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E7805 NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3) V.2

New England Biolabs[1]

[1]New England Biolabs

New England Biolabs (NEB) NEBNext

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ABSTRACT

The NEBNext Ultra II FS DNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of intact gDNA, cDNA, plasmids and amplicons* into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow also has minimal hands-on time.
*Fragmentation times for cDNA, plasmids and amplicons may vary from gDNA times.

Note: The Ultra II FS Kit is not compatible with bisulfite conversion workflows and FFPE DNA.
See the following FAQ's for more information:
Is the NEBNext Ultra II FS DNA library prep kit for Illumina compatible with all sample types?
Can enzymatically fragmented DNA be used as input material for EM-seq?

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 OEM & Customized Solutions at NEB. Please contact custom@neb.com for further information.

Figure 1. Workflow demonstrating the use of NEBNext Ultra II FS DNA Library Prep Kit Illumina

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

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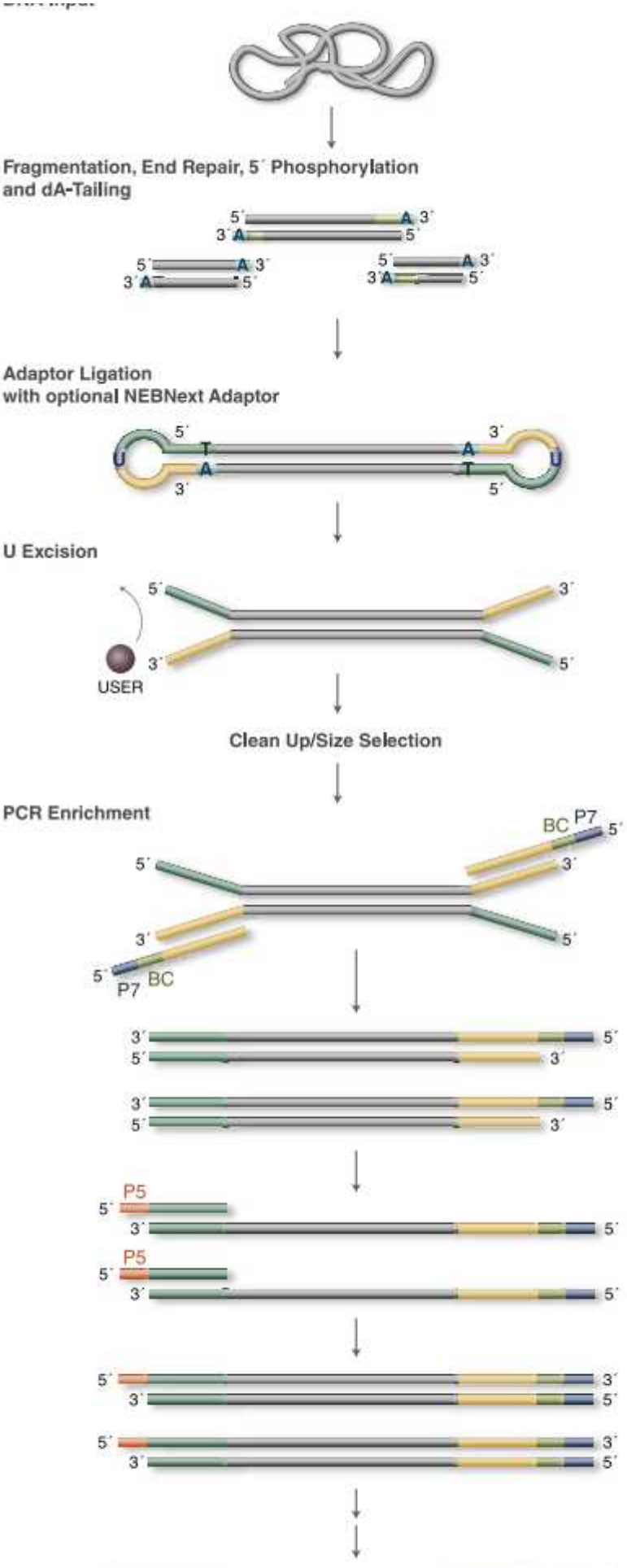
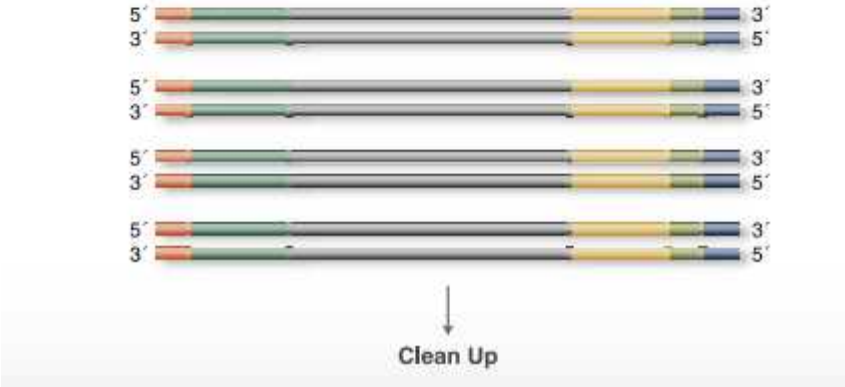




