NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina®

for use with NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA)

NEB #E7805S/L, #E6177S/L

24/96 reactions

Version 2.0_5/23

Table of Contents

The Library Kit Includes

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°C. Colored bullets represent the color of the cap of the tube containing the reagent.

Package 1: Store at –20°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 Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA). NEBNext Oligo Kit options can be found at www.neb.com/oligos
- Magnetic rack/stand
- PCR machine
- Vortex Mixer
- Microcentrifuge

For NEB #E7805 only:

- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

Overview

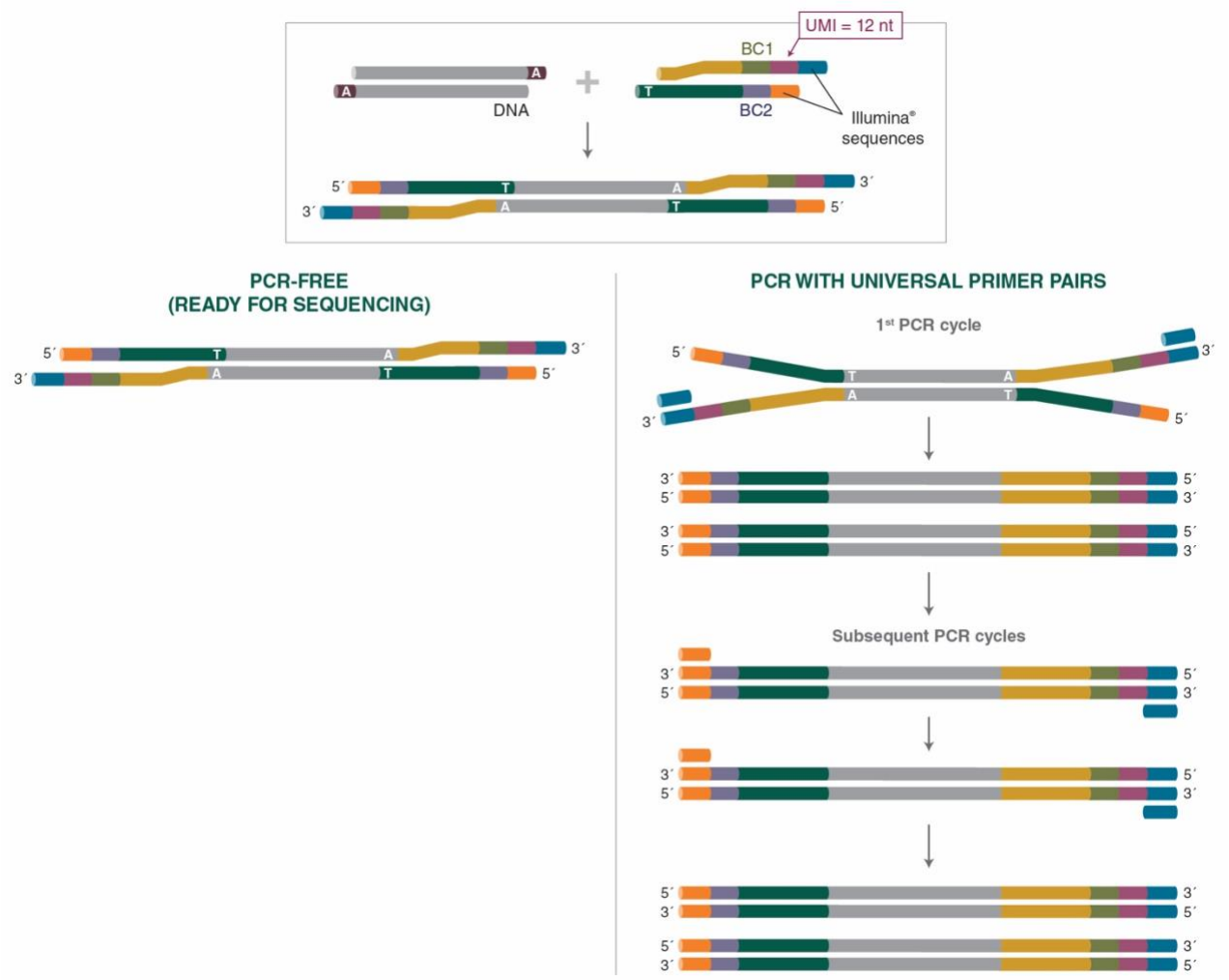
The NEBNext Ultra II FS DNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of DNA into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow also has minimal hands-on time.

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 custom@neb.com for further information.

Figure 1. Workflow demonstrating the use of NEBNext Ultra II FS DNA Library Prep Kit for Illumina with NEBNext Multiplex Oligos (Unique Dual Index UMI Adaptors DNA).



Section 1
Protocol for use with Inputs ≤ 100 ng

Symbols

This is a point where you can safely stop the protocol.

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.

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_2O 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.

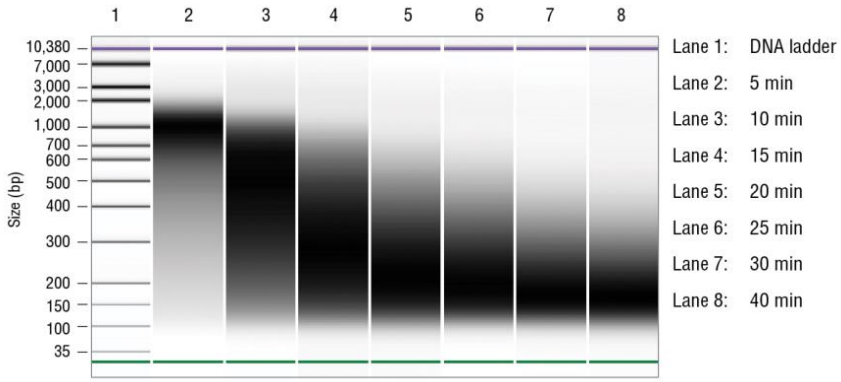
FRAGMENTATION SIZE	INCUBATION @ 37°C	OPTIMIZATION
100 bp–250 bp	30 min	30–40 min
150 bp–350 bp	20 min	20–30 min
200 bp–450 bp	15 min	15–20 min
300 bp–700 bp	10 min	5–15 min
500 bp–1 kb	5 min	5–10 min

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 thermal cycler, 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 NEBNext UMI Adaptors for Illumina in the UMI Dilution Buffer provided in the kit 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	20 µM
5 ng–99 ng	10-Fold (1:10)	2 µM
1 ng to < 5 ng	50-Fold (1:50)	0.4 µM
< 1 ng	100-Fold (1:100)	0.2 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
NEBNext UMI Adaptors for Illumina*	2.5 µl
• (red) NEBNext Ultra II Ligation Master Mix**	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**68.5 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (page 1). Please refer to corresponding oligo manual for valid barcode combinations.

** Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

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 thermal cycler with the heated lid off.

Samples can be stored overnight at –20°C.

1.3. Cleanup of Adaptor-ligated DNA

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

Note: 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 (68.5 µl; Step 1.2.3.). 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 41 µl (0.6X) 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 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 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.

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 22 µl 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 20 µl to a new PCR tube.

Samples are ready for sequencing on the Illumina platform and can be stored at –20°C. We recommend qPCR based methods (NEBNext Library Quant Kit for Illumina, NEB #E7630) for quantification of PCR-free libraries.

Please proceed to Section 1.4 if PCR amplification is required.

1.4. PCR Enrichment of Adaptor-ligated DNA

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

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 1.3.11.)	20 µl
• (blue) NEBNext Primer Mix*	5 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Total Volume	**50 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (page 1).

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 thermal cycler 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	3-14*
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	13–14

* 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 40 µl (0.8X) 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 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 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.

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 µl 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 µl to a new PCR tube and store at –20°C.

1.6. Assess Library Quality on a Bioanalyzer

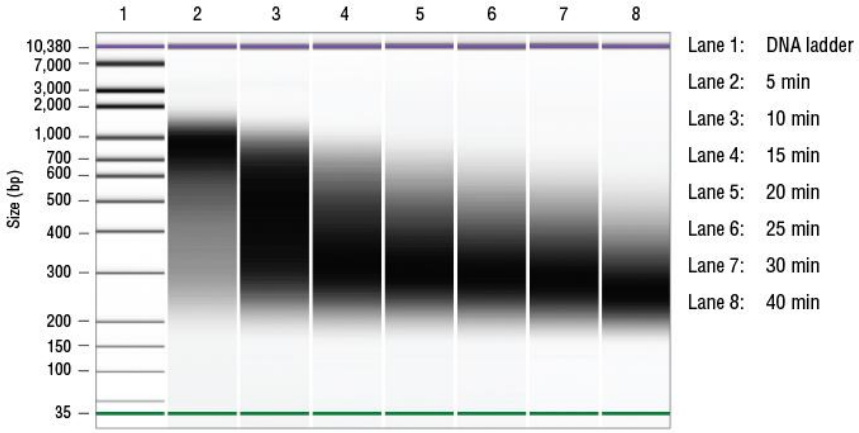
1.6.1. Dilute library (from Step 1.5.11.) 5-fold in 0.1X TE Buffer (inputs ≤ 1 ng may not require dilution to run on a Bioanalyzer).

1.6.2. Run 1 µl 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 ~146 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 1.5.11.) to 50 µl 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 ≤ 1 ng.

Figure 1.2. Bioanalyzer traces representing final library size distributions without size selection. Human DNA (NA 19240).



Section 2
Protocol for use with Inputs ≥ 100 ng

Symbols

This is a point where you can safely stop the protocol.

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.

Note: Follow the protocol in this chapter for inputs ≥ 100 ng, as size selection is recommended for this input range. Follow the protocol in Chapter 1 for inputs ≤ 100 ng, as size selection is <u>not</u> 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.

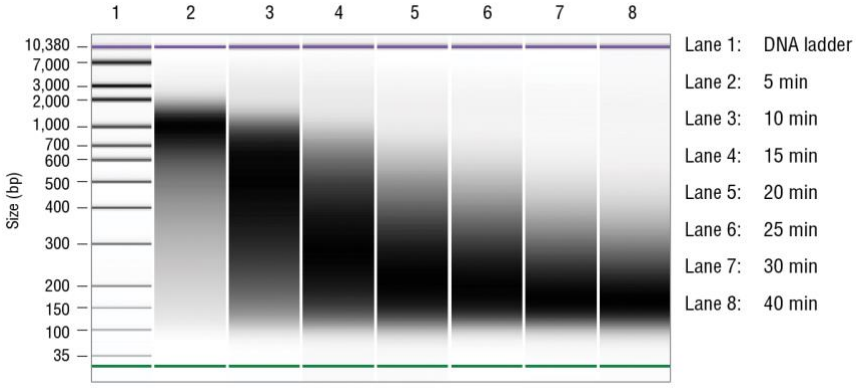
Starting Material: 100–500 ng purified, genomic DNA. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or H_2O are also acceptable. If the input DNA is less than 26 µl, add TE (provided) to a final volume of 26 µl.

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	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, and quickly 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 thermal cycler, 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.

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 µl
NEBNext UMI Adaptors for Illumina*	2.5 µl
● (red) NEBNext Ultra II Ligation Master Mix**	30 µl
● (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**68.5 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (page 1). Please refer to corresponding oligo manual for valid barcode combinations.

** Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

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.

2.2.2. 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).**

2.2.3. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

Samples can be stored overnight at –20°C.

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

If the starting material is ≥ 100 ng, follow the protocol for size selection below. For inputs < 100 ng, size selection is not recommended. Follow the protocol for cleanup without size selection in Chapter 1 Section 1.3. 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 (68.5 µl; Step 2.2.3.). 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 bp inserts only. For libraries with different size fragment inserts, refer to Table 2.3.1. below for the appropriate volumes of beads to be added. The size selection protocol is based on a starting volume of 100 µl. Size selection conditions were optimized with SPRIselect or NEBNext Sample Purification Beads; however, AMPure XP beads can be used following the same conditions. If using AMPure XP beads, please allow the beads to warm to room temperature for at least 30 minutes before use.

To select a different insert size than 150 bp, please use the volumes in this table:

Table 2.3.1: Recommended Conditions for Bead-based Size Selection

	APPROXIMATE INSERT SIZE	100-150 bp	150-300 bp	250-400 bp	300-550 bp
LIBRARY PARAMETER	Approx. Final Library Size (insert + adaptor)	250-300 bp	300-450 bp	400-550 bp	450-700 bp
BEAD VOLUME TO BE ADDED (µl)	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 µl by adding 31.5 µl 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 µl (~ 0.4X) of resuspended beads to the 100 µl 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 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 µl) 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 µl (~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 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 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.

2.3.11. Repeat Step 2.3.10. 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.

2.3.12. 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 22 µl 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 20 µl to a new PCR tube.

Samples are ready for sequencing on the Illumina platform and can be stored at –20°C. We recommend qPCR based methods (NEBNext Library Quant Kit for Illumina, NEB #E7630) for quantification of PCR-free libraries.

Please proceed to Section 2.4 if PCR amplification is required.

2.4. PCR Enrichment of Adaptor-ligated DNA

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

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 2.3.15.)	20 µl
• (blue) NEBNext Primer Mix*	5 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Total Volume	**50 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (page 1)..

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 thermal cycler 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 F OR 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.

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 40 µl (0.8X) 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 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.

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 µl 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 µl to a new PCR tube and store at –20°C.

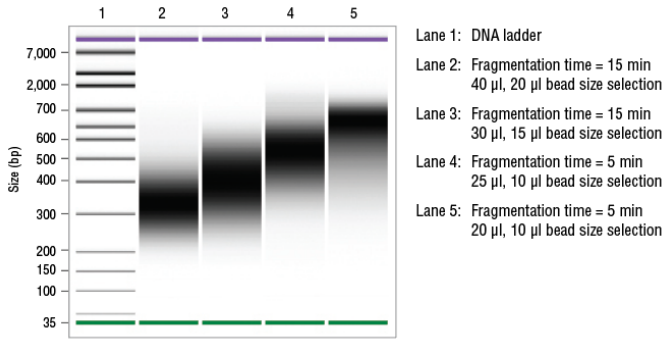
2.6. Assess Library Quality on a Bioanalyzer

2.6.1. Dilute library (from Step 2.5.11.) 5-fold in 0.1X TE Buffer.

2.6.2. Run 1 µl 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 (Figure 2.2).
Note: If a peak ~146 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 2.5.11.) to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 2.5.

Figure 2.2. Bioanalyzer traces representing final library size distributions with size selection. Human DNA (NA 19240) was fragmented for 5 or 15 minutes.



Section 3
Protocol for Large Fragment Sizes (> 550 bp)

Symbols

This is a point where you can safely stop the protocol.

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.

Note: Follow the protocol in this chapter for inputs ≥ 100 ng, and fragment sizes > 550 bp. Follow the protocol in Chapter 2 for inputs ≥ 100 ng and fragment sizes ≤ 550 bp. Follow the protocol in Chapter 1 for inputs ≤ 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_2O are also acceptable. If the input DNA is less than 26 µl, add TE (provided) to a final volume of 26 µl.

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, and quickly 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 µl
○ (yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
○ (yellow) NEBNext Ultra II FS Enzyme Mix	2 µl
Total Volume	**35 µl**

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

3.1.5. In a thermal cycler, 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
NEBNext UMI Adaptors for Illumina*	2.5 µl
• (red) NEBNext Ultra II Ligation Master Mix**	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**68.5 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to "Required Materials Not Included" section (page 1). Please refer to corresponding oligo manual for valid barcode combinations.

** Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

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 thermal cycler with the heated lid off.

Samples can be stored overnight at –20°C.

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

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 (68.5 µl; Step 3.2.3.). 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 µl by adding 31.5 µl 0.1X TE (dilute 1X TE Buffer 1:10 with water).

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

3.3.3. 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.

3.3.4. Incubate samples for 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 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.

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 µl 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 µl to a new PCR tube.

3.3.13. 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.

3.3.14. Incubate samples at room temperature for 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 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.

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 22 µl 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 20 µl to a new PCR tube.

3.3.23. Proceed to PCR Enrichment of Adaptor-ligated DNA in Section 3.4.

Samples are ready for sequencing on the Illumina platform and can be stored at –20°C. We recommend qPCR based methods (NEBNext Library Quant Kit for Illumina, NEB #E7630) for quantification of PCR-free libraries.

3.4. PCR Enrichment of Adaptor-ligated DNA

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

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments (Step 3.3.22.)	20 µl
• (blue) NEBNext Primer Mix*	5 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Total Volume	**50 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. For oligo purchasing options refer to “Required Materials Not Included” section (page 1).

3.4.2. 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.

3.4.3. Place the tube on a thermal cycler 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 non-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 non-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 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.

3.5.3. Incubate samples on bench top for 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 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.

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 µl 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 µl to a new PCR tube and store at –20°C.

3.6. Assess Library Quality on a Bioanalyzer

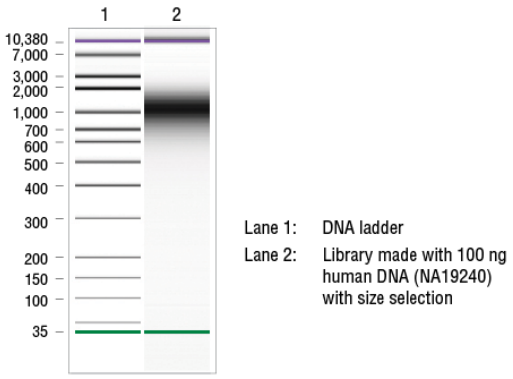
3.6.1. Dilute library (from Step 3.5.11.) 5-fold in 0.1X TE Buffer.

3.6.2. Run 1 µl 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 (Figure 3.1).

Note: If a peak ~146 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 3.5.11.) to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 3.5.

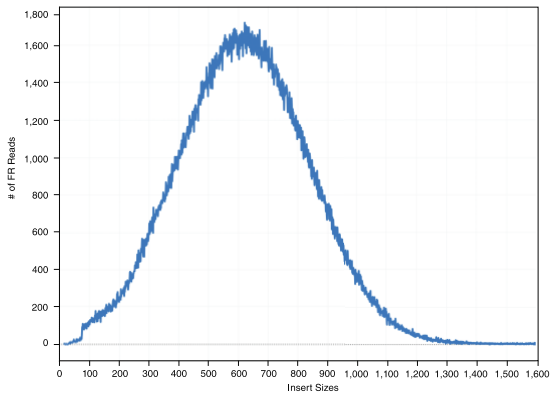
Figure 3.1. A Bioanalyzer trace representing final library size distributions without size selection. 100 ng Human DNA (NA 19240) was fragmented for 5 minutes.



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	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	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	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	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

Checklist

1. NEBNext Fragmentation/End Prep

[_] 1.1. Add Fragmentation/End Prep Reagents to 26 µl of DNA sample:

- [_] Vortex the Ultra II FS Reaction Buffer to mix
- [_] Vortex the Ultra II FS Enzyme Mix 5–8 seconds
- [_] 7 µl FS Reaction Buffer
- [_] 2 µl FS Enzyme Mix

[_] 1.2. Vortex the reaction for 5 seconds, quick spin

[_] 1.3. Thermal cycle (Heated lid ≥ 75°C; 5-30 min at 37°C, 30 min at 65°C, Hold at 4°C)

2. Adaptor Ligation

[_] 2.1. Dilute UMI adaptors if necessary

[_] 2.2. Add Ligation reagents to sample:

- [_] 2.5 µl UMI adaptor
- [_] 30 µl Ligation Master Mix
- [_] 1 µl Ligation Enhancer

[_] 2.3. Pipette mix 10 times with pipette set to 60 µl, quick spin

[_] 2.4 Incubate 15 min at 20°C (heated lid off)

3. Cleanup, Size Selection or Large Fragment Insert (> 550 bp)

3A. Cleanup of Adaptor-ligated DNA without Size Selection

[_] 3A.1. Vortex beads

[_] 3A.2. Add 41 µl of beads to sample and mix by pipetting 10 times

[_] 3A.3. Incubate for 5 min

[_] 3A.4. Place tubes on magnet

[_] 3A.5. Wait 5 min and remove supernatant (keep the beads)

[_] 3A.6. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3A.7. Repeat Step 3A.6. once

[_] 3A.8. Air dry beads, do not overdry

[_] 3A.9. Off magnet add 22 µl 0.1X TE

[_] 3A.10. Mix by pipetting 10 times. Incubate 2 min

[_] 3A.11. Place tubes on magnet. Wait 5 min and transfer 20 µl to a new tube

Skip to 4.1

3B. Size Selection of Adaptor-ligated DNA

[_] 3B.1. Add 31.5 µl 0.1X TE to sample for 100 µl volume

[_] 3B.2. Vortex beads

[_] 3B.3. Add __ µl of beads to sample and mix by pipetting 10 times.

[_] 3B.4. Incubate 5 min

[_] 3B.5. Place tubes on magnet

[_] 3B.6. Wait 5 min then transfer the supernatant to a new tube (keep the supernatant)

[_] 3B.7. Add __ µl of beads to the supernatant and mix by pipetting 10 times. Incubate 5 min.

[_] 3B.8. Place tubes on magnet

[_] 3B.9. Wait 5 min then remove the supernatant (keep the beads)

[_] 3B.10. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3B.11. Repeat Step 3B.10. once

[_] 3B.12. Air dry beads, do not overdry

[_] 3B.13. Off magnet add 22 µl 0.1X TE

[_] 3B.14. Mix by pipetting 10 times. Incubate 2 min.

[_] 3B.15. Place tubes on magnet. Wait 5 min and transfer 20 µl to a new tube

Skip to 4.1

3C. Protocol for Large Fragment Sizes (> 550 bp)

[_] 3C.1. Add 31.5 µl 0.1X TE to sample for 100 µl volume

[_] 3C.2. Vortex beads

[_] 3C.3. Add 40 µl of beads to sample and mix by pipetting 10 times.

[_] 3C.4. Incubate 5 min

[_] 3C.5. Place tubes on magnet

[_] 3C.6. Wait 5 min and remove supernatant (keep the beads)

[_] 3C.7. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3C.8. Repeat Step 3C.7. once

[_] 3C.9. Air dry beads, do not overdry

[_] 3C.10. Off magnet add 102 µl 0.1X TE

[_] 3C.11. Mix by pipetting 10 times. Incubate 2 min

[_] 3C.12. Place tubes on magnet. Wait 5 min and transfer 100 µl to a new tube

[_] 3C.13. Add 50 µl of beads to the supernatant and mix by pipetting 10 times. Incubate 5 min.

[_] 3C.14. Place tubes on magnet

[_] 3C.15. Wait 5 min then remove the supernatant (keep the beads)

[_] 3C.16. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 3C.17. Repeat Step 3C.16. once

[_] 3C.18. Air dry beads, do not overdry

[_] 3C.19. Off magnet add 22 µl 0.1X TE

[_] 3C.20. Mix by pipetting 10 times. Incubate 2 min.

[_] 3C.21. Place tubes on magnet. Wait 5 min and transfer 20 µl to a new tube

Skip to 4.1

4. PCR Enrichment of Adaptor-ligated DNA

4.1. PCR Amplification

[_] 4.1.1. Add PCR Reagents to sample

[_] 5 µl NEBNext Primer Mix

[_] 25 µl Q5 Master Mix

[_] 4.1.2. Pipette mix 10 times with pipette set to 40 µl, quick spin

[_] 4.1.3. Thermal cycle (Heated lid ≥ 103°C; 98°C 30 sec; 3-15 cycles of 98°C for 10 sec and 65°C for 75 sec; 65°C for 5 min, Hold at 4°C)

5. Cleanup of PCR Reaction

[_] 5.1. Vortex beads

[_] 5.2. [_] Add 40 µl of beads to sample and mix by pipetting 10 times (Standard fragment sizes)

or

[_] Add 30 µl of beads to sample and mix by pipetting 10 times (> 550 bp large fragment sizes)

[_] 5.3. Incubate for 5 min

[_] 5.4. Place tubes on magnet

[_] 5.5. Wait 5 min and remove supernatant (keep the beads)

[_] 5.6. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 5.7. Repeat Step 5.6. once

[_] 5.8. Air dry beads, do not overdry

[_] 5.9. Off magnet add 33 µl 10 mM Tris-HCl or 0.1X TE

[_] 5.10. Mix by pipetting 10 times. Incubate 2 min.

[_] 5.11. Place tubes on magnet. Wait 5 min and transfer 30 µl to a new tube

[_] 5.12. Check size distribution on Bioanalyzer

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	5/20
2.0	Updated to remove reference to specific UMI set. Also updated table formatting and legal footnote	5/23

www.neb.com

New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com