Ramaciotti Centre for Genomics

Protocol for archaeal 16S (A16S) rRNA amplification and sequencing on the Illumina MiSeq

1.0 Introduction

This protocol details the procedure for the amplification and sequencing of a 16S rRNA region preferential to archaeal targets, using the Illumina MiSeq platform.

Preparation of archaeal 16S (A16S) enriched libraries uses a twol round PCR strategy. The first round of PCR uses locusl specific primers with overhang adapters (A2F_Nex and 519R_Nex). The locusl specific region of the forward primer was based on the A2F primer from Reysenbach et al.(1995), which is specific to archaeal targets. The reverse primer has a locusl specific region that is universal for prokaryotes. The second round PCR and subsequent steps of library preparation and sequencing follow the "16S Metagenomic Sequencing Library Preparation" guidelines from Illumina.

Reysenbach AL, Pace NR. In: Robb, F.T., Place, A.R. (Eds.), Archaea: A Laboratory Manual Thermophiles. CSHLP, 101l 107 (1995).

2.0 Amplification of A2F and 519R region of the 16S rRNA gene

2.1 Primers for amplification of A2F and 519R region of the 16S rRNA gene

Amplification primers

A2F_Nex PCR Primer Sequence – Forward primer

1. Forward overhang
2. Locus specific sequence (A2F primer from Reysenbach et al.1995)

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTCCGGTTGATCCYGCCGGA

519R_Nex – Reverse primer

1. Reverse overhang
2. Locus specific sequence (16S universal primer)

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GWATTACCGCGGCKGCT

2.2 Preparation of master mix for amplification of A2F and 519R region of the 16S rRNA gene

Component	Volume 1 rxn
PCR Grade H2O[a]	17.55 µL
10x immoBuffer[b]	2.5 µL
50mM MgCl2[b]	0.75 µL
10mM dNTPs	0.5 µL
A2F Nex primer (10µM)	1.25 µL
519R Nex primer (10µM)	1.25 µL
Immolase NA olymerase[b]	0.2 µL
Template DNA	1.0 µL
Total reaction volume	25.0 µL

Notes:

(a) PCR grade water was purchased from MoBio Laboratories (MoBio Labs: Item#17000l 11)
(b) IMMOLASE DNA Polymerase Kit (Cat number: BIO-2146)
(c) Final primer concentration in reaction: 0.5 µM

2.3 Thermocycler Conditions for amplification of A2F and 519R region of the 16S rRNA gene

	Temperature	Time (mm:ss)
Activation	95°C	10:00
Amplification (35 cycles)	95°C	00:30
	60°C	00:15
	72°C	00:50
Final Extension	72°C	5:00
HOLD	4°C	∞

2.4 Process

2.4.1 Use undiluted NA for first attempt, and 1:10 dilution for second attempt (failed samples).

2.4.2 Amplify samples with conditions outlined above.

2.4.3 Run amplicons on an agarose gel. Expected band size is roughly 520 bp

2.4.4 If there is no band present, repeat PCR using a 1:10 dilution of the sample. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used at higher volumes.

2.4.5 Clean amplicons with Agencourt AMPure XP beads, according to manufacturer's instructions.

2.4.6 Perform a second round PCR (Index PCR) following Illumina's *16S Metagenomic Sequencing Library Preparation*, section *Index PCR (part #15044223)*. A halfl reaction 5ul total reaction volume) can be used.

2.4.7 Clean and normalize the PCR products equalPrep Normalization plates (Invitrogen, A1051001) according to manufacturer instructions.

2.4.8 Pool equal volumes of each normalized amplicon.

2.4.9 Perform QC on pool using Qubit (concentration) and Tapestation (size) and calculate molarity

3.0 Sequencing of A2F and 519R region of the 16S rRNA gene

3.1 Sequencing Setup

3.1.1 Dilute pool prepared in step 2.4.7 to 4nM.

3.1.2 Denature according to Ilumina protocol, with increased PhiX control spike as recommended for low diversity libraries. see *Preparing Libraries for Sequencing on the MiSeq (part #15039740)*.

3.1.3 Prepare MiSeq Reagent Cartridge (v3 600 cycles). see *MiSeq Reagent Kit v3 Reagent Preparation Guide (part # 15044983).*

3.1.4 Load 600 µL of library pool into the MiSeq reagent cartridge in designated sample well.

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