Takara Bio USA, Inc.

SMART-Seq® mRNA LP User Manual

Cat. Nos. 634768, 634769, & 634771
(012125)

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I. Introduction

SMART-Seq mRNA LP (Cat. Nos. 634768, 634769 & 634771) is designed to generate high-quality, Illumina® sequencing-ready libraries directly from 1–1,000 intact cells or 10 pg–100 ng of total RNA, in a convenient input volume of 1–10 µl. The cDNA synthesis protocol can be completed in five hours, and the entire library construction protocol can be completed within two days (Figure 1).

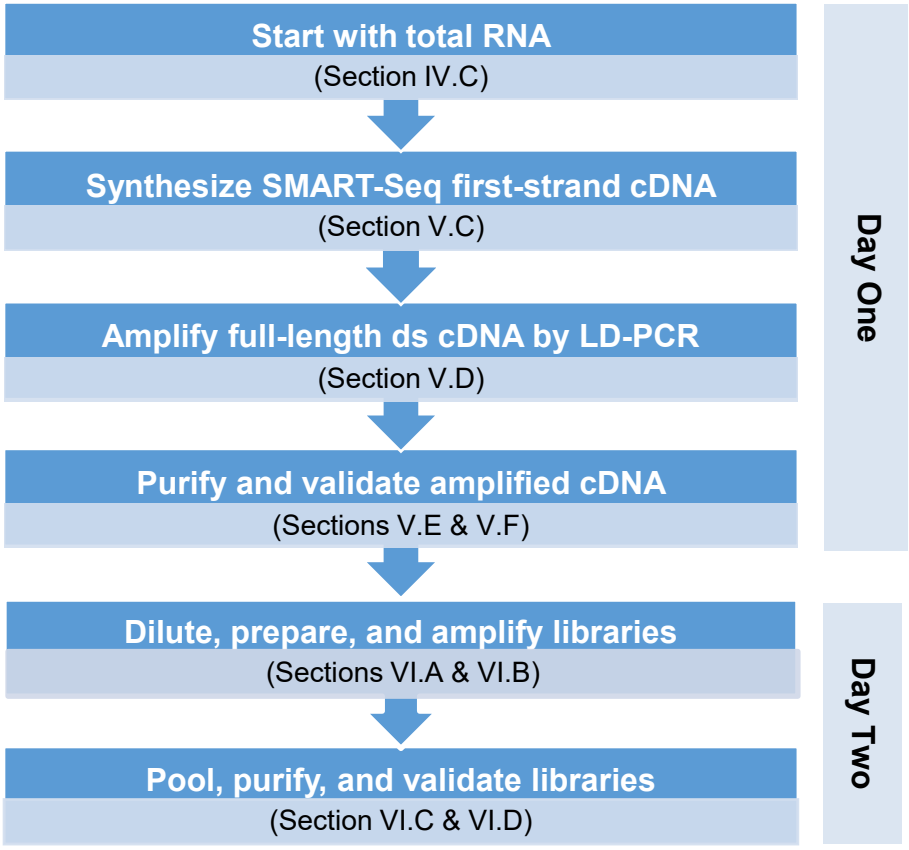

Figure 1. SMART-Seq mRNA LP protocol overview.

SMART-Seq mRNA technology, which uses oligo(dT) priming, provides the excellent sensitivity needed to obtain full-length transcript information from high-quality total RNA (RIN ≥8) or intact cells. Through even gene-body coverage and accurate representation of GC-rich transcripts, this chemistry enables reliable analysis of transcript isoforms, gene fusions, point mutations, and more. SMART-Seq mRNA technology improves upon the Smart-seq2 method (Picelli et al. 2013) by incorporating locked nucleic acid (LNA) technology in the template-switching oligo for more efficient template switching, allowing for the identification of higher numbers of genes relative to other methods.

The SMART-Seq mRNA LP incorporates our proprietary SMART® (**S**witching **M**echanism **a**t 5′ end of **R**NA **T**emplate) technology (Figure 2). 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.

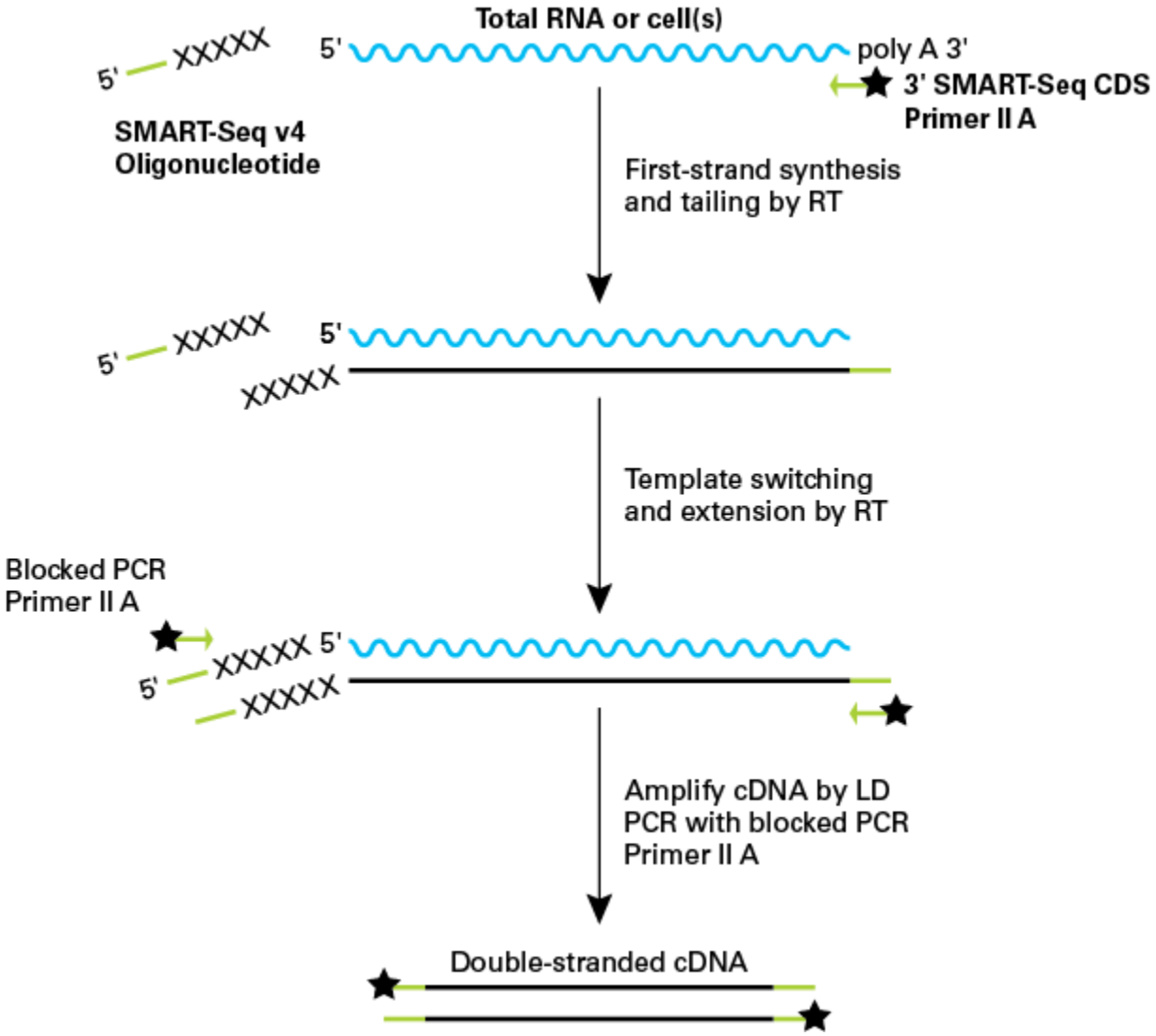

Figure 2. Schematic of the cDNA synthesis technology in the SMART-Seq mRNA LP. SMART technology is used in a ligation-free workflow to generate full-length cDNA. Nontemplated nucleotides (indicated by Xs) added by the SMARTScribe™ Reverse Transcriptase (RT) hybridize to the SMART-Seq v4 template-switching oligonucleotide (SMART-Seq v4 Oligonucleotide), which provides a new template for the RT. The SMART adapters used for amplification during PCR—added by the oligo(dT) primer (3′ SMART-Seq CDS Primer II A) and SMART-Seq v4 Oligonucleotide—are indicated in green. Chemical modifications to block ligation (if using a ligation-based library preparation method) are present on some primers (indicated by black stars).

Once full-length cDNA is synthesized, libraries are prepared through an enzymatic fragmentation method, followed by ligation of stem-loop adapters and nick extension to create primer binding sites for indexed Illumina adapters. The option of four 96-well unique dual index products (Takara Bio, Cat. Nos. 634752– 634756) tested with the kit allows for multiplexing of up to 384 samples using unique dual indexes (UDIs, Figure 3).

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.

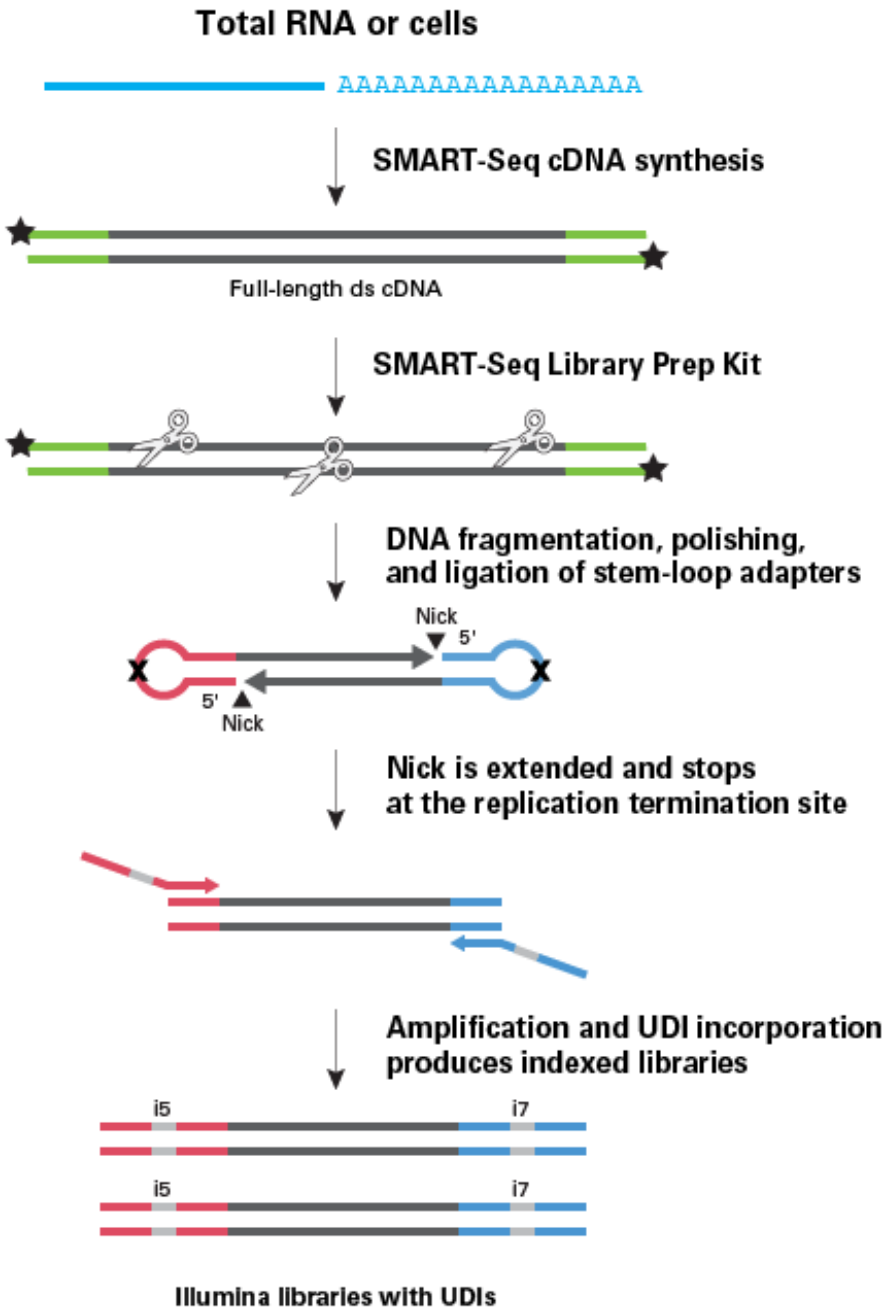

Figure 3. Schematic of the SMART-Seq mRNA LP library preparation workflow. Double-stranded cDNA generated using SMART-Seq mRNA chemistry is enzymatically fragmented and stem-loop adapters ligated, all in a single step. Libraries are then amplified and indexed, generating Illumina-compatible libraries 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. Sequencing output from Illumina sequencers can be analyzed by our free to use Cogent™ NGS Analysis Pipeline (CogentAP) and Cogent NGS Discovery Software (CogentDS).

II. List of Components

The SMART-Seq mRNA LP contains sufficient reagents to prepare up to 24, 96, or 384 reactions. Freeze-thaw of reagents should be limited to ≤6 times. The components in the SMART-Seq mRNA LP 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.

NOTE: Indexing primers are not included in the kit and need to be purchased separately (Unique Dual Index Kits, Takara Bio, Cat. Nos. 634752–634756).

Table 1. SMART-Seq mRNA LP components.

SMART-Seq mRNA LP	634768 (24 rxns)	634769 (96 rxns)	634771 (384 rxns)
SMART-Seq mRNA	**634772***	**634773***	**4 x 634773***
Box 1 (Store at –70°C)			
Control Total RNA (1 µg/µl)	5 µl	5 µl	4 x 5 µl
Box 2 (Store at –20°C)			
SMART-Seq v4 Oligonucleotide (48 µM)	24 µl	96 µl	4 x 96 µl
PCR Primer II A (12 µM)	24 µl	96 µl	4 x 96 µl
5X Ultra® Low First Strand Buffer	96 µl	384 µl	4 x 384 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	48 µl	192 µl	4 x 192 µl
3′ SMART-Seq CDS Primer II A (12 µM)	48 µl	192 µl	4 x 192 µl
RNase Inhibitor (40 U/µl)	60 µl	240 µl	4 x 240 µl
Nuclease-Free Water	2 x 1 ml	4 ml	4 x 4 ml
10X Lysis Buffer†	460 µl	1.85 ml	4 x 1.85 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)‡	2 x 1.7 ml	2 x 6.8 ml	8 x 6.8 ml
SeqAmp™ DNA Polymerase	50 µl	200 µl	4 x 200 µl
SeqAmp CB PCR Buffer (2X)	1.25 ml	5 ml	4 x 5 ml
SMART-Seq Library Prep Kit (Store at –20°C)	**634764§**	**R400747§**	**4 x R400747§**
10X FE	24 µl	60 µl	4 x 60 µl
FE Dilution Buffer	250 µl	1 ml	4 x 1 ml
Lib Prep Buffer	120 µl	480 µl	4 x 480 µl
Lib Prep Enzyme	70 µl	280 µl	4 x 280 µl
Rxn Enhancer	100 µl	400 µl	4 x 400 µl
Stem Loop Adapters	120 µl	480 µl	4 x 480 µl
Amplification Buffer	600 µl	2 x 1.2 ml	8 x 1.2 ml
PrimeSTAR® HS DNA Polymerase (5 U/µl)	30 µl	120 µl	4 x 120 µl
Nuclease-Free Water	1 ml	4 x 1 ml	16 x 1 ml

*Also sold separately.
†Store 10X Lysis Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
‡Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at room temperature.
§Not sold separately.

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, 200 µl, and 1,000 µl
- Eight-channel or 12-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Minicentrifuge for 1.5-ml tubes
- Minicentrifuge for 0.2-ml tubes or strips
- 96-well PCR chiller rack, such as IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4) or 96-well Aluminum Block (Light Labs, Cat. No. A-7079)
- Unique Dual Index Kits (Cat. Nos. 634752–634756)
- NucleoMag NGS Clean-up and Size Select Beads (Takara Bio, Cat. Nos. 744970.50, 744970.5, or 744970.500)

  If the NucleoMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter, Cat. Nos. A63880 & A63881) is an appropriate substitute.

NOTES:
- The kit has been specifically validated with the beads listed above. Please do not make any substitutions as it may lead to unexpected results.
- We strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment and decreases the chances of contamination.
- Magnetic beads should only be used at room temperature. Individual aliquoted tubes can be removed 30 minutes before an experiment to bring the beads to room temperature.

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 additional plates

For cDNA Synthesis and Amplification

- Deionized water
- Two thermal cyclers with heated lids
  - One thermal cycler used only for first-strand cDNA synthesis (Section V.A)
  - One thermal cycler used only for double-stranded cDNA amplification by PCR (Section V.B) and library amplification (Section VI.B)

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, Cat. No.1402-4700) or similar nuclease-free, PCR-grade, thin-wall PCR tubes, strips, or 96-well plates
- Nuclease-free, low-adhesion 1.5-ml tubes (USA Scientific, Cat. No. 1415-2600), DNA LoBind tubes (Eppendorf, Cat. No. 022431021), or similar nucleic acid low-binding tubes
- Thermo Scientific Adhesive PCR Plate Seals (Thermo Fisher, Cat. No. AB0558) for 96-well plates or cap strips (Thermo Fisher, Cat. No. AB0784/AB0850) for 8-tube strips

For cDNA and Amplified Library Purifications

- 80% ethanol: freshly made for each experiment from molecular-biology-grade 100% ethanol
- Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates, such as:
  - For 12–24 samples:
    - SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011); accommodates two 8-tube or 12-tube strips
    - Minicentrifuge for 0.2-ml tubes or strips
  - For 24–96 or more samples:
    - Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher Scientific, Cat. No. AB0558)
    - Low-speed benchtop centrifuge for a 96-well plate
  - For 1.5 ml tubes (for pooling sequencing libraries):
    - Magnetic Stand (Takara Bio, Cat. No. 631964)
  - 8-tube strips (Thermo Fisher Scientific, Cat. No. AB0264) or other nuclease-free, PCR-grade tube strips secured into a PCR rack, or 96-well plates that have been validated to work with your FACS instrument

For cDNA and Illumina Library Quantification (As Required)

- Instrument and reagents for assessing library quality (may be used in both Sections V.C. and VI.D):
  - Agilent 2100 Bioanalyzer and Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
    or
  - Agilent Fragment Analyzer System and Agilent HS Large Fragment Kit (Agilent, Cat. No. DNF-493-0500)
    or
  - Tapestation instrument (Agilent) and D5000 High Sensitivity ScreenTape (Agilent Cat. No. 5067-5588) with D5000 Reagents (5067-5589)
- 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.D 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, first-strand cDNA synthesis (Section V.A) and set up prior to amplification (Sections V.B).

  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 amplification (Section V.B, Step 4), perform cDNA purification (Section V.C), measure cDNA concentration (Section V.D), and prepare the sequencing libraries (Section VI).

B. General Requirements

- **IMPORTANT!** For products Cat. No. 634769 (96 rxns) and Cat. No. 634771 (4 x 96 rxns), we recommend performing a **minimum** of 12 reactions per protocol run to ensure sufficient reagents to utilize 96 (or 384) reactions per kit.
- **The success of your experiment depends on the quality of your input RNA. Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants.**
- The assay is very sensitive to variations in pipetting volume. Please make sure all your pipettes are calibrated for reliable delivery.
- All lab supplies related to the cDNA synthesis reaction need to be stored in a nucleic-acid-free and nuclease-free closed cabinet.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not change the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the cDNA synthesis reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that the kit components are working properly.

C. Sample Recommendations and Requirements

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

1. Cell Inputs

- This protocol has been validated to generate cDNA starting from intact cells sorted by fluorescence-activated cell sorting (FACS) into 96-well plates or PCR strips. **It cannot be used with cells that have undergone fixation.**
- For the removal of media prior to dilution or FACS, bulk cell suspensions should be washed and resuspended in Mg^{2+}- and Ca^{2+}-free PBS, as the presence of media can interfere with the

first-strand synthesis. It is best to perform at least two washes. If necessary, test the effect of your media or FACS buffer on cDNA synthesis by performing a reaction with control RNA and the estimated amount of media that you expect to accompany your cells (See Appendix A).

- Following appropriate washes, cells can be diluted in BD FACS Pre-Sort Buffer (BD Biosciences, Cat. No. 563503) to maintain cells in a single-cell suspension before FACS.
- **Cells should be sorted into 12.5 µl of our recommended sorting buffer (see Section V.A). If you do not wish to include the CDS IIA oligo in the lysis buffer or if cells are aliquoted in a different buffer, please follow the recommendations in Appendix A, Sections A and B, respectively.**

2. Total RNA Input

- **RNA should be in a maximum volume of 10.5 µl**.
- This protocol has been optimized for cDNA 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 100 ng). Purified total RNA should be in nuclease-free water and free of contamination.
- The sequence complexity and the average length of the cDNA are dependent on the quality of the starting RNA material. Due to the limited sample size, most traditional RNA isolation methods may not be applicable. Several commercially available products enable purification of total RNA preparations from extremely small samples [e.g., 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 to ensure the kit performs as expected and essential for troubleshooting experiments. The best positive control has a similar RNA input mass as your experimental samples (e.g., 10 pg of RNA is a good estimate for a cell). Until you are comfortable with the protocol, you may want to test two positive control inputs (e.g., 10 pg and 100 pg). Serial dilution is essential to ensure an accurate concentration of the final dilution. Follow the guidelines below to reach a single cell equivalent of 10 pg. When used with 17 cycles of PCR, 10 pg of the Control Total RNA included in the kit should generate a cDNA yield of at least 200 pg/µl.

NOTE: Make fresh dilutions before each use. If desired, make single-use aliquots of the 50 ng/µl dilution generated in Step 2 (below) and store at –80˚C. When needed, thaw an aliquot, further dilute (Steps 3–6), and throw away any leftover. Make sure to change pipette tips for each dilution step described below. Use low nucleic acid binding tubes for all dilutions.

1. Prepare RNase Inhibitor Water (RI Water) by combining 396 µl of Nuclease-Free Water with 4 µl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.
2. Dilute Control Total RNA (mouse brain) to 50 ng/µl by mixing 38 µl of RI Water with 2 µl of Control Total RNA (1 µg/µl) in a sterile microcentrifuge tube.
3. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of RI Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.
4. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of RI Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.
5. Further dilute Control Total RNA to 10 pg/µl by mixing 120 µl of RI Water with 5 µl of 0.25 ng/µl Control Total RNA in a sterile microcentrifuge tube.
6. For example, use 1 µl of 10 pg/µl (from Step 5) Control Total RNA as a positive control RNA input for the kit and process along with the other samples.

V. cDNA Synthesis

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

A. Protocol: 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 first-strand cDNA synthesis 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.

   NOTE: Due to small pipetting volumes, prepare no less than 250 µl of sorting buffer, which is enough for up to 18 wells. Scale up as needed. Be sure to count any negative control reactions you wish to include.

   In this protocol, we are assuming that FACS sorting of the cells will not change the volume of liquid in the plate wells. If your sorter dispenses a non-negligible amount of sheath fluid, adjust the volume of the CSS mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 12.5 µl per well.

   Table 2. CDS Sorting Solution recipe.

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

	Per well	1–18 wells*
10X Lysis Buffer	0.95 µl	19.0 µl
RNase Inhibitor	0.05 µl	1.0 µl
3′ SMART-Seq CDS Primer II A†	2 µl	40.0 µl
Nuclease-Free Water†	9.5 µl	190.0 µl
Total volume	12.5 µl	250.0 µl

*Volumes include ~10% extra for overage and are based on use of 2 µl of 3′ SMART-Seq CDS Primer II A and 9.5 µl Nuclease-Free Water.

[†]If you are sorting single cells or performing ≥17 PCR cycles for cDNA amplification (see Section V.D, Table 4 for PCR cycling guidelines), add 1 µl of the 3′ SMART-Seq CDS Primer II A. Keep the total volume at 12.5 µl by increasing the volume of Nuclease-Free Water to 10.5 µl. Keep the volume of 10X Lysis Buffer and the RNase Inhibitor unchanged, regardless of the number of PCR cycles.

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

When ready to sort:

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

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

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

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

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

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

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

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

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

19 µl	10X Lysis Buffer
1 µl	RNase Inhibitor
20 µl	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 3 below for guidelines on setting up your positive and negative controls alongside your test samples. Transfer 1–9.5 µl of purified total RNA, cell sample, or control sample 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 3. Sample preparation guidelines

Components*	Negative control	Positive control	Experimental sample
10X Reaction Buffer	1 µl	1 µl	1 µl
Nuclease-Free Water†	9.5 µl	Up to 8.5 µl	Up to 8.5 µl
Diluted Control RNA‡	–	1–9.5 µl	–
Sample	–	–	1–9.5 µl
Total volume	10.5 µl	10.5 µl	10.5 µl

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

†If you are performing ≥17 PCR cycles (see Table 4 for PCR cycling guidelines), use 1 µl of the 3′ SMART-Seq CDS Primer II A. Keep the final reaction volume, which includes the primer, at 12.5 µl by increasing the volume of your sample (or control) to 10.5 µl, either by adding additional Nuclease-Free Water or increasing the volume of your sample. Keep the volume of 10X Reaction Buffer at 1 µl regardless of the number of PCR cycles.

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

3. Place the samples on ice and add 3′ SMART-Seq CDS Primer II A according to the note below. Mix well by gentle vortexing and then briefly centrifuge the plate/strips to collect the contents at the bottom of the tube.

   NOTE:

   – If you are performing ≤17 cycles, use 2 µl of 3′ SMART-Seq CDS Primer II A.

   – If you are sorting single cells or performing ≥17 PCR cycles for cDNA amplification (see Section V.D, Table 4), add 1 µl of primer.

4. Immediately proceed to Section V.C.

C. Protocol: First-Strand cDNA Synthesis

First-strand cDNA synthesis is primed by the 3′ SMART-Seq CDS Primer II A and uses the SMART-Seq v4 Oligonucleotide for template switching at the 5′ end of the transcript.

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

1. At room temperature, thaw the 5X Ultra Low First-Strand Buffer. On ice, thaw all the remaining reagents (except the enzyme) needed for first-strand cDNA synthesis. Gently vortex each reagent to mix and spin down briefly. Store all reagents on ice except the 5X Ultra Low First-Strand Buffer.

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

2. **(Optional)** If starting with frozen samples containing CSS, remove the samples (tubes or PCR strips containing the sorted cells) from the freezer and briefly spin to collect the contents at the bottom of the tube.

3. Incubate samples (either from Step 2 or Section V.B, Step 4) at 72°C in a preheated, hot-lid thermal cycler for 3 minutes. Immediately after the 3-min incubation at 72°C, place the samples on ice for at least 2 min (but no more than 10 min).

   NOTE: Prepare the RT Master Mix (Step 4) while your tubes are incubating. The SMARTScribe Reverse Transcriptase will be added just before use (Step 7). Steps 8 & 9 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 7.

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

   RT Master Mix:

4 µl	5X Ultra Low First-Strand Buffer
1 µl	SMARTSeq v4 Oligonucleotide (48 µM)
0.5 µl	RNase Inhibitor (40 U/µl)
2 µl	SMARTScribe Reverse Transcriptase*
7.5 µl	Total volume

   *Add the SMARTScribe Reverse Transcriptase during Step 7, just prior to use.

5. Preheat the thermal cycler to 42°C.

6. Add the SMARTScribe Reverse Transcriptase to the RT Master Mix.

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

7. Add 7.5 µl of the Master Mix to each sample. Mix the contents of the tubes by gently vortexing and spin briefly to collect the contents at the bottom of the tubes.

8. Place the tubes in a thermal cycler preheated to 42°C. The thermocycler lid should preheated to at least 10°C higher than 70°C to accommodate the inactivation step. Run the following program:

42°C	90 min
70°C	10 min
4°C	forever

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

D. Protocol: cDNA Amplification by LD-PCR

The PCR Primer II A amplifies the cDNA by priming to the sequences introduced by the 3′ SMART-Seq CDS Primer II A and the SMART-Seq v4 Oligonucleotide.

IMPORTANT: Table 4 provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for the first-strand cDNA synthesis. These guidelines were determined using the Control Mouse Brain Total RNA. Typical cycle numbers are provided as a rough guide for working with small amounts of RNA. Optimal parameters may vary for different templates, different cell types, and different thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles. See Appendix B for PCR optimization suggestions.

Table 4. Cycling guidelines based on amount of starting material

Input amount of Total RNA	Input amount of cells	Typical number of PCR cycles
100 ng	—	5–6
10 ng	1,000 cells	7–8
1 ng	100 cells	10–11
100 pg	10 cells	14–15
10 pg	1 cell	17–18

1. Thaw SeqAmp CB PCR Buffer and PCR Primer II A on ice. Do not thaw SeqAmp DNA Polymerase. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Prepare enough PCR Master Mix for all the reactions, plus 10% of the total reaction mix volume. Combine the following reagents in the order shown:

PCR Master Mix:

25 µl	SeqAmp CB PCR Buffer
1 µl	PCR Primer II A
3 µl	Nuclease-Free Water
1 µl	SeqAmp DNA Polymerase
30 µl	Total volume

NOTE: Remove the SeqAmp DNA Polymerase from the freezer, gently mix the tube without vortexing, and add to the PCR Master Mix just before use. Mix the master mix well by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube.

3. Add 30 µl of PCR Master Mix to each tube containing 20 µl of first-strand cDNA product from Section V.C. Mix well by gently vortexing, and briefly spin to collect the contents at the bottom of the tube.

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

4. Place the tubes in a preheated in a thermal cycler with a block preheated to 95°C and the lid heated to 105°C. Run the following program:

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 Table 4 for the recommended number of PCR cycles.

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

E. 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. 744970.50, 744970.5, or 744970.500) beads following the protocol outlined in steps below.

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

NOTES:

- Before each use, bring a 1.5 ml aliquot of NucleoMag NGS Clean-up and Size Select beads and the Elution Buffer to room temperature for at least 30 min. Mix the beads well by vortexing.
- Bead:sample ratio is 1:1.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- You will need 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 50 µl of beads to each sample. Mix thoroughly by vortexing for 3–5 sec or pipetting the entire volume up and down at least 10 times. Proceed to Step 2.

   (Optional) If you are performing purification with the Thermo Fisher Magnetic Stand-96 (recommended if processing 48–96 samples), cDNA samples need to be transferred to a 96-well V-bottom plate. Distribute 50 µl of beads to each well of the 96-well V-bottom plate, and then use a multichannel pipette to transfer the cDNA. Pipette the entire volume up and down at least 10 times to mix thoroughly. Proceed to Step 2.
2. Incubate mixture at room temperature for 8 min to let the cDNA bind to the beads.
3. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. (Centrifugation is generally not necessary if using a 96-well V-bottom plate as described in Step 1.)
4. Place the samples on the magnetic separation device for ~5 minutes or longer, until the liquid appears completely clear and there are no beads left in the supernatant.
5. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.

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

   NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you would like additional assistance with this determination.

   https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

   If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

10. Once the beads are dry, remove the samples from the magnetic separation device and add 17 µl of Elution Buffer to cover the bead pellet. Mix thoroughly by pipetting or gently vortexing to resuspend the beads.
11. Incubate at room temperature for at least 2 min to rehydrate.
12. Briefly spin the samples to collect the liquid from the sides of the tubes or plate wells. Place the samples back on the magnetic separation device for 1 min or longer, until the solution is completely clear.
13. Transfer clear supernatant (~15 µl) containing purified cDNA from each tube/well to a new tube/plate. Do not pool samples at this point. Take care not to carry over any beads with your sample.
14. Proceed to validation immediately or store at –20°C.

F. Protocol: Determination of cDNA Quality and Quantity

1. Aliquot 1 µl of the amplified cDNA for validation using an Agilent 2100 Bioanalyzer with an Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) a TapeStation instrument with a High Sensitivity D5000 ScreenTape (Agilent; 5067-5592). Please refer to the respective user manuals of these products for instructions.
2. Compare the results for your samples and controls (see Figure 4) to verify whether the sample is suitable for further processing.

   With the Agilent 2100 Bioanlayzer/Aligent Sensitivity DNA Kit, successful cDNA synthesis and amplification should yield no product in the negative control (Figure 4, Panel B) and a distinct peak spanning 400 bp to 10,000 bp, peaked at ~2,000–2,500 bp for the positive control RNA sample (Figure 4, Panel A).

   With Tapestation/High Sensitivity D5000 Screentape, successful cDNA synthesis, and amplification should yield no products in the negative control (Figure 4, Panel D) and a broad smear with a peak spanning 1,000 bp to 2,500 bp (Figure 4, Panel C).

3. Proceed to "Library Preparation for Illumina Sequencing" (Section VI).

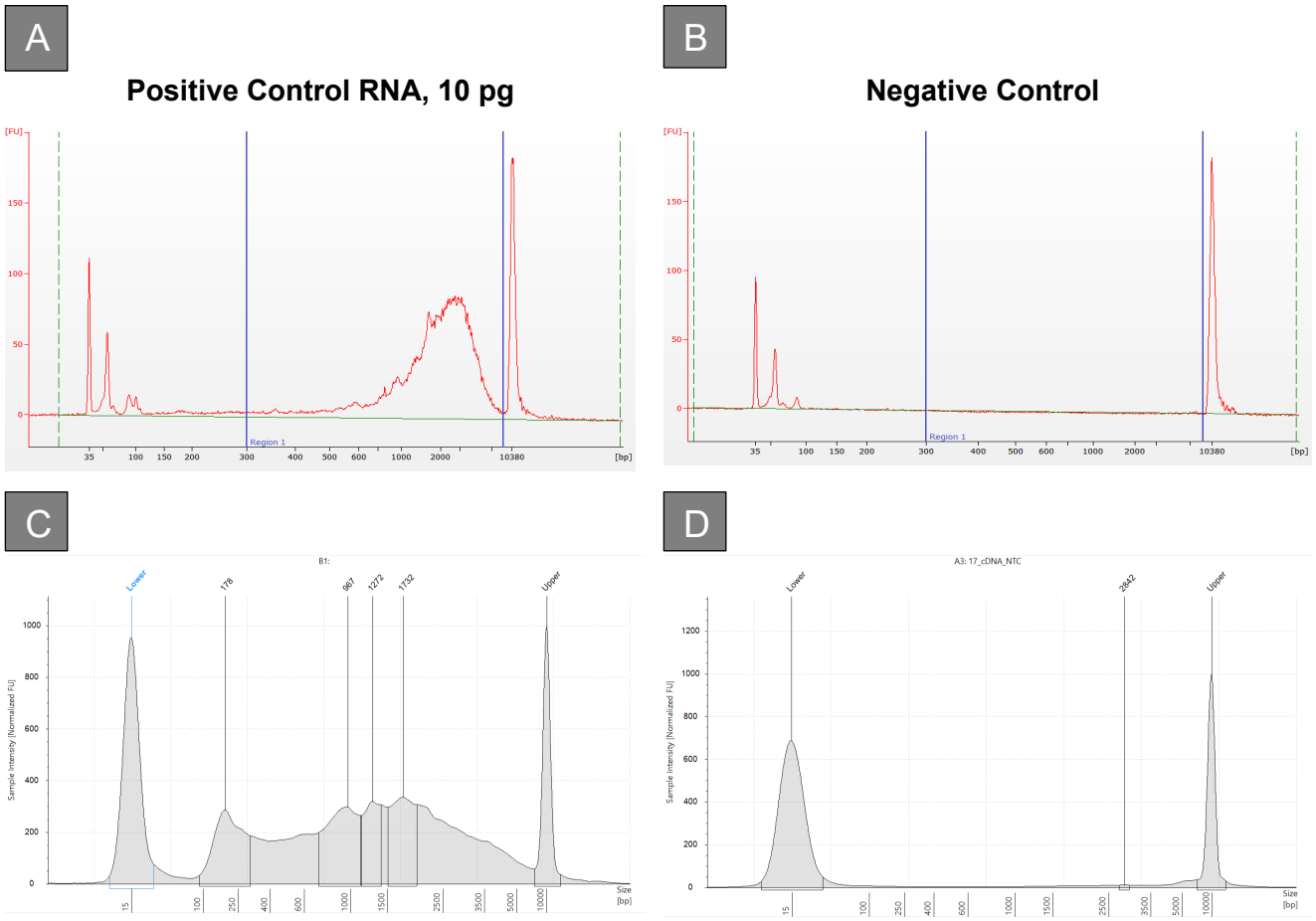

Figure 4. Example electropherogram analysis of cDNA after amplification and purification. All samples were subjected to SMART cDNA synthesis and amplification as described in the protocol. **Panel A.** Agilent 2100 Bioanalyzer/High Sensitivity DNA Kit electropherogram showing a clean product following cDNA synthesis and amplification (17 PCR cycles). **Panel B.** Aligent 2100 Bioanalyzer/High Sensitivity DNA Kit electropherogram shows no product in the negative control following cDNA synthesis and amplification (17 PCR cycles). **Panel C**. Tapestation 4150/High Sensitivity D5000 ScreenTape eletropherogram showing the cDNA synthesis and amplification products (17 PCR cycles) **Panel D.** Tapestation 4150/High Sensitivity D5000 ScreenTape eletropherogram showing no product in the negative control following cDNA synthesis and amplification (17 PCR cycles).

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 Kits (Cat. Nos. 634752–634756) are used in Section VI.B. Before starting, please read the:

- entire set of protocols in Section VI, below,
- Unique Dual Index Kits Protocol-at-a-Glance, that includes the instructions for UDI use, and
- Unique Dual Index Kit Indexes and Plate Map specific for the UDI plate product you are using. This XLSX file includes a plate map and barcode sequences for the UDIs included on the plate and can be accessed either on our website or through hyperlinks in the UDI Kit protocol document, above.

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

- 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 Kits (Cat. Nos. 634752–634756)
  - SMART-Seq Library Prep Kit components: FE Dilution Buffer, Lib Prep Buffer, Rxn Enhancer, Stem-Loop Adapters, and Amplification Buffer
  - SMART-Seq mRNA: Elution Buffer
- Keep the 10X FE, Library Prep Enzyme, and PrimeSTAR HS DNA Polymerase (5 U/µl) in the –20°C freezer until used. Use a benchtop cooler to keep the enzymes cold while working with them at the bench.
- We recommend using 8 µl 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.

NOTE: If stopping at the end of Section VI.A, keep the Unique Dual Index Kit , Amplification Buffer, and PrimeSTAR HS DNA Polymerase (5 U/µl) in the –20°C freezer until ready to begin Section VI.B.

1. 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.
2. On ice, add 4 µl of Stem-Loop 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.
3. Calculate the amounts of cDNA and Elution Buffer needed for each sample to achieve a concentration of between 0.125 ng/µl and 1.25 ng/µl in an 8 µl input volume.

   NOTE: The concentration of cDNA for each sample does not have to be the same as this may not be possible. However, if you plan to pool libraries prior to bead purification (Section VI.C and VI.D), we recommend the same cDNA input/concentration be used for each sample.
4. Dilute the cDNA of each sample as calculated in Step 3 using Elution Buffer.

   NOTES:
   - Always use a minimum of 2 µl of cDNA to perform the dilution. Samples containing less than 100 pg/µl cDNA can be used without dilution, but you may get fewer reads than for other samples if you pool your samples during bead clean-up.
   - The number of PCR cycles performed during library amplification (Section IV.B, Step 4) depends on the input cDNA amount. Refer to Table 5 for more information.
5. Add freshly diluted cDNA sample or Elution Buffer (as a negative control) from Step 4 to a tube/well containing the 4 µl of Stem-Loop Adapters (from Step 2); the total volume per reaction should be 12 µl.
6. On ice, prepare 1X FE by diluting the 10X FE in cold FE Dilution Buffer (~4°C) in a 1:9 ratio (1 part 10X FE to 9 parts FE Dilution Buffer). Prepare enough material to accommodate the Library Prep Master Mix in Step 6, plus 10% of the total reaction mix volume.

   NOTE: To allow for greater accuracy pipetting the 10X FE, a minimum of 40 µl of 1X FE Preparation should be prepared, which is enough for 36 rxns.

1X FE Preparation

	1 rxn	1–36 rxns*
FE Dilution Buffer	0.9 µl	36.0 µl
10X FE	0.1 µl	4.0 µl
Total volume	1.0 µl	40.0 µl

*Volumes include ~10% extra for overage.

Mix gently by pipetting up and down 10 times. Spin down and keep on ice. Immediately proceed with assembling the Library Prep Master Mix in Step 6.

6. On ice, prepare the Library Prep Master Mix for all reactions, plus 10% of the total reaction mix volume. Combine the following reagents in the order shown below.

Library Prep Master Mix:

4 µl	Library Prep Buffer
3.5 µl	Rxn Enhancer
2 µl	Library Prep Enzyme
1 µl	1X FE
10.5 µl	**Total volume per reaction**

NOTES:

- **Library Prep Master Mix is very viscous.** Ensure adequate mixing by gently 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/Stem-Loop Adapters mix or negative control/Stem-Loop 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 Stem-Loop Adapters and the Library Prep Master Mix.

8. Perform the library preparation reaction by placing the tubes/plate in a thermal cycler with a block precooled to 20°C and the lid heated to 105°C. Run 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 transferred to –20°C for up to a week.

B. Protocol: Library Amplification

1. If the samples were stored at –20°C, thaw the samples and the following items on ice:

- The Unique Dual Index Kit being used
- Amplification Buffer from the SMART-Seq Library Prep Kit

NOTE: Keep the PrimeSTAR HS DNA Polymerase (5 U/µl) in the –20°C freezer until used. Use a benchtop cooler to keep the enzyme cold while working with it at the bench.

2. On ice, prepare the Library Amplification Master Mix for all reactions, plus 10% of the total reaction mix volume.

Library Amplification Master Mix:

21.5 µl	Amplification Buffer
1 µl	PrimeSTAR HS DNA Polymerase (5 U/µl)
3 µl	Deionized water
25.5 µl	**Total volume per reaction**

BEFORE PROCEEDING: Please refer to the Unique Dual Index Kits Protocol-at-a-Glance, which contains more information about the use of the Unique Dual Index Kit.

3. Add 25.5 µl of the Library Amplification Master Mix to each reaction from Section VI.A, Step 8.
4. Add 2 µl of a different index from the Unique Dual Index Kit 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.
5. Place the tubes in a thermal cycler with a heated lid (105°C) and perform PCR amplification using the following program:

72°C		3 min
85°C		2 min
98°C		2 min
12–16 cycles:*		
	98°C	20 sec
	60°C	75 sec
68°C		5 min
4°C		Hold

*Please consult Table 5 (next page) for the recommended number of PCR cycles based on cDNA input amount.

Table 5. 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.

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

C. Protocol: Pooling and Purification of Amplified Libraries Using Double-Sided Bead Clean-Up

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).

After pooling, the PCR-amplified libraries are purified using NucleoMag NGS Clean-up and Size Select Beads. We provide protocols for both double-sided bead clean-up (below) and single-sided bead clean-up (Section VI.D). Double-sided bead clean-up will result in a narrower library size range and more consistent sequencing performance; however, many customers have successfully used a single-sided bead clean-up protocol, which is an adequate option for users who prefer it.

If you prefer to perform single-sided bead clean-up, please proceed to Section VI.D.

NOTES:

- 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.
- The ratio of beads to sample/supernatant for each side of the double-sided bead cleanup is 0.4X:1X.
- Prepare fresh 80% ethanol for each experiment. You need ~400 µl 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 the Unique Dual Index Kits Protocol-at-a-Glance, which contains more information about pooling strategies.

   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 µl are appropriate. See examples in Table 6.

   Table 6. Example volumes of pooled libraries and beads for double-sided bead cleanup.

Number of libraries to be pooled	Volume per library (µl)	Total pool volume (µl)	Bead volume: first round*	Supernatant transfer volume (µl)	Bead volume: second round*
8	8	64	25.6	80	32
12	4	48	19.2	60	24
16	4	64	25.6	80	32
24	2	48	19.2	60	24
32	2	64	25.6	80	32
48	2	96	38.4	125	50
96	2	192	76.8	260	104

*The bead volume is approximately 40% of the total pool volume (first round) or supernatant transfer volume (second round).

NOTES:

- Do not use less than 2 µl per sample to ensure greater accuracy.
- If pooling 96 samples, make sure to use a 1.5-ml tube.

2. Add a first volume of beads representing 40% of the volume of the pooled libraries. See Table 6 for guidance.

   NOTE: If cleaning up libraries individually, add 20 µl of beads to each 50 µl sample.

3. 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.

4. Incubate at room temperature for 5 min to let the library bind to the beads
5. 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.
6. While the samples are on the magnetic separation device, transfer the supernatant according to Table 6 to a clean tube. Take care not to disturb the beads.

   NOTE: If cleaning up libraries individually, transfer 60 µl of supernatant.

7. Add a second volume of beads representing 40% of the volume of the transferred supernatant. See Table 6 for guidance.

   NOTE: If cleaning up libraries individually, add 24 µl of beads to each supernatant that was collected.

8. Mix well by vortexing or pipetting the entire mixture up and down 10 times.
9. Incubate at room temperature for 5 min to let the library bind to the beads.
10. 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.
11. While the samples are on the magnetic separation device, remove and discard the supernatant. Take care not to disturb the beads.
12. 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.
13. Repeat the ethanol wash (Step 12) once more.
14. 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.
15. Incubate the samples at room temperature for ~5–15 min, until the pellet is no longer shiny, but before cracks appear.

    NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

    https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

    If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

16. 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, according to Table 7.

Table 7. Example volumes of Nuclease-Free Water to resuspend libraries in.

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

*Nuclease-Free Water volume is 50% of the original pool volume (Column 3, Table 6).

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

17. 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.

18. 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.

    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: Pooling and Purification of Amplified Libraries Using Single-Sided Bead Clean-Up

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).

After pooling, the PCR-amplified libraries are purified using NucleoMag NGS Clean-up and Size Select Beads. We provide protocols for both double-sided bead clean-up (below) and single-sided bead clean-up (Section VI.C). Double-sided bead clean-up will result in a narrower library size range and more consistent sequencing performance; however, many customers have successfully used a single-sided bead clean-up protocol, which is an adequate option for users who prefer it.

If you have already performed double-sided bead clean-up, please proceed to Section VI.E. If you would like to perform double-sided bead clean-up instead of single-sided bead clean-up, please see Section VI.C.

NOTES:

- 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.
- The ratio of beads to sample is 0.8X.
- Prepare fresh 80% ethanol for each experiment. You need ~400 µl 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 the Unique Dual Index Kits Protocol-at-a-Glance, which contains more information about pooling strategies.

   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 µl are appropriate. See examples in Table 8.

   Table 8. Example volumes of pooled libraries and beads for single-sided bead cleanup.

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

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

   NOTES:
   - Do not use less than 2 µl per sample to ensure greater accuracy.
   - If pooling 96 samples, make sure to use a 1.5-ml tube.

2. Add a volume of beads representing 80% of the volume of the pooled libraries. See Table 8 for guidance.

   NOTE: If cleaning up libraries individually, add 40 µl of beads to each 50-µl sample.

3. 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.

4. Incubate at room temperature for 5 min to let the library bind to the beads.
5. 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.
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 sec, and then remove and discard the supernatant, taking care not to disturb the beads. The library remains bound to the beads during washing.
8. Repeat the ethanol wash (Step 8) once more.
9. 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.

10. Incubate the samples at room temperature for ~5–15 min, until the pellet is no longer shiny, but before cracks appear.

NOTE: Check the pellet frequently during this time and continue to Step 10 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

11. 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, according to Table 7.

Table 9. Example volumes of Nuclease-Free Water to resuspend libraries in.

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

*Nuclease-Free Water volume is 50% of the original pool volume (Column 3, Table 8).

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

12. 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.
13. 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.
14. 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.

E. 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 quantifying sequencing libraries.

A fragment analyzer, such as the Agilent 2100 Bioanalyzer or TapeStation instrument (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 either an Agilent 2100 Bioanalyzer/Agilent High Sensitivity DNA Kit or a Tapestation instrument/High Sensitivity D5000 ScreenTape. Please refer to the respective user manuals of these products for instructions.

For loading on Illumina NGS platforms, follow the "Denature and Dilute Libraries Guide" specific to Illumina sequencer you are using.

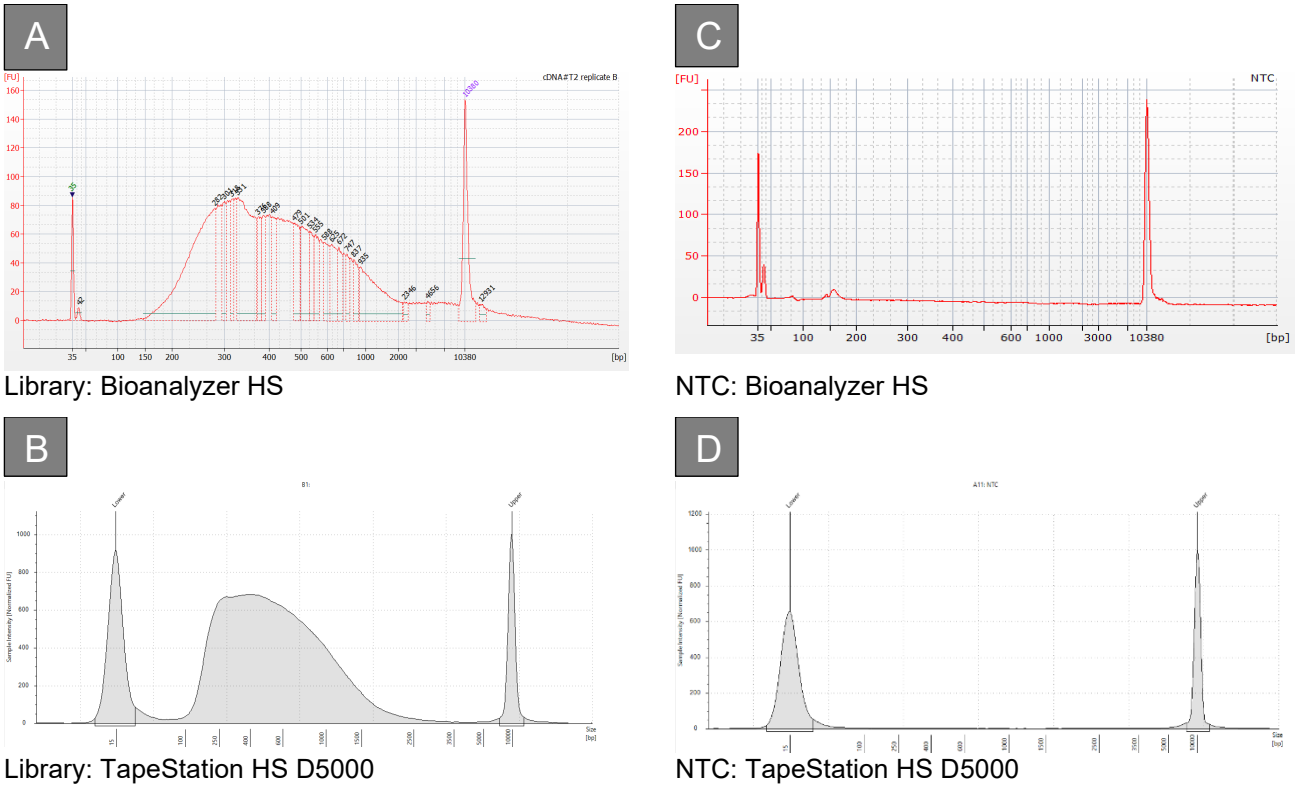

Figure 5. Example electropherogram analysis of libraries generated with the SMART-Seq Library Prep Kit. 3 ng of libraries or 1 µl of NTC control were analyzed using an Agilent 2100 Bioanalyzer/Agilent High Sensitivity DNA Kit (Bioanalyzer HS) or a TapeStation 4200/High Sensitivity D5000 ScreenTape (TapeStation 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**). Adapter dimers are observed below 200 bp when the NTC was ran on the TapeStation (**Panel D**). Libraries were prepared using double-sided bead clean-up (Section VI.C); however, using the single-sided bead clean-up protocol (Section VI.D) yielded similar results.

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 µl PBS. For recommendations on how to proceed after sorting into ≤5 µl of PBS, see Section B, below.

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

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

In this protocol, cells are sorted in 10.5 µl of buffer.

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

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

   - Due to small pipetting volumes, prepare no less than 210 µl of sorting buffer, which is enough for 18 wells
   - Scale up as needed for all reactions; include an additional 10% of the total reaction mix volume for overage
   - Be sure to include negative control reactions in the count for the number of wells to prepare

   Table 10. Plain Sorting Solution recipe

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

	Per well	1–18 wells*
10X Lysis Buffer	0.95 µl	19.0 µl
RNase Inhibitor	0.05 µl	1.0 µl
Nuclease-Free Water†	9.5 µl	190.0 µl
Total volume	10.5 µl	210.0 µl

   *Volumes include ~10% extra for overage and are based on use of 2 µl of 3′ SMART-Seq CDS Primer II A and 9.5 µl Nuclease-Free Water.
   †If you are sorting single cells or performing ≥17 PCR cycles for cDNA amplification (see Section V.D, Table 4 for PCR cycling guidelines), add 1 µl of the 3′ SMART-Seq CDS Primer II A in Section V.B, Step 3. Increase the total volume of the PSS to 11.5 µl by increasing the volume of Nuclease-Free Water to 10.5 µl. Keep the volume of 10X Lysis Buffer and the RNase Inhibitor unchanged, regardless of the number of PCR cycles.

2. Briefly mix gently, then spin down.

   NOTES:
   - The 10X Lysis Buffer contains a detergent; it is critical to avoid bubbles when mixing.
   - If you need to sort large numbers of cells compared to the number of cDNA reactions you plan to prepare, you have the option to purchase the 10X Lysis Buffer (Takara Bio, Cat. No. 635013) separately.

3. Aliquot 10.5 µl of PSS from Step 1 into the appropriate number of wells of PCR tube strips or a 96-well plate.

   NOTES:
   - To minimize bubble formation, set single- or multi-channel pipettes to 11.6 µl and pipette only to the first stop when aliquoting. Changing tips often also minimizes bubble formation.

- If using 1 µl of the 3′ SMART-Seq CDS Primer II A in Section V.B, Step 3, aliquot 11.5 µl PSS.

4. Seal the plate/tube strips with Microplate film, 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 min at a minimum and up to 24 hr. As the volume of sorting buffer is small, the tubes/plate should be kept at –20°C until just before sorting.

When ready to sort:

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

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

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

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

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

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

IMPORTANT: Since the PSS does not include the 3′ SMART-Seq CDS Primer II A, you need to add it in Section V.B, Step 3.

B. FACS Sorting into Nonvalidated Buffers

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

If using an alternative buffer, we recommend performing a pilot experiment using Control Total RNA and the estimated amount of buffer that you expect to accompany your cell(s) to determine its impact on cDNA synthesis. Sorting cells in 1 µl of 1X PBS is acceptable and will not interfere with kit performance. If it is desired to sort cells in >1 µl of 1X PBS, it is critical to keep the PBS volume below 5 µl. PBS is not an optimal sorting buffer as it interferes with the efficiency of cDNA synthesis and PCR amplification; thus, kit performance (cDNA yield and sensitivity) will be impacted (when using >1 µl of PBS). If you must use 2–5 µl of PBS, the cDNA yield will be lower, and you should consider adding one to three extra PCR cycles to the recommended number of cycles in Table 4 (Section V.D). In addition, you must use

PBS without Ca^{2+} and Mg^{2+} (e.g., Sigma, Cat. No. D8537). The addition of RNase Inhibitor in a ratio similar to what is recommended for CDS and PSS may be helpful, although not essential.

When your samples are not in a recommended FACS sorting buffer, we still recommend flash freezing samples on dry ice as quickly as possible after collection and storing them at –80°C until processing. Follow the instructions outlined in Section V.B. for preparing a 10X Reaction Buffer, setting up control reactions, and adding 3′ SMART-Seq CDS Primer II A.

Appendix B. PCR Optimization

If you have a sufficient amount of starting material (>1 ng total RNA), we recommend optimizing the PCR cycling parameters for your experiment. If you have a very limited amount of material or your sample is unique, use a similar source of RNA or cells to perform PCR cycle optimization prior to using your actual sample. Choosing the optimal number of PCR cycles ensures that the amplification will remain in the exponential phase. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA can result in a less representative cDNA library. Undercycling, on the other hand, results in a lower cDNA yield. The optimal number of cycles for your experiment is one cycle fewer than is needed to reach the plateau. Be conservative; when in doubt, it is better to use too few cycles than too many.

To perform PCR cycle optimization, prepare several tubes containing an amount of RNA equal to your sample amount. Subject each tube to a different range of PCR cycles. For example, if you have 1 ng of RNA, subject one tube to the recommended a number of cycles. Subject the other two tubes to 2–3 cycles fewer or more than the first tube (e.g., 12, 10, and 8 cycles, *N*) for a 1 ng sample.

1. Use the following program for thermal cycling:

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

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

Appendix C. Expected Results When Using a Single Cell as Input

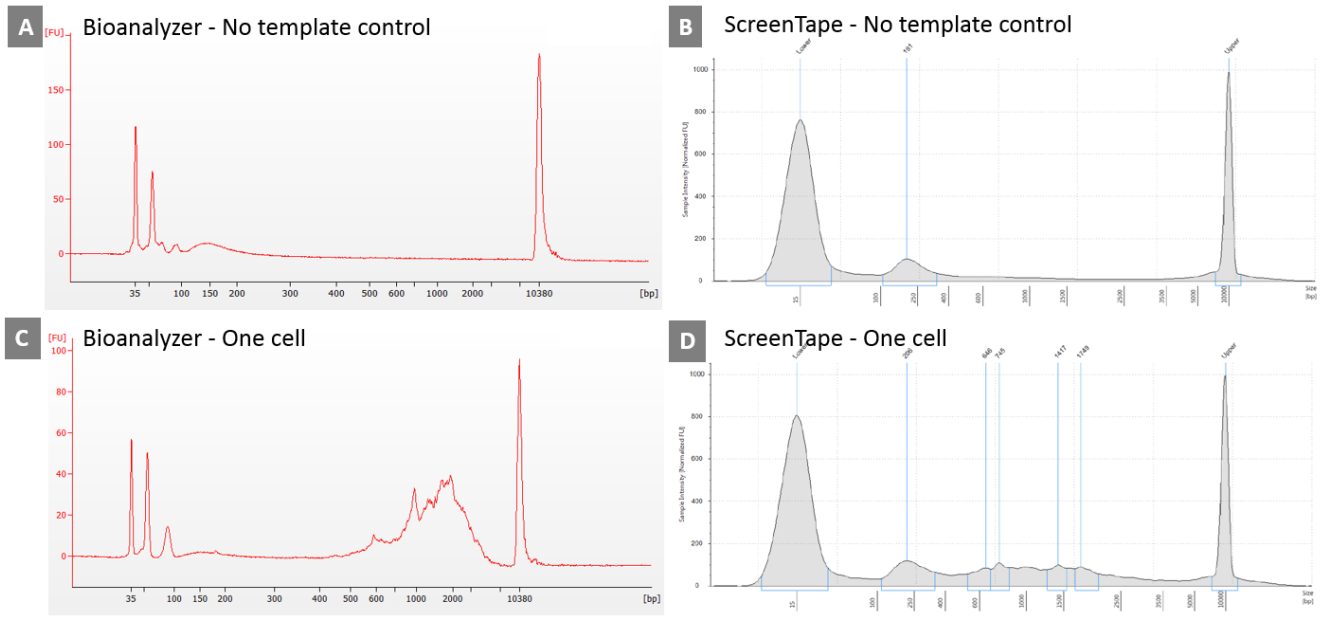

Figure 6. Example electropherogram analysis of single-cell input cDNA produced using SMART-Seq technology. All samples were subjected to one-step RT-PCR as described in the SMART-Seq HT Kit (Takara Bio, Cat. No. 634437) protocol, using 18 PCR cycles. (cDNA was not generated with SMART-Seq mRNA LP, but rather the SMART-Seq HT Kit, which features a similar technology compatible with cDNA synthesis from single cells and generates a cDNA profile similar to SMART-Seq mRNA LP.) After bead purification, 1 µl of the sample was analyzed using Agilent's Bioanalyzer High Sensitivity DNA Kit (**Panels A and C**) or the High Sensitivity D5000 ScreenTape (**Panels B and D**). The cDNA generated with the SMART-Seq HT Kit may show a small amount of PCR product between 100 and 200 bp, as shown in the example here, but cDNA generated with SMART-Seq mRNA LP should be relatively flat in this region.

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 the 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 150 bp.	Presence of remaining primer dimers due to insufficient bead cleanup.	Perform bead cleanup using a bead:sample ratio of 0.7x.

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