

# OptiPrep™ Application Sheet M09

## Analysis of protein complex formation, microtubules and cytoskeleton

- ◆ 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 Macromolecules and Macromolecular Complex Index; key Ctrl “F” and type the M-Number in the Find Box
- ◆

### 1. Background

Determination of the sedimentation coefficient of a protein, traditionally in a 5-20% sucrose gradient, in order to determine its molecular mass, is a technique that was developed many years ago. Although alternative methods, notably polyacrylamide gel electrophoresis, have achieved a wide popularity, there are certain situations that are better suited to density gradients, for example the analysis of heavily glycosylated proteins, which run anomalously on gels. Another area that may be better suited to density gradient analysis is the study of protein-protein interactions.

The standard sucrose gradient analysis, which is carried out in swinging-bucket rotors for 4-16 h, may be less than ideal for determining protein-protein interactions. Macromolecular complexes may be insufficiently stable to survive these long centrifugation times and they may be intolerant of the high hydrostatic pressures generated in a swinging-bucket rotor [1,2]. In addition Timasheff [3] pointed out that solutions of low water activity (high osmolality) can remove bound water from proteins and could cause changes in stability of the protein and its propensity to aggregate. The use Nycodenz® will reduce the osmolality (raise the water activity) of gradients considerably, while with iodixanol gradients can be made isoosmotic throughout the entire useful density range. Gradient made from one or other of these solutes may therefore be an important advantage in studying protein-protein interactions in density gradients. Some reports also highlight the functional problems associated with sucrose gradients; for example these caused proteolysis of a kinesin-related motor protein, while in iodixanol gradients there was no proteolysis whatsoever [4]. [This Application Sheet covers the following topics:](#)

- ◆ Section 2: Alzheimer’s A $\beta$  peptide and other neurodegenerative disease-associated proteins
- ◆ Section 3: Non-muscle myosin II (NMII)
- ◆ Section 4: Dimerization of the kinesin-related motor protein KIF1A
- ◆ Section 5:  $\alpha$ -Synuclein aggregation
- ◆ Section 6: Microtubule fragment size analysis
- ◆ Section 7: Cytoskeleton analysis
- ◆ Section 8: Prion proteins and fibrils
- ◆ Section 9: Association of *Drosophila* proteins with lipophorin
- ◆ Section 9: Other applications

### 2. Alzheimer’s disease amyloid-beta (A $\beta$ ) peptide

#### 2a. Aggregation (oligomerization) studies

##### 2a-1. Introduction

Moir et al [5] studied the aggregation of the A $\beta$  peptide by Zn<sup>2+</sup>, Cu<sup>2+</sup>, EDTA or a pH 5.5 buffer. The incubations were simply layered over a series of Nycodenz® barriers of increasing density; the concentrations were 43%, 44%, 44.6%, 46.8% and 47% (w/v) in microcentrifuge tubes and centrifuged at 16,000 g for 10 min. Tubes were then analyzed by rapid freezing to -170°C for 1 h and “fractionated” by cutting the tube. All the treatments, except EDTA, allowed the aggregates to sediment through the 43 and 44% barriers but only the Zn<sup>2+</sup> induced aggregates sedimented through the 44.6 and 46.8% barriers.

The analysis of oligomerization of the  $\beta$ -amyloid A $\beta$  peptide in iodixanol gradients, using a high performance near-vertical rotor, developed by Ward et al [6], may be applicable to any protein oligomerization studies and is thus given here in some detail. The short sedimentation path length of the rotor (18 mm) means that the centrifugation time can be reduced to 3 h and the hydrostatic pressure is correspondingly low. The authors used the gradients to analyze the interactions of the  $\beta$ -amyloid (A $\beta$ ) peptide that is implicated in Alzheimer's disease, but the strategy could be used or adapted to any protein oligomerization or protein-protein interaction study. The protocol described below is adapted from ref 6. **For Notes see Section 2a-5**

### 2a-2. Solutions required

A. OptiPrep™

B. OptiPrep™ diluent: for the study of the oligomerization of  $\beta$ -amyloid (A $\beta$ ) peptide [6] the diluent used was phosphate-buffered saline (PBS), but any diluent compatible with the protein-protein interaction may be used (see Note 1). **Include protease inhibitors in solutions as required.**

### 2a-3. Ultracentrifuge rotor requirements

A near-vertical (e.g. Beckman NVT100, NVT90 or NVT65.2) or vertical (e.g. Beckman VTi90, VTI65.2 or Sorvall TV1665 or 70V6) rotor: all these rotors accommodate tubes for approx 5 ml total volume (see Note 2).

