proBDNF Isoform Detection and Quantification by ELISA

The Development of proBDNF-Specific ELISA Assays – A Challenging Task

Brain-derived Neurotrophic Factor (BDNF) was identified, isolated and sequenced from pig brain in 1989. This followed many years of research seeking the cause of its biological activity in brain and nerve extracts. Sequencing demonstrated close homology to Nerve Growth Factor (NGF), the first growth factor isolated and sequenced that led to the award of the Nobel Prize to Rita Levi-Montalcini and Stanley Cohen in 1986. Like NGF, BDNF is produced as a precursor molecule (proBDNF), which is cleaved to produce the biologically active mature BDNF. Surprisingly, over the past 20 years both NGF and BDNF full-length precursor (proBDNF) molecules have been shown to have a number of biological actions that are often acting in opposition to the mature isoforms. For example, the mature forms are strong stimulators of neuronal survival and growth, whereas the full-length precursors trigger neuronal intracellular death pathways. Twenty years ago, a third isoform of BDNF was uncovered from gene sequence analysis and shown to exist in cell cultures. The 28 kD isoform is produced from cleavage within the pro-domain of full-length BDNF. However, although this truncated form of BDNF has been shown by Western blot to exist in disease-free human blood, to date no biological function has been identified for this isoform.

BDNF and proBDNF remain the most highly studied neurotrophic factors with thousands of new publications appearing each year. In addition to the study of biological activities, various immunological assays are popular including western blot, immunohistochemistry and ELISA assays.

Despite this intense scrutiny, results are often confounded by assay specificity and accuracy. Assays and antibodies that can distinguish between the separate isoforms are therefore essential when making conclusions about function from the various immunoassays commonly used. Importantly, validation experiments are essential to determine whether an ELISA assay is ‘fit-for-purpose’ for a particular sample matrix such as human serum. Validation will ensure that matrix interference and false-positive or false-negative readings are minimized, and that protein concentrations are estimated accurately.

Validation Experiments to Assess Accurate proBDNF Detection in Human Serum

The complex nature of BDNF biology necessitates the development of specific, accurate and reproducible ELISA assays to reliably quantify proBDNF in fluid samples such as human serum. However, not all ELISA assays are created equal. For instance, Biosensis has addressed specific detection of proBDNF (as opposed to the mature BDNF isoform) by matching an ELISA antibody pair that provides specific capturing of the full-length proBDNF isoform via the pro-domain, and detection via the mature domain (Figure 1).

