INSTRUCTION MANUAL

PROCEDURE FOR THE PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PELLETS FROM WHOLE BLOOD

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I. INTRODUCTION

Before Beginning

The procedure outlined in this manual has strict requirements regarding both time and temperature. Further, correct performance of this procedure requires the user to have access to special equipment.

PLEASE READ AND UNDERSTAND ALL STEPS IN THIS MANUAL BEFORE BEGINNING. IN ADDITION, HAVE ALL THE NECESSARY REAGENTS AND EQUIPMENT PRESENT AND AT THE PROPER TEMPERATURES BEFORE BEGINNING.

Intended Use

FOR IN VITRO USE ONLY

Summary and Explanation

The technique of separating peripheral blood mononuclear cells (PBMCs) from whole blood is not new, having been developed over 30 years ago (1,2). This technique has been used worldwide to collect and study lymphocytes and monocytes from a variety of subjects and in a variety of diseases (3-5).

Modern advances in biochemistry and molecular biology now allow researchers and clinicians to investigate diseases at the molecular level. Accordingly, to preserve the integrity of specimens for analysis, new techniques must be developed to ensure accurate and reproducible assay results.

In a recent finding, a novel protein has been identified in patients with a subset of Chronic Fatigue Syndrome (6). The current assay for this protein requires that PBMCs be separated from whole blood within hours after drawing, and the subsequent PBMC "pellet" be frozen at –70°C until assayed.

Method Description

The isolation of PBMCs requires separation of whole blood by centrifugation through a density gradient. Whole blood is layered onto a sterile aqueous medium containing ficoll and sodium diatrizoate at a predetermined density of 1.077 g/ml at 25°C. Gentle centrifugation at room temperature results in the separation of PBMCs at the blood/ficoll interface, with the other white blood cells (WBCs) and red blood cells (RBCs) passing through the interface and collecting at the bottom of the tube.

The PBMC interface is the collected and washed with sterile Phosphate Buffered Saline (PBS) to remove any contaminating separation medium. The PBMCs are then subjected to a brief treatment with a hypotonic aqueous medium containing

ammonium chloride to lyse any contaminating RBCs present. The PBMCs are pelleted by centrifugation and washed with PBS. Finally, the PBMCs are transferred to a microcentrifuge tube and centrifuged briefly to form a tight pellet. Any remaining liquid is completely removed and the resulting “pellet” is frozen at –70°C until use.

NOTE THAT STORAGE AND SHIPMENT OF PELLETS AT –70°C IS CRITICAL TO ENSURE SPECIMEN INTEGRITY

II. REAGENTS

Note: All reagents should be stored at 2-8°C

1. PBMC Separation Medium

This mixture of ficoll and sodium diatrizoate is available from many manufacturers under the following trade names:

Histopaque-1077	Trademark of Sigma Chemicals
Ficoll-Paque	Trademark of Pharmacia
Lymphodex	Trademark of Fresenius
Lymphoprep	Trademark of Nycomed
Leucosep	Trademark of Esquire
LSM	Trademark of Organon-Teknika

The PBMC separation medium should have a density of 1.077 g/ml at 25°C and be kept sterile.
BEFORE USE: Warm medium to room temperature (remove from refrigerator at least one hour prior to use).
FAILURE TO BRING PBMC SEPARATION MEDIUM TO ROOM TEMPERATURE WILL RESULT IN LIMITED RECOVERY OF MONONUCLEAR CELLS.

2. Sterile Phosphate Buffered Saline (PBS)

PBS is available from many manufacturers as a 1X liquid, 10X liquid, pre-measured powder or tablet. [Final reagent formula is a 1X solution containing 154 mM NaCl, 1.54 mM KH_2PO_4, 2.7 mM Na_2HPO_4; pH=7.4] BEFORE USE: Warm bottle to room temperature (remove bottle from refrigerator at least one hour prior to use).

3. Sterile Red Blood Cell Lysing Solution

Sterile RBC Lysing Solution has a reagent formula = 155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA; pH=7.4. The solution is used cold at 2-8°C and has a shelf life of six months when made and kept sterile.

Note: A list of suppliers and chemical manufacturers is located in Appendix A

III. MATERIALS AND EQUIPMENT REQUIRED

1. Laboratory marking pen
2. Sterile pipettes (10ml, 5ml, pasteur/transfer pipettes)
3. Pipette bulb or automatic pipettor
4. Centrifuge capable of accommodating 50 ml conical tubes and achieving 500 x g
5. Sterile 50 ml conical centrifuge tubes
6. Container for waste liquids
7. Clock or timer
8. Wet ice
9. Microcentrifuge
10. Microcentrifuge tubes (1.5 ml)
11. –70°C freezer

In addition, other routine laboratory materials and safety equipment should be in place before beginning this procedure.

IV. WARNINGS & PRECAUTIONS

FOR IN VITRO USE ONLY
NOT FOR INTERNAL OR EXTERNAL USE IN HUMANS OR ANIMALS

Handling of Human Blood and Blood Products

Treat all human blood and blood products as potentially infectious. It is strongly recommended that good laboratory practices be employed when handling any human specimens: Wear protective clothing, protective eyewear, and gloves. Dispose of all contaminated materials according to local, state, and national laws.

