

Archaea V3-5 16S rRNA Amplicon Sequencing

Standard Protocol

Version 1.0

Skill Prerequisites: DNA handling, gel electrophoresis, DNA concentration measurement, polymerase chain reaction (PCR)

Introduction

This protocol describes how to prepare archaea V3-5 16S rRNA amplicon sequencing libraries used for microbial community analysis in anaerobic digesters. The V3-5 primers also target some bacteria.

The protocol is based on the Illumina Nextera library preparation approach. A two-step PCR approach is used to first amplify the V3-5 16S rRNA gene fragment and to subsequently attach Illumina adapters and barcodes necessary for sequencing to the V3-5 amplicons.

Due to the V3-5 region being around 575 bp long, merging of sequencing reads is not possible, and therefore only read 1 is used.

	V3-5
Fwd primer (aV35-A-f)	CCCTAHGGGGYGCAACA
Rv primer (aV35-A-r)	GWGCYCCCCGYCAATTC
<i>E. coli</i> positions sequenced	340-915
Fragment size ¹	575 (682)
Archaea taxonomic coverage ²	82.5%
Literature	Pinto <i>et al.</i> 2013

Materials

Instruments

- Nanodrop1000 (Thermo Fisher Scientific)
- Qubit® 2.0 Fluorometer (Thermo Fisher Scientific)
- Infinite® M1000 PRO (Tecan) or similar
- Thermocycler
- MagneSphere® Technology Magnetic Separation Stand (Promega) or Magentic Stand-96 (AM10027, Ambion)
- TapeStation2200 (Agilent)
- Pipettes (Range 1 µL to 1000 µL)

Reagents/consumables

- DNase free tips (10 µL, 300 µL and 1000 µL)

¹ First size is the size of the target variable region in *E. coli*. Size in brackets is size of the variable region with attached Illumina adaptors. Depending on the target species these sizes might vary with as much as +/- 75 bp.

² Archaea taxonomic coverage calculated using Silva TestPrime v1.0 and Silva RefNR SSU 128 (2016-11-15)

- DNase free tubes (1.5 mL)
- 96-well PCR plates (#82006-664, VWR)
- PCR strip caps
- OptiPlate-96 Black (Perkin Elmer)
- Nuclease Free H₂O (Qiagen)
- Qubit dsDNA HS assay kit (Thermo Fisher Scientific)
- Qubit dsDNA BR assay kit (Thermo Fisher Scientific)
- Platinum® Taq DNA Polymerase High Fidelity kit (Thermo Fisher Scientific)
- dNTP mix
- Tailed aV35-A primer mix (1 μM of each forward and reverse tailed primer)
- Barcoded Nextera adapter mixes (1 μM of each forward and reverse adaptor)
- Agencourt AMPure XP (Beckman Coulter)
- EtOH, 99 %
- D1000 Screentape (Agilent)
- Genomic DNA Screentapes (Agilent)

Method

Planning

1. Assign barcoded adapters to samples
 1. Contact person in charge of sequencing and inform about number of samples and what variable regions are to be analyzed. You will be assigned appropriate adapters.
 2. Barcode and adapter names/sequences are available on the [MiDAS website](#).
2. Help regarding reagents and this protocol
 1. Contact person in charge of sequencing or mail@midasfieldguide.org

Input sample material

IMPORTANT: Samples must be processed using the exact same DNA extraction procedure if they are to be compared. For WWTP activated sludge samples and anaerobic digester samples use respective **standard protocols**.

Sample DNA QC

Pick at least 5 out of i.e. 96 samples for QC screening. If samples are known to be problematic perform QC on all samples.

1. Nanodrop UV-Vis Quality and Concentration Check
 1. Use standard protocol.
 2. A260/280 should be 1.8 to 2.0. A260/230 2.0 to 2.2.
 3. Also compare the UV-vis absorbance curve to that of pure DNA³ to detect anomalies/contamination.
2. TapeStation gel electrophoresis
 1. Use Genomic DNA Screentapes and follow standard guidelines.
 2. Qualitative range of the tapes is 3-300 ng/μL. Dilution might be necessary.

³ DNA extracted with 'FastDNA spin kit for soil' is known to contain contamination producing a peak around 230 nm. The contaminant does not inhibit steps in this protocol but may result in overestimation of the DNA concentration.

