Prepare Small-Cell cDNA Libraries with the C1 Single-Cell mRNA Seq HT IFC, 5–10 μm

For use with: C1™ mRNA Seq HT scripts Rev B on Script Hub™

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Introduction

What’s New

Revised scripts are available for the small-cell (5–10 μm) C1™ mRNA Seq HT integrated fluidic circuit (IFC) that improve the row-to-row, cell loading, and harvest uniformity.

Before you proceed, make sure to:

• Download the Rev B scripts from Script Hub™: mRNA Seq HT: Prime Rev B (1911x), mRNA Seq HT: Cell Load Rev B (1911x), and mRNA Seq HT: RT & Amp Rev B (1911x).
• Note that there are changes to the IFC pipetting maps (see pages 8, 12, and 19) that are for use only with the Rev B scripts.
• Use this document only with the Rev B scripts.

Overview of mRNA Seq HT Chemistry

This document describes how to use the C1 mRNA Seq HT Reagent Kit v2 with the small-cell C1 mRNA Seq HT IFC and the C1 system to capture up to 800 cells, and perform universal amplification of the cDNA for 3’ end-counting mRNA sequencing (mRNA Seq)* on Illumina® MiSeq, HiSeq, or NextSeq systems.

Figure 1 on page 3 shows an overview of the mRNA Seq HT chemistry used in this document. In this 3’ end-counting approach, the cell barcode is applied across the row of the HT IFC during the reverse transcription step, while the Nextera index is used to define the column containing the cell during the 3’ end enrichment (library preparation) step.

* 3’ end-counting is a technique used to determine the number of transcripts present per gene within an individual cell.
1. Reverse transcription

![Diagram of reverse transcription](image)

- RT primer
- Polyadenylated mRNA
- Cell barcode primer
- Preamp primer

2. Preamplification

![Diagram of preamplification](image)

- Cell barcode primer
- Linker 1
- Linker 2
- Nextera index (column)
- Unenriched
- Enrichment primer
- Amplify
- Amplification adapter
- Preamplification adapter

3. Library tagmentation

![Diagram of library tagmentation](image)

4. 3’ end enrichment

![Diagram of 3’ end enrichment](image)

- Unenriched
- Linker 2
- Nextera index (column)
- Enrichment primer
- Linker 1

5. Prepared next-generation sequencing template

![Diagram of prepared sequencing template](image)

- Nextera index (column)
- cDNA sequence
- Cell barcode (row)
- Linker 2
- Read primer site 2
- Read primer site 1
- Linker 1

6. Demultiplexed paired-end sequencing reads

- R1 reads
  - 5’
  - Poly T
  - 3’
  - Cell barcode (row)
  - cDNA sequence

- R2 reads
  - 5’
  - Poly A
  - 3’

Figure 1. Overview of mRNA Seq HT chemistry
**Materials**

**IMPORTANT** Read and understand the safety data sheets (SDSs) before handling chemicals.

**Required Reagents**

**IMPORTANT** Store reagents as soon as they are received, according to manufacturer’s storage recommendations. The part numbers below provide the necessary reagents to run mRNA Seq HT chemistry on five C1 HT IFCs.

**Required Reagents from Fluidigm**

The C1 Single-Cell mRNA Seq HT Reagent Kit v2 (C1 HT Kit) is shipped in five boxes (modules). When ordering the modules from Fluidigm, use the kit part number: 101-3473.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Part Number</th>
<th>Source</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 Single-Cell mRNA Seq HT Reagent Kit v2</td>
<td>101-3473</td>
<td>Fluidigm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Module 1:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>–20 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Module 2:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>–20 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Module 3:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>–80 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Module 4:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>–20 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Module 5:</td>
</tr>
</tbody>
</table>

**Required Reagents from Other Suppliers**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Part Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeqAmp™ DNA Polymerase</td>
<td>638504</td>
<td>Takara Bio, Inc.</td>
</tr>
<tr>
<td>Nextera XT DNA Library Preparation Kit (96 Samples)</td>
<td>FC-131-1096</td>
<td>Illumina</td>
</tr>
<tr>
<td>Nextera XT Index Kit v2 (96 Indices, 384 Samples)</td>
<td>FC-131-2001</td>
<td>(Set A) and FC-131-2002 (Set B)</td>
</tr>
<tr>
<td>Qubit® dsDNA HS Assay Kit</td>
<td>Q32851</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td></td>
<td>or Q32854</td>
<td></td>
</tr>
<tr>
<td>Agencourt® AMPure XP</td>
<td>A63880</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td><strong>NOTE</strong> You will need three bottles of PN A63880 or one bottle of PN A63881 to run five C1 HT IFCs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Suspension Buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0)*</td>
<td>T0227</td>
<td>Teknova</td>
</tr>
<tr>
<td>Ethanol, 200 proof</td>
<td>–</td>
<td>Major laboratory supplier (MLS)</td>
</tr>
</tbody>
</table>

* Recommended if Teknova is not available in your location: 1X TE Buffer (Thermo Fisher Scientific, PN 12090015).
**Required Consumables**

<table>
<thead>
<tr>
<th>Product</th>
<th>Part Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 Single-Cell mRNA Seq HT IFC, 5–10 μm</td>
<td>101-0219 (1 IFC)</td>
<td>Fluidigm</td>
</tr>
<tr>
<td>High Sensitivity DNA Kit</td>
<td>5067-4626</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>Qubit Assay Tubes</td>
<td>Q32856 (500 tubes)</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>MAXYMum Recovery® Microtubes (1.5 mL)</td>
<td>MCT-150-L-C</td>
<td>Axygen Scientific</td>
</tr>
<tr>
<td>MicroAmp® Clear Adhesive Film</td>
<td>4306311</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>PCR Sealing Film</td>
<td>82018-844</td>
<td>VWR International</td>
</tr>
<tr>
<td>96-well PCR plates*</td>
<td>–</td>
<td>MLS</td>
</tr>
<tr>
<td>0.2 mL PCR 8-tube strips</td>
<td>–</td>
<td>MLS</td>
</tr>
<tr>
<td>Filtered pipette tips</td>
<td>–</td>
<td>MLS</td>
</tr>
<tr>
<td>Low-lint cloth</td>
<td>–</td>
<td>MLS</td>
</tr>
</tbody>
</table>

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

**Required Equipment**

<table>
<thead>
<tr>
<th>Product</th>
<th>Part Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 system</td>
<td>100-7000</td>
<td>Fluidigm</td>
</tr>
<tr>
<td>2100 Bioanalyzer®</td>
<td>G2940CA</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>Qubit 3.0 Fluorometer</td>
<td>Q33216</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>96-well PCR plate thermal cycler</td>
<td>–</td>
<td>MLS</td>
</tr>
<tr>
<td>Three centrifuges: one picofuge, one for microtubes, one for 96-well PCR plates</td>
<td>–</td>
<td>MLS</td>
</tr>
<tr>
<td>Vortexer</td>
<td>–</td>
<td>MLS</td>
</tr>
<tr>
<td>Select the appropriate magnet for your 96-well PCR plate:</td>
<td></td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>• DynaMag™-96 Side Magnet (recommended) – for use with semi-skirted plates (see above) and with non-skirted plates</td>
<td>• 12331D or 12027</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>• DynaMag™-96 Side Skirted Magnet – for use with full-skirted plates</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IMPORTANT** PCR plates may vary. Make sure to test your plate for compatibility before use.

<table>
<thead>
<tr>
<th>Product</th>
<th>Part Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic stand for microtubes*</td>
<td>–</td>
<td>MLS</td>
</tr>
</tbody>
</table>

* Recommended: DynaMag™-2 Magnet (Thermo Fisher Scientific PN 12321D).

**Suggested Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Part Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhiX Control v3</td>
<td>FC-110-3001</td>
<td>Illumina</td>
</tr>
<tr>
<td>1X Phosphate-buffered saline (PBS) or similar wash buffer</td>
<td>–</td>
<td>MLS</td>
</tr>
</tbody>
</table>
Before You Begin

To ensure reliable results, we recommend that you do the following:

- Make sure to install C1 system software v2.2.3 or later to run the mRNA Seq HT: Rev B (1911x) scripts available for download from Script Hub™. For more information, see the C1 System Software Release Notes (PN 101-5841) and Updating the C1 System Software Quick Reference (PN 100-6217). To download the scripts, go to fluidigm.com/c1openapp/scripthub.

- Use good laboratory practices to minimize contamination of samples. Use a new pipette tip for every new sample. Change gloves before and after handling the cell barcodes (see page 13 for barcode preparation).

