FLUIDIGM®

PN 100-4904 K1

Using C1 to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays

PROTOCOL

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About This Guide

CAUTION ABBREVIATED SAFETY ALERTS. Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For complete safety information, see the safety appendix on page 42.

For detailed instructions on instrument and software operation, refer to the C1 System User Guide (PN 100-4977).

Safety Alert Conventions

This guide uses specific conventions for presenting information that may require your attention. Refer to the following safety alert conventions.

Safety Alerts for Chemicals

Fluidigm follows the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) for communicating chemical hazard information. GHS provides a common means of classifying chemical hazards and a standardized approach to chemical label elements and safety data sheets (SDSs). Key elements include:

- Pictograms that consist of a symbol on a white background within a red diamond shaped frame. Refer to the individual SDS for the applicable pictograms and warnings pertaining to the chemicals being used.

- Signal words that alert the user to a potential hazard and indicate the severity level. The signal words used for chemical hazards under GHS:

  DANGER Indicates more severe hazards.

  WARNING Indicates less severe hazards.

Safety Alerts for Instruments

For hazards associated with instruments, this guide uses the following indicators:

- Pictograms that consist of a symbol on a white background within a black triangle shaped frame.

- Signal words that alert the user to a potential hazard and indicate the severity level. The signal words used for instrument hazards:

  DANGER Indicates an imminent hazard that will result in severe injury or death if not avoided.

  WARNING Indicates a potentially hazardous situation that could result in serious injury or death.

  CAUTION Indicates a potentially hazardous situation that could result in minor or moderate personal injury.

  IMPORTANT Indicates information necessary for proper use of products or successful outcome of experiments.

Safety Data Sheets

Read and understand the SDSs before handling chemicals. To obtain SDSs for chemicals ordered from Fluidigm Corporation, either alone or as part of this system, go to fluidigm.com/sds and search for the SDS using either the product name or the part number.

Some chemicals referred to in this user guide may not have been provided with your system. Obtain the SDSs for chemicals provided by other manufacturers from those manufacturers.

Revision History

Revision	Date	Description of change
K1	26 May 2016	• Updated formatting. • Changed all instances of Life Technologies to Thermo Fisher Scientific (various pages throughout). • Changed all Fluidigm instances of PreAmp to Preamp (various pages throughout). • Updated the priming and loading/staining script times (see page 10, page 22, and page 25). • Updated the tube control processing times (see page 10 and page 35). • Updated the C1 reagent kit name (see page 11 and page 39). • Updated the C1 IFC name (see page 12). • Changed the Single Cell Lysis Solution storage temperature to 4 °C (see page 14). • Updated the small-cell staining recommendations (see page 22). • Updated the input cell concentration range (see page 23). • Updated cell suspension ratio recommendation (see page 24). • Increased cell mix load volume to 6 µL (see page 24 and page 38). • Reorganized steps for running cell load scripts (see page 25). • Clarified post-PCR workflow (see page 33).
J1	29 May 2015	Updated safety and technical support contact information.
I1	15 April 2015	• Improved formatting and added new capture site map. (See Harvest the Amplified Products on page 28.) • Changed “C1 Loading Reagent” to “Loading Reagent,” “C1 Cell Wash Buffer” to “Cell Wash Buffer,” and “C1 Suspension Reagent” to “Suspension Reagent.” (See Required Reagents on page 11.)
H1	3 November 2014	Updated product names, illustrations, and legal boilerplate to new branding specifications.
G1	22 July 2014	• Replaced the photographs of the kit modules with diagrams. (See Appendix D: C1 Single-Cell Auto Prep Reagent Kit, PN 100-5319 on page 39.) • Corrected the cross-reference to the Fluidigm Real-Time PCR Analysis User Guide (PN 68000088) from Appendix A to Appendix B and further specified that exonuclease treatment is not needed when “Preparing the Sample Pre-Mix and Samples”. (See Run Reaction Products on a 96.96 Dynamic Array IFC on page 33.)
F1	10 April 2014	• Updated name from “C1 Module 1 Kit” to “Module 1” and from “C1 Module 2 Kit” to “Module 2 (PreAmp).” • Changed “MSDSs” to “SDSs”. (See Appendix G: Safety on page 42.) • Moved the reagent retrieval table to before the reagent mixes section. (See Reagent Retrieval to Perform Preamplification Using Delta Gene Assays on page 14.)

Revision	Date	Description of change
E1	23 December 2013	• Changed the volume of C1 Harvest Reagent to use from 150 µL to 180 µL. (See Run Lysis, Reverse Transcription, and Preamplification on C1 on page 26.) • Updated descriptions of the scripts used for preamplification protocol. (See Appendix E: IFC Types and Related Scripts on page 40.) • Replaced “chip” with “IFC” where appropriate. • Reformatted the tube control tables for clarity. (See Appendix A: Run the Tube Controls on page 34.)

Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays

Introduction

This protocol allows the user to capture cells and perform targeted preamplification using the Fluidigm® C1™ and C1 integrated fluidic circuits (IFCs). This protocol explains all steps, including: capturing cells, staining for viability, imaging cells, lysing cells, performing reverse transcription and preamplification, and harvesting the amplified products. It describes the procedure to evaluate the RNA content of cells. Gene expression analysis of preamplified amplicons is then performed with 48.48 or 96.96 Dynamic Array™ IFCs using the Biomark™ or Biomark HD as recommended in Appendix B of the Fluidigm Real-Time PCR Analysis User Guide (PN 68000088) .

Targeted preamplification enriches samples for loci of interest, maintains relative abundance between loci, and permits quantitative C_q information to be derived. Quantitative PCR is performed on the preamplified targets using a DNA binding dye (EvaGreen® dye). For more information on preamplification, see Devonshire et al., *BMC Genomics* 12 (2011): 118.

Figure 1 shows reverse transcription and preamplification using the Single Cell-to-CT™ Kit (Thermo Fisher Scientific).

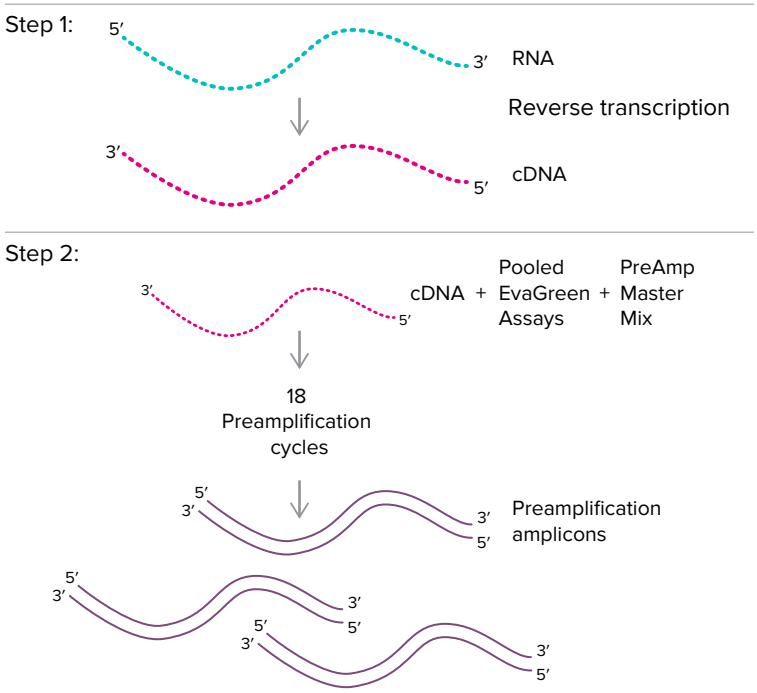


Figure 1. Overview of the reverse transcription and preamplification protocol with EvaGreen assays

Overview of Experimental Workflow

Table 1. Overview of the experimental workflow

	Reagent handling	Automated steps	Time
1	Prepare reagent pre-mixes		15 minutes
2	Pipet priming solutions into the IFC		5 minutes
3		Prime the IFC on C1	• Small- or large-cell IFC: 10 min • Medium-cell IFC: 12 min
4	Pipet cells into the IFC		5 minutes
5		Load cells on C1	Small-cell IFC: • 30 minutes if staining • 20 minutes if not staining Medium-cell IFC: • 65 min if staining • 35 min if not staining Large-cell IFC: • 60 min if staining • 30 min if not staining
6	Image cells with a microscope		15–30 minutes
7	Pipet lysis, reverse transcription, and preamplification chemistry into the IFC		5 minutes
8		Run the Preamp script on C1. This includes lysis, reverse transcription, preamplification, and harvest.	• 330 minutes for small-cell IFC • 360 minutes for medium- or large-cell IFC You can run the script overnight with a pause between Preamp 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 minutes

IMPORTANT If you are running the optional tube controls, add ≈3 hours to the total time to complete the experimental workflow.

