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❸ HiDEF-seq V.1

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We use this protocol and it's
working

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Abstract

This is the HiDEF-seq library preparation protocol for bulk, single-molecule fidelity, long-read sequencing. This version of the HiDEF-seq protocol is designed for high-quality DNA. 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 v2 protocol with A-Tailing used for high-quality DNA as assessed by fragment size analysis (e.g., TapeStation). For low-quality DNA samples, refer to our paper for the relevant protocol: Liu & Costa et al. DNA mismatch and damage patterns revealed by single-molecule sequencing.

2 Reagent List:

A	В	С	D	E
Reagent	Supplier	Product #	Kit	Kit Product #
PB Elution Buffer	Pacific Biosciences	101-159-800	SMRTbell express template prep kit 2.0	100-938-900
Ligation Mix	Pacific Biosciences	101-654-100	SMRTbell express template prep kit 2.0	100-938-900
Ligation Additive	Pacific Biosciences	101-654-200	SMRTbell express template prep kit 2.0	100-938-900
Ligation Enhancer	Pacific Biosciences	101-654-300	SMRTbell express template prep kit 2.0	100-938-900
Enzyme A	Pacific Biosciences	101-741-100	SMRTbell enzyme cleanup kit 1.0	101-746-400
Enzyme B	Pacific Biosciences	101-741-700	SMRTbell enzyme cleanup kit 1.0	101-746-400
Enzyme C	Pacific Biosciences	101-741-400	SMRTbell enzyme cleanup kit 1.0	101-746-400
Enzyme D	Pacific Biosciences	101-741-500	SMRTbell enzyme cleanup kit 1.0	101-746-400
Stock PB Ampure Beads	Pacific Biosciences	100-265-900		
Barcoded Overhang Adapter Kit 8A	Pacific Biosciences	101-628-400		



A	В	С	D	E
Barcoded Overhang Adapter Kit 8B	Pacific Biosciences	101-628-500		
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher	Q33231		
Genomic DNA ScreenTape	Agilent	5067-5365		
High Sensitividy D5000 ScreenTape	Agilent	5097-5592		
100mM dATP	Thermo Fisher	R0141		
10mM ddNTP Bundle	Jena Bioscience	NU-1019		
10X CutSmart Buffer	NEB	B7204		
Hpy166II	NEB	R0616S		
10X rCutSmart Buffer	NEB	B6004S		
β-Nicotinamide adenine dinucleotide (NAD+)	NEB	B9007S		
E. Coli DNA Ligase	NEB	M0205S		
10X NEBuffer 4	NEB	B7004S		
Klenow Fragment (3'→5' exo-)	NEB	M0212S		

Reagent Preparation

3 If necessary, create 75% PB Ampure Bead dilution as follows:

A	В
Component	Volume (µL)
Stock PB Ampure Beads	2250
PB Elution Buffer	750
Total	3000



- Vortex mix
- 4 Create fresh 80% Ethanol as follows:

A	В
Component	Volume (mL)
Ethanol	8
Nuclease Free Water	2
Total	10

- Vortex mix
- 5 Create 10mM Tris pH8 as follows:

A	В
Component	Volume (µL)
Nuclease Free Water	990
1M Tris pH8	10
Total	1000

- Vortex mix
- 6 If necessary, create 500µM aliquots of NAD+ as follows:

	A	В
	Component	Volume (µL)
	Nuclease Free Water	198
Г	50mM NAD+	2
Г	Total	200

- Pipette mix and spin down
- Split into 10µL aliquots and store at -80C
- 7 If necessary, dilute 100mM stock dATP to 10mM dATP as follows:



	A	В
	Component	Volume (µL)
	Nuclease Free Water	9
Г	100mM dATP	1
	Total	10

- Pipette mix and spin down
- 8 If necessary, make 1mM dATP/ddBTP Mix as follows:

	A	В
Γ	Component	Volume (µL)
	Nuclease Free Water	60
Γ	10mM dATP	10
Γ	10mM ddCTP	10
Γ	10mM ddGTP	10
Γ	10mM ddTTP	10
	Total	100

- Pipette mix and spin down
- 9 Take out DNA from freezer
 - Thaw, vortex, and spin down
- 10 Measure concentration of DNA samples with Qubit.

11 Measure DNA size distribution and quality with Genomic DNA ScreenTape.

Restriction Enzyme Digestion

12 Prepare Restriction Enzyme Digestion:



■ Input 1500ng of gDNA into a 70µL reaction as follows:



A	В	С	D
Component	Starting Concentration	Input (µL)	Final Amount
Nuclease Free Water		62 - X	
10X Cutsmart Buffer	10X	7	1X
gDNA Sample		X	1500ng
Hpy166II	10U/μL	1	10U
Total		70	

Calculation for gDNA Sample Input Volume (x) = 1500ng / gDNA Sample Concentration

- Pipette mix and spin down
- 13 Run Restriction Enzyme Digestion Thermocycler Protocol:
 - Lid: 🖁 105 °C
- 14 Dilute the reaction to a DNA concentration of 10ng/µL by adding 80µL of Nuclease Free Water
 - Note: If more or less than 1500ng of DNA was input into the library preparation, calculate the amount of water to add to obtain 10ng/µL of DNA concentration and then adjust the subsequent bead cleanup volume accordingly.
 - Vortex mix and spin down
- 15 Perform a 0.8X Bead Clean:
 - Add 120µL of 75% PacBio Ampure Beads.
 - Note: If more or less than 1500ng of DNA was input into the library preparation, calculate the amount of 0.8X relative bead volume according to the prior step's post-dilution volume.
 - Continue with a standard bead clean up, with two 80% Ethanol washes.
 - Elute in 22µL of 10mM Tris pH8
- 16 Measure concentration of DNA samples by inputting 1µL into Qubit.

Mq′

20m

E. Coli Nick Ligation



17 Prepare E. Coli Nick Ligation reaction as follows:



A	В	С	D
Component	Starting Concentration	Input (µL)	Final Amount
Eluted DNA		21	
Nuclease Free Water		2.94	
rCutSmart Buffer	10X	3	1X
NAD+	500µM	1.56	26µM
E. Coli DNA Ligase	10U/μL	1.5	15U
Total		30	

- Pipette mix and spin down
- 18 Run E. Coli Nick Ligation Thermocycler Protocol:





- Dilute the samples to a maximum of 10ng/µL based on the post Restriction Enzyme Digest Clean Up Qubit values, using the following equation:
 - Volume to add = (Qubit Concentration)(21μL)/(10ng/μL) 30μL
 - Vortex mix and spin down
- 20 Perform a 0.75X Bead Clean with 75% PacBio Ampure Beads



- Calculate bead volume relative to the post-dilution volume of the sample after completing the prior step
- Wash twice with 80% Ethanol
- Elute in 22µL of 10mM Tris pH8
- 21 Measure concentration of DNA samples by inputting $1\mu L$ into Qubit.



A-Tailing

1h

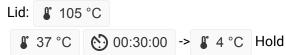
22 Prepare A-Tailing reaction as follows:

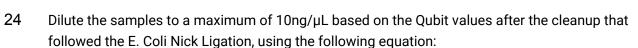




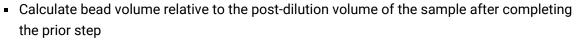
A	В	С	D
Component	Starting Concentration	Input (µL)	Final Amount
Eluted DNA		21	
Nuclease Free Water		1.5	
NEBuffer 4	10X	3	1X
dATP/ddBTP Mix	1mM	3	0.1mM
Klenow Fragment (3'→5' exo-)	5U/μL	1.5	7.5U
Total		30	

- Pipette mix and spin down
- 23 Run A-Tailing Thermocycler Protocol:





- Volume to add = (Qubit Concentration)(21μL)/(10ng/μL) 30μL
- Vortex mix and spin down
- 25 Perform a 0.75X Bead Clean with 75% PacBio Ampure Beads



- Wash twice with 80% Ethanol
- Elute in 22µL of 10mM Tris pH8
- 26 Add to the sample 3µL of 10X NEBuffer 4 and 5µL of Nuclease Free Water

Adaptor Ligation

30m

27 Prepare Adaptor Ligation reaction as follows:

А	В
Component	Input (µL)





A	В
Eluted DNA with NEBuffer 4	30
PacBio Hairpin Barcode Overhang Adapter	2.5
Ligation Mix	15
Ligation Additive	0.5
Ligation Enhancer	0.5
Total	48.5

- Pipette mix and spin down
- 28 Run Adaptor Ligation Thermocycler Protocol:
 - Heated lid off



Nuclease Treatment



29 Prepare Nuclease Treatment Master Mix as follows:



A	В
Component	Input (μL)
Enzyme A	2
Enzyme B	0.5
Enzyme C	0.5
Enzyme D	1
Total	4

- Pipette mix and spin down
- 30 Prepare Nuclease Treatment reaction as follows::

	Α	В
	Component	Input (µL)
	Ligated DNA	48.5
	Nuclease Treatment Mastermix	4
	Total	52.5



- Pipette mix and spin down
- 31 Run Nuclease Digestion Thermocycler Protocol:
 - Lid: **å** 105 °C

1h



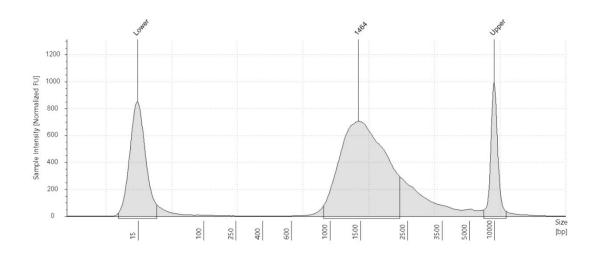
- 32 Perform a 1.2X Bead Clean by adding 63µL 75% PacBio Ampure Beads
 - Wash twice with 80% Ethanol
 - Elute in 24µL of 10mM Tris pH8

QC

- 33 Measure concentration of library by inputting $1\mu L$ into Qubit.
 - Expected concentration: 2.5 7.5ng/μL



- 34 Measure DNA size distribution with High Sensitivity D5000 ScreenTape.
 - Example size distribution:



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