E7805 NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® Protocol for use with Inputs ≤ 100 ng V.2

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We use this protocol and it's working

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ABSTRACT

The NEBNext Ultra II FS DNA Module 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 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.

GUIDELINES

Safe Stop: This is a point where you can safely stop the protocol and store the sample prior to proceeding to the next step.

Caution: 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.

Color: Indicates the cap color of the reagent to be added to a reaction.

The NEBNext Ultra II FS DNA Module is Designed for use with the Following:
NEBNext Ultra II Ligation Module (NEB #E7595)
NEBNext Ultra II Q5® Master Mix (NEB #M0544)
NEBNext Singleplex or Multiplex Oligos for Illumina® www.neb.com/oligos
This protocol is written for **non-UMI adaptors**

MATERIALS

Materials

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

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

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

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

NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs Catalog #M0544L**

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

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 DUEL INDEX UMI ADAPTORS.

- Magnetic rack (NEB #ES1515S), 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.

Starting Material

1 100 pg – 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

2 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 1.1 for a typical fragmentation pattern.

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

3 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.

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

Note

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

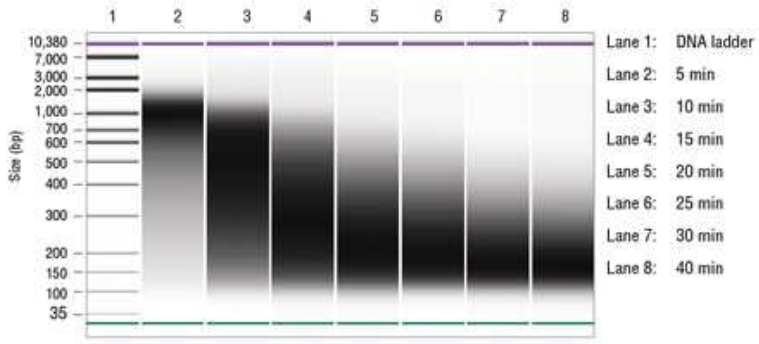
4 Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice.

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

Note

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

Figure 1.1: Example of size distribution on a Bioanalyzer®. Human DNA (NA19240) was fragmented for 5-40 min.



5 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

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

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

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

7 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

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

8 Determine whether adaptor dilution is necessary.

Caution: *If DNA input is <* 100 ng*, dilute the (red) NEBNext Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl as indicated in Table 1.2.1.*

Table 1.2.1: Adaptor Dilution

INPUT	ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL VOLUME)	WORKING ADAPTOR CONCENTRATION
100 ng–500 ng	No Dilution	15 µM
5 ng–99 ng	10-Fold (1:10)	1.5 µM
less than 5 ng	25-Fold (1:25)	0.6 µM

Note

Note: The appropriate adaptor dilution for your sample input and type may need to be optimized experimentally. The dilutions provided here are a general starting point.

9 Add the following components directly to the FS Reaction Mixture

	A	B
	COMPONENT	VOLUME
	FS Reaction Mixture (Step 7)	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 to the reaction.
** The NEBNext adaptor is provided in the NEBNext Oligo Kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

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**

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.**

10 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.)

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

12 Add 3 µL of (red or blue) USER® Enzyme to the ligation mixture from the previous step.
NEB USER® Enzyme **New England Biolabs Catalog #M5505S/L**

Note

Note: Steps 12 and 13 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 in not needed. Please see corresponding manual for use with UMI on the NEB #E7395 product page under the protocols, manuals, and usage tab.

13 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 or Cleanup of Adaptor-ligated DNA

14 **Caution: The following section is for cleanup of the ligation reaction for inputs ≤ 100 ng. If your input DNA is > 100 ng, follow the size selection protocol in Chapter 2, Section 2.3. If you want fragment sizes > 550 bp and your input is ≥ 100 ng, follow the entire protocol in Chapter 3.**

Note

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 (71.5 µL previous 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 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.

15 Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

16 Add 57 µL (0.8X) resuspended beads to the Adaptor Ligation 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.

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

18 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.

19 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).

20 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

21 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.

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

Note

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.

