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HiDEF-seq V.4

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We use this protocol and it's working

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Abstract

This is the HiDEF-seq v3.1 library preparation protocol for bulk, single-molecule fidelity, long-read sequencing. This version of the HiDEF-seq protocol is designed for whole-genome profiling via random fragmentation and larger fragments than in our prior protocol for increased cost-efficiency. See our paper "DNA mismatch and damage patterns revealed by single-molecule sequencing" (Liu & Costa et al.) for more information.

Introduction

1 This is the HiDEF-seq v3 protocol for single-molecule sequencing with single-molecule fidelity on PacBio sequencers.

This version of the protocol enables genome-wide profiling by random fragmentation to an average size of ~4 kb. The protocol can be performed either with A-Tailing (for high-quality DNA per TapeStation analysis) or without A-Tailing (for low-quality / fragmented DNA).

Required Materials

2 **Instruments**

	Instrument	Supplier	Product #
	Megaruptor 3	Diagenode	B06010003
	NanoDrop One	Thermo Scientific	ND-ONE-W
	Qubit 4 Fluorometer	Invitrogen	Q33226
	4150 TapeStation System	Agilent	G2992AA
	96S Super Magnet	Alpaqua	A001322
	Mastercycler X50l Thermal Cycler	Eppendorf	-
	ThermoMixer C	Eppendorf	-
	Centrifuge 5424 R	Eppendorf	-
	BenchMixer Vortex Mixer	Benchmark Scientific	BV1000
	Corning Mini Centrifuge	Corning	6770

Reagents and consumables

	Item	Supplier	Product #	Kit	Kit #
	DNA LoBind Tube 0.2mL	Axygen	PCR-02-L-C		
	DNA LoBind Tube 0.5mL	Eppendorf	022431005		

	Item	Supplier	Product #	Kit	Kit #
	DNA LoBind Tube 1.5mL	Eppendorf	022431021		
	Nuclease free water	Thermo Fisher	AM9937		
	Molecular-grade ethanol	Fisher	BP2818100		
	1M Tris pH 8	Invitrogen	AM9855G		
	5M NaCl	Promega	V4221		
	10% SDS solution	Invitrogen	AM9822		
	0.5M EDTA	Invitrogen	AM9260G		
	50mM β-Nicotinamide adenine dinucleotide (NAD+)	NEB	B9007S		
	100mM dATP	Thermo Fisher	R0141		
	10mM ddCTP	Jena Bioscience	NU-1016	10mM ddNTP Bundle	NU-1019
	10mM ddGTP	Jena Bioscience	NU-1017	10mM ddNTP Bundle	NU-1019
	10mM ddTTP	Jena Bioscience	NU-1018	10mM ddNTP Bundle	NU-1019
	Buffer SRE	Pacific Biosciences	102-280-900	SRE Kit	102-208-300
	Megaruptor 3 Shearing Kit	Hologic Diagenode	E07010003		
	Hydropore - short (2-9 kb)	Hologic Diagenode	E07010001		
	10X NEBuffer r1.1	NEB	B6001S		
	10X NEBuffer 4	NEB	B7004S		
	10X rCutSmart Buffer	NEB	B6004S		
	10X T4 DNA Ligase Buffer	NEB	B0202S		
	10X Mung Bean Nuclease Buffer	Takara	2420A		

	Item	Supplier	Product #	Kit	Kit #
	Mung Bean Nuclease	Takara	2420A		
	Nuclease P1	NEB	M0660S		
	T4 Polynucleotide Kinase (PNK)	NEB	M0201S		
	E. Coli DNA Ligase	NEB	M0205S		
	Klenow Fragment (3'→5' exo-)	NEB	M0212S		
	NEBNext Ultra II Ligation Master Mix	NEB	E7648A	NEBNext Ultra II Ligation Module	E7595S
	NEBNext Ligation Enhancer	NEB	E7374A	NEBNext Ultra II Ligation Module	E7595S
	Exonuclease I (E. coli)	NEB	M0293S		
	RecJf	NEB	M0264S		
	T7 Exonuclease	NEB	M0263S		
	Ampure PB Beads	Pacific Biosciences	100-265-900		
	Elution Buffer	Pacific Biosciences	101-633-500		
	SMRTbell adapter index plate 96A (for A-tailed libraries)	Pacific Biosciences	102-009-200		
	Barcoded non-overhang adapters (for non-A-tailed libraries)	IDT	Custom Order (see below table)		
	Qubit 1X dsDNA HS Assay Kit	Thermo Fisher	Q33231		
	Qubit Assay Tubes	Thermo Fisher	Q32856		
	Genomic DNA Ladder for ScreenTape	Agilent	5190-6292	Genomic DNA Reagents	5067-5366
	Genomic DNA Sample Buffer for ScreenTape	Agilent	5190-6293	Genomic DNA Reagents	5067-5366