Adaptor trimming sequences
The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TruSeq:
AdaptorRead1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
AdaptorRead2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

GUIDELINES

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

Caution: This signifies a step in the protocol that has multiple paths that lead to the same end point but is dependent on a user variable like the amount of input DNA.

Color: A color listed before or after a reagent name indicates the cap color of the reagent to be added to a reaction.

MATERIALS

This Library Kit Includes
The volumes provided are sufficient for preparation of up to 24 reaction (NEB #E7805S/#E6177S) and 96 reactions (NEB #E7805L/#E6177L). All reagents should be stored at -20°C. Color words in parenthesis represent the color o the cap of the tube containing the reagent.

Package 1: Store at -20°C.
(yellow)
NEBNext Ultra II FS Enzyme Mix **New England Biolabs Catalog #E7806**
(yellow)
NEBNext Ultra II FS Reaction Buffer **New England Biolabs Catalog #E7807**
(red)
NEBNext Ultra II Ligation Master Mix **New England Biolabs Catalog #E7648**

(red) NEBNext Ligation Enhancer **New England Biolabs Catalog #E7374**

(blue)

NEBNext Ultra II Q5 Master Mix - 50 rxns **New England Biolabs Catalog #M0544S**

TE Buffer (1X) **New England Biolabs Catalog #E7808**

Package 2: Store at room temperature. Do not freeze.
Supplied only with NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads, NEB #E6177.
NEBNext Sample Purification Beads

Required Materials Not Included
NEBNext Oligo Kit options can be found at www.neb.com/oligos
Alternatively, customer supplied adaptor and primers can be used, please see information in the link below:
www.neb.com/faq-nonNEB-adaptors

Please Note: Separate instructions exist for UNIQUE DUAL INDEX UMI ADAPTORS.

- Magnetic rack (NEB #S1515S), magnetic plate (Alpaqua® cat. #A001322) or equivalent
- 80% Ethanol (freshly prepared)
- Nuclease-free water
- Thin wall 200 µl PCR tubes (For example Tempassure PCR flex-free 8-tube strips USA Scientific #1402-4708)
- PCR machine
- Vortex
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- Microcentrifuge

For NEB #E7805 only:
- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

Optional:
- 10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl (for adaptor dilution of DNA input < 100 ng) or NEB #B1430S

PROTOCOL MATERIALS

NEBNext Ultra II Ligation Master Mix **New England Biolabs Catalog #E7648**

Materials, Step 6

USER Enzyme - 50 units **New England Biolabs Catalog #M5505S** Step 9

NEBNext Ultra II Q5 Master Mix **New England Biolabs Catalog #E7649** Step 36

NEBNext Ultra II Q5 Master Mix - 50 rxns **New England Biolabs Catalog #M0544S**

Materials

TE Buffer (1X) **New England Biolabs Catalog #E7808** In Materials and 5 steps

NEBNext Ultra II FS Reaction Buffer **New England Biolabs Catalog #E7807**

Materials, Step 3

NEBNext Ultra II FS Enzyme Mix **New England Biolabs Catalog #E7806**

Materials, Step 3

NEBNext Ligation Enhancer **New England Biolabs Catalog #E7374**

Materials, Step 6

NEBNext Adaptor for Illumina **New England Biolabs** Step 6

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

In 4 steps

BEFORE START INSTRUCTIONS

Note: Follow this protocol for inputs ≥ 100 ng and fragment sizes > 550 bp.

Follow the protocol in Chapter 1 for inputs ≤ 100 ng
https://www.protocols.io/view/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-k8tczwn

Follow the protocol in Chapter 2 for inputs ≥ 100 ng and fragments ≤ 550 bp.
https://www.protocols.io/view/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-nwxdffn

For 100 ng inputs, either the no size selection protocol (Chapter 1) or a size selection protocol (Chapter 2 or 3) can be followed.

