OptiPrep™ Application Sheet S48

Fractionation of acidocalcisomes, contractile vacuoles and other organelles from trypanosomes and other microorganisms

- 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 Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- Important technical notes, information regarding the extension of the technology to a variety of organisms and membrane analysis are contained in the "Technical Notes and Review" (Section 5).
- **Important information on the use of other organisms is in Sections 5.3, 5.4 and 5.5**
- See also Application Sheet S56 for other organelle isolation methods

1. Background

Acidocalcisomes, the electron-dense, acidic, calcium-storing organelles, which are rich in calcium and polyphosphate, were originally identified in *Trypanosoma cruzi* by Docampo et al [1] in 1995. They have now been detected in a range of prokaryotic and eukaryotic cells but there are significant differences in their characteristics from organism to organism. Although strict parallels in mammalian and non-mammalian cells may not exist, some dense granules in human platelets [2] and sea urchin eggs [3] share the same high levels of calcium and polyphosphate and may have similar functions.

Scott and Docampo [4] developed a discontinuous iodixanol gradient for purifying acidocalcisomes that effectively replaces the previous Percoll® method and overcomes many of the disadvantages associated with the use of Percoll®. The more general problem, observed with the recovery of mammalian organelles (e.g. lysosomes and peroxisomes) free from the colloidal silica particles with which they tend to co-sediment, is more serious in the case of acidocalcisomes because of their higher density and more rapid rate of sedimentation. Iodixanol, being a true solute, does not pose this problem at all. Moreover, since it is non-light-scattering, most spectrophotometric assays and electrophoresis can be carried out directly on gradient fractions without the need to remove the medium, so long as the concentration of the organelle in the gradient fraction is sufficiently high for accurate analysis. In some cases the gradient is able to provide simultaneous resolution of some other organelles.

- Protocol 4a describes the method for isolation of the acidocalcisomes only, adapted from ref 4. Protocol 4b describes a later modification for the simultaneous isolation of acidocalcisomes and contractile vacuoles, adapted from ref 5.

2. Solutions required (see Section 5.1)

A. OptiPrep™ (60% w/v, iodixanol)

B. Lysis buffer: 0.125 M sucrose, 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 5 mM dithiothreitol (DTT), 20 mM Hepes-KOH, pH 7.2

C. OptiPrep™ diluent: 0.125 M sucrose, 0.3 M KCl, 24 mM $MgCl_2$, 3.0 mM EDTA, 30 mM DTT, 120 mM Hepes-KOH, pH 7.2

D. Iodixanol (50% w/v) working solution: mix 5 vol. of OptiPrep™ + 1 vol. of Solution C

Keep the following stock solutions at 4°C:

1 M Hepes (free acid)	23.8 g per 100 ml water
1 M KCl	7.45 g per 100 ml water
1 M $MgCl_2 \bullet 6H_2O$	20.3 g per 100 ml water
100 mM EDTA(K_2)	3.68 g per 100 ml water
100 mM DTT	1.54 g per 100 ml water

Solution B: Dissolve 8.55 g sucrose in 100 ml water; add 4 ml, 10 ml, 0.8 ml, 1 ml and 10 ml respectively of Hepes, KCl, $MgCl_2$, EDTA and DTT stock solutions; adjust to pH 7.2 with 1 M KOH and make up to 200 ml.

Solution C: Dissolve 4.28 g sucrose in 30 ml of KCl stock solution; add 12 ml, 2.4 ml, 3 ml and 30 ml respectively of Hepes, $MgCl_2$, EDTA and DTT stock solutions; adjust to pH 7.2 with 1 M KOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Ultracentrifuge with swinging-bucket rotors to accommodate approx 30 ml thick-walled tubes, e.g. Beckman SW28, Sorvall AH629 or similar (see Section 5.2)

4. Protocol

Carry out all operations at 0-4°C.

4a. Isolation of acidocalcisomes

1. Lyse the washed epimastigotes by grinding with silicon carbide using standard procedures (see ref 3 for further details).

