OptiPrep™ Application Sheet C24

Purification of hepatic non-parenchymal cells on a density barrier

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **Axis-Shield Mini-Review "Hepatic non-parenchymal (stellate, Kupffer and endothelial) cells –a short methodological survey" (MC08)** compares some of the current methodologies
- To access **MC08** return to the initial list of Folders and select "**Mini-Reviews**"
- Note that there are two related Application Sheets: **C33 "Preparation of stellate cells from liver and pancreas"** and **C50 - "Enrichment of hepatic Kupffer cells in a discontinuous gradient".**
- 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

Parenchymal and non-parenchymal cells (PC and NPC) may be prepared by collagenase digestion of the liver using a tissue perfusion system. The PC are then separated from the NPC by differential pelleting at 50 *g* for 1-4 min. It is however necessary to repeat this centrifugation (maybe twice more) to remove PC from the supernatant; moreover the NPC yield is usually low. It is both more common and more effective to carry out the 50 *g* centrifugation once; to harvest all the cells from the supernatant by centrifugation at a higher *g*-force and then use a density barrier prepared from one of the of iodinated density gradient media to resolve the two types of cell. Many workers prefer a modified perfusion strategy, which involves a mixture of collagenase and Pronase or *Clostridium perfringens* enterotoxin to destroy the PC selectively [1,2].

The purification of NPC is a starting point for the isolation of Kupffer and endothelial cells; the NPC are then processed further by centrifugal elutriation to obtain reasonably pure populations of these important cells, although there are instances of the reverse situation in which the elutriation is the first step (e.g. ref. 3). Although metrizamide was widely used for the density barrier step prior to 1984, Nycodenz® subsequently became more popular (over 200 papers) because of its lower toxicity to cells. More recently iodixanol gradients have become a popular choice and because of the ease of gradient solution preparation from OptiPrep™, the following methodology is based solely on use of this medium.

- Note that two-layer discontinuous gradients are able to improve the purity of Kupffer cells, **see Application Sheet C50.**

2. Solution selection and preparation

2a. Buffer salt solution

A crude NPC preparation may be suspended in a routine balanced salt solution such as Hank's Balances Salt Solution (HBSS) or more often this is an NPC customized medium such as Gey's balanced salt solution and this may be prepared as described in the box. The chosen solution is also used in the preparation of the gradient solutions. Unless the solution contains 10% serum, the density of any balanced salt solution (or culture medium) is likely to be very similar, if not identical, to GBSS or HBSS (i.e. approx 1.006 g/ml). A medium containing 10% serum has a density of approx 1.009 g/ml and the amount of iodixanol should be adjusted to account for this. For more information about making up density gradient solutions. For more information on the preparation of gradient solutions see **Application Sheet C01.**

GBSS
Dissolve the following in 500 ml water:
7.0 g NaCl
0.37 g KCl
70 mg $MgSO_4.7H_2O$
150 mg $Na_2HPO_4.2H_2O$
220 mg $CaCl_2.2H_2O$
2.27 g $NaHCO_3$
30 mg KH_2PO_4
210 mg $MgCl_2.6H_2O$
1.0 g glucose

Make up to 1 liter of water and gas with 5% CO_2/air. The pH should be 7.4.

2b. Gradient solution preparation

The rationale regarding the preparation of iodixanol solutions is given in **Application Sheet C01**. A major advantage of the use of this medium over Nycodenz® is the ease of solution preparation; for NPC enrichment OptiPrep™ is simply diluted with either HBSS or GBSS to give the appropriate density and all the solutions of any density will be isoosmotic. Nycodenz® and iodixanol solutions of the same % (w/v) have almost identical densities. It is highly likely that iodixanol can substitute directly for Nycodenz® in any protocol if the latter solutions are isoosmotic, but as far as we know, direct comparisons between the efficacies of the two solutes have not been made. Therefore, for completeness, some Nycodenz® variants are described.

3. Protocols

3a. Density barrier format

The crude cell suspension may be layered over a barrier of the chosen density or the suspension may be adjusted to the chosen density and a small volume of GBSS or HBSS layered on top (this is often termed a "Mixer Format"). In the former all the cells will sediment to or through the barrier; in the latter cells will float to the interface; remain in the barrier or sediment to form a pellet. The layer of salt solution in the second format prevents the floating cells from banding at an air/liquid interface and aids recovery of the cell layer. Most centrifugations are carried out at 4°C.

