Takara Bio USA

SMART-Seq® Total RNA Single Cell (ZapR® Mammalian) User Manual

Cat. Nos. 634360, 634361 & 634362
(021925)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

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I. Introduction

SMART-Seq Total RNA Single Cell (ZapR Mammalian) (Cat. Nos. 634360, 634361 & 634362) includes all components needed to generate total RNA sequencing (RNA-seq) libraries suitable for next-generation sequencing (NGS) on any Illumina® platform starting directly from 1–1000 intact cells, or 10 pg–10 ng of mammalian total RNA. The kit includes components for both cDNA synthesis and ribosomal RNA (rRNA)-derived cDNA removal. The kit incorporates SMART® (**S**witching **M**echanism **a**t 5′ end of **R**NA **T**emplate) cDNA synthesis technology followed by the use of ZapR technology to remove rRNA-derived cDNA transcripts corresponding to mature mammalian rRNAs (5S, 5.8S, 18S, and 28S) and human mitochondrial rRNA (12S and 16S). Up to 384 indexed libraries can be prepared using the Unique Dual Index Kits (Cat. Nos. 634752–634756, sold separately). The entire library construction protocol, starting with single cells or total RNA, can be completed in about 6 hr 20 min (Figure 1). Single-cell or low-input total RNA-seq libraries generated with the kit enable the analysis of both coding and non-coding RNA species and provide information on strand orientation, transcript isoforms, gene fusions, single nucleotide variations (SNV), etc.

