

## LIBRARY PREPARATION

# NEBNEXT® Ultra™ II FS DNA Library Prep Kit for Illumina®

### Instruction Manual

NEB #E7805S/L, #E6177S/L  
24/96 reactions  
Version 1.0 08/17



be INSPIRED  
drive DISCOVERY  
stay GENUINE

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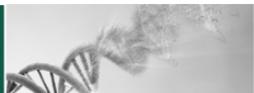
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## The Library Kit Includes:

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*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7805S/#E6177S) and 96 reactions (NEB #E7805L/#E6177L). All reagents should be stored at  $-20^{\circ}\text{C}$ . Colored bullets represent the color of the cap of the tube containing the reagent.*

### Package 1: Store at $-20^{\circ}\text{C}$ .

- (yellow) NEBNext Ultra II FS Enzyme Mix
- (yellow) NEBNext Ultra II FS Reaction Buffer
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (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:

80% Ethanol (freshly prepared)

Nuclease-free water

0.2 ml thin wall PCR tubes

NEBNext Singleplex or Multiplex Oligos for Illumina

(NEB #E7350, #E7335, #E7500, #E6609, #E7710, #E7730 or #E7600)

Magnetic rack/stand

PCR machine

Vortex

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 8.0 with 10 mM NaCl  
(for adaptor dilution of DNA input < 100 ng)

## Overview:

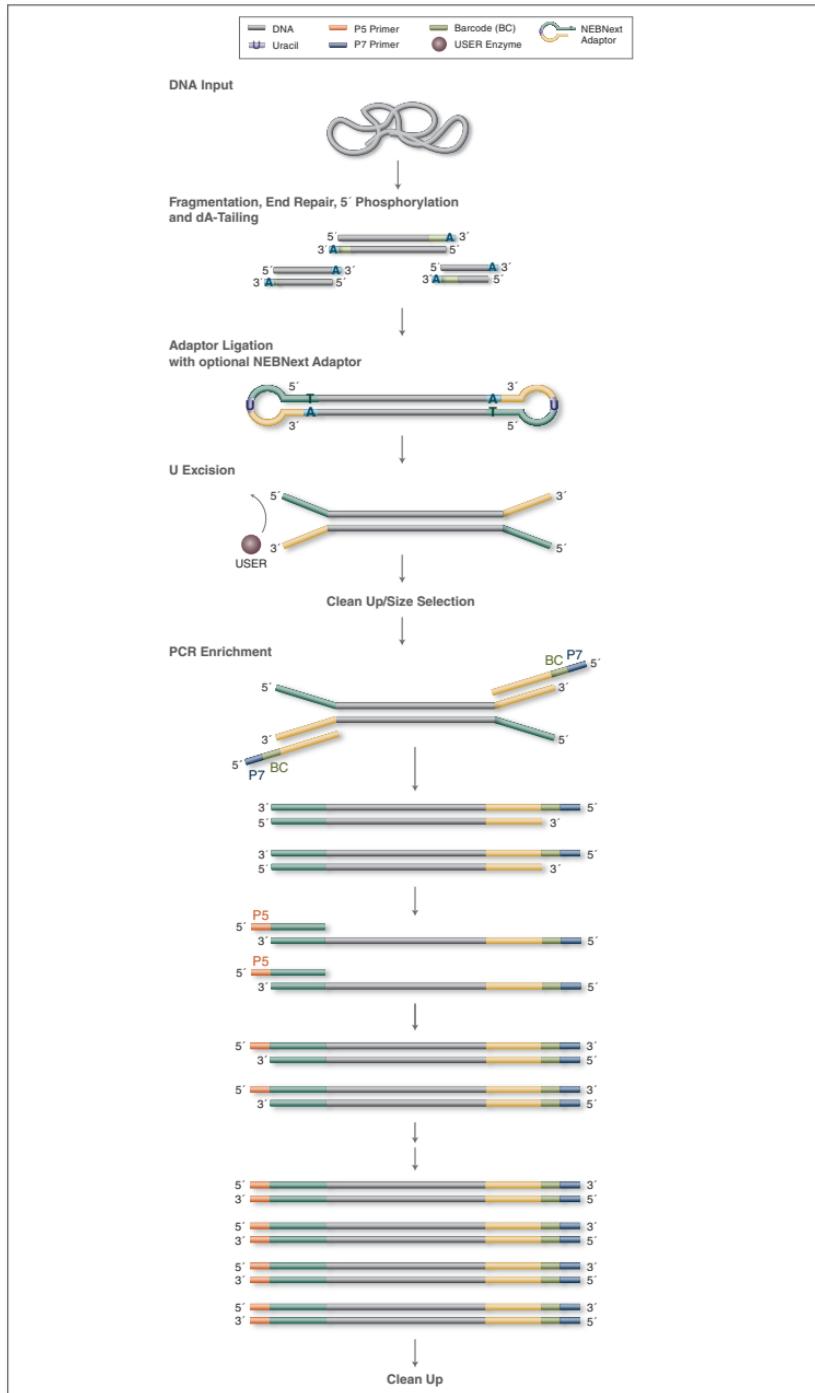
The NEBNext Ultra II FS DNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of DNA into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow also has minimal hands-on time.

**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.

Figure 1: Workflow demonstrating the use of NEBNext Ultra II FS DNA Library Prep Kit for Illumina



# 1

## Protocol for use with Inputs $\leq$ 100 ng

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NEBNext Ultra II  
FS DNA Library Prep Kit for Illumina  
Instruction Manual

## Symbols



*This caution sign 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.*



*Colored bullets indicate the cap color of the reagent to be added to a reaction.*



*Stopping points in the protocol.*

**Note:** Follow the protocol in this chapter for inputs ≤ 100 ng, as size selection is not recommended for this input range. Follow the protocol in Chapter 2 for inputs ≥ 100 ng, as size selection is recommended for this input range. Follow the protocol in Chapter 3 for inputs ≥ 100 ng and fragment sizes > 550 bp. 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 pg–100 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<sub>2</sub>O are also acceptable. If the input DNA is less than 26 µl, add TE (provided) to a final volume of 26 µl.

### 1.1. Fragmentation/End Prep

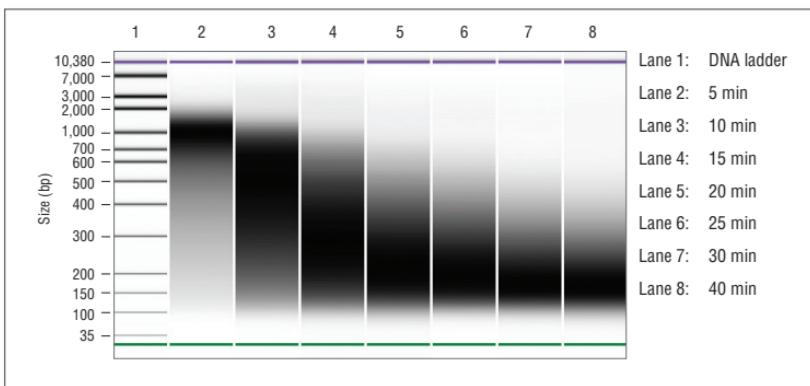
Fragmentation occurs during the 37°C incubation step. Use the chart below to determine the incubation time required to generate the desired fragment sizes. Incubation time may need to be optimized for individual samples. See Figure 1.1 for a typical fragmentation pattern.

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

- 1.1.1. 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.
- 1.1.2. 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.

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



- 1.1.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

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

- 1.1.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.
- 1.1.5. In a thermocycler, with the heated lid set to 75°C, run the following program:
- 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 adaptor ligation before stopping.

## 1.2. Adaptor Ligation

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.**

### 1.2.1. Add the following components directly to the FS Reaction Mixture:

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 NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600, #E7535 and #E6609) 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. We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.**

### 1.2.2. 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).

- 1.2.3. Incubate at 20°C for 15 minutes in a thermocycler with the heated lid off.
- 1.2.4. Add 3  $\mu$ l of ● (red) USER® Enzyme to the ligation mixture from Step 1.2.3.

**Note: Steps 1.2.4. and 1.2.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600 and #E6609) Oligos for Illumina.**

- 1.2.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to  $\geq 47^\circ\text{C}$ .



**Samples can be stored overnight at  $-20^\circ\text{C}$ .**

### 1.3. Size Selection or Cleanup of Adaptor-ligated DNA



The following section is for cleanup of the ligation reaction for inputs  $\leq 100$  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  $\geq 100$  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  $\mu$ l; Step 1.2.5.). 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.**

- 1.3.1. Vortex SPRiselect or NEBNext Sample Purification Beads to resuspend.
- 1.3.2. Add 57  $\mu$ l (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.
- 1.3.3. Incubate samples at room temperature for at least 5 minutes.

- 1.3.4. 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.
  - 1.3.5. 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**).
  - 1.3.6. Add 200  $\mu$ l 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.
  - 1.3.7. Repeat Step 1.3.6. 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.
  - 1.3.8. 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.**
- 1.3.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17  $\mu$ l 0.1X TE (dilute 1X TE Buffer 1:10 in water).
  - 1.3.10. 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.
  - 1.3.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 15  $\mu$ l to a new PCR tube.
  - 1.3.12. Proceed to PCR Enrichment of Adaptor-ligated DNA in Section 1.4.

 **Samples can be stored at -20°C.**

## 1.4. PCR Enrichment of Adaptor-ligated DNA



**Follow Section 1.4.1A. if you are using the following oligos (10 µM primer):**

**NEBNext Singleplex Oligos for Illumina (NEB #E7350)**

**NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)**

**NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)**

**NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)**

**NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)**

**NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)**

**Follow Section 1.4.1B. if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609)**

- 1.4.1. Add the following components to a sterile strip tube:

1.4.1A.	<b>Forward and Reverse Primers <u>not already combined</u></b>	1.4.1B. <b>Forward and Reverse Primers already combined</b>		
	Adaptor Ligated DNA Fragments (Step 1.3.12.)	Adaptor Ligated DNA Fragments (Step 1.3.12.)		
	• (blue) NEBNext Ultra II Q5 Master Mix	15 µl	15 µl	
	• (blue) Index Primer/i7 Primer*, **	25 µl	25 µl	
	• (blue) Universal PCR Primer/i5 Primer*, ***	5 µl	• (blue) Index/Universal Primer****	10 µl
	Total volume	50 µl	Total volume	50 µl

\* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

\*\* For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one index primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 primer per reaction.

\*\*\* For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

\*\*\*\* The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

- 1.4.2. 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.

1.4.3. 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	3–13*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

\* The number of PCR cycles recommended in Table 1.4.1 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples. Use Table 1.4.2 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 1.4.1.

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

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

Table 1.4.2.

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

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

1.4.4. Proceed to Cleanup of PCR reaction in Section 1.5.

## 1.5. Cleanup of PCR Reaction

**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 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.**

- 1.5.1. Vortex SPRiselect or NEBNext Sample Purification Beads to resuspend.
- 1.5.2. Add 45  $\mu$ l (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.
- 1.5.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 1.5.4. 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.
- 1.5.5. 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**).
- 1.5.6. Add 200  $\mu$ l 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.
- 1.5.7. Repeat Step 1.5.6. 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.

- 1.5.8. 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.**

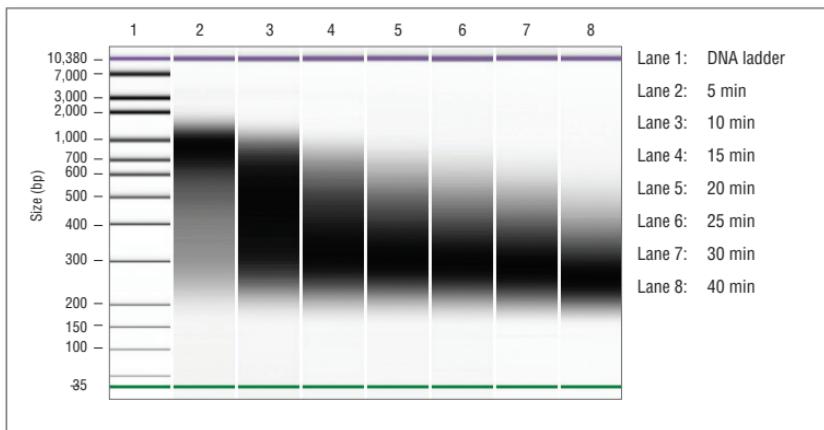
- 1.5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33  $\mu$ l of 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 1.5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 1.5.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30  $\mu$ l to a new PCR tube and store at  $-20^{\circ}\text{C}$ .

## 1.6. Assess Library Quality on a Bioanalyzer

- 1.6.1. Dilute library (from Step 1.5.11.) 5-fold in 0.1X TE Buffer (inputs  $\leq$  1 ng may not require dilution to run on a Bioanalyzer).
- 1.6.2. Run 1  $\mu$ l on a DNA High Sensitivity Chip.
- 1.6.3. Check that the library size shows a narrow distribution with an expected peak size based on fragmentation time (Figure 1.2).

**Note: If a peak  $\sim$ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 1.5.11.) to 50  $\mu$ l with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 1.5. You may see adaptor-dimer when starting with inputs  $\leq$  1 ng.**

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



# 2

## Protocol for use with Inputs $\geq$ 100 ng

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NEBNext Ultra II  
FS DNA Library Prep Kit for Illumina  
Instruction Manual

## Symbols



*This caution sign 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.*



*Colored bullets indicate the cap color of the reagent to be added to a reaction.*



*Stopping points in the protocol.*

**Note:** Follow the protocol in this chapter for inputs  $\geq$  100 ng, as size selection is recommended for this input range. Follow the protocol in Chapter 1 for inputs  $\leq$  100 ng, as size selection is not recommended for this input range. Follow the protocol in Chapter 3 for inputs  $\geq$  100 ng and fragment sizes  $> 550$  bp. For 100 ng inputs, either the no size selection protocol (Chapter 1) or a size selection protocol (Chapter 2 or 3) can be followed.

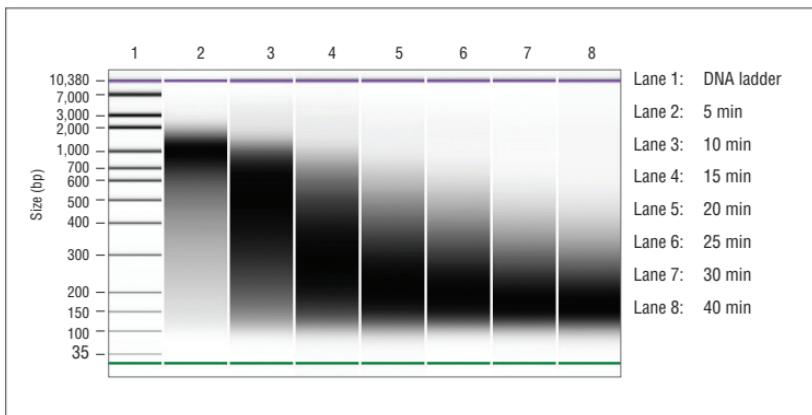
**Starting Material:** 100–500 ng purified, genomic DNA. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or H<sub>2</sub>O are also acceptable. If the input DNA is less than 26  $\mu$ l, add TE (provided) to a final volume of 26  $\mu$ l.

### 2.1. Fragmentation/End Prep

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.

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 distribution on a Bioanalyzer®. Human DNA (NA19240) was fragmented for 5-40 min.



- 2.1.1. 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.
- 2.1.2. 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.**
- 2.1.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

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

- 2.1.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.
- 2.1.5. In a thermocycler, with the heated lid set to 75°C, run the following program:
- 5-30 min @ 37°C**  
**30 min @ 65°C**  
**Hold @ 4°C**



If necessary, samples can be stored at  $-20^{\circ}\text{C}$ ; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.

## 2.2. Adaptor Ligation

### 2.2.1. Add the following components directly to the FS Reaction Mixture:

COMPONENT	VOLUME
FS Reaction Mixture (Step 2.1.5)	35 $\mu\text{l}$
● (red) NEBNext Ultra II Ligation Master Mix*	30 $\mu\text{l}$
● (red) NEBNext Ligation Enhancer	1 $\mu\text{l}$
● (red) NEBNext Adaptor for Illumina**	2.5 $\mu\text{l}$
Total volume	68.5 $\mu\text{l}$

- \* 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 NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600, #E7535 and #E6609) 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 @  $4^{\circ}\text{C}$ . We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.**

### 2.2.2. Set a 100 $\mu\text{l}$ or 200 $\mu\text{l}$ pipette to 50 $\mu\text{l}$ 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).**

### 2.2.3. Incubate at $20^{\circ}\text{C}$ for 15 minutes in a thermocycler with the heated lid off.

### 2.2.4. Add 3 $\mu\text{l}$ of ● (red) USER® Enzyme to the ligation mixture from Step 2.2.3.

**Note: Steps 2.2.4. and 2.2.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600 and #E6609) Oligos for Illumina.**

### 2.2.5. Mix well and incubate at $37^{\circ}\text{C}$ for 15 minutes with the heated lid set to $\geq 47^{\circ}\text{C}$ .

**Samples can be stored overnight at  $-20^{\circ}\text{C}$ .**



## 2.3. Size Selection of Adaptor-ligated DNA for DNA Input $\geq$ 100 ng



If the starting material is  $\geq$  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 1.3. If you want fragment sizes  $>$  550 bp and your input is  $\geq$  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  $\mu$ l; Step 2.2.5.). 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.



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  $\mu$ l. 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
BEAD VOLUME TO BE ADDED ( $\mu$ l)	Approx. Final Library Size Distribution (insert + adaptor + primers)	270-370 bp	320-470 bp	400-600 bp	470-800 bp
	1st Bead Addition	40	30	25	20
	2nd Bead Addition	20	15	10	10

- 2.3.1. Bring the volume of the reaction up to 100  $\mu$ l by adding 28.5  $\mu$ l 0.1X TE (dilute 1X TE Buffer 1:10 with water).
- 2.3.2. Vortex SPRiselect Beads or NEBNext Sample Purification Beads to resuspend.
- 2.3.3. Add 40  $\mu$ l (~ 0.4X) of resuspended beads to the 100  $\mu$ l sample from Step 2.3.1. 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.
- 2.3.4. Incubate samples for at least 5 minutes at room temperature.
- 2.3.5. 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.
- 2.3.6. After 5 minutes (or when the solution is clear), carefully transfer the supernatant (~ 140  $\mu$ l) containing your DNA to a new tube (**Caution: do not discard the supernatant**). Discard the beads that contain the unwanted large fragments.
- 2.3.7. Add 20  $\mu$ l (~0.2X) resuspended SPRiselect or Sample Purification Beads to the supernatant and mix at least 10 times. Be careful to expel all of the liquid from the tip during the last mix. Incubate samples on the bench top for at least 5 minutes at room temperature.
- 2.3.8. 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.
- 2.3.9. 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 beads**).
- 2.3.10. Add 200  $\mu$ l 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.
- 2.3.11. Repeat Step 2.3.10. 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.

- 2.3.12. 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 overdry 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.**

- 2.3.13. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 17  $\mu$ l 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 2.3.14. 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 sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 2.3.15. Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15  $\mu$ l to a new PCR tube.
- 2.3.16. Proceed to PCR Enrichment of Adaptor-ligated DNA in Section 2.4.

**Samples can be stored at  $-20^{\circ}\text{C}$ .**



## 2.4. PCR Enrichment of Adaptor-ligated DNA



**Follow Section 2.4.1A. if you are using the following oligos (10 µM primer):**

**NEBNext Singleplex Oligos for Illumina (NEB #E7350)**

**NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)**

**NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)**

**NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)**

**NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)**

**NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)**

**Follow Section 2.4.1B. if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609)**

- 2.4.1. Add the following components to a sterile strip tube:

2.4.1A.	<b>Forward and Reverse Primers not already combined</b>	2.4.1B. <b>Forward and Reverse Primers already combined</b>		
	Adaptor Ligated DNA Fragments (Step 2.3.16.)	Adaptor Ligated DNA Fragments (Step 2.3.16.)		
	• (blue) NEBNext Ultra II Q5 Master Mix	15 µl	15 µl	
	• (blue) Index Primer/i7 Primer*, **	25 µl	25 µl	
	• (blue) Universal PCR Primer/i5 Primer*, ***	5 µl	• (blue) Index/Universal Primer****	10 µl
	Total volume	50 µl	Total volume	50 µl

\* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

\*\* For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one index primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 primer per reaction.

\*\*\* For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

\*\*\*\* The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

- 2.4.2. 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.

2.4.3. 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	3–7*
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.

2.4.4. Proceed to Cleanup of PCR reaction in Section 2.5.

## 2.5. Cleanup of PCR Reaction

**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 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.

- 2.5.1. Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.
- 2.5.2. Add 45  $\mu$ l (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.
- 2.5.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 2.5.4. 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.
- 2.5.5. 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**).
- 2.5.6. Add 200  $\mu$ l 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.
- 2.5.7. Repeat Step 2.5.6. 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.
- 2.5.8. 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.**

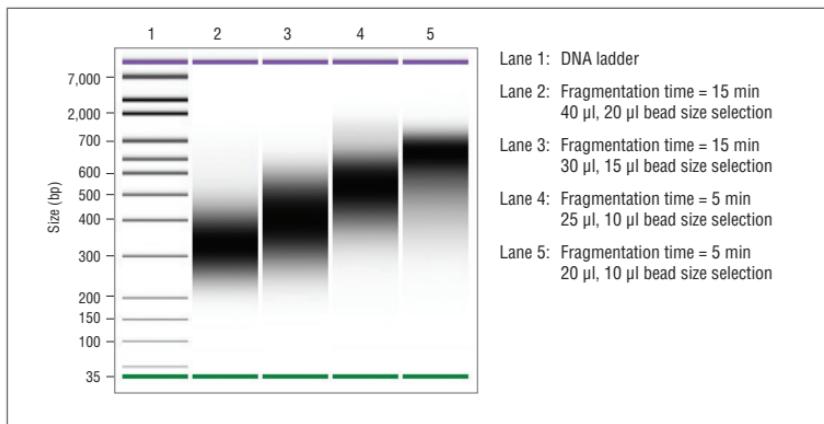
- 2.5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33  $\mu$ l of 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 2.5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 2.5.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30  $\mu$ l to a new PCR tube and store at  $-20^{\circ}\text{C}$ .

## 2.6. Assess Library Quality on a Bioanalyzer

- 2.6.1. Dilute library (from Step 2.5.11.) 5-fold in 0.1X TE Buffer.
- 2.6.2. Run 1  $\mu$ l on a DNA High Sensitivity Chip.
- 2.6.3. 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).

**Note: If a peak  $\sim$ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 2.5.11.) to 50  $\mu$ l with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 2.5.**

Figure 2.2: Example of final library size distributions with size selection. Human DNA (NA19240) was fragmented for 5 or 15 minutes.



# 3

## Protocol for Large Fragment Sizes (> 550 bp)

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NEBNext Ultra II  
FS DNA Library Prep Kit for Illumina  
Instruction Manual

## Symbols



*This caution sign 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.*



*Colored bullets indicate the cap color of the reagent to be added to a reaction.*



*Stopping points in the protocol.*

**Note:** Follow the protocol in this chapter for inputs  $\geq$  100 ng, and fragment sizes  $>$  550 bp. Follow the protocol in Chapter 2 for inputs  $\geq$  100 ng and fragment sizes  $\leq$  550 bp. Follow the protocol in Chapter 1 for inputs  $\leq$  100 ng. 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<sub>2</sub>O are also acceptable. If the input DNA is less than 26  $\mu$ l, add TE (provided) to a final volume of 26  $\mu$ l.

### 3.1. Fragmentation/End Prep

3.1.1. 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.

3.1.2. 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.

3.1.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

COMPONENT	VOLUME PER ONE LIBRARY
DNA	26 $\mu$ l
● (yellow) NEBNext Ultra II FS Reaction Buffer	7 $\mu$ l
● (yellow) NEBNext Ultra II FS Enzyme Mix	2 $\mu$ l
Total Volume	35 $\mu$ l

3.1.4. Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

- 3.1.5. 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.**

## 3.2. Adaptor Ligation

- 3.2.1. Add the following components directly to the FS Reaction Mixture:

COMPONENT	VOLUME
FS Reaction Mixture (Step 3.1.5)	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 NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600, #E7535 and #E6609) 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. We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.**

- 3.2.2. 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).**

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

- 3.2.4. Add 3 µl of ● (red) USER® Enzyme to the ligation mixture from Step 3.2.3.

**Note: Steps 3.2.4. and 3.2.5. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600 and #E6609) Oligos for Illumina.**

- 3.2.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to  $\geq 47^{\circ}\text{C}$ .



