OptiPrep™ Application Sheet S16

Light mitochondrial fraction analysis (mitochondria, lysosomes and peroxisomes) in self-generated 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 **2020SMemapp** file and select the appropriate S-number.
- **OptiPrep™ Application Sheet S14a** provides a protocol review of papers reporting the use of OptiPrep™ for the fractionation and analysis of a total, heavy or light mitochondrial fraction.
- **Reference List RS03** focuses on the isolation of **mitochondria**, **RS04 - lysosomes** and **RS02 - peroxisomes**: to access return to the initial list of Folders and select "**Reference Lists**"
- Centrifugation of a post-nuclear supernatant (PNS) at 3000 *g* for 10 min produces the heavy mitochondrial fraction (HMF) containing principally mitochondria plus some of the lysosomes and peroxisomes. The light mitochondrial fraction (LMF) is the material that sediments from the 3000 *g* supernatant at 12-20,000*g* for 10-20 min and contains mitochondria, lysosomes, peroxisomes and some of the microsomes. For a total mitochondrial fraction the 3,000*g* step is omitted.

1. Background

This Application Sheet describes the fractionation principally of lysosomes, mitochondria, and peroxisomes from mammalian liver in self-generated gradients of iodixanol. Usually an LMF is prepared for the gradient, but sometimes the material includes the HMF (i.e. the 3000g step of the standard differential centrifugation protocol is omitted). The primary use of the gradients is an analytical one, but they may in certain circumstances also be used preparatively.

In iodixanol gradients the densities of lysosomes is slightly lower than those in gradients of Nycodenz®; the density of mitochondria is much lower (ρ = 1.13-1.15 against 1.18-1.20 g/ml) while the density of peroxisomes is virtually the same [1]. Note in some published methods other membranes such as Golgi, ER and/or plasma membrane may be identified.

The formation of self-generated gradients requires higher *g*-forces than would be used with a pre-formed gradient. But, because the sedimentation path length of the rotors used for self-generated gradients (vertical, near-vertical or some fixed-angle rotors) is much lower than that of a swinging-bucket rotor, the hydrostatic pressure on the organelles is rather similar in the two systems. Compared to pre-formed gradients, there are several major advantages to the use of self-generated gradients for organelle fractionation. (1) Multiple samples can be handled far more easily. (2) To maximize resolution, high concentrations of particles in the sample and the build-up of particles at interfaces during centrifugation should be avoided. With pre-formed gradients this can be achieved only by incorporating the sample into the gradient (not always very convenient) - but this aim is a natural consequence for self-generated gradients as the sample is simply mixed with a suitable solution of iodixanol. (3) Gradient density profiles and hence fractionations are far more reproducible than with pre-formed gradients.

- Section 2 of this Application Sheet describes the preparation of the LMF or HMF+LMF by differential centrifugation. For more information on differential centrifugation see **Application Sheet S07**.
- Section 3 describes the fractionation of LMF or HMF+LMF in self-generated iodixanol gradients.
- Section 4 describes how modulation of the centrifugation conditions affects the generated density profile and hence the separation of the organelles. For more information on self-generated gradient generation see **Application Sheet S04**. It also has important information on the analysis of membranes from iodixanol gradients.

- The self-generated gradients were developed for mouse liver but have been adapted to a variety of tissues and cells; some of the variations in protocol are summarized in Section 5.

2. Preparation of LMF or HMF+LMF

2a. Solutions required (see Box 1)

Homogenization medium (HM): 0.25 M sucrose, 1 mM EDTA, 20 mM Hepes-NaOH, pH 7.4 (see Section 2c, Notes 1 and 2)

Box 1
Keep the following stock solutions at 4°C:
1 M Hepes (free acid); 23.8 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$); 3.72 g per 100 ml water

HM: Dissolve 17 g sucrose in 100 ml water; add 2.0 ml and 4.0 ml respectively of EDTA stock and Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml.

2b. Protocol

Carry out all operations at 0-4°C.

To prepare the HMF+LMF omit steps 6-8 and use the combined 1000 *g* supernatants (from step 5) instead of the 3000 *g* supernatant in step 9.

1. *For soft tissues:* Mince the tissue very finely with scissors (or with a tissue chopper) and transfer to a Potter-Elvehjem (Teflon and glass) homogenizer with the HM (use 10 ml for every 2.5 g tissue). Homogenize using approx 6 strokes of the pestle at 500-700 rpm (**see Section 2c, Note 3**).

2. *For cells:* Wash 1-3x10^8 cells in 5 ml of phosphate buffered saline and again with 5 ml of HM. Suspend the cells in 3 ml of HM and homogenize in a ball-bearing homogenizer using five passes (**see Section 2c, Note 4**).

3. Centrifuge the homogenate at 1000 g_{av} for 5 min to pellet the nuclei (do not use the brake to decelerate the rotor); then carefully decant the supernatant or aspirate it using a syringe and metal cannula and retain on ice.

4. Resuspend the pellet in 10 ml (5 ml for cells) of HM using 2-3 gentle strokes of the pestle of a loose-fitting Dounce homogenizer (**see Section 2c, Note 5**).

5. Repeat the centrifugation and combine the supernatants.

6. To pellet the HMF centrifuge the suspension at 3000 g_{av} for 10 min, then aspirate or decant the supernatant and retain on ice.

7. Resuspend the 3000 *g* pellet (HMF) in about half the original volume of HM.

8. Gently homogenize the pellet using a loose-fitting Dounce homogenizer and repeat step 6.

9. Centrifuge the combined 3000 *g* supernatants at 17,000 g_{av} for 10-15 min.

10. Resuspend the pellet (LMF) in a small volume (approx. 2 ml) of HM using a loose-fitting Dounce homogenizer (**see Section 2c, Notes 6 and 7**).

2c. Notes

1. Protease inhibitors may be included in the HM at the operator's discretion.
2. Any suitable buffered isoosmotic solution may be used and there is considerable variation in the detailed composition of the HM in the literature. Media, which are most "mitochondria-friendly", are based on 0.25 mM mannitol rather than sucrose and the EDTA is often replaced with 0.1 mM EGTA for rat liver. Alternatively peroxisome-specific media often contain 0.1% (v/v) ethanol. MOPS is another frequently used buffer (**see Section 4a**).
3. The described methodology applies to tissues such as rodent liver and kidney. Other tissues such as skeletal and cardiac muscle, intestine and brain require special treatments and the operator should consult relevant texts. For more information **see Application Sheet S05**.

4. The ball-bearing homogenizer (cell cracker) is generally regarded as one of the best devices for cultured cells; delicate organelles are best preserved by this technique. If one is not available, shearing by several passages through a syringe needle may be a reliable alternative. For more information **see Application Sheet S06**.
5. The nuclei may be very fragile since any homogenization medium containing EDTA is not well suited to the preservation of these organelles. The pellet must be washed by very gently.
6. The LMF may be washed to remove trapped microsomes by suspension to the original volume with HM and repeating steps 9 and 10.

Box 2
See Box 1 for stock solutions

OD: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml and 12 ml respectively of EDTA and Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3. Self-generated gradient fractionation

3a. Solutions required (**see Section 3d, Note 1**)

OptiPrep™

OptiPrep™ diluent (OD): 0.25 M sucrose, 6 mM EDTA, 120 mM Hepes-NaOH, pH 7.4 (**see Box 2**)

Working Solution (WS): 50% iodixanol (ρ = 1.272 g/ml): 5 vol. of OptiPrep™solution A + 1 vol. of OD

Homogenization medium (HM): **see Section 2a**

3b. Ultracentrifuge rotor requirements

A vertical or near vertical rotor with a tube capacity of 10-14 ml or a fixed-angle rotor (tube angle <24°) with a tube capacity <10 ml. The rotor should be able to achieve an RCF of ≥ 180,000g (**see Section 3d, Note 2**).

3c. Protocol

Carry out all operations at 0-4°C.

1. Mix the resuspended light mitochondrial pellet with WS to the chosen final concentration of iodixanol: 15%, 17.5% or 20% (w/v) (**see Section 3d, Note 3**).

2. Transfer to tubes for a vertical, near-vertical or low-angle fixed angle rotor (approx 20°). Fill about 90-95% of the tube volume with sample and then layer HM on top to fill the tube.

3. Centrifuge a vertical or near-vertical rotor at 353,000 g_{av} for 1-2 h or a fixed-angle at 270,000 g_{av} for 3 h; allow the rotor to decelerate from 2000 rpm without the brake or use a slow deceleration program (**see Section 3d, Note 4**).

4. Unload the gradients in 0.5-1.0 ml fractions by upward displacement or, if this is impractical, use tube puncture or aspiration from the bottom. For more information on harvesting gradients, **see Application Sheet S08**.

