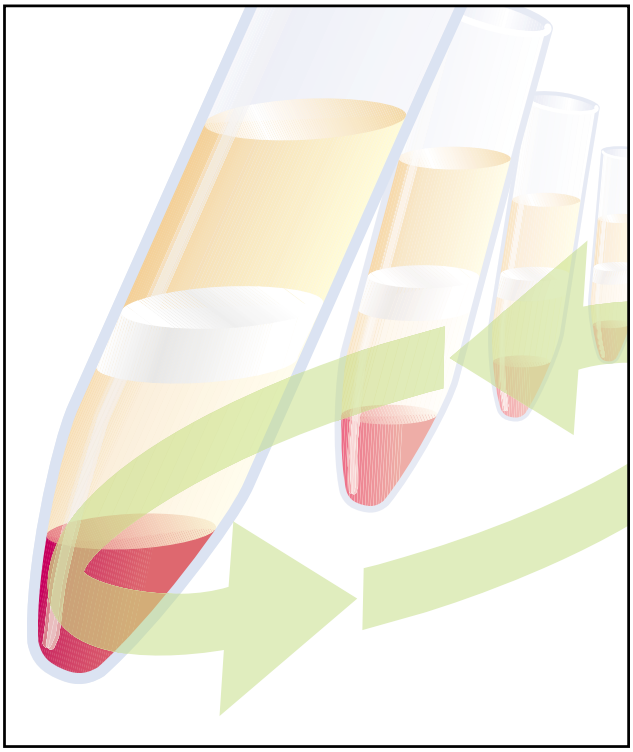
Ficoll-Paque PLUS

For *in vitro* isolation of lymphocytes



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Introduction

Isolation of lymphocytes from whole human blood is often required in such clinical investigations as histocompatibility testing and the assay of cell-mediated immune responses, as well as in many areas of immunological research.

Ficoll-Paque™ PLUS is a sterile, ready to use density gradient medium for purifying lymphocytes 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 PLUS can also be used to prepare purified lymphocytes from sources other than human peripheral blood.

Ficoll-Paque PLUS from Amersham Biosciences is:

- A sterile endotoxin tested (<0.12 EU/ml) solution of Ficoll™ 400 and sodium diatrizoate with a density of 1.077 ± 0.001 g/ml.
- Recommended for small- or large-scale isolation of viable lymphocytes in high yield from whole human peripheral blood.
- Subjected to rigorous quality control function testing, which guarantees 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 x 100 ml for research requirements and 6 x 500 ml for daily routine lymphocyte isolation. Detailed instructions for use are included with each pack.
- Stable for at least 3 yr when stored at 4–25 °C and protected from light.

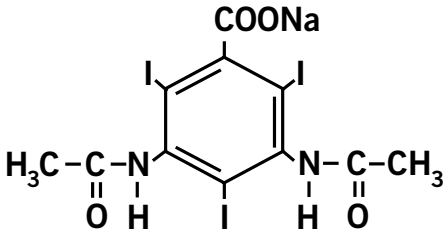
Separation of normal whole human peripheral blood by the procedure recommended in this booklet typically yields a lymphocyte preparation with:

- 60 ± 20% recovery of the lymphocytes present in the original blood sample,
- 95 ± 5% monocular cells,
- >90% viability of the separated cells,
- 3 ± 2% granulocytes,
- 5 ± 2% erythrocytes and
- <0.5% of the total platelet content of the original blood sample.

Ficoll-Paque PLUS

Ficoll-Paque PLUS is an aqueous solution of density 1.077 ± 0.001 g/ml containing 5.7 g Ficoll 400 and 9 g sodium diatrizoate with 0.0231 g calcium disodium ethylenediamintetraacetic acid in every 100 ml. Ficoll 400 is a synthetic high molecular weight (M_w 400 000) polymer of sucrose and epichlorohydrin which is readily soluble in water. The molecules of Ficoll 400 are highly branched, approximately spherical and compactly coiled with a Stokes' radius of a about 10 nm. Ficoll 400 has a low intrinsic viscosity (17 ml/g) compared with linear polysaccharides of the same molecular weight (cf. dextran M_w 400 000: /η/ 49 ml/g) and solutions of Ficoll 400 have low osmotic pressures.

