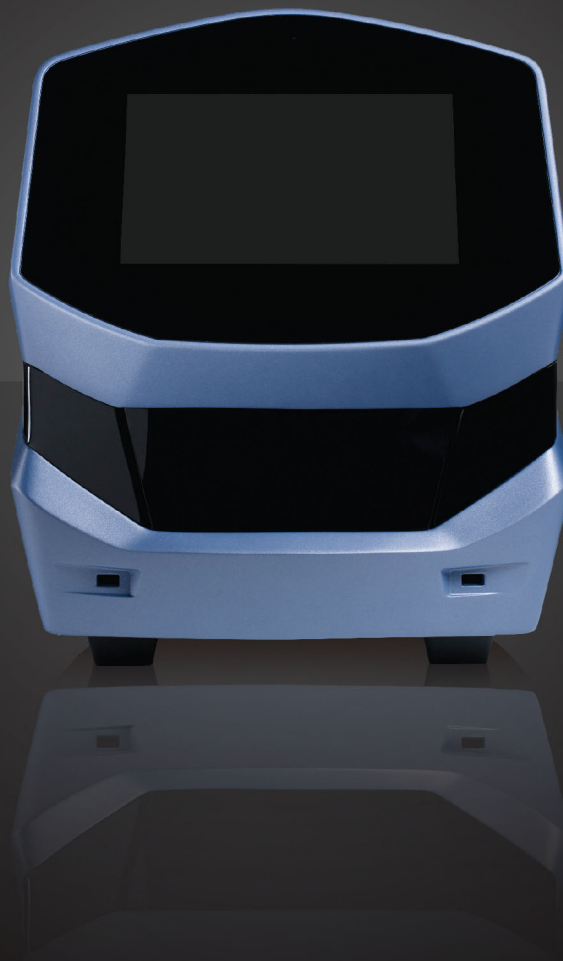


Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing

PROTOCOL



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Contents

5	Introduction	26	Running Lysis, Reverse Transcription and PCR on the C1 System
7	Revision History	28	(Optional) Continuing the Tube Control: mRNA Sequencing PCR
8	Overview of Cell Capture Process	28	Harvesting the Amplified Products
9	References	32	Library Preparation for Illumina Sequencing
9	Required Consumables	32	Introduction
10	Suggested Consumables	32	References
10	Required Reagents for Tube Controls	32	Required Equipment
11	Required Reagents for cDNA Synthesis	33	Reagent Retrieval for Illumina Sequencing
12	Required Reagents for Illumina Sequencing Library Preparation	34	Quantify and Dilute Harvest Amplicons
13	Required Equipment	34	cDNA Sample Dilution
13	Recommended Equipment	35	Tagmentation
14	IFC Types and Related Scripts	35	Preparation of cDNA for Tagmentation
15	Safety	36	PCR Amplification
16	Reagent Retrieval for cDNA Synthesis	38	Library Pooling and Cleanup
17	Reagent Mixes	40	Repeat Cleanup
18	(Optional) ArrayControl RNA Spikes	42	Appendix A: Running the Tube Controls
19	(Optional) Diluting RNA Spikes for Lysis Final Mix	42	Sample Preparation
19	Lysis Mix—Mix A	42	Protocol A: Extraction and Purification
20	Reverse Transcription (RT) Reaction Mix—Mix B	43	Protocol B: Washing the Cells
20	PCR Mix—Mix C	43	mRNA Sequencing Reaction
21	(Optional) Preparing LIVE/DEAD Cell Staining Solution	45	Dilute Products
21	Using the IFC Map Loading Plate	46	Appendix B: RNA Spike Assays
22	Priming the IFC	47	Appendix C: IFC Pipetting Map
24	Preparing the Cells	47	Overview of IFC Pipetting
24	Cell Mix—Mix D (Prepared While Priming the IFC)	48	Appendix D: Library Prep Examples
25	Loading the Cells	48	Typical Agilent Bioanalyzer Trace
26	(Optional) Starting the Tube Control: Lysis and Reverse Transcription	49	Appendix E: C1 Reagent Kit for mRNA Seq, PN 100-6201
26	Imaging the Cells		

Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing

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® C1™ system and C1 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 sequencing, the full-length cDNA must first be converted to a sequencing library. The final steps of library generation from cDNA are described in 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 the Fluidigm Real-Time PCR Analysis User Guide (PN 68000088) using Delta Gene™ 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 9). 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 guanines 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 Ultra™ Low RNA Kit for the Fluidigm C1 system (Clontech PN 634833):

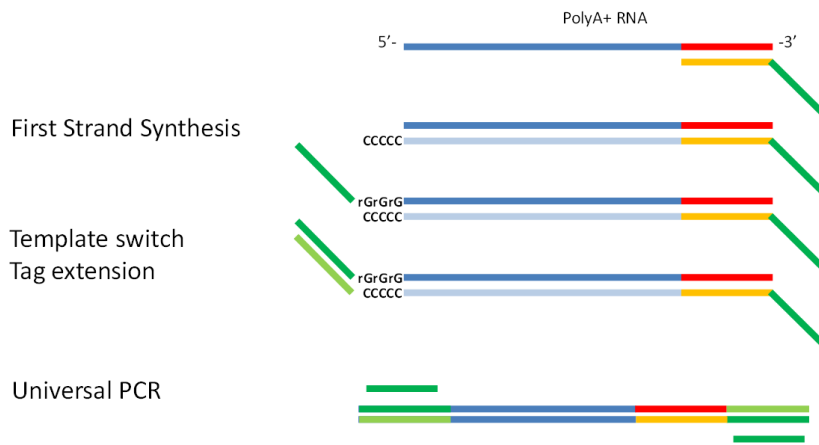


Figure 1: Overview SMARTer Ultra Low RNA Kit for the Fluidigm C1 system

The protocol also describes the modified Illumina® Nextera™ XT DNA sample preparation protocol for single-cell mRNA sequencing library preparation for sequencing from cDNA acquired from the C1 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
G1	3 November 2014	Updated product names, illustrations, and legal boilerplate to new branding specifications.
F1	1 August 2014	<ul style="list-style-type: none"> Replaced “DNA Suspension Buffer” with “C1 DNA Dilution Reagent” (see “Library Preparation for Illumina Sequencing” on page 32). Replaced the photographs of the kit modules with diagrams (see “Appendix E: C1 Reagent Kit for mRNA Seq, PN 100-6201” on page 49). Replaced Index 2 Primer “S501” with “S517” according to an update from Illumina, Inc. (see “PCR Amplification” on page 36). Corrected the temperature for use of C1 Cell Wash Buffer (see “Reagent Retrieval for cDNA Synthesis” on page 16).
E1	10 April 2014	<ul style="list-style-type: none"> Updated name from “C1 Single-Cell Auto Prep Module 1 Kit” to “Module 1” and from “C1 Single-Cell Auto Prep Module 2 Kit” to “Module 2 (mRNA Seq).” Provided more specific kit names in the Reagent Retrieval table (see “Reagent Retrieval for cDNA Synthesis” on page 16). Moved the reagent retrieval table to before the reagent mixes section (see “Reagent Retrieval for cDNA Synthesis” on page 16).
D1	19 March 2014	<ul style="list-style-type: none"> Corrected the source company for PN 100-6201 from Clontech to Fluidigm (see “Required Consumables” on page 9). Changed “MSDS” to “SDS” (safety data sheet; see “Safety” on page 15).
C1	29 January 2014	Updated title of protocol from Using the C1 Single-Cell Auto Prep System to Generate mRNA from Single Cells and Libraries for Sequencing Protocol to Using the C1 Single-Cell Auto Prep System to Generate Single-Cell cDNA Libraries for mRNA Sequencing Protocol.
B1	23 December 2013	<ul style="list-style-type: none"> Updated the name of the Clontech kit to SMARTer Ultra Low RNA Kit for the Fluidigm C1 System (see “Required Consumables” on page 9). Replaced “chip” with “IFC” where appropriate. Updated descriptions of the scripts used for mRNA sequencing (see “IFC Types and Related Scripts” on page 14). Correctly arranged the product names according to storage temperature, as specified by the product labels (see “Required Reagents for cDNA Synthesis” on page 11). Reformatted the tube control tables for clarity (see “Appendix A: Running the Tube Controls” on page 42).
A2	1 November 2013	Corrected graphic so that it illustrates harvest amplicons transferred to columns 1–6 in the 96-well plate.
A1	4 October 2013	New document on use of the new C1 IFC for mRNA sequencing with small cells (5–10 μm).

Overview of Cell Capture Process

Table 1: Overview of the cell capture procedure

Reagent handling	Automated steps	Time
mRNA seq prep		
1	Prepare reagent pre-mixes	60 min
2	Pipet priming solutions into the IFC	5 min
3	Prime the IFC on C1	10 min
4	Pipet cells into the IFC	5 min
5	Load cells on C1	Small-cell IFC: 30 min if staining 20 min if not staining Medium- or large-cell IFC: 60 min if staining 30 min if not staining
6	Image cells with a microscope	15–30 min*
7	Pipet lysis, reverse transcription, and PCR chemistry into the IFC	5 min
8	Run the mRNA sequencing script on C1. This includes lysis, reverse transcription, PCR, and harvest.	467 min for small-cell IFC 515 min for medium- or large-cell IFC You can run the script overnight with a pause between PCR and harvest functions. You have up to 60 minutes after the script finishes to transfer the cDNA from the IFC to a plate.
9	Harvest amplicons from the IFC	10 min* (Optional) Run 96.96 Dynamic Array IFC*
Library prep		
1	Quantify and dilute harvest amplicons	30 min–2 hours
2	Tagmentation	30 min
3	Limited cycle PCR amplification	10 min
4	PCR amplification on thermal cycler	30 min
5	Library pooling, cleanup, and quantification	40 min
6	Agilent® Bioanalyzer analysis	40 min

! **IMPORTANT** If you are running the optional tube controls, add up to 4 hours to the time estimate. The asterisks (*) in the workflow denote where you would start, continue, and finish the tube controls steps.

References

- 1 Chenchik, A., Zhu, Y.Y., Diatchenko, L. et al. “Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR,” in Gene Cloning and Analysis of RT-PCR (BioTechniques Books, 1998)
- Fluidigm Biomark HD Data Collection User Guide (PN 100-2451)
 - Fluidigm Real-Time PCR Analysis User Guide (PN 68000088)
 - Fluidigm C1 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, 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
C1 Reagent Kit for mRNA seq	Fluidigm	100-6201
SMARTer Ultra Low RNA Kit for the Fluidigm C1 System, 10 IFCs	Clontech	634833
C1 IFC for mRNA seq (5–10 µm)	Fluidigm	100-5759
C1 IFC for mRNA seq(10–17 µm)		100-5760
C1 IFC for mRNA seq (17–25 µm)		100-5761
Nextera XT DNA Sample Preparation Kit	Illumina	FC-131-1096
Nextera XT DNA Sample Preparation Index Kit		FC-131-1002
(96 indices, 384 samples)		
Agencourt® AMPure® XP	Agencourt Bioscience Corp.	A63880

Suggested Consumables

Product Name	Company	Part Number
ArrayControl RNA Spikes	Life Technologies	AM1780
THE RNA Storage Solution		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	Qiagen	74034
QIAshredder disposable cell lysate homogenizers		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 C1 Kit for mRNA Seq, Module 1 and Module 2 (mRNA Seq), from Fluidigm, use the parent part number: 100-6201.

See a diagram of the C1 Reagent Kit in “Appendix E: C1 Reagent Kit for mRNA Seq, PN 100-6201” on page 49.

Stored at **–80 °C**

- (Suggested) ArrayControl RNA Spikes (Life Technologies, PN AM1780)

NOTE The SMARTer Kit is for 10 runs. Clontech also offers a kit for two runs (Clontech, PN 634832).

Stored at **–20 °C**

- SMARTer Ultra Low RNA Kit for the Fluidigm C1 system, Box 1 of 2 (Clontech PN 634835; not sold separately; sold as part of Clontech PN 634833)
- SMARTer Ultra Low RNA Kit for the Fluidigm C1 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 runs.

- Module 2 (mRNA sequencing) (Fluidigm, PN 100-6209)
 - C1 DNA Dilution Reagent
 - C1 Loading Reagent
 - C1 Preloading Reagent
 - C1 Harvest Reagent

! **IMPORTANT** Store Module 2 (mRNA Seq) (PN 100-6209) in a –20 °C freezer upon receipt.

- (Optional) LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells (Life Technologies, PN L-3224)

Stored at 4 °C

- Module 1 (Fluidigm, PN 100-5518)
 - C1 Suspension Reagent
 - C1 Blocking Reagent
 - C1 Cell Wash Buffer

! IMPORTANT

- Store Module 1 (PN 100-5518) at 4 °C upon receipt.
- Do not freeze Module 1.

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 Note storage conditions for each component in the SMARTer Kit and Advantage 2 Kit and store appropriately.

Required Reagents for Illumina Sequencing Library Preparation

-20 °C

- 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)
- C1 DNA Dilution Reagent (Fluidigm, PN 100-5317)

4 °C

- 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 Kit Reagents (Agilent Technologies, PN 5067)
- Agencourt AMPure XP (Agencourt BioScience Corp., PN A63880)

Room Temperature

- Ethanol, 500 mL (Sigma-Aldrich, PN E70235)[™]

Required Equipment

- C1 system
- C1 IFCs for mRNA sequencing (barcodes 1771x, 1772x, or 1773x)
- 96-well PCR Plate (USA Scientific, TempPlate™ semi-skirted, PN 1402-9700)
- Two centrifuges: one for Eppendorf tubes, one for 96-well plates
- Vortexer
- MicroAmp™ clear adhesive film (Life Technologies, PN 4306311)
- Agilent Bioanalyzer
- Agilent High Sensitivity DNA Kit chips (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

- Two biocontainment hoods to prevent DNA contamination of lab and samples
- Imaging equipment compatible with C1 IFCs. See Minimum Specifications for Single-Cell Imaging Specification Sheet, PN 100-5004.

IFC Types and Related Scripts

There are currently three C1 system-compatible IFCs for small, medium, and large single cells:

Table 2: IFCs and related scripts

Cell Size (Median)	Barcode (prefix)	Script Names	Description
Small (5–10 μm) C1 IFC for mRNA sequencing (5–10 μm) PN 100-5759	1771x	mRNA Seq: Prime (1771x)	Priming the control line and cell capture channels of the 5–10 μm IFC (1771x)
		mRNA Seq: Cell Load (1771x)	Cell loading and washing without staining for PCR of 5–10 μm diameter cells (1771x)
		mRNA Seq: Cell Load & Stain (1771x)	Cell loading, staining, and washing for mRNA sequencing of 5–10 μm diameter cells (1771x)
		mRNA Seq: RT & Amp (1771x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA amplification for mRNA sequencing of 5–10 μm diameter cells (1771x)
Medium (10–17 μm) C1 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 IFC (1772x)
		mRNA Seq: Cell Load (1772x)	Cell loading and washing without staining for PCR of 10–17 μm diameter cells (1772x)
		mRNA Seq: Cell Load & Stain (1772x)	Cell loading, staining, and washing for mRNA sequencing 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 mRNA sequencing of 10-17 μm diameter cells (1772x)
Large (17–25 μm) C1 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 IFC (1773x)
		mRNA Seq: Cell Load (1773x)	Cell loading and washing without staining for mRNA sequencing 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 mRNA sequencing of 17–25 μm diameter cells (1773x)

Safety

It is the individual's responsibility to review all safety data sheets (SDSs) for chemicals used in this procedure before running the test.

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

- Wear personal protective equipment (PPE): safety glasses, fully enclosed shoes, and gloves.
- Know the locations of all safety equipment (fire extinguishers, spill kits, eyewashes/showers, first aid kits, 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.

! WARNING HOT SURFACE. The C1 thermal cycler chuck gets hot and can burn your skin. Use caution when working near the chuck.



! WARNING PINCH HAZARD. The C1 door and shuttle can pinch your hand. Make sure your fingers, hand, shirt sleeve, etc. are clear of the door and shuttle when loading or ejecting an IFC.







! WARNING BIOHAZARD. If you are putting live cells on the C1, use the appropriate personal protective equipment and follow your lab's safety protocol to limit biohazard risks.



Reagent Retrieval for cDNA Synthesis

Table 3: Reagent supplies

	Required Reagents	Preparation	Kit Name	
1	IFC Priming	C1 Preloading Reagent 	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Module 2 (mRNA Seq) (Fluidigm)
		C1 Harvest Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Module 2 (mRNA Seq) (Fluidigm)
		C1 Blocking Reagent 	Remove from 4 °C and thaw to room temperature in a DNA-free hood	Module 1 (mRNA Seq) (Fluidigm)
2	(Optional) RNA Spikes	ArrayControl RNA Spikes	Remove from -80 °C, thaw on ice, and keep on ice	ArrayControl RNA Spikes (Life Technologies)
		THE RNA Storage Solution	Keep at room temperature	THE RNA Storage Solution (Life Technologies)
3	Lysis Mix—Mix A	C1 Loading Reagent 	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Module 2 (mRNA Seq) (Fluidigm)
		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

		Required Reagents	Preparation	Kit Name
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	—
		C1 Suspension Reagent 	Remove from 4 °C and vortex well	Module 1 (mRNA Seq) (Fluidigm)
7	(Optional) LIVE/DEAD Cell Staining	C1 Cell Wash Buffer	Remove from 4 °C and equilibrate to room temperature	Module 1 (mRNA Seq) (Fluidigm)
		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
8	Harvest the Amplified Products	C1 DNA Dilution Reagent	Remove from -20 °C and thaw to room temperature in a DNA-free hood	Module 2 (mRNA Seq) (Fluidigm)

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) ArrayControl RNA Spikes” on page 18
- “(Optional) Diluting RNA Spikes for Lysis Final Mix” on page 19
- “Lysis Mix—Mix A” on page 19
- “Reverse Transcription (RT) Reaction Mix—Mix B” on page 20
- “PCR Mix—Mix C” on page 20
- “(Optional) Preparing LIVE/DEAD Cell Staining Solution” on page 21
- “Cell Mix—Mix D (Prepared While Priming the IFC)” on page 24

(Optional) ArrayControl RNA Spikes

RNA spikes serve as a positive control for thermal cycling of the C1 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 IFCs. Due to the low volume pipetted, we highly recommend making this mix in bulk and aliquoting for future use.
- 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 Pipet the following in three tubes:

Table 4: RNA spikes

Tube	A	B	C
THE RNA Storage Solution	13.5 μ L	12.0 μ L	148.5 μ L
RNA Spikes	No. 7, 1.5 μ L	No.4, 1.5 μ L	No. 1, 1.5 μ L

- 3 Vortex briefly tube A and spin to collect contents. Pipet 1.5 μ L from tube A into tube B. Discard tube A.
- 4 Vortex briefly tube B and spin to collect contents. Pipet 1.5 μ L 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 μ L volumes and store at -80 °C until use. One tube is necessary for each C1 run.


NOTE For ordering information for RNA spike assays, see “Appendix B: RNA Spike Assays” on page 46.

(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:

Table 5: RNA spike dilution

Reagent	Volume (μL)
RNA Spikes mixture (Life Technologies)	1.0
C1 Loading Reagent (Fluidigm) 	99.0
Total	100.0

NOTE You can serially dilute twice 1 μL of the RNA spikes mixture to 9 μL of C1 Loading Reagent. Vortex after each dilution.


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

Lysis Mix—Mix A

NOTE If you are not using RNA spikes, just add 1 μL of C1 Loading Reagent. For detailed instructions, see the tables in this section and succeeding sections.

- 1 Mix the following reagents in a tube labeled “A” to create the Lysis Mix:

Table 6: Cell lysis mix

Components	Volume (μL)
C1 Loading Reagent (Fluidigm)  or (Optional) RNA Spikes mixture prepared in Table 5	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

- 2 Pipet the cell lysis mix up and down a few times to mix. Keep on ice until use.

Reverse Transcription (RT) Reaction Mix—Mix B

- Mix the following reagents in a tube labeled “B” to create the RT reaction mix:

Table 7: 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

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

PCR Mix—Mix C

- 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.

Table 8: PCR final mix

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
C1 Loading Reagent (Fluidigm)	4.5
Total	90.0

- 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 .
- Cell staining solution may be prepared up to 2 hours before loading into the C1 IFC. Keep on ice and away from light before pipetting into the IFC.

! **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 9).

- 1 Vortex the dyes well before pipetting.
- 2 Prepare the LIVE/DEAD stain:

Table 9: Staining solution

Components	Volume (μL)
C1 Cell Wash Buffer (Fluidigm) (30-mL bottle)	1,250.0
Ethidium homodimer-1 (LIVE/DEAD kit, Life Technologies/Molecular Probes)	2.5
Calcein AM (LIVE/DEAD kit, Life Technologies/Molecular Probes)	0.625
Total	1,253.125

- 3 Vortex the C1 LIVE/DEAD staining solution well before pipetting into the IFC.

Using the IFC Map Loading Plate

A black IFC map loading plate accessory can be used to assist IFC pipetting.

- 1 Obtain an IFC map loading plate:

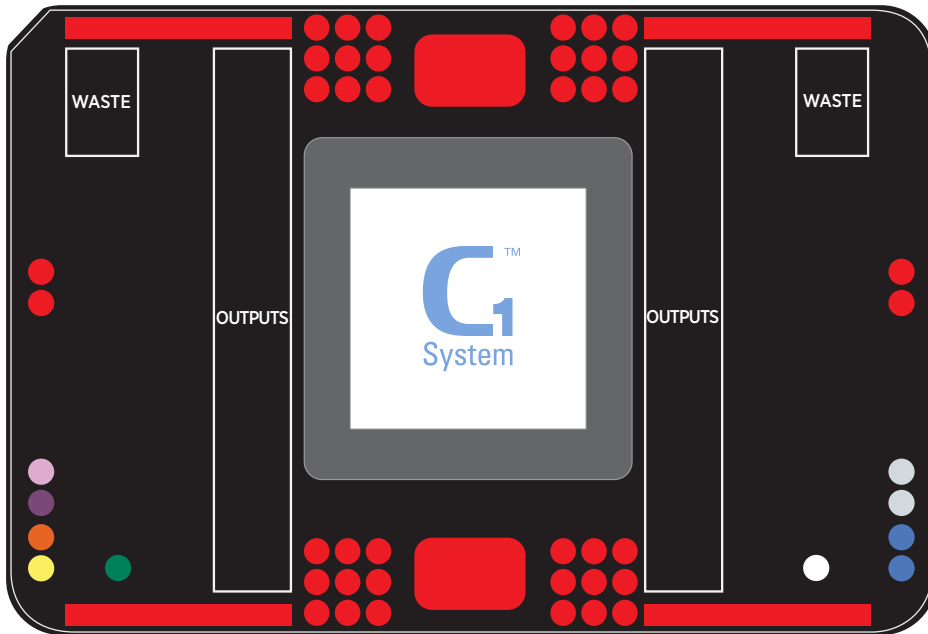


Figure 2: IFC map loading plate

NOTE The IFC map loading plate is supplied with the C1 system. If you do not have an IFC map loading plate, contact Fluidigm technical support.

- 2 Place the C1 IFC onto the IFC map loading plate. For details on IFC loading, see “Appendix C: IFC Pipetting Map” on page 47.
- 3 Pipet the reagents (see “Priming the IFC” on page 22).

Priming the IFC

NOTE When pipetting into the C1 IFC, 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 IFC.

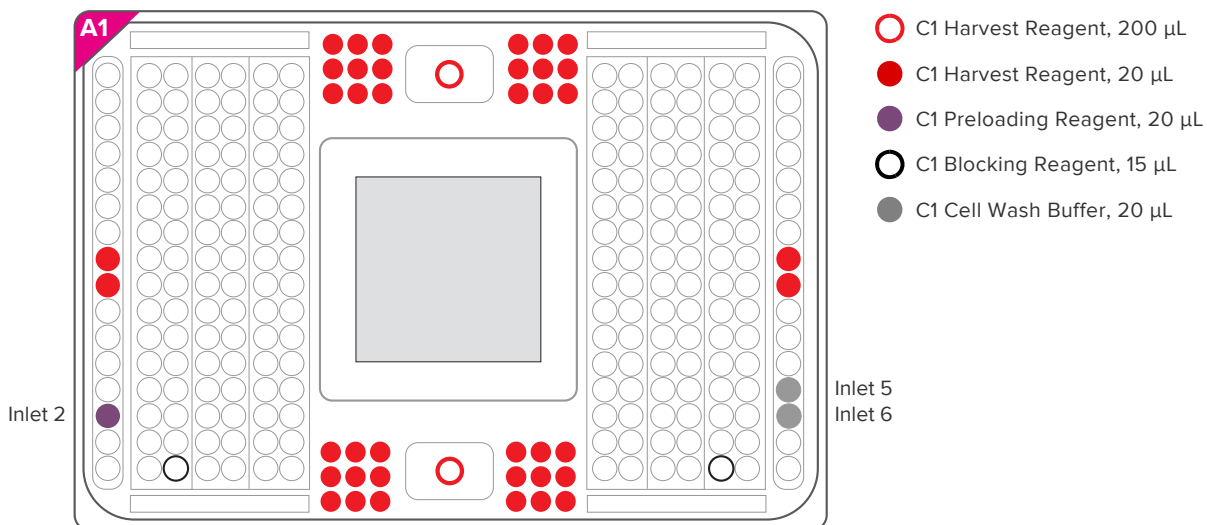


Figure 3: C1 IFC priming pipetting map

- 1 Add 200 μL of C1 Harvest Reagent from 4 mL bottle into accumulators marked with red outlined circles in Figure using a pipette tip.
- 2 Pipet 20 μL of C1 Harvest Reagent into inlets marked with solid red circles on each side of the accumulators (36 total).
- 3 Pipet 20 μL of C1 Harvest Reagent into the two inlets marked with solid red circles in the middle of the outside columns of inlets on each side of the IFC. These wells are marked on the bottom of the IFC with a notch to ensure they are easily located.
- 4 Pipet 20 μL of C1 Preloading Reagent into inlet 2, marked with a purple dot.
- 5 Pipet 15 μL of C1 Blocking Reagent into the cell inlet and outlet marked with white dots.
- 6 Pipet 20 μL of C1 Cell Wash Buffer into inlets 5 and 6, marked with dark gray dots.
- 7 Peel off white tape on bottom of IFC.
- 8 Place the IFC into the C1 system. Run the **mRNA Seq: Prime (1771x/1772x/1773x)** script. Priming takes approximately 10 minutes. When the Prime script has finished tap **EJECT** to remove the primed IFC from the instrument.

NOTE After priming the IFC, you have up to one hour to load the IFC with the C1 system.

Preparing the Cells

Prepare a cell suspension of a concentration of 166–250 K/mL in native medium prior to mixing with C1 Suspension Reagent and loading into the IFC. This will ensure a total cell count pipetted on IFC of approximately 500–750 cells. As few as 200 cells total, from 66,000 mL in native medium, may be loaded on the IFC. 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 IFC 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 incyto.com for instructions for use.


Cell Mix—Mix D (Prepared While Priming the IFC)

- 1 Prepare Cell Mix D by combining cells with C1 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 μ L of cell mix is necessary for the IFC. Maintain a ratio of cells to C1 Suspension Reagent of 3:2.

Table 10: Cell Mix D

Components	Volume (μ L)
Cells 166,000–250,000 mL	60
C1 Suspension Reagent (Fluidigm) 	40
Total	100

- 2 Pipet 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 incyto.com for instructions for use.

Loading the Cells

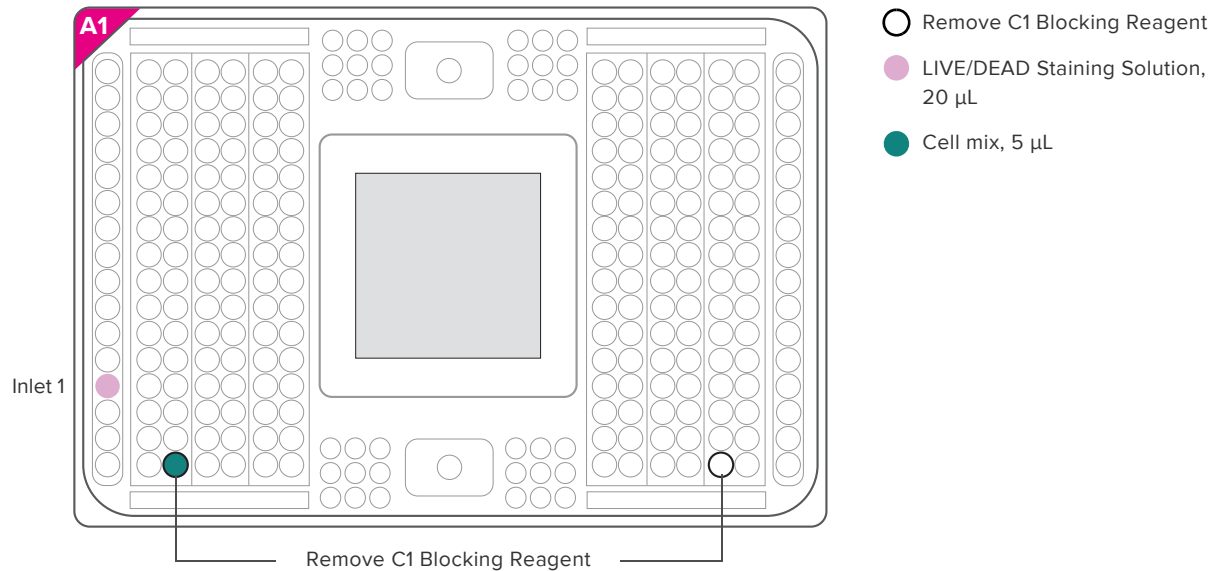


Figure 4: C1 IFC loading pipetting map

- 1 Remove blocking solutions from cell inlet and outlet marked with teal and white dots in Figure 4.
 - 2 Pipet 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 Pipet 5 µL of Cell Mix D into the cell inlet marked with the teal dot. You may pipet up to 20 µL of cell mix, however only 5 µL will enter the IFC.
 - 4 Perform one of these tasks:
 - Staining cells: Vortex the C1 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 C1 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 IFC into the C1. 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 Table 1 on page 8.
 - 6 When the script has finished tap **EJECT** to remove the IFC from the C1 system.

(Optional) Starting the Tube Control: Lysis and Reverse Transcription

If you are running tube controls, see “Appendix A: Running the Tube Controls” on page 42 for instructions.

Imaging the Cells

Cells may be imaged on a microscope compatible with C1 IFCs. Guidelines for the selection of a microscope are outlined in Minimum Specifications for Single-Cell Imaging, PN 100-5004. Contact technical support for this document or with any questions. For phone or email contact information, see page 2

Running Lysis, Reverse Transcription and PCR on the C1 System

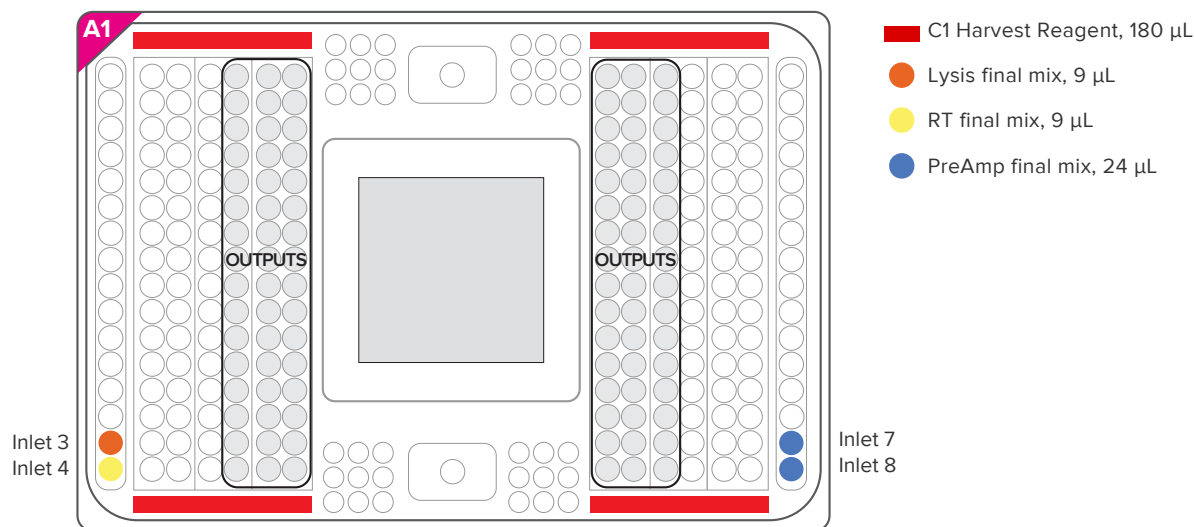


Figure 5: C1 IFC Lysis, RT and PCR pipetting map

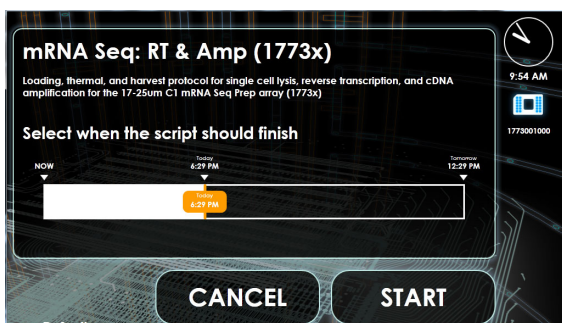
- 1 Pipet 180 μ L of C1 Harvest Reagent into the four reservoirs marked with large solid red rectangles in Figure 5.
- 2 Pipet 9 μ L of lysis Mix A in inlet 3, marked with an orange dot.
- 3 Pipet 9 μ L of RT Mix B in inlet 4, marked with a yellow dot.
- 4 Pipet 24 μ L of PCR Mix C in inlets 7 and 8, marked with blue dots.

- 5 Place the IFC into the C1 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:

Table 11: Thermal cycling protocols

Lysis		PCR		
Temperature	Time	Temperature	Time	Cycles
72 °C	3 min	95 °C	1 min	1
4 °C	10 min	95 °C	20 sec	5
25 °C	1 min	58 °C	4 min	
Reverse Transcription		68 °C	6 min	
Temperature	Time	95 °C	20 sec	9
42 °C	90 min	64 °C	30 sec	
70 °C	10 min	68 °C	6 min	
		95 °C	30 sec	7
		64 °C	30 sec	
		68 °C	7 min	
		72 °C	10 min	1

(Optional) Continuing the Tube Control: mRNA Sequencing PCR

If you are running tube controls, see “Appendix A: Running the Tube Controls” on page 42 for instructions.

Harvesting the Amplified Products

- 1 When the mRNA sequencing preparation script has finished, tap **EJECT** to remove the IFC from the instrument.
NOTE The IFC may remain in the C1 system for up to one hour after harvest before removing products from their inlets.
- 2 Transfer the C1 IFC to a post-PCR lab environment.
- 3 Label a new 96-well plate “Diluted Harvest Plate.”
- 4 Aliquot 10 μ L 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 IFC using the plastic removal tool:

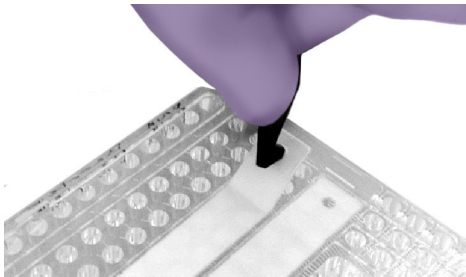


Figure 6: Tape removal

- 6 Using an eight-channel pipette, pipet the harvested amplicons from the inlets according to Figure 7 and Table 12 on page 29 and place in the diluted harvest plate:

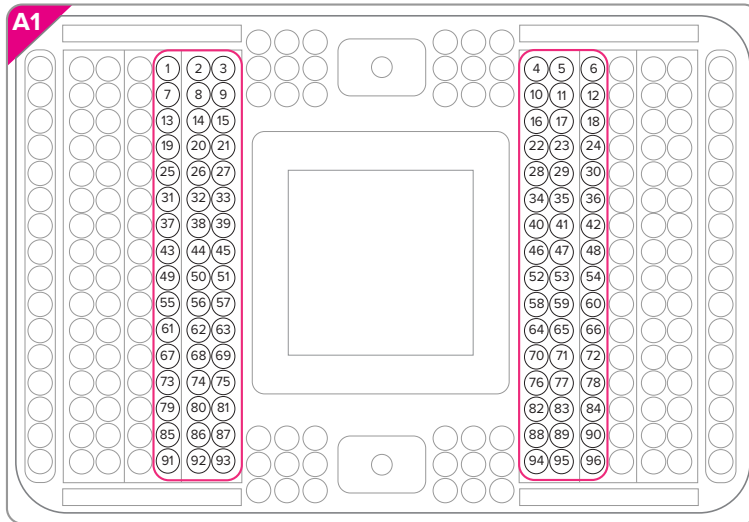


Figure 7: Pipette map of reaction products on the C1 IFC

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

Table 12: Harvest amplicon dilution

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

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 C1 harvest amplicons out of the left-side wells of the C1 IFC into the 10 μ L of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

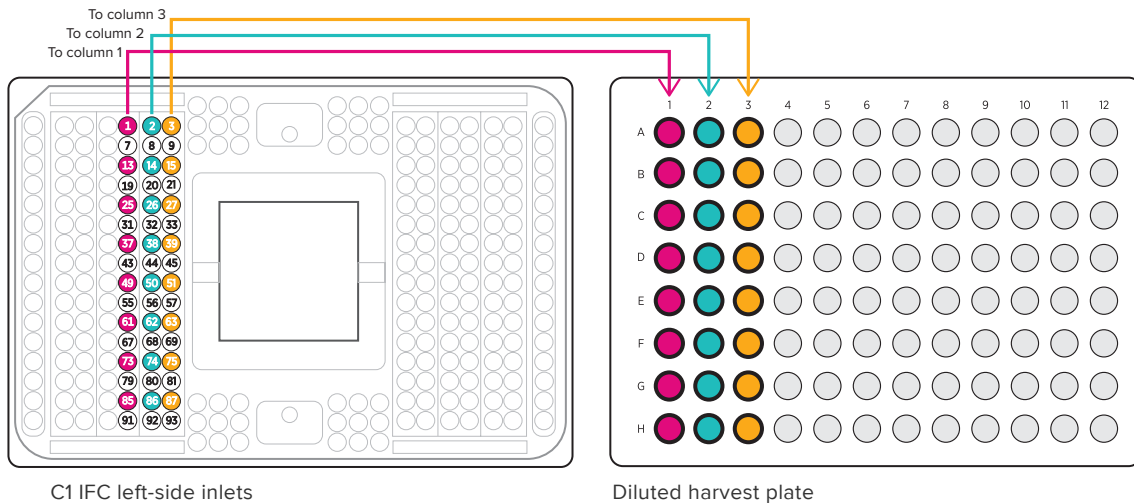


Figure 8: First three harvest product pipette steps

- 8 Pipet the entire volume of C1 harvest amplicons out of the right-side wells of the C1 IFC into the 10 μ L of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

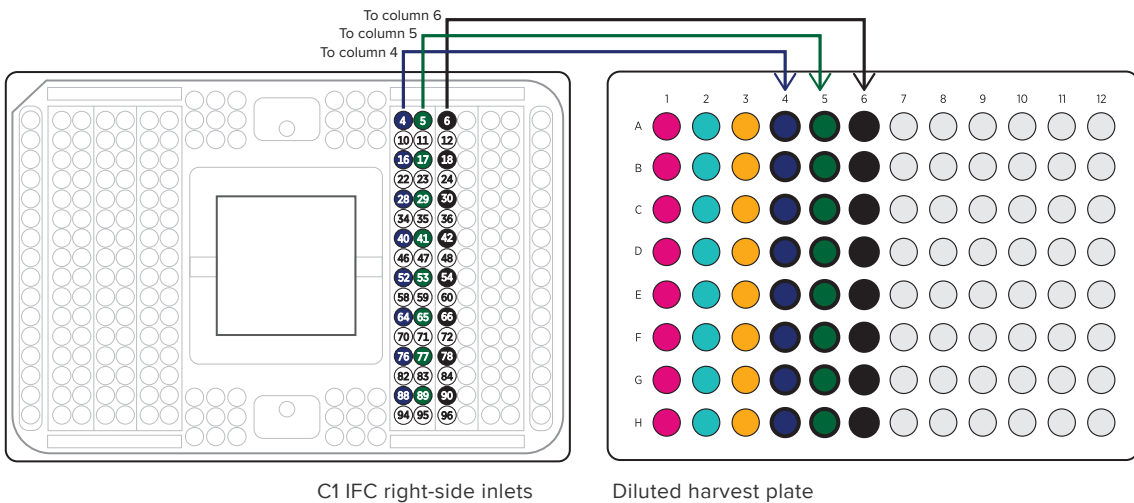


Figure 9: Fourth, fifth, and sixth pipetting steps

- 9 Pipet the entire volume of C1 harvest amplicons out of the left-side wells of the C1 IFC into the 10 μ L of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

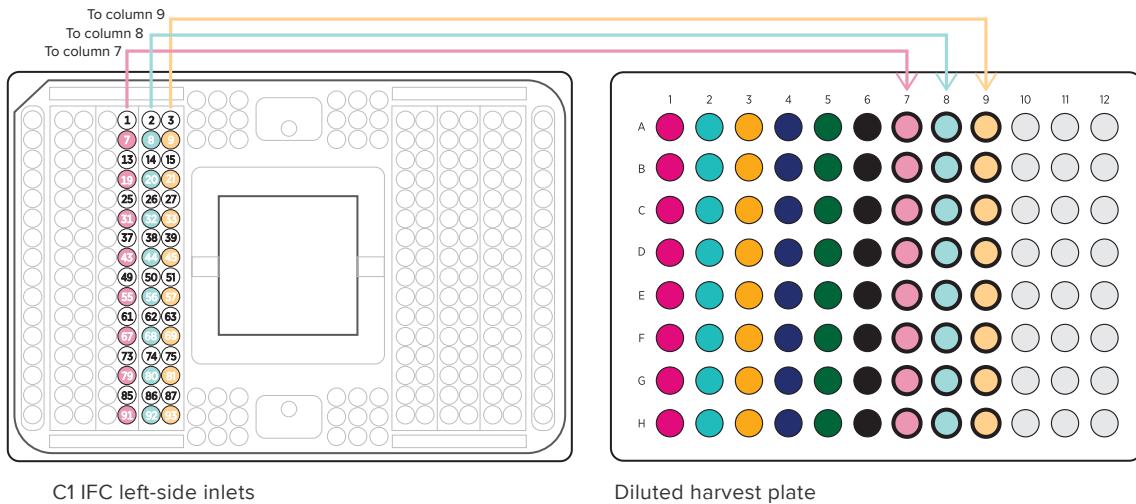


Figure 10: Seventh, eighth, and ninth pipetting steps

- 10 Pipet the entire volume of C1 harvest amplicons out of the right-side wells of the C1 IFC into the 10 μ L of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

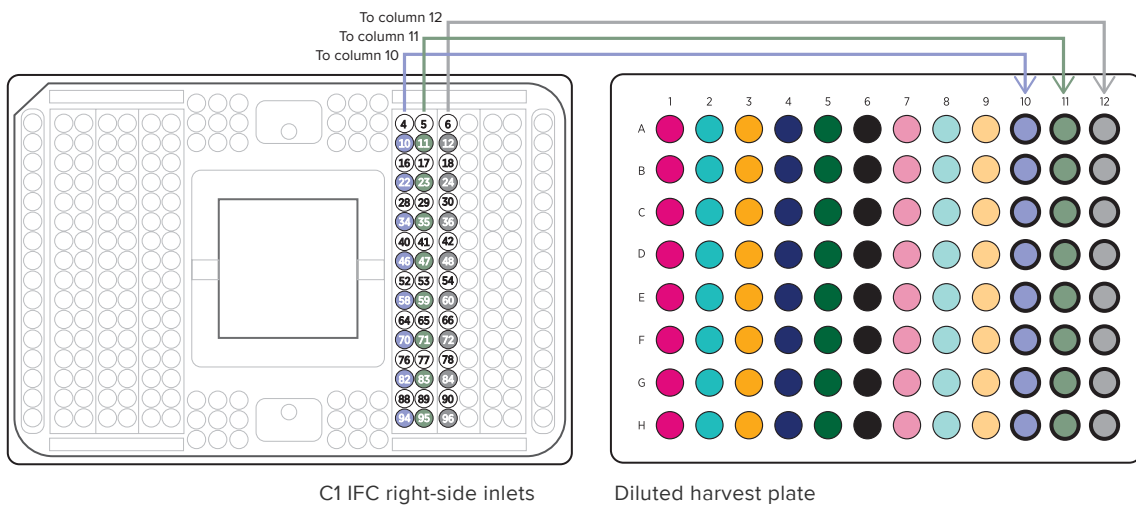


Figure 11: Tenth, eleventh, and twelfth pipetting steps

- 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 Delta Gene 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 sequencing library preparation for sequencing from cDNA acquired from the C1 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 sequencing 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 Kit 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

Table 13: Reagent supplies

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
C1 DNA Dilution Reagent	Remove from -20°C and thaw to room temperature in a DNA-free hood	Module 2 (mRNA Seq) (Fluidigm)
C1 Harvest Reagent	Remove from -20°C and thaw to room temperature in a DNA-free hood	Module 2 (mRNA Seq) (Fluidigm)
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 (S502-S508 and S517)	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
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

Quantify and Dilute Harvest Amplicons

cDNA Sample Dilution

A. cDNA concentrations yielded from the C1 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 and dilution have 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 C1 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 100–0,000 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 Pipet the appropriate amount of C1 Harvest Reagent to each well of the diluted samples plate listed in the table per determined sample dilution:

Table 14: C1 Harvest Reagent required at different sample dilutions

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

- 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 In a 1.5 mL PCR tube, combine the components of the PreMix:

Table 15: Tagmentation reaction

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

- 4 Vortex at low speed for 20 seconds and spin down all components.
- 5 Aliquot equal amounts of PreMix into each tube of an eight-tube strip.
- 6 Pipet 3.75 μL of the PreMix to each well of the library prep plate using an 8-channel pipette.
- 7 Pipet 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. 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.

Table 16: Thermal cycle protocol

Temperature	Time
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. For 96 samples:

Table 17: NT buffer addition

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	—

- 11** Once the sample reaches 10 °C, pipet 1.25 µL of the NT Buffer to each of the tagged samples to neutralize the samples.
- 12** Seal plate and vortex at medium speed. 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 tagged cDNA.

- 1 Aliquot equal volumes of Nextera PCR Master Mix (NPM) into each tube of an 8-tube strip.
- 2 Pipet 3.75 µL of the aliquoted NPM to each well of the library prep plate using an eight-channel pipette:

Table 18: Tagment reaction plus NPM

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	—

- 3 Pipet 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 eight-channel pipette as shown in Table 19.
- 4 Pipet 1.25 μL of Index Primer 2 (S502-S508 and S517) to the corresponding well of **each column** of the library prep plate using an eight-channel pipette:

Table 19: Index primer pipette map and index assignment

		N701	N702	N703	N704	N705	N706	N707	N708	N709	N710	N711	N712
		1	2	3	4	5	6	7	8	9	10	11	12
S517	A	S517/ N701	S517/ N702	S517/ N703	S517/ N704	S517/ N705	S517/ N706	S517/ N707	S517/ N708	S517/ N709	S517/ N710	S517/ N711	S517/ 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

- 5 Seal the plate with adhesive film and vortex at medium speed for 20 seconds. Centrifuge at 4,000 rpm for 2 minutes.
- 6 Place the plate into a thermal cycler and perform PCR amplification:

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

Table 20: Thermal cycle protocol

Temperature	Time	Cycles
72 °C	3 min	1
95 °C	30 sec	1
95 °C	10 sec	12
55 °C	30 sec	
72 °C	60 sec	
72 °C	5 min	1
10 °C	hold	—

- 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 21 according to the determined number of samples to be pooled:

Table 21: Sample volume to be pooled for different pool sizes and AMPure beads required

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

- 4** To the pooled library add the required amount of AMPure XP beads listed in the Table 21.
- 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 9–10.
- 12** Allow the beads to air-dry on bench for 10–15 minutes.
- 13** Elute the samples by adding the required volume of C1 DNA Dilution Reagent per number of samples pooled:

Table 22: Elution buffer required for libraries pooled from different number of samples (elution buffer volume equal to pooled library volume)

Number of libraries pooled	Volume of C1 DNA Dilution Reagent (volume of original sample pool; μL)
8	32
12	48
16	32
24	48
32	32
48	48
96	96

- 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:

Table 23: Elution buffer required for libraries pooled from different number of samples

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

- 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 6–7.

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 C1 DNA Dilution Reagent per number of samples pooled:

Table 24: Final elution buffer required for libraries pooled from different number of samples

Number of libraries pooled	Volume of C1 DNA Dilution Reagent (1.5X of original pool volume; μL)
8	48
12	66
16	48
24	66
32	48
48	66
96	144

- 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. 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 A: 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 μ L β -ME at 14.3 M to 1 mL of Buffer RLT Plus. Alternatively, you may use 20 μ L 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/ μ L in media.
- 3 Combine 20 μ L of cells in media to 350 μ L Buffer RLT Plus with β -ME. If you have less than 20 μ L of material available, simply add the buffer directly to remaining volume.
- 4 Vortex mixture for 1 minute at high speed.
- 5 (Optional) Include a QIAshredder step if primary tissue cells are used:
 - a Pipet the total volume into QIAshredder 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 “mRNA Sequencing Reaction” on page 43, 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 C1 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 C1 Cell Wash Buffer by pipetting up and down at least 5 times.
- 6 Pellet cells again and remove supernatant.
- 7 Resuspend cells in C1 Cell Wash Buffer to approximately 90% original volume to keep original concentration, assuming a 10% loss.
- 8 Dilute your cell suspension to 100–200 cells/ μ L using C1 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 incyto.com for instructions for use.

mRNA Sequencing Reaction

- 1 Prepare two tube controls by combining lysis reagents and thermal cycling them:
 - a Prepare cell lysis mix:

Components	Tube 1: Positive Control (μ L)	Tube 2: NTC (no template control; μ L)
Prepared cells	1.0	—
C1 Cell Wash Buffer	—	1.0
Lysis final mix	2.0	2.0
Total	3.00	3.00

- 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:

Table 25: Tube controls

Components	Tube 1: Positive Control (μL)	Tube 2: NTC (μL)
Cell lysis mix (see step 1 on page 43)	3.0	3.0
RT final mix	4.0	4.0
Total	7.00	7.00

- b Vortex briefly and spin to collect contents.
- c In a PCR thermal cycler, run the following protocol for reverse transcription:

Table 26: RT hold parameters

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

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:

Table 27: PCR reaction

Components	Tube 1: Positive Control (μL)	Tube 2: NTC (μL)
PCR Mix C	9.0	9.0
RT reaction (see step 2)	1.0	1.0
Total	10.0	10.0

4 In a PCR thermal cycler, run:

Table 28: PCR thermal cycling protocol

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	

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:

Table 29: Dilution of PCR products

Components	Volume (µL)
C1 DNA Dilution Reagent (Fluidigm)	45
PCR product	1
Total	46

- 4 Continue with quantification protocol for library preparation (see “Quantify and Dilute Harvest Amplicons” on page 34).