5. Analyze the gradients as required, **see Application Sheet S09**.

3d. Notes

1. Protease inhibitors may be included in OD at the operator's discretion. Strategies for preparing gradient solutions for mammalian tissues are given in **Application Sheet S01**.
2. The efficacy of a rotor for self-generated gradient formation should always be assessed by determining the density profile of a blank gradient and comparing it with those described in the Figures in the Section 4 below. The density of the collected fractions is most accurately determined by refractive index (RI) measurement. RI values are given in **Application Sheet S01**. If a refractometer is not available, then absorbance measurement is another option, for information **see Application Sheet S09**.
3. The optimum final concentration of iodixanol will depend on the requirements of the operator (i.e. whether it is necessary to resolve all of the organelles or one organelle in particular), **see Section 5**.

4. The optimum centrifugation condition will depend on the rotor and the required density profile; **see Section 5**.

4. Gradient Analysis

The gradient (**see Figure 1**) gives acceptable resolution of all the major organelles from mammalian liver and would be ideal for use in an analytical mode. Any ER (not shown) in the light mitochondrial fractions sometimes bands between the mitochondria and the lysosomes, sometimes between the lysosomes and the Golgi (depending on the tissue or cell type). It is also important that the specific activity profiles of the four enzyme markers (not shown) are very similar to the percentage distribution, i.e. yield and purity go together (see ref 1 for more information).

The relative separation of the organelles can be modulated by changing the starting concentration of the iodixanol. If, for example, it is required that the separation of the denser lysosomes from the mitochondria should be improved, at the expense of the resolution of the lighter lysosomes from the Golgi, this can be achieved by increasing the iodixanol starting concentration to 20% (w/v) – **see Figure 2**. Note the shallower central region of the gradient caused by the use of a lower g-force of 180,000g_{av} rather than 270,000g_{av}. The same low-angle fixed-angle rotor was used in all the separations described.

If the g-force is maintained at the lower value of 180,000g_{av} and the starting iodixanol concentration is reduced to 15% (w/v) then the gradient may be used principally to purify the lighter Golgi membranes, while the denser organelles are largely confined to the bottom third of the gradient (Figure 3). A fourth variation is raising the iodixanol concentration to 25%, **see also Application Sheet S13**.

Enzyme activities can be measured directly on the gradient fractions as iodixanol neither inhibits enzymes nor does it interfere with spectrophotometric assays above 340 nm [2]. If however, it is necessary to remove the gradient medium, fractions can be diluted with an equal volume of buffer; pelleted at approx 30,000g_{av} for 10 min and resuspended in a suitable buffer. For more information on analyzing gradients **see Application Sheet S09**.

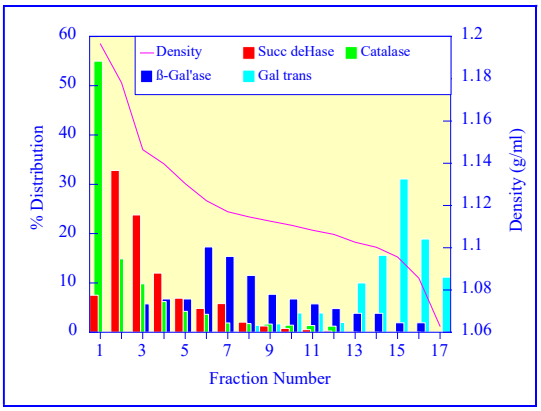

Figure 1: Separation of major organelles from a mouse liver LMF in a self-generated iodixanol (17.5%) gradient, centrifuged at 270,000g_{av}, for 3 h in a fixed-angle rotor (10 ml tube, 20° angle): enzyme distribution. Succ deHase = succinate dehydrogenase; ß-Gal'ase = ß galactosidase; Gal trans = galactosyl transferase.

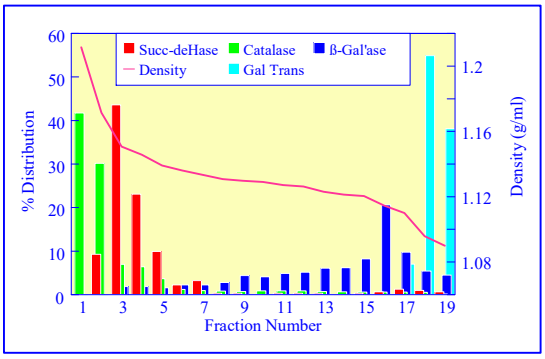

Figure 2: Separation of major organelles from a mouse liver LMF in a self-generated iodixanol (20%) gradient, centrifuged at 180,000g_{av}, for 3 h in a fixed-angle rotor (10 ml tube, 20° angle): enzyme distribution: for abbreviations see legend to Figure 1.

