Takara Bio USA

SMART-Seq® mRNA Long Read User Manual

Cat. Nos. 634376 & 634377
(022725)

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 mRNA Long Read (Cat. Nos. 634376 & 634377, referred to as SMART-Seq LR in this manual) generates high-quality, barcoded cDNA directly from 1–1,000 intact cells or 10 pg–100 ng of high integrity total RNA (RIN ≥ 8) in a convenient input volume of 1–9.5 µl. The full workflow can be completed within 2 days (Figure 1).

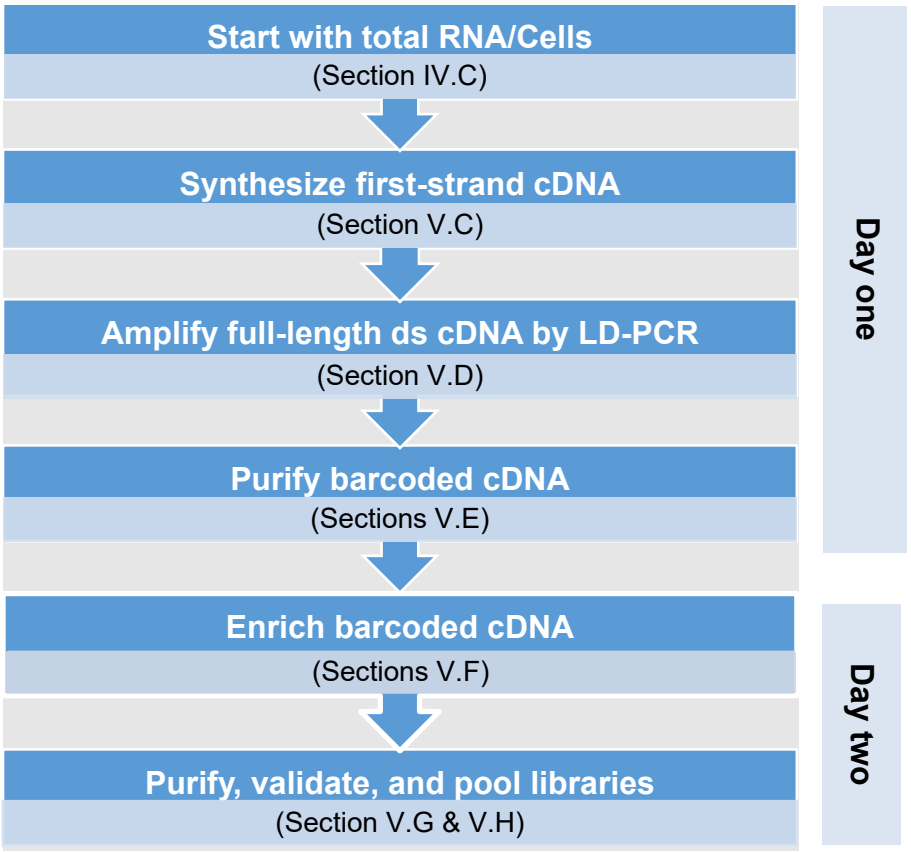

Figure 1. SMART-Seq mRNA Long Read protocol overview.

SMART-Seq LR uses oligo(dT) priming, which provides the excellent sensitivity needed to obtain full-length transcript information from high-integrity total RNA (RIN ≥ 8) or intact cells. Through even gene-body coverage and accurate representation of GC-rich transcripts, this chemistry enables reliable analysis of transcript isoforms, gene fusions, and more. This kit has been validated to work with library preparation using the Oxford Nanopore Technologies (ONT) Ligation Sequencing Kit V14 (SQK-LSK114). This kit is compatible for analysis with bioinformatic tools for ONT; refer to Section VII, "Guidelines for Data Analysis", for more information.

The SMART-Seq LR kit incorporates our proprietary SMART® (**S**witching **M**echanism **a**t the 5′ end of **R**NA **T**emplate) technology (Figure 2). This technology relies on the template-switching activity of the reverse transcriptase to enrich for full-length cDNAs and to directly add defined PCR adapters to both ends of the first-strand cDNA (Chenchik et al. 1998). SMART technology offers unparalleled sensitivity and unbiased amplification of cDNA transcripts and allows direct cDNA synthesis from intact cells.

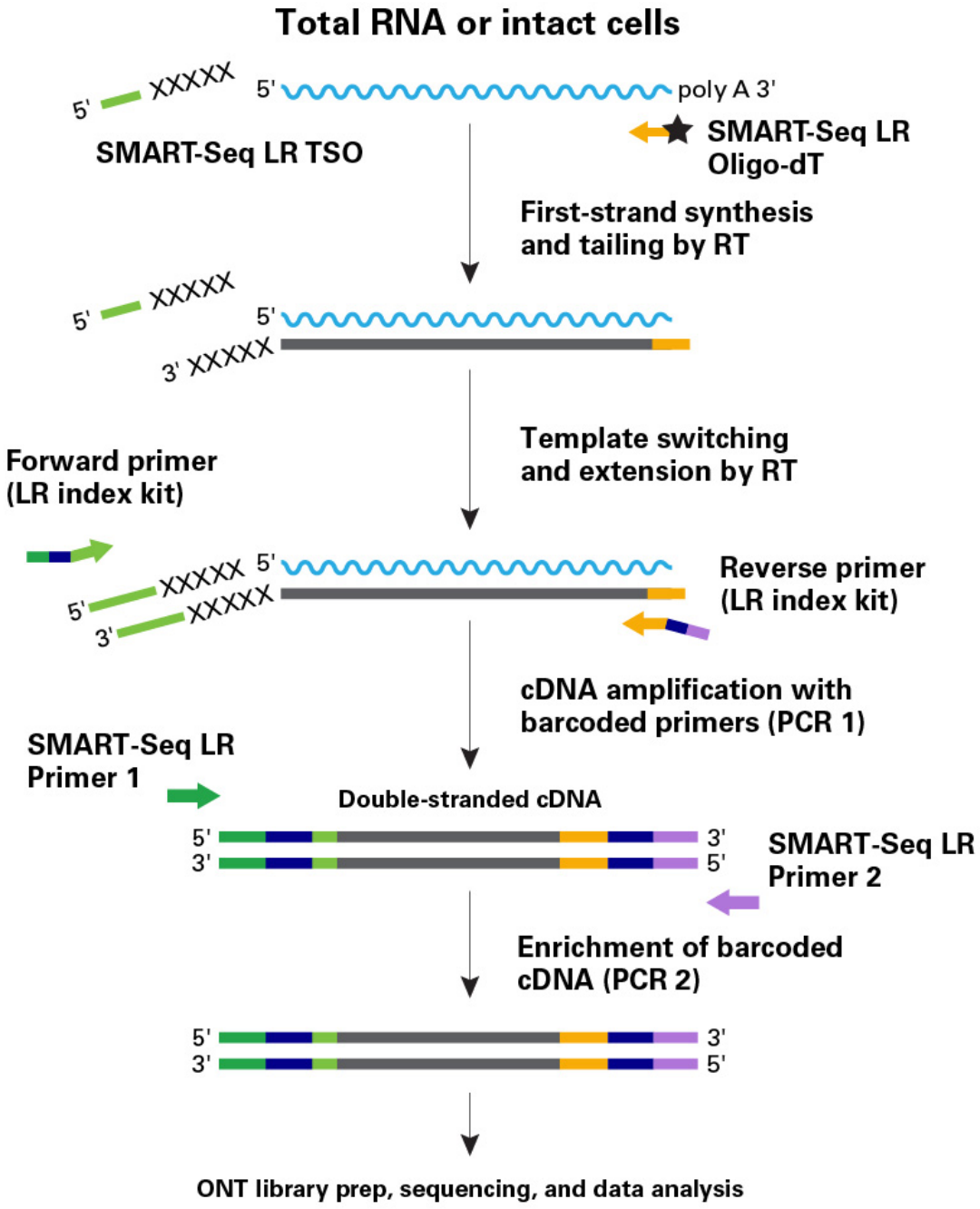

Figure 2. Schematic of the SMART-Seq mRNA Long Read workflow. SMART technology is used to generate full-length cDNA. Nontemplated nucleotides (indicated by Xs) added by the SMARTScribe™ Reverse Transcriptase (RT) hybridize to the SMART-Seq LR template-switching oligonucleotide (SMART-Seq LR TSO), which provides a new template for the RT. The SMART adapters containing barcodes used for amplification during PCR—added by the oligo(dT) primer (SMART-Seq LR OligodT) and SMART-Seq LR TSO —are indicated in purple and green, respectively. A second PCR enriches the barcoded cDNA fragments. Barcodes are shown in dark blue. SMART-Seq adapter sequences are shown (left-to-right) in dark green, light green, yellow, and purple.

II. List of Components

SMART-Seq mRNA Long Read contains sufficient reagents to prepare up to 24 (Cat. No. 634377) or 96 (Cat. No. 634376) reactions. The 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.

Table 1. SMART-Seq mRNA Long Read components.

SMART-Seq mRNA Long Read	634377 (24 rxns)	634376 (96 rxns)
Package 1 (Store at –70°C)		
Control Total RNA (1 µg/µl)	5 µl	5 µl
Package 2 (Store at –20°C)		
5X Ultra® Low First-Strand Buffer (5X)	96 µl	384 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	48 µl	192 µl
RNase Inhibitor (40 U/µl)	60 µl	240 µl
Nuclease-Free Water	2 x 1 ml	4 ml
10X Lysis Buffer (10X)*	460 µl	1.85 ml
Elution Buffer†	2 x 1.7 ml	2 x 6.8 ml
SeqAmp™ DNA Polymerase	50 µl	200 µl
SeqAmp CB PCR Buffer (2X)	1.25 ml	5 ml
SMART-Seq LR Oligo-dT (10 µM)	48 µl	192 µl
SMART-Seq LR TSO (100 µM)	12 µl	48 µl
SMART-Seq LR Primer 1 (12 µM)	24 µl	96 µl
SMART-Seq LR Primer 2 (12 µM)	24 µl	96 µl
TRH	10 µl	20 µl
SMART-Seq LR Index Plate (12.5 µM)‡	5 µl x 24	5 µl x 96

*Store 10X Lysis Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
†Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at room temperature.
‡Do not freeze-thaw SMART-Seq LR Index Plate more than 12 times.

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)

For single cell sorting

- 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) 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.B)
  - One thermal cycler used only for double-stranded cDNA amplification by PCR (Section V.C) and library amplification (Section V.E)

NOTE: 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, please set the lid temperature manually to 105°C. .

- 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 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 µl size: Cat. No. A63880; 60 µl 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 min). 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 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 quantification

- High Sensitivity DNA Kit (Agilent, 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.G and VI.D)
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854) or Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Cat. No. P11496) (may be used in Sections V.D and Appendix D)
- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2 ml PCR 8-tube strip; Thermo Fisher Scientific, Cat. No. AB0264) 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

IV. General Considerations or Precautions

A. General Requirements

- **IMPORTANT!** For products Cat. No. 634377 (24 rxns) and Cat. No. 634376 (96 rxns), we recommend performing a **minimum** of 8 reactions per protocol run to ensure sufficient reagents to utilize 96 reactions per kit and enough yield for sequencing library preparation for Oxford Nanopore sequencing.
- **The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is of high integrity, intact, and free of contaminants, carriers, or co-precipitants.**
- The assay is very sensitive to variations in pipetting volume. Please make sure all your pipettes are calibrated for reliable delivery.

- All lab supplies related to the cDNA synthesis reaction need to be stored in a nucleic-acid-free and nuclease-free closed cabinet.
- 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 reaction component. The amounts and concentrations have been carefully optimized for the cDNA synthesis reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that the kit components are working properly.

B. Requirements for Preventing Contamination

Before you set up the experiment, **make sure you have two physically separated workstations**:

- **A PCR clean workstation** for all pre-PCR experiments that require cleanroom conditions, i.e., sample preparation, first-strand cDNA synthesis (Section V.B) and set up prior to amplification (Sections V.C).

  NOTE: The PCR clean workstation should be in a clean room with positive airflow, as contamination may occur very easily. Once contamination occurs, it can be difficult to remove. Strictly obey cleanroom operation rules.

- **A second workstation located in the general laboratory** where you perform cDNA amplification (Section V.C), perform cDNA purification (Section V.D), measure cDNA concentration (Section V.G), and prepare the sequencing libraries (Section VI).

C. Sample Recommendations and Requirements

SMART-Seq LR works with intact cells (1–1,000) or ultra-low amounts of total RNA (10 pg–100 ng).

1. Total RNA Input

- **RNA should be in a maximum volume of 10.5 µl for 10 pg sample and 9.5 µl for any other input**.
- This protocol has been optimized for cDNA synthesis starting from as little as 10 pg of total RNA. However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 100 ng). Purified total RNA should be in nuclease-free water and free of contaminants.
- The sequence complexity and the average length of the cDNA are dependent on the quality of the starting RNA material. Due to the limited sample size, most traditional RNA isolation methods may not be applicable. Several commercially available products enable purification of total RNA preparations from extremely small samples (e.g., the NucleoSpin RNA XS kit [Takara Bio, Cat. No. 740902.10] for purification of RNA from ≥100 cells). When choosing a purification method or kit, ensure that it is appropriate for your sample amount. **Input RNA should be free from poly(A) carrier RNA that interferes with oligo(dT)-primed cDNA synthesis**.

