

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

NEB #E7805S/L, #E6177S/L

24/96 reactions

Version 4.0_7/23

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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

- NEBNext Oligo Kit options can be found at www.neb.com/oligos
- Alternatively, customer supplied adaptor and primers can be used, please see www.neb.com/faq-nonNEB-adaptors.

Please note: Separate instructions exist for UNIQUE DUAL INDEX UMI ADAPTORS.

- Magnetic rack (NEB #S1515S), magnetic plate (Alpaqua® cat. #A001322) or equivalent
- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- Thin wall 200 µl PCR tubes (For example Tempassure PCR flex-free 8-tube strips USA Scientific #1402-4708)
- PCR machine
- Vortex
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables
- 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 7.5-8.0 with 10 mM NaCl (for adaptor dilution of DNA input < 100 ng) or NEB #B1430S

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 intact gDNA, cDNA, plasmids and some amplicons* into high quality libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow also has minimal hands-on time.

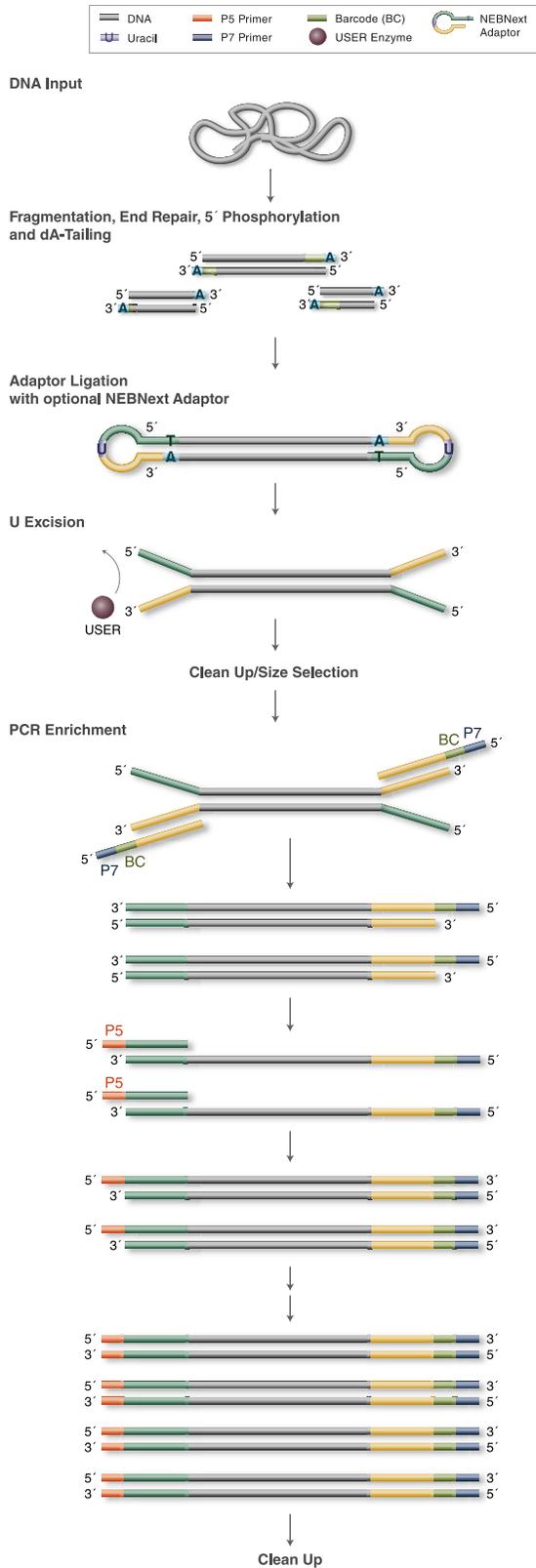
* Fragmentation times for cDNA, plasmids and amplicons may vary from gDNA times.

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

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 OEM & Customized Solutions 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 Illumina.



Adaptor trimming sequences:

The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TruSeq:

AdaptorRead1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

AdaptorRead2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Section 1

Protocol for use with Inputs \leq 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 \leq 100 ng, as size selection is not recommended for this input range. Follow the protocol in Chapter 2 for inputs \geq 100 ng, as size selection is 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 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 H₂O are also acceptable. If the input DNA is less than 26 μ l, add TE (provided) to a final volume of 26 μ l.

