DEMONSTRATED PROTOCOL

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

## Overview

This protocol outlines cryopreservation and thawing of human peripheral blood mononuclear cells (PBMCs) for use with 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 the 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 using fresh human PBMCs from AllCells.

## Preparation-Buffers

| Cryopreservation | |
|---|---|
| **Media/Buffers** | **Composition** |
| Resuspension Medium (maintain at **4°C**) | 40% FBS in cell culture media (e.g., IMDM/RPMI) |
| 2X Freezing Medium (maintain at **4°C**) | 30% DMSO in cell culture media (e.g., IMDM/RPMI) containing 40% FBS |

| Thawing & Resuspension | |
|---|---|
| **Media/Buffers** | **Composition** |
| Complete Growth Medium (maintain at **37°C**) | 10% FBS in cell culture media (e.g., IMDM/RPMI) |
| PBS + 0.04% BSA (maintain at **room temperature**) | |

## Specific Reagents & Consumables

| Vendor | Item | Part Number |
|---|---|---|
| Thermo Fisher Scientific | Gibco IMDM | 12440-053 |
| | 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 | AMAQAF1000 |
| | 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 |
| Bel-Art | Flowmi Cell Strainer, 40 µm (alternative to Miltenyi product) | H13680-0040 |
| Corning | Corning RPMI 1640 | 10-040-CM |
| | Phosphate-Buffered Saline without Calcium & Magnesium | 21-040-CV |
| 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 |
| Eppendorf | DNA LoBind Tubes, 2.0 ml | 022431048 |

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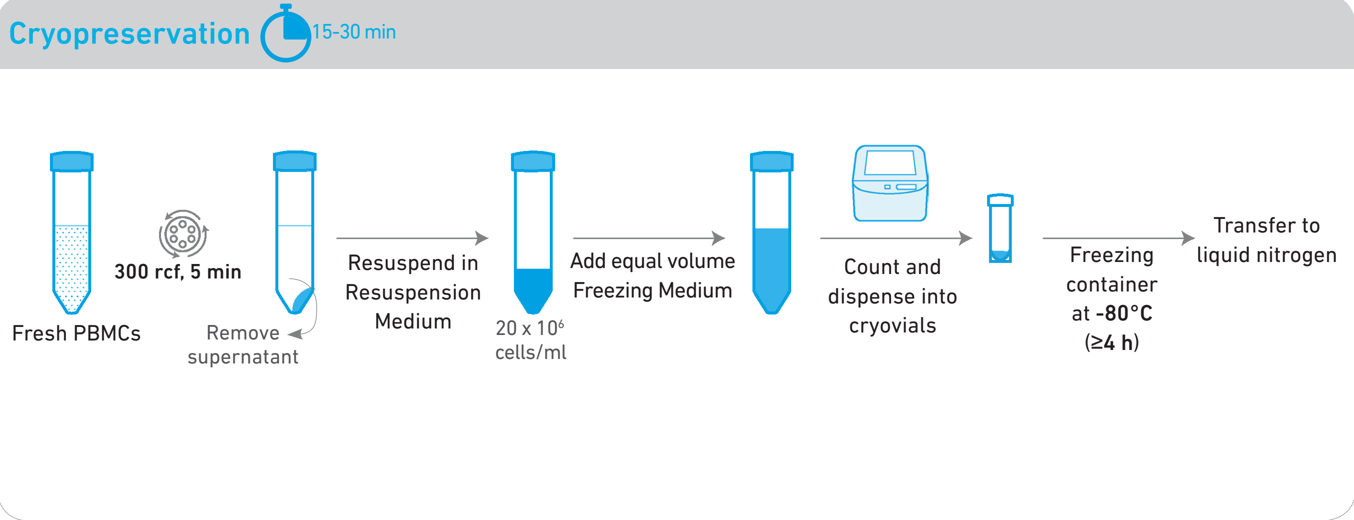
# Protocol Overview




## Thawing & Resuspension

30-45 min

Thaw

Transfer

Sequential dilution 5x (see Appendix)

300 rcf, 5 min

Remove supernatant leaving ~1 ml

Resuspend in media (9 ml)

Count

Transfer 2 x 10⁶ cells

300 rcf, 5 min

Remove supernatant

Resuspend (1 ml)*, transfer

300 rcf, 5 min

Remove supernatant

Resuspend* (700-1,200 cells/μl)

OPTIONAL

Strain cells to remove debris

Count

Proceed to 10x Genomics Single Cell protocols

*Resuspensions are in PBS + 0.04% BSA

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# Protocol

## Cryopreservation

Pre-cool a cell freezing container by placing it on ice before starting cryopreservation.

**a.** Place PBMCs on ice.

**b.** Gently mix the cells.

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

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

**e.** Remove the supernatant.

**f.** Resuspend the cell pellet in an appropriate volume of chilled Resuspension Medium to achieve a concentration of 20 x $10^6$ cells/ml.
Maintain the cells on ice.

**g.** Add an equivalent volume of chilled 2X Freezing Medium to achieve a concentration of 10 x $10^6$ cells/ml.
Gently mix the cells.

