Takara Bio USA, Inc.

SMART-Seq® Total RNA Mid Input User Manual

Cat. Nos. 635048, 635049 & 635050
(120523)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

Table of Figures

Table of Tables

I. Introduction

SMART-Seq Total RNA Mid Input (Cat. Nos. 635048, 635049 & 635050) can be used to generate indexed total RNA sequencing (RNA-seq) libraries suitable for next-generation sequencing (NGS) on any Illumina® platform, starting from as little as 100 pg of poly(A)-purified or ribosomal RNA-depleted RNA. Up to 384 indexed libraries can be prepared using our Unique Dual Index Kits (Takara Bio, Cat. Nos. 634752–634756, sold separately).

Ribosomal RNA depletion can be performed using RiboGone™ Mammalian (Takara Bio, Cat. Nos. 634846 & 634847, sold separately) which removes ribosomal RNA (rRNA) (*5S*, *5.8S*, *18S*, and *28S*) and some mitochondrial rRNA (*12S*) sequences from 10–100 ng of full-length or sheared total RNA from human, mouse, or rat.

The entire library construction protocol can be completed in less than 4 hr (Figure 1). The kit utilizes our proprietary SMART® (**S**witching **M**echanism **A**t 5′ end of **R**NA **T**emplate) technology, coupled with PCR amplification, to generate Illumina-compatible libraries without the need for enzymatic clean-up or adapter ligations. 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.

