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Single-Cell Library Preparation of iPSC-Derived Neural Stem Cells

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Abstract

Single-cell RNA-seq technology allows for the identification of heterogeneous cell populations, measures stochastic gene expressions, and identifies highly variable genes. Thus, with this technology it is possible to identify relevant pathways involved in development or in disease progression. Herein, we describe a protocol to capture and process single-cell transcriptomes that will be used for RNA sequencing. This chapter discusses the use of the Fluidigm C1 System and Integrated Fluidic Circuit microfluidics system, TapeStation 4200, SMART-Seq v4, Nextera XT Library Preparation Kit, and AMPure XP beads.

Keywords

Single cell; Neural stem cells; Fluidigm; C1; Library preparation; RNA-seq; Single-cell RNA-seq; Nextera XT; Next-generation sequencing; SMART-Seq

1 Introduction

Next-generation sequencing (NGS) is a high-throughput and cost-effective means of sequencing genomic material [1]. With NGS technology, sequencing whole transcriptomes has become possible. RNA sequencing (RNA-seq) is used to measure average expression levels for genes across a population of cells. It is useful for comparative transcriptomics and quantifying expression patterns. However, each cell is unique with its own gene expression profile. As bulk RNA-seq represents a large population of cells, there may be bias toward a subpopulation with high expression and therefore be insufficient when dealing with heterogeneous systems [2]. Averaging of these expressions does not represent the individual cells, and rare cell types may remain hidden. Single-cell RNA-seq (scRNA-seq) is a relatively new technology that solves these issues with bulk RNA-seq. Unlike bulk RNA-seq, scRNA-seq measures the distribution of gene expression levels in single cells, which allows for the observation of cell-specific transcriptomic changes. scRNA-seq allows us to identify cell types in heterogeneous cell populations, measure the stochastic expression profile of individual cells, and identify highly variable genes across the sample population [3]. Furthermore, scRNA-seq enables the identification of pathways involved in cell development, physiology, or disease pathologies.

Single-cell technology has found a niche in stem cell biology and can be used for studying cellular diversity, identifying subpopulations in heterogeneous culture systems and stem cell-based disease models. There are many methods available for capturing single cells for analysis. Manual cell picking, fluorescence-activated cell sorting (FACS) [4], magnetic-activated cell sorting (MACS) [5], laser capture microdissection (LCM) [6], or microfluidics [7] are some examples. Single-cell analyses tend to be challenging to properly perform in capturing single cells and in working with small-scale template material amplification for high-throughput sequencing. Importantly, having a reliable pipeline will ensure the single-cell analysis is reliable.

To begin preparing the samples for sequencing, first extract genomic material (exome, transcriptome, or whole-genome) from the individual cells for the generation of cDNA libraries. This chapter covers the use of Fluidigm's C1 Integrated Fluidic Circuits (IFC) microfluidic system with SMART-Seq v4 chemistry and Illumina Nextera XT Library Preparation Kit for the generation of sequencing libraries. It will cover every step from cell preparation to sequencing (Fig. 1).

2 Materials

2.1 Thawing Reagents

1. Warm Cell Wash Buffer, C1 Preloading Reagents, C1 Blocking Reagents, and C1 Harvest Reagent to room temperature.
2. Thaw C1 Suspension Buffer on ice and vortex well before use.
3. Thaw 5× Ultra Low First-Strand Buffer at room temperature.
4. Thaw all remaining reagents for first-strand cDNA syntheses (except enzyme) on ice.
5. Gently vortex each reagent and spin down.
6. Store all but 5× Ultra Low First-Strand Buffer on ice.

2.2 Cell Buoyancy Test

When utilizing the C1 microfluidic system, it is imperative that cells float in suspension and it is essential to test buoyancy of single cells.

1. Dissociate the stem cell colonies or clusters into single cells with enzyme of choice.
2. Centrifuge at 1000 rpm ($200 \times g$) for 5 min.
3. Perform a serial dilution of the cell suspension with Fluidigm's Suspension Reagent. Refer to Table 1.
4. In a clear, flat-bottomed 384-well plate, add in 20 μ L per well of the cell suspension mix.
5. Let suspensions sit for 10 min, and exam each well for even cell distribution throughout the entire volume of the well.