In addition, it is strongly recommended that a biosafety cabinet be used to 1) help keep the operator safe when working with blood and blood products, and 2) to help keep the reagents sterile.

Indications of Possible Deterioration of Reagents

The reagents listed above must be kept sterile. Contamination may be present if 1) there is present any abnormal particulate material, or 2) if cloudiness or haziness is present upon gentle mixing of the reagent. If any contamination is suspected, the reagent should be discarded.

V. SPECIMEN REQUIREMENTS

Thirty (30) mLs of anticoagulated whole blood are required.

Collection and Storage of Whole Blood

Collect blood by venipuncture into evacuated collection tubes containing sodium heparin as anticoagulant. Collect a total volume of approximately 30 ml. Mix gently and thoroughly immediately after collection.

Anticoagulated blood may be stored up to six hours at room temperature before preparing a PBMC pellet. DO NOT REFRIGERATE WHOLE BLOOD. DO NOT STORE WHOLE BLOOD LONGER THAN SIX HOURS BEFORE PREPARING A “PELLET.”

VI. PROCEDURE

HAVE ALL THE NECESSARY REAGENTS, MATERIALS, AND EQUIPMENT PRESENT AND AT THE PROPER TEMPERATURE BEFORE BEGINNING

Note: Steps #1-3 for 50 ml conical tubes without frit

1. Label tubes appropriately with patient identification. Use one 50 ml conical tube per patient.

2. Pipette 20 ml of PBMC Separation Medium into each 50 ml tube.

3. Carefully layer 30 ml of whole blood onto Separation Medium taking care not to disrupt the interface. [See Procedural Comment #1 if using less than 30 ml whole blood] **Proceed to step #8**.

Note: Steps #4-7 for 50 ml conical tubes with frit (tubes available from Greiner; see Appendix A)

4. Label tubes appropriately with patient identification. Use one 50 ml conical tube per patient.

5. Pipette 15 ml of PBMC Separation Medium into each 50 ml tube.

6. Centrifuge tubes for two minutes at 100 x g*. All PBMC separation medium must be below the frit before the blood is added.

7. Add 30 ml of whole blood by directly pouring blood onto frit [See Procedural Comment #1 if using less than 30 ml whole blood].

8. Centrifuge the filled conical tubes at 500 x g* for 30 minutes at 20-25°C.

9. After centrifugation an interface should be present. The interface rests above the frit and below the top of the plasma layer and is cloudy-white in color. This interface contains the mononuclear cells. With a pipette, gently remove and discard the upper, plasma layer, leaving approximately 1 cm of liquid above the interface. DO NOT DISTURB THE INTERFACE!

10. With a pipette, gently collect the interface layer containing the PBMCs. Transfer this interface to a sterile 50 ml conical tube [See Procedural Comment #2].

11. Add Sterile PBS (Reagent #2) to the collected PBMCs. Fill the conical tube to the 50 ml mark. Close cap tightly and invert to mix. [See Procedural Comment #3].

12. Centrifuge the tubes for 5 minutes at 500 x g* at 20-25°C.

13. Discard the supernatant by inversion.

14. Using a pipette, re-suspend the PBMCs in 10 ml of cold (2-8°C) RBC Lysing Solution (Reagent #3). Close tubes tightly and place in ice water bath, or on wet ice, for 5 minutes.

15. Centrifuge the tubes for 5 minutes at 500 x g* at 20-25°C.

16. Discard the supernatant by inversion.

17. Using a pipette, re-suspend the PBMCs in 20 ml of PBS (Reagent #2).

18. Centrifuge the tubes for 5 minutes at 500 x g* at 20-25°C.

19. Discard the supernatant by inversion.

20. Re-suspend the PBMCs in 1 ml of PBS (Reagent #2) and transfer these cells to a pre-labeled microcentrifuge tube.

21. Microcentrifuge the tubes for 1 minute at 20-25°C at highest speed so as to form a tight cell pellet.

22. Discard the supernatant by inversion and blot the tube dry by gently tapping the inverted tube on absorbent paper. [See Procedural Note #4]

23. Freeze the "pellet" at –70°C.

*[$g = (1118 \times 10^{-8})(\text{radius in cm})(\text{RPM})^2$]

Procedural Comments

a) Addition of PBS to whole blood to balance tubes

When preparing more than one specimen, it may be the case that the amount of blood collected from one or more individuals is not exactly 30 ml. To make sure that the tubes balance for centrifugation, PBS (Reagent #2) may be added to equalize the volumes. Add the PBS gently to the whole blood so as not to disturb the blood-separation medium interface.

b) Amount of interface volume transferred

Most frequently, between 7-12 ml of interface are collected. Collecting more than 12 mLs may lead to RBC and granulocyte contamination of the PBMCs.

c) Dilution of PBMC separation medium post-separation

It is essential that the PBMCs be washed free of separation medium after being collected from the interface. Use PBS and fill the collection tube to the 50 ml mark (interface included) to ensure adequate washing.

d) Complete removal of PBS before freezing pellet

After the final microcentrifugation step, the supernatant must be completely removed. This can be accomplished by blotting the tube (still inverted after discarding the supernatant) on a piece of absorbent material to remove the last traces of liquid. Discard any used absorbent materials as you would any contaminated dry waste.

