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

NEB #E7420S/L 24/96 reactions

Version 10.1_5/20

## Table of Contents



## The Library Prep Kit Includes

*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7420S) and 96 reactions (NEB #E7420L). All reagents should be stored at -20°C.*

- (pink) NEBNext First Strand Synthesis Reaction Buffer
- (pink) Random Primers
- (pink) ProtoScript II Reverse Transcriptase
- (pink) Murine RNase Inhibitor
- (orange) NEBNext Second Strand Synthesis Enzyme Mix
- (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix
- (green) NEBNext End Prep Enzyme Mix
- (green) NEBNext End Repair Reaction Buffer (10X)
- (red) Blunt/TA Ligase Master Mix

Nuclease-free Water

- (blue) NEBNext Q5® Hot Start HiFi PCR Master Mix
- (blue) NEBNext USER® Enzyme

## Required Materials Not Included

- NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) or NEBNext rRNA Depletion Kit (NEB #E6310)
- NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina or customer supplied oligos
- NEBNext. Magnetic Separation Rack (NEB #S1515)
- 80% Ethanol (freshly prepared)
- 0.1X TE, pH 8.0
- 10 mM Tris-HCl, pH 7.5–8.0
- 10 mM NaCl (optional)
- Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Actinomycin D (Sigma #A1410, dissolved in dimethylsulfoxide[DMSO] to 5 µg/µl). See page 5, 14 or 22 for details.
- Additional required materials not included for use with NEBNext rRNA Depletion Kit (NEB #E6310): Agencourt RNAClean® XP (Beckman Coulter, Inc., Cat #A63987).

## Applications

The NEBNext Ultra RNA Directional Library Prep Kit for Illumina contains enzymes and buffers that are ideally suited for cDNA library preparation for next-generation sequencing. Each of these components must pass rigorous quality control standards and are lot controlled, both individually and as a set of reagents.

**Lot Control:** The lots provided in the NEBNext Ultra RNA Directional Library Prep Kit for Illumina are managed separately and are qualified by additional functional validation. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria in the additional quality controls listed on each individual component page.

**Functionally Validated:** Each set of reagents is functionally validated together through construction and sequencing of a transcriptome library on an 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.

# Section 1
# Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)

**Symbols**

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


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

●

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

*The protocol has been optimized using high quality Universal Human Reference Total RNA. For PolyA mRNA selection, high quality RNA with RIN score > 7 (measured by Agilent Bioanalyzer®) is required.*

**RNA Sample Recommendations:**
The RNA sample should be free of salts (e.g., $Mg^{2+}$, or guanidinium salts, divalent cation chelating agents (e.g., EDTA or EGTA) or organics (e.g., phenol or ethanol).

Treat the RNA sample with DNase I to remove all traces of DNA. Remove DNase I after treatment.

**AMPure XP Beads are required throughout the protocol. Allow beads to reach room temperature prior to use.**

**Starting Material:**
Total RNA (100 ng–1 µg) quantified by Bioanalyzer.

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

### 1.1. Preparation of First Strand Reaction Buffer and Random Primer Mix

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

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

You can prepare the first strand synthesis reaction buffer later in the protocol, but it is important that it is ready before the elution in Step 1.2.37. The beads should not be allowed to dry out.

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

**Note: Keep the mix on ice until mRNA is purified.**

### 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. Aliquot 20 µl of NEBNext Oligo $d(T)_{25}$ beads into a nuclease-free 0.2 ml PCR tube.

1.2.3. Wash the beads by adding 100 µl of RNA Binding Buffer (2X) to the beads. Pipette the entire volume up and down 6 times to mix thoroughly.

1.2.4. Place the tubes on the magnetic rack at room temperature for 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. Repeat Steps 1.2.3.–1.2.6.

1.2.8. Resuspend the beads in 50 µl of RNA Binding Buffer (2X) and add the 50 µl of total RNA sample from Step 1.2.1.

1.2.9. Place the tube on a thermal cycler and close the lid. Heat the sample at 65°C for 5 minutes and hold at 4°C to denature the RNA and facilitate binding of the poly-A mRNA to the beads.

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

1.2.11. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.

1.2.12. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.

1.2.13. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.

1.2.14. Incubate for 5 minutes at room temperature to allow the RNA to bind to the beads.

1.2.15. Place the tube on the magnetic rack at room temperature for 2 minutes to separate the poly-A mRNA bound to the beads from the solution.

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. 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 for 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 to each tube. Gently pipette the entire volume 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 to elute the Poly-A 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. Gently pipette the entire volume up and down 6 times to mix thoroughly.

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

1.2.28. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.

1.2.29. Incubate for 5 minutes at room temperature to allow the RNA to bind to the beads.

1.2.30. Place the tube on the magnetic rack at room temperature for 2 minutes.

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

1.2.32. Remove the tube from the magnetic rack.

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

1.2.34. Place the tube on the magnetic rack at room temperature for 2 minutes.

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

**Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip remove all of the wash buffer. Caution: Do not disturb beads that contain the mRNA.**

1.2.36. Remove the tube from the magnetic rack.

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**Note: For RNA insert sizes > 200 nt, refer to Section 5 (Appendix A) for recommended fragmentation times in Step 1.2.37.**

1.2.37. Elute mRNA from the beads by adding 15.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer mix prepared in Step 1.1.1. incubating the sample at 94°C for 15 minutes. Immediately, place the tubes on the magnetic rack.

1.2.38. Collect the purified mRNA by transferring 13.5 µl of the supernatant to a clean nuclease-free PCR Tube.

1.2.39. Place the tube on ice and proceed directly to first strand cDNA synthesis.

### 1.3. First Strand cDNA Synthesis

*Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.*

*Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.*

1.3.1. To the fragmented and primed mRNA (13.5 µl from above Step 1.2.38) add the following components and mix by gentle pipetting:.

| COMPONENT | VOLUME |
|---|---|
| ○ (pink) Murine RNase Inhibitor | 0.5 µl |
| Actinomycin D (0.1 µg/µl) | 5 µl |
| ○ (pink) ProtoScript II Reverse Transcriptase | 1 µl |
| Total Volume | 20 µl |

**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 in Step 1.3.2.**

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

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

1.3.3. Immediately, perform Second Strand cDNA Synthesis reaction.

### 1.4. Perform Second Strand cDNA Synthesis

1.4.1. Add the following reagents to the First Strand Synthesis reaction (20 µl).

| COMPONENT | VOLUME |
|---|---|
| Nuclease-free Water | 48 µl |
| • (orange) Second Strand Synthesis Reaction Buffer | 8 µl |
| • (orange) Second Strand Synthesis Enzyme Mix | 4 µl |
| Total Volume | 80 µl |

1.4.2. Mix thoroughly by gentle pipetting.

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

### 1.5. Purify the Double-stranded cDNA using 1.8X Agencourt AMPure XP Beads

1.5.1. Vortex AMPure XP Beads to resuspend.

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

1.5.3. Incubate for at 5 minutes at room temperature.

1.5.4. Quickly spin the tube in a microcentrifuge to collect any sample from the sides of the tube. 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.

