OptiPrep™ Application Sheet S60

Isolation of organelles from plants and plant 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 **2020SMemapp** file and select the appropriate S-number.
- The purification of plant protoplasts that is often a prelude to subcellular membrane fractionation is covered in **Application C19** (see **Mammalian and Non-mammalian Cell Index**)
- **This Application Sheet covers the purification of the following organelles: chloroplasts, peroxisomes and glyoxysomes, mitochondria, amyloplasts, leucoplasts and vacuoles. For nuclei see Application Sheet S10**
- **Reference List RS01 "Purification of nuclei from tissues and cells in iodixanol gradients"** lists all the published papers. The methodology has been adapted to various plant sources. To access **RS01** return to the initial list of Folders and select **"Reference Lists".**
- **Application Sheet S61 describes methods for analysis of membrane trafficking, protein processing and proteomic analysis; it covers plasma membrane, ER, Golgi and mitochondria.**

1. Introduction

Until recently Nycodenz® was used for most plant organelle separations. It is not known if iodixanol can be substituted directly for Nycodenz® in any of the Nycodenz® applications. Certainly the availability of iodixanol as a 60% (w/v) solution (OptiPrep™) makes gradient solution preparation much easier than is the case with Nycodenz®. Iodixanol and Nycodenz® solutions of the same % (w/v) concentration have almost identical densities, but solutions of Nycodenz® are hyperosmotic above 1.15 g/ml, in contrast to those of iodixanol which can be made isoosmotic at all densities. Whether the osmolality of Nycodenz® solutions plays an important role in achieving the separations described in this Application Sheet is not known. Comparisons can only be made empirically. For the preparation of iodixanol gradient solutions see **Application Sheet S01**.

2. Chloroplasts

Laganowsky et al [1] purified and pelleted chloroplasts from a filtered homogenate of *Arabidopsis thaliana* through a 36% (w/v) iodixanol cushion, containing 0.27 M sucrose, 2 mM EDTA-Na_2, 1 mM $MgCl_2$, 0.2% bovine serum albumin, 50 mM HEPES-NaOH, pH 7.6, centrifuged at 1200 *g* for 10 min at 4°. The authors commented that there was no difference between iodixanol-purified chloroplasts and the more traditional Percoll™-purified organelles in terms of electron transport properties. It is important to point out however that add-on procedures such as SDS-PAGE require the removal of residual Percoll; however in the case of iodixanol, this is only true for electron microscopy..

3. Peroxisomes/glyoxysomes

Although peroxisomes and glyoxysomes are essentially very similar in structure, the latter being often referred to as a type of the former, it is not clear if all the methods published are equally applicable to both organelles.

3a. Nycodenz® gradients

Tobacco leaf peroxisomes can be purified from a leaf homogenate in 0.5 M sucrose, 10 mM KCl, 1 mM EDTA, 25 mM MES-KOH, pH 6.0 in a discontinuous gradient of 1 ml each of 17.5% and 25% and 1.55 ml of 35% (w/v) Nycodenz® [2]. Make up the 35% Nycodenz® solution as follows: place 50 ml of the homogenization medium (HM) in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 35 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and make up to 100 ml with HM. It may be filter-sterilized if required. Dilute this further with HM to give the lower density solutions. In a swinging-bucket rotor such as the Beckman SW50.1, a 1000 *g* (10 min) supernatant is layered on top and centrifuged at 190,000 *g* for 2.5 h. The peroxisomes band sharply in the bottom half of the gradient and are well separated from mitochondria and other membranes (see comments in Section 1).

Peroxisomes from etiolated Soybean cotyledons have now been isolated in 15.5-36% (w/v) iodixanol gradients (approx 13 ml) + a 2 ml 50% iodixanol cushion; 2 ml of the crude fraction were layered on top and centrifuged for 2.5 h at 100,000 *g* in a swinging-bucket rotor [3].

Sunflower seeds have been used widely as the plant source for glyoxysomes [4-7], but there is no obvious reason why the gradient might not have a wider applicability. The homogenization medium and Nycodenz® solutions are made up exactly as described in Section 3, but the 17.5% (w/v) Nycodenz® is omitted. The 1000 *g* supernatant is layered (to fill the tube) over 10 ml of 35% and 8 ml of 25% (w/v) Nycodenz® in tubes for a Beckman SW 28 rotor gradients; the tubes are centrifuged at approx. 120,000 *g* for 2.5 h. Unload the gradients dense-end first in 1 ml fractions (for more information on gradient collection see **Application Sheet S08**). The glyoxysomes are recovered close to the top of the 25% Nycodenz®.

3b. OptiPrep™

The first paper to report the use of OptiPrep™ for the isolation of peroxisomes from etiolated soybean cotyledons [8] used an initial Percoll™ gradient followed by a layering of the partially purified organelles on to a 15.5-36% (w/v) iodixanol gradient in 0.3M sucrose, 1mM EDTA, 0.1% ethanol and 5mM MOPS-HCl pH 7.2; underlayered with a cushion of 50% (w/v) iodixanol containing 25mM sucrose, 0.5mM EDTA, 0.05% ethanol, 2.5mM MOPS-HCl pH 7.2. After centrifugation at 100,000 *g* for 2.5 h the peroxisomes banded very sharply close to the cushion. Whether the Percoll™ gradient can be omitted is not clear. Several reviews have pointed out the advantages of the Arai et al [8] method [9-11]. Hossain and Komatsu [12] stressed the advantages of using iodixanol.

4. Mitochondria

Hartman et al [13] analysed the mitochondria from cultures of *Arabidopsis thaliana* by homogenizing the cells in a routine organelle medium of 0.25 M sucrose, 1 mM EDTA, HEPES-NaOH, pH 7.4. After removing unbroken cells, cell wall fragments and nuclei at 2,200 *g* for 5 min (the supernatant was centrifuged under the same conditions), the remaining organelles were concentrated on to a 16% (w/v) iodixanol cushion at 100,000 *g* for 2h. Whether the *g*-force can be reduced should be tested experimentally. The membrane band was collected; readjusted to 16% (w/v) iodixanol and the membranes banded in a self-generated gradient (approx. 11 ml tubes in a vertical rotor at 350,000 *g* for 3 h. The mitochondria banded approx. three-quarters of the way down the gradient.

- Note that mitochondria are also often identified in iodixanol gradients that are used in proteomic analysis; see **Application Sheet S61**

5. Amyloplasts

Amyloplasts are rapidly sedimenting organelles that can be isolated without a centrifuge; a wide variety of gradient systems using Nycodenz® have however been used. Most use a discontinuous gradient, although one of the earliest published methods used a continuous gradient.

5a. Continuous gradients (adapted from refs 14-16)

5a-1. Gradient solutions

Make up a 60% (w/v) Nycodenz® stock solution as follows: Place 50 ml of 50 mM Hepes-NaOH, pH 7.5 in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 60 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and make up to 100 ml with buffer. It may be filter-sterilized if required. Make dilutions of this 60% Nycodenz® with 0.5 M sucrose, 50 mM Hepes-NaOH, pH 7.5 to give solutions of 40%, 20% and 10% (w/v) Nycodenz®.

5a-2. Gradient separation

In a glass tube layer 6 ml each of the four Nycodenz® solutions and allow a continuous gradient to form by diffusion at room temperature. For methods describing the construction of continuous gradients see **Application Sheet S03**. Bring the gradients to 4°C and layer 8 ml of a plant protoplast lysate on top and leave for 4-6 h at 4°C. The amyloplasts band a third to half way down the gradient.

5b. Discontinuous gradients

The amyloplasts are allowed to sediment through a 2% (w/v) Nycodenz® in 0.8 M sorbitol, 1 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, 50 mM Hepes-NaOH, pH 7.5 solution on to a 1% agar cushion at 30 *g* for 10 min [17-19]. Note however that although this same medium was used for *Hordeum spontaneum*, for *Hordeum murinum* the Nycodenz® concentration was reduced to 0.5% [20]. This is a widely used methodology [21-25].

In a variant of this method, the agar cushion is replaced by 60% (w/v) Nycodenz® and the sedimentation is carried at 1 *g*. The Nycodenz® solutions were prepared in 1 M sucrose, 1 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.2% BSA, 5 mM DTT, 50 mM Hepes-NaOH, pH 7.6 [26,27].