6. The best ratio of cell mix and suspension reagent will be used for the actual experiment.

2.3 Prepare RNA Spike-in Mix

The concentration of RNA spike-in varies depending on the cell type. To determine required dilution, consult pertinent literature. For neural stem cells, the concentration used is anywhere between 1:100000 and 1:400000. When using the ERCC RNA spike-in mix (ThermoFisher), first dilute the stock spike-in mix. Follow the series dilution in the table below (Table 2). Note that only 1 μ L of spike-in is needed for the cell lysis mix.

2.4 Prepare 10 $\times$ Reaction Buffer

1. Mix the following reagents and keep on ice. Refer to Table 3.
2. Pipet up and down to mix and spin down briefly. Keep on ice until ready to use.

2.5 Prepare Cell Lysis Mix

1. Mix the following in a tube labeled “lysis” and keep on ice (*see* Note 1). Refer to Table 4.
2. Pipet the cell lysis mix up and down a few times to mix and spin down. Keep on ice until ready to use.

2.6 Prepare RT Mix

1. Mix together all *except* SMARTScribe Reverse Transcriptase in a tube labeled “RT,” and keep on ice. Add in SMARTScribe Reverse Transcriptase just before pipetting RT mixture into IFC. Refer to Table 5.
2. Gently vortex and spin down. Keep on ice until use (*see* Note 2).

2.7 Prepare PCR Mix

1. Mix together in a tube labeled “PCR” and keep on ice. Refer to Table 6.
2. Gently vortex for 3 s and centrifuge briefly. Keep on ice until ready to use.

3 Methods

3.1 Priming IFC Chip

Required reagents: C1 Harvest Reagent, C1 Preloading Reagent, C1 Blocking Reagent, and Cell Wash Buffer. Avoid creating air bubbles in inlets of chip during the priming process.

1. Place C1 IFC onto the provided IFC map. Refer to Fig. 2 for Subheading 3.1.
2. Add 200 μ L of C1 Harvest Reagent into the accumulators which are marked with red circles (2 total). Push down on the O-ring with the pipet tip and inject reagent.

¹-If using an RNA spike-in, add 1 μ L of RNA spike mix instead of C1 loading reagent.

²-Do not vigorously vortex the RT mixture.

3. Swirl IFC gently to distribute the reagent evenly.
4. Pipet 20 μ L of C1 Harvest Reagent into inlets marked with solid red circles on each side of the accumulators (36 total).
5. Pipet 20 μ L of C1 Harvest Reagent into the two inlets marked with small red circles in the middle of the outside columns of inlets on each side of the IFC (4 total).
6. Pipet 15 μ L of C1 Blocking Reagent into the cell inlet and outlet marked with yellow dots (2 total).
7. Peel off white tape on bottom of IFC. Do not peel the four pieces of tape that cover the harvest inlets.
8. Place the IFC into the C1 system.
9. Run the SMART-seq v4: Prime (1861x/1862x/1863x) script.
10. Priming takes approximately 10 min.
11. When the Prime script has finished, tap EJECT to remove the primed IFC from the instrument (*see* Note 3).

3.2 Cell Preparation

This process can be performed while the IFC chip is being primed.

1. Isolate neurospheres from culture.
2. Centrifuge to form a pellet and remove supernatant.
3. Wash with phosphate-buffered saline (PBS).
4. Centrifuge to form a pellet and remove supernatant.
5. Add 1 mL of Accutase and resuspend the pellet.
6. Incubate the suspension at 37 °C for 5 min.
7. Triturate cell suspension and add 5 mL of fresh media.
8. Centrifuge to form pellet and remove supernatant.
9. Resuspend cells with 1 mL of fresh media.
10. Count the cells and measure viability with method of choice e.g. Countess (ThermoFisher).
11. Use 10 μ L of the cell suspension with 10 μ L of Trypan blue. Load the suspension into cell counting chamber slides and quantify with Countess.
12. Prepare a cell suspension in native medium of 66,000–333,000 cells/mL (*see* Note 4).

³.After priming the IFC, there is up to 1 h to load the IFC with the C1 system.

⁴.The recommended concentration range ensures that a total of 200–1000 cells are loaded into the IFC.

13. Suspend the cells in a final volume of 0.5–1 mL to ensure enough cells are viable for the IFC and tube controls.
14. Prepare the cell mix by combining cells with suspension reagent at the ratio optimized in advance (*see* Note 5).

3.3 Loading Cells

1. Use pipet to remove blocking reagent from inlets (marked with teal and gray dots). Refer to Fig. 3 for Subheading 3.3.
2. Set a pipet to 12 μ L and slowly pipet cell mix 5–10 times to mix and dissociate any clumps (*see* Note 6).
3. Pipet 24 μ L of C1 Preloading Reagent into Inlet 2 on the left side of chip (marked with purple dot).
4. Pipet 7 μ L of Cell Wash Buffer into Inlet 6 on the right side of the chip (marked with pink dot).
5. Pipet 5 μ L of cell mix into inlet marked with teal dot (*see* Note 7).
6. Place the IFC into the C1.
7. Run the SMART-Seq v4: Cell Load (1861 $\times$ /1862 $\times$ /1863 $\times$).
8. When the script has finished, tap EJECT to remove the IFC from the C1 system.
9. At this point, check each capture chamber under a phase contrast microscope, and note which chambers exhibit successful single-cell capture and which were unsuccessful.

3.4 Run Lysis, Reverse Transcription, and PCR on the C1

1. Aspirate solution in the flowthrough outlet (marked with gray dot). Refer to Fig. 4 for Subheading 3.4.
2. Pipet 180 μ L of C1 Harvest Reagent into the four reservoirs marked with large solid red rectangles.
3. Pipet 7 μ L of lysis Mix A in Inlet 3 (marked with an orange dot).
4. Pipet 8 μ L of RT Mix in Inlet 4 (marked with a yellow dot).
5. Pipet 24 μ L of PCR Mix C in Inlets 7 and 8 (marked with blue dots).
6. Place the IFC into the C1 system.
7. Run the SMART-Seq v4: Sample Prep (1861 $\times$ /1862 $\times$ /1863 $\times$) script. Run time for this operation is 8 h and can be performed overnight (*see* Note 8)

⁵. Many cell types use the standard suspension ratio of 3:2.

⁶. Do not vortex and avoid bubbles.

⁷. Pipet up to 20 μ L of cell mix, but only 5 μ L will enter the IFC.

⁸. Harvest should be performed as soon as the Sample Prep script has completed. There is a possibility of evaporation if left for more than 8 h. Designate the time to complete the operation when setting up the script.

3.5 Harvest Amplified Products

Pre-warm C1 DNA Dilution Reagent to room temperature. If running the Sample Prep script overnight, a 96-well plate with 10 μ L aliquots of dilution reagent can be prepared the day before. This should be sealed and put into 4°C overnight and pre-warmed to room temperature before using.

1. When the SMART-Seq v4: Sample Prep script has finished, tap EJECT to remove the IFC from the instrument.
2. Transfer the C1 IFC to a post-PCR lab environment.
3. Label a new 96-well plate as “Diluted Harvest Plate.”
4. Aliquot 10 μ L of C1 DNA Dilution Reagent into each well of the diluted harvest plate.
5. Carefully pull back the tape covering the harvesting inlets of the IFC using the plastic removal tool.
6. Using an eight-channel pipet (set to 3.5 μ L), pipet the harvested amplicons from the inlets (*see* Note 9). Use the bottom schematic to map the harvest (Fig. 5). Start with the top left well and work to the right. Then move to the well below the starting point and again work to the right. This should complete the harvest if using an eight-channel pipet.
7. Pipet the entire volume of C1 harvest amplicons into the 10 μ L of C1 DNA Dilution Reagent in each well of the “Diluted Harvest” plate.
8. Seal and vortex the plate for 20 s, and then centrifuge at $350 \times g$ for 1 min.
9. Samples can be stored up to a week in 4 °C or long term in -20°C .

3.6 cDNA Sample Dilution

Measure the concentrations of the libraries using a NanoDrop or similar device, such as PicoGreen assay. If the NanoDrop is not sensitive enough to detect low concentrations, then dilute harvested amplicons to optimal working concentration. The optimal concentration for Nextera XT library preparation is 0.10–0.30 ng/ μ L.

1. Label a new 96-well PCR plate as “Diluted for Sequencing.”
2. Pipet the appropriate amount of C1 Harvest Reagent to each well of the plate listed in the table per determined sample dilution (Table 7).
3. Transfer 2 μ L of the harvest sample from the “Diluted Harvest” plate to the “Diluted for Sequencing” plate.
4. Seal the plate with adhesive film.
5. Vortex at medium speed for 20 s, and centrifuge at 1500 rpm ($450 \times g$) for 1 min.

⁹It is imperative to use a well-calibrated eight-channel pipet to harvest as much of the amplified product as possible. Replace tips after each use. Also, it is absolutely important to remove the samples in order according to the map provided by Fluidigm. This can be found in their written protocol for the C1. This is to ensure that the harvested amplicons can be traced back to the sample IDs.

3.7 Tagment Genomic DNA

Use the following tagmentation program for the thermal cycler:

Choose the preheat lid option.

55 °C for 5 min.

Hold at 10 °C

1. Thaw Amplicon Tagment Mix on ice.
2. Warm Tagment DNA Buffer and NT Buffer to room temperature. Vortex NT Buffer if precipitate is present.
3. Mix reagents by inverting thawed tubes 3–5 times followed by brief centrifugation.
4. Label a new 96-well PCR plate as “Library Prep.”
5. In a 1.5 mL PCR tube, combine the components of the tagmentation premix. Each sample will require 2.5 µL of Tagment DNA Buffer and 1.25 µL Amplification Tagment Mix. Make a master mix with the calculated amount of reagents for the number of samples.
6. Vortex gently for 20 s, followed by brief centrifugation.
7. Aliquot equal amounts of premix into each tube of an eight-tube strip.
8. Using an eight-channel pipet, pipet 3.75 µL of the premix into each well of the “Library Prep” PCR plate.
9. Transfer 1.25 µL of each diluted sample from the “Diluted for Sequencing” plate to the “Library Prep” plate.
10. Seal and vortex the plate at medium speed for 20 s. Then centrifuge at 2000 rpm ($800 \times g$) for 5 min to remove any air bubbles.
11. Place the plate onto the thermal cycler and run the tagmentation program. Immediately proceed to the next step once the thermal cycler reaches 10 °C.
12. Aliquot equal amounts of NT Buffer into each tube of an eighttube strip. 1.25 µL of NT Buffer will be required for each sample.
13. Once the thermal cycler again reaches 10 °C, pipet 1.25 µL of NT Buffer into each of the samples for neutralization.
14. Seal and vortex the plate at medium speed for 20 s, and centrifuge at 2000 rpm ($800 \times g$) for 5 min.

3.8 Amplify Libraries

Use the following amplifying program for the thermal cycler. Choose the preheat lid option:

72 °C for 3 min

95 °C for 30 s

12 cycles of:

95 °C for 10 s

55 °C for 30 s

72 °C for 30 s.

72 °C for 5 min

Hold at 10 °C.

1. Thaw Index 1 (N7) and 2 (S5) primers at room temp for 20 min. Invert and centrifuge briefly.
2. Thaw Nextera PCR Master Mix on ice for 20 min.
3. Aliquot equal volumes of Nextera PCR Master Mix into each tube of an eight-tube strip. Each sample will require 3.75 µL of master mix so calculate accordingly.
4. Using an eight-channel pipet, pipet 3.75 µL of master mix into each well of the “Library Prep” plate.
5. Arrange Index 1 (N7) adapters in columns 1–12.
6. Arrange Index 2 (S5) adapters in rows A–H.
7. Add 1.25 µL of each Index 1 (N7) adapter down each column. Replace the cap on each i7 adapter tube with a new orange cap (*see Note 10*).
8. Add 1.25 µL of each Index 2 (S5) adapter across each row. Replace the cap on each i5 adapter tube with a new white cap.
9. Seal and vortex plate at medium speed for 20 s. Then centrifuge at 2000 rpm ($800 \times g$) for 2 min.
10. Place on the thermal cycler and run the amplifying program (*see Note 11*).
11. Amplified products can be store long term in $-20\text{ }^{\circ}\text{C}$.

