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Preparing multiplexed 16S rRNA gene amplicons (with fusion primers) for the Illumina MiSeq

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Protocol status: Working

We use this protocol and it's working, but do not hesitate to reach out if you have any questions, as they might help us improve clarity in the protocol.

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Abstract

The following protocol is for the generation of paired-end sequencing reads of 16S rRNA gene (V4 or V4V5) amplicons with dual barcodes (i.e.: "indexes") on the Illumina MiSeq machine using v3 600 cycle chemistry.

We use this protocol to make MiSeq libraries from DNA extracted from a variety of environmental samples, including seawater, freshwater, and swabs from hosts or surfaces of interest. This protocol is modified from [dx.doi.org/10.17504/protocols.io.4r3l277k3g1y/v1](https://doi.org/10.17504/protocols.io.4r3l277k3g1y/v1) and makes use of 'fusion' primers or PCR primers that include not only the primer sequence, but also the Illumina adapter and Nextera index. This allows samples to be indexed for amplicon sequencing using a single PCR rather than using a two-step PCR approach described [here](#).

Many thanks to André Comeau and the Integrated Microbiome Resource at Dalhousie University for so clearly describing methods and allowing for reproducibility. The resources provided here https://github.com/LangilleLab/microbiome_helper/wiki and in their publication <https://journals.asm.org/doi/10.1128/msystems.00127-16> were instrumental in developing our in-house protocols.

Note: This protocol leverages combinatorial dual indexes. For other Illumina instruments (e.g. NextSeq), unique dual indexes may improve data quality and reduce index hopping. For a bit more information about the difference between unique dual indexes and combinatorial dual indexes, check out [this resource](#).

If you have any questions, please don't hesitate to contact us!



Materials

Consumables:

5 mL eppendorf tubes

1.7 mL eppendorf tubes

96-well PCR plates

8-well strip tubes

Filter pipet tips

Molecular Biology Grade Water

96-well PCR-cooler block

Microtube racks

96-well plate racks

Cooler racks for 2.0 and 15 mL tubes

Molecular Biology Grade Agarose

RedSafe Nucleic Acid Staining Solution

SpeedBeads magnetic beads

(additional reagents listed below in *Protocol Materials*)

Equipment:

Below are suggestions based on equipment we have in the lab. You may have an equivalent piece of equipment that will do the same job!

PCR Workstation

Vortex

Mini-centrifuge

Pipets (p10, P20, P200, P1000 and single and multichannel pipets)

BioRad T100 Thermal Cycler

BioRad Gel Doc XR+ System

Gel Electrophoresis Systems, gel caster and gel combs

BMG CLARIOStar

Qubit Fluorometer

Illumina MiSeq

Magnetic plate for bead clean-up



Protocol materials

✕ Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

✕ NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs Catalog #E7630S**

✕ Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**

✕ QIAxcel DNA Fast Analysis Kit **Qiagen Catalog #929008**

✕ Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **GE Healthcare Catalog #44152105050350**

✕ MiSeq v3 Sequencing Reagents (600 cycles) **Illumina, Inc. Catalog #MS-102-3003**

✕ PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001**

✕ Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #Q32850**

✕ Quant-it™ PicoGreen® dsDNA Assay Kit **Life Technologies Catalog #P7589**

✕ 10X TBE Buffer **Bio-Rad Laboratories Catalog #1610770**

✕ Certified Molecular Biology Agarose **Bio-Rad Laboratories Catalog #1613101**

✕ RedSafe Nucleic Acid Staining Solution **Froggabio Catalog #21141**

✕ Taq FroggaMix **Froggabio Catalog #FBTAQM96**

✕ Corning® 100 mL Molecular Biology Grade Water Tested to USP Sterile Purified Water Specifications **Corning Catalog #46-000-CI**

✕ Recombinant Albumin, Molecular Biology Grade **New England Biolabs Catalog # B9200S**



Before You Get Started

1 Make DNA Template Plates

We aliquot sample DNA into 96-well plates to use for PCRs (called our **DNA template plates**). For our samples, we have found that a concentration of 2.5 ng/ul typically yields successful amplification from our seawater samples (depths ranging from 0 m to 250 m; coastal setting). Some people prefer doing a 1:10 dilution of sample DNA rather than targeting a specific input concentration. All this to say, that for 16S rRNA PCRs from coastal seawater, some form of sample dilution is often needed, depending on the DNA extraction protocol used, how efficient that extraction was, and how deep the sample was collected from.

In the process of creating these DNA template plates, we always leave well 12H empty (and/or add PCR-grade water to it) to ensure space for a PCR negative with each plate of samples to be amplified.

Barcoded PCRs

2 Day-of Preparation:

30m

Several hours before getting started, make sure you have a couple PCR cooler plates and cool racks for larger tubes in the freezer. These will be used to keep your reagents cool during the PCR setup.

We set up PCRs in a clean room, in a PCR workstation. Before getting started,

UV for  00:30:00 the following:

- 96-well PCR plates
- 8-well strip tubes
- 5 mL and 1.5 mL eppendorf tubes
- Sharpie
- Pipette tips
- Multichannel pipettes
- Pipettes
- Sterile Nuclease-Free Water
- Any necessary racks for holding tubes

Note

Do NOT UV your DNA template plates or your PCR reagents!



3 Let's get started:

On ice or in a cooler block, prepare the following PCR master mix for sample **PCR Plate 1** in a 5 mL Eppendorf tubes. **Primer plates** were prepared using the primer format described here [Preparing Indexed Primer Plates \(IDT Ultramers\) for the Illumina MiSeq - Nextera Dual Indices](#). **This protocol assumes that both your template DNA and fusion PCR primers have already been aliquoted into 96-well plates.** This allows for use of multichannel pipettes and for working in a more high-throughput format.

Note

Samples are amplified in duplicate, so if you feel so compelled, prepare enough master mix for 2 PCR plates (x210 rxns column below).

Reagent	25 ul rxn	x105 rxns	x210 rxns
PCR-grade Water	5.5 μ L	578 μ L	1155 μ L
2x Taq FroggaMix	12.5 μ L	1313 μ L	2625 μ L
BSA (10 mg/ml)	1 μ L	105 μ L	210 μ L
F primer (2.5 μ M)	2 μ L	added after	added after
R primer (2.5 μ M)	2 μ L	added after	added after
Template (sample DNA)	2 μ L	added after	added after

Reagent Details:

 Taq FroggaMix **Froggabio Catalog #FBTAQM96**

 Recombinant Albumin, Molecular Biology Grade **New England Biolabs Catalog # B9200S**

Note

We have recently switched away from BSA to Recombinant Albumin, because traditional molecular biology grade BSA was resulting in amplification in PCRs for other genes (namely 12S-MiFish). We dilute to 10 mg/mL before use.



Corning® 100 mL Molecular Biology Grade Water Tested to USP Sterile Purified Water Specifications **Corning Catalog #46-000-CI**

Primer Details:

Note

The actual oligo/ fusion primer sequences used are much longer and described in **Preparing Indexed Primer Plates (IDT Ultramers) for the Illumina MiSeq - Nextera Dual Indices** but the specific primers, that are embedded in the fusion primer are listed below, just for reference. Please do not use these shorter primers in this protocol! We do use the shorter 'normal' primers to test how well the samples will amplify or troubleshoot samples that are tricky to amplify before using the more expensive fusion primers to prepare the library.

Also, don't hesitate to reach out if you have any questions!

	Primer Name	Sequence (5'-3')	Target Gene Region	Publication
	515F-Y	GTGYCAGCMGCCGCGG TAA	16S-V4; 16S-V4V5	Parada et al. 2016; https://doi.org/10.1111/1462-2920.13023
	806R	GGACTACNVGGGTWTCT AAT	16S-V4	Apprill et al. 2015, https://doi.org/10.3354/ame01753
	926R	CCGYCAATTYMTTTRAG TTT	16S-V4V5	Parada et al. 2016; https://doi.org/10.1111/1462-2920.13023

- 4 Pipet 125 µL of the master mix into each of the 8 wells of 4 sets of strip tubes. From these strip tubes, you will transfer the master mix into the **PCR plate(s)** using a multichannel pipette (MCP).
- 5 Pipet 19 µL of master mix from the master mix strip tubes into each well of the PCR plate (which is held in a PCR cooler plate to keep all reagents cold throughout this process), one column at a time with the MCP.

