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Optimized Mung Bean Nuclease Nanoseq Library Preparation Protocol for Whole Genome Sequencing

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We use this protocol and it's working

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Abstract

Duplex Sequencing (DS) is an ultra-accurate next-generation sequencing method that independently sequences both parent DNA strands, identifying true mutations shared between them and eliminating errors from artifacts or misincorporation. There are multiple duplex sequencing protocols, Nanoseq being one such method (PMID: 33911282). NanoSeq enhances duplex sequencing accuracy by combining BotSeqS (PMID: 27528664) with a restrictive end-repair method using dideoxy nucleotides, overcoming limitations of shallow sequencing and targeted panels for high-sensitivity applications. Here we describe step-by-step, the Nanoseq method using the mung bean nuclease, an enzyme that preferentially cleaves single-stranded DNA while preserving double-stranded DNA. Combining use of this enzyme following mechanical fragmentation of genomic DNA ensures nearly 2x more genome coverage compared to using the HpyCH4V enzyme for genomic DNA fragmentation. This method has been optimized and tested across >15 human tissues, including heart, muscle, colon, blood, cord blood, liver, and brain etc. to generate reproducible results.

Guidelines

Additional Notes

The accuracy of the qPCR-1 is critical quantification for optimizing the complexity of the sequencing library given the desired sequencing coverage, maximizing duplex coverage.

High adapter dimmer in step 7 library PCR could results in inefficient amplification.

Materials

Reagents

- TE Buffer **Thermo Fisher Scientific** Catalog # 12090015
- Mung Bean Nuclease **New England Biolabs** Catalog # M0250S
- Mung bean nuclease buffer **New England Biolabs** Catalog # B0250S
- UltraPure™ SDS Solution, 10% **Thermo Fisher Scientific** Catalog # 15553027
- T4 Polynucleotide Kinase **New England Biolabs** Catalog # M0201S
- NEBuffer™ 4 **New England Biolabs** Catalog # B7004S
- Klenow Fragment (3'→5' exo-) **New England Biolabs** Catalog # M0212L
- Dideoxynucleoside Triphosphate Set **Millipore Sigma** Catalog # 3732738001
- dATP Solution (100mM) **New England Biolabs** Catalog # N0440S
- T4 DNA Ligase **New England Biolabs** Catalog # M0202L
- Adenosine 5'-Triphosphate (ATP, 10mM) **New England Biolabs** Catalog # P0756S
- CS adapter (15 µM) **Integrated DNA Technologies (IDT)** Catalog # 1080799
- xGen™ UDI 10nt Primer Plates 1-4 **Integrated DNA Technologies (IDT)** Catalog # 10008052
- NEBNext® Ultra™ II Q5® Master Mix **New England Biolabs** Catalog # M0544L
- AMPure XP **Beckman Coulter** Catalog # A63882
- DEPC-Treated Water **Thermo Fisher Scientific** Catalog # AM9906
- 10 mM Tris-HCl Elution buffer **Invitrogen** Catalog# A33566
- DNA 7500 kit **Agilent Technologies** Catalog# 5067-1506
- High Sensitivity DNA Kit **Agilent Technologies** Catalog# 5067-4626
- KAPA Library Quant Complete kit (ABI Prism®) **Roche** Catalog # 07960204001
- Nuclease free water, **Thermo Fisher Scientific**, Catalog# AM9937
- 100 µM NanoqPCR primer 1: 5′-ACACTCTTTCCCTACACGAC-3′ **IDT HPLC grade**
- 100 µM NanoqPCR primer 2: 5′-GTGACTGGAGTTCAGACGTG-3′ **IDT HPLC grade**
- Ethanol absolute (200 Proof) **Avantor** Catalog # 89125-170

Equipments

- Covaris E220 Focused-ultrasonicator system **Covaris, Catalog # 500239**
- QuantStudio™ 6 Flex Real-Time PCR System, 384-well **Thermo Fisher Catalog # 4485691**
- PTC-200 Peltier Thermo Cycler **MJ Research Catalog # 8252-30-0001**

1 x TE Buffer **Thermo Fisher Scientific Catalog #**12090015

Mung Bean Nuclease - 1,500 units **New England Biolabs Catalog #**M0250S

UltraPure™ SDS Solution, 10% **Thermo Fisher Catalog #**15553027

T4 Polynucleotide Kinase - 500 units **New England Biolabs Catalog #**M0201S

NEBuffer 4 - 5.0 ml **New England Biolabs Catalog #**B7004S

Klenow Fragment (3'-5' exo-) - 1,000 units **New England Biolabs Catalog #**M0212L