3b. Removal of erythrocytes and cell debris from a crude NPC fraction

Protocol 3b-1 (all iodixanol concentrations are %, w/v)

1. Layer the NPC suspension over a barrier of ρ = 1.14-1.16 g/ml; published methods report the use of 28% [4] or 24% [5] iodixanol. Higher Nycodenz® concentrations (approx 29%) have also been used [6-8].

2. Centrifuge to band the NPC at the interface; a wide range of conditions have been used - 5 min at 310 *g* [4], 15 min at 500 *g* [5] and 15 min at 1500 *g* [6].

Protocol 3b-2

1. Mix the NPC suspension with OptiPrep™ (volume ratio of 3.4:2.6 respectively).

2. Layer 0.5-1.0 ml of GBSS on top; centrifuge at 400 *g* for 15 min [9] and collect the cells at the interface.

3c. Purification of NPC

Because of the simplicity of using OptiPrep™ in a Mixer Format, the following protocols describe the use of this strategy. Some protocol variations and comments, together with a brief summary of the major Nycodenz® based methods are given in the Section 3c-3.

3c-1. Collagenase-dispersed cells (adapted from refs 10 and 11)

1. Prepare a 12.6% iodixanol solution: mix OptiPrep™ with any balanced salt solution or culture medium (12.6 vol. + 47.4 vol. respectively).

2. Centrifuge a suspension of the collagenase-dispersed cells at 50 *g* for 3 min.

3. Recover the supernatant and centrifuge it again at 50 *g*.

4. Recover the supernatant and centrifuge it at 850 *g* for 10 min.

5. Remove and discard all of the supernatant and resuspend the pellet in the 12.6% iodixanol.

6. Layer 1-2 ml of balanced salt solution or culture medium on top and centrifuge at 3300 *g* for 30 min to pellet the residual PC cells and erythrocytes.

7. Allow the rotor to decelerate without the brake.

8. Harvest the non-PC cells from top of the barrier.

3c-2. Collagenase/Pronase-dispersed cells (adapted from refs 1 and 2)

1. Make a solution of 40% iodixanol: mix 4 vol. of OptiPrep™ and 2 vol. of GBSS.

2. Suspend the crude non-parenchymal cells in GBSS.

3. Mix 40% iodixanol with the cell suspension, thoroughly (but gently) so that the final concentration of iodixanol is 17% (w/v) iodixanol solution (ρ = 1.096 g/ml).

4. Layer 1-2 ml of GBSS on top and centrifuge at 400 *g* for 15 min at 20°C.

5. Allow the rotor to decelerate without the brake.

6. Collect the non-PC cells, which band at the interface between GBSS and the 17% iodixanol layer.

3c-3. Protocol variations

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Note that the two protocols (3c-1 and 3c-2) describe two ways of processing the sample. In 3c-1 the cells are sedimented as a pellet and then resuspended in the solution of the required density, while in 3c-2 this pelleting step is omitted and the suspension mixed with a high-density solution. In some instances the cell suspension is simply mixed with OptiPrep™ rather than a 40% iodixanol working solution. The final concentrations of iodixanol are variable, e.g. 11.5% [12] 13.2%, [13,14] and 16.8% [15]. A wide variation in centrifugation conditions has been reported, from 200 *g* for 20 min to 1500 *g* for 25 min.

A rather more sophisticated gradient was recommended by Yovchev et al [16]. OptiPrep™ is diluted with a HEPES- buffered saline, pH 7.4 containing 0.2% bovine serum albumin to produce solutions of 11%, 13%, 16% and 18% (w/v) iodixanol. The NPC pellets were suspended in 11% iodixanol and each 10 ml of suspension was underlayered with 10 ml each of the 13% and 16% iodixanol solutions and 5 ml of 18% iodixanol. The gradients were centrifuged at 6,500 *g* for 30 min and were allowed to decelerate without the brake. NPC's banded at the 11-13% and 13%-16% interfaces. The lower density interface was particularly enriched in stellate and oval cells. For full details see ref 16.

Nycodenz®

The Nycodenz® literature reveals use of both the more traditional layering of the crude fraction over a density barrier and also the Mixer Format. Nycodenz® density barriers of approx 15.7% [17,18], 16% [19] and 16.75% [20-22] are commonly used; centrifugation conditions vary from 600-1700 *g* for 15-20 min. In the Mixer Format 28-30% Nycodenz® has been added to the cell suspension to adjust it to 15.8% [23-24], 16.7% [25-28] or 17.5% [29,30]; the centrifugation conditions are generally 1500-1700 *g* for 15-20 min.

