Takara Bio USA, Inc.

SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian User Manual

Cat. Nos. 634411, 634412, 634413, 634414
(063017)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@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

SMARTer cDNA Synthesis for Illumina® Sequencing Platforms

The **SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian** (Cat. Nos. 634411, 634412, 634413, 634414) includes all components needed to generate indexed cDNA libraries suitable for next-generation sequencing (NGS) on any Illumina platform, with a recommended input range of 250 pg to 10 ng of mammalian total RNA. This kit is an updated version of the original SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian (Cat. Nos. 635005, 635006, 635007) that has been further optimized to provide improved sequencing performance. While the original kit requires the inclusion of a PhiX control spike-in at a 10–20% concentration for optimum sequencing performance, this kit does not require any PhiX spike-in. The kit also features a new PCR buffer (SeqAmp™ CB PCR buffer), specifically optimized to make the handling of AMPure beads easier.

The entire library construction protocol, starting with total RNA, can be completed in about 6 hours (Figure 1). This kit incorporates SMART® (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) cDNA synthesis technology (Chenchik et al. 1998) and generates Illumina-compatible libraries via PCR amplification, avoiding the need for adapter ligation. The directionality of the template-switching reaction preserves the strand orientation of the original RNA, making it possible to obtain strand-specific sequencing data from the synthesized cDNA. Illustrated below are the cDNA library construction process and technologies employed by the kit (Figure 2), and the structural details of final libraries (Figure 3). For sequencing libraries produced with this kit, Read 1 generates sequences antisense to the original RNA. This contrasts with libraries produced by the original SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian, for which Read 1 generates sequences sense to the original RNA.

Ribosomal RNA (rRNA) comprises a significant proportion (~90% or more) of all RNA molecules in 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. However, with very low input amounts, initial rRNA depletion from total RNA is not very effective and often leaves an insufficient amount of material for preparation of high-quality libraries. The workflow used in this kit takes advantage of a novel technology allowing removal of ribosomal cDNA (cDNA fragments originating from rRNA molecules) after cDNA synthesis using probes specific to mammalian rRNA. These R-Probes target ribosomal RNA and mitochondrial rRNA sequences; however, the mitochondrial R-Probes are derived from the human mitochondrial genome and are therefore strictly human-specific. The rRNA depletion method used in this kit makes it especially well-suited for working with very small quantities of total RNA.

The SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian is compatible with picogram inputs of total RNA from high-quality or degraded samples (250 pg–10 ng). Most RNA samples will perform best in the 250 pg to 10 ng range. Inputs higher than 10 ng generate libraries of excellent quality, but may yield more rRNA-associated reads than inputs ≤10 ng. RNA samples with chemical modifications, such as those extracted from FFPE tissue, typically generate lower cDNA yields and produce the best sequencing results when analyzed in the 5 to 50 ng range. Please refer to Section IV.C for more details. In addition to purified total RNA, the kit can also be used for processing intact-cell inputs (50–1,000 cells). The protocol for cell inputs is available upon request.

In order to generate library inserts of an appropriate size for compatibility with Illumina sequencing, RNA molecules obtained from high-quality or partially degraded samples must be fragmented prior to cDNA synthesis. For highly degraded, low-quality starting material, the RNA fragmentation step should be skipped. Please refer to Option 2 for Protocol A (Section V.A) for guidance on how to proceed if you are skipping the fragmentation step.

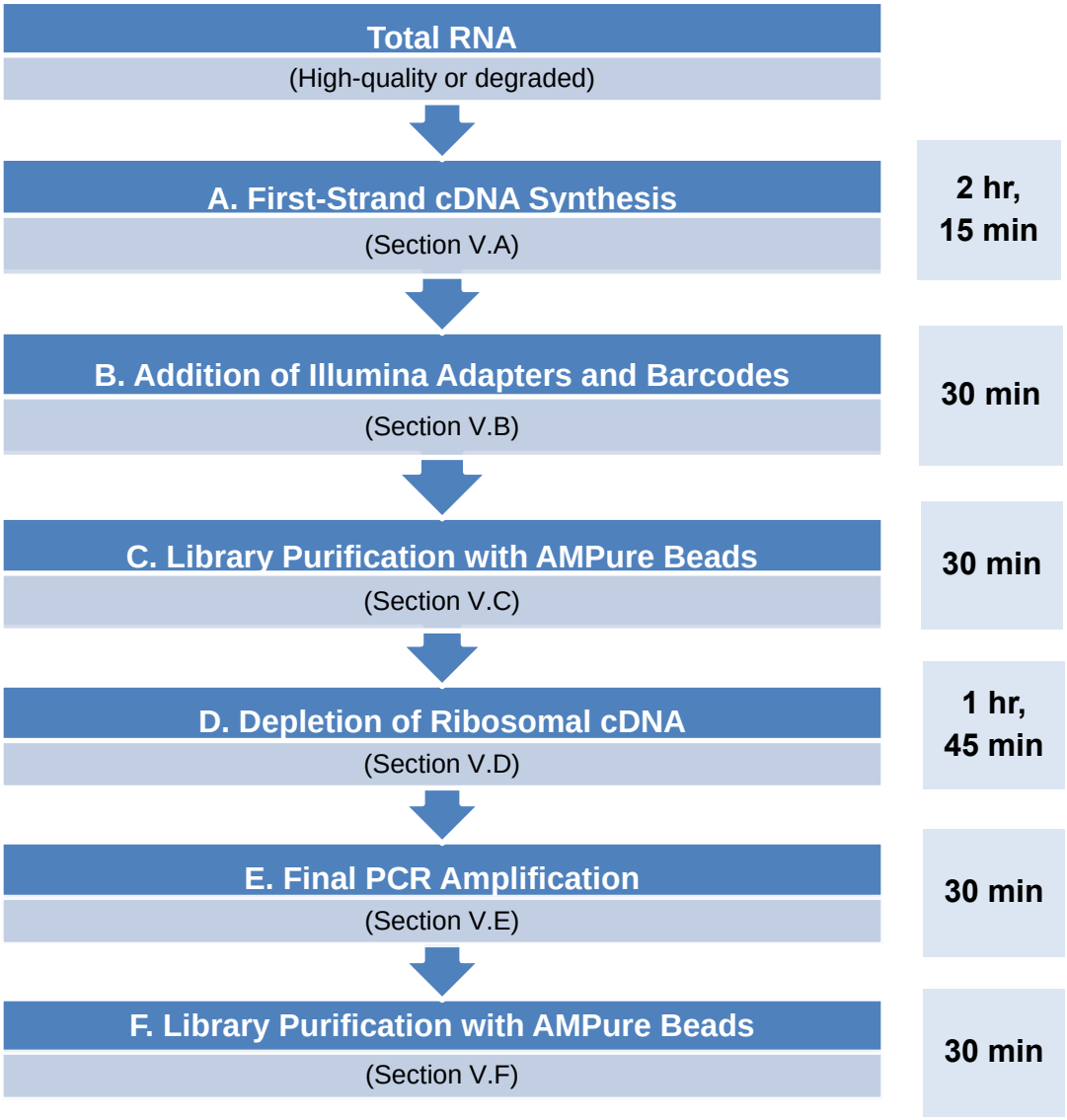

Figure 1. SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian protocol overview. This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries in approximately 6 hr. First, total RNA is converted to cDNA (Protocol A), and then adapters for Illumina sequencing (with specific barcodes) are added through PCR using only a limited number of cycles (Protocol B). The PCR products are purified (Protocol C), and then ribosomal cDNA is depleted (Protocol D). The cDNA fragments from Protocol D are further amplified (Protocol E) with primers universal to all libraries. Lastly, the PCR products are purified once more to yield the final cDNA library (Protocol F). Actual processing time may vary depending on the number of samples and cycling conditions (e.g., Protocol E takes more than 30 min if using 16 cycles of PCR or if using a thermal cycler with a slow ramping time), but a set of 24–48 samples can be easily processed from A to F within an 8-hr working day. As outlined in Section V, the kit workflow includes three safe stopping points following the completion of Protocols A, B, and E, respectively.

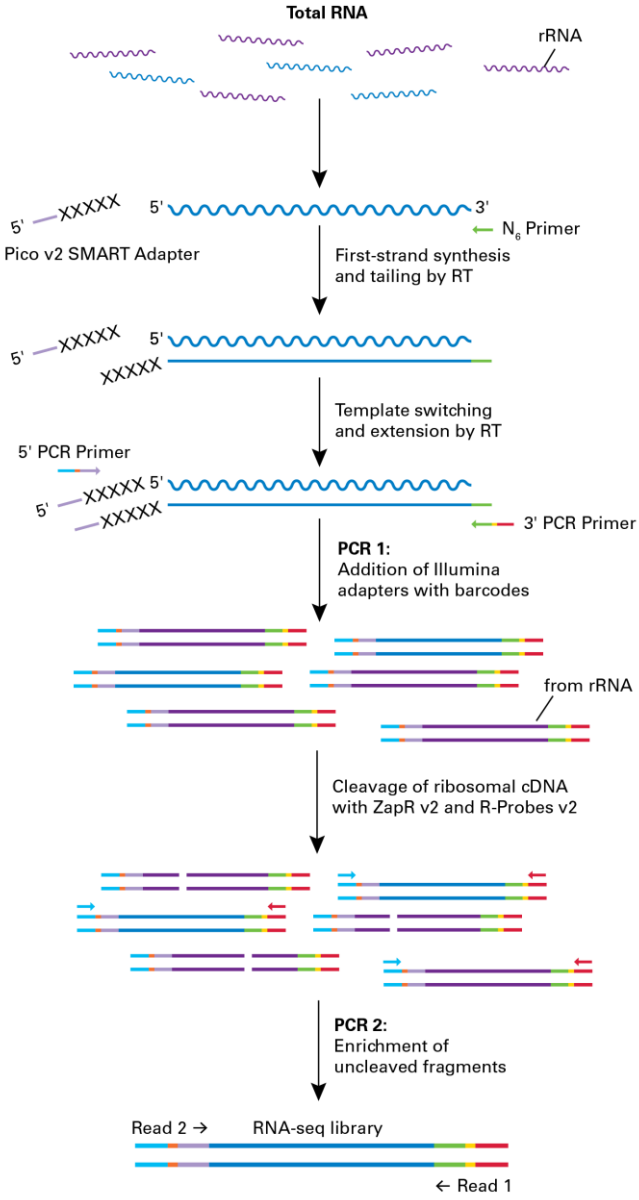

Figure 2. Schematic of technology in the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian. SMART technology is used in this ligation-free protocol to preserve strand-of-origin information. Random priming (represented as the green N6 Primer) allows the generation of cDNA from all RNA fragments in the sample, including rRNA. 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 (shown as Xs). The carefully designed Pico v2 SMART Adapter (included in the SMART TSO Mix v2) base-pairs with the nontemplated nucleotide stretch, creating an extended template to enable the RT to continue replicating to the end of the oligonucleotide. The resulting cDNA contains sequences derived from the random primer and the Pico v2 SMART Adapter used in the reverse transcription reaction. In the next step, a first round of PCR amplification (PCR1) adds full-length Illumina adapters, including barcodes. The 5' PCR Primer binds to the Pico v2 SMART Adapter sequence (light purple), while the 3' PCR Primer binds to sequence associated with the random primer (green). The ribosomal cDNA (originating from rRNA) is then cleaved by ZapR in the presence of the mammalian-specific R-Probes. This process leaves the library fragments originating from non-rRNA molecules untouched, with priming sites available on both 5' and 3' ends for further PCR amplification. These fragments are enriched via a second round of PCR amplification (PCR2) using primers universal to all libraries. The final library contains sequences allowing clustering on any Illumina flow cell (see details in Figure 3).

Figure 3. Structural features of final libraries generated with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian. The adapters added using 5' PCR Primer HT and 3' PCR Primer HT contain sequences allowing clustering on any Illumina flow cell (P7 shown in light blue, P5 shown in red), Illumina TruSeq® HT indexes (Index 1 [i7] sequence shown in orange, and Index 2 [i5] sequence shown in yellow), as well as the regions recognized by sequencing primers Read Primer 2 (Read 2, purple) and Read Primer 1 (Read 1, green). Read 1 generates sequences antisense to the original RNA, while Read 2 yields sequences sense to the original RNA (orientation of original RNA denoted by 5' and 3' in dark blue). The first three nucleotides of the second sequencing read (Read 2) are derived from the Pico v2 SMART Adapter (shown as Xs). These three nucleotides must be trimmed prior to mapping if performing paired-end sequencing.

II. List of Components

The SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian consists of the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian Components (not sold separately) and the Indexing Primer Set HT for Illumina v2 (not sold separately). **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. Please make sure to spin down tubes to collect all the liquid at the bottom before first use.

SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian		**634411** (12 rxns)	**634412** (48 rxns)	**634413** (96 rxns)	**634414** (192 rxns)
SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)					
Cap Color	**Package 1 (Store at –70°C)**				
Brown	SMART TSO Mix v2[1, 2]	55 µl	225 µl	450 µl	900 µl
Light Blue	R-Probes v2[1]	20 µl	80 µl	160 µl	320 µl
	Control Total RNA[3] (1 µg/µl)	5 µl	5 µl	5 µl	5 µl
	Package 2 (Store at –20°C)				
Clear	ZapR v2	20 µl	80 µl	160 µl	320 µl
Pink	SMART Pico Oligos Mix v2[1]	15 µl	55 µl	110 µl	220 µl
Red	5X First-Strand Buffer	50 µl	200 µl	400 µl	800 µl
Purple	SMARTScribe RT (100 U/µl)	25 µl	100 µl	200 µl	400 µl
White	RNase Inhibitor (40 U/µl)	8 µl	25 µl	50 µl	100 µl
Blue	ZapR Buffer (10X)	50 µl	200 µl	400 µl	800 µl
Orange	Tris Buffer (5 mM)	1.25 ml	1.25 ml	2 x 1.25 ml	5 ml[4]
Khaki	PCR2 Primers v2[5]	50 µl	200 µl	400 µl	800 µl
	Nuclease-Free Water	1.25 ml	4 x 1.25 ml	10 ml	2 x 10 ml
Green	SeqAmp DNA Polymerase[6]	50 µl	200 µl	2 x 200 µl	4 x 200 µl
	SeqAmp CB PCR Buffer (2X)[7]	1.25 ml	4 x 1.25 ml	10 ml	2 x 10 ml

[1] Takara Bio proprietary sequences

[2] SMART TSO Mix v2 contains the Pico v2 SMART Adapter (a.k.a., template-switching oligo).

[3] Control Total RNA is from human brain.

[4] The 5-ml allotment of Tris Buffer is provided in an 8-ml bottle with a clear cap instead of a tube with an orange cap.

[5] Do not freeze-thaw the PCR2 Primers v2 more than 10 times.

[6] SeqAmp DNA Polymerase is a hot-start enzyme.

[7] Do not substitute regular SeqAmp PCR Buffer for SeqAmp CB PCR Buffer.

WARNING: Do not freeze/thaw R-Probes v2 more than 3 times! We recommend aliquoting R-Probes v2 into multiple vials to avoid repeated freeze-thaw cycles.

WARNING: The Nuclease-Free Water is used in Sections V.A, V.B, and V.E. When using this kit for the first time, set aside a small amount of water to be used only for first-strand synthesis (Section V.A). This helps to avoid contamination during the kit's subsequent uses from previously introduced molecules with library adapters from Sections V.B and V.E.

Indexing Primer Sets:

Kit Cat. No.		634411	634412	634413	634414
Indexing Primer Set Version		**HT for Illumina v2 - 12**	**HT for Illumina v2 - 48**	**HT for Illumina v2 - 96**	**2 x HT for Illumina v2 - 96**
Size		**12 rxns**	**48 rxns**	**96 rxns**	**192 rxns**

(Not sold separately. Store at –20°C.)

3' PCR Primers 12.5 µM Full names of primers have been shortened[1]	3' 1[2]		20 µl	20 µl	2 x 20 µl
	3' 2	20 µl	20 µl	20 µl	2 x 20 µl
	3' 3		20 µl	20 µl	2 x 20 µl
	3' 4		20 µl	20 µl	2 x 20 µl
	3' 5			20 µl	2 x 20 µl
	3' 6			20 µl	2 x 20 µl
	3' 7			20 µl	2 x 20 µl
	3' 8			20 µl	2 x 20 µl
5' PCR Primers 12.5 µM Full names of primers have been shortened[1]	5' 1	15 µl	15 µl	15 µl	2 x 15 µl
	5' 2	15 µl	15 µl	15 µl	2 x 15 µl
	5' 3	15 µl	15 µl	15 µl	2 x 15 µl
	5' 4	15 µl	15 µl	15 µl	2 x 15 µl
	5' 5	15 µl	15 µl	15 µl	2 x 15 µl
	5' 6	15 µl	15 µl	15 µl	2 x 15 µl
	5' 7	15 µl	15 µl	15 µl	2 x 15 µl
	5' 8	15 µl	15 µl	15 µl	2 x 15 µl
	5' 9	15 µl	15 µl	15 µl	2 x 15 µl
	5' 10	15 µl	15 µl	15 µl	2 x 15 µl
	5' 11	15 µl	15 µl	15 µl	2 x 15 µl
	5' 12	15 µl	15 µl	15 µl	2 x 15 µl

[1] Full names of primers have been shortened: for example, 3' PCR Primer HT Index 1 has been shortened to 3' 1 and 5' PCR Primer HT Index 1 has been shortened to 5' 1.

[2] 3'1 to 3'8 correspond to Illumina TruSeq HT indexes D501–D508; 5'1 to 5'12 correspond to Illumina TruSeq HT indexes D701–D712 (see table below).

Indexing Primer Set HT for Illumina v2 Adapter Sequences:

i5 Index (Tube Label)	i5 Illumina Index Name	i5 Bases for Sample Sheet MiSeq®, HiSeq® 2000/2500	i5 Bases for Sample Sheet MiniSeq™, NextSeq®, HiSeq 3000/4000	i7 Index (Tube Label)	i7 Illumina Index Name	i7 Bases for Sample Sheet
3' 1	D501	TATAGCCT	AGGCTATA	5' 1	D701	ATTACTCG
3' 2	D502	ATAGAGGC	GCCTCTAT	5' 2	D702	TCCGGAGA
3' 3	D503	CCTATCCT	AGGATAGG	5' 3	D703	CGCTCATT
3' 4	D504	GGCTCTGA	TCAGAGCC	5' 4	D704	GAGATTCC
3' 5	D505	AGGCGAAG	CTTCGCCT	5' 5	D705	ATTCAGAA
3' 6	D506	TAATCTTA	TAAGATTA	5' 6	D706	GAATTCGT
3' 7	D507	CAGGACGT	ACGTCCTG	5' 7	D707	CTGAAGCT
3' 8	D508	GTACTGAC	GTCAGTAC	5' 8	D708	TAATGCGC
				5' 9	D709	CGGCTATG
				5' 10	D710	TCCGCGAA
				5' 11	D711	TCTCGCGC
				5' 12	D712	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.

- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No. 1402-4700)
- Single-channel pipette: 10 µl, 20 µl, and 200 µl, two each (one for pre-PCR amplification steps and one dedicated for PCR amplification)
- Hot-lid PCR thermal cyclers: two (one dedicated for pre-PCR amplification steps and one dedicated for PCR amplification)

NOTE: The final RNA-seq library amplification (Section V.E) is intended to be carried out with thermal cyclers that can accommodate 100-µl sample volumes. If your thermal cyclers only accommodate ≤50-µl sample volumes, we recommend splitting each reaction equally into two tubes so the PCR proceeds optimally.

- Multi-channel pipettes: 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
- Quickspin Minicentrifuge for 0.2-ml tubes
- 96-well PCR chiller rack: IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4) or 96 Well Aluminum Block (Light Labs, Cat. No. A-7079)

NOTE: A PCR chiller rack is essential to keep samples cold during several steps of the protocol. Be sure to decontaminate the ice bucket and the PCR chiller rack before each use.

For PCR Amplification & Validation:

- Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
- Qubit dsDNA HS Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- 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 Purification Using AMPure Beads:

- Agencourt AMPure XP PCR purification system (5 ml; Beckman Coulter, Item No. A63880 or 60 ml; Beckman Coulter, Item No. A63881). Kit size needed depends on the number of reactions performed.

NOTE: Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, 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, allowing them to come to room temperature more quickly (~30 min). This aliquoting process is also essential for minimizing the chances of bead contamination.

NOTE: Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

- Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)

  IMPORTANT: Very strong magnets produce the best-quality libraries with the highest yields. The recommended magnetic separation device has been successfully tested with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian protocol. In order to prevent cross-contamination, we strongly recommend using separate magnetic separation devices for the initial rounds (Sections V.C. and V.D) and final round of library purification (Section V.F).

- 80% ethanol: freshly made for each experiment

IV. General Considerations

A. Recommendations for Preventing Contamination

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

   - **A PCR-clean work station** for all pre-PCR experiments that require clean room conditions, such as first-strand cDNA synthesis (Section V.A.)
   - **A second work station located in the general laboratory** where you will perform PCR1 (Section V.B.) and PCR2 (Section V.E.), and cleave ribosomal cDNA with ZapR v2 and R-Probes v2 (Section V.D.)
   - **A third work station located in the general laboratory** where you will purify the library (Sections V.C, V.D., and V.F.) and measure its concentration (Section V.G.)

     IMPORTANT: We recommend three separate work areas in order to avoid contaminating samples with PCR products from previous reactions. Since the PCR primers recognize sequences common to all libraries, setting up new reactions in the same area where the final library cleanup occurs increases the risk of contamination. The PCR-clean 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. While the use of three separate work areas is not an absolute requirement, it can greatly minimize contamination and ensure the preparation of high-quality libraries every time.

2. **Guidelines for PCR-clean work station operation:**

   - Only move materials/supplies from the PCR-clean work station to the general lab, NOT the other way around. Do not share any equipment/reagents between the PCR-clean work station and the general lab work stations.
   - Use a separate PCR thermal cycler (dedicated to first-strand cDNA synthesis) inside the PCR-clean work station for first-strand 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 purity 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 pipettes are calibrated for reliable reagent delivery and that nothing adheres to the outsides of the tips when dispensing liquids.
- All lab supplies related to SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Ideally, reagents for SMARTer cDNA synthesis should 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 (without RNA) and positive (with provided Control Total RNA) control reactions.
- Because of the large volume or viscosity of mixtures subject to purification using AMPure beads, each round of purification requires a very strong magnet, particularly the final purification step (Section V.F). Never assume that bead separation will be completed within a given timeframe; when in doubt, leave samples on the magnet long enough beyond the recommended 5-min period for the samples to be completely clear.

C. Sample Requirements

Input RNA Quality

- Degraded, partially degraded, or high-quality RNA can be analyzed with this kit. Please determine the quality of your RNA (RIN score or DV200) before starting the workflow using the **Agilent RNA 6000 Pico Kit** (Cat. No. 5067-1513). The section of this manual describing first-strand cDNA synthesis (Section V.A) includes alternate protocols for processing RNA inputs of varying quality; **Option 1** includes a fragmentation step and is intended for partially degraded or high-quality RNA, while **Option 2** proceeds without fragmentation and is intended for highly degraded RNA, such as material extracted from FFPE samples. Please refer to Section V.A for further guidance. This kit will offer the best performance for RNA samples with DV200 >50%; however, good quality libraries have been obtained from RNA with DV200 values as low as 25%.

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. Samples should have been treated with DNase I prior to use with this kit, as the random priming used in this protocol may lead to amplification of any DNA present in the starting material.