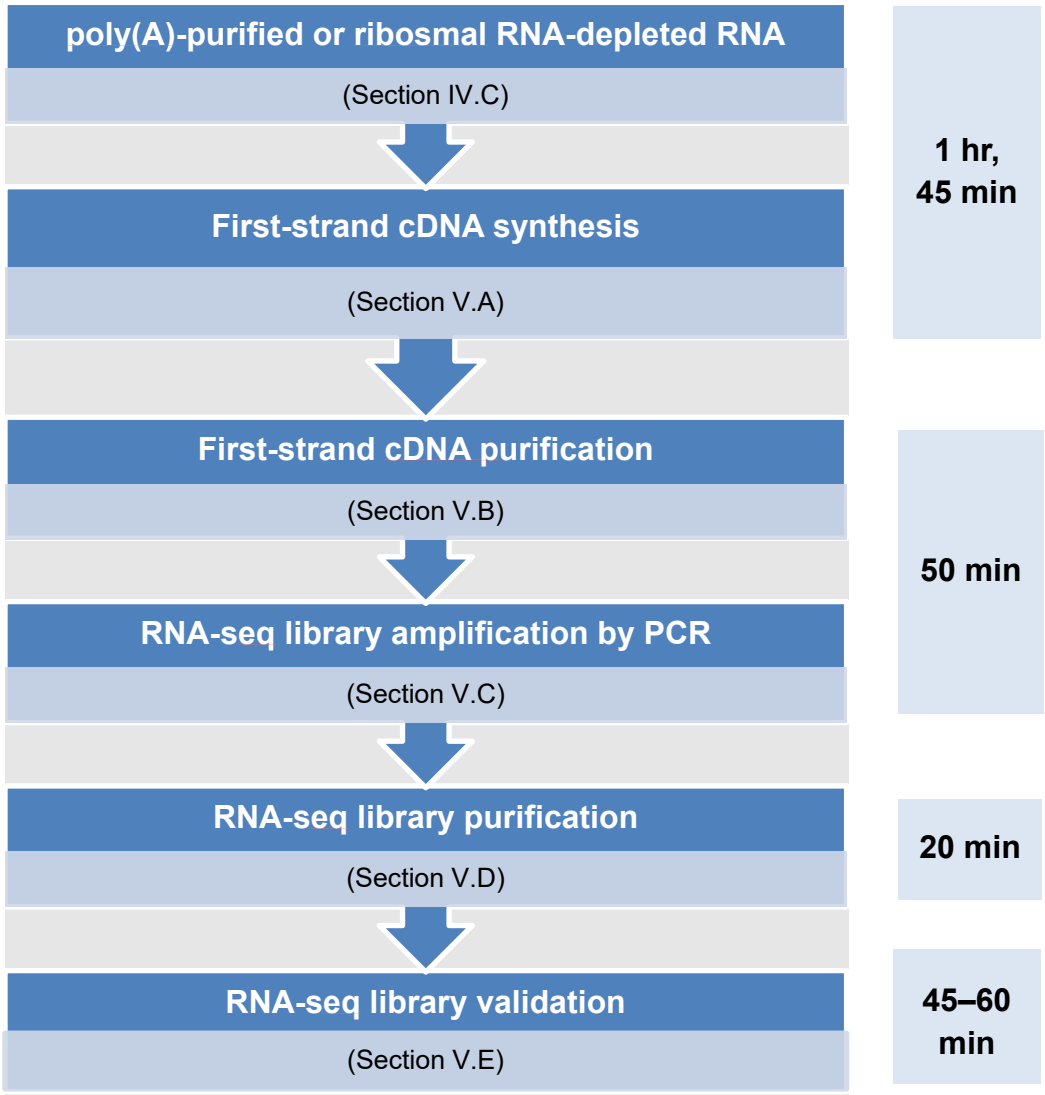

Figure 1. SMART-Seq Total RNA Mid Input protocol overview. This protocol can be completed in less than 4 hr.

SMART-Seq Total RNA Mid Input starts with sub-nanogram amounts of RNA. The RNA is heat fragmented in the presence of Mg^{2+} to generate fragments required for the construction of Illumina-compatible libraries. A modified N6 primer (the SMART Stranded N6 Primer) primes the first-strand synthesis reaction (Figure 2).

NOTE: If your sample is degraded or of low quality (RNA Integrity Number; RIN <3), see Appendix A for a fragmentation-free protocol.

When the SMARTScribe™ Reverse Transcriptase (RT) reaches the 5′ end of the RNA fragment, the enzyme's terminal transferase activity adds a few nontemplated nucleotides to the 3′ end of the cDNA. The carefully-designed SMARTer® Stranded Oligonucleotide base-pairs with the non-templated nucleotides, creating an extended template to enable the SMARTScribe RT to continue replicating to the end of the oligonucleotide (Chenchik et al. 1998). The resulting full-length, single-stranded cDNA contains the complete 5′ end of the mRNA, as well as sequences that are complementary to the SMARTer Stranded Oligo.

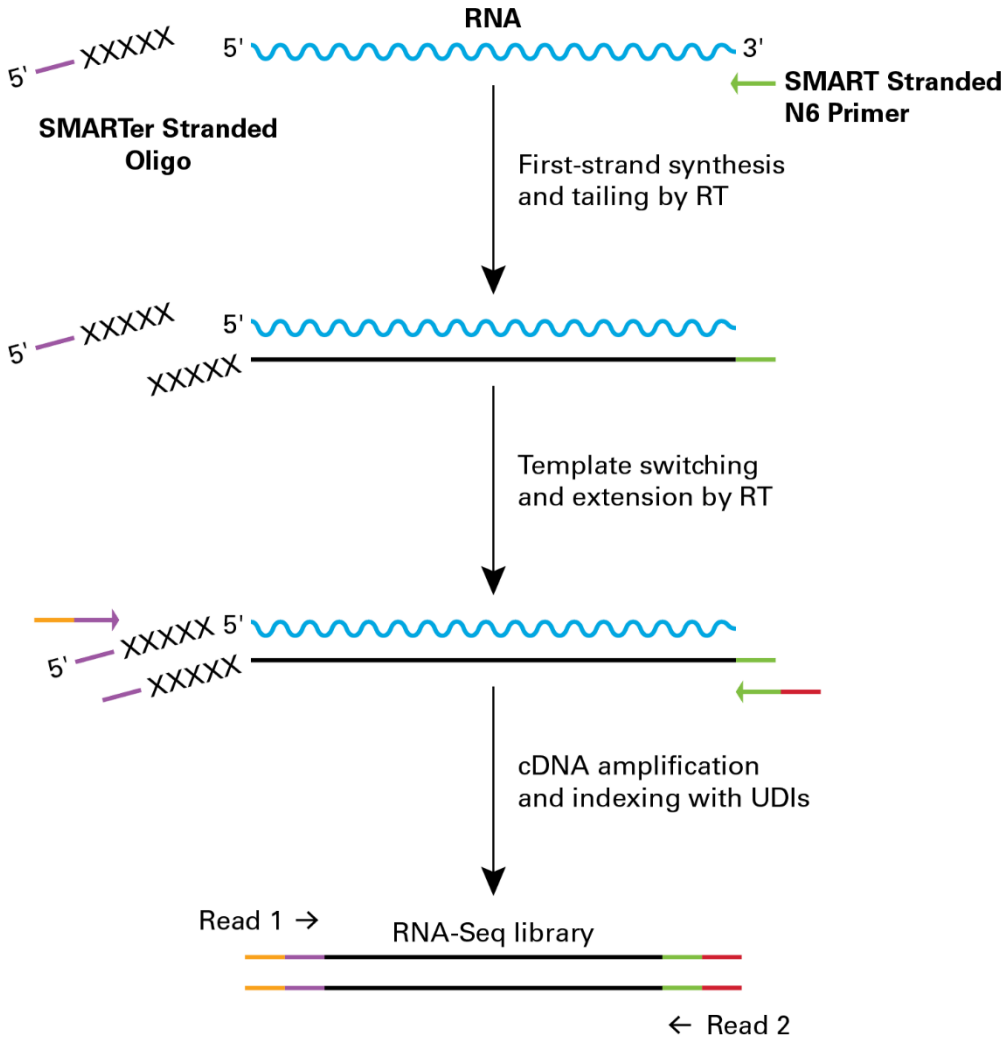

Figure 2. Flowchart of SMART-Seq Total RNA Mid Input library generation.

II. List of Components

The components of SMART-Seq Total RNA Mid Input have been specifically designed to work together and are optimized for this particular 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). You 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 Mid Input components.

SMART-Seq Total RNA Mid Input	Cap color	635049 (24 rxns)	635050 (96 rxns)	635048 (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)				
SMART Stranded N6 Primer (12 µM)*	Green	24 µl	96 µl	4 x 96 µl
First-Strand Buffer (RNase-Free) (5X)	Red	96 µl	384 µl	4 x 384 µl
dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)	Green	48 µl	192 µl	4 x 192 µl
Dithiothreitol (DTT; 100 mM)	Orange	24 µl	96 µl	4 x 96 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	48 µl	192 µl	4 x 192 µl
Nuclease-Free Water	-	1 ml	3 x 1 ml	12 x 1 ml
RNase Inhibitor (40 U/µl)	White	55 µl	55 µl	4 x 55 µl
Stranded Elution Buffer†	-	1 ml	4 ml	4 x 4 ml
SeqAmp™ DNA Polymerase	White	50 µl	100 µl	4 x 100 µl
SeqAmp PCR Buffer (2X)	-	1.25 ml	2.5 ml	4 x 2.5 ml

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

III. Additional Materials Required

The following materials are required but not supplied. They 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
- Eight-channel or 12-channel pipette (recommended): 10 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

For PCR Amplification & Validation:

- One dedicated PCR thermal cycler used only for first-strand synthesis
- High Sensitivity DNA Kit (Agilent Technology, Cat No. 5067-4626)

- 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

For cDNA and library Bead Purification:

- 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.
- 80% ethanol, freshly made.
- 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 8-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 purification of first-strand cDNA (Section V.B) and purification of the RNA-seq library (Section V.D).

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 first-strand cDNA synthesis (Section V.A) and purification of first-strand cDNA (Section V.B).
   - **A second workstation located in the general laboratory** where you will perform PCR to amplify the RNA-seq library (Section V.C), purify the RNA-seq library (Section V.D), and measure its concentration (Section V.E).

