Bacterial V1-3 16S rRNA Amplicon Sequencing

Standard Protocol

Version 1.2

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

Introduction

This protocol describes how to make amplicon sequencing libraries of the V1-3 variable region of the bacterial 16S rRNA gene. **The MIDAS amplicon data is produced using the V1-3 region.**

The 16S rRNA gene is used as a universal phylogenetic marker for distinguishing and classifying bacteria. Targeting the 16S rRNA gene enables whole bacterial community analysis with a resolution ranging from taxonomic family to species level depending on the variable region used and the phylogenetic branches investigated. The library preparation is PCR based and relies on PCR primers targeting conserved sequence regions between the variable regions in the bacterial 16S rRNA gene. This means that the analysis will not be able to capture bacteria whose sequences deviate from the consensus sequences of the targeted conserved regions. The use of the V1-3 primers described here does not capture Archaea.

	V1-3
Fwd primer	AGAGTTTGATCCTGGCTCAG
Rv primer	ATTACCGCGGCTGCTGG
E. coli positions sequenced	28-516
Fragment size[1]	488 (614)
Taxonomic coverage[2]	96.6%
Literature	HMP Consortium, 2010

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)
- Magentic Stand-96 (AM10027, Ambion)
- Tapestation 2200 (Agilent)
- Pipettes (Range 1 µL to 1000 µL)
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[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] Bacterial coverage evaluated in *Karst et al*, bioRxiv 070771; doi: http://dx.doi.org/10.1101/070771

Reagents/consumables

- DNAse free tips (10 µL, 300 µL and 1000 µL)
- DNAse free tubes (1.5 mL)
- 96-well PCR plates (#82006-664, VWR)
- PCR strip caps
- OptiPlate-96 Black (Perkin Elmer)
- Nuclease Free H_2O (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
- Barcoded 16S V1-3 adapter mixes (1 µM of each forward and reverse adapter)
- Agencourt AMPure XP (Beckman Coulter)
- EtOH, 99 %
- D1000 Screentapes (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 digester sludge 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 guidelines provided by the vendor.
    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 DNA[3].
2. Tapestation gel electrophoresis
    1. Use Genomic DNA Screentapes and follow standard guidelines.
    2. Qualitative range of the tapes is 3-300 ng/uL. 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 apply a 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 heavily degraded and it might affect the analysis.

Sample dilution

1. Measure DNA concentration using Qubit dsDNA BR assay kit.
    1. Follow standard protocol.
    2. Use 1 µL of sample.
    3. Perform single measurement of each sample.
2. Based on Qubit measurements dilute the samples to 5 ng/µl with nuclease free water.

Library PCR

1. Library PCR reaction is run in duplicate for each sample. Remember negative control (nuclease free water) and positive control (sample run before)[4].
2. Prepare master mix:

Reagents	Final conc.	1 reaction [µL]	X Reactions
nuclease free water	-	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	
Platinum® Taq DNA Polymerase High Fidelity (5 U/µl)	1U	0.1	
Total volume		13	

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

3. Transfer **13 ul** of mastermix to the wells of a 96-well PCR plate.
4. **Spin down 96-well plate containing amplicon adaptors to prevent cross contamination.**[5]
5. Add **10 µl** of assigned barcoded 16S adapter mix (1 µM) to each tube. Final conc. is 400 nM.
6. Seal adaptor plate wells with **new** 8-strip caps to prevent cross contamination.
7. Add 2 µl of template (ca. 10 ng total DNA).

[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] Adapter solutions needs to be spun down, otherwise we risk of cross contamination between the different adapter mixes, when opening the lids.

8. Run PCR program:

V1-3		
Temp		Time
95°C		2 min
30 cycles		
95°C		20 sec
56°C		30 sec
72°C		60 sec
72°C		5 min
4°C		Forever

9. Pool the duplicates.
10. After the PCR, samples are referred to as “libraries”.

Library 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 library (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 10 µL pipette.
9. Air dry for approximately 5-10 min to evaporate the EtOH[6].
10. Remove library container from magnetic rack. Add 23 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 20 µL of the liquid to a new container.

Library QC screen

1. Measure DNA concentration of each individual library using Qubit dsDNA HS assay kit
    1. Follow standard protocol.
    2. Single measurement per sample.[7]
    3. Use 2 µL library for each measurement.
2. Tapestation Gel electrophoresis

[6] Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.
[7] Perform duplicate measurement if more correct estimate is required.

