

NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina®

for use with NEBNext Multiplex Oligos for Illumina

(Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416)

NEB #E7760S/L, #E7765S/L

24/96 reactions

Version 1.0_7/20

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The Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7760S/#E7765S) and 96 reactions (NEB #E7760L/#E7765L).

Package 1: Store at -20°C.

- (lilac) NEBNext First Strand Synthesis Reaction Buffer
- (lilac) Random Primers
- (lilac) NEBNext First Strand Synthesis Enzyme Mix
- (brown) NEBNext Strand Specificity Reagent
- (orange) NEBNext Second Strand Synthesis Enzyme Mix
- (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)
- (green) NEBNext Ultra II End Prep Enzyme Mix

- (green) NEBNext Ultra II End Prep Reaction Buffer

- (red) NEBNext Ultra II Ligation Master Mix

- (red) NEBNext Ligation Enhancer

- (blue) NEBNext USER® Enzyme

- (blue) NEBNext Ultra II Q5® Master Mix

NEBNext Adaptor Dilution Buffer

(0.1X) TE Buffer

Nuclease-free Water

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

Supplied only with NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads, NEB #E7765.

NEBNext Sample Purification Beads

Required Materials Not Included

- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Magnetic Rack (Alpaqua®, cat. #A001322 or equivalent)
- 80% Ethanol (freshly prepared)
- Thermocycler
- Any thin wall 200 µl PCR tube
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables

For NEB #E7760 only:

- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- In NEB #E7765 beads are included [Sample Purification Beads (NEB #E7767)]

For use with NEBNext Poly(A) mRNA Magnetic Isolation Module:

- NEB #E7490 (not included)
- 96-well 0.2 ml PCR Plates and Microseal® 'B' Adhesive Sealer (BioRad MSB-1001) or 0.2 ml RNase-free tube
- 1.5 ml Microcentrifuge tube and NEB #S1506 Magnet stand or equivalent
(for washing beads only)

For use with NEBNext Ribosomal Depletion Kit:

- NEB #E6310 (not included) Agencourt® RNAClean® XP Beads (Beckman Coulter, Inc. #A63987)
- In NEB #E6350, beads are included [RNA Sample Purification Beads (NEB #E6351)]

Considerations on Choosing an RNA-seq Library Preparation Method

The library preparation protocol should be chosen based on the goals of the project and quality of the RNA sample. Total cellular RNA is mainly composed of rRNA and often is not of interest. rRNA can be removed from total cellular RNA by one of two common methods. The first method uses oligo d(T) beads, which bind to the poly(A) tail of eukaryotic mRNA. Alternatively, rRNA can be depleted using rRNA specific probes. NEB offers the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) and the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310) for the enrichment of non-ribosomal RNA.

In the oligo d(T) approach, only mRNA with poly(A) tails will be enriched; other cellular RNA without a poly(A) tail, such as non-coding RNA or mRNA lacking poly(A) will not bind to the beads. In addition, mRNA from some organisms (e.g., prokaryotes) or degraded RNA (e.g., FFPE RNA) do not have poly(A) tails and will not be captured by oligo d(T) beads. On the other hand, the probe based rRNA depletion kit will remove the targeted ribosomal RNA, but it will preserve other biologically relevant cellular RNA such as non-coding RNA or mRNA.

The quality of an RNA sample should also be considered when deciding on a library preparation protocol. The NEBNext Poly(A) mRNA Magnetic Isolation Module should only be used with high quality RNA samples (RIN > 7), since degradation results in a loss of poly(A)

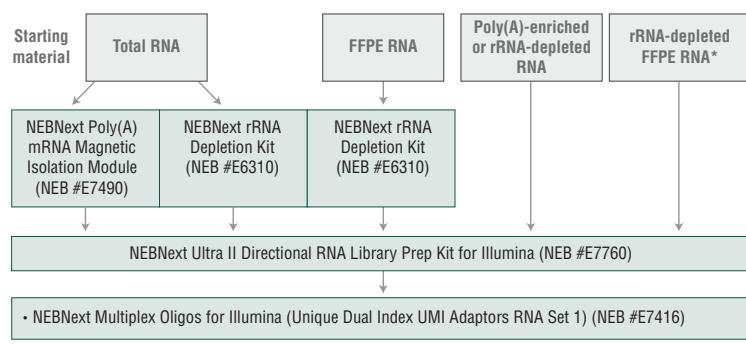
tails from mRNA molecules. For partially degraded or heavily degraded samples (e.g., RIN \leq 7, FFPE RNA), the NEBNext rRNA Depletion Kit should be used. For libraries without any enrichment or depletion of total RNA, use Section 4 for high quality RNA (RIN > 7) or Section 5 for degraded or FFPE RNA (RIN < 7).

Please refer to the product page on NEB.com for FAQs about this product.

NEBNext Ultra II Directional RNA Product Selection Guide

The library preparation protocol should be chosen based on the goals of the project and quality of the RNA sample. Total cellular RNA is mainly composed of rRNA and often is not of interest. rRNA can be removed from total cellular RNA by one of two common methods. The first method uses oligo d(T) beads, which bind to the poly(A) tail of eukaryotic mRNA. Alternatively, rRNA can be depleted using rRNA specific probes. NEB offers the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) and the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310) for the enrichment of non-ribosomal RNA.

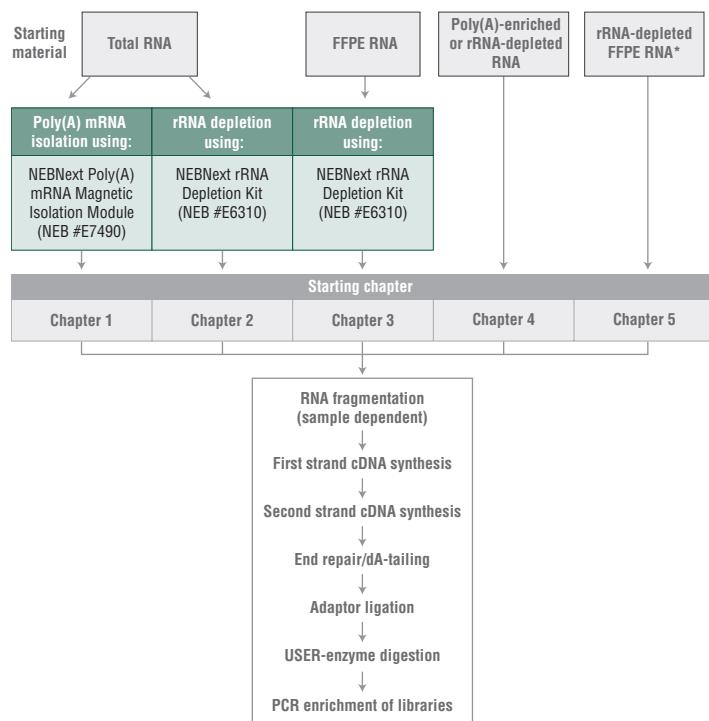
Figure 1. NEBNext Ultra II Directional RNA Product Selection Guide.



NEBNext Ultra II Directional RNA Protocol Selection Guide

Use the following chart to determine the most suitable protocol in this manual. Every Section in this manual contains a different protocol based on the starting material. More detailed information is available at the beginning of each Section. Please read the RNA sample recommendations and input amount requirements in its entirety before starting the protocol.

Figure 2. NEBNext Ultra II Directional RNA Protocol Selection Guide.



Overview

The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of RNA into high quality directional (strand-specific) libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow has minimal hands-on time and is compatible with poly(A) mRNA enrichment and rRNA depletion methods. This manual is optimized for use of the Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765) in combination with the NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors for RNA) (NEB #7416).

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of an indexed transcriptome library on the 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 Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)

Symbols



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



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.



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

The protocol has been optimized using high quality Universal Human Reference Total RNA.

RNA Sample Requirements

RNA Integrity:

Assess the quality of the Input RNA by running the RNA sample on an Agilent Bioanalyzer® RNA 6000 Nano/Pico Chip. For PolyA mRNA enrichment, high quality RNA with a RIN score > 7 is required.

RNA Sample Requirements:

The RNA sample should be free of salts (e.g., Mg²⁺ and guanidinium salts), divalent cation chelating agents (e.g., EDTA and EGTA) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (DNase is not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. Any residual activity of the DNase I may degrade the oligos necessary for the enrichment. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation.

Input Amount Requirements

10 ng–1 µg DNA-free total RNA quantified by Qubit® Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 bp RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A (Section 6) for recommended fragmentation times and size selection conditions.

Keep all the buffers on ice, unless otherwise indicated.

1.1.



Preparation of First Strand Reaction Buffer and Random Primer Mix

- 1.1.1. Prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) in a nuclease-free microcentrifuge tube as follows:

COMPONENT	VOLUME
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	8 µl
• (lilac) Random Primers	2 µl
Nuclease-free Water	10 µl
Total Volume	20 µl

You can prepare the first strand synthesis reaction buffer later in the protocol, but it is important that it is ready before the elution in Step 1.2.36. The beads should not be allowed to dry out.

- 1.1.2. Mix thoroughly by pipetting up and down ten times.

Note: Keep the mix on ice until mRNA is purified. It will be used in Step 1.2.36 and 1.2.39.

1.2. mRNA Isolation, Fragmentation and Priming Starting with Total RNA

- 1.2.1. Dilute the total RNA with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.

