

NEBNext UltraShear™ FFPE DNA Library Prep Kit

NEB #E6655

24/96 reactions

Version 2.1_10/26

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* Please review **Indexing Strategy** row found in the [NEBNext Multiplex Oligo Selection Chart](#).

The NEBNext UltraShear FFPE DNA Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E6655S) and 96 reactions (NEB #E6655L). All reagents should be stored at -20°C. Colored bullets represent the color of the tube cap containing the reagent.

Package 1: Store at -20°C

- (lilac) NEBNext FFPE DNA Repair Mix v2
- (lilac) NEBNext Thermolabile Proteinase K
- (yellow) NEBNext UltraShear Reaction Buffer
- (yellow) NEBNext UltraShear
- (green) 500 mM DTT
- (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

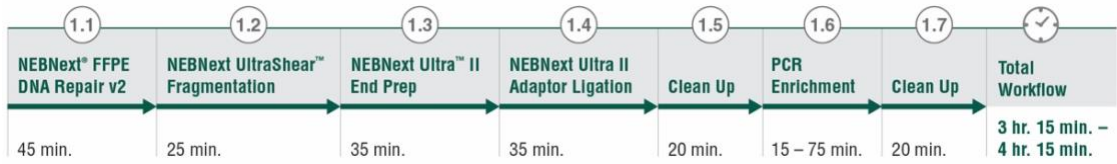
Required Materials Not Included

- 80% Ethanol
- Nuclease-free Water
- 1X TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 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
- Thermal cycler
- Magnetic rack/stand (NEB #S1515S, Alpaqua® cat. #A001322, or equivalent)
- Agilent® Bioanalyzer® or TapeStation® and associated reagents and consumables
- Adaptor Dilution Buffer NEB #B1430S (10 mM Tris-HCl, 10 mM NaCl, pH 7.5 @ 25°C) or NEBNext Unique Dual Index UMI Adaptor Dilution Buffer (supplied with NEBNext Multiplex Oligos Unique Dual Index UMI Adaptor DNA Sets – NEB #E7395/E7874/E7876/E7878)

Overview

The NEBNext UltraShear FFPE DNA Library Prep Kit contains the enzymes and buffers required to convert 5 to 250 ng of FFPE DNA into high quality libraries for next generation sequencing. The kit includes a mix of enzymes optimized to repair FFPE DNA, an enzymatic fragmentation solution ideal for challenging samples such as FFPE DNA, and library prep reagents with a fast protocol and minimal hands-on time.

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



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* (Dual Index, or Unique Dual Index Primers)

* Please see the [NEBNext Multiplex Oligo Selection Chart](#) for the full list of compatible multiplex oligos.

This section is for use with non-indexed adaptors and PCR primers that add sample indices.

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 FFPE DNA. We recommend that DNA be diluted in 1X TE. Alternatively, samples can be diluted with 0.1X TE, 10 mM Tris pH 8.0, or nuclease-free water.

1.1. NEBNext FFPE DNA Repair v2

1.1.1. Ensure that the NEBNext UltraShear Reaction Buffer is completely thawed and vortexed before use.

1.1.2. Add the following components to a 0.2 ml thin wall PCR tube on ice (38 µl final volume):

COMPONENT	VOLUME
FFPE DNA	29 µl
• (yellow) NEBNext UltraShear Reaction Buffer	7 µl
• (lilac) NEBNext FFPE DNA Repair Mix v2	2 µl
Total Volume	38 µl

1.1.3. Set a 100 µl or 200 µl pipette to 25 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.

1.1.4. 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

1.1.5. Add 2 µl of • (lilac) NEBNext Thermolabile Proteinase K to the reaction on ice.

1.1.6. Set a 100 µl or 200 µl pipette to 25 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.

1.1.7. 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

1.1.8. Place samples on ice and proceed to NEBNext UltraShear Enzymatic Fragmentation.

1.2. NEBNext UltraShear Enzymatic Fragmentation

1.2.1. Vortex the • (yellow) NEBNext UltraShear for 5–8 seconds prior to use and place on ice.

1.2.2. Add 4 µl • (yellow) NEBNext UltraShear to repaired FFPE DNA from Step 1.1.8. on ice.

1.2.3. Vortex the reaction for 5 seconds and perform a quick spin to collect all liquid from the sides of the tube.

- 1.2.4. Place in a thermal cycler, with the heated lid set to 75°C, and run the following program:
 5 minutes at 37°C
 15 minutes at 65°C
 Hold at 4°C
- 1.2.5. Place samples on ice and proceed to End Prep.

1.3. NEBNext Ultra II End Prep

- 1.3.1. Add the following components directly to the end-prepped DNA on ice.

COMPONENT	VOLUME
Fragmented DNA from Step 1.2.5.	44 µl
• (green) 500 mM DTT	2 µl
• (green) Ultra II End Prep Enzyme Mix	3 µl
Total Volume	49 µl

- 1.3.2. Set a 100 µl or 200 µl pipette to 35 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.
- 1.3.3. 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
- 1.3.4. Place samples on ice and proceed to Adaptor Ligation.

1.4. 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

* Please see the [NEBNext Multiplex Oligo Selection Chart](#) for the full list of compatible oligos.

- 1.4.1. Determine which adaptor dilution is necessary.
Based on input DNA amount (ng), dilute the NEBNext Adaptor for Illumina in NEBNext Adaptor Dilution Buffer (NEB# B1430) or 10 mM Tris-HCl, 10 mM NaCl, pH 7.5 @ 25°C, as indicated in Table 1.4.1.

Table 1.4.1. 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

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.

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

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 1.3.4)	49 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	82.5 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 NEBNext 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.

- 1.4.3. Set a 100 µl or 200 µl pipette to 60 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).**
- 1.4.4. Place in a thermal cycler with the heated lid off, and run the following program:
20°C for 15 minutes
Hold at 4°C
- 1.4.5. Add 3 µl of • (red) USER® Enzyme to the ligation mixture from Step 1.4.4.
Note: Steps 1.4.5. through 1.4.6. are only required for use with NEBNext Adaptors supplied in all NEBNext Multiplex Oligo kits, except NEBNext Unique Dual Index UMI Adaptors DNA Sets. USER enzyme is supplied with the NEBNext Multiplex Oligos for Illumina.
- 1.4.6. 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

1.5. 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.

- 1.5.1. Vortex NEBNext Sample Purification Beads to resuspend.
- 1.5.2. Add **77 µl (0.9X)** resuspended beads to the Adaptor Ligation reaction (85.5 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.
- 1.5.3. Incubate samples on bench top for 5 minutes at room temperature.
- 1.5.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 1.5.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads)**.
- 1.5.6. 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.
- 1.5.7. Repeat Step 1.5.6. once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

- 1.5.8. Air 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.**
- 1.5.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **17 µl of 0.1X TE**.
- 1.5.10. Mix well by pipetting 10 times or by vortexing for 3–5 seconds on high. 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.
- 1.5.11. 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.

1.6. PCR enrichment of Adaptor-ligated DNA



PCR Setup Options A and B:

Use Option A (Step 1.6.1A.): 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 (Step 1.6.1B.): 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.

1.6.1A. Forward and Reverse Primer Not Already Combined

COMPONENT	VOLUME (µl) PER REACTION
Adaptor-Ligated DNA Fragments (Step 1.5.11.)	15 µl
• (blue) Index Primer/i7 Primer*, **	5 µl
• (blue) Universal PCR Primer/i5 Primer*, **	5 µl
• (blue) NEBNext MSTC FFPE Master Mix	25 µl
Total Volume	50 µl

1.6.1B. Forward and Reverse Primer Already Combined

COMPONENT	VOLUME (µl) PER REACTION
Adaptor-Ligated DNA Fragments (Step 1.5.11.)	15 µl
NEBNext i5/i7 primers combined in 96 well plate format*	10 µl
• (blue) NEBNext MSTC FFPE 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.

- 1.6.2. 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.

- 1.6.3. Place the tube on a thermal cycler with lid temperature set to 105°C and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	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 1.6.4 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples.

- 1.6.4. 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	7–10
100 ng	6–8	8–11
50 ng	8–12	–
25 ng	9–13	–
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.

