RNA based library preparation for high through-put 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 full-length SSU rRNA sequences from all domains of life without primer bias.

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

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Guidelines

Primers and adaptors used for library preparation:

Oligo name	sequence	Purification
SSU_rRNA_RT1	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNTCGCCTTAACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT2	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNCTAGTACGACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT3	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNTTCTGCCTACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT4	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNGCTCAGGAACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT5	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNAGGAGTCCACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT6	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNCATGCCTAACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT7	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNGTAGAGAGACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT8	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNCCTCTCTGACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT9	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNAGCGTAGCACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_RT10	AGGGGGGCAAAGATGAAGATNNNNNNNNNNNNNNNNCAGCCTCGACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	PAGE
SSU_rRNA_s	/5Phos/GGGCAATATCAGCACCAACAGAAA/3SpC3/	HPLC
SSU_rRNA_l	CTCCACCCAGACTCATCCATNNNNNNNNNNNNNNNNATAGAGGCTTTCTGTTGGTGCTGATATTGC	PAGE
SSU_rRNA_pcr_rv	AGGGGGGCAAAGATGAAGAT	HPLC
SSU_rRNA_pcr_fw	CTCCACCCAGACTCATCCAT	HPLC
SSU_rRNA_readtag_rv	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGGGGGCAAAGATGAAGAT	PAGE
SSU_rRNA_readtag_fw	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCCACCCAGACTCATCCAT	PAGE
SSU_rRNA_linktag_rv	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTGCAATATCAGCACCAACAGAAA	PAGE
SSU_rRNA_linktag_fw	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGATAGAGCGACAGGCAAGT	PAGE
SSU_rRNA_read2_rv	GCTCTTCCGATCTAGGGGGGCAAAGATGAAGAT	HPLC
SSU_rRNA_read2_fw	GCTCTTCCGATCTCTCCACCCAGACTCATCCAT	HPLC

Protocol

SSU rRNA size selection

Step 1.

Dilute the RNA to 40 ng/µL with nuclease-free water and transfer 25 µL to a 200 µL PCR tube.

SSU rRNA size selection

Step 2.

Heat denature the RNA at 70°C for 5 min. Snap cool the sample on an ice block for 2 min to prevent RNA from refolding. Immediately proceed to the next step.

SSU rRNA size selection

Step 3.

Isolated the SSU rRNA from total RNA by size selection on an E-gel electrophoresis system with a precast E-Gel CloneWell gel (Thermo Fisher Scientific):

- Use 20 µL of the heat denatured RNA (800 ng total RNA)
- 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 SSU rRNA peak.
- Pool every two aliquots to obtain 8 pooled aliquots per sample.

SSU rRNA size selection

Step 4.

Analyze the pooled aliquots on an Agilent 2200 Tapestation with the High Sensitivity RNA Screen tape and pool the aliquots that contains the SSU rRNA.

SSU rRNA size selection

Step 5.

Purify the sample using RNAClean XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 32 µL nuclease-free water

Poly(A) tailing of SSU rRNA

Step 6.

Prepare the poly(A) tailing reaction in a 200 µL PCR tube:

- 30 µL sample from above (10 – 200 ng SSU rRNA)
- 4 µL 10X *E. coli* Poly(A) Polymerase Reaction Buffer (New England Biolabs)
- 4 µL 10 mM ATP (New England Biolabs)
- 1.5 µL *E. coli* Poly(A) Polymerase (New England Biolabs)
- 1 µL RiboLock RNase Inhibitor (Thermo Fisher Scientific)

Poly(A) tailing of SSU rRNA

Step 7.

Perform polyadenylation at 37°C for 30 min.

Poly(A) tailing of SSU rRNA

Step 8.

Purify the sample using RNAClean XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Use 1.0x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 15 µL nuclease-free water

Poly(A) tailing of SSU rRNA

Step 9.

Validate the polyadenylation on an Agilent 2200 Tapestation using RNA Screentape. Use the untreated RNA from step 5 as a reference.

Poly(A) tailing of SSU rRNA

Step 10.

Dilute the sample to 2 ng/µL with nuclease-free water based on the gel electrophoresis results.

NOTES

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If the concentration of the sample is below 2 ng/µL use it as it is.

We have prepared successful libraries with as little as 0.45 ng/µL.

First strand cDNA synthesis

Step 11.

Prepare the adaptor priming reaction in a 200 µL PCR tube:

- 11.5 µL sample from above (5 - 23 ng polyadenylated SSU rRNA)
- 0.5 µL 100 µM SSU_rRNA_RT
- 1 µL 10 mM dNTP mix

First strand cDNA synthesis

Step 12.

Incubate the sample at 70°C for 5 min. Snap cooled on an ice block for 2 min. Proceed immediately to the next step.

First strand cDNA synthesis

Step 13.

Prepare the first strand cDNA synthesis reaction in a 200 µL PCR tube:

- 13 µL sample from above
- 4 µL 5x SSIV buffer (Thermo Fisher Scientific)
- 1 µL 200 U/µL SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific)
- 1 µL 100 mM DTT (Thermo Fisher Scientific)
- 1 µL RiboLock RNase Inhibitor (Thermo Fisher Scientific)

First strand cDNA synthesis

Step 14.

Perform cDNA synthesis at 50°C for 50 min, inactivate the reaction at 80°C for 10 min, and finally cool the sample to 37°C. Proceed immediately to the next step.