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

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

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

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

1.5.8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 µl 0.1X TE Buffer or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down. Quickly 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 55.5 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. Mix the following components in a sterile nuclease-free tube:

| END PREP REACTION | VOLUME |
|---|---|
| Purified double stranded cDNA (Step 1.5.9) | 55.5 µl |
| • (green) NEBNext End Repair Reaction Buffer | 6.5 µl |
| • (green) NEBNext End Prep Enzyme Mix | 3 µl |
| Total Volume | 65 µl |

1.6.2. 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

1.6.3. Proceed immediately to Adaptor Ligation.

### 1.7. Perform Adaptor Ligation

**Dilute the • (red) NEBNext Adaptor* for Illumina (15 µM) to 1.5 µM with a 10-fold dilution (1:9) with 10 mM Tris-HCl and 10 mM NaCl for immediate use.**

1.7.1. Add the following components directly to the end prep reaction mixture. **(Caution: do not pre-mix the components to prevent adaptor-dimer formation):**

| COMPONENT | VOLUME |
|---|---|
| End Prep Reaction | 65 µl |
| • (red) Blunt/TA Ligase Master Mix | 15 µl |
| Diluted NEBNext Adaptor* | 1 µl |
| Nuclease-free Water | 2.5 µl |
| Total Volume | 83.5 µl |

*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. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

1.7.3. Incubate 15 minutes at 20°C in a thermal cycler. Turn off the heated lid on the thermal cycler.

Note: This step is only required for use with NEBNext Adaptors. USER Enzyme can be found in the NEBNext SinglePlex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

**A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while purifying the ligation reaction. Once thawed, gently mix by inverting the tube several times.**

### 1.8. Purify the Ligation Reaction Using AMPure XP Beads

**Note: If you are selecting for larger insert size libraries (> 200 nt) follow the size selection recommendations in Appendix A, Section 4.**

1.8.1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl.

**Note: X refers to the original sample volume of 100 µl from the above step.**

1.8.2. Add 100 µl (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

1.8.3. Incubate for 5 minutes at room temperature.

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

1.8.5. 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.

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

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

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

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

1.8.9. Remove the tube from the rack. Elute DNA target from the beads with 52 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

1.8.10. Transfer 50 µl supernatant to a clean PCR tube. Discard beads.

1.8.11. To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

1.8.12. Incubate for 5 minutes at room temperature.

1.8.13. 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).

1.8.14. 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.

1.8.15. Repeat Step 1.8.14 once for a total of 2 washing steps.

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

1.8.17. Completely remove the residual ethanol, and air dry 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 target.**

1.8.18. Remove the tube from the rack. Elute DNA targtet from the beads with 19 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 minutes at room temperature.Put the tube in the magnetic rack until the solution is clear.

1.8.19. Without disturbing the bead pellet, transfer 17 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

## 1.9. PCR Enrichment of Adaptor Ligated DNA

**Follow Section 1.9A if you are using the following oligos (10 µM primer):**

**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 1.9B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).**

### 1.9.1A. PCR Library Enrichment

1.9.1A.1. To the cDNA (17 µl) from Step 1.8.19 add the following components and mix by gentle pipetting:

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| • (blue) NEBNext USER Enzyme | 3 µl |
| • (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 µl |
| • (blue) Index (X) Primer/i7 Primer*, ** | 2.5 µl |
| • (blue) Universal PCR Primer/i5 Primer*, *** | 2.5 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 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.

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

1.9.1A.2. PCR Cycling Conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| USER Digestion | 37°C | 15 minutes | 1 |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 seconds | 12–15*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid over-amplification.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

1.9.1A.3. Proceed to Section 1.10 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

### 1.9.1B. PCR Library Enrichment

1.9.1B.1. To the cDNA (17 µl) from Step 1.8.19 add the following components and mix by gentle pipetting:

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| • (blue) NEBNext USER Enzyme | 3 µl |
| • (blue) Index/Universal Primer Mix* | 5 µl |
| • (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 µl |
| Total Volume | 50 µl |

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Refer to NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

1.9.1B.2. PCR Cycling Conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| USER Digestion | 37°C | 15 minutes | 1 |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 seconds | 12–15*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid over-amplification.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

1.9.1B.3. Proceed to Section 1.10 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

### 1.10. Purify the PCR Reaction using Agencourt AMPure XP Beads

**Note: X refers to the original sample volume from the above step.**

1.10.1. Vortex Agencourt AMPure XP Beads to resuspend.

1.10.2. Add 45 µl (0.9X) of resuspended Agencourt AMPure XP 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 target.**

1.10.8. Remove the tube from the rack. Elute the DNA target from the beads into 23 µl 0.1X TE. 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 it 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 High Sensitivity Chip)

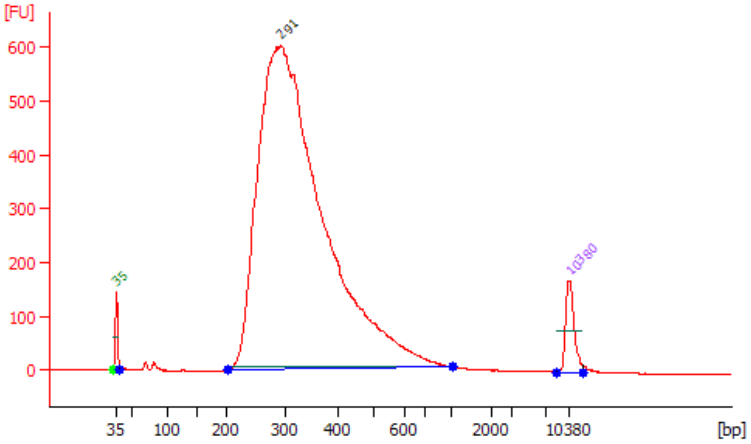
1.11.1. Dilute 2–3 µl of the library in 10 mM Tris or 0.1X TE.

