OptiPrep™ Application Sheet C14

Fractionation of a mixed population of cells

- 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

If neither an OptiPrep™ or a Nycodenz® based method is not presently available for the purification or enrichment of a particular cell type from a body fluid or lavage or from a mechanically or enzymically-dissociated tissues, this Application Sheet contains some simple suggestions for the design of a new gradient system. Although there may be a published method for the isolation of a particular cell type using one of the polysaccharide or colloidal silica (Percoll®) media, the use of OptiPrep™ will provide several important advantages. The very low endotoxin levels of OptiPrep™ and the lack of interaction of iodixanol with cells are important properties not exhibited by these other media. This Application Sheet will confine its recommendations to the use of OptiPrep™ because of the ease of preparation of gradient solutions (simple dilution of OptiPrep™ with saline); Nycodenz® gradient solutions must be prepared from Nycodenz® powder.

Although positive selection with antibody-bound magnetic beads may provide highly purified cells very easily, the interaction of beads with the cell surface may lead to unpredictable functional changes. Even if an OptiPrep™ based gradient cannot provide the purity of positive selection, a preliminary enrichment of particular cell type can make the subsequent use of negative selection with antibody-bound beads a more economical and very attractive alternative strategy.

Although the "traditional" means of applying a cell suspension to pre-formed gradient is simply to layer it on top, there alternative strategy of layering it in a dense solution under the gradient is an increasingly used alternative that can often provide improved resolution. This is particularly the case if the aim is to isolate a minor population of low-density cells from a mixture of predominant denser cells.

- Any non-viable cells should be removed from the suspension during the preliminary stages of any gradient fractionation; see **Application Sheet C13**.

2. Solutions and reagents required (see Note 1)

A. OptiPrep™

B. OptiPrep™ Diluent: 0.85% (w/v) NaCl, 40 mM HEPES-NaOH, pH 7.4

C. Working Solution (WS) Diluent: 0.85% (w/v) NaCl, 20 mM HEPES-NaOH, pH 7.4

Keep HEPES (free acid) as 100 mM stock solution at 4°C; 2.38 g HEPES per 100 ml water.

Solution B: Dissolve 0.85 g NaCl in 50 ml water, add 40 ml of buffer stock solution; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml.

Solution C: Dissolve 0.85 g NaCl in 50 ml water, add 20 ml of buffer stock solution; adjust to pH 7.4 with 1 M NaOH; make up to 100 ml.

3. Protocols

3a. Iodixanol working solution preparation

1. Shake the bottle of OptiPrep™ gently before use.
2. Make a 30% (w/v) iodixanol working solution (approx ρ = 1.16 g/ml) by mixing equal volumes of OptiPrep™ and Solution B. Dilute further with Solution C to produce solutions of lower density.

3b. Preparation of cell suspension for gradient loading

3b-1. Non-viable cells removed using Strategy 1 of Application Sheet C13

If in the subsequent fractionation, the cells are to be layered on top of the gradient: harvest the cell band (taking as little of the density barrier as possible). Dilute the suspension with 2 vol. of Solution C and pellet the cells at approx 250-400g for 10-20 min. Resuspend the pellet in Solution C.

If in the subsequent fractionation, the cells are to be layered beneath the gradient: discard as much of the top layer as possible and harvest the cell band, together with most of the density barrier, but avoiding the pellet. Then mix gently to resuspend the cells: the density of the suspension should be approx 1.12 g/ml.

3b-2. Non-viable cells removed using Strategy 2 of Application Sheet C13

If in the subsequent fractionation, the cells are to be layered on top of the gradient: harvest the cell band in the top layer of culture medium (taking as little of the 1.12 g/ml layer as possible) and dilute with 3 volumes of Solution C or culture medium. If the density of the cell suspension is not less than that of the top of the subsequent gradient, the cells will have to be pelleted and resuspended (see above).

If in the subsequent fractionation, the cells are to be layered beneath the gradient: discard as much of the top layer of culture medium and harvest the cell band in 4-5 ml of the 1.12 g/ml layer. The density of this suspension may need to be increased by mixing with a small volume of the 1.16 g/ml iodixanol Working Solution.

3c. Fractionation by buoyant density

1. Prepare a preformed, continuous gradient, with a density range of 1.03 to 1.10 g/ml (this is approximately equivalent to 5-20% (w/v) iodixanol. Alternatively construct a discontinuous gradient with several layers covering the same density range (see Notes 4 and 5).

2. Layer the recovered cell sample (see Note 4) either under or on top of the gradient and centrifuge at 800-1000 *g* for 20-30 min at 20°C (or 4°C) in a swinging bucket rotor (see Note 6).

3. Identify the cell types in each band, which, during centrifugation, will band at respective their buoyant densities (see Notes 7-9).

- With a little patience and experimentation, a suitable separation method can be developed. Once the banding characteristics have been determined, it may be possible to devise a simplified density barrier or two-step discontinuous gradient.

3d. Fractionation on the basis of cell size

1. If two types of cell, with the same buoyant density, but of different sizes are present, the larger will sediment (or float up) more quickly than the smaller until they reach the point in the gradient equivalent to their buoyant density. With continuing centrifugation, the smaller cells will reach the same point. So time of centrifugation is important in separations by size.

2. Suspend the cells in Solution C and layer them (see Note 10) on top of a preformed continuous gradient (approx 1.03-1.09 g/ml).

3. Centrifuge at 600 g_{av} for 10 min and examine to determine if a separation has been achieved. If not try other centrifugation times (8, 12, 15 min etc).

4. Notes

1. All gradient solutions prepared as described in Section 2 will have an osmolality in the range 290-305 mOsm. Solution B has double the buffer concentration of that of Solution C so that the buffer concentration in the gradient solutions (and hence in the gradient) is constant. The same principle could be applied to any other low concentration additive that might be deemed an important

component of the gradient for maintenance of cell viability, e.g. $MgCl_2$ and/or $CaCl_2$ at 1-2 mM. If this is unimportant then Solutions B and C may be identical and be any isotonic buffered saline, balanced salt solution or a routine culture medium; none of these substitutions will have a significant effect on the final density or osmolality of the gradient solutions. If 10% serum is included then this will slightly increase the density of all the solutions. For more information see **Application Sheet C01**.

2. There are some instances in which the cells may benefit from and increase in ionic strength of the gradient solutions, see for example **Application Sheet C09**; or the use of a significantly hyperosmotic medium maybe beneficial to the separation of a particular cell type, see for example **Application Sheet C15**.
3. For information on the preparation of discontinuous and continuous gradients for cell separations see **Application Sheet C02**.
4. If the step to remove non-viable cells is omitted the cells can be suspended directly in a high-density solution (approx 22.5% (w/v) iodixanol or Nycodenz®) for bottom-loading or in Solution C for top-loading.
5. It may be beneficial to carry out the centrifugation at 4°C in some cases, in which case it may be necessary to increase the time of centrifugation.
6. The resolving power of a discontinuous gradient depends on the density interval of adjacent layers. Cells that band at any interface will have a range of densities between those of the two layers.
7. If the cells of interest co-band with other types, then the gradient may be too steep to resolve them, in which case a shallower gradient (or layers covering a smaller density interval) may be required. Alternatively different cell types may have the same density, and only be separable on the basis of size.
8. If all of the cells band either towards the top or bottom of the gradient, its density range should be modulated to avoid this.
9. Resolution on the basis of sedimentation (or flotation) rate is inversely proportional to the depth of the sample; sample volume must therefore be kept to a minimum.

Application Sheet C14; 7th edition, May 2016

AXIS-SHIELD
Innovation for Life