UCSD

Single Cell Analysis Protocol Version 2

Sep 30, 2014

Nuclei Preparation and Sorting

&

cDNA Preparation and NexteraXT Library Preparation

Protocol_UCSD_2

1. **Nuclei Preparation and Sorting**
   - According to attached *Subprotocol_1*
2. **cDNA Preparation and NexteraXT Library Preparation**
   - According to the *Subprotocol_2*: *Fluidigm C1 RNA-Seq*. With the following modifications:
     - ERCC RNA Spike-In Mix (4456740, Ambion) used at 1:40,000 dilution in lysis reaction buffer
     - BSA (1%) added to the C1 blocking buffer
     - For loading, C1 Suspension buffer added in a 7 (nuclei) to 3 (C1 Suspension RGT) ratio
     - C1 Cell Wash Buffer was replaced with C1 DNA Seq Cell Wash Buffer (P/N 100-7158)
     - cDNA quantification using Quant-iT PicoGreen dsDNA Assay Kit (P7589, Life Technologies)
     - PCR Cycles used as indicated in **NUM_PCR_CYCLES_cDNA** and **NUM_PCR_CYCLES_Nextera**
   - C1_ADD_Reagents:
     - **Randomer** added to Lysis Reaction Mix, (3.5ul of 12uM primer diluted in Clontech Dilution Buffer - replacing equal volume of Clontech Dilution Buffer from original protocol)
     - **PolydIdC** (250ng/ul, Sigma) added to Lysis Reaction Mix (1.33ul PolydIdC + 0.67ul 0.6% NP-40 - replacing equal volume of Clontech Dilution Buffer from original protocol)

Subprotocol_1: Nuclei isolation

1. Clean all instruments and trays that contact human material with RNase Away. Rinse with DEPC-water.
2. Move human brain sample from -80oC and place at -20^{o}C for 15 minutes (cryostat chamber is acceptable).
3. Use either razor blade or cryostat to cut off appropriate piece. Place into microfuge tube with ice-cold PBS+2 mM EDTA (PBSE). Mince with scissors, then transfer to 6-well plate.
4. Place onto rotator for 20 minutes on ice.
5. Triturate tissue. Pass through 40 um cell filter. Spin down 250-300xg for 5 minutes.
6. Add 1% NP40 + PBSE and mix well to liberate nuclei. Place onto ice for 7 minutes.
7. Spin down 250-300xg for 5 minutes.
8. Discard supernatant, resuspend nuclei into PBSE+1% BSA (alternatively PBSE + 3% BSA) into control and experimental FACS tubes. Block for 5 minutes.
9. Add primary antibody (anti-NeuN 1:1000) to experimental tube and rotate for 20 minutes.
10. Add secondary antibody (anti-rabbit-APC 1:1000) to both tubes. Stain for 15 minutes.
11. Add DAPI to both tubes and sort.
12. Sort strategy:
    a. Gate FSc and SSc. There should be a distinct nuclear population.
    b. Verify nuclei by gating for DAPI+ populations.
    c. Remove doublets by sequential FSc-A/FSc-W and SSc-A/SSc-W doublet discrimination.
    d. Examine NeuN+ (APC+) population. Sort NeuN+ high and DAPI+ population.

Using the C1™ Single-Cell Auto Prep System to Generate mRNA from Single Cells and Libraries for Sequencing

PN 100-7168 Rev. A1

Protocol

Introduction

This protocol allows the user to capture cells, convert polyA+ RNA into full-length cDNA, and perform universal amplification of the cDNA using the Fluidigm C_1™ Single-Cell Auto Prep System and C_1™ Single-Cell Auto Prep Array Integrated Fluidic Circuits (IFCs). The protocol explains all steps performed during cDNA synthesis, including: capturing cells, staining for viability, imaging cells, lysing cells, performing reverse transcription and long-distance PCR, and harvesting the amplified cDNA. To perform analysis by mRNA Seq, the full-length cDNA must first be converted to a sequencing library, and the final steps of library generation from cDNA are described in a the *mRNA Seq Library Preparation for Sequencing Protocol* (Fluidigm PN 100-5989). If desired, direct gene expression analysis of full-length cDNA can also be performed through qPCR on the 48.48 or 96.96 Dynamic Array™ IFCs using the BioMark™ or BioMark™ HD System as recommended in *Fluidigm® Real-Time PCR Analysis Software User Guide* (PN 68000088) using DELTAgene™ Assays.

The SMARTer® chemistry described in this protocol uses a modified oligo (dT) primer to prime first-strand synthesis, and selects for polyA+ RNA in a sample (see reference 1 in "References" on page 5). When the reverse transcriptase (RT) reaches the 5′ end of the mRNA, the enzyme's terminal transferase activity adds a few non-templated deoxycytidines to the 3′ end of the cDNA. The template-switch primer contains a few guanosines at its 3′ end that base-pair with the non-templated deoxycytidines on the cDNA to create an extended template. The RT then extends to the end of the template-switch primer, producing single-stranded cDNA that contains the SMARTer® universal tag sequence, the 3′ end of the mRNA, the full-length transcript up to the 5′ end of the mRNA, and the reverse complement of the SMARTer® universal tag sequence. Prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from RNA without polyA tail will not contain universal tag at both ends and will not be exponentially amplified during long-distance PCR. However, degraded RNAs present in low quality RNA that still have polyA tails may be amplified, yielding shorter cDNA fragments with incomplete coverage at the 5′ end of the transcript. Full-length transcripts are enriched during PCR because the SMARTer® tag, found at the 5′ end of the cDNA, can pair with its own reverse complement. The reverse complement is found at the 3′ end of short cDNAs and prevents amplification of short cDNAs. Figure 1 shows the template-switch chemistry used in the SMARTer® Kit (Clontech PN 634833):

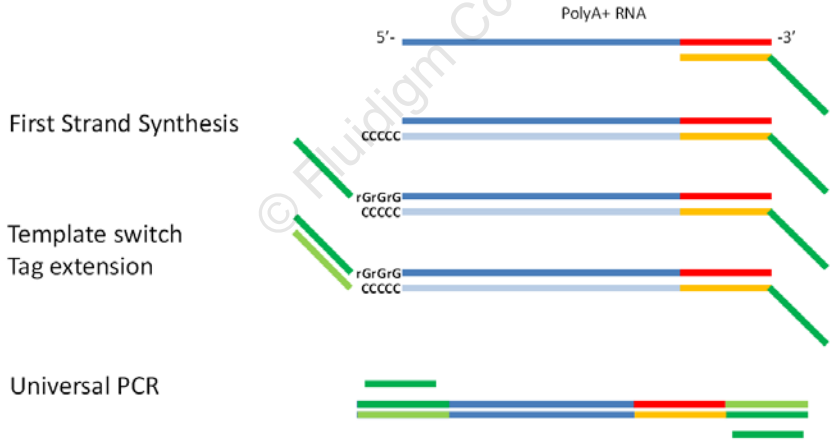

Figure 1 Overview SMARTer® Kit

The protocol also describes the modified Illumina® Nextera XT DNA sample preparation protocol for single-cell mRNA Seq library preparation for sequencing from cDNA acquired from the C_1™ Single-Cell Auto Prep System (see "Library Preparation for Illumina Sequencing" on page 32). Before proceeding with this protocol, we highly recommend that you carefully read the *Nextera XT DNA Sample Preparation User Guide*.

Revision History

Revision	Date	Description of change
A1	5 August 2013	New document on use of the new $C_1^{™}$ IFC for mRNA seq (5-10 µm) with small cells.

Overview of Cell Capture Process

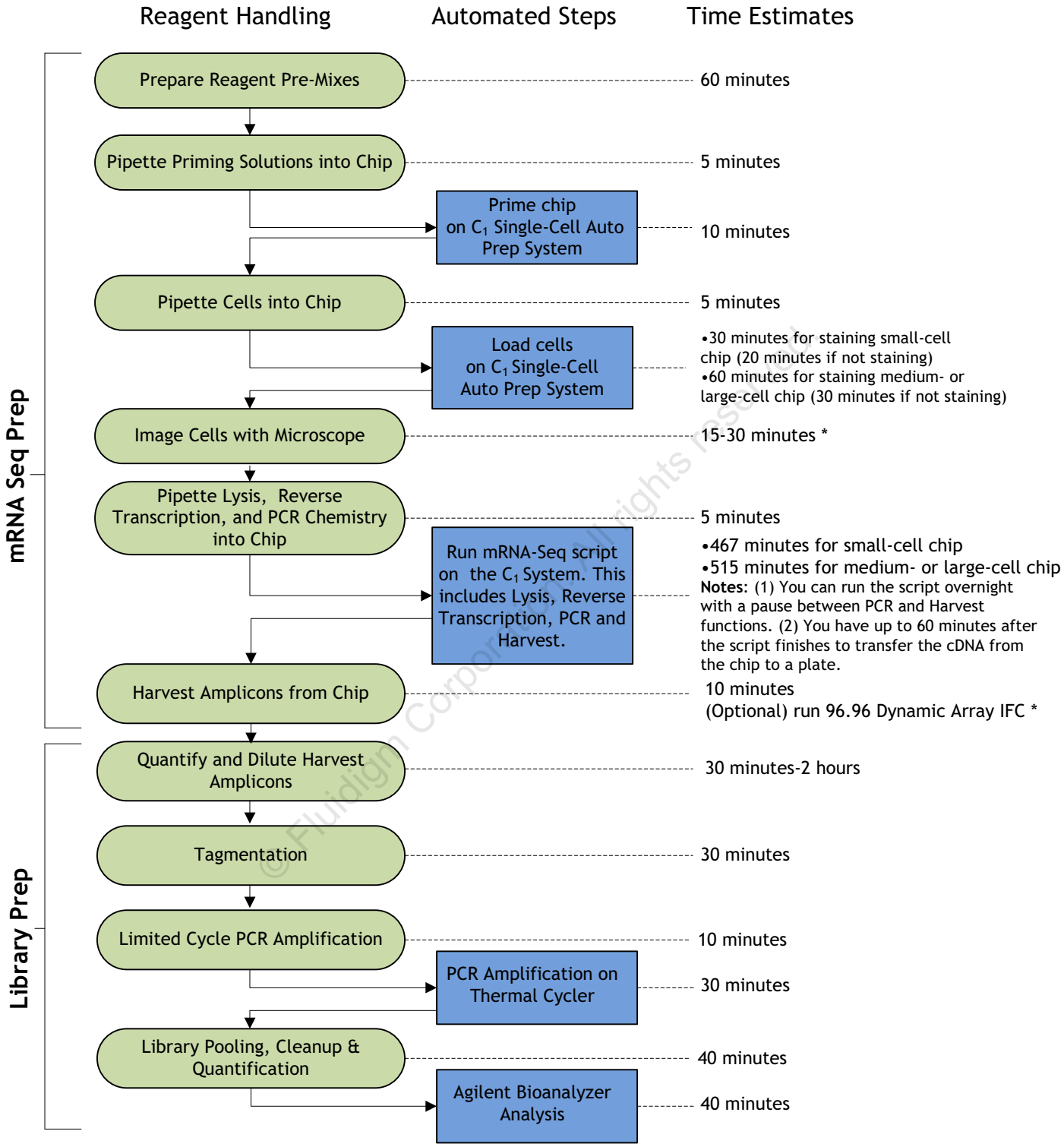

Figure 2 Overview of the cell capture procedure

IMPORTANT: If you are running the optional tube controls, please add up to 4 hours to the time estimate. The asterisks above denote where you would start, continue and finish the tube controls steps.