Reagents

IMPORTANT

- Thaw reagents on ice unless directed to thaw them at room temperature. Store reagents as soon as they are received according to manufacturer's storage recommendations. Vortex and then centrifuge reagents as directed.
- The names of these reagents have changed:

Previous Reagent Name	New Reagent Name
C1 Loading Reagent	Loading Reagent
C1 Cell Wash Buffer	Cell Wash Buffer
C1 Suspension Reagent	Suspension Reagent

Use the reagents as before. Only use the reagents provided in the required kit. Do not swap reagents between kits.

Required Reagents

NOTE When ordering Module 1 and Module 2 (Preamp) from Fluidigm, use the parent part number: 100-5319.

See a diagram of the C1 Single-Cell Auto Prep Reagent Kit in Appendix D: C1 Single-Cell Auto Prep Reagent Kit, PN 100-5319 on page 39.

Product Name	Company	Part Number	Storage
C1 Single-Cell Auto Prep Reagent Kit	Fluidigm	100-5319	Module 1: 4 °C Module 2: –20 °C

Product Name	Company	Part Number
Ambion Single Cell-to-CT™ qRT-PCR Kit	Thermo Fisher Scientific	4458237
Delta Gene™ assays or other 100 µM F+R primers	Fluidigm or major laboratory supplier (MLS)	—

Suggested Reagents

Product Name	Company	Part Number
LIVE/DEAD Viability/Cytoxicity Kit, for mammalian cells	Thermo Fisher Scientific	L-3224
ArrayControl RNA Spikes	Thermo Fisher Scientific	AM1780
The RNA Storage Solution	Thermo Fisher Scientific	AM7000

Consumables

Required Consumables

Product Name	Company	Part Number
Select the IFC needed: • C1 Single-Cell Auto Prep IFC for Preamp (5–10 µm) • C1 Single-Cell Auto Prep IFC for Preamp (10–17 µm) • C1 Single-Cell Auto Prep IFC for Preamp (17–25 µm)	Fluidigm	 • 100-5757 • 100-5749 • 100-5758
96-well PCR plates	MLS*	—
Lint-free cloth	MLS	—

* Recommended: TempPlate® semi-skirted 96-well PCR plates (USA Scientific, PN 1402-9700)

Suggested Consumables

Product Name	Company	Part Number
INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved)	INCYTO	DHC-N01

Equipment

Required Equipment

Product Name	Company	Part Number
C1 System	Fluidigm	100-7000
Two centrifuges: one for microcentrifuge tubes and one for 96-well plates	MLS	—
Vortexer	MLS	—

Suggested Equipment

Product Name	Company	Part Number
Two biocontainment hoods*	MLS	—
Imaging equipment compatible with C1 IFCs†	MLS	—

* To prevent DNA contamination of lab and samples.

† Refer to the Minimum Specifications for Single-Cell Imaging Specification Sheet, PN 100-5004.

Best Practices

- Use good laboratory practices to minimize contamination of samples. Use a new pipette tip for every new sample. Whenever possible, separate pre- and post-PCR activities. Dedicate laboratory materials to designated areas.
- Thaw reagents on ice unless directed to thaw them at room temperature.

Reagent Retrieval to Perform Preamplification Using Delta Gene Assays

Table 2. Reagent supplies

	Required Reagents	Preparation	Kit Name
(Optional) RNA Spikes	ArrayControl RNA Spikes	Remove from −80 °C and thaw to room temperature in a DNA-free hood	ArrayControl RNA Spikes (Thermo Fisher Scientific)
	THE RNA Storage Solution	Keep at room temperature	THE RNA Storage Solution (Thermo Fisher Scientific)
Pooled Primers (500 nM)	100 µM stock F+R primer plate	Remove from −20 °C and thaw to room temperature in a DNA-free hood	Delta Gene Assays (Fluidigm) or other primer stock
	C1 DNA Dilution Reagent	Remove from −20 °C and thaw to room temperature in a DNA-free hood	Module 2 (Preamp) (Fluidigm)
Lysis Final Mix	Single Cell Lysis Solution	Remove from 4 °C and keep on ice	Single Cell-to-CT Kit (Thermo Fisher Scientific)
	C1 Lysis Plus Reagent	Remove from −20 °C and thaw to room temperature	Module 2 (Preamp) (Fluidigm)
RT Final Mix	Stop Solution	Remove from −20 °C, thaw, and keep on ice	Single Cell-to-CT Kit
	Single Cell VILO™ RT	Remove from −20 °C, thaw, and keep on ice	Single Cell-to-CT Kit
	Single Cell SuperScript® RT	Remove from −20 °C, thaw, and keep on ice	Single Cell-to-CT Kit
	Loading Reagent	Remove from −20 °C and thaw to room temperature	Module 2 (Preamp) (Fluidigm)
Preamp Final Mix	Single Cell PreAmp Mix	Remove from −20 °C, thaw, and keep on ice	Single Cell-to-CT Kit
	C1 Preamp Dilution Reagent	Remove from −20 °C and thaw to room temperature	Module 2 (Preamp) (Fluidigm)
(Optional) LIVE/DEAD Cell Staining	Cell Wash Buffer	Remove from 4 °C, thaw, and keep on ice	Module 1 (Fluidigm)
	Ethidium homodimer-1	Remove from −20 °C, and keep in the dark as much as possible	LIVE/DEAD Viability/ Cytotoxicity Kit (Thermo Fisher Scientific)
	Calcein AM	Remove from −20 °C, and keep in the dark as much as possible	LIVE/DEAD Viability/ Cytotoxicity Kit (Thermo Fisher Scientific)

	Required Reagents		Preparation	Kit Name
Priming	C1 Blocking Reagent	○	Remove from 4 °C and equilibrate to room temperature	Module 1 (Fluidigm)
	C1 preloading Reagent	●	Remove from −20 °C and thaw to room temperature	Module 2 (Preamp) (Fluidigm)
Cell Loading	Suspension Reagent	●	Remove from 4 °C and thaw to room temperature	Module 1 (Fluidigm)

Prepare Reagent Mixes

The following instructions prepare reagents sufficient for one IFC. The 500 nM pooled primer mix and RNA spikes can be made in advance and stored. All other reagents can be scaled up if running multiple IFCs simultaneously:

- (Optional) RNA Spikes Mix
- Pool Primers (500 nM) on page 17
- Lysis Final Mix on page 18
- Reverse Transcription (RT) Final Mix on page 18
- Preamp Final Mix on page 19

IMPORTANT Remove C1 DNA Dilution Reagent and C1 Harvest Reagent (Fluidigm) from −20 °C freezer well before they are needed. These reagents must equilibrate to room temperature prior to use.

(Optional) RNA Spikes Mix

IMPORTANT It is essential to obtain the assay primers to target the RNA Spikes mix before proceeding with running preamplification on the C1 system. The oligo ordering information is in Appendix B: RNA Spike Assays on page 37.

RNA Spikes mix serves as a positive control for thermal cycling of the C1 system independent of cell capture. Although this standard is not required, it is highly recommended.

NOTE

- The RNA Spikes 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.

Prepare the RNA Spikes Mix

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 3. RNA Spikes mix

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

3. Vortex tube A for 3 seconds and centrifuge to collect contents. Pipet 1.5 µL from tube A into tube B. Discard tube A.
4. Vortex tube B for 3 seconds and centrifuge to collect contents. Pipet 1.5 µL from tube B into tube C. Discard tube B.
5. Vortex tube C for 3 seconds and centrifuge to collect contents. Tube C is the concentrated RNA Spikes mix 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 IFC run.