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

- **Volume and amount of input RNA:** This kit accommodates up to 8 µl of input RNA. This protocol has been optimized for cDNA synthesis from 250 pg–10 ng of mammalian total RNA.

  NOTE: Higher input amounts ranging from 10–50 ng can also be used to generate high-quality sequencing libraries with this kit, but may yield more rRNA-associated reads than inputs ≤10 ng.

RNA extracted from FFPE samples contain chemical modifications that decrease the efficiency of reverse transcription and overall cDNA yield. Therefore, we recommend an input range of 5–50 ng for analysis of FFPE samples. Inputs higher than 50 ng have not been validated. It is strongly recommended that working conditions for your samples be established before trying inputs beyond the recommended range. For total RNA inputs <250 pg, the yield may be low and is highly dependent on the RNA source (e.g., the mRNA content in a particular sample/cell type/tissue) and the size of the RNA (high-quality versus highly degraded). Libraries generated from inputs <250 pg will also contain a significantly higher amount of PCR duplicates due to the low complexity of the starting material and are more likely to contain undesirable environmental contaminants.

Diluting the Control Total RNA

1. Dilute Control Total RNA (human brain) to 50 ng/µl by mixing 38 µl of Nuclease-Free Water with 2 µl of Control Total RNA (1 µg/µl) in a sterile microcentrifuge tube.

   NOTE: Fresh dilutions should be made before use. If desired, make single-use aliquots of the 50 ng/µl dilution and store at –70˚C until needed, then further dilute.

2. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of Nuclease-Free Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.
3. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of Nuclease-Free Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.
4. Use 1 µl or more of 0.25 ng/µl Control Total RNA as a positive control RNA input for the kit and proceed alongside your samples.

   NOTE: Try to match the input amount of Control Total RNA to the input amount of your own samples, and use the same number of PCR cycles. Due to the high quality of the Control Total RNA (RIN >8), a 4-min fragmentation is recommended regardless of the amount of fragmentation needed for your experimental samples. However, for experiments involving sample fragmentation times in the range of 3–4 min, a corresponding fragmentation time within the range of 3–4 min should be sufficient for the Control Total RNA.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

Fragmentation of RNA into a size appropriate for sequencing on Illumina platforms is performed in the first step of the cDNA synthesis protocol. Fragmentation time is adjusted depending on the quality of the RNA input. **Option 1 (with fragmentation)** should be used when starting from samples with RIN ≥4 or DV200 ≥60%, and **Option 2 (without fragmentation)** should be used when starting from samples that are already severely degraded. For samples with RIN ~4 and below, the RIN value is often unreliable, and DV200 is a better metric for evaluating the quality of the RNA input. Please refer to Table 1 (below) for guidelines.

NOTE: When planning to use inputs >10 ng, please be aware that the proportion of rRNA-associated reads in sequencing data may be higher than for inputs ≤10 ng. However, inputs as high as 50 ng can be used safely for FFPE RNA samples.

Table 1. Recommended Fragmentation Protocol Options and Fragmentation Times.

RNA quality	Use protocol	Fragmentation time (min)
RIN ≥7	Option 1	4
RIN 5–6	Option 1	3
RIN 4/DV200 ≥60%	Option 1	2[1]
DV200 25–60% and all FFPE samples[2]	Option 2	–

[1] In most cases, samples with RIN ~4 will be optimally processed after 90 sec–2 min of fragmentation. However, we recommend optimizing the fragmentation time, as overfragmentation may lead to reduced performance due to inefficient ribosomal cDNA depletion in Section V.D. When in doubt, choose a shorter fragmentation time or Option 2.
[2] Option 2 is recommended for all FFPE samples regardless of RIN or DV200 values.

For this protocol, you will need the following components: SMART Pico Oligos Mix v2, 5X First-Strand Buffer, Nuclease-Free Water, SMART TSO Mix v2, RNase Inhibitor, and SMARTScribe RT.

Option 1 (With Fragmentation): Starting from High-Quality or Partially Degraded RNA

1. Mix the following components on ice:

1–8 µl	RNA
1 µl	SMART Pico Oligos Mix v2
4 µl	5X First-Strand Buffer
0–7 µl	Nuclease-Free Water
13 µl	Total volume per reaction

2. Incubate the tubes at **94°C** in a preheated, hot-lid thermal cycler for the amount of time recommended in Table 1 or for an experimentally determined, optimal amount of time, then immediately place the samples on an ice-cold PCR chiller rack for 2 min.

NOTE: The samples should be taken out of the thermal cycler immediately after the time indicated to avoid overfragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples.

NOTE: The next reaction steps (Steps 4–5) are critical for first-strand synthesis and should not be delayed after Step 2. Start Step 3, preparing the First-Strand Master Mix, while your tubes are incubating (Step 2), or have it almost ready before starting Step 2.

3. Prepare enough First-Strand Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

4.5 µl	SMART TSO Mix v2
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe Reverse Transcriptase
7 µl	Total volume per reaction

NOTE: The SMART TSO Mix v2 is very viscous—make sure to homogenize the First-Strand Master Mix very well by pipetting up and down 10 times with a pipette set at a volume larger than the final master mix volume.

4. Add 7 µl of the First-Strand Master Mix to each reaction tube from Step 2. Mix the contents of the tubes by vortexing for ~2 sec, then spin the tubes briefly to collect the contents at the bottom.

NOTE: The First-Strand Master Mix is very viscous—make sure to homogenize the content of the tubes very well.

5. Incubate the tubes in a preheated hot-lid thermal cycler with the following program:

42°C	90 min
70°C	10 min
4°C	forever

6. Leave the samples in the thermal cycler at 4°C until the next step.

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

Option 2 (Without Fragmentation): Starting from Highly Degraded RNA

1. Mix the following components on ice:

1–8 µl	RNA
1 µl	SMART Pico Oligos Mix v2
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 exactly 3 min, then immediately place the samples on an ice-cold PCR chiller rack for 2 min.
3. Prepare enough First-Strand Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown.

