Excellence in Separations

DENSITY GRADIENT MEDIA

Axis-Shield has the solutions

AXIS-SHIELD
Innovation for Life

Contents

10. edition
October 2013

Preface

Much of modern cell and molecular biology relies on the isolation of pure, functionally intact populations of cells, subcellular organelles and macromolecules in high yield and both research and clinical applications provide an impetus for the development of improved separation technology.

The most commonly used method of separating suspensions of mixed populations of biological particles is centrifugation in density gradients - and the choice of medium used to form the gradients is critical for the retention of particle function. Since the early sixties Nycomed have been developing and producing iodinated compounds as X-ray imaging agents. It was recognized early on that these compounds were also well suited to the formation of density gradients and offered new opportunities for improved methods of separating biological particles, especially osmotically-sensitive cells and subcellular organelles. Nycomed has therefore been actively involved over the last 30 years in the research and development of density gradient media.

A new chapter opened in Nycomed's history, when in February 2000, Axis-Shield took over responsibility for these products. Today, Axis-Shield offers a range of iodinated density gradient media, produced in compliance with FDA and EU cGMP standards and regulatory requirements, which can be used for the purification not only of cells and organelles but also membrane vesicles, viruses, proteins, nucleic acids and lipoproteins. The use of these compounds as contrast media demanded that they be rigorously tested to ensure their safety and suitability for clinical use. Axis-Shield density gradient media are therefore proven to be non-toxic; have a low osmolality and are sufficiently dense to permit the purification of all types of biological particle.

- **No other density gradient media conform to such strict clinical criteria.**

For further information regarding our Density Gradient Media please go to: www.axis-shield-density-gradient-media.com

Iodinated Density Gradient Media

Molecular structure

Iodinated media are derivatives of triiodobenzoic acid and capable of forming solutions that are uniquely capable of banding any biological particle, according to its buoyant density, often under isoosmotic conditions. In 1968 Bøyum (1) published his famous density barrier method for the isolation of mononuclear cells from human blood that used sodium metrizoate (Figure1) and Ficoll®. This technique received universal approval and Nycomed was the first company to introduce a ready-made medium in 1973 based on his original formula. The modern version is called Lymphoprep™ and more recently metrizoate has been replaced by diatrizoate (Figure 2).

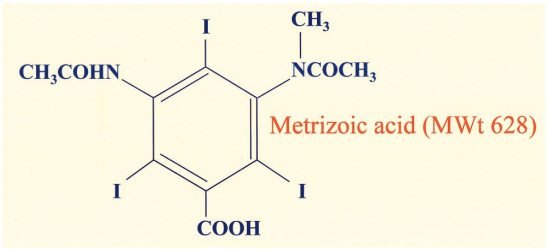

Figure 1 Molecular structure of metrizoic acid

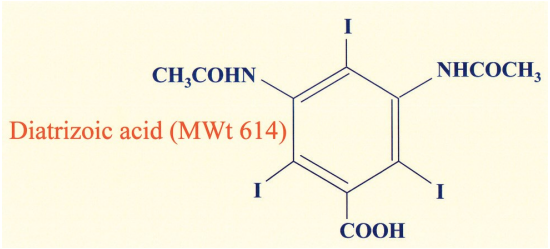

Figure 2 Molecular structure of diatrizoic acid

Both metrizoate and diatrizoate are ionic compounds which are able to interact with other charged groups on biological particles and also influence the distribution of ions across membranes, thus the development of non-ionic iodinated compounds was the next important step. Metrizamide was introduced in 1973 (2,3) and iohexol in 1982 (4). Both of these non-ionic gradient media can form solutions of high density (>1 .30 g/ml at 60% w/v); in metrizamide the carboxyl group present in metrizoic acid is linked to glucosamine, while in iohexol the carboxyl group is linked to the amine group of 3-amino-1,2-propanediol. This difference gives iohexol some advantages over metrizamide, notably its lower toxicity towards cells and autoclavability. The systematic name of iohexol is 5-(N-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-N,N'-bis(2,3-dihydroxypropyl)isophthalamide whose molecular structure is given in Figure 3. Its molecular mass is 821.

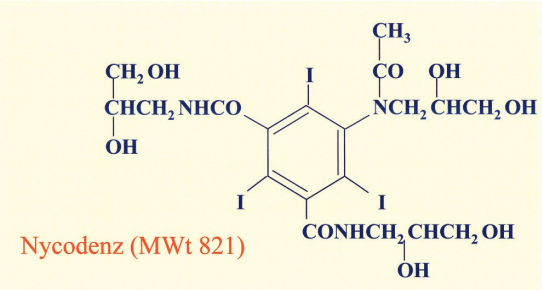

Figure 3 Molecular structure of Nycodenz®

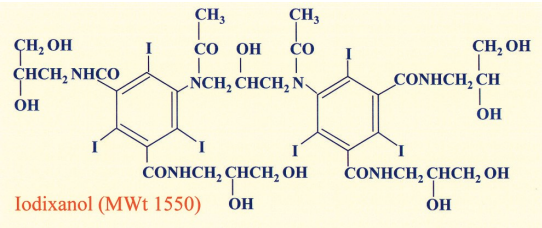

Figure 4 Molecular structure of iodixanol

The latest addition to Axis-Shield's nonionic iodinated compounds is iodixanol. The systematic name of iodixanol is 5,5 -[(2-hydroxy-1,3-propanediyl)-bis (acetylimino)]bis-[N,N bis(2,3dihydroxypropyl)-2,4,6-triiodo-benzenedicarboxamide] whose molecular structure is given in Figure 4. Its molecular mass is 1550 and it is essentially a dimer of Nycodenz®.

- **OptiPrep™ is the trademark name for a sterile 60% (w/v) solution of iodixanol in water**
- **Nycodenz® is the trademark name for iohexol**

The chemical properties and stability of Nycodenz® and iodixanol are related to their structure (Figures 3 and 4). Their high density derives from the presence of the substituted triiodobenzene rings which are linked to a number of hydrophilic groups.

Solubility, stability and spectroscopic properties

The highly hydrophilic side chains of **Nycodenz®** and **iodixanol** make these media readily soluble in water (5). At pHs below 9 solutions are stable almost indefinitely. Above pH 9 there is a very slow generation of inorganic iodine, but even after 12 months this represents a degradation of only 0.01% of the compound. Concentrations in water up to 80% (w/v), equivalent to a density of approx 1.426 g/ml can be prepared. Nycodenz® and iodixanol are also soluble in formamide, dimethylformamide and in ethanolic solutions; concentrations up to 50% (w/v) are possible. Non-aqueous media may be of particular use for minimizing loss of water soluble molecules from some biological particles, particularly nuclei and mitochondria.

Prolonged exposure of Nycodenz® and iodixanol to sunlight over a period of months, and to a lesser extent, to artificial light, may lead to the release of iodine from these compounds, but the extent of the reaction is generally insignificant when working with solutions on a day to day basis.

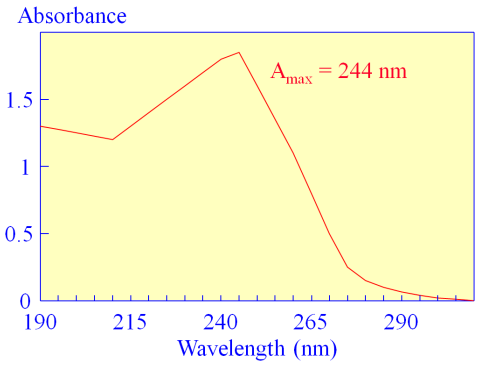

Figure 5 Absorbance spectrum of Nycodenz® (0.05 mg/ml in water)

The iodinated aromatic nucleus absorbs strongly in the ultraviolet region of the spectrum (Figure 5) with an absorbance maximum of approx 244 nm; this can be used as a direct measurement of the concentration of the medium in gradient fractions.

