



# THE SINGLE-CELL PREPARATION GUIDE

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# HOW TO USE THE FLUIDIGM SINGLE-CELL PREPARATION GUIDE

The secret to successful single-cell studies is the preparation of high-quality single-cell suspensions. Whether you are working with cultured or primary cells, adherent cells, or cells in suspension, the condition of the cells is a critical factor in ensuring high cell capture efficiency and optimal chemistry performance.

In this guide, we provide optimized protocols for dissociation, enrichment, staining, and quality control that are essential to the preparation of high-quality single-cell suspensions for use with the C1™ system.

The guide begins with workflows that are organized by sample. See the Sample Preparation Workflow below. Click a step in the appropriate workflow to find a protocol that best matches the sample that you are using in your research. Protocols in this guide are developed for use with the Fluidigm C1 system and the Biomark™ HD system. The protocols can be adapted for use with your specific sample types. For example, use the guide to enrich B cells from human blood or dissociate neural tissue.

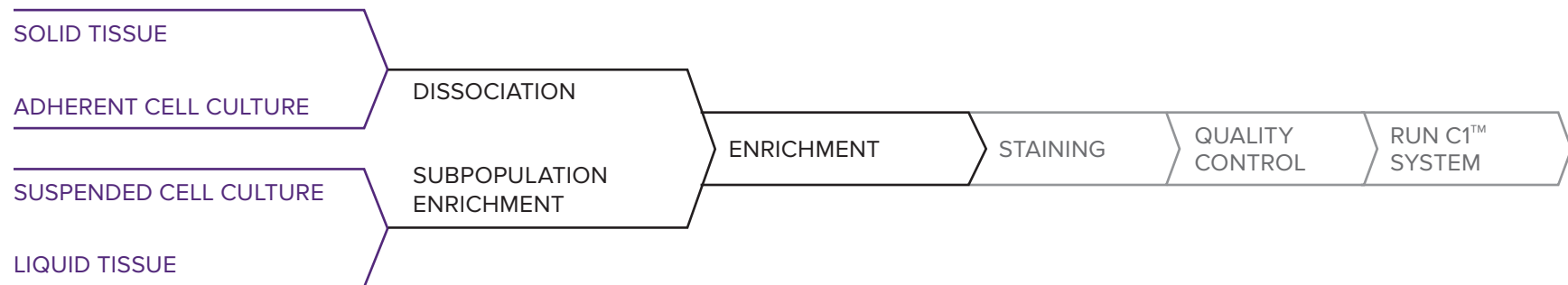
Use of the protocols assumes that you will use good laboratory practices and common molecular biology equipment, supplies, and reagents to minimize cross-contamination of products. Store reagents according to manufacturers' recommendations. For more information, see the experimental method disclaimer.

You can learn single-cell preparation techniques from real-life examples in the guide entitled "At the Bench" (for an example, [see page 5](#)). "At the Bench" examples are complete experiments involving the dissociation, enrichment, and staining of relevant tissues and cells. In each example, read the checkpoints for additional and pertinent steps to improve experimental design or perform quality control.

To overcome experimental obstacles, see "[Troubleshooting](#)" on [page 26](#).

We welcome your contributions to the Single-Cell Preparation Guide. Email your cell preparation methods to [techsupport@fluidigm.com](mailto:techsupport@fluidigm.com)

## Sample Preparation Workflow



Click the workflow step to view the associated protocols.

## At the Bench: Liquid Tissue

### ENRICHMENT OF B LYMPHOCYTES FROM HUMAN WHOLE BLOOD FOR GENE EXPRESSION ANALYSIS






<b>Cell Type:</b>	Human CD19-positive B cells from human whole blood
<b>Staining:</b>	LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells, Life Technologies
<b>Enrichment:</b>	Whole blood fractionation with Ficoll-Paque™ PLUS followed by <a href="#">FACS</a> (as described in <a href="#">Subpopulation Enrichment on page 13</a> )
<b>Processing:</b>	C1 system with a 5–10 µm IFC
<b>Detection:</b>	qPCR on the Biomark HD system

## Introduction

The human B lymphocyte, a critical component of the humoral immune response, can be studied according to the unique signature of every B cell. Each B cell expresses a single clonal antibody and is activated by a single antigen. Because each B cell presents only one antibody gene expression profile, and that profile varies from cell to cell, the genetic signature of each cell is unique. Therefore, single-cell genomic analysis of populations of B cells can characterize a specific humoral response.

Here, we enrich and isolate B cells from whole blood. B cells comprise 0.7%–2.0% of cells in whole blood, requiring two steps of enrichment before analysis. Density separation using Ficoll enriches B cells by shrinking red blood cells and separating them from white blood cells, leaving a concentrated population of peripheral blood mononuclear cells (PBMCs). The B cells are further enriched from the PBMC population by labeling with a fluorescent anti-CD19 antibody and sorting by flow cytometry. Individual CD19-positive B cell isolations, cell lysis, reverse transcription, preamplification, and harvesting of amplified products were performed using the C1 system. For more information, see [Using the C1™ System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene™ Assays \(Fluidigm, PN 100-4904\)](#). Gene expression profiles of the isolated B cells were compared to PBMCs from the parental suspension using data generated from the Biomark HD system.

### LIQUID TISSUE WORKFLOW

Method	Time
<b>1 Perform subpopulation enrichment by density centrifugation of whole blood.</b> Use Ficoll to separate blood for PBMC isolation. 	1 hr
<b>2 Use FACS to select for human CD19+ B cells.</b> Use FACS to positively select anti-CD19-labeled PBMCs. 	1 hr
<b>3 Perform quality assessment of FACS-sorted cells.</b> Confirm the size, concentration, and viability of cells. 	15 min
<b>4 Prime the C1 IFC, load and image the cells.</b> Load cells in the C1 IFC and stain them before microscopy. 	1 hr
<b>5 Run the C1 system reverse transcription and preamplification.</b> Reinsert the C1 into the system for automated preamplification. 	6 hr
<b>6 Harvest cDNA and perform qPCR on the Biomark HD system.</b> Harvest the amplified cDNA and load it onto the Biomark HD system for qPCR analysis. Analyze using the Singular™ Analysis Toolset.	1 hr

## Method Overview

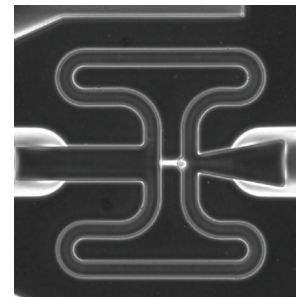
- 1 PBMCs were enriched using Ficoll-Paque PLUS (see “[Density Centrifugation of Human Blood to Produce Concentrated PBMCs](#)” on page 13). ☒
- 2 FACS was used to enrich for CD19+ B cells (see “[FACS Selection for Human CD19+ B Cells](#)” on page 15). ☒
- 3 Cell viability and concentration were determined by hemocytometry (see “[Cell Size and Viability](#)” on page 21 and “[Cell Concentration](#)” on page 23). ☒
- 4 Cell lysis, reverse transcription, and preamplification were performed on the C1 system on CD19+ B cells and PBMCs. All standard procedures were followed to process the cells in the C1 IFCs. For more information, see [Using the C1 System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays](#) (Fluidigm, PN 100-4904).

## Check Points

- ☒ When working with blood, be aware of factors that could inhibit PCR. For example, the anticoagulant heparin may inhibit PCR.
- ☒ Process whole blood no more than three hours post-collection. Consult a flow cytometry expert on the best sorting speed for your sample type. Use the slowest sorting flow rate possible in order to improve cell quality.
- ☒ Perform quality control steps. Viable single-cell suspensions are required to generate high-quality data (see [Quality Control](#) on page 21).

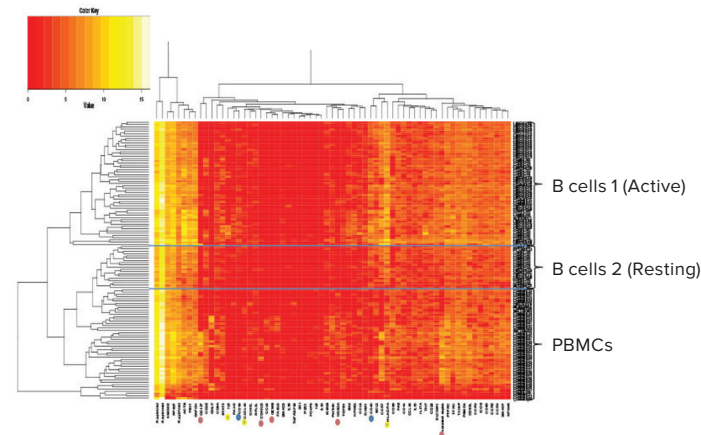
## Data and Results

Results were compiled and collected from three separate C1 runs. C19+ B cells were sorted for the first and second C1 runs, with capture rates of 76% and 73%, respectively. PBMCs from the parental suspension were captured at 76% efficiency (Figure 1). 750 cells in 5  $\mu$ L were loaded in each IFC run. Gene expression profiles were collected using the Biomark HD system and the data analyzed with the Fluidigm. Real-Time PCR Analysis software (Figure 2). Principal component analysis (PCA), violin plots, and hierarchical clustering (shown below) were generated using the Fluidigm. Singular Analysis Toolset (PN 100-5066).



**Figure 1.**  
A single CD19+ B cell nested in the C1 IFC capture site

### Hierarchical Clustering Comparing CD19-positive B Cells and Resting PBMCs



**Figure 2.**  
Hierarchical clustering (HC) map comparing gene expression profiles between CD19+ B cells (activated and resting) and resting PBMCs. B cell-specific genes (blue), activated B cell genes (yellow: *IL6*, *CXCL10*, *HLA-DRA*), and non-B cell (red: *CD127*, *CD40LG*, *GZMB*, *NOS2A*, *TRC beta*) are highlighted.

## At the Bench: Cultured Adherent Cells

### SURFACE ANTIBODY STAINING IN CULTURED INDUCED PLURIPOTENT STEM CELLS (iPSCs)






<b>Cell Type:</b>	Human induced pluripotent stem cells (iPSCs) and human neural progenitor cells (NPCs)
<b>Staining Method:</b>	StainAlive™ TRA-1-60 Antibody and CellTracker™ Orange CMRA
<b>Dissociation Method:</b>	Accutase® Cell Detachment Solution
<b>Processing:</b>	C1 system with a 10–17 µm IFC
<b>Detection:</b>	qPCR on the Biomark HD system

### Introduction

A significant challenge in stem cell studies is ensuring the identity, growth, and status of a cell population. For instance, in vitro embryonic and pluripotent stem cells may differentiate spontaneously, while in directed differentiation studies a subset of the cell population can choose alternate differentiation pathways. In both models, stem cells can be tracked for “cell stemness,” their proliferation analyzed, and their viability monitored. Thus, cell staining and gene expression profiles are important tools for characterizing heterogeneous cell populations (see “Cell Size and Viability” on page 21).

Here, we used an automated cell staining with StainAlive TRA-1-60 Antibody (DyLight™ 488, Stemgent) to assess pluripotency and CellTracker™ Orange CMRA (Life Technologies) to determine viability on the C1 system. We interrogated the product from the C1 by qPCR on the Biomark HD system and analyzed the data with the Singular Analysis Toolset. We found that human induced pluripotent stem cells (iPSCs) could be distinguished from human neural progenitor cells (NPCs).

### CULTURED ADHERENT CELLS WORKFLOW

Method	Time
<b>1 Prepare single-cell suspensions.</b> Treat iPSCs with Accutase Cell Detachment Solution and perform single-cell suspension QC. 	1.5 hr
<b>2 Prepare reagents and stains.</b> Prepare C1™ reagent mixes and TRA-1-60 and CellTracker™ Orange stains. 	30 min
<b>3 Prime the C1 IFC, load and image the cells.</b> Load the cells onto the C1 IFC and stain them before microscopy. 	1 hr
<b>4 Visualize cells in the C1 IFC.</b> Record viability and pluripotency data using an inverted microscope. 	1 hr
<b>5 Run the C1 system reverse transcription and preamplification.</b> Reinsert the C1 IFC into the C1 system for automated preamplification. 	6 hr
<b>6 Harvest cDNA and perform qPCR on the Biomark HD.</b> Harvest the amplified cDNA and load it onto the Biomark HD system for qPCR analysis. Analyze using the SINGuLAR Analysis Toolset.	1 hr

## Method Overview

- 1 Human iPSCs were passaged with 0.5  $\mu$ M EDTA and maintained with Essential 8™ Medium (Life Technologies) on Matrigel® (1:80 in DPBS). iPSCs were freshly dissociated into single-cell suspension with [Accutase Cell Detachment Solution](#) for 5 to 7 minutes at 37 °C. ☒
- 2 iPSCs were differentiated to NPCs using the small molecules LDN-193189 and SB-431542 for dual-SMAD inhibition and the Hedgehog inhibitor cyclopamine (Chambers, S.M. et al. 2009). Differentiation was confirmed by immunostaining for PAX6, an NPC-specific marker.
- 3 Suspensions were optimized for increased single-cell capture. ☒  
iPSCs and NPCs are more buoyant than the cell lines used to develop the standard protocol (K562, BJ fibroblasts, and HL60S). To adjust for buoyancy, single-cell suspensions of iPSCs and NPCs in media were resuspended in C1 Suspension Reagent at 70:30 (cells:reagent). Then the suspension was loaded into a cell-input inlet of a 10–17  $\mu$ m C1 IFC (Fluidigm, PN 100-5479).
- 4 Cells were stained in the C1 IFC to determine pluripotency with StainAlive TRA-1-60 (see [“Surface Marker Staining in the IFC” on page 20](#)) and CellTracker Orange to determine viability.
- 5 A set of Delta Gene assays was used for preamplification and interrogation of both iPSC and NPC samples. Delta Gene assays use EvaGreen® dye—an intercalating, fluorescent dye—for quantitation (see [Real-Time PCR Analysis User Guide, PN 68000088](#)). The assays included six endogenous controls and 90 pluripotency, differentiation, and stemness genes commonly used to characterize undifferentiated human stem cells (ISCI et al. 2007).
- 6 All standard procedures were followed to stain and lyse cells and to perform reverse transcription and preamplification in the C1 IFCs. For more information, see [Using the C1 System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays](#) (Fluidigm, PN 100-4904).
- 7 Cells and signal from labeling were observed by fluorescence microscopy at 10X magnification.

- 8 All gene expression analysis was performed using the Biomark HD system. Data analysis was performed using the [Singular Analysis Toolset](#) (Fluidigm, PN 100-5066).

## Check Points

- ☒ Dissociation of cells can impact cell viability and the quality of the cell suspension. After preparing the single-cell suspension, perform quality control on the cells (see [“Quality Control” on page 21](#)).
- ☒ Determine whether your cells are neutrally buoyant or require optimization before loading into the C1 IFC (see [“Optimizing Cell Buoyancy for the C1” on page 24](#)).



# Data and Results

To study surface marker staining within the C1 system, 10 C1 IFCs were loaded with either human iPSCs or NPCs and stained with StainAlive TRA-1-60. The capture sites of each C1 IFC were scanned and imaged using a fluorescent microscope with an automated stage. Minimal fluorescence was observed in captured NPCs (see Table 1). Captured iPSCs, however, demonstrated strong fluorescence in at least 87% and up to 96% of cells.

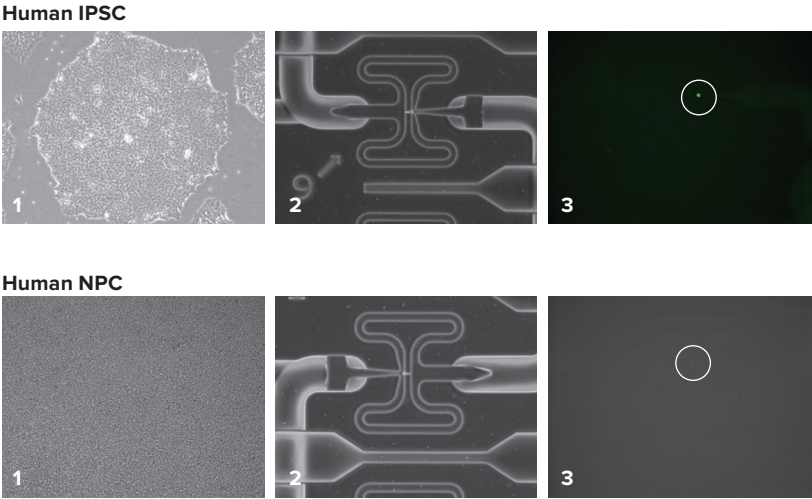
Phase contrast and fluorescence microscopy (Figure 3) confirms both cell capture and pluripotency.

	Chip Run and Cell Type	% Cell Occupancy	% TRA-1-60 + Cells
1	iPSC	94	87
2	iPSC	100	96
3	iPSC	93	96
4	iPSC	95	90
5	iPSC	93	96
6	iPSC	83	89
7	iPSC	91	87
8	iPSC	88	88
9	NPC	98	6
10	NPC	100	3

**Table 1.**

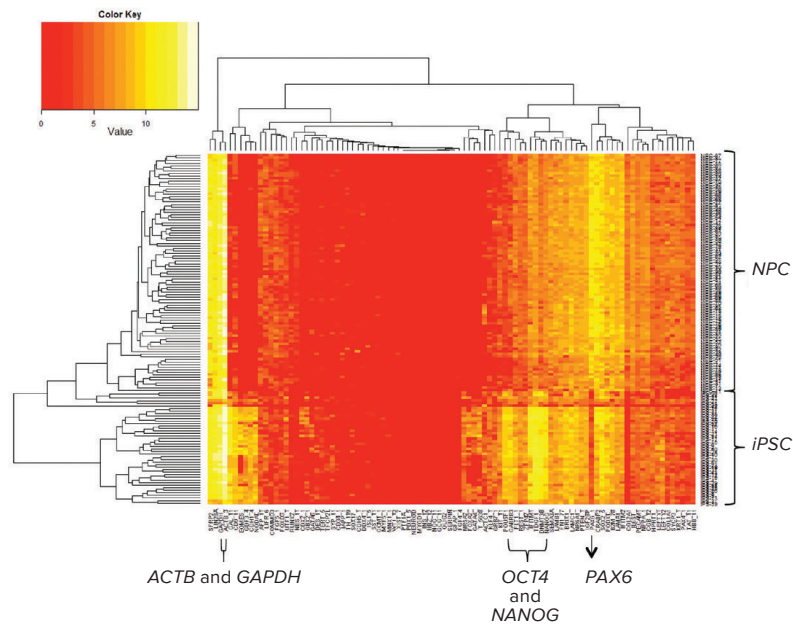
TRA-1-60 Antibody Staining. In 10 individual C1 IFC experiments, iPSCs and NPCs were stained with TRA-1-60. In each IFC run, 1,000 cells in 5  $\mu$ L were loaded. Cell occupancy is the percentage of occupied capture sites containing a single cell. In IFCs loaded with iPSCs, up to 96% of cells captured were TRA-1-60-positive. As expected, IFCs loaded with NPCs showed a significantly lower percentage of fluorescent cells

Gene expression analysis (Figure 4, next page) confirms the undifferentiated state of iPSCs when compared to NPCs. Principal component analysis (PCA, Figure 5, next page) demonstrates distinct clustering of NPCs and iPSCs. Combining single-cell gene expression profiling with fluorescent markers allows for correlation of the two readouts and analysis of rare subpopulations such as the TRA-1-60+ NPCs (Table 1). For more data and analysis, see the Technical Note [Automated Cell Staining of Induced Pluripotent Stem Cells on the C1 System \(Fluidigm, PN 100-6857\)](#).



