OptiPrep™ Application Sheet C27

Purification of protozoa

- 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
- This Application Sheet includes the following sections:
  (1) A simple density barrier for recovering protozoa (*Cyclospora*) from foodstuffs
  (2) A discontinuous gradient for purification of *Cryptosporidium* oocysts
  (3) A discontinuous gradient for separation of morphologically distinct merozoites (*Sarcocystis*)
  (4) Comments on other pathogens
- For *Toxoplasma* applications see **Application Sheets C34 and C52**
- For *Plasmodium* see **Application Sheet C41**

1. Isolation of protozoa from foodstuffs

1a. Background

A simple density barrier prepared from OptiPrep™, which permitted the identification of *Cyclospora* in frozen raspberries [1], should be more generally applicable.

1b. Solutions required

A. OptiPrep™ (shake gently before use)

B. Salt buffer: 150 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl. pH 8.0

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$): 3.72 g per 100 ml water

Solution B: To 100 ml water; add 30 ml of NaCl stock and 20 ml each of Tris and EDTA stocks; adjust to pH 8.0 with 1 M HCl and make up to 200 ml.

1c. Protocol (from ref 1)

1. Carry out any pre-treatment of the sample that is necessary, then wash the crude cells twice in Solution B and resuspend in the same solution.

2. Mix equal volumes of OptiPrep™ and Solution B and transfer 4 ml to tube for a low speed centrifuge and overlay with 4 ml of the crude suspension.

3. Centrifuge at 250 *g* for 15 min at 15°C and harvest the parasites by aspirating the top layer plus the top 1.5 ml of the density barrier.

2. Purification of *Cryptosporidium* oocysts

2a. Background

Cryptosporidium parvum and *Cryptosporidium meleagridis* oocysts have been purified from fecal matter, usually from bovine or porcine sources, generally in simple two-layer Nycodenz® gradients [2-9], although for the partial separation of Type 1 and Type 2 oocysts linear gradients of the same solute have been used [10]. Chesnot and Schwartzbrod [11] noted that Nycodenz® gradients were superior to those of Percoll® for oocyst purification, when considering both "recovery and particulate load". The Nycodenz® solutions have been made up either in phosphate-buffered saline [2] or water [6]; there will be a small difference in density between the two types of solvent, but otherwise it is unlikely that the solvent will have any significant effect on the separation.

2b. Sample preparation

Widmer et al [2] first removed coarse debris from the fecal matter by filtration through gauze or low speed centrifugation. The suspension was then mixed with 2 vol. of saturated NaCl and centrifuge at 1000 *g* for 15 min. After diluting the supernatant with 3 vol. of water, the crude oocysts were pelleted at 4000 *g* for 15 min before resuspension in a small volume of water.

Akiyoshi et al [7] concentrated the fecal material by centrifugation at 4000 *g* for 10-15 min. The pellet was resuspended in 0.5% Tween 80 and filtered through gauze to remove debris. After vortexing with an equal volume of diethyl ether the suspension was recentrifuged at 4000 *g* and all of the ether and aqueous layers aspirated. The crude oocysts were again finally resuspended in small volume of water.

2c. Gradient purification

Widmer et al [2] used a two-layered gradient of 15% and 30% (w/v) Nycodenz® with centrifugation at 100,000 *g* for 1 h; this method was also reported in ref 3-6. Akiyoshi et al [7] and Chappell et al [8] used layers of 10% and 25% and much milder centrifugation conditions of 4000 *g* for 30 min. Zuckerman et al [9] used the same gradient but a higher *g*-force – 20,000 *g*.

To make 100 ml of a stock solution of either 25% or 30% (w/v) Nycodenz® place approx. 50 ml of water or phosphate-buffered saline (PBS) in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 25 g or 30 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and then make up to 100 ml with the chosen solvent. Filter sterilize if required. Dilute the stock solution with water (or PBS) to make 10% or 15% (w/v) solutions, then layer 2.5 ml of each of the Nycodenz® solutions in tubes for a swinging-bucket rotor, followed by the sample and centrifuge as required. The oocysts band at the interface between the two Nycodenz® solutions.

To separate Type 1 and Type 2 oocysts use either a linear 15-30% or 15-25% (w/v) Nycodenz® continuous gradient and centrifuged at 50,000 *g* for 1 h [10]. Type 2 oocysts are recovered from the top half of the gradient, while although Type 1 oocysts are mainly in the bottom half, there are significant numbers of Type 2 here also.

♦ As far as we know OptiPrep™ has not been investigated for any of these separations

3. Isolation of morphologically distinct forms of *Sarcocystis neurona*

3a. Background

To be able to study the cell and molecular biological characteristics of the parasites that cause debilitating diseases in a variety of livestock requires an effective system for their growth in, and release from, cultured cells. Subsequent density gradient centrifugation is often an important add-on technique for the separation of the parasites from cell debris and for resolving morphologically distinct forms of the parasite.

Sarcocystis neurona grows relatively slowly in host cells and the merozoites are also released from the cells rather slowly. Ellison et al [12,13] have described a procedure that uses the calcium ionophore A23187 to cause rapid and synchronous release of the merozoites. A discontinuous iodixanol gradient was also developed by Ellison et al [12] to resolve the different morphological forms of the parasite.

Although the merozoite release and density gradient are customized to *Sarcocystis neurona,* the density gradient strategy may have a wider application to the fractionation of merozoites from any parasite-infected cell monolayers. This Application Sheet is therefore concerned solely with these procedures rather than the cell culture itself.

3b. Solutions required

A. OptiPrep™ (shake gently before use)
B. Any isoosmotic solution: RPMI, DMEM, Hank's balanced salt solution, phosphate-buffered saline, etc.
C. A23187 in Solution B (1 µM).

Dissolve A23187 in DMSO or ethanol at 1 mM concentration (5.2 mg/10 ml) before diluting into chosen solution.

3c. Protocol (adapted from ref 12)

1. After washing the infected cell monolayer three times in Solution B, incubate in Solution C for 40 min at 37° C in 5% CO_2/95% air (see Note 1).
2.
3. Prepare three density gradient solutions of 1.03, 1.04 and 1.06 g/ml (equivalent to 5.4%, 6.4% and 10.3%, w/v iodixanol) by diluting Optiprep™ with Solution B (see Note 2).
4. Transfer 4 ml of the 10.3% iodixanol solution to a 15 ml centrifuge tube and overlayer with the same volume of the two other gradient solutions.
5. Centrifuge the merozoite-containing solution at 300-500 *g* for 10 min and resuspend the pellet gently in Solution B.

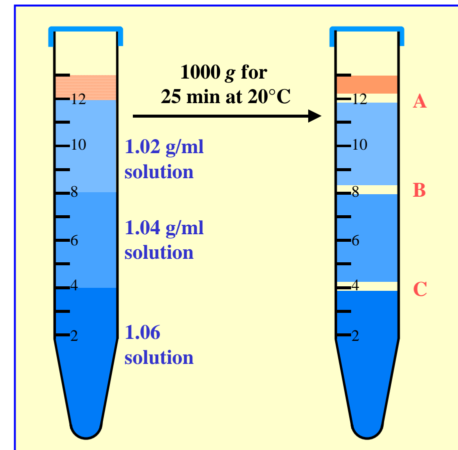

Figure 1: Fractionation of *Sarcocystis neurona* in a discontinuous iodixanol gradient. For further information see text

6. Layer 1 ml of the suspension on top of the gradient and centrifuge at 1000 *g* for 25 min at 20°C (see Note 3).
7. Harvest the banded material and any pellet from the gradient and process as required. To pellet the recovered material dilute the sample with two volumes of Solution B and centrifuge at 300-500g for 10 min (see Figure 1 and Notes 4 and 5).

3d. Notes

1. Any suitable strategy for the efficient release of merozoites should be used.
2. The density of the various gradient solutions may require modulation for the fractionation of parasites from other sources. This can only determined in the light of experience.
3. If the host cell debris persistently contaminates the merozoites then it may be beneficial to layer the suspension in a dense solution (e.g. 1.08 g/ml) beneath the gradient and allow the merozoites to band by flotation. For more information on the preparation of density gradient solutions see **Application Sheet C01**.
4. The morphology and the nucleus:cytoplasm ratio of the three forms of merozoites that banded in the gradient were distinctively different (see Figure 1). The parasites that banded at interface A were tear-shaped to oblong, while those at interface B were more rounded with a lower nucleus:cytoplasm ratio. The early merozoites banded at interface C along with host cells. For more detailed information about the identity and properties of the banded material see ref 12.

4. Other pathogens

A final continuous 10-50% (w/v) iodixanol gradient was added to an earlier Percoll™ barrier and continuous sucrose gradient [14-16] in order to improve the purification of spores of the protozoan *Enterocytozoon bieneusi* (a parasite found in the faeces of primates causing major gastrointestinal symptoms, particularly in patients with AIDS) . Moreover this additional purification step did not cause any loss of recovery (a problem associated with earlier density gradient steps). The gradient was a 10-50% (w/v) iodixanol (OptiPrep™ diluted with 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.4) centrifuged at 30,000g for 60 min. The densest spores from a previous gradient [14] were further resolved in the iodixanol gradient into two distinct populations of median density 1.15 and 1.16 g/ml. As with the *Sarcocystis neurona* separations described above, iodixanol gradients demonstrate an ability to resolve different cell types that is lacking in other density gradient media. Gradients produced from OptiPrep™ have also been used to purify *Mattesia orzaephili* oocysts [17].

5. References

1. Ho, A.Y., Lopez, A.S., Eberhart, M.G., Levenson, R., Finkel, B.S., da Silva, A.J., Roberts, J.M., Orlandi, P.A., Johnson, C.C. and Herwaldt, B.L. (2002) *Outbreak of cyclosporiasis associated with imported raspberries, Philadelphia, Pennsylvania, 2000* Emerging Infect. Dis., **8**, 783-788

2. Widmer, G., Tchack, L., Chappell, C.L. and Tzipori, S. (1998) *Sequence polymorphism in the β-tubulin gene reveals heterogeneous and variable population structures in Cryptosporidium parvum* Appl. Environ. Microbiol., **64**, 4477-4481
3. Widmer, G., Orbacz, E.A. and Tzipori, S. (1999) *β-tubulin mRNA as a marker of Cryptosporidium parvum oocyst viability* Appl. Envir. Microbiol., **65**, 1584-1588
4. Widmer, G., Akiyoshi, D., Buckholt, M.A., Feng, X., Rich, S.M., Deary, K.M., Bowman, C.A, Xu, P., Wang, Y., Wang, X., Buck, C.A. and Tzipori, S. (2000) *Animal propagation and genomic survey of a genotype 1 isolate of Crystosporidium parvum* Mol. Biochem. Parasitol., **108**, 187-197
5. Feng, X., Rich, S.M., Akiyoshi, D., Tumwine, J.K., Kekitiinwa, A., Nabukeera, N., Tzipori, S. and Widmer, G. (2000) *Extensive polymorphism in Cryptosporidium parvum identified by multilocus microsatellite analysis* Appl. Envir. Microbiol., **66**, 3344-3349
6. Feng, X., Rich, S.M., Tzipori, S. and Widmer, G. (2002) *Experimental evidence for genetic recombination in the opportunistic pathogen Cryptosporidium parvum* Mol. Biochem. Parasitol., **119**, 55-62
7. Akiyoshi, D.E., Dilo, J., Pearson, C., Chapman, S., Tumwine, J. and Tzipori, S.et al (2003) *Characterization of Cryptosporidium meleagridis of human origin passaged through different host species* Infect. Immun., **71**, 1828-1832
8. Chappell, C.L. Okhuysen, P.C., Langer-Curry, R., Widmer, G., Akiyoshi, D.E., Tanriverdi, S. and Tzipori, S. (2006) *Cryptosporidum hominis: experimental challenge of healthy adults* Am. J. Trop. Med., **75**, 851-857
9. Zuckerman, U., Armon, R., Tzipori, S. and Gold, D. (1999) *Evaluation of a portable differential continuous flow centrifuge for concentration of Cryptosporidium oocysts and Giardia cysts from water* J. Appl. Microbiol., **86**, 955-961
10. Tanriverdi, S., Özkan Arslam, M., Akiyoshi, D.E., Tzipori, S. and Widmer, G. (2003) *Identification of genotypically mixed Cryptosporidium parvum populations in humans and calves* Mol. Biochem. Parasitol., **130**, 13-22
11. Chesnot, T. and Schwartzbrod, J. (2004) *Quantitative and qualitative comparison of density-based purification methods for detection of Cryptosporidium oocysts in turbid environmental matrices* J. Microbiol. Meth., **58**, 375-386
12. Ellison, S.P., Greiner, E. and Dame, J.B. (2001) *In vitro culture and synchronous release of Sarcocystis neurona merozoites from host cells* Vet. Parasitol., **95**, 251-261
13. Ellison, S.P., Greiner, E., Brown, K.W. and Kennedy, T. (2004) *Experimental infection of horses with culture-derived Sarcocystis neurona merozoites as a model for equine protozoal myeloencephalitis* Intern. J. Appl. Res. Vet. Med., **2**, 79-89
14. Zhang, Q., Singh, I., Sheoran, A., Feng, X., Nunnari, J., Carville, A. and Tzipori, S. (2005) *Production and characterization of monoclonal antibodies against Enterocytozoon bieneusi purified from rhesus macaques* Infect. Immun., **73**, 5166-5172
15. Akiyoshi, D.E., Morrison, H.G., Lei, S., Feng, X., Zhang, Q., Corradi, N., Mayanja, H., Tumwine, J.K., Keeling, P.J., Weiss, L.M. and Tzipori, S. (2009) *Genomic survey of the non-cultivatable opportunistic human pathogen, Enterocytozoon bieneusi* PLoS Pathog., **5**: e1000261
16. Zhang, Q., Feng, X., Nie, W., Golenbock, D.T., Mayanja-Kizza, H., Tzipori, S. and Feng, H. (2011) *MyD88-dependent pathway is essential for the innate immunity to Enterocytozoon bieneusi* Parasite Immunol., **33**, 217–225
17. Lord, J.C. (2007) *Detection of Mattesia oryzaephili (Neogregarinorida: Lipotrophidae) in grain beetle laboratory colonies with an enzyme-linked immunoadsorbent assay* J. Invertebr. Pathol., **94,** 74-76

Application Sheet C27; 6th edition, June 2016