References

1 A. Chenchik, Y. Zhu, L. Diatchenko, R. Li, J. Hill, and P. Siebert, "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* (BioTechniques Books, 1998)

- *Fluidigm® BioMark™ HD Data Collection Software User Guide* (PN 100-2451)
- *Fluidigm® Real-Time PCR Analysis Software User Guide* (PN 68000088)
- *Fluidigm® C1™ Single-Cell Auto Prep System User Guide* (PN 100-4977)
- *Fluidigm® Application Guidance: Single-Cell Analysis* (PN 100-5066)
- ArrayControl™ Spots and Spikes (Life Technologies, PN AM1781)
- LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells (Life Technologies, PN L-3224)
- *Minimum Specifications for Single-Cell Imaging* (Fluidigm, PN 100-5004)
- INCYTO Disposable Hemocytometer, www.incyto.com/product/product02_detail.php
- *Illumina® Nextera XT DNA Sample Preparation User Guide*
- *Single-Cell WTA PicoGreen® Template* (Fluidigm, PN 100-6260)
- Agilent Bioanalyzer user guide

Required Consumables

Product Name	Company	Part Number
Clontech SMARTer® Kit Designed for the C1™ System, 10 IFCs	Clontech	634833
C1™ IFC for mRNA seq (5-10 µm) C1™ IFC for mRNA seq (10-17 µm) C1™ IFC for mRNA seq (17-25 µm) C1™ Single-Cell Auto Prep Reagent Kit for mRNA Seq	Fluidigm	100-5759 100-5760 100-5761 100-6201
Nextera XT DNA Sample Preparation Kit Nextera XT DNA Sample Preparation Index Kit (96 indices, 384 samples)	Illumina	FC-131-1096 FC-131-1002
Agencourt AMPure® XP	Agencourt Bioscience Corp	A63880

Suggested Consumables

Product Name	Company	Part Number
ArrayControl™ RNA Spikes THE RNA Storage Solution	Life Technologies	AM1780 AM7000
LIVE/DEAD® Viability/Cytotoxicity Kit	Life Technologies	L-3224
High Sensitivity DNA Chips and Reagents	Agilent Technologies	5067-4626
Quant-IT™ PicoGreen® dsDNA Assay Kit	Life Technologies	P11496
RNeasy Plus Micro Kit QIAshredder disposable cell-lysate homogenizers	Qiagen	74034 79654

Required Reagents for Tube Controls

- RNeasy Plus Micro Kit (Qiagen, PN 74035)

NOTE: Store the RNeasy MinElute spin columns immediately upon receipt at 2–8 °C. Store the remaining components of the kit dry at room temperature (15–25 °C). All kit components are stable for at least 9 months under these conditions.

- 14.3 M β-mercaptoethanol (β-ME; commercially available solutions are usually 14.3 M) or, alternatively, 2 M dithiothreitol (DTT) in water
- Ethanol (70% and 80%) (Do not use denatured ethanol)
- QIAshredder disposable cell-lysate homogenizers (Qiagen, PN 79654)

Required Reagents for cDNA Synthesis

NOTE: When ordering the C_1™ Single-Cell Auto Prep Kit for mRNA Seq, Module 1 and Module 2 kits, from Fluidigm, use the parent part number: 100-6201.

Stored at –80 °C

- (Suggested) ArrayControl™ RNA Spikes (Life Technologies, PN AM1780)
- Clontech SMARTer® Kit Designed for the C_1™ System, Box 1 of 2 (Clontech PN 634835; not sold separately; sold as part of Clontech PN 634833)

NOTE: The above SMARTer® kit is for 10 chip runs. Clontech also offers a kit for 2 chip runs (Clontech, PN 634832).

Stored at –20 °C

- Clontech SMARTer® Kit Designed for the C_1™ System, Box 2 of 2 (Clontech PN 634835; not sold separately; sold as part of Clontech PN 634833). **Note**: After the first thaw, you can store the Dilution Buffer at 4 °C.
- Advantage® 2 PCR Kit (Clontech PN 639207). Sold as part of Clontech PN 634833 and 634832.

NOTE: The Advantage® 2 PCR Kit is sufficient for >10 chip runs.

- C_1™ Single-Cell Auto Prep Kit for mRNA Seq, Module 2 (Fluidigm, PN100-6209)
- (*Optional*) LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells (Life Technologies, PN L-3224)

Stored at 4 ºC

- C_1™ Single Cell Auto Prep Reagent Kit, Module 1 (Fluidigm, PN 100-5319)

Stored at room temperature

- 70% Ethanol in a squirt bottle
- (Suggested) THE RNA Storage Solution (Life Technologies, PN AM7000)
- (Optional) INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved, PN DHC-N01)

NOTE: Please note storage conditions for each component in the SMARTer® and Advantage® 2 kits and store appropriately.

Required Reagents for Illumina Sequencing Library Preparation

–20 ºC

- Nextera XT DNA Sample Preparation Kit (Illumina, PN FC-131-1096) (Box 1 of 2)
- Nextera XT DNA Sample Preparation Index Kit (96 Indices, 385 Samples) (Illumina, PN FC-131-1002)
- Lambda DNA (Life Technologies, PN 25250-010)
- $C_1^{™}$ DNA Dilution Reagent (Fluidigm, PN 100-5317)

4 ºC

- Nextera XT DNA Sample Preparation Kit (Illumina, PN FC-131-1096) (Box 2 of 2)
- Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, PN P11496)
- High Sensitivity DNA Reagents (Agilent Technologies, PN 5067-4626)
- Agencourt AMPure® XP (Agencourt BioScience Corp., PN A63880)

Room Temperature

- Ethanol, 500 mL (Sigma-Aldrich, PN E70235)

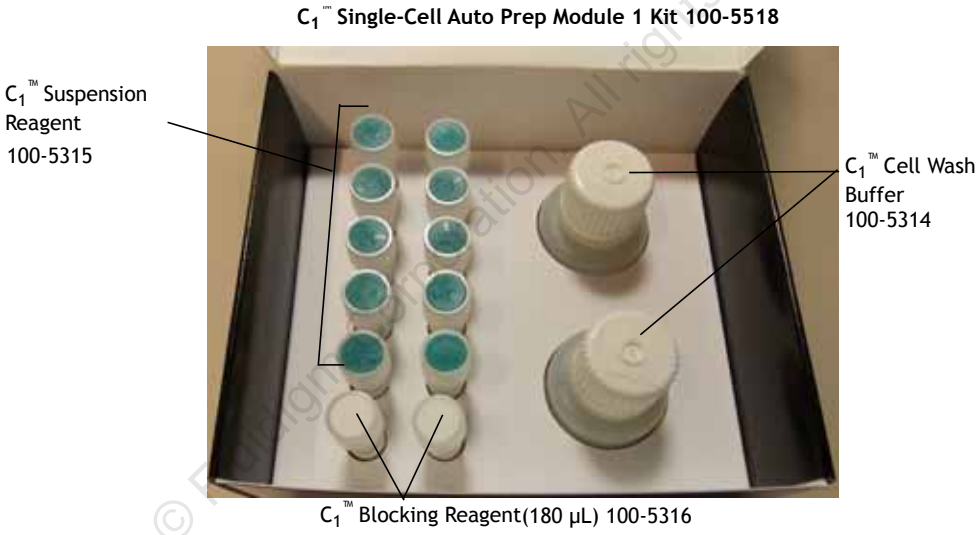

Figure 3 $C_1^{™}$ Single-Cell Auto Prep Module 1 Kit

IMPORTANT:

- Store the $C_1^{™}$ Single-Cell Auto Prep Module 1 kit at 4 ºC upon receipt.
- Do not freeze Module 1.

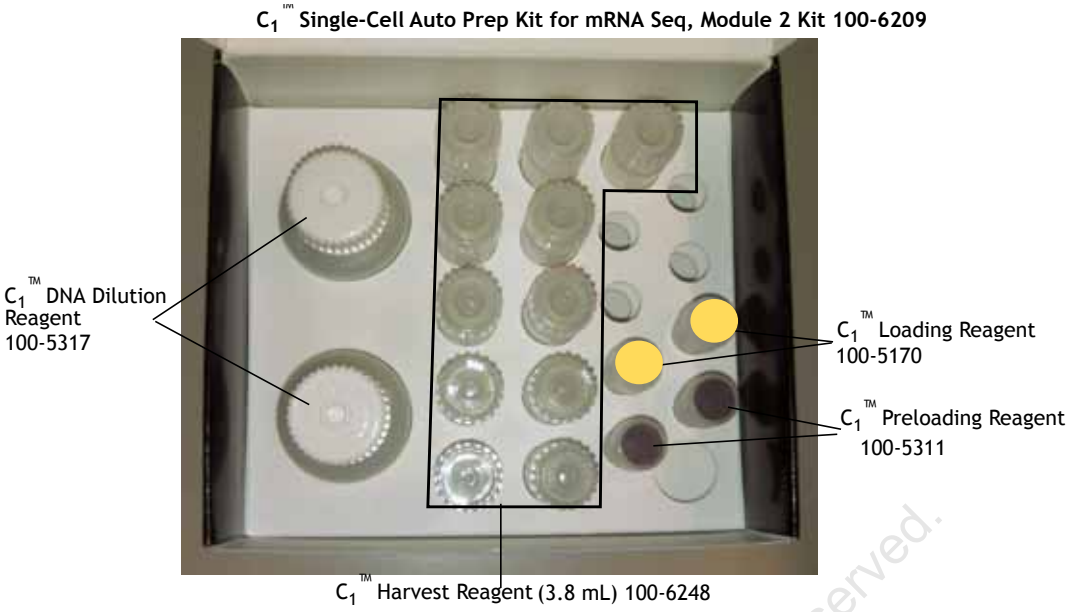

Figure 4 C1™ Single-Cell Auto Prep Module 2 Kit

IMPORTANT: Store the C1™ Single-Cell Auto Prep Module 2 kit in a -20 ºC freezer upon receipt.

Required Equipment

- C1™ Single-Cell Auto Prep System
- C1™ Single-Cell Auto Prep Array IFCs for mRNA Seq (barcodes 1771x, 1772x, or 1773x)
- 96-well PCR Plate (USA Scientific, TempPlate™ semi-skirted, PN 1402-9700)
- 2 centrifuges: 1 for Eppendorf tubes, 1 for 96-well plates
- Vortexer
- MicroAmp™ clear adhesive film (Life Technologies, PN 4306311)
- Agilent Bioanalyzer
- Agilent High Sensitivity DNA Chips and reagents (Agilent Technologies, PN 5067-4626)
- Thermal cycler
- Magnetic stand for PCR tubes
- Fluorometer (for PicoGreen® assay)
- Low-lint cloth
- Corning® 384 Well Low Flange Black Flat Bottom Polystyrene Not Treated Microplate (Corning, PN 3573)

Recommended Equipment

- 2 biocontainment hoods to prevent DNA contamination of lab and samples
- Imaging equipment compatible with C1™ Auto Prep Array integrated fluidic circuits (IFCs). See *Minimum Specifications for Single-Cell Imaging Specification Sheet*, PN 100-5004.

Chip Types and Related Scripts

There are currently three C1™ System-compatible IFCs for medium and large single cells:

Cell Size (Median)	Barcode (prefix)	Script Names	Description
Small (5-10 µm) C1™ Single-Cell Auto Prep IFC for mRNA Seq (5-10 µm) PN 100-5759	1771x	mRNA: Prime (1771x)	Priming the control line and cell capture channels of the 5–10 µm array (1771x)
		mRNA: Cell Load (1771x)	Cell loading and washing for PCR of 5-10 µm diameter cells (1771x)
		mRNA: Cell Load & Stain (1771x)	Cell loading, staining, and washing for mRNA Seq of 5-10 µm diameter cells (1771x)
		mRNA: RT & Amp (1771x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA amplification for the 5-10 µm C1™ mRNA Seq (1771x)
Medium (10-17 µm) C1™ Single-Cell Auto Prep IFC for mRNA Seq (10-17 µm) PN 100-5760	1772x	mRNA Seq: Prime (1772x)	Priming the control line and cell capture channels of the 10-17 µm array (1772x)
		mRNA Seq: Cell Load (1772x)	Cell loading and washing for PCR of 10-17 µm diameter cells (1772x)
		mRNA Seq: Cell Load & Stain (1772x)	Cell loading, staining, and washing for mRNA Seq Prep of 10-17 µm diameter cells (1772x)
		mRNA Seq: RT & Amp (1772x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA amplification for the 10-17 µm C1™ mRNA Seq Prep array (1772x)
Large (17-25 µm) C1™ Single-Cell Auto Prep IFC for mRNA Seq (17-25 µm PN 100-5761	1773x	mRNA Seq: Prime (1773x)	Priming the control line and cell capture channels of the 17-25 µm array (1773x)
		mRNA Seq: Cell Load (1773x)	Cell loading and washing for mRNA Seq Prep of 17-25 µm diameter cells (1773x)
		mRNA Seq: Cell Load & Stain (1773x)	Cell loading, staining, and washing for PCR of 17-25 µm diameter cells (1773x)
		mRNA Seq: RT & AMP (1773x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA amplification for the 17-25 µm C1™ mRNA Seq Prep array (1773x)

Table 1 IFCs and related scripts

Safety

It is the individual's responsibility to review all MSDS for chemicals used in this procedure before running the test.