- Establish a working cell dissociation protocol for each new cell type you will run on the HT IFC before proceeding (see page 10 for cell mix requirements).

- Practice microscopy imaging before loading the HT IFC for the first time (see page 16 for cell imaging recommendations). If you practice with an unused IFC, cover the IFC with PCR sealing film to prevent contamination during imaging.

- Use the pipetting maps provided with this document (see page 8 for the first map). Note that there are changes to the pipetting maps that are for use only with the Rev B scripts. Follow the instructions for each map, and make sure to remove any remaining reagents before loading the reagents in the order shown.
Generate High-Throughput Single-Cell cDNA Libraries for mRNA Sequencing

Prime the HT IFC

Dilute the Blocking Reagent

Prepare two dilutions of the 10X Blocking Reagent before priming the HT IFC: 1X and 0.01X.

1X Blocking Reagent

1. Combine the following reagents in a microtube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Rinsing Reagent (C1 HT Kit Module 1)</td>
<td></td>
</tr>
<tr>
<td>10X Blocking Reagent (C1 HT Kit Module 1)</td>
<td>15</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>150</strong></td>
</tr>
</tbody>
</table>

2. Gently vortex and centrifuge briefly to collect contents. Keep at room temperature.

0.01X Blocking Reagent

1. Combine the following reagents in a microtube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Rinsing Reagent (C1 HT Kit Module 1)</td>
<td></td>
</tr>
<tr>
<td>1X Blocking Reagent dilution (from step 2 above)</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>400</strong></td>
</tr>
</tbody>
</table>

2. Gently vortex and centrifuge briefly to collect contents. Keep at room temperature.

Before You Pipet into the HT IFC

- Ensure that the notch (A1 position) is at the top-left corner of the HT IFC and the barcode faces to the left.
- Always stop at the first stop on the pipette to avoid creating bubbles in the HT IFC inlets. If a bubble is introduced, ensure that it floats to the top of the well.
• The viscosity of some solutions may vary. Pipet high viscosity solutions slowly and carefully to avoid bubbles. Pipet low viscosity solutions quickly and carefully to avoid leaks into unintended HT IFC inlets.

• Make sure to keep the HT IFC as level as possible, and evenly distribute reagents over the bottom surfaces of the accumulators and reservoirs.

• To evenly distribute reagents and avoid creating bubbles in reservoirs, pipet outward from edge closest to the center of the HT IFC and do not allow a pipette tip to touch the inlet hole ( ).

Prime the HT IFC

Load the reagents as shown in Figure 2.

Figure 2. HT IFC priming pipetting map

Add these:
- Actuation Fluid, 180 μL (2 accumulators)
- Stability Solution, 20 μL (3 inlets)
- Valve Fluid v2, 20 μL (33 inlets)
- 1X Blocking Reagent, 20 μL (5 inlets)
- 0.01X Blocking Reagent, 150 μL (2 reservoirs)
- DNA Suspension Buffer, 150 μL (1 reservoir)
- Empty well

1 Peel off white tape on bottom of HT IFC.

2 Using a P200 pipette, carefully pipet 180 μL of Actuation Fluid into each of the accumulators (tan outlined circles ), avoiding spills. Use the pipette tip to gently press down on the black O-ring, insert the tip to one side of the accumulator, and then release the fluid.

**IMPORTANT** Actuation Fluid is a low surface tension solution. Make sure to use a P200 pipette. Make sure to keep the HT IFC as level as possible, and evenly distribute the Actuation Fluid over the bottom surface of the accumulators. Use a lint-free wipe to remove any excess fluid around the accumulator.
3 Using a P200 pipette, **slowly** and carefully pipet 20 μL of Stability Solution into each of the 3 inlets (blue circles), **avoiding spills**.

**IMPORTANT** The Stability Solution is viscous. **Do not vortex**. Make sure to use a P200 pipette.

4 Using a P20 pipette, pipet 20 μL of Valve Fluid v2 into each of the 33 control line inlets near the accumulators (solid gold circles).

5 Pipet 150 μL of DNA Suspension Buffer into the top-left reservoir (black outlined rectangle).

**IMPORTANT** Make sure to keep the HT IFC as level as possible, and evenly distribute the DNA Suspension Buffer over the bottom surface of the reservoir.

6 Make sure to dilute the Blocking Reagent (see page 7) and then:
   a Pipet 20 μL of 1X Blocking Reagent into each of the 5 inlets (solid dark violet circles).
   b Pipet 150 μL of 0.01X Blocking Reagent into each of the 2 wash reservoirs at the bottom of the HT IFC (solid light violet rectangles).

**IMPORTANT** Make sure to keep the HT IFC as level as possible, and evenly distribute the 0.01X Blocking Reagent over the bottom surface of the reservoirs.

7 Place the IFC into the C1 system, and then run the **mRNA Seq HT: Prime Rev B (1911x)** script. Priming takes approximately 55 minutes.

**NOTE** See the C1 System User Guide (PN 100-4977) for instructions for use and safety guidelines. If a priming reagent pipetting error has occurred, wait for the Prime script to finish (do not press ABORT), pipet new priming reagents, and then run the prime script again.

8 During HT IFC priming, prepare the cell mix for loading (see **Prepare and Load Cells on page 10**). If time allows, you can also start preparing the reagent mixes and diluted barcodes (see **Prepare Reagent Mixes for cDNA Synthesis on page 13**).

**NOTE** After priming the HT IFC, you have up to 1 hour to load cells. We recommend that you keep the primed IFC in the instrument until you are ready to load cells.

9 When the Prime script has finished, tap **EJECT** to remove the primed IFC from the instrument.
Prepare and Load Cells

The center panel of the HT IFC is divided into two sections, left and right, with 10 columns of 40 capture sites in each section. You can load the same or different cell mixes into each section through the corresponding cell inlets.

Prepare the Cell Mix While Priming the HT IFC

Prepare the Single-Cell Suspension

**IMPORTANT** Establish a working cell dissociation protocol for each new cell type you will run on the HT IFC before proceeding. For more information, see the Fluidigm Single-Cell Preparation Guide (PN 100-7697).

1. Prepare a cell suspension of **2,500 cells/μL** for small cells (5–10 μM) in native medium using an established protocol for your cell type.

   **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.

2. Keep the cell suspension at the appropriate storage condition for your cell type.

Prepare the Cell Mix for the HT IFC

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 for loading into the HT IFC.

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

1. Use a P200 pipette to **gently and slowly** add the cells to the Suspension Reagent in a microtube. Many cell types use the standard suspension ratio of 3:2 as shown below.

   **NOTE** The volume of cell mix may be scaled depending on volume of cells available. You will load 10 μL of cell mix into each cell inlet in the HT IFC (see Figure 4 on page 12).

2. (Optional) If performing a tube control, label the tube containing the remaining cells “PC” and keep at the appropriate storage condition for your cell type, for later use in the tube control reactions (see Wash the Cells on page 37).

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume for each cell inlet (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension Reagent (C1 HT Kit Module 1)</td>
<td>20</td>
</tr>
<tr>
<td>Cells (from step 1 above)</td>
<td>30</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>
Load Cells

Step 1. Remove Reagents

Before you continue, make sure to remove the reagents as shown in Figure 3.

- Use a P200 set to 150 μL to remove the 0.01X Blocking Reagent from the 2 wash reservoirs at the bottom of the HT IFC (solid light violet rectangles ). Some fluid can remain in the reservoir.
- Use a P20 set to 20 μL to remove the 1X Blocking Reagent from each of the 5 inlets (solid dark violet circles ).

Figure 3. HT IFC remove before loading pipetting map
Step 2. Load Reagents

Before you continue, make sure to remove the reagents as shown in Figure 3, and then proceed to load the reagents as shown in Figure 4.

Figure 4. HT IFC loading pipetting map

1. Set a P200 pipette to 90% of the total cell mix volume (see step 1 on page 10), and then slowly pipet the cell mix up and down 3–5 times to mix, depending on whether the cells tend to clump. Do not vortex. Avoid creating bubbles.

2. Pipet 10 μL of cell mix into the 2 cell inlets (solid magenta circles ●).
   **NOTE** You can load the same cell mix into both cell inlets, or a different cell mix into each cell inlet (see Figure 4).

3. Pipet 20 μL of Cell Rinsing Reagent into the 3 rinse inlets (solid purple circles ●).

4. Pipet 150 μL of Cell Rinsing Reagent into each of the 2 wash reservoirs at the bottom of the HT IFC (solid purple rectangles □).
   **IMPORTANT** Make sure to keep the HT IFC as level as possible, and evenly distribute the Cell Rinsing Reagent over the bottom surface of the reservoirs.

