Takara Bio USA, Inc.

SMARTer® Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian User Manual

Cat. Nos. 634485, 634486, 634487 & 634488
(072121)

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I. Introduction

SMARTer cDNA Synthesis for Illumina® Sequencing Platforms

The **SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian** includes all components needed to generate 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 v2 - Pico Input Mammalian (Cat. Nos. 634411, 634412, 634413, and 634414) that has been further optimized to provide improved sequencing performance without the additional need of PhiX spike-in and features a new PCR buffer (SeqAmp™ CB PCR Buffer) specifically for library purification using NucleoMag NGS Clean-up and Size Select (Takara Bio; Cat. Nos. 744970.5, 744970.50, or 744970.500). Additionally, SMARTer RNA Unique Dual Index Kit - 24U (Takara Bio, Cat. No. 634451) is supplied with this kit and contains PCR primers for amplification of indexed Illumina-compatible multiplexed libraries. This updated kit also adds an 8 nucleotide (nt) unique molecular identifier (UMI) through the reverse-transcription step to mitigate potential PCR bias as well as to provide customers with additional information for transcript quantification, specifically for true variants and rare mutations. This kit is designed for analysis with Takara Bio's Cogent™ NGS Analysis Pipeline Software, which removes PCR duplicates and errors based on the UMIs.

The entire library construction protocol, starting with total RNA, can be completed in about 8 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).

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 (originating from rRNA) after cDNA synthesis using probes specific to mammalian rRNA. These R-Probes target nuclear 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 v3 - Pico Input Mammalian is compatible with picogram inputs of total RNA from high-quality or degraded samples. 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 of 10 ng or less. 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 0.5 to 10 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 (10–1,000 cells).

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 Section V.A for guidance on how to proceed if you are skipping the fragmentation step.