Dilute the RNA Spikes Mix for the Lysis Final Mix

IMPORTANT Diluted RNA does not store well. Do not dilute RNA more than an hour before you load the IFC. Only store concentrated aliquots long term.

1. Thaw the RNA Spikes mix.
2. Dilute by combining:

Table 4. RNA Spikes mix dilution

Components	Volume (µL)
RNA Spikes mix	1
C1 DNA Dilution Reagent (Fluidigm; 30-mL bottle)	99
Total	**100**

3. Vortex the diluted RNA Spikes mix for 3 seconds and centrifuge briefly to collect contents.

Pool Primers (500 nM)

We recommend using Delta Gene Assays from Fluidigm. These assays are provided as forward and reverse primer mixes with each primer at a concentration of 100 µM. If you obtain primers from another source, combine the primers for each assay so the concentration of each primer is 100 µM before proceeding to step 1.

1 After 100 µM stock F+R primer plate is thawed, vortex the plate for 10 seconds and then centrifuge at 2,000 rpm for 1 minute.

2 In a DNA-free hood, make the pooled primers (500 nM):

a Combine equal volumes of each 100 µM primer pair.

b Add C1 DNA Dilution Reagent to a final concentration of 500 nM.

c Vortex for 5 seconds and centrifuge briefly to collect contents.

d Keep on ice until use.

Table 5. Pooled primers (500nM)

Components	Volume (µL)
1 µL each primer pair (100 µM each)	1 (x 93 = 93 µL)
(Optional) RNA spike primers	1 (x 3 = 3 µL)
C1 DNA Dilution Reagent (Fluidigm) (30 mL bottle)	104
Total	**200**

NOTE The pooled primers (500 nM) can be made in advance and pre-aliquoted prior to use. The mix pool can be stored for up to six months at −20 °C.

Lysis Final Mix

1 Once the Single Cell Lysis Solution from the Single Cell-to-CT Kit is thawed, vortex for 3 seconds and centrifuge briefly to collect contents.

2 Prepare 18 µL of lysis final mix by combining the following components:

Table 6. Lysis final mix

Components	Volume (µL)
Diluted RNA Spikes mix*	0.90
Single Cell Lysis Solution (Thermo Fisher Scientific)	12.75
C1 Lysis Plus Reagent (Fluidigm)	4.35
Total	**18.00**

* You can substitute 0.90 µL C1 DNA Dilution Reagent (Fluidigm) for the diluted RNA Spikes mix.

3 Vortex for 3 seconds and centrifuge briefly to collect contents. Keep on ice until use.

Reverse Transcription (RT) Final Mix

1 Once the stop solution and Single Cell VILO RT Mix from the Single Cell-to-CT Kit are thawed, vortex each for 3 seconds and centrifuge briefly to collect contents.

2 In a DNA-free hood, prepare 12 µL of RT (reverse transcription) final mix by mixing according to the table below.

Table 7. RT final mix

Components	Volume (µL)
Stop Solution (Thermo Fisher Scientific)	1.94
Single Cell VILO RT Mix (Thermo Fisher Scientific)	5.84
Single Cell SuperScript RT (Thermo Fisher Scientific)	3.62
Loading Reagent (Fluidigm)	0.60
Total	**12.00**

3 Vortex the RT final mix for 5 seconds and centrifuge briefly to collect contents. Keep on ice until use.

Preamp Final Mix

1 In a DNA-free hood, prepare 60 µL of Preamp final mix according to the table below.

Table 8. Preamp final mix

Components	Volume (µL)
Single Cell PreAmp Mix (Thermo Fisher Scientific)	12
C1 Preamp Dilution Reagent (Fluidigm)	42
Pooled primers (500 nM)	6
Total	**60**

2 Vortex the Preamp final mix for 3 seconds and centrifuge to collect contents before use. Keep on ice until ready to use.

Use the IFC Map Loading Plate

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

1 Obtain an IFC map loading plate:

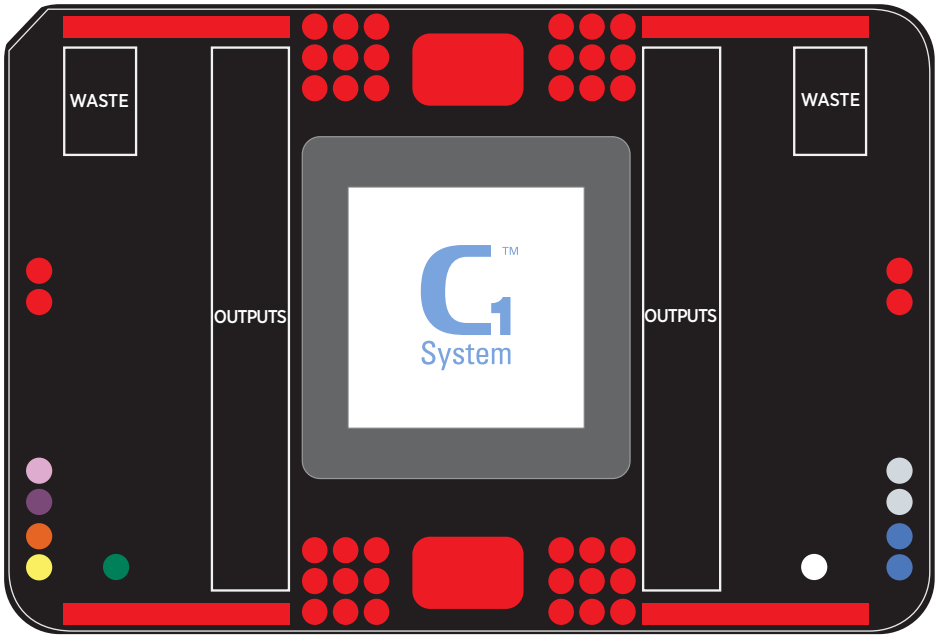


Figure 2. IFC map loading plate

2 Place the C1 IFC onto the IFC map loading plate. For more details on IFC loading, see Appendix C: IFC Pipetting Map on page 38.

Prime the IFC

NOTE

- Vortex and then centrifuge all reagents before pipetting into the IFC.
- 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 inlet.

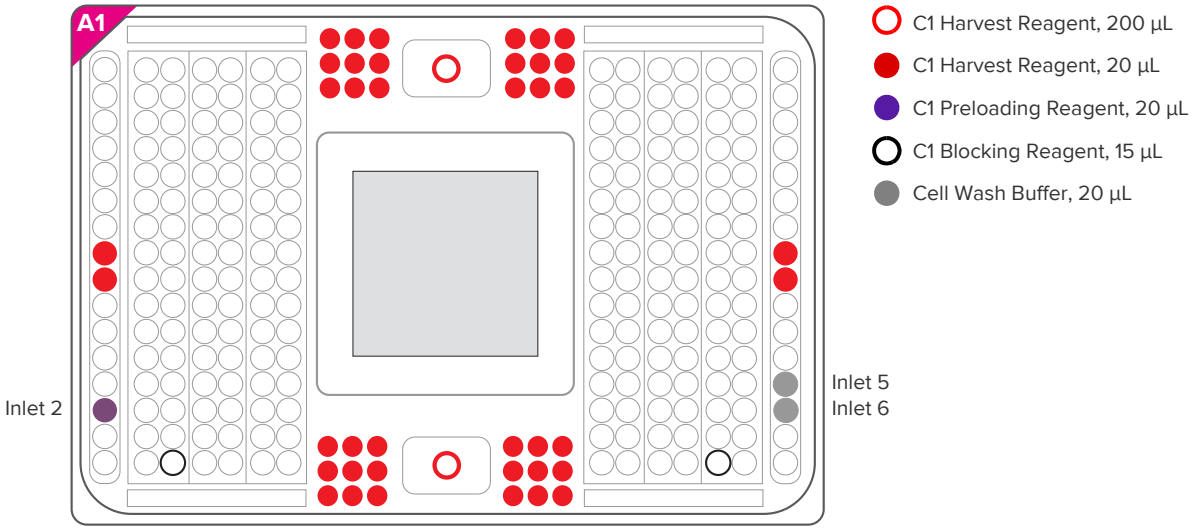


Figure 3. C1 IFC priming pipetting map

1. Pipet 200 µL of C1 Harvest Reagent from 4 mL bottle into the accumulators marked with red outlined circles in Figure 3.
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 on each side of the IFC in the middle of the outside columns of inlets marked with solid red circles. These inlets 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 Cell Wash Buffer (from 30 mL bottle) into inlets 5 and 6, marked with dark gray dots.

