NEBNext® Ultra™ II Directional RNA Library Prep for Element Biosciences Sequencing

New England Biolabs

Library preparation methods need to be flexible as users begin to adopt newly emerging sequencing platforms. The Element AVITI system and Cloudbreak chemistry promise high-quality sequencing data with an affordable, user-friendly benchtop system. The commercial availability of Element barcoded sequencing adaptors enables users to apply their preferred library prep method and still perform native sequencing on the Element instrument.

The NEBNext Ultra II Directional RNA Library Prep Kit is an optimal choice for producing high-quality RNA sequencing libraries from a broad range of species, input amounts, and sample types. The Element Elevate Adapter Kits can be easily incorporated into the NEBNext Ultra II Directional RNA Library Kit workflow downstream of either poly(A) mRNA enrichment or rRNA depletion protocols. Sample purification bead cleanup recommendations are used to remove excess adaptor dimer and optimize the library size for efficient loading of the AVITI instrument.

PROTOCOL

Section 1

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

RNA Sample Requirements

RNA Integrity:

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer® RNA 6000 Nano/Pico Chip or Agilent TapeStation High Sensitivity RNA ScreenTape®. For poly(A) mRNA enrichment, high-quality RNA with a RIN score > 7 is required.

RNA Sample Requirements:

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

Input Amount Requirements:

100 ng–1 µg DNA-free total RNA quantified by Qubit® Fluorometer and quality checked by Bioanalyzer or TapeStation.

The protocol is optimized for approximately 200 bp RNA inserts.

Keep all the buffers on ice, unless otherwise indicated.

MATERIALS

- High-quality total RNA (100 ng to 1 µg)
- NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760)
- NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) or NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400, #E7405)
- RNAClean XP beads (Beckman Coulter, Inc. #A63987). Required only for use with the NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400)
- Element Elevate™ Long UDI Adapter Kit Set A (Element Biosciences #830-00010)* or Element Elevate Index and Adapter Kit (Element Biosciences #830-00005)
- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- Nuclease-free water
- 80% ethanol (freshly prepared)
- Pipettes with disposable tips
- Thin wall 200 µl PCR tubes (For example TempAssure® PCR flex-free 8-tube strips USA Scientific #1402-4708)
- Magnetic rack (NEB #S1515S), magnetic plate (Alpaqua® #A001322) or equivalent
- 1.5ml tube Magnetic rack (NEB #S1506) or equivalent. For use with NEBNext poly(A) mRNA Magnetic Isolation Module only.
- Thermal cycler
- Microcentrifuge
- Vortex Mixer
- User-supplied SP5 and SP27 outer PCR primer mix, 5 µM each. Required only for use with the Element Elevate Long UDI Adapter Kit set A (Element Biosciences #830-00010).

  Primer sequences:

  SP5 5' Phosphate-CATGTAATGCACGTACTTTCAGGGT

  SP27 5' GATCAGGTGAGGCTGCGACGACT
- Bioanalyzer®, TapeStation® (Agilent Technologies, Inc.) or similar fragment analyzer and consumables

1.1 Preparation of First Strand Reaction Buffer and Random Primer Mix

1.1.1 Prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix in a nuclease-free microcentrifuge tube as follows:

COMPONENT	VOLUME
● (lilac) NEBNext First Strand Synthesis Reaction Buffer	8 µl
● (lilac) Random Primers	2 µl
Nuclease-free Water	10 µl
Total Volume	**20 µl**

You can prepare the first strand synthesis reaction buffer later in the protocol, but it is important that it is ready before the elution in Step 1.2.26. The beads should not be allowed to dry out.

1.1.2 Mix thoroughly by pipetting up and down ten times.
Note: Keep the mix on ice until mRNA is purified. It will be used in Step 1.2.26 and 1.2.29.

1.2 mRNA Isolation, Fragmentation and Priming Starting with Total RNA

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

1.2.2 Add 20 µl of NEBNext Oligo d(T)$_{25}$ beads per reaction to a 1.5 ml tube. If preparing multiple libraries, beads for up to 24 samples can be added to a single 1.5 ml tube for subsequent washes (use magnetic rack 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.
Note: Vortex all buffers before use. Do not vortex RNA or beads. Mix the beads well before using by flicking or inverting the tube to ensure a homogeneous suspension.

1.2.3 Place the tube on the magnetic rack at room temperature for 2 minutes or until the solution is clear.

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

1.2.5 Remove the tube from the magnetic rack.

1.2.6 Add 50 µl of NEBNext RNA Binding Buffer (2X) to the beads and wash by pipetting up and down 6 times. If preparing multiple libraries, add 50 µl RNA Binding Buffer per sample. The Binding Buffer does not have to be diluted.

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

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

1.2.9 Remove the tube from the magnetic rack and add 50 µl of 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 of RNA Binding Buffer per sample. The Binding Buffer does not have to be diluted.

1.2.10 Add 50 µl of beads to each RNA sample from Step 1.2.1. Mix thoroughly by pipetting up and down 6 times. This first binding step removes most of the non-target RNA.

1.2.11 Heat the sample to denature the RNA and to facilitate binding of the poly(A) RNA to the beads. Place in a thermal cycler with the heated lid set to ≥ 90°C and run the following program:

- 2 minutes at 80°C
- 5 minutes at 25°C
- Hold at 25°C

1.2.12 Remove the tube from the thermal cycler when the temperature reaches hold at 25°C.

1.2.13 Place the tube on the magnetic rack at room temperature for 2 minutes or until the solution is clear to separate the poly(A) RNA bound to the beads from the solution.

1.2.14 Remove and discard all of the supernatant. Take care not to disturb the beads. Do not remove the tube from the magnetic rack.

1.2.15 While still on the magnet rinse the beads by gently adding 200 µl of NEBNext Wash Buffer to the tube to remove unbound RNA.

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

1.2.17 Remove the tube from the magnetic rack.

1.2.18 Add the following to each tube containing mRNA-bound beads to allow the poly(A) RNA to rebind to the same beads. Mix thoroughly by gently pipetting up and down 6 times.

COMPONENT	VOLUME
NEBNext Tris Buffer	50 µl
NEBNext RNA Binding Buffer (2X)	50 µl
Total Volume	100 µl

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

1.2.19 Place the tube in a thermal cycler, with the heated lid set to ≥ 90°C, and run the following program:

- 2 minutes at 80°C
- 5 minutes at 25°C
- Hold at 25°C

1.2.20 Remove the tube from the thermal cycler when the temperature reaches hold at 25°C.

1.2.21 Place the tube on the magnetic rack at room temperature for 2 minutes or until the solution is clear.

1.2.22 Remove and discard all of the supernatant from each tube. Take care not to disturb the beads. Do not remove the tube from the magnetic rack.

1.2.23 While still on the magnetic rack, rinse the beads by gently adding 200 µl of NEBNext Wash Buffer to the tube to remove unbound RNA.

1.2.24 Remove and discard all of the supernatant from the tube. Take care to remove all of the NEBNext Wash Buffer and avoid disturbing the beads that contain the mRNA.
Note: It is important to remove all of the supernatant to successfully use the RNA in downstream steps. Spin down the tube. Place the tube on the magnetic rack and using a 10 µl tip remove all of the NEBNext Wash Buffer. (Caution: Do not disturb beads that contain the mRNA). Avoid letting the beads dry out before adding elution buffer.

1.2.25 Remove the tube from the magnetic rack.

1.2.26 To elute the mRNA from the beads and fragment, add 11.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer Mix prepared in Step 1.1.2, pipette up and down six times to resuspend the beads.

1.2.27 Place the tube in a thermal cycler, with the heated lid set at 105°C, and run the following program:

- 15 minutes at 94°C
- Hold at 4°C*

*Immediately transfer the tube to ice for 1 minute as soon as it is cool enough to handle (~65°C)

1.2.28 Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (~1-2 minutes).

1.2.29 Collect the fragmented mRNA by transferring 10 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.
Note 1: If the supernatant volume recovered is less than 10 µl for any reason, bring the volume up to 10 µl by adding the First Strand Synthesis Reaction Buffer and Random Primer Mix prepared in Step 1.1.2 and continue with the protocol.
Note 2: Avoid transferring any of the magnetic beads.

1.2.30 Place the tube on ice and proceed directly to First Strand cDNA Synthesis.

1.3 First Strand cDNA Synthesis

1.3.1 Assemble the first strand cDNA synthesis reaction on ice by adding the following components to the fragmented and primed RNA from Step 1.2.30.

COMPONENT	VOLUME
Fragmented and primed RNA (Step 1.2.30)	10 µl
● (brown) NEBNext Strand Specificity Reagent	8 µl
● (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

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

1.3.3 Place the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C, and run the following program:

- 10 minutes at 25°C
- 15 minutes at 42°C
- 15 minutes at 70°C
- Hold at 4°C

1.3.4 Immediately perform Second Strand cDNA Synthesis once the temperature reaches 4°C.

1.4 Second Strand cDNA Synthesis

1.4.1 Assemble the second strand cDNA synthesis reaction on ice by adding the following components into the first strand synthesis reaction product from Step 1.3.4.

COMPONENT	VOLUME
First Strand Synthesis Product (Step 1.3.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
● (orange) NEBNext First Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

1.4.2 Keep the tube on ice and mix thoroughly by pipetting the reaction up and down at least 10 times.

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

We recommend proceeding to the cleanup step.

1.5 Cleanup of Double-stranded cDNA

1.5.1 Vortex SPRIselect Beads or AMPure XP beads to resuspend. If using AMPure XP Beads, remove from 4°C, keep at room temperature for 30 minutes, and resuspend prior to use.

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

1.5.3 Incubate for at least 5 minutes at room temperature.

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

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

1.5.6 Repeat Step 1.5.5 once for a total of 2 washing steps.

1.5.7 Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.
Caution: Do not over-dry the beads. This may result in lower recovery of the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

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

1.5.9 Remove **50 µl** of the supernatant and transfer to a clean nuclease-free PCR tube.
Note: If you need to stop at this point in the protocol, samples can be stored at –20°C.

1.6 End Prep of cDNA Library

1.6.1 Assemble the end prep reaction on ice by adding the following components to the Second Strand cDNA Synthesis Product from Step 1.5.9.

COMPONENT	VOLUME
Second Strand cDNA Synthesis Product (Step 1.5.9)	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

1.6.2 Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.
Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

1.6.3 Incubate the sample in a thermal cycler with the heated lid set at ≥ 75°C and run the following program:

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

1.6.4 Proceed immediately to Adaptor Ligation.

1.7 Adaptor Ligation

1.7.1 Dilute each Elevate Long UDI Adapter (if using Element Elevate Long UDI Adapter Kit) or Ligation Adapters (if using Element Elevate Index and Adapter Kit) prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer (provided) and keep the diluted adapter on ice.

Table 1.7.1: Adaptor dilution based on total RNA input amount

TOTAL RNA INPUT	DILUTION REQUIRED
250 ng – 1 µg	5-fold dilution in Adaptor Dilution Buffer
100 ng – 249 ng	25-fold dilution in Adaptor Dilution Buffer

1.7.2 Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 1.6.4.

COMPONENT	VOLUME
End Prepped DNA (Step 1.6.4)	60 µl
Diluted Adaptor* (Step 1.7.1)	2.5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

**Note: This protocol is compatible with both Element Elevate Long UDI Adapter Kit Set A (Element Biosciences #830-00010) or Element Elevate Index and Adapter Kit (Element Biosciences #830-00005). Please ensure that the PCR primers used in step 1.9 are correct for the adaptor of choice.*

1.7.3 Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.
Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

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

1.7.5 Add 3 µl (● blue) USER Enzyme to the ligation mixture from Step 1.7.4, resulting in total volume of 96.5 µl.

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

1.7.7 Proceed immediately to the Cleanup of adaptor-ligated DNA.

1.8 Cleanup of adaptor-ligated DNA

1.8.1 Vortex SPRIselect Beads or AMPure XP beads to resuspend. If using AMPure XP Beads, remove from 4°C, keep at room temperature for 30 minutes, and resuspend prior to use.

1.8.2 Add **67.5 µl (0.7X)** of resuspended SPRIselect beads or AMPure XP beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

1.8.3 Incubate for 5 minutes at room temperature.

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

1.8.5 Remove the tube from the magnet and add **50 µl** 0.1X TE to the beads. Mix well on a vortex mixer or by pipetting up and down.

1.8.6 Add **45 µl (0.9X)** of resuspended SPRIselect Beads or AMPure XP beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

1.8.7 Incubate for 5 minutes at room temperature.

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

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

1.8.10 Repeat Step 1.8.8 once for a total of 2 washing steps.

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

1.8.12 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 the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

1.8.13 Remove the tube from the magnet. Elute DNA target from the beads by adding **17 µl** 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

1.8.14 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.*

1.9 PCR Enrichment of Adaptor-Ligated DNA

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

COMPONENT	VOLUME
Adaptor Ligated DNA (Step 1.8.13)	15 µl
PCR Primer Mix*	10 µl
● (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Total Volume	50 µl

**User-supplied SP5 and SP27 outer PCR primer mix, 5µM each (if using Element Elevate Long UDI Adapter Kit) or Element Unique Index Pair (if using Element Elevate Index and Adapter Kit)*

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

1.9.3 Place the tube on a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 1.9.3A and Table 1.9.3B):

Table 1.9.3A: PCR cycling conditions

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	7-14*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 seconds	1
Hold	4°C	∞	

** The number of PCR cycles should be adjusted based on RNA input (Table 1.9.3B).*

*** 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.*

Table 1.9.3B: Recommended PCR cycles based on total RNA input amount

TOTAL RNA INPUT	PCR CYCLES
1 µg	7-9
100 ng	12-14

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

1.10 Cleanup of PCR Reaction

1.10.1 Vortex SPRIselect Beads or AMPure XP beads to resuspend. If using AMPure XP Beads, remove from 4°C, keep at room temperature for 30 minutes, and resuspend prior to use.

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

1.10.3 Incubate for up to 5 minutes at room temperature.

1.10.4 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.

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

1.10.6 Repeat Step 1.10.5 once for a total of 2 washing steps.

1.10.7 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 the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

1.10.8 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.

1.10.9 Transfer **20 µl** of the supernatant to a clean PCR tube, and store at –20°C. This is the final library.

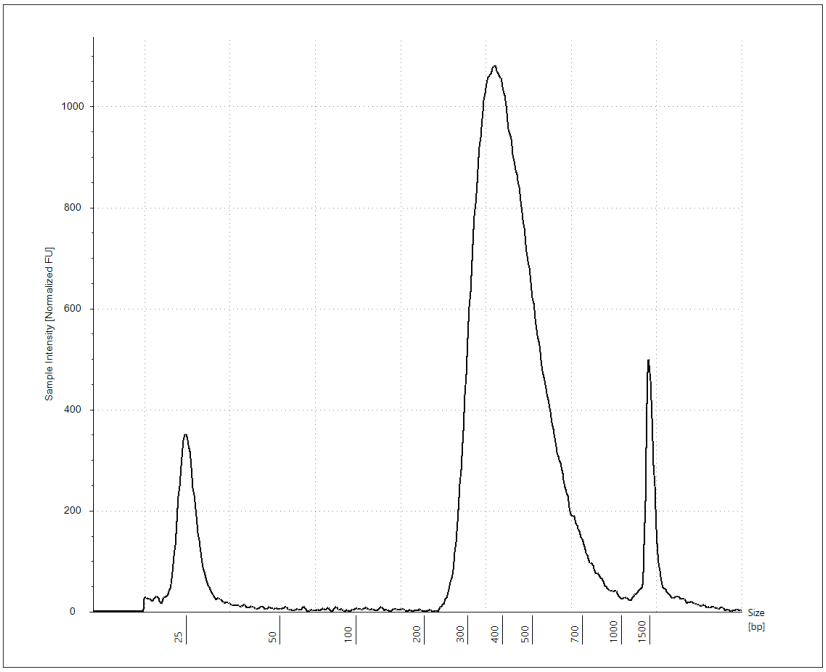
1.11 Assessment of Library Yield and Size

