Fractionation and analysis of normal erythrocytes, sickle cells and reticulocytes; removal of erythrocytes from blood and bone marrow

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- To access other Application Sheets referred to in the text return to the Cell Index; key Ctrl "F" and type the C-Number in the Find Box

1. Background

1a Reticulocytes

A number of density gradient strategies have been developed for the fractionation of human erythrocytes according to their age. As the cells age, so their density tends to increase; reticulocytes therefore have the lowest densities. Reticulocytes have frequently been partially purified on discontinuous gradients of arabinogalactan; the actual density range being quite variable, sometimes broad, e.g. 1.066-1.220 g/ml [1], sometimes narrow, e.g. 1.083-1.124 g/ml [2]. These gradients have entailed the use of unusually high RCFs of 27,000g [1] 74,000g [2] for approx 1 h. Such high RCFs are rarely used for other types of cell and are often detrimental to cell function; the viscosity of arabinogalactan gradients maybe a contributory factor. Continuous gradients of metrizamide (12%-27%) equivalent to a density range of approx 1.07-1.15 g/ml were used by Lodish et al [3] to purify rabbit reticulocytes and again a relatively high RCF was used – approx 27,000 *g* for 1 h.

1b Erythrocytes

Percoll® gradients have also been used to fractionate erythrocytes on the basis of density in self-generated gradients, again RCFs more associated with the banding of smaller particles (33,000g) were used [4]. Moreover importantly, the properties of reticulocytes, which control their release from bone marrow, i.e. their deformability and rigidity, may be compromised by the use of Percoll®. There is evidence that the colloidal silica particles of Percoll® adhere to the surface of erythrocytes (even after washing) and cause progressive hemolysis; the elongation index of erythrocytes is thus difficult to measure and their deformability is affected [5].

2 Nycodenz® applications

2-1 Comparison with OptiPrep™

Many of the published Nycodenz® methods report the use of an isoosmotic solution of density 1.15 g/ml (NycoPrep™ 1.15); this is no longer commercially available. The two options are to prepare the solutions from Nycodenz® powder or to substitute iodixanol. The latter is available as a sterile 60% (w/v) solution (OptiPrep™) that can simply be diluted with a buffered saline; all dilutions will be isoosmotic. Nycodenz® and iodixanol solutions of the same % (w/v) have an almost identical density and at densities <1.15 g/ml, they will also have a very similar osmolality. It is unlikely that substitution of Nycodenz® by iodixanol at such densities will have any affect on the behaviour of the erythrocytes, but no comparative data is available. For density tables of iodixanol solutions **see Application Sheet C01.**

2-2. Erythrocyte density determination

Erythrocytes washed three times in a buffered saline were layered over a single Nycodenz® solution (ρ = 1.095-1.105 g/ml) and centrifuge at 600 *g* for 20 min. By observing the distribution of cells above, below and within the Nycodenz® solution, Tong and Caldwell [6] could determine whether a particular *in vitro* treatment modulates the density of erythrocytes.

2-3 Maintenance of erythrocytes in suspension

Nycodenz® solutions gave also been used to maintain erythrocytes in suspension during video microscopic observation [7] although physical measurements on such cells are now widely carried out using an iodixanol solution (**see "Cells in suspension, maintenance of", Application Sheet C38 in index**).

2-4 Separation of erythrocytes and leukocytes

A simple Nycodenz® density barrier (1.11 g/ml: approx. equivalent to 19.5% w/v Nycodenz®) can also be used to separate leukocytes and erythrocytes; it was used in immune complex binding studies [8].

2-5 Separation of dense and light sickle erythrocytes in a two-layer discontinuous gradient [9]

After washing the erythrocytes three times in PBS, the cells are suspended to an 80% haematocrit in PBS. One ml of the cell suspension is then layered over 1.5 ml each of 27.5% (w/v) Nycodenz® (ρ = 1.145 g/ml) and 15.7% (w/v) Nycodenz® (ρ = 1.105 g/ml). After centrifugation at 1000 *g* for 20 min (slow acceleration and no brake to decelerate the rotor) the light and dense erythrocyte layers are harvested; diluted with at least 3 vol. of PBS and the cells collected by centrifugation at 2000 *g* for 8 min.

3 OptiPrep™ applications

3-1 Introduction

Gradients of iodixanol are able to resolve, at least partially, different sub-populations of normal human erythrocytes, sickle erythrocytes and reticulocytes. One of the first publications reporting the use of OptiPrep™ by Kamiyama et al [10] described the use of discontinuous gradients of iodixanol (covering the density range 1.097-1.104 g/ml) and RCFs of 1000 *g* for 30 min to separate younger from older human erythrocytes in their studies on the correlation of deformability and CR1 activity. Holtzclaw et al [11] employed a similar gradient (1.077-1.107 g/ml) to separate sickle erythrocytes on the basis of density in order to analyze the production of low-density cells from high-density cells by oxygenation or oxygenation/deoxygenation cycles. Again the RCF used was 1000 *g*. In both of these studies the erythrocyte fraction was layered on top of the gradient. More recently Inaba et al [12] have used a discontinuous 1.077-1.100 g/ml gradient to obtain a reticulocyte-enriched fraction by flotation from whole blood, layered beneath the gradient after adjustment of the plasma to 1.105 g/ml.

Sections 3-2 and 3-3 describe the execution of the methods described in refs 10-12; there is also an extensive Notes Section (3-4) containing brief comments on some methodological variations. Section 4 summarizes a few of the most recently published methods.

3-2. Iodixanol solution preparation

To keep the buffer concentration in all gradient solutions constant and their osmolality approx 290-305 mOsm, make a 50% (w/v) iodixanol working solution before making further dilutions with a buffered saline as follows:

A. OptiPrep™(shake the bottle gently before use)
B. OptiPrep™ Diluent: 0.85% NaCl, 60 mM HEPES-NaOH, pH 7.4
C. Working Solution (50% iodixanol): mix 5 vol. of Solution A with 1 vol. of Solution B
D. Buffered saline: 0.85% (w/v) NaCl, 10 mM HEPES-NaOH, pH 7.4

> Keep the following stock solution at 4°C:
> 500 mM HEPES (free acid): 11.9 g per 100 ml water.
>
> Solution B: Dissolve 0.85 g NaCl in 50 ml water; add 12 ml of buffer stock solution; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.
>
> Solution D: Dissolve 0.85 g NaCl in 50 ml water; add 2 ml of buffer stock solution; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3-3 Protocols

3-3-1. Fractionation of erythrocytes in a four-step discontinuous gradient

1. For sickle cell studies make up four gradient solutions of 1.077, 1.087, 1.097 and 1.107 g/ml [11] by mixing Solution C with Solution D, equivalent to approx. 13.5%, 15.5%, 17.5% and 21.5% (w/v) iodixanol respectively (see Notes 1, 2, 7 and 9).

   For studies on normal human erythrocytes make up four gradient solutions of 1.097, 1.099, 1.101, 1.104 g/ml [10] equivalent to approx. 17.5%, 17.9%, 18.2% and 18.8% (w/v) iodixanol respectively (see Notes 1 and 2).

2. Wash the erythrocytes three times in PBS and then resuspend in this solution to 50% haematocrit.

3. Produce a discontinuous gradient from 2.5 ml of each of the density solutions, by underlayering using a syringe and metal cannula (see Note 3).

4. Layer 1 ml of the blood cells on top of the gradient and centrifuge at 1000 *g* for 30 min at 20-22°C (see Note 4).

5. Collect specific density fractions by aspiration or harvest the entire gradient by upward displacement with a dense medium or aspiration form the bottom of the tube (see Notes 5-9).

3-3-2. Isolation of a reticulocyte-rich fraction in a four-step discontinuous gradient

1. Make up four gradient solutions of 1.077, 1.092, 1.095 and 1.100 g/ml [12] equivalent to approx. 13.5%, 16.5%, 17%, and 18% (w/v) iodixanol respectively (see Notes 1, 2, 10,11).