5. Place the HT IFC into the C1 system. Run the mRNA Seq HT: Cell Load Rev B (1911x).
   Approximate time: cell loading takes 75 minutes.

6. During cell loading, you can prepare reagent mixes (see below).

7. When the script has finished, tap **EJECT** to remove the HT IFC from the C1 system.
Prepare Reagent Mixes for cDNA Synthesis

During cell loading and imaging, prepare the following reagent mixes for lysis, RT, and preamplification.

Lysis Mix—Mix A

1. Combine the following reagents in a microtube labeled “A.”

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-Free Water (C1 HT Kit Module 5)</td>
<td>206.4</td>
</tr>
<tr>
<td>10X Lysis Buffer - v3 (do not vortex) (C1 HT Kit Module 5)</td>
<td>45.6</td>
</tr>
<tr>
<td>RNase Inhibitor (C1 HT Kit Module 5)</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>254.4</strong></td>
</tr>
</tbody>
</table>

2. Gently pipet up and down a few times to mix (do not vortex). Avoid creating bubbles. Centrifuge briefly to collect contents. Keep on ice until use.

Lysis Mix A Plus Diluted Barcodes

The Cell Barcode Plate contains concentrated cell (row) barcodes that are single-use only (see page 43 for the plate map). You must first prepare a diluted barcodes plate, then prepare a plate containing Lysis Mix A plus the diluted barcodes as shown in Figure 5.

![Figure 5. Cell barcode dilution process](image-url)

**IMPORTANT** To avoid contamination: do not remove the foil cover on the Cell Barcode Plate, do not vortex plates containing barcodes, use fresh pipette tips for each well, and change gloves before and after handling barcodes.
**Prepare Reagent Mixes for cDNA Synthesis**

**Prepare Plate 2: Diluted Barcodes**

1. Centrifuge the original Cell Barcode Plate at \( \sim 3,000 \times g \) for 3 minutes (do not vortex to avoid contamination). This is Plate 1.

2. Label a new 96-well PCR plate “Diluted Barcodes 1:40”. This is Plate 2.

3. Aliquot 200 µL of Teknova DNA Suspension Buffer into each tube of two 8-tube strips, then use a multichannel pipette to pipet 78 µL of the buffer into the first 5 columns of the Diluted Barcodes plate (40 wells total).

4. Using a multichannel pipette with fresh P200 pipette tips for each well, pierce the foil for the first 5 columns of the Cell Barcode Plate. Do not remove the foil and do not pipet any liquid with the P200 pipette to avoid contamination.

5. Using a P20 or P10 pipette, pipet 2 µL of the barcodes column by column into the same 40 wells of the Diluted Barcodes plate and pipet up and down 10 times to mix. Do not vortex and pipet carefully to avoid contamination. Keep the Diluted Barcodes plate on ice until use in step 3 below.

**Prepare Plate 3: Lysis Mix A Plus Diluted Barcodes**

1. Label a new 96-well PCR plate “Lysis Mix A Plus Diluted Barcodes”. This is Plate 3.

2. Aliquot 30 µL of Lysis Mix A into each tube of an 8-tube strip, and then carefully pipet the following, avoiding bubbles:
   - For samples – Pipet 4.6 µL of Lysis Mix A into the first 5 columns of the new Lysis Mix A Plus Diluted Barcodes plate (40 total wells).
   - (Optional) For tube controls – Pipet 3.8 µL of Lysis Mix A into wells A12, B12, and C12 of the new Lysis Mix A Plus Diluted Barcodes plate (one well for each tube control, see Figure 5 on page 13).
3 Use a P10 pipette to carefully pipet the following, gently pipetting up and down a few times to mix and using fresh pipette tips for each well (avoid bubbles and do not vortex to avoid contamination):

- For samples – Pipet 1.5 μL of the diluted barcodes from the Diluted Barcodes plate column by column into the 40 wells of the new Lysis Mix A Plus Diluted Barcodes plate.
- (Optional) For tube controls – Pipet 1.25 μL of the diluted barcodes from wells A1, B1, and C1 of the Diluted Barcodes plate into wells A12, B12, and C12 of the new Lysis Mix A Plus Diluted Barcodes plate (see Figure 5 on page 13).

4 Cover the Lysis Mix A Plus Diluted Barcodes plate with PCR sealing film. Centrifuge the plate at ~ 3,000 x g for 3 minutes (do not vortex to avoid contamination). Keep on ice until use.

Reverse Transcription (RT) Reaction Mix—Mix B

1 Combine the following reagents in a microtube labeled “B.”

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume for samples (μL)</th>
<th>Volume for samples + 2 tube controls (μL)</th>
<th>Volume for samples + 3 tube controls (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First-Strand Buffer (RNase-free) (C1 HT Kit Module 5)</td>
<td>11.4</td>
<td>15.5</td>
<td>17.6</td>
</tr>
<tr>
<td>Nuclease-Free Water (C1 HT Kit Module 5)</td>
<td>3.6</td>
<td>4.9</td>
<td>5.6</td>
</tr>
<tr>
<td>dNTP Mix (20 mM each) (C1 HT Kit Module 5)</td>
<td>2.9</td>
<td>3.9</td>
<td>4.4</td>
</tr>
<tr>
<td>RNase Inhibitor (C1 HT Kit Module 5)</td>
<td>1.4</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Dithiothreitol (DTT) (C1 HT Kit Module 5)</td>
<td>1.4</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Reverse Transcription Primer (C1 HT Kit Module 4)</td>
<td>2.9</td>
<td>3.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Loading Reagent (C1 HT Kit Module 2)</td>
<td>1.0</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Reverse Transcriptase (C1 HT Kit Module 5)</td>
<td>5.8</td>
<td>7.8</td>
<td>8.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>30.4</strong></td>
<td><strong>41.2</strong></td>
<td><strong>46.6</strong></td>
</tr>
</tbody>
</table>

2 Gently pipet up and down a few times to mix (do not vortex). Avoid creating bubbles. Centrifuge briefly to collect contents. Keep on ice until use.
Preamplification Mix—Mix C

1. Combine the following reagents in a microtube labeled “C.”

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume for samples (μL)</th>
<th>Volume for samples + 2 tube controls (μL)</th>
<th>Volume for samples + 3 tube controls (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeqAmp PCR Buffer (2X) (Takara Bio USA, Inc.)</td>
<td>135.0</td>
<td>150.0</td>
<td>157.5</td>
</tr>
<tr>
<td>Loading Reagent (C1 HT Kit Module 2)</td>
<td>4.6</td>
<td>5.1</td>
<td>5.35</td>
</tr>
<tr>
<td>Preamp Primer (C1 HT Kit Module 2)</td>
<td>1.4</td>
<td>1.5</td>
<td>1.75</td>
</tr>
<tr>
<td>SeqAmp DNA Polymerase (Takara Bio USA, Inc.)</td>
<td>5.4</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>146.3</strong></td>
<td><strong>162.6</strong></td>
<td><strong>170.9</strong></td>
</tr>
</tbody>
</table>

2. Gently pipet up and down a few times to mix *(do not vortex)*. *Avoid creating bubbles.* Centrifuge briefly to collect contents. Keep on ice until use.

Image Cells

1. We highly recommend that you image all of the cell capture sites in the center panel of the HT IFC (see Figure 6) using a microscope compatible with IFCs, and then perform quality control (scoring) on the cells using an established protocol for your cell type(s).
While the vast majority of cells will be in the capture site (as shown in Figure 7), cells may occasionally be outside the capture site but still within the capture chamber where chemistry will occur. Ensure that you are imaging the larger outlined area in Figure 7 to visualize all possible captured cells.

**NOTE** See the following for additional imaging guidelines:

- Viewing cell capture sites – Using a Microscope with an Automated Stage Quick Reference (PN 100-6130).

2 (Optional) If time allows, you can start the tube controls during imaging of loaded cells (see Appendix A on page 37 for instructions).
Run Lysis, Reverse Transcription, and Preamplification on the C1 System

Step 1. Remove Reagents

Remove the reagents as shown in Figure 8, using a fresh pipette tip for each well.

- Use a P200 set to 150 μL to remove the Cell Rinsing Reagent from the 2 wash reservoirs at the bottom of the HT IFC (solid purple rectangles). Some fluid can remain in the reservoir.
- Use a P20 set to 20 μL to remove the cell mix from the 2 cell inlets (solid magenta circles).
- Use a P20 set to 20 μL to remove the Cell Rinsing Reagent from the 3 rinse inlets (solid purple circles).

