OptiPrep™ Application Sheet C42

Isolation of renal cells: (1) Proximal tubule cells and (2) Interstitial cells and thin loop of Henlé 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
- Nycodenz® gradients have been used routinely for the fractionation of renal cells (see Sections 1 and 2); it is only very recently that papers reporting the use of OptiPrep™ have been published (see Section 4).

1. Proximal tubule cells

1a. Background

Proximal tubule cells are used as an *in vitro* model for studies on nephrotoxicity and the method developed by Boogaard et al [1,2] yields 90-95% of proximal tubule cells with a high viability (97%). The perfusion technique is an important part of the methodology and is provided in the protocol, but for information about the preliminary ligation of blood vessels, the operator should refer to ref 1. As with all isolations of cells from tissues, the release of viable cells as a single cell suspension by disaggregation of the tissue with collagenase is a critical part of the procedure.

The following protocol is adapted from ref 1 (see Section 3, Notes 1 and 2).

1b. Solutions required (see Note 3)

A. Perfusion medium 1: Calcium-free Hank's Balanced Salt Solution containing 0.5 mM EGTA and 25 mM HEPES

B. Perfusion Medium 2: As solution A without EGTA.

C. Perfusion Medium 3: As Solution B containing 4 mM $CaCl_2$ and 0.12% collagenase

D. Perfusion Medium 4: As Solution B containing 2.5% (w/v) bovine serum albumin (BSA)

E. Nycodenz® stock: 40% (w/v) in water (see Note 4)

F. Nycodenz® diluent: 67 mM KCl, 12.2 mM $CaCl_2$, 100 mM HEPES-NaOH, pH 7.4

G. Nycodenz® (34%, w/v) solution: Mix 3.4 vol. of Solution E with 0.6 vol. of Solution F and 2 vol. of water

H. Low density barrier solution: mix 1 vol. of solution G with 4 vol. of Solution D (see Note 5).

Keep the following stock solutions at 4°C
1 M HEPES (free acid): 23.8 g per 100 ml water; adjust to pH 7.4 with 1 M NaOH before making up to final volume.
100 mM EGTA: 3.80 g per 100 ml water (pH 11-12)
1 M KCl: 7.46 g per 100 ml water
1 M $CaCl_2 \bullet 2H_2O$: 14.7 g per 100 ml water

Prepare Solutions A-D from commercially available 10x calcium-free Hank's Balanced Salt Solution. Dilute this with approx half of the required water before adding the HEPES stock solution plus the other supplements as appropriate. Then make up to the full volume with water. Gas the solutions with 5% CO_2/air, the pH should be adjusted to 7.4.

Solution F: To 50 ml of water add 10 ml, 6.7 ml and 1.22 ml respectively of the HEPES, KCl and $CaCl_2$ stock solutions; adjust to pH 7.4 and make up to 100 ml with water

1c. Protocol

Carry out Steps 1-3 at 37°C and step 5 onwards at 0-4°C. Keep Solutions D, G and H at 0-4°C

1. Perfuse the kidneys with 150 ml of Solution A at 37°C at 10 ml/min; once all the blood has been washed out reduce the flow rate to 7.5 ml/min.

2. After removing kidneys, continue perfusion with 25 ml of Solution B.

3. Perfuse in a recirculating system with Solution C for 18 min.

4. Wash out Solution C with 10 ml Solution D.

5. Remove the capsule and disperse the tissue in Solution D.

6. Filter the cell suspension through two layers of nylon gauze (80 mesh).

7. Centrifuge the cells at 80 *g* for 3 min and wash the pellet three times in Solution D (see Note 6).

8. Resuspend the cells in 4 ml of Solution D and mix with 2 ml of Solution G.

9. Overlayer 3 ml of the cell suspension with 1 ml of Solution H and 0.5 ml of Solution D (see Note 7).

10. Centrifuge at 2300 *g* for 3 min (see Note 8).

11. Harvest the proximal tubule cells from the lower interface.

2. Interstitial cells and thin loop of Henlé cells

2a. Background

Interstitial cells and thin limb of Henlé cells from the disaggregated inner medulla have been purified in two steps. Firstly, they are separated from inner medullary collecting duct cells using magnetic beads coated with *Dolichos biflorus* Agglutinin, which binds almost exclusively to the collecting duct cells. Subsequently purification of the non-collecting duct cells is achieved in a continuous Nycodenz® gradient with a density range of 1.052-1.093 g/ml; this is approximately equivalent to 9.4%-17% Nycodenz® [3-6]. In the following truncated protocol only the gradient centrifugation is described; for information regarding the preparation of the crude medullary cell suspension see ref 5. The method is taken from ref 5.

