OptiPrep™ Application Sheet S44

Endocytosis in cultured cells: analysis of endosomes, lysosomes and plasma membrane by buoyant density

- 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 Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5)
- A summary of some of the more recent papers is given in Section 5.7
- There are two relevant **OptiPrep™ Mini-Reviews: MS15 – "Endocytosis analysis – a review of density gradient methods"** and **MS16 "Endocytosis – a bibliographical review"** which lists all the relevant papers reporting the use of OptiPrep™: to access these files return to the initial list of Folders and select "**Mini-Reviews**".

1. Background

The protocol in this Application Sheet was devised by Sheff et al [1] for the separation of two types of endosomes from transfected MDCK cells: peripheral early endosomes and perinuclear recycling endosomes in a study of transferrin internalization. These endosomes are involved in the sorting of proteins for delivery to the apical and basolateral domains of polarized epithelial cells and are differentially associated with rab4, rab11 and the transferrin receptor [1]. The method uses a conventional approach of a pre-formed gradient, centrifuged over night to equilibrium density in a swinging-bucket rotor.

Although there are many published methods in which buoyant density gradient centrifugation is carried out in 1-3 h at 150,000-200,000g, there is evidence that longer centrifugation times at lower RCFs, as used in this protocol, are required for true equilibrium density banding of membrane vesicles and that this strategy achieves their optimal separation. If times of 12-18 h are used, then the RCFs should be <100,000g to prevent significant gradient density profile distortion by the sedimentation of iodixanol molecules.

The following protocol is adapted from ref 1.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Optiprep™ diluent: 235 mM KCl, 12 mM $MgCl_2$, 25 mM $CaCl_2$, 30 mM EGTA, 150 mM Hepes-NaOH pH 7.0

C. 40% iodixanol working solution (WS): 2 vol. of Solution A + 1 vol. of Solution B

D. WS diluent: 78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-NaOH pH 7.0

E. Homogenization medium: 0.25 M sucrose, 78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-NaOH pH 7.0

Keep the following stock solutions at 4°C:

1 M Hepes (free acid)	23.8 g per 100 ml water
1 M KCl	7.45 g per 100 ml water
1 M $MgCl_2 \bullet 6H_2O$	20.3 g per 100 ml water
1 M $CaCl_2 \bullet 2H_2O$	14.7 g per 100 ml water
100 mM EGTA (free acid)	3.80 g per 100 ml water (pH 11-12)

Solution B: Mix together 15 ml, 23.5 ml, 1.2 ml, 2.5 ml and 30 ml respectively of the Hepes, KCl, $MgCl_2$, $CaCl_2$ and EGTA stock solutions; adjust to pH 7.0 with 1 M KOH and make up to 100 ml.

Solution D: Mix together 5 ml, 7.8 ml, 0.4 ml, 0.84 ml and 10 ml respectively of the Hepes, KCl, $MgCl_2$, $CaCl_2$ and EGTA stock solutions; adjust to pH 7.0 with 1 M KOH and make up to 100 ml.

Solution E: Dissolve 8.5 g sucrose in Solution D before adjusting pH and making up to 100 ml.

3. Ultracentrifuge rotor requirements (see Section 5.2)

Swinging-bucket rotor with tube size approx 14 ml, e.g. Beckman SW41Ti or Sorvall TH641

4. Protocol

Following any experimental procedures to allow binding, uptake and processing of a ligand or other functional manipulations, all operations must be carried out at 4°C.

1. Remove any unbound ligand or other components from the cell surface by washing the cell monolayer twice in any solution compatible with the study and then scrape the cells into 1 ml of Solution E.

2. Homogenize the cells using a ball-bearing homogenizer (cell cracker); four passes of the cell suspension should be sufficient. Check for adequate homogenization by phase contrast microscopy. If such a device is not available then use several passages through a fine gauge syringe needle or a Dounce homogenizer (**see Section 5.3**).

3. Centrifuge the homogenate in a swinging-bucket rotor at 1000 *g* for 5 min to pellet the nuclei and any cell debris. The pellet may be washed with Solution E if necessary and the two supernatants combined.

