INSTRUCTION MANUAL

NEBNext UltraExpress™ RNA Library Prep Kit

NEB #E3330S/L

24/96 reactions

Version 2.0_2/24

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The Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3330S) and 96 reactions (NEB #E3330L)

Package 1: Store at –20°C.

- (lilac) NEBNext UltraExpress RNA Fragmentation Mix
- (lilac) NEBNext UltraExpress First Strand Enzyme Mix
- (brown) NEBNext UltraExpress Strand Specificity Reagent
- (orange) NEBNext UltraExpress Second Strand Master Mix
- (green) NEBNext UltraExpress End Prep Enzyme Mix
- (green) NEBNext UltraExpress End Prep Reaction Buffer
- (red) NEBNext UltraExpress Ligation Master Mix
- (blue) NEBNext UltraExpress USER® Enzyme
- (blue) NEBNext MSTC High Yield Master Mix
- (white) NEBNext Adaptor Dilution Buffer
- (white) 0.1X TE
- (white) Nuclease-free Water
- (white) NEBNext Bead Reconstitution Buffer

Required Materials Not Included

- NEBNext Multiplex Oligos for Illumina®
- NEBNext Multiplex Oligos options can be found at www.neb.com/oligos. Alternatively, customer supplied adaptor and primers can be used; please see information in link below: https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb
- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Magnetic Rack (NEB S1515S, Alpaqua® cat. #A001322, or equivalent)
- 80% Ethanol (freshly prepared)
- Thermal cycler
- DNase-, RNase-free PCR strip tubes, for example TempAssure® PCR flex-free 8-tube strips (USA Scientific® #1402-4708)
- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables

For use with NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490):

- 1.5 ml Microcentrifuge tube and NEB #S1506 Magnet stand or equivalent (for washing beads only)

For use with NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400) and other NEBNext RNA depletion kits that do not include beads (NEB #E7750, E7850, E7865):

- Agencourt® RNAClean® XP Beads (Beckman Coulter, Inc. #A63987)

Considerations on Selecting Poly(A) mRNA Enrichment or rRNA Depletion

The library preparation protocol should be chosen based on the goals of the project and the quality of the RNA sample. Total cellular RNA is mainly composed of ribosomal RNA (rRNA) and often is not of interest. rRNA can be removed from total cellular RNA with either 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 v2 (Human/Mouse/Rat) with or without RNA Sample Purification Beads (NEB #E7400/ #E7405) 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 rRNA, 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 ≤ 7, FFPE RNA), the NEBNext rRNA Depletion Kit should be used.

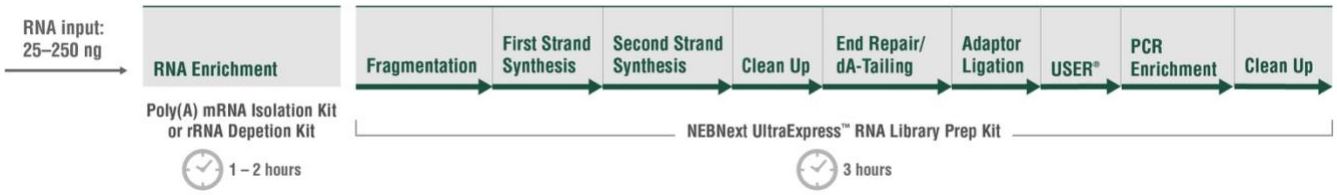
Overview

The NEBNext UltraExpress RNA Library Prep Kit contains the enzymes and buffers required to to rapidly convert 25–250 ng of total RNA into high-quality libraries for next-generation sequencing on the Illumina platform. The fast, simple workflow features minimal hands-on time and allows use of a single adaptor dilution and PCR cycling condition across the entire input range. In addition to the standard protocol, an appendix is included that details customized adaptor and cycling recommendations for varying RNA input amounts, if further optimization for library yields is required. If a stopping point is required between mRNA enrichment/ribosomal RNA depletion and library preparation, please refer to the FAQs tab for considerations and recommendations.

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

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

Figure:1 NEBNext UltraExpress RNA Workflow



Section 1
Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

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

●

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 (UHRR).

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 Poly(A) 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., Mg^{2+}), or guanidinium salts, divalent cation chelating agents (e.g., EDTA or 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 I 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

25–250 ng DNA-free total RNA quantified by Qubit® Fluorometer or spectrophotometer and quality checked by Bioanalyzer.
The protocol is optimized for approximately 200 bp RNA inserts.

Keep all the buffers on ice, unless otherwise indicated.

Prior to Starting Enrichment

Remove the following reagents from –20°C freezer to thaw on ice:

- NEBNext UltraExpress RNA Fragmentation Mix
- NEBNext UltraExpress Second Strand Master Mix (Note: do not vortex reagent, mix only by inversion)

1.1. Preparation of 1X Fragmentation Mix for RNA elution

1.1.1 Thaw the Fragmentation Master Mix (2X) and prepare 1X composition per sample as follows:

COMPONENT	VOLUME
• (lilac) NEBNext UltraExpress RNA Fragmentation Mix	4 µl
Nuclease-free Water	4 µl

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

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 from the table below 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. Include at least one reaction overage if preparing beads in bulk as mixing buffer can cause frothing that results in volume loss (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.

Note: Vortex all buffers before use. Do not vortex RNA or beads. Mix the beads well before using by flicking or inverting the tube to ensure a homogeneous suspension.