- Dideoxynucleoside Triphosphate Set **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**03732738001
- dATP Solution - 25 umol **New England Biolabs** **Catalog #**N0440S
- T4 DNA Ligase - 100,000 units **New England Biolabs** **Catalog #**M0202L
- Adenosine-5 Triphosphate (ATP) - 1 ml **New England Biolabs** **Catalog #**P0756S
- xGen™ NGS Adapters & Indexing Primers for Illumina® sequencing **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**1080799
- xGen™ NGS Adapters & Indexing Primers for Illumina® sequencing **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**10008052
- NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs** **Catalog #**M0544L
- AMPure XP **Bechman Coulter** **Catalog #**A63882
- DEPC-Treated Water **Thermo Fisher** **Catalog #**AM9906
- Elution Buffer for Dynabeads™ mRNA Purification Kits **Thermo Fisher** **Catalog #**A33566
- Bioanalyzer DNA Analysis DNA 7500 Kit **Agilent Technologies** **Catalog #**5067-1506
- Agilent High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626
- KAPA Library Quantification Kits **Roche** **Catalog #**07960204001
- Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific** **Catalog #**AM9937

- Ethanol absolute, KOPTEC, meets analytical specification of BP, Ph. Eur., USP (200 Proof) **Avantor Sciences** **Catalog #**89125-176

Equipment

E220 Focused-ultrasonicator	NAME
Covaris	BRAND
500239	SKU
https://www.covaris.com/e220-focused-ultrasonicator-500239	LINK

Equipment

QuantStudio™ 6 Flex Real-Time PCR System, 384-well, laptop
NAME

Applied Biosystems™
BRAND

4485691
SKU

https://www.thermofisher.com/order/catalog/product/4485691
LINK

Equipment

PTC-200 Thermal Cycler
NAME

MJ Research
BRAND

8252-30-0001
SKU

https://www.gmi-inc.com/product/mj-research-ptc-200-thermal-cycler/?srsltid=AfmBOopNoU-2KhYScE8×1qQsrdebGEXKkaNSnnebjUbsH9pBJbJhxn0J
LINK

Library Preparation: DNA Shearing

5m

1 Prepare 50-100 ng DNA sample into 50 µL TE buffer in PCR plate.

2 Gently pipette up and down to mix.

3 Transfer entire sample volume into microTUBE plate (Covaris, 520078), spin plate at 600 x g, 00:05:00 .

5m

4 Shear DNA to about 450-bp on Covaris E220 Focused-ultrasonicator system (Covaris, Inc. Woburn, MA) with the following setting:

	A	B
	Water level	6
	Power	140
	Duty cycle	10%
	Cycle Per Burst	200
	Treatment time	40 seconds
	Temperature	7°C+/-2°C

Library Preparation: DNA sample purification

13m

5 Spin the microTUBE plate and transfer sheared DNA samples into PCR strip tube.

6 Purify sheared DNA by adding 90 µL (1.8X) AMPure XP beads (Beckman Coulter Catalog # A63882) to tube and pipette up and down 8 times followed by 00:08:00 incubation at Room temperature .

8m

7 Put sample tube on magnet stand for 00:05:00 . 5m

8 Remove and discard the supernatant.

9 Wash beads twice by adding 150 µL 80% ethanol.

10 Dry beads for 3-5 min at Room temperature .

11 Elute DNA into 22 µL DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906).

12 DNA QC on Bioanalyzer2100: optional *

12.1 Dilute sheared DNA 10X using DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906).

12.2 Load 1 µL diluted DNA on Agilent HS DNA kit and QC on Bioanalyzer 2100.

Mung Bean Nuclease Treatment 45m

13 Dilute Mung Bean Nuclease to 0.25 µL .

	A	B
	Mung Bean Nuclease (New England Biolabs Catalog # M0250S)	1 µL
	Mung bean nuclease buffer (New England Biolabs Catalog # B0250S)	4 µL
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	35 µL

13.1 Mix well by pipetting up and down 10 times.

14 Prepare 0.3% SDS Solution.

	A	B
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	32 µL
	UltraPure™ SDS Solution, 10% (Thermo Fisher Scientific Catalog # 15553027)	1 µL

15 Mung bean nuclease reaction.

	A	B
	Sheared DNA	20 µL
	Mung bean nuclease buffer (New England Biolabs Catalog # B0250S)	2.8 µL
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	5.2 µL
	diluted Mung bean nuclease (0.25 U/ µL)	2.0 µL

15.1 Mix well by pipetting up and down 10 times.

15.2 Incubate reaction at 30 °C 00:30:00 on thermocycler. 30m

15.3 At the end of incubation, add 1 µL 0.3% SDS immediately and pipetting up and down to mix.

16 Sample Purification.

16.1
- Add 60 µL (2X) AMPure XP beads (Beckman Coulter Catalog # A63882) to sample.
- Pipetting up and down 8 times to mix.

