High-throughput microbiome library preparation (16S+ITS1)

Datum: 2024-03-18
Tags: Microbiome
Erstellt von: Eddy Mendoza

This protocol allows creating of amplicons, indexing and ligate adapters in one-time PCR. The resulting libraries have to be cleaned, normalized and pooled, and they are ready to send for sequencing. The length of the resulting amplicons is thought to allow almost full overlapping of paired reads using a 2x250 MiSeq instrument.

The procedure and primer design were built as a consensus from a Bio-protocol (BP; Sundareson Lab, UC Davis), the standard Earth Microbiome protocol (EM) and the Standard Operating Procedure (SOP) from the Schloss lab (the group that designed the original primer structure, ref. Kozik 2013).

The primer structure is represented as follows:

1. **P5/P7 flow cell adapters**
2. **i5/i7 in-line indexes .** 8bp.
3. **Pad and linker .** The pad is a 10-nt sequence to boost the sequencing primer melting temperatures.The link is a 2-nt sequence that is anti-complementary to the known sequences.
4. **Targeting primers .** For prokaryotes, it targets the V4 region of the 16S ribosomal gene (~250 bp), using the primers 515F and 806R.For (mostly) fungi, it targets the ITS1 region (~230 to >600bp), using the primers ITS1F and ITS2R.

To standardize PCR variation, each sample must be amplified in technical triplicates.

NOTE: Most of the recipes and procedures are thought to be used for blocks of ~100 samples (a 96-well plate). Calculate quantities and times accordingly if your number of samples is larger than this.

Material that may have to be ordered: (Check availability in the lab first)

- DNeasy 96 PowerSoil Pro Kit (384).
- Q5 polymerase (including buffer).
- Qubit kit.
- dNTPs.
- mPNAs + pPNAs.
- SequalPrep Normalization Plate Kit, 96-well.

PROCEDURE:

Microbiome harvesting

Before you start:

- Prepare ~1.5 L of sterile NaCl solution 0.9% w/v (for 100 samples)
- Prepare ~2 L of sterile water (for 100 samples)
- Sterilize a scalpel and tweezers, at least two each, to change between samples.

Minimum lab material expected to be used	• For each sample: ◦ 1 labeled 15mL falcon tube with ~10 mL of NaCl Buffer ◦ 1 labeled sterile 1.5 mL Eppendorf tube. Use Safe-Lock tubes to avoid tube explosion (and sample loss) when freezing. • Paper towels, cut in half (sterile if possible, one per sample) • Liquid nitrogen • Gloves
Time required	One person would do around 100 samples a day
Recommendations	Use a clean lab coat, and disinfect the bench before working. Additionally would be isolating the workspace (block doors) and wearing a mask.

Steps:

The full procedure can be found in another eLab entry:

> Detailed harvesting protocol

My recommendations:

- When working with biological replicates of the same treatment/genotype, change gloves for each treatment/sample. If possible, even for each sample. The way the microbiome is harvested can significantly impact the outcome. Try to reduce cross-sample contamination as much as possible.
- After removing the soil, lay the whole plant on a paper towel and split the rosette and the root with the scalpel. Use the same paper towel to dry the roots and shoots after washing. Discard the towel and use a new one for each sample. This would avoid cleaning many times.
- When washing the roots, it is okay to use the same water for many samples. Later, disinfection steps would be performed. Also, if collecting DNA/RNA, it is better to freeze the roots directly.
- It is easier to harvest the rhizosphere when the soil has not been watered for one day. After two days, the soil can get very dry, and most of the particles will get stuck to the roots. If the soil is too wet, it is complicated to find and not break the little roots. In brief: not too wet and not too dry.

Sample preparation

Before you start:

- Prepare 500 mL of sterile distilled water (for 100 samples).

- Prepare 150 mL of 2% v/v sodium hypochlorite (or commercial bleach; for 100 samples). Use sterile water for the solution.
- Prepare 150 mL 70% EtOH (for 100 samples). Use sterile water for the solution.
- If the rhizosphere samples were frozen, let them thaw on the bench before working with them.
- Endosphere samples should not be thawed at room temperature; try to keep them between -20°C and 4°C or on ice.

Minimum lab material expected to be used	For each endosphere sample: • One labeled sterile Safe-Lock Eppendorf tube. Importantly: Use **2 mL** Eppendorf tubes (1.5 mL tubes can't be used to disrupt the tissue). • 3 blue (1000uL) tips. Prepare an additional box in case of mistakes. • One sterile collection tube. Prepare extra in case of mistakes. Collection tubes are sold in 96-tube boxes with 12 strips of 8 tubes each. Split the tubes and sterilize them before use. For each rhizosphere: • 1 blue (1000 uL) tip. Prepare an additional box in case of mistakes.
Time required	Expect to invest around one hour for the root washing for 16-20 samples. Expect one whole day if processing ~100 samples. For the rhizosphere, expect to invest around 2 hours for 100 samples. For the root tissue disruption, expect to invest around two hours for ~60 samples. Processing the root samples can take a long time.
Equipment	Book a sterile hood. Fire is necessary for the endosphere only.