2b. Solutions required (see Notes 3 and 9)

A. Nycodenz® Stock Solution, 28% (w/v), $\rho = 1.15$ g/ml (see step 1 of Protocol)

B. Diluent: 3mM KCl, 0.3 mM $CaNa_2$-EDTA, 5 mM Tricine-NaOH, pH 7.4

C. 7.45% (w/v) sucrose in 5 mM Tricine-NaOH, pH 7.4

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 B: To 40 ml water; add 5 ml, 3 ml and 0.3 ml respectively of Tricine, KCl and EDTA stock solutions; adjust to pH 7.4 with 0.1 M NaOH; make up to 100 ml

Solution C: Dissolve 7.45 g of sucrose in 50 ml water; add 5 ml of Tricine stock solution; adjust to pH 7.0 with 1 M NaOH and make up to 100 ml

2c. Protocol (density gradient only)

1. To make 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 28 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and then make up to 100 ml with Solution B. Filter sterilize if required.

2. Dilute the 28% Nycodenz® solution with Solution C to produce solutions of 8% and 20% (w/v) Nycodenz®.

3. In a 15 ml centrifuge tube layer 2 ml of the 8% Nycodenz® over 3 ml of the 20% Nycodenz® solution and allow a continuous gradient to form by diffusion (see Note 10).

4. Layer the crude cell suspension on top and centrifuge at 1,500 *g* for 45 min.

5. Allow the rotor to decelerate without the brake.

6. The interstitial cells band at 1.081-1.093 g/ml, approx. equivalent to 15-17% Nycodenz® [3,4]. In this same gradient, the thin limb of Henlé cells band predominantly at 1.052-1.069 g/ml, approx equivalent to 9.4%-12.5% Nycodenz® [5,6].

7. Recover the appropriate layers using a syringe and metal cannula or unload the gradient in a series of equal volume fractions (see Note 11)

3. Notes

1. A number of other published papers have also reported the use of this methodology for the isolation of rat renal proximal tubule cells [7-18], more or less as described by Boogaard et al [1,2]. There have however, been some small modifications to both the buffers and the gradient. Schaaf et al [19] used a Hank's Balanced Salt Solution (HBSS) containing PIPES rather than HEPES; these workers also suspended the cells in 14% (w/v) Nycodenz® rather than 11.3% Nycodenz® and 9% Nycodenz® was layered on top rather than 6.8% Nycodenz®.
2. Kruidering et al [20] obtained the cells from pigs and used a perfusion medium (Eurocollins, pH 7.4) comprising 177 mM glucose, 10 mM $NaHCO_3$, 15 mM KCl, 42 mM K_2HPO_4, 15 mM KH_2PO_4 and 2 mM glycine. In this case, the minced cortex was washed with Ca^{2+}/Mg^{2+}-free HBSS containing 25 mM HEPES and 2 mM glycine. Disaggregation of the minced tissue was achieved in 0.07% collagenase in the same HBSS solution supplemented with 4 mM $CaCl_2$ and 1 mM deferoxamine. The density gradient was rather different, being composed of 8.5% (5 ml), 11.3% (10 ml) and 17% (w/v) Nycodenz® (5 ml); the cells being in the 11.3% Nycodenz® layer. The centrifugation time was 6 min and the proximal tubule cells were recovered from the upper interface.
3. It is not known if iodixanol can be substituted for Nycodenz® in these separations; certainly its availability as a sterile 60% (w/v) solution (OptiPrep™) would make solution preparation more easy – see **Application Sheet C01**.
4. Keeping Nycodenz® as a sterile stock solution of 40% (w/v) in water is a convenient source for preparation of a broad range of gradients. To make the stock solution place approx. 50 ml of water in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 40 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.
5. This 6.8% Nycodenz® solution has a density of approx 1.037 g/ml.
6. Carry out the resuspension of the cells after each centrifugation very gently, with the minimum of shearing forces.
7. For more information about making gradients see **Application Sheet C02**.
8. Do not use the brake to decelerate the rotor.
9. Although in ref 5 Tris was used as the buffer, the more "cell-friendly" Tricine has been substituted in this Application Sheet. The approx. isoosmotic solution of sucrose (Solution C) used as the diluent for the stock Nycodenz® solution has a density of approx 1.28 g/ml. In view of the known toxicity of sucrose to mammalian cells, a variant might be to use an isoosmotic 4.5% (w/v) solution of Nycodenz®, which has the same density. This can be produced by dilution of Solution A with any normal buffered saline solution.
10. Diffusion was allowed to occur after the tube was capped and rotated to a horizontal position; for more details on the formation of continuous gradients see **Application Sheet C02**.
11. A gradient unloading device such as the Beckman Fraction Recovery System may also be used. Information for calculating density from refractive index and absorbance measurements can be found in **Application Sheet S09 (Subcellular membranes index)**.