2. Adjust the density of whole blood to 1.105 g/ml (for blood with an haematocrit of 45% mix 2.1 ml of OptiPrep™ with 10 ml of blood) OR for a total erythrocyte fraction in buffered saline, then at a 45% “haematocrit”, mix 2.6 ml of OptiPrep™ with 10 ml of the suspension (see Note 12).

3. Layer 1-2 ml of sample under the gradient and centrifuge at 1000 *g* for 30 min at 20-22°C (see Notes 4 and 13).

4. Collect specific density fractions by aspiration or harvest the entire gradient by upward displacement with a dense medium or aspiration form the bottom of the tube (see Notes 14-16).

3-3-3. Separation of leukocytes and erythrocytes from human blood

A mixture containing 12% (w/v) iodixanol and 1.66 % (w/v) Methocel in 130 mM NaCl effectively removes erythrocytes [13,14] for the subsequent separation of PBMCs and PMNs using an iodixanol discontinuous gradient **(see “Polymorphonuclear leukocytes” Application Sheet C12 in index)**. Austin et al [15] removed the erythrocytes from a suspension of mouse bone marrow cells by centrifugation at 900 *g* for 15 min over a density barrier of 16 % (w/v) iodixanol in 10 mM HEPES-NaOH, pH 7.4 (containing 0.1% azide)

3-4. Notes

1. Check the density of the solutions by refractive index; if a refractometer is not available optical absorbance is an alternative way of determining density: **see General Methods: “Analysis of gradients” Application Sheet C51 in index**.
2. It may be necessary to customize the gradient density range to the operator’s requirements, once the efficacy of one or other of these gradients has been determined.
3. Discontinuous gradients are normally most easily prepared by underlayering (i.e. low density first) using a syringe (2 ml) and a long metal cannula; overlayering solutions, particularly those which differ in density by only a small amount, is more difficult. One alternative for overlayering is to use a small volume (low-pulsating) peristaltic pump; first to take up the required volume of solution into the attached tubing and second, reverse the flow, to expel it slowly on to a denser

layer in the centrifuge tube. For more information about preparing gradients **see Application Sheet C02**.

4. Do not use the brake to decelerate the rotor.
5. Any appropriate means of harvesting the gradient may be used.
6. Using the 1.097-1.104 g/ml gradient [10]; erythrocytes that banded in the 1.097 g/ml layer exhibited approx four times the CR1 activity and greater (approx 40%) elongation index than those in the 1.104 g/ml layer.
7. Using the 1.077-1.107 g/ml gradient [11,16]; high-density sickle erythrocytes band in the 1.107 g/ml layer.
8. Huang et al [17] employed an interesting alternative approach that avoids the requirement of making shallow gradients for the fractionation of sickle venous blood. By mixing the blood with an isoosmotic solution of iodixanol prepared from OptiPrep™, phosphate-buffered saline and dextran, the authors separated a dense population (~ 15%) that sedimented from the remainder, which floated. The latter was collected and adjusted to a lower density and re-centrifuged to produce a second pellet (~45%) and another floating fraction. In this manner, three fractions of light, median and high density, each of which had a distinctive deformability coefficient.
9. Using a discontinuous gradient Arnold et al [18] studied two fractions; 1.093-1.100 g/ml (normal) and >1.120 g/ml (sickle). More recently Barber et al [19] used a discontinuous gradient with densities of 1.073, 1.087, 1.093, 1.100 and 1.120 g/ml (1000 *g* for 30 min) to resolve hydrated sickle cells (≥1.093 g/ml) and pathologically dehydrated sickle cells (≥1.120 g/ml)
10. Joiner et al [20] used a 1.075, 1.080, 1.085, 1.090, 1.095, 1.100 g/ml gradient to enrich for reticulocytes and fractionate cells according to age; the blood was top-loaded and centrifuged at 3000 *g* for 5 min. In the method of Sentürk et al [21] the densities were 1.075, 1.085, 1.095, 1.105 and 1.115 g/ml and the centrifugation carried out at 2,500 *g* for 25 min.
11. Gifford et al [22] prepared a very shallow continuous gradient of iodixanol of 16-19.6% (w/v) in a 15 ml tube; the blood was bottom-loaded; centrifugation was carried out at 3100 *g* for 20 min and the topmost layer was collected as a reticulocyte fraction.
12. In the method to purify a reticulocyte-enriched fraction, Inaba et al [12] used whole blood, but the method may work equally well with a total erythrocyte-fraction.
13. Inaba et al [12] used 800 *g* for 30 min.
14. Using the 1.077-1.100 g/ml gradient the reticulocytes band close to the top interface [12].
15. Larkin et al [23] also collected reticulocytes as a <1.077 g/ml fraction.
16. The Inaba et al gradient [12] was also used by Sarachana et al [24] and Vu et al [25] to isolate a pure fraction of mature erythrocytes for their studies on the role of small non-coding RNAs in blood storage lesions; the fraction contained no leukocytes and only 0-2 reticulocytes per 1000 cells.

