Takara Bio USA, Inc.

SMART-Seq® Total RNA High Input (RiboGone™ Mammalian) User Manual

Cat. Nos. 635045, 635046 & 635047
(112723)

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I. Introduction

SMART-Seq Total RNA High Input (RiboGone Mammalian) (Cat. Nos. 635045, 635046 & 635047) can be used to generate indexed total RNA sequencing (RNA-seq) libraries suitable for next-generation sequencing (NGS) on any Illumina® platform. The kit contains components for both ribosomal RNA (rRNA) removal and cDNA synthesis. It incorporates RiboGone - Mammalian for the removal of rRNA (*5S*, *5.8S*, *18S*, and *28S* nuclear rRNA; *12S* mitochondrial rRNA) sequences from human, mouse, or rat full-length or sheared total RNA, followed by cDNA synthesis using SMART® (**S**witching **M**echanism **a**t 5′ end of **R**NA **T**emplate) technology. Up to 384 indexed libraries can be prepared using Unique Dual Index Kits (Cat. Nos. 634752–634756, sold separately). The entire library construction protocol, starting with total RNA, can be completed in about 5 hours (Figure 1).

The kit is designed to work with input ranges from 100 ng–1 µg of total RNA of either high or low quality [RNA integrity number (RIN) 3–10]. Total RNA-seq libraries generated with the kit enable the analysis of both coding and non-coding RNA species, and provide information on strand orientation, transcript isoforms, gene fusions, single nucleotide variations (SNV), etc.

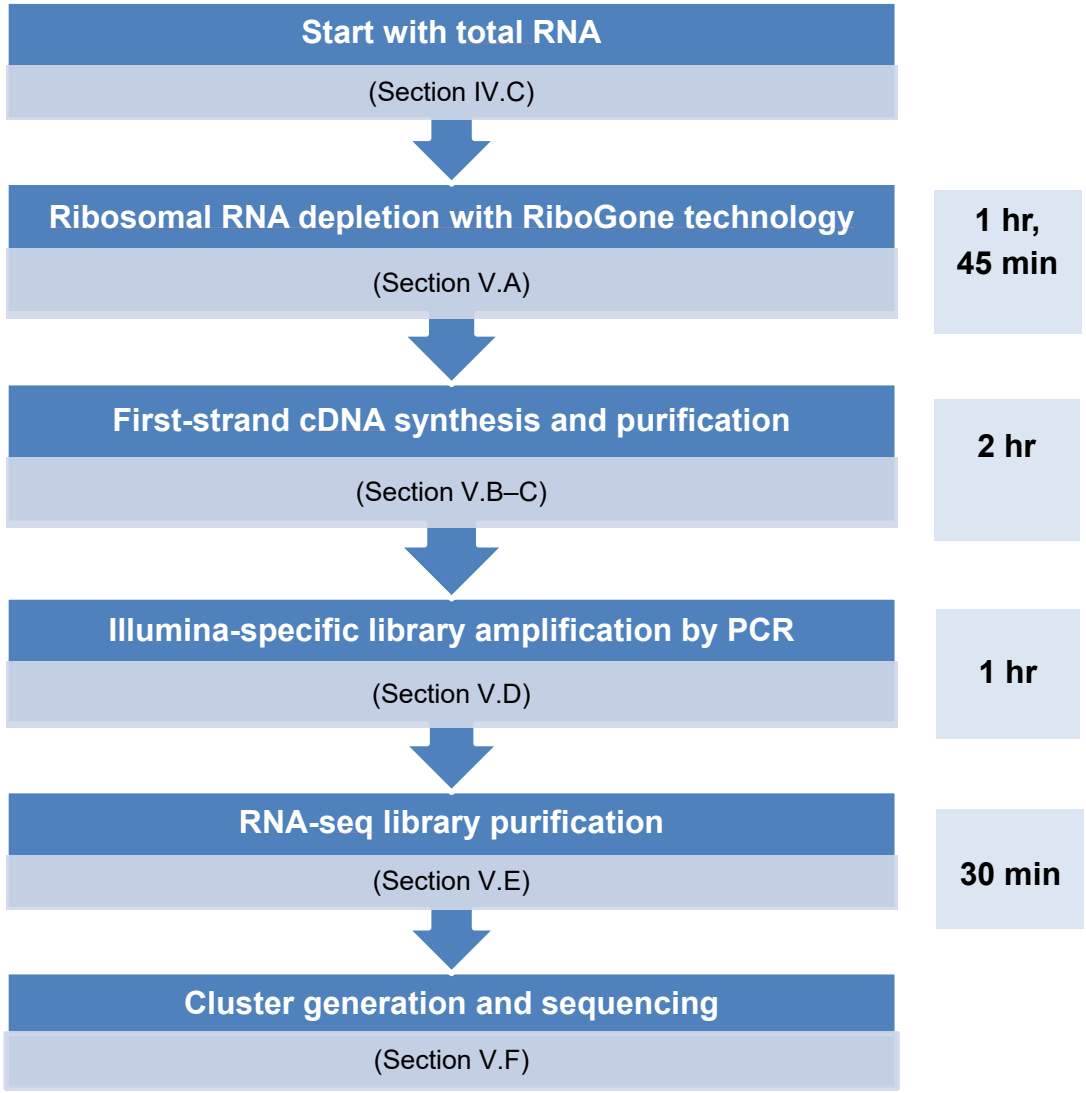

Figure 1. SMART-Seq Total RNA High Input (RiboGone Mammalian) protocol overview. Illumina-compatible RNA-seq libraries can be generated in around 5 hr.

Ribosomal RNA (rRNA) comprises a signification proportion (~90%) of total RNA samples. Depleting these abundant transcripts from total RNA samples prior to generating libraries provides benefit by lowering sequencing costs and improving mapping statistics. RiboGone technology (Figure 2, next page; section A) allows for the specific depletion of nuclear rRNA sequences (*5S*, *5.8S*, *18S*, and *28S*), as well as some mitochondrial rRNA sequences (*12S*), from human, mouse, or rat total RNA (Morlan et al. 2012). Sample incubation with the Total RNA Hyb Buffer allows the RiboGone oligos (Figure 2; wavy, dark green lines) to specifically bind to rRNA and deplete it by RNase H-mediated digestion. For more information on RiboGone technology, please visit our website.

After enzymatic cleanup, samples processed using the RiboGone reagents are ready for first-strand cDNA synthesis with random primers (Figure 2; section B). Prior to cDNA synthesis, RNA is heat fragmented in the presence of Mg^{2+} to unwind RNA secondary structures and facilitate the annealing of the modified N6 primer (the SMART Stranded N6 Primer, included in the 10X Total RNA First-Strand Buffer). As part of the template switching mechanism, when the PrimeScript™ Reverse Transcriptase (RT) reaches the 5′ end of the RNA fragment, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3′ end of the cDNA. The carefully-designed SMARTer® Stranded Oligonucleotide base-pairs with the non-templated nucleotide stretch, creating an extended template to enable PrimeScript RT to continue replicating to the end of the oligonucleotide (Chenchik et al. 1998). The resulting single-stranded cDNA contains the complete 5′ end of the mRNA as well as sequences that are complementary to the SMARTer Stranded Oligo. During the RT reaction, the SMARTer Stranded Oligo and SMART Stranded N6 Primer incorporate the Illumina Read Primer 1 (Figure 2; purple fragment) and Illumina Read Primer 2 (Figure 2; light green fragment) sequences, respectively. The directionality of the template-switching reaction preserves the strand orientation of the RNA, making it possible to obtain strand-specific sequencing data from the synthesized cDNA.

Indexed Illumina-compatible sequencing libraries are then generated by PCR amplification (Figure 2; section C) using the Unique Dual Index kits. This reaction makes use of PCR primers that contain the forward cluster-generating sequence P5 and indexing sequences (Figure 2; orange fragment) and the reverse cluster-generating sequence P7 and indexing sequences (Figure 2; red fragment). The number of indexes and specific sequences will depend on the Unique Dual Index kit you have purchased.

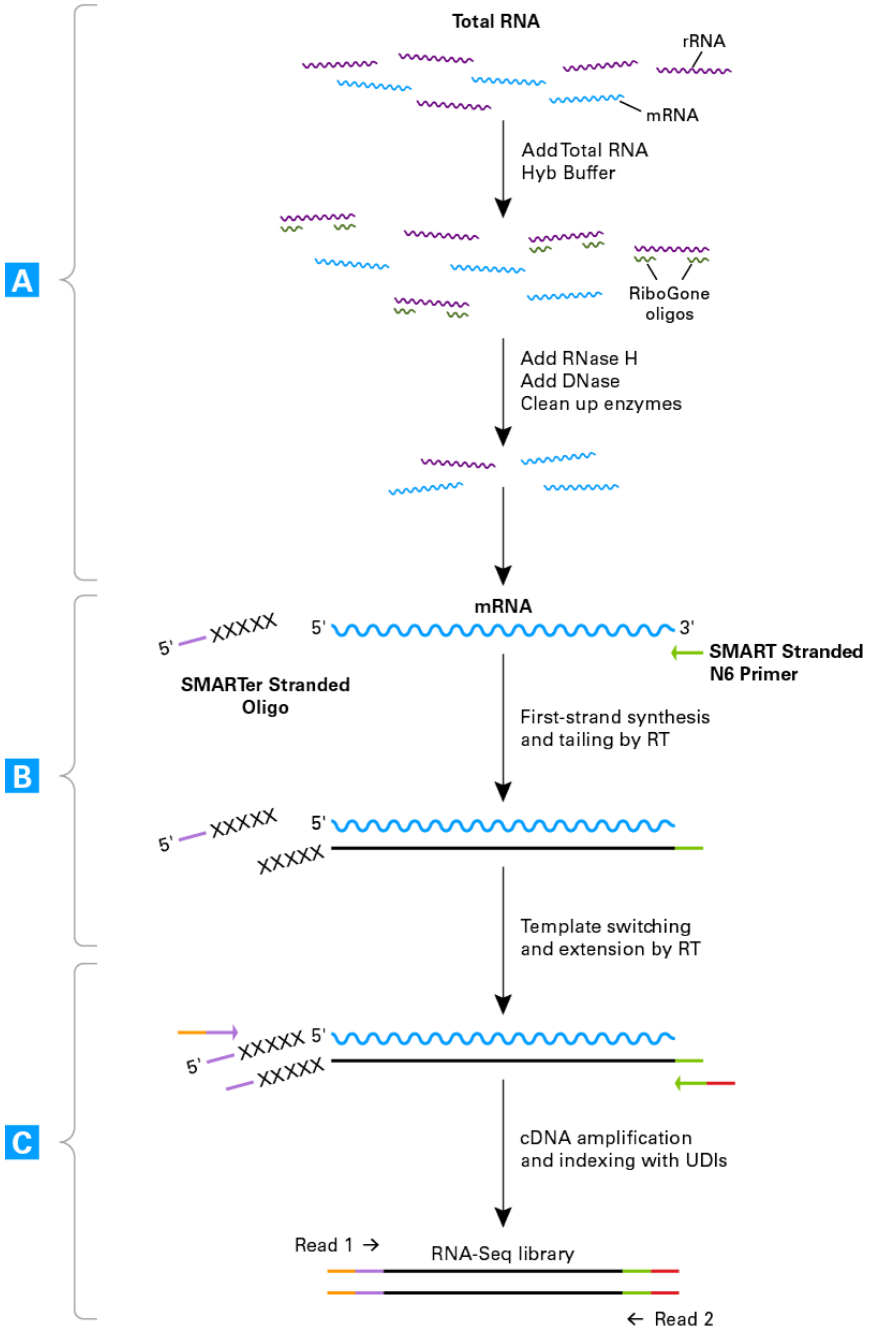

Figure 2. Flowchart of SMART-Seq Total RNA High Input (RiboGone Mammalian) library generation. RiboGone technology depletes rRNA from total RNA samples prior to library generation. Illumina-specific, indexed libraries are generated with SMART technology that eliminates enzymatic cleanup and adapter ligation. Primer fragments shown: Illumina Read Primer 1 in purple, Illumina Read Primer 2 in light green, cluster-generating sequence P5 in orange, cluster-generating sequence P7 in red.

II. List of Components

The components of SMART-Seq Total RNA High Input (RiboGone Mammalian) have been specifically designed to work together and are optimized for this protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or modification of the protocol may lead to unexpected results.

NOTE: UDI kits are not included and sold separately (Cat. Nos. 634752–634756). The user may select the appropriate size UDI kit depending on the number of libraries to be pooled; the UDI kits allow for preparing and pooling up to 384 Illumina-compatible libraries.

Table 1. SMART-Seq Total RNA High Input (RiboGone Mammalian) components.

SMART-Seq Total RNA High Input (RiboGone Mammalian)	Cap color	635046 (24 rxns)	635047 (96 rxns)	635045 (384 rxns)
Package 1 (Store at –70°C)				
Control Mouse Liver Total RNA (1 µg/µl)	Yellow	5 µl	5 µl	4 x 5 µl
SMARTer Stranded Oligonucleotide (12 µM)*	Pink	48 µl	192 µl	4 x 192 µl
Package 2 (Store at –20°C)				
Total RNA First-Strand Buffer (10X)	Red	48 µl	192 µl	4 x 192 µl
Total RNA Hyb Buffer (10X)	Clear	24 µl	96 µl	4 x 96 µl
RNase H	Blue	48 µl	192 µl	4 x 192 µl
RNase H Buffer (10X)	Purple	34 µl	136 µl	4 x 136 µl
RNase Inhibitor (40 U/µl)	White	110 µl	440 µl	4 x 440 µl
DNase I (5 U/µl)	Orange	96 µl	384 µl	4 x 384 µl
Stranded Elution Buffer†	-	1 ml	4 ml	4 x 4 ml
PrimeScript Reverse Transcriptase (200 U/µl)	Green	48 µl	192 µl	4 x 192 µl
Nuclease-Free Water	-	1 ml	3 ml	4 x 3 ml
SeqAmp™ DNA Polymerase	White	50 µl	200 µl	4 x 200 µl
SeqAmp PCR Buffer (2X)	-	1.25 ml	5 ml	4 x 5 ml

*Takara Bio proprietary sequences.
†Once thawed, store Stranded Elution Buffer at room temperature.

III. Additional Materials Required

The following reagents are required but not supplied. These materials have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- Single channel pipette: 10 µl, 20 µl, and 200 µl, two each (one for pre-amplification steps and one dedicated for PCR amplification)
- Eight-channel or 12-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 10 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips
- PCR thermal cyclers: two (one dedicated for pre-amplification steps and one dedicated for PCR amplification)

- NucleoMag NGS Clean-up and Size Select beads (Takara Bio, Cat. Nos. 744970.50, 744970.5, or 744970.500). If the NucleoMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter, Cat. Nos. A63880 & A63881) is an appropriate substitute.

  NOTES:
  - The kit has been specifically validated with the beads listed above. Please do not make any substitutions as it may lead to unexpected results.
  - We strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment and decreases the chances of contamination.
  - Magnetic beads should only be used at room temperature. Individual aliquoted tubes can be removed 30 minutes before an experiment to bring the beads to room temperature.

- 80% ethanol, freshly-made
- 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR-grade tube strips secured in a PCR rack, or 96-well plates (e.g., Bio-Rad iQ 96-Well PCR Plates, Cat. No. 2239441)
- Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates, such as:
  - For 12–24 samples:
    - SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011); accommodates two eight-tube or 12-tube strips
    - Minicentrifuge for 0.2 ml tubes or strips
  - For 24–96 or more samples:
    - Magnetic Stand-96 (Thermo Fisher Scientific, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher Scientific, Cat. No. AB0558)
    - Low-speed benchtop centrifuge for a 96-well plate

  NOTE: To prevent cross-contamination, we strongly recommend using separate magnets for pre-PCR steps (rRNA removal and purification of first-strand cDNA, Section V.A and V.C), and post-PCR purification of the RNA-seq library (Section V.E).

For Sample Preparation

- 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR-grade tube strips secured in a PCR rack, or 96-well plates (e.g., Bio-Rad iQ 96-Well PCR Plates, Cat. No. 2239441)
- Aluminum single tab foil seal (USA Scientific, Cat. No. 2938-4100) or cap strips (Thermo Fisher Scientific, Cat. No. AB0784/AB0850) for sealing tubes/plates

For PCR Amplification & Validation:

- Unique Dual Index Kit (Takara Bio, Cat. Nos. 634752–634756)
- High Sensitivity DNA Kit (Agilent Technologies, Cat No. 5067-4626) for Bioanalyzer
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854)
- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2 ml PCR 8-tube strip; Thermo Fisher Scientific, Cat. No. AB0264) or similar nuclease-free, PCR-grade, thin-wall PCR tubes, strips, or 96-well plates
- Nuclease-free, low-adhesion 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes

IV. General Considerations

A. Recommendations for Preventing Contamination

1. **Before you set up the experiment, it is advisable to have two physically separated workstations:**

   - **A PCR clean workstation** for all pre-PCR experiments that require clean room conditions such as rRNA depletion (Section V.A), first-strand cDNA synthesis (Section V.B), and purification of first-strand cDNA (Section V.C).
   - **A second workstation located in the general laboratory** where you will perform PCR to amplify the RNA-seq library (Section V.D), purify the RNA-seq library (Section V.E), and measure its concentration (Section V.F).

   IMPORTANT: The PCR workstation must be in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.

2. **Guidelines for clean room operation:**

   - Only move materials/supplies from the clean room to the general lab, NOT the other way around. Don't share any equipment/reagents between the clean room and the general lab.
   - Use a separate PCR machine inside the PCR workstation for cDNA synthesis.
   - Wear gloves and sleeve covers throughout the procedure to protect your RNA samples from degradation by contaminants and nucleases. Be sure to change gloves and sleeve covers between each section of the protocol.