COMPONENT	VOLUME PER ONE LIBRARY
Oligo dT Beads $d(T)_{25}$	20 µl
• NEBNext 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 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 NEBNext RNA Binding Buffer (2X) to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 100 µl NEBNext 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 NEBNext 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 NEBNext RNA Binding Buffer (2X) per sample.

1.2.11. Add 50 µl of well-mixed bead slurry to each RNA sample from Step 1.2.1. Mix thoroughly by pipetting up and down six times. This first binding step removes most of the non-target RNA.

1.2.12. Heat the sample to denature the RNA and facilitate binding of the mRNA to the beads. Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program:
5 minutes at 65°C
Hold at 4°C

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 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 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. For the first elution of the mRNA from the beads. Place in a thermocycler, with the heated lid set to ≥ 90°C, and run the following program:
2 minutes at 80°C
Hold at 25°C

1.2.25. Remove the tube from the thermocycler when the temperature reaches 25°C.

1.2.26. Add 50 µl of NEBNext 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 the supernatant from the tube. Take care not to disturb the beads that contain the mRNA.

Note: It is important to remove all 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 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, see Appendix D.

1.2.36. To elute the mRNA from the beads and fragment, add 6.5 µl of the 1X Fragmentation Master Mix (from step 1.1.1.) pipette up and down six times to resuspend the beads.

1.2.37 Place in a thermocycler, with the heated lid set at 105°C, and run the following program:
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 5 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note 1: If the supernatant volume recovered is less than 5 µl for any reason, bring the volume up to 5 µl by adding additional 1X Fragmentation Master Mix 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.)	5 µl
• (brown) NEBNext UltraExpress Strand Specificity Reagent	4 µl
• (lilac) NEBNext UltraExpress First Strand Enzyme Mix	1 µl
Total Volume	**10 µl**

1.3.2. If processing multiple samples, prepare a master mix.

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

1.3.4. Place in a thermocycler, with the heated lid set to ≥ 80°C, and run the following program:
10 minutes at 25°C
10 minutes at 42°C
5 minutes at 70°C
Hold at 4°C

1.3.5. Immediately perform Second Strand cDNA Synthesis.

1.4. Second Strand cDNA Synthesis

1.4.1. Thaw the second strand master mix on ice and mix by inverting tube ten times, perform a quick spin to collect all liquid from the sides of the tube and place back on ice. Add the following components into the first strand synthesis reaction product from Step 1.3.5.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 1.3.5.)	10 µl
• (orange) NEBNext UltraExpress Second Strand Master Mix	30 µl
Total Volume	**40 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. Place in a thermocycler, with the heated lid set at ≤ 40°C (or off), and run the following program:
30 minutes at 16°C
Hold at 4°C

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

Note: If using AMPure Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

1.5.1. Vortex SPRIselect Beads or AMPure Beads to resuspend.

1.5.2. Add 72 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~40 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Alternatively, vortex on high for 3–5 seconds. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

1.5.3. Incubate samples on bench top for 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 two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

1.5.7. 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/plate from the magnetic stand. Elute the DNA target from the beads by adding 22 µl 0.1X TE Buffer (provided) to the beads.

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

1.5.10. Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 20 µl to a new 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 end prep reactions on ice. Add the following components to second strand synthesis product from Step 1.5.10.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 1.5.10)	20 µl
• (green) NEBNext UltraExpress End Prep Reaction Buffer	2.5 µl
• (green) NEBNext UltraExpress End Prep Enzyme Mix	1.5 µl

1.6.2. If processing multiple samples, prepare a master mix.

1.6.3. Set a 20 µl or 100 µl pipette to 20 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.

1.6.4. Place in a thermocycler, with the heated lid set at ≥ 75°C, and run the following program:
5 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

1.6.5. Proceed immediately to Adaptor Ligation.

1.7. Adaptor Ligation

1.7.1. Dilute the • (red) NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
25–250 ng	50-fold dilution in Adaptor Dilution Buffer

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

Note: If customized adaptor titration per input is preferred use recommendations in Table 1 Appendix A.

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 1.6.5.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 1.6.5.)	24 µl
Diluted Adaptor (Step 1.7.1.)	2 µl
• (red) NEBNext UltraExpress Ligation Master Mix	12 µl
Total Volume	**38 µl**

Do not premix the Ligation Master Mix and adaptor prior to use in the Adaptor Ligation Step.

1.7.3. Set a 100 µl pipette to 35 µl and then pipette the entire volume up at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

⚠

Caution: NEBNext UltraExpress 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. Place in a thermocycler, with the heated lid set at ≤ 40°C (or off), and run the following program:
15 minutes at 20°C
Hold at 4°C

1.7.5 Add 2 µl (• blue) NEBNext UltraExpress USER Enzyme to the ligation mixture from Step 1.7.4, resulting in total volume of 40 µl. Mix well by gently pipetting up and down ten times at 35 µl volume.

1.7.6 Place in a thermocycler, with the heated lid set at ≥ 45°C, and run the following program:
5 minutes at 37°C
Hold at 4°C

1.7.7 Proceed immediately to PCR Enrichment of Adaptor Ligated DNA.

1.8. PCR Enrichment of Adaptor Ligated DNA

⚠

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

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

1.8.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

1.8.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 1.7.7.)	40 µl
• (blue) NEBNext MSTC High Yield Master Mix	50 µl
Index (X) Primer/i7 Primer*, **	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	**100 µl**

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

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

1.8.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 1.7.7.)	40 µl
• (blue) NEBNext MSTC High Yield Master Mix	50 µl
Index Primer Mix*	10 µl
Total Volume	**100 µl**

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

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

1.8.3. 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 1.8.3A):

Table 1.8.3A:

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

** PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality. If using customized cycling conditions per input is preferred, use recommendations in Table 2.1 in Appendix C.

