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

NEB #E7750S/L/X, #E7755S/L/X 6/24/96 reactions

Version 4.0_6/24

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The Kit Includes

The volumes provided are sufficient for preparation of up to 6 reactions (NEB #E7750S/#E7755S) 24 reactions (NEB #E7750L/#E7755L) and 96 reactions (NEB #E7750X/#E7755X).

Package 1: Store at –20°C.

- (white) NEBNext Globin and rRNA Depletion Solution
- (white) 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 Globin and rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads, NEB #E7755.

NEBNext RNA Sample Purification Beads

Required Materials Not Included

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

For NEB #E7750 only:

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

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

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

For NEB #E7760 & NEB #E7770:

- SPRISelect Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- In NEB #E7765 & NEB #E7775, beads are included [Sample Purification Beads (NEB #E7767)]

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

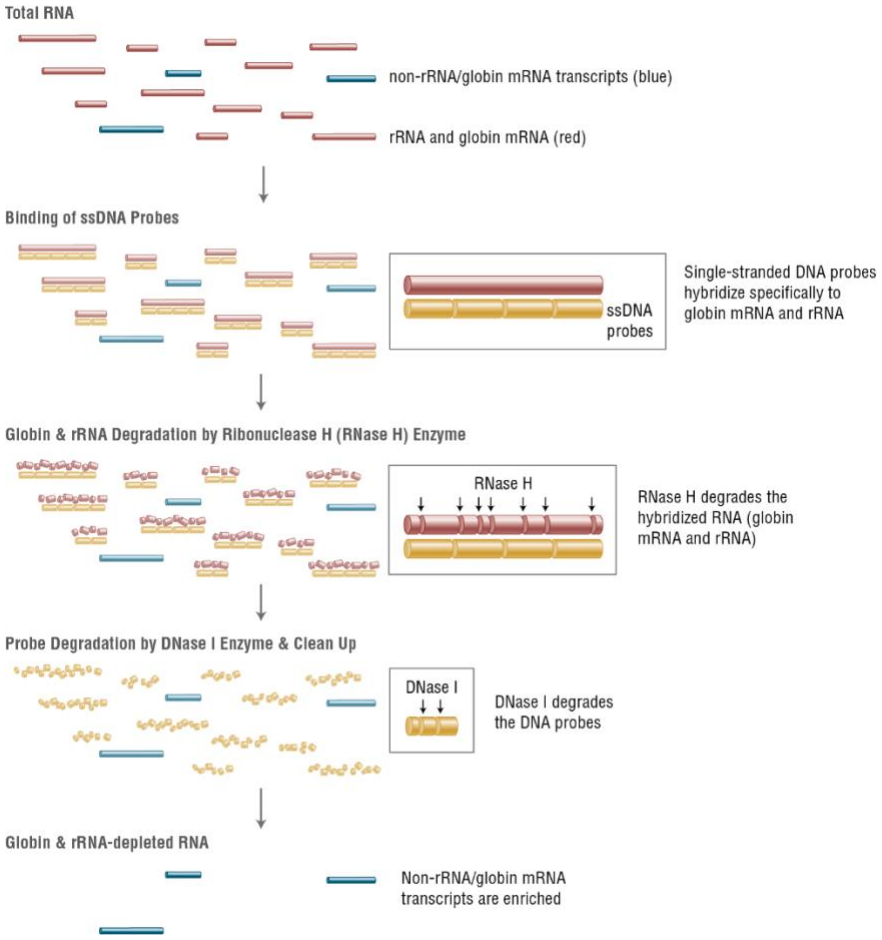
The NEBNext Globin and rRNA Depletion Kit (Human/Mouse/Rat) employs the NEBNext RNase H-based RNA Depletion Workflow to deplete the following:

- Globin mRNA (HBA 1/2, HBB, HBD, HBM, HBG1/2, HBE, HBQ1 and HBZ)
- Cytoplasmic rRNA (5S, 5.8S, 18S, 28S, ITS and ETS)
- Mitochondrial rRNA (12S and 16S)

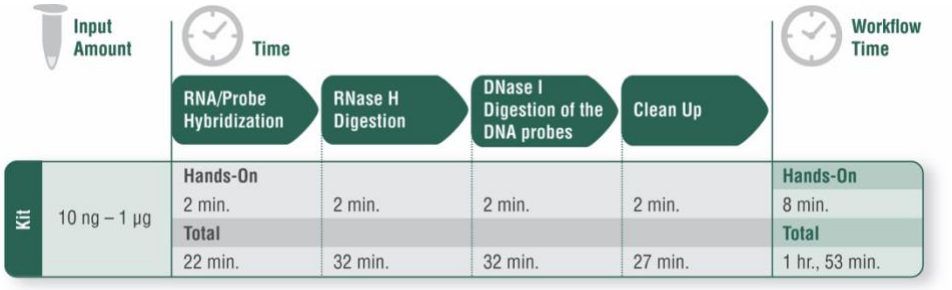
The kit is effective with human, mouse and rat total RNA preparations, both intact and degraded.

The kit can also be used following poly (A) mRNA enrichment (e.g., using the NEBNext poly(A) mRNA Magnetic Isolation Module, NEB #E7490), so that the final depleted RNA contains only mRNA of interest and not non-coding RNA.

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



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



The protocol supports globin mRNA and rRNA depletion from 10 ng–1 µg total RNA and can be completed in approximately two hours.

Applications

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

When using the NEBNext Globin and rRNA Depletion Kit (Human/Mouse/Rat; NEB #E7750 or #E7755) 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), Section 2
- NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770), Section 3

Section 1
Protocol for use with Non-Indexed Adaptor, NEBNext Globin & rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E7750, NEB #E7755)

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.

Master 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 size and 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). In the RNA-Seq library preparation application, RNA with different RIN values require different fragmentation times or no fragmentation at all.

RNA Sample Requirements:

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

Input Amount Requirements:

This protocol requires 10 ng–1 µg total RNA (DNA free) in a maximum of 10 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. Hybridize the Probes to the RNA

1.1.1. Dilute 10 ng-1 µg of total RNA with nuclease-free water to a final volume of 10 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)	10 µl
∘ (white) NEBNext Globin and rRNA Depletion Solution	3 µl
∘ (white) 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: Its 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 the tube in a pre-heated thermal cycler and run the following program with the heated lid set at 105°C. This program will take approximately 15–20 minutes to complete:

TEMPERATURE	TIME
95°C	2 minutes
Ramp down to 22°C	0.1°C/sec
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 the 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) NEBNext Thermostable RNase H	2 µl
∘ (white) RNase H Reaction Buffer	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 gently 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 at 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 the 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 the tube in a pre-heated thermal cycler for **30 minutes at 37°C** with the lid set at 40°C (or off).

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

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 the tube for **15 minutes on ice** to bind the 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 target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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

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

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

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

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

Section 2

Protocol for use with Non-Indexed Adaptor Library Preparation with NEBNext Globin & rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E7750, NEB #E7755) 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.

This protocol has been optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 4) for recommended fragmentation times and size selection conditions.

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 size and 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). In the RNA-Seq library preparation application, RNA with different RIN values require different fragmentation times or no fragmentation at all.

RNA Sample Requirements:

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

Input Amount Requirements:

This protocol requires 10 ng–1 µg total RNA (DNA free) in a maximum of 10 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 10 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)	10 µl
∘ (white) NEBNext Globin and rRNA Depletion Solution	3 µl
∘ (white) 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 gently pipetting up and down at least 10 times. **Note: Its 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 the tube in a pre-heated thermal cycler and run the following program with the heated lid set at 105°C. This program will take approximately 15–20 minutes to complete

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

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

2.2. RNase H Digestion

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

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 2.1.6.)	15 µl
∘ (white) NEBNext Thermostable RNase H	2 µl
∘ (white) RNase H Reaction Buffer	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 gently pipetting up and down at least 10 times.

2.2.3. Briefly spin down the tube in a microcentrifuge.

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

2.2.5. Briefly spin down the tube in a microcentrifuge, and place **on ice**. Proceed immediately to the 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 sample in a microcentrifuge.

2.3.4 Incubate the tube in a pre-heated thermal cycler for **30 minutes at 37°C** with the lid set at 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 the RNA Purification step.

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 the tube for **15 minutes on ice** to bind the 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 target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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

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

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

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

2.4.13. Place the 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.

2.5. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Tables 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 5) 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
Ribosomal 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 sample on a 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 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	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 samples.

Note: Refer to Appendix (Section 5) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix 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 sample in a pre-heated thermal cycler with the heated lid set at ≥ 80°C as follows:

Note: If you are following recommendations in Appendix (Section 5) for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

2.6.4. Proceed directly to Second Strand cDNA Synthesis.

2.7. Second Strand cDNA Synthesis

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

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 at ≤ 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 washing steps.

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

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

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

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

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

2.9. End Prep of cDNA Library

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

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

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

2.9.4. Proceed immediately to Adaptor Ligation.

2.10. Adaptor Ligation

2.10.1. 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
1,000–101 ng	5-fold dilution in Adaptor Dilution Buffer
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.

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** in a thermal cycler with the heated lid off.

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 5.

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

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

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

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

2.11.7. Completely remove the residual ethanol, and air dry beads 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 (Table 2.12.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 6.2 in Section 6).

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

***** The PCR cycles are recommended based on high quality human whole blood total RNA. To prevent over-amplification, the number of cycles may require optimization based on the sample quality and the fraction of globin mRNA. For RNA where globin mRNA is > than 50% of the transcripts (once rRNA is removed), follow the higher cycle recommendation for that input.

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

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

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

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

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

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

2.13.6. Repeat Step 2.13.5. once for a total of 2 washing steps.

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

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

2.13.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding **23 µl 0.1X TE** (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down 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.

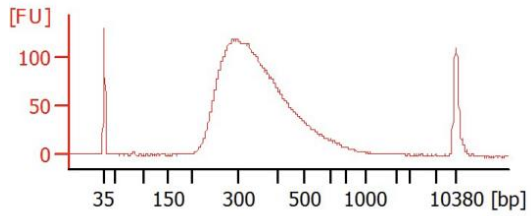
2.14. Library Quantification

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.

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 2.14.1 Example of RNA library size distribution on a Bioanalyzer.



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

Section 3

Protocol for use with Library Preparation with NEBNext Globin & rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E7750, NEB #E7755) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

Symbols

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

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

●

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

This protocol has been optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 4) for recommended fragmentation times and size selection conditions.

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, 3.5.1, 3.6.1, 3.7.1, 3.9.1. immediately before use.

RNA Sample Requirements

RNA Integrity:

Assess the size and 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). In the RNA-Seq library preparation application, RNA with different RIN values require different fragmentation times or no fragmentation at all.

RNA Sample Requirements:

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

Input Amount Requirements:

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

3.1. Probe Hybridization to RNA

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

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

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
Total RNA in nuclease-free water (10 ng–1 µg)	10 µl
∘ (white) NEBNext Globin and rRNA Depletion Solution	3 µl
∘ (white) 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 gently pipetting up and down at least 10 times. **Note: Its 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 the tube in a pre-heated thermal cycler and run the following program with the heated lid set at 105°C. This program will take approximately 15–20 minutes to complete.

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

3.1.6. Briefly spin down the sample 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) NEBNext Thermostable RNase H	2 µl
∘ (white) RNase H Reaction Buffer	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 gently pipetting up and down at least 10 times.

3.2.3. Briefly spin down the tube in a microcentrifuge.

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

3.2.5. Briefly spin down the tube in a microcentrifuge and place **on ice**. Proceed immediately to the 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 sample in a microcentrifuge.

3.3.4 Incubate the tube in a pre-heated thermal cycler for **30 minutes at 37°C** with the lid set at 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 the RNA Purification step.

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 the tube for **15 minutes on ice** to bind the 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 target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

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

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

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

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

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

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

3.5. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Tables 3.5.3A. and 3.5.3B. The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 5) for recommended fragmentation times and size selection conditions.

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

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

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

3.5.2. Mix thoroughly by pipetting up and down 10 times. Briefly spin down the tube in a microcentrifuge.

3.5.3. Place the sample on a thermal cycler and incubate as follows:

Incubate the sample at 94°C with the heated lid set to 105°C.

Hold at 4°C.

Follow the recommendations in Table 3.5.3A. and 3.5.3B. below for libraries with inserts ~200 nt. If ribosomal RNA peaks are present, suggesting higher quality, follow recommendations in Table 3.5.3A. If ribosomal peaks are not present, then follow the DV200 recommendations in Table 3.5.3B.

Table 3.5.3A. 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 3.5.3B. 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 samples.

Note: Refer to Appendix (Section 5) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix only apply for intact RNA.

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

3.6. First Strand cDNA Synthesis

3.6.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 3.5.4.:

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

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

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

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

Note: If you are following recommendations in Appendix (Section 5) for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

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

3.6.4. Proceed directly to Second Strand cDNA Synthesis.

3.7. Second Strand cDNA Synthesis

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

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 3.6.3.)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

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 at least 10 times.

3.7.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C (or off). 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 rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

3.8.6. Repeat Step 3.8.5 once for a total of **2 washes**.

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

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

3.8.8. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding **53 µl 0.1X TE Buffer** (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear (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 cDNA Synthesis Product (Step 3.8.9.)	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

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

3.9.4 Proceed immediately to Adaptor Ligation.

3.10. Adaptor Ligation

3.10.1. 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
1,000–101 ng	5-fold dilution in Adaptor Dilution Buffer
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.

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** in a thermal cycler with the heated lid off.

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

Note: USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed / UMI adaptor, USER is not needed. Please see corresponding manual for use with indexed/ UMI adaptors.

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

Note: If you are selecting for libraries with larger insert size (> 200 nt) follow the size selection recommendations in the Appendix, Section 5.

3.11.1. Add **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 (~ 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 until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

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

3.11.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding **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).

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	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 (Table 3.12.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 6.2 in Section 6).

Table 3.12.3B. Recommended PCR cycles based on total RNA input amount:

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

***** The PCR cycles are recommended based on high quality human whole blood total RNA. To prevent over-amplification, the number of cycles may require optimization based on the sample quality and the fraction of globin mRNA. For RNA where globin mRNA is > than 50% of the transcripts (once rRNA is removed), follow the higher cycle recommendation for that input.

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

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

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

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

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

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

3.13.6. Repeat Step 3.13.5. once for a total of 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.

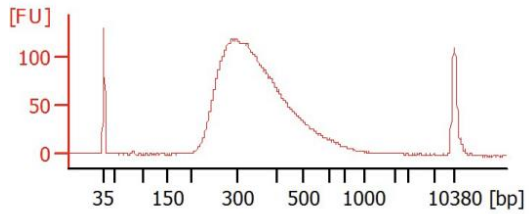
3.14. Library Quantification

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.

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 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 3.13.).

Figure 3.14.2 Example of RNA library size distribution on a Bioanalyzer.



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

Section 4

Protocol for Enrichment of mRNAs, Excluding Globin mRNA, from Whole Blood Total RNA using Non -Indexed adaptors, NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490), NEBNext Globin & rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E7750, #E7755) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, #E7765)

Symbols

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

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

●

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

This protocol has been optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 4) for recommended fragmentation times and size selection conditions.

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

Master Mixes can be prepared for steps 4.2.1, 4.3.1, 4.4.1, 4.6.1, 4.7.1, 4.8.1, 4.10.1 immediately before use.

RNA Sample Requirements

RNA Integrity:

Assess the size and 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 Poly(A) mRNA enrichment, high quality RNA with RIN Score > 7 is required.

RNA Sample Requirements:

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 (not provided in this kit) to remove all traces of DNA. After treatment, the DNase I should be removed from the sample. Any residual DNase I may degrade the oligos necessary for the enrichment.

Input Amount Requirements:

This protocol has been tested with 100 ng human whole blood total RNA (DNA-free) in a maximum of 50 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (Qubit®) and quality checked by Bioanalyzer.

Keep all buffers on ice, unless otherwise indicated.

4.1. Poly(A) mRNA Enrichment using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)

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

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

COMPONENT	VOLUME
NEBNext Oligo $d(T)_{25}$ Beads	20 µl
NEBNext RNA Binding Buffer (2X)	100 µl
Total Volume	**120 µl**

4.1.3. Wash the beads by pipetting up and down 6 times.

4.1.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

4.1.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

4.1.6. Remove the tube from the magnetic rack.

4.1.7. Add 100 µl NEBNext RNA Binding Buffer (2X) to the beads and wash by pipetting up and down 6 times. If preparing multiple libraries, add 100 µl NEBNext RNA Binding Buffer (2X) per sample. The Binding Buffer does not have to be diluted.

4.1.8. Place the tubes on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

4.1.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

4.1.10. Remove the tubes from the magnet and add 50 µl NEBNext RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl NEBNext RNA Binding Buffer (2X) per sample.

4.1.11. Add 50 µl beads to each RNA sample from Step 4.1.1. Mix thoroughly by pipetting up and down 6 times. This binding step removes most of the non-target RNA.

4.1.12. Place the tube in a thermal cycler and close the lid. Heat the sample at 65°C for 5 minutes and cool to 4°C with the heated lid set at ≥ 75°C. This step will denature the RNA and facilitate binding of the mRNA to the beads.

4.1.13. Remove the tube from the thermal cycler when the temperature reaches 4°C.

4.1.14. Mix thoroughly by pipetting up and down 6 times. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.

4.1.15. Place the tube on the magnetic rack at room temperature until the solution is clear (~ 2 minutes).

4.1.16. Remove and discard all of the supernatant. Take care not to disturb the beads.

4.1.17. Remove the tube from the magnetic rack.

4.1.18. To remove unbound RNA add 200 µl of NEBNext Wash Buffer to the tube. Gently pipette the entire volume up and down 6 times to mix thoroughly.

4.1.19. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

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

4.1.20. Place the tube on the magnetic rack at room temperature until the solution is clear (~ 2 minutes).

4.1.21. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads containing the mRNA.

4.1.22. Remove the tube from the magnetic rack.

4.1.23. Repeat steps 4.1.18.–4.1.21.

4.1.24. Add 11 µl of nuclease-free water to each tube. Gently pipette up and down 6 times to mix thoroughly.

4.1.25. Place the tube in the thermal cycler. Close the lid and heat the samples at 80°C for 2 minutes, then cool to 25°C with the heated lid set at ≥ 90°C to elute the mRNA from the beads.

4.1.26. Remove the tube from the thermal cycler when the temperature reaches 25°C.

4.1.27. Immediately place the tube on the magnet at room temperature until the solution is clear (~ 2 minutes).

4.1.28. Collect the purified mRNA by transferring 10 µl of the supernatant to a clean nuclease-free PCR tube.

4.1.29. Place the RNA on ice and proceed to the Globin and rRNA Depletion in Step 4.2.

4.2. Probe Hybridization to RNA

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

COMPONENT	VOLUME PER ONE LIBRARY
∘ (white) mRNA in Nuclease-free Water (Step 4.1.29.)	10 µl
∘ (white) NEBNext Globin and rRNA Depletion Solution	3 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.

4.2.2. Mix thoroughly by gently pipetting up and down at least 10 times. **Note: It's crucial to mix well at this step.**

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

4.2.4. Place the tube in a pre-heated thermal cycler and run the following program with the heated lid set at 105°C. This program will take approximately 15–20 minutes to complete.

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

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

4.3. RNase H Digestion

4.3.1. Assemble the following RNase H Digestion reaction on ice:

RNASE H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 4.2.5.)	15 µl
∘ (white) NEBNext Thermostable RNase H	2 µl
∘ (white) RNase H Reaction Buffer	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.

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

4.3.3. Briefly spin down the tube in a microcentrifuge.

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

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

4.4. DNase I Digestion

4.4.1. Assemble the following DNase I Digestion reaction **on ice**:

DNASE I DIGESTION REACTION	VOLUME
RNase H treated RNA (Step 4.3.5.)	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.

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

4.4.3. Briefly spin down the sample in a microcentrifuge.

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

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

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

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

4.5.2. Add **90 µl (1.8X)** beads to the RNA Sample from Step 4.4.5. and mix thoroughly by pipetting up and down at least 10 times.

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

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

4.5.5. 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.

4.5.6. 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.

4.5.7. Repeat Step 4.5.6. once for a total of 2 washes.

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

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

4.5.9. 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.

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

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

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

4.5.13. 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.

4.6. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 3.5.3. The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to the Appendix (Section 5) for recommended fragmentation times and size selection conditions.

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

FRAGMENTATION AND PRIMING REACTION	VOLUME
Enriched mRNA Sample (Step 4.5.13.)	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.

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

Note: The next step provides a fragmentation incubation time resulting in an RNA insert of ~200nt. Refer to Appendix A (Section 6 of the NEBNext Ultra II Directional RNA Library Prep for Illumina Manual) for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp).

4.6.3. Incubate the sample for 15 minutes at 94°C in a thermal cycler with the heated lid set at 105°C.

4.6.4. Immediately transfer the tube to ice for 1 minute.

4.6.5 Perform a quick spin to collect all liquid from the sides of the tube and proceed to First Strand cDNA Synthesis.

4.7. First Strand cDNA Synthesis

4.7.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 4.6.5.:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 4.6.5.)	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.

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

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

Note: If you are following recommendations in Appendix (Section 5) for libraries with longer inserts (> 200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

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

4.7.4. Proceed directly to Second Strand cDNA Synthesis.

4.8. Second Strand cDNA Synthesis

4.8.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis product from Step 4.7.3.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 4.7.3.)	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.

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

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

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

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

4.9.2. Add **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.

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

4.9.4. 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).**

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

4.9.6. Repeat Step 4.9.5. once for a total of **2 washes**.

4.9.7. 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.

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

4.9.9. 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.

4.10. End Prep of cDNA Library

4.10.1. Assemble the end prep reaction **on ice** by adding the following components to the second strand synthesis product from Step 4.9.9.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 4.9.9.)	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.

4.10.2. 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.

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

4.10.4. Proceed immediately to Adaptor Ligation.

4.11. Adaptor Ligation

4.11.1. 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
100 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.

4.11.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 4.10.3.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 4.10.3.)	60 µl
Diluted Adaptor (Step 4.11.1.)	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.

4.11.3. 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.

4.11.4. Incubate **15 minutes at 20°C** in a thermal cycler with the heated lid off.

4.11.5. Add 3 µl • (blue or red) USER Enzyme to the ligation mixture from Step 4.11.4, resulting in total volume of 96.5 µl.

Note: USER enzyme can be found in most NEBNext oligo kits. If you are using the indexed / UMI adaptor, USER is not needed. Please see corresponding manual for use with indexed/ UMI adaptors.

4.11.6. 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.

4.12. 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, Appendix A of the NEBNext Ultra II Directional RNA Library Prep for Illumina Manual.

4.12.1. 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.

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

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

4.12.4. 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.

4.12.5. Repeat Step 4.12.4. once for a total of 2 washes.

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

4.12.7. 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.

4.12.8. 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).

4.12.9. 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.

4.13. 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).

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

4.13.1A.Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.12.9.)	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

4.13.1B.Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.12.9.)	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.

4.13.2. 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.

4.13.3. 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 4.13.3A and Table 4.13.3B):

Table 4.13.3A.

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

* The number of PCR cycles should be adjusted based on RNA input (Table 4.13.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 6.2 in Section 6).

Table 4.13.3B. Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES*
100 ng	14–15

***** The PCR cycles are recommended based on high quality human whole blood total RNA. To prevent over-amplification, the number of cycles may require optimization based on the sample quality and the fraction of globin mRNA. For RNA where globin mRNA is > than 50% of the transcripts (once rRNA is removed), follow the higher cycle recommendation for that input.

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

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

4.14.2. 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.

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

4.14.4. 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).**

4.14.5. 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.

4.14.6. Repeat Step 4.14.5. once for a total of 2 washes.

4.14.7. 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.

4.14.8. 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).

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

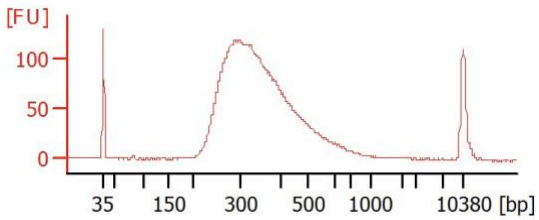
4.15. Library Quantification

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

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

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 4.14.9.) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 4.14.).

Figure 4.15.2 Example of RNA library size distribution on a Bioanalyzer.



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

Section 5
Appendix for use with NEBNext Ultra II RNA Library Prep Kits for Illumina (NEB #E7760, NEB #E7765) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

Symbols

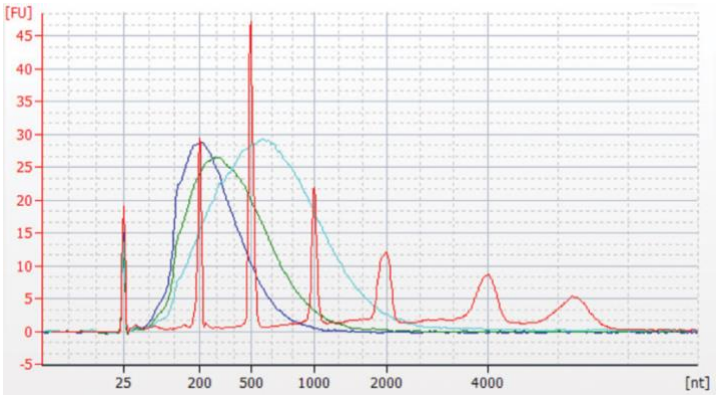
This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

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

5.1. Fragmentation

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

Figure 5.1. Modified fragmentation times for longer RNA inserts.



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

Modified fragmentation times for longer RNA inserts. Bioanalyzer traces of RNA as shown in an RNA Pico Chip. mRNA isolated from Universal Human Reference RNA and fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (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.

5.2. Size Selection of Adaptor Ligated DNA

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

The size selection protocol is based on a starting volume of 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 5.2. The protocol below is for libraries with a 300 bp insert size (420 bp final library size).

Table 5.2. Recommended size selection conditions for libraries with insert sizes larger than 300 bp.

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

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

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

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

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

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

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

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

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

5.2.8. Repeat Step 5.2.7. once for a total of 2 washes.

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

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

5.2.10. Remove the tube/plate from the magnetic rack. Elute the DNA target from the beads by adding 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.

5.2.11. 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.

5.3. PCR Enrichment of Size-selected Libraries

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

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).

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

5.3.1A.Forward and Reverse Primers Separate

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

5.3.1B.Forward and Reverse Primers Combined

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

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

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

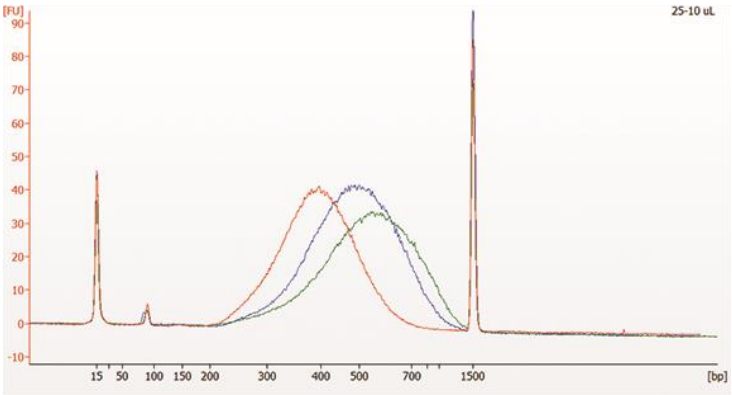
CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	variable*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

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

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 6.2 in Section 6).

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

Figure 5.3: Bioanalyzer traces of size selected DNA libraries.



50 ng mRNA was fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix at 94°C for 10 or 5 minutes. 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 5.3.

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

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

Section 6
Troubleshooting Guide for use with NEBNext Ultra II RNA Library Prep Kits for Illumina (NEB #E7760, NEB #E7765) and NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770, NEB #E7775)

OBSERVATIONS	POSSIBLE CAUSES	EFFECT	SUGGESTED SOLUTIONS
Presence of Bioanalyzer peaks < 85 bp (Figure 6.1)	• Presence of Primers remaining after PCR clean up	Primers cannot cluster or be sequenced, but can bind to flowcell and reduce cluster density	• Clean up PCR reaction again with 0.9X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield)
Presence of ~127 bp adaptor-dimer Bioanalyzer peak (Figure 6.1)	• 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 6.2)	• 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 6.1.

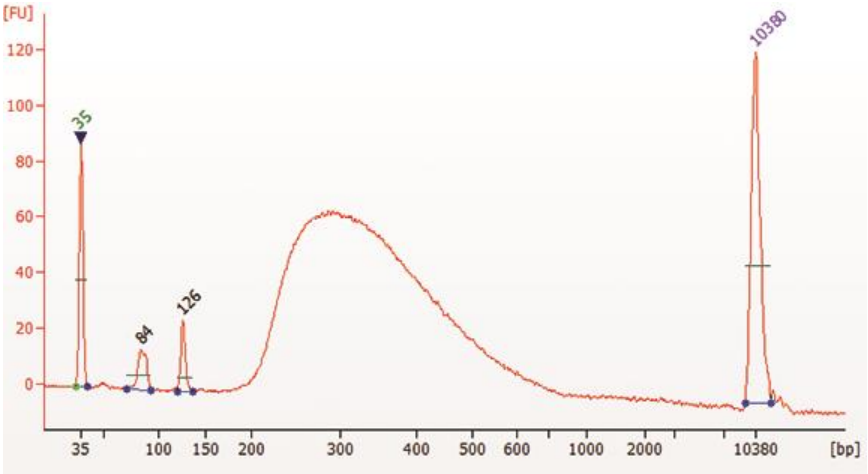
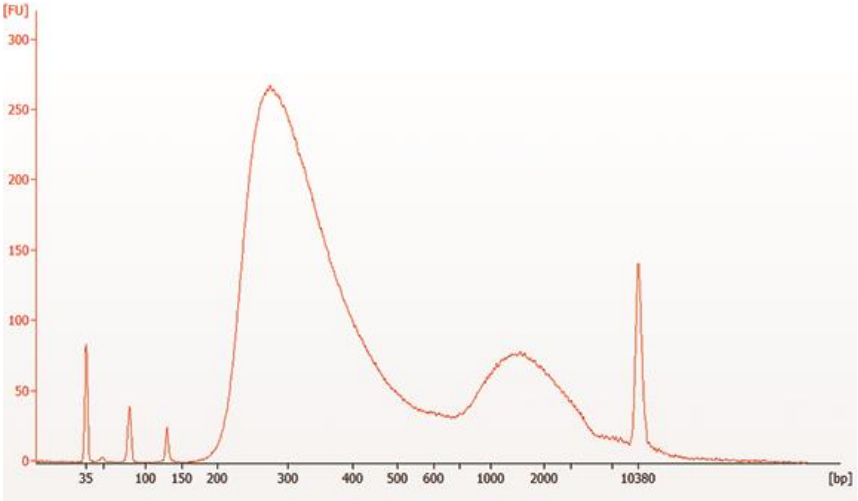


Figure 6.2.



Checklist: Globin and rRNA Depletion Kit

1. Hybridize the Probes to the RNA

1.1. Assemble Probe/RNA Hybridization Reaction

[_] 1.1.1.Total RNA in nuclease-free water 10 µl

[_] 1.1.2NEBNext Globin and rRNA Depletion Solution 3 µl

[_] 1.1.3.Probe Hybridization Buffer 2 µl

[_] 1.2.Mix 10 times

[_] 1.4.Quick spin

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

[_] 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 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

[_] 3.1.3.Nuclease-free water 22.5 µl

[_] 3.2.Mix 10 times

[_] 3.3.Quick spin

[_] 3.4. Incubate in 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 #E7750S Table of Components

NEB #	PRODUCT	VOLUME
E7752-2	NEBNext Thermostable RNase H	0.012 ml
E6312-2	RNase H Reaction Buffer	0.012 ml
E7751-2	NEBNext Globin and rRNA Depletion Solution	0.018 ml
E6314-2	NEBNext Probe Hybridization Buffer	0.012 ml
E7753-2	NEBNext DNase I	0.015 ml
E6315-2	DNase I Reaction Buffer	0.03 ml
E6317-2	Nuclease-free Water	0.4 ml

NEB #E7750L Table of Components

NEB #	PRODUCT	VOLUME
E7752-3	NEBNext Thermostable RNase H	0.048 ml
E6312-3	RNase H Reaction Buffer	0.048 ml
E7751-3	NEBNext Globin and rRNA Depletion Solution	0.072 ml
E6314-3	NEBNext Probe Hybridization Buffer	0.048 ml
E7753-3	NEBNext DNase I	0.06 ml
E6315-3	DNase I Reaction Buffer	0.120 ml
E6317-3	Nuclease-free Water	1.5 ml

NEB #E7750X Table of Components

NEB #	PRODUCT	VOLUME
E7752-4	NEBNext Thermostable RNase H	0.192 ml
E6312-4	RNase H Reaction Buffer	0.192 ml
E7751-4	NEBNext Globin and rRNA Depletion Solution	0.288 ml
E6314-4	NEBNext Probe Hybridization Buffer	0.192 ml
E7753-4	NEBNext DNase I	0.24 ml
E6315-4	DNase I Reaction Buffer	0.48 ml
E6317-4	Nuclease-free Water	6.0 ml

NEB #E7755S Table of Components

NEB #	PRODUCT	VOLUME
E7752-2	NEBNext Thermostable RNase H	0.012 ml
E6312-2	RNase H Reaction Buffer	0.012 ml
E7751-2	NEBNext Globin and rRNA Depletion Solution	0.018 ml
E6314-2	NEBNext Probe Hybridization Buffer	0.012 ml
E7753-2	NEBNext DNase I	0.015 ml
E6315-2	DNase I Reaction Buffer	0.03 ml
E6317-2	Nuclease-free Water	0.4 ml
E6351S	NEBNext RNA Sample Purification Beads	0.66 ml

NEB #E7755L Table of Components

NEB #	PRODUCT	VOLUME
E7752-3	NEBNext Thermostable RNase H	0.048 ml
E6312-3	RNase H Reaction Buffer	0.048 ml
E7751-3	NEBNext Globin and rRNA Depletion Solution	0.072 ml
E6314-3	NEBNext Probe Hybridization Buffer	0.048 ml
E7753-3	NEBNext DNase I	0.06 ml
E6315-3	DNase I Reaction Buffer	0.120 ml
E6317-3	Nuclease-free Water	1.5 ml
E6351L	NEBNext RNA Sample Purification Beads	2.64 ml

NEB #E7755X Table of Components

NEB #	PRODUCT	VOLUME
E7752-4	NEBNext Thermostable RNase H	0.192 ml
E6312-4	RNase H Reaction Buffer	0.192 ml
E7751-4	NEBNext Globin and rRNA Depletion Solution	0.288 ml
E6314-4	NEBNext Probe Hybridization Buffer	0.192 ml
E7753-4	NEBNext DNase I	0.24 ml
E6315-4	DNase I Reaction Buffer	0.48 ml
E6317-4	Nuclease-free Water	6.0 ml
E6351X	NEBNext RNA Sample Purification Beads	10.6 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	5/19
2.0	Updated protocols.	11/20
3.0	Updated protocols.	8/22
4.0	Updated protocols. Also updated header and footer with new logo. Updated legal footnote.	6/24