- 1.2.2. To wash the Oligo dT Beads, add the following to a 1.5 ml nuclease-free tube. If preparing multiple libraries, beads for up to 10 samples can be added to a single 1.5 ml tube for subsequent washes (use magnet NEB #S1506 for 1.5 ml tubes). The purpose of this step is to bring the beads from the storage buffer into the binding buffer. The 2X Binding Buffer does not have to be diluted for this step.

COMPONENT	VOLUME PER ONE LIBRARY
Oligo dT Beads d(T) ₂₅	20 µl
RNA Binding Buffer (2X)	100 µl
Total Volume	120 µl

- 1.2.3. Wash the beads by pipetting up and down six times.
1.2.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).
1.2.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
1.2.6. Remove the tube from the magnetic rack.
1.2.7. Add 100 µl RNA Binding Buffer (2X) to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 100 µl RNA Binding Buffer (2X) per sample. The Binding Buffer does not have to be diluted.
1.2.8. Place the tubes on the magnet and incubate at room temperature until the solution is clear (~2 minutes).
1.2.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.
1.2.10. Add 50 µl RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl RNA Binding Buffer (2X) per sample. This first binding step removes most of the non-target RNA.
1.2.11. Add 50 µl beads to each RNA sample from Step 1.2.1. Mix thoroughly by pipetting up and down six times.
1.2.12. Place the tube in a thermocycler and close the lid. Heat the sample at **65°C for 5 minutes and cool to 4°C with the heated lid set at ≥ 75°C** to denature the RNA and facilitate binding of the mRNA to the beads.
1.2.13. Remove the tube from the thermocycler when the temperature reaches 4°C.
1.2.14. Mix thoroughly by pipetting up and down six times. Place the tube on the bench and incubate at **room temperature for 5 minutes** to allow the mRNA to bind to the beads.
1.2.15. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).
1.2.16. Remove and discard all of the supernatant. Take care not to disturb the beads.
1.2.17. Remove the tube from the magnetic rack.
1.2.18. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.
1.2.19. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

- 1.2.20. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
- 1.2.21. Remove the tube from the magnetic rack.
- 1.2.22. Repeat steps 1.2.18–1.2.21.
- 1.2.23. Add 50 µl of Tris Buffer (provided in NEB #E7490 kit) to each tube. Gently pipette up and down 6 times to mix thoroughly.
- 1.2.24. Place the tube on the thermocycler. Close the lid and heat the samples at **80°C for 2 minutes, then cool to 25°C with the heated lid set at ≥ 90°C** to do the first elution of the mRNA from the beads.
- 1.2.25. Remove the tube from the thermocycler when the temperature reaches 25°C.
- 1.2.26. Add 50 µl of RNA Binding Buffer (2X) to the sample to allow the mRNA to re-bind to the beads. Mix thoroughly by gently pipetting up and down six times.
- 1.2.27. Incubate the tube at **room temperature for 5 minutes**.
- 1.2.28. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).
- 1.2.29. Remove and discard the supernatant from the tube. Take care not to disturb the beads.
- 1.2.30. Remove the tube from the magnetic rack.
- 1.2.31. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.
- 1.2.32. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

Note: It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.

- 1.2.33. Place the tube on the magnet at room temperature until the solution is clear (~2 minutes).
- 1.2.34. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads that contain the mRNA.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip, remove all of the wash buffer. (Caution: Do not disturb beads that contain the mRNA). Avoid letting the beads dry out before adding elution buffer.

- 1.2.35. Remove the tube from the magnetic rack.



Note: The next step provides a fragmentation incubation time resulting in an RNA insert size of ~ 200 nt. For RNA insert sizes > 200 nt, refer to Section 6 (Appendix A) for recommended fragmentation times in Step 1.2.37.

- 1.2.36. To elute the mRNA from the beads and fragment, add 11.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1.1.2, pipette up and down six times to resuspend the beads.
- 1.2.37. Incubate the sample in a thermocycler with the heated lid set at 105°C as follows:

15 minutes at 94°C
Hold at 4°C*

*Immediately transfer the tube to ice for 1 minute as soon as it is cool enough to handle (~65°C)
- 1.2.38. Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (~1-2 minutes).
- 1.2.39. Collect the fragmented mRNA by transferring 10 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note 1: If the supernatant volume recovered is less than 10 µl for any reason, bring the volume up to 10 µl by adding the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1.1.2 and continue with the protocol.

Note 2: Avoid transferring any of the magnetic beads.
- 1.2.40. Place the tube on ice and proceed directly to First Strand cDNA Synthesis.

1.3. First Strand cDNA Synthesis

- 1.3.1. Assemble the first strand cDNA synthesis reaction **on ice** by adding the following components into fragmented and primed RNA from Step 1.2.40.

FIRST STRAND cDNA SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 1.2.40)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

- 1.3.2. Mix thoroughly by pipetting up and down at least 10 times.



- 1.3.3. Incubate the sample in a preheated thermocycler with the heated lid set at $\geq 80^{\circ}\text{C}$ as follows:

Note: If you are following recommendations in Appendix A, for longer RNA fragments (creating inserts > 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

- 1.3.4. Immediately, perform Second Strand cDNA Synthesis.

1.4. Second Strand cDNA Synthesis

- 1.4.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis reaction product from Step 1.3.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 1.3.4)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

- 1.4.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down at least 10 times.

- 1.4.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at $\leq 40^{\circ}\text{C}$ (or off).

1.5. Purification of Double-stranded cDNA using SPRIselect Beads or NEBNext Sample Purification Beads

- 1.5.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.
- 1.5.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- 1.5.3. Incubate for at least **5 minutes at room temperature**.
- 1.5.4. Briefly spin the tube in a microcentrifuge to collect any sample from the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. (**Caution: do not discard beads**).
- 1.5.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 1.5.6. Repeat Step 1.5.5 once for a total of 2 washing steps.
- 1.5.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack 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.

- 1.5.8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Briefly spin the tube and **incubate for 2 minutes at room temperature**. Place the tube on the magnetic rack until the solution is clear.

- 1.5.9. Remove 50 µl of the supernatant and transfer to a clean nuclease-free PCR tube.



Note: If you need to stop at this point in the protocol, samples can be stored at -20°C.

1.6. End Prep of cDNA Library

- 1.6.1. Assemble the end prep reaction on ice by adding the following components to second strand synthesis product from Step 1.5.9.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 1.5.9)	50 µl
● (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
● (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

- 1.6.2. 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.

- 1.6.3. Incubate the sample in a thermocycler with the heated lid set at $\geq 75^{\circ}\text{C}$ as follows:

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C

- 1.6.4. Proceed immediately to Adaptor Ligation.

1.7. Adaptor Ligation



- 1.7.1. Dilute the NEBNext Unique Dual Index UMI RNA Adaptor* prior to setting up the ligation reaction in ice-cold UMI Adaptor Dilution Buffer and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
1,000 ng–250 ng	No dilution
249 ng–100 ng	10-fold dilution in UMI Adaptor Dilution Buffer
99 ng–10 ng	50-fold dilution in UMI Adaptor Dilution Buffer

*The UMI RNA Adaptors and UMI Adaptor Dilution Buffer must be purchased separately (NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416). Do not use the Adaptor Dilution Buffer provided with the Ultra II Directional RNA Library Prep Kit for diluting Unique Dual Index UMI Adaptors.

- 1.7.2. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 1.6.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 1.6.3)	60 µl
Diluted Adaptor (Step 1.7.1)	5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	96 µl

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

- 1.7.3. Set a 100 μ l or 200 μ l pipette to 80 μ l 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.

- 1.7.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermocycler.

- 1.7.5. Add 3 μ l (blue) USER Enzyme to the ligation mixture from Step 1.7.4, resulting in total volume of 99 μ l.

- 1.7.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to $\geq 45^\circ\text{C}$.

- 1.7.7. Proceed immediately to Purification of the Ligation Reaction.

1.8. Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads



Note: If you are selecting for larger insert size libraries (> 200 nt) follow the size selection recommendations in Appendix A, Section 6.

- 1.8.1. Add 70 μ l (0.7X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 1.8.2. Incubate for **10 minutes at room temperature**.

- 1.8.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (~ 5 minutes), discard the supernatant that contain unwanted fragments (**Caution: do not discard beads**).

- 1.8.4. Add 200 μ l of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

- 1.8.5. Repeat Step 1.8.4 once for a total of 2 washing steps.

- 1.8.6. Briefly spin the tube and put the tube back in the magnetic rack.

- 1.8.7. Completely remove the residual ethanol and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack 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.

- 1.8.8. Remove the tube from the magnet. Elute DNA target from the beads by adding 22 μ l 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for **2 minutes at room temperature**. Put the tube in the magnetic rack until the solution is clear.

- 1.8.9. Without disturbing the bead pellet, transfer 20 μ l of the supernatant to a clean PCR tube and proceed to PCR enrichment.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C .

1.9. PCR Enrichment of Adaptor Ligated DNA

- 1.9.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 1.8.9)	20 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
NEBNext Primer Mix*	5 µl
Total Volume	50 µl

* NEBNext Primer Mix is provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adapters RNA Set 1, NEB #E7416)

- 1.9.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.
- 1.9.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 1.9.3A and Table 1.9.3B):

Table 1.9.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	8–17*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input (Table 1.9.3B).

** It is important to limit the number of PCR cycles to avoid overamplification.

If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.1 on page 44).