Note

You can use the same set of 8 tips to aliquot your master mix into all columns of your PCR plate.

- 6 Remove the protective film from **column 1** of the **Forward Set 1 Primer Plate (F1)**, align it horizontally on the bench to the left of **PCR Plate 1** and pipet  **2 µL** into from each well of **column 1** of **Primer Plate F1** into **column 1** of your **PCR Plate** using the MCP. Now, remove more of the film (or caps, if not using a sealing film) from the next column (**column 2**) of **Primer Plate F1** and pipet  **2 µL** into **column 2** of your **PCR plate**. Do this one **column** at a time using the MCP until you have aliquoted contents from each column of your primer plate into the corresponding column of your PCR plate.

Note

In the forward primer plates, each row contains a different index.

Note

Remember to **change your pipet tips** after each column to prevent cross contamination.

- 7 Uncover one **column** of the **Reverse Set 1 Primer Plate (R1)**, align it horizontally on the bench to the left of your **PCR Plate 1** and and dispense  **2 µL** into each well, one **column** at a time, using the MCP.

Note

In the reverse primer plates, each column contains the same index.

Note

Remember to **change your pipet tips** after each column to prevent cross contamination.



- 8 Uncover the **DNA Template Plate 1**, align it along the top (or beside, depending on your preference!) of **PCR Plate 1** (which now contains master mix, forward primers and reverse primers) and dispense  **2 µL** of template DNA into each corresponding well, **one column at a time** using the MCP. Seal the **PCR Plate 1** with PCR film and either keep on ice until both PCR plates are ready for the thermal cyclers or place in the thermal cycler right away, as per below.

Note

Remember to **change tips** after every column.

- 9 **Repeat steps 4 - 8** for your replicate PCR plate, if preparing it at the same time (recommended since everything is thawed!).
- 10 Once PCR setup is complete, seal the plates with PCR film, place in thermal cyclers (we have BioRad T100 Thermal Cyclers) and run the following program for a total of **30 total cycles**:

	A	B	C
	Initial denaturation	94°C	3 min
	Denaturation	94°C	45 s
	Annealing	50°C	60 s
	Extension	72°C	90 s
	GO TO Step 2		29 times
	Final Extension	72°C	10 min
	Hold	4°C	forever

- 11 Once the two PCRs for **Plate 1** are complete, repeat **steps 2-10** to prepare **PCR Plates** for **DNA Template Plate 2** using **Forward Set 1 Primer Plate (F1)** and **Reverse Set 2 Primer Plate (R2)**.
- 12 Once the two PCRs for **DNA Plate 1 and 2** are complete, repeat **steps 2-10** to prepare **PCR Plates** for **DNA Template Plate 3** using **Forward Set 1 Primer Plate (F2)** and **Reverse Set 2 Primer Plate (R1)**.



- 13 Once the two PCRs for **DNA Plate 1, 2 and 3** are complete, repeat **steps 2-10** to prepare **PCR Plates** for **DNA Template Plate 4** using **Forward Set 1 Primer Plate (F2)** and **Reverse Set 2 Primer Plate (R2)**.

Note

We typically only multiplex 3 plates of samples for a MiSeq Run, but with the available index combinations (F1R1, F1R2, F2R1, F2R2), it is possible to multiplex 4.

Gel Verification

14

Note

We are transitioning away from using traditional gel verification of PCR success to using our **Qiaxcel System** for this step but given that not all labs would have access to this system or something similar (e.g. Agilent Tapestation), we describe the more 'traditional' gel verification method here.

We typically run completed PCRs on a 2% agarose gel to verify their success (or mourn their failure). To optimize time, make your gel while the PCRs are running. We have a BioRad Sub-Cell Model 192 Cell Electrophoresis system (among others). Sadly this is now discontinued but it allows you to run 2 PCR plates of samples simultaneously with the 25 × 10 cm and 25 × 15 cm trays.

