Isolation of Leukocytes, Bone Marrow and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing

Introduction

This protocol outlines methods to obtain leukocytes, bone marrow mononuclear cells (BMMCs), and peripheral blood mononuclear cells (PBMCs) for use with 10x Genomics Single Cell RNA Sequencing protocols. The protocol was demonstrated using whole blood and bone marrow aspirate collected in various BD Vacutainer tubes containing anticoagulants. Comparable post-isolation cell viability was observed between various collection tubes with a range of anticoagulants. For best granulocyte quality and viability, whole blood should be processed within 2 h of collection.

Additional Guidance

Consult Demonstrated Protocol – Cell Preparation Guide (Document CG000053) for Tips & Best Practices during sample preparation and for more information on 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.

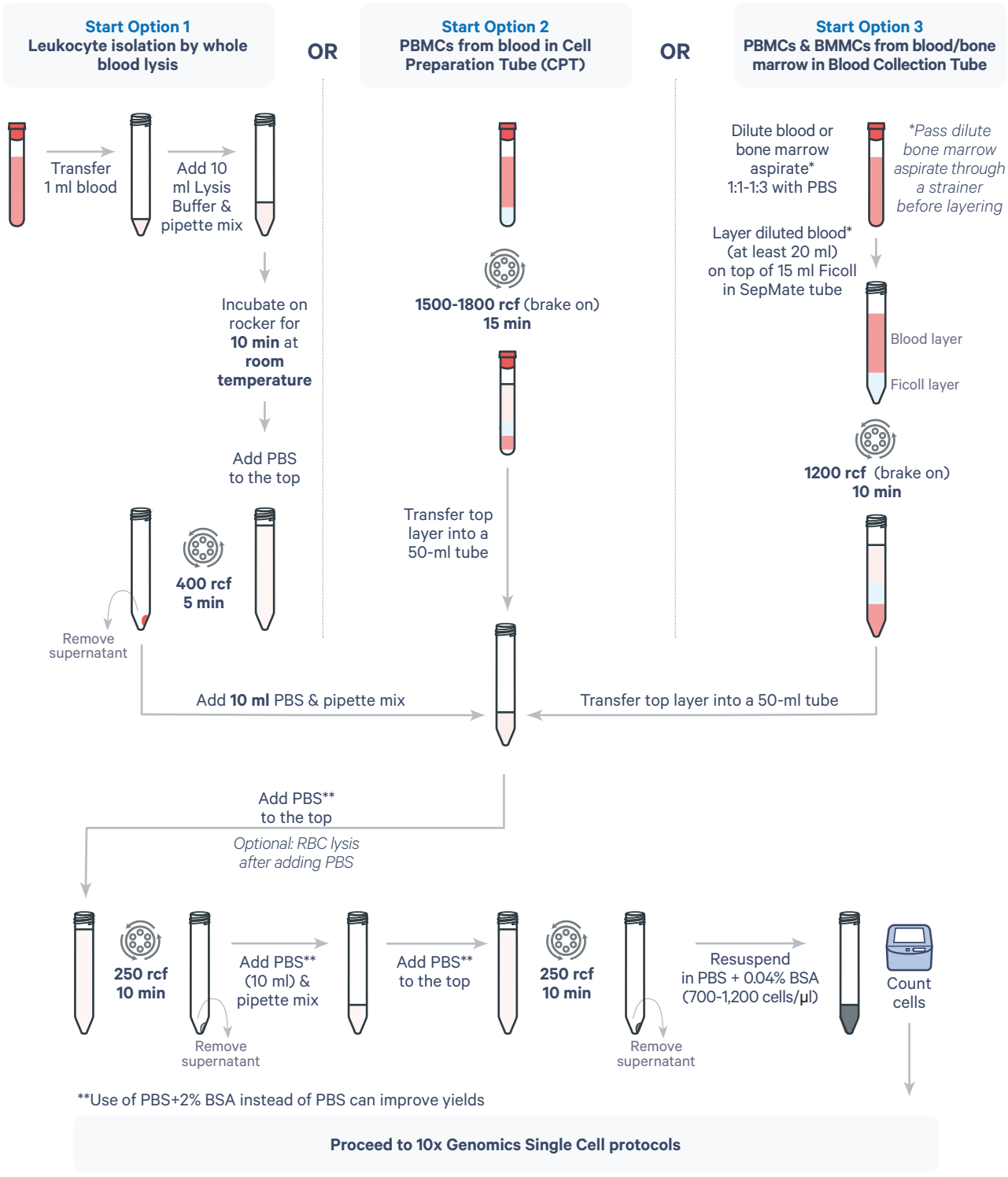
*Refer to the Appendix for cell counting guidance

Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Countess II FL Automated Cell Counter*	AMQAF1000
	Countess II FL Automated Cell Counting Chamber Slides*	C10228
	eBioscience 1X RBC Lysis Buffer	00-4333-57
Fisher Scientific	*All tubes below were tested with comparable post-isolation cell viability. Choose one.*	
	BD Vacutainer Glass Blood Collection Tubes with Sodium Heparin	366480
	BD Vacutainer Glass Blood Collection Tubes: Buffered Sodium Citrate	369714
	BD Vacutainer Plastic Blood Collection Tubes with K_2EDTA: Hemogard Closure	366643
	BD Vacutainer Glass Blood Collection Tubes with Acid Citrate Dextrose (ACD)	364606
	BD Vacutainer Glass Mononuclear Cell Preparation (CPT) Tubes	362753
	BD Vacutainer Glass Mononuclear Cell Preparation (CPT) Tubes	362761
	UltraPure BSA (50 mg/mL) *Alternative to Millipore Sigma*	AM2616
Stemcell Technologies	SepMate-50 (IVD) *(for blood collection in non-CPT tube)*	85450
Millipore Sigma	Ficoll Paque Plus	17-1440-020
	Bovine Serum Albumin in DPBS (10%)	A1595
Corning-Cellgro	Phosphate-Buffered Saline 1X without Calcium & Magnesium	21-040-CV
Miltenyi Biotec	MACS SmartStrainers (70 μm) *(for bone marrow aspirate)*	130-098-462

Protocol Overview

Isolation of Leukocytes, BBMCs & PBMCs *(see Appendix for additional options)*



Cell Sourcing

Vendor	Table Header	Part Number
StemExpress	40 mL Custom Fresh Bone Marrow-K2EDTA, NaHep, NaC, ACD-A	CUSBM01
Body	Whole Blood Vacutainer-ACD-A	PBACD010F
	Whole Blood Vacutainer-Custom	PBCUS010F
	Whole Blood Vacutainer-EDTA	PBEDT010F
	Whole Blood Vacutainer-NaHep	PBNAH010F
	Whole Blood Vacutainer-NaC	PBSC004.5F

Preparation - Buffers

OPTIONAL: Prepare PBS + 0.04% BSA for cell resuspension (alternatively only PBS may be used)

Protocol

Choose protocol start option 1, 2, or 3 based on sample collection/processing method.
See Appendix for additional options.

Start Option 1: Leukocyte isolation by whole blood lysis

- Transfer **1 ml** whole blood to a 50-ml tube.
- Add **10 ml** 1x RBC lysis buffer
- Incubate on a rocker for **10 min** at **room temperature**.
- Add PBS to the top of the tube.
- Centrifuge at **400 rcf** for **5 min**.
- Pour off the supernatant. Add **10 ml PBS**. Gently pipette mix 10x to resuspend the pellet.
- Add PBS to the top of the of the 50-ml tube
- Proceed directly to either optional **step g** for additional RBC lysis or directly to **step h**.

Start Option 2: PBMCs from blood collected in BD Vacutainer CPT

- Centrifuge at 1,500 -1,800 rcf (brake on) for 15 min at room temperature in a horizontal rotor (swing-out head). If transporting the sample, centrifugation is recommended prior to transportation.
- Proceed directly to **step f**.

Start Option 3. PBMCs & BMMCs from blood/bone marrow collected in BD Vacutainer Blood Collection Tube

a. Add **15 ml** Ficoll PAQUE Plus (1.077 mg/ml) to a SepMate tube through the SepMate insert center hole without introducing bubbles.

b. Dilute collected blood **1:1-1:3** with 1x PBS (e.g. 10 ml blood diluted with 10-30 ml PBS). Minimum recommended input volume for a 50-ml SepMate tube is **20 ml** diluted blood. For bone marrow aspirate, pass the diluted aspirate through a **70 µm** strainer to remove debris.

c. Pipette diluted blood slowly down the side of the SepMate tube.

d. Centrifuge at **1,200 rcf** for **10 min** (brake on). For samples collected **>24 h** before processing, centrifugation for **20 min** is recommended.

e. Pour top layer into a new 50-ml centrifuge tube in single smooth motion.

TIPS *Use of PBS+2%BSA instead of PBS in the following steps can improve cell yields.*

f. Add PBS to the top of the of the 50-ml tube.

