NEBNext® rRNA Depletion Kit (Bacteria)

for use with NEBNext Multiplex Oligos for Illumina®
(Unique Dual Index UMI Adaptors RNA Set 1, NEB #E7416)

NEB #E7850 S/L/X, NEB #E7860 S/L/X 6/24/96 reactions

Version 2.0_12/22

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The Kit Includes

The volumes provided are sufficient for preparation of up to 6 reactions (NEB #E7850S/#E7860S), 24 reactions (NEB #E7850L/#E7860L) and 96 reactions (NEB #E7850X/#E7860X).

Package 1: Store at –20°C

- (white) NEBNext Bacterial rRNA Depletion Solution
- (white) NEBNext Probe Hybridization Buffer
- (white) NEBNext Thermostable RNase H
- (white) RNase H Reaction Buffer
- (white) NEBNext DNase I
- (white) DNase I Reaction Buffer

Nuclease-free Water

Package 2: Store at 4°C. Do not freeze.

Supplied only with the NEBNext rRNA Depletion Kit (Bacteria) with RNA Sample Purification Beads, NEB #E7860.

NEBNext RNA Sample Purification Beads E6351

Required Materials Not Included

- Magnetic rack/stand (NEB #S1515S, Alpaqua®, cat. #A001322 or equivalent)
- 80% Ethanol (freshly prepared)
- Microcentrifuge
- Vortex Mixer
- Thermal cycler
- Any thin wall 200 µl PCR tube (for example Tempassure PCR flex-free 8 tube strips USA Scientific #1402-4708)
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables

For NEB #E7850 only:

- Agencourt® RNAClean® XP Beads (Beckman Coulter, Inc. #A63987)
- In NEB #E7860 beads are included

For use with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760/E7765) & NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770/E7775):

- NEBNext Multiplex Oligos (Unique Dual Index UMI Adaptors RNA Set 1) (NEB #E7416)

For NEB #E7760 & NEB #E7770:

- SPRISelect Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- (In NEB #E7765 & NEB #E7775, beads are included)

Adaptor trimming sequences:
The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TruSeq:

Adaptor Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
Adaptor Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

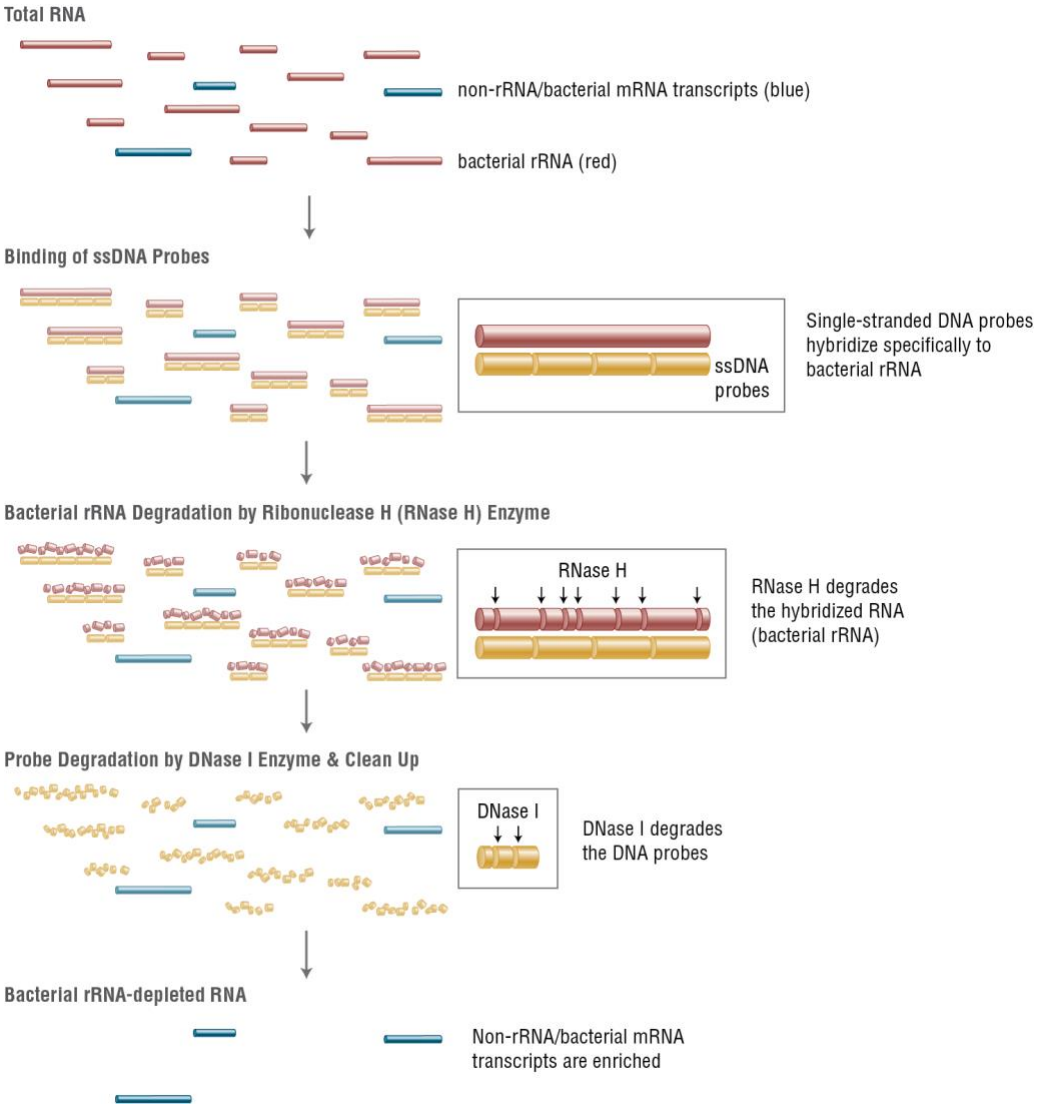
Overview

Total cellular RNA is mainly composed of rRNA and often is not of interest. rRNA can be depleted using rRNA specific probes. NEB offers the NEBNext rRNA Depletion Kit (Bacterial) (NEB #E7850, NEB #E7860) for the enrichment of non-ribosomal RNA from bacterial species. The depletion method works on both intact and degraded RNA, in monocultures or a sample with mixed bacterial species (e.g., Metatranscriptome).