Figure 8. HT IFC remove before run pipetting map
Step 2. Load Reagents

Before you continue, make sure to remove the reagents as shown in Figure 8, and then proceed to load the reagents as shown in Figure 9. Use a fresh pipette tip for each well.

**IMPORTANT** Make sure to keep the HT IFC as level as possible, and evenly distribute the reagents over the bottom surface of the reservoirs.

![Figure 9. HT IFC lysis, RT and preamplification pipetting map](image)

1. Pipet 160 μL of C1 Harvest Reagent into the bottom left harvest reservoir (solid gray rectangle).
2. Pipet 150 μL of Preloading Reagent into the bottom right wash reservoir (solid purple rectangle).
3. Pipet 130 μL of Preamplification Mix (Mix C, see page 16) into the top right reservoir (solid lime rectangle).
4. Pipet 20 μL of Preloading Reagent into each of 5 wells (outlined purple circle).
5. Pipet 24 μL of RT Mix B (see page 15) into the RT well (solid red circle).
6. Pipet 20 μL of Valve Fluid v2 into each of 2 wells (solid gold circles).
7. Aliquot 35 μL of Preloading Reagent into each tube of an 8-tube strip, then use a multichannel pipette to pipet 10 μL of Preloading Reagent into each of the 24 inlets (solid purple circles).
8. Use a multichannel pipette with fresh pipette tips for each column to very carefully transfer, column by column, 5 μL of the Lysis Mix A containing diluted barcodes (see page 13) into each of the corresponding wells marked 1–5 in Figure 9 ( ).

Avoid creating bubbles. Make sure to transfer the barcodes in the correct order. To avoid cross-contamination, do not allow a pipette tip to touch another well.

**IMPORTANT** For proper demultiplexing to occur, it is critical to avoid cross-contamination. If a pipette tip touches another well, make sure to note the affected wells for use during data analysis.
9 Place the HT IFC into the C1 system and immediately run the mRNA Seq HT: RT & Amp Rev B (1911x) script. Approximate run time: ~10 hours (see Appendix A for the thermal cycling protocol).

**STOPPING POINT** The script may be run overnight with a user-defined pause between PCR and harvest functions. Slide the orange box (end time) to the desired time to program the protocol to harvest at a convenient time. For example, the harvest function could be programmed to next morning.

**NOTE** To abort the script, tap ABORT. Do not tap ABORT unless you need to stop the experiment, as the HT IFC will no longer be usable and you must start a new experiment with a new IFC.

10 Make sure to store any required reagents or optional tube controls as directed elsewhere in this document.

**(Optional) Start the Tube Control**

If you are running tube controls, see Appendix A on page 37 for instructions. We recommend that you start to run the tube control reaction after you start the mRNA Seq HT: RT & Amp Rev B (1911x) script.

**NOTE** If time allows, you can start the tube controls during imaging of loaded cells (see page 16 for imaging instructions).

**Harvest and Clean up off the C1 System**

**Transfer and Dilute Harvest Amplicons**

1 When the mRNA Seq HT: RT & Amp Rev B (1911x) script has finished, tap EJECT to remove the HT IFC from the instrument.

**NOTE** The HT IFC may remain in the C1 system for up to 1 hour after harvest before you remove products from their inlets.

2 Transfer the HT IFC to a post-PCR lab environment.
3 Label a new 96-well PCR plate “Harvest Cleanup Plate 1”.

4 Aliquot 100 μL of DNA Suspension Buffer into each tube of an 8-tube strip, and then use a multichannel pipette to pipet 25 μL of buffer into the first 3 columns of the plate.

5 Carefully pull back the tape covering the harvesting inlets on the left and right side of the HT IFC using the plastic removal tool:

6 Using a P20 multichannel pipette set to 8 μL, transfer the harvested amplicons from each column of HT IFC inlets and into the corresponding wells of the harvest plate (see Figure 10) containing DNA Suspension Buffer, for a total volume of ~30 μL in each well. The exact volume harvested from each well may vary. The expected volume is 3–8 μL.

7 (Optional) If performing a tube control, transfer 5 μL of the product from the tube control preamplification reactions (see Process the Tube Controls with Harvest Samples on page 40) into the recommended wells of the 96-well harvest plate (see Figure 11 on page 22), then continue with the procedure.
Harvest and Clean up off the C1 System

STOPPING POINT You can store the harvest plate overnight at 4 ºC or immediately proceed to cleanup.

Clean Up Harvest Samples

Before You Begin

Before proceeding, see the Agencourt AMPure XP PCR Purification Instructions for Use Guide (PN B37419AA) for further information and troubleshooting tips.

Special Handling for Magnetic Beads

- Make sure to test your PCR plate and magnet for 96-well PCR plates for compatibility before use (see page 5 for recommendations). Depending on your plate/magnet combination, beads may be captured in a different area of the well than is shown in this document.

- Vortex the beads immediately before use and pipet carefully to ensure proper bead:sample ratios. After vortexing, the bead suspension should appear homogeneous and uniform in color. Expel any beads left in the pipette tip by pipetting the suspension up and down 5–10 times.

- Samples may be located in the supernatant or on the beads. When on the magnet, make sure the supernatant is clear and do not disturb the beads when pipetting. Change tips between samples to avoid contamination.

- Do not over dry the beads as this may decrease elution efficiency. (If over dried, beads look cracked.)

Special Handling for Ethanol

Always prepare fresh 75% ethanol (EtOH) from absolute EtOH immediately before use in wash steps, and make sure to tightly close all EtOH containers when not in use. (EtOH can absorb water over time leading to a lower concentration.)
First Cleanup

1. Prepare ~20 mL of 75% EtOH by measuring each component separately and mixing immediately before use. **Do not** top off.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-certified water</td>
<td>5</td>
</tr>
<tr>
<td>EtOH (200 proof, anhydrous)</td>
<td>15</td>
</tr>
</tbody>
</table>

2. Warm AMPure XP beads up to room temperature and vortex for 1 minute immediately before use.

3. Aliquot 100 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to pipet 27 μL of beads to each harvest sample (for a total volume of ~57 μL in each well), mixing well by pipetting up and down 5–10 times and changing tips between each sample.

**IMPORTANT** Ensure beads are completely mixed and do not adhere to the side of the wells.

4. Incubate the bead mix at room temperature for 5 minutes.

5. Mix again by pipetting up and down 5–10 times, changing tips between each sample. Incubate again at room temperature for 5 minutes.

6. Place the plate on the appropriate magnet (see page 5) until the solution is clear (approximately 3–5 minutes).

7. Keep the plate on the magnet and use a P200 pipette set to 47 μL to slowly and carefully remove and discard the **supernatant** from each well without disturbing the beads. We recommend that you leave ~10 μL of the supernatant behind.

**IMPORTANT** The harvest samples are on the beads. Discard the supernatant only. Visually check for captured beads.

8. Keeping the plate on the magnet: use a multichannel pipette with fresh tips for each sample to slowly add 180 μL of freshly prepared 75% EtOH to each well (**do not mix**), incubate at room temperature for 30 seconds, and then use a P200 pipette to remove the EtOH without disturbing the beads.

9. Repeat step 8.

10. Keep the plate on the magnet and use a P20 pipette set to 20 μL to remove any remaining traces of EtOH without disturbing the beads. Excess carryover of EtOH may inhibit downstream reactions.

11. Remove the plate from the magnet and allow the beads to air-dry on bench for approximately 3–5 minutes. Visually check the beads frequently to **avoid over-drying**.
12 Aliquot 115 μL of Teknova DNA Suspension Buffer into each tube of an 8-tube strip, and then use a multichannel pipette to elute the samples by adding 32 μL of buffer to each well, mixing well by pipetting up and down 5–10 times until all the beads are in suspension and changing tips between each sample.

**NOTE** If the beads are over-dry and do not easily go into suspension, continue to pipet up and down until mixed.

13 Incubate at room temperature for 2 minutes.

14 Mix again by pipetting up and down 5–10 times, changing tips between each sample. Incubate again at room temperature for 2 minutes.

15 Place the plate on the magnet until the solution is clear (approximately 3–5 minutes).

16 Label a new 96-well PCR plate “Harvest Cleanup Plate 2.”

17 Slowly and carefully transfer 30 μL of the **supernatant** from each well to the new plate without disturbing the beads.

