INSTRUCTION MANUAL

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

NEB #E7430S/L, #E7435S/L 24/96 reactions

Version 2.0_6/25

Table of Contents

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7430S/#E7435S) and 96 reactions (NEB #E7430L/#E7435L). 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
- (yellow) DTT (100 mM)
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer

TE Buffer (1X)

Package 2: Store at room temperature. Do not freeze.

Supplied only with NEBNext Ultra II FS PCR-free DNA Library Prep with Sample Purification Beads, NEB #E7435.

NEBNext Sample Purification Beads

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- Nuclease-free water
- 0.2 ml thin wall PCR tubes, for example TempAssure PCR flex-free 8-stube strips (USA Scientific #1402-4708)
- Magnetic rack/stand (NEB #S1515S; Alpaqua®, cat. #A001322 or equivalent)
- Thermal Cycler
- Vortex Mixer
- Bioanalyzer®, TapeStation® (Agilent Technologies, Inc.) or similar instrument and consumables
- Microcentrifuge
- NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA Set 1-4) NEB #E7395, NEB #E7874, NEB #E7876, NEB #E7878

Adaptor trimming sequences:

- The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TruSeq:
- Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
- Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

For NEB #E7430 only:

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

Overview

The NEBNext Ultra II FS PCR-free 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 without PCR amplification. The fast, user-friendly workflow also has minimal hands-on time.

Note: The Ultra II FS PCR-free Kit is not compatible with Enzymatic Methyl-seq (EM-seq™) and 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.

Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more

Section 1
Protocol for use with High Inputs (100 ng – 500 ng)

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

We strongly recommend to start PCR-free library prep with as much DNA input as possible due to the stringent cleanup conditions for large insert fragments.

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

Starting Material: 100 ng–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 25 µl, add TE (provided) to a final volume of 25 µl.

1.1. Fragmentation/End Prep

1.1.1. Ensure that the Ultra II FS Reaction Buffer and DTT 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.

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

COMPONENT	VOLUME PER ONE LIBRARY
DNA	25 µl
○ (yellow) NEBNext Ultra II FS Reaction Buffer	7 µl
○ (yellow) DTT	1 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:

INSERT SIZE	FS PROGRAM
350 bp	10 minutes at 37°C 30 minutes @ 65°C Hold at 4°C
450 bp	8 minutes at 37°C 30 minutes @ 65°C Hold at 4°C

Proceed immediately to **Adaptor ligation** once the reaction temperature reaches 4°C.

1.2. Adaptor Ligation

1.2.1. Add the following components directly to the FS Reaction Mixture in the order listed:

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**

* The NEBNext UMI adaptors are provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA Set 1-4). Please refer to the appropriate Oligo manual or NEBNext® Index Oligo Selector 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. Do not premix the adaptor with the Ligation Master Mix and Ligation Enhancer.

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 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. Proceed immediately to Step 1.3 or freeze.

Safe Stopping Point: Samples can be stored overnight at –20°C.

1.3. Cleanup of Adaptor-ligated DNA

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.). These volumes may not work properly for a cleanup at a different step in the workflow, or if this is a second cleanup. For cleanup of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

The following cleanup protocol is for libraries with ~ 350 bp or ~ 450 bp inserts only. Size selection conditions were optimized with NEBNext Sample Purification Beads and SPRIselect 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.

1.3.1. Add 40 µl 0.1X TE (dilute 1X TE Buffer 1:10 with water) to the ligation reaction.

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

1.3.3. Add 50 µl of resuspended NEBNext Sample Purification Beads or SPRIselect beads to the sample from Step 1.3.1. 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.

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

1.3.5. 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.

1.3.6. When the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard the beads)**.

1.3.7. 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.

1.3.8. Repeat Step 1.3.7. once.

1.3.9. 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.

1.3.10. 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 target. 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.

1.3.11. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 102 µl of 0.1X TE. Mix well by pipetting up and down 10 times, or on a vortex mixer.

1.3.12. Incubate for 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.

1.3.13. Place the tube/plate on a magnetic stand After the solution is clear (about 5 minutes), transfer 100 µl to a new PCR tube without disturbing the bead pellet.