1.11.1 Check the yield and size distribution on Agilent High Sensitivity D1000 Tapestation ScreenTape or Agilent High Sensitivity Bioanalyzer chip.

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

Note: If a peak at ~ 85 bp (primers) or 156 bp (adaptor-dimer) is visible in the TapeStation traces, bring up the sample volume (from Step 1.10.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead Cleanup Step (Section 1.10). Optionally, this additional cleanup can be performed after creating an equimolar pool of multiple libraries. Adaptor dimer peak range is derived from values observed on TapeStation. Peaks may appear shifted on other library analysis instruments.

FIGURE 1.11.1: **Example of RNA library size distribution on a TapeStation.**



Section 2

Protocol for use with NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400, #E7405)

RNA Sample Requirements

RNA Integrity:

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip or Agilent TapeStation High Sensitivity RNA ScreenTape to determine the RNA Integrity Number (RIN). This protocol can be performed with intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7).

RNA Purity:

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

Input Amount:

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

2.1 Probe Hybridization to RNA

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

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (100 ng–1 µg)	11 µl
O (white) NEBNext v2 rRNA Depletion Solution	2 µl
O (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	15 µl

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

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

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

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

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

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

2.2 RNase H Digestion

2.2.1 Assemble the following RNase H digestion reaction on ice:

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

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

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

2.2.3 Briefly spin down the tube in a microcentrifuge.

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

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

2.3 DNase I Digestion

2.3.1 Assemble the following DNase I digestion reaction on ice:

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

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

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

2.3.3 Briefly spin down the tube in a microcentrifuge.

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

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

2.4 Cleanup of rRNA-depleted RNA

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

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

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

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

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

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

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

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

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

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

2.4.10 Incubate for 2 minutes at room temperature.

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

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

2.4.13 Place the tube on ice and proceed to RNA 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 are listed in Table 2.5.3.

2.5.1 Assemble the following fragmentation and priming reaction on ice:

COMPONENT	VOLUME
Ribosomal RNA Depleted Sample (Step 2.4.13)	5 µl
● (lilac) First Strand Synthesis Reaction Buffer	4 µl
● (lilac) Random Primers	1 µl
Total Volume	10 µl

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

2.5.2 Mix thoroughly by pipetting up and down 10 times.

2.5.3 Place the tube in a thermal cycler with heated lid set at 105°C and run the following program:

- Incubate at 94°C for the suggested fragmentation time (see Table 2.5.3)
- Hold at 4°C

Table 2.5.3. Suggested fragmentation times (at 94°C) based on RIN value of RNA input
Note: The Table 2.5.3 recommendations result in libraries with inserts ~200nt long.

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

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:

COMPONENT	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 preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

- 10 minutes at 25°C
- 15 minutes at 42°C
- 15 minutes at 70°C
- 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 to the first strand synthesis product from Step 2.6.3.

COMPONENT	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 Cleanup of Double-stranded cDNA

2.8 Cleanup of Double-stranded cDNA

2.8.1 Vortex SPRIselect Beads or AMPure XP Beads to resuspend. If using AMPure XP Beads, remove from 4°C, keep at room temperature for 30 minutes, and resuspend prior to use.