Table 1.9.3B: Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
1,000 ng	8–9
100 ng	12–13
10 ng	15–17

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

1.10. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

- 1.10.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.
- 1.10.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- 1.10.3. Incubate for up to **5 minutes at room temperature**.
- 1.10.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- 1.10.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 1.10.6. Repeat Step 1.10.5 once for a total of 2 washing steps.
- 1.10.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack 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.

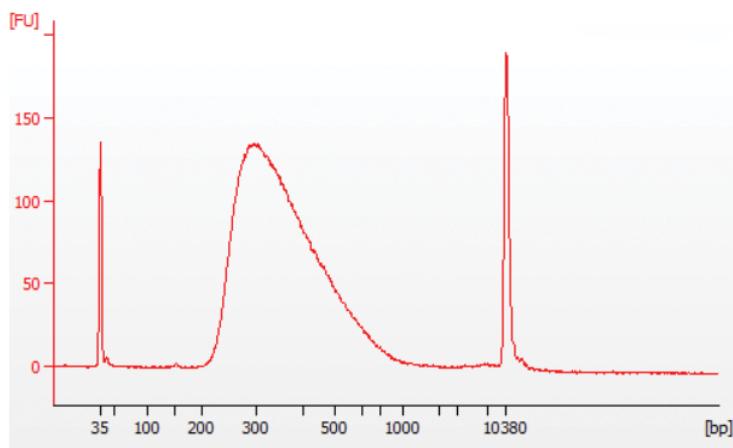
- 1.10.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube in a microcentrifuge and incubate for **2 minutes at room temperature**. Place the tube in the magnetic rack until the solution is clear.
- 1.10.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at -20°C .

1.11 Assess Library Quality on an Agilent Bioanalyzer DNA Chip

- 1.11.1. Use a Bioanalyzer or Tape Station to determine the size distribution and concentration of the libraries.
- 1.11.3. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 1.10.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 1.10).

Figure 1.11.1. Example of a representative RNA library size distribution on a Bioanalyzer.



Section 2

Protocol for use with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)

Symbols



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



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.



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

This protocol has been optimized using Universal Human Reference Total RNA.

RNA Sample Requirements

RNA Integrity:

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 (current Section). See Table 2.5.1. for the recommended fragmentation times, based on RIN.

For highly degraded samples (RIN = 1 to 2) (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3.

RNA Sample Requirements:

The RNA sample should be free of salts (e.g., Mg²⁺ and guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation.

Input Amount Requirements

5 ng–1 µg total RNA (DNA free) in a 12 µl total volume, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A (Section 6) for recommended fragmentation times and size selection conditions.

Keep all of the buffers on ice, unless otherwise indicated.

2.1. Probe Hybridization to RNA

2.1.1. Dilute the total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA on ice.

2.1.2. Prepare RNA/Probe master mix as follows:

RNA/PROBE MASTER MIX	VOLUME
NEBNext rRNA Depletion Solution	1 µl
Probe Hybridization Buffer	2 µl
Total Volume	3 µl

2.1.3. Add 3 µl of the above mix to 12 µl total RNA (from Step 2.1.1), resulting in a total volume of 15 µl.

2.1.4. Mix thoroughly by pipetting up and down at least 10 times.

2.1.5. Briefly spin down the sample in a microcentrifuge.

- 2.1.6. Place samples in a thermocycler and run the following program with the heated lid set at 105°C.
 This will take approximately 15-20 minutes to complete.

TEMPERATURE	TIME
95°C	2 minutes
Ramp down to 22°C	0.1°C/sec
Hold at 22°C	5 minutes

- 2.1.7. Briefly spin down the sample in a microcentrifuge, and place on ice. Proceed immediately to RNase H Digestion Step.

2.2. RNase H Digestion

- 2.2.1. Assemble the RNase H master mix **on ice** as follows.

RNASE H MASTER MIX	VOLUME
NEBNext RNase H	2 µl
NEBNext RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	5 µl

- 2.2.2. Mix thoroughly by pipetting up and down at least 10 times.
 2.2.3. Briefly spin down the samples in a microcentrifuge.
 2.2.4. Add 5 µl of the RNase H master mix to the RNA sample from Step 2.1.7, resulting in a total volume of 20 µl.
 2.2.5. Mix thoroughly by pipetting up and down at least 10 times.
 2.2.6. Incubate the sample in a thermocycler for **30 minutes at 37°C** with the lid set to 40°C (or off).
 2.2.7. Briefly spin down the samples in a microcentrifuge and place on ice. Proceed immediately to DNase I Digestion to prevent non-specific degradation of RNA.

2.3. DNase I Digestion

- 2.3.1. Assemble the DNase I master mix **on ice** in a nuclease-free tube.

DNASE I MASTER MIX	VOLUME
DNase I Reaction Buffer	5 µl
DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	30 µl

- 2.3.2. Mix thoroughly by pipetting up and down at least 10 times.
 2.3.3. Briefly spin down the sample in a microcentrifuge.
 2.3.4. Add 30 µl of DNase I master mix to 20 µl RNA sample from Step 2.2.7, resulting in a total volume of 50 µl.
 2.3.5. Mix thoroughly by pipetting up and down ten times.
 2.3.6. Incubate the sample in a thermocycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).
 2.3.7. Briefly spin down the sample in a microcentrifuge and place on ice. Proceed immediately to RNA Purification.

2.4 RNA Purification Using Agencourt RNAClean® XP Beads or NEBNext RNA Sample Purification Beads

- 2.4.1. Vortex the RNAClean XP or RNA Sample Purification Beads to resuspend.
 2.4.2. Add 110 µl (2.2X) beads to the RNA sample from Step 2.3.7 and mix thoroughly by pipetting up and down at least 10 times.
 2.4.3. Incubate the sample for **15 minutes on ice** to bind RNA to the beads.
 2.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads which contain RNA.
 2.4.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA.

- 2.4.6. Repeat Step 2.4.5 once for a total of 2 washing steps.
- 2.4.7. Completely remove residual ethanol and air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.
- Caution: Do not over-dry the beads. This may result in lower recovery of RNA 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.**
- 2.4.8. Remove the tube from the magnet. Elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting up and down at least 10 times and briefly spin the tube.
- 2.4.9. Incubate for **2 minutes at room temperature**. Place the tube in the magnet until the solution is clear (~2 minutes).
- 2.4.10. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.
- 2.4.11. Place the sample on ice and proceed to RNA Fragmentation and Priming.

2.5. RNA Fragmentation and Priming



RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 2.5.1.

- 2.5.1. Assemble the following fragmentation and priming reaction **on ice**:

FRAGMENTATION AND PRIMING REACTION	VOLUME
Ribosomal RNA Depleted Sample (Step 2.4.11)	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	10 µl

- 2.5.2. Mix thoroughly by pipetting up and down ten times.
- 2.5.3. Place the sample on a thermocycler and incubate the sample at **94°C** following the recommendations in Table 2.5.1 below for libraries with inserts ~200 nt.

Table 2.5.1. Suggested fragmentation times based on RIN value of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	> 7	15 min. at 94°C
Partially Degraded RNA	2–6	7–8 min. at 94°C

Note: Refer to Appendix A (Section 6) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A only apply for intact RNA.

- 2.5.4. Immediately transfer the tube to ice and proceed to First Strand cDNA Synthesis.

2.6. First Strand cDNA Synthesis

- 2.6.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 2.5.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 2.5.4)	10 µl
● (brown) NEBNext Strand Specificity Reagent	8 µl
● (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

- 2.6.2. Mix thoroughly by pipetting up and down ten times.



- 2.6.3. Incubate the sample in a preheated thermocycler with the heated lid set at $\geq 80^{\circ}\text{C}$ as follows:

Note: If you are following recommendations in Appendix A (Section 6), for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

- 2.6.4. Proceed directly to Second Strand cDNA Synthesis.

2.7. Second Strand cDNA Synthesis

- 2.7.1. Assemble the second strand cDNA synthesis reaction on ice by adding the following components into the first strand synthesis product from Step 2.6.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 2.6.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
● (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

- 2.7.2. Keeping the tube on ice, mix thoroughly by pipetting up and down at least 10 times.

- 2.7.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at $\leq 40^{\circ}\text{C}$ (or off).

2.8. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads

- 2.8.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

- 2.8.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 2.8.3. Incubate for **5 minutes at room temperature**.

- 2.8.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnet to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. (**Caution: do not discard beads**).

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

- 2.8.6. Repeat Step 2.8.5 once for a total of 2 washing steps.

- 2.8.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with 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.

2.8.8. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Quickly spin the tube and incubate for **2 minutes at room temperature**. Place the tube on the magnetic rack until the solution is clear.

2.8.9. Remove 50 µl of the supernatant and transfer to a clean nuclease-free PCR tube.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

2.9. End Prep of cDNA Library

2.9.1. Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product from Step 2.8.9.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 2.8.9)	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

2.9.2. 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.

2.9.3. Incubate the sample in a thermocycler with the heated lid set at $\geq 75^{\circ}\text{C}$ as follows.

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C .

2.9.4. Proceed immediately to Adaptor Ligation.

2.10. Adaptor Ligation



2.10.1. Dilute the NEBNext Unique Dual Index UMI RNA Adaptor* prior to setting up the ligation reaction in ice-cold UMI Adaptor Dilution Buffer and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
1,000 ng–101 ng	No dilution
100 ng–10 ng	10-fold dilution in UMI Adaptor Dilution Buffer
5 ng	50-fold dilution in UMI Adaptor Dilution Buffer

*The UMI RNA Adaptors and UMI Adaptor Dilution Buffer must be purchased separately (NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416). Do not use the Adaptor Dilution Buffer provided with the Ultra II Directional RNA Library Prep Kit for diluting Unique Dual Index UMI Adaptors.

- 2.10.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 2.9.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 2.9.4)	60 µl
Diluted Adaptor (Step 2.10.1)	5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	96 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

- 2.10.3. 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.

- 2.10.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermocycler.

- 2.10.5. Add 3 µl • (blue) USER Enzyme to the ligation mixture from Step 2.10.4, resulting in total volume of 99 µl.

- 2.10.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to $\geq 45^\circ\text{C}$.

- 2.10.7. Proceed immediately to Purification of the Ligation Reaction.

2.11. Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads



Note: If you are selecting for libraries with larger insert size (> 200 nt) follow the size selection recommendations in Appendix A, Section 6.

- 2.11.1. Add 70 µl (0.7X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 2.11.2. Incubate for **10 minutes at room temperature**.

- 2.11.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (~ 5 minutes), discard the supernatant that contains unwanted fragments. (**Caution: do not discard beads**).

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

- 2.11.5. Repeat Step 2.11.4 once for a total of 2 washing steps.

- 2.11.6. Briefly spin the tube and put the tube back in the magnetic rack.

- 2.11.7. Completely remove the residual ethanol and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack 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.

- 2.11.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 22 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for **2 minutes at room temperature**. Put the tube in the magnet until the solution is clear.

- 2.11.9. Without disturbing the bead pellet, transfer 20 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

2.12. PCR Enrichment of Adaptor Ligated DNA

- 2.12.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.11.9)	20 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
NEBNext Primer Mix*	5 µl
Total Volume	50 µl

* NEBNext Primer Mix is provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adapters RNA Set 1, NEB #E7416).

- 2.12.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

- 2.12.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 2.12.3A and Table 2.12.3B):

Table 2.12.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	7–17*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input.

** It is important to limit the number of PCR cycles to avoid overamplification.

If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.1 on page 44).

Table 2.12.3B: Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
1,000 ng	7–8
100 ng	11–12
10 ng	14–16
5 ng	16–17

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

2.13. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

- 2.13.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.
- 2.13.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- 2.13.3. Incubate for **5 minutes at room temperature**.
- 2.13.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. (**Caution: do not discard beads**).
- 2.13.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 2.13.6. Repeat Step 2.13.5 once for a total of 2 washing steps.
- 2.13.7. Air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack 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.

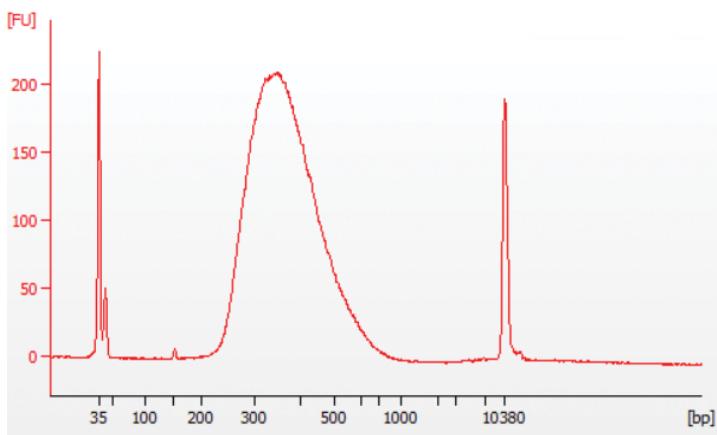
- 2.13.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube in a microcentrifuge and incubate for **2 minutes at room temperature**. Place the tube in the magnetic rack until the solution is clear.
- 2.13.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at -20°C.

2.14. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

- 2.14.1. Use a Bioanalyzer or Tape Station to determine the size distribution and concentration of the libraries.
- 2.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the bioanalyzer traces, bring up the sample volume (from Step 2.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 2.13).

Figure 2.14.1. Example of a representative RNA library size distribution on a Bioanalyzer.



Section 3

Protocol for use with FFPE RNA, NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)

Symbols



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



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.



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

This protocol has been optimized using Universal Human Reference Total RNA.

RNA Sample Requirements

RNA Integrity:

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2. See Table 2.5.1. for the recommended fragmentation times, based on RIN.

For highly degraded samples (RIN = 1 to 2) (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3 (current Section).

RNA Sample Requirements:

The RNA sample should be free of salts (e.g., Mg²⁺ and guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation.

Input Amount Requirements

10 ng–100 ng FFPE RNA (DNA free) in a 12 µl total volume, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts.

Keep all of the buffers on ice, unless otherwise indicated.

3.1.1. Probe Hybridization to RNA

3.1.1. Dilute the total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA **on ice**.

3.1.2. Prepare RNA/Probe master mix as follows:

RNA/PROBE MASTER MIX	VOLUME
NEBNext rRNA Depletion Solution	1 µl
Probe Hybridization Buffer	2 µl
Total Volume	3 µl

3.1.3. Add 3 µl of the above mix to 12 µl total RNA sample (from Step 3.1.1), resulting in a total volume of 15 µl.

3.1.4. Mix thoroughly by pipetting up and down ten times.

3.1.5. Briefly spin down the sample in a microcentrifuge.

- 3.1.6. Place samples in a thermocycler, and run the following program with the heated lid set at 105°C.
 This will take approximately 15-20 minutes to complete:

TEMPERATURE	TIME
95°C	2 minutes
Ramp down to 22°C	0.1°C/sec
Hold at 22°C	5 minutes

- 3.1.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to RNase H Digestion Step.

3.2. RNase H Digestion

- 3.2.1. Assemble the RNase H master mix **on ice** as follows.

RNASE H MASTER MIX	VOLUME
NEBNext RNase H	2 µl
NEBNext RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	5 µl

- 3.2.2. Mix thoroughly by pipetting up and down ten times.
 3.2.3. Briefly spin down the samples in a microcentrifuge.
 3.2.4. Add 5 µl of the RNase H master mix to the RNA sample from Step 3.1.7, resulting in a total volume of 20 µl.
 3.2.5. Mix thoroughly by pipetting up and down ten times.
 3.2.6. Incubate the sample in a thermocycler for **30 minutes at 37°C** with the lid set to 40°C (or off).
 3.2.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to DNase I Digestion to prevent non-specific degradation of RNA.

3.3. DNase I Digestion

- 3.3.1. Assemble DNase I digestion master mix **on ice** in a nuclease-free tube.

DNASE I MASTER MIX	VOLUME
DNase I Reaction Buffer	5 µl
DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	30 µl

- 3.3.2. Mix thoroughly by pipetting up and down ten times.
 3.3.3. Briefly spin down the samples in a microcentrifuge.
 3.3.4. Add 30 µl of DNase I digestion master mix to 20 µl RNA sample from Step 3.2.7, resulting in a total volume of 50 µl.
 3.3.5. Mix thoroughly by pipetting up and down ten times.
 3.3.6. Incubate the samples in a thermocycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).
 3.3.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to RNA purification.

3.4. RNA Purification Using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

- 3.4.1. Vortex the RNAClean XP or RNA Sample Purification Beads to resuspend.
 3.4.2. Add 110 µl (2.2X) beads to the RNA sample from Step 3.3.7 and mix thoroughly by pipetting up and down at least 10 times.
 3.4.3. Incubate the sample for **15 minutes on ice** to bind RNA to the beads.
 3.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain RNA.

- 3.4.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant
- 3.4.6. Repeat Step 3.4.5 once for a total of 2 washing steps.
- 3.4.7. Completely remove residual ethanol and air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack with the lid open.
- Caution: Do not over-dry the beads. This may result in lower recovery of RNA 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.**
- 3.4.8. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting up and down ten times and briefly spin the tube.
- 3.4.9. Incubate for **2 minutes at room temperature**. Place the tube in the magnet until the solution is clear (~2 minutes).
- 3.4.10. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.
- 3.4.11. Place the sample on ice and proceed to Priming of Highly Degraded RNA.

3.5. Priming of Highly Degraded RNA (FFPE) Which has a RIN ≤ 2 and Does not Require Fragmentation

- 3.5.1. Assemble the Priming Reaction **on ice** by adding the following components:

PRIMING REACTION	VOLUME
rRNA Depleted Sample (Step 3.4.11)	5 µl
• (brown) Random Primers	1 µl
Total Volume	6 µl

- 3.5.2. Mix thoroughly by pipetting up and down ten times.
- 3.5.3. Briefly spin down the samples in a microcentrifuge.
- 3.5.4. Incubate the sample in a preheated thermocycler as follows.
5 minutes at 65°C, with heated lid set at 105°C.
Hold at 4°C.
- 3.5.5. Transfer the tube directly to ice and proceed to First Strand cDNA Synthesis.

3.6. First Strand cDNA Synthesis

- 3.6.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the primed RNA from Step 3.5.5:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 3.5.5)	6 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

- 3.6.2. Keeping the tube on ice, mix thoroughly by pipetting up and down ten times.



- 3.6.3. Incubate the sample in a preheated thermocycler with the heated lid set at ≥ 80°C as follows:

Note: If you are following recommendations in Appendix A, for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

- 3.6.4. Proceed directly to Second Strand cDNA Synthesis Reaction.

