JAN 04, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.5jyl8pm26g2w/v1

Protocol Citation: Nhu Nguyen, Sophia Lee, Andrew Lin 2024. High-throughput cultivation and identification of soil bacteria. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl8pm26g2w/v1

High-throughput cultivation and identification of soil bacteria

Andrew
Nhu Nguyen[1], Sophia Lee[1], Lin[1]

[1]University of Hawai'i at Mānoa

Sophia Lee: Sophia helped develop and tested this protocol when she was an undergraduate researcher in the Nguyen Lab.
Andrew Lin: Andrew helped develop and tested this protocol when he was an undergraduate researcher in the Nguyen Lab.

Nguyen Lab

Nhu Nguyen

ABSTRACT

This protocol uses a dilution to extinction technique to isolate bacteria from soil but also applicable to other substrates. The isolation takes place in 96-well plates, thus allowed us to replicate the isolation in a high-throughput manner. The second part of the protocol uses high-throughput DNA sequencing to identify the samples. We amplified and sequenced >900 isolates within one month working non-continuously. The estimated cost (without technician time) for each sample is approximately $3.50 and the cost will drop with more samples added to a single MiSeq run.

Protocol status: Working

We use this protocol and it's working. We amplified and sequenced >900 isolates within one month working non-continuously. The estimated cost (without technician time) for each sample is approximately $3.50 and the cost will drop with more samples added to a single MiSeq run.

Created: Jun 30, 2023

Last Modified: Jan 04, 2024

PROTOCOL integer ID:
84276

Funders Acknowledgement:
National Institutes of Health
Grant ID: 5P20GM125508-03

Media and Calculations

1 **Media:** This is a big topic and determines the outcome of what is being isolated. General laboratory broth media contain high amounts of nutrients and isn't necessarily reflective of the soil environment. It is recommended that a dilute medium be used for isolation to mimic slightly more natural conditions. We typically use a 1/10 dilution of tryptic soy broth for general isolation, and more specialized medium like soil infusion or root exudates for more specific microbes.

2 **Calculations:** Cultivation can occur in any number of multi-well microtiter plates. We generally use 96-well round bottom plates for ease of growth observation. Depending on the volume of each well, calculate the total amount of media for each plate. For a 96-well plate, we add 150 µL of media to each well, so you will need 14.4 mL of media total. We use 20 mL per plate to be safe.

3 **Dilutions**: This protocol uses a dilution to extinction method to try and isolate single cells into each well of a microtiter plate. Since each sample can contain drastically different amounts of living cells, each experiment needs to test the appropriate dilution to achieve 1/3 to 1/4 of the plate being colonized. This is to minimize the chance of multiple organisms growing within any single well.

Sample preparation

1w 1d 2h 42m 25s

4 Collect the soil (or sample) of interest. It is best to do the cultivation immediately after collection, but 1d materials can be stored in the refrigerator overnight. Longer storage can affect the outcome if you are using this method for quantification.

5 Prepare the collected soil. Any materials in the soil that is not the target of interest (e.g. roots, detritus) should be removed. 1h

6 Homogenize the sample, including breaking of aggregates into fine particles. 30m

7 Weigh out 1 g of the homogenized sample. 1m

Inoculation

1w 1d 2h 42m 25s

8 Add the homogenized sample into 5 mL of sterile medium.

9 Vortex for 00:01:00 to suspend the soil particles in the medium. 1m

10 Allow the heavier particles to sink for 00:00:15. 15s

11 Pipet 1 mL of the suspension into 1L of medium. Do not include any large particles. Shake well to mix.

12 Allow heavier particles to sink for 00:00:15. Observe carefully to make sure that this is happening. No large particles should be visible. 15s

13 Add the appropriate amount of the sample suspension prepared above to the growth medium to mal 30m
total of 20 mL per dilution series. These 4 dilutions series should be enough for most soil samples.

- 10^{-1} or 1/10 dilution (2000 µL into 18000 µL medium)
- 10^{-2} or 1/100 dilution (200 µL into 19800 µL medium)
- 10^{-3} or 1/1000 dilution (20 µL into 19980 µL medium)
- 10^{-4} or 1/10000 dilution (2 µL into 19998 µL medium)

Troubleshoot: If you did not get any growth on the 10^{-1} dilution, use the undiluted (10^{0}) sample, dilute the original 1 g of soil into 100 mL medium, or increase the soil sample to 10 g.

14 Pour a dilution into a sterile pipetting reservoir. Use a multichannel pipet to dispense 150 µL int 10m
each well of a 96-well plate.

15 Repeat for all 4 dilutions. 30m

16 Wrap at least two layers of parafilms around the plate, and secure the lid with lab tape. Place the plate 1m
into a ziplock bag to minimize moisture loss. Make sure your plates are labeled properly.

17 Incubate at the desired temperature and check periodically. For soil bacteria, we incubate them for at 1w
least 1 week, sometimes up to 2. However, you risk evaporation of your medium for longer or warmer incubations. For warm and/or longer term incubations, place the ziplock bag into a moist chamber inside the incubator.