VII. EXPECTED RESULTS

The expected result of preparing a PBMC pellet from 30 ml of anticoagulated whole blood is a small, white pellet, approximately 0.25-0.50 cm^3, at the bottom of the microcentrifuge tube.

Normal Range

The size of the mononuclear cell pellet will vary from individual to individual based on a number of factors:

- The amount of blood used
- The number of circulating mononuclear cells in the patient at the time of blood draw
- The ability of the technician to completely remove the mononuclear cell interface after the separation step
- Any cell loss occurring from one or more washing steps

Separation of normal human peripheral blood by the protocol as outlined in this manual typically yields a mononuclear cell preparation with the following characteristics:

- 60 $\pm$ 20 percent recovery of the mononuclear cells present in the original blood sample
- 95 $\pm$ 5 percent mononuclear cells
- >90 percent viability
- < 5 percent contaminating granulocytes

The use of RBC Lysing Solution eliminates any contaminating RBCs and platelets.

VIII. TROUBLESHOOTING GUIDE

From time to time certain specimens may prove more difficult to work with than others. In addition, two uncommon occurrences are also addressed.

Occurring Problems

A. No definite interface after the separation step

This occurs for two reasons: First, the centrifugation time was not sufficient. Replace the tube in the centrifuge and centrifuge for an additional 30 minutes. If, however, a faint interface can be seen, it is likely due to the second reason, i.e. cloudy or opaque plasma that interferes with the visualization of the interface. Should this occur, leave two centimeters of plasma on top of where the interface appears to begin, then collect the interface with the excess plasma.

NOTE: If the interface collected is more than 20 ml, this volume should be divided into two 50 ml conical tubes. Fill both tubes with PBS to the 50 ml mark and centrifuge as in Step #12.

B. First wash step yields small (or no) pellet

It is probable that too much interface was collected and that the amount of PBS added did not sufficiently dilute the separation medium present in the wash. Divide the volume into two 50 ml conical tubes and fill each to the 50 ml mark with PBS (Reagent #2). Repeat the centrifugation in step #12. The resulting pellets may be re-combined into one tube for the RBC Lysing step (Step #14).

C. PBMC pellet resistant to re-suspension

This is common and is not really a problem. Treatment of PBMCs with ammonium chloride (in the RBC Lysing Solution) causes the cell membranes to become “sticky” and the cells adhere to one another. Vigorous re-suspension is not necessary; small clumps of cells are allowed.

Uncommon Problems

A. Clotted blood in original blood collection tubes

Clotted blood results from incomplete mixing of anticoagulant after blood drawing. Clots will result in lower PBMC yields. If clotting is present in more than half the blood volume, the PBMC pellet procedure should not be performed and the blood should be re-drawn. If only a small amount of the blood is clotted, the technician may proceed with the procedure, but should note the presence of any clots detected on his/her worksheet.

B. Disruption of the interface layer before collection

A violent disturbance to the interface layer may occasionally occur (dropping the centrifuge tube or giving it a “hard bump”). If the interface disperses, remove the entire amount of liquid above the frit and place into a sterile 50 ml conical tube. Dilute the liquid with PBS (Reagent #2) to the 50 ml mark. Add this material to two, pre-labeled, pre-filled separation tubes (25 ml to each) and repeat the centrifugation as outlined in step #8.

IX. REFERENCES

1. Bøyum, A., *Scand. J. Clin. Lab. Invest*. **21** Suppl. 97, 31 (1968)
2. Bøyum, A., *Scand. J. Clin. Lab. Invest*. **21** Suppl. 97, 77 (1968)
3. Hokland, P. and I. Heron, *J. Immunol. Methods* **32**:31 (1980)
4. Kaplan, J., et al., *J. Immunol. Methods* **50**:187 (1982)
5. Hirvonen, H., et al., *Blood* **78**:3012 (1991).
6. Suhadolnik, R., et al., J. Interferon & Cytokine Res. **17**:377 (1997)

APPENDIX 1 – List of Suppliers

This list is to act as a helpful reference only. Listing of the names and products below does not represent an endorsement for use of these products by R.E.D. Laboratories NV. In addition, there are other manufacturers of these items that have not been listed due to space considerations. Please check the catalogue numbers and pricing with the manufacturer before ordering any reagents.

For PBMC Separation Medium

Histopaque-1077	Trademark of Sigma Chemicals
Ficoll-Paque	Trademark of Pharmacia
Lymphodex	Trademark of Fresenius
Lymphoprep	Trademark of Nycomed
Leucosep	Trademark of Esquire
LSM	Trademark of Organon-Teknika

For PBS (ready made)

Life Technologies (GIBCO/BRL)
Sigma Chemicals

For Chemicals Used in PBS and RBC Lysing Buffer

Sigma Chemicals

For Centrifuge Tubes With Frits

Greiner Labortechnik