Protocol to isolate, cryopreserve, and fix mouse PBMCs for IGVF V.2

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

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ABSTRACT

This protocol describes isolation of peripheral blood mononuclear cells (PBMCs, tissue ID: 20) from whole blood of 10 week old mice, cryopreservation, thawing, and fixation for single-cell RNA-seq using the Parse Biosciences protocol (Split-seq). For 8-10 samples, PBMC isolation and cryopreservation take about 3 hours from start to finish, while thawing and fixation take around 1 hour.

The final result is 1 aliquot of a fixed single-cell suspension for Parse Bio scRNA-seq ("Split-seq") for each sample at >= 2,500 cells/ul stored at -80C. The intermediate result is approximately 1 million cryopreserved PBMCs per sample in 1 mL 10% DMSO + FBS, stored in liquid nitrogen until thawing and fixing.

The first part of the protocol describes isolation and cryopreservation. Due to low input volumes (ranging from 100-1,000 ul), whole blood is diluted 1:2 in PBS and Ficoll-Paque density gradient centrifugation is performed in 2 mL tubes or blood collection tubes. Buffy coat is collected and red blood cells are lysed before counting on a hemocytometer. Plasma is also collected and stored in 200 ul aliquots at -20C. PBMCs are cryopreserved in liquid nitrogen in 10% DMSO FBS. Cryopreserved PBMCs are suitable for fixation for a variety of downstream protocols including single-cell RNA-seq, but **not** cell culture. If PBMCs are meant to be grown after isolation, perform all steps after the initial blood collection in a cell culture hood / biosafety cabinet with sterile technique. The second part of the protocol describes thawing and fixation. Cells are gently thawed and washed with culture media and PBS. Due to low cell recovery, we modify the original Parse Biosciences Evercode fixation protocol (attached) by using half volumes of all fixation reagents.

ATTACHMENTS

Thawing-Cryopreserved-PBMCs-2022.pdf

SO+10122022_Evercode+Fixation+v2.0.2+User+Manual.pdf

MATERIALS

PROTOCOL integer ID: 90520

Keywords: PBMC, Fixation, Cell fixation, Parse Biosciences, scRNA-seq, Split-seq, Evercode, Mouse, Mortazavi, IGVF, UCI

	Name	Manufacturer	Cat. #
	Ficoll-Paque	Cytiva	17144002
	EDTA tubes	BD-Vacutainer	367856
	7.5% BSA	Life Technologies	15260037
	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	MDH-2N1-50PK
	Mr. Frosty	Sigma-Aldrich	635639
	5 mL DNA/RNA LoBind tubes	Eppendorf	0030108310
	2 mL DNA/RNA LoBind tubes	Eppendorf	022431048
	NucBlue Fixed Cell ReadyProbes	Thermo Fisher	R37606
	Cell Fixation Kit v2	Parse Biosciences	ECF2001
	DMEM	Sigma-Aldrich	D5796
	Penicillin-Streptomycin	Gibco	15070063

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	

	A	B	C	D
	20% DMSO in FBS	DMSO	1.2 mL	20%
		FBS	4.8 mL	
	20% FBS DMEM	DMEM	450 mL	
		FBS	100 mL	20%
		Pen-strep (100x)	5 mL	1x

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 15 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 (biosafety cabinet), 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 and keep tube on rotating platform as other dissections are underway.

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** 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 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 **10 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.

Thawing and fixation setup

34 Prepare complete medium by adding **20% FBS** and **1% penicillin-streptomyci**n to your media of choice (we use **DMEM**, but RPMI works too). Warm culture medium to 37°C.

35 Aliquot **10 mL** of pre-warmed culture medium into a 15 mL polypropylene tube.

36 Open 1 new **Parse Biosciences Cell Fixation kit**. Add **550 µL** of **Cell Fixation Additive** directly into the **Cell Fixation Solution**. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL. To record the addition of **Cell Fixation Additive**, mark the cap of the **Cell Fixation Solution** tube, and store on ice.

37 Add **50 µL** of **RNase Inhibitor** directly into the **Cell Prefixation Buffer** tube. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL. To record the addition of **RNase Inhibitor**, mark the cap of the **Cell Prefixation Buffer** tube, and store on ice.

38 Add **17 µL** of **RNase Inhibitor** directly into the **Cell Buffer** tube. Mix thoroughly by pipetting up and down 5x with a P1000 set to 750 µL. To record the addition of **RNase Inhibitor**, mark the cap of the **Cell Buffer** tube, and store on ice.

