OptiPrep™ Application Sheet C49

Purification of bacteria and bacterial minicells from cultured 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
- Note that purification of bacteria from soil, clinical specimens, host species/tissues and food is described in **Application Sheet C39.**

1. Introduction

Although Nycodenz® has been widely used in the purification of a broad range of bacteria from soil samples (and other biological matrices), **see Application Sheet C39**, there are in addition some other specific examples of the purification of bacteria, which have been primarily grown in either mammalian or non-mammalian cells (or organisms), using both Nycodenz® and OptiPrep™. Some of these methods are summarized in this Application Sheet. The majority of the published methodology is concerned with obligate intracellular bacteria and two of these, *Chlamydophila abortus* and *Piscirickettsia salmonis* are considered in Sections 2 and 3. Sections 4-10 summarize some of the other important applications of OptiPrep™ for a broad range of other bacteria. **The interesting development of the possible clinical uses of bacterial minicells is described in Section 10.**

2. Elementary bodies of *Chlamydophila abortus*

2a. Introduction

To purify the elementary bodies of *Chlamydophila abortus* Everson et al [1] used a density barrier of 18% (w/v) Nycodenz® containing 0.13 M NaCl, 3 mM KCl, 0.3 mM $CaNa_2EDTA$, 5 mM Tris-HCl, pH 7.2. It was prepared from Nycoprep™ 1.15 (an isoosmotic solution of 27.6% Nycodenz® containing 3mM KCl, 0.3 mM $CaNa_2$-EDTA, 5 mM Tris-HCl, pH 7.4, ρ = 1.15 g/ml). This is no longer commercially available so all solutions must be made from Nycodenz® powder as described below. The $CaNa_2$-EDTA in the Nycoprep™ 1.15 was present only to increase solution stability during autoclaving and may not be necessary for the separation. It may either be omitted or replaced by Na_2-EDTA. The Tris, in the example below, has been replaced by the more cell-friendly buffer Tricine. It is highly probable that Nycodenz® can be replaced by iodixanol, but this has not been verified experimentally.

Keep the following stock solutions at 4°C:

100 mM Tricine	1.79g per 100 ml water
100 mM EDTA($CaNa_2$)	3.74 g per 100 ml water
100 mM KCl	0.74 g per 100 ml water

Solution A: Dissolve 1.52 g of NaCl in 40 ml water; add 10 ml, 6 ml, 0.6 ml and respectively of Tricine, KCl and EDTA stock solutions; adjust to pH 7.2 with 0.1 M NaOH; make up to 100 ml

2b. Density barrier solution preparation

2b-1 Nycodenz®

To make up the density barrier solution place 50 ml of Solution A, which contains 0.26 M NaCl, 6 mM KCl, 0.6 mM $CaNa_2EDTA$, 10 mM Tricine-HCl, pH 7.2. (see box), in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 18 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and then make up to 100 ml with water. Filter sterilize if required.

2b-2 Iodixanol

If the iodixanol option is chosen dilute 1.8 vol. of OptiPrep™ with 2.1 vol. of Solution A and 2.1 vol. of water (see Section 2e, Note 1).

2. Centrifuge requirements

Ultracentrifuge with a swinging-bucket rotor, e.g. Beckman SW28; a similar volume rotor in a high-speed centrifuge, e.g. Sorvall HB4, can be substituted but the centrifugation time may have to be extended to make up for the slightly lower *g*-force capability (see Section 2e, Note 2).

2d. Protocol (adapted from ref 1)

1. Detach the cultured cell monolayer by the normal exposure to trypsin-EDTA in DMEM.

2. Harvest the cells by centrifugation and wash twice in medium containing 10% FCS.

3. Suspend the cells in a 10x dilution of phosphate-buffered saline and disrupted the swollen cells by homogenization in a Dounce homogenizer.

4. Remove cell debris at 250 *g* for 5 min, then raise the osmolality of the homogenate by addition of an equal volume of phosphate buffer containing 0.4 M sucrose.

5. Load the suspension on to a solution of 18% (w/v) Nycodenz® containing 0.13 M NaCl, 3 mM KCl, 0.3 mM $CaNa_2EDTA$, 5 mM Tris-HCl, pH 7.2 and centrifuged at 35,000 *g* for 40 min (or 27,000 *g* for 1 h) in a swinging-bucket rotor for an ultracentrifuge or high-speed centrifuge respectively.

6. Harvest the elementary bodies from the interface and wash in phosphate-buffered saline.

2e. Notes

1. It is likely that one of the main determinants of the density of bacteria is the osmolality of the medium; thus the NaCl strength in the OptiPrep™ diluent may require modulation to optimize separations.
2. Most modern high-speed rotors with tube capacities of < 50 ml are capable of approx 27,000 *g*

3. *Piscirickettsia salmonis*

3a. Introduction

This is an obligate intracellular bacterium that requires fish host cells to replicate. Both diatrizoate (methylglucamine salt) and Percoll® have been used for the purification [2] but yields are poor and contamination by host cell components remains a problem [2]. Iodixanol gradients give better yields and improved resolution from host cell components. The methodology, although developed for *Piscirickettsia* may be more widely applicable for other obligate intracellular organisms. After growth of the organism in cultured cells, the bacteria are harvested from the cell culture fluid. If sufficient numbers of bacteria are not expressed into the culture fluid, it may be necessary to disrupt the cells.

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
200 mM EDTA ($Na_2 \bullet 2H_2O$): 7.44 g per 100 ml water
100 mM $MnCl_2 \bullet 4H_2O$: 1.96 g per 100 ml water

Solution B: To 100 ml water; add 30 ml, 24 ml, 2ml and 1ml respectively of the NaCl, $MnCl_2$, Tris and EDTA stocks; adjust to pH 7.6 with 1 M HCl and make up to 200 ml.

Solution C: To 50 ml water; add 15 ml, 3 ml and 1.5 ml respectively of the NaCl, Tris and EDTA stocks; adjust to pH 7.6 with 1 M HCl and make up to 100 ml.

Solution E: As solution B without the $MnCl_2$

3b. Solutions required

A. OptiPrep™ (shake gently before use)
B. Salt buffer: 150 mM NaCl, 12 mM $MnCl_2$, 1 mM EDTA, 10 mM Tris-HCl pH 7.6
C. OptiPrep™ diluent: 150 mM NaCl, 3 mM EDTA, 30 mM Tris-HCl pH 7.6
D. Working Solution (WS) of 40% (w/v) iodixanol: mix 4 vol. of OptiPrep™ with 2 vol. of Solution C.
E. WS diluent: 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.6

3c. Ultracentrifuge requirements
Swinging-bucket rotor with 17 ml tubes (e.g. Beckman SW28 or 28.1 or Sorvall AH629 (see Section 3e, Note 1)

3d. Protocol (adapted from refs 2 and 3)
Carry out all operations (except step 5) at 4°C

1. Make up three gradient solutions of 22%, 24% and 26% (w/v) iodixanol by diluting Solution D with Solution E and in 17 ml tubes make up discontinuous gradients from 4.5 ml of each solution (see Section 3e, Note 2).

2. Allow the gradients to become linear by diffusion for 1 h at room temperature then bring them to 4°C (see Section 3e, Note 2).

3. Collect the bacteria-containing fluid and centrifuge at 200 *g* for 10 min to remove host cell debris.

4. Concentrate the bacteria by centrifugation of the 200 *g* supernatant at 10,000 *g* for 45 min.

5. Suspend the pellet in 6 ml of Solution B and incubate with 20 U of DNase I at 30°C for 1 h.

6. Stop the reaction by adding 0.6 ml of the 200 mM EDTA stock solution.

7. Layer 3 ml on top of each gradient and centrifuge at 25,000 *g* for 3 h. Then harvest the sharp band of bacteria from the top third of the gradient (see Section 3e, Note 3).

3e. Notes
1. If an ultracentrifuge is not available, the g-force required is sufficiently low that a swinging bucket rotor for a high-speed centrifuge may be acceptable (e.g. Beckman JS24.15).
2. Alternatively a continuous gradient might be made using a two-chamber gradient maker or a Gradient Master™. For more information about preparing both discontinuous and continuous gradients **see Application Sheet C02**.
3. *Piscirickettsia salmonis* bands at a density of approx. 1.13 g/ml; other bacteria may have different banding densities and it may be necessary to modify the gradient density range in some cases. Contaminant organelles from the host cell band at higher densities.
4. Isla et al [4] used the same gradient strategy.

4. *Listeria monocytogenes* [5]

This bacteria are grown intracellularly in monocyte culture and released from the cells by distilled water lysis. The bacteria are then purified away from cell debris and cytosol by centrifugation through a discontinuous gradient of 60, 30 and 20% (w/v) iodixanol at 45,000 *g* for 1 h [5].