4. Prepare the low and high density gradient solutions of 5% and 20% (w/v) iodixanol by diluting Solution C with Solution D.

5. In tubes for the swinging-bucket rotor prepare 12-13 ml 5-20% (w/v) iodixanol gradients using either a two-chamber gradient maker or a Gradient Master™ (**see Section 5.4**).

6. Layer the 1000*g* supernatant(s) on top of the gradient and centrifuge at 90,000 g_{av} for 18-20 h (**see Section 5.5**).

7. Collect the gradient in approx 0.25 ml fractions either by upward displacement with a dense liquid, tube puncture or aspiration from the meniscus. For more information on harvesting gradients **see Application Sheet S08.**

8. If it is necessary to remove the iodixanol, fractions can be pelleted at 200,000 *g* for 20 min after dilution with 2 vol of Solution E. For more information **see Section 5.6**.

♦ Information regarding the analysis of endocytic compartments can be found in **Section 5.7**.

5. Technical Notes and Review

5.1 Homogenization media and gradient solutions

The homogenization medium often has to be tailored to the tissue or cell type. Organic osmotic balancers such as sucrose, mannitol and sorbitol were introduced for their compatibility in functional studies on subcellular membranes; moreover these low ionic strength HMs and gradient solutions permit the direct use of fractions for SDS-PAGE. Although 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and containing EDTA or EGTA is still a widely used HM for both tissues and cultured cells, for the latter in particular, supplementation with inorganic salts, as in this protocol, is becoming increasingly common and can reduce ionic interactions, aggregation between membranes and combat any raised viscosity of the homogenate due to cytoskeletal proteins. Some media that omit sucrose entirely use either NaCl or KCl or both as the principal osmotic balancer(s). The composition of the HM should also be compatible with any subsequent analytical process. The inclusion of divalent cations can guard against nuclear breakage; stabilize membranes generally, but may lead to aggregation.

The use of an Optiprep™ diluent (solution B) containing 235 mM KCl, 12 mM $MgCl_2$, 25 mM $CaCl_2$, 30 mM EGTA, 150 mM Hepes-NaOH pH 7.0 to produce a 40% (w/v) iodixanol working solution ensures that the concentrations of KCl, $MgCl_2$, $CaCl_2$, EGTA and buffer remain constant in the gradient when this solution is diluted with Solution D. Indeed the osmolality of the gradient will also

be approximately the same as in the HM (Solution E) the iodixanol and the sucrose providing almost identical osmotic contributions to the solutions. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

The same homogenization medium and gradient solutions as those described in Section 2 have been used for PC12 cells [2], HeLa cells [3-6], CHO cells [7], COS-7 cells [9] HEK293 cells [10], renal epithelial cells [11], a fibrosarcoma cell line [12], polarized human airway epithelial cells [13] and cultured monocytes [14,15]. Other homogenization media are given in Table 1.

Table 1: Homogenization media variations

Cell/tissue type	Homogenization medium	Ref. #
Brain (mouse)	0.5 M sucrose, 2 mM EDTA, 2 mM EGTA, 40 mM Tris-HCl, pH 7.4	16
Caco-2	0.25 M sucrose, 50 mM Tris-HCl, pH 7.4	17
CHO	0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8	8
Daudi	0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine-HCl, pH 7.8	18
HeLa	0.25 M sucrose, 3 mM imidazole, 1 mM EDTA, pH 7.4	19
	0.25 M sucrose, 10 mM HEPES-NaOH, pH 7.4	20
HepG2	0.25 M sucrose, 1 mM EDTA, 10 mM DTT, 10 mM HEPES, pH 7.4	21
Human airway epithelial	0.25 M sucrose, 20 mM Tricine-NaOH, pH 7.8	22
MCF-7	0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine pH 7.8	23
Neuroblastoma	150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4	24

If a hypoosmotic medium has to be used to swell the cells in order to achieve an adequate degree of homogenization it is important to return the homogenate to isoosmotic conditions as soon as possible. Other examples of homogenization media are given in **Application Sheets S05 (tissues) and S06 (cells)**.

- Protease inhibitors may be included in Solutions B, D and E at the operator's discretion.

