C1 HT Medium-Cell RNA Expression and Protein Sequencing (REAP-Seq)

For use with the C1 Single-Cell mRNA Seq HT IFC, 10–17 µm

Introduction

The RNA expression and protein sequencing (REAP-seq) assay uses DNA-barcoded antibodies to measure protein expression levels in conjunction with gene expression on the same single cells. This method leverages the DNA polymerase activity of reverse transcriptase to extend antibody barcodes (Ab BC) containing a poly(dT) tail, and synthesize cDNA from mRNA in the same reaction.

The C1™ mRNA Seq HT (high-throughput) v2 system from Fluidigm can be used to run REAP-seq assays in conjunction with downstream processing of Ab BC in order to characterize the unique levels of mRNA and protein expression in individual cells. REAP-seq on C1 HT uses the C1 mRNA Seq HT v2 chemistry for 3' end counting gene expression while simultaneously measuring protein expression through antibody-specific barcode detection from the same single cell.

To download a presentation containing more information on REAP-seq assay design and experimental results on C1 HT, go to fluidigm.com/c1openapp.

Overview of REAP-Seq Assays on C1 HT

Figure 1 summarizes the generation of both cDNA and libraries using REAP-seq assays on C1 HT.

1 Antibody Labeling: TotalSeq™ DNA-conjugated antibodies (BioLegend®) include a Read 2 sequencing primer, a 15 bp Ab BC, and a poly(dA) tail.

2 Lysis and Reverse Transcription: When cells are lysed, the poly(dA) tail on the Ab BC hybridizes to the poly(dT) of the reverse transcription (RT) primer with cell barcode (cell BC) used on C1 HT. The Ab BC are now labeled with their integrated fluidic circuit (IFC) row of origin. Reverse transcriptase binds to the RT primer on the strand containing the cell BC and to the poly(dA) on the Ab BC strand for extension.

3 Preamplification: After reverse transcriptase forms complementary double strands, the resulting fragments are amplified. To facilitate this process, an antibody primer that binds to the partial Read 2 sequence primer binding site is added to amplify both strands.

4 Library Preparation:

   - The P5 primer (i5xx) includes a flow cell adapter for next-generation sequencing (NGS) and the partial Read 1 sequencing primer sequences. A set of 8 P5 custom primers with unique barcode indices can be used for multiplexing cDNA and Ab BC libraries from up to 4 IFCs, in the same sequencing run.

   - The P7 primer (i7xx) includes a flow cell adapter, an IFC column index, and the partial Read 2 sequencing primer sequences in order to differentiate the column of origin for each cell.

5 Prepared NGS Template: After denaturation and pooling, the final REAP-seq library is ready for sequencing on Illumina® NextSeq™ 500/550 systems.

Figure 1. Overview of REAP-seq chemistry on C1 HT
Workflow Overview

DAY 1: On-IFC Workflow

1. Label medium-size cells (10–17 µm) with DNA-barcoded antibodies.

2. Load cells on C1 mRNA Seq HT IFC, 10–17 µm.

3. Run lysis, reverse transcription, and preamplification with C1 Single-Cell mRNA Seq HT Reagent Kit v2 on C1.

DAY 2: Off-IFC Workflow

1. Harvest REAP-seq amplicons from C1 HT IFC.

2. Separate amplified Ab BC in supernatant from cDNA on beads. > 500 bp cDNA < 200 bp Ab BC

3. Perform AMPure® bead cleanup of harvested amplicons.

4. Prepare Ab BC and cDNA libraries separately.

5. Pool and sequence Ab BC and/or cDNA libraries.

Illumina NextSeq 500/550 systems
Materials

**IMPORTANT** Store reagents as soon as they are received, according to manufacturer’s storage recommendations.

**Part 1: Materials to Label Cells with Ab BC and Run on C1**

**Required Sample**
- At least $5 \times 10^5$ to $>1 \times 10^6$ medium (10–17 µm) cells in native medium (user-supplied)

**Required Reagents from Fluidigm**
- C1 Single-Cell mRNA Seq HT Reagent Kit v2 (PN 101-3473)
  
  **NOTE** The C1 HT Kit v2 is shipped in 5 boxes (modules). See the product insert (101-3808) for kit contents.
- 4X TSP Master Mix Kit (PN 101-9621)

**Required Reagents from Other Suppliers**
- Agencourt® AMPure XP [Beckman Coulter A63880 (5 mL) or A63881 (60 mL)]
  
  **NOTE** You will need 3 bottles of PN A63880 or 1 bottle of PN A63881 to run 5 C1 HT IFCs with this protocol.
- SeqAmp™ DNA Polymerase [Takara Bio PN 638504 (50 reactions)]
- DNA Suspension Buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0), [PN Teknova T0227 (100 mL)]
  
  **NOTE** Recommended if Teknova is not available in your location: 1X TE Buffer (Thermo Fisher Scientific, PN 12090015).
- LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific PN L3224)

**Required Consumables**
- C1 Single-Cell mRNA Seq HT IFC, 10–17 µm [Fluidigm PN 101-4981 (1 IFC) or 101-4982 (5 IFCs)]

**Recommended suppliers:**
- Cell Staining Buffer (BioLegend PN 420201)
- Human TruStain FcX™ (Fc Receptor Blocking Solution) (BioLegend PN 422301)
- TotalSeq-A Antibody Oligonucleotide Conjugates (BioLegend, variable PNs)
- Custom Ab BC preamplification primer (Integrated DNA Technologies, see Appendix B)
- Ethanol, 200 proof [Major laboratory supplier (MLS)]

**Recommended Equipment**
- C1 system (Fluidigm PN 100-7000)
- Select the appropriate magnet for your 96-well PCR plate:
  - DynaMag™-96 Side Magnet (recommended) (Thermo Fisher Scientific PN 12331D). For use with semi-skirted plates and with non-skirted plates
  
  **IMPORTANT** PCR plates may vary. Make sure to test your plate for compatibility before use.
- Imaging equipment compatible with C1 mRNA Seq HT IFCs (MLS, see Appendix D)
Part 2: Materials to Prepare Ab BC Amplicons for Sequencing

Required Reagents from Other Suppliers

- Qubit® dsDNA HS Assay Kit [Thermo Fisher Scientific PN Q32851 (100 assays) or Q32854 (500 assays)]

Recommended suppliers:

- Custom P5 (i5xx) and P7 (i7xx) primers (Integrated DNA Technologies, see Appendix B)

- Exonuclease I Reaction Buffer (10X) (New England Biolabs® PN B0293S)

- Exonuclease I (E. coli) (New England Biolabs® PN M0293L)

Required Consumables

- High Sensitivity DNA Kit (Agilent® Technologies PN 5067-4626)

- Qubit Assay Tubes [Thermo Fisher Scientific PN Q32856 (500 tubes)]

Required Equipment

- 2100 Bioanalyzer® (Agilent Technologies PN G2940CA)

- Qubit 3.0 Fluorometer (Thermo Fisher Scientific PN Q33216)

Part 3: Materials to Prepare cDNA Amplicons for Sequencing

Required Reagents from Other Suppliers

- Nextera® XT DNA Library Preparation Kit (96 samples) [Illumina PN FC-131-1096 (Box 1 and Box 2)]

- Nextera XT Index Kit v2 (96 indices, 384 samples) [Illumina PN FC-131-2001 (Set A) and FC-131-2002 (Set B)]

- PhiX Control v3 (Illumina PN FC-110-3001)

Required Equipment

- Magnetic stand for microtubes (MLS)

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

General Materials

Required Consumables

- MAXYMum Recovery™ Microtubes (1.5 mL) (Axygen® Scientific PN MCT-150-L-C)

- MicroAmp® Clear Adhesive Film (Thermo Fisher Scientific PN 4306311)

- PCR Sealing Film (VWR® International PN 82018-844)

- 96-well PCR plates (MLS)

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

- 0.2 mL PCR 8-tube strips (MLS)

- Filtered pipette tips (MLS)

- Low-lint cloth (MLS)

Required Equipment

- 96-well PCR plate thermal cycler (MLS)

- 3 centrifuges: 1 picofuge, 1 for microtubes, 1 for 96-well PCR plates (MLS)

- Vortexer (MLS)

Before You Begin

General Guidelines

IMPORTANT Before using reagents, read and understand the detailed instructions and safety guidelines in this document. For complete safety information, see Appendix E.