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 (see size in introduction) and that no unspecific products are present.[8]

Library Pooling

1. Calculate the required volume of each sample
    1. Libraries with a concentration of less than 1 ng/µL should be excluded (either leave out or rerun PCR).
    2. Detect the sample with the lowest concentration and multiply this concentration with 15 µL (e.g. 1 ng/µL × 15 µL = 15 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 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) :

$$nM = \frac{c}{bp \cdot 650} \cdot 1\,000\,000$$

c = concentration in ng/µL
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.

[8] Unspecific products are most likely primer dimers. These need to be completely removed since they have a negative impact on sequencing.

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

Huttenhower, C., Fah Sathirapongsasuti, J., Segata, N., Gevers, D., Earl, A. M., Fitzgerald, M. G., … Segre, J. A. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. http://doi.org/10.1038/nature11234

Revision History

V1.00 2015-2-14

- Merged protocols “140922_EBstd_Bacteria 16S rRNA V4 Amplicon Sequencing v1.03”, “140922_EBstd_Bacteria 16S rRNA V3-4 Amplicon Sequencing v1.03” and “140922_EBstd_Bacteria 16S rRNA V1-3 Amplicon Sequencing v1.05”.
- Protocol has been optimized for high throughput preparation.
- Sample DNA QC: Reduced to only be a screen of a subset of samples. Normal gel electrophoresis exchanged for Genomic DNA screentapes.
- Sample Dilution: Removed 2 step dilution strategy. Only one dilution required based on DNA concentration measurement made with Quant-iT dsDNA Assay Kit, broad range.
- Library Cleanup: Re-introduced individual cleanup of each library.
- Library pooling: Explanation simplified.
- Alternative Strategies: Added for advanced users.

V1.1 2015-3-16

- Changed volume of barcode working solution. Changing the relative volumes between mastermix and barcode working solution will make it easier to see if barcode has been added to the wells of a 96-well pcr plate.
- Added not regarding spin down of adaptors to prevent cross contamination.

V1.2 2016-11-11

- Clarification of dNTP concentration in stock and final PCR reaction. The total dNTP concentration of the stock is 5 mM (1.25 mM of each dATP, dCTP, dGTP, dTTP), and the final PCR reaction concentration is therefore 0.100 mM of each.
- Removed description of V4 and bV34 library preparation, as these are made with nextera two step PCR setup in the future.
- Changed title to reflect focus on V1-3.
- Major changes to ‘Library Pooling’ wording to make it easier to understand the concept.
- Minor changes to wording.

Alternative Strategy for Standard Samples

This alternative protocol is recommended for standard samples that are known to perform well i.e. wastewater activated sludge or digester sludge. These changes trade a large reduction in time and cost for a little higher risk of sample fall out. Usually sample fallout isn't a problem in big studies since these can be re-prepped with another sample batch.

After PCR do the following:

Library QC screen

This is done to ensure that the PCR reaction has been successful.

1. Tapestation Gel electrophoresis
    1. Pick 2 random libraries and positive and negative control libraries (= total of 4 libraries)
    2. Run Tapestation 2200 with D1000 Screentapes. Follow standard protocol. No replicates.
    3. Ensure that the target amplicon is present (see the size in introduction)[9] .

Library Pooling

1. Transfer 5 µL of each sample and pool together.

Library Pool Cleanup

1. Gently shake Agencourt AMPure XP Bottle to resuspend beads, remove required amount and let it equilibrate to room temperature before use.
2. Transfer 80 µL of bead solution to a 1.5 mL eppendorf tube, add 100 µL of library pool to the beads and mix with pipette (10 times up and down).
3. Incubate for 5 minutes at room temperature.
4. Place sample Eppendorf tube in magnetic rack for Eppendorf tubes 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 aspirating 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 10 µL pipette.
9. Dry for approximately 10 min to evaporate the EtOH[10].
10. Remove sample Eppendorf tube from magnetic rack. Add 23 µL of nuclease free water and mix with pipette (>10 times up and down) to resuspend the beads.
11. Place sample Eppendorf tube back on the magnetic rack and wait until the liquid clears.
12. Transfer 20 µL of the liquid to a new Eppendorf tube. This is your library pool.

Library Pool QC

1. Measure DNA concentration of library pool with Qubit dsDNA HS assay kit
    4. Follow standard protocol.
    5. Measure in triplicate and calculate mean.

[9] Depending on the PCR efficiency there can be significant contamination present <300 bp.
[10] Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.

6. Use 2 µL sample.

2. Tapestation Gel electrophoresis
    3. Run Tapestation 2200 with D1000 Screentapes. Follow standard protocol. No replicates.
    4. **Only** the target amplicon (see size above) should be present on the gel.