	Item	Supplier	Product #	Kit	Kit #
	Genomic DNA ScreenTape	Agilent	5067-5365		
	High Sensitivity D5000 Ladder for ScreenTape	Agilent	5190-7747	High Sensitivity D5000 Reagents	5067-5593
	High Sensitivity D5000 Sample Buffer for ScreenTape	Agilent	5190-7745	High Sensitivity D5000 Reagents	5067-5593
	High Sensitivity D5000 ScreenTape	Agilent	5067-5592		
	Optical Tube Caps, 8x for ScreenTape	Agilent	401425		
	Optical Tube Strips, 8x for ScreenTape	Agilent	401428		

3 Barcoded non-overhang adapters (**only for non-A-tailed libraries**):

- Order with HPLC purification

	Adapter ID	Sequence	Barcode
	bcAd2001	/5Phos/ATACTCGTCGCACGATCTTTTCCTCCTCCTCCGTTGTTGTTGTTGATCGTGCGACGAGTAT	ATCGTGCGACGAGTAT
	bcAd2002	/5Phos/ATACTCATGACATGCACTTTTCCTCCTCCTCCGTTGTTGTTGTTGTGCATGTCATGAGTAT	TGCATGTCATGAGTAT
	bcAd2003	/5Phos/ATACTCGAGCACTCGTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGACGAGTGCTCGAGTAT	ACGAGTGCTCGAGTAT
	bcAd2004	/5Phos/ATACTCGAGCACTGCACTTTTCCTCCTCCTCCGTTGTTGTTGTTGTGCAGTGCTCGAGTAT	TGCAGTGCTCGAGTAT
	bcAd2005	/5Phos/ATACTCGATCGAGTCACTTTTCCTCCTCCTCCGTTGTTGTTGTTGTGACTCGATCGAGTAT	TGACTCGATCGAGTAT
	bcAd2006	/5Phos/ATACTCAGATCGCATGCTTTTCCTCCTCCTCCGTTGTTGTTGTTGCATGCGATCTGAGTAT	CATGCGATCTGAGTAT

	Adapter ID	Sequence	Barcode
	bcAd2007	/5Phos/ATACTCAGATGCTAGTCTTTTCCTCC TCCTCCGTTGTTGTTGTTGACTAGCATCTGAG TAT	ACTAGCATCTGAGTA T
	bcAd2008	/5Phos/ATACTCAGACTAGCGTCTTTTCCTCC TCCTCCGTTGTTGTTGTTGACGCTAGTCTGA GTAT	ACGCTAGTCTGAGTA T

DNA Quality Control

4 Measure sample input DNA library concentration, A260/A280, and A260/A230 using a NanoDrop instrument.

5 Measure sample input DNA library concentration using the Qubit 1X dsDNA HS Assay (Q33231).

6 Evaluate DNA quality:
- Pure DNA will have an A260/280 absorbance ratio of 1.8, and an A260/230 ratio of 2.0 or higher.
- Evaluate discordance between NanoDrop and Qubit measurement to assess for RNA contamination, and treat with RNase and cleanup if needed.
- For challenging sample types that fail sequencing due to the presence of co-extracted inhibitors, use the DNeasy PowerClean Pro Cleanup Kit (Qiagen) to further clean the DNA per PacBio's recommendation.
- See this note for additional details:

Technical-Note-Preparing-DNA-for... 4.4MB

7 Measure sample input DNA size distribution with the Genomic DNA ScreenTape assay (5067-5366 and 5067-5593).
- Samples with low DNA Integrity Numbers (i.e., fragmented DNA) should be prepared as non-A-tailed libraries to avoid fragmentation-related ssDNA artifacts.
- For highly degraded DNA, skip the 'SRE cleanup' step and perform instead the '0.75 X Size Selection with 75% Diluted Beads' step.
- Samples with high DNA Integrity Numbers can also be prepared as non-A-tailed libraries if sufficient input DNA is available (see SRE cleanup section for recommended input DNA amounts).

