INSTRUCTION MANUAL

NEBNext UltraExpress™ DNA Library Prep Kit

NEB #E3325S/L 24/96 reactions

Version 3.1_11/26

Table of Contents

Overview....2

Protocol for DNA Inputs 10 ng–200 ng....3

Appendix A: Recommendations for Customized Adaptor Dilutions and Cycling per Input (0.5 ng–200 ng)....7

Appendix B: Recommendations for Customized Cleanup to Alter Library Size Distributions....8

Kit Components....9

Revision History....9

The Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3325S) and 96 reactions (NEB #E3325L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

Package 1: Store at –20°C.

- (green) NEBNext UltraExpress End Prep Enzyme Mix
- (green) NEBNext UltraExpress End Prep Reaction Buffer
- (red) NEBNext UltraExpress Ligation Master Mix
- (blue) NEBNext MSTC™ High Yield Master Mix
- (white) 0.1X TE
- (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 adaptors and primers can be used; please see information in link below: https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-nebhttps://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)
- Thermal cycler
- Covaris® instrument (ME220 and ML230)
- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables
- UV/Vis Absorbance Spectrophotometer [for example, NanoDrop® or Lunatic® (Unchained Labs®)]

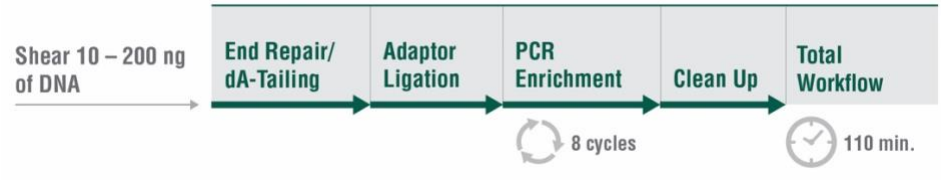
Overview

The NEBNext UltraExpress DNA Library Prep Kit contains the enzymes and buffers required to rapidly convert 10–200 ng of DNA into high-quality libraries for sequencing on the Illumina platform. Recommendations for lower input are now available in Appendix A. The fast, simple workflow features reduced cleanup steps and minimal hands-on time, and allows use of a single adaptor dilution and 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) cleanup conditions for altering library size distribution, if required.

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 DNA Library Prep Kit



Protocol for DNA 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 input DNA fragmented to 200 bp range. We recommend that DNA be sheared in 1X TE, with a total volume of 18 µl and 15 µl of sheared DNA to be transferred to End Prep reaction.

1. End Prep

1.1. Add the following components to a sterile nuclease-free tube:

COMPONENT	VOLUME
● (green) NEBNext UltraExpress End Prep Reaction Buffer	2.3 µl
● (green) NEBNext UltraExpress End Prep Enzyme Mix	1.0 µl
0.1X TE	1.7 µl
Fragmented DNA	15 µl
Total Volume	**20 µl**

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

1.2. Place in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program:
15 minutes at 20°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.

2. Adaptor Ligation

2.1. Add the following components directly to the End Prep Reaction Mixture:

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 1.2. in Section 1)	20 µl
• (red) NEBNext Adaptor for Illumina**	2 µl
• (red) NEBNext UltraExpress Ligation Master Mix	10 µl
Total Volume	**32 µl**

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

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

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 30 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 amount 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 2 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 with the heated lid set to ≥ 47°C.

Samples can be stored at –20°C for up to 3 days.

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. Primers are supplied at 10 µM combined (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)	34 µl
• (blue) NEBNext MSTC High Yield Master Mix	40 µl
• (blue) Index Primer/i7 Primer*,**	3 µl
• (blue) Universal PCR Primer/i5 Primer*,**	3 µl
Total Volume	**80 µl**

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

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.5)	34 µl
• (blue) NEBNext MSTC High Yield Master Mix	40 µl
• (blue) Index/Universal Primer*,**	6 µl
Total Volume	**80 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 70 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 thermocycler 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	8
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

PCR amplification with 8 cycles can generate sufficient library yields for sequencing from DNA inputs ranging from 10 ng to 200 ng and allow one PCR condition for the entire input range.

Note:

(1) For DNA input higher than 25 ng, 8 cycles of PCR will result in amplification beyond the linear phase. This non-linear amplification phase during the late cycles can cause inaccurate library quantification on migration-based methods like Bioanalyzer and TapeStation. In this case, we recommend using Nanodrop or Lunatic for library quantification. An example is shown on Figure 4.1.

(2) The adaptor amount and PCR cycle numbers are validated using high quality genomic DNA and NEBNext adaptor for Illumina. For other DNA input type, adaptor amount and PCR cycle optimization is recommended.

(3) For more specific library yield requirement, follow Appendix 1 for recommended adaptor dilution and 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.

3.1.4. 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 e.g., post ligation recommendations will not apply to samples post PCR. 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 NEBNext Bead Reconstitution Buffer at room temperature.

4.2. Add 56 µl (0.7X) resuspended beads to the 80 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. Alternatively, vortex on high for 3–5 seconds. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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: do not need ethanol wash at this step)**. Add 50 µl 0.1 X TE to resuspend the beads. Mix well by pipetting up and down at least 10 times. Add 40 µl (0.8X) NEBNext Bead Reconstitution Buffer to the 50 µl 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. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

4.8. 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.

4.9. 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)**.

4.10. Add 200 µl of 80% freshly prepared 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.

4.11. Repeat Step 4.10. 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.

4.12. 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.

4.13. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE.

4.14. 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.

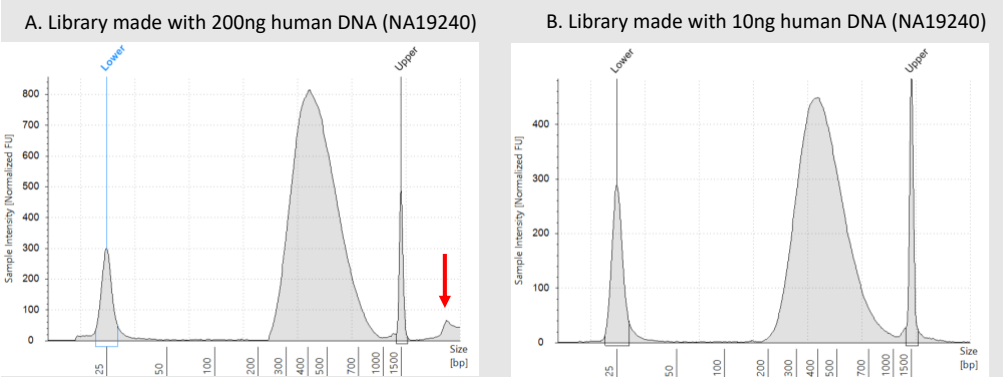
4.15. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube and store at –20°C.

4.16. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA Chip or Agilent High Sensitivity D1000 Screen Tape. The sample may need to be diluted before loading.



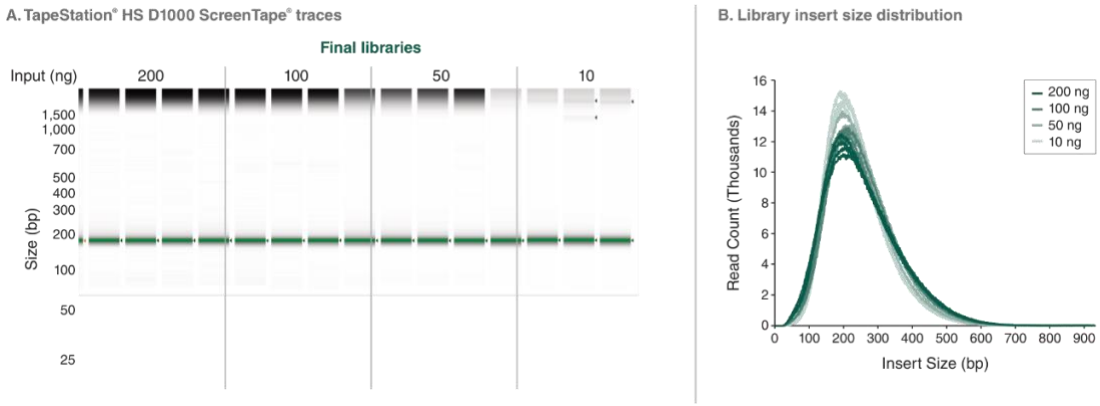
Samples can be stored at –20°C.