Iodixanol and Nycodenz® in solution are stable to heat and may be autoclaved; stability to autoclaving is enhanced by the presence of millimolar concentrations of an organic buffer such as Tris and also Ca/Na-EDTA.

Density, osmolality and viscosity

These important physicochemical properties of Nycodenz® and iodixanol solutions have been fully investigated and some of the data is summarized in Figure 6, which shows the relationships between density, osmolality, viscosity and concentration of aqueous solutions, measured at 20°C. Comparative data for some other media in common use for the fractionation of biological material is also included.

Expressed in terms of % (w/v), for any given concentration, solutions of Nycodenz® are denser than those of sucrose or Polysucrose™ 400, less dense those of CsCl or Percoll® and almost identical to those of iodixanol.

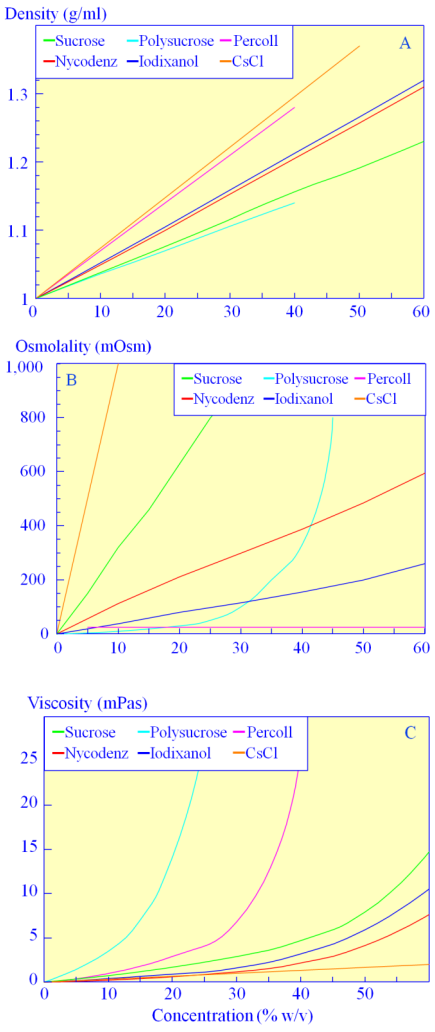

Figure 6 Physiochemical characteristics of gradient media; A density, B osmolality, C viscosity

The osmotic behaviour of solutions is often critical when choosing a gradient medium. When separating cells (lacking a rigid cell wall) or subcellular organelles it is most important to maintain a constant osmolality throughout the gradient. Exposing cells, organelles and membrane vesicles to changing osmolality will alter not only their volume and hence their density, but their viability and function might also be impaired. The resolution of these biological particles depends on differences in density, making osmotic conditions in the gradient an important factor. Less obviously, the density of many viruses, nucleic acids and proteins, and consequently their banding and resolution in gradients, may also be significantly affected by the osmolality of the medium. Moreover changes in the hydration of nucleic acids and proteins which occur in media of high osmolality may affect their molecular stability (6).

The use of polysaccharides such as Polysucrose™ 400 is limited not only by their density range but also by the non-linear relationship between concentration and osmolality. Sucrose, Nycodenz® and iodixanol exhibit a near linear relationship between concentration and osmolality. The osmolality of Nycodenz® solutions is considerably lower than that of sucrose (and CsCl) but above a density of approx 1.16 g/ml (approx 30% w/v) Nycodenz® solutions are hyperosmotic, although even at 50% (w/v), which is equivalent to a density of 1.265 g/ml (greater than that of the great majority of biological particles) the osmolality is only 485 mOsm. At the densities which are commonly used to isolate subcellular organelles, sucrose (and inorganic salt solutions) have osmolalities in excess of 1500 mOsm. Percoll® solutions in water do not exhibit any significant osmolality since Percoll®, is a colloid rather than a true solute.

Because of the higher molecular mass of iodixanol, the osmolality of its solutions (calculated from freezing point depression measurements) is less than half that of Nycodenz® solutions of the same density. It is technically difficult to measure the osmolality of concentrations of iodixanol above 50% (w/v). Vapour pressure measurements for example give a lower value (170 mOsm) than freezing point measurements (approx 260 mOsm) at 60% (w/v). The reason for this is not clear but may be related to the tendency of iodixanol to form oligomers at these higher concentrations and thus reduce the effective number of particles in solution. This has no effect on the density but it affects colligative properties such as osmolality. However when OptiPrep™ is diluted with an iso-osmotic medium to provide a solution containing 40- 50% (w/v) iodixanol, the osmolality is easily measured and reproducible and in the range 285-305 mOsm. In this manner working solutions can be produced which are iso-osmotic with the biological particles being fractionated.

Solutions of Nycodenz®, are less viscous (Figure 6) than any other gradient medium, except CsCl, thus particles sediment very rapidly in this medium. When very dense gradients are required, the viscosity can be reduced even further by using deuterium oxide (D_2O) as the gradient solvent ($\rho = 1.105$ g/ml). However, partial substitution of D_2O for H_2O in the hydration shell of particles is likely to lead to an increase in their effective buoyant densities.

Although the viscosity of iodixanol solutions is slightly higher than those of Nycodenz®, it is lower than those of sucrose. Since however many particles exhibit densities in iodixanol that are much lower than those in sucrose (and often lower than those in Nycodenz®), the density range of gradients of iodixanol required to band the particles is also lower. Consequently these gradients usually have a very low viscosity.

Biological Properties

A number of lines of evidence point to the lower toxicity of Nycodenz® and iodixanol compared to all other gradient media. Both Nycodenz® and iodixanol, as widely used X-ray contrast media (Omnipaque™ and Visipaque™ respectively), have undergone extensive clinical testing. Comparative clinical studies with other X-ray contrast media have shown the non-toxic nature of Nycodenz® (7,8). In clinical trials iodixanol has been shown to have extremely low acute toxicity in rodents and the LD_{50} was higher than for any other X-ray contrast medium tested (9) Upon injection it is rapidly excreted by the kidneys in an unchanged form (10) and in clinical trials iodixanol showed a lower frequency of adverse effects, compared to other media (11,12).

Non-ionic media such as iodixanol have been shown to induce markedly less abnormalities (the formation of cytoplasmic vacuoles) in cultured renal epithelial cells compared to ionic media such as metrizoate (13). This maybe related to the fact that metrizoate is an impermeant ion which could affect the Gibbs Donnan equilibrium of ions across the cell membrane. Induction of vacuolization however is not related to kidney function in rats or monkeys (14). In other studies (15) rat glial cells showed

only a transient and small reduction in viability when exposed to iodixanol with no changes in intracellular morphology, although Nycodenz® did affect viability and induced vacuolization. No teratogenic potential of iodixanol has been observed in rats or rabbits, nor any genotoxicity (four separate standard tests) or antigenic potential in passive cutaneous anaphylactic or active systemic anaphylactic tests in guinea pigs (14).

Studies have shown that iodixanol does not bind to proteins in human plasma (10) and neither iodixanol (16) or Nycodenz® (17) have significant effects on cell morphology or on cell growth, nor are they metabolized by cells. MOLT-4 T cells have been grown as monolayers in a standard RPMI medium supplemented with 5% (w/v) iodixanol. After 1-72 h the increase in viable cell number, as judged by the MTT Test was identical to control cells grown in the absence of the medium (16). Confluent monolayers of human embryo lung fibroblasts can be exposed to 30% (w/v) iodixanol in culture medium for up to 3 days without any change in cell viability or subsequent plating efficiency. Nycodenz® solutions have also been shown to be very resistant to bacterial degradation.

A wide variety of membrane-bound enzymes show little or no inhibition in the presence of Nycodenz® or iodixanol and considerably less inhibition compared to that observed in the presence of sucrose or metrizamide.

