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E7805 NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® Protocol to use with Inputs ≥ 100 ng (Chapter 2) V.2

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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 DNA into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow also has minimal hands-on time.

Note: The Ultra II FS Kit is not compatible with bisulfite conversion workflows.

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/Bulks department at NEB. Please contact OEM@neb.com for further information.

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

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MATERIALS

Materials

Package 1: Store at -20°C.
(yellow) NEBNext Ultra II FS Enzyme Mix
(yellow) NEBNext Ultra II FS Reaction Buffer
(red) NEBNext Ultra II Ligation Master Mix
(red) NEBNext Ligation Enhance
(blue) NEBNext Ultra II Q5 Master Mix
TE Buffer (1X)

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: www.neb.com/faq-nonNEB-adaptors

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

- Manetic 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

BEFORE START INSTRUCTIONS

Note: Follow this protocol for inputs ≥ 100 ng and size selection for inserts up to 550 bp.

Follow the protocol in Chapter 1 for inputs ≤ 100 ng, as size selection is not recommended for this input range. https://www.protocols.io/view/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-14egnypmv5dy/v2

Follow the protocol in Chapter 3 for inputs ≥ 100 ng and fragment sizes > 550 bp. https://www.protocols.io/view/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-n2idgce

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

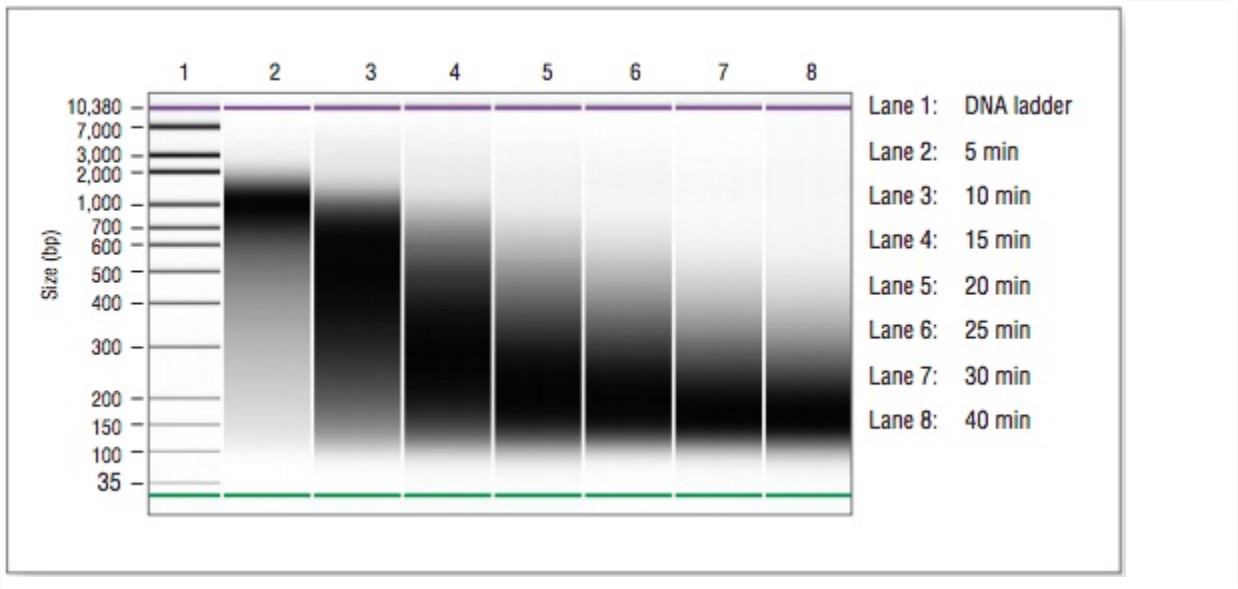
Starting Material: 100–500 ng purified, genomic DNA. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or H_2O are also acceptable. If the input DNA is less than 26 µl, add TE (provided) to a final volume of 26 µl.

Fragmentation/End Prep

1 Fragmentation occurs during the 37°C incubation step. Use the chart below to determine the incubation time required to generate the desired fragment sizes. Incubation time may need to be optimized for individual samples. See Figure 2.1 for a typical fragmentation pattern.

	A	B	C
	Fragmentation Size	Incubation @ 37°C	Optimization
	100 bp-250 bp	30 min	30-40 min
	150 bp-350 bp	20 min	20-30 min
	200 bp-450 bp	15 min	15-20 min
	300 bp-700 bp	10 min	5-15 min
	500 bp-1 kb	5 min	5-10 min

Figure 2.1: Example of size distrubibtion on a Bioanalyzer®. Human DNA (NA19240) was fragmented for 5-40 min.



2 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.

Note

There are short videos on NEB.com about handling the FS reagents:
Preparing FS Reagents: https://www.neb.com/tools-and-resources/video-library/quick-tips---preparing-the-nebnext-ultra-ii-fs-dna-reaction-buffer-and-enzyme-mix
FS master mix: https://www.neb.com/tools-and-resources/video-library/quick-tips---preparing-nebnext-ultra-ii-fs-dna-reaction-buffer-and-enzyme-mix-master-mix

3 Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice.

Note

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

4 Add the following competents to a 0.2 ml thin wall PCR tube on ice:

	A	B

	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

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

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

	A	B
	Duration	Temp
	5-30 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 the adaptor ligation before stopping.

Adaptor Ligation

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

	A	B
	Component	Volume
	FS Reaction Mixture (Previous Step)	35 µl
	(red) NEBNext Ultra II Ligation Master Mix *	30 µl

	A	B
	(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 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. Please see www.neb.com/oligios for additional information.