**Samples can be stored overnight at  $-20^{\circ}\text{C}$ .**

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

**Note: the volumes of SPRIdeselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step (71.5  $\mu\text{l}$ ; Step 3.2.5.). 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.**

- 3.3.1. Bring the volume of the reaction up to 100  $\mu\text{l}$  by adding 28.5  $\mu\text{l}$  0.1X TE (dilute 1X TE Buffer 1:10 with water).
- 3.3.2. Vortex SPRIdeselect or NEBNext Sample Purification Beads to resuspend.
- 3.3.3. Add 40  $\mu\text{l}$  (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.
- 3.3.4. Incubate samples for at least 5 minutes at room temperature.
- 3.3.5. 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.
- 3.3.6. 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**).

- 3.3.7. Add 200  $\mu$ l 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.
- 3.3.8. Repeat Step 3.3.7. 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.
- 3.3.9. 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.**
- 3.3.10. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 102  $\mu$ l 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 3.3.11. 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.
- 3.3.12. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 100  $\mu$ l to a new PCR tube.
- 3.3.13. Add 50  $\mu$ l (~ 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.
- 3.3.14. Incubate samples at room temperature for at least 5 minutes.
- 3.3.15. 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.
- 3.3.16. 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 beads).**

- 3.3.17. Add 200  $\mu$ l 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.
- 3.3.18. Repeat Step 3.3.17. 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.
- 3.3.19. 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 overdry 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.**
- 3.3.20. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 17  $\mu$ l 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 3.3.21. 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 sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 3.3.22. Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15  $\mu$ l to a new PCR tube.
- 3.3.23. Proceed to PCR Enrichment of Adaptor-ligated DNA in Section 3.4.

 **Samples can be stored at -20°C.**

### 3.4. PCR Enrichment of Adaptor-ligated DNA



Follow Section 3.4.1A. if you are using the following oligos (10  $\mu$ M primer):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 3.4.1B. if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609)

- 3.4.1. Add the following components to a sterile strip tube:

3.4.1A. Forward and Reverse Primers <u>not already combined</u>	3.4.1B. Forward and Reverse Primers already combined		
Adaptor Ligated DNA Fragments (Step 3.3.23.)	Adaptor Ligated DNA Fragments (Step 3.3.23.)		
• (blue) NEBNext Ultra II Q5 Master Mix	15 $\mu$ l	15 $\mu$ l	
• (blue) Index Primer/i7 Primer*, **	25 $\mu$ l	25 $\mu$ l	
• (blue) Universal PCR Primer/i5 Primer*, ***	5 $\mu$ l	• (blue) Index/Universal Primer****	10 $\mu$ l
Total volume	50 $\mu$ l	Total volume	50 $\mu$ l

\* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

\*\* For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one index primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 primer per reaction.

\*\*\* For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

\*\*\*\* The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

- 3.4.2. Set a 100  $\mu$ l or 200  $\mu$ l pipette to 40  $\mu$ l 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.

3.4.3. 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 determined 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 selected libraries.

3.4.4. Proceed to Cleanup of PCR reaction in Section 3.5.

### 3.5. Cleanup of PCR Reaction

**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 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.

- 3.5.1. Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.
- 3.5.2. Add 30  $\mu$ l (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.
- 3.5.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 3.5.4. 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.
- 3.5.5. 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**).
- 3.5.6. Add 200  $\mu$ l 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.
- 3.5.7. Repeat Step 3.5.6. 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.
- 3.5.8. 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.**

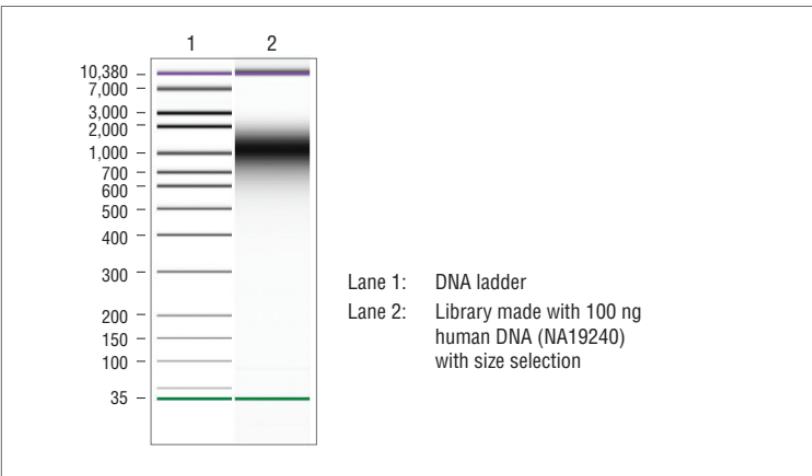
- 3.5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33  $\mu$ l of 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 3.5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 3.5.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30  $\mu$ l to a new PCR tube and store at -20°C.

