

Takara Bio USA, Inc.

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

Cat. Nos. 634357, 634358 & 634359  
(021925)

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## I. Introduction

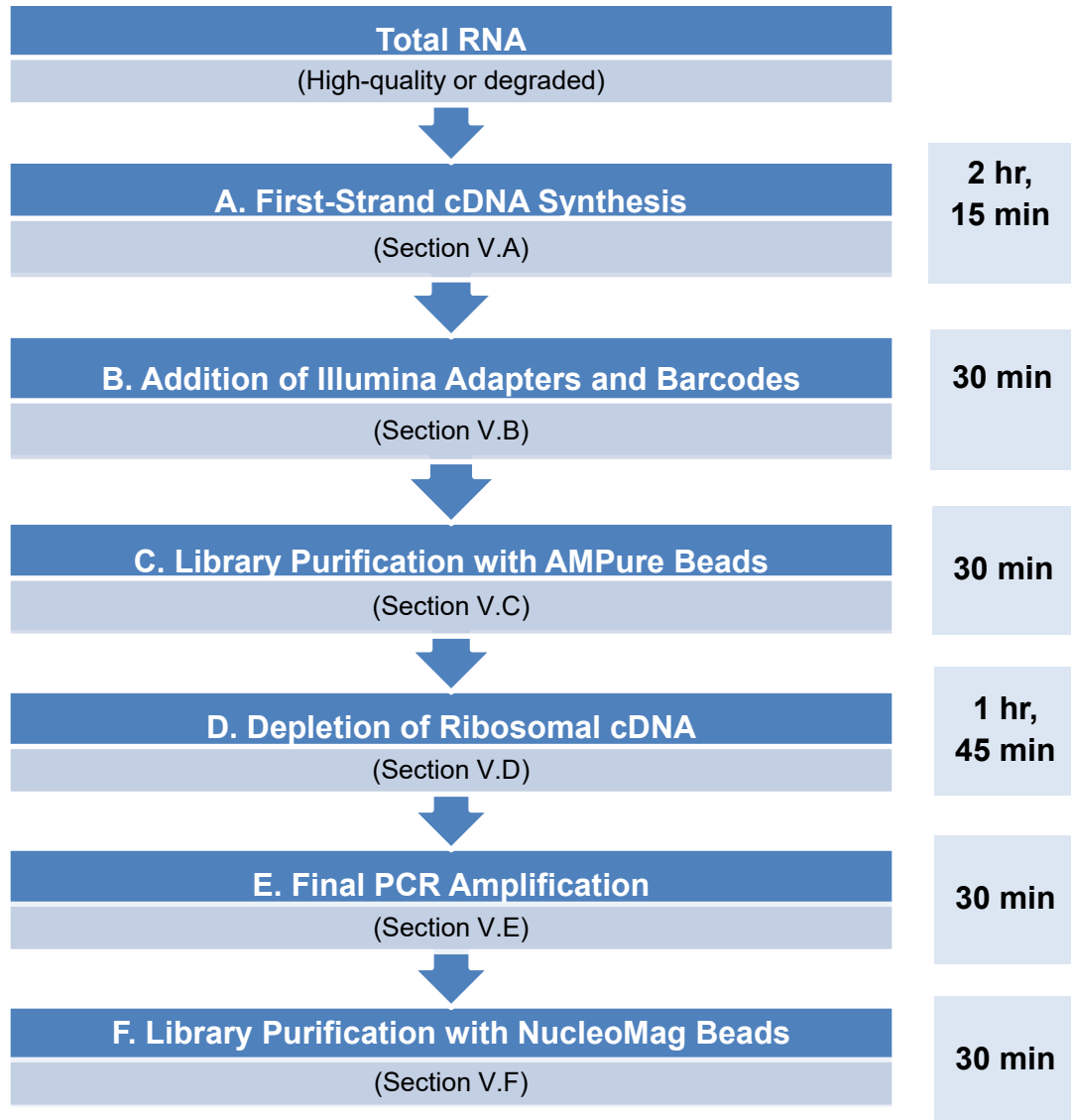
**SMART-Seq Total RNA Pico Input (ZapR Mammalian)** (Cat. Nos. 634357, 634358 & 634359) includes all components needed to generate cDNA libraries suitable for next-generation sequencing (NGS) on any Illumina® platform, with a recommended input range of 250 pg–10 ng of mammalian total RNA. The entire library construction protocol, starting with total RNA, can be completed in about 6 hours (Figure 1).

