Mature BDNF Isoform Detection and Quantification by ELISA

BDNF Isoforms – A Challenge for Specific and Accurate Detection and Quantification

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, 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. Assays and antibodies that can distinguish between the separate isoforms are therefore essential when making conclusions about function from the various immunoassays commonly used. The choice of immunological technique, and appropriate validation experiments are crucial to determine which BDNF isoform(s) a particular method can detect and quantify.

Validation of BDNF Isoform Detection in ELISA Assay – Why the Choice of Immunological Technique Matters

Validation of an antibody for a particular immunological technique is essential to demonstrate whether it is fit-for-purpose. Taking a BDNF antibody raised against the mature BDNF domain and Flow Cytometry as an example, this method is only able to show BDNF-immunoreactivity (BDNF-IR). However, whether the antibody detects the full-length (proBDNF) and/or mature and truncated isoforms cannot easily be answered using this validation approach. As for BDNF isoform detection, Western Blotting is useful to identify a BDNF antibody's ability to detect proBDNF, mature BDNF, truncated BDNF and potentially other BDNF isoforms due to known molecular weights. Dot blot is another technique that can be used to show an antibody's ability to bind BDNF isoforms if appropriate protein standards are available.

While the aforementioned methods rely on one single primary antibody, antibody validation becomes more complex when choosing an antibody pair for ELISA assays. Protein conformation, epitope localization, among other factors, may affect which BDNF isoforms are detected with a given pair of BDNF antibodies. Thus, careful evaluation of antibodies and validation by ELISA assay is required to determine an ELISA assay's ability to detect specific BDNF isoforms. Figure 1 (adapted from Polacchini *et. al.*, 2015) exemplifies the necessity to choose the correct validation method to determine BDNF isoform detection.

In their 2015 Scientific Reports publication, Polacchini and colleagues compared 5 different commercially available ELISA assays for BDNF quantification in human serum. Their analysis by Line Blot (a technique similar to dot blot), using the ELISA kit's detection antibodies, suggested that the Biosensis Mature BDNF *Rapid*™ ELISA kit (cat# BEK-2211) detects 'total' BDNF (proBDNF and mature BDNF isoforms). The authors assessed antibody immunoreactivity (IR) towards mature and full-length BDNF proteins (Figure 1). Figure 1A demonstrates that proBDNF protein (10 pg loading) yields slight IR for the Biosensis antibody, while proBDNF-IR for the Aviscera antibody appears lower. However, the relative IR for mature/proBDNF binding for each antibody was not assessed. This is important, as different antibody concentrations may result in different levels of IR. With a 'qualitative eye', the ratio of mature BDNF over proBDNF detection appears very similar for both antibodies, with overall stronger IR for the Biosensis antibody. Figure 1B, which represents an overexposed image of mature BDNF/proBDNF-IR confirms this (alternate) interpretation of the researcher's data.

In Figure 1C, Polacchini *et al.* (2015) confirm that the Biosensis ELISA assay detection antibody reacts with proBDNF protein by blotting increased amounts of proBDNF protein (~64 kDa), and equal amounts (not moles!) of mature BDNF (~28 kDa). Of note, the same analysis has not been performed for the Aviscera antibody. Yet, the data presented in Figure 1 mislead the researchers to believe that the Biosensis Mature BDNF ELISA assay quantifies total BDNF, a conclusion that has been carried forward to their 2019 publication (Sartori *et al.*). Their finding contradicts our results of in-house validation, presented in our kit protocol, which shows preferential quantification of mature BDNF isoform in the Mature BDNF *Rapid*™ ELISA. Instead of dot/line blot analysis, we used the ELISA

assay to assess proBDNF cross-reactivity and interference of proBDNF with mature BDNF quantification, by spiking proBDNF protein into assay buffer and human serum samples.

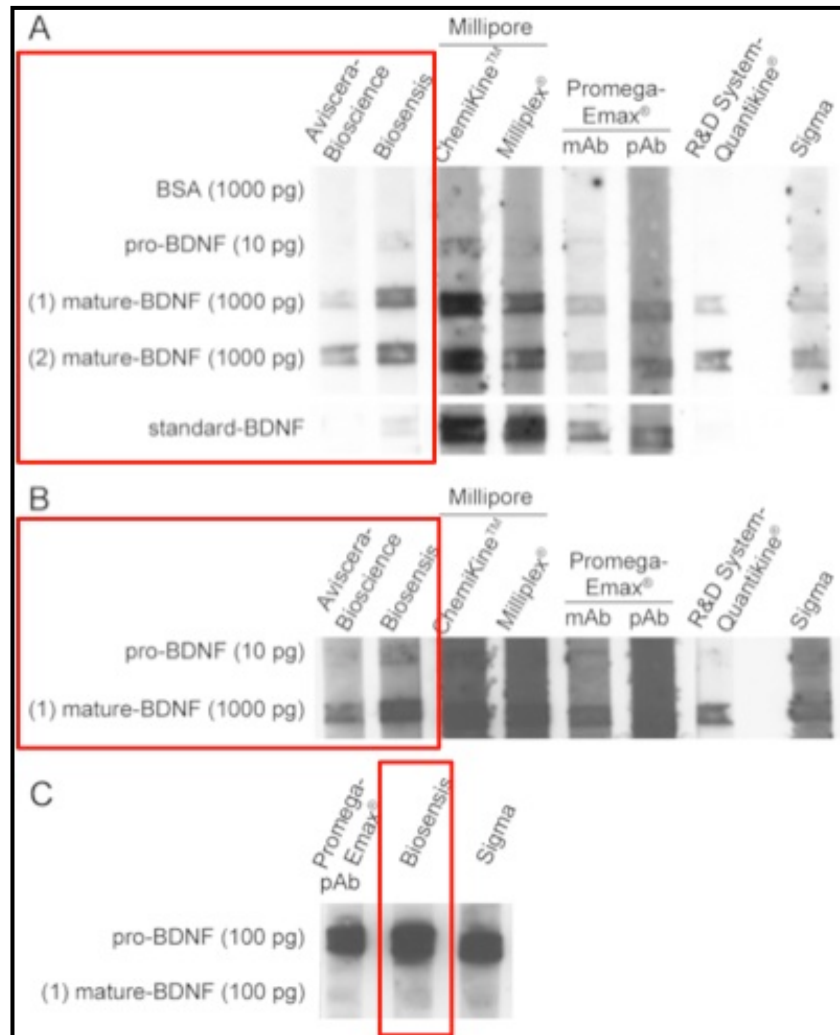

Figure 1: Line-blot for qualitative analysis of anti-BDNF antibodies specificity.
(A) The antibodies from each ELISA kit were tested for specificity against pro-BDNF or mature BDNF. The BDNF standards blotted were commercial pro-BDNF (Alomone; 10 pg/lane), mature BDNF (1 and 2 from Alomone and Sigma, respectively; both 1000 pg/lane) and the standard BDNF protein included in each kit (Aviscera-Bioscience and Biosensis: 10 pg/lane; Millipore-ChemiKine™, Millipore-Milliplex®- and R&D System-Quantikine®: 100 pg/lane; Promega-Emax®: 1000 pg/lane). BSA (1000 pg/lane) was used as a negative control. The mouse monoclonal anti-BDNF antibody, (1:1000; Sigma) was tested as a control. (**B**) Central region of the same blot shown in A, from an overexposed film to better visualize the reactivity against pro-BDNF. (**C**) Reactivity of antibodies from Biosensis, Promega-Emax® pAb and Sigma on a dot blot in which the same quantity of pro-BNDF and mature BDNF were spotted (100 pg each). Each antibody from the ELISA kits was used at the dilution suggested by the manufacturer's instructions. mAb: Promega-Emax® monoclonal capture antibody for plate coating. pAb: Promega-Emax® polyclonal detection antibody. Figure adapted from Polacchini A *et al.*, 2015.

In light of both publications, we therefore re-assessed performance of our mature BDNF assay kit (BEK-2211), and compared it under identical conditions with Aviscera's Human BDNF ELISA Kit (cat# SK00752-01).

Preferential Quantification of Mature BDNF in Human Serum With Biosensis' Mature BDNF *Rapid*™ ELISA

Human serum samples were assayed and additional validation experiments with proBDNF proteins were performed, comparing Aviscera's Human BDNF ELISA Kit (cat# SK00752-01), claimed to be mature BDNF-specific, and Biosensis' Mature BDNF *Rapid*™ ELISA Kit (cat# BEK-2211), claimed to measure total BDNF (Polacchini *et al.*, 2015; Sartori *et al.*, 2019). Assay procedure and sample preparation were followed as outlined by the manufacturer's protocols. Table 1 summarizes assay performance parameters as given in the kit manual.

Table 1: ELISA Kit Specifications

Parameter	Biosensis	Aviscera
Assay Duration	1 h 45 min	5 hours
Range	7.8– 500 pg/mL	23 – 1,500 pg/mL
Sensitivity	< 2 pg/mL	5 – 8 pg/mL
Calibrator Protein	Mature BDNF, E.coli (WHO reference material)	Mature BDNF, CHO-derived
Intra-Assay Precision	2 – 7%	4 – 6%
Inter-Assay Precision	1 – 9%	8 – 10%
Recommended MRD* for human serum	1:50 or higher	1:40 or higher
Kit Storage	12 months at 2-8C	1 month at 2-8C

*MRD: Minimum required dilution

Serum samples were tested in the Biosensis ELISA kit at dilutions between 1:40 and 1:400, and between 1:40 and 1:160 in the Aviscera assay. Two to 4 dilutions per serum sample were prepared and assayed in duplicate wells. Accuracy of quantification was assessed by calculating % recovery of BDNF concentration at subsequent dilution, in relation to previous dilution (acceptable range: 80 – 120%). Additionally, mature BDNF calibrator proteins were compared in each assay, five different recombinant proBDNF proteins spiked into assay buffer were tested for cross-reactivity, and interference of mature BDNF quantification in human serum was assessed by spiking proBDNF protein at 3 concentrations into human serum.

Differences in assay performance were observed when comparing reactivity of mature BDNF calibrator proteins in each assay (Figure 2). Using Biosensis' ELISA kit (Figure 2, Left), both Biosensis BDNF protein and Aviscera BDNF protein reacted comparably. In Aviscera's assay (Figure 2, Right), assay antibodies reacted more strongly with Aviscera's protein as compared to the Biosensis calibrator protein.

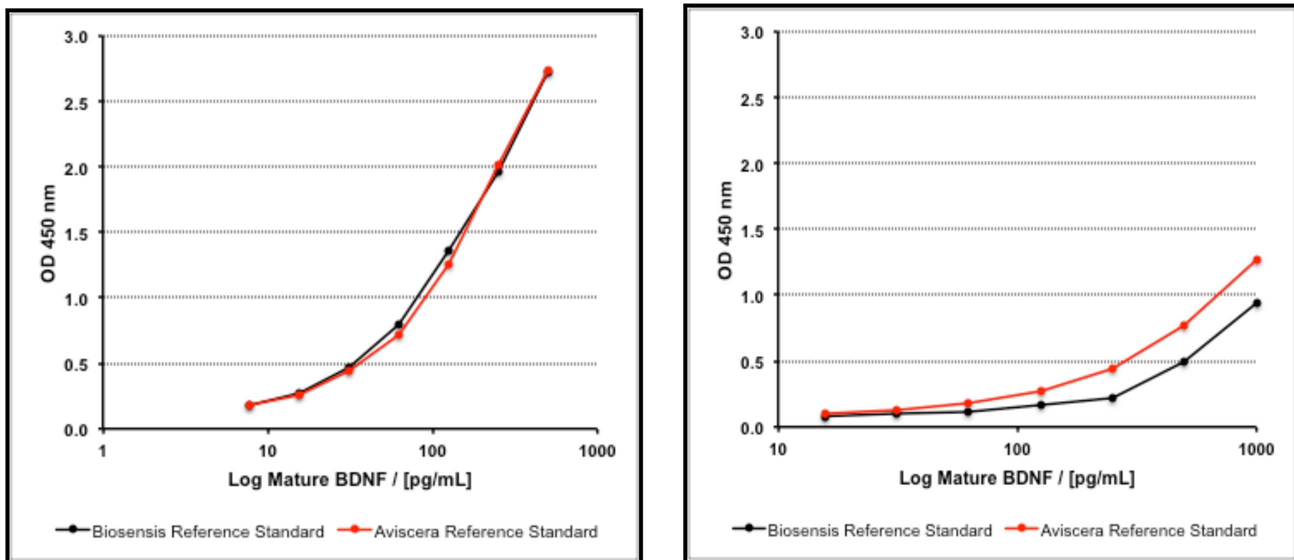

Figure 2: Comparison of ELISA kit reference calibrator proteins in Biosensis ELISA (left) and Aviscera ELISA (right). Reactivity of reference standards is comparable with Biosensis assay antibodies, while Aviscera assay antibodies react more strongly with Aviscera mature BDNF protein.

Serum samples were then assayed at minimum of 2 dilutions in duplicate wells with both kits, and quantified against the reference standard provided in each kit. Dilution-corrected BDNF concentrations were within 80-120% of previous dilution for all samples and both assays, demonstrating accurate BDNF quantification (data not shown). Overall, BDNF concentrations in human serum assayed with both kits agreed well (Figure 3). BDNF content quantified with both kits were within +/- 10% for 8 out of 9 serum samples.

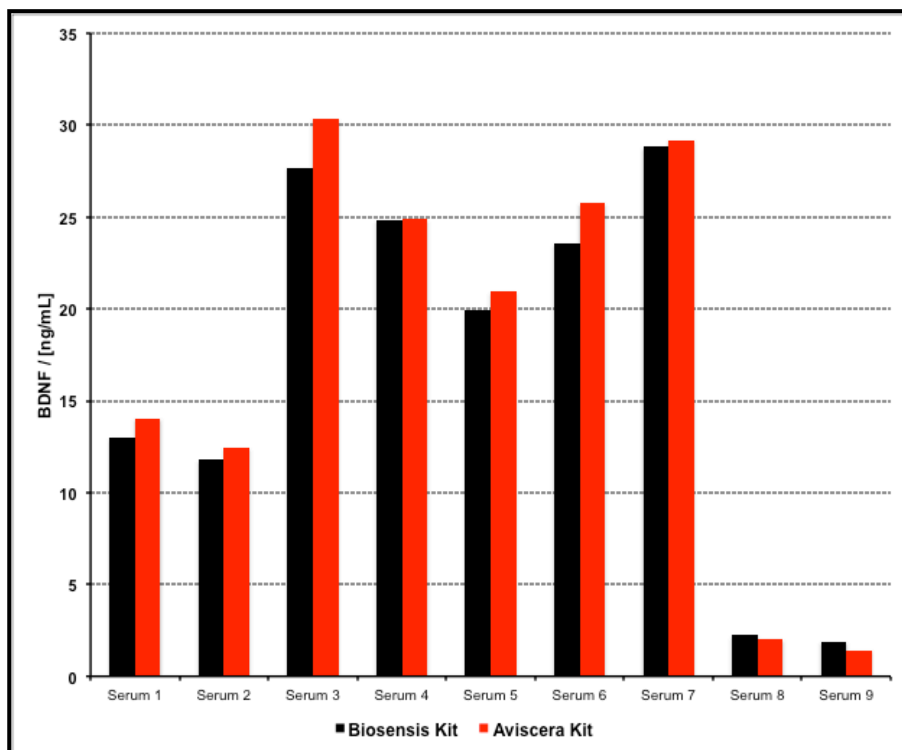

Figure 3: Quantification of human serum BDNF with Biosensis (black) and Aviscera (red) kits. Assayed values agree well between both kits.

In the next experiment, 5 different proBDNF proteins from 4 suppliers (Table 2) were spiked into each kit's sample diluent buffer at 3-5 ng/mL (6.9-11.4 pmol/L) and quantified against mature BDNF in each ELISA assay. Due to the difference in molecular weight of the two isoforms (mature BDNF: ~28 kDa; proBDNF: ~64 kDa), molar %-cross-reactivity was calculated (Table 2 and Figure 4).

All 5 tested proBDNF proteins demonstrated cross-reactivity with both kit's assay antibodies, however, levels varied from 0.42 – 22.0% for the Biosensis kit (average: 8.62%), and 2.12 – 78.2% for the Aviscera kit (average: 39.2%). Overall, Aviscera's BDNF ELISA showed a ~4-fold higher cross-reactivity with proBDNF protein, demonstrating that, while none of the ELISA is truly 100% mature BDNF specific, the Biosensis kit shows a clear preference for mature BDNF protein over full-length proBDNF. Of note, the observed low cross-reactivity of Aviscera's HEK-derived proBDNF protein agrees with data presented in their kit manual. However, results presented here demonstrate that the use of HEK-derived proBDNF may not reflect true proBDNF cross-reactivity in their BDNF ELISA assay.

Table 2: Molar % Cross-Reactivity of proBDNF Proteins in Mature BDNF ELISA Assays.

Protein	Expression System	Supplier	% Cross-Reactivity (molar)	
			Biosensis	Aviscera
proBDNF-mut	Mammalian (CHO)	Biosensis	22.0	61.7
proBDNF-?	Mammalian (HEK293)	Aviscera	0.42	2.12
proBDNF-mut	Bacterial (E.coli)	Alomone Labs	6.22	24.6
proBDNF-wt	Bacterial (E.coli)	Alomone Labs	8.23	29.3
proBDNF-wt	Mammalian (CHO)	R&D Systems	6.23	78.2
		Average	**8.62**	**39.2**

mut: mutated/cleavage-resistant proBDNF protein; wt: wild-type

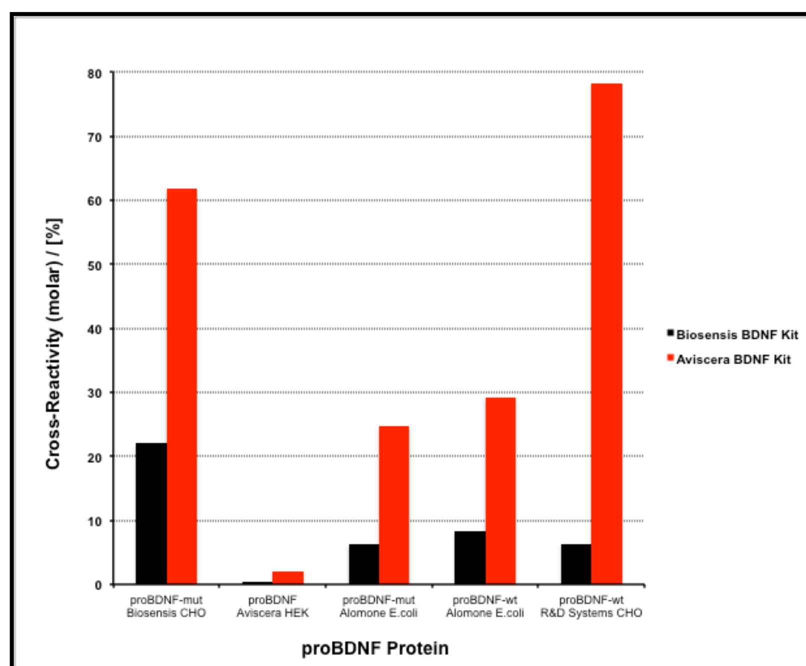

Figure 4: Molar % cross-reactivity of 5 proBDNF proteins in BDNF ELISA assays. Large differences exist among these proteins in their reactivity with BDNF assay antibodies, with Aviscera's assay antibodies showing stronger reactivity towards proBDNF.

In the next step, interference of proBDNF in mature BDNF quantification was assessed by spiking proBDNF into a typical human serum sample (~ 30 ng/mL) at typical sample dilution (1:100). proBDNF was spiked into serum at concentration levels of 20 pg/mL, 50 pg/mL, and 100 pg/mL, corresponding to 2 ng/mL, 5 ng/mL and 10 ng/mL proBDNF in serum after correction for dilution. Mutated proBDNF (Biosensis, CHO-derived) was used as model protein as it showed the highest cross-reactivity potential in the Biosensis kit, and also strong reactivity in the Aviscera BDNF kit. For each assay, 3 independent dilutions were prepared for each unspiked control and spiked serum sample, and assayed in duplicate wells. A significant increase of mean BDNF concentrations upon spiking of proBDNF was assessed with Student's t-test (unpaired, one-tailed, unequal variance, $\alpha = 0.05$).

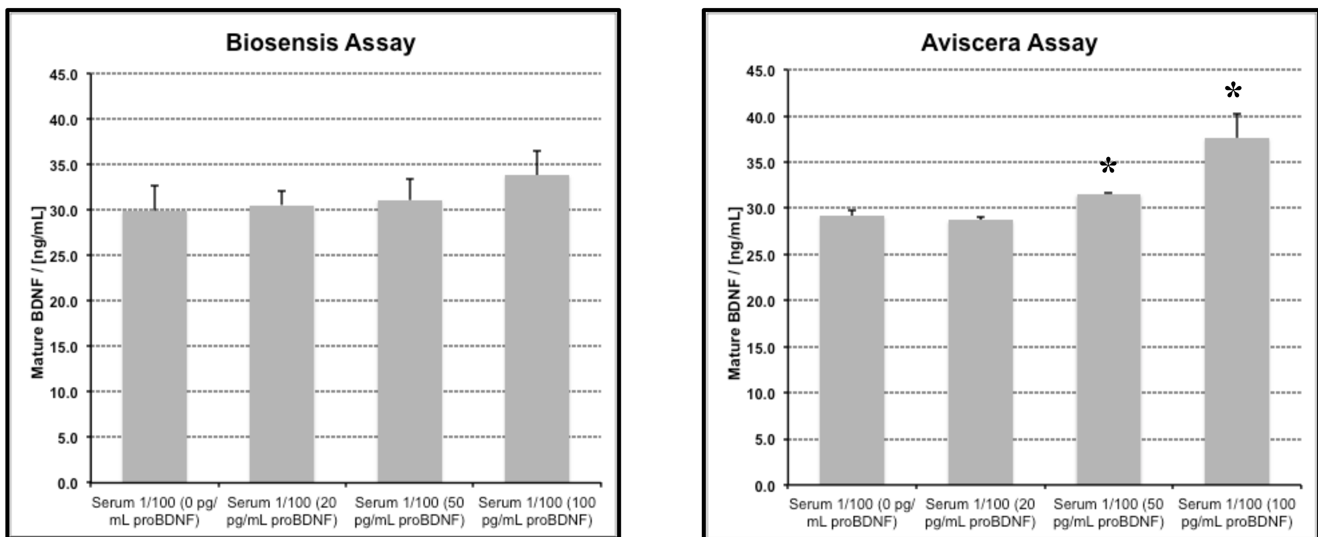

Figure 4: Interference of proBDNF spiked into human serum with mature BDNF quantification. Left: Biosensis Mature BDNF ELISA Kit. Right: Aviscera BDNF ELISA Kit. Significant differences between mature BDNF values of 3 independently prepared dilutions for each assay of unspiked control and spiked samples were assessed with Student's t-test (unpaired, one-tailed, unequal variance). *$P<0.05$.

The Biosensis Mature BDNF ELISA assay showed a minor trend towards higher BDNF concentrations with increase in proBDNF spike level (Figure 4, left). However, even at highest spike of 100 pg/mL proBDNF (equal to 10 ng/mL proBDNF in undiluted serum) no significant increase in BDNF concentrations was observed ($P = 0.176$). In contrast, proBDNF spiked into human serum caused a significant increase in mature BDNF concentrations at 50 pg/mL ($P = 0.007$) and 100 pg/mL ($P = 0.012$) spike level in the Aviscera assay (Figure 4, right).

In conclusion, quantification of BDNF with Biosensis and Aviscera ELISA kits resulted in serum BDNF concentrations in close agreement (within 10% for 8 out of 9 samples). Interestingly, Biosensis' assay antibodies reacted equally with both protein standards, while differences were observed in the Aviscera assay. This observation correlates to results shown in Figure 1A above (Polacchini *et al.*, 2015), which shows equal reactivity of the Biosensis detection antibody towards mature BDNF protein from 2 different sources in line blots. This might add to the robustness of the Biosensis assay, as it is less prone to differences in mature BDNF protein structure.

Experiments with spiked proBDNF protein from 5 different sources into assay buffer and human serum revealed clear differences in BDNF assay performance. Across all conditions tested, the Biosensis Mature BDNF ELISA showed less susceptibility to proBDNF interference as compared to the Aviscera BDNF assay. Neither assay showed 100% cross-reactivity with any proBDNF protein, making neither kit suitable to measure total BDNF. However, the Biosensis BDNF ELISA demonstrated clear preferential quantification of the mature BDNF isoform over proBDNF at typical serum sample dilution, with minimal proBDNF interference, thus ensuring accurate mature BDNF quantification. This clear result is not mitigated by the fact that proBDNF interference observed at such high spike levels may not reflect "real-world" conditions in blood, since actual proBDNF concentrations in serum are unknown, and that it remains to be determined which of the proBDNF proteins tested resembles blood proBDNF most closely. It is also worthwhile mentioning that the reactivity of the third BDNF isoform, 28 kDa truncated BDNF, remains unclear at this stage. Importantly, detection of truncated BDNF in the Biosensis Mature BDNF ELISA cannot be assumed purely on the choice of assay antibodies, which are known to react with the mature domain of BDNF protein.

It becomes clear, therefore, that other immunological techniques such as dot/line blots cannot be used to determine BDNF isoform cross-reactivity in ELISA assays as published by Polacchini *et al.* (2015), and referenced in Sartori *et al.* (2019). BDNF isoform cross-reactivity in an ELISA assay, such as the Biosensis Mature BDNF *Rapid*™ ELISA (BEK-2211) must be determined in the ELISA assay, designing the most suitable experiments to assess interference of structural similar isoforms.

References

Polacchini A *et al.* (2015) A method for reproducible measurements of serum BDNF: comparison of the performance of six commercial assays. Sci Rep. 10;5:17989.

Sartori A *et al.* (2019) Interferon-beta, but not Glatiramer Acetate treatment induces gender-specific increase in BDNF serum levels in relapsing-remitting multiple sclerosis female patients. Res J Neuro N Disord. 1:5-18

About Biosensis' Mature BDNF *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. Additionally, Biosensis used a novel antibody for both capture and detection allowing only the homodimeric form of BDNF to be detected, added a WHO-type standard, and performed extensive validation on real samples, both in-house and through independent, leading academic laboratories. The kit was also unique in being assessed to ensure low cross-reactivity against the proBDNF isoform. This also allowed Biosensis researchers to manufacture 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.