NEBNext® rRNA Depletion Kit (Human/Mouse/Rat)

NEB #E6310S/L/X, #E6350S/L/X

6/24/96 reactions

Version 8.0_8/22

Table of Contents

The Kit Includes

The volumes provided are sufficient for preparation of up to 6 reactions (NEB #E6310S/#E6350S), 24 reactions (NEB #E6310L/#E6350L) and 96 reactions (NEB #E6310X/#E6350X).

Package 1: Store at –20°C.

NEBNext RNase H

RNase H Reaction Buffer

NEBNext rRNA Depletion Solution

NEBNext Probe Hybridization Buffer

DNase I (RNase-free)

DNase I Reaction Buffer

Nuclease-free Water

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

Supplied only with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads, NEB #E6350.

NEBNext RNA Sample Purification Beads

Please Note: For superior performance, the NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400) is now available.

Required Materials Not Included

- Pipettes
- Magnetic rack (NEB #S1515S), magnetic plate (Alpaqua® cat. #A001322) or equivalent
- 80% Ethanol (freshly prepared)
- Thin wall 200 µl PCR tubes (For example Tempassure PCR flex-free 8-tube strips USA Scientific #1402-4708)
- Microcentrifuge
- Vortex mixer
- Thermal cycler
- Bioanalyzer®, TapeStation® (Agilent Technologies, Inc.) or similar instrument and consumables

For NEB #E6310 only:

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

For NEB #E7760 & NEB #E7770:

- SPRISelect Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

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 Adaptors and Primers:
  - www.neb.com/oligos
  - Alternatively, customer supplied adaptor and primers www.neb.com/faq-nonNEB-adaptors

Please note: Separate instructions exist for UNIQUE DUAL INDEX UMI ADAPTORS. Please contact Technical Support at info@neb.com

Adaptor trimming sequences:

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

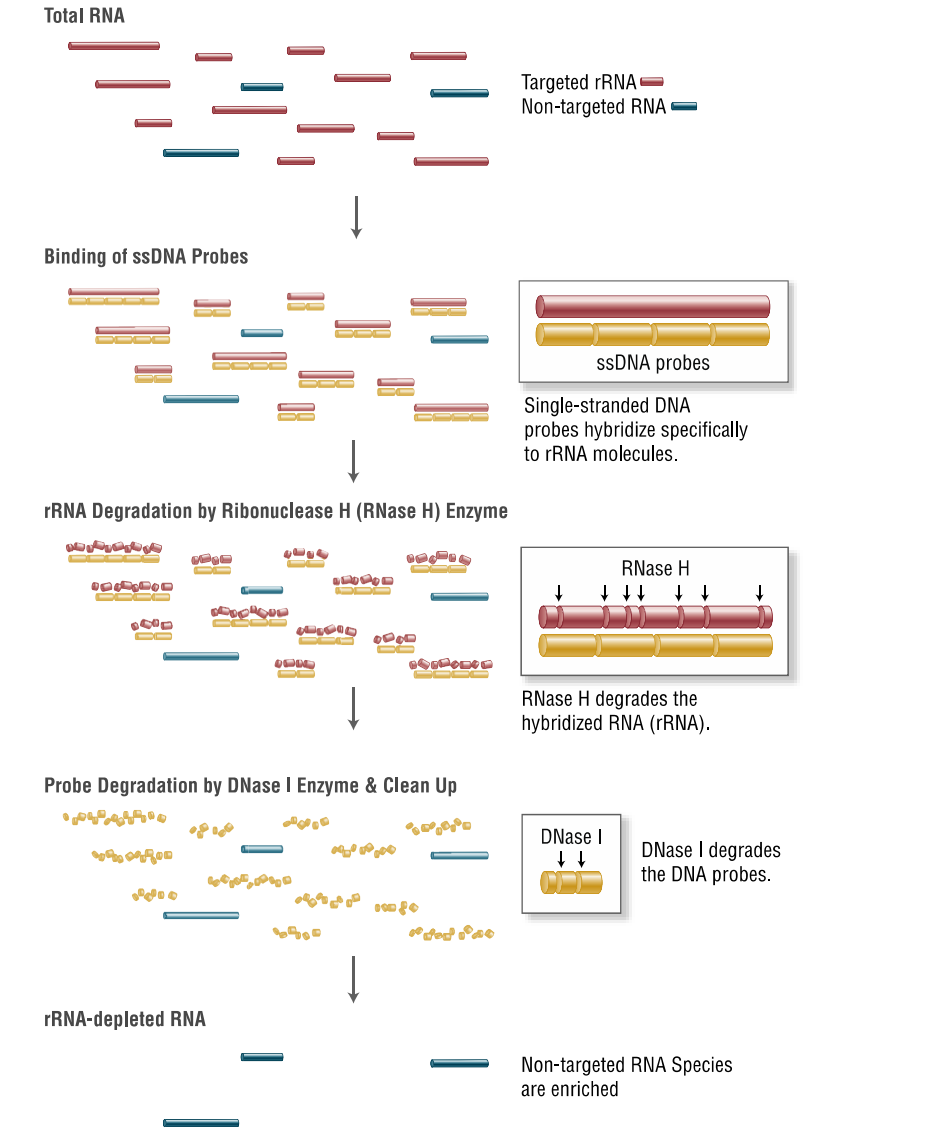
Adaptor Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Adaptor Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Overview

The NEBNext rRNA Depletion Kit (Human/Mouse/Rat) depletes both cytoplasmic (5S rRNA, 5.8S rRNA, 18S rRNA and 28S rRNA) and mitochondrial ribosomal RNA (12S rRNA and 16S rRNA) from human, mouse and rat total RNA preparations. This product is suitable for both intact and degraded RNA (e.g., FFPE RNA).

Figure 1. NEBNext RNase H-based rRNA Depletion Workflow.



Input Amount	Time: RNA/Probe Hybridization	RNase H Digestion	DNase I Digestion of the DNA probes	Clean Up	Workflow Time
Kit	Hands-On				Hands-On
5 ng – 1 µg	2 min.	2 min.	2 min.	2 min.	8 min.
	Total				Total
	22 min.	32 min.	32 min.	27 min.	1 hr., 53 min.