1.7. Cleanup of PCR Amplification

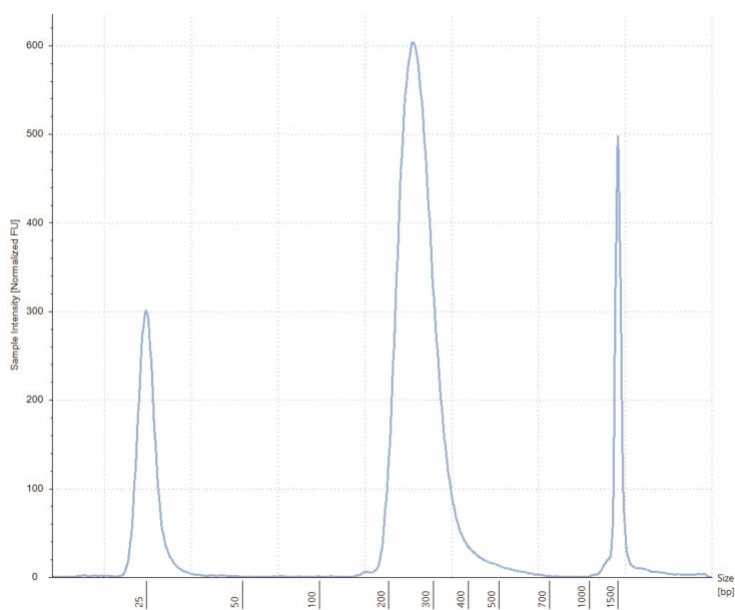
Note: The volumes of NEBNext Sample Purification Beads provided here are for use with the sample contained in the exact buffer at this step. These volumes may not work properly for a cleanup at a different step in the workflow. For cleanups of samples contained in different buffer conditions, the volumes may need to be experimentally determined.

- 1.7.1. Vortex NEBNext Sample Purification Beads to resuspend.
- 1.7.2. 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.
- 1.7.3. Incubate samples on bench top for 5 minutes at room temperature.
- 1.7.4. 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.
- 1.7.5. 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**).
- 1.7.6. 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.
- 1.7.7. Repeat Step 1.7.6. once for a total of 2 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.
- 1.7.8. 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.

- 1.7.9. 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).
- 1.7.10. Mix well by pipetting up and down 10 times or by vortexing for 3–5 seconds on high. 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.
- 1.7.11. 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.
- 1.7.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip or Agilent TapeStation HSD1000 screentape. The sample may need to be diluted before loading.
- 1.7.13. 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 1.7.1. to 1.7.11. 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 1.7.1: 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 the full list of compatible multiplex oligos. This section is for use with indexed adaptors and universal PCR primers.

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 FFPE DNA. We recommend that DNA be diluted in 1X TE. Alternatively, samples can be diluted with 0.1X TE, 10 mM Tris pH 8.0, or nuclease-free water.

2.1. NEBNext FFPE DNA Repair v2

- 2.1.1. Ensure that the NEBNext UltraShear Reaction Buffer is completely thawed and vortexed before use.
- 2.1.2. Mix the following components in a sterile nuclease-free tube (38 µl final volume):

COMPONENT	VOLUME
FFPE DNA	29 µl
• (yellow) NEBNext UltraShear Reaction Buffer	7 µl
• (lilac) NEBNext FFPE DNA Repair Mix v2	2 µl
Total Volume	38 µl

- 2.1.3. Set a 100 µl or 200 µl pipette to 25 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.
- 2.1.4. 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
- 2.1.5. Add 2 µl of • (lilac) NEBNext Thermolabile Proteinase K.
- 2.1.6. Set a 100 µl or 200 µl pipette to 25 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.
- 2.1.7. 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
- 2.1.8. Place samples on ice and proceed to UltraShear Enzymatic Fragmentation.

2.2. NEBNext UltraShear Enzymatic Fragmentation

- 2.2.1. Vortex the • (yellow) NEBNext UltraShear for 5–8 seconds prior to use and place on ice.
- 2.2.2. Add 4 µl of • (yellow) NEBNext UltraShear to the repaired FFPE DNA from Step 2.1.8. on ice.
- 2.2.3. Vortex the reaction for 5 seconds and perform a quick spin to collect all liquid from the sides of the tube.

- 2.2.4. Place in a thermal cycler, with the heated lid set to 75°C, and run the following program:
 5 minutes at 37°C
 15 minutes at 65°C
 Hold at 4°C
- 2.2.5. Place samples on ice and proceed to End Prep.

2.3. NEBNext Ultra II End Prep

- 2.3.1. Add the following components to a 0.2 ml thin wall PCR tube on ice

COMPONENT	VOLUME
Fragmented DNA from Step 2.2.5.	44 µl
• (green) 500 mM DTT	2 µl
• (green) Ultra II End Prep Enzyme Mix	3 µl
Total Volume	49 µl

- 2.3.2. Set a 100 µl or 200 µl pipette to 35 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.

- 2.3.3. 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

- 2.3.4. Place samples on ice and proceed to Adaptor Ligation.

2.4. NEBNext Ultra II Adaptor Ligation using NEBNext Multiplex Oligos for Illumina Index Adaptor DNA Sets*

* Please see the [NEBNext Multiplex Oligo Selection Chart](#) for the full list of compatible multiplex oligos.

- 2.4.1 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 2.4.1.

Table 2.4.1. 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

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

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 2.3.4)	49 µl
Diluted NEBNext UMI Adaptor*	2.5 µl
• (red) NEBNext Ultra II Ligation Master Mix**	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	82.5 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 is stable for at least 8 hours at 4°C. Do not premix the adaptor with the Ligation Master Mix and Ligation Enhancer.

- 2.4.3. Set a 100 µl or 200 µl pipette to 60 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).**
- 2.4.4. Place in a thermal cycler with the heated lid off, and run the following program:
20°C for 15 minutes
Hold at 4°C.

2.5. 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 sample purification bead additions according to the protocol below.

- 2.5.1. Vortex NEBNext Sample Purification Beads to resuspend.
- 2.5.2. Add **58 µl (0.7X)** resuspended beads to the Adaptor Ligation reaction (82.5 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.
- 2.5.3. Incubate samples on bench top for 5 minutes at room temperature.
- 2.5.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 2.5.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads)**.
- 2.5.6. Remove the tube or plate from the magnetic stand and add **50 µl 0.1X TE** to the sample purification beads.
- 2.5.7. Add **45 µl (0.9X)** resuspended sample purification beads. Mix well by pipetting up and down at least 10 times or by vortexing for 3–5 seconds on high. Be careful to expel all the liquid out of the tip during the last mix.
- 2.5.8. Incubate samples on bench top for 5 minutes at room temperature.
- 2.5.9. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 2.5.10. 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)**.
- 2.5.11. 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.
- 2.5.12. Repeat Step 2.5.11. once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

2.5.13. 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.

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

2.5.15. Mix well by pipetting 10 times or by vortexing for 3–5 seconds on high. 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.

2.5.16. 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.

2.6. PCR-enrichment of Adaptor-ligated DNA

2.6.1 PCR reaction setup:

COMPONENT	VOLUME (µl) PER REACTION
Adaptor-Ligated DNA Fragments (Step 2.5.16.)	20 µl
• (blue) NEBNext Primer Mix*	5 µl
• (blue) NEBNext MSTC FFPE Master Mix	25 µl
Total Volume	50 µl

* NEBNext Primer Mix is supplied with NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors, DNA Sets – NEB #E7395/E7874/E7876/E7878)

2.6.2. 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.

2.6.3. Place the tube on a thermal cycler with lid temperature set to 105°C and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	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 2.6.4 are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples.

2.6.4. 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	7–10
100 ng	5–8	8–11
50 ng	8–12	–
25 ng	9–13	–
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.