* This kit can also be used for plasmids, cDNA and some amplicons. It is not recommended for use with FFPE DNA. See FAQ <https://www.neb.com/faqs/2020/10/16/is-the-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina-compatible-with-all-sample-types>.

For FFPE DNA we recommend NEBNext Ultra II DNA Library Pre Kit for Illumina + Covaris or NEBNext FFPE DNA Library Prep Kit (NEB #E6650) or NEBNext UltraShear FFPE DNA Library Prep Kit (NEB #E6655).

1.1. Fragmentation/End Prep

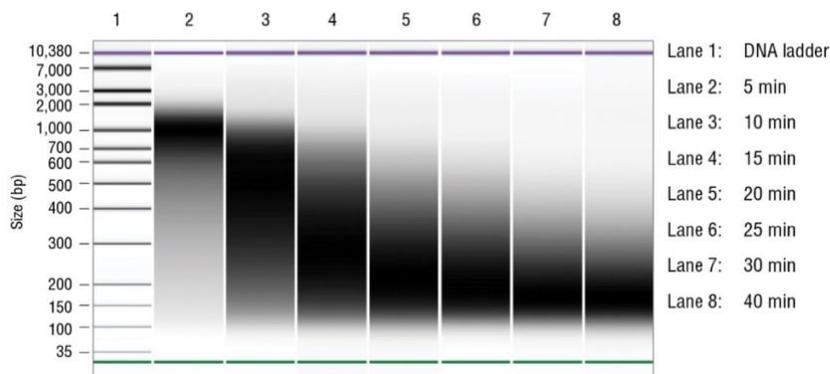
Fragmentation occurs during the 37°C incubation step. Use the chart below to determine the incubation time required to generate the desired fragment sizes. Incubation time may need to be optimized for individual samples. See Figure 1.1 for a typical fragmentation pattern.

| FRAGMENTATION | 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 μ l |
| ● (yellow) NEBNext Ultra II FS Reaction Buffer | 7 μ l |
| ● (yellow) NEBNext Ultra II FS Enzyme Mix | 2 μ l |
| Total Volume | 35 μl |

* Intact gDNA, cDNA, plasmids and some amplicons

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 minutes @ 37°C

30 minutes @ 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 7.5-8.0 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 μ M |
| 5 ng–99 ng | 10-Fold (1:10) | 1.5 μ M |
| less than 5 ng | 25-Fold (1:25) | 0.6 μ M |

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 μ l |
| • (red) NEBNext Ultra II Ligation Master Mix* | 30 μ l |
| • (red) NEBNext Ligation Enhancer | 1 μ l |
| • (red) NEBNext Adaptor for Illumina** | 2.5 μ l |
| 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 Oligo kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

- 1.2.2. Set a 100 μ l or 200 μ l pipette to 50 μ 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.**)

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

- 1.2.4. Add 3 μ l of • (red or blue) 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 non indexed NEBNext Adaptor. USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed UMI adaptor, USER is not needed. Please see corresponding manual for use with UMI on the Unique Dual Index UMI Adaptor Sets 1-4 Product page under the protocols, manuals, and usage tab.

- 1.2.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to \geq 47°C.



Samples can be stored overnight at –20°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 μ 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 μ 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 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 17 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 15 µl 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 Step 1.4.1A. if you are using the following oligos:

Use Option A for any NEBNext Oligo Kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at 10 µM each.

Follow Step 1.4.1B. if you are using the following oligos:

Use Option B for any NEBNext Oligo Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined (5 µM each).

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

1.4.1A. Forward and Reverse Primers not already combined

| COMPONENT | VOLUME |
|---------------------------------------------|--------------|
| Adaptor Ligated DNA Fragments (Step | 15 µl |
| • (blue) NEBNext Ultra II Q5 Master Mix | 25 µl |
| • (blue) Index Primer/i7 Primer*,** | 5 µl |
| • (blue) Universal PCR Primer/i5 Primer*,** | 5 µl |
| Total Volume | 50 µl |

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

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

1.4.1B. Forward and Reverse Primers already combined

| COMPONENT | VOLUME |
|-----------------------------------------|--------------|
| Adaptor Ligated DNA Fragments (Step | 15 µl |
| • (blue) NEBNext Ultra II Q5 Master Mix | 25 µl |
| • (blue) Index Primer Mix* | 10 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 with the heated lid set to 105°C 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.