Starting Material: 100–500 ng purified, genomic DNA*. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or H_2O are also acceptable. If the input DNA is less than 26 µl, add TE (provided) to a final volume of 26 µl.

* This kit can also be used for plasmids, cDNA and some amplicons. It is not recommended for use with FFPE DNA. See FAQ: https://www.neb.com/faqs/2020/10/16/is-the-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina-compatible-with-all-sample-types

For FFPE DNA we recommend NEBNext Ultra II DNA Library Pre Kit for Illumina + Covaris or NEBNext FFPE DNA Library Prep Kit (NEB #E6650) or NEBNext UltraShear FFPE DNA Library Prep Kit (NEB #E6655).

Fragmentation/End Prep

1 Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

2 Vortex the Ultra II FS Enzyme Mix 00:00:05 - 00:00:08 prior to use and place on ice. 13s

Note

It is important to vortex the enzyme mix prior to use for optimal performance.

3 Add the following components to a 0.2 ml thin wall PCR tube on ice:

	A	B
	COMPONENT	VOLUME PER ONE LIBRARY
	DNA*	26 µL
	(yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
	(yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
	Total Volume	35 µl

*Intact gDNA, cDNA, plasmids and some amplicons.

NEBNext Ultra II FS Reaction Buffer **New England Biolabs Catalog #E7807**

NEBNext Ultra II FS Enzyme Mix **New England Biolabs Catalog #E7806**

4 Vortex the reaction for 00:00:05 and briefly spin in a microcentifuge. 5s

5 In a thermal cycler, with the heated lid set to 75 °C, run the following program:

	A	B
	Time	Temperature (°C)
	5 min	37°C
	30 min	65°C
	Hold	4°C

Note

Safe Stop: 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.

Adaptor Ligation

6 Add the following components directly to the FS Reaction Mixture:

	A	B
	Component	Volume
	FS Reaction Mixture (Step 5)	35 µl
	(red) NEBNext Ultra II Ligation Master Mix*	30 µl
	(red) NEBNext Ligation Enhancer	1 µl
	(red) NEBNext Adaptor for Illumina**	2.5 µl
	Total Volume	68.5 µl

* Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding 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. Please see www.neb.com/oligios for additional information.

Note

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

NEBNext Ultra II Ligation Master Mix **New England Biolabs Catalog #E7648**

NEBNext Ligation Enhancer **New England Biolabs Catalog #E7374**

NEBNext Adaptor for Illumina **New England Biolabs**

7 Set a 100 µl or 200 µl pipette to 50 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 Ultra II 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.

8 Incubate at 20 °C for 00:15:00 in a thermal cycler with the heated lid off. 15m

9 Add 3 µL of (red or blue) USER Enzyme to the ligation mixture from the previous step.

Note

Steps 9 and 10 are only required for use with non indexed NEBNext Adaptor. USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed UMI adaptor, USER enzyme is not needed. Please see corresponding manual for use with UMI on the Unique Dual Index UMI Adaptor Sets 1-4 product page under the protocols, manuals, and usage tab.

USER Enzyme - 50 units **New England Biolabs Catalog #M5505S**

10 Mix well and incubate at 37 °C for 00:15:00 with the heated lid set to ≥ 47 °C. 15m

Note

Safe Stop: Samples can be stored overnight at -20 °C.

Size Selection of Adaptor-ligated DNA for Fragment Sizes > 5...

11 **Caution: The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for us** 30m **with the sample contained in the exact buffer at this step (71.5 µL ; Step 10). AMPure XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to room temperature for at least 00:30:00 before use. These bead volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup at this step. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.**

12 Bring the volume of the reaction up to 100 µL by adding 28.5 µL 0.1X TE (dilute 1X TE Buffer 1:10 with water).

TE Buffer (1X) **New England Biolabs Catalog #E7808**

13 Vortex SPRIselecct or NEBNext Sample Purification Beads to resuspend

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

14 Add 40 µL (0.4X) resuspended beads to the Adaptor Ligation reaction. Mix well by pipetting up an 8s down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 00:00:03 - 00:00:05 on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

15 Incubate samples for at least 00:05:00 at room temperature. 5m

16 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.

17 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be 5m careful not to disturb the beads that contain DNA targets.

Caution: Do not discard the beads.

