Takara Bio USA, Inc.

SMARTer® Stranded Total RNA Sample Prep Kit - HI Mammalian User Manual

Cat. Nos. 634873, 634874, 634875, 634876, 634877, 634878
(092617)

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I. Introduction

SMARTer cDNA Synthesis for the Illumina® Sequencing Platform

The **SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian** (Cat. Nos. 634873, 634874, 634875, 634876, 634877, 634878) includes the components needed to generate indexed cDNA libraries suitable for next-generation sequencing (NGS) on any Illumina platform, with input ranges from 100 ng to 1 µg of total mammalian RNA. The kits consist of the SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian Components, SeqAmp™ DNA Polymerase, and the Indexing Primer Set HT for Illumina (PCR primers for the amplification of indexed, paired-end Illumina-compatible sequencing libraries, which enable multiplexing of NGS library analysis).

The entire library construction protocol, starting with total RNA, can be completed in about 5 hours (Figure 1). This kit incorporates both RiboGone™ and SMART® (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) technologies, with PCR amplification, to generate Illumina-compatible libraries without the need for 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.

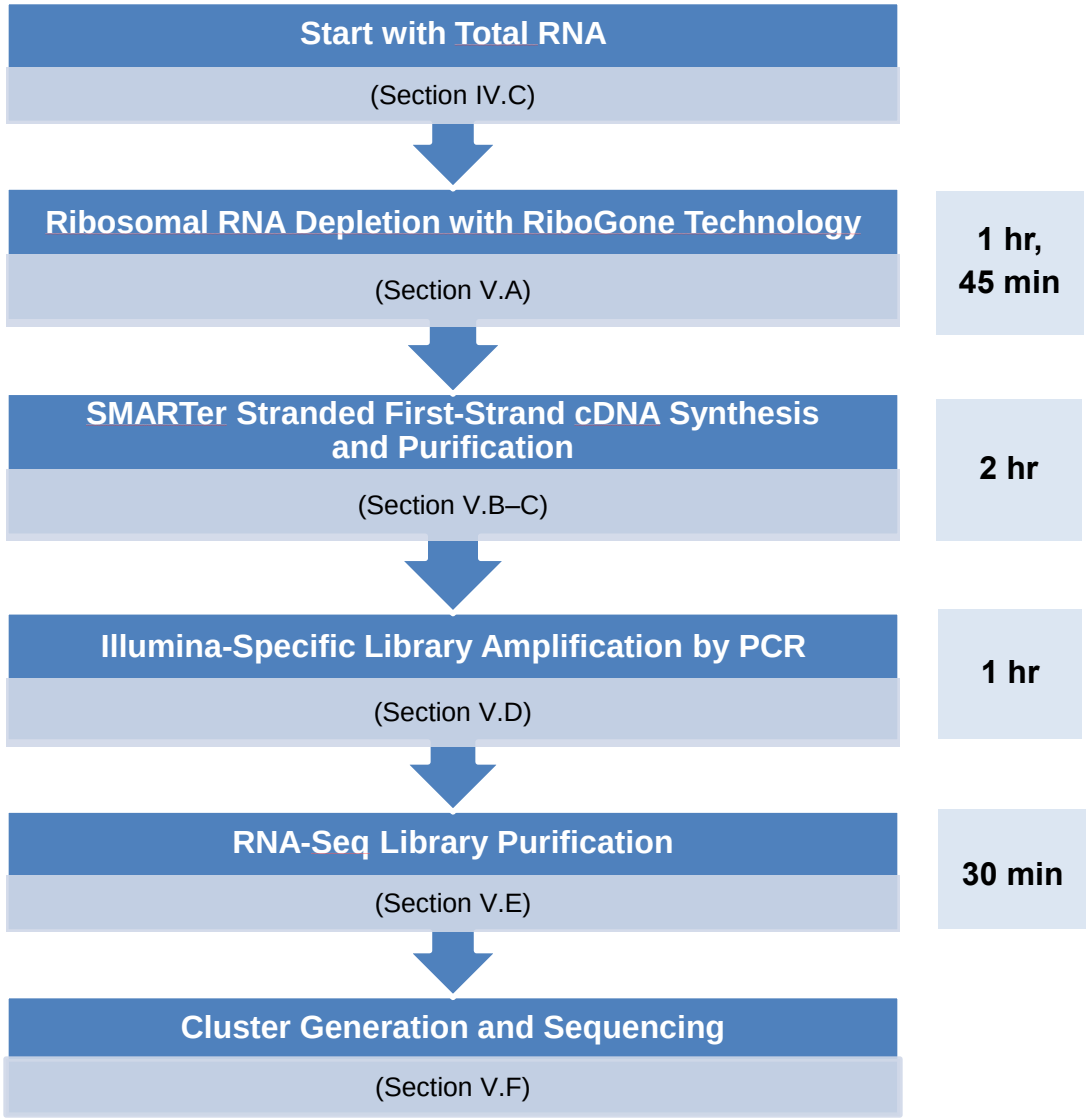

Figure 1. SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian protocol overview. Illumina-compatible RNA-seq libraries can be generated in around 5 hr.

The SMARTer Stranded Total RNA Sample Prep Kit – HI Mammalian starts with total RNA, and is designed for use with high-input samples (100 ng–1 µg of total RNA) of either high or low quality.

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 benefits by lowering sequencing costs and improving mapping statistics. RiboGone technology (Figure 2; 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 depletes them 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). A modified N6 primer (the SMART Stranded N6 Primer, included in the 10X Total RNA First-Strand Buffer) primes the first-strand synthesis reaction. For added simplicity, the RNA is chemically fragmented during denaturation.

As part of the template switching mechanism, when the PrimeScript™ Reverse Transcriptase 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 Oligo base-pairs with the non-templated nucleotide stretch, creating an extended template to enable the PrimeScript RT to continue replicating to the end of the oligonucleotide (Chenchik, *et al.*, 1998). The resulting single-stranded (ss) 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.

Sequencing libraries with Illumina indexes (and cluster generation sequences) are then generated by PCR amplification (Figure 2; section C). 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 particular kit you have purchased.

