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The use of the Fluidigm C1 for RNA expression analyses of single cells

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Abstract

Understanding the transcriptional heterogeneity that occurs on the level of a single cell is critical to understanding the gene regulatory mechanisms underlying development and disease. Population-level whole-transcriptome profiling approaches average gene expression across thousands to millions of cells and are unable to delineate the transcriptional signature of individual cells. Considerable biological heterogeneity between individual cells arises from differences in cell lineage, environment, or response to stimulus. The development of single-cell RNA-sequencing (RNA-seq) enabled a high-resolution and unbiased, analysis of cell transcriptomes. This unit describes a procedure utilizing an automated microfluidic platform, the Fluidigm C1 system, to simultaneously isolate dozens of single cells in a size- and shape-dependent manner. The microfluidic platform processes cells in individual nanoliter-scale reactions to convert their contents into double-stranded cDNA. This cDNA is used to make dual-indexed libraries using the Illumina Nextera XT library preparation kit for eventual RNA-seq analysis.

Keywords

Single-cell; RNA-seq; Fluidigm C1

INTRODUCTION

This unit contains two protocols which describe the use of the Fluidigm C1 system for isolating single cells for RNA-seq analysis. The first protocol details how to prime and load with cells the Integrated Fluidic Circuit (IFC) (Figure 1A). The IFC is placed in the

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INTERNET RESOURCES

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

<https://www.fluidigm.com/binaries/content/documents/fluidigm/consumables/pages/single-cell-mrna-sequencing/single-cell-mrna-sequencing/fluidigm%3Aresources%5B4%5D/c1-mrna%2%80%90seq-pr-100%2%80%907168/fluidigm%3Afile>

Detailed protocol from the manufacturer which contains information on which capture sites correlate to which output well on the C1, how to implement RNA-spike ins, and use a live/dead stain to visualize cell viability on the IFC.

Fluidigm C1 Cell Preparation Guide

<https://www.fluidigm.com/binaries/content/documents/fluidigm/marketing/single-cell-preparation-guide-ebook/single-cell-preparation-guide-ebook/fluidigm%3Afile>

Detailed guide from Fluidigm which covers how to optimize cell viability, density, and buoyancy. The protocol also covers how to use ImageJ to measure cell size.

Nextera XT DNA Library Preparation Guide

https://support.illumina.com/downloads/nextera_xt_sample_preparation_guide_15031942.html

Detailed guide from manufacturer on the best practices to follow during library preparation as well as how to select index primers.

Fluidigm C1 machine and the cells are flowed through microfluidic channels for size- and shape-dependent capture. Each captured cell is visualized and cataloged before the IFC is reloaded in the C1 machine. A selected automated process begins that lyses the cells, performs reverse transcription and first-strand cDNA synthesis, followed by Polymerase Chain Reaction (PCR) amplification of resultant cDNA. The amplified double-stranded cDNA is harvested from the C1 and quantified.

The second protocol describes making dual-indexed cDNA libraries using the Illumina Nextera XT library preparation kit (Figure 1B). This procedure covers the cDNA being tagged with Illumina primers and simultaneously cut with transposons before being tagged with dual indexes which serve to give each cell a unique barcode. These individual barcoded cDNA libraries are then pooled, cleaned, and quantified before submission for next generation sequencing.

The importance of cell viability and selecting the appropriate IFC for capturing cells of a specific range of sizes is discussed.

BASIC PROTOCOL 1: Capturing cells with the Fluidigm C1 System

This protocol covers the isolation of single cells using the Fluidigm C1 machine. A fluidigm IFC is primed and loaded with a single-cell suspension. The IFC is inserted into the Fluidigm C1 machine to undergo a capture cycle. After the cells have been flowed through the IFC, the capture state of each of the 96 capture sites is visually ascertained using a microscope. The IFC is then reloaded onto the Fluidigm C1 machine and the appropriate script launched to initiate an automated process that will perform cell lysis, reverse transcription and first-strand cDNA synthesis, and amplification. These cDNA are harvested from the IFC from 96 individual wells and the amount of cDNA quantified. Below is an abbreviated protocol. For more details, plate maps, and instructions for tube controls, spike-ins, and live-dead staining see “Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing” (see electronic references).

Materials

C1 Single-Cell Reagent Kit for mRNA Seq (Fluidigm Catalogue number: 100–6201)

Contains:

C1 Preloading Reagent (purple lid)

C1 Blocking Reagent (white lid)

Loading Reagent (yellow lid)

Suspension Reagent (green lid)

C1 Harvest Reagent

SMARTer Ultra Low RNA Kit for the Fluidigm C1 System (Takara, Catalogue number: 634833) Contains:

RNase Inhibitor

3'SMART CDS Primer IIA

SMARTer Dilution Buffer

5x First-Strand Buffer (RNase-Free)

Dithioereitol (DTT)

dNTP Mix

SMARTer IIA oligonucleotide

SMARTScribe Reverse Transcriptase

Advantage 2 PCR Kit (Takara, Catalogue number: 639206) Contains:

50x Advantage 2 Polymerase Mix

10X Advantage 2 PCR Buffer

50X dNTP Mix

IS PCR Primer

PCR-Grade Water

C1 IFC for mRNA seq (5–10 μ m, 10–17 μ m, or 17–25 μ m) (Fluidigm) in 3 capture site sizes:

5–10 μ m (Catalogue number: 100–5759)

10–17 μ m (Catalogue number: 100–5760)

17–25 μ m (Catalogue number: 100–5761)

C1 System (Fluidigm, Catalogue number: 100–7000)

0.2 mL PCR Tubes, Nuclease-Free

1.5 mL Eppendorf Tubes, Nuclease-Free

Agilent 2100 Bioanalyzer

Hot-lid thermal cycler

PCR Hood

8 Channel Pipette

Magnetic Stand for 1.5 mL Eppendorf Tubes

Centrifuge for 1.5-mL tubes

Centrifuge for 96-well plates

Prepare Reagents for C1 operation

1. Thaw reagents

Thaw on ice: RNase inhibitor, 3'SMART CDS Primer IIA, 5X First-Strand Buffer, Dithiothreitol, dNTP Mix, SMARTScribe Reverse Transcriptase, 10X

Advantage 2 PCR Buffer, 50X dNTP Mix, IS PCR Primer, 50X Advantage 2 Polymerase Mix

Equilibrate to room temperature: C1 Preloading Reagent, C1 Harvest Reagent, C1 Blocking Reagent, Loading Reagent, SMARTer dilution buffer, SMARTer IIA Oligonucleotide, Suspension Reagent, C1 DNA Dilution Reagent

Note: It is important to minimize the time enzymes are not at normal storage temperature to preserve activity.

2. Prepare the Lysis Mix using the reagents in Table 1. Add in order shown. Pipet to mix and keep on ice until use.
3. Prepare the Reverse Transcription mixture by adding the reagents listed in Table 2 in the order shown. Then vortex mix for 3 seconds, centrifuge briefly, and keep on ice until use.
4. Prepare the PCR Mixture by mixing the reagents in Table 3 in the order shown. Then vortex mix for 3 seconds, centrifuge briefly, and keep on ice until use.

To preserve enzyme activity, return reagents used in step 2–4 to manufacturer's recommended storage condition after use.

Prime the IFC

5. Remove the IFC from its packaging in a pre-amplification environment PCR hood and place on a dust-free surface.

An IFC Map loading plate is included with Fluidigm C1 System and provides a helpful visual guide for loading. A more detailed guide is present in, "Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing". This protocol will refer to Figure 2.

The box containing the IFC contains a plastic tape removal tool which should be set aside for later.

6. Add 200 μ L of C1 Harvest Reagent to the 2 wells marked with red square (Figure 2). Gently press down on the lid until the pipette tip enters the accumulator chamber, then slowly disperse the C1 Harvest Reagent.
7. Add 20 μ L of C1 Harvest reagent into all wells highlighted in blue (40 in total) (Figure 2).

For this and all subsequent loaded wells, rest the pipette tip gently on the bottom, then slowly raise it while dispensing the liquid. It is CRITICAL to ensure that there are no bubbles in the bottom of the wells. If a bubble is observed, use a pipette tip to gently remove it. Carefully holding the IFC over a dark surface and using one hand to shield the IFC from light can help visualize any bubbles.

8. Add 20 μ L of C1 Preloading Reagent into the well highlighted in purple (Figure 2).

9. Add 15 μ L of C1 Blocking Reagent into the wells highlighted in green (Figure 2).
10. Add 20 μ L of Cell Wash Buffer into the wells highlighted in grey (Figure 2).
11. Peel off the white tape on the bottom of the IFC.
12. Insert the IFC into the C1 system.

Make sure the C1 is on, and then select open tray. Place the IFC on the tray, aligning the notch in the top left corner of the IFC with the top left corner of the tray. Then hit the “Load” button on the screen.
13. Run the mRNA Seq: Prime program

The C1 System should autodetect the correct set of scripts to run for each size category of IFC (with a different label at the end of each script, 1771x, 1772x, or 1773x). The first time running an IFC, make sure the appropriate script is selected. Priming will take between 10–12 minutes depending on the IFC. CRITICAL: After priming is finished, there is a 1 hour window to load the chip. To ensure the cells are loaded during this period begin step 15 while the IFC is priming.
14. When the priming script has finished hit “Eject” and remove the IFC. Place it into the PCR hood again until ready to load the cells.

Loading Cells and Inventorying Capture Sites

15. Combine cells with Suspension Reagent following Table 4 and then pipette to mix. Keep the mix on ice until loading.

Cells should be at a concentration of 66,000–333,000 cells/mL in native medium. Check the cells under a light microscope to ensure they are in a single cell suspension with no visible clumps. If there are enough cells, check the viability of the cells. Concentrations near or below 66,000 cells/mL may result in a lower number of captured cells, potentially necessitating a repeat capture cycle. Using cell concentrations higher than 330,000 cells/mL may result in clogging the channels of the IFC. If there are sufficient cells, make the cell suspension in 1 mL total volume. If there are a limiting number of cells, a smaller volume of cells to reach the desired concentration. In this case, maintain the 3:2 ratio of cells to Suspension Reagent.
16. Remove blocking solutions from green highlighted wells on IFC.

This step occurs after the IFC has been primed at placed back in a PCR hood.
17. Add 6 μ L of the Cells and Suspension Reagent mix to the well with the white arrow pointing towards it with the words “Load Cells” (Figure 2).

Pipette mix Cells and Suspension Reagent mix 10 times before loading to ensure the cells are not clumped and evenly mixed.
18. Add 20 μ L of Cell Wash Buffer into the well highlighted in pink (Figure 2).

19. Place IFC into the C1 and select the script “mRNA Seq: Cell Load”

Just as during IFC priming, the “mRNA Seq: Cell Load” script is numbered 1771x/1772x/1773x depending on the type of IFC run. The type of IFC also determines how long cell loading takes. 5–10 μ M takes 15 minutes. 10–17 μ M and 17–25 μ M take approximately 30 minutes. When the script is finished running an Eject button will appear. Stopping the cell loading script before this will compromise the experiment.

20. Remove the IFC from the C1 system and image the cells

The capture sites are located in the center of the IFC (Figure 2, black outline). A standard light microscope with a 10x or 20x lens can image the cells. Alternately, microscopes with automated platforms can be programmed to scan all of the capture sites. Regardless, an inventory of the occupancy of the capture sites is necessary to determine how to proceed. Make log with 96 entries, one for each capture sites, and list the contents at each site (example: 0 cells, 1 cell, multiple cells, debris, presence of a fluorescent label, live cell, dead cell, big cell, small cell). It is important to take this inventory as quick as possible.

If a sufficient number of cells have been captured for the experiment or if there are no vacant capture sites (capture sites can contain single cells, multiple cells, or potentially even debris), then proceed to the Cell Harvest steps. An inventory of the observed contents of each capture site is critical for interpreting the data in later steps.

If an insufficient number of cells have been captured (can depend on the needs of the experiment, the fragility of the cells) then cell loading may be repeated. Make sure that the capture sites are unoccupied with no visible signs of clogs (clumps of cells blocking the channels will prevent a repeat loading from being effective). To rerun, add another 6 μ L of cells to the well with the white arrow pointing to it with the words “Load Cells.” Place the IFC back into the C1 system and rerun the “mRNA Seq: Cell Load script.” After the script has run again (it will take the same time as before) reimage the capture sites and inventory their contents as before, even the capture sites that had a single cell. In the reloading process, these cells may have been knocked loose or an additional cell may have entered the capture site.

Reverse transcription and amplification of the IFC

21. Add 180 μ L of C1 Harvest Reagent into the four larger wells outlined in red and highlighted in blue (Figure 2).

Perform this and the subsequent IFC loading steps in a preamplification PCR hood. When pipetting, place the tip by the inlet (on the side of the each of the four wells closest to the capture sites of the IFC) and slowly add liquid while drawing the pipette to the far side of the well. Again, ensure there are no bubbles on the inlet of the well. Bubbles on the surface of the liquid will not impede IFC function.

22. Add 9 μ L of Lysis Mix to the well labeled 3 (Figure 2, highlighted in orange with red outline).
23. Add 9 μ L of Reverse Transcription Mix prepared earlier to the well labeled 4 (Figure 2, highlighted in yellow with red outline).
24. Add 24 μ L of PCR Mix prepared earlier to wells 7 and 8 (Figure 2, highlighted in black with red outline).
25. Check all loaded wells for bubbles. Remove any bubbles using a pipette tip.
26. Insert IFC into the C1 system.
27. Run the “mRNA Seq: RT & Amp” script.

When running the RT & Amp script it will ask for an ending time. The approximate minimal run time is 7.75 hours for the 5–10 μ M IFC and 8.5 hours for the 10–17 μ M and 17–25 μ M IFCs. The script can also be set to run overnight. Critical: the IFC must be removed from the C1 System and the output harvested within an hour of the selected end time. The thermocycler protocol run by the C1 at this step can be found in the “Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing.”

28. Remove the IFC from the C1 System.

The IFC should be taken to and all subsequent steps should be undertaken in a post-amplification environment.

29. Add 10 μ L of C1 DNA Dilution Reagent into each well of a 96-well plate.

The double stranded cDNA output from the IFC will be diluted on this 96-well plate. Different cell types can have different mRNA content and thus may have either higher or lower cDNA yields when isolated by the Fluidigm C1 system. If the cells being used have low cDNA yield then the volume of DNA Dilution Reagent should be lowered. A minimum of 0.1 ng/ μ L of cDNA per cell after dilution is needed for input for library preparation.

30. Use the tape removal tool set aside in step 5 to remove the white tape covering the outlet wells (Figure 3).

Place the pointed end of the removal tool in the circular opening of each strip of white tape (4 in total) and pry up the edge of the tape. Peel the tape slowly off. It is important to not jostle or otherwise cause cross contamination between wells at this point. If the removal tool is lost. It is possible to use the edge of a straight razor to carefully peel up the corner of tape.

31. Use an 8-channel pipette set to 3.5 μ L to carefully remove the cDNA from all of the wells labeled with a black 1 (Figure 3) and pipette into the first column of the 96-well plate from step 29.

A standard 8 channel pipette made for 96-well plates should have its tips spaced to skip every other well on the IFC. The volume removed from each well may vary so some of the pipette tips may have less liquid in them than others. It is

imperative to carefully remove the liquid from the output wells so as to not cross contaminate the samples. Pipette tips must be changed immediately after pipetting the output into the 96-well plate.

32. Repeat step 31 for each set of wells labeled with a number (Figure 3) in increasing order.

The wells labeled 2 should be pipetted into column 2 of the 96-well plate from step 29. The wells labeled 3 should be pipetted into column 3 and so on for all 12 sets of output wells. It is important to use the order shown so that each well can be traced back to a specific capture site. For which capture site corresponds to which well please see “Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing.”

Use fresh pipette tips for each set of numbers. If one of the pipette tips has no liquid, only use a fresh, clean tip to attempt to remove cDNA from the corresponding well.

33. Seal the plate using 96-well plate adhesive film.

Firmly seal the plate by applying pressure between the tops of wells. It is critical to ensure that there is no contamination between wells and that low volume (~13 μ L) of liquid does not evaporate.

34. Vortex the 96-well plate for 10 seconds and centrifuge briefly to collect the contents of each well.

This plate may be stored long term at -20°C or for a week at 4°C .

35. Using a small aliquot (~1 μ L) quantify the amount of cDNA from each well.

Several methods are available, such as the High Sensitivity DNA Kit for the Agilent 2100 Bioanalyzer. See the Agilent High Sensitivity DNA Kit User Manual for instructions. Other methods of quantifying cDNA concentration can also be used, such as PicoGreen (Quant-IT, PicoGreen dsDNA Assay kit) or Tape Station (Agilent Technologies, High Sensitivity D5000 ScreenTape). See the appropriate manufacturer's protocol.

BASIC PROTOCOL 2: Nextera XT Library Preparation for Next Generation Sequencing

In this protocol the double-stranded cDNA generated from individual cells in the IFC is diluted to begin DNA library preparation. The cDNA from each outlet well of the IFC is simultaneously fragmented and adaptor sequences are added to the ends of the fragments to enable subsequent PCR amplification. This PCR amplification provides sufficient material for sequencing while also attaching Nextera XT index sequences that enable the libraries of each outlet well to be distinguished. After amplification, the barcoded libraries from outlet wells are pooled and then cleaned using AMPure beads. The pooled library is then quantified using a bioanalyzer or tape station before undergoing next generation sequencing. For details on the original Nextera XT protocol see “Nextera XT DNA Library Preparation

Guide.” For details on this protocol modified for single cell use see “Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing.”

Materials

cDNA from Basic Protocol 1

Nextera XT DNA Sample Preparation Kit (Illumina, FC-131–1096) Contains:

TD (Tagment DNA Buffer)
ATM (Amplicon Tagment Mix)
NT (Neutralize Tagment Buffer)
NPM (Nextera PCR Master Mix)
RSB (Resuspension Buffer)

Nextera XT DNA Library Preparation Index Kit (Illumina, FC-131–1002) Contains:

Index 1 Primers
Index 2 Primers

Agencourt AMPure XP PCR purification kit (60 ml, Beckman Coulter, cat. no. A63881)

80% (v/v) ethanol made with nuclease-free H₂O

Diluting output cDNA from Fluidigm C1 to 100–300 pg/μL

1. Label a new 96-well plate “Dilution Plate” and use an 8-channel pipette to transfer 2 μL of each sample from the harvest plate to the Dilution Plate

It is critical to keep track of which sample corresponds to which well on the output plate and thus which capture site. To simplify this, pipette samples from the output plate into the same well (column and row) of the Dilution Plate if possible. If this is not feasible (for example, many of the output plate wells yielded no cDNA), then make a careful inventory of which wells of the output plate are being transferred to which wells of the dilution plate. During this process, it is still critical to avoid cross contamination. Always use fresh pipette tips when transferring the sample.
2. Dilute each sample to achieve a final concentration of 100–300 pg/μL cDNA per well by adding the corresponding volume of cDNA dilution reagent

For example, if the concentration of a sample is 900 pg/μL, then it needs to be diluted 3x to achieve an appropriate concentration.
3. Seal the Dilution Plate with adhesive film, vortex mix at medium speed for 20 seconds, and collect the contents of the wells by centrifuging at 275 xg for 5 minutes.

The contents of the dilution plate will be stable long term at –20°C or up to a week at 4°C. Keep at 4°C until proceeding with tagmentation.

Tagmentation

4. Thaw reagents, then mix contents by gently inverting tubes, and centrifuge briefly to collect.

Thaw Tagment DNA buffer and NT buffer to at room temperature. If precipitate is observed in the NT buffer, vortex until precipitate is gone and centrifuge to collect.

5. Prepare the following tagmentation reaction mix in a 1.5 mL PCR tube, adding components in order shown in Table 5. Then vortex mix for 20 seconds on low speed, and centrifuge briefly.

An overage of 25% is recommended by Fluidigm when prepping multiple samples (the most common use case). For example, for 96 samples, use 300 μL of Tagment DNA buffer. The calculation is as follows: $2.5 \mu\text{L}/\text{sample} \times 96 \text{ samples} = 240 \mu\text{L}$. Then, $240 \mu\text{L} \times 25\% \text{ overage} = 60 \mu\text{L}$. Finally, $60 \mu\text{L} + 240 \mu\text{L} = 300 \mu\text{L}$ total). To use reagents more efficiently, the percentage of overage may be cautiously reduced to 10%.

6. Pipet 3.75 μL of tagmentation reaction mix into a 96-well plate labeled “Library Prep.”

For processing more than 8 samples, aliquot equal volumes of the tagmentation reaction mix into an eight-tube strip for ease of use with an 8-channel pipette. For example, if processing 96 samples using 300 μL of tagmentation reaction mix add 50 μL per tube in the eight-tube strip ($300 \mu\text{L} / 8 \text{ tubes} = 50 \mu\text{L}/\text{tube}$). To simplify keeping track of which samples came from which capture site on the IFC, add the 3.75 μL of tagmentation reaction mix to the wells in the library prep plate that correspond to the wells of the dilution plate with samples.

7. Add 1.25 μL of the diluted sample from the diluted sample plate to the library prep plate.

The timing of the tagmentation reaction is critical therefore steps 8 through 14 must be done briskly with minimal pause.

8. Seal library prep plate with adhesive cover and vortex at medium speed for 20 seconds.

9. Centrifuge library prep plate at 1,968 $\times g$ for 5 minutes.

10. Immediately put library prep plate into a thermal cycler and run the program in table 6.

The lid of the thermal cycler should be heated to prevent condensation. Remove plate immediately once sample temperature has reached 10°C .

11. As the library prep plate incubates, aliquot NT buffer into an eight-tube strip. The total volume needed is 1.25 μL of NT buffer per sample plus 25% overage.

For example, 96 samples would require 150 μL of NT buffer. To calculate: $1.25 \mu\text{L}/\text{Sample} \times 96 \text{ Samples} = 120 \mu\text{L}$ NT buffer. To add overage, 120 μL NT buffer

+ 30 μL (25% of 120 μL) = 150 μL . To aliquot, 150 μL / 8 tubes = 18.75 μL /tube of NT buffer in the eight-tube strip.

12. Immediately after sample reaches 10°C remove from thermocycler and add 1.25 μL of NT buffer each sample to neutralize the tagmentation reaction using an 8-channel pipette.
13. Seal library prep plate with adhesive film, vortex at medium speed, and centrifuge at 1,968 xg for 5 minutes.

Amplify the DNA

14. Add 3.75 μL of NPM to each sample in the library prep plate.

First, aliquot the NPM into each tube of an eight-tube strip. For example, to calculate for 96 samples: 3.75 μL /sample $\times$ 96 samples = 360 μL . To add overage to account for pipetting errors use 25% overage, so .25 $\times$ 360 μL = 90 μL . The total volume to aliquot is 360 μL + 90 μL overage = 450 μL of NPM.

Note: NPM is the limiting reagent in the Nextera kit. It is possible to use a smaller overage (15%) or to carefully save the excess NPM in a labeled PCR tube after adding required amounts to the library plate.

NPM should be taken out of -20°C storage just before this step and kept on ice. To ensure enzyme activity is not diminished, return NPM to storage as soon as possible.

15. Select an Index 1 primer (N7xx) for each column of the library prep plate which contains samples. Then select an Index 2 primer (N5xx) for each row of the library prep plate which contains samples.

If all 96 wells contain sample, then 12 Index 1 primers and 8 Index 2 primers will be selected. Refer to the “Illumina Nextera XT DNA Library Preparation Guide” for information on which index primers to select when using fewer Index 1 or Index 2 primers.

16. Add 1.25 μL of the selected Index 1 Primers (N7xx) to each well in the corresponding row (labeled A-H) of the library prep plate using an 8- or 12-channel pipette.

It is critical to not cross contaminate the Index primers and extreme care must be taken while pipetting. Additionally, if many Index 1 or Index 2 primers are being used, discard the white and orange caps and replace them after steps 17 and 18 with fresh caps from the Index Primer kit from Illumina which comes with extra white and orange caps. Additional extra caps may be ordered as well if many experiments are to be performed. While the caps are off the index primer tubes the tubes should be covered if library prep is not done in a PCR hood. The lid of a box of pipette tips can serve this role.

17. Add 1.25 μL of the selected Index 2 Primers (N5xx) to each well in the corresponding column (labeled 1–12 on a 96 well plate) of the library prep plate using an 8-channel pipette.

18. Seal library prep plate with adhesive film, vortex at medium speed for 20 seconds, and centrifuge at 1,968 xg for 2 minutes.
19. Place plate into thermal cycler and run the program on Table 7.
Reaction volume is 12.5 μ L. Lid should be heated to avoid condensation.
After amplification is complete product may be stored at -20°C .

Pooling and cleaning the library

20. Warm Agencourt AMPure XP beads to room temperature. Vortex beads for 1 minute to resuspend them.
21. Choose the number of samples to pool.
Samples can be pooled after cleaning and quantification to ensure only successfully made libraries are submitted for sequencing.
22. Pool samples together using a volume dependent on samples to be pooled. For up to 12 samples use 4 μ L of each sample to pool. For up to 24 samples use 1 μ L of each sample to pool. For up to 96 samples use 1 μ L to pool. Make note of the final pooled sample volume.
For example, for 96 samples 1 μ L of each sample the final volume would be 96 μ L.
23. Add a volume of AMPure beads equal to 90% of pooled sample volume.
For example, for 96 samples pooled in one 96 μ L pooled sample, add 87 μ L of AMPure beads. Vortex mix beads for 1 minute before adding to pooled sample. For processing sample individually without pooling, use the full volume of each sample, 12.5 μ L, and add 11.25 μ L of AMPure beads.
24. Mix samples by pipetting up and down 5 times.
25. Incubate samples at room temperature for 5 min.
26. Place the tube on a magnetic stand for 2 minutes.
The solution should clear and a brown pellet will form on the PCR tube wall nearest the magnet. For processing samples individually, a magnet accommodating 96-well plate is required.
27. Without removing the sample from the magnetic stand, add 180 μ L of freshly prepared 70% ethanol and incubate for 30 seconds.
The PCR tube's lid should be closed or covered for all incubation steps. For processing individual samples in a 96 well plate, cover the plate during incubation when not performing library prep in a PCR hood. Using an 8- or 12-channel pipette will expedite library clean up.
28. Carefully remove and discard the ethanol.
29. Repeat steps 28 and 29.

- 30.** Allow beads to air-dry for 10–15 minutes.
- Do not close tube during incubation but do cover it with a pipette tip box lid.
- 31.** Add a volume of C1 DNA dilution reagent to the beads equal to the initial pooled library volume to elute the sample.
- 32.** Vortex mix the tube for 3 seconds, flick tube downwards to collect contents at the bottom of the tube, and incubate it at room temperature for 2 minutes.
- 33.** Place pooled sample tube on magnetic stand for 2 minutes.
- 34.** Transfer supernatant to a new PCR tube.
- Aspirate supernatant slowly to ensure no beads are taken up with it. If the bead pellet is disturbed and beads are observed in the pipette after aspiration return it to the tube on the magnetic plate and repeat steps 34 and 35. The brown beads can be visualized by holding pipette tip over a white background.
- 35.** Repeat cleanup by repeating steps 24–35 one time, except on step 32, use 1.5x the original pool volume of C1 DNA Dilution Reagent.
- For example, for 96 samples with a pooled sample volume of 96 μL , use 144 μL of C1 DNA Dilution Reagent.
- 36.** Quantify pooled library using Agilent Tape Station or Agilent Bioanalyzer. Refer to manufacturer's protocol for details.
- Refer to the appropriate manual for the model of Illumina sequencer to be used for desired final library concentration. Diluting pooled library to this concentration can be done with C1 DNA Dilution Reagent.
- For quantification of individual libraries, high sensitivity Agilent Bioanalyzer chips or Agilent Tape Station Tapes can be used. For larger numbers of individual libraries (>11), use the Agilent Tape Station to achieve higher throughput. After quantification, individual libraries may be pooled as desired.
- Typical cDNA libraries will be between 150 to 1500 base pairs in size. If a significant primer dimer is observed (Figure 4), then cleanup steps can be repeated to reduce it.

COMMENTARY

Background Information

RNA sequencing is broadly an approach wherein RNA from cells is converted into cDNA libraries which are analyzed using next-generation sequencing to determine the quantity and make up of the individual transcripts contained within the transcriptome of a biological sample. The initial development of RNA sequencing provided many key insights into the heterogeneity of RNA species and the dynamic nature of the transcriptome. The amount of RNA needed for these protocols necessitated that RNA from hundreds-of-thousands to millions of cells be pooled together for a single experiment. This requirement limited the

ability of pooled RNA sequencing approaches to resolve the considerable transcriptional heterogeneity that has long been appreciated to exist between individual cells.

Not long after first bulk RNA sequencing experiments were performed, though, a protocol to sequence transcriptomes from single cells was developed (Tang et al., 2009). In the following years more protocols were developed, with different strategies to improve the quality of the libraries and account for the PCR amplification required by the vanishingly small starting amounts of RNA (Hashimshony, Wagner, Sher, & Yanai, 2012; Islam et al., 2014; Picelli et al., 2013). These advances in the library preparation were eventually met and enabled similar advances in cell isolation strategies which allowed a higher throughput than the initial micromanipulation techniques (Tang et al., 2009; Xue et al., 2013). High throughput cell isolation strategies range from Fluorescence Activated Cell sorting (FACs) cells into multi-well plates (Hayashi et al., 2010; Jaitin et al., 2014), capturing cells in a microfluidic device (Pollen et al., 2014), or encapsulation of cells in micro-droplets (Klein et al., 2015; Macosko et al., 2015; Zheng et al., 2017).

Thus, modern single-cell RNA-seq approaches enable a high-resolution, unbiased, and high-throughput method to investigate the transcriptomes of individual cells. Currently, in the rapidly developing field of single-cell RNA-seq there is no consensus best protocol but rather specific niches for each methodology governed by the biological system and the specific questions being asked (reviewed in (Haque, Engel, Teichmann, & Lonnberg, 2017; Kolodziejczyk, Kim, Svensson, Marioni, & Teichmann, 2015; Ziegenhain et al., 2017). This also holds true for the bioinformatic tools developed to address the unique challenges presented by single-cell RNA-seq data sets that are not found in their bulk sequencing counterparts (Dal Molin, Baruzzo, & Di Camillo, 2017).

Critical Parameters

Cell Viability—An effective cell isolation protocol that yields viable and single cells is critical for the success of single-cell RNA-seq experiments. Dead or dying cells can have degraded mRNA which yields low complexity libraries. Additionally, masses of dying cells can cause contamination in the sample with leaked mRNA or hard to see blebs from dying cells contaminating the capture sites of otherwise viable cells. To mitigate this risk, it is necessary to confirm ahead of the single-cell experiment that the isolation procedure used for the experiment yields high (>80%) viability cells. If not, then the procedure must be modified or a method of separating the viable from the dead cells, such as FACs sorting, should be implemented. For primary cells, this may involve changing the enzyme mixture, using less harsh conditions, and/or keeping the cells at 4°C whenever possible. Alternatively, if the digestion protocol is too gentle it may result in clumps of cells. These clumps may clog the inlet of the IFC or lead to multiple cell occupancy of capture sites, preventing the isolation of single cells.

Cell Size—Cell size is an important variable when designing an experiment for the Fluidigm C1 system. Depending on the range of cell sizes present, one of the 3 IFCs (5–10 µM, 10–17 µM, 17–25 µM) may be used. It is very possible that the range of cell sizes, particularly in heterogenous cell populations, may overlap the size ranges one IFC (example:

cells 8–12 μM in size). In this case it may be necessary to perform multiple experiments using different sizes of IFCs to fully capture the whole cell population or to utilize a different cell isolation strategy that is more size agnostic, like tube or droplet based single-cell capture strategies. Generally, loading cells too small for a given IFC will result in poor capture efficiencies as the cells are too small to be trapped in the capture sites. Conversely loading cells larger than the listed IFC size range may result in clogging of the chip. This can be observed under a microscope by focusing on the well cells are pipetted into and following the channel with cells to the capture sites. Additionally, the sizes listed for each IFC assume a rounded cell and that cells with irregular shapes, like adult cardiomyocytes which are rectangular, may not be captured efficiently and require an alternate isolation strategy.

RNase—RNase is an omnipresent enzyme that degrades RNA and can compromise RNA quality during any RNA-seq experiment. Thus, when working with RNA it is critical to keep an RNase free environment in which to work. RNase neutralizing chemicals should be used to clean the bench surface and pipettes before the relevant steps of the protocol are undertaken.

Troubleshooting—See Table 8.

Understanding Results—The end result of this protocol is a pooled cDNA library which can be quantified using an Agilent Tape Station or Bioanalyzer. A successful run will produce a library with cDNA detected between 150 and 1500 bp (Figure 3). If there are issues with the library preparation protocol than little or no cDNA may be observed. If no library is observed, then the experiment may need to be repeated.

Time Considerations—The first protocol takes approximately 2 hours until the Fluidigm C1 is loaded with the IFC for reverse transcription, cDNA synthesis, and amplification. This script takes between 467 or 515 minutes depending on the IFC to run, but can be delayed until the next morning.

The second protocol takes between 2 to 4 hours to complete.

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Figure 1:

Overview of using the Fluidigm C1 system to capture single-cells and generate cDNA libraries for next-generation sequencing as described in (A) Basic Protocol 1 and (B) Basic Protocol 2. Sequences are not drawn to scale.

Figure 2:
Loading guide for Fluidigm C1

Figure 3:
Sample harvest guide for Fluidigm C1

Figure 4:

Expected results from the quantification of pooled single-cell libraries. Pooled libraries from embryonic heart cells quantified using an Agilent Tape Station D1000 high sensitivity screen tape.

Table 1:

Lysis Mixture

Component	Volume (μL)
Loading Reagent	1
RNase Inhibitor	0.5
3' SMART CDS Primer IIA	7
Clonotech Dilution Buffer	11.5
Total Volume	20

Table 2:

Reverse Transcription Mixture

Component	Volume (μL)
Loading Reagent	1.2
5X First-Strand Buffer	11.2
Dithiothreitol	1.4
SMARTer IIA Oligonucleotide	5.6
RNase Inhibitor	1.4
SMARTScribe Reverse Transcriptase	5.6
Total Volume	32

Table 3:

PCR Mixture

Component	Volume (μl)
PCR-Grade Water	63.5
10X Advantage 2 PCR Buffer	10
50X dNTP Mix	4
IS PCR primer	4
50X Advantage 2 Polymerase Mix	4
Loading Reagent	4.5
Total Volume	90

Table 4:

Cell Suspension

Component	Volume (μL)
Cells	60
Suspension Reagent	40
Total Volume	100

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Table 5:

Tagmentation Mixture

Volume per sample (μL)	Component
2.5	Tagment DNA Buffer
1.25	Amplification Tagment Mix
3.75	Total Volume Per Sample

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Table 6:

Nextera Incubation

Temperature	Time
55°C	10 min
10°C	Hold

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Table 7:

Nextera Thermal Cycler Program

Temp	Time	Cycles
72°C	3 min	1
95°C	30 sec	1
95°C	10 sec	12
55°C	30 sec	12
72°C	60 sec	12
72°C	5 min	1
10°C	Hold	1

Table 8:

Trouble Shooting

Issue	Possible Cause	Action
No cells in capture site	Possibly caused by clogs in inlet, too few cells loaded, cells too small for chip size.	If no clog is visible and all reagents were properly loaded then rerun capture cycle up to 2 times after carefully recalculating cell concentration. Recalculate cell size to ensure correct IFC is being used.
Too many cells in capture site, clumps	Loading too many cells, loading cells too large for selected chip, incomplete digestion of sample.	Reduce cell concentration for future experiments with given cell type. Adjust cell isolation protocol to minimize clumps. Select size appropriate IFC. Use FACS to separate to pre-isolate cells of appropriate size.
No liquid in outlet wells of IFC	Clog, failure to properly prime IFC, failure to add harvest reagent to all appropriate wells, bubbles in IFC loading wells.	If clumps observed, adjust cell isolation protocol. At any step before loading IFC into Fluidigm C1 machine carefully check each well that reagents were added to ensure that they are appropriately loaded and that no bubbles are present at the bottom of the wells.
No cDNA detected after IFC amplification for individual sample(s)	Poor cell viability, presence of debris, Rnase contamination, low RNA content of cell	Check viability of cells and revise isolation protocol if necessary to maximize viability while reducing debris. FACS, Density separation, or other sorting may be done to remove debris. Ensure work area is clean with Rnase degrading chemicals.
No cDNA detected after IFC amplification in all samples	Clog, reagent missing mixture or not loaded into IFC, loss of cell viability during preparation.	Check for clog, cell viability, that cells are of the appropriate size for IFC, and that each well is appropriately loaded and that no bubbles are present at the bottom of the wells.