**IMPORTANT** The harvest samples are in the supernatant.

**Second Cleanup**

1 Vortex the beads for 1 minute immediately before use.

2 Aliquot 100 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to pipet 27 μL of beads to each sample in the Harvest Cleanup Plate 2 (for a total volume of ~57 μL in each well), mixing well by pipetting up and down 5–10 times and changing tips between each sample.

**IMPORTANT** Ensure beads are completely mixed and do not adhere to the side of the wells.

3 Incubate the bead mix at room temperature for 5 minutes.

4 Mix again by pipetting up and down 5–10 times, changing tips between each sample. Incubate again at room temperature for 5 minutes.

5 Place the plate on the magnet until the solution is clear (approximately 3–5 minutes).

6 Keep the plate on the magnet and use a P200 pipette set to 47 μL to slowly and carefully remove and discard the **supernatant** from each well without disturbing the beads. We recommend that you leave ~10 μL of the supernatant behind.

**IMPORTANT** The harvest samples are on the beads. Discard the supernatant only. Visually check for captured beads.
7 Keeping the plate on the magnet: use a multichannel pipette with fresh tips for each sample to slowly add 180 μL of freshly prepared 75% EtOH to each well (do not mix), incubate at room temperature for 30 seconds, and then use a P200 pipette to remove the EtOH without disturbing the beads.

8 Repeat step 7.

9 Keep the plate on the magnet and use a P20 pipette set to 20 μL to remove any remaining traces of EtOH without disturbing the beads. Excess carryover of EtOH may inhibit downstream reactions.

10 Remove the plate from the magnet and allow the beads to air-dry on bench for approximately 3–5 minutes. Visually check the beads frequently to avoid over-drying.

11 Aliquot 40 μL of DNA Suspension Buffer into each tube of an 8-tube strip, and then use a multichannel pipette to elute the samples by adding 11 μL of buffer to the beads on the side of each well, mixing well by pipetting up and down 5–10 times until all the beads are in suspension and changing tips between each sample.

**IMPORTANT** Make sure the beads are completely submerged and in suspension. For best results, align the pipette tip to the top of the bead pellet, and then slowly release the 11 μL of buffer until all of the beads are fully submerged and they drop to the bottom of the well. You can place the plate on a light color surface to easily visualize the beads. If the beads are over-dry and do not easily go into suspension, continue to pipet up and down until mixed.

12 Incubate at room temperature for 2 minutes.

13 Mix again by pipetting up and down 5–10 times, changing tips between each sample. Incubate again at room temperature for 2 minutes.

14 Place the plate on the magnet until the solution is clear (approximately 3–5 minutes).

15 Label a new 96-well PCR plate “Harvest Cleanup Plate FINAL.”

16 Use a single-channel pipette to slowly and carefully transfer 9 μL of the supernatant from each well to the new plate without disturbing the beads.

**IMPORTANT** The harvest samples are in the supernatant.

**STOPPING POINT** The samples are now ready for library preparation for sequencing (see Prepare Next Generation Sequencing Libraries on page 26). You can store the harvest amplicons overnight at 4 °C, and then proceed to Quantify and Dilute Harvest Amplicons on page 26.
Preparing Small-Cell cDNA Libraries with the C1 Single-Cell mRNA Seq HT IFC, 5–10 μm Technical Note

Introduction

Perform the modified Illumina Nextera XT DNA library preparation procedure for single-cell mRNA sequencing on MiSeq, HiSeq®, or NextSeq systems using cDNA acquired from the HT IFC.

**IMPORTANT** The Illumina Nextera XT DNA Library Preparation Guide provides detailed instructions for next generation sequencing (NGS) 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 Library Preparation Guide to familiarize yourself with the basic concepts and handling instructions before proceeding with this modified procedure.

**NOTE** From here, each diluted column harvest from the HT IFC is referred to as a “sample.”

Quantify and Dilute Harvest Amplicons

cDNA concentrations yielded from the C1 system may vary with cell types and cell treatments. Library yield and size distribution also vary with input cDNA/DNA concentrations. To minimize library prep variation and to achieve high library quality, carefully determine the harvest concentration and dilution.

Determine the concentration of all column pool samples harvested from the HT IFC (and optional tube controls, if desired) on the Qubit 3.0 Fluorometer.

**NOTE** Optionally determine the cDNA quality and size range for a subset of samples on the Agilent® Bioanalyzer. To maximize workflow efficiency, you can run the samples on the Qubit Fluorometer while the Agilent Bioanalyzer is running.

Before You Begin

For best results, briefly centrifuge the “Harvest Cleanup Plate FINAL” (see page 25) and then place the plate on the appropriate magnet to ensure the solution is clear of any beads before pipetting samples from the plate.

Determine the Concentration of All Samples

1. With the “Harvest Cleanup Plate FINAL” on the magnet, pipet a minimum sample volume of 1 μL into the Qubit dsDNA HS Assay for use on the Qubit Fluorometer. [See the Qubit 3.0 Fluorometer User Guide (Thermo Fisher Scientific PN MAN0010866) for instructions.]

2. Determine the effective cDNA concentration of each sample and document the results in the C1 HT IFC Workbook (Fluidigm PN 101-5976).
Dilute the Samples

1. Label a new plate “Diluted Libraries (0.2 ng/µL)".

2. With the “Harvest Cleanup Plate FINAL” on the magnet, pipet at least 2 µL of each harvested sample in the appropriate volume of DNA Suspension Buffer to achieve a final concentration of 0.2 ng/µL (based on the effective concentration in the C1 HT IFC Workbook), and pipet up and down 5–10 times to mix (do not vortex).

STOPPING POINT The samples are now ready for tagmentation (see Perform Tagmentation on page 27). Keep the Diluted Libraries plate on ice until ready to use. Diluted libraries can also be stored overnight at 4 °C or long term at –20 °C.

Perform Tagmentation

NOTE If you are running the optional tube controls (see Appendix A on page 37), prepare enough extra volume in the following procedures (plus 25% overage) for the number of tube controls you are running.

Prepare cDNA for Tagmentation

IMPORTANT Warm Tagment DNA Buffer and Neutralize Tagment (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.

1. After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by centrifuging the tubes briefly to collect the contents.

   NOTE If you stored the Diluted Libraries plate at –20 °C, thaw the plate on ice, and then vortex at medium speed for 20 seconds. Centrifuge at ~ 3,000 x g for 3 minutes to remove bubbles.

2. Label a new 96-well PCR plate “Library Prep” and place it on ice.

3. Set up the following thermal cycling protocol, and ensure that the thermal cycler lid is heated during the incubation:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 ºC</td>
<td>Preheat and pause</td>
</tr>
<tr>
<td>55 ºC</td>
<td>10 min</td>
</tr>
<tr>
<td>10 ºC</td>
<td>Hold</td>
</tr>
</tbody>
</table>
Perform Tagmentation

4 In a 0.5 mL microtube on ice, combine the components of the tagmentation pre-mix. You will need enough pre-mix for each sample you wish to sequence, plus 25% overage.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per Sample (μL)</th>
<th>Volume for 20 Samples (plus 25% overage; μL)</th>
<th>Volume for 20 Samples and 2 Tube Controls (plus 25% overage; μL)</th>
<th>Volume for 20 Samples and 3 Tube Controls (plus 25% overage; μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE-MIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tagment DNA Buffer</td>
<td>2.5</td>
<td>62.5</td>
<td>68.75</td>
<td>71.9</td>
</tr>
<tr>
<td>Amplicon Tagment Mix</td>
<td>1.25</td>
<td>31.25</td>
<td>34.4</td>
<td>35.9</td>
</tr>
<tr>
<td>Diluted Sample (0.2 ng/μL)</td>
<td>1.25</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>5.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

5 Vortex at low speed for 20 seconds and centrifuge the tube to collect contents.

6 Pipet 3.75 μL of the pre-mix into each of the assigned wells of the Library Prep plate (for the 20 samples and optional tube controls). If using a multichannel pipette, aliquot 14 μL of pre-mix into each tube of an 8-tube strip before transferring to wells.

**NOTE** We recommend that you maintain the same plate layout for tracking purposes (see Figure 11 on page 22 and Figure 12 on page 30).

7 Pipet 1.25 μL of the diluted sample at 0.2 ng/μL from the diluted sample plate (see page 27) to the Library Prep plate.

**NOTE** After you transfer the samples, you can store the diluted sample plate at −20 °C long term, if desired.

8 Seal the Library Prep plate and vortex at medium speed for 20 seconds. Centrifuge at ~ 3,000 x g for 3 minutes to remove bubbles.