B. General Requirements

- **IMPORTANT!** For products Cat. No. 635047 (96 rxns) and Cat. No. 635045 (4 x 96 rxns), we recommend performing a minimum of 12 reactions per protocol run to ensure sufficient reagents to utilize 96 (or 384) reactions per kit.
- **The success of your experiment depends on the quality of your starting RNA sample. Prior to rRNA removal and cDNA synthesis, please make sure that your RNA is free of contaminants.**
- The assay is very sensitive to variations in pipette volume, etc. Please make sure that all your pipettes are calibrated for reliable delivery, and that nothing adheres to the outside of the tips.
- All lab supplies related to cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not change the amount or concentration of any of the components in the reactions; they have been carefully optimized for the amplification reagents and protocol.
- If you are using this protocol for the first time, we **strongly recommend** that you perform negative and positive control reactions. The positive control should be at the same concentration as the sample RNA used in your experiment, and the negative control should have the same number of cycles as your samples.

C. Sample Requirements

1. Input RNA Length

- SMART-Seq Total RNA High Input (RiboGone Mammalian) was developed for total RNA with a RIN between 3–10.
- After RNA extraction, if your sample amount is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Cat. No. 5067-1511).

2. Input RNA Purity and Quantity

- **Purity of input RNA:** Input RNA should be free from genomic or carrier DNA and contaminants that would interfere with oligo annealing.

  IMPORTANT: Purified total RNA should be resuspended in Nuclease-Free Water, **not in TE or other buffers containing EDTA.** Chelation of divalent cations by EDTA will interfere with RNA fragmentation.

- **Volume and amount of input RNA:** This kit accommodates up to 9 µl of input RNA. This protocol has been optimized for rRNA removal and cDNA synthesis with 100 ng–1 µg of total mammalian RNA.

D. Sample Preparation

The sequence complexity and the average length of cDNA produced by this protocol is noticeably dependent on the quality of starting RNA material.

- There are several commercially available products that enable purification of total RNA preparations [e.g., Takara Bio offers the NucleoSpin RNA XS Kit (Cat. No. 740902.10)].
- If feasible, additional DNase treatment of purified RNA followed by purification/RNA revalidation is advisable, since column-based removal of DNA may not be sufficient. Single-stranded DNA (present in the RNA prep or generated during RNA heat fragmentation) may be randomly primed and used as a template by the reverse transcriptase, incorporating genomic DNA into the final library.
- When choosing a purification method (kit), ensure that it is appropriate for your sample amount.

V. Protocols

A. Protocol: rRNA Removal

This step removes specific nuclear rRNA sequences (*5S*, *5.8S*, *18S*, and *28S*), as well as mitochondrial rRNA sequences (*12S*), from human, mouse, or rat total RNA. Samples processed with this section of the protocol are ready for cDNA synthesis with the included random primers.

NOTES:

- Bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min and mix well by vortexing.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. In nuclease-free 0.2 ml PCR-grade tubes, strip tubes, or 96-well plate, mix the following components on ice:

1–9 µl	Total RNA sample (100 ng–1 µg)
0–8 µl	Nuclease-Free Water*
1 µl	10X Total RNA Hyb Buffer
10 µl	**Total volume per reaction**

*Adjust the volume of Nuclease-Free Water proportional to the volume of sample to ensure the total volume per reaction is 10 µl.

2. Incubate the reactions in a thermal cycler at 95°C for 2 min then decrease the temperature at a rate of –0.1°C/sec, down to 25°C (11 min, 40 sec).
3. Add 2 µl of RNase H, 1 µl of Rnase Inhibitor, and 1.4 µl of 10X Rnase H Buffer to the reaction; mix well by vortexing and centrifuge briefly (~2,000*g*) to collect the liquid at the bottom of each tube.
4. Incubate the reactions in a thermal cycler at 37°C for 30 min.
5. Add 4 µl of Dnase I to each reaction and incubate at 37°C for an additional 15 min.
6. Add 33 µl of NucleoMag NGS Clean-up and Size Select beads to each reaction and mix by pipetting the entire volume up and down 7–8 times.

   NOTE: The beads are viscous; pipette the entire volume and push it out slowly.
7. Incubate the reactions at room temperature for 8 min.
8. Place the reaction tubes on a magnetic separation device for 3–5 min, or until all the reactions have become completely clear. While the tubes are sitting on the magnetic stand, carefully pipette out the supernatants.
9. Add 200 µl of freshly prepared 80% ethanol to each sample without disturbing the bead pellets. Wait for 30 sec and carefully pipette out the supernatants.
10. Repeat Step 9 once.
11. Centrifuge briefly (~2,000*g*) to collect the residual ethanol at the bottom of the tubes. Place the tubes on the magnetic stand for approximately 30 sec, then remove all remaining ethanol with a pipette.
12. Allow the tubes to stand at room temperature for 3–5 min until all the bead pellets appear dry.
13. Resuspend each bead pellet in 15 µl of Nuclease-Free Water by slowly pipetting the entire volume up and down 7–8 times.
14. Return the tubes to the magnetic separation device until they are completely clear.
15. Transfer 13.5 µl of each supernatant into a fresh tube or plate. These are your final samples for use in cDNA synthesis (Section V.B).