Sodium diatrizoate is a convenient compound to use with Ficoll 400 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, Ficoll-Paque PLUS must be stored protected from light. The function of sodium diatrizoate in Ficoll-Paque PLUS is to provide the optimal density and osmolarity necessary for the efficient removal of other cells from the lymphocytes.

Ficoll-Paque PLUS is supplied as a sterile solution in a bottle with a rubber septum closure. To maintain sterility, aseptic techniques should be used when withdrawing solution and the rubber septum should not be removed. Ficoll-Paque PLUS should be stored between 4 °C and 25 °C and protected from direct light. Storage of unopened bottles in the dark will increase their shelf-life. Deterioration of Ficoll-Paque PLUS is indicated by the appearance of a distinct yellow colour or particulate material in the clear solution. Ficoll-Paque PLUS showing such deterioration should be discarded.

The separation principle

Lymphocyte isolation using Ficoll-Paque PLUS is based on methodology established through the extensive studies of Bøyum (1,2,3) and investigations carried out in our own laboratories. Separation media consisting of a mixture of Ficoll 400 and an iodinated density gradient medium such as sodium diatrizoate have been very widely used for purifying human lymphocytes following the publication of Bøyum's pioneering work in 1968.

For lymphocyte separation, defibrinated or anticoagulant-treated blood is diluted with an equal volume of balanced salt solution and layered carefully over Ficoll-Paque PLUS (without intermixing) in a centrifuge tube. After a short centrifugation at room temperature (typically at 400 g_{av} for 30–40 min) lymphocytes, together with monocytes and platelets, are harvested from the interface between the Ficoll-Paque PLUS and sample layers. This material is then centrifuged twice in balanced salt solution to wash the lymphocytes and to remove the platelets.

Several factors contribute to the success of this separation. On centrifugation, cells in the blood sample sediment towards the blood/Ficoll-Paque PLUS interface, where they come in contact with the Ficoll 400 present in Ficoll-Paque PLUS. 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 lymphocytes. Granulocytes also sediment to the bottom of the Ficoll-Paque PLUS layer. This process is facilitated by an increase in their densities caused by contact with the slightly hypertonic Ficoll-Paque PLUS medium. Thus, on completion of centrifugation, both granulocytes and red blood cells are found at the bottom of the tube, beneath the Ficoll-Paque PLUS.

Lymphocytes, monocytes, and platelets are not dense enough to penetrate into the Ficoll-Paque PLUS layer. These cells therefore collect as a concentrated band at the interface between the original blood sample and the Ficoll-Paque PLUS. This banding enables the lymphocytes to be recovered with high yield in a small volume with little mixing with the Ficoll-Paque PLUS medium. Washing and centrifugation the harvested cells subsequently removes platelets, any contaminating Ficoll-Paque PLUS and plasma. The resulting cell suspension then contains highly purified, viable lymphocytes and monocytes and is suitable for further studies.

A recommended standard method

Lymphocyte purification using Ficoll-Paque PLUS 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. Because of its rapidity and simplicity it is also the method of choice for emergency tissue typing procedures (4). For maximum reproducibility of separation it is recommended that a standardized procedure be used. The following procedure has been evaluated in our laboratories and is recommended for separation of normal blood samples on Ficoll-Paque PLUS. Simple changes can easily be made to suit a particular centrifugation system.

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 lymphocytes depend to a considerable extent 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 to trap lymphocytes 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 enhanced at higher temperatures (37 °C), which consequently decreases the yield of lymphocytes. 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 lymphocytes. A compromise temperature of 18–20 °C gives optimal results.

Equipment and solutions required

1. Two 10 ml glass test-tubes for each blood sample to be processed. The test-tubes should be siliconized (see "Notes", page 10).
2. 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

		Conc. g/l
Anhydrous D-glucose	5.5×10^{-3} M (0.1%)	1.0
$CaCl_2 \cdot 2H_2O$	5.0×10^{-3} M	0.0074
$MgCl_2 \cdot 6H_2O$	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 conc. HCl until pH is 7.6 before adjusting the volume to 1 l.

Solution B

		Conc. 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.