3.7. Second Strand cDNA Synthesis

- 3.7.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 3.6.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 3.6.4)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

- 3.7.2. Keeping the tube on ice, mix thoroughly by pipetting up and down ten times.

- 3.7.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at $\leq 40^\circ\text{C}$ (or off).

3.8. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads

- 3.8.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

- 3.8.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 3.8.3. Incubate for up to **5 minutes at room temperature**.

- 3.8.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnet to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. (**Caution: do not discard beads**).

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

- 3.8.6. Repeat Step 3.8.5 once for a total of 2 washing steps.

- 3.8.7. Air dry the beads for up to 5 minutes while the tube is on the magnet with 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.

- 3.8.8. Remove the tube from the magnet. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube and incubate for **2 minutes at room temperature**. Place the tube on the magnetic rack until the solution is clear.

- 3.8.9. Remove 50 µl of the supernatant and transfer to a clean nuclease free PCR tube.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

3.9. End Prep of cDNA Library

- 3.9.1. Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product from Step 3.8.9.

END PREP REACTION	VOLUME
Second Strand Synthesis Product (Step 3.8.9)	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

- 3.9.2. 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.

- 3.9.3. Incubate the sample in a thermocycler with the heated lid set at $\geq 75^{\circ}\text{C}$ as follows.

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C .

- 3.9.4. Proceed immediately to Adaptor Ligation.

3.10. Adaptor Ligation



- 3.10.1. Dilute the NEBNext Unique Dual Index UMI RNA Adaptor* prior to setting up the ligation reaction in ice-cold UMI Adaptor Dilution Buffer and keep the diluted adaptor on ice.

FFPE RNA	DILUTION REQUIRED
100 ng–10 ng	10-fold dilution in UMI Adaptor Dilution Buffer

*The UMI RNA Adaptors and UMI Adaptor Dilution Buffer must be purchased separately (NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416). Do not use the Adaptor Dilution Buffer provided with the Ultra II Directional RNA Library Prep Kit for diluting Unique Dual Index UMI Adaptors.

- 3.10.2. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 3.9.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 3.9.4)	60 µl
Diluted Adaptor (Step 3.10.1)	5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	96 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C . We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

- 3.10.3. 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.

- 3.10.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermocycler.

- 3.10.5. Add 3 µl • (blue) USER Enzyme to the ligation mixture from Step 3.10.4, resulting in total volume of 99 µl.

- 3.10.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to $\geq 45^{\circ}\text{C}$.

- 3.10.7. Proceed immediately to Purification of the Ligation Reaction.

3.11. Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads



Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Section 6.

- 3.11.1. Add 70 µl (0.7X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
- 3.11.2. Incubate for **10 minutes at room temperature**.
- 3.11.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments. (**Caution: do not discard beads**).
- 3.11.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 3.11.5. Repeat Step 3.11.4 once for a total of 2 washing steps.
- 3.11.6. Briefly spin the tube and put the tube back in the magnetic rack.
- 3.11.7. Completely remove the residual ethanol and air dry beads until the beads are dry for **up to 5 minutes** while the tube is on the magnetic rack 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.
- 3.11.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 22 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down, incubate for **2 minutes at room temperature**. Put the tube in the magnet until the solution is clear.
- 3.11.9. Without disturbing the bead pellet, transfer 20 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

3.12. PCR Enrichment of Adaptor Ligated DNA

- 3.12.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 3.11.9)	20 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
NEBNext Primer Mix*	5 µl
Total Volume	50 µl

* NEBNext Primer Mix is provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416).

- 3.12.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.
- 3.12.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 3.12.3A and Table 3.12.3B):

Table 3.12.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12–17*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. The recommendation of PCR cycles are based on internal tests for FFPE RNA.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.1 on page 44).

Table 3.12.3B: Recommended PCR cycles based on input amount:

FFPE RNA INPUT	RECOMMENDED PCR CYCLES
100 ng	12–14
10 ng	15–17

3.13. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

- 3.13.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.
- 3.13.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- 3.13.3. Incubate for **up to 5 minutes** at room temperature.
- 3.13.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. (**Caution: do not discard beads**).
- 3.13.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 3.13.6. Repeat Step 3.13.5 once for a total of 2 washing steps.
- 3.13.7. Air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack 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.**
- 3.13.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times, quickly spin the tube in a microcentrifuge and incubate for **2 minutes at room temperature**. Place the tube in the magnetic rack until the solution is clear.

3.13.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

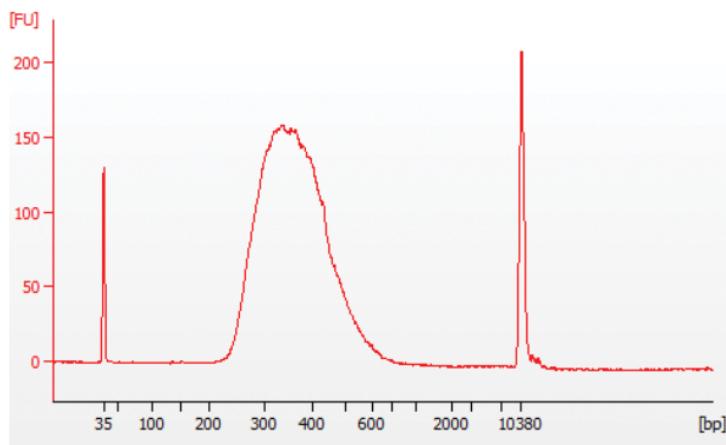
3.14. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

3.14.1. Use a Bioanalyzer or Tape Station to determine the size distribution and concentration of the libraries.

3.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the bioanalyzer traces, bring up the sample volume (from Step 3.13.9) to 50 µl with 1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 3.13). Adaptor-dimer is common for low inputs of FFPE samples and if observed, a second cleanup is recommended.

Figure 3.14.1. Example of a representative RNA library size distribution on a Bioanalyzer.



Section 4

Protocols for use with Purified mRNA or rRNA Depleted RNA

Symbols



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



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.



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

RNA Sample Requirements

This Section can be used for libraries without any enrichment or depletion of total RNA with RIN scores > 7.

RNA Integrity:

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. In this case, we recommend that the fragmentation time is empirically determined if the RNA sample is suspected to be low quality. **The following recommendation apply to the total RNA samples only.**

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 4 (current Section). See Table 4.1.1 for the recommended the fragmentation times.

For highly degraded samples (RIN = 1 to 2) (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 5.

RNA Sample Requirements:

The RNA sample should be free of salts (e.g., Mg²⁺ and guanidinium salts), divalent cation chelating agents (e.g., EDTA and EGTA) or organics (e.g., phenol or ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (DNase is not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation.

Input Amount Requirements

1 ng – 100 ng purified mRNA or rRNA depleted RNA that is **quantified after the purification**. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A (Section 6) for recommended fragmentation times and size selection conditions.

This protocol has been optimized using Universal Human Reference Total RNA.

4.1. RNA Fragmentation and Priming



RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 4.1.1.

- 4.1.1. Assemble the fragmentation and priming reaction **on ice** in a nuclease-free tube by adding the following components:

FRAGMENTATION AND PRIMING MIX	VOLUME
Purified mRNA or rRNA Depleted RNA	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	10 µl

- 4.1.2. Mix thoroughly by pipetting up and down ten times.

- 4.1.3. Place the sample in a thermocycler and incubate the sample at 94°C following the recommendations in Table 4.1.1 below for fragment sizes ~200 nt.

Table 4.1.1 Suggested fragmentation times based on RIN value of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	> 7	15 min. at 94°C
Partially Degraded RNA	2–6	7–8 min. at 94°C

Note: Refer to Appendix A (Section 6) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A (Section 6) only apply for intact RNA.

- 4.1.4. Immediately transfer the tube to ice and proceed to First Strand cDNA Synthesis.

4.2 First Strand cDNA Synthesis Reaction

- 4.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 4.1.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and Primed RNA (Step 4.1.4)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

- 4.2.2. Mix thoroughly by pipetting up and down ten times.



- 4.2.3. Incubate the sample in a preheated thermocycler with the heated lid set at ≥ 80°C as follows:

Note: If you are following recommendations in Appendix A, (Section 6), for longer RNA fragments (creating inserts > 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

- 4.2.4. Proceed directly to Second Strand cDNA Synthesis.

4.3. Second Strand cDNA Synthesis

- 4.3.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 4.2.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 4.2.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
● (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

- 4.3.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down ten times.

- 4.3.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at $\leq 40^\circ\text{C}$ (or off).

4.4. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads

- 4.4.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

- 4.4.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 4.4.3. Incubate for **5 minutes at room temperature**.

- 4.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. (**Caution: do not discard beads**).

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

- 4.4.6. Repeat Step 4.4.5 once for a total of 2 washing steps.

- 4.4.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with 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.

- 4.4.8. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube and incubate for **2 minutes at room temperature**. Place the tube on the magnetic rack until the solution is clear.

- 4.4.9. Remove 50 µl of the supernatant and transfer to a clean nuclease free PCR tube.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

4.5. End Prep of cDNA Library

- 4.5.1. Assemble the end prep reaction **on ice** by adding the following components to second strand synthesis product from Step 4.4.9.

END PREP REACTION	VOLUME
Second Strand Synthesis Product (Step 4.4.9)	50 µl
● (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
● (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

- 4.5.2. 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.

