Ramaciotti Centre for Genomics

Amplification and Illumina Sequencing of the V4 region of the 18S rRNA gene

1.0 Introduction

The protocol detailed here is designed to amplify the V4 region of the 18S rRNA gene for paired-end 18S community sequencing on the Illumina MiSeq platform. This protocol is based on Illumina's 16S Metagenomic Sequencing Library Preparation guide and the protocol used by Ocean Sampling Day, modified to amplify the target and add indexed adapter sequences in a single PCR step.

2.0 Amplification of the V4 region of the 18S rRNA gene

2.1 Primers for amplification of V4 region of the 18S rRNA gene

Amplification primers
See Appendix 1 for full list of primer sequences.

Forward primer
Field number (space-delimited), description:
5' Illumina adapter
Nextera XT i5 index sequence
Illumina forward overhang sequence
18S V4 forward

AATGATACGGCGACCACCGAGATCTACAC XXXXXXXX TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCAGCASCYGCGGTAATTCC

Reverse primer
Field number (space-delimited), description:
Reverse complement of 3' Illumina adapter
Nextera XT i7 index sequence
Illumina reverse overhang sequence
18S V4 reverse

CAAGCAGAAGACGGCATACGAGAT XXXXXXXX GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTTCGTTCTTGATYRATGA

2.2 Master mix for amplification of V4 region of the 18S rRNA gene

Component	Volume 1 rxn
KAPA HiFi Hot Start Readymix (2x) [(a)]	12.5
H_2O	9
Forward primer (10 µm)	1.25
Reverse primer (10 µm)	1.25
Template	1
Total Volume	**25**

Kit code KK2601 or KK2602

2.3 Thermocycler Conditions for amplification of V4 region of the 18S rRNA gene (96 well thermocyclers)

	Temperature	Time (mm:ss)
Activation	98°C	0:30
Amplification (10 cycles)	98°C	0:10
	44°C	0:30
	72°C	0:15
Amplification (20 cycles)	98°C	0:10
	62°C	0:30
	72°C	0:15
Final Extension	72°C	7:00
HOLD	4°C	∞

2.4 Process

2.4.1 Use undiluted DNA as a first attempt, and 1:10 diluted for repeats/failed reactions

2.4.2 Amplify samples with conditions outlined above.

2.4.3 Run amplicons on an agarose gel. Expected band size for 18S-V4 is approximately 536bp.

2.4.4 Clean and normalize the PCR products using SequalPrep Normalization plates according to manufacturer's instructions (Invitrogen cat no. A10510-01)

2.4.5 Pool equal volumes of each normalized amplicon.

2.4.6 Perform QC on pool using Qubit (concentration) and Tapestation (size) and calculate molarity of pool.

3.0 Sequencing of V4 region of the 18S rRNA gene

3.1 Sequencing Setup

3.2 Dilute pool prepared in **step 2.4.6** to **4nM**.

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

3.4 Prepare MiSeq Reagent Cartridge (v2 500-cycles). See *MiSeq Reagent Kit v2 - Reagent Preparation Guide* (part # 15034097).

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

3.6 Prepare sample sheet to include the appropriate index sequences.

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

References

16S Metagenomic Sequencing Library Preparation (Illumina Part # 15044223 Rev. B) available here: http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

LifeWatch Italy Ocean Sampling Day 2014 Protocol – available here: http://mb3is.megx.net/osd-files/download?path=/2014/protocols&files=OSD2014_protocol_B_18S_V4andV9_Sequencing_LifeWath_MoBiLab_BARI.pdf

Appendix 1. Primer Sequences

Primer Name	Primer Sequence
18S-V4f_S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S510	AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S511	AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S513	AATGATACGGCGACCACCGAGATCTACACTCGACTAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S515	AATGATACGGCGACCACCGAGATCTACACTTCTAGCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S516	AATGATACGGCGACCACCGAGATCTACACCCTAGAGTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S517	AATGATACGGCGACCACCGAGATCTACACGCGTAAGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S518	AATGATACGGCGACCACCGAGATCTACACCTATTAAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S520	AATGATACGGCGACCACCGAGATCTACACAAGGCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S521	AATGATACGGCGACCACCGAGATCTACACGAGCCTTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC
18S-V4f_S522	AATGATACGGCGACCACCGAGATCTACACTTATGCGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAGCASCYGCGGTAATTCC

18S-V4Lr_N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N707	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N710	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N711	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N712	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N714	CAAGCAGAAGACGGCATACGAGATTCATGAGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N715	CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N716	CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N718	CAAGCAGAAGACGGCATACGAGATGTAGCTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N719	CAAGCAGAAGACGGCATACGAGATTACTACGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N720	CAAGCAGAAGACGGCATACGAGATAGGCTCCGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N721	CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N722	CAAGCAGAAGACGGCATACGAGATCTGCGCATGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N723	CAAGCAGAAGACGGCATACGAGATGAGCGCTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N724	CAAGCAGAAGACGGCATACGAGATCGCTCAGTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N726	CAAGCAGAAGACGGCATACGAGATGTCTTAGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N727	CAAGCAGAAGACGGCATACGAGATACTGATCGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N728	CAAGCAGAAGACGGCATACGAGATTAGCTGCAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA
18S-V4Lr_N729	CAAGCAGAAGACGGCATACGAGATGACGTCGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTTCGTTCTTGATYRATGA