OptiPrep™ Application Sheet C40

Isolation of mononuclear cells from tissues

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **OptiPrep™ Application Sheet C03** "Purification of mononuclear cells, monocytes and polymorphonuclear leukocytes – a methodological review" compares all of the currently available methodologies
- **OptiPrep™ Reference List RC01** "Purification of mononuclear cells, monocytes and polymorphonuclear leukocytes – a bibliographical review" provides a comprehensive bibliography of all the published papers reporting the use of OptiPrep™
- To access **RC01** return to the initial list of Folders and select "**Reference Lists**".
- 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

Mononuclear cells (MCs) from a variety of tissues, predominantly liver, spleen, intestine and bone marrow, have been purified by using a strategy similar to that used for the isolation of these cells from blood, namely sedimentation onto a density barrier. This density barrier has commonly been a Nycodenz® solution, often in the form of one of the ready-made Nycoprep™ solutions; Nycoprep™ 1.077 [1,2] for human blood, Nycoprep™ 1.077A [3-8] for rodent blood: the former was formulated for isolation of MC from human blood, the latter from rodent blood. Neither of these ready-made solutions is now commercially available, However solutions of the same density and osmolality may very easily, be prepared from OptiPrep™ (see Section 3).

More recently MCs from liver have been have been purified on an iodixanol density barrier of approx ρ = 1.084 g/ml [9,10] or banded between a two-layer gradient of 1.051 and 1.077 g/ml [11]. Other tissues from which MCs have been prepared using sedimentation onto an iodixanol density barrier are rat spleen [12], spinal cord [12] and bone marrow [13-15]. As far as is known, these barrier sedimentation methods have not been executed using a low-osmolality density barrier. The iodixanol barrier solutions have been produced by dilution of OptiPrep™ with regular saline or culture medium.

In the alternative "mixer" strategy the sample is simply adjusted to a density just higher than that of the MCs so that the latter float to the top during the centrifugation, introduced for human blood by Ford and Rickwood [16] using Nycodenz®. This was later adapted to the use of OptiPrep™ and extended to both mouse and rat blood. It has now been used successfully for the isolation of MCs from liver [17-22] and spleen [23,24]. This technology should be applicable to any mouse or rat tissue.

The most commonly used tissues for the isolation of lymphocytes are bone marrow, spleen, intestine and liver. Since this Application Sheet was first prepared however, the use of iodixanol gradients has additionally been reported for their isolation from brain, heart and lung tissue. Although the general gradient strategies have not changed, there is considerable variation in some of the details, some of which will be indicated in Section 6.

- This Application Sheet presents the density gradient methods for resolving the lymphocytes and not the methods that are used to disaggregate the tissues. **Methods for preparing a total non-parenchymal cell fraction are described in Application Sheet C26**
- Section 2 describes the options for sedimentation on to a density barrier
- Section 3 describes the two-layer gradient
- Section 4 describes the flotation strategy
- Section 5 contains important notes to Sections 2, 3 and 4
- Section 6 briefly describes some of the methodological variations reported in recent papers

2. Sedimentation on to a density barrier

2a. Solution preparation

A. OptiPrep™ (60%, w/v iodixanol) – shake the bottle gently before use

B. Buffered saline

Keep Hepes (free acid) or Tricine as a 100 mM stock solution at 4°C; Hepes (2.38 g) or Tricine (1.79 g) per 100 ml water.

Solution B: Dissolve 0.85 g of NaCl in 50 ml water; add 10 ml of Hepes or Tricine stock solution; adjust to pH 7.2-7.4 with 1 M NaOH and make up to 100 ml.

2a-1 Isoosmotic barrier solution

Dilute Solution A with Solution B to obtain isoosmotic solutions of lower density. The density of this barrier solution may be modulated to improve either the purity or yield of mononuclear cells. For more information on the density of iodixanol solutions **see Application Sheet C01**. The published methods quote % (w/v) iodixanol concentrations from 12.6% (see ref 17), which has a density of approx. 1.072 g/ml, to 15% (e.g. see refs 9, 10, 25) with a density of approx. 1.084 g/ml and also includes the standard 1.077 g/ml barrier [26] and for intestinal cells a much lower density barrier (1.055 g/ml) was employed at 1700 *g* for 10 min [27]. Centrifugation conditions are normally in the range 600-1000 *g* for 10-20 min.

2a-2 Hypoosmotic 1.077 g/ml barrier solution

Dilute Solution B with water (2.5 vol. + 0.5 vol.) and then mix 2.7 vol. of Solution A with 9.3 vol. of the diluted saline solution (see Note 1).

2b. Protocol (adapted from refs 9 and 10)

1. Layer 2 vol. of cell suspension (see Section 2) on top of 1 vol. of the density barrier (see Note 2).

2. Centrifuge at 750 *g* for 20 min at room temperature (see Note 3).

3. Allow the rotor to decelerate without the brake and harvest the MCs from the interface.

3. Sedimentation in a two-layer gradient

3a. Solutions required

A. OptiPrep™ (60%, w/v iodixanol)

B. Culture medium (RPMI 1640)

3b. Protocol (adapted from ref 11)

1. Shake the OptiPrep™ gently before use.

2. Make up two solutions of 1.051 and 1.078 g/ml by mixing Solutions A and B in the following volume ratios (1:5.8) and (1:3.5) respectively (see Note 4)

3. Suspend the cells in the 1.051 g/ml solution and layer over an equal volume of the 1.078 g/ml solution (see Note 2).

4. Centrifuge at 750 *g* for 20 min at room temperature.

5. Allow the rotor to decelerate without the brake and harvest the MCs from the interface (see Note 5).

4. Flotation strategy

4a. Solutions required

A. OptiPrep™ (60%, w/v iodixanol)

B. Culture medium (RPMI 1640)

4b. Protocol (adapted from ref 20)

1. Shake the OptiPrep™ gently before use and mix 4 vol. of Solution A with 2 vol. of Solution B to produce a solution of density 1.215 g/ml (see Note 7).

2. Suspend the cells in 3.9 ml of Solution B and mix gently but thoroughly with 2.1 ml of the 1.215 g/ml solution (see Note 8 and 9).

3. Layer 1 ml of Solution B on top and centrifuge at 1500 *g* for 20 min at 4°C (see Notes 5 and 10).

4. Allow the rotor to decelerate without the brake and harvest the MCs from the interface.

5. Notes

1. This solution is equivalent to Nycoprep™ 1.077A and has a density of 1.077 g/ml and an osmolality of approx 265 mOsm.
2. The relative volumes of sample and density barrier are probably not critical but the given ratio is widely used.
3. The separation on an isoosmotic barrier should not be temperature dependent but lower temperatures may require a further 5 min of centrifugation because of the increased viscosity at lower temperatures. Use of the hypoosmotic medium should be carried out at room temperature because the movement of water across an osmotic gradient is reduced at low temperatures.
4. The density of the 1.078 g/ml solution might be modulated upwards if too many MCs are lost to the pellet. For intestine and liver mononuclear cells Zellweger et al used densities of 1.052 and 1.076 g/ml [28]. This was also very similar to the method of Dai et al [29]
5. The separation should not be temperature dependent but lower temperatures may require a further 5 min of centrifugation because of the increased viscosity at lower temperatures.
6. The ratio of Solution A:Solution B may be modulated in the light of data on the recovery of MCs. In step 3 it is possible to mix the sample directly with OptiPrep™ rather than with the ρ = 1.215 g/ml medium; the latter is the chosen method because complete mixing of OptiPrep™ with the sample requires rather more vigorous agitation, which may be deleterious to the cells.
7. The final v/v ratio of OptiPrep™ in the sample is 22%, variations include 21% [17,18], 21.5% [21,22] and 24% [23]. So the final concentration of iodixanol varies from 12.6% (w/v) to 14.4% (w/v), equivalent to densities of approx. 1.072-1.082 g/ml.
8. The small layer of culture medium on top of the sample does not influence the separation, but it prevents the MCs from banding at an air/liquid interface, which causes aggregation problems.
9. For the isolation of mononuclear cells from mouse bone marrow, Mukai et al [30] floated the mononuclear cells from a dense sample layer (1.090 g/ml), through a 1.08 g/ml layer using very mild centrifugation conditions of 100 *g* for 20 min.
10. For bone marrow cells, 30 ml of cell suspension was mixed with 10.1 ml of OptiPrep™ and 4 ml of water; then overlaid with 5 ml of PBS and centrifuged at 1000 *g* for 30 min [31] – note that the final iodixanol concentration in the sample was 13.7% (w/v).

6. Recent methods and variations in methodology

- Lung lymphocytes have been purified on both the normal density barrier [32] and a slightly denser barrier of approx 16.5% (w/v) iodixanol (described in the paper as 27.5% (v/v) OptiPrep™) [33] similar to that used for intestinal cells [34].
- Two-layer gradients of 15% (w/v) and 11% (w/v) iodixanol (equivalent to 1.085 and 1.063 g/ml density) were applied to resolve the lymphocytes in the top zone of the 15% layer and macrophages + dendritic cells just above the interface [35]
- Brain lymphocytes have only relatively recently been isolated using OptiPrep™ using a three-layer gradient of 5%, 10% and 18% (w/v) iodixanol at 800 *g* for 30 min; the leukocytes collected at the 10-18% interface [36-38]
- Recently published papers describe the use of iodixanol gradients for the isolation of mononuclear cells from bone marrow [39,40], liver [41] and spleen [42].

7. References

1. Zhang, Z., Kaptanoglu, L., Haddad, W., Ivancic, D., Alnadjim, Z., Hurst, S., Tishler, D., Luster, A.D., Barrett, T.A. and Fryer, J. (2002) *Donor T cell activation initiates small bowel allograft rejection through an IFN-γ-inducible protein-10-dependent mechanism* J. Immunol., **168**, 3205-3212
2. Fukutome, K., Watarai, S., Mukamoto, M. and Kodama, H. (2001) *Intestinal mucosal immune response in chickens following intraocular immunization with liposome-associated Salmonella enterica serovar enteritidis antigen* Devel. Comp. Immunol., **25**, 475-484
3. Medana, I., Li, Z., Flügel, A., Tschopp, J., Wekerle, H. and Neumann, H. (2001) *Fas ligand (CD95L) protects neurons against perforin-mediated T lymphocyte cytotoxicity* J. Immunol., **167**, 674-681
4. Villey, I., Caillol, D., Selz, F., Ferrier, P. and de Villartay, J-P. (1996) *Defect in rearrangement of the most 5'TCR-Jα following targeted deletion of T early α (TEA): implications for TCRα locus accessibility* Immunity, **5**, 331-342
5. Wurbel, M-A., Malissen, M., Guy-Grand, D., Meffre, E., Nussenzweig, M.C., Richelme, M., Carrier, A. and Malissen, B. (2001) *Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor γδ$^+$ gut intraepithelial lymphocytes* Blood, **98**, 2626-2632
6. Ćupić, V., Čolić, M., Pavičić, L., Vučević, D. and Varagić, V.M. (2001) *Immunomodulatory effect of xylazine, an α_2 adrenergic agonist, on rat spleen cells in culture* J. Neuroimmunol., **113**, 19-29
7. Becker, J.C., Varki, N., Gillies, S.D., Furukawa, K. and Reisfeld, R.A. (1996) *Long-lived and transferable tumor immunity in mice after targeted interleukin-2 therapy* J. Clin. Invest., **98**, 2801-2804
8. Arstila, T., Arstila, T.P., Calbo, S., Selz, F., Malassis-Seris, M., Vassalli, P., Kourlisky, P. and Guy-Grand, D. (2000) *Identical T cell clones are located within the mouse gut epithelium and lamina propria and circulate in the thoracic lymph duct* J. Exp. Med., **191**, 823-834
9. Obhari, J.S., Oberbarnscheidt, M.H., Hand, T.W., Diggs, L., Chalasani, G. and Lakkis, F.G. (2006) *Effector T cell differentiation and memory T cell maintenance outside secondary lymphoid organs* J. Immunol., **176**, 4051-4058
10. Nasr, I.W., Reel, M., Oberbarnscheidt, M.H., Mounzer, R.H., Baddoura, F.K., Ruddle, N.H. and Makkis, F.G. (2007) *Tertiary lymphoid tissues generate effector and memory T cells that lead to allograft rejection* Am. J. Transplant., **7**, 1071-1079
11. Lian Z-X., Okada, T., He, X-S., Kita, H., Liu, Y-J., Ansari, A.A., Kikuchi, K., Ikehara, S. and Gershwin, M.E. (2003) *J. Immunol.*, **170**, 2323-2330
12. Flügel, A., Berkowicz, T., Ritter, T., Labeur, M., Jenne, D.E., Li, Z., Ellwart, J.W., Willem, M., Lassmann, H. and Wekerle, H. (2001) *Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis* Immunity, **14**, 547-560
13. Whetton, A.D., Lu, Y., Pierce, A., Carney, L. and Spooncer, E. (2003) *Lysophospholipids synergistically promote primitive hematopoietic cell chemotaxis via a mechanism involving Vav1* Blood, **102**, 2798-2802
14. Evans, C.A., Tonge, R., Blinco, D., Pierce, A., Shaw, J., Lu, Y., Hanzah, H.G., Gray, A., Downes, C.P., Gaskell, S.J., Spooncer, E. and Whetton, A.D. (2004) *Comparative proteomics of primitive hematopoietic cell populations reveals differences in expression of proteins regulating motility* Blood, **103**, 3751-3759
15. Unwin, R.D., Smith, D.L., Blinco, D., Wilson, C.L., Miller, C.J., Evans, C.A., Jaworska, E., Baldwin, S.A., Barnes, K., Pierce, A., Spooncer, E. and Whetton, A.D. (2006) *Quantitative proteomics reveals posttranslational control as a regulatory factor in primary hematopoietic stem cells* Blood, **107**, 4687-4694
16. Ford, T. C. and Rickwood, D. (1990) *A new one-step method for the isolation of human mononuclear cells* J. Immunol. Meth., **134**, 237-241
17. Mehal, W., Sheikh, S.Z., Gorelik, L. and Flavell, R.A. (2005) *TGF-β signaling regulates CD8+ T cell responses to high- and low-affinity TCR interactions* Int. Immunol., **17**, 531-538
18. John, B. and Crispe, I.N. (2005) *LR-4 regulates CD8$^+$ T cell trapping in the liver* J. Immunol., **175,** 1643-1650
19. Klein, I. and Crispe, I.N. (2006) *Complete differentiation of CD8$^+$ T cells activated locally within the transplanted liver* J. Exp. Med., **203**, 437-447
20. Wuensch, S.A., Pierce, R.H. and Crispe, I.N. (2006) *Local intrahepatic CD8+ T cell activation by a non-self- antigen results in full functional differentiation* J. Immunol., **177**, 1689-1697
21. Au-Yeung, B.B. and Fowell, D.J. (2007) *A key role for Itk in both IFNγ and IL-4 production by NKT cells* J. Immunol., **179**, 111-119
22. Polakos, N.K., Klein, I., Richter, M.V., Zaiss, D.M., Giannandrea, M., Crispe, I.N. and Topham, D.J. (2007) *Early intrahepatic accumulation of CD8$^+$ T cells provides a source of effectors for nonhepatic immune responses* J. Immunol., **179**, 201-210
23. DiJoseph, J.F., Dougher, M.M., Kalyandrug, L.B., Armellino, D.C., Boghaert, E.R., Hamann, P.R., Moran, J.K. and Damle, N.K. (2006) *Antitumor efficiacy of a combination of CMC-544 (inotuzumab ozogamicin), a CD22-targeted cytotoxic immunoconjugate of calicheamicin, and rituximab against non-Hodgkin's B-cell lymphoma* Clin. Cancer Res., **12**, 242-249
24. Kivi, G., Teesalu, K., Parik, J., Kontkar, E., Ustav Jr, M., Noodla, L., Ustav, M. and Männik, A. (2016) *HybriFree: a robust and rapid method for the development of monoclonal antibodies from different host species* BMC Biotechnol., **16**: 2
25. Dobaczewski, M., Xia, Y., Bujak, M., Gonzalez-Quesada, C. and Frangogiannis, N.G. (2010) *CCR5 signaling suppresses inflammation and reduces adverse remodeling of the infarcted heart, mediating recruitment of regulatory T cells* Am. J. Pathol., **176**, 2177–2187
26. Aliotta, J.M., Pereira, M., Johnson, K.W., de Paza, N., Dooner, M.S., Puente, N., Ayala, C., Brilliant, K., Berza, D., Lee, D., Ramratnam, B., McMillan, P.N., Hixson, D.C., Josic, D. and Quesenberry, P.J. (2010) *Microvesicle entry into*

