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

New England Biolabs[1]

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1 *Works for me* dx.doi.org/10.17504/protocols.io.nwxdffn

New England Biolabs (NEB)
Tech. support phone: **+1(800)632-7799** email: **info@neb.com**

Isabel Gautreau
New England Biolabs

SUBMIT TO PLOS ONE

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.

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OWNERSHIP HISTORY

Mar 19, 2018 Lauren Moran New England Biolabs

Dec 21, 2018 Isabel Gautreau New England Biolabs

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GUIDELINES

The NEBNext Ultra II FS DNA Library Prep Kit For Illumina Designed for use with the Following:
NEBNext Singleplex or Multiplex Oligos for Illumina® www.neb.com/oligos
This protocol is written for **non-UMI adaptors**

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

MATERIALS TEXT

Required Materials Not Included

•80% Ethanol (freshly prepared)
•Nuclease-free water
•0.2 ml thin wall PCR tubes
•NEBNext Multiplex Oligos www.neb.com/oligos
•Magnetic rack/stand (NEB #S1515; Alpaqua®, cat. #A001322 or equivalent)
•PCR machine
•Vortex
•Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
•Microcentrifuge
• NEB #B1430 (10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl for adaptor dilution of DNA input < 100 ng)

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

BEFORE STARTING

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-k8tczwn

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

1 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

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 beed 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-250 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

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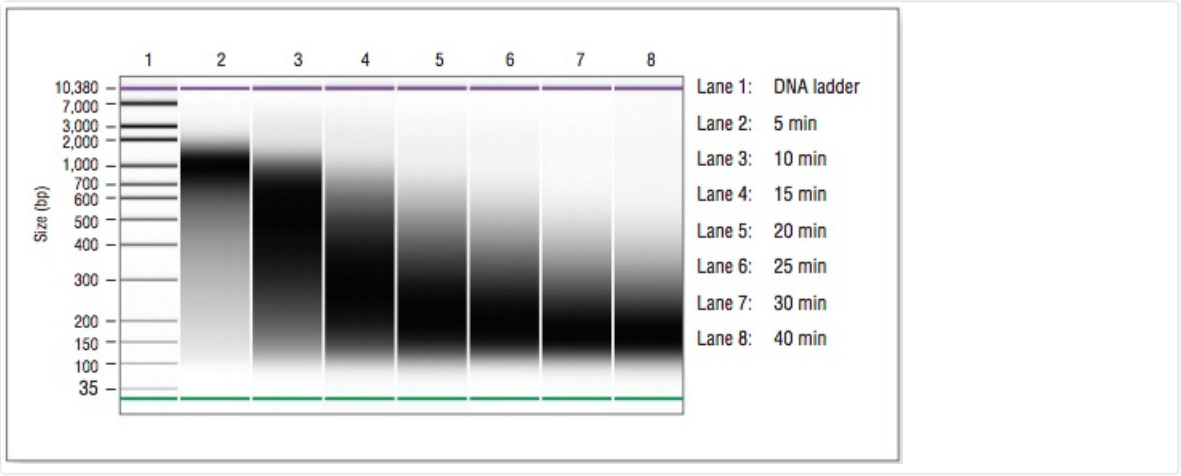
Figure 2.1: Example of size distrubibtion on a Bioanalyzer. Human DNA (NA19240) was fragmented for 5-40 min.



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, followed by a quick vortex to mix. Place on ice until use.

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.

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

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
NEBNext Ultra II FS Enzyme Mix	2 µl
Total Volume	35 µl

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

7 In a thermocycler, with the heated lid set to 70°C, run the following program:

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol to use with Inputs ÃçÂÂ¥ 100 ng (Chapter 2). https://dx.doi.org/10.17504/protocols.io.nwxdffn

A	B
Duration	**Temp**
5-30 min	37°C
30 min	65°C
Hold	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 the adaptor ligation before stopping.

Adaptor Ligation

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

A	B
Component	**Volume**
FS Reaction Mixture (Previous Step)	35 µl
NEBNext Ultra II Ligation Master Mix *	30 µl
NEBNext Ligation Enhancer	1 µl
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.

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 Adaptor in the Adaptor Ligation Step.