B. Protocol: First-Strand cDNA Synthesis

During this step, RNA is fragmented and converted to single-stranded cDNA.

IMPORTANT: The following protocol is designed for total RNA with a RIN value of 3–10. The first two steps will simultaneously fragment and prime the RNA for cDNA synthesis.

1. Add 2 µl of 10X Total RNA First-Strand Buffer to the 13.5 µl of supernatant resulting from Section V.A (after Step 15).

   NOTE: 10X Total RNA First-Strand Buffer is cloudy when frozen. When fully thawed, vortex to completely resuspend all components before use.

2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler for 3 min, then place the samples on ice for 2 min.

   IMPORTANT: While the samples are incubating, perform Step 3 (preparing your master mix), except for adding the PrimeScript Reverse Transcriptase. You will want to be prepared to proceed to Step 4 with no delay when the cooldown is complete, as the following steps are critical for first-strand cDNA synthesis.

3. In a nuclease-free, 1.5 ml PCR-grade tube, prepare enough master mix for all reactions, plus 10% of the total volume, by combining the following reagents on ice, in the order shown.

2 µl	SMARTer Stranded Oligonucleotide (12 µM)
0.5 µl	RNase Inhibitor
2 µl	PrimeScript Reverse Transcriptase (200 U/µl)*
4.5 µl	**Total volume per reaction**

   *Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing for 3–5 sec and spin the tube briefly in a microcentrifuge to collect the contents at the bottom.

4. Add 4.5 µl of the master mix to each reaction tube from Step 2. Mix the contents of the tubes by gently pipetting and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated thermal cycler at 42°C for 60 min.
6. Terminate the reaction by heating the tubes at 70°C for 10 min, then leave them in the thermal cycler at 4°C.

SAFE STOPPING POINT: The tubes can be stored at 4°C overnight.

C. Protocol: Purification of First-Strand cDNA

The first-strand cDNA selectively binds to NucleoMag NGS Clean-up and Size Select beads, leaving unincorporated nucleotides and small (<100 bp) cDNA fragments in solution which are removed by a magnetic separation. The beads are then directly used for RNA-seq library amplification.

NOTES:
- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min and mix well by vortexing.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. If purification is performed directly in the PCR tubes or strips using the Takara Bio SMARTer-Seq Magnetic Separator - PCR Strip, add 20 µl of beads to each sample. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 2.

   (Optional) If you are performing purification with the Thermo Fisher Magnetic Stand-96 (recommended if processing 48–96 samples), cDNA samples need to be transferred to a 96-well V bottom plate. Distribute 20 µl of beads to each well of the 96-well V-bottom plate, and then use a multichannel pipette to transfer the cDNA. Pipette the entire volume up and down at least 10 times to mix thoroughly. Proceed to Step 2.

   NOTE: The beads are viscous; pipette up the entire volume and push it out slowly.

2. Incubate at room temperature for 8 min to let cDNA bind to the beads.

3. Briefly spin the sample tubes or plate to collect the liquid from the walls of the tube/wells. Place the sample tubes/plate on the magnetic separation device for 5 min or longer, until the solution is completely clear.
4. While the tubes/plate are sitting on the magnetic stand, pipette out the supernatant.
5. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant.
6. Repeat Step 5 once.
7. Perform a brief spin of the tubes or plate (~2,000*g*) to collect the remaining ethanol at the bottom of each tube/well. Place the tubes/plate on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes/plate rest open at room temperature for ~3–5 min until the pellet appears dry.

NOTE: Check the pellet frequently during this time and continue to the next section when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you would like additional assistance with this determination.

https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

If the beads are overdried, there will be cracks in the pellet. If this occurs, PCR efficiency will be reduced, resulting in lower yields.

D. Protocol: RNA-Seq Library Amplification by PCR

The purified first-strand cDNA is amplified into RNA-seq libraries using SeqAmp DNA Polymerase and the unique dual-indexed primers from the Unique Dual Index Kit.

IMPORTANT:

– Optimal parameters may vary with different templates and cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend performing a range of cycles.

– It is not recommended to perform more than 14 cycles. If 14 cycles is determined to be the optimal number of cycles for your sample and conditions, elute the first-strand cDNA in 20 µl of Nuclease-Free Water and perform the entire first-strand cDNA purification (Section V.C) a second time before proceeding to PCR amplification (Section V.D).

