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

New England Biolabs[1]

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1 Works for me dx.doi.org/10.17504/protocols.io.k8tczwn

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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 intact DNA into fragmented DNA with 5´ phosphorylated 3´ dA-tailed ends. The module is optimized for use with the NEBNext Ultra II Ligation Module (NEB #E7595) and with the NEBNext Ultra II Q5 Master Mix (NEB #M0544) if amplification is required. The fast, user-friendly workflow has minimal hands on time.

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PROTOCOL CITATION

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OWNERSHIP HISTORY

Dec 12, 2017 Lenny Teytelman protocols.io

Dec 12, 2017 Isabel Gautreau New England Biolabs

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9203

GUIDELINES

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 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)

ABSTRACT

The NEBNext Ultra II FS DNA Module contains the enzymes and buffers required to convert a broad range of input amounts of intact DNA into fragmented DNA with 5´ phosphorylated 3´ dA-tailed ends. The module is optimized for use with the NEBNext Ultra II Ligation Module (NEB #E7595) and with the NEBNext Ultra II Q5 Master Mix (NEB #M0544) if amplification is required. The fast, user-friendly workflow has minimal hands on time.

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.

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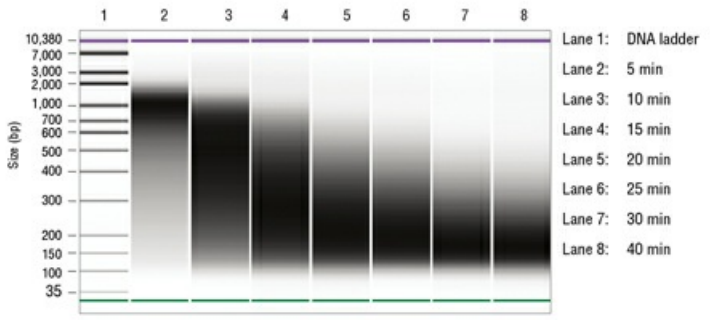
FRAGMENTATION SIZE	INCUBATION @ 37°C	OPTIMIZATION
100 bp-250 bp	30 min	30-40 min
150 bp-350 bp	20 min	20-30 min
200 bp-450 bp	15 min	15-20 min
300 bp-700 bp	10 min	5-15 min
500 bp-1 kb	5 min	5-10 min

Figure 1.1: Example of size distribution 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, 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

4 Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice. 5s **00:00:05 Mixing**

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:

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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

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

7 In a Thermocycler, with heated lid set to 75°C, run the following program:
5-30 min @ 37°C
30 min @65°C
Hold @4°C

Note: 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 using the NEBNext Ultra II Ligation Module (NEB #E7595) before stopping.

Adaptor Ligation

8 Determine whether adaptor dilution is necessary.

If DNA input is < 100 ng, dilute the• (red) NEBNext Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5 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: 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

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A	B
COMPONENT	VOLUME
FS Reaction Mixture (Step 1.1.5)	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 to the reaction.
** The NEBNext adaptor is provided in the NEBNext Oligos for Illumina.

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 Adaptor 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 15 minutes in a thermocycler with the heated lid off.
00:15:00 Incubate

12 Add 3 µl of

(red) USER® Enzyme to the ligation mixture from the previous step.

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

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

Samples can be stored overnight at -20°C.

Size Selection or Cleanup of Adaptor-ligated DNA

14 **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 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.**

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 30 minutes 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

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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 5 minutes.
00:05:00 Incubate

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 remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets

(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 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
00:00:30 Incubate

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 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.

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).

24 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.

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

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

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.
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.

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. 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 thermocycler 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	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles recommended in Table 1 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples. Use the 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).

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

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

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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 5 minutes at room temperature.

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 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.

37 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.

00:00:30 Incubate

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 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.

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 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.

42 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

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.

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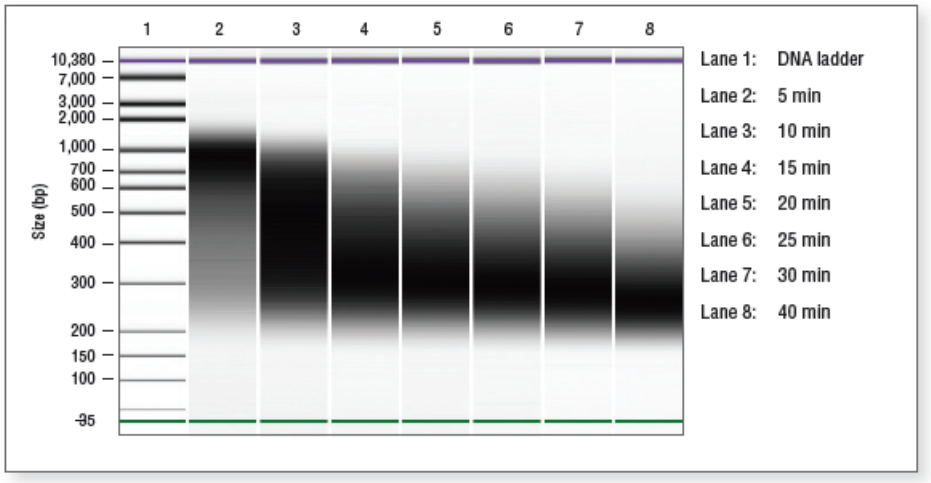
45 Check that the library size shows a narrow distribution with an expected peak size based on fragmentation time (Figure 1.2).

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



If a peak ~80 bp (primers) or 128 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.

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