubator-shaker (Eppendorf, cat. no. M1299-0090) for root washing
- Microplate centrifuge (MilliporeSigma, cat. no. 40161701)
- Thermal cycler (Eppendorf Mastercycler EP Thermal Cycler, model no. 8125-30-1025)
- Electrophoresis system (Bio-Rad, cat. no. 1704405)
- Gel Doc XR+ Imaging system (Azure Biosystems Inc. Azure 300 AZI300-01)
- High-speed freezing centrifuge (Eppendorf, model no. 5810R)
- DynaMag-2 magnet (Thermo Fisher Scientific, cat. no. AM10027)
- Tabletop high-speed micro centrifuge (Thermo Fisher Scientific, model no. Heraeus Pico 17)
- -80 °C Upright Ultra-Low Temperature Freezers (Thermo Fisher Scientific, model no. 907)
- -20 °C freezer (VWR Lab HC Manual Def -20 CF, cat. no. 75836-238)

Computer requirements

- Operating system requirements: The bioinformatic pipeline was developed and tested on a 64-bit RedHat Enterprise Linux version 8+ system, but any operating system capable of running a modern version of the R language – Linux, MacOS, or Windows – should work.
- Hardware requirements: 16+ GB of RAM is recommended for most datasets. RAM requirements increase with number of plates per analysis. If analyzing only a few plates, 4-8 GB of RAM may be sufficient.

Software

- R 4.1+ (R Core Team, 2024) with the argparser (Shih DJH, 2024), tidyverse (Wickham et al., 2019), and DADA2 (Callahan et al., 2016) packages. Older versions of R may work, but have not been tested.
- RStudio 2024+ (<https://posit.co/download/rstudio-desktop/>) for running the R notebook version of the pipeline. Older versions of RStudio may work, but have not been tested.

Input data files

- Sequence data: NCBI SRA under the BioProject PRJNA1220177
- Silva 16S taxonomy database (Quast et al., 2012) downloaded from <https://www.arb-silva.de/download/archive/>. Testing was done with v138.1, but other versions may be used as well.

Primers used in the protocol

Primers for well barcoding (PCR1 High-Throughput Cultivation) for step 15

Name	Sequence
V4_F_BC1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTAGTGCCAGCMGCCGC GGTAA
V4_F_BC2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTGTGTGCCAGCMGCCG CGGTAA
V4_F_BC3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGTCTGGTGCCAGCMGCC GCGGTAA
V4_F_BC4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCAGTGCCAGCMGCCGC GGTAA
V4_F_BC5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACGAGTGCCAGCMGCCG CGGTAA
V4_F_BC6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGTGGTGCCAGCMGCC GCGGTAA
V4_F_BC7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCTGTGCCAGCMGCCGC GGTAA
V4_F_BC8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGTTGTGCCAGCMGCCG CGGTAA
V4_F_BC9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACATGTGTGCCAGCMGCC GCGGTAA
V4_F_BC1 0	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCGGGTGCCAGCMGCCG CGGTAA
V4_F_BC11	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTTGAGTGCCAGCMGCCG CGGTAA
V4_F_BC1 2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGGCTGTGCCAGCMGCC GCGGTAA
V4_R_BC1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTCACTACHVGGGTATC TAATCC
V4_R_BC2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAGTACTACHVGGGTAT CTAATCC
V4_R_BC3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGATCACTACHVGGGTA TCTAATCC
V4_R_BC4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCACTACHVGGGTATC TAATCC
V4_R_BC5	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTA ACTACHVGGGTAT CTAATCC
V4_R_BC6	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATAA CACTACHVGGGTA TCTAATCC



Name	Sequence
V4_R_BC7	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGAACTACHVGGGTATC TAATCC
V4_R_BC8	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGTTCACTACHVGGGTAT CTAATCC

Primers for plate barcoding (PCR2 High-Throughput Cultivation) for step 35

Name	Sequence
F1_MetalIndex	AATGATACGGCGACCACCGAGATCTACACTATAGCCTTCGTCGGCAGCG TC
F2_MetalIndex	AATGATACGGCGACCACCGAGATCTACACATAGAGGCTCGTCGGCAGCG TC
F3_MetalIndex	AATGATACGGCGACCACCGAGATCTACACCCTATCCTTCGTCGGCAGCG TC
F4_MetalIndex	AATGATACGGCGACCACCGAGATCTACACGGCTCTGATCGTCGGCAGC GTC
F5_MetalIndex	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGTCGTCGGCAGC GTC
F6_MetalIndex	AATGATACGGCGACCACCGAGATCTACACTAATCTTATCGTCGGCAGCG TC
F7_MetalIndex	AATGATACGGCGACCACCGAGATCTACACCAGGACGTTTCGTCGGCAGC GTC
F8_MetalIndex	AATGATACGGCGACCACCGAGATCTACACGTAAGTCTGACTCGTCGGCAGCG TC
F9_MetalIndex	AATGATACGGCGACCACCGAGATCTACACTGAACCTTTCGTCGGCAGC GTC
F10_MetalIndex	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCG TC
F11_MetalIndex	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCG TC
F12_MetalIndex	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCG TC
F13_MetalIndex	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCG TC
F14_MetalIndex	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGC GTC
F15_MetalIndex	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCG TC