3. Remember to use ladder, as no in silico ladder is available for use with Genomic tapes.
4. The majority of DNA should be in the range of 10 kb and above. If it is below the DNA is degraded and it might affect the analysis.

Sample dilution

1. Measure DNA concentration using Qubit dsDNA Assay Kit, broad range
 1. Follow standard protocol.
 2. Use 1 μ L of sample.
 3. Perform single measurement of each sample.
2. Based on Qubit measurements dilute the samples to 5 ng/ μ L with nuclease free water.


Amplicon PCR

1. Duplicate PCR reaction is run for each sample⁴. Remember negative control (nuclease free H₂O) and positive control (sample run before).
2. Prepare master mix:

Reagents	Final conc.	1 reaction [μ L]	X Reactions
nuclease free H ₂ O	-	7.65	
x10 buffer Platinum® High Fidelity	x1	2.5	
dNTP (5 mM total, 1.25 mM of each)	0.1 mM of each	2	
MgSO ₄ (50mM)	1.5 mM	0.75	
aV3-5-A primer Mix (1 μ M)	400 nM	10	
Platinum® Taq DNA Polymerase High Fidelity (5 U/ μ l)	1U	0.1	
Total volume		23	

NB: When handling many samples prepare for $n(\text{samples}) \times 2 + 5$, so the multipipette can be used.

3. Transfer 23 ul of mastermix to the wells of a 96-well PCR plate.
4. Add 2 μ l of template (10 ng DNA).
5. Run PCR program:

aV35 PCR ⁵		
Temp	Time	
95°C	2 min	
35 cycles		
95°C		20 sec

⁴ Samples are run in duplicate to mitigate the effects of PCR drift and to obtain enough total Library amplicons for sequencing and storage.

⁵ Values in red should be changed to match target amplicon.

50 °C		30 sec
72°C		60 sec
72°C		5 min
4°C		Forever

6. Pool duplicates.

Amplicon Cleanup

1. Gently shake Agencourt AMPure XP Bottle to resuspend the beads, remove required volume and let it equilibrate to room temperature.
2. Transfer 40 μL of bead solution to each pooled PCR product (50 μL) and mix with pipette (10 times up and down).
3. Incubate for 5 minutes at room temperature.
4. Place the library container in magnetic rack for 2-4 minutes, until liquid is cleared.
5. Remove liquid with pipette and discard it.
6. Wash bead-pellet with 200 μL **freshly** prepared 80 % EtOH by gently dispensing it over the beads with a pipette. Let it rest for 30 s and then remove the liquid.
7. **Repeat** step 6.
8. Ensure no excess liquid is left after the washes. If there is, remove it with a pipette.
9. Dry for approximately 5-10 min to evaporate the EtOH⁶.
10. Remove library container from magnetic rack. Add 33 μL of nuclease free water and mix with pipette (>10 times up and down) to resuspend the beads.
11. Place library container back on the magnetic rack and wait until the liquid clears.
12. Transfer 30 μL of the liquid to a new container.

Amplicon QC screen

1. Measure DNA concentration using Qubit dsDNA HS Assay Kit
 1. Follow standard protocol.
 2. Use 2 μL of sample for each measurement.
 3. Perform single measurement of each sample.
2. Tapestation Gel electrophoresis
 1. Run Tapestation 2200 with D1000 Screentapes on the positive control, negative control and 1-2 regular samples. Follow standard protocol. No replicates.
 2. Ensure that the target amplicon is present (see size in introduction) and not too much unspecific product.⁷
3. If the concentration is higher than 5 ng/ μL dilute to 5 ng/ μL .

Library PCR

1. Single PCR reaction is run.
2. Prepare master mix:


⁶ Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.

⁷ Unspecific products are most likely primer dimers.

Reagents	Final conc.	1 reaction [μL]	X Reactions
nuclease free water	-	7.5	
X5 PCR BIO Reaction buffer (includes Mg ²⁺ and dNTPs)	x1	5	
PCR BIO Hifi Polymerase (2 U/μl)	1U	0.5	
Total volume		13	

NB: When handling many samples prepare for n(samples) x 2 + 5, so the multipipette can be used.

- Transfer 13 μl of mastermix to the wells of a 96-well PCR plate.
- Add 10 μL of nextera adaptor mix.
- Add 2 μl of purified Amplicon PCR product (ca. 5 ng/μL).