Prepare reagents

8 80% Ethanol:

	Component	Starting Concentration	Final Concentration	Input (mL)
	Ethanol (BP2818100)	100 %	80 %	8
	Nuclease Free Water (AM9937)			2
	Total			10

- Vortex mix

9 10 mM Tris pH8:

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			990
	1 M Tris pH 8 (AM9855G)	1000 mM	10 mM	10
	Total			1000

- Vortex mix

10 10 X Annealing Buffer:

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			400
	1 M Tris pH 8 (AM9855G)	1000 mM	100 mM	50
	5 M NaCl (V4221)	5000 mM	500 mM	50
	Total			500

- Vortex mix

11 1 X Shearing Buffer :

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			988
	1 M Tris pH 8 (AM9855G)	1000 mM	10 mM	10
	5 M NaCl (V4221)	5000 mM	10 mM	2
	Total			1000

- Vortex mix

12 1 % SDS:

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			900
	SDS, 10 % Solution, RNase-free (AM9822)	10 %	1 %	100
	Total			1000

- Pipette mix and spin down

13 500 µM NAD+:

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			198
	β-Nicotinamide adenine dinucleotide (NAD+) (B9007S)	50 mM	500 µM	2
	Total			200

- Pipette mix and spin down
- Split into 10 µL aliquots and store at -80C

14 1 mM dATP / ddBTP Mix (**only for A-tailed libraries**):

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			69
	100 mM dATP (R0141)	100 mM	1 mM	1
	10 mM ddCTP (NU-1016)	10 mM	1 mM	10
	10 mM ddGTP (NU-1017)	10 mM	1 mM	10
	10 mM ddTTP (NU-1018)	10 mM	1 mM	10
	Total			100

- Pipette mix and spin down
- Store at -20C

15 1 mM ddBTP Mix (**only for non-A-tailed libraries**):

	Component	Starting Concentration	Final Concentration	Input (µL)
	Nuclease Free Water (AM9937)			21
	10 mM ddCTP (NU-1016)	10 mM	1 mM	3
	10 mM ddGTP (NU-1017)	10 mM	1 mM	3
	10 mM ddTTP (NU-1018)	10 mM	1 mM	3
	Total			30

- Pipette mix and spin down
- Store at -20C

16 75% Diluted Ampure PB Beads (**only for highly degraded DNA libraries that skip 'SRE Cleanup'**):

	Component	Input (uL)
	Elution Buffer (101-633-500)	250
	Ampure PB Beads (100-265-900)	750
	Total	1000

- Vortex mix

Prepare barcoded non-overhang adapters (only for non-A-tailed libraries)

17 If not previously performed, reconstitute adapters to 100 µM as follows:
- Add 10 * (nmoles listed on tube) µL of nuclease-free water
- Let sit at room temperature for 3 min to avoid losing dry oligo
- Vortex mix to ensure capture of all dry oligo
- Spin down
- Split into aliquots in DNA LoBind tubes to limit each aliquot to 10 freeze-thaw cycles

18 Make 20 µM annealed adapters in 0.2 mL DNA LoBind tubes (perform for each barcoded adapter) as follows:

	Components	Starting Concentration	Final Concentration	Input (µL)
	Nuclease-Free Water (AM9937)			14.00
	10 X Annealing Buffer	10 X	1 X	2.00
	100 µM Barcoded non-overhang adapter	100 µM	20 µM	4.00
	Total			20.00

- Pipette mix and spin down

Run Hairpin Annealing thermocycler protocol:
- Heated lid on at 105°C
- 95 °C hold

Place the 20 µM adapter dilution on the thermocycler for **3 mins**, and then immediately transfer the adapter to **room temperature for 30 mins**.

- Measure volume and add enough nuclease free water to bring the final volume back to 20 µL.
- Transfer volume into a 1.5 mL DNA LoBind tube and store at -20C.
- Limit annealed adapters to 2 freeze-thaw cycles.

SRE Cleanup (removes small DNA fragments)

19
- **Important:** For highly degraded DNA, skip this 'SRE cleanup' step, since this step would lose most of the DNA. Instead perform the '0.75X Size Selection with 75% Diluted Beads' step.