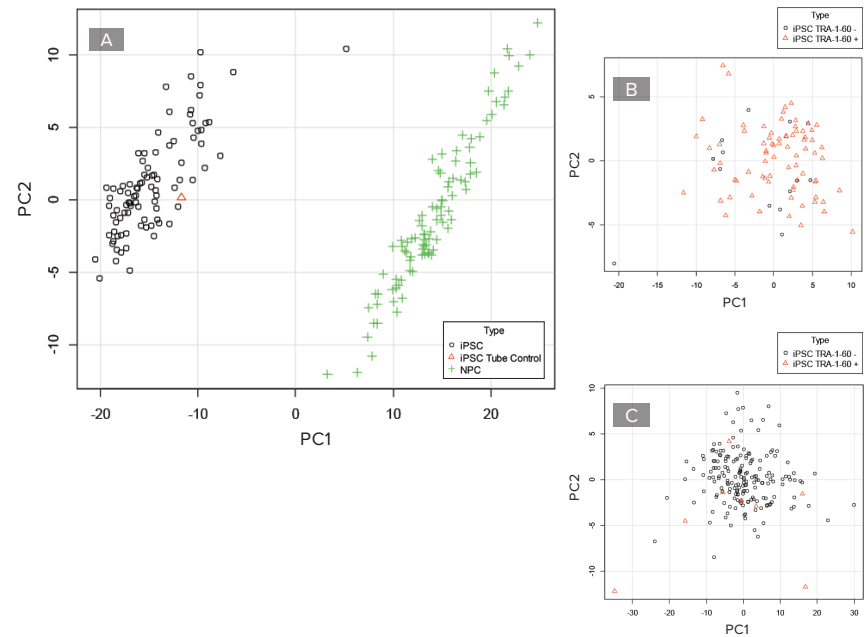
**Figure 3.** iPSC and NPC Capture Images. 1. Cells prior to single-cell dissociation observed under phase contrast microscopy. 2. An isolated single cell captured in the C1 IFC and imaged under phase contrast microscopy. 3. The same cell imaged with fluorescence microscopy for TRA-1-60 antibody signal.

## Hierarchical Clustering of iPSCs and NPCs



**Figure 4.**

Gene Expression Analysis of iPSC vs. NPC. Both iPSCs and NPCs show similar expression of housekeeping genes, *ACTB* and *GAPDH*. iPSCs have significantly higher expression levels of stemness genes *OCT4* and *NANOG*, as expected: a 5.69  $C_t$  difference for *OCT4* and a 2.96  $C_t$  difference for *NANOG* when averages of iPSCs and NPCs are compared. NPCs have significantly higher levels of *PAX6*, as expected, expressing approximately 80x more copies, a 6.32  $C_t$  difference



**Figure 5.**

Principal Component Analysis (PCA) of iPSCs and NPCs. A. Distinct clusters display different states of differentiation for iPSC and NPC cell types. B. iPSCs positive and negative for TRA-1-60 signal cluster together when profiled for gene expression. C. NPCs positive and negative for TRA-1-60 signal cluster together when profiled for gene expression.

More At the Bench examples, including single-cell neural, tumor, and other solid tissue data, are coming soon.

## DISSOCIATION

Tissues and cell lines from different origins require specific methods of dissociation to produce high-quality single-cell suspensions. Use the appropriate dissociation method to prepare a high-quality single-cell suspension according to tissue or cell type:

Tissue Type	Protocol
Adherent cell culture	TrypLE™ Select (1X) for the preparation of single-cell suspensions
Stem cells in culture	Accutase Cell Detachment Solution for preparation of single-cell suspensions

## TrypLE Select (1X) for the Preparation of Single-Cell Suspensions

### CUSTOMER-SUPPLIED MATERIALS

- Calcium- and magnesium-free 1X Dulbecco's Phosphate Buffered Saline [1X DPBS (-/-), Life Technologies, PN 14190-144]
- TrypLE Select (1X) (Life Technologies, PN 12563029)
- Incubator (37 °C)
- Adherent cells in culture

## Prepare Reagents

### 1X DPBS (-/-)

Use 1X DPBS (-/-) at room temperature.

### TrypLE SELECT (1X)

Use the TrypLE Select (1X) at room temperature.

## Procedure

- 1 Aseptically remove the entire volume of spent medium from the culture vessel.
- 2 Gently dispense 0.2 mL/cm<sup>2</sup> of 1X DPBS (-/-) on the cell monolayer. For example, dispense 5 mL of buffer into a 25 cm<sup>2</sup> T25 flask containing a cell monolayer.
- 3 Gently swirl the culture vessel with DPBS (-/-) seven or eight times to rinse the cells.
- 4 Aseptically remove the entire volume of DPBS.
- 5 Dispense 0.03 mL/cm<sup>2</sup> of TrypLE Select (1X). For example, dispense 0.75 mL for a T25 flask.
- 6 Swirl the vessel several times to ensure that the cell monolayer is covered with TrypLE Select (1X).
- 7 Incubate the cells at 37 °C. Check the cells every 2 minutes for up to 8 minutes under a microscope until 90% of the cells are detached. If the cells remain attached to the vessel, very gently tap the vessel until the cells detach.
- 8 Dilute the cells in TrypLE Select (1X) with an equal volume of complete growth medium. As a general rule when following this protocol, a culture vessel containing cells at 80% confluence yields 200,000–400,000 cells/mL.
- 9 Perform quality control on the cells (see “Quality Control” on page 21).

# Accutase™ Cell Detachment Solution for Preparation of Single-Cell Suspensions

## CUSTOMER-SUPPLIED MATERIALS

- Calcium- and magnesium-free 1X Dulbecco's Phosphate Buffered Saline (DPBS) [1X DPBS (-/-), Life Technologies, PN 14190-144]
- 1X Accutase Cell Detachment Solution (BD Biosciences, PN 561527)
- Complete growth medium
- Incubator (37 °C)
- 1 mL serological pipette
- 15 mL conical tube
- Centrifuge
- Stem cells in culture

## Prepare Reagents

### 1X DPBS (-/-)

Use 1X DPBS (-/-) at room temperature.

### ACCUTASE CELL DETACHMENT SOLUTION

Store Accutase Cell Detachment Solution at 4 °C, then warm to 37 °C just before use.

### COMPLETE GROWTH MEDIUM

Store complete growth medium at 4 °C, then warm to 37 °C just before use.

## Procedure

**NOTE** When necessary, centrifuge cells at the appropriate g-force, temperature, and time.


- 1 Aseptically remove the entire volume of spent medium from the cell culture vessel.
- 2 Gently dispense 0.2 mL/cm<sup>2</sup> of 1X DPBS (-/-) on the cell monolayer. For example, gently dispense 5 mL of buffer into a T25 flask containing a cell monolayer.
- 3 Gently swirl the vessel once to rinse the cells.
- 4 Aseptically remove the entire volume of DPBS rinse.
- 5 Dispense 0.2 mL/cm<sup>2</sup> 1X Accutase at 37 °C onto the cell monolayer.
- 6 Swirl the vessel several times to ensure that the cell monolayer is covered with 1X Accutase Cell Detachment Solution.
- 7 Incubate the cells at 37 °C. Check the cells every 30 seconds under a microscope until the colonies detach. If the colonies remain attached to the wells, gently tap the vessel until the cells detach.
- 8 Use a 1 mL serological pipette to gently pipet the cells up and down three to four times. Do not generate bubbles in the suspension while pipetting.
- 9 Incubate the suspended colonies at 37 °C. Check the cells every two minutes to verify colony dissociation. Continue to incubate the cells until the colonies are fully dissociated. To fully dissociate 70% confluent iPSCs takes approximately seven minutes.
- 10 Pipet 10 mL of complete growth medium (37 °C) into a new 15 mL conical tube.
- 11 Transfer the cell suspension from Step 9 to the 15 mL conical tube with the complete growth medium.
- 12 Centrifuge the cells to pellet them, then remove and discard the supernatant.
- 13 Resuspend the pellet in 10 mL of complete growth medium (37 °C).
- 14 Centrifuge the cells to pellet them, then remove and discard the supernatant.
- 15 Resuspend the pellet in 4 mL of complete growth medium (37 °C).
- 16 Perform quality control on the cells (see [“Quality Control” on page 21](#)).