Name	Sequence
F16_MetalIndex	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
R13_MetalIndex	CAAGCAGAAGACGGCATACGAGATGTCGTGATGTCTCGTGGGCTCGG
R14_MetalIndex	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTCTCGTGGGCTCGG
R15_MetalIndex	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTCTCGTGGGCTCGG
R16_MetalIndex	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTCTCGTGGGCTCGG
R17_MetalIndex	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTCTCGTGGGCTCGG
R18_MetalIndex	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTCTCGTGGGCTCGG
R19_MetalIndex	CAAGCAGAAGACGGCATACGAGATACGAATTCGTCTCGTGGGCTCGG
R20_MetalIndex	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTCTCGTGGGCTCGG
R21_MetalIndex	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTCTCGTGGGCTCGG
R22_MetalIndex	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTCTCGTGGGCTCGG
R23_MetalIndex	CAAGCAGAAGACGGCATACGAGATTTTCGCGGAGTCTCGTGGGCTCGG
R24_MetalIndex	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTCTCGTGGGCTCGG
R1_MetalIndex	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
R2_MetalIndex	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
R3_MetalIndex	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
R4_MetalIndex	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
R5_MetalIndex	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
R6_MetalIndex	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
R7_MetalIndex	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG
R8_MetalIndex	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGG
R25_MetalIndex	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTCTCGTGGGCTCGG



	Name	Sequence
	R10_MetalIndex	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG
	R11_MetalIndex	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG
	R12_MetalIndex	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG

Primers for Full-Length 16S rRNA Gene Amplification for step 64

	Name	Sequence
	27F	AGAGTTTGATCCTGGCTCAG
	1492R	TACGGCTACCTTGTTACGACTT

Primers to sequence the slurry microbiome (PCR1) for step 71

	A	B
	V4_515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA
	V4_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC

Safety warnings



1. Risk of Contamination

- Cross-contamination between wells and plates can affect the accuracy of microbial isolation and sequencing.
- Work in a laminar flow hood when handling cultures and setting up PCR reactions.
- Always use sterile pipette tips, nuclease-free water, and dedicated PCR reagents to minimize contamination risks.

2. Handling of Microbial Cultures

- Some environmental bacteria may pose biosafety risks, especially opportunistic pathogens.
- Always wear appropriate personal protective equipment (PPE) (lab coat, gloves, eye protection) when handling cultures.
- Dispose of biohazardous waste properly according to institutional guidelines.

3. Alkaline Lysis DNA Extraction Precautions

- NaOH in the lysis buffer is highly caustic and can cause skin and eye irritation.
- Handle alkaline lysis solutions with care and wear gloves and protective eyewear.
- If accidental contact occurs, rinse immediately with copious amounts of water and seek medical assistance if necessary.

4. Safe Handling of Ethanol and Magnetic Beads

- 80% ethanol is highly flammable—keep away from open flames and heat sources.
- Magnetic beads can clump if not resuspended properly, leading to inefficient DNA purification. Always vortex before use and follow recommended incubation times.

5. Storage and Freezing Precautions

- Bacterial glycerol stocks at -80°C must be handled carefully to prevent repeated freeze-thaw cycles, which can reduce viability.
- Ensure proper mixing of glycerol and bacterial culture before freezing to maintain even distribution.

6. Sequencing-Related Issues

- Degraded DNA or poor PCR performance can lead to low sequencing depth or biased results.
- Ensure quality control checks at key points:
 - Verify PCR amplification by gel electrophoresis before pooling libraries.
 - Normalize DNA concentration before sequencing to avoid uneven sequencing depth.

Before start

Reagent setup

- **PBS stock, pH 7**

Concentrated solution (10x): PBS concentrated stock is prepared by adding 1.3 M NaCl, 70 mM Na₂HPO₄ and 30 mM NaH₂PO₄ (pH 7). Autoclave and store at room temperature (22–25 °C) for up to 3 months.

Working solution (1x): Prepare 1 L by adding 100 mL of concentrated stock 10x PBS to 900 mL of deionized water. Store at room temperature for up to 3 months.

- **1x Liquid TSB medium**

Prepare 1 L as follows. Dissolve 30 g of TSB in 1 L of deionized water and distribute into final containers. Sterilize by autoclaving at 121 °C for 15 min. The medium can be stored at 4°C for up to 1 week.

- **10mM MgCl₂ Solution**

Prepare 1 M MgCl₂ stock by adding 20.33 g of MgCl₂·6H₂O to 100 mL of deionized water. Then, add 1 mL of 1 M MgCl₂ stock to 99 mL of deionized water. Sterilize by filtration to avoid precipitation and store at 4°C for up to 2 months.

- **80% (vol/vol) glycerol**

Prepare 1 L by adding 800 mL of glycerin to 200 mL of deionized water. Autoclave twice and store at room temperature for up to 3 months.

- **Sterile water**

Prepare 7 bottles of 1L capacity with 450mL each and autoclave it at 121 °C for 15 min. These bottles will be used to prepare the 10x liquid TSB medium for the serial dilutions and negative control. Additionally, autoclave 1 L of water that will be used to wash the roots.

