INTEGRATED BIOREPOSITORY OF H3AFRICA UGANDA

MAKERERE UNIVERSITY
COLLEGE OF HEALTH SCIENCES

STANDARD OPERATING PROCEDURE

TITLE: **ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS**	**PAGE 1 of 8**

SOP #: **IBRH$_3$AU-SOP-BSP-006.1**	Effective Date: **09/01/2014**	Next Rev: **DEC 2014**
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VALIDATION AND RETIREMENT

	NAME	DATE
Validated by:		
Retired by:		

ACKNOWLEDGEMENT OF READING AND UNDERSTANDING

I have received and understood the training on this SOP. If I have not understood the training I have asked the trainer to retrain me to ensure that I completely understand all the requirements.

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Table of Contents

1 SCOPE

This document details the isolation of peripheral blood mononuclear cells (PBMCs) from anti-coagulated whole blood.

2 PURPOSE

This standard procedure describes how PBMCs are isolated from whole blood.

3 ABBREVIATIONS AND TERMS

3.1 PBMCs: Peripheral Blood Mononuclear Cells

3.2 CSF: Cerebral Spinal Fluid

3.3 FBS: Fetal Bovine Serum.

3.4 FCS: Fetal Calf Serum

3.5 PBS: Phosphate Buffered Saline

3.6 Complete RPMI medium

4 RESPONSIBILITIES

Personnel	**Role**
Laboratory Technologist	Performs isolation of PBMCs

5 MATERIALS AND EQUIPMENT

5.1 Sterile 50 ml polypropylene tubes

5.2 Sterile 15 ml polypropylene tubes

5.3 Sterile 1.5ml Nalgene/Corning cryotubes

5.4 Sterile disposable serological pipettes (1,5,10,25,50 ml)

5.5 Pipetting aid

5.6 Pasteur pipettes

5.7 Pipette tips (10, 200, 1000µl)

5.8 Ficoll Paque/Histopaque

5.9 RPMI medium (**to a 500ml bottle of RPMI add 5ml of Pen Strep, 5ml of**

L- Glutamine and 12.5 ml of HEPES buffer)

5.10 Phosphate Buffered Saline

5.11 Fetal Bovine Serum/ Fetal Calf Serum

5.12 Penicillin-streptomycin solution

5.13 1M HEPES buffer

5.14 20mM L-glutamine

5.15 Absolute Isopropanol

5.16 Mr. Frosty

5.17 AIM-V

5.18 Dimethyl Sulfoxide (DMSO)

5.19 Swing out rotor centrifuge

5.20 Biosafety cabinet /hood

5.21 Liquid Nitrogen Tanks

5.22 Trypan blue

5.23 Disposable counting chambers

5.24 Automated counting machine/Microscope

6 SAFETY AND ENVIRONMENT

6.1 Perform all steps using the Universal Precautions for handling human blood and CSF in a biological safety hood.

6.2 Perform procedure under aseptic conditions.

7 PROCEDURE

7.1 Isolation of PBMCs using Ficoll over-layering method

7.1.1 All reagents to be used should be at room temperature i.e. 18-25°C before proceeding with assay.

7.1.2 Label cryotubes for storage of plasma with sample I.D, date and patient's name initials; number of required tubes depends on client specifications or sample volume.

7.1.3 Label polypropylene tubes with the sample I.D; one for each sample.

7.1.4 Centrifuge collected blood tubes at 1200 rpm for 10 minutes at 18°C. Collect off the plasma and aliquot into previously labeled cryo-tubes. Store plasma at -80°C.

7.1.5 Transfer the remaining blood into a labeled 15ml or 50 ml polypropylene tube and dilute blood with an equal volume of PBS i.e. ratio of blood: PBS is 1:1.

7.1.6 Dispense 4ml of Ficoll Hypaque into another 15ml polypropylene tube OR 15ml of Ficoll Hypaque into a 50ml polypropylene tube. The amount of Ficoll and size of tube used depends on the volume of diluted blood.