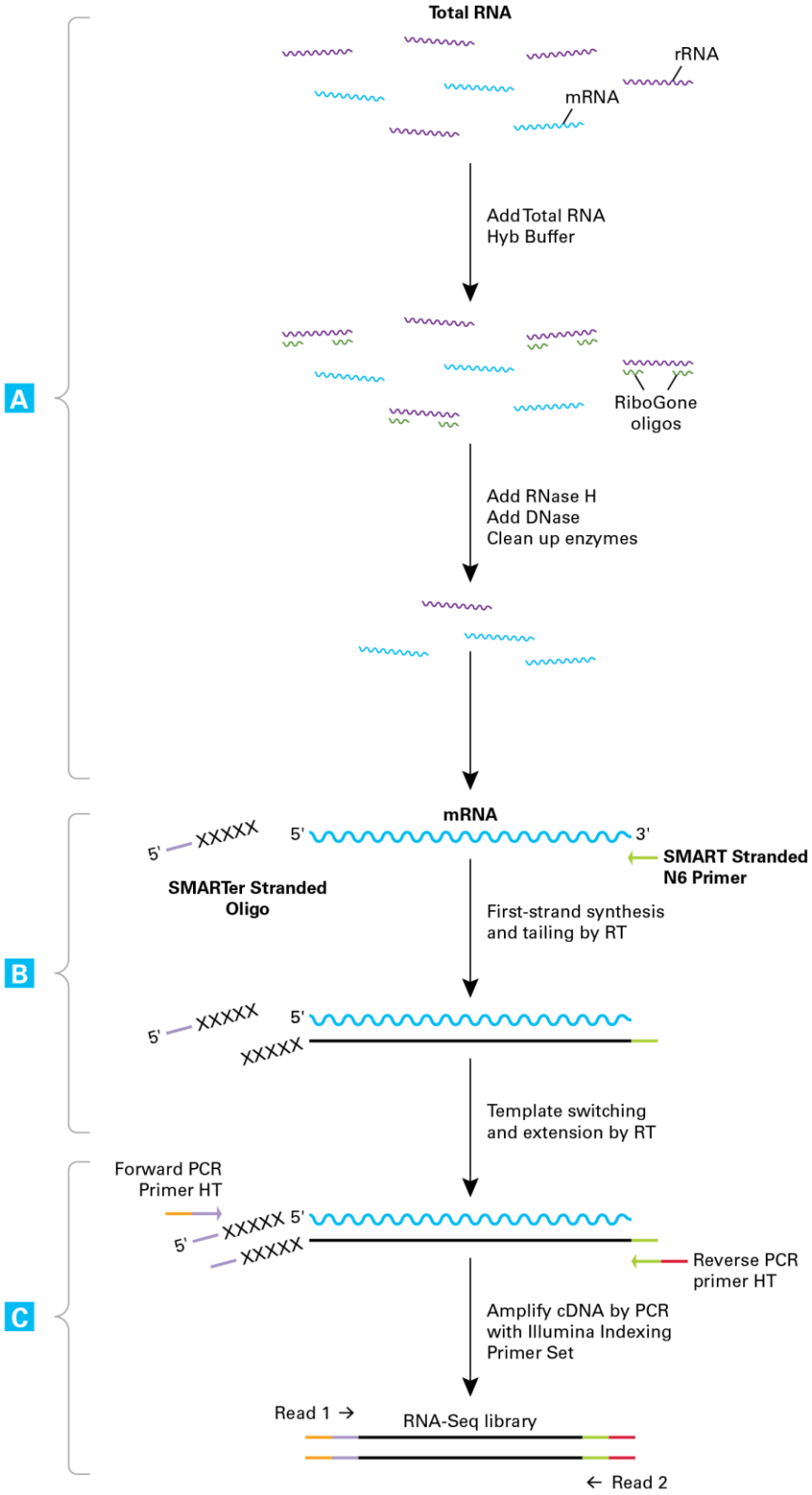

Figure 2. Flowchart of SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian library generation. RiboGone technology depletes rRNA from total RNA samples prior to library generation. Illumina-specific, indexed libraries are generated with SMART technology that eliminates enzymatic cleanup and adapter 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 SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian consists of the SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian Components (not sold separately), the Illumina Indexing Primer Set HT for Illumina (not sold separately), and SeqAmp DNA Polymerase. **These components 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 a modification of the protocol may lead to unexpected results.

SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian	**634873** (12 rxns)	**634874** (24 rxns)	**634875** (48 rxns)	**634876** (96 rxns)	**634877** (192 rxns)	**634878** (480 rxns)
SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)						
Package 1 (Store at –70°C.)						
SMARTer Stranded Oligonucleotide (12 µM)*	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