- **10x diluted liquid TSB medium**

Prepare in a laminar flow hood by adding 50 mL of the 1x liquid TSB medium into the bottles containing 450mL of sterile water. This is the media that will be used for preparing the serial dilutions.

NOTE: Prepare fresh before cultivation to minimize contamination.

- **Alkaline lysis buffer, pH 12**

Concentrated stock: Preparing a stock solution of 100 mM NaOH and 100 mM Na₂-EDTA.

Working solution: Alkaline lysis buffer containing 25 mM NaOH and 0.2 mM Na₂-EDTA at pH 12. To prepare, add 100 mL of NaOH concentrated stock and 0.8 mL of Na₂-EDTA concentrated stock to 299.2 mL of deionized water and use that as a working solution. Autoclave and store at 4°C for up to 3 months.



- **Neutralization buffer, pH 7.5**

Neutralization buffer containing 40 mM Tris-HCl at pH 7.5. To prepare 400 mL, dissolve 1.936 g of Tris-HCl in 400 mL of deionized water. Autoclave and store at 4°C for up to 3 months.

- **80% (vol/vol) ethanol**

80% (vol/vol) ethanol is prepared by diluting absolute ethanol with molecular biology grade water.

NOTE: Always prepare fresh on the day of DNA purification with Mag-Bind beads and prepare just the necessary volume as it can not be reused for the same purpose.

- **TAE solution for electrophoresis**

Concentrated solution (50x): 1 L of 50x concentrated solution is prepared by dissolving 242 g of Tris and 37.2 g of Na₂-EDTA·2H₂O in 800 mL of deionized water, add 57.1 mL of acetic acid until fully dissolved and fill water to 1 L. The solution can be stored at room temperature for up to 3 months.

Working solution: To prepare 1X TAE buffer, add 20 mL of 50X TAE stock solution to 980 mL of deionized water. Mix thoroughly before use. Store at room temperature for short-term use or at 4°C for up to 6 months.

Equipment setup

Downloading and installing software

Download the R code for the bioinformatic pipeline by cloning the GitHub repository:

git clone <https://github.com/NDSU-Geddes-Lab/cultured-microbe-identification.git>

The pipeline requires R version 4.1.0 or later. Earlier versions of R may also work but have not been tested. In addition, several R packages are required: tidyverse, argparser, BiocManager, and DADA2. These can be installed with the following R commands:

```
install.packages(c("tidyverse","argparser","BiocManager"))  
BiocManager::install("dada2")
```

Preparation of dilutions from fresh root samples

1w 5d 8h 18m

- 1 Harvest at least three fresh field plants and collect roots from all those plants.

15m

NOTE: If your plant is a legume, carefully separate the nodules from the roots to avoid the bias of growing an unbalanced number of Rhizobia, the main inhabitants of legume nodules.

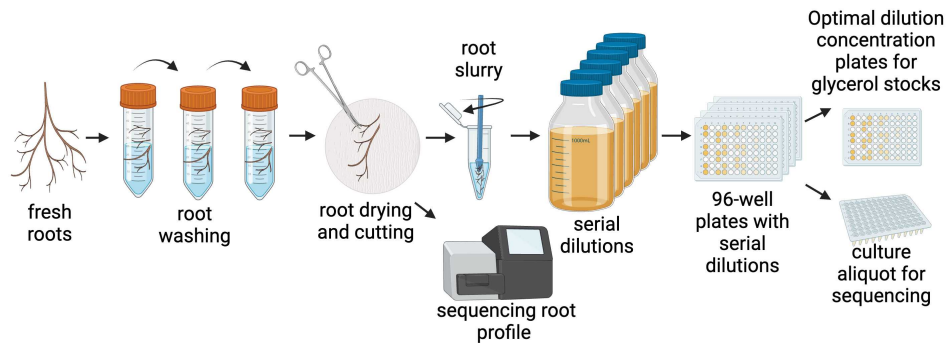


Figure 1. Steps to prepare serial dilutions from fresh root samples for high-throughput bacterial cultivation. Roots from field-grown plants are harvested and washed before being processed into a homogenized root slurry. A dilution series is prepared by transferring specific volumes of the root slurry into bottles containing 10% TSB to generate six different dilution factors (2,000× to 486,000×). The diluted microbial suspensions are distributed into 96-well plates and incubated for 12 days. Optimal dilution concentration (ODC) is determined by ~40% of wells show visible bacterial growth. Aliquots of the culture are transferred to 96-well PCR plates for downstream molecular identification and the rest is preserved in 80% glycerol at -80°C for storage.

- 1.1 Place the roots in a sterile 50-mL Falcon tube, we recommend that for a representative composite of roots at least 2 g are collected.

3m

- 2 Wash the roots with sterile water until no soil particles are visible around the roots.

7m

CRITICAL STEP: Avoid excessive soil contamination as it may introduce environmental microbes that could bias the culture composition.



- 2.1 Transfer the washed roots into a new 50-mL Falcon tube containing 25 ml of sterile 1× PBS and shake at room temperature in a shaker at 180 r.p.m. for 15 min. Repeat this process three times.

20m

- 2.2 Remove the roots from the Falcon tube and distribute them on sterile filter paper to dry the PBS.

