INSTRUCTION MANUAL

NEBNext® RNA Library Prep Kit for MGI®

NEB #E9710S/L

24/96 reactions

Version 1.0_06/24

Table of Contents

Required Materials Not Included 2

Considerations on Selecting an RNA-seq Library Preparation Method 2

Overview 3

Section 1A
Express Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module 4

Section 1B
Standard Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module 12

Section 2
Protocol for use with NEBNext rRNA Depletion Kits 20

Section 3
Protocol for use with FFPE RNA 28

Kit Components 36

Revision History 37

The Library Prep Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E9710S) or up to 96 reactions (NEB #E9710L).

Store at –20°C

- (lilac) NEBNext RNA Fragmentation Buffer
- (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
- (green) NEBNext End Prep Reaction Buffer
- (green) NEBNext End Prep Enzyme Mix
- (red) NEBNext Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (blue) NEBNext USER® Enzyme
- (blue) NEBNext MSTC™ High Yield Master Mix
- (white) (0.1X) TE Buffer
- (white) Nuclease-free Water

Required Materials Not Included

- NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1) (#E9725)
- NEBNext Circularization Module for MGI (#E9720)
- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Magnetic Rack (NEB S1515S, Alpaqua® cat. #A001322, or equivalent)
- 80% Ethanol (freshly prepared)
- Thermal cycler
- DNase-, RNase-free PCR strip tubes, for example TempAssure® PCR flex-free 8-tube strips (USA Scientific® #1402-4708)
- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables

For use with NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490):

- 1.5 ml microcentrifuge tube and NEB #S1506 Magnet stand or equivalent (for washing beads only)

For use with NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400) and other NEBNext rRNA depletion kits that do not include beads (NEB #E7750, E7850, E7865):

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

Considerations on Selecting an RNA-seq Library Preparation Method

The library preparation protocol should be chosen based on the goals of the project and the quality of the RNA sample. Total cellular RNA is mainly composed of ribosomal RNA (rRNA) and often is not of interest. rRNA can be removed from total cellular RNA with either of two standard 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 v2 (Human/Mouse/Rat) with or without RNA Sample Purification Beads (NEB #E7400/#E7405) 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 rRNA, but it will preserve other biologically 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.

The new Express protocol for poly(A) mRNA enrichment (Section 1A) enables comparable performance (to our current/standard protocol, Section 1B) using a substantially faster and more streamlined workflow.

Overview

The NEBNext RNA Library Prep Kit for MGI contains the enzymes and buffers required to convert 10 ng–1 µg of total RNA into high-quality directional (strand-specific) libraries for next-generation sequencing on the MGI DNBSEQ® platforms. The fast, user-friendly workflow has minimal hands-on time and is compatible with poly(A) mRNA enrichment and rRNA depletion methods.

The new Express protocol for poly(A) mRNA enrichment (Section 1A) enables comparable performance (to our current/standard protocol, Section 1B) using a substantially faster and more streamlined workflow.

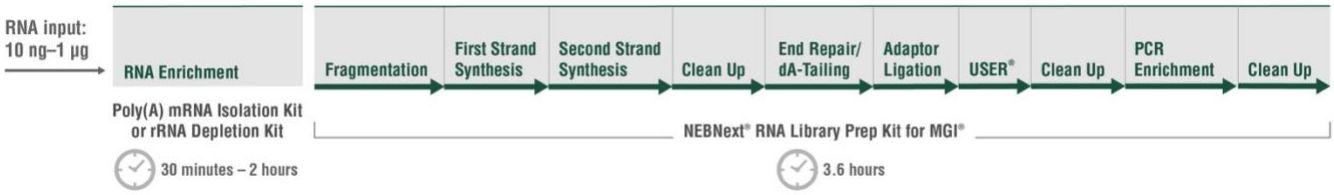
This kit is used in conjunction with NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1) (#E9725) and libraries are circularized using the NEBNext Circularization Module for MGI (#E9720) before sequencing.

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

Please refer to the product page on NEB.com for FAQs about this product.

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

Figure 1: NEBNext RNA Library Prep Kit for MGI



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

Symbols

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

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

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

Keep all buffers and enzymes on ice, unless otherwise indicated.

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

RNA Sample Requirements

RNA Integrity

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

RNA Sample Requirements

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

Input Amount Requirements

- 10 ng–1 µg DNA-free total RNA quantified by Qubit® Fluorometer and quality checked by Bioanalyzer or TapeStation.
- The protocol is optimized for approximately 200 bp RNA inserts.
- Keep all the buffers on ice, unless otherwise indicated.

1A.1. Preparation of 1X Fragmentation Buffer for RNA elution

1A.1.1. Thaw the NEBNext RNA Fragmentation Buffer (2X).

1A.1.2. Add an equivalent amount of nuclease-free water (10 µl per sample) to 2X Fragmentation Buffer for 1X concentration, as shown in table below.

COMPONENT	VOLUME
• (lilac) NEBNext RNA Fragmentation Buffer (2X)	10 µl
Nuclease-free water	10 µl
Total Volume	**20 µl**

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

Note: Keep the mix on ice until mRNA is purified. It will be used in Steps 1A.2.26 and 1A.2.29.

1A.2. mRNA Isolation, Fragmentation and Priming Starting with Total RNA

1A.2.1. Dilute the total RNA (10 ng–1µg) with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.

1A.2.2. Resuspend Oligo $d(T)_{25}$ Beads by inversion or gentle vortexing.

1A.2.3. Add 20 µl Oligo $d(T)_{25}$ beads per reaction to a 1.5 ml tube. If preparing multiple libraries, beads for up to 24 samples can be added to a single 1.5 ml tube for subsequent washes (use magnet NEB #S1506 for 1.5 ml tubes). The purpose of this step is to bring the beads from the storage buffer into the binding buffer. The 2X Binding Buffer does not have to be diluted for this step.

1A.2.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

1A.2.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

1A.2.6. Remove the tube from the magnetic rack.

1A.2.7. Add 50 µl 2X RNA Binding Buffer to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 50 µl RNA Binding Buffer per sample. The Binding Buffer does not have to be diluted.

1A.2.8. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

1A.2.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

1A.2.10. Remove the tube from the magnet, add 50 µl 2X RNA Binding Buffer to the beads and mix by pipetting up and down six times until beads are homogenous. If preparing multiple libraries, add 50 µl RNA 2X Binding Buffer per sample.

1A.2.11. Add 50 µl beads to each RNA sample from Step 1A.2.1. Mix thoroughly by pipetting up and down six times. This first binding step removes most of the non-target RNA.

1A.2.12. Heat the sample to denature the RNA and facilitate mRNA binding to the beads. Place in a thermocycler with the heated lid set to ≥ 90°C, and run the following program:
2 minutes at 80°C
5 minutes at 25°C
Hold at 25°C

1A.2.13. Remove the tube from the thermocycler when the temperature reaches hold at 25°C.

1A.2.14. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1A.2.15. Remove and discard all of the supernatant. Take care not to disturb the beads. Do not remove the tube from the magnetic rack.

1A.2.16. While still on the magnet, rinse the beads and tube by gently adding 200 µl of Wash Buffer to the tube to remove unbound RNA.

1A.2.17. Gently remove and discard all of the supernatant. Take care not to disturb the beads.

1A.2.18. Remove the tube from the magnet and add the following to each tube containing RNA-bound beads to bind poly(A) mRNA a second time. Mix thoroughly by gently pipetting up and down six times.

COMPONENT	VOLUME PER ONE LIBRARY
Tris Buffer	50 µl
RNA Binding Buffer (2X)	50 µl
Total Volume	**100 µl**

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

1A.2.19. Place the tube in a thermocycler with the heated lid set to ≥ 90°C, and run the following program:
2 minutes at 80°C
5 minutes at 25°C
Hold at 25°C

1A.2.20. Remove the tube from the thermocycler when the temperature reaches hold at 25°C.

1A.2.21. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1A.2.22. Remove and discard all of the supernatant. Take care not to disturb the beads. Do not remove the tube from the magnetic rack.

1A.2.23. While still on the magnet rinse the beads by gently adding 200 µl of Wash Buffer to the tube to remove unbound RNA.

1A.2.24. Remove and discard all of the supernatant from the tube. Be sure to remove all of the wash buffer and do not disturb the beads, which contain the mRNA.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. If necessary, while the tubes are on the magnetic rack, remove any remaining Wash Buffer with a 10 µl tip. (Caution: Do not disturb beads that contain the mRNA). Avoid letting the beads dry out before adding elution buffer.

1A.2.25. 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

1A.2.26. To elute the mRNA from the beads and fragment, add 11.5 µl of the 1X Fragmentation Mix (from step 1A.1.1.), pipette up and down six times to resuspend the beads.

1A.2.27 Place in a thermocycler with the heated lid set at 105°C, and run the following program:
15 minutes at 94°C
Hold at 4°C*

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

1A.2.28. Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (~1-2 minutes).

1A.2.29. Collect the fragmented mRNA by transferring 10 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note 1: If the supernatant volume recovered is less than 10 µl for any reason, bring the volume up to 10 µl by adding additional 1X NEBNext RNA Fragmentation Buffer (from step 1A.1.1.), and continue with the protocol.

Note 2: Avoid transferring any of the magnetic beads.

1A.2.30. Place the tube on ice and proceed directly to First Strand cDNA Synthesis.

1A.3. First Strand cDNA Synthesis

1A.3.1. Assemble the first strand cDNA synthesis reaction on ice by adding the following components into fragmented and primed RNA from Step 1A.2.30.

COMPONENT	VOLUME
Fragmented and primed RNA (Step 1A.2.30.)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

1A.3.2. If processing multiple samples, prepare a master mix.

1A.3.3. Mix thoroughly by pipetting up and down at least 10 times.

1A.3.4. Place in a thermocycler with the heated lid set to ≥ 80°C, and run the following program:
10 minutes at 25°C
10 minutes at 42°C
15 minutes at 70°C
Hold at 4°C

1A.3.5. Immediately perform Second Strand cDNA Synthesis.

1A.4. Second Strand cDNA Synthesis

1A.4.1. Add the following components into the first strand synthesis reaction product from Step 1A.3.4.

COMPONENT	VOLUME
First-Strand Synthesis Product (Step 1A.3.4.)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

1A.4.2. If processing multiple samples, prepare a master mix.

1A.4.3. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down at least 10 times.

1A.4.4. Place in a thermocycler with the heated lid set to ≤ 40°C (or off), and run the following program:
30 minutes at 16°C
Hold at 4°C

1A.4.5. Proceed immediately to SPRIselect Bead/AMPure XP Bead cleanup.

1A.5. Purification of Double-stranded cDNA using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

1A.5.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

1A.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.

1A.5.3. Incubate for at least 5 minutes at room temperature.

1A.5.4. Briefly spin the tube in a microcentrifuge to collect any sample from the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. **(Caution: do not discard beads).**

1A.5.5. While in the magnetic stand, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1A.5.6. Repeat Step 1A.5.5. once for a total of 2 washing steps.

1A.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. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

1A.5.8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Briefly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

1A.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 overnight.

1A.6. End Prep of cDNA Library

1A.6.1. Thaw the End Prep Enzyme Mix and End Prep Reaction Buffer on ice. Add the following components to second strand synthesis product from Step 1A.5.9.

COMPONENT	VOLUME
Second Strand cDNA Synthesis Product (Step 1A.5.9.)	50 µl
• (green) NEBNext End Prep Reaction Buffer	7 µl
• (green) NEBNext End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

1A.6.2. If processing multiple samples, prepare a master mix.

1A.6.3. Set a 100 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.

1A.6.4. Place in a thermocycler with the heated lid set to ≥ 75°C, and run the following program:
5 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

1A.6.5. Proceed immediately to Adaptor Ligation.

1A.7. Adaptor Ligation

1A.7.1. Dilute the NEBNext Adaptor for MGI/Complete Genomics®* before setting up the ligation reaction in ice-cold NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics* and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
500 ng–1 µg	no dilution
100 ng–499 ng	2-fold dilution
10 ng–99 ng	4-fold dilution

*The NEBNext Adaptor for MGI/Complete Genomics and NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics are provided in NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1, #E9725). The concentration of the adaptor provided is 40 µM.

1A.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 1A.6.4.

COMPONENT	VOLUME
End Prepped DNA (Step 1A.6.4.)	60 µl
Diluted Adaptor (Step 1A.7.1.)	5 µl
• (red) NEBNext Ligation Master Mix	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**96 µl**

Do not premix the NEBNext Ligation Master Mix and adaptor before use in the Adaptor Ligation Step.

1A.7.3. Set a 100 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 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.

1A.7.4. Place in a thermocycler with the heated lid off, and run the following program:
15 minutes at 20°C
Hold at 4°C

1A.7.5 Add 3 µl (• blue) NEBNext USER Enzyme to the ligation mixture from Step 1A.7.4, resulting in total volume of 99 µl.

1A.7.6 Mix well and place in a thermocycler with the heated lid set to ≥ 45°C, and run the following program:
5 minutes at 37°C
Hold at 4°C

1A.7.7 Proceed immediately to SPRIselect Bead/AMPure XP Bead cleanup.

1A.8. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

1A.8.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

1A.8.2. Add 70 µl (0.7X) of resuspended beads to ligation reaction (~ 100 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

1A.8.3. Incubate for up to 5 minutes at room temperature.

1A.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.

1A.8.5. While in the magnetic rack, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1A.8.6. Repeat Step 1A.8.5 once for a total of 2 washing steps.

1A.8.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

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

1A.8.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 21 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten 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.

1A.8.9. Transfer 19 µl of the supernatant to a clean PCR tube.

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

1A.9. PCR Enrichment of Adaptor Ligated DNA

Note: Use NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1) and follow the index guidelines from the manual (#E9725).

1A.9.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME
Adaptor Ligated DNA (Step 1A.8.9.)	19 µl
NEBNext Dual Index Primer Pairs for MGI/Complete Genomics	6 µl
• (blue) NEBNext MSTC High Yield Master Mix	25 µl
Total Volume	**50 µl**

1A.9.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

1A.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 1A.9.3A and Table 1A.9.3B):

Table 1A.9.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	8–16*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input (Table 1A.9.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak at ~ 1,000 bp will appear on the Bioanalyzer trace, and ~ 2,000 bp on TapeStation D1000.

Table 1A.9.3B: Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
500 ng–1 µg	8-9
100–499 ng	10-12
10–99 ng	13-16

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. Depending on the sample quality, optimization may be required.

1A.10. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes prior to use.

1A.10.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

1A.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.

1A.10.3. Incubate for up to 5 minutes at room temperature.

1A.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.

1A.10.5. While in the magnetic rack, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1A.10.6. Repeat Step 1A.10.5 once for a total of 2 washing steps.

1A.10.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

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

1A.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 ten 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.

1A.10.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

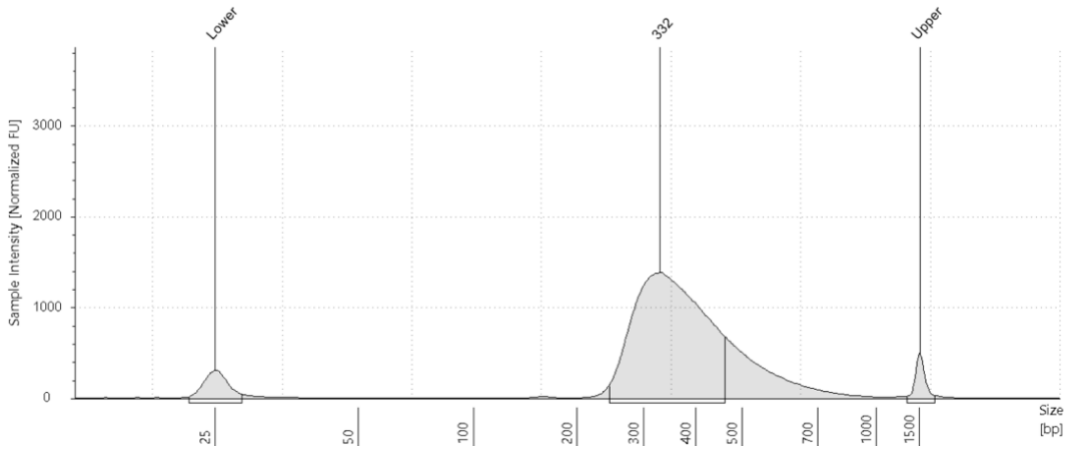
1A.11. Assess Library Quality on an Agilent Bioanalyzer DNA Chip or Agilent TapeStation D1000 Tape

1A.11.1. Run appropriate library volume on a Bioanalyzer DNA 1000 chip or TapeStation D1000 Tape. If the library yield is too low to quantify on this Bioanalyzer chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA chip.

1A.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 132 bp (adaptor-dimer) is visible in the Bioanalyzer traces, increase the sample volume (from Step 1A.10.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect or AMPure XP Bead Cleanup Step (Section 1A.10).

Figure 1A. Example of library made using 10 ng of Universal Human Reference Total RNA on TapeStation D1000 Tape



Follow NEBNext Circularization Module for MGI (NEB #E9720) protocol to make single-strand circular DNA libraries. Then follow DNBSEQ instructions to make DNA nanoballs (DNB) using single-strand circular DNA libraries (ssCircular DNA). We recommend using 80 fmol ssCircular DNA in each DNB making reaction when using NEBNext RNA Library Prep Kit for MGI (NEB #E9710). This has been tested on DNBSEQ-G400RS FCL PE100 reagent and DNBSEQ-G99RS with FCL PE150 reagent. For other DNBSEQ platforms and sequencing reagents, we recommend doubling the ssDNA input as suggested in the DNBSEQ instructions. For example, if the DNBSEQ instructions recommend 40 fmol ssDNA input for a 100 µl DNB reaction, we recommend doubling the ssDNA input to 80 fmol to prepare the 100 µl DNB reaction.

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

Symbols

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

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

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

Keep all buffers and enzymes on ice, unless otherwise indicated.

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

RNA Sample Requirements

RNA Integrity

Assess the quality of the Input RNA by running the RNA sample on an Agilent Bioanalyzer (or similar) RNA 6000 Nano/Pico Chip. For Poly(A) mRNA enrichment, high quality RNA with a RIN score > 7 is required.

