NEBNext® FFPE DNA Repair v2 Module

NEB #E7360S/L

24/96 reactions

Version 1.0_8/21

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The NEBNext FFPE DNA Repair v2 Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7360S) and 96 reactions (NEB #E7360L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (lilac) NEBNext FFPE DNA Repair Mix v2
- (lilac) NEBNext Thermolabile Proteinase K
- (lilac) NEBNext FFPE DNA Repair Buffer v2

Required Materials Not Included

- NEBNext Ultra II DNA Library Prep Kit (NEB #E7645S, #E7645L, #E7103S or #E7103L) for Illumina, or other
- 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
- DNA LoBind® Tubes (Eppendorf #022431021)
- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- NEBNext Multiplex Oligos for Illumina (www.neb.com/oligos)
- Magnetic rack/stand (NEB #S1515, 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 NEB #E7935S/L

Overview

The NEBNext FFPE DNA Repair v2 Module contains enzymes and buffers that are ideal to repair FFPE DNA or other damaged DNA samples 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 v2 Module 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 II DNA Library Prep Kit for Illumina, and is 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, #E7103) and all NEBNext Multiplex Oligo Kits except Unique Dual Index UMI DNA Adaptors (NEB #E7395)

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 – 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 nuclease-free tube on ice (55 µl final volume):

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

1.1.2 Mix by pipetting 10 times followed by 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. Mix by pipetting 10 times followed by 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

1.2. 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 1.1.6 on ice.

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

Follow this protocol if using the NEBNext Adaptor for Illumina supplied with the following Non-indexed Adaptor NEBNext Multiplex Oligos for Illumina (e.g., NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600, #E7780, #E6640, #E6442, #E6444, #E6446)

1.3.1. Determine whether 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 in Adaptor Dilution Buffer or Tris/NaCl, pH 8.0 as indicated in Table 1.3a.

Table 1.3a 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 on ice:

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 1.2.3)	60 µl
● (red) 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 oligos 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. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

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 1.3.7 are only required for use with NEBNext Adaptors supplied in all NEBNext Multiplex Oligo kits except indexed/UMI Adaptor NEB #E7395. USER enzyme is supplied with the NEBNext Multiplex Oligos for Illumina.

1.3.6. Mix well by pipetting 10 times. Perform a quick spin to collect all liquid from the sides of the tube.

1.3.7. 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

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

1.4. 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. 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 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.

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 multiplex oligo kit where the forward and reverse primers are supplied in separate tubes (e.g., NEB #E7335, #E7500, #E7710, #E7730, #E7600, #E7780).

Use Option B for any NEBNext multiplex oligo kit where the forward and reverse (i7 and i5) primers are combined in a 96-well plate format (e.g., NEB #E6609, #E6640, #E6442, #E6444, #E6446).

1.5.1A. Forward and Reverse Primer Not Already Combined

Add the following components to a sterile strip tube on ice:

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) 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 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	4-13*
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**	3-5	5-7
100 ng	5-7	8-10
50 ng	8-10	9-11
10 ng	10-12	-
5 ng	11-13	-

* The number of cycles may 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.

***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.

1.6. 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.

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 of the liquid from 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.

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.

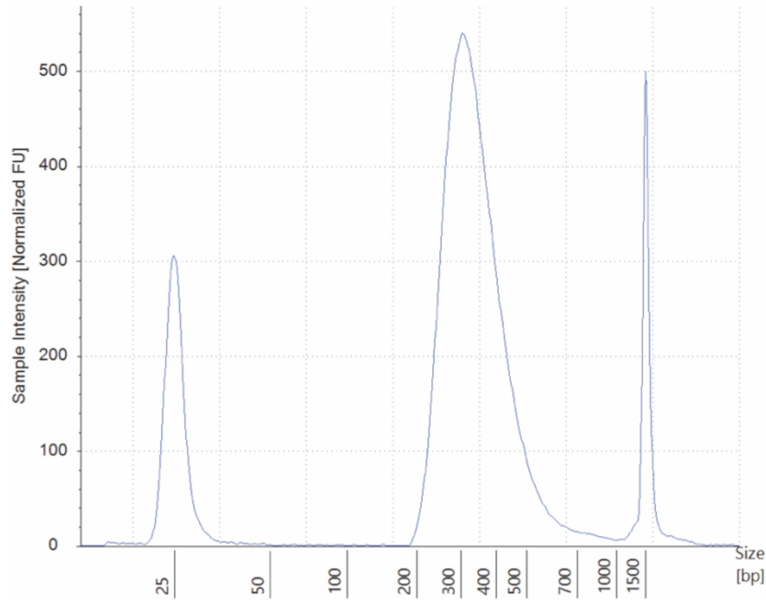
1.6.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube for and store at –20°C.

1.6.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip or Agilent TapeStation High Sensitivity D1000 screentape. 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 TapeStation or Agilent Bioanalyzer corresponds to adaptor-dimer. We recommend 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 (DIN 2.0) on Agilent TapeStation High Sensitivity D1000 Assay



Section 2
Protocol for use with NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB #E7645, #E7103) and NEBNext Multiplex Oligos for Illumina Unique Dual Index UMI DNA Adaptors (NEB #E7395)

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 –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 on ice (55 µl final volume):

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

2.1.2 Mix by pipetting 10 times followed by 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 • (lilac) NEBNext Thermolabile Proteinase K on ice.