Control Mouse Liver Total RNA (1 µg/µl)	5 µl	5 µl	5 µl	5 µl	2 x 5 µl	5 x 5 µl
Package 2 (Store at –20°C. Once thawed, store Stranded Elution Buffer at Room Temperature. Continue to store all other reagents at –20°C)						
10X Total RNA Hyb Buffer	12 µl	24 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl
RNase Inhibitor (40 U/µl)	55 µl	110 µl	220 µl	440 µl	2 x 440 µl	5 x 440 µl
RNase H	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
10X RNase H Buffer	17 µl	34 µl	68 µl	136 µl	2 x 136 µl	5 x 136 µl
DNase I (5 U/µl)	48 µl	96 µl	192 µl	348 µl	2 x 348 µl	5 x 384 µl
Nuclease-Free Water	1 ml	1 ml	2 ml	3 ml	2 x 3 ml	5 x 3 ml
10X Total RNA First-Strand Buffer	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
PrimeScript Reverse Transcriptase (200 U/µl)	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
Stranded Elution Buffer	240 µl	480 µl	960 µl	1920 µl	2 x 1920 µl	5 x 1920 µl
SeqAmp DNA Polymerase (Store at –20°C.)						
SeqAmp DNA Polymerase	50 µl	50 µl	2 x 50 µl	200 µl	200 µl	3 x 200 µl
SeqAmp PCR Buffer (2x)	1.25 ml	1.25 ml	2 x 1.25 ml	4 x 1.25 ml	4 x 1.25 ml	12 x 1.25 ml

