DNA based library prepartion for full-length small ribosomal RNA sequencing on the Illumina MiSeq and HiSeq platforms

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Abstract

This protocol describes the preparation of Illumina libraries for high through-put sequencing of near full-length bacterial 16S rRNA sequences. The method is based on the universal 27F and 1492R primers designed by Lane *et al*.*

Sequences for primers and adaptor used can be found under guidelines.

*Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt & M. Goodfellow (Eds.), *Nucleic Acid Techniques in Bacterial Systematics* (pp. 115–175). Chichester, United Kingdom: John Wiley and Sons.

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Guidelines

Primers and adaptors used for library preparation:

Oligo name	sequence	Purification
B16S_rDNA_PCR1_fw1	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNTGCCTCTTAGAGTTTGATCMTGGCTCAG	PAGE
B16S_rDNA_PCR1_fw2	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNTCCTCTACAGAGTTTGATCMTGGCTCAG	PAGE
B16S_rDNA_PCR1_fw3	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNTCATGAGCAGAGTTTGATCMTGGCTCAG	PAGE
B16S_rDNA_PCR1_fw4	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNCCTGAGATAGAGTTTGATCMTGGCTCAG	PAGE
B16S_rDNA_PCR1_fw5	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNTAGCGAGTAGAGTTTGATCMTGGCTCAG	PAGE
B16S_rDNA_PCR1_fw6	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNGTAGCTCCAGAGTTTGATCMTGGCTCAG	PAGE
B16S_rDNA_PCR1_rv	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNCGTACTAGTACGGYTACCTTGTTACGACTT	PAGE
B16S_rDNA_PCR2_fw	CTCCACCCAGACTCATCCAT	PAGE
B16S_rDNA_PCR2_rv	AGGGGGGCAAAGATGAAGAT	PAGE
B16S_rDNA_readtag_rv	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGGGGGCAAAGATGAAGAT	PAGE
B16S_rDNA_readtag_fw	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCCACCCAGACTCATCCAT	PAGE
B16S_rDNA_linktag_fw	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTGAGCCAKGATCAAACTCT	PAGE
B16S_rDNA_linktag_rv	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGTCGTAACAAGGTARCCGTA	PAGE
B16S_rDNA_read2_fw	GCTCTTCCGATCTCTCCACCCAGACTCATCCAT	HPLC

Protocol

Adaptor annealing

Step 1.

Adaptors containing defined primer binding sites and unique molecular tags are added to each end of the SSU genes by PCR.

Prepare the following reaction in a 200 µL PCR tube:

- 10 µL 10x PCR buffer (Qiagen)
- 2 µL 10 mM dNTP (Qiagen)
- 5 µL 10 µM B16S_rDNA_pcr1_fw
- 5 µL 10 µM B16S_rDNA_pcr1_rv
- 4 µL 25 mM $MgCl_2$ (Qiagen)
- 72.5 µL nuclease-free water
- 1 µL of genomic DNA
- 0.5 µL 5 U/µL Taq polymerase (Qiagen)

Adaptor annealing

Step 2.

Perform the PCR with an initial denaturation at 94°C for 3 min, followed by 2 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 3 min and then a final extension at 72°C for 5 min.

Adaptor annealing

Step 3.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 0.6x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 11 µL nuclease-free water

Library amplification

Step 4.

Prepare a library amplification reaction in a 200 µL PCR tube:

- 10 µL sample from above
- 63.5 µL nuclease-free water
- 10 µL 10x PCR buffer (Qiagen)
- 2 µL 10 mM dNTP (Qiagen)
- 5 µL 10 µM B16S_rDNA_pcr2_fw

- 5 µL 10 µM B16S_rDNA_pcr2_rv
- 4 µL 25 mM MgCl (Qiagen)
- 0.5 µL 5 U/µL Taq polymerase (Qiagen)

NOTES

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The adaptor annealed molecules are amplified by PCR to obtain enough product for validation, size selection, and quantification.

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B16S_rDNA_pcr2_fw and B16S_rDNA_pcr2_rv are identical to SSU_rRNA_pcr_fw and SSU_rRNA_pcr_rv from the RNA based protocol, respectively.

Primary ibrary amplification

Step 5.

Perform the PCR with an initial denaturation at 94°C for 3 min, followed by 20-25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 3 min and then a final extension at 72°C for 5 min.

NOTES

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The number of PCR cycles depends on the number of adaptor annealed template molecules. Too many PCR cycles results in loss of the PCR product and the formation of unspecific products. We usually perform two PCR reactions, one with 20 cycles and one with 25. We then choose the best based on analysis on a D5000 screentape.

Primary library amplification

Step 6.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 0.6x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 20 µL nuclease-free water

Primary ibrary amplification

Step 7.

Analyze the PCR product on an Agilent 2200 Tapestation using a D5000 screen tape.

Primary library size selection

Step 8.

Isolated the full-length SSU PCR products by size selection on an E-gel electrophoresis system with precast E-Gel CloneWell gels (Thermo Fisher Scientific):

- Use 20 µL of sample from above (100-300 ng of PCR product)
- Use 500 ng GeneRuler 1 kB DNA ladder (Thermo Fisher Scientific) as a reference.
- Run the gel until the SSU peak (ca. 1,500 bp) is approximately 1 mm from the the elution well.
- Now collect 20 µL elution aliquots every 15 seconds, up to a total of 16 aliquots, and the visible passing of the full-length SSU cDNA peak.
- Pool every two aliquots to obtain 8 pooled aliquots per sample.

NOTES

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Presence of unspecific products and template DNA is frequently observed after the PCR. To increase the final yield of full-length SSU rRNA amplicons, a size selection on a precast E-Gel CloneWell gel is performed.

Primary library size selection

Step 9.

Analyze the pooled aliquots on an Agilent 2200 Tapestation with the High Sensitivity D5000 Screentape and pool the aliquots that contains the full-length SSU amplicons.

Primary library size selection

Step 10.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 0.6x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 15 µL nuclease-free water

Primary library size selection

Step 11.

Determine the quality and the concentration of the final library on an Agilent 2200 Tapestation with the High Sensitivity D5000 Screentape.

Clonal library preparation

Step 12.

Dilute the primary libraries based on the tapestation data to 10,000 (MiSeq) or 100,000 (HiSeq) copies/µL with nuclease-free water.

NOTES

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The number of amplicons in the primary library can be calculated using the following formula:

number = (amount in ng * 6.022×10^{23}) / (length in bp * 1×10^{9} * 650) =>

number = (amount in ng * $6.022x10^{23}$)/ (1800 * $1x10^{9}$ * 650) =>

number = amount in ng * $5.15x10^{8}$ copies/ng

Clonal library preparation

Step 13.

Prepare a clonal library amplification reaction in a 200 µL PCR tube:

- 10 µL sample from above
- 63.5 µL nuclease-free water
- 10 µL 10x PCR buffer (Qiagen)
- 2 µL 10 mM dNTP (Qiagen)
- 5 µL 10 µM B16S_rDNA_pcr2_fw
- 5 µL 10 µM B16S_rDNA_pcr2_rv
- 4 µL 25 mM MgCl (Qiagen)
- 0.5 µL 5 U/µL Taq polymerase (Qiagen)

NOTES

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B16S_rDNA_pcr2_fw and B16S_rDNA_pcr2_rv are identical to SSU_rRNA_pcr_fw and SSU_rRNA_pcr_rv from the RNA based protocol, respectively.

Clonal library preparation

Step 14.

Perform the PCR with an initial denaturation at 94°C for 3 min, followed by 25 (MiSeq) or 20 (HiSeq) cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 2 min and then a final extension at 72°C for 5 min.

Clonal library preparation

Step 15.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 0.6x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 20 µL nuclease-free water

Clonal library preparation

Step 16.

Determine the quality of the clonal library on an Agilent 2200 Tapestation with the D5000 Screentape.

Clonal library preparation

Step 17.

Determine the concentration of the clonal library on a Qubit 2.0 fluorometer with the Qubit dsDNA HS

Assay kit.

- Use 1 µL sample
- Perform duplicate measurements

Clonal library preparation

Step 18.

Dilute the clonal library to 4.35 ng/µL with nuclease-free water

Read-tag library preparation

Step 19.

A Nextera library preparation kit (Illumina) is used to prepare a paired-end read-tag sequencing library from the clonal library using a customized protocol.

Prepare a tagmentation reaction in a 200 µL PCR tube:

- 23 µL diluted clonal library from above
- 12.5 µL tagment DNA buffer (Illumina)
- 1.5 µL tagment DNA enzyme (Illumina)

Read-tag library preparation

Step 20.

Perform the tagmentation incubated at 55°C for 5 min. Immediately proceed to the next step.

Read-tag library preparation

Step 21.

Read-tag library preparation

Step 22.

The tagmentation products are PCR amplified using two separate PCRs (A and B). PCR A selectively amplified fragments containing the 5’ termini of the cDNA amplicons and PCR B selectively amplified fragments containing the 3’ termini.

Prepare the PCR reactions in 200 µL PCR tubes:

- 20 µL of sample from above
- 5 µL N504 nextera adaptor (Illumina)
- 5 µL 10 µM B16S_rDNA_readtag_fw (PCR A) or B16S_rDNA_readtag_rv (PCR B)
- 15 µL Nextera PCR master mix (Illumina)
- 5 µL PCR primer cocktail (Illumina)

NOTES

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B16S_rDNA_readtag_fw and B16S_rDNA_readtag_rv are identical to SSU_rRNA_readtag_fw and SSU_rRNA_readtag_rv from the RNA based protocol, respectively.

Read-tag library preparation

Step 23.

Perform the PCR with an initial elongation at 72°C for 3 min, denaturation at 98°C for 3 s, followed by 10 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min and then a final extension at 72°C for 5 min.