1.3.14. Add appropriate amounts of resuspended Sample Purification Beads or SPRIselect beads to the sample for the desired insert sizes. 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.

INSERT SIZE	BEADS VOLUME
350 bp	65 µl
450 bp	58 µl

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

1.3.16. 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.

1.3.17. When the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard beads)**.

1.3.18. 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.

1.3.19. Repeat Step 1.3.18 once.

1.3.20. 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.

1.3.21. 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 target. 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.

1.3.22. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 22 µl of 0.1X TE. Mix well by pipetting up and down 10 times, or on a vortex mixer.

1.3.23. Incubate for 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.

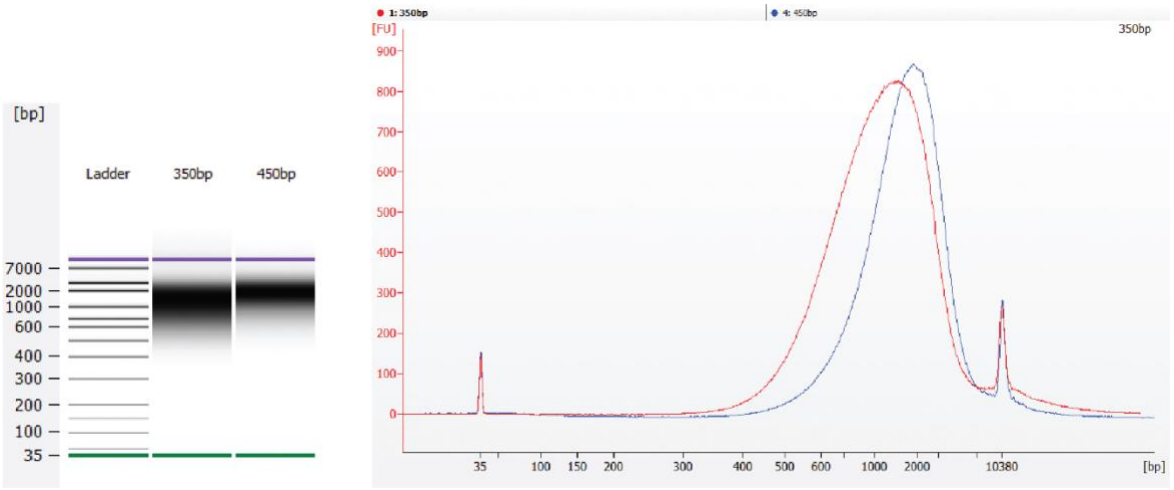
1.3.24. Place the tube/plate on a magnetic stand . After the solution is clear (about 5 minutes), transfer 20 µl to a new tube.

1.3.25. Quantitate the library using qPCR (NEBNext Library Quant Kit for Illumina, NEB # E7630S/L).

1.3.26. Verify fragment size by checking the library size distribution on an Agilent Bioanalyzer or TapeStation. Run 1µl library on a DNA High Sensitivity Chip (Bioanalyzer) or High Sensitivity D5000 ScreenTape® (TapeStation). See Figure 1 for an example.

Safe Stopping Point: It is safe to store the library at -20°C.

Figure 1: Examples of Ultra II FS PCR-free libraries on a Bioanalyzer. Please note that PCR-free libraries migrate slower due to the single strand regions of the adaptors, thus appearing significantly larger than the actual fragment sizes.



Section 2
Protocol for use with Low Inputs (50 ng to 99 ng)

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

Note: This low-input protocol is being provided to support users for whom obtaining sufficient sample for the standard protocol is not possible. While NEB strongly recommends inputs of 100 ng–500 ng of DNA, lower inputs (50 ng–99 ng) may be used. Starting with lower inputs may result in shorter fragments and lead to lower mapping quality. It may also result in slightly higher adaptor dimer levels.