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Industrial production of density gradient media

Axis-Shield density gradient media are produced by Fresenius Kabi Norge AS, a subsidiary of Fresenius AG.

With its comprehensive range of products in combination with responsive services Fresenius Kabi has a unique position in its business fields and is the European leader in infusion and nutrition therapy.

Dedication to quality is an integral part of the culture and values at Fresenius Kabi. They provide thorough evaluation and testing of all manufactured materials in modern, fully equipped, quality-control laboratories. Their facilities operate under strict FDA and EU cGMP compliance. The use of highly automated production equipment combined with experienced personnel who have a total commitment to cGMP, further ensure product quality and customer satisfaction.

The production operations follow the routines prescribed in the production documentation, where all steps in the process are defined.

Cleaning

The mixing tanks, the pipelines and the filling equipment are cleaned with Water for Injection. All cleaning procedures are thoroughly tested and validated to ensure clean tanks between manufacturing of different products.

In order to conserve the microbiological integrity of the product, the entire production line is treated with water for injection ≥ 80°C at regular intervals.

Preparation of the solution

The production takes place in grade C areas.
Mixing is performed in large stainless steel tanks. The mixing tank, which is placed on weight cells is filled with Water for Injection. Active ingredients and the required excipients are added and the mixture is agitated until completely dissolved. After a fixed stirring time, samples are taken for physical, chemical and microbiological inprocess analyses. When the product has achieved the required physical and chemical specifications, the product is ready for filtration and filling.

Preparation of the bottle

The bottle is made of pharmaceutical grade polypropylene The bottles are moulded according to an injection blow moulding principle following four steps:

• Heating of the granulate to approx. 220°C to form the parison and to perform the treads and bottle body
• Conditioning and adjustment of temperature
• Blow moulding: The parison is stretched lengthwise by a stretch pin and blown to its final shape
• Ejection of finished bottles from the machine onto the conveyor belt.

Filling

The transport of the bottles from the moulding machine, filling and capping of bottles are carried out under conditions given in PIC regulations grade A.

The solution is filtrated through a membrane filter before filling. The pre-cleaned filling line, filter and filling machine are flushed with solution prior to filling.

The bottles are filled to a volume not less than nominal volume. The filling is performed according to a mass flow meter principle. The bottles are capped immediately after filling. After filling and capping, the bottles are stacked in autoclave racks and are ready for sterilization.
At specified intervals samples are withdrawn in order to verify correct filling volumes and functional testing. Samples for bioburden – and endotoxin testing are collected during the process. A filter integrity test is performed before and after filling.

Sterilization

The filled bottles are subjected to a validated steam sterilization cycle on the day of mixing and filling. The autoclave is computer controlled and operates by means of clean steam, distilled water and compressed air. It gives a sterilization cycle at 121°C which ensures that a minimum of F^0 value = 12 is delivered to the product.

Packaging

The filled sterilized units are visually inspected, labelled and packed into transport cartons.

Container/Closure information

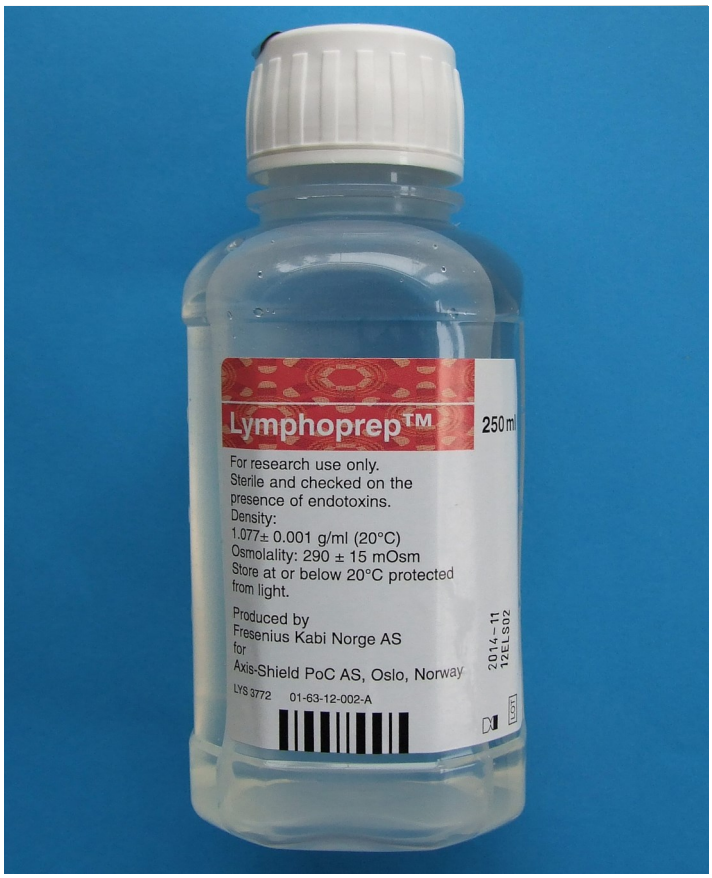
The material of the bottles is a copolymer of polypropylene and ethylene. The bottleneck is especially designed to support the excellent and controlled pouring qualities and the octagonal shaped body of the bottle supports a firm grip. The bottles are supplied with a screw cap. The design of the screw cap ensures that the bottle can be reopened several times. It has a pilfer-proof ring, which is broken when the bottle is opened for the first time. The

screw cap consists of a white polypropylene shell and a polypropylene plug or a perfusable cap with white shell with an aluminium pealable lid and a chlorobutyl rubber stopper that seals the bottle. The perfusable screw cap is designed to be used as an ordinary screw cap or allow for a syringe needle to be inserted through the top of the cap and further down through the rubber plug. The top of the cap is covered with a protective aluminium lid, which is pealed off before use. The caps are pre-assembled by the manufacturer.

Quality control/Quality assurance

After production the products are controlled in the QC laboratory for pH, density and osmolality. The specification on density is within ± 0.001 g/ml of the desired value. The specification on osmolality is within ± 15 mOsm of the desired value. The content of endotoxin should be less than 1.0 EU/ml (according to European Pharmaceutical Standards). However, our goal is to produce batches with endotoxin content less than 0.13 EU/ml.

The Fresenius Kabi quality system meets the requirements of the NS-EN ISO 9001.



Lymphoprep™

A simple and effective method for the isolation of mononuclear cells from human blood was reported by Dr. Arne Bøyum in 1968. For more than 35 years a commercial medium known as Lymphoprep™ has been widely used for isolating these cells.

Mononuclear cells (monocytes and lymphocytes) have a lower buoyant density than the erythrocytes and the polymorphonuclear (PMN) leukocytes (granulocytes). The vast majority of mononuclear cells have densities below 1.077 g/ml. These cells can therefore be isolated by centrifugation on an isoosmotic medium with a density of 1.077 g/ml, which allows the erythrocytes and the PMNs to sediment through the medium while retaining the mononuclear cells at the sample/medium interface.

The described method is rapid, simple and reliable and gives excellent results with blood samples from normal individuals and patients.

To obtain the maximum yield it is important that the blood sample is diluted 1:1 with physiological saline before being applied to the Lymphoprep™

The contamination of erythrocytes in the mononuclear cell suspension is usually between 3-10% of the total cell number.

Some immature PMNs may band with the lymphocytes during intense immunosuppressive therapy. It is essential to remove most of the platelets from mononuclear cell preparations in order to avoid inhibition in the cytotoxicity test.