Weigh out  3.6 g of

 Certified Molecular Biology Agarose **Bio-Rad Laboratories Catalog #1613101** into a large Erlenmeyer flask (e.g. 500 mL). Add  180 mL of 1X TBE buffer (we make a 1X stock from  10X TBE Buffer **Bio-Rad Laboratories Catalog #1610770**) and swirl to mix.

- 15 Microwave for  00:02:00 or until boiling. With a protective glove on, swirl flask.

2m

Note

Be careful when you swirl the flask as some steam will likely come out of the flask.



- 16 Microwave for another  00:01:00 and allow the solution to boil until it is clear, and the agarose is completely dissolved. 1m
- 17 Using a protective glove, remove the flask with melted agarose solution from the microwave and allow to cool slightly.
- 18 Assemble gel tray in gel caster.
- 19 When flask has cooled enough that you can pick it up without it being too hot, add  9 μL of  RedSafe Nucleic Acid Staining Solution **Froggabio Catalog #21141** to the flask and gently swirl to mix. Now you can gently pour the liquid agarose solution into the gel tray. Add 4 $\times$ 51-well combs to the gel tray.
- 20 Allow gel to cool and set completely, for at least  00:30:00 . 30m
- 21 Once the PCRs are complete and the gel is completely set, remove the gel from the gel caster and place it in the gel electrophoresis box. Add 1x TBE until it completely covers the gel with a couple centimeters of liquid above the gel. Remove the combs.
- 22 Remove the PCR plates from the thermocycler and briefly spin the plate to remove any liquid from the foil. Gently pull back the seal, and load  2 μL (or up to 5 μL) of each sample into gel wells using a MCP.

Note

Our PCR master mix from FroggaBio already has loading dye in it, so we can load the PCR product directly to the wells, without first mixing an aliquot with any sort of loading dye.

Also, with our set-up and the 51-well combs, the MCP loads samples into every other well. So you load column 1 of your PCR plate into the gel with the samples being loaded into every other well. Then you load column 2, where column 2A will be loaded right next to column 1A in the gel.

- 23 Run your gel at a voltage and for a duration of time appropriate for the size of gel you are running. For example, for a large 2% gel we might run at 100 V for  00:30:00 30m



- 24 After checking that your samples amplified successfully on your Gel Visualization system (e.g. [BioRad XR Gel Documentation System](#)) pool the replicate PCR plates (make sure the PCR plates are oriented the same way before doing this!) using a MCP. Add the contents of column 1 from one PCR replicate plate to the contents of column 1 in the other replicate plate. Continue this process until every column and sample has been pooled. This is now your **aggregate PCR plate**.
- 25 Repeat **steps 14-24** for **PCR Plates 2**.
- 26 Repeat **steps 14-24** for **PCR Plates 3**.
- 27 If you are intending to multiplex 4 plates of samples, repeat **steps 14-24** for **PCR Plates 4**.

PCR Clean-up

- 28 Clean and concentrate the remaining ~  45 μL of **Aggregated PCR Plate 1**.

Preparations

Note

Prepare the purification in the post-PCR working space. Size selection can be achieved using different ratios of magnetic beads to sample. A ratio of bead to a sample of 0.8-1.5 will efficiently purify the amplicons away from primer dimers and allow the selection of fragments larger than 200 bp.

Materials

- Sera-Mag SPRI beads. We are currently using



Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **GE Healthcare Catalog #44152105050350**

If not already prepared follow this protocol. [Serapure Beads Preparation and Testing](#)

- Magnetic 96-well plate stand (for example: <https://www.thermofisher.com/order/catalog/product/12331D>)
- Anhydrous Ethanol to make a fresh 80% ethanol solution
- Molecular grade water

**UV for 30 minutes the following:**

- 96-well PCR plates (or 8-strip tubes)
- Sharpie
- Pipette tips
- Multichannel pipettes
- Pipettes
- Sterile Nuclease-Free Water

Remove the magnetic beads from the fridge (allow 30 min to reach room temperature).