Steps:

For Rhizosphere samples:

- Centrifuge the falcon tubes at maximum speed for 5 minutes. Use the large centrifuge from the Weiberg group. Before centrifugation, mix up the samples to avoid the soil particles getting stuck in the tube walls.
- Move to the sterile hood.
- Remove the supernatant.
- Add 800 uL of buffer CD1 to the tube (Qiagen Power Soil Kit).
- Resuspend the soil with the buffer.

 CHECKPOINT - You can store the samples at 4°C overnight. If a longer time is needed, store at -80°C.

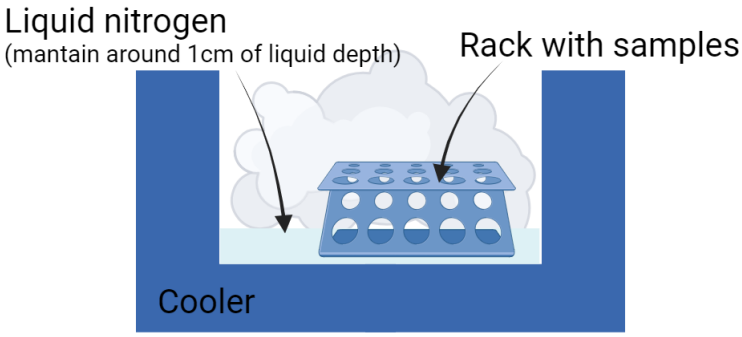
For Endosphere samples:

- Move to a sterile hood with a fireboy.
- For each sample:
  - Add ~800 uL of 2% NaOCl (bleach).
  - Mix for 30 seconds.
  - Discard the liquid (can be done with the same tip).
  - Add ~800 uL of 70% EtOH.
  - Mix for 15 seconds.

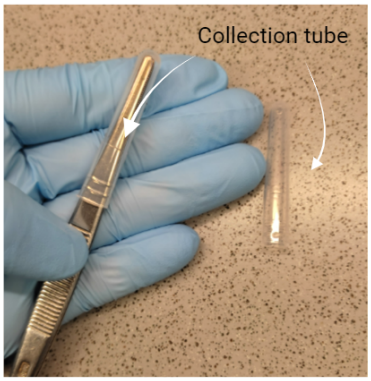
- Discard the liquid (can be done with the same tip).
- Do three washes with sterile water. Use a pair of tweezers to hold the roots inside the tube while discarding the water. You can use one tip per sample using the tweezers this way.
- Transfer the roots to a new sterile **2 mL** Eppendorf tube.

 CHECKPOINT - You can store the samples at -80°C. Do not store them at warmer temperatures. If possible, quickly freeze the samples with liquid nitrogen after washing.

- Prepare a cold chamber as follows: (use a metal rack)



- Before working with each sample, prepare a plastic "mortar":



- Maintain all samples frozen at all times.
- Disrupt the root tissues by smashing them vigorously with the tip of the collection tube. Keep the sample tube in touch with the liquid nitrogen. Handling the tubes with a pair of tweezers allows easy manipulation and to avoid contamination. The diameter of the collection tubes is almost as large as that of the Eppendorf tube, which should be enough to avoid the entrance of external contaminants during the process.
- Make sure the roots are fully disrupted; you should observe a thin white powder. If needed, spin the tubes to remove the tissue that got stuck in the walls. Discard the collection tube.

 CHECKPOINT - Store the samples at -80°C.

My recommendations:

- In the hood, work with blocks of 32 samples and keep the rest frozen to avoid cell death.
- It is also possible to skip the maceration part and put the roots in the tissue-layer plate. In my

experience, disrupting the root tissues is necessary to get enough DNA concentrations (the beads cannot disrupt the tissues).

DNA extraction

Before you start:

Thaw samples if frozen. Prepare

Minimum lab material expected to be used	DNA extraction in plates with kit. For a plate of 96 samples: • Two boxes of blue tips (1000 uL). • Three boxes of big purple tips (192 boxes; 1250 uL). • Three boxes of yellow tips. • Sterilize a pair of scissors.
Time required	One whole day
Equipment	Multichannel pipettes Centrifuge from the Genomics Unit (to fit the S-block + QIAmp Plate)

Steps:

In a sterile hood:

- Add 800 uL of solution CD1 to each root sample (assuming the tissue has been disrupted) and transfer to a PowerBead Pro plate.
- Transfer 800 uL of the rhizosphere re-suspension to the PowerBead plate (assuming it was re-suspended with solution CD1).
- NOTE: The soil particles may be too big to enter a blue tip. For this, use a sterile pair of scissors to cut around 4 mm of the tip.

