OSUCCC LTB Laboratories Procedure Mononuclear cell separation of bone marrow aspirate and peripheral blood using Ficoll-paque Plus® [1]			Effective: 10/1/1997
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1.0 PRINCIPLE

Ficoll-Paque PLUS (Amersham Pharmacia Biotech) is an aqueous solution of density 1.077 ± 0.001 g/ml; consisting of 5.7 g Ficoll 400 and 9g sodium diatrizoate with calcium EDTA per 100 ml. [1] The density and osmolality have been optimized for the isolation of lymphocytes from whole blood and bone marrow. Anticoagulated venous blood or bone marrow is diluted and layered onto Ficoll-Paque PLUS **(FPP)**. Typical recovery is 60 ± 20% of lymphocytes from the original blood sample; 95 ± 5% of cells present in the lymphocyte fraction are mononuclear leukocytes; > 90% viability (measured by trypan blue exclusion).[2] During centrifugation, erythrocytes and granulocytes are aggregated and rapidly sediment whereas lymphocytes and other mononuclear cells form a distinct band at the FPP/plasma interface.[3] This band may be removed and further purified by washing in an isotonic buffer such as phosphate buffered saline.

2.0 SPECIMEN

Fresh peripheral blood, bone marrow or products of leukapheresis are collected with anticoagulation reagent (heparin, EDTA) that is specified in the sample collection protocol. Specimens should be procured as soon as they are received by the lab and preferably within 24 hours of being procured from the patient/subject. Alliance or OSU treatment protocols specify collection methods for samples requiring mononuclear cell isolation. Samples for all Alliance studies should be collected into the appropriate collection tube indicated in the study protocol. Any problems or comments regarding sample collection, shipment and receipt will be noted by the technician on the sample procurement form. For study samples, non-Alliance or Alliance, collected at OSU, the LTB lab will be notified that a patient sample is ready for pick up and the location of sample.

3.0 MATERIALS AND REAGENTS

Ficoll-paque plus (FPP)
Sterile 15 or 50cc conical tube
Sterile pipets (5ml, 10ml and 25ml)
Sterile Dulbecco's PBS (Invitrogen # 19140-144) Sterile Fetal Bovine Serum (Invitrogen#/16140-071)
Cell freezing Medium (see cell freezing medium protocol) Sterile 4x4 gauze
Sterile pipet tips (200µl and 1000 µl)
Micropipetters (20-200µl and 1000 µl) ViCell specimen cup
Beckman Coulter – Isoton Solution
Zapoglobin solution (if using Beckman Coulter®Counter)
70% isopropyl alcohol

4.0 EQUIPMENT

Biosafety cabinet
Benchtop centrifuge with swinging bucket rotors to hold 15 and 50cc conical tubes
Beckman Coulter ViCell automated viable cell counter
Beckman Coulter Z1 Coulter ® Counter, particle counter

5.0 QUALITY CONTROL AND SAFETY

Ficoll-Paque PLUS is provided as a sterile solution, which is stable for at least 3 years when stored between 4°C and 25°C and protected from light. Deterioration of FPP is indicated by the appearance of a yellow color or particulate material in the solution. It is recommended that specimen collection be carried out in accordance with NCCLS document M29T2. No known test sample can offer complete assurance that human blood samples will not transmit infection. Therefore, all derivatives are potentially infectious. Always spray alcohol on the caps before opening solutions. The alcohol can be dried off using gauze. If you have been out of the hood for a while and are wearing the same pair of gloves, use a new pair of gloves. Remember not to touch the sides (inside or outside) of any bottles with your pipette – if you do, dispose of the pipette and start again.

6.0 PROCEDURE

6.0.1 Record all necessary sample information, carefully and clearly, in the written sample record including: patient name, protocol number, accession number, diagnosis, sample type, sample period, and the number/type of tubes received.