16.2 Incubate at Room temperature for 00:10:00, then put on magnet stand for 00:05:00. 15m

16.3 ▪ Wash beads with 150 µL with 80% Ethanol.
▪ Dry beads at Room temperature for 3-5 min.

16.4 Elute DNA with 11.5 µL DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906).

17 **Sample QC**: Optional.

17.1 Dilute MBN treated DNA sample 10X and load 1 µL on High Sensitivity DNA Kit (Agilent Technologies Catalog# 5067-4626).

T4 PNK Treatment

30m

18 Prepare T4PNK reaction master mix:

	A	B
	NEBuffer™ 4 (New England Biolabs Catalog # B7004S)	1.5 µL
	Adenosine 5'-Triphosphate (ATP) (New England Biolabs Catalog # P0756S)	1.5 µL
	T4 Polynucleotide Kinase (New England Biolabs Catalog # M0201S)	1.0 µL
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	1.0 µL

19 T4PNK reaction

	A	B
	MBN-treated DNA	10 µL
	T4PNK master mix	5 µL

19.1 Gently pipetting up and down to mix.

19.2 Incubate at 37 °C for 00:30:00 on a thermocycler. 30m

A-Tailing 30m

20 Prepare [M] 1 millimolar (mM) equimolar dATP/ddBTPs.

	A	B
	dATP Solution (New England Biolabs Catalog # N0440S)	2.5 µL
	ddTTP Solution Dideoxynucleoside Triphosphate Set (Millipore Sigma Catalog # 3732738001)	25 µL
	ddCTP Solution Dideoxynucleoside Triphosphate Set (Millipore Sigma Catalog # 3732738001)	25 µL
	ddGTP Solution Dideoxynucleoside Triphosphate Set (Millipore Sigma Catalog # 3732738001)	25 µL
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	172.5 µL

20.1 Mix by gentle vortex.

21 Prepare A-Tailing reaction mix:

	A	B
	NEBuffer™ 4 (New England Biolabs Catalog # B7004S)	0.5 µL
	Klenow Fragment (3'→5' exo-) (New England Biolabs Catalog # M0212L)	1.5 µL
	1 mM equimolar dATP/ddBTPs	1.5 µL
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	1.5 µL

21.1 Mix by gently pipetting up and down 6X.

22 A-Tailing reaction

	A	B
	DNA sample from previous step.	15 µL
	A-Tailing reaction mix	5.0

22.1 Mix gently by pipetting up and down.

22.2 Incubate reaction at 37 °C for 00:30:00 on a thermocycler. 30m

Adapter Ligation 20m

23 Prepare Ligation master mix.

	A	B
	NEBuffer™ 4 (New England Biolabs Catalog # B7004S)	1.74 µL
	Adenosine 5'-Triphosphate (10mM ATP) (New England Biolabs Catalog # P0756S)	4.0 µL
	CS adapter (Integrated DNA Technologies (IDT) Catalog # 1080799)	0.4 µL
	DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906)	9.76 µL

23.1 Mix gently by pipetting up and down 6 times.

24 Adapter ligation reaction

	A	B
	DNA sample from A-Tailing step	20 µL
	Ligation master mix.	15.9 µL
	T4 DNA Ligase (New England Biolabs Catalog # M0202L)	1.5 µL

24.1 Mix gently by pipetting up and down 6 times.

24.2 Incubate reaction at 20 °C for 00:20:00 followed by 4 °C hold on a thermocycler. 20m

24.3 Purify sample with 1X (37.4 µL) AMPure XP beads (Beckman Coulter Catalog # A63882) and eluted into 25 µL H_2O.

24.4 **Optional**: Dilute 1 µL adapter-ligated DNA 5X and load 1 µL library on High Sensitivity DNA Kit (Agilent Technologies Catalog# 5067-4626).

Note

If high adapter dimmer was seen on QC, perform a second AMPure XP beads purification.

Post Adapter-Ligation qPCR-1 Quantification

25 Prepare qPCR master mix.

	A	B
	Primer Mix (KAPA Library Quant Kit, Roche Catalog # 07960204001)	1 mL
	KAPA SYBR qPCR Master Mix (KAPA Library Quant Kit, Roche Catalog # 07960204001)	5 mL
	100 µM Nanoseq PCR primer1: 5′-ACACTCTTTCCCTACACGAC-3′ (IDT, HPLC grade)	20 µL
	100 µM Nanoseq qPCR primer2: 5′-GTGACTGGAGTTCAGACGTG-3′ (IDT, HPLC grade)	20 µL

26 Dilute adapter-ligated DNA 20x by adding 2 µL adapter-ligated DNA to 38 µL DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906).