### 2a-4. Protocol

1. Carry out all operations at 0-4°C or any temperature compatible with the interactions under investigation.
2. Dilute OptiPrep™ with PBS to produce the solutions used in the next step (see Note 1).
3. Layer sequentially in tubes for the chosen rotor: 0.65 ml of 50%, 40% and 30% (w/v) iodixanol, 1.95 ml of 20%, 0.65 ml of 10% and 0.3 ml of 5% (w/v) iodixanol (see Note 3)
4. Layer 0.3 ml of the protein solution on top and centrifuge at 350,000 g for 3 h at 0-4°C, using controlled acceleration and deceleration over the 0-2000 rpm range (see Note 4).
5. Collect the gradient in 0.3-0.35 ml fractions either by upward displacement with Maxidens™ and an Axis-Shield Gradient Collector, tube puncture or by automatic aspiration from the meniscus using a Labconco Auto Densi-flow® (see Notes 5 and 6).

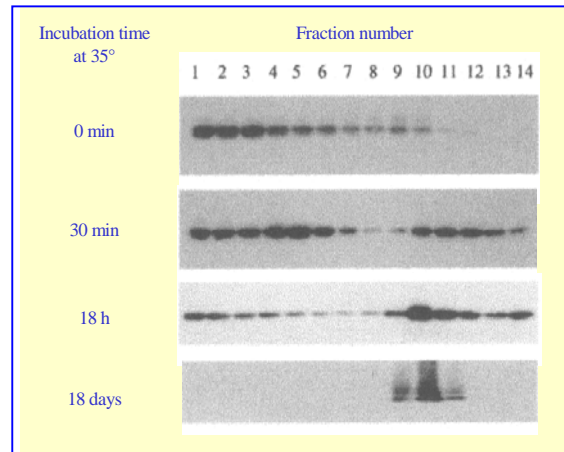
◆ See Notes 7-10 for other examples of the use of iodixanol gradients for A $\beta$  peptide studies

### 2a-5. Notes

1. If it is important to maintain a constant background of buffer concentration (e.g. 20 mM Tris-HCl, pH 8.0) and low concentration of some other additive (e.g. 1 mM EDTA and 1 mM DTT); then a 50% (w/v) iodixanol working solution (WS) should first be prepared by mixing 5 vol. of OptiPrep™ with 1 vol. of 6 mM EDTA, 6 mM DTT, 120 mM Tris-HCl, pH 8.0. Further dilutions are then obtained by dilution of the WS with 20 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA. All gradient solutions will then contain the requisite concentration of buffer and additive. For more information on the preparation of density gradient solutions **see Application Sheet M01.**
2. Vertical or near-vertical rotors are ideal for sedimentation velocity separations because, (a) the sample layer achieves a very narrow zone-width after reorientation and (b) the sedimentation path length is short. If other rotors (fixed-angle or swinging-bucket) are used the sample volume must never be greater than 10% of the gradient volume and the centrifugation time will need increasing to take account of the longer sedimentation path length (particularly in larger volume swinging-bucket rotors).
3. Because of the short path length, the gradient will become more or less continuous within 30 min. An alternative approach may be to produce a linear continuous gradient in the centrifuge tube

before layering the sample (see [Application Sheet M02](#)); however the irregular density profile achieved by using the non-uniform layer volumes described in Step 3 of the Protocol will be lost. The consequences of this are not clear.