Note

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 08:00:00 @ 4 °C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

8 Set a 100 µl or 200 µl 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 the 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.

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

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

Note

Steps 11 and 12 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 is not needed. Please see corresponding manual for use with UMI on the NEB #E7395 product page under protocols, manuals, and usage tab.

11 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 DNA Input ≥ 100 ...

12 **Caution: If the starting material is ≥ 100 ng, follow the protocol for size selection below. For inputs < 100 ng, size selection is not recommended. Follow the protocol for cleanup without size selection in Chapter 1: Section Size Selection or Cleanup of Adaptor-Ligated DNA in the Ultra II FS DNA manual. If you want fragment sizes > 550 bp and your input is ≥ 100 ng follow the entire protocol in Chapter 3 in the Ultra II FS DNA manual.** 30m

Note

Caution: 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 (71.5 µl; Step 12). These volumes may not work properly for a size selection at a different step in the workflow, or if this is a second size selection. For size selection of samples contained in different buffer conditions bead volumes may need to be experimentally determined.

Caution: The following size selection protocol is for libraries with 150-200 bp inserts only. For libraries with different size fragment inserts, refer to Table 2.3.1. below for the appropriate volumes of beads to be added. The size selection protocol is based on a starting volume of 100 µl. Size selection conditions were optimized with SPRIselect or NEBNext Sample Purification Beads; however, AMPure XP beads can be used following the same conditions. If using AMPure

XP beads, please allow the beads to warm to room temperature for at least 00:30:00 **before use.**

To select a different insert size than 200 bp, please use the volumes in this table:

Table 2.3.1: Recommended conditions for bead based size selection:

LIBRARY PARAMETERS	APPROXIMATE INSERT SIZE DISTRIBUTION	150-250 bp	200-350 bp	275-475 bp	350-600 bp
	Approx. Final Library Size Distribution (insert + adaptor + primers)	270-370 bp	320-470 bp	400-600 bp	470-800 bp
BEAD VOLUME TO BE ADDED (µl)	1st Bead Addition	40	30	25	20
	2nd Bead Addition	20	15	10	10

13 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).

14 Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

15 Add 40 µL (~ 0.4X) of resuspended beads to the 100 µL sample from Step 12. 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.

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

17 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.

18 After 5 minutes (or when the solution is clear), carefully transfer the supernatant (~ 140 µL) containing your DNA to a new tube **(Caution: do not discard the supernatant.)** Discard the beads that contain the unwanted large fragments.

19 Add 20 µL (~0.2 X) resuspended SPRIselect or Sample Purification Beads to the supernatant and mix at least 10 times. Be careful to expel all the liquid from the tip during the last mix. Incubate samples on the bench top for at least 00:05:00 at room temperature. 5m

20 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.

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

Caution: Do not discard the beads.

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

23 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.

24 Air dry the beads for up to 00:05:00 while the tube/plate is on the magnetic stand with the lid open. 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 as evaporated. When the beads turn lighter brown and start to crack, they are too dry.

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

26 Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least 00:02:00 at room temperature. If necessary, quickly spin the samples to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 2m

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

28 Proceed to PCR Enrichment or Adaptor-ligated DNA in the next section: **PCR Enrichment of Adaptor-ligated DNA**.

Note

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

PCR Enrichment of Adaptor-ligated DNA

29 Use **Option A** for any kit where NEBNext index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate 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).

30 Add the following components to a sterile tube:

Option A: Forward and Reverse Primers NOT already combined:

	A	B
	Not Combined Primers	Amount
	Adaptor Ligated DNA Fragments (Step 28)	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 separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (Materials section). Refer to the corresponding NEBNext Oligo 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.

Option B: Forward and Reverse Primers Already combined:

	A	B
	Combined Primers	Amount
	Adaptor Ligated DNA Fragments (Step 28)	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" section (Materials section).

31 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.

32 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:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	3-7*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles recommended in Table 2.4.1 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples. Use Table 2.4.2 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 2.4.1.

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

* Cycle number was determined for size selected libraries.
** NEBNext adaptors contain a unique truncated design. Libraries constructed with NEBNext adaptors require a minimum of 3 amplification cycles to add the complete adaptor sequences for downstream processes.

Table 2.4.2.

INPUT DNA IN THE END PREP REACTION	# OF CYCLES REQUIRED FOR TARGET ENRICHMENT LIBRARY PREP (YIELD ~750 ng–1 µg)*
500 ng	4–5
200 ng	5–6
100 ng	6

* Cycle number was determined for size selected libraries.

33 Proceed to Cleanup of PCR reaction in the next section.

Cleanup of PCR Reaction

34 **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** 00:30:00 **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.** 30m

35 Vortex SPRIselect or NEBNext Sample Purificiation Beads to resuspend.

36 Add 45 µL (0.9X) resuspended beads to the 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. 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.

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

38 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.

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

Caution: Do not discard the beads.

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

41 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.

42 Air dry the beads for up to 00:05:00 while the tube/plate is on the magnetic stand with the lid open. 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.

43 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).

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

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

Assess Library Quality on a Bioanalyzer

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

47 Run 1 µL on a DNA High Sensitivity Chip.

48 Check that the library size shows a narrow distribution with an expected peak size based on fragmentation time (See Figure 2.2).

Note

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 46) to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in the previous section.

Figure 2.2: Example of final library size distributions with size selection. Human DNA (NA 19240) was fragmented for 5 or 15 minutes.