1.11.2. Run 1 µl in 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 shown in the Bioanalyzer traces, bring up the sample volume (Step 1.10.9) to 50 µl exactly with nuclease-free water and repeat the AMPure XP Bead clean up step (Section 1.10).**

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



## Section 2
## Protocol for use with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)

**Symbols**

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

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

●

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

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

**RNA Sample Recommendations**

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).

Treat the RNA sample with DNase I to remove all traces of DNA. Remove DNaseI after treatment.

**Typical Yield of rRNA-depleted RNA from a Reaction**

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

**RNA Input**

100 ng to 1 µg total RNA in up to 12 µl total volume.

*Note: The NEBNext rRNA Depletion Kit can be used with as low as 10 ng total RNA, however, for RNAseq samples we recommend using total RNA inputs 100 ng–1 µg to increase library complexity and reduce sequencing duplication rates.*

*Assess quality of the input RNA by running input RNA on an Agilent Bioanalyzer to determine the RIN number (RIN). Highly degraded samples (RIN #1–2) (FFPE) or partially degraded samples (RIN #2–7) will require different fragmentation times (Section 2.5).*

### 2.1. Hybridize the Probes to the RNA

2.1.1. 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 |

2.1.2. Add 3 µl of the above mix to 12 µl total RNA sample.

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

2.1.4. Spin down briefly in a tabletop centrifuge, and immediately proceed to the next step

2.1.5. 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.

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

2.1.6. 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. On ice, prepare a master mix according to the following, and mix by pipetting up and down; use immediately.

| 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. Add 5 µl of the above mix to the RNA sample from Step 2.1.6.

2.2.3. Mix by pipetting up and down.

2.2.4. Place samples in a thermal cycler (with lid at 40°C) and incubate at 37°C for 30 minutes.

2.2.5. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent non-specific degradation of RNA.

**2.3. DNase I Digestion**

2.3.1. On ice, prepare a DNase I Digestion Master Mix according to the following table, and mix by pipetting up and down; use immediately

| 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. Add 30 µl of the above mix to the RNA sample from Step 2.2.5, and mix by pipetting up and down.

2.3.3. Place samples in a thermal cycler (with lid at 40°C) and incubate at 37°C for 30 minutes.

2.3.4. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

**2.4 RNA Purification after rRNA Depletion Using Agencourt RNAClean® XP**

2.4.1. Add 2.2X (110 µl) Agencourt RNAClean XP Beads to the RNA sample from the previous section (Step 2.3.4) and mix by pipetting up and down.

2.4.2. Incubate samples on ice for 15 minutes.

2.4.3. Place the tube on an appropriate magnetic rack to separate beads from the supernatant.

2.4.4. When the solution is clear (about 5 minutes), discard the supernatant.

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 washes.

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

2.4.8. Completely remove the residual ethanol, and air dry beads.

2.4.9. Remove the tube from the magnetic rack. Elute RNA from the beads with 8 µl nuclease-free water.

2.4.10. Mix well by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.

2.4.11. Transfer 6 µl of the supernatant to a clean PCR tube.

2.4.12. Place the sample on ice and proceed to Section 2.5.

**2.5. RNA Fragmentation, Priming and First Strand cDNA Synthesis**

⚠

**RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 2.1. Follow protocol in 2.5A to set up the reaction. For highly degraded RNA (FFPE Samples) which do not require fragmentation proceed to Step 2.5B.**

### 2.5A RNA Fragmentation and Priming Starting from Intact or Partially Degraded RNA:

2.5A.1. Set up the following reaction and mix by gentle pipetting:

| FRAGMENTATION AND PRIMING REACTION | VOLUME |
|---|---|
| Ribosome Depleted RNA | 5 µl |
| ○ (pink) NEBNext First Strand Synthesis Reaction Buffer | 4 µl |
| ○ (pink) Random Primers | 1 µl |
| Total Volume | 10 µl |

2.5A.2. Incubate the sample at 94°C following the recommendations in Table 2.1 for fragments sizes ~200 nt.

Table 2.1. Suggested fragmentation times based on RIN number 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 |

**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.**

2.5A.3. Transfer the tube to ice.

**2.5A.4. First Strand cDNA Synthesis**

*Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.*

*Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.*

2.5A.5. To the fragmented and primed mRNA from Step 2.5A.3 (10 µl), add the following components and mix by gentle pipetting:

| FIRST STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| ○ (pink) Murine RNase Inhibitor | 0.5 µl |
| Actinomycin D (0.1 µg/µl) | 5 µl |
| ○ (pink) ProtoScript II Reverse Transcriptase | 1 µl |
| Nuclease-free Water | 3.5 µl |
| Total Volume | 20 µl |

**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 in Step 2.5A.6.**

2.5A.6. Incubate the sample in a preheated thermal cycler with the heated lid set at 105°C as follows:

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

2.5A.7. Proceed directly to Second Strand cDNA Synthesis, Section 2.6.

### 2.5B Priming of Highly Degraded RNA (FFPE) which has a RIN ≤ 2 and does not Require Fragmentation:

2.5B.1. Set up the following priming reaction and mix by gentle pipetting:

| FRAGMENTATION AND PRIMING REACTION | VOLUME |
|---|---|
| Ribosome Depleted RNA | 5 µl |
| ○ (pink) Random Primers | 1 µl |
| Total Volume | 6 µl |

2.5B.2. Incubate the sample in a preheated thermal cycler with the heated lid set at 105°C as follows:

5 minutes at 65°C
Hold at 4°C

2.5B.3. Transfer the tube directly to ice.

**2.5B.4. First Strand cDNA Synthesis**

*Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.*

*Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.*

2.5B.5. To the primed RNA from 2.5B.3 (6 µl), add the following components and mix by gentle pipetting:

| FRAGMENTATION AND PRIMING REACTION | VOLUME |
|---|---|
| ○ (pink) NEBNext First Strand Synthesis Reaction Buffer | 4 µl |
| ○ (pink) Murine RNase Inhibitor | 0.5 µl |
| Actinomycin D (0.1 µg/µl) | 5 µl |
| ○ (pink) Protoscript II Reverse Transcriptase | 1 µl |
| Nuclease-free Water | 3.5 µl |
| Total Volume | 20 µl |

**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 in Step 2.5B.6.**

2.5B.6. Incubate the sample in a preheated thermal cycler with the heated lid set at 105°C as follows:

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

2.5B.7. Proceed directly to Second Strand cDNA Synthesis, Section 2.6.