3. Pasteur pipettes (3 ml). One for each sample to be processed. These pipettes should be siliconized (see "Notes", page 10).
4. A low speed centrifuge.
5. Glass centrifuge tubes. Two centrifuge tubes for each blood sample to be processed. Internal diameter approximately 1.3 cm, volume 15 ml. The centrifuge tubes should be siliconizied (see "Notes", page 10). For larger or smaller samples see "Notes", page 10.
6. Ficoll-Paque PLUS. 3 ml for each sample being processed. For larger or smaller samples see "Notes", p 10.
7. Syringe with needle. Needed for withdrawing Ficoll-Paque PLUS from the bottle under aseptic conditions.

Preparation of the sample

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

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 the buffer in and out of a Pasteur pipette.

Procedure for isolation of lymphocytes

1. Remove the blue cap on the bottle of Ficoll Paque PLUS (Fig. 1).
2. Invert the bottle of Ficoll-Paque PLUS several times to ensure mixing. Using the syringe with needle attached, pierce the septum and withdraw the required volume of Ficoll-Paque PLUS (3 ml for each centrifuge tube) from the inverted bottle (Fig 2). If this method is employed, each bottle

will deliver at least 100 ml Ficoll-Paque PLUS.

3. Add Ficoll-Paque PLUS (3 ml) to the centrifuge tube.
4. Carefully layer the diluted blood sample (4 ml) onto the Ficoll-Paque PLUS (Fig 3).
   Important. When layering the sample do not mix the Ficoll-Paque PLUS and the diluted blood sample.
5. Centrifuge at 400g_{av} for 30–40 min at 18–20 °C.
6. Draw off the upper layer using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface (Fig 4 and Fig 5). Care should be taken not to disturb the lymphocyte layer.
   The upper layer, which contains the plasma, may be saved for later use.

Fig 1.

Fig 2.

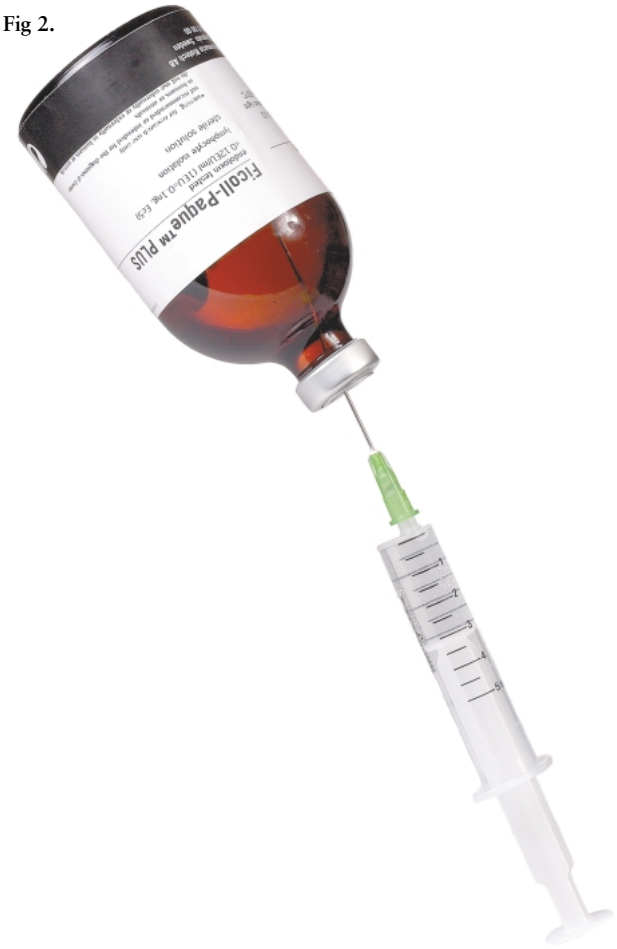


Fig 3.

Fig 4.

Fig 5.

Washing lymphocytes free of platelets

1. Using a clean Pasteur pipette transfer the lymphocyte layer to a clean centrifuge tube. It is critical to remove all the material at the interface but in a minimum volume. Removing excess Ficoll-Paque PLUS causes granulocyte contamination; removing excess supernatant results in platelet contamination.
2. Add at least 3 volumes (6 ml) of balanced salt solution to the lymphocytes in the test-tube.
3. Suspended the cells by gently drawing them in and out of a Pasteur pipette.
4. Centrifuge at 60–100 g_{av} for 10 min at 18–20 °C.
5. Discard the supernatant.
6. Suspend the lymphocytes in 6–8 ml balanced salt solution by gently drawing them in and out of a Pasteur pipette.
7. Centrifuge at 60–100 g_{av} for 10 min at 18–20 °C.
8. Discard the supernatant. The lymphocytes should now be suspended in the medium appropriate to the application.