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5.2 Ultracentrifuge rotors

The method may scaled up or down to the use of larger or smaller volume swinging-bucket rotors to accommodate other samples sizes.

5.3 Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elevhjem homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells use of 5-15 passages through a 27- or 25-gauge syringe needle, sometimes preceded by Dounce homogenization, is more common. The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices. Ideally the procedure should be as gentle and reproducible as possible, the aim being to cause at least 95% cell disruption without damage to the major organelles, particularly the nuclei and lysosomes. The type and severity of the homogenization process will have consequences for the integrity of the organelles and the size of the vesicles produced from tubular structures in the cytoplasm. Therefore the pattern of membrane banding in any subsequent gradient may not be easily predicted. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells).**

- Ref 25 describes the use of a complex treatment of PC12 cells for internalization of nerve growth factor, followed by mechanical permeabilization of the cells (one pass through a ball-bearing homogenizer) to release the internal membrane vesicles.

5.4 Gradients and centrifugation conditions

If neither of these gradient-making devices is available then a continuous gradient can be prepared by diffusion of a discontinuous gradient. For more information on gradient construction see Application Sheet S03. If necessary, adjust all volumes proportionately so that tubes (after sample application) are properly filled according to the manufacturer's instructions.

The long-spin 5-20% (w/v) iodixanol gradients described in the above protocol have now been used for a variety of cell types with small variations in *g*-force and centrifugation time (90,000-125,000 *g* for 15-20h). The cell types include including CHO [7,8], COS-7 [9], HeLa [3-6,19], HepG2 [21] human airway epithelial cells [13] and PC-12 [2]. Gradients covering other density ranges and/or centrifuged for different times have also been used (see Table 2).

Table 2: Continuous gradient formats - variations

Cell type	Iodixanol gradient (%w/v)/*g*-force/time	Other conditions/notes	Ref #
Brain (mouse)	2-30%/100,000 *g*/20 h	Sample under-layered in 40%	16
BSC1 Renal epithelial	8-25%/100,000 *g*/20 h		11
Caco2	9-30%/52,000 *g*/1.5 h	Sample under-layered in 35%	17
Daudi	0-20%/200,000 *g*/2 h		18
Fibrosarcoma line	5-20%/100,000 *g*/3 h		12
HeLa	2.5-25%/133,000 *g*/3 h		20
HepG2	0-30%/114,000 *g*/16 h	Compared with standard 5-20%	21
Human airway epithelial	15-25%/100,000 *g*/3h	Sample loaded in 5%	22
Monocytes	5-30%/90,000 *g*/20h		14,15
Neuroblastoma	5-30%/90,000 *g*/16 h		24

Other strategies

Idkowiak-Baldys et al [10] used a discontinuous gradient of 5%, 10%, 15% and 20% (w/v) iodixanol for HEK cells, rather than the recommended 5-20% continuous gradient, but since the gradient is centrifuged at a relatively low *g*-force for at least 16 h the gradient will become continuous and more or less linear by diffusion.

Lin et al [25] used PC12 cells, permeabilized by a single passage through a ball-bearing homogenizer to isolate a vesicle-containing fraction. This was first separated on a 0-30% (w/v) iodixanol sedimentation velocity gradient and fractions from this gradient adjusted to 32% iodixanol and further fractionated by flotation through a long-spin 0-30% iodixanol gradient at 133,000 *g* for 18 h. The authors were investigating the trafficking of the TrkA neurotrophin receptor and were able to completely resolve one population of vesicles (ρ = 1.12 g/ml) which contained both TrkA and the TrkA-associated protein APPL1 from a denser one (ρ = 1.2 g/ml) which contained only APPL1. **See Application Sheet S42 (Section 5.7)** for other examples of this double gradient strategy.

Li et al [23] used a discontinuous gradient of 2%, 24% and 32% (w/v) iodixanol (with the sample in the latter), centrifuged at 83,000 *g* for 2 h to separate (in order of increasing density) the plasma membrane, early/recycling endosomes, mitochondria and peroxisomes from MCF7 cells.

5.5 Removal of iodixanol from gradient samples

Large pore size dialysis tubing, Maxi GeBAflex (www.geba.org) dialysis tubes (highest MWt cut off), centrifugal ultrafiltration cones or a G25 Sephadex column may be used. More information about the removal of iodixanol can be found in **Section 7 of Application Sheet S09.**

5.6 Analytical review

Sheff et al [1] carried out a number of elegant experiments to investigate the endosomal pathways of transfected MDCK cells. In one of these, the uptake of ^{125}I-transferrin into MDCK cells transfected with human transferrin receptor was studied by a classical pulse-chase technique. The data is summarized in Figure 1. The early endosomes

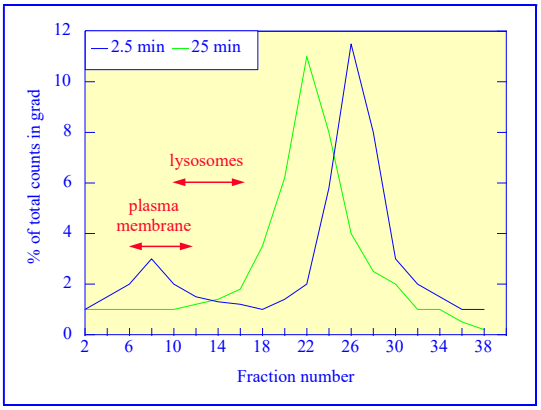

Figure 1 Distribution of transferrin (radiolabel) in iodixanol gradient: effect of chase time. Low density is on the left. Adapted from ref 1 with kind permission of authors and The Rockefeller University Press.

(2.5 min) band at a significantly higher density than the recycling endosomes (25 min) and the equilibrium density of both of these are distinctively higher than either plasma membrane or lysosomes. The position of the latter was determined by ß-hexosaminidase activity [1].

The gradients are generally able to resolve plasma membrane from early and late endosomes, although the relative position of the plasma membrane is variable; it is usually lighter than the endosomes, but there may be instances where it is denser, see for example refs 4 and 8. Sugii et al [8] also demonstrated that there was also a distinctive banding of the TGN (denser than the late endosomes) from CHO cells. In nearly all instances early endosomes are reported as being less dense than the late endosomes and there may be some distinctive banding of recycling endosomes, although there is usually significant overlap between the latter and early endosomes. Sheff et al [1] reported a slightly lower density for recycling endosomes (see Figure 1).

5.7 Summary of other papers reporting use of this continuous gradient technology

- Urbanska et al [26] compared the resolving power of sucrose and iodixanol gradients in the characterization of APPL endosomes from HeLa cells. The distinctive banding patterns in iodixanol gradients of EEA-1, APPL1, AP50 and Rab5 were not seen in sucrose gradients.
- The 5-20% (w/v) iodixanol gradient format described above achieved a complete separation of plasma membrane and late endosomes/lysosomes from brain tissue [27] and early endosomes and late endosomes/lysosomes from HeLa cells [28].
- Keith et al [29] did not use a post-nuclear supernatant (PNS) from HEK cells as the gradient input; instead the PNS was centrifuged at 12,000 *g* for 20 min; the pellet resuspended in lysis buffer and layered on a 2.5%-17% (w/v) iodixanol gradient, which was centrifuged at 90,000 *g* for 7 h. The gradient achieved effective resolution of early endosomes (EEA1), recycling endosomes (Rab11) and late endosomes (Rab7).
- Niu et al [30] studied the infection of HL60 cells by the obligate intracellular bacterium *Anaplasma phagocytophilum* (*Ap*) The continuous iodixanol gradient was able to resolve the characteristic *Ap* inclusions, from early endosomes (EEA1), late endosomes+lysosomes (LAMP2), ER (calnexin) and autophagosomes (LC3). Interestingly in the infected cells the ER was shifted to slightly higher densities compared to that in uninfected cells.