This kit incorporates SMART® (Switching Mechanism at 5' end of RNA Template) 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).

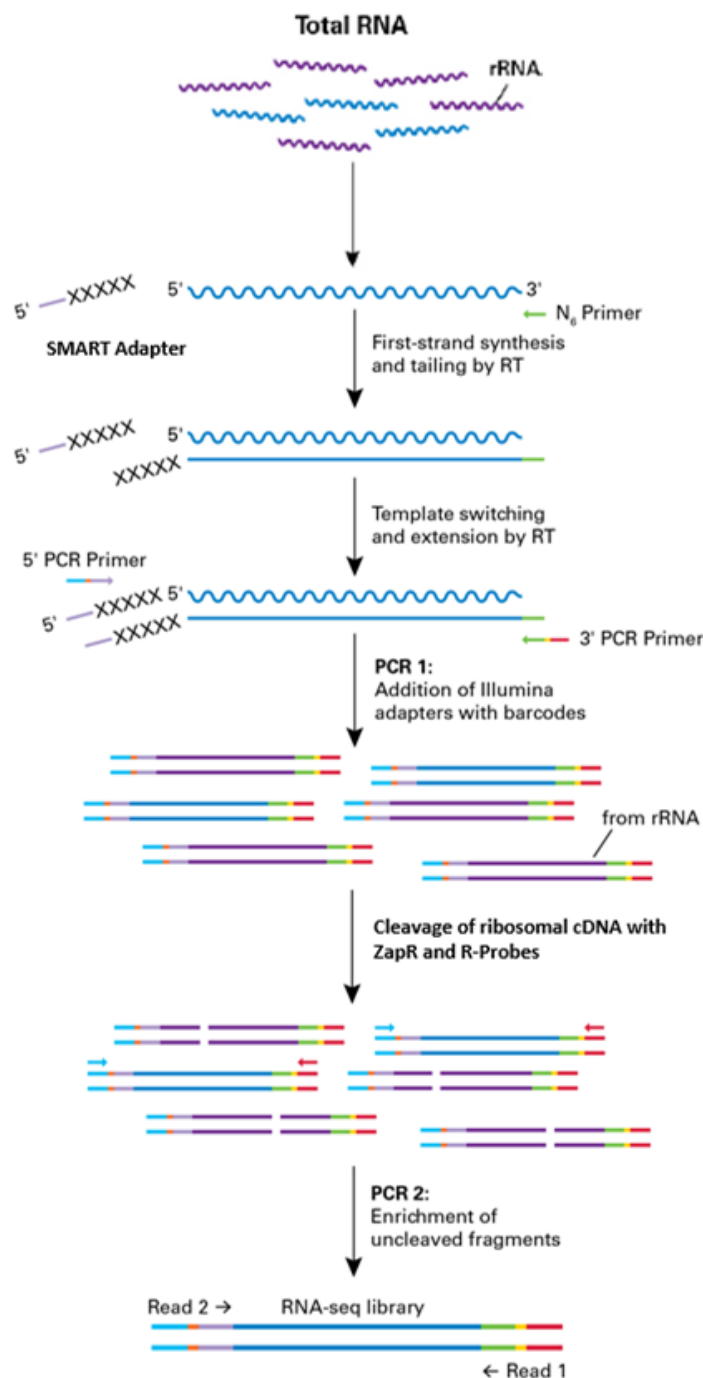
Ribosomal RNA (rRNA) comprises a significant proportion (~90% or more) of all RNA molecules in total RNA samples. Depleting these abundant transcripts from total RNA samples prior to generating libraries provides benefits by lowering sequencing costs and improving mapping statistics. However, with very 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 ZapR technology allowing removal of ribosomal cDNA (cDNA fragments originating from rRNA molecules) after cDNA synthesis using probes specific to mammalian rRNA. These R-Probes target 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 (ZapR 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–10 ng range. Inputs >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 yield and produce the best sequencing results when analyzed in the 5–50 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-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. Protocols for both options are provided in Section V.A.