20 Prepare DNA sample:

- In 0.5 mL DNA LoBind tubes

	Component	Input (µL)	Final Amount (ng)
	Nuclease Free Water (AM9937)	50 - X	
	DNA sample	X	See recommended input below
	Total	50	

Calculation for DNA Sample volume (X) = [ng input] / [DNA Sample Concentration in ng / µL]

- Recommended input for A-tailing samples: 3000 ng
- Recommended input for non-A-tailing samples: 4000 ng

Note: For less concentrated DNA, SRE reaction volume can be increased to allow DNA input at the recommended amount. Scale Buffer SRE volume accordingly to maintain a 1:1 ratio to DNA volume.

- Add 50 µL of Buffer SRE (102-280-900). Vortex and spin down.

21 Incubate on Thermomixer:

- 01:00:00 | 37 °C | 0 rpm

22 Spin Down on Centrifuge

- 10000 x g, 24°C, 00:30:00

23 Resuspend SRE Pellet

- Carefully remove the supernatant from tube without disturbing the DNA pellet

- Add 90 µL of 1 X Shearing Buffer to the bottom of the tube without touching the bottom of the tube with pipette tip. Incubate at **room temperature for 10 minutes.**
- Pipet mix 20x. Spin down briefly. Incubate at **room temperature for 5 minutes.**
- Vortex tube at ¾ of max speed for 10 seconds and then spin down briefly.
- Measure the sample volume and add additional 1 X Shearing Buffer if necessary for a final sample volume of 100 µL.
- Pipet mix 20x. Spin down briefly.
- Keep DNA on ice.

0.75X Size Selection with 75% Diluted Beads

24 **Important:** Perform this step only for highly degraded DNA, instead of the 'SRE cleanup' step.

25
- Add 75% Diluted Ampure PB Beads at a 0.75X bead to sample volume ratio.
- Wash twice with 180 µL 80% Ethanol
- Elute with 101 µL of 1X Shearing Buffer and transfer 100 µL to a new 0.2 mL DNA LoBind tube

Fragmentation

26 Below we provide instructions on fragmenting DNA to ~4kb using the Megaruptor 3 Instrument.

27 Resources:

- Megaruptor 3 Manual: Megaruptor 3 Manual.pdf 3.2MB
- Setting up samples in Megaruptor 3: https://www.youtube.com/watch?v=zVhqnkQL8zo

28 Megaruptor pre-assembled consumables contain long hydropores (E07010002, black). Replace the long hydropores with short hydropores (E07010001, white) as follows:
- Holding the top of the syringe, gently and steadily twist the long hydropore in a counterclockwise motion to remove the hydropore.
- Holding the top of the syringe, fasten a short hydropore to the syringe by gently and steadily twisting in a clockwise motion.
- Ensure the syringe plunger is fully compressed to remove any air.
- Tighten the syringe, adaptor, and hydropore components together securely.

Components of the megaruptor consumable:

Megaruptor Consumable.png 46.7KB

29 Transfer the DNA sample to the hydro tube.

30 Prepare Megaruptor shearing parameters as follows:

Standard Megaruptor

	Concentration (ng/µL)	Volume (µL)	Speed
	40	100	65

Ultra-Fast Protocol (UFP) Activated Megaruptor

	Volume (µL)	Speed	Passes	Pause (s)
	100	65	20	5

31 Run shearing protocol on Megruptor:

- Connect the hydro tube to the hydropore syringe by inserting it carefully—no screwing required.
- Load the syringe with the sample on the instrument.
- For Standard Megaruptor: run the protocol 3 times (total shearing time is ~90 minutes, 30 minutes per run)
- For Ultra-Fast Protocol (UFP) Activated Megaruptor: run the protocol 5 times (total shearing time is ~15 minutes, 3 minutes per run)

Attaching the hydrotube to the megaruptor consumable:

Hydropore Attachment.png 61.3KB

32 Remove samples from Megaruptor

- Hold down the plunger while removing the syringe assembly from the megaruptor instrument.
- While holding down the plunger, vigorously flick down the consumable so that liquid pools at the bottom of the hydropore.
- While holding down the plunger, unplug the hydropore from the hydrotube slowly by tilting the syringe (hold the hydropore). Pull the tubing slowly from the liquid surface.
- Press the syringe plunger maximally in order to recover the remaining volume of sample inside the hydropore tubing.
- Slowly lower the hydropore tubing until it comes in contact with the surface of the liquid. The drop will be instantly added to the sample.
- Additional volume may be removed with a P10 pipette from the lower part of the syringe after unscrewing the syringe from the adapter.
- Spin down hydro tubes for 5 seconds.
- Pipette the sheared sample from the hydro tube into a new 0.2 mL DNA LoBind tube.