5. *Anaplasma phagocytophilum* [6]

Anaplasma phagocytophilum is grown in the human promyelocytic cell line (HL-60). To purify the bacteria released by homogenization of the cells the crude fraction is layered atop a discontinuous iodixanol gradient of 30% (6 ml), 25%, (5 ml), 20% and then 3.5 ml each of 17.5%, 15%, 12.5% (w/v) iodixanol (in an isoosmotic buffer). The gradient is centrifuged at 87,000 *g* for 74 min at 4°C. The bacteria band at the lowermost interface.

6. *Rickettsia*

Rickettsia typhi was grown in L929 mouse fibroblasts; cell lysates were first centrifuged at 1000 *g* for 5 min to remove cell nuclei and partially broken cells and then at 14,000 *g* for 10 min to pellet the bacteria [7]. After suspension of the pellet in a phosphate-buffered 218 mM sucrose (containing 4.9 mM potassium glutamate) the bacteria are pelleted through a 20% (w/v) iodixanol cushion (prepared by dilution of OptiPrep™ with the same buffered sucrose) at 14,000 *g* for 10 min. Pelc et al [8] used a

similar strategy. Smalley et al [9] used a discontinuous gradient of 20%, 26% and 32% OptiPrep™ for purification of *Rickettsia australis* but did not give any more detailed information. A similar gradient was used by Bechelli et al [10].

7. *Chlamydia trachomatis* (different morphological forms); see refs 11-13

The persistent form, reticulate body and elementary body from *Chalmydia trachomatis* have been separated in a gradient of 54%, 44%, 40%, 34% and 24% (v/v) OptiPrep™, formed by dilution of OptiPrep™ with the widely used phosphate-buffered 218 mM sucrose (containing 4.9 mM potassium glutamate). The top loaded sample was centrifuged at 100,000 *g* for 1 h at 4°C: the persistent form banded above the 24-34% interface, the reticulate body above the 34-40% and the elementary body in the 44% layer. Allbritton et al [13] used the same methodology as that described in ref 11.

8. Cyanobacteria

Fujishiro et al [14] have published a paper on the establishment of a pure culture of the cyanobacterium *Aphanothece sacrum* that required the isolation of the organism in a very pure form. A complex medium of inorganic salts was used to suspend the organism and aggregates were filtered; sonication was used to remove the dense exopolysaccharide matrix.

After sonication, the crude cell suspension was centrifuged at 14,000 *g* for 10 min; the cell pellet was resuspended in complex medium (see ref 8 for details of the composition of the medium). The suspension was loaded on to a 40-45% (w/v) iodixanol gradient and the cyanobacterium banded sharply in the middle of the gradient after centrifugation at 112,700 *g* for 2 h [14]. Thus the banding density of the cyanobacterium was approx. 1.22 g/ml.

9. Density determination of bacteria and bacterial protoplasts

Nycodenz® gradients (1.10-1.46 g/ml), or sequential centrifugation in Nycodenz® solutions of increasing density, are used to determine the density of bacterial spore protoplasts (e.g. refs 15 and 16). Lewis et al [17] stressed the importance of understanding how bacteria such as *Escherichia coli* O157:H7 and *Listeria innocua* survive and proliferate. The authors investigated the buoyant masses of live and dead cells using a suspended microchannel resonator (SMR). Preparation of solutions from OptiPrep™ that were either more or less dense than the cells and which minimized any osmolarity changes were integral to the measurements.

- A review of some of the methodology for purifying a variety of pathogenic bacteria is given in refs 18 and 19.

10. Bacterial minicells

Minicells are the products of an aberrant division of the bacterial cell that contains RNA and protein, but no chromosomal DNA. They are *"capable of delivering heterologous antigens to the class I antigen presentation pathway stimulating immune responses both in vitro and in vivo"*. Carleton et al [20]. Briefly, bacteria were removed from the culture at 2000 *g* for 10 min and the minicells sedimented from the supernatant at 10,000 *g* for 30 min; the pellet was resuspended in medium and applied to a 5–20% (w/v) iodixanol gradient, centrifuged at 20,000 *g* for 20 min. The minicells that were recovered from the gradient contained <0.001% bacteria. Similar conditions were used by MacDiarmid et al [21].

11. References

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