   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 Cat. No. 635050 (96 rxns) and Cat. No. 635048 (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 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 is attached 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 increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations 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 to verify that kit components are working properly. 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 all of your samples. It can also be useful to perform an additional control reaction starting with your method of rRNA depletion or poly(A) enrichment and using the Control Mouse Liver Total RNA processed along with your test samples.

C. Sample Requirements & Preparation

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

- There are several commercially available products that enable purification of total RNA preparations from extremely small sample amounts [e.g., we offer the **NucleoSpin RNA XS Kit** (Cat. No. 740902.10)].
- When choosing a purification method (kit), ensure that it is appropriate for your sample amount.

1. Ribosomal RNA (rRNA) Depletion

Prior to cDNA synthesis using this kit (Section V.A), we strongly recommend removing rRNA from the sample. For 10–100 ng of input total RNA, we recommend the RiboGone - Mammalian (Cat. Nos. 634846 & 634847) rRNA depletion kit. This kit specifically and efficiently degrades *5S*, *5.8S*, *18S*, and *28S* nuclear rRNA and *12S* mtRNA from mouse, rat, and human total RNA samples.

2. Input RNA Integrity

- SMART-Seq Total RNA Mid Input is compatible with full-length or compromised RNA. Fragmentation times for RNA of intermediate lengths are provided in Section V.A. An alternative protocol for degraded samples is available in Appendix A.
- After RNA extraction, if your sample amount is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Agilent Technology, Cat. No. 5067-1513).

3. Input RNA Purity

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

  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.

- **If feasible:** additional DNase treatment of purified RNA, followed by purification and 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.

4. Input RNA Quantity

- Sample input should be 100 pg–100 ng poly(A)-purified, rRNA-depleted, or otherwise-purified RNA samples, or 10 ng–100 ng total RNA, after rRNA depletion with RiboGone.
- The protocol has been optimized for cDNA synthesis starting from 100 pg (0.1 ng) of RNA. However, if your RNA sample is not limiting, we recommend that you start with more than 1 ng of RNA.
- This kit accommodates up to 8 µl of input RNA.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

During this step, RNA is fragmented and converted to single-stranded (ss) cDNA that contains sequences complementary to the SMARTer Stranded Oligo.

IMPORTANT:

– This protocol was designed to fragment RNA for a final mean library insert size of ~180 nucleotides (nt). The first two steps will simultaneously fragment and prime the RNA for cDNA synthesis. See instructions below on RNA shearing conditions, depending on the RIN or its equivalent.

– For some RNA samples or sequencing applications, it may be appropriate to titrate the fragmentation time to achieve optimal yield and library size.

– **When working with degraded RNA samples with RIN <3,** use the "First-Strand cDNA Synthesis Protocol for Degraded Samples" in Appendix A instead of this step, because additional fragmentation is unnecessary and will result in lower library yields.

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

1–8 µl	Sample RNA (0.1–100 ng)
1 µl	SMART Stranded N6 Primer (12 µM)
4 µl	5X First-Strand Buffer (RNase-Free)
0–7 µl	Nuclease-Free Water
13 µl	**Total volume per reaction**

2. Incubate the tubes/plate at 94°C in a preheated, hot-lid thermal cycler for an appropriate time depending on the RIN of the RNA sample, then place the samples on ice for 2 min.

Table 2. Incubation time for RNA samples by RIN value

RIN	Time
>7	5 min
4–7	4 min
3	3 min

NOTE: Steps 4–6 should not be delayed after completing Step 2, since they are critical for first-strand cDNA synthesis. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 3), while your tubes are incubating (Step 2) in order to jump start the cDNA synthesis.

3. Prepare enough master mix for all reactions, plus 10% of the total volume, by combining the following reagents in the order shown on ice.