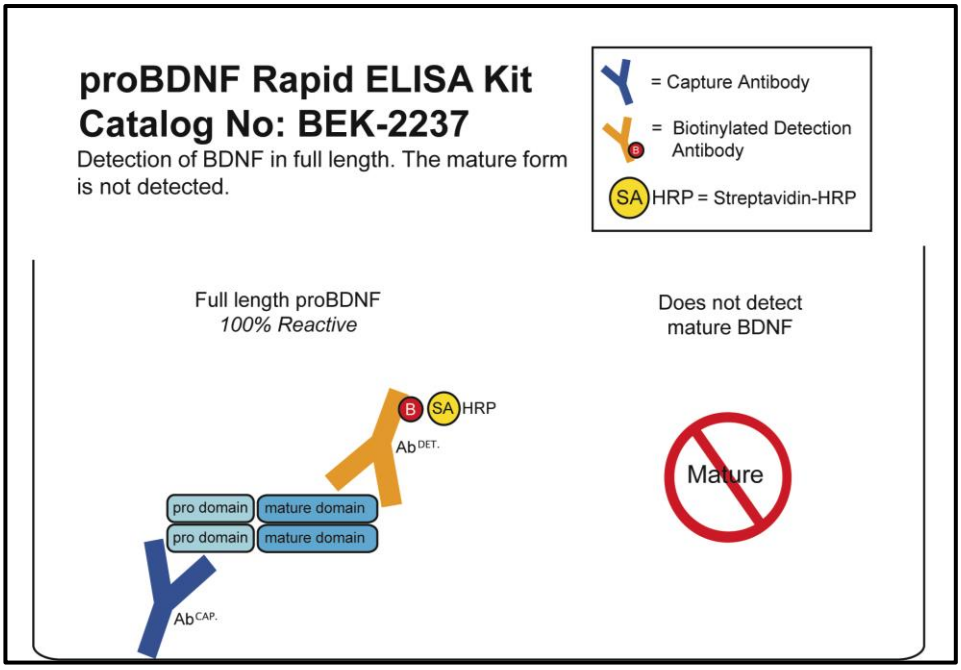

Figure 1: Assay Setup for Biosensis' proBDNF *Rapid*™ ELISA Assay Kit
This assay has been developed to reduce mature BDNF interference by choosing an appropriate capture antibody, which binds the pro-domain of full-length proBDNF. The detection with an anti-mature domain BDNF antibody ensures detection and quantification of the full-length protein.

ELISA assay development does not stop at finding a matched antibody pair. Validation experiments are essential to "fine-tune" an assay's suitability to accurately quantify the target of interest. Optimization work can include, but is not limited to:

- Optimization of sample diluent composition to allow accurate quantification at the lowest possible minimum required dilution (MRD) of the biological sample
- Evaluation of immunoblockers to reduce the likelihood of false-positive readings

The most important tool to assess accuracy is to perform parallelism experiments with biological samples over a range of sample dilutions. Parallelism between diluted reference standard and diluted samples confirms the absence of matrix interference and the suitability of the chosen reference standard and assay antibodies for a particular sample matrix. Essentially, this demonstrates an assay being "fit-for-purpose". Please refer to our Technical Note #1 for further information on ELISA validation experiments.

The Biosensis proBDNF *Rapid*™ ELISA Kit (BEK-2237) has been validated to allow accurate quantification of proBDNF in human serum. In this Technical Note, we will be comparing assay performance with a competitor assay to demonstrate the pitfalls of running ELISA assays in general, but more specifically the challenges associated with proBDNF quantification. Table 1 summarizes and compares ELISA kit parameters.

Table 1: ELISA Kit Specifications

Parameter	Biosensis	Competitor
Assay Duration	3 hours	5 hours
Range	15.6 – 1,000 pg/mL	391 – 25,000 pg/mL
Sensitivity	6 pg/mL	100 pg/mL
Calibrator Protein	Human proBDNF, CHO-derived (cleavage-resistant)	Human proBDNF, HEK293-derived (wt)
Intra-Assay Precision	< 7 %	4-6 %
Inter-Assay Precision	< 7 %	8-10 %
Recommended MRD* for human serum	1:10 or higher	1:2 or higher
Kit Storage	12 months at 2-8°C	1 month at 2-8°C

*MRD: Minimum required dilution; wt: wild-type

Quantification of proBDNF in Human Serum – An ELISA Kit Comparison

In the first experiment, four human serum samples were diluted 1:10 and 1:20 and run in the Biosensis proBDNF ELISA kit (BEK-2237). Protein reference standards from the Biosensis and Competitor ELISA kits were run in parallel in the Biosensis assay to evaluate differences in reference protein recognition and the effect on proBDNF quantification (Figure 2 and Table 2).