2. Centrifuge the lysed cells in Solution B at 144 *g* for 5 min (see Section 5.3).

3. Decant the supernatant and centrifuge this at 325 *g* for 10 min (see Section 5.3).

4. Decant and retain the supernatant.

5. Resuspend the pellet in Solution B and repeat the centrifugation at 325 *g* for 10 min.

6. Combine the two supernatants and centrifuge at 10,500 *g* for 30 min (see Section 5.4).

7. Resuspend the pellet in Solution B (4 ml) by repeated passages through the 22-gauge needle of a syringe (see Section 5.4).

8. Prepare density gradient solutions containing 24%, 28%, 34%, 37% and 40% (w/v) iodixanol by diluting Solution D with Solution B (see Section 5.5).

9. In tubes for the chosen rotor, layer 4 ml of each of the density gradient solutions and layer the resuspended 10,500 *g* pellet on top of the discontinuous gradient, to fill the tube (see Section 5.6).

10. Centrifuge at 50,000 *g* for 60 min.

11. The acidocalcisomes form a pellet at the bottom of the gradient. If the primary interest is isolation of the acidocalcisomes, aspirate the gradient and resuspend the pellet in a suitable medium. If the aim is to analyze other organelles as well, then collect the gradient in a series of 15-20 equal volume fractions (see Sections 5.7 and 5.8).

4b. Isolation of acidocalcisomes and contractile vacuoles

1. Clarify the lysate sequentially using two centrifugations at 36 *g* for 5 min and one at 144 *g* for 10 min (see Section 5.3).

2. Centrifuge the final supernatant at 100,000 *g* for 1 h.

3. Resuspend the pellet in Solution B (2 ml) by repeated passages through the 22-gauge needle of a syringe (see Section 5.4).

4. Mix the suspension with an equal volume of Solution D and 0.15 ml of Solution B to adjust it to 24% (w/v) iodixanol.

5. Prepare density gradient solutions containing 15%, 20%, 28%, 34%, 37% and 40% (w/v) iodixanol by diluting Solution D with Solution B.

6. In tubes for the chosen rotor, layer 4 ml of each of the density gradient solutions, including the sample in 24% iodixanol (see Section 5.6).

7. Centrifuge at 50,000 *g* for 60 min.

8. Collect the gradient in a series of 15-20 equal volume fractions (see Section 5.7 and 5.8).

5. Technical Notes and Review

5.1 Homogenization media

Solutions are commonly buffered with Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and it is unlikely if the type of buffer significantly influences the fractionation.

The preparation of a Working Solution as described, ensures that the concentrations of KCl, $MgCl_2$, EDTA, DTT and the buffer (Hepes-KOH, pH 7.2) are constant throughout the gradient. If this is deemed unimportant the iodixanol solutions may be prepared simply by diluting OptiPrep™ with Solution B. Strategies for preparing working solutions are given in Application Sheet S01.

Protease inhibitors may be included in Solutions B and C at the operator's discretion.

5.2 Ultracentrifuge rotors

The gradient + sample volume is 20-24 ml, thus thick-walled tubes which may be partially filled are the recommended ones for the rotor (total tube volume = approx. 30 ml). There is no obvious reason however why the gradient cannot be scaled up to allow the use of thin-walled tubes, which may be more convenient if complete unloading of the gradient into multiple fractions is envisaged. Similarly the procedure may be scaled down for use in rotors with a smaller tube volume.

5.3 Clarification of the lysate

In many cases of acidocalcisome preparation, for example from ***Toxoplasma gondii*** [6-8], the 144 *g* and 325 *g* steps have been replaced by 36 *g* and 144 *g* as in Protocol 4b, while for bacteria (***Rhodospirillum rubrum*** and ***Agrobacterium tumerfaciens***) the lysate was clarified at 1000*g* for 5 min [8,9].

