GE Healthcare

Isolation of mononuclear cells

Methodology and applications

Ficoll-Paque

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Introduction

Ficoll-Paque™ products are sterile, ready-to-use density gradient media for isolating mononuclear cells in high yield and purity from small or large volumes of human peripheral blood, using a simple and rapid centrifugation procedure based on the method developed by Bøyum (1). Ficoll-Paque products can also be used to prepare purified mononuclear cells from sources other than human peripheral blood.

Ficoll-Paque products from GE Healthcare are:

- Sterile (moist heat sterilization), endotoxin tested (< 0.12 EU/ml) solutions of Ficoll™ PM400 and sodium diatrizoate available in different densities.
- Recommended for small- or large-scale isolation of viable mononuclear cells in high yield from whole human peripheral blood, bone marrow, and umbilical cord blood.
- Subjected to rigorous quality control testing, which ensures reproducible performance from batch to batch.
- Supplied in bottles sealed with a rubber septum closure, which facilitates aseptic withdrawal of solution.
- Available in convenient pack sizes: 6 × 100 ml and 6 × 500 ml. Detailed instructions for use are included with each pack.
- Stable for at least 3 yr when stored at 4°C to 30°C and protected from light. When opened the bottles should be stored at 4°C to 8°C.

Separation of normal whole human peripheral blood by the procedure recommended in this booklet for Ficoll-Paque PLUS and Ficoll-Paque PREMIUM typically yields a mononuclear cell preparation with:

- 60 ± 20% recovery of the mononuclear cells present in the original blood sample
- 95 ± 5% mononuclear cells
- > 90% viability of the separated cells
- Maximum 5% granulocytes
- Maximum 10% erythrocytes

Ficoll-Paque PLUS (see page 10) is intended for research use only and has a density of 1.077 ±0.001 g/ml at 20°C.

In October 2005, GE Healthcare launched Ficoll-Paque PREMIUM. This product (see page 10) is based on Ficoll-Paque PLUS but is manufactured in a GMP compliant environment (48), certified to ISO 13485:2003 standard, and is intended for the preparation of human mononuclear cells for clinical applications. Ficoll-Paque PREMIUM, with a density of 1.077± 0.001 g/ml, can be incorporated into existing procedures where Ficoll-Paque PLUS is used, enabling the isolation of human mononuclear cells from bone marrow, peripheral blood, and umbilical cord blood.

Ficoll-Paque PREMIUM 1.084 and Ficoll-Paque PREMIUM 1.073 (see page 13), have densities of 1.084 ± 0.001 g/ml and 1.073 ± 0.001 g/ml, respectively. They are both manufactured in a GMP-compliant environment and certified to ISO 13485:2003 standard as for the Ficoll-Paque PREMIUM product. These newer density products were launched in December 2007.

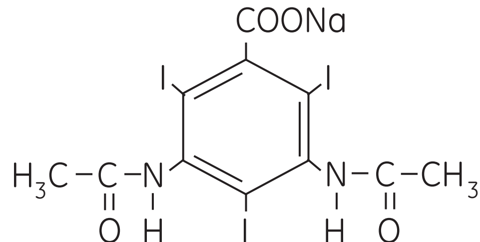
Ficoll-Paque PREMIUM 1.073 can be used when isolating lower density human mononuclear cells, for example mesenchymal stromal/stem cells or monocytes. The higher density lymphocytes and granulocytes will sediment through Ficoll-Paque PREMIUM 1.073 to the bottom of the tube, thereby enriching the lower density cells at the interface. Ficoll-Paque PREMIUM 1.073 has been found superior to Ficoll-Paque PREMIUM for isolating mesenchymal stem cells from human bone marrow (56).

Ficoll-Paque PREMIUM 1,084 can be used for preparation of cell fractions including higher density human mononuclear cells or for isolating lymphocytes that form rosettes with autologous red blood cells (15). It can also be used for separating blood cells from mice and rats, since the lymphocytes in rodents have a slightly higher average density than lymphocytes in humans (50, 51).

Ficoll-Paque density gradient media

Ficoll-Paque media products are aqueous solutions containing Ficoll PM400 and sodium diatrizoate with calcium disodium ethylenediamine-tetraacetic acid. Ficoll PM400 is a synthetic high molecular weight (M_r 400 000) polymer of sucrose and epichlorohydrin which is readily soluble in water. The molecules of Ficoll PM400 are highly branched, approximately spherical, and compactly coiled with a Stokes' radius of about 10 nm. Ficoll PM400 has a low intrinsic viscosity (17 ml/g) compared with linear polysaccharides of the same molecular weight (cf. dextran M_r 400 000:/intrinsic viscosity (η)/49 ml/g) and solutions of Ficoll PM400 have low osmotic pressures.