### 2.6. Second Strand cDNA Synthesis

2.6.1. Add the following reagents to the First Strand Synthesis reaction (20 µl):

| SECOND STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| Nuclease-free Water | 48 µl |
| • (orange) Second Strand Synthesis Reaction Buffer (10X) | 8 µl |
| • (orange) Second Strand Synthesis Enzyme Mix | 4 µl |
| Total Volume | 80 µl |

2.6.2. Mix thoroughly by gentle pipetting.

2.6.3. Incubate in a thermal cycler for 1 hour at 16°C, with heated lid set at ≤ 40°C.

### 2.7. Purify the Double-stranded cDNA Using 1.8X Agencourt AMPure XP Beads

2.7.1. Vortex AMPure XP Beads to resuspend.

2.7.2. Add 144 µl (1.8X) of resuspended AMPure XP 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.

2.7.3. Incubate for 5 minutes at room temperature.

2.7.4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from 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.

2.7.5. 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.

2.7.6. Repeat Step 2.7.5 once for a total of 2 washing steps.

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

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

2.7.8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 µl 0.1X TE Buffer or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

2.7.9. Remove 55.5 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.**

### 2.8. Perform End Prep of cDNA Library

2.8.1. Mix the following components in a sterile nuclease free tube:

| END PREP REACTION | VOLUME |
|---|---|
| Purified double-stranded cDNA (Step 2.7.9) | 55.5 µl |
| • (green) NEBNext End Repair Reaction Buffer | 6.5 µl |
| • (green) NEBNext End Prep Enzyme Mix | 3 µl |
| Total Volume | 65 µl |

2.8.2. 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

2.8.3. Proceed immediately to Adaptor Ligation.

### 2.9. Adaptor Ligation

Dilute the • (red) NEBNext Adaptor* prior to setting up the ligation reaction.

| TOTAL RNA INPUT | DILUTION REQUIRED |
|---|---|
| 100 ng | 30-fold dilution in 10 mM Tris-HCl with 10 mM NaCl |
| > 100 ng up to 1 µg | 10-fold dilution in 10 mM Tris-HCl with 10 mM NaCl |

2.9.1. Add the following components directly to the End Prep Reaction
**(Caution: Do not pre-mix the components to prevent adaptor-dimer formation):**

| LIGATION REACTION | VOLUME |
|---|---|
| End Prep Reaction | 65 µl |
| • (red) Blunt/TA Ligase Master Mix | 15 µl |
| Diluted NEBNext Adaptor* | 1 µl |
| Nuclease-free Water | 2.5 µl |
| Total Volume | 83.5 µl |

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

2.9.2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

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

**A precipitate can form upon thawing of the NEBNext Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while purifying the ligation reaction. Once thawed, gently mix by inverting the tube several times.**

**2.10. Purify the Ligation Reaction Using AMPure XP Beads**

**Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Section 4.**

2.10.1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl. It is important to ensure the final volume is 100 µl prior to adding AMPure XP Beads.
**Note: X refers to the original sample volume of 100 µl from the above step.**

2.10.2. Add 100 µl (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.10.3. Incubate for 5 minutes at room temperature.

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

2.10.5. 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.

2.10.6. Repeat Step 2.10.5 once for a total of 2 washing steps.

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

2.10.8. Completely remove the residual ethanol, and air dry beads 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 target.**

2.10.9. Remove the tube from the magnet. Elute DNA target from the beads with 52 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

2.10.10. Transfer the 50 µl supernatant to a clean PCR tube. Discard beads.

2.10.11. To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.10.12. Incubate for 5 minutes at room temperature.

2.10.13. 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**).

2.14.14. 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.

2.10.15. Repeat Step 2.14.14 once for a total of 2 washing steps.

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

2.10.17. Completely remove the residual ethanol, and air dry beads 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 target.**

2.10.18. Remove the tube from the magnet. Elute DNA target from the beads with 19 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

2.10.19. Without disturbing the bead pellet, transfer 17 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

## 2.11. PCR Enrichment of Adaptor Ligated DNA

**Follow Section 2.11A if you are using the following oligos (10 µM primer):**
**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 2.11B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).**

### 2.11A. PCR Library Enrichment

2.11A.1. To the cDNA (17 µl) from Step 2.10.19 add the following components and mix by gentle pipetting:

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| • (blue) NEBNext USER Enzyme | 3 µl |
| • (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 µl |
| • (blue) Index (X) Primer/i7 Primer*, ** | 2.5 µl |
| • (blue) Universal PCR Primer/i5 Primer*, *** | 2.5 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 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.

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

2.11A.2. PCR Cycling Conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| USER Digestion | 37°C | 15 minutes | 1 |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 seconds | 12–15*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid overamplification.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

2.11A.3. Proceed to Section 2.12 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

### 2.11.1B. PCR Library Enrichment

2.11B.1. To the cDNA (17 µl) from Step 2.10.19 add the following components and mix by gentle pipetting:

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| • (blue) NEBNext USER Enzyme | 3 µl |
| • (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 µl |
| • (blue) Index/Universal Primer Mix* | 5 µl |
| Total Volume | 50 µl |

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Refer to NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions

2.11B.2. PCR Cycling Conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| USER Digestion | 37°C | 15 minutes | 1 |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 seconds | 12–15*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid overamplification.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

2.11B.3. Proceed to Section 2.12 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

## 2.12. Purify the PCR Reaction using Agencourt AMPure XP Beads

**Note: X refers to the original sample volume from the above step.**

2.12.1. Vortex Agencourt AMPure XP Beads to resuspend.

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

2.12.3. Incubate for 5 minutes at room temperature.

2.12.4. 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.

2.12.5. 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.

2.12.6. Repeat Step 2.12.5 once for a total of 2 washing steps.

2.12.7. Air dry the beads 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 target.**

2.12.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads into 23 µl 0.1X TE. 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.