We also offer a UMI-based library preparation approach for total RNA-seq from picogram inputs of total RNA or 10-1,000 single cells with SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian).



**Figure 1. SMART-Seq Total RNA Pico Input (ZapR Mammalian) protocol overview.** This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries in approximately 6 hr. 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 only a limited number of cycles (Protocol B). The PCR products are purified (Protocol C), and then ribosomal cDNA is depleted (Protocol D). The cDNA fragments from Protocol D are further amplified (Protocol E) with 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 (ZapR Mammalian).** SMART technology is used in this ligation-free protocol to preserve strand-of-origin information. Random priming (represented by the green N<sub>6</sub> Primer) allows the generation of cDNA from all RNA fragments in the sample, including rRNA. When the SMARTScribe™ Reverse Transcriptase (RT) reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity adds a few nontemplated nucleotides to the 3' end of the cDNA (shown as XXXXX). The SMART Adapter (included in the SMART TSO Mix v2) base-pairs with the nontemplated nucleotide stretch, creating an extended template to enable the RT to continue replicating to the end of the oligonucleotide. 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 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 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 Illumina 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 (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 three nucleotides of the second sequencing read (Read 2) are derived from the SMART Adapter (shown as XXX). These three nucleotides must be trimmed prior to mapping if performing paired-end sequencing.

## II. List of Components

The SMART-Seq Total RNA Pico Input (ZapR Mammalian) contains sufficient reagents to prepare 24 (Cat. No. 634357), 96 (Cat. No. 634358), or 384 (Cat. No. 634359) 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 (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 (ZapR Mammalian) components.**

SMART-Seq Total RNA Pico Input (ZapR Mammalian)	634357 (24 rxns)	634358 (96 rxns)	634359 (384 rxns)
<b>SMART-Seq Total RNA Pico Input</b>	<b>634365*</b>	<b>634366*</b>	<b>4 x 634366*</b>
<b>Package 1 (Store at –70°C)</b>			
SMART TSO Mix v2†	110 µl	450 µl	4 x 450 µl
<b>Package 2 (Store at –20°C)</b>			
SMART Pico Oligos Mix v2†	30 µl	110 µl	4 x 110 µl
5X First-Strand Buffer	100 µl	400 µl	4 x 400 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	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 v2	100 µl	400 µl	4 x 400 µl
Nuclease-Free Water	2 x 1.25 ml	10 ml	4 x 10 ml
<b>ZapR Mammalian rRNA Depletion Kit</b>	<b>634369*</b>	<b>634670*</b>	<b>4 x 634670*</b>
<b>Package 1 (Store at –70°C)</b>			
R-Probes†‡	40 µl	160 µl	4 x 160 µl
Control Total RNA (1 µg/µl)	5 µl	5 µl	4 x 5 µl
<b>Package (Store at –20°C)</b>			
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 v2 more than 10 times.
- 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 TSO Mix v2 contains the SMART Adapter (a.k.a., 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, and 200 µl, two each (one for pre-PCR amplification steps and one dedicated for PCR amplification)
- Eight-channel or 12-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 10 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips
- 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)

### 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

### For cDNA Synthesis and Amplification

- Two thermal cyclers with heated lids
  - One thermal cycler used only for first-strand cDNA synthesis (Section V.A) and addition of Illumina adapters and indexes (Section V.B)
  - One thermal cycler used only for library amplification (Section V.E)

### **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 the Addition of Illumina Adapters and Indexes**

- 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)
- 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)
- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2 ml PCR 8-tube strip; USA Scientific, Item No. 1402-4700 or similar)
- 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

## **IV. General Considerations**

### **A. Recommendations for Preventing Contamination**

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

- **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 and R-Probes (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. 634358 (96 rxns) and Cat. No. 634359 (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

### 1. 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 (Agilent Technologies, 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
  - **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%.