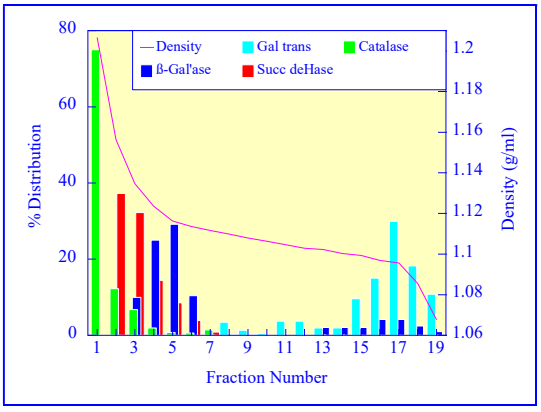

Figure 3 Purification of Golgi membranes from a mouse liver LMF in a self-generated iodixanol (15%) gradient, centrifuged at 180,000g_{av}, for 3 h in a fixed-angle rotor (10 ml tube, 20° angle): for abbreviations see legend to Figure 1.

Schmidt et al [19] noted that the extensive washing of organelles that was required for organelles purified in Percoll™ led to a serious loss of functionality.

5. Summary of published gradient conditions

Published papers reporting the use of the method described in this Application Sheet (or a modification of the method) have been sorted alphabetically according to tissue or cell source in Table 1). Each entry has a summary of the iodixanol gradient and centrifugation conditions used, the type of rotor that was used, whether a LMF or LMF+HMF fraction was analyzed and also an indication of the organelles that were identified in the gradient.

Table 1 Self-generated iodixanol gradient literature summary

Tissue/cell type	Fraction	Iodixanol[1]	RCF/time	Rotor[2]	Organelle analysis[3]	Ref #
Bovine liver	HMF+LMF	0+20	100,000 *g*/3h	SW28[4]	Golgi, lys, mit, perox	3
Carcinoma	HMF+LMF[4]	25	362,000 *g*/1h	S100	Golgi, lys,	4
	LMF	17.5	ns	ns	Lys, mit, perox, ER	24
	Lysosome frac[7]	19	150,000 *g*/	ns	Lys	29
COS7	LMF	20	180,000 *g*/3h	VTi65	ER, lys, mit	5
Fibroblasts	LMF	33	380,000 *g*/4h	vertical	Lys, vesicles	6
HEK cells	HMF	17.5	270,000 *g*/	70Ti	Mit	28
Human liver	LMF	20	180,000 *g*/3h	VTi65	ER, lys, mit	5
Human glioma	LMF	17.5	380,000 *g*/1.5h	TLV100	Lys	23
Human promyeloid	LMF	20	180,000 *g*/3h	VTi80	Lys	7
Lymphocyte	HMF+LMF	15	350,000 *g*/3h	VTi65.1	Lys, mit, Golgi	20
Lymphoma	LMF[5]	20	180,000 *g*/3h	ns	Lys	8
	LMF	20	208,000 *g*/18h	ns	Lys	9
Mouse liver	HMF+LMF	25	180,000 *g*/3h	ns	Mit, perox	22
Mouse macrophage	HMF	ns	320,000 *g*/3h	VTi65.1	Lys, ER, end	21
Mouse mammary	LMF	17.5	180,000 *g*/3h	ns	PM, Golgi	10
Mouse myoblasts	HMF+LMF	20	ns	ns	Mit	11
Mouse skelet. muscle	HMF+LMF	20	ns	ns	Mit	11
Neuroblastoma	LMF	20	27,000 *g*/3h	ns	Lys	25
Rat heart	HMF+LMF[6]	17.5	154,000 *g*/5h	NVT65	ER, Golgi, mit	12
Rat hepatoma	HMF+LMF	17.5	180,000 *g*/3h	ns	ER, Golgi, lys, mit	13
Rat liver	LMF	17.5	230,000 *g*/4h	ns	ER, lys, mit	14,15
	HMF	25	180,000 *g*/3h	T865-1	Mit	16