4. Recent publications on erythrocyte/reticulocyte fractionation

4-1 Simple mixer technique

Mixing OptiPrep™ with whole blood (1:8 v/v) raises the density of the plasma sufficiently to allow a total erythrocyte fraction to be harvested by centrifugation at 2000 *g* for 15 min [26].

4-2. Age-related effects

More recently fractionations of erythrocytes were carried out on discontinuous iodixanol gradients of the following densities 1.085, 1.090, 1.095, 1.100, 1.105 and 1.110 (equivalent to 15%, 16%, 17%, 18%, 19% and 20% w/v iodixanol) obtaining 7 erythrocyte subfractions [27] that demonstrated an age-related density and IgG increase together with a loss of phospholipid asymmetry. A review of techniques for studying erythrocyte aging is documented in ref 28. Studies on exercise have also been assisted by the ability of iodixanol gradients to discriminate erythrocytes on the basis of age [29].

4-3. Sickle cell studies

A similar range of gradient densities (1.075, 1.085, 1.090, 1.095, 1.100 and 1.105 g/ml) centrifuged at 1875 *g* for 30 min was used in studies of ROS production in sickle cells [30 Other gradients covering, broadly, the same density range (sometimes with fewer layers) have also been shown to be effective in sickle cell studies. The mean corpuscular haemoglobin content was shown to rise over four fractions of increasing density from 27.3 to 50 $g.dl^{-1}$ [31]. Four populations of sickle cells (1.081-1.091, 1.091-1.100, 1.100-1.110 and >1.110 g/ml) were also studied by Du et al [32]. Hosseini et al [33] described a similar gradient of 1.081, 1.091, 1.100 and 1.111 g/ml, which was top-layerd with the blood sample and centrifuged at 820 *g* for 30 min. Cells were observed at each interface plus a pellet; the sickle cells in the lowermost interface and pellet were not observed in samples from healthy donors. Light, intermediate and dense fractions were obtained by Hannemann et al [34] who interestingly tailored the gradient density ranges to the individual, HbSC or HbSS.

More recently Kiser et al [35] used a four layer iodixanol gradient of densities 1.077, 1.087, 1.097 and 1.107 g/ml to fractionate erythrocytes in a study of neutrophil activation.

4-4. Red cell progenitor subsets

Gradient solutions of Ficoll™ and OptiPrep™ of densities 1.091, 1.109 and 1.117 g/ml have been reported [36].

4-5. Reticulocytes

In a study of microcytic anaemia in mice by Blanc et al [37] an iodixanol gradient of 1.083, 1.087, 1.091, 1.095 and 1.100 g/ml centrifuged at 141,000 *g* for 30 min was used. Reticulocytes were very clearly resolved in the lower density fractions and there was a pronounced and significant shift towards these lighter density cells in the knock-out mice compared to the normal mice. Mouse reticulocytes were also isolated in iodixanol gradients by Shimizua et al [38]

5. Acoustic wave studies

An isotonic solution of iodixanol has been used to maintain erythrocytes in suspension for microscopic acoustic wave studies [39].

6. Recent studies using highly purified erythrocytes

More recently iodixanol-gradient purified erythrocytes have been used in extracellular vesicle studies [40]; in studies that required leukocyte-free preparation [41] and in migration velocity measurements [42].

7. References

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