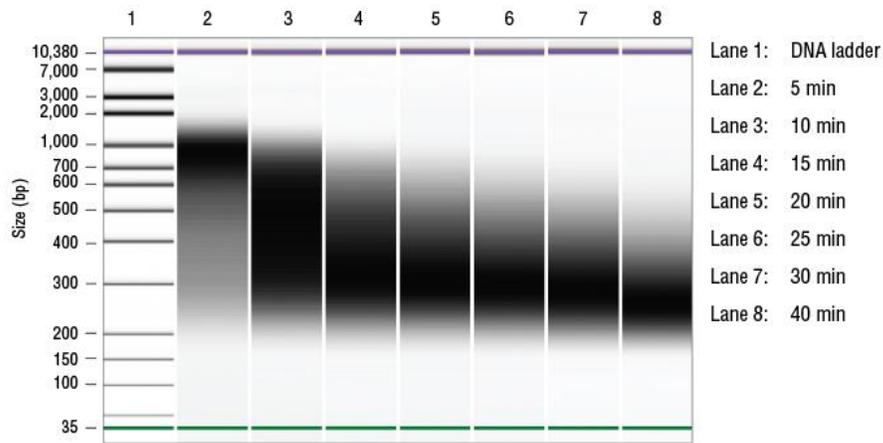
- 1.5.1. Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.
- 1.5.2. Add 45 μ l (0.9X) resuspended beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high 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 μ 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 μ 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 μ l 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 \leq 1 ng may not require dilution to run on a Bioanalyzer).
- 1.6.2. Run 1 μ 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 ~80 bp (primers) or ~128-140 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 1.5.11.) to 50 μ 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.



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 not 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 H₂O are also acceptable. If the input DNA is less than 26 μ l, add TE (provided) to a final volume of 26 μ l.

* This kit can also be used for plasmids, cDNA and some amplicons. It is not recommended for use with FFPE DNA. See FAQ <https://www.neb.com/faqs/2020/10/16/is-the-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina-compatible-with-all-sample-types>.

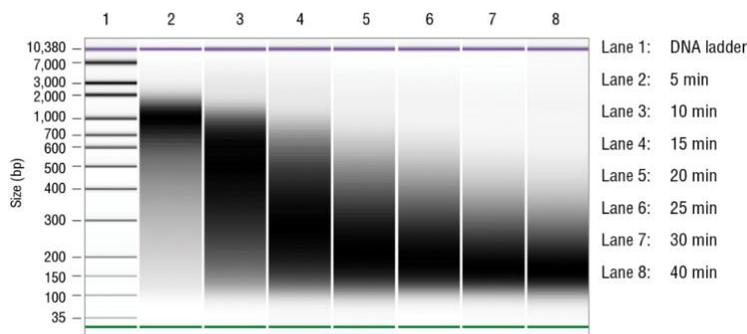
For FFPE DNA we recommend NEBNext Ultra II DNA Library Prep Kit for Illumina + Covaris or NEBNext FFPE DNA Library Prep Kit (NEB #E6650) or NEBNext UltraShear FFPE DNA Library Prep Kit (NEB #E6655).

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, 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 | 7 µl |
| ● (yellow) NEBNext Ultra II FS Enzyme Mix | 2 µl |
| Total Volume | 35 µl |

* Intact gDNA, cDNA, plasmids and some amplicons

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 minutes @ 37°C

30 minutes @ 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 |
| ● (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 Oligo kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use 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.

2.2.4. Add 3 µl of ● (red or blue) 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 non indexed NEBNext Adaptor. USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed UMI adaptor, USER is not needed. Please see corresponding manual for use with UMI on the Unique Dual Index UMI Adaptor Sets 1-4 Product page under the protocols, manuals, and usage tab.

2.2.5. 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°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 (71.5 μl ; 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 μl . Size selection conditions were optimized with SPRIselect or NEBNext Sample Purification Beads; however, AMPure XP beads can be used following the same conditions. If using AMPure XP beads, please allow the beads to warm to room temperature for at least 30 minutes before use.