Tips for sample recovery from megaruptor:

Recover_maximum_sample_volume... 450.9KB

33 Sample may be stored at 4C for up to 24 hours before proceeding to End Repair.

Nuclease P1 Reaction

34 **Important:** Perform *either* this Nuclease P1 end repair step *or* the Takara Mung Bean Nuclease end repair step, not both. Takara Mung Bean Nuclease typically has higher yield compared to Nuclease P1. Nuclease P1 is recommended when Takara Mung Bean Nuclease is unavailable, as Takara enzyme is not readily available in the United States and NEB Mung Bean Nuclease does not perform as well.

35 Prepare Nuclease P1 reaction as follows:

- Measure the volume of each sample post-shearing and update the table accordingly

Note: Expect around 10 % volume loss

	Component	Starting Concentration	Final Concentration	Input (µL)
	Fragmented DNA			X
	Nuclease Free Water (AM9937)			115 - X - 11.5 - 0.58
	NEBuffer r1.1 (B6001S)	10 X	1 X	11.50
	Nuclease P1 (M0660S)	100 U / µL	0.5 U / µL	0.58
	Total			115

- Pipette mix and spin down

36 Run Nuclease P1 Reaction on the thermocycler:

- Heated lid off
- 37 °C 00:30:00 → 4 °C Hold

37 Inactivate Nuclease P1 Reaction

- Add 4.5 µL of 0.5 M EDTA (AM9260G)
- Pipette mix and spin down
- Immediately add 1.2 µL of 1 % SDS (AM9822)
- Pipette mix and spin down

38 Perform a 1.5 X bead cleanup:

- Transfer sample to a new 0.5mL DNA LoBind tube
- Add 181 µL Ampure PB Beads (100-265-900)
- Wash twice with 360 µL 80 % Ethanol
- Elute with 22 µL of 10 mM Tris pH 8 and transfer 22 µL to a new 0.2 mL DNA LoBind tube

Takara Mung Bean Reaction

39 **Important:** Perform *either* this Takara Mung Bean Nuclease end repair step *or* the Nuclease P1 end repair step, not both. Takara Mung Bean Nuclease typically has higher yield compared to Nuclease P1.

40 Prepare Takara Mung Bean reaction as follows:

- Measure the volume of each sample post-shearing and update the table accordingly

Note: Expect around 10 % volume loss

	Component	Starting Concentration	Final Concentration	Input (µL)
	Fragmented DNA			X
	Nuclease Free Water (AM9937)			115 - X - 11.5 - 1.44
	10X Mung Bean Nuclease Buffer (Takara 2420A)	10 X	1 X	11.50
	Mung Bean Nuclease (Takara 2420A)	40 U / µL	0.5 U / µL	1.44
	Total			115

- Pipette mix and spin down

41 Run Mung Bean Nuclease Reaction on thermocycler:

- Heated lid off
- 37 °C 00:10:00 → 4 °C Hold

42 Inactivate Takara Mung Bean Reaction

- Add 4.5 µL of 0.5 M EDTA (AM9260G)
- Pipette mix and spin down
- Immediately add 1.2 µL of 1 % SDS (AM9822)
- Pipette mix and spin down

43 Perform a 1.5 X bead cleanup:

- Transfer sample to a new 0.5mL DNA LoBind tube
- Add 181 µL Ampure PB Beads (100-265-900)
- Wash twice with 360 µL 80 % Ethanol
- Elute with 22 µL of 10 mM Tris pH 8 and transfer 22 µL to a new 0.2 mL DNA LoBind tube

E. Coli Nick Ligation

44 Prepare E. Coli Nick Ligation reaction as follows:

	Component	Starting Concentration	Final Concentration	Input (µL)
	Post-End Repair DNA			22.00
	Nuclease Free Water (AM9937)			1.14
	rCutSmart Buffer (B6004S)	10 X	1 X	2.90
	500 µM NAD+ dilution	500 µM	26 µM	1.51
	E. Coli DNA Ligase (M0205S)	10 U / µL	0.5 U / µL	1.45
	Total			29.00

- Pipette mix and spin down

45 Run E. Coli Nick Ligation Thermocycler Protocol:

- Heated lid off
- 16 °C 00:30:00 → 4 °C Hold

Klenow Fragment 3'→5'/PNK Reaction

46 Prepare Klenow/PNK reaction as follows:

- Add dATP/ddBTP Mix for A-tailed libraries and ddBTP Mix for non-A-tailed libraries.