Lymphoprep™ is a ready-made, sterile and endotoxin tested solution with the following specifications:

Sodium diatrizoate: 9.1% (w/v)

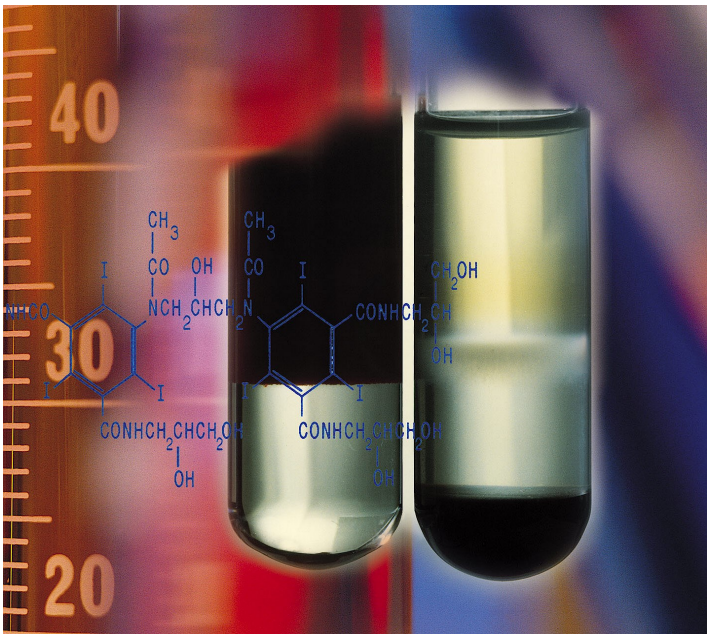
Polysaccharide: 5.7% (w/v)

Density: 1.077 ± 0.001 g/ml

Osmolality: 290 ± 15 mOsm

Endotoxins: < 1.0 EU/ml

Each batch of Lymphoprep™ is checked on the level of endotoxins using a specific LAL test. Our goal is to produce batches with an endotoxin level lower or equal to 0.13 EU/ml. For every batch produced a Certificate of Analysis showing the actual values of density, osmolality and endotoxins is made available at www.axis-shield-density-gradient-media.com. We also claim sterility according to Ph.Eur



Lymphoprep™ is manufactured, packed and released in compliance with:

1. Current EU guide to Good Manufacturing Practice
2. Fresenius Kabi AS Quality System
3. Fresenius Kabi AS Manufacturing Licence

Lymphoprep™ has the same specifications as the expensive PLUS or PREMIUM media from other manufacturers.

Lymphoprep™ can be used for the preparation of pure lymphocyte suspensions for tissue typing, antilymphocyte sera and immunological research. Thorsby and Brattelie used this technique with only slight modifications in the preparation of pure lymphocyte suspensions for cytotoxicity tests and lymphocyte cultures.

Lymphoprep™ is supplied as a sterile solution in the following package sizes:

Prod. no. 1114544	1x250ml	
Prod. no. 1114545	4x250ml	
Prod. no 1115754	1x500ml	
Prod. no. 1114547	6x500ml	
Prod. no. 1115757	20x250ml	(Econo-pack)
Prod. no. 1115758	12x500ml	(Econo-pack)

Bøyum, A. (1968)
Separation of leucocytes from blood and bone marrow
Scand. J. Clin. Lab. Invest., ***21***, *suppl.97*

Lymphoprep™ Tube

A simple and effective method for the isolation of mononuclear cells from human blood was reported by Dr. Arne Bøyum in 1968. For more than 35 years a commercial medium known as Lymphoprep™ has been widely used for isolating these cells.

Mononuclear cells (monocytes and lymphocytes) have a lower buoyant density than the erythrocytes and the polymorphonuclear (PMN) leukocytes (granulocytes). The vast majority of mononuclear cells have densities below 1.077 g/ml. These cells can therefore be isolated by centrifugation on an isoosmotic medium with a density of 1.077 g/ml, which allows the erythrocytes and the PMNs to sediment through the medium while retaining the mononuclear cells at the sample/medium interface.

The described method is rapid, simple and reliable and gives excellent results with blood samples from normal individuals and patients.

The success of the standard method for isolation mononuclear cells using Lymphoprep™ depends to a large extent on the careful layering of the diluted blood sample on top of the centrifugation medium to maintain a sharp interface between the two layers. This procedure requires some practise and can be time-consuming with large numbers of samples.

Lymphoprep™ Tube is a sterile tube in which the Lymphoprep™ is contained below a plastic filter disc. This allows blood to be poured simply and directly into the tube, the disc preventing any mixing with the separation medium. The erythrocytes displace the Lymphoprep™ upwards to allow mononuclear cells to band above the filter disc.

Lymphoprep™ is a ready-made, sterile and endotoxin tested solution with the following specifications:

Sodium diatrizoate: 9.1% (w/v)

Polysaccharide: 5.7% (w/v)

Density: 1.077 ± 0.001 g/ml

Osmolality: 290 ± 15 mOsm

Endotoxins: < 1.0 EU/ml

Each batch of Lymphoprep™ is checked on the level of endotoxins using a specific LAL test. Our goal is to produce batches with an endotoxin level lower or equal to 0.13 EU/ml. For every batch produced a Certificate of

Analysis showing the actual values of density, osmolality and endotoxins is made available at www.axis-shield-density-gradient-media.com. We also claim sterility according to Ph.Eur.

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Lymphoprep™ Tube can be used for the preparation of pure lymphocyte suspensions for tissue typing, antilymphocyte sera and immunological research. Thorsby and Brattelie used this technique with only slight modifications in the preparation of pure lymphocyte suspensions for cytotoxicity tests and lymphocyte cultures.

Lymphoprep™ Tube is supplied as a sterile solution in the following package sizes:

Prod. no. 1019817	30 tubes (each filled with 2ml)
Prod. no. 1019818	18 tubes (each filled with 10ml)

NycoPrep™ 1.077

In 1983 Dr. Arne Bøyum devised a new separation medium for the density barrier isolation of human mononuclear cells. This medium is based on Nycodenz®, a triiodinated derivative of benzoic acid with three aliphatic, highly hydrophilic side chains with a molecular weight of 821. In NycoPrep™ 1.077, Nycodenz® replaces sodium diatrizoate of the Ficoll/Hypaque method and a buffered sodium chloride solution maintains the correct osmotic environment. Importantly no Ficoll or other polysaccharide is added. Thus the components of NycoPrep™ 1.077 are unlikely to mask receptors or other marker molecules on the surface of lymphocytes. Mitogen stimulation of the mononuclear cells recovered is found to give results similar to or better than, cells isolated by the Ficoll/ Hypaque method.

Using NycoPrep™ 1.077, the erythrocytes do not form such a compact pellet and if resuspended, appear as single, unclumped cells of good morphological appearance.

To obtain the maximum yield it is important that the blood sample is diluted 1:1 with physiological saline before being applied to the NycoPrep™ 1.077.

The contamination of erythrocytes in the mononuclear cell suspension is usually between 3-10% of the total cell number.

Some immature PMNs may band with the lymphocytes during intense immunosuppressive therapy. It is essential to remove most of the platelets from mononuclear cell preparations in order to avoid inhibition in the cytotoxicity test.

NycoPrep™ 1.077 is a ready-made, sterile and endotoxin tested solution with the following specifications:

Nycodenz: 14.1% (w/v)

NaCl: 0.44% (w/v)

Tricine/NaOH, pH 7.0: 5mM

Density: 1.077 ± 0.001 g/ml

Osmolality: 295 ± 15 mOsm

Endotoxins: < 1.0 EU/ml

Each batch of NycoPrep 1.077™ is checked on the level of endotoxins using a specific LAL test. Our goal is to produce batches with an endotoxin level lower or equal to 0.13 EU/ml.

For every batch produced a Certificate of Analysis showing the actual values of density, osmolality and endotoxins is made available at www.axis-shield-density-gradient-media.com. We also claim sterility according to Ph.Eur.

NycoPrep™ 1.077 is manufactured, packed and released in compliance with:

1. Current EU Guide to Good Manufacturing Practice

2. Fresenius Kabi AS Quality System

3. Fresenius Kabi AS Manufacturing Licence

NycoPrep™ 1.077 can be used for the preparation of pure lymphocyte suspensions for tissue typing, antilymphocyte sera and immunological research.

The described method is rapid, simple and reliable and gives excellent results with blood samples from normal individuals and patients.

NycoPrep™ 1.077 is supplied as a sterile solution in the following package size:

Prod. no. 1114550 4x250ml

Bøyum, A. (1983)
In Iodinated Gradient Media, a practical approach (ed. D. Rickwood). 147-171, IRL Press

Polymorphprep™

With the exception of the basophils, polymorphonuclear leukocytes (PMNs) have a much greater buoyant density than the mononuclear cells, > 1.085 g/ml. Unfortunately, the buoyant density of the erythrocytes tends to be from 1.09-1.11 g/ml, this makes a separation from whole blood using a density barrier similar to that used for mononuclear cells, more difficult. A number of procedures have been developed in an effort to overcome these difficulties.

The high osmolality of Polymorphprep™ causes erythrocytes to lose water and shrink, thus increasing their effective buoyant densities. This allows the dextran aggregated erythrocytes to sediment rapidly through the dense medium.

Because the osmotic gradient between the medium and the erythrocytes declines as the cells sediment further into the medium (ie the water loss from the erythrocytes is greatest at the top of the Polymorphprep™ and progressively decreases as they sediment further) – a gradient of diatrizoate forms within the density barrier. The PMNs band within this density gradient while the mononuclear cells remain at the sample/medium interface.

Polymorphprep™ is a ready-made, sterile and endotoxin tested solution with the following specifications:

Sodium diatrizoate: 13.8% (w/v)
Dextran 500: 8.0% (w/v)
Density: 1.113 ± 0.001 g/ml
Osmolality: 445 ± 15 mOsm
Endotoxins: < 1.0 EU/ml

Each batch of Polymorphprep™ is checked on the level of endotoxins using a specific LAL test. Our goal is to produce batches with an endotoxin level lower or equal to 0.13 EU/ml.

The method is effective only with whole undiluted human blood not with a leukocyte-rich fraction or blood from animal species.

The temperature is important to obtain optimal results, as changes in temperature effect the density and viscosity of the Polymorphprep™ solution. The temperature of the blood sample and the medium should be kept between 18 -22°C.

Analysis of the top and bottom bands from the Polymorphprep™ separation using a Coulter STKR Cell Analyzer is shown in the figure beneath. The analyzer determines the number of cells in the sample (ordinate) as a function of cell volume (abscissa). Relative cell number is the number of cells of a particular volume expressed as a fraction of the total in each sample. The top band contains only lymphocytes (40-80 fl) and monocytes (80-130 fl); all of the PMNs (150-320 fl) are in the bottom band which has negligible contamination from mononuclear cells. Contamination of the PMN band by erythrocytes is between 2-6% of the total cell number.

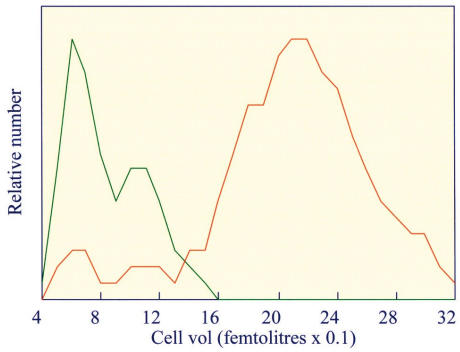


Figure 7 Coulter STKR analysis of the two bands from a Polymorphprep™ separation. The upper (–) band contains all of the mononuclear cells (MC); the lower (–) band contains PMNs and less than 2% MCs.

Polymorphprep™ is supplied as a sterile solution in the following package size:

Prod. no. 1114683 1x250ml

Polysucrose™ 400

Product description

Polysucrose™ 400 is a synthetic high molecular weight polymer made by the copolymerization of sucrose and epichlorohydrin. The molecules have a branched structure with a high content of hydroxyl groups giving a good solubility in aqueous solutions. The product is similar to Ficoll® 400 from GE Healthcare.

The reactivity and stability of Polysucrose™ 400 are determined by its hydroxyl groups and the glycosidic bonds in the sucrose residues. Polysucrose™ is stable in alkaline and neutral solutions. At pH values lower than 3, it is rapidly hydrolyzed, especially at elevated temperatures. In neutral solutions, Polysucrose™ 400 can be sterilized by autoclaving at 110^0C for 30 minutes without any degradation.

Polysucrose™ 400 is readily soluble in aqueous solutions when added slowly to the liquid with constant stirring. Concentrations up to 50% (w/v) can easily be obtained.

Applications

Using sodium metrizoate and a polysaccharide Bøyum (1968) developed a one-step centrifugal technique for isolation of lymphocytes (Lymphoprep™). In this method the polysaccharide aggregates the erythrocytes, thereby increasing their sedimentation rate. Polysucrose™ 400 has also been used as a density gradient medium for the purification of other cells and in membrane fractionation.

Non-ionic high molecular weight solutes such as polysucrose are required for a number of other research scenarios. Polysucrose™ 400 may be used as a stabilizing agent in protein solutions and it can function as an immuno-logically inert carrier for low molecular weight haptens in immunological studies. Polysucrose™ 400 is also used to reduce non-specific binding of labelled probes to nitrocellulose membranes during nucleic acid hybridization. It also simplifies the loading of nucleic acids into the sample wells of agarose gels for electrophoresis.

Technical data

Specific optical rotation $(\alpha)_{D,20}$	53 –59°
Intrinsic viscosity (20° C)	0.14 –0.20
Average molecular weight (Mw)	450,000 ± 100,000
Mw distribution by GPC	conforms to standard
Loss on drying (%)	<5.0%
pH (10% w/v aqueous solution)	7.0 –9.0
Sulphated ash	<0.3%
Content of chloride (ppm)	<500 ppm
Microbiological contamination	<100 CFU/g
	<10 yeasts and mould/g

Availability

Polysucrose™ 400 is available in the following package sizes:

Prod. no. 1026582	1x500g
Prod. no. 1006031	1x 5kg
Prod. no. 1017120	1x25kg

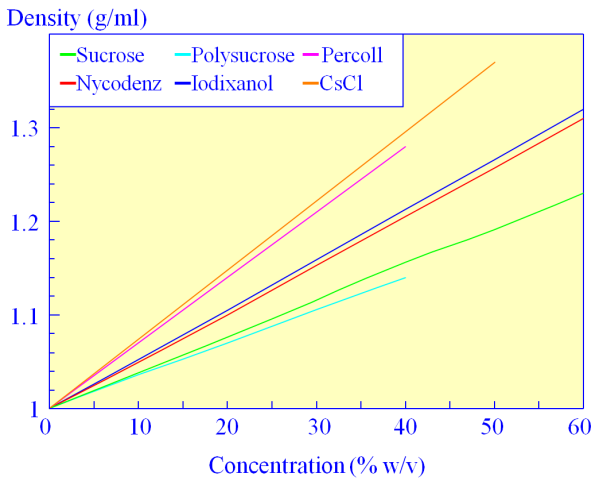
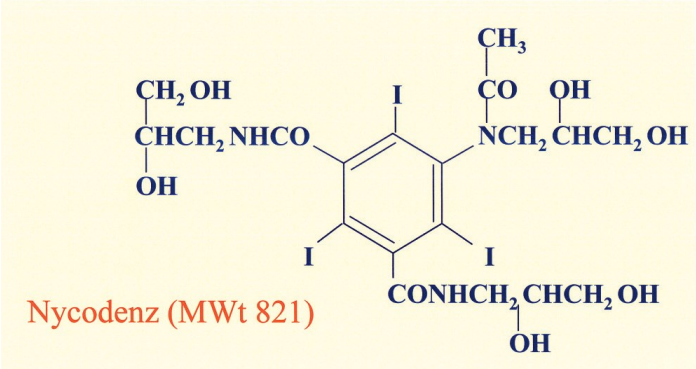


Figure 8 Concentration vs. Density for some density solutions

Ficoll is a trademark of GE Healthcare companies

Nycodenz®

Nycodenz® is an off-white powder, freely soluble in water. Solutions up to 80% (v/w) with a density of 1.426 g/ml can be prepared.



