INSTRUCTION MANUAL

NEBNext® RNA Depletion Core Reagent Set

NEB #E7865 S/L/X, #E7870 S/L/X

6/24/96 reactions

Version 5.0_10/25

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Kit Includes

The volumes provided are sufficient for preparation of up to 6 reactions (NEB #E7865S/#E7870S) 24 reactions (NEB #E7865L/#E7870L) and 96 reactions (NEB #E7865X/#E7870X).

Package 1: Store at –20°C.

- (white) NEBNext Probe Hybridization Buffer
- (white) NEBNext Thermostable RNase H
- (white) RNase H Reaction Buffer
- (white) NEBNext DNase I
- (white) DNase I Reaction Buffer

Nuclease-free Water

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

Supplied only with NEBNext RNA Depletion Core Reagent Set with RNA Sample Purification Beads, NEB #E7870.

NEBNext RNA Sample Purification Beads

Required Materials Not Included

- Pipettes
- Custom RNA Depletion Probe Pool
- 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 #E7865 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):

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

Please note: This manual is not for use with UNIQUE DUAL INDEX UMI ADAPTORS.

Adaptor trimming sequences:

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

Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

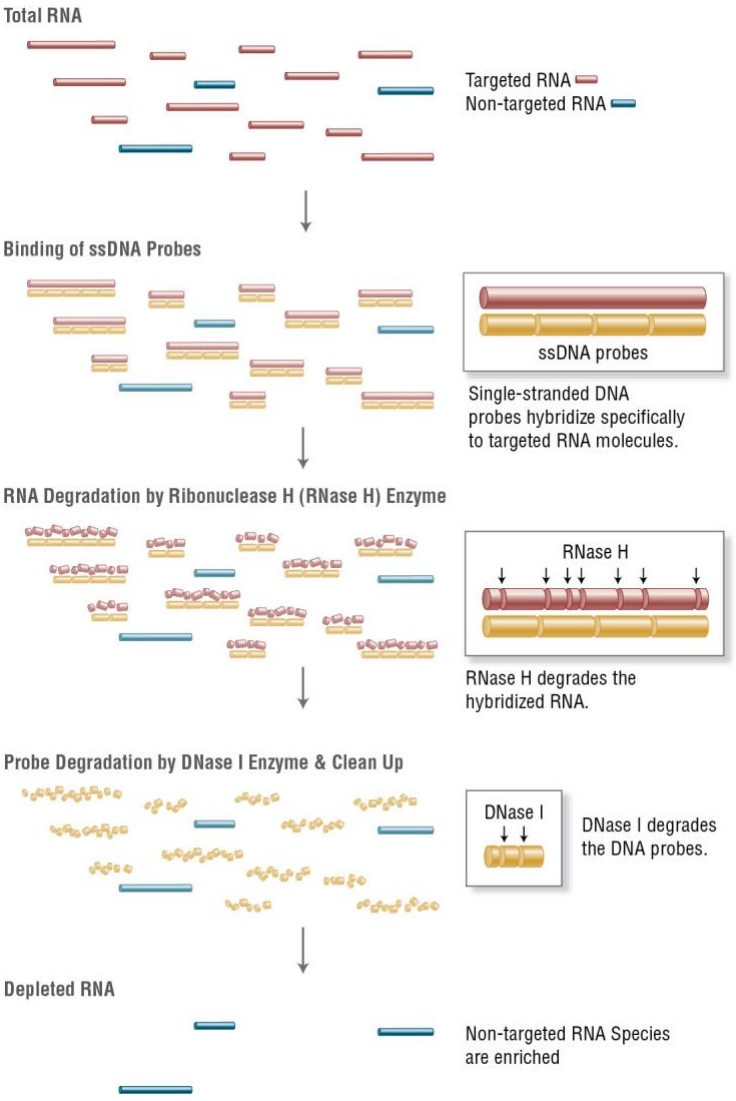
Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Overview

The NEBNext RNA Depletion Core Reagent Set employs the NEBNext RNase H-based RNA Depletion Workflow (Figure 1) to remove unwanted RNA species. Depletion of unwanted RNA allows for increased sequencing coverage of non-targeted transcripts.

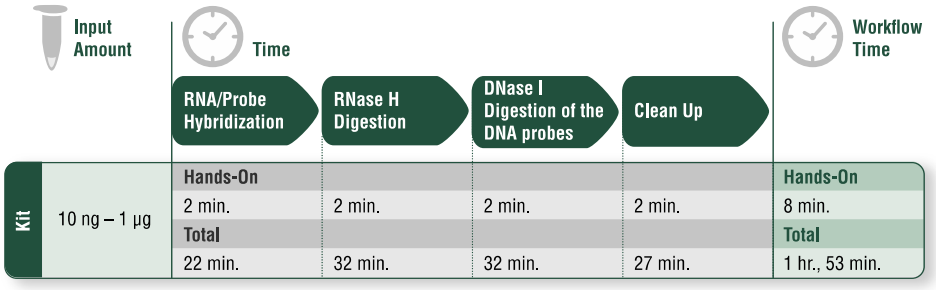
The NEBNext RNA Depletion Core Reagent Set is used in combination with a user-supplied RNA depletion probe pool. Refer to the *Guidelines for Use of Custom RNA Depletion Probe Pools with the NEBNext RNA Depletion Core Reagent Set* section for details on how to design and prepare this pool.

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



DNA probes designed against unwanted RNAs are hybridized to total RNA, followed by an RNase H digestion where the enzyme recognizes the RNA:DNA hybrid and degrades the targeted RNA. Finally, the DNA probes are digested with DNase I and the reaction is cleaned using magnetic beads.

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



Applications

The resulting 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 RNA 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 Kit (NEB #E7770, #E7775) has a non-directional, streamlined and automatable workflow.

When using the NEBNext RNA Depletion Core Reagent Set (NEB #E7865, #E7870) 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)]

Please note: This manual is not for use with UNIQUE DUAL INDEX UMI ADAPTORS.

Every section in this manual contains a different protocol based on the starting material and application. Please read "RNA Sample Recommendations" and "Input Amount" sections in their 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.

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

Guidelines for use of Custom RNA Depletion Probe Pools with the NEBNext RNA Depletion Core Reagent Set

Design of Custom RNA Depletion Probes

DNA probes refer to ssDNA oligonucleotides used to remove unwanted RNAs from total RNA in the NEBNext RNase H-based RNA Depletion workflow. To facilitate the design of probes we have created a Custom RNA Depletion Design Tool at https://depletion-design.neb.com/. The input to the tool is the sequence of the unwanted/targeted RNA, 5′ to 3′ in FASTA format (Figure 2). The input sequence should only contain A, C, G or T nucleotides. The tool outputs and emails a list of probe sequences antisense to the targeted RNA sequence (Figure 3).