First strand cDNA synthesis

Step 15.

Add 1 µL 10 U/µL Ribonuclease H, from *E. coli* (cloned) (Thermo Fisher Scientific) to the cDNA from above, and incubate the sample at 37°C for 20 min to remove the RNA template.

First strand cDNA synthesis

Step 16.

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 11 µL nuclease-free water

Adaptor ligation

Step 17.

Prepare the single stranded adapter ligation reaction in a 200 µL PCR tube:

- 8.25 µL sample from above
- 2.5 µL 10 µM SSU_rRNA_s
- 5 µL 10x custom T4 RNA ligase buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM hexamminecobalt(III) chloride, 200 µM ATP, pH 8.0)
- 31.25 µL 40% (wt/wt) PEG 8000 (Thermo Fisher Scientific)
- 0.5 µL 1 mg/mL BSA (Thermo Fisher Scientific)

- 2.5 µL 10 U/µL T4 RNA ligase (Thermo Fisher Scientific)

Adaptor ligation

Step 18.

Perform ligation at 22°C overnight.

Adaptor ligation

Step 19.

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 50 µL nuclease-free water
- Repeat the purification with 1.0x beads and elude in 12 µL nuclease-free water

NOTES

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High amounts of PEG-8000 in the samples prevents stringent size selection during the AMPure XP purification. A two step AMPure purification is therefore performed to ensure the removal of free adaptor oligoes.

Second strand synthesis

Step 20.

Prepare the second strand cDNA synthesis reaction in a 200 µL PCR tube:

- 10 µL sample from above
- 26.75 µL nuclease free water
- 5 µL 10x PCR Buffer (Qiagen)
- 1 µL 10 mM dNTP (Qiagen)
- 2.5 µL 10 µM SSU_rRNA_l
- 2.5 µL 10 µM SSU_rRNA_pcr_rv
- 2 µL 25 mM $MgCl_2$
- 0.25 µL 5 U/µL Taq polymerase (Qiagen)

ANNOTATIONS

Elvira Blume 20 Apr 2018

Why do you use a non-proofreading polymerase? Have you ever tried using a proofreading polymerase e.g. Phusion or Q5? Is there any distinct disadvantage using these?

Second strand synthesis

Step 21.

Perform the second strand cDNA syntheis 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.

Second strand synthesis

Step 22.

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 25 µL nuclease-free water

Second strand synthesis

Step 23.

Prepare a single-standed DNA digestion reaction in a 200 µL PCR tube:

- 23 µL sample from above
- 3 µL 10X S1 nuclease buffer (Thermo Fisher Scientific)
- 3 µL 3 M NaCl (Thermo Fisher Scientific)
- 1 µL 10 U/µL S1 nuclease (Thermo Fisher Scientific)

NOTES

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This reaction removes the remaining single-stranded cDNA, which would otherwise hamper the following PCR.

Second strand synthesis

Step 24.

Perform digestion at at 25 °C for 25 min. Terminate the reaction by addition of 2 µL 0.5 M EDTA and heating to 70°C for 10 min.

Second strand synthesis

Step 25.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Dilute the sample to 50 µL with nuclease-free water (18 µL)
- Use 0.6x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 12 µL nuclease-free water

Primary library amplification

Step 26.

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

NOTES

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The ds cDNA is amplified by PCR to obtain enough product for validation, size selection, and quantification.

Primary library amplification

Step 27.

Perform the PCR with an initial denaturation at 94°C for 3 min, followed by 20 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.

Primary library amplification

Step 28.

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 library amplification

Step 29.

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

Primary library size selection

Step 30.

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.

Primary library size selection

Step 31.

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 cDNA peak.

Primary library size selection

Step 32.

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 33.

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 34.

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 * $6.022x10^{23}$) / (length in bp * $1x10^{9}$ * 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 35.

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 SSU_rRNA_pcr_fw

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

Clonal library preparation

Step 36.

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 37.

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 38.

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

Clonal library preparation

Step 39.

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 40.

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

Read-tag library preparation

Step 41.

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 42.

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

Read-tag library preparation

Step 43.

Purify the sample using AMPure XP beads:

- Perform purification in a 2 mL Eppedorf tube
- Dilute the sample to 100 µL with nuclease-free water (63 µL)
- Use 0.6x beads
- Wash 2x with freshly prepared 80% ethanol
- Elude in 42 µL nuclease-free water

Read-tag library preparation

Step 44.

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 SSU_rRNA_readtag_fw (PCR A) or SSU_rRNA_readtag_rv (PCR B)
- 15 µL Nextera PCR master mix (Illumina)
- 5 µL PCR primer cocktail (Illumina)

Read-tag library preparation

Step 45.

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 46.

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 47.

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 48.

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 49.

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 50.

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 51.

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 52.

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

Read-tag library preparation

Step 53.

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 54.

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

- 4.6 µL diluted clonal library from step 40
- 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 55.

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

Linked-tag library preparation

Step 56.

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 57.

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 58.

Perform ligation at 16°C for 1 hr.

Linked-tag library preparation

Step 59.

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 60.

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_rRNA_linktag_fw
- 2.5 µL 10 µM SSU_rRNA_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 61.

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 62.

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 63.

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

Linked-tag library preparation

Step 64.

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 65.

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 66.

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