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



Libraries made from (A) 200 ng and (B) 10 ng human DNA (NA19240) with 8 PCR cycles were loaded on TapeStation using HSD1000 ScreenTape®. Figure A shows a peak above upper marker resulting from non-linear amplification phase for the 200 ng at late PCR cycles. Although the molecules generated at non-linear amplification phase are sequencible to generate high-quality data, 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.

Figure 4.2: Example of library and insert size distributions for 200, 100, 50 and 10 ng Human gDNA (Promega #G3041) inputs following the Covaris® ML230 Focused- ultrasonicator 8 AFA-TUBE TPX Strip 175 bp protocol using the NEBNext UltraExpress DNA Library Prep Kit.



(A)TapeStation® HS D1000 ScreenTape® traces of final libraries made from Human Genomic DNA (Promega #G3041). (B) Graphical depiction of library insert size distributions as determined by Picard CollectinsertSizeMetrics tool.

Appendix A
Recommendations for Customized Adaptor Dilutions and Cycling per Input (0.5 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 customized adaptor dilutions and PCR cycle numbers at given DNA inputs.

Follow PCR cycle numbers in **Table A1 to reach 100 ng library yield** using undiluted NEBNext Adaptor for Illumina for all input.

Table A1: For 100 ng library yield

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

Follow adaptor dilution recommendation and PCR cycle number for each input in **Table A2 to reach 1 µg library yield** for target enrichment application. 10 ng DNA input is not recommended for this application. Adaptor dilution is recommended for high PCR cycle numbers to avoid elevated adaptor dimers.

Table A2: For 1 µg library yield

INPUT DNA IN 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)
200 ng	No Dilution	7–8
100 ng	No Dilution	8–9
50 ng	5-Fold (1:5)	10–12

Appendix B
Recommendations for Customized 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 phased final library cleanup conditions for varying final library size distributions.

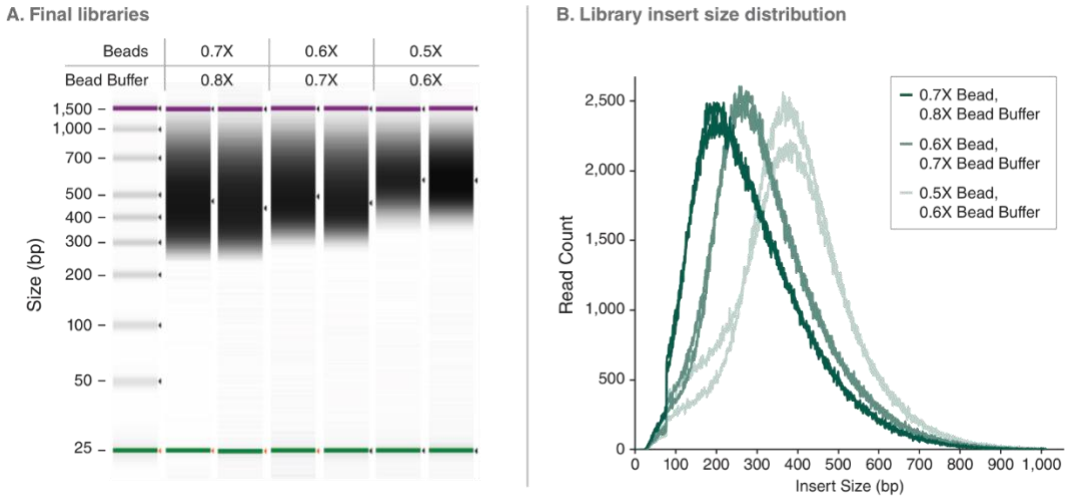
Note: Phased cleanups with less than 0.5X bead addition result in decreased library yields. The following recommendations are for libraries made from DNA fragmented to the 350 bp range with an input mass of 200 ng with 8 cycles of PCR. To apply custom phased cleanups on inputs of 10–100 ng, we recommend additional PCR cycling prior to cleanup (see Table A2).

Table B1: Approximate size distributions for libraries and inserts with custom phased cleanups.