4. References

1. Boyum, A., Berg, T. and Blomhoff, R. (1983) *Fractionation of mammalian cells* In Iodinated density gradient media – a practical approach (ed. Rickwood, D.) IRL Press at Oxford University Press, Oxford, UK, pp 147-171
2. Brouwer, A., Hendricks, H. F. J., Ford, T. and Knook, D. L. (1991) *Centrifugation separations of mammalian cells* In Preparative centrifugation – a practical approach (ed. Rickwood, D.) IRL Press at Oxford University Press, Oxford, UK, pp 271-314
3. Gaustad, R., Berg, T. and Fonnum, F. (1992) *Heterogeneity of carboxylesterases in rat liver cells* Biochem. Pharmacol., **44**, 827-829

4. Malerød, L., Juvet, L.K., Gjøen, T. and Berg, T. (2002) *The expression of scavenger receptor class B, type I (SR-BI) and caveolin-1 in parenchymal and nonparenchymal liver cells* Cell Tissue Res., **307**, 173-180
5. Shao, B., Lu, M., Katz, S.C., Varley, A.W., Hardwick, J., Rogers, T.E., Ojogun, N., Rockey, D.C., DeMatteo, R.P. and Munford, R.S. (2007) *A host lipase detoxifies bacterial lipopolysaccharides in the liver and spleen* J. Biol. Chem., **282**, 13726-13735
6. Hong, G., Bazin-Redureau, M., Gires, P. and Scherrmann, J.M. (1998) *Hepatic disposition and toxicity of cationized goat immunoglobulin G and Fab fragments in isolated perfused rat liver* Drug. Metab. Dispos., **26**, 661-669
7. Milosevic, N., Schawalder, H. and Maier, P. (1999) *Kupffer cell-mediated differential down-regulation of cytochrome P450 metabolism in rat hepatocytes* Eur. J. Pharmacol., **368**, 75-87
8. Upadhya, G.A. and Strasberg, S.M. (1999) *Evidence that actin disassembly is a requirement of matrix metalloproteinase secretion by sinuosoidal endothelial cells during cold preservation in the rat* Hepatology, **30**, 169-176
9. Katz, S.C., Pillarisetty, V.G., Bleier, J.I., Shah, A.B. and DeMatteo, R.P. (2004) *Liver sinusoidal endothelial cells are insufficient to activate T cells* J. Immunol., **173**, 230-235
10. Nedredal, G. I., Elvevold, K. H., Ytrebo, L. M., Olsen, R., Revhaug, A. and Smedsrod, B. (2003) *Liver sinusoidal endothelial cells represent an important blood clearance system in pigs* Comp. Hepatol., **2**:1.
11. Elvevold, K.H., Nedredal, G.I., Revhaug, A. and Smedsrød, B. (2004) *Scavenger properties of cultivated pig liver endothelial cells* Comp. Hepatol., **3**, 1-11
12. Zhu, J., Huang, X. and Yang, Y. (2007) *Innate immune response to adenoviral vectors is mediated by both toll-like receptor-dependent and -independent pathways* J. Virol., **81**, 3170-3180
13. Kuniyasu, Y., Qamar, A., Sheikh, S.Z., Jhandier, M.N., Hakim, W. and Mehal, W.Z. (2005) *Blocking intrahepatic deletion of activated $CD8^+$ T cells by an altered peptide ligand* Cell. Immunol., **238**, 31-37
14. Klein, I., Cornejo, J.C., Polakos, N.K., Beena, J., Wuensch, S.A., Topham, D.J., Pierce, R.H. and Crispe, N.J. (2007) *Kupffer cell heterogeneity: functional properties of bone marrow-derived and sessile hepatic macrophages* Blood, **110**, 4077-4085
15. Zelnickova, P., Matiasovic, J., Pavlova, B., Kudlackove, H., Kovaru, F. and Faldyna, M. (2008) *Quantitative nitric oxide production by rat, bovine and porcine macrophages* Nitric Oxide, **19**, 36-41
16. Yovchev, M.I., Dabeva, M.D. and Oertel, M. (2013) *Isolation, characterization, and transplantation of adult liver progenitor cells* In Methods Mol. Biol., **976**, Stem Cells and Aging: Methods and Protocols, (ed. Tursen, K.) Springer Science+Business Media, LLC pp 37-51
