INSTRUCTION MANUAL

NEW ENGLAND Biolabs®

NEBNext UltraExpress™ FS DNA Library Prep Kit

NEB #E3340S/L

24/96 reactions

Version 4.0_7/26

Table of Contents

Overview....................3

Protocol for Inputs 10 ng–200 ng....................4

Appendix A: Recommendations for Customized Adaptor Dilutions and Cycling per Input (0.1 ng–200 ng)....................9

Appendix B: Recommendations for Customized Fragmentation Time and/or Cleanup to Alter Library Size Distributions....................10

Kit Components....................11

Revision History....................11

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3340S) and 96 reactions (NEB #E3340L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (yellow) NEBNext UltraExpress FS Enzyme Mix
- (yellow) NEBNext UltraExpress FS Reaction Buffer
- (red) NEBNext UltraExpress Ligation Master Mix
- (blue) NEBNext® MSTC™ High Yield Master Mix
- (white) TE Buffer (1X)
- (white) NEBNext Bead Reconstitution Buffer

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind® Tubes (Eppendorf® #022431021)
- DNase-, RNase-free PCR strip tubes
- NEBNext Singleplex or Multiplex Oligos for Illumina®

  NEBNext Multiplex Oligos options can be found at www.neb.com/oligos. Alternatively, customer supplied adaptor and primers can be used; please see information in link below:
  https://www.neb.com/en-us/faqs/2023/10/06/can-this-kit-be-used-with-adaptors-and-primers-from-suppliers-other-than-neb

  Note: this protocol is compatible with NEB's non-indexed loop adaptor, as well as third party non-indexed adaptors that have a T overhang. Please contact Technical Support at info@neb.com with any questions regarding alternate adaptors.
- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Magnetic rack/stand (NEB #S1515, Alpaqua® cat. #A001322, or equivalent)
- Vortex
- Thermal cycler

- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables
- NanoDrop® or Lunatic® (Unchained Labs®) and associated consumables
- NEBNext Adaptor for Illumina Overview
  The following sequences are used for adaptor trimming of NEBNext adaptors for Illumina:
  - Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
  - Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Overview

The NEBNext UltraExpress FS DNA Library Prep Kit contains the enzymes and buffers required to rapidly convert 10–200 ng of intact DNA into high-quality libraries for sequencing on the Illumina platform. The fast and simple workflow features reduced cleanup steps, minimal hands-on time, no adaptor dilutions, and a single PCR cycling condition across the entire input range. The protocol also allows for library preparation in a single tube, minimizing plastic consumables waste. In addition to the standard protocol, the appendices detail: (A) cycling and adaptor dilution recommendations for optimizing library yields from varying DNA input amounts and (B) fragmentation times and cleanup conditions for altering library size distribution, if required.

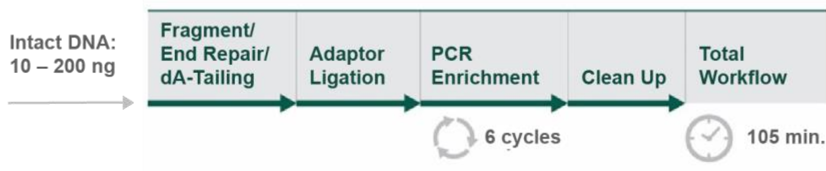
Note: The UltraExpress FS DNA Library Prep Kit is not compatible with bisulfite or enzymatic conversion workflows and FFPE DNA.

Each kit component must pass rigorous quality control standards and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of indexed libraries on an Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the Customized Solutions Team at NEB. Please contact custom@neb.com for further information.

Please refer to the product page on NEB.com for FAQs about this product.

Figure 1. Workflow demonstrating the use of NEBNext UltraExpress FS DNA Library Prep Kit



Protocol for Inputs 10 ng–200 ng

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

Colored bullets indicate the cap color of the reagent to be added to a reaction.

Starting Material: 10–200 ng of intact DNA. If the DNA volume is less than 15 µl, add TE Buffer (1X) to a final volume of 15 µl. Alternatively, samples can be diluted with 10 mM Tris-HCl, pH 8.0; low EDTA TE; 0.1X TE; or Nuclease-free water.

1. Fragmentation/End Prep

Note:

(1) Ensure that the FS Reaction Buffer is completely thawed. If precipitate is seen in the buffer, pipette up and down several times to break it up, vortex to mix, and place on ice prior to use.

(2) It is important to vortex the FS enzyme mix prior to use for optimal performance.

(3) See Figure 1.1 for typical fragment, library, and insert size distributions.

(4) Fragmentation occurs during the 37°C incubation step in a time-dependent manner; optionally, see Appendix A for varying fragmentation time and library cleanup conditions to alter library size distributions.

1.1. Vortex the UltraExpress FS Enzyme Mix and Reaction Buffer 5–8 seconds prior to use and place on ice. A master mix of the enzyme mix and buffer may be prepared immediately prior to use.

1.2. Add the following components to a sterile nuclease-free tube on ice:

COMPONENT	VOLUME
Intact DNA*	15 µl
● (yellow) NEBNext UltraExpress FS Reaction Buffer	4 µl
● (yellow) NEBNext UltraExpress FS Enzyme Mix	1 µl
Total Volume	**20 µl**

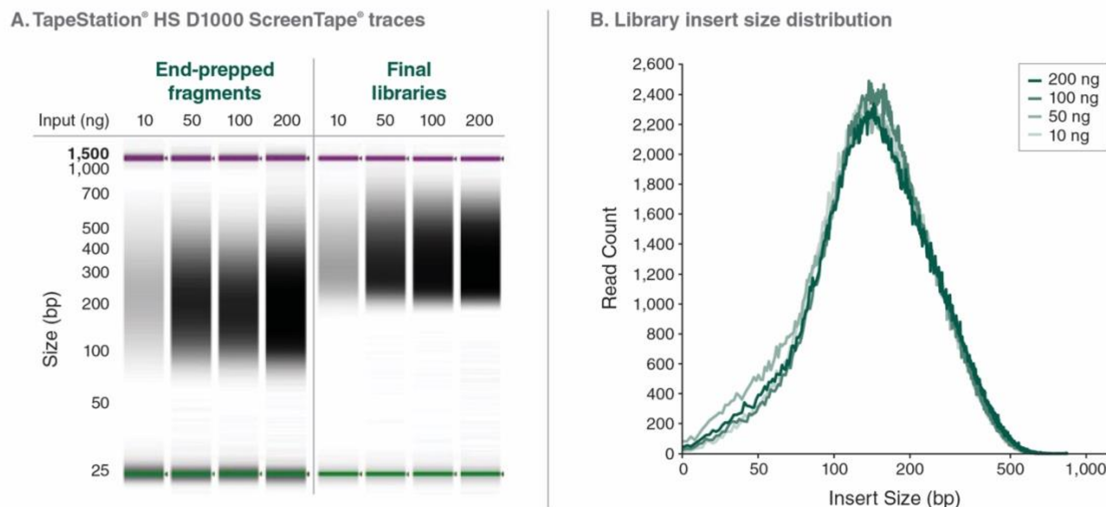
*Intact gDNA, cDNA, plasmids, and amplicons

1.3. Vortex the reaction for 5 seconds and briefly spin down. A small number of bubbles in the reaction will not inhibit performance.

1.4. Place in a thermal cycler, with the heated lid set to ≥ 75°C or on, and run the following program:
20 minutes at 37°C
15 minutes at 65°C
Hold at 4°C

If necessary, samples can be stored at –20°C; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.

Figure 1.1: Example of fragment, library, and insert size distributions for 10, 50, 100 and 200 ng Human gDNA (NA19240) inputs following 20 minutes of enzymatic fragmentation using the NEBNext UltraExpress FS DNA Library Prep Kit.