7 Place the IFC into the C1 system. Run the **STA: Prime (1782x/1783x/1784x)** script. (See Appendix E: IFC Types and Related Scripts on page 40.) Priming small- or large-cell IFCs takes 10 minutes, and priming medium-cell IFCs takes 12 minutes. When the STA: Prime script has finished tap **EJECT** to remove the primed IFC from the instrument.

NOTE After priming the IFC, you have up to 1 hour to load the IFC with the C1 system.

Prepare Cells

Follow these protocols to prepare the cell mix for loading into the C1 IFC:

- (Optional) Prepare LIVE/DEAD Cell Staining Solution
- Prepare the Cell Mix on page 23
- Load Cells on page 24
- (Optional) Start the Tube Control: Lysis and Reverse Transcription on page 25
- Image the Cells on page 25

(Optional) Prepare 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 airtight bag with desiccant pack at −20 °C.
- Cell staining solution may be prepared up to two hours before loading onto the C1 IFC. Keep on ice and protected from light before pipetting into IFC.

IMPORTANT Approximate staining times: small cells (5–10 µm) takes 30 minutes, medium cells (10–17 µm) takes 65 minutes, and large cells (17–25 µm) cells takes 60 minutes. To easily visualize small cells, you may need to double the amount of stain used.

1 Vortex the dyes for 10 seconds and then centrifuge them before pipetting.

2 Prepare the LIVE/DEAD stain by combining reagents in this order:

Components	Volume (µL)
Cell Wash Buffer (Fluidigm) (30 mL bottle)	1250.0
Ethidium homodimer-1 (LIVE/DEAD kit, Thermo Fisher Scientific/Molecular Probes)	2.5
Calcein AM (LIVE/DEAD kit, Thermo Fisher Scientific/Molecular Probes)	0.625
Total	**1253.125**

3 Vortex the LIVE/DEAD staining solution for 10 seconds before pipetting into the IFC.

Prepare the Cell Mix

IMPORTANT Vortex the Suspension Reagent for 5 seconds before use. If Suspension Reagent contains particulate, ensure they are properly removed by vortexing. **Do not vortex** the cells.

1 Ensure that you have begun priming the IFC. (See Prime the IFC on page 21.)

2 Before mixing cells with Suspension Reagent and loading them into the IFC, prepare a cell suspension in native medium of 66,000–333,000 cells/mL. The recommended concentration range ensures that a total of 200–1000 cells are loaded into the IFC. You can prepare a cell suspension with a minimum concentration of 66,000 cells/mL, but fewer cells will be loaded and captured in the IFC. Preparing a cell suspension of >333,000 cells/mL may clog the fluidic channels. Suspend the cells in a final volume of 0.5–1 mL to ensure enough cells are available for the IFC and 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/product/product02_detail.php for instructions for use.
- Make sure to record your final cell concentration.

3 Prepare the cell mix by combining cells with Suspension Reagent ● at a ratio optimized in advance for your cell type, to create a neutrally buoyant cell suspension. Many cell types use the standard ratio of 3:2 as shown below.

Table 9. Cell mix

Components	Volume (µL)
Cells 66,000–333,000/mL	60
Suspension Reagent (Fluidigm) ●	40
Total	**100**

NOTE

- For more information, see the Fluidigm Single-Cell Preparation Guide (PN 100-7697).
- The total volume of cell mix may be scaled depending on volume of cells available. You will load 6 µL of the cell mix into the IFC (see Figure 4).

4 Set a P200 pipette to 60 µL, and then pipet the cell mix 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.

Load Cells

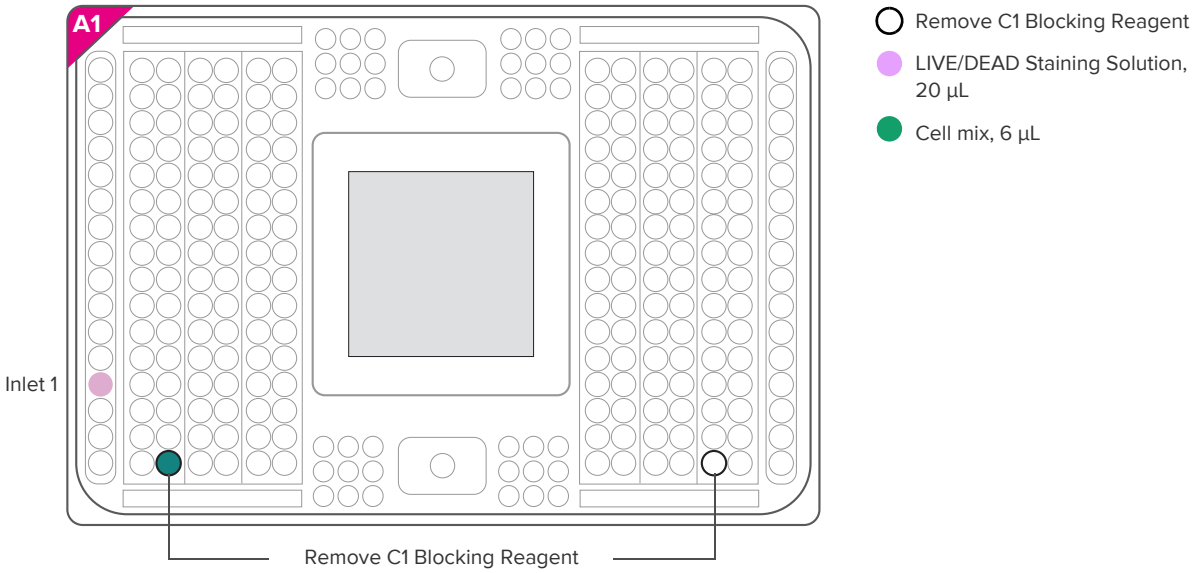


Figure 4. C1 IFC loading pipetting map

1 Use a pipette and tip to remove blocking solutions from cell inlet and outlet marked with teal and white dots in Figure 4.

2 Set a P200 pipette to 60 µL, and then pipet the cell mix 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.

3 Pipet 6 µL of cell mix into the cell inlet marked with the teal dot.

4 Perform one of these tasks:
- Staining cells: Vortex the LIVE/DEAD staining solution for 10 seconds, and then pipet 20 µL of the solution into inlet 1, marked with a pink dot.
- Not staining cells: Pipet 20 µL of Cell Wash Buffer into inlet 1, marked with a pink dot.

5 Place the IFC into the C1 system. Run the **STA: Cell Load (1782x/1783x/1784x)** or **STA: Cell Load & Stain (1782x/1783x/1784x)** script. (See Appendix E: IFC Types and Related Scripts on page 40.)

NOTE Approximate staining times: small cells (5–10 µm) takes 30 minutes, medium cells (10–17 µm) takes 65 minutes, and large (17–25 µm) cells takes 60 minutes.

6 When the script has finished, tap **EJECT** to remove the IFC from the C1 System.

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

If you choose to start tube controls, see Appendix A: Run the Tube Controls on page 34 for instructions.