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

**2.13 Assess library quality on a Bioanalyzer (Agilent High Sensitivity Chip).**

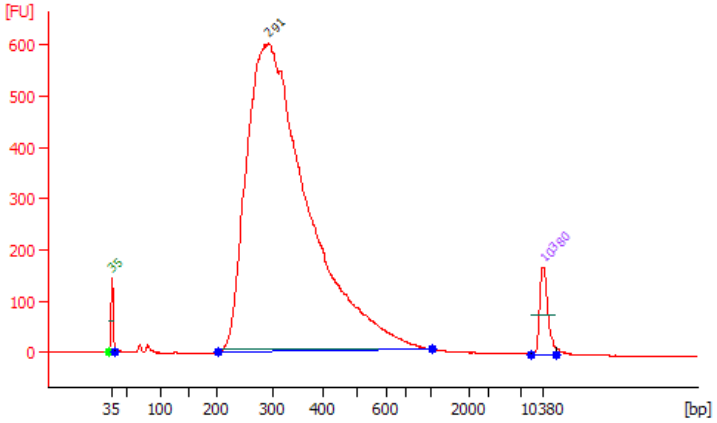
2.13.1. Dilute 2–3 µl of the library in 10 mM Tris or 0.1X TE.

2.13.2. Run 1 µl in a DNA High Sensitivity chip

2.13.3. 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 shown in the Bioanalyzer traces, bring up the sample volume (Step 2.12.9) to 50 µl exactly with nuclease-free water and repeat the AMPure XP Bead clean up step (Section 2.12).**

**Figure 2.13.1: Example of RNA library size distribution on a Bioanalyzer.**



## Section 3
## Protocol for use with Purified mRNA or Ribosome Depleted RNA

**Symbols**

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

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

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

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

### 3.1 RNA Fragmentation, Priming and First Strand cDNA Synthesis

**RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 3.1. Follow protocol in 3.1A to set up the reaction. For highly degraded RNA (FFPE Samples) which do not require fragmentation proceed to Step 3.1B.**

### 3.1A RNA Fragmentation and Priming Starting from Intact or Partially Degraded RNA:

3.1A.1. Set up the following reaction and mix by gentle pipetting:

| FRAGMENTATION AND PRIMING REACTION | VOLUME |
|---|---|
| Purified mRNA or Ribosome depleted RNA (10-100 ng) | 5 µl |
| ○ (pink) NEBNext First Strand Synthesis Reaction Buffer | 4 µl |
| ○ (pink) Random Primers | 1 µl |
| Total Volume | 10 µl |

3.1A.2. Incubate the sample at 94°C following the recomendations in Table 3.1 for fragments sizes ~200 nt.

**Table 3.1. Suggested fragmentation times based on RIN number 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 |

**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.**

3.1A.3. Transfer the tube to ice.

**First Strand cDNA Synthesis**

*Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.*

*Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.*

3.1A.4. To the fragmented and primed mRNA from Step 3.1A.3 (10 µl) add the following components and mix by gentle pipetting:

| FIRST STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| ○ (pink) Murine RNase Inhibitor | 0.5 µl |
| Actinomycin D (0.1 µg/µl) | 5 µl |
| ○ (pink) ProtoScript II Reverse Transcriptase | 1 µl |
| Nuclease-free Water | 3.5 µl |
| Total Volume | 20 µl |

**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 in Step 3.1A.5.**

3.1A.5. Incubate the sample in a preheated thermal cycler (with the heated lid set at 105°C) as follows:

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

3.1A.6. Proceed directly to Second Strand cDNA Synthesis, Step 3.2.1.

## 3.1B. Priming of Highly Degraded RNA (FFPE) which has a RIN ≤ 2 and does not Require Fragmentation:

3.1B.1. Set up the following priming reaction and mix by gentle pipetting:

| FRAGMENTATION AND PRIMING REACTION | VOLUME |
|---|---|
| Purified mRNA or Ribosome Depleted RNA (10-100 ng) | 5 µl |
| ○ (pink) Random Primers | 1 µl |
| Total Volume | 6 µl |

3.1B.2. 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.

3.1B.3. Transfer the tube directly to ice.

**First Strand cDNA Synthesis**

*Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.*

*Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.*

3.1B.4. To the primed RNA from Step 3.1B.3 (6 µl) add the following components and mix by gentle pipetting:

| FIRST STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| ○ (pink) NEBNext First Strand Synthesis Reaction Buffer | 4 µl |
| ○ (pink) Murine RNase Inhibitor | 0.5 µl |
| Actinomycin D (0.1 µg/µl) | 5 µl |
| ○ (pink) ProtoScript II Reverse Transcriptase | 1 µl |
| Nuclease-free Water | 3.5 µl |
| Total Volume | 20 µl |

**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 in Step 3.1B.5.**

3.1B.5. Incubate the sample in a preheated thermal cycler (with the heated lid set at 105°C) as follows:

10 minutes at 25°C

15 minutes at 42°C

15 minutes at 70°C

Hold at 4°C

3.1B.6. Proceed directly to Second Strand cDNA Synthesis, Step 3.2.

**3.2. Perform Second Strand cDNA Synthesis**

3.2.1. Add the following reagents to the First Strand Synthesis reaction (20 µl):

| SECOND STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| Nuclease-free Water | 48 µl |
| • (orange) Second Strand Synthesis Reaction Buffer | 8 µl |
| • (orange) Second Strand Synthesis Enzyme Mix | 4 µl |
| Total Volume | 80 µl |

3.2.2. Mix thoroughly by gentle pipetting.

3.2.3. Incubate in a thermal cycler for 1 hour at 16°C, with heated lid set at ≤ 40°C.

**3.3. Purify the Double-stranded cDNA Using 1.8X Agencourt AMPure XP Beads**

3.3.1. Vortex AMPure XP Beads to resuspend.

3.3.2. Add 144 µl (1.8X) of resuspended AMPure XP 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.

3.3.3. Incubate for 5 minutes at room temperature.

3.3.4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from 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.

3.3.5. 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.