- Vortex and centrifuge all reagents according to manufacturer’s instructions.
• Use good laboratory practices to minimize contamination of samples.
• For detailed instructions on instrument and software operation, see the C1 System User Guide (100-4977).

**Ab BC Pooling and Cell Labeling Volume Guidelines**

- Each BioLegend TotalSeq DNA-conjugated antibody (Ab BC, see Required Reagents from Other Suppliers) is provided in a stock concentration of 0.5 µg/µL.
- We recommend labeling with each Ab BC at a final concentration of 0.5 µg in 100 µL of cell labeling volume (includes Fc receptor blocking solution and cells; see Table 1 for volume guidelines and Table 2 for specific examples). For best results, perform a titration experiment to find the optimal Ab BC concentration for your cells.

Table 1. Ab BC pooling and cell labeling volume guidelines

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab BC pool (N = number of Ab BC in pool)</td>
<td>N × 1</td>
</tr>
<tr>
<td>Human TruStain FcX (X)</td>
<td>5</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>(100 – X) – (N × 1)</td>
</tr>
<tr>
<td><strong>Total Cell Labeling Volume</strong></td>
<td>100</td>
</tr>
</tbody>
</table>

2 Vortex well. Keep solution protected from light until ready to load into the HT IFC.

**C1 HT REAP-Seq Protocol**

**Part I: Label Cells with Ab BC and Run on C1**

**Prepare Cells for REAP-Seq**

1 Note the number and identity of the Ab BCs you wish to pool for use in cell labeling.

**NOTE** You will prepare enough Ab BC pool volume to run 4 REAP-Seq experiments.

2 Remove the appropriate BioLegend stock Ab BC tubes from storage and place on ice.

3 Manually flick the bottom of each stock Ab BC tube with your forefinger 5 times to mix (do not vortex).
4 Centrifuge the stock Ab BC tubes at 2,000 x g for 1 min at room temperature to collect contents.

5 Prepare the Ab BC pool:
   a Label a new 1.5 mL microtube Ab BC Pool.
   b Using a P20 pipette, pipet 4 μL of each stock Ab BC to the new tube.
   c Manually flick the bottom of the Ab BC pool tube with your forefinger 10 times to mix (do not vortex).
   d Centrifuge the Ab BC pool at 14,000 x g for 10 min at 2–8 °C to collect contents.

STOPPING POINT Store stock Ab BC and Ab BC pools at 4 °C when not in use.

Harvest, Wash, and Block the Cells

1 Harvest and wash cells:
   a Remove the BioLegend Cell Staining Buffer from storage and place on ice.
   b Label a new 15 mL conical tube.
   c Retrieve a medium cell (10–17 μm) suspension in native medium and then transfer at least 5 x 10^5 cells into the new tube.
   d Wash the cells by pipetting 10 mL of cold Cell Staining Buffer into the tube.
   e Centrifuge the tube at 350 x g at 4 °C for 5 min.
   f Use a 10 mL serological pipet to carefully remove and discard all but approximately 20 μL of the supernatant without disturbing the cell pellet.
   g Pipet 100 μL of Cell Staining Buffer into the tube, and then gently pipet up and down to break up the cell pellet.

2 Count the cells:
   a Pipet 10 μL of the cell suspension into a hemocytometer and then count the number of cells according to the manufacturer’s instructions.

NOTE Dilute cells with Cell Staining Buffer before adding to hemocytometer if the cell density is very high.

b Adjust the cell concentration to <1 x 10^6 cells/100 μL of Cell Staining Buffer.

c Pipet the appropriate volume of cell suspension into a new 15 mL conical tube according to the pooling guidelines in Table 1 and Table 2.

3 Block cells before adding Ab BC:
   a Pipet the appropriate volume of Human TruStain FcX (Fc receptor blocking solution) to the cell suspension according to the pooling guidelines in Table 1 and Table 2.
   b Very gently pipet up and down to mix, and then incubate at 4 °C or on ice for 10 min.

Label Cells with Ab BC

1 After the 10 min incubation of cells with Fc receptor blocking solution is complete, add the appropriate Ab BC pool volume to the blocked cell mixture according to the pooling guidelines in Table 1 and Table 2.

NOTE When transferring the Ab BC pool to the cell suspension, make sure to carefully withdraw the pool without touching the pipette tip to the bottom of the tube, in order to avoid any sediment that may have formed after centrifugation of the pool.

2 Incubate the Ab BC plus blocked cell mixture for 30 min on ice. During centrifugation, proceed to prime the HT IFC.

Prime the HT IFC

Dilute the Blocking Reagent

Combine the following reagents in a 1.5 mL microtube, gently vortex, and keep at room temperature.
Table 3. 1X Blocking Reagent

<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>135</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>

Table 4. 0.01X Blocking Reagent

<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>396</td>
</tr>
<tr>
<td>1X Blocking Reagent (from Table 3)</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>400</strong></td>
</tr>
</tbody>
</table>

**Load the Reagents**

![HT IFC priming pipetting map](image)

Add volume to each:
- 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, 180 µL (2 reservoirs)
- Empty well

**Figure 2. HT IFC priming pipetting map**

Load reagents onto the IFC as shown in Figure 2.

**IMPORTANT** Ensure that the A1 notch is in the proper orientation. Stability Solution is viscous. **Do not vortex.** Make sure to keep the HT IFC as level as possible, and evenly distribute the reagents over the bottom surface of the reservoirs.

**Run the Prime Script on C1**

1. Place the HT IFC into the C1 system, and then run the mRNA Seq HT: Prime (1912x) script. Priming takes approximately 24 min.

   **NOTE** If a priming reagent pipetting error has occurred, wait for the Prime script to finish (do not press ABORT), pipet new priming reagents, and run the prime script again.

2. During priming, proceed to **Prepare the Cell Mix for Loading.** If time allows, you can also start preparing the reagent mixes and diluted barcodes.

3. When the Prime script has finished, tap EJECT to remove the IFC.

   **NOTE** After priming, you have up to 1 hr to load cells. Keep the primed IFC in the instrument until you are ready to load cells.

**Prepare the Cell Mix for Loading**

**Wash and Count the Cells**

1. After the 30 min incubation of the Ab BC plus blocked cell mixture is complete, wash the cells:
   a. Pipet 10 mL of cold Cell Staining Buffer into the 15 mL conical tube containing the Ab BC plus blocked cell mixture.
   b. Centrifuge the mixture at 350 x g at 4 °C for 5 min.
   c. Use a 10 mL serological pipet to carefully remove and discard all but approximately 20 µL of the supernatant without disturbing the cell pellet.
   d. Pipet 500 µL of cold Cell Staining Buffer into the tube containing washed cells, and then gently pipet up and down to break up the cell pellet.
2 (Optional) Filter the cells if clumps are observed:

a Set a P1000 pipette to 500 μL and aspirate the entire volume of labeled cells. Do not elute the cells.

b With the cells still inside the pipette tip, carefully push the tip into a Flowmi™ Cell Strainer, and then press down to secure the pipette tip to the cell strainer.

c Carefully dispense the cells from the pipette so they flow through the cell strainer and into a new 15 mL microtube.

d Eject and discard the used pipette tip with the attached cell strainer.

3 Count the cells using a hemocytometer, and then resuspend cells to 400 cells/μL in the appropriate volume of Cell Staining Buffer.

Prepare the Cell Mix to Load into the HT IFC

1 Combine cells with Suspension Reagent at a ratio optimized for your cell type to create a neutrally buoyant cell suspension.

a Vortex the Suspension Reagent for 5 sec until particulates are not visible.

b Use a P200 pipette to gently add cells to Suspension Reagent in a 1.5 mL microtube. Many cell types use the standard 3:2 suspension ratio:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension Reagent (C1 HT Kit Module 1)</td>
<td>20</td>
</tr>
<tr>
<td>400 cells/μL (from Step 3 above)</td>
<td>30</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

2 Add 1 μL of 50X LIVE/DEAD staining solution (from page 6) to the cell mix and then incubate at room temperature for 10 min.

3 Pipet 10 μL of the cell mix into a hemocytometer to check cell viability using a fluorescence microscope. Note the ratio of live/dead cells.

Remove Reagents and Load Cells

1 Use a P20 set to 20 μL to remove the 1X Blocking Reagent from each of the 4 inlets shown in Figure 3.

Figure 3. HT IFC pipetting map for removal of priming reagents

2 Load reagents and cells into the IFC as shown in Figure 4. You can load the same cells mix or a different cell mix into each cell inlet.

NOTE The center panel of the IFC is divided into 2 sections, left and right, allowing you to load the same or 2 different cell mixes into the IFC.
**Prepare Reagent Mixes for cDNA Synthesis**

**Prepare Lysis Mix A**

1. Combine the following reagents in a 0.5 mL microtube labeled **Mix A**.

<table>
<thead>
<tr>
<th>Reagent</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>
</tbody>
</table>

Total: 254.4

2. Gently pipet up and down a few times to mix, centrifuge briefly to collect contents, and then keep on ice until use.

**Prepare Lysis Mix A Plus Diluted Barcodes**

**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 Plate 1: Cell Barcode Plate**

Centrifuge the original Cell Barcode Plate (C1 HT Kit Module 3) at 3,000 x g for 3 min. This is **Plate 1**.
Prepare Plate 2: Diluted Barcodes

1. Label a new 96-well PCR plate as **Plate 2: Diluted BC**.

2. Aliquot 100 μL of Teknova DNA Suspension Buffer into each tube of an 8-tube strip, and then use a multichannel pipet to aliquot 18 μL of the buffer into the first 5 columns (40 wells) of Plate 2.

3. Using a multichannel P200 pipet, pierce the foil for the first 5 columns of Plate 1.

4. Pipet 2 μL of the cell barcodes column by column into the corresponding first 5 columns of Plate 2, and then pipet up and down 10 times to mix.

Prepare Plate 3: Lysis Mix A Plus Diluted Barcodes

1. Label a new 96-well PCR plate as **Plate 3: Mix A + Diluted BC**.

2. Aliquot 30 μL of Lysis Mix A into each tube of an 8-tube strip, and then carefully pipet the following:
   - For samples: Pipet 4.6 μL of Lysis Mix A into the first 5 columns of the new Plate 3.
   - (Optional) For tube controls: Pipet 3.8 μL of Lysis Mix A into wells A12, B12, and C12 of Plate 3.

3. Use a P10 pipette to carefully pipet the following, and then gently pipet up and down 5 times to mix.
   - For samples: Pipet 1.5 μL of diluted barcodes column by column from Plate 2 into the corresponding first 5 columns of Plate 3.
   - (Optional) For tube controls: Pipet 1.25 μL of diluted barcodes from wells A1, B1, and C1 of Plate 2 into wells A12, B12, and C12 of Plate 3.

4. Seal Plate 3, centrifuge at 3,000 x g for 3 min, and then keep on ice.

Prepare RT Reaction Mix B

1. Combine the following reagents in a 0.5 mL microtube labeled **Mix B**.

---

### Table: RT Reaction Mix B Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol. for Samples (µL)</th>
<th>Vol. for Samples + 2 Tube Controls (µL)</th>
<th>Vol. 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>9.5</td>
<td>13.6</td>
<td>15.65</td>
</tr>
<tr>
<td>Nuclease-Free Water (C1 HT Kit Module 5)</td>
<td>3.0</td>
<td>4.3</td>
<td>4.95</td>
</tr>
<tr>
<td>dNTP Mix (20 mM each) (C1 HT Kit Module 5)</td>
<td>2.4</td>
<td>3.4</td>
<td>3.9</td>
</tr>
<tr>
<td>RNase Inhibitor (C1 HT Kit Module 5)</td>
<td>1.2</td>
<td>1.7</td>
<td>1.95</td>
</tr>
<tr>
<td>Dithiothreitol (DTT) (C1 HT Kit Module 5)</td>
<td>1.2</td>
<td>1.7</td>
<td>1.95</td>
</tr>
<tr>
<td>Reverse Transcription Primer (C1 HT Kit Module 4)</td>
<td>2.4</td>
<td>3.4</td>
<td>3.9</td>
</tr>
<tr>
<td>Loading Reagent (C1 HT Kit Module 2)</td>
<td>0.8</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Reverse Transcriptase (C1 HT Kit Module 5)</td>
<td>4.8</td>
<td>6.8</td>
<td>7.8</td>
</tr>
</tbody>
</table>

**Total**                                      | 25.3                  | 36.1                                   | 41.5                                   |
Prepare Preamplification Mix C

1. Combine the following reagents in a 0.5 mL microtube labeled Mix C.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vol. (µL)</th>
<th>Vol. for 22 Samples (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeqAmp PCR buffer 2X (Takara Bio)</td>
<td>135</td>
<td>150</td>
</tr>
<tr>
<td>Loading Reagent (C1 HT Kit Module 2)</td>
<td>3.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Preamp Primer (cDNA) (C1 HT Kit Module 2)</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Custom preamplification primer, 100 µM (Ab BC) (IDT)</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>SeqAmp DNA Polymerase (Takara Bio)</td>
<td>5.4</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>146.4</strong></td>
<td><strong>162.6</strong></td>
</tr>
</tbody>
</table>

2. Gently pipet up and down a few times to mix, centrifuge briefly to collect contents, and then 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 5) using a microscope compatible with IFCs, and then perform quality control (scoring) on the cells using an established protocol for your cell types.

2. (Optional) If time allows, you can start the tube controls during imaging of loaded cells (see Appendix A).

Run Lysis, Reverse Transcription, and Preamplification on C1

Remove Reagents

Remove the reagents as shown in Figure 7.

- Use a P200 set to 180 µL to remove the 0.01X Blocking Reagent from the 2 wash reservoirs.
- Use a P20 set to 20 µL to remove the cell mix, Cell Rinsing Reagent, and 1X Blocking Reagent from their respective inlets.
Load Reagents

Load the reagents as shown in Figure 8, paying particular attention to the following:

1. 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 solid purple inlets.

2. Use a multichannel pipette with fresh pipette tips for each column to very carefully transfer, column by column, 5 μL from the first 5 columns of Plate 3: Mix A + Diluted BC into each of the corresponding wells marked 1–5.

**IMPORTANT** 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.

3. Place the HT IFC into the C1 system and immediately run the mRNA Seq HT: RT & Amp (1912x) script. The run time is approximately 8.5 hr.

**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.

4. (Optional) If you are running tube controls, make sure to save the remaining reagent mixes. You can start to run the tube control reaction after you start the mRNA Seq HT: RT & Amp (1912x) script (see Appendix A for instructions).
Harvest and Clean Up Amplicons off the C1 HT IFC

Make sure to change tips between samples to avoid contamination.

Transfer and Dilute Harvest Amplicons

1. Up to 1 hr after the mRNA Seq HT: RT & Amp (1912x) script is finished, tap EJECT to remove the HT IFC from the instrument and then move the IFC to a post-PCR environment.

2. Label a new 96-well PCR plate Harvest Plate cDNA + Ab BC.

3. Aliquot 150 μL of DNA Suspension Buffer into each tube of an 8-tube strip, and then pipet 45 μL of the buffer into the first 3 columns of the Harvest Plate.

4. Use the plastic removal tool to pull back the tape covering the harvesting inlets on both sides of the IFC.

5. Using a multichannel pipette set to 6 μL, transfer the harvested amplicons from each column of IFC inlets (expected volume 3–6 μL) into the Harvest Plate wells containing buffer, for a total volume of approximately 50 μL in each well.

6. (Optional) If performing a tube control, transfer 5 μL of tube control amplicons into wells F1, G1, and H1 of the Harvest Plate.