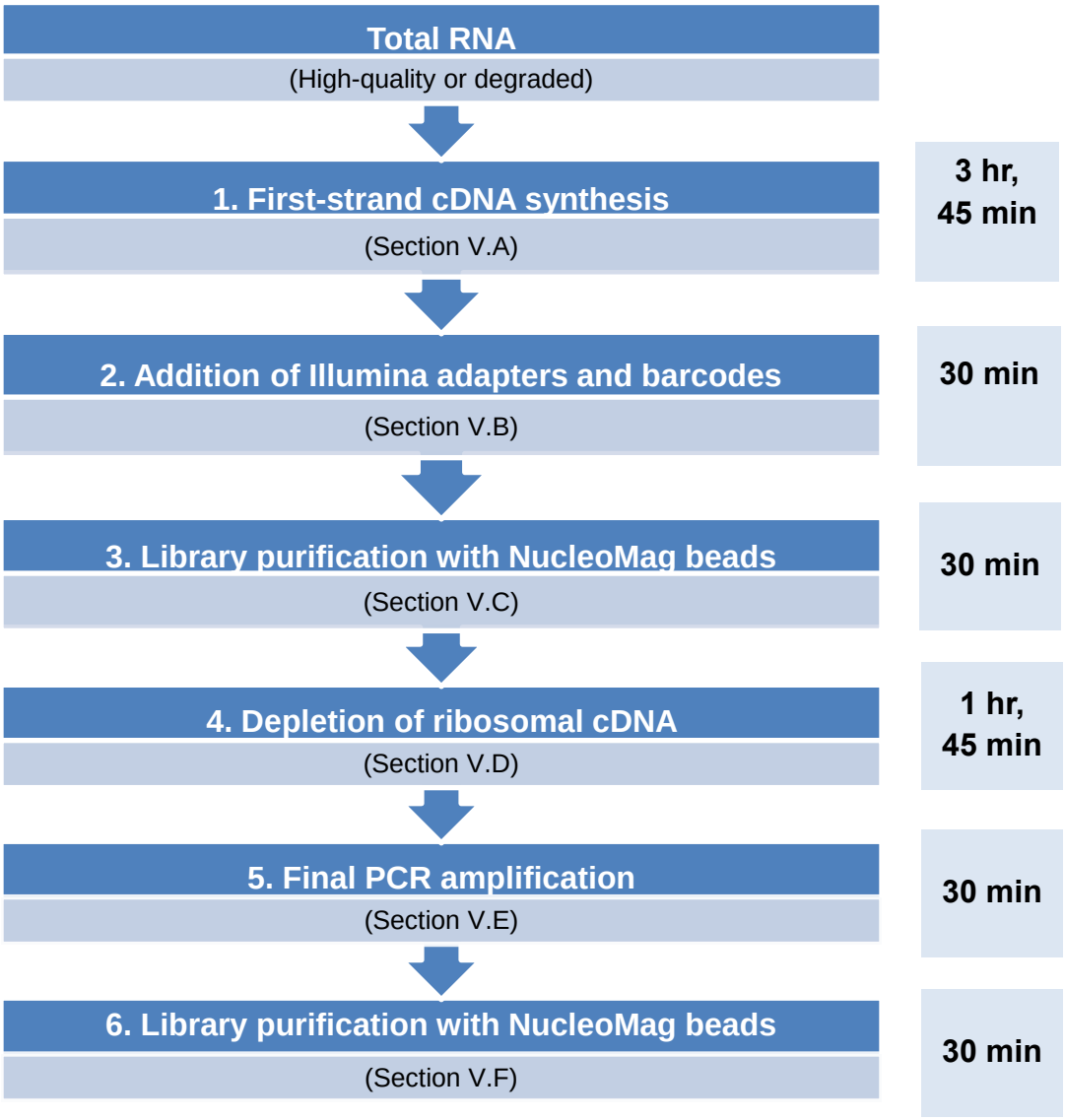

Figure 1. SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian protocol overview. This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries in approximately 7 hr 30 min. Actual processing time may vary depending on the number of samples and cycling conditions (e.g., Protocol E takes more than 30 min if using 16 cycles of PCR or if using a thermal cycler with a slow ramping time). First, total RNA is converted to cDNA (Protocol A), and then adapters for Illumina sequencing (with specific barcodes) are added through PCR using 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). As outlined in Section V, the kit workflow includes three safe stopping points following the completion of Protocols A, B, and E, respectively.