17. Knolle, P., Schlaak, J., Uhrig, A. Kempf, P. Meyer zum Büschenfelde, K.H. and Gerken, G. (1995) *Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge* J. Hepatol., **22**, 226-229
18. Schmitz, F., Bresciani, R., Hartmann, H. and Braulke, T. (1995) *Effect of insulin-like growth factor II on uptake of arylsulfatase A by cultured rat hepatocytes and Kupffer cells* J. Hepatol., **22**, 356-363
19. Yoshioka, M., Nakajima, Y., Ito, T. Mikarni, O. Tanaka, S., Miyazaki, S. and Motoi, Y. (1997) *Primary culture and expression of cytokine mRNAs by lipopolysaccharide in bovine Kupffer cells* Vet. Immunol. Immunopathol., **58**, 155-163
20. Petermann, H., Heymann, S., Vogl, S. and Dargel, R. (1966) *Phagocytic function and metabolite production in thioacetamide-induced liver cirrhosis: a comparative study in perfused livers and cultured Kupffer cells* J. Hepatol., **24**, 468-477
21. Petermann, H., Vogl, S., Schulze, E. And Dargel, R. (1999) *Chronic liver injury alters basal and stimulated nitric oxide production and ^{3}H-thymidine incorporation in cultured sinusoidal endlthelial cells from rats* J. Hepatol., **31**, 284-292
22. Novosyadlyy, R., Dargel, R. and Scharf, J-G. (2005) *Expression of insulin-like growth factor-I ans insulin-like growth factor binding proteins during thioacetamide-induced liver cirrhosis in rats* Growth Horm. IGF Res., **15**, 313-323
23. Ten Hagen, T.L.M., Van Vianen, V. and Bakker-Woudenberg, I.A.J.M. (1996) *Isolation and characterization of murine Kupffer cells and splenic macrophages* J. Immunol. Meth., **193**, 81-91
24. Luedde, T., Assmus, U., Wüstefeld, T., zu Vilsendorf, A.M., Roskams, T., Schmidt-Supprian, M., Rajewsky, K., Brenner, D.A., Manns, M.P., Pasparakis, M. and Trautwein, C. (2005) *Deletion of IKK2 in hepatocytes does not sensitize these cells to TNF-induced apoptosis but protects from ischemia/reperfusion injury* J. Clin. Invest., **115**, 849-859
25. Juillerat, M., Marceau, N., Coeytaux, S., Sierra, F., Kolodziejczyk, E. and Guigoz, Y. (1997) *Expression of organ-specific structures and functions in long-term cultures of aggregates from adult rat liver cells* Toxicol. in Vitro, **11**, 57-69
26. Pestel, S., Jungermann, K., Götze, O. and Schieferdecker, L. (2002) *Inhibition by prostaglandin E_2 of anaphylatoxin C5a-but not zymosan-induced prostanoid release from rat Kupffer cells* Lab. Invest., **82**, 463-471

27. Pestel, S., Schlaf, G., Götze, O., Jungermann, K. and Schieferdecker, H.L. (2003) *Differences in the involvement of prostanoids from Kupffer cells in the meditation of anaphylylatoxin C5a-, zymosan-, and lipopolysaccharide-dependent hepatic glucose output and flow reduction* Lab. Invest., **83**, 1733-1741
28. Pestel, S., Jungermannm, K. and Schieferdecker, H.L. (2005) *Re-evaluation of thin layer chromatography as an alternative method for the quantification of prostaglandins from rat Kupffer cells* Prostaglandins Other Lipid Mediat., **75**, 123-139
29. De Rijke, Y.B., Jurgens, G., Hessels, e.M., Hermann, A. and van Berkel, T.J. (1992) *In vivo fate and scavenger receptor recognition of oxidized lipoprotein (a) isoforms in rats* J. Lipid Res., **33**, 1315-1325
30. Ling, W., Lougheed, M., Suzuki, H., Buchan, A., Kodama, T., Steinbrecher, U.P. (1997) *Oxidized or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of the scavenger receptor class A type I/II gene* J. Clin. Invest., **100**, 244-252

Application Sheet C24; 8th edition, May 2016

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