3m



- 3 To grind the roots into a homogeneous slurry, proceed to cut the roots into ~2-mm fragments and mix. Place around 0.025 g of mixed root tissue into a 1.5-mL Eppendorf tube. 15m
- 3.1 In a laminar flow hood, resuspend the root tissue in 1000 μ L of 10 mM sterile $MgCl_2$ and grind the root material in the Eppendorf tube with a sterile pestle until it gets homogenized. 10m
- NOTE: Avoid the use of liquid nitrogen or any harsh smashing tool like bead beating to avoid cell lysis which will decrease the isolation efficiency. For harder plant tissues like seeds or cereal heads the samples can be first crush with a sterile mortar and if necessary, again with the sterile pestle.*
- 4 Keep the rest of the root slurry frozen at $-20^{\circ}C$ for cross-referencing cultivated bacteria with root microbiota profiling by sequencing. 3m
- 5 Transfer the root slurry into a sterile Falcon tube containing 24 mL of 10 mM $MgCl_2$, mix well and incubate the samples for 15 min at room temperature to release the bacteria from the root tissue. 20m 
- 6 To prepare the dilution series, resuspend the root slurry and transfer 250 μ L, 83 μ L, 28 μ L, 9 μ L, 3 μ L and 1 μ L of the root slurry to six bottles containing 500 mL of 10% TSB to generate a 2,000 $\times$, 6,000 $\times$, 18,000 $\times$ and 54,000 $\times$, 162,000 $\times$ and 486,000 $\times$ dilution series. 25m  
- CRITICAL STEP: Accurate pipetting is essential to maintain proper dilution factors. Inconsistent dilutions can lead to incorrect growth estimates and affect microbial diversity.*
- 7 Distribute the diluted root slurries into 96-well cell culture plates. 2m
- NOTE: For each dilution in the series, shake the bottle and transfer 50 mL of medium containing the diluted root slurry to a plastic reservoir that fits the multichannel pipette.*
- 7.1 Place 160 μ L of sample into each well of ten 96-well cell culture plates per dilution using a multichannel pipette in a laminar flow hood. In total, 63 96-well cell culture plates are going to be used, including three 96-well cell culture plates with sterile medium as the negative control. 3h  
- 6 dilutions x 10 plates each + 3 negative controls = 63 plates
Each dilution is plated into 10 separate 96-well plates (total of 60 plates), plus 3 additional plates as negative controls, resulting in a total of 63 plates.



NOTE: More plates can be prepared if needed.

NOTE: The laminar flow hood used to perform this step must be as clean as possible to avoid microbial contamination from the environment. Pipetting should be slow to avoid liquid splashing out of wells.

- 8 Incubate the samples. Seal the edge of each cell culture plate with Parafilm. Stack the plates and incubate them in the dark at room temperature for 12 days.

1w 5d



CRITICAL STEP: Plates must be properly sealed with Parafilm to prevent evaporation and contamination.

- 8.1 Observe bacterial growth and determine the dilution at which ~40% of wells in 96-well cell culture plates show visible bacterial growth. This is the optimal dilution concentration (ODC) for high-throughput bacterial isolation.

15m

CRITICAL STEP: Identifying the correct dilution where ~40% of wells show visible bacterial growth is key for maximizing cultivable diversity. Misjudging this step can bias results toward fast-growing bacteria and will complicate further culturing steps.

- 9 Pipette 12 μ L of the bacterial solution in each well of the 96-well cell culture plate into each well of a 96-well PCR plate for bacterial identification, seal them and store them at -20°C .

1h 30m



PAUSE POINT: Bacterial cultures in 96-well PCR plates can be stored at -20°C for several months before DNA extraction and sequencing.

NOTE: It is advisable to centrifuge the plates before using them on a microplate centrifuge for 10s to place any liquid from the seal or the walls into the bottom for the 96-well plate and to avoid cross-contamination between wells. Pipetting should be done carefully to avoid liquid splashing out of wells.

CRITICAL STEP: Cross-contamination must be avoided when transferring cultures for sequencing. Centrifuging plates before pipetting minimizes carryover contamination.

- 10 Add 140 μ L of 80% (vol/vol) glycerol to the remaining bacterial culture in each well of the 96-well cell culture plate and store at -80°C .

1h 30m



PAUSE POINT: Long-term storage: Bacterial stocks can be stored at -80°C indefinitely for future revival and sequencing.

DNA extraction from the cultivated bacteria using the alkaline lysis method

12h 10m

- 11 To extract DNA from the cultivated bacteria using the alkaline lysis method add 16.6 μ L of working solution lysis buffer to 12 μ L of bacterial cultures in the 96-well PCR plates

1h 40m

produced in Step 10.

- 12 Mix the lysis buffer and bacterial culture with a multichannel pipette and incubate in a PCR machine at 95°C for 30 min.

5h 30m



CRITICAL STEP: Overheating during 95°C incubation may degrade DNA. Ensure proper sealing to prevent evaporation.

- 13 Cool the plates to room temperature, add 16.6 μ L of neutralization buffer to each well, cover the PCR plates with polyester sealing film and store at -20°C.

5h



PAUSE POINT: DNA samples can be stored at -20°C for several months before continuing with PCR.

