

Ficoll-Paque PREMIUM

Intended use

For *in vitro* preparation of human mononuclear cells from peripheral blood, bone marrow, and umbilical cord blood. Not for *in vitro* diagnostic use.

Introduction and summary of the method

Ficoll-Paque™ PREMIUM is based on Ficoll-Paque PLUS, which has a proven track record as a sterile density medium for the isolation of high yields of mononuclear cells. Ficoll-Paque PREMIUM differs from Ficoll-Paque PLUS in that it is manufactured in a strictly controlled environment compliant with ISO and in accordance with GMP (Good Manufacturing Practice) guidelines (1) and the recommendations of the United States Pharmacopeia (2) for the manufacture of cell therapy products.

Early techniques for separating mononuclear cells 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 mononuclear cells could then be collected from the upper part of the tube (3, 4). The disadvantages of these techniques are that lengthy, repeated procedures are required to obtain purified suspensions of mononuclear cells, and yield is low.



Bøyum (5) noted that the low viscosity of Ficoll™, compared to other polymeric erythrocyte aggregating agents, makes it possible to devise a mononuclear cell isolation procedure involving a short, low-speed centrifugation. 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 low speed for a short time. The erythrocytes and granulocytes sedimented to the bottom of the tube, and the purified mononuclear cells could be collected from the interface between the two phases. Sodium diatrizoate has been successfully substituted for sodium metrizoate in this procedure by numerous workers (6, 7).

The following procedure has been evaluated in our laboratories for preparation of mononuclear cells from normal blood samples on Ficoll-Paque PLUS. It has also been used with success to prepare mononuclear cells from bone marrow and umbilical cord blood (8–11).

Ficoll-Paque PREMIUM provides a GMP manufactured, sterile, ready to use Ficoll/sodium diatrizoate solution of the proper density, viscosity, and osmotic pressure for use in a simple and rapid isolation procedure for mononuclear cells. Ficoll-Paque PREMIUM also provides the additional advantage of low levels of endotoxin.

Note: Use aseptic procedures at all times as Ficoll-Paque PREMIUM does not contain antibiotics or preservatives.

Principle of the procedure

Defibrinated or anticoagulant-treated blood is layered on the Ficoll-Paque PREMIUM 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 in the Ficoll-Paque PREMIUM layer. The layer immediately above the erythrocyte layer contains mostly granulocytes, which at the osmotic pressure of the Ficoll-Paque PREMIUM solution, attains a density great enough to migrate through the Ficoll-Paque PREMIUM layer.

Because of their lower density, mononuclear cells are found at the interface between the plasma and the Ficoll-Paque PREMIUM layer, with other slowly sedimenting particles (platelets). Mononuclear cells are then recovered from

the interface and subjected to a short washing step with a balanced salt solution to remove any platelets, density gradient medium, and plasma.

Reagents

Each 100 ml of steam-sterilized Ficoll-Paque PREMIUM contains Ficoll PM400, 5.7 g, Diatrizoate Sodium, 9.0 g, with Edetate Calcium Disodium in Water For Injection (WFI).

Precautions

Upon contact with human source materials, treat all reagents and equipment as potentially biohazardous. Dispose of waste observing all local, national and international laws and regulations.

All glass has the potential for breakage, therefore, precautionary measures should be taken during handling.

Precautions should be taken to prevent injury when pulling off the metal seal.

Storage

Ficoll-Paque PREMIUM should be stored between +4°C and +30°C and protected from direct light. The medium is stable for 3 yr under the recommended storage conditions.

Indications of instability

Deterioration of the Ficoll-Paque PREMIUM 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 mononuclear cells. Prepare sample at 18–20°C as follows:

1. To a 10-ml centrifuge 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) can be used. Defibrinated blood requires no anticoagulant.*

Procedure

Materials provided

See under "Reagents".

Materials required but not provided

Sterile Balanced Salt Solution or other standard salt solutions. See under "Preparation of reagents";

Centrifuge with swing-out rotor capable of producing 60–400 × g;

Sterile tubes and pipettes;

Sterile needles and syringes.

Parameters of the method

Sample volume	4 ml total
Defibrinated or anticoagulant-treated blood	2 ml
Balanced salt solution	2 ml } Mix

Larger blood samples: Larger volumes of blood may also be processed with the same efficiency of separation. This is achieved by increasing the diameter of the centrifuge tube while maintaining approximately the same height of the Ficoll-Paque PREMIUM (2.4 cm) and of blood sample (3.0 cm) in the centrifuge tube (3).

Smaller blood samples: Smaller quantities of blood can be processed rapidly by a modification of the recommended procedure (4).

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.

Stock solution A		Conc. (g/l)
Anhydrous D-glucose	0.1%	1.0
CaCl ₂ × 2H ₂ O	5.0 × 10 ⁻⁵ M	0.0074
MgCl ₂ × 6H ₂ O	9.8 × 10 ⁻⁴ M	0.1992
KCl	5.4 × 10 ⁻³ M	0.4026
Tris	0.145 M	17.565
Conc. HCl	10 N	to pH 7.6
Distilled water*		to 1 l

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

Stock solution B		Conc. (g/l)
NaCl	0.14 M	8.19

To prepare the balanced salt solution mix 1 volume Solution A with nine volumes Solution B and sterilize. Other sterile standard salt solutions may be used.