Note: Take out NEBNext Bead Reconstitution Buffer and bring up to room temperature prior to Phased Bead Cleanup. Allow the buffer (and beads if using AMPure XP) to warm to room temperature for at least 30 minutes before use.

1.9. Phased Bead Cleanup of PCR Reaction

Note: The SPRIselect/AMPure Beads ratio recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g. post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

1.9.1. Vortex SPRIselect Beads or AMPure Beads to resuspend.

1.9.2. Add 70 µl (0.7X) of resuspended beads to the PCR reaction (~100 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

1.9.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

1.9.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

1.9.6. Remove the tube/plate from the magnetic stand (Note: do not need ethanol wash at this step). Add 50 µl 0.1 X TE and resuspend the beads by pipetting. Add another 40 µl (0.8X) NEBNext Bead Reconstitution Buffer to the 50 µl resuspended beads. Mix well by pipetting up and down at least 10 times. **Be careful to expel all the liquid out of the tip during the last mix.** Vortex for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

1.9.8. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

1.9.9. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

1.9.10. Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.9.11. Repeat Step 1.9.10. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

1.9.12. 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.

1.9.13. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads.

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

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

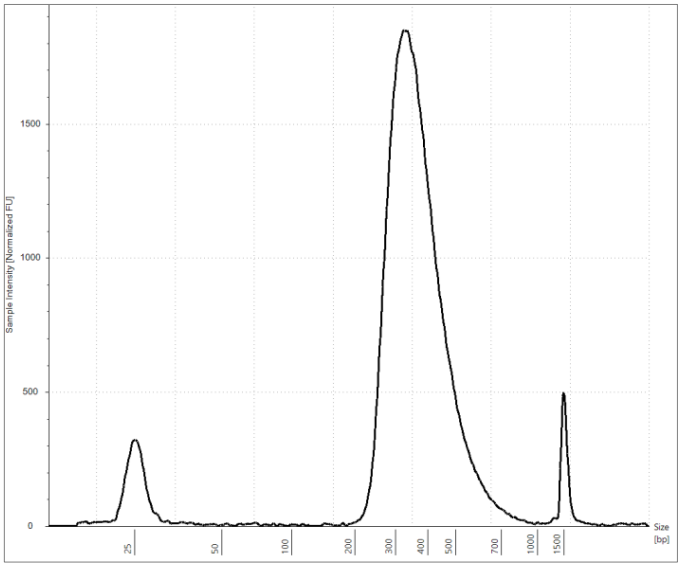
1.10. Assess Library Quality on an Agilent Bioanalyzer DNA Chip or TapeStation

1.10.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA chip.

1.10.3. 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 traces, bring up the sample volume (from Step 1.9.15) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step using 0.9X (45 µl) standard bead clean-up.

Figure 1.10.3.1 Example of library made using 250ng of UHRR on a TapeStation.



Section 2
Protocol for use with NEBNext rRNA Depletion Kits (NEB #E7400, #E7405)

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

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

●

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

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

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). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 (current section). For highly degraded samples (e.g., FFPE) which do not require fragmentation, follow the library preparation protocol recommendations in Appendix B.

RNA Purity

The RNA sample should be free of salts (e.g., Mg^{2+}, or 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. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free™ Kit TURBO™ DNase Treatment and Removal Reagents do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

25–250 ng DNA-free total RNA quantified by Qubit Fluorometer or spectrophotometer and quality checked by Bioanalyzer.
The protocol is optimized for approximately 200 bp RNA inserts.

Prior to Starting Enrichment

Remove the following components to thaw on ice:

- NEBNext UltraExpress RNA Fragmentation Mix
- NEBNext UltraExpress Second Strand Master Mix (Note: do not vortex reagent, mix only by inversion)

1.1. Preparation of 1X Fragmentation Mix for RNA elution

1.1.1. Thaw the Fragmentation Master Mix (2X) and prepare 1X composition as follows:

COMPONENT	VOLUME
• (lilac) NEBNext UltraExpress RNA Fragmentation Mix	4 µl
Nuclease-free Water	4 µl

Note 1: Keep the mix on ice until RNA is purified. It will be used in Step 2.4.9.

2.1. Probe Hybridization to RNA

2.1.1. Dilute 25–250 ng of total RNA with Nuclease-free Water to a final volume of 11 µl in a PCR tube. Keep the RNA on ice.

