OptiPrep™ Application Sheet C24

Isolation of a progenitor cell-enriched fraction

- 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
- Section 1 describes the more traditional sedimentation from a cell suspension on to a density barrier; Section 2 describes the use of the sample itself as the density barrier; Section 3 briefly describes a strategy for neural tissue progenitors

1. Sedimentation on to a density barrier

1a Introduction

The study of the proliferation and maturation of stem cells from bone marrow is a major area of cell and molecular biology research. Usually the resolving power of simple density barriers is insufficient to allow progenitor cells to be isolated in a sufficiently pure form for further analysis and culture. Nevertheless, the low-density cell fraction harvested from the barrier interface can provide a progenitor cell-enriched population that allows a more effective and economical use of immuno-magnetic beads to remove lineage cells (using a cocktail of lineage-specific monoclonal antibodies).

Although metrizamide was used for density barriers prior to 1984, the lower cell toxicity of Nycodenz®, made this the medium of choice. Mayanagi et al [1] also emphasized the lack of toxicity of Nycodenz® (compared to Percoll®) and the avoidance of positive selection with antibody-beads was important in recovering viable progenitor cells. Bertoncello et al [2,3] reported the use of a 1.085 g/ml Nycodenz® barrier to isolate low-density cells from a suspension of nucleated bone marrow cells; 3 ml containing 10^8 cells were layered over 5 ml of the density barrier and centrifuged at 100 *g* for 30 min, at 4°C and the progenitor cell-enriched fraction harvested from the interface. A barrier of the same density was used by Erlich et al [4] and Sitnicka et al [5]. Lower density barriers, 1.080 g/ml [6], 1.077 g/ml [7-10] and 1.068 g/ml [11] have also been used and frequently the centrifugation is carried out at room temperature rather than at 4°C. In some cases, a shorter centrifugation time (20 min) has been employed at 1000 *g* [12]; in others a g-force of 400 *g* was used [5,6]. The 1.077 g/ml density barrier has been widely used since ready-made solutions of this density were previously available as NycoPrep™ 1.077 and NycoPrep™ 1.077A, the latter being the more popular for murine samples (see also Section 3). The density barrier approach has since been adapted to OptiPrep™; several publications report the use of a ρ = 1.08 g/ml iodixanol barrier [13-17]. As with the Nycodenz® methodology however, there are a number of variations for the density of the iodixanol barrier: 1.077 g/ml [18-21] and 1.074 g/ml [22].

Progenitor-enriched fractions have also been isolated from human blood [23] and cord blood [24] using NycoPrep™ 1.077; those from murine blood [25,26] and fetal liver [27-30] on NycoPrep™ 1.077A. Again the *g*-force is rather variable – 400-1000 *g*. Using OptiPrep™, progenitor cells have been enriched from blood (1.072 g/ml) [31] and thymus (1.05 g/ml) [32-34].

- **Important note**: As many Nycodenz® gradient solutions were routinely used as NycoPrep™ 1.077A or diluted from NycoPrep™ 1.15, neither of which are now commercially available, the following methodology is based on the use of OptiPrep™ (a sterile solution of 60% w/v iodixanol) to avoid making up solutions from Nycodenz® powder.

- Modified density barriers, in which the density of the sample is raised before layering on the barrier, or the use of discontinuous gradients, may provide higher enrichments of progenitor cells than a simple density barrier. For more information on the advantages of using such techniques **see "Hepatic Kupfer cells", Application Sheet C28 in index.**

1b. Solutions required (see Note 1)

A. OptiPrep™ (shake the bottle gently before use)
B. Iscove's modified Dulbecco's medium Hank's (IMDM)
C. Fetal calf serum (FCS)
D. Phosphate buffered saline (PBS) **OR**
E. Tricine buffered saline

Keep Tricine as 100 mM stock solution at 4°C; 1.79g per 100 ml water.

Solution E: Dissolve 0.85g NaCl in 50 ml water; add 10 ml of Tricine stock solution; adjust to pH 7.0 with 1 M NaOH and make up to 100 ml.

1c. Protocol

Carry out all operations at room temperature (see Note 2)

1. Harvest bone marrow material in Solution B containing 2% FCS.

2. Dissociate the bone marrow cells by passing three times through a 25-gauge needle.

3. Filter the suspension through 200 μm nylon mesh.

4. To prepare a density barrier solution of approx 395 mOsm, dilute OptiPrep™ directly with Solution D or E (with or without 5% FCS) to give a solution of the chosen density (see Table 1 and Notes 3 and 4).

Table 1 Density of iodixanol solutions prepared by dilution of OptiPrep with Solutions D or E

Density (g/ml)	OptiPrep	Solution D/E
1.050	8.5 vol.	51.5 vol.
1.063	11.0 vol.	49.0 vol.
1.068	12.0 vol.	48.0 vol.
1.077	13.5 vol.	46.5 vol.
1.080	14.0 vol.	46.0 vol.
1.085	15.0 vol.	45.0 vol.
1.090	16.0 vol.	44.0 vol.