RNA Sample Requirements

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

Input Amount Requirements

- 10 ng–1 µg DNA-free total RNA quantified by Qubit Fluorometer and quality checked by Bioanalyzer.
- The protocol is optimized for approximately 200 bp RNA inserts.
- Keep all the buffers on ice, unless otherwise indicated.

1B.1. Preparation of 1X Fragmentation Mix for RNA elution

1B.1.1. Thaw the NEBNext RNA Fragmentation Buffer (2X) and prepare 1X composition by adding nuclease-free water in 1:1 ratio.

1B.1.2. Add an equivalent amount of nuclease-free water (10 µl per sample) to 2X Fragmentation Buffer for 1X concentration.

COMPONENT	VOLUME
• (lilac) NEBNext RNA Fragmentation Buffer (2X)	10 µl
Nuclease-free water	10 µl
Total Volume	**20 µl**

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

Note: Keep the mix on ice until mRNA is purified. It will be used in Steps 1B.2.36 and 1B.2.39.

1B.2. mRNA Isolation, Fragmentation and Priming Starting with Total RNA

1B.2.1. Dilute the total RNA (10 ng–1µg) with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.

1B.2.2. Resuspend Oligo d(T)$_{25}$ Beads by inversion or gentle vortexing. 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 (use magnet NEB #S1506 for 1.5 ml tubes). The purpose of this step is to bring the beads from the storage buffer into the binding buffer. The 2X Binding Buffer does not have to be diluted for this step.

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

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

1B.2.3. Wash the beads by pipetting up and down six times.

1B.2.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

1B.2.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

1B.2.6. Remove the tube from the magnetic rack.

1B.2.7. Add 100 µl 2X RNA Binding Buffer to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 100 µl 2X RNA Binding Buffer per sample. The Binding Buffer does not have to be diluted.

1B.2.8. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

1B.2.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

1B.2.10. Add 50 µl 2X RNA Binding Buffer to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl 2X RNA Binding Buffer per sample.

1B.2.11. Add 50 µl beads to each RNA sample from Step 1B.2.1. Mix thoroughly by pipetting up and down six times. This first binding step removes most of the non-target RNA.

1B.2.12. Heat the sample to denature the RNA and facilitate mRNA binding to the beads. Place in a thermocycler with the heated lid set to ≥ 75°C, and run the following program:
5 minutes at 65°C
Hold at 4°C

1B.2.13. Remove the tube from the thermocycler when the temperature reaches 4°C.

1B.2.14. Mix thoroughly by pipetting up and down six times. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.

1B.2.15. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1B.2.16. Remove and discard all of the supernatant. Take care not to disturb the beads.

1B.2.17. Remove the tube from the magnetic rack.

1B.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.

1B.2.19. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1B.2.20. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

1B.2.21. Remove the tube from the magnetic rack.

1B.2.22. Repeat steps 1B.2.18–1B.2.21.

1B.2.23. Add 50 µl of Tris Buffer (provided in NEB #E7490 kit) to each tube. Gently pipette up and down 6 times to mix thoroughly.

1B.2.24. For the first elution of the mRNA from the beads. Place in a thermocycler with the heated lid set to ≥ 90°C, and run the following program:
2 minutes at 80°C
Hold at 25°C

1B.2.25. Remove the tube from the thermocycler when the temperature reaches 25°C.

1B.2.26. Add 50 µl of 2X RNA Binding Buffer to the sample to allow the mRNA to re-bind to the beads. Mix thoroughly by gently pipetting up and down six times.

1B.2.27. Incubate the tube at room temperature for 5 minutes.

1B.2.28. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1B.2.29. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

1B.2.30. Remove the tube from the magnetic rack.

1B.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.

1B.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.

1B.2.33 Place the tube on the magnet at room temperature until the solution is clear (~2 minutes).

1B.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. 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). Avoid letting the beads dry out before adding elution buffer.

1B.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.

1B.2.36. To elute the mRNA from the beads and fragment, add 11.5 µl of the 1X Fragmentation Mix (from step 1B.1.1.), pipette up and down six times to resuspend the beads.

1B.2.37 Place in a thermocycler with the heated lid set at 105°C, and run the following program:
15 minutes at 94°C
Hold at 4°C*

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

1B.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).

1B.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 additional 1X NEBNext RNA Fragmentation Buffer and continue with the protocol.

Note 2: Avoid transferring any of the magnetic beads.

1B.2.40. Place the tube on ice and proceed directly to First Strand cDNA Synthesis.

1B.3. First Strand cDNA Synthesis

1B.3.1. Assemble the first strand cDNA synthesis reaction on ice by adding the following components into fragmented and primed RNA from Step 1B.2.40.

COMPONENT	VOLUME
Fragmented and primed RNA (Step 1B.2.40.)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

1B.3.2. If processing multiple samples, prepare a master mix.

1B.3.3. Mix thoroughly by pipetting up and down at least 10 times.

1B.3.4. Place in a thermocycler with the heated lid set to ≥ 80°C, and run the following program:
10 minutes at 25°C
10 minutes at 42°C
15 minutes at 70°C
Hold at 4°C

1B.3.5. Immediately perform Second Strand cDNA Synthesis.

1B.4. Second Strand cDNA Synthesis

1B.4.1. Add the following components into the first strand synthesis reaction product from Step 1B.3.4.

COMPONENT	VOLUME
First-Strand Synthesis Product (Step 1B.3.4.)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

1B.4.2. If processing multiple samples, prepare a master mix.

1B.4.3. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down at least 10 times.

1B.4.4. Place in a thermocycler with the heated lid set to ≤ 40°C (or off), and run the following program:
30 minutes at 16°C
Hold at 4°C

1B.4.5. We recommend proceeding to SPRIselect Bead/AMPure XP Bead cleanup.

1B.5. Purification of Double-stranded cDNA using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

1B.5.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

1B.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.

1B.5.3. Incubate for at least 5 minutes at room temperature.

1B.5.4. Briefly spin the tube in a microcentrifuge to collect any sample from the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. **(Caution: do not discard beads).**

1B.5.5. While in the magnetic stand, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1B.5.6. Repeat Step 1B.5.5. once for a total of 2 washing steps.

1B.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. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

1B.5.8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Briefly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

1B.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 overnight.

1B.6. End Prep of cDNA Library

1B.6.1. Thaw the end prep enzyme and buffer on ice. Add the following components to second strand synthesis product from Step 1B.5.9.

COMPONENT	VOLUME
Second Strand cDNA Synthesis Product (Step 1B.5.9.)	50 µl
• (green) NEBNext End Prep Reaction Buffer	7 µl
• (green) NEBNext End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

1B.6.2. If processing multiple samples, prepare a master mix.

1B.6.3. Set a 100 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.

1B.6.4. Place in a thermocycler with the heated lid set to ≥ 75°C, and run the following program:
5 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

1B.6.5. Proceed immediately to Adaptor Ligation.

1B.7. Adaptor Ligation

1B.7.1. Dilute the NEBNext Adaptor for MGI/Complete Genomics* before setting up the ligation reaction in ice-cold NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics* and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
500 ng–1 µg	no dilution
100–499 ng	2-fold dilution
10–99 ng	4-fold dilution

* The NEBNext Adaptor for MGI/Complete Genomics and NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics are provided in NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1, #E9725). The concentration of the adaptor provided is 40 µM.

1B.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 1B.6.4.

COMPONENT	VOLUME
End Prepped DNA (Step 1B.6.4.)	60 µl
Diluted Adaptor (Step 1B.7.1.)	5 µl
• (red) NEBNext Ligation Master Mix	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**96 µl**

Do not premix the NEBNext Ligation Master Mix and adaptor before use in the Adaptor Ligation Step.

1B.7.3. Set a 100 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 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.

1B.7.4. Place in a thermocycler with the heated lid off, and run the following program:
15 minutes at 20°C
Hold at 4°C

1B.7.5. Add 3 µl (• blue) NEBNext USER Enzyme to the ligation mixture from Step 1B.7.4, resulting in total volume of 99 µl.

1B.7.6. Place in a thermocycler with the heated lid set to ≥ 45°C, and run the following program:
5 minutes at 37°C
Hold at 4°C

1B.7.7 Proceed immediately to SPRIselect Bead/AMPure XP Bead cleanup.

1B.8. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

1B.8.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

1B.8.2. Add 70 µl (0.7X) of resuspended beads to ligation reaction (~100 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

1B.8.3. Incubate for up to 5 minutes at room temperature.

1B.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.

1B.8.5. While in the magnetic rack, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1B.8.6. Repeat Step 1B.8.5. once for a total of 2 washing steps.

1B.8.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

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

1B.8.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 21 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten 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.

1B.8.9. Transfer 19 µl of the supernatant to a clean PCR tube.

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

1B.9. PCR Enrichment of Adaptor Ligated DNA

Note: Use NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1) and follow the index guidelines from the manual (#E9725).

1B.9.1. Set up the PCR reaction as described below.

COMPONENT	VOLUME
Adaptor Ligated DNA (Step 1B.8.9.)	19 µl
NEBNext Dual Index Primer Pairs for MGI/Complete Genomics	6 µl
• (blue) NEBNext MSTC High Yield Master Mix	25 µl
Total Volume	**50 µl**

1B.9.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

1B.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 1B.9.3A and Table 1B.9.3B):

Table 1B.9.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	8–16*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input (Table 1B.9.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak at ~ 1,000 bp will appear on the Bioanalyzer trace, and ~ 2,000 bp on TapeStation D1000.

Table 1B.9.3B: Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
500 ng–1 µg	8–9
100 ng–499 ng	10–12
10 ng–99 ng	13–16

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. Depending on the sample quality, optimization may be required.

1B.10. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

1B.10.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

1B.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.

1B.10.3. Incubate for up to 5 minutes at room temperature.

1B.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.

1B.10.5. While in the magnetic rack, add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1B.10.6. Repeat Step 1B.10.5. once for a total of 2 washing steps.

1B.10.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

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

1B.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 ten 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.

1B.10.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

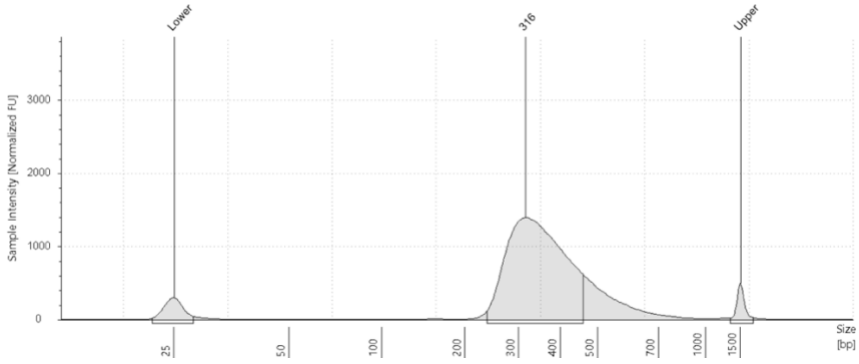
1B.11. Assess Library Quality on an Agilent Bioanalyzer DNA Chip or Agilent TapeStation D1000 Tape

1B.11.1. Run appropriate volume of library on a Bioanalyzer DNA 1000 chip or TapeStation D1000 Tape. If the library yield is too low to quantify on this Bioanalyzer chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA chip.

1B.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 132 bp (adaptor-dimer) is visible in the Bioanalyzer traces, increase the sample volume (from Step 1B.10.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect or AMPure Bead Cleanup Step (Section 1B.10).

Figure 1B. Example of library made using 10ng of Universal Human Reference Total RNA on TapeStation D1000 Tape



Follow NEBNext Circularization Module for MGI (#E9720) protocol to make single-strand circular DNA libraries.
Then follow DNBSEQ instructions to make DNA nanoballs (DNB) using single-strand circular DNA libraries (ssCircular DNA).
We recommend using 80 fmol ssCircular DNA in each DNB making reaction when using NEBNext RNA Library Prep Kit for MGI (NEB #E9710). This has been tested on DNBSEQ-G400RS FCL PE100 reagent and DNBSEQ-G99RS with FCL PE150 reagent. For other DNBSEQ platforms and sequencing reagents, we recommend doubling the ssDNA input as suggested in the DNBSEQ instructions. For example, if the DNBSEQ instructions recommend 40 fmol ssDNA input for a 100 µl DNB reaction, we recommend doubling the ssDNA input to 80 fmol to prepare the 100 µl DNB reaction.