The NEBNext rRNA Depletion Kit (Bacteria) employs the NEBNext RNase H-based RNA Depletion Workflow (Figure 1) to deplete bacterial rRNA.

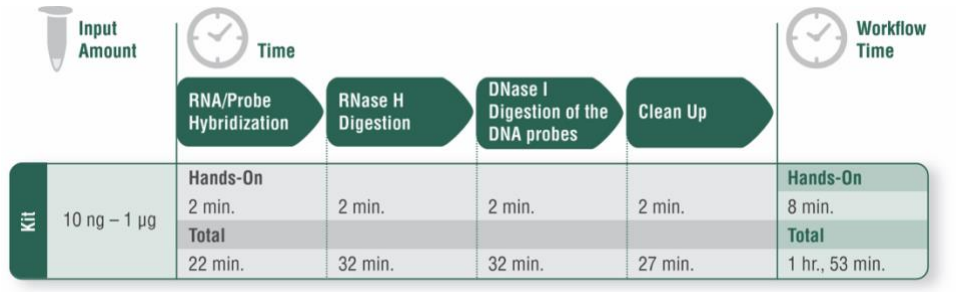
- Targets bacterial rRNA (5S, 16S, 23S)
- Compatible with both Gram positive and Gram negative organisms
- Works on monocultures and mix of bacterial species (e.g., Metatranscriptome)
- Depletion works on bacterial total RNA preparations, both intact and degraded
- The kit contains all enzymes and buffers required to deplete rRNA from bacterial RNA samples

Figure 1. NEBNext RNase H-based rRNA Depletion (Bacteria) Workflow



Total RNA is hybridized with DNA probes targeting unwanted abundant RNAs (e.g., rRNA), followed by an RNase H digestion where the enzyme recognizes the RNA:DNA hybrid and degrades the targeted RNA. Finally, the DNA probes are digested with DNaseI and the reaction is cleaned using magnetic beads.

The protocol supports bacterial rRNA depletion from 10 ng–1 µg total RNA (intact or partially degraded) or 100 ng–1 µg total RNA (degraded) and can be completed in approximately two hours.



Applications

The resulting rRNA-depleted RNA is suitable for RNA-Seq, random-primed cDNA synthesis, or other downstream RNA analysis applications.

NEBNext RNA-Seq Product and Protocol Selection Guide

Following depletion the rRNA depleted material can be used in RNA-Seq applications. The library preparation protocol should be chosen based on the goals of the project and quality of the RNA sample. NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760/#E7765) for Illumina uses the dUTP method to retain strand specificity and has a streamlined, automatable workflow. NEBNext Ultra II RNA Libray Prep (NEB #E7770/#E7775) is a Non-Directional, streamlined and automatable workflow.

When using the NEBNext rRNA Depletion Kit (Bacteria; NEB #E7850 or #E7860) for RNA-Seq library preparation with the NEBNext kits listed below please follow the appropriate section in this manual.

- NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760/#E7765), Section 1 (Intact RNA) and Section 2 (Degraded RNA)
- NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770/#E7775), Section 3 (Intact RNA) and Section 4 (Degraded RNA)

Please note: A separate manual exists for NON INDEXED ADAPTORS.

Every Section in this manual contains a different protocol based on the starting material and application. Please read the RNA sample recommendations and input amount requirements in its entirety before starting the protocol.

Each kit component must pass rigorous quality control standards. 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 Library Preparation of Intact RNA using Indexed- UMI Adaptor, NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850, NEB #E7860) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765)

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.

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). 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 1 or 3. For highly degraded samples (RIN = 1 to 2), which do not require fragmentation, follow the library preparation protocol in Section 2 or 4.

RNA Purity:

The RNA sample should be free of salts (e.g., Mg^{2+} and guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant in 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, the DNase I should be removed from the sample. 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. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion.

Input Amount:

10 ng–1 µg intact or partially degraded 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.

1.1. Probe Hybridization to RNA

1.1.1. Dilute 10 ng–1 µg of total RNA with Nuclease-free Water to a final volume of 11 µl in a PCR tube. Keep the RNA on ice.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 5) for recommended fragmentation times and size selection conditions.
Keep all of the buffers on ice, unless otherwise indicated.

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

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (10 ng–1 µg)	11 µl
○ (white) NEBNext Bacterial rRNA Depletion Solution	2 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

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

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

1.1.5. Place tube in a preheated thermal cycler and run the following program with the heated lid set to 105°C. This program 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

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

1.2. RNase H Digestion

1.2.1. Assemble the following RNase H digestion reaction **on ice**:

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 1.1.6)	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**

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

1.2.3. Briefly spin down the tube in a microcentrifuge.

1.2.4. Incubate in a thermal cycler for **30 minutes at 50°C** with the lid set to 55°C.

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

1.3. DNase I Digestion

1.3.1. Assemble the following DNase I digestion reaction **on ice**:

DNASE I MASTER MIX	VOLUME
RNase H treated RNA (Step 1.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) NEBNext DNase I	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

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

1.3.3. Briefly spin down the tube in a microcentrifuge.

1.3.4. Incubate in a thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).

1.3.5. Briefly spin down the tube in a microcentrifuge and place on ice. Proceed immediately to RNA Purification.

1.4 RNA Purification using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

1.4.1. Vortex the Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads to resuspend.

1.4.2. Add 90 µl (1.8X) beads to the RNA sample from Step 1.3.5 and mix thoroughly by pipetting up and down at least 10 times.

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

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

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

1.4.6. 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.

1.4.7. Repeat Step 1.4.6 once for a total of two washes.

1.4.8. 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.

1.4.9. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl of Nuclease-free Water. Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube.

1.4.10. Incubate for **2 minutes at room temperature**.

1.4.11. Place the tube on the magnetic rack until the solution is clear (~ 2 minutes).

1.4.12. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.

1.4.13. Place the tube on ice and proceed to RNA Fragmentation and Priming.

Note: If you need to stop at this point in the protocol samples can be stored at –80°C.

1.5. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 1.5.3.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 5) for recommended fragmentation times and size selection conditions.

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

FRAGMENTATION AND PRIMING REACTION	VOLUME
Ribosomal RNA Depleted Sample (Step 1.4.13)	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	**10 µl**

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

1.5.3. Place the tube on a pre-heated thermal cycler with the heated lid set to 105°C and incubate at **94°C** following the recommendations in Table 1.5.3 for libraries with inserts ~200 nt.

Table 1.5.3. 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 (Section 5) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix (5.1) only apply for intact RNA.

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

1.6. First Strand cDNA Synthesis

1.6.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 1.5.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 1.5.4)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

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

1.6.3. Incubate the tube in a preheated thermal cycler with the heated lid set to ≥ 80°C as follows:

Note: If you are following recommendations in Appendix (Section 5), 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

1.6.4. Proceed directly to Second Strand cDNA Synthesis.

1.7. Second Strand cDNA Synthesis

1.7.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis product from Step 1.6.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 1.6.3)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

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

1.7.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set to ≤ 40°C (or off).

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

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

1.8.2. 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.

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

1.8.4. 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).**

1.8.5. 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.

1.8.6. Repeat Step 1.8.5 once for a total of two washes.

1.8.7. 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.

1.8.8. Remove the tube from the magnetic rack. Elute the cDNA 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.

1.8.9. 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.

1.9. End Prep of cDNA Library

1.9.1. Assemble the end prep reaction **on ice** by adding the following components to the second strand synthesis product from Step 1.8.9.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 1.8.9)	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.

1.9.2. 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.

1.9.3. Incubate in a thermal cycler with the heated lid set to ≥ 75°C as follows:

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

1.9.4. Proceed immediately to Adaptor Ligation.

1.10. Adaptor Ligation

Thaw the NEBNext UMI RNA Adaptor Plate on ice and briefly spin, if necessary. **It is important to keep the adaptor cold at all times.**

1.10.1. 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

*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.**

1.10.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 1.9.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 1.9.3)	60 µl
Diluted Adaptor (Step 1.10.1)	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. Do not premix the Ligation Master Mix, Ligation Enhancer and Adaptor prior to use in the Adaptor Ligation Step.

1.10.3. 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.

1.10.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermal cycler.

1.10.5. Add 3 µl ● (blue or red) USER® Enzyme to the ligation mixture from Step 1.10.4, resulting in total volume of 99 µl.

1.10.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C.

1.10.7. Proceed immediately to Purification of the Ligation Reaction.

1.11. 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 the Appendix, Section 5.

1.11.1. 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.

1.11.2. Incubate for **10 minutes at room temperature**.

1.11.3. 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).**

1.11.4. 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.

1.11.5. Repeat Step 1.11.4 once for a total of two washes.

1.11.6. Briefly spin the tube and put the tube back in the magnetic rack.

1.11.7. Completely remove the residual ethanol and air dry 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.

1.11.8. 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.

1.11.9. 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.

1.12. PCR Enrichment of Adaptor Ligated DNA

1.12.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
UMI Adaptor Ligated DNA (Step 1.11.9)	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)

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

1.12.3. Place the tube on a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 1.12.3A and Table 1.12.3B):

Table 1.12.3A:

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.

** 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 6.1 in Section 6).

Table 1.12.3B: 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 Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

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

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

1.13.2. 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.

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

1.13.4. 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).**

1.13.5. 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.

1.13.6. Repeat Step 1.13.5 once for a total of two washes.

1.13.7. 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.

1.13.8. 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.

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

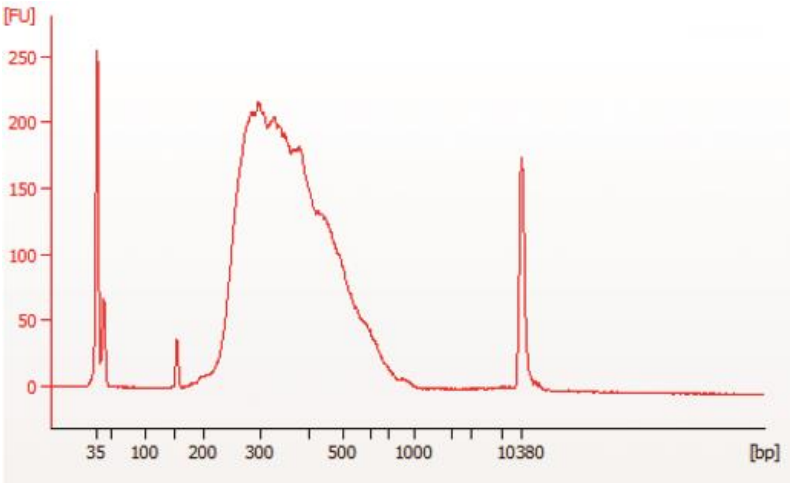
1.14. Library Quantification

1.14.1. Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries.

1.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp (Figure 1.14).

Note: If a peak at ~ 40 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 1.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 1.13).

Figure 1.14. Example of a representative RNA library size distribution on a Bioanalyzer.



Please read the FAQ section on NEB.com for additional information about this product.

Section 2:
Protocol for Library Preparation of Degraded RNA using Indexed- UMI Adaptor, NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850, NEB #E7860) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765)

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.

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). 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 1 or 3. For highly degraded samples (RIN = 1 to 2), which do not require fragmentation, follow the library preparation protocol in Section 2 or 4.

RNA Purity:
The RNA sample should be free of salts (e.g., Mg^{2+} and guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant in 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, the DNase I should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). Contaminating DNA can cause inaccurate RNA quantification and impede proper globin mRNA and rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion.

Input Amount:
100 ng–1 µg degraded 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.

2.1. Probe Hybridization to RNA

2.1.1. Dilute 100 ng–1 µg 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 (100 ng–1 µg)	11 µl
○ (white) NEBNext Bacterial 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 preheated thermal cycler and run the following program with the heated lid set to 105°C. This program 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 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 preheated thermal cycler for **30 minutes at 50°C** with the lid set to 55°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 MASTER MIX	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 preheated thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).

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 7 µl of Nuclease-free Water. Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube.

2.4.10. Incubate for **2 minutes at room temperature**.

2.4.11. Place the tube on the magnetic rack until the solution is clear (~ 2 minutes).

2.4.12. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.

2.4.13. Place the tube on ice and proceed to RNA Priming.

Note: If you need to stop at this point in the protocol samples can be stored at –80°C.

2.5. Priming of Highly Degraded RNA which has a RIN ≤ 2 and Does Not Require Fragmentation

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

PRIMING REACTION	VOLUME
rRNA Depleted Sample (Step 2.4.13)	5 µl
• (lilac) Random Primers	1 µl
Total Volume	**6 µl**

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

2.5.3. Briefly spin down the tube in a microcentrifuge.

2.5.4. Incubate in a preheated thermal cycler as follows:

5 minutes at 65°C, with heated lid set to 105°C
Hold at 4°C

2.5.5. Transfer the tube directly 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 primed RNA from Step 2.5.5:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 2.5.5)	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**

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

2.6.3. Incubate in a preheated thermal cycler with the heated lid set to ≥ 80°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

2.6.4. Proceed directly to Second Strand cDNA Synthesis Reaction.

2.7. Second Strand cDNA Synthesis

2.7.1 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 2.6.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 2.6.3)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	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 ten times.

2.7.3 Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set to ≤ 40°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 stand. 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 two washes.

2.8.7. 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.

2.8.8. Remove the tube from the magnet. Elute the cDNA 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.

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 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 in a thermal cycler with the heated lid set to ≥ 75°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

Thaw the NEBNext UMI RNA Adaptor Plate on ice and briefly spin, if necessary. **It is important to keep the adaptor cold at all times.**

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**.

DEGRADED RNA	DILUTION REQUIRED
100 ng–1 µg	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.**

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 2.9.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 2.9.3)	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. Do not premix 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 thermal cycler.

2.10.5. Add 3 µl • (blue or red) 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 ≥ 45°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

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 (about 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 two washes.

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 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, 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 Adaptors 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 thermal cycler 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	9–14*, **
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. The recommendation of PCR cycles are based on internal tests using degraded 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 6.1, Section 6).

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

DEGRADED RNA	RECOMMENDED PCR CYCLES
1 µg	9–10
100 ng	13–14

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 two washes.

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.

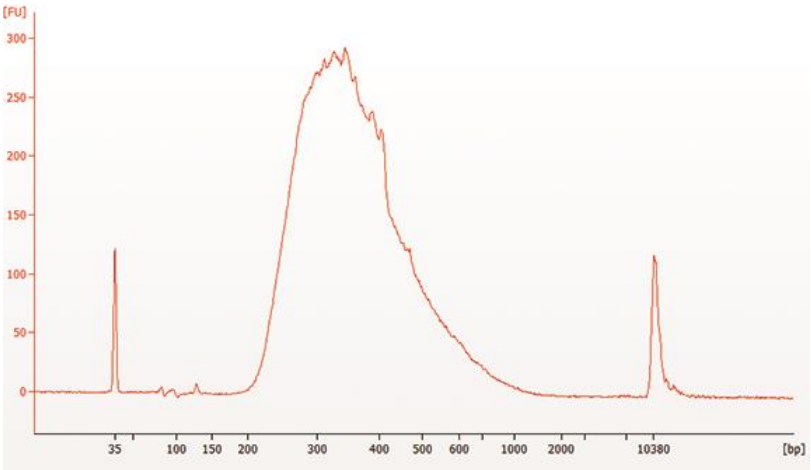
2.14. Library Quantification

2.14.1. Use a Bioanalyzer or TapeStation 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 (Figure 2.14).

Note: If a peak at ~ 40 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 1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 2.13).

Figure 2.14. Example of a representative RNA library size distribution on a Bioanalyzer.



Please read the FAQ section on NEB.com for additional information about this product.

Section 3
Protocol for Library Preparation of Intact RNA using Indexed- UMI Adaptor, NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850, NEB #E7860) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

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 for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 5) for recommended fragmentation times and size selection conditions.

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). 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 1 or 3. For highly degraded samples (RIN = 1 to 2), which do not require fragmentation, follow the library preparation protocol in Section 2 or 4.

RNA Purity:
The RNA sample should be free of salts (e.g., Mg^{2+} and guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant in 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, the DNase I should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). Contaminating DNA can cause inaccurate RNA quantification and impede proper globin mRNA and rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion.

Input Amount:
10 ng–1 µg intact or partially degraded 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.

3.1. Probe Hybridization to RNA

3.1.1. Dilute 10 ng–1 µg total RNA with Nuclease-free Water to a final volume of 11 µl in a PCR tube. Keep the RNA on ice.

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

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (10 ng–1 µg)	11 µl
○ (white) NEBNext Bacterial rRNA Depletion Solution	2 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

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

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

3.1.5. Place tube in a preheated thermal cycler and run the following program with the heated lid set to 105°C. This program 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

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

3.2. RNase H Digestion

3.2.1. Assemble the following RNase H digestion reaction **on ice**:

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 3.1.6)	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**

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

3.2.3. Briefly spin down the tube in a microcentrifuge.

3.2.4. Incubate in a thermal cycler for **30 minutes at 50°C** with the lid set to 55°C.

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

3.3. DNase I Digestion

3.3.1. Assemble the following DNase I digestion reaction **on ice**:

DNASE I MASTER MIX	VOLUME
RNase H treated RNA (Step 3.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) NEBNext DNase I	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

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

3.3.3. Briefly spin down the tube in a microcentrifuge.

3.3.4. Incubate in a thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).

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

3.4.2. Add 90 µl (1.8X) beads to the RNA sample from Step 3.3.5 and mix thoroughly by pipetting up and down at least 10 times.

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

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

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

3.4.6. 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.

3.4.7. Repeat Step 3.4.6 once for a total of two washes.

3.4.8. 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.

3.4.9. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl of Nuclease-free Water. Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube.

3.4.10. Incubate for **2 minutes at room temperature**.

3.4.11. Place the tube on the magnetic rack until the solution is clear (~ 2 minutes).

3.4.12. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.

3.4.13. Place the sample on ice and proceed to RNA Fragmentation and Priming.

Note: If you need to stop at this point in the protocol samples can be stored at –80°C.

3.5. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 3.5.3.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 5) for recommended fragmentation times and size selection conditions.

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

FRAGMENTATION AND PRIMING REACTION	VOLUME
Ribosomal RNA Depleted Sample (Step 3.4.13)	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	**10 µl**

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

3.5.3. Place the tube on a preheated thermal cycler with the heated lid set to 105°C and incubate at 94°C following the recommendations in Table 3.5.3 for libraries with inserts ~200 nt.

Table 3.5.3. 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 (Section 5) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix (Section 5.1) only apply for intact RNA.

3.5.4. Immediately transfer the tube 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 fragmented and primed RNA from Step 3.5.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and Primed RNA (Step 3.5.4)	10 µl
Nuclease-free Water	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

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

3.6.3. Incubate the tube in a preheated thermal cycler with the heated lid set to ≥ 80°C as follows:

Note: If you are following recommendations in Appendix (Section 5), 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.

3.7. Second Strand cDNA Synthesis

3.7.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis product from Step 3.6.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 3.6.3)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer	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 at least 10 times.

3.7.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set to ≤ 40°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 **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 rack. 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 two washes.

3.8.7. 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.

3.8.8. Remove the tube from the magnetic rack. Elute the cDNA 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.

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 cDNA 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 preheated thermal cycler with the heated lid set to ≥ 75°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

Thaw the NEBNext UMI RNA Adaptor Plate on ice and briefly spin, if necessary**. It is important to keep the adaptor cold at all times.**

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**.

TOTAL RNA INPUT	DILUTION REQUIRED
1,000 ng–101 ng	No dilution
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 3.9.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 3.9.3)	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. Do not premix 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 thermal cycler.

3.10.5. 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 libraries with larger insert size (> 200 nt) follow the size selection recommendations in the Appendix, Section 5.

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 (~ 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 two washes.

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 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. Quickly spin the tube and 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 thermal cycler 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	7–16*, **
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 6.1, Section 6).

Table 3.12.3B: 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

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

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 **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 two washes.

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.

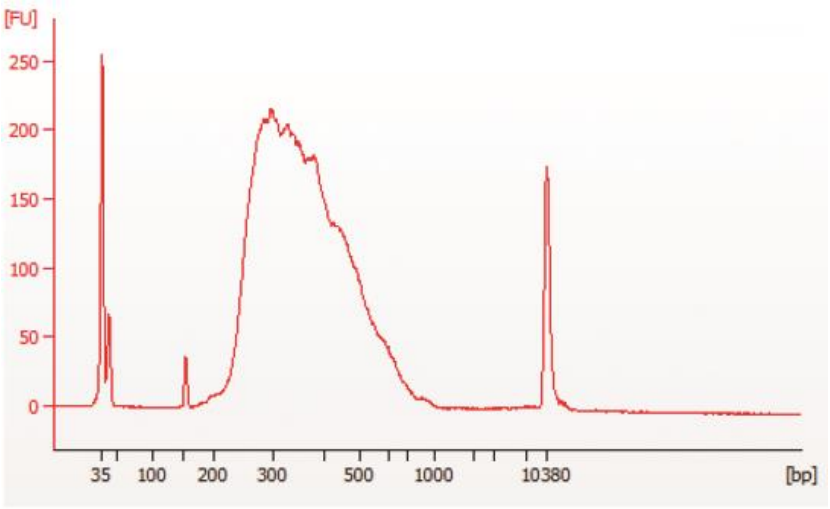
3.14. Library Quantification

3.14.1. Use a Bioanalyzer or TapeStation 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 (Figure 3.14).

Note: If a peak at ~ 40 bp (primers) or ~ 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 3.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 3.13).

Figure 3.14. Example of a representative RNA library size distribution on a Bioanalyzer.



Please read the FAQ section on NEB.com for additional information about this product.

Section 4
Protocol for Library Preparation of Degraded RNA using Indexed- UMI Adaptor NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850, NEB #E7860) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

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.

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). 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 1 or 3. For highly degraded samples (RIN = 1 to 2), which do not require fragmentation, follow the library preparation protocol in Section 2 or 4.

RNA Purity:
The RNA sample should be free of salts (e.g., Mg^{2+} and guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant in 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, the DNase I should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). Contaminating DNA can cause inaccurate RNA quantification and impede proper globin mRNA and rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion.

Input Amount:
100 ng–1 µg degraded 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.

4.1. Probe Hybridization to RNA

4.1.1. Dilute 100 ng–1 µg total RNA with Nuclease-free Water to a final volume of 11 µl in a PCR tube. Keep the RNA on ice.