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. The 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. The NEBNext Ultra II RNA Library Prep (NEB #E7770, #E7775) has a non-directional, streamlined and automatable workflow. When using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310, #E6350) 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 2 (Intact or Partially Degraded RNA) and Section 3 [Degraded RNA (e.g., FFPE)]
- NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, #E7775), Section 4 (Intact or Partially Degraded RNA) and Section 5 [Degraded RNA (e.g., FFPE)]

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 an 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.

Typical Yield of rRNA-depleted RNA from a Reaction

The actual yield is dependent on the quality of the input RNA, the rRNA content of the sample, and the method used to purify the rRNA-depleted RNA. Recoveries of 3%–10% of the input RNA are typical.

Section 1
Protocol for NEBNext rRNA Depletion using the NEBNext rRNA Depletion Kit
(NEB #E6310, #E6350)

Symbols

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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). Both intact and degraded RNA can be used in the depletion protocol. However, processing of samples for different downstream applications may be impacted by the RIN scores.

RNA Purity

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) and organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I, the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free™ Kit, TURBO™ DNase Treatment and Removal Reagents, do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

5 ng–1 µg total RNA (DNA free) in a 12 µl total volume of nuclease free water, quantified by an RNA-specific dye-assisted fluorometric method (e.g., Qubit, RiboGreen), and quality checked by Bioanalyzer. If the total RNA may contain gDNA contamination, treat the RNA sample with DNase I to remove all traces of DNA, then purify the treated RNA to remove DNase I.

1.1. Hybridize the Probes to the RNA

1.1.1. Dilute 5 ng–1 µg of total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA on ice.

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (5 ng – 1 µg)	12 µl
NEBNext rRNA Depletion Solution	1 µl
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 **pre-heated** thermal cycler and run the following program with the heated lid set to 105°C.

This will take approximately 15–20 minutes to complete.

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

1.1.6. Briefly spin down the samples 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 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 pre-heated thermal cycler for **30 minutes at 37°C** with lid set to **40°C** (or off).

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:

COMPONENT	VOLUME
RNase H treated RNA (Step 1.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) DNase I (RNase-free)	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 pre-heated 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 after rRNA Depletion Using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

1.4.1. Vortex Agencourt RNAClean XP Beads or NEBNext **RNA** Sample Purification Beads until fully resuspended.

1.4.2. Add 110 µl (2.2X) resuspended beads to the RNA Sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

1.4.3. Incubate samples **on ice** for 15 minutes.

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

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

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

1.4.7. Repeat Step 1.4.6 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

1.4.8. Air dry the beads for **up to 5 minutes** while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of 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/plate from the magnetic stand. Elute the RNA from the beads by adding 8 µl of nuclease free water.

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

1.4.10. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 6 µl to a new PCR tube.

1.4.11. Place the tube on ice and proceed with NGS library construction or other downstream application. Alternatively, the sample can be stored at -80°C.

Optional: To make sure rRNA is efficiently depleted, you can design RT qPCR primers for the sample species rRNA and primers for a housekeeping gene. Compare rRNA content before and after ribosomal depletion to assess the rRNA removal efficiency.

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

Section 2
Protocol for Library Preparation of Intact or Partially Degraded RNA using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310, #E6350) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7660, 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.

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

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

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

RNA Sample Requirements

RNA Integrity

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 (current Section) or 4. For highly degraded samples (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3 or 5.

RNA Sample Requirements

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) and organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I, the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free Kit, TURBO DNase Treatment and Removal Reagents do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

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

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

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

2.1. Hybridize the Probes to the RNA

2.1.1. Dilute 5 ng–1 µg of total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA on ice.

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (5 ng–1 µg)	12 µl
○ (white) NEBNext rRNA Depletion Solution	1 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	15 µl

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

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

2.1.5. Place tube in a **pre-heated** thermal cycler and run the following program with the heated lid set to 105°C.

This will take approximately 15-20 minutes to complete.

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

2.1.6. Briefly spin down the samples 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 RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	20 µl

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

2.2.3. Briefly spin down the tube in a microcentrifuge.

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

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**:.

COMPONENT	VOLUME
RNase H treated RNA (Step 2.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) 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 sample in a microcentrifuge.

2.3.4. Incubate the sample in a 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 sample in a microcentrifuge, and place on ice. Proceed immediately to RNA Purification.

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

2.4.1. Vortex the RNAClean XP or **RNA** Sample Purification Beads until fully resuspended.

2.4.2. Add 110 µl (2.2X) 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 the sample for **15 minutes on ice** to bind RNA to the beads.

2.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA **(Caution: do not discard the beads)**.

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

2.4.6. Repeat Step 2.4.5. once for a total of two washes.

2.4.7. Completely remove residual ethanol, and air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of RNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

2.4.8. Remove the tube from the magnet. Elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting up and down at least 10 times and briefly spin the tube.

2.4.9. Incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear (~2 minutes).

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

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

2.5. RNA Fragmentation and Priming

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

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

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

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

2.5.3. Place the sample in a thermal cycler with the heated lid set to 105°C and incubate the sample at 94°C following the recommendations in Table 2.5.1 below for libraries with inserts ~200 nt.

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

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

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

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

2.6. First Strand cDNA Synthesis

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

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

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

2.6.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

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

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

2.6.4. Proceed directly to Second Strand cDNA Synthesis.

2.7. Second Strand cDNA Synthesis

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

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

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

2.7.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 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 until fully resuspended.

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

2.8.3. Incubate for 5 minutes at room temperature.

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

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

2.8.6. Repeat Step 2.8.5. once for a total of two washes.

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

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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

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

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

2.9. End Prep of cDNA Library

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

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

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

2.9.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

2.9.3. Incubate the sample in a thermal cycler with the heated lid set at ≥ 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

2.10.1. Thaw the ● (red) NEBNext Adaptor* on ice and once thawed, dilute the adaptor in ice-cold Adaptor Dilution Buffer prior to setting up the ligation reaction. Keep both the adaptor and the adaptor dilution on ice at all times.

TOTAL RNA INPUT	DILUTION REQUIRED
1,000 ng–101 ng	5-fold dilution in Adaptor Dilution Buffer
100 ng–10 ng	25-fold dilution in Adaptor Dilution Buffer
< 10 ng	200-fold dilution in Adaptor Dilution Buffer

* The NEBNext adaptor is provided as part of one of various NEBNext Multiplex Oligo Kits that are supplied separately from the library prep kit.

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

LIGATION REACTION	VOLUME
End Prepped DNA (Step 2.9.4)	60 µl
Diluted Adaptor (Step 2.10.1)	2.5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours at 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and diluted 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 96.5 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

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

2.11.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

2.11.2. Add 87 µl (0.9X) 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.

2.11.3. Incubate for 10 minutes at room temperature.

2.11.4. 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 (~ 5 minutes), discard the supernatant that contains unwanted fragments. **(Caution: do not discard beads).**

2.11.5. 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.

2.11.6. Repeat Step 2.11.5 once for a total of two washes.

2.11.7. Briefly spin the tube, and put the tube back in the magnetic rack.

2.11.8. Completely remove the residual ethanol, and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

2.11.9. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 17 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 magnetic rack until the solution is clear.

2.11.10. Without disturbing the bead pellet, transfer 15 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

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

Use Option B for any NEBNext Multiplex Oligos Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined, 5 µM each.

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

2.12.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X) Primer/i7 Primer*, **	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	50 µl

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

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

2.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer Mix*	10 µl
Total Volume	50 µl

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

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 in 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	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 7.2 in Section 7).

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

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
1,000 ng	7–8
100 ng	11–12
10 ng	14–15
5 ng	15–16

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

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

2.13.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

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.

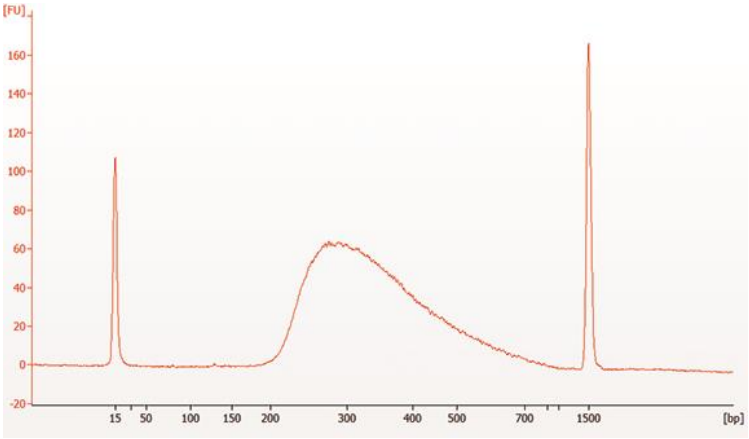
2.14. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

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

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

Note: If a peak at ~ 80 bp (primers) or ~128–140 bp (adaptor-dimer) is visible in the bioanalyzer traces, bring up the sample volume (from Step 2.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 2.13). Adaptor dimer peak range is derived from values observed on BioAnalyzer. Peaks may appear shifted on other library analysis instruments such as TapeStation or Fragment Analyzer.

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



Section 3
Protocol for Library Preparation of Degraded RNA (e.g., FFPE) using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310, #E6350) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, #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.

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

RNA Sample Requirements

RNA Integrity

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 or 4. For highly degraded samples (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3 (current section) or 5.

RNA Sample Requirements

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) and organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I, the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free Kit, TURBO DNase Treatment and Removal Reagents do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

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

The protocol is optimized for approximately 200 nt RNA inserts.

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

3.1. Probe Hybridization to RNA

3.1.1. Dilute 10 ng–100 ng of total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA on ice.

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (10 ng – 100 ng)	12 µl
○ (white) NEBNext rRNA Depletion Solution	1 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 **pre-heated** thermal cycler and run the following program with the heated lid set to 105°C.

This will take approximately 15–20 minutes to complete.

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

3.1.6. Briefly spin down the samples in 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 RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	20 µl

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

3.2.3. Briefly spin down the tube in a microcentrifuge.

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

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**:

COMPONENT	VOLUME
RNase H treated RNA (Step 3.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) DNase I (RNase-free)	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 pre-heated 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 RNAClean XP or RNA Sample Purification Beads until fully resuspended.

3.4.2. Add 110 µl (2.2X) 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 the sample for **15 minutes on ice** to bind RNA to the beads.

3.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA **(Caution: do not discard the beads)**.

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

3.4.6. Repeat Step 3.4.5 once for a total of two washes.

3.4.7. Completely remove residual ethanol, and air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of RNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

3.4.8. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting up and down ten times and briefly spin the tube.

3.4.9. Incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear (~2 minutes).

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

3.4.11. Place the sample on ice and proceed to Priming of Highly Degraded RNA.

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

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

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

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

3.5.3. Briefly spin down the samples in a microcentrifuge.

3.5.4. Incubate the sample in a preheated thermal cycler as follows.

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

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

3.6. First Strand cDNA Synthesis

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

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

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

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

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

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

3.6.4. Proceed directly to Second Strand cDNA Synthesis Reaction.

3.7. Second Strand cDNA Synthesis

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

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 3.6.3)	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

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

3.7.3 Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 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 until fully resuspended.

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

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

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

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

3.8.6. Repeat Step 3.8.5 once for a total of two washes.

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

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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

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

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

3.9. End Prep of cDNA Library

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

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

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

3.9.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

3.9.3. Incubate the sample in a thermal cycler with the heated lid set at ≥ 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

3.10.1. Thaw the • (red) NEBNext Adaptor* on ice and once thawed, dilute the adaptor in ice-cold Adaptor Dilution Buffer prior to setting up the ligation reaction. Keep both the adaptor and the adaptor dilution on ice at all times.

FFPE RNA	DILUTION REQUIRED
100 ng–10 ng	25-fold dilution in Adaptor Dilution Buffer

*The NEBNext adaptor is provided as part of one of various NEBNext Multiplex Oligo Kits that are supplied separately from the library prep kit.

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)	2.5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours at 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and diluted 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** (lid off) in a thermal cycler.

3.10.5. Add 3 µl (blue or red) USER Enzyme to the ligation mixture from Step 3.10.4, resulting in total volume of 96.5 µl.

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

3.10.7. Proceed immediately to Purification of the Ligation Reaction.

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

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

3.11.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

3.11.2. Add 87 µl (0.9X) 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.

3.11.3. Incubate for 10 minutes at room temperature.

3.11.4. 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), discard the supernatant that contains unwanted fragments. **(Caution: do not discard beads)**.

3.11.5. 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.

3.11.6. Repeat Step 3.11.5 once for a total of two washes.

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

3.11.8. Completely remove the residual ethanol, and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

3.11.9. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 17 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 magnetic rack until the solution is clear.

3.11.10. Without disturbing the bead pellet, transfer 15 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

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

Use Option B for any NEBNext Multiplex Oligos Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined, 5 µM each.

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

3.12.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 3.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X) Primer/i7 Primer*, **	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	50 µl

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

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

3.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 3.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer Mix*	10 µl
Total Volume	50 µl

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

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 in 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	12–16*, **
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. The recommendation of PCR cycles are based on internal tests for FFPE RNA.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.2 in Section 6).

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

FFPE RNA INPUT	RECOMMENDED PCR CYCLES
100 ng	12–13
10 ng	15–16

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 until fully resuspended.

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

3.13.3. Incubate for up to 5 minutes at room temperature.

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

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

3.13.6. Repeat Step 3.13.5 once for a total of 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.

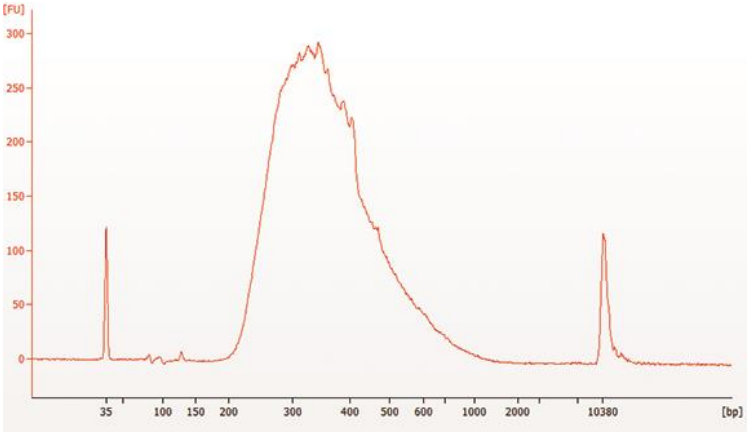
3.14. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

3.14.1. Run 1 µl library on a DNA High Sensitivity Chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

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

Note: If a peak at ~ 80 bp (primers) or ~128–140 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). Adaptor dimer peak range is derived from values observed on Bioanalyzer. Peaks may appear shifted on other library analysis instruments such as TapeStation or Fragment Analyzer.

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



Section 4
Protocol for Library Preparation of Intact or Partially Degraded RNA using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310, #E6350) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, #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 using Universal Human Reference Total RNA.

RNA Sample Requirements

RNA Integrity

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 or 4 (current section). For highly degraded samples (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3 or 5.

RNA Sample Requirements

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) and organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I, the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free Kit, TURBO DNase Treatment and Removal Reagents do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

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

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

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

4.1. Hybridize the Probes to the RNA

4.1.1. Dilute 5 ng–1 µg of total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA on ice.

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (5 ng – 1 µg)	12 µl
○ (white) NEBNext rRNA Depletion Solution	1 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 **pre-heated** thermal cycler and run the following program with the heated lid set to 105°C.

This will take approximately 15–20 minutes to complete.

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

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

4.2. RNase H Digestion

4.2.1. Assemble the RNAse H digestion reaction **on ice** as follows.

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 4.1.6)	15 µl
○ (white) RNase H Reaction Buffer	2 µl
○ (white) NEBNext 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 pre-heated thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off).

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**:

COMPONENT	VOLUME
RNase H treated RNA (Step 4.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) DNase I (RNase-free)	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 pre-heated 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 RNAClean XP or **RNA** Sample Purification Beads until fully resuspended.

4.4.2. Add 110 µl (2.2X) 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 the sample for **15 minutes on ice** to bind RNA to the beads.

4.4.4. Place the ○ (white) tube on a magnetic rack to separate beads from the supernatant. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA **(Caution: do not discard the beads)**.

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

4.4.6. Repeat Step 4.4.5 once for a total of two washes.

4.4.7. Completely remove residual ethanol, and air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of RNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

4.4.8. Remove the tube from the magnet. Elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting up and down at least 10 times and briefly spin the tube.

4.4.9. Incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear (~2 minutes).

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

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

4.5. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 4.5.1.

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

FRAGMENTATION AND PRIMING REACTION	VOLUME
Ribosomal RNA Depleted Sample (Step 4.4.11)	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	10 µl

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

4.5.3. Place the sample on a thermal cycler with the heated lid set to 105°C and incubate the sample at 94°C following the recommendations in Table 4.5.1 below for libraries with inserts ~200 nt.

Table 4.5.1. Suggested fragmentation times based on RIN value of RNA input.

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

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

4.5.4. Immediately transfer the tube 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 fragmented and primed RNA from Step 4.5.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 4.5.4)	10 µl
Nuclease-free Water	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

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

4.6.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:
Note: If you are following recommendations in Appendix A (Section 6), for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

4.6.4. Proceed directly to Second Strand cDNA Synthesis.

4.7. Second Strand cDNA Synthesis

4.7.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis 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 at least 10 times.

4.7.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 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 until fully resuspended.

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 rack. 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 magnetic rack with lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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

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 cDNA 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 the sample in a thermal cycler with the heated lid set at ≥ 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

4.10.1. Thaw the • (red) NEBNext Adaptor* on ice and once thawed, dilute the adaptor in ice-cold Adaptor Dilution Buffer prior to setting up the ligation reaction. Keep both the adaptor and the adaptor dilution on ice at all times.

TOTAL RNA INPUT	DILUTION REQUIRED
1,000 ng–101 ng	5-fold dilution in Adaptor Dilution Buffer
100 ng–10 ng	25-fold dilution in Adaptor Dilution Buffer
< 10 ng	200-fold dilution in Adaptor Dilution Buffer

* The NEBNext adaptor is provided as part of one of various NEBNext Multiplex Oligo Kits that are supplied separately from the library prep kit.

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)	2.5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours at 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and diluted 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** in a thermal cycler.

4.10.5. Add 3 µl (red or blue) USER Enzyme to the ligation mixture from Step 4.10.4, resulting in total volume of 96.5 µl.

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

4.10.7. Proceed immediately to Purification of the Ligation Reaction.

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

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

4.11.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

4.11.2. Add 87 µl (0.9X) 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.

4.11.3. Incubate for 5 minutes at room temperature.

4.11.4. 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 (~ 5 minutes), discard the supernatant that contains unwanted fragments. **(Caution: do not discard beads).**

4.11.5. 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.

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

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

4.11.8. Completely remove the residual ethanol, and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

4.11.9. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 17 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 magnetic rack until the solution is clear.

4.11.10. Without disturbing the bead pellet, transfer 15 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

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

Use Option B for any NEBNext Multiplex Oligos Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined, 5 µM each.

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

4.12.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X) Primer/i7 Primer*, **	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	50 µl

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

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

4.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer Mix*	10 µl
Total Volume	50 µl

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

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 in 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	6–15*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

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

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.2 in Section 6).

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

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
1,000 ng	6–7
100 ng	10–11
10 ng	13–14
5 ng	14–15

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

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

4.13.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

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.

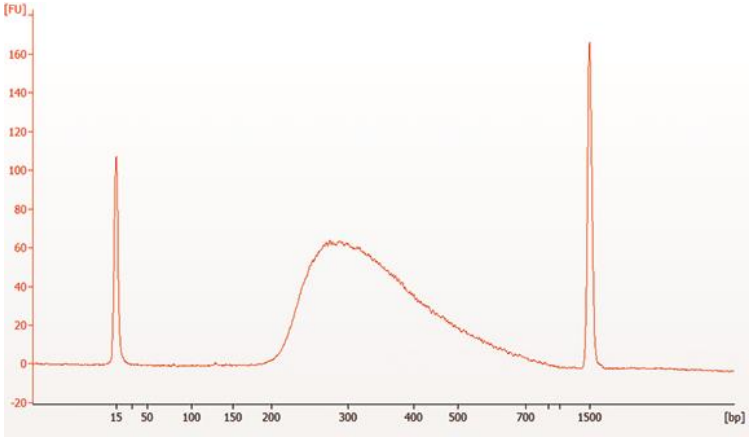
4.14. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

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

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

Note: If a peak at ~ 80 bp (primers) or ~128–140 bp (adaptor-dimer) is visible in the bioanalyzer traces, bring up the sample volume (from Step 4.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 4.13). Adaptor dimer peak range is derived from values observed on Bioanalyzer. Peaks may appear shifted on other library analysis instruments such as TapeStation or Fragment Analyzer.

Figure 4.14.1 Example of RNA library size distribution on a Bioanalyzer.



Section 5
Protocol for Library Preparation of Degraded RNA (e.g., FFPE) using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310, #E6350) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, #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 using fragmented Universal Human Reference Total RNA and FFPE RNA.

RNA Sample Requirements

RNA Integrity

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 or 4. For highly degraded samples (e.g., FFPE), which do not require fragmentation, follow the library preparation protocol in Section 3 or 5 (current section).

RNA Sample Requirements

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) and organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I, the enzyme should be removed from the sample. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation. Prior to depletion the RNA must be in nuclease free water. Some products, e.g., TURBO DNA-free Kit, TURBO DNase Treatment and Removal Reagents do not produce RNA in nuclease free water and are not compatible with NEBNext rRNA depletion.

Input Amount

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

The protocol is optimized for approximately 200 nt RNA inserts.

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

5.1. Hybridize the Probes to the RNA

5.1.1. Dilute 10 ng–100 ng of total RNA with Nuclease-free Water to a final volume of 12 µl in a PCR tube. Keep the RNA on ice.

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (10 ng–100 ng)	12 µl
○ (white) NEBNext rRNA Depletion Solution	1 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	15 µl

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

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

5.1.5. Place tube in a **pre-heated** thermal cycler and run the following program with the heated lid set to 105°C.

This will take approximately 15-20 minutes to complete.

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

5.1.6. Briefly spin down the samples in microcentrifuge and place on ice. Proceed immediately to RNase H Digestion.

5.2. RNase H Digestion

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

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 5.1.6)	15 µl
○ (white) RNase H Reaction Buffer	2 µl
○ (white) NEBNext RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	20 µl

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

5.2.3. Briefly spin down the tube in a microcentrifuge.

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

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

5.3. DNase I Digestion

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

COMPONENT	VOLUME
RNase H treated RNA (Step 5.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	50 µl

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

5.3.3. Briefly spin down the tube in a microcentrifuge.

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

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

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

5.4.1. Vortex the RNAClean XP or **RNA** Sample Purification Beads until fully resuspended.

5.4.2. Add 110 µl (2.2X) beads to the RNA sample from Step 5.3.5 and mix thoroughly by pipetting up and down at least 10 times.

5.4.3. Incubate the sample for **10 minutes on ice** to bind RNA to the beads.

5.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA **(Caution: do not discard the beads)**.

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

5.4.6. Repeat Step 5.4.5 once for a total of two washes.

5.4.7. Completely remove residual ethanol, and air dry the beads for **up to 5 minutes** while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of RNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

5.4.8. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl Nuclease-free Water. Mix well by pipetting up and down ten times and briefly spin the tube.

5.4.9. Incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear (~2 minutes).

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

5.4.11. Place the sample on ice and proceed to Priming of Highly Degraded RNA.

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

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

PRIMING REACTION	VOLUME
rRNA Depleted Sample (Step 5.4.11)	5 µl
• (lilac) Random Primers	1 µl
Total Volume	6 µl

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

5.5.3. Briefly spin down the samples in a microcentrifuge.

5.5.4. Incubate the sample in a preheated thermal cycler as follows.

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

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

5.6. First Strand cDNA Synthesis

5.6.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the primed RNA from Step 5.5.5:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 5.5.5)	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

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

5.6.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

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

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

5.6.4. Proceed directly to Second Strand cDNA Synthesis Reaction.

5.7. Second Strand cDNA Synthesis

5.7.1 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 5.6.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 5.6.3)	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

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

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

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

5.8.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

5.8.2. 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.

5.8.3. Incubate for up to 5 minutes at room temperature.

5.8.4. 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).**

5.8.5. 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.

5.8.6. Repeat Step 5.8.5 once for a total of two washes.

5.8.7. 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.

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

5.8.9. 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.

5.9. End Prep of cDNA Library

5.9.1. Assemble the end prep reaction on ice by adding the following components to the second strand synthesis product from Step 5.8.9.

END PREP REACTION	VOLUME
Second Strand Synthesis Product (Step 5.8.9)	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.

5.9.2. 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.

5.9.3. Incubate the sample in a thermal cycler with the heated lid set at ≥ 75°C as follows.

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

5.9.4. Proceed immediately to Adaptor Ligation.

5.10. Adaptor Ligation

5.10.1. Thaw the • (red) NEBNext Adaptor* on ice and once thawed, dilute the adaptor in ice-cold Adaptor Dilution Buffer prior to setting up the ligation reaction. Keep both the adaptor and the adaptor dilution on ice at all times.

FFPE RNA	DILUTION REQUIRED
100 ng–10 ng	25-fold dilution in Adaptor Dilution Buffer

* The NEBNext adaptor is provided as part of one of various NEBNext Multiplex Oligo Kits that are supplied separately from the library prep kit.

5.10.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 5.9.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 5.9.3)	60 µl
Diluted Adaptor (Step 5.10.1)	2.5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

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

5.10.3. 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.

5.10.4. Incubate **15 minutes at 20°C** in a thermal cycler.

5.10.5. Add 3 µl (red or blue) USER Enzyme to the ligation mixture from Step 5.10.4, resulting in total volume of 96.5 µl.

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

5.10.7. Proceed immediately to Purification of the Ligation Reaction.

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

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

5.11.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

5.11.2. Add 87 µl (0.9X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

5.11.3. Incubate for 5 minutes at room temperature.

5.11.4. 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), discard the supernatant that contains unwanted fragments. **(Caution: do not discard beads)**.

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

5.11.6. Repeat Step 5.11.5 once for a total of two washes.

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

5.11.8. Completely remove the residual ethanol, and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

5.11.9. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 17 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 magnetic rack until the solution is clear.

5.11.10. Without disturbing the bead pellet, transfer 15 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.

5.12. PCR Enrichment of Adaptor Ligated DNA

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

Use Option B for any NEBNext Multiplex Oligos Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined, 5 µM each.

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

5.12.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 5.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X) Primer/i7 Primer*, **	5 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Total Volume	50 µl

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

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

5.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 5.11.10)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer Mix*	10 µl
Total Volume	50 µl

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

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

5.12.3. Place the tube in a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 5.12.3A and Table 5.12.3B):

Table 5.12.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12–16*, **
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. The recommendation of PCR cycles are based on internal tests for FFPE RNA.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.2 in Section 7).

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

FFPE RNA INPUT	RECOMMENDED PCR CYCLES
100 ng	12–13
10 ng	15–16

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

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

5.13.2. 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.

5.13.3. Incubate for up to 5 minutes at room temperature.

5.13.4. 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).**

5.13.5. 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.

5.13.6. Repeat Step 5.13.5 once for a total of 2 washing steps.

5.13.7. 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.

5.13.8. 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.

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

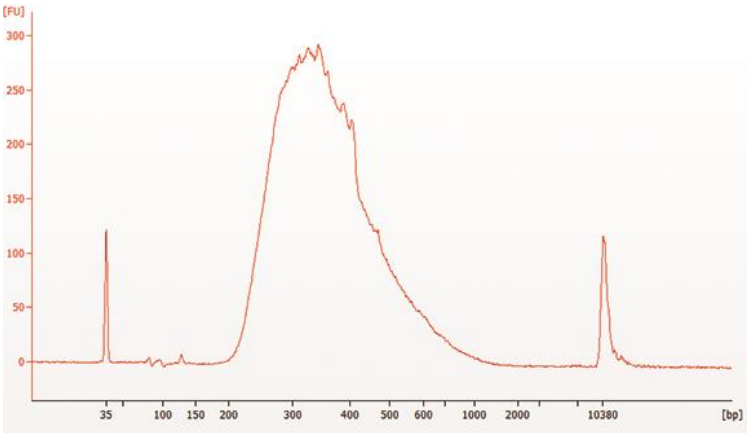
5.14. Assess Library Quality on an Agilent Bioanalyzer DNA Chip

5.14.1. Run 1 µl library on a DNA High Sensitivity Chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

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

Note: If a peak at ~ 80 bp (primers) or ~128–140 bp (adaptor-dimer) is visible in the bioanalyzer traces, bring up the sample volume (from Step 5.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 5.13). Adaptor dimer peak range is derived from values observed on Bioanalyzer. Peaks may appear shifted on other library analysis instruments such as TapeStation or Fragment Analyzer.

Figure 5.14.1 Example of FFPE RNA library size distribution on a Bioanalyzer.

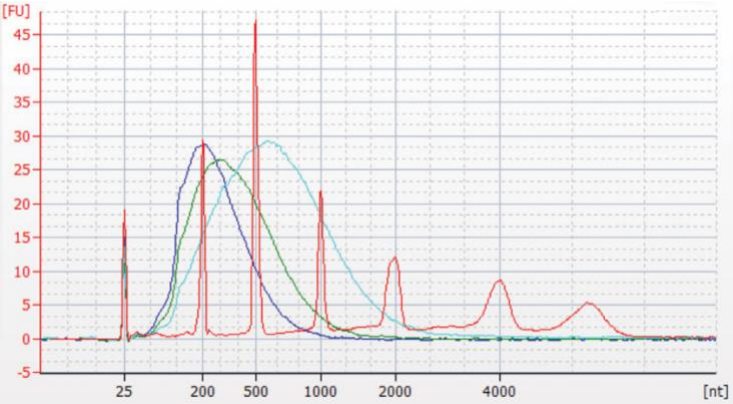


Section 6
Appendix for use with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, #E7765) or NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, #E7775)

6.1. Fragmentation

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

Figure 6.1. Modified fragmentation times for longer RNA inserts.



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

Modified fragmentation times for longer RNA inserts. Bioanalyzer traces of RNA as shown in an RNA Pico Chip. mRNA isolated from Universal Human Reference RNA and fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 5, 10 or 15 minutes, and purified using 2.2X volume of Agencourt® RNAClean® XP Beads.

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

For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes and remember to increase the incubation at 42°C from 15 to 50 minutes during the first strand cDNA synthesis reaction.

6.2. Size Selection of Adaptor Ligated DNA

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

The size selection protocol is based on a starting volume of 96.5 µl. Size selection conditions were optimized with SPRIselect Beads and NEBNext Sample Purification Beads; however, AMPure XP Beads can be used following the same conditions. If using Ampure XP Beads, please allow the beads to warm to room temperature for at least 30 minutes before use.

Please adjust recommended bead volumes for each target size according to Table 6.2. The protocol below is for libraries with a 300 bp insert size (420 bp final library size).

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

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

LIBRARY PARAMETER	APPROXIMATE INSERT SIZE	300 bp	400 bp	450 bp
	Approx. Final Library Size	420 bp	520 bp	570 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.

6.2.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads until fully resuspended.

6.2.2. Add 25 µl of resuspended beads to the 96.5 µl ligation reaction. Mix well by pipetting up and down at least 10 times.

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

6.2.4. Place the tube on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic rack. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube **(Caution: do not discard the supernatant).** Discard the beads that contain the unwanted large fragments.

6.2.5. Add 10 µl resuspended beads to the supernatant, mix well by pipetting up and down at least 10 times and incubate for 5 minutes at room temperature.

6.2.6. Place the tube/plate on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic rack. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard beads).**

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

6.2.8. Repeat Step 6.2.7 once for a total of two washes.

6.2.9. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

6.2.10. Remove the tube/plate from the magnetic rack. Elute the DNA target from the beads by adding 17 µl of 0.1 X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube and incubate for **2 minutes at room temperature**.

6.2.11. Place the tube on a magnetic rack. After the solution is clear (about 5 minutes), transfer 15 µl to a new PCR tube for amplification.

6.3. PCR Enrichment of Size-selected Libraries

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

⚠

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

Use Option B for any NEBNext Multiplex Oligos Kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at 10 µM combined, 5 µM each.

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

6.3.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 6.2.11)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Index (X) Primer/i7 Primer*, **	5 µl
Total Volume	50 µl

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

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

6.3.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 6.2.11)	15 µl
• (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer Mix*	10 µl
Total Volume	50 µl

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

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

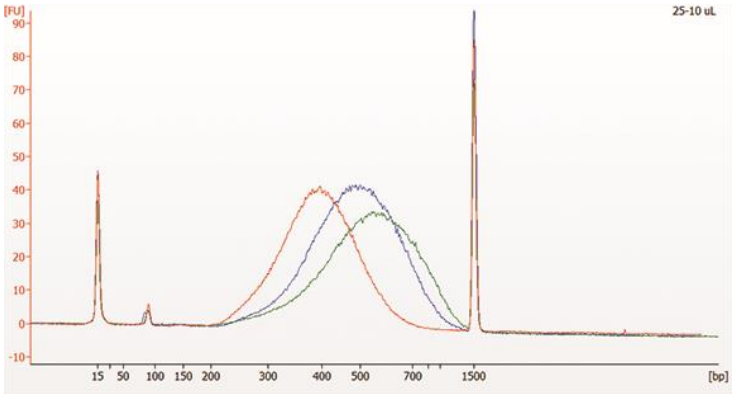
6.3.3. Place the tube in a thermal cycler with the heated lid set to 105°C. Perform PCR amplification using the following PCR cycling conditions:

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 7.2 in Section 7).

Figure 6.3.3: Bioanalyzer traces of size selected RNA libraries.



50 ng mRNA was fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 5 and 10 minutes, respectively. Libraries were size-selected as described in Table 6.3, then amplified by PCR and run on Agilent Bioanalyzer DNA 1000 chip. Fragmentation times and corresponding size selection conditions are shown in the table below.

Table 6.3:

LIBRARY SAMPLE	FRAGMENTATION TIME	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

Section 7
Troubleshooting Guide for use with the NEBNExt Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, #E7765) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, #E7775)

OBSERVATIONS	POSSIBLE CAUSES	EFFECT	SUGGESTED SOLUTIONS
Presence of Bioanalyzer peaks <85 bp (Figure 7.1)	• 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 ~127 bp adaptor-dimer Bioanalyzer peak (Figure 7.1)	• Addition of non-diluted adaptor • RNA input was too low • RNA was over fragmented or lost during fragmentation • Inefficient Ligation • Ligation was not setup on ice	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 • Setup ligation reaction on ice • Clean up PCR reaction again with 0.9X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield)
Presence of additional Bioanalyzer peak at higher molecular weight than the expected library size (~ 1,000 bp) (Figure 7.2)	• 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 7.1:

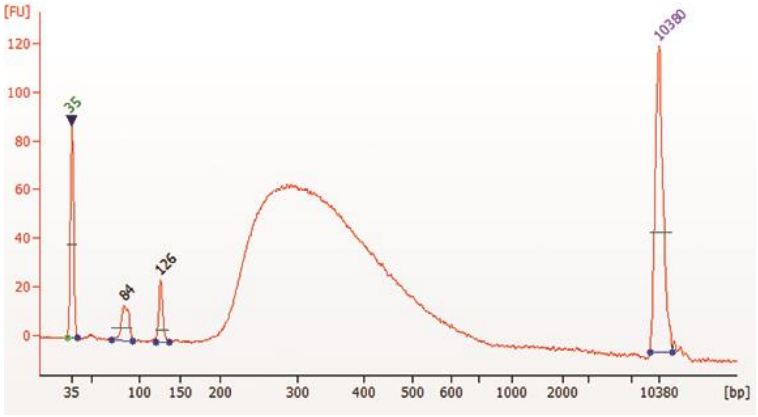
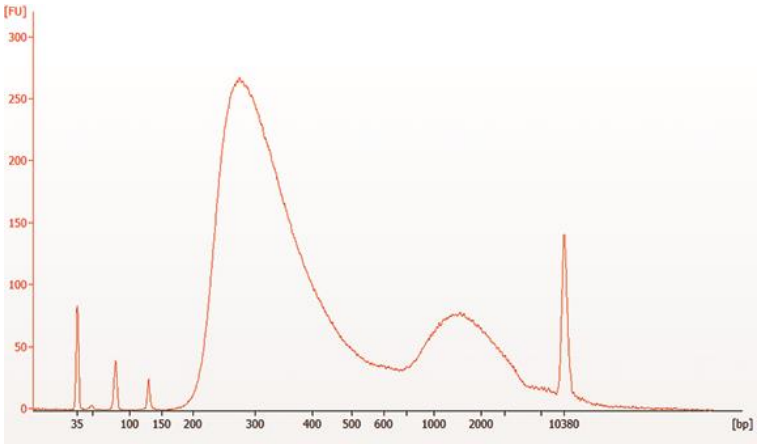


Figure 7.2:



Kit Components

NEB #E6310S Table of Components

NEB #	PRODUCT	VOLUME
E6318-2	NEBNext RNase H	0.012 ml
E6312-2	RNase H Reaction Buffer	0.012 ml
E6313-2	NEBNext rRNA Depletion Solution	0.010 ml
E6314-2	NEBNext Probe Hybridization Buffer	0.012 ml
E6316-2	DNase I (RNase-free)	0.015 ml
E6315-2	DNase I Reaction Buffer	0.03 ml
E6317-2	Nuclease-free Water	0.4 ml

NEB #E6310L Table of Components

NEB #	PRODUCT	VOLUME
E6318-3	NEBNext RNase H	0.048 ml
E6312-3	RNase H Reaction Buffer	0.048 ml
E6313-3	NEBNext rRNA Depletion Solution	0.024 ml
E6314-3	NEBNext Probe Hybridization Buffer	0.048 ml
E6316-3	DNase I (RNase-free)	0.06 ml
E6315-3	DNase I Reaction Buffer	0.120 ml
E6317-3	Nuclease-free Water	1.5 ml

NEB #E6310X Table of Components

NEB #	PRODUCT	VOLUME
E6318-4	NEBNext RNase H	0.192 ml
E6312-4	RNase H Reaction Buffer	0.192 ml
E6313-4	NEBNext rRNA Depletion Solution	0.096 ml
E6314-4	NEBNext Probe Hybridization Buffer	0.192 ml
E6316-4	DNase I (RNase-free)	0.24 ml
E6315-4	DNase I Reaction Buffer	0.48 ml
E6317-4	Nuclease-free Water	6.0 ml

NEB #E6350S Table of Components

NEB #	PRODUCT	VOLUME
E6318-2	NEBNext RNase H	0.012 ml
E6312-2	RNase H Reaction Buffer	0.012 ml
E6313-2	NEBNext rRNA Depletion Solution	0.010 ml
E6314-2	NEBNext Probe Hybridization Buffer	0.012 ml
E6316-2	DNase I (RNase-free)	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 #E6350L Table of Components

NEB #	PRODUCT	VOLUME
E6318-3	NEBNext RNase H	0.048 ml
E6312-3	RNase H Reaction Buffer	0.048 ml
E6313-3	NEBNext rRNA Depletion Solution	0.024 ml
E6314-3	NEBNext Probe Hybridization Buffer	0.048 ml
E6316-3	DNase I (RNase-free)	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 #E6350X Table of Components

NEB #	PRODUCT	VOLUME
E6318-4	NEBNext RNase H	0.192 ml
E6312-4	RNase H Reaction Buffer	0.192 ml
E6313-4	NEBNext rRNA Depletion Solution	0.096 ml
E6314-4	NEBNext Probe Hybridization Buffer	0.192 ml
E6316-4	DNase I (RNase-free)	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

CheckList

1. Hybridize the Probes to the RNA

[_] 1.1. Prepare Master Mix

- [_] 1.1.1. NEBNext rRNA Depletion Solution 1 µl
- [_] 1.1.2. Probe Hybridization Buffer 2 µl

[_] 1.2. Add 3 µl of mix to 12 µl total RNA

[_] 1.3. Mix 10 times

[_] 1.4. Quick Spin

[_] 1.5. Put in Thermal cycler (95°C for 2 min, 95-22°C 0.1°C/sec, 22°C 5 min)

[_] 1.6. Quick spin, place on ice

2. RNase H Digestion

[_] 2.1. Prepare Master Mix and mix

- [_] 2.1.1. RNase H 2 µl
- [_] 2.1.2. RNase H Reaction Buffer 2 µl
- [_] 2.1.3. Nuclease-free Water 1 µl

[_] 2.2. Add 5 µl of master mix to sample

[_] 2.3. Mix 10 times

[_] 2.4. Quick Spin

[_] 2.5. Put in Thermal cycler (37°C for 30 min)

[_] 2.6. Quick spin, place on ice

3. DNase I Digestion

[_] 3.1. Prepare Master Mix and mix

- [_] 3.1.1. DNase I Reaction Buffer 5 µl
- [_] 3.1.2. DNase I 2.5 µl
- [_] 3.1.3. Nuclease-free Water 22.5 µl

[_] 3.2. Add 30 µl of master mix to sample

[_] 3.3. Mix 10 times

[_] 3.4. Quick Spin

[_] 3.5. Put in Thermal cycler (37°C for 30 mn)

[_] 3.6. Quick spin, place on ice

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

[_] 4.1. Add 110 µl of beads and mix 10 times

[_] 4.2. Incubate on ice 15 min

[_] 4.3. Place on Magnet 5 min

[_] 4.4. Remove Supernatant

[_] 4.5. Add 200 µl 80% ethanol, remove after 30 seconds

[_] 4.6. Repeat Step 4.5 once

[_] 4.7. Air dry for up to 5 min

[_] 4.8. Add 8 µl of Nuclease-free Water and mix 10 times; wait 2 min

[_] 4.9. Place on magnet 5 min

[_] 4.10. Transfer 6 µl to new tube

[_] 4.11. Place on ice or store

Revision History

REVISION #	DESCRIPTION	DATE
2.0	Component change: The name, part # and formulation of RNase H has changed.	11/15
3.0	Protocol edits, renumbering and Quick Checklist	8/16
4.0	E6350 Kit was created and merged with E6310.	4/17
5.0	Required materials not included has additional materials added. Workflow diagram was updated. RNA Sample Recommendations was updated and new text added. Protocol was updated. FAQs were deleted but kept on web page. Kit components were placed in table styles for E6310 as well as E6350.	12/18
6.0	Updated to new manual format.	2/20
7.0	Added section 2-5 protocols.	12/21
8.0	Updated protocols.	8/22

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