7.1.7 Carefully over-lay Ficoll Hypaque with diluted blood. The ratio of blood to Ficoll should be 2:1, for example 30ml of diluted laid over 15ml of Ficoll Hypaque. Blood and Ficoll layers should not mix.

7.1.8 Carefully transfer the tubes to centrifuge and spin at 1500 rpm for 30 minutes with no brakes applied.

7.1.9 Using a sterile pipette for each sample, harvest the PBMC band (cloudy interface between Ficoll and plasma) into a labeled 15 ml polypropylene tube. Care should be taken not to aspirate any more Ficoll Hypaque than necessary.

7.1.10 Wash PBMCs by topping off with sterile PBS and centrifuging at 1500 rpm for 10 minutes.

7.1.11 Decant off the supernatant and re-suspend cell pellet by gently tapping the tube. Dilute in 5ml of R-10 media.

7.1.12 Count cells by trypan blue dye exclusion method.

7.2 Isolation of PBMCs using Ficoll under-layering method

7.2.1 See steps 7.1.1 to 7.1.5.

7.2.2 Aliquot blood into labeled 50 ml polypropylene tubes, Introduce serological pipette containing an appropriate volume of Ficoll Hypaque (final ratio of blood to Ficoll should be 2:1) at the bottom of tube containing blood.

7.2.3 Dispense Ficoll-Hypaque carefully at the bottom of blood OR remove pipetting aid and allow gravity to push Ficoll- Hypaque at the bottom of blood thus under-layering. Blood and Ficoll-Hypaque layers should not mix.

7.2.4 See steps 7.1.8 to 7.1.12

7.3 Isolation of PBMCs from blood collected in CPT tubes

7.3.1 See steps 7.1.1 to 7.1.3.

7.3.2 Invert CPT tubes gently in order to mix the blood.

7.3.3 Spin CPT tubes at 2500 rpm for 25 minutes at 18-25°C.

7.3.4 Harvest plasma into cryo-tubes and store at -80°C.

7.3.5 Harvest cloudy band or buffy coat i.e. PBMC band into a labeled 15ml polypropylene tubes

7.3.6 Wash cells by centrifuging in 5ml of RPMI.

7.4 Manual Cell Counting

7.4.1 Prepare a 1:5 dilution of cells by adding 10ul of the cell (7.3.6 above) suspension to 40ul of 0.4% Trypan blue.

7.4.2 Pipette 10ul of the dilution onto a fast read disposable counting slide and count viable cells in 4x4 grid using a suitable microscope .(each grid holds a volume of 0.1mm.

7.4.3 Care should be taken to ensure the cell suspension is mixed thoroughly prior to removal of aliquots for cell counting.

7.4.4 Calculate the number of cells present using

7.4.4.1 ***Cell concentration = cells counted x dilution factor x volume of suspension x 10^4***

Where cells counted = n cells in 4x4 grid

Dilution factor = 5

Volume of suspension = 5

7.5 Cryopreservation

7.5.1 Centrifuge the 5ml suspension **(from 7.3.6 above)** at 365g for 7 minutes

7.5.2 Pour off the supernatant and re-suspend in freezing media.

7.5.3 Transfer the 0.5ml of the cell/freezing media suspension into cryovials (0.5ml freezing media per 1.0 2.0 x 10^6 cells separated).

7.5.4 Place the cryovials into a starter cooler (Mr. Frosty with cooling rate of -1^0C/minute and screw tightly with cover).

7.5.5 Store overnight at -80^0C

7.5.6 The following day, transfer the cells from the -80^0C freezer to LN2 tanks for long term storage.

7.6 Freezing Media preparation

DMSO (ml)	1	2	3	4	5
FBS (ml)	9	18	27	36	45
Total Volume (ml)	10	20	30	40	50

8 REVISION HISTORY

Revision No	Effective Date	Description of Changes Made from Preceding Revision	Approved by/ Date

ANNEX 1: DOCUMENTATION OF SUGGESTED CHANGES TO THIS SOP

CLAUSE	SUGGESTION	BY	DATE