The Art of Obtaining Accurate ELISA Results – The Importance of Parallelism, Linearity-of-Dilution and Spike-and-Recovery Experiments

Introduction:

Accurately measuring the amounts of a specific analyte present in a biological sample is one of the greatest concerns for any analytical experiment. The method of enzyme-linked immunosorbent assay (ELISA) is just one analytical method that is being routinely used both in research laboratories and in diagnostic applications to measure analyte (the 'target') levels in complex biological samples. However, to ensure that an ELISA accurately quantifies a specific target in a complex biological sample (the 'matrix'), it is first necessary to conduct a series of specific developmental and calibration experiments, which are designed to demonstrate the particular assay's effectiveness and accuracy for measuring the desired target in the desired matrix. The most common and essential pre-measurement experiments are called, Parallelism, Spike-and-recovery and Linearity-of-dilution experiments. These essential studies assess and validate the ability of the ELISA assay to measure the true (i.e. accurate) amount of target in the sample. Additionally, performing these experiments will reveal any unwanted interference issues or sample matrix issues that may result in the reporting of false-positive or false-negative results.

Interference types:

Non-specific interference is associated with binding of components in the ELISA to non-specific binding sites such as polystyrene plates, or cross-reactivity of ELISA assay reagents. Observing high assay background OD readings is often a result of non-specific interference that will limit the quantification range of the assay and reduce assay sensitivity.

Specific interference refers to the unwanted interaction of sample constituents with components of the ELISA assay (generally the ELISA's antibodies) resulting in either a false-positive, or false-negative result. There are numerous excellent publications describing the causes of immunoassay interference and resulting artefactual results (see for example, Kragstrup *et al*., 2013; Tate and Ward, 2004). For example, a false-positive result can be because of the presence of an unknown protein in the sample binding to the capture and/or detection antibodies in the absence of target antigen, thereby providing a bridge between the assay antibodies. In contrast, a false-negative result can occur if an unknown protein in the sample binds to the capture antibody and competes with antigen binding. Well-described examples include rheumatoid factor (RF) and heterophilic antibodies (HA) such as human anti-mouse antibodies (HAMA), all of which can complex with the specific ELISA antibodies and cause detection and sensitivity difficulties. "Spike-and-recovery" as well as "Linearity-of-dilution" and "Parallelism" experiments help to detect such interferences and thus are an indispensable tool for validating ELISA assays.

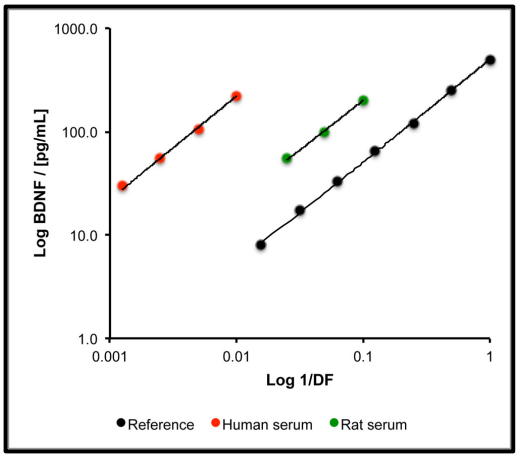
Matrix Interference:

Sample specific interference: Every complex sample matrix (such as human blood, urine, tissue extract) contains a different mix of specific and non-specific interfering factors. Even the same sample type obtained from different humans, or even from the same human but taken at different ages can potentially have different kinds and levels of interfering compounds. **Thus, an ELISA validated for a specific sample type, say serum, may not give accurate results even when used on other human serum samples. Nor can it be assumed that an ELISA validated for serum will be valid for plasma taken at the same time from the same individual.** It is for these reasons that Biosensis has done the hard work and optimized the *Rapid*™ ELISA kits to minimize interference for a range of validated matrices, using multiple samples of each matrix type. Should you wish to use a Biosensis® ELISA kit to assay samples that our scientific staff have not yet validated, the following guide provides a short introduction to how best to ensure the accuracy of your data. This guide also assists you in verifying accurate measurements in validated sample matrices and should be consulted for ***every*** ELISA experiment undertaken.

1. ***Linearity-of-Dilution and Parallelism*** experiments follow the same principle, but differ in their use of spiked blank matrix (*Linearity-of-Dilution*) or biological sample (*Parallelism*) with sufficiently high levels of endogenous target to perform at least 3 (better 4 or more) serial dilutions within the range of the reference (calibration) curve. While Parallelism and Linearity-of-dilution experiments both can demonstrate absence of matrix effects, parallelism experiments provide additional information about the comparability between the reference protein (often a recombinant protein) and the native form of the target, and the ability of the assay antibodies to detect both in equal manner and stable equilibrium. A coefficient of variation (CV) of ≤ 30% among the serially diluted sample is usually considered to be sufficient, however, more stringent requirements might be chosen on a case-by-case basis. Parallelism can be demonstrated and presented in simple ways in form of a data table or graph (Table 1 and Figure 1). More sophisticated data analysis can be performed by statistically evaluating whether sample and reference curve slopes are significantly different, and we refer to literature of data analysis software packages for further guidance.

Table 1 & Figure 1: Parallelism for mature BDNF in human and rat serum (BEK-2211). Parallelism is demonstrated by dilutional linearity (concentration of BDNF is within 80-120% of previous dilution) and an intra-sample CV of ~6%. Figure 1 confirms parallelism graphically.

Sample	Dilution Factor (DF)	BDNF (pg/mL)	BDNF Corrected for DF (ng/mL)	% Conc. of Prev. Dilution	Mean BDNF / [ng/mL]	CV (%)
Human Serum	100	223.3	22.3	100	22.5	5.9
	200	105.5	21.1	94		
	400	55.5	22.2	105		
	800	30.3	24.3	109		
Rat Serum	10	202.7	2.03	100	2.08	6.0
	20	99.6	1.99	98		
	40	55.6	2.22	112		



Linearity-of-dilution experiments are important if samples with sufficiently high endogenous target concentrations cannot be obtained. However, this validation experiment will not provide sufficient information regarding the compatibility between reference vs. native target, and any potential differences in assay antibodies detecting reference and native protein. The type of experiment involves serially diluting a sample matrix where endogenous target is absent or at very low levels, spiked with a known concentration of exogenous analyte. The reference protein is usually spiked into the lowest dilution of the sample matrix, then serial dilutions are performed, and the antigen concentration is then measured against the standard curve. It is good practice to serially dilute the un-spiked sample as well, to subtract concentrations of endogenous target that may be present at each dilution.

In both types of experiments, data is analysed in the same manner. Once adjusted for the dilution factor, the analyte concentration at each dilution should be 100% (+/- 20%) of the concentration measured at the previous dilution, which then demonstrates dilutional linearity. The dilution factor where the change in concentration from previous dilution is between 80-120% of expected sample recovery, and remains constant with further dilutions, becomes the Minimum Required Dilution (MRD) for *that* particular sample (Table 2). A serially diluted sample that is not affected by matrix effects should be parallel with the expected calibration curve.

Calculation of Linearity-of-dilution/Parallelism:

Example: Serial 1:2 dilution starting with 1:4 sample dilution.

$$\%\ \text{change in concentration from previous dilution (1:8)} = \frac{c\ (analyte\ at\ 1{:}8\ dilution)}{c\ (analyte\ at\ 1{:}4\ dilution)}\ x\ 100\%$$

$$\%\ \text{change in concentration from previous dilution (1:16)} = \frac{c\ (analyte\ at\ 1{:}16\ dilution)}{c\ (analyte\ at\ 1{:}8\ dilution)}\ x\ 100\%$$

etc. for all other subsequent dilutions.

Note: Analyte concentrations are adjusted for the sample dilution factor.

Table 2: Dilutional linearity and recovery for exogenous BDNF spiked in pooled human milk (BEK-2211). The sample diluent provides excellent recovery and dilutional linearity for quantification of BDNF in human milk. The OD signal is greatly suppressed at 1:2 dilution illustrating interference; thus, false-negative results would be reported if 1:2 dilution was used. Data analysis suggests a Minimum Required Dilution (MRD) of at least 1:4 for human milk. For more consistent results, higher dilutions are recommended if the concentration of endogenous BDNF is sufficiently high to be read on the calibration curve.

Dilution	Endogenous BDNF (pg/mL)	Spiked BDNF (pg/mL)	% Concentration from prev. dilution	% Recovery
1:2	< LOD	91	-	10
1:4	< LOD	731	805	81
1:8	< LOD	838	115	93
1:16	< LOD	910	109	101
1:32	< LOD	918	101	101
1:64	< LOD	930	101	103

2. ***Spike-and-recovery*** assays involve adding ('spiking') a known concentration of exogenous protein analyte to your diluted sample and testing this sample in the ELISA against an identical concentration of spike added to the sample diluent (Blank). Spikes should be performed at the MRD of a particular sample, The sample should also be assayed without spike to allow quantification of endogenous analyte. The spiked sample, spiked blank and sample without spike are each measured in the ELISA and the concentrations calculated against the standard curve. The aim is to achieve identical responses for both the blank and spiked sample (100% recovery). A range between 80-120% recovery is considered acceptable and indicates any matrix affect has been overcome (Figure 2). Recoveries outside this range indicate interference from sample components possibly due to incorrect choice of sample diluent.

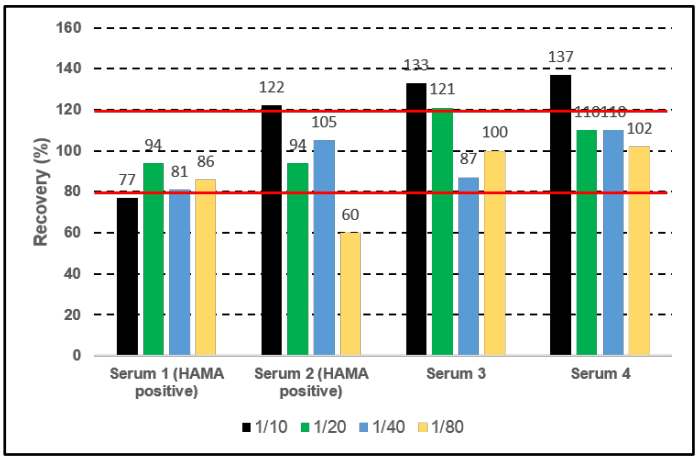


Figure 2: Recovery of spiked human proNGF in four serum samples at four dilutions (BEK-2226). This data demonstrates that under the given assay conditions with the chosen sample diluent a minimum sample dilution of 1:20 (green bars) is required to achieve accurