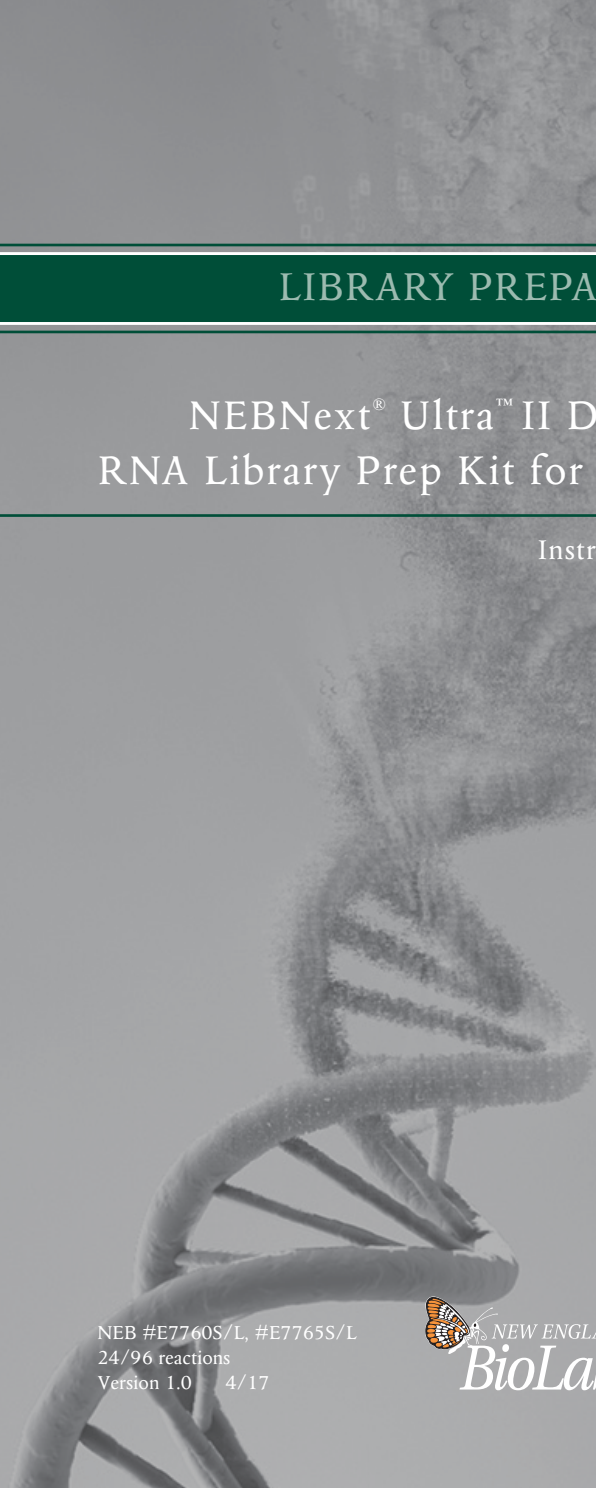
LIBRARY PREPARATION

NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®

Instruction Manual



NEB #E7760S/L, #E7765S/L
24/96 reactions
Version 1.0 4/17

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be INSPIRED
drive DISCOVERY
stay GENUINE

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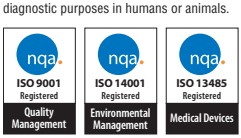

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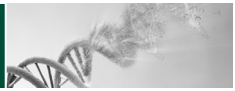

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The Library Prep Kit Includes:

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7760S/#E7765S) and 96 reactions (NEB #E7760L/#E7765L).

Package 1: Store at –20°C.

- (lilac) NEBNext First Strand Synthesis Reaction Buffer
- (lilac) Random Primers
- (lilac) NEBNext First Strand Synthesis Enzyme Mix
- (brown) NEBNext Strand Specificity Reagent
- (orange) NEBNext Second Strand Synthesis Enzyme Mix
- (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)
- (green) NEBNext Ultra II End Prep Enzyme Mix
- (green) NEBNext Ultra II End Prep Reaction Buffer (10X)
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (blue) NEBNext USER™ Enzyme
- (blue) NEBNext Ultra II Q5® Master Mix

NEBNext Adaptor Dilution Buffer

TE Buffer (0.1X)

Nuclease-free Water

Package 2: Store at room temperature. Do not freeze.

Supplied only with NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads, NEB #E7765.

NEBNext Sample Purification Beads

Required Materials Not Included:

NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina or customer supplied oligos

Magnetic Rack (Alpaqua®, cat. #A001322 or equivalent)

80% Ethanol (freshly prepared)

Thermal Cycler

For NEB #E7760 only:

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

Considerations on Choosing an RNA-seq Library Preparation Method

The library preparation protocol should be chosen based on the goals of the project and quality of the RNA sample. Total cellular RNA is mainly composed of rRNA and often is not of interest. rRNA can be removed from total cellular RNA by one of two common methods. The first method uses oligo d(T) beads, which bind to the poly(A) tail of eukaryotic mRNA. Alternatively, rRNA can be depleted using rRNA specific probes. NEB offers the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) and the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310) for the enrichment of non-ribosomal RNA.

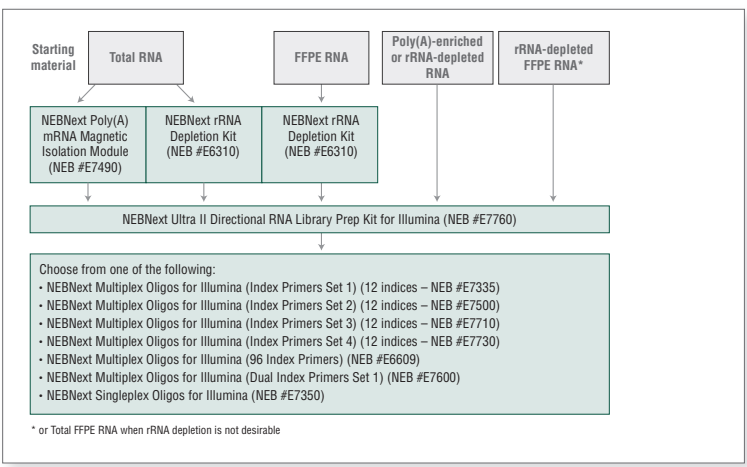
In the oligo d(T) approach, only mRNA with poly(A) tails will be enriched; other cellular RNA without a poly(A) tail, such as non-coding RNA or mRNA lacking poly(A) will not bind to the beads. In addition, mRNA from some organisms (e.g., prokaryotes) or degraded RNA (e.g., FFPE RNA) do not have poly(A) tails, and will not be captured by oligo d(T) beads. On the other hand, the probe based rRNA depletion kit will remove the targeted ribosomal RNA, but it will preserve other biologicaly relevant cellular RNA such as non-coding RNA or mRNA.

The quality of an RNA sample should also be considered when deciding on a library preparation protocol. The NEBNext Poly(A) mRNA Magnetic Isolation Module should only be used with high quality RNA samples (RIN > 7), since degradation results in a loss of poly(A) tails from mRNA molecules. For partially degraded or heavily degraded samples (e.g., RIN ≤ 7, FFPE RNA), the NEBNext rRNA Depletion Kit should be used, or total RNA should be directly converted to RNA-seq libraries without any enrichment or depletion.

NEBNext Ultra II Directional RNA Product Selection Guide

Use the following chart to determine the required kits to make RNA libraries based on starting input material. The chart contains kit recommendations only for the protocols described in this manual. For other RNA library needs or related products, please refer to www.nebnext.com.

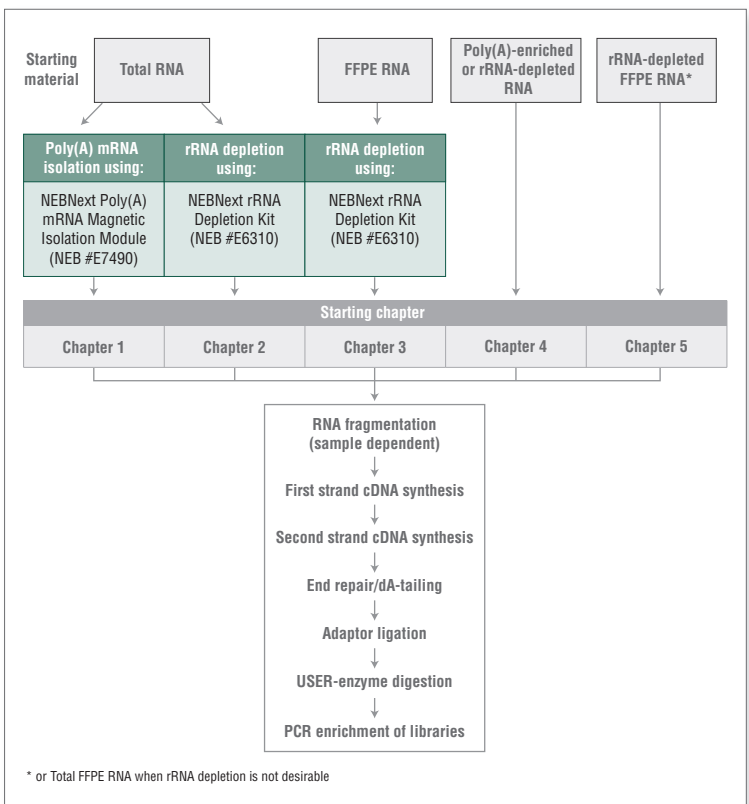
Figure 1. NEBNext Ultra II Directional RNA Product Selection Guide.



NEBNext Ultra II Directional RNA Protocol Selection Guide

Use the following chart to determine the most suitable protocol in this manual. Every chapter in this manual contains a different protocol based on the starting material. More detailed information is available at the beginning of each chapter. Please read the RNA sample recommendations and input amount requirements in its entirety before starting the protocol.

Figure 2. NEBNext Ultra II Directional RNA Protocol Selection Guide.



Overview

The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina contains the enzymes and buffers required to convert a broad range of input amounts of RNA into high quality directional (strand-specific) libraries for next-generation sequencing on the Illumina platform. The fast, user-friendly workflow has minimal hands-on time and is compatible with poly(A) mRNA enrichment and rRNA depletion methods.

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of an indexed transcriptome library on the Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

1

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

NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual

Symbols

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.

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

The protocol has been optimized using high quality Universal Human Reference Total RNA.

RNA Sample Recommendations

RNA Integrity:
Assess the quality of the Input RNA by running the RNA sample on an Agilent Bioanalyzer® RNA 6000 Nano/Pico Chip. For PolyA mRNA enrichment, high quality RNA with a RIN score > 7 is required.

RNA Purity:
Treat the RNA sample with DNase I to remove all traces of DNA. Remove DNase I after treatment. The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Input Amount Requirement: 10 ng–1 µg total RNA quantified by Qubit® Fluorometer and quality checked by Bioanalyzer.

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

Keep all the buffers on ice, unless otherwise indicated.

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 (2X) in a nuclease-free microcentrifuge tube as follows:

COMPONENT	VOLUME
(lilac) NEBNext First Strand Synthesis Reaction Buffer (5X)	8 µl
(lilac) NEBNext Random Primers	2 µl
Nuclease-free water	10 µl
Total Volume	20 µl

1.1.2. Mix thoroughly by pipetting up and down several times.

Note: Keep the mix on ice until mRNA is purified. It will be used in Step 1.2.36 and 1.2.39.

1.2. mRNA Isolation, Fragmentation and Priming Starting with Total RNA

1.2.1. 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.

1.2.2. To wash the Oligo dT Beads, add the following 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.

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

1.2.3. Wash the beads by pipetting up and down several times.

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

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

1.2.6. Remove the tube from the magnetic rack.

1.2.7. Add 100 µl RNA Binding Buffer (2X) to the beads and wash by pipetting up and down. If preparing multiple libraries, add 100 µl RNA Binding Buffer (2X) per sample.

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

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

1.2.10. Add 50 µl 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 RNA Binding Buffer (2X) per sample.

1.2.11. Add 50 µl beads to each RNA sample from Step 1.2.1. Mix thoroughly by pipetting up and down several times.

1.2.12. 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** to denature the RNA and facilitate binding of the mRNA to the beads.

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

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

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

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

1.2.17. Remove the tube from the magnetic rack.

1.2.18. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.

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

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

1.2.21. Remove the tube from the magnetic rack.

1.2.22. Repeat steps 1.2.18–1.2.21.

1.2.23. Add 50 µl of Tris Buffer (provided) to each tube. Gently pipette up and down 6 times to mix thoroughly.

1.2.24. Place the tube on the thermal cycler. Close the lid and heat the samples at **80°C for 2 minutes, then hold at 25°C with the heated lid set at ≥ 90°C** to do the first elution of the mRNA from the beads.

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

1.2.26. Add 50 µl of RNA Binding Buffer (2X) to the sample to allow the mRNA to re-bind to the beads. Mix thoroughly by gently pipetting up and down several times.

1.2.27. Incubate the tube at **room temperature for 5 minutes**.

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

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

1.2.30. Remove the tube from the magnetic rack.

1.2.31. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.

1.2.32. 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 Wash Buffer in subsequent steps.

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

1.2.34. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads that contains the mRNA.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps.

1.2.35. Remove the tube from the magnetic rack.

⚠ **Note: The next step provides a fragmentation incubation time resulting in an RNA insert size of ~ 200 nt. For RNA insert sizes > 200 nt, refer to Chapter 6 (Appendix A) for recommended fragmentation times in Step 1.2.37.**

1.2.36. To elute the mRNA from the beads and fragment, add 11.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1.1.2, pipette up and down several times to resuspend the beads.

1.2.37 Incubate the sample in a thermal cycler with the heated lid set at 105°C as follows:

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)

1.2.38. 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).

1.2.39. 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 (2X) prepared in Step 1.1.2 and continue with the protocol.

Note 2: Avoid transferring the magnetic beads.

1.2.40. 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 into fragmented and primed RNA from Step 1.2.40.

FIRST STRAND cDNA SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 1.2.40)	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 several times.

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

Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.

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

1.3.4. Immediately, perform Second Strand cDNA Synthesis.

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.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 1.3.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
● (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

1.4.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down several times.

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

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

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

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 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.

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

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

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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 several 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 second strand synthesis product from Step 1.5.9.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 1.5.9)	50 µl
● (green) NEBNext Ultra II End Prep Reaction Buffer (10X)	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 as follows:

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

TOTAL RNA INPUT	DILUTION REQUIRED
1,000 ng–250 ng	5-fold dilution in Adaptor Dilution Buffer
249 ng–100 ng	25-fold dilution in Adaptor Dilution Buffer
99 ng–10 ng	100-fold dilution in Adaptor Dilution Buffer

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

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.

LIGATION REACTION	VOLUME PER ONE LIBRARY
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

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

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

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 Purification of the Ligation Reaction.

1.8 Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

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

1.8.1. 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.

1.8.2. Incubate for 10 minutes at room temperature.

1.8.3. 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 contain unwanted fragments **(Caution: do not discard the beads)**.

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

1.8.5. Repeat Step 1.8.4 once for a total of 2 washing steps.

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

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

1.8.8. 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.

1.8.9. 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.

1.9. PCR Enrichment of Adaptor Ligated DNA

Check and verify that the concentration of your oligos is 10 µM on the label.

Follow Section 1.9.1A if you are using the following oligos (10 µM):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 1.9.1B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

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

1.9.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 1.8.9)	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

1.9.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 1.8.9)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/Universal Primer Mix****	10 µl
Total Volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7710, #E7730 or #E7500) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

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 thermocycler 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:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	8–16*, **
Final Extension	65°C	5 minutes	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 (see page 76).

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

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

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

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

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

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 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 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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 several 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.

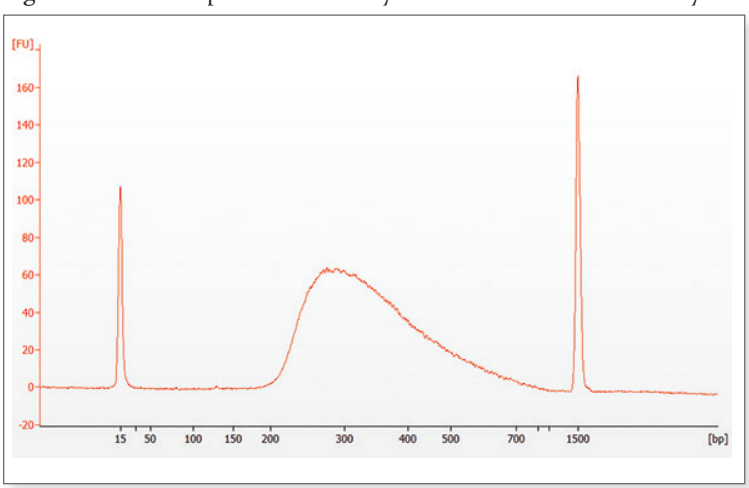
1.11 Assess Library Quality on a Bioanalyzer (Agilent DNA 1000 Chip)

1.11.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip.

1.11.3. 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 1.10.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 1.10).

Figure 1.11.1: Example of RNA library size distribution on a Bioanalyzer.



2

Protocol for use with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)

NEBNext Ultra II Directional
RNA Library Prep Kit for Illumina
Instruction Manual

Symbols

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.

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

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

RNA Sample Recommendations

RNA Integrity:

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

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Chapter 2 (current chapter). See Table 2.5.1. for the recommended fragmentation times, based on RIN.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Chapter 3.

RNA Purity:

Treat the RNA sample with DNase I to remove all traces of DNA. Remove DNase I after treatment. The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Input Amount Requirement

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

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

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

2.1. Probe Hybridization to RNA

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

2.1.2. Prepare a RNA/Probe master mix as follows:

RNA/PROBE MASTER MIX	VOLUME
NEBNext rRNA Depletion Solution	1 µl
Probe Hybridization Buffer	2 µl
Total Volume	3 µl

2.1.3. Add 3 µl of the above mix to 12 µl total RNA (from Step 2.1.1), resulting in a total volume of 15 µl.

2.1.4. Mix thoroughly by pipetting up and down several times.

2.1.5. Briefly spin down the sample in a microcentrifuge.

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

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

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

2.2. RNase H Digestion

2.2.1. Assemble the RNAse H master mix **on ice** as follows.

RNASE H MASTER MIX	VOLUME
NEBNext RNase H	2 µl
NEBNext RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	5 µl

2.2.2. Mix thoroughly by pipetting up and down several times.

2.2.3. Briefly spin down the samples in a microcentrifuge.

2.2.4. Add 5 µl of the RNase H master mix to the RNA sample from Step 2.1.7, resulting in a total volume of 20 µl.

2.2.5. Mix thoroughly by pipetting up and down several times.

2.2.6. Incubate the sample in a thermal cycler for **30 minutes at 37°C** with the lid set to 40°C.

2.2.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to DNase I Digestion to prevent non-specific degradation of RNA.

2.3. DNase I Digestion

2.3.1. Assemble the DNase I master mix **on ice** in a nuclease-free tube.

DNASE I MASTER MIX	VOLUME
DNase I Reaction Buffer	5 µl
DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	30 µl

2.3.2. Mix thoroughly by pipetting up and down several times.

2.3.3. Briefly spin down the sample in a microcentrifuge.

2.3.4. Add 30 µl of DNase I master mix to 20 µl RNA sample from Step 2.2.7, resulting in a total volume of 50 µl.

2.3.5. Mix thoroughly by pipetting up and down several times.

2.3.6. Incubate the sample in a thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C.

2.3.7. Briefly spin down the sample in a microcentrifuge, and place on ice. Proceed immediately to RNA Purification.

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

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

2.4.2. Add 110 µl (2.2X) beads to the RNA sample from Step 2.3.7 and mix thoroughly by pipetting up and down at least 10 times.

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

2.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain RNA.

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

2.4.6. Repeat Step 2.4.5 once for a total of 2 washing steps.

2.4.7. Completely remove residual ethanol, and air dry the beads for ~2-5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of RNA.

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

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

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

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

2.5. RNA Fragmentation and Priming

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

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

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

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

2.5.3. Place the sample on a thermal cycler and incubate the sample at 94°C following the recommendations in Table 2.5.1 below for fragment sizes ~200 nt.

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

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

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

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

2.6. First Strand cDNA Synthesis

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

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

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

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

Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.

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

2.6.4. Proceed directly to Second Strand cDNA Synthesis.

2.7. Second Strand cDNA Synthesis

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

SECOND STRAND SYNTHESIS REACTION	VOLUME
First Strand Synthesis Product (Step 2.6.4)	20 µl
(orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP (10X)	8 µl
(orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

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

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

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

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

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

2.8.3. Incubate for 5 minutes at room temperature.

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

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 5 minutes while the tube is on the magnetic rack with lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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 several times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

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

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

2.9. End Prep of cDNA Library

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

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

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 ng–101 ng	5-fold dilution in Adaptor Dilution Buffer
100 ng–10 ng	25-fold dilution in Adaptor Dilution Buffer
5 ng	200-fold dilution in Adaptor Dilution Buffer

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

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

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

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing 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.

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.

2.10.7. Proceed immediately to Purification of the Ligation Reaction.

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

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

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

2.11.2. Incubate for 10 minutes at room temperature.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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.

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

Check and verify that the concentration of your oligos is 10 µM on the label.

Follow Section 2.12.1A if you are using the following oligos (10 µM):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 2.12.1B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

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

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 (X)/Universal Primer Mix****	10 µl
Total Volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

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 thermocycler 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 Annealing/Extension	98°C 65°C	10 seconds 75 seconds	7–16*, **
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

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

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (see page 76).

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

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

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

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

2.13.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads 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.

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 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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 several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

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

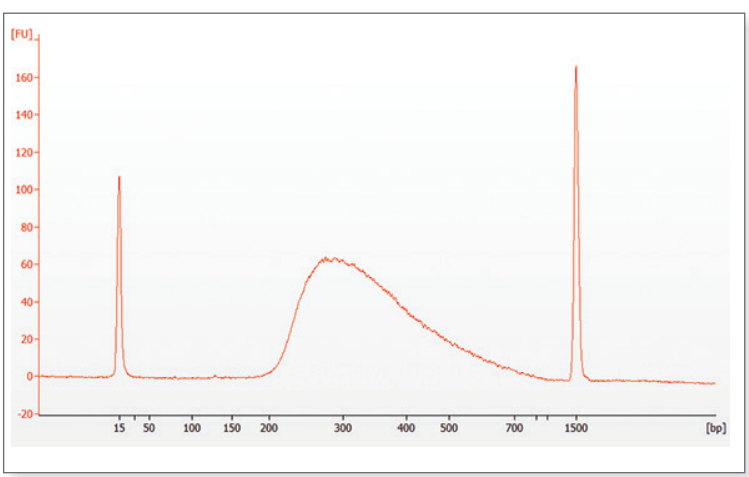
2.14. Assess Library Quality on a Bioanalyzer (Agilent DNA 1000 Chip)

2.14.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip.

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.



3

Protocol for use with FFPE RNA, NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)

NEBNext Ultra II Directional
RNA Library Prep Kit for Illumina
Instruction Manual

Symbols

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.

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

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

RNA Sample Recommendations

RNA Integrity:

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

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Chapter 2.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which does not require fragmentation, follow the library preparation protocol in Chapter 3 (current chapter).

RNA Purity:

Treat the RNA sample with DNase I to remove all traces of DNA. Remove DNase I after treatment. The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Input Amount Requirement

10 ng – 100 ng FFPE RNA in up to 12 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts.

3.1.1. Probe Hybridization to RNA

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

3.1.2. Prepare a RNA/Probe master mix as follows:

RNA/PROBE MASTER MIX	VOLUME
NEBNext rRNA Depletion Solution	1 µl
Probe Hybridization Buffer	2 µl
Total Volume	3 µl

3.1.3. Add 3 µl of the above mix to 12 µl total RNA sample (from Step 3.1.1), resulting in a total volume of 15 µl.

3.1.4. Mix thoroughly by pipetting up and down several times.

3.1.5. Briefly spin down the sample in a microcentrifuge.

3.1.6. Place samples in a thermal cycler, and run the following program with the heated lid set at 105°C. This will take approximately 15-20 minutes to complete:

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

3.1.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to RNase H Digestion Step.

3.2. RNase H Digestion

3.2.1. Assemble the RNAse H master mix **on ice** as follows.

RNASE H MASTER MIX	VOLUME
NEBNext RNase H	2 µl
NEBNext RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	5 µl

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

3.2.3. Briefly spin down the samples in a microcentrifuge.

3.2.4. Add 5 µl of the RNase H master mix to the RNA sample from Step 3.1.7, resulting in a total volume of 20 µl.

3.2.5. Mix thoroughly by pipetting up and down several times.

3.2.6. Incubate the sample in a thermal cycler for **30 minutes at 37°C** with the lid set to 40°C.

3.2.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to DNase I Digestion to prevent non-specific degradation of RNA.

3.3. DNase I Digestion

3.3.1. Assemble DNase I digestion master mix **on ice** in a nuclease-free tube.

DNASE I DIGESTION MASTER MIX	VOLUME
DNase I Reaction Buffer	5 µl
DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	30 µl

3.3.2. Mix thoroughly by pipetting up and down several times.

3.3.3. Briefly spin down the samples in a microcentrifuge.

3.3.4. Add 30 µl of DNase I digestion master mix to 20 µl RNA sample from Step 3.2.7, resulting in a total volume of 50 µl.

3.3.5. Mix thoroughly by pipetting up and down several times.

3.3.6. Incubate the samples in a thermal cycler for **30 minutes at 37°C** with the heated lid set to 40°C.

3.3.7. Briefly spin down the samples in a microcentrifuge, and place on ice. Proceed immediately to RNA purification.

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

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

3.4.2. Add 110 µl (2.2X) beads to the RNA sample from Step 3.3.7 and mix thoroughly by pipetting up and down at least 10 times.

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

3.4.4. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain RNA.

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

3.4.6. Repeat Step 3.4.5 once for a total of 2 washing steps.

3.4.7. Completely remove residual ethanol, and air dry the beads for ~2-5 minutes while the tube is on the magnetic rack with lid open.

Caution: Do not overdry the beads. This may result in lower recovery of RNA.

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

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

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

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

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

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

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

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

3.5.3. Briefly spin down the samples in a microcentrifuge.

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

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

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

3.6. First Strand cDNA Synthesis

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

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 3.5.5)	6 µl
● (lilac) NEBNext First Strand Synthesis Reaction Buffer (5X)	4 µl
● (brown) NEBNext Strand Specificity Reagent	8 µl
● (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

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

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

Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.

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

3.6.4. Proceed directly to Second Strand cDNA Synthesis Reaction.

3.7. Second Strand cDNA Synthesis

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

SECOND STRAND SYNTHESIS REACTION	VOLUME
First Strand Synthesis Product (Step 3.6.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP (10X)	8 µl
● (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

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

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

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

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

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

3.8.3. Incubate for 5 minutes at room temperature.

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

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

3.8.6. Repeat Step 3.8.5 once for a total of 2 washing steps.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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

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

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

3.9. End Prep of cDNA Library

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

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

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 diluted adaptor on ice.**

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

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

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 3.9.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 3.9.4)	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 @ 4°C. We do not recommend premixing 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.

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

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

3.10.7. Proceed immediately to Purification of the Ligation Reaction.

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

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

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

3.11.2. Incubate for 10 minutes at room temperature.

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

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 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.

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

3.11.9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

SAFE STOP **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

⚠ Check and verify that the concentration of your oligos is 10 µM on the label.

⚠ **Follow Section 3.12.1A** if you are using the following oligos (10 µM):
NEBNext Singleplex Oligos for Illumina (NEB #E7350)
NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)
NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)
NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)
NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)
NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

⚠ **Follow Section 3.12.1B** if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

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

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 (X)/Universal Primer Mix****	10 µl
Total Volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

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

3.12.3. Place the tube on a thermocycler 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 Annealing/Extension	98°C 65°C	10 seconds 75 seconds	12–16*, **
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. The recommendation of PCR cycles are based on internal tests for FFPE RNA.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (see page 76).

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

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

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

3.13.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads 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.

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 washing steps.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

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

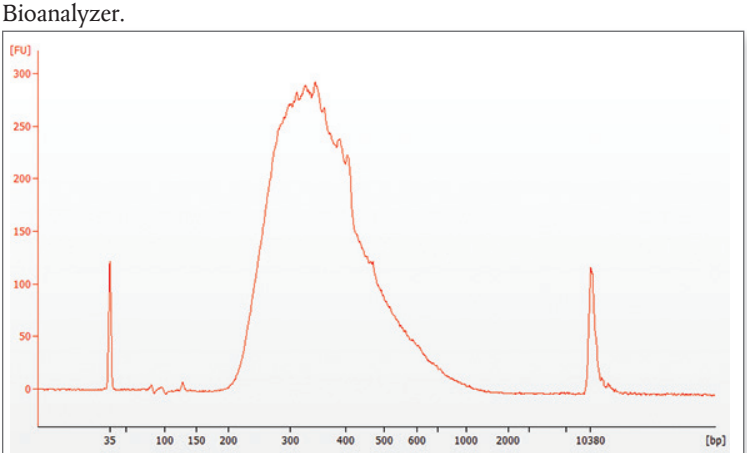
3.14. Assess Library Quality on a Bioanalyzer (Agilent High Sensitivity Chip)

3.14.1. Run 1 µl library on a DNA High Sensitivity Chip.

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 1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 3.13).

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



4

Protocol for use with Purified mRNA or rRNA Depleted RNA

NEBNext Ultra II Directional
RNA Library Prep Kit for Illumina
Instruction Manual

Symbols

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.

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

RNA Sample Recommendations

RNA Integrity:

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. In this case, we recommend that the fragmentation time is empirically determined if the RNA sample is suspected to be low quality. **The following recommendation apply to the total RNA samples only.**

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

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Chapter 4 (current chapter). See Table 4.1.1 for the recommended the fragmentation times.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Chapter 5.

RNA Purity:

The RNA sample should be free of DNA, salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Input Amount Requirement

1 ng – 100 ng purified mRNA or rRNA depleted RNA that is **quantified after the purification**. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

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

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

4.1. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 4.1.1.

4.1.1. Assemble the fragmentation and priming reaction **on ice** in a nuclease-free tube by adding the following components:

FRAGMENTATION AND PRIMING MIX	VOLUME
Purified mRNA or rRNA Depleted RNA	5 µl
(lilac) NEBNext First Strand Synthesis Reaction Buffer (5X)	4 µl
(lilac) Random Primers	1 µl
Total Volume	10 µl

4.1.2. Mix thoroughly by pipetting up and down several times.

4.1.3. Place the sample in a thermal cycler and incubate the sample at 94°C following the recommendations in Table 4.5.1 below for fragment sizes ~200 nt.

Table 4.1.1 Suggested fragmentation times based on RIN value of RNA input.

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

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

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

4.2 First Strand cDNA Synthesis Reaction

4.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 4.1.4:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 4.1.4)	10 µl
(brown) NEBNext Strand Specificity Reagent	8 µl
(lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

4.2.2. Mix thoroughly by pipetting up and down several times.

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

Note: If you are following recommendations in Appendix A (Chapter 6), for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.

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

4.2.4. Proceed directly to Second Strand cDNA Synthesis.

4.3. Second Strand cDNA Synthesis

4.3.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 4.2.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First Strand Synthesis Product (Step 4.2.4)	20 µl
● (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
● (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

4.3.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down several times.

4.3.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C.

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

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

4.4.2. 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.

4.4.3. Incubate for 5 minutes at room temperature.

4.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on 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.

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

4.4.6. Repeat Step 4.4.5 once for a total of 2 washing steps.

4.4.7. Air dry the beads for **5 minutes** while the tube is on the magnetic rack with lid open.

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

4.4.8. 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 several times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

4.4.9. 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.

4.5. End Prep of cDNA Library

4.5.1. Assemble the end prep reaction **on ice** by adding the following components to second strand synthesis product from Step 4.4.9.

END PREP REACTION	VOLUME
Second strand cDNA Synthesis Product (Step 4.4.9)	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

4.5.2. 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.

4.5.3. 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

4.5.4. Proceed immediately to Adaptor Ligation.

4.6. Adaptor Ligation

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

PURIFIED RNA	DILUTION REQUIRED
100 ng–11 ng	5-fold dilution in Adaptor Dilution Buffer
10 ng–1 ng	25-fold dilution in Adaptor Dilution Buffer

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

4.6.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 4.5.4:

LIGATION REACTION	VOLUME PER ONE LIBRARY
End Prepped DNA (Step 4.5.4)	60 µl
Diluted Adaptor (Step 4.6.1)	2.5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

4.6.3. 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.**

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

4.6.5. Add 3 µl ● (blue) USER Enzyme to the ligation mixture from Step 4.6.4, resulting in total volume of 96.5 µl.

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

4.6.7. Proceed immediately to Purification of the Ligation Reaction.

4.7 Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

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

4.7.1. 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.

4.7.2. Incubate for 10 minutes at room temperature.

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

4.7.4. 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.

4.7.5. Repeat Step 4.7.4 once for a total of 2 washing steps.

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

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

4.7.8. 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 several times, and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

4.7.9. 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.

4.8. PCR Enrichment of Adaptor Ligated DNA

⚠ Note: Check and verify that the concentration of your oligos is 10 µM on the label.

⚠ **Follow Section 4.8.1A** if you are using the following oligos (10 µM):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 4.8.1B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

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

4.8.1A Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.7.9)	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

4.8.1B Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 4.7.9)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/Universal Primer Mix****	10 µl
Total Volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7710, #E7730 or #E7500) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

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

4.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 4.8.3A and Table 4.8.3B):

Table 4.8.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	6–13*, **
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

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

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (see page 76).

Table 4.8.3B: Recommended PCR cycles based on input amount:

PURIFIED mRNA or rRNA DEPLETED RNA (QUANTIFIED AFTER PURIFICATION)	RECOMMENDED PCR CYCLES
100 ng	6–7
50 ng	7–8
10 ng	9–10
1 ng	12–13

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

4.9. Purification of the PCR Reaction 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 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.

4.9.3. Incubate for 5 minutes at room temperature.

4.9.4. 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.

