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ssUMI: high-throughput long-read sequencing workflow for highly-accurate near full-length 16S rRNA genes on the ONT platform

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ABSTRACT

This is the online protocol for near full-length 16S rRNA amplicon sequencing with unique molecule identifiers (ssUMI) on the Nanopore platform. Sequencing libraries are prepared with Oxford Nanopore Native Barcoding kit 96 V12 or V14 (SQK-NBD112.96 or SQK-NBD114.96) for sequencing on PromethION platforms in super high accuracy mode (260 bps).

The near full-length 16S rRNA amplicons are generated with primers 8F: AGRGTTYGATYMTGGCTCAG and 1391R: GACGGGCGGTGWGTRCA; the UMI design was adapted from (Karst and Ziels, 2021) and the Oxford Nanopore community protocol.

For more details, please check our preprint:
https://www.biorxiv.org/content/10.1101/2023.06.19.544637v1

MANUSCRIPT CITATION:
Lin, X., Waring, K., Tyson, J. & Ziels, R. M. High-accuracy meets high-throughput for microbiome profiling with near full-length 16S rRNA amplicon sequencing on the Nanopore platform. 2023.06.19.544637 Preprint at https://doi.org/10.1101/2023.06.19.544637 (2023).

Protocol status: Working
We use this protocol and it's working

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MATERIALS

Primers and probe (order from IDT)

1. ddPCR

ddPCR-8F: AGRGTTYGATYMTGGCTCAG

ddPCR-1391R: GACGGGCGGTGWGTRCA

ddPCR-515F-FAM: (FAM)-TGYCAGCMG-(ZEN)-CCGCGGTAA-(IBFQ)

2. ssUMI-PCR

ssUMI-8F-UMI: GTATCGTGTAGAGACTGCGTAGG NNNYRNNNYRNNNYRNNNA GRGTTYGATYMTGGCTCAG (PAGE purified)

ssUMI-1391R-UMI: AGTGATCGAGTCAGTGCGAGTG NNNYRNNNYRNNNYRNNN GACGGGCGGTGWGTRCA (PAGE purified)

ssUMI-Universal-F: GGTGCTGAAGAAAGTTGTCGGTGTCTTTGTGTTAACCGTATCGTGTAGAGACTGCGTAGG

ssUMI-Universal-R: GGTGCTGAAGAAAGTTGTCGGTGTCTTTGTGTTAACCAGTGATCGAGTCAGTGCGAGTG

PROTOCOL MATERIALS

Ultrapure Water **Thermofisher Catalog #10977023** Step 1

ddPCR 96-well plates **Bio-Rad Laboratories Catalog #12001925** Step 2.1

PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #1814040** Step 2.2

Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** In 6 steps

NEB Blunt/TA Ligase Master Mix **Catalog #M0367** Step 11

NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #E7546L** Step 10

NEBNext Quick Ligation Reaction Buffer (5X) - 2.0 ml **New England Biolabs Catalog #B6058S** Step 14

Quick T4 DNA Ligase **New England Biolabs Catalog #E7180S** Step 14

BEFORE START INSTRUCTIONS

This protocol was developed with 96 samples, and we highly recomand using multi-channel pipettes and/or an automatic liquid handler.

Time can be saved by preparing master mixes first, before PCR steps. The master mix for PCR steps should be prepared in a Master Mix (PCR) Hood. To avoid cross-contamination make sure that your original stock reagents have no contact with any amplified DNA material.

A Negative Control (nuclease-free H_2O) should be included.

Keep the enzymes on ice and thaw the other reagents at room temperature, mix, then spin down before placing on ice.

All steps after ssUMI-EarlyPCR2 should be performed in post-PCR area.

Sample pre-dilution and ddPCR quantification of starting material

12h

1 Estimate the full-length 16S rRNA concentration in the DNA extract (e.g., sample), and dilute the sample to 2h [M] 20000 copies/µL with Ultrapure Water **Thermofisher Catalog #10977023** .

Note

The concentration of full-length 16S rRNA copy numbers can be estimated based on previous experiments, literature, or qPCR. It's important to pre-dilute the DNA sample to ensure the 16S rRNA copy number is within the dynamic range of ddPCR instrument (1-125,000 copies/µl).

2 Run ddPCR quantification with 2.5 µL diluted DNA sample.

2.1 Prepare the ddPCR mastermix in a PCR hood, vortex to mix and dispense 19.5 µL into ea... 10m well of the ddPCR 96-well plates **Bio-Rad Laboratories Catalog #12001925** . Each

component is thawed at Room temperature , vortexed to mix, then spun down. A 10% pipetting loss should be included when preparing mastermix for multiple samples.