	Component	Starting Conc.	Final Conc.	A-tailed lib input (µL)	Non A-tailed lib input (µL)
	Post Nick Ligation DNA			29.00	29.00

	Component	Starting Conc.	Final Conc.	A-tailed lib input (µL)	Non A-tailed lib input (µL)
	Nuclease Free Water (AM9937)			2.35	2.35
	10X T4 DNA Ligase Buffer (B0202S)	10 X	1 X	4.50	4.50
	dATP/ddBTP Mix	1 mM each	0.1 mM each	4.50	-
	ddBTP Mix	1 mM each	0.1 mM each	-	4.50
	Klenow Fragment (3'→5' exo-) (M0212S)	5 U / µL	0.35 U / µL	3.15	3.15
	T4 PNK (M0201S)	10 U / µL	0.33 U / µL	1.50	1.50
	Total			45.00	45.00

- Pipette mix and spin down

47 Run Klenow/PNK Thermocycler Protocol:

- Heated lid off
- 37 °C 00:30:00 → 4 °C Hold

Adaptor Ligation

48 Prepare Adaptor Ligation reaction as follows:

- The type of adapter to use depends on the library type: Use 'SMRTbell adapter index plate 96A' adapters for A-tailed libraries and annealed non-overhang adapters for non-A-tailed libraries. Both adapter types are 20 µM, yielding 0.88 µM in the ligation reaction.

	Component	Input (µL)
	Post Klenow Reaction DNA	45.00

	Component	Input (µL)
	Nuclease Free Water (AM9937)	0.56
	Barcoded adapter (type depends on A-tailed vs non-A-tailed library)	3.19
	NEBNext Ultra II Ligation Master Mix (E7648A)	23.00
	NEBNext Ligation Enhancer (E7374A)	0.75
	Total	72.50

- Pipette mix and spin down

49 Run Adaptor Ligation Thermocycler Protocol:

- Heated lid off
- 20 °C 01:00:00 → 4 °C Hold

50 Perform a 1.2 X bead cleanup:

- Add 87 µL Ampure PB Beads (100-265-900)
- Incubate with beads for **15 minutes**.
- Wash twice with 200 µL 80% Ethanol
- Elute with 41 µL of 10 mM Tris pH 8 and transfer 40 µL to a new 0.2 mL DNA LoBind tube

Nuclease Treatment

51 Prepare Nuclease Treatment reaction as follows:

	Component	Starting Concentration	Final Concentration	Input (µL)
	Ligated DNA			40.0
	NEBuffer 4 (B7004S)	10 X	1 X	5.00
	Exonuclease I (M0293S)	20 U / µL	0.29 U / µL	0.73
	RecJF (M0264S)	30 U / µL	0.86 U / µL	1.43
	T7 Exonuclease	10 U / µL	0.57 U / µL	2.85

	Component	Starting Concentration	Final Concentration	Input (µL)
	(M0263S)			
	Total			50.0

- Pipette mix and spin down

52 Run Nuclease Digestion on thermocycler:

- Heated lid off
- 37 °C 00:30:00 → 4 °C Hold

53 **For A-tailed libraries:**
Perform a 1 X Bead Clean

- Add 50 µL Ampure PB Beads (100-265-900)
- Incubate with beads for **15 minutes**.
- Wash twice with 80 % Ethanol
- Elute with 24 µL of 10 mM Tris pH 8 and transfer 24 µL to a new 0.5 mL DNA LoBind tube

For non-A-tailed libraries:
Perform a 0.9 X Bead Clean

- Add 45 µL Ampure PB Beads (100-265-900)
- Incubate with beads for **15 minutes**.
- Wash twice with 80 % Ethanol
- Elute with 24 µL of 10 mM Tris pH 8 and transfer 24 µL to a new 0.5 mL DNA LoBind tube