Run the TissueLyzer at 2 X 30Hz for 4 min. Rotate the plates after the first cycle. If possible, rest the plates until they cool down. The movement of the TissueLyzer can induce heating, and the plates (or the covers) may break. Do not use the silicone pads, they won't fit.

Follow the kit's instructions.

My recommendations:

- After adding the solution CD2 and centrifuging, transfer the supernatant with yellow tips. Those will give you more control. See further recommendations in my notebook.
- Use microtest plates to elute DNA. A box of collection tubes won't fit in any centrifuge.

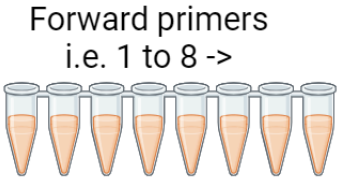
PCR

NOTE: All recipes include 10% extra volume to consider pipetting mistakes and errors.

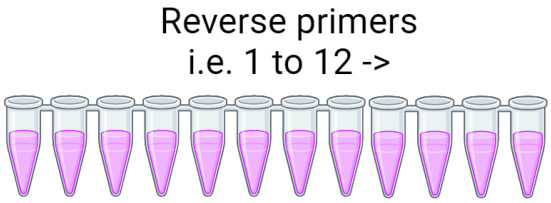
The volume of each reaction is 20 uL. Each sample will be done in triplicates.

Before you start:

- Measure DNA concentrations for each sample/plate well. Nanodrop is usually enough. Dilute DNA to **1-2 ng/uL.** More concentrated DNA might be needed for endosphere samples or fungi.
- Get and extract control DNA:
    - ITS1 positive control: Yeast or any other fungus (ask around the department). Dilute DNA.
    - 16S positive control: Any bacterial colony. Ask around the lab. Dilute DNA.
- Prepare a mPNA/pPNA mix with 1nM each. Heat it up to 60°C **before use** and let it cool down to ~25°C.
- If primer dilutions are not done, dilute each primer to 10 nM.
- Prepare primer strips (to dispense primers over the plate, aiming to add 0.5 uL of each primer in each PCR reaction):
    - Forward primers (**ROWS; each primer will do 12x3 reactions**):
        - Mix **19.8 uL** of each primer dilution (assuming they are at 10 nM) and **167.4** uL of sterile miliQ/PCR water.



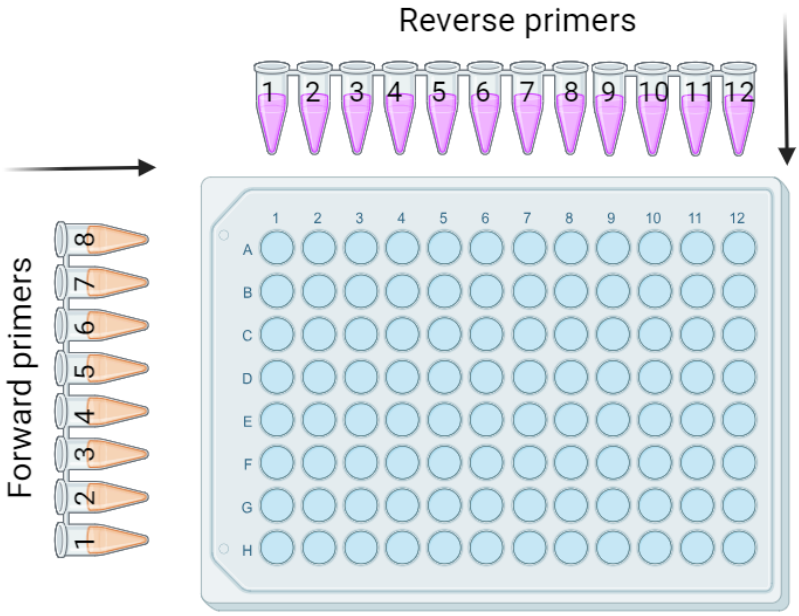
    - Reverse primers (**COLUMNS; each primer will do 8x3 reactions**):
        - Mix **13.2 uL** of each primer dilution (assuming they are at 10 nM) and **111.6 uL** of sterile miliQ/PCR water.



