

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 two-round PCR strategy. The first round of PCR uses locus-specific primers with overhang adapters (A2F_Nex and 519R_Nex). The locus-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 locus-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, 101-107 (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

Field number (space-delimited), description:

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 H ₂ O ^a	17.55 µL
10x immoBuffer ^b	2.5 µL
50mM MgCl ₂ ^b	0.75 µL
10mM dNTPs	0.5 µL
A2F-Nex primer (10µM)	1.25 µL
519R-Nex primer (10µM)	1.25 µL
Immolase DNA polymerase ^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#17000-11)
- (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 (96 well thermocyclers)

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

2.4 Process

- 2.4.1 Use undiluted DNA.
- 2.4.2 Amplify samples with conditions outlined above.
- 2.4.3 Run amplicons on an agarose gel. Expected band size for A2F-Nex/519R-Nex 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 half-reaction (25ul total reaction volume) can be used.
- 2.4.7 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:
- Perform a bead clean-up following manufacturer's instructions.
 - 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).
 - Run clean amplicons on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
 - Normalize 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.
 - Pool equal volumes of each normalized amplicon.
- If using SequalPrep Normalization plates:*
- Perform a plate normalization following manufacturer's instructions.
 - Pool equal volumes of each normalized amplicon.
 - Run clean amplicon pool on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
- 2.4.8 Optional: If spurious bands were present on gel (in step 2.4.3), or in the clean amplicons (step 2.4.7c) or amplicon pool (step 2.4.7h), a portion of the final amplicon pool can go through a gel extraction or a second round of bead clean up. A lower bead to template ratio will get rid of small bands.
- 2.4.9 Measure concentration of the final clean pool using Qubit or picogreen.

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

3.1 Sequencing Setup

- Dilute pool prepared in step 2.4.7 to 4nM.
- Denature according to Illumina protocol, with increased PhiX control spike-in as recommended for low diversity libraries. See *Preparing Libraries for Sequencing on the MiSeq (part #15039740)*.
- Prepare MiSeq Reagent Cartridge (v3 600-cycles). See *MiSeq Reagent Kit v3 - Reagent Preparation Guide (part # 15044983)*.
- Load 600 µL of library pool into the MiSeq reagent cartridge in designated sample well.
- Start sequencing run following *MiSeq System User Guide (part # 15027617)*.