OptiPrep™ Application Sheet C17

Purification and processing of animal (non-human) and avian spermatozoa

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **For human spermatozoa see Application Sheet C18**
- 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
- Section 2 describes the optimal method for the enrichment of viable spermatozoa from bovine semen using OptiPrep™.
- Sections 3-5 summarize some of the other published methods for sperm from rodents (3), equine sperm (4), and *Xenopus laevis* sperm and spermatids (5).
- Section 6 describes the use of OptiPrep™ for cryopreservation of sperm from a number of species.

1. Introduction

In ejaculates, viable spermatozoa of normal morphology are sometimes a very low percentage of the total cell population. This Application Sheet presents a detailed protocol for the recovery of a highly viable fraction of bovine spermatozoa for use in fertilization. The recommended strategy involves, in the first instance, adjustment of the density of a semen sample to approx. 1.17 g/ml. Ideally, two lower density solutions are then layered on top, so that the viable semen of normal morphology band at the interface between theses two layers. These cells are thus completely separated from both the non-viable cells (and any soluble material released from partially broken cells), which all remain in the load zone, and any morphologically abnormal cells that band at the top of the least dense layer. This result is depicted in Figure 1.

Iodixanol is the gradient solute of choice: firstly, all of the solutions are easily prepared by dilution of OptiPrep™ with any buffered saline or a special diluent formulated for the maintenance of sperm viability, while Nycodenz® solutions must be prepared from Nycodenz® powder. Secondly, to raise the density of the ejaculate to approx 1.170 g/ml it is necessary to mix it with a high-density medium (usually >1.26 g/ml). Nycodenz® solutions are hyperosmotic above ρ = 1.16 g/ml, thus the seminal fluid would also become hyperosmotic. Consequently with Nycodenz® the semen has to be loaded at a lower density in the middle, or top of the gradient. This is not the case with iodixanol; OptiPrep™ or a dense solution prepared from OptiPrep™ and the chosen diluent, can be added to a raw ejaculate without increasing its osmolality (see Notes 1 and 2).

2. Purification of bovine spermatozoa

2a. Solutions required

A. OptiPrep™ (shake gently before use)

B. Diluent**:** Hanks Buffered Salt Solution (HBSS) or other suitable ambient temperature diluent such as Ruthin Diluent (RD); see also Note 2.

2b. Protocol

1. Assess a freshly taken ejaculate for viability and then mix with an equal volume of Solution A to raise its density to approx. 1.170 g/ml.

2. Prepare the two gradient solutions: 1.119 g/ml (4 vol. OptiPrep™ + 7 vol. HBSS) and 1.154 g/ml (9 vol. OptiPrep™ + 10 vol. HBSS). Using RD mix respectively 4 vol. + 8 vol. and 9 vol. + 11 vol. (see Notes 2 and 3).

3. In a suitable tube (50 ml) layer 10 ml of each of the two gradient solutions and underlayer these with 10-15 ml of the sample-OptiPrep™ mixture (density approximately 1.17 g/ml), to form a three-step gradient (see Figure 1 and Notes 4 and 5).

4. Centrifuge the gradient in a swinging-bucket rotor at 1500 g_{av} for 20 min at approx. 20°C.

5. After centrifugation, deformed sperm, cytoplasmic droplets, detached heads and tails band at the top of the gradient (A). Motile cells of normal morphology band at the 1.119/1.154 g/ml interface (B) while in the loading area a pellet (D) and some particulate material in suspension (C) contain immotile sperm (see Figure 1). The sperm cells from interface B can then be checked for viability and fertility and stored (see Notes 6-9).

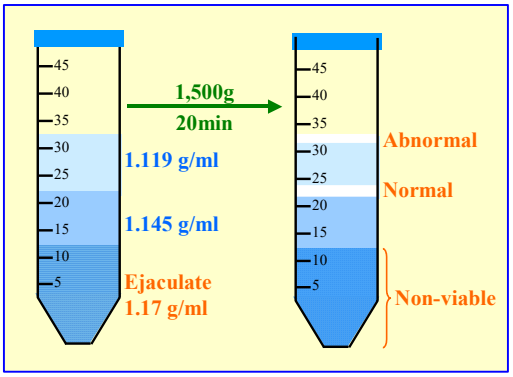

Figure 1: Purification of normal motile sperm cells in a discontinuous iodixanol gradient