2.1.2. Assemble the following RNA/Probe hybridization reaction on ice:

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (25–250 ng)	11 µl
◦ (white) NEBNext v2 rRNA Depletion Solution	2 µl
◦ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

2.1.3. Mix thoroughly by pipetting up and down at least 10 times. Note: It is crucial to mix well at this step.

2.1.4. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

2.1.5. Place tube in a pre-heated thermocycler and run the following program with the heated lid set to 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

2.1.6. Briefly spin down the tube in a microcentrifuge and place on ice. Proceed immediately to the RNase H Digestion.

2.2. RNase H Digestion

2.2.1. Assemble the following RNase H digestion reaction on ice:

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 2.1.6)	15 µl
◦ (white) RNase H Reaction Buffer	2 µl
◦ (white) NEBNext Thermostable RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

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

2.2.3. Briefly spin down the tube in a microcentrifuge.

2.2.4. Place in a pre-heated thermocycler, with the heated lid set to 55°C, and run the following program:
30 minutes at 50°C
Hold at 4°C

2.2.5. Briefly spin down the tube in a microcentrifuge and place on ice. Proceed immediately to DNase I Digestion.

2.3. DNase I Digestion

2.3.1. Assemble the following DNase I digestion reaction on ice:

DNASE I DIGESTION REACTION	VOLUME
RNase H treated RNA (Step 2.2.5)	20 µl
◦ (white) DNase I Reaction Buffer	5 µl
◦ (white) NEBNext DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

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

2.3.3. Briefly spin down the tube in a microcentrifuge.

2.3.4. Place in a thermocycler, with the heated lid set at ≥ 45°C, and run the following program:
30 minutes at 37°C
Hold at 4°C

2.3.5. Briefly spin down the tube 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 Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads to resuspend.

2.4.2. Add 90 µl (1.8X) beads to the RNA sample from Step 2.3.5 and mix thoroughly by pipetting up and down at least 10 times.

2.4.3. Incubate for 15 minutes on ice to bind RNA to the beads.

2.4.4. Place the tube on a magnetic rack to separate the beads from the supernatant.

2.4.5. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA.

2.4.6. 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.

2.4.7. Repeat Step 2.4.6 once for a total of two washes.

2.4.8. 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.

2.4.9. Remove the tube from the magnetic rack. Resuspend the RNA from the beads by adding 6.5 µl of Fragmentation Master Mix (1X). Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube. Incubate samples for 2 minutes on bench top following elution and proceed to on-bead fragmentation.

Note: The next step provides a fragmentation incubation time resulting in an RNA insert size of ~ 200 nt. For RNA insert sizes > 200 nt, see Appendix D.

2.4.13. Place in a thermocycler, with the heated lid set at 105°C, and run the following program:
15 minutes at 94°C
Hold at 4°C

2.4.14. 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).

2.4.15. Collect the fragmented RNA by transferring 5 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note 1: If the supernatant volume recovered is less than 5 µl for any reason, bring the volume up to 5 µl by adding additional 1X Fragmentation Master Mix and continue with the protocol.

Note 2: Avoid transferring any of the magnetic beads.

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

2.5. First Strand cDNA Synthesis

2.5.1. Assemble the first strand synthesis reaction on ice by adding the following components to the fragmented and primed RNA from Step 2.4.16.:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 2.4.16.)	5 µl
• (brown) NEBNext UltraExpress Strand Specificity Reagent	4 µl
• (lilac) NEBNext UltraExpress First Strand Enzyme Mix	1 µl
Total Volume	**10 µl**

2.5.2. If processing multiple samples, prepare a master mix.

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

2.5.4. Place in a thermocycler, with the heated lid set at ≥ 80°C, and run the following program:
10 minutes at 25°C
10 minutes at 42°C
5 minutes at 70°C
Hold at 4°C

2.5.5. Proceed directly to Second Strand cDNA Synthesis.

2.6. Second Strand cDNA Synthesis

2.6.1. Thaw the second strand master mix on ice and mix by inverting tube ten times, perform a quick spin to collect all liquid from the sides of the tube and place back on ice. Add the following components into the first strand synthesis reaction product from Step 2.5.5.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 2.5.5.)	10 µl
• (orange) NEBNext UltraExpress Second Strand Master Mix	30 µl
Total Volume	40 µl

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

2.6.3. Place in a thermocycler, with the heated lid set at ≤ 40°C (or off), and run the following program:
30 minutes at 16°C
Hold at 4°C

2.7. Purification of double-stranded cDNA using SPRIselect Beads or AMPure Beads

Note: If using AMPure Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

2.7.1 Vortex SPRIselect Beads or AMPure Beads to resuspend.

2.7.2. Add 72 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~40 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

2.7.4. 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**).

2.7.5. 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.

2.7.6. Repeat Step 2.7.5. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

2.7.7. 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.

2.7.8. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 22 µl 0.1X TE Buffer (provided) to the beads.

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

2.7.10. Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 20 µl to a new PCR tube.

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

2.8. End Prep of cDNA Library

2.8.1 Thaw the end prep master mix on ice. Add the following components to second strand synthesis product from Step 2.7.10.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 2.7.10.)	20 µl
• (green) NEBNext UltraExpress End Prep Reaction Buffer	2.5 µl
• (green) NEBNext UltraExpress End Prep Enzyme Mix	1.5 µl

2.8.2 If processing multiple samples, prepare a master mix.

2.8.3. Set a 20 µl or 100 µl pipette to 20 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.

2.8.4. Place in a thermocycler, with the heated lid set at ≥ 75°C, and run the following program:
5 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

2.8.5. Proceed immediately to Adaptor Ligation.

2.9. Adaptor Ligation

2.9.1. Dilute the • (red) NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
25–250 ng	50-fold dilution in Adaptor Dilution Buffer

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

Note: If customized adaptor titration per input is preferred use recommendations in Table 1 Appendix A.

2.9.2. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 2.8.5.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 2.8.5.)	24 µl
Diluted Adaptor (Step 2.9.1.)	2 µl
• (red) NEBNext UltraExpress Ligation Master Mix	12 µl
Total Volume	**38 µl**

Do not premix the Ligation Master Mix and adaptor prior to use in the Adaptor Ligation Step.