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

Separate the Harvest Amplicons: cDNA and Ab BC

Before you begin, warm AMPure XP beads up to room temperature, and vortex for 1 min before use.

1. Label a new 96-well PCR plate Harvest Cleanup Plate 1: Ab BC.

2. Aliquot 100 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to pipet 30 μL beads to each sample in the Harvest Plate (0.6X beads in 20 wells, for a total volume of approximately 80 μL in each well).

3. Mix well by pipetting up and down 5–10 times, and then incubate bead mix at room temperature for 5 min.

4. Repeat Step 3.

5. Place the Harvest Plate on a plate magnet until the solution is clear (approximately 3–5 min).

6. Keep the Harvest Plate on the magnet and use a P200 pipette set to 75 μL to carefully transfer the supernatant to the Harvest Cleanup Plate 1: Ab BC.

IMPORTANT Make sure to save both beads and supernatant. The cDNA samples (>300 bp) are on the beads in the Harvest Plate and the amplified Ab BC (<190 bp) are in the supernatant of the Harvest Cleanup Plate 1: Ab BC. Do not discard either plate.
1. Keeping the Harvest Plate: cDNA + Ab BC on the magnet, cross out Ab BC from the plate label. This plate now only contains AMPure beads bound to cDNA, since Ab BC were separated into the supernatant of Harvest Cleanup Plate 1: Ab BC in the previous section.

2. Use a multichannel pipet to slowly add 180 μL of 75% ethanol to each sample, incubate for 30 sec at room temperature, and then use a P200 pipette to remove the ethanol without disturbing the beads.

3. Repeat Step 2.

4. Keep the plate on the magnet and use a P200 pipette set to 20 μL to remove any remaining ethanol without disturbing the beads.

5. Remove the plate from the magnet, and then air-dry beads on bench for 3–5 min. Avoid overdrying.

6. Aliquot 115 μL of Teknova DNA Suspension Buffer into each tube of an 8-tube strip.

7. Use a multichannel pipette to elute the cDNA by adding 32 μL of buffer to each harvest well, and then mix well by pipetting up and down 5–10 times until all beads are in suspension.

8. Incubate at room temperature for 2 min.

9. Mix again by pipetting up and down 5–10 times, and then incubate again at room temperature for 2 min.

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

11. Label a new 96-well PCR plate Harvest Cleanup Plate 1: cDNA.

12. Carefully transfer 30 μL of the supernatant from each well of Harvest Plate: cDNA to the new Harvest Cleanup Plate 1: cDNA without disturbing the beads.

**IMPORTANT** The harvested and purified cDNA amplicons are in the supernatant. Discard the beads only.

## Part 2: Prepare Ab BC Amplicons for Sequencing

Before you begin, prepare 40 mL of fresh 80% ethanol from absolute ethanol.

### Perform First Cleanup of Ab BC

1. Retrieve Harvest Cleanup Plate 1: Ab BC from Separate the Harvest Amplicons (see Step 6 on page 14).

2. Aliquot 180 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to pipet 48 μL beads to each sample well (0.6X beads, for a total volume of approximately 123 μL in each well).

3. Mix well by pipetting up and down, and then incubate bead mix at room temperature for 5 min.

4. Repeat Step 3.

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

6. Keep the plate on the magnet and use a P200 pipette to carefully remove and discard the supernatant without disturbing the beads.

7. Keeping the plate on the magnet, use a multichannel pipette to slowly add 200 μL of 80% ethanol to each sample, incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.

8. Repeat Step 7.
Perform single

Use exonuclease enzyme to remove any remaining ethanol without disturbing the beads.

Remove the plate from the magnet, and then air-dry beads on bench for 3 min. Avoid overdrying.

Aliquot 120 μL of Teknova DNA Suspension Buffer into each tube of an 8-tube strip.

Use a multichannel pipette to elute the amplified Ab BC by adding 36 μL of buffer to each harvest sample, and then mix well by pipetting up and down until all beads are in suspension.

Incubate at room temperature for 2 min.

Mix by pipetting up and down, and then incubate again at room temperature for 2 min.

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

Label a new 96-well PCR plate Harvest Cleanup Plate 2: Ab BC.

Carefully transfer 34 μL of the supernatant from each well of Plate 1 to the new Plate 2 without disturbing the beads.

IMPORTANT The harvested and purified Ab BC amplicons are in the supernatant. Discard the beads only.

Clean Up to Remove Unbound BC

Use exonuclease enzyme to remove excess unbound single-stranded cell BC or Ab BC.

Perform Exonuclease Cleanup

1 Set up the following exonuclease protocol, and ensure that the thermal cycler lid is heated:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 °C</td>
<td>30 min</td>
<td>1</td>
<td>Incubation</td>
</tr>
<tr>
<td>80 °C</td>
<td>15 min</td>
<td>1</td>
<td>Deactivation</td>
</tr>
<tr>
<td>4 °C</td>
<td>∞</td>
<td>1</td>
<td>Hold</td>
</tr>
</tbody>
</table>

2 Combine the following reagents in a 0.5 mL microtube and keep on ice:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per Well (μL)</th>
<th>Volume for 24 Wells (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exonuclease I</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>Reaction Buffer (10X)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exonuclease I (20 units/μL)</td>
<td>2</td>
<td>48</td>
</tr>
</tbody>
</table>

Total 6 144

* Includes 20% overage for sample wells. If performing tube controls, prepare extra volume.

3 Keeping the Harvest Cleanup Plate 2: Ab BC on ice, pipet 6 μL of exonuclease mix into each well containing 34 μL of purified Ab BC.

4 Place the plate in the preheated thermal cycler and run the exonuclease protocol.

Perform Bead Cleanup

1 Aliquot 200 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to pipet 60 μL of beads to each exonuclease-treated sample well in the Harvest Cleanup Plate 2 (1.5X AMPure beads, for a total volume of approximately 100 μL in each well).

2 Mix well by pipetting up and down, and then incubate at room temperature for 5 min.

3 Repeat Step 2.

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

5 Keep the plate on the magnet and use a P200 pipette to carefully remove and discard the supernatant without disturbing the beads.

6 Keeping the plate on the magnet, use a multichannel pipette to add 200 μL of 80% ethanol to each sample, incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.

7 Repeat Step 6.

8 Keep the plate on the magnet and use a P20 pipette to remove any remaining ethanol without disturbing the beads.
9 Remove the plate from the magnet, and then air-dry beads on bench for 3 min. Avoid over-drying.

10 Aliquot 120 μL of Teknova DNA Suspension Buffer into each tube of an 8-tube strip.

11 Use a multichannel pipette to elute the purified Ab BC by adding 34 μL of buffer to each sample, and then mix well by pipetting up and down until all beads are in suspension.

12 Incubate at room temperature for 2 min.

13 Mix by pipetting up and down, and then incubate again at room temperature for 2 min.

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

15 Label a new 96-well PCR plate Library Prep Plate 1: Ab BC.

16 Carefully transfer 30 μL of the supernatant from each well of the Harvest Cleanup Plate 2 to Library Prep Plate 1 without disturbing the beads.

**Add Custom Primers to Ab BC**

The custom P5 primer (i5xx) can be used to amplify and index cDNA or Ab BC, while the custom P7 primers (i7xx) are used to amplify and index cDNA only. See Appendix B for custom primer sequences.

**IMPORTANT** Select one custom P5 primer (i5xx) per Ab BC library and make sure to track the custom primer assignments for each well during this procedure. We recommend the layout shown in Figure 10.

