OptiPrep™ Application Sheet C32

Analysis of apoptotic events in 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

A simple density barrier technique is available for the removal of non-viable cells either from tissue digests or from cultured cells. Most viable mammalian cells have a buoyant density in the range ρ = 1.07-1.12 g/ml in isoosmotic gradients of iodixanol. Non-viable cells, which no longer enclose an osmotic space, are much denser (ρ>1.15 g/ml) and thus are easily separated from the viable cells across a density barrier; for details **see "Viable/non-viable cells" Application Sheet C13 in index**.

A natural extension of this strategy is to the analysis of apoptotic events in cells. Francois et al [1] have now developed a protocol for studying these events in asynchronous cultures of rat pheochromocytoma (PC12) cells (a line of neuronal cells). The use of density gradients to help analyze these events overcomes the disadvantages of other techniques; the artificiality of synchronization; the need to use cell-free systems or the restrictions imposed by observations on single cells. Francois et al [1] have developed a multi-step discontinuous gradient. The multi-step approach permits the sample to be made part of one of the steps in the middle of the gradient. By adopting this strategy, the lower density cells can migrate to their banding density by flotation, while other ones may either remain close to the load zone or sediment to denser regions of the gradient. This strategy should provide for optimal resolution as it prevents the inevitable build-up and possible aggregation of cells, which all move in the same direction in the centrifugal field, when either top- or bottom-loaded on to a gradient.

Although the method has been developed for a specific cell type, there is no reason why the strategy cannot be extended to other cell systems, although it may be necessary to modulate the density of one or more of the steps in order to optimize the resolving power of the gradient.

In principle, it may be possible to use a continuous gradient, but to achieve the same sort of resolution it would be necessary to mix the cell suspension with the low- and high-density solutions that are used to create the gradient. This could be conveniently carried out in a Gradient Master™ in which the centrifuge tube is sealed after layering one solution on top of the other (i.e. sterile conditions can be easily maintained) but not in a two-chamber gradient maker. For more information on the Gradient Master™ **see Application Sheet C02**.

The following protocol is adapted from ref 1.

2. Solutions required

A. OptiPrep™ (shake gently before use)
B. Phosphate buffered saline (PBS)
C. Cell suspension medium: 0.1% (w/v) glucose, 0.1% (w/v) bovine serum albumin (BSA) in PBS (see Note 1)
D. OptiPrep™ Diluent: 0.3% (w/v) glucose, 0.3% (w/v) (BSA) in PBS (see Notes 2 and 3)
E. Working Solution (40% w/v iodixanol): Mix 4 vol. of Solution A with 2 vol. of Solution D

3. Protocol

1. Prepare the following density gradient solutions by diluting Solution E with Solution C: 37.5%, 31.8%, 26.0%, 22.2%, 18.3%, 14.5%, 10.6% (w/v) iodixanol; these are equivalent to the following densities: 1.20, 1.17, 1.14, 1.12, 1.10, 1.08 and 1.06 g/ml and bring to 4°C.

2. After treatment of the cell culture as required (e.g. serum withdrawal), wash them once Solution D and resuspend in the 18.3 % iodixanol solution (see Notes 4 and 5).

3. Prepare a discontinuous gradient from equal volumes (1 ml) of each of the iodixanol solutions; underlayering is probably the easiest way of doing this (see Figure 1 and Notes 6-9).

4. Centrifuge at 1000 *g* for 30 min at 4°C (see Note 10).

5. Collect the gradient in approx. twenty fractions and analyze as required (see Note 11).

4. Notes

1. Solution C may be any suitable isoosmotic balanced salt solution compatible with the cells of choice.
2. Preparing a Working Solution (D), and diluting this with Solution C to make the gradient solutions, keeps the concentration of glucose and BSA in the gradient constant.
3. For more information on the preparation of solutions for density gradients, **see Application Sheet C01**.
4. Keep any cell washing to a minimum prior to loading the cells on the gradient, in order to reduce any physical assaults on the cells, which might cause loss of cell viability and thus compromise the observed effect of apoptosis.

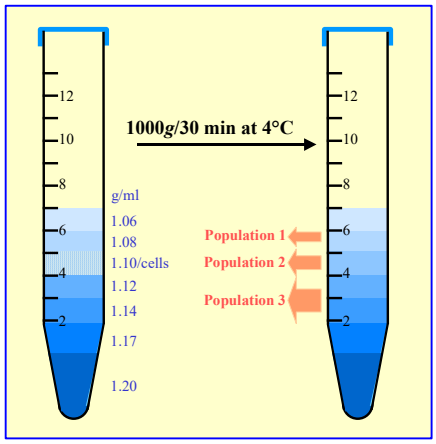

Figure 1: Analysis of apopotosis in discontinuous iodixanol gradient (see Note 10)

5. Because of the small size of the gradient steps (1-2 ml) and the small difference in density between adjacent steps, it is important that the iodixanol solution (1.10 g/ml) that is used to suspend the cells is not significantly diluted by any residual medium above the pellet. It may be more reliable to resuspend the cells in a small volume of medium and then to mix 1 vol. of cell suspension with 1 vol. of 36.6% (w/v) iodixanol.
6. Underlayering using a metal cannula attached to a 1-2 ml syringe is the best way of creating multiple step gradients. Alternatively a small volume ("low-pulse") peristaltic pump might be used to introduce each layer, dense end first. Use the pump to take up the aliquot of solution and then reverse the flow to expel into the centrifuge tube. For more information about preparing gradients **see Application Sheet C02**.
7. The choice of tube type will depend to a large extent on the mode of gradient collection. The vast majority of cell density gradient separations are carried out in routine thick-walled polypropylene centrifuge tubes. These tubes can be used for this application if the collection of the gradient is carried out by manual aspiration from the meniscus (using a 1 ml syringe attached to narrow-bore flat-tipped metal cannula). Aspiration from the meniscus can also be carried automatically using the Auto Densi-Flow™, produced by the Labconco Corporation comprises a hollow metal tube that terminates in a small collection head. Although this device is no longer produced by Labconco, many remain available in laboratories and second hand machines are available from instrument "recycling" companies. The only other alternative is to introduce a narrow tube to the bottom of the centrifuge tube and to aspirate the gradient dense-end first using a peristaltic pump.
8. If a thin-walled (round-bottomed) tube is used, then collection from the bottom of the tube is feasible using tube puncture or upward displacement with a dense medium. For more information on all these gradient collection methods **see "Virus Application Sheet Index" - Application Sheet V04. Note that although V04 is part of the Virus Application data, the methods apply to the harvesting of any gradients**.

9. During the preparation and centrifugation of the gradients the interfaces between each of the layers will become less well-defined due to the small amount of mixing that is bound to occur during the setting up and centrifugation and to diffusion.
10. Do not use the brake to decelerate the rotor.
11. Such a device is incorporated into the Beckman Fraction Recovery System.
12. Francois et al [1] analyzed each fraction by refractive index (in order to determine the density) and a number of markers for apoptosis (see Figure 1). Population 3 cells (median density approx 1.07 g/ml) exhibited no chromatin condensation, which was significant in Population 2 (median density approx 1.085 g/ml) and increased as the density of the cells increased. Oligonucleosomal DNA fragmentation was most noticeable in Population 1 (median density approx 1.11 g/ml) but barely detectable in Population 2, and absent in Population 3.

5. References

1. Francois, F., Godinho, M. J., Dragunow, M. and Grimes, M. L. (2001) *A population of PC12 cells that is initiating apoptosis can be rescued by nerve growth factor* Mol. Cell Neurosci., **18**, 347-362

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