Typical results from our laboratories

Lymphocytes:	60 ± 20%	recovery 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)
Other cells:	3 ± 2%	granulocytes
	5 ± 2%	erythrocytes
	<0.5%	of the total platelet content of the original blood sample

Notes

Preparation of glassware. All glassware that comes in contact with the sample should be siliconized before use. Glassware should be immersed in a 1% silicone solution for 10 seconds (where no specific coating procedure is recommended by the manufacturer), washed thoroughly with distilled water and dried in an oven. The best siliconizing fluids are those based on dimethyldichlorosilane dissolved in an organic solvent.

Examples of suitable fluids are:

Sigmacote	Sigma Chemicals Co., Cat. No. SL-2.
Repelcote	Hopkins and Williams, Cat. No. 9962-70
Dimethyldichlorosilane	BDH, Cat. No. 33164
Prosil-28	PCR Research Chemicals.
Silicone Oil	Midland Silicones Ltd., Cat. No. MS 1107, use as 2—5% (v/v) solution in ethyl acetate.
Siliclad	Clay-Adams, Cat. No. 1950.

Alternatively, tissue culture plasticware may be used.

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 lymphocyte yield and may cause increased contamination by red cells (3). It also causes selective loss of monocytes. Bøyum has found that a slightly purer

lymphocyte preparation is obtained using EDTA instead of heparin as anticoagulant (3). It has also been noted in the purification of lymphocytes from sources other than peripheral blood that addition of heparin may cause gelling of cell suspensions (5).

Larger blood samples. 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 PLUS (2.4 cm) and blood sample (3.0 cm) as in standard method described above. Increasing the tube diameter does not affect the separation time required.

Smaller blood samples. Smaller volumes of blood can be processed rapidly by a modification of the method of Bøyum (6). A micromethod suitable for use in tissue typing of blood cells from cadavers has also been described (4).

Sample storage. Blood samples should be processed as soon as possible after collection to ensure optimal results. 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 (7).

Pathological blood samples. The standard method described above has been developed for the purification of lymphocytes from peripheral blood of normal, healthy, human donors. Different results may be obtained with samples taken from donors with infections or other pathological conditions, e.g. cancer (see “Further Applications”, page 13).

Platelet removal. The washing procedure described in this standard method will give efficient removal of platelets from the lymphocytes in the majority of cases. If difficulty is experienced, centrifugation through a 4–20% sucrose gradient layered over Ficoll-Paque PLUS may be used to remove platelets (8). Alternatively, the platelets may be removed by aggregation with adenosine-5-diphoshate (ADP) before separating the lymphocytes (9).

Troubleshooting inadequate performance

If used according to the recommended standard procedure, Ficoll-Paque PLUS may be expected to give trouble-free isolation of human peripheral blood lymphocytes with results as shown under "Typical results from our laboratories", page 10. 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 density of Ficoll-Paque PLUS is greater at low temperature and red blood cells are aggregated less well, so granulocytes and red blood cells are prevented from entering the Ficoll-Paque PLUS layer. Raise the temperature to 18-20 °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 lymphocytes.	Temperature too high.	Ficoll-Paque PLUS is less dense at high temperature and some lymphocytes may penetrate into the Ficoll-Paque PLUS layer. Cell viability may also be affected. Reduce the temperature to 18-20 °C.
Low yield of lymphocytes with normal viability.	Blood not diluted 1:1 with balanced salt solution; unusually high hematocrit.	The high cell density results in a large numbers of lymphocytes being trapped by red blood cell aggregates. Dilute the blood sample further.
Low yield of lymphocytes with increased granulocyte contamination.	Vibration of the centrifuge rotor, leading to stirring of the gradient.	Vibration may cause broadening of the lymphocyte 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 lymphocytes, 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, or samples from sources other than peripheral blood. Percoll™, a medium for density gradient centrifugation, may be more suitable than Ficoll-Paque PLUS for such separations.