Image the Cells

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

Run Lysis, Reverse Transcription, and Preamplification on C1

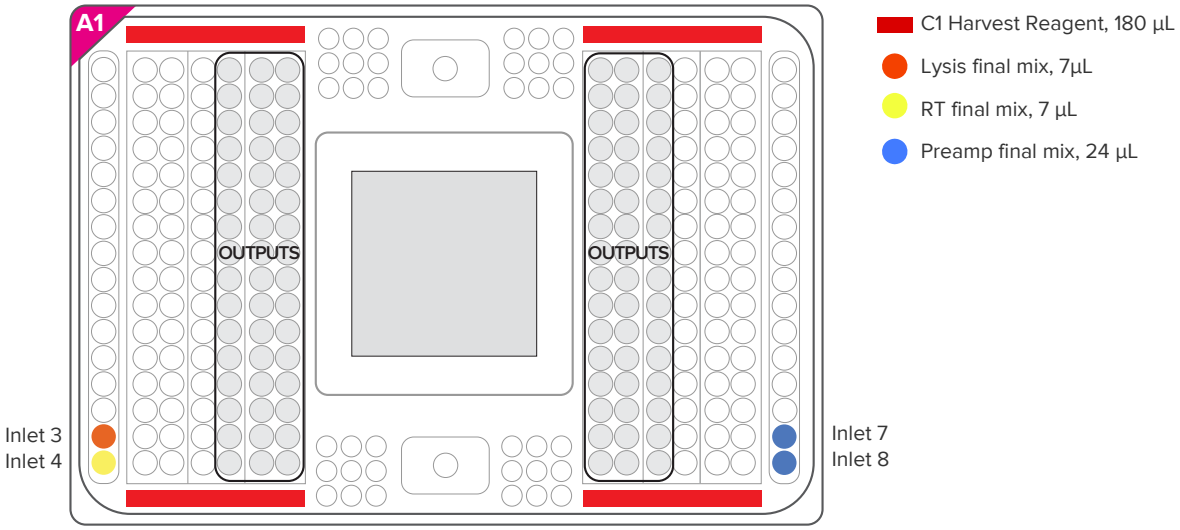


Figure 5. C1 IFC lysis, RT, and preamplification pipetting map

1 Pipet 180 µL of Harvest Reagent into the four reservoirs marked with large solid red rectangles in Figure 5.

2 Pipet 7 µL of lysis final mix in inlet 3, marked with an orange dot.

3 Pipet 7 µL of RT final mix in inlet 4, marked with a yellow dot.

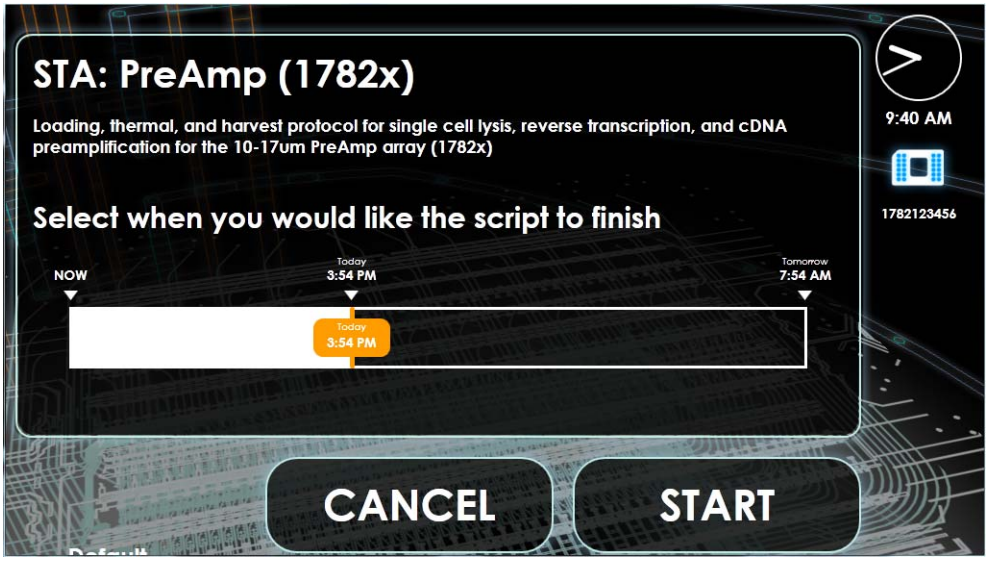
4 Pipet 24 µL of Preamp final mix in inlets 7 and 8, marked with blue dots.

5 Place the IFC into the C1 system. Choose the **STA: Preamp (1782x/1783x/1784x)** script, and then tap **START.** (See Appendix E: IFC Types and Related Scripts on page 40.)

NOTE The STA: Preamp (1782x/1783x/1784x) script may be run overnight. Approximate run times are:

- Small-cell IFC: ~5.5 hours (4.5 hours for lysis, reverse transcription, and preamplification; and 1 hour for harvest)
- Medium- and large-cell IFCs: ~6 hours (4 hours for lysis, reverse transcription, and preamplification; 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 can be programmed to complete next morning:



NOTE To abort the harvest, tap **ABORT**. The IFC will no longer be usable. Start a new experiment with a new IFC.

The STA: Preamp (1782x/1783x/1784x) script contains the following thermal cycling protocols:

Table 10. Thermal cycling protocols

Reverse Transcription	
Temperature	**Time**
25 °C	600 sec
42 °C	3,600 sec
85 °C	300 sec

Preamplification			
Stage	**Temperature**	**Time**	**Cycles**
Enzyme activation/ RT inactivation	95 °C	600 sec	1
Denature	95 °C	15 sec	18
Anneal/Extend	60 °C	240 sec	
Hold	4 °C	Hold	Hold

(Optional) Continue the Tube Controls: Preamplification

If you are running tube controls, see Appendix A: Run the Tube Controls on page 34 for instructions.

Harvest the Amplified Products

1. When the STA: Preamp script has finished, tap **EJECT** to remove the IFC from the instrument.

   NOTE The IFC may remain in the C1 system for up to 1 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 25 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 IFC using the plastic removal tool.

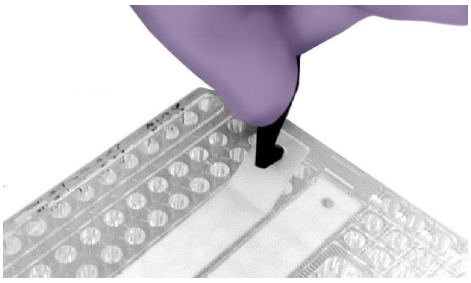


Figure 6. Tape removal

6 Using an eight-channel pipette, pipet the harvested amplicons from the inlets according to Figure 7 and Table 11 and place in the diluted harvest plate.

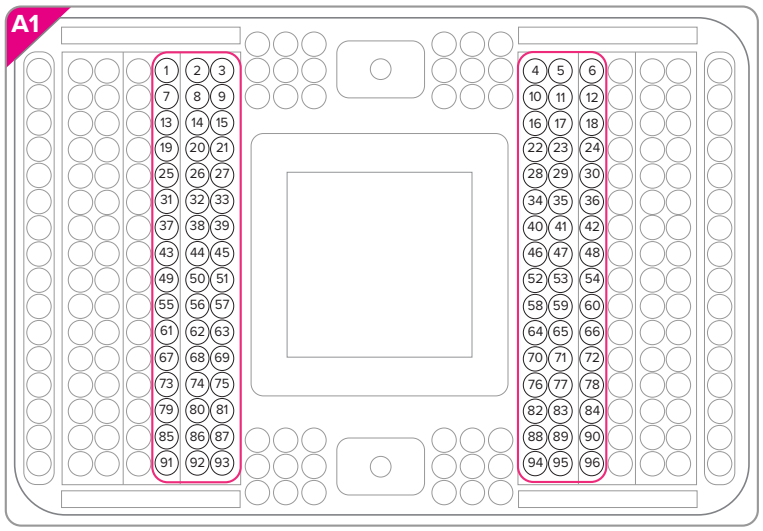


Figure 7. Pipetting map of reaction products on the C1 IFC

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

Table 11. Harvest amplicon dilution

Components	Volume (µL)
C1 DNA Dilution Reagent (Fluidigm) (30 mL bottle)	25
C1 harvest amplicons	~3
Total	**~28**

NOTE These preamplified samples are now ready for analysis on Biomark or Biomark HD, following Appendix B of the Fluidigm Real-Time PCR Analysis User Guide (PN 68000088) .

