# NEBNext® FFPE DNA Library Prep Kit

NEB #E6650

24/96 reactions

Version 1.0_6/23

## Table of Contents



** Please review **Indexing Strategy row** found in the NEBNext Multiplex Oligo Selection Chart.*

## The NEBNext FFPE DNA Library Prep Kit Includes

*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E6650S) and 96 reactions (NEB #E6650L). 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**

- (lilac) NEBNext FFPE DNA Repair Mix v2
- (lilac) NEBNext Thermolabile Proteinase K
- (lilac) NEBNext FFPE DNA Repair Buffer v2
- (green) NEBNext Ultra II End Prep Enzyme Mix
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (blue) NEBNext MSTC FFPE Master Mix

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

NEBNext Sample Purification Beads

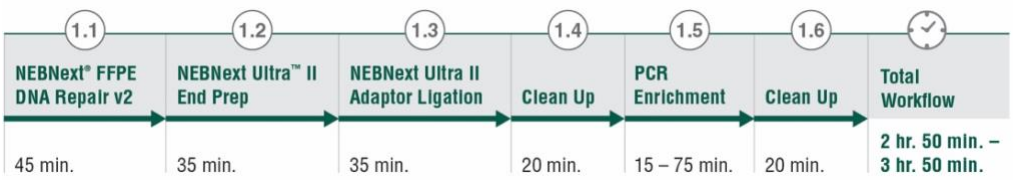
## Required Materials Not Included

- 80% Ethanol
- Nuclease-free Water
- 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- DNase-, RNase-free PCR strip tubes
- NEBNext Multiplex Oligos for Illumina® (www.neb.com/oligos)
- Magnetic rack/stand (NEB #S1515S, Alpaqua® cat. #A001322, or equivalent)
- Thermal cycler
- Agilent® Bioanalyzer® or TapeStation® and associated reagents and consumables
- Adaptor Dilution Buffer NEB #B1430S or NEBNext Unique Dual Index UMI Adaptor Dilution Buffer supplied with NEBNext Unique Dual Index UMI Adaptor DNA Sets (NEB #E7395/E7874/E7876/E7878)

## Overview

The NEBNext FFPE DNA Library Prep Kit contains the enzymes and buffers required to convert 5 to 250 ng of pre-sheared FFPE DNA into high quality libraries for next generation sequencing. The kit includes a mix of enzymes optimized to repair FFPE DNA, and library prep reagents with a fast protocol and minimal hands-on time.

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.




**NEB has determined that there are safe stopping points after each step in this protocol (store at -20°C overnight). Please use the time estimates above to assist you.**

## Section 1

## Protocol for use with all NEBNext Multiplex Oligos Index Primers* (Single Index, Dual Index, or Unique Dual Index Primers)

** Please see the NEBNext Multiplex Oligo Selection Chart for more details on the different options for multiplex oligos.*

**Symbols**

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

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

**Starting Material:** 5 ng – 250 ng fragmented FFPE DNA. We recommend that DNA be sheared in 0.1X TE. If the DNA volume after shearing is less than 46 µl, add 0.1X TE to a final volume of 46 µl. Alternatively, samples can be diluted with 1X TE or nuclease-free water.

**1.1 NEBNext FFPE DNA Repair v2**

1.1.1. Mix the following components in a sterile, 0.2 ml nuclease-free tube (55 µl final volume):

| COMPONENT | VOLUME |
|---|---|
| FFPE DNA | 46 µl |
| • (lilac) NEBNext FFPE DNA Repair Buffer v2 | 7 µl |
| • (lilac) NEBNext FFPE DNA Repair Mix v2 | 2 µl |
| **Total Volume** | **55 µl** |

1.1.2 Set a 100 µl or 200 µl pipette to 40 µl and then pipet 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.

1.1.3. Place in a thermal cycler, with a heated lid set to 50°C, and run the following program:
15 minutes at 37°C
Hold at 4°C

1.1.4. Add 2 µl of • (lilac) NEBNext Thermolabile Proteinase K on ice.

1.1.5. Set a 100 µl or 200 µl pipette to 40 µl and then pipet 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.

1.1.6. Place in a thermal cycler, with a heated lid set to 75°C, and run the following program:

15 minutes at 37°C

5 minutes at 65°C

Hold at 4°C

1.1.7. Place samples on ice and proceed to End Prep.

### 1.2. NEBNext Ultra II End Prep

1.2.1. Add 3 µl of ● (green) NEBNext Ultra II End Prep Enzyme Mix directly to the repaired reaction mixture from Step 1.1.7.

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.

**Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.**

1.2.3. Place in a thermal cycler, with the heated lid set to 75°C, and run the following program:

15 minutes at 20°C

15 minutes at 65°C

Hold at 4°C

1.2.4. Place samples on ice and proceed to Adaptor Ligation.

### 1.3. NEBNext Ultra II Adaptor Ligation

**Follow this protocol if using the non-indexed, NEBNext Adaptor for Illumina supplied with any NEBNext Multiplex Oligo Index Primers* (e.g., NEB #E7335, #E7600, #E6440, etc.)**

** Please see the NEBNext Multiplex Oligo Selection Chart for oligo options.*

1.3.1. Determine which adaptor dilution is necessary.

*Note: Due to the varying degree of quality of FFPE DNA, adaptor dilution may need to be further optimized. The dilutions provided here are a general starting point. Excess adaptor should be removed prior to PCR enrichment.*

*Based on input DNA amount (ng), dilute the NEBNext Adaptor for Illumina in Tris/NaCl, pH 8.0 or NEBNext Adaptor Dilution Buffer as indicated in Table 1.3.*

**Table 1.3 NEBNext Adaptor Dilution**

| INPUT | ADAPTOR DILUTION (VOLUME ADAPTOR:TOTAL VOLUME) | WORKING ADAPTOR CONCENTRATION |
|---|---|---|
| 101–250 ng | 2-fold (1:2) | 7.5 µM |
| 25–100 ng | 10-fold (1:10) | 1.5 µM |
| 5–24 ng | 25-fold (1:25) | 0.6 µM |

1.3.2. Add the following components directly to the repaired/end-prepped DNA:

| COMPONENT | VOLUME |
|---|---|
| End Prep Reaction Mixture (Step 1.2.4) | 60 µl |
| ● (red) Diluted NEBNext Adaptor for Illumina* | 2.5 µl |
| ● (red) NEBNext Ultra II Ligation Master Mix** | 30 µl |
| ● (red) NEBNext Ligation Enhancer | 1 µl |
| **Total Volume** | **93.5 µl** |

* The NEBNext adaptor is provided in the NEBNext Multiplex Oligos Index Primers kit. NEB has several oligo kit options, which are supplied separately from the library prep kit.

** 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.**

1.3.3. Set a 100 µl or 200 µl pipette to 80 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).**

1.3.4. Place in a thermal cycler with the heated lid set to off, and run the following program:

20°C for 15 minutes

Hold at 4°C

1.3.5. Add 3 µl of ● (red) USER® Enzyme to the ligation mixture from Step 1.3.4

**Note: Steps 1.3.5 through 1.3.6 are only required for use with NEBNext Adaptors supplied in all NEBNext Multiplex Oligo Index Primer kits, except NEBNext Unique Dual Index UMI Adaptors DNA Sets. USER enzyme is supplied with the NEBNext Multiplex Oligos for Illumina.**

1.3.6. Mix well and place in a thermal cycler with the heated lid set to >45°C, and run the following program:

37°C for 15 minutes

Hold at 4°C

**1.4. Cleanup of Adaptor-ligated DNA**

**Note: The volumes of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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.**

1.4.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.4.2. Add **87 µl (0.9X)** resuspended beads to the Adaptor Ligation reaction (96.5 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all 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.

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

1.4.4. 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.

1.4.5. 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 beads).**

1.4.6. Add 200 µl of freshly prepared 80% ethanol to the tube/plate while on 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.

1.4.7. Repeat Step 1.4.6 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.

1.4.8. 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 the 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.**

1.4.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **17 µl of 0.1X TE**.

1.4.10. Mix well by pipetting 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect all liquid from the sides of the tube before placing back on the magnetic stand.

1.4.11. 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.

### 1.5. PCR-enrichment of Adaptor-ligated DNA

**Use Option A:** for any NEBNext Multiplex Oligo Index Primer kits where the forward and reverse primers (i5 and i7) are supplied in separate tubes. Primers are supplied at 10 µM each.

**Use Option B:** for any NEBNext Multiplex Oligo Index Primer kits where the forward and reverse (i5 and i7) primers are combined in a 96-well plate format. Primers are supplied at 10 µM combined (5 µM each).

See **NEBNext Multiplex Oligo Selection Chart** for the full list of oligo options.

#### 1.5.1A. Forward and Reverse Primer Not Already Combined

| COMPONENT | VOLUME (µl) PER REACTION |
|---|---|
| Adaptor-Ligated DNA Fragments (Step 1.4.11) | 15 µl |
| • (blue) NEBNext MSTC FFPE Master Mix | 25 µl |
| Index Primer/i7 Primer*,** | 5 µl |
| Universal PCR Primer/i5 Primer*, ** | 5 µl |
| **Total Volume** | **50 µl** |

#### 1.5.1B. Forward and Reverse Primer Already Combined

| COMPONENT | VOLUME (µl) PER REACTION |
|---|---|
| Adaptor-Ligated DNA Fragments (Step 1.4.11) | 15 µl |
| • (blue) NEBNext MSTC FFPE Master Mix | 25 µl |
| NEBNext i5/i7 primers combined in 96 well plate format* | 10 µl |
| **Total Volume** | **50 µl** |

*NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

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

1.5.2. 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.

1.5.3. Place the tube on a thermocycler and perform PCR amplification using the following PCR cycling conditions:

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation | 98°C | 10 seconds | 5-14* |
| Annealing/Extension | 65°C | 75 seconds | |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* 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). The number of PCR cycles recommended in Table 1.5.4 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples.

1.5.4. Recommended Number of PCR Cycles*

| FFPE DNA INPUT | STANDARD RECOMMENDED NUMBER OF PCR CYCLES | RECOMMENDED NUMBER OF PCR CYCLES FOR TARGET ENRICHMENT ** |
|---|---|---|
| 250 ng | 5-7 | 7-11 |
| 100 ng | 6-8 | 8-12 |
| 50 ng | 8-12 | - |
| 25 ng | 9-14 | - |
| 10 ng | 10-14 | - |
| 5 ng | 11-14 | - |

* The number of cycles may need to be determined experimentally by the user depending upon the quality of the FFPE DNA used.

** Target enrichment input amounts vary by vendor often ranging from 200 ng to 1500 ng of PCR-amplified library. Cycle numbers may need to be optimized to achieve specific input requirements of a user-supplied target enrichment kit, while minimizing over-cycling artifacts and duplication rates.

**1.6. Cleanup of PCR Amplification**

**Note: The volumes of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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.**

1.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

1.6.2. 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 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.

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

1.6.4. 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.

1.6.5. 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 beads).**

1.6.6. Add 200 µl of freshly prepared 80% ethanol to the tube/ plate while on 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.

1.6.7. Repeat Step 1.6.6 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.

1.6.8. 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 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.**

1.6.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **33 µl of 0.1X TE**.

**Note: Alternative elution buffer or water can be used if required by downstream application (e.g., target enrichment).**

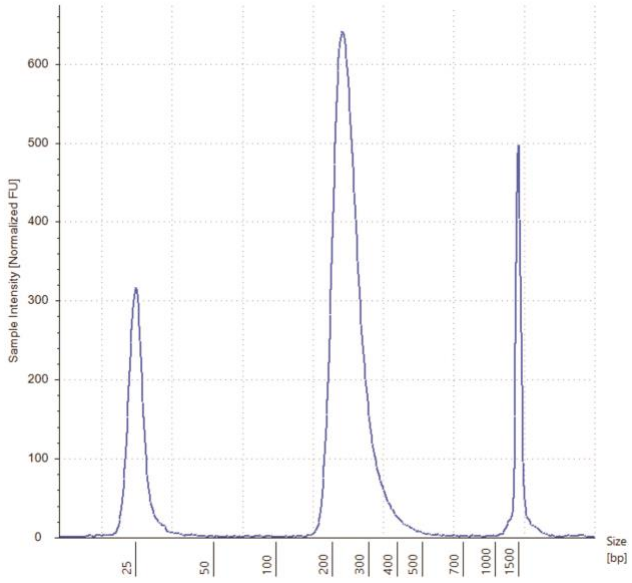
1.6.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

1.6.11. 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 for and store at -20°C.

