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PBMC Isolation & Cryopreservation from Whole Blood

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ABSTRACT

The purpose of this procedure is to isolate and cryopreserve peripheral blood mononuclear cells (PBMC's) from 30 mL of freshly drawn whole blood.

ATTACHMENTS

PBMC Isolation & Cryopreservation from Whole Blood.pdf

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MATERIALS TEXT

Materials and Equipment

1. RPMI 1640 (**Room temperature** i.e. **19 °C** - **25 °C**)

   Ficoll-Paque

2. PLUS **Cytiva Catalog #17144003** (**Room temperature** for assay, but stored in fridge)
3. Freezing Medium (FBS (heat inactivated) with [M]**10 % DMSO** – **500 mL FBS** add **50 mL DMSO**)

   Store unopened FBS in **-80 °C** and Freezing medium at **4 °C** (in the fridge); DMSO is stored at **Room temperature** .
4. [M]**0.1 % Trypan blue** (dilute stock in PBS to [M]**0.1 %** final concentration)
5. Serological Pipette/Pipet Gun/p100 micropipette/p1000 micropipette
6. Pasteur Pipettes
7. 50 mL and 15 mL Conical Falcon Tubes
8. Hemocytometer
9. Mr. Frosty container (stored in the fridge, with [M]**100 % Isopropyl alcohol** according to manufacturer's instructions)

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

Disinfect area with [M]**70 % EtOH** and wipe off blood tubes.

Step 1: Prepare materials and supplies

1 Place RPMI media and Ficoll in water bath. Bring to **Room temperature** (**19 °C** - **25 °C**).

Step 2: First Spin 15m

45m

2 

Spin tubes at **1850 rpm, 00:15:00** (breaks off; 5 acc 0 brake) for plasma removal.

Total time for spin including acceleration and deceleration – **00:30:00** .

Step 3: Plasma Aliquot

3 

Pipette **15 mL plasma** from the spun blood. Leave ~ **1 mL** - **2 mL plasma** above the buffy coat/RBC layers.

4 Store sample ID labeled Plasma aliquot in the -80˚C freezer.

Step 4: Preparing Ficoll tubes (15 mL of Ficoll to 35 mL of diluted blood) for density gradient centrifugation

5 Prepare **two sterile 50 mL** conical tubes and label with sample ID.

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6 

Use pipette gun to distribute **15 mL Ficoll** into each tube.

Note to step 5-9

7 **Use a maximum of 10-12 Ficoll tubes at one single time.**
If more samples – repeat step 5-9 with remainder while the first batch waits for step 10 (Fourth spin). Try to limit the time the cells are in contact with Ficoll.

Step 5: Dilute Blood Ficoll overlay

8 Aspirate remaining plasma with Pasteur pipette vacuum from all blood tubes until 1 mL - 2 mL of plasma remain above RBC layers.

9 Dilute remaining blood in all tubes with RPMI media to 50 mL.

10 

Gently pipet diluted blood to homogenize.

> Important to pipet slowly.

11 Use pipette gun to slowly dispense **35 mL diluted blood** onto each Ficoll tube, without breaking the interface or mixing. (**35 mL diluted blood** to **15 mL Ficoll**)

12 Use small amount of RPMI media to rinse empty blood tubes and overlay over Ficoll.

13 Secure all caps to avoid leaks.

Step 6: Second spin 40m

14 Spin tubes at **1850 rpm, 00:25:00** (breaks off). Total time for spin including acceleration and deceleration – **00:40:00** . 1h 5m

Step 7: Harvesting PBMC buffy coat and washing cells

15 Prepare and label new sterile conical tube for PBMC's; 2 isolated layers per conical tube
(i.e. 2 ficoll tubes / 2 layers = 1 50ml conical needed).

16 Retrieve Ficoll tubes from centrifuge. Tubes will have four distinct layers.

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17 Aspirate media/plasma (top layer) from all tubes until **10 mL** remain over PBMC buffy coat layer.

18 

Pipette PBMC buffy coat layers into sterile 50 mL conical tubes (combine 2 isolated layers per tube).

19 Rinse conical tube containing PBMC's with RPMI media by adding RPMI to 50 mL.

Step 8: Third spin 10m

20 Spin tubes at **1850 rpm, 00:10:00** (breaks low). 10m

Step 9: Washing cells

21 Aspirate supernatant and resuspend cell pellets in **10 mL RPMI media** .

22 

Wash cells by diluting to 50 mL with RPMI media.

23 

Repeat step 5-9 as needed depending on number of samples being processed simultaneously.

Step 10: Fourth spin 10m

24 Spin tubes at **1850 rpm, 00:10:00** (breaks ON; acceleration at 5). 10m

Step 11: Washing cells and counting

25 Aspirate supernatant and resuspend cell pellets in **10 mL RPMI media** .

26 Combine cell pellets into one conical tube and dilute cell suspension to 30 mL.

27 Cap and invert tube to ensure cell suspension is homogenous.

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28 Use p100 micropipette and aliquot **20 µL cell suspension** for counting.

29 Do a 1:2 dilution with **0.1 % trypan blue** and count in hemocytometer (i.e. **20 µL cell suspension** with **20 µL 0.1% trypan blue**).

29.1 Count the viable cells in 4 corner squares and middle square of hemocytometer.

29.2 Count the number of dead (blue) cells in the same squares.

29.3 Calculate cell density, total cells yielded and viability (no. of dead cells divided by total number of cells).

29.4 Calculate number of cryovials needed based on specified freezing density.

Step 12: Fifth spin 10m

30 Spin tubes at **800 rpm, 00:10:00** (breaks OFF). 10m

Step 13: Cryopreservation 1d

31 Print labels with sample ID, cell density, date, and initial. Prepare and label cryovials and Mr. Frosty container.

32 Freeze at a density of ~10 million cells per vial with a maximum of 3 vials per sample for 30 ml sample.

33 Aspirate supernatant.

34 Resuspend cell pellet in appropriate amount of fridge cold Freezing media (FBS with **10 % DMSO**) at **1 mL** per vial *(i.e. 3 vials = resuspend in* **3 mL FBS** *).*

Important to work quickly and in batches for this step. DMSO is toxic for the cells.

34.1 

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** pipette cell suspension into cryovials quickly and efficiently to prevent loss of viability.

34.2 Aim for 8-10 million cells per vial.

- 15 million or less cells – 1 vial,
- 16-23 million cells – 2 vials,
- 24 million and above – 3 vials.

34.3 

1d

Place cryovials in frosty container and freeze in -80˚C freezer **Overnight** (**24:00:00**).

34.4 Transfer cells to nitrogen tank for long term storage (work quickly to avoid temperature changes).

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