4 µl	5X First-Strand Buffer
4.5 µl	SMART TSO Mix v2
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe Reverse Transcriptase
11 µl	Total volume per reaction

NOTE: The SMART TSO Mix v2 is very viscous—make sure to homogenize the First-Strand Master Mix very well by pipetting up and down 10 times with a pipette set at a volume larger than the final master mix volume.

4. Add 11 µl of the First-Strand Master Mix to each reaction tube from Step 2. Mix the contents of the tubes by vortexing for ~2 sec, then spin the tubes briefly to collect the contents at the bottom.

NOTE: The First-Strand Master Mix is very viscous—make sure to homogenize the content of the tubes very well.

5. Incubate the tubes in a preheated hot-lid thermal cycler with the following program:

42°C	90 min
70°C	10 min
4°C	forever

6. Leave the samples in the thermal cycler at 4°C until the next step.

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

B. Protocol: PCR1—Addition of Illumina Adapters and Indexes

The indexes (barcodes) that are used to distinguish pooled libraries from each other after sequencing are added at this step. Great care must be taken to select the right indexes.

For this protocol, you will need the following components: Nuclease-Free Water, SeqAmp CB PCR Buffer (2X), SeqAmp DNA Polymerase, and 5' and 3' PCR Primer HT sets.

NOTE: If library purification (Section V.C) will be performed immediately following PCR1, remove aliquots of AMPure beads from the refrigerator to allow them to reach room temperature.

1. Prepare a PCR1 Master Mix for all reactions. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge:

2 µl	Nuclease-Free Water
25 µl	SeqAmp CB PCR Buffer (2X)
1 µl	SeqAmp DNA Polymerase
28 µl	Total volume per reaction

NOTE: If the 3' index (i5) is going to be the same for all libraries, the 3' PCR Primer can also be added to the PCR1 Master Mix (1 µl/reaction). Typically, a single i5 index can be used if fewer than 12 libraries will be pooled for sequencing.

2. Add 28 µl (29 µl if the 3' PCR Primer is included) of PCR Master Mix to each sample from Step A.6.
3. Add 1 µl of each 5' and 3' PCR Primer HT to each sample. Mix by gentle vortexing or tapping of the tubes, then spin down briefly.
4. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C	1 min
5 cycles:	
98°C	15 sec
55°C	15 sec
68°C	30 sec
68°C	2 min
4°C	forever

SAFE STOPPING POINT: Samples can be left for up to 1 hr in the thermal cycler at 4°C. If not processed within the next hour, freeze the PCR products at –20°C for up to 2 weeks.

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

The amplified RNA-seq library is purified by immobilization onto AMPure beads. The beads are then washed with 80% ethanol, and the cDNA is eluted in Nuclease-Free Water. The purification is then performed a second time, starting in Section V.C and finishing in Section V.D.

IMPORTANT: Do not start Section V.C if you do not have enough time to perform all steps up to Section V.E.

For this protocol, you will need the following components: AMPure beads (at room temperature), 80% ethanol (freshly prepared), Nuclease-Free Water, and a magnetic separation device.

IMPORTANT: Remove ZapR Buffer from –20°C storage and thaw it at room temperature in preparation for Section V.D.

1. Allow AMPure beads to come to room temperature before use (~30 min). Add 40 µl of AMPure beads to each sample.

   NOTE: Mix by vortexing for 5 sec (recommended) or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up and then out slowly.

2. Incubate at room temperature for 8 min to allow the DNA to bind to the beads.
3. Briefly spin the sample tubes to collect the liquid at the bottom. 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 separation device, pipette out the supernatant and discard.
5. Keeping the tubes on the magnetic separation device, 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 and discard the supernatant. cDNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.
7. Briefly spin the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
8. Let the open sample tubes rest at room temperature for 3–5 min until the pellets appear dry.

   NOTE: You may see a tiny crack in each pellet when dry. Do not overdry.

9. Once the beads are dry, add 52 µl of Nuclease-Free Water to cover the beads. Remove the tubes from the magnetic separation device and mix thoroughly by pipetting up and down until all the beads have been washed off the sides of the tubes.
10. Incubate at room temperature for 5 min to rehydrate.
11. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
12. Pipette 50 µl of supernatant from each sample into respective wells of a new 8-well strip.
13. Add 40 µl of AMPure beads to each sample and mix well.

    NOTE: Mix by vortexing for 5 sec (recommended) or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up and then out slowly.

14. Incubate at room temperature for 8 min to allow the DNA to bind to the beads. During the incubation time, proceed immediately to Section V.D.

D. Protocol: Depletion of Ribosomal cDNA with ZapR v2 and R-Probes v2

In this section, the library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by ZapR v2 in the presence of R-Probes v2 (mammalian-specific). These R-Probes hybridize to ribosomal RNA and mitochondrial rRNA sequences; however, the mitochondrial sequences are derived from the human mitochondrial genome and are therefore strictly human-specific.

For this protocol, you will need the following components: R-Probes v2, ZapR v2, ZapR Buffer, Nuclease-Free Water, and a magnetic separation device.

1. Thaw R-Probes v2 and ZapR Buffer at room temperature. Place R-Probes v2 on ice as soon as it is thawed, but keep ZapR Buffer at room temperature. ZapR v2 should be kept on ice at all times and returned to the freezer immediately after use.
2. Preheat the thermal cycler in anticipation of Step D.5.

3. Upon completion of the 8-min incubation in Step C.14, briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.

   NOTE: It is acceptable—and in some cases necessary—to leave the tubes on the magnetic separation device for more than 5 min.

4. During the 5-min incubation time in Step D.3, pipette into a pre-chilled PCR tube a sufficient volume of R-Probes v2 for the number of reactions to be performed—1.5 µl per reaction, see Step D.12—plus 10% to account for pipetting errors. Keep the PCR tube containing R-Probes v2 on ice and immediately return the remaining unused R-Probes v2 to a –70°C freezer.
5. Incubate the PCR tube containing R-Probes v2 at 72°C in a preheated hot-lid thermal cycler using the following program:

72°C	2 min
4°C	forever

6. Leave the R-Probes v2 tube in the thermal cycler at 4°C for at least 2 min, but for no more than 10–15 min, before using it in the next step (Step D.12).
7. Once the 5-min incubation on the magnetic separation device is complete (Step D.3) and the samples are clear, pipette out the supernatant and discard, while keeping the tubes sitting on the magnetic separation device.
8. Keeping the tubes on the magnetic separation device, 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 and discard the supernatant. cDNA will remain bound to the beads during the washing process.
9. Repeat Step 8 once.
10. Briefly spin the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
11. Let the open sample tubes rest at room temperature until the pellets appear dry.