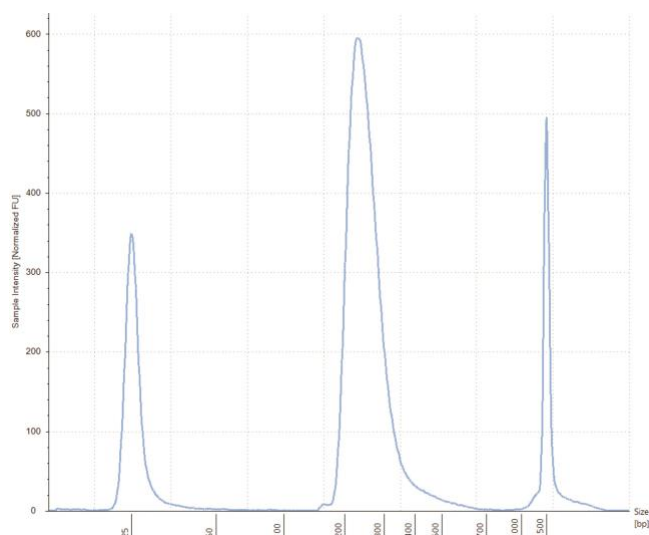
** Target enrichment input amounts vary by vendor often ranging from 200 ng to 1500 ng of PCR-amplified library. Cycle numbers may need to be optimized to achieve specific input requirements of a user-supplied target enrichment kit, while minimizing over-cycling artifacts.

2.7. 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 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.

- 2.7.1. Vortex NEBNext Sample Purification Beads to resuspend.
- 2.7.2. 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.
- 2.7.3. Incubate samples on bench top for 5 minutes at room temperature.
- 2.7.4. 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.
- 2.7.5. 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**).
- 2.7.6. 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.
- 2.7.7. Repeat Step 2.7.6. once for a total of 2 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.
- 2.7.8. 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.
- 2.7.9. 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).
- 2.7.10. Mix well by pipetting up and down 10 times or by vortexing for 3–5 seconds on high. 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.
- 2.7.11. 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.
- 2.7.12. 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.
- 2.7.13. A sharp peak at 146 bp on Agilent Bioanalyzer or TapeStation corresponds to adaptor-dimer. We recommend repeating Steps 2.7.1. to 2.7.11. 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 2.7.1: 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 Sample Quality Agnostic Workflow (DIN 1-9)

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 DNA. We recommend that DNA be diluted in 1X TE.

3.1. NEBNext FFPE DNA Repair v2

- 3.1.1. Ensure that the NEBNext UltraShear Reaction Buffer is completely thawed and vortexed before use.
- 3.1.2. Add the following components to a 0.2 ml thin wall PCR tube on ice (38 µl final volume):

COMPONENT	VOLUME
FFPE DNA	29 µl
● (yellow) NEBNext UltraShear Reaction Buffer	7 µl
● (lilac) NEBNext FFPE DNA Repair Mix v2	2 µl
Total Volume	38 µl

- 3.1.3. Set a 100 µl or 200 µl pipette to 25 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.
- 3.1.4. Place in a thermal cycler, with a heated lid set to 50°C, and run the following program:
15 min at 37°C
Hold at 4°C
- 3.1.5. Add 2 µl of ● (lilac) NEBNext Thermolabile Proteinase K to the reaction on ice.
- 3.1.6. Set a 100 µl or 200 µl pipette to 25 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.
- 3.1.7. Place in a thermal cycler, with a heated lid set to 75°C, and run the following program:
15 min at 37°C
5 min at 65°C
Hold at 4°C
- 3.1.8. Place samples on ice and proceed to NEBNext UltraShear Enzymatic Fragmentation.

3.2. NEBNext UltraShear Enzymatic Fragmentation

- 3.2.1. Vortex the ● (yellow) NEBNext UltraShear for 5–8 seconds on high speed prior to use and place on ice.

Table 3.2.1. Fragmentation time



Determine required fragmentation time based on desired sequenced insert size and adaptor used.

	NEBNext NON-INDEXED ADAPTOR		NEBNext UMI UDI ADAPTOR	
Sequenced Insert Size	150 bp	350 bp*	150 bp	350 bp*
Fragmentation Time	30 min	20 min	30 min	20 min

*may not be possible to achieve with highly damaged DNA

- 3.2.2. Add 4 µl (yellow) NEBNext UltraShear to repaired FFPE DNA from Step 3.1.8. on ice.
- 3.2.3. Vortex the reaction for 5 seconds on high speed and perform a quick spin to collect all liquid from the sides of the tube.
- 3.2.4. Place in a thermal cycler, with the heated lid set to 75°C, and run the following program:
 ___ min at 37°C (according to Table 3.2.1. above)
 15 min at 65°C
 Hold at 4°C
- 3.2.5. Place samples on ice and proceed to End Prep.