Nycodenz (MWt 821)

Nycodenz® was originally developed as an X-ray contrast medium and has therefore been subjected to rigorous clinical testing.

Nycodenz® is non-ionic, non-toxic to cells and metabolically inert.

Nycodenz® can be used for the isolation of cells, subcellular organelles and membranes, macromolecules and viruses.

Nycodenz® forms true solutions. It is therefore easy to remove the medium from the cells after fractionation.

Nycodenz® is resistant to bacterial degradation.

Nycodenz® solutions can be autoclaved.

Nycodenz® is the trademark name for iohexol, whose systematic name is 5-(*N*-2,3-dihydroxypropylacetamido) 2,4,6-tri-iodo-*N*-*N'*-bis(2,3-dihydroxypropyl)isophthalamide. It has a molecular weight of 821. The chemical properties and stability of Nycodenz® are related to its structure. Its high density derives from the presence of a substituted triiodobenzene ring linked to a number of hydrophilic groups which are responsible for the high water solubility of Nycodenz®. It is a non-ionic derivative of metrizoic acid; the carboxyl group present in metrizoic acid is linked to the amine group of 3-amino-1,2-propanediol. The dihydroxypropylacetamido side chain is responsible for the very low toxicity of Nycodenz® compared to metrizamide. The iodinated aromatic nucleus absorbs strongly in the ultraviolet region of the spectrum with an absorption maximum of 244 nm.

Gradients of Nycodenz® can be generated by:

- Diffusion of a discontinuous gradient; simple preparation within 45 minutes.
- Tilted tube rotation (Gradient Master™).
- Two-chamber gradient maker
- Freezing/thawing of a solution of uniform density
- Centrifugation (self-generated gradient)

The density of Nycodenz® in solution can be determined by measuring the refractive index or it can be calculated from absorbance measurements

Nycodenz® is a non-colloidal medium therefore the distribution of cells in gradients can be determined using a haemocytometer, electronic particle counter or by light scattering measurements using a spectrophotometer.

Nycodenz® does not interfere with the orcinol and diphenylamine reactions for the estimation of nucleic acids nor with the very sensitive dye binding assays for protein and DNA.

Polysaccharides and sugars can be determined in the presence of Nycodenz® using the phenol/H_2SO_4 assay. Fluorimetric assays of nucleic acids and proteins can also be carried out in the presence of Nycodenz®. Nycodenz® does not interfere with most assays for the marker enzymes of subcellular components, also commercial scintillants are compatible with Nycodenz®.

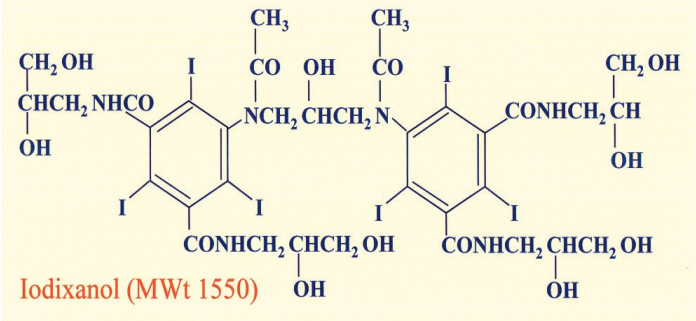
Nycodenz® can be removed from samples by dialysis, ultrafiltration or gel filtration. Cells, subcellular organelles and other particulate matter can be isolated from Nycodenz® by centrifugation without the risk of contaminating the pellet with Nycodenz®.

Nycodenz® is supplied as a powder in the following package size:

Prod. No. 1002424 1x500g

OptiPrep™

OptiPrep™ is a sterile endotoxin tested solution of 60% iodixanol in water with a density of 1.32 g/ml.



Iodixanol was developed as an X-ray contrast medium an has therefore been subjected to rigorous clinical testing. **Iodixanol** is non-ionic, non-toxic to cells and metabolically inert.

Iodixanol is approximately a dimer of Nycodenz® and its biological and physicochemical properties are very similar, see p16. **However, iodixanol solutions have osmolalities approx. half that of Nycodenz® solutions of the same density.**

Iodixanol solutions can be made isoosmotic at all densities and OptiPrep™ can be added directly to a cell or organelle suspension without changing its osmolality, thus facilitating their purification in a flotation gradient.

Because of its higher molecular mass, iodixanol forms self-generated gradients more quickly than Nycodenz®.

Mammalian and non-mammalian cells purified in iodixanol gradients have high viability.

Organelles and membrane vesicles are effectively fractionated in either pre-formed or self-generated gradients.

Viruses purified in iodixanol gradients show an infectivity: particle ratio at least 100x higher than those from CsCl gradients.

Protein-protein and protein-nucleic acid complexes are better preserved in iodixanol than in sucrose or glycerol gradients.

Plasma lipoproteins analyzed in self-generated gradients within 4h.

OptiPrep™ is a ready-made, sterile and endotoxin tested solution with the following specifications:

Iodixanol: 60% (w/v)

Density: 1.320 ± 0.001 g/ml

Osmolality: approx. 170 mOsm

Endotoxins: < 1.0 EU/ml

Each batch of OptiPrep™ is checked on the level of endotoxins using a specific LAL test. Our goal is to produce batches with an endotoxin level lower or equal to 0.13 EU/ml.

For every batch produced a Certificate of Analysis showing the actual values of density and endotoxin levels is made available at www.axis-shield-density-gradient-media.com. We also claim sterility according to Ph.Eur.

OptiPrep™ is manufactured, packed and released in compliance with:

1. Current EU Guide to Good Manufacturing Practice
2. Fresenius Kabi AS Quality System
3. Fresenius Kabi AS Manufacturing Licence

OptiPrep™ is supplied as a sterile solution in the following package size:

Prod. no. 1114542 1x250ml

Application Sheet example

Excellence in Separations

Application Sheet S09

Purification of mammalian peroxisomes in a continuous gradient

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- For links to other relevant files click on the double blue arrow in the following text.
- For links to the Axis-Shield OptiPrep™ and Nycodenz® Abstract Databases see Section 6 (page 5)

1. Background

A huge number of published papers have reported the use of Nycodenz® gradients for the purification of peroxisomes, mainly from a large range of mammalian tissues but also some types of cultured cells, principally fibroblasts. Percoll® gradients were also a popular choice, largely because of their ease of use in a self-generated format, but Hiltunen et al [1] commented that to reduce contamination from mitochondria Nycodenz® was the gradient medium of choice and many groups have used a Nycodenz® gradient to increase the purity of Percoll®-isolated organelles. Moreover in Percoll® gradients peroxisomes and endoplasmic reticulum (ER) have a very similar banding density; in Nycodenz® and iodixanol the ER has a much lower density. In both Nycodenz® and iodixanol the peroxisomes are the densest of the major subcellular organelles (ρ = 1.19-1.23 g/ml) and normally well-separated from the other organelles (mitochondria, lysosomes and ER) in the light mitochondrial fraction. In iodixanol the density of these other organelles is much lower; mitochondria have a median density of approx 1.145 g/ml and lysosomes approx 1.12 g/ml; slightly lower than the figures in Nycodenz®; the separation from peroxisomes is thus potentially greater in iodixanol.

If the concentration of peroxisomes in the gradient fraction(s) is sufficiently high for the analytical technique to provide reliable information, it is usually unnecessary to remove the gradient medium. Unlike Percoll®, neither Nycodenz® or iodixanol interfere with any spectrophotometric assays in the visible region of the spectrum nor do they affect the proper running of SDS-polyacrylamide gels.