3.9 Clean Up Libraries

To perform library cleanup with AMPure XP beads, a magnetic separation device for 1.5 mL tubes will be needed. Before each use, bring bead aliquots to room temperature for at least 30 min. Make sure to mix well.

1. Prepare fresh 70% EtOH (*see Note 12*). About 800 µL per tube will be needed.
2. Aliquot AMPure XP beads into 1.5 mL tubes, and bring to room temperature and vortex for 1 min.

¹⁰It is important to replace the cap of the index tubes with a new cap after every use.

¹¹At this point, it is a safe stopping point. If stopping, seal the plate, and store at $2\text{--}8\text{ }^{\circ}\text{C}$ for up to 2 days. Alternatively, leave on the thermal cycler overnight.

¹²It is important to make fresh 80% EtOH every time.

3. Pool the libraries by pipetting the volumes designated in the table below into a 1.5 mL tube (Table 8).
4. Add the designated amount of AMPure XP beads to the pooled libraries.
5. Pipet mixture slowly five times to mix.
6. Incubate bead mixture at room temperature for 5 min.
7. Briefly spin down the mixture, and collect the mixture from the side of the tube.
8. Place the tube on a magnetic stand for 2 min, until the liquid appears completely clear and no beads are present in the supernatant.
9. While the tube is left on the magnetic stand, pipet out the supernatant and discard.
10. Keep the tube on the magnetic stand, and add 180 μ L of 70% EtOH to the sample without disturbing the beads.
11. Wait for 30 s and carefully pipet out the supernatant and discard.
12. Repeat the EtOH wash one more time, but this time do not remove supernatant.
13. Remove the tube from the magnetic stand and spin down briefly.
14. Place the tube back on the magnetic stand for 30 s, and then remove all remaining ethanol.
15. Leave the tube on the magnetic stand, and air-dry the beads for 10–15 min or until the pellet is no longer shiny but before a crack appears (*see* Note 13).
16. Once the pellet is properly dried, add the designated amount of C1 DNA Dilution Reagent according to the table below (Table 9).
17. Remove the tube from the magnetic stand and vortex for 3 s to mix.
18. Incubate the sample at room temperature for 2 min to rehydrate the pellet.
19. Spin down the sample briefly, and place back on the magnetic stand until the solution becomes clear.
20. Transfer the supernatant to another tube. This will contain the purified cDNA libraries.
21. Repeat steps 4–15.
22. Elute the pellet with the following volumes of C1 DNA Dilution Reagent. Refer to Table 10.
23. Remove the tube from the magnetic stand and vortex for 3 s.
24. Incubate the sample at room temperature for 2 min.
25. Place the tube back onto the magnetic stand until the supernatant becomes clear.

¹³. If the pellet is under-dry, there will still be ethanol present in the sample. This will reduce the recovery yield. Also, if the pellet is over-dry, it will take longer than 2 min to rehydrate and may also reduce yield. Keep an eye on the pellet for a matte finish.

26. Carefully transfer the supernatant to a new 1.5 mL tube labeled “cleaned library.”

3.10 Quality Check Libraries

1. Allow Genomic DNA Reagents to equilibrate at room temperature for 30 min.
2. Prepare tube strip or 96-well plate. Add 1 μ L of Genomic DNA Ladder to 10 μ L of Genomic DNA Sample Buffer into the A1 position of a tube strip or 96-well plate. Add 1 μ L of cDNA to 10 μ L of Genomic DNA Sample Buffer (*see Note 14*).
3. Vortex at 2000 rpm for 1 min and spin down samples.
4. Power on the laptop and wait for it to be fully booted, and launch the 4200 TapeStation Control Software.
5. Turn on the TapeStation 4200 and allow the machine to initialize.
6. Open the lid and clean out any tip waste and used ScreenTape devices.
7. Install the tip rack and remove the lid.
8. Flick the ScreenTape device to move any air bubbles into the buffer chamber.
9. Place first ScreenTape into the ScreenTape nest. The label should be facing to the front.
10. Insert extra ScreenTape into racks (*see Note 15*).
11. Centrifuge samples to remove air bubbles.
12. Insert sample tube strips and remove the lids (*see Note 16*).
13. In the software, select samples for analysis. Ladder is provided in location A1 of the samples.
14. Start the run.
15. Results will appear when the run has finished. Results can be saved as a pdf.
16. Pooled libraries can now be sent for sequencing.