Sodium diatrizoate is a convenient compound to use with Ficoll PM400 since it forms solutions of low viscosity with high density. Sodium diatrizoate (M_r 635.92) is the sodium salt of 3,5-diacetamido-2,4,6-triiodobenzoic acid.



Since sodium diatrizoate is light-sensitive, all Ficoll-Paque products must be stored protected from light. The function of sodium diatrizoate in Ficoll-Paque products is to provide the optimal density and osmolarity necessary for the efficient removal of other cells from the mononuclear cells.

Ficoll-Paque products are supplied as sterile solutions in a bottle with a rubber septum closure. To maintain sterility, aseptic techniques should be used when withdrawing solution and the rubber septum closure should not be removed.

The separation principle

Mononuclear cell isolation using Ficoll-Paque separation media is based on methodology established through the extensive studies of Bøyum (1, 2, 3) and investigations carried out in our own laboratories.

Defibrinated or anticoagulant-treated blood is diluted with an equal volume of a balanced salt solution, layered carefully over Ficoll-Paque product (without intermixing) and centrifuged for 30 to 40 min. Differential migration of cells during centrifugation results in the formation of layers containing different cell types:

- The bottom layer contains erythrocytes, which have been aggregated by Ficoll PM400 and therefore sediment completely in the Ficoll-Paque density gradient media layer.
- The layer immediately above the erythrocyte layer contains mostly granulocytes, which at the osmotic pressure of the Ficoll-Paque media solution, attain a density high enough to migrate through the Ficoll Paque media layer.
- At the interface between the plasma and the Ficoll-Paque layer, mononuclear cells are found together with other slowly sedimenting particles (e.g., platelets) with low density.

Several factors contribute to the success of this separation. On centrifugation, cells in the blood sample sediment towards the blood/Ficoll-Paque media interface, where they come in contact with the Ficoll PM400 present in Ficoll-Paque products. Red blood cells are efficiently aggregated by this agent at room temperature. Aggregation increases the rate of sedimentation of the red cells, which rapidly collect as a pellet at the bottom of the tube, where they are well separated from mononuclear cells. Granulocytes also sediment to the bottom of the Ficoll-Paque media layer. This process is facilitated by an increase in their densities caused by contact with the slightly hypertonic Ficoll-Paque media. Thus, on completion of centrifugation, both granulocytes and red blood cells are found at the bottom of the tube, beneath the Ficoll-Paque product.

Lymphocytes, monocytes, and platelets are not dense enough to penetrate into the Ficoll-Paque media layers having densities of 1.077 and 1.084 g/ml. These cells therefore collect as a concentrated band at the interface between the original blood sample and the Ficoll-Paque products having densities of 1.077 and 1.084 g/ml. This banding enables the mononuclear cells to be recovered with high yield in a small volume with little mixing with the Ficoll-Paque media. When Ficoll-Paque PREMIUM 1.073 is used, some lymphocytes with densities >1.073 will enter the Ficoll-Paque media layer and the resulting cell preparation will be enriched for lower density cells like mesenchymal stromal/stem cells and monocytes. Washing and centrifuging the harvested cells subsequently removes platelets, any contaminating Ficoll-Paque media, and plasma. The resulting cell suspension then contains highly purified, viable lymphocytes, monocytes, mesenchymal stromal/stem cells, and is suitable for further studies.

A recommended standard method

Cell separation using Ficoll-Paque products can be carried out over a wide range of blood sample volumes. With its high yield, this method can be adapted to the processing of very small amounts of blood, such as may be obtained from children. For maximum reproducibility of separation it is recommended that a standardized procedure be used. The following procedure has been evaluated in our laboratories with Ficoll-Paque PLUS and is recommended for separation of normal blood samples. Simple changes can easily be made to suit a particular centrifugation system. The same procedure is recommended when separating cells using Ficoll-Paque PREMIUM, Ficoll-Paque PREMIUM 1.084, and Ficoll-Paque PREMIUM 1.073.

To standardize the technique, blood volume and diameter of the centrifuge tube should be chosen first. These factors determine 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 is, however, not appreciably affected by changing the diameter of the tube. Hence a larger volume can be separated with the same degree of purification in a tube of larger diameter if the height of the blood sample in the tube and the separation time are kept constant.

The yield and degree of purity of the mononuclear cells depend to a considerable extent on the efficiency of red cell removal.

When erythrocytes in whole blood are aggregated, some mononuclear cells are trapped in the clumps and therefore sediment with the erythrocytes. This tendency to trap mononuclear cells is reduced by diluting the blood. Dilution gives a better yield of mononuclear cells and reduces the size of the red cell clumps. Aggregation of erythrocytes is enhanced at higher temperatures (37°C), which consequently decreases the yield of mononuclear cells. At lower temperatures (4°C); however, the rate of aggregation is decreased but the time of separation is increased, which also decreases the yield of mononuclear cells. A compromise temperature of 18°C to 20°C gives optimal results.

Equipment and solutions required but not provided

1. Sterile balanced salt solution or other standard salt solutions (see Preparation of reagents).
2. Centrifuge with swing-out rotor (brake should be off).
3. Sterile centrifuge tubes and pipettes.
4. Sterile needles and syringes.
5. Red blood cell lysis solution of choice (if isolating granulocytes).

Preparation of reagents

Diluent and washing solution

The balanced salt solution for dilution of the blood and cell washing can be made according to the instruction below. Other diluents and washing fluids such as isotonic Ca^{2+}/Mg^{2+} free phosphate buffered saline (e.g. Dulbecco's PBS), salt solutions (e.g., Hank's) or cell culture media (e.g., RPMI 1640) may also be used. .

Balanced salt solution

To prepare the balanced salt solution, mix one volume stock solution A with nine volumes stock solution B and sterilize. At least 20 ml for each sample should be processed. Other sterile standard salt solutions may also be used.

Solution A		Concentration (g/l)
Anhydrous D-glucose	5.5×10^{-3} M (0.1%)	1.0
$CaCl_2 \cdot 2H_2O$	5.0×10^{-5} M	0.0074
$MgCl_2 \cdot 6H_2 0$	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 of distilled water and add concentrated HCl until the pH is 7.6 before adjusting the volume to 1 l.

Solution B		Concentration (g/l)
NaCl	0.14 M	8.19

To prepare the balanced salt solution, mix 1 volume of solution A with 9 volumes of solution B. Prepare the solution freshly each week.
Other standard salt solutions may be used.

Ficoll-Paque product

Warm the Ficoll-Paque density gradient media to 18°C to 20°C before use. For samples larger than 3 ml, see Notes on page 8.

Preparation of the sample

Fresh blood should be used to ensure high viability of isolated mononuclear cells. Prepare the sample at 18°C to 20°C.

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 the blood and buffer by inverting the tube several times or by drawing the mixture in and out of a pipette.

Procedure for isolation of mononuclear cells

1. Invert the Ficoll-Paque media bottle several times to ensure thorough mixing.

   For withdrawal of Ficoll-Paque media by syringe:

   Snap-off the polypropylene cap and insert the syringe needle through the septum (Fig 1).

   For withdrawal of Ficoll-Paque media by pipette:

   Remove the snap-off polypropylene cap. Lift the aluminum ring. Pull off the metal seal. Remove the silver ring. Remove the rubber closure. Using aseptic techniques, withdraw the required volume of Ficoll-Paque media.
2. Add Ficoll-Paque media (3 ml) to the centrifuge tube.
3. Carefully layer the diluted blood sample (4 ml) onto the Ficoll-Paque media solution (Fig 3).
   Important: When layering the sample do not mix the Ficoll-Paque media solution and the diluted blood sample.
4. Centrifuge at 400 g for 30 to 40 min at 18°C to 20°C (brake should be turned off).
5. Draw off the upper layer containing plasma and platelets using a sterile pipette, leaving the mononuclear cell layer undisturbed at the interface (Fig 4 and Fig 5). The upper layer, which contains the plasma, may be saved for later use.
6. Transfer the layer of mononuclear cells to a sterile centrifuge tube using a sterile pipette.

Washing the cell isolate

1. Estimate the volume of the transferred mononuclear cells. Add at least 3 volumes (~ 6 ml) of balanced salt solution to the mononuclear cells in the centrifuge tube.
2. Suspend the cells by gently drawing them in and out of a pipette.
3. Centrifuge at 400 to 500 × g for 10 to 15 min at 18°C to 20°C.

Note: A centrifugation at high speed increases the mononuclear cell recovery. However, if it is important to also get rid of platelets a lower centrifugation speed is recommended (60 to 100 × g).

