OptiPrep™ Application Sheet C18

Purification of intact plant protoplasts by density gradient flotation

- 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

Once the cellulose walls are removed from plant cells, they become sensitive to osmotic changes in the environment, shrinking or swelling in response to the environment, with consequent changes to their buoyant densities. In the preparation of plant protoplasts the osmolality of the medium used to digest the plant walls is therefore important: use of a digest mixture whose osmolality is 1.8 x that of the living plant tissue is a widely used guideline [1].

Similar attention must be given to devising a suitable density gradient for the purification of the intact protoplasts away from the debris of the digested walls, broken protoplasts and the digestion medium itself. Nycodenz® or iodixanol solutions can be adjusted to an appropriate osmolality by dissolution of a suitable salt or by addition of a sorbitol or mannitol solution of known osmolality (see Note 1). Important osmoticum components can be added while still maintaining an appropriate physiological osmolality. The osmolality of the gradient solutions should be the same as that of the digesting solutions and will depend on the source material.

- In this Application Sheet a detailed method for the isolation of barley or wheat leaf protoplasts using a simple discontinuous iodixanol gradient is given (Section 2)
- Section 3 summarizes some of the Nycodenz® methods

2. Isolation of protoplasts for leaf tissue using OptiPrep™

2a. Solutions required

A. Plasmolysing solution: 5 mM MES, 1 mM KH_2PO_4, 0.44 M D-sorbitol, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM L-arginine, 1 mM dithiothreitol (DTT), 0.1% (w/v) polyvinylpyrrolidone (PVP-10), 2 mM glutathione (GSH), 2 mM L-ascorbic acid, 0.01% (w/v) soybean trypsin inhibitor, 1300 U /ml catalase, adjusted to pH 5.7.

B. Digest solution: 2%(w/v) Cellulysin™ and 0.5%(w/v) macerase in Solution A.

C. Isolation buffer: as Solution A without the catalase or trypsin inhibitor.

D. OptiPrep™(shake the bottle gently before use)

E. Working Stock: dissolve 0.6 g KCl in 100 ml Solution D (500 mOsm).

Keep the following Stock Buffer at 4°C:
Dissolve 1.95 g MES (final conc. 100 mM), 0.27 g KH_2PO_4 (20 mM), 1.47 g $CaCl_2 \bullet 2H_2O$ (100 mM), 0.81 g $MgCl_2 \bullet 6H_2O$ (40 mM), 0.79 g $MnCl_2 \bullet 4H_2O$ (40 mM) in 100ml water.

Keep the following Additive Solution at -20°C: Dissolve 0.21 g L-arginine•HCl (100 mM final conc.), 0.15 g DTT (100 mM), 0.35 g L-ascorbic acid (200 mM) and 0.62 g GSH (200 mM) in 10 ml water.

For 100 ml of Solution A: Dissolve 8.3 g of sorbitol and 0.1 g PVP-10 in 50 ml of water; add 5 ml of Stock Buffer and 1 ml of Additive Solution; adjust pH to 5.7; add 0.01 g soya bean trypsin inhibitor and 130,000 U catalase; make up to 100 ml. Check pH.

- If a sterile preparation is required, all solutions should be filter sterilized.

2b. Protocol

2b-1. Determination of leaf osmolality

1. Pulverize leaf tissue (3 g is convenient) after freezing in liquid N_2.

2. After thawing in a sealed tube, to exclude condensation, centrifuge the mixture at 30,000g for 20 min at 4°C. Measure the osmolality of the supernatant by depression of freezing point.

3. The osmolality of the digest and gradient solutions is then set at 1.8x that of the tissue.

2b-2. Sterilization

If required, before processing, the leaf blades should be surface sterilized by sequential washing in 1% sodium hypochlorite containing 0.01% (v/v) Tween-80 (5 min); sterile distilled water (x3), 70% (v/v) ethanol (2 min) and finally sterile distilled water again (x3). This procedure not only surface sterilizes the tissue but also weakens the cuticle thus aiding protoplast release [1].

2b-3. Protoplast preparation (adapted from ref 1)

1. Place the leaf tissue in Solution A (50 ml /g of tissue) for 30 min at 20°C.

2. Remove the leaves from the solution and cut in to 0.5-1 mm pieces and place in 9 cm Petri dishes (2 g of tissue per dish) containing Solution B (10 ml /g of tissue).

3. Digest the tissue at 20°C for 3 h, with shaking at 40 rpm for the first and last 30 min.

4. After digestion filter the contents of each culture plate through Nylon mesh (pore size 100 µm). Wash off the tissue retained by the mesh in isolation buffer; mash lightly to release more protoplasts, and filter again.

5. Wash the mesh through with the buffer and make up the volume of filtrate from each plate to 30 ml in 50 ml Sterilin centrifuge tubes.