	A	B	C
	Component	Volume (per rxn)	Volume (96 rxn)
	ddPCR-8F (10 µM)	1.98 µL	217.8 µL
	ddPCR-1391R (10 µM)	1.98 µL	217.8 µL
	ddPCR-515F-FAM (10 µM)	0.55 µL	60.5 µL
	Nuclease-free water	3.99 uL	438.9 µL
	ddPCR Supermix for Probes (No dUTP)	11 µL	1210 µL
	Total volume	19.5 µL	2145 µL

2.2 Add 2.5 µL diluted DNA sample to each well, seal the plate with PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #1814040** , vortex to mix, and spin down. 10m

2.3 Load the plates on the QX200 AutoDG Droplet Digital PCR System, and run droplet generation. 40m

2.4 Run PCR in a deep-well thermalcycler using the following program (set total volume as 40 µL) : 6h

	A	B	C	D	E
	Step	Temperature	Ramp rate	Time	Cycles
	Enzyme Activation	95°C	2°C/sec	10 min	1
	Denaturation Annealing Extension	94°C 60°C 72 °C	2°C/sec	30 sec 1 min 4 min	50
	Enzyme Deactivation	98 °C	2°C/sec	10 min	1
	Hold	4°C	2°C/sec	∞	-

Note

ddPCR requires using a doubled reaction volume to prevent droplet burst during the PCR reaction

2.5 Count droplets in QX200 AutoDG Droplet Digital PCR System using manufacturer's protocols. 2h

ssUMI-PCR1: UMI tagging and cleanup 2h 10m

3 In this step, the near full-length 16S rRNA gene primers containing UMIs are annealed to both ends of th 1h 10m DNA template using 2 rounds of PCR. Only one copy of a dual-end UMI-tagged amplicon will be generated for each input molecule.

3.1 Adjust the input DNA concentration to [M] 5000 copies/µL with nuclease-free water (100,000 16S rRNA gene copies in 20 µL) based on the ddPCR measured near full-length 16S rRNA gene copy numbers (see Step 2.5). 30m

3.2 Prepare ssUMI-PCR1 mastermix in a PCR hood, flick the tube to mix and dispense 30 µL into each well of a 96-well PCR plate. Each component is thawed On ice , mixed by flicking the tube, then spun down. A 10% pipetting loss should be included when preparing mastermix for multiple samples. 10m

	A	B	C
	Component	Volume (per rxn)	Volume (96 rxn)
	2X Platinum™ SuperFi™ II Green PCR Master Mix	25 µL	2750 µL
	100 µM UMI_8F (500 nM final)	0.25 µL	27.5 µL
	100 µM UMI_1391R (500 nM final)	0.25 µL	27.5 µL
	Nuclease-free water	4.5 µL	495 µL
	Total volume	30 µL	3300 µL

3.3 Add 20 µL DNA samples from step 3.1 (100,000 full-length 16S rRNA gene copies) into each well of a 96-well PCR plate, 5m

3.4 Run PCR in a thermocycler using the following program (total volume: 50 µL) : 22m

	A	B	C	D	E
	Step	Temperature	Ramp rate	Time	Cycles
	Initial denaturation	98°C	max	3 min	1
	Denaturation Annealing Extension	98°C Touchdown from 66°C to 60°C 72°C	max 0.2°C/sec max	30 sec 90 sec 3 min	2
	Final extension	72 °C	max	5 min	1
	Hold	4 °C	max	∞	-

4 ssUMI-PCR1 cleanup using Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** with a bead-to-sample ratio of 0.6. 1h

4.1 Homogenize Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** solution by vortexing.

4.2 Add 30 µL bead solution to the 50 µL ssUMI-PCR1 product (ratio 0.6) and mix by pipetting or brief vortexing.

4.3 Spin down, and then incubate at Room temperature for 00:10:00 . 10m

4.4 Place the plate on a magnetic rack to pellet the beads, and wait until the solution is clear on the side of each well (~5 min).

4.5 Keep the plate on the magnetic rack, carefully discard the supernatant.

4.6 Wash beads by adding 200 µL fresh 80% ethanol along the opposite side of the beads.

4.7 Wait 30 seconds and discard the ethanol.

4.8 Repeat the washing steps (Steps 4.5-4.7).

4.9 Spin down and place the plate back on the magnetic rack, then remove residual ethanol with a smaller pipette.

> **Note**
>
> Not removing residual ethanol could cause primer and UMI carryover and high chimera rates.

4.10 Let the beads air dry for a maximum of 2 min (but do not overdry the beads to the point of cracking).

4.11 Remove the plate from the magnetic rack and elute the purified DNA by adding 20 µL nuclease-free water and mix by pipetting.

4.12 Incubate at Room temperature for 00:05:00 . 5m

4.13 Place the plate on the magnetic rack to pellet the beads and wait until the eluate is clear and colourless.

4.14 Pipette off the 18 µL supernatant to a new 96-well PCR plate.

ssUMI-EarlyPCR2: amplification of UMI-tagged amplicons 2h 20m

5 In this step, the dual-UMI-tagged near full-length 16S rRNA gene amplicons are further amplified with P 1h 20m

5.1 Prepare ssUMI-PCR2 mastermix in PCR hood, flick the tube to mix. Each component is thawed 10m On ice , mixed by flicking the tube, then spun down. A 10% pipetting loss should be included when preparing mastermix for multiple samples.

	A	B	C
	Component	Volume (per rxn)	Volume (96 rxn)
	2X Platinum™ SuperFi™ II Green PCR Master Mix	25 µL	2750 µL
	100 µM UMI_8F (500 nM final)	0.5 µL	55 µL
	100 µM UMI_1391R (500 nM final)	0.5 µL	55 µL

	A	B	C
	25 mM MgCl (1mM final)	2 µL	220 µL
	Nuclease-free water	4 µL	440 µL
	Total volume	32 µL	3520 µL

5.2 Dispense 32 µL into each well of the 96-well PCR plate containing 18 µL cleaned ssUMI-PCR1 product. 5m

5.3 Spin down and run PCR in a thermocycler using the following program (total volume: 50 µL) : 1h 5m

	A	B	C	D	E
	Step	Temperature	Ramp rate	Time	Cycles
	Initial denaturation	98°C	max	3 min	1
	Denaturation Annealing Extension	98°C Touchdown from 70°C to 63°C 72°C	max 0.2°C/sec max	20 sec 45 sec 3 min 30s	5
	Denaturation Extension	98°C 72°C	max max	20 sec 4 min	5
	Final extension	72 °C	max	5 min	1
	Hold	4 °C	max	∞	-

6 ssUMI-EarlyPCR2 cleanup using Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** with a bead-to-sample ratio of 0.6. 1h

Note

This step should be conducted in a post-PCR area to prevent amplicon contamination.

6.1 Homogenize Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** solution by vortexing.

6.2 Add 30 µL bead solution to the 50 µL ssUMI-PCR1 product (ratio 0.6) and mix by pipetting or brief vortexing.

6.3 Spin down, and then incubate at Room temperature for 00:10:00 . 10m

6.4 Place the plate on a magnetic rack to pellet the beads, and wait until the solution is clear on the side of each well (~5 min).

6.5 Keep the plate on the magnetic rack, carefully discard the supernatant.

6.6 Wash beads by adding 200 µL fresh 70% ethanol along the opposite side of the beads.

6.7 Wait 30 seconds and discard the ethanol.

6.8 Repeat the washing steps (Steps 6.5-6.7).

6.9 Spin down and place the plate back on the magnetic rack, then remove residual ethanol with a smaller pipette.

6.10 Let the beads air dry for a maximum of 2 min (but do not overdry the beads to the point of cracking).

6.11 Remove the plate from the magnetic rack and elute the purified DNA by adding 20 µL nuclease-free water and mix by pipetting.

6.12 Incubate at Room temperature for 00:05:00 . 5m

6.13 Place the plate on the magnetic rack to pellet the beads and wait until the eluate is clear and colourless.

6.14 Pipette off the 18 µL supernatant to a new 96-well PCR plate.

Note

This is a safe stopping point.
For short-term storage, samples can be stored at 4 °C overnight;
For long-term storage, samples should be stored at -20 °C .

ssUMI-LatePCR2: amplification of UMI-tagged amplicons

2h 40m

7 Similar to ssUMI-EarlyPCR2, the dual-UMI-tagged near full-length 16S rRNA gene amplicons are further amplified with 15 cycles of PCR.

7.1 Prepare the same mastermix as in Step 5.1 (ssUMI-PCR2 mastermix) in PCR hood, flick the tub 10m to mix. Each component is thawed On ice , mixed by flicking the tube, then spun down. A 10% pipetting loss should be included when preparing mastermix for multiple samples.

	A	B	C
	Component	Volume (per rxn)	Volume (96 rxn)
	2X Platinum™ SuperFi™ II Green PCR Master Mix	25 µL	2750 µL
	100 µM UMI_8F (500 nM final)	0.5 µL	55 µL
	100 µM UMI_1391R (500 nM final)	0.5 µL	55 µL
	25 mM MgCl (1mM final)	2 µL	220 µL
	Nuclease-free water	4 µL	440 µL
	Total volume	32 µL	3520 µL

7.2 Dispense 32 µL into each well of the 96-well PCR plate containing 18 µL cleaned ssUMI-EarlyPCR2 product. 5m

Note

This step should be conducted in a post-PCR area to prevent amplicon contamination.

