PROTOCOL FOR ISOLATION AND CRYOPRESERVATION OF DOG (LT-DTM) PERIPHERAL BLOOD MONONUCLEAR CELLS

PRINCIPLE:

Differences in cell density are exploited to separate granulocytes and erythrocytes from PBMCs. Granulocytes and erythrocytes have a higher density at the osmotic pressure of Lymphoprep™ and sediment through the Lymphoprep™ layer during centrifugation. The polysaccharide in Lymphoprep™ enhances erythrocyte aggregation, thereby increasing erythrocyte sedimentation. PBMCs, with lower densities, remain at the plasma: Lymphoprep™ interface.

PROTOCOL FOR ISOLATION OF PBMCs

1. Warm Lymphoprep™ to room temperature (15 - 25°C) before use.
2. Dilute blood with an equal amount of Dulbecco's Phosphate Buffered Saline with 2% Fetal Bovine Serum (PBS + 2% FBS).
3. Layer blood on top of LymphoprepTM; with the amount of LymphoprepTM equal to the volume of the diluted blood mixture.
4. Care to be taken to avoid mixing of blood with Lymphoprep™.
5. Centrifuge at 800 x g for 20 minutes at room temperature (15 - 25°C) with brake off.
6. Remove and discard upper plasma layer without disturbing the plasma: Lymphoprep™ interface.
7. Remove and retain PBMC layer at the plasma: Lymphoprep™ interface without disturbing the erythrocyte/granulocyte pellet.
8. Wash PBMCs with RPMI-1640 medium (15% FBS, antibiotic antimycotic solution, ciprofloxacin, erythromycin and centrifuge at 2000rpm for 5mins.
9. Again, wash twice with cold PBS at 850rpm for 5 mins.

MAINTENANCE OF PBMCs

Freshly isolated PBMCs are inactive and less proliferative in nature. Hence, media containing mitogens are added to activate and induce proliferation. Some of the mitogens are Lipopolysaccharide (LPS) = 1µg/ml, phytohemagglutinin (PHA) = 10µg/ml, concanavalin = 2.5µg/ml, IL-2 (specific for T cells) = 10pg/ml.

Composition of Cell Growth Media: RPMI-1640 with 10% FBS and antibiotics.

Growth Conditions: Temperature 37 ºC; Carbon dioxide 5% atmosphere; Humidity – 80-90%.

Note: Typically, PBMCs takes 48-72hrs to activate and proliferate.

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PROTOCOL FOR CRYOPRESERVATION OF PBMCs

MATERIALS:

1. PBMCs
2. Cryopreservation medium: Fetal Bovine Serum (FBS)
   Dimethyl sulfoxide (DMSO)
3. Cryogenic vials (cryochill vial cell standing sterile)
4. Pipette
5. Pipette tips

EQUIPMENTS:

1. Centrifuge
2. Inverted microscope
3. -80°C freezer (Biocare).
4. Freezing container (Mr. Frosty)
5. Liquid nitrogen container.

PROCEDURE:

1. Ensure that media was cold prior to starting of this protocol.
2. Aliquot FBS and FBS+DMSO cocktails separately as direct addition of DMSO may cause cell damage (stored in 4°C for 10-15 mins).
3. Cryomedia should include 90% of FBS and 10% of DMSO.
4. Cryogenic vials are labelled.
5. Ensure PBMCs to be in a single-cell suspension.
6. Centrifuge cells at 2000 rpm for 5 minutes to obtain a cell pellet.
7. Remove supernatant carefully with a pipette
8. Add require amount of cold FBS to make suspension.
9. Later, add cold FBS+DMSO cocktail drop wise to the cell suspension and mix gently.
10. Maintain the final ratio of FBS and DMSO i.e., 90:10 (for 1ml).
11. Add 1ml of the suspension into cryovials.
12. Place cryogenic vials in isopropanol freezing container (Mr. Frosty).
13. Container was placed in -80°C freezer for overnight.
14. Transfer vials into liquid nitrogen containing tank for long-term storage.

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