### **3.6. Assess Library Quality on a Bioanalyzer**

- 3.6.1. Dilute library (from Step 3.5.11.) 5-fold in 0.1X TE Buffer.
- 3.6.2. Run 1  $\mu$ l on a DNA High Sensitivity Chip.
- 3.6.3. Check that the library size shows a narrow distribution with an expected peak size > 700 bp (see Figure 3.1).

**Note: If a peak ~80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 3.5.11.) to 50  $\mu$ l with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 3.5.**

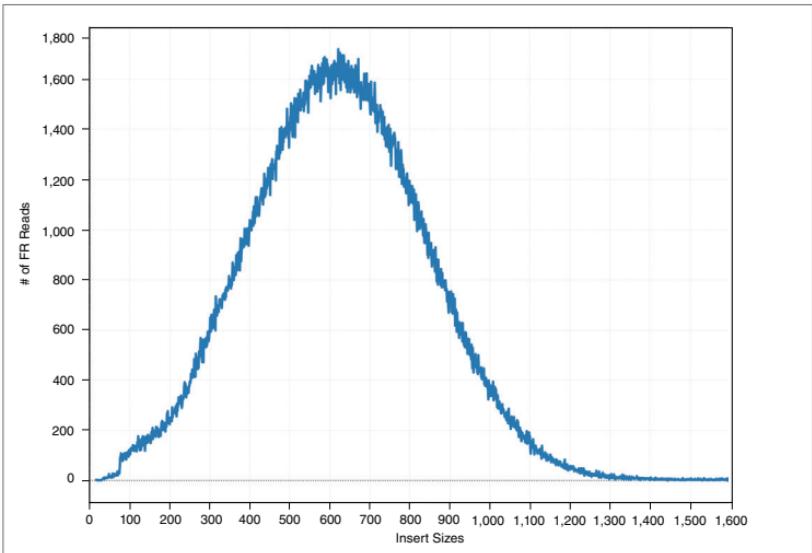
Figure 3.1: Example of final library size distribution after size selection. 100 ng Human DNA (NA19240) was fragmented for 5 minutes.



**Note: Due to the preference of the Illumina sequences to preferentially cluster smaller fragments, the average insert size from the sequence data may be smaller than expected (see Figure 3.2.).**

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.



# Kit Components

## NEB #E7805S Table of Components

NEB #	PRODUCT NAME	VOLUME
E7808A	TE Buffer (1X)	1.1 ml
E7807A	NEBNext Ultra II FS Reaction Buffer	0.168 ml
E7806A	NEBNext Ultra II FS Enzyme Mix	0.048 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml

## NEB #E7805L Table of Components

NEB #	PRODUCT NAME	VOLUME
E7808AA	TE Buffer (1X)	4.3 ml
E7807AA	NEBNext Ultra II FS Reaction Buffer	0.672 ml
E7806AA	NEBNext Ultra II FS Enzyme Mix	0.192 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	2 x 1.2 ml

## NEB #E6177S Table of Components

NEB #	PRODUCT NAME	VOLUME
E7808A	TE Buffer (1X)	1.1 ml
E7807A	NEBNext Ultra II FS Reaction Buffer	0.168 ml
E7806A	NEBNext Ultra II FS Enzyme Mix	0.048 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml
E6178S	NEBNext Sample Purification Beads	3.6 ml

## NEB #E6177L Table of Components

NEB #	PRODUCT NAME	VOLUME
E7808AA	TE Buffer (1X)	4.3 ml
E7807AA	NEBNext Ultra II FS Reaction Buffer	0.672 ml
E7806AA	NEBNext Ultra II FS Enzyme Mix	0.192 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	2 x 1.2 ml
E6178L	NEBNext Sample Purification Beads	4 x 3.6 ml

## Revision History:

REVISION #	DESCRIPTION	DATE
1.0	N/A	08/17



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