Processing and Storage

18 For each soil sample, determine the dilution plates that have visible growth in less than 30% of the wells. Plates with higher percentages of growth tend to have more mixed-culture wells. Discard plates that have higher number of wells with growth.

Tip: To inspect for growth in your plate, set up an diffused light panel and place the plate on top. This will allow you to see which well has growth and which doesn't. Mark wells on the lid of the plate as appropriate. These lights can commonly be found in the lighting section of hardware stores or specialty

lighting stores.

19 Transfer all the contents of any wells with growth into a new 96-well plate.

19.1 It is generally recommended that you refresh the cells so that you don't lose any cultures. Use a 96-well replicating tool to inoculate a new 96-well plate with fresh medium. Incubate using the same parameters as isolation.

20 **Storage:** Add enough glycerol (or preferred cryopreservative) to each well so that they contain ~ [M] 30 % (v/v) glycerol. Freeze at -80 °C.

21 **Post-identification purification:** After identification and determination of purity of a sample, wells with mixed cultures of interest should be streaked out, isolated, and re-identified. Since working with 96 well-plates increases the chance of cross-contamination, any cultures from a well used in future experiments should be streaked out to ensure purity of isolate.

High-throughput identification

22 This protocol allows high-throughput identification of soil bacteria in 96-well plate format. It starts with an alkaline lysis of cells, followed by a 2-step PCR method that amplifies and barcodes the samples, then Illumina MiSeq sequencing of a targeted variable region(s) of the rRNA gene, and a bioinformatic workflow that cleans up the sequences and identifies the samples. In our preliminary proof of concept trial, we amplified and sequenced >900 isolates within one month working non-continuously. The final estimated cost (without technician time) for each sample is $3.50 and the cost will drop with more samples added to a single MiSeq run. We expect that a single run could handle 1500 isolates.

DNA Extraction

3m

23 Transfer 100 µL of cell cultures grown in liquid medium to a fresh 96-well PCR plate.

24 Spin down at 4000 x g for 00:03:00. 3m

25 Pipet out as much of the growth medium as possible. We find that it's difficult to pipet out more than 75 µL using a multichannel pipet and not disrupt the cell pellet. A bit of medium left over should not interfere with PCR.

26 Add 75 µL of Extraction Solution (ES) to each well. Seal the plate with a sealing film and gently vortex to mix the cells into solution. Spin down quickly but do not pellet the cells.

26.1 Add 75 µL of Dilution Solution (DS) so that the final ES:ED solution ratio is 1:1.

Note: The recipes for Extraction and Dilution Solutions is available elsewhere.

26.2 Pellet the cells by centrifuging at 4000 x g for 3 minutes. The final DNA concentration is ~25 ng/µl. Use 1-2 µl of the clear solution for PCR (avoid the precipitate). For bacteria and yeast samples, we typically use 2 µL template in a 15 µL PCR reaction.

PCR

27 For PCR, we use a two-step dual barcoding method to amplify and barcode each sample. For detailed methods, please see our lab's Illumina high-throughput sequencing protocol.

28 Perform the 1st PCR using the gene primer with overhangs for the second primer. Samples should amplify well, and there should be little to no primer dimers left over. Check all samples for amplification using gel electrophoresis.

Total reaction volume: 15 µL
Mastermix: Any good quality mastermix with relatively high-fidelity polymerase.
Template: 2 µL of crude extract
Primers: [M] 0.3 micromolar (µM) ([M] 0.15 micromolar (µM) each) final concentration of premixed pairs of primers.

29 Perform the second PCR using index primer mixes. If you have products in the first PCR, there is a very high chance that it will amplify in the second PCR. We do random spot checks (8 wells) across the plate to make sure that there is good amplification across the plate.

Total reaction volume: 10 µL
Mastermix: Any good quality mastermix with relatively high-fidelity polymerase.
Template: 1 µL of first PCR
Primers: [M] 0.2 micromolar (µM) ([M] 0.1 micromolar (µM) each) final concentration of premixed pairs of primers. See the Nguyen Lab Index Primer setup.
PCR parameters: Same as the first PCR, except primer annealing is at 52 °C.

Preparing the library

30 Combine all samples in equal volume. Here we assume that all samples have been amplified equally, and in our experience, amplification from the 2nd PCR tends to be uniform. We typically only use 2 µl per sample in this step.

31 Perform SPRI bead cleanup on the final sample. Depending on how many samples you have, the final volume will exceed the limits of the tubes. For this step, we usually clean up 500 µL of the combined library. The cheapest way to do this is to buy a small, rare-earth magnet and set it up in such a way that it can interact with your tube in a rack. We simply tape it to the rack when needed.

32 Submit the sample to your favorite sequencing facility to be sequenced using Illumina MiSeq 300PE. Generally, we find that 1500 samples isn't enough for a run so we combine them with other microbiome amplicon libraries.

Bioinformatics

33 Use your favorite bioinformatics pipeline to identify the resulting cultures. Interpret your results carefully. Consider these points:

- Illumina sequencing can produce a good bit of noise, so an isolate may appear to be mixed but it isn't. In that case, you will need to determine what portion of the sequence is actual data and what portion is noise.
- You will find that certain isolates have many copies of the 16 rRNA variants. This is not noise, but the true biology of the organism.