7.3 Spin down and run PCR in a thermocycler using the following program (total volume: 50 µL 1h 25m):

	A	B	C	D	E
	Step	Temperature	Ramp rate	Time	Cycles
	Initial denaturation	98°C	max	3 min	1
	Denaturation Extension	98°C 72°C	max max	20 sec 4 min	15

	A	B	C	D	E
	Final extension	72 °C	max	5 min	1
	Hold	4 °C	max	∞	-

8 ssUMI-LatePCR2 cleanup using Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** with a bead-to-sample ratio of 0.6. 1h

> **Note**
>
> This step should be conducted in a post-PCR area to prevent amplicon contamination.

8.1 Homogenize Mag-Bind® TotalPure NGS beads **Omega Biotek Catalog #M1378-01** solution by vortexing.

8.2 Add 30 µL bead solution to the 50 µL ssUMI-PCR1 product (ratio 0.6) and mix by pipetting or brief vortexing.

8.3 Spin down , and then incubate at Room temperature for 00:10:00 . 10m

8.4 Place the plate on a magnetic rack to pellet the beads, and wait until the solution is clear on the side of each well (~5 min).

8.5 Keep the plate on the magnetic rack, carefully discard the supernatant.

8.6 Wash beads by adding 200 µL fresh 70% ethanol along the opposite side of the beads.

8.7 Wait 30 seconds and discard the ethanol.

8.8 Repeat the washing steps (Steps 8.5-8.7).

8.9 Spin down and place the plate back on the magnetic rack, then remove residual ethanol with a smaller pipette.

8.10 Let the beads air dry for a maximum of 2 min (but do not overdry the beads to the point of cracking).

8.11 Remove the plate from the magnetic rack and elute the purified DNA by adding 20 µL nuclease-free water and mix by pipetting.

8.12 Incubate at Room temperature for 00:05:00 . 5m

8.13 Place the plate on the magnetic rack to pellet the beads and wait until the eluate is clear and colourless.

8.14 Pipette off the 18 µL supernatant to a new 96-well PCR plate.

9 Quantify 1 µL of the cleaned ssUMI-LatePCR2 product using a Qubit fluorometer with Qubit dsDNA HS Assay Kit. Amplicons should have concentration higher than [M] 4 ng/µL

Note

This is a safe stopping point.
For short-term storage, samples can be stored at 4 °C overnight;
For long-term storage, samples should be stored at -20 °C .

Nanopore sequencing library preparation using Native Barcoding Kit 12/14

4h

10 DNA End-prep with

1h 30m

NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #E7546L**

Reagent preparation before starting this step

1. Thaw the NEBNext Ultra II End Prep Reaction Buffer On ice , check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise all precipitate.
2. Place the Ultra II End Prep Enzyme Mix On ice

Note

Do NOT vortex the Ultra II End Prep Enzyme Mix.

10.1 In a clean 96-well plate, aliquot 50 fmol (46.4 ng) of amplicon per sample (based on quantification in Step 9).

10.2 Make up each sample to 12.5 µL using nuclease-free water. Mix gently by pipetting and spin down.

10.3 Prepare End-prep mastermix, mix by pipetting 10-20 times. A 10-15% pipetting loss should be included when preparing mastermix for multiple samples.

	A	B	C
	Reagent	Volume (per rxn)	Volume (96 rxn)
	Ultra II End-prep Reaction Buffer	1.75 µl	210 µl
	Ultra II End-prep Enzyme Mix	0.75 µl	90 µl
	Total	2.5 µl	300 µl

10.4 Add 2.5 µL End-prep mastermix to each well containing DNA amplicons, pipetting 10-20 times to mix and spin down briefly.

10.5 Using a thermocycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

Note

Take forward the end-prepped DNA into the native barcode ligation step.

If users want to pause the library preparation here, we recommend cleaning up your sample with 1X AMPure XP Beads (AXP) and eluting in nuclease-free water before storing at 4 °C .

11 Native barcode ligation with Native Barcoding Kit 12 or Kit 14. 1h

Reagent preparation before starting this step

1. Thaw NEB Blunt/TA Ligase Master Mix **Contributed by users Catalog #M0367** at Room temperature, spin down and mix by performing 10 full volume pipette mixes, then place On ice.