5. To prepare a density barrier solution of 1.077 g/ml and osmolality approx 265 mOsm (equivalent to Nycoprep 1.077A) start by diluting Solution E with water (volume ratio 2.5:0.5 respectively); this solution has an osmolality of approx 242 mOsm. Dilute OptiPrep™ with the 242 mOsm solution using a volume ratio of 2.7:9.3 respectively.

6. Layer 5 ml of the bone marrow cell suspension (10^7 cells/ml) over 3 ml of the chosen density barrier (see Note 5).

7. Centrifuge at 450 *g* for 20 min; allow the rotor to decelerate without the brake (see Notes 6 and 7).

8. Collect the cells from the interface; dilute with an equal volume of Solution D or E (+ 5% FCS); wash twice and resuspend in the same medium prior to lineage committed cell depletion.

1d. Notes

1. To dilute the OptiPrep™ use any suitable medium, two examples are given as Solutions D or E; culture medium may also be used. As long as the solution is isoosmotic, then the dilutions will also be isoosmotic.
2. Some papers report the use of centrifugation temperatures of 4°C.
3. Step 5 describes the preparation of an alternative reduced osmolality OptiPrep™ density solution.
4. Dilutions of OptiPrep™ with any isotonic medium (including culture medium) will give the same densities as those shown in Table 1. These diluents all have a density of approx 1.006 g/ml. Inclusion of FCS will however modulate the density slightly upwards. FCS has a density of approx 1.032 g/ml, so any balanced salt solution containing 10% FCS for example will have a density of 1.009 g/ml. The density of the OptiPrep™/saline mixtures given in Table 1 will be increased by 0.002-0.003 g/ml. If 5% FCS is used the density difference is proportionately less. For more information on preparing gradient solutions see **Application Sheet C01**.
5. Since the megakaryocytic progenitor cells are less dense than the majority of other cell types, it may be worth considering using a flotation strategy in which the bone marrow cell suspension is adjusted to the barrier density (e.g. 1.085 g/ml) and the progenitor cells harvested from the top of the solution. This approach has been used for fetal liver cells [35].

6. The published g-force, time and temperature values are rather variable. What effect lower or higher values might have on the separation can only be determined by experimentation.
7. Using the brake may create a vortex in the liquid and cause loss of definition of the interfacial band and even contamination from denser cells.

2. Progenitor cells from blood using a mixer strategy

This was first introduced for the isolation of mononuclear cells from human blood and later rat and mouse blood (**see "Mononuclear cells" Application Sheets C05, C07 and C08 respectively in index**). It has also been applied to the isolation of progenitor cells. The crude cell suspension is PBS + 5% FCS (30 ml) was mixed with 10.1 ml of OptiPrep™ and 4 ml of water. This adjusts the cell suspension medium to approx 1.077 g/ml. A small volume of buffered saline is placed on top to prevent the low-density cells from banding at an air-liquid interface during centrifugation [36-41]. The strategy avoids the build up and possible aggregation of cells at the interface between the sample and the density barrier in the standard barrier sedimentation strategy (see Section 1). It allows the denser cells to sediment while the low-density cells float. The 4 ml of water used in the preparation of the sample could certainly be replaced with saline if required, without affecting the separation.

- In a symposium abstract Hu et al [42] also reported a new OptiPrep™-based method for the purification of blood-derived mesenchymal stem cells.

3. Discontinuous gradients

A novel method [43,44] used gradient solutions produced by mixing OptiPrep™ with NycoPrep 1.077A. The latter is now commercially unavailable but a solution of the same density and reduced osmolality can be produced from OptiPrep™ by dilution with a reduced osmolality saline solution **see "Mononuclear cells" Application Sheet C43 in index for more information**) Solutions can be prepared as follows: use 0.7% (w/v) NaCl, 10 mM Tricine, pH 7.0 to dilute OptiPrep™ to produce solutions of 1.050, 1.080, 1.090 g/ml (approx. iodixanol concentrations if 8.4, 14 and 16% w/v). The gradient is centrifuged at 400 *g* for 15 min to isolate a low density fraction.

The iodixanol density gradient devised by Brewer et al [45] for the isolation of hippocampal motoneurons is also used as a gradient for purifying neural progenitor cells [46-50]. The method is described in detail in **"Brain motoneurons" Application Sheet C36 in index**. Abbosh et al [50] reported a two-step gradient of 1.058 and 1.11 g/ml for postnatal hippocampal precursors; He and Shen [52] and Nunan et al [53] also used a discontinuous iodixanol gradient for the isolation of glial progenitor cells, but gave no details.

The oval cells of liver, which are considered to be the equivalent of progenitor cells in the liver, may potentially be propagated *in vitro* and used restoratively in some liver diseases. Three layer gradients of 13%, 16 and 18% (w/v) iodixanol (OptiPrep™ diluted with HBSS, supplemented with 0.2% BSA (10 ml, 10 ml and 5 ml respectively) are overlaid with the non-parenchymal cells (see Application Sheet C24) in 11% iodixanol and centrifuged at 6,500 *g* for 30 min [54]. The oval cells are enriched at the 11/13% interface.

4. References

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