6. Leucoplasts

6.1 Gradient solutions

Protoplasts are homogenized in 21% (w/v) sucrose, 10 mM KCl, 1mM $MgCl_2$, 1 mM EDTA, 50 mM Tricine-KOH, pH 7.4 (Solution A). This has an osmolality of approx. 800 mOsm and the 0-40% (w/v) Nycodenz® gradient is constructed in such a way as maintain the same osmolality [28,29]. Make up the 40% Nycodenz® solution as follows: place 50 ml of the 0.3 M sucrose, 10 mM KCl, 1mM $MgCl_2$, 1 mM EDTA, 50 mM Tricine-KOH, pH 7.4 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 make up to 100 ml with the same medium. It may be filter-sterilized if required.

6.2 Centrifugation

In tubes for a Beckman SW28 swinging-bucket rotor (or equivalent) construct a continuous gradient (approx 30 ml) from equal volumes of the 40% Nycodenz® and Solution A using a two-chamber gradient maker or a Gradient Master™. For methods describing the construction of continuous gradients see **Application Sheet S03**. Layer 7-8 ml of the protoplast lysate on top and centrifuge at 90,000 *g* for 90 min. Unload the gradients low-density end first in 1 ml fractions (for more information on gradient collection see **Application Sheet S08**). The leucoplasts are recovered close to the bottom of the gradient.

7. Vacuoles

These particles can be isolated by flotation through low-density solutions under very low *g*-forces. Protoplasts in 550 mM sorbitol, 10 mM Hepes-KOH, pH 7.4 [29] are osmotically shocked by rapid addition of 1 ml to 1.75 ml of a low osmolality solution of 7.9 mM EDTA, 10 mM Hepes-KOH, pH 7.3 [30] or 5 mM EDTA, 25 mM MES-Tris, pH 7.3 [31]. Sometimes 0.5 % BSA is included in the solutions. After maintenance at 4°C for 10-30 min, with occasional shaking, the lysate is mixed with 10 ml of 350 mM sorbitol, 8.5% (w/v) Nycodenz® in the same low osmolality medium. After layering 1 ml of the protoplast medium on top, the tubes are centrifuged at 160 *g* for 3 [30] or 10 [31] min. The vacuoles are recovered from the top half of the protoplast medium.

Sometimes crude vacuoles from plant tissues (rather than protoplasts) are pelleted at 2000 *g* for 10 min from a homogenate before suspension in 5ml of 15% (w/v) Nycodenz®, 1.2 M sorbitol, 1 mM EDTA, 25 mM MES-Tris, pH 8.0 [32]. This is overlaid with 5 ml of 10% Nycodenz® in the same medium and 2 ml of medium and after centrifugation at 650 *g* for 5 min the vacuoles collect at the top interface.

In a few published methods very different centrifugation conditions are reported. A filtered suspension (16 ml) of an 8,000 *g* –15 min pellet (from a cauliflower bud homogenate) in 15% (w/v) Nycodenz®, 1.5 M sorbitol, 1mM EDTA, 4 mM DTT, 10 mM Tris-HCl, pH 7.6 is overlaid with 5 ml of 8% (w/v) Nycodenz® in the same medium and 4 ml of medium [32]. After centrifugation at 100,000 *g* for 45 min (very slow acceleration and deceleration) the vacuoles band at the top interface [33].

8. Tonoplasts

The micrsomal pellet from a *Pteris vittata* homogenate was adjusted to 15% (w/v) iodixanol; ayered beneath an 8% (w/v) iodixanol solution and the tube filled with an upper layer of isolation medium. After centrifugation at ed (100,000 *g*, for 45 min, the tonoplasts banded at the 0% and 8% iodixanol interface [34]. In a modified method, a pellet from an 8000 *g* supernatant was suspended in 15% (w/v) iodixanol (in suspension medium), overlaid with suspension medium and centrifuged at 100,000 *g* for 1 h. The interfacial material (in 15% iodixanol) was overlaid with 8%, 4% (w/v) iodixanol and the suspension medium. After repeating the centrifugation, the tonoplast vesicles banded at the top interface [35].