4. If the method is being used to study the oligomerization of other proteins the RCF or centrifugation time may need modulation according to the size of the protein particles.
5. An example of the resolution, which is achievable with this gradient system, is given in Figure 1. Freshly-prepared hexafluoro-2-propanol (HFIP) treated  $\beta$ -amyloid ( $A\beta$ ) peptide oligomerizes during incubation at 35°C and the distribution of this peptide across the gradient was analyzed by SDS-PAGE after 0 min, 30 min, 18 h and 18 days of incubation at this temperature. The position of the peptide in the gradient reflects the extent and type of association of the peptide monomers.
6. For more information on methods of harvesting gradients see [Application Sheet M04](#).
7. The method has also been adapted to small volume swinging-bucket rotors: Rzepecki et al [7] used a similar gradient, which was scaled down to use in a Beckman TLS55 (2.2 ml tubes) with centrifugation at 259,000 g for 4 h and Lockhart et al [8] used a Beckman MLS50 (5 ml tubes) for studying the formation of  $\beta$ -amyloid fibrils at 268,000 g for 3 h. The Rzepecki et al [7] gradient was also used by Funke et al [9] to study the effect of the D-enantiomeric peptide D3, which caused a shift of the  $A\beta$  peptide from an oligomeric form to much larger aggregates and the  $A\beta$  binding peptide L3 [10].
8. More recent studies of the oligomerization procedure [11-13] have also used the TLS55 rotor under more or less similar centrifugation conditions; the gradients were however slightly modified; a discontinuous gradient of 0.26 ml each of 50%, 40% and 30% iodixanol, 0.78 ml of 20%, 0.26 ml of 10% and 0.1 ml of 5% iodixanol. The gradients were used to analyse  $A\beta$  (1-42) aggregates using 100 $\mu$ l of samples, which was layered on top.
9. Sehlin et al [14] used an iodixanol gradient similar to that of Ward et al [6] each step was 0.65 ml except for the 30% (w/v) iodixanol (1.95 ml) and omitted the 5% layer. The gradient effectively resolved  $A\beta$  peptide aggregates of different sizes.
10.  $A\beta$  prions have also been extensively purified in a multi-step method. In the primary fractionation of a brain homogenate the latter was adjusted to 18% (w/v) iodixanol and layered over 30% and 35% iodixanol. After centrifugation at 60,000 g for 20 min the top lipid layer was discarded and the material within the denser two layers collected. After dilution with buffer, this was layered over a second gradient of 26% and 35% iodixanol and centrifuged at the same speed for 40 min. Again the top layer was discarded and the material in the two denser layers recovered prior to further processing [15].



**Figure 1:** Iodixanol gradient sedimentation velocity analysis of  $\beta$ -amyloid ( $A\beta$ ) peptide oligomerization: effect of time of incubation (low density on left). For more details see text. Reproduced from ref 6 with kind permission of the authors, ©The Biochemical Society

## 2b. Other neurodegenerative disease-related protein studies

### 2b-1. Fibrillar structures

Levy et al [16] adapted the method described in Section 2 to an analysis of islet amyloid fibril formation using a 3 ml pre-formed continuous 0-60% (w/v) iodixanol gradient, generated from equal volumes of OptiPrep™ and water (pH 2) in tubes for a Beckman SW60Ti swinging-bucket rotor. The gradients were loaded with 0.6 ml of sample and centrifuged at 150,000 g for 24 h. The gradient separated a series of low molecular mass pre-fibrillar assemblies in the top half of the gradient from the fibrils at the bottom. More recently Frenzel et al [17] separated fibrils from smaller aggregates and monomers by iodixanol density gradient centrifugation. The gradient comprised layers of 50%, 40%, 30%, 20%, 10% and 5% (v/v) OptiPrep™ iodixanol in 10 mM phosphate buffer pH 7.4; the sample was

top-loaded. Centrifugation in a small volume (2.2 ml tubes) swinging-bucket rotor for 3 h at 259,000 g, resolved the three forms of the protein.

- ◆ See Section 3 for a related method for separating monomeric and filamentous protein forms.

The method developed by Ward et al [6] has been adapted to the slightly larger tubes (6 ml) of the Sorvall TV865 by Khlistunova et al [18] for studying the Tau protein; usually a soluble protein, but in some neurodegenerative diseases it forms “paired helical filaments” and associates with microtubules. The gradient was generated from 0.85 ml of 50% and 40%, 2.2 ml of 30%, 0.85 ml of 10% and 0.3 ml of 5% (w/v) iodixanol. Sarkosyl-insoluble fractions from neuronal tissue have also been purified in an iodixanol gradient [19].

### 3. Non-muscle myosin II (NMII)

This investigation was reported by Shutova et al [20]. Detergent-soluble cell lysates was layered on to a discontinuous gradient of 50%, 25%, 12%, and 6% OptiPrep™ layers. After centrifugation at 80,000 rpm for 1 h the gradient resolved monomeric and filamentous forms of NMII which had sedimentation coefficients of approx 7S and 16S respectively. The monomeric form was found to be increased to a greater extent in blebbistatin-treated cells.

### 4. Kinesin-related motor protein KIF1A dimerization

Rashid et al [4] used a 3.8 ml continuous gradient of 5-40% (w/v) iodixanol overlaid by 0.2 ml of sample in a Beckman SW60Ti swinging-bucket rotor at 100,000  $g_{av}$  for 16 h at 4°C, to identify dimers of KIF1A.

### 5. $\alpha$ -Synuclein aggregation

Rather larger protein aggregates of cytoplasmic  $\alpha$ -synuclein from COS7 cells have been analyzed in discontinuous gradients of 2.5%, 25% and 35% (w/v) iodixanol, centrifuged at only 50,000 g for 30 min [21].

### 6. Microtubule fragment size analysis

MacCormick et al [22] characterized a large protein complex containing a nerve-growth-factor-activated ERK (extracellular-signal-regulated kinase) and MEK. Part of the fractionation process included the isolation of a non-ionic detergent insoluble fraction that also contained microtubule fragments that contained some bound kinases. Glycerol gradients were not effective in resolving the larger microtubule fragments; the following iodixanol gradient method devised by MacCormick et al [22] however provided excellent resolution of these particles.

1. Permeabilize PC12 cells by a single passage through a ball-bearing homogenizer.
2. Release the intracellular material by the permeabilization and separate from the residual “cells” by centrifugation at 1000 g for 10 min.
3. Concentrate the supernatant material by pelleting at 100,000 g through a cushion of 10% (w/v) sucrose in 20 mM MOPS (pH 7.2) containing 1 mM EGTA and 1 mM  $\text{Na}_3\text{VO}_4$ .
4. Suspend the pellet in buffer containing potassium aspartate, potassium gluconate and potassium glutamate (all 38 mM), 20 mM MOPS, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EDTA, 1 mM EGTA, pH 7.1.
5. Prepare a 30% (w/v) iodixanol solution in the same buffer and make a continuous 0-30% iodixanol prepared using a two-chamber gradient maker or Gradient Master™. If neither of these devices is available make up a discontinuous gradient from equal volumes of 0%, 10%, 20% and 30% (w/v) and allow to diffuse overnight at 4°C. For more information about preparing continuous iodixanol gradients see [Application sheet M02](#).
6. Layer the sample on top of the gradient (sample volume <10% of total gradient volume). The gradient separation is a sedimentation velocity one, so its resolving power is inversely proportional to the sample volume. Centrifuge at 200,000 g for 1-3 h (turn off the brake during deceleration from 2000 rpm or use a controlled deceleration program).

7. Collect the gradient either by tube puncture or collection from the meniscus. For more information about gradient fractionation [see Application Sheet M05](#). MacCormick et al [22] collected the gradient in 27 fractions.
  - ◆ After a centrifugation for 1 h the microtubule fragments from PC12 cells has been resolved on the basis of size into four discrete fractions, as determined by the  $\alpha$ -tubulin distribution [22] but ERK was only associated with three of them.

## 7. Cytoskeleton analysis

Plasma membrane and cytoskeleton in *Drosophila* extracts have been effectively separated on 10-40% (w/v) iodixanol gradients prepared by dilution of OptiPrep™ with 0.25 M Sucrose, 10 mM Tris-HCl, pH 8.0 centrifuged at 250,000 g for 3 h [23]. It provided a very clear separation of the syntaxin 1A (plasma membrane marker) the denser myosin II heavy chain (cytoskeleton marker).

A widely used discontinuous sedimentation velocity gradient originally devised by Majoul et al [24], comprising 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 30% (w/v) iodixanol was used by Chen et al [25] who studied the cytoskeleton structure in a 65,000 g (1 h) fraction from a post-nuclear supernatant of a mouse spinal cord homogenate. The gradients were allowed to diffuse at 4°C for 3-4 h solution before loading the fraction and centrifuging at 170,000 g for 90 min. Both actin and tubulin showed a biphasic distribution, being concentrated in the top quarter of the gradient but also demonstrating a sharp band about three-quarters of the way down the gradient. Mice deficient in a presenilin binding protein called “modifier of cell adhesion” (MOCA) lacked the sharp denser band.

