10x Genomics®

Sample Preparation Demonstrated Protocol

Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing

10x GENOMICS®

10xGenomics.com

Notices

Manual Part Number

CG00039 Rev C

Legal Notices

Customer Information and Feedback

For technical information or advice, please contact our Customer Technical Support Division online at any time.

Email: support@10xgenomics.com

10x Genomics

7068 Koll Center Parkway

Suite 401

Pleasanton, CA 94566 USA

Table of Contents

Demonstrated Protocol 1

1. Overview 2
2. Getting Started 2
2.1. Tips & Safety 2
2.2. General Materials 3
2.3. Preparation – Buffers & Media 4
3. Cryopreservation of Human PBMCs 4
3.1. Cryopreservation Protocol 4
4. Thawing & Washing Human PBMCs 5
4.1. Preparation 5
4.2. Thawing, Washing & Counting Cells 5
5. Viability Results 7

Demonstrated Protocol

Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing

1. Overview

This Demonstrated Protocol outlines (i) how to generate cryopreserved human peripheral blood mononuclear cells (PBMCs) and (ii) how to thaw, wash, and count cryopreserved human PBMCs in preparation for use in 10x Genomics® Single Cell Protocols.

While this Demonstrated Protocol is specific to PBMCs, the Protocol may be used as a basis for handling other primary cells in preparation for use in 10x Genomics Single Cell Protocols.

2. Getting Started

2.1. Tips & Safety

Best practices for handling any cell line include using sterile technique, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips where possible to minimize cell damage.

To determine accurate cell counts, best practices include sampling the cell suspension at least twice and at least two counts on each sample (*i.e.* a minimum of four counts in total, based on two independent draws from the cell suspension).

CRITICAL!

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

2.2. General Materials

Supplier	Description	Part Number (US)
-	Refrigerated Benchtop Centrifuge for 15 ml and 50 ml tubes	-
	Microcentrifuge for 2 ml LoBind tubes	-
	Heated Water Bath, 2l	-
Rainin	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 1ML Fltr RT-L1000WFLR	17014297
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
Sigma-Aldrich	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Miltenyi	MACS SmartStrainers, 30 µm	130-098-458
Bel-Art	Flowmi™ Cell Strainer, 40 µm	H13680-0040
Thermo Fisher Sci	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml) (alternative to Sigma-Aldrich product)	AM2616
	Trypan Blue Stain (0.4%) for use with the Countess® Automated Cell Counter	T10282
	Countess® II Automated Cell Counter	AMQAX1000
	Countess® II Automated Cell Counting Chamber Slides	C10228
	Nunc™ Biobanking and Cell Culture Cryogenic Tubes, 1.8 ml	368632
	Dimethyl Sulfoxide (DMSO), for molecular biology	AC327182500
Eppendorf	DNA LoBind Tubes 2.0 ml*	022431048
Integra	PIPETBOY acu 2	155018
VWR	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 15 ml	21008-103
	10 ml Serological Pipette	89130-898
	75 cm^2 Cell Culture Flask, Canted Neck	46610-078
	Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
ATCC	IMDM	30-2005
Biocision	CoolCell® FTS30 Cell Freezing Container	BSC-170

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations.

2.3. Preparation – Buffers & Media

a) Prepare chilled (4°C) resuspension medium: 40% FBS in medium (*e.g.* IMDM).

b) Prepare chilled (4°C) 2X freezing medium: 30% DMSO in medium (*e.g.* IMDM) containing 40% FBS.

c) Chill a CoolCell® FTS30 or equivalent cell freezing container in a 4°C refrigerator.

3. Cryopreservation of Human PBMCs

3.1. Cryopreservation Protocol

This Protocol is based on working with fresh human PBMCs suspended in 10% FBS in IMDM and targets a concentration of 10 x 10^6 cells per vial.

NOTE

The ideal total cell number for cryopreservation is 10 x 10^6 cells per vial. However, down to 1 x 10^6 cells per vial can be accommodated if cell number is limiting.

a) Obtain and place fresh PBMCs on ice.

NOTE

This Protocol was developed using fresh PBMCs from AllCells (www.allcells.com; PN-PB001 or PN-PB002) shipped on ice. The cells were used immediately upon receipt.

b) Gently mix the cells with a serological pipette.

c) Determine cell viability and total cell number using a Countess® II Automated Cell Counter.

d) Centrifuge the cells at **300 rcf** for **5 min** at **4°C**.

e) Resuspend the cell pellet in an appropriate volume of chilled resuspension medium to achieve a cell concentration of 20 x 10^6 cells/ml. Maintain the cells on ice.

f) Gently add an equivalent volume of chilled 2X freezing medium to achieve a cell concentration of 10 x 10^6 cells/ml.

g) Gently mix the cells.

h) Place cryovials in a rack on ice.

i) Dispense **1 ml** aliquots of cell suspension into cryovials.

j) Remove the chilled CoolCell® FTS30 from the 4°C refrigerator and place the cryovials inside.

k) Place the chilled CoolCell® FTS30 in a **–80°C** freezer for at least **4 h**, ensure that the bottom and top vents are not obstructed to allow for adequate air flow.

l) After **4 h**, transfer the cryovials to either liquid nitrogen or vapor-phase nitrogen for long-term storage.

4. Thawing & Washing Human PBMCs

NOTE

This Protocol assumes the human PBMCs were cryopreseved according to the Protocol outlined in Section 3.

4.1. Preparation

a) Warm a water bath to 37°C prior to commencing the thawing Protocol.

b) Prepare 35 ml warm complete growth medium (*e.g.* 10% FBS in IMDM) per sample by incubating in a 37°C incubator prior to use.

c) Prepare 1X PBS with 0.04% BSA (400 µg/ml) solution.

4.2. Thawing, Washing & Counting Cells

NOTE

Perform cell washes at room temperature.

a) Remove cryovial(s) from liquid or vapor-phase nitrogen storage and immediately thaw in the water bath at **37°C** for **2-3 min**. Do not to submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

b) After thawing is complete, clean the vial with 70% alcohol and KimWipes.

c) In a biosafety hood, gently transfer thawed cells to a 50 ml conical tube using a **wide-bore** pipette tip.

d) Using a **wide-bore** pipette tip, rinse the cryovial with **1 ml** warm complete growth medium.

e) Using a **wide-bore** pipette tip, add the rinse medium **dropwise** (1 drop per 5 sec) to the 50 ml conical tube while gently shaking the tube.

NOTE

Dropwise addition of medium allows cells sufficient time for gradual loss of DMSO and therefore prevents osmotic lysis.

f) Serially dilute cells with complete growth medium a total of 5 times by 1:1 volume additions with ~1 min wait between additions. Add complete growth medium at a speed of 3-5 ml/sec.

EXAMPLE

For example, slowly add 2 ml medium into the existing 2 ml cell suspension, gently swirl for 5 sec, and leave the tube at room temperature for ~1 min. This is considered the second serial dilution. Repeat 3 more times to achieve a final volume of ~32 ml.

g) Centrifuge cells at **300 rcf** for **5 min**.

NOTE

Some primary cells need to be centrifuged at higher speed (e.g. up to 400 rcf) or for longer time (e.g. up to 10 or 15 min) to minimize cell loss due to inefficient pelleting. However, be careful not to over-centrifuge as increased centrifugation speed can compromise the cell integrity and viability.

CRITICAL!

Depending on the rotor type, the cell pellet forms on the side or on the bottom of the conical vial. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be difficult to see.

h) Remove most of the supernatant, leaving ~1 ml and resuspend cell pellet in this volume using a **regular-bore** pipette tip. Save the removed supernatant in another tube until the Protocol is complete.

i) Add an additional **9 ml** complete growth medium (at a speed of 3-5 ml/sec) to achieve a total volume of ~10 ml.

j) Determine the cell concentration using a Countess® II Automated Cell Counter. Calculate the total cell number (N) based on the total volume (V) and concentration (C) where N = C x V.

Example:

If the cell concentration is 0.5 x 10^6 cells/ml and the total volume is 10 ml, the total number of cells in the tube is ~5 million cells

N = C x V = 0.5 x 10^6 cells/ml x 10 ml = 5 x 10^6 cells

k) If total cell number is ≤2 x 10^6 cells, use the entire sample for washing. If total cell number is >2 x 10^6 cells, transfer ~2 million cells into a new tube for washing.

Example continued:

For example, with 5 million cells in hand at a concentration of 0.5 x 10^6 cells/ml

$$V = \frac{N}{C} = \frac{2 \times 10^6 \text{cells}}{0.5 \times 10^6 \text{ cells/ml}} = 4 \text{ ml}$$

Excess cells can be processed for cryopreservation by repeating this section 3.1 of this Protocol, or be used for other purposes.

l) Centrifuge cells at **300 rcf** for **5 min**.

m) Remove supernatant without disrupting the cell pellet. Save the removed supernatant in another tube until the Protocol is complete.

n) Using a **wide-bore** pipette tip, add **1 ml** 1X PBS with 0.04% BSA (400 µg/ml) and gently pipette mix 5 times. Transfer suspended cells into a 2 ml Eppendorf tube. Rinse the 50 ml conical tube with 0.5 ml 1X PBS with 0.04% BSA and transfer the rinse into the 2 ml tube containing cells. Finish suspending cells by inverting the tube.

o) Centrifuge cells at **300 rcf** for **5 min**.

p) Remove supernatant without disrupting the cell pellet. Save the removed supernatant in another tube until the Protocol is complete.

q) Using a **regular-bore** pipette tip, add **1 ml** 1X PBS with 0.04% BSA or an appropriate volume to achieve a cell concentration of ~1 x 10^6 cells/ml. Gently pipette mix 10-15 times or until cells are completely suspended.

NOTE

Do not invert the tube in this step, as cells can stick to the sides of the tube, thereby changing the cell concentration.

r) Use a cell strainer to remove cell debris and large clumps. For low volume, a 40 µm Flowmi™ Tip Strainer is recommended to minimize loss of sample volume.

s) Determine the cell concentration using a Countess® II Automated Cell Counter. If cell concentration is >1 x 10^6 cells/ml, adjust the volume accordingly.

t) Once a cell concentration of ~1 x 10^6 cells/ml is obtained, place the cells on ice.

u) Proceed with the 10x Genomics® Single Cell Protocol.

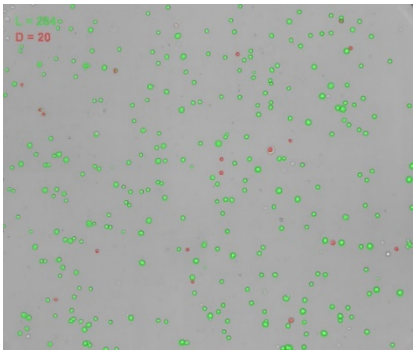
5. Viability Results

The viability of PBMCs depends on both the freezing and thawing protocols, in addition to the total cell number per vial used during cryopreservation. The typical percent PBMCs viability obtained by following this Protocol ranges from high 80s to low 90s (88 – 93%) based on both trypan blue staining and live/dead staining (using a Thermo Fisher Live/Dead® Viability/Cytotoxicity Kit, for example).

Trypan Blue Staining on Countess® II

Live = Green = 92%

Dead = Red = 8%



Live/Dead Viability Staining

Live = Green = 93.4%

Dead = Red = 6.6%