- After RNA extraction, if your sample quantity is not limited, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513) or an equivalent microfluidics-based automated electrophoresis system. Refer to the manufacturer's instructions for information on how to use the Agilent RNA 6000 Pico Kit.
- Because the initiation of reverse transcription relies on oligo(dT) priming to polyadenylated RNA, this kit is not suitable for degraded RNA samples such as RNA extracted from FFPE or body fluids.

2. Cell Input

- This protocol has been validated to generate cDNA starting from 1–1,000 intact cells sorted by fluorescence-activated cell sorting (FACS) into 96-well plates or PCR strips. **It cannot be used with cells that have undergone fixation.**
- For the removal of media prior to dilution or FACS, bulk cell suspensions should be washed and resuspended in Mg^{2+}- and Ca^{2+}-free PBS, as the presence of media can interfere with the first-strand synthesis. It is best to perform at least two washes. If necessary, test the effect of your media or FACS buffer on cDNA synthesis by performing a reaction with control RNA and the estimated volume of media that you expect to accompany your cells (See Appendix B).
- Following appropriate washes, cells can be diluted in BD FACS Pre-Sort Buffer (BD Biosciences, Cat. No. 563503) to maintain cells in a single-cell suspension before FACS.
- Verify cell integrity prior to sorting—damaged cells will contain compromised RNA.
- Cells should be sorted into 12.5 µl of CDS Sorting Solution (see Section V.A.2). If you do not wish to include the SMART-Seq LR Oligo-dT in the lysis buffer or if cells are aliquoted in a different buffer, please follow the recommendations in Appendix B, Sections A and B, respectively.
- If sorting more than one cell per SMART-Seq cDNA synthesis reaction, it is advisable to perform a preliminary cell sorting study to determine sheath fluid carryover, which should not exceed 5 µl but will differ depending on the cell diameter and quantity.

  Depending on the predicted transfer volume of the sheath fluid, decrease the volume of nuclease-free water in the cell collection buffer per collection well/tube to ensure that the final volume of collected cells does not exceed the recommended sample input volume.
- Collect cells into the low binding plate/tube strip compatible with the SMART-Seq cDNA synthesis. Transferring cells or cell lysate from a collection plate/tube into a new plate/tube may result in the loss of the material.

D. Diluting the Control RNA

Positive control reactions are invaluable to ensure the kit performs as expected and are essential for troubleshooting experiments. The best positive control has a similar RNA input mass as your experimental samples (e.g., 10 pg of RNA is a good estimate for a single cell). Until you are

comfortable with the protocol, you may want to test two positive control inputs (e.g., 10 pg and 100 pg). Serial dilution is essential to ensure an accurate concentration of the final dilution. Follow the guidelines below to reach a single cell equivalent of 10 pg. When used with 19 cycles of PCR, 10 pg of the Control Total RNA included in the kit should generate a cDNA yield of at least 25 fmol.

NOTE: Make fresh dilutions before each use. If desired, make single-use aliquots of the 50 ng/µl dilution generated in Step 2 (below) and store them at –80˚C. When needed, thaw an aliquot, further dilute (Steps 3–6), 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 396 µl of Nuclease-Free Water with 4 µl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.
2. Dilute Control Total RNA (mouse 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 10 pg/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. For example, use 1 µl of 10 pg/µl (from Step 5) Control Total RNA as a positive control RNA input for the kit and process along with the other samples.

V. Protocol: cDNA Synthesis

NOTE: Please read the entire set of protocols before starting. The protocols in this user manual have been optimized for cDNA synthesis from multiple cell inputs (1–1,000 intact cells) or ultra-low input amounts of total RNA (10 pg–100 ng). Due to the sensitivity of these protocols, the input material needs to be collected and purified under cleanroom conditions to avoid contamination. The whole process of cDNA synthesis should be carried out in a PCR clean workstation under cleanroom conditions.

A. Protocol: Sample and Control Preparation

IMPORTANT: Sample preparation using the protocol in this section is dependent on the expected number of cycles for the PCR 1 reaction (Section V.C, "Protocol: cDNA Amplification by LD-PCR (PCR 1)"). Before you start, we recommend you perform PCR optimization (Appendix C)

If you have a sufficient amount of starting material (>1 ng total RNA), we recommend performing the procedure in Appendix C with your sample. If you have a very limited amount of material or your sample is unique, use a similar source of RNA or cells to perform the optimization.

NOTES:

- The 10X Lysis Buffer contains a detergent; it is critical to avoid bubbles when mixing.
- The SMART-Seq LR Oligo-dT provided with each kit is sufficient to collect the same number of cells as the number of reactions in the kit (e.g., a 96-reaction kit contains enough to prepare one 96-well plate).

  If you need to sort large numbers of cells compared to the number of cDNA reactions you plan to prepare, we recommend that you purchase the 10X Lysis Buffer (Takara Bio, Cat. No. 635013) for additional lysis buffer. However, additional SMART-Seq LR Oligo-dT is currently not available separately.

1. Positive Controls or if Starting with RNA or Cells Sorted into Non-CSS Buffer

- If you are starting from purified total RNA or cells resuspended in nonvalidated buffers (including Mg^{2+}- and Ca^{2+}-free PBS), follow the protocol below.
- For further instructions on processing cells suspended in nonvalidated buffers, see Appendix B, "FACS Sorting Recommendations", before starting.
- For users starting from cells sorted into CSS, skip ahead to Section V.A.2.

1. Prepare a stock solution of 10X reaction buffer (scale up as needed for use in Step 2, plus 10% of the total reaction mix volume for overage):

   10X reaction buffer:

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

2. Briefly mix the 10X reaction buffer gently, and then spin down.

   NOTE: Lysis Buffer contains a detergent, so it is critical to avoid bubbles when mixing.

3. Determine the number of cycles you will need for PCR 1, based on your starting input amount and Table 2.

   Table 2. Cycling guidelines based on amount of starting material.

Input amount of Total RNA	Input amount of cells	Typical number of PCR cycles
100 ng	—	5–6
10 ng	1,000 cells	7-8
1 ng	100 cells	11-12
100 pg	10 cells	14-15
10 pg	1 cell	18-19

4. Based on the expected number of cycles, use Table 3 to set up your positive and negative controls alongside your test samples (RNA or cells sorted into non-CSS buffer), plus 10% of the total reaction mix volume, depending on the results of your PCR optimization (Appendix C).

Table 3. Preparation for positive controls or samples and negative controls of RNA or cells sorted into non-CSS buffer.

	<17 PCR 1 cycles			≥17 PCR 1 cycles		
Component	**Negative control**	**Positive control**	**Experimental sample**	**Negative control**	**Positive control**	**Experimental sample**
Sample	—	—	1–9.5 µl	—	—	1–10.5 µl
Diluted Control RNA*	—	1–9.5 µl	—	—	1–10.5 µl	—
Nuclease-Free Water†	9.5 µl	Up to 8.5 µl	Up to 8.5 µl	10.5 µl	Up to 9.5 µl	Up to 9.5 µl
10X reaction buffer	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Total volume per reaction	**10.5 µl**	**10.5 µl**	**10.5 µl**	**11.5 µl**	**11.5 µl**	**11.5 µl**

*The Control Total RNA is supplied at a concentration of 1 µg/µl. It should be diluted to match the concentration of your test sample using serial dilutions as described in Section IV.D.