To select a different insert size than 200 bp, please use the volumes in this table:

Table 2.3.1: Recommended Conditions for Bead Based Size Selection

| LIBRARY PARAMETERS | APPROXIMATE INSERT SIZE DISTRIBUTION | 150–250 bp | 200–350 bp | 275–475 bp | 350–600 bp |
|-------------------------------------------------|-------------------------------------------------------------------------|------------|------------|------------|------------|
| | Approx. Final Library Size Distribution (insert + adaptor + primers) | 270–370 bp | 320–470 bp | 400–600 bp | 470–800 bp |
| BEAD VOLUME TO BE ADDED (μl) | 1st Bead Addition | 40 | 30 | 25 | 20 |
| | 2nd Bead Addition | 20 | 15 | 10 | 10 |

- 2.3.1. Bring the volume of the reaction up to 100 μl by adding 28.5 μl 0.1X TE (dilute 1X TE Buffer 1:10 with water).
- 2.3.2. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.
- 2.3.3. Add 40 μl ($\sim 0.4\text{X}$) 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 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 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 ($\sim 0.2\text{X}$) 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 μ 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 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 17 μ 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 μ 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°C .

2.4. PCR Enrichment of Adaptor-ligated DNA



Follow Step 2.4.1A. if you are using the following oligos:

Use Option A for any NEBNext Oligo Kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at 10 μ M each.

Follow Step 2.4.1B. if you are using the following oligos:

Use Option B for any NEBNext Oligo Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 μ M combined (5 μ M each).

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

2.4.1A. Forward and Reverse Primers not already combined

| COMPONENT | VOLUME |
|----------------------------------------------|-----------------------------|
| Adaptor Ligated DNA Fragments (Step 2.3.16.) | 15 μ l |
| • (blue) NEBNext Ultra II Q5 Master Mix | 25 μ l |
| • (blue) Index Primer/i7 Primer*,** | 5 μ l |
| • (blue) Universal PCR Primer/i5 Primer*,** | 5 μ l |
| 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). Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

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

2.4.1B. Forward and Reverse Primers already combined

| COMPONENT | VOLUME |
|----------------------------------------------|--------------|
| Adaptor Ligated DNA Fragments (Step 2.3.16.) | 15 µl |
| • (blue) NEBNext Ultra II Q5 Master Mix | 25 µl |
| • (blue) Index Primer Mix* | 10 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 with the heated lid set to 105°C 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 μl (0.9X) resuspended beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.
- 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.

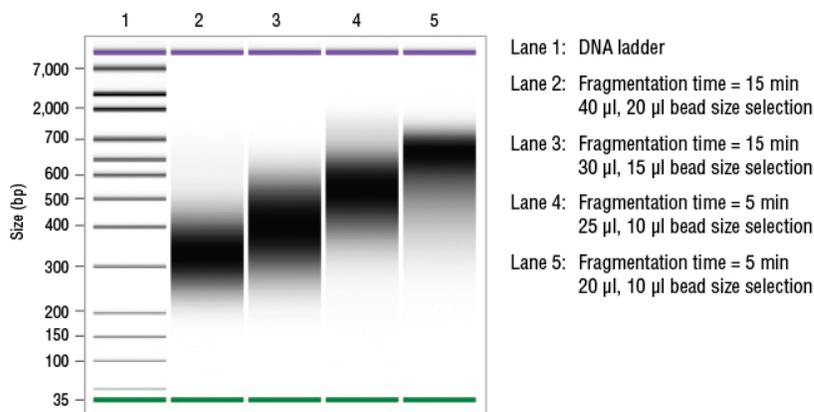
- 2.5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 μl of 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 2.5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly 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 ~80 bp (primers) or 128-140 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: Example of 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 H₂O are also acceptable. If the input DNA is less than 26 μ l, add TE (provided) to a final volume of 26 μ l.

* This kit can also be used for plasmids, cDNA and some amplicons. It is not recommended for use with FFPE DNA. See FAQ <https://www.neb.com/faqs/2020/10/16/is-the-nebnext-ultra-ii-fs-dna-library-prep-kit-for-illumina-compatible-with-all-sample-types>.

For FFPE DNA we recommend NEBNext Ultra II DNA Library Pre Kit for Illumina + Covaris or NEBNext FFPE DNA Library Prep Kit (NEB #E6650) or NEBNext UltraShear FFPE DNA Library Prep Kit (NEB #E6655).