4. Remove the supernatant.
5. Resuspend the mononuclear cells in 6 to 8 ml balanced salt solution.
6. Centrifuge at 400 to 500 × g (or 60 to 100 × g for removal of platelets) for 10 min at 18°C to 20°C.
7. Remove the supernatant.
8. Resuspend the cell pellet in media appropriate for the application.

Fig 1.

Fig 2.

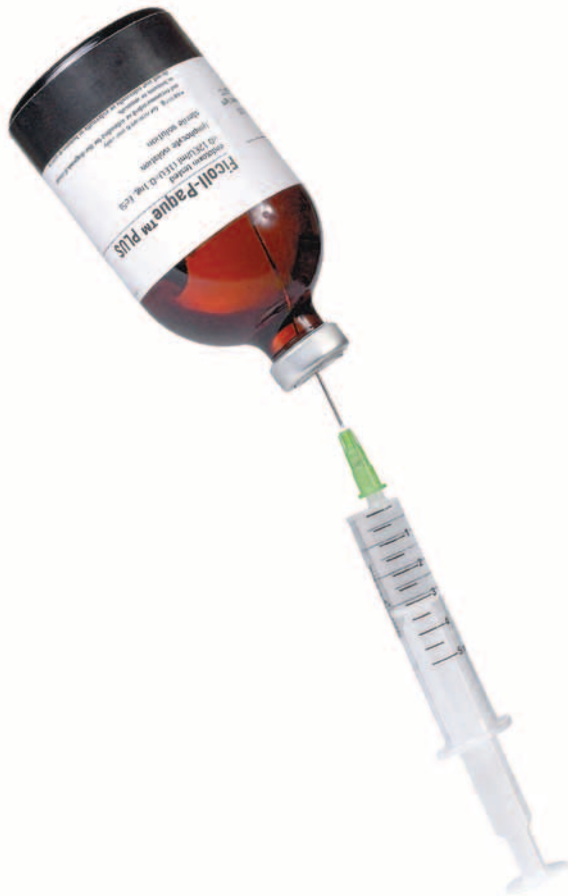


Fig 3.

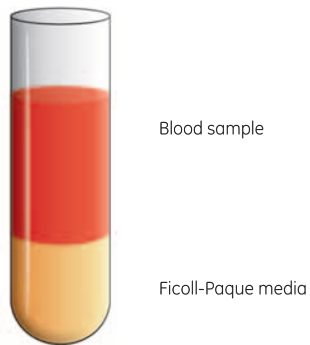


Fig 4.

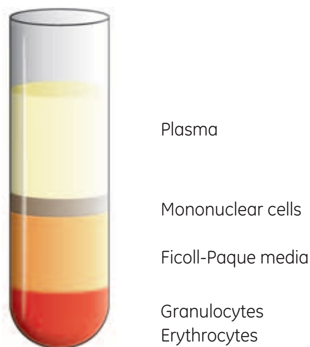
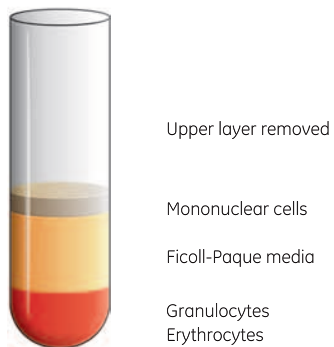


Fig 5.



Procedure for isolation of granulocytes

1. Perform density gradient centrifugation using Ficoll-Paque media as described above in Procedure for isolation of mononuclear cells, steps 1 to 6.
2. Draw off the upper layer of Ficoll-Paque media using a sterile pipette, leaving the white cell layer of granulocytes above the red blood cell layer undisturbed.
3. Collect the thin white cell layer of granulocytes with a pipette and transfer to a sterile centrifuge tube.
4. Resuspend the cells in at least five volumes of balanced salt solution and centrifuge at 400 × g for 15 min.
5. Lyse remaining red blood cells with any red blood cell lysis solution of choice.
6. Centrifuge the granulocytes at 400 to 500 × g for 10 to 15 min at 18°C to 20°C.
7. Remove the supernatant.
8. Resuspend the granulocytes in 6 to 8 ml balanced salt solution.
9. Centrifuge at 400 to 500 × g for 10 min at 18°C to 20°C.
10. Remove the supernatant.
11. Resuspend the cell pellet in media appropriate for the application.