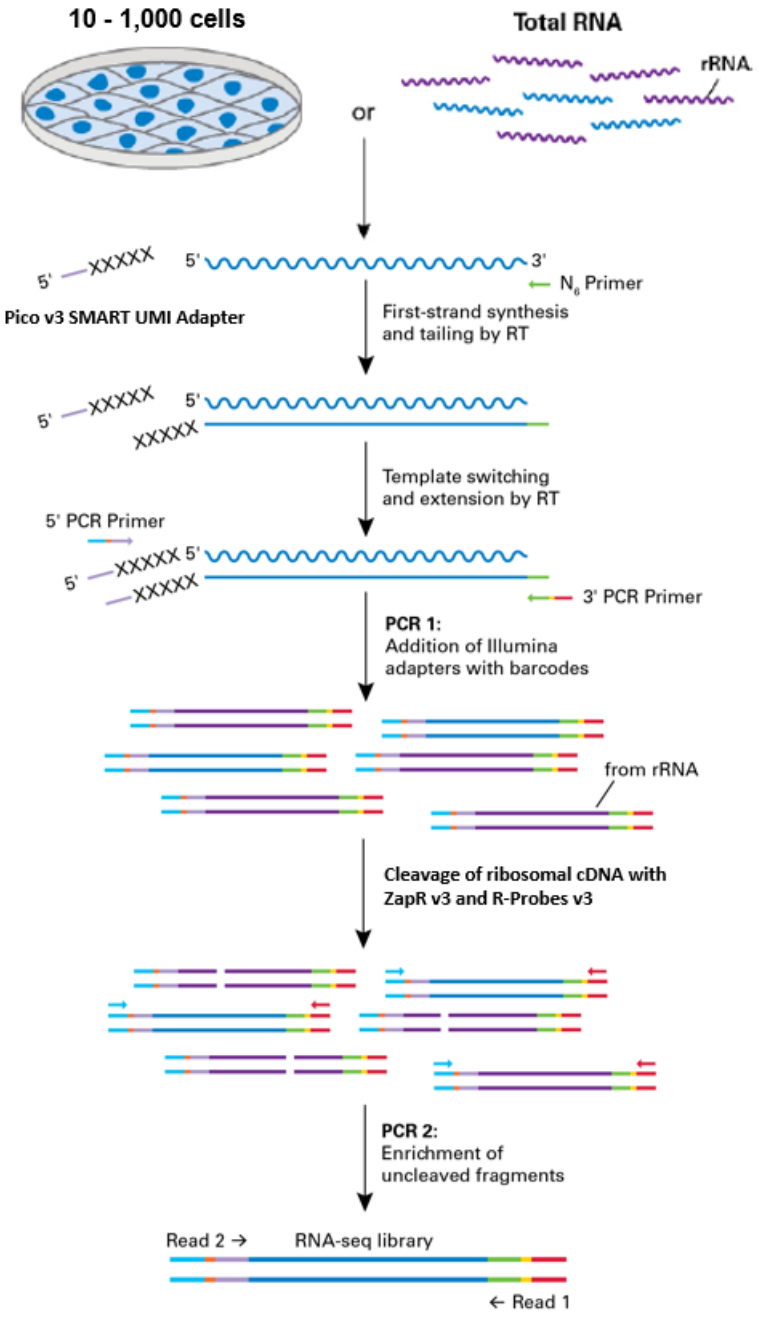

Figure 2. Schematic of technology in the SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian. SMART technology is used in this ligation-free protocol to preserve strand-of-origin information. Random priming (represented by the green N6 Primer) allows the generation of cDNA from all RNA fragments in the sample, including rRNA. When the SMARTScribe™ II 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 XXXXX). The Pico v3 SMART UMI Adapter (included in the SMART UMI-TSO Mix v3) 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 Pico v3 SMART UMI Adapter also contains 8 random nt which function as the unique molecular identifiers for downstream data analysis. In the next step, a first round of PCR amplification (PCR 1) adds full-length Illumina adapters, including barcodes. The 5′ PCR Primer binds to the Pico v3 SMART UMI Adapter sequence (light purple), while the 3′ PCR Primer binds to sequence associated with the random primer (green). The ribosomal cDNA is then cleaved by ZapR v3 in the presence of the mammalian-specific R-Probes. The resulting cDNA contains sequences derived from the random primer and the Pico v3 SMART UMI Adapter used in the reverse transcription reaction. 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 (PCR 2) 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 v3 - Pico Input Mammalian. The adapters added using the SMARTer RNA Unique Dual Index Kit - 24U (Takara Bio, Cat. No. 634451) contain sequences allowing clustering on any Illumina flow cell (P7 shown in light blue, P5 shown in red, 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 8 nt of the second sequencing read (Read 2) are UMIs (dark purple) followed by 3 nucleotides of UMI-linker (shown as NNN) and 3 nucleotides derived from the Pico v3 SMART UMI Adapter (shown as XXX).

II. List of Components

The SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian consists of the SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian Components (Takara Bio, Cat. Nos. 634489, 634490, 634491, and 634492; not sold separately) and the SMARTer RNA Unique Dual Index Kit - 24U (Takara Bio, Cat. No. 634451). **These components have been specifically designed to work together and are optimized for this protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or 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.

Component breakdown		**634485** (24 rxns)	**634486** (48 rxns)	**634487** (96 rxns)	**634488** (192 rxns)
SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian Components					
Package 1 (Store at –70°C)	**Cap color**				
SMART UMI-TSO Mix v3 [1]	Brown	110 µl	225 µl	450 µl	900 µl
R-Probes v3[2]	Light blue	40 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)	**Cap color**				
ZapR v3	Clear	40 µl	80 µl	160 µl	320 µl
SMART Pico Oligos Mix v3	Pink	30 µl	55 µl	110 µl	220 µl
5X First-Strand Buffer	Red	100 µl	200 µl	400 µl	800 µl
SMARTScribe II Reverse Transcriptase	Purple	50 µl	100 µl	200 µl	400 µl
RNase Inhibitor (40 U/µl)	White	30 µl	60 µl	100 µl	200 µl
ZapR Buffer (10X)	Blue	100 µl	200 µl	400 µl	800 µl
Tris Buffer	Orange	1.25 ml	1.25 ml	2 x 1.25 ml	5 ml[4]
PCR2 Primers v3[5]	Khaki	100 µl	200 µl	400 µl	800 µl
Nuclease-free water	Clear	2 x 1.25 ml	4 x 1.25 ml	10 ml	2 x 10 ml
SeqAmp DNA Polymerase[6]	Green	100 µl	200 µl	2 x 200 µl	4 x 200 µl
SeqAmp CB PCR Buffer (2X)[7]	Clear	2 x 1.25 ml	4 x 1.25 ml	10 ml	2 x 10 ml
10X Lysis Buffer	Clear	0.5 ml	1 ml	1 ml	2 x 1ml
SMARTer RNA Unique Dual Index Kit - 24U		1 kit	1 kit	1 kit	2 kits

[1] SMART UMI-TSO Mix v3 contains the Pico v3 SMART UMI Adapter (i.e., template-switching oligo).
[2] Takara Bio proprietary sequences
[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 v3 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

WARNINGS:

- **Do not freeze/thaw R-Probes v3 more than 3 times!** We recommend aliquoting R-Probes v3 into multiple vials to avoid repeated freeze-thaw cycles.
- 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.

SMARTer RNA Unique Dual Index Kit - 24U (Store at –20°C)	**Volume**
U25 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U26 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U27 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U28 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U29 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U30 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U31 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U32 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U33 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U34 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U35 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U36 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U37 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U38 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U39 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U40 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U41 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U42 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U43 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U44 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U45 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U46 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U47 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl
U48 - SMARTer RNA Unique Dual Index (12.5 µM)	8 µl

More unique dual index primers can be purchased separately:

- SMARTer RNA Unique Dual Index Kit - 96U Set A (Takara Bio, Cat. No. 634452)
- SMARTer RNA Unique Dual Index Kit - 96U Set B (Takara Bio, Cat. No. 634457)

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 might not obtain the expected results.

- UDI primers†:
  - SMARTer RNA Unique Dual Index Kit - 96U Set A (Takara Bio, Cat. No. 634452)
  - SMARTer RNA Unique Dual Index Kit - 96U Set B (Takara Bio, Cat. No. 634457)

†**NOTE:** As SMARTer RNA Unique Dual Index Kit - 24U (Takara Bio, Cat. No. 634451) is supplied with the SMARTer Stranded Pico v3, the primers above are only recommended for purchase if pooling more than 24 samples on a sequencer.

- 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 Beads Purification:

- NucleoMag NGS Clean-up and Size Select (available from Takara Bio; 5 ml size: Cat. No. 744970.5, 50 ml size: Cat. No. 744970.50, 500 ml size: Cat. No. 744970.500)

NOTES

- If the above NuceloMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter; 5-ml size: Cat. No. A63880; 60-ml size: Cat. No. A63881) is an appropriate substitute.
- The 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.
- 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 v3 - 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 workstations:
    - **A PCR-clean workstation** for all pre-PCR experiments that require clean room conditions, such as first-strand cDNA synthesis (Section V.A.)
    - **A second workstation located in the general laboratory** where you will perform PCR 1 (Section V.B.) and PCR 2 (Section V.E.), and cleave ribosomal cDNA with ZapR v3 and R-Probes v3 (Section V.D.)
    - **A third workstation 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 workstation must be located in a clean room with positive air flow, as contamination can 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 workstation operation:**

   - Only move materials/supplies from the PCR-clean workstation to the general lab, NOT the other way around. Do not share any equipment/reagents between the PCR-clean workstation and the general lab workstations.
   - Use a separate PCR thermal cycler (dedicated to first-strand cDNA synthesis) inside the PCR-clean workstation 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 NucleoMag 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 if necessary) 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: It is strongly recommended that working conditions for your samples be established before trying inputs beyond the recommended range.

  Input amounts higher than **10 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 would.

  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 **0.5–10 ng** for analysis of FFPE samples. Inputs higher than 10 ng have not been validated.

  For total RNA inputs **<250 pg** (**<500 pg** RNA extracted from FFPE samples), 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 (<500 pg FFPE sample RNA) will also contain a significantly higher amount of amplification artifacts and primer dimers due to the low complexity of the starting material and are more likely to contribute to low mapping rate.

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 –80˚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 include it 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.

Cell Input

- This protocol has been validated to generate libraries starting from intact cells. The cDNA synthesis protocol has been tested with 10–1,000 cultured K562 cells. It cannot be used with cells that have undergone fixation.
- When working with cultured cells, it is important to select a cell culture medium that does not inhibit first-strand cDNA synthesis. The protocol in this user manual was validated with cultured cells washed in cell-culture-grade PBS (Ca^{2+} and Mg^{2+}-free).

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 RNA samples with RIN ≥4 and DV200 ≥60% or intact cells.
- **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 I (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.

Table 1. Recommended Fragmentation Protocol Options and Fragmentation Times.

RNA quality	Use protocol	Fragmentation
RIN ≥7	Option 1	4min at 94°C
RIN 5–6	Option 1	3min at 94°C
RIN 4/DV200 ≥60%	Option 1	2min at 94°C
10–1,000 cells	Option 1	6min at 85°C
DV200 25–60% and all FFPE samples[2]	Option 2	–

[1] In most cases, samples with RIN ~4 will be optimally processed after 1.5–2 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 v3, 5X First-Strand Buffer, Nuclease-free water, SMART UMI-TSO Mix v3, RNase Inhibitor, and SMARTScribe II Reverse Transcriptase.

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

1. *To start with RNA samples*, mix the following components on ice:

1–8 µl	RNA
1 µl	SMART Pico Oligos Mix v3
4 µl	5X First-Strand Buffer
0–7 µl	Nuclease-free water
13 µl	Total volume per reaction

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.

2. *To start with cells:*
   a. Prepare RNase Inhibitor Water (RI Water) by combining 199 µl of Nuclease-free water with 1 µl of RNase Inhibitor (scale up as needed). Mix by vortexing and keep on ice until needed.
   b. Prepare a stock solution of 10X Lysis Mix by combining 19 µl of 10X Lysis Buffer and 1 µl of RNase Inhibitor (scale up as needed). Mix by vortexing and keep on ice until needed.
   c. Make sure your cells are in a total volume of 7 µl, in 0.2-ml PCR tubes or strip tubes. If input volume is less than 7 µl, complete with RI Water. Keep sample on ice.

      NOTE: Make sure to include a negative control with 7 µl RI Water, in addition to any other mock or no-cell control.

   d. Prepare enough Shearing Master Mix for all reactions, plus 10%, by combining following:

1 µl	10X Lysis Mix
1 µl	SMART Pico Oligos Mix v3
4 µl	5X First-Strand Buffer
6 µl	Total volume per reaction

   e. Add 6 µl of the Shearing Master Mix to each of your 7 µl cell suspension prepared in Step 2c, and mix by tapping gently, then spin down.
   f. Incubate the tube at 85°C in a preheated, hot-lid thermal cycler for **6 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:

4.5 µl	SMART UMI-TSO Mix v3
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe II Reverse Transcriptase
7 µl	Total volume per reaction

NOTE: The SMART UMI-TSO Mix v3 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 1 (RNA sample) or Step 2 (cells). 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	180 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 v3
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 UMI-TSO Mix v3
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe II Reverse Transcriptase
11 µl	Total volume per reaction

NOTE: The SMART UMI-TSO Mix v3 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	180 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: PCR 1—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 SMARTer RNA Unique Dual Index Kits (Cat. Nos. 634451, 634452, and 634457).

NOTES:

- The SMARTer RNA Unique Dual Index Kit - 24U (Takara Bio, Cat. No. 634451) is supplied with this kit, however SMARTer RNA Unique Dual Index Kits (Cat. Nos. 634452and 634457), if needed, will be required for purchase for use with the component kits.
- If library purification (Section V.C) will be performed immediately following PCR 1, remove aliquots of NucleoMag beads from the refrigerator to allow them to reach room temperature.

1. Prepare a PCR 1 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

2. Add 28 µl of PCR 1 Master Mix to each sample from Step A.6.
3. Add 2 µl of the premixed SMARTer RNA Unique Dual Index primers. 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 or 10 cycles*:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
68°C		2 min
4°C		forever

***Use 5 cycles for 0.25 ng–10 ng regular RNA samples.**
Use 10 cycles for 0.5 ng–10 ng FFPE RNA samples.

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 RNA-Seq Library Using NucleoMag Beads

IMPORTANT:
- Do not start this protocol if you do not have enough time to perform all steps up to Section V.E.
- Remove ZapR Buffer from –20°C storage and thaw it at room temperature in preparation for Section V.D.

The amplified RNA-seq library is purified by immobilization onto NucleoMag NGS Clean-up and Size Select 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.

