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SOP –UNITS-4.0

Author:
O. Schillaci;
A. C. Boncristiani
Approved:
S. Schoeftner

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ISOLATION AND CRYO-CONSERVATION OF PBMCs

Purpose

The purpose of SOP-UNITS-4.0 is to describe the procedures for the isolation and cryopreservation of peripheral blood mononuclear cells (PBMCs) from human individuals.

Scope

This SOP will be applied to whole blood that is separated to cells suitable for cell culture.

Introduction

Blood samples from patients with lung primary tumors or metastatic lung cancer have been collected by the Anatomia Patologica Unit of Cattinara Hospital. Samples were passed to UNITS collaborators to allow the isolation and cryopreservation of PBMCs.

1. Cell culture media, reagents and solutions

- Human blood samples
- RPMI 1640 medium (Euroclone cat.no. ECB2000)
- Penicillin G sulfate 100 mg/ml (Sigma-Aldrich cat. no. P3032)
- Streptomycin sulfate 100 mg/ml (Sigma-Aldrich cat. no. S9137)
- Fetal bovine serume (FBS) (Opticlone cat.no. ECS0183L)
- 1x PBS sterile, pH 7,4 (Life Technologies, cat. no. 14190-094)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- Ficoll –Paque PREMIUM 1.084 (Sigma-Aldrich cat. no.17-5446-02)

2. Equipment

- Cell culture incubator with 5% CO_2, 37 °C
- High-speed centrifuge (Eppendorf centrifuge 5810R)
- Pipette aid (Primo® mate)
- Serological pipettes 5ml (Euroclone, cat. no. EPS05N), 10ml (Euroclone, cat. no. EPS010N) and 25 ml (Euroclone, cat. no. EPS025N)
- Laboratory test tube 15 ml (Euroclone, cat. no. ET5015B) and 50 ml (Euroclone, cat. no. ET5050B)
- Cryo-conservation vials; 2ml (VWR, cat. no. 479-0287)
- Blood collection tubes 7 ml, with EDTA K3, (VACUETTE® 455036)
- Mr. Frosty™ Freezing Container (Thermo Fisher Scientific cat. no 5100-0001)

3. Reagent setup

- Complete cell culture medium: RPMI 1640 medium supplemented with Glutamine 1%, Penicillin/Streptomycin 1% and FBS 10%.
- Freezing medium: 90% of FBS serum supplemented with DMSO 10%.

4. Safety

Use universal safety precautions when handling human samples and personal protective equipment (e.g., face mask with shield, gloves, lab-coat). Dispose of all solutions and supplies in contact with human blood in biohazardous waste.

Enrolled patients were previously tested negative for virus infections including SARS-CoV-2, HIV and Hepatitis virus.

5. Procedure

- 7 ml blood was collected in blood collection tubes, with EDTA K3. EDTA is an anti-coagulant, thus preventing blood clotting.
- Transfer the blood sample in a 50 ml tube.
- Dilute the blood sample to a 1:1 volume ratio with complete cell culture medium.
- Mix contents of tube by gently inverting 5 to 8 times.
- Prepare Ficoll–Paque PREMIUM 1.084 in a new 50 ml tube in a 1:2-volume ratio compared to diluted blood sample (e.g. 15 ml Ficoll + 30 ml diluted blood).
- Gently apply the diluted blood sample on top of Ficoll –Paque PREMIUM 1.084. Two separate phases should form. Take care not to mix the two layers.
- Centrifuge at 700 x g for 20 minutes, at room temperature using the Eppendorf centrifuge 5810R. Chose the "deceleration= brake OFF" and "acceleration= 1" programs. Centrifugation will generated four different layers from the top to the bottom: 1. Plasma and platelets; 2. PBMCs; 3: Ficoll and 4. Granulocytes and Erythrocytes (See *Figure 1*).

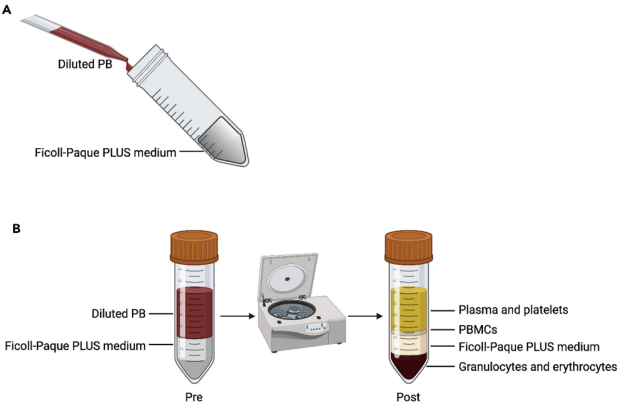

Figure 1. Schematic representation of PBMC isolation (STAR Protocols, Michelozzi et al., 2022)

- Aspirate off top plasma layer to within 1 cm from the PBMCs ring and collect it in a new 50ml tube.
- Carefully collect PBMCs ring using a p1000 pipette in a new 15 ml tube.
- Discard the remaining material in the appropriate biohazardous waste container inside the cell culture hood.

- Add sterile 10 ml complete medium to PBMCs falcon tube.
- Centrifuge at 400 x g for 10 minutes, at room temperature using the Eppendorf centrifuge 5810R, chose the "medium deceleration" and "medium acceleration" programs and discard the supernatant.
- Add sterile 1x PBS to the PBMCs until reaching a final volume of 10 ml.
- Centrifuge at 400 x g for 10 minutes at room temperature with "medium deceleration" and "medium acceleration"
- Discard the supernatant and repeat the washing step using 10 ml sterile 1xPBS, if erythrocytes appear on the bottom.
- Resuspend the cells in cold freezing medium and transfer in cryoconservation tube.
- Immediately place in Mr. Frosty™ Freezing Container in a -80°C freezer overnight.
- The following day, transfer the cryovial into the liquid nitrogen cryotank.

6. Applicable references

Mallone R. et al. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabete Society. Clinical and Experimental Immunology. 2010.

Michelozzi et al. High-dimensional functional phenotyping of preclinical human CAR T cells using mass cytometry. STAR Protocols. 2022.