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

2.1.6. 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

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 2.1.6 on ice.

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:

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

2.3. NEBNext Ultra II Adaptor Ligation using Indexed/UMI Adaptor Multiplex Oligo Kits (e.g. NEB #E7395)

2.3.1 Determine whether 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 UMI Adaptor Dilution Buffer (supplied with e.g., NEB #E7395) as indicated in Table 2.3a.

Table 2.3A. NEBNext Unique Dual Index UMI Adaptor Dilution

INPUT	ADAPTOR DILUTION (VOLUME ADAPTOR: TOTAL VOLUME)	WORKING ADAPTOR CONCENTRATION
250 ng	2-fold (1:2)	10 µM
100 ng	5-fold (1:5)	4 µM
50 ng	10-fold (1:10)	2 µM
25 ng	10-fold (1:10)	2 µM
10 ng	25-fold (1:25)	0.8 µM
5 ng	25-fold (1:25)	0.8 µM

2.3.2 Add the following components directly to the repaired/end-prepped DNA on ice:

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 2.2.3)	60 µl
• (red) NEBNext UDI-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 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 are 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. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off.

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.

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

2.4.2. Add 84 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 from 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 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.

2.4.7. 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 containing 45 µl (0.9X) SPRIselect or NEBNext Sample Purification Beads.

2.4.12. 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.

2.4.13. 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 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.

2.4.17. 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.

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

2.5. PCR-enrichment of Adaptor-ligated DNA

2.5.1 Add the following components to a sterile strip tube on ice:

COMPONENT	VOLUME (µl) PER REACTION
Adaptor Ligated DNA Fragments (Step 2.4.21)	20 µl
• (blue) NEBNext Ultra II Q5 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 (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 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	4-13*
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**	4-5	6-9
100 ng	5-7	8-11
50 ng	8-10	-
10 ng	10-12	-
5 ng	11-13	-

* 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 SPRIselect or NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. AMPure XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use. These volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

2.6.1. Vortex SPRIselect or 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 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.

2.6.3. 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 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.

2.6.7. 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.

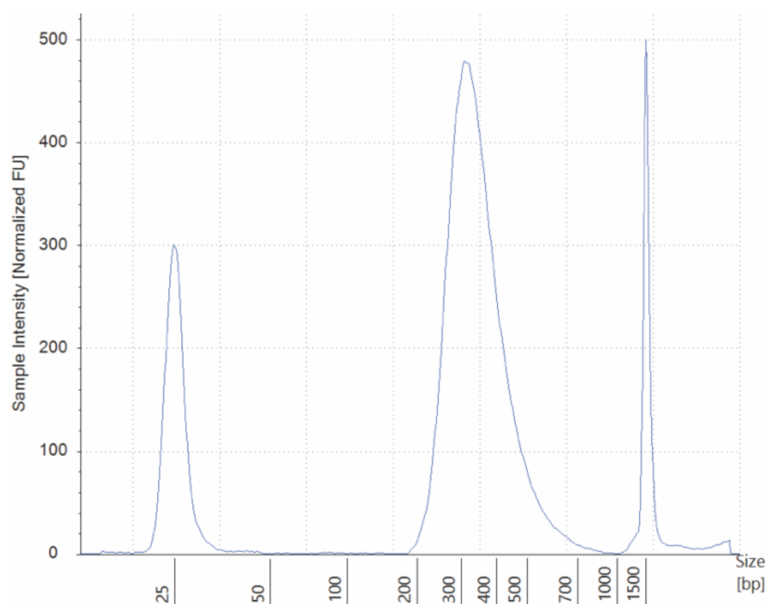
2.6.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 µl to a new PCR tube for and store at –20°C.

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

2.6.13. A sharp peak at 146 bp on Agilent TapeStation or Agilent Bioanalyzer corresponds to adaptor-dimer. We recommend repeating Steps 2.6.1 to 2.6.11 if this occurs.

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

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



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 DNA Repair v2

Input amount should be determined based on recommendations by end user-supplied library preparation kits. The NEBNext FFPE DNA Repair v2 Module was validated for FFPE DNA inputs ranging from 5 ng – 250 ng. If acoustic shearing (e.g., Covaris) is used, proceed with NEBNext FFPE DNA Repair v2 after shearing. Alternatively, FFPE DNA can be repaired upstream of other library workflows that include enzymatic fragmentation.

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

COMPONENT	VOLUME
FFPE DNA	51 µl
• (lilac) FFPE DNA Repair Buffer v2	7 µl
• (lilac) NEBNext FFPE DNA Repair Mix v2	2 µl
Total Volume	60 µl

3.1.2 Bring reaction volume to 60 µl with nuclease-free water, 0.1X TE, or 1X TE.

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

3.1.4. 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

3.2. Cleanup Using SPRI Beads

3.2.1. 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.

3.2.2. Add 90 µl (1.5X) of resuspended beads to the repair reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

3.2.3. Incubate for 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 for a total of two washes.

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 32 µl 0.1X TE to the beads (**Note: please consult manual of user-supplied library preparation kit for appropriate elution buffer composition and volume).** 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 30 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 #E7360S 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

NEB #E7360L 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

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	

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