Bacterial 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 make amplicon sequencing libraries of the V1-3, V3-4 and V4 variable regions 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 and for distinguishing and classifying bacteria. Using the 16S rRNA enables whole community analysis with a resolution ranging from 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 genes. This means that the analysis will not target bacteria whose sequences deviate from the consensus sequences of these conserved regions. It is not recommended to use this protocol for Archaea.

<table>
<thead>
<tr>
<th>V1-3</th>
<th>V3-4</th>
<th>V4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fwd primer</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>CCTACGGGNGGCAGCCCA</td>
</tr>
<tr>
<td>Rv primer</td>
<td>ATTACCGCGGCTGCTGG</td>
<td>GACTACHVGGGTATCTAATCC</td>
</tr>
<tr>
<td>E. coli positions</td>
<td>28-516</td>
<td>357-784</td>
</tr>
<tr>
<td>Fragment size a</td>
<td>488 (614)</td>
<td>427 (550)</td>
</tr>
<tr>
<td>Taxonomic coverage b</td>
<td>78.5%</td>
<td>82%</td>
</tr>
<tr>
<td>Literature</td>
<td>HMP Consortium, 2010</td>
<td>Herlemann et al., 2011</td>
</tr>
</tbody>
</table>

a 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 variable region and target species these sizes might vary with as much as +/- 75 bp.

b Bacterial coverage evaluated using Silva TestPrime, max 1 mismatch, in SILVA SSU-114 RefNR database (2014-9-19).

Materials

- Nanodrop1000 (Thermo Scientific)
- Qubit® 2.0 Fluorometer (Life Technologies)
- Infinite® M1000 PRO (Tecan) or similar
- Thermocycler
- MagneSphere® Technology Magnetic Separation Stand (Promega)
- 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)
- 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)
- Quibit dsDNA HS assay kit (Life Technologies)
- Quant-iT dsDNA Assay Kit, broad range (Life Technologies)
- Quant-iT dsDNA Assay Kit, high sensitivity (Life Technologies)
- Platinum® Taq DNA Polymerase High Fidelity kit (Life Technologies)
- dNTP mix
- Barcoded 16S adaptor mixes (5 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 adaptors to samples
   1. Contact person in charge of sequencing and inform about number of samples and what variable regions is to be analyzed. You will be assigned appropriate adaptors.
   2. Barcode and adaptor 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: DNA samples must be extracted using the exact same procedure if they are to be compared. For WWTP activated sludge samples and digester sludge samples use respective standard protocols. Preferably perform DNA extraction of biological triplicates and perform independent amplicon analysis of each replicate.

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.

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.

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1 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.
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

3. Measure DNA concentration using Quant-iT dsDNA Assay Kit, broad range
   1. Follow standard protocol.
   2. Use 2 µL of sample.
   3. Perform single measurement of each sample.

4. Based on Quant-iT 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 (DNA H2O) and positive control (sample run before).

2. Prepare master mix:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final conc.</th>
<th>1 reaction [µL]</th>
<th>X Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclease free water</td>
<td>-</td>
<td>15.65</td>
<td></td>
</tr>
<tr>
<td>x10 buffer Platinum® High Fidelity</td>
<td>x1</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>dNTP (5 mM)</td>
<td>400 nM</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ (50 mM)</td>
<td>1.5 mM</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Platinum® Taq DNA Polymerase High Fidelity (5 U/µl)</td>
<td>1U</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

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

3. Transfer 21 µl of mastermix to the wells of a 96-well PCR plate.
4. Add 2 µl of assigned barcoded 16S adaptor mix (5 µM) to each tube. Final conc. is 400 nM.
5. Add 2 µl of template (ca. 10 ng total DNA).

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2 Single point measurements add some uncertainty to the DNA concentration estimate. However the protocol has shown robustness towards varying template DNA concentrations. No significant difference could be found between 1-50 ng of template in activated sludge (Albertsen et al, in prep).