Section 2 of this Application Sheet describes the OptiPrep™ method as applied to rat liver in detail; it is adapted from the method of Van Veldhoven et al [2,3]. Section 3 describes some of the procedural variations and Section 4 summarizes the principal Nycodenz® methodology.

2. Iodixanol gradient methodology

2a. Solutions Required (see Section 2d, Note 1)

A. OptiPrep™.

B. Homogenization medium: 0.25 M sucrose, 1mM EDTA, 0.1% (v/v) ethanol, 5 mM Mops pH 7.2.

C. 6 mM EDTA, 0.6% ethanol, 30 mM Mops, pH 7.2.

D. 1 M sucrose.

E. Gradient solutions: Make up from solutions A, C, D and water using respectively, these ratios by volume:
1: 5 + 0.6 + 0.4 + 0.0 (50% iodixanol)
2: 4 + 0.6 + 0.7 + 0.7 (40% iodixanol)
3: 2 + 0.6 + 1.1 + 2.3 (20% iodixanol)

Keep the following stock solutions at 4°C:
500 mM Mops 10.45 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$) 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 0.2 ml, 2 ml and 2 ml respectively of ethanol, EDTA stock and Mops stock; adjust to pH 7.2 with 1 M NaOH and make up to 200 ml.

Solution C: Add 0.6 ml, 6 ml and 6 ml respectively of ethanol, EDTA stock and Mops stock to 50 ml water; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml.

Solution D: Dissolve 34.2 g sucrose in 50 ml water and make up to 100 ml.

2b. Ultracentrifuge rotor requirements

Any 30-50 ml fixed-angle rotor for an ultracentrifuge, capable of approx 100,000 *g* (see Section 3)

2c. Protocol

Carry out all operations at 0-4°C.

1. Mince the liver very finely with scissors and transfer to a Potter-Elvehjem homogenizer with Solution B (use 10 ml medium for every 2.5 g tissue). Homogenize using approx 6 strokes of the pestle (500-700 rpm) (see Section 2d, Note 2).
2. Centrifuge the homogenate at 3000 g_{av} in a fixed-angle rotor for 10 min to pellet the nuclei and heavy mitochondria. This pellet may be rehomogenized in solution B and the centrifugation repeated.
3. Centrifuge the supernatant(s) at 17,000 g_{av} for 10-15 min.
4. Resuspend the 17,000 *g* pellet in solution B using a loose-fitting Dounce homogenizer (2-3 strokes of the pestle). Adjust to a volume of about 0.5 ml per g of tissue.
5. Use a two chamber gradient maker or a Gradient Master to prepare a linear gradient from 9 ml each of gradient solutions E2 and E3 in thick-walled polycarbonate tubes for a 36-40 ml fixed-angle rotor and underlayer each gradient with 2 ml of gradient solution E1 (see Section 2d, Note 3).
6. Layer 3 ml of the suspension over each gradient and centrifuge at 105,000 g_{av} for 1 h.
7. Allow the rotor to decelerate from 1000 rpm without the brake, the collect the gradient in 1 ml fractions dense end first (see Section 2d, Note 4).

2d. Notes

1. The variable volume of 1 M sucrose maintains each solution isoosmotic. Keep these solutions, and carry out all subsequent operations, at 0-4°C. Protease inhibitors may be included in any or all of the media at the operator's discretion.
2. For more information on homogenization of tissues and cells and differential centrifugation of an homogenate see respectively Application Sheets S05, ➔➔ S06 ➔➔ and S07 ➔➔.
3. Thin-walled tubes can be used but may require some capping or sealing device. For more details on the preparation of pre-formed iodixanol gradients see Application Sheet S02. ➔➔
4. Gradients can be unloaded dense end first by carefully introducing a narrow metal cannula (connected to a peristaltic pump) to the bottom of the tube. Thin-walled tubes can be collected by tube puncture. For more information on unloading gradients see Application Sheet S52. ➔➔

2e. Analysis

Iodixanol does not significantly inhibit any enzyme so far tested. Standard spectrophotometric methods (carried out above 340 nm), for measuring organelle enzyme markers can be performed directly on gradient fractions [4]. Protein can also be measured directly by any Coomassie blue-based method [4]. If it is necessary to remove the gradient medium, dilute fractions with an equal volume of buffer; pellet at approx 30,000g_{av} for 10 min and resuspend in a suitable buffer.

A typical result with rat liver is shown in Figure 1, which shows the distribution of marker enzymes across the gradient. The catalase (peroxisome) band is well separated from all of the mitochondria (glutamate dehydrogenase) and lysosomes (acid phosphatase). The endoplasmic reticulum (glucose-6-

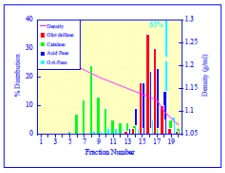


Figure 1 Isolation of peroxisomes: enzyme distribution in gradient. Glut deHase = glutamate dehydrogenase, Acid Pase = acid phosphatase; G-6-Pase = glucose-6-phosphatase. Adapted from ref 2 with kind permission of the authors and Academic Press.

phosphatase) is the least dense membrane type. This is in contradistinction to Percoll® gradients in which ER and peroxisomes always overlap. Similar separations of peroxisomes are obtained with mouse kidney [5] and hepatocytes [6].

The yield of peroxisomes is 80-90% with no detectable contamination from any other organelle. Yields of peroxisomes from Percoll® gradients are often low due to loss of material during the final centrifugation step to remove the Percoll®. This is normally obligatory as Percoll® frequently interferes with subsequent analyses.

3. Alternative centrifugation/gradient formats for iodixanol gradient separations

1. Joly et al [7] used exactly the same iodixanol gradient as described in this OptiPrep™ Application Sheet but carried out the centrifugation at 125,000 *g* for 1 h in a Beckman SW41 swinging-bucket rotor. An almost identical distribution of organelle markers was reported. The authors investigated the localization of malonyl-CoA decarboxylase.
2. **Light and dense peroxisomes** have been resolved in a continuous exponential gradient [8-10]. It was generated from a discontinuous one of 1.12, 1.155, 1.19, 1.225 and 1.26 g/ml (equivalent to 18.5%, 26%, 32.5%, 40.5% and 47.5% iodixanol) by freezing in liquid nitrogen (and storage at -20°C) followed by rapid thawing (approx. 30 min). After centrifugation at 39,000 g_{max} for 30 min in a Beckman VTi50 rotor the light peroxisomes banded at approx 1.21 g/ml and the dense peroxisomes at approx. 1.24 g/ml. Islinger and Weber [10] showed that iodixanol gradients could even identify a very low density population. Note that using a vertical rotor is widely used practice with Nycodenz® gradients (see Section 4) and the short sedimentation path lengths of such rotors, compared to either swinging-bucket or fixed-angle rotors permits both the use of lower *g*- forces and shorter times. This also means that the fragile organelles are exposed to much lower hydrostatic pressures.
3. Antonenkov et al [11] used an iodixanol gradient as a final step for the production of highly purified peroxisomes, virtually devoid of any contamination. An initially discontinuous gradient comprised 6 ml each of 20%, 25%, 30% and 35% (w/v) iodixanol, together with 4 ml each of 40% and 50% iodixanol, in tubes for the Beckman VTi50 rotor. After being allowed to diffuse overnight at 4°C, 8-9 ml of crude peroxisomes was layered on top and centrifuged at 65,000 g_{av} for 1 h. The peroxisomes produced by this procedure were used in physical [11], membrane transport [12] and fatty acid binding protein studies [13]. In a more recent paper the centrifugation conditions were 100,000 *g* for 90 min [14].