1.6.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip. The sample may need to be diluted before loading.

1.6.13. A sharp peak at 128 bp (single barcode) or 146 bp (dual barcodes) on Agilent Bioanalyzer or TapeStation corresponds to adaptor-dimer. We recommend repeating Steps 1.6.1 to 1.6.11 if this occurs. For target enrichment libraries, additional cleanup may not be required but consider the accuracy of quantification if performing a Qubit® dsDNA assay (ThermoFisher) to assess library input amount for hybrid capture.

**Figure 1.1: Example of a library prepared with normal human liver FFPE DNA (DIN 2.0) on Agilent TapeStation High Sensitivity D1000 Assay.**



## Section 2

## Protocol for use with NEBNext Multiplex Oligos Index Adaptors* (Unique Dual Index UMI Adaptors DNA Sets)

** Please see the NEBNext Multiplex Oligo Selection Chart for more details on the different options for multiplex oligos.*

**Symbols**

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

●

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

**Starting Material:** 5 ng–250 ng fragmented FFPE DNA. We recommend that DNA be sheared in 0.1X TE. If the DNA volume after shearing is less than 46 µl, add 0.1X TE to a final volume of 46 µl. Alternatively, samples can be diluted with 0.1X TE or nuclease-free water.

### 2.1. NEBNext FFPE DNA Repair v2

2.1.1. Mix the following components in a sterile nuclease-free tube (55 µl final volume):

| COMPONENT | VOLUME |
|---|---|
| FFPE DNA | 46 µl |
| ● (lilac) NEBNext FFPE DNA Repair Buffer v2 | 7 µl |
| ● (lilac) NEBNext FFPE DNA Repair Mix v2 | 2 µl |
| **Total Volume** | **55 µl** |

2.1.2 Set a 100 µl or 200 µl pipette to 40 µl and then pipet 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.

2.1.3. Place in a thermal cycler, with a heated lid set to 50°C, and run the following program:
15 minutes at 37°C
Hold at 4°C

2.1.4. Add 2 µl of NEBNext Thermolabile Proteinase K (●) on ice.

2.1.5. Set a 100 µl or 200 µl pipette to 40 µl and then pipet 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.

2.1.6. Place in a thermocycler, with a heated lid set to 75°C, and run the following program:
15 minutes at 37°C
5 minutes at 65°C
Hold at 4°C

2.1.7. Place samples on ice and proceed to End Prep.

### 2.2. NEBNext Ultra II End Prep

2.2.1. Add 3 µl of ● (green) NEBNext Ultra II End Prep Enzyme Mix directly to the repaired reaction mixture from Step 2.1.7.

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.

**Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.**

2.2.3. Place in a thermal cycler, with the heated lid set to 75°C, and run the following program:
15 minutes at 20°C
15 minutes at 65°C
Hold at 4°C

2.2.4 Place samples on ice and proceed to Adaptor Ligation.

### 2.3. NEBNext Ultra II Adaptor Ligation using NEBNext Multiplex Oligos for Illumina Index Adaptors DNA Sets* (e.g., NEB #E7395, etc.)

** Please see the NEBNext Multiplex Oligo Selection Chart for more details on the different options for multiplex oligos.*

2.3.1 Determine which adaptor dilution is necessary.

*Note: Due to the varying degree of quality of FFPE DNA, adaptor dilution may need to be further optimized. The dilutions provided here are a general starting point. Excess adaptor should be removed prior to PCR enrichment.*

*Based on input DNA amount (ng), dilute adaptors on ice using the NEBNext UMI Adaptor Dilution Buffer (supplied with NEBNext Multiplex Oligos Unique Dual Index UMI Adaptor DNA Sets – NEB #E7395/E7874/E7876/E7878) as indicated in Table 2.3.*

**Table 2.3 NEBNext Unique Dual Index UMI Adaptor Dilution**

| INPUT | ADAPTOR DILUTION (VOLUME ADAPTOR : TOTAL VOLUME) | WORKING ADAPTOR CONCENTRATION |
|---|---|---|
| 201-250 ng | 2-fold (1:2) | 10 µM |
| 100-200 ng | 5-fold (1:5) | 4 µM |
| 25-99 ng | 10-fold (1:10) | 2 µM |
| 5-24 ng | 25-fold (1:25) | 0.8 µM |

2.3.2 Add the following components directly to the repaired/End-prepped DNA:

| COMPONENT | VOLUME |
|---|---|
| End Prep Reaction Mixture (Step 2.2.4) | 60 µl |
| Diluted NEBNext Unique Dual Index UMI Adaptor* | 2.5 µl |
| • (red) NEBNext Ultra II Ligation Master Mix** | 30 µl |
| • (red) NEBNext Ligation Enhancer | 1 µl |
| **Total Volume** | **93.5 µl** |

* The NEBNext Unique Dual Index UMI Adaptors are supplied in a 96-well plate format.

** 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.**

2.3.3. Set a 100 µl or 200 µl pipette to 80 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).**

2.3.4. Place in a thermal cycler with the heated lid off, and run the following program:

20°C for 15 minutes

Hold at 4°C

### 2.4. Cleanup of adaptor-ligated DNA

**Note: For optimal removal of excess Unique Dual Index UMI adaptor and PCR amplification of FFPE DNA libraries we recommend performing two sequential 0.9X bead cleanups according to the protocol below. The volumes of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. These bead 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.**

2.4.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.4.2. Add **84 µl (0.9X)** resuspended beads to the Adaptor Ligation reaction (93.5 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all 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.

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

2.4.4. 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.

2.4.5. 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 beads).**

2.4.6. Add 200 µl of freshly prepared 80% ethanol to the tube/plate while on 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.

2.4.7. Repeat Step 2.4.6 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.

2.4.8. Air the dry 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 the 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.**

2.4.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **52 µl of 0.1X TE.**

2.4.10. Mix well by pipetting 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect all liquid from the sides of the tube before placing back on the magnetic stand.

2.4.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer **50 µl** to a new PCR tube or plate containing **45 µl (0.9X)** NEBNext Sample Purification Beads.

2.4.12. Mix well by pipetting up and down at least 10 times. Be careful to expel all 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.

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

2.4.14. 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.

2.4.15. 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 beads).**

2.4.16. Add 200 µl of freshly prepared 80% ethanol to the tube/plate while on 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.

2.4.17. Repeat Step 2.4.16 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.

2.4.18. Air the dry 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 the 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.**

2.4.19. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **22 µl of 0.1X TE.**

2.4.20. Mix well by pipetting 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect all liquid from the sides of the tube before placing back on the magnetic stand.

2.4.21. 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.

**2.5. PCR-enrichment of Adaptor-ligated DNA**

2.5.1 PCR reaction setup:

| COMPONENT | VOLUME (µl) PER REACTION |
|---|---|
| Adaptor-Ligated DNA Fragments (Step 2.4.21) | 20 µl |
| • (blue) NEBNext MSTC FFPE Master Mix | 25 µl |
| • (blue) NEBNext Primer Mix* | 5 µl |
| **Total Volume** | **50 µl** |

*NEBNext Primer Mix is supplied with NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors DNA Sets, e.g., NEB #E7395)

2.5.2. 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.

2.5.3. Place the tube on a thermocycler and perform PCR amplification using the following PCR cycling conditions:

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation | 98°C | 10 seconds | 4-14* |
| Annealing/Extension | 65°C | 75 seconds | |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* 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). The number of PCR cycles recommended in Table 2.5.4 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples.

2.5.4. Recommended Number of PCR Cycles*

| FFPE DNA INPUT | STANDARD RECOMMENDED NUMBER OF PCR CYCLES | RECOMMENDED NUMBER OF PCR CYCLES FOR TARGET ENRICHMENT** |
|---|---|---|
| 250 ng | 4-7 | 7-11 |
| 100 ng | 5-8 | 8-12 |
| 50 ng | 8-12 | - |
| 25 ng | 9-14 | |
| 10 ng | 10-14 | - |
| 5 ng | 11-14 | - |

* The number of cycles may need to be determined experimentally by the user depending upon the quality of the FFPE DNA used.