- 4.5.3. Incubate the sample in a thermocycler with the heated lid set at $\geq 75^{\circ}\text{C}$ as follows:

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

- 4.5.4. Proceed immediately to Adaptor Ligation.

4.6. Adaptor Ligation



- 4.6.1. Dilute the NEBNext Unique Dual Index UMI RNA Adaptor* prior to setting up the ligation reaction in ice-cold UMI Adaptor Dilution Buffer and keep the diluted adaptor on ice.

PURIFIED RNA	DILUTION REQUIRED
100 ng–11 ng	No dilution
10 ng–1 ng	10-fold dilution in UMI Adaptor Dilution

*The UMI RNA Adaptors and UMI Adaptor Dilution Buffer must be purchased separately (NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416). Do not use the Adaptor Dilution Buffer provided with the Ultra II Directional RNA Library Prep Kit for diluting Unique Dual Index UMI Adaptors.

- 4.6.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 4.5.4:

LIGATION REACTION	VOLUME
End Prepped DNA (Step 4.5.4)	60 μl
Diluted Adaptor (Step 4.6.1)	5 μl
• (red) NEBNext Ligation Enhancer	1 μl
• (red) NEBNext Ultra II Ligation Master Mix	30 μl
Total Volume	96 μl

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C . We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

- 4.6.3. 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.

- 4.6.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermocycler.

- 4.6.5. Add 3 μl • (blue) USER Enzyme to the ligation mixture from Step 4.6.4, resulting in total volume of 99 μl .

- 4.6.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to $\geq 45^{\circ}\text{C}$.

- 4.6.7. Proceed immediately to Purification of the Ligation Reaction.

4.7. Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads



Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Section 6.

- 4.7.1. Add 70 μl (0.7X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 4.7.2. Incubate for **10 minutes at room temperature**.
- 4.7.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments (**Caution: do not discard beads**).
- 4.7.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 4.7.5. Repeat Step 4.7.4 once for a total of 2 washing steps.
- 4.7.6. Briefly spin the tube and put the tube back in the magnetic rack.
- 4.7.7. Completely remove the residual ethanol and air dry beads until the beads are dry **for up to 5 minutes** while the tube is on the magnetic rack 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.**
- 4.7.8. Remove the tube from the magnet. Elute DNA target from the beads by adding 22 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times and incubate for **2 minutes at room temperature**. Put the tube in the magnetic rack until the solution is clear.
- 4.7.9. Without disturbing the bead pellet, transfer 20 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

4.8. PCR Enrichment of Adaptor Ligated DNA



Check and verify that the concentration of your oligos is 40 µM on the label.

- 4.8.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.7.9)	20 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
NEBNext Primer Mix*	5 µl
Total Volume	50 µl

* NEBNext Primer Mix is provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416).

- 4.8.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.
- 4.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 4.8.3A and Table 4.8.3B):

Table 4.8.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	6–14*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input.

** It is important to limit the number of PCR cycles to avoid overamplification.

If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See page 44).

Table 4.8.3B: Recommended PCR cycles based on input amount:

PURIFIED mRNA or rRNA DEPLETED RNA (QUANTIFIED AFTER PURIFICATION)	RECOMMENDED PCR CYCLES
100 ng	6–7
50 ng	7–8
10 ng	9–11
1 ng	12–14

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

4.9. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

- 4.9.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.
- 4.9.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- 4.9.3. Incubate for **5 minutes at room temperature**.
- 4.9.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. (**Caution: do not discard beads**).
- 4.9.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- 4.9.6. Repeat Step 4.9.5 once for a total of 2 washing steps.
- 4.9.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack 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.

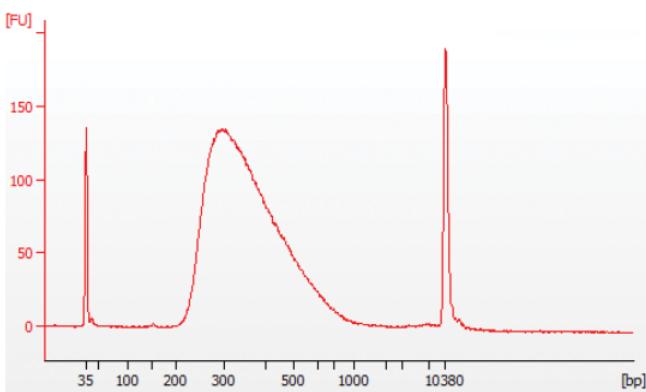
- 4.9.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube in a microcentrifuge and incubate for **2 minutes at room temperature**. Place the tube in the magnetic rack until the solution is clear.
- 4.9.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

4.10. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

- 4.10.1. Use a Bioanalyzer or Tape Station to determine the size distribution and concentration of the libraries.
- 4.10.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the Bioanalyzer traces; Bring up the sample volume (from Step 4.9.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 4.9).

Figure 4.10.1. Example of a representative RNA library size distribution on a Bioanalyzer.



Section 5

Protocol for use with rRNA Depleted FFPE RNA

Symbols



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



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.



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

The protocol has been optimized using high quality Universal Human Reference Total RNA.

RNA Sample Requirements

This Section can be used for libraries without any enrichment or depletion of total RNA with RIN scores 1–2.

RNA Integrity:

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. The following recommendation apply to the total RNA samples only.

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 4.

For highly degraded samples (RIN = 1 to 2) (e.g., FFPE), which does not require fragmentation, follow the library preparation protocol in Section 5 (current Section).

RNA Sample Requirements:

The RNA sample should be free of salts (e.g., Mg²⁺ and guanidinium salts), divalent cation chelating agents (e.g., EDTA and EGTA) or organics (e.g., phenol or ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (DNase is not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation.

Starting Material: 1 ng – 100 ng alternative rRNA depleted FFPE RNA that is quantified after rRNA depletion. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

5.1. Priming of Highly Degraded RNA (FFPE) Which has a RIN ≤ 2 and Does not Require Fragmentation

5.1.1. Assemble the Priming Reaction on ice by adding the following components:

PRIMING REACTION	VOLUME
rRNA Depleted FFPE RNA (1 ng–100 ng)	5 µl
• (lilac) Random Primers	1 µl
Total Volume	6 µl

5.1.2. Mix thoroughly by pipetting up and down several times.

5.1.3. Briefly spin down the samples in a microcentrifuge.

5.1.4. Incubate the sample in a preheated thermocycler as follows.

 5 minutes at 65°C, with heated lid set at 105°C

 Hold at 4°C

5.1.5. Transfer the tube directly to ice and proceed to First Strand cDNA Synthesis.

5.2. First Strand cDNA Synthesis

- 5.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 5.1.5)	6 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

- 5.2.2. Keeping the tube on ice, mix thoroughly by pipetting up and down several times.



- 5.2.3. Incubate the sample in a preheated thermocycler with the heated lid set at $\geq 80^{\circ}\text{C}$ as follows:

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

- 5.2.4. Proceed directly to Second Strand cDNA Synthesis.

5.3. Second Strand cDNA Synthesis

- 5.3.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 5.2.4:

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 5.2.4)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

- 5.3.2. Keeping the tube on ice, mix thoroughly by pipetting up and down several times.

- 5.3.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at $\leq 40^{\circ}\text{C}$ (or off).

5.4. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads

- 5.4.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

- 5.4.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

- 5.4.3. Incubate for **5 minutes at room temperature**.

- 5.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. (**Caution: do not discard beads**).

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

- 5.4.6. Repeat Step 5.4.5 once for a total of 2 washing steps.

- 5.4.7. Air dry the beads for up to 5 minutes while the tube is on the magnet with 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.

- 5.4.8. Remove the tube from the magnet. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube and incubate for **2 minutes at room temperature**. Place the tube on the magnetic rack until the solution is clear.
- 5.4.9. Remove 50 µl of the supernatant and transfer to a clean nuclease free PCR tube.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

5.5. End Prep of cDNA Library

- 5.5.1. Assemble the end prep reaction **on ice** by adding the following components to the second strand synthesis product from Step 5.4.9.

END PREP REACTION	VOLUME
Second Strand Synthesis Product (Step 5.4.9)	50 µl
● (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
● (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

- 5.5.2. 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.

- 5.5.3. Incubate the sample in a thermocycler with the heated lid set at $\geq 75^{\circ}\text{C}$ as follows.

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C .

- 5.5.4. Proceed immediately to Adaptor Ligation.

5.6. Adaptor Ligation



- 5.6.1. Dilute the NEBNext Unique Dual Index UMI RNA Adaptor* prior to setting up the ligation reaction in ice-cold UMI Adaptor Dilution Buffer and keep the diluted adaptor on ice.

PURIFIED RNA	DILUTION REQUIRED
100 ng–11 ng	No dilution
10 ng–1 ng	10-fold dilution in UMI Adaptor Dilution Buffer

*The UMI RNA Adaptors and UMI Adaptor Dilution Buffer must be purchased separately (NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416). Do not use the Adaptor Dilution Buffer provided with the Ultra II Directional RNA Library Prep Kit for diluting Unique Dual Index UMI Adaptors.

- 5.6.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 5.5.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 5.5.4)	60 µl
Diluted Adaptor (Step 5.6.1)	5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	96 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

- 5.6.3. Set a 100 μ l or 200 μ l pipette to 80 μ l 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.