marrow cells mediates tissue-specific changes in mRNA by direct delivery of mRNA and induction of transcription Exp. Hematol., **38**, 233–245
27. Wang, X., O'Gorman, M.R.G., Bu, H-F., Koti, V., Zuo, X-L. and Tan, X-D. (2009) *Probiotic preparation VSL#3 alters the distribution and phenotypes of dendritic cells within the intestinal mucosa in 57BL/10J mice* J. Nutr. **139**, 1595–1602
28. Zellweger, R.M., Prestwood, T.R. and Shresta, S. (2010) *Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease* Cell Host Microbe **7**, 128–139
29. Dai, K., Huang, L., Sun, X., Yang, L. and Gong, Z. (2015) *Hepatic CD206-positive macrophages express amphiregulin to promote the immunosuppressive activity of regulatory T cells in HBV infection* J. Leukoc. Biol., **98**, 1071–1080
30. Mukai, M., Suruga, N., Saeki, N. and Ogawa, K. (2017) *EphA receptors and ephrin-A ligands are upregulated by monocytic differentiation/maturation and promote cell adhesion and protrusion formation in HL60 monocytes* BMC Cell Biol., **18**: 28
31. Liu, L., Papa, E.F., Dooner, M.S., Machan, J.T., Johnson, K.W., Goldberg, L.R., Quesenberry, P.J. and Colvin, G.A. (2012) *Homing and long-term engraftment of long- and short-term renewal hematopoietic stem cells* PLoS One, **7**: e31300
32. Licona-Limón, P., Henao-Mejia, J., Temann, A.U., Gagliani, N., Licona-Limón, I., Ishigame, H., Hao, L., Herbert, D.R. and Flavell, R.A. (2013) *Th9 cells drive host immunity against gastrointestinal worm infection* Immunity, **39**, 744–757
33. Koyama, S., Akbay, E.A., Li, Y.Y., Herter-Sprie, G.S., Buczkowski, K.A., Richards, W.G., Gandhi, L., Redig, A.J., Rodig, S.J. et al (2016) *Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints* Nat. Comm., **7**: 10501
34. Goodyear, A.W., Kumar, A., Dowa, S. and Ryan, E.P. (2014) *Optimization of murine small intestine leukocyte isolation for global immune phenotype analysis* J. Immunol. Methods, **405**, 97–108
35. Kitazawa, Y., Ueta, H., Hünig, T., Sawanobori, Y. and Matsuno, K. (2015) *A novel multicolor immunostaining method using ethynyldeoxyuridine for analysis of in situ immuno-proliferative response* Histochem. Cell Biol., **144**, 195–208
36. Kim, J.H., Choi, J.Y., Kim, S.B., Uyangaa, E., Patil, A.M., Han, Y.W., Park, S-Y., Lee, J.H., Kim, K. and Eo, S.K. (2015) *CD11chi dendritic cells regulate Ly-6Chi monocyte differentiation to preserve immune-privileged CNS in lethal neuroinflammation* Sci. Rep., **5**: 17548
37. Kim, S.B., Choi, J.Y., Kim, J.H., Uyangaa, E., Patil, A.M., Park, S-Y., Lee, J.H., Kim, K., Han, Y.W. and Eo, S.K. (2015) *Amelioration of Japanese encephalitis by blockage of 4-1BB signaling is coupled to divergent enhancement of type I/II IFN responses and Ly-6Chi monocyte differentiation* J. Neuroinflamm., **12**: 216
38. Kim, S.B., Choi, J.Y., Uyangaa, E., Patil, A.M., Hossain, F.M.A., Hur, J., Park, S-Y. et al (2016) *Blockage of indoleamine 2,3-dioxygenaseregulates Japanese encephalitis via enhancement of type I/II IFN innate and adaptive T-cell responses* J. Neuroinflam. **13**: 79
39. Aliotta, J.M., Pereira, M., Sears, E.H., Dooner, M.S., Wen, S., Goldberg, L.R. and Quesenberry, P.J. (2015) *Lung-derived exosome uptake into and epigenetic odulation of marrow progenitor/stem and ifferentiated cells* J. Extracell. Vesicles, **4:**26166
40. Wen, S., Dooner, M., Cheng, Y., Papa, E., Del Tatto, M., Pereira, M., Deng, Y., Goldberg, L., Aliotta1, J., Chatterjee, D. et al (2016) *Mesenchymal stromal cell-derived extracellular vesicles rescue radiation damage to murine marrow hematopoietic cells* Leukemia, **30**, 2221–2231
41. King, A., Houlihan, D.D., Kavanagh, D., Haldar, D., Luu, N., Owen, A., Suresh, S., Than, N.N. et al (2017) *Sphingosine-1-phosphate prevents egress of hematopoietic stem cells from liver to reduce fibrosis* Gastroenterology, **153**, 233–248
42. Liang, Y., Song, D-Z., Liang, S., Zhang, Z-F., Gao, L-X. and Fan, X-H. (2017) *The hemagglutinin-neuramidinase protein of Newcastle disease virus upregulates expression of the TRAIL gene in murine natural killer cells through the activation of Syk and NF-κB* PLoS One, **12**: e0178746

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