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

Overview

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 and Technical Note – Guidelines on Accurate Target Cell Counts (Document CG0000091) 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.

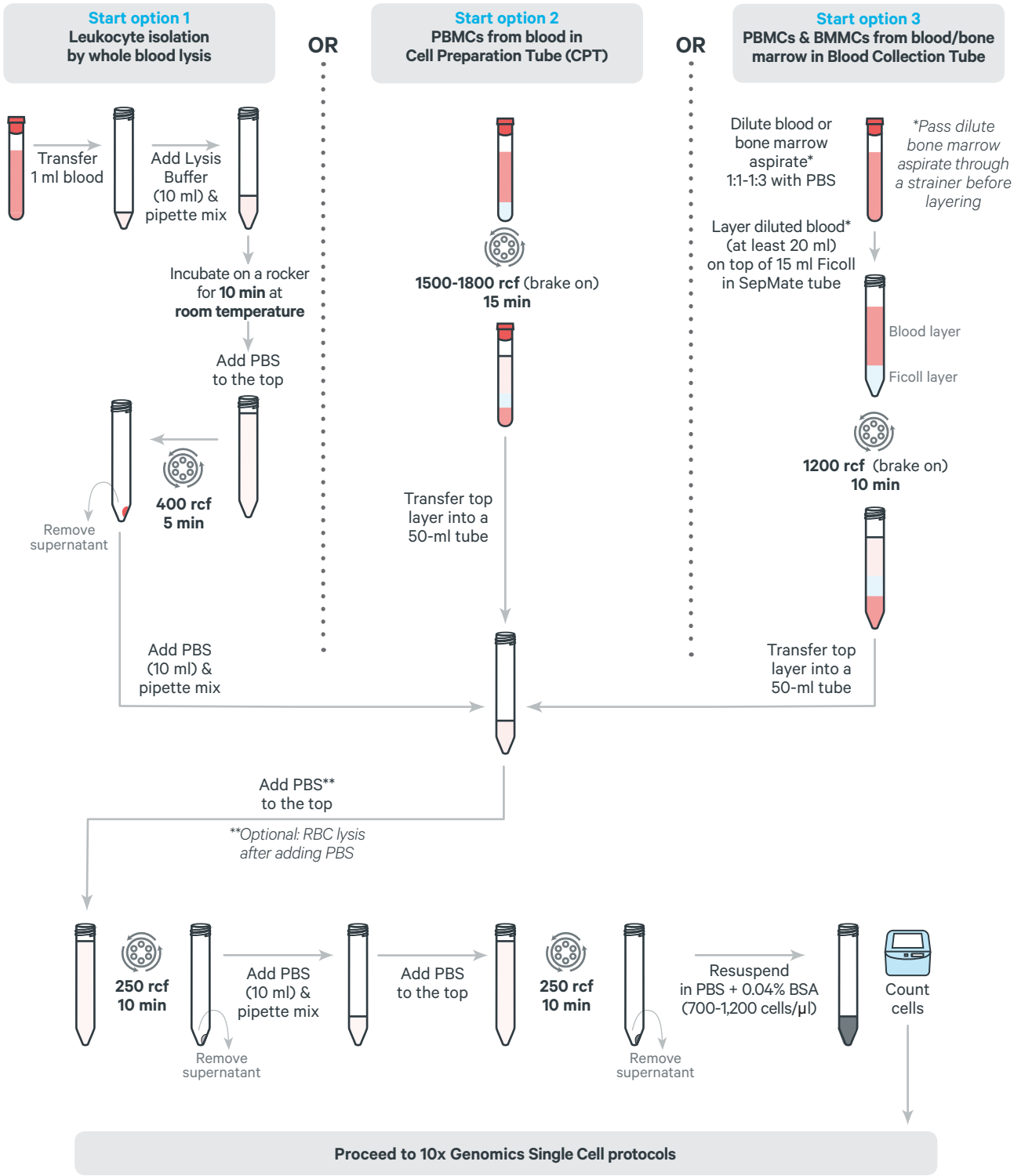
*Alternatively, a fluorescence counter and fluorescent nuclei stain may be used, such as, Cellaca MX High-throughput Automated Cell Counter with ViaStain™ AOPI Staining Solution (CS2-0106-5mL) from Nexcelom Biosciences.

Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Trypan Blue Stain (0.4%)*	T10282*
	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 the listed tubes were tested for this protocol, resulting in 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
Millipore Sigma	Ficoll Paque Plus	17-1440-020
	Bovine Serum Albumin in DPBS (10%)	A1595
Stemcell Technologies	SepMate-50 (IVD) *(use for blood collected in non-CPT tube)*	85450
Corning-Cellgro	Phosphate-Buffered Saline 1X without Calcium & Magnesium	21-040-CV
Miltenyi Biotec	MACS SmartStrainers (70 µm) *(only if processing bone marrow aspirate)*	130-098-462

Protocol Overview

Isolation of Leukocytes, BMMCs, & PBMCs



Cell Sourcing

Vendor	Item	Part Number
StemExpress	40 mL Custom Fresh Bone Marrow-K2EDTA, NaHep, NaC, ACD-A	CUSBM01
	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.

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.

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 sec**.

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 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.

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.

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