- 5.6.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermocycler.
5.6.5. Add 3 μ l • (blue) USER Enzyme to the ligation mixture from Step 5.6.4, resulting in total volume of 99 μ l.
5.6.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to $\geq 45^\circ\text{C}$.
5.6.7. Proceed immediately to Purification of Ligation Reaction.

5.7. Purification of Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

- 5.7.1. Add 70 μ l (0.7X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
5.7.2. Incubate for **10 minutes at room temperature**.
5.7.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments. (**Caution: do not discard beads**).
5.7.4. Add 200 μ l of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
5.7.5. Repeat Step 5.7.4 once for a total of 2 washing steps.
5.7.6. Briefly spin the tube and put the tube back in the magnetic rack.
5.7.7. Completely remove the residual ethanol and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack 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.
5.7.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 22 μ l 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube and incubate for **2 minutes at room temperature**. Put the tube on the magnet until the solution is clear.
5.7.9. Without disturbing the bead pellet, transfer 20 μ l of the supernatant to a clean PCR tube and proceed to PCR enrichment.



Note: If you need to stop at this point in the protocol samples can be stored at -20°C.

5.8. PCR Enrichment of Adaptor Ligated DNA

5.8.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 5.7.9)	20 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
NEBNext Primer Mix*	5 µl
Total Volume	50 µl

* NEBNext Primer Mix is provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416).

5.8.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

5.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 5.8.3A and Table 5.8.3B):

Table 5.8.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	7–15*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. The recommendation of PCR cycles are based on internal tests for FFPE RNA.

** It is important to limit the number of PCR cycles to avoid overamplification.

If overamplification occurs, a second peak ~1,000 bp will appear on the Bioanalyzer trace (See Figure 7.1 on page 44).

Table 5.8.3B: Recommended PCR cycles based on input amount:

rRNA DEPLETED FFPE RNA (QUANTIFIED AFTER rRNA PURIFICATION)	RECOMMENDED PCR CYCLES
100 ng	7–8
50 ng	8–9
10 ng	10–12
1 ng	13–15

Note: PCR cycles are recommended based on internally tested FFPE RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

5.9. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

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

5.9.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

5.9.3. Incubate for **5 minutes at room temperature**.

5.9.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

5.9.6. Repeat Step 5.9.5 once for a total of 2 washing steps.

5.9.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack 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.

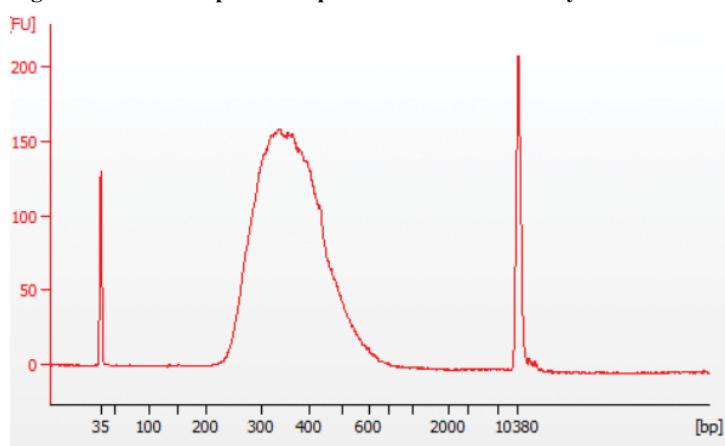
- 5.9.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube in a microcentrifuge and incubate for **2 minutes at room temperature**. Place the tube on the magnetic rack until the solution is clear.
- 5.9.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at -20°C.

5.10. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

- 5.10.1. Use a Bioanalyzer or Tape Station to determine the size distribution and concentration of the libraries.
- 5.10.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the bioanalyzer traces, bring up the sample volume (from Step 5.9.9) to 50 µl with 0.1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 5.9).

Figure 5.10.1. Example of a representative RNA library size distribution on a Bioanalyzer.



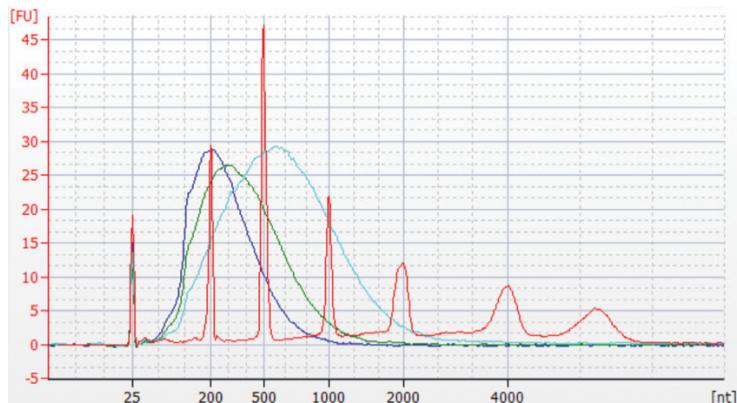
Section 6

Appendix A

6.1. Fragmentation

Note: These recommendations have been optimized using Universal Human Reference Total RNA. Other types of RNA may require different fragmentation times.

Figure 6.1. Modified fragmentation times for longer RNA inserts.



- Red Ladder
Blue 150-300 bp, mRNA fragmented for 15 minutes at 94°C
Green 200-500 bp mRNA fragmented for 10 minutes at 94°C
Cyan 400-1,000 bp mRNA fragmented for 5 minutes at 94°C

Modified fragmentation times for longer RNA inserts. Bioanalyzer traces of RNA as shown in an RNA Pico Chip. mRNA isolated from Universal Human Reference RNA and fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 5, 10 or 15 minutes, and purified using 2.2X volume of Agencourt® RNAClean® XP Beads. For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes and remember to increase the incubation at 42°C from 15 to 50 minutes during the first strand cDNA synthesis reaction.

6.2. Size Selection of Adaptor Ligated DNA

Note: Size selection should be done after adaptor ligation and USER digestion.



The size selection protocol is based on a starting volume of 96 µl. Size selection conditions were optimized with SPRIselect Beads and NEBNext Sample Purification Beads; however, AMPure XP Beads can be used following the same conditions. If using Ampure XP Beads, please allow the beads to warm to room temperature for at least 30 minutes before use.



Please adjust recommended bead volumes for each target size according to Table 6.2. The protocol below is for libraries with a 300 bp insert size (450 bp final library size).

Table 6.2: Recommended size selection conditions for libraries with insert sizes larger than 300 bp.



Note: Size selection for < 100 ng total RNA input is not recommended.

LIBRARY PARAMETER	APPROXIMATE INSERT SIZE	300 bp	400 bp	450 bp
	Approx. Final Library Size	450 bp	550 bp	600 bp
BEAD VOLUME TO BE ADDED (µl)	1 st Bead Selection	25	20	15
	2 nd Bead Selection	10	10	10

Note: Any differences in insert sizes between the Agilent Bioanalyzer and that obtained from paired end sequencing can be attributed to the higher clustering efficiency of smaller sized fragments.

- 6.2.1. Vortex SPRiselect Beads or NEBNext Sample Purification Beads to resuspend.
 - 6.2.2. Add 25 µl of resuspended beads to the 99 µl ligation reaction. Mix well by pipetting up and down at least 10 times.
 - 6.2.3. Incubate for **5 minutes at room temperature**.
 - 6.2.4. Place the tube on an appropriate magnetic rack 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 rack. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube (**Caution: do not discard the supernatant**). Discard the beads that contain the unwanted large fragments.
 - 6.2.5. Add 10 µl resuspended beads to the supernatant, mix well by pipetting up and down at least 10 times and incubate for 5 minutes at room temperature.
 - 6.2.6. Place the tube/plate on an appropriate magnetic rack 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 rack. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets (**Caution: do not discard beads**).
 - 6.2.7. Add 200 µl of 80% freshly prepared ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
 - 6.2.8. Repeat Step 6.2.7 once for a total of 2 washing steps.
 - 6.2.9. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic rack 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.**
- 6.2.10. Remove the tube/plate from the magnetic rack. Elute the DNA target from the beads by adding 22 µl of 0.1 X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube and incubate for **2 minutes at room temperature**.
 - 6.2.11. Place the tube on a magnetic rack. After the solution is clear (about 5 minutes), transfer 20 µl to a new PCR tube for amplification.

6.3. PCR Enrichment of Size-selected Libraries

Note: Size-selected libraries require 2 additional PCR cycles due to loss during size selection steps compared to non-size-selected libraries.

- 6.3.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 6.2.11)	20 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
NEBNext Primer Mix*	5 µl
Total Volume	50 µl

* NEBNext Primer Mix is provided in NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416).

- 6.3.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

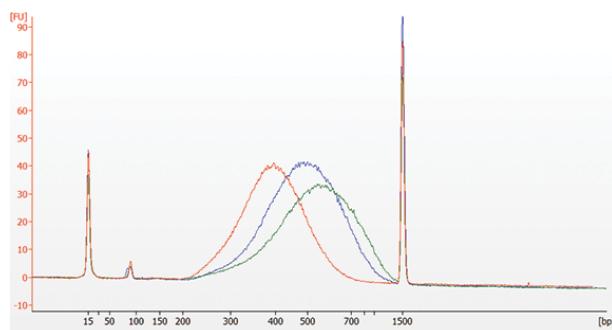
6.3.3. Place the tube in a thermocycler with the heated lid set to 105°C. Perform PCR amplification using the following PCR cycling conditions:

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

* The number of PCR cycles should be adjusted based on RNA input. Size-selected libraries require additional 2 PCR cycles and should be adjusted accordingly. For example if a non-size selected library requires 8 PCR cycles, the size-selected library should be amplified for 10 cycles (8 + 2) after the size selection.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.1 on page 44).