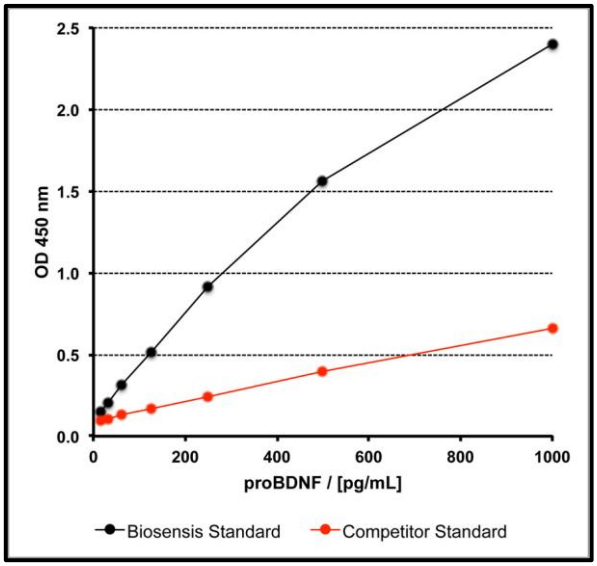

Figure 2: Reference Standard Curves in the Biosensis ELISA
Biosensis and Competitor proBDNF proteins were compared in the Biosensis proBDNF ELISA assay (BEK-2237). The assay antibodies show better recognition of the Biosensis proBDNF protein.

The Biosensis kit showed better recognition of the Biosensis proBDNF protein, producing a more sensitive calibration curve. Differences in reference protein binding directly affected proBDNF quantification (Table 2). When quantified against the Competitor proBDNF protein, the assay showed an approximately 6-fold higher proBDNF "concentration" as compared to the Biosensis proBDNF protein.

Table 2: Serum proBDNF levels assayed against two reference proteins in the Biosensis proBDNF ELISA (BEK-2237).

Sample #	proBDNF (pg/mL) Quantification Against Reference Protein from		Ratio proBDNF Concentration
	Biosensis	Competitor	
1	450.7	2556.6	5.7
2	2079.3	12181.4	5.9
3	2786.4	16588.3	6.0
4	334.7	1943.3	5.8

While these initial results confirmed the common "semi-quantitative nature" of ELISA assays and the dependency of target concentrations on assay antibodies and reference protein source, the next set of experiments were designed to evaluate each kit's ability to accurately quantify proBDNF in human serum within each kit's assay parameters. To assess accuracy, parallelism experiments were performed with 4 sample dilutions. An inter-dilution CV for each sample was calculated, with an industry-wide accepted CV of less than 20% demonstrating accurate quantification.

Calibration curves with recombinant proBDNF proteins were generated with each individual ELISA assay kit (Figure 3). Both kits produced standard curves at considerably different calibration ranges in agreement with stated kit performance (Table 1).

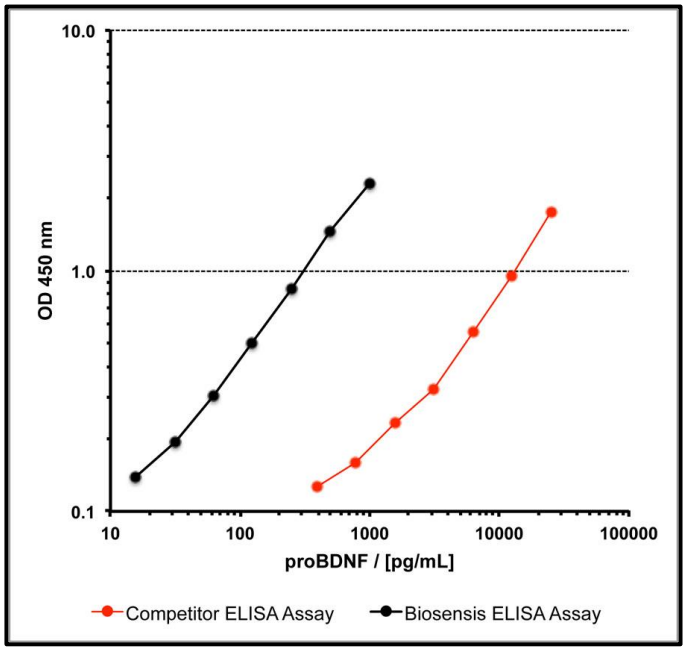

Figure 3: proBDNF Calibration Curves for Each Individual ELISA Kit
Standard curves were generated using the individual kit proteins. Both kits produced calibration curves at different calibration ranges (Biosensis: 15.6 – 1,000 pg/mL; Competitor: 391 – 25,000 pg/mL).