(A) TapeStation® HS D1000 ScreenTape® traces of end prepped fragments cleaned up with 2X beads prior to adaptor ligation and final libraries made from Human gDNA (NA19240). (B) Graphical depiction of library insert size distributions as determined by Picard CollectInsertSizeMetrics tool.

2. Adaptor Ligation

2.1. Add the following components directly to the FS Reaction Mixture:

COMPONENT	VOLUME
FS Reaction Mixture (Step 1.4. in Section 1)	20 µl
• (red) NEBNext Adaptor for Illumina*	2 µl
• (red) NEBNext UltraExpress Ligation Master Mix**	20 µl
Total Volume	**42 µl**

* The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which are supplied separately from the library prep kit. www.neb.com/oligos

** Mix the UltraExpress Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

Note: Do not premix the NEBNext UltraExpress Ligation Master Mix and Adaptor prior to use in the Adaptor Ligation Step.

2.2. Set a 100 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. **(Caution: The NEBNext UltraExpress Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small number of bubbles will not interfere with performance).**

2.3. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

Note: Steps 2.4. and 2.5. are only required for use with NEBNext Adaptor (loop adaptor). USER® enzyme can be found in NEBNext Multiplex Oligos for Illumina sets.

2.4. Add 3 µl of • (red) USER Enzyme to the ligation mixture from Step 2.3.

2.5. Mix well and incubate at 37°C for 5 minutes in a thermal cycler with the heated lid set to ≥ 47°C or on.

Samples can be stored at –20°C overnight.

3. PCR Enrichment of Adaptor-ligated DNA

Use Option A (3.1.1A): for any NEBNext Index Primers where the forward and reverse primers are supplied separately in tubes. Primers are supplied at 10 µM.

Use Option B (3.1.1B): for any NEBNext Index Primers where index primers are supplied with the forward and reverse primers (i5 and i7) premixed in a 96-well plate format. These primers are supplied at a 10 µM combined concentration (5 µM each).

3.1. PCR Amplification

3.1.1. Add the following components to a sterile strip tube:

3.1.1A. Forward and Reverse Primers not already combined (Option A)

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.5.)	45 µl
• (blue) NEBNext MSTC High Yield Master Mix	45 µl
• (blue) Index Primer/i7 Primer*,**	5 µl
• (blue) Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	**100 µl**

3.1.1B. Forward and Reverse Primers already combined (Option B)

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.5.)	45 µl
• (blue) NEBNext MSTC High Yield Master Mix	45 µl
Index/Universal Primer*,**	10 µl
Total Volume	**100 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Multiplex Oligos Kit manual for determining valid barcode combinations.

** Use only one i7 primer/index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

3.1.2. Set a 100 µl or 200 µl pipette to 90 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

3.1.3. Place the tube in a thermal cycler with the heated lid set to 105°C or on and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	6*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* PCR amplification with 6 cycles can generate sufficient library yields for sequencing from DNA inputs ranging from 10–200 ng and allows for one PCR condition across the entire input range.

Note:

(1) For DNA inputs higher than 100 ng, 6 cycles of PCR can result in amplification beyond the linear phase during the late cycles. This can cause inaccurate library quantification on migration-based methods like Bioanalyzer and TapeStation (See Figure 4.1). In this case, we recommend using Nanodrop or Lunatic for library quantification.

(2) The adaptor amount and PCR cycle numbers were validated using high quality genomic DNA and NEBNext adaptor for Illumina. For other DNA input types, we recommend optimization of adaptor amount and PCR cycles.

(3) For more specific library yield requirements, you may choose to follow Appendix B for recommended number of PCR cycles.

(4) Take out NEBNext Bead Reconstitution Buffer and bring up to room temperature prior to Phased Bead Cleanup. Allow the buffer (and beads if using AMPure XP) to warm to room temperature for at least 30 minutes before use.

Proceed to Cleanup of PCR Amplification in Section 4.

4. Phased Bead Cleanup of PCR Reaction

Note:The SPRIselect/Ampure Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using Ampure Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

4.1. Vortex SPRIselect/Ampure Beads to resuspend. Thaw and mix NEBNext Bead Reconstitution Buffer at room temperature and afterwards it may be stored in a sealed container at room temperature for future use.