9 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 efficienty. The presence of a small amount of bubbles will not interfere with performance.

10 Incubate at 20°C for 15 minutes in a thermocycler with the heated lid off.

00:15:00 Incubate

11 Add 3 µl of USER Enzyme to the ligation mixture from the previous step.

USER enzyme addition and incubation are only required for use with NEBNext Standard Adaptors. USER enzyme can be found in the NEBNext Oligos for Illumina.

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

00:15:00 Incubate

Samples can be stored overnight at –20°C.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂ¢ II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol to use with Inputs ÇÂÂ¥ 100 ng (Chapter 2). https://dx.doi.org/10.17504/protocols.io.nwxdffn

Size Selection of Adaptor-ligated DNA for DNA Input ≥ 100 ng

13 **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.**

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. 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.

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 30 minutes 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

14 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).

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

16 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.

17 Incubate samples for at least 5 minute at room temperature.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol to use with Inputs ÃçÂÂ¥ 100 ng (Chapter 2). https://dx.doi.org/10.17504/protocols.io.nwxdffn

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 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.

20 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 5 minutes at room temperature.

21 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.

22 After 5 mintues (or when the solution is clear), carefully remove and sicard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA.

Caution: Do not discard the beads.

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

24 Repeat the previous step once. 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.

25 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 as evaporated. When the beads turn lighter brown and start to crack, they are too dry.

26 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).

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

28 Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl to a new PCR tube.

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

Samples can be stored at –20°C.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol to use with Inputs ÃçÂÂ¥ 100 ng (Chapter 2). https://dx.doi.org/10.17504/protocols.io.nwxdffn

PCR Enrichment of Adaptor-ligated DNA

30 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.

Use **Option B** for any kit where NEBNext index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined.

31 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
NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer/i7 Primer *, **	5 µl
Universal PCR Primer/i5 Primer	5 µl
Total Volume	50 µl

Option B: Forward and Reverse Primers Already combined:

A	B
Combined Primers	**Amount**
Adaptor Ligated DNA Fragments (Step 28)	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index/ Universal Primer*	10 µl
Total Volume	50 µl

* NEBNext Oligos must be purchased separately from the library prep kit. 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.

32 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.

33 Place the tube of 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	3-7*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

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* 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.
** Standard NEBNext adaptors contain a unique truncated design. Libraries constructed with Standard 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.

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

Cleanup of PCR Reaction

35 **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.**

36 Vortex SPRIselect or NEBNext Sample Purificiation Beads to resuspend.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol to use with Inputs ÃçÂÂ¥ 100 ng (Chapter 2). https://dx.doi.org/10.17504/protocols.io.nwxdffn

37 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.

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

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

40 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.

41 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.

42 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.

43 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.

44 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).

45 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.

46 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.

Assess Library Quality on a Bioanalyzer

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

48 Run 1 µl on a DNA High Sensitivity Chip.

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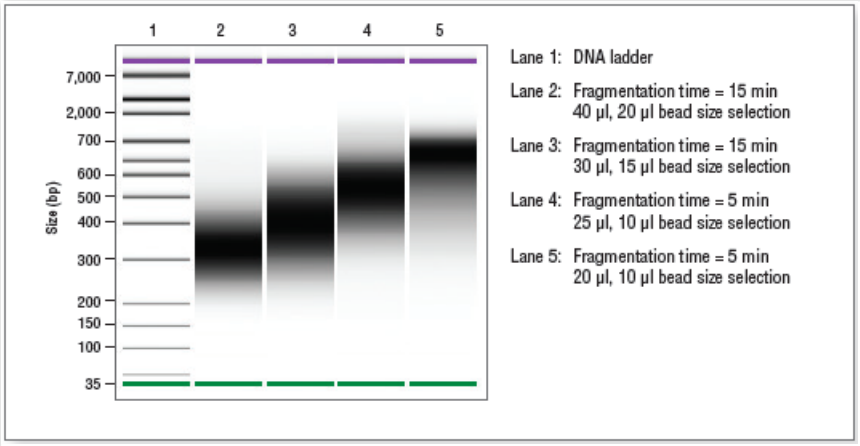
If a peak ~80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 46) to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section: PCR Enrichment of Adaptor-ligated DNA.

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

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



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