DEMONSTRATED PROTOCOL

Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing

Overview

The ability of the 10x Genomics Single Cell Solutions to partition single cells in a heterogeneous population can be verified by profiling a mixture of cells from two different species. Ideally, all sequence reads from a single Gel Bead-in-emulsion (GEM) will be unambiguously mapped to the transcriptome of only one of the two species. The fraction of GEMs containing a mixture of human and mouse transcripts is used to infer doublet rates (see Appendix).

10x Genomics routinely uses a 1:1 mixture of human and mouse cells to validate the technical performance of the 10x Genomics Single Cell Solutions. This Demonstrated Protocol outlines cryopreservation and thawing of 1:1 mixtures of human and mouse cells in preparation for use in 10x Genomics Single Cell protocols.

Additional Guidance

Consult Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices on handling cells and Technical Note Guidelines for Accurate Target Cell Counts using 10x Genomics Single Cell Solutions (Document CG000091) for determining accurate cell counts.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Cell Sourcing

This protocol was demonstrated by preparing a 1:1 mixture of 293T/17 and NIH/3T3 cells.

Cell Type Used	Species	Supplier
293T/17 (CRL-11268)	Human	ATCC
NIH/3T3 (CRL-1658)	Mouse	ATCC

NIH/3T3 cells will often be the limiting reagent as they grow to a lower density than the 293T/17 cells. Approximately 5 times more NIH/3T3 culture flasks are required to achieve balanced cell numbers (e.g., 4 confluent T75 flasks of 293T/17s will require ~20 nearly confluent T75 flasks of NIH/3T3 cells).

Preparation - Buffers

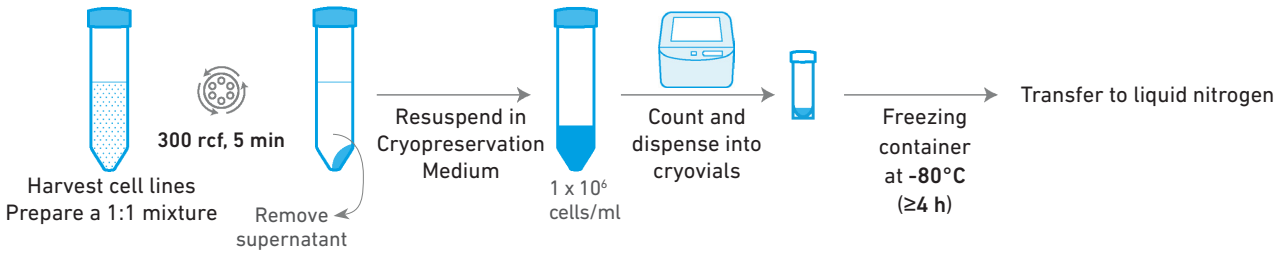
Cryopreservation (maintain at 4°C)	
Media	**Composition**
Cryopreservation Medium	20% FBS + 10% DMSO in cell culture media (e.g., DMEM)
Thawing (maintain at room temperature)	
PBS + 0.04% BSA	