Section 2
Protocol for use with NEBNext rRNA Depletion Kits (NEB #E7400, #E7405)

Symbols

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

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

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

Keep all buffers and enzymes on ice, unless otherwise indicated.

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

RNA Sample Requirements

RNA Integrity

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer (or similar) RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2 (current section). For highly degraded samples (e.g., FFPE) which do not require fragmentation, follow the library preparation protocol in Section 3.

RNA Purity

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

Input Amount

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

2.1. Probe Hybridization to RNA

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

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (10 ng–1µg)	11 µl
◦ (white) NEBNext v2 rRNA Depletion Solution	2 µl
◦ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

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

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

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

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

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

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

2.2. RNase H Digestion

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

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

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

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

2.2.3. Briefly spin down the tube in a microcentrifuge.

2.2.4. Place in a pre-heated thermocycler with the heated lid set to 55°C, and run the following program:
30 minutes at 50°C
Hold at 4°C

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

2.3. DNase I Digestion

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

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

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

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

2.3.3. Briefly spin down the tube in a microcentrifuge.

2.3.4. Place in a thermocycler with the heated lid set at ≥ 45°C, and run the following program:
30 minutes at 37°C
Hold at 4°C

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

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

Note: Thaw the • (lilac) NEBNext RNA Fragmentation Buffer. Keep the mix on ice until RNA is purified.

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

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

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

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

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

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

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

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

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

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

2.4.10. Incubate for 2 minutes at room temperature.

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

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

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

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

2.5. RNA Fragmentation and Priming

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 2.5.3.

2.5.1. Add 5 µl of (• lilac) NEBNext RNA Fragmentation Buffer to 5 µl of sample containing RNA.

2.5.2. Mix well and proceed with fragmentation.

2.5.3. Place in a thermocycler with the heated lid set to 105°C, and run the following program:
Incubate at 94°C, for the suggested fragmentation time (Table 2.5.3A.)
Hold at 4°C

Follow the recommendations in Table 2.5.3A for libraries with inserts ~200 nt. If ribosomal RNA peaks are present, suggesting higher quality, follow recommendations in Table 2.5.3A. For samples with lower integrity or are from FFPE, please go to Section 3.

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

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

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

2.6. First Strand cDNA Synthesis

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

COMPONENT	VOLUME
Fragmented and primed RNA (Step 2.5.4.)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

2.6.2. If processing multiple samples, prepare a master mix.

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

2.6.4. Place in a thermocycler with the heated lid set to ≥ 80°C, and run the following program:
10 minutes at 25°C
10 minutes at 42°C
15 minutes at 70°C
Hold at 4°C

2.6.5. Proceed directly to Second Strand cDNA Synthesis.

2.7. Second Strand cDNA Synthesis

2.7.1. Add the following components into the first strand synthesis reaction product from Step 2.6.5.

COMPONENT	VOLUME
First Strand Synthesis Product (Step 2.6.5.)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

2.7.2. If processing multiple samples, prepare a master mix.

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

2.7.4. Place in a thermocycler with the heated lid set to ≤ 40°C (or off), and run the following program:
30 minutes at 16°C
Hold at 4°C

2.7.5. We recommend proceeding to SPRIselect Bead/AMPure XP Bead cleanup.

2.8. Purification of Double-stranded cDNA using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

2.8.1 Vortex SPRIselect Beads or AMPure XP 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 at least 5 minutes at room temperature.

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

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

2.8.6. Repeat Step 2.8.5. once for a total of 2 washing steps.

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

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

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

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 overnight.

2.9. End Prep of cDNA Library

2.9.1 Add the following components to second strand synthesis product from Step 2.8.9.

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

2.9.2 If processing multiple samples, prepare a master mix.

2.9.3. Set a 100 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.

2.9.4. Place in a thermocycler with the heated lid set to ≥ 75°C, and run the following program:
5 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

2.9.5. Proceed immediately to Adaptor Ligation.

2.10. Adaptor Ligation

2.10.1. Dilute the NEBNext Adaptor for MGI/Complete Genomics* before setting up the ligation reaction in ice-cold NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics* and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
500 ng–1 µg	no dilution
100 ng–499 ng	2-fold dilution
10 ng–99 ng	4-fold dilution

* The NEBNext Adaptor for MGI/Complete Genomics and NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics are provided in NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1, #E9725). The concentration of the adaptor provided is 40 µM.

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

COMPONENT	VOLUME
End Prepped DNA (Step 2.9.4.)	60 µl
Diluted Adaptor (Step 2.10.1.)	5 µl
• (red) NEBNext Ligation Master Mix	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**96 µl**

Do not premix the NEBNext Ligation Master Mix and adaptor before use in the Adaptor Ligation Step.

2.10.3. If processing multiple samples, prepare a master mix.

2.10.4. Set a 100 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 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.

2.10.5. Place in a thermocycler with the heated lid off, and run the following program:
15 minutes at 20°C
Hold at 4°C

2.10.6. Add 3 µl (• blue) NEBNext USER Enzyme to the ligation mixture from Step 2.10.5., resulting in total volume of 99 µl.

2.10.7. Place in a thermocycler with the heated lid set to ≥ 45°C, and run the following program:
5 minutes at 37°C
Hold at 4°C

2.10.8. Proceed immediately to SPRIselect Bead/AMPure XP Bead cleanup.

2.11. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

2.11.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

2.11.2. Add 70 µl (0.7X) of resuspended beads to ligation reaction (~ 100 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.11.3. Incubate for up to 5 minutes at room temperature.

2.11.4. 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.

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

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

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

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

2.11.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 21 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten 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.

2.11.9. Transfer 19 µl of the supernatant to a clean PCR tube.

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

2.12. PCR Enrichment of Adaptor Ligated DNA

Note: Use NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1) and follow the index guidelines from the manual (#E9725).

2.12.1 Set up the PCR reaction as described below.

COMPONENT	VOLUME
Adaptor Ligated DNA (Step 2.11.9.)	19 µl
NEBNext Dual Index Primer Pairs for MGI/Complete Genomics	6 µl
• (blue) NEBNext MSTC High Yield Master Mix	25 µl
Total Volume	**50 µl**

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	98°C	10 seconds	7–15*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input (Table 2.12.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak at ~ 1,000 bp will appear on the Bioanalyzer trace, and ~ 2,000 bp on TapeStation D1000.