0.5 µl	DTT (100 mM)
0.5 µl	RNase Inhibitor
2 µl	dNTP Mix (10 mM)
2 µl	SMARTer Stranded Oligo (12 µM)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
7 µl	**Total volume per reaction**

*Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing and spin the tubes briefly in a microcentrifuge.

4. Add 7 µl of the master mix from Step 3 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 90 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 until the next step (Section V.B).

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

B. 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 is 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.

– Clean-up must be performed using the recommended beads. **Spin columns do not adequately remove adapter-dimers from the reactions and will result in experimental failure!**

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 DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the walls of the tube. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.
4. While the tubes 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, in order 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 (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes 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.

9. If using less than 10 ng of input RNA or if performing more than 12 PCR cycles, elute the cDNA in 20 µl of Nuclease-Free Water as described below (Steps 9a–d), this ensures complete removal of adapter primers.
   a. Add 20 µl Nuclease-Free Water to the pellet from Step 8.
   b. Thoroughly resuspend the beads and allow to rehydrate for 2 min.
   c. Briefly spin the sample tubes to collect the liquid from the walls of the tube. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
   d. Transfer the supernatant to a fresh 0.2 ml tube.
   e. Repeat Steps 1–8 above before proceeding to section V.C.

C. 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 thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles.

Table 3. Cycling Guidelines Based on the Input Amount of rRNA-depleted or poly(A)-enriched RNA.

Amount of Input RNA (ng)	Typical Number of PCR Cycles
0.1	18
1	16
10	12
100	9

1. Prepare a PCR Master Mix for all reactions. **Separate master mixes should be prepared for different library indexes.** Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge:

   PCR Master Mix:

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

2. Add 50 µl of PCR Master Mix to each tube containing cDNA (bound to the beads) resulting from Section V.B. 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	
*N** cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C	forever	

   *The number of cycles (*N*) depends on the amount of input RNA. See Table 3 (above) for guidelines.

D. 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 on the magnetic separation device for 5 min or longer, until the solution is completely clear.
4. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
5. Keep the tubes 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 (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes 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. Once the beads are dried, add 20 µl of Stranded Elution Buffer to cover the beads. Remove the tubes 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 min or longer, until the solution is completely clear.
13. Transfer the clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free, low-adhesion tube.

E. Protocol: Validation Using the Agilent 2100 Bioanalyzer

1. Dilute 1 µl of the amplified RNA-seq library with 3 µl Stranded Elution Buffer.
2. Use 1 µl of the diluted sample for validation using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from the High Sensitivity DNA Kit (Agilent Technology, Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
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 150–1,000 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 RNA-seq library yield should be >7.5 nM (depending on the input and number of cycles).