Test protocol

Procedure for isolation of mononuclear cells

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

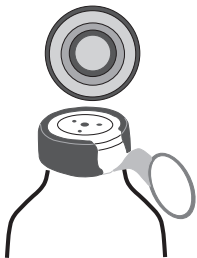


Fig 1. Opening the cap of a Ficoll-Paque PREMIUM bottle.

Snap-off the polypropylene cap (Fig 1). Insert the syringe needle through the septum (Fig 2). Inject air from the syringe to equalize pressure. Invert the bottle and withdraw the required volume of Ficoll-Paque PREMIUM.

B. For withdrawal of Ficoll-Paque PREMIUM by pipette

Remove the snap-off polypropylene cap. Lift the aluminum ring. Pull off the metal seal. Remove the silver ring. Remove the rubber septum. Using aseptic techniques, withdraw the required volume of Ficoll-Paque PREMIUM.



Fig 2. Withdrawal of Ficoll-Paque PREMIUM by syringe.

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

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

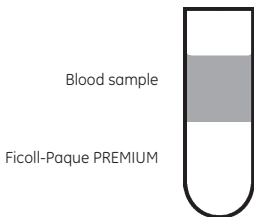


Fig 3. Layering the diluted blood sample on Ficoll-Paque PREMIUM.

4. Centrifuge at $400 \times g$ for 30–40 min at 18–20°C.
5. Draw off the upper layer containing plasma and platelets using a sterile Pasteur pipette, leaving the layer of mononuclear cells undisturbed at the interface (Figs 4 and 5); care should be taken not to disturb this layer. The upper layer of plasma, which is essentially free of cells, may be saved for later use.

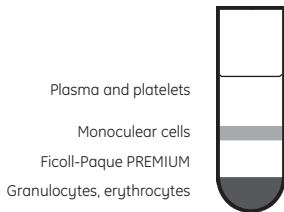


Fig 4. After centrifugation, remove the upper layer.

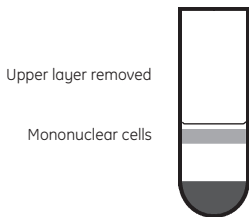


Fig 5. Mononuclear cells remain when upper layer is removed.

Procedure for washing the mononuclear cells to remove platelets

1. Using a sterile Pasteur pipette transfer the layer of mononuclear cells to a sterile centrifuge tube. It is critical to remove all of the interface but a minimal amount of Ficoll-Paque PREMIUM and supernatant. Removing excess Ficoll-Paque PREMIUM causes granulocyte contamination, removing excess supernatant results in unnecessary contamination by platelets and plasma proteins.
2. Add at least three volumes (6 ml) of balanced salt solution to the mononuclear cells in the centrifuge tube.
3. Suspend the cells by gently drawing them in and out of a Pasteur pipette.
4. Centrifuge at $60-100 \times g$ for 10 min at $18-20^{\circ}\text{C}$.
5. Remove the supernatant.
6. Suspend the mononuclear cells in 6–8 ml balanced salt solution by gently drawing them in and out of the Pasteur pipette.
7. Centrifuge at $60-100 \times g$ for 10 min at $18-20^{\circ}\text{C}$.
8. Remove the supernatant.
9. The mononuclear cells should now be suspended in the medium appropriate to the application.

Expected results

Typical results from our laboratory

Mononuclear cells	95 ± 5% of cells present in fraction are mononuclear cells, 95 ± 5% viability ¹ , 60 ± 20% recovery of mononuclear cells from the original blood ² sample.
Other cells	Max. 5% granulocytes ³ , max. 10% erythrocytes, < 0.5% of total platelets in the original blood sample remain.

¹ *Mononuclear cell viability was determined by the Trypan blue exclusion test (12).*

² *The white blood cell count on the starting blood sample was done in a hemacytometer (13). 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 (13).*

Factors affecting the isolation of mononuclear cells using Ficoll-Paque PREMIUM

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 erythrocyte contamination. The separation, however, is not appreciably affected by the diameter of the tube. As a result, 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 mononuclear cells depend on the efficiency of erythrocyte removal. When erythrocytes in whole blood are aggregated, some mononuclear cells are trapped in the clumps and, therefore, sediment with the erythrocytes. This tendency is reduced by diluting the blood. Dilution gives a better yield of mononuclear cells and reduces the size of the erythrocyte aggregates. 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 PREMIUM will effectively remove the platelet contamination (14). Platelets will remain at the top of the sucrose gradient, and mononuclear cells will sediment through the sucrose gradient to the top of the Ficoll-Paque PREMIUM layer.

Ordering information

Product	Pack size	Code No.
Ficoll-Paque PREMIUM	6 × 100 ml	17-5442-02
Ficoll-Paque PREMIUM	6 × 500 ml	17-5442-03

References

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