10x Genomics®
Sample Preparation
Demonstrated Protocol
Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing
NOTICES

Notices

Manual Part Number
CG00039 Rev C

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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).

*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

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Description</th>
<th>Part Number (US)</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>Refrigerated Benchtop Centrifuge for 15 ml and 50 ml tubes</td>
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<tr>
<td></td>
<td>Microcentrifuge for 2 ml LoBind tubes</td>
<td>-</td>
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<tr>
<td></td>
<td>Heated Water Bath, 2l</td>
<td>-</td>
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<tr>
<td>Rainin</td>
<td>Tips LTS 1 ML Filter RT-L1000FLR</td>
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<td></td>
<td>Tips LTS W-O 1 ML Fltr RT-L1000WFLR</td>
<td>17014297</td>
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<td>Pipet-Lite LTS Pipette L-1000XLS+</td>
<td>17014382</td>
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<tr>
<td>Sigma-Aldrich</td>
<td>Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)</td>
<td>SRE0036</td>
</tr>
<tr>
<td>Milttenyi</td>
<td>MACS SmartStrainers, 30 µm</td>
<td>130-098-458</td>
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<tr>
<td>Bel-Art</td>
<td>Flowmi™ Cell Strainer, 40 µm</td>
<td>H13680-0040</td>
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<tr>
<td>Thermo Fisher Sci</td>
<td>UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml) (alternative to Sigma-Aldrich product)</td>
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<tr>
<td></td>
<td>Trypan Blue Stain (0.4%) for use with the Countess® Automated Cell Counter</td>
<td>T10282</td>
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<td></td>
<td>Countess® II Automated Cell Counter</td>
<td>AMQAX1000</td>
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<td>Countess® II Automated Cell Counting Chamber Slides</td>
<td>C10228</td>
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<td></td>
<td>Nunc™ Biobanking and Cell Culture Cryogenic Tubes, 1.8 ml</td>
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<tr>
<td></td>
<td>Dimethyl Sulfoxide (DMSO), for molecular biology</td>
<td>AC327182500</td>
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<tr>
<td>Eppendorf</td>
<td>DNA LoBind Tubes 2.0 ml*</td>
<td>022431048</td>
</tr>
<tr>
<td>Integra</td>
<td>PIPETBOY acu 2</td>
<td>155018</td>
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<tr>
<td>VWR</td>
<td>Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml</td>
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<td></td>
<td>Sterile Polypropylene Centrifuge Tubes with Flat Caps, 15 ml</td>
<td>21008-103</td>
</tr>
<tr>
<td></td>
<td>10 ml Serological Pipette</td>
<td>89130-898</td>
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<tr>
<td></td>
<td>75 cm² Cell Culture Flask, Canted Neck</td>
<td>46610-078</td>
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<tr>
<td></td>
<td>Seradigm Premium Grade Fetal Bovine Serum (FBS)</td>
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<td>ATCC</td>
<td>IMDM</td>
<td>30-2005</td>
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<tr>
<td>Biocision</td>
<td>CoolCell® FTS30 Cell Freezing Container</td>
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</tbody>
</table>

*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.*

<table>
<thead>
<tr>
<th>NOTE</th>
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<tbody>
<tr>
<td>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.</td>
</tr>
</tbody>
</table>

a) Obtain and place fresh PBMCs on ice.

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

This Protocol assumes the human PBMCs were cryopreserved 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

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.

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.

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.

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.

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 x 10^6 cells}{0.5 x 10^6 cells/ml} = 4 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.

Do not invert the tube in this step, as cells can stick to the sides of the tube, thereby changing the cell concentration.
DEMONSTRATED PROTOCOL  Fresh Frozen Human Peripheral Blood Mononuclear Cells

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](image1)

- Live = Green = 92%
- Dead = Red = 8%

![Live/Dead Viability Staining](image2)

- Live = Green = 93.4%
- Dead = Red = 6.6%