2c. Notes

1. Iodixanol is essentially a dimer of Nycodenz®, it therefore has approximately twice the molecular mass and solutions have half the osmolality of Nycodenz®
2. The diluent solution may be any solution thought appropriate by individual workers. The final density of the diluted fractions will depend upon the density of the diluent. Common physiological salt solutions, such as phosphate-buffered saline, Hanks Buffered Salt Solution (HBSS) or a more complex medium such as RPMI, have densities close to 1.005 - 1.006 g/ml. It may however be preferable to use a medium designed to preserve the viability and motility of sperm cells at room temperature. These so-called ambient temperature diluents (for example Ruthin Diluent) frequently contain polyhydric alcohols such as sorbitol and consequently have a slightly higher density (ρ = 1.018 g/ml). Details of the Ruthin Diluent can be obtained from Dr Stuart Revell, Genus Freezing Unit, Llanrhydd, Ruthin, Denbighshire, LL15 2UP, UK.
3. The volume of OptiPrep™ and medium required to prepare the density solutions will vary with the density of the medium. For more information about preparing density gradient solutions for mammalian cells see **Application Sheet C01**.
4. For more information on preparation of discontinuous gradients see **Application Sheet C02**.
5. More recently Garrett et al [1] simplified the gradient: the semen (diluted first with Eqcellsire™) was mixed with an equal volume of OptiPrep™; 8 ml of this suspension was then overlaid with 1 ml of a 1.15 g/ml solution and centrifuged at 1000 *g* for 15 min. The viable sperm banded at the top of the gradient.
6. The quality of the semen has been assessed by using membrane integrity as an indicator of general cell function and viability. The Osmotic Resistance Test (ORT) described by Revell and Mrode [2] and the fluorescent analysis method described by Harrison and Vickers [3] have been used to check membrane integrity. The motile band from the 1.119/1.154 g/ml interface shows over 95% viability by these tests, while the pelleted material and particulate material remaining in the loading layer are found to be 99% non-viable cells by these methods.
7. Routinely, the viable, motile spermatozoa are diluted in skimmed milk, glucose and glycerol, to provide $1.5x10^7$ per A.I. straw and deep-frozen. When the straws are subsequently thawed and subjected to ORT, 74% of the sperm are still viable as judged by membrane integrity and activity.
8. Ejaculates from other species have shown similar but not identical banding characteristics: small changes to the precise densities of the layers may be required. The solutions and protocol in this Application Sheet will serve as a useful starting point from which adjustments to the final densities of the gradient layers can be made, to optimize the fractionation of material from other species.
9. This methodology has also been reported in ref 4.

3. Rodent sperm

Separation of viable and non-viable sperm cells was achieved on double layer iodixanol gradient of 15% and 24% (w/v) iodixanol; after centrifugation at 400 *g* for 20 min the viable sperm banded at the interface of the two iodixanol solutions [5-8].

4. Equine sperm

Stuhtmann et al [9] and Heutelbeck et al [10,11] used similar two-layer gradients for stallion sperm of 1.090 and 1.165 g/ml or 1.090 and 1.170 g/ml and commented that these gradients promoted better retention of morphology and progressive motility and that the gradient purification was more effective if carried out just after collection rather then immediately before cryopreservation.

5. *Xenopus laevis* sperm and spermatids

A discontinuous iodixanol gradient of 12%, 20% and 30% (w/v) iodixanol was used to separate the lighter spermatids from the denser mature semen [12].

6. Concentration of semen on to a dense cushion, prior to freezing

Compared with simple pelleting, sedimentation on to a dense cushion of iodixanol prior to cooling and freezing considerably improves the recovery and motility of viable sperm. The cushion is usually OptiPrep™ itself and the centrifugation conditions vary from 800 *g* for 10 min to 1000 *g* for 20 min. To harvest the band of spermatozoa, a narrow metal cannula is used to remove the liquids above and below the cells. The spermatozoa band is often described as a "pellet"; a "sharp band" is the more accurate description. The method has been widely used for equine sperm [13-33]; this has also been observed for gazelle semen [34], elephant semen [35-38], boar semen [39,40], donkey semen [41], buffalo semen [42], porcine semen [43], ram semen [44] and rodent semen [45,46].

Some more recent references reporting the use of OptiPrep™ are listed in Section 7: refs. # 47-53

Important technical note: It is not known if any of the published methods using the iodinated density gradient medium Nycodenz® can be translated direrctly to the use of OptiPrep™. Although solutions of the same % (w/v) will have the same density, the osmolality of iodixanol solutions will be approx. half those of Nycodenz®. Unlike those of Nycodenz®, iodixanol solutions can be made approx. isoosmotic with plasma at all densities.

7. References

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