Figure 2. Example of input sequence for the Custom RNA Depletion Design Tool.

>MT-ATP8-201_ENSE00001435286|protein_coding
ATGCCCCAACTAAATACTACCGTATGGCCCACCATAATTACCCCCATACTCCTTACACTATTCCTCATCACCCAACTAAA
AATATTAAACACAAACTACCACCTACCTCCCTCACCAAAGCCCATAAAAATAAAAAATTATAACAAACCCTGAGAACC
AAAATGAACGAAAATCTGTTCGCTTCATTCATTGCCCCCACAATCCTAG

Figure 3. Example of probes designed against the MT-ATP8-201 transcript using the Custom RNA Depletion Design Tool.

MT-ATP8-201_ENSE000014:26-81 TTTAGTTGGGTGATGAGGAATAGTGTAAGGAGTATGGGGGTAATTATGGTGGGCC
MT-ATP8-201_ENSE000014:63-113 AGGGAGGTAGGTGGTAGTTTGTGTTTAATATTTTTAGTTGGGTGATGAGG
MT-ATP8-201_ENSE000014:94-134 TTATTTTTATGGGCTTTGGTGAGGGAGGTAGGTGGTAGTT
MT-ATP8-201_ENSE000014:129-178 ACAGATTTTCGTTCATTTTGGTTCTCAGGGTTTGTTATAATTTTTTATT
MT-ATP8-201_ENSE000014:160-200 GTGGGGGCAATGAATGAAGCGAACAGATTTTCGTTCATTT

The NEBNext Custom RNA Depletion Design Tool designs probes based on the RNA sequence provided. Therefore, non-targeted transcripts with sequence homology to the targeted transcript will also be depleted. To identify potential unintended depletion, it is recommended to check for sequence homology between the targeted sequence and other transcripts. This can be done by using standard sequence homology search tools (e.g., NCBI BLAST, Bowtie2, etc.). Transcripts with high sequence similarity to the targeted RNA will likely be depleted. We have observed depletion of transcripts with ~ 70% homology (or greater), however depletion efficiency will also be influenced by the relative abundance of all homologous sites in your sample.

Ordering and Pooling of the Custom RNA Depletion Probes

Order the single stranded DNA probes from a trusted oligonucleotide synthesis provider. Standard desalting purification is sufficient, and no 5′ or 3′ end modifications are necessary.

Pool the DNA probes to create the Custom RNA Depletion Probe Pool which is used in the NEBNext RNA Depletion Core Reagent Set protocols.

We recommend preparing an equimolar probe pool, in which each probe is present at a final concentration of 2 µM. Use 2 µl of this pool per RNA depletion reaction.

Note: Optimization of the probe pool composition and amount might be required to achieve efficient depletion of your target RNA species. The following steps outline one approach for preparing an equimolar probe pool at a final concentration of 2 µM per probe. This approach involves normalizing all the probes to the same concentration and pooling equal volumes to achieve equimolar representation.

1. Calculate Probe Quantity

The amount of probe required depends on the total number of reactions planned and the target final concentration for each probe. In this protocol we use 2 µl of probe per reaction of an equimolar probe pool with each probe at a concentration of 2 µM (2 pmol/µl). This means each reaction requires 4 pmol of each probe:
Amount of each probe required per reaction (pmol): 2 µl × 2 pmol/µl = 4 pmol

To determine how much of each probe to order, multiply 4 pmol by the total number of reactions:
Total amount of each probe to order (pmol) = 4 pmol/reaction × number of reactions

It is recommended to order a small excess of each probe to account for potential loss during pipetting, aliquoting, or other handling steps. When placing an order, confirm that the required amount of oligo falls within the manufacturer's guaranteed yield for the selected synthesis scale.

2. Determine Minimum Stock Concentration

Each probe must be concentrated enough so that, when pooled in equal volumes, the final concentration of each individual probe in the mix is 2 µM.

Use the formula below to calculate the minimum required stock concentration for each probe:
Minimal stock concentration (µM) = concentration of each probe in final pool (2 µM) × number of probes in the pool

3. Resuspend Probes

Probes may be supplied pre-resuspended or lyophilized. Ordering pre-resuspended probes is preferable as it reduces hands on time and lowers the risk of cross-contamination. When ordering pre-resuspended probes, request each probe to be normalized to the minimal stock concentration calculated in Step 2 (or higher), and resuspended using 10 mM Tris, 0.1 mM EDTA, pH 7.5.

If ordering lyophilized probes, spin down the tube or plate to collect the material before resuspending in 10 mM Tris, 0.1 mM EDTA, pH 7.5 to achieve the minimal stock concentration calculated in Step 2 (or higher). Ensure that each probe is fully resuspended prior to pooling, and verify the concentration of each probe using a spectrophotometer. To prevent cross-contamination probes should be pooled in a PCR hood.

Calculate the volume required to resuspend your lyophilized probes to the stock concentration with this formula:
Resuspension volume (µl) = amount of probe (pmol)/probe stock concentration (µM = pmol/µl)

4. Pool Probes Equimolarly

To prepare an equimolar probe pool first confirm that all the probes are normalized to same concentration, then pipette equal volumes of each probe into a single tube. Mix well.

If the probes were normalized to the minimal stock concentration (calculated in Step 2), each probe in the pool will be at the desired 2 µM each final concentration.

If the probes were normalized to a higher stock concentration, calculate the concentration of each probe in the pool and then dilute to 2 µM using 10 mM Tris, 0.1 mM EDTA, pH 7.5.:
Concentration of each probe in pool (µM)* = stock concentration (µM) / number of probes in the pool
*when equal volumes of probes at the same stock concentration are pooled

It might be practical to pool only a percentage of each of the resuspended oligos so that the amount of individual oligos can be adjusted by spiking in more of those oligos if necessary.

The final custom RNA Depletion Probe Pool can be stored at -20°C.

Follow the steps on the protocol sections to use the custom pool with the NEBNext RNA Depletion Core Reagent Set. If combining the Custom RNA Depletion Probe Pool with an existing NEBNext depletion solution, please refer to the next section

Example Calculations for a 250-Probe Design Requiring 10,000 Reactions

1. Amount of Probe

Total amount of each probe to order (pmol) = 4 pmol/reaction × number of reactions
Total amount of each probe to order (pmol) = 4 pmol/reaction × 10,000 reactions = 40,000 pmol

A minimum of 40 nmol per oligo is required to prepare a probe pool suitable for 10,000 reactions.

2. Probe Stock Concentration

Minimal stock concentration (µM) = concentration of each probe in final pool (2 µM) × number of probes in the pool
Minimal stock concentration (µM) = 2 µM × 250 probes = 500 µM

Each of the 250 probes must be ordered (if pre-resuspended) or resuspended (if lyophilized) to a concentration of at least 500 µM to allow for equimolar pooling at 2 µM per probe.

3. Resuspension Volume

Resuspension volume (µl) = amount of probe (pmol)/probe stock concentration (µM = pmol/µl)
Resuspension volume (µl) = 40,000 pmol/500 µM = 80 µl

Resuspending 40,000 pmol of probes in 80 µl of will result in a stock probe concentration of 500 µM.

4. Probe Pooling

Pipette equal volumes of each of the 250 probes (with the stock concentration at 500 µM) to create a probe pool in which each probe is present at a final concentration of 2 µM.

Concentration of each probe in pool (µM) = stock concentration (µM)/number of probes in the pool
Concentration of each probe in pool (µM) = 500 µM/250 probes = 2 µM

For example, pooling 80 µl per probe will generate 20,000 µl of a 2 µM each probe pool. This is sufficient to process 10,000 reactions.

Probe pool volume (µl) = number of probes × volume for each probe
Probe pool volume (µl) = 250 probes × 80 µl/probe = 20,000 µl

Number of reactions processed with probe pool volume = probe pool volume/volume of probe pool per reaction
Number of reactions processed with probe pool volume = 20,000 µl/2 µl per reaction

Combining a Custom RNA Depletion Probe Pool with Other NEBNext Depletion Solutions

It's possible to combine a Custom RNA Depletion Probe Pool with depletion solutions from the following NEBNext RNA depletion kits:

1. NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat), (NEB #E7400, #E7405)
2. NEBNext rRNA Depletion Kit (Bacteria), (NEB #E7850, #E7860)
3. NEBNext Globin and rRNA Depletion Kit (Human/Mouse/Rat), (NEB #E7750, #E7755)

To do so, set up the probe hybridization reaction (Steps 1.1.2, 2.1.2, 3.1.2, 4.1.2 or 5.1.2) as suggested in Table 1, and continue to follow the protocol (in Steps 1.1.3, 2.1.3, 3.1.3, 4.1.3 or 5.1.3) in the corresponding section of this manual, using the reagents in the NEBNext RNA Depletion Core Reagent Set, or the depletion reagents included in the kits.

Table 1. Probe hybridization reaction setup when combining a custom RNA depletion probe pool with existing NEBNext depletion solutions.

	Core Reagent Set (NEB #E7865) **Custom Probe Pool Only**	**rRNA HMR v2** (NEB #E7400) **+ Custom Probe Pool**	**Bacteria** (NEB #E7860) **+ Custom Probe Pool**	**Globin & rRNA HMR** (NEB #E7550) **+ Custom Probe Pool**
Total RNA in Nuclease-free Water	11	9	9	8
Hybridization Buffer	2	2	2	2
Kit's Depletion Solution	–	2	2	3
User Supplied Custom RNA Depletion Probe Pool	2	2	2	2
Total Volume	**15**	**15**	**15**	**15**

Note: The above recommendations are based on equal abundance of target sequences to be depleted (e.g., 1:1, rRNA: target RNA). If the expected target sequence ratio is variable, the ratio of probes to be combined can be optimized. The ideal probe ratio will vary by sample, and is best determined experimentally.

The recommended volume of user supplied custom RNA depletion probe pool is 2 µl. If a higher or lower volume is desired, adjust the volume of water in the reaction. The total volume of the RNA/Probe Hybridization Reaction must remain at 15 µl.

The adaptor dilution and number of PCR cycles recommended in the library preparation sections of this manual were calculated based on depletion of rRNA, which comprises ~80-90% of total RNA in most samples. Consider the abundance of the depleted RNA in the sample when selecting the adaptor dilution and PCR cycles to use. Additional PCR cycles may be necessary if you deplete other highly abundant RNAs in addition to rRNA.

Section 1
Protocol for RNA depletion using the NEBNext RNA Depletion Core Reagent Set (NEB #E7865, NEB #E7870)

Symbols

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

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

●

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

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

Mast Mixes can be prepared for steps 1.1.2, 1.2.1, 1.3.1. immediately before use.

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

Input Amount:

10 ng–1 µg total RNA (DNA free) in a maximum of 11 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (e.g., Qubit®, RiboGreen®), and quality checked by Bioanalyzer.

1.1. Probe Hybridization to RNA

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

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

1.1.3. Mix thoroughly by gently 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 program will take approximately 15–20 minutes to complete.

TEMPERATURE	TIME
95°C	2 minutes
Ramp down to 22°C	0.1°C/second
22°C	5 minutes
Hold at 4°C	

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

1.2. RNase H Digestion

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

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 1.1.6.)	15 µl
∘ (white) RNase H Reaction Buffer	2 µl
∘ (white) NEBNext Thermostable RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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 the tube in a pre-heated thermal cycler for **30 minutes at 50°C** with the lid set to 55°C, and hold at 4°C.

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

1.3. DNase I Digestion

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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), and hold at 4°C.

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

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

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

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

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

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

1. 4.5. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

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

1.4.7. Repeat Step 1.4.6. once for a total of 2 washes.

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

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

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

1.4.10. Incubate for 2 minutes at room temperature.

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

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

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

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

Section 2
Protocol for Library Preparation of Intact RNA using the NEBNext RNA Depletion Core Reagent Set (NEB #E7865, NEB #E7870) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765)

Symbols

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

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

●

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

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

Master Mixes can be prepared for steps 2.1.2, 2.2.1, 2.3.1, 2.5.1, 2.6.1, 2.7.1, 2.9.1, immediately before use.

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 Purity:

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). 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. Contaminating DNA can cause inaccurate RNA quantification and impede proper rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the depletion.

Input Amount:

10 ng–1 µg intact or partially degraded total RNA (DNA free) in a maximum of 11 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (e.g., Qubit, RiboGreen), and quality checked by Bioanalyzer.

2.1. Probe Hybridization to RNA

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

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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
22°C	5 minutes
Hold at 4°C	

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

2.2. RNase H Digestion

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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 50°C** with the lid set to 55°C, hold at 4°C.

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

2.3. DNase I Digestion

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

2.3.3. Briefly spin down the tube in a microcentrifuge.

2.3.4. Incubate in a pre-heated thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C (or off) and hold at 4°C.

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

2.4. RNA Purification using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

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

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

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

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

2.4.5. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

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

2.4.7. Repeat Step 2.4.6. once for a total of 2 washes.

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

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

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

2.4.10. Incubate for 2 minutes at room temperature.

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

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

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

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

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 2.5.3A. and 2.5.3B.

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

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

FRAGMENTATION AND PRIMING REACTION	VOLUME
RNA Depleted Sample (Step 2.4.13.)	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	**10 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

2.5.3. Place the tube on a pre-heated thermal cycler and incubate as follows:

Incubate at 94°C, with the heated lid set at 105°C

Hold at 4°C

Follow the recommendations in Tables 2.5.3A. and 2.5.3B. for libraries with inserts ~200 nt. If ribosomal RNA peaks are present, suggesting higher quality, follow recommendations in Table 2.5.3A. If ribosomal peaks are not present, then follow the DV200 recommendations in Table 2.5.3B.

Table 2.5.3A. Suggested fragmentation times (at 94°C) based on RIN value of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	≥ 7	8–15 minutes
Partially Degraded RNA	2–6	8 minutes

Table 2.5.3B. Suggested fragmentation times (at 94°C) based on DV200 of RNA input.

RNA TYPE	RIN	FRAG. TIME
Partially Degraded RNA	DV200 between 30 and 70	5 minutes
Highly Degraded RNA	DV200 ≤ 30	5 minutes*

* Results may vary due to the high degradation level of these samples.

Note: Refer to Appendix (Section 6) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix (6.1) 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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

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

Note: If you are following recommendations in Appendix (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

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 2.6.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 2.6.3.)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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 to ≤ 40°C (or off). Proceed to Purification of Double-stranded cDNA.

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

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

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

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

2.8.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnet to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. **(Caution: do not discard beads).**

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

2.8.6. Repeat Step 2.8.5. once for a total of 2 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 cDNA from the beads by adding **53 µl 0.1X TE Buffer** (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear (about 2 minutes).

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample. If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

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

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

2.9.3. Incubate in a thermal cycler with the heated lid set to ≥ 75°C as follows:
30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C

Proceed immediately to adaptor ligation.

2.10. Adaptor Ligation

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

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

* The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which 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 2.9.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 2.9.3.)	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 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 by pipetting up and down at least 10 times and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C, hold at 4°C. 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 the Appendix, Section 6.

2.11.1. 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.

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

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

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

2.11.5. Repeat Step 2.11.4. once for a total of 2 washes.

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

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

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

2.11.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding **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 magnet until the solution is clear (about 2 minutes).

2.11.9. 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 oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (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 2.11.9.)	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.

2.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.11.9.)	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. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

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

Table 2.12.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	8–16*, **
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 8.2 in Section 8).

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

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
1,000 ng	8–9
100 ng	12–13
10 ng	15–16

Note: PCR cycles are recommended based on high quality Total RNA with about 80-90% of RNA depleted. It may require optimization based on the sample quality to prevent PCR over-amplification.

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

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

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

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

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

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

2.13.6. Repeat Step 2.13.5. once for a total of 2 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 10 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 (about 2 minutes).

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

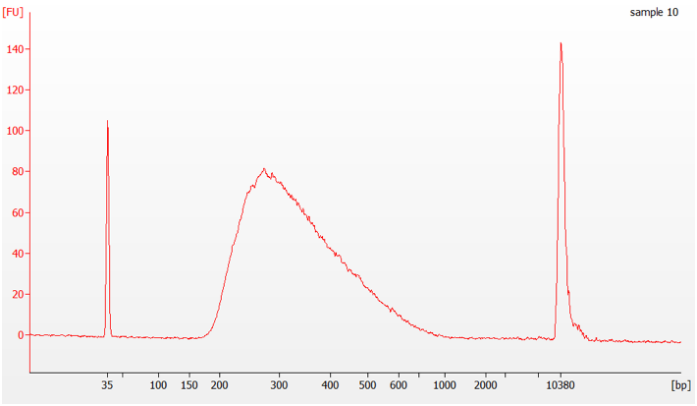
2.14. Library Quantification

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

2.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp (Figure 2.14).

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 2.13.9.) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 2.13.).

Figure 2.14 Example of library size distribution on a Bioanalyzer.



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

Section 3
Protocol for Library Preparation of Degraded RNA (e.g., FFPE) using the NEBNext RNA Depletion Core Reagent Set (NEB #E7865, NEB #E7870) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765)

Symbols

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

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

●

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

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

Master Mixes can be prepared for steps 3.1.2, 3.2.1, 3.3.1, 3.6.1, 3.7.1, 3.9.1, immediately before use.

RNA Sample Requirements

RNA Integrity:

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

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 or 4. For highly degraded samples (RIN = 1 or 2) (e.g., FFPE) which do not require fragmentation, follow the library preparation protocol in Section 3 (current section) or 5.

RNA Purity:

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). 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. Contaminating DNA can cause inaccurate RNA quantification and impede proper rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the depletion.

Input Amount:

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

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 11 µl in a PCR tube. Keep the RNA on ice.

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

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (10–100 ng)	11 µl
User Supplied Custom RNA Depletion Probe Pool	2 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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/second
22°C	5 minutes
Hold at 4°C	

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

3.2. RNase H Digestion

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

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 3.1.6.)	15 µl
∘ (white) RNase H Reaction Buffer	2 µl
∘ (white) NEBNext Thermostable RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

3.2.3. Briefly spin down the tube in a microcentrifuge.

3.2.4. Incubate in a pre-heated thermal cycler for **30 minutes at 50°C** with the lid set to 55°C, hold at 4°C.

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

3.3. DNase I Digestion

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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), and hold at 4°C.

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

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

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

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

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

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

3.4.5. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

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

3.4.7. Repeat Step 3.4.6. once for a total of 2 washes.

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

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

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

3.4.10. Incubate for 2 minutes at room temperature.

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

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

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

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

3.5. Priming of Highly Degraded RNA 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
Depleted Sample (Step 3.4.13.)	5 µl
• (lilac) Random Primers	1 µl
Total Volume	**6 µl**

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

3.5.3. Briefly spin down the tube in a microcentrifuge.

3.5.4. Incubate in a pre-heated thermal cycler as follows:
5 minutes at 65°C, with heated lid set to 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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

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

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

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 3.6.3.