Notes

Anticoagulants: Heparin, EDTA, citrate, acid citrate dextrose (ACD), and citrate phosphate dextrose (CPD) may be used as anticoagulants for the blood sample. Defibrinated blood requires no anticoagulant. Defibrination, however, results in a lower mononuclear cell yield and may cause increased contamination by red cells (3). It also causes selective loss of monocytes. Bøyum has found that a slightly purer mononuclear cell preparation is obtained using EDTA instead of heparin as anticoagulant (3). It has also been noted in the purification of mononuclear cells from sources other than peripheral blood that addition of heparin may cause gelling of cell suspensions (4). Citrate-stabilized blood may result in better quality RNA and DNA than other anticoagulants, and produce a higher yield of mononuclear cells. Heparin affects T-cell proliferation and binds to many proteins. EDTA is good for DNA based assays, but influences Mg^{2+} concentration and causes problems for cytogenetic analysis (5). It has also been shown that the RNA yield is higher in EDTA-blood than in heparin-blood (6).

Blood volume: Larger volumes of blood may be processed with the same efficiency of separation by using centrifuge tubes of increased diameter while maintaining approximately the same heights of Ficoll-Paque media (2.4 cm) and blood sample (3.0 cm) as in the standard method described above. Increasing the tube diameter does not affect the separation time required.

Blood sample storage: Blood samples should be free of clots and processed as soon as possible after collection to ensure optimal results. Delays in processing the blood can result in loss of viability, lower cell recoveries and more contaminating granulocytes and/or erythrocytes. Indeed, storage for 24 h at room temperature has been reported to result in reduced lymphocyte yield, altered expression of surface markers, and reduced response to mitogenic stimulation (5, 7, 8).

Dead cell removal: Dead cells are removed during cell isolation using Ficoll-Paque PLUS (9, 10, 58).

Density and temperature: The temperature at which density gradient separations are carried out are naturally affected by room temperature, centrifuge temperature, temperature of the density gradient media, and temperature of the liquid sample. There can, at times, also be confusion regarding what is meant by room temperature (e.g., in Europe it can be 20°C and in North America > 20°C). It is known that the densities of Ficoll-Paque products decrease with increase in temperature. For instance Ficoll-Paque PREMIUM (1.077 g/ml) exhibits a density of 1.0772, 1.0767, and 1.0758 at 20°C, 22°C, and 25°C, respectively.

Pathological blood samples: The standard method described above has been developed for the purification of mononuclear cells from peripheral blood of normal, healthy, human donors. Different results may be obtained with samples taken from donors with infections or other pathological conditions, such as cancer (see Further applications of Ficoll-Paque PLUS and Ficoll-Paque PREMIUM, page 12).

Platelet removal: If it is important to remove all platelets from the mononuclear cell fraction a second centrifugation in a 4% to 20% sucrose gradient layered over Ficoll-Paque PLUS can be applied. This procedure will effectively remove any platelet contamination (11). 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 PLUS layer. Alternatively, the platelets may be removed by aggregation with adenosine-5'-diphoshate (ADP) before separating the mononuclear cells (12).

Troubleshooting inadequate performance

If used according to the recommended standard procedure, Ficoll-Paque PLUS and Ficoll-Paque PREMIUM may be expected to give trouble-free isolation of human peripheral blood mononuclear cells with results as shown on page 2. As mentioned earlier, Ficoll-Paque PREMIUM 1.073 and Ficoll-Paque PREMIUM 1.084 will lead to isolation of cell preparations having slightly different density subsets of mononuclear cells. However, deviations in certain experimental parameters may lead to poor results and the troubleshooting chart given here is intended to assist in the rapid identification and correction of the problem causing reduced performance.

Deviation in performance	Likely source of problem	Comments
Increased red blood cell and contamination of the lymphocytes.	A. Temperature too low.	The densities of all Ficoll-Paque products are greater at low temperature (see *Notes*, Density and temperature) and red blood cells are aggregated less well, so granulocytes and red blood cells are prevented from entering the Ficoll-Paque media layer. Raise the temperature to 18°C to 20°C.
	B. Centrifugation speed too slow and/ or centrifugation time too short.	Adequate time and g-force must be used to ensure complete sedimentation of non-lymphoid cells.
Low yield and viability of mononuclear cells.	Temperature too high.	Ficoll-Paque products are less dense at high temperature and some lymphocytes may penetrate into the Ficoll-Paque media layer. Cell viability may also be affected. Reduce the temperature to 18°C to 20°C.
Low yield of mononuclear cells with normal viability.	Blood not diluted 1:1 with balanced salt solution; unusually high hematocrit.	The high cell density results in large numbers of lymphocytes being trapped by red blood cell aggregates. Dilute the blood sample further.
Low yield of mononuclear cells with increased granulocyte contamination.	Vibration of the centrifuge rotor, leading to stirring of the gradient.	Vibration may cause broadening of the mononuclear cell band and mixing with the underlying cells. Check that the rotor is properly balanced. Choose rotor speed to avoid natural resonant frequencies.
Low yield of mononuclear cells, low viability, and contamination by other cell types.	Sample contains cells with abnormal densities; densities different from those in normal human blood.	May be encountered with pathological blood samples, non-human blood samples, old blood samples, or samples from sources other than peripheral blood. Percoll™ PLUS, a medium for density gradient centrifugation, may be more suitable than Ficoll-Paque products for such separations.

Ficoll-Paque PLUS

Ficoll-Paque PLUS consists of a mixture of Ficoll PM400 and sodium diatrizoate at a density of 1.077 g/ml, which is optimized for the isolation of mononuclear cells from human peripheral blood. It has also been used in many other applications as described below. For over 30 years it has been trusted to deliver consistent performance and reproducible results in research laboratories. Ficoll-Paque PLUS as well as the Ficoll-Paque PREMIUM products are sterile. For the purpose of ensuring sterility, all Ficoll-Paque products are subject to terminal moist heat sterilization.

Ficoll-Paque PREMIUM products

Ficoll-Paque PREMIUM (density 1.077 g/ml) is a density gradient media used as a reagent in cell therapy applications for the preparation of human mononuclear cells. Based on Ficoll-Paque PLUS, it was developed for both large- and small-scale purification of mononuclear cells from human peripheral blood using a simple and rapid centrifugation technique developed by Bøyum (1). Ficoll-Paque PREMIUM 1.084 and Ficoll-Paque PREMIUM 1.073, have densities of 1.084 ± 0.001 g/ml and 1.073 ± 0.001 g/ml, respectively. These may be used when higher or lower densities than the standard 1.077 g/ml are required.

The methodology for using Ficoll-Paque PREMIUM 1.084 and Ficoll-Paque PREMIUM 1.073 is the same as for all Ficoll-Paque products described earlier. Ficoll-Paque PREMIUM 1.084 can be used for preparation of cell fractions including higher-density human mononuclear cells, and for separating blood cells from mice or rats. Ficoll-Paque PREMIUM 1.073 can be used when preparing lower density mononuclear cells, for example mesenchymal stromal cells or monocytes. The higher density lymphocytes and granulocytes will sediment through Ficoll-Paque PREMIUM 1.073 to the bottom of the tube, thereby enriching the lower density cells at the interface.

Ficoll-Paque PREMIUM products differ from Ficoll-Paque PLUS in that they are manufactured in a strictly controlled environment compliant with ISO 13485:2003 and GMP (Good Manufacturing Practice) (48), and in accordance with the recommendations of the United States Pharmacopeia (49) on ancillary materials for the manufacture of cell therapy products. ISO 13485 and GMP compliance requires stringency in validation and documentation of manufacturing procedures.

Ficoll-Paque PREMIUM products offer the following benefits:

- Manufactured according to GMP and ISO standards
- Manufactured in accordance to USP <1043> recommendations for ancillary materials
- Sterile (moist heat sterilized), ready-to-use reagent
- Low levels of endotoxin (< 0.12 EU/ml) secured and tested
- Osmolality tested
- RSF (Regulatory Support File) available as support for clinical applications to regulatory agencies
- No cytotoxicity according to USP Method <87> and ISO 10993-5 guidelines

Properties of mononuclear cells isolated by the Ficoll-Paque method

Since its introduction in 1968, the mononuclear cell separation method described by Bøyum (1, 2) has been used in numerous immunological investigations. This widespread adoption indicates the superior results obtained with this technique and its freedom from impairment of lymphocyte function. Nevertheless, certain effects of the separation procedure have been seen and these are noted below, since research situations may arise in which they are of significance.

Separation with Ficoll-Paque PLUS has been reported to lead to adsorption of cytophilic IgG to the mononuclear leukocytes (13), resulting in erroneously high estimates of the number of Ig-bearing lymphocytes and too low estimates of the number of cells bearing Fc receptors. This interference can be avoided by washing the blood cells with balanced salt solution before isolation, thus removing the IgG present in the plasma that gives rise to these artifacts.

Selective loss of a population of lymphocytes that form rosettes with autologous red blood cells has been reported to occur using the standard procedure (14, 15) and evidence was found that this is the result of a specific lymphocyte-red blood cell interaction, not a non-specific trapping (15). This population was found to account for about 6% of the lymphocytes initially present in the blood sample and could be recovered almost quantitatively by resuspending the red cell pellet in medium and recentrifuging over a gradient of slightly higher density than normal (i.e. 1.083 g/ml) (15).