As with all procedures, the following general safety guidelines apply:

- Personal Protective Equipment (PPE): safety glasses, fully-enclosed shoes, gloves.
- Know the locations of all safety equipment (fire extinguishers, spill kits, eyewashes/ showers, first aid kits, material safety data sheets, etc.), emergency exit locations, and emergency/injury reporting procedures.
- No eating, drinking, or smoking in lab areas
- Maintain clean work areas.
- Wash hands before leaving the lab.

HOT SURFACE! The $C_1^{™}$ Single-Cell Auto Prep System thermal cycler chuck gets hot and can burn your skin. Please use caution when working near the chuck.

PINCH HAZARD! The $C_1^{™}$ Single-Cell Auto Prep System door and shuttle can pinch your hand. Please make sure your fingers, hand, shirt sleeve, etc. are clear of the door and shuttle when loading or ejecting a chip.

BIOHAZARD! If you are putting live cells on the $C_1^{™}$ Single-Cell Auto Prep System, please use personal protective equipment and your lab's safety protocol to limit biohazard risks.

mRNA Sequencing Preparation—cDNA Synthesis

Reagent Retrieval for cDNA Synthesis

	Required Reagents	Preparation	Kit Name
1. Chip Priming	$C_1^{™}$ Preloading Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Fluidigm $C_1^{™}$ Kit
	$C_1^{™}$ Harvest Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Fluidigm $C_1^{™}$ Kit
	$C_1^{™}$ Blocking Reagent	Remove from 4 °C and thaw to room temperature in a DNA-free hood	Fluidigm $C_1^{™}$ Kit
2. Optional RNA Spikes	Array Control RNA Spikes	Remove from -80 °C, thaw on ice, and keep on ice	Array Control RNA Spikes (Life Technologies)
	THE RNA Storage Solution	Keep at room temperature	THE RNA Storage Solution (Life Technologies)
3. Lysis Mix - Mix A	$C_1^{™}$ Loading Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Fluidigm $C_1^{™}$ Kit
	RNase Inhibitor	Remove from -20 °C, thaw on ice, and keep on ice	SMARTer® Kit
	3′SMART CDS Primer IIA	Remove from -20 °C and thaw on ice. Keep on ice.	SMARTer® Kit
	SMARTer® Dilution Buffer	Remove from -20 °C (or 4 °C after first thaw) and keep at room temperature in a DNA-free hood	SMARTer® Kit
4. Reverse Transcription Mix - Mix B	5X First-Strand Buffer (RNase-free)	Remove from -20 °C, thaw on ice, and keep on ice	SMARTer® Kit
	Dithiothreitol	Remove from -20 °C, thaw on ice, and keep on ice	SMARTer® Kit
	dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)	Remove from -20 °C, thaw on ice, and keep on ice	SMARTer® Kit
	SMARTer® IIA Oligonucleotide	Remove from **-80 °C** and thaw to room temperature in a DNA-free hood	SMARTer® Kit
	SMARTScribe™ Reverse Transcriptase	Remove from -20 °C, thaw on ice, and keep on ice	SMARTer® Kit
5. PCR Mix - Mix C	PCR-Grade Water	Keep at room temperature	Advantage® 2 PCR Kit
	10X Advantage® 2 PCR Buffer (*not* SA, short amplicon)	Remove from -20 °C, thaw on ice, and keep on ice	Advantage® 2 PCR Kit
	50X dNTP Mix	Remove from -20 °C thaw on ice, and keep on ice	Advantage® 2 PCR Kit
	IS PCR primer	Remove from -20 °C, thaw on ice, and keep on ice	SMARTer® Kit
	50X Advantage® 2 Polymerase Mix	Remove from -20 °C, thaw on ice, and keep on ice	Advantage® 2 PCR Kit
6. Cells Mix - Mix D	Cells	Count and resuspend to appropriate concentration	—
	$C_1^{™}$ Suspension Reagent	Remove from 4 °C and vortex well	Fluidigm $C_1^{™}$ Kit
7. Optional LIVE/DEAD® Cell Staining	$C_1^{™}$ Cell Wash Buffer	Keep at room temperature	Fluidigm $C_1^{™}$ Kit
	Ethidium homodimer-1	Remove from -20 °C and keep in the dark as much as possible	LIVE/DEAD® Kit, Life Technologies
	Calcein AM	Remove from -20 °C and keep in the dark as much as possible	LIVE/DEAD® Kit, Life Technologies

Table 2 Reagent supplies

Reagent Mixes

IMPORTANT: Allow C1™ Cell Wash Buffer, C1™ DNA Dilution Reagent, and C1™ Harvest Reagent (Fluidigm) to equilibrate to room temperature prior to use.

- "Optional Array Control RNA Spikes"
- "Optional: Diluting RNA Spikes for Lysis Final Mix" on page 14
- "Lysis Mix – Mix A" on page 15
- "Reverse Transcription (RT) Reaction Mix – Mix B" on page 15
- "PCR Mix – Mix C" on page 16
- "Optional: Preparing LIVE/DEAD® Cell Staining Solution" on page 16
- "Cell Mix – D (Prepared while Priming the Chip)" on page 21

Optional Array Control RNA Spikes

RNA Spikes serve as a positive control for thermal cycling of the C1™ Single-Cell Auto Prep System that is independent of cell capture. Although this standard is not required, it is highly recommended.

NOTE: This reagent mix is sufficient for 125 C1™ chips. Due to the low volume pipetted, we highly recommend making this mix in bulk and aliquoting for future use.

NOTE: ArrayControl™ RNA Spikes contain eight RNA transcripts. We will use only three.

1 After the ArrayControl™ RNA Spikes have thawed, remove spikes 1, 4 and 7 from the box.

2 Pipette the following in three tubes:

Tube	A	B	C
THE RNA Storage Solution	13.5 µL	12.0 µL	148.5 µL
RNA Spikes	#7 - 1.5 µL	#4 - 1.5 µL	#1 - 1.5 µL

Table 3 RNA spikes

3 Vortex briefly tube A and spin to collect contents. Pipette 1.5 µL from tube A into tube B. Discard tube A.

4 Vortex briefly tube B and spin to collect contents. Pipette 1.5 µL from tube B into tube C. Discard tube B.

5 Vortex briefly tube C and spin to collect contents. Tube C is the concentrated RNA Standard that may be aliquoted and frozen for future use.

6 Aliquot in tubes containing 1.25 µL volumes and store at -80 °C until use. One tube is necessary for each C1™ chip run.

NOTE: For ordering information for RNA spike assays, see "Appendix 2: RNA Spike Assays" on page 45.

Optional: Diluting RNA Spikes for Lysis Final Mix

NOTE: Do not dilute and store RNA spike mixture at final concentration. Only store concentrated aliquots long term.

1 Thaw the RNA spikes mixture.

2 Dilute by combining:

Reagent	Volume (µL)
RNA Spikes mixture (Life Technologies)	1.0
$C_1^{™}$ Loading Reagent (Fluidigm)	99.0
Total	**100.0**

Table 4 RNA spike dilution

NOTE: You can serially dilute twice 1 µL of the RNA spikes mixture to 9 µL of $C_1^{™}$ Loading Reagent. Vortex after each dilution.

3 Vortex for 3 seconds and spin briefly to collect contents.

Lysis Mix – Mix A

NOTE: If not using RNA spikes, just add 1 µL of C1™ Loading Reagent. See tables below for detailed instructions.

1 Mix the following reagents in a tube labeled "A" to create the Lysis Mix:

Components	Volume (µL)
C1™ Loading Reagent (Fluidigm) or **Optional RNA Spikes mixture prepared in Table 4 above**	1.0
RNase Inhibitor (Clontech)	0.5
3′ SMART CDS Primer IIA (Clontech) (stored at -20 °C)	7.0
Clontech Dilution Buffer (**brown bottle**) (Do not vortex)	11.5
Total	**20.0**

Table 5 Cell Lysis Mix

2 Pipette up and down a few times to mix. Keep on ice until use.

Reverse Transcription (RT) Reaction Mix – Mix B

1 Mix the following reagents in a tube labeled "B" to create the RT Reaction Mix:

Components	Volume (µL)
C1™ Loading Reagent (Fluidigm)	1.2
5X First-Strand Buffer (RNase-free) (Clontech)	11.2
Dithiothreitol (Clontech)	1.4
dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM) (Clontech)	5.6
SMARTer® IIA Oligonucleotide (Clontech) (stored at -80 °C)	5.6
RNase Inhibitor (Clontech)	1.4
SMARTScribe™ Reverse Transcriptase (Clontech)	5.6
Total	**32.0**

Table 6 RT Reaction Mix

2 Vortex briefly and spin briefly to collect contents. Keep on ice until use.

PCR Mix – Mix C

1 Mix the following reagents in tube labeled "C" to create PCR Mix:

IMPORTANT: The Clontech kit contains two PCR buffers. Do *not* use the short amplicon or "SA" buffer. Staining small cells (5–10 µm) takes 30 minutes, and staining medium (10–17 µm) or large (17–25 µm) cells takes 60 minutes.

Components	Volume (µL)
PCR-Grade Water (Advantage® 2 PCR Kit)	63.5
10X Advantage® 2 PCR Buffer (*not* SA, short amplicon) (Advantage 2 Kit)	10.0
50X dNTP Mix (Advantage® 2 PCR Kit)	4.0
IS PCR primer (Clontech SMARTer® Kit)	4.0
50X Advantage® 2 Polymerase Mix (Advantage® 2 PCR Kit)	4.0
C_1™ Loading Reagent (Fluidigm)	4.5
Total	**90.0**

Table 7 PCR Final Mix

2 Briefly vortex and spin to collect contents before use. Keep on ice until ready to use.

Optional: Preparing LIVE/DEAD® Cell Staining Solution

The optional live/dead cell staining step uses the LIVE/DEAD® Viability/Cytotoxicity Kit, which tests the viability of a cell based on the integrity of the cell membrane. This test contains two chemical dyes. The first dye is green-fluorescent calcein-AM, which stains live cells. This dye is cell permeable and tests for active esterase activity in live cells. The second dye is red-fluorescent ethidium homodimer-1, which will stain cells only if the integrity of the cell membrane has been lost.

NOTE: 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 dark, airtight bag with desiccant pack at -20 °C.

NOTE: Cell staining solution may be prepared up to 2 hours before loading onto the C_1™ chip. Keep on ice and away from light before pipetting into chip.

IMPORTANT: Staining small cells (5–10 µm) takes 30 minutes, and staining medium (10–17 µm) or large (17–25 µm) cells takes 60 minutes. Optimize staining of small cells (see Table 8).

1 Vortex the dyes well before pipetting.

2 Prepare the LIVE/DEAD® stain:

Components	Volume (µL)
C1™ Cell Wash Buffer (Fluidigm) (30 mL bottle)	1250
Ethidium homodimer-1 (LIVE/DEAD® kit, Life Technologies/Molecular Probes)	2.5
Calcein AM (LIVE/DEAD® kit, Life Technologies/Molecular Probes)	0.625
Total	**1253.125**

Table 8 Staining solution

3 Vortex the C1™ LIVE/DEAD® staining solution well before pipetting onto chip.

Using the Chip Map Loading Plate

A black Chip Map Loading Plate accessory can be used to assist chip pipetting.

1 Obtain a Chip Map Loading Plate:

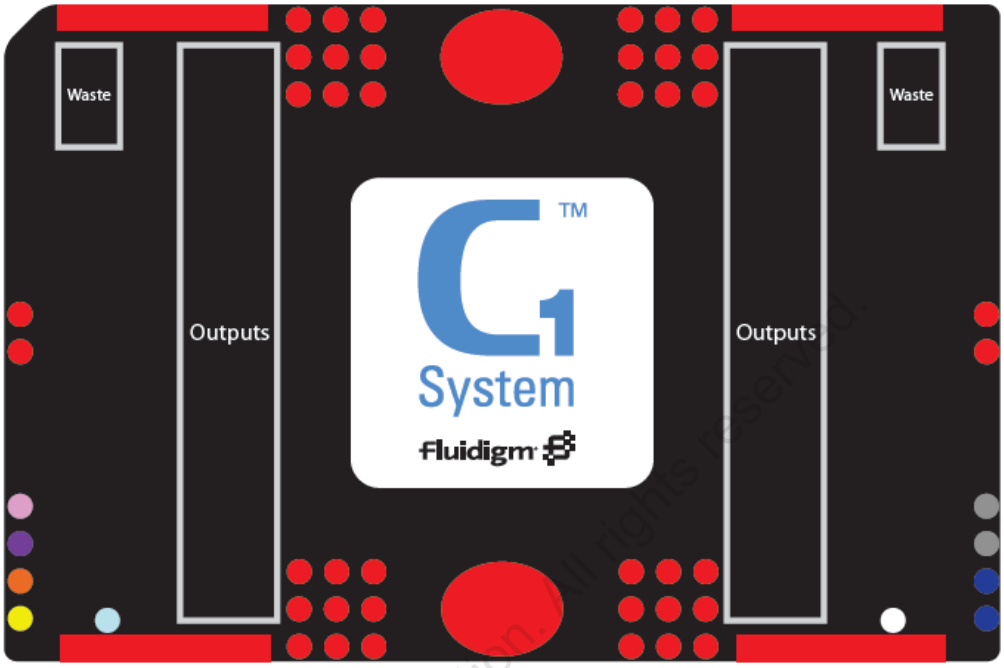

Figure 5 Chip Map Loading Plate

2 Place the $C_1^{™}$ Single-Cell Auto Prep IFC onto the Chip Map Loading Plate. For details on chip loading, see "Appendix 3: Chip Pipetting Map" on page 46.

3 Pipet the reagents (see "Priming the Chip" on page 19).

Priming the Chip

NOTE: When pipetting into the $C_1^{™}$ chip, always stop at the first stop on the pipette to avoid creating bubbles in the inlets. If a bubble is introduced, ensure that it floats to the top of the well.

NOTE: Vortex and spin all reagent mixes before pipetting into the chip.

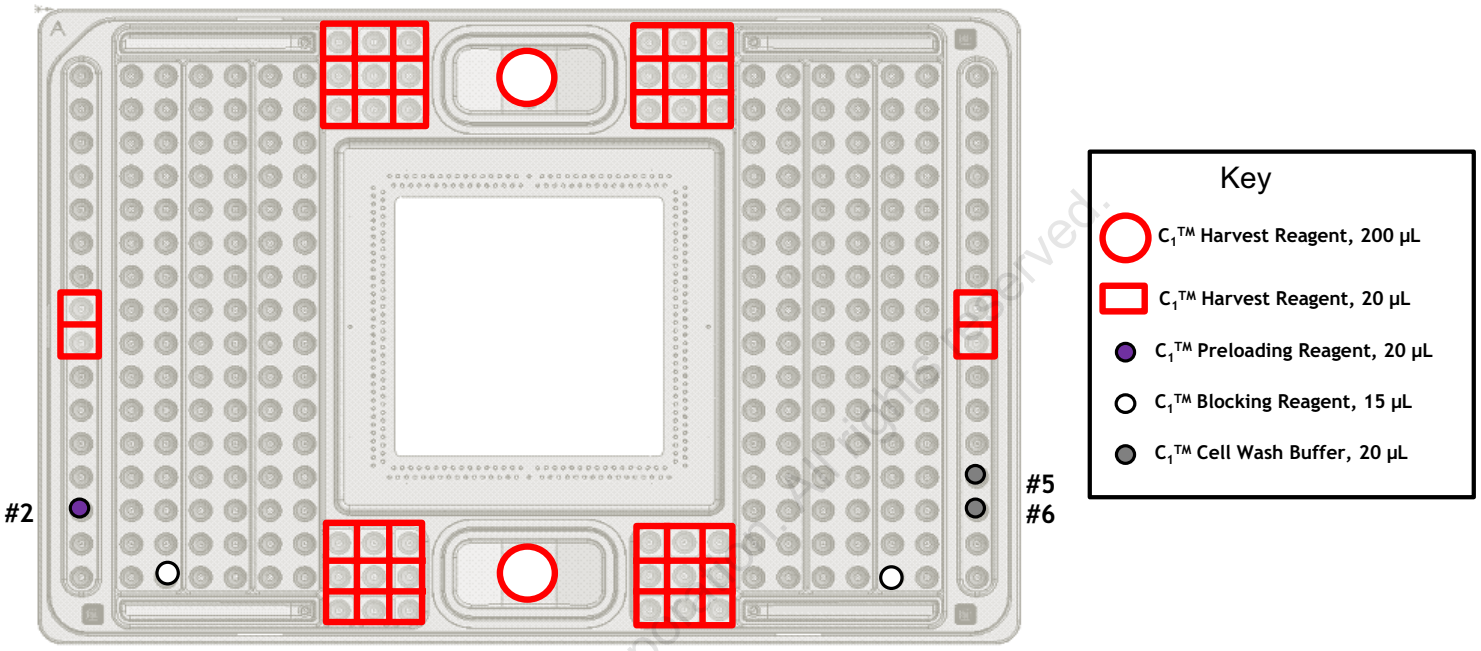

Figure 6 $C_1^{™}$ Chip priming pipetting map

1. Add 200 µL of $C_1^{™}$ Harvest Reagent from 4 mL bottle into accumulators marked with red circles in Figure 6 using a pipette tip.
2. Pipette 20 µL of $C_1^{™}$ Harvest Reagent into wells marked with red squares on each side of the accumulators (36 total).
3. Pipette 20 µL of $C_1^{™}$ Harvest Reagent into the two wells on each side of the chip marked with red squares. These wells are marked on the bottom of the chip with a notch to ensure they are easily located.
4. Pipette 20 µL of $C_1^{™}$ Preloading Reagent into inlet 2 marked with a purple dot.
5. Pipette 15 µL of $C_1^{™}$ Blocking Reagent into the Cell Inlet and Outlet marked with white dots.
6. Pipette 20 µL of $C_1^{™}$ Cell Wash Buffer into inlets 5 and 6 marked with dark gray dots.

7 Peel off white tape on bottom of chip.

8 Place the chip into the C1™ Single-Cell Auto Prep System then run the **mRNA Seq: Prime (1771x/1772x/1773x)** script. Priming takes approximately 10 minutes. When the Prime script has finished press **EJECT** to remove the primed chip from the instrument.

NOTE: After priming the chip, you have ≤1 h to load the chip with the C1™ Single-Cell Auto Prep System.

Preparing the Cells

Prepare a cell suspension of a concentration of 166-250 K/mL in native medium prior to mixing with C1™ Cell Suspension Reagent and loading onto the chip. This will ensure a total cell count pipetted on chip of approximately 500-750 cells. As few as 200 cells total, from 66 K/mL in native medium, may be loaded on the chip. Fewer cells loaded may yield fewer captured cells. A final volume of 0.5–1 mL is desirable so that there are enough cells for both the chip and the tube controls.

NOTE: Cells may be counted by any preferred method. If an established cell counting protocol does not exist, we suggest using the disposable hemocytometer C-Chip by INCYTO. See www.incyto.com for instructions for use.

Cell Mix – D (Prepared while Priming the Chip)

1 Prepare Cell Mix D by combining cells with C1™ Cell Suspension Reagent at a ratio of 3:2. For example

CAUTION! Vortex the C1™ Suspension Reagent thoroughly prior to use. If C1™ Suspension Reagent contains particulate, ensure they are properly removed by vortexing. Do NOT vortex the cells.

NOTE: The volume of Cell Mix may be scaled depending on volume of cells available. A minimum volume of 5 µL of Cell Mix is necessary for the chip. Maintain a ratio of cells to C1™ Cell Suspension Reagent of 3:2.

Components	Volume (µL)
Cells 166-250K/mL	60
C1™ Cell Suspension Reagent (Fluidigm)	40
Total	**100**

Table 9 Cell Mix D

2 Pipette Cell Mix D up and down 5–10 times to mix, depending on whether the cells tend to clump. Do not vortex the cell mix. Avoid bubbles when mixing as these may cause load failures.

NOTE: Cells may be counted by any preferred method. If an established cell counting protocol does not exist, we suggest using the disposable hemocytometer C-Chip by INCYTO. See www.incyto.com for instructions for use.

Loading the Cells

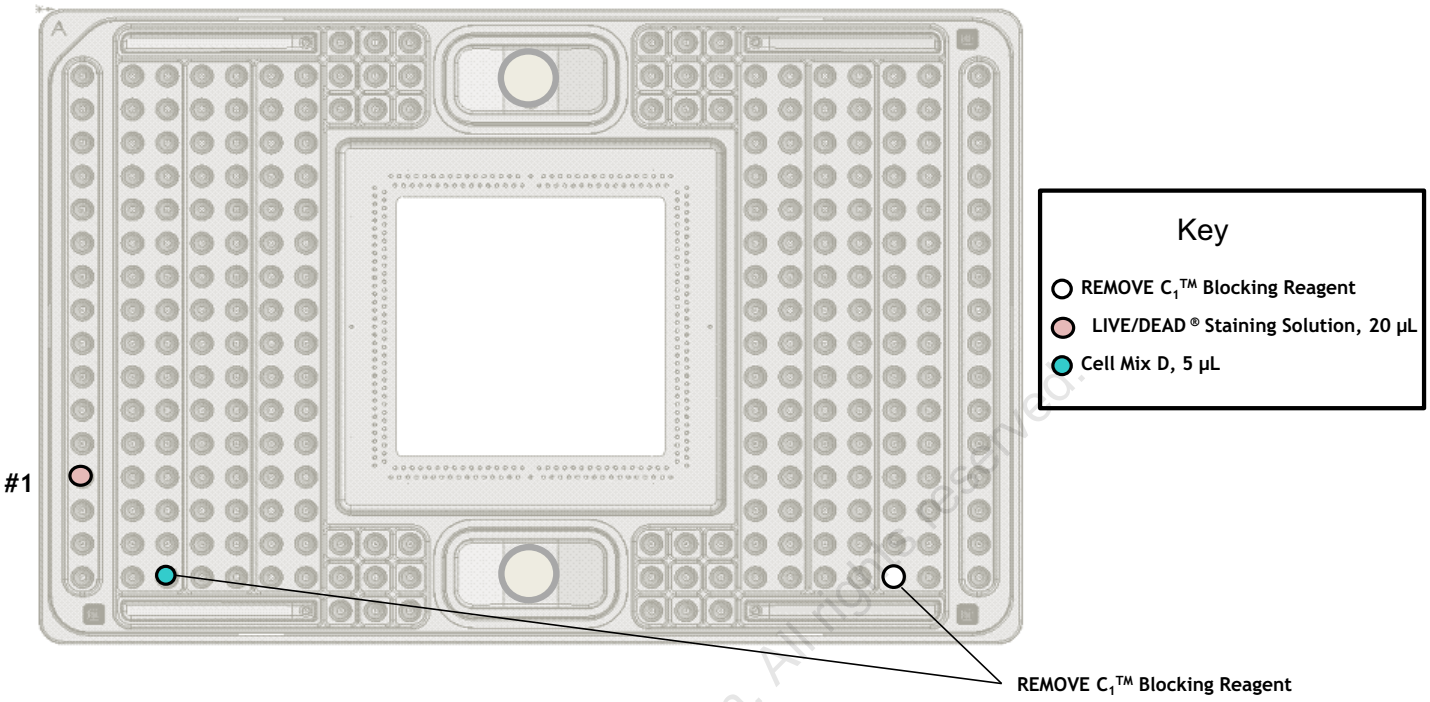

Figure 7 $C_1^{™}$ Chip loading pipetting map

1. Remove blocking solutions from cell inlet and outlet marked with teal and white dots in Figure 7.
2. Pipette Cell Mix D up and down 5–10 times to mix, depending on whether the cells tend to clump. Do not vortex the Cell Mix. Avoid bubbles when mixing, as these may cause load failures.
3. Pipette 5 µL of Cell Mix - D into the cell inlet marked with the teal dot. You may pipette up to 20 µL of Cell Mix, however only 5 µL with enter the chip.
4. Perform one of these tasks:
   - Staining cells: Vortex the $C_1^{™}$ LIVE/DEAD® staining solution well, then pipet 20 µL of the solution into inlet 1 marked with a pink dot.
   - Not staining cells: Pipet 20 µL of $C_1^{™}$ Cell Wash Buffer into inlet 1 marked with a pink dot.

NOTE: Staining small cells (5–10 µm) takes 30 minutes, and staining medium (10–17 µm) or large (17–25 µm) cells takes 60 minutes.

5 Place the chip into the $C_1^{™}$ Single-Cell Auto Prep System then run the **mRNA Seq: Cell Load (1771x/1772x/1773x)** or **mRNA Seq: Cell Load & Stain (1771x/1772x/1773x)** script. For loading and staining times, see Figure 2.

6 When the script has finished press **EJECT** to remove the chip from the $C_1^{™}$ Single-Cell Auto Prep System.

Optional: Starting the Tube Control: Lysis + Reverse Transcription

If you are running tube controls, please see "Appendix 1: Running the Tube Controls" on page 41 for instructions.

Imaging the Cells

Cells may be imaged on a microscope compatible with $C_1^{™}$ chips. Guidelines for the selection of a microscope are outlined in *Minimum Specifications for Single-Cell Imaging*, PN 100-5004. Please contact Technical Support for this document or with any questions. Call 1-866-358-4354 (within U.S.) or 1-650-266-6100 (outside U.S.), or email techsupport@fluidigm.com.

Running Lysis, Reverse Transcription and PCR on the C1™ System

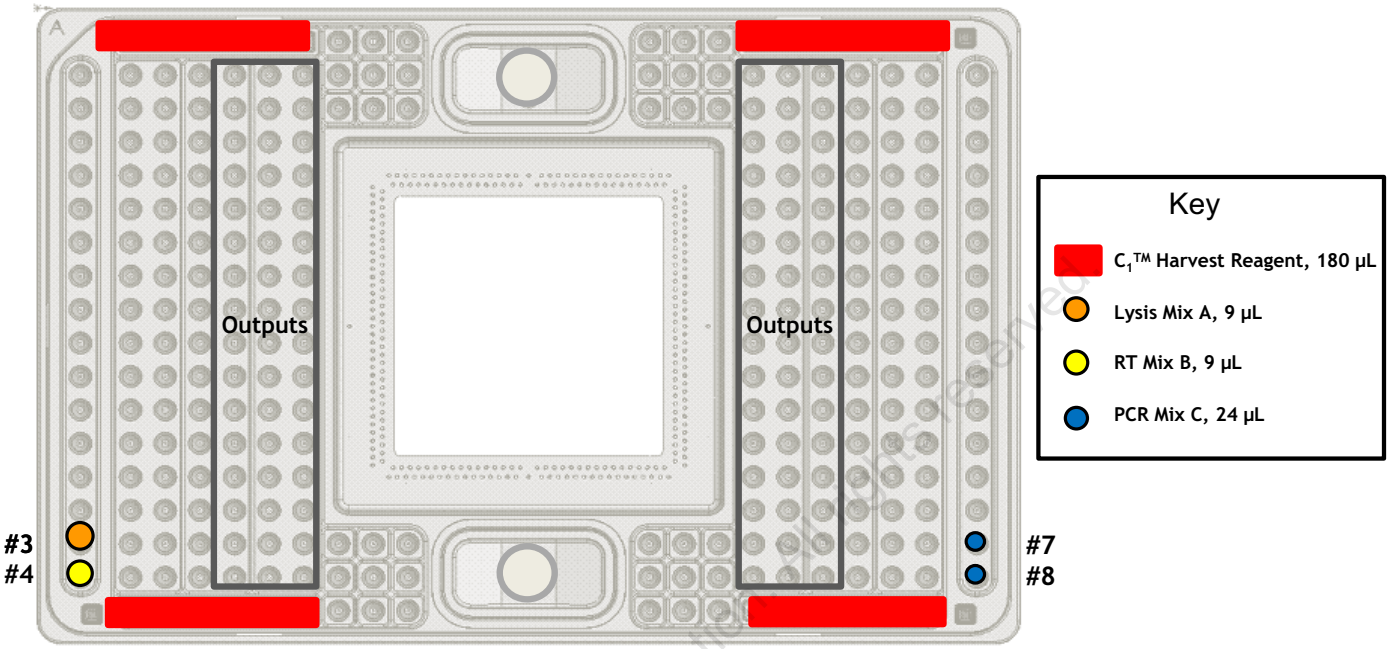

Figure 8 C1™ chip Lysis, RT and PCR pipetting map

1. Pipette 180 µL of C1™ Harvest Reagent into the four reservoirs marked with large solid red rectangles in Figure 8.
2. Pipette 9 µL of Lysis Mix A in well #3 marked with an orange dot.
3. Pipette 9 µL of RT Mix B in well #4 marked with a yellow dot.

4 Pipette 24 µL of PCR Mix C in wells #7 and #8 marked with blue dots.

5 Place the Chip into the $C_1^{™}$ Single-Cell Auto Prep System and run the **mRNA Seq: RT + Amp (1771x/1772x/1773x)** script.

NOTE: The mRNA Seq: RT + Amp (1771x/1772x/1773x) script may be run overnight. Approximate run times are:

- Small-cell IFC: ~7.75 hours (6.5 hours for lysis, reverse transcription, and amplification; and 1.25 hours for harvest)
- Medium- and large-cell IFCs: ~8.5 hours (6.5 hours for lysis, reverse transcription, and amplification; and 2 hours for harvest)

This protocol can be programmed to harvest at a convenient time. Slide the orange box (end time) to the desired time. For example, the harvest function could be programmed to next morning:

The PCR (1771x/1772x/1773x) script contains the following thermal cycling protocols:

Lysis	
Temperature	Time
72 °C	3 min
4 °C	10 min
25 °C	1 min
Reverse Transcription	
Temperature	Time
42 °C	90 min
70 °C	10 min

PCR		
Temperature	Time	Cycles
95 °C	1 min	1
95 °C	20 sec	5
58 °C	4 min	
68 °C	6 min	
95 °C	20 sec	9
64 °C	30 sec	
68 °C	6 min	
95 °C	30 sec	7
64 °C	30 sec	
68 °C	7 min	
72 °C	10 min	1

Table 10 Thermal cycling protocols

Optional: Continuing the Tube Control: mRNA Seq PCR

If you are running tube controls, please see "Appendix 1: Running the Tube Controls" on page 41 for instructions.

Harvesting the Amplified Products

1 When the mRNA Seq Prep script has finished, press **EJECT** to remove the chip from the instrument.

NOTE: The chip may remain in the C1™ Single-Cell Auto Prep System for up to one hour after harvest before removing products from their inlets.

2 Transfer the C1™ chip to a post-PCR lab environment.

3 Label a new 96-well plate "DILUTED HARVEST PLATE."

4 Aliquot 10 µL of C1™ DNA Dilution Reagent into each well of the DILUTED HARVEST PLATE.

5 Carefully pull back the tape covering the harvesting inlets of the chip using the plastic removal tool:

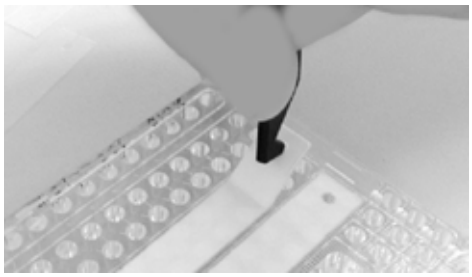

Figure 9 Tape removal

6 Using an 8-channel pipette, pipette the harvested amplicons from the inlets according to the diagram below and place in the DILUTED HARVEST PLATE.

NOTE: Harvest volumes may vary. Set pipette to 3.5 µL to ensure entire volume is extracted.

Components	Volume (µL)
C1™ DNA Dilution Reagent (Fluidigm)	10
C1™ harvest amplicons	~3
Total	**~13**

Table 11 Harvest amplicon dilution

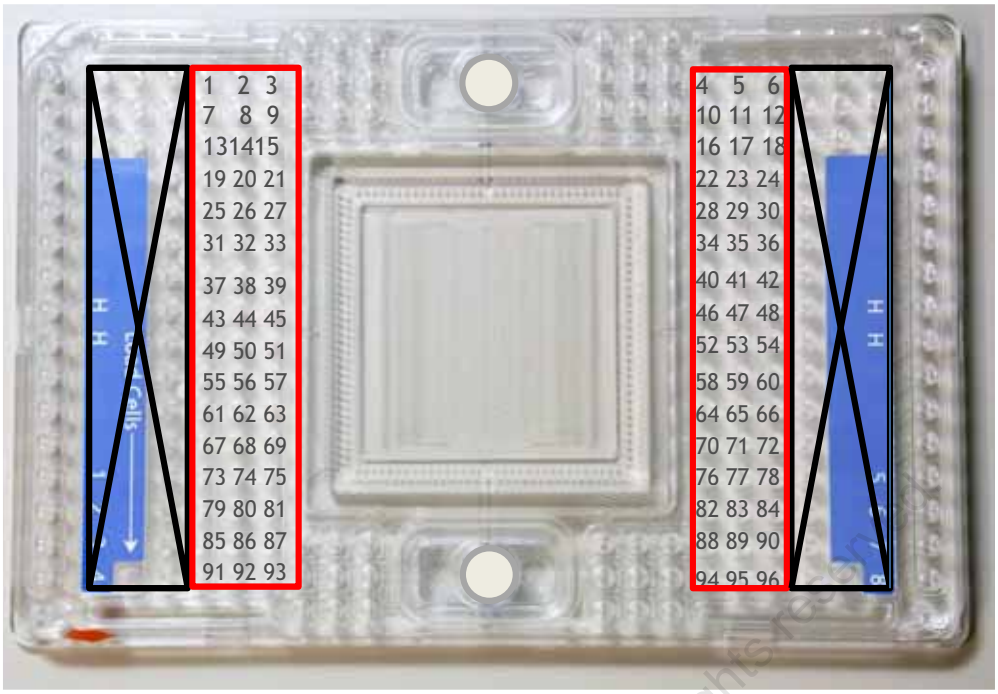

Figure 10 Pipette map of reaction products on the $C_1^{™}$ chip

NOTE: For detailed instructions on pipetting the harvested aliquots to the "DILUTED HARVEST PLATE," proceed to steps 7–10.

7 Pipet the entire volume of $C_1^{™}$ harvest amplicons out of the left-side wells of the $C_1^{™}$ chip into the 10 µL of $C_1^{™}$ DNA Dilution Reagent in each well of the DILUTED HARVEST PLATE:

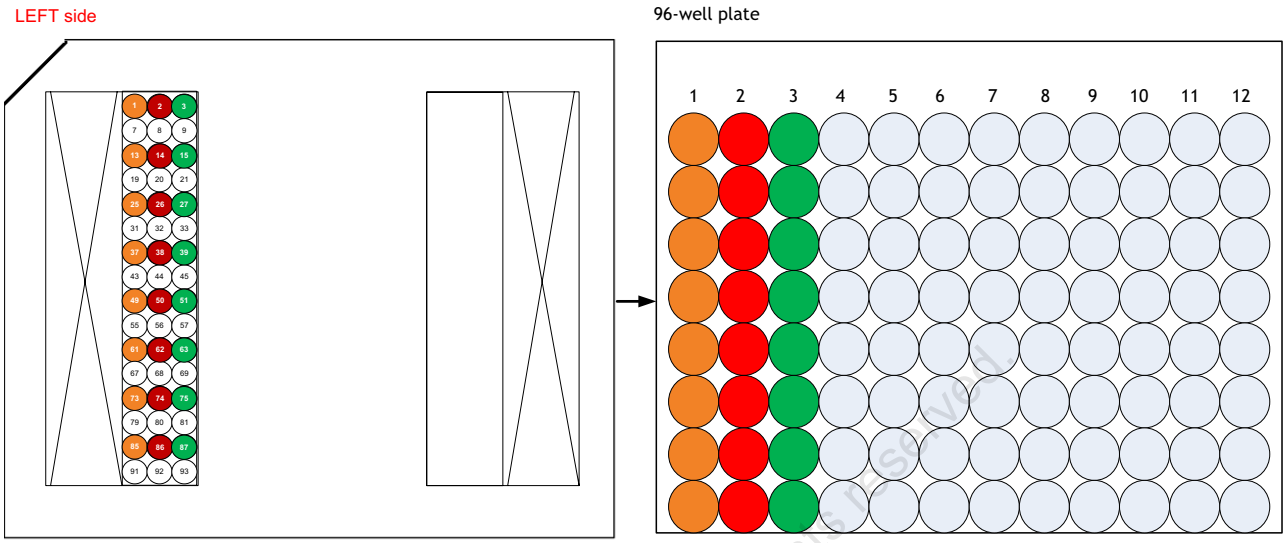

Figure 11 First three harvest product pipette steps

8 Pipet the entire volume of $C_1^{™}$ harvest amplicons out of the right-side wells of the $C_1^{™}$ chip into the 10 µL of $C_1^{™}$ DNA Dilution Reagent in each well of the DILUTED HARVEST PLATE:

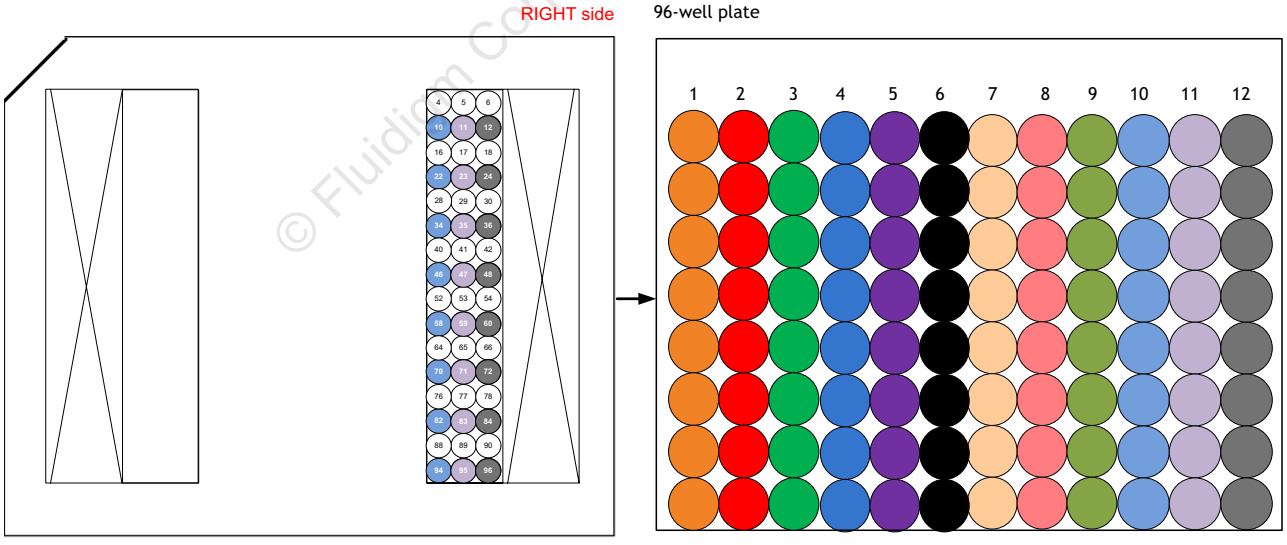

Figure 12 Fourth, fifth, and sixth pipetting steps

9 Pipet the entire volume of $C_1^{™}$ harvest amplicons out of the left-side wells of the $C_1^{™}$ chip into the 10 µL of $C_1^{™}$ DNA Dilution Reagent in each well of the DILUTED HARVEST PLATE:

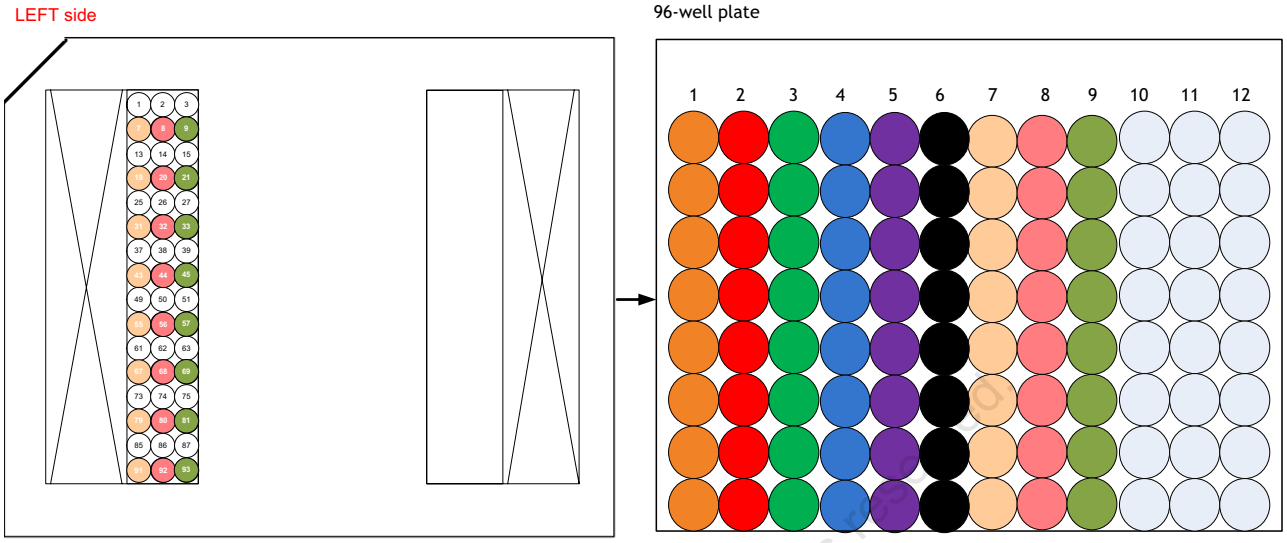

Figure 13 Seventh, eighth, and ninth pipetting steps

10 Pipet the entire volume of $C_1^{™}$ harvest amplicons out of the right-side wells of the $C_1^{™}$ chip into the 10 µL of $C_1^{™}$ DNA Dilution Reagent in each well of the DILUTED HARVEST PLATE:

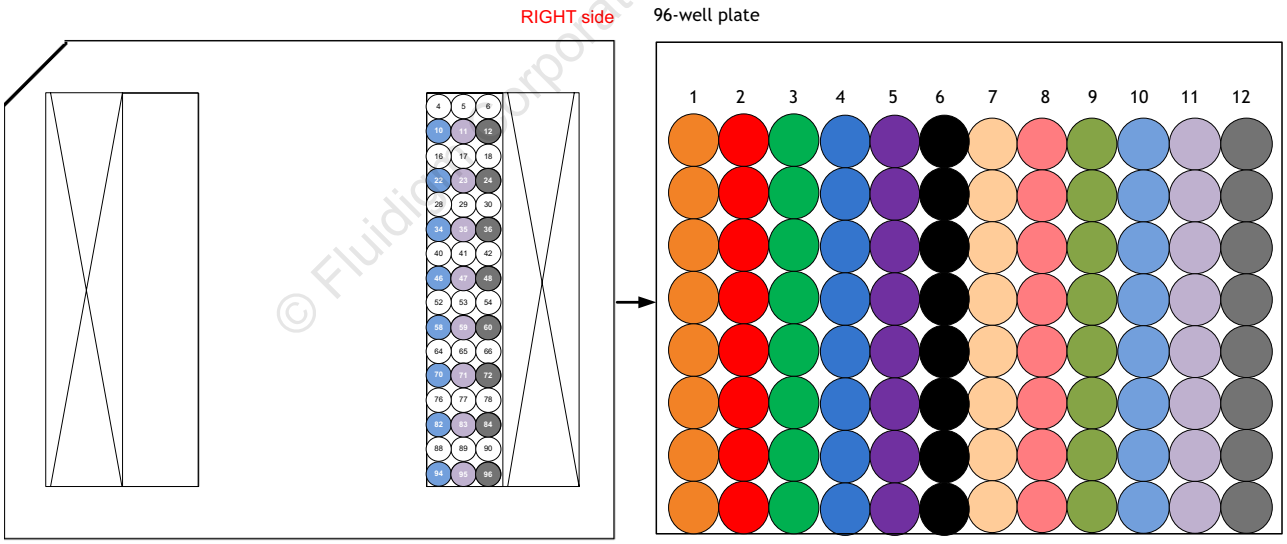

Figure 14 Tenth, eleventh, and twelfth pipetting steps

11 Seal, vortex, and spin down plate.

NOTE: These samples are now ready for library preparation for sequencing (see "Library Preparation for Illumina Sequencing" on page 32). Samples can be stored for up to 1 week at 4 °C or at –20 °C for long-term storage. Samples can also be run with DELTAgene™ Assays on a 96.96 Dynamic Array™ IFC using the *Fast EvaGreen Gene Expression Analysis on the BioMark™* protocol in the *Fluidigm Real-Time PCR User Guide*, PN 68000088.

Library Preparation for Illumina Sequencing

Introduction

This protocol describes the modified Illumina® Nextera XT DNA sample preparation protocol for single-cell mRNA Seq library preparation for sequencing from cDNA acquired from the C_1™ Single-Cell Auto Prep System. The *Illumina® Nextera XT DNA Sample Preparation User Guide* provides detailed instructions for library preparation; however, modifications have been made in order to adapt the Nextera XT chemistry to the single-cell mRNA Seq application. We highly recommend that you carefully read the *Nextera XT DNA Sample Preparation User Guide* before proceeding with this protocol.

References

- *Illumina® Nextera XT DNA Sample Preparation User Guide*
- *Single-Cell WTA PicoGreen® Template* (Fluidigm, PN 100-6260)
- Agilent Bioanalyzer user guide

Required Equipment

- Agilent Bioanalyzer
- Agilent High Sensitivity DNA Chips and reagents (Agilent Technologies, PN 5067-4626)
- 96-well PCR plates
- Vortexer
- Centrifuge
- Thermal cycler
- Magnetic stand for PCR tubes
- Fluorometer (for PicoGreen® assay)
- Corning® 384 Well Low Flange Black Flat Bottom Polystyrene Not Treated Microplate (Corning, PN 3573)

Reagent Retrieval for Illumina Sequencing

Required Reagents	Preparation	Kit Name
Amplicon Tagment Mix	Remove from -20 °C and keep on ice	Nextera XT Kit, Box 1
Tagment DNA Buffer	Remove from -20 °C and keep on ice	Nextera XT Kit, Box 1
NT Buffer	Remove from 4 °C and thaw to room temperature in a DNA-free hood	Nextera XT Kit, Box 2
Harvest Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Fluidigm C_1™ Kit
Nextera PCR Master Mix (NPM)	Remove from -20 °C and keep on ice	Nextera XT Kit, Box 1
Nextera XT Index Primer 1 (N701-N712)	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Nextera Index Kit
Nextera XT Index Primer 2 (S501-S508)	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Nextera Index Kit
AMPure® XP Beads	Remove from 4 °C and thaw to room temperature in a DNA-free hood	Agencourt Kit
C_1™ DNA Dilution Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Fluidigm C_1™ Kit
Quant-IT™ PicoGreen® dsDNA Assay Kit	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Life Technologies
Lambda DNA	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Life Technologies
High Sensitivity DNA Reagents	Remove from 4 °C and thaw to room temperature in a DNA-free hood	Agilent Technologies
Ethanol	Keep at room temperature	Sigma-Aldrich

Table 12 Reagent supplies

Quantify and Dilute Harvest Amplicons

cDNA Sample Dilution

A. cDNA concentrations yielded from the C_1™ Single-Cell Auto Prep System may vary with cell types and cell treatments. Both the library yield and size distribution also vary with input cDNA/DNA concentrations. To minimize library prep variation and to achieve high library quality, the harvest concentration + dilution has to be carefully determined.

1. We suggest using the PicoGreen® assay to determine the concentration of cDNA samples; however, alternate methods can be used.
2. We suggest using the Microsoft® Excel worksheet, *Single-Cell mRNA Seq PicoGreen® Template* (Fluidigm, PN 100-6260), to quantify the library.
3. The optimal concentration for Nextera XT library preparation is 0.10-0.3 ng/µL. Dilute each sample with the appropriate dilution factor to fall within this range. This can be done with a single- or multiple-dilution steps.

B. If a 384-well fluorometer is not available, an Agilent Bioanalyzer can be used. Samples from a C_1™ IFC should be run on the Agilent Bioanalyzer with the High Sensitivity DNA Chip. The concentration of each sample is estimated with a size range of 100-10,000 bp.

Using the Single-Cell mRNA Seq PicoGreen® Template with a Qubit® fluorometer is also an option. Input values into Concentration Estimate Table on the “Example Results” tab of the template.

1 Label a new 96-well PCR plate “Diluted Samples.”
2 Pipette the appropriate amount of $C_1^{™}$ Harvest Reagent to each well of the “Diluted Samples” plate listed in table below per determined sample dilution:

cDNA Sample Dilution	1:2	1:3	1:4	1:5	1:6	1:8	1:10	1:12
Volume of $C_1^{™}$ Harvest Reagent required	2 µL	4 µL	6 µL	8 µL	10 µL	14 µL	18 µL	22 µL

Table 13 $C_1^{™}$ Harvest Reagent required at different sample dilutions

3 Transfer 2 µL of the harvest sample from the harvest sample plate to the “Diluted Samples” plate.
4 Seal the plate with adhesive film.
5 Vortex at medium speed for 20 seconds and centrifuge at 1,500 rpm for 1 minute.

Tagmentation

Preparation of cDNA for Tagmentation

IMPORTANT: Bring NT Buffer to room temperature. Visually inspect NT buffer to ensure there is no precipitate. If there is precipitate, vortex until all particulates are resuspended.

IMPORTANT: Warm Tagment DNA Buffer to room temperature before starting this step.

1 After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief spin in a microcentrifuge.
2 Label a new 96-well PCR plate “Library Prep.”
3 To a 1.5 mL PCR tube, combine the components of the Pre-Mix:

	Reagent	Volume per Sample (µL)	Volume for 96 Samples (25% overage) (µL)
Pre-Mix	Tagment DNA Buffer	2.5	300.0
	Amplification Tagment Mix	1.25	150.0
	Diluted Sample	1.25	
	Total	5.0	

Table 14 Tagmentation reaction

4 Vortex at low speed for 20 seconds and spin down all components.
5 Aliquot equal amounts of Pre-Mix into each tube of an 8-tube strip.
6 Pipette 3.75 µL of the Pre-Mix to each well of the “Library Prep” plate using an 8-channel pipette.

7 Pipette 1.25 µL of the diluted sample from the “Diluted Sample” plate to the “Library Prep” plate.

8 Seal plate and vortex at medium speed for 20 seconds and centrifuge at 4,000 rpm for 5 minutes to remove bubbles.

9 Place the “Library Prep” plate in a thermal cycler and run the following program:

NOTE: Ensure that the thermal cycler lid is heated during the incubation.

Temperature	Time
55 °C	10 minutes
10 °C	Hold

Table 15 Thermal cycle protocol

10 Aliquot equal amounts of NT buffer into each tube of an 8-tube strip. You will need 1.25 µL of NT buffer for each sample plus 25% overage. For 96 samples:

Reagent	Volume per Sample (µL)	Volume per 96 Samples (25% overage) (µL)
Library Prep Plate	5.0	
NT Buffer	1.25	150.0
Total	**6.25**	

Table 16 NT buffer addition

11 Once the sample reaches 10 °C, pipette 1.25 µL of the NT buffer to each of the tagmented samples to neutralize the samples.

12 Seal plate and vortex at medium speed and centrifuge at 4,000 rpm for 5 minutes.

PCR Amplification

Carefully read *Illumina® Nextera XT DNA Sample Preparation User Guide* for Index primer selection and instructions before proceeding to PCR amplification of the tagmented cDNA.

1 Aliquot equal volumes of Nextera PCR Master Mix (NPM) into each tube of an 8-tube strip.

2 Pipette 3.75 µL of the aliquoted NPM to each well of the "Library Prep" plate using an 8-channel pipette:

Reagent	Volume per Sample (µL)	Volume per 96 Samples (25% overage) (µL)
Library Prep Plate	6.25	
NPM	3.75	450.0
Total	10.0	

Table 17 Tagment reaction plus NPM

3 Pipette 1.25 µL of Index Primer 1 (N701-N712) to the corresponding well of **Each Row** of the "Library Prep" plate using a 12- or 8-channel pipette as shown in Figure 15.

4 Pipette 1.25 µL of Index Primer 2 (S501-S508) to the corresponding well of **Each Column** of the "Library Prep" plate using an 8-channel pipette:

		N701	N702	N703	N704	N705	N706	N707	N708	N709	N710	N711	N712
		1	2	3	4	5	6	7	8	9	10	11	12
S501	A	S501/ N701	S501/ N702	S501/ N703	S501/ N704	S501/ N705	S501/ N706	S501/ N707	S501/ N708	S501/ N709	S501/ N710	S501/ N711	S501/ N712
S502	B	S502/ N701	S502/ N702	S502/ N703	S502/ N704	S502/ N705	S502/ N706	S502/ N707	S502/ N708	S502/ N709	S502/ N710	S502/ N711	S502/ N712
S503	C	S503/ N701	S503/ N702	S503/ N703	S503/ N704	S503/ N705	S503/ N706	S503/ N707	S503/ N708	S503/ N709	S503/ N710	S503/ N711	S503/ N712
S504	D	S504/ N701	S504/ N702	S504/ N703	S504/ N704	S504/ N705	S504/ N706	S504/ N707	S504/ N708	S504/ N709	S504/ N710	S504/ N711	S504/ N712
S505	E	S505/ N701	S505/ N702	S505/ N703	S505/ N704	S505/ N705	S505/ N706	S505/ N707	S505/ N708	S505/ N709	S505/ N710	S505/ N711	S505/ N712
S506	F	S506/ N701	S506/ N702	S506/ N703	S506/ N704	S506/ N705	S506/ N706	S506/ N707	S506/ N708	S506/ N709	S506/ N710	S506/ N711	S506/ N712
S507	G	S507/ N701	S507/ N702	S507/ N703	S507/ N704	S507/ N705	S507/ N706	S507/ N707	S507/ N708	S507/ N709	S507/ N710	S507/ N711	S507/ N712
S508	H	S508/ N701	S508/ N702	S508/ N703	S508/ N704	S508/ N705	S508/ N706	S508/ N707	S508/ N708	S508/ N709	S508/ N710	S508/ N711	S508/ N712

Figure 15 Index primer pipette map and index assignment

5 Seal the plate with adhesive film and vortex at medium speed for 20 seconds and centrifuge at 4,000 rpm for 2 minutes.

6 Place the plate onto a thermal cycler and perform PCR amplification:

NOTE: Ensure that the thermal cycler lid is heated during the incubation.

Temperature	Time	Cycles
72 °C	3 minutes	1
95 °C	30 seconds	1
95 °C	10 seconds	12
55 °C	30 seconds	
72 °C	60 seconds	
72 °C	5 minutes	1
10 °C	hold	–

Table 18 Thermal cycle protocol

7 Amplified products can be stored at -20 °C for long-term storage.

Library Pooling and Cleanup

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

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

2 Warm Agencourt AMPure® XP beads up to room temperature and vortex.

3 Make library pool by pipetting the appropriate volume from each sample listed in Table 19 according to the determined number of samples to be pooled:

Number of samples to be pooled	Volume per sample (µL)	Total pool volume (µL)	AMPure® bead volume for cleanup (µL) (90% of total pool volume)
8	4	32	29
12	4	48	44
16	2	32	29
24	2	48	44
32	1	32	29
48	1	48	44
96	1	96	87

Table 19 Sample volume to be pooled for different pool sizes and AMPure® beads required

4 To the pooled library add the required amount of AMPure® XP beads listed in the Table 19.
5 Mix well by pipetting up and down 5 times.
6 Incubate the bead mix at room temperature for 5 minutes.
7 Place the tube on a magnetic stand for 2 minutes.
8 Carefully remove the supernatant without disturbing the beads.
9 Add 180 µL of freshly prepared 70% ethanol and incubate for 30 seconds on the magnetic stand.
10 Remove the ethanol.
11 Repeat steps 9 and 10.
12 Allow the beads to air dry on bench for 10–15 minutes.

13 Elute the samples by adding the required volume of DNA Suspension Buffer per number of samples pooled:

Number of libraries pooled	Volume of DNA Suspension Buffer (volume of original sample pool; µL)
8	32
12	48
16	32
24	48
32	32
48	48
96	96

Table 20 Elution buffer required for libraries pooled from different number of samples (Elution buffer volume equal to pooled library volume)

14 Vortex and incubate the tube for 2 minutes at room temperature.

15 Plate the tube on a magnetic stand for 2 minutes.

16 Transfer the entire volume of supernatant to another PCR tube.

Repeat Cleanup

1 Add the required amount of AMPure® XP beads:

Number of libraries pooled	AMPure® bead volume for cleanup (90% of total pool volume; µL)
8	29
12	44
16	29
24	44
32	29
48	44
96	87

Table 21 Elution buffer required for libraries pooled from different number of samples

2 Mix well by pipetting up and down 5 times.

3 Incubate the bead mix 5 minutes at room temperature.

4 Place the tube on a magnetic stand for 2 minutes.
5 Carefully remove the supernatant without disturbing the beads.
6 Add 180 µL of freshly prepared 70% ethanol and incubate for 30 seconds on the magnetic stand.
7 Remove the ethanol.
8 Repeat Steps 6 and 7.

NOTE: Some beads may be lost during ethanol cleanup.

9 Allow beads to air dry on bench for 10–15 minutes.
10 Elute the samples by adding the required volume of DNA suspension buffer per number of samples pooled:

Number of libraries pooled	Volume of DNA Suspension Buffer (1.5X of original pool volume; µL)
8	48
12	66
16	48
24	66
32	48
48	66
96	144

Table 22 Final elution buffer required for libraries pooled from different number of samples

11 Remove the tube from the magnetic stand and vortex the tube.
12 Incubate at room temperature for 2 minutes.
13 Place the tube on the magnetic stand for 2 minutes.
14 Carefully transfer the supernatant to another PCR tube labeled as “SC Lib”.
15 Perform Agilent Bioanalyzer analysis in triplicates using High Sensitivity DNA Chip for library size distribution and quantitation.Please refer to the Agilent Bioanalyzer user guide for this step.
16 Refer to the Illumina sequencing manual to determine the appropriate library concentration for sequencing.

Appendix 1: Running the Tube Controls

Sample Preparation

Large numbers (hundreds) of cells in the tube control may inhibit the reaction chemistry. As such, we recommend an extraction and purification step as described in Protocol A prior to RT to ensure reliable tube controls.

NOTE: For cell types that do not exhibit inhibition, sample preparation may also be performed according to Protocol B.

Protocol A: Extraction and Purification

IMPORTANT: Review Qiagen RNeasy Plus Micro Kit Protocol for proper usage and handling of material before proceeding. Some components contain guanidine thiocyanate which can form highly reactive compounds when combined with bleach. Special care in handling and disposal must be taken.

1. Make a mixture of Buffer RLT Plus and β-mercaptoethanol per Qiagen recommendation, 10 µL β-ME at 14.3 M to 1 mL of Buffer RLT Plus. Alternatively, you may use 20 µL DTT (dithiothreitol) at 2 M to 1 mL Buffer RLT Plus. This mixture is recommended for cell lines rich in RNases.
2. Dilute cells as needed for a final concentration of 100–200 cells/µL in media.
3. Combine 20 µL of cells in media to 350 µL Buffer RLT Plus with β-ME. If you have less than 20 µL of material available, simply add the buffer directly to remaining volume.
4. Vortex mixture for 1 minute at high speed.
5. Optional: Include a QIA Shredder step if primary tissue cells are used:
   a. Pipette total volume into QIA Shredder Column.
   b. Centrifuge for 2 minutes at top speed in table-top centrifuge.
6. Transfer eluent to gDNA column and follow Qiagen Quick Start protocol steps 2–9.
7. Proceed to the mRNA reaction below, using the elution as the “prepared cells” for the lysis reaction.

NOTE: Even though cells are lysed, continue with the lysis reaction as written, since the 3′ SMART primer is incorporated during this step.

Protocol B: Washing the Cells

1. Pellet remaining cells. Speeds and durations may vary. We suggest spinning cells at 300 x *g* for 5 minutes.
2. Remove buffer from pellet by gently pipetting out the supernatant media without disturbing the cell pellet.
3. Resuspend cells in 1 mL $C_1^{™}$ Cell Wash Buffer by pipetting up and down at least 5 times. This is wash 1.
4. Pellet cells again and remove supernatant.

5 Wash a second time by resuspending in 1 mL of $C_1^{™}$ Cell Wash Buffer by pipetting up and down at least 5 times.

6 Pellet cells again and remove supernatant.

7 Resuspend cells in $C_1^{™}$ Cell Wash Buffer approximately 90% original volume to keep original concentration, assuming a 10% loss.

8 Dilute your cell suspension to 100–200 cells/µL using $C_1^{™}$ 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 disposable hemocytometer C-Chip by INCYTO. See www.incyto.com for instructions for use.

mRNA Seq Reaction

1 Prepare two tube controls by combining lysis reagents and thermal cycling them:

a Lysis:

Tube 1 - Positive Control	Tube 2 - NTC	Volume (µL)
Prepared Cells	$C_1^{™}$ Cell Wash Buffer	1.0
Lysis Final Mix A	Lysis Final Mix A	2.0
Total	—	**3.0**

b In a PCR thermal cycler, run the Lysis Thermal Cycle:

Temperature	Time
72 °C	3 min
4 °C	10 min
25 °C	1 min
4 °C	hold

2 Combine RT final mix with lysis thermal products from step 1.

a RT reaction:

Tube 1 - Positive Control	Tube 2 - NTC	Volume (µL)
Cell Lysis Mix	Cell Lysis Mix	3.0 (from table above)
RT Final Mix B	RT Final Mix B	4.0
Total	—	**7.0**

Table 23 Tube controls

b Vortex briefly and spin to collect contents.

c In a PCR thermal cycler, run the following protocol for reverse transcription:

Temperature	Time
42 °C	90 min
70 °C	10 min
4 °C	hold

Table 24 RT hold parameters

NOTE: This is a potential stopping point. PCR mix and RT reaction products can be stored at 4 °C on a thermal cycler overnight and prepared the following morning.

3 Once thermal cycle protocol has finished, combine the following in two tubes of an unused PCR strip:

Tube 1 – Positive Control	Tube 2 - NTC	Volume (µL)
PCR Mix C	PCR Mix C	9.0 µL
RT Reaction	RT Reaction	1.0 µL
Total	—	**10.0 µL**

Table 25 PCR reaction

4 In a PCR thermal cycler, run:

PCR		
Temperature	Time	Cycles
95 °C	1 min	1
95 °C	20 sec	5
58 °C	4 min	
68 °C	6 min	
95 °C	20 sec	9
64 °C	30 sec	
68 °C	6 min	
95 °C	30 sec	7
64 °C	30 sec	
68 °C	7 min	
72 °C	10 min	1
4 °C	hold	

Table 26 PCR thermal cycling protocol

Dilute Products

1 Transfer prepared material to Post PCR Room.
2 Briefly vortex the prepared products and spin to collect content.
3 Combine the following reagents:

Components	Volume (µL)
$C_1^{™}$ DNA Dilution Reagent (Fluidigm)	45
PCR Product	1
Total	**46**

Table 27 Dilution of PCR products

4 Continue with quantification protocol for library preparation (see "Quantify and Dilute Harvest Amplicons" on page 33).

Appendix 2: RNA Spike Assays

If ordering DELTAgene™ Assays, order:

Reverse Assay Primer	Sequence
C_1™ Single Cell Auto Prep RNA Standards Kit	100-5582

Table 28 Fluidigm part numbers

Appendix 3: Chip Pipetting Map

Overview of chip pipetting

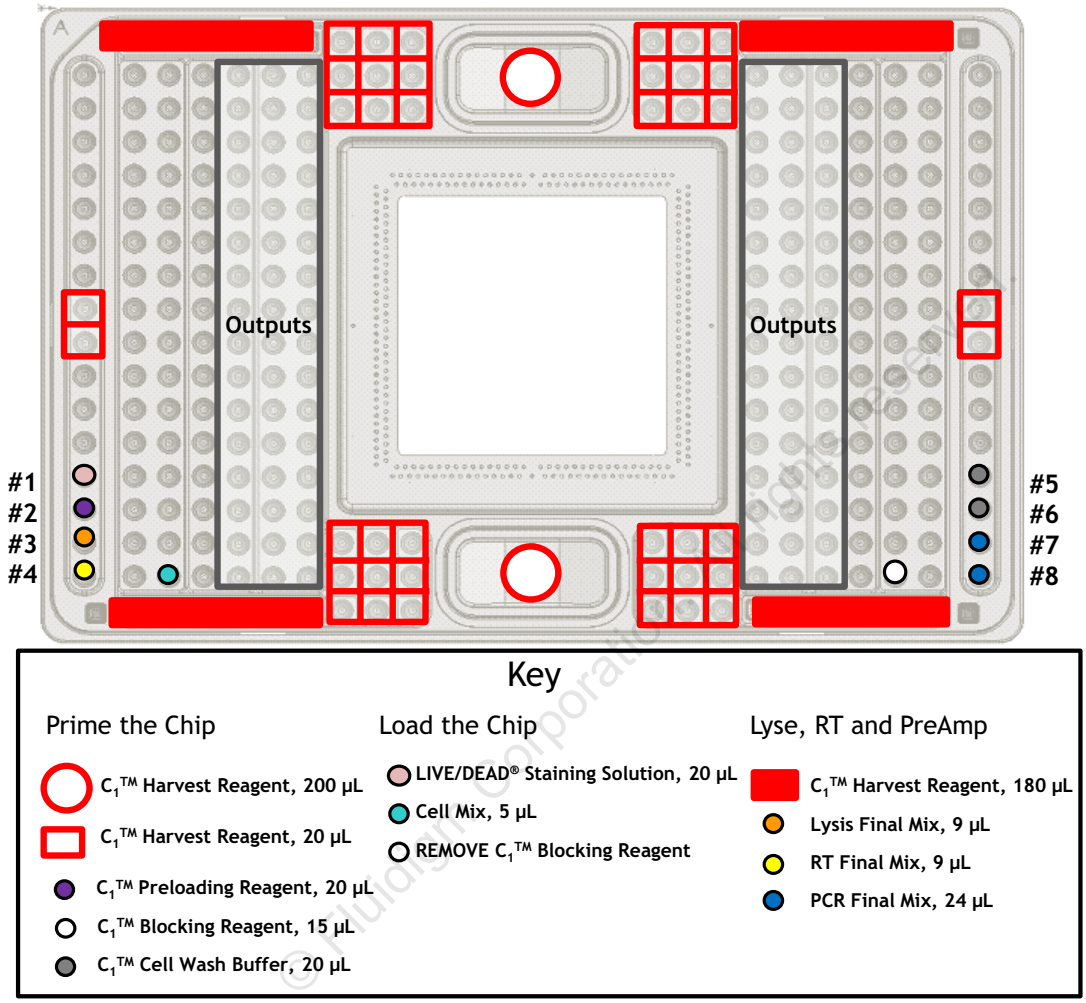

Figure 16 Chip pipetting map

Appendix 4: Library Prep Examples

Typical Agilent Bioanalyzer Trace

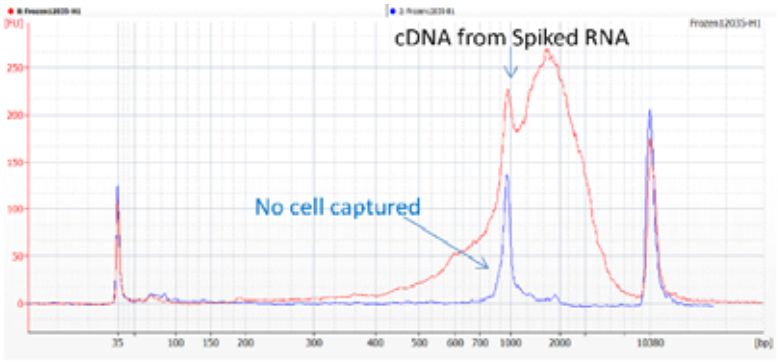

Figure 17 A typical cDNA harvested from chip

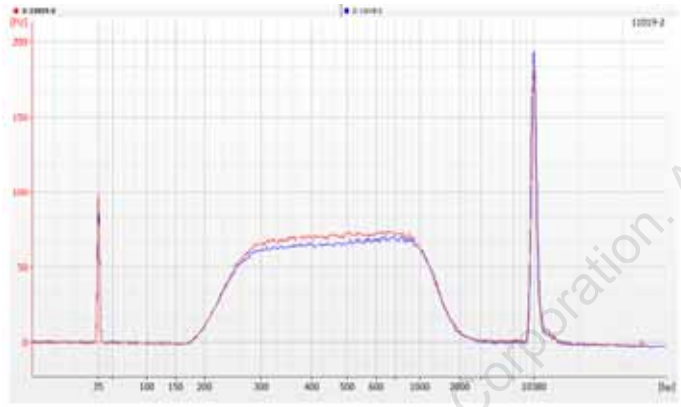

Figure 18 Typical library size distribution after purification

For More Information

To find out more about the information in this or any other Fluidigm Protocol, contact techsupport@fluidigm.com or call:

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