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

Starting Material: 50 ng to 99 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

2.1.1. Ensure that the Ultra II FS Reaction Buffer and DTT 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) DTT	1 µl
(yellow) NEBNext Ultra II FS Enzyme Mix	1 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 to obtain 300 bp inserts:

INSERT SIZE	FS PROGRAM
300 bp	20 minutes at 37°C 30 minutes @ 65°C Hold at 4°C

Proceed immediately to **Adaptor ligation** once the reaction temperature reaches 4°C.

2.2. Adaptor Ligation

Thaw the NEBNext UMI DNA Adaptor Plate on ice and briefly spin, if necessary. It is important to keep the adaptor cold at all times. Dilute the NEBNext UMI Adaptors for Illumina in the UMI Dilution Buffer provided in the oligo kit as indicated in Table 2.2.1.

INPUT	ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL	WORKING ADAPTOR CONCENTRATION
50 ng–99 ng	10-Fold (1:10)	2 µM

Note: The appropriate adaptor dilution for your sample input and type may need to be optimized experimentally. The dilution provided here is a general starting point.

2.2.1. Add the following components directly to the FS Reaction Mixture in the order listed:

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**

* The NEBNext UMI adaptors are provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA Set 1-4). Please refer to the appropriate Oligo manual or NEBNext® Index Oligo Selector 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 at 4°C. Do not premix the adaptor with the Ligation Master Mix and Ligation Enhancer.

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 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. Move immediately to Step 2.3 or freeze.

Safe Stopping Point: Samples can be stored overnight at –20°C.

2.3. Cleanup of Adaptor-ligated DNA

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 cleanup at a different step in the workflow, or if this is a second cleanup. For cleanup of samples contained in different buffer conditions the volumes may need to be experimentally determined.

The following cleanup protocol is for libraries with ~ 300 bp inserts only. Size selection conditions were optimized with NEBNext Sample Purification Beads and SPRIselect 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.

2.3.1. Add 50 µl 0.1X TE (dilute 1X TE Buffer 1:10 with water) to the ligation reaction.

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

2.3.3. Add 50 µl of resuspended NEBNext Sample Purification Beads or SPRIselect beads to the 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 on the bench top 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. When the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard beads)**.

2.3.7. 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.

2.3.8. Repeat Step 2.3.7 once.

2.3.9. 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.

2.3.10. 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 target. 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.

2.3.11. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 17 µl of 0.1X TE. Mix well by pipetting up and down 10 times, or on a vortex mixer.

2.3.12. Incubate for 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.

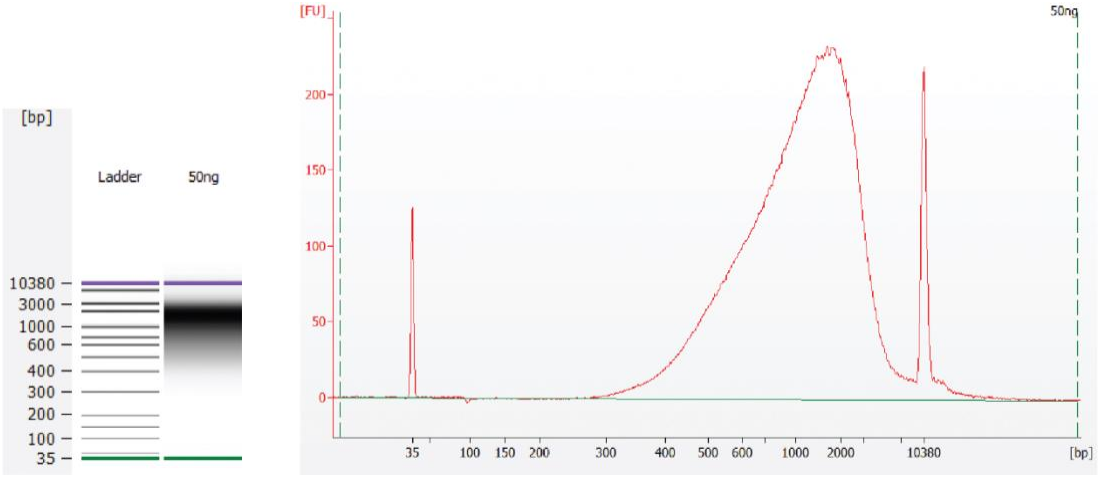
2.3.13. Place the tube/plate on a magnetic stand. After the solution is clear (about 5 minutes),, transfer 15 µl to a new tube.