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

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

24 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

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

26 Proceed to PCR Enrichment of Adaptor-ligated DNA in Next Section.

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

PCR Enrichment of Adaptor-ligated DNA

27

Select PCR Reaction Setup
Step 27 includes a Step case.
Option A
Option B

step case

Option A

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 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).

Add the following components to a sterile strip tube:

	A	B

	A	B
	COMPONENT	VOLUME (µl) PER REACTION
	Adaptor Ligated DNA Fragments (Step 25)	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 (page 1). 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.

28 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.

29 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	3-13*
	Annealing/Extension	65°C	75 seconds	3-13*
	Final Extension	65°C	5 minutes	1
	Hold	4°C	∞	

* The number of PCR cycles recommended in the Table 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 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).

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

	A	B
	100 ng	3–4
	50 ng	4-5
	10 ng	6-7
	5 ng	7-8
	1 ng	8-9
	0.5 ng	8-10
	0.1 ng	12-13

TABLE 1

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

	A	B
	INPUT DNA IN THE FS REACTION	# OF CYCLES REQUIRED FOR TARGET ENRICHMENT LIBRARY PREP (YIELD ~750 ng–1 µg)*:
	100 ng	4-5
	50 ng	5-6
	10 ng	8-9
	5 ng	9-10
	1 ng	11-12
	0.5 ng	12-13
	0.1 ng	N/A

TABLE 2

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

30 Proceed to Cleanup of PRC reaction in next section.

Cleanup of PCR Reaction

31

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.

32 Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

33 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.

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

35 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.

36 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.

37 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

38 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.

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

Note

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.

40 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).

41 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

42 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 45m

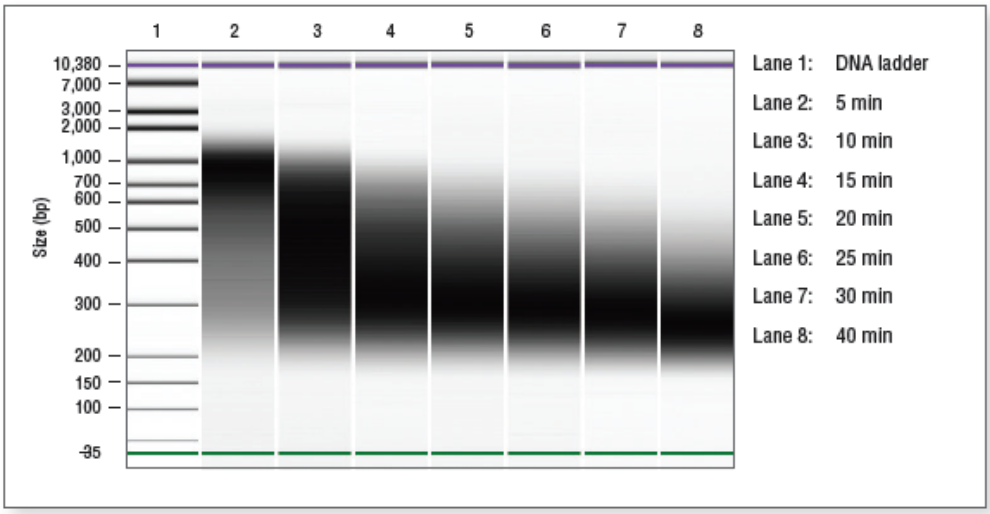
43 Dilute library (from the previous step) 5-fold in 0.1X TE Buffer (inputs ≤ 1 ng may not require dilution to run on a Bioanalyzer).

44 Run 1 µL on a DNA High Sensitivity Chip.

45 Check that the library size shows a narrow distribution with an expected peak size based on fragmentation time (Figure 1.2).

Figure 1.2: Example of final library size distribution without size selection. Human DNA (NA 19240) was fragmented for 5-40 min.

Figure 1.2: Example of final library size distributions without size selection. Human DNA (NA 19240) was fragmented for 5-40 minutes.



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 42) to 50 µL with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section "Cleanup of PCR reaction". You may see adaptor-dimer when starting with inputs ≤ 1 ng.

Step 45 includes a Step case.