4.2. Add 70 µl (0.7X) of resuspended beads to the 100 µl PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

4.3. Incubate samples on bench top for at least 5 minutes at room temperature.

4.4. Place the tube/ plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

4.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

4.6. Remove the tube/ plate from the magnetic stand **(Note: no ethanol wash at this step)**. Add 100 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in water) to resuspend the beads. Mix well by pipetting up and down at least 10 times.

4.7. Add 80 µl (0.8X) of NEBNext Bead Reconstitution Buffer to the 100 µl of resuspended beads. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

4.8. Incubate samples on bench top for at least 5 minutes at room temperature.

4.9. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

4.10. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

4.11. Add 200 µl of freshly prepared 80% ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

4.12. Repeat Step 4.11. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

4.13. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

4.14. Remove the tube/ plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in water).

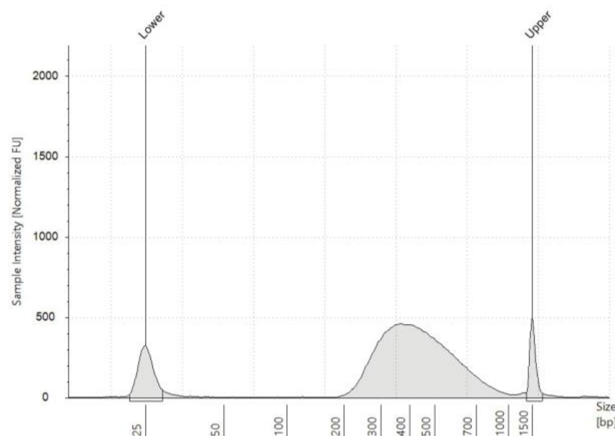
4.15. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

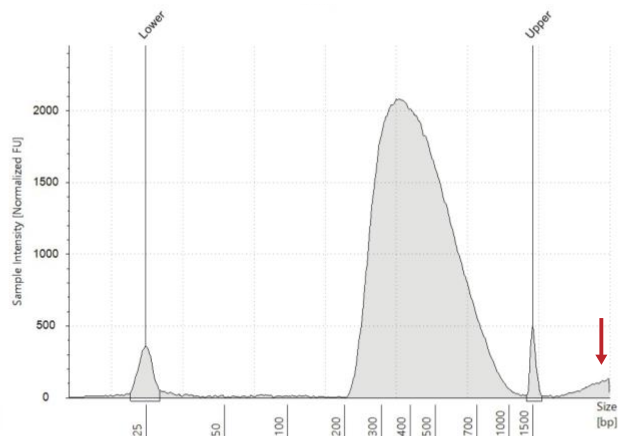
4.16. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube and store at –20°C.

4.17. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip or TapeStation D1000 HS ScreenTape. The sample may need to be diluted before loading.

Samples can be stored at –20°C.

Figure 4.1: Examples of libraries prepared with human DNA (NA19240).





Libraries made from (A) 10 ng and (B) 200 ng human gDNA (NA19240) with 6 PCR cycles were diluted 1:3 and run on a TapeStation HSD1000 ScreenTape. Figure 4.1.B shows a peak above the upper marker (indicated by the red arrow) resulting from non-linear amplification phase for the 200 ng sample at late PCR cycles. Although the molecules generated during the non-linear amplification phase are sequencible, their different migration speed on ScreenTapes causes inaccurate quantification of the library concentration. In this case, we recommend measuring library concentration on NanoDrop or Lunatic using 400 bp or the intended library size to convert the concentration values to nanomolar. The following equation can be used to convert concentration from ng/µl to nM: [(Library Concentration in ng/µl) / (660 g/mol x Library Size in bp)] x 10^6 *= Concentration in nM.*

Note: If excess adaptor dimer peak is observed at 150–180 bp, an additional 0.8X bead cleanup can be performed on individual or pooled libraries. The additional cleanup will result in a reduction in yield and slightly larger libraries, which will not affect sequencing results.