Cofilin-actin rods have been isolated from cultured nerve cells in a double gradient strategy [26]. All steps were performed at 4°C. A low-speed supernatant was layered over 10% and 15% (w/v) iodixanol and centrifuged at 6,650 g for 10 min to band the rods above the interface between the two iodixanol layers. The collected fraction was diluted with 13% iodixanol (vol. ratio of approx 0.4:1.7) and centrifuged at 166,000 g for 2 h to create a self-generated gradient – a small volume vertical rotor is best suited to self-generated gradient formation ([see OptiPrep Application Sheet M03 for more information on self-generated gradients](#)). The rods band close to the bottom of the gradient. Once recovered, the rods in the fraction (after dilution with an equal volume of buffer) may be concentrated on a 25% iodixanol cushion at 5,000 g for 15 min [26].

Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) controls its influence on F-actin-related processes and Lin et al [27] studied them in *Dictyostelium*. A discontinuous iodixanol gradient of 5%, 10%, 15% and 20% (w/v) iodixanol centrifuged at approx 150,000 g for 18 h. WASP banded broadly in the bottom half of the gradient but in the presence of VASP, the WASP distribution shifted significantly to a higher density.

## 8. Prion proteins

Tixador et al [28] solubilized the proteins from a mouse brain homogenate in a buffered solution of dodecyl- $\beta$ -D-maltoside and *N*-lauryl sarcosine. The material was layered on top of a continuous 10-25% (w/v) iodixanol gradient (4.8 ml total volume) and centrifuged 285,000 g for 45 min. The host-encoded prion protein (PrP<sup>C</sup>) banded close to the top of the gradient, while the PrP<sup>Sc</sup>, the multimeric misfolded conformer PrP<sup>Sc</sup> banded about half-way down the gradient. Discontinuous iodixanol gradients have also been used as part of the procedure in the purification of prion fibrils from mouse brain [29]. Sarcosyl-containing 10-54% (w/v) iodixanol gradients (200,000 g for 1 h) have also been used to determine the size of aggregates containing prion protein [30].

An extensive study of the quaternary structure of prion proteins was carried out by Laferrière et al [31] using both sedimentation velocity and equilibrium iodixanol density gradients. Mouse brain homogenates were solubilized in a buffered saline containing EDTA, DTT, dodecyl- $\beta$ -D-maltoside and *N*-lauryl sarcosine.

- ◆ For sedimentation velocity analysis the samples were layered on top of a continuous 10–25% iodixanol gradient and centrifuged at 285 000 g for 45 min. In some studies the time was increased to 90 min; this provided additional resolution of the proteins. Rapidly- and slowly-sedimenting ovine strains were identified
- ◆ For equilibrium gradients the sample was adjusted to 40% iodixanol and made part of a discontinuous gradient spanning the range 10-60% iodixanol and centrifuged at 115,000 g for 17 hours.
- ◆ Similar sedimentation velocity gradients (5-25% iodixanol) were used by Coleman et al [32] in the identification of small soluble protease-sensitive prion proteins as the major source of infectivity in mouse brain.
- ◆ Wenborn et al [33] were able to purify prion protein from brain homogenates that was devoid of ferritin and other contaminating proteins by a combination of filtration and a series of centrifugations through 17.5% (w/v) iodixanol at 16,100 g.
- ◆ Herrmann et al [34] used 10-40% and 2-20% iodixanol gradients (52,000 rpm for 90 min) to show an impoverishment of the larger molecular weight prion proteins (from mouse brain) during drug treatment.

## 9. Association of *Drosophila* proteins with lipophorin

This methodology was first reported by Eugster et al [35]: a homogenate of larvae was centrifuged at 120,000 g and the supernatant adjusted to adjusted to 50% (w/v) iodixanol and layered beneath gradients of 10%–20% to 35%–45% (w/v) iodixanol. They were centrifuged at 4°C, at 285,000 g, for 16 h in a swinging-bucket rotor (2.2 ml tube volume). There is no obvious reason why the separation cannot be scaled up. Proteins not bound to the lipophorin lipoprotein remained at the bottom of the gradient, while lipophorin- bound proteins floated into the top half of the gradient. The method was used to study the interaction of the lipophorin with various morphogens via heparan sulphate. Similar gradients for studying lipophorin interactions have been used by Palm et al [36, 37].