1. Prepare a PCR master mix for all reactions. **Separate master mixes should be prepared for different library indexes.**

   For each PCR master mix, combine the following reagents in the order shown, plus 10% of the total master mix volume, then mix well and spin the tube briefly in a microcentrifuge:

25 µl	SeqAmp PCR Buffer (2X)
2 ul	UDI
1 µl	SeqAmp DNA Polymerase
22 µl	Nuclease-Free Water
50 µl	**Total volume per reaction**

2. Add 50 µl of a PCR master mix to each tube containing cDNA (bound to the beads) from Section V.C., Step 8. Mix well, making sure that the beads are uniformly resuspended.

3. Place the tube in a preheated thermal cycler with a heated lid. Start thermal cycling using the following program:

94°C		1 min
12–14 cycles		
(see note):		
	98°C	15 sec
	55°C	15 sec
	68°C	1 min
4°C		forever

NOTE: If the starting sample is 1 µg, use 12 cycles. If the starting sample is 100 ng, use 14 cycles.

E. Protocol: Purification of the RNA-Seq Library

The amplified RNA-seq library is purified by immobilizing it onto NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol and eluted in Stranded Elution Buffer. The following steps should be performed on a bench designated for post-PCR work.

NOTES:
- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min and mix well by vortexing.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a second magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. If purification is performed directly in the PCR tubes or strips using the Takara Bio SMARTer-Seq Magnetic Separator - PCR Strip, add 50 µl of beads to each sample. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 2.

   (Optional) If you are performing purification with the Thermo Fisher Magnetic Stand-96 (recommended if processing 48–96 samples), cDNA samples need to be transferred to a 96-well V bottom plate. Distribute 50 µl of beads to each well of the 96-well V-bottom plate, and then use a multichannel pipette to transfer the cDNA. Pipette the entire volume up and down at least 10 times to mix thoroughly. Proceed to Step 2.

   REMINDER: The beads are viscous; pipette up the entire volume and push it out slowly.

2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes/plate on the magnetic separation device for 5 min or longer, until the solution is completely clear.
4. While the tubes/plate are sitting on the magnetic stand, pipette out the supernatant.
5. Keep the tubes/plate on the magnetic stand. Add 200 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.

7. Perform a brief spin of the tubes/plate (~2,000*g*) to collect the remaining ethanol at the bottom of each tube/well. Place the tubes/plate on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes/plate rest open at room temperature for ~3–5 min until the pellet appears dry.

   NOTE: Check the pellet frequently during this time and continue to Step 9 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you would like additional assistance with this determination.

   https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

   If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

9. Add 20 µl of Stranded Elution Buffer to cover the beads. Remove the tubes/plate from the magnetic stand and mix thoroughly to resuspend the beads.

   NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube. We recommend vortexing or directly pipetting the beads up and down to ensure complete dispersion.

10. Incubate at room temperature for 2 min to rehydrate.
11. Mix by pipetting up and down 10 times to elute the DNA from the beads.
12. Place the tubes/plate back on the magnetic stand for 1 minute or longer, until the solution is completely clear.
13. Transfer the clear supernatant containing the purified RNA-seq library from each tube to a new PCR tube or plate.

F. Protocol: Determination of Amplified Library Quality and Quantity

1. Quantify libraries using fluorescence-detection-based methods such as the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Cat. No. Q32854).
2. Evaluate library size distribution by running samples on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) or an equivalent microfluidic device/kit. Dilute libraries to about 1.5 ng/µl prior to loading the chip (for a consistent library-to-library profile).
3. Compare the results for your samples and controls (if performed) to determine whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield a distinct peak spanning 200–1,000 bp, peaked at ~300 bp for the positive control RNA sample (Figure 3, Panel A), and no product in the negative control (Figure 3, Panel B). The library yield should be 2–10 ng/µl (depending on the input and number of cycles).