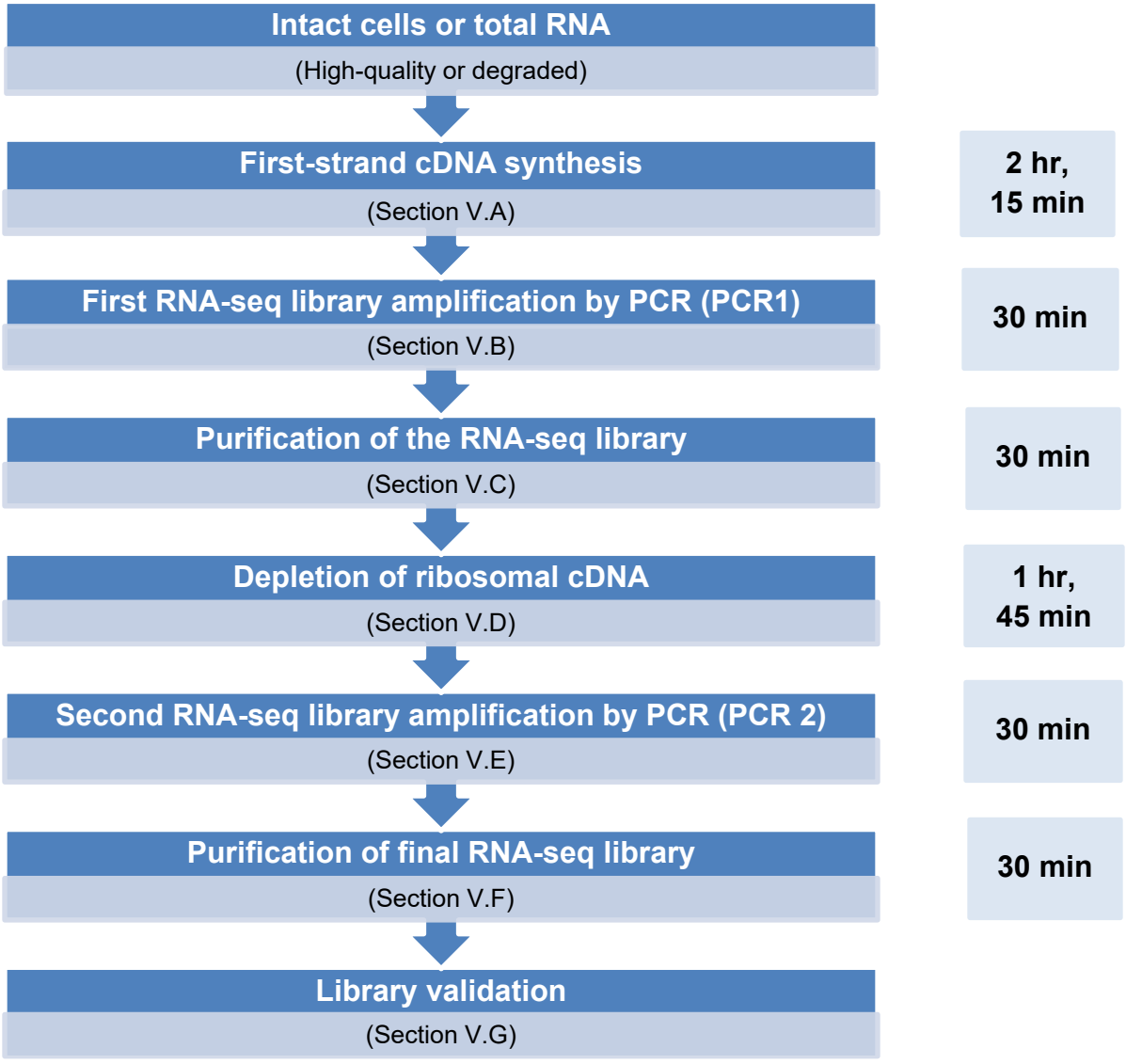

Figure 1. SMART-Seq Total RNA Single Cell (ZapR Mammalian) protocol overview. This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries in approximately 6 hr 30 min. Actual processing time may vary depending on the number of samples and cycling conditions (e.g., Protocol E takes more than 30 min if using 16 cycles of PCR or if using a thermal cycler with a

slow ramping time). First, total RNA is converted to cDNA (Protocol A), and then adapters for Illumina sequencing (with specific barcodes) are added through PCR using a limited number of cycles (Protocol B). The PCR products are purified (Protocol C), and then ribosomal cDNA is depleted (Protocol D). The cDNA fragments from Protocol D are further amplified (Protocol E) with primers universal to all libraries. Lastly, the PCR products are purified once more to yield the final cDNA library (Protocol F). As outlined in Section V, the kit workflow includes three safe stopping points following the completion of Protocols A, B, and E, respectively.

Ribosomal RNA (rRNA) comprises a considerable proportion (~90% or more) of all RNA molecules in cells and is readily captured by random priming. Depleting these abundant transcripts from cell lysates or total RNA samples prior to generating libraries relying on random priming provides benefits by lowering sequencing costs and improving mapping statistics. However, with very low-input amounts, initial rRNA depletion from total RNA often leaves insufficient material for preparing high-quality libraries. In addition, initial rRNA depletion from the lysate of single cells is impossible. ZapR technology (Figure 2) allows for the removal of rRNA-derived cDNAs following cDNA synthesis.

Indexed Illumina-compatible sequencing libraries are then generated by PCR amplification (Figure 2) using the Unique Dual Index kits. This reaction makes use of PCR primers that contain the forward cluster-generating sequence P5 and indexing sequences (Figure 2; orange fragment) and the reverse cluster-generating sequence P7 and indexing sequences (Figure 2; red fragment). The number of indexes and specific sequences will depend on the Unique Dual Index kit you have purchased.