3.3. NEBNext Ultra II End Prep

- 3.3.1. Assemble the End Prep reaction on ice by adding the following components to the UltraShear reaction from Step 3.2.5.

COMPONENT	VOLUME
Fragmented DNA from Step 3.2.5.	44 µl
• (green) 500 mM DTT	2 µl
• (green) Ultra II End Prep Enzyme Mix	3 µl
Total Volume	49 µl

- 3.3.2. Set a 100 µl or 200 µl pipette to 35 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.
- 3.3.3. 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
- 3.3.4. Place samples on ice and proceed to Adaptor Ligation.

3.4. NEBNext Ultra II Adaptor Ligation

* Please see the www.neb.com/oligos for the full list of compatible oligos.

- 3.4.1. Determine which adaptor dilution is necessary.



Based on input DNA amount (ng), dilute the NEBNext non-indexed Adaptor for Illumina in NEBNext Adaptor Dilution Buffer NEB #B1430S (10 mM Tris-HCl, 10 mM NaCl, pH 7.5 @ 25°C). If using NEBNext UMI UDI Adaptor in dilute in NEBNext UMI Adaptor Dilution Buffer (supplied with NEB #E7395 etc.) as indicated in Table 3.4.1.

Table 3.4.1 Adaptor Dilution

INPUT	NEBNext NON-INDEXED ADAPTOR DILUTION	NEBNext UMI UDI ADAPTOR DILUTION
101–250 ng	2-fold (1:2)	2-fold (1:2)
25–100 ng	10-fold (1:10)	10-fold (1:10)
5–24 ng	25-fold (1:25)	25-fold (1:25)

Note: Due to the varying degrees of quality of some types of DNA, such as 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. Higher quality samples may obtain better library yield if higher adaptor concentration is used.

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

COMPONENT	VOLUME
End Prep Reaction Mixture (Step 3.3.4)	49 µl
• (red) Diluted NEBNext Non-Indexed Adaptor for Illumina or NEBNext UMI UDI Adaptor (plate format)	2.5 µl
• (red) NEBNext Ultra II Ligation Master Mix*	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	82.5 µl

* Mix the NEBNext 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.

3.4.3. Set a 100 µl or 200 µl pipette to 60 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. **(Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance).**

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

20°C for 15 minutes

Hold at 4°C

3.4.5. Add 3 µl of • (red or blue) USER® Enzyme to the ligation mixture from Step 3.4.4.

Note: Steps 3.4.5 through 3.4.6 are only required for use with NEBNext Non-indexed Adaptors supplied in all NEBNext Multiplex Oligo kits, except NEBNext UMI UDI DNA Adaptors. The USER enzyme is supplied with these NEBNext Multiplex Oligos Index Primers for Illumina.

3.4.6. 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 min

Hold at 4°C

3.5. Cleanup of Adaptor-ligated DNA



SPRI Cleanup Options A and B:

Use **Option A (Step 3.5A)**: to achieve the maximum library yield and complexity.

Use **Option B (Step 3.5B)**: should be followed to achieve a specific sequenced insert size.

3.5A: Protocol for maximum yield and library complexity



Determine bead volumes and elution volumes based on adaptor used.

For samples of DNA Integrity Score (DIN) <3, this protocol is recommended for achieving maximum library complexity. Section 5B can be used for samples of DIN 1-9 but significant sample loss may be observed for samples that are already highly degraded.

Table 3.5A.1.

	NEBNext NON-INDEXED ADAPTOR	NEBNext UMI UDI ADAPTOR
SPRI Bead Volume	77 µl	74 µl
Elution Volume (0.1X TE)	17 µl	22 µl
Volume used for PCR	15 µl	20 µl

3.5A.1. Vortex NEBNext Sample Purification Beads to resuspend. SPRIselect or AMPure XP beads can be used as well. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

- 3.5A.2. Add ___ μ l resuspended beads according to Table 3.5A.1. to the Adaptor Ligation reaction from Step 3.4.4. or 3.4.6., depending on which adaptor was used. 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.
- 3.5A.3. Incubate samples on bench top for 5 minutes at room temperature.
- 3.5A.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 3.5A.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**).
- 3.5A.6. Add 200 μ l 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.
- 3.5A.7. Repeat Step 3.5A.6. once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.
- 3.5A.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.
- 3.5A.9. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding ___ μ l of 0.1X TE according to Table 3.5A.1.
- 3.5A.10. Mix well by pipetting 10 times or by vortexing for 3–5 seconds on high. 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.
- 3.5A.11. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer ___ μ l (volume used for PCR) to a new PCR tube according to Table 3.5A.1.

3.5B: Protocol to achieve specific sequenced insert sizes



Determine bead and elution volumes based on desired sequenced insert size and adaptor used.

Note: for samples of DNA Integrity Score (DIN) <3, library yield and complexity loss may be observed when selecting for a larger sequenced insert size. For samples with DIN >6, sequenced insert sizes of 150 bp or 350 bp were observed using the following protocol parameters. For larger insert sizes, bead volumes can be further reduced. For samples of DIN <6 the sequenced insert size will depend on the level of fragmentation and crosslinking of the input sample.

Table 3.5B.1.

	NEBNext NON-INDEXED ADAPTOR		NEBNext UMI UDI ADAPTOR	
Sequenced Insert Size	150 bp	350 bp	150 bp	350 bp
SPRI Bead Volume	70 μ l	45 μ l	55 μ l	40 μ l
Elution Volume (0.1X TE)	17 μ l	17 μ l	22 μ l	22 μ l
Volume used for PCR	15 μ l	15 μ l	20 μ l	20 μ l

- 3.5B.1. Vortex NEBNext Sample Purification Beads to resuspend. SPRIselect or AMPure XP beads can be used as well. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.
- 3.5B.2. Add **30 μ l** of nuclease free water to the Adaptor Ligation reaction from Step 3.4.4. or 3.4.6., depending on which adaptor was used.
- 3.5B.3. Add ___ μ l resuspended SPRI beads according to Table 3.5B.1. 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.
- 3.5B.4. Incubate samples on bench top for 5 minutes at room temperature.
- 3.5B.5. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

- 3.5B.6. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard beads**).
- 3.5B.7. 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.
- 3.5B.8. Repeat Step 3.5B.7. once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.
- 3.5B.9. 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.
- 3.5B.10. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **___ µl of 0.1X TE according to Table 3.5B.1.**
- 3.5B.11. Mix well by pipetting 10 times or by vortexing for 3–5 seconds on high. 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.
- 3.5B.12. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer **___ µl (Volume used for PCR) to a new PCR tube according to Table 3.5B.1.**

3.6. PCR-enrichment of Adaptor-ligated DNA



PCR Setup Options A and B:

Use Option A (Step 3.6.1A.): 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).

Use Option B (Step 3.6.1B.): for any NEBNext Unique Dual Index UMI Adaptor kits where the non-indexed forward and reverse (p5 and p7) primers are combined in a single tube as the NEBNext Primer Mix. Primers are supplied at 40 µM combined (20 µM each).

3.6.1A. Reaction setup for NEBNext Non-Indexed Adaptor

COMPONENT	VOLUME (µl) PER REACTION
Adaptor-Ligated DNA Fragments (Step 3.5A.11)	15 µl
NEBNext i5/i7 primers combined in 96 well plate format*	10 µl
• (blue) NEBNext MSTC FFPE 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.

3.6.1B. Reaction setup for NEBNext UMI UDI Adaptor

COMPONENT	VOLUME (µl) PER REACTION
Adaptor-Ligated DNA Fragments (Step 3.5B.12)	20 µl
NEBNext Primer Mix*	5 µl
• (blue) NEBNext MSTC FFPE Master Mix	25 µl
Total Volume	50 µl

* NEBNext Primer Mix is supplied with NEBNext UMI UDI Adaptors

- 3.6.2. 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.

- 3.6.3. Place the tube on a thermal cycler with lid temperature set to 105°C and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	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 3.6.4. are to be seen as a starting point to determine the number of PCR cycles best for standard library prep samples.