## 10. Other applications

Iodixanol gradients have also been used to study the size of complexes formed by the Circadian clock PERIOD complex [38] and using a small volume step gradient of 20%, 15%, 10%, 5%, centrifuged for 18 h at approx 150,000  $g_{av}$  4°C, Lin et al [39] were able to demonstrate an association of VASP and GST-WASP proteins from *Dictyostelium*. The gradient would be continuous within about 3 h of the centrifugation.

## 11. References

1. Marcum, J.M. and Borisy, G.G. (1978) *Sedimentation velocity analyses of the effect of hydrostatic pressure on the 30 S microtubule protein oligomer* J. Biol. Chem., **253**, 2852-2857 J. Biol. Chem., **253**, 2852-2857
2. Hauge, J.G. (1971) *Pressure-induced dissociation of ribosomes during ultracentrifugation* FEBS Lett., **17**, 168-172
3. Timasheff, S.N. (1993) *The control of protein stability and association by weak interactions with water: how do solvents affect these processes* Annu. Rev. Biophys. Biomol. Struct., **22** 67-97
4. Rashid, D.J., Bononi, J., Tripet, B.P., Hodges, R.S. and Pierce, D.W. (2005) *Monomeric and dimeric states exhibited by the kinesin-related motor protein KIF1A* J. Pept. Res., **65**, 538-549
5. Moir, R.D., Romano, D.M., Laurans, M.H., Huang, X., Bush, A.I., Smith, J.D. and Tanzi, R.E. (1999) *Differential effects of apolipoprotein E isoforms on metal-induced aggregation of A $\beta$  using physiological concentrations* Biochemistry, **38**, 4595-4603
6. Ward, R.V., Jennings, K.H., Jepras, R., Neville, W., Owen, D.E., Hawkins, J., Christie, G., Davis, J.B., George, A., Karran, E.H. and Howlett, D.R. (2000) *Fractionation and characterization of oligomeric, protofibrillar and fibrillar forms of  $\beta$ -amyloid peptide* Biochem. J., **348**, 137-144
7. Rzepecki, P., Nagel-Steger, L., Feuerstein, S., Linne, U., Molt, O., Zadnarski, R., Aschermann, K., Wehner, M., Schrader, T. and Riesner, D. (2005) *Prevention of Alzheimer's disease-associated A $\beta$  aggregation by rationally designed nonpeptidic  $\beta$ -sheet ligands* J. Biol. Chem., **279**, 47497-47505
8. Lockhart, A., Ye, L., Judd, D.B., Merritt, A.T., Lowe, P.N., Morgenstern, J.L., Hong, G., Gee, A.D. and Brown, J. (2005) *Evidence for the presence of three distinct binding sites for the thioflavin T class of Alzheimer's disease PET imaging agents on  $\beta$ -amyloid peptide fibrils* J. Biol. Chem., **280**, 7677-7684
9. Funke, S.A., van Groen, T., Kadish, I., Bartnik, D., Nagel-Steger, L., Brener, O., Sehl, T., Batra-Safferling, R., Moriscot, C., Schoehn, G., Horn, A.H.C., Müller-Schiffmann, A., Korth, C., Sticht, H. and Willbold, D. (2010) *Oral treatment with the D-enantiomeric peptide D3 improves the pathology and behavior of Alzheimer's disease transgenic mice* ACS Chem. Neurosci., **1**, 639–648