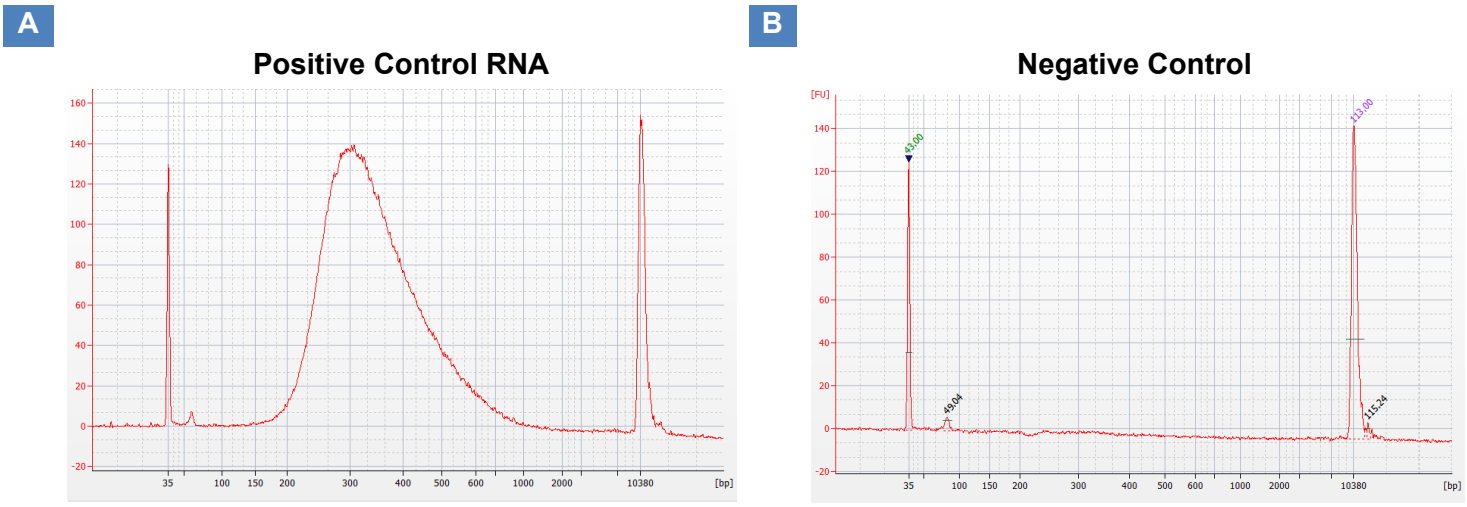

Figure 3. Example electropherogram results from Agilent 2100 Bioanalyzer.

VI. References

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

Appendix A: First-Strand cDNA Synthesis Protocol (for Degraded Samples)

Our typical protocol for first-strand cDNA synthesis (Section V.A) includes simultaneous RNA fragmentation. If your RNA has a RIN <3 (or equivalent), use this alternative protocol for first-strand cDNA synthesis.

1. Mix the following components on ice:

1–8 µl	Sample RNA (0.1–100 ng)
1 µl	SMART Stranded N6 Primer (12 µM)
0–7 µl	Nuclease-Free Water
9 µl	**Total volume per reaction**

2. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min, then put the samples on ice for 2 min.

NOTE: Steps 4–6 should not be delayed after completing Step 2, since they are critical for first-strand cDNA synthesis. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 3), while your tubes are incubating (Step 2) in order to jump start the cDNA synthesis.

3. Prepare enough master mix for all reactions, plus 10% of the total volume, by combining the following reagents in the order shown on ice.

4 µl	5X First Strand Buffer
0.5 µl	DTT (100 mM)
0.5 µl	RNase Inhibitor
2 µl	dNTP Mix (10 mM)
2 µl	SMARTer Stranded Oligo (12 µM)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
11 µl	**Total volume per reaction**

*Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing and spin the tubes briefly in a microcentrifuge.

4. Add 11 µl of the master mix from Step 3 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 90 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 until the next step (Section V.B).

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

Appendix B: 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 corresponds 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 library size as determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for this conversion: http://www.molbiol.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using the 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 errors, 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 errors.
3. Depending on the Illumina sequencing library preparation protocol, use a 5 µl aliquot (for the 10 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 4) 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 spike-in (Illumina, Cat. No. FC-110-3001). Make sure to use a fresh, 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 3).

Table 4. 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 PhiX spike-in can be beneficial, it does not significantly improve overall performance.

C. Extra Precautions When Using NextSeq and MiniSeq Instruments

- Both systems use automatic adapter trimming by default. This can unexpectedly shorten your reads and cause the reads to change from the original sequence to a poly(N) sequence because of the default mask setting in BaseSpace. The minimum mask length is 35 cycles, and any trimmed reads shorter than 35 bases will become poly(N) reads. This can be problematic if the RNA input was very fragmented.

  We strongly recommend that you turn off automatic adapter trimming by creating a custom library prep kit program without adapter trimming. More information can be found on the Illumina website.

- Due to the algorithm's sensitivity to low complexity (found in the first three nucleotides of Read 1), NextSeq and MiniSeq runs without PhiX spike-ins may result in low-quality sequencing reads and incorrect base calling. Therefore, we strongly recommend adding 20% of PhiX spike-in when using current NextSeq 500/550 v2 or MiniSeq sequencing reagent kits.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com/service	web: takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

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