3.6.4. Recommended Number of PCR Cycles*

DNA INPUT	STANDARD RECOMMENDED NUMBER OF PCR CYCLES	RECOMMENDED NUMBER OF PCR CYCLES FOR TARGET ENRICHMENT**
250 ng	5–7	7–10
100 ng	6–8	8–11
50 ng	8–12	–
25 ng	9–13	–
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.

3.7. Cleanup of PCR Amplification



SPRI Cleanup Options A and B:

Use Option A (Step 3.7A): to achieve the maximum library yield and complexity.

Use Option B (Step 3.7B): should be followed to achieve a specific sequenced insert size.

3.7A: Protocol for maximum yield and library complexity



Determine cleanup protocol based on desired sequenced insert size and adaptor used.

- 3.7A.1. Vortex NEBNext Sample Purification Beads to resuspend. SPRIselect or AMPure XP beads can be used as well. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.
- 3.7A.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.
- 3.7A.3. Incubate samples on bench top for 5 minutes at room temperature.
- 3.7A.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 3.7A.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**).
- 3.7A.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.

- 3.7A.7. Repeat Step 3.7A.6. once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.
- 3.7A.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.
- 3.7A.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 nuclease free water can be used, if required by downstream application (e.g. target enrichment).
- 3.7A.10. Mix well by pipetting up and down 10 times or by vortexing for 3–5 seconds on high. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 3.7A.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.
- 3.7A.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip or Agilent TapeStation HSD1000 screentape. The sample may need to be diluted before loading.
- 3.7A.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 3.7A.1. to 3.7A.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.

3.7B: Protocol to achieve specific sequenced insert sizes



Determine cleanup protocol based on desired sequenced insert size and adaptor used.

Note: for samples of DNA Integrity Score (DIN) <3, library yield and complexity loss may be observed when selecting for a larger sequenced insert size. For samples with DIN >6, sequenced insert sizes of 150 bp or 350 bp were observed using the following protocol parameters. For larger insert sizes, bead volumes can be further reduced. For samples of DIN <6 the sequenced insert size will depend on the level of fragmentation and crosslinking of the input sample.

Table 3.7B.1.

	NEBNext NON -INDEXED ADAPTOR		NEBNext UMI UDI ADAPTOR	
Sequenced Insert Size	150 bp	350 bp	150 bp	350 bp
SPRI Bead Volume	45 µl	30 µl	45 µl	30 µl

- 3.7B.1. Vortex NEBNext Sample Purification Beads to resuspend. SPRIselect or AMPure XP beads can be used as well. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.
- 3.7B.2. Add **__ µl** resuspended beads according to Table 3.7B.1. to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.
- 3.7B.3. Incubate samples on bench top for 5 minutes at room temperature.
- 3.7B.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
- 3.7B.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**).
- 3.7B.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.
- 3.7B.7. Repeat Step 3.7B.6. once for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

- 3.7B.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.**
- 3.7B.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).**
- 3.7B.10. Mix well by pipetting up and down 10 times or by vortexing for 3-5 seconds on high. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
- 3.7B.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.
- 3.7B.12. Check the size distribution on an Agilent Bioanalyzer High Sensitivity DNA chip or Agilent TapeStation HSD1000 screentape. The sample may need to be diluted before loading.
- 3.7B.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 3.7A.1. to 3.7A.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.

Kit Components

NEB #E6655S Table of Components

NEB #	PRODUCT	VOLUME
E7361A	NEBNext FFPE DNA Repair Mix v2	0.048 ml
E7362A	NEBNext Thermolabile Proteinase K	0.048 ml
E6656A	NEBNext UltraShear	0.096 ml
E6657A	NEBNext UltraShear Reaction Buffer	0.168 ml
E6658A	500 mM DTT	0.048 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 #E6655L Table of Components

NEB #	PRODUCT	VOLUME
E7361AA	NEBNext FFPE DNA Repair Mix v2	0.192 ml
E7362AA	NEBNext Thermolabile Proteinase K	0.192 ml
E6656AA	NEBNext UltraShear	0.384 ml
E6657AA	NEBNext UltraShear Reaction Buffer	0.672 ml
E6658AA	500 mM DTT	0.192 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
2.0	Added Section 3, updated protocols.	7/26
2.1	Added dashes to input ranges in Table 1.4.1.	10/26

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