Ficoll-Paque® and Ficoll-Paque ET

INSTRUCTIONS

Intended Use

For *in vitro* isolation of lymphocytes.

Introduction and Summary of the Method

There is a need for a rapid, simple and reliable method of isolating lymphocytes from whole blood in such areas of scientific endeavor as histocompatability testing, *in vitro* cell-mediated immunity assays, and other procedures requiring pure lymphocyte preparations.

Early techniques for separating lymphocytes from other blood cell types involved mixing the blood with some erythrocyte aggregating agent, thereby causing the erythrocytes to clump and sediment to the bottom of the tube. The lymphocytes could then be collected from the upper part of the tube (1, 2). The disadvantages of these techniques are that lengthy, repeated procedures are required to obtain purified lymphocyte suspensions, and the yield of recovered lymphocytes is low.

Bøyum noted that the low viscosity of Ficoll®, compared to the other polymeric erythrocyte aggregating agents, makes it possible to devise a lymphocyte isolation procedure involving a short, low speed centrifugation (3). A solution of Ficoll and sodium metrizoate of the proper density and osmotic strength was placed in a centrifuge tube. Blood was layered on top, and the two-phase system was centrifuged at a low speed for a short time. The erythrocytes and granulocytes sedimented to the bottom of the tube, and the purified lymphocytes could be collected from the interface between the two phases. Other authors, using slight modifications of this procedure, have emphasized that the Ficoll-sodium metrizoate centrifugation procedure is an easy one-step, rapid, reproducible method for the preparation of viable lymphocytes in high yield (4–7). Sodium diatrizoate has been successfully substituted for sodium metrizoate in this procedure by numerous workers (8-12).

Ficoll-Paque, produced by Pharmacia Biotech, provides a sterile, ready-to-use Ficoll-sodium diatrizoate solution of the proper density, viscosity, and osmotic pressure for use in a simple and rapid lymphocyte isolation procedure.

Principle of the Procedure

Defibrinated or anticoagulant-treated blood is layered on the Ficoll-Paque solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated by the Ficoll and, therefore, sediment completely through the Ficoll-Paque. The layer immediately above the erythrocyte layer contains mostly granulocytes which at the osmotic pressure of the Ficoll-Paque solution attain a density great enough to migrate through the Ficoll-Paque layer.

Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll-Paque with other slowly sedimenting particles (platelets and monocytes). The lymphocytes are then recovered from the interface and subjected to a short washing step with a balanced salt solution to remove any platelets, Ficoll-Paque and plasma.

Since this method can be adapted to very small volumes of blood, it is particularly suitable for isolation of lymphocytes where only limited quantities of blood are available, such as from cadavers and small children. Because of the rapidity and simplicity of the method, it is also the method of choice in emergency tissue typing procedures (8).

Reagents

Each 100 ml contains Ficoll 400, 5.7 g, Diatrizoate Sodium, 9.0 g, with Edetate Calcium Disodium in Water for Injection U.S.P.

71-7167-00
Edition AA

Pharmacia Biotech

This product is intended for *in vitro* isolation of lymphocytes for research applications.

Storage

Ficoll-Paque should be stored between 4 °C and 25 °C and protected from direct light. Storage in the cold will increase the shelf life.

Indications of Instability

Deterioration of the Ficoll-Paque is indicated by the appearance of a distinct yellow color or particulate material in the clear solution.

Specimen Collection and Handling

Fresh blood should be used to ensure high viability of isolated lymphocytes. Prepare sample at 18-20 °C as follows:

1. To a 10 ml test-tube add 2 ml of defibrinated or anticoagulant-treated blood* and an equal volume of balanced salt solution. (Final volume 4 ml.)
2. Mix by drawing the blood and buffer in and out of a Pasteur pipette.

* Anticoagulants: Heparin, EDTA, citrate, acid citrate dextrose (ACD) and citrate phosphate dextrose (CPD) may be used. Defibrinated blood requires no anticoagulant.

PROCEDURE

Materials Provided

See under "Reagents."

Materials Required but not Provided

Item	No.	Size
Test tubes*	2/sample	10 ml
Balanced salt solution	>20 ml/sample	-
Pasteur pipettes*	1/sample	3 ml
Centrifuge tubes*	2/sample	15 ml; internal diameter 1.3 cm
Centrifuge	-	capable of producing 60-400 x g and maintaining 18-20 °C temperature
Syringe with needle	-	-
Silicone solution, 1%	-	-
Distilled water	-	-

* Tissue culture plasticware or pretreated glassware. Preparation of glassware: All glassware which comes in contact with the sample should be siliconized before use. The glassware should be immersed in a 1% silicone solution for 10 seconds, washed with distilled water (6 times) and then dried in an oven.

Parameters of the Method

Sample volume: 4 ml total

Defibrinated or anticoagulant-treated blood	2 ml	Mix
Balanced salt solution	2 ml	

Larger blood samples: Larger volumes of blood may also be processed with the same efficiency of separation. This is effected by increasing the diameter of the centrifuge tube while maintaining approximately the same height of the Ficoll-Paque (2.4 cm) and of blood sample (3.0 cm) in the centrifuge test tube (1).

Smaller blood samples: Smaller quantities of blood can be processed rapidly by a modification of the recommended procedure (2).

Preparation of Reagents

Balanced Salt Solution. At least 20 ml for each sample to be processed. The balanced salt solution may be prepared from two stock solutions, A and B.

Solution A		Con. g/l
Anhydrous D-glucose	0.1 percent	1.0
$CaCl_2 \times 2H_2O$	5.0×10^{-5} M	0.0074
$MgCl_2 \times 6H_2O$	9.8×10^{-4} M	0.1992
KCl	5.4×10^{-3} M	0.4026
TRIS	0.145 M	17.565

Dissolve in approximately 950 ml distilled water and add 10 N HCl until pH is 7.6 before adjusting the volume to 1 litre.

Solution B		Con. g/l
NaCl	0.14 M	8.19

To prepare the balanced salt solution mix 1 volume Solution A with 9 volumes solution B.

Prepare the solution fresh each week. Other standard salt solutions may be used.

Test Protocol

Procedure for isolation of lymphocytes

1. Invert the Ficoll-Paque bottle several times to ensure thorough mixing.
   A. For withdrawal of Ficoll-Paque by syringe.

   Snap-off the polypropylene cap (Fig.1).

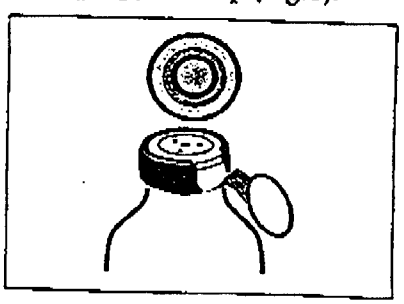

Fig. 1.

Insert the syringe needle through the septum. Inject air from the syringe to equalize pressure. Invert the bottle (Fig. 2) and withdraw the required volume of Ficoll-Paque.

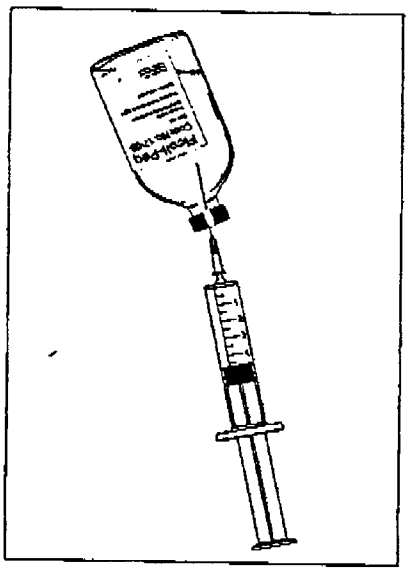

Fig. 2.

B. For withdrawal of Ficoll-Paque by pipette.

100 ml bottles – Remove the snap-off polypropylene cap. Lift the aluminium ring. Pull off the metal seal. Remove the silver ring. Remove the rubber septum. Using aseptic techniques, withdraw the required volume of Ficoll-Paque.

500 ml bottles – Remove the snap-off polypropylene cap. Lift the aluminium ring. Pull off the metal seal. Remove the silver ring. Remove the rubber septum. Using aseptic techniques, withdraw the required volume of Ficoll-Paque.

2. Add Ficoll-Paque (3 ml) to the centrifuge tube.
3. Carefully layer the diluted blood sample (4 ml) on Ficoll-Paque (Fig. 3)

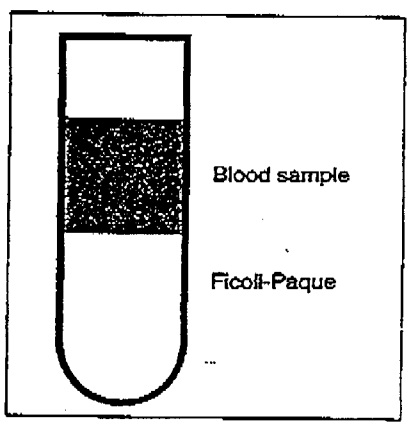

Fig. 3.

Important: When layering the sample do not mix Ficoll-Paque and the diluted blood sample.

4. Centrifuge at 400 x g for 30-40 minutes at 18–20 °C.
5. Draw off the upper layer using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface (Fig. 4, 5). Care should be taken not to disturb the lymphocyte layer. The upper layer of plasma, which is essentially free of cells, may be saved for later use.

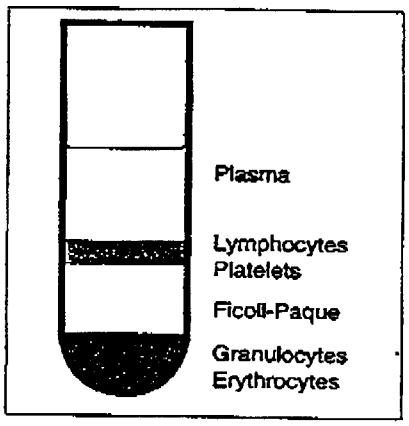

Fig. 4.

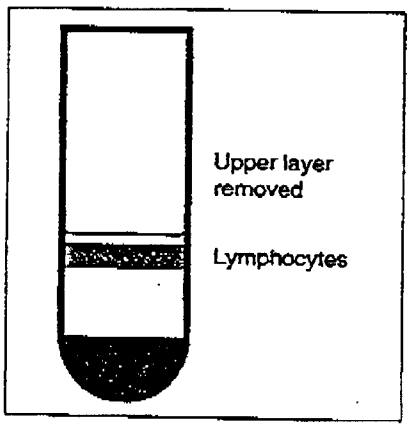

Fig. 5.

Procedure for washing the lymphocytes to remove platelets.

1. Using a clean Pasteur pipette transfer the lymphocyte layer to a clean centrifuge tube. It is critical to remove all of the interface but a minimum amount of Ficoll-Paque and supernatant. Removing excess Ficoll-Paque causes granulocyte contamination, removing excess supernatant results in unnecessary contamination by platelets and plasma proteins.
2. Add at least 3 volumes (6 ml) of balanced salt solution to the lymphocytes in the test tube.
3. Suspend the cells by gently drawing them in and out of a Pasteur pipette.

18-20 °C.

5. Remove the supernatant.
6. Suspend the lymphocytes in 6-8 ml balanced salt solution by gently drawing them in and out of the Pasteur pipette.
7. Centrifuge at 60-100 x g for 10 minutes at 18-20 °C.
8. Remove the supernatant.
9. The lymphocytes should now be suspended in the medium appropriate to the application.

Expected Value

Typical results from our laboratory.

Lymphocytes	95 ± 5% of cells present in fraction are mononucleocytes
	95 ± 5% viability[1]
	50 ± 15% recovery of lymphocytes from the original blood[2] sample
Other cells	3 ± 2% granulocytes[3]
	5 ± 2% erythrocytes
	<0.5% of total platelets in the original blood sample remain

[1] Lymphocyte viability was determined by the Trypan blue exclusion test (14).
[2] The white blood cell count on the starting blood sample was done in a hemacytometer (15). A differential count of the white blood cells was then performed to determine the amount of agranulocytes in the starting blood sample.
[3] The differential cell count was obtained from a smear of the lymphocyte fraction treated with Wright's Stain (15).

Factors Affecting Lymphocyte Isolation by the Ficoll-Paque Procedure

The blood volume and tube diameter are factors determining the height of the blood sample in the tube and, consequently, the centrifugation time. Increasing the height of the blood sample in the tube increases red cell contamination. The separation, however, is not appreciably affected by the diameter of the tube.

Hence, a larger volume can be separated in a tube of larger diameter, chosen so that the height of the blood sample in the tube and the separation time are constant.

The yield and the degree of purity of the lymphocytes depend on the efficiency of red cell removal. When erythrocytes in whole blood are aggregated, some lymphocytes are trapped in the clumps and, therefore, sediment with the erythrocytes. This tendency is reduced by diluting the blood. Dilution gives a better lymphocyte yield and reduces the size of the red cell clumps. Aggregation of erythrocytes is, however, enhanced at higher temperatures (37 °C) which decreases yield, but at low temperatures (4 °C) the rate of aggregation is decreased, increasing the time of separation. A temperature of 18 °C gives optimum results.

If problems are encountered when removing platelets from the lymphocyte fraction by the washing procedure, a second centrifugation in a 4-20% sucrose gradient layered over Ficoll-Paque will effectively remove the platelet contamination (13). The platelets will remain at the top of the sucrose gradient, and the lymphocytes will sediment through the sucrose gradient to the top of the Ficoll-Paque layer.

How Supplied

Ficoll-Paque is supplied as a sterile solution in bottles of 100 ml and 500 ml.

References

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