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illumina MiSeq Dual Index Amplicon Sequencing Sample Preparation Bacterial 16S rRNA gene

Forked from a private protocol

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

Use at your own risk

Abstract

Preparation of PCR products for amplicon sequencing

Guidelines

We have a common stock of barcoded primers stored in the -80C freezer number 3. The primers are in an orange box, which box can be found in the position indicated on the following picture:

In the box from 1 to 20 are the forward primers and from 37 to 56 are the reverse primers. The last line has extras from some primers. If you run out of a barcode, check those first before ordering. Check primer ordering file on common server for primer ordering.

Sequence of barcodes to fill in the SampleSheet file for sequencing**:**

	Index 2 (i5)	Index 2 (i5) Sequence	Index 1 (i7)	Index 1 (i7) Sequence
	Illu_N 501F	TAGA TCGC	Illu_N 701R	TCGC CTTA
	Illu_N 502F	CTCT CTAT	Illu_N 702R	CTAG TACG
	Illu_N 503F	TATC CTCT	Illu_N 703R	TTCT GCCT
	Illu_N 504F	AGAG TAGA	Illu_N 704R	GCTC AGGA
	Illu_N 505F	GTAA GGAG	Illu_N 705R	AGGA GTCC
	Illu_N 506F	ACTG CATA	Illu_N 706R	CATG CCTA
	Illu_N 507F	AAGG AGTA	Illu_N 707R	GTAG AGAG
	Illu_N 508F	CTAA GCCT	Illu_N 708R	CCTC TCTG
	Illu_N 521F	CTTG CTTT	Illu_N 709R	AGCG TAGC
	Illu_N 522F	GGCT TCAA	Illu_N 710R	CAGC CTCG
	Illu_N 523F	AATC GGCA	Illu_N 711R	TGCC TCTT
	Illu_N 524F	GGTT CAAA	Illu_N 712R	TCCT CTAC
	Illu_N 525F	ACTT CGAC	Illu_N 733R	GGTA TAAG
	Illu_N 526F	TGAC TTGC	Illu_N 735R	CAGC TAGA
	Illu_N 527F	TAGG ACCT	Illu_N 736R	CCAT AGCA
	Illu_N 528F	GGAG ACTT	Illu_N 738R	GGTA TAGC
	Illu_N 529F	AGGT TACG	Illu_N 739R	GGTT ATGC
	Illu_N 530F	AATT CGCT	Illu_N 740R	TAGG CAAG

	Illu_N 531F	TCAG CTAA	Illu_N 741R	TTGT CCAT
	Illu_N 532F	GCGA TATG	Illu_N 743R	TCTA GGCA

Note: the sequence of the reverse barcodes to fill in the SampleSheet is the reverse complement of their sequence of in the primers.

The 1st part of the protocol is performed in the pre-PCR room.

The 2nd part in the post-PCR room.

Never bring back PCR products to the pre-PCR room.

Materials

MATERIALS

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

Protocol materials

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

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #**M0491L Step 1

Before start

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

1 Perform the first PCR (triplicates/duplicates of each sample) using Illumina adaptor attached primers that target the gene of your choice. Here we present the protocol using the Bacteria primers 341F and 805RN. For the forward primer cite: **Herlemann et al., 2011** and for the reverse primer cite based on: **Apprill et al., 2015** but the one here is not exactly as the one in that paper.

Illumina adapter-N4-341F:
5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN**CCTACGGGNGGCWGCAG**-3'
Illumina adapter-805NR:
5'-AGACGTGTGCTCTTCCGATCT**GACTACNVGGGTATCTAATCC**-3'

First PCR reactions

	Components	Working conc.	Final conc.	1 reaction (20 µl)	(N) reactions	
	5xQ5 Reaction Buffer	5X	1X	4 µl		
	Forward Primer (illu-ada-341F)	10 µM	0.25 µM	0,5 µl		
	Reverse Primer (illu-ada-805NR)	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

	STEP	TEMP.	TIME
	Initial Denaturation	98°C	**3 minutes***
	20 cycles	98°C	10 seconds
	48°C**	30 seconds	
	72°C	30 seconds/kb	
	Final Extension	72°C	2 minutes
	Hold	6°C	∞

* In the latest protocols this was reduced to 30 seconds but in order to have complete denaturation of long high GC genome fragments, we increased it to 3 minutes, which is in accordance with the manufacturers recommendation.
** First temperature ever used was 55C. Then it changed to 62C. We decided on 48 based on suggestion by Anna Szekely. 48C gets unbiased product amounts of non-mismatch and 3-mismatch isolates. If you use this profile cite: **addressing PCR biases** or **effect of annealing temperature**

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

3 Pool PCR duplicate samples together and perform purification with magnetic beads (Agencourt AMPure or similar)- Optional: run Agarose gel electrophoresis (1%)

4 1. A 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

(We have 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. To find the common stock of the barcoded second step primers check the guidelines of this protocol)

Table 2: 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

	STEP	TEMP.	TIME
	Initial Denat	98°C	30 secon

	uration		ds
	15 cycles	98°C	10 seconds
	66°C	30 seconds	
	72°C	30 seconds/kb	
	Final Extension	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 (optional) run Agarose gel electrophoresis (1%)

7 Quantification: using gel analyzer program or PicoGreen assay

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 tube with a mix of all the samples 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 Gel purify the pool and requantify with PicoGreen before submitting to sequencing facility.