2.9.3. Set a 100 µl pipette to 35 µl and then pipette the entire volume up and down at moderate speed at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

⚠

Caution: NEBNext UltraExpress 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.

2.9.4. Place in a thermocycler, with the heated lid set at ≤ 40°C (or off), and run the following program:
15 minutes at 20°C
Hold at 4°C

2.9.5 Add 2 µl (• blue) NEBNext UltraExpress USER Enzyme to the ligation mixture from Step 2.9.4, resulting in total volume of 40 µl. Mix well by gently pipetting up and down ten times at 35 µl volume.

2.9.6 Place in a thermocycler, with the heated lid set at ≥ 45°C, and run the following program
37°C for 5 minutes
Hold at 4°C

2.9.7 Proceed immediately to PCR Enrichment of Adaptor Ligated DNA.

2.10. PCR Enrichment of Adaptor Ligated DNA

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

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i5and i7) primers combined.

2.10.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

2.10.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.9.7.)	40 µl
• (blue) NEBNext MSTC High Yield Master Mix	50 µl
Index (X) Primer/i7 Primer*, **	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	**100 µl**

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

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

2.10.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.9.7)	40 µl
• (blue) NEBNext MSTC High Yield Master Mix	50 µl
Index Primer Mix*	10 µl
Total Volume	**100 µl**

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

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

2.10.3. 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 2.10.3A and Table 2.10.3B):

Table 2.10.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	11**
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

** PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality. If using customized cycling conditions per input is preferred, use recommendations in Table 2.2 in Appendix C.

NOTE: Take out NEBNext Bead Reconstitution Buffer and bring up to room temperature prior to Phased Bead Cleanup. Allow the buffer (and beads if using AMPure XP) to warm to room temperature for at least 30 minutes before use.

2.11. Phased Bead Cleanup of PCR Reaction

Note: The SPRIselect/AMPure Bead ratio recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g. post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

2.11.1. Vortex SPRIselect Beads or AMPure Beads to resuspend.

2.11.2. Add 70 µl (0.7X) of resuspended beads to the PCR reaction (~100 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

2.11.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

2.11.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

2.11.6. Remove the tube/plate from the magnetic stand (**Note: do not need ethanol wash at this step**). Add 50 µl 0.1 X TE and resuspend the beads by pipetting. Add another 40 µl (0.8X) NEBNext Bead Reconstitution Buffer to the 50 µl resuspended beads. Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortex for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

2.11.8. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

2.11.9. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

2.11.10 Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.11.11. Repeat Step 2.11.10. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

2.11.7. 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.

2.11.8. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads.

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

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

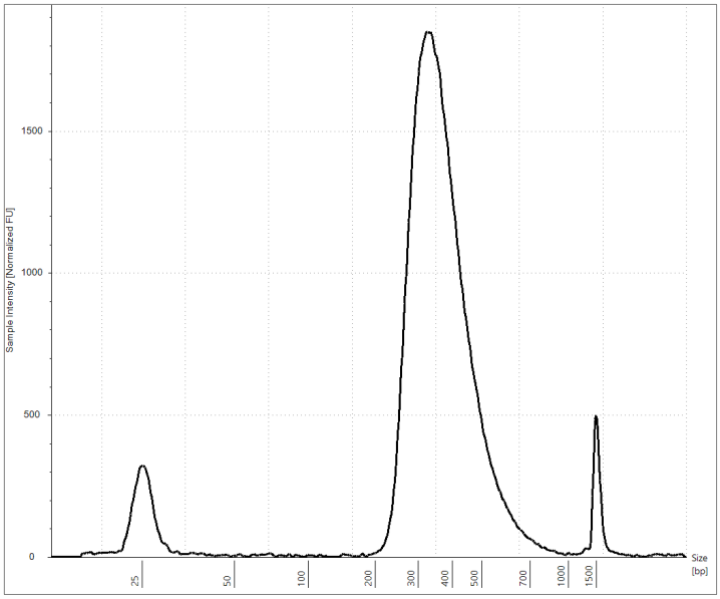
2.12. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

2.12.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

2.12.2. 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 traces, bring up the sample volume (from Step 2.11.10) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step using 0.9X (45 µl) standard bead clean-up.

Figure 2.12.2.1 Example of library made using 250ng of UHRR on a TapeStation.



Appendix A
Recommendations for customized adaptor dilutions and cycling per input

NEBNext UltraExpress RNA Library Prep kit was designed with ease of use and flexibility in mind. This library protocol allows users to use a single adaptor dilution and PCR cycling condition over the entire input range of the kit. In addition to our standard protocol, we have added this appendix detailing customized adaptor and cycling recommendations for varying RNA input amounts, if further optimization for library yields is required.

1. Adaptor Dilutions

1.1. Adaptor Dilutions for poly(A) enriched mRNA or ribosomal RNA depleted total RNA

Table 1

TOTAL RNA INPUT	DILUTION REQUIRED
76–250 ng	20-fold dilution in Adaptor Dilution Buffer
25–75 ng	100-fold dilution in Adaptor Dilution Buffer

2. PCR Enrichment

2.1. PCR recommendations for poly(A) enriched mRNA

Table 2.1 Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
76–250 ng	10–12**
25–75 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.