## SUBPOPULATION ENRICHMENT

### Density Centrifugation of Human Blood to Produce Concentrated PBMCs

#### CUSTOMER-SUPPLIED MATERIALS

- Whole blood drawn in EDTA or sodium citrate vacuum collection tubes. This protocol is written for 30 mL of blood but can be scaled for higher or lower volumes.
- 50 mL Leucosep™ tubes (Geiner Bio-One, PN 227 290)
- Ficoll-Paque PLUS (GE Healthcare Life Sciences, PN 17-1440-02)
- Calcium- and magnesium-free Hank's Buffered Salt Solution [HBSS (-/-), Life Technologies, PN 14175-095]
- Swinging-bucket rotor centrifuge
- Collection tubes
- (Optional) INCYTO C-Chip™ Disposable Hemocytometer (Neubauer Improved, PN DHC-N01)

 **WARNING** BIOHAZARD. Human-derived whole blood and byproducts are a known biohazard risk. Use personal protective equipment and your lab's safety protocol to limit biohazard risks.



#### Procedure

- 1 Add 15 mL of fresh Ficoll-Paque PLUS to each of two Leucosep tubes. Centrifuge the tubes at 800 x g for 1 minute with the brake ON.
- 2 Add 5 mL of HBSS (-/-) to each Leucosep tube, then decant a maximum of 15 mL of blood into each tube with the HBSS (-/-).
- 3 Wash one blood collection tube with 10 mL of HBSS (-/-), then add the wash to one of the Leucosep tubes. Repeat the 10 mL wash with the second collection tube.
- 4 Immediately centrifuge the Leucosep tubes at 800 x g for 15 minutes with the brake OFF.
- 5 Remove the Leucosep tubes from the centrifuge, then decant the entire contents of one tube above the frit into a new 50 mL conical tube. Decant the contents of the second Leucosep tube into a second new 50 mL conical tube.
- 6 Centrifuge the 50 mL tubes at 250 x g for 10 minutes with the brake ON.
- 7 Remove the supernatant from each 50 mL tube with a serological pipette.
- 8 Resuspend each pellet in 5 mL of HBSS (-/-), then combine the cells in one tube.
- 9 Centrifuge the cells at 200 x g, then remove and discard the supernatant.
- 10 Gently tap the pellet to dislodge and resuspend the cells, and then add 5 mL of HBSS (-/-) to completely resuspend the cells with a serological pipette.
- 11 Centrifuge the cells at 200 x g, then remove and discard the supernatant.
- 12 Repeat steps 10–11 once.
- 13 Count the cells (see [“Cell Concentration” on page 23](#)).
- 14 Perform quality control on the cells (see [“Quality Control” on page 21](#)). If you are going to use cells for further enrichment, proceed immediately to [“Enrichment” on the next page](#).

## ENRICHMENT

Use one of these methods as appropriate for your cells to enrich dissociated solid tissue (free of debris) or suspended cells:

- Magnetic bead selection (Miltenyi Biotec)
- FACS selection (see “FACS Selection for Human CD19+ B Cells” on the next page)

In this example, we outline protocols to isolate human B cells from PBMCs. However, the protocols can be adapted to enrich for other cell types from different sources.

### Magnetic Bead Selection (Miltenyi Biotec)

#### FLUIDIGM-SUPPLIED MATERIALS

- C1 system (PN 100-7000)
- C1 IFC (5–10 µm, PN 100-5757)

#### CUSTOMER-SUPPLIED MATERIALS

- BSA, Fraction V (Sigma, PN A9418)
- CD19 MicroBeads conjugated to monoclonal anti-human CD19 antibodies (Miltenyi Biotec, PN 130-050-301)
- 50 mL conical tube
- Calcium- and magnesium-free HBSS [HBSS (-/-), Life Technologies, PN 14175-095]
- 500 mM EDTA (Sigma, PN 03690)
- Peripheral blood mononuclear cells (PBMCs)
- MidiMACS™ Separator and MACS MultiStand (Miltenyi Biotec, PN 130-042-302 and PN 130-042-303)
- Axygen® Aerosol Barrier Filter Tips, Wide-Bore, Presterilized, 100–1,000 µL (Axygen, PN TF-1005-WB-L-R-S)
- 0.22 µm Tube Top Filters (Corning, PN 430320)

- LS Column (Miltenyi Biotec, PN 130-042-401)
- (Optional) INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved, PN DHC-N01)
- Vortexer
- Centrifuge
- Rotator
- 15 mL conical tubes

**! IMPORTANT** Do not use this protocol with large µm beads, such as Dynabeads® products.

**! WARNING BIOHAZARD.** Human-derived whole blood and byproducts are a known biohazard risk. Use personal protective equipment and your lab's safety protocol to limit biohazard risks.



## Procedure

- 1 In a new 50 mL tube, prepare fresh cell handling buffer for each new experiment by combining the following components:

Component	Quantity
HBSS (-/-)	49.8 mL
500 mM EDTA	0.2 mL
BSA, Fraction V	0.250 g
<b>Total</b>	<b>50.0 mL</b>

- 2 Vortex the cell handling buffer until the BSA dissolves. Filter the solution through a 0.22 µm filter, then put the buffer on ice.
- 3 Ensure that you have pre-enriched the PBMCs by density centrifugation (see “Density Centrifugation of Human Blood to Produce Concentrated PBMCs” on page 13). Count the cells.

- 4 Centrifuge the PBMCs at 300 x *g* for 10 minutes. Resuspend pellet at a maximum cell density of 10<sup>7</sup> cells/80 µL in cell handling buffer.
- 5 Add 20 µL of anti-target beads per 10<sup>7</sup> cells.
- 6 Gently rotate cells and controls for 20 minutes at 4 °C. As the cells rotate, proceed to the next step.
- 7 Install an LS Column by placing it wing-side forward into the MidiMACS Separator. Place a waste container under the column.
- 8 Wash the column with 3 mL of ice-cold cell handling buffer.
- 9 Centrifuge the cells at 200 x *g* at room temperature for 5 minutes, then discard the supernatant.
- 10 Pipet 500 µL ice-cold cell handling buffer onto the pellet, then resuspend the cells by pipetting them up and down gently with a wide-bore pipette tip.
- 11 Pipet the cells up and down one or two times to suspend them with a regular pipette tip.
- 12 Add the cells immediately to the prepared column. Proceed immediately to the next step.
- 13 After the cells have completely passed through the column (and all liquid is drained from the column), wash the column with 3 mL of ice-cold cell handling buffer. Completely drain the column.
- 14 Wash the column two more times with 3 mL of ice-cold cell handling buffer. After each wash, drain the column completely.
- 15 Place the column in a new 15 mL conical tube.
- 16 Add 5 mL of ice-cold cell handling buffer to the column.
- 17 Insert the plunger slowly into the column. Depress the plunger very gently until the buffer has passed through the column. Do not force air through the column, as this could damage cells.
- 18 Centrifuge the enriched cells at 200 x *g* for 5 minutes. Discard the supernatant. If necessary, use dark paper behind the tube to increase contrast and leave 50 µL of supernatant to minimize cell loss.
- 19 Gently resuspend the cells in 1 mL of cell handling buffer.

- 20 Perform quality control on the cells (see [“Quality Control” on page 21](#)) and adjust the concentration for loading on a C1 IFC (5–10 µm).
- 21 Immediately perform single-cell capture and preamplification of loci of interest on the C1. For more information, see the protocol [Using the C1 System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays](#) (Fluidigm, PN 100-4904).

## FACS Selection for Human CD19+ B Cells

### FLUIDIGM-SUPPLIED MATERIALS

- C1 system (PN 100-7000)
- C1 IFC (5–10 µm, PN 100-5757)

### Customer-Supplied Materials

- Heat-inactivated FBS (HI-FBS, Life Technologies, PN 10082139), thawed
- Alexa Fluor® 488 Mouse Anti-Human CD19 (BD Biosciences, PN 557697)
- Alexa Fluor 647 Mouse Anti-Human CD3 (BD Biosciences, PN 557706)
- Anti-Mouse Ig, kappa/Negative Control BD™ CompBead Particles (anti-mouse beads, BD Biosciences, PN 552843)
- LIVE/DEAD. Fixable Violet Dead Cell Stain Kit, for 405 nm excitation (Life Technologies, PN L34955)
- ArC™ Amine Reactive Compensation Bead Kit (Life Technologies, PN A-10346)
- Aluminum foil
- • 50 mL conical tube
- Calcium- and magnesium-free Hank’s Buffered Salt Solution [HBSS (-/-), Life Technologies, PN 14175-095]
- 500 mM EDTA (Sigma, PN 03690)
- Axygen Snaplock Microtubes, Homopolymer, MAXYmum Recovery™ 1.5 mL (Axygen, PN MCT-150-L-C)
- 0.22 µm Tube Top Filters (Corning, PN 430320)

- Strainer cap FACS tubes (BD Biosciences, PN 352235)
- Vortexer
- (Optional) INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved, PN DHC-N01)
- Freshly isolated PBMCs

**! WARNING** BIOHAZARD. Human-derived whole blood and byproducts are a known biohazard risk. Use personal protective equipment and your lab's safety protocol to limit biohazard risks.