    NOTE: The beads will dry more quickly than in Step C.8. 1–2 min may be sufficient, but the beads can be left to dry for up to 5 min during preparation of the ZapR Master Mix in Step D.12.

12. While the beads are drying, prepare the ZapR Master Mix. Prepare enough Master Mix for all reactions, plus 10%, by combining the following reagents at room temperature in the order shown. Make sure to add the preheated and chilled R-Probes v2 from Step D.6 last. Return ZapR v2 to a –20°C freezer immediately after use. Mix the components well by vortexing briefly, and spin the tubes briefly in a microcentrifuge.

16.8 µl	Nuclease-Free Water
2.2 µl	10X ZapR Buffer
1.5 µl	ZapR v2
1.5 µl	R-Probes v2
22 µl	Total volume per reaction

13. To each tube of dried AMPure beads from Step D.11, add 22 µl of the ZapR Master Mix. Remove the tubes from the magnetic separation device and mix thoroughly to resuspend the beads.
14. Incubate at room temperature for 5 min to rehydrate.

15. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
16. Pipet out 20 µl of supernatant, being careful not to disturb the beads, into a new PCR strip.
17. Incubate the tubes in a preheated hot-lid thermal cycler using the following program:

37°C	60 min
72°C	10 min
4°C	forever

NOTE: Samples can be left in the thermal cycler at 4°C for up to 1 hr. However, we recommend proceeding immediately to Section V.E.

E. Protocol: PCR2—Final RNA-Seq Library Amplification

In this section, the library fragments not cleaved by the ZapR reaction in Section V.D will be further enriched in a second round of PCR. Since barcodes have already been added to the libraries, a single pair of primers can be used for all libraries.

For this protocol, you will need the following components: Nuclease-Free Water, SeqAmp CB PCR Buffer (2X), PCR2 Primers v2 and SeqAmp DNA Polymerase.

1. Prepare a PCR2 Master Mix for all reactions (plus 10%). Combine the following reagents in the order shown, then mix well and spin the tubes briefly in a microcentrifuge:

26 µl	Nuclease-Free Water
50 µl	SeqAmp CB PCR Buffer
2 µl	PCR2 Primers v2
2 µl	SeqAmp DNA Polymerase
80 µl	Total volume per reaction

NOTE: DO NOT reduce the reaction volume. The 100-µl final volume is important for yield. If your thermal cycler cannot accommodate 100-µl sample volumes, it is important to equally divide each sample into two tubes (containing ~50 µl each) *after* the PCR Master Mix has been added, mixed, and spun down (prior to Step 3).

2. Add 80 µl of PCR2 Master Mix to each tube from Step D.17. Mix by tapping gently, then spin down.
3. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C		1 min
9–16 cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

NOTE: The actual number of cycles varies depending on the starting material. The guidelines below must be validated with your material. We do not recommend performing more than 16 cycles, as it may lead to background amplification. We recommend that you perform a pilot experiment with a small number of samples to determine the optimal number of cycles for your input material.

Table 2. Cycling Guidelines Based on Amount of Starting Material.

Amount of input RNA (ng)	Typical number of PCR cycles Regular RNA	FFPE RNA*
50	9–10	13
10	12	15–16
1	14–15	16
0.25	16	-

*Typically, FFPE RNA requires extra PCR cycles for achieving adequate yield. For inputs below 10 ng, 16 cycles should be used. An input of 1 ng can generate enough material for sequencing, but a minimum of 5 ng is recommended.

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed within the next day, freeze the PCR products at –20°C for up to 2 weeks.

F. Protocol: Purification of Final RNA-Seq Library Using AMPure Beads

The amplified RNA-seq library is purified by immobilization onto AMPure beads. The beads are then washed with 80% ethanol and eluted in Tris Buffer.

For this protocol, you will need the following components: AMPure beads (at room temperature), 80% ethanol (freshly prepared), Tris Buffer, and a magnetic separation device.

1. Allow AMPure beads to come to room temperature before use (~30 min). Add 100 µl of AMPure beads to each sample.

   NOTE: Mix by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up, and then out slowly.

2. Incubate at room temperature for 8 min to let the cDNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 5–10 min or longer, until the solution is completely clear.

   NOTE: This step will take more time than in Protocol C (Section V.C) due to the high volume.

4. While the tubes are sitting on the magnetic separation device, pipette out the supernatant and discard.
5. Keep the tubes on the magnetic separation device. Without disturbing the beads, add 200 µl of **freshly made** 80% ethanol to each sample to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. cDNA 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 separation device for 30 sec, then carefully remove all remaining ethanol with a pipette, without disturbing the beads.
8. Let the sample tubes rest open at room temperature for ~5–7 min until the pellets appear dry.

   NOTE: You may see a tiny crack in each pellet. Do not overdry.

9. Once the beads are dry, add 20 µl of Tris Buffer to cover the beads. Remove the tubes from the magnetic separation device and mix thoroughly by pipetting up and down several times until all the beads have been washed off the sides of the tubes.

   NOTE: Consider eluting in 12 µl instead of 20 µl if anticipated yield is low.

10. Incubate at room temperature for 5 min to rehydrate.

11. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 2 min or longer, until the solution is completely clear.
12. Transfer the supernatants to nonsticky tubes. Proceed to validation immediately or store at –20°C.