3.3.6. Repeat Step 3.3.5 once for a total of 2 washing steps.

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

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

3.3.8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 µl 0.1X TE Buffer or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

3.3.9. Remove 55.5 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.**

### 3.4. Perform End Prep of cDNA Library

3.4.1. Mix the following components in a sterile nuclease free tube:

| END PREP REACTION | VOLUME |
|---|---|
| Purified double-stranded cDNA (Step 3.3.9) | 55.5 µl |
| • (green) NEBNext End Repair Reaction Buffer | 6.5 µl |
| • (green) NEBNext End Prep Enzyme Mix | 3 µl |
| Total Volume | 65 µl |

3.4.2. 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

3.4.3. Proceed immediately to Adaptor Ligation.

### 3.5. Perform Adaptor Ligation

**Dilute the • (red) NEBNext Adaptor* for Illumina (15 µM) to 1.5 µM with a 10-fold dilution (1:9) with 10 mM Tris-HCl and 10 mM NaCl for immediate use.**

3.5.1. Add the following components directly to the End Prep Reaction **(Caution: Do not pre-mix the components to prevent adaptor-dimer formation)**:

| LIGATION REACTION | VOLUME |
|---|---|
| End Prep Reaction | 65 µl |
| • (red) Blunt/TA Ligase Master Mix | 15 µl |
| Diluted NEBNext Adaptor* | 1 µl |
| Nuclease-free Water | 2.5 µl |
| Total Volume | 83.5 µl |

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

3.5.2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

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

**A precipitate can form upon thawing of the NEBNext Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while purifying the ligation reaction. Once thawed, gently mix by inverting the tube several times.**

### 3.6. Purify the Ligation Reaction Using AMPure XP Beads

**Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Section 4.**

3.6.1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl. It is important to ensure the final volume is 100 µl prior to adding AMPure XP Beads.

**Note: X refers to the original sample volume of 100 µl from the above step.**

3.6.2. Add 100 µl (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

3.6.3. Incubate for 5 minutes at room temperature.

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

3.6.5. 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.

3.6.6. Repeat Step 3.2.5 once for a total of 2 washing steps.

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

3.6.8. Completely remove the residual ethanol, and air dry beads 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 target.**

3.6.9. Remove the tube from the magnet. Elute DNA target from the beads with 52 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

3.6.10. Transfer the 50 µl supernatant to a clean PCR tube. Discard beads.

3.6.11. To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

3.6.12. Incubate for 5 minutes at room temperature.

3.6.13. 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)**.

3.6.14. 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.

3.6.15. Repeat Step 3.6.14 once for a total of 2 washing steps.

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

3.6.17. Completely remove the residual ethanol, and air dry beads 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 target.**

3.6.18. Remove the tube from the magnet. Elute DNA target from the beads with 19 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

3.6.19. Without disturbing the bead pellet, transfer 17 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

## 3.7. PCR Enrichment of Adaptor Ligated DNA

**Follow Section 3.7A if you are using the following oligos (10 µM primer):**

**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 3.7B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).**

### 3.7A. PCR Library Enrichment

3.7A.1. To the cDNA (17 µl) from Step 3.6.19 add the following components and mix by gentle pipetting:

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| • (blue) NEBNext USER Enzyme | 3 µl |
| • (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 µl |
| • (blue) Index (X) Primer/i7 Primer*, ** | 2.5 µl |
| • (blue) Universal PCR Primer/i5 Primer*, *** | 2.5 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 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.

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

3.7A.2. PCR Cycling Conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| USER Digestion | 37°C | 15 minutes | 1 |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 seconds | 12–15*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid overamplification.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

3.7A.3. Proceed to Section 3.8 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

### 3.7B. PCR Library Enrichment

3.7B.1. To the cDNA (17 µl) from Step 3.6.19 add the following components and mix by gentle pipetting:

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| • (blue) NEBNext USER Enzyme | 3 µl |
| • (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix | 25 µl |
| • (blue) Index/Universal Primer Mix* | 5 µl |
| Total Volume | 50 µl |

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Refer to NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions

3.7B.2. PCR Cycling Conditions

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| USER Digestion | 37°C | 15 minutes | 1 |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 seconds | 12–15*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid overamplification.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

3.7B.3. Proceed to Section 3.8 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

**3.8. Purify the PCR Reaction using Agencourt AMPure XP Beads**

**Note: X refers to the original sample volume from the above step.**

3.8.1. Vortex Agencourt AMPure XP Beads to resuspend.

3.8.2. Add 45 µl (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 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. 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.

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

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

3.8.7. Air dry the beads 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 target.**

3.8.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads into 23 µl 0.1X TE. 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.

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

**3.9 Assess library quality on a Bioanalyzer (Agilent High Sensitivity Chip).**

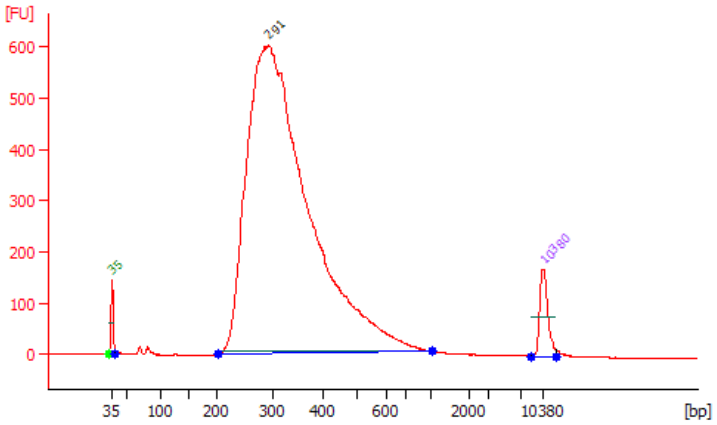
3.9.1. Dilute 2–3 µl of the library in 10 mM Tris or 0.1X TE.

3.9.2. Run 1 µl in a DNA High Sensitivity chip

3.9.3. 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 shown in the Bioanalyzer traces, bring up the sample volume (Step 3.8.9) to 50 µl exactly with nuclease-free water and repeat the AMPure XP Bead clean up step (Section 3.8).**

**Figure 3.9.1: Example of RNA library size distribution on a Bioanalyzer.**

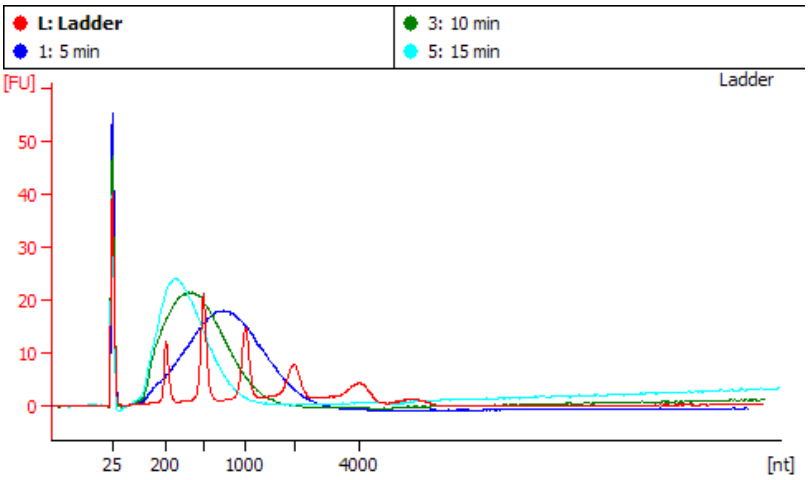


# Section 4
# Appendix A

## 4.1. Fragmentation

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

**Figure 4.1. Modified fragmentation times for longer RNA inserts.**




*Bioanalyzer traces of RNA as shown in RNA Pico Chip. mRNA isolated from Universal Human Reference RNA (1 µg) using the NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB #E7490) and Fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix at 94°C for 5, 10 or 15 minutes.* ***For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes.***