9 Place the Library Prep plate in the preheated thermal cycler and skip the pause to start the protocol (see step 3 above).

10 While the protocol is running, aliquot equal volumes of room temperature NT Buffer into each tube of an 8-tube strip.

**NOTE** You will need 1.25 μL of NT Buffer for each sample you wish to sequence (see step 11), plus 20% overage. For 20 samples (entire HT IFC), use 4.5 μL aliquots.

11 Once the sample reaches 10 °C, immediately pipet 1.25 μL of the NT Buffer to each of the tagmented samples, and then pipette up and down 10 times to mix and quickly neutralize the samples.

12 Seal plate and vortex at medium speed for 20 seconds. Centrifuge briefly at ~ 3,000 x g to collect contents.
Amplify the Tagmented cDNA

Before You Begin

- For Index Primer selection criteria and handling instructions, carefully read the Nextera XT DNA Library Preparation Guide before proceeding to PCR amplification of the tagmented cDNA. **Do not** touch cap threads and discard and replace caps with each use.

- We recommend that you maintain the same plate layout throughout for tracking purposes (see also Figure 11 on page 22).

Amplify the Tagmented DNA

1. In a microtube, combine the components of the PCR pre-mix. You will need enough pre-mix for each sample you wish to sequence, plus 25% overage.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per Sample (µL)</th>
<th>Volume for 20 Samples (plus 25% overage; µL)</th>
<th>Volume for 20 Samples and 2 Tube Controls (plus 25% overage; µL)</th>
<th>Volume for 20 Samples and 3 Tube Controls (plus 25% overage; µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nextera PCR Master Mix (NPM)</td>
<td>3.75</td>
<td>93.75</td>
<td>103.1</td>
<td>107.8</td>
</tr>
<tr>
<td>Enrichment Primer (EP)</td>
<td>1.25</td>
<td>31.25</td>
<td>34.4</td>
<td>35.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.0</strong></td>
<td><strong>125.0</strong></td>
<td><strong>137.5</strong></td>
<td><strong>143.7</strong></td>
</tr>
</tbody>
</table>

2. Vortex at low speed for 20 seconds and centrifuge the tube to collect contents.

3. To avoid bubbles, use a single channel P20 pipette to pipet 5 µL of PCR pre-mix into each sample well of the Library Prep plate.

4. To avoid contamination, use a multichannel pipette to carefully pipet 1.25 µL of the appropriate Index Primer from Set A or Set B to each of the 20 sample libraries on the Library Prep plate (see Figure 12 on page 30). We recommend using the following indices (only 20 of the 24 total indices with orange caps are required):
   - **Set A:** N701–N707, N710–N712, and N714–N715
   - **Set B:** N716, and N718–N724

Each sample well should now contain a total volume of 12.5 µL.
Perform Tagmentation

5. (Optional) If performing a tube control (see Appendix A on page 37), select and document 2–3 of the unused indices from Set B (we recommend N726–N728) and pipet 1.25 μL of the appropriate Index Primer to each of the recommended wells (see Figure 12). Make sure to track the positions and indices carefully.

6. Seal the plate with adhesive film and vortex at medium speed for 20 seconds. Centrifuge at ~3,000 x g for 3 minutes.

7. Place the plate into a thermal cycler and perform PCR amplification, making sure the thermal cycler lid is heated during the incubation:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 °C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>95 °C</td>
<td>30 sec</td>
<td>1</td>
</tr>
<tr>
<td>95 °C</td>
<td>10 sec</td>
<td>12</td>
</tr>
<tr>
<td>55 °C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>60 sec</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>10 °C</td>
<td>Hold</td>
<td>—</td>
</tr>
</tbody>
</table>

**STOPPING POINT** The PCR amplification protocol takes ~45 minutes to complete and may be run overnight. Amplified products can be stored overnight at 4 °C or long-term at ~20 °C.
Clean Up and Pool the Library

Equivolume Pooling

Pool an equal volume of each sample into a single microtube, clean up, and then quantify.

Before You Begin

- Always prepare fresh 75% ethanol (EtOH) from absolute EtOH immediately before use in wash steps, and make sure to tightly close all EtOH containers when not in use.
- If you are running the optional tube controls (see Appendix A on page 37), prepare enough extra volume in the following procedures (plus 25% overage) for the number of tube controls you are running.

Pool by Volume and First Cleanup

1. Prepare ~20 mL of 75% EtOH by measuring each component separately and mixing immediately before use. Do not top off:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-certified water</td>
<td>5</td>
</tr>
<tr>
<td>EtOH (200 proof, anhydrous)</td>
<td>15</td>
</tr>
</tbody>
</table>

2. Keep the amplified sample plate (see page 30) at room temperature.

3. Determine number of samples to be pooled based on desired sequencing depth and sequencer throughput. Keep in mind that each column pool sample from the HT IFC will include up to 40 cells.

4. Warm AMPure XP beads up to room temperature and vortex for 1 minute immediately before use.

5. In a 1.5 mL low bind microtube, make the library pool as shown below:
   a. Pipet the appropriate volume from each sample (column 2) according to the number of samples to be pooled (column 1).
   b. Vortex at low speed for 20 seconds and centrifuge the tube to collect contents.
   c. Add the required amount of AMPure XP beads (column 4) to the pooled library, and mix well by pipetting up and down 5–10 times until all the beads are in suspension.

   IMPORTANT Ensure beads are completely mixed and do not adhere to the side of tube.

<table>
<thead>
<tr>
<th>1. Number of Samples to be Pooled</th>
<th>2. Volume per Sample (µL)</th>
<th>3. Total Library Pool Volume (µL)</th>
<th>4. AMPure Bead Volume for Cleanup (~75% of total library pool volume; µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>4</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>48</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>20 (entire HT IFC)</td>
<td>2</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>
6 Incubate the bead mix at room temperature for 5 minutes.

7 Place the tube on a magnetic stand until the solution is clear (approximately 3–5 minutes).

8 Keep the tube on the magnet and carefully remove and discard all but "10 μL of the supernatant without disturbing the beads.

   IMPORTANT The samples are on the beads. Discard the supernatant only. Visually check for captured beads.

9 Keep the tube on the magnet and slowly add 180 μL of freshly prepared 75% EtOH (do not mix), incubate at room temperature for 30 seconds, and then use a P200 pipette set to 200 μL to remove the EtOH without disturbing the beads.

10 Repeat step 9.

11 Keep the tube on the magnet and use a P20 pipette set to 20 μL to remove any remaining traces of EtOH without disturbing the beads. Excess carryover of EtOH may inhibit downstream reactions.

12 Remove the tube from the magnet and allow the beads to air-dry at room temperature for approximately 3–5 minutes. Visually check the beads frequently to avoid over-drying.

13 Elute the samples by adding the required volume of Teknova DNA Suspension Buffer according to the number of samples pooled, mixing well by pipetting up and down 5–10 times until all the beads are in suspension:

<table>
<thead>
<tr>
<th>Number of Samples Pooled</th>
<th>DNA Suspension Buffer Volume for Elution (= original total library pool volume; μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>20 (entire HT IFC)</td>
<td>40</td>
</tr>
</tbody>
</table>

   NOTE If the beads are over-dry and do not easily go into suspension, continue to pipet up and down until mixed.

14 Incubate for 2 minutes at room temperature.

15 Mix again by pipetting up and down 5–10 times. Incubate again at room temperature for 2 minutes.

16 Place the tube on a magnetic stand until the solution is clear (approximately 3–5 minutes).

17 Keep the tube on the magnet, and slowly and carefully transfer the entire volume of supernatant to another microtube without disturbing the beads.

   IMPORTANT The samples are in the supernatant.
Second Cleanup

1 Vortex the beads for 1 minute immediately before use.

2 Add the required volume of AMPure XP beads to the eluted samples according to the number of samples pooled, mixing well by pipetting up and down 5–10 times until all the beads are in suspension.

**IMPORTANT** Ensure beads are completely mixed and do not adhere to the side of the tube.

<table>
<thead>
<tr>
<th>Number of Samples Pooled</th>
<th>AMPure Bead Volume for Cleanup (~75% of elution volume; μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>20 (entire HT IFC)</td>
<td>30</td>
</tr>
</tbody>
</table>

3 Incubate the bead mix at room temperature for 5 minutes.

4 Place the tube on a magnetic stand until the solution is clear (approximately 3–5 minutes).

**NOTE** If you observe beads adhering to the sides of the tube, centrifuge the tube briefly to collect the contents. Placing the tube on the magnet for longer than 5 minutes may result in binding of unwanted products.

