Takara Bio USA, Inc.

SMART-Seq® mRNA HT User Manual

Cat. Nos. 634791, 634795 & 634796
(032124)

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 HT (Cat. Nos. 634791, 634795 & 634796) is designed to be an automation-friendly solution for the generation of high-quality, full-length cDNA directly from 1–100 cells or 10 pg–1 ng of high-integrity total RNA (RIN ≥8). The cDNA synthesis protocol can be completed in four hours (Figure 1).

IMPORTANT: Users looking for a complete solution for cDNA synthesis and library prep should use the SMART-Seq mRNA HT LP (Cat. Nos. 634792, 634793 & 634794). In addition to SMART-Seq mRNA HT (the cDNA core kit covered in this user manual), these kits include our recommended library preparation kit.

Used along with the Unique Dual Index Kits (Cat. Nos. 634752–634756), the SMART-Seq mRNA HT LP kits can be used to generate up to 384 Illumina-compatible libraries that can be sequenced on Illumina® HiSeq®, MiSeq®, MiniSeq™, NextSeq® and NovaSeq™ systems.

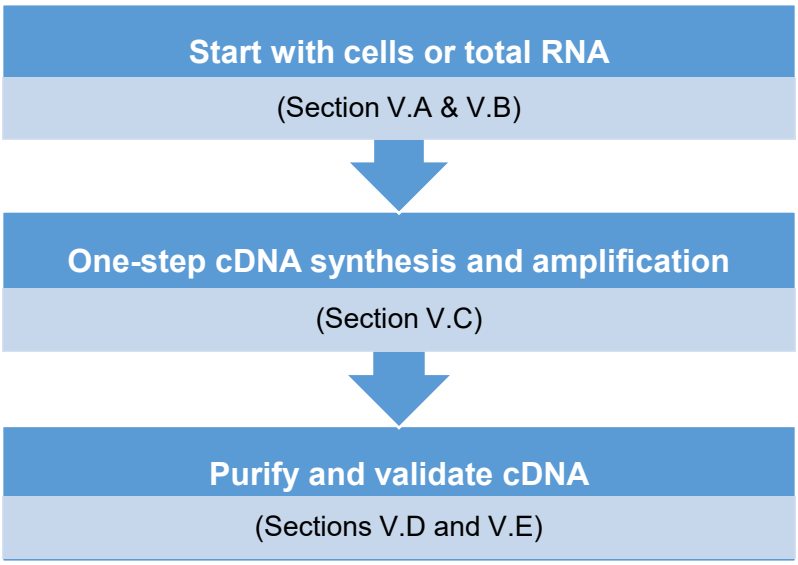

Figure 1. SMART-Seq mRNA HT protocol overview. This kit features a streamlined workflow that generates full-length cDNA with minimal hands-on time. While the workflow is primarily designed to accommodate cells isolated using FACS, it can also be used with purified total RNA.

SMART-Seq mRNA HT incorporates our patented SMART® (**S**witching **M**echanism **a**t 5′ End of **R**NA **T**emplate) technology. This technology relies on the template-switching activity of reverse transcriptase to enrich for full-length cDNAs and to add defined PCR adapters directly to both ends of the first-strand cDNA (Chenchik et al. 1998). This ensures that the final cDNA libraries contain the 5′ end of the mRNA and maintain a true representation of the original mRNA transcripts; these factors are critical for transcriptome sequencing and gene expression analysis. SMART technology offers unparalleled sensitivity, unbiased amplification of cDNA transcripts, and allows direct cDNA synthesis from intact cells.

SMART-Seq mRNA HT is designed to reduce handling time for greater ease of use without compromising on performance. SMART-Seq mRNA HT features a streamlined protocol (Figure 2), which combines the reverse transcription and PCR amplification into a single step. The user simply needs to set up the one-step RT-PCR and walk away until the double-stranded cDNA is amplified and ready for purification. With the fewer steps, fewer reagents are needed, making SMART-Seq mRNA HT very automation friendly. SMART-Seq mRNA HT still continues to have high sensitivity and reproducibility.

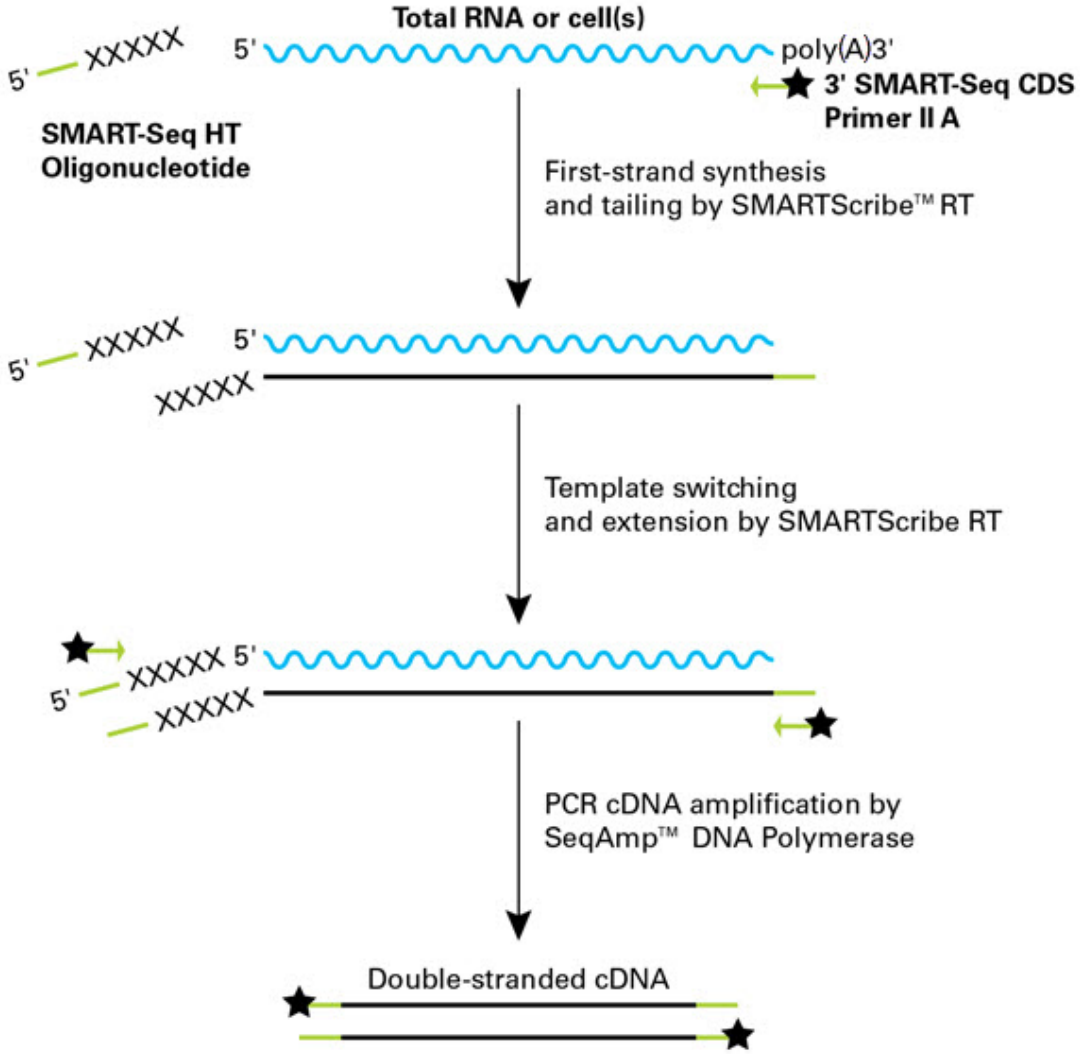

Figure 2. Schematic of technology in SMART-Seq mRNA HT. SMART technology is used in a ligation-free workflow to generate full-length cDNA. The reverse transcriptase (RT) adds non-templated nucleotides (indicated by Xs) which hybridize to the SMART-Seq HT Oligonucleotide, providing a new template for the RT. Chemical modifications to block ligation during sequencing library preparation are present on some primers (indicated by black stars). The SMART adapters, added by the oligo (dT) primer (SMART-Seq CDS Primer IIA) and SMART-Seq HT Oligonucleotide and used for amplification during PCR, are indicated in green. The one-step RT-PCR is set up as a single reaction so that all the reagents are mixed together but then used sequentially. SeqAmp polymerase is a hot-start DNA polymerase activated only after the reverse transcription/template-switching step is complete.

II. List of Components

The components in SMART-Seq mRNA HT have been specifically designed to work together and are optimized for this protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or modifications of the protocol may lead to unexpected results. Freeze-thaw of reagents should be limited to ≤6 times.

NOTE: No library preparation solution is provided with this kit. Users looking for a complete solution for cDNA synthesis and library prep should use SMART-Seq mRNA HT LP (Cat. Nos. 634792, 634793, & 634794)

SMART-Seq mRNA HT	634795 (24 rxns)	634796 (96 rxns)	634791 (384 rxns)
Box 1 (Store at –70°C)			
Control Total RNA* (1 µg/µl)	5 µl	5 µl	4 x 5 µl
Box 2 (Store at –20°C)			
SMART-Seq HT Oligonucleotide	24 µl	96 µl	4 x 96 µl
3' SMART-Seq CDS Primer II A	48 µl	192 µl	4 x 192 µl
RNase Inhibitor (40 U/µl)	60 µl	150 µl	4 x 150 µl
SMARTScribe Reverse Transcriptase (100 U/ µl)	48 µl	192 µl	4 x 192 µl
One-Step Buffer	250 µl	1 ml	4 x 1 ml
SeqAmp DNA Polymerase	20 µl	50 µl	4 x 50 µl
10X Lysis Buffer†‡	500 µl	1.85 ml	4 x 1.85 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)§	1.7 ml	6.8 ml	4 x 6.8 ml
Nuclease-Free Water‡	1 ml	4 ml	4 x 4 ml

*The Control Total RNA is from mouse brain.
†Store 10X Lysis Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
‡Depending on the product size, these components may be packaged in a 1.5 ml tube, an 8 ml bottle (10X Lysis Buffer), or a 15 ml bottle (Nuclease-Free Water in Cat. No. 634790).
§Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at room temperature.

III. Additional Materials Required

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

- Single-channel pipette: 10 µl, 20 µl, and 200 µl
- Eight-channel or twelve-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips
- 96-well PCR chiller rack: IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4) or 96-Well Aluminum Block (Light Labs, Cat. No. A-7079)

For Sample Preparation

- 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR grade tube strips secured into a PCR rack, or 96-well plates that have been validated to work with your FACS instrument (e.g., Bio-Rad iQ 96-Well PCR Plates, Cat. No. 2239441)
- 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 (Takara Bio, Cat. No. 634439) or 10X Lysis Buffer (Takara Bio, Cat. No. 635013) for sorting extra plates

For One-Step cDNA Synthesis and Amplification

- If starting from purified RNA:
  - 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, Item 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
- Thermal cycler with a heated lid

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, you may want to follow the manufacturer's instructions to choose a lower lid temperature for the reverse-transcription step.

For Bead Purification

- NucleoMag NGS Clean-up and Size Select (Takara Bio, Cat. Nos. 744970.5, 744970.50 or 744970.500)
  If the NucleoMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter, Cat. Nos. A63880 or 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.
- 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 and decreases the chances of contamination.
- Magnetic beads should only be used at room temperature. Individual aliquoted tubes can be removed 30 minutes before an experiment to bring the beads to room temperature.

- 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 Scientific, 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 in a PCR rack, or 96-well plates (e.g., Bio-Rad iQ 96-Well PCR Plates, Cat. No. 2239441)

For cDNA Quantification (As Required; Section V.E)

- High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) for Bioanalyzer or equivalent high-sensitivity microfluidic device or kit.
- Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Cat. No. P11496) or Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854)
- Library Quantification Kit (Takara Bio, Cat. No. 638324)
- 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

A. 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 and first-strand cDNA synthesis (Sections V.B & C).

  NOTE: The PCR clean workstation should be in a clean room with positive air flow, 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 purification (Section V.D), measure cDNA concentration (Section V.E).

B. General Requirements

- **IMPORTANT!** For Cat. No. 634796 (96 rxns) and Cat. No. 634791 (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 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 one-step RT-PCR 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 one-step RT-PCR 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.

C. Sample Recommendations and Requirements

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

Cell Inputs

- This protocol has been validated to generate cDNA starting from 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 amount of media that you expect to accompany your cell(s) (See Appendix A).
- 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.
- **Cells should be sorted into 12.5 µl of our recommended sorting buffer (see Section V.A). If you do not wish to include the CDS IIA oligo in the lysis buffer or if cells are aliquoted in a different buffer, please follow the recommendations in Appendix A, Sections A and B, respectively.**

Total RNA Inputs

- **RNA should be in a maximum volume of 10.5 µl**.
- This protocol has been optimized for one-step RT-PCR synthesis starting from 10 pg of high-integrity total RNA (RIN ≥8). However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 1 ng). Purified total RNA should be in nuclease-free water and free of contamination.
- The sequence complexity and the average length of the cDNA generated during the one-step RT-PCR 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., we offer the NucleoSpin RNA XS kit (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 and/or other co-precipitants that interfere with oligo(dT)-primed cDNA synthesis.
- After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513). 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.

D. Diluting the Control RNA

Positive control reactions are invaluable to ensure the kit performs as expected and 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 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 17 cycles of PCR, 10 pg of the Control Total RNA included in the kit should generate a cDNA yield of at least 200 pg/µl.

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 at –80˚C. When needed, thaw an aliquot, further dilute

(Steps 3–6), and throw away any leftover. 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. Further dilute Control Total RNA to 1 pg/µl by mixing 45 µl of RI Water with 5 µl of 10 pg/µl Control Total RNA in a sterile microcentrifuge tube.

7. Use 2–10 µl of 10 pg/µl (from Step 5) or 10 µl of the 1 pg/µl (from Step 6) Control Total RNA as a positive control for the kit and process along with the other samples.

V. Protocols

SMART-Seq mRNA HT can be used with either purified total RNA or intact whole cells. If you are sorting single cells using FACS, begin with Section V.A then proceed to Section V.C. If you are starting from purified total RNA, begin with Section V.B and then proceed to Section V.C.

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

A. Protocol: 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 3′ SMART-Seq CDS Primer II A, which is suitable for quick setup of the one-step RT-PCR in Section V.C. This treatment is not a requirement for successful cDNA synthesis. To see recommendations for alternative sorting buffers, see Appendix A. For users starting from purified RNA, skip ahead to Section V.B.

1. Prepare enough CSS to prefill the 96-well plate or PCR strips, as indicated below. Scale up as needed, making sure to count any negative control reactions you wish to include, plus an additional 10% of the total reaction mix volume for overage.

   NOTE: Due to small pipetting volumes, prepare no less than 250 µl of sorting buffer, which is enough for up to 18 wells.

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 mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 12.5 µl per well.

CDS Sorting Solution (CSS; with 3′ SMART-Seq CDS Primer II A):

	Per well	1–18 wells*
10X Lysis Buffer	0.95 µl	19 µl
RNase Inhibitor	0.05 µl	1 µl
3′ SMART-Seq CDS Primer II A	1 µl	20 µl
Nuclease-Free Water	10.5 µl	210 µl
Total volume	12.5 µl	250 µl

*Volumes include ~10% extra for overage.

Mix briefly, and then spin down.

NOTES:
- The 10X Lysis Buffer contains a detergent; it is critical to avoid bubbles when mixing.
- The 3′ SMART-Seq CDS Primer II A 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 a 96-well plate). However, 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 SMART-Seq HT Lysis Components (Takara Bio, Cat. No. 634439) separately.

2. Aliquot 12.5 µl of 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 multi-channel pipettes to 12.6 µl and pipette only to the first stop when aliquoting. Changing tips often also minimizes bubble formation.

3. Seal the plate/tube strips Microplate film, and briefly spin to collect the sorting buffer at the bottom of the wells.

4. 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:

5. Unseal the prepared plate/tube strips and sort cells into the sorting solution according to the FACS system manual and desired parameters.
6. 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.

7. Immediately after sorting the cells and sealing the plate, spin briefly to collect the cells at the bottom of each well in the CDS Sorting Solution.
8. 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.

9. Store sorted samples at –80°C until ready to proceed with cDNA synthesis (Section V.C, 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, proceed to Section V.B. Otherwise, proceed directly to Section V.C.

B. Protocol: If Starting with RNA or Cells Sorted into Non-CSS Buffer

If you are starting from purified total RNA or cells resuspended in a nonvalidated buffers (including PBS), follow the protocol below. For further instructions on processing cells suspended in nonvalidated buffers, see Appendix A before starting.

1. Prepare a stock solution of 10X Reaction Buffer (scale up as needed for use in Step 2):

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

Mix the 10X Reaction Buffer briefly, and then spin down.

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

2. See Table 1 below for guidelines on setting up your positive and negative controls alongside your test samples. Transfer 1–10.5 µl of purified total RNA to a nuclease-free 96-well PCR plate or tube strips. If necessary, bring the total volume to 10.5 µl with Nuclease-Free Water. Add 1 µl of 10X Reaction Buffer to each sample.

Table 1. Sample preparation guidelines

Components*	Negative control	Positive control	Experimental sample
10X Reaction Buffer	1 µl	1 µl	1 µl
Nuclease-Free Water	10.5 µl	Up to 9.5 µl	Up to 9.5 µl
Diluted Control RNA†	–	1–10.5 µl	–
Sample	–	–	1–10.5 µl
Total volume	11.5 µl	11.5 µl	11.5 µl

*Volumes are per reaction. When scaling up, include ~10% extra for overage.

†The Control Total RNA is supplied at a concentration of 1 µg/µl. It should be diluted in Nuclease-Free Water with RNase Inhibitor (1 µl of RNase Inhibitor in a final volume of 50 µl of water) to match the concentration of your test sample (see "Diluting the Control RNA" under Section IV.D).

3. Place the samples on ice and add 1 µl of 3′ SMART-Seq CDS Primer II A. Mix well by gentle vortexing and then briefly centrifuge the plate/strips to collect the contents at the bottom of the tube.
4. Immediately proceed to Section V.C.

C. Protocol: One-Step First-Strand cDNA Synthesis and Double-Stranded cDNA Amplification

First-strand cDNA synthesis (from total RNA or cells) is primed by the 3′ SMART-Seq CDS Primer II A and uses the SMART-Seq HT Oligonucleotide for template switching at the 5′ end of the transcript. **If you did not include the 3′ SMART-Seq CDS Primer II A in the sorting buffer (e.g., if you sorted your**

cells in Plain Sorting Solution [PSS] or PBS; see Appendix A), you will need to add it to your samples in the optional Step 2 below.

IMPORTANT: To avoid introducing contaminants into your samples, the first part of the cDNA synthesis protocol requires the use of a PCR clean workstation, ideally in a clean room.

1. At room temperature, thaw the One-Step Buffer. On ice, thaw all the remaining reagents (except the enzymes) needed for first-strand cDNA synthesis: Nuclease-Free Water, SMART-Seq HT Oligonucleotide, RNase Inhibitor, and 3′ SMART-Seq CDS Primer II A (if needed). Gently vortex each reagent to mix and spin down briefly. Store all reagents on ice except the One-Step Buffer.

   NOTE: The One-Step Buffer may not be frozen solid at –20°C.

2. **(Optional)** If starting from sorted cells, take out the plate or PCR strips from the freezer and briefly spin to collect the contents at the bottom of the tubes.

   CAUTION: If you did not include the 3′ SMART-Seq CDS Primer II A in the sorting buffer, add 1 µl of the 3′ SMART-Seq CDS Primer II A to each sample. Keep samples cold during this step.

3. Incubate the samples (either from Step 2 or Section V.B) at 72°C in a preheated thermal cycler with a heated lid for 3 min.

   NOTE: Prepare One-Step Master Mix (Step 4) while your samples are incubating. Enzymes should be added just before use (Step 6). Steps 7 and 8 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 6.

4. Prepare enough One-Step Master Mix for all the reactions, plus 10% of the total reaction mix volume, by combining the following reagents in the order shown at room temperature.

   NOTE: For SeqAmp DNA Polymerase, it is critical to pipette the exact volume specified. Great care must be taken to avoid using a volume larger than specified. For that reason, it is recommended to prepare the master mix for **12 or more samples**.

   One-Step Master Mix:

0.7 µl	Nuclease-Free Water
8 µl	One-Step Buffer
1 µl	SMART-Seq HT Oligonucleotide
0.5 µl	RNase Inhibitor
0.3 µl	SeqAmp DNA Polymerase
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
12.5 µl	**Total volume per reaction**

   Mix well by gentle vortexing and then spin the tube(s)/plate briefly to collect the contents at the bottom of the tube/plate.

5. Immediately after the 3-min incubation at 72°C, place the samples on ice for at least 2 min (but no more than 10 min).
6. Preheat the thermal cycler to 42°C.
7. Add the SMARTScribe Reverse Transcriptase and SeqAmp DNA Polymerase to the One-Step Master Mix.

   NOTE: Add just prior to use. Mix well by gently vortexing, and then spin the tube briefly in a minicentrifuge to collect the contents at the bottom of the tube.

8. Add 12.5 µl of the One-Step Master Mix to each sample. Mix the contents of the tubes/plate by gently vortexing and spin briefly to collect the contents at the bottom of the tubes. Apply an adhesive seal to the plate or cap strips to the 8-tube strips.
9. Place the plate/tubes in a thermal cycler with a heated lid, preheated to 42°C. Run this program:

42°C	90 min
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

*Please consult Tables 2 and 3 below for the recommended number of PCR cycles.

Table 2. General recommendations for the number of PCR cycles for cell lines and purified RNA

Input amount of total RNA	Input amount of cells	Recommended PCR cycles*
1 ng	100 cells	10–11
100 pg	10 cells	14–15
10 pg	1 cell	17–19

*We do not recommend going above 20 cycles.

Table 3. Recommended number of PCR cycles for single cells.

Sample type	Approximate RNA content	Recommended PCR cycles*
K562/HEK293	10 pg	17–18
Jurkat	5 pg	18–19
B or T cells	2 pg	20
PBMCs	1 pg	20

*We do not recommend going above 20 cycles.

SAFE STOPPING POINT: The samples may be stored at 4°C overnight or –20°C for a more extended period until the next step, Section V.D (below).

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.

IMPORTANT: Do not pool the samples at the cDNA purification step. If pooling is desired, it can be performed during library preparation (Section VI).

NOTES:

- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads and the Elution Buffer 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 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 25 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), the cDNA samples need to be transferred to a 96-well V-bottom plate. Distribute 25 µl of beads to each well of the 96-well V-bottom plate, 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 cDNA bind to the beads.
3. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. (Centrifugation is generally not necessary if using a 96-well V-bottom plate as described in Step 1).
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 tube 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 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 dry, remove the samples from the magnetic 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 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 containing purified cDNA from each tube/well to new tube/plate. Do not pool samples at this point. Take care not to carry over any beads with your samples.

14. Proceed to validation immediately or store at –20°C.

E. Protocol: Determination of cDNA Quality and Quantity

Determine cDNA Quality

It is recommended to evaluate the profile of the cDNA using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626), or the Fragment Analyzer and High Sensitivity Large Fragment Analysis Kit (Agilent, Cat. No. DNF-464). The cDNA profile obtained on a TapeStation Automated Electrophoresis Systems using a High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067-5592) is not as informative as the other two options, but can still deliver usable information, including cDNA yield (see Appendix B for more details). The cDNA profile obtained on a LabChip (PerkinElmer) instrument is typically unsatisfactory and difficult to interpret due to the lack of sensitivity. Evaluation of the cDNA profile is particularly important when performing initial optimization.

When evaluating cDNA quality, use 1 µl of each amplified cDNA for validation using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (see the Agilent High Sensitivity DNA Kit User Manual for instructions). Successful cDNA synthesis and amplification should yield a distinct peak spanning 400–10,000 bp, with a summit at around 2,500 bp for the positive control RNA sample (Figure 3, Panel A). Profiles obtained from cells or purified total RNA of lower quality than the control RNA provided in the kit may yield profiles that are more variable, sometimes with a peak slightly smaller (around 2,000 bp; Figure 3, Panel B). In any case, the negative control should be totally flat with no product visible (Figure 3, Panel C), although a very small amount of product between 100–300 bp may be occasionally visible (Figure 3, Panel D).

For quantification, perform smear analysis using a 300–9,000 bp range.

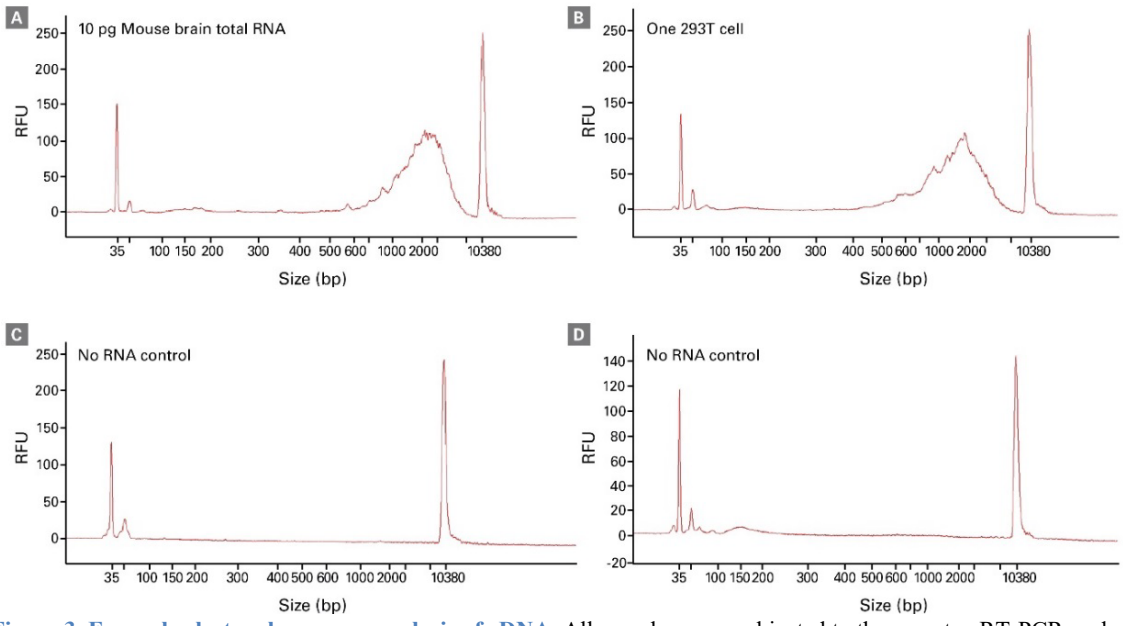

Figure 3. Example electropherogram analysis of cDNA. All samples were subjected to the one-step RT-PCR as described in the protocol, using 17 PCR cycles. After bead purification, 1 µl of cDNA was analyzed using Agilent's High Sensitivity DNA Kit and an Agilent 2100 Bioanalyzer. Panel A. Example produced from 10 pg of mouse brain total RNA control included in the kit, showing a peak around 2,500 bp. Panel B. Example generated from one 293T cell (isolated using FACS). Note the smaller size of the peak around 2,000 bp compared to the example in Panel A, suggesting a small amount of RNA degradation occurring during the process of cell sorting. Panels C and D. Examples generated from no RNA controls, showing either a totally flat profile (Panel C) or a small number of unspecific products between 100–300 bp (Panel D). Similar low molecular weight products can also be detected in the positive samples and do not affect the quality of the sequencing data.

Determine cDNA Quantity

cDNA concentrations obtained with SMART-Seq mRNA HT may vary between different cell types and treatments. To minimize library prep variation and to achieve high sequencing library quality, the concentration of the amplified cDNAs must be carefully determined. Several options are available for quantification. We recommend using Agilent's High Sensitivity DNA Kit for reliable double-stranded (ds) cDNA quantification. Select the region encompassing 400–9,000 bp to estimate the concentration of ds cDNA.

For processing 96 or more samples, the Quant-iT PicoGreen dsDNA Assay Kit (PicoGreen) is a very sensitive tool that can be fast and convenient (see below). However, if using either of these methods, we suggest also performing cDNA quantitation for a subset of the samples on the Bioanalyzer for more accurate results. Another option is to quantify the cDNA using Qubit dsDNA HS Assay (Qubit). Refer to the manufacturer's instructions for information on how to use the PicoGreen or Qubit assay reagents.

When using the Qubit or PicoGreen assay for quantification, the negative control may generate an apparent yield up to 200 pg/µl (for 20 cycles of PCR), even if the same sample run on the Bioanalyzer appears flat, because low molecular weight primers or primer dimers are detected. A higher number of cycles generates a higher background. For this reason, it is critical to include negative controls that can be used to subtract the background and get a more accurate quantification of the cDNA yield.

1. If using PicoGreen or Qubit assay reagents for quantification, refer to the manufacturer's instructions for information. Remember that it is critical to subtract the value of the negative control to obtain an accurate quantification.
2. If cDNA quantity was measured, compare the results for your samples and controls (see Figure 3, above) to verify whether the sample is suitable for further processing.
3. Proceed to Library Preparation for Illumina Sequencing (Section VI).

VI. Library Preparation for Illumina Sequencing

For generation of Illumina-compatible libraries we recommend using the SMART-Seq Library Prep Kit, sold as part of SMART-Seq mRNA HT LP (Cat. Nos. 634792, 634793 & 634794). Refer to the note in the Introduction (Section I) of this user manual for more details. For more information about SMART-Seq mRNA HT LP, please refer to the product page on the takarabio.com website.

VII. References

Chenchik, A., Zhu, Y. Y., Diatchenko, L., Li, R. & Hill, J. Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. in *Gene cloning Anal. by RT-PCR* (Eds. Siebert, P. D. & Larrick, J. W.) 305–319 (Eaton Pub Co, 1998).

Appendix A: Alternative FACS Sorting Recommendations

When sorting the cells, we strongly recommend including the oligo required for priming the reverse transcription (3′ SMART-Seq CDS Primer II A) in the sorting buffer (see CDS Sorting Solution described in Section V.A). This eliminates a pipetting step when setting up the reverse transcription. However, this is not an absolute requirement for successful cDNA synthesis. For recommendations on sorting in a buffer not containing the 3′ SMART-Seq CDS Primer II A, proceed to Section A, below. Alternatively, sort cells in a maximum volume of 5 µl PBS. However, PBS is *not* an optimal sorting buffer as it can interfere with the efficiency of the one-step RT-PCR reaction. For recommendations on how to proceed after sorting into ≤5 µl of PBS, see Section B, below.

A. FACS Sorting into a Buffer Not Containing CDS Primer II A

In this protocol, cells are sorted in 11.5 µl of buffer.

1. Prepare enough sorting buffer to prefill the 96-well plate or PCR strips as indicated below.

   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 PSS mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 11.5 µl per well.

   - Due to small pipetting volumes, prepare no less than 230 µl of sorting buffer, which is enough for 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

   Plain Sorting Solution (PSS; without 3′ SMART-Seq CDS Primer II A):

	Per well	1–18 wells*
10X Lysis Buffer	0.95 µl	19 µl
RNase Inhibitor	0.05 µl	1 µl
Nuclease-Free Water	10.5 µl	210 µl
Total volume	11.5 µl	230 µl

   *Volumes include ~10% extra for overage.

   Mix briefly, 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.

2. Aliquot 11.5 µl of PSS 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 multi-channel pipettes to 11.6 µl and pipette only to the first stop when aliquoting. Changing tips often also minimizes bubble formation.

3. Seal the plate/tube strips and briefly spin to ensure the sorting buffer collects at the bottom of the wells.
4. 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:

5. Unseal the prepared plate/tube strips and sort cells into the sorting solution according to the FACS system manual and desired parameters.
6. 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 aluminum foil, but are not practical when sorting a large number of samples.

7. Immediately after sorting the cells and sealing the plate, spin briefly to collect the cells at the bottom of each well in the PSS.

8. 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.

9. 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 3′ SMART-Seq CDS Primer II A, you need to add it when you thaw your samples—see Section V.C, Step 2.

B. FACS Sorting into Nonvalidated Buffers

Sorting into CSS (CDS Sorting Solution) or PSS (Plain Sorting Solution) 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 Control Total RNA and the estimated amount of buffer that you expect to accompany your cell(s) 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 may be lower, and you should consider adding one to three extra PCR cycles to the recommended number of cycles in Table 3 (Section V.B). 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 CDS 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.C for preparing a One-Step Master Mix, setting up control reactions, and adding 3′ SMART-Seq CDS Primer II A.

Appendix B: PCR Optimization

If you have a sufficient amount of starting material (>1 ng total RNA), we recommend optimizing the PCR cycling parameters 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 cycles ensures that the amplification will remain in the exponential phase. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA can result in a less representative cDNA library. Undercycling, on the other hand, 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.

To perform PCR cycle optimization, prepare several tubes containing an amount of RNA equal to your sample amount. Subject each tube to a different range of PCR 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., 12, 10, and 8 cycles, *N*) for a 1 ng sample.

1. 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

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

Appendix C: Expected Results When Analyzing cDNA with TapeStation

The Agilent 2100 Bioanalyzer used with the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) offers the best option for visualization of cDNA profiles generated with the SMART-Seq HT Kit. Another good option, particularly for processing a large number of samples, is the Fragment Analyzer and High Sensitivity Large Fragment Analysis Kit (Agilent, Cat. No. DNF-464).

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 to ~2,500 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. The SMART-Seq HT Kit should generate cDNA yields higher than 200 pg/µl and the cDNA profile should be detected relatively easily.

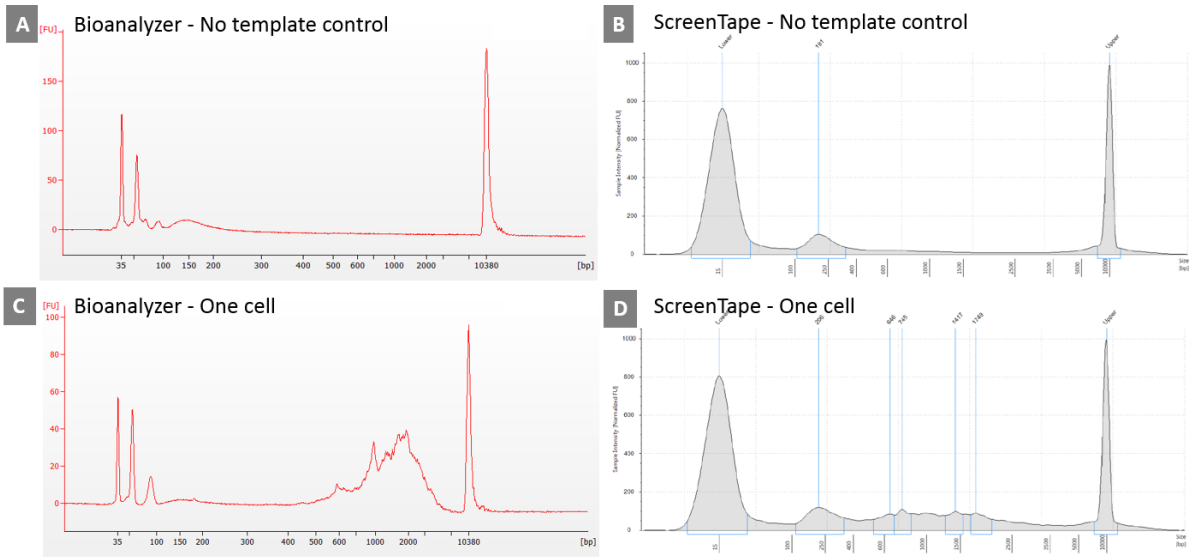

Figure 4. Comparison of electropherogram results from Agilent 2100 Bioanalyzer and TapeStation Automated Electrophoresis Systems. All samples were subjected to the one-step RT-PCR as described in the protocol, using 18 PCR cycles. After bead purification, 1 µl of the sample was analyzed using the Agilent's Bioanalyzer High Sensitivity DNA Kit (**Panels A and C**) or the High Sensitivity D5000 ScreenTape (**Panels B and D**).

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