* Takara Bio proprietary sequences

The Illumina primers you receive will depend on your particular kit:

SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian
(Cat. No. 634873 and 634874; 12 rxns and 24 rxns, respectively)

Indexing Primer Set HT for Illumina - 12
(Not sold separately. Store at –20°C.)

Forward PCR Primer HT Index 2 (F2; 12.5 µM)	2 x 15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian
(Cat. No. 634875; 48 rxns)

Indexing Primer Set HT for Illumina - 48 A
(Not sold separately. Store at –20°C.)

Forward PCR Primer HT Index 1 (F1; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 2 (F2; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 3 (F3; 12.5 µM)	2 x 15 µl
Forward PCR Primer HT Index 4 (F4; 12.5 µM)	2 x 15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian
(Cat. No. 634876, 634877, and 634878; 96 rxns, 192 rxns, and 480 rxns, respectively)

Indexing Primer Set HT for Illumina
(Not sold separately. Store at –20°C.)

Forward PCR Primer HT Index 1 (F1; 12.5 µM)	15 µl
Forward PCR Primer HT Index 2 (F2; 12.5 µM)	15 µl
Forward PCR Primer HT Index 3 (F3; 12.5 µM)	15 µl
Forward PCR Primer HT Index 4 (F4; 12.5 µM)	15 µl
Forward PCR Primer HT Index 5 (F5; 12.5 µM)	15 µl
Forward PCR Primer HT Index 6 (F6; 12.5 µM)	15 µl
Forward PCR Primer HT Index 7 (F7; 12.5 µM)	15 µl
Forward PCR Primer HT Index 8 (F8; 12.5 µM)	15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

Indexing Primer Set HT for Illumina sequences:

Index (tube label)	Barcode	Index (tube label)	Barcode
F1	TATAGCCT	R1	ATTACTCG
F2	ATAGAGGC	R2	TCCGGAGA
F3	CCTATCCT	R3	CGCTCATT
F4	GGCTCTGA	R4	GAGATTCC
F5	AGGCGAAG	R5	ATTCAGAA
F6	TAATCTTA	R6	GAATTCGT
F7	CAGGACGT	R7	CTGAAGCT
F8	GTACTGAC	R8	TAATGCGC
		R9	CGGCTATG
		R10	TCCGCGAA
		R11	TCTCGCGC
		R12	AGCGATAG

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)
- PCR thermal cyclers: two (one dedicated for pre-amplification steps and one dedicated for PCR amplification)
- Multi-channel pipette: 20 µl and 200 µl, (eight- or twelve-channel pipettes are recommended when performing multiple reactions in a single experiment)
- Filter pipette tips: 10 µl, 20 µl, and 200 µl
- One QuickSpin minicentrifuge for 0.2 ml tubes

For PCR Amplification & Validation:

- High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)
- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No.1402-4700)
- Nuclease-free nonsticky 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600)

For SPRI Bead Purification:

- Agencourt AMPure PCR Purification Kit (5 ml Beckman Coulter, Part No. A63880; 60 ml Beckman Coulter, Part No. A63881). This kit contains the magnetic SPRI beads to be used in this protocol.

  NOTE: SPRI beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5 ml Eppendorf tubes upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). This process will also decrease the chances of bead contamination. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

- Magnetic separation device for 0.2 ml tubes (see Appendix A)

  NOTE: In order 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).

- 80% ethanol

IV. General Considerations

A. Recommendations for Preventing Contamination

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

    - **A PCR Clean Work Station** 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 work station 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 work station must be located 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

- **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 SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMARTer 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 SMARTer 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.