6.0.2 In biosafety cabinet, mix samples by inversion 5-6 times; transfer samples into a clean, sterile 15 or 50cc conical tube, depending upon initial sample volume (A 50cc tube should contain no more than 20ccs of the original sample and a 15cc tube should contain no more than 8ccs). Make sure the tube is labeled with the accession number on both the side and cap.

6.0.3 Dilute sample with sterile Dulbecco's PBS+ 2% FBS up to 9 or 35ml (for 15 or 50cc conical tubes, respectively.). Mix by inversion.
Note: If sample volume is less than 5ml use the following dilution and ficoll schema (15cc conical tube)
1/3 volume = sample + 1/3 = Dulbecco's PBS+ 2% FBS + 1/3 = FPP This allows for better concentration and recovery of buffy coat.

6.0.4 Using a sterile pipet, layer FPP *under* the diluted sample (SLOWLY) for total volume of 15 or 50cc.

6.0.5 Cap tube tightly and spin at room temp for 30 minutes at 300 x *g*.
Centrifuge brake should be disengaged for Ficoll spin.

6.0.6 Obtain clean, sterile, 15 or 50cc conical tubes, label tubes as above.

6.0.7 With a sterile pipet, remove the mononuclear layer (buffy coat), which will appear as a white floating disk at the interphase of FPP and plasma. Be careful not to touch the FPP layer, which is below the disk. Transfer this cellular fraction into the new tube. The left over liquid may be disposed of after replacing the lid.

6.0.8 Dilute buffy coat with sterile PBS to 15 or 40cc (for 15 or 50cc tubes, respectively.) and centrifuge for 10 min at 1000 x *g* (with full braking).

6.0.9 Pour off supernatant. Be careful not to let the pellet pour out with it.
Resuspend pellet in 2 to 20ml of DPBS depending on the pellet size. (If the pellet is very large resuspend pellet in a larger volume of DPBS to obtain accurate cell count.)

6.1 SAMPLE EVALUATION

6.1.1 A hemacytometer or automated cell counter may be used to obtain a cell count.

6.1.2 If using the ViCell Counter, prepare a ViCell cup with 2ml of Isoton® II.

6.1.3 If using the Coulter ® Counter prepare a counting vial with 20ml of Isoton® II; add 3 drops of Zap-oglobin® to vial and mix gently by inversion. Zap-oglobin is not used with the ViCell.

6.1.4 Add 40µl of mixed sample to the prepared vial (ViCell or Coulter®Counter).

6.1.5 Count sample and record count on sample worksheet.

6.1.6 Cell count is given in cells/ml. To obtain total cell number, multiply cell/ml x total volume of cell suspension, e.g. count result = 1.9×10^7

cells/ml resuspended in 5ml PBS or 1.9x107cells/ml x 5 ml = $9.5x10^7$ total cells.

6.1.7 Total cell count will be affected by certain special procedures. Before recording the total cell count to be banked as viables, subtract the number to be used in other procedures (ex. RNA-stat, cytospins, RNA-Qiagen). See reference sheet posted in the lab.

6.1.8 Prepare cryovials for LN2 storage based on total cell count. Approximately 5-10 million cells/ml freezing medium/tube. Total cell count per vial varies by protocol. Always use the information posted in the lab. Never resuspend more than 35 million cells/ml freezing medium/tube. Label cryotubes with the assigned accession number only (see Brady labeler instructions). DO NOT USE THE PATIENT'S NAME ON THE STORAGE TUBE.

6.1.9 Centrifuge sample to pellet cells as before. Remove supernatant and resuspend pellet in freezing media, adding 1ml of freezing media for each cryovial labeled.

6.1.10 Place vials in a slow rate freezing container, place containers in –80°C freezer overnight. Record the number vials and the concentration in the appropriate space in log sheet.