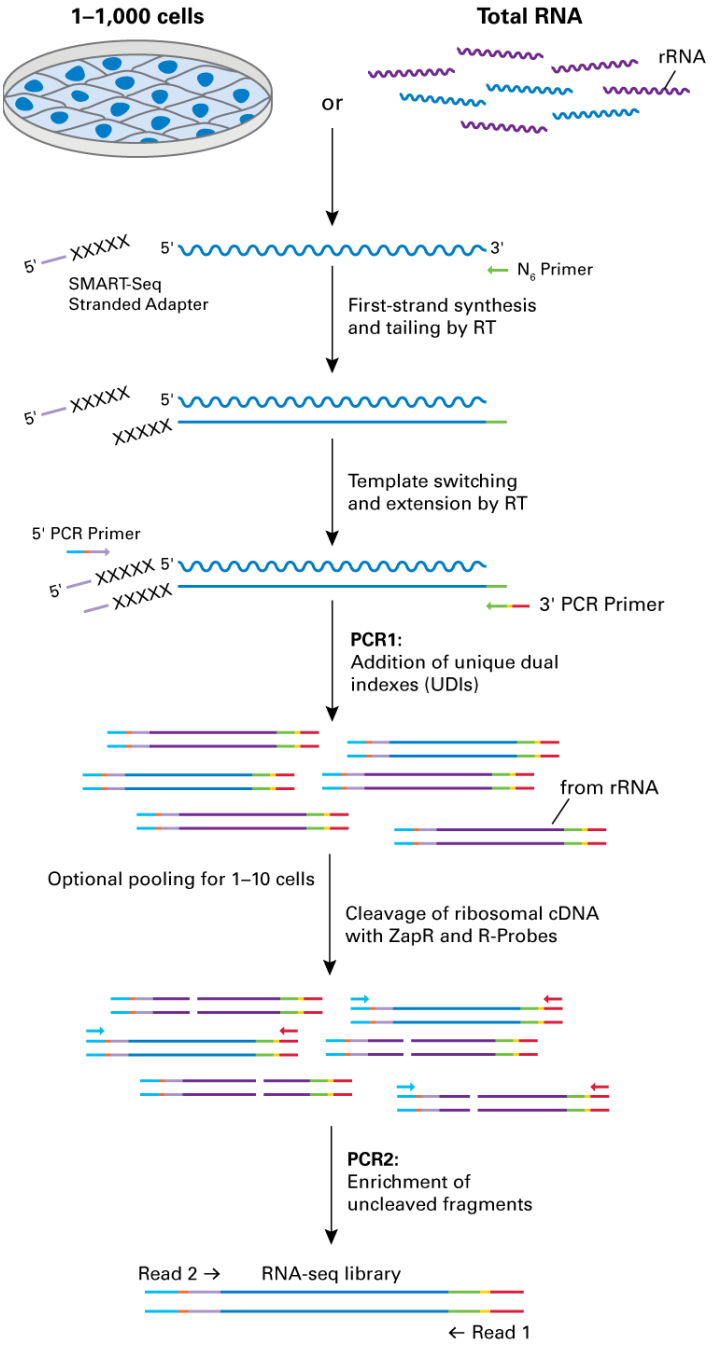

Figure 2. Schematic of technology in SMART-Seq Total RNA Single Cell (ZapR Mammalian). SMART technology is used in a ligation-free protocol to preserve strand-of-origin information. Random priming (through the SMART scN6 Primer) allows the generation of cDNA from all RNA fragments in the sample, including 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 carefully designed SMART-Seq Stranded Adapter (included in the SMART scTSO Mix) base-pairs with the nontemplated nucleotide stretch, creating an extended template to enable the RT to continue replicating to the end of the oligonucleotide. The resulting cDNA contains sequences derived from the SMART scN6 Primer and the SMART-Seq Stranded Adapter. In the next step, a first round of PCR amplification (PCR 1) adds unique dual indexes. The 5′ PCR Primer binds to the SMART-Seq Stranded Adapter sequence (light purple), while the 3′ PCR Primer binds to sequence associated with the SMART scN6 sequence (green). Ribosomal cDNA (originating from rRNA) is cleaved by ZapR Enzyme in the presence of the mammalian-specific R-Probes. This process leaves the library fragments originating from non-rRNA molecules untouched, with priming sites available on both 5′ and 3′ ends for further PCR amplification. These fragments are enriched via a second round of PCR amplification (PCR 2) using primers universal to all libraries. An optional pooling of up to 12 samples after PCR 1 allows for greater ease of use by minimizing the number of samples to be processed downstream.

II. List of Components

SMART-Seq Total RNA Single Cell (ZapR Mammalian) contains sufficient reagents to prepare 24 (Cat. No. 634360), 96 (Cat. No. 634361), or 384 (Cat. No. 634362) 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 Single Cell (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 Single Cell (ZapR Mammalian) components.

SMART-Seq Total RNA Single Cell (ZapR Mammalian)	634360 (24 rxns)	634361 (96 rxns)	634362 (384 rxns)
SMART-Seq Total RNA Single Cell	**634367***	**634368***	**4 x 634368***
Package 1 (Store at –70°C)			
SMART scTSO Mix†	110 µl	450 µl	4 x 450 µl
Package 2 (Store at –20°C)			
SMART scN6	30 µl	110 µl	4 x 110 µl
scRT 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	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 x1 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 2 (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 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 scTSO Mix contains the SMART-Seq Stranded 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 Enzyme 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. 634361 (96 rxns) and Cat. No. 634362 (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 quality of your starting RNA samples. Prior to cDNA synthesis, please make sure 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 Single Cell (ZapR Mammalian) has been optimized for 1–1,000 intact mammalian cells, or 10 pg–10 ng of DNA-free mammalian total RNA.

1. Total RNA Input

- **RNA should be in a maximum volume of 7 µl.**
- Compromised or high-integrity RNA can be analyzed with this kit.
- This protocol has been optimized for cDNA synthesis from 10 pg–10 ng of mammalian total RNA. Purified total RNA should be in nuclease-free water and free of contamination.
- 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 DNA or any carrier 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 kit accommodates 1–1,000 intact mammalian cells in a volume of 7 µl.
- 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>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.