Slight differences in phenotype compositions have been seen between fresh samples of whole blood and samples purified using Ficoll-Paque PLUS (16, 17). Fresh samples of whole blood show lower percentages of CD4+, CD19+, and CD4+CD45RA+ cells, and higher percentages of CD8+ and CD4+CD29+ subsets than fresh lymphocyte purified on a Ficoll-Paque PLUS gradient (15).

Ficoll-Paque density gradient separation (FDS) was associated with significantly higher percentages of CD3+/CD62L+ and CD3+/CD11b+ lymphocytes in young children and adults alike, while the percentages of CD3+/CD54+ cells from adults was not affected by FDS. The percent expression of CD54, CD62L, and CD11b on T cells from both children and adults were significantly higher following FDS, with a greater increase in CD11b expression on T cells from young children, reaching adult levels (16).

Lymphocytes separated by the Bøyum procedure have been reported (18) to show enhanced stimulation in mixed lymphocyte cultures as compared with lymphocytes in "leukocyte-rich plasma" (not exposed to Ficoll-Paque PLUS). This enhanced reaction was postulated to depend at least partially on the removal in the Ficoll-Paque PLUS method of neutrophils that appear otherwise to have a suppressive effect on the mixed lymphocyte reaction (18).

Further applications of Ficoll-Paque PLUS and Ficoll-Paque PREMIUM

A great many modifications and extensions of the method have come into use following the introduction of the technique described by Bøyum in 1968 and its subsequent widespread adoption. For example, monocytes (which are recovered in the mononuclear cell fraction, using the standard procedure described in this booklet) can be removed, if desired, by incubating the blood sample with iron (or iron carbonyl) before separation on Ficoll-Paque PLUS. The monocytes phagocytose the iron particles and become denser, with the result that they sediment through the Ficoll-Paque PLUS layer on centrifugation and collect in the red blood cell pellet at the bottom of the tube (3).

An important and widely used extension of the original technique is its application, in combination with selective rosetting (clustering), to the isolation of lymphocyte subclasses. Selective rosetting is presented in RosetteSep™ products marketed by StemCell Technologies Inc (19, 20, 21, 59). These products contain tetrameric antibody complexes (TAC) that crosslink unwanted cells to multiple red blood cells present in the sample, forming immunorosettes. Upon centrifugation over Ficoll-Paque product, the immunorosettes sediment to the bottom of the tube together with the red blood cells. Alternatively, the purified lymphocytes obtained by the standard procedure (with or without monocyte removal) can be incubated with an excess of sheep red blood cells (ratio of red blood cells to lymphocytes at least 50:1), whereupon the T lymphocytes spontaneously form rosettes with the sheep red blood cells. On centrifugation for a second time over Ficoll-Paque PLUS, the T lymphocyte rosettes sediment to the bottom of the tube together with the excess red blood cells, leaving the other (non-rosetting) lymphocytes at the interface (3).

Such techniques for the separation of lymphocyte subclasses, as well as the standard method for isolating the entire lymphocyte population, have been widely applied to studies of lymphocyte functions and surface markers in disease states as compared to normal controls. Caution is, however, necessary in applying the Ficoll-Paque PLUS technique to pathological blood specimens, since it has been found that the resulting mononuclear cell layer may be contaminated with immature granulocytes in patients with certain infections (22), and particularly cancer (9, 23). In the latter case, elevated numbers of monocytes may also be present (24).

Ficoll-Paque PLUS has been used with success to separate cells from a variety of sources other than peripheral blood, even though its properties have been optimized specifically for blood mononuclear cell isolation. Thus, separation over Ficoll-Paque PLUS facilitated detection and identification of malignant cells in abdominal and pleural fluids (23,25). Separation on Ficoll-Paque PLUS has also been reported to assist in establishing cultures of amniotic fluid cells and to facilitate their subsequent cytogenic analysis (26). Ficoll-Paque PLUS and Ficoll-Paque PREMIUM have also been used for the isolation of cord blood derived hematopoietic stem cells, cord blood derived pluripotent stem cells for regenerative medicine clinical applications, and bone marrow mesenchymal stem cells (27, 28, 60, 61). Ficoll-Paque products are also suitable for granulocyte isolation (29, 30, 31).