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

1. Allow NucleoMag beads to come to room temperature before use (~30 min). Add 40 µl of NucleoMag 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. Remove the tubes from the magnetic separation device. Briefly spin them (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes back on the magnetic separation device for 30 sec, then—with the tubes still on the device—carefully remove any remaining ethanol with a pipette, without disturbing the beads.
8. Let the open sample tubes rest on the magnetic device 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 NucleoMag 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 v3 and R-Probes v3

In this section, the library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by ZapR v3 in the presence of R-Probes v3 (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 v3, ZapR v3, ZapR Buffer, Nuclease-free water, and a magnetic separation device.

1. Thaw R-Probes v3 and ZapR Buffer at room temperature. Place R-Probes v3 on ice as soon as it is thawed but keep ZapR Buffer at room temperature. ZapR v3 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 prechilled PCR tube a sufficient volume of R-Probes v3 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 v3 on ice and immediately return the remaining unused R-Probes v3 to a –70°C freezer.

5. Incubate the PCR tube containing R-Probes v3 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 v3 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 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—with the tubes still on the device—carefully remove any remaining ethanol with a pipette, without disturbing the beads.
11. Let the open sample tubes rest at room temperature on the magnetic separation device 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 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 v3 from Step D.6 last. Return ZapR v3 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 v3
1.5 µl	R-Probes v3
22 µl	Total volume per reaction

13. To each tube of dried NucleoMag beads from Step D.11, add 22 µl of the ZapR Master Mix (Step D.12). 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. With tubes on the magnetic separation device, 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: PCR 2—Final RNA-Seq Library Amplification

In this section, the library fragments not cleaved by the ZapR v3 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 v3 and SeqAmp DNA Polymerase.

1. Prepare a PCR 2 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 v3
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 2 Master Mix has been added, mixed, and spun down (prior to Step 3).

2. Add 80 µl of PCR 2 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 (Table 2) 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
5–10	12	10
0.5–1	14–15	13
0.25	16	-

*Lower inputs (<0.25 ng regular RNA and <0.5 ng FFPE RNA) may generate acceptable libraries by increasing PCR2 cycles but need to be evaluated by the user on a case-by-case-basis.

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 NucleoMag Beads

The amplified RNA-seq library is purified by immobilization onto NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol and eluted in Tris Buffer.

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

1. Allow NucleoMag beads to come to room temperature before use (~30 min). Add 100 µl of NucleoMag 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 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—with the tubes still on the device—carefully remove all remaining ethanol with a pipette, without disturbing the beads.
8. Let the sample tubes rest open on the magnetic separation device 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 22 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.
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 20 µl of supernatants to new tubes.
13. Add 20 µl of NucleoMag beads to perform a second round of beads clean-up by repeating Step 2–8.
14. Once the beads are dry, add 12 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.
15. Incubate at room temperature for 5 min to rehydrate.
16. 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.
17. Transfer 10 µl of supernatants to new tubes, and proceed to next step immediately or store in –20°C.

G. Protocol: Validation Using the Agilent 2100 Bioanalyzer

1. Quantify libraries with Qubit dsDNA HS kit (Thermo Fisher Scientific). A yield ≥4 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 20 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 2 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 cleanup (Section V.F) if an excessive amount of products <200 bp in size is present.

NOTE: Library preparation adds 150 bp to the size of the original RNA molecules.

