Takara Bio USA, Inc.

SMART-Seq® Total RNA Pico Input with UMIs (ZapR® Mammalian) User Manual

Cat. Nos. 634354, 634355 & 634356
(021925)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) (Cat. Nos. 634354, 634355 & 634356) is designed to prepare strand-specific, ribosomal RNA (rRNA) depleted Illumina® sequencing libraries from picogram inputs of high-integrity or compromised total RNA (250 pg–10 ng) or from 10–1,000 intact cells. The kit 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 Cogent™ NGS Analysis Pipeline Software (CogentAP), which filters out PCR duplicates and errors based on the UMIs.

The entire library construction protocol, including rRNA depletion starting with total RNA, can be completed in less than 7 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 4).

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 ZapR technology allowing removal of ribosomal cDNA (originating from rRNA) after cDNA synthesis using probes specific to mammalian rRNA. These R-Probes target mammalian nuclear 28S, 18S, 5.8S, and 5S rRNA and human mitochondrial m16S and m12S rRNA sequences (nonhuman mitochondrial rRNA sequences will not be removed); The rRNA depletion method used in this kit makes it especially well-suited for working with very small quantities of total RNA.

SMART-Seq Total RNA Pico input with UMIs (ZapR Mammalian) performs best with total RNA in the 250 pg–10 ng range, however inputs >10 ng also generates 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 0.5–10 ng range. Please refer to Section IV.C for more details.

To generate library inserts of an appropriate size for compatibility with Illumina sequencing, RNA molecules obtained from high-integrity or partially degraded samples must be fragmented prior to cDNA synthesis. For highly degraded, low-integrity starting material, the RNA fragmentation step should be skipped. Protocols for both options are provided in Section V.A.