C. Sample Preparation

The sequence complexity and the average length of SMARTer cDNA 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)].
- When choosing a purification method (kit), ensure that it is appropriate for your sample amount.

D. Sample Requirements

Input RNA Length

- The SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian was developed for total RNA with a RIN (RNA integrity number) 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** (Cat. No. 5067-1511).

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.

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.

NOTE: You will need a Magnetic Separation Device for 0.2 ml tubes. If you do not have such a device, we recommend constructing one using the instructions in Appendix A.

1. Mix the following components on ice:

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

2. Incubate the reactions in a thermal cycler at 95°C for 2 min; then ramp down the temperature (–0.1°C/sec) to 25°C.
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 x 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 SPRI beads to each reaction and mix by pipetting the entire volume up and down 7–8 times.

   NOTE: Use the previously aliquoted SPRI beads and allow them to come to room temperature for 30 min prior to use. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them 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. Then, 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 x 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 pipetting the entire volume up and down 7–8 times.
14. Return the tubes to the Magnetic Separation Device until they are completely clear. Then transfer 13.5 µl of each supernatant into a fresh tube. 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 (ss) 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 from Section V.A.14.

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

   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 PrimeScript 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%, 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 and spin the tubes briefly in a microcentrifuge.

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 until the next step (Section V.C.1).

   NOTE: If desired, you may stop here and store the reaction tubes at 4°C overnight before proceeding to Section V.C.

C. Protocol: Purification of First-Strand cDNA Using SPRI AMPure Beads

The first-strand cDNA selectively binds to SPRI 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:
- Use previously aliquoted SPRI beads and allow them to come to room temperature for 30 min prior to use, and mix well to disperse.
- You will need a Magnetic Separation Device for 0.2 ml tubes. If you do not have such a device, we recommend constructing one using the instructions in Appendix A.
- Clean-up of SMARTer reactions must be performed using Ampure XP beads. **Spin columns do not adequately remove adapter-dimers from the reactions and will result in experimental failure!**

Follow this procedure for each reaction tube:

1. Add 20 µl of SPRI AMPure beads to each sample using a 20 µl pipetter.
   - Mix by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times.
   - 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 x 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. You may see a tiny crack in the pellet.

   NOTE: Under- or over-drying the beads will reduce PCR efficiency, 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 Forward and the Reverse PCR Primers from the Illumina Indexing Primer Set HT for Illumina.

IMPORTANT: Do not perform more than 14 cycles with this protocol. If using more than 14 cycles, please elute the first-strand cDNA in 20 µl of Nuclease-Free Water, and perform the entire first-strand cDNA purification a second time (Section V.C) before proceeding to PCR amplification (Section V.D).

NOTE: 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.

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:

25 µl	2X SeqAmp PCR Buffer
1 µl	Forward PCR Primer HT (12.5 µM)
1 ul	Reverse PCR Primer HT (12.5 µM)
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 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 cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	1 min
4°C		forever

E. Protocol: Purification of the RNA-Seq Library Using SPRI AMPure Beads

The amplified RNA-seq library is purified by immobilizing it onto SPRI 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.

1. Add 50 µl of SPRI AMPure beads to each sample.
    - Mix by vortexing 5 sec or by pipetting the entire volume up and down at least 10 times.
    - 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 x 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. You may see a tiny crack in the pellet.

    NOTE: Be sure to dry the pellet enough.
    - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your RNA-seq library recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, it will take longer than 2 min to rehydrate (Step V.E.9, below).
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 incubate at room temperature for 2 min to rehydrate.

10. Mix by pipetting up and down 10 times to elute the DNA from the beads, then place the tubes back on the magnetic stand for 1 minute or longer, until the solution is completely clear.

11. Transfer the clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free nonsticky tube.

F. Protocol: Validation Using the Agilent 2100 Bioanalyzer

1. Use 1 µl of the cDNA library for validation using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from Agilent's **High Sensitivity DNA Kit** (Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.

