Feb 10, 2021

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

New England Biolabs[1]

[1]New England Biolabs

1 Works for me dx.doi.org/10.17504/protocols.io.n2idgce

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.

EXTERNAL LINK

https://www.neb.com/products/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina#Product%20Information

DOI

dx.doi.org/10.17504/protocols.io.n2idgce

EXTERNAL LINK

https://www.neb.com/products/e7805-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina#Product%20Information

PROTOCOL CITATION

New England Biolabs 2021. E7805 NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2idgce

CREATED

Mar 26, 2018

LAST MODIFIED

Feb 10, 2021

OWNERSHIP HISTORY

Mar 26, 2018 Lauren Moran New England Biolabs

Dec 21, 2018 Isabel Gautreau New England Biolabs

PROTOCOL INTEGER ID

11050

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 fragment sizes > 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 2 for inputs ≥ 100 ng and inserts up to 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.

Fragmentation/End Prep

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.

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.

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

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂ¢ II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

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

4 Vortex the reaction for 5 seconds and briefly spin in a microcentifuge.

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

5 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 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 NEBNext or Multiplex Oligos for Illumina. 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.

Set a 100 µl or 200µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

7 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 15 minutes in a thermocycler with the heated lid off.

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

Steps 9 and 10 are only required for use with standard NEBNext Adaptors. USER Enzyme can be found in the NEBNext Oligos for Illumina.

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

Samples can be stored overnight at –20°C.

Size Selection of Adaptor-ligated DNA for Fragment Sizes > 550 bp

11 **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 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 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 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).

13 Vortex SPRIselecct or NEBNext Sample Purification Beads to resuspend

14 Add 40 µl (0.4X) 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.

15 Incubate samples for at least 5 minutes at room temperature.

16 Place the tube/plate on an appropriate magnetic stand to separate the bdads 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 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.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

Caution: Do not discard the beads.

18 Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperautre for 30 seconds, and then carefuly 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 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.

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).

22 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.

23 Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 100 µl to a new PCR tube.

24 Add 50 µl (~ 0.5X) of resuspended SPRIselect or Sample Purification Beads to the sample. 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.

25 Incubate samples at room temperautre for at least 5 minutes.

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

27 After 5 minutes (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.

Caution: Do not discard the beads.

28 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 thatcontain DNA targets.

00:00:30 Incubate

29 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.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

30 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.

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).

32 Mix well on a vortex mixer or by pipetting up and down at least 10 times. 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.

33 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.

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

PCR Enrichment of Adaptor-ligated DNA

35 • 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.

36 Add the following components to a sterile strip tube:

Option A: Forward and Reverse Primers NOT Already Combined:

A	B
Component	**Volume per One Library**
Adaptor Ligated DNA Fragments (Step 33)	15 µl
(blue) 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
Component	Volume per One Library
Adaptor Ligated DNA Fragments (Step 33)	15 µl
(blue) 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.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

**Use only one i7 primer/ index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

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 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	3-8*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles recommended in Table 3.4.1 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples. Use Table 3.4.2 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 3.4.1.

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 3.4.2.

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
100 ng	7–8

* Cycle number was determined for size delected libraries.

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

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

Cleanup of PCR Reaction

40 **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.

42 Add 30 µl (0.6X) 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.

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

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 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).

46 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.

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 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.

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).

50 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.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂ¢ II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce

51 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

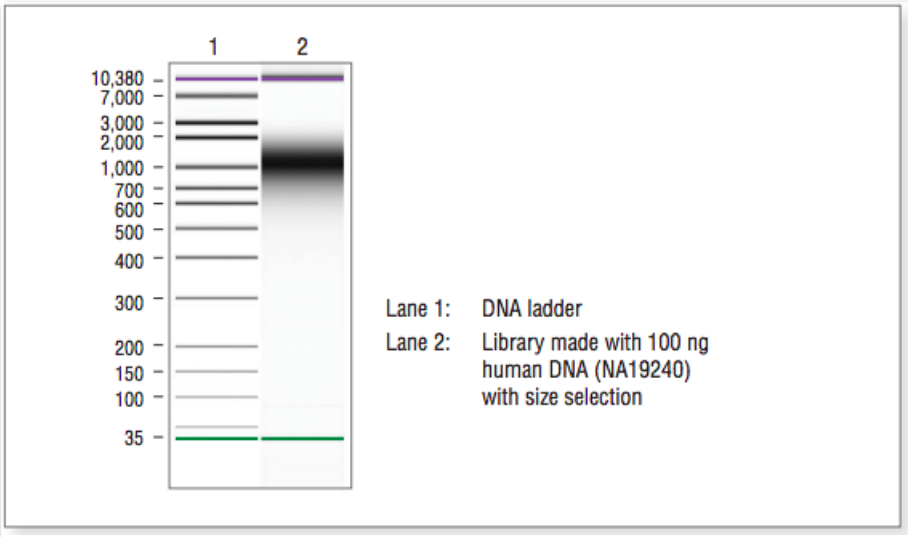
52 Dilute library (from previous step) 5-fold in 0.1X TE Buffer.

53 Run 1 µl on a DNA High Sensitivity Chip.

54 **If a peak ~80 bp (primers) or 128 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 Section Cleanup of PCR Reaction.**

Check that the library size shows a narrow distribution with an expected peak size > 700 bp (See Figure 3.1)

Figure 3.1: Example of final library size distribution after size selection. 100 ng Human DNA (NA19240) was fragmented for 5 minutes.

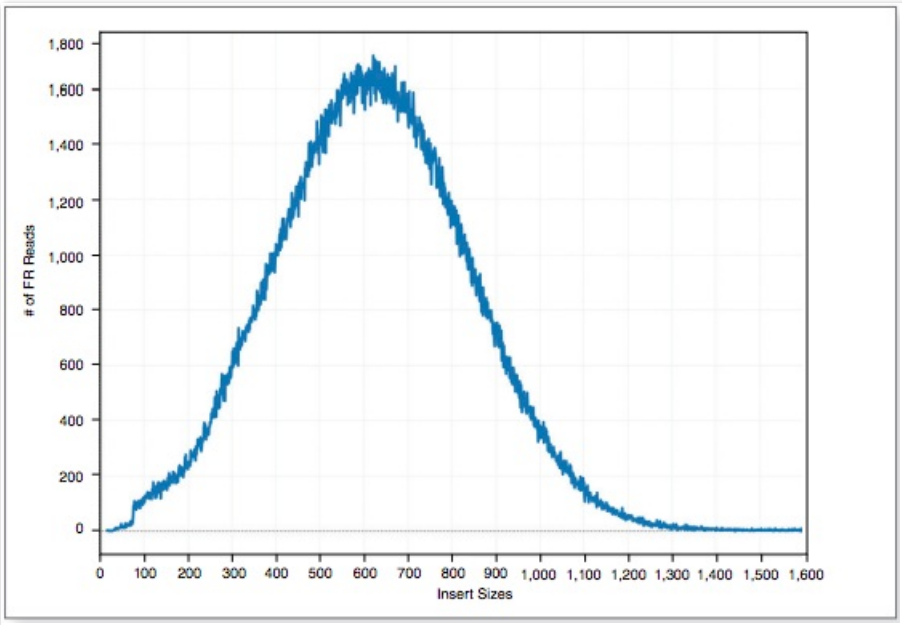


(Note: Due to the preperence of the Illumina sequences to preperentially cluster smaller fragments, the average insert sizes from the sequence data may be smaller than expected (See Figure 3.2. below).

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

Figure 3.2: Example of insert size distribution after bead-based size selection.

Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce



Citation: New England Biolabs (02/10/2021). E7805 NEBNextÃÂ® UltraÇÂÂç II FS DNA Library Prep Kit for IlluminaÃÂ® Protocol for Large Fragment Sizes (> 550 bp) (Chapter 3). https://dx.doi.org/10.17504/protocols.io.n2idgce