FEB 24, 2020

OPEN ACCESS

DOI:
[dx.doi.org/10.17504/protocols.io.468gzhw](https://doi.org/10.17504/protocols.io.468gzhw)

External link:
<https://doi.org/10.1038/s41598-020-59182-1>

Protocol Citation: Máté Vass, Anna Székely 2020. Sample Preparation for Illumina MiSeq Dual Index Amplicon Sequencing. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.468gzhw>

MANUSCRIPT CITATION:
 Vass M, Székely AJ, Lindström ES, Langenheder S, Using null models to compare bacterial and microeukaryotic metacommunity assembly under shifting environmental conditions. Scientific Reports doi: [10.1038/s41598-020-59182-1](https://doi.org/10.1038/s41598-020-59182-1)

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Protocol status: Working

Sample Preparation for Illumina MiSeq Dual Index Amplicon Sequencing

Forked from a private protocol

Scientific Reports

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ABSTRACT

Amplicon sequencing sample preparation for bacteria and microeukaryotes

MATERIALS

MATERIALS

Q5 High-Fidelity DNA Polymerase - 100 units **New England Biolabs Catalog #M0491S**

STEP MATERIALS

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

BEFORE START INSTRUCTIONS

Put pipettes and tips in the UV chamber for 10 mins.

Clean bench with MQ and EtOH.

Created: Jul 05, 2019

Last Modified: Feb 24, 2020

PROTOCOL integer ID:
25536

- 1 Perform the first PCR (duplicates of each sample) using Illumina adaptor attached primers that target the gene of your choice. Here we present the protocol using the bacterial primers 341F and 805RN, and eukaryotic primers 574*f and 1132r.

Bacterial primers ([Herlemann et al., 2011](#)):

Illumina adapter-N4-341F:

5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCCTACGGGNGGCWGCAG-3'

Illumina adapter-805R:

5'-AGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC-3'

Eukaryotic primers ([Hugerth et al., 2014](#)):

Illumina adapter-4N-574*f:

5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCGGTAAYTCCAGCTCYV-3'

Illumina adapter-1132r:

5'-AGACGTGTGCTCTTCCGATCTCCGTCAATTHCTTYAART-3'

First PCR reactions

	Com pone nts	Work ing conc.	Final conc.	1 react ion (20 µl)	(N) reactions	
	5xQ5 Reacti on Buffer	5X	1X	4 µl		
	Forwa rd Prime r	10 µM	0.25 µM	0.5 µl		
	Rever se Prime r	10 µM	0.25 µM	0.5 µl		
	dNTP s	2 mM	200 µM	2 µl		

Q5 HF DNA polymerase	2 U/ μ l	0.02 U/ μ l	0.2 μ l		
Template DNA			1 μ l		
Nuclease-Free water			11.8 μ l		
Σ			20 μ l		

First PCR program for bacteria

STEP	TEMP.	TIME
Initial Denaturation	98°C	30 seconds
20 cycles	98°C	10 seconds
Annealing temp.	48°C*	30 seconds
	72°C	30 seconds/kb
Final elongation	72°C	2 minutes
Hold	6°C	∞

First PCR program for eukaryote

STEP	TEMP.	TIME
Initial Denaturation	98°C	1 minute
20 cycles	98°C	10 seconds
Annealing temp.	51°C	30 seconds

		72°C	1 minute/kb
	Final elongation	72°C	2 minutes
	Hold	6°C	∞

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

2 Check PCR products with Agarose gel electrophoresis (1%) - optional

3 Pool PCR duplicate samples together
Optional: run Agarose gel electrophoresis (1%)

4 Second PCR is conducted for attaching standard illumina handles and index primers

Multiplex_fwd

AATGATACGGCGACCACCGAGA{TCTACAC}-[i5 index] ACACTCTTTCCCTACACGACG

Multiplex_rev

CAAGCAGAAGACGGCATACGAGAT-[i7 index]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

(In total 20 different forward index/barcode primers and 20 different reverse index/barcode primers. By combining both primers (20X20), it can possible to generate 400 tags in one final pool for sequencing)

Second PCR reactions

Com pone nts	Work ing conc.	Final conc.	1 react ion (20 µl)	(N) react ions	
5xQ5 Reacti on Buffer	5X	1X	4 µl		
Forwa rd index (i5, illu- N501- N508)	5 µM	0.25 µM	1 µl		

Reverse index (i7, illu-N701-N712)	5 μ M	0.25 μ M	1 μ l		
dNTPs	2 mM	200 μ M	2 μ l		
Q5 HF DNA polymerase	2 U/ μ l	0.02 U/ μ l	0.2 μ l		
Template from 1 st PCR			2 μ l		
Nuclease-Free water			9.8 μ l		
Σ			20 μ l		

Second PCR program for both bacteria and eukaryote

STEP	TEMP.	TIME
Initial Denaturation	98°C	30 seconds
15 cycles	98°C	10 seconds
	66°C	30 seconds
	72°C	30 seconds/kb
Final Elongation	72°C	2 minutes
Hold	6°C	∞

5 Check second PCR products with Agarose gel electrophoresis (1%)

00:01:00

- 6 Perform purification with magnetic beads (Agencourt AMPure) and run Agarose gel electrophoresis (1%)
- 7 Quantification: PicoGreen assay (Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen)
- 8 Calculate PCR samples concentration and volume before pooling
- 9 Pool the PCR samples in equal DNA amount (ng) or for unequal length amplicons, in equal molecule amount (mol).
You will get one-one tube with a mix of all the samples (bacteria and eukaryote separately) in it.

To calculate the volume of each sample to be pooled (DNA amount mixing):
 - Use the lowest concentration sample to define the minimum amount of DNA (ng) that you have available from a single sample: the DNA concentration (ng/μL) of the lowest concentration sample multiplied with its volume (μL). This will be your **target DNA amount** for each sample.
 - Calculate how many μLs of each sample you need to achieve the **target DNA amount**: divide the **target DNA amount** with the concentration of each sample.
 - Pipette into one tube the calculated volume of each sample. Aim to use the same pipette for all samples (dilute or pipette multiple times) to avoid pipette calibration errors.
- 10 Requantify with PicoGreen before submitting to sequencing facility.