*DO NOT hold the SepMate tube in an inverted position for more than **1 second**.*

g. OPTIONAL For RBC Lysis: Centrifuge at **400 rcf** for **5 min**. Pour off the supernatant and resuspend the cell pellet in **10 ml** 1x RBC lysis buffer. Incubate on a rocker for **10 min** at **room temperature**. Add PBS to the top of the 50-ml tube with the sample.

h. Centrifuge at **250 rcf** for **10 min** at **room temperature**.

i. Pour off the supernatant. Add **10 ml** PBS. Gently pipette mix 10x to resuspend the pellet.

j. Add PBS to the top of the of the 50-ml tube.

k. Centrifuge at **250 rcf** for **10 min** at **room temperature**.

Additional RBC lysis may be performed (as described in optional **step g)** if the pellet looks reddish and/or the supernatant is reddish and opaque (if RBC lysis is complete, the pellet will not be reddish while the supernatant will be reddish but clear).

l. Pour off the supernatant.

m. Resuspend cell pellet in equal volume PBS/PBS+0.04% BSA (or in other buffers compatible with 10x Genomics Single Cells assays, such as PBS+1-10% BSA) to original whole blood volume. For example, if the input was **10 ml** whole blood, resuspend in 10 ml PBS for **~1x10^6 cells/ml**.

Lower volume may be used if more concentrated cell suspension is desired.

n. Determine cell concentration and viability using an Automated Cell Counter (Countess II /Cellaca MX) or a hemocytometer. Cellaca MX with AOPI Staining Solution was used for this protocol.

If needed, add an appropriate volume of PBS/PBS+0.04% BSA to obtain a concentration of **700-1,200 cells/µl**.

Count only nucleated cells. DO NOT count RBCs that account for ~55% reads in mononucleated cell data. Alternatively, a fluorescence counter and fluorescent nuclei stain may be used for accurate counting. DO NOT use Trypan Blue. See Appendix for additional guidance.

o. Once the final cell concentration is achieved, maintain cells at **room temperature**. DO NOT place on ice as it may result in granulocyte lysis.

p. Proceed **immediately** to the 10x Genomics Single Cell protocol.

Appendix

Compatible Isolation Kit & Protocol

PBMCs and Leukocytes can also be isolated using the EasySep Direct Human PBMC Isolation Kit and RBC Depletion Reagent Kit (STEMCELL Technologies). The EasySep protocols have not been tested for BMMCs.

Tested products and part numbers (with associated protocols) are listed below.

- EasySep Direct Human PBMC Isolation Kit (19654)
- EasySep RBC Depletion Reagent Kit (18170)
- Magnets
  (all the listed magnets are compatible; choose one)
  - EasySep Magnet 18000
  - The Big Easy EasySep Magnet (18001)
  - Easy 50 EasySep Magnet (18002)
  - EasyEights EasySep Magnet (18103)

When using the above products & associated protocols, note two key deviations:

- Resuspend the final cell pellet in PBS+0.4%BSA or in a compatible buffer as specified in **step m**.
- The third bead separation step in the protocol is optional.

Cell Counting

Accurate sample counting is critical for optimal assay performance. Cells should be stained with an appropriate dye and counted using an automated cell counter. See below for the dye recommendation for a specific counter.

Counter	Dye Recommended
Cellaca MX	AO/PI staining solution
Countess 3 FL	PI staining solution
Cellometer K2	**Nucspot 470

*** Dilute stock to 1:100 and mix 1:1 with the sample. For example, add 10 µl diluted dye to 10 µl sample.*

The following section provides counting guidance using AO/PI staining solution and the Cellaca counter. For counting guidance using other dyes/counters, refer to manufacturer's instructions.

Counting using AO/PI Staining Solution: This protocol provides instructions for counting samples using AO/PI staining solution and the Cellaca counter to enable accurate quantification even in the presence of subcellular debris. The optimal cell concentration for the Cellaca counter is 100-10,000 cells/µl. Refer to manufacturer's instructions for details on operations.

- Add **25 µl** AO/PI staining solution into Mixing Row of Cellaca plate
- Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add **15 µl** cell suspension to **15 µl** PBS.
- Add **25 µl** sample to Mixing Row of plate containing AO/PI staining solution. Gently pipette mix 8x.
- Transfer stained sample to Loading Row of Cellaca plate.

TIPS *DO NOT use trypan blue for counting. This will count RBCs as cells.*

Document Revision Summary

Document Number	CG000392
Title	Isolation of Leukocytes, Bone Marrow and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing
Revision	Rev A to Rev B
Revision Date	August 2024
Description of Changes	• Updated to include use of PBS + 2% BSA for post-Ficoll wash steps • Updated to include Appendix with compatible isolation kit and cell counting guidance • Updated for general minor consistency of language, format, and terms throughout

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