5.4 Preparation of a crude acidocalcisome fraction

In the case of ***Toxoplasma gondii*** [6,7] the crude acidocalcisomes were sedimented at 15,000 *g* rather than 10,000 *g*, while for bacteria 14,500*g* was used [9,10]. Thus to maximize yields and purity of the acidocalcisomes it may be necessary to optimize the differential centrifugation of the homogenate.

Suspension of the crude fraction should be carried out as gently as possible to avoid damage not only to the organelles of interest but also to any other organelles present – particularly those which may release degradative enzymes. If median loading of the sample in the gradient is chosen, rather than top-loading, suspend the pellet in no more than approx 2 ml of Solution B so the volume after adjustment of the density with Solution D remains manageable.

5.5 Gradient format (all iodixanol concentrations are % w/v)

In some instances, for example in the isolation of acidocalcisomes from ***Dictylostelium discoideum*** the 40% iodixanol layer has been omitted [11]. To avoid any possible loss of material due to the rapid accumulation of particles at the sample/24% iodixanol interface in Protocol 4a, it may be preferable to suspend the crude pellet in one of the gradient layers, e.g. 24% or 28% iodixanol as in Protocol 4b. In Protocol 4b the lower density layers (15% and 20% iodixanol) are included to improve the resolution of the contractile vacuole from the denser mitochondria, glycosomes and lysosomes; such a format may generally be beneficial in any studies in which a more complete fractionation is required. Bacteria suspensions (***Rhodospirillum rubrum*** and ***Agrobacterium tumerfaciens***) were adjusted to 30% iodixanol and made part of a 24%, 28%, 30%, 35%, 40% iodixanol gradient [9,10] Note that in some bacteria the acidocalcisomes may be termed volutin granules [10]. For ***Toxoplasma gondii*** the gradient comprised 10%, 15%, 20% (sample), 25% and 30% iodixanol [6,7], while for isolation of acidocalcisomes from the plant trypanosomatid ***Phytomonas françai*** Protocol 4a was used [12]. In proteomic analytical studies of acidocalcisomes, Ferella et al [13] used a discontinuous 20-50% (w/v) iodixanol gradient and in a similar study of the contractile vacuole the discontinuous gradient comprised 15, 20, 25, 30, 34, 37 and 40% iodixanol, with the sample in the 25% layer [14]. A 14,500 g (10 min) fraction from a ***Corynebacterium matruchotii*** lysate was also median loaded as the 30% (w/v) step in a 24%, 28%, 30%, 35%, 40% (w/v) iodixanol gradient and centrifuged at 27,000 g_{av} for 1 h. The organelles banded at the 30%-35% interface [15].

5.6 Forming the discontinuous gradient

Although overlayering (i.e. starting with the densest layer) is the most common means of creating a discontinuous gradient, underlayering (i.e. starting with the least dense layer) with a syringe and metal cannula is more reliable and the recommended method for making discontinuous gradients. For more information on gradient construction see Application Sheet S03. If necessary, adjust all volumes proportionately so that tubes (after sample application) are properly filled according to the manufacturer's instructions.

5.7 Fractionating the gradient

If thick-walled tubes are used then aspiration from the bottom of the tube or from the meniscus (with a Labconco Auto Densi-flow device) are acceptable methods for harvesting gradients. Unloading by upward displacement with a dense liquid may be less acceptable since the pellet may become dispersed. So long as the acidocalcisomes do not form a too firmly-packed pellet, tube puncture may be satisfactory for a thin-walled tube. For more information on harvesting gradients see Application Sheet S52.

5.8 Analysis

Protocol 4a has been used preparatively for the isolation of acidocalcisomes from *Trypanosoma cruzi* [4,16-18], *Trypanosoma brucei* [18] and *Leishmania major* [18] in studies on Ca^{2+} and phosphate metabolism. This protocol also permits the partial resolution of some other organelles [19], although glycosomes, lysosomes and the vacuolar compartment tend to overlap close to the top interface between the sample and the top layer of the gradient.