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¹⁴For 16 or more samples, use 2 μ L of Genomic DNA Ladder and 20 μ L of Genomic DNA Sample Buffer. If using a 96-well plate, apply a foil seal to the sample plate.

¹⁵Only use ScreenTape devices of identical assays.

¹⁶Be sure not to create bubbles when removing the lids. If using the foil-sealed 96-well plate, do not remove the foil.

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Fig. 1.

Workflow for single-cell RNA-seq using iPSC-derived NSCs with the C1 auto prep system. NSCs derived from iPSCs grow as neurospheres. Neurospheres are dissociated into single cells and loaded into the C1 IFC. Following the single-cell cDNA generation, libraries are amplified and indexed with the Nextera XT Library Preparation Kit. Generated libraries are then cleaned with AMPure XP beads and quality checked with the Agilent TapeStation 4200. Cleaned libraries are then sequenced with an Illumina-based next-generation sequencing platform. Raw reads are then demultiplexed and analyzed

Fig. 2.
Integrated Fluidic Circuits (IFC) priming reagent map

Fig. 3.
IFC loading cell map

Fig. 4.
IFC sample prep map

Fig. 5.
Harvesting sequence map

Table 1

Serial dilution for cell buoyancy test

		50%	55%	60%	65%	70%	75%	80%	85%
Cells (μL)	20	10	11	12	13	14	15	16	17
Suspension reagent (μL)	0	10	9	8	7	6	5	4	3

Table 2

Dilution of spike-in mix

Dilution	Spike-in Mix	Nuclease-free Water
1:10	1 μ L undiluted	9 μ L
1:100	1 μ L of 1:10	9 μ L
1:1000	1 μ L of 1:100	9 μ L
1:10000	1 μ L of 1:1000	9 μ L
1:100000	1 μ L of 1:10000	9 μ L

Table 3

10× Reaction Buffer mix

Component	Volume (μL)
10× Lysis Buffer	19
RNase Inhibitor	1
Total	20

Table 4

Cell lysis mix

Component	Volume (μL)
C1 Loading Reagent (20×)	1
3' SMART-Seq CDS Primer II A (12 μM)	2.4
Nuclease-free Water	14
10× Reaction Buffer	2.6
Total	20

Table 5

Reverse transcriptase mix

Component	Volume (μL)
C1 Loading Reagent (20×)	1.2
5× Ultra Low First-strand Buffer (RNase-free)	11.2
SMART-seq v4 Oligonucleotide (48 uM)	2.8
Rnase Inhibitor	1.4
Nuclease-free Water	9.8
SMARTScribe Reverse Transcriptase (100 U/μL)	5.6
Total	32

Table 6

PCR mix

Component	Volume (μL)
C1 Loading Reagent (20×)	4.5
Nuclease-free Water	4.4
PCR Primer II A (12 μM)	3
SeqAmp PCR Buffer (2×)	75.2
SeqAMP DNA Polymerase (1.25 U/μL)	2.9
Total	90

Table 7

cDNA sample dilution for sequencing

cDNA Sample dilution	1:2	1:3	1:4	1:5	1:6	1:8	1:10	1:12
Volume of C1 harvest reagent required	2 Ml	4 µL	6 µL	8 µL	10 µL	14 µL	18 µL	22 µL

Table 8

Library cleanup with AMPure beads

Number of libraries pooled	Volume per library (μL)	Total pool volume (μL)	AMPure bead volume (μL)
8	4	32	29
12	4	48	44
16	2	32	29
24	2	48	44
32	1	32	29
48	1	48	44
96	1	96	87

Table 9

First elution of pellet during library cleanup

Number of libraries pooled	C1 Dilution Reagent volume (μL)
8	32
12	48
16	32
24	48
32	32
48	48
96	96

Table 10

Second elution of pellet for library cleanup

Number of libraries pooled	C1 Dilution reagent volume (μL)
8	48
12	72
16	48
24	72
32	48
48	72
96	144