6. References

1. Sheff, D. R., Daro, E. A., Hull, M. and Mellman, I. (1999) *The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions* J. Cell Biol., **145**, 123-139
2. Li, Y., Chin, L-S., Levey, A.I. and Li, L. (2002) *Huntingtin-associated protein 1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate and functions in endosomal trafficking* J. Biol. Chem., **277**, 28212-28221
3. Chin, L-S., Raynor, M. C., Wei, X., Chen, H-Q. and Li, L. (2001) *Hrs interacts with sorting nexin 1 and regulates degradation of epidermal growth factor receptor* J. Biol. Chem., **276**, 7069-7078
4. Meyers, J. M. and Prekeris, R. (2002) *Formation of mutually exclusive Rab11 complexes with members of the family of Rab11-interacting proteins regulates Rab11 endocytic targeting and function* J. Biol. Chem., **277**, 49003-49010
5. Proikas-Cezanne, T., Gaugel, A., Frickey, T. and Nordheim, A. (2006) *Rab14 is part of the early endosomal clathrin-coated TGN microdomain* FEBS Lett., **580**, 5241-5246
6. Barroso-González, J., Machado, J-D., García-Expósito, L. and Valenzuela-Fernández, A. (2009) *Moesin regulates the trafficking of nascent clathrin-coated vesicles* J. Biol. Chem., **284**, 2419–2434
7. Daro, E., Sheff, D., Gomez, M., Kreis, T. and Mellman, I. (1997) *Inhibition of endosome function in CHO cells bearing a temperature-sensitive defect in the coatomer (COPI) component ∈-COP* J. Cell Biol., **139**, 1747-1759
8. Sugii, S., Reid, P.C., Ohgami, N., Du, H. and Chang, T-Y. (2003) *Distinct endosomal compartments in early trafficking of low density lipoprotein-derived cholesterol* J. Biol. Chem., **278**, 27180-27189
9. Shen, X., Xu, K-F., Fan, Q., Pacheco-Rodriguez, G., Mos, J. and Vaughan, M. (2006) *Association of brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2) with recycling endosomes during transferring uptake* Proc. Natl. Acad. Sci. USA, **103**, 2635-2640
10. Idkowiak-Baldys, J., Becker, K.P., Kitatani, K. and Hannun, Y.A. (2006) *Dynamic sequestration of the recycling compartment by classical protein kinase C* J. Biol. Chem., **281**, 22321-22331
11. McKenzie, J., Johannes, L., Taguchi, T.and Sheff, D. (2009) *Passage through the Golgi is necessary for Shiga toxin B subunit to reach the endoplasmic reticulum* FEBS J., **276**, 1581–1595
12. Molle, D., Segura-Morales, C., Camus, G., Berlioz-Torrent, C., Kjems, J., Basyuk, E. and Bertrand, E. (2009) *Endosomal trafficking of HIV-1 Gag and genomic RNAs regulates viral egress* J. Biol. Chem., **284**, 19727-19743