Read-tag library preparation

Step 24.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 20 µL nuclease-free water

Read-tag library preparation

Step 25.

Size selection the read-tag libraries on an E-gel electrophoresis system with precast E-Gel CloneWell gels (Thermo Fisher Scientific):

- Use 20 µL of sample from above
- Use 500 ng GeneRuler 1 kB DNA ladder (Thermo Fisher Scientific) as a reference.
- Run the gel until the 500 bp marker is approximately 1 mm from the the elution well.
- Now collect 20 µL elution aliquots every 15 seconds, up to a total of 32 aliquots, and the visible passing of the 1500 bp marker.
- Pool every two aliquots to obtain 16 pooled aliquots per sample.

NOTES

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This size selection is perform in order to normalize the read-tag libraries with respect to sequencing efficiency. This results in even coverage of the full-length SSU molecules during the assembly.

Read-tag library preparation

Step 26.

Determine the size and the concentration of each fraction on an Agilent 2200 Tapestation with the High Sensitivity D1000 Screentape.

Read-tag library preparation

Step 27.

Calculate the effective sequencing concentration for each fraction based on the tapestation data and

the empirical formula:

C_{seq} = Peak molarity [pmol/l] * (-0.0124*(peak size [bp] – 215 bp) + 10.332).

Read-tag library preparation

Step 28.

Pool equal sequencing capacity of each fractions with an average size of 550-1200 bp.

NOTES

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We use the whole sample with an average size of approximately 1000 bp. The amounts of the other fractions are determined based on the sequencing concentration and volumen of this sample.

Read-tag library preparation

Step 29.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 12 µL nuclease-free water

Read-tag library preparation

Step 30.

Validate the final read-tag libraries on an Agilent 2200 Tapestation with the High Sensitivity D1000 Screentape.

Read-tag library preparation

Step 31.

Determine the concentration of the read-tag libraries on a Qubit 2.0 fluorometer with the Qubit dsDNA HS Assay kit.

- Use 1 µL sample
- Perform duplicate measurements

Linked-tag library preparation

Step 32.

Prepare a clonal library end-repair reaction in a 200 µL PCR tube:

- 4.6 µL diluted clonal library from step 18
- 16.65 µL nuclease-free water

- 2.5 µL 10X NEBNext End repair Reaction Buffer (New England Biolabs)
- 1.25 µL NEBNext End Repair Enzyme Mix (New England Biolabs)

Linked-tag library preparation

Step 33.

Perform end-repair at 20°C for 30 min.

Linked-tag library preparation

Step 34.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 10 µL nuclease-free water

Linked-tag library preparation

Step 35.

Prepare a intramolecular blunt-end ligation reaction in a 200 µL PCR tube:

- 2 µL sample from above
- 150 µL nuclease-free water
- 20 µL 50% (w/w) PEG 4000 solution (New England Biolabs)
- 20 µL 10X T4 DNA ligase buffer (New England Biolabs)
- 8 µL T4 DNA ligase (New England Biolabs)

Linked-tag library preparation

Step 36.

Perform ligation at 16°C for 1 hr.

Linked-tag library preparation

Step 37.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 10 µL nuclease-free water

Linked-tag library preparation

Step 38.

Prepare a junction amplification reaction in a 200 µL PCR tube:

- 8 µL sample from above
- 5 µL 10x PCR buffer (Qiagen)

- 1 µL 10 mM dNTP (Qiagen)
- 2.5 µL 10 µM SSU_rDNA_linktag_fw
- 2.5 µL 10 µM SSU_rDNA_linktag_rv
- 3µL 25 mM MgCl (Qiagen)
- 30.25 µL nuclease-free water
- 0.25 µL 5 U/µL Taq polymerase (Qiagen)

Linked-tag library preparation

Step 39.

Perform the PCR with an initial denaturation at 94°C for 3 min, followed by 20 cycles of denaturation at 94°C for 20 s, annealing at 56°C for 20 s, and extension at 72°C for 20 s and then a final extension at 72°C for 3 min.

Linked-tag library preparation

Step 40.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 12 µL nuclease-free water

Linked-tag library preparation

Step 41.

Validate the final read-tag libraries on an Agilent 2200 Tapestation with the High Sensitivity D1000 Screentape.

Linked-tag library preparation

Step 42.

Determine the concentration of the linked-tag library on a Qubit 2.0 fluorometer with the dsDNA HS Assay kit.

- Use 1 µL sample
- Perform duplicate measurements

Pooling of the read- and linked-tag libraries

Step 43.

Dilute the read- and linked-tag libraries as follows:

Platform	Read-tag libraries	Linked-tag library
MiSeq	1.3 ng/µL	0.3 ng/µL
HiSeq	0.93 ng/µL	0.21 ng/µL

Pooling of the read- and linked-tag libraries

Step 44.

Pool 4.6 µL of each read-tag library with 0.8 µL of the linked-tag library to create the sequencing ready library.