Appendix A
Recommendations for Customized Adaptor Dilutions and Cycling per Input (0.1 ng–200 ng)

The standard protocol described elsewhere in this Instruction Manual is designed to offer fast, streamlined library prep. However, for cases where customization is desired, we provide the following recommendations for adaptor dilutions and PCR cycle numbers based on the amount of input DNA.

Follow recommended adaptor dilutions and PCR cycle numbers for the different input amounts provided in **Table A1 to obtain 100 ng library yield**.

Table A1. For a library yield of 100 ng.

INPUT DNA IN FRAGMENTATION/ END PREP REACTION	ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL VOLUME)	# OF CYCLES REQUIRED FOR STANDARD LIBRARY PREP Yield ~ 100 ng (> 15 nM final cleanup 30 µl library)
≥ 100 ng	No Dilution	3–4
50 ng	No Dilution	5–6
10 ng	No Dilution	6–7
5 ng	25-Fold (1:25)	8–9
1 ng	25-Fold (1:25)	11–12
0.5 ng	25-Fold (1:25)	12–13
0.1 ng	25-Fold (1:25)	13–14

Follow recommended PCR cycle numbers for different input amounts provided in **Table A2 to reach 1µg library yield** using undiluted NEBNext Adaptor for Illumina.

Table A2. For a library yield of 1 µg.

INPUT DNA IN FRAGMENTATION/ END PREP REACTION	ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL VOLUME)	# OF CYCLES REQUIRED FOR TARGET ENRICHMENT LIBRARY PREP: Yield ~ 1 µg (~150 nM final cleanup 30 µl library)
≥ 100 ng	No Dilution	5–6
50 ng	No Dilution	7–8
10 ng	No Dilution	9–10

Appendix B
Recommendations for Customized Fragmentation Time and/or Cleanup to Alter Library Size Distributions

The standard protocol described elsewhere in this Instruction Manual is designed to offer fast, streamlined library prep. However, for cases where customization is desired, we provide the following recommendations for customized fragmentation times at 37°C and/or phased final library cleanup conditions for varying final library size distributions.

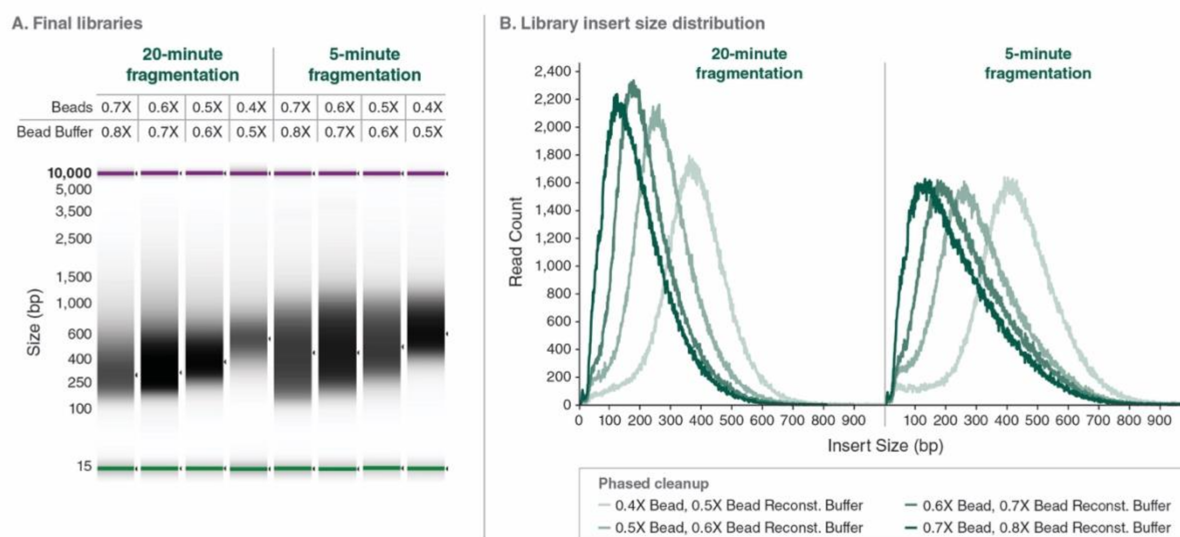
Note: Phased cleanups with less than 0.5X bead addition result in decreased library yields. The following recommendations are for libraries made from DNA > 1 kb in size or intact gDNA inputs of at least 100 ng following the 6-cycle PCR protocol (Step 3.1.3.). To apply custom phased cleanups on inputs of 10–200 ng, we recommend additional PCR cycling prior to cleanup (see Appendix A).