**Table 4.1: Recommended Conditions for libraries with insert sizes larger than 300 bp.**

| APPROXIMATE INSERT SIZE DISTRIBUTION | 250-400 bp | 300-450 bp | 400-600 bp | 500-700 bp |
|---|---|---|---|---|
| Approx. Final Library Size Distribution (insert + adaptor + primers) | 350-500 bp | 400-550 bp | 500-700 bp | 600-800 bp |
| 1st Bead Addition | 45 | 40 | 35 | 30 |
| 2nd Bead Addition | 20 | 20 | 15 | 15 |

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.

## 4.2. Size Selection of Adaptor Ligated DNA

⚠

**For libraries with different size fragment inserts, refer to Table 4.1 for the appropriate volume of beads to be added. The size selection protocol is based on a starting volume of 100 µl. The protocol below is for libraries with a 300–450 bp insert size.**

4.2.1. Vortex AMPure XP Beads to resuspend.

4.2.2. Adjust the final volume after ligation by adding nuclease free water for a 100 µl total volume.

4.2.3. Add 40 µl of resuspended AMPure XP Beads to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.

4.2.4. Incubate for 5 minutes at room temperature.

4.2.5. Quickly spin the tube and place the tube on an appropriate magnetic stand to separate the beads from the supernatant. 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.

4.2.6. Add 20 µl resuspended AMPure XP Beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

4.2.7. Quickly spin the tube and place it on an appropriate magnetic stand to separate the beads from the supernatant. 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)**.

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

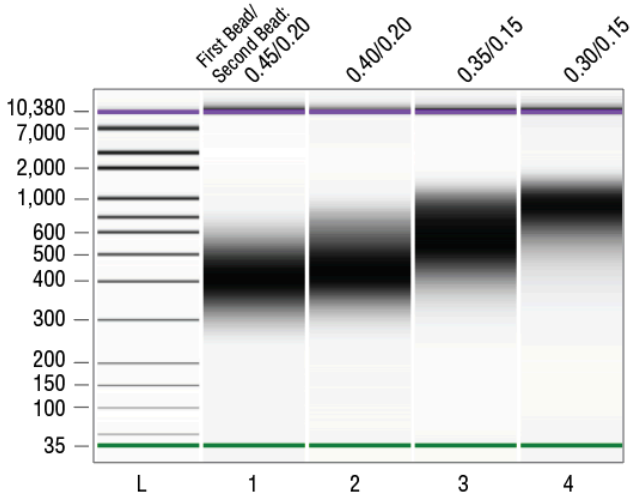
4.2.9. Repeat Step 4.2.8 once for a total of two washes.

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

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

4.2.11. Elute the DNA target from the beads into 19 µl of 10 mM Tris-HCl or 0.1 X TE. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 17 µl to a new PCR tube for amplification.

**Figure 4.2.1: Recommended size selection conditions for libraries with insert sizes > 300 bp.**




*RNA libraries made from Universal Human Reference Total RNA (500 ng) and size selected using different bead/DNA rations as indicated in Table 4.1. RNA was fragmented at 94°C for 5 minutes.*

## Section 5
## Troubleshooting Guide

| OBSERVATIONS | POSSIBLE CAUSES | EFFECT | SUGGESTED SOLUTIONS |
|---|---|---|---|
| Presence of Bioanalyzer peaks < 85 bp (Figure 5.1) | • 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 AMPure Beads (second clean up may result in reduction of library yield) |
| Presence of ~127 bp adaptor-dimer Bioanalyzer peak (Figure 5.1) | • Addition of non-diluted adaptor<br>• RNA input was too low<br>• RNA was over fragmented or lost during fragmentation<br>• 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 (10 fold dilution) before setting up ligation reaction<br>• Clean up PCR reaction again with 1.0X AMPure 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 5.2) | • 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 (Figure 5.3) | • Under-fragmentation of the RNA | Library size will contain longer insert sizes | • Increase RNA fragmentation time |