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

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (100 ng–1 µg)	11 µl
○ (white) NEBNext Bacterial rRNA Depletion Solution	2 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

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

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

4.1.5. Place tube in a preheated thermal cycler and run the following program with the heated lid set to 105°C. This program 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

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

4.2. RNase H Digestion

4.2.1. Assemble the following RNase H digestion reaction **on ice**:

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 4.1.6)	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**

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

4.2.3. Briefly spin down the tube in a microcentrifuge.

4.2.4. Incubate in a preheated thermal cycler for **30 minutes at 50°C** with the lid set to 55°C.

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

4.3. DNase I Digestion

4.3.1. Assemble the following DNase I digestion reaction **on ice**:

DNASE I MASTER MIX	VOLUME
RNase H treated RNA (Step 4.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) NEBNext DNase I	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

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

4.3.3. Briefly spin down the tube in a microcentrifuge.

4.3.4. Incubate in a thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).

4.3.5. Briefly spin down the tube in a microcentrifuge and place on ice. Proceed immediately to RNA Purification.

4.4 RNA Purification using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

4.4.1. Vortex the Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads to resuspend.

4.4.2. Add 90 µl (1.8X) beads to the RNA sample from Step 4.3.5 and mix thoroughly by pipetting up and down at least 10 times.

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

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

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

4.4.6. 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.

4.4.7. Repeat Step 4.4.6 once for a total of two washes.

4.4.8. 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.

4.4.9. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl of Nuclease-free Water. Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube.

4.4.10. Incubate for **2 minutes at room temperature**.

4.4.11. Place the tube on the magnetic rack until the solution is clear (~ 2 minutes).

4.4.12. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.

4.4.13. Place the tube on ice and proceed to RNA Priming.

Note: If you need to stop at this point in the protocol samples can be stored at –80°C.

4.5. Priming of Highly Degraded RNA which has a RIN ≤ 2 and Does Not Require Fragmentation

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

PRIMING REACTION	VOLUME
rRNA Depleted Sample (Step 4.4.13)	5 µl
• (lilac) Random Primers	1 µl
Total Volume	**6 µl**

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

4.5.3. Briefly spin down the tube in a microcentrifuge.

4.5.4. Incubate in a preheated thermal cycler as follows:

5 minutes at 65°C, with heated lid set to 105°C
Hold at 4°C

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

4.6. First Strand cDNA Synthesis

4.6.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the primed RNA from Step 4.5.5:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 4.5.5)	6 µl
Nuclease-free Water	8 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

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

4.6.3. Incubate in a preheated thermal cycler with the heated lid set to ≥ 80°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

4.6.4. Proceed directly to Second Strand cDNA Synthesis Reaction.

4.7. Second Strand cDNA Synthesis

4.7.1 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 4.6.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 4.6.3)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

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

4.7.3 Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set to ≤ 40°C (or off).

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

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

4.8.2. 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.

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

4.8.4. 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).**

4.8.5. 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.

4.8.6. Repeat Step 4.8.5 once for a total of two washes.

4.8.7. 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.

4.8.8. 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.

4.8.9. 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.

4.9. End Prep of cDNA Library

4.9.1. Assemble the end prep reaction **on ice** by adding the following components to the second strand synthesis product from Step 4.8.9.

END PREP REACTION	VOLUME
Second Strand Synthesis Product (Step 4.8.9)	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.

4.9.2. 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.

4.9.3. Incubate in a thermal cycler with the heated lid set to ≥ 75°C as follows:

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

4.9.4. Proceed immediately to Adaptor Ligation.

4.10. Adaptor Ligation

Thaw the NEBNext UMI RNA Adaptor Plate on ice and briefly spin, if necessary. **It is important to keep the adaptor cold at all times.**

4.10.1. 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**.

DEGRADED RNA	DILUTION REQUIRED
100 ng–1 µg	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.**

4.10.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 4.9.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 4.9.3)	60 µl
Diluted Adaptor (Step 4.10.1)	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. Do not premix the Ligation Master Mix, Ligation Enhancer and Adaptor prior to use in the Adaptor Ligation Step.

4.10.3. 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.

4.10.4. Incubate **15 minutes at 20°C** (heated lid off) in a thermal cycler.

4.10.5. Proceed immediately to Purification of the Ligation Reaction.

4.11. Purification of the Ligation Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

4.11.1. 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.

4.11.2. Incubate for **10 minutes at room temperature**.

4.11.3. 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)**.

4.11.4. 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.

4.11.5. Repeat Step 4.11.4 once for a total of two washes.

4.11.6. Briefly spin the tube and put the tube back in the magnetic rack.

4.11.7. Completely remove the residual ethanol and air dry 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.

4.11.8. 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.

4.11.9. 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.

4.12. PCR Enrichment of Adaptor Ligated DNA

4.12.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.11.9)	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).

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

4.12.3. Place the tube on a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 4.12.3A and Table 4.12.3B):

Table 4.12.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	8–13*, **
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 using degraded 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 6.1, Section 6).

Table 4.12.3B: Recommended PCR cycles based on input amount:

DEGRADED RNA	RECOMMENDED PCR CYCLES
1 µg	8–9
100 ng	12–13

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

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

4.13.2. 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.

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

4.13.4. 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).**

4.13.5. 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.

4.13.6. Repeat Step 4.13.5 once for a total of two washes.

4.13.7. 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.

4.13.8. 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.

