May 17, 2024 Version 1

HiDEF-seq V.1

DOI
dx.doi.org/10.17504/protocols.io.kxygxy9mwl8j/v1

Mei Hong Liu[1], Benjamin M. Costa[1], Emilia C. Bianchini[1], Una Choi[1], Rachel C. Bandler[1], Emilie Lassen[2], Marta Grońska-Pęski[1], Adam Schwing[1], Zachary R. Murphy[1], Daniel Rosenkjær[2], Shany Picciotto[3], Vanessa Bianchi[4], Lucie Stengs[4], Melissa Edwards[4], Nuno Miguel Nunes[4], Caitlin A. Loh[1], Tina K. Truong[1], Randall E. Brand[5], Tomi Pastinen[6], J. Richard Wagner[7], Anne-Bine Skytte[2], Uri Tabori[4], Jonathan E. Shoag[3], Gilad D. Evrony[1]

[1]New York University Grossman School of Medicine; [2]Cryos International Sperm and Egg Bank; [3]Case Western Reserve University School of Medicine; [4]The Hospital for Sick Children; [5]University of Pittsburgh School of Medicine; [6]Children's Mercy Kansas City; [7]Université de Sherbrooke

Benjamin M. Costa
NYU Grossman School of Medicine

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.kxygxy9mwl8j/v1

Protocol Citation: Mei Hong Liu, Benjamin M. Costa, Emilia C. Bianchini, Una Choi, Rachel C. Bandler, Emilie Lassen, Marta Grońska-Pęski, Adam Schwing, Zachary R. Murphy, Daniel Rosenkjær, Shany Picciotto, Vanessa Bianchi, Lucie Stengs, Melissa Edwards, Nuno Miguel Nunes, Caitlin A. Loh, Tina K. Truong, Randall E. Brand, Tomi Pastinen, J. Richard Wagner, Anne-Bine Skytte, Uri Tabori, Jonathan E. Shoag, Gilad D. Evrony 2024. HiDEF-seq. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.kxygxy9mwl8j/v1**Version created by **Benjamin M. Costa**

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: May 13, 2024

Last Modified: May 17, 2024

Protocol Integer ID: 99842

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	B	C	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	B	C	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	B
	Component	Volume (µL)
	Stock PB Ampure Beads	2250
	PB Elution Buffer	750
	Total	3000

- Vortex mix

4 Create fresh 80% Ethanol as follows:

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

- Vortex mix

5 Create 10mM Tris pH8 as follows:

	A	B
	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	B
	Component	Volume (µL)
	Nuclease Free Water	198
	50mM NAD+	2
	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	B
	Component	Volume (µL)
	Nuclease Free Water	9
	100mM dATP	1
	Total	10

- Pipette mix and spin down

8 If necessary, make 1mM dATP/ddBTP Mix as follows:

	A	B
	Component	Volume (µL)
	Nuclease Free Water	60
	10mM dATP	10
	10mM ddCTP	10
	10mM ddGTP	10
	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	B	C	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: 20m

- Lid: 105 °C
- 37 °C 00:20:00 -> 4 °C Hold

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.

E. Coli Nick Ligation

17 Prepare E. Coli Nick Ligation reaction as follows:

	A	B	C	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: 30m

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

19 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 1µL into Qubit.

A-Tailing 1h

22 Prepare A-Tailing reaction as follows:

	A	B	C	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: 30m

Lid: 105 °C

37 °C 00:30:00 -> 4 °C Hold

24 Dilute the samples to a maximum of 10ng/µL based on the Qubit values after the cleanup that followed the E. Coli Nick Ligation, using the following equation:

- 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

- 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

26 Add to the sample 3µL of 10X NEBuffer 4 and 5µL of Nuclease Free Water

Adaptor Ligation 1h

27 Prepare Adaptor Ligation reaction as follows:

	A	B
	Component	Input (µL)

	A	B
	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: 1h

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

Nuclease Treatment 1h

29 Prepare Nuclease Treatment Master Mix as follows:

	A	B
	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::

	A	B
	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: 1h

- Lid: 105 °C
- 37 °C 01:00:00 -> 4 °C Hold

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 1µL into Qubit.

- Expected concentration: 2.5 - 7.5ng/µL

34 Measure DNA size distribution with High Sensitivity D5000 ScreenTape.

- Example size distribution: