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Protocol to isolate and cryopreserve fresh mouse PBMCs

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ABSTRACT

This protocol describes isolation of cells from adult mouse PBMCs, preparation of a single cell suspension, and cryopreservation. This protocol takes about 3 hours from start to finish.

The results are approximately 1 million cryopreserved PBMC in 1 mL 10% DMSO + FBS, stored in liquid nitrogen until thawing and fixing.

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 86988

MATERIALS

	Name	Manufacturer	Cat. #
	Ficoll-Paque	Cytiva	17144002
	EDTA tubes	BD-Vacutainer	367856
	BSA (optional)	Sigma-Aldrich	A9418
	FBS	Omega Scientific	FB-01
	PBS	Cytiva	BSS-PBS-1X6
	EDTA	Sigma-Aldrich	E6511
	DMSO	Sigma-Aldrich	D2650
	Red Blood Cell LysisSolution	Miltenyi	130-094-183
	DEPC water	Invitrogen	750023
	NucBlue Live ReadyProbes	Thermo Fisher	R37605
	Millicell Disposable Hemocytometer	Millipore Sigma	MDH-2N1-50PK
	Mr. Frosty	Sigma-Aldrich	635639
	5 mL DNA/RNA LoBind tubes	Eppendorf	0030108310
	2 mL DNA/RNA LoBind tubes	Eppendorf	022431048

Reagents/equipment, manufacturer and catalog number

	A	B	C	D
	1% BSA-DEPC (optional)	BSA	1 g	1%
		DEPC water	100 mL	
	PBS-EDTA	EDTA	0.146 g	1 mM
		PBS	500 mL	
	PBMC-RSB	PBS-EDTA	490 mL	
		FBS	10 mL	2%
	1x RBC lysis	10x Red Blood Cell LysisSolution	1.2 mL	1x
		DEPC water	10.8 mL	
	20% DMSO in FBS	DMSO	1.2 mL	20%
		FBS	4.8 mL	

Buffers

Setup

1 Set centrifuge to 19C.

2 Prepare 1 ice bucket.

3 Thaw and filter FBS with a cell culture filter unit and aliquot into 50 mL conical tubes.

4 Prepare PBS-EDTA by adding EDTA powder to PBS and filter with a cell culture filter unit. 500 mL of PBS-EDTA should be enough for around 16 samples. Store at room temperature, or 4C if not

using that week.

5 Prepare PBMC-RSB and 1x RBC lysis buffer at room temperature.

6 Prepare 20% DMSO FBS on ice.

7 Distribute 20 ul dye into 10 PCR tubes for cell counting.

8 Optional: Prepare 1% BSA-DEPC stock tubes by adding BSA powder to 100 mL DEPC. Coat 5 mL tubes by adding 5 mL 1% BSA-DEPC in the cell culture hood, incubating for 30 minutes, emptying the tubes, and drying for 30 more minutes. More detailed instructions are posted on the cell culture hood. We typically prepare many tubes ahead of time and store at 4C.

PBMC isolation and cryopreservation

9 Collect blood in labeled EDTA-coated tubes at the vivarium during dissection. Cap and invert tubes so that all the blood touches the walls of the EDTA-coated tubes to prevent clotting.

10 At the lab, briefly spin tubes to collect any remaining blood from the walls/cap.

11 Measure blood volume with a 1 mL pipette and record.

12 Dilute blood 1:2 with PBS. E.g. 900 ul blood + 900 ul PBS.

13 Aliquot appropriate amount of Ficoll-Paque density gradient media.
Multiple final diluted blood volume by 0.75x. E.g. 1800 u diluted bloodl x 0.75 = 1350 ul Ficoll-Paque. Use the smallest possible tube. If total blood volume is >500 ul, use another EDTA-coated blood collection tube. If total blood volume is <500 ul, use a 2 mL tube.

14 Layer diluted blood on top of Ficoll-Paque.

15 Centrifuge 400g for 30 minutes at 19°C with no brake. Be careful to balance the centrifuge.

16 Collect plasma and distribute in PCR tubes, 200 ul each. Label and store in IGVF Plasma box at -20C.

17 Collect PBMC layer in ~1 mL into a labeled 5 mL tube (1% BSA-coating optional). Fill tube to 5 mL with PBMC-RSB or just PBS to wash cells and remove Ficoll-Paque. Discard gradient tube with remaining Ficoll-Paque and red blood cells in biohazard trash.

18 Centrifuge 400g at 19°C for 8 minutes with full brake.

19 Remove supernatant until 100 uL PBMC-RSB is remaining in the tube.

20 Add 1 mL 1x RBC lysis buffer to 100 uL cells. Vortex cells at low speed for 5 seconds and incubate for 10 minutes at room temperature.

21 Centrifuge 400g at room temperature for 8 minutes with full brake.

22 Remove supernatant and resuspend cells in 500 uL PBMC-RSB for counting.
Do not disturb the pellet. Do not count cells later when they're in FBS, the proteins in the FBS cause background autofluorescence under the microscope.

Counting

23 Aliquot 20 ul from each 5 mL tube containing 500 uL cells into the PCR tubes containing 20 ul dye.

24 Label each side of a disposable hemocytometer with the sample number.

25 At an angle, pipette 20 ul of cells in dye into the divots on the slide. Use 1 side per sample.

26 Under EVOS FL Auto 2 microscope at 10x resolution (PBMCs are small), count cells in 2 squares on opposite sides of the grid. Use the DAPI channel to tell PBMCs from red blood cells or debris.

27 Sum up squares (accounts for the 1:2 dilution), and convert to cells/mL by multiplying by 10^4.
E.g. (45 + 63)*10^4= 1,080,000 cells per mL.

28 Take pictures on the microscope for your records.

29 Centrifuge for the last time at 400g at room temperature for 8 minutes with full brake.

30 Remove supernatant and resuspend cells in 500 uL cold FBS. Move the cells to labeled cryotubes.

31 Slowly add 500 uL 20% DMSO in FBS for a final volume of 1 mL. Mix gently after all 500 uL are added. Keep cryotubes on ice.Final concentration is 10% DMSO in FBS.

32 Move tubes to Mr. Frosty and to the -80C freezer.

33 The next day, move cryotubes to liquid nitrogen racks and record location.