2. 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 no product in the negative control (Figure 3, Panel B), and a distinct peak spanning 200–1,000 bp, peaked at ~300 bp for the positive control RNA sample (Figure 3, Panel A), yielding 2 ng/µl–10 ng/µl RNA-seq library (depending on the input and number of cycles).

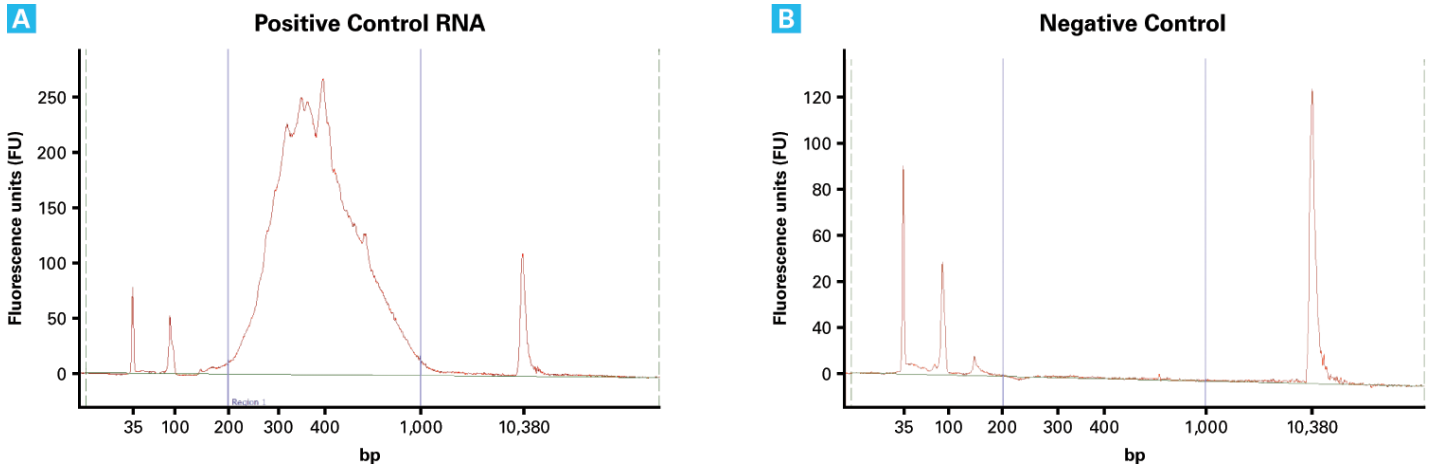

Figure 3. Electropherogram example results from Agilent 2100 Bioanalyzer. Samples were treated according to the protocol for the SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian. FU = fluorescence absorption units. **Panel A** shows the expected product starting from 1 µg of Control Mouse Liver Total RNA (included in the kit). **Panel B** shows no product in the negative control, using water in place of control RNA.

VI. References

Morlan, J.D., *et al.* (2012). Selective depletion of rRNA enables whole transcriptome profiling of archival fixed tissue. *PLOS One*, **7**(8):e42882.

Chenchik, A., *et al*. (1998). In *RT-PCR Methods for Gene Cloning and Analysis*. (BioTechniques Books, MA), pp. 305-319.

Appendix A: Constructing a Magnetic Separation Device for 0.2 ml PCR Tubes

It can be difficult to find magnetic separation devices designed specifically to handle 0.2 ml PCR strip tubes. Often, one can place strip tubes in a column/row of a magnetic separation device designed for use with 96-well plates. Alternatively, one can construct a suitable low-cost separation device from common laboratory materials.