1 Set up the following thermal cycling protocol, and ensure that the thermal cycler lid is heated:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 °C</td>
<td>15 min</td>
<td>1</td>
<td>Hot start</td>
</tr>
<tr>
<td>95 °C</td>
<td>15 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 °C</td>
<td>90 sec</td>
<td>9</td>
<td>PCR</td>
</tr>
<tr>
<td>68 °C</td>
<td>90 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68 °C</td>
<td>3 min</td>
<td>1</td>
<td>Final</td>
</tr>
<tr>
<td>4 °C</td>
<td>∞</td>
<td>1</td>
<td>extension</td>
</tr>
</tbody>
</table>

2 In a 0.5 mL microtube on ice, combine the components of the PCR Pre-Mix:

**Table 7. PCR Pre-Mix**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Conc. (μM)</th>
<th>Final Conc. (μM)</th>
<th>Vol. per Well (μL)</th>
<th>Vol. for 20 Wells (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X TSP Master Mix (101-5786)</td>
<td>4X 1X</td>
<td>12.5</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Custom P5 primer (i5xx)*</td>
<td>10 0.3</td>
<td>1.5</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>—</td>
<td>4.5</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>—</td>
<td><strong>18.5</strong></td>
<td><strong>444</strong></td>
<td></td>
</tr>
</tbody>
</table>

* Includes 20% overage for sample wells. If performing tube controls, prepare extra volume.

† The stock concentration of P5 primer varies based on vendor (recommended: Integrated DNA Technologies).
3 Pipet 18.5 μL of PCR Pre-Mix containing custom P5 primer (i5xx) to each sample well on the Library Prep Plate 1: Ab BC (see Figure 10).

4 To avoid contamination, use a multichannel pipette to pipet 1.5 μL of the appropriate custom P7 primer (i7xx) to each sample well on the Library Prep Plate (see Figure 10). Each sample well should now contain a total volume of 50 μL.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Conc. (μM)</th>
<th>Final Conc. (μM)</th>
<th>Vol. per Well (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Custom P7 primer (i7xx)*</td>
<td>10</td>
<td>0.3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* The stock concentration of P7 primer varies based on vendor (recommended: Integrated DNA Technologies).

**NOTE** If performing optional tube controls, select 2–3 unused custom P7 primers (i7xx) and pipet 1.25 μL of the appropriate primer to each of the tube control wells (see Figure 10).

5 Seal the plate and vortex for 3 min at medium speed. Centrifuge for 1 min at 3,000 x g.

6 Place the plate in the thermocycler with preheated lid and run the protocol.

**Perform Post-Indexing Cleanup**

Before you begin, prepare 20 mL of fresh 80% ethanol from absolute ethanol.

**Perform Ab BC Cleanup 1**

1 Vortex the AMPure XP beads for 1 min immediately before use.

2 Aliquot 160 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to add 50 μL beads to each sample well of the Library Prep Plate 1: Ab BC (1X beads, for a total volume of approximately 100 μL in each well).

3 Mix well by pipetting up and down, and then incubate at room temperature for 5 min.

4 Repeat Step 3.

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

6 Keep the plate on the magnet and use a P200 pipette to carefully remove and discard the supernatant without disturbing the beads.

7 Keeping the plate on the magnet, use a multichannel pipette to add 200 μL of 80% ethanol to each sample, incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.

8 Repeat Step 7.

9 Keep the plate on the magnet and use a P20 pipette to remove any remaining ethanol without disturbing the beads.

10 Remove the plate from the magnet, and then air-dry beads on bench for 3 min. **Avoid overdrying.**

11 Aliquot 110 μL of DNA Suspension Buffer into each tube of an 8-tube strip.

12 Use a multichannel pipette to elute the indexed Ab BC by adding 34 μL of buffer to each sample, and then mix well by pipetting up and down until all beads are in suspension.

13 Incubate at room temperature for 2 min.

14 Mix by pipetting up and down, and then incubate again at room temperature for 2 min.

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

16 Label a new 96-well PCR plate **Library Prep Plate 2: Ab BC Cleanup.**

17 Carefully transfer 32 μL of the supernatant from each well of Plate 1 to the new Plate 2 without disturbing the beads.

**Perform Ab BC Cleanup 2**

1 Aliquot 100 μL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to add 32 μL beads to each sample well of Library
Prep Plate 2: Ab BC Cleanup (1X beads, for a total volume of approximately 64 μL in each well).

2 Mix well by pipetting up and down, and then incubate at room temperature for 5 min.

3 Repeat Step 2.

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

5 Keep the plate on the magnet and use a P200 pipette to carefully remove and discard the supernatant without disturbing the beads.

6 Keeping the plate on the magnet, use a multichannel pipette to add 200 μL of 80% ethanol to each sample, incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.

7 Repeat Step 6.

8 Keep the plate on the magnet and use a P20 pipette to remove any remaining ethanol without disturbing the beads.

9 Remove the plate from the magnet and then air-dry beads on bench for 3 min. Avoid overdrying.

10 Aliquot 110 μL of DNA Suspension Buffer into each tube of an 8-tube strip.

11 Use a multichannel pipette to elute the indexed and purified Ab BC by adding 32 μL of buffer to each sample, and then mix well by pipetting up and down until all beads are in suspension.

12 Incubate at room temperature for 2 min.

13 Mix by pipetting up and down, and then incubate again at room temperature for 2 min.

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

15 Label a new 96-well PCR plate Library Prep Plate 3: Ab BC Final.

16 Carefully transfer 30 μL of the supernatant from each well of Plate 2 to the new Plate 3 without disturbing the beads.

Quantify and Dilute Ab BC Libraries

Determine the Concentration of All Libraries

Use a Qubit Fluorometer to measure and record the concentration in ng/μL of every well in the Library Prep Plate 3: Ab BC Final, following the manufacturer’s instructions (see Table 8).

Table 8. Qubit Fluorometer measurements (see Figure 9 for Harvest Plate layout)

<table>
<thead>
<tr>
<th>Harvest Column</th>
<th>Conc. (ng/μL)</th>
<th>Column Conc. (ng/μL)</th>
<th>Column Conc. (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>8</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>7</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>6</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>5</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Determine the cDNA Quality for a Subset of Libraries

Randomly select 5 wells in the Library Prep Plate 3: Ab BC Final and run 1 μL of each library in duplicate on an Agilent HS DNA chip according to the Agilent High Sensitivity DNA Kit instructions. (If performing optional tube controls, use the remaining chip well to run a positive control.) Assess the cDNA quality and verify the library size distribution, using Table 9 to record your results. The amplicon size should be approximately 180–190 bp (see Figure 11).
Table 9. Agilent Bioanalyzer measurements

<table>
<thead>
<tr>
<th>Well Origin</th>
<th>Well Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS DNA chip</td>
<td>1 2 3</td>
</tr>
<tr>
<td>Library plate</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip</td>
<td>4 5 6</td>
</tr>
<tr>
<td>Library plate</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip</td>
<td>7 8 9</td>
</tr>
<tr>
<td>Library plate</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip</td>
<td>10 11*</td>
</tr>
</tbody>
</table>

* (Optional) If performing tube controls, run 1 µL of PC1 or PC2 in well 11 of the Agilent HS DNA chip.

4 Pipet 5 µL of each library into a 1.5 mL microtube labeled Ab BC Pool to create the library pool for sequencing.

STOPPING POINT Store the Library Prep Plate 3: Ab BC Final, Diluted Ab BC Libraries Plate, and Ab BC pool overnight at 4 °C or long-term at –20 °C.

Part 3: Prepare cDNA Amplicons for Sequencing

Perform Second Cleanup of cDNA

Before you begin, prepare 20 mL of fresh 75% ethanol from absolute ethanol.

1 Retrieve the Harvest Cleanup Plate 2: cDNA from Perform First Cleanup of cDNA (see Step 12 on page 15).

2 Vortex the AMPure XP beads for 1 min immediately before use.

3 Aliquot 100 µL of beads into each tube of an 8-tube strip, and then use a multichannel pipette to add 27 µL of beads to each sample in Plate 2, for a total volume of approximately 57 µL in each well.

4 Mix well by pipetting up and down, and then incubate at room temperature for 5 min.

5 Repeat Step 4.

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

7 Keep the plate on the magnet and then carefully remove and discard the supernatant from each well without disturbing the beads. Leave approximately 10 µL of the supernatant behind.

IMPORTANT The harvest samples are on the beads. Discard the supernatant only.

8 Keeping the plate on the magnet, use a multichannel pipette to add 180 µL of 75% ethanol to each well, incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.
9 Repeat Step 8.

10 Keep the plate on the magnet and use a P20 pipette to remove any remaining ethanol without disturbing the beads.

11 Remove the plate from the magnet and air-dry beads on bench for 3–5 min. Avoid overdrying.

12 Aliquot 40 µL of DNA Suspension Buffer into each well of an 8-tube strip.

13 Use a multichannel pipette to elute the purified cDNA by adding 11 µL of buffer to each sample, and then mix well by pipetting up and down until all beads are in suspension.

14 Incubate at room temperature for 2 min.

15 Mix by pipetting up and down, and then incubate again at room temperature for 2 min.

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

17 Label a new 96-well PCR plate Harvest Cleanup Plate 3: cDNA.

18 Use a single-channel pipette to slowly and carefully transfer 9 µL of the supernatant from each well of Plate 2 to the new Plate 3 without disturbing the beads.

IMPORTANT The harvest samples are in the supernatant. Discard beads only.

Quantify and Dilute cDNA Libraries

Determine the Concentration of All Libraries

Use a Qubit Fluorometer to measure and record the concentration in ng/µL of every well in the Harvest Cleanup Plate 3: cDNA, following the manufacturer’s instructions (see Table 10).

Table 10. Qubit Fluorometer measurements (see Figure 9 for Harvest Plate layout)

<table>
<thead>
<tr>
<th>Harvest Column</th>
<th>Conc. (ng/µL)</th>
<th>Column 1</th>
<th>Conc. (ng/µL)</th>
<th>Column 12</th>
<th>Conc. (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>8</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>7</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>6</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>5</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Determine the cDNA Quality for a Subset of Libraries

Randomly select 5 wells in the Harvest Cleanup Plate 3: cDNA and run 1 µL of each library in duplicate on an Agilent HS DNA chip according to the manufacturer’s instructions. (If performing optional tube controls, use the remaining chip well to run a positive control.) Assess the cDNA quality and verify the library size distribution, using Table 11 to record your results. The amplicon size should be approximately 200–9,000 bp (see Figure 12).
Table 11. Agilent Bioanalyzer measurements

<table>
<thead>
<tr>
<th>Well Origin</th>
<th>Well Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS DNA chip</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Library plate</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Library plate</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Library plate</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11*</td>
</tr>
</tbody>
</table>

* (Optional) If performing tube controls, run 1 μL of PC1 or PC2 in well 11 of the Agilent HS DNA chip.

Figure 12. Typical cDNA library size distribution (after harvest and purification)

Dilute the cDNA Libraries

1. Label a new plate **Diluted cDNA Libraries Plate (0.2 ng/μL)**.

2. With the Harvest Cleanup Plate 3: cDNA on the magnet, pipet at least 2 μL of each cDNA library into the appropriate volume of DNA Suspension Buffer in the respective wells of the Diluted cDNA Libraries Plate to achieve a final concentration of 0.2 ng/μL.

3. Pipet up and down 5–10 times to mix (do not vortex).

**STOPPING POINT** Store the Harvest Cleanup Plate 3: cDNA and Diluted cDNA Libraries Plate overnight at 4 °C or long-term at –20 °C.

Perform Tagmentation

Before You Begin

- If running optional tube controls, prepare extra reagent volume in the following procedures.
- Thaw the Diluted cDNA Libraries Plate (0.2 ng/μL) and place on ice.
- Warm Tagment DNA Buffer and Neutralize Tagment (NT) Buffer to room temperature. Inspect NT Buffer for precipitate and vortex if precipitate is observed.
- Determine the P7 index configuration to use, and thaw the selected Nextera XT Index Primers (N7xx) to room temperature. If performing optional tube controls, select and document 2–3 unused P7 index primers. Make sure to note which index primer is assigned to each well. We recommend the following layout.

![Figure 13. Primer assignments for Library Prep Plate](image)

**NOTE** The P7 Nextera XT Index Primers (N7xx) are used to amplify cDNA only, while the custom P5 primer (i5xx) can be used to amplify cDNA or Ab BC.

- If you plan to sequence both Ab BC and cDNA on the same Illumina cartridge, select and thaw to room temperature a different custom P5 primer (i5xx) than the one you used to amplify Ab BC.
(see Add Custom Primers to Ab BC on page 17), in order to separate the sequencing products during analysis. Make sure to select one custom P5 primer (i5xx) per cDNA library. See Appendix B for custom primer sequences.

- Set up the following thermal cycling protocol, and ensure that the thermal cycler lid is heated:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 °C</td>
<td>Preheat and pause</td>
</tr>
<tr>
<td>55 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>10 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**Tagment the cDNA**

1. Label a new 96-well PCR plate **Library Prep Plate: cDNA** and place on ice.

2. In a 0.5 mL microtube on ice, combine the components of the Tagmentation Pre-Mix.

   Table 12. Tagmentation Pre-Mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per Sample (µL)</th>
<th>Volume for 20 Samples (µL)*</th>
<th>Volume for 20 Samples and 2 Tube Controls (µL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tagment DNA Buffer</td>
<td>2.5</td>
<td>62.5</td>
<td>68.75</td>
</tr>
<tr>
<td>Amplicon Tagment Mix</td>
<td>1.25</td>
<td>31.25</td>
<td>34.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>3.75</strong></td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Includes 25% overage

3. Vortex at low speed for 10 sec and centrifuge briefly to collect contents.

4. Use a multichannel pipette to aliquot 14 µL of pre-mix into each tube of an 8-tube strip, and then pipet 3.75 µL of the pre-mix into the Library Prep Plate using the same plate layout as the Diluted cDNA Libraries Plate.

5. Pipet 1.25 µL of each 0.2 ng/µL sample from the Diluted cDNA Libraries Plate to the corresponding wells in the Library Prep Plate, for a total volume of 5.0 µL in each well.

6. Seal the Library Prep Plate and vortex at medium speed for 10 sec. Centrifuge at 3,000 x g for 1 min.

7. Place the Library Prep Plate in the preheated thermal cycler and skip the pause to start the protocol.

**During and After Thermal Cycling**

1. While the protocol is running, aliquot 4.5 µL of room temperature NT Buffer into each tube of an 8-tube strip.

2. Once the sample reaches 10 °C, remove the plate, plate it on ice, and then **immediately** pipet 1.25 µL of the NT Buffer to each of the tagmented samples, pipetting up and down 10 times to mix and quickly neutralize the samples.

3. Seal plate and incubate at room temperature for 5 min. Centrifuge at 3,000 x g for 3 min.

**Amplify the Tagmented cDNA**

1. Set up the following thermal cycling protocol:

<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></td>
</tr>
<tr>
<td>55 °C</td>
<td>30 sec</td>
<td>12</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>

2. In a 0.5 mL microtube on ice, combine the components of the PCR Pre-Mix (see Table 13).
**Technical Note**

**Table 13. PCR Pre-Mix**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Conc.</th>
<th>Final Conc.</th>
<th>Vol. per Well (μL)</th>
<th>Vol. for 20 Wells (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nextera PCR Master Mix (NPM)</td>
<td>N/A</td>
<td>1X</td>
<td>3.75</td>
<td>90</td>
</tr>
<tr>
<td>Custom P5 primer (i5xx)*</td>
<td>10 μM</td>
<td>0.3 μM</td>
<td>1.5</td>
<td>36</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>—</td>
<td>—</td>
<td>5.25</td>
<td>126</td>
</tr>
</tbody>
</table>

* Includes 20% overage
† The stock concentration of P5 primer varies based on vendor (recommended: Integrated DNA Technologies).

3 Vortex at low speed for 10 sec and then briefly centrifuge to collect contents.

4 To avoid bubbles, use a single-channel pipette to pipet 5.25 μL of PCR Pre-Mix into each sample well of the Library Prep Plate.

5 To avoid contamination, use a multichannel pipette to carefully pipet 1.25 μL of the appropriate P7 Nextera XT Index Primer (N7xx) to each of the sample libraries and optional tube controls on the Library Prep Plate, for a total volume of 12.75 μL in each sample well. Make sure to note which index is assigned to each well. We recommend the following layout.