4. OptiPrep™

More recently there has been considerable interest in the use OptiPrep™ in the selection of renal cells for transplantation, the well-established innocuous nature of the gradient medium being cited as being of prime importance.

The first reports of the use of OptiPrep™ for rodent kidney cells involved studies on chronic kidney disease using cultured kidney cells [21]. First of all a 15% (w/v) iodixanol barrier (ρ = 1.085 g/ml) was used to remove erythrocytes from the initial cell suspension of UNFX cells. After an initial period of culture the cells were fractionated on a four-step discontinuous iodixanol gradient of 7%, 11%, 13% and 16% (w/v) iodixanol (approx. ρ = 1.042, 1.064, 1.074 and 1.090 g/ml). After centrifugation at 800 *g* for 20 min at room temperature, the cells were resolved into four major subfractions. Epithelial cells of the tubular and collecting ducts were enriched in the second band across the 7%/11% iodixanol interface (designated 1.045-1.063 g/ml), while non-tubular cells were enriched in the fourth band across the 11%/16% iodixanol interface (designated 1.073-1.091 g/ml). The technique was later extended and

optimized to kidney cells of other species [22]: for canine kidney the four iodixanol concentrations were 7%, 10%, 11% and 16% (w/v) and for human 7%, 9%, 11% and 16% (w/v).

Centrifugation of a suspension of urothelial cells (neurogenic and non-neurogenic tissue) from patient bladder biopsy material considerably facilitated the recovery of viable cells for culture [23]. More recently Genheimer et al [24] used a similar gradient system of 16%, 13%, 11% and 7% (w/v) iodixanol; OptiPrep™ was diluted with un-supplemented keratinocyte serum-free medium (KSFM), and the same centrifugation conditions, for rat kidney cells. The authors employed a mixture of cells from the second and fourth band (at a 97:3 ratio) as a source of injectable bioactive renal tubular cells.

For the use of viable tubule cells in a regenerative medicine approach to "neurogenic bladder disease that is refractory to medical treatment" Presnell et al [22] and Bruce et al [23] have stressed the importance of the use a density gradient medium (iodixanol) that is manufactured according to cGMP and which, as an X-ray imaging agent, is approved for clinical use. These publications [21-24] have highlighted the potential use of renal tubular epithelial cells for transplantation into patients with chronic kidney disease.

Detailed methodologies have been published by Bruce et al [25] and Halberstadt et al [26]. Bruce et al [25] used a 30% (w/v) iodixanol stock from equal volumes of OptiPrep™ and KSFM, which was mixed 1:1 with the crude cell suspension and a small volume of PBS layered on top. Cells were collected from the interface and pellet after centrifugation at 800 *g* for 15 min. After further culture of the cells they were resolved by sedimentation through 7% and 16% (w/v) iodixanol (800 *g* for 20 min. The cells at the sample/7% iodixanol layer were enriched in tubular epithelial cells and collecting duct cells, while proximal tubular cells were resolved at the lower interface. Halberstadt et al [26] used a second gradient of 7%, 11%, 13% and 16% (w/v) iodixanol at 800 *g* for 20 min.

5. References

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