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. Prepare RNase Inhibitor Water (RI water) by combining 398 µl of Nuclease-Free Water with 2 µl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.
2. Dilute Control Total RNA (human brain) to 50 ng/µl by mixing 38 µl of RI Water with 2 µl of Control Total RNA (1 µg/µl) in a sterile microcentrifuge tube.
3. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of RI Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.
4. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of RI Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.
5. Further dilute Control Total RNA to 0.01 ng/µl by mixing 120 µl of RI Water with 5 µl of 0.25 ng/µl Control Total RNA in a sterile microcentrifuge tube.

6. Use 1 µl or more of 0.01 ng/µl Control Total RNA as a positive control RNA input for the kit and include it alongside your samples.

E. Choosing Between the Ultra-Low-Input and Low-Input Workflow

SMART-Seq Total RNA Single Cell (ZapR Mammalian) offers two workflow choices which depend on input level, divided between ultra-low (1–50 cells or 10–500 pg total RNA) and low (50–1,000 cells or 0.5–10 ng total RNA) inputs, with variances in pooling, PCR cycling, and cleanup steps (Figure 3 and Table 2, next page). As a rule, ultra-low inputs require 10 cycles in the initial PCR (PCR 1), and two NucleoMag bead cleanups after the final PCR (PCR 2), while low inputs require only 5 cycles in PCR 1 and a single NucleoMag bead cleanup after PCR 2.

Furthermore, the ultra-low-input workflow offers the possibility to pool samples after PCR 1 if inputs are less than 10 cells or 100 pg total RNA. See Appendix A for extra protocol associated with pooling. Please note that while pooling is convenient when working with single cells, it is not required to achieve high-quality libraries, particularly for experienced users.

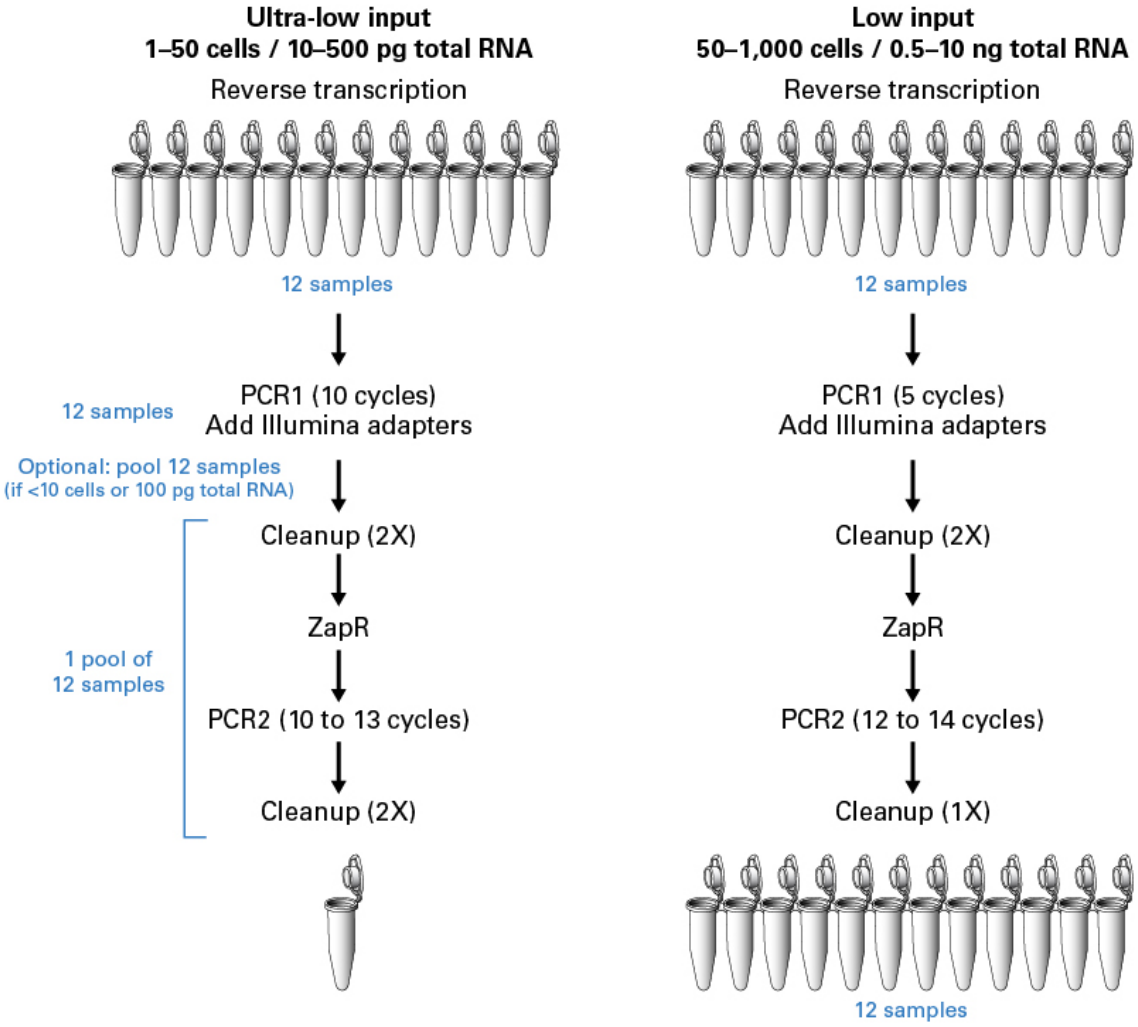

Figure 3. Summary of SMART-Seq Total RNA Single Cell (ZapR Mammalian) workflows based on input amounts. SMART-Seq Total RNA Single Cell (ZapR Mammalian) can accommodate ultra-low input amounts (1–50 cells or 10–500 pg total RNA) as well as low input amounts (50–1,000 cells or 0.5–10 ng total RNA). The number of PCR cycles in PCR 1 and PCR 2 vary between the two options. The ultra-low input requires two rounds of bead cleanup after PCR 2, instead of a single

cleanup in the low-input workflow. When following the ultra-low-input workflow with 1–10 cells or <100 pg total RNA, an optional pooling of 8–12 samples after PCR 1 significantly reduces hands-on time for downstream steps.

Table 2. Summary of SMART-Seq Total RNA Single Cell (ZapR Mammalian) protocols.

Input category	Cell input	RNA input	Post-PCR 1 Pooling	PCR 1 # of cycles	PCR 2 # of cycles	# of final cleanups	Final elution volume (µl)
Ultra-low	1	10 pg	Yes	10	14–15	2	12
			No	10	15		
	10	100 pg	Yes	10	12		
			No	10	13–14		
	10–50	100–500 pg	No	10	12		
Low	50–100	0.5–1 ng	No	5	16	1	22
	500	5 ng	No	5	15–16		
	1,000	10 ng	No	5	14		

The guidelines in Table 2 must be validated with your material. When optimizing cycling conditions, only PCR 2 cycles should be modified. We do not recommend performing more than 13 cycles for PCR 2 in the ultra-low-input category, and no more than 16 cycles for PCR 2 in the low-input category, as it will lead to substantial background amplification. We recommend that you perform a pilot experiment with a small number of samples to determine the optimal number of PCR 2 cycles for your input material. In particular, an input of 500–1,000 cells may require 1 extra PCR 2 cycle because inhibitors associated with high cell input may make the library preparation less efficient than for lower cell inputs.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

During this step, RNA is fragmented (if needed), and first-strand cDNA synthesis is randomly primed through the SMART scN6 primer and uses the adaptors within the SMART scTSO Mix for template-switching at the 5′ end of the transcript.

- **Option 1 (with fragmentation)** should be used with RNA samples with a 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 3 (below) for guidelines.

Table 3. 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%	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 overfragmentation may lead to reduced performance due to inefficient ribosomal cDNA depletion in Section V.D. When in doubt, choose a shorter fragmentation time or Option 2.

Before You Start:

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). Mix by vortexing and keep on ice until needed.
2. Prepare a stock solution of 10X Lysis Mix by mixing 10X Lysis Buffer with RNase Inhibitor as indicated below (scale up as needed). Mix by pipetting up and down and keep on ice until needed.

19 µl	10X Lysis Buffer
1 µl	RNase Inhibitor
20 µl	**Total volume**

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

1. Make sure RNA or cell input are in a total volume of 7 µl, in 0.2 ml PCR tubes or strip tubes.
   - If RNA input is less than 7 µl, complete with RI Water. Keep samples on ice.
   - If cell input is less than 7 µl, complete with Ca^{2+}/Mg^{2+}-free PBS. **DO NOT** bring up the volume of cell input with RI Water. Keep samples on ice.
2. Prepare enough shearing master mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

Shearing master mix:

1 µl	10X Lysis Mix
1 µl	SMART scN6
4 µl	scRT Buffer
6 µl	**Total volume per reaction**

3. Add 6 µl of the shearing master mix to each of your 7 µl cell RNA or cell suspensions prepared in Step 1. Mix by tapping gently, then spin down.

6 µl	Shearing master mix (Step 2)
7 µl	RNA or cell suspensions (Step 1)
13 µl	**Total volume per reaction**

4. Incubate the tubes at 85°C in a preheated, hot-lid thermal cycler for 6 min if starting from cells; if starting from purified total RNA, follow the recommendations in Table 3 or use an experimentally determined optimal amount of time.
5. After the incubation in complete, place the samples on an ice-cold PCR chiller rack for 2 min. **Proceed immediately to Step 6**.
6. On ice, prepare enough first-strand master mix for all reactions, plus 10%, by combining the following reagents in the order shown:

First-strand master mix:

4.5 µl	SMART scTSO Mix
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe RT
7 µl	**Total volume per reaction**

NOTE: The SMART scTSO Mix is very viscous—it may be left at room temperature after thawing to facilitate accurate pipetting. Make sure to homogenize the first-strand master mix very well by vortexing for ~5 seconds followed by a brief spin-down.

7. Add 7 µl of the first-strand master mix to each reaction tube from Step 5. Mix the contents of the tubes by vortexing for ~5 sec, then spin the tubes briefly to collect the contents at the bottom.

   NOTE: The samples will be viscous—make sure to homogenize the content of the tubes very well.

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

42°C	90 min
70°C	10 min
4°C	forever

9. 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.

Option 2 (Without Fragmentation): Starting from Compromised RNA

1. Make sure your RNA is in a total volume of 7 µl, in 0.2 ml PCR tubes. If input is less than 7 µl, complete with RI Water. Keep samples on ice.
2. On ice, prepare enough annealing master mix for all reactions, plus 10%, by combining the following reagents in the order shown:

   Annealing master mix:

1 µl	10X Lysis Mix
1 µl	SMART scN6
2 µl	**Total volume per reaction**

3. Add 2 µl of the Annealing Master Mix to each reaction tube and mix by tapping gently, then spin down.

2 µl	Annealing master mix (Step 2)
7 µl	RNA suspensions (Step 1)
9 µl	**Total volume per reaction**

4. 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.
5. On ice, prepare enough first-strand master mix for all reactions, plus 10%, by combining the following reagents in the order shown.

   First-strand master mix:

4 µl	scRT Buffer
4.5 µl	SMART scTSO Mix
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe RT
11 µl	**Total volume per reaction**

   NOTE: The SMART scTSO Mix is very viscous—it may be left at room temperature after thawing to facilitate accurate pipetting. Make sure to homogenize the First-Strand Master Mix very well by vortexing for 5 seconds followed by a brief spin-down.

6. Add 11 µl of the first-strand master mix to each reaction tube from Step 6. Mix the contents of the tubes by vortexing for ~2–3 sec, then spin the tubes briefly to collect the contents at the bottom.

7. 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

8. 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 the unique dual-indexed primers from the Unique Dual Index Kit.

1. In a 1.5 ml microcentrifuge 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 from Section V.A, Step 8.

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

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

4. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the following program, making sure to choose the appropriate number of cycles for your input level (either 5 or 10 cycles, depending).

94°C	1 min
5 or 10 cycles*:	
98°C	15 sec
55°C	15 sec
68°C	30 sec
68°C	2 min
4°C	forever

*Use 5 cycles if following the low-input workflow; use 10 cycles if following the ultra-low-input workflow (see Table 2).

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 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:

- **If inputs are less than 10 cells or 100 pg**: it is possible to pool the samples (grouping 8–12 samples) prior to library preparation. Skip this section and follow the recommendations in Appendix A instead, then resume the protocol at Section V.D.
- 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 0.7:1
- 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 35 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 50 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 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 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 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.

D. Protocol: Depletion of Ribosomal cDNA

Library fragments originating from rRNA and mitochondrial rRNA are cut by the ZapR Enzyme in the presence of R-Probes (mammalian-specific). These R-Probes target mammalian nuclear 28S, 18S, 5.8S, and 5S rRNA sequences 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 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.

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 from Step 1. 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 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 ~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 3 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 ZapR master mix (from Step 9).
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 1.5ml 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
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
12–16 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. See Table 2 for the recommended number of cycles based on the amount of starting material.

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.

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 ~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 minute 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 supernatants to new tubes, and proceed to next step immediately or store 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

peak spanning 200–2,000 bp, peaked at ~300–450 bp for the positive control RNA sample (Figure 4, Panel A & Panel B) and no product or very minimal background over the corresponding range 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).

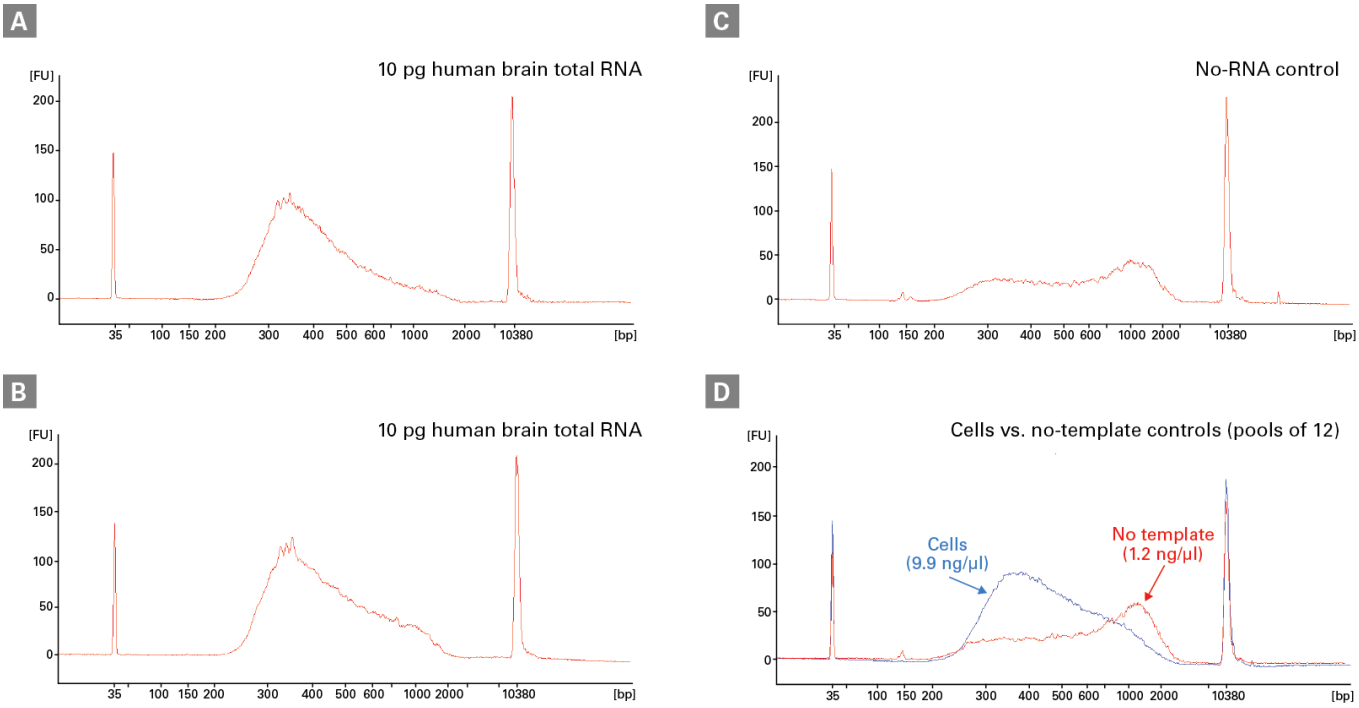

Figure 4. Example electropherogram results from the Agilent 2100 Bioanalyzer. **Panels A & B**. Libraries were generated using 10 pg of Control Total RNA (human brain) using the ultra-low workflow, without any pooling and 13 cycles in PCR 2. **Panel C.** Libraries were generated using a no-RNA control using the ultra-low workflow, without any pooling and 13 cycles in PCR 2. **Panel D**. Example of libraries generated directly from single cells and a no-template control using the ultra-low workflow, with pooling of 12 samples and 12 cycles in PCR 2; the no-template control was prepared with pooling of 12 samples but generated a yield significantly lower than the yield obtained from the pool of 12 cells. All libraries were diluted to 1.5 ng/µl (based on Qubit quantification) prior to analysis with a High Sensitivity DNA Kit (Agilent), except for the negative controls which were analyzed without dilution. Note that the no-RNA control exhibits a small amount of background <200 bp, and typically shows some amount of PCR product between 200–1,000 bp, in addition to a larger amount of product >1,000 bp. This background is typically unavoidable but acceptable if the libraries from the RNA samples contain a significantly larger amount of material (at least three times more, as measured by Qubit).

Appendix A. Pooling Strategy for Single-Cell Applications

When processing single cells, it can be convenient to pool samples after PCR 1 in order to minimize the number of samples to process downstream. Some users may find that the pooling helps them to get better, more consistent data from single cells because sample loss is minimized. One caveat is that if resequencing of particular samples is going to be required, the entire pool will need to be resequenced. Failed wells—those that did not receive a cell or in which the cell was dead—are likely to have a yield similar to the no-template control. In general, we find that failed samples are only a small fraction of the reads, thus there should be no concern over wasting reads by sequencing failed samples using the pooling method. If the viability of the sorted cells is a concern, a small number of cells can be processed for a small-scale evaluation prior to processing a large number of samples.

We recommend pooling no less than 8 samples and no more than 12 samples at a time. Pooling more than 12 samples has not been validated and is not recommended. For example, 48 samples can be processed as 4 groups of 12 samples (or 6 groups of 8 samples), resulting in only 4 (or 6) samples to handle in subsequent steps. The final samples will then represent pools of 12 (or 8) individuals and will not be distinguishable from each other

except through sequencing. In this example, the 4 or 6 samples should be carefully quantified and further mixed in equimolar amounts (see recommendations in Appendix C) prior to sequencing.

IMPORTANT:

- Pooling after PCR 1 has been fully validated only for inputs ranging from 1–10 cells, or 10–100 pg. Do not use this pooling strategy for higher inputs. It is also critical to ensure that each sample receive a distinct set of Unique Dual Indexes during PCR 1.
- **The pooling strategy still requires the use of the entire PCR 1 reaction volume (50 µl) for each sample. Do not attempt to use less than the entire reaction volume**. Any loss of material at this stage will affect the quality of the final library. The input amounts are so low that every effort should be taken to recover every µl of each sample while pooling.

The protocol below replaces the protocol in Section V.C if you are pooling samples. When you are finished with this protocol, continue with Section V.D.

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.
- 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. After completion of PCR 1 in Section V.B, Step 5, transfer the entire volume (50 µl) into a 1.5 ml LoBind tube; transfer no less than 8 and no more than 12 samples. The volume should be between 400–600 µl of pooled PCR product.
2. Add beads to each pooled PCR product as shown in Table 4. The beads are viscous; even when using such a large volume, it is critical to pipette the entire volume up and then out very slowly. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 3.

Table 4. Bead volume for various numbers of pooled samples.

Number of samples in pool	Total sample volume (µl)	Bead volume (µl)
8	400	260
9	450	293
10	500	325
11	550	358
12	600	390

3. Incubate at room temperature for 8 min to let the DNA bind to the beads.
4. 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 10 min or longer, until the solution is completely clear.

   NOTE: Because of the large volume involved, a very strong magnet is required. Make sure the solution is completely clear before moving on to the next step. Failure to recover all of the beads will lead to low-yield and low-quality libraries.

5. While the tubes are sitting on the magnetic stand, pipette out the supernatant.

6. Keep the tubes on the magnetic stand. Add 1 ml of freshly made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 1 min and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.
7. Repeat Step 6 once.
8. 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.
9. Let the sample tubes rest open at room temperature for ~10 min until the pellet appears dry.

   NOTE: Check the pellet frequently during this time and continue to Step 10 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.
10. 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.
11. Incubate at room temperature for 5 min to rehydrate.
12. Mix by pipetting up and down 10 times to elute the DNA from the beads.
13. Place the tubes/plate back on the magnetic stand for 1 min or longer, until the solution is completely clear.
14. Transfer 50 µl of clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free, low-adhesion tube.
15. 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.
16. 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.

Appendix B. Illumina UDIs

Appropriate combinations of Illumina indexes are necessary to ensure enough nucleotide diversity and allow for discrimination between samples when sequencing a pool of two or more libraries on a single flow cell lane. Consult the Illumina literature (e.g., TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines.

SMART-Seq Total RNA Single Cell (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 C. 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.

NOTE: If samples were pooled after PCR 1 (see Appendix A), all pool groups should be normalized to 2 or 4 nM and then combined in equimolar amounts as described.

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 Single Cell (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.

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 the SMART-Seq Total RNA Single Cell (ZapR Mammalian) cluster very efficiently and care must be taken to avoid overclustering. The guidelines in Table 5 (below) are a good starting point and have been fully validated.

Table 5. 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	1.1–1.2

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

Libraries generated with the SMART-Seq Total RNA Single Cell (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 D. Guidelines for Data Analysis

We recommend using Cogent™ NGS Analysis Pipeline Software to analyze the sequencing results from this workflow. 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.
- Trim the 3 nt from the SMART-Seq Stranded Adapter from Read 2 prior to mapping.

Figure 5. Structural features of final libraries generated with SMART-Seq Total RNA Single Cell (ZapR Mammalian). The adapters added during PCR 1 contain sequences allowing clustering on Illumina flow cells (P7 shown in light blue, P5 shown in red), indexes for pooling multiple samples in a single sequencing lane (Index 1 [i7] sequence shown in orange and associated with the P7 sequence, and Index 2 [i5] sequence shown in orange and associated with P5 sequence), 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-Seq Stranded Adapter (shown as XXX). It is best to trim these three nucleotides prior to mapping if performing paired-end sequencing.

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