10. Funke, S.A., Liu, H., Sehl, T., Bartnik, D., Brener, O., Nagel-Steger, L., Wiesehan, K. and Willbold, D. (2012) *Identification and characterization of an A $\beta$  oligomer precipitating peptide that may be useful to explore gene therapeutic approaches to Alzheimer disease* Rejuvenation Res., **15**, 144-147
11. Brener, O., Dunkelmann, T., Gremer, L., van Groen, T., Mirecka, E.A., Kadish, I., Willuweit, A., Kutzsche, J., Jürgens, D. et al (2015) *QIAD assay for quantitating a compound's efficacy in elimination of toxic A $\beta$  oligomers* Sci. Rep., **5**: 13222
12. Rudolph, S., Klein, A.N., Tusche, M., Schlosser, C., Elfgen, A., Brener, O., Teunissen, C., Gremer, L., Funke, S.A., Kutzsche, J. and Willbold, D. (2016) *Competitive mirror image phage display derived peptide modulates amyloid beta aggregation and toxicity* PLoS One, **11**: e0147470
13. Klein, A.N., Ziehm, T., Tusche, M., Buitenhuis, J., Bartnik, D., Boeddrich, A., Wiglenda T., Wanker, E., Funke, S.A. et al (2016) *Optimization of the All-D Peptide D3 for A $\beta$  Oligomer Elimination* PLoS One, **11**, e015035
14. Sehlin, D., Englund, H., Simu, B., Karlsson, M., Ingelsson, M., Nikolajeff, F., Lannfelt, L. and Pettersson, F.E. (2012) *Large aggregates are the major soluble Ab species in AD brain fractionated with density gradient ultracentrifugation* PLoS One, **7**: e32014
15. Stöhr, J., Watts, J.C., Mensinger, Z.L., Oehler, A., Grillo, S.K., DeArmond, S.J., Prusiner, S.B. and Giles, K. (2012) *Purified and synthetic Alzheimer's amyloid beta (A $\beta$ ) prions* Proc. Natl. Acad. Sci. USA, **109**, 11025-11030
16. Levy, M., Porat, Y., Macharach, E., Shalev, D.E. and Gazit, E. (2008) *Phenolsulfophthalein but not phenolphthalein inhibits amyloid fibril formation: implications for the modulation of amyloid self-assembly* Biochemistry, **47**, 5896-5904
17. Frenzel, D., Glück, J.M., Brener, O., Oesterhelt, F., Nagel-Steger, L. and Willbold, D. (2014) *Immobilization of homogeneous monomeric, oligomeric and fibrillar A $\beta$  species for reliable SPR measurements* PLoS One, **9**: e89490
18. Khlistunova, I., Biernat, J., Wang, Y., Pickhardt, M., von Bergen, M., Gazova, Z., Mandelkow, E. and Mandelkow, E-M. (2006) *Inducible expression of Tau repeat domain in cell models of tauopathy* J. Biol. Chem., **281**, 1205-1214
19. Mocanu, M-M., Nissen, A., Eckermann, K., Khlistunova, I., Biernat, J., Drexler, D., Petrova, O., Schönin, K., Bujard, H., Mandelkow, E., Zhou, L., Rune, G. and Mandelkow, E-M. (2008) *The potential for  $\beta$ -structure in the repeat domain of Tau protein determines aggregation, synaptic decay, neuronal loss and coassembly with endogenous Tau in inducible mouse models of Tauopathy* J. Neurosci., **16**, 737-748
20. Shutova, M., Yang, C., Vasiliev, J.M. and Svitkina, T. (2012) *Functions of nonmuscle myosin II in assembly of the cellular contractile system* PLoS One, **7**: e40814
21. Lee, H-J. and Lee, S-J. (2002) *Characterization of cytoplasmic  $\alpha$ -synuclein aggregates* J. Biol. Chem., **277**, 48976-48983
22. MacCormick, M., Modersheim, T., van der Salm, L.W M., Moore, A., Pryor, S.C., McCaffrey, G. and Grimes, M.L. (2005) *Distinct signaling particles containing ERK/MEK and B-Raf in PC12 cells* Biochem. J., **387**, 155-164
23. Betschinger, J., Eisenhaber, F. and Knoblich, J.A. (2005) *Phosphorylation-induced autoinhibition regulates the cytoskeletal protein lethal (2) giant larvae* Curr. Biol., **15**, 276-282
24. Majoul, I.V., Bastiaens, P.I.H. and Soling H-D (1996) *Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells* J. Cell Biol., **133**, 777-789