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

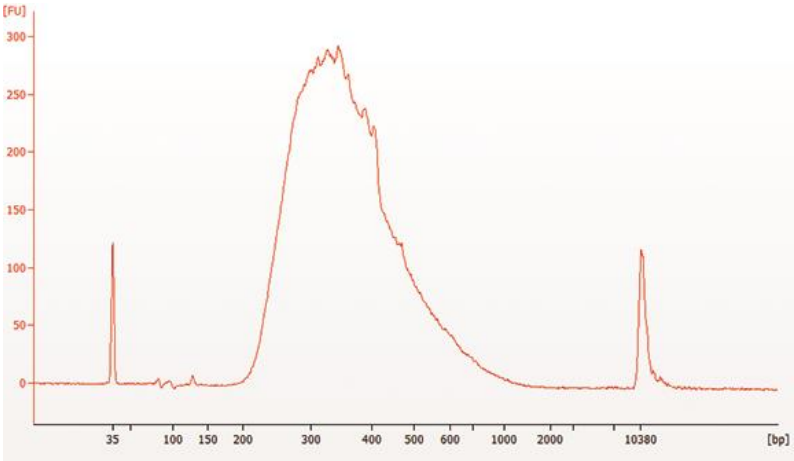
4.14. Library Quantification

4.14.1. Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries.

4.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp (Figure 4.14).

Note: If a peak at ~ 40 bp (primers) or ~ 150 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 4.13.9) to 50 µl with 1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 4.13).

Figure 4.14. Example of a representative RNA library size distribution on a Bioanalyzer.



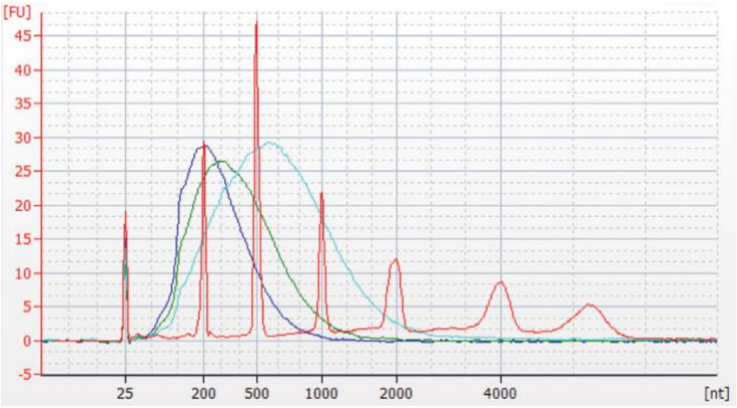
Please read the FAQ section on NEB.com for additional information about this product.

Section 5
Appendix

5.1. Fragmentation

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

Figure 5.1. 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 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.***

5.2. 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 99 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 5.2. The protocol below is for libraries with a 300 bp insert size (450 bp final library size).

Table 5.2: 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**	**450 bp**	**500 bp**
BEAD VOLUME TO BE ADDED (µl)	1st Bead Selection	25	20	15
	2nd 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.

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

5.2.2. Add **25 µl of resuspended beads** to the 99 µl ligation reaction. Mix well by pipetting up and down at least 10 times.

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

5.2.4. Place the tube on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the tube 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.

5.2.5. 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**.

5.2.6. Place the tube/plate on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the tube 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).**

5.2.7. 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.

5.2.8. Repeat Step 5.2.7 once for a total of two washes.

5.2.9. 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.

5.2.10. 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**.

5.2.11. 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.

5.3. 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.

5.3.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME PER ONE LIBRARY
UMI Adaptor Ligated DNA (Step 5.2.11)	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).

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

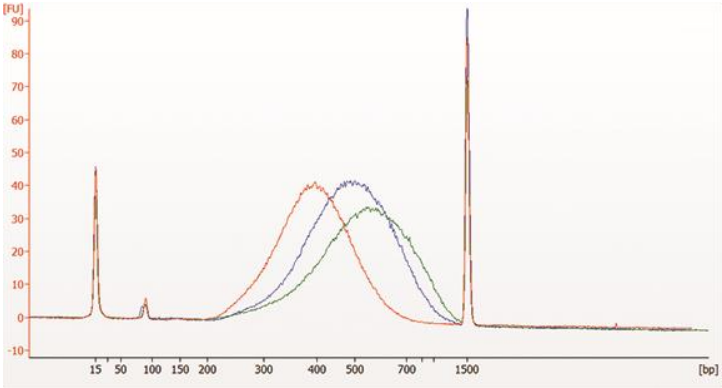
5.3.3. Place the tube in a thermal cycler with the heated lid set to 105°C. Perform PCR amplification using the following PCR cycling conditions:

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 6.1, Section 6).

Figure 5.3: Bioanalyzer traces of size selected DNA libraries.



50 ng mRNA was fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix at 94°C for 10 or 5 minutes and using different size selection conditions. Libraries were size-selected as described in Table 5.2, 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 5.3:

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

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 6
Troubleshooting Guide

OBSERVATIONS	POSSIBLE CAUSES	EFFECT	SUGGESTED SOLUTIONS
Presence of Bioanalyzer peaks < 85 bp (Figure 6.1)	• Presence of Primers remaining after PCR clean up	Primers cannot cluster or be sequenced, but can bind to flowcell and reduce cluster density	• 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 6.2)	• 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.	• 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 6.1)	• 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	• Reduce number of PCR cycles
Broad library size distribution	• Under-fragmentation of the RNA	Library size will contain longer insert sizes	• Increase RNA fragmentation time

Figure 6.1:

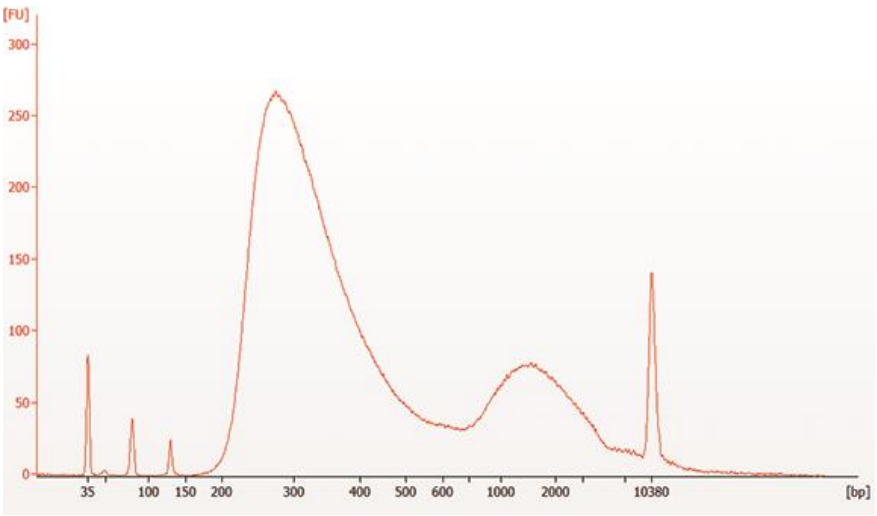
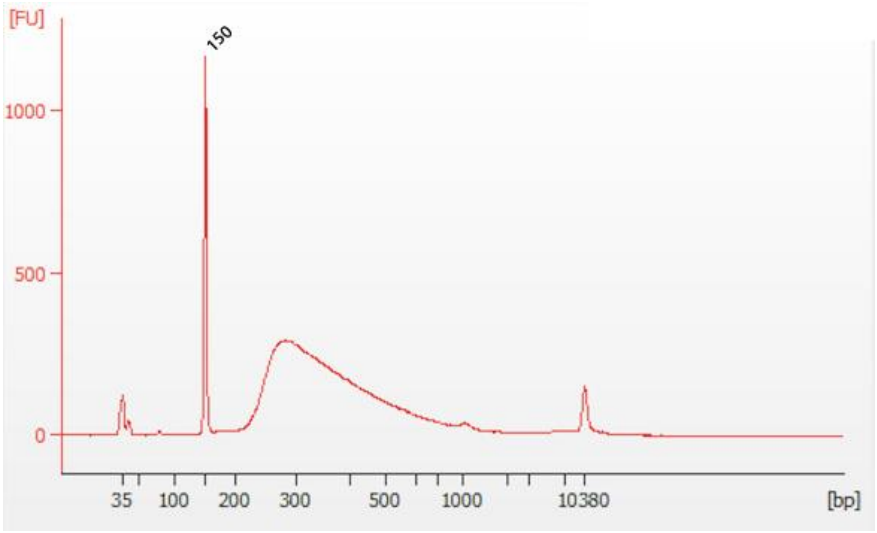


Figure 6.2:



Kit Components

NEB #E7850S Table of Components

NEB #	PRODUCT	VOLUME
E7752-2	NEBNext Thermostable RNase H	0.012 ml
E6312-2	RNase H Reaction Buffer	0.012 ml
E7851-2	NEBNext Bacterial rRNA Depletion Solution	0.018 ml
E6314-2	NEBNext Probe Hybridization Buffer	0.012 ml
E7753-2	NEBNext DNase I	0.015 ml
E6315-2	DNase I Reaction Buffer	0.03 ml
E6317-2	Nuclease-free Water	0.4 ml

NEB #E7850L Table of Components

NEB #	PRODUCT	VOLUME
E7752-3	NEBNext Thermostable RNase H	0.048 ml
E6312-3	RNase H Reaction Buffer	0.048 ml
E7851-3	NEBNext Bacterial rRNA Depletion Solution	0.072 ml
E6314-3	NEBNext Probe Hybridization Buffer	0.048 ml
E7753-3	NEBNext DNase I	0.06 ml
E6315-3	DNase I Reaction Buffer	0.120 ml
E6317-3	Nuclease-free Water	1.5 ml

NEB #E7850X Table of Components

NEB #	PRODUCT	VOLUME
E7752-4	NEBNext Thermostable RNase H	0.192 ml
E6312-4	RNase H Reaction Buffer	0.192 ml
E7851-4	NEBNext Bacterial rRNA Depletion Solution	0.288 ml
E6314-4	NEBNext Probe Hybridization Buffer	0.192 ml
E7753-4	NEBNext DNase I	0.24 ml
E6315-4	DNase I Reaction Buffer	0.48 ml
E6317-4	Nuclease-free Water	6.0 ml

NEB #E7860S Table of Components

NEB #	PRODUCT	VOLUME
E7752-2	NEBNext Thermostable RNase H	0.012 ml
E6312-2	RNase H Reaction Buffer	0.012 ml
E7851-2	NEBNext Bacterial rRNA Depletion Solution	0.018 ml
E6314-2	NEBNext Probe Hybridization Buffer	0.012 ml
E7753-2	NEBNext DNase I	0.015 ml
E6315-2	DNase I Reaction Buffer	0.03 ml
E6317-2	Nuclease-free Water	0.4 ml
E6351S	NEBNext RNA Sample Purification Beads	0.66 ml

NEB #E7860L Table of Components

NEB #	PRODUCT	VOLUME
E7752-3	NEBNext Thermostable RNase H	0.048 ml
E6312-3	RNase H Reaction Buffer	0.048 ml
E7851-3	NEBNext Bacterial rRNA Depletion Solution	0.072 ml
E6314-3	NEBNext Probe Hybridization Buffer	0.048 ml
E7753-3	NEBNext DNase I	0.06 ml
E6315-3	DNase I Reaction Buffer	0.120 ml
E6317-3	Nuclease-free Water	1.5 ml
E6351L	NEBNext RNA Sample Purification Beads	2.64 ml

NEB #E7860X Table of Components

NEB #	PRODUCT	VOLUME
E7752-4	NEBNext Thermostable RNase H	0.192 ml
E6312-4	RNase H Reaction Buffer	0.192 ml
E7851-4	NEBNext Bacterial rRNA Depletion Solution	0.288 ml
E6314-4	NEBNext Probe Hybridization Buffer	0.192 ml
E7753-4	NEBNext DNase I	0.24 ml
E6315-4	DNase I Reaction Buffer	0.48 ml
E6317-4	Nuclease-free Water	6.0 ml
E6351X	NEBNext RNA Sample Purification Beads	10.6 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	7/20
2.0	Update Protocols	12/22

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