OptiPrep™ Application Sheet C18

Purification of viable human spermatozoa in iodixanol or Nycodenz® gradients

- 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

1. Background

Human ejaculates contain variable proportions of viable spermatozoa of normal morphology, sometimes a very low percentage of the total cell population but Mortimer [1] stressed the need to avoid direct pelleting of sperm cells in any fertilization studies. The use of various density gradient techniques for the enrichment of motile sperm media has therefore been investigated and this application sheet presents some of the recommended methods. Although gradients of Percoll® became popular in the early nineteen-eighties for the enrichment of motile spermatozoa from human semen, several groups of workers recognized that the use of an iodinated density gradient medium that was already approved for human injection as an X-ray contrast agent offered a much more attractive solution for this procedure. Earlier papers reported the use of Nycodenz® (Section 2), but more recently OptiPrep™ (Section 3) has been used for this purpose.

- Gellert-Mortimer et al [2] showed that 60% of the sperm isolated from a four-step Nycodenz® gradient retained their motility after 21 h, while this figure was only 5% with a Percoll® gradient. The method is summarized in Section 2. Mortimer [3] also stressed that, particularly in the case of the semen from asthenozoospermic individuals, a Nycodenz® gradient gave superior results.

2. Nycodenz®

2a. Solutions and reagents required

A. Nycodenz® powder
B. Diluent: 6 mM KCl, 10 mM Tricine (or HEPES) - NaOH, pH 7.4
C. Any buffered saline balanced salt solution or culture medium

Keep the following stock solutions at 4°C
100 mM HEPES (free acid): 2.38 g per 100 ml water
OR 100 mM Tricine: 1.79 g per 100 ml water
100 mM KCl: 0.74 g per 100 ml water

Solution B: To 40 ml water; add 10 ml and 6 ml respectively of HEPES (or Tricine) and KCl stock solutions; adjust to pH 7.4 with 0.1 M NaOH; make up to 100 ml.

2b. Solution preparation

Published papers report the use of Nycoprep™ 1.15, a solution of 27.6% (w/v) Nycodenz® containing 3mM KCl, 0.3 mM $CaNa_2$-EDTA, 5 mM Tris-HCl, pH 7.4. This isoosmotic solution is no longer commercially available, but a similar stock solution can be made up easily from Nycodenz® powder. In this Application Sheet the Tris is replaced by either Tricine of HEPES (see box), which are much more cell-friendly, and the EDTA omitted. To make 100 ml of the 1.15 g/ml stock solution place approx. 50 ml of Solution B in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 27.6 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 (see Note 1).

2c. Protocol

Gellert-Mortimer et al [2] diluted the 27.6% Nycodenz® stock solution with a Ham's F10 medium supplemented with 1mM calcium lactate, 20 mM $NaHCO_3$, 5 mM $KHCO_3$ and 0.5 mM $MgSO_4$, to produce the gradient solutions. Sbracia et al [4] used Human Tubule Fluid (HTF) containing 0.5% bovine serum albumin (BSA).

1. Measure out aliquots of 4.85 ml, 6.9 ml and 9.0 ml of the 27.6% Nycodenz® stock solution and dilute each with the supplemented Ham's F10 medium to 13.8 ml. This produces solutions of 9.7%, 13.8% and 18% (w/v) Nycodenz® (see Note 2).

2. Form discontinuous gradients from 1.6 ml, 1.6 ml, 3.2 ml and 1.6 ml of the 9.7%, 13.8%, 18% and 27.6% (w/v) Nycodenz® solutions (see Notes 2 and 3).

3. Dilute the liquefied semen with an equal volume of the supplemented Ham's F10 medium and layer it on top of the gradient (see Note 2).

4. Centrifuge at 350 *g* for 12 min (see Note 2).

5. Collect the motile semen that band at the bottom of the 18% Nycodenz® layer (see Notes 2 and 4).

2d. Notes on the Nycodenz® methodology

1. There is no obvious reason why iodixanol should not be substituted for Nycodenz® in these methods; it would certainly be an easier option. To produce a stock solution of density 1.15 g/ml dilute 27.6 ml of OptiPrep™ to 60 ml with the chosen diluent (see Section 2c).
2. Sbracia et al [4] used a simplified two-layer gradient from 2 ml each of 9.7% and 18% (w/v) Nycodenz®. The semen was diluted in the HTF+BSA medium prior to loading and the gradient was centrifuged at 400 *g* for 20 min. In this format the semen form a pellet.
3. For the construction of discontinuous gradients see **Application Sheet C02**.
4. Sbracia et al [4] reported that the long-term retention of sperm motility (after 24 h) was substantially improved in Nycodenz® compared to Percoll®: 35% versus 26% with 60% versus 40% of the initial motility respectively. Nycodenz® samples also exhibited a higher retention of total motile sperm. The sperm motility index, a multiple of velocity and motility in the sample (a measure of the efficiency of the sperm population in sperm-oocyte interaction, was 75% higher in the Nycodenz®. Refs 5 and 6 review some of the current technology.