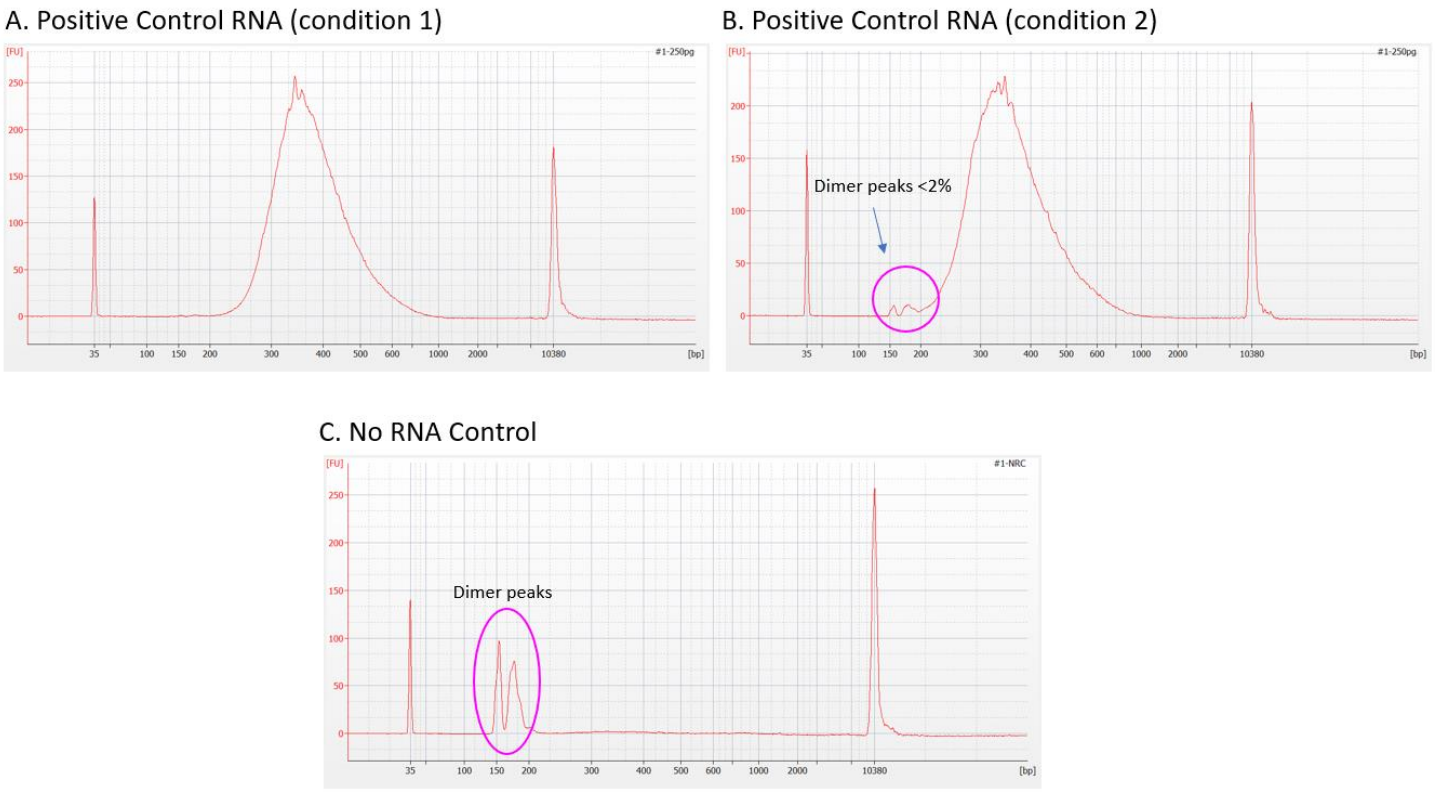

Figure 4. Example electropherogram results from the Agilent 2100 Bioanalyzer. Libraries were generated using 0.25 ng Control Total RNA (human brain; **Panel A, B**—library diluted to 2 ng/µl based on Qubit quantification) and a no-RNA control (**Panel C**—no library dilution). For both examples, PCR 2 was performed using 16 cycles. Note that in certain experimental conditions, small dimer peaks might be observed from positive libraries (Panel B), but the peak region should not exceed 2% of total library. No RNA control libraries often show obvious dimer peaks.

VI. References

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

Appendix A: Optional DNase pretreatment to remove genomic DNA

For RNA materials containing genomic DNA contamination, we recommend an optional DNase pretreatment to remove the residual DNA. We have validated the Heat&Run gDNA removal kit (ArcticZymes, Cat. No. 80200-250) for our workflow.

1. Take 10X reaction Buffer and HL-dsDNase out from –20°C and leave them on ice.
2. Prepare gDNA Removal Mix by combining following:

1– 5.2 µl	RNA
0.7 µl	10X reaction Buffer (ArcticZymes)
1.0 µl	HL-dsDNase (ArcticZymes)
0.1 µl	RNase Inhibitor
7 µl	Total volume per reaction

3. Incubate at 37°C for 10 min, and then at 58°C for 5 min.
4. The 7 µl RNA is now gDNA free and can be used directly as RNA input for Section V.A. (First-Strand cDNA Synthesis).

Appendix B: Illumina UDIs

Appropriate combinations of Illumina indexes are necessary to ensure enough nucleotide diversity and allow for discrimination between samples when sequencing a pool of two or more libraries on a single flow cell lane. Please consult the following resources for component information, best practices, pooling strategies, an index plate map, and index sequences.

- Unique Dual Index Kit Protocol-At-A-Glance
- Unique Dual Index Sequence Information

The SMARTer RNA Unique Dual Index Kit - 24U contains indexed PCR primers corresponding to the IDT for Illumina-TruSeq DNA and RNA UD Indexes - UDI0001–UDI0096 according to the Illumina Adapter Sequences Document (1000000002694 v10) and offers up to 24 unique dual indexes (Table 3). Dual-index sequencing on a paired-end flow cell follows one of two workflows. Use i5 in the Table 3 depending on the system. The indexed PCR primers are supplied predispensed in 8-tube PCR strips (individually labeled U25 to U48).

Table 3. SMARTer RNA Unique Dual Index sequences (SMARTer RNA Unique Dual Index Kit - 24U)

Index name	i7 bases for sample sheet	i5 bases for sample sheet (MiSeq®, NovaSeq™, HiSeq ® 2000/2500)	i5 bases for sample sheet (MiniSeq™, NextSeq®, HiSeq 3000/4000)
U25	ACTAAGAT	AACCGCGG	CCGCGGTT
U26	GTCGGAGC	GGTTATAA	TTATAACC
U27	CTTGGTAT	CCAAGTCC	GGACTTGG
U28	TCCAACGC	TTGGACTT	AAGTCCAA
U29	CCGTGAAG	CAGTGGAT	ATCCACTG
U30	TTACAGGA	TGACAAGC	GCTTGTCA
U31	GGCATTCT	CTAGCTTG	CAAGCTAG
U32	AATGCCTC	TCGATCCA	TGGATCGA
U33	TACCGAGG	CCTGAACT	AGTTCAGG
U34	CGTTAGAA	TTCAGGTC	GACCTGAA
U35	AGCCTCAT	AGTAGAGA	TCTCTACT
U36	GATTCTGC	GACGAGAG	CTCTCGTC
U37	TCGTAGTG	AGACTTGG	CCAAGTCT
U38	CTACGACA	GAGTCCAA	TTGGACTC
U39	TAAGTGGT	CTTAAGCC	GGCTTAAG
U40	CGGACAAC	TCCGGATT	AATCCGGA
U41	ATATGGAT	CTGTATTA	TAATACAG
U42	GCGCAAGC	TCACGCCG	CGGCGTGA
U43	AAGATACT	ACTTACAT	ATGTAAGT
U44	GGAGCGTC	GTCCGTGC	GCACGGAC
U45	ATGGCATG	AAGGTACC	GGTACCTT
U46	GCAATGCA	GGAACGTT	AACGTTCC
U47	GTTCCAAT	AATTCTGC	GCAGAATT
U48	ACCTTGGC	GGCCTCAT	ATGAGGCC

Appendix C: Sequencing Guidelines

A. Sequencing Analysis Guidelines

- 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.
- First eight cycles of Read 2 belong to UMIs followed by 3 nucleotides of UMI-linker and 3 nucleotides derived from the Pico v3 SMART UMI Adapter.
- Cogent NGS Analysis Pipeline Software collapses UMIs and trims 3 nucleotides of UMI linker and 3 nucleotides derived from the Pico v3 SMART UMI Adapter. To obtain the Cogent NGS Analysis Pipeline Software, please visit takarabio.com/ngs-cogentap.
- If not using Cogent NGS Analysis Pipeline, trim **8 nt UMIs + 3 nt UMI linker + 3 nt from Pico v3 SMART UMI Adapter** from Read2 prior to mapping**.**

B. Pooling Recommendations

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained with the Qubit with the average library size determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for the conversion: http://www.molbiol.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 at a concentration 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 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. For Illumina sequencers with patterned flow cells, include 10% PhiX spike-in.

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.

C. Loading Guidelines for Various Illumina Instruments

Libraries generated with the SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian cluster very efficiently and care must be taken to avoid overclustering. The guidelines in Table 4 (below) are a good starting point and have been fully validated.

Table 4. 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

D. Extra Precautions When Using NextSeq and MiniSeq Instruments

Libraries generated with the SMARTer Stranded Total RNA-Seq Kit v3 - 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 v3 - 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.

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