Library Quality Control

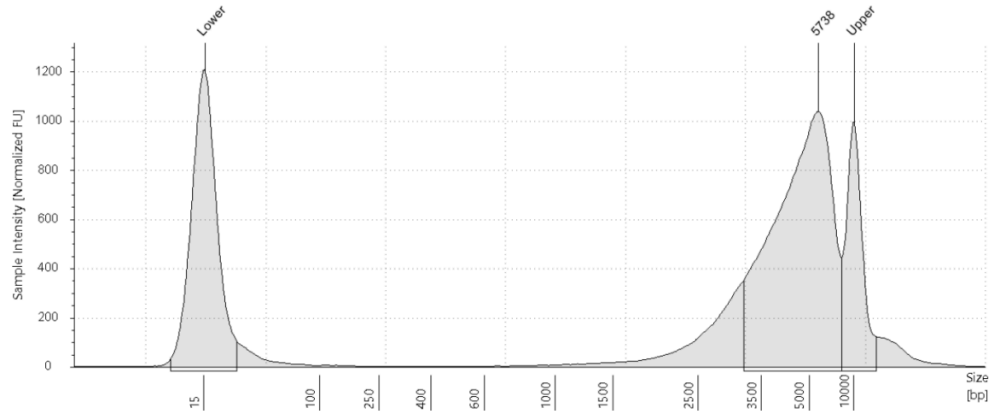
54 Measure library concentration by inputting 1 µL into the Qubit 1X dsDNA HS Assay (Q33231).
Expected concentration: 1 - 5 ng / µL

- Combine 190 µL Qubit Working Solution with 10 µL Qubit Standard 1 in a Qubit assay tube
- Combine 190 µL Qubit Working Solution with 10 µL Qubit Standard 2 in a Qubit assay tube
- Combine 199 µL Qubit Working Solution with 1 µL Library in a Qubit assay tube
- Vortex mix and incubate at room temperature for 2 minutes, then proceed to quantification

55 Measure library size distribution with High Sensitivity D5000 ScreenTape (5067-5592).

- Combine 2 µL High Sensitivity D5000 Sample Buffer (5190-7745) with 2 µL High Sensitivity D5000 Ladder (5190-7747) in the first well of a tapestation optical strip tube
- For each sample, combine 2 µL High Sensitivity D5000 Sample Buffer (5190-7745) + 2 µL Library in remaining wells
- Vortex at 2000 rpm for 1 min and spin down briefly, then proceed to tapestation measurement

- Example result:



- If there is a small amount of residual adapter dimers, these will be removed during the clean-up of the polymerase binding step prior to sequencing. However, if there is concern the adapter dimer is too much, then an additional 1 X bead cleanup can be performed.

56 Library may be stored at 4C for up to 24 hours. Place at -20C for long term storage.

Sequencing

57 Multiplex libraries using Qubit quantification results.

58 Perform ABC (sequencing polymerase annealing/binding/cleanup) per the PacBio SMRTbell prep kit 3.0 manual.

59 Run on PacBio Revio instrument per the following example sample CSV configuration:
- Modify this as needed if running > 1 SMRTcell in the same run

[Run Settings]
Instrument Type,Revio

```
Run Name,[Run name]
Plate 1,[Plate ID]
CSV Version,1

[SMRT Cell Settings],1_A01
Well Name,[Run name]
Application,Other
Library Type,Standard
Movie Acquisition Time (hours),30
Insert Size (bp),[Average size from ScreenTape]
Assign Data To Project,1
Library Concentration (pM),300
Include Base Kinetics,TRUE
Bio Sample Name,[Run name]
Sample is indexed,FALSE
Use Adaptive Loading,TRUE
Consensus Mode,strand
Full Resolution Base Qual,TRUE
Subread To HiFi Pileup,TRUE
```

Protocol references

DNA mismatch and damage patterns revealed by single-molecule sequencing
Liu MH*, Costa B*, Bianchini EC, Choi U, Bandler RC, Lassen E, Grońska-Pęski M, Schwing A, Murphy ZR, Rosenkjær D, Picciotto S, Bianchi V, Stengs L, Edwards M, Nunes NM, Loh CA, Truong TK, Brand RE, Pastinen T, Wagner JR, Skytte AB, Tabori U, Shoag JE, Evrony GD
Nature | 2024