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I. **Introduction**

**cDNA Synthesis Using Template Switching Technology**

The **SMART-Seq Single Cell Kit** (Cat. Nos. 634470, 634471 & 634472) is designed to generate high-quality, full-length cDNA directly from single cells, especially those with low RNA content. This kit provides the benefit of generating cDNA that is compatible with Illumina® platform-specific library preparation kits. The cDNA synthesis protocol can be completed within one day, and the entire library construction protocol can be completed within two days (Figure 1).

**Figure 1. SMART-Seq Single Cell Kit protocol overview.**

The SMART-Seq Single Cell Kit incorporates our proprietary SMART® (Switching Mechanism at 5’ end of RNA Template) technology, which 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 allows direct cDNA synthesis from intact cells.

The SMART-Seq Single Cell Kit has been specifically created for single-cell applications and to generate mRNA-seq libraries that clearly outperform previously published protocols (including Smart-seq2 [Picelli et al. 2013]) and existing commercial kits. The SMART-Seq Single Cell Kit has been optimized to provide improved performance for cells with very low RNA content, such as PBMCs. Each lot of the kit has been validated with 2 pg of mouse brain total RNA as input.
For more information on SMART technology and to find application data for the SMART-Seq Single Cell Kit, please visit takarabio.com. A schematic outline of the technology is shown in Figure 2.

Figure 2. Schematic of the technology in the SMART-Seq Single Cell Kit. SMART technology is used in a ligation-free workflow to generate full-length cDNA. Non-templated nucleotides (indicated by Xs) added by the SMARTScribe II Reverse Transcriptase (RT) hybridize to the SMART-Seq single-cell template-switching oligonucleotide (SMART-Seq sc TSO), 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 TSO—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).
II. **List of Components**

The components in the SMART-Seq Single Cell 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.

<table>
<thead>
<tr>
<th>SMART-Seq Single Cell Kit</th>
<th>634470 (12 rxns)</th>
<th>634471 (48 rxns)</th>
<th>634472 (96 rxns)</th>
</tr>
</thead>
</table>

**Box 1 (Store at –70°C)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Total RNA*</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

**Box 2 (Store at –20°C)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Lysis Buffer</td>
<td>230 µl</td>
<td>920 µl</td>
</tr>
<tr>
<td>RNase Inhibitor (40 U/µl)</td>
<td>30 µl</td>
<td>120 µl</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>1 ml</td>
<td>2 x 1 ml</td>
</tr>
<tr>
<td>SMART-Seq sc TSO</td>
<td>12 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>3’ SMART-Seq CDS Primer II A</td>
<td>25 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>SMARTScribe II Reverse Transcriptase</td>
<td>25 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>SMART-Seq sc First Strand Buffer (5X)</td>
<td>50 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>SeqAmp DNA Polymerase</td>
<td>50 µl</td>
<td>2 x 50 µl</td>
</tr>
<tr>
<td>SeqAmp CB PCR Buffer (2X)</td>
<td>1.25 ml</td>
<td>2 x 1.25 ml</td>
</tr>
<tr>
<td>PCR Primer</td>
<td>12 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Elution Buffer (10 mM Tris-Cl, pH 8.5)</td>
<td>1.7 ml</td>
<td>1.7 ml</td>
</tr>
</tbody>
</table>

*The Control Total RNA is from mouse brain.

**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 12-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, such as 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 Scientific, Cat. No. AB0264) or other nuclease-free, PCR-grade tube strips secured in a PCR rack, or 96-well plates that have been validated to work with your FACS instrument (e.g., Bio-Rad iQ 96-Well PCR Plates, Cat. No. 2239441)
- Microplate film (USA Scientific, Cat. No. 2920-0010) for sealing tube strips/plates before sorting
- Aluminum single tab foil seal (USA Scientific, Cat. No. 2938-4100) or cap strips (Thermo Fisher Scientific, 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 (Takara Bio, Cat. No. 634439) or 10X Lysis Buffer (Takara Bio, Cat. No. 635013) for sorting additional plates

For cDNA Synthesis & 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, 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

For Bead Purifications

- Recommended: Agencourt AMPure XP PCR purification kit (Beckman Coulter; 5-ml size, Cat. No. A63880 or 60-ml size, Cat. No. A63881)
NOTE:
- The SMART-Seq Single Cell Kit has been specifically validated with AMPure XP beads. Please do not make any substitutions as this 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 from the fridge 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 Scientific, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher Scientific, Cat. No. AB0558)
    - Low-speed benchtop centrifuge for a 96-well plate
  - For 1.5-ml tubes (for pooling Nextera® sequencing libraries):
    - Magnetic Stand (Takara Bio, 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.D and Appendix B)
- 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, i.e., sample preparation and first-strand cDNA synthesis (Section V.A)

  **NOTE:** 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 amplification (Section V.B) and cDNA purification (Section V.C), 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 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 Single Cell Kit works best with single cells. This protocol is not suitable for processing large pools of cells or purified RNA >100 pg. If you wish to use this kit for higher inputs, consult our tech support for recommendations.

- We recommend processing at least 12 samples at a time so the reagents can be pipetted accurately.

- This protocol has been validated to generate cDNA starting from intact cells sorted by fluorescence-activated cell sorting (FACS) into 96-well plates or PCR strips. **It cannot be used with cells that have undergone fixation.**

- For the removal of media prior to dilution or FACS, bulk cell suspensions should be washed and resuspended in Mg\(^{2+}\)- and Ca\(^{2+}\)-free PBS, as the presence of media can interfere with the first-strand synthesis. It is best to perform at least two washes. If necessary, test the effect of your media or FACS buffer on cDNA synthesis by performing a reaction with control RNA and the estimated amount of media that you expect to accompany your cell(s).

- 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.

- Cells should be sorted into 12.5 µl of our recommended sorting buffer (see Appendix A, Section 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, Sections B and C, respectively.
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. For example, 2 pg of RNA is a good starting point for single immune cells, while 10–15 pg may be more appropriate for many cell lines (e.g., K-562, HEK293, etc.). Serial dilution is essential to ensure accurate concentration of the final dilution. Follow the guidelines below to reach a concentration of 1 pg/μl. When used with 19 cycles of PCR, 2 pg of the Control Total RNA included in the kit should generate a cDNA yield of at least 500 pg/μl.

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

1. Prepare RNase Inhibitor Water (RI Water) by combining 396 μl of Nuclease-Free Water with 4 μl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.

2. Dilute Control Total RNA (mouse brain) to 50 ng/μl by mixing 38 μl of RI Water with 2 μl of Control Total RNA (1 μg/μl) in a sterile microcentrifuge tube.

3. Further dilute Control Total RNA to 5 ng/μl by mixing 45 μl of RI Water with 5 μl of 50 ng/μl Control Total RNA in a sterile microcentrifuge tube.

4. Further dilute Control Total RNA to 0.25 ng/μl by mixing 95 μl of RI Water with 5 μl of 5 ng/μl Control Total RNA in a sterile microcentrifuge tube.

5. Further dilute Control Total RNA to 10 pg/μl by mixing 120 μl of RI Water with 5 μl of 0.25 ng/μl Control Total RNA in a sterile microcentrifuge tube.

6. Further dilute Control Total RNA to 1 pg/μl by mixing 45 μl of RI Water with 5 μl of 10 pg/μl Control Total RNA in a sterile microcentrifuge tube.

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

NOTE: Please read the entire set of protocols before starting. The protocols described in this user manual have been optimized for cDNA synthesis, using the SMART-Seq Single Cell Kit only, from single intact cells. Due to the sensitivity of these protocols, cells need to be collected under the cleanest environment possible to avoid contamination. The whole process of cDNA synthesis should be carried out in a PCR clean work station under clean-room conditions.

A. 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 sc TSO for template switching at the 5’ end of the transcript.

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

1. At room temperature, thaw the SMART-Seq sc First Strand Buffer. On ice, thaw all the remaining reagents (except the enzyme) needed for first-strand cDNA synthesis: 10X Lysis Buffer, RNase Inhibitor, Nuclease-Free Water, SMART-Seq sc TSO, and 3’ SMART-Seq CDS Primer II A (if needed; see Table I, below). Gently vortex each reagent to mix and spin down briefly. Store all reagents on ice except the SMART-Seq sc First Strand Buffer.

NOTE: The SMART-Seq sc First Strand Buffer forms precipitates. Keep this buffer at room temperature until ready to use. Make sure to vortex before using to ensure all components are completely in solution.

2. Prepare a stock solution of 10X Reaction Buffer (scale up as needed):

\[
\begin{align*}
19 \mu l & \quad 10X \text{ Lysis Buffer} \\
1 \mu l & \quad \text{RNase Inhibitor} \\
20 \mu l & \quad \text{Total volume}
\end{align*}
\]

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.

3. See Table I below for guidelines on setting up your positive and negative controls alongside your cell samples. Prepare each sample (12.5 µl total volume) in 8-well strips or a 96-well plate.
Table I. Sample preparation guidelines.

<table>
<thead>
<tr>
<th>Components</th>
<th>Negative control</th>
<th>Positive control</th>
<th>Cells in CSS*</th>
<th>Cells in PSS†</th>
<th>Cells in other buffer‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Reaction Buffer (Step 2)</td>
<td>1 µl</td>
<td>1 µl</td>
<td>–</td>
<td>–</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>10.5 µl</td>
<td>8.5 µl</td>
<td>–</td>
<td>–</td>
<td>Up to 9.5 µl</td>
</tr>
<tr>
<td>Diluted Control Total RNA§</td>
<td>–</td>
<td>2 µl</td>
<td>12.5 µl</td>
<td>11.5 µl</td>
<td>1–9.5 µl</td>
</tr>
<tr>
<td>Cell sample</td>
<td>–</td>
<td>–</td>
<td>12.5 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>3′ SMART-Seq CDS Primer II A</td>
<td>1 µl</td>
<td>1 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
<td>12.5 µl</td>
</tr>
</tbody>
</table>

* CDS Sorting Solution (CSS) is described in Appendix A, Section A. This buffer already contains the 3′ SMART-Seq CDS Primer II A and 10X Reaction Buffer.
† Plain Sorting Solution (PSS) is described in Appendix A, Section B. PSS does not contain the 3′ SMART-Seq CDS Primer II A, so it must be added here.
‡ Other buffers will not generate optimal results and are not recommended. See Appendix A, Section C for guidance if you must use a non-validated buffer.
§ 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. To minimize pipetting errors, use 2 µl of the diluted control RNA. For example, dilute to 1 pg/µl and use 2 µl.

4. When ready to start, remove the samples (plate or PCR strips containing the sorted cells) from the freezer and briefly spin to collect the contents at the bottom of the tubes.

5. (Optional) If cells were not sorted in CSS, place the samples on ice and add any necessary remaining reagents, including 1 µl of 3′ SMART-Seq CDS Primer II A. Mix well by gently vortexing and then spin the tube(s) briefly to collect the contents at the bottom of the tube.

NOTE: If more than one reagent needs to be added (i.e., the 10X Reaction Buffer, the 3′ SMART-Seq CDS Primer II A, and extra Nuclease-Free Water), they can be pooled in a master mix and incorporated into each sample as a single addition.

6. Immediately incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min.

NOTE: Prepare your RT Master Mix (Step 7) while your tubes are incubating. The SMARTScribe II Reverse Transcriptase will be added just before use (Step 10). Steps 11 & 12 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 10.

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

RT Master Mix:

<table>
<thead>
<tr>
<th></th>
<th>1 rxn</th>
<th>12 rxns*</th>
<th>48 rxns*</th>
<th>96 rxns*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMART-Seq sc First Strand Buffer</td>
<td>4 µl</td>
<td>52.8 µl</td>
<td>211.2 µl</td>
<td>422.4 µl</td>
</tr>
<tr>
<td>SMART-Seq sc TSO</td>
<td>1 µl</td>
<td>13.2 µl</td>
<td>52.8 µl</td>
<td>105.6 µl</td>
</tr>
<tr>
<td>RNase Inhibitor (40 U/µl)</td>
<td>0.5 µl</td>
<td>6.6 µl</td>
<td>26.4 µl</td>
<td>52.8 µl</td>
</tr>
<tr>
<td>SMARTScribe II Reverse Transcriptase†</td>
<td>2 µl</td>
<td>26.4 µl</td>
<td>105.6 µl</td>
<td>211.2 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>7.5 µl</td>
<td>99 µl</td>
<td>396 µl</td>
<td>792 µl</td>
</tr>
</tbody>
</table>

* Includes 10% overage.
† Add the SMARTScribe II Reverse Transcriptase in Step 10, just prior to use.

8. 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).
9. Preheat the thermal cycler to 42°C.

10. Add the SMARTScribe II 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.

11. Add 7.5 μl of the RT 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.

12. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

    - 42°C  180 min
    - 70°C  10 min
    - 4°C   forever

    **STOPPING POINT:** The tubes can be stored at 4°C overnight.
B. Protocol: cDNA Amplification by LD PCR

The PCR Primer amplifies the cDNA by priming to the sequences introduced by the 3’ SMART-Seq CDS Primer II A and the SMART-Seq sc TSO.

**IMPORTANT:** Table II provides guidelines for the number of PCR cycles appropriate for the cell type used for the first-strand cDNA synthesis. When using good-quality control RNA such as the Control Total RNA from the kit (mouse brain total RNA), 2 pg will generate ~500–1,500 pg/µl if using 19 cycles. It is best to choose a number of cycles that will yield <1,500 pg/µl.

### Table II. Cycling guidelines based on cell type.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Approximate RNA equivalent (per cell)</th>
<th>Typical number of PCR cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-562 or HEK293 cells</td>
<td>10–15 pg</td>
<td>17</td>
</tr>
<tr>
<td>PBMCs</td>
<td>1–5 pg</td>
<td>20</td>
</tr>
<tr>
<td>Jurkat cells</td>
<td>5 pg</td>
<td>17</td>
</tr>
<tr>
<td>Lymphoblastoid cells</td>
<td>2–15 pg</td>
<td>17–19</td>
</tr>
</tbody>
</table>

1. Thaw SeqAmp CB PCR Buffer and PCR Primer 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:**

<table>
<thead>
<tr>
<th>SeqAmp CB PCR Buffer (2X)</th>
<th>1 rxn</th>
<th>12 rxns*</th>
<th>48 rxns*</th>
<th>96 rxns*</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl</td>
<td>330 µl</td>
<td>1,320 µl</td>
<td>2,640 µl</td>
<td></td>
</tr>
<tr>
<td>1 µl</td>
<td>13.2 µl</td>
<td>52.8 µl</td>
<td>105.6 µl</td>
<td></td>
</tr>
<tr>
<td>1 µl</td>
<td>13.2 µl</td>
<td>52.8 µl</td>
<td>105.6 µl</td>
<td></td>
</tr>
<tr>
<td>3 µl</td>
<td>39.6 µl</td>
<td>158.4 µl</td>
<td>316.8 µl</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>30 µl</td>
<td>396 µl</td>
<td>1,584 µl</td>
<td>3,168 µl</td>
</tr>
</tbody>
</table>

   *Includes 10% overage.

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

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

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

   - 95°C 1 min
   - 98°C 10 sec
   - 65°C 30 sec
   - 68°C 3 min
   - 72°C 10 min
   - 4°C forever

   *Consult Table II for PCR cycle number guidelines.

**STOPPING POINT:** The tubes may be stored at 4°C overnight.
C. Protocol: Purification of Amplified cDNA using AMPure Beads

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

NOTES:
- Aliquot AMPure beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well by vortexing.
- Bead:sample ratio is 0.8:1.
- 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.
- Do not pool the samples at the cDNA purification step. If pooling is desired, it can only be performed in Section VI.C.

1. If purification is performed directly in the PCR tubes or strips using the Takara Bio SMARTer-Seq Magnetic Separator - PCR Strip, add 40 µl of AMPure 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 40 µl of AMPure 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 3.

3. Incubate the bead-cDNA mixture at room temperature for 8 min to let the cDNA bind to the beads.

4. Briefly spin the samples to collect the liquid from the side of the tubes. (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 freshly made 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec. Then, carefully remove and discard the supernatant. 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 tubes or plate wells. 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 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 will look matte with no shine.
- If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce the amplified cDNA recovery rate and ultimately the cDNA yield. Allow the plate to sit at room temperature until the pellet is dry.
- If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 min to rehydrate (Step 12) and may reduce amplified cDNA recovery and yield.

11. Once the beads are dry, remove the samples from the magnetic 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.

12. Incubate at room temperature for at least 2 min to rehydrate.

13. Briefly spin the samples to collect the liquid from the side of the tubes or plate wells. Place the samples back on the magnetic separation device for 1 min or longer, until the solution is completely clear.

14. Transfer clear supernatant (~15 μl) containing purified cDNA from each tube/well to a new tube/plate. Take care not to carry over any beads with your sample.

15. Proceed to validation immediately or store at −20°C.

D. Protocol: Validation and Quantification of Amplified cDNA

cDNA concentrations obtained with the SMART-Seq Single Cell 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 the 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 manufacturers’ 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 Technologies, 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 an Agilent TapeStation system using a High Sensitivity D5000 ScreenTape (Agilent Technologies, 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 details. The cDNA profile obtained on a LabChip (PerkinElmer) instrument is typically unsatisfactory and difficult to interpret due to the lack of sensitivity. Evaluation of the cDNA profile is particularly important when performing initial optimization. For routine experiments by experienced users, evaluation of the cDNA profile may not be necessary or may be restricted to a few randomly selected samples combined with a positive and a negative control.

When using the Qubit or PicoGreen assay for quantification, the negative control may generate an apparent yield up to 200 pg/μl (for 20 cycles of PCR), even if the same sample run on the Bioanalyzer appears flat, because low-molecular-weight primers or primer dimers are detected. A lower number of cycles generates a lower 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 the negative controls generate ~150 pg/μl, a sample with an apparent yield of 550 pg/μl is in reality no more than 400 pg/μl. Use this background-subtracted value for planning the dilution to be used for library preparation in Section VI.A. Regardless of the quantification method, the SMART-Seq Single Cell Kit should yield >500 pg/μl (after background subtraction, if applicable) from an input of 2 pg of Control Total RNA using 19 PCR cycles.
1. If using PicoGreen or Qubit assay reagents for quantification, refer to the manufacturers’ instructions for information. Remember that it is critical to subtract the value of the negative control to obtain an accurate quantification.

2. If evaluating the 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.

   **NOTE:** Other options are available for evaluating the cDNA profile (see above and Appendix B). Do not attempt to run the cDNA on a regular agarose gel.

3. 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 300–10,000 bp, with a summit around 2,000 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 slightly smaller peak summit. In any case, the negative control should be almost flat with little or no product visible (Figure 3, Panel B). **For quantification, perform smear analysis using a 200–9,000 bp range.**

4. Proceed to Sequencing Library Preparation Protocols (Section VI).

---

**Figure 3. Example electropherogram results from Agilent 2100 Bioanalyzer.** All samples were subjected to cDNA synthesis and amplification as described in the protocol. **Panel A** shows a clean product (~1,050 pg/μl) following cDNA synthesis and amplification with 19 PCR cycles. The spiky profile with the Control Total RNA is normal. The level of spikiness will vary depending on the cell or RNA origin. **Panel B** shows almost no product in the negative control following cDNA synthesis and amplification with 20 PCR cycles. A very small amount of background (~25 pg/μl) may be present when using 20 cycles, as shown in this example. **Panels C** shows a representative cDNA profile obtained from a single K-562 cell (17 PCR cycles; 1,600 pg/μl), and **Panel D** shows a representative cDNA profile obtained from a single GM12878 cell (19 PCR cycles; 2,300 pg/μl).
VI. Sequencing Library Preparation Protocols

The following sections describe a modified Illumina Nextera XT DNA library preparation protocol (Nextera reagents not included; see Section III) that has been fully validated to work with the SMART-Seq Single Cell Kit. The reaction size has been reduced to a quarter volume of what is recommended by Illumina. Read the Nextera XT DNA Library Prep Kit Reference Guide (https://support.illumina.com/sequencing/sequencing_kits/nextera_xt_dna_kit/documentation.html) before proceeding, as it provides extra detailed instructions for library preparation.

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 cleanup (Section VI.C) and a relatively uniform amount of sequencing reads will be obtained. However, sample-to-sample read coverage varies, and you may observe some underrepresented or overrepresented samples with the pooling option.

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 350g 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 cleanup (Section VI.C). If negative controls are going to be sequenced, they 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 sec and centrifuge the tube briefly.

**Tagmentation Premix:**

<table>
<thead>
<tr>
<th></th>
<th>1 rxn</th>
<th>12 rxns*</th>
<th>48 rxns*</th>
<th>96 rxns*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tagment DNA Buffer</td>
<td>2.5 μl</td>
<td>37.5 μl</td>
<td>150 μl</td>
<td>300 μl</td>
</tr>
<tr>
<td>Amplification Tagment Mix</td>
<td>1.25 μl</td>
<td>18.8 μl</td>
<td>75 μl</td>
<td>150 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>3.75 μl</td>
<td>56.3 μl</td>
<td>225 μl</td>
<td>450 μl</td>
</tr>
</tbody>
</table>

*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 2,000g 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:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>55°C</td>
<td>10 min</td>
</tr>
<tr>
<td>10°C</td>
<td>forever</td>
</tr>
</tbody>
</table>

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 number of samples, aliquot equal amounts of NT Buffer into each tube of an 8-tube strip, and then use an eight-channel pipette to distribute the NT Buffer.

10. Seal the plate and vortex at medium speed, and then centrifuge at 2,000g for 1 min.

11. Incubate at room temperature for 5 min.

**B. Protocol: Amplify 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, and 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) into 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 2,000g for 2 min.

4. Place the “Library Prep” plate into a thermal cycler and perform PCR amplification using the following program:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td>95°C</td>
<td>30 sec</td>
</tr>
</tbody>
</table>

**12 cycles:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>55°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>10°C</td>
<td>forever</td>
</tr>
</tbody>
</table>

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.
### Protocol: Pool and Clean Up the Libraries

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

**NOTES:**
- Aliquot AMPure beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 min and mix well by vortexing.
- Bead: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 or PCR 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 1.5-ml tube or PCR tube. Volumes between 2 and 4 μl are appropriate. See examples in the table below. Do not use less than 2 μl per sample to ensure greater accuracy.

<table>
<thead>
<tr>
<th>Number of libraries to be pooled</th>
<th>Volume per library</th>
<th>Total pool volume</th>
<th>AMPure bead volume*</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>4 μl</td>
<td>32 μl</td>
<td>19 μl</td>
</tr>
<tr>
<td>12</td>
<td>4 μl</td>
<td>48 μl</td>
<td>29 μl</td>
</tr>
<tr>
<td>16</td>
<td>2 μl</td>
<td>32 μl</td>
<td>19 μl</td>
</tr>
<tr>
<td>24</td>
<td>2 μl</td>
<td>48 μl</td>
<td>29 μl</td>
</tr>
<tr>
<td>32</td>
<td>2 μl</td>
<td>64 μl</td>
<td>38 μl</td>
</tr>
<tr>
<td>48</td>
<td>2 μl</td>
<td>96 μl</td>
<td>58 μl</td>
</tr>
</tbody>
</table>

   *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 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 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 sec, and then remove and discard the supernatant, taking care not to disturb the beads. The cDNA remains bound to the beads during washing.

9. Repeat the ethanol wash (Step 8) once.
10. Briefly centrifuge the samples to collect the liquid from the side of the tube or plate well. Place the samples on the magnetic separation device for 30 sec, then remove any residual ethanol with a pipette.

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

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

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

<table>
<thead>
<tr>
<th>Number of libraries pooled</th>
<th>Nuclease-Free Water volume*</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>48 µl</td>
</tr>
<tr>
<td>12</td>
<td>72 µl</td>
</tr>
<tr>
<td>16</td>
<td>48 µl</td>
</tr>
<tr>
<td>24</td>
<td>72 µl</td>
</tr>
<tr>
<td>32</td>
<td>96 µl</td>
</tr>
<tr>
<td>48</td>
<td>144 µl</td>
</tr>
</tbody>
</table>

* 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 Technologies, 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 below for an example of a successful library.

17. Refer to the Nextera XT DNA Library Prep Kit Reference Guide (https://support.illumina.com/sequencing/sequencing_kits/nextera_xt_dna_kit/documentation.html) to determine the appropriate library concentration for sequencing.
Figure 4. Example of a Nextera XT sequencing library. cDNA generated from 2 pg of Control Total RNA was quantified by smear analysis after electrophoresis using Agilent’s High Sensitivity DNA Kit as described in Section V.D and used as the input for Nextera XT library construction (125 pg per sample). 1 μl of the bead-purified library was analyzed on the Bioanalyzer using Agilent’s High Sensitivity DNA Kit.

VII. References


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 A, below). 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 B, below. Alternatively, you can sort cells in a maximum volume of 5 μl PBS. PBS is not an optimal sorting buffer, as it interferes with the efficiency of cDNA synthesis and PCR amplification. For recommendations on how to proceed after sorting into ≤ 5 μl of PBS, see Section C, below.

Please note that the amount of water in the recipes below may need to be adjusted if single cells are isolated with a method other than FACS. When using FACS, the amount of liquid surrounding each cell (sheath fluid) is typically negligible. This may not be the case when dispensing cells with other methods.

A. FACS Sorting into a Buffer Containing the CDS Oligo

In this protocol, cells are sorted into 12.5 μl of buffer.

1. Prepare enough sorting buffer to prefill the 96-well plate or PCR strips as indicated below. Due to small pipetting volumes, prepare no less than 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):**

<table>
<thead>
<tr>
<th></th>
<th>Per well</th>
<th>18 wells*</th>
<th>48 wells*</th>
<th>96 wells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Lysis Buffer</td>
<td>0.95 μl</td>
<td>19 μl</td>
<td>52.25 μl</td>
<td>104.5 μl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.05 μl</td>
<td>1 μl</td>
<td>2.75 μl</td>
<td>5.5 μl</td>
</tr>
<tr>
<td>3' SMART-Seq CDS Primer II A</td>
<td>1 μl</td>
<td>20 μl</td>
<td>55 μl</td>
<td>110.0 μl</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>10.5 μl</td>
<td>210 μl</td>
<td>577.5 μl</td>
<td>1,155.0 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>12.5 μl</td>
<td>250 μl</td>
<td>687.5 μl</td>
<td>1,375.0 μl</td>
</tr>
</tbody>
</table>

   *Volumes include ~10% extra for overage.

Mix briefly, and then spin down.

**NOTES:**
- The 10X Lysis Buffer contains a detergent; it is critical to avoid bubbles when mixing.
- The 3' SMART-Seq CDS Primer II A provided with each kit is sufficient to collect twice the number of cells than the number of reactions in the kit (e.g., a 96-reaction kit contains enough to prepare two plates of 96 wells). 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 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 aluminum foil, but are not practical when sorting a large number of samples.

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

**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.

B. **FACS Sorting into a Buffer Not Containing the CDS Oligo**

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

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):**

<table>
<thead>
<tr>
<th></th>
<th>Per well</th>
<th>18 wells*</th>
<th>48 wells*</th>
<th>96 wells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Lysis Buffer</td>
<td>0.95 µl</td>
<td>19 µl</td>
<td>52.25 µl</td>
<td>104.5 µl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.05 µl</td>
<td>1 µl</td>
<td>2.75 µl</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>10.5 µl</td>
<td>210 µl</td>
<td>577.5 µl</td>
<td>1,155.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>11.5 µl</td>
<td>230 µl</td>
<td>632.5 µl</td>
<td>1,265.0 µl</td>
</tr>
</tbody>
</table>

*Volumes include ~10% extra for overage.

Mix briefly, then spin down.

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

2. Aliquot 11.5 µl of PSS from Step 1 into the appropriate number of wells of PCR tube strips or a 96-well plate.

**NOTE:** To minimize bubble formation set single- or multi-channel pipettes to 11.6 µl and pipette only to the first stop when aliquoting. Changing tips often also minimizes bubble formation.
3. Seal the plate/tube strips and briefly spin to ensure the sorting buffer collects at the bottom of the wells.

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

**When ready to sort:**

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

6. Seal the plate/tube strips with an aluminum foil seal or PCR strip caps. Ensure the plate/tube strips are sealed firmly to minimize any evaporation.

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

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

   **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.

   **IMPORTANT:** Since the PSS does not include the 3’ SMART-Seq CDS Primer II A, you need to add it when you thaw your samples—see Section V.A, Step 5.

C. **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 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, and kit performance (cDNA yield and sensitivity) will be impacted (except if using only 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 II (Section V.B). In addition, you must use PBS without Ca²⁺ and Mg²⁺ (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: Expected Results When Analyzing cDNA with TapeStation

The Agilent 2100 Bioanalyser 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 Single Cell Kit. Another good option, particularly for processing a large number of samples, is the Fragment Analyzer and High Sensitivity Large Fragment Analysis Kit (Advanced Analytical Technologies, Inc., Cat. No. DNF-493).

If these instruments are not available, an Agilent TapeStation system can be used with a High Sensitivity D5000 ScreenTape (Agilent, Cat. No. 5067-5592). Because the scale is very different—as Figure 5 shows—the cDNA profile on the TapeStation may look quite different than the profile on the Bioanalyzer, particularly for yields below 500 pg/μl. However, if the ScreenTape shows a broad smear going from ~600 to ~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. The SMART-Seq Single Cell Kit should generate cDNA yields higher than 500 pg/μl and the cDNA profile should be detected relatively easily.

![Figure 5](image-url)

Figure 5. Comparison of electropherogram results from Agilent 2100 Bioanalyzer and Agilent TapeStation system. 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 the SMART-Seq Single Cell Kit, 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 the SMART-Seq Single Cell Kit.) 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 the SMART-Seq Single Cell Kit should be relatively flat in this region.
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