1. Iodixanol concentration in % (w/v)
2. All rotors are Beckman except S100 and T865-1 (Sorvall)
3. Lys = lysosomes, mit = mitochondria, perox = peroxisomes, ER = endoplasmic reticulum, PM = plasma membrane, end = endosomes
4. Swinging-bucket rotors are rarely, if ever, used for self-generated gradients, but by overlayering the sample with Solution D, the density gradient was generated partly by self-generation and partly by diffusion in this rare case
5. The organelles from a post-nuclear supernatant were concentrated by sedimentation on to a cushion of 22% (w/v) iodixanol by centrifugation at 100,000 *g* for 1,5 h [20]
6. This material also contained the total microsomes
7. The lysosome fraction was initially purified in a dicontinuous iodixanol gradient

ns = not stated

A strategy first reported by Zhang et al [17,18], who used a large-volume low-angle fixed-angle rotor rather than a vertical or near-vertical rotor. The gradient was first generated from 21 ml of 30% Nycodenz® (layered on top of 1.5 ml of 60% sucrose) at 60,000 *g* for 24 h. The LMF was then layered on top of the gradient and re-centrifuged for 1 h at 76,000 *g*. Only a fixed-angle rotor with open-topped tubes allows this novel approach to be executed. Excellent resolution of mitochondria and peroxisomes (rat liver) was observed. In a way it combines the best of both worlds, easy and highly reproducible gradient formation and low *g*-forces for the organelle separation. It could certainly be adapted to the use of OptiPrep™ but this has not been reported.

Occasionally a total post-nuclear supernatant is used, for example as described for retinal pigment epithelial cells [26] and rat hepatoma cells [27].

6. References

1. Graham, J., Ford, T. and Rickwood, D. (1994) *The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol* Anal. Biochem., **220**, 367-373

2. Ford, T., Graham, J. and Rickwood, D. (1994) *Iodixanol: A nonionic iso-osmotic centrifugation medium for the formation of self generated gradients* Anal. Biochem., **220**, 360-366
3. Tschantz, W. R., Zhang, L. and Casey, P. (1999) *Cloning, expression, and cellular localization of a human prenylcysteine lyase* J. Biol. Chem., **274**, 35802-35808
4. Liang, X-J., Shen, D-W., Garfield, S. and Gottesman, M. M. (2003) *Mislocalization of membrane proteins associated with multidrug resistance in cisplastin-resistant cancer cell lines* Cancer Res., **63**, 5909-5916
5. Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., Lefrancois, S., Morales, C.R. and Pshezhetsky (2001) *Intracellular distribution of lysosomal sialidase is controlled by the internalisation signal in its cytoplasmic tail* J. Biol. Chem., **276**, 46172-46181
6. Bär, S., Daeffler, L., Rommelaere, J. and Nüesch, J.P.F. (2008) *Vesicular egress of non-enveloped lytic parvoviruses depends on gelsolin functioning* PloS Pathog., **4**:e1000126
7. Nathanson, C-M, Wasselius, J., Wallin, H. and Abrahamson, M. (2002) *Regulated expression and intracellular localization of cystatin F in human U937 cells* Eur. J. Biochem., **269**, 5502-5511
8. Prigozy, T.I., Naidenko, O., Qasba, P., Elewaut, D., Brossay, L., Khurans, A., Natori, T., Koezuka, Y., Kulkarni, A. and Kronenberg, M. (2001) Glycolipid antigen processing for presentation by CD1d molecules *Science*, **291**, 664-667
9. Kung Sutherland, M.S., Sanderson, R.J., Gordon, K.A., Andreyka, J., Cerveny, C.G., Yu, C., Lewis, T.S., Meyer, D.L., Zabinski, R.F., Doronina, S.D., Senter, P.D., Law, C-L., Wahl, A.F. (2006) *Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by peptide-linked anti-CD30-auristatin conjugates* J. Biol. Chem., **281**, 10540-10547
10. Nemeth, B.A., Tsang, S.W.Y., Geske, R.S. and Haney, P. (2000) *Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland* Pediatr Res., **47**, 444-450
11. Flierl, A., Chen, Y., Coskun, P.E., Samulski, R.J. and Wallace, D.C. (2005) *Adeno-associated virus-mediated gene transfer of the heart/muscle adenine nucleotide translocator (ANT) in mouse* Gene Ther., **12**, 570-578
12. Bensimon, M., Chang, A.I., Kuroski de Bold, M.L., Ponce, A., Carreras, D. and de Bold A.J. (2004) *Participation of G proteins in natriuretic peptide hormone secretion from heart atria* Endocrinology, **145**, 5313-5321
13. Sultan, A.S., Miyoshi, E., Ihara, Y., Nishikawa, A., Tsukada, Y. and Taniguchi, N. (1997) *Bisecting GlcNac structures act as negative sorting signals for cell surface Glycoproteins in forskolin-treated rat hepatoma cells* J. Biol. Chem., **272**, 2866-2872
14. Gille, L. and Nohl, H. (2000) *The existence of a lysosomal redox chain and the role of ubiquinone* Arch. Biochem. Biophys., **375**, 347-354
15. Nohl, H. and Gille, L. (2002) *The biofunctional activity of ubiquinone in lysosomal membranes* Biogerontology, **3**, 125-131
16. Yu, W., Liang, X., Ensenauer, R.E., Vockley, J., Sweetman, L. and Schultz, H. (2004) *Leaky β-oxidation of a trans –fatty acid* J. Biol. Chem., **279**, 52160-52167
17. Zhang, D., Liang, X., He, X-Y., Alipui, O. D., Yang, S-Y. and Schulz, H. (2001) *$\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase is a multifunctional isomerase* J. Biol. Chem., **276**, 13622-13627
18. Zhang, D., Yu, W., Geisbrecht, B. V., Gould, S., Sprecher, H. and Schulz, H. (2002) *Functional characterization of Δ^3,Δ^2-enoyl-CoA isomerases from rat liver* J. Biol. Chem., **277**, 9127-9132
19. Schmidt, H., Gelhaus, C., Lucius, R., Nebendahl, M. Leippe, M. and Janssen, O. (2009) *Enrichment and analysis of secretory lysosomes from lymphocyte populations* BMC Immunol., **10**:41
20. Hall, S.L., Hester, S., Griffin, J.L., Lilley, K.S. and Jackson, A.P. (2009) *The organelle proteome of the DT40 lymphocyte cell line* Mol.Cell. Proteom., **8**, 1295–1305
21. DiMezzo, T.L., Ruthel, G., Brueggemann, E.E., Hines, H.B., Ribot, W.J., Chapman, C.E., Powell, B.S. and Welkos, S.L. (2009) *In vitro intracellular trafficking of virulence antigen during infection by Yersinia pestis* PLoS One, **4**:e6281
22. Nakamura, Y., Ogura, M., Ogura, K., Tanaka, D. and Inagaki, N. (2012) *SIRT5 deacetylates and activates urate oxidase in liver mitochondria of mice* FEBS Lett., **586**, 4076–4081
23. Di Piazza, M., Mader, C., Geletneky, K., Herrero y Calle, M., Weber, E., Schlehofer, L. and Rommelaere, J. (2007) *Cytosolic activation of cathepsins mediates parvovirus H-1-induced killing of cisplatin and TRIAL-resistant glioma cells* J. Virol., **81**, 4186-4198
24. Li, N., Zheng, Y., Chen, W., Wang, C., Liu, X., He, W., Xu, H. and Cao, X. (2007) *Adaptor protein LAPF recruits phosphorylated p53 to lysosomes and triggers lysosomal destabilization in apoptosis* Cancer Res., **67**, 11176-11185
25. Tringali, C., Cirillo, F., Lamorte, G., Papini, N., Anastasia, L., Lupo, B., Silvestri, I., Tettamanti, G. and Venerando, B. (2012) *NEU4L sialidase overexpression promotes β-catenin signaling in neuroblastoma cells, enhancing stem-like malignant cell* growth Int. J. Cancer, **131**, 1768–1778

26. Soni, L.E., Warren, C.M., Bucci, C., Orten, D.J. and Hasson, T. (2005) *The unconventional myosin-VIIa associates with lysosomes* Cell Motil. Cytoskeleton, **62**, 13-26
27. Kidane, T.Z., Sauble, E. and Linder, M.C. (2006) *Release of iron from ferritin requires lysosomal activity* Am. J. Physiol. Cell Physiol., **291**, C445-C455
28. Shneyer, B.I., Ušaj, M. and Henn, A. (2016) *Myo19 is an outer mitochondrial membrane motor and effector of starvation-induced filopodia* J. Cell Sci., **129**, 543-556
29. Bertoli, F., Davies, G-L., Monopoli, M.P., Moloney, M., Gunko, Y.K., Salvati, A. and Dawson, K.A. (2014) *Magnetic nanoparticles to recover cellular organelles and study the time resolved nanoparticle-cell interactome throughout uptake* Small, **10**, 3307–3315

OptiPrep™ Application Sheet S16; 8th edition, January 2020