**Figure 5.1:**

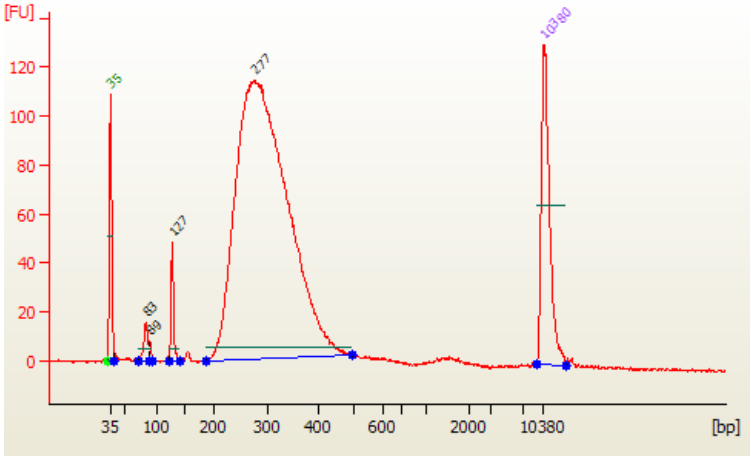


**Figure 5.2:**

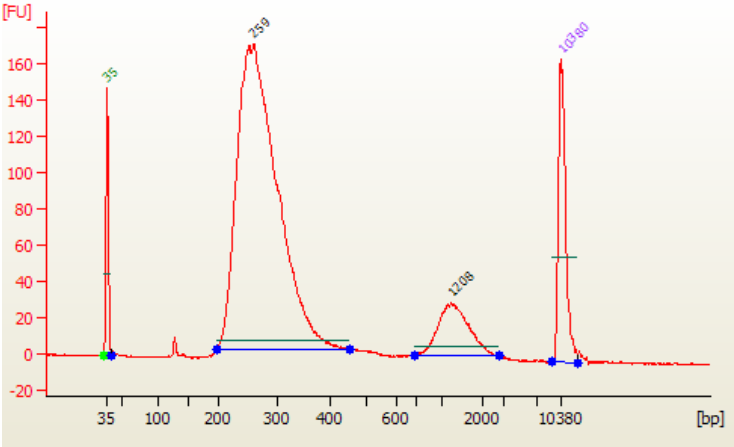
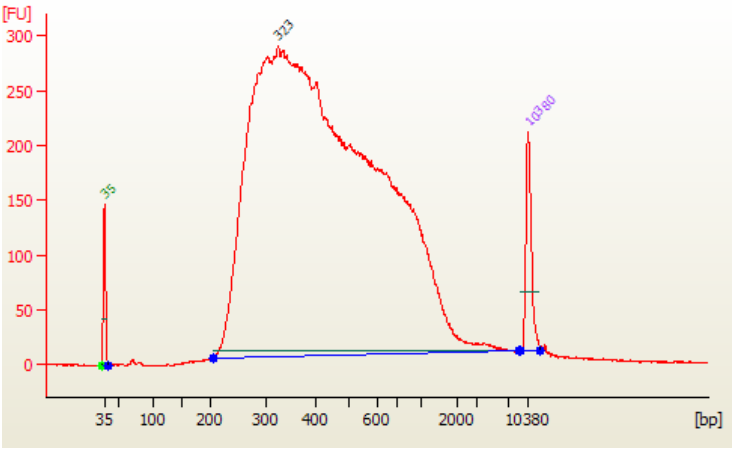



**Figure 5.3:**




## Section 6
## Frequently Asked Questions (FAQs)

*Q.* *What is the difference between the NEBNext Ultra Directional RNA library prep kit for Illumina (NEB #E7420) and the NEBNext Ultra RNA library prep kit for Illumina (NEB #E7530)?*

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

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

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

*Q.* *Where do I have to start the protocol if I have purified mRNA or Ribosome-depleted RNA?*

A. If starting material is purified mRNA or ribosome-depleted RNA, proceed to Section 3 of the manual.

*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.* *Does the kit provide adaptor and primers?*

A. No. Adaptors and primers are provided in the NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E6609, #E7710, #E7730 or #E7600) Oligos for Illumina.

## Kit Components

NEB #E7420S Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7421A | NEBNext First Strand Synthesis Reaction Buffer | 0.192 ml |
| E7422A | Random Primers | 0.048 ml |
| E7423A | ProtoScript II Reverse Transcriptase | 0.024 ml |
| E7424A | Murine RNase Inhibitor | 0.015 ml |
| E7425A | NEBNext Second Strand Synthesis Enzyme Mix | 0.096 ml |
| E7426A | NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix | 0.192 ml |
| E7371A | NEBNext End Prep Enzyme Mix | 0.072 ml |
| E7372A | NEBNext End Repair Reaction Buffer | 0.156 ml |
| E7373A | Blunt/TA Ligase Master Mix | 0.360 ml |
| E7431A | Nuclease-free Water | 8 ml |
| E6625A | NEBNext Q5 Hot Start HiFi PCR Master Mix | 0.6 ml |
| E7428A | NEBNext USER® Enzyme | 0.072 ml |

NEB #E7420L Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7421AA | NEBNext First Strand Synthesis Reaction Buffer | 0.768 ml |
| E7422AA | Random Primers | 0.192 ml |
| E7423AA | ProtoScript II Reverse Transcriptase | 0.096 ml |
| E7424AA | Murine RNase Inhibitor | 0.048 ml |
| E7425AA | NEBNext Second Strand Synthesis Enzyme Mix | 0.384 ml |
| E7426AA | NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix | 0.768 ml |
| E7371AA | NEBNext End Prep Enzyme Mix | 0.288 ml |
| E7372AA | NEBNext End Repair Reaction Buffer | 0.624 ml |
| E7373AA | Blunt/TA Ligase Master Mix | 1.44 ml |
| E7431AA | Nuclease-free Water | 30 ml |
| E6625AA | NEBNext Q5 Hot Start HiFi PCR Master Mix | 2.4 ml |
| E7428AA | NEBNext USER Enzyme | 0.288 ml |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 2.0 | Added RNA input recommendations, removed the size selection for 200 bp fragments - replaced with clean up step. Added additional recommendation for larger insert sizes (Appendix A), Troubleshooting Guide, FAQs. Removed additional washing step in PolyA Isolation Protocol. Moved stopping point from after Second Strand cDNA Synthesis to follow the clean up step.Changed First Strand cDNA Synthesis conditions from 50 minutes at 42°C to 15 minutes at 42°C. Added recommendation to dilute the NEBNext adaptor. | |
| 2.1 | Renamed "elution buffer" in text to "Tris Buffer". | |
| 3.0 | Added protocol for use with NEB #E6310. Changed NEBNext adaptor dilution recommendations. Changed AMPure Bead drying time to 5 minutes. Updated final library elution and dilution for Bioanalyzer to 10 mM Tris or 0.1X TE. Changed ratio of AMPure Beads to 0.9X in final clean up after PCR reaction. | |
| 4.0 | Included protocol for use with NEBNext Q5 Hot Start HiFi PCR Master Mix. Include protocol for changes in concentration of NEBNext Singleplex and Multiplex Oligos for Illumina. Changed all AMPure Bead elutions to 0.1X TE or 10 mM Tris-HCl. Added 2 minute incubation after eluting DNA from AMPure Beads. | |
| 5.0 | Removed protocol for use with NEBNext High-Fidelity 2X PCR Master Mix. Include protocol for use with NEBNext Multiplex Oligos (96 Index Primers, NEB #E6609). | |
| 6.0 | Volume of beads increased from 15 µl to 20 µl. Additional mixing and incubation steps were added after each thermal cycler incubation. Elution volume changed before fragmentation. Updated table on PCR Cycling Conditions 3.7A.2. | |
| 7.0 | Component change: The name, part # and formulation of RNase H has changed. | |
| 8.0 | Protocol updated to include NEB #E7710 and NEB #E7730. Correction of typos and clarifications in several places. Section C in the PCR setup step was removed because all of the 25 µM primers are now expired. | 6/16 |
| 9.0 | Create "Kit Component – Table of Components" for small and large size kits. Delete individual component information pages. | 4/18 |
| 10.0 | New Format Applied | 4/20 |
| 10.1 | Updated product license information. Updates to protocols applied. | 6/20 |