2.2. PCR recommendations for ribosomal RNA depleted Total RNA

Table 2.2. Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
76–250 ng	9–11**
25–75 ng	11–13**

** Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality.

Appendix B
Recommendations for use with Indexed UMI Adaptor

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

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

●

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

Keep all the buffers on ice, unless otherwise indicated.

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 Poly(A) 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., Mg^{2+}, or guanidinium salts, divalent cation chelating agents (e.g., EDTA or 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. 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

25–250 ng DNA-free total RNA quantified by Qubit Fluorometer or spectrophotometer and quality checked by Bioanalyzer.
The protocol is optimized for approximately 200 bp RNA inserts.

Keep all the buffers on ice, unless otherwise indicated.

*** Please note that this protocol starts with ligation of UMI RNA adaptors (Unique Dual Index UMI Adaptors RNA Set 1; NEB #7416), follow the appropriate enrichment chapter for poly(A) isolation of mRNA or rRNA depletion up to End Prep of cDNA Library prior to using this section.**

1.1. Adaptor Ligation

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

TOTAL RNA INPUT	DILUTION REQUIRED
76–250 ng	4-fold dilution in UMI Adaptor Dilution Buffer
25–76 ng	20-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). Please see www.neb.com/oligos for additional information. Do not use the Adaptor Dilution Buffer provided with the UltraExpress RNA Library Prep Kit for diluting Unique Dual Index UMI Adaptors.

1.1.2. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Section 1 or Section 2.

LIGATION REACTION	VOLUME
End Prepped DNA (Section 1 or Section 2)	24 µl
Diluted UMI Adaptor (Step 1.1.1)	2.0 µl
• (red) NEBNext UltraExpress Ligation Master Mix	12 µl
Total Volume	**40 µl**

Do not premix the Ligation Master Mix and adaptor prior to use in the Adaptor Ligation Step.

1.1.3. Set a 100 µl pipette to 35 µl and then pipette the entire volume up and down at moderate speed at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The NEBNext UltraExpress 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.

1.1.4. Place in a thermocycler, with the heated lid set at ≤ 40°C (or off), and run the following program:
15 minutes at 20°C
Hold at 4°C

1.1.5 Add 2 µl (• blue) NEBNext UltraExpress USER Enzyme to the ligation mixture from Step 1.1.4., resulting in total volume of 40 µl. Mix well by gently pipetting up and down ten times at 35 µl volume.

1.1.6 Place in a thermocycler, with the heated lid set at ≥ 45°C, and run the following program
37°C for 5 minutes
Hold at 4°C

1.1.7 Proceed immediately to PCR Enrichment of UMI Adaptor Ligated DNA.

2.1. PCR Enrichment of UMI Adaptor Ligated DNA

2.1.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

2.1.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
UMI Adaptor Ligated DNA (Step 1.1.7.)	40 µl
NEBNext MSTC High Yield Master Mix	45 µl
NEBNext Primer Mix*	5 µl
Total Volume	**90 µl**

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

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

2.1.3. 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 2.1.3A and Table 2.1.3B):

Table 2.1.3A.

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	9-14*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* PCR amplification cycles are based on RNA enrichment method and total RNA input.

Table 2.1.3B. Recommended PCR cycle for protocol

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES POLY(A)	RECOMMENDED PCR CYCLES DEPLETION
76–250 ng	10–12**	9–11**
25–75 ng	12–14**	11–13**

** PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality.

Note: Take out NEBNext Bead Reconstitution Buffer and bring up to room temperature prior to Phased Bead Cleanup. Allow the buffer (and beads if using AMPure XP) to warm to room temperature for at least 30 minutes before use.

3.1. Phased Bead Cleanup of PCR Reaction

Note: The SPRIselect/AMPure Bead ratio recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

3.1.1. Vortex SPRIselect Beads or AMPure Beads to resuspend.

3.1.2. Add 70 µl (0.7X) of resuspended beads to the PCR reaction (~100 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

3.1.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

3.1.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

3.1.6. Remove the tube/plate from the magnetic stand (Note: do not need ethanol wash at this step). Add 50 µl 0.1X TE and resuspend the beads by pipetting. Add another 40 µl (0.8X) NEBNext Bead Reconstitution Buffer to the 50 µl resuspended beads. Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortex for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

3.1.8. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

3.1.9. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).

3.1.10 Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

3.1.11. Repeat Step 3.1.10. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

3.1.12. 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.

3.1.13. Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads.

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

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

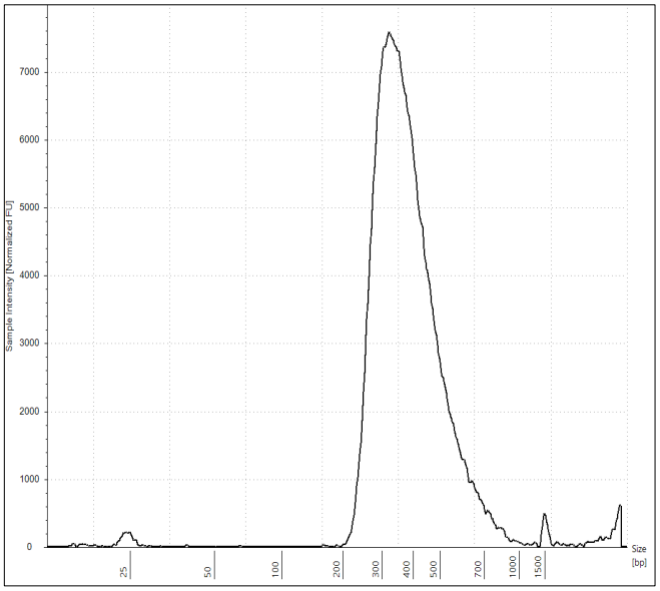
4.1. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

4.1.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

4.1.2. 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 traces, bring up the sample volume (from Step 3.1.15.) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step using 0.9X (45 µl) standard bead clean-up.

