STANDARD OPERATING PROCEDURE 7

TITLE: PROCEDURE FOR THE ISOLATION OF PBMCs FROM HIV INFECTED BLOOD (using method for HIRD Study)

Purpose The purpose of this SOP is to describe the procedure to be followed to isolate viable lymphocytes from HIV-infected blood.

Scope BioBank Staff

Responsibilities Working with HSE and HTA guidelines as well as local rules (e.g. CL3)

Materials Lymphoprep, 1xPBS, DMSO, Heat-Inactivated AB plasma

Equipment Centrifuge, Automated pipette, Gilson pipettes

Consumables 15ml and 50ml centrifuge tubes, 5ml, 10ml and 25ml sterile pipettes, sterile P1000, P200 and P20 filter tips, Sterile 3.5ml Pasteur pipettes, disposable haemocytometer chambers, 2ml screw-top cryovials

General Procedures Before Processing Blood

1. Biobottles holding either sodium heparin or ACD vacutainers (according to individual study protocols) will be removed from the cardboard boxes and taken into the Class II microbial safety cabinets (MSC) within room 3.27 of the CL3 facility. Samples will be inspected for evidence of spillage by removal of the Biobottle top and by inspection of the clear plastic bags. In the event of leakage, the spillage procedure (SOP 4) will be enacted.
3. 'MSC' refers to the Class II microbiological safety cabinet. All processing of blood should be carried out in a MSC within the CL3 unit and the working surface of the cabinet should be wiped down with 70% IMS before use.
4. A 'waste pot' containing 50% P3 Sterile diluted with tap water (1/3 to 1/2full) should be made up for use in the MSC and an autoclave bag used as a liner for the dry waste bucket.
5. The lymphoprep datasheet located in the BioBank folder in Room 3.27 of the CL3 unit should be completed.

Procedure: Isolation of Viable PBMCs

1. Collect samples & record the following details on Biobank booking in form: i) patient and visit number if appropriate, ii) date and time sample received, iii) sample volume(s). (SOP2)

2. In a safety cabinet, add 15 ml of Lymphoprep to a 50ml centrifuge tube, one for each patient to

be processed. If larger volumes of blood are received prepare more Lymphoprep tubes as appropriate.

3. In a separate 50ml Falcon tube, transfer blood from heparin tubes and add an equal volume (*i.e.* 15ml with 15mls) of 1xPBS (Ca & Mg free). Cap the tube and then invert gently to mix.
4. Layer 30ml of the diluted blood carefully on top of the 15ml Lymphoprep and centrifuge at 450g for 30mins (Heraeus Multifuge 4KR, Program 8 in CL3).
5. After centrifugation, carefully remove tubes from the rotor, and without disturbing the layers place them in a rack.
6. Carefully aspirate most of the top layer (consisting of plasma and PBS) from each tube without disturbing the buffy layer and if not harvesting, discard.
7. Into a new 50ml conical tube, carefully aspirate/transfer the buffy coat layer. To wash bring the total volume up to 50ml with 1x PBS and mix/invert the tube several times.

   NB It is recommended that the volume of PBS used is 3x that of the buffy coat.
8. Place tubes in the centrifuge and spin for 10mins at 200g at room temperature (Heraeus Multifuge 4KR, Program 9 in CL3). Whilst cells are spinning, aliquot 10µl of 0.4% Trypan Blue stain into the necessary number of eppendorfs (1 per sample to be analysed).
9. After centrifugation, pour off the top layer (consisting of PBS) and resuspend the remaining cell pellet in 10ml of 1x PBS. Mix by gently pipetting up and down several times.
10. Determine the cell recovery & viability by mixing 10µl of the above cell suspension with 10µl of 0.4% Trypan Blue stain. Add ~6.6µl to a haemocytometer slide chamber & count as quickly as possible, using a hand tally counter to count the number of cells in each 9x9 grid, in all **four** corners of the chamber. Count only live cells (unstained by trypan blue) **BUT** note the number of dead cells.
11. Calculate the viable cell count by using the following equation and record on the data sheet:

    *Average no.viable cells in **one** 9x9 square **X** dilution factor **X** 10^4 **X** no. of ml = total no. cells.*

12. Spin down cells at 4°C at 1400 rpm for 10mins. Whilst cells are spinning prepare part (ii) of freezing medium (step 13) and ensure that Nalgene Mr Frosty freezing container is at room temperature & filled with isopropanol (also known as isopropyl alcohol, 2-propanol) to the correct volume. Label an appropriate number of cryovials for each subject with the following information: Study ID, sample and visit number, date of sample, cell type.

13. Prepare freezing medium – (i) neat filtered heat inactivated (HI) AB sera* and (ii) a mixture of 80% filtered HI-AB sera + 20% high purity DMSO (Sigma D2650) – prepare 500µl of each per vial of cells to be frozen. Freezing medium should be prepared daily.

***AB sera should have PREVIOUSLY been heat inactivated at 56**°C **for 1hr and then filtered using a 0.8 micron filter and a syringe within a MSC II safety cabinet before storing at -20°C as 10ml aliquots in 15ml falcon tubes.**

14. Once cells have spun, decant supernatant and flick tube gently to loosen cell pellet. Add 500µl neat AB plasma per 10^7 cells. Then add an equal volume of AB/20% DMSO drop wise over 1-2 minutes. Mix slowly with each drop; repeatedly swirling tube gently to mix.

15. Pipette 1ml (10^7 cells max) into each cryovial. Work quickly during step 14 and 15 if there are many cryovials. Transfer to Mr Frosty and immediately store at -80°C overnight before transfer to liquid nitrogen the next day (SOPs 8 and 9).

Note that each Mr Frosty can only accommodate 18 vials.

Cross Referenced SOPS : SOP2: Tracking samples, SOP4: Spillage Procedure, SOP8: Long term storage of PBMCs in liquid nitrogen, CL3 codes of practice, SOP 9: Transport for long-term storage.