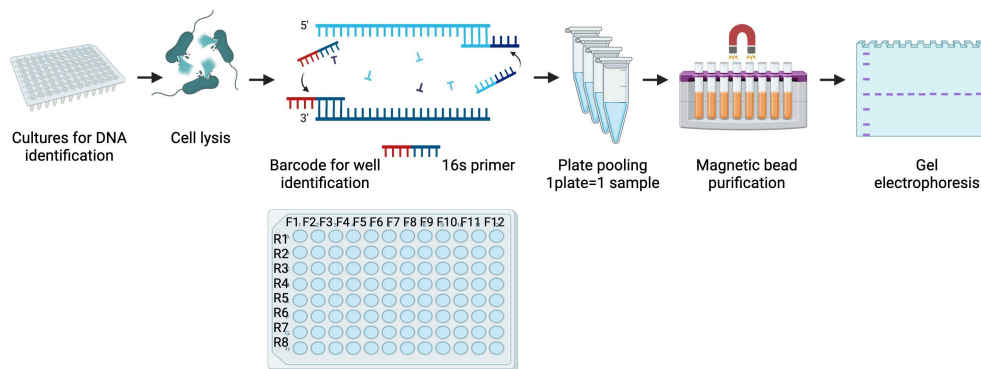


Figure 2. Steps following the transfer of bacterial culture to PCR plates for sequencing identification. Once part of the culture is transferred into PCR 96-well plates, we proceed to cell lysis. The DNA will be then used as template for the first step PCR used for the barcoding of wells from individual 96-well plates. The next step is to pool each well from the 96-well plate into individual samples and clean them up using magnetic beads. Finally, a gel electrophoresis is performed to corroborate the presence and size of the PCR product. Each pooled plate should yield a PCR product of 360bp.

First step PCR1 (well barcoding)

1d 5h 35m

- 14 Examine 38 DNA random samples from all plates (2 left for ladder) by 1.5% (wt/vol) agarose gel electrophoresis using 3 μ L of PCR product and 3 μ L of loading buffer. If the DNA is visible, proceed to the next step.

2h 30m



- 15 Set up PCR1 (well barcoding) reaction mixture for each well on a laminar flow hood as indicated below. Use polymerase without bacterial DNA contamination, we used KAPA Hotstart polymerase (Roche Diagnostics, USA) with great success.

6h 40m



	x1 reaction
Polymerase KAPA	5
Forward primer 1uM	1.5
Reverse primer 1uM	1.5
DNA	1.3
H ₂ O	1
Total	10.3

NOTE: There are 12 forward primers for each column in the 96well plate and 8 reverse primers for each row in the 96well plate. Therefore, each well in every 96well plate will have a unique set of barcodes associated to their forward and reverse primer. The list of primers used for this PCR is provided in the Materials section under Primers.

CRITICAL STEP: Use DNA-free polymerase to avoid contamination. Incorrect primer selection could lead to low amplification efficiency.

- 16 Perform PCR using the following cycling conditions:

16h 40m



Cycle number	Denaturation	Annealing	Extension
1	95 °C, 3 m		
2–26	95°C, 30 s	55 °C, 30 s	72 °C, 30 s
27			72 °C, 5 m

- 17 Examine the PCR products by 1.5% (wt/vol) agarose gel electrophoresis using 3 µL of PCR product and 3 µL of loading buffer. Successful reactions should yield a single 360-bp product for wells with cultures and a blank result for wells without a product.

2h

- 18 Take 5µL of the each of the products/wells from the first PCR from one plate and mix in a 1.5-mL Eppendorf tube. Do this for each individual plates.

1h 40m



- 19 Aliquot 30µL of the pooled PCR1 product from each plate into a new 1.5-mL Eppendorf tube.

5m

NOTE: One Eppendorf tube for each plate.





PAUSE POINT: Pooled PCR1 products can be stored at -20°C for several weeks before proceeding to purification.

Purification of PCR1 product

1h 29m

- 20 Remove Mag-Bind beads from the 4°C refrigerator and allow them to reach room temperature before using them.

5m

NOTE: The next steps uses Mag-Bind beads to purify the PCR1 product away from unattached primers and primer dimer.

Prepare the follow consumables:

Item	Quantity
Ultra-pure water	40 μL per plate/sample
Mag-Bind beads	30 μL per plate/sample
Freshly Prepared 80% Ethanol	400 μL per plate/sample

- 21 Vortex the Mag-Bind beads for 30 seconds to make sure that the beads are evenly dispersed.

1m



- 22 Transfer 30 μL of Mag-Bind beads into the 1.5-mL Eppendorf tube with the aliquoted 30 μL of PCR1 product (step 19).

10m

- 23 Briefly vortex the mixture

3m



- 24 Incubate at room temperature without vortexing for 5 minutes.

5m



- 25 Place each Eppendorf tube containing the PCR1 product and the magnetic beads mixture on a magnetic stand for 2 minutes or until the supernatant has cleared.

3m

- 26 With the PCR1 product and the magnetic beads mixture in the Eppendorf tube still on the magnetic stand pipette to remove and discard the supernatant. Change tips between samples.

8m

- 27 With the Eppendorf tube still on magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

- 27.1 Add 200 μL of freshly prepared 80% ethanol to each sample tube.