2b-4. Purification of protoplasts

1. Add 7.5 ml of Solution E to each 30 ml of digest to make a final density of close to 1.07 g/ml (see Note 2).

2. Dilute 2 ml of Solution E with 20 ml of Solution C (approx 1.03 g/ml). Overlayer the digest mixture with 10 ml of this solution.

3. Finally, layer 2-3 ml of the Solution C on top.

4. Centrifuge the tubes at 200 *g* for 4 min in a swinging-bucket rotor at 4°C (see Note 3).

5. After centrifugation, a band of material is found at the top of the medium and in the overlying buffer. The medium from this band down to the 1.03/1.07 g/ml interface is clear of material. The 1.07 g/ml layer contains particulate material and there is also a pellet.

6. Harvest the band at the top using a plastic Pasteur pipette with the tip cut off to increase the size of the orifice and thus reduce damage to the delicate protoplasts.

7. The top band contains over 95% intact protoplasts, with the remainder just showing signs of lysing and releasing chloroplasts. The number of intact protoplasts remaining in the 1.07 g/ml layer is insignificant (see Notes 4-8).

2c. Notes

1. For more information on the preparation of working solutions and gradient solutions of the appropriate density and osmolality see **Application Sheet S02** (Subcelluar membranes index).
2. Plant material other than wheat and barley leaves may require small modifications to the densities of the two gradient layers. When calculating the volumes of Solutions C and E required to produce a particular density, the densities of both solutions must be taken into account (see Note 1).
3. Centrifugation is actually not necessary for this preparation: the size of the protoplasts means that they will float to the top of the density barrier in about 30 min at 1 *g*.
4. Recovery of protoplasts with barley is approx $4x10^6$ per gram of tissue; using wheat, the yield is significantly lower.

5. The harvested protoplasts suspension contains a small amount of 1.03 g/ml layer (about 2% iodixanol) which has no effect whatsoever on the protoplasts.
6. The protoplasts are in high concentration and free of any residual enzyme activity, thus eliminating the need for washing. Washing any cell (but particularly delicate plant protoplasts) by pelleting and resuspending them several times is very damaging. This protocol eliminates this requirement.

This protocol was developed for wheat or barley leaves. It has also been used to purify protoplasts from the grass *Glyceria fluitans* [2]. The flotation method was also used for *Gracilaria gracilis (Gracilariales, Rhodophyta)* although the gradient was modified [3]: the crude fraction was suspended in 34.8 % (w/v) iodixanol (approx 1,189 g/ml) and solutions of 19.2 % iodixanol (approx. 1.105 g/ml) and 0% iodixanol layered on top (all solutions contained artificial sea water). After centrifugation at 160 *g* for 10 min the protoplasts banded at the 0%/19.2% iodixanol interface. For more details regarding the preparation of the protoplasts see ref. 3.

3. Use of Nycodenz® gradients

3a. Protoplasts from barley grain aleurone

In the method developed by Bethke et al [4] Gamborg B5 medium (minimal organics) was used in the preparation of the protoplasts, but for the gradient isolation this was supplemented with a variety of additives. Three gradient solutions of densities 1.26 g/ml (5 g Nycodenz® + 10 g of medium), 1.18 g/ml (3 g Nycodenz® + 10 g of medium) and 1.08 g/ml (sorbitol in medium) were produced with an osmotic pressure of 1150-1200 $mmol.kg^{-1}$. Protoplasts were very gently suspended in the densest medium and the two lower density solutions layered on top. Because of the small volume of each step (350 μl) the protoplasts gather at the top interface after only 10 min (see also refs 5-9 for other papers reporting use of this method). A similar three-layered gradient uses slightly higher concentrations of Nycodenz® of 70% and 50% (w/v) [10-12].

3b. Protoplasts from barley endosperm

A pellet of crude protoplast, obtained at 40-50 *g* for 2-4 min, was suspended in 2 ml of 40% (w/v) Nycodenz® (in 50 mM $CaCl_2$, 25 mM MES, pH 5.5), overlaid with 1 ml each of 26.6%, 13.3% and 6.6% Nycodenz® (produced by dilution of the 40% Nycodenz® solution with the protoplast medium). After centrifugation at 40-50 *g* for 4min, two bands of protoplasts were obtained at the top of the gradient [13].

3c. Protoplasts from *Amaranthus*

A Nycodenz®-sorbitol gradient has been used for the isolation of *Amaranthus* cotyledon protoplasts [14]. The gradient contained three layers of (1) 20.5% (w/v) Nycodenz®, 0.25 M sorbitol, (2) 8.2% Nycodenz®, 0.4 M sorbitol and (3) 4.1% Nycodenz®, 0.45 M sorbitol. All solutions contained 10 mM $CaCl_2$, 1% BSA (w/v) and 25 mM MES, pH 6.0. In this case the crude protoplast suspension was layered on top and after centrifugation at 200 *g* for 4 min, the protoplasts were recovered from the lowest interface.

3d. Protoplasts from Scots pine bud callus

Flotation through a layer of 6% (w/v) Nycodenz® was used [15].

4. References

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