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 washing steps.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

4.9.8. 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 several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

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

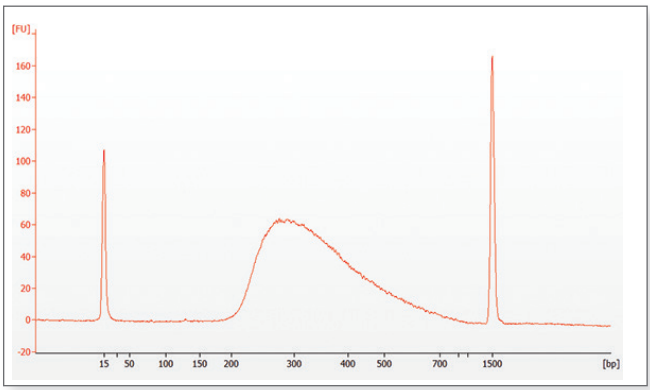
4.10. Assess Library Quality on a Bioanalyzer (Agilent DNA 1000 Chip)

4.10.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip.

4.10.2. 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 4.9.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 4.9).

Figure 4.10.1: Example of RNA library size distribution on a Bioanalyzer.



5

Protocol for use with rRNA Depleted FFPE RNA

NEBNext Ultra II Directional RNA Library Prep Kit for Illumina Instruction Manual

Symbols

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.

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

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

RNA Sample Recommendations

RNA Integrity:

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. **The following recommendation apply to the total RNA samples only.**

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

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Chapter 4.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which does not require fragmentation, follow the library preparation protocol in Chapter 5 (current chapter).

RNA Purity:

The RNA sample should be free of DNA, salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Starting Material: 1 ng – 100 ng alternative rRNA depleted FFPE RNA that is **quantified after rRNA depletion**. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

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

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

PRIMING REACTION	VOLUME
rRNA Depleted FFPE RNA (1 ng–100 ng)	5 µl
(lilac) Random Primers	1 µl
Total Volume	6 µl

5.1.2. Mix thoroughly by pipetting up and down several times.

5.1.3. Briefly spin down the samples in a microcentrifuge.

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

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

Hold at 4°C

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

5.2. First Strand cDNA Synthesis

5.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Primed RNA (Step 5.1.5)	6 µl
(lilac) NEBNext First Strand Synthesis Reaction Buffer (5X)	4 µl
(brown) NEBNext Strand Specificity Reagent	8 µl
(lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

5.2.2. Keeping the tube on ice, mix thoroughly by pipetting up and down several times.

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

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

5.2.4. Proceed directly to Second Strand cDNA Synthesis.

5.3. Second Strand cDNA Synthesis

5.3.1 Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 5.2.4:

SECOND STRAND SYNTHESIS REACTION	VOLUME
First Strand Synthesis Product (Step 5.2.4)	20 µl
(orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP (10X)	8 µl
(orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

5.3.2 Keeping the tube on ice, mix thoroughly by pipetting up and down several times.

5.3.3 Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C.

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

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

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

5.4.3. Incubate for 5 minutes at room temperature.

5.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on 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.

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

5.4.6. Repeat Step 5.4.5 once for a total of 2 washing steps.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

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

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

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

5.5. End Prep of cDNA Library

5.5.1. Assemble the end prep reaction **on ice** by adding the following components to the second strand synthesis product from Step 5.4.9.

END PREP REACTION	VOLUME
Second Strand Synthesis Product (Step 5.4.9)	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

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

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

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

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

5.5.4. Proceed immediately to Adaptor Ligation.

5.6. Adaptor Ligation

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

PURIFIED RNA	DILUTION REQUIRED
100 ng–11 ng	5-fold dilution in Adaptor Dilution Buffer
10 ng–1 ng	25-fold dilution in Adaptor Dilution Buffer

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

5.6.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 5.5.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 5.5.4)	60 µl
Diluted Adaptor (Step 5.6.1)	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 @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

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

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

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

5.6.5. Add 3 µl ● (blue) USER Enzyme to the ligation mixture from Step 5.6.4, resulting in total volume of 96.5 µl.

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

5.6.7. Proceed immediately to Purification of Ligation Reaction.

5.7. Purification of Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

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

5.7.2. Incubate for 10 minutes at room temperature.

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

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

5.7.5. Repeat Step 5.7.4 once for a total of 2 washing steps.

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

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

5.7.8. 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 several times. Quickly spin the tube and incubate for 2 minutes at room temperature. Put the tube on the magnet until the solution is clear.

5.7.9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

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

5.8. PCR Enrichment of Adaptor Ligated DNA

⚠ Check and verify that the concentration of your oligos is 10 µM on the label.

⚠ **Follow Section 5.8.1A** if you are using the following oligos (10 µM):
NEBNext Singleplex Oligos for Illumina (NEB #E7350)
NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)
NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)
NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)
NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)
NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

⚠ **Follow Section 5.8.1B** if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

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

5.8.1A Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor ligated DNA (Step 5.7.9)	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

5.8.1B Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor ligated DNA (Step 5.7.9)	15 µl
● (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X)/Universal Primer Mix****	10 µl
Total Volume	50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

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

5.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 5.8.3A and Table 5.8.3B):

Table 5.8.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	7–13*, **
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. The recommendation of PCR cycles are based on internal tests for FFPE RNA.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (see page 76).

Table 5.8.3B: Recommended PCR cycles based on input amount:

rRNA DEPLETED FFPE RNA (QUANTIFIED AFTER rRNA DEPLETION)	RECOMMENDED PCR CYCLES
100 ng	7-8
50 ng	8-9
10 ng	10-11
1 ng	13-14

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

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

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

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

5.9.3. Incubate for 5 minutes at room temperature.

5.9.4. 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.

5.9.5. 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.

5.9.6. Repeat Step 5.9.5 once for a total of 2 washing steps.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA.

5.9.8. 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 several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

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

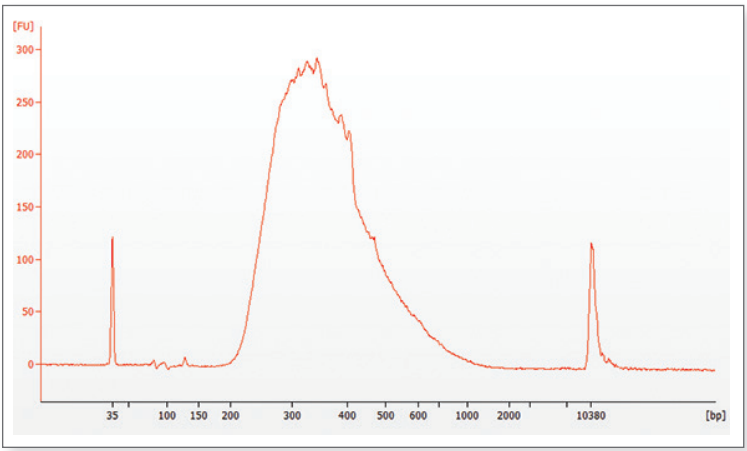
5.10. Assess Library Quality on a Bioanalyzer (Agilent High Sensitivity Chip)

5.10.1. Run 1 µl library on a DNA High Sensitivity Chip.

5.10.2. 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 5.9.9) to 50 µl with 0.1X TE Buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 5.9).

Figure 5.10.1: Example of FFPE RNA library size distribution on a Bioanalyzer.



6

Appendix A

NEBNext Ultra II Directional
RNA Library Prep Kit for Illumina
Instruction Manual

6.1. Fragmentation

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

Modified fragmentation times for longer RNA inserts.

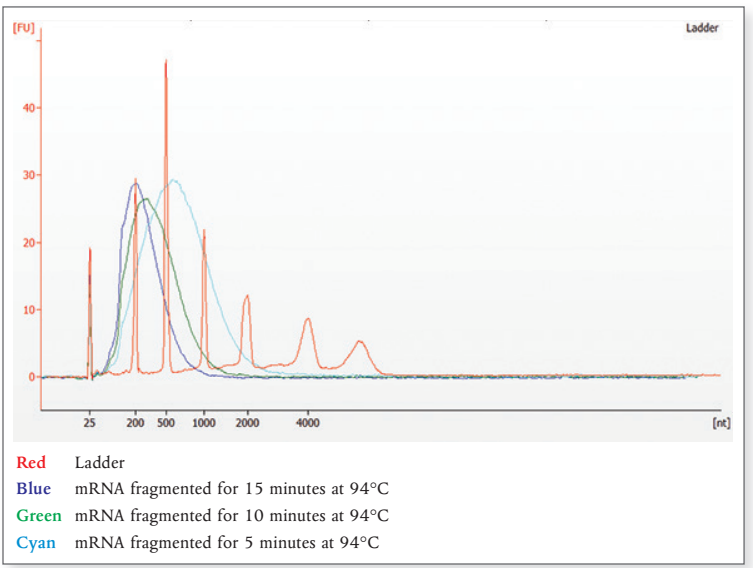


Figure 6.1: *Modified fragmentation times for longer RNA inserts. Bioanalyzer traces of RNA as shown in an RNA Pico Chip. mRNA isolated from Universal Human Reference RNA and fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 5, 10 or 15 minutes, and purified using 2.2X volume of Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads.* ***For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes.***

6.2. Size Selection of Adaptor Ligated DNA

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

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

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

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

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

LIBRARY PARAMETER	APPROXIMATE INSERT SIZE	300 bp	400 bp	450 bp
	Approx. Final Library Size	420 bp	520 bp	570 bp
BEAD VOLUME TO BE ADDED (µl)	1st Bead Selection	25	20	15
	2nd Bead Selection	10	10	10

Note: Any differences in insert sizes between the Agilent Bioanalyzer and that obtained from paired end sequencing can be attributed to the higher clustering efficiency of smaller sized fragments.

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

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

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

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

6.2.5. Add 10 µl resuspended beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

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

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

6.2.8. Repeat Step 6.1.7 once.

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

Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

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

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

6.3. PCR Enrichment of Size-selected Libraries

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

⚠ Check and verify that the concentration of your oligos is 10 µM on the label.

⚠ **Follow Section 6.3.1A** if you are using the following oligos (10 µM):

NEBNext Singleplex Oligos for Illumina (NEB #E7350)

NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)

NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)

NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)

NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

⚠ **Follow Section 6.3.1B** if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

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

6.3.1A Forward and Reverse Primers Separate

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

6.3.1B Forward and Reverse Primers Combined

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

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

*** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.

**** The primers are provided in NEBNext Multiplex Oligos for Illumina (NEB #E6609). Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

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

6.3.3. Place the tube in a thermocycler 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 Annealing/Extension	98°C 65°C	10 seconds 75 seconds	variable*, **
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 page 76).

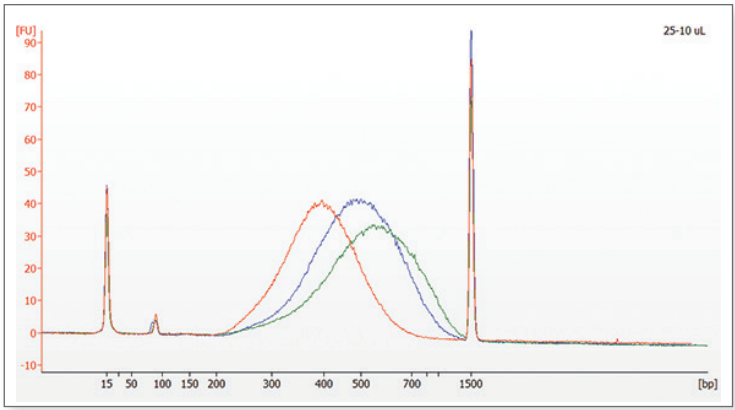


Figure 6.3: *Bioanalyzer traces of size selected DNA libraries. 50 ng mRNA was fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 15, 10 or 5 minutes. Libraries were size-selected as described in Table 6.2, then amplified by PCR, and run on Agilent Bioanalyzer DNA 1000 chip. Fragmentation times and corresponding size selection conditions are shown in the table below.*

Table 6.3:

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

7

Troubleshooting Guide

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OBSERVATIONS	POSSIBLE CAUSES	EFFECT	SUGGESTED SOLUTIONS
Presence of Bioanalyzer peaks <85 bp (Figure 7.1)	• Presence of Primers remaining after PCR clean up	Primers cannot cluster or be sequenced, but can bind to flowcell and reduce cluster density	• Clean up PCR reaction again with 1.0X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield)
Presence of ~127 bp adaptor-dimer Bioanalyzer peak (Figure 7.1)	• Addition of non-diluted adaptor • RNA input was too low • RNA was over fragmented or lost during fragmentation • Inefficient Ligation	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 1.0X SPRIselect Beads or NEBNext Sample Purification Beads (second clean up may result in reduction of library yield).
Presence of additional Bioanalyzer peak at higher molecular weight than the expected library size (~ 1,000 bp) (Figure 7.2)	• PCR artifact (over-amplification). Represents single-stranded library products that have self-annealed. If the PCR cycle number (or PCR input amount) is too high; in the late cycles of PCR the primers become limiting. Therefore, the adaptor sequences on either end of the fragment anneal to each other. This creates heteroduplexes with different insert sequences that run slower in the Bioanalyzer.	If ratio is low compared to library, may not be a problem for sequencing	• Reduce number of PCR cycles.
Broad library size distribution	• Under-fragmentation of the RNA	Library size will contain longer insert sizes	• Increase RNA fragmentation time

Figure 7.1:

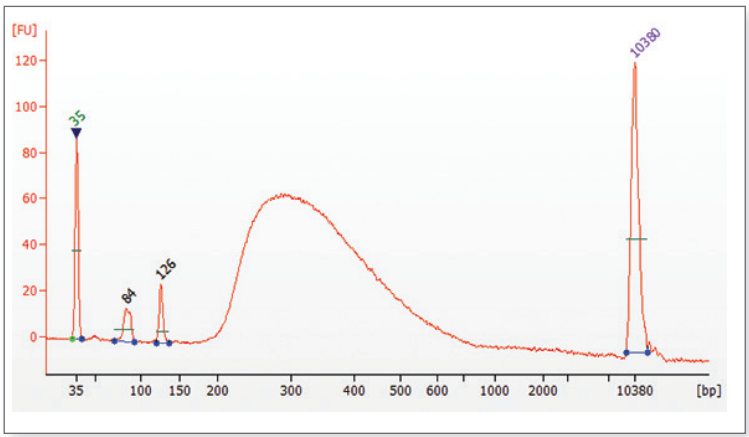
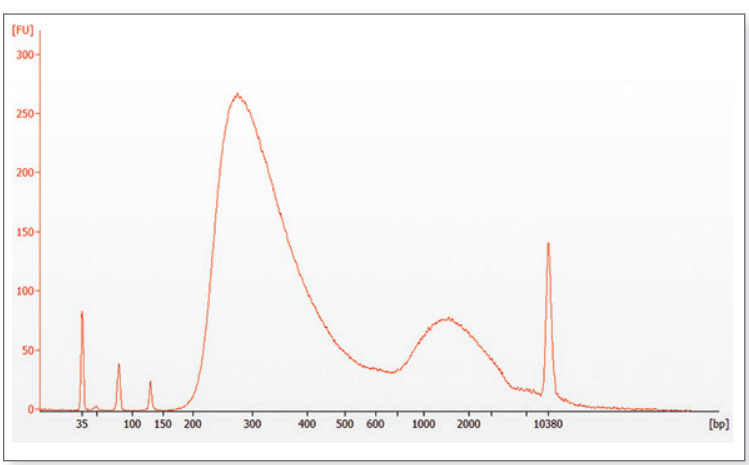


Figure 7.2:



8

Frequently Asked Questions (FAQs)

NEBNext Ultra II Directional
RNA Library Prep Kit for Illumina
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Q. What is the difference between the NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760) and the NEBNext Ultra II RNA Library Prep Kit (NEB #E7770)?

A. The NEBNext Ultra II Directional RNA Library Prep workflow preserves information about RNA strand orientation while the NEBNext Ultra II RNA Library Prep does not. The NEBNext Ultra II Directional RNA Library Prep contains dUTP in the second strand synthesis buffer that allows labeling the second strand cDNA and subsequent excision with USER enzyme.

Q. What is the starting material I need to use when preparing libraries using the NEBNext Ultra II Directional RNA kit?

A. The starting material is Total RNA (5/10 ng-1 µg); previously isolated mRNA (1-100 ng) or ribosomal-depleted RNA (1-100 ng).

Q. Which kit can I use to isolate Poly (A) mRNA from total RNA?

A. To isolate poly (A) mRNA from total RNA use the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490).

Q. Which kit can I use to deplete rRNA from human, mouse or rat?

A. To deplete rRNA from human, mouse or rat, use the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310).

Q. Does the kit provide adaptor and primers?

A. No. The primers are provided in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600 or #E6609) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

Kit Components

Each set of reagents is functionally validated and compared to the previous lot through construction of libraries using the minimum and maximum amount of Universal Human Reference Total RNA. The previous and current lots are sequenced together on the same Illumina flow cell and compared across various sequence metrics including individual transcript abundances, 5´→3´ transcript coverage, and fraction of reads mapping to the reference

NEB #E7760S Table of Components

NEB #	PRODUCT NAME	VOLUME
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7421A	NEBNext First Strand Synthesis Reaction Buffer	0.192 ml
E7422A	Random Primers	0.048 ml
E7425A	NEBNext Second Strand Synthesis Enzyme Mix	0.096 ml
E7426A	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.192 ml
E7428A	NEBNext USER Enzyme	0.072 ml
E7646A	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
E7647A	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.720 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml
E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
E7762A	NEBNext Adaptor Dilution Buffer	2.4 ml
E7763A	TE Buffer (0.1X)	2.78 ml
E7764A	Nuclease-free Water	1.25 ml
E7766A	NEBNext Strand Specificity Reagent	0.192 ml

Kit Components (cont.)

NEB #E7760L Table of Components

NEB #	PRODUCT NAME	VOLUME
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7421AA	NEBNext First Strand Synthesis Reaction Buffer	0.768 ml
E7422AA	Random Primers	0.192 ml
E7425AA	NEBNext Second Strand Synthesis Enzyme Mix	0.384 ml
E7426AA	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.768 ml
E7428AA	NEBNext USER Enzyme	0.288 ml
E7646AA	NEBNext Ultra II End Prep Enzyme Mix	0.288 ml
E7647AA	NEBNext Ultra II End Prep Reaction Buffer	0.672 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	2 x 1.2 ml
E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
E7762AA	NEBNext Adaptor Dilution Buffer	9.6 ml
E7763AA	TE Buffer (0.1X)	11.5 ml
E7764AA	Nuclease-free Water	5.7 ml
E7766AA	NEBNext Strand Specificity Reagent	0.768 ml

Kit Components (cont.)

NEB #E7765S Table of Components

NEB #	PRODUCT NAME	VOLUME
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7421A	NEBNext First Strand Synthesis Reaction Buffer	0.192 ml
E7422A	Random Primers	0.048 ml
E7425A	NEBNext Second Strand Synthesis Enzyme Mix	0.096 ml
E7426A	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.192 ml
E7428A	NEBNext USER Enzyme	0.072 ml
E7646A	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
E7647A	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.720 ml
E7649A	NEBNext Ultra II Q5 Master Mix	0.6 ml
E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
E7762A	NEBNext Adaptor Dilution Buffer	2.4 ml
E7763A	TE Buffer (0.1X)	2.78 ml
E7764A	Nuclease-free Water	1.25 ml
E7766A	NEBNext Strand Specificity Reagent	0.192 ml
E7767S	NEBNext Sample Purification Beads	8.65 ml

Kit Components (cont.)

NEB #E7765L Table of Components

NEB #	PRODUCT NAME	VOLUME
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7421AA	NEBNext First Strand Synthesis Reaction Buffer	0.768 ml
E7422AA	Random Primers	0.192 ml
E7425AA	NEBNext Second Strand Synthesis Enzyme Mix	0.384 ml
E7426AA	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.768 ml
E7428AA	NEBNext USER Enzyme	0.288 ml
E7646AA	NEBNext Ultra II End Prep Enzyme Mix	0.288 ml
E7647AA	NEBNext Ultra II End Prep Reaction Buffer	0.672 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	3 x 0.960 ml
E7649AA	NEBNext Ultra II Q5 Master Mix	2 x 1.2 ml
E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
E7762AA	NEBNext Adaptor Dilution Buffer	9.6 ml
E7763AA	TE Buffer (0.1X)	11.5 ml
E7764AA	Nuclease-free Water	5.7 ml
E7766AA	NEBNext Strand Specificity Reagent	0.768 ml
E7767L	NEBNext Sample Purification Beads	4 x 8.65 ml

Revision History:

Revision #	Description
1.0	N/A

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