9. References

1. Laganowsky, A., Gómez, S.M., Whitelegge, J.P., Nishio, J.N. (2009) *Hydroponics on a chip: Analysis of the Fe deficient Arabidopsis thylakoid membrane proteome* J. Proteom., **72**, 397-415
2. Onyeocha, I., Behari, R., Hill, D. and Baker, A. (1993) *Targeting of castor bean glyoxysomal isocitrate lyase to taco leaf peroxisomes* Plant Mol. Biol., **22**, 385-396
3. Arai, Y., Hayashi, M. and Nishimura, M. (2008) *Proteomic analysis of highly purified peroxisomes from etiolated Soybean cotyledons* Plant Cell Physiol., **49**, 526-539
4. Behari, R. and Baker, A. (1993) *The carboxyl terminus of isocitrate lyase is not essential for import into glyoxysomes in an in vitro system* J. Biol. Chem., **268**, 7315-7322
5. Horng, J-T., Behari, R., Bruke, L.E.C-A. and Baker, A. (1995) *Investigation of the energy requirement and targeting signal for the import of glycolate oxidase into glyoxysomes* Eur. J. Biochem., **230**, 157-163
6. Pool, M.R., Lopez-Huertas, E., Homg, J-T- and Baker, A. (1998) *NADPH is a specific inhibitor of protein import into glyoxysomes* Plant J., **15**, 1-14
7. Tugal, H.B., Pool, M. and Baker, A. (1999) *Arabidopsis 22-kilodalton peroxisomal membrane protein. Nucleotide sequence analysis and biochemical characterization* Plant Physiol., **120**, 309-320
8. Arai, Y., Hayashi, M. and Nishimura, M. (2008) *Proteomic analysis of highly purified peroxisomes from etiolated Soybean cotyledons* Plant Cell Physiol., **49**, 526-539
9. Komatsu, S. and Ahsan, N. (2009) *Soybean proteomics and its application to functional analysis* J. Proteom., **72**, 325-336
10. Palma, J.M., Corpas, F.J. and del Rio, L.A. (2009) *Proteome of plant peroxisomes: new perspectives on the role of these organelles in cell biology* Proteomics, **9**, 2301-2312
11. Reumann, S. (2011) *Toward a definition of the complete proteome of plant peroxisomes: Where experimental proteomics must be complemented by bioinformatics* Proteomics **11**, 1764–1779
12. Hossain, Z. and Komatsu, S. (2014) *Soybean proteomics* In Plant Proteomics: Methods Mol. Biol., **1072** (ed. Jorrin-Novo, J.V. et al), Springer Science+Business Media, LLC, pp 315-331
13. Hartman, N.T., Sicilia, F., Lilley, K.S. and Dupree, P. (2007) *Proteomic complex detection using sedimentation* Anal. Chem., **79**, 2078-2083
14. Entwistle, G., Tyson, R.H. and ap Rees, T (1988) *Isolation of amyloplasts from wheat endosperm* Phytochemistry, **27**, 993-996
15. Entwistle, G. and Rees, T.A. (1988) *Enzymic capacities of amyloplasts from wheat (Triticum aestivum) endosperm* Biochem. J., **255**, 391-396
16. Coates, S. and ap Rees, T. (1994) *Metabolism of glucose monophosphates by leucoplasts and amyloplasts from soybean suspension cultures* Phytochemistry, **35**, 881-883
17. Tetlow, I.J., Blissett, K.J. and Emes, M.J. (1993) *A rapid method for the isolation of purified amyloplasts from wheat endosperm* Planta, **189**, 597-600
18. Tetlow, I.J., Bowsher, C.G. and Emes, M.J. (1996) *Reconstitution of the hexose phosphate translocation from the envelope membranes of wheat endosperm amyloplasts* Biochem. J., **319**, 717-723
19. Wischmann, B., Nielsen, T.H. and Møller, B.L. (1999) *In vitro biosynthesis of phosphorylated starch in intact potato amyloplasts* Plant Physiol., **119**, 455-462
20. Beckles, D.M., Smith, A.M. and ap Rees, T. (2001) *A cytosolic ADP-glucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs* Plant Physiol., **125**, 818-827
21. Davies, E.J., Tetlow, I.J., Bowsher, C.G. and Emes, M.J. (2003) *Molecular and biochemical character-ization of cytosolic phosphoglucomutase in wheat endosperm (Triticum aestivum L. cv. Axona)* J. Exp. Bot., **54**, 1351-1360
22. Tetlow, I.J., Davies, E.J., Vardy, K.A., Bowsher, C.G., Burrell, M.M. and Emes, M.J. (2003) *Subcellular localization of ADPglucose pyrophosphorylatase in developing wheat endosperm and analysis of the properties of a plastidial isoform* J. Exp. Bot., **54**, 715-725
23. Balmer, Y., Vensel, W.H., Cai, N., Manieri, W., Schürmann, P., Hurkman, W.J. and Buchanan, B.B. (2006) *A complete ferrodoxin/thioredoxin system regulates fundamental processes in amyloplasts* Proc. Natl. Acad. Sci, USA, **103**, 2988-2993

24. Balmer, Y., Vensel, W.H., DuPont, F.M., Buchanan, B.B. and Hurkman, W.J. (2006) *Proteome of amyloplasts isolated from developing wheat endosperm presents evidence of broad metabolic capability* J. Exp. Bot., **57**, 1591-1602
25. Hurkmann, W.J. and Tanaka, C.K. (2007) *Extraction of wheat endosperm proteins for proteome analysis* J. Chromatogr. B, **849**, 344-350
26. Sweetlove, L.J:, Burrell, M.M. and ap Rees, T. (1996) *Characterization of transgenic popato (Solanum tuberosum) tubers with increased ADPglucose pyrophosphorylase* Biochem. J., **320**, 487-492
27. Naeem, M., Tetlow, I.J. and Emes, M.J. (1997) *Starch synthesis in amyloplasts purified from developing potato tubers* Plant J., **11**, 1095-1103
28. Coates, S.A. and ap Rees, T. (1993) *Carbohydrate oxidation by leucoplasts from suspension cultures of soybean (Glycine max L.)* Planta, **189**, 516-521
29. Coates, S. and ap Rees, T. (1994) *Metabolism of glucose monophosphates by leucoplasts and amyloplasts from soybean suspension cultures* Phytochemistry, **35**, 881-883
30. Renaudin, J.P., Brown, S.C., Barbier-Brygoo, H. and Guern, J. (1986) *Quantitative characterization of protoplasts and vacuoles from suspension-cultured cells of Catharanthus roseus* Physiol. Plantarium, **68**, 695-703
31. Sottomayor, M., de Pinto, M.C., Salema, R., DiCosmo, F., Pedreno, M.A. and Barcelo, A.R. (1996) *The vacuolar localization of a basic peroxidase isoenzyme responsible for the synthesis of α-3^{1},4^{1}-anhydrovinblastine in Catharanthus roseus (L.) G. Don leaves* Plant Cell Envir., **19**, 761-767
32. Orsomando, G., de la Garza, R.D., Green, B.J., Peng, M., Rea, P.A., Ryan, T.J., Gregory, J.F. and Hanson, A.D. (2005) *Plant γ-glutamyl hydrolyses and folate polyglutamates* J. Biol. Chem., **280**, 28877-28884
33. Schmidt, U.G., Endler, A., Schlebert, S., Brunner, A., Schnell, M., Neuhaus, H.E., Marty-Mazars, D., Marty, F., Baginsky, S. and Martinoia,E.(2007) *Novel tonoplast transporters identified using a proteomic approach with vacuoles isolated from cauliflower buds* Plant Physiol., **145**, 216-229
34. Shen, H., He, Z., Yan, H., Xing, Z., Chen, Y., Xu, W., Xu, W. and Ma, M. (2014) T*he fronds tonoplast quantitative proteomic analysis in arsenic hyperaccumulator Pteris vittata* L. J. Proteom., **105**, 46–57
35. Liu, R., Wang, Y., Qin, G. and Tian, S. (2016) *iTRAQ-based quantitative proteomic analysis reveals the role of the tonoplast in fruit senescence* J. Proteom., **146**, 80–89

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