- 29 Vortex the beads before use.
- Add 36 μ l beads to 45 μ l of PCR product to obtain a ratio of 0.8.
 - Pipette up and down ten times (or until the solution is well mixed – you will see that the color changes).
 - Spin tubes down to remove drops from the walls.
- 30 Incubate at room temperature without shaking for  00:05:00 . 8m
- Then, place the plate on the magnetic stand until the supernatant has cleared (~  00:03:00).
- 31 Remove the supernatant with a MCP, ensuring to not disturb the beads.
- 32 With the samples on the magnetic rack, wash the beads by adding 180 μ l of freshly prepared 80% ethanol and incubate for 30s. Carefully remove the supernatant without disturbing the beads.
- 33 Repeat the washing step.  [go to step #32](#)
- 34 Remove all residual ethanol using a pipette and air dry, leaving the samples on the magnetic stand (~  00:05:00). 5m

Note

The drying time depends on the type of the magnetic rack – the O-ring magnet dries faster than the side magnet. Keep an eye on the beads and do not over-dry. Otherwise, you will not get an efficient DNA recovery.

- 35 Remove the plate from the magnetic stand and add 40 µl of nuclease-free water for elution. Gently pipet up and down ten times to resuspend the beads. Incubate the plate at room temperature for 5 min.
- 36 Place the plate back on the magnetic rack for at least 5 min or until the supernatant is cleared.
- 37 Carefully transfer 30 µl of the clear supernatant to a new plate. Seal the plate.
- 38 Name the plate. For example: Project, Gene_name + Primer Plate Combo Used, Cleaned PCRs, Date, Initials.

Samples can be stored in the fridge for the short term (e.g. overnight before quantification), but should be placed in the freezer (-20 °C) for long-term storage.
- 39 Repeat **steps 29-38** for **Aggregated PCR Plate 2**.
- 40 Repeat **steps 29-38** for **Aggregated PCR Plate 3**.
- 41 Repeat **steps 29-38** for **Aggregated PCR Plate 4**.

Quantification

- 42 Use a fluorometric quantification method that uses dsDNA dyes to measure the concentration of your libraries. If doing a few samples, we use a Qubit. If quantifying an entire plate of samples (or more) we often use a microplate reader, like a BMG LabTech CLARIOstar. If using Qubit, give preference to the broad range kit if you visualize a strong band in the gel. For either method, follow manufacturers instructions for how to use the kit and the instrument (Qubit or plate reader).

 Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #Q32850**

 Quant-it™ PicoGreen® dsDNA Assay Kit **Life Technologies Catalog #P7589**

- 43 Calculate sample volume to have a final amount of 10-40 ng per sample. This amount may vary depending on the overall quantification. For example, if, on average, the concentration of your samples is about 3 ng/µl and you have 20 µl of product, you



can add up to 60 ng of cleaned per sample (although it is ideal to aim for less than the max possible).

You will want to pool approximately the same amount of cleaned, amplified DNA for each sample to have a more even read count among samples in the library.

- 44 After determining amount of DNA to pool for each sample, it is time to pool the DNA for each sample! This takes a bit of time, so get comfortable.

1h

Note

Check the final volume that you will get after pooling by adding all of the samples volumes you will pool – sometimes you will end up with 2 mL or more. Then use the proper Eppendorf tube for pooling (1.5, 2.0, or 5 mL).

- 45 Following manufacturers instructions, measure the final library pool concentration on Qubit using

 Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

- 46 Label tube. For example: Project_Name-Gene_name, Pooled Amplicons. Date, Initials, pool concentration.

Illumina Sequencing Parameters

- 47 Library fragment size (BP) is determined using

 Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**

OR

 QIAxcel DNA Fast Analysis Kit **Qiagen Catalog #929008**

- 48 Molarity of the final amplicon library pool is assessed using

 NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs Catalog #E7630S**



- 49 The library is then sequenced, according the manufacturers instructions, on an Illumina MiSeq using



MiSeq v3 Sequencing Reagents (600 cycles) **Illumina, Inc. Catalog #MS-102-3003**

with pair-end setup (2*300 bp), spiked with 10-15%



PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001**

Protocol references

Comeau AM, Douglas GM, Langille MGI. 2017. Microbiome Helper: a Custom and Streamlined Workflow for Microbiome Research. mSystems 2:10.1128/msystems.00127-16.

<https://doi.org/10.1128/msystems.00127-16>

https://github.com/LangilleLab/microbiome_helper/wiki