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

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
500 ng–1 µg	7–9
100 ng–499 ng	10–12
10 ng–99 ng	13–15

Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. Depending on the sample quality, optimization may be required.

2.13. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

2.13.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

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

2.13.3. Incubate for 5 minutes at room temperature.

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

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

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

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

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

2.13.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten 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 an Agilent Bioanalyzer DNA Chip or TapeStation D1000

2.14.1. Run appropriate volume of library on a Bioanalyzer DNA 1000 chip or TapeStation D1000 Tape. If the library yield is too low to quantify on this Bioanalyzer chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA 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 132 bp (adaptor-dimer) is visible in the Bioanalyzer traces, increase the sample volume (from Step 2.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect or AMPure Bead Cleanup Step (Section 2.13)

Follow NEBNext Circularization Module for MGI (#E9720) protocol to make single-strand circular DNA libraries. Then follow DNBSEQ instructions to make DNA nanoballs (DNB) using single-strand circular DNA libraries (ssCircular DNA). We recommend using 80 fmol ssCircular DNA in each DNB making reaction when using NEBNext RNA Library Prep Kit for MGI (NEB #E9710). This has been tested on DNBSEQ-G400RS FCL PE100 reagent and DNBSEQ-G99RS with FCL PE150 reagent. For other DNBSEQ platforms and sequencing reagents, we recommend doubling the ssDNA input as suggested in the DNBSEQ instructions. For example, if the DNBSEQ instructions recommend 40 fmol ssDNA input for a 100 µl DNB reaction, we recommend doubling the ssDNA input to 80 fmol to prepare the 100 µl DNB reaction.

Section 3
Protocol for use with FFPE RNA, NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400, #E7405)

Symbols

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

This caution sign signifies a step in the protocol with two paths leading to the same endpoint 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.

Keep all buffers and enzymes on ice, unless otherwise indicated.

RNA Sample Requirements

RNA Integrity

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer (or similar) RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all. For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 2. For highly degraded samples (RIN = 1 or 2) (e.g., FFPE) which do not require fragmentation, follow the library preparation protocol in Section 3 (current section).

RNA Purity

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

Input Amount

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

3.1. Probe Hybridization to RNA

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

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

COMPONENT	VOLUME
Total RNA in Nuclease-free Water (10–100 ng)	11 µl
○ (white) NEBNext v2 rRNA Depletion Solution	2 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

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

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

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

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

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

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

3.2. RNase H Digestion

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

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

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

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

3.2.3. Briefly spin down the tube in a microcentrifuge.

3.2.4. Place in a thermocycler with the heated lid set to 55°C, and run the following program:
30 minutes at 50°C
Hold at 4°C

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

3.3. DNase I Digestion

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

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

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

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

3.3.3. Briefly spin down the tube in a microcentrifuge.

3.3.4. Place in a thermocycler with the heated lid set at ≥ 45°C, and run the following program:
30 minutes at 37°C
Hold at 4°C

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

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

Note: Thaw the • (lilac) NEBNext RNA Fragmentation Buffer. Keep the mix on ice until RNA is purified.

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

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

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

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

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

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

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

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

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

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

3.4.10. Incubate for 2 minutes at room temperature.

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

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

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

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

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

3.5.1. Add 5 µl of (• lilac) NEBNext RNA Fragmentation Buffer to 5 µl of RNA and mix thoroughly by pipetting up and down ten times.

3.5.2. Place in a thermocycler with the heated lid set at 105°C, and run the following program:
5 minutes at 65°C
Hold at 4°C.

3.5.3. Immediately remove tube from thermal cycler once it reaches 4°C.

3.5.4. 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 fragmented and primed RNA from Step 3.5.4.:

COMPONENT	VOLUME
Primed RNA (Step 3.5.4.)	10 µl
• (brown) NEBNext Strand Specificity Reagent	8 µl
• (lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

3.6.2. If processing multiple samples, prepare a master mix.

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

3.6.4. Place in a thermocycler with the heated lid set to ≥ 80°C, and run the following program:
10 minutes at 25°C
10 minutes at 42°C
15 minutes at 70°C
Hold at 4°C

3.6.5. Proceed directly to Second Strand cDNA Synthesis.

3.7. Second Strand cDNA Synthesis

3.7.1. Add the following components into the first strand synthesis reaction product from Step 3.6.5.

COMPONENT	VOLUME
First Strand Synthesis Product (Step 3.6.5.)	20 µl
• (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	8 µl
• (orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

3.7.2. If processing multiple samples, prepare a master mix.

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

3.7.4. Place in a thermocycler with the heated lid set to ≤ 40°C (or off), and run the following program:
30 minutes at 16°C
Hold at 4°C

3.7.5. We recommend proceeding to SPRIselect Bead/AMPure XP Bead cleanup.

3.8. Purification of Double-stranded cDNA using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

3.8.1 Vortex SPRIselect Beads or AMPure XP 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 at least 5 minutes at room temperature.

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

3.8.5. While in the magnetic stand, add 200 µl of freshly prepared 80% ethanol to the tube. 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 up to 5 minutes while the tube is on the magnetic rack with the lid open.

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

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

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 overnight.

3.9. End Prep of cDNA Library

3.9.1. Thaw the end prep enzyme and buffer on ice. Add the following components to second strand synthesis product from Step 3.8.9.

COMPONENT	VOLUME
Second Strand cDNA Synthesis Product (Step 3.8.9.)	50 µl
• (green) NEBNext End Prep Reaction Buffer	7 µl
• (green) NEBNext End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

3.9.2. If processing multiple samples, prepare a master mix.

3.9.3. Set a 100 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.

3.9.4. Place in a thermocycler with the heated lid set to ≥ 75°C, and run the following program:
5 minutes at 20°C
10 minutes at 65°C
Hold at 4°C

3.9.5. Proceed immediately to Adaptor Ligation.

3.10. Adaptor Ligation

3.10.1. Dilute the NEBNext Adaptor for MGI/Complete Genomics* before setting up the ligation reaction in ice-cold NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics* and keep the diluted adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
10–100 ng	4-fold dilution

* The NEBNext Adaptor for MGI/Complete Genomics and NEBNext Adaptor Dilution Buffer for MGI/Complete Genomics are provided in NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1, #E9725). The concentration of the adaptor provided is 40 µM.

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 3.10.4.

COMPONENT	VOLUME
End Prepped DNA (Step 3.9.5.)	60 µl
Diluted Adaptor (Step 3.10.1.)	5 µl
• (red) NEBNext Ligation Master Mix	30 µl
• (red) NEBNext Ligation Enhancer	1 µl
Total Volume	**96 µl**

Do not premix the NEBNext Ligation Master Mix and adaptor before use in the Adaptor Ligation Step.

3.10.3. If processing multiple samples, prepare a master mix.

3.10.4. Set a 100 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 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.

3.10.5. Place in a thermocycler with the heated lid off, and run the following program:
15 minutes at 20°C
Hold at 4°C

3.10.6. Add 3 µl (• blue) NEBNext USER Enzyme to the ligation mixture from Step 3.10.5, resulting in total volume of 99 µl.

3.10.7. Place in a thermocycler with the heated lid set to ≥ 45°C, and run the following program:
5 minutes at 37°C
Hold at 4°C

3.10.8. Proceed immediately to SPRIselect Bead/AMPure XP Bead cleanup.

3.11. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4ºC and keep at room temperature for 30 minutes before use.

3.11.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

3.11.2. Add 70 µl (0.7X) of resuspended beads to ligation reaction (~ 100 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

3.11.3. Incubate for up to 5 minutes at room temperature.

3.11.4. 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.

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

3.11.6. Repeat Step 3.11.5. once for a total of 2 washing steps.

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

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

3.11.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 21 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten 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.

3.11.9. Transfer 19 µl of the supernatant to a clean PCR tube.

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

3.12. PCR Enrichment of Adaptor Ligated DNA

Note: Use NEBNext Multiplex Oligos for MGI (Dual Index Primer Pairs Set 1) and follow the index guidelines from the manual (#E9725).

3.12.1 Set up the PCR reaction as described below.

COMPONENT	VOLUME
Adaptor Ligated DNA (Step 3.11.9.)	19 µl
NEBNext Dual Index Primers for MGI/Complete Genomics	6 µl
• (blue) NEBNext MSTC High Yield Master Mix	25 µl
Total Volume	**50 µl**

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	98°C	10 seconds	15–18*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input (Table 3.12.3B).

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak at ~ 1,000 bp will appear on the Bioanalyzer trace, and ~ 2,000 bp on TapeStation D1000

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

TOTAL RNA INPUT	RECOMMENDED PCR CYCLES
10–100 ng	15–18

Note: PCR cycles are recommended based on highly degraded samples (RIN = 1 or 2). Depending on the sample quality, optimization may be required.

3.13. Purification of the PCR Reaction using SPRIselect Beads or AMPure XP Beads

Note: The SPRIselect/AMPure XP Beads ratios recommended in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols. Please adhere to these guidelines and not those recommended by other sources or for other kits. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes before use.

3.13.1. Vortex SPRIselect Beads or AMPure XP Beads to resuspend.

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

3.13.3. Incubate for 5 minutes at room temperature.

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

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

3.13.6. Repeat Step 3.14.5. once for a total of 2 washing steps.

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

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

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

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 an Agilent Bioanalyzer DNA Chip or TapeStation D1000 Tape.

3.14.1. Run appropriate volume of library on a Bioanalyzer DNA 1000 chip or TapeStation D1000 Tape. If the library yield is too low to quantify on this Bioanalyzer chip, please run the samples on a DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA 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 132 bp (adaptor-dimer) is visible in the Bioanalyzer traces, increase the sample volume (from Step 3.13.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect or AMPure XP Bead Cleanup Step (Section 3.13)

Follow NEBNext Circularization Module for MGI (#E9720) protocol to make single-strand circular DNA libraries. Then follow DNBSEQ instructions to make DNA nanoballs (DNB) using single-strand circular DNA libraries (ssCircular DNA). We recommend using 80 fmol ssCircular DNA in each DNB making reaction when using NEBNext RNA Library Prep Kit for MGI (NEB #E9710). This has been tested on DNBSEQ-G400RS FCL PE100 reagent and DNBSEQ-G99RS with FCL PE150 reagent. For other DNBSEQ platforms and sequencing reagents, we recommend doubling the ssDNA input as suggested in the DNBSEQ instructions. For example, if the DNBSEQ instructions recommend 40 fmol ssDNA input for a 100 µl DNB reaction, we recommend doubling the ssDNA input to 80 fmol to prepare the 100 µl DNB reaction.

Kit Components

NEB #E9710S Table of Components

NEB #	COMPONENT	VOLUME
E9618A	NEBNext RNA Fragmentation Buffer	0.24 ml
E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
E7766A	NEBNext Strand Specificity Reagent	0.192 ml
E7425A	NEBNext Second Strand Synthesis Enzyme Mix	0.096 ml
E7426A	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.192 ml
E9611A	NEBNext End Prep Reaction Buffer	0.168 ml
E9612A	NEBNext End Prep Enzyme Mix	0.072 ml
E9613A	NEBNext Ligation Master Mix	0.72 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E9619A	NEBNext USER Enzyme	0.072 ml
E9615A	NEBNext MSTC High Yield Master Mix	0.6 ml
E7763A	0.1X TE	2.78 ml
E7764A	Nuclease-free Water	1.25 ml

NEB #E9710L Table of Components

NEB #	COMPONENT	VOLUME
E9618AA	NEBNext RNA Fragmentation Buffer	0.96 ml
E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
E7766AA	NEBNext Strand Specificity Reagent	0.768 ml
E7425AA	NEBNext Second Strand Synthesis Enzyme Mix	0.384 ml
E7426AA	NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix	0.768 ml
E9611AA	NEBNext End Prep Reaction Buffer	0.672 ml
E9612AA	NEBNext End Prep Enzyme Mix	0.288 ml
E9613AA	NEBNext Ligation Master Mix	3 x 0.96 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E9619AA	NEBNext USER Enzyme	0.288 ml
E9615AA	NEBNext MSTC High Yield Master Mix	2x 1.2 ml
E7763AA	0.1X TE	9.4 ml
E7764AA	Nuclease-free Water	5.7 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	6/24

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