G. Protocol: Validation Using the Agilent 2100 Bioanalyzer

1. Quantify libraries with Qubit dsDNA HS kit (Thermo Fisher Scientific). A yield >2 ng/µl will provide enough material for further library validation and sequencing. Consider adding one PCR cycle in subsequent experiments if the yield is insufficient, or reducing cycles if the yield is more than 10 ng/µl. Eluting the final libraries in a smaller volume (e.g., 12 µl instead of 20 µl) is also a simple way to achieve more concentrated libraries.
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). See Figure 4 for an example of a successful library.
3. Compare the results for your samples and controls (if performed) to determine whether samples are suitable for further processing. Successful cDNA synthesis and amplification should produce a distinct curve spanning 200–1,000 bp, with a local maximum at ~300–400 bp, in the positive control RNA sample (see Figure 4A) and no product or very minimal background over the corresponding range in the negative control (see Figure 4B). A small amount of products ~150–200 bp in size such as those found in the example in Figure 4A will not interfere with sequencing. However, consider repeating the final clean-up (Section V.F) if an excessive amount of products <200 bp in size is present.

NOTE: Library preparation adds 139 bp to the size of the original RNA molecules.

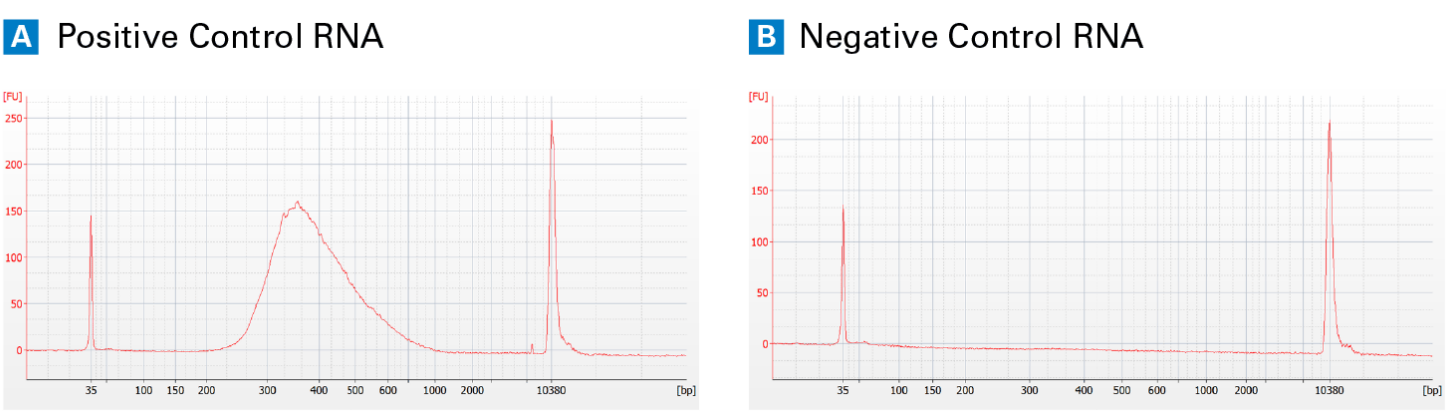

Figure 4. Example electropherogram results from the Agilent 2100 Bioanalyzer. Libraries were generated using 0.25 ng Control Total RNA (human brain; Panel A—library diluted to 1.5 ng/µl based on Qubit quantification) and a no-RNA control (Panel B—no library dilution). For both examples, PCR2 was performed using 16 cycles. Note that the no-RNA control exhibits a small amount of background (indicated by minimal product in the 200–1,000 bp range), which is acceptable as long as the libraries from the RNA samples contain a significantly larger amount of material.

VI. References

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

Appendix A: Sequencing Guidelines

Sequencing Analysis Guidelines

IMPORTANT: When performing paired-end sequencing, the first three nucleotides of the second sequencing read (Read 2) are derived from the Pico v2 SMART Adapter. These three nucleotides must be trimmed prior to mapping.

- Read 1 matches the antisense sequence of the input RNA.
- If you are performing paired-end sequencing, Read 2 will correspond to the sense strand.

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 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 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. Illumina recommends the systematic inclusion of ~1% PhiX to help assess run performance and troubleshooting. Libraries generated with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian do not require the inclusion of extra PhiX beyond the typical 1%. However, we cannot guarantee that your particular sample type and RNA input amount will display the well-balanced nucleotide diversity required for base calling. If in doubt, include 5–10% PhiX.

- Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Make sure to use a fresh and reliable stock of the PhiX control library.

Loading Guidelines for Various Illumina Instruments

Libraries generated with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian cluster very efficiently and care must be taken to avoid overclustering. The guidelines in Table 3 (below) are a good starting point and have been fully validated.

Table 3. Library Loading Guidelines for Various Illumina Sequencing Instruments.

Sequencing instrument	Loading concentration (pM)
MiSeq – v2 chemistry	8
MiSeq – v3 chemistry	10
MiniSeq	1.2
NextSeq 500/550	1.3

Extra Precautions When Using NextSeq and MiniSeq Instruments

Libraries generated with the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian perform extremely well when sequenced on NextSeq and MiniSeq instruments. However, care must be taken to ensure that you get the most out of the sequencing run.

- Both systems use automatic adapter trimming by default. This can unexpectedly shorten your reads and cause your 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, considering that the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian is good at retaining small inserts. Therefore, 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 nt of Read 2), NextSeq and MiniSeq runs may display incorrect base calling in the first three bases. Because those bases are trimmed, it does not affect the quality of the data obtained. However, we still strongly recommend avoiding overloading of the instrument.

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	web: takarabio.com
e-mail: ordersUS@takarabio.com	e-mail: techUS@takarabio.com

Notice to Purchaser

Our products are to be used for research purposes only. They may not be used for any other purpose, including, but not limited to, use in drugs, *in vitro* diagnostic purposes, therapeutics, or in humans. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without prior written approval of Takara Bio USA, Inc.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

©2017 Takara Bio Inc. All Rights Reserved.

All trademarks are the property of Takara Bio Inc. or its affiliate(s) in the U.S. and/or other countries or their respective owners. Certain trademarks may not be registered in all jurisdictions. Additional product, intellectual property, and restricted use information is available at takarabio.com.

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