Clontech Laboratories, Inc.

SMART-Seq® v4 Ultra® Low Input RNA Kit for the Fluidigm® C1™ System, IFCs User Manual

Cat. Nos. 635025 & 635026
(032416)

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I. Introduction

cDNA Synthesis from Single Cells Using Template-Switching Technology

The **SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs** (Cat. Nos. 635025 & 635026) allows high-quality cDNA synthesis starting from up to 96 single cells that have been isolated and processed with the Fluidigm C1 Single-Cell Auto Prep System. This protocol details the use of the C1 system, C1 integrated fluidic circuits (IFCs), and required reagents to capture cells, convert polyA+ RNA into full-length cDNA, and amplify the cDNA using SMART® technology (Table 1 and Figure 1). The protocol also includes a modified Illumina® Nextera® XT DNA sample preparation protocol for single-cell mRNA sequencing library preparation from cDNA acquired using the C1 system. Using this protocol, up to 384 single-cell samples (from 4 IFCs) can be pooled for a single sequencing reaction. cDNA libraries generated with this kit have been tested for compatibility with Illumina sequencing platforms.

Hands-on aspects of the cDNA synthesis protocol can be completed in approximately two hours, followed by running the eight-hour SMART-Seq v4 script on the C1 instrument. The entire library construction protocol can be completed within two working days (Table 1).

Table 1. Protocol overview. Once you start the experiment, proceed with each step as soon as possible.

Procedure	Time
cDNA synthesis	
Prepare reagent mixes	40 min
Pipet priming reagents into the IFC	10 min
Run the Prime script on the C1 *(automated step)*	~10 min
Pipet cells into the IFC	5 min
Run Cell Load script or Cell Load & Stain script on the C1 *(automated step; latter script includes optional cell staining protocol)*	15–30 min*
Image and count the cells on the IFC	15–30 min
Pipet lysis, RT, and PCR mixes into the IFC	5 min
Run the Sample Prep script (lysis, RT, and PCR) on the C1 *(automated step)*	8 hr
Run optional tube controls while running the Sample Prep script	~6 hr
Harvest cDNA from the IFC	10 min
Library prep	
Quantify cDNA concentration	
Quantify and dilute the cDNA	2 hr
Prepare Illumina sequencing libraries	
Tagment the cDNA	30 min
Amplify the libraries	50 min
Pool, clean up, and quantify the libraries	90 min
Quantify sequencing libraries and determine size distribution	
Analyze each library using the Agilent Bioanalyzer	50 min

Note:
* If performing staining, loading times are increased to 30–55 min.

SMART technology was initially adapted to next-generation sequencing (NGS) in the SMARTer® Ultra Low RNA Kit for Illumina Sequencing (for ultra-low input mRNA-seq) and published as the SMART-Seq method (Ramsköld *et al.*, 2012). Our current SMART-Seq v4 products improve upon several previous generations by incorporating the novel use of locked nucleic acid (LNA) technology into an optimized template-switching oligo, as well as other advancements developed by Clontech scientists.

As a result of these improvements, SMART-Seq v4 kits, including the SMART-Seq v4 Ultra Low RNA Kit for the Fluidigm C1 System, IFCs, produce single-cell mRNA-seq libraries that clearly outperform previously-published protocols and older products. SMART-Seq v4 kits provide higher reproducibility and sensitivity (meaning more genes are identified from libraries produced with these kits) and exhibit significantly lower background than other methods. Additionally, due to a change in the polymerase used in SMART-Seq v4 kits, genes with high GC content show better representation than in previous-generation SMARTer kits. A schematic outline of the SMART-Seq v4 technology and workflow is shown in Figure 1. For more information on SMART technology, please visit www.clontech.com/NGS.

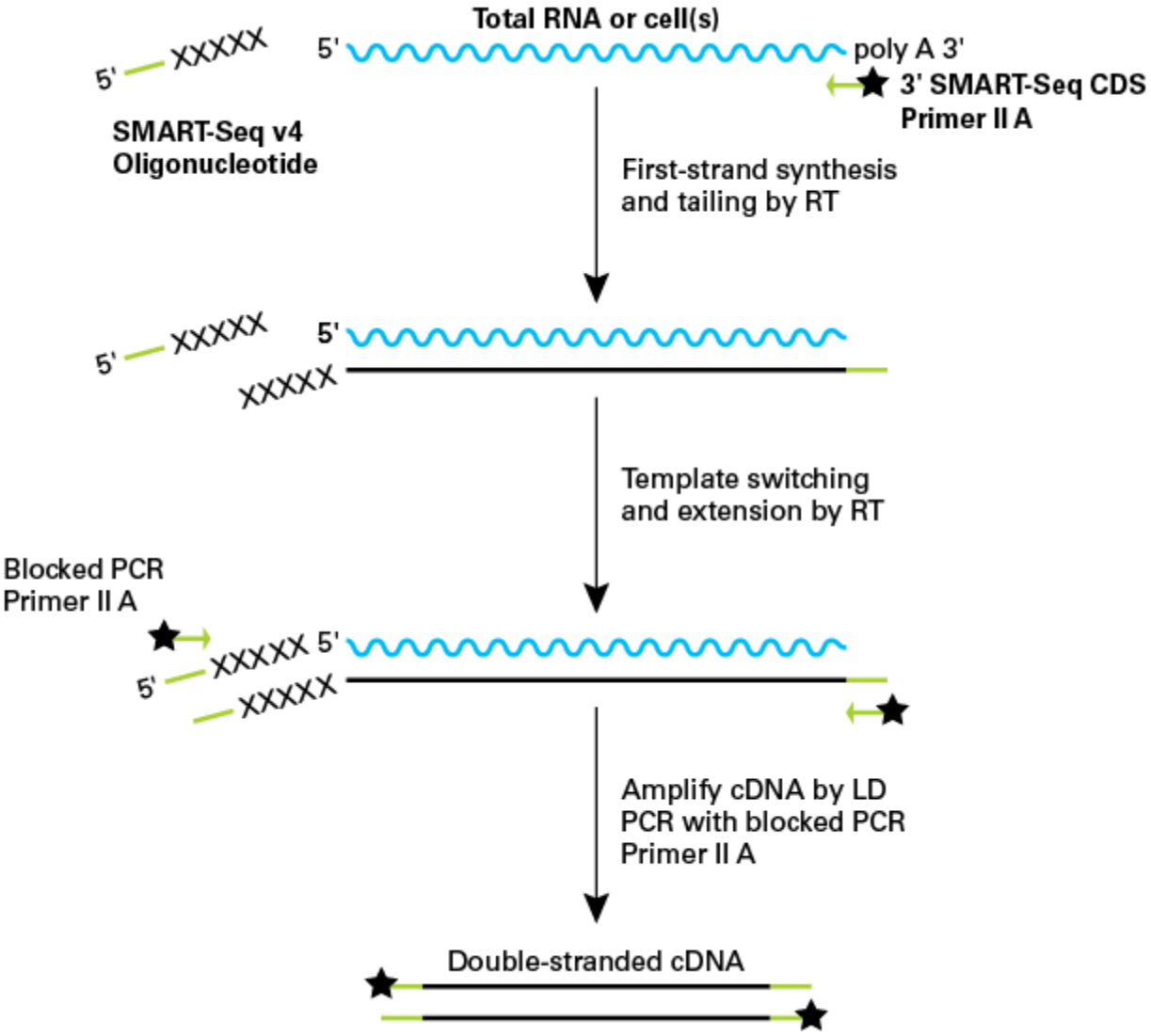

Figure 1. Flowchart of SMART cDNA synthesis. The SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs incorporates SMART (**S**witching **M**echanism **a**t 5' End of **R**NA **T**emplate) technology, which offers unparalleled sensitivity and unbiased amplification of cDNA transcripts, and allows direct cDNA synthesis from intact cells. This technology relies on the template-switching activity of reverse transcriptases 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 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. The SMART-Seq v4 Oligonucleotide, 3' SMART-Seq CDS Primer II A, and PCR Primer II A all contain a stretch of identical sequence. The black star indicates a chemical block on the 5' end of the oligonucleotide.

II. List of Components

The SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs consists of the SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs Components (not sold separately) and SeqAmp™ DNA Polymerase. **These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs	635025 (4 IFCs)	635026 (10 IFCs)
SeqAmp DNA Polymerase (Store at –20°C.)		
SeqAmp DNA Polymerase	50 µl	50 µl
SeqAmp PCR Buffer (2X)	1.25 ml	1.25 ml
SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs Components (Not sold separately.)		
(Store at –20°C. Once thawed, store 10X Lysis Buffer at 4°C. Continue to store all other reagents at –20°C.)		
SMART-Seq v4 Oligonucleotide (48 µM)	12 µl	28 µl
PCR Primer II A (12 µM)	12 µl	30 µl
5X Ultra Low First-Strand Buffer	45 µl	120 µl
SMARTScribe™ Reverse Transcriptase (100 U/µl)	25 µl	60 µl
3' SMART-Seq CDS Primer II A (12 µM)	10 µl	24 µl
RNase Inhibitor (40 U/µl)	10 µl	24 µl
10X Lysis Buffer	100 µl	200 µl
Nuclease-Free Water	120 µl	300 µl

III. Additional Materials

The following reagents and materials are required or recommended but not supplied. They have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- C1 System (Fluidigm, Part No. 100-7000)
- SMART-Seq v4 script (available for download from the Fluidigm C1 Open App Script Hub, https://www.fluidigm.com/c1openapp/scripthub)
  - This script can be run with the C1 Single-Cell Auto Prep IFC for mRNA Seq or the C1 Single-Cell Auto Prep IFC for Open App.
- Imaging equipment compatible with C1 IFCs
- Thermal cycler for library amplification (Section VI.C) and for optional tube controls (Appendix A)
- Agilent 2100 Bioanalyzer
- Fluorometer (for PicoGreen assay)
- Single-channel pipette: 10 µl, 20 µl, and 200 µl
- Eight-channel pipette: 20 µl and 200 µl
- Twelve-channel pipette: 20 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
- Centrifuge for 96-well plates

For cDNA synthesis:

- C1 Single-Cell Auto Prep Reagent Kit (Fluidigm, Part No. 100-6201)
  Store Module 1 (Part No. 100-5518) at 4°C and Module 2 (Part No. 100-6209) at –20°C.
- The appropriate Fluidigm IFC (mRNA Seq or Open App can be used):
  - C1 Single-Cell Auto Prep IFC for mRNA Seq, 5–10 µm (Fluidigm, Part No. 100-5759)
  - C1 Single-Cell Auto Prep IFC for mRNA Seq, 10–17 µm (Fluidigm, Part No. 100-5760)
  - C1 Single-Cell Auto Prep IFC for mRNA Seq, 17–25 µm (Fluidigm, Part No. 100-5761)
  - C1 Single-Cell Auto Prep IFC for Open App, 5–10 µm (Fluidigm Part No. 100-8133)
  - C1 Single-Cell Auto Prep IFC for Open App, 10–17 µm (Fluidigm, Part No. 100-8134)
  - C1 Single-Cell Auto Prep IFC for Open App, 17–25 µm (Fluidigm, Part No. 100-8135)

For sequencing library preparation (Section VI):

- C1 DNA Dilution Reagent (Fluidigm, Part No. 100-5317; store at –20°C.)
- Nextera XT DNA Library Prep Kit (Illumina, Cat. No. FC-131-1096)
- Nextera XT DNA Library Prep Index Kit (Illumina; choose the appropriate kit from the following Cat. Nos.: FC-131-1002, FC-131-2001, FC-131-2002, FC-131-2003, FC-131-2004)
- Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Cat. No. P11496)
- Agilent High Sensitivity DNA Kit (Agilent Technologies, Cat. No. 5067-4626)
- Corning 384 Well Low Flange Black Flat Bottom Polystyrene Not Treated Microplate (Corning, Prod. No. 3573)
- 96-well PCR plates
- MicroAmp Clear Adhesive Film (Thermo Fisher Scientific, Cat. No. 4306311)

For SPRI (Solid Phase Reversible Immobilization) bead purifications (Section VI.D):

- Agencourt AMPure XP (Beckman Coulter; 5 ml Kit, Item No. A63880; 60 ml Kit, Item No. A63881)
  - Use this kit for pooled library purification.

  NOTE: AMPure XP beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5-ml tubes upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). This will also decrease the chance of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.
- 100% ethanol (molecular biology grade)
- Magnetic separation device for 1.5-ml tubes

Additional, recommended items:

- Recommended for cell counting: INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved; INCYTO, Prod. No. DHC-N01)
- Recommended for staining: LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific, Cat. No. L3224)
- Recommended for preparing the positive control:
  - ArrayControl RNA Spikes (Thermo Fisher Scientific, Cat. No. AM1780)
  - THE RNA Storage Solution (Thermo Fisher Scientific, Cat. No. AM7000)
- Recommended for preparing the tube controls: NucleoSpin® RNA XS (Clontech, Cat. No. 740902.10)

IV. General Considerations

A. Requirements for Preventing Contamination

Before you set up the experiment, **make sure you have two physically separated work stations**:

- **A PCR Clean Work Station** for all pre-PCR experiments that require clean-room conditions: preparing reagent mixes (Section V.A) and tube controls (Appendix A).

  NOTES:
  - The PCR Clean Work Station must be located in a room with 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 work station located in the general laboratory** where you will perform the remainder of the cDNA synthesis protocol (Sections V.B–V.E) and library preparation (Section VI).

B. General Requirements

- The assay is very sensitive to variations in pipette volume. Please make sure all pipettes are calibrated for reliable delivery, and make sure nothing is attached to the outside of the tips.
- All lab supplies related to SMART cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMART cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR products.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the provided amplification reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive tube control reactions to verify that kit components are working properly.

C. Sample Requirements

This protocol has been validated to generate cDNA starting from intact, cultured cells, including cryopreserved cells that have not been lysed during the freeze-thaw process. **It cannot be used with cells that have undergone fixation.**

V. cDNA Synthesis Protocols

NOTES: Please read the all of the protocols in this manual before starting. The protocols in this section are optimized for cDNA synthesis from single cells.

- Prepare reagent mixes (Section V.A) and optional tube controls (Appendix A) in a PCR Clean Work Station under clean-room conditions.
- For the best results, once you begin, proceed without delay through the entire experiment. Refer to Table 1 in the Introduction for approximate times to complete each general step.
- The protocols described in this user manual have been optimized for cDNA synthesis using the SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs only.
- In Section V.C.2, the recipe used to prepare the Cell Mix varies based on the buoyancy of your cell type, which you can determine ahead of time. Cell buoyancy should be determined for each new cell line. To maximize cell capture, cells must not float or sink unevenly in the Suspension Reagent. A 3:2 ratio of cell solution to Suspension Reagent normally gives good buoyancy for many cultivable cells, but the ratio can vary drastically depending on the cell type, especially for primary cells. For guidelines on optimizing this ratio, consult the Fluidigm Single-Cell Preparation Guide. [See Appendix B for instructions on how to download the Fluidigm Single-Cell Preparation Guide (PN 100-7697).]

A. Protocol: Prepare Reagent Mixes (Perform in PCR Clean Work Station)

Prepare the following reagent mixes:

- RNA Spikes Mix (optional)
- Lysis Mix
- Reverse Transcription (RT) Mix
- PCR Mix

NOTE: The lysis, RT, and PCR master mixes include enough reagents to perform the optional tube controls.

1. Prewarm Buffers and Reagents

IMPORTANT: Prewarm Cell Wash Buffer, C1 Preloading Reagent, C1 Blocking Reagent, and C1 Harvest Reagent (from the C1 Single-Cell Auto Prep Reagent Kit [Fluidigm, Part No. 100-6201]) to room temperature prior to use. Thaw C1 Suspension Buffer on ice and vortex well before use.

2. Prepare the RNA Spikes Mix (Optional Positive Control)

RNA spikes serve as a positive control for thermal cycling of the C1 system independent of cell capture. This control is highly recommended, but not required.

NOTES:

- The bulk volume of RNA Spikes Mix is sufficient for 125 C1 IFCs. Aliquot unused mix and store at –80°C for future use.
- Only three out of the eight RNA transcripts contained within the ArrayControl RNA Spikes set will be used: #1, #4, and #7.

a. Prepare the concentrated RNA Spikes Mix.

i. Pipet the following into three tubes:

Tube A:

13.5 µl	THE RNA Storage Solution
1.5 µl	RNA spikes #7

Tube B:

12 µl	THE RNA Storage Solution
1.5 µl	RNA spikes #4

Tube C:

148.5 µl	THE RNA Storage Solution
1.5 µl	RNA spikes #1

ii. Vortex tube A for 3 sec and spin down briefly to collect contents at the bottom of the tube. Pipet 1.5 µl from tube A into tube B.

iii. Vortex tube B for 3 sec and spin down briefly. Pipet 1.5 µl from tube B into tube C.

iv. Vortex tube C for 3 sec and spin down briefly. Tube C is the concentrated RNA standard that will be run on the C1 instrument.

v. Aliquot tube C by pipetting 1.25 µl into fresh tubes. Store aliquots at −80ºC until use. Discard tubes A and B.

b. Dilute the RNA Spikes Mix (for use with the Lysis Mix).

NOTES:

- If you are using RNA spikes, this diluted mix replaces C1 Loading Reagent in the Lysis Mix at Step 4.b, below.
- Do not dilute RNA more than an hour before you load the IFC. Do not store diluted RNA spikes for long-term use.

i. Thaw an aliquot of the RNA Spikes Mix (from Step 2.a.v, above) and dilute as follows:

1 µl	RNA Spikes Mix (Tube C)
99 µl	Loading Reagent (Fluidigm)
100 µl	Total volume

ii. Vortex the diluted RNA Spikes Mix for 3 sec and spin down briefly.

3. Thaw 5X Ultra Low First-Strand Buffer

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

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

4. Prepare Lysis Mix

a. Prepare 10X Reaction Buffer as follows:

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

Pipet the solution up and down a few times or gently vortex to mix, then spin down briefly. Keep the solution on ice until use.

NOTE: Lysis Buffer contains a detergent. It is critical to avoid bubbles when mixing. Do not vortex the lysis buffer vigorously.

b. Mix the following reagents in a tube labeled "Lysis":

1 µl	C1 Loading Reagent (20X)*
2.4 µl	3′ SMART-Seq CDS Primer II A (12 µM)
14 µl	Nuclease-Free Water
2.6 µl	10X Reaction Buffer
20 µl	Total volume

* If you are using RNA spikes, add 1 µl of the diluted RNA Spikes Mix (see Section A.2.b above) instead of the C1 Loading Reagent (20X).

c. Pipet the solution up and down a few times to mix, or vortex gently, and then briefly spin down. Keep the solution on ice until use.

5. Prepare Reverse Transcription (RT) Mix

a. Mix the following reagents **except** SMARTScribe Reverse Transcriptase in a tube labeled "RT" and keep the solution on ice. Add SMARTScribe Reverse Transcriptase just before pipetting the mixture into the IFC.

NOTE:

- Ensure that no precipitate is present in the 5X Ultra Low First-Strand Buffer.
- Do not vigorously vortex SMARTScribe Reverse Transcriptase.

1.2 µl	C1 Loading Reagent (20X)
11.2 µl	5X Ultra Low First-Strand Buffer (RNase-free)
2.8 µl	SMART-Seq v4 Oligonucleotide (48 µM)
1.4 µl	RNase Inhibitor (40 U/µl)
9.8 µl	Nuclease-Free Water
5.6 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
32 µl	Total volume

b. Gently vortex the Reverse Transcription (RT) Mix for 3 sec and centrifuge briefly to collect contents. Keep on ice until use.

REMINDER: Do not vigorously vortex SMARTScribe Reverse Transcriptase.

6. Prepare PCR Mix

a. Mix the following reagents in a tube labeled "PCR":

4.5 µl	C1 Loading Reagent (20X)
4.4 µl	Nuclease-Free Water
3 µl	PCR Primer II A (12 µM)
75.2 µl	SeqAmp PCR Buffer (2X)
2.9 µl	SeqAmp DNA Polymerase (1.25 U/µl)
90 µl	Total volume

b. Gently vortex the PCR Mix for 3 sec and centrifuge briefly to collect contents. Keep on ice until ready to use.

B. Protocol: Prime the IFC

Vortex gently and then centrifuge all reagents before pipetting into the C1 IFC. Be very careful when pipetting reagents: It is essential to avoid creating bubbles in the inlets of the IFC.

NOTES:

- To avoid creating bubbles, always stop at the first stop on the pipette.
- If a bubble is introduced, ensure that it floats to the top of the well, or remove the reagent from the inlet with a pipette and reload the inlet with the same fluid that was removed.
- If necessary, load more reagent (up to 25 µl) into the inlet to make it easier to pipet the reagent without bubbles. Excess reagent will not be used; only the amount specified in this protocol will actually be used for the reactions that occur on the IFC.

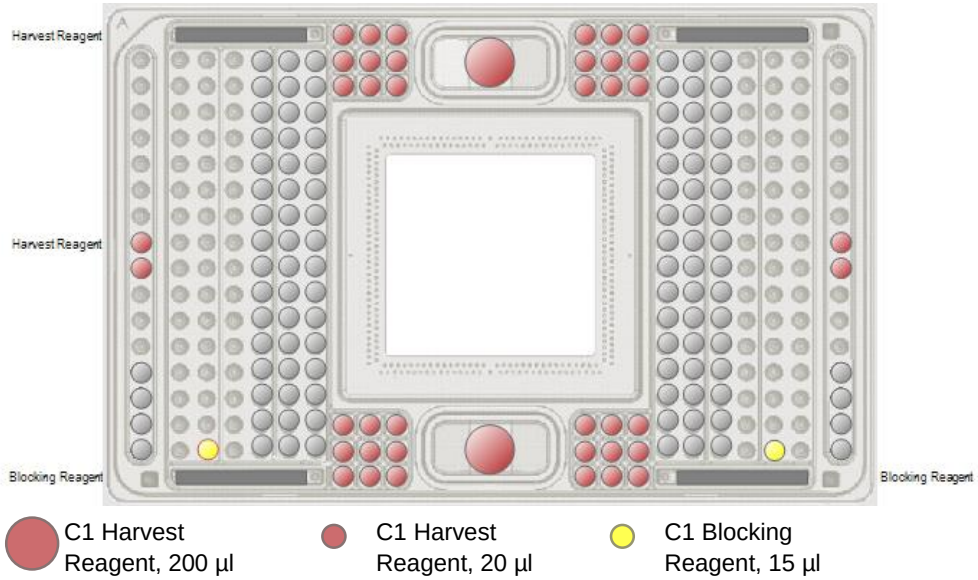

Figure 2. C1 IFC Priming pipetting map.

1. Pipet 200 µl of C1 Harvest Reagent into each of the accumulators marked with a large red circle in Figure 2 (2 total). Push down the O-ring with the pipette tip to inject the reagent into each inlet. After injection, swirl the IFC gently to distribute the reagent evenly in each inlet.
2. Pipet 20 µl of C1 Harvest Reagent into each of the inlets marked with small red circles on each side of the accumulators (36 total).
3. Pipet 20 µl of C1 Harvest Reagent into the pairs of inlets marked with small red circles in the middle of the outside columns of inlets on each side of the IFC (4 total). These inlets are marked on the bottom of the IFC with notches.
4. Pipet 15 µl of C1 Blocking Reagent into the cell inlet and outlet marked with yellow dots (2 total).
5. Peel the white tape off the *bottom* of the IFC.

   IMPORTANT: Do not remove the four pieces of tape that cover the harvest inlets on the top of the IFC. These strips will be removed later, during the harvest step.
6. Place the IFC into the C1 system and tap the **LOAD** button. Run the **SMART-Seq v4: Prime (1861x/1862x/1863x)** script. Priming takes 11 min for the small-cell IFC and 12 min for the medium- and large-cell IFCs. When the Prime script has finished, tap **EJECT** to remove the primed IFC from the C1.

   NOTE: After priming the IFC, you have up to one hour to load the IFC on the C1 system for the Cell Load (and Stain) step (Section V.C.3, below).

C. Protocol: Cell Load (and Stain) on the IFC

The following protocols will be used to prepare the reagents and cells, load the cells into the C1 IFC, and visualize the loaded cells:

- Prepare the LIVE/DEAD Cell Staining Solution (optional)
- Prepare the Cell Mix (while priming the IFC)
- Load the cells into the IFC
- Image the cells in the IFC

1. Prepare the LIVE/DEAD Cell Staining Solution (Optional)

The optional live/dead cell staining step uses the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific) to test the viability of a cell based on the integrity of its membrane. This test contains two chemical dyes: a green dye that stains live cells and a red dye that only stains cells that have lost cell membrane integrity. See Appendix B for more information on accessing the manual and protocol (Cat. No. L-3224) for the LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells.

NOTES:

- Keep the dye tubes closed and in the dark as much as possible, as they can hydrolyze over time. When not in use, store in a dark, airtight bag with a desiccant pack at −20ºC.
- Prepare the cell staining solution up to 2 hr before loading into the C1 IFC. Keep on ice and protected from light before pipetting into the IFC.

a. Vortex the dyes for 10 sec and spin down.

b. Prepare the LIVE/DEAD staining solution:

1,250 µl	Cell Wash Buffer (Fluidigm)
2.5 µl	Ethidium homodimer-1 (LIVE/DEAD kit, Thermo Fisher)
0.625 µl	Calcein AM (LIVE/DEAD kit, Thermo Fisher)
1,253.125 µl	Total volume

c. Vortex the LIVE/DEAD staining solution thoroughly before pipetting into the IFC.

2. Prepare the Cell Mix (While Priming the IFC)

IMPORTANT:

- Vortex the Suspension Reagent for 5 sec before use. If Suspension Reagent contains particulates, ensure they are properly removed by vortexing. **Do not vortex the cells.**
- To maximize the single-cell capture rate, the buoyancy of the cell suspension should be optimized by adding the proper amount of Suspension Reagent. Conduct the buoyancy test following the Fluidigm Single-Cell Preparation Guide (PN 100-7697) and as described in the introduction to Part V, above. Preparing 20 µl of Cell Mix as instructed in Step V.C.2.d (using a 3:2 ratio of cell solution to Suspension Reagent) may give good results; however, you should adjust the recipe in this step if your buoyancy test indicates a different ratio.

a. Ensure that you have begun priming the IFC. (See Section V.B, "Prime the IFC.")

b. Determine your cell concentration. Cells may be counted using any preferred method. If an established cell counting protocol does not exist, we suggest using the INCYTO C-Chip disposable hemocytometer.

c. Prepare 0.5 ml of a cell solution in native medium (enough for the IFC and tube controls). The recommended concentration varies depending on the size of cells you are using.

   - If you are using medium-sized cells (10–17 µm diameter) or large-sized cells (17–25 µm diameter), make a cell suspension containing 166,000–250,000 cells/ml. The recommended concentration range ensures that a total of 500–750 cells are pipetted into the cell loading inlet of the IFC.
   - If you are using small-sized cells (5–10 µm diameter), a higher concentration of cells (~400,000–800,000 cells/ml) may increase the single-cell capture rate. The recommended concentration range ensures that a total of 1,200–2,400 cells are pipetted into the cell loading inlet of the IFC.

d. Prepare the Cell Mix by combining the cell solution with Suspension Reagent at 1) a 3:2 ratio or 2) a ratio determined by a buoyancy test. **Do not vortex the cells**; slowly pipet up and down a few times to mix. For example, using a 3:2 ratio for a total volume of 20 µl, mix:

12 µl	Cell solution (from Step V.C.2.c)
8 µl	Suspension Reagent (Fluidigm)
20 µl	Total volume

e. Keep remaining cell solution on ice until necessary for tube control preparation (Appendix A).

3. Load the Cells

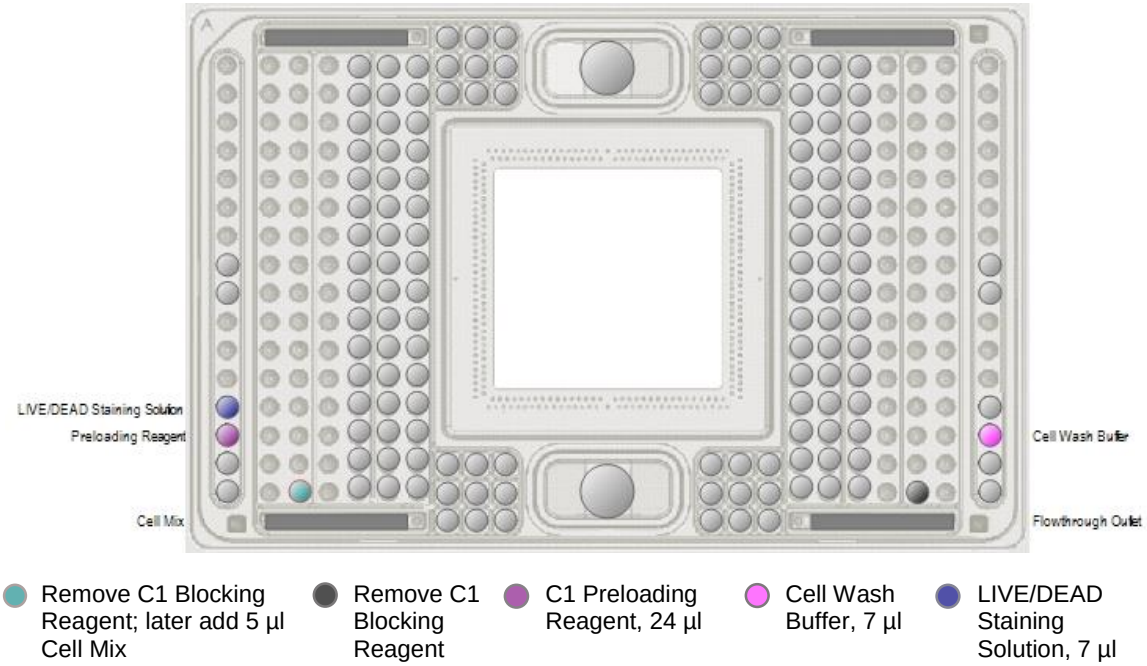

Figure 3. C1 IFC Cell Load pipetting map.

a. Use a pipette to remove the blocking reagent from the cell inlet C1 (marked with a teal dot; 1 total) and outlet C2 (marked with a dark gray dot; 1 total) in Figure 3.

IMPORTANT: It is critical to remove the blocking reagent before proceeding.

b. Set a pipette to 12 µl, then slowly pipet the Cell Mix up and down 5–10 times to mix and dissociate any clumped cells. **Do not vortex the Cell Mix.** Avoid bubbles when mixing.

c. Pipet 24 µl of C1 Preloading Reagent into Inlet 2 on the left side of the chip (marked with a purple dot; 1 total).

d. Pipet 7 µl of Cell Wash Buffer into Inlet 6 on the right side of the chip (marked with a pink dot; 1 total).

e. Pipet 5 µl of the Cell Mix into the inlet marked with the teal dot (1 total). You may pipet up to 20 µl of Cell Mix, but only 5 µl will enter the IFC.

f. **If you are performing the optional live/dead cell staining**, vortex the LIVE/DEAD staining solution well, and then pipet 7 µl of the solution into Inlet 1 (marked with a dark blue dot; 1 total). Choose the script that includes a staining step.

NOTE: Staining takes an additional 16–25 minutes, depending on the size of the cells and IFC used: Loading times are increased to 31, 55, or 51 min for the small-cell IFC, medium-cell IFC, or large-cell IFC, respectively.

g. Place the IFC into the C1. Run the **SMART-Seq v4: Cell Load (1861x/1862x/1863x)** or **SMART-Seq v4: Cell Load & Stain (1861x/1862x/1863x)** script. For loading and staining times, see Table 1 in the Introduction.

h. When the script has finished, tap **EJECT** to remove the IFC from the C1 system.

4. Image the Cells

Examine all 96 capture sites on the IFC using microscopy. Record how many cells are captured in each capture site. Check the viability of the cells if the LIVE/DEAD staining step was performed.

D. Protocol: Sample Prep (Run Lysis, Reverse Transcription, and PCR) on the C1 System

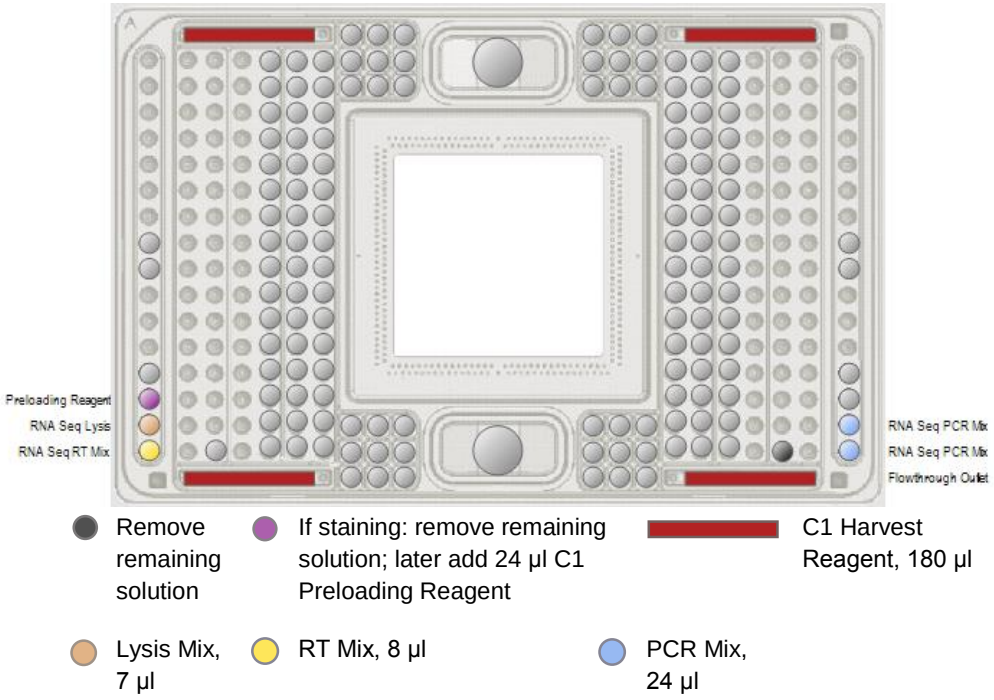

Figure 4. C1 IFC Lysis, RT, and PCR pipetting map.

1. Aspirate any solution remaining in the flowthrough outlet C2 (marked with a dark gray dot; 1 total). If you are using the script that includes a staining step, also aspirate Inlet 2 (marked with a purple dot; 1 total).
2. Pipet 180 µl of C1 Harvest Reagent into each of the reservoirs (marked with large solid red rectangles; 4 total) in Figure 4.
3. Pipet 7 µl of Lysis Mix into Inlet 3 (marked with an orange dot; 1 total).
4. Pipet 8 µl of Reverse Transcription (RT) Mix into Inlet 4 (marked with a yellow dot; 1 total).
5. Pipet 24 µl of PCR Mix into Inlets 7 and 8 (marked with blue dots; 2 total).
6. If you used the script that includes a staining step, pipet 24 µl of Preloading Reagent into Inlet 2 (marked with a purple dot; 1 total).
7. Place the IFC into the C1 system and run the **SMART-Seq v4: Sample Prep (1861x/1862x/1863x)** script.

   NOTE: The SMART-Seq v4: Sample Prep (1861x/1862x/1863x) script can be run overnight. Run time is 8 hr.

   IMPORTANT: Harvest should be performed as soon as the Sample Prep script is finished. Otherwise, small volumes are prone to loss by evaporation. If >8 hr will elapse between the start of sample prep and harvest (for example, when running the script overnight), designate the time to complete the script by sliding the orange bar in the dialog box to "Select when the script should finish."
8. If you are running the optional tube controls, start them now. See Appendix A for instructions on running the tube controls.

NOTE: Thermal-cycling conditions for PCR during sample prep can be found in Appendix C.

E. Protocol: Harvest the Amplified cDNA

Throughout Section E, we will refer to diagrams in the Fluidigm protocol titled "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing." The Fluidigm protocol is available for download at https://www.fluidigm.com/productsupport/c1-support-hub underneath Documents > Protocols > C1 System for mRNA Seq.

NOTE: Prewarm the C1 DNA Dilution Reagent (Fluidigm, Part No. 100-5317) to room temperature. If you plan to run the Sample Prep script overnight, you can aliquot 10 µl of the dilution reagent into each well of a 96-well plate the day before, store the sealed plate in the refrigerator overnight, and prewarm to room temperature in the morning.

1. When the SMART-Seq v4: Sample Prep script has completed, tap **EJECT** to retrieve the IFC from the instrument.
2. Label a new 96-well plate "Diluted Harvest" along with the experiment name, dilution factor, and date.
3. Pipette 10 µl of C1 DNA Dilution Reagent into each well of the "Diluted Harvest" plate.
4. Carefully pull back the four pieces of tape covering the harvesting inlets of the IFC using the plastic removal tool as shown in Figure 6 on page 30 of the Fluidigm protocol.
5. Set an eight-channel pipette to 3.5 µl. For detailed instructions on pipetting the harvested aliquots to the "Diluted Harvest" plate, proceed to steps 7–10 of the Fluidigm protocol. According to the exact plate maps in Figures 8–10 on pages 32 and 33 of the Fluidigm protocol, pipet the cDNA from the IFC inlets into the "Diluted Harvest" plate containing 10 µl dilution reagent per well.

   IMPORTANT: It is critical to follow the maps while transferring the cDNAs from the IFC to the "Diluted Harvest" plate in order to trace the sample IDs. After completing this transfer, the cDNAs generated from the capture sites will be arranged on the "Diluted Harvest" plate as shown in Figure 11 on page 34 of the Fluidigm protocol.
6. Seal and vortex the "Diluted Harvest" plate for 10 sec at medium speed and then spin it down.

   NOTE: Samples can be stored for up to one week at 4ºC or at –20ºC long term.

VI. Library Preparation Protocols

These protocols describe a modified Illumina Nextera XT DNA library preparation protocol for single-cell mRNA sequencing library preparation from cDNA acquired via the C1 system. The Illumina Nextera XT DNA Library Preparation Guide provides detailed instructions for library preparation and we highly recommend that you read it before proceeding; however, keep in mind that modifications have been made in order to adapt the Nextera XT chemistry to the single-cell mRNA sequencing application.

A. Protocol: Quantify and Dilute Harvested cDNA

cDNA concentrations obtained with the C1 system may vary with cell types and treatments. Library yield and size distribution may also vary with input DNA concentration. To minimize library prep variation and to achieve high library quality, the concentration of the harvested cDNA for dilution must be carefully determined.

NOTE: The optimal cDNA concentration for Nextera XT library preparation is 0.10–0.3 ng/µl.

1. Determine cDNA concentration. You may use the PicoGreen assay to determine the concentration of the cDNA samples: The Microsoft Excel worksheet, Single-Cell mRNA Seq PicoGreen Template (Fluidigm, Part No. 100-6260), is useful for quantifying the cDNA.
   Alternate methods can also be used to quantify the cDNA:
   - If a 384-well fluorometer is not available, concentrations can be determined using an Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit. Estimate the concentration of each sample using a 150–10,000 bp range.
   - Concentrations can also be measured using a Qubit fluorometer and the Single-Cell mRNA Seq PicoGreen Template. Input values into the Concentration Estimate Table on the "Example Results" tab of the aforementioned Fluidigm template (PN 100-6260).
2. Based on the optimal dilution determined from the Concentration Estimate Table, dilute each sample so it falls within the optimal range of concentrations for Nextera XT library preparation as follows:
   a. Label a new 96-well PCR plate "Diluted for Sequencing."
   b. Pipet the appropriate amount of C1 Harvest Reagent into each well of the "Diluted for Sequencing" plate per determined sample dilution:

cDNA Sample Dilution	Volume of C1 Harvest Reagent Required
1:2	2 µl
1:3	4 µl
1:4	6 µl
1:5	8 µl
1:6	10 µl
1:8	14 µl
1:10	18 µl
1:12	22 µl

   c. Transfer 2 µl of each harvest sample from the "Diluted Harvest" plate to the "Diluted for Sequencing" plate.
   d. Seal the "Diluted for Sequencing" plate with adhesive film.
   e. Vortex at medium speed for 20 sec and centrifuge at 350*g* for 1 min.

B. Protocol: Prepare cDNA for Tagmentation

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 until all particulates are resuspended.

1. After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by centrifuging the tubes briefly.
2. Label a new 96-well PCR plate "Library Prep."
3. In a 1.5-ml PCR tube, combine the components of the tagmentation premix. Calculate the amount of premix needed based on your number of samples, or prepare enough premix for 96 samples:

Volume per sample:		Master mix for 96 samples*	
2.5 µl	Tagment DNA Buffer	300 µl	Tagment DNA Buffer
1.25 µl	Amplification Tagment Mix	150 µl	Amplification Tagment Mix
1.25 µl	Diluted sample	—	Diluted sample
5 µl	Total volume of premix	—	Total volume of premix

* Calculated based on a 25% overage.

4. Vortex gently for 20 sec and centrifuge the tube briefly.
5. Aliquot equal amounts of premix into each tube of an eight-tube strip.
6. Using an eight-channel pipette, pipet 3.75 µl of the premix into each well of the "Library Prep" plate.
7. Transfer 1.25 µl of each diluted sample from the "Diluted for Sequencing" plate to the "Library Prep" plate.
8. Seal the plate and vortex at medium speed for 20 sec. Centrifuge at 2,000*g* for 5 min to remove bubbles.
9. Place the "Library Prep" plate in a hot-lid thermal cycler and run the following program:

55°C	10 min
10°C	Hold

10. Aliquot equal amounts of NT Buffer into each tube of an eight-tube strip. You will need 1.25 µl of NT Buffer for each sample plus 25% overage. Calculate the amount of NT Buffer needed based on your number of samples, or prepare enough NT Buffer for 96 samples:

Volume per sample		Master mix for 96 samples*	
5 µl	Library Prep sample	—	Library Prep plate
1.25 µl	NT Buffer	150 µl	NT Buffer
6.25 µl	Total volume	—	Total volume

* Calculated based on a 25% overage.

11. Once the thermal cycler reaches 10°C, pipet 1.25 µl of NT Buffer into each of the tagmented samples to neutralize the samples.
12. Seal the plate and vortex at medium speed. Centrifuge at 2,000*g* for 5 min.

C. Protocol: Amplify the Tagmented cDNA

IMPORTANT: Read the Illumina protocol, "Nextera XT DNA Library Prep Reference Guide" carefully for index primer selection criteria before proceeding to PCR amplification of the tagmented cDNA. See Appendix B for more information.

1. Aliquot equal volumes of Nextera PCR Master Mix (NPM) into each tube of an eight-tube strip. Calculate the amount of NPM needed based on your number of samples, or prepare enough NPM for 96 samples:

Volume per sample		Volume for 96 samples*	
6.25 µl	Library Prep sample	—	Library Prep plate
3.75 µl	NPM	450 µl	NPM
10 µl	Total volume	—	Total volume

* Calculated based on a 25% overage.

2. Pipet 3.75 µl of the aliquoted NPM into each well of the "Library Prep" plate using an eight-channel pipette.
3. Select appropriate Index 1 (N7xx) and Index 2 (S5xx) primers for the number of samples in your experiment. Each Index 1 Primer corresponds to a column of the 96-well plate and each Index 2 Primer corresponds to a row, as shown in Figure 3 on Page 11 of the aforementioned Illumina protocol:
    a. Pipet 1.25 µl of Index 1 Primers (N7xx) into the corresponding wells of **each row** of the "Library Prep" plate using a 12-channel pipette. As a result, each of the 12 wells in row "A" will contain different Index 1 Primers.
    b. Pipet 1.25 µl of Index 2 Primers (S5xx) to the corresponding wells of **each column** of the "Library Prep" plate using an eight-channel pipette. As a result, each of the 8 wells in column "1" will contain different Index 2 Primers.
4. Seal the plate with adhesive film and vortex at medium speed for 20 sec. Centrifuge at 2,000*g* for 2 min.
5. Place the "Library Prep" plate into a thermal cycler and perform PCR amplification.

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

6. Amplified products can be stored at −20ºC long term.

D. Protocol: Pool and Clean Up the Libraries

PCR-amplified, tagmented cDNA is purified by immobilization on AMPure XP beads (Agencourt). The beads are then washed with 70% ethanol and cDNA is eluted with C1 DNA Dilution Reagent.

NOTES:
- Aliquot AMPure XP beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 minutes and mix well to disperse.
- Prepare fresh 70% ethanol for each experiment. You will need ~800 µl per sample.
- You will need a magnetic separation device for 1.5-ml tubes.

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

   NOTE: If preferred, libraries can be cleaned up individually prior to pooling.

2. Warm AMPure XP beads to room temperature and vortex for 1 min.
3. Pool the libraries by pipetting the appropriate volume from each sample into a 1.5-ml tube, according to the number of libraries to be pooled:

Number of libraries to be pooled	Volume per library	Total pool volume	AMPure bead volume*
8	4 µl	32 µl	29 µl
12	4 µl	48 µl	44 µl
16	2 µl	32 µl	29 µl
24	2 µl	48 µl	44 µl
32	1 µl	32 µl	29 µl
48	1 µl	48 µl	44 µl
96	1 µl	96 µl	87 µl

* The bead volume is approximately 90% of the total pool volume.

4. Add the required amount of AMPure XP beads to the pooled libraries according to the table above.
5. Mix well by vortexing or pipetting the entire mixture up and down five times.
6. Incubate the bead mix at room temperature for 5 min to let the cDNA libraries bind to the beads.

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

7. Briefly spin the sample to collect the liquid from the side of the tube. Place the tube on a magnetic stand for 2 min, until the liquid appears completely clear, and there are no beads left in the supernatant.
8. While the sample is on the magnetic separation device, pipette the supernatant and discard.
9. Keep the sample on the magnetic separation device. Add 180 µl of freshly made 70% ethanol to the sample without disturbing the beads. Wait for 30 sec and carefully pipette and discard the supernatant containing contaminants. cDNA libraries will remain bound to the beads during the washing process.
10. Repeat the ethanol wash (Step 9) once.
11. Briefly spin the sample to collect the liquid from the side of the tube. Place the sample on the magnetic separation device for 30 sec, then remove all remaining ethanol with a pipette.
12. Allow the beads to air-dry for 10–15 min, until the pellet is no longer shiny, but before a crack appears.

NOTE: 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. The ethanol will reduce your recovery rate and ultimately your yield. Allow the sample to sit at room temperature until the pellet is dry.
- If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 minutes to rehydrate (Step 13) and may reduce amplified library recovery and yield.

13. Once the beads are dry, elute the pooled, purified libraries by adding the required volume of C1 DNA Dilution Reagent, based on the number of samples pooled.

Number of libraries pooled	C1 Dilution Reagent volume*
8	32 µl
12	48 µl
16	32 µl
24	48 µl
32	32 µl
48	48 µl
96	96 µl

* C1 Dilution Reagent volume is equal to the original pool volume.

14. Remove from the magnetic separation device and vortex the tube for 3 sec to mix thoroughly. Incubate at room temperature for 2 min to rehydrate the beads.
15. 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.
16. Transfer the entire volume of clear supernatant containing purified cDNA libraries to another PCR tube.

Repeat Cleanup:

1. Repeat Steps 4–12 in the previous section.
2. Elute the sample by adding the required volume* of C1 DNA Dilution Reagent, based on the number of libraries pooled:

Number of libraries pooled	C1 Dilution Reagent volume*
8	48 µl
12	72 µl
16	48 µl
24	72 µl
32	48 µl
48	72 µl
96	144 µl

* C1 Dilution Reagent volume is 1.5 times the original pool volume.

3. Remove the tube from the magnetic stand and vortex the tube for 3 sec.
4. Incubate at room temperature for 2 min.
5. Place the tube on the magnetic stand for 2 min.
6. Carefully transfer the supernatant to another 1.5-ml tube labeled “Cleaned Lib.”

7. To determine library size distribution and quantitation, perform Agilent Bioanalyzer analysis in triplicate using the Agilent High Sensitivity DNA Kit. Refer to the Agilent Bioanalyzer user guide (see Appendix B). Alternatively, you can quantify the library using a Qubit fluorometer.
8. Refer to the "Nextera XT DNA Library Preparation Guide" to determine the appropriate library concentration for sequencing.

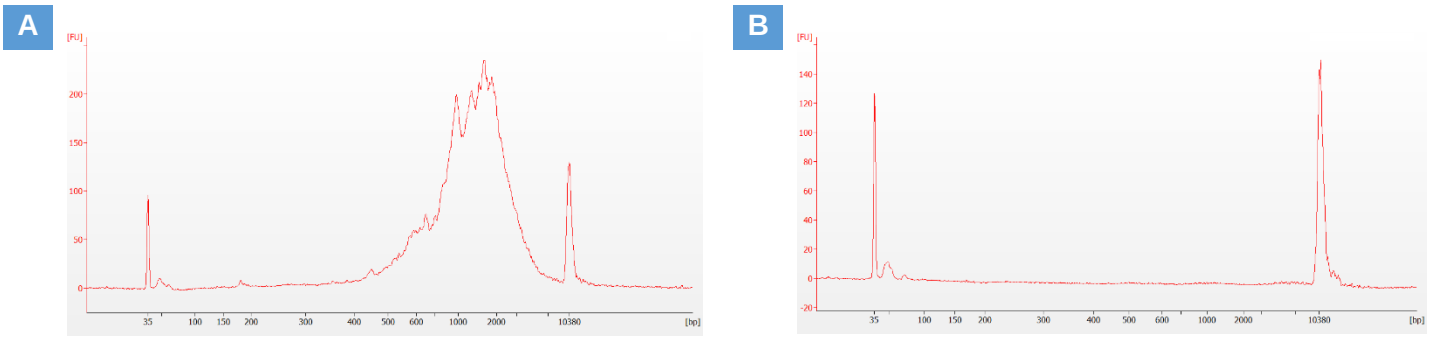

Figure 5. Examples of cDNA harvested from the IFC (electropherogram results from the Agilent 2100 Bioanalyzer). One µl of the harvested cDNA (from 3–3.5 µl cDNA diluted in 10 µl C1 Dilution Reagent) generated from a K562 cell was analyzed using an Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit and 2100 Expert software. Most cDNAs ranged between 400 and 10,000 bp in length. **Panel A** shows a clean product following cDNA synthesis and amplification. **Panel B** shows the product derived from a capture site where no cell was captured, following cDNA synthesis and amplification.

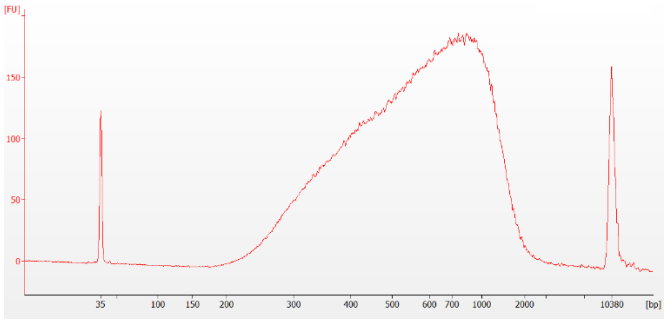

Figure 6. An example of Nextera XT sequencing library size distribution from 12 pooled cDNA libraries. cDNAs from twelve K562 cells were harvested and quantified, and an average of 230 pg of each cDNA was used as the input for Nextera XT library construction. Nextera XT libraries were created from these samples, pooled, and bead purified following the protocol. One µl of the bead-purified libraries was analyzed on the Bioanalyzer to yield this curve.

VII. References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. (1998) Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis*. Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319.

Ramsköld, D., Luo, S., Wang, Y. C., Li, R., Deng, Q., Faridani, O. R., Daniels, G. A., Khrebtukova, I., Loring, J. F., Laurent, L. C., Schroth, G. P. & Sandberg, R. (2012) Full-length mRNA-seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology* **30**(8):777–782.

Appendix A: Run the Tube Controls
(Prepare Samples and Perform Tube Control Reactions in PCR Clean Workstation)

Prepare Samples

Large numbers (hundreds) of cells in the tube control may inhibit the reaction chemistry. Therefore, we recommend performing a positive tube control from purified RNA (Protocol A) or from whole cells (Protocol B), as well as a negative tube control.

Protocol A: Tube Controls with Purified RNA

IMPORTANT: Review the NucleoSpin RNA XS Kit protocol before proceeding. Some components contain guanidinium thiocyanate, which can form highly reactive compounds when combined with bleach. Special care must be taken in handling and disposal.

1. Dilute cells in media to a final concentration of 100–200 cells/µl.
2. Isolate RNA from 20–100 µl of cell solution (from Section V.C.2.e), following the NucleoSpin RNA XS Kit protocol.
3. Proceed to "Perform the Tube Control Reactions" below.

   NOTE: Even though cells are lysed, perform the lysis reaction as directed, because the 3′ SMART primer is added during the lysis step.

Protocol B: Tube Controls with Whole Cells

1. Pellet cells from the cell solution (from Section V.C.2.e). Speeds and durations may vary. We suggest centrifuging cells at 300*g* for 5 min.
2. Gently pipette and discard the supernatant without disturbing the cell pellet.
3. Resuspend cells in 1 ml Cell Wash Buffer by pipetting up and down at least 5 times.
4. Pellet cells again and remove supernatant.
5. Wash a second time: Resuspend cells in 1 ml Cell Wash Buffer by pipetting up and down at least 5 times.
6. Pellet cells again and remove supernatant.
7. Resuspend cells in Cell Wash Buffer to approximately 90% original volume (to keep the original concentration, assuming a 10% loss).
8. Determine the concentration and dilute the cell suspension to 100–200 cells/µl with Cell Wash Buffer.

   NOTE: Cells may be counted by any preferred method. If an established cell counting protocol does not exist, we suggest using the INCYTO C-Chip disposable hemocytometer.
9. Proceed to "Perform the Tube Control Reactions," below.

Perform the Tube Control Reactions

1. Prepare the tube controls by combining and then thermal cycling the lysis reagents:
   a. Prepare the cell lysis mix:

Components	Positive control (from purified RNA)	Positive control (from cells)	No-template control
Purified RNA (Protocol A)	1 µl	—	—
Prepared whole cells (Protocol B)	—	1 µl	—
Cell Wash Buffer	—	—	1 µl
Lysis Mix	2 µl	2 µl	2 µl
Total volume	3 µl	3 µl	3 µl

   b. In a thermal cycler, run the following protocol:

72°C	3 min
4°C	10 min
25°C	1 min
4°C	Hold

IMPORTANT: As soon as this step finishes, quickly proceed to the next step.

2. Combine the RT Mix with the cell lysis mix from Step 1:
   a. RT reaction:

Components	Positive control	No-template control
Cell lysis mix	3 µl	3 µl
RT Mix	4 µl	4 µl
Total volume	7 µl	7 µl

   b. Vortex the tube controls for 3 sec and spin down briefly.
   c. In a thermal cycler, run the following protocol for reverse transcription. Preheat the thermal cycler to 42ºC:

42°C	90 min
70°C	10 min
4°C	Hold

NOTE: This is a potential stopping point. RT reaction products can be stored at 4ºC in the thermal cycler overnight and prepared for PCR the following morning.

3. Once reverse transcription has finished, combine the following in two tubes:

Components	Positive control	No-template control
PCR Mix	9 µl	9 µl
RT reaction products	1 µl	1 µl
Total volume	10 µl	10 µl

4. In a thermal cycler, run the following protocol:

98°C		1 min
5 cycles:		
	98°C	20 sec
	59°C	4 min
	68°C	6 min
9 cycles:		
	95°C	20 sec
	65°C	30 sec
	68°C	6 min
7 cycles:		
	95°C	30 sec
	65°C	30 sec
	68°C	7 min
72°C		10 min
4°C		Hold

Dilute Products

IMPORTANT: Transfer the samples from the PCR Clean Work Station to the general lab. All downstream processes should be performed in the general lab.

1. Transfer prepared material to a post-PCR room.
2. Vortex the PCR products for 3 sec and centrifuge to collect contents.
3. Combine the following reagents:

45 µl	C1 DNA Dilution Reagent (Fluidigm)
1 µl	PCR product
46 µl	Total volume

4. Continue with the quantification protocol for library preparation. (See "Quantify and Dilute Harvest cDNA," Section VI.A)

Appendix B: Related Documentation

cDNA Synthesis

- **Protocol (Part No. 100-7168), "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing"**
  This Fluidigm protocol is available for download at https://www.fluidigm.com/productsupport/c1-support-hub underneath Documents > Protocols > link : C1 System for mRNA Seq
- **C1 System User Guide (Part No. 100-4977)**
  This Fluidigm user guide is available for download at https://www.fluidigm.com/productsupport/c1-support-hub underneath Documents > User Guides > C1 System
- **Single-Cell Preparation Guide (Part No. 100-7697)**
  This Fluidigm guide is available for download at https://www.fluidigm.com/products/c1-system underneath Resources > E-Book > link : Single-Cell Preparation Guide ebook
- **SMART-Seq v4 script for the C1 System**
  This script is available for download at https://www.fluidigm.com/c1openapp/scripthub
- **Manual and Protocol (Cat. No. L-3224), LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells**
  A Thermo Fisher Scientific manual is available for download at https://www.thermofisher.com/order/catalog/product/L3224 underneath Manuals > Manuals & protocols > LIVE/DEAD Viability/Cytotoxicity Kit
- **Protocol (Prod. No. DHC-N01), "Using a hemacytometer."**
  This INCYTO user manual is available for download at http://incyto.com/product/product02_detail.php underneath Useful References > link : Using a hemacytometer
- **User Manual (Part No. G2938-90321), "Agilent High Sensitivity DNA Kit Guide"**
  This Agilent Technologies user manual is available for download at http://www.genomics.agilent.com/en/Bioanalyzer-DNA-RNA-Kits/High-Sensitivity-DNA-Analysis-Kits/?cid=AG-PT-105&tabId=AG-PR-1069&crumbAction=push&contentType=User+Manual by clicking Manuals & Protocols > link : Agilent High Sensitivity DNA Kit Guide (Rev. B)

DNA Sequencing

- **Protocol (Part No. 15031942 v01), "Nextera XT DNA Library Prep Reference Guide"**
  This Illumina protocol is available for download at http://support.illumina.com/downloads/nextera_xt_sample_preparation_guide_15031942.html underneath File Name > link : Nextera XT DNA Library Prep Reference Guide (15031942 v01)
- **Single-Cell WTA PicoGreen Template (Fluidigm, Part No. 100-6260)**

Appendix C: SMART-Seq v4 Script Thermal-Cycling Conditions

The Sample Prep (1861x/1862x/1863x) script, which is a part of the SMART-Seq v4 script, contains the following thermal-cycling conditions:

Lysis:

72°C	3 min
4°C	10 min
25°C	1 min

Reverse Transcription:

42°C	90 min
70°C	10 min

PCR:

1 cycle:

98°C	1 min

5 cycles:

98°C	20 sec
59°C	4 min
68°C	6 min

9 cycles:

95°C	20 sec
65°C	30 sec
68°C	6 min

7 cycles:

95°C	30 sec
65°C	30 sec
68°C	7 min

1 cycle:

72°C	10 min
10°C	Hold

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