6.1.11 Transfer tubes to LN2 storage, recording their location on the sample worksheet.

7.0 LIMITATIONS OF THE PROCEDURE

1. The purity of cell population obtained by this procedure may be determined by performing a Romanowsky stain (Wright stain) on a smear or cytospin slide of material from step 14. Viability may be determined by treatment with Trypan blue.
2. If cell viability is <80% replacement of DPBS with tissue culture medium such as RPMI 1640 with fetal bovine serum, 5% may be helpful.
3. If T- and B- cell enumeration is to be performed, sample should not be stored for more than several hours.[4] Addition of blood to certain tissue culture media may allow storage for up to 72 hours without alteration of T-and B-cell values.
4. Prediluted blood may be used with this procedure. Blood may be diluted with PBS or other appropriate medium, recommended ratio: 1 part blood to 1 part medium. Roughly equal volumes of FPP and blood should be used to optimize cell recovery. Dilution is appropriate for specimens with hematocrits above normal.
5. Removing excess amounts of FPP with the mononuclear band increases granulocyte contamination from residual granulocytes, which may remain at the mononuclear interface.
6. Removing excess amounts of supernatant with the mononuclear band may promote contamination by plasma proteins.
7. Use of volumes of prediluted or whole blood other than those recommended may result in decreased recovery.
8. To remove all contaminating platelets, a second centrifugation in 4-20% sucrose gradient layered over FPP can be performed. The sucrose gradient will effectively isolate the platelets while the mononuclear cells will penetrate to the FPP layer.

9. Failure to centrifuge Ficoll Paque PLUS at room temperature may present limited recovery of mononuclear cells.

8.0 VICELL PROCEDURE[5]

The ViCell particle counter is employed here as an in-process control in the LTB, in that it is used to evaluate the affect of the sample processing procedure on patient samples.

8.0.1 Turn on the computer and double click **ViCell** icon to open program. Once the program is s tarted up, you should hear a pump initializing the instrument.

8.0.2. Prepare sample a cup by labeling it with the sample accession number and adding 2ml Isoton counting solution.

8.0.3. Add 40µl of the diluted sample to the prepared cup.

8.0.4. Place sample cups in carousel. The samples do not have to start in position 1

8.0.5. Click **Log in Sample** and follow on screen instructions.

8.0.6. Type in sample accession number.

8.0.6.1 Select **PBMCs** as cell type. This identifies the analysis parameters for ficolled blood and bone marrow samples.

8.0.6.2 Use dilution factor of **50**.

8.0.6.3 If counting more than one sample, select **Next Sample**, otherwise, select **OK**.

8.0.7. Click **Start Queue** to begin counting.

8.0.8. Record the cell counts and viability on the appropriate worksheet.

8.0.9. The ViCell is self-cleaning and will dispose of the sample cups after counting. When finished, leave the ViCell on for next use. Remove used reagent pack:

8.0.9.1. Attach color-coded tubing to each corresponding reagent pack bottle.

8.0.9.2 Place reagent pack inside ViCell compartment with the open window of the pack facing the door. Attach waste line to waste container and place inside the ViCell.

8.0.9.3. Click main screen toolbar **Instrument and Replace Reagent Pack**, this allows the lines to prime and will reset the maximum number of runs.

8.0.9.4. If more Reagent Packs are needed outside our regular order, please contact Beckman-Coulter. **1-800-523-3713 ext. 3826**.

9.0 REFERENCES

1. Amersham Pharmacia Biotech Product bulletin revised December 1999.
2. Isolation of mononuclear cells and granulocytes from human blood. (Paper IV). Bøyum, A. Scand. J. Clin. Lab Invest. 21 Suppl, 97, 77-89 (1968)
3. Ficoll-paque PLUS technical manual 18-1152-69. Amersham Pharmacia Biotech 2001.
4. Hofman FM et al: Stability of T-and B-cell numbers in human peripheral blood. Am. J Clin. Pathol. 77:710, 1982.
5. ViCell® Operator's Manual. Beckman Coulter Corporation, March 2003
6. Coulter® Model Z1 Operator's Manual. Beckman Coulter Corporation, August 1997.