Figure 4.1.2.1. Example of library made using 250ng of UHRR and UMI adaptors on a TapeStation.



Appendix C
Recommendations for use with FFPE RNA

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

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

●

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

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

RNA Sample Requirements

NEBNext UltraExpress RNA Library Prep Kit was designed for use with high quality RNA samples. Libraries with an input of 100 ng FFPE total RNA have been successfully generated, however library quality can be variable for challenging samples.

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). For highly degraded samples (e.g., FFPE) which do not require fragmentation, follow the guidance below when indicated in Section 2.

Input Amount

100 ng or more of degraded (e.g., FFPE) total RNA (DNA free) in a maximum of 11 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (e.g., Qubit, RiboGreen), and quality checked by Bioanalyzer.

Prior to Starting Enrichment

Remove the following components to thaw on ice:

- NEBNext UltraExpress RNA Fragmentation Mix
- NEBNext UltraExpress Second Strand Master Mix (Note: do not vortex reagent, mix only by inversion)

1.1. Preparation of 1X Fragmentation Mix for RNA elution

1.1.1. Thaw the Fragmentation Master Mix (2X) and prepare 1X composition per sample as follows:

COMPONENT	VOLUME
• (lilac) NEBNext UltraExpress RNA Fragmentation Mix	4 µl
Nuclease-free Water	4 µl

Note : Keep the mix on ice until RNA is purified. It will be used in Step 2.4.9.

2.1. Probe Hybridization to RNA

2.1.1. Dilute 25–250 ng of total RNA with Nuclease-free Water to a final volume of 11 µl in a PCR tube. Keep the RNA on ice.

2.1.2. Assemble the following RNA/Probe hybridization reaction on ice:

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (25–250 ng)	11 µl
◦ (white) NEBNext v2 rRNA Depletion Solution	2 µl
◦ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

2.1.3. Mix thoroughly by pipetting up and down at least 10 times. Note: It is crucial to mix well at this step.

2.1.4. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

2.1.5. Place tube in a pre-heated thermocycler and run the following program with the heated lid set to 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

2.1.6. Briefly spin down the tube in a microcentrifuge and place on ice. Proceed immediately to the RNase H Digestion.

2.2. RNase H Digestion

2.2.1. Assemble the following RNase H digestion reaction on ice:

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 2.1.6.)	15 µl
◦ (white) RNase H Reaction Buffer	2 µl
◦ (white) NEBNext Thermostable RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

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

2.2.3. Briefly spin down the tube in a microcentrifuge.

2.2.4. Incubate in a pre-heated thermocycler with the lid set to 55°C:
30 minutes at 50°C
Hold at 4°C

2.2.5. Briefly spin down the tube in a microcentrifuge and place on ice. Proceed immediately to DNase I Digestion.

2.3. DNase I Digestion

2.3.1. Assemble the following DNase I digestion reaction on ice:

DNASE I DIGESTION REACTION	VOLUME
RNase H treated RNA (Step 2.2.5.)	20 µl
◦ (white) DNase I Reaction Buffer	5 µl
◦ (white) NEBNext DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

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

2.3.3. Briefly spin down the tube in a microcentrifuge.

2.3.4. Incubate in a pre-heated thermocycler for the heated lid set to 40°C (or off):
30 minutes at 37°C
Hold at 4°C

2.3.5. Briefly spin down the tube 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 Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads to resuspend.

2.4.2. Add 90 µl (1.8X) beads to the RNA sample from Step 2.3.5 and mix thoroughly by pipetting up and down at least 10 times.

2.4.3. Incubate for 15 minutes on ice to bind RNA to the beads.

2.4.4. Place the tube on a magnetic rack to separate the beads from the supernatant.

2.4.5. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

2.4.6. 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 which contain the RNA.

2.4.7. Repeat Step 2.4.6. once for a total of two washes.

2.4.8. 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.

2.4.9. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 6.5 µl of Fragmentation Master Mix (2X). Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube. Do not remove supernatant from beads. Incubate samples for 2 minutes on ice following elution. Keep samples on bead and proceed with priming of highly degraded RNA which does not require fragmentation.

2.5. RNA primer annealing

2.5.1. Incubate the sample on-bead in a preheated thermocycler as follows with lid set at 105°C:
5 minutes at 65°C
Hold at 4°C.

2.5.2. Immediately remove tube from thermal cycler once it reaches 4°C.

2.5.3. Place on a magnetic rack to separate the beads and transfer 5 µl to a new tube on ice. Proceed with library preparation from First Strand cDNA synthesis through PCR enrichment beginning from step 2.5. in Section 2 using the following adaptor dilution and cycling recommendations in Table 2.4.12A and 2.4.12B:

Adaptor Dilutions for FFPE total RNA when using NEBNext Adaptor for Illumina

Table 2.4.12A

TOTAL RNA INPUT	DILUTION REQUIRED
100 ng or above*	100-fold dilution in Adaptor Dilution Buffer

* The adaptor dilution may need to be optimized for variable quality or higher input samples PCR recommendations for FFPE Total RNA

Table 2.4.12B. Recommended PCR cycles based on total RNA input amount.