Library PCR ⁸	
Temp	Time
95°C	2 min
8 cycles	
95°C	20 sec
55 °C	30 sec
72°C	60 sec
	
72°C	5 min
4°C	Forever

- After the PCR, samples are referred to as “libraries”.

Library Cleanup

- Gently shake Agencourt AMPure XP Bottle to resuspend the beads, remove required volume and let it equilibrate to room temperature.
- Add **20 μL** of bead solution to each library (25 μL) and mix with pipette (10 times up and down).
- Incubate for 5 minutes at room temperature.
- Place the library container in magnetic rack for 2-4 minutes, until liquid is cleared.
- Remove liquid with pipette and discard it.
- Wash bead-pellet with 200 μL **freshly** prepared 80 % EtOH by gently dispensing it over the beads with a pipette. Let it rest for 30 s and then remove the liquid.
- Repeat** step 6.
- Ensure no excess liquid is left after the washes. If there is, remove it with 10 μL pipette.
- Dry for approximately 5-10 min to evaporate the EtOH⁹.
- Remove library container from magnetic rack. Add 20 μL of nuclease free water and mix with pipette (>10 times up and down) to resuspend the beads.
- Place library container back on the magnetic rack and wait until the liquid clears.

⁸ Values in red should be changed to match target amplicon.

⁹ Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.

12. Transfer 18 μL of the liquid to a new container.

Library QC

1. Measure DNA concentration of each individual library using Qubit dsDNA HS Assay Kit
 1. Follow standard protocol.
 2. Single measurement for each sample.
 3. Use 2 μL library for each measurement.
2. Tapestation Gel electrophoresis
 1. Based on the measured DNA concentration, pick libraries with low DNA concentrations (potentially failed), as well as 2 good libraries and the negative and positive control.
 2. Run Tapestation 2200 with D1000 Screentapes. Follow standard protocol. No replicates.
 3. Ensure that the target amplicon is present and that no unspecific products are present.¹⁰

Library Pooling

1. Calculate the required volume of each sample
 1. Libraries with a concentration of less than 1 $\text{ng}/\mu\text{L}$ should be excluded (either leave out, rerun PCR or discuss with your supervisor).
 2. Detect the sample with the lowest concentration and multiply this concentration with 15 μL (e.g. 1 $\text{ng}/\mu\text{L} \times 15 \mu\text{L} = 15 \text{ ng}$). This is the amount of library wanted from each library.
 3. Calculate the volumes required to obtain the same amount of library for each of the other libraries.
 4. If volumes less than 1 μL are required for some libraries, consider diluting the libraries and recalculate the required volumes.
2. Pool libraries
 1. Use a new Eppendorf tube (1.5 mL).
 2. Transfer the calculated volume of each sample to the tube.
 3. Mix well after all the samples have been added.

Library Pool QC

1. Measure DNA concentration of library pool with Qubit dsDNA HS assay kit
 1. Follow standard protocol.
 2. Measure in triplicate and calculate mean.
 3. Use 2 μL sample for each measurement.
2. Tapestation Gel electrophoresis
 1. Run Tapestation 2200 with D1000 Screentapes. Follow standard protocol. No replicates.
 2. **Only** the target amplicon (see the size above) should be present on the gel.

Submit for sequencing

1. Calculate sample pool concentration in nanomolar (nM) :

¹⁰ Unspecific products are most likely primerdimers. These need to be completely removed since they have a negative impact on sequencing.

$$nM = \frac{c}{bp \cdot 650} \cdot 1000\ 000$$

c = concentration in ng/ μ L
bp = fragment size in basepairs

2. Name your library pool tube: “<yymmdd>-16S-<your_initials>-<supervisor_initials>” ie. 130812-16S-SMK-PHN and write on the nM concentration
3. Follow local guidelines for sequencing submission.

Literature

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, 108 Suppl , 4516–22. doi:10.1073/pnas.1000080107

Caporaso, J. G., Lauber, C. L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, 6(8), 1621–4. doi:10.1038/ismej.2012.8

Illumina 2015, 16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. B

Revision History

V1.0 2016-11-15

- Protocol based on “161111_EBstd_Nextera Amplicon Sequencing v1.3”
- Introduction
 - aV35-A primer specific information added
- All protocol
 - Generic amplicon considerations replaced by aV35-A specific information.