7 Place the plate into the thermal cycler and start the protocol, making sure the thermal cycler lid is heated during the incubation.

**STOPPING POINT** The PCR amplification takes approximately 45 min to complete and may be run overnight. The amplified products in the Library Prep Plate: cDNA can be stored overnight at 4 °C or long-term at −20 °C.

**Pool and Clean Up the cDNA Library**

**Before You Begin**

- Prepare 20 mL of fresh 75% ethanol from absolute ethanol.
- If running optional tube controls, prepare extra reagent volume in the following procedures.
- Warm AMPure XP beads up to room temperature and vortex for 1 min immediately before use.

**Pool by Volume and Perform First Cleanup**

1 Retrieve the Library Prep Plate: cDNA and keep at room temperature.

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

3 Pool the libraries into a 1.5 mL low-bind microtube labeled **cDNA Pool: Cleanup 1**:
  
  a Pipet the appropriate volume from each sample (Table 14, column 2) based on total number of samples to be pooled (column 1).
  
  b Vortex at low speed for 10 sec and centrifuge briefly to collect contents.
  
  c Add the required volume of AMPure XP beads (column 4) to the library pool, and then mix well by pipetting up and down until all the beads are in suspension.

6 Seal the plate and vortex at low speed for 10 sec. Centrifuge at 3,000 x g for 3 min.
4 Incubate bead mix at room temperature for 5 min.
5 Place the tube on a magnetic stand until the solution is clear (approximately 3–5 min).
6 Keep the tube on the magnet and 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.
7 Keep the tube on the magnet and slowly add 180 µL of 75% ethanol (do not mix), incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.
8 Repeat Step 7.
9 Keep the tube on the magnet and use a P20 pipette to remove any remaining ethanol without disturbing the beads.
10 Remove the tube from the magnet and air-dry beads on bench for 3–5 min. Avoid overdrying.
11 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 until all the beads are in suspension.

### Perform Second Cleanup

1 Vortex the AMPure beads for 1 min immediately before use.
2 Add the required volume of AMPure XP beads to the eluted samples, and then mix well by pipetting up and down until all the beads are in suspension.

**Table 14. Sample pooling**

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Samples to be Pooled</td>
<td>Vol. per Sample (µL)</td>
<td>Total Library Pool Vol. (µL)</td>
<td>AMPure Bead Vol. for Cleanup (~75% of total library pool; µL)</td>
</tr>
<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>

<table>
<thead>
<tr>
<th>Number of Samples Pooled</th>
<th>DNA Suspension Buffer for Elution (volume = original total library pool; 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>

12 Incubate at room temperature for 2 min.
13 Mix by pipetting up and down, and then incubate again at room temperature for 2 min.
14 Place the tube on a magnetic stand until solution is clear (approximately 3–5 min).
15 Keep the tube on the magnet and carefully transfer the entire volume of supernatant to a new 0.5 mL microtube labeled cDNA Pool: Cleanup 2 without disturbing the beads.
   **IMPORTANT** The samples are in the supernatant. Discard the beads only.

### Perform Second Cleanup

1 Vortex the AMPure beads for 1 min immediately before use.
2 Add the required volume of AMPure XP beads to the eluted samples, and then mix well by pipetting up and down until all the beads are in suspension.

<table>
<thead>
<tr>
<th>Number of Samples Pooled</th>
<th>AMPure Bead Volume for Cleanup (~75% of total library pool; 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 bead mix at room temperature for 5 min.
4 Centrifuge the tube briefly to dislodge adherent beads and then place on a magnetic stand until solution is clear (maximum of 5 min).

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.

6 Keep the tube on the magnet and slowly add 180 μL of 75% ethanol (do not mix), incubate at room temperature for 30 sec, and then use a P200 pipette to remove the ethanol without disturbing the beads.

7 Repeat Step 6.

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

9 Remove the tube from the magnet and air-dry beads on bench for 3–5 min. **Avoid overdrying.**

10 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 until all the beads are in suspension.

<table>
<thead>
<tr>
<th>Number of Samples Pooled</th>
<th>AMPure Bead Volume for Cleanup (“75% of total library pool; 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>

11 Incubate bead mix at room temperature for 2 min.

12 Mix by pipetting up and down, and then incubate again at room temperature for 2 min.

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

14 Carefully transfer approximately 90% of the supernatant volume to a new tube labeled cDNA Pool: Final without disturbing the beads. **IMPORTANT** The samples are in the supernatant. Discard beads only.

**STOPPING POINT** Keep the final pooled and cleaned cDNA library at 4 °C until ready to use, or store at −20 °C long-term.

**Size and Quantify the Pooled Libraries**

Verify the library size distribution and concentration of each pooled REAP-seq library (Ab BC and cDNA) prior to sequencing. Make sure to place each pooled sample on a magnet to ensure that the solution is clear of any beads before pipetting into the Agilent HS DNA chip or Qubit Assay Tube. **IMPORTANT** We highly recommend that you include the PhiX Control v3 library in your sequencing run. 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 pooled library samples to verify that the PhiX concentration is correct before you combine this control with your samples in the recommended ratio for sequencing (see Table 17).

**Determine the Library Size Distribution of the Pooled Libraries**

Run 1 μL of each pooled library in triplicate on an Agilent HS DNA chip to check for library size distribution and quantity (see Figure 11 and Figure 12) following the manufacturer’s instructions and using Table 15 to record your results.
Table 15. Agilent Bioanalyzer measurements

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS DNA chip 1</td>
<td>1</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 2</td>
<td>2</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 3</td>
<td>3</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 4</td>
<td>4</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 5</td>
<td>5</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 6</td>
<td>6</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 7</td>
<td>7</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 8</td>
<td>8</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 9</td>
<td>9</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 10</td>
<td>10</td>
</tr>
<tr>
<td>Library pool</td>
<td></td>
</tr>
<tr>
<td>HS DNA chip 11*</td>
<td>11*</td>
</tr>
</tbody>
</table>

* (Optional) If performing tube controls, run 1 μL of PC1 or PC2 in well 11 of the Agilent HS DNA chip.

Determine the Concentration of the Pooled Libraries

Use a Qubit Fluorometer to measure and record the concentration in ng/μL of each pooled library in duplicate following the manufacturer’s instructions, and using Table 16 to record your results (a subset is shown).

Table 16. Subset of duplicate Qubit Fluorometer measurements

<table>
<thead>
<tr>
<th>Pooled Library</th>
<th>Conc. 1 (ng/μL)</th>
<th>Conc. 2 (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA Pool 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDNA Pool x...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab BC Pool 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab BC Pool x...</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequencing Parameters

Library Concentration for Sequencing

Denature each pooled REAP-seq library (Ab BC, cDNA) and PhiX Control v3 library with sodium hydroxide according to the NextSeq System Denature and Dilute Libraries Guide (Illumina,15048776). After denaturing, we recommend diluting each library to a final concentration of 1.8 pM before combining the libraries in the ratio listed below.

Table 17. Sequencing library concentration

<table>
<thead>
<tr>
<th>Library</th>
<th>Conc. (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>67</td>
</tr>
<tr>
<td>Ab BC</td>
<td>8</td>
</tr>
<tr>
<td>PhiX Control v3</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>

Sequencing Read Length

We recommend that you sequence REAP-seq libraries on the Illumina NextSeq system using the NextSeq 500/550 High Output Kit v2.5 (150 cycles, Illumina PN 20024907).

For each of the sequencer reads, we recommend that you run the following number of cycles in the order shown:

- **Read 1**: Use 26 cycles to sequence the cell BC, 8 cycles for the i7 column index, and 8 cycles for the i5 primer.
- **Read 2**: Use 75 cycles to sequence the Ab BC and cDNA.
Sequencing Sample Naming

For best results, run the C1 mRNA Sequencing High Throughput Demultiplexer Script (v2.0.1 or later) to separate the HT IFC pooled samples into single-cell samples (see Appendix C), and use the following sequencing sample naming recommendations:

- 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 “HT1912123456ControlCells-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.)
Appendix A: (Optional) Run the Tube Controls

The tube controls are used as positive and negative controls for the C1™ Single-Cell mRNA Seq HT reagents and workflow performed off the HT IFC. Prepare 2 types of tube controls: 1 or more with cells (positive control or PC), and 1 without cells (no template control, or NTC). Perform the tube control reactions (lysis, reverse transcription, and preamplification) off the IFC using the same chemistry you use to process the single cells on the IFC.