There are however quite clear differences in the manner in which organelles from different organisms behave using Protocol 4a. Figure 1 shows the distribution of organelles from a 10,500g pellet prepared from a post-nuclear supernatant of a *Dictyostelium discoideum* homogenate, which was layered on top of the gradient, as in Protocol 4a, but omitting the 40% iodixanol layer [11].

Alkaline phosphodiesterase shows a clear concentration at the top of the gradient (Figure 1); this is an established marker for contractile vacuoles from a number of microorganisms. The vacuole invariably bands at a lower density than any other organelle. On the other hand both pyrophosphatase and the vacuolar H^+-ATPase (V-H^+ ATPase) are present in both the vacuole and the dense acidocalcisome, confirming a functional link between these two particles [11]. Acid phosphatase (lysosomes) and succinate cytochrome c reductase (mitochondria) each show distinctive profiles. The distribution of the mitochondria is broad (compared to the usual pattern from mammalian cells); nevertheless fractions 8-10 which demonstrate the highest succinate-cytochrome c reductase are also significantly impoverished in markers for other organelles. It is also notable that the lysosomes from *Dictyostelium* are denser than are the bulk of the mitochondria (the reverse is true in all mammalian cells so far studied).

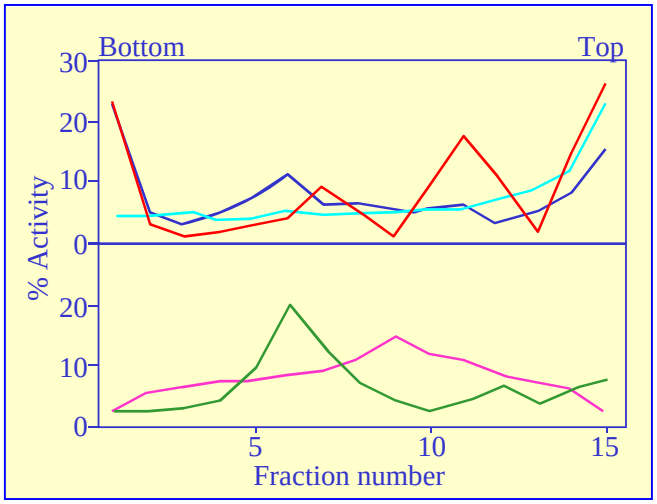

Figure 1. Fractionation of *Dictyostelium* 10,000g pellet: pyrophosphatase (—); V-H^+ ATPase (—), alkaline phosphodiesterase (—), acid phosphatase (—), succinate-cyt c reductase (—). Adapted from ref 8 with kind permission of the authors and The American Society of Biochemistry and Molecular Biology.

In the case of *Chlamydomonas reinhardtii* Protocol 4a was also able to resolve very clearly an acidocalcisome fraction and in this case the mitochondria were also well resolved about a third of the way down the gradient, but the chloroplasts were rather broadly distributed [20].

Protocol 4b provides a much more clear resolution in the case of *Toxoplasma gondii* [6,7] and *Trypanosoma cruzi* [5] of the vacuole, lysosomes and glycosomes and the acidocalcisomes which band towards the top, middle and bottom of the gradient respectively.

- A review of the functional significance of acidocalcisomes is to be found in ref 21
- Other papers using this methodology for *Trypanosoma brucei* report that modulation of polyphosphate alters acidocalcisome biogenesis and function [22]; acidocalcisome lipids and glycolipids [23] and that the inositol 1,4,5-trisphosphate receptor present on acidocalcisomes has an important role in growth and infectivity [24].
- A very thorough proteomic analysis of acidocalcisomes from a variety of sources, using variants of the above technology, see ref 25.
- **IMPORTANT NOTE: Mini-review MS03 gives and up-to-date bibliography of all the published papers reporting the use of iodixanol gradient methodology for the purification of organelles from non-mammalian eukaryotes.**

6. References

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