Takara Bio USA, Inc.

SMART-Seq® HT Kit User Manual

Cat. Nos. 634437, 634438, 634436, 634455, 634456

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I. Introduction

cDNA Synthesis Using Template Switching Technology

The **SMART-Seq HT Kit** (Cat. Nos. 634436, 634437, 634438, 634455, and 634456) is designed to generate high-quality, full-length cDNA directly from 1–100 cells or 10 pg–1 ng of total RNA. The kit generates cDNA compatible with Illumina® HiSeq®, MiSeq®, MiniSeq™, and NextSeq® systems after Illumina Nextera® XT sequencing library preparation. A detailed protocol for Nextera XT library preparation is provided in Section VI of this user manual. The cDNA synthesis protocol can be completed in four hours, and the entire library construction protocol can be completed within two working days (Figure 1, below).

The SMART-Seq HT Kit 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.

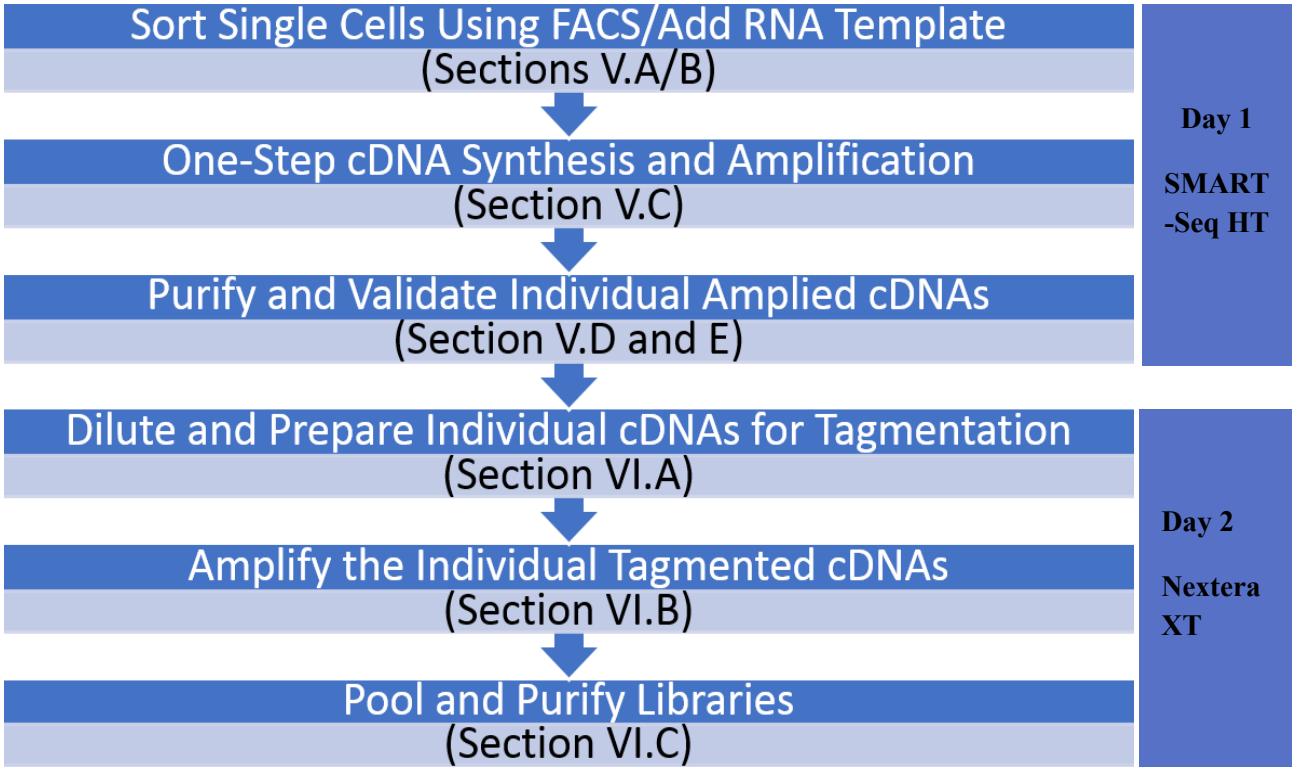

Figure 1. SMART-Seq HT Kit protocol overview. This kit features a streamlined workflow that generates full-length cDNA with minimal hands-on time. While the workflow is primarily designed to accommodate cells isolated using FACS, it can also be used with purified total RNA.

Adaptations to SMART technology for next-generation sequencing (NGS) were incorporated into the first generation of our kit for ultra-low input mRNA-seq (the SMARTer® Ultra® Low RNA Kit for Illumina Sequencing) and published as the SMART-Seq method (Ramsköld et al. 2012). Improvements continued in subsequent generations of SMARTer Ultra low kits, and the SMART-Seq method was updated to SMART-Seq2 method (Picelli et al. 2013). The SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (SMART-Seq v4)

improved upon the SMART-Seq2 method by incorporating both the novel use of locked nucleic acid (LNA) technology into an optimized template switching oligo and other advancements developed by Takara scientists. The enhancements in the SMART-Seq v4 kit resulted in single-cell mRNA-seq libraries that outperform previously published protocols (including SMART-Seq2 method) and existing kits. The SMART-Seq v4 kit has higher sensitivity and reproducibility, meaning more genes are identified, in addition to generating significantly lower background than the SMART-Seq2 method.

The new SMART-Seq HT Kit evolved from the SMART-Seq v4 kit and is designed to reduce handling time for greater ease of use without compromising on performance. Indeed, the SMART-Seq HT Kit has the same high sensitivity and reproducibility that characterize the SMART-Seq v4 kit. The difference from the SMART-Seq v4 kit is that it features a streamlined protocol (Figure 1, above), 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.

For more information on SMART technology, please visit www.takarabio.com.

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II. List of Components

The components in the SMART-Seq HT 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 a modification of the protocol may lead to unexpected results.

SMART-Seq HT Kit	Cap color and label	634455 (12 rxns)	634456 (48 rxns)	634437 (96 rxns)	634438 (192 rxns)	634436 (480 rxns)
Control Total RNA (1 µg/µl)	N/A	5 µl	5 µl	5 µl	2 x 5 µl	5 x 5 µl
SMART-Seq HT Oligonucleotide	Pink, HT Oligo	12 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl
3′ SMART-Seq CDS Primer II A	Blue, CDS IIA	24 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
One-Step Buffer	Orange, 1-Step Buffer	125 µl	500 µl	1 ml	2 x 1 ml	5 x 1 ml
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple, SMARTScribe	24 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
SeqAmp DNA Polymerase	Green, SeqAmp	20 µl	20 µl	50 µl	2 x 50 µl	5 x 50 µl
RNase Inhibitor (40 U/µl)	White, RRI	30 µl	75 µl	150 µl	300 µl	5 x 150 µl
10X Lysis Buffer	N/A	230 µl	920 µl	1.85 ml	2 x 1.85 ml	5 x 1.85 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)	N/A	1.7 ml	2 x 1.7 ml	6.8 ml	2 x 6.8 ml	5 x 6.8 ml
Nuclease-Free Water	N/A	1 ml	2 x 1 ml	4 ml	2 x 4 ml	5 x 4 ml

Storage Conditions

- Store Control Total RNA at –70°C.
- Store all other reagents at –20°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 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 Cell Sorting:

- 8-tube strips (Thermo Fisher, 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, Cat No. AB0784/AB0850)
- 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, Cat No. 563503)
- **(Optional)** SMART-Seq HT Kit Lysis Components (Cat No. 634439) for sorting extra plates

For One-Step RT-PCR & Validation:

- 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.

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

For SPRI (Solid Phase Reversible Immobilization) Bead Purifications:

- Agencourt AMPure XP PCR purification kit (5-ml size: Beckman Coulter Item No. A63880; 60-ml size: Beckman Coulter Item No. A63881)

NOTES:

- The kit has been specifically validated with AMPure XP beads. Please do not make any substitutions as it may lead to unexpected results.
- AMPure 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 Nextera sequencing libraries before purification):
  - Takara Bio USA, Inc. Magnetic Stand (Cat. No. 631964) or NEB Magnetic Separation Rack (NEB, Cat. No. S1506S or S1509S)

For cDNA Quantification:

- High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) or equivalent high sensitivity electrophoresis method (Section V.E)
- Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Cat. No. P11496) or Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854)

For Sequencing Library Generation:

- Nextera XT DNA Library Preparation Kit (Illumina, Cat. Nos. FC-131-1024, FC-131-1096)
- Nextera XT Index Kit (Illumina, Cat. No. FC-131-2001) or other Nextera-compatible indexes

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 clean room conditions, e.g., one-step RT-PCR (Section V.C)

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

- **A second workstation located in the general laboratory** where you perform cDNA purification, measure cDNA concentration (Section V.D) and prepare the Nextera 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- 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 Kit works with cells or purified RNA. We recommend processing at least 12 samples at a time so that the reagents can be pipetted accurately.

Cell Input

- **Cells should be in 12.5 µl of our recommended sorting buffer (see Section V.A). If you do not wish to include the CDS oligo in the lysis buffer or if cells are aliquoted in a different buffer, please follow the recommendations in Appendix A for appropriate volumes.**
  If using an alternative buffer, we recommend doing a pilot experiment to determine its impact on cDNA synthesis by performing a reaction with control RNA and the estimated amount of buffer that you expect to accompany your cell(s).
- 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.**
- After dispensing the cells into tubes/plates containing our recommended sorting buffer using dilution or FACS, the cells can either be processed immediately (ideally within no more than 5 minutes) or snap frozen on dry ice and stored at –80°C until processing.
- 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).
- Following appropriate washes, cells can be diluted in BD FACS Pre-Sort Buffer (BD, Cat No. 563503) to maintain cells in a single-cell suspension before FACS.

Total RNA Input

- **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 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 ≥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 for ensuring the kit behaves as expected and 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 starting point for single cells). 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 should be used to ensure 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 (RRI 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 RRI 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 RRI 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 RRI Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.
5. Further dilute Control Total RNA to 0.01 ng/µl by mixing 120 µl of RRI Water with 5 µl of 0.25 ng/µl Control Total RNA in a sterile microcentrifuge tube.
6. Use 1 µl or more of 0.01 ng/µl Control Total RNA as a positive control RNA input for the kit and process along with your samples.

V. cDNA Synthesis Protocols

The SMART-Seq HT 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 protocol before starting. We have optimized this protocol for cDNA synthesis from 1–100 intact cells or ultra-low input amounts of total RNA (10 pg–1 ng). Due to the sensitivity of the protocol, 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: Sort Single Cells Using FACS

This section provides guidance for sorting cells directly in a buffer suitable for quick set-up of the one-step RT-PCR in Section V.C. For users starting from purified RNA, skip ahead to Section V.B. The recommendations below include 3′ SMART-Seq CDS Primer II A in the sorting buffer to streamline later cDNA synthesis reaction set up. However, this is not a requirement for successful cDNA synthesis. To see recommendations for alternative sorting buffers, see Appendix A.

Important: If you do not include the 3′ SMART-Seq CDS Primer II A in the sorting buffer, you need to add it when you thaw your samples, before the first step of Section V.C.

1. Prepare 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.

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

	Per well	18 wells*	48 wells*	96 wells*
10X Lysis Buffer	0.95 µl	19 µl	52.25 µl	104.5 µl
RNase Inhibitor	0.05 µl	1 µl	2.75 µl	5.5 µl
3′ SMART-Seq CDS Primer II A	1 µl	20 µl	55 µl	110.0 µl
Nuclease-Free Water	10.5 µl	210 µl	577.5 µl	1155.0 µl
Total volume	12.5 µl	250 µl	687.5 µl	1375.0 µl

*Volumes include ~10% extra for overage.

2. Mix briefly, 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 the kit is sufficient to prepare at least 96 wells for sorting. 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 SMART-Seq HT Lysis Components (Cat No. 634439) separately.

3. 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 first stop when aliquoting. Changing tips often also minimizes bubble formation.

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

When ready to sort:

4. Unseal the prepared plate/tube strips. Sort cells into the sorting solution according to the FACS system manual and desired parameters.
5. Seal the plate with an aluminum foil seal or PCR strip caps. Ensure the plate is 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.

6. 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.
7. Place the plate on dry ice to flash-freeze the sorted cells.

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

8. Store sorted samples at –80°C until ready to proceed with cDNA synthesis.

NOTES:
- To use PCR strips sealed with an aluminum foil, 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.

9. If preparing positive control reactions, proceed to Section V.B. Otherwise, proceed directly to Section V.C.

B. Protocol: If Starting with RNA, Prepare RNA Template

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

1. Prepare a stock solution of 10X Reaction Buffer by mixing the 10X Lysis Buffer with the RNase Inhibitor as indicated below (scale up as needed):

10X Reaction Buffer:

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

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

2. Mix briefly, then spin down.
3. See Table I for guidelines on setting up your control and 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 I. 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

*The Control 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. Proceed to Section V. C, below.

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 (i.e., if you sorted your cells in PSS), you need to add it to your samples in the optional Step 2 below**.

IMPORTANT: The first part of the cDNA synthesis protocol should be completed in a PCR clean workstation, ideally in a clean room to avoid introducing contaminants into your RNA/cell sample.

1. Thaw the One-Step Buffer at room temperature. Thaw the remaining reagents (except the enzymes) on ice, then gently vortex and briefly spin them down. Store all reagents on ice until use.

   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 (i.e., if you sorted your cells in PSS), 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 from Section V.A or Section V.B at 72°C in a preheated, thermal cycler with a heated lid for 3 minutes. Then immediately place the samples on ice for 2 minutes.

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

4. Prepare enough One-Step Master Mix for all the reactions, plus 10% of the total reaction mix volume. 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*	48 rxns*	96 rxns*
Nuclease-Free Water	0.7 µl	9.2 µl	37.1 µl	74.2 µl
One-Step Buffer	8 µl	105.6 µl	424 µl	848 µl
SMART-Seq HT Oligonucleotide	1 µl	13.2 µl	53 µl	106 µl
RNase Inhibitor	0.5 µl	6.6 µl	26.5 µl	53 µl
SeqAmp DNA Polymerase	0.3 µl	4 µl	15.9 µl	31.8 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	2 µl	26.4 µl	106 µl	212 µl
Total volume	12.5 µl	165 µl	662.5 µl	1,325 µl

*Volumes include ~10% extra for overage.

NOTES:
- Add the SMARTScribe Reverse Transcriptase and SeqAmp DNA Polymerase to the Master Mix just before use. Mix the Master Mix well by gentle pipetting or vortexing and spin the tube briefly in a minicentrifuge.
- For SeqAmp DNA Polymerase, it is particularly 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.

5. Preheat the thermal cycler to 42°C.
6. Add 12.5 µl of the Master Mix from Step 4 to each sample. Mix the contents of the tubes/plate by gentle pipetting or vortexing and spin them briefly to collect the contents at the bottom of the tubes.

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

42°C	90 min	
95°C	1 min	
10 to 20 cycles*:		
	98°C	10 sec
	65°C	30 sec
	68°C	3 min
72°C	10 min	
4°C	Forever	

*Please consult Tables II and III below for the recommended number of PCR cycles.

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

Table II. 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 III. 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 cells or T cells	2 pg	20
PBMCs	1 pg	20

*We do not recommend going above 20 cycles of PCR.

D. Protocol: Purification of Amplified cDNA using the Agencourt AMPure XP Kit

PCR-amplified cDNA is purified by immobilization on AMPure XP beads. The beads are then washed with 80% ethanol, and cDNA is eluted with Elution Buffer.

NOTES:
- Aliquot AMPure XP 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.
- 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.

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 AMPure XP 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), cDNA samples need to be transferred to a 96-well V-bottom plate. Distribute 25 µl of AMPure XP 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 AMPure XP beads-cDNA mixture at room temperature for 8 minutes to let the cDNA bind to the beads.
4. Briefly spin the samples to collect the liquid from the side of the tubes (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 minutes 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 fresh 80% ethanol to each sample without disturbing the beads. Incubate for 30 seconds. Then, 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.
9. Briefly centrifuge the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for 30 seconds, then remove any residual ethanol with a pipette.
10. Incubate the samples at room temperature for ~2–2.5 minutes 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 your amplified cDNA recovery rate and ultimately your 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 might take longer than 2 minutes to rehydrate (Step 12) and may reduce amplified cDNA recovery and yield.
11. Once the beads are dry, add 17 µl of Elution Buffer to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads.
12. Incubate at room temperature for 2 minutes to rehydrate.
13. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic separation device for 1 minute or longer, until the solution is completely clear.
14. Transfer clear supernatant containing purified cDNA from each well to new tubes/plate. Do not pool samples at this point. Take care not to carry over any beads with your samples. Label each tube with sample information. Proceed to validation immediately or store at –20°C.

E. Protocol: Validation and quantification of amplified cDNA

cDNA concentrations obtained with the SMART-Seq HT Kit may vary between different cell types and treatments. The yield and size distribution of the Nextera XT sequencing library preparation (Section VI) also varies depending on input cDNA concentration. 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.

However, neither the PicoGreen nor Qubit assays provide information on the size of the cDNA. Therefore, 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 cDNA profile is particularly important when performing initial optimization. For routine experiments by experienced users, evaluation of cDNA profile may not be necessary or may be restricted to a few randomly selected samples combined with a positive and a no-RNA control.

When using the Qubit or PicoGreen assay for quantification, the negative control may generate an apparent yield up to 100 pg/µl (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.

For example, if you perform 19 cycles of PCR and find that your negative controls generate about 200 pg/µl, a sample with an apparent yield of 350 pg/µl is in reality no more than 150 pg/µl, and this is the concentration you should use for library preparation in Section VI.A. Regardless of the quantification method used, the SMART-Seq HT Kit should yield >200 pg/µl when used on 10 pg of the Control Total RNA with 17 PCR cycles after background subtraction, if applicable.

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 evaluating cDNA profile, 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.
   - Compare the results for your samples and controls (see Figure 3, below) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield a distinct peak spanning 400–10,000 bp, with a summit 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 summit slightly smaller (around 2000 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.**

3. Proceed to **Library Preparation for Sequencing on Next-Generation Sequencing Protocols** (Section VI).

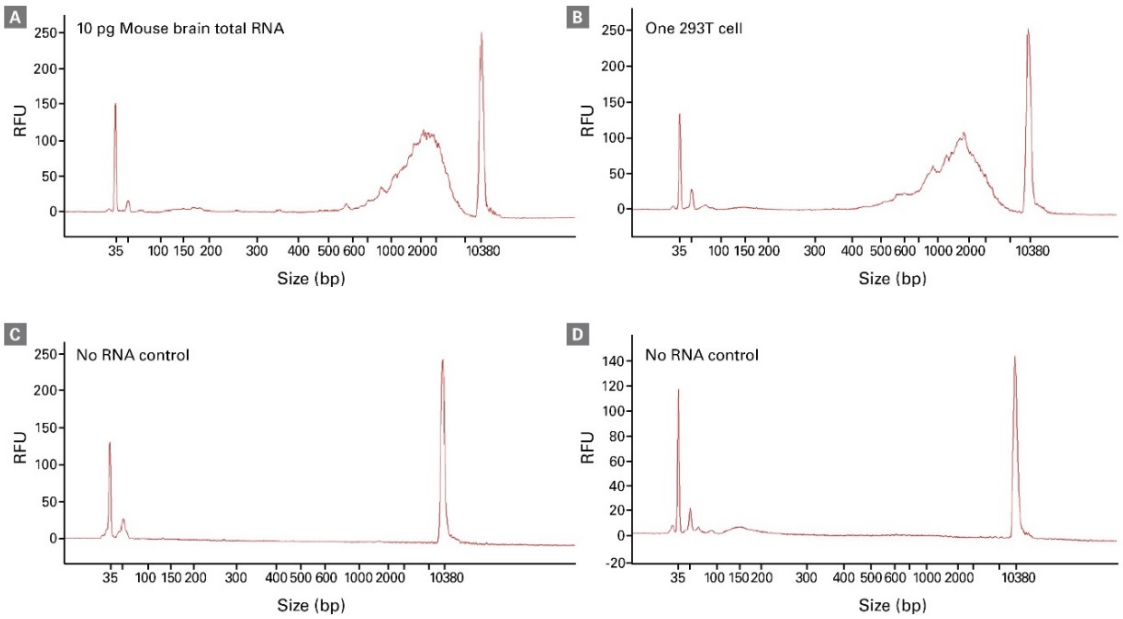

Figure 2. Example electropherogram results from Agilent 2100 Bioanalyzer. All samples were subjected to the one-step RT-PCR as described in the protocol, using 17 PCR cycles. After bead purification, 1 µl of cDNA was analyzed using Agilent's High Sensitivity DNA Kit. **Panel A.** Example produced from 10 pg of Mouse Brain Total RNA control included in the kit, showing a peak summit around 2,500 bp. **Panel B.** Example generated from one 293T cell (isolated using FACS). Note the smaller size of the summit 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.

VI. Library Preparation for Next-Generation Sequencing

These protocols describe a modified Illumina Nextera XT DNA library preparation protocol that was adapted for single-cell mRNA sequencing library preparation at one-quarter the volume recommended by Illumina. The Illumina Nextera XT DNA Library Preparation Guide provides detailed instructions for library preparation, and we highly recommend that you read it before proceeding. Keep in mind that the modifications included in the protocol below have been made to adapt the Nextera XT chemistry to work downstream of the SMART-Seq HT Kit.

A. Protocol: Dilute and Prepare cDNA for Tagmentation

NOTES:

- The optimal cDNA input for Nextera XT library preparation is 100–300 pg. A larger amount of cDNA will generate libraries that are too large for sequencing on an Illumina instrument. The protocol below uses 125 pg of cDNA (in a volume of 1.25 µl), but any input between 100–300 pg will work.
- If all samples are correctly quantified and normalized to a uniform input amount before Nextera XT library preparation, sequencing libraries can be pooled before clean-up and a relatively uniform amount of sequencing reads will be obtained.

1. Dilute each cDNA to 100 pg/µl with Nuclease-Free Water in a plate or PCR strips. Do not pool at this step. Vortex at medium speed for 20 sec and centrifuge at 350*g* for 1 min.

   NOTES:

   - Always use a minimum of 2 µl of cDNA to perform dilution.
   - Samples containing less than 100 pg/µl can still be used without dilution, but you may get fewer reads than for other samples if you pool for clean-up (Section VI.C). Negative controls should be used without dilution.

   IMPORTANT: Warm Tagment DNA Buffer and NT Buffer to room temperature. Visually inspect NT Buffer to ensure that there is no precipitate. If there is a precipitate, vortex the buffer until all particles are resuspended.

2. After thawing, gently invert the tubes 3–5 times, followed by centrifuging the tubes briefly, to ensure all reagents are adequately mixed.
3. Label a new 96-well PCR plate "Library Prep."
4. In a 1.5-ml PCR tube, combine the components of the Tagmentation Premix as described below. Vortex gently for 20 secs and centrifuge the tube briefly.

   Tagmentation Premix:

	1 rxn	12 rxns*	48 rxns* (µl)	96 rxns* (µl)
Tagment DNA Buffer	2.5 µl	37.5 µl	150 µl	300 µl
Amplification Tagment Mix	1.25 µl	18.8 µl	75 µl	150 µl
Total volume	3.75 µl	56.3 µl	225 µl	450 µl

*Calculated based on a 25% excess.

5. Distribute 3.75 µl of the Tagmentation Premix into each well of the "Library Prep" plate.

   NOTE: If processing a large volume of samples, aliquot equal amounts of Tagmentation Premix into each tube of an 8-tube strip and then use an eight-channel pipette to distribute the Tagmentation Premix.

6. Transfer 1.25 µl of each diluted cDNA sample to the "Library Prep" plate.
7. Seal the plate and vortex at medium speed for 20 sec. Centrifuge at 2,000*g* for 5 min to remove bubbles.
8. Place the "Library Prep" plate in a thermal cycler with a heated lid and run the following program:

55°C	10 min
10°C	Hold

9. Once the thermal cycler reaches 10°C, pipette 1.25 µl of NT Buffer into each of the tagmented samples to neutralize the samples.

NOTE: If processing a large volume of samples, aliquot equal amounts of NT Buffer into each tube of an 8-tube strip, then use an eight-channel pipette to distribute the NT Buffer.

10. Seal the plate and vortex at medium speed, then centrifuge at 2,000*g* for 1 min.
11. Incubate at room temperature for 5 min.

B. Protocol: Amplify the Tagmented cDNA

IMPORTANT: Consult Illumina literature (Index Adapters Pooling Guide 1000000041074) for proper index primer selection before proceeding to PCR amplification of the tagmented cDNA.

1. Pipette 3.75 µl of Nextera PCR Master Mix (NPM) into each well of the "Library Prep" plate using an eight-channel pipette.

NOTE: If processing a large number of samples, aliquot equal amounts of NPM into each tube of an 8-tube strip, then use an eight-channel pipette to distribute the NPM.

2. Select appropriate Index 1 (N7xx) and Index 2 (S5xx) primers for the number of samples in your experiment.
   a. Pipette 1.25 µl of Index 1 Primers (N7xx) into the corresponding wells of **each row** of the "Library Prep" plate. As a result, each of the 12 wells in row "A" will contain different Index 1 Primers.
   b. Pipette 1.25 µl of Index 2 Primers (S5xx) to the corresponding wells of **each column** of the "Library Prep" plate. As a result, each of the 8 wells in column "1" will contain different Index 2 Primers.
3. Seal the plate with adhesive film and vortex at medium speed for 20 sec. Centrifuge at 2,000*g* for 2 min.
4. Place the "Library Prep" plate into a thermal cycler and perform PCR amplification using the following program:

72°C		3 min
95°C		30 sec
12 cycles:		
	95°C	10 sec
	55°C	30 sec
	72°C	60 sec
72°C		5 min
10°C		Hold

5. Samples can be left overnight in the thermal cycler at 4°C. If not processed within the next day, freeze the PCR products at –20°C.

C. Protocol: Pool and Clean up the Libraries

PCR-amplified, tagmented cDNA is purified by immobilization on AMPure XP beads. The beads are then washed with 80% ethanol and cDNA is eluted with Nuclease-Free Water.

NOTES:
- Aliquot AMPure XP 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.
- Beads:sample ratio is 0.6:1.
- Prepare fresh 80% ethanol for each experiment. You need ~800 µl per sample.
- You will need a magnetic separation device for 1.5-ml tubes.

1. Determine the number of libraries to be pooled based on the desired sequencing depth and sequencer throughput.

   NOTE: If preferred, clean up libraries individually before pooling.

2. Pool the libraries by pipetting a fixed volume from each sample into a PCR tube or 1.5-ml tube. Volumes between 2 to 4 µl are appropriate. See examples in the table below. Do not use less than 2 µl per sample to ensure greater accuracy.

Number of libraries to be pooled	Volume per library	Total pool volume	Ampure bead volume*
8	4 µl	32 µl	19 µl
12	4 µl	48 µl	29 µl
16	2 µl	32 µl	19 µl
24	2 µl	48 µl	29 µl
32	2 µl	64 µl	38 µl
48	2 µl	96 µl	58 µl

*The bead volume is approximately 60% of the total pool volume.

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

3. Add a volume of AMPure XP beads representing 60 % of the volume of the pooled libraries. See the table above for guidance.

   NOTE: If cleaning up libraries individually, add 7.5 µl of AMPure XP beads to each 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 cDNA libraries 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 seconds then, remove and discard the supernatant taking care not to disturb the beads. The cDNA remains bound to the beads during the washing process.
9. Repeat the ethanol wash (Step 8) once.

10. Briefly centrifuge the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for 30 seconds, 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 your 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 minutes 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, based on the number of samples pooled.

Number of libraries pooled	Nuclease-Free Water volume*
8	48 µl
12	72 µl
16	48 µl
24	72 µl
32	96 µl
48	144 µl

*Nuclease-Free Water volume is 1.5 times the original pool volume.

NOTE: If cleaning up libraries individually, elute in 18 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 entire volume of clear supernatant containing purified cDNA libraries to another tube.
16. Evaluate library size distribution by running samples on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) or an equivalent microfluidic device/kit. Dilute libraries to about 1.5 ng/µl before loading the chip for a consistent library-to-library profile. See Figure 4 for an example of a successful library.
17. Refer to the Illumina Nextera XT DNA Library Preparation Guide to determine the appropriate library concentration for sequencing.

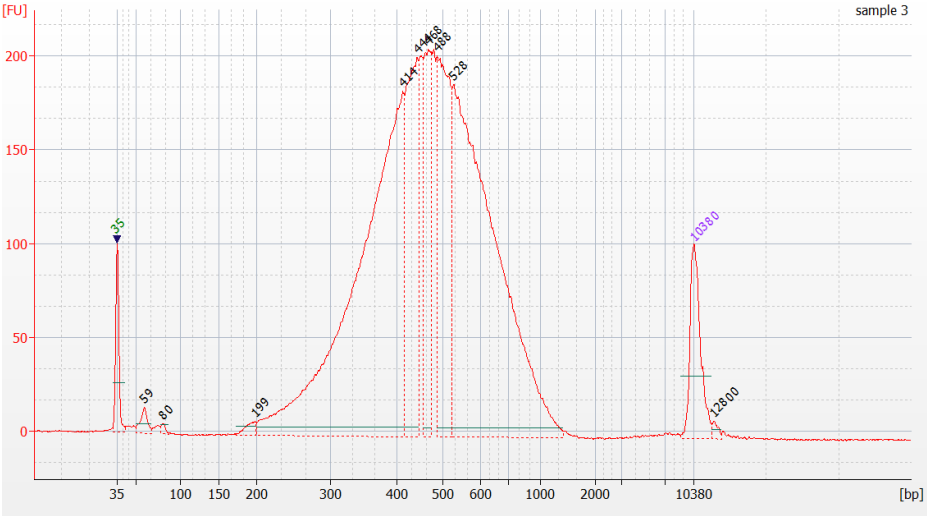

Figure 3. Example of a Nextera XT sequencing library. cDNA generated from 10 pg of Mouse Brain Total RNA control included in the kit was quantified by smear analysis after electrophoresis using Agilent's High Sensitivity DNA Kit as described in Section V.E and used as the input for Nextera XT library construction (125 pg per cell). One µl of the bead-purified library was analyzed on the Bioanalyzer using Agilent's High Sensitivity DNA Kit

VII. References

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Picelli, S., Björklund, Å. K., Faridani, O. R., Sagasser, S., Winberg, G., Sandberg, R. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat. Methods* **10,** 1096–1098 (2013).

Ramsköld, D., Luo, S., Wang, Y.-C., Li, R., Deng, Q., Faridani, O.R., Daniels, G.A., Khrebtukova, I., Loring, J.F., Laurent, L.C., Schroth, G.P. & Sandberg, R. Full-length mRNA-seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology* **30,** 777–782 (2012).

Appendix A: Alternative FACS Sorting Recommendations

When sorting the cells, it is possible to include 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 and may help recover more transcripts by minimizing the handling of the samples in this sensitive step of the protocol. However, this is not a requirement for successful cDNA synthesis. For recommendations 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 the CDS oligo

1. Prepare 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.

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

	Per well	18 wells*	48 wells*	96 wells*
10X Lysis Buffer	0.95 µl	19 µl	52.25 µl	104.5 µl
RNase Inhibitor	0.05 µl	1 µl	2.75 µl	5.5 µl
Nuclease-Free Water	10.5 µl	210 µl	577.5 µl	1155.0 µl
Total volume	11.5 µl	230 µl	632.5 µl	1245.0 µl

*Volumes include ~10% extra for overage

2. 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, we recommend that you purchase the SMART-Seq HT Lysis Components (Cat No. 634439) separately.

3. 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 pipette 11.6 µl and pipette only to first stop when aliquoting. Changing tips often also minimizes bubble formation.

4. Seal the plate/tube strips and briefly spin to ensure the sorting buffer collects at the bottom of the wells.
5. Store the plate/tube strips at –20°C for 10 minutes at a minimum and up to 24 hours. As the volume of sorting buffer is small, the tubes/plate should be kept at –20°C until just before sorting.
6. Proceed to Step 4 in Section V.A.

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, before the first step of Section V.C**.

B. FACS Sorting into Non-Validated Buffers

Sorting into CSS (CDS Sorting Solution) or PSS (Plain Sorting Solution) delivers the best performance from cells isolated with FACS. 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 the buffer that can be added to a cDNA synthesis reaction is 10.5 µl.

We also strongly recommend testing the effect of your FACS buffer or media on cDNA synthesis by performing a reaction with control RNA and the estimated amount of buffer that you expect to accompany your cell(s).

For best cDNA yield and sensitivity, we recommend that you use the Lysis Buffer (either the CSS or PSS) for cell sorting. If it is desired to sort cells in 1X PBS, it is acceptable as long as the volume is kept below 5 µl. PBS is not an optimal sorting buffer as it can interfere with the efficiency of the one-step RT-PCR reaction, and the performance (cDNA yield and sensitivity) may be impacted, particularly with single cells. Higher cell inputs (e.g., 100 cells) may not be impacted as strongly as single cells. If you must use PBS and your cDNA yield is low, consider adding one extra PCR cycle. In addition, you must use PBS without Ca^{2+} and Mg^{2+} (for example, Sigma Cat. No. D8537).

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.B. for preparing a 10X Reaction buffer, setting up control reactions, and adding 3′ SMART-Seq CDS Primer II A.

NOTE: Long-term storage at –80°C may impact the efficiency of cDNA synthesis, especially if not stored in an appropriate buffer.

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 kit. Another 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, the TapeStation Automated Electrophoresis Systems can be used with a High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067-5592). Because the scale is very different, the cDNA profile on the TapeStation may look quite different than the profile on the Bioanalyzer, particularly for yield below 500 pg/µl. However, if the TapeStation shows a broad smear going from ~600 bp to ~2500 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 except for a smear with a summit ~200 bp.

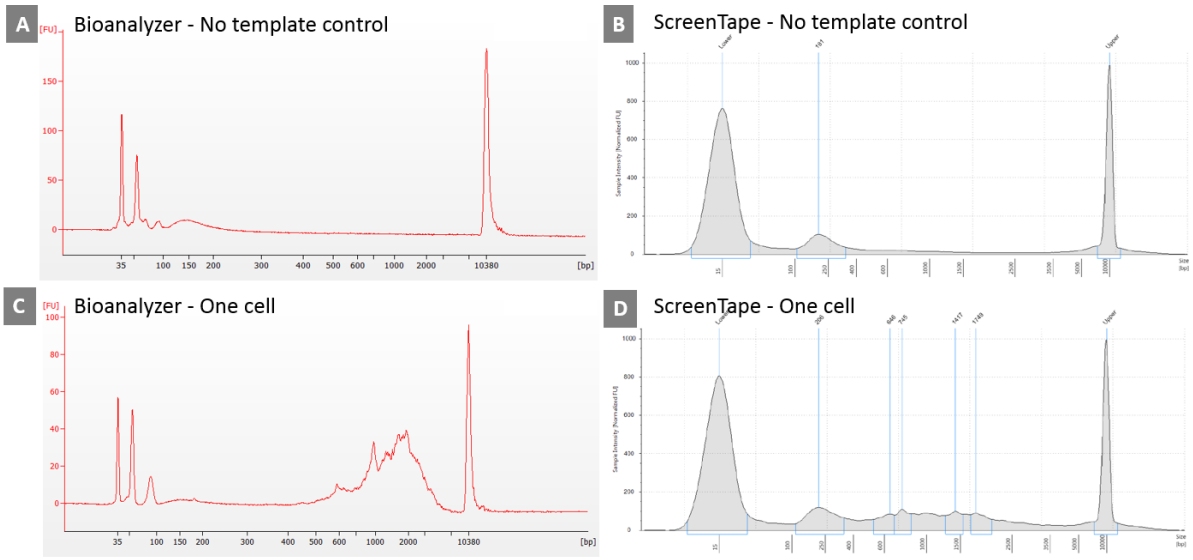

Figure 4. Comparison of electropherogram results from Agilent 2100 Bioanalyzer and TapeStation Automated Electrophoresis Systems. All samples were subjected to the one-step RT-PCR as described in the protocol, using 18 PCR cycles. After bead purification, 1 µl of the sample was analyzed using the Agilent's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

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