Properties of lymphocytes isolated by the Ficoll-Paque PLUS method

Since its introduction in 1968, the lymphocyte separation method of Bøyum (1,2) has been used in numerous immunological investigations as well as in routine diagnostic studies. 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 (10), resulting in erroneously high estimates of the number of Ig-bearing lymphocytes and too low estimates of the number of cells bearing F_c 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 (11,12) and evidence was found that this is the result of a specific lymphocyte-red blood cell interaction, not a non-specific trapping (12).
This population was found to account for ca. 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 (12).

Lymphocytes separated by the Bøyum procedure have been reported (13) 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 (13). Ficoll-Paque PLUS-separated lymphocytes have also been reported to show increased levels of "spontaneous" blastogenesis, as measured by ^{3}H-thymidine incorporation in cultures not stimulated by mitogens, but the cause of this enhancement was not established (14). Diminished response of Ficoll-Paque PLUS-separated lymphocytes to mitogenic stimulation by phytohaemagglutinin (PHA) as compared to lymphocytes prepared by centrifugal elutriation has been reported in one instance (15).

Further applications of Ficoll-Paque PLUS

A great many modifications and extensions of the method have come into use follwing the introduction of the technique by Bøyum in 1968 and its subsequent widespread adoption. For example, monocytes (which are recovered in the lymphocyte fraction, using the standard procedure decribed 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. In the most often used case, the purified lymphocytes obtained by the standard procedure (with or without monocyte removal) are 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" (clusters) 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. For such comparative studies it is important that the lymphocyte purification method should not lead to preferential enrichment or loss of any particular lymphocyte subclass. Evidence that the standard Ficoll-Paque PLUS procedure is free from this kind of distortion has been presented by Häyry *et al.* (16), although the existence of the minor subset of autologous rosette-forming lymphocytes described by Hokland and Heron (11) was not recognized in the earlier work. Caution is, however, necessary in applying the Ficoll-Paque PLUS technique to pathological blood specimens, since it has been found that the resulting lymphocyte layer may be contaminated with immature granulocytes in patients with certain infections (17), and particularly cancer (18,19). In the latter case, elevated numbers of monocytes may also be present (20). However, in a study of immunocompromised patients with aplastic anemia or acute leukemia, lymphocyte isolation proceeded normally using Ficoll-Paque PLUS and resulted, in combination with purification of granulocytes from the red blood cell pellet by dextran sedimentation, in substantially increased rates of virus recovery from the blood samples (21).

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 lymphocyte isolation. Thus, separation over Ficoll-Paque PLUS facilitated detection and identification of malignant cells in abdominal and pleural fluids (22) and similar conclusions have been drawn using Ficoll-Paque PLUS mixtures of densities other than 1.077 g/ml (23,24). 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 (25).

Ficoll-Paque PLUS can also be used to isolate lymphocytes 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 lymphocytes, may not give optimal yield and purity of lymphocytes from other species. However, isolation methods using Ficoll-Paque PLUS of standard density have been described for mouse (26), dog (27), monkey (28), cow (29,30), rabbit (31), horse (32), pig (32,33), and even fish (34) lymphocytes. Where it is desired to work with solutions of densities other than 1.077 g/ml it may be convenient to use the alternative centrifugation medium Percoll™ (a descriptive handbook is available free on request), since iso-osmotic solutions of different densities are very easily prepared with this medium, facilitating the optimization of a particular separation. Separation with Percoll has also been reported to give improved lymphocyte yields and purities in some cases (35–37).

Availability and storage

Ficoll-Paque PLUS is available in packs of 6 x 100 ml (Code No. 17-1440-02) and 6 x 500 ml (Code No. 17-1440-03) as a sterile, ready to use liquid in glass bottles with rubber septum caps. Full instructions for use are included with each pack. Ficoll-Paque PLUS should be stored between 4 °C and 25 °C protected from light, under which conditions it will maintain sterility and stability for 3 yr. Storage in the cold will prolong shelf-life. Freezing of this product is not recommended, but if frozen accidentally, the bottle should be inverted several times after thawing to ensure a homogeneous solution.

Precautionary note

This material is intended for *in vitro* diagnostic use for the isolation of lymphocytes and other research applications.

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