

Takara Bio USA, Inc.

# SMART-Seq® HT PLUS Kit User Manual

Cat. Nos. R400748 & R400749

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**Takara Bio USA, Inc.**

1290 Terra Bella Avenue, Mountain View, CA 94043, USA

U.S. Technical Support: [techUS@takarabio.com](mailto:techUS@takarabio.com)

United States/Canada  
800.662.2566

Asia Pacific  
+1.650.919.7300

Europe  
+33.(0)1.3904.6880

Japan  
+81.(0)77.565.6999

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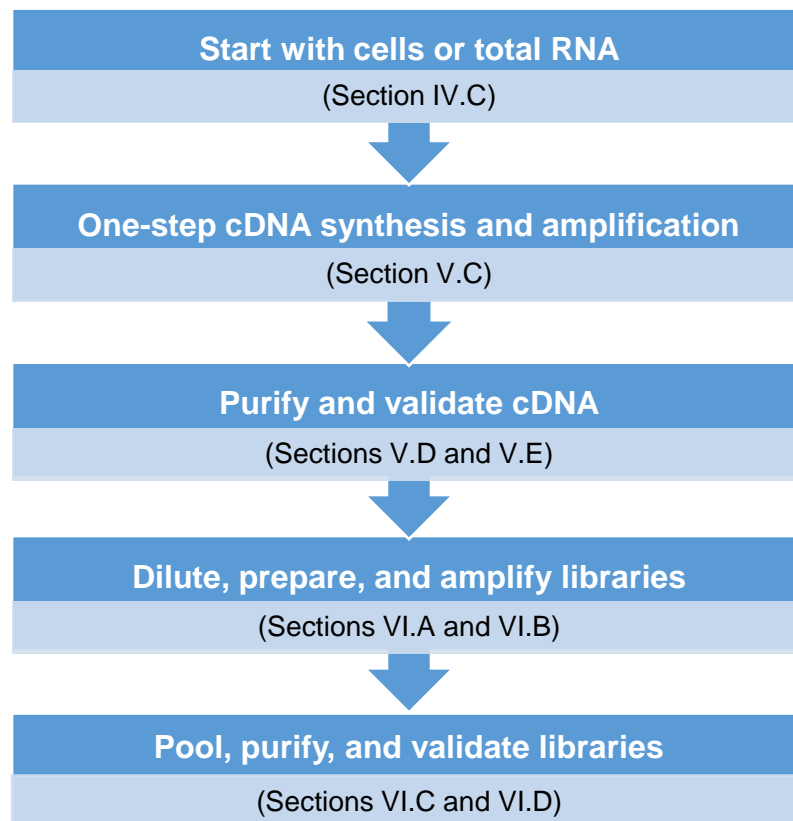
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## I. Introduction

The **SMART-Seq HT PLUS Kit** (Cat. Nos. R400748 and R400749) is designed to generate high-quality, Illumina® sequencing-ready libraries directly from 1–100 intact cells or ultra-low amounts of total RNA (10 pg–1 ng). The cDNA synthesis protocol can be completed in four hours, and the library can be generated in an easy two-step protocol within two hours. (Figure 1).



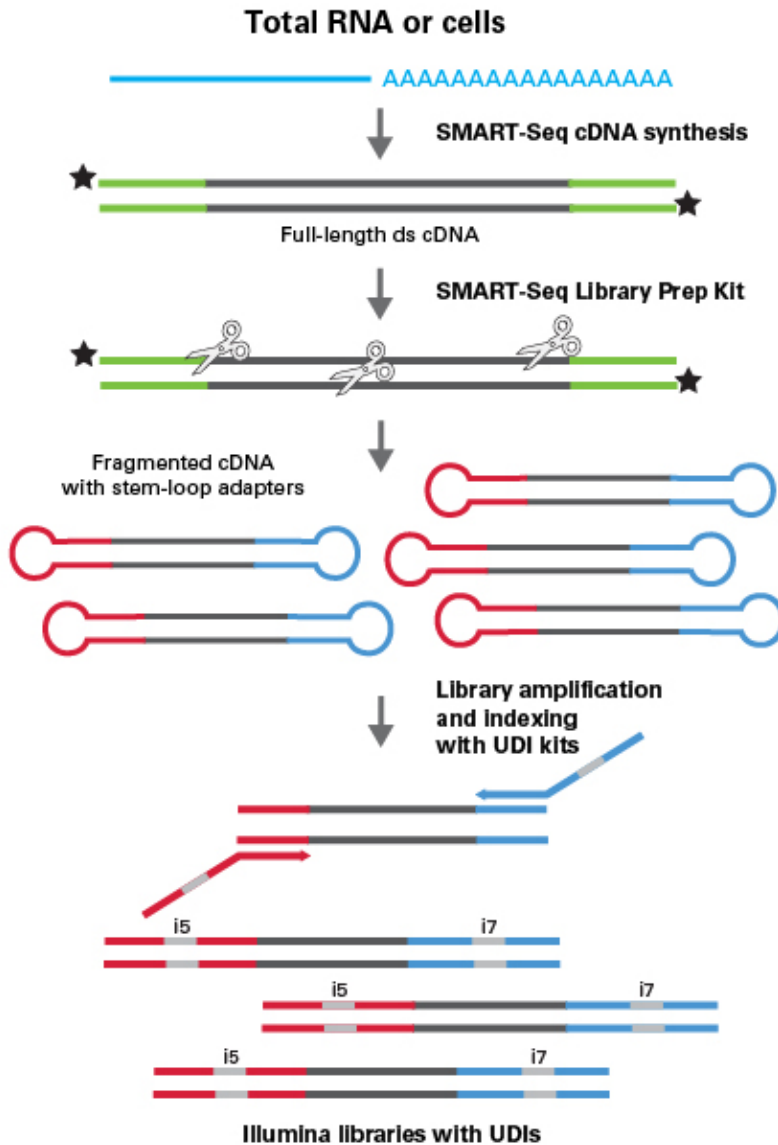
**Figure 1. SMART-Seq HT PLUS Kit protocol overview.** This kit features a streamlined workflow that generates Illumina sequencing libraries with minimal hands-on time. While the workflow is primarily designed to accommodate cells isolated using FACS, it can also be used with purified total RNA.

The SMART-Seq HT PLUS Kit incorporates our patented SMART® (Switching Mechanism at 5' End of RNA Template) 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 and unbiased amplification of cDNA transcripts, and it allows direct cDNA synthesis from intact cells.

The SMART-Seq HT PLUS Kit is designed to reduce handling time for greater ease of use without compromising on performance. An advantage of SMART-Seq HT cDNA library generation is that it 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.

Once full-length cDNA is synthesized, libraries are prepared through an enzymatic fragmentation method, followed by library amplification and indexing of sequencing libraries, allowing multiplexing of up to 96 samples

using unique dual indexes (Figure 2). Sequencing library preparation from full-length cDNA incorporates Takara Bio’s patented library preparation chemistry. Unlike other sequencing library preparation kits, which are based on ligation of Y-adapters, our technology uses stem-loop adapters to construct high-quality libraries. The workflow (fragmentation, repair, ligation, amplification, and indexing) takes place in a single tube in about 2 hours. No intermediate purification steps or sample transfers are necessary, which minimizes handling errors (such as sample mix up) and sample loss.



**Figure 2. Schematic of the SMART-Seq HT PLUS Kit library preparation workflow.** Double-stranded cDNA generated using SMART-Seq HT chemistry is enzymatically fragmented and stem-loop adapters ligated in a single step. Libraries are then amplified and indexed, generating Illumina-compatible libraries with unique dual indexes (UDIs; light gray).

Once purified and quantified, the resulting libraries are ready for Illumina NGS instruments using standard Illumina sequencing reagents and protocols.

## II. List of Components

The SMART-Seq HT PLUS Kit contains sufficient reagents to prepare up to 48 (Cat. No. R400748) or 96 (Cat. No. R400749) reactions. Freeze-thaw of reagents should be limited to  $\leq 6$  times. The components in the SMART-Seq HT PLUS Kit 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 modification of the protocol may lead to unexpected results.

SMART-Seq HT PLUS Kit		R400748 (48 rxns)	R400749 (96 rxns)
SMART-Seq HT Kit	Cap color and label	634456	634437
<b>Box 1 (store at <math>-70^{\circ}\text{C}</math>)</b>			
Control Total RNA (1 $\mu\text{g}/\mu\text{l}$ )*	-	5 $\mu\text{l}$	5 $\mu\text{l}$
<b>Box 2 (store at <math>-20^{\circ}\text{C}</math>)</b>			
SMART-Seq HT Oligonucleotide	Pink, HT Oligo	48 $\mu\text{l}$	96 $\mu\text{l}$
3' SMART-Seq CDS Primer II A	Blue, CDS IIA	96 $\mu\text{l}$	192 $\mu\text{l}$
One-Step Buffer	Orange, 1-Step Buffer	500 $\mu\text{l}$	1 ml
SMARTScribe™ Reverse Transcriptase (100 U/ $\mu\text{l}$ )	Purple, SMARTScribe	96 $\mu\text{l}$	192 $\mu\text{l}$
SeqAmp™ DNA Polymerase	Green, SeqAmp	20 $\mu\text{l}$	50 $\mu\text{l}$
RNase Inhibitor (40 U/ $\mu\text{l}$ )	White, RRI	75 $\mu\text{l}$	150 $\mu\text{l}$
10X Lysis Buffer	Neutral/-†	920 $\mu\text{l}$	1.85 ml
Elution Buffer (10 mM)	-	2 x 1.7 ml	6.8 ml
Nuclease-Free Water	-†	2 x 1 ml	4 ml
<b>SMART-Seq Library Prep Kit (not sold separately; store at <math>-20^{\circ}\text{C}</math>)</b>		<b>R400746</b>	<b>R400747</b>
10X FE	White	45 $\mu\text{l}$	60 $\mu\text{l}$
FE Dilution Buffer	White	500 $\mu\text{l}$	1 ml
Lib Prep Buffer	Blue	240 $\mu\text{l}$	480 $\mu\text{l}$
Lib Prep Enzyme	Blue	140 $\mu\text{l}$	280 $\mu\text{l}$
Rxn Enhancer	Red	200 $\mu\text{l}$	400 $\mu\text{l}$
Adapters	Yellow	120 $\mu\text{l}$	240 $\mu\text{l}$
Amplification Buffer	Violet	1.2 ml	2 x 1.2 ml
PrimeSTAR® HS DNA Polymerase (5 U/ $\mu\text{l}$ )	Green	60 $\mu\text{l}$	120 $\mu\text{l}$
Nuclease-Free Water	Neutral	2 x 1 ml	4 x 1 ml
<b>Unique dual index kit - 48U or 96U (store at <math>-20^{\circ}\text{C}</math>)‡</b>		<b>R400744</b>	<b>R400745</b>
Dual-indexed primers in 96-well plate	-	48 indexes	96 indexes

\*The Control Total RNA is from mouse brain.

†Depending on the product size, these components may be packaged in a 1.5-ml tube with the indicated cap color or in an 8-ml bottle.

‡See Appendix C for important information about using these kits.

## Storage Conditions

- Store Control Total RNA at  $-70^{\circ}\text{C}$ .
- Store components in Box 2 of the SMART-Seq HT Kit at  $-20^{\circ}\text{C}$ . Once thawed, 10X Lysis Buffer may be stored at  $4^{\circ}\text{C}$  and Elution Buffer may be stored at room temperature. Continue to store all other reagents at  $-20^{\circ}\text{C}$ .
- Store components of the SMART-Seq Library Prep Kit at  $-20^{\circ}\text{C}$ . Once thawed, FE Dilution Buffer may be stored at  $4^{\circ}\text{C}$  and Nuclease-Free Water may be stored at room temperature. Continue to store all other reagents at  $-20^{\circ}\text{C}$ .

## 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  $\mu\text{l}$ , 20  $\mu\text{l}$ , 200  $\mu\text{l}$ , and 1,000  $\mu\text{l}$
- Eight-channel or twelve-channel pipette (recommended): 20  $\mu\text{l}$  and 200  $\mu\text{l}$
- Filter pipette tips: 2  $\mu\text{l}$ , 20  $\mu\text{l}$ , 200  $\mu\text{l}$ , and 1,000  $\mu\text{l}$
- 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
- Microplate film (USA Scientific, Cat. No. 2920-0010) for sealing tubes/plates before sorting
- Aluminum single tab foil seal (USA Scientific, Cat. No. 2938-4100) or cap strips (Thermo Fisher Scientific, Cat. No. AB0784/AB0850) for sealing tubes/plates after sorting
- Low-speed benchtop centrifuge for 96-well plates or tube strips
- Dry ice in a suitable container for flash freezing cells
- **(Optional)** BD FACS Pre-Sort Buffer (BD Biosciences, Cat. No. 563503)
- **(Optional)** SMART-Seq HT Kit Lysis Components (Cat. No. 634439) or 10X Lysis Buffer (Takara Bio, Cat. No. 635013) for sorting extra plates

### For One-Step RT-PCR

- 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  $105^{\circ}\text{C}$ . 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 (available from 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 above NucleoMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter; 5-ml size: Cat. No. A63880; 60-ml size: Cat. No. A63881) is an appropriate substitute

#### **NOTES:**

- The kit has been specifically validated with the beads listed above. Please do not make any substitutions as it may lead to unexpected results.
  - Beads need to come to room temperature before the container is opened. We strongly recommend aliquoting the beads into 1.5-ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). Aliquoting is also instrumental in decreasing the chances of bead contamination.
  - Immediately before use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.
- 80% ethanol: freshly made for each experiment from molecular-biology-grade 100% ethanol
  - Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates, such as:
    - For 12–24 samples:
      - SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011); accommodates two 8-tube or 12-tube strips
      - Minicentrifuge for 0.2-ml tubes or strips
    - For 24–96 or more samples:
      - Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher, Cat. No. AB0558)
      - Low-speed benchtop centrifuge for a 96-well plate
    - For 1.5-ml tubes (for pooling sequencing libraries before purification):
      - 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 and Illumina Library Quantification (As Required)**

- High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) for Bioanalyzer or equivalent high-sensitivity electrophoresis method (may be used in Sections V.E and VI.D)
- Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Cat. No. P11496) or Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854) (may be used in Sections V.E and VI.D)
- Library Quantification Kit (Takara Bio, Cat. No. 638324) (may be used in Section VI.D)
- Nuclease-free, PCR-grade, thin-wall PCR strips (0.2-ml PCR 8-tube strip; 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

### For Illumina Library Preparation

- 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
- Benchtop cooler, such as VWR CryoCoolers (VWR, Cat. No. 414004-286)

## 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) and prepare the sequencing libraries (Section VI).

### B. General Requirements

- 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

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

#### Cell Inputs

- We recommend processing no less than 12 reactions to ensure sufficient reagent quantities for all the intended reactions within the kit format.
- 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<sup>2+</sup>- and Ca<sup>2+</sup>-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  $\mu$ l 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  $\mu$ l.**
- This protocol has been optimized for one-step RT-PCR synthesis starting from 10 pg of total RNA. 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  $\geq 100$  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 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  $-80^{\circ}\text{C}$ . 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. For example, 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. cDNA Synthesis

The SMART-Seq HT PLUS Kit 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 Buffer Containing CSS

This section provides guidance for sorting cells directly into 12.5 µl of a buffer containing the CDS II A Oligo, 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 sorting buffer to prefill the 96-well plate or PCR strips, as indicated below. Due to small pipetting volumes, prepare no less than 250 µl of sorting buffer, which is enough for 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 12.5 µl per well.

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

	Per well	18 wells*	48 wells*	96 wells*
<b>10X Lysis Buffer</b>	0.95 µl	19 µl	52.25 µl	104.5 µl
<b>RNase Inhibitor</b>	0.05 µl	1 µl	2.75 µl	5.5 µl
<b>3' SMART-Seq CDS Primer II A</b>	1 µl	20 µl	55 µl	110.0 µl
<b>Nuclease-Free Water</b>	10.5 µl	210 µl	577.5 µl	1,155.0 µl
<b>Total volume</b>	12.5 µl	250 µl	687.5 µl	1,375.0 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  $-20^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  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 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):

19 $\mu\text{l}$	10X Lysis Buffer
1 $\mu\text{l}$	RNase Inhibitor
20 $\mu\text{l}$	Total volume

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  $\mu\text{l}$  of purified total RNA to a nuclease-free 96-well PCR plate or tube strips. If necessary, bring the total volume to 10.5  $\mu\text{l}$  with Nuclease-Free Water. Add 1  $\mu\text{l}$  of 10X Reaction Buffer to each sample.

Table 1. Sample preparation guidelines

Components	Negative control	Positive control	Experimental sample
10X Reaction Buffer	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
Nuclease-Free Water	10.5 $\mu$ l	Up to 9.5 $\mu$ l	Up to 9.5 $\mu$ l
Diluted Control RNA*	–	1–10.5 $\mu$ l	–
Sample	–	–	1–10.5 $\mu$ l
<b>Total volume</b>	11.5 $\mu$ l	11.5 $\mu$ l	11.5 $\mu$ l

\*The Control Total RNA is supplied at a concentration of 1  $\mu$ g/ $\mu$ l. It should be diluted in Nuclease-Free Water with RNase Inhibitor (1  $\mu$ l of RNase Inhibitor in a final volume of 50  $\mu$ l of water) to match the concentration of your test sample (see “Diluting the Control RNA” under Section IV.D).

- Place the samples on ice and add 1  $\mu$ l 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.
- 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.

- 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  $-20^{\circ}\text{C}$ .

- (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  $\mu$ l of the 3' SMART-Seq CDS Primer II A to each sample. Keep samples cold during this step.

- Incubate the samples from Section V.A or Section V.B at  $72^{\circ}\text{C}$  in a preheated thermal cycler with a heated lid for 3 minutes. Then, immediately place the samples on ice for 2 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.

- Prepare enough One-Step Master Mix for all the reactions.\* Mix well by gentle vortexing and then spin the tube(s)/plate briefly to collect the contents at the bottom of the tube/plate:

**One-Step Master Mix:**

	1 rxn	12 rxns <sup>†</sup>	48 rxns <sup>†</sup>	96 rxns <sup>†</sup>
<b>Nuclease-Free Water</b>	0.7 µl	9.2 µl	37.1 µl	74.2 µl
<b>One-Step Buffer</b>	8 µl	105.6 µl	424 µl	848 µl
<b>SMART-Seq HT Oligonucleotide</b>	1 µl	13.2 µl	53 µl	106 µl
<b>RNase Inhibitor</b>	0.5 µl	6.6 µl	26.5 µl	53 µl
<b>SeqAmp DNA Polymerase</b>	0.3 µl	4 µl	15.9 µl	31.8 µl
<b>SMARTScribe Reverse Transcriptase (100 U/µl)</b>	2 µl	26.4 µl	106 µl	212 µl
<b>Total volume</b>	12.5 µl	165 µl	662.5 µl	1,325 µl

\*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.

†Volumes include ~10% extra for overage.

- Preheat the thermal cycler to 42°C.
- Add the SMARTScribe Reverse Transcriptase and SeqAmp DNA Polymerase to the One-Step Master Mix. Mix well by gently vortexing and then spin briefly to collect the contents at the bottom of the tube.
- 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.
- 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
  - X 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.

**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).

**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

**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.

**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:**

- Aliquot beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 minutes and mix well by vortexing. Use room-temperature Elution Buffer for this protocol.
- Beads:sample ratio is 1:1.
- Prepare fresh 80% ethanol for each experiment. You 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 only be performed in Section VI.C.

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 3.
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 3.
3. Incubate the beads-cDNA mixture at room temperature for 8 min to let the cDNA bind to the beads.
4. 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 2).
5. 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.
6. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.
7. 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.
8. Repeat the ethanol wash (Step 7) once more.
9. 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.
10. Incubate the samples at room temperature for ~2–2.5 min until the pellet is no longer shiny, but before cracks appear.

**NOTES:**

- Be sure to dry the pellet only until it is just dry. The pellet should look matte with no shine.
- If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol might reduce the amplified cDNA recovery rate and ultimately the cDNA yield. Allow the plate to sit at room temperature until the pellet is dry.

- If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 min to rehydrate (Step 12) and may reduce amplified cDNA recovery and yield.
11. Once the beads are dry, remove the samples from the magnetic device and add 17  $\mu$ l of Elution Buffer to cover the bead pellet. Mix thoroughly by pipetting or gently vortexing to resuspend the beads.
  12. Incubate at room temperature for 2 min to rehydrate.
  13. 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.
  14. 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.
  15. Proceed to validation immediately or store at  $-20^{\circ}\text{C}$ .

## E. Protocol: Determination of cDNA Quality and Quantity

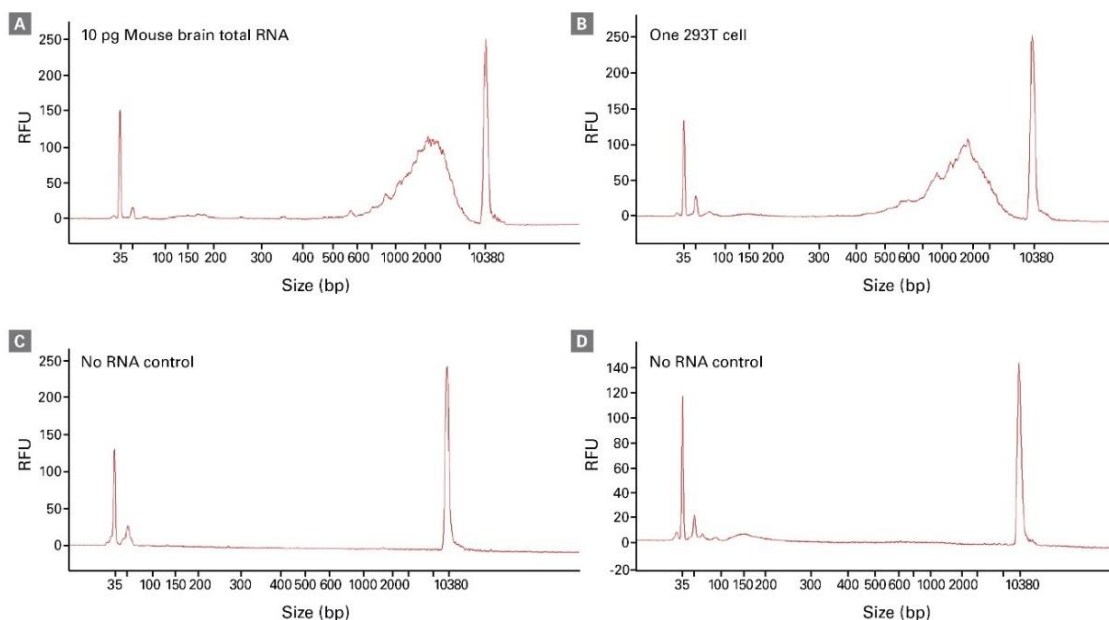
### Determine cDNA Quality

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 (Advanced Analytical Technologies, Inc., Cat. No. DNF-493). 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  $\mu$ l 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  $\mu$ l 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 the SMART-Seq HT PLUS Kit 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. 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). 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 100 pg/ $\mu$ l (for 17 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 quality 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

Components from the SMART-Seq Library Prep Kit are used in Sections VI.A & B, and the Unique dual index kit - 48U or 96U is used in Section VI.B. Before starting, please read the entire set of protocols in Section VI as well as Appendix C for other documentation on the unique dual index kit that contain instructions for use, a plate map, and barcode sequences.

**NOTE:** If all samples are correctly quantified and normalized to a uniform input amount before library preparation (Section VI.A, Step 4), sequencing libraries can be pooled after amplification and before cleanup (in Section VI.C), and a relatively uniform amount of sequencing reads will be obtained.

### A. Protocol: cDNA Dilution and Library Preparation

1. If performing library preparation (Section VI.A) and amplification (Section IV.B) on the same day, thaw the following items on ice:
  - Unique dual index kit - 48U or 96U
  - SMART-Seq Library Prep Kit components: FE Dilution Buffer, Lib Prep Buffer, Rxn Enhancer, Adapters, and Amplification Buffer
  - SMART-Seq HT Kit: Elution Buffer

Keep the 10X FE, Library Prep Enzyme, and PrimeSTAR HS DNA Polymerase (5 U/ $\mu$ l) in the  $-20^{\circ}\text{C}$  freezer until used. Use a benchtop cooler to keep the enzymes cold while working with them at the bench.

**NOTE:** If stopping at the end of Section VI.A, keep the Unique dual index kit - 48U or 96U, Amplification Buffer, and PrimeSTAR HS DNA Polymerase (5 U/ $\mu$ l) in the  $-20^{\circ}\text{C}$  freezer until ready to begin Section VI.B.

2. Mix all components by gentle vortexing, and then spin down the tubes briefly to collect the contents at the bottom of the tubes. Keep on ice.
3. On ice, add 2  $\mu$ l of Adapters to each tube/well of PCR tubes, 8-tube PCR strips, or a 96-well PCR plate, according to the number of reactions to be performed.
4. Add a total of 10  $\mu$ l of a freshly diluted cDNA sample or Elution Buffer as a negative control to a tube/well containing the 2  $\mu$ l of Adapters (Step 3); the total volume per reaction should be 12  $\mu$ l. Calculate the amounts of cDNA and diluent (Elution Buffer, a component of the SMART-Seq HT Kit) needed for each sample to achieve a fixed concentration (one value between 0.1 ng/ $\mu$ l and 1 ng/ $\mu$ l) for all samples. Add the cDNA and diluent sequentially (or negative control only) to the tubes containing the adapters. Use the following notes as guidelines.

#### NOTES:

- Keep in mind that the number of PCR cycles performed during library amplification (Section IV.B, Step 4) depends on the input cDNA amount. Refer to Table 4 for guidelines that may inform cDNA dilution in the current step.
- Always use a minimum of 2  $\mu$ l of cDNA to perform dilution.
- Samples containing less than 100 pg/ $\mu$ l can still be used without dilution, but you may get fewer reads than for other samples if you pool for cleanup (Section VI.C).
- We recommend using 10  $\mu$ l of Elution Buffer as the negative control. Alternatively, you can use the negative control produced during cDNA synthesis (Section V). Negative controls should be used without dilution.

5. On ice, prepare the 1X FE by diluting the 10X FE in the cold (~4°C) FE Dilution Buffer, as described below. Mix gently by pipetting up and down 10 times. Spin down and keep on ice. Immediately proceed with assembling the library preparation master mix in Step 6.

	1–36 rxns	48 rxns*	96 rxns*
<b>FE Dilution Buffer</b>	36 µl	54 µl	99 µl
<b>10X FE</b>	4 µl	6 µl	11 µl
<b>Total volume</b>	40 µl	60 µl	110 µl

\*Volumes include ~10% extra for overage.

6. On ice, prepare the Library Prep Master Mix for all reactions. Combine the following reagents in the order shown below.

**Library Prep Master Mix:**

	1 rxn	12 rxns*	48 rxns*	96 rxns*
<b>Library Prep Buffer</b>	4 µl	52.8 µl	211.2 µl	422.4 µl
<b>Rxn Enhancer</b>	3.5 µl	46.2 µl	184.8 µl	369.6 µl
<b>Library Prep Enzyme</b>	2 µl	26.4 µl	105.6 µl	211.2 µl
<b>1X FE</b>	1 µl	13.2 µl	52.8 µl	105.6 µl
<b>Total volume</b>	10.5 µl	138.6 µl	554.4 µl	1,108.8 µl

\*Volumes include ~10% extra for overage.

**NOTES:**

- **Library Prep Master Mix is very viscous.** Ensure adequate mixing by vortexing for 5 sec, and then spin the tubes briefly to collect the contents at the bottom of the tubes/plate. If necessary, vortex for an additional 5 sec and spin down again. Keep on ice.
- Discard leftover 1X FE. Do not reuse.

7. On ice, assemble the library preparation reaction. To each tube/well containing 12 µl cDNA/Adapters mix or negative control/Adapters mix from Step 4, add 10.5 µl of the Library Prep Master Mix prepared in Step 6. Mix by vortexing for 5 sec, and then spin the tubes briefly to collect the contents at the bottom of the tubes/wells.

**NOTES:**

- During this step, keep the samples cold (4°C) until placed in the thermal cycler.
- Do not create a master mix with the Adapters and the Library Prep Master Mix.

8. Perform the library preparation reaction by placing the tubes/plate in a precooled thermal cycler and running the following program:

20°C 40 min

85°C 10 min

4°C Hold

Proceed to library amplification, Section VI.B.

**SAFE STOPPING POINT:** The samples can be stored in the thermal cycler at 4°C overnight, or they can be transferred to –20°C for up to a week.

## B. Protocol: Library Amplification

- If the samples were stored at  $-20^{\circ}\text{C}$ , thaw the samples and the following items on ice:
  - Unique dual index kit - 48U or 96U
  - Amplification Buffer from the SMART-Seq Library Prep Kit

Keep the PrimeSTAR HS DNA Polymerase (5 U/ $\mu\text{l}$ ) in the  $-20^{\circ}\text{C}$  freezer until used. Use a benchtop cooler to keep the enzyme cold while working with it at the bench.

- On ice, prepare the Library Amplification Master Mix for all reactions.

### Library Amplification Master Mix:

	1 rxn	12 rxns*	48 rxns*	96 rxns*
<b>Amplification Buffer</b>	21.5 $\mu\text{l}$	283.8 $\mu\text{l}$	1,135.2 $\mu\text{l}$	2,270.4 $\mu\text{l}$
<b>PrimeSTAR HS DNA Polymerase (5 U/<math>\mu\text{l}</math>)</b>	1 $\mu\text{l}$	13.2 $\mu\text{l}$	52.8 $\mu\text{l}$	105.6 $\mu\text{l}$
<b>Total volume</b>	22.5 $\mu\text{l}$	297 $\mu\text{l}$	1,188 $\mu\text{l}$	2,376 $\mu\text{l}$

\*Volumes include  $\sim 10\%$  extra for overage.

**BEFORE PROCEEDING:** Please refer to Appendix C, which contains more information about the use of the unique dual index kit.

- Add 22.5  $\mu\text{l}$  of the Library Amplification Master Mix to each reaction from Section VI.A, Step 8. Then, add 5  $\mu\text{l}$  of a different index from the Unique dual index kit - 48U or 96U to each reaction. Mix by vortexing for 5 sec, and then spin the tubes briefly to collect the contents at the bottom of the tubes/plate.
- Place the tubes in a thermal cycler with a heated lid ( $105^{\circ}\text{C}$ ) and perform PCR amplification using the following program:

72 $^{\circ}\text{C}$	3 min
85 $^{\circ}\text{C}$	2 min
98 $^{\circ}\text{C}$	2 min
<u>12–16 cycles:*</u>	
98 $^{\circ}\text{C}$	20 sec
60 $^{\circ}\text{C}$	75 sec
68 $^{\circ}\text{C}$	5 min
4 $^{\circ}\text{C}$	Hold

\*Please consult Table 4 for the recommended number of PCR cycles based on cDNA input amount.

**STOPPING POINT:** The samples can be stored in the thermal cycler at  $4^{\circ}\text{C}$  overnight, or they can be transferred to  $-20^{\circ}\text{C}$  for up to a week.

**Table 4. General recommendations for the number of PCR cycles for library amplification.**

Input amount of cDNA	Recommended PCR cycles
1 ng*	15–16
2 ng	14–15
5 ng	13–14
10 ng	12–13

\*Contact Technical Support if cDNA input is lower than 1 ng.

### C. Protocol: Pooling and Purification of Amplified Libraries

PCR-amplified libraries can be purified individually or, optionally, the libraries can be pooled if the input cDNA was quantified and normalized to a uniform input amount before library preparation (Section VI.A, Step 4). The libraries are then 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 then the library is eluted with Nuclease-Free Water.

#### NOTES:

- Aliquot beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well by vortexing. Use room-temperature Nuclease-Free Water for this protocol.
- Bead:sample ratio is 0.8:1.
- Prepare fresh 80% ethanol for each experiment. You need ~400  $\mu$ l per sample.
- You will need a magnetic separation device for 0.2-ml tubes, 1.5-ml tubes, strip tubes, or a 96-well plate.

1. Determine the number of libraries to be pooled based on the desired sequencing depth and sequencer throughput. If preferred, clean up libraries individually before pooling.

**NOTE:** Please refer to Appendix C, which contains more information about pooling strategies.

2. Pool the libraries by pipetting a fixed volume from each sample into a 1.5-ml tube or PCR tube. Volumes between 2 and 8  $\mu$ l are appropriate. See examples in the table below. Do not use less than 2  $\mu$ l per sample to ensure greater accuracy.

**Table 5. Example volumes of pooled libraries and beads**

Number of libraries to be pooled	Volume per library	Total pool volume	Bead volume*
8	8 $\mu$ l	64 $\mu$ l	52 $\mu$ l
12	4 $\mu$ l	48 $\mu$ l	39 $\mu$ l
16	4 $\mu$ l	64 $\mu$ l	52 $\mu$ l
24	2 $\mu$ l	48 $\mu$ l	39 $\mu$ l
32	2 $\mu$ l	64 $\mu$ l	52 $\mu$ l
48	2 $\mu$ l	96 $\mu$ l	77 $\mu$ l
96	2 $\mu$ l	192 $\mu$ l	154 $\mu$ l

\*The bead volume is approximately 80% of the total pool volume.

**NOTE:** If pooling 96 samples, make sure to use a 1.5-ml tube.

3. Add a volume of beads representing 80% of the volume of the pooled libraries. See the table above for guidance.

**NOTE:** If cleaning up libraries individually, add 40  $\mu$ l of beads to each 50- $\mu$ l sample.

4. Mix well by vortexing or pipetting the entire mixture up and down 10 times.

**NOTE:** The beads are viscous; pipette the entire volume and push it out slowly.

5. Incubate at room temperature for 5 min to let the library bind to the beads.
6. Briefly spin the sample to collect the liquid from the side of the tube. Place the tube on a magnetic stand for ~2 min or until the liquid appears completely clear and there are no beads left in the supernatant.
7. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.

8. Keep the samples on the magnetic separation device. Add 200 µl of fresh 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec, and then remove and discard the supernatant, taking care not to disturb the beads. The library remains bound to the beads during washing.
9. Repeat the ethanol wash (Step 8) once more.
10. Briefly centrifuge the samples to collect the liquid from the side of the tube or plate well. Place the samples on the magnetic separation device for 30 sec, then remove any residual ethanol with a pipette.
11. Incubate the samples at room temperature for ~5–15 min, until the pellet is no longer shiny, but before cracks appear.

**NOTES:**

- Be sure to dry the pellet only until it is just dry. The pellet should look matte with no shine.
- If you under-dry the pellet, ethanol will remain in the sample. The ethanol will reduce your recovery rate and, ultimately, the library yield. Allow the sample to sit at room temperature until the pellet is dry.
- If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 min to rehydrate (Step 13) and may reduce amplified library recovery and yield.
- The pooled samples requiring higher bead volumes take longer to dry.

12. Once the beads are dry, elute the pooled, purified libraries by adding the required volume of Nuclease-Free Water (provided), based on the number of samples pooled.

**Table 6. Example volumes of Nuclease-Free Water to resuspend libraries.**

<b>Number of libraries pooled</b>	<b>Nuclease-Free Water*</b>
<b>8</b>	32 µl
<b>12</b>	24 µl
<b>16</b>	32 µl
<b>24</b>	24 µl
<b>32</b>	32 µl
<b>48</b>	48 µl
<b>96</b>	96 µl

\*Nuclease-Free Water volume is 50% of the original pool volume (Column 3, Table 5).

**NOTE:** If cleaning up libraries individually, elute in 25 µl of Nuclease Free Water.

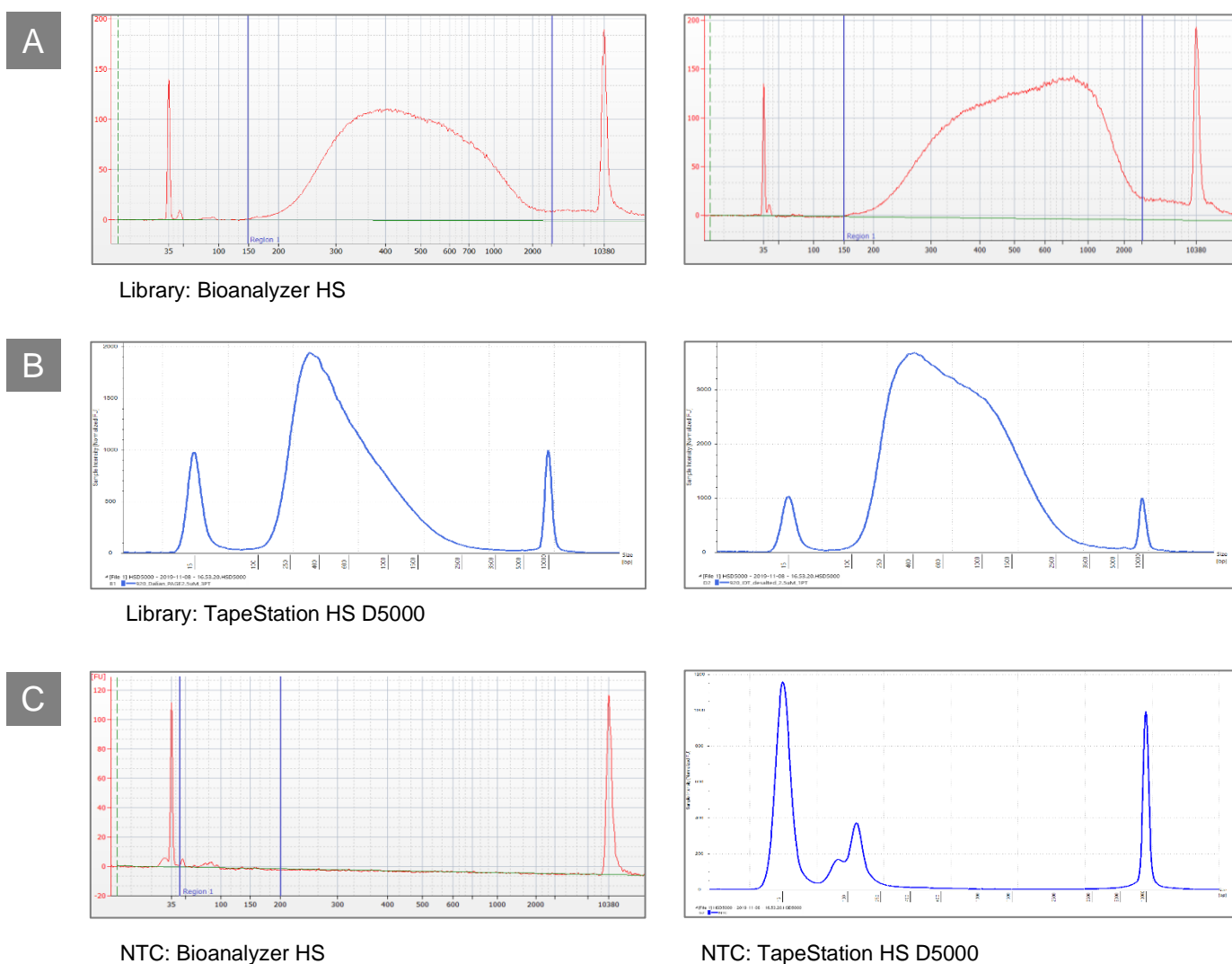
13. Remove from the magnetic separation device and vortex the tube for 3 sec to mix thoroughly. Incubate at room temperature for ~5 min to rehydrate the beads.
14. Briefly spin to collect the liquid from the side of the tube. Place the tube back on the magnetic separation device for ~2 min or longer until the solution is completely clear.
15. Transfer the clear supernatant containing purified libraries to a nuclease-free, low-adhesion tube. Label each tube with sample information. The purified libraries can be stored at –20°C.

### D. Protocol: Determination of Amplified Library Quality and Quantity

Library quantification can be performed using fluorescence-detection-based methods, such as the Qubit dsDNA HS Assay or Quant-iT PicoGreen dsDNA Assay Kit. A qPCR assay like the Library Quantification Kit (Takara Bio, Cat. No. 638324) is also recommended for the quantification of the sequencing libraries.

A fragment analyzer, such as the Agilent 2100 Bioanalyzer or TapeStation system (see Figure 5 for expected Bioanalyzer or TapeStation profiles), can be used to assess the quality of the libraries. We recommend diluting an aliquot of each library in TE buffer 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 (see the Agilent High Sensitivity DNA Kit User Manual for instructions).

For loading on Illumina NGS platforms, follow the “Denature and Dilute Libraries Guide” specific to your system.



**Figure 4. Example electropherogram analysis of SMART-Seq HT PLUS Kit libraries.** 3 ng of libraries or 1 μl of NTC control were analyzed using Agilent’s High Sensitivity DNA Kit and an Agilent 2100 Bioanalyzer (Bioanalyzer HS) or the TapeStation 4200 and Agilent High Sensitivity D5000 ScreenTape System (TapeStation HS D5000). Shown are examples of successful library profiles from Bioanalyzer HS (**Panel A**) and TapeStation HS D5000 (**Panel B**). The NTC control showed little or no amplification when run on the Bioanalyzer (**Panel C, left**). Adapter dimers are observed below 200 bp when the NTC was run on the TapeStation (**Panel C, right**).

## VII. References

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## Appendix A: 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, above). 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, you can sort cells in a maximum volume of 5  $\mu$ l PBS. PBS is *not* an optimal sorting buffer, as it interferes with the efficiency of cDNA synthesis and PCR amplification. For recommendations on how to proceed after sorting into  $\leq 5$   $\mu$ l of PBS, see Section B, below.

### A. FACS Sorting into a Buffer Not Containing the CDS IIA Oligo

In this protocol, cells are sorted in 11.5  $\mu$ l of buffer.

1. Prepare enough sorting buffer to prefill the 96-well plate or PCR strips as indicated below; due to small pipetting volumes, prepare no less than 230  $\mu$ l of sorting buffer, which is enough for 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 PSS mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 11.5  $\mu$ l per well.

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

	Per well	18 wells*	48 wells*	96 wells*
<b>10X Lysis Buffer</b>	0.95 $\mu$ l	19 $\mu$ l	52.25 $\mu$ l	104.5 $\mu$ l
<b>RNase Inhibitor</b>	0.05 $\mu$ l	1 $\mu$ l	2.75 $\mu$ l	5.5 $\mu$ l
<b>Nuclease-Free Water</b>	10.5 $\mu$ l	210 $\mu$ l	577.5 $\mu$ l	1,155.0 $\mu$ l
<b>Total volume</b>	11.5 $\mu$ l	230 $\mu$ l	632.5 $\mu$ l	1,265.0 $\mu$ l

\*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  $\mu$ l 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  $\mu$ l 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  $-20^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  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  $\mu\text{l}$ .

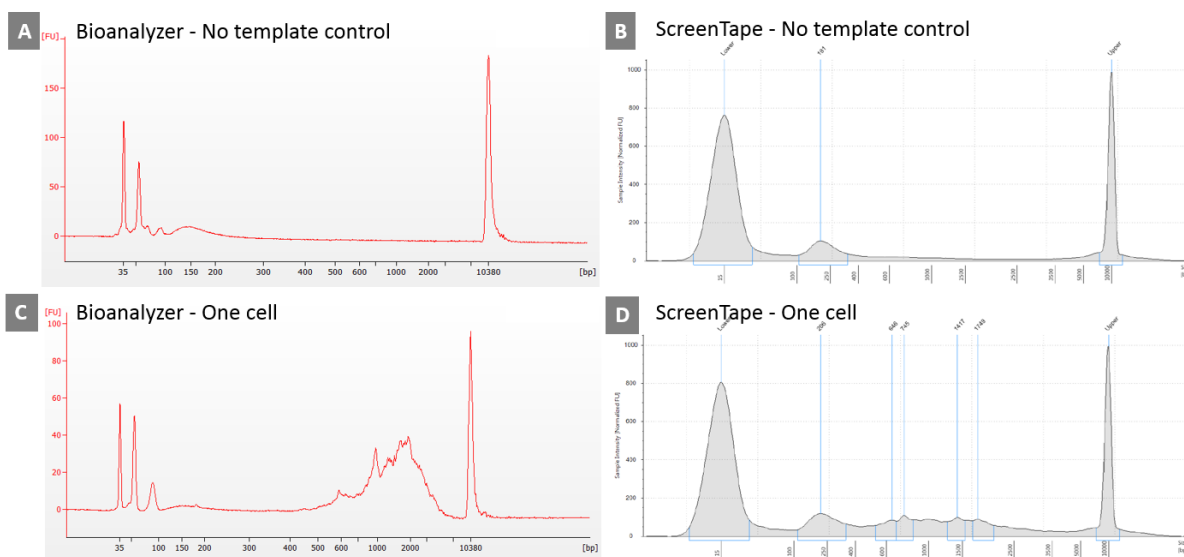
If using an alternative buffer, we recommend performing a pilot experiment using Control Total RNA and the estimated amount of buffer that you expect to accompany your cell(s) to determine its impact on cDNA synthesis. Sorting cells in 1  $\mu\text{l}$  of 1X PBS is acceptable and will not interfere with kit performance. If it is desired to sort cells in  $>1$   $\mu\text{l}$  of 1X PBS, it is critical to keep the PBS volume below 5  $\mu\text{l}$ . 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  $>1$   $\mu\text{l}$  of PBS). If you must use 2–5  $\mu\text{l}$  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 2 (Section V.B). In addition, you must use PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (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  $-80^{\circ}\text{C}$  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: 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 PLUS Kit. Another good option, 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 5—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 PLUS Kit should generate cDNA yields higher than 200 pg/μl and the cDNA profile should be detected relatively easily.



**Figure 5. 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**).

## Appendix C: Indexing Recommendations and Sequences

The SMART-Seq HT PLUS Kit includes the Unique Dual Index Kit - 48U (48 rxns; Cat. No. R400744) or Unique Dual Index Kit - 96U (96 rxns; Cat. No. R400745). The included indexes are 8-nt long and employ “IDT for Illumina TruSeq® UD Indexes” i5 and i7 dual index sequences. In both versions of the index kit, the primers are provided in a 96-well plate format; the indexes in the Unique Dual Index Kit - 48U are a subset of Unique Dual Index Kit - 96U. Please consult the following resources for component information, best practices, pooling strategies, an index plate map, and index sequences.

- Unique Dual Index Kit Protocol-At-A-Glance (download at [takarabio.com/UDI-kit-protocol](http://takarabio.com/UDI-kit-protocol))
- Unique Dual Index Sequence Information (Excel file) (download at [takarabio.com/UDI-sequence-info](http://takarabio.com/UDI-sequence-info))

## Appendix D: Troubleshooting Guide for Final Libraries

### A. Broad peak(s) in Bioanalyzer traces

Description of problem	Possible explanation	Solution
After purification of the amplified library, the Bioanalyzer traces show broad peak(s) extending from <1,000 bp to >10,000 bp	The library 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 Bioanalyzer run.

### B. High quantity of small molecules in Bioanalyzer traces

Description of problem	Possible explanation	Solution
After purification of the amplified library, 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.

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