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Sample Preparation for Illumina MiSeq Dual Index Amplicon Sequencing

Forked from illumina MiSeq Dual Index Amplicon Sequencing Sample Preparation

Scientific Reports

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1 Works for me dx.doi.org/10.17504/protocols.io.468gzhw

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ABSTRACT

Amplicon sequencing sample preparation for bacteria and microeukaryotes

EXTERNAL LINK

https://doi.org/10.1038/s41598-020-59182-1

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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

MATERIALS

NAME	CATALOG #	VENDOR
Q5 High-Fidelity DNA Polymerase - 100 units	M0491S	New England Biolabs

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Q5 High-Fidelity DNA Polymerase - 500 units	M0491L	New England Biolabs

BEFORE STARTING

Put pipettes and tips in the UV chamber for 10 mins.
Clean bench with MQ and EtOH.

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'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN**CGGTAAYTCCAGCTCYV-3'**
Illumina adapter-1132r:
5'-AGACGTGTGCTCTTCCGATCT**CCGTCAATTHCTTYAART-3'**

First PCR reactions

Components	Working conc.	Final conc.	1 reaction (20 µl)	(N) reactions	
5xQ5 Reaction Buffer	5X	1X	4 µl		
Forward Primer	10 µM	0.25 µM	0.5 µl		
Reverse Primer	10 µM	0.25 µM	0.5 µl		
dNTPs	2 mM	200 µM	2 µl		
Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0.2 µl		
Template DNA			1 µl		
Nuclease-Free water			11.8 µl		
∑			20 µl		

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
by 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

Components	Working conc.	Final conc.	1 reaction (20 µl)	(N) reactions	
5xQ5 Reaction Buffer	5X	1X	4 µl		
Forward index (i5, illu-N501-N508)	5 µM	0.25 µM	1 µl		
Reverse index (i7, illu-N701-N712)	5 µM	0.25 µM	1 µl		
dNTPs	2 mM	200 µM	2 µl		
Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0.2 µl		
Template from 1st PCR			2 µl		
Nuclease-Free water			9.8 µl		
∑			20 µl		

Second PCR program for both bacteria and eukaryote

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

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.