†If planning to perform <17 cycles during PCR 1 (Section V.C), add enough Nuclease-Free Water to bring the total volume with the sample up to 9.5 µl (10.5 µl total volume). If planning to perform ≥17 cycles, add enough to bring the total volume with the sample up to 10.5 µl (11.5 µl total volume).

5. Place the samples on ice and add 2.0 µl SMART-Seq LR Oligo-dT **if** planning <17 PCR 1 cycles or 1.0 µl if planning ≥17 cycles for a final reaction volume of 12.5 µl.:

	Number of PCR 1 cycles	
	<17	**≥17**
From Step 3 (Table 3)	10.5 µl	11.5 µl
SMART-Seq LR Oligo-dT	2 µl	1 µl
Total volume per reaction	12.5 µl	12.5 µl

Mix well by gentle vortexing then briefly centrifuge the plate/strips to collect the contents at the bottom of the tube.

6. Immediately proceed to Section V.B, "Protocol: First-Strand cDNA Synthesis".

2. If Starting with Cells Sorted into CDS Sorting Solution (CSS)

This section provides guidance for sorting cells directly into 12.5 µl of a buffer containing the SMART-Seq LR Oligo-dT, which is suitable for quick setup of the first-strand cDNA synthesis in Section V.B. This treatment is not a requirement for successful cDNA synthesis. To see recommendations for alternative sorting buffers, see Appendix B.

NOTES:

- Due to small pipetting volumes, prepare no less than 230 µl of sorting buffer, plus ~10% extra for overage (total of 250 µl), which is enough for up to 18 wells. Scale up as needed.
- Be sure to count any negative control reactions you wish to include.
- In this protocol, we are assuming that FACS sorting of the cells will not change the volume of liquid in the plate wells. If your sorter dispenses a non-negligible amount of sheath fluid, adjust the volume of the CSS mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 11.5 µl per well.

1. Determine the number of cycles you will need for PCR 1, based on your starting input amount and Table 4.

Table 4. Cycling guidelines based on amount of starting material.

Input amount of cells	Typical number of PCR cycles*
1,000 cells	7-8
100 cells	11-12
10 cells	14-15
1 cell	18-19†

2. Based on the expected number of cycles, use the guidelines in Table 5 to prepare enough CSS to prefill the 96-well plate or PCR strips, plus 10% of the total reaction mix volume.

Table 5. Preparation of CSS with SMART-Seq LR Oligo-dT.

	<17 PCR 1 cycles		≥17 PCR 1 cycles	
Component	**Per well**	**1–18 wells***	**Per well**	**1–18 wells***
10X Lysis Buffer	0.95 µl	19.0 µl	0.95 µl	19.0 µl
RNase Inhibitor	0.05 µl	1.0 µl	0.05 µl	1.0 µl
SMART-Seq LR Oligo-dT†	2 µl	40.0 µl	1 µl	20.0 µl
Nuclease-Free Water†	9.5 µl	190.0 µl	10.5 µl	210.0 µl
Total volume	**12.5 µl**	**250.0 µl**	**12.5 µl**	**250.0 µl**

*Volumes include ~10% extra for overage.

†SMART-Seq LR Oligo-dT and Nuclease-Free Water volumes vary depending on the number of PCR 1 cycles that will be used.

Briefly mix the CSS gently, and then spin down.

3. Aliquot 12.5 µl of the mixed CSS from Step 1 into the appropriate number of wells of PCR tube strips or a 96-well plate.

NOTE: To minimize bubble formation, set single- or multichannel pipettes to 12.6 µl and pipette only to the first stop when aliquoting. Changing tips often also minimizes bubble formation.

4. Seal the plate/tube strips with microplate film and briefly spin to collect the sorting buffer at the bottom of the wells.
5. Store the plate/tube strips at –20°C for 10 min at a minimum and up to 24 hr. As the volume of sorting buffer is small, the tubes/plate should be kept at –20°C until just before sorting.

When ready to sort:

6. Unseal the prepared plate/tube strips and sort cells into the sorting solution according to the FACS system manual and desired parameters.
7. Seal the plate/tube strips with an aluminum foil seal or PCR strip caps. Ensure the plate/tube strips are sealed firmly to minimize any evaporation.

NOTE: When using PCR strips, strip caps can be used instead of an aluminum foil seal but are not practical when sorting a large number of samples.

8. Immediately after sorting the cells and sealing the plate, spin briefly to collect the cells at the bottom of each well in the CSS.
9. Place the plate on dry ice to flash-freeze the sorted cells.

   NOTE: If using PCR strips, leave them secured on the PCR rack for freezing.

10. Store sorted samples at –80°C until ready to proceed with cDNA synthesis (Section V.B, Step 2).

    NOTES:

    - To use PCR strips sealed with an aluminum foil seal, use a clean razor blade to separate the individual strips, then push up slightly on the tubes from under the PCR rack to loosen them before taking out the desired number of strips.
    - Long-term storage at –80°C may impact the efficiency of cDNA synthesis; however, it is safe to store the cells for several weeks prior to cDNA synthesis.
    - If preparing positive control reactions, go back to Section V.A.1. Otherwise, proceed directly to Section V.B.

B. Protocol: First-Strand cDNA Synthesis

First-strand cDNA synthesis is primed by the SMART-Seq LR Oligo-dT and uses the SMART-Seq LR TSO for template-switching at the 5′ end of the transcript.

IMPORTANT: To avoid contaminating your sample, the first part of the cDNA synthesis protocol requires the use of a PCR clean workstation, ideally in a cleanroom.

1. Thaw the 5X Ultra Low First-Strand Buffer at room temperature.

   NOTE: The 5X Ultra Low First-Strand Buffer forms precipitates. Thaw this buffer at room temperature and vortex before using it to ensure all components are completely in solution before using.

   Thaw all the remaining reagents (except the SMARTScribe Reverse Transcriptase) needed for the first-strand cDNA synthesis on ice. Keep the SMARTScribe Reverse Transcriptase at –20°C until just prior to use.

2. Gently vortex each reagent to mix and spin down briefly. Store all reagents on ice except the 5X Ultra Low First-Strand Buffer.

   NOTE: The 5X Ultra Low First-Strand Buffer forms precipitates. Ensure all components are completely in solution before using.

3. **(Optional)** If starting with frozen cell samples collected in CSS, remove the samples (tubes or PCR strips containing the sorted cells) from the freezer and briefly spin to collect the content at the bottom of the tube.
4. Incubate samples (either the controls, RNA, and/or non-CSS collected cells from Section V.A.1or cells collected in CSS, from the step immediately preceding this one) at 72°C in a preheated, hot-lid thermal cycler for 3 min.