Calibration curves were then used to quantify proBDNF in 10 human serum samples for both ELISA assays (Table 3). Data analysis was performed applying the following methodology:

- Sample dilutions with concentrations higher than the highest standard were disregarded
- Sample dilutions with concentrations lower than the lowest standard, but higher than the stated LOD, were disregarded and labelled as "< LOQ"
- Sample dilutions with concentrations below the stated LOD were disregarded and labelled as "< LOD"
- To calculate the average proBDNF concentration and the inter-dilution CV% for each sample, only sample dilutions were considered were the concentration was within 80 – 120% of the previous dilution

proBDNF concentrations could be measured in 7 out of 10 serum samples (70 %) with the Biosensis kit (Table 3). Concentrations varied between ~0.3 – 2.0 ng/mL, while proBDNF was undetectable (< LOD) in 3 serum samples. Importantly, interpolated concentrations were accurate as determined by the percentage of proBDNF concentration in relation to the previous dilution (80 – 120 %), and by the inter-dilution CV%, which ranged from 1.6 % to 17.7 % (target: < 20 %). Graphically, this resulted in parallel curves between the reference standard and sample dilutions (Figure 4).

When quantifying proBDNF in these 7 samples, at least 2 sample dilutions were usable to determine proBDNF concentrations, with more than half of these samples allowing 3-4 samples dilutions, thus providing more confidence in data accuracy.

In contrast, the Competitor proBDNF ELISA produced accurate proBDNF concentration for Sample 10 only. While samples that had undetectable levels of proBDNF, as determined with the Biosensis kit, also showed undetectable levels with the Competitor kit, Serum 8 was an exception. The Biosensis kit accurately determined proBDNF in this sample, while the Competitor kit was not able to detect it.

At least 50% of assayed samples showed non-linear/non-parallel behaviour in the Competitor assay when diluted across the suggested dilution range of 1:2 or higher. In one example, Sample 5 read 1023 pg/mL proBDNF at a sample dilution of 1:2. If this concentration was accurate, the 1:4 dilution would give a quantifiable concentration between 409 – 614 pg/mL, yet, the observed concentration was "< LOD". In another example, proBDNF quantified at 1:2 dilution of Serum 12 gave a concentration of 1788 pg/mL, while the subsequent 1:4 dilution read 197 pg/mL. Taken together, assay results demonstrated that the Biosensis kit is superior to the Competitor assay in determining proBDNF concentration in human serum.

Table 3: Serum proBDNF levels assayed in Biosensis and Competitor proBDNF ELISA kits.

	Biosensis ELISA							Competitor ELISA						
Sample #	**OD450**	**DF**	**pg/mL**	**pg/mL (DF corr)**	**Mean (pg/mL)**	**% of prev dil**	**CV%**	**OD450**	**DF**	**pg/mL**	**pg/mL (DF corr)**	**Mean (pg/mL)**	**% of prev dil**	**CV%**
5	0.347	5	63.8	319.1	**298.2**	100	**10.0**	0.183	2	1023.0	2045.9	**2045.9**	100	-
	0.204	10	27.7	277.2		87		0.094	4	< LOD	< LOD		-	
	0.146	20	< LOQ	< LOQ		-		0.098	8	< LOD	< LOD		-	
	0.117	40	< LOQ	< LOQ		-		0.108	16	< LOD	< LOD		-	
6	0.106	5	< LOD	< LOD	**<LOD**	-	-	0.091	2	< LOD	< LOD	**< LOD**	-	-
	0.093	10	< LOD	< LOD		-		0.095	4	< LOD	< LOD		-	
	0.087	20	< LOD	< LOD		-		0.098	8	< LOD	< LOD		-	
	0.086	40	< LOD	< LOD		-		0.105	16	< LOD	< LOD		-	
7	0.094	5	< LOD	< LOD	**<LOD**	-	-	0.090	2	< LOD	< LOD	**< LOD**	-	-
	0.085	10	< LOD	< LOD		-		0.102	4	< LOD	< LOD		-	
	0.083	20	< LOD	< LOD		-		0.103	8	< LOD	< LOD		-	
	0.083	40	< LOD	< LOD		-		0.104	16	< LOD	< LOD		-	
8	1.607	5	445.3	2226.3	**1406.9**	-	**17.7**	0.101	2	< LOD	< LOD	**< LOD**	-	-
	0.729	10	166.1	1661.4		100		0.100	4	< LOD	< LOD		-	
	0.370	20	69.7	1394.1		84		0.112	8	< LOD	< LOD		-	
	0.209	40	29.1	1165.3		84		0.115	16	< LOD	< LOD		-	
9	1.677	5	470.7	2353.6	**1940.4**	100	**15.2**	3.715	2	61133.2	122266.4	**28711.2**	-	**101.9**
	0.827	10	194.2	1942.3		83		1.982	4	29050.1	116200.2		-	
	0.443	20	88.6	1771.4		91		0.541	8	6175.1	49400.7		100	
	0.262	40	42.4	1694.4		96		0.146	16	501.4	8021.7		16	
10	0.844	5	199.1	995.4	**700.0**	-	**3.2**	>4.000	2	-	-	**8215.4**	-	**3.7**
	0.377	10	71.6	715.7		100		1.146	4	15366.3	61465.2		-	
	0.230	20	34.2	684.3		96		0.185	8	1054.0	8431.7		100	
	0.133	40	< LOQ	< LOQ		-		0.146	16	500.0	7999.2		95	

11	0.088	10	< LOD	< LOD	**< LOD**	-	-	0.098	2	< LOD	< LOD	**< LOD**	-	-
	0.084	20	< LOD	< LOD		-		0.096	4	< LOD	< LOD		-	
	0.088	40	< LOD	< LOD		-		0.103	8	< LOD	< LOD		-	
	0.090	80	< LOD	< LOD		-		-	-	-	-		-	
12	0.879	10	258.8	2588.3	**1384.1**	-	**8.4**	0.237	2	1788.4	3576.7	**789.3**	-	-
	0.335	20	76.0	1519.1		100		0.124	4	197.3	789.3		100	
	0.198	40	33.0	1318.4		87		0.113	8	< LOQ	< LOQ		-	
	0.146	80	16.4	1314.9		100		0.114	16	< LOQ	< LOQ		-	
13	1.607	10	570.1	5700.6	**1999.5**	-	**2.7**	>4.000	2	N/A	N/A	**111073.7**	-	**77.3**
	0.548	20	144.6	2891.8		-		3.669	4	60232.0	240928.0		-	
	0.256	40	50.9	2037.3		100		1.528	8	21476.7	171813.9		100	
	0.171	80	24.5	1961.6		96		0.333	16	3145.8	50333.4		29	
14	0.962	10	289.4	2893.9	**2126.6**	-	**1.6**	0.150	2	553.3	1106.5	**1106.5**	100	-
	0.424	20	104.3	2086.9		100		0.098	4	< LOQ	< LOQ		-	
	0.265	40	53.8	2150.3		103		0.097	8	< LOQ	< LOQ		-	
	0.179	80	26.8	2142.5		100		0.102	16	< LOQ	< LOQ		-	

DF: Dilution Factor; LOD: Limit of Detection; LOQ: Limit of Quantification.

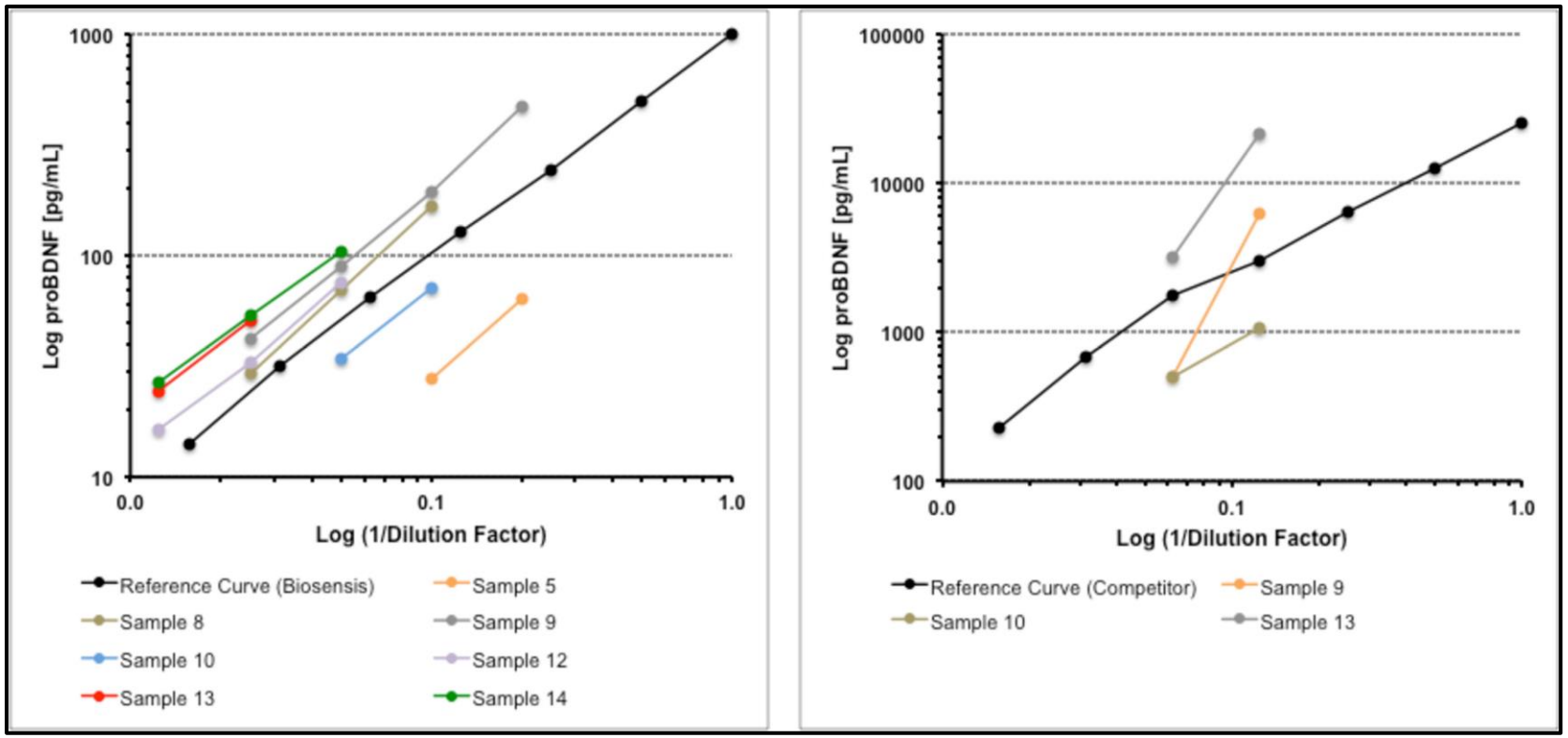

Figure 4: Graphical Presentation of Serum Parallelism

Parallelism graphs were produced for both ELISA kits (left: Biosensis; right: Competitor). Accurate quantification with the Biosensis kit was achieved for 7 out of 10 samples, while proBDNF was undetectable (< LOD) in the remaining 3 samples. In contrast, the Competitor kit showed poor performance when assaying human serum samples. Only Sample 10 appeared to produce an accurate proBDNF concentration as judged by 2 usable sample dilutions. Only another 2 samples (9 and 13) allowed plotting 2 concentration points, however, absence of parallelism clearly showed that the proBDNF concentration is inaccurate.