## Prepare Reagents

### FACS BUFFER

- 1 In a new 50 mL conical tube, combine:

Component	Quantity
HBSS (-/-)	49.8 mL
500 mM EDTA	00.2 mL
BSA, Fraction V	00.250 g
<b>Total</b>	<b>50.0 mL</b>

- 2 Vortex the FACS buffer for 10 seconds.
- 3 Filter the buffer using 0.22 µm tube-top vacuum filter, then place the FACS buffer on ice.

## LIVE/DEAD Fixable Violet Dead Cell Stain

**! IMPORTANT!** Prepare the LIVE/DEAD Fixable Violet Dead Cell Stain fresh before use. Keep the stain on ice and protect it from light.

- 1 Add 50 µL of Component B to a new tube of Component A.
- 2 Vortex the solution immediately, then keep at room temperature until use.

## Antibody Staining Solutions

**! IMPORTANT!** Prepare the antibody staining solutions fresh before use. Keep the stain on ice and protect it from light.

Sample Type	Antibody Conjugate (µL)	FACS Buffer (µL)	Number of Tubes ()	For Number of Cells
Single stains, compensation cell controls	5	100	(1) CD3, (1) CD19	10 <sup>6</sup> for each stain
Single stains, compensation bead controls	5	100	(1) CD3, (1) CD19	
Dual stain, compensation cell controls	5+5	100	(1) CD3 + CD19	10 <sup>6</sup>
Dual stain, compensation bead controls	5+5	100	(1) CD3 + CD19	
Dual stain, experimental sample	50+50	1,000	(1) CD3 + CD19	10 <sup>7</sup>

**Table 2.**  
Antibody staining solutions

## Procedure

### DISPENSE THE CELLS AND BEADS INTO LABELED TUBES

- 1 Ensure that you have freshly isolated human peripheral blood mononuclear cells (PBMCs) suspended in HBSS (-/-) with no BSA or FBS (see [“Density Centrifugation of Human Blood to Produce Concentrated PBMC”](#) on page 13).
- 2 Count the total number of cells.
- 3 Label 1.5 mL MAXYmum Recovery microcentrifuge tubes for all antibody compensation controls, including a no-stain control for compensation beads and cells, as well as for the cells to be sorted. For the samples that will be required, see Table 3 in the next step.



4 Dispense cells and well-vortexed beads into the labeled tubes:

Antibody-Fluorophore Conjugate Controls	Number of PBMCs	Volume (cell concentration) or Volume of Bead Reagent	Volume of FACS buffer (μL)
CD3 cell control	10 <sup>6</sup>	100 μL (10 <sup>7</sup> /mL)	—
CD19 cell control	10 <sup>6</sup>	100 μL (10 <sup>7</sup> /mL)	—
Dual-labeled cell control	10 <sup>6</sup>	100 μL (10 <sup>7</sup> /mL)	—
No-label cell control	10 <sup>6</sup>	100 μL (10 <sup>7</sup> /mL)	—
CD3 bead control	—	2 drops (anti-mouse beads)	100
CD19 bead control	—	2 drops (anti-mouse beads)	100
Dual-labeled bead control	—	2 drops (anti-mouse beads)	100
No-label bead control	—	2 drops (anti-mouse beads)	100
— — —	—	—	—
Viability controls, microtube	—	—	—
LIVE/DEAD Fixable Violet Dead Cell Stain beads	—	2 drops ArC beads	<b>IMPORTANT!</b> Do not add FACS buffer to these beads.
Cell viability control	10 <sup>6</sup>	100 μL (10 <sup>7</sup> /mL); add 900 μL HBSS (-/-) so the total volume is 1 mL.	
Sample to be sorted, 15 mL conical	—	—	—
Cells, all stains	10 <sup>7</sup>	1000 μL (10 <sup>7</sup> /mL)	—

**Table 3.**  
Required samples.

5 Place all tubes on ice. Work on ice for all incubations.


## Stain for Viability and Viability Controls

- 1 To the ArC Amine Reactive Compensation beads (Component A), add 1 μL of prepared LIVE/DEAD Fixable Violet Dead Cell Stain per drop, mix the solution well, then incubate the beads at room temperature for 20 minutes.
- 2 To the viability compensation control cells and the cells to be sorted, add 1 μL of prepared LIVE/DEAD Fixable Violet Dead Cell Stain per mL of buffer present, mix the solutions well, then incubate the cells at room temperature for 20 minutes.
- 3 To the ArC beads, add 1 mL of HBSS (-/-).
- 4 Centrifuge the cells and ArC beads at 300 x *g* for 5 minutes, then remove and discard the supernatant.
- 5 Add 1 mL of HBSS (-/-) to each tube of beads and cells, centrifuge the tubes at 300 x *g* for 5 minutes, then remove and discard the supernatant.
- 6 From the ArC beads, remove the supernatant and resuspend in 300 μL of FACS buffer. Add one drop of ArC negative beads (Component B) to the tube. Keep the ArC beads on ice until needed and protect from light.
- 7 Resuspend the viability control cells in 300 μL of FACS buffer. Keep the viability control cells on ice and protected from light until use.
- 8 Immediately proceed to “Stain for Sorting and Compensation Controls.”

## Stain for Sorting and Compensation Controls

- 1 Centrifuge the control cells and anti-mouse beads at 300 x *g* for 5 minutes, then remove and discard the supernatant.
- 2 For all cells and beads, remove and discard supernatants and resuspend the cells according to Table 3, using appropriate staining solution from Table 2. For no-stain controls, use 100 μL of FACS buffer.
- 3 Incubate the beads and cells on ice for 30 minutes. Protect the beads and cells from light.
- 4 Centrifuge the tubes at 200 x *g* for 5 minutes, then remove and discard the supernatant.
- 5 Add 1 mL of FACS buffer to each tube, then centrifuge the tubes at 200 x *g* for 5 minutes. Remove and discard the supernatant.

- 6 Resuspend pellets in FACS buffer (300  $\mu$ L for compensation controls and 1 mL for the cells to be sorted), then strain into FACS tubes. Place tubes on ice and protect them from light.
- 7 Sort the cells immediately.
- 8 Perform quality control on the cells (see “Quality Control” on page 21).

 **IMPORTANT!** Immediately after FACS use the sorted cells on the C1 system for preamplification.

For more information, see the protocol [Using the C1 System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays \(Fluidigm, PN 100-4904\)](#). For cells derived from PBMC populations, use the C1 IFC (5–10  $\mu$ m).

## CELL STAINING

### Staining Cells with Nuclear Stain, Hoechst 33342

Use the Hoechst 33342 to stain all cells (dead and live) before loading cells into the IFC. Changes in gene expression due to Hoechst 33342 staining have not been evaluated.

**NOTE** Adjust centrifugation parameters for your cell type.

**!** **IMPORTANT!** Do not use the Hoechst 33342 staining protocol with cells that will be used for whole genome amplification.

#### FLUIDIGM-SUPPLIED MATERIALS

- C1 Cell Wash Buffer

#### CUSTOMER-SUPPLIED MATERIALS

- Ethidium homodimer-1 (in LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells, Life Technologies, PN L-3224)
- Axygen Snaplock Microtubes, Homopolymer, MAXYMum Recovery 1.5 mL (Axygen, PN MCT-150-L-C)
- Hoechst 33342, 10 mg/mL in DMSO (Life Technologies, Cat. No. V13244)
- Aluminum foil
- Cells suspended in appropriate cell culture medium

#### PREPARE REAGENTS

Combine in a new 1.5 mL Axygen Snaplock Microtube in this order, and protect from light:

Component	Volume (μL)
C1™ Cell Wash Buffer	1,250
Hoechst 33342, 10 mg/mL	1
Ethidium homodimer-1	2.5
<b>Total</b>	<b>1,253.5</b>

### Procedure

- 1 In new 1.5 mL Axygen Snaplock Microtube, centrifuge 500,000–1,000,000 cells at the appropriate g-force, temperature, and time. Then, remove and discard the supernatant.
- 2 Resuspend the cells in 1 mL of C1 Cell Wash Buffer.
- 3 Centrifuge cells at the appropriate g-force, temperature, and time. Then, remove and discard the supernatant.
- 4 Gently pipet the entire volume of the prepared Hoechst 33342 with ethidium homodimer-1 stain to the cells, then gently mix the suspension.
- 5 Incubate the cells at room temperature for 15–20 minutes.
- 6 Centrifuge cells at appropriate g-force, temperature, and time. Then, remove and discard the supernatant.
- 7 Resuspend the pellet in Fluidigm C1 Cell Wash Buffer at 166,000–250,000 cells/mL.
- 8 View the cells under a microscope.

## Surface Marker Staining in the IFC

As an example of surface marker staining, we use TRA-1-60 DyLight 488 antibody to stain human iPSCs. The general steps of the protocol may be applicable to different surface antibodies and different cell types.

### FLUIDIGM-SUPPLIED MATERIALS

- C1 Cell Suspension Reagent (PN 100-5319, 100-6201, or 100-7357)
- C1 system (PN 100-7000)
- C1 IFC (10–17  $\mu\text{m}$ , PN 100-5479)

### CUSTOMER-SUPPLIED MATERIALS

- StainAlive TRA-1-60 Antibody (Dylight 488) stock (Stemgent, PN 09-0068)
- Essential 8 cell culture medium (Life Technologies, PN A1517001; supplement stored at  $-20^{\circ}\text{C}$ )
- Microscope with FITC filter
- Cells suspended in appropriate cell culture medium
- FITC filter set

## Procedure

- 1 Prime the IFC.
- 2 Warm Essential 8 cell culture medium to room temperature.
- 3 Prepare staining solution:

Staining Solution Component	Volume ( $\mu\text{L}$ )
StainAlive TRA-1-60 Antibody (Dylight 488) stock	.05
Essential 8 medium (or dilution buffer)	24.5
<b>Total volume</b>	<b>25</b>
<b>Final concentration</b>	<b>10 <math>\mu\text{g}/\text{mL}</math></b>

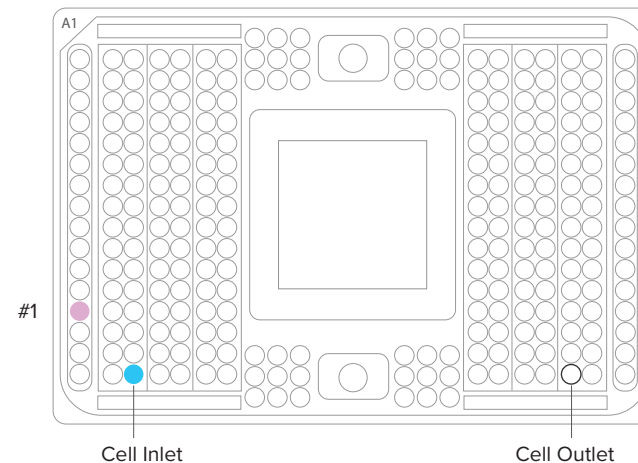
- 4 Resuspend the stem cells in Essential 8 cell culture medium at 166,000–250,000/mL.

- 5 Prepare the cell mix:

**NOTE** This formulation is specific for iPSCs.

Cell Mix Component	Volume ( $\mu\text{L}$ )
Cells (166,000–255,000/mL)	70
C1™ Cell Suspension Reagent (Fluidigm)	30
<b>Total</b>	<b>100</b>

- 6 Pipet the cell mix up and down five to ten times to mix. Do not vortex the cell mix. Avoid bubbles.
- 7 Remove blocking solutions from the cell inlet (teal dot) and cell outlet (white dot) of the C1 IFC.
- 8 Pipet 5–20  $\mu\text{L}$  of the cell mix into the cell inlet (teal dot).
- 9 Pipet 20  $\mu\text{L}$  of Antibody Staining Solution into inlet 1 (pink dot).
- 10 Place the IFC into the C1, then run the Cell Load & Stain script.
- 11 After the run, tap **Eject** to remove the IFC.
- 12 Image the cells on a microscope compatible with the C1 IFC.
- 13 A FITC filter set can be used to observe Dylight 488.



## QUALITY CONTROL

Check the quality of single cells according to these properties:

- Cell size and viability.
- Cell concentration on [page 23](#)
- Cell buoyancy on (see “Optimizing Cell Buoyancy for the C1” on [page 24](#))

## Cell Size and Viability

Follow these procedures to characterize cells in order to choose the appropriate C1 IFC:

- Stain cells with viability stain.
- Image the cells by hemocytometer.
- Measure the cells by imageJ software.

You can also measure cell size and count cells with commercial, automated instruments, such as the Scepter™ 2.0 Handheld Automated Cell Counter (Millipore) or the Countess® Automated Cell Counter (Life Technologies).

### CUSTOMER-SUPPLIED MATERIALS

- Cells suspended in appropriate cell culture medium
- LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Life Technologies, PN L-3224)
- Appropriate buffer/medium for cells of interest
- Benchtop centrifuge
- INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved, PN DHC-N01)
- Inverted microscope equipped for epifluorescence and phase contrast, digital image capture required (CCD camera), and 10X or 20X objective
- ImageJ software. To download go to [rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/)
- Microsoft® Excel® software

**NOTE** Where necessary, centrifuge cells at the appropriate g-force, temperature, and time.

## Procedure

### PREPARE CELLS

- 1 Dilute the cells to 150–200 cells/μL in their cell culture medium (a total volume of 500–1,000 μL is required for viability staining).
- 2 Stain cells with viability stain.

Cell Mix Component	Volume (μL)
C1™ Cell Wash Buffer	1250
Ethidium homodimer-1	2.5
Calcein AM	0.625
<b>Total</b>	<b>1253.125</b>

- ! IMPORTANT!** Staining solution must be used in less than 1 hour. Prepare fresh staining solution as needed.
- 3 Centrifuge 500–1,000 μL of cells in a new 2.0 mL microcentrifuge tube to pellet the cells.
  - 4 Remove and discard as much medium as possible without disturbing the pellet.
  - 5 Resuspend the pellet in 500 μL of staining solution.
  - 6 Leave the suspension on the bench for 5–10 minutes.
  - 7 Centrifuge the suspension to pellet the cells.
  - 8 Remove and discard as much solution as possible without disturbing the pellet.
  - 9 Resuspend the cells in 200 μL of C1 Cell Wash Buffer.

## IMAGE CELLS

- 1 Load ~10  $\mu\text{L}$  to the INCYTO C-Chip by capillary action.
- 2 Observe the fluorescent signals from the cells with the microscope. Count cells using both FITC and TRITC filter sets (referred to in the table below). For more information, see Minimum Specifications for Single-Cell Imaging, PN 100-5004. Passing viability is >90%.

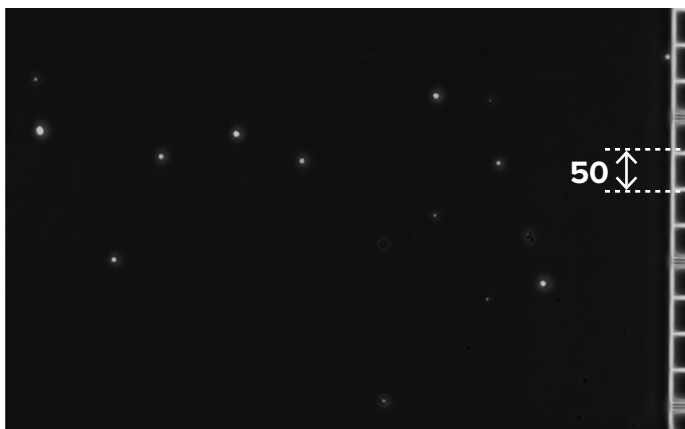
Fluorescence Emission (nm) Viability

517 (FITC, green) Live

635 (TRITC, orange-red) Dead

**NOTE** If the cells do not meet the viability specification, see [troubleshooting](#).

- 3 Observe cells in phase contrast using a 20X objective.
- 4 Photograph >150 distinct (no overlapping) cells on the same microscope at the same magnification, avoiding grid lines in the image.
- 5 The smallest-division grid line (50 $\mu\text{m}$ ) is noted in order to provide a scaling reference:



## MEASURE THE CELLS BY IMAGEJ SOFTWARE

Measure the Cells by ImageJ software. Download at [rsbweb.nih.gov/ij](http://rsbweb.nih.gov/ij).

Using the images you captured above:

- 1 Draw two principal axes for each cell (see red crosshatch).



a. Measure and record each axis in pixels by using the command-CTRL-M keys on each axis.

b. Measure 150 cells.

### ImageJ result table

Results						
File	Edit	Font	Results			
	Area	Mean	Min	Max	Angle	Length
1	40	19543.735	10610.777	29051.529	-111.251	38.873
2	37	20517.882	11777	31160.309	148.496	35.901
3	34	20805.485	8933.783	32302.840	-114.305	33.440
4	32	21384.264	10212.556	33554.109	155.095	31.013
5	34	22261.102	11521	36349.840	-117.350	32.796
6	28	19409.555	12270.037	32535.475	151.557	27.455
7	54	18548.550	9878.432	29918.814	-59.982	52.595
8	39	15362.740	9098.337	27851.656	-146.725	38.459
9	41	16693.789	11521	23976.254	162.897	40.486
10	40	17194.808	12545	24245.766	-107.969	39.215

2 principal axis measurements per cell are made (ImageJ)

← Length of 2 principal axis for cell 1

← Length of 2 principal axis for cell 2

- 2 Calculate the size ( $\mu\text{m}$ ) of cells:

a. Determine the scale for measurement (number of  $\mu\text{m}$  per pixels) by referring to the appropriate manual from the optics manufacturer or by using a micrometer to calculate the distance, or verify by measuring a known feature such as the grid of the hemocytometer.

- b. In Microsoft Excel software, convert the principal axis of each cell from pixels to microns.
- c. Calculate the average of the two scaled principal axis measurements. Call this value the “cell diameter.”

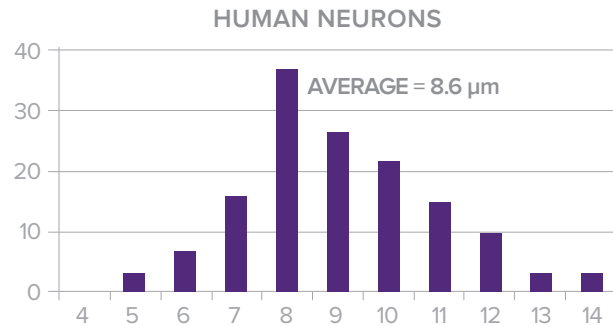
**Excel Processing Table**

Axis values are scaled, averaged, and plotted.