Figure 6.3: Bioanalyzer traces of size selected DNA libraries.



50 ng mRNA was fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 15, 10 or 5 minutes. Libraries were size-selected as described in Table 6.2, then amplified by PCR, and run on Agilent Bioanalyzer DNA 1000 chip. Fragmentation times and corresponding size selection conditions are shown in the table below.

Table 6.3:

LIBRARY SAMPLE	FRAGMENTATION TIME	1 st BEAD SELECTION	2 nd BEAD SELECTION
Red	10 minutes	25 μ l	10 μ l
Blue	5 minutes	20 μ l	10 μ l
Green	5 minutes	15 μ l	10 μ l

For libraries with longer inserts (> 200 bp), remember to increase the incubation at 42°C from 15 to 50 minutes during the First Strand cDNA Synthesis reaction.

Section 7

Troubleshooting Guide

OBSERVATIONS	POSSIBLE CAUSES	EFFECT	SUGGESTED SOLUTIONS
Presence of Bioanalyzer peaks < 85 bp (Figure 7.1)	<ul style="list-style-type: none"> • Presence of Primers remaining after PCR clean up 	Primers cannot cluster or be sequenced, but can bind to flowcell and reduce cluster density	<ul style="list-style-type: none"> • Clean up PCR reaction again with 0.9X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield)
Presence of ~150 bp adaptor-dimer Bioanalyzer peak (Figure 7.2)	<ul style="list-style-type: none"> • Addition of non-diluted adaptor • RNA input was too low • RNA was over fragmented or lost during fragmentation • Inefficient Ligation 	Adaptor-dimer will cluster and be sequenced. If ratio is low compared to library, may not be a problem but some reads will be dimers.	<ul style="list-style-type: none"> • Dilute adaptor before setting up ligation reaction • Clean up PCR reaction again with 0.9X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield)
Presence of additional Bioanalyzer peak at higher molecular weight than the expected library size (~ 1,000 bp) (Figure 7.1)	<ul style="list-style-type: none"> • PCR artifact (over-amplification). Represents single-stranded library products that have self-annealed. If the PCR cycle number (or PCR input amount) is too high; in the late cycles of PCR the primers become limiting. Therefore, the adaptor sequences on either end of the fragment anneal to each other. This creates heteroduplexes with different insert sequences that run slower in the Bioanalyzer. 	If ratio is low compared to library, may not be a problem for sequencing	<ul style="list-style-type: none"> • Reduce number of PCR cycles.
Broad library size distribution	<ul style="list-style-type: none"> • Under-fragmentation of the RNA 	Library size will contain longer insert sizes	<ul style="list-style-type: none"> • Increase RNA fragmentation time

Figure 7.1:

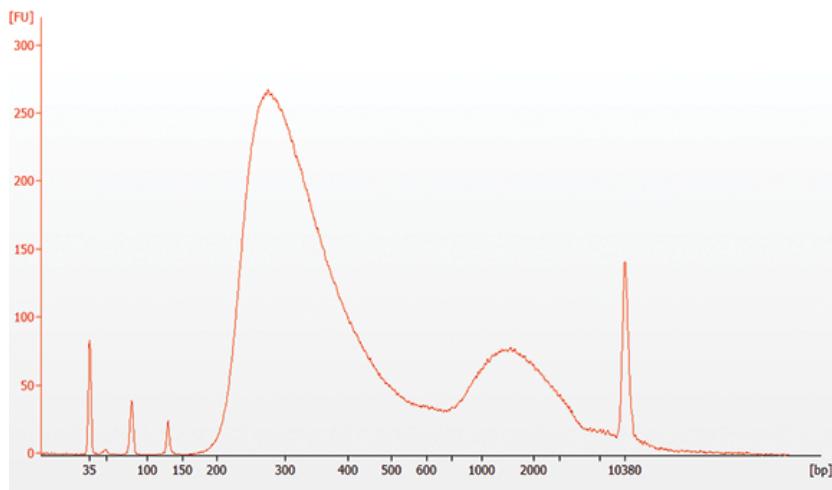
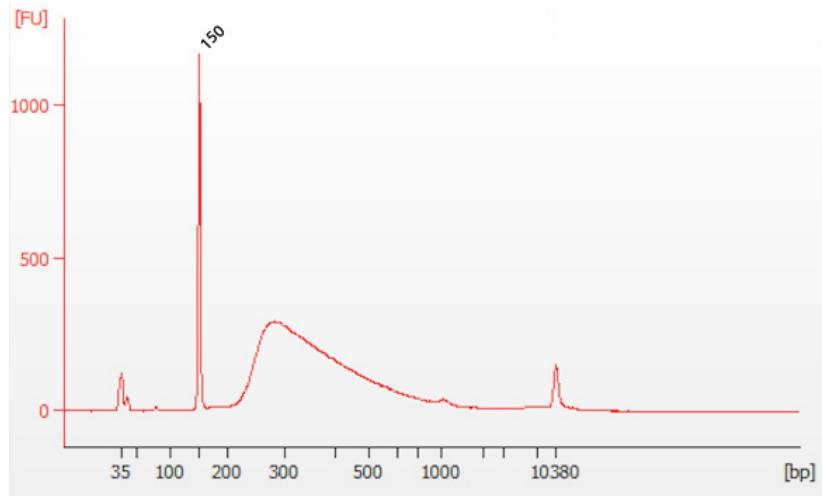


Figure 7.2:



Kit Components

Each set of reagents is functionally validated and compared to the previous lot through construction of libraries using the minimum and maximum amount of Universal Human Reference Total RNA. The previous and current lots are sequenced together on the same Illumina flow cell and compared across various sequence metrics including individual transcript abundances, 5' → 3' transcript coverage, and fraction of reads mapping to the reference

NEB #E7760S Table of Components

NEB #	PRODUCT	VOLUME
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7421A	NEBNext First Strand Synthesis Reaction Buffer	0.192 ml
E7422A	Random Primers	0.048 ml
E7425A	NEBNext Second Strand Synthesis Enzyme Mix	0.096 ml
E7426A	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.192 ml
E7428A	NEBNext USER Enzyme	0.072 ml
E7646A	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
E7647A	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.720 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml
E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
E7762A	NEBNext Adaptor Dilution Buffer	2.4 ml
E7763A	(0.1X) TE Buffer	2.78 ml
E7764A	Nuclease-free Water	1.25 ml
E7766A	NEBNext Strand Specificity Reagent	0.192 ml

NEB #E7760L Table of Components

NEB #	PRODUCT	VOLUME
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7421AA	NEBNext First Strand Synthesis Reaction Buffer	0.768 ml
E7422AA	Random Primers	0.192 ml
E7425AA	NEBNext Second Strand Synthesis Enzyme Mix	0.384 ml
E7426AA	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.768 ml
E7428AA	NEBNext USER Enzyme	0.288 ml
E7646AA	NEBNext Ultra II End Prep Enzyme Mix	0.288 ml
E7647AA	NEBNext Ultra II End Prep Reaction Buffer	0.672 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	2 x 1.2 ml
E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
E7762AA	NEBNext Adaptor Dilution Buffer	9.6 ml
E7763AA	(0.1X) TE Buffer	11.5 ml
E7764AA	Nuclease-free Water	5.7 ml
E7766AA	NEBNext Strand Specificity Reagent	0.768 ml

NEB #E7765S Table of Components

NEB #	PRODUCT	VOLUME
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7421A	NEBNext First Strand Synthesis Reaction Buffer	0.192 ml
E7422A	Random Primers	0.048 ml
E7425A	NEBNext Second Strand Synthesis Enzyme Mix	0.096 ml
E7426A	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.192 ml
E7428A	NEBNext USER Enzyme	0.072 ml
E7646A	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
E7647A	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.720 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml
E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
E7762A	NEBNext Adaptor Dilution Buffer	2.4 ml
E7763A	(0.1X) TE Buffer	2.78 ml
E7764A	Nuclease-free Water	1.25 ml
E7766A	NEBNext Strand Specificity Reagent	0.192 ml
E7767S	NEBNext Sample Purification Beads	8.65 ml

NEB #E7765L Table of Components

NEB #	PRODUCT	VOLUME
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7421AA	NEBNext First Strand Synthesis Reaction Buffer	0.768 ml
E7422AA	Random Primers	0.192 ml
E7425AA	NEBNext Second Strand Synthesis Enzyme Mix	0.384 ml
E7426AA	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.768 ml
E7428AA	NEBNext USER Enzyme	0.288 ml
E7646AA	NEBNext Ultra II End Prep Enzyme Mix	0.288 ml
E7647AA	NEBNext Ultra II End Prep Reaction Buffer	0.672 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	2 x 1.2 ml
E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
E7762AA	NEBNext Adaptor Dilution Buffer	9.6 ml
E7763AA	(0.1X) TE Buffer	11.5 ml
E7764AA	Nuclease-free Water	5.7 ml
E7766AA	NEBNext Strand Specificity Reagent	0.768 ml
E7767L	NEBNext Sample Purification Beads	4 x 8.65 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	7/20

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