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

3.7.3 Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set to ≤ 40°C (or off). Proceed to Purification of Double-stranded cDNA.

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

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

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

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

3.8.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnet to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. **(Caution: do not discard beads).**

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

3.8.6. Repeat Step 3.8.5. once for a total of 2 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 cDNA from the beads by adding **53 µl 0.1X TE Buffer** (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down 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 (about 2 minutes).

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample. 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 in a thermal cycler with the heated lid set to ≥ 75°C as follows:

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C

Proceed immediately to Adaptor Ligation.

3.10. Adaptor Ligation

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

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

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

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 Adaptor prior to use in the Adaptor Ligation Step.

3.10.3. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

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

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

3.10.6. Mix well by pipetting up and down at least 10 times and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C, hold at 4°C. Proceed immediately to Purification of the Ligation Reaction.

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

3.11.1. 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.

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

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

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

3.11.5. Repeat Step 3.11.4. once for a total of 2 washes.

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

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

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

3.11.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding **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 magnet until the solution is clear (about 2 minutes).

3.11.9. 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 oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (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 3.11.9.)	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.

3.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 3.11.9.)	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. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

3.12.3. Place the tube on a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 3.12.3A and Table 3.12.3B):

Table 3.12.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	13–17*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR should be adjusted based on RNA input, RNA quality and based on what percentage of RNA is depleted. The recommendation of PCR cycles are based on internal tests using degraded RNA with 80-90% depletion.

** 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 8.2 in Section 8).

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

DEGRADED RNA INPUT	RECOMMENDED PCR CYCLES
100 ng	13–14
10 ng	16–17

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

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

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

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

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

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

3.13.6. Repeat Step 3.13.5. once for a total of 2 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 10 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 (about 2 minutes).

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

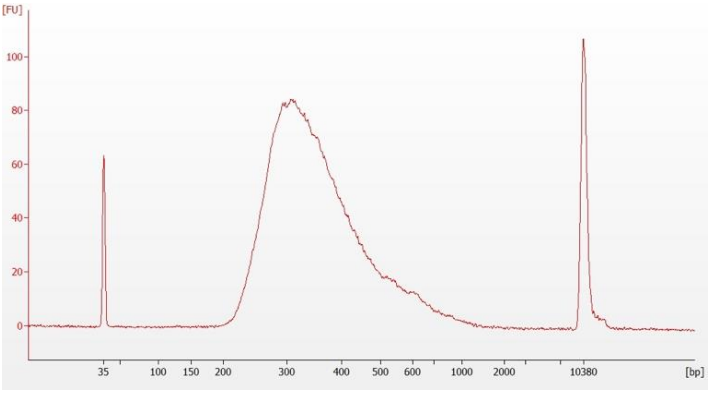
3.14. Library Quantification

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

3.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp (Figure 3.14).

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 3.13.9.) to 50 µl with 1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 3.13.).

Figure 3.14 Example of library size distribution on a Bioanalyzer.



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

Section 4
Protocol for Library Preparation of Intact or Partially Degraded RNA using the NEBNext RNA Depletion Core Reagent Set (NEB #E7865, NEB #E7870) and the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

Symbols

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

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

●

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

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

Master Mixes can be prepared for steps 4.1.2, 4.2.1, 4.3.1, 4.5.1, 4.6.1, 4.7.1, 4.9.1, immediately before use.

RNA Sample Requirements

RNA Integrity:

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all. For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 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 Purity:

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). 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. Contaminating DNA can cause inaccurate RNA quantification and impede proper rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the depletion.

Input Amount:

10 ng–1 µg intact or partially degraded total RNA (DNA free) in a maximum of 11 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (e.g., Qubit, RiboGreen), and quality checked by Bioanalyzer.

4.1. Probe Hybridization to RNA

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

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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
22°C	5 minutes
Hold at 4°C	

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

4.2. RNase H Digestion

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

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 4.1.6.)	15 µl
∘ (white) RNase H Reaction Buffer	2 µl
∘ (white) NEBNext Thermostable RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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 50°C** with the lid set to 55°C, hold at 4°C.

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

4.3. DNase I Digestion

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

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

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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) and hold at 4°C.

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

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

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

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

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

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

4.4.5. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

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

4.4.7. Repeat Step 4.4.6. once for a total of 2 washes.

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

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

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

4.4.10. Incubate for 2 minutes at room temperature.

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

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

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

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

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 4.5.3A. and Table 4.5.3B.

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

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

FRAGMENTATION AND PRIMING REACTION	VOLUME
RNA Depleted Sample (Step 4.4.13.)	5 µl
• (lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
• (lilac) Random Primers	1 µl
Total Volume	**10 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

4.5.3. Place the tube on a pre-heated thermal cycler and incubate as follows:
Incubate at 94°C with the heated lid set to 105°C
Hold at 4°C

Follow the recommendations in Tables 4.5.3A. and 4.5.3B. for libraries with inserts ~200 nt. If ribosomal RNA peaks are present, suggesting higher quality, follow recommendations in Table 4.5.3A. If ribosomal peaks are not present, then follow the DV200 recommendations in Table 4.5.3B.

Table 4.5.3A. Suggested fragmentation times (at 94°C) based on RIN value of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	≥ 7	8–15 minutes
Partially Degraded RNA	2–6	8 minutes

Table 4.5.3B. Suggested fragmentation times (at 94°C) based on DV200 of RNA input.

RNA TYPE	DV200	FRAG. TIME
Partially Degraded RNA	DV200 between 30 and 70	5 minutes
Highly Degraded RNA	DV200 ≤ 30	5 minutes*

* Results may vary due to the high degradation level of these sample.

Note: Refer to Appendix (Section 6) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix (Section 6.1) 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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

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

Note: If you are following recommendations in Appendix (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

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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 to ≤ 40°C (or off). Proceed to Purification of Double-stranded cDNA.

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

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

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

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

4.8.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnet to separate beads from the supernatant. After the solution is clear (about 5 minutes), 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 2 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 cDNA from the beads by adding **53 µl 0.1X TE Buffer** (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear (about 2 minutes).

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample. 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 to ≥ 75°C as follows:
30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C.

Proceed immediately to Adaptor Ligation.

4.10. Adaptor Ligation

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

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

* The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which 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 Adaptor prior to use in the Adaptor Ligation Step.

4.10.3. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

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

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

4.10.6. Mix well by pipetting up and down at least 10 times and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C, hold at 4°C. 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, Section 6.

4.11.1. 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.

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

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

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

4.11.5. Repeat Step 4.11.4. once for a total of 2 washes.

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

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

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

4.11.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding **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 magnet until the solution is clear (about 2 minutes).

4.11.9. 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 oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (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 4.11.9.)	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.

4.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.11.9.)	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. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

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

Table 4.12.3A:

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

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

** 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 8.2 in Section 8).

Table 4.12.3B: 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

Note: PCR cycles are recommended based on high quality Total RNA with about 80-90% of RNA depleted. 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 to resuspend.

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

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

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

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

4.13.6. Repeat Step 4.13.5. once for a total of 2 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 10 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 (about 2 minutes).

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

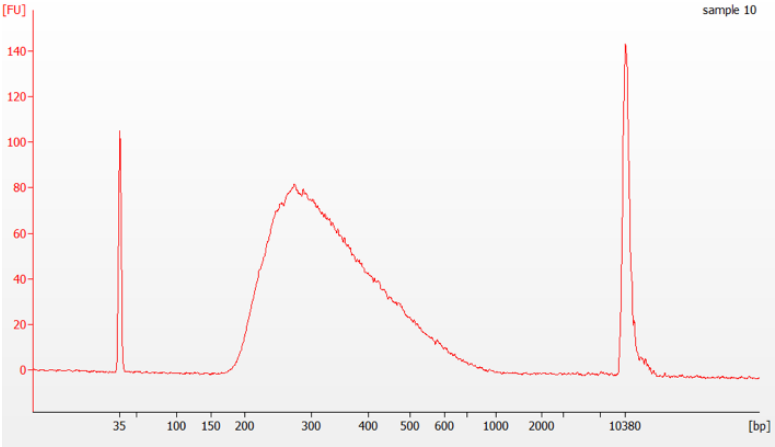
4.14. Library Quantification

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

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

Note: If a peak at ~ 80 bp (primers) or 128 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.).

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



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

Section 5
Protocol for Library Preparation of Degraded RNA (e.g., FFPE) using the NEBNext RNA Depletion Core Reagent Set (NEB #E7865, NEB #E7870) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

Symbols

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

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

●

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

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

Master Mixes can be prepared for steps 5.1.2, 5.2.1, 5.3.1, 5.6.1, 5.7.1, 5.9.1, immediately before use.

RNA Sample Requirements

RNA Integrity:

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

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 or 4. For highly degraded samples (RIN= 1 or 2) (e.g., FFPE) which do not require fragmentation, follow the library preparation protocol in Section 3 or 5 (current section).

RNA Purity:

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/ chloroform extraction and ethanol precipitation or silica column methods such as the Monarch RNA Cleanup Kit (NEB #T2030). 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. Contaminating DNA can cause inaccurate RNA quantification and impede proper rRNA removal. Any residual activity of DNase I will degrade the single stranded DNA probes necessary for the depletion.

Input Amount:

10–100 ng degraded (e.g., FFPE) total RNA (DNA free) in a maximum of 11 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (e.g., Qubit, RiboGreen), and quality checked by Bioanalyzer.

5.1. Probe Hybridization to RNA

5.1.1. Dilute 10–100 ng total RNA with Nuclease-free Water to a final volume of 11 µl in a PCR tube. Keep the RNA on ice.

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

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in Nuclease-free Water (10–100 ng)	11 µl
User Supplied Custom RNA Depletion Probe Pool	2 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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
22°C	5 minutes
Hold at 4°C	

5.1.6. Briefly spin down the tube in a 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 Thermostable RNase H	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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 50°C** with the lid set to 55°C and hold at 4°C.

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**:

DNASE I DIGESTION REACTION	VOLUME
RNase H treated RNA (Step 5.2.5.)	20 µl
∘ (white) DNase I Reaction Buffer	5 µl
∘ (white) NEBNext DNase I	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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) and hold at 4°C.

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

5.4.2. Add **90 µl (1.8X)** 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 for **15 minutes on ice** to bind RNA to the beads.

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

3.4.5. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

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

5.4.7. Repeat Step 5.4.6. once for a total of 2 washes.

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

Caution: Do not over-dry the beads. This may result in lower recovery of RNA. 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.

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

5.4.10. Incubate the tube for 2 minutes at room temperature.

5.4.11. Place the tube on the magnetic rack until the solution is clear (about 2 minutes).

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

5.4.13. Place the tube on ice and proceed to Priming of Highly Degraded RNA.

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

5.5. Priming of Highly Degraded RNA 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
Depleted Sample (Step 5.4.13.)	5 µl
• (lilac) Random Primers	1 µl
Total Volume	**6 µl**

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

5.5.3. Briefly spin down the tube in a microcentrifuge.

5.5.4. Incubate in a pre-heated thermal cycler as follows:

5 minutes at 65°C, with heated lid set to 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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

5.6.3. Incubate in a pre-heated thermal cycler with the heated lid set to ≥ 80°C as follows:
Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample.

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

5.7.3 Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set to ≤ 40°C (or off). Proceed to Purification of Double-stranded cDNA.

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 to resuspend.

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 **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 (about 5 minutes), 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 2 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 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 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 (about 2 minutes).

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**

For multiple reactions, a master mix of the reaction components can be prepared on ice before addition to the sample. 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 in a thermal cycler with the heated lid set to ≥ 75°C as follows:

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C

Proceed immediately to Adaptor Ligation.

5.10. Adaptor Ligation

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

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

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

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 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** (heated lid off) in a thermal cycler.

5.10.5. Add 3 µl ● (blue or red) NEBNext 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, hold at 4°C. Proceed immediately to Purification of the Ligation Reaction.

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

5.11.1. 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.

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

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

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

5.11.5. Repeat Step 5.11.4. once for a total of 2 washes.

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

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

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

5.11.8. 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 magnet until the solution is clear (about 2 minutes).

5.11.9. 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 oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (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 5.11.9.)	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.

5.12.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 5.11.9.)	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. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

5.12.3. Place the tube on a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 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 using degraded RNA.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 8.2 in Section 8).

Table 5.12.3B: Recommended PCR cycles based on input amount:

DEGRADED 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 **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 washes.

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 10 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 (about 2 minutes).