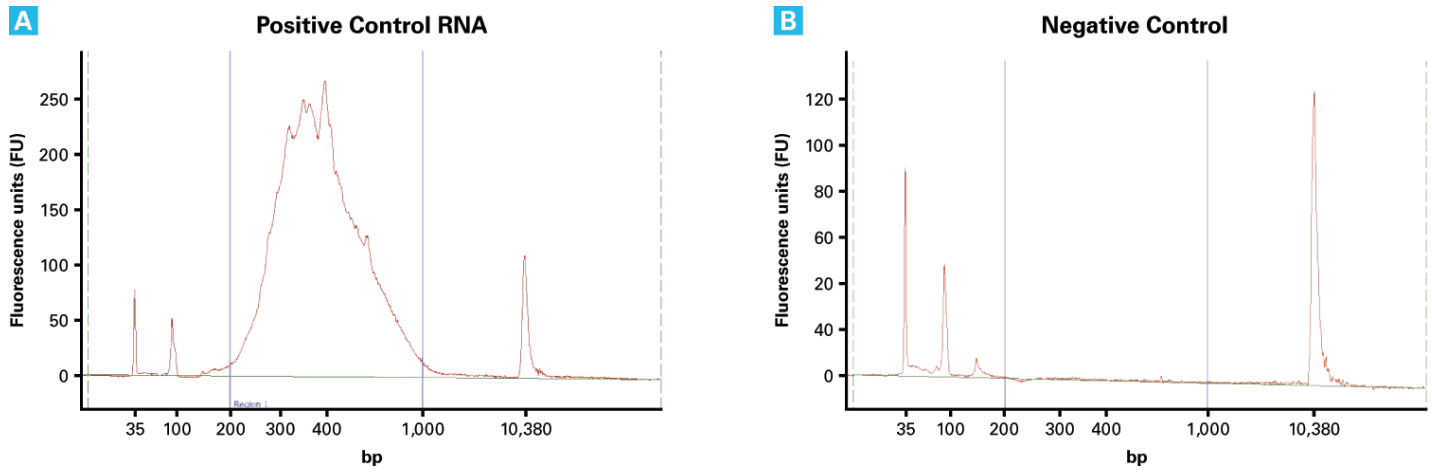

Figure 3. Electropherogram example results from Agilent 2100 Bioanalyzer. Samples were treated according to the protocol for the SMART-Seq Total RNA High Input (RiboGone Mammalian). FU = fluorescence absorption units. **Panel A**. The expected product starting from 1 µg of Control Mouse Liver Total RNA. **Panel B**. No peak is visible for the negative control (Nuclease-Free Water in place of control RNA).

VI. References

Chenchik, A., Zhu, Y., Diatchenko, L., R, L. & Hill, J. RT-PCR Methods for Gene Cloning and Analysis. *Biotechniques* 305–319 (1998).

Morlan, J. D., Qu, K. & Sinicropi, D. V. Selective depletion of rRNA enables whole transcriptome profiling of archival fixed tissue. (2012).

Appendix: Sequencing Guidelines

A. Sequencing Analysis Guidelines

- The first three nucleotides of the first sequencing read (Read 1) are derived from the template-switching oligo. These three nucleotides must be trimmed prior to mapping.
- Read 1 is derived from the sense strand of the input RNA.
- If you are performing paired-end sequencing, Read 2 will correspond to the antisense strand.

B. Pooling Recommendations

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained with the Qubit with the average library size determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for the conversion: http://www.molbiol.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using our Library Quantification Kit (Takara Bio, Cat. No. 638324).

Most Illumina-sequencing library preparation protocols require libraries with a final concentration of 2 nM or 4 nM, depending on the sequencing platform. Lower concentrations can also be accommodated, depending on the instrument.

Prepare a pool of 2 nM (or 4 nM) as follows:

1. Dilute each library to 2 nM (or 4 nM) in Nuclease-Free Water. To avoid pipetting error, use at least 2 µl of each original library for dilution.

2. Pool the diluted libraries by combining an equal amount of each library in a low-bind 1.5 ml tube. Mix by vortexing at low speed or by pipetting up and down. Use at least 2 µl of each diluted library to avoid pipetting error.
3. Depending on the Illumina sequencing library preparation protocol, use a 5 µl aliquot (for the 4 nM concentration) or a 10 µl aliquot (for the 2 nM concentration) of the pooled libraries. Follow the library denaturation protocol according to the latest edition of your Illumina sequencing instrument's user guide.

If you are planning to include a PhiX control spike-in, make sure to combine the aliquot with an appropriate amount of the PhiX control. See our recommendations below (Table 2) regarding the amount of PhiX control to include with stranded libraries.

Follow Illumina guidelines on how to denature, dilute and combine a PhiX control library with your own pool of libraries.

PhiX Control Spike-In Recommendations

Illumina cluster detection algorithms are optimized around a balanced representation of A, T, G, and C nucleotides. SMART-Seq stranded libraries can have a lower-than-average pass filter rate due to the low complexity observed in the first three cycles. To alleviate this issue, libraries should be combined with a PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in. Make sure to use a fresh and reliable stock of the PhiX control library. Spike in a PhiX control at about 10% or more of the total library pool, depending on the instrument (see Table 2).

Table 2. PhiX Control Spike-in Guidelines for Various Illumina Sequencing Instruments.

Sequencing instrument	PhiX (%)
MiSeq®	5–10*
HiSeq® 1500/2000/2500	10
HiSeq 3000/4000	20
NextSeq®/MiniSeq™	20

*Optional. A typical MiSeq run generates a high passing filter rate. While the inclusion of a PhiX spike-in can be beneficial, it does not significantly improve overall performance.

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This document has been reviewed and approved by the Quality Department.