Amplification and Illumina Sequencing of 27F and 519R region of the 16S rRNA gene

1.0 Introduction

The protocol detailed here is for designed to amplify the 27F and 519 region of the 16S rRNA gene Primers for paired-end 16s community sequencing on the Illumina MiSeq platform using.

2.0 Amplification of 27F and 519R region of the 16S rRNA gene

2.1 Primers for amplification of 27F and 519R region of the 16S rRNA gene

ILM_27F_Uv3 (forward)

AATGATACGGCGACCACCGAGATCTACAC TATGGCGAGT GA AGAGTTTGATCMTGGCTCAG

ILM_519R_NNNN (reverse)*

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG GG GWATTACCGCGGCKGCTG

* This primer includes a 12 base Golay barcode as described by Caporaso et al.

2.2 Preparation of master mix for amplification of 27F and 519R region of the 16S rRNA gene

Component	Volume 1 rxn	Final Conc.
10x ImmoBuffer [(a)]	2.5	1 x
10 mM dNTP	0.5	200 nM
50mM MgCl2	1.25	2.5 mM
ILM_27F_Uv3 (forward) (**5 µM)**	2.5	500 nM
ILM_519R_XXXX (**5µM**)	2.5	500 nM
Immolase DNA Polymerase (5U/µL) [(a)]	0.2	1 Unit
H_2O	14.55	-
Template	1	-
Total Volume	25	-

[a] IMMOLASE™ DNA Polymerase (Bioline, #BIO-21047)

2.3 Thermocycler Conditions for amplification of 27F and 519R region of the 16S rRNA gene (96 well thermocyclers)

	Temperature	Time (mm:ss)
Activation	95°C	10:00
Amplification (35 cycles)	94°C	00:30
	55°C	00:10
	72°C	00:45
Final Extension	72°C	10:00

2.3 Process

2.3.1 Dilute DNA 1:10.

2.3.2 Amplify samples with conditions outlined above.

2.3.3 Run amplicons on an agarose gel. Expected band size for 27F/519R is approx. 530 bp.

2.3.4 If there is no band present, repeat PCR using either the undiluted DNA or a 1:100 dilution. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used neat.

2.3.5 Clean and normalize the PCR products. For this step, both Agencourt AMPure XP bead-clean-up and SequalPrep Normalization plates (Invitrogen, A1051001) are acceptable.

If using Agencourt AMPure XP beads:

a. Perform a bead clean-up following manufacturer's instructions.
b. Quantify amplicon yields. Acceptable methods include Picogreen (see manufacturers protocol; Invitrogen Item #P11496) or the high sensitivity Quant-iT™ DNA Assay Kit (Life Technologies, Q-33120).
c. Run clean amplicons on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
d. Normalise amplicons by diluting to 10nM with variable volume of buffer (EB buffer, or 10mM Tris pH 8.5) and a set volume of the amplicons.
e. Pool equal volumes of each normalized amplicon.

If using SequalPrep Normalization plates:

f. Perform a plate normalization following manufacturer's instructions.
g. Pool equal volumes of each normalized amplicon.
h. Run clean amplicon pool on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.

2.3.6 Optional: If spurious bands were present on gel (in step 2.3.3), or in the clean amplicons (step 2.3.5c) or amplicon pool (step 2.3.5h), a portion of the final pool can be run on a gel and then gel extracted to select only the target bands or put through another round of bead cleanup. A lower bead to template ratio will get rid of small bands.

2.3.7 Measure concentration using Qubit or picogreen of final pool that has been cleaned and check the 260/280 ratio. For best results the 260/280 should be between 1.8-2.0.

3.0 Sequencing of 27F and 519R region of the 16S rRNA gene

3.1 Sequencing Primers

Read 1 Primer

ACACTATGGCGAGTGAAGAGTTTGATCMTGGCTCAG

Read 2 Primer

AGTCAGTCAGGGGWATTACCGCGGCKGCTG

Index Primer

CAGCMGCCGCGGTAATWCCCCTGACTGACT

3.2 Sequencing Setup

3.2.1 Dilute pool prepared in **step 2.3.7** to **4nM**.

3.2.2 Denature according to Ilumina protocol. See *Preparing Libraries for Sequencing on the MiSeq (part #15039740)*.

3.2.3 Prepare MiSeq Reagent Cartridge (v3 600-cycles). See *MiSeq Reagent Kit v3 - Reagent Preparation Guide (part # 15044983).*

3.2.4 Using an extra long pipette tip set to 75 µL, add 3.4 µL of Read1 sequencing primer (100 µM) into well 12 of the MiSeq Reagent Cartridge and mix 10 times. Repeat adding the Index Primer into well 13 and the Read2 sequencing primer into well 14.

3.2.5 Load 600 µl of library pool into the MiSeq reagent cartridge in designated reservoir

3.2.6 Modify sample sheet to include the custom index sequences.

3.2.7 Start sequencing run following *MiSeq System User Guide (part # 15027617).*

References

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J

LANE, DJ. 1991. 16S/23S rRNA sequencing, p 115–175. In Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. Wiley, New York, NY.

Lane DJ, et al. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. U. S. A. 82:6955–6959.