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

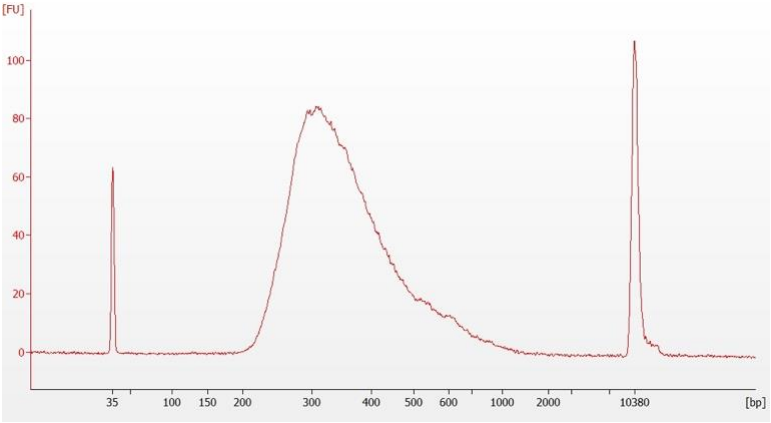
5.14. Library Quantification

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

5.14.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp (Figure 5.14).

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 5.13.9.) to 50 µl with 1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 5.13.).

Figure 5.14 Example of library size distribution on a Bioanalyzer.



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

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)

Symbols

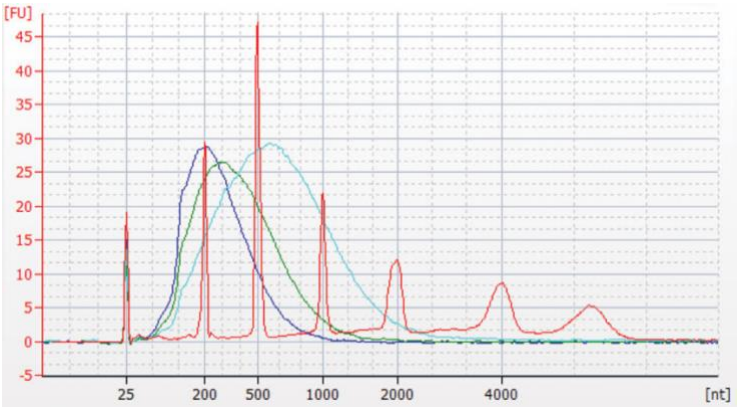
This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

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

6.1. Fragmentation

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

Figure 6.1. Modified fragmentation times for longer RNA inserts.



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

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

6.2. Size Selection of Adaptor Ligated DNA

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

The size selection protocol is based on a starting volume of 96.5 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.

	APPROXIMATE INSERT SIZE	300 bp	400 bp	450 bp
LIBRARY PARAMETER	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 to resuspend.

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 2 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 10 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 2 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 oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

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

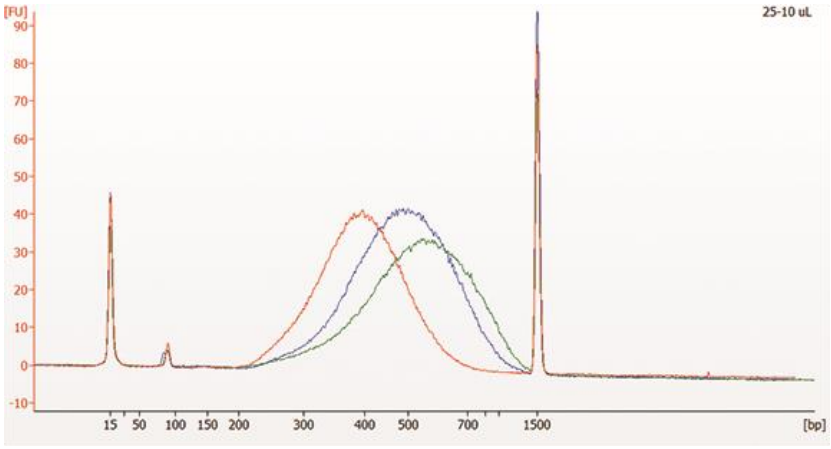
CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	variable*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR should be adjusted based on RNA input, RNA quality and based on what percentage of RNA is depleted. 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 8.2 in Section 8).

Perform the step "Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads" from your respective chapter.

Figure 6.3: Bioanalyzer traces of size selected DNA libraries.



50 ng mRNA was fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix at 94°C for 10 or 5 minutes. Libraries were size-selected as described in Table 4.2, then amplified by PCR, and run on Agilent Bioanalyzer DNA 1000 chip. Fragmentation times and corresponding size selection conditions are shown in the table below.

Table 6.3:

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

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

Section 7
Troubleshooting Guide for use with the NEBNext RNA Depletion Core Reagent Set

OBSERVATION	POSSIBLE CAUSES	SOLUTIONS
% of reads mapping to the targeted sequence did not decrease after depletion.	• Target sequence used as input in the NEBNext Custom RNA Depletion Design tool is not RNA, but cDNA.	• Verify your sequence source.
	• Probes are not covering the area being evaluated for depletion.	• Align the probes against the target sequence using your preferred aligner (e.g., Bowtie or bwa) and visualize the probe alignments along with your aligned reads (e.g., IGV or IGB). Probes should be reverse complement to the target sequences. • Look for gaps in the area covered by the probes. Do these gaps correspond to the areas with high read coverage? • If so, design probes against the gap region using the Custom RNA Depletion Tool. • The additional probes can be spiked into the original pool for testing. • We recommend running a no-treatment control for comparison. • Note that small gaps (~15<nt) between probes and overlap are expected in the design when using the tool. Such short fragments will be eliminated during library cleanup.
	• Probe integrity is compromised.	• Order the probes from a trusted oligo synthesis provider and store appropriately. • Evaluate your pool using a single-stranded DNA size estimation method to ensure that the length distribution is between 40 and 60 nt.
	• DNA Contamination of input RNA.	• Contaminating DNA can cause inaccurate RNA quantification and impede proper RNA removal. • If the total RNA sample may contain DNA contamination, treat the sample with DNase I (not provided in this kit) to remove all traces of DNA. 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 Contamination of input RNA.	• Purify the RNA from DNase I enzyme after treatment. Residual activity of DNase I will degrade the single stranded DNA probes necessary for the ribosomal depletion.
	• Other Contaminants of input RNA.	• Ensure the RNA samples are free of salts (e.g., Mg2+, or guanidinium salts) or organics (e.g., phenol and ethanol), and in nuclease-free water.
	• Probe hybridization step was not successful.	• Ensure that the temperature ramp down during the probe hybridization step occurs at 0.1°C/s. This step should take approximately 20 min.
Depletion is not uniform across targeted sequences.	• Probe amount needs to be optimized for your sample.	• Titrate the amount of probe pool used. If specific targeted regions are not being depleted, increase the relative probe amount of that region in the pool.
	• Target sequence used to design the probes is different from that used to evaluate depletion.	• Genome annotations are constantly evolving. Check for differences between the transcriptome/genome version used to extract the target RNA sequence and that used to evaluate depletion.

Section 8
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 8.1)	• 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 8.1)	• Addition of non-diluted adaptor • RNA input was too low • RNA was over fragmented or lost during fragmentation • Inefficient Ligation	Adaptor-dimer will cluster and be sequenced. If ratio is low compared to library, may not be a problem but some reads will be dimers.	• Dilute adaptor before setting up ligation reaction • Clean up PCR reaction again with 0.9X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield)
Presence of additional Bioanalyzer peak at higher molecular weight than the expected library size (~ 1,000 bp) (Figure 8.2)	• 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 8.1:

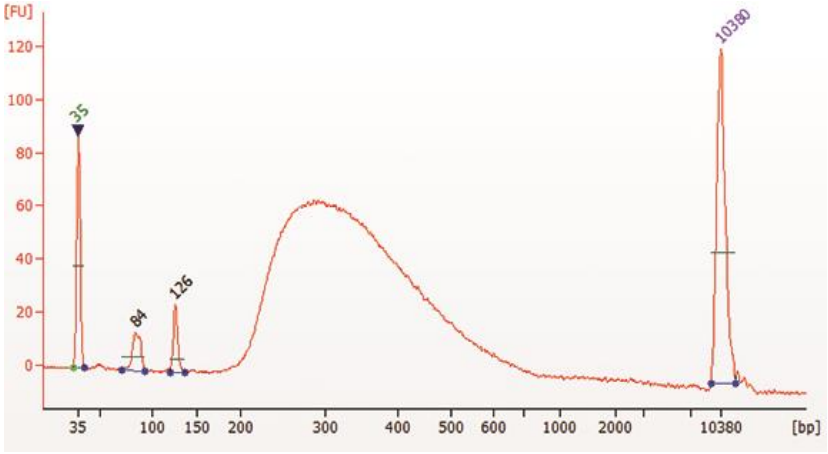
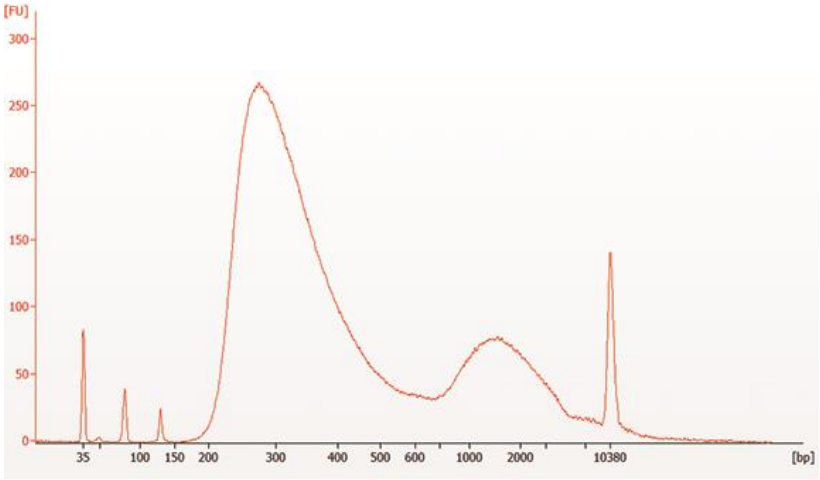


Figure 8.2:



Checklist for NEBNext RNA Depletion Core Reagent Set (NEB #E7865, NEB #E7870)

1. Hybridize the Probes to the RNA

1.1. Dilute total RNA to a volume of 11 µl

1.2. Assemble Probe/RNA Hybridization Reaction

[_] 1.2.1. Total RNA in nuclease-free water 11 µl

[_] 1.2.2. User Supplied Custom RNA Depletion Probe Pool 2 µl

[_] 1.2.3. NEBNext Probe Hybridization Buffer 2 µl

[_] 1.3. Mix 10 times

[_] 1.4. Quick spin

[_] 1.5. Run in pre-heated thermal cycler (95°C for 2 min, 95-22°C 0.1°C/sec, 22°C 5 min; heated lid 105°C)

[_] 1.6. Quick spin, place on ice

2. RNase H Digestion

2.1. Assemble RNaseH digestion reaction

[_] 2.1.1. Hybridized RNA 15 µl

[_] 2.1.2. NEBNext Thermostable RNase H 2 µl

[_] 2.1.3. RNase H Reaction Buffer 2 µl

[_] 2.1.4. Nuclease-free water 1 µl

[_] 2.2. Mix 10 times

[_] 2.3. Quick spin

[_] 2.4. Incubate in pre-heated thermal cycler (50°C for 30 min)

[_] 2.5. Quick spin, place on ice

3. DNase I Digestion

3.1. Assemble DNase I digestion reaction

[_] 3.1.1. RNase H treated RNA 20 µl

[_] 3.1.2. DNase I Reaction Buffer 5 µl

[_] 3.1.3. NEBNext DNase I 2.5 µl

[_] 3.1.4. Nuclease-free water 22.5 µl

[_] 3.2. Mix 10 times

[_] 3.3. Quick spin

[_] 3.4. Incubate in pre-heated thermal cycler (37°C for 30 min)

[_] 3.5. Quick spin, place on ice

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

[_] 4.1. Add 90 µl of beads and mix 10 times

[_] 4.2. Incubate on ice 15 min

[_] 4.3. Place on magnetic rack until solution is clear

[_] 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 7 µl of Nuclease-free water and mix 10 times; wait 2 min

[_] 4.9. Place on magnet 5 min

[_] 4.10. Transfer 5 µl to new tube

[_] 4.11. Place on ice or store

Kit Components

NEB #E7865S Table of Components

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

NEB #E7865L Table of Components

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

NEB #E7865X Table of Components

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

NEB #E7870S Table of Components

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

NEB #E7870L Table of Components

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

NEB #E7870X Table of Components

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

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	7/20
2.0	Update protocols	11/20
3.0	Update protocols	8/22
4.0	Added updated DV200 recommendations. Updated protocols.	8/25
5.0	A mistake in the probe ordering instructions was corrected	10/25

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