2. Thaw the AMPure XP Beads (AXP) at Room temperature and mix by vortexing. Keep the beads at Room temperature.

3. Thaw the EDTA (provided with the sequencing kit) at Room temperature and mix by vortexing. Then spin down and place On ice.

4. Thaw the Native Barcodes NB01-96 at Room temperature. Individually mix the barcodes by pipetting, spin down, and place them On ice.

11.1 In a new 96-well plate, add the reagents in the following order per well (select unique barcode for each sample on the plate). Between each addition, pipette mix 10 times.

	A	B
	Reagent	Volume
	End-prepped DNA	3.75 µL
	Native Barcode (NB01-96)	1.25 µL
	Blunt/TA Ligase Master Mix	5 µL
	Total	10 µL

11.2 Thoroughly mix the reaction by gently pipetting 10 times and then briefly spin down.

11.3 Incubate for 00:20:00 at Room temperature. 20m

11.4 Add 1 µL EDTA to each well and mix thoroughly by pipetting and spin down briefly.

11.5 Pool all barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

12 Native Barcoding reaction cleanup with 0.4x AMPure XP Beads. 30m

Before start of this step:

1. Prepare 2 mL fresh 80% ethanol in nuclease-free water.
2. Pre-heat an incubator to 37 °C .

12.1 Resuspend the AMPure XP Beads (AXP) by vortexing.

12.2 Add 422 µL (for 96 samples) AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting for a 0.4x clean.

12.3 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at Room temperature . 10m

12.4 Spin down the sample and pellet on a magnet for 00:05:00 . Keep the plate on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant. 5m

12.5 Keep the tube on the magnetic rack and wash the beads with 700 µL freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

> **Note**
>
> If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

12.6 Repeat the previous step (step 12.5).

12.7 Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not overdry the pellet to the point of cracking.

12.8 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water 35 µl nuclease-free water by gently flicking or pipetting.

12.9 Incubate for 00:10:00 at 37 °C . Every 2 minutes, agitate the sample by gently flicking 10m for 10 seconds to encourage DNA elution.

12.10 Pellet the beads on a magnetic rack until the eluate is clear and colourless.

12.11 Remove and retain 35 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

13 Quantify 1 µL of the eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit.

Note

This is a safe stopping point.

Samples can be stored at 4 °C overnight

14 Nanopore sequencing adapter ligation 30m

Reagent preparation before starting this step

1. Thaw the NEBNext Quick Ligation Reaction Buffer (5X) - 2.0 ml **New England Biolabs Catalog #B6058S** at Room temperature, spin down and mixed by performing 10 full volume pipette mixes.

2. Spin down the Adapter Mix II (AMII H, Kit 12) or Native Adapter (NA, Kit 14) and Quick T4 DNA Ligase **New England Biolabs Catalog #E7180S**, pipette mix and place on ice.

3. Thaw the Elution Buffer (EB) and Short Fragment Buffer (SFB) at Room temperature and mix by vortexing. Then spin down and place On ice.

Note

Do NOT vortex the Quick T4 DNA Ligase.

14.1 In a 1.5 ml Eppendorf LoBind tube, mix in the following order. Between each addition, pipette mix 10 times.

	A	B
	Reagent	Volume
	Pooled barcoded sample	30 µL
	Native Adapter (NA)	5 µL

	A	B
	NEBNext Quick Ligation Reaction Buffer (5x)	10 µL
	Quick T4 DNA Ligase	5 µL
	Total	50 µL

14.2 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

14.3 Incubate the reaction for 00:20:00 at Room temperature . 20m

15 Adapter Ligation reaction cleanup with 0.4x AMPure XP Beads. 30m

Before start of this step:
Pre-heat an incubator to 37 °C .

15.1 Resuspend the AMPure XP Beads (AXP) by vortexing.

15.2 Add 20 µL of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

15.3 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at Room temperature . 10m

15.4 Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

15.5 Wash the beads by adding 125 µL Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

15.6 Repeat the previous step (Step 15.5).

15.7 Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

15.8 Remove the tube from the magnetic rack and resuspend pellet in 25 µL Elution Buffer (EB).

15.9 Spin down and incubate for 00:10:00 at 37 °C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution. 10m

15.10 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

15.11 Remove and retain 25 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

16 Quantify 1 µL of the eluted sample using a Qubit fluorometer with Qubit dsDNA HS Assay Kit. Then make up the library to 32 µl at 10-20 fmol (18.4 ng).

Note

This is a safe stop point.

For short-term storage, sequencing libraries can be stored in Eppendorf DNA LoBind tubes at 4 °C For single use and long-term storage of more than 3 months, we recommend storing libraries at -80 °C in Eppendorf DNA LoBind tubes.