5 Keep the tube on the magnet and carefully remove and discard all but ~10 μL of the supernatant without disturbing the beads.

**IMPORTANT** The samples are on the beads. Discard the supernatant only. Visually check for captured beads.

6 Keep the tube on the magnet and slowly add 180 μL of freshly prepared 75% EtOH (do not mix), incubate at room temperature for 30 seconds on the magnetic stand, and then use a P200 pipette set to 200 μL to remove the EtOH without disturbing the beads.

7 Repeat step 6.

8 Keep the tube on the magnet and use a P20 pipette set to 20 μL to remove any remaining traces of EtOH without disturbing the beads. Excess carryover of EtOH may inhibit downstream reactions.

9 Remove the tube from the magnet and allow the beads to air-dry at room temperature for approximately 3–5 minutes. Visually check the beads frequently to avoid over-drying.
10 Elute the samples by adding the required volume of DNA Suspension Buffer according to the number of samples pooled, mixing well by pipetting up and down 5–10 times until all the beads are in suspension:

<table>
<thead>
<tr>
<th>Number of Samples Pooled</th>
<th>Volume of DNA Suspension Buffer for Elution (~90% of original pool volume; μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>28.8</td>
</tr>
<tr>
<td>12</td>
<td>43.2</td>
</tr>
<tr>
<td>16</td>
<td>28.8</td>
</tr>
<tr>
<td>20 (entire HT IFC)</td>
<td>36</td>
</tr>
</tbody>
</table>

**NOTE** If the beads are over-dry and do not easily go into suspension, continue to pipet up and down until mixed.

11 Incubate at room temperature for 2 minutes.

12 Mix again by pipetting up and down 5–10 times. Incubate again at room temperature for 2 minutes.

13 Place the tube on the magnetic stand until the solution is clear (approximately 3–5 minutes).

14 Set a P200 pipette to ~90% of the elution volume and carefully transfer the supernatant to another microtube without disturbing the beads. (For example, for 20 samples you will transfer 33 μL of supernatant.)

**IMPORTANT** The sample is in the supernatant.

15 Run 1 μL of the pooled sample in triplicate on an Agilent HS DNA Chip to check for library size distribution and quantity. Place the pooled sample on the magnet to ensure the solution is clear of any beads before pipetting into the Agilent HS DNA Chip. (See the Agilent 2100 Bioanalyzer 2100 Expert User’s Guide for this step.)

**IMPORTANT** We highly recommend that you include the PhiX Control library in your sequencing run (see page 35). For best results, we recommend that you verify the PhiX library concentration (either diluted or undiluted) on an Agilent HS DNA Chip in parallel with your library samples, to verify that the PhiX concentration is correct before you combine this control with your samples in the recommended ratio for your sequencer.

**STOPPING POINT** Keep the pooled and cleaned library at 4 °C until ready to use, or store at −20 °C long term.
Requirements and Recommendations for Illumina Sequencing

Library Concentration for Sequencing

- Base diversity can facilitate cluster calling. We highly recommend that you add the following PhiX spike-in concentrations to your samples, depending on the Illumina sequencer and version of Real-Time Analysis (RTA) software you are using.

<table>
<thead>
<tr>
<th>Illumina Sequencer</th>
<th>PhiX Concentration (% of total sample concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiSeq</td>
<td>1–5%</td>
</tr>
<tr>
<td></td>
<td>Although not required for base diversity on MiSeq, a small percentage of PhiX is recommended for estimating sequencing metrics and for troubleshooting sequencing runs.</td>
</tr>
<tr>
<td>HiSeq 2500</td>
<td>5%</td>
</tr>
<tr>
<td>HiSeq 3000/4000</td>
<td>10–20%</td>
</tr>
<tr>
<td>NextSeq® 500/550</td>
<td>20–40%</td>
</tr>
</tbody>
</table>

- See the denature and dilute libraries guide for your Illumina sequencer to determine the appropriate loading concentration for sequencing and dilute the pooled sample library and the PhiX library as needed.

**NOTE** The PhiX Control is provided at a 10 nM concentration. It may be necessary to dilute this library to ~2 nM or lower prior to combining with your pooled library. Make sure to verify the concentration of your sample library and the PhiX control library on the Agilent Bioanalyzer prior to combining (see page 34).

- We recommend that you first combine the PhiX library with the sample library in the recommended concentration as shown above, and then denature as instructed in the denature and dilute libraries guide for your Illumina sequencer.

Sample Naming and Sequencing Read Length

Sample Naming Recommendations

For best results with the C1 mRNA Sequencing High Throughput Demultiplexer Script (v2.0.1 or later, see Appendix B on page 41), we recommend that you follow the sequencing sample naming recommendations below, using the C1 HT IFC Workbook (PN 101-5976) as a guide.

- Begin each Sample ID in the Illumina sample sheet with “<samplename>-COL<N>”, where:
  - `<samplename>` = your sample name (alphanumeric, no spaces)
  - `-` (hyphen) = allowed separator
  - `COL<N>` = column number from the HT IFC (0-padded to 2 digits)

All other characters are invalid. For example, if you are interested in sequencing a sample harvested from Column 1 in the HT IFC, an entry of “HT1911123456-ControlCells-COL01” in the Sample ID field of the sample sheet is acceptable in order to generate a FASTQ file starting with the recommended information.
• If you use the Illumina Experiment Manager (IEM) software to set up your Illumina sample sheet, make sure to set the Index Reads field to 1. The number of Index Cycles is automatically set to 8 based on this selection. (See the Illumina website for more information.)

**Sequencing Read Length Recommendations**

For accurate paired-end sequencing results, we recommend that you run the following number of cycles for each of the Illumina sequencer reads (see Figure 1 on page 3):

• **Read 1 (R1)** – Use a minimum of 26 cycles, to identify the sample based on the row barcode sequence, and for cluster identification. Increasing the number of cycles for Read 1 will not provide additional sequencing information.

• **Read 2 (R2)** – Use a minimum of 75 cycles, to generate sufficient read length for accurate sequence alignment.
Appendices

Appendix A: (Optional) Run the Tube Controls

Introduction

The tube controls are used as positive and negative controls for the C1 Single-Cell mRNA Seq HT v2 reagents and workflow performed off the HT IFC. The results from the multicellular positive tube control can also be compared with the single-cell samples harvested from the IFC. You prepare two types of tube controls: one or more with cells (positive control or PC), and one without cells (no template control or NTC). You perform the tube control reactions (lysis, RT, and preamplification) off the IFC using the same chemistry you use to process the single cells on the IFC.

Wash the Cells

Before use in the tube control reactions, wash the cells you reserved in the tube labeled “PC” (see step 2 on page 10) using 1X PBS or similar user-supplied wash buffer appropriate for your cell type:

1. Pellet the reserved cells in the tube labeled “PC”. Speeds and durations may vary. We suggest centrifuging the 1.5 mL microtube containing the reserved cells at 300 x g for 5 minutes.

2. Gently remove the supernatant without disturbing the cell pellet.

   IMPORTANT The cell pellet may be difficult to see at lower volumes or cell concentrations. Make sure to pipet slowly and carefully to avoid accidental transfer of cells into the supernatant.

3. Resuspend cells in 1 mL of user-supplied wash buffer by gently pipetting up and down at least 5 times. This is wash 1.

4. Repeat steps 1–2.

5. Resuspend cells again in 1 mL of user-supplied wash buffer by gently pipetting up and down at least 5 times. This is wash 2.

6. Repeat steps 1–2.

7. Resuspend cells in user-supplied wash buffer to approximately 50% of original volume to achieve a concentration of 250–500 cells/μL.

   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.

8. Keep the washed cells on ice until use.
Appendix A: (Optional) Run the Tube Controls

### Perform the Tube Control Reactions

You can prepare two or three tube controls, either one NTC and one PC, or one NTC and two PCs (one for each section of the HT IFC).

#### Lysis

Prepare the tube control reactions by combining lysis reagents and thermal cycling them.

1. **Prepare cell lysis mix in 2 or 3 tubes of an unused 8-tube strip:**

<table>
<thead>
<tr>
<th>Components</th>
<th>NTC (no template control; μL)</th>
<th>PC (positive control; μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed cells (see page 37)</td>
<td>—</td>
<td>2.1</td>
</tr>
<tr>
<td>Cell Rinsing Reagent</td>
<td>2.1</td>
<td>—</td>
</tr>
<tr>
<td>Lysis Mix A plus diluted barcodes</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>(Plate 3 from step 4 on page 15)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   **Total**                                           | **5.7**                      | **5.7**                     |

   * Include a second positive control if you load two different cell mixes into the HT IFC (see page 12).