18 Add 200 µL of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incub 30s at room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

19 Repeat the previous step 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.

20 Air dry the beads for up to 00:05:00 while the tube/ plate is on the magnetic stand with the lid op 5m

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.

21 Remove the tube/ plate from the magnetic stand. Elute the DNA target from the beads by adding 102 µL 0.1X TE (dilute 1X TE Buffer 1:10 in water).

TE Buffer (1X) **New England Biolabs Catalog #E7808**

22 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 2m 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.

23 Place the tube/ plate on the magnetic stand. After 00:05:00 (or when the solution is clear), trans 5m 100 µL to a new PCR tube.

24 Add 50 µL (~ 0.5X) of resuspended SPRIselect or Sample Purification Beads to the sample. Mix w 8s 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 00:00:03 – 00:00:05 on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

25 Incubate samples at room temperature for at least 00:05:00. 5m

26 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.

27 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant that 5m contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA.

Caution: Do not discard the beads.

28 Add 200 µL of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incub 30s

at room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

29 Repeat the previous step 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.

30 Air dry the beads for up to 00:05:00 while the tube/ plate is on the magnetic stand with the lid op 5m

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.

31 Remove the tube/ plate from the magnetic stand. Elute the DNA target from the beads into 17 µL 0.1X TE (dilute 1X TE Buffer 1:10 in water).

TE Buffer (1X) **New England Biolabs Catalog #E7808**

32 Mix well on a vortex mixer or by pipetting up and down at least 10 times. Incubate for at least 2m 00:02:00 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.

33 Place the tube/ plate on a magnetic stand. After 00:05:00 (or when the solution is clear), transfe 5m 15 µL to a new PCR tube.

34 Proceed to PCR Enrichment of Adaptor-ligated DNA in next Section: **PCR Enrichment of Adaptor-ligated DNA**

Safe Stop Point: Samples can be stored at -20 °C

PCR Enrichment of Adaptor-ligated DNA

35 • Use **Option A** for any NEBNext Oligo Kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in serparate tubes. Primers are supplied at 10 µM each.

• Use **Option B** for any NEBNext Oligo Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined (5 µM each).

36 Add the following components to a sterile strip tube:

Option A: Forward and Reverse Primers NOT Already Combined:

	A	B
	Component	Volume
	Adaptor Ligated DNA Fragments (Step 33)	15 µl
	(blue) NEBNext Ultra II Q5 Master Mix	25 µl
	(blue) Index Primer/i7 Primer *,**	5 µl
	(blue) Universal PCR Primer/i5 Primer *,**	5 µl
	Total Volume	50 µl

* NEBNext Oligos must be purchased seperately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section in the materials section. Refer to the corresponding NEBNext Oligo kit manuals 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.

Option B: Forward and Reverse Primers Already Combined

	A	B
	Component	Volume
	Adaptor Ligated DNA Fragments (Step 33)	15 µl
	(blue) NEBNext Ultra II Q5 Master Mix	25 µl
	(blue) Index Primer Mix*	10 µl
	Total Volume	50 µl

*NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" in the materials section.

NEBNext Ultra II Q5 Master Mix **New England Biolabs Catalog #E7649**

37 Set a 100 µl or 200 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.

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

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	3-8*
	Annealing/ Extension	65°C	75 seconds	
	Final Extension	65°C	5 minutes	1
	Hold	4°C		

* The number of PCR cycles recommended in Table A are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples. Use Table B for applications requiring high library yields, such as target enrichment. The number of PCR cycles should be chosen based on input amount and sample type. Thus, samples prepared with a different method prior to library prep may require re-optimization of the number of PCR cycles. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on Bioanalyzer).

Table A

	A	B
	INPUT DNA IN THE FS REACTION	# OF CYCLES REQUIRED FOR STANDARD LIBRARY PREP: YIELD ~100 ng (5-35 nM)*
	500 ng	3-4
	200 ng	4-5
	100 ng	5-7

* Cycle number was determine for size selected libraries.

Table B

	A	B
	INPUT DNA IN THE FS REACTION	# OF CYCLES REQUIRED FOR TARGET ENRICHMENT LIBRARY PREP (YIELD ~1 µg)*
	500 ng	4-5
	200 ng	5-6

	A	B
	100 ng	7-8

* Cycle number was determined for non-size selected libraries.