5m



27.2 Incubate the tube on the magnetic stand for 30 seconds.

1m

27.3 Carefully remove and discard the supernatant.

5m

28 With the tube on the magnetic stand, perform a second ethanol wash as follows:

28.1 Add 200 μ L of freshly prepared 80% ethanol to each sample tube.

5m



28.2 Incubate the Eppendorf tube on the magnetic stand for 30 seconds.

1m

28.3 Carefully remove and discard the supernatant.

5m



28.4 With a small volume pipette remove excess ethanol.

5m



29 With the Eppendorf tube still on the magnetic stand, allow the beads to air-dry for 5 minutes.

5m

30 Remove the PCR1 tube from the magnetic stand.

1m

31 Add 40 μ L of Ultra-pure water to each PCR1 Eppendorf tube and pipet up and down 20 times or vortex for 30 seconds.

5m



32 Incubate at room temperature for 2-3 minutes.

3m



33 Place the PCR1 Eppendorf tube on the magnetic stand to magnetize the Mag-Bind and let it sit at room temperature until the solution is completely cleared from beads.

3m





- 34 Carefully transfer 38 μL of the supernatant from the PCR1 Eppendorf tube to a new 1.5-mL Eppendorf tube. Change tips between samples to avoid cross-contamination.

10m

II

PAUSE POINT: Purified PCR1 products can be stored at -20°C for later use.

Second step PCR2 (plate barcoding and Illumina sequencing adaptors)

2h 30m

- 35 Set up reaction mixture for each well on a laminar flow hood as indicated below. Use polymerase without bacterial DNA contamination, we used KAPA Hotstart polymerase (Roche Diagnostics, USA) with great success.

30m

		x1 reaction
KAPA Hotstart polymerase		12.5
Forward primer (1uM)		2.5
Reverse primer (1uM)		2.5
DNA		2.5
H ₂ O		5
Total		25

Note: The list of primers used for PCR2 is provided in the Materials section under Primers.

- 36 Perform PCR 2 using the following cycling conditions:

30m

	Cycle number	Denaturation	Annealing	Extension
	1	95 $^{\circ}\text{C}$, 3 m		
	2–9	95 $^{\circ}\text{C}$, 30 s	55 $^{\circ}\text{C}$, 30 s	72 $^{\circ}\text{C}$, 30 s
	10			72 $^{\circ}\text{C}$, 5 m

- 37 Examine the PCR products by 1.5% (wt/vol) agarose gel electrophoresis using 3 μL of PCR product and 3 μL of loading buffer. Successful reactions should yield a single 400-bp product for wells with cultures and a blank result for well without a product.

1h 30m

Purification PCR2 product

1h 25m

- 38 Remove Mag-Bind beads from the 4 $^{\circ}\text{C}$ refrigerator and allow them to reach room temperature before using them.

5m

NOTE: The next steps uses Mag-Bind beads to purify the PCR2 product away from unattached primers and primer dimer.



Prepare the follow consumables:

Item	Quantity
Ultra-pure water	33.5µL per plate/sample
Mag-Bind beads	35 µL per plate/sample
Freshly Prepared 80% Ethanol	400 µL per plate/sample

- 39 Vortex the Mag-Bind beads for 30 seconds to make sure that the beads are evenly dispersed. 1m
- 40 Take the total PCR2 reaction (25µL) of the pooled PCR2 product into a new 1.5-mL Eppendorf tube and add 25µL of Mag-Bind beads. Do this separately for each pooled plate. If you started with 10 plates you will have 10 Eppendorf tubes. 10m
- 41 Briefly vortex the mixture 3m
- 42 Incubate at room temperature without vortexing for 5 minutes. 5m
- 43 Place each Eppendorf tube containing the PCR2 product and the magnetic beads mixture on a magnetic stand for 2 minutes or until the supernatant has cleared. 3m
- 44 With the PCR2 product and the magnetic beads mixture in the Eppendorf tube still on the magnetic stand pipette to remove and discard the supernatant. Change tips between samples. 8m
- 45 With the Eppendorf tube still on magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
 - 45.1 Add 200 µL of freshly prepared 80% ethanol to each sample tube. 5m
 - 45.2 Incubate the tube on the magnetic stand for 30 seconds. 1m
 - 45.3 Carefully remove and discard the supernatant. 5m
- 46 With the tube on the magnetic stand, perform a second ethanol wash as follows:
 - 46.1 Add 200 µL of freshly prepared 80% ethanol to each sample tube.



5m

46.2 Incubate the Eppendorf tube on the magnetic stand for 30 seconds.

1m

46.3 Carefully remove and discard the supernatant.

5m

46.4 With a small volume pipette remove excess ethanol.

5m

47 With the Eppendorf tube still on the magnetic stand, allow the beads to air-dry for 5 minutes.

5m

48 Remove the PCR2 tube from the magnetic stand.

1m

49 Add 33.5 μ L of Ultra-pure water to each PCR2 Eppendorf tube and pipet up and down 20 times or vortex for 30 seconds.

5m

50 Incubate at room temperature for 2 minutes.

2m

51 Place the PCR2 Eppendorf tube on the magnetic stand to magnetize the Mag-Bind and let it sit at room temperature until the solution is completely cleared from beads.

52 Carefully transfer 31 μ L of the supernatant from the PCR2 Eppendorf tube to a new 1.5-mL Eppendorf tube. Change tips between samples to avoid cross-contamination.