4. Use of Nycodenz® gradients

4a. Solution preparation

Nycodenz® is only available commercially as a powder. To make up a dense stock solution of 50% (w/v) Nycodenz® place 50 ml of water or a suitable buffer (e.g. 1 mM EDTA, 10 mM Hepes-NaOH, pH 7.4) in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 50 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and then make up to 100 ml with buffer or water (additions of small volumes of concentrated DTT solutions and ethanol may be added before the final volume adjustment). Filter sterilize if required. This stock solution of Nycodenz® will have an osmolality of approx 490 mOsm, so there should be no osmotic balancer present. This stock solution is then usually diluted with the homogenization medium to make lower density solutions.

4b. Gradients and centrifugation

There are too many published papers for a comprehensive discussion of all the technology (see Section 6) but it is worth noting that many of the current techniques are based on methods that were developed more than 15 years ago. Nevertheless one of the earliest papers [15] described a method that has been used with relatively minor modifications up to the present day. Pryde et al [15] used a 10-48% (w/v) Nycodenz® gradient in a Beckman VTi 50 rotor, centrifuging it at 35,000 g_{av} for 75 min. Appelkvist et al [16] changed the gradient to 1.12-1.27 g/ml (approx. equivalent to 20%-50% Nycodenz®) – the same gradient was used by Wolvetang et al [17] and Pedersen et al [18] used a 15-

45% gradient. The latter also used a technique (also adopted by other groups) of including a 2 ml cushion of the very dense (1.9 g/ml) non-aqueous inert Maxidens™ (normally used for unloading gradients), which prevented any very dense particle sedimenting to the wall of the tube. When vertical tubes are unloaded any such particles are likely to contaminate the entire gradient when it is unloaded. This can also be avoided by using a near-vertical rotor rather than a vertical rotor. Maxidens™ is no longer available from Axis-Shield, Oslo, but perfluorodecalin (identical to Maxidens™) can currently be purchased from F2 Chemicals Ltd, Lea Lane, Lea Town, Preston PR4 0RZ, UK (tel: +44 (0)1772 775802, fax +44 (0)1772 775808); contact name Gerry May (gerry.may@fluoros.co.uk). Similar solutions, under the trade name "Fluorinert" are also available from Sigma-Aldrich Chemicals.

5. References

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6. The Axis-Shield Abstract Database

To access comprehensive lists of both Nycodenz® and OptiPrep™ abstracts return to the Subcellular Membranes Application Sheet Index and follow the instructions at the top of page 1.

Contact information

Product Management

Bjørn Henriksen, MSc

Axis-Shield PoC is a company based in Oslo, developing, producing and marketing in vitro diagnostic products. Axis-Shield PoC develops and produces (among other products) density gradient media based on iodinated X-ray contrast compounds.

Bjørn Henriksen graduated with a M.Sc in biochemistry at the University of Oslo in 1974. His M.Sc. concerned the effect of 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) on the nucleic acid synthesis in Erlich ascites cells.

After working 5 years in a local Norwegian company marketing products from Bio-Rad Laboratories, Sigma Chemical, Dako, Dynatech and others, Mr. Henriksen joined Nyegaard & Co (later Nycomed) in 1979.

Bjørn Henriksen was appointed International Product Group Manager in 1986, and has since then worked full time developing and marketing new density gradient media.

His responsibilities also includes the day to day business regarding media already on the market.

Since 1986 Nycomed has introduced products like Polymorphprep™, the NycoPrep™ line and OptiPrep™.

In February 2000, Axis-Shield took over responsibilities for these products.

Bjørn Henriksen can be reached in Oslo on phone no: +47 24 05 60 00, fax no: +47 24 05 60 01 or via e-mail: bjh@axis-shield.com

Product Development

John Graham, PhD

Dr. John Graham graduated in biochemistry (1965) at the University of Liverpool and his PhD research at the same university concerned the binding of sterols in membranes. This work involved the purification of all the subcellular organelles from mammalian liver and it laid the foundation for Dr. Graham's interest in fractionation techniques using centrifugation.

A period of two years in the Department of Biological Chemistry at Harvard Medical School (Boston, US) was followed by 5 years at the Imperial Cancer Research Fund in London.

After a long collaboration with Professor Charles Pasternak at the University of Oxford Biochemistry Department , Professor Pasternak invited Dr. Graham to join him in the new Department of Biochemistry of St. George's Hospital Medical School, where he stayed from 1975 until 1989. In 1978 he began an association with Dr. David Rickwood and later, Mr. Terry Ford at the University of Essex. This association began with the running of technical training courses in centrifugation techniques and later with the development of applications related to Nycomed's iodinated density gradient media.

Dr. Graham has since 1994 been a consultant for Nycomed and Axis-Shield PoC.

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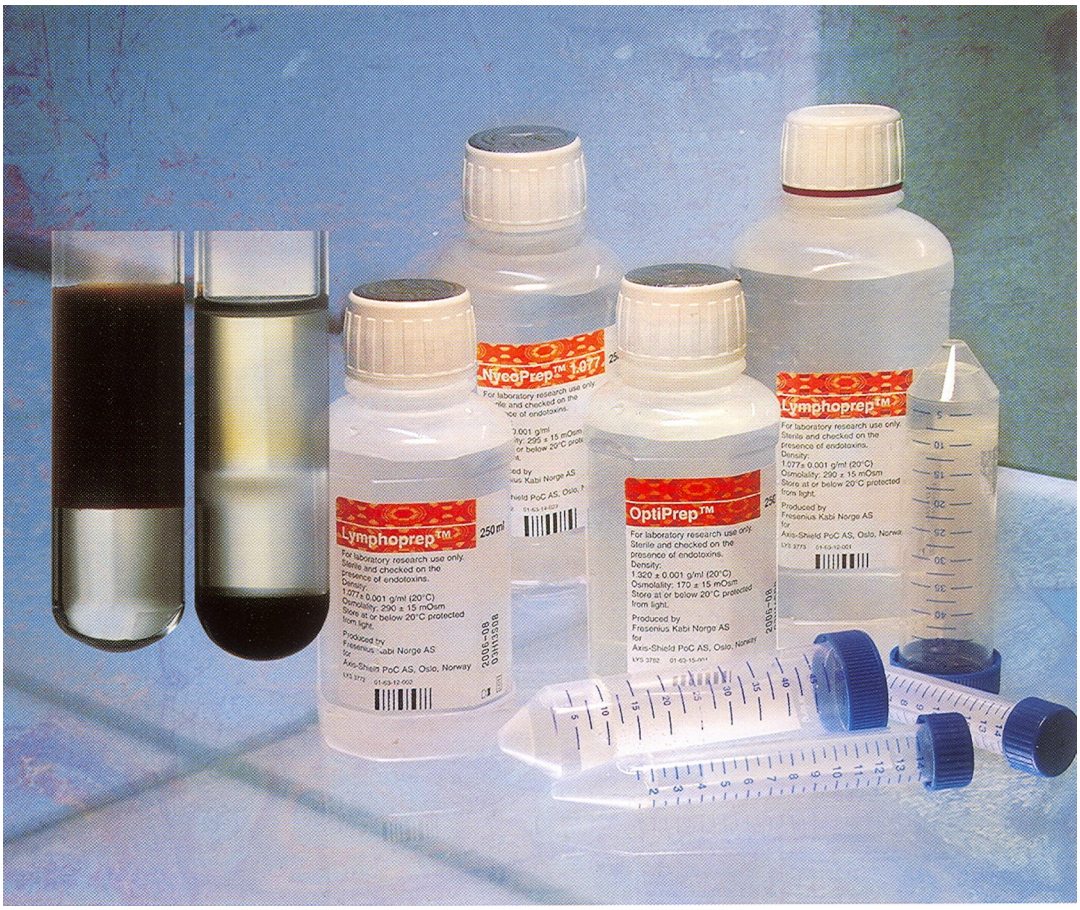

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