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

2.8.3 Incubate for 5 minutes at room temperature.

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

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

2.8.6 Repeat Step 2.8.5 once for a total of 2 washes.

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

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

2.8.8 Remove the tube from the magnetic rack. Elute the 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).

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.

COMPONENT	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 prepared, 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 each Elevate Long UDI Adapter (if using Element Elevate Long UDI Adapter Kit) or Ligation Adapters (if using Element Elevate Index and Adapter Kit) prior to setting up the ligation reaction in ice-cold Adapter Dilution Buffer (provided) and keep the diluted adapter on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
250 ng – 1 µg	5-fold dilution in Adapter Dilution Buffer
100 ng - 249 ng	25-fold dilution in Adapter Dilution Buffer

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

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

Note: This protocol is compatible with both Element Elevate Long UDI Adapter Kit Set A (Element Biosciences #830-00010) or Element Elevate Index and Adapter Kit (Element Biosciences #830-00005). Please ensure that PCR primers used in step 2.12 are correct for the adaptor of choice.

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and are 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) USER Enzyme to the ligation mixture from Step 2.10.4, resulting in total volume of 96.5 µl.

2.10.6 Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 45°C, hold at 4°C. Proceed immediately to Purification of the Ligation Reaction.

2.11 Cleanup of adaptor-ligated DNA

2.11.1 Vortex SPRIselect Beads or AMPure XP beads to resuspend. If using AMPure XP Beads, remove from 4°C, keep at room temperature for 30 minutes, and resuspend prior to use.

2.11.1 Add **67.5 µl (0.7X)** of resuspended SPRIselect Beads or AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.11.2 Incubate for 5 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 Remove the tube from the magnet and add 50 µl 0.1X TE to the beads. Mix well on a vortex mixer or by pipetting up and down.

2.11.5 Add **45 µl (0.9X)** of resuspended SPRIselect Beads or AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.11.6 Incubate for 5 minutes at room temperature.

2.11.7 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)***.

2.11.8 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.

2.11.9 Repeat Step 2.11.8 once for a total of 2 washes.

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

2.11.11 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.

2.11.12 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),

2.11.13 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

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

COMPONENT	VOLUME PER ONE
Adaptor Ligated DNA (Step 2.11.13)	15 µl
PCR Primer Mix*	10 µl
● (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Total Volume	50 µl

* Element Universal PCR Primer Mix (if using Element Elevate Long UDI Adapter Kit) or Element Unique Index Pair (if using Element Elevate Index and Adapter Kit)

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

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

Table 2.12.3A: PCR cycling conditions

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	7-14*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 seconds	1
Hold	4°C	∞	

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

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace.

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

TOTAL RNA	PCR CYCLES
1 µg	7–9
100 ng	12–14

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

2.13 Cleanup of PCR Reaction

2.13.1 Vortex SPRIselect Beads or AMPure XP Beads to resuspend. If using AMPure XP Beads, remove from 4°C, keep at room temperature for 30 minutes, and resuspend prior to use.

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

2.13.3 Incubate for 5 minutes at room temperature.

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

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

2.13.6 Repeat Step 2.13.5 once for a total of 2 washes.

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

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

2.13.8 Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding **23 µl** 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear (about 2 minutes).

2.13.9 Transfer **20 µl** of the supernatant to a clean PCR tube, and store at –20°C. This is the final library.

2.14 Assessment of Library Yield and Size

2.14.1 Check the yield and size distribution on Agilent High Sensitivity D1000 Tapestation ScreenTape or Agilent High Sensitivity Bioanalyzer chip.

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

Note: *If a peak at ~ 85 bp (primers) or 156 bp (adaptor-dimer) is visible in the TapeStation 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 AMPure XP Bead Cleanup Step (Section 2.13). Optionally, this additional cleanup can be performed after creating an equimolar pool of multiple libraries. Adaptor dimer peak range is derived from values observed on TapeStation. Peaks may appear shifted on other library analysis instruments.*

FIGURE 2.14.1: **Example of RNA library size distribution on a TapeStation.**