Example 1: Using a 96-well separation device with strip tubes

As seen in Figure 4, you may place the tubes in the top part of an inverted P20 or P200 Rainin Tip Holder which is taped to a MagnaBlot II Magnetic Separator (Promega Part No. V8351)

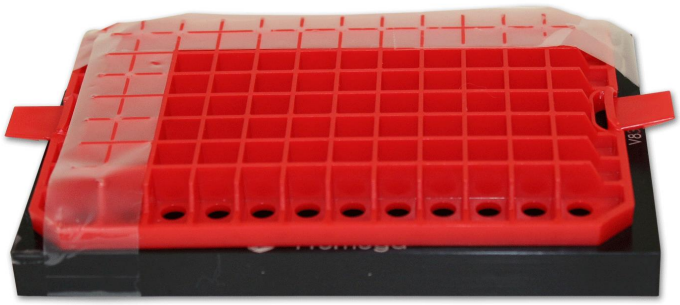

Figure 4. Setup for positioning 0.2 ml tubes containing first-strand cDNA on a MagnaBlot II Magnetic Separator.

Example 2: Building a 0.2ml tube magnetic separation device from rare earth bar magnets and a tip rack

As seen in Figure 5, neodymium bar magnets are taped together on the underside of the top section of a 20 µl tip rack (Panel A), and the rack is inverted so the tubes can be inserted (Panel B).

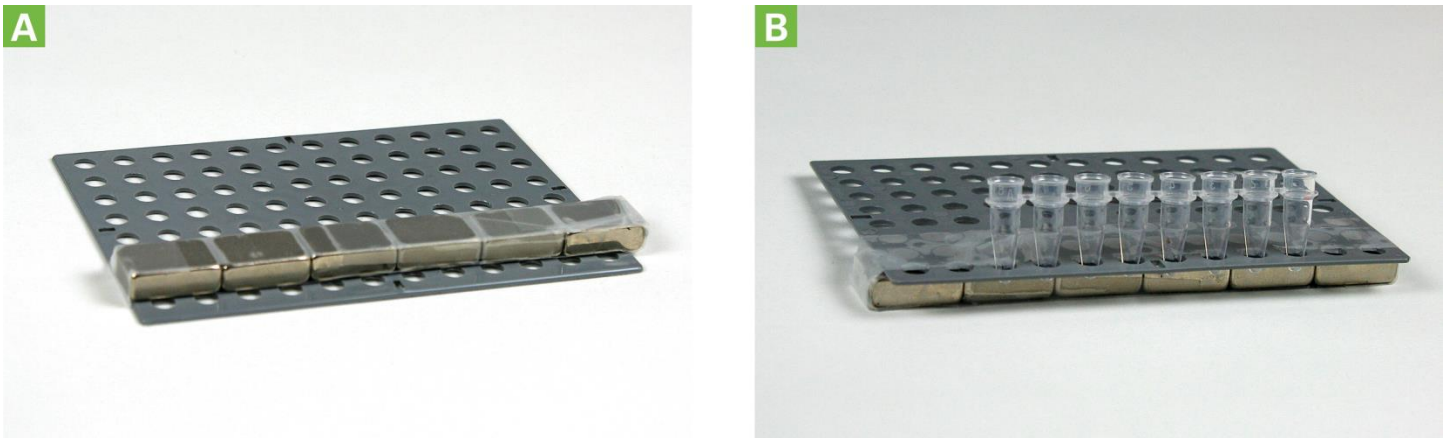

Figure 5. Constructing a magnetic separation device for 0.2 ml tubes from rare earth magnets. **Panel A** shows six 0.75" x 0.25" x 0.5" neodymium bar magnets (Applied Magnets Model # NB026) taped together on the underside of the top section of a 20 µl tip rack. **Panel B** shows the upright rack, into which an 8-tube strip of 0.2 ml tubes has been inserted.

Appendix B: Sequencing Guidelines

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.edu.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using our Library Quantification Kit (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 I) regarding the amount of PhiX control to include with SMARTer 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 nt. SMARTer 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.

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

Sequencing instrument	PhiX (%)
MiSeq	5–10 (optional[1])
HiSeq 1500/2000/2500	10
HiSeq 3000/4000	20
NextSeq/MiniSeq	20

[1] 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.

the problem.

Sequencing Analysis Guidelines

IMPORTANT: 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.

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