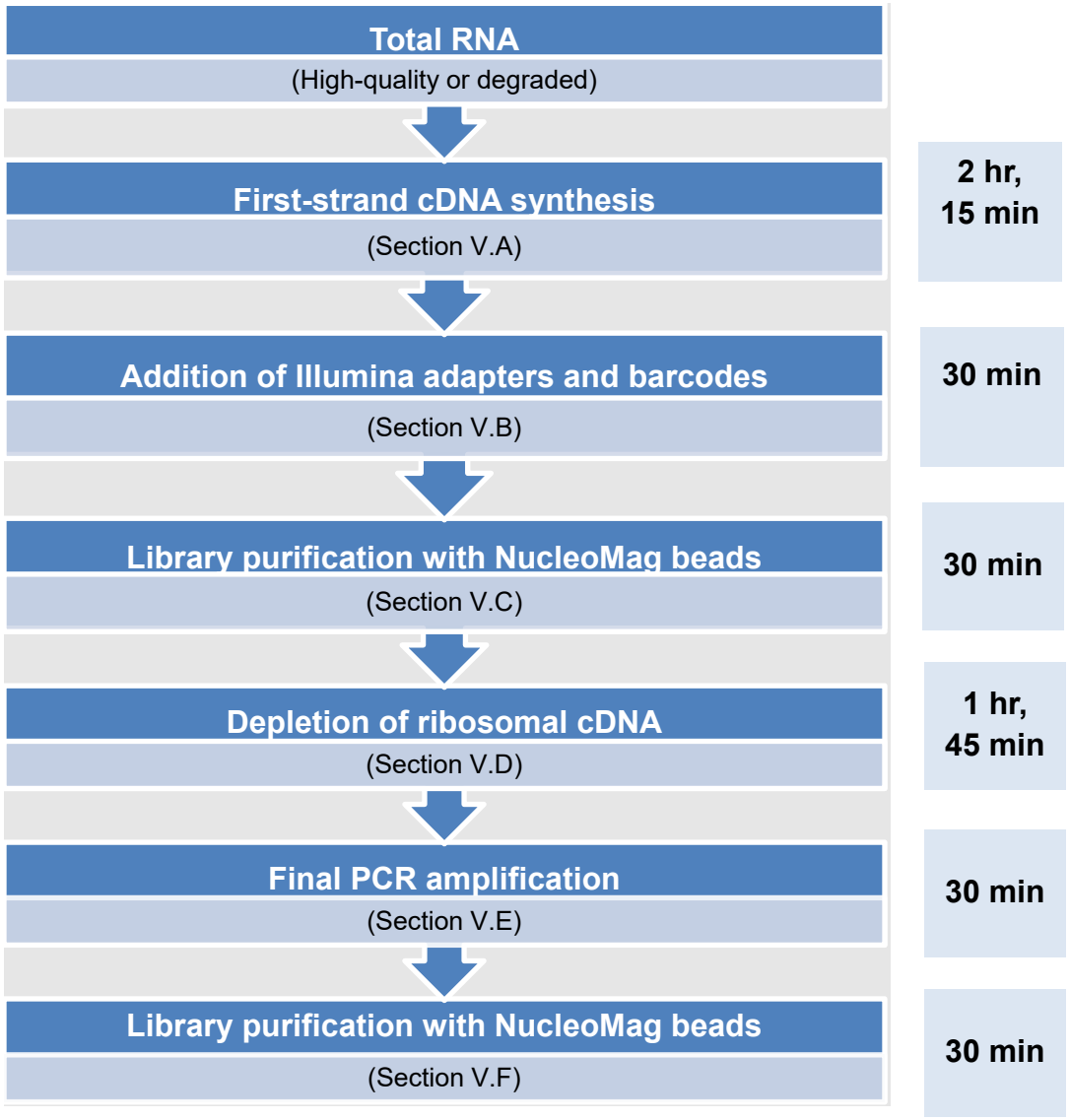

Figure 1. SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) protocol overview. This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries in approximately 6 hr 30 min. Actual processing time may vary depending on the number of samples and cycling conditions (e.g., Protocol E takes more than 30 min if using 18 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 p5/p7 primers universal to all Illumina 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.

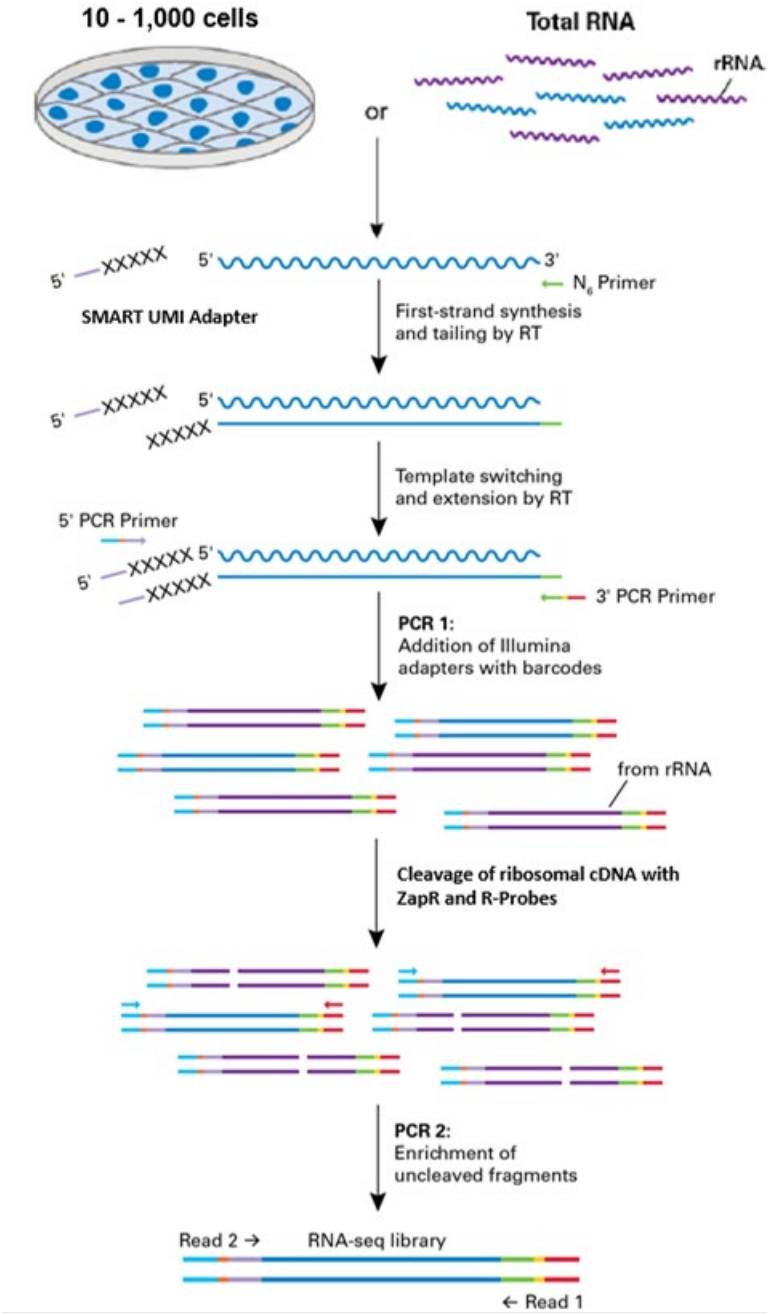

Figure 2. Schematic of technology in SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian). SMART technology is used in this ligation-free protocol to preserve strand-of-origin information. Random priming (represented by the green N6 Primer) allows the generation of cDNA from all RNA fragments in 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 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 SMART UMI Adapter also contains 8 random nts which function as the unique molecular identifiers (UMIs) for the 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 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 Enzyme in the presence of the mammalian-specific R-Probes. The resulting cDNA contains sequences derived from the random primer and the 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 p5/p7 primers universal to all Illlumina 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 SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian). The adapters added during PCR 1 using a Unique Dual Index Kit (Takara Bio, Cat. Nos. 634752–634756) 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 nontemplated nucleotides derived from the SMART UMI Adapter (shown as XXX).