Ficoll-Paque PLUS can also be used to isolate mononuclear cells from species other than man. In some cases (e.g. cow, goat, and rabbit) it may be necessary to alter the standard procedure to achieve good results (3) and it should be remembered that the density of Ficoll-Paque PLUS (1.077 g/ml), although optimized for the isolation of human mononuclear cells, may not give optimal yield and purity of mononuclear cells from other species. However, isolation methods using Ficoll-Paque PLUS for preparation of mononuclear cells from peripheral blood (PB) and bone marrow (BM) have been described for mouse ((32(PB), 33 (BM)), dog (34 (PB), 35(BM)), monkey (36 [PB], 37 [BM]), cow (38 [PB], 39 [PB]), rabbit (36 [BM], 40 [PB]), horse (41 [PB]), pig (42 [PB]), and even fish (43). Where it is desired to work with solutions of densities other than 1.077 g/ml it may be convenient to use Ficoll-Paque PREMIUM 1.084, Ficoll-Paque PREMIUM 1.073, or the alternative centrifugation media Percoll/Percoll PLUS since iso-osmotic solutions of different densities are very easily prepared with these media, facilitating the optimization of a particular separation. In research studies, separation with Percoll has also been reported to give improved lymphocyte yields and purities in some cases (44 to 47).

Applications using Ficoll-Paque PREMIUM 1.084

Ficoll-Paque PREMIUM 1.084 can be used for isolating higher density cells. It has for instance been shown that the lymphocytes in rodents have a slightly higher average density than lymphocytes in humans (50, 51) and a fraction of these will therefore be lost into the pellet after a standard 1.077 g/ml density gradient centrifugation, contaminating the granulocyte layer and decreasing the mononuclear cell recovery. Dog, bovine, rabbit, mouse, and rat blood cells have successfully been separated using a 1.084 g/ml density gradient media such as Percoll or Ficoll-diatrizoate solutions (52,53).

It has been reported that selective loss of lymphocytes that form rosettes with autologous blood cells (as described above) may occur by the standard procedure using a 1.077 g/ml Ficoll-Paque products (14, 15) and these lymphocytes have been shown to be recovered almost quantitatively by resuspending the red cell pellet in medium and recentrifuging over a gradient of slightly higher density than normal (i.e., 1.083 g/ml) (15). Thus, Ficoll-Paque PREMIUM 1.084 could potentially be used for this application, although this remains to be shown.

Applications using Ficoll-Paque PREMIUM 1.073

Ficoll-Paque PREMIUM 1.073 can be used for enriching lower density cells such as monocytes from peripheral blood, or mesenchymal stem cells from bone marrow or placenta (54, 55, 56, 57).

Grisendi et al. directly compared Ficoll-Paque PREMIUM and Ficoll-Paque PREMIUM 1.073 for isolating and expanding mesenchymal stem cells from human bone marrow aspirate and convincingly demonstrated that density gradient separation using the lower density Ficoll-Paque PREMIUM 1.073 was associated with an enrichment of mesenchymal stem cell (MSC) subtypes characterized by a higher proliferation potential. Thus, Ficoll-Paque PREMIUM 1.073 can ultimately benefit clinical applications based on these cells. Brooke et al. recently described a successful manufacturing process for isolating and expanding placenta-derived human MSC using Ficoll-Paque PREMIUM 1.073 for a clinical trial (57).

Availability and storage

Ficoll-Paque PLUS and Ficoll-Paque PREMIUM are available in packs of 6 × 100 ml (code numbers 17-1440-02 and 17-5442-02 respectively) and 6 × 500 ml (code numbers 17-1440-03 and 17-5442-03 respectively) as sterile (moist heat sterilized), ready-to- use liquids in glass bottles with rubber septum closures. The Ficoll-Paque PREMIUM products of different densities, Ficoll-Paque PREMIUM 1.084 and Ficoll-Paque PREMIUM 1.073, are available in packs of 6 × 100 ml (code numbers 17-5446-02 and 17-5446-52 respectively). The different densities of each Ficoll-Paque product is clearly reflected in the colors of the polypropylene caps where density 1.077 g/ml is shown in blue, density 1.084 g/ml in red, and density 1.073 g/ml in gray.

Full instructions for use are included with each pack. Ficoll-Paque products should be stored between 4°C and 30°C protected from light, under which conditions they will maintain sterility and stability for 3 yr. When opened, the bottles should be stored in the cold between 4°C and 8°C. Freezing is not recommended, but if frozen accidentally, the bottles should be inverted several times after thawing to ensure a homogeneous solution.

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