13. Bomberger, J.M., Guggino, W.B. and Stanton, B.A. (2011) *Methods to monitor cell surface expression and endocytic trafficking of CFTR in polarized epithelial cells* In Cystic Fibrosis, Methods Mol. Biol. (eds. Amaral, M.D. and Kunzelmann, K.) Springer Science+Business Media, pp 271-283
14. Gibbings, D.J., Ciaudo, C., Erhardt, M. and Voinnet, O. (2009) *Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity* Nat. Cell Biol., 11, 1143-1149
15. Gibbings, D. (2011) *Continuous density gradients to study argonaute and GW182 complexes associated with the endocytic pathway* In Argonaute Proteins: Methods and Protocols, Methods Mol. Biol., **725**, (ed. Hobman. T.C. and Duchaine, T.F.) Springer Science+Business Media, pp 63-76
16. Shinohara, M., Sato, N., Kurinami, H., Takeuchi, D., Takeda, S., Shimamura, M., Yamashita, T., Uchiyama, Y., Rakugi, H. and Morishita, R. (2010) *Reduction of brain β-amyloid (Aβ) by fluvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, through increase in degradation of amyloid precursor protein C-terminal fragments (APP-CTFs) and Aβ clearance* J. Biol. Chem., **285**, 22091–22102
17. Orlandi, P.A (1997) *Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line* J. Biol. Chem., **272**, 4591-4599 (1997)
18. Payelle-Brogard, B. and Pellegrini, S. (2010) *Biochemical monitoring of the early endocytic traffic of the type I interferon receptor* J. Interferon Cytokine Res., **30**, 89-98
19. Mairhofer, M., Steiner, M., Salzer, U. and Prohaska, R. (2009) *Stomatin-like protein-1 interacts with stomatin and is targeted to late endosomes* J. Biol. Chem., **284**, 29218-29229
20. Tagami, S., Okochi, M., Yanagida, K., Ikuta, A., Fukumori, A., Matsumoto, N., Ishizuka-Katsura, Y., Nakayama, T., Itoh, N., Jiang, J., Nishitomi, K., Kamino, K., Morihara, T., Hashimoto, R., Tanaka, T., Kudo, T., Chiba, S. and Takeda, M. (2008) *Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1* Mol. Cell. Biol., **28**, 165-76
21. Manunta, M., Izzo, L., Duncan, R. and Jones, A.T. (2007) *Establishment of subcellular fractionation techniques to monitor the intracellular fate of polymer therapeutics II: Identification of endosomal and lysosomal compartments in HepG2 cells combining single-step subcellular fractionation and fluorescent imaging* J. Drug Target., **15**, 37-50
22. Bomberger, J.M., Ye, S., MacEachran, D.P., Koeppen, K., Barnaby, R.L., O'Toole, G.A. and Stanton, B.A. (2011) *A Pseudomonas aeruginosa toxin that hijacks the host ubiquitin proteolytic system* PLoS Pathog., **7**: e1001325
23. Li, Q., Harraz, M.M., Zhou, W., Zhang, L.N., Ding, W., Zhang, Y., Eggleston, T., Yeaman, C., Banfi, B. and Engelhardt, J.F. (2006) *Nox2 and Rac1 regulate H_2O_2-dependent recruitment of TRAFg to endosomal interleukin-1 receptor complexes* Mol. Cell. Biol., **26**, 140-154
24. Wiesinger, J.A., Buwen, J.P., Cifelli, C.J., Unger, E.L., Jones, B.C. and Beard, J.L. (2007) *Down-regulation of dopamine transporter by iron chelation in vitro is mediated by altered trafficking, not synthesis* J. Neurochem., **100**, 167-179
25. Lin, D.C., Quevedo, C., Brewer, N.E., Bell, A., Testa, J.R., Grimes, M.L., Miller, F.D. and Kaplan, D.R. (2006) *APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction* Mol. Cell. Biol., **26**, 8928-8941
26. Urbanska, A., Sadowski, L., Kalaidzidis, Y. and Miaczynska, M. (2011) *Biochemical characterization of APPL endosomes: the role of annexin A2 in APPL membrane recruitment* Traffic, **12**, 1227–1241
27. Takamura, A., Higaki, K., Ninomiya, H., Takai, T., Matsuda, J., Iida, M., Ohno, K., Suzuki, Y. and Nanba, E. (2011) *Lysosomal accumulation of Trk protein in brain of G_{M1}-gangliosidosis mouse and its restoration by chemical chaperone* J. Neurochem., **118**, 399–406
28. Huotari, J., Meyer-Schaller, N., Hubner, M., Stauffer, S., Katheder, N., Horvath, P., Mancini, R., Helenius, A. and Peter, M. (2012) *Cullin-3 regulates late endosome maturation* Proc. Natl. Acad. Sci. USA, **109**, 823–828
29. Keith, D.J., Wolfrum, K., Eshleman, A.J. and Janowsky, A. (2012) *Melittin initiates dopamine transporter internalization and recycling in transfected HEK-293 cells* Eur. J. Pharmacol., **690**, 13–21
30. Niu, H., Xiong, Q., Yamamoto, A., Hayashi-Nishino, M. and Rikihisa, Y. (2012) *Autophagosomes induced by a bacterial Beclin 1 binding protein facilitate obligatory intracellular infection* Proc. Natl. Acad. Sci. USA, **109**, 20800–20807

7. Acknowledgements

Alere Technologies AS wish to thank Dr David Sheff, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520, USA, for his kind cooperation in the preparation of this Application Sheet.

Application Sheet S44; 8th edition, February 2018

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