** Target enrichment input amounts vary by vendor often ranging from 200 ng to 1500 ng of PCR-amplified library. Cycle numbers may need to be optimized to achieve specific input requirements of a user-supplied target enrichment kit, while minimizing over-cycling artifacts

**2.6. Cleanup of PCR Amplification**

**Note: The volumes of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. 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.**

2.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

2.6.2. 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 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.

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

2.6.4. 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.

2.6.5. 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 beads).**

2.6.6. Add 200 µl of freshly prepared 80% ethanol to the tube/ plate while on 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.

2.6.7. Repeat Step 2.6.6 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.

2.6.8. 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 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.**

2.6.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **33 µl** of 0.1X TE.

**Note: Alternative elution buffer or water can be used if required by downstream application (e.g. target enrichment).**

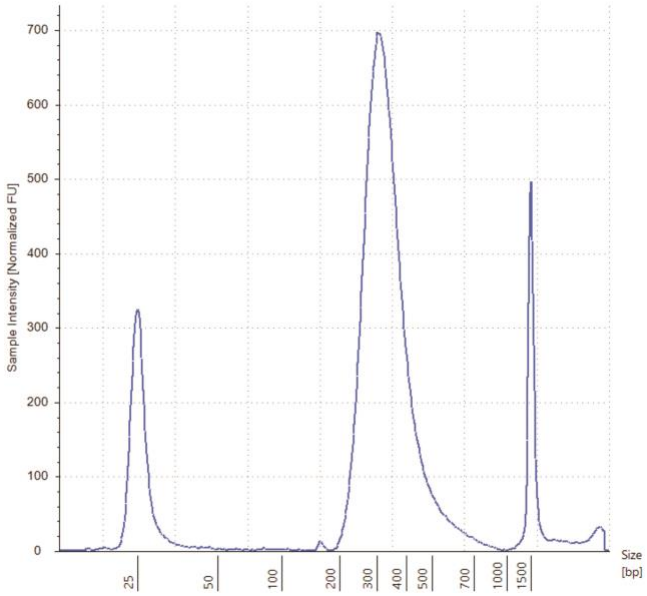
2.6.10. Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

2.6.11. 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 for and store at -20°C.

2.6.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip or Agilent HS D1000 TapeStation screen tape. The sample may need to be diluted before loading.

2.6.13. A sharp peak at 146 bp on Agilent Bioanalyzer or TapeStation corresponds to adaptor-dimer. We recommend repeating Steps 2.6.1 to 2.6.11 if this occurs. For target enrichment libraries, additional cleanup may not be required but consider the accuracy of quantification if performing a Qubit dsDNA assay (ThermoFisher) to assess library input amount for hybrid capture.

**Figure 2.1: Example of a library prepared with normal human liver FFPE DNA (DIN 2.0) on Agilent TapeStation High Sensitivity D1000 Assay.**



## Kit Components

NEB #E6650S Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7361A | NEBNext FFPE DNA Repair Mix v2 | 0.048 ml |
| E7362A | NEBNext Thermolabile Proteinase K | 0.048 ml |
| E7363A | NEBNext FFPE DNA Repair Buffer v2 | 0.168 ml |
| E7646A | NEBNext Ultra II End Prep Enzyme Mix | 0.072 ml |
| E7648A | NEBNext Ultra II Ligation Master Mix | 0.72 ml |
| E7374A | NEBNext Ligation Enhancer | 0.024 ml |
| E6651A | NEBNext MSTC FFPE Master Mix | 0.6 ml |
| E6652A | NEBNext Sample Purification Beads | 4.3 ml |

NEB #E6650L Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7361AA | NEBNext FFPE DNA Repair Mix v2 | 0.192 ml |
| E7362AA | NEBNext Thermolabile Proteinase K | 0.192 ml |
| E7363AA | NEBNext FFPE DNA Repair Buffer v2 | 0.672 ml |
| E7646AA | NEBNext Ultra II End Prep Enzyme Mix | 0.288 ml |
| E7648AA | NEBNext Ultra II Ligation Master Mix | 3 x 0.96 ml |
| E7374AA | NEBNext Ligation Enhancer | 0.096 ml |
| E6651AA | NEBNext MSTC FFPE Master Mix | 2 x 1.2 ml |
| E6652AA | NEBNext Sample Purification Beads | 17 ml |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 1.0 | N/A | 6/23 |





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