	A	B	C	D	E	F	G	H	I
		Area	Mean	Min	Max	Angle	axis length (pixels)	axis length (microns)	average
1									
2	1	40	19543.74	10610.78	29051.53	-111.251	38.873	17.91268	
3	2	37	20517.88	11777	31160.31	148.496	35.901	16.54318	17.22793 ← Average diameter of cell 1
4	3	34	20805.49	8933.783	32302.84	-114.305	33.44	15.40915	
5	4	32	21384.26	10212.56	33554.11	155.095	31.013	14.29079	14.84997 ← Average diameter of cell 2
6	5	34	22261.1	11521	36349.84	-117.35	32.796	15.1124	
7	6	28	19409.56	12270.04	32535.48	151.557	27.455	12.65126	13.88183
8	7	54	18548.55	9878.432	29918.81	-59.982	52.595	24.23578	
9	8	39	15362.74	9098.337	27851.66	-146.725	38.459	17.72191	20.97884
10	9	41	16693.79	11521	23976.25	162.897	40.486	18.65595	
11	10	40	17194.81	12545	24245.77	-107.969	39.215	18.07027	18.36311

### 3 Report the cell size distribution:

- a. In Microsoft Excel, create a histogram displaying the distribution of cell sizes:



- b. Calculate the average cell size.
  - c. Match the average cell size to the appropriate IFC size (5–10  $\mu\text{m}$ , 10–17  $\mu\text{m}$ , or 17–25  $\mu\text{m}$ ).
- 4 Dispose of the hemocytometer in biohazardous waste.

## Cell Concentration

To avoid clogging the IFC and experiencing poor cell capture, it is important to use the appropriate cell concentration. Use this protocol to measure the cell concentration before proceeding to “[Optimizing Cell Buoyancy for the C1.](#)”

For more detailed information on cell counting, go to [hemocytometer.wordpress.com](http://hemocytometer.wordpress.com)

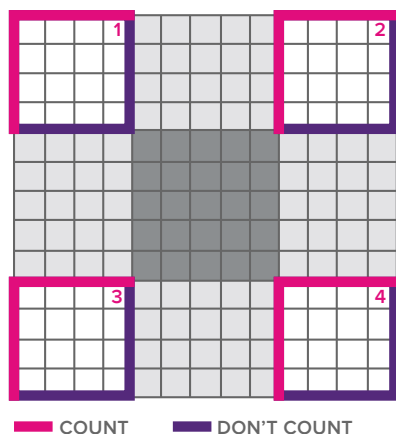
### CUSTOMER-SUPPLIED MATERIALS

- (Optional) INCYTO C-Chip Disposable Hemocytometer (Neubauer Improved, PN DHC-N01)
- Inverted microscope equipped for phase contrast and 10X, 20X, or higher objective; (optional) digital image capture (CCD camera)
- Cells suspended in appropriate cell culture medium

## Procedure

- 1 If necessary, dilute the cells.
- 2 If necessary, add viability dyes at the appropriate concentration for the cells.
- 3 Pipet 10  $\mu\text{L}$  of sample into the hemocytometer through one of the inlets. The cells wick under the coverslip by capillary action.

- Count cells in the four corner squares on the hemocytometer (1–4). To avoid overcounting cells that are half-in and half-out of the squares, consistently count cells that are touching only two of the four boundaries of any square. Record the counts of the four squares. Use this representation of a hemocytometer to help you:



- Calculate: Cells/mL = [(Total cell counts in 4 squares)/4] x dilution factor x  $10^4$
- Dispose of the hemocytometer in biohazardous waste.
- Proceed to [Optimizing Cell Buoyancy for the C1](#).

## Optimizing Cell Buoyancy for the C1

Use this protocol to measure the buoyancy of cells in any single-cell suspension.

Follow the protocol to create a buoyant, single-cell suspension for loading into a C1™ IFC and for determining the optimal ratio of cells to C1 Suspension Reagent.

### FLUIDIGM-SUPPLIED MATERIALS

- C1 Suspension Reagent

- C1 system (PN 100-7000)
- C1 IFC (5–10  $\mu\text{m}$ , PN 100-5757)

### CUSTOMER-SUPPLIED MATERIALS

- 384-well microplate (optional)
- Inverted microscope equipped for phase contrast and 10X; (optional) digital image capture (CCD camera)
- Cells at 166–250 cells/ $\mu\text{L}$  in appropriate cell culture medium

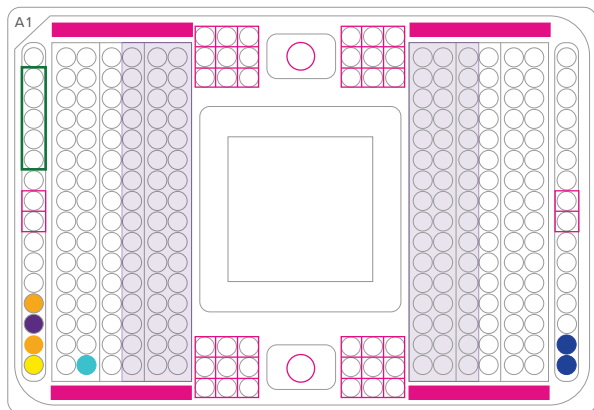
## Procedure

- Mix cells with C1™ Cell Suspension Reagent in individual tubes. Pipet the suspensions up and down to mix:

Ratio	Volume Cells ( $\mu\text{L}$ )	Volume Cell Wash Buffer ( $\mu\text{L}$ )	Volume of C1 Suspension Reagent ( $\mu\text{L}$ )
5:5	5	0	5
6:4	5	1	4
7:3	5	2	3
8:2	5	3	2
9:1	5	4	1
10:0	5	5	0
<b>Total</b>	<b>30</b>	<b>15</b>	<b>15</b>

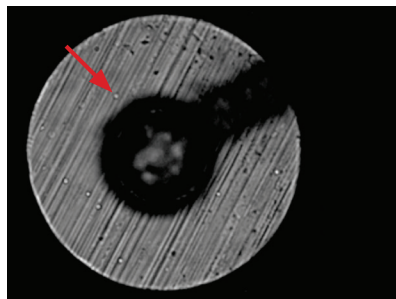


- Transfer 9  $\mu\text{L}$  of each cell mix to unused inlets of a C1™ IFC (green box) or individual wells of a 384-well microplate. Do not generate bubbles.

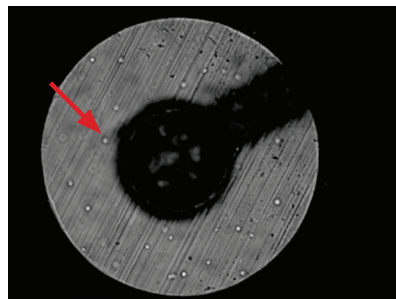


- Immediately examine the cells at 10X magnification by focusing on the bottom of the inlet or well. Then, scanning up through the liquid column, verify that there is an even distribution of cells from bottom to top and no air bubbles. Keep a record of the cell count, or capture an image of the cells.
- Wait 10 minutes, and then re-examine the cells (as described in Step 3). At the optimal ratio of cells to cell suspension buffer the distribution of cells should be unchanged after 10 minutes:

**Example of optimal ratio cells evenly distributed**

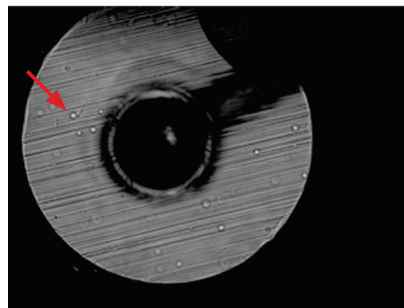


Time = 0 min

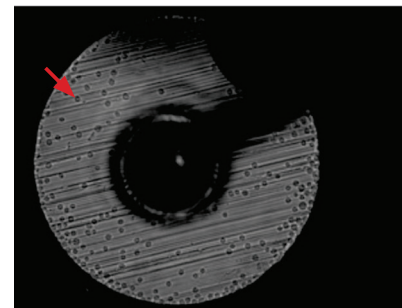


Time = 10 min

**Example of an incorrect ratio with insufficient suspension reagent: cells at bottom**

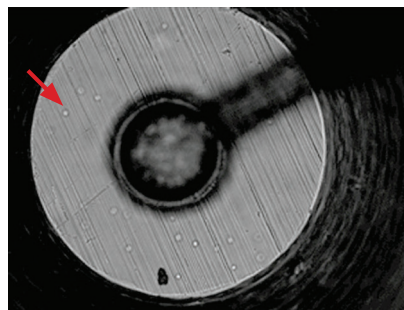


Time = 0 min

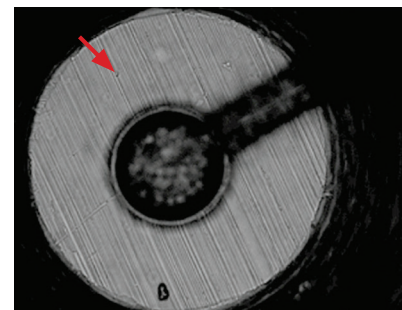


Time = 10 min

**Example of an incorrect ratio with excessive suspension reagent: cells on surface**



Time = 0 min



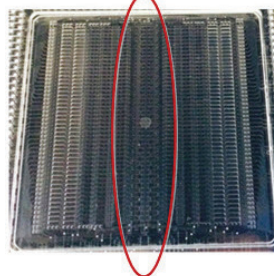
Time = 10 min

- Load the cells in the optimal ratio of cells to C1 Suspension Reagent into a C1 IFC. For more information, see page 20 in the protocol [Using the C1 System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays](#) (Fluidigm, PN 100-4904).