27 qPCR reaction setup.

	A	B
	20X diluted library	1 µL
	NFW Nuclease free water (Thermo Fisher Scientific, Catalog# AM9906)	3 µL
	qPCR master mix	6 µL

27.1 Mix by pipetting up and down 10X.

27.2 Set reactions as triplicates in 384 well plates.

28 qPCR Standards.

	A	B
	Standard 1-6 (KAPA Library Quant Kit, Roche Catalog # 07960204001)	2 µL
	NFW Nuclease free water (Thermo Fisher Scientific, Catalog# AM9906)	2 µL
	qPCR master mix	6 µL

28.1 Set reactions as triplicates in 384 well plates.

28.2 Mix by pipetting up and down 10X.

29 qPCR amplification.
Run qPCR reactions on QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™) using the following PCR program:

	A	B	C	D
	Step	Temp	Time	Cycle #

	A	B	C	D
	1	95 °C	5 min	1
	2	95°C	30 s	35
		60°C	45 s	
	3	95°C	15 s	1
	4	65°C	1 min	1
	5	95°C	15 s	1

30 Determining the sample concentration.

The concentration (nM (fmol/µl)) is determined as follows:

mean of sample concentration × dilution factor (20)× 452/573/1,000.

Note

where 452 is the size of the standard in bp; 573 is the proxy for the average library size in bp.

Library PCR

31 Based on qPCR quantification result, dilute [M] 0.1 Mass Percent adapter-ligated DNA in 23.5 µL using DEPC-Treated Water (Thermo Fisher Scientific Catalog # AM9906).

32 Set up library amplification mix.

	A	B
	adapter-ligated DNA (0.1 fmol)	23.5 µL
	IDT Index primer, xGen™ UDI 10nt Primers (IDT Catalog # 10008052)	1.5 µL

	A	B
	NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs Catalog # M0544L)	25 µL

Oligonucleotides sequences

P7 CAAGCAGAAGACGGCATACGAGAT[i7-NNNNNNNNNN]GTCTCGTGGGCTCGG

P5 AATGATACGGCGACCACCGAGATCTACAC[i5-NNNNNNNNNN]TCGTCGGCAGCGTC

33 Library amplification program.

	A	B	C	D
	Step	Temp	Time	Cycle #
	1	98 °C	30 s	1
	2	98°C	10 s	16*
		65°C	75 s	
	3	65°C	5 min	1
	4	4°C	Hold	1

*The number of PCR cycles is dependent on the input: 0.1 fmol, 16 cycles; 0.3 fmol, 14 cycles; 0.6 fmol, 13 cycles; 5 fmol, 10 cycles.

34 Library Purification.

34.1 Purify amplified library twice with 0.75X AMPure XP beads (Beckman Coulter Catalog # A63882).

34.2 Elute library into 20 µL [M] 10 millimolar (mM) Tris-HCl Elution buffer (Invitrogen Catalog# A33566).

35 Library Quantification and normalization.

35.1 QC 1 µL library on DNA 7500 kit (Agilent Technologies Catalog# 5067-1506).

35.2 Dilute library to [M] 5 nanomolar (nM) in [M] 10 millimolar (mM) Tris-HCl Elution buffer (Invitrogen Catalog# A33566) for pooling.

Final Library Quantification by qPCR-2

36 Library dilution.

36.1 Dilute [M] 5 nanomolar (nM) library 3000X by sequential 100 X and 30X dilutions using Nuclease free water (Thermo Fisher Scientific, Catalog# AM9937).

37 Prepare qPCR-2 master mix.

	A	B
	Primer Mix (KAPA Library Quant Kit, Roche Catalog # 07960204001)	1 mL
	KAPA SYBR qPCR Master Mix (KAPA Library Quant Kit, Roche Catalog # 07960204001)	5 mL

38 qPCR-2 reaction setup.

	A	B
	3000x diluted library or Standard 1-6 (KAPA Library Quant Kit) Roche Catalog # 07960204001	4 µL
	qPCR master mix	6 µL

38.1 Set reactions as triplicates in 384 well plates.

38.2 Mix by pipetting up and down 10X.

39 qPCR-2 amplification.
Carry out qPCR reactions on QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™) using the following PCR program:

	A	B	C	D
	Step	Temp	Time	Cycle #
	1	95 °C	5 min	1
	2	95°C	30 s	35
		60°C	45 s	
	3	95°C	15 s	1
	4	65°C	1 min	1
	5	95°C	15 s	1

Library Pooling

40 Pool libraries at equimolar concentrations based on qPCR-2 results and sequence libraries on Illumina platform for 30X coverage.

Protocol references

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