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 μ l |
| ● (yellow) NEBNext Ultra II FS Reaction Buffer | 7 μ l |
| ● (yellow) NEBNext Ultra II FS Enzyme Mix | 2 μ l |
| Total Volume | 35 μl |

* Intact gDNA, cDNA, plasmids and some amplicons

- 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 minutes @ 37°C
30 minutes @ 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 Oligo kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use 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.

3.2.4. Add 3 µl of • (red or blue) 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 non indexed NEBNext Adaptor. USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed UMI adaptor, USER is not needed. Please see corresponding manual for use with UMI on the Unique Dual Index UMI Adaptor Sets 1-4 product page under the protocols, manuals, and usage tab.

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°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 (71.5 µl; 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 µl by adding 28.5 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 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 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 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 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 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.
- 3.3.19. 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 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 15 µl 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 Step 3.4.1A. if you are using the following oligos:

Use Option A for any NEBNext Oligo Kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at 10 μ M each.

Follow Step 3.4.1B. if you are using the following oligos:

Use Option B for any NEBNext Oligo Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 μ M combined (5 μ M each).

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

3.4.1A. Forward and Reverse Primers not already combined

| COMPONENT | VOLUME |
|----------------------------------------------|-----------------------------|
| Adaptor Ligated DNA Fragments (Step 3.3.23.) | 15 μ l |
| • (blue) NEBNext Ultra II Q5 Master Mix | 25 μ l |
| • (blue) Index Primer/i7 Primer*,** | 5 μ l |
| • (blue) Universal PCR Primer/i5 Primer*,** | 5 μ l |
| 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). Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

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

3.4.1B. Forward and Reverse Primers already combined

| COMPONENT | VOLUME |
|----------------------------------------------|-----------------------------|
| Adaptor Ligated DNA Fragments (Step 3.3.23.) | 15 μ l |
| • (blue) NEBNext Ultra II Q5 Master Mix | 25 μ l |
| • (blue) Index Primer Mix* | 10 μ l |
| 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 μ l or 200 μ l pipette to 40 μ 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 thermal cycler with the heated lid set to 105°C 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 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 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.

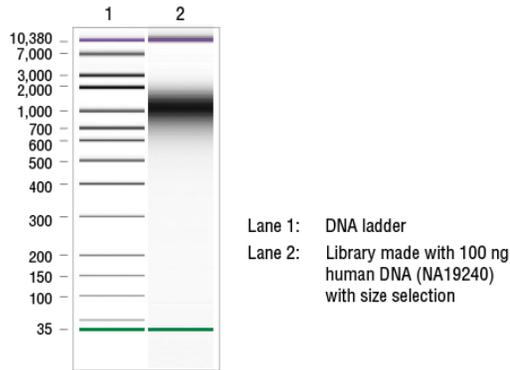
- 3.5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in water).
- 3.5.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly 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 ~ 80 bp (primers) or ~ 128 -140 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.

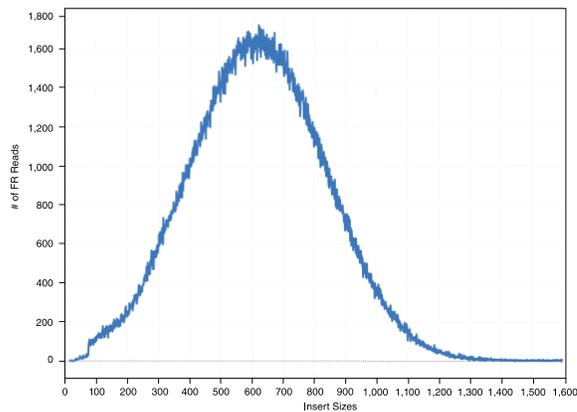
Figure 3.1: Example of final library size distributions without size selection. 100 ng Human DNA (NA 19240) was fragmented for 5 minutes.



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 |

Revision History

| REVISION # | DESCRIPTION | DATE |
|------------|----------------------------------------------------------------------------------------|------|
| 1.0 | N/A | 8/17 |
| 2.0 | New formatted manual. | 4/20 |
| 2.1 | Updated product license information. | 5/20 |
| 2.2 | Added hyperlink to “Required Materials Not Included”. | 6/20 |
| 3.0 | Updated protocols. | 9/22 |
| 4.0 | Updated information on UMI adaptors. Also updated table formatting and legal footnote. | 7/23 |

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