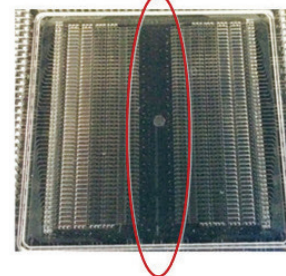
# TROUBLESHOOTING

Observations	Possible Causes	Recommended Actions
No cells in any capture site of the C1™ IFC	<ul style="list-style-type: none"> <li>Incorrect input concentration</li> <li>Wrong IFC type</li> <li>Cell viability is &lt;90%</li> <li>Ratio of cells to C1™ Suspension Reagent sub-optimal</li> </ul>	<ul style="list-style-type: none"> <li>Confirm the quality of the samples.</li> <li>Always determine the sample concentration, cell size, and viability before loading the cells into the C1™ IFC.</li> <li>Use the IFC type that is appropriate for your cell size.</li> <li>Add cells to the C1™ IFC at the recommended concentration (see <a href="#">“Cell Concentration” on page 23</a>).</li> <li>If cells are at an appropriate concentration and still there are no cells in any capture site, it is possible that the cells are not neutrally buoyant at the standard suspension ratio. In a standard workflow, Fluidigm recommends combining 60 µL of cells with 40 µL of C1 Suspension Reagent. It might be necessary to increase or decrease this ratio if cell capture fails. Find the optimal ratio of cells to C1™ Suspension Reagent (see <a href="#">“Optimizing Cell Bouyancy for the C1” on page 24</a>).</li> </ul>
	<ul style="list-style-type: none"> <li>Incorrect priming. For example, the IFC on the left is not primed, while the one on the right is primed.</li> </ul>	<ul style="list-style-type: none"> <li>Prime the IFC according to the instructions. Add the correct reagents to the designated inlets and process the IFC in the C1 with the appropriate Prime script.</li> <li>Ensure that in medium and large C1™ IFCs, the Cell Input well has a bubble after priming. The bubble is normal and created with the movement of reagents throughout the channels. If there is no bubble, check all inputs, refill inputs if necessary, then reprime the IFC.</li> <li>Ensure that the C1™ Harvest Reagent, which is added to the accumulator tanks, is used entirely, with only a trace amount remaining in the tanks.</li> <li>Ensure that all priming reagents were added to the IFC [see the protocol <a href="#">Using the C1 System to Capture Cells from Cell Culture and Perform Preamplification Using Delta Gene Assays</a> (Fluidigm, PN 100-4904)].</li> </ul>

Unprimed IFC



Primed IFC



The 10X phase contrast:

Unprimed IFC



Primed IFC



Observations	Possible Causes	Recommended Actions
	Incompatible beads used for enrichment	<ul style="list-style-type: none"> <li>Use beads that are &lt;50 nm. The Miltenyi Biotec beads are validated for use with the C1. Do not use Dynabeads.</li> <li>Enrichment with some beads or nanoparticles can affect cell buoyancy, which might lower the capture rate. To improve results, follow the buoyancy protocol (see <a href="#">“Optimizing Cell Buoyancy for the C1” on page 24</a>).</li> </ul>
	Cell clumps clogging channels	<ul style="list-style-type: none"> <li>Under the microscope, view capture sites 48 and 96 to look for cell clumps clogging channels.</li> <li>Follow dissociation methods to declump cells (see <a href="#">“Dissociation” on page 11</a>).</li> <li>Very gently pipet cells up and down before combining them with C1 Suspension Buffer and transferring the cell suspension mix to the cell input inlet.</li> <li>Use a 40 µm cell strainer to remove debris and clumps before mixing with the suspension reagent.</li> </ul>
	C1™ Suspension Reagent or C1 Blocking Reagent frozen or heated above room temperature.	<ul style="list-style-type: none"> <li>Discard reagents and use new reagents.</li> <li>Store the new reagents at 4 °C. Do not freeze them.</li> </ul>
Capture rate is low (also see <a href="#">“No cells in any capture site of the C1™ IFC” on page 26</a> )	<ul style="list-style-type: none"> <li>Cell quality poor</li> <li>Input concentration too low</li> <li>Cell size not appropriate for IFC type</li> </ul>	<ul style="list-style-type: none"> <li>Confirm the quality of the samples: Always determine the sample concentration, cell size, and viability before loading the cells into the C1™ IFC (see <a href="#">“Quality Control” on page 21</a>).</li> <li>Add cells to the C1™ IFC at the recommended concentration (see <a href="#">“Cell Concentration” on page 23</a>).</li> <li>Use the IFC type that is appropriate to the cell size.</li> <li>Use freshly prepared, viable cells.</li> </ul>
	Cells not neutrally buoyant in the cell suspension	<ul style="list-style-type: none"> <li>Find the optimal ratio of cells to C1™ Suspension Reagent (see <a href="#">“Optimizing Cell Buoyancy for the C1” on page 24</a>).</li> </ul>
Most or all cells stain red using the LIVE/DEAD viability stain	<ul style="list-style-type: none"> <li>Sample quality sub-optimal</li> <li>Cells stored too long at room temperature</li> <li>Cells stored too long in incompatible buffer</li> <li>Enrichment (such as FACS) completed too quickly or slowly</li> </ul>	<ul style="list-style-type: none"> <li>Minimize the time between FACS sorting, processing, and loading cells into the C1.</li> <li>Sort the cells into their native media for viability and maintain collection vials at the optimal temperature.</li> <li>Sort the cells slowly to minimize stress on FACS-sorted cells. Higher sort rates may lower cell-viable recoveries.</li> </ul>
	Low viability of cell sample	<ul style="list-style-type: none"> <li>Remove dead or dying cells from the sample before loading in the C1 to improve capture rates, reproducibility, and data accuracy.</li> <li>There are several ways to remove dead cells from a sample. For example, use Miltenyi Biotec’s Dead Cell Removal Kit (Cat. No. 130-090-101), a magnet-based method. Or stain dead cells with a dye, such as SYTOX® or LIVE/DEAD Fixable Dead Cell Stain (both from Life Technologies), then sort the dead or dying cells by FACS.</li> </ul>

Observations	Possible Causes	Recommended Actions
	Cell damaged	<ul style="list-style-type: none"> <li>Avoid cell death by freeze-thaw. Follow the appropriate cryostorage and thawing protocols according to cell line.</li> <li>Gently dissociate cells with reagents such as Accutase Cell Detachment Solution instead of trypsin or papain.</li> <li>Handle cells gently. Do not vortex cells, and use a pipette to gently dislodge cells.</li> </ul>
	<ul style="list-style-type: none"> <li>DMSO</li> </ul>	<ul style="list-style-type: none"> <li>Verify if frozen cells were preserved in DMSO. If there is residual DMSO in the residual suspension, false dead cell detection is possible.</li> <li>If the cells stain green, they are live cells and can still be used for analysis.</li> </ul>
Most or all cells stain red-green using the LIVE/ DEAD viability stain (see more above).	<ul style="list-style-type: none"> <li>Cell membrane damaged</li> <li>DMSO</li> </ul>	<ul style="list-style-type: none"> <li>Check for cell death. Dying cells may stain with both dyes. Ethidium homodimer enters the cell and emits a red fluorescence.</li> <li>Note which cells stain red or green. Continue to analyze the cells and use the gene expression profile to guide your analysis.</li> </ul>
Cells visible under phase contrast, but there is no fluorescent signal to indicate living or dead cells or specific signal from antibody staining.	<ul style="list-style-type: none"> <li>LIVE/DEAD Kit stored improperly</li> <li>LIVE/DEAD Staining Solution was not made fresh</li> </ul>	<ul style="list-style-type: none"> <li>Store dye vials at <math>-20^{\circ}\text{C}</math> in a sealed bag containing the desiccant provided by the manufacturer.</li> <li>Prepare fresh working solution and keep in the dark. Use the working solution no more than one hour after preparation.</li> <li>Use aliquots of Calcein AM (live dye), which is sensitive to hydrolysis and freeze-thawing.</li> </ul>
	Small cells require twice as much dye	<ul style="list-style-type: none"> <li>Double the concentration of dye used on small cells (<math>5\text{--}10\text{ }\mu\text{m}</math>) relative to the concentration used on larger cells.</li> </ul>
	Cy5 is outside visible spectrum for human eye	<ul style="list-style-type: none"> <li>If you are using dyes with emission spectra above <math>630\text{ nm}</math>, check the fluorescent signal using a camera rather than the ocular lenses.</li> <li>Increase the exposure time.</li> <li>Turn off the lights or create “walls” around the instrument to block light</li> </ul>



## EXTERNAL RESOURCES

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., et al. "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling." *Nature Biotechnology* 27 (2009): 275–80.

International Stem Cell Initiative et al. "Characterization of human embryonic stem cell lines," *Nature Biotechnology* 17 (2007): 803–816.

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