39 Record today's date on the Cell Fixation Reagents kit box.
Note: After mixing reagents, Evercode Cell Fixation kits should only be freeze-thawed once and stored for up to 1 month at -20°C. Longer storage or additional freeze-thaws will compromise data quality.

40 Prepare **Cell Prefixation Buffer + BSA.** Add 200 µL of 7.5% BSA to 3 mL Cell Prefixation Buffer. **Cell Prefixation Buffer + BSA** should be prepared fresh and used the same day. Mix thoroughly by pipetting up and down 5x and store on ice.

Thawing cryopreserved PBMCs

41 Transport cryovials containing PBMCs from the freezer to the water bath on dry ice. We prepare 8 samples at a time.

42 Hold the lower half of the vials in a **37°C** water bath for **30–45 sec,** flicking intermittently.
Note: Do not submerge the vial completely under water. Do not thaw completely. Allow about 20% of the ice crystals to remain intact.

43 While 20% of the ice crystal is still visible, take the vials out of the water bath and wipe them with 70% ethanol.

44 Transfer the cell suspension immediately from the cryovial to the **15 mL tube containing culture medium** using a P1000 pipette. Note: Dispense the culture media dropwise from the 15 mL tube to the cryovial to slowly thaw the ice crystals. Aseptic technique not necessary since we are proceeding directly to fixation.

45 Transfer all the contents in the cryovial to the culture medium in the 15 mL tube. Use an additional **1 mL** of culture medium to rinse out the cryovial and ensure maximum recovery of PBMCs.

46 Gently invert the 15 mL polypropylene tube 3–4 times for even cell distribution and centrifuge at **350g** for **5 min** at **room temperature (21°C).**

47 Carefully remove the supernatant. Leave a small amount of media at the bottom to re-suspend the pellet obtained after centrifugation.

48 Gently loosen the cell pellet by flicking. Add **10 mL** of **culture medium.** Repeat steps 41 and 42 in this section with PBS rather than culture media to wash off media. Resuspend cells in 375 ul **Cell Prefixation Buffer + BSA** with a P1000.

Cell Fixation

49 Pipette cells through a 40 µm strainer into a new 15 mL polypropylene centrifuge tube with a P1000 and store on ice.

Critical! To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the filter and steadily depress down the pipette plunger. All of the liquid should pass through the strainer in ~1 second.

50 Add **125 µL** of **Cell Fixation Solution** to the 15 mL tube and mix immediately by pipetting up and down exactly 3x with a P1000 set to 125 µL. Return the tube to ice.

Critical! Do NOT perform additional mixing at this step. Also, ensure the **Cell Fixation Solution** contains **Cell Fixation Additive**, as indicated by a mark on the tube cap.

51 Incubate on ice for **10 minutes**

52 Add **40 µL** of **Cell Permeabilization Solution** to the 15 mL tube and mix thoroughly by pipetting up and down 3x with a P1000 set to 125 µL. Return the tube to ice.

53 Incubate on ice for **3 minutes**.
*Note: Do NOT vortex the **Cell Neutralization Buffer**. Prior to use, invert the tube 5x to mix.*

54 Add **2 mL** of **Cell Neutralization Buffer** to the 15 mL tubes. Gently invert the 15 mL tube once to mix and return to ice.

55 Centrifuge the 15 mL tube in a swinging bucket rotor for **10 minutes** at **750g** at **4C**.

56 Remove and discard the supernatant. Fully resuspend each pellet in **100 µL** of cold **Cell Buffer** with a P200 set to 100 µL and return to ice.

57 Take a **10 ul aliquot** to dilute 1:2 with prepared **10 ul dye** to manually count with a disposable hemacytometer and record numbers.

58 Count cells. **Use 1:2 dilution factor**, 10 ul + 10 ul dye.

59 Re-concentrate: spin cells **750g** for **5 minutes** and carefully take off supernatant until **15-25 ul** are remaining. Resuspend (hopefully visible) pellet in the remaining ul and measure exact volume.

60 Add DMSO: **0.5 ul into 25 ul samples** and gently flick tubes to mix. One minute later, add another **0.5 ul** and flick to mix, then after another minute add a final **0.5 ul** for a total volume of **1.5 ul.** Mix by gently pipetting 5x. Avoid creating bubbles.

61 Place tubes in a Mr. Frosty for storage at -80C. The next day, move tubes to boxes in -80C racks.