39 Proceed to Cleanup of PCR reaction in next Section: **Cleanup of PCR Reaction**.

Cleanup of PCR Reaction

40 **Note: The volumes of SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. AMPure XP beads can be used as well. If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use. These volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.**

41 Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

42 Add 30 µL (0.6X) resuspended beads to the PCR reaction. Mix well by pipetting up and down at 8s least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 00:00:03 - 00:00:05 on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

NEBNext Sample Purification Beads **New England Biolabs Catalog #E7767**

43 Incubate samples on bench top for at least 00:05:00 at room temperature. 5m

44 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.

45 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be 5m careful not to disturb the beads that contain DNA targets
Caution: do not discard the beads.

46 Add 200 µL of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incub 30s at room temperature for 00:00:30, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

47 Repeat the previous step 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.

48 Air dry the beads for up to 00:05:00 while the tube/ plate is on the magnetic stand with the lid op 5m

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.

49 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).

TE Buffer (1X) **New England Biolabs Catalog #E7808**

50 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 2m 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.

51 Place the tube/ plate on the magnetic stand. After 00:05:00 (or when the solution is clear), trans 5m 30 µL to a new PCR tube and store at -20 °C.

Assess Library Quality on a Bioanalyzer

52 Dilute library (from previous step) 5-fold in 0.1X TE Buffer.

TE Buffer (1X) **New England Biolabs Catalog #E7808**

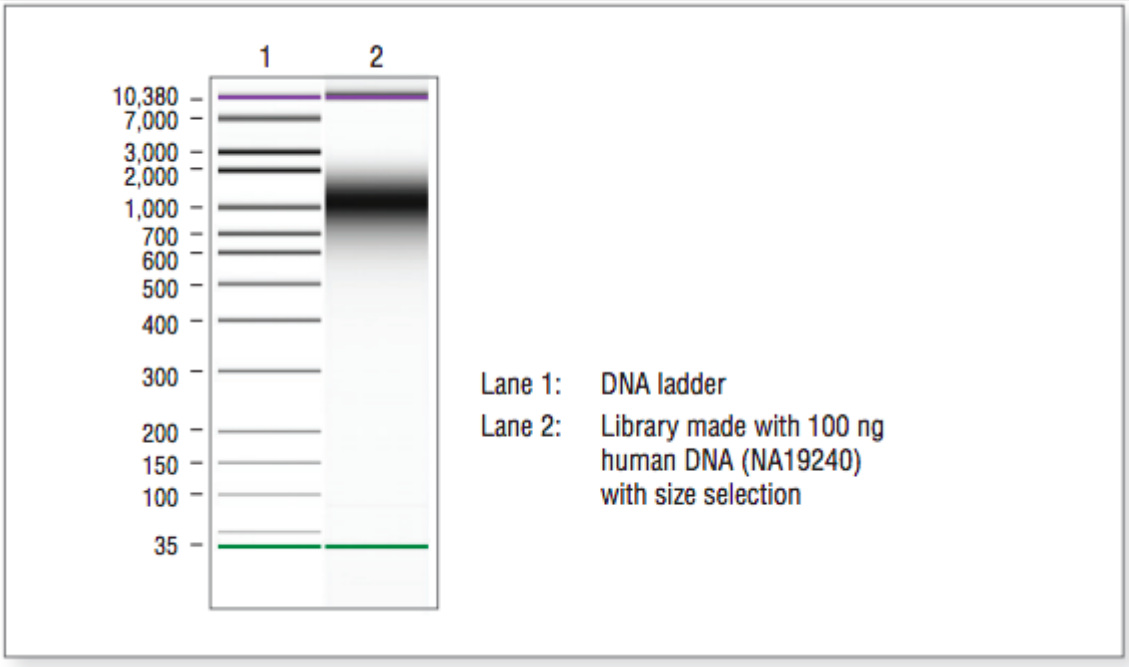
53 Run 1 µL on a DNA High Sensitivity Chip.

54 Check that the library size shows a narrow distribution with an expected peak size > 700 bp (See Figure 1)

Note

If a peak ~80 bp (primers) or ~128-140 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 51) to 50 µL with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in the previous section: Cleanup of PCR Reaction.

Figure 1: Example of final library size distributions without size selection. 100 ng Human DNA (NA 19240) was fragmented for 5 minutes.



Note

Due to the preference of the Illumina sequences to preferentially cluster smaller fragments, the average insert size from the sequence data may be smaller than expected (see Figure 2).

We recommend gel size selection if you need an average sequence insert size > 700 bp.

Figure 2: Example of insert size distribution after bead-based size selection.