Table B1. Approximate size distributions for libraries and inserts with custom fragmentation time and/or phased cleanups.

The approximate library and insert sizes were determined using TapeStation traces and sequence data analysis with the Picard CollectInsertSizeMetrics tool.

FRAGMENTATION TIME (MINUTES)	PHASED CLEANUP REAGENT VOLUMES TO BE ADDED (µl)		APPROXIMATE LIBRARY PARAMETERS		
	BEAD ADDITION (STEP 4.2.)	BEAD RECONSTITUTION BUFFER ADDITION (STEP 4.7.)	LIBRARY SIZE DISTRIBUTION (bp)	INSERT SIZE DISTRIBUTION (bp)	PEAK INSERT SIZE (bp)
20	70 (0.7X)	80 (0.8X)	200–720	80–600	125
	60 (0.6X)	70 (0.7X)	220–770	100–650	175
	50 (0.5X)	60 (0.6X)	250–820	130–700	250
	40 (0.4X)	50 (0.5X)	350–920	230–800	375
5	70 (0.7X)	80 (0.8X)	200–820	80–700	150
	60 (0.6X)	70 (0.7X)	220–850	100–730	200
	50 (0.5X)	60 (0.6X)	250–920	130–800	275
	40 (0.4X)	50 (0.5X)	350–1,000	230–880	400

Figure B1. Example of library and insert size distributions for 100 ng Human gDNA (NA19240) input following 5- or 20-minute enzymatic fragmentation and varying phased cleanup conditions.



TapeStation HS D5000 ScreenTape traces of (A) final libraries made from 100 ng Human gDNA (NA19240) fragmented for 20 minutes or 5 minutes and cleaned using a standard or custom phased cleanup. The first TapeStation lane on the left is the library trace for the standard fragmentation and cleanup condition. (B) Graphical depiction of library insert size distribution as determined by the Picard CollectInsertSizeMetrics tool.

Kit Components

NEB #E3340S Table of Components

NEB #	COMPONENT	VOLUME
E3347A	TE Buffer (1X)	0.72 ml
E3344A	NEBNext UltraExpress FS Reaction Buffer	0.096 ml
E3343A	NEBNext UltraExpress FS Enzyme Mix	0.024 ml
E3345A	NEBNext UltraExpress Ligation Master Mix	0.48 ml
E3346A	NEBNext MSTC High Yield Master Mix	1.08 ml
E3348A	NEBNext Bead Reconstitution Buffer	1.92 ml

NEB #E3340L Table of Components

NEB #	COMPONENT	VOLUME
E3347AA	TE Buffer (1X)	2.88 ml
E3344AA	NEBNext UltraExpress FS Reaction Buffer	0.384 ml
E3343AA	NEBNext UltraExpress FS Enzyme Mix	0.096 ml
E3345AA	NEBNext UltraExpress Ligation Master Mix	2 x 0.96 ml
E3346AA	NEBNext MSTC High Yield Master Mix	4.32 ml
E3348AA	NEBNext Bead Reconstitution Buffer	7.68 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	12/23
2.0	Updated figure legend to match graph. Updated header and footer with new logo.	4/24
3.0	Updated text in Required Materials Not Included; NEBNext singleplex or multiplex oligos for Illumina.	9/25
4.0	Updated Appendices.	7/26

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