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 inlets of the C1 IFC into the 25 µL of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

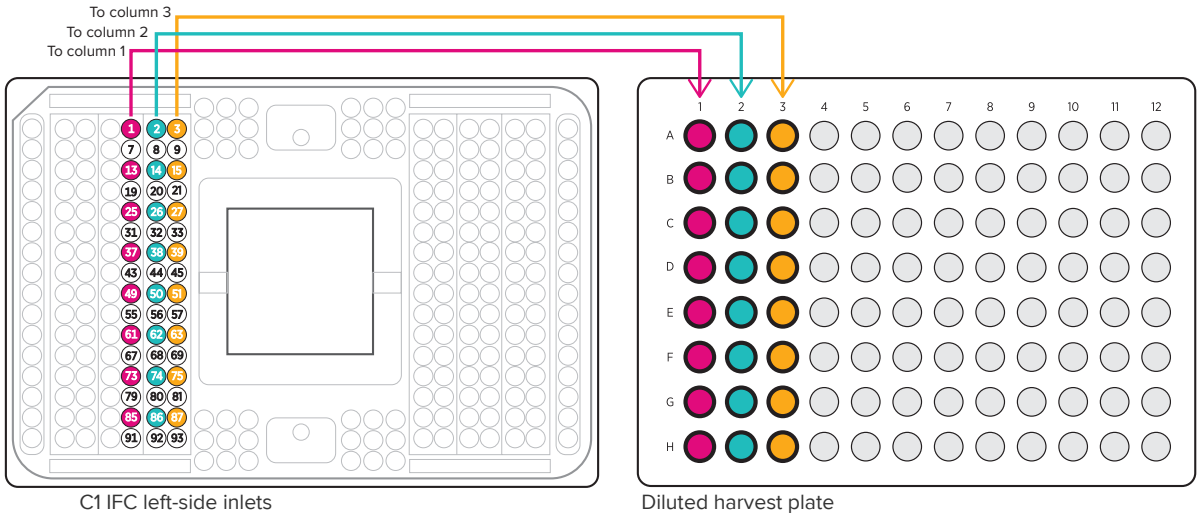


Figure 8. First, second, and third pipetting steps

8 Pipet the entire volume of C1 harvest amplicons out of the right-side inlets of the C1 IFC into the 25 µL of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

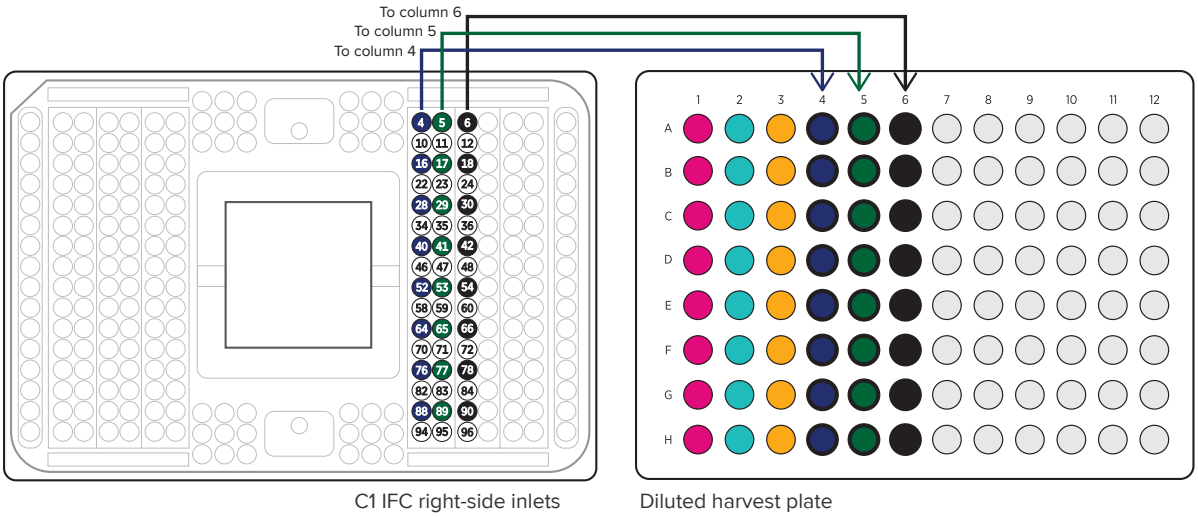


Figure 9. Fourth, fifth, and sixth pipetting steps

9 Pipet the entire volume of C1 harvest amplicons out of the left-side inlets of the C1 IFC into the 25 µL of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

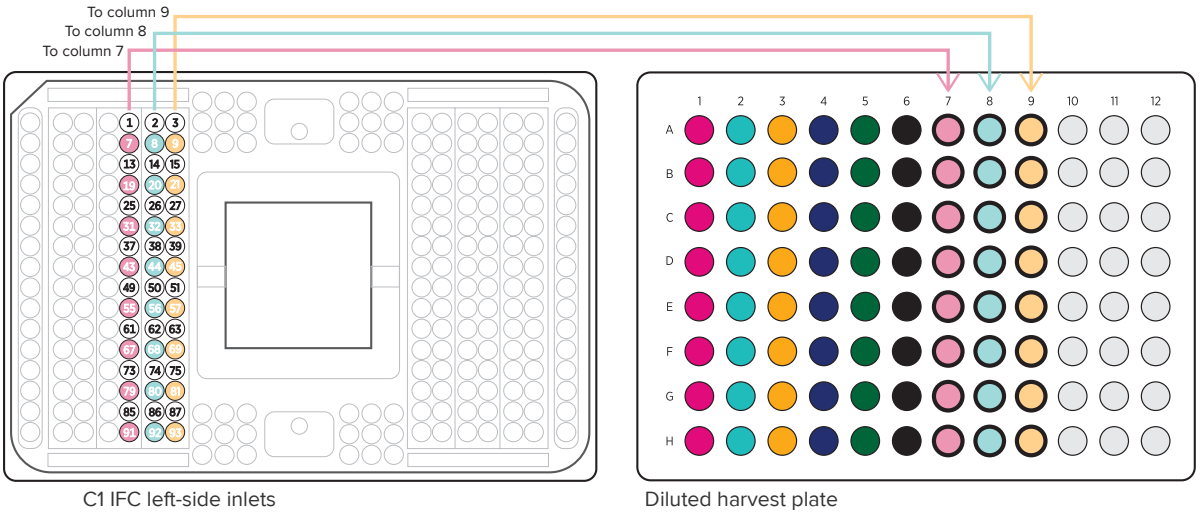


Figure 10. Seventh, eighth, and ninth pipetting steps

10 Pipet the entire volume of C1 harvest amplicons out of the right-side inlets of the C1 IFC into the 25 µL of C1 DNA Dilution Reagent in each well of the diluted harvest plate:

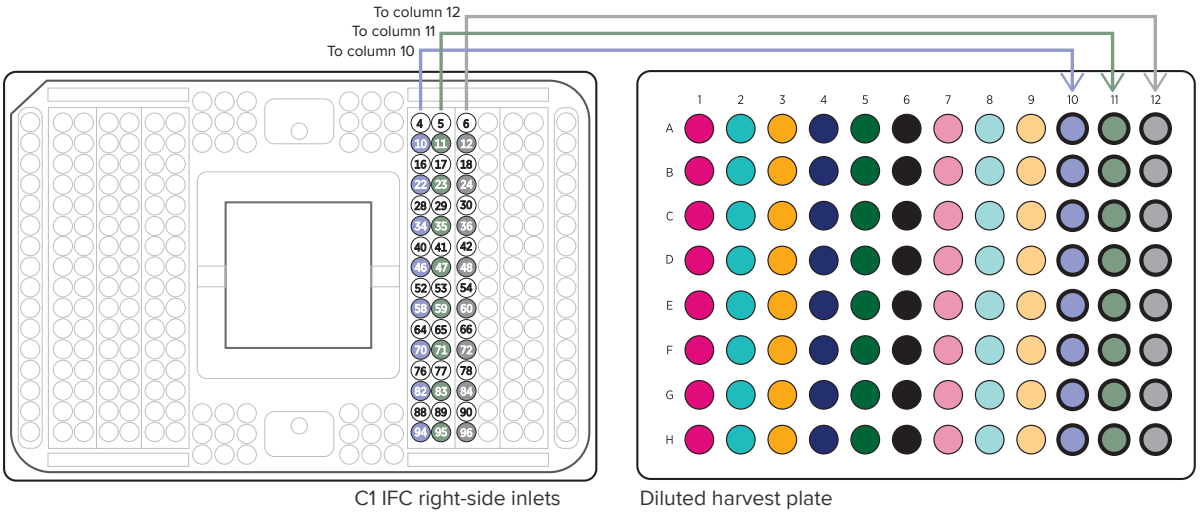
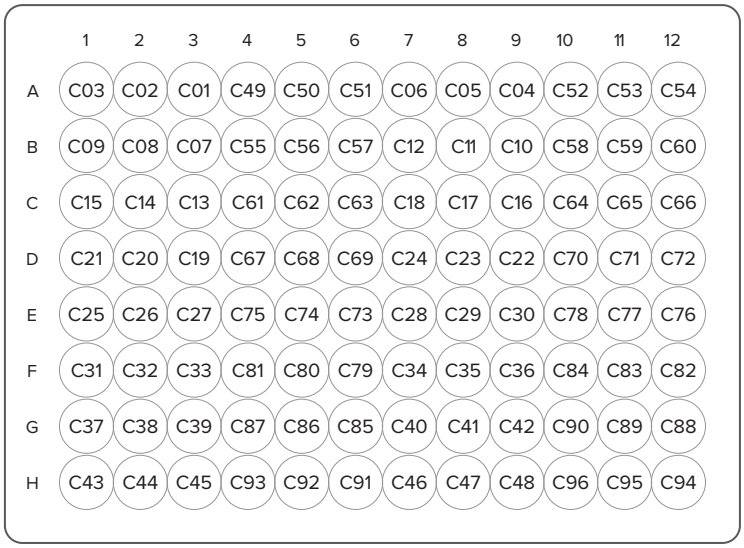