10m

PAUSE POINT: Purified PCR2 products can be stored at -20°C until ready for sequencing.

Confirmation of PCR 2 product size and concentration

1h

53 Perform a gel electrophoresis with all purified samples from PCR2 (1 per plate) by 1.5% (wt/vol) agarose gel electrophoresis using 3 μ L of PCR purified product and 3 μ L of loading buffer.

54 Quantify all the purified samples using a Quant-iT PicoGreen dsDNA Assay Kit.

1h

- 55 Continue the standard protocol for NGS with an Illumina MiSeq sequencer (Benz, Brooke R., et al. Improved Efficiency of Two-Step Amplicon PCR Using an Acoustic Liquid Handler. *bioRxiv* (2024): 2024-12).

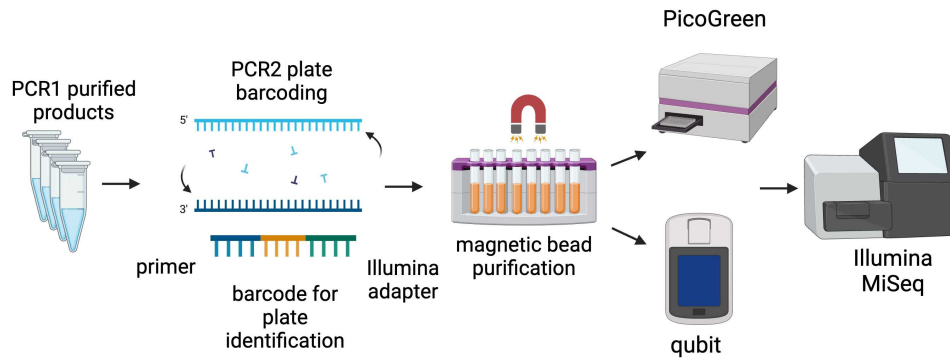


Figure 3. Steps following the pooled PCR1 products until samples get sequenced in the Illumina MiSeq sequencer. The purified PCR1 products are then subjected to a second-step PCR (PCR2), where Illumina sequencing adapters and plate-specific barcodes are added. Following another bead-based purification step, the final PCR2 products are quantified using a fluorometric assay (PicoGreen) and normalized for library preparation. The prepared libraries are then loaded onto the Illumina MiSeq sequencer for high-throughput sequencing and taxonomic identification of cultivated bacterial isolates.

Bioinformatic analysis of amplicon sequencing to identify unique cultivated bacteria

27m

- 56 Launch a command line terminal and navigate to the directory cloned from GitHub (see "Downloading and installing software" in the "Before starting section").
NOTE: The following instructions are accurate as of the time of writing of this manuscript. Updates to these instructions after publication will be reflected in the GitHub repository: <https://github.com/NDSU-Geddes-Lab/cultured-microbe-identification>.

10m



- 57 Run the microbeID.R script with the -h flag to print the help menu for the workflow:

2m



Command:

```
Rscript microbeID.R -h
```

Output:

```
usage: microbeID.R [--]
[--help] [--db DB] [--barcodes BARCODES] [--fwd FWD] [--rev REV] [--hits HITS]
[--outdir OUTDIR] [--multithread MULTITHREAD] fastq_dir
```

Cultured Microbe ID

positional arguments:

fastq_dir	Directory containing input sequences
(.fastq.gz)	

flags:

-h, --help show this help message and exit

optional arguments:

```
-d, --db          Path to taxonomy database [default:
./db/silva_nr99_v138.1_train_set.fa.gz]
-b, --barcodes   Path to barcode plate map (.csv) [default: ./BC_to_well2.csv]
-f, --fwd        Forward primer [default: GTGCCAGCMGCCGCGGTAA]
-r, --rev        Reverse primer [default: GACTACHVGGGTATCTAATCC]
--hits           Number of hits to report (top n wells) [default: 5]
-o, --outdir     Output directory [default: ./output]
-m, --multithread Set to FALSE for Windows [default: TRUE]
```

58 To run the workflow, execute the Rscript with the path to the directory containing your sequence files. For example:

```
Rscript microbelD.R /path/to/fastq/directory
```

The input directory is the only required parameter. Other parameters have built-in defaults. You may override any of these additional parameters by using the appropriate command line arguments. In particular, if you are running the workflow on Windows, you may want to set `--multithread FALSE` to turn off multithreading for certain functions known to cause problems on Windows.

The script expects the following to be present in the same directory, unless otherwise specified at runtime using the appropriate help menu options:

- SILVA Database: ./db/silva_nr99_v138.1_train_set.fa.gz
- Barcode plate well map (provided in the GitHub repository): ./BC_to_well2.csv

59 Results from the workflow will be placed in the output directory within the current working directory (unless an alternative output location was specified). This directory will contain the following:

- `asv_analysis_results.csv`: The main results file, containing the list of ASVs, their taxonomy, and the count and purity for the top n plate wells (by count).
- `filtered`: Directory containing filtered reads generated by DADA2 during execution of the workflow.
- `Rplots.pdf`: PDF file containing any graphical outputs generated by the workflow, e.g. sequence quality plots from DADA2.