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

PHASED CLEANUP REAGENT VOLUMES TO BE ADDED (µl)		APPROXIMATE LIBRARY PARAMETERS		
BEAD ADDITION (STEP 4.2.)	BEAD RECONSTITUTION BUFFER ADDITION (STEP 4.6.)	LIBRARY SIZE DISTRIBUTION (bp)	INSERT SIZE DISTRIBUTION (bp)	PEAK INSERT SIZE (bp)
56 (0.7X)	40 (0.8X)	250–1000	125–875	~180
48 (0.6X)	35 (0.7X)	300–1000	175–875	~250
40 (0.5X)	30 (0.6X)	400–1000	275–875	~400

Figure B1: Example of library and insert size distributions for 200 ng Human gDNA (NA19240) input fragmented with the Covaris ML230 350 bp 8 AFA-TUBE TPX Strip protocol.



TapeStation HS D1000 ScreenTape traces of (A) final libraries made from 200 ng Human gDNA (NA19240) fragmented with the Covaris ML230 350 bp 8 AFA-TUBE TPX Strip protocol and cleaned using a standard or custom phased cleanup. The first TapeStation lane on the left is the library trace for the standard cleanup condition. (B) Graphical depiction of library insert size distribution as determined by Picard CollectInsertSizeMetrics tool.

Kit Components

NEB #E3325S Table of Components

NEB #	COMPONENT	VOLUME
E3324A	NEBNext UltraExpress End Prep Reaction Buffer	0.056 ml
E3326A	NEBNext UltraExpress End Prep Enzyme Mix	0.024 ml
E3327A	NEBNext UltraExpress Ligation Master Mix	0.24 ml
E3328A	NEBNext MSTC High Yield Master Mix	0.96 ml
E3339A	NEBNext Bead Reconstitution Buffer	0.96 ml
E3341A	0.1X TE	2.4 ml

NEB #E3325L Table of Components

NEB #	COMPONENT	VOLUME
E3324AA	NEBNext UltraExpress End Prep Reaction Buffer	0.224 ml
E3326AA	NEBNext UltraExpress End Prep Enzyme Mix	0.096 ml
E3327AA	NEBNext UltraExpress Ligation Master Mix	0.96 ml
E3328AA	NEBNext MSTC High Yield Master Mix	3.9 ml
E3339AA	NEBNext Bead Reconstitution Buffer	3.9 ml
E3341AA	0.1X TE	9.6 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	9/23
1.1	Updated to remove storage information in Step 4.1, page 4.	10/23
2.0	Updated text in Required Materials Not Included; NEBNext singleplex or multiplex oligos for Illumina.	9/25
3.0	Update table of contents. Update title Appendix A and add Appendix B. Add figure B1 and 4.2.	7/26
3.1	Updated Table B1 in Appendix B.	11/26

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

Products and content are covered by one or more patents, trademarks and/or copyrights owned or controlled by New England Biolabs, Inc (NEB). The use of trademark symbols does not necessarily indicate that the name is trademarked in the country where it is being read; it indicates where the content was originally developed. See www.neb.com/trademarks. The use of these products may require you to obtain additional third-party intellectual property rights for certain applications. For more information, please email busdev@neb.com.

AGILENT®, BIOANALYZER®, TAPESTATION® and SCREENTAPE® are registered trademarks of Agilent Technologies, Inc.
ALPAQUA® is a registered trademark of Alpaqua Engineering, LLC.
BECKMAN COULTER® and AMPURE® are registered trademarks of Beckman Coulter, Inc. SPRISELECT™ is a trademark of Beckman Coulter, Inc.
EPPENDORF® and LOBIND® are registered trademarks of Eppendorf SE
COVARIS® is a registered trademark of Covaris, LLC
ILLUMINA® is registered trademarks of Illumina, Inc.
NANODROP® is a registered trademark of NanoDrop Technologies, Inc.
UNCHAINED LABS® and LUNATIC® are registered trademarks of Unchained Labs
B CORPORATION® is a registered trademark of B Lab IP, LLC, Inc.
New England Biolabs is an ISO 9001, ISO 14001 and ISO 13485 certified facility

© Copyright 2026, New England Biolabs, Inc.; all rights reserved