### 2. Input RNA Purity

- **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.
- Input RNA should be free from genomic or any 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.

### 3. Input RNA Quantity

- This kit accommodates up to 8 µl of input RNA.
- The protocol has been optimized for cDNA synthesis from 250 pg–10 ng of mammalian total RNA.
- It is **strongly recommended** that working conditions for your samples should be established before trying inputs beyond the recommended range.

- Higher input amounts ranging from 10–50 ng can also be used to generate high-quality sequencing libraries with this kit but may yield more rRNA-associated reads than inputs  $\leq 10$  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 5–50 ng for analysis of FFPE samples. Inputs higher than 50 ng have not been validated.
- For total RNA inputs  $< 250$  pg, 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 RNA integrity (high-quality versus highly degraded). Libraries generated from inputs  $< 250$  pg will also contain a significantly higher amount of PCR duplicates due to the low complexity of the starting material and are more likely to contain undesirable environmental contaminants.

## 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  $> 7$ ), 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.

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.

**NOTE: Fresh dilutions should be made before use.** If desired, make single-use aliquots of the 50 ng/ $\mu$ l dilution and store at  $-80^{\circ}\text{C}$  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/ $\mu$ l by mixing 38  $\mu$ l of Nuclease-Free Water with 2  $\mu$ l of Control Total RNA (1  $\mu$ g/ $\mu$ l) in a sterile microcentrifuge tube.
2. Further dilute Control Total RNA to 5 ng/ $\mu$ l by mixing 45  $\mu$ l of Nuclease-Free Water with 5  $\mu$ l of 50 ng/ $\mu$ l Control Total RNA in a sterile microcentrifuge tube.
3. Further dilute Control Total RNA to 0.25 ng/ $\mu$ l by mixing 95  $\mu$ l of Nuclease-Free Water with 5  $\mu$ l of 5 ng/ $\mu$ l Control Total RNA in a sterile microcentrifuge tube.
4. Use 1  $\mu$ l or more of 0.25 ng/ $\mu$ l 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

Fragmentation of RNA into a size appropriate for sequencing on Illumina platforms is performed in the first step of the cDNA synthesis protocol. Fragmentation time is adjusted depending on the quality of the RNA input.

- **Option 1 (with fragmentation)** should be used when starting from samples with RIN  $\geq 4$  or DV200  $\geq 60\%$
- **Option 2 (without fragmentation)** should be used when starting from samples that are already significantly degraded. For samples with RIN  $\sim 4$  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  $\leq 10$  ng. However, inputs as high as 50 ng can be used safely for FFPE RNA samples.

**Table 2. Recommended fragmentation protocol options and fragmentation times.**

RNA quality	Use protocol	Fragmentation time
RIN $\geq 7$	Option 1	4 min
RIN 5–6	Option 1	3 min
RIN 4/DV200 $\geq 60\%$	Option 1	2 min*
DV200=25–60% & all FFPE samples <sup>†</sup>	Option 2	–

\*In most cases, samples with RIN  $\sim 4$  will be optimally fragmented after 1.5–2 min. 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.

<sup>†</sup>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

1. On ice, mix the following components in a 1.5 ml centrifuge tube:

1–8 $\mu$ l	RNA
1 $\mu$ l	SMART Pico Oligos Mix v2
4 $\mu$ l	5X First-Strand Buffer
0–7 $\mu$ l	Nuclease-Free Water
<b>13 <math>\mu</math>l</b>	<b>Total volume per reaction</b>

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.

**NOTE:** Steps 5–6 are critical for first-strand synthesis and should not be delayed after Step 3. Start Step 4, preparing the first-strand master mix, while your tubes are incubating (Step 2), or have it almost ready before starting Step 3.

3. At the expiration of that time, immediately place the samples on an ice-cold PCR chiller rack for 2 min.

- On ice, prepare enough first-strand master mix for all reactions, plus 10% of the total reaction mix volume (in the order shown). Mix by gentle vortexing or pipetting and keep on ice until needed.

**First-strand master mix:**

4.5 µl	SMART TSO Mix v2
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe Reverse Transcriptase

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**7 µl Total volume per reaction**

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

- Add 7 µl of the first-strand master mix to each reaction tube for plate well from Step 3.

13 µl	Sample mix (from Step 3)
7 µl	First-strand master mix (From Step 4)

---

**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.

- 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

- 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

- Mix the following components on ice:

**Sample mix:**

1–8 µl	RNA
1 µl	SMART Pico Oligos Mix v2
0–7 µl	Nuclease-Free Water*

---

**9 µl Total volume per reaction**

\*Use enough Nuclease-Free Water to bring the total volume to equal 9 µl after adding the SMART Pico Oligos Mix v2 to your sample RNA.

- 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.

- 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 TSO Mix v2 is very viscous—make sure to homogenize the first-strand master mix very well by pipetting up and down 10 times with a pipette set at a volume larger than the final master mix volume.

- 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**

- 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

- 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: 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.

- 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 PCR1 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)
<b>48 µl Total volume per reaction</b>	

3. Add 2 µl of the appropriate UDI from a 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
<b>50 µl Total volume per reaction</b>	

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

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

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

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

## C. Protocol: Purification of the RNA-Seq Library

**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 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, 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 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. 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. cDNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.
7. Perform a brief spin of the tubes (~2,000g) 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 open sample tubes rest on the magnetic device 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 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.

**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 or well 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.



15. 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.

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

## D. Protocol: Depletion of Ribosomal cDNA

Library fragments originating from nuclear rRNA and mitochondrial rRNA are cut by ZapR Enzyme in the presence of target-specific R-Probes. 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.

**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, 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. cDNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.

7. Briefly spin the tubes (~2,000g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec, then remove any 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 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: 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 v2
2 µl	SeqAmp DNA Polymerase

**80 µl Total volume per reaction**

**NOTE:** DO NOT reduce the reaction volume. The 100 µl final volume is important for yield. If your thermal cycler cannot accommodate 100 µl sample volumes, it is important to equally divide each sample into two tubes (containing ~50 µl each) after the 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) 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. Cycling guidelines based on amount of starting material.**

Amount of input RNA (ng)	Typical number of PCR cycles	
	Regular RNA	FFPE RNA*
50	9–10	13
10	12	15–16
1	15–16	18
0.25†	18	-

\*Typically, FFPE RNA requires extra PCR cycles to achieve adequate yield. For inputs below 10 ng, 18 cycles should be used. An input of 1 ng can generate enough material for sequencing, but a minimum of 5 ng is recommended.

†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 at the bottom. 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 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 (~2,000g) 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 dry, add 20 µl of Tris Buffer 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.

**NOTES:**

- 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.
- Consider eluting in 12 µl of Tris Buffer instead of 20 µl if anticipated yield is low.

10. Incubate at room temperature for 5 min to rehydrate.
11. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 2 min or longer, until the solution is completely clear.
12. Transfer the clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free, low-adhesion tube.

**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

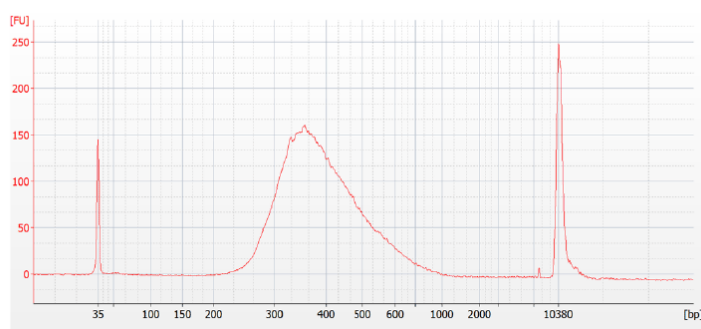
- analyzing and validating final libraries using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851 or Q32854) and
- evaluating the libraries' size distributions with an Agilent 2100 Bioanalyzer and the DNA High Sensitivity Kit (Agilent Technologies, Cat.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

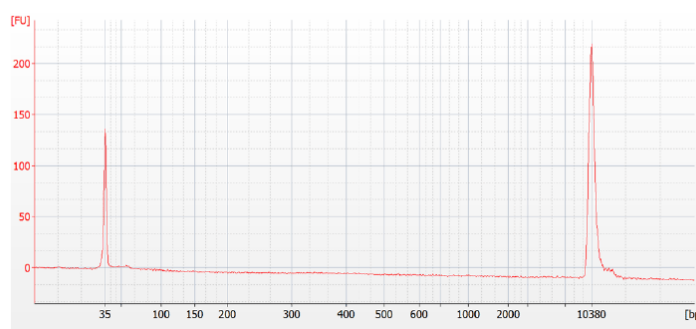
1. Qubit: A yield >2 ng/µl will provide enough material for further library validation and sequencing. Consider adding one PCR cycle in subsequent experiments if the yield is insufficient, or reducing cycles if the yield is more than 10 ng/µl. Eluting the final libraries in a smaller volume (e.g., 12 µl instead of 20 µl) is also a simple way to achieve more concentrated libraries.
2. Bioanalyzer: Dilute libraries to about 1 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, peaked at ~300–400 bp, in the positive control RNA sample (Figure 4A) and no product or very minimal background over the corresponding range in the negative control (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 139 bp to the size of the original RNA molecules.

## A Positive Control RNA



## B Negative Control RNA



**Figure 4. Example electropherogram results from the Agilent 2100 Bioanalyzer.** Libraries were generated using 0.25 ng Control Total RNA (human brain; **Panel A**—library diluted to 1.5 ng/μl based on Qubit quantification) and a no-RNA control (**Panel B**—no library dilution). For both examples, PCR 2 was performed using 16 cycles. Note that the no-RNA control exhibits a small amount of background (indicated by minimal product in the 200–1,000 bp range), which is acceptable as long as the libraries from the RNA samples contain a significantly larger amount of material.

## VI. References

Chenchik A, *et al.* RT-PCR Methods for Gene Cloning and Analysis. 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 (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.edu.ru/eng/scripts/01\\_07.html](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. Libraries generated with SMART-Seq Total RNA Pico Input (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, especially for patterned flow cells. For patterned flowcells, include 5–10% PhiX.

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

## B. Loading Guidelines for Various Illumina Instruments

Libraries generated with SMART-Seq Total RNA Pico Input (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 (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 SMART-Seq Total RNA Pico Input (ZapR Mammalian) is good at retaining small inserts. Therefore, we strongly recommend that you turn off automatic adapter trimming by creating a custom library prep kit program without adapter trimming. More information can be found on the Illumina website.
- Due to the algorithm's sensitivity to low complexity (found in the first three nt of Read 2), NextSeq and MiniSeq runs may display incorrect base calling in the first three bases. Because those bases are trimmed, it does not affect the quality of the data obtained. However, we still strongly recommend avoiding overloading of the instrument.

## Appendix C. Guidelines for Data Analysis

We recommend using Cogent™ NGS Analysis Pipeline Software to analyze the sequencing results from this workflow. CogentAP collapses and trims the 3 nucleotides derived from the SMART Adapter. To obtain CogentAP, please visit [takarabio.com/ngs-cogentAP](http://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.

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



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



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