10m



5m





Here is an example of the count/purity information, for a top hit, produced by the workflow (ASV sequences and phylogeny omitted):

● Plate1_A2 | 183 | 93.85

For each hit, the workflow reports the plate and well where the ASV was identified, the frequency of the ASV in that well, and the purity of the ASV, with respect to the total number of sequences coming from that well.

Purification and preservation of cultivated bacteria

1w 5d 17h 30m

- | | | |
|----|---|---|
| 60 | For each unique ASV identified in Step 59, select 2 wells with the highest purity and reads and then scratch the surface of the frozen bacterial culture with a pipette tip and streak it onto a 1/2 TSB plate. | 15m |
| 61 | After 3–5 d of incubation at room temperature, pick a single colony and streak it onto a new 1/2 TSB plate. Repeat this process two more times.

<i>CRITICAL STEP: Owing to the diverse growth rates of bacteria, the time for this step may vary from 9 to 15 d. It is important to pick single colonies to ensure that the bacterial culture is pure. If different colonies are growing on a single plate, pick the different colonies and purify them separately on plates containing the same bacterial medium until pure culture plates are reached.</i> | 5d
  |
| 62 | Pick a single colony from the bacterial medium plate, incubate the colony in 1/2 TSB liquid medium and shake at 28 °C at 180 r.p.m. for 5–7 d. | 1w
 |
| 63 | When the bacterial liquid culture is turbid, transfer 10 µL of the culture into a PCR tube for DNA extraction following Steps 11–13. | 12h 15m
  |
| 64 | Validate the bacterial 16S rRNA gene Illumina sequences using Sanger sequencing. Amplify the full-length 16S rRNA gene by PCR using primers 27F and 1492R, following Steps 15 and 16. Increase the annealing time to 1 minute instead of 30 seconds.

<i>NOTE: The list of primers used for this PCR is provided in the Materials section under Primers.</i> | 5h
  |
| 65 | Preserve the bacteria in glycerol stocks (Steps 66–69) when the Sanger sequencing results are consistent with the high-throughput Illumina sequencing results obtained in Step 59.

<i>PAUSE POINT: Glycerol stocks can be maintained at -80°C indefinitely for future strain recovery and studies.</i> |    |

Glycerol stocks of unique ASVs

20m

- 66 Centrifuge 30 mL of bacterial solution from Step 63 for 10 min at 2,900g at room temperature. 10m
- 67 Discard the excess culture medium to retain 1 mL of concentrated bacterial cells. 2m
- 68 Vortex to completely suspend the bacteria in the remaining medium and combine 800 μ L of bacterial solution and an equal volume of 80% sterile glycerol in a cryopreservation tube. 5m
- 69 Mix the solutions evenly by pipetting and store at -80°C . 3m

CRITICAL STEP: Glycerol stocks must be thoroughly mixed before freezing at -80°C to prevent uneven distribution and loss of viability upon revival.

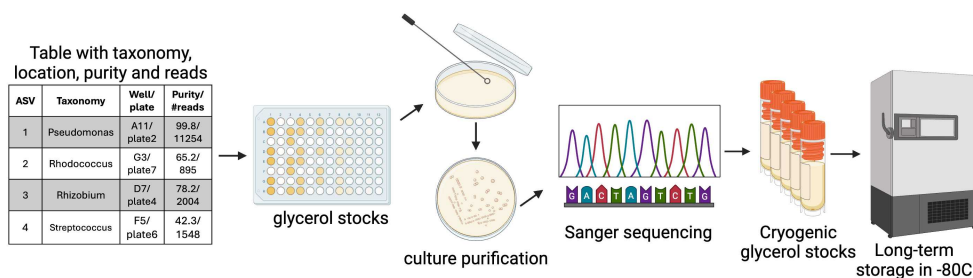


Figure 4. Steps to follow once the sequencing results have been analyzed through the bioinformatic pipeline. After sequencing results are processed, unique amplicon sequence variants (ASVs) are identified and linked to specific wells in 96-well plates. Selected bacterial isolates are retrieved from glycerol stock plates and streaked onto 1/2 TSB solid Petri dishes to obtain pure cultures. After three consecutive transfers to ensure purity, isolates are subjected to Sanger sequencing for further validation. Successfully identified and confirmed isolates are cultured in liquid media, and glycerol stocks are prepared for long-term storage at -80°C , preserving the cultivated bacterial collection for future studies.

Root slurry microbiome profiling

11h

- 70 Isolate DNA from the root slurry (stored at -20°C , Step 4) using the DNeasy Plant Pro Kit, following the manufacturer's instructions. This DNA will be used for microbiome profiling PCR to compare the native root microbial community with cultivated isolates. 3h



- 71 Follow Benz, Brooke R., et al. (2024) (See References) for library preparation, sequencing, and bioinformatics analysis.

8h

NOTE: Samples from root slurry microbiome profiling (Step 4 and 70) and high-throughput cultivated bacterial plates (step 55) can be processed and sequenced together to ensure direct comparison.

Protocol references

Benz, B. R., Echarte, E. L., Whitaker, B. K., Baldwin, T. & Geddes, B. A. Improved Efficiency of Two-Step Amplicon PCR Using an Acoustic Liquid Handler. 2024.12.06.627172 Preprint at <https://doi.org/10.1101/2024.12.06.627172> (2024).

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