2.3.14. Quantitate the library using qPCR (NEBNext Library Quant Kit for Illumina, NEB # E7630S/L).

2.3.15. Verify fragment size by checking the library size distribution on an Agilent Bioanalyzer or TapeStation. Run 1µl library on a DNA High Sensitivity Chip (Bioanalyzer) or High Sensitivity D5000 ScreenTape (TapeStation). See Figure 2 for an example.

Safe Stopping Point: It is safe to store the library at -20°C.

Figure 2: Examples of Ultra II FS PCR-free libraries (low input) on a Bioanalyzer. Please note that PCR-free libraries migrate slower due to the single strand regions of the adaptors, thus appearing significantly larger than the actual fragment sizes.



Kit Components

NEB #E7430S Table of Components

NEB #	PRODUCT	VOLUME
E7807A	NEBNext Ultra II FS Reaction Buffer	0.168 ml
E7806A	NEBNext Ultra II FS Enzyme Mix	0.048 ml
E7139AA	DTT (100 mM)	1 x 0.5 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7808A	TE Buffer (1X)	1.1 ml

NEB #E7430L Table of Components

NEB #	PRODUCT	VOLUME
E7807AA	NEBNext Ultra II FS Reaction Buffer	0.672 ml
E7806AA	NEBNext Ultra II FS Enzyme Mix	0.192 ml
E7139AA	DTT (100 mM)	1 x 0.5 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7808AA	TE Buffer (1X)	4.3 ml

NEB #E7435S Table of Components

NEB #	PRODUCT	VOLUME
E7807A	NEBNext Ultra II FS Reaction Buffer	0.168 ml
E7806A	NEBNext Ultra II FS Enzyme Mix	0.048 ml
E7139AA	DTT (100 mM)	1 x 0.5 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7808A	TE Buffer (1X)	1.1 ml
E7104S	NEBNext Sample Purification Beads	4 ml

NEB #E7435L Table of Components

NEB #	PRODUCT	VOLUME
E7807AA	NEBNext Ultra II FS Reaction Buffer	0.672 ml
E7806AA	NEBNext Ultra II FS Enzyme Mix	0.192 ml
E7139AA	DTT (100 mM)	1 x 0.5 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7808AA	TE Buffer (1X)	4.3 ml
E7104L	NEBNext Sample Purification Beads	4 x 4 ml

Checklist for High Input Protocol (100 ng – 500 ng)

1.1. NEBNext Fragmentation/End Prep

[_] 1.1.1 Add Fragmentation/End Prep Reagents to 25 µl of DNA sample:

- [_] Vortex the Ultra II FS Reaction Buffer and DTT to mix
- [_] Vortex the Ultra II FS Enzyme Mix 5–8 seconds
- [_] 7 µl FS Reaction Buffer
- [_] 1 µl DTT
- [_] 2 µl FS Enzyme Mix

[_] 1.1.2. Vortex the reaction for 5 seconds, quick spin

[_] 1.1.3. Thermal cycling (choose the program based on library insert size)

INSERT SIZE	FS PROGRAM
350 bp	10 minutes at 37°C 30 minutes @ 65°C Hold at 4°C
450 bp	8 minutes at 37°C 30 minutes @ 65°C Hold at 4°C

1.2. Adaptor Ligation

[_] 1.2.1. Add Ligation reagents to sample:

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

[_] 1.2.2. Pipette mix 10 times with pipette set to 50 µl, quick spin

[_] 1.2.3. Incubate 15 minutes at 20°C (heated lid off)

1.3. Size Selection of Adaptor-ligated DNA

[_] 1.3.1. Add 40 µl of 0.1X TE to sample

[_] 1.3.2. Vortex beads

[_] 1.3.3. Add 50 µl of beads to sample and mix by pipetting 10 times

[_] 1.3.4. Incubate 5 min at room temperature

[_] 1.3.5. Place tubes on magnet

[_] 1.3.6. Wait 5 min then then remove and discard the supernatant (keep the beads)