3 Samples are run in duplicate for historical reasons – presumably to A) To even out possible variances between PCR reactions B) To obtain enough total Library amplicons for sequencing and storage.
6. Run variable region specific PCR program:

<table>
<thead>
<tr>
<th>V1-3</th>
<th>V3-4</th>
<th>V4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp</td>
<td>Time</td>
<td>Temp</td>
</tr>
<tr>
<td>95°C</td>
<td>2 min</td>
<td>95°C</td>
</tr>
<tr>
<td>30 cycles</td>
<td></td>
<td>30 cycles</td>
</tr>
<tr>
<td>95°C</td>
<td>20 sec</td>
<td>95°C</td>
</tr>
<tr>
<td>56°C</td>
<td>30 sec</td>
<td>50°C</td>
</tr>
<tr>
<td>72°C</td>
<td>60 sec</td>
<td>72°C</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
<td>72°C</td>
</tr>
<tr>
<td>4°C</td>
<td>Forever</td>
<td>4°C</td>
</tr>
</tbody>
</table>

7. Pool the duplicates.
8. After the PCR, samples are referred to as “libraries”.

Library Pool 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 a container (spintube or 96-well plate), add 50 µL of library to the beads 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 70 % 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 7.
8. Ensure no excess liquid is left after the washes. If there is, remove it with 10 µL pipette.
9. Dry for approximately 10 min to evaporate the EtOH.
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 Quant-iT dsDNA Assay Kit, high sensitivity
   1. Follow standard protocol.
   2. Measure in duplicate and calculate mean.
   3. Use 2 µL library.

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4 Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.
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.
   3. Ensure that the target amplicon is present (see size in introduction) and that no unspecific products are present.\(^5\)

Library Pooling

1. Pool libraries in equimolar concentrations. The simplest way is to add a fixed amount of DNA from each library to the pool. Different volumes of library is pooled but the pool contains the same amount of DNA from each library. For example if 10 ng DNA is wanted from each library, then 2 µL library is needed if the concentration is 5 ng/µL or 5 µL library is needed if the concentration is 2 ng/µL.

Library Pool QC

1. Measure DNA concentration of library pool with Quibit dsDNA HS assay kit
   1. Follow standard protocol.
   2. Measure in triplicate and calculate mean.
   3. Use 2 µL sample.
2. Tapestation Gel electrophoresis
   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): \[ nM = \frac{c}{bp} \cdot 650 \cdot 1000000 \]
   - c = concentration in ng/µL
   - bp = fragmentsize 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.

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\(^5\) Unspecific products are most likely primerdimers. These need to be completely removed since they have a negative impact on sequencing.
Literature


Revision History

V1.00 2013-2-14

- 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 screens.
- 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.
Alternative Strategy for Standard Samples

This protocol change 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 3 random libraries and positive and negative control libraries (= total of 5 libraries)
   3. Ensure that the target amplicon is present (see the size in introduction)\(^6\).

Library Pooling

2. Simply take 5 µL of each sample and pool together.

Library Pool Cleanup

3. Gently shake Agencourt AMPure XP Bottle to resuspend beads, remove required amount and let it equilibrate to room temperature.
4. Transfer 80 µl of bead solution to a 1.5 mL spintube, add 100 µl of library pool to the beads and mix with pipette (10 times up and down).
5. Incubate for 5 minutes at room temperature.
6. Place sample spintube in magnetic rack for spintubes for 2-4 minutes, until liquid is cleared.
7. Remove liquid with pipette and discard it.
8. Wash bead-pellet with 200 µl freshly prepared 70 % EtOH by gently aspirating it over the beads with a pipette. Let it rest for 30 s and then remove the liquid.
9. Repeat step 7.
10. Ensure no excess liquid is left after the washes. If there is, remove it with a 10 µL pipette.
11. Dry for approximately 10 min to evaporate the EtOH\(^7\).
12. Remove sample spintube from magnetic rack. Add 23 µl of nuclease free water and mix with pipette (>10 times up and down) to resuspend the beads.
13. Place sample spintube back on the magnetic rack and wait until the liquid clears.
14. Transfer 20 µl of the liquid to a new spintube. This is your library pool.

Library Pool QC

3. Measure DNA concentration of library pool with Quibit dsDNA HS assay kit
   4. Follow standard protocol.
   5. Measure in triplicate and calculate mean.
   6. Use 2 µL sample.

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\(^6\) Depending on the PCR efficiency there can be significant contamination present <300 bp.

\(^7\) Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.
4. Tapestation Gel electrophoresis
   4. **Only** the target amplicon (see size above) should be present on the gel.