II. List of Components

SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) contains sufficient reagents to prepare 24 (Cat. No. 634354), 96 (Cat. No. 634355), or 384 (Cat. No. 634356) reactions. Freeze-thaw of the R-Probes should be limited to a total of 3 times, while freeze-thaw of all other reagents should be limited to ≤6 times. The SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) components have been specifically designed to work together and are optimized for this protocol.

IMPORTANT: Please do not make any substitutions. The substitution of reagents in the kit and/or modification of the protocol may lead to unexpected results.

NOTE: Indexing primers are not included in this kit and need to be purchased separately (Unique Dual Index Kit, Takara Bio, Cat. Nos. 634752–634756).

Table 1. SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) components.

SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian)	634354 (24 rxns)	634355 (96 rxns)	634356 (384 rxns)
SMART-Seq Total RNA Pico Input with UMIs	**634363***	**634364***	**4 x 634364***
Package 1 (Store at –70°C)			
SMART UMI-TSO Mix v3†	110 µl	450 µl	4 x 450 µl
Package 2 (Store at –20°C)			
SMART Pico Oligos Mix v3†	30 µl	110 µl	4 x 110 µl
5X First-Strand Buffer	100 µl	400 µl	4 x 400 µl
SMARTScribe II Reverse Transcriptase	50 µl	200 µl	4 x 200 µl
RNAse Inhibitor (40 U/µl)	30 µl	100 µl	4 x 100 µl
SeqAmp™ DNA Polymerase	100 µl	2 x 200 µl	8 x 200 µl
SeqAmp CB PCR Buffer (2X)	2 x 1.25 ml	10 ml	4 x 10 ml
Tris Buffer (5 mM)	1.25 ml	5 ml	4 x 5 ml
PCR2 Primers v3	100 µl	400 µl	4 x 400 µl
Nuclease-Free Water	2 x 1.25 ml	10 ml	4 x 10 ml
10X Lysis Buffer	0.5 ml	1 ml	4 x 1 ml
ZapR Mammalian rRNA Depletion Kit	**634369***	**634370***	**4 x 634370***
Package 1 (Store at –70°C)			
R-Probes‡	40 µl	160 µl	4 x 160 µl
Control Total RNA (1 µg/µl)	5 µl	5 µl	4 x 5 µl
Package (Store at –20°C)			
ZapR Enzyme	75 µl	300 µl	4 x 300 µl
ZapR Buffer (10X)	100 µl	400 µl	4 x 400 µl

*Not sold separately.
†Takara Bio proprietary sequences.
‡See important note on the next page regarding freeze-thaw of R-Probes.

Additional component information:

- **IMPORTANT: Do not freeze thaw R-Probes more than 3 times!** We recommend aliquoting R-Probes into multiple vials to avoid repeated freeze-thaw cycles.
- Do not freeze-thaw the PCR2 Primers v3 more than 10 times.
- Store 10X Lysis Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
- 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.
- SMART UMI-TSO Mix v3 contains SMART UMI Adapter (i.e. template-switching oligo).
- SeqAmp DNA Polymerase is a hot-start enzyme.
- Control Total RNA is from human brain.
- Make sure to spin down tubes to collect all the liquid at the bottom before first use.

III. Additional Materials Required (Not Provided)

The following reagents and materials are required but not supplied. The specified brands have been validated to work with this protocol.

- Single-channel pipette: 10 µl, 20 µl, 200 µl, and 1,000 µl
- Eight-channel or 12-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips
- 96-well PCR chiller rack, such as IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4) or 96-Well Aluminum Block (Light Labs, Cat. No. A-7079)
- Benchtop coolers, such as VWR CryoCoolers (VWR, Cat. No. 414004-286)

For Sample Preparation

- 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR grade tube strips secured into a PCR rack, or 96-well plates that have been validated to work with your FACS instrument
- Microplate film (USA Scientific, Cat. No. 2920-0010) for sealing tubes/plates before sorting
- Aluminum single tab foil seal (USA Scientific, Cat. No. 2938-4100) or cap strips (Thermo Fisher Scientific, Cat. No. AB0784/AB0850) for sealing tubes/plates after sorting
- Low-speed benchtop centrifuge for 96-well plates or tube strips
- Dry ice in a suitable container for flash freezing cells
- **(Optional)** BD FACS Pre-Sort Buffer (BD Biosciences, Cat. No. 563503)
- **(Optional)** SMART-Seq HT Kit Lysis Components (Cat. No. 634439) or 10X Lysis Buffer (Takara Bio, Cat. No. 635013) for sorting extra plates

For cDNA Synthesis and Amplification

- Two thermal cyclers with heated lids
  - One thermal cycler used only for first-strand cDNA synthesis (Section V.A)
  - One thermal cycler used only for double-stranded cDNA amplification by PCR (Section V.B) and library amplification (Section VI.B)

NOTES:

- The thermal cycler should always be used with the heated lid option turned on. If prompted to input a specific temperature, use 105°C. Most thermal cyclers with heated lids will automatically adjust the lid temperature just above the highest block temperature within a cycling program. However, if your thermal cycler does not make this automatic adjustment, you may want to follow the manufacturer's instructions to choose a lower lid temperature for the reverse transcription step.
- 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.

- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2 ml PCR 8-tube strip; USA Scientific, Cat. No.1402-4700) or similar nuclease-free, PCR-grade, thin-wall PCR tubes, strips, or 96-well plates
- Nuclease-free, low-adhesion 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes
- Thermo Scientific Adhesive PCR Plate Seals (Thermo Fisher, Cat. No. AB0558) for 96-well plates or cap strips (Thermo Fisher, Cat. No. AB0784/AB0850) for 8-tube strips

For Illumina Library Preparation (Section V.B)

- Unique Dual Index Kits
  - 96 indexes: Takara Bio, Cat. Nos. 634752, 634753, 634754, and/or 634755
  - 24 indexes: Takara Bio, Cat. No. 634756

For Bead Purifications

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

NOTES:

- The kit has been specifically validated with the beads listed above. Please do not make any substitutions as it may lead to unexpected results.
- Beads need to come to room temperature before the container is opened. 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 minutes). Aliquoting is also instrumental in decreasing the chances of bead contamination.
- Immediately before 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.

- 80% ethanol: freshly-made for each experiment from molecular-biology-grade 100% ethanol

- Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates, such as:
  - For 12–24 samples:
    - SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011); accommodates two 8-tube or 12-tube strips
    - Minicentrifuge for 0.2 ml tubes or strips
  - For 24–96 or more samples:
    - Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher Scientific, Cat. No. AB0558)
    - Low-speed benchtop centrifuge for a 96-well plate
  - For 1.5 ml tubes (for pooling sequencing libraries):
    - Magnetic Stand (Takara Bio, Cat. No. 631964)
    - 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR grade tube strips secured into a PCR rack, or 96-well plates that have been validated to work with your FACS instrument

For cDNA and Illumina Library Quantification (As Required)

- High Sensitivity DNA Kit (Agilent Technologies, Cat. No. 5067-4626) for Bioanalyzer, Agilent 2200 TapeStation system with a High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067-5592), or an equivalent high-sensitivity electrophoresis method (may be used in Sections V.D and VI.D)
- Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Cat. No. P11496) or Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or Q32854) (may be used in Sections V.D and VI.D)
- Library Quantification Kit (Takara Bio, Cat. No. 638324) (may be used in Section VI.D)

IV. General Considerations

A. Recommendations for Preventing Contamination

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.)

NOTE: The PCR-clean workstation should 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.

B. General Requirements

- **IMPORTANT!** For products Cat. No. 634355 (96 rxns) and Cat. No. 634356 (4 x 96 rxns), we recommend performing a **minimum** of 12 reactions per protocol run to ensure sufficient reagents to utilize 96 (or 384) reactions per kit.

- **The success of your experiment depends on the 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 SMART cDNA synthesis need to be stored in a DNA-free, closed cabinet. Ideally, reagents for SMART 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 SMART 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.

C. Sample Recommendations and Requirements

SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) works picogram inputs (250 pg–10 ng) of total RNA from high-quality or degraded samples or intact cells (10–1,000).

1. Total RNA Input

- **RNA should be in a maximum volume of 8 µl.**
- Degraded, partially degraded, or high-integrity RNA can be analyzed with this kit.
- This protocol has been optimized for cDNA synthesis from 250 pg–10 ng of mammalian total RNA. Purified total RNA should be in nuclease-free water and free of contamination.
    - 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 RNA content in a particular sample/cell type/tissue) and the length of the RNA (high-integrity versus highly degraded). Libraries generated from inputs <250 pg (<500 pg FFPE sample RNA) may 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.
- Before starting the workflow, evaluate total RNA integrity using the Agilent RNA 6000 Pico Kit (Agilent Technologies, Cat. No. 5067-1513) or an equivalent microfluidics-based automated electrophoresis system to determine if fragmentation will be necessary.
- Input RNA should be free from genomic or carrier DNA and contaminants that would interfere with oligo annealing, cDNA synthesis Samples should be 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.

2. 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).

D. Diluting the Control Total RNA

Positive control reactions are invaluable to ensure the kit performs as expected and are essential for troubleshooting experiments. 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.

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. When needed, thaw an aliquot, further dilute (Steps 2–4), and throw away any leftovers. Make sure to change pipette tips for each dilution step described below. Use low-nucleic acid-binding tubes for all dilutions.

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
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.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

During this step, RNA is fragmented (if needed) and first-strand cDNA synthesis is primed by the SMART Pico Oligos and uses the SMART UMI-TSO for template-switching at the 5′ end of the transcript.

- **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 2 (below) for guidelines.

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 2. Recommended fragmentation protocol options and fragmentation times.

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

*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 over-fragmentation 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.
†Option 2 is recommended for all FFPE samples regardless of RIN or DV200 values.

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

Follow the appropriate initial steps depending on your starting material.

a) Starting with RNA samples

1. 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**

2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler for the amount of time recommended in Table 2 or for an experimentally determined, optimal amount of time.
3. At the expiration of that time, immediately place the samples on an ice-cold PCR chiller rack for 2 min. **Proceed immediately to c) Protocol**.

b) Starting with cells

1. Prepare RNase Inhibitor Water (RI Water) by combining 199 µl of Nuclease-Free Water with 1 µl of RNase Inhibitor (scale up as needed, depending on the volume needed in Step 3). Mix by vortexing and keep on ice until needed.
2. 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, depending on the quantity needed in Step 4). Mix by vortexing and keep on ice until needed.
3. Make sure your cells are in a total volume of 7 µl, in 0.2 ml PCR tubes or strip tubes or a 96-well plate. If input volume is less than 7 µl, add enough Ca^{2+}/Mg^{2+}-free PBS to make the volume 7 µl. Keep sample on ice.

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

4. Prepare enough shearing master mix for all reactions, plus 10% of the total reaction mix volume (in the order shown below) Mix by gentle vortexing or pipetting and keep on ice until needed.

Shearing master mix:

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

5. Add 6 µl of the shearing master mix to each of your 7 µl cell suspensions prepared in Step 3 (the order shown). Mix by tapping gently, then spin down.

6 µl	Shearing master mix (Step 4)
7 µl	Cell suspension (Step 3)
13 µl	**Total volume per reaction**

6. Incubate the tubes at 85°C in a preheated thermal cycler for 6 min.
7. After 6 min, immediately place the samples on an ice-cold PCR chiller rack for 2 min. **Proceed immediately to c) Protocol**.

c) Protocol

1. On ice, prepare enough first-strand master mix for all reactions, plus 10% of the total reaction mix volume, by combining the following reagents in the order shown:

First-strand master mix (with fragmentation):

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.

2. Add 7 µl of the first-strand master mix to each reaction tube or plate well resulting from a) (RNA samples) or b) (Cells) from above.

13 µl	Sample mix [from a) or b)]
7 µl	First-Strand master mix (From Step 1)
20 µl	**Total volume per reaction**

Mix the contents of the tubes by vortexing for ~2 sec, then spin the tubes briefly to collect the contents at the bottom.

3. Incubate the tubes in a preheated thermal cycler with the following program:

42°C	90 min
70°C	10 min
4°C	forever

4. Leave the samples in the thermal cycler at 4°C until the next step (Section V.B).

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.

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

1. On ice, mix the following components in 0.2 ml PCR tubes, strip tubes, or 96-well plate.

Sample mix:

1–8 µl	RNA
1 µl	SMART Pico Oligos Mix v3
0–7 µl	Nuclease-Free Water*
9 µl	**Total volume per reaction**

*Use enough Nuclease-Free Water that to bring the total volume, after adding the SMART Pico Oligos Mix v3 to your sample RNA, to equal 9 µl.

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. On ice, prepare enough first-strand master mix for all reactions, plus 10% of the total reaction mix volume, by combining the following reagents in a 1.5 ml centrifuge tube in the order shown.

First-strand master mix (without fragmentation):

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.

9 µl	Sample mix (From Step 1)
11 µl	First-strand master mix (From Step 3)
20 µl	**Total volume per reaction**

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 (Section V.B).

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: First RNA-Seq Library Amplification by PCR (PCR 1)

The first-strand cDNA is amplified into RNA-seq libraries using SeqAmp DNA Polymerase and Illumina adapters and indexes are incorporated via the unique dual-indexed primers from the Unique Dual Index Kits.

NOTE: 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. In a 1.5 ml centrifuge tube, prepare a PCR1 master mix for all reactions, plus 10% of the total reaction mix volume. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge. Keep the master mix on ice until use:

   PCR1 master mix:

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 resulting from Step A.

20 µl	First-strand cDNA (from Section V.A)
28 µl	PCR1 master mix (from Step 1)
48 µl	**Total volume per reaction**

3. Add 2 µl of the appropriate UDI from the Unique Dual Index Kit (12.5 µM) to each sample tube or well to make a library amplification mix.

48 µl	PCR1 sample mix (from Step 2)
2 µl	UDI
50 µl	**Total volume per reaction**

   Mix by gentle vortexing or tapping of the tubes, then spin down briefly.

4. Place the tubes or plate in a preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C	1 min	
5–10 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 first hour, freeze the PCR products at –20°C for up to 2 weeks.

C. Protocol: Purification of the RNA-Seq Library

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

The amplified RNA-seq library is purified by immobilizing it onto NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol and the cDNA is eluted in Nuclease-Free Water prior to depletion of ribosomal cDNAs.

NOTES:

- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min and mix well by vortexing.
- Bead:sample ratio is 0.8:1
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.
- **Thaw ZapR Buffer at room temperature in preparation for Section V.D.**

1. If purification is performed directly in the PCR tubes or strips using the Takara Bio SMARTer-Seq Magnetic Separator - PCR Strip, add 40 µl of beads to each sample. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 2.

   (Optional) If you are performing purification with the Thermo Fisher Magnetic Stand-96 (recommended if processing 48–96 samples), cDNA samples need to be transferred to a 96-well V bottom plate. Distribute 40 µl of beads to each well of the 96-well V-bottom plate, and then use a multichannel pipette to transfer the cDNA. Pipette the entire volume up and down at least 10 times to mix thoroughly. Proceed to Step 2.
2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.
4. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
5. Keep the tubes on the magnetic stand. Add 200 µl of freshly-made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.
7. Perform a brief spin of the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for ~3–5 min until the pellets appear dry.

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

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

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

9. Once the beads are dried, add 52 µl of Nuclease-Free Water to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly to resuspend the beads.

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

10. Incubate at room temperature for 5 min to rehydrate.
11. Mix by pipetting up and down 10 times to elute the DNA from the beads.
12. Place the tubes/plate back on the magnetic stand for 1 min or longer, until the solution is completely clear.
13. Transfer 50 µl of clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free, low-adhesion tube.
14. Add 40 µl of NucleoMag beads to each sample. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 15.
15. Incubate at room temperature for 8 min to let the DNA bind to the beads. During the incubation time, proceed immediately to Section V.D.

NOT A SAFE STOPPING POINT: Continue on to Section V.D, "Depletion of Ribosomal cDNA".

D. Protocol: Depletion of Ribosomal cDNA

Library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by ZapR Enzyme in the presence of R-Probes (mammalian-specific). These R-Probes target mammalian nuclear 28S, 18S, 5.8S, and 5S rRNA and human mitochondrial m16S and m12S rRNA sequences. Non-human mitochondrial rRNA sequences will not be removed.

NOTES: Before starting:

- Thaw R-Probes and ZapR Buffer at room temperature.
- Place R-Probes on ice as soon as it is thawed but keep ZapR Buffer at room temperature.
- Prechill an empty 0.2 ml PCR tube on ice.
- ZapR Enzyme should be kept on ice at all times and returned to the freezer immediately after use.
- Preheat the thermal cycler in anticipation of Step 3.

1. Upon completion of the steps in Section V.C, briefly spin the sample tubes or plate to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for an additional ~3–5 min or 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.

2. During the incubation time in Step 1, on ice, pipette a sufficient volume of R-Probes for the number of reactions to be performed (1.5 µl per reaction, see Step 10), plus 10% of the total reaction volume, into the prechilled PCR tube.

   NOTE: Immediately return the remaining unused R-Probes to a –70°C freezer.

3. Incubate the R-Probes at 72°C in a preheated hot-lid thermal cycler using the following program:

72°C	2 min
4°C	forever

Leave the R-Probes 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 10.

NOTE: Perform Steps 4–9 while the R-Probes are incubating (Step 3).

4. Once the 5-min incubation on the magnetic separation device is complete (Step 1) and the samples are clear, pipette out and safely discard the supernatant from the sample tubes. Keep the tubes on the magnetic stand.
5. Add 200 µl of freshly-made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 sec and carefully pipette out and discard the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.
7. Perform a brief spin of the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette without disturbing the beads.
8. Let the sample tubes rest open at room temperature for ~1–2 min until the pellet appears dry.

NOTES:
- The beads will dry more quickly than in Section V.C, Step 8. 1–2 min may be sufficient, but the beads can be left to dry for up to 5 min.
- Perform Step 9 while the beads are drying.

9. At room temperature, prepare enough ZapR master mix for all reactions, plus 10% of the total reaction volume, by combining the following reagents at room temperature in the order shown.

IMPORTANT:
- Make sure to add the preheated and chilled R-Probes from Step 6 last.
- Return ZapR enzyme to a –20°C freezer immediately after use.

ZapR master mix:

15.5 µl	Nuclease-free water
2.2 µl	10X ZapR Buffer
2.8 µl	ZapR enzyme
1.5 µl	R-Probes
22 µl	**Total volume per reaction**

Mix the components well by vortexing briefly and spin the tubes briefly in a microcentrifuge.

10. To each tube of dried NucleoMag beads (from Step 8), add 22 µl of the ZapR master mix.
11. Remove the tubes from the magnetic separation device and mix thoroughly to resuspend the beads.
12. Incubate at room temperature for 5 min to rehydrate.
13. 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.
14. With tubes on the magnetic separation device, pipette out 20 µl of supernatant into new PCR tubes, strips, or plate wells, being careful not to disturb the beads.

15. Incubate the tubes in a preheated 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: Second RNA-Seq Library Amplification by PCR (PCR 2)

Library fragments not cleaved during the ZapR reaction are enriched in a second round of PCR. Since barcodes have already been added to the libraries, a single pair of primers can be used.

1. Prepare the PCR2 master mix for all reactions, plus 10% of the total reaction mix volume in a 1.5 ml centrifuge tube. Combine the following reagents in the order shown, then mix well and spin the tubes briefly in a microcentrifuge:

PCR2 master mix:

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 PCR2 master mix has been added, mixed, and spun down (prior to Step 3).

2. Add 80 µl of PCR2 master mix to each tube of depleted samples and controls resulting from Section V.D. 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
9–18 cycles*:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

*The actual number of cycles varies depending on the starting material.

The guidelines below (Table 3, next page) must be validated with your material. We do not recommend performing more than 18 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 3. PCR 2 cycling guidelines based on the amount of starting material.

Amount of input RNA (ng)	Typical number of PCR cycles	
	Regular RNA	FFPE RNA
5–10	14	12
0.5–1	16-17	16
0.25*	18	-

*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

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 with Tris Buffer.

NOTES:
- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads to room temperature for at least 30 min and mix well by vortexing.
- Bead:sample ratio is 1:1
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- Use a second magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate.

1. If purification is performed directly in the PCR tubes or strips using the Takara Bio SMARTer-Seq Magnetic Separator - PCR Strip, add 100 µl of beads to each sample. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 2.

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

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

3. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.

   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 stand, pipette out the supernatant and safely discard it.

5. Keep the tubes on the magnetic stand. Add 200 µl of freshly-made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 sec and carefully pipette out and discard the supernatant. DNA will remain bound to the beads during the washing process.

6. Repeat Step 5 once.

7. Perform a brief spin of the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.

8. Let the sample tubes rest open at room temperature for ~5–7 min until the pellet appears dry.

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

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

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

9. Once the beads are dried, add 22 µl of Nuclease-Free Water to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly by pipetting up and down several times until all the beads have been washed off the sides of tubes.

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

10. Incubate at room temperature for 5 min to rehydrate.
11. Briefly spin the sample tubes. Place the tubes/plate back on the magnetic stand for 1 min or longer, until the solution is completely clear.
12. Transfer 20 µl of clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free, low-adhesion tube.
13. Add 20 µl of beads to perform a second round of beads clean-up by repeating Steps 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.

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

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 each supernatant to new nuclease-free, low-adhesion tubes.

SAFE STOPPING POINT: Proceed to library validation step immediately or store tubes at –20°C.

G. Protocol: Library Validation

To assess the success of library preparation, purification, and size selection, we recommend

- analyzing and validating final libraries using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or Q32854) and
- evaluating the libraries' size distributions with an Agilent 2100 Bioanalyzer and the DNA High Sensitivity Kit (Agilent Technologies, Cat. No. 5067-4626) or Tapestation and High Sensitivity D5000 ScreenTape (Agilent Technologies, Cat. No. 5067-5588).

Please refer to the corresponding user manuals for detailed instructions.

1. Qubit: Use 1 µl of undiluted library for quantification using a Qubit 2.0 Fluorometer (see manufacturer's instructions for more details).
2. Bioanalyzer: To validate libraries using the DNA High Sensitivity Kit, dilute libraries to a concentration of 1 ng/µl. Use 1 µl of the diluted sample for validation using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from the High Sensitivity DNA Kit (Agilent Technology, Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
3. Compare the results for your samples and controls (if performed) to determine whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield a distinct curve spanning 150–1,000 bp, peaked at ~300 bp for the positive control RNA sample (Figure 4, Panels A & B) and no product in the negative control (Figure 4, Panel C). The RNA-seq library yield should be >7.5 nM (depending on the input and number of cycles).

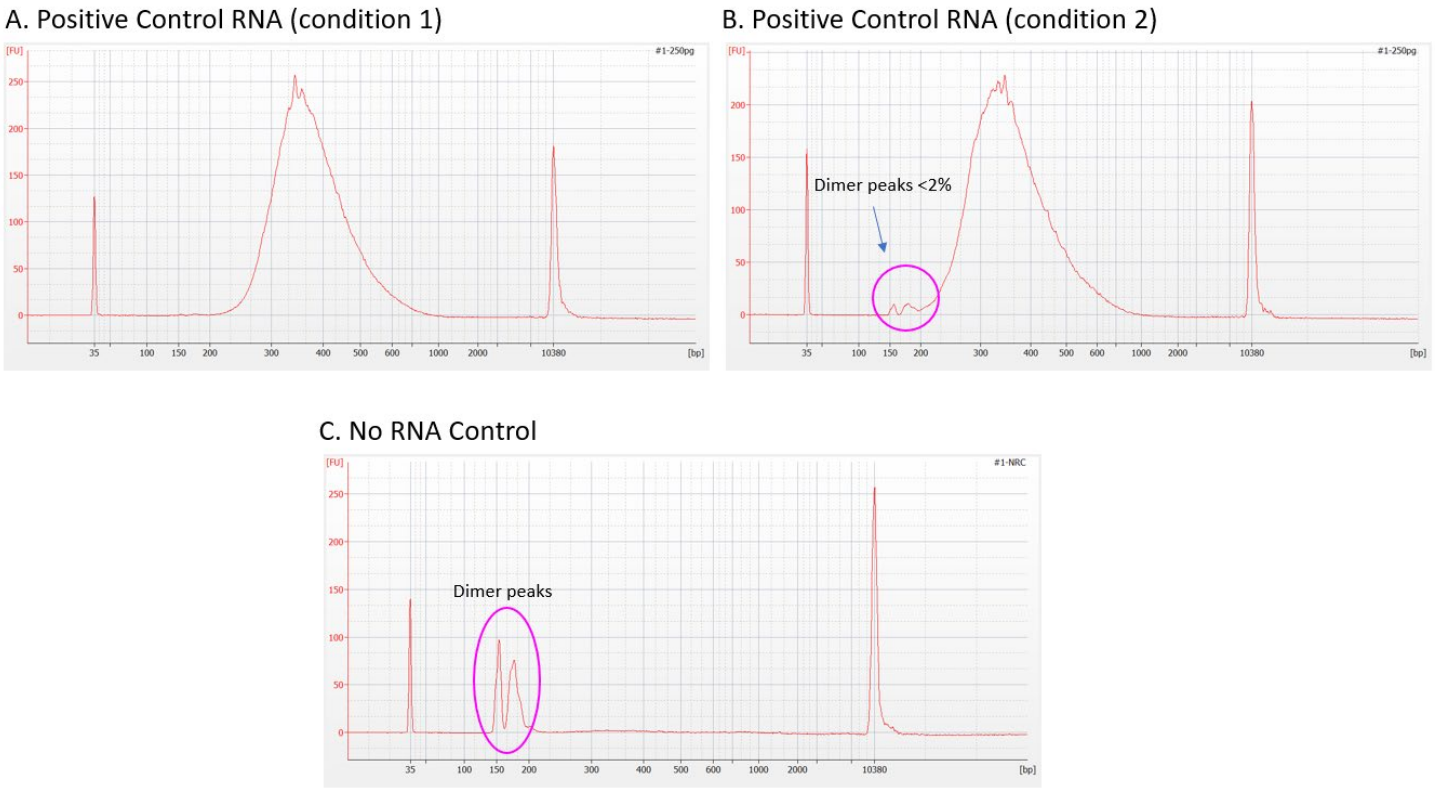

Figure 4. Example electropherogram results from the Agilent 2100 Bioanalyzer. For all examples, PCR 2 was performed using 18 cycles. **Panels A & B**. Libraries were generated using 0.25 ng Control Total RNA (human brain; libraries diluted to 1 ng/µl based on Qubit quantification). **Panel B**. Under certain experimental conditions, small dimer peaks might be observed from positive libraries, but the peak region should not exceed 2% of total library for non-patterned flow cells and not exceed 0.5% for patterned flow cells. **Panel C.** Libraries were generated using 0.25 ng a no-RNA control with no dilution. 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. 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. Consult the Illumina literature (e.g., TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines.

SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) requires use of the Unique Dual Index Kits (Cat. Nos, 634752–634756, sold separately). The indexes are 8-nt long and employ "IDT for Illumina TruSeq UD Indexes" i5 and i7 dual index sequences. In all versions of the UDI kits, the primers are provided in a 96-well plate format; the indexes in Unique Dual Index Kit (1-24) (Cat. No. 634756) are a subset of Unique Dual Index Kit (1-96) (Cat. No. 634752). Please consult the following resources for component information, best practices, pooling strategies, an index plate map, and index sequences.

- Unique Dual Index Kits Protocol-At-A-Glance (download)
- Indexes and plate maps (Excel files)
  - Unique Dual Index Kit (1-96) Indexes and Plate Map (download)
  - Unique Dual Index Kit (97-192) Indexes and Plate Map (download)
  - Unique Dual Index Kit (193-288) Indexes and Plate Map (download)
  - Unique Dual Index Kit (289-384) Indexes and Plate Map (download)
  - Unique Dual Index Kit (1-24) Indexes and Plate Map (download)

Appendix B. Guidelines for Library Sequencing

A. Pooling Recommendations

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained with the Qubit with the average library size determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for the conversion: http://www.molbiol.ru/eng/scripts/01_07.html . Alternatively, libraries can be quantified by qPCR using 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. Libraries generated with SMART-Seq Total RNA Pico Input with UMIs (ZapR 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. 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.

B. Loading Guidelines for Various Illumina Instruments

Libraries generated with SMART-Seq Total RNA Pico Input with UMIs (ZapR 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

C. Extra Precautions When Using NextSeq 500/550 and MiniSeq Instruments

Libraries generated with SMART-Seq Total RNA Pico Input with UMIs (ZapR 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 workflow 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.

Appendix C. Guidelines for Data Analysis

We recommend using Cogent NGS Analysis Pipeline Software to analyze the sequencing results from this workflow. CogentAP collapses UMIs and trims 3 nucleotides of UMI linker and 3 nucleotides derived from the Pico v3 SMART UMI Adapter. To obtain CogentAP, please visit takarabio.com/ngs-cogentAP.

If not using CogentAP, use the following information when analyzing the results:

- 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.
- Trim **8 nt UMIs + 3 nt UMI linker + 3 nt from the SMART UMI Adapter** from Read2 prior to mapping**.**

Figure 5. Structure of final libraries generated with SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian). For more information, see the caption on Figure 3.

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