25. Chen, Q., Peto, C.A., Shelton, G.D., Mizisin, A., Sawchenko, P.E. and Schubert, D. (2009) *Loss of modifier of cell adhesion reveals a pathway leading to axonal degeneration* J. Neurosci., **29**, 118-130
26. Minamide, L.S., Maiti, S., Boyle, J.A., Davis, R.C., Coppinger, J.A., Bao, Y., Huang, T.Y., Yates, J., Bokoch, G.M. and Bamberg, J.R. (2010) *Isolation and characterization of cytoplasmic cofilin-actin rods* J. Biol. Chem., **285**, 5450-5460
27. Lin, W-H., Nelson, S.E., Hollingsworth, R.J. and Chung, C.Y. (2010) *Functional roles of VASP phosphorylation in the regulation of chemotaxis and osmotic stress response* Cytoskeleton, **67**, 259-27
28. Tixador, P., Herzog, L., Reine, F., Jaumain, E., Chapuis, J., Le Dur, A., Laude, H. and Béringue, V. (2010) *The physical relationship between infectivity and prion protein aggregates is strain-dependent* PLoS Pathogens, **6**: e1000859
29. Sim, V.L. and Caughey, B. (2009) *Ultrastructures and strain comparison of under-glycosylated scrapie prion fibrils* Neurobiol. Aging, **30**, 2031-2042
30. Sigurdson, C.J., Joshi-Barr, S., Bett, C., Winson, O., Manco, G., Schwarz, P., Rüllicke, T., Nilsson, P.R., Margalith, I., Raeber, A., Peretz, D., Hornemann, S., Wüthrich, K. and Aguzzi, A. (2011) *Spongiform encephalopathy in transgenic mice expressing a point mutation in the  $\beta$ 2- $\alpha$ 2 loop of the prion protein* J. Neurosci., **31**, 13840-13847
31. Laferrière, F., Tixador, P., Moudjou, M., Chapuis, J., Sibille, P., Herzog, L., Reine, F., Jaumain, E., Laude, H., Rezaei, H. and Béringue, V. (2013) *Quaternary structure of pathological prion protein as a determining factor of strain-specific prion replication dynamics* PLoS Pathog., **9**: e1003702
32. Coleman, B.M., Harrison, C.F., Guo, B., Masters, C.L., Barnham, K.J., Lawson, V.A. and Hill, A.F. (2014) *Pathogenic mutations within the hydrophobic domain of the prion protein lead to the formation of protease-sensitive prion species with increased lethality* J. Virol., **88**, 2690-2703
33. Wenborn, A., Terry, C., Gros, N., Joiner, S., D'Castro, L., Panico, S., Sells, J., Cronier, S., Linehan, J.M., Brandner, S., Saibil, H.R., Collinge, J. and Wadsworth, J.D.F. (2015) *A novel and rapid method for obtaining high titre intact prion strains from mammalian brain* Sci. Rep. **5**: 10062
34. Herrmann, U.S., Schütz, A.K., Shirani, H., Huang, D., Saban, D., Nuvolone, M., Li, B., Ballmer, B., Åslund, A.K.O. et al (2015) *Structure-based drug design identifies polythiophenes as anti-prion compounds* Sci. Transl. Med., **7**, 299ra123
35. Eugster, C., Panáková, D., Mahmoud, A. and Eaton, S. (2007) *Lipoprotein-heparan sulfate interactions in the Hh pathway* Devel. Cell, **13**, 57-71
36. Palm, W., Sampaio, J.L., Brankatschk, M., Carvalho, M., Mahmoud, A., Shevchenko, A. and Eaton, S. (2012) *Lipoproteins in Drosophila melanogaster—assembly, function, and influence on tissue lipid composition* PLoS Genet., **8**: e1002828

37. Palm, W., Swierczynska, M.M., Kumari, V., Ehrhart-Bornstein, M., Bornstein, S.R. and Eaton, S. (2013) *Secretion and signaling activities of lipoprotein-associated hedgehog and non-sterol-modified hedgehog in flies and mammals* PLoS Biol., **11**: e1001505
38. Padmanabhan, K., Robles, M.S., Westerling, T. and Weitz, C.J. (2012) *Feedback regulation of transcriptional termination by the mammalian circadian clock PERIOD complex* Science, **337**, 599-602
39. Lin, W-H., Nelson, S.E., Hollingsworth, R.J. and Chung, C.Y. (2010) *Functional roles of VASP phosphorylation in the regulation of chemotaxis and osmotic stress response* Cytoskeleton, **67**, 259–271

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