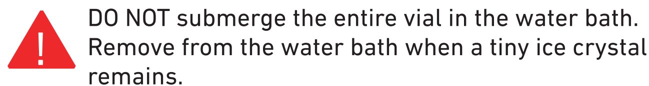
**h.** Dispense cell suspension aliquots into the pre-cooled cryovials and place the cryovials inside a pre-cooled cell freezing container e.g., CoolCell FTS30.

**i.** 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 are performed at **room temperature**.

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



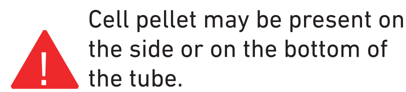
DO NOT submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

**b.** In a biosafety hood, slowly transfer thawed cells to a 50-ml conical tube using a **wide-bore** pipette tip. Rinse the cryovial with **1 ml** warm complete growth medium and add the rinse **dropwise** (1 drop per 5 sec) to the 50-ml conical tube while gently shaking the tube.

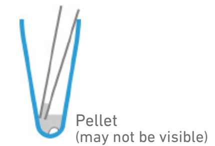
**c.** Sequentially dilute cells in the 50-ml conical tube by incremental 1:1 volume additions of media for a total of 5 times (including dilution at step b) with **~1 min** wait between additions (see Appendix).
Add media at a speed of 1 ml/3-5 sec to the tube and swirl.

**d.** Centrifuge at **300 rcf** for **5 min.**

**e.** Remove most of the supernatant, leaving ~**1 ml** and resuspend cell pellet in this volume using a **regular-bore** pipette tip.



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




**f.** Add an additional **9 ml** complete growth medium (at a speed of 1 ml/ 3-5 sec) to achieve a total volume of **~10 ml**.

**g.** Determine the cell concentration using a Countess II Automated Cell Counter.

**h.** Transfer ~2 x $10^6$ cells into a new 50-ml tube.

**i.** Centrifuge at **300 rcf** for **5 min**.

**j.** Remove the supernatant without disrupting the cell pellet.

**k.** Using a **wide-bore** pipette tip, add **1 ml** 1X PBS + 0.04% BSA and gently pipette mix 5x.

**l.** Transfer the cells into a 2-ml microcentrifuge tube. Rinse the 50-ml conical tube with **0.5 ml** 1X PBS + 0.04% BSA and transfer the rinse into the 2-ml tube containing cells.

**m.** Centrifuge at **300 rcf** for **5 min**.

**n.** Remove the supernatant without disrupting the cell pellet.

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

**OPTIONAL** If cell debris and large clumps are present, pass the sample through a 40 µm Flowmi Cell Strainer.

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

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

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

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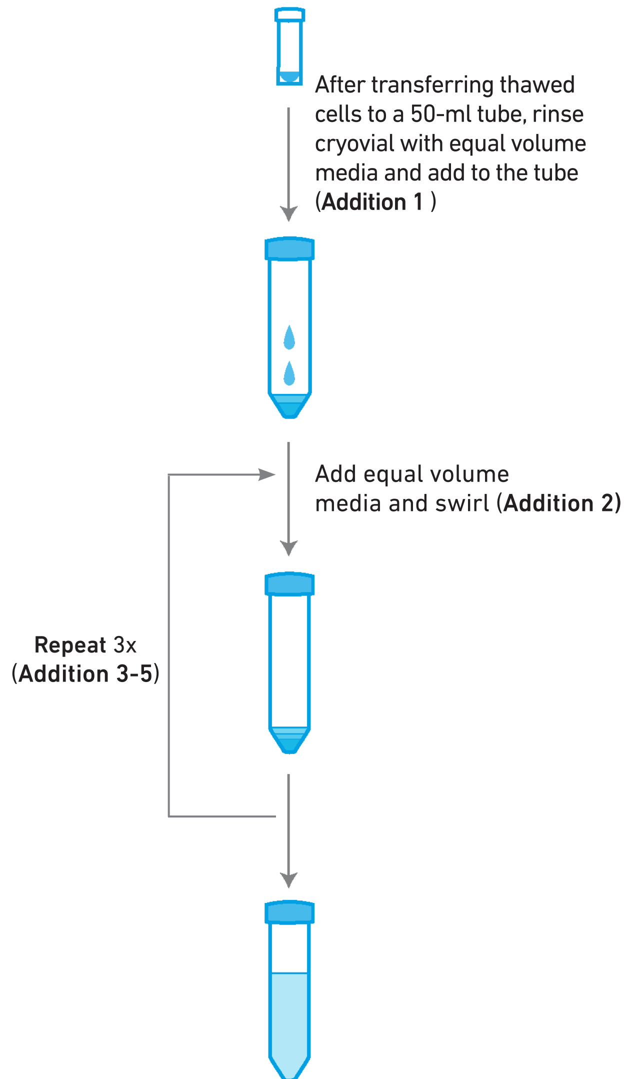
## Appendix

**Sequential Dilution with Media**




## 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 88-93% based on both trypan blue and live/dead staining.

**Example of Trypan Blue Staining**

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