Perform the Tube Control Reactions

You can prepare 2 or 3 tube controls: either 1 NTC and 1 PC, or 1 NTC and 2 PCs (1 for each section of the HT IFC).

**NOTE** Include extra volume for a second PC in the following reaction mixes if you choose to load 2 different cell mixes into the HT IFC.

Perform Lysis

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

<table>
<thead>
<tr>
<th>Component</th>
<th>NTC (µL)</th>
<th>PC (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC tube containing cells reserved after loading IFC (see page 10)</td>
<td>—</td>
<td>2.1</td>
</tr>
<tr>
<td>Cell Rinsing Reagent (C1 HT Kit Module 1)</td>
<td>2.1</td>
<td>—</td>
</tr>
<tr>
<td>Lysis Mix A plus diluted barcodes (see page 10)</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.7</strong></td>
<td><strong>5.7</strong></td>
</tr>
</tbody>
</table>

2. Mix gently and centrifuge briefly to collect contents.

3. In a thermal cycler, run the following cell lysis protocol (takes approximately 15 min to complete).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 °C</td>
<td>3 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>25 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**Perform Reverse Transcription (RT)**

1. After the cell lysis protocol is finished, add 6.4 µL of RT Mix B (reserved from page 13) to the cell lysis products, for a total volume of 12.1 µL.

2. Mix gently and centrifuge briefly to collect contents.

3. In a thermal cycler, run the following RT protocol (takes approximately 2 hr to complete).

<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** You can store the RT reaction products in the thermal cycler overnight at 4 °C until you are ready to run the PCR reaction.

Perform Preamplification (PCR)

1. After the RT protocol is finished, prepare the preamplification reaction by combining the RT reaction products and Preamplification Mix C in the appropriate tubes of an 8-tube strip.

<table>
<thead>
<tr>
<th>Component</th>
<th>NTC (µL)</th>
<th>PC (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT reaction products (see Step 3, above)</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Preamplification Mix C (see page 12)</td>
<td>7.25</td>
<td>7.25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>13.25</strong></td>
<td><strong>13.25</strong></td>
</tr>
</tbody>
</table>

2. Mix gently and centrifuge briefly to collect contents.
3 In a thermal cycler, run the following PCR protocol (takes approximately 2 hr 10 min to complete).

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

4 When the reaction is complete, centrifuge briefly to collect contents.

**Process the Tube Controls with Harvest Samples**

1 Transfer the PCR products (from Step 4 above) to a post-PCR room.

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

3 Transfer 5 μL of the tube control samples to the appropriate wells of the Harvest Plate (see Figure 9), then continue with the protocol and process the tube control samples along with the harvested samples.
## Appendix B: IDT Custom Primer Sequences

A set of 8 custom P5 primers (i5xx) with unique barcode indices can be used to multiplex cDNA and Ab BC libraries from up to 4 IFCs in the same sequencing run. A set of 24 custom P7 primers (i7xx) with unique barcode indices are used to index and amplify Ab BC libraries and optional tube controls.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Index Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preamplification primer (Ab BC)</td>
<td>CCTTGGCACCCGAGAATTCCA</td>
<td>21</td>
<td>—</td>
</tr>
</tbody>
</table>

### Custom P5 Primers for Multiplexing Ab BC and cDNA Libraries or Multiple IFCs (i5xx)*

<table>
<thead>
<tr>
<th>Name (i5xx)</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Index Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>i501</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>TATAGCCT</td>
</tr>
<tr>
<td>i502</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>ATAGAGGC</td>
</tr>
<tr>
<td>i503</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>CCTATCCT</td>
</tr>
<tr>
<td>i504</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>GGCTCTGA</td>
</tr>
<tr>
<td>i505</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>AGGCAGAG</td>
</tr>
<tr>
<td>i506</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>TAATCTTA</td>
</tr>
<tr>
<td>i507</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>CAGGACGT</td>
</tr>
<tr>
<td>i508</td>
<td>AATGATA CGCGACCACCAGAGCTCTACTACACTTTCCCTACAGACGCTCTTCGATC*T</td>
<td>70</td>
<td>GTACTGAC</td>
</tr>
</tbody>
</table>

### Custom P7 Primers for Indexing 20 Columns of Ab BC Libraries (i7xx)

<table>
<thead>
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## Appendix B: IDT Custom Primer Sequences

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(Optional) Custom P7 Primers for Tube Controls (Ab BC)

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<th>Index Sequence*</th>
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<td>GACGTCGA</td>
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</tbody>
</table>

* Oligonucleotide sequences © 2018 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.

† For information on ordering additional custom P5 primers (i5xx), visit techsupport.fluidigm.com.
Appendix C: Data Analysis

Stand-Alone Script for Demultiplexing

Initially, only the column pool samples loaded onto the Illumina sequencer are demultiplexed from the 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 programing 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 2 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 28 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 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 HT1912123456-ControlCells-COL01, the script outputs HT1912123456-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.
cDNA Analysis

cDNA Analysis Workflow

Workflow for analysis of RNA sequencing data from REAP-seq on C1 HT:

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

Primary analysis recommendations:
 detention by TopHat2
 Gene expression calculation by Cufflink/rssem

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

- Before aligning R2 reads to the reference genome, trim the poly(A) 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 cDNA Analysis Workflow.

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

Antibody Analysis Recommendations

For antibody barcode analysis, we recommend using the CITE-seq-Count algorithm (github.com/Hoohm/CITE-seq-Count). Please refer to the manual for details. Here is an example call:

```
CITE-seq-Count -R1 TAGS_R1.fastq.gz -R2 TAGS_R2.fastq.gz -t TAG_LIST.csv -cbf X1 -cbl X2 -umif Y1 -umil Y2 -cells EXPECTED CELLS -o OUTFOLDER
```

Demultiplexing of HT column FASTQ files occurs when parameters -cbf and -cbl are set to 1 and 6, respectively, in the command. The demultiplexed data is reflected in the call outputs. We recommend writing a custom script that concatenates each HT column matrix output into a single protein expression table for your experiment and samples renamed to match the gene expression names.

Downstream analysis and visualization of the protein expression table can then be imported into Seurat. Refer to the vignette for multimodal data at satijalab.org/seurat/multimodal_vignette.html.
Appendix D: Related Documents

Go to fluidigm.com to download these related documents.

<table>
<thead>
<tr>
<th>Title</th>
<th>Document Number</th>
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<tbody>
<tr>
<td>C1™ System User Guide</td>
<td>100-4977</td>
</tr>
<tr>
<td>Minimum Specifications for Imaging Cells in Fluidigm Integrated Fluidic Circuits</td>
<td>100-5004</td>
</tr>
<tr>
<td>Using a Microscope with an Automated Stage Quick Reference</td>
<td>100-6130</td>
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<tr>
<td>The Single-Cell Preparation Guide</td>
<td>100-7697</td>
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<tr>
<td>C1 Single-Cell mRNA Seq HT Reagent Kit v2 Product Insert</td>
<td>101-3808</td>
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</table>

Appendix E: 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 the appropriate personal protective equipment (PPE): safety glasses, fully enclosed shoes, lab coats, and gloves, according to your laboratory safety practices.
- 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

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

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

**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), a publication 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 online at cdc.gov/biosafety/publications/index.htm.

Chemical Safety

The responsible individuals must take the necessary precautions to ensure that the surrounding workplace is safe and that instrument operators are not exposed to hazardous levels of toxic substances. When working with any chemicals, refer to the applicable safety data sheets (SDSs) provided by the manufacturer or supplier.

Disposal of Products

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