Minimum lab material expected to be used	For 96 samples (three replicates each): • Seven boxes of red tips. • One box of yellow tips. • Three 96-well PCR plates and covers. • One strip of PCR tubes.
Time required	One day
Equipment	96-well PCR machines (Book in the Protein Room or the Genomics Unit Floor 2)

Steps:

- Dispense **5 uL of DNA** into three 96-well PCR plates.
- NOTE : I use the wells H11 and H12 for the positive control and negative control (water instead of DNA), respectively.
- Dispense **4.72 uL** from the primer strips as follows:



- For example, well A1 will receive the Forward primer 8 and the Reverse primer 1. The well H12 will receive the Forward primer 1 and the Reverse Primer 12, and so on.

 CHECKPOINT - Until here, we have only added DNA. You can seal the plates and store them for later.

- Prepare Master Mix (one for each plate, one at a time):

	For 1 reaction	**For a plate (105 reactions including 10% extra)**
Q5 reaction buffer	4 µL	420uL
dNTP mix	0.5 µL	52.5uL
PNA mix *	1 µL	105uL
Q5 polymerase	0.04 uL	5.25uL

* Only for 16S. For ITS1, replace it with water.

- Distribute 72.8 uL of master mix into a PCR strip. This will allow a multichannel pipette to be used to distribute the mix across the plate.
- Distribute **5.55 uL of master mix** in each well.
- Run the PCR cycles.

PCR conditions:

Step	Target region			
	16p		ITS1	
	Temperature	Time	Temperature	Time
Pre-denaturation	95°C	2 minutes	94°C	1 minute
Denaturation	95°C	30 seconds	94°C	30 seconds
Annealing	55°C	30 seconds	55°C	30 seconds
Extension	72°C	4 minutes	68°C	30 seconds
Number of cycles	25	-	35	-
Final extension	72°C	10 minutes	68°C	10 minutes
rest	18°C	-	18°C	-

- After the cycles, run a gel to visualize the controls. If the controls look good in the three plates, mix the replicates from each sample. Run a gel to visualize each sample, repeat individual samples if necessary.

My recommendations:

- Prepare the PCR plates in a cooler with ice. Use a metal rack to keep the wells always cold. Use aluminum foil to cover the plate while pipetting; it is also helpful to guide you through the plate.
- The ITS1 conditions need to be optimized .

Pooling (this part needs to be improved)

- A simple option is to use a SequalPrep Normalization Plate Kit (Thermo Fisher) to clean and normalize the amplicons. Nevertheless, people report very low and variable dsDNA concentrations afterwards. In our lab it hasn't been tried.
- **What I did:** Measure the dsDNA concentration of 10 random samples over the plate using Qubit. Estimate the mean concentration of the plate and pool equal volumes of each sample. I used 10 uL. If a fraction of samples has low-quality amplicons (usually the fungal and endosphere samples), use equivalent volume to compensate for the concentration.
- Clean the pools using magnetic beads. Follow the instructions from the kit. A concentration of **0.8X beads** is good for removing overaimplification and primer dimers. 0.6X seems to do a better job; nevertheless, the amplicon concentration is reduced significantly as well (6 times lower).
- Based on an experiment, approximately 1/3 of the PCR product would get to the pool. On average, a standard amplicon should produce ~12 ng/uL of dsDNA, which would be reduced to ~4ng/uL after cleaning and pooling. Pooling after or before cleaning does not affect the final concentration.

Sequencing

Use the BioAnalyzer (Genomics Unit) to evaluate the quality of the pools. Save the results.

If sequencing is done in the Genomics Unit at the Biozentrum, notify Prof. Andreas Brachmann to please follow the protocol from the Scholss Lab.

DEMULTIPLEXING:

The first index will be the **reverse complement** of the Reverse Barcode, followed by the Forward Barcode. Example:

Forward Barcode	Reverse Barcode	First Index	Second Index	Tag in the read
GACACCGT	AACTCTCG	CGAGAGTT	GACACCGT	CGAGAGTT+GACACCGT

The table of the Primers is Attached to this protocol.

References

- Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79:5112–5120. [Design of the Primers]
- Walters, W., E. R. Hyde, D. Berg-Lyons, G. Ackermann, G. Humphrey, A. Parada, J. A. Gilbert, J. K. Jansson, J. G. Caporaso, J. A. Fuhrman, A. Apprill, and R. Knight. 2016. Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. mSystems 1. [ITS1 primers]

Angehängte Dateien

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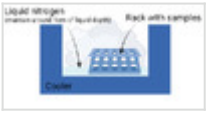

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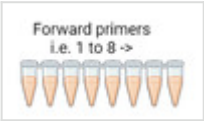

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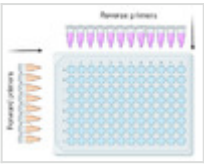

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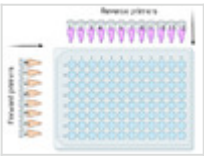

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