NEBNext® FFPE DNA Repair Mix

NEB #M6630S/L

24/96 reactions

Version 7.0_12/22

Table of Contents

The NEBNext FFPE DNA Repair Mix Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E6330S) and 96 reactions (NEB #E6330L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (green) NEBNext FFPE DNA Repair Buffer
- (green) NEBNext FFPE DNA Repair Mix

Please note: For superior performance, the NEBNext FFPE DNA Repair v2 Module (NEB #E7360) is now available.

Required Materials Not Included

- NEBNext Ultra II DNA Library Prep Kit (NEB #E7645S, E7645L, E7103S or E7103L, E7370S/L) for Illumina, or other
- 80% Ethanol
- Nuclease-free Water
- 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- Tempassure PCR flex-free 8-tube strips (USA Scientific® #1402-4708)
- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317), AMPure® XP Beads (Beckman Coulter, Inc. #A63881), or NEBNext Sample Purification Beads (from NEB #E7103 kit)
- Magnetic rack/stand (NEB #S1515S, Alpaqua®, cat. #A001322 or equivalent)
- PCR Machine
- Bioanalyzer and associated reagents and consumables
- Optional: 10 mM Tris-HCl, pH 8.0 with 10 mM NaCl or NEB #B1430S (for adaptor dilution of DNA input less than 100 ng)
- NEBNext Oligo kit options can be found at neb.com/oligos

Alternatively, customer supplied adaptor and primers can be used, please see information in link below:
https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb

Please note: This manual is not for use with UNIQUE DUAL INDEX UMI ADAPTORS.

Applications

The NEBNext FFPE DNA Repair Mix contains enzymes and buffers that are ideal to repair FFPE DNA for downstream NGS library construction and sequencing. Each of these components must pass rigorous quality control standards and are lot controlled.

Lot Control: The lots provided in the NEBNext FFPE DNA Repair Mix are managed separately and qualified by additional functional validation. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria in the additional quality controls listed on each individual component page.

Functionally Validated: Each lot of reagents is functionally validated with FFPE DNA and the NEBNext Ultra DNA Library Prep Kit for Illumina, and sequenced on an Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available
by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

Section 1
Protocol for use with NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB #E7645)

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 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–1 µg fragmented FFPE DNA. NEB recommends that DNA be sheared in 1X TE. If the DNA volume post-shearing is less than 50 µl, add 1X TE to a final volume of 50 µl. Alternatively, samples can be diluted with 10 mM Tris-HCl, pH 8.0 or 0.1X TE.

1.1. NEBNext FFPE DNA Repair

1.1.1. Mix the following components in a sterile nuclease-free tube (57 µl final volume):

COMPONENT	VOLUME
FFPE DNA	48 µl
● (green) FFPE DNA Repair Buffer	3.5 µl
● (green) NEBNext Ultra II End Prep Buffer	3.5 µl
● (green) NEBNext FFPE DNA Repair Mix	2 µl
Total Volume	**57 µl**

1.1.2 Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

1.1.3. Incubate at 20°C for 30 minutes (with the heated lid off).

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 FFPE repair reaction mixture from Step 1.1.3.

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:

30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C
Proceed to Adaptor Ligation

1.3. NEBNext Ultra II Adaptor Ligation

1.3.1. Determine whether adaptor dilution is necessary.

If DNA input is ≤ 100 ng, dilute the NEBNext Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5-8.0 with 10 mM NaCl as indicated in Table 1.3.

Table 1.3 Adaptor Dilution

INPUT	ADAPTOR DILUTION (VOLUME ADAPTOR: TOTAL VOLUME)	WORKING ADAPTOR CONCENTRATION
1 µg–101 ng	No dilution	15 µM
100 ng–5 ng	10-fold (1:10)	1.5 µM
Less than 5 ng	25-fold (1:25)	0.6 µM

*** 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.**

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

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 1.2.3)	60 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	**93.5 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 the NEBNext oligos kit. NEB has several oligo kit 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 at 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

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

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

Note: Steps 1.3.5 and 1.3.6 are only required for use with NEBNext loop adaptors. USER enzyme can be found in the NEBNext Multiplex Oligos for Illumina. If you are using the Unique Dual Index UMI Adaptors, USER is not needed so please see the manual for E7395.

1.3.6. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

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

1.4. Cleanup of Adaptor-ligated DNA

Note: Size selection is not recommended for FFPE DNA since it contains a large amount of small DNA fragments that would be lost in the size selection process. A cleanup will preserve more of the library.

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 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 SPRIselect or NEBNext Sample Purification Beads to resuspend.

1.4.2. Add 87 µl (0.9X) 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.

1.4.3. 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 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.

1.4.7. 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 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.

1.4.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of 10 mM Tris-HCl or 0.1X TE (pH 8.0).

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.

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

1.5. PCR-enrichment of Adaptor-ligated DNA

Use Option A for any NEBNext oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos 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.

1.5.1A. Forward and Reverse Primer Not Already Combined

Add the following components to a sterile strip tube:

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 1.4.11)	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**

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 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. 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 thermal cycler and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	3-12*
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	RECOMMENDED NUMBER OF PCR CYCLES*
1 µg	3-4**
500 ng	5-6
100 ng	6-7
50 ng	7-8
10 ng	10-11
5 ng	11-12

* The higher end of the recommended cycle number for the Ultra II DNA Library Prep Kit was used for FFPE DNA due to the lower fraction of starting DNA that can be converted into a library. The number of cycles will need to be determined experimentally by the user depending upon the quality of the FFPE DNA used.

** 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.

1.6. 1X SPRI Bead Cleanup of PCR Amplification

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.

1.6.1. Vortex SPRIselect or Sample Purification Beads to resuspend. 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.

1.6.2. Add 50 µl (1X) resuspended SPRIselect 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.

1.6.3. 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 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.

1.6.7. 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.

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.

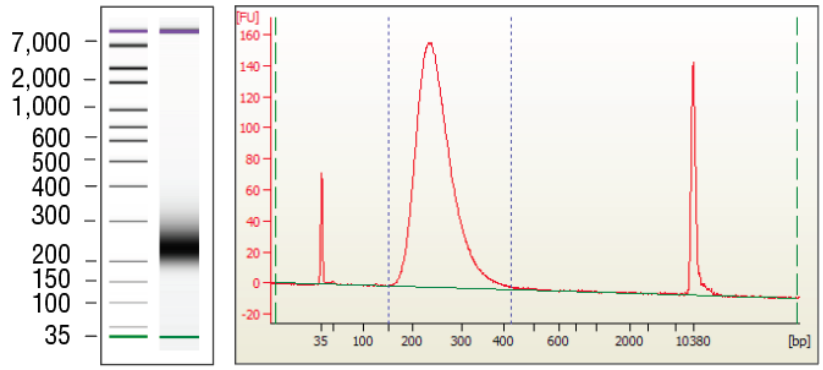
1.6.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube for and store at –20°C.

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 corresponds to adaptor-dimer. NEB recommends repeating Steps 1.6.1 to 1.6.11 if this occurs.

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

Figure 1.1: Example of a library prepared with normal human liver FFPE DNA



Adaptor Trimming Sequences

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

Adaptor Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Adaptor Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Section 2
Protocol for use with NEBNext Ultra DNA Library Prep Kit for Illumina (NEB #E7370)

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 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–1 µg fragmented FFPE DNA.

2.1. NEBNext FFPE Repair

2.1.1. Mix the following components in a sterile nuclease-free tube:

COMPONENT	VOLUME
FFPE DNA	53.5 µl
• (green) FFPE DNA Repair Buffer	6.5 µl
• (green) NEBNext FFPE DNA Repair Mix	2 µl
Total Volume	**62 µl**

2.1.2 Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

2.1.3. Incubate at 20°C for 15 minutes.

2.2. Cleanup Using AMPure XP Beads

2.2.1. Vortex AMPure XP Beads to resuspend. SPRIselect can be used as well.

2.2.2. Add 186 µl (3X) of resuspended AMPure XP Beads to the repair reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

2.2.4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

2.2.5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.2.6. Repeat Step 2.2.5 once.

2.2.7. Air dry beads for up to 5 minutes while the tube/PCR 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.

2.2.8. Remove the tube/plate from the magnet. Elute DNA target by adding 60 µl 0.1X TE to the beads. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature. Put the tube/PCR plate in the magnetic stand until the solution is clear.

2.2.9. Without disturbing the bead pellet, carefully transfer 55.5 µl of the supernatant to a fresh, sterile microfuge tube.

2.3. NEBNext End Prep

2.3.1. Add the following directly to the FFPE repair reaction mixture:

COMPONENT	VOLUME
• (green) End Repair Reaction Buffer	6.5 µl
• (green) End Prep Enzyme Mix	3.0 µl
Repaired DNA from above (Step 2.2.9)	55.5 µl
Total Volume	**65 µl**

2.3.2. Mix by pipetting at least 10 times followed by a quick spin to collect all liquid from the sides of the tube.

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

30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C

2.4 Adaptor Ligation

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If DNA input is < 100 ng, dilute the NEBNext Adaptor for Illumina (provided at 15 µM) 10 fold in 10 mM Tris-HCl pH 7.5 with 10 mM NaCl to a final concentration of 1.5 µM; use immediately.

2.4.1. Add the following components directly to the End Prep reaction mixture:

COMPONENT	VOLUME
• (red) Blunt/TA Ligase Master Mix *	15 µl
• (red) NEBNext Adaptor for Illumina (15 µM)**	2.5 µl
• (red) Ligation Enhancer	1 µl
Total Volume	**83.5 µl**

* Mix the Blunt/TA Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.

** The NEBNext adaptor is provided in the NEBNext oligos kit. NEB has several oligo kit options, which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information.

Note: The Ligation Enhancer and Blunt/TA Ligase Master Mix can be mixed ahead of time and is stable for at least 8 hours at 4°C. Do not add adaptor to a premix in the Adaptor Ligation Step. For best results add adaptor last and mix well immediately or premix adaptor and sample and then add the other ligation reagents.

Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. **(Caution: The Blunt/TA Ligase 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).**

2.4.2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

2.4.3. Incubate at 20°C for 15 minutes in a thermal cycler.

2.4.4. Add 3 µl of • (red or blue) USER Enzyme to the ligation mixture from Step 2.4.3. Mix well and incubate at 37°C for 15 minutes.

Note: This step is for use with NEBNext adaptors only. USER Enzyme can be found in the NEBNext Multiplex Oligos for Illumina.

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A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while performing cleanup of adaptor-ligated DNA. Once thawed, gently mix by inverting the tube several times.

2.5 Cleanup of Adaptor-ligated DNA

2.5.1. Vortex AMPure XP Beads to resuspend.

2.5.2. Add 86.5 µl (1X) resuspended AMPure XP Beads to the 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.

2.5.3. Incubate for at least 5 minutes at room temperature.

2.5.4. Quickly spin the tube and place it on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads).**

2.5.5. Add 200 µl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.5.6. Repeat Step 2.5.5 once.

2.5.7. Air the dry beads for up to 5 minutes while the tube 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.

2.5.8. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding 22 µl of 10 mM Tris-HCl, pH 8.0 or 0.1X TE.

2.5.9. Mix well by pipetting up and down, or on a vortex mixer.

2.5.10. 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 on a magnetic stand. After the solution is clear (about 5 minutes), transfer 20 µl to a new PCR tube for amplification.

2.5.12. Proceed to PCR Amplification.

2.6. PCR-Amplification

Use Option A for any NEBNext oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined.

2.6.1A. Forward and Reverse Primer Supplied Separately

Add the following components to sterile strip tubes:

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 2.5.11)	15 µl
• (blue) Index Primer/i7 Primer*,**	5 µl
• (blue) Universal PCR Primer/i5 Primer*, **	5 µl
• (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 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.

2.6.2A. Proceed to Step 4.2.

2.6.1B. Forward and Reverse Primer Already Combined

Add the following components to sterile strip tubes:

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 2.5.11)	15 µl
• (blue) Index/Universal Primer*	10 µl
• (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 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.

2.6.2B. 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.

2.6.3B. Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	4-15*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* We suggest 4-6 cycles for 1 µg DNA input, 8-9 cycles for 100 ng, 11-12 cycles for 20 ng, and 13-15 cycles for 5 ng. Further optimization of PCR cycle number may be required.

2.6.4B. Proceed to cleanup of PCR Amplification

2.7 Cleanup of PCR Amplification

2.7.1. Vortex AMPure XP Beads to resuspend.

2.7.2. Add 50 µl of resuspended AMPure XP Beads to the PCR reactions (~ 50 µl). Mix well by pipetting up and down at least 10 times.

2.7.3. Incubate for at least 5 minutes at room temperature.

2.7.4. Quickly spin the tube and place it on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution do not discard beads).**

2.7.5. Add 200 µl of 80% ethanol to the PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.7.6. Repeat Step 2.7.5 once.

2.7.7. Air dry the beads for up to 5 minutes while the PCR plate is on the magnetic stand with the lid open.

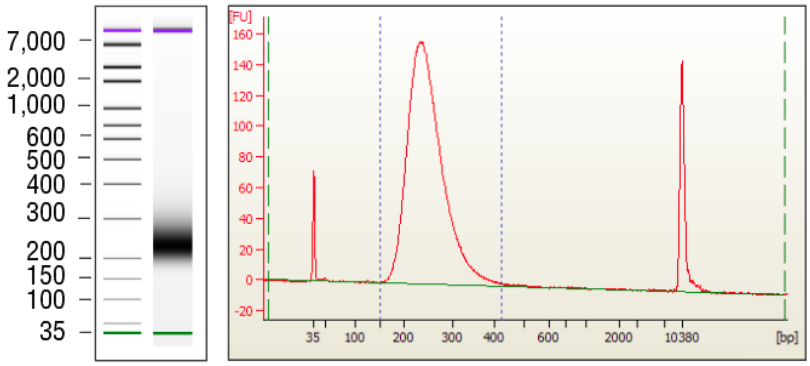
Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

2.7.8. Remove the tube/plate from the magnet. Elute DNA target from beads into 33 µl of 0.1X TE. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and place it on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer 28 µl supernatant to a new PCR tube. Libraries can be stored at –20°C.

2.7.9. Dilute 2–3 µl of the library 5 fold with 10 mM Tris or 0.1X TE and check the size distribution on an Agilent Bioanalyzer® (High Sensitivity Chip).

2.7.10. A sharp peak at 128 bp corresponds to adaptor-dimer. NEB recommends repeating Steps 2.7.1 to 2.7.9 if this occurs.

Figure 2.1: Example of a library prepared with normal human liver FFPE DNA



Section 3
Protocol for use with Other User-supplied Library Construction Reagents

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 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.

3.1. NEBNext FFPE Repair

Input amount should be determined based on recommendations by end user supplied library preparation kits.

3.1.1. Mix the following components in a sterile nuclease-free tube:

COMPONENT	VOLUME
FFPE DNA	53.5 µl
• (green) FFPE DNA Repair Buffer (10X)	6.5 µl
• (green) NEBNext FFPE DNA Repair Mix	2 µl
Total Volume	**62 µl**

3.1.2 Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

3.1.3. Incubate at 20°C for 15 minutes.

3.2. Cleanup Using AMPure XP Beads

3.2.1. Vortex AMPure XP Beads to resuspend. SPRIselect can be used as well.

3.2.2. Add 186 µl (3X) of resuspended AMPure XP Beads to the repair reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

3.2.4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

3.2.5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

3.2.6. Repeat Step 3.2.5 once.

3.2.7. Air dry beads for up to 5 minutes while the tube/PCR 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.

3.2.8. Remove the tube/plate from the magnet. Elute DNA target by adding 40 µl 0.1X TE to the beads. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature. Put the tube/PCR plate in the magnetic stand until the solution is clear.

3.2.9. Without disturbing the bead pellet, carefully transfer 32 µl of the supernatant to a fresh, sterile microfuge tube.

3.2.10. Proceed to library construction using end-user supplied reagents.

Kit Components

NEB #M6630S Table of Components

NEB #	PRODUCT	VOLUME
M6630S	NEBNext FFPE DNA Repair Mix	0.048 ml
E6622A	NEBNext FFPE DNA Repair Buffer (10X)	0.156 ml

NEB #M6630L Table of Components

NEB #	PRODUCT	VOLUME
M6630L	NEBNext FFPE DNA Repair Mix	0.192 ml
E6622AA	NEBNext FFPE DNA Repair Buffer (10X)	0.624 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	
2.0	Remove protocol for use with NEBNext High-Fidelity 2X PCR Master Mix.	
2.1	Updated title of 2.8B Protocol to "PCR Amplification"	
3.0	Protocol updated to include NEB #E7710 and NEB #E7730. Section C in the PCR setup step was removed because all of the 25 µM primers are now expired.	
4.0	Change chapter one to the protocol for use with E7645. Update protocols in Chapter 2, 3 and 4.	4/18
5.0	Change title of Chapter 2 protocol. Create "Kit Component – Table of Components" for small and large size kits. Delete individual component information pages.	9/18
6.0	Change to new manual format.	1/20
7.0	Update protocol and required materials not included.	12/22

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