Conclusions

Assaying proBDNF in human serum is indispensable to delineate specific roles of various BDNF isoforms in (patho-)physiological conditions. This study has highlighted the need of high-quality ELISA assays and assay optimization for particular sample matrixes to ensure accurate quantification. Without thorough validation using biological samples, the applicability of an ELISA for a particular sample type cannot be established.

The Biosensis proBDNF *Rapid*™ ELISA (BEK-2237) detected proBDNF in 7 out of 10 serum samples and was able to accurately quantify proBDNF content. Most samples produced accurate proBDNF concentrations at a dilution factor of 1:10, while some samples allowed a 1:5 sample dilution, and others required a minimum dilution of 1:20. This highlights the importance of running several dilutions when assaying unknown samples, particularly because proBDNF concentrations appear to vary quite considerably, which is in contrast to mature BDNF that is released from platelets at typical serum concentrations of 20 – 40 ng/mL. It remains unknown whether undetectable levels of proBDNF in some serum samples are a real physiological phenomenon, are an artefact of sample preparation, or are due to insufficient assay sensitivity. Further research in the proBDNF field will likely provide an answer in the future.

In contrast to the superior Biosensis kit, assay results clearly demonstrated that the Competitor kit – despite its stated use for human serum – is not 'fit-for-purpose' and that human serum cannot be accurately measured for proBDNF content in the recommended dilution range. The occurrence of very high OD readings at low dilutions (eg., Samples 9 and 10) suggest instead that this assay is prone to false-positive readings, and any serum sample needs to be assayed at a minimum of 3 sample dilutions to detect false-positives, and to determine accuracy via parallelism experiments.

Importantly, the Biosensis assay produced higher sample OD readings at similar sample dilutions in comparison with the Competitor assay. This finding implicates that the Biosensis assay antibodies detect natural proBDNF with higher sensitivity, thus challenging the concept of sensitivity, which traditionally only refers to assay concentrations, expressed as Limit of Detection (LOD). This highlights the importance of choosing assay antibodies that not only detect recombinant protein standards, but also natural proBDNF with sufficient sensitivity in biological samples.

In reference to ELISA assays as an immunological technique for quantifying protein targets, this study has also emphasized the general semi-quantitative nature of ELISA assays. The first experiment clearly demonstrated that different protein standards run in one assay can produce target protein levels in samples that are considerably different, here up to 6-fold in case of proBDNF. It remains unknown whether this is due to the proBDNF protein itself, or the characteristics of the assay antibodies, but this observation triggers the question about real proBDNF concentrations in human serum. Complementary techniques, such as mass spectrometry, will need to be applied to estimate absolute proBDNF levels. In absence of such data, scientific literature will need to be evaluated with caution regarding absolute and accurate proBDNF concentrations in human serum, or any other biological matrix as such.

About Biosensis' proBDNF *Rapid*™ ELISA Kit

Founders of Biosensis created in 2002/03 the first commercial ELISA BDNF kit with Chemicon International, one of the earliest, and world leader in antibody commercialisation and innovation. These same founders re-built that first BDNF kit into the Biosensis *Rapid*™ Mature BDNF ELISA, launched in 2014 (BEK-2211). The kit is unique in being assessed to ensure low cross-reactivity against the proBDNF isoform. This allowed Biosensis researchers to develop a complementary ELISA kit to specifically quantify the proBDNF isoform independently from mature BDNF (proBDNF *Rapid*™ ELISA Kit, BEK-2237). Researchers now use these two complementary kits to achieve independent analysis of mature BDNF and proBDNF in the same sample. Biosensis continues to develop these well-established assays further to achieve higher sensitivity and better accuracy, in an effort to address urgent needs of the research community.