3. OptiPrep™

3a. Purification by flotation

The optimal protocol for the isolation of viable sperm cells of normal morphology from bovine sperm is to adjust the density of the raw ejaculate to approx 1.170 g/ml and place this beneath a two-layer gradient (1.154 and 1.119 g/ml). OptiPrep™ can be added to a raw ejaculate without increasing its osmolality. In this respect OptiPrep™ offers an advantage over Nycodenz® as solutions of this solute are hyperosmotic above ρ = 1.16 g/ml. Viable cells float upwards to their buoyant banding density [7]. Those of normal morphology band at the 1.154 and 1.119 g/ml interface and non-viable cells either pellet or remain in the load zone, see **Application Sheet C17**. This methodology was very successfully adapted by Smith et al [8,9] to human semen; the two upper layers of iodixanol were adjusted to 1.05 and 1.15 g/ml interface. The method described in below is adapted from refs 8 and 9.

3a-1. Solutions required

A. OptiPrep™ (shake the bottle gently before use)
B. Modified Human Tubal Fluid (mHTF)

3a-2. Protocol

1. Dilute OptiPrep™ with mHTF to obtain solutions of ρ = 1.15 and 1.05 g/ml - approx 27.5% and 8%, w/v iodixanol respectively (see Notes 1 and 2 in Section 3c).

2. Mix the liquefied semen with OptiPrep™ (4 vol. + 6.5 vol. respectively) to raise its density to approx ρ = 1.17 g/ml.

3. Prepare the discontinuous gradients from 3 ml each of the ρ = 1.15 and 1.05 g/ml solutions in a 15 ml tube (see Note 3 in Section 3c).

4. Layer the dense semen suspension below the gradient and centrifuge at 1500 *g* for 40 min.

5. Collect the viable sperm cells of normal morphology from the 1.05/1.15 g/ml interface.

6. Dilute the 5 vol. of mHTF and harvest the cells by centrifugation at 500 *g* for 15 min (see Note 4 Section 3c).

3b. Purification by sedimentation

The protocol described below takes account of the fact that the customary procedure for purifying human sperm cells involves layering the semen on top of the gradient rather than below it in a dense solution. It should be regarded as a trial procedure rather than a definitive procedure and may require modification to suit the particular medium used and/or in the light of experience. The density of the top layer has been increased to allow for possible variation in the density of the applied sample. It is adapted from ref. 10.

3b-1. Protocol

1. Dilute OptiPrep™ with mHTF (see Note 2) to obtain solutions of ρ = 1.09 and 1.132 g/ml (approx 16% and 24%, w/v iodixanol respectively). See Notes 5-9 in Section 3c for more information on density selection and quality of the purified semen.

2. Layer the liquefied ejaculate on top of equal volumes (1 ml was recommended in ref 8) of the two density barriers.

3. Centrifuge at 400 *g* for 20 min at room temperature.

4. Remove and discard the upper layers containing abnormal cells and harvest the motile normal cells from the lower interface.

5. Harvest the cells after dilution as in Section Protocol 3a-2.

4. Notes

1. The osmolality of these solutions is approx 280 mOsm.
2. The volume of OptiPrep™ and medium required to prepare the density solutions will vary with the density of the medium. If the density of the diluent is significantly different to that of mHTF it may be necessary to adjust the volumes of OptiPrep™ and diluent required to produce a particular density. To determine the amounts of OptiPrep™ and medium to mix together use the equation described in **Application Sheet C01**.
3. For information on the preparation of discontinuous and continuous gradients for cell separations see **Application Sheet C02**.
4. Smith et al [8] reported that 78% of the motile and 99% of the morphologically normal sperm cells were recovered in the interfacial band and they, and other workers [10-14] have concluded that the method was a suitable nontoxic alternative to Percoll®.
5. Harrison [10] reported that these two densities produced best recovery of viable sperm (30-34%) but layers of 18% and 27% (w/v) iodixanol, equivalent to densities of 1.100 and 1.148 g/ml were almost as effective. A lower layer with a density as high as approx ρ = 1.16 g/ml should still allow non-viable cells to pellet and this higher density may improve the recovery of viable cells. The recommended density of the upper layer may also require modulation to suit the operator's requirements. The aim of this layer is to separate the viable sperm cells, which sediment through it and band at 1.09/1.132 g/ml interface, from abnormal cells which band at the sample/1.09 g/ml interface or within the 1.09 g/ml layer.
6. Van den Bergh et al [11] used a three-layer gradient of 7.5%, 15% and 30% (w/v) iodixanol.

7. Kaftani et al [12] compared a 10.5% and 21% (w/v) iodixanol gradient (approx. equivalent to 1.062 and 1.117 g/ml) with the standard Percoll gradient and found no significant difference between the two gradient media in terms of recovery of % motile sperm or morphology.
8. There are other published methods in which the sedimentation strategy (Protocol B) has been variously modified to take account of particular laboratory or clinical requirements, some of which use polysucrose as an additive to the iodixanol solution [13-15]. A number of publications have compared some or all of the available methods, for example ref. 16.
9. More recently Araki et al [17] used a two layer gradient of 16% and 24% (w/v) iodixanol to purify human sperm.

5. Other OptiPrep™ applications

The use of OptiPrep™ as a cushion on to which the sperm may be concentrated prior to cryopreservation, which has been widely used for non-human sperm applications (**see OptiPrep™ Application Sheet C17**), has more recently been extended to human samples [18]. Jallouk et al [19] used nanoparticles to reduce sperm and vaginal epithelium cytotoxicity and subsequently concentrated the modified sperm cells on to an OptiPrep™ cushion, which also allowed the unbound nanoparticles to sediment.

Iodixanol solutions have also been used to separate sperm cells from leukocyte in studies on individuals with HIV infection [20,21] and more recently used in cryopreservation studies [22].

A recent publication has reviewed the technologies used for purifying and cryopreservation of human sperm [23].

6. References

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