Appendix B: RNA Spike Assays

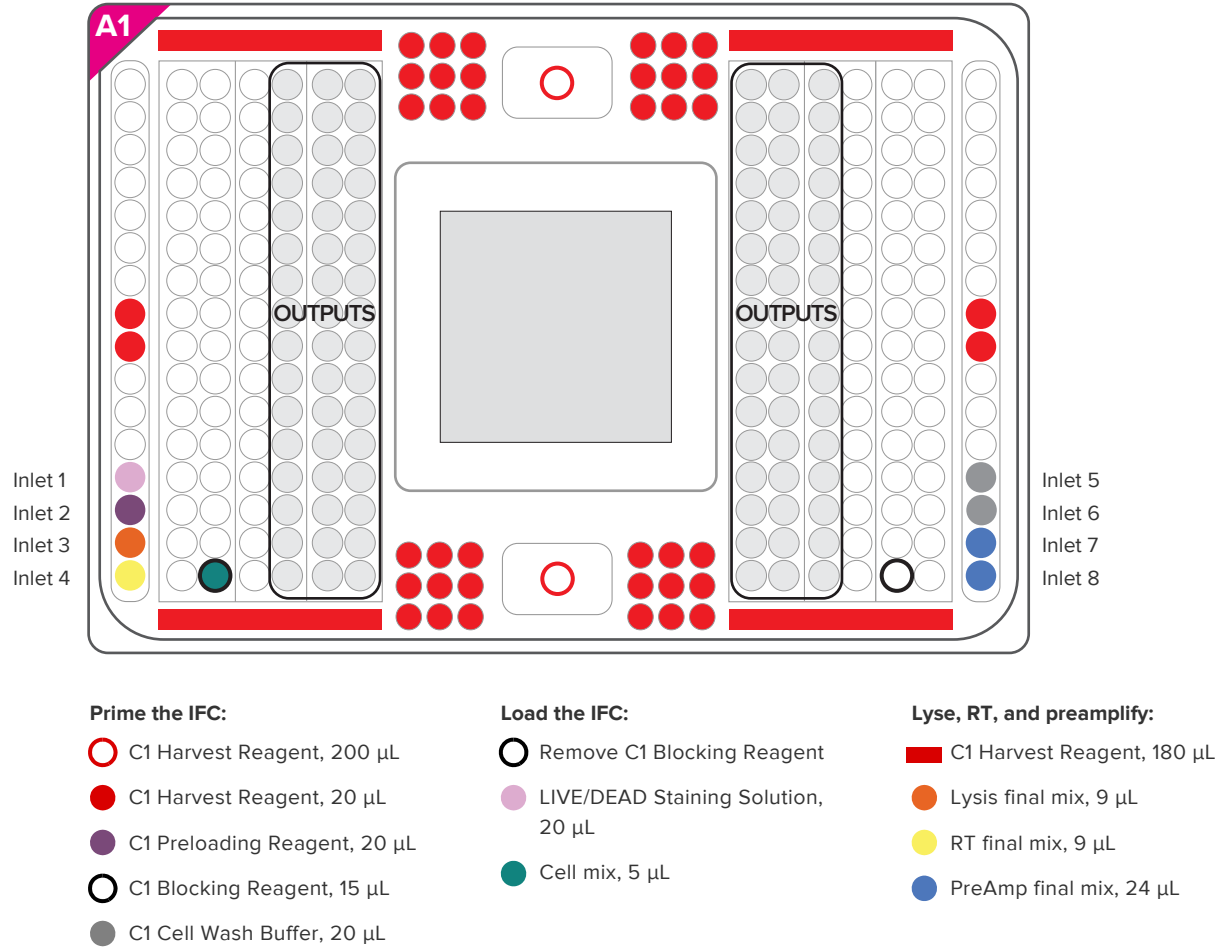
If ordering Delta Gene™ assays, order:

Table 30: Fluidigm part numbers

Reverse Assay Primer	Sequence
C1 RNA Standards Kit	100-5582

Appendix C: IFC Pipetting Map

Overview of IFC Pipetting



Appendix D: Library Prep Examples

Typical Agilent Bioanalyzer Trace

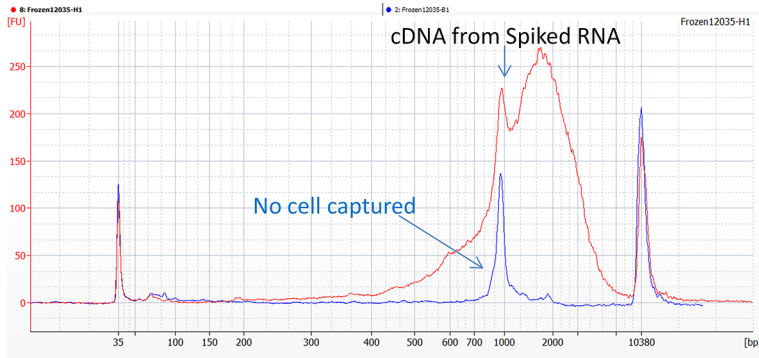


Figure 12: A typical cDNA harvested from IFC

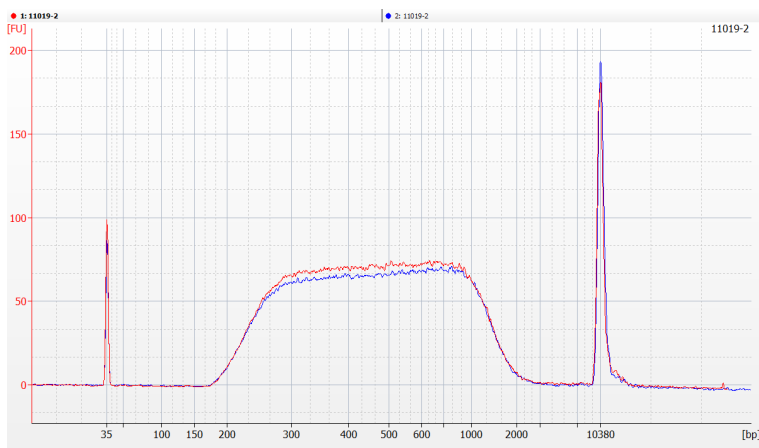
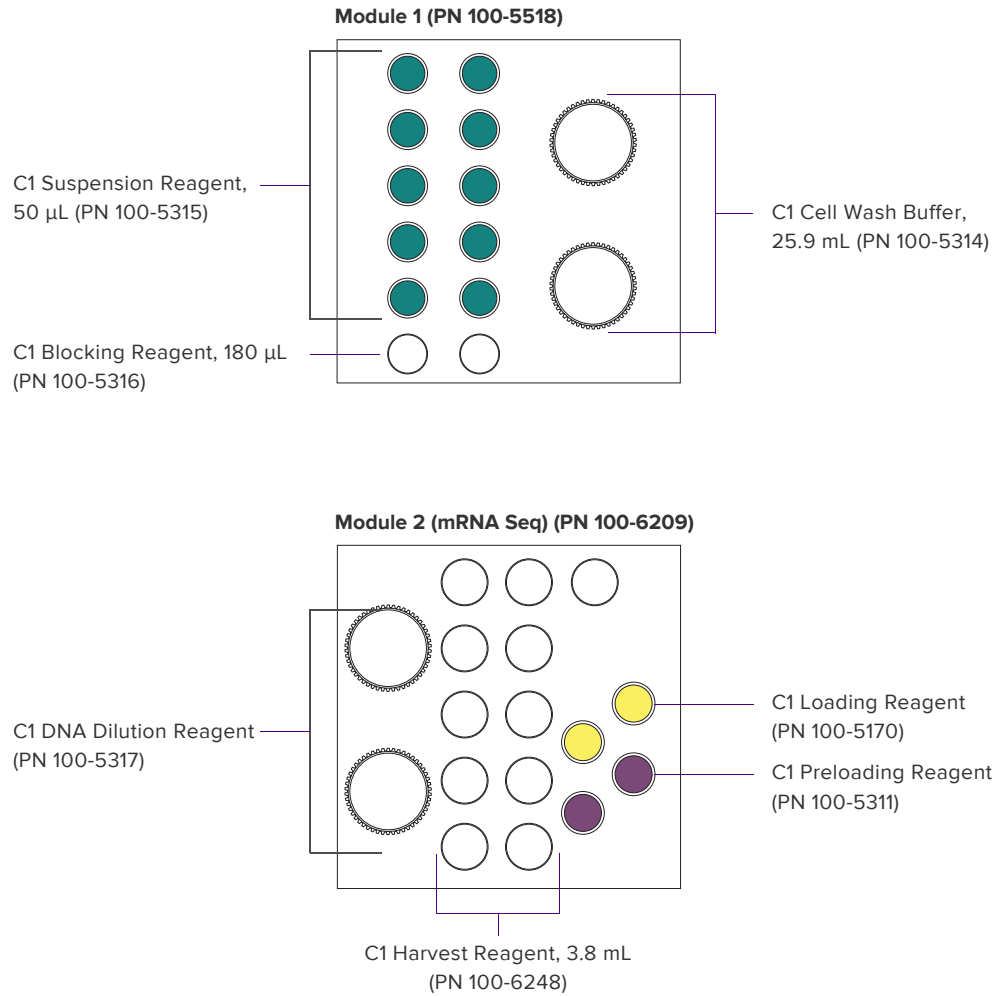


Figure 13: Typical library size distribution after purification

Appendix E: C1 Reagent Kit for mRNA Seq, PN 100-6201

For storage conditions, see “Required Reagents for cDNA Synthesis” on page 11.





For technical support visit fluidigm.com/support

