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

Introduction

The protocol detailed here is 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.

Primers for amplification of 27F (Lane 1991) and 519R (Lane et al. 1993) region of the 16S rRNA gene

ILM_*27F*_Uv3 –forward primer

Field number (space-delimited), description:
1. 5' Illumina adapter
2. Forward primer pad
3. Forward primer linker
4. Forward primer (1391f)

AATGATACGGCGACCACCGAGATCTACAC TATGGCGAGT GA ***AGAGTTTGATCMTGGCTCAG***

ILM_*519R*_NNNN – reverse primer

1. Reverse complement of 3' Illumina adapter
2. Golay barcode*
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (EukBr)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX *AGTCAGTCAG GG* ***GWATTACCGCGGCKGCTG***

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

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)

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

Process

1. Dilute DNA 1:10.
2. Amplify samples with conditions outlined above
3. Clean amplicon using AMpure XP according to the manufacturer protocol (Beckman Item # 5067-1504)
4. Run amplicons on an agarose gel. Expected band size for 27F/519R is approx. 530 bp.
5. Quantify amplicons with Picogreen (see manufacturers protocol; Invitrogen Item # P11496).
6. Run on Agilent Bioanalyzer chip, Screen Tape, or Lab Chip to get accurate sizing information.
7. Combine equimolar amounts of amplicons into a single, sterile tube.
8. Optional: If spurious bands were present on gel (in step 3), ½ of the final pool can be run on a gel and then gel extracted to select only the target bands.
9. 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.

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

Sequencing Primers

Read 1 Primer

ACACTATGGCGAGTGA**AGAGTTTGATCMTGGCTCAG**

Read 2 Primer

AGTCAGTCAGGG**GWATTACCGCGGCKGCTG**

Index Primer

CAGCMGCCGCGGTAATWCCCCTGACTGACT

Sequencing Setup

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

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

2. Prepare MiSeq Reagent Cartridge. *See MiSeq Reagent Preparation Guide (part # 15044983).*
3. Add custom sequencing primers into reservoirs 12-14. See *Using Custom Primers on the Miseq (part # 15041638).*
4. Load 600 µl of library pool into the MiSeq reagent cartridge in designated reservoir
5. Modify sample sheet to include custom primer's sequence/indexes (see index sequences in appendix 2) – Optional Set Workflow to "Metagenomics"
6. 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.*