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
100 ng or above*	12–14

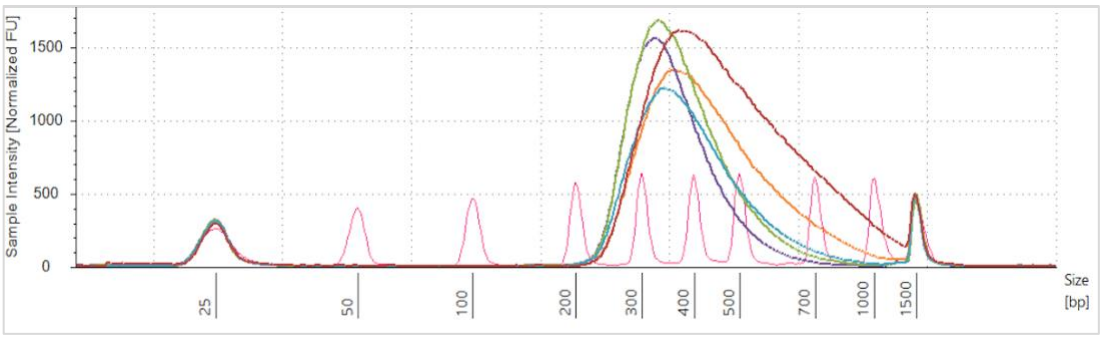
* Note: PCR cycles are recommended based on 100 ng FFPE Total RNA. It may require optimization based on the sample quality or higher inputs.

Appendix D
Recommendations for fragmentation times for longer RNA inserts

1.1. Fragmentation

Note: These recommendations have been optimized using Universal Human Reference Total RNA. Other types of RNA may require different fragmentation times. These recommendations are not intended for low quality (< 2 RIN) RNA samples.

Figure 1.1. Modified fragmentation times for longer RNA libraries.



Pink Electronic Ladder
Red 600–1,500 bp library made from mRNA fragmented for 3 minutes at 94°C
Orange 400–1,000 bp library made from mRNA fragmented for 6 minutes at 94°C
Cyan 300–700 bp library made from mRNA fragmented for 9 minutes at 94°C
Green 200–500 bp library made from mRNA fragmented for 12 minutes at 94°C
Purple 150–300 bp library made from mRNA fragmented for 15 minutes at 94°C

Modified fragmentation times for longer RNA inserts. TapeStation traces of libraries made from 100 ng UHRR shown. mRNA isolated from Universal Human Reference RNA and fragmented in 1X NEBNext UltraExpress Fragmentation Mix at 94°C for 3, 6, 9, 12 or 15 minutes. ***For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes.***

Note: Size selection is not recommended for NEBNext UltraExpress RNA libraries, however, it can be performed to isolate larger libraries if desired. We would recommend starting with the high input for this workflow.

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´ to 3´ transcript coverage, and fraction of reads mapping to the reference.

NEB #E3330S Table of Components

NEB #	PRODUCT	VOLUME
E3329A	NEBNext UltraExpress RNA Fragmentation Mix	0.096 ml
E3331A	NEBNext UltraExpress First Strand Enzyme Mix	0.024 ml
E3332A	NEBNext UltraExpress Strand Specificity Reagent	0.096 ml
E3333A	NEBNext UltraExpress Second Strand Master Mix	0.72 ml
E3334A	NEBNext UltraExpress End Prep Enzyme Mix	0.036 ml
E3335A	NEBNext UltraExpress End Prep Reaction Buffer	0.06 ml
E3336A	NEBNext UltraExpress Ligation Master Mix	0.288 ml
E3337A	NEBNext UltraExpress USER Enzyme	0.048 ml
E3338A	NEBNext MSTC High Yield Master Mix	1.2 ml
E7762A	NEBNext Adaptor Dilution Buffer	2.4 ml
E3341A	0.1X TE	2.4 ml
E3342A	Nuclease-free Water	0.096 ml
E3339A	NEBNext Bead Reconstitution Buffer	0.96 ml

NEB #E3330L Table of Components

NEB #	PRODUCT	VOLUME
E3329AA	NEBNext UltraExpress RNA Fragmentation Mix	0.384 ml
E3331AA	NEBNext UltraExpress First Strand Enzyme Mix	0.096 ml
E3332AA	NEBNext UltraExpress Strand Specificity Reagent	0.384 ml
E3333AA	NEBNext UltraExpress Second Strand Master Mix	2.88 ml
E3334AA	NEBNext UltraExpress End Prep Enzyme Mix	0.144 ml
E3335AA	NEBNext UltraExpress End Prep Reaction Buffer	0.24 ml
E3336AA	NEBNext UltraExpress Ligation Master Mix	1.16 ml
E3337AA	NEBNext UltraExpress USER Enzyme	0.192 ml
E3338AA	NEBNext MSTC High Yield Master Mix	4.8 ml
E7762AA	NEBNext Adaptor Dilution Buffer	9.6 ml
E3341AA	0.1X TE	9.6 ml
E3342AA	Nuclease-free Water	0.384 ml
E3339AA	NEBNext Bead Reconstitution Buffer	3.84 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	9/23
1.1	Updated value in first row of Table 2.2 (p. 20).	12/23
2.0	Updated input values from high-to-low in tables (p. 20). Updated with new NEB logos/footer.	02/24