Figure 11. Tenth, eleventh, and twelfth pipetting steps

11 Seal, vortex the harvest plate for 10 seconds, and then centrifuge it to collect harvest products.

After harvesting, material from the capture sites is arranged on the harvest plate as follows:



12 Run the diluted amplicons on Biomark or the Biomark HD. (See Run Reaction Products on a 96.96 Dynamic Array IFC.)

Run Reaction Products on a 96.96 Dynamic Array IFC

IMPORTANT Always handle the reaction products from the C1 IFC in the post-PCR lab.

Refer to Appendix B of the Fluidigm Real-Time PCR Analysis User Guide (PN 68000088) . Follow “Preparing the Sample Pre-Mix and Samples” onwards for reagent preparation of the 48.84 Dynamic Array IFC and the 96.96 Dynamic Array IFC. Do *not* follow the procedures for preamplification and exonuclease treatment.

Appendix A: Run the Tube Controls

Wash Cells

1. Pellet remaining cells (1 mL volume is easiest). Speeds and durations may vary. We suggest centrifuging 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 of Cell Wash Buffer by pipetting up and down at least 5 times.
4. Pellet cells again and remove supernatant.
5. Wash a second time by resuspending in 1 mL by pipetting up and down 5 times.
6. Pellet cells a third time and remove supernatant.
7. Resuspend cells in Cell Wash Buffer to approximately 90% original volume, to keep original concentration, assuming a 10% loss.
8. Prepare two tube controls in new tubes:

Table 1. Tube controls without RT final mix

Components	Tube 1: Positive control (µL)	Tube 2: NTC (no template control; µL)
Washed cells	1.0	—
Cell Wash Buffer	—	1.0
Lysis final mix	2.0	2.0
Total	**3.0**	**3.0**

9. Incubate the tube controls at room temperature for 5 minutes.

10 Add to each tube control:

Table 2. Tube controls with all reagents

Components	Tube 1: Positive control (µL)	Tube 2: NTC (µL)
Incubated tube control (from previous step)	3.0	3.0
RT final mix	2.0	2.0
Total	**5.0**	**5.0**

11 Vortex the tube controls for 3 seconds and centrifuge to collect contents.

12 In a PCR thermal cycler, run the following protocol:

Table 3. Thermal cycling protocol

Reverse Transcription	
Temperature	**Time**
25 °C	10 min
42 °C	60 min
85 °C	5 min

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

Table 4. Preamplification reaction

Components	Volume (µL)
Preamp final mix	3.33
RT reaction (See previous step.)	0.35

14 In a PCR thermal cycler, run the following protocol:

Table 5. Preamplification thermal cycling protocol

Preamplification			
Stage	**Temperature**	**Time**	**Cycles**
Enzyme activation/ RT inactivation	95 °C	10 min	1
Denature	95 °C	15 sec	18
Anneal/extend	60 °C	4 min	
Hold	4 °C	Hold	Hold

Dilute Products and Heat Denature the Enzyme

1 Transfer prepared material to post-PCR lab.

2 Vortex the prepared products for 3 seconds and centrifuge to collect contents.

3 Combine the following reagents according to the table below.

Table 6. Dilution of preamplification products

Components	Volume (µL)
C1 DNA Dilution Reagent (Fluidigm)	99
Preamplification product	1
Total	**100**

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

Table 7. Thermal cycling parameters

Denature the Enzyme		
Temperature	**Time**	**Cycles**
95 °C	10 min	1

5 Once denature is completed, vortex for 3 seconds and centrifuge to collect contents.

6 Store the diluted preamplification products at −20 °C until use.

Run Reaction Products on a 96.96 Dynamic Array IFC

See Run Reaction Products on a 96.96 Dynamic Array IFC on page 33.

Appendix B: RNA Spike Assays

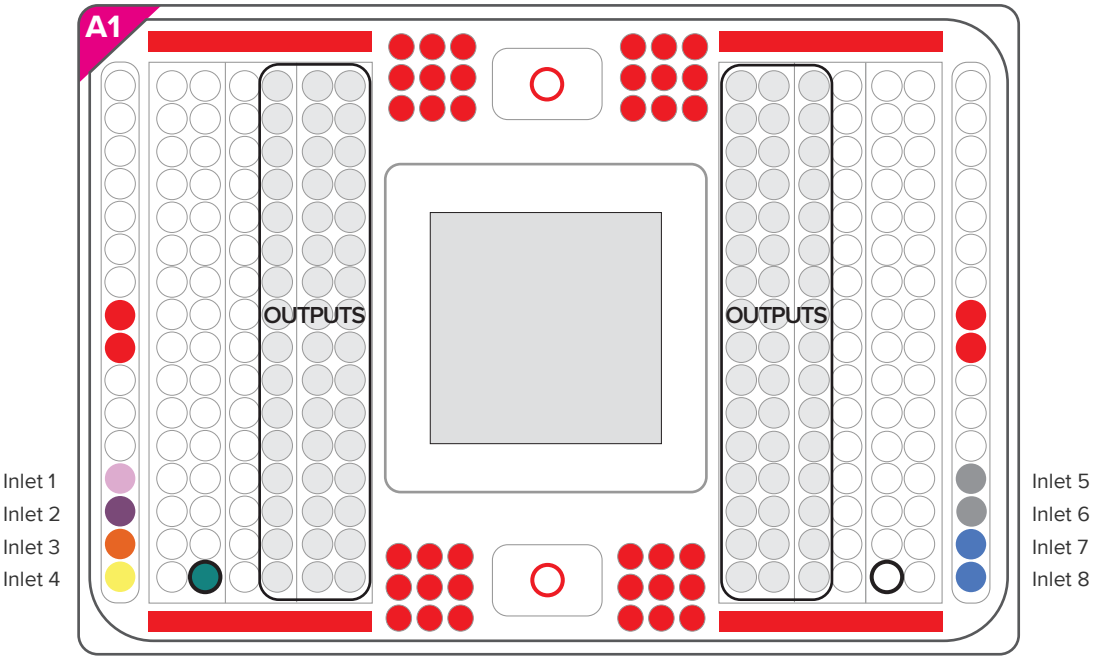
If ordering Delta Gene Assays, order with the following part numbers:

Table 1. Fluidigm part numbers

Reverse Assay Primer	Part Number
RNA Spikes Assays Kit	100-5582

Appendix C: IFC Pipetting Map

Overview of IFC pipetting



Prime the IFC:

- C1 Harvest Reagent, 200 µL
- C1 Harvest Reagent, 20 µL
- C1 Preloading Reagent, 20 µL
- C1 Blocking Reagent, 15 µL
- Cell Wash Buffer, 20 µL