2. **Mix gently and centrifuge briefly to collect contents.**

3. **In a thermal cycler, run the following cell lysis protocol:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 ºC</td>
<td>3 min</td>
</tr>
<tr>
<td>4 ºC</td>
<td>10 min</td>
</tr>
<tr>
<td>25 ºC</td>
<td>1 min</td>
</tr>
<tr>
<td>4 ºC</td>
<td>Hold</td>
</tr>
</tbody>
</table>
Reverse Transcription (RT)

1. After the cell lysis protocol has finished, prepare the RT reaction by adding RT Mix B to the appropriate tubes containing cell lysis products (from step 3 above):

<table>
<thead>
<tr>
<th>Components</th>
<th>NTC (µL)</th>
<th>PC (µL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysis products (from step 3 above)</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>RT Mix (Mix B, see page 15)</td>
<td>6.4</td>
<td>6.4</td>
</tr>
</tbody>
</table>

**Total** 12.1 12.1

* Include a second positive control if you load two different cell mixes into the HT IFC (see page 12).

2. Mix gently and centrifuge briefly to collect contents.

3. In a thermal cycler, run the following RT protocol:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>42 °C</td>
<td>120 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**STOPPING POINT** If you program to harvest the next day (see page 20), you can store the RT reaction and the Preamplification Mix C (see page 16) overnight at 4 °C until you are ready to run the PCR reaction.

Preamplification (PCR)

1. After the RT protocol has finished, prepare the preamplification reaction by combining the RT reaction products (from step 3 above) and Preamplification Mix C in the appropriate tubes of an unused 8-tube strip:

<table>
<thead>
<tr>
<th>Components</th>
<th>NTC (µL)</th>
<th>PC (µL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT reaction (from step 3 above)</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Preamplification Mix (Mix C, see page 16)</td>
<td>7.25</td>
<td>7.25</td>
</tr>
</tbody>
</table>

**Total** 13.25 13.25

* Include a second positive control if you load two different cell mixes into the HT IFC (see page 12).
Appendix A: (Optional) Run the Tube Controls

2 Mix gently and centrifuge briefly to collect contents, then run the following PCR protocol:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 ºC</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>98 ºC</td>
<td>10 sec</td>
<td>4</td>
</tr>
<tr>
<td>59 ºC</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>68 ºC</td>
<td>6 min</td>
<td></td>
</tr>
<tr>
<td>98 ºC</td>
<td>10 sec</td>
<td>14</td>
</tr>
<tr>
<td>59 ºC</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>68 ºC</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>72 ºC</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>4 ºC</td>
<td>Hold</td>
<td>1</td>
</tr>
</tbody>
</table>

3 Centrifuge briefly to collect contents.

Process the Tube Controls with Harvest Samples

1 Transfer the PCR products (from step 3 above) to a post-PCR room.

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

3 Transfer 5 µL of the tube control samples to the appropriate wells of the harvest plate (see Harvest and Clean up off the C1 System on page 20), then continue with the procedure and process the tube control samples along with the harvested samples.
**Appendix B: Data Analysis**

**Analysis Workflow**

The workflow for analysis of RNA sequencing data from the C1 mRNA Seq HT IFC is as follows:

1. **RNA sequencing data from C1 mRNA Seq HT IFC**
   - (fastq of column pool)

2. **Stand-alone API script for demultiplexing row barcodes of column sample using R1 reads**
   - (fastq of individual cell)

3. **Trim and align R2 reads to reference genome**
   - (sam/bam)

4. **Calculate gene expression in reads per million (RPM)**
   - (exp table)

5. **Generate metrics for performance evaluation**
   - (QC metric tables)

   For example:
   - Debarcoded reads per cell
   - Reads mapping to reference genome
   - Reads mapping to refseq genes
   - Reads mapping to 3UTR, CDS
   - Detected genes, accs
   - Reads mapping to rRNAs
   - Reads mapping to mtDNA
   - Junk reads
   - Reads of insertion size

6. **Downstream differential gene expression analysis**
   - (Singular™ Analysis Toolset v3.5.x or later)

   or clustering visualizations

**Sequencing data:**
- HT Demultiplex script available from fluidigm.com/software

**Primary analysis recommendations:**
- Alignment by Tophat/rsem
- Gene expression calculation by Cufflink/rsem
Stand-alone Script for Demultiplexing

Initially, only the column pool samples loaded onto the sequencer are demultiplexed from the Illumina MiSeq or HiSeq sequencer reads (R1 and R2) because the sequencer only demultiplexes Nextera indices (column pools). The C1 mRNA Sequencing High Throughput Demultiplexer Script is available for download from the Fluidigm Software products page as a Perl script application programming interface (API) for use on a Linux™ operating system.

The script allows you to automatically:

- Demultiplex the individual single-cell samples from each column using the cell barcodes on the R1 reads.
- Separate the large FASTQ file generated from each column pool into 40 pairs (R1 and R2) of FASTQ files, one pair for each row in the HT IFC.
- If running script v2.0.1 or later:
  - Combine FASTQ files for the same sample if run in multiple lanes on the sequencer.
  - Generate a demultiplex report of the number of reads for each cell sample.

Download and Install the Script

1. Go to fluidigm.com/software and download the “C1_mRNA_Seq_HT_Demultiplex_Script_[version].zip” file.
2. Unzip the downloaded file, then copy the “C1_mRNA_Seq_HT_Demultiplex_Script_[version]” folder to a server location of your choice.
3. Within this folder, create two new folders to contain the individual FASTQ files:
   - input – input directory where raw FASTQ files for each column pool are placed
   - output – output directory of demultiplexed (individual cell) FASTQ files for each row (this directory is empty prior to running the script for the first time)

Run the Script

1. Make sure the raw FASTQ files from each column are in the “input” folder (see page 35 for naming recommendations, for example: “<samplename>-COL<N>”).
2. Navigate to and run the C1 mRNA Sequencing High Throughput Demultiplexer Perl script API ("mRNASeqHT_demultiplex.pl") with this command:

```perl
perl mRNASeqHT_demultiplex.pl -i input -o output
```

3. (v2.0.1 or later) The script demultiplexes the raw FASTQ files in the "input" folder into multiple FASTQ files, appended with "_ROW<N>", and saves these processed files to the "output" folder.

For example, if a raw FASTQ file is named "HT1911123456-ControlCells-COL01", the script will output HT1911123456-ControlCells-COL01_ROW<N> into 40 pairs of FASTQ files (where N is 0-padded to 2 digits).

**NOTE** Contact the IT or bioinformatics representative at your site if you require assistance to run this script with your analysis pipeline.

### Additional Analysis Recommendations

- Before aligning R2 reads to the reference genome, trim the polyA stretch from the 3′ end of the R2 reads.
- Use only R2 reads for the downstream analysis for end-counting in transcriptome expression, as described in the analysis workflow (see page 41).

For more information on downstream differential gene expression analysis, see the Singular Analysis Toolset User Guide (version 3.5.x or later).

### Appendix C: Cell Barcode Plate

Each Cell Barcode Plate contains 40 concentrated cell (row) barcodes that are single-use only. The numbers below are the HT IFC row assignments for each well in the plate.

![Cell Barcode Plate, 40 barcodes (PN 101-0164)](image)
Appendix D: Related Documentation

cDNA Synthesis

- Agencourt AMPure XP PCR Purification Instructions for Use Guide (Beckman Coulter, PN B37419AA)
- C1 mRNA Sequencing DataSheet (Fluidigm PN 101-3386)
- C1 System User Guide (Fluidigm PN 100-4977)
- Fluidigm Single-Cell Preparation Guide (Fluidigm PN 100-7697)
- INCYTO Disposable Hemocytometer, incyto.com/product/product02_detail.php
- Minimum Specifications for Imaging Cells in Fluidigm Integrated Fluidic Circuits Specification Sheet (Fluidigm PN 100-5004)
- Singular Analysis Toolset User Guide (Fluidigm PN 100-5066)
- Using a Microscope with an Automated Stage Quick Reference (Fluidigm PN 100-6130)
- C1 HT IFC Workbook (Fluidigm PN 101-5976)

DNA Sequencing

- Illumina Nextera XT DNA Library Preparation Guide (Illumina PN 15031942)