   NOTE: Prepare the RT premix (Step 5) while your tubes are incubating. The SMARTScribe Reverse Transcriptase will be added just before use (Step 7). Steps 8 & 9 below are critical for the first-strand cDNA synthesis and should not be delayed after completing Step 7.

5. Prepare enough RT premix for all the reactions, plus 10% of the total reaction mix volume, by combining the following reagents at room temperature in the order shown:

   RT premix:

4 µl	5X Ultra Low First-Strand Buffer
0.5 µl	SMART-Seq LR TSO (100 µM)
0.5 µl	RNase Inhibitor (40 U/µl)
0.5 µl	Nuclease-Free Water
5.5 µl	**Total volume per reaction**

6. Immediately after the 3-min incubation at 72°C (Step 4), place the samples on ice for at least 2 min (but no more than 10 min).

7. Preheat the thermal cycler to 42°C using program of Step 10 and the lid to 105°C.

8. Remove the SMARTScribe II Reverse Transcriptase from –20°C storage and add to the RT Premix, plus 10% of the total reaction mix volume, to make the RT master mix.

   RT master mix:

5.5 µl	RT premix
2.0 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
7.5 µl	**Total volume per reaction**

   Mix well by gently vortexing, and then spin the tube briefly in a minicentrifuge to collect the content at the bottom of the tube.

9. Add 7.5 µl of the RT master mix to each sample. Mix the contents of the tubes by gently pipetting and spin briefly to collect the contents at the bottom of the tubes.

   Sample mix:

12.5 µl	Sample (from Step 4)
7.5 µl	RT Master Mix
20.0 µl	**Total volume per reaction**

10. Place the tubes in the preheated thermal cycler. Run the following program:

42°C	90 min
70°C	10 min
4°C	forever

SAFE STOPPING POINT: The tubes can be stored at 4°C overnight.

C. Protocol: cDNA Amplification by LD-PCR (PCR 1)

The SMART-Seq LR Indexes amplify the cDNA by priming to the sequences introduced by the SMART-Seq LR Oligo-dT and the SMART-Seq LR TSO.

IMPORTANT: Table 7 provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for the first-strand cDNA synthesis. These guidelines were determined using the Control Mouse Brain Total RNA. Typical cycle numbers are provided as a rough guide for working with small amounts of RNA. Optimal parameters may vary for different templates, different cell types, and different thermal cyclers. To determine the optimal number of cycles for

your sample and conditions, we strongly recommend that you perform a range of cycles. See Appendix C for PCR optimization suggestions.

1. Program the thermocycler with the program in Step 7, run it, then hold it at the first step of the PCR program in Step 7 to preheat the thermal cycler.
2. Thaw SeqAmp CB PCR Buffer and SMART-Seq LR Index Plate on ice. Gently vortex each reagent to mix and spin down briefly. Store on ice.

   NOTE: Do **NOT** thaw SeqAmp DNA Polymerase and TRH. Keep at –20°C until just prior to use.

3. Add 4 µl of SMART-Seq LR indexes, one index per sample, to each tube containing 20 µl of first-strand cDNA product from Section V.B.

   Sample/index mix:

20 µl	First-strand cDNA product (from Section V.B)
4 µl	SMART-Seq LR index
24 µl	**Total volume per reaction**

Table 6. SMART-Seq LR Index Plate map for Cat. No. 634376 (96 rxns). The plate includes indexes BC01–BC96.

	1*	2*	3*	4	5	6	7	8	9	10	11	12
A	BC01	BC09	BC17	BC25	BC33	BC41	BC49	BC57	BC65	BC73	BC81	BC89
B	BC02	BC10	BC18	BC26	BC34	BC42	BC50	BC58	BC66	BC74	BC82	BC90
C	BC03	BC11	BC19	BC27	BC35	BC43	BC51	BC59	BC67	BC75	BC83	BC91
D	BC04	BC12	BC20	BC28	BC36	BC44	BC52	BC60	BC68	BC76	BC84	BC92
E	BC05	BC13	BC21	BC29	BC37	BC45	BC53	BC61	BC69	BC77	BC85	BC93
F	BC06	BC14	BC22	BC30	BC38	BC46	BC54	BC62	BC70	BC78	BC86	BC94
G	BC07	BC15	BC23	BC31	BC39	BC47	BC55	BC63	BC71	BC79	BC87	BC95
H	BC08	BC16	BC24	BC32	BC40	BC48	BC56	BC64	BC72	BC80	BC88	BC96

*The SMART-Seq LR Index Plate for Cat. No. 634377 (24 rxns) includes only indexes BC01–BC24 in columns 1–3.

4. Remove the SeqAmp DNA Polymerase and TRH from the freezer and gently mix the tube without vortexing. Immediately proceed to the next step.
5. Prepare enough PCR1 master mix for all the reactions, plus 10% of the total reaction mix volume. Combine the reagents in the order shown:

   PCR1 master mix:

25.0 µl	SeqAmp CB PCR Buffer
0.1 µl	TRH
1.0 µl	SeqAmp DNA Polymerase
26.1 µl	**Total volume per reaction**

   Mix the master mix well by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube.

6. Add 26 µl of PCR1 master mix to each tube containing 24 µl of first-strand cDNA product and SMART-Seq Long Read Indexes.

PCR1/sample/index mix:

24 µl	Sample/index mix (from Step 2)
26 µl	PCR master mix (from Step 4)
50 µl	**Total volume per reaction**

Mix well by gentle pipetting and briefly spin to collect the contents at the bottom of the tube.

7. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

95°C	1 min
N cycles*:	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

*For *N*, please consult Table 7 for the recommended number of PCR cycles.

Table 7. Cycling guidelines based on amount of starting material

Input amount of Total RNA	Input amount of cells	Typical number of PCR cycles*
100 ng	—	5–6
10 ng	1,000 cells	7-8
1 ng	100 cells	11-12
100 pg	10 cells	14-15
10 pg	1 cell	18-19†

SAFE STOPPING POINT: The tubes may be stored at 4°C overnight.

IMPORTANT: Transfer the samples from the PCR clean workstation to the general lab. All downstream processes starting with Section D should be performed in the general lab.

D. Protocol: Purification of Amplified cDNA

PCR-amplified cDNA is purified by immobilization on NucleoMag NGS Clean-up and Size Select (available from Takara Bio, Cat. No. 744970) beads. The beads are then washed with 80% ethanol, and cDNA is eluted with Elution Buffer.

NOTES:

- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well by vortexing. Use room-temperature Elution Buffer for this protocol.
- 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.
- Do not pool the samples at the cDNA purification step. If pooling is desired, it can be performed in Section VI.

1. 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.
2. Incubate the beads-cDNA mixture at room temperature for 8 min to let the cDNA bind to the beads.
3. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells.
4. Place the samples on the magnetic separation device for ~5 min or longer until the liquid appears completely clear and there are no beads left in the supernatant.
5. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.
6. Keep the samples on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec. Then, carefully remove and discard the supernatant, taking care not to disturb the beads. The cDNA remains bound to the beads during the washing process.
7. Repeat the ethanol wash (Step 7) once more.
8. Briefly centrifuge the samples to collect the liquid from the side of the tubes or plate wells. Place the samples on the magnetic separation device for 30 sec, then remove any residual ethanol with a pipette.
9. Incubate the samples at room temperature for ~2–2.5 min, until the pellet is no longer shiny, but before cracks appear.

   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'd 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 dry, remove the samples from the magnetic separation device and add 23 µl of Elution Buffer to cover the bead pellet. Mix thoroughly by pipetting or gently vortexing to resuspend the beads.
11. Incubate at room temperature for at least 2 min to rehydrate.
12. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. Place the samples back on the magnetic separation device for 1 min or longer until the solution is completely clear.
13. Transfer clear supernatant (20 µl) containing purified cDNA from each tube/well to a new tube/plate.

    NOTES:
    - Do not pool samples at this point
    - Take care not to carry over any beads with your sample.

SAFE STOPPING POINT: Proceed to PCR2 (next section) immediately or store purified products at –20°C.

E. Protocol: cDNA Enrichment by LD-PCR (PCR 2)

The SMART-Seq LR Primer 1 and Primer 2 amplify the cDNA by priming to the sequences introduced by the SMART-Seq LR Indexes.

1. Program, run, then hold at the first step of the PCR program in Step 5 to preheat the thermal cycler.
2. Thaw SeqAmp CB PCR Buffer and SMART-Seq LR Primers on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.

   NOTE: Do **NOT** thaw SeqAmp DNA Polymerase and TRH. Keep at –20°C until just prior to use.

3. Prepare enough PCR2 master mix for all the reactions, plus 10% of the total reaction mix volume. Combine the reagents in the order shown:

   PCR2 master mix:

25.0 µl	SeqAmp CB PCR Buffer
1.9 µl	Nuclease-Free Water
1.0 µl	SMART-Seq LR Primer 1
1.0 µl	SMART-Seq LR Primer 2
0.1 µl	TRH
1.0 µl	SeqAmp DNA Polymerase
30.0 µl	**Total volume per reaction**

   NOTE: Remove the SeqAmp DNA Polymerase and TRH from the freezer, gently mix the tubes without vortexing, and add to the PCR2 master mix just before use.

   Mix the master mix well by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube.

4. Add 30 µl of PCR master mix to each tube containing 20 µl of purified cDNA from Section V.D. Mix well by gentle pipetting, and briefly spin to collect the contents at the bottom of the tube.
5. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

95°C	1 min
6 cycles:	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

SAFE STOPPING POINT: The tubes may be stored at 4°C overnight.

F. Protocol: Purification of Enriched cDNA

PCR-enriched cDNA is purified by immobilization on NucleoMag NGS Clean-up and Size Select beads. The beads are then washed with 80% ethanol, and cDNA is eluted with Elution Buffer.

NOTES:

- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well by vortexing. Use room-temperature Elution Buffer for this protocol.
- 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.
- Do not pool the samples. If pooling is desired, it can be performed in Section VI.

1. 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.
2. Incubate the beads-cDNA mixture at room temperature for 8 min to let the cDNA bind to the beads.
3. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells.
4. Place the samples on the magnetic separation device for ~5 min or longer until the liquid appears completely clear and there are no beads left in the supernatant.
5. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.
6. Keep the samples on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec. Then, carefully remove and discard the supernatant, taking care not to disturb the beads. The cDNA remains bound to the beads during the washing process.
7. Repeat the ethanol wash (Step 6) once more.
8. Briefly centrifuge the samples to collect the liquid from the side of the tubes or plate wells. Place the samples on the magnetic separation device for 30 sec, then remove any residual ethanol with a pipette.
9. Incubate the samples at room temperature for ~2–2.5 min, until the pellet is no longer shiny, but before cracks appear.

   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'd 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 dry, remove the samples from the magnetic separation device and add 17 µl of Elution Buffer to cover the bead pellet. Mix thoroughly by pipetting or gently vortexing to resuspend the beads.
11. Incubate at room temperature for at least 2 min to rehydrate.

12. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. Place the samples back on the magnetic separation device for 1 min or longer until the solution is completely clear.
13. Transfer clear supernatant (~15 µl) containing purified enriched cDNA from each tube/well to a new tube/plate.

NOTES:
- Do not pool samples at this point.
- Take care not to carry over any beads with your sample.

SAFE STOPPING POINT: Purified cDNA can be stored at –20°C, or you can proceed immediately to the next step, "Protocol: Determination of cDNA Quality and Quantity".

G. Protocol: Determination of cDNA Quality and Quantity

Quantify cDNA using fluorescence-detection-based methods, such as the Qubit dsDNA HS Assay or the Quant-iT PicoGreen dsDNA Assay Kit.

1. Dilute a 1 µl aliquot of the amplified cDNA (from Section V.F) to ~3 ng/µl. Use 1 µl of this diluted sample for validation using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). See the Agilent High Sensitivity DNA Kit User Manual for instructions.
2. Compare the results for your samples and controls (see Figure 3) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield a distinct peak spanning 400–10,000 bp, with an apex of ~2,500 bp for the 10 pg positive control RNA sample (Figure 3 Panel A) and no product in the negative control (Figure 3, Panel B).
3. Proceed to Section VI, "Sample Pooling and Library Preparation for ONT Sequencing", when ready to sequence.

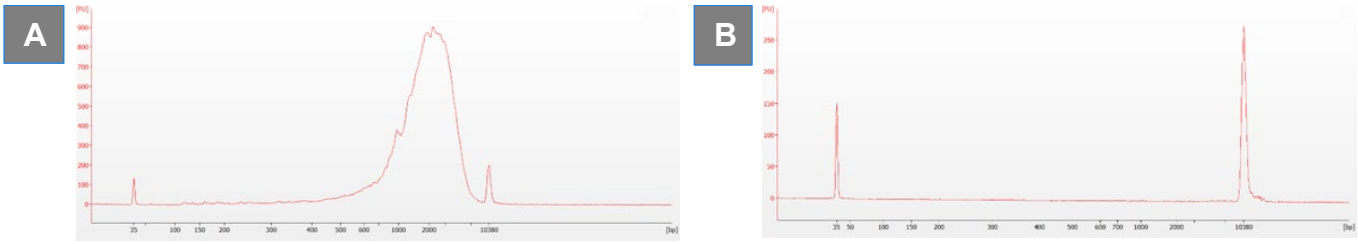

Figure 3. Example electropherogram results from Agilent 2100 Bioanalyzer. All samples were subjected to SMART cDNA synthesis and amplification described in the protocol for 19 PCR cycles. **Panel A**. Electropherogram shows a clean product following cDNA synthesis and amplification (10 pg input). **Panel B.** Result shows no product in the negative control following cDNA synthesis and amplification.

VI. Sample Pooling and Library Preparation for ONT Sequencing

Barcoded and enriched cDNA can be pooled to perform sequencing library preparation with the Ligation Sequencing Kit V14 (ONT, Cat. No. SQK-LSK114).

NOTE: If all samples are correctly quantified and normalized to a uniform input amount before library preparation a relatively uniform amount of sequencing reads will be obtained.

1. Pool a minimum of 8 cDNA samples (per library) and quantify the pool with Qubit and Bioanalyzer or Tapestation.
2. Continue with library preparation according to the Ligation sequencing amplicons V14 (SQK-LSK114) protocol starting with cDNA repair and end-prep.
3. Use 100–200 fmol of cDNA in up to 50 µl for the cDNA repair and end-prep if you choose to omit the DNA CS.

NOTE: It is recommended to use the Short Fragment Buffer to retain all DNA fragments. Using the Long Fragment Buffer may lead to the loss of a large portion of the sample.

VII. Guidelines for Data Analysis

A guide on how to demultiplex SMART-Seq LR Indexes can be found on takarabio.com. The protocol requires additional files available for download on our website as input into the demux pipelines. please visit the tool website to find the instructions for use and to sign-up to download the additional required files.

VIII. References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis*. Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319 (1998).

Appendix A. Troubleshooting Guide for cDNA

Table 8. Issue: Broad peaks in Bioanalyzer traces.

Description of problem	Possible explanation	Solution
After purification of the amplified cDNA, the Bioanalyzer traces show broad peak(s) extending from <1,000 bp to >10,000 bp.	The cDNA was overamplified.	Perform fewer PCR cycles during the library amplification reaction.
	The Bioanalyzer chip was overloaded.	This is a common problem for high-sensitivity chips. Load ~1–5 ng/µl, and then repeat the Bioanalyzer run.

Table 9. Issue: High quantity of small molecules in Agilent Bioanalyzer and TapeStation traces.

Description of problem	Possible explanation	Solution
After purification of the amplified cDNA, the Bioanalyzer trace shows a high quantity of small molecules between 35 and 200 bp.	Bead:sample ratio was higher than the suggested 0.8:1.	Perform an additional bead purification using a ratio of 0.8:1 or 0.7:1.

Appendix B. FACS Sorting Recommendations

When sorting the cells, we strongly recommend including the oligo required for priming the reverse transcription (SMART-Seq LR Oligo-dT) in the sorting buffer (see CDS sorting solution described in Section V.A). Including the oligo eliminates a pipetting step when setting up the reverse transcription. However, this is not an absolute requirement for successful cDNA synthesis. Alternatively, you can sort cells in a maximum volume of 5 µl PBS.

NOTE: PBS is **NOT** an optimal sorting buffer, as it interferes with the efficiency of cDNA synthesis and PCR amplification.

- For sorting in a buffer not containing the SMART-Seq LR Oligo-dT, proceed to Section A.
- For how to proceed after sorting into ≤5 µl of PBS, see Section B.

A. FACS Sorting into a Buffer Not Containing SMART Seq LR Oligo-dT

In this protocol, cells are sorted in 10.5 µl of buffer. We are assuming that FACS sorting of the cells will not change the volume of liquid in the plate wells. If your sorter dispenses a non-negligible amount of sheath fluid, adjust the volume of the Plain Sorting Solution (PSS) mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 10.5 µl per well.

1. Determine the number of cycles you will need for PCR 1, based on your starting input amount and Table 3 in Section V.A.1, "Positive Controls or if Starting with RNA or Cells Sorted into Non-CSS Buffer".
2. Based on the expected number of cycles, use the guidelines in Table 10 to prepare enough buffer to prefill the 96-well plate or PCR strips as indicated below.

NOTES:
- Due to small pipetting volumes, prepare sorting buffer for a minimum of 18 wells. Scale up as needed for all reactions; include an additional 10% of the total reaction mix volume for overage.
- Be sure to include negative control reactions in the count for the number of wells to prepare.

Table 10. Plain Sorting Solution (PSS); without SMART Seq mRNA Oligo dT.

	<17 PCR 1 cycles		≥17 PCR 1 cycles	
Component	**Per well**	**1–18 wells***	**Per well**	**1–18 wells***
10X Lysis Buffer	0.95 µl	19.0 µl	0.95 µl	19.0 µl
RNase Inhibitor	0.05 µl	1.0 µl	0.05 µl	1.0 µl
Nuclease-Free Water†	8.5 µl	168.3 µl	9.5 µl	190.0 µl
Total volume	9.5 µl	188.3 µl	10.5 µl	210.0 µl

*Volumes include ~10% extra for overage.
†Nuclease-Free Water volume depends on the number of PCR 1 cycles that will be performed.

Briefly mix gently, then spin down.

NOTES:
- The 10X Lysis Buffer contains a detergent; it is critical to avoid bubbles when mixing.

- If you need to sort large numbers of cells compared to the number of cDNA reactions you plan to prepare, you have the option to purchase the 10X Lysis Buffer (Takara Bio, Cat. No. 635013) separately.

1. Aliquot PSS from Step 1 into the appropriate number of wells of PCR tube strips or a 96-well plate, depending on the number of PCR 1 cycles are being used:
   - For <17 cycles, aliquot 10.5 µl of PSS.
   - For ≥17 cycles, use 11.5 µl of PSS.

   NOTE: To minimize bubble formation, set single- or multichannel pipettes to 11.6 µl and pipette only to the first stop when aliquoting. Changing tips often also minimizes bubble formation.
2. Seal the plate/tube strips with microplate film, and briefly spin to ensure the sorting buffer collects at the bottom of the wells.
3. Store the plate/tube strips at –20°C for 10 min at a minimum and up to 24 hr. As the volume of sorting buffer is small, the tubes/plate should be kept at –20°C until just before sorting.

When ready to sort:

4. Unseal the prepared plate/tube strips and sort cells into the sorting solution according to the FACS system manual and desired parameters.
5. Seal the plate/tube strips with an aluminum foil seal or PCR strip caps. Ensure the plate/tube strips are sealed firmly to minimize any evaporation.

   NOTE: When using PCR strips, strip caps can be used instead of an aluminum foil seal but are not practical when sorting a large number of samples.
6. Immediately after sorting the cells and sealing the plate, spin briefly to collect the cells at the bottom of each well in the PSS.
7. Place the plate on dry ice to flash-freeze the sorted cells.

   NOTE: If using PCR strips, leave them secured on the PCR rack for freezing.
8. Store sorted samples at –80°C until ready to proceed with cDNA synthesis.

   NOTES:
   - To use PCR strips sealed with an aluminum foil seal, use a clean razor blade to separate the individual strips, then push up slightly on the tubes from under the PCR rack to loosen them before taking out the desired number of strips.
   - Long-term storage at –80°C may impact the efficiency of cDNA synthesis; however, it is safe to store the cells for several weeks prior to cDNA synthesis.

   IMPORTANT: Since the PSS does not include the SMART-Seq LR Oligo-dT, you will need to add it in Section V.A.1, Step 4.

B. FACS Sorting into Nonvalidated Buffers

Sorting into CSS (Section V.A) or PSS (Appendix B, Section A) delivers the best performance from cells isolated with FACS or other single-cell isolation methods. However, it may not always be possible to use these buffers. In such cases, we recommend minimizing the volume of the buffer being carried into the cDNA synthesis reaction. The maximum volume of buffer that can be added to a cDNA synthesis reaction is 10.5 µl.

If using an alternative buffer, we recommend performing a pilot experiment using the Control Total RNA and the estimated amount of buffer that you expect to accompany your cell(s) through sorting to determine its impact on cDNA synthesis. Sorting cells in 1 µl of 1X PBS is acceptable and will not interfere with kit performance. If it is desired to sort cells in >1 µl of 1X PBS, it is critical to keep the PBS volume below 5 µl. PBS is not an optimal sorting buffer as it interferes with the efficiency of cDNA synthesis and PCR amplification; thus, kit performance (cDNA yield and sensitivity) will be impacted (when using >1 µl of PBS). If you must use 2–5 µl of PBS, the cDNA yield will be lower, and you should consider adding one to three extra PCR cycles to the recommended number of cycles in Table 7. Cycling guidelines based on amount of starting material(Section V.C). In addition, you must use PBS without Ca^{2+} and Mg^{2+} (e.g., Sigma, Cat. No. D8537). The addition of RNase Inhibitor in a ratio similar to what is recommended for CSS and PSS may be helpful, although not essential.

When your samples are not in a recommended FACS sorting buffer, we still recommend flash freezing samples on dry ice as quickly as possible after collection and storing them at –80°C until processing. Follow the instructions outlined in Section V.A.1, "Positive Controls or if Starting with RNA or Cells Sorted into Non-CSS Buffer", for preparing a 10X reaction buffer, setting up control reactions, and adding SMART-Seq LR Oligo-dT.

Appendix C. PCR Optimization

If you have a sufficient amount of starting material (>1 ng total RNA), we recommend optimizing the PCR 1 cycling parameters (Section V.C) for your experiment. If you have a very limited amount of material or your sample is unique, use a similar source of RNA or cells to perform PCR cycle optimization prior to using your actual sample. Choosing the optimal number of PCR 1 cycles ensures that the amplification will remain in the exponential phase. When the yield of PCR 1 products stops increasing with more cycles, the reaction has reached its plateau.

- Overcycled cDNA can result in a less representative cDNA library
- Undercycling results in a lower cDNA yield

The optimal number of cycles for your experiment is one cycle fewer than is needed to reach the plateau. Be conservative; when in doubt, it is better to use too few cycles than too many.

NOTE: PCR cycle optimization should be performed for PCR 1 only. Do **NOT** change PCR 2 cycles.

To perform PCR cycle optimization:

1. Prepare several tubes containing an amount of RNA equal to your sample amount.
2. Subject each tube to a different range of PCR1 cycles. For example, if you have 1 ng of RNA, subject one tube to the recommended a number of cycles. Subject the other two tubes to 2–3 cycles fewer or more than the first tube (e.g., 13, 11, and 9 cycles, *N*) for a 1 ng sample.

3. Use the following program for thermal cycling:

95°C	1 min
N cycles:	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

4. Perform "Protocol: Purification of Amplified cDNA" (Section V.D).
5. Run the samples on an Agilent High Sensitivity DNA Chip using the Agilent 2100 Bioanalyzer to evaluate DNA output (Section V.G). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
6. Determine the optimal number of PCR 1 cycles required for each experimental and control sample. We recommend using the lowest PCR 1 cycle number that generates enough material for library construction.

Appendix D. Expected Results When Analyzing cDNA with TapeStation

A good option for visualization of cDNA profiles generated with SMART-Seq LR, particularly for processing a large number of samples, is the Fragment Analyzer and High Sensitivity Large Fragment Analysis Kit (Advanced Analytical Technologies, Inc., Cat. No. DNF-493).

If these instruments are not available, an Agilent TapeStation system can be used with a High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067-5592). Because the scale is very different—as shown in Figure 4—the cDNA profile on the TapeStation may look quite different than the profile on the Bioanalyzer, particularly for yields below 500 pg/µl. However, if the ScreenTape shows a broad smear going from ~600–3,000 bp, the cDNA synthesis can be considered successful, particularly if the negative control, performed with the same number of PCR cycles, shows a relatively flat profile. SMART-Seq mRNA LR should generate cDNA yields higher than 200 pg/µl, and the cDNA profile should be detected relatively easily.

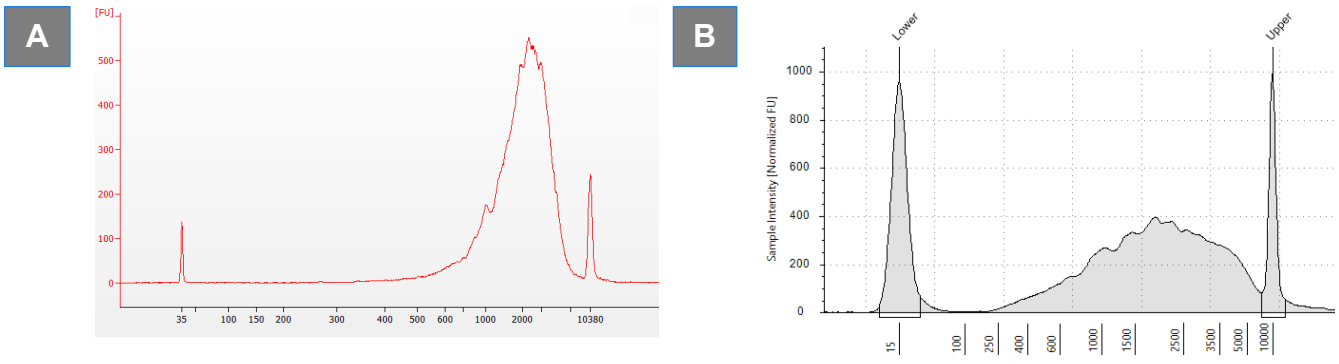

Figure 4. Comparison of electropherogram results from Agilent 2100 Bioanalyzer and Agilent TapeStation system. All samples were subjected to SMART cDNA synthesis and amplification described in the protocol for 18 PCR cycles. **Panel A.** Electropherogram shows Bioanalyzer trace following cDNA synthesis and amplification (10 pg input). **Panel B.** Electropherogram shows Tapestation trace following cDNA synthesis and amplification (10 pg input).

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