[_] 1.3.7. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 1.3.8. Repeat Step 1.3.7. once. Remove all visible liquid

[_] 1.3.9. Air dry the beads for up to 5 minutes on the magnetic stand

[_] 1.3.10. Elute DNA from the beads into 102 µl of 0.1X TE off the magnetic stand and mix by pipetting 10 times. Incubate 2 min

[_] 1.3.11. Place tubes on magnet

[_] 1.3.12. Wait 2 min then then transfer 100 µl supernatant to a new tube

[_] 1.3.13. Add 65 µl (350 bp insert) or 58µl (450 bp insert) of beads to the supernatant and mix by pipetting 10 times.

[_] 1.3.14. Incubate 5 min at room temperature

[_] 1.3.15. Place tubes on magnet

[_] 1.3.16. Wait 5 min then remove and discard the supernatant (keep the beads)

[_] 1.3.17. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 1.3.18. Repeat Step 1.3.17. once. Remove all visible liquid

[_] 1.3.19. Air dry the beads for up to 5 minutes on the magnetic stand

[_] 1.3.20. Elute DNA from the beads into 22 µl of 0.1X TE off the magnetic stand and mix by pipetting 10 times. Incubate 2 min

[_] 1.3.21. Place tubes on magnet

[_] 1.3.22. Wait 2 min then transfer 20 µl supernatant to a new tube

[_] 1.3.23. Store at -20°C

Checklist for Low Input Protocol (50 ng – 99 ng)

2.1. NEBNext Fragmentation/End Prep

[_] 2.1.1. Add Fragmentation/End Prep Reagents to 26 µl of DNA sample:

- [_] Vortex the Ultra II FS Reaction Buffer and DTT to mix
- [_] Vortex the Ultra II FS Enzyme Mix 5–8 seconds
- [_] 7 µl FS Reaction Buffer
- [_] 1 µl DTT
- [_] 1 µl FS Enzyme Mix

[_] 2.1.2. Vortex the reaction for 5 seconds, quick spin

[_] 2.1.3. Thermal cycling

INSERT SIZE	FS PROGRAM
300 bp	20 minutes at 37°C 30 minutes @ 65°C Hold at 4°C

2.2. Adaptor Ligation

[_] 2.2.1. Dilute UMI adaptors 10-fold

[_] 2.2.2. Add Ligation reagents to sample:

- [_] 2.5 µl NEBNext UMI adaptor (1:10 dilution)
- [_] 30 µl Ultra II Ligation Master Mix
- [_] 1 µl Ligation Enhancer

[_] 2.2.3. Pipette mix 10 times with pipette set to 50 µl, quick spin

[_] 2.2.4. Incubate 15 minutes at 20°C (heated lid off)

2.3. Cleanup of Adaptor-ligated DNA

[_] 2.3.1. Add 50 µl of 0.1x TE to sample

[_] 2.3.2. Vortex beads

[_] 2.3.3. Add 50 µl of beads to sample and mix by pipetting 10 times

[_] 2.3.4. Incubate 5 min at room temperature

[_] 2.3.5. Place tubes on magnet

[_] 2.3.6. Wait 5 min then then remove and discard the supernatant (keep the beads)

[_] 2.3.7. On magnet add 200 µl 80% ethanol, wait 30 seconds and remove

[_] 2.3.8. Repeat Step 2.3.7. once. Remove all visible liquid

[_] 2.3.9. Air dry the beads for up to 5 minutes on the magnetic stand

[_] 2.3.10. Elute DNA from the beads into 17 µl of 0.1X TE off the magnetic stand and mix by pipetting 10 times. Incubate 2 min

[_] 2.3.11. Place tubes on magnet

[_] 2.3.12. Wait 2 min then transfer 15 µl supernatant to a new tube

[_] 2.3.13. Store at -20°C

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	9/21
2.0	Updated protocols and legal disclaimer. Also added new NEB logo.	6/25

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

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