Load the IFC:

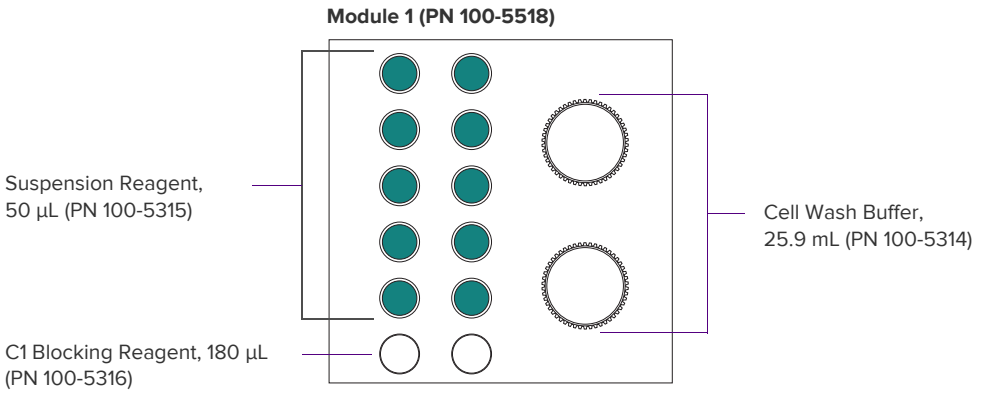
- Remove C1 Blocking Reagent
- LIVE/DEAD Staining Solution, 20 µL
- Cell mix, 6 µL

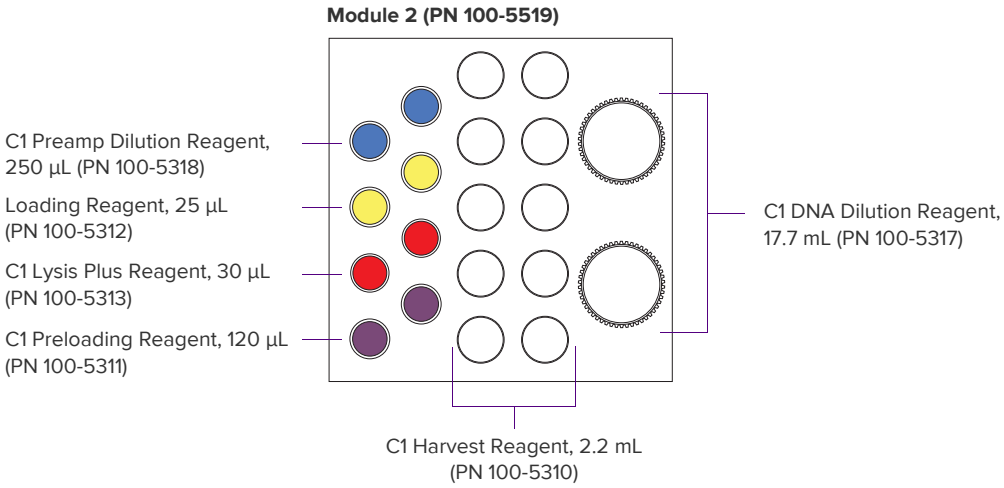
Lyse, RT, and preamplify:

- C1 Harvest Reagent, 180 µL
- Lysis final mix, 7µL
- RT final mix, 7 µL
- Preamp final mix, 24 µL

Appendix D: C1 Single-Cell Auto Prep Reagent Kit, PN 100-5319

For storage conditions, see Reagents on page 11.





Appendix E: IFC Types and Related Scripts

Cell size/IFC Name and Part Numbers	**Barcode (prefix)**	**Scripts**	**Description**
Small (5–10 µm) C1 IFC for Preamp (5–10 µm) PN 100-5757	1784x	STA: Prime (1784x)	Priming the control line and cell capture channels of the 5–10 µm Preamp IFC (1784x)
		STA: Cell Load (1784x)	Cell loading and washing without staining for preamplification of 5–10 µm diameter cells (1784x)
		STA: Cell Load & Stain (1784x)	Cell loading, staining, and washing for preamplification of 5–10 µm diameter cells (1784x)
		STA: Preamp (1784x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA preamplification for the 5–10 µm Preamp IFC (1784x)
Medium (10–17 µm) C1 IFC for Preamp (10–17 µm) PN 100-5479	1782x	STA: Prime (1782x)	Priming the control line and cell capture channels of the 10–17 µm Preamp IFC (1782x)
		STA: Cell Load (1782x)	Cell loading and washing without staining for preamplification of 10–17 µm diameter cells (1782x)
		STA: Cell Load & Stain (1782x)	Cell loading, staining, and washing for preamplification of 10–17 µm diameter cells (1782x)
		STA: Preamp (1782x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA preamplification for the 10–17 µm Preamp IFC (1782x)
Large (17–25 µm) C1 IFC for Preamp (17–25 µm) PN 100-5758	1783x	STA: Prime (1783x)	Priming the control line and cell capture channels of the 17–15 µm Preamp IFC (1783x)
		STA: Cell Load (1783x)	Cell loading and washing without staining for preamplification of 17–25 µm diameter cells (1783x)
		STA: Cell Load & Stain (1783x)	Cell loading, staining, and washing for preamplification of 17–25 µm diameter cells (1783x)
		STA: Preamp (1783x)	Loading, thermal, and harvest protocol for single-cell lysis, reverse transcription, and cDNA preamplification for the 17–25 µm Preamp IFC (1783x)

Appendix F: Related Documentation

- LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific, PN L-3224)
- Biomark HD Data Collection Software User Guide (Fluidigm, PN 100-2451)
- C1 System User Guide (Fluidigm, PN 100-4977)
- Singular Analysis Toolset User Guide (Fluidigm, PN 100-5066)
- Mao et al., *BMC Biotechnology* 2007, 7: 76, for further information on the physicochemical properties of EvaGreen dye
- Devonshire et al. "Applicability of RNA standards for evaluating RT-qPCR assays and platforms." *BMC Genomics* 2011, 12: 118
- ArrayControl™ Spots and Spikes (Thermo Fisher Scientific, PN AM1781)
- Minimum Specifications for Single-Cell Imaging (Fluidigm, PN 100-5004)
- INCYTO C-Chip™ Disposable Hemocytometer, at incyto.com/product/product02_detail.php

Appendix G: Safety

General Safety

In addition to your site-specific safety requirements, Fluidigm recommends the following general safety guidelines in all laboratory and manufacturing areas:

- Use personal protective equipment (PPE): safety glasses, fully enclosed shoes, lab coats, 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.
- Do not eat, drink, or smoke in lab areas.
- Maintain clean work areas.
- Wash hands before leaving the lab.

Instrument Safety

WARNING Do not modify this device. Unauthorized modifications may create a safety hazard.

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

CAUTION 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 a IFC.

WARNING BIOHAZARD. If you are putting biohazardous material on the instrument, use appropriate personal protective equipment and adhere to *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) from the Centers for Disease Control and Prevention and to your lab's safety protocol to limit biohazard risks. If biohazardous materials are used, properly label the equipment as a biohazard. For more information, see the BMBL guidelines at: cdc.gov/biosafety/publications/index.htm.

For a full list of the symbols on the instrument, refer to the C1 System User Guide (PN 100-4977).

Chemical Safety

Read and comprehend all safety data sheets (SDSs) by chemical manufacturers before you use, store, or handle any chemicals or hazardous materials.

Wear personal protective equipment (gloves, safety glasses, fully enclosed shoes, lab coats) when handling chemicals.

Do not inhale fumes from chemicals. Use adequate ventilation, and return caps to bottles immediately after use.

Check regularly for chemical spills or leaks. Follow SDS recommendations for cleaning up spills or leaks.

Disposal of Products

Used IFCs should be handled and disposed of in accordance with federal, state, regional, and local laws for hazardous waste management and disposal.

Do not dispose of this product in unsorted municipal waste. This equipment may contain hazardous substances that could affect health and the environment. Use appropriate take-back systems when disposing of materials and equipment.

Learn more at fluidigm.com/compliance.