Specific Reagents & Consumables

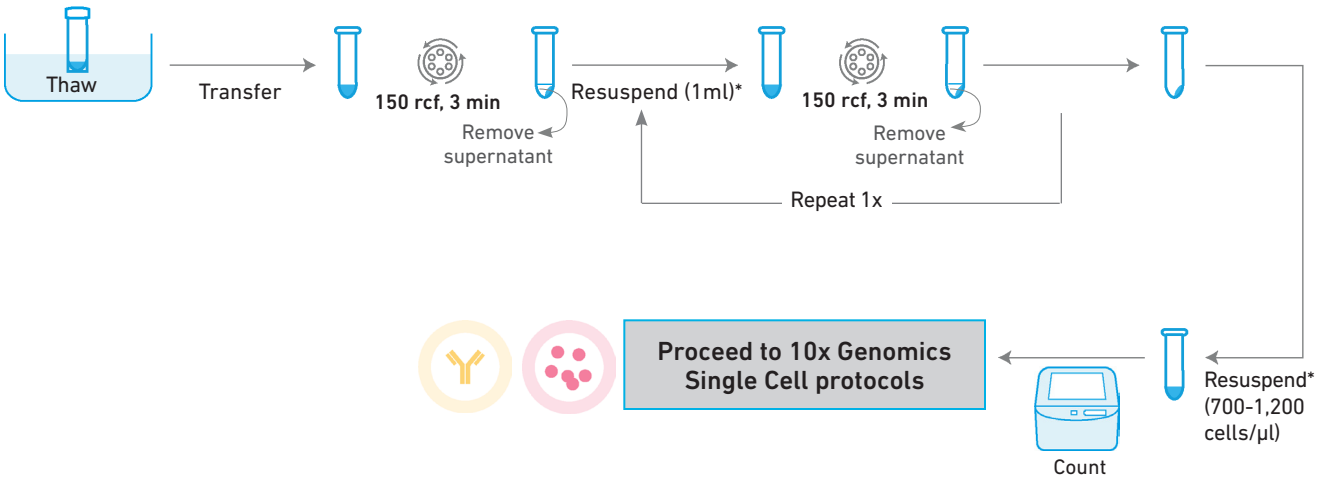
Vendor	Item	Part Number
Thermo Fisher Scientific	Gibco DMEM	11965-092
	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Trypan Blue Stain (0.4%)	T10282
	Nunc Biobanking & Cell Culture Cryogenic Tubes, 1.8 ml	368632
	Countess II FL Automated Cell Counter	AMQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228
Fisher Scientific	Dimethyl Sulfoxide (DMSO), for molecular biology	ICN19141880
Millipore-Sigma	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Miltenyi	MACS SmartStrainers, 30 µm	130-098-458
Eppendorf	DNA LoBind Tubes, 2.0 ml	022431048
VWR	Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
Biocision	CoolCell FTS30 Cell Freezing Container	BSC-170
Corning	Phosphate-Buffered Saline without Calcium & Magnesium	21-040-CV

Protocol Overview

Cryopreservation 15-30 min



Thawing & Resuspension 30-45 min



*Resuspensions are in PBS + 0.04% BSA

Protocol

Cryopreservation

Cell Harvesting

Remove medium from cultured 293T/17 and NIH/3T3 cells, rinse culture vessels and, detach cells using trypsin. Centrifuge cells and resuspend cell pellets in growth medium by pipette mixing 10x. Pass through a cell strainer to remove clumps and to ensure single cell suspensions. Determine the cell concentration using a Countess II Automated Cell Counter or a hemocytometer.

Consult Demonstrated Protocol Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing (Document CG00054) for more details on harvesting adherent cells.

Preparation of 1:1 Human-Mouse Cell Line Mixture

- Based on total volumes (V) and concentrations (C) of each cell, calculate total cell number (N = C x V) for each type to identify limiting cell type (cell line with lower cell number).
- Determine the volume of non-limiting cell type to be added to the limiting cell type to achieve a 1:1 ratio (see Appendix).
- Add appropriate volume of both cell lines to achieve a 1:1 ratio.

a. Centrifuge the 1:1 mixture at **300 rcf** for **5 min** at **4°C**.

b. Remove the supernatant.

c. Resuspend the cell pellet in an appropriate volume of chilled Cryopreservation Medium to obtain a cell concentration of 1 x 10^6 cells/ml.

d. Dispense cell suspension aliquots into pre-cooled cryovials and place the cryovials inside a pre-cooled cell freezing container e.g., CoolCell FTS30.

e. Place the cell freezing container in a –80°C freezer for **≥4 h**. After **4 h**, transfer the cryovials to liquid nitrogen for **long-term** storage.

Thawing & Resuspension

Set up a water bath to **37°C** before staring cell thawing. All cell washes were performed at **room temperature**.

a. Remove cryovials from storage and **immediately** thaw in the water bath at **37°C** for **2-3 min**.

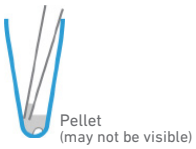
DO NOT submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

b. Pipette mix the cells and transfer the entire volume to a 2-ml tube.

c. Centrifuge at **150 rcf** for **3 min.**

d. Remove supernatant without disrupting the pellet.

Cell pellet may be present on the side or on the bottom of the tube.



e. Using a **wide-bore** pipette tip, add **1 ml** 1X PBS + 0.04% BSA to each tube and gently pipette mix 5x to resuspend cell pellet. Pool the tubes if necessary.

f. Centrifuge at **150 rcf** for **3 min.** Remove the supernatant.

g. Repeat e-f for a total of two washes.

h. Based on starting cell concentration and assuming ~50% cell loss, add an appropriate volume 1X PBS + 0.04% BSA to obtain a cell concentration of 700-1,200 cells/µl. Gently pipette mix using a **regular-bore** pipette tip until a single cell suspension is achieved.

DO NOT invert the tube, as cells can stick to the sides of the tube, thereby changing the cell concentration.

i. Determine the cell concentration using a Countess II Automated Cell Counter or a hemocytometer. The targeted final cell concentration is 700-1,200 cells/µl.

j. Once the final cell concentration is achieved, place cells on ice.

k. Proceed **immediately** to the 10x Genomics Single Cell protocols.

Appendix

Example Calculation for Preparation of Cell Line Mixture

Example Calculation

Cell Line	Volume (ml)	Concentration (cells/ml)	Total Number
293T/17	10	3 x10^6	3 x 10^7
NIH/3T3	6	2 x10^6	1.2 x 10^7

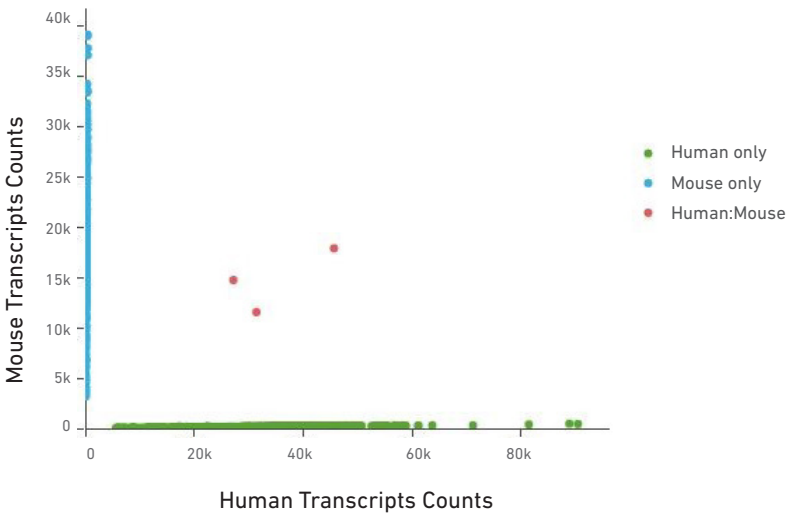
NIH/3T3 is the limiting and 293T/17 is the non-limiting type.

$$\text{Volume of non-limiting} = \frac{\text{Total cell number of limiting cell type}}{\text{Concentration of non-limiting type}}$$

$$\text{Volume of 293T/17} = \frac{1.2 \times 10^7 \text{ cells}}{3 \times 10^6 \text{ cells/ml}} = 4 \text{ ml}$$

4 ml 293T/17 cells are added to 6 ml NIH/3T3 cells to generate 10 ml of a 1:1 mixture.

Inferring Doublet Rate using a Human-Mouse Cell Line Mixture



Approximately 1000 cells from a 1:1 mixture of human (293T/17) and mouse (NIH/3T3) cells were profiled using the Chromium Single Cell Gene Expression Solution. 99.7% of the cell-containing GEMs resulted in sequencing reads mapping to only one species. This implies a total doublet rate (including human:human and mouse:mouse doublets) of 0.6% in this experiment.

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