Bacterial 16S rRNA gene PCR for illumina MiSeq dual index amplicon sequencing

1. Perform the first PCR in duplicate (or triplicate) reactions using the following bacterial primer constructs:

   Illumina adapter-NNNN-341F:
   5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN**CCTACGGGNGGCWGCAG** -3'
   Illumina adapter-805R:
   5'- AGACGTGTGCTCTTCCGATCT**GACTACHVGGGTATCTAATCC** -3'

Table 1: First PCR reactions

Components	Working conc.	Final conc.	1 reaction (20 µl)
Q5 reaction buffer	5×	1×	4 µl
Forward primer (illu-ada-341F)	10 µM	0.5 µM	1 µl
Reverse primer (illu-ada-805R)	10 µM	0.5 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 DNA			1 µl
Nuclease-free water			10.8 µl
∑			20 µl

First PCR program

STEP	TEMP.	TIME
Initial Denaturation	98 °C	1 minute
20 cycles	98 °C	10 seconds
	54 °C	30 seconds
	72 °C	30 seconds
Final Extension	72 °C	2 minutes
Hold	6 °C	∞

2. Check PCR products with agarose gel electrophoresis (1%)
3. Pool duplicates and perform purification with magnetic beads (Agencourt AMPure). Optional: run agarose gel electrophoresis (1%)
4. 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), we can generate 400 samples with individual barcodes that can be pooled and added to a single sequencing run. Later these can be demultiplexed and analyzed using standard bioinformatics pipeline.

Table 2: Second PCR reactions

Components	Working conc.	Final conc.	1 reaction (20 µl)	(N) reactions
Q5 Reaction Buffer	5×	1×	4 µl	
Forward index (i5, illu-N501-N532)	5 µM	0.25 µM	1 µl	
Reverse index (i7, illu-N701-N743)	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 Denaturation	98°C	30 seconds
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%)
6. Perform purification with magnetic beads (Agencourt AMPure). Optional: run agarose gel electrophoresis (1%)
7. Quantification using PicoGreen assay (Quant-iT PicoGreen, Invitrogen).
8. Calculate sample concentration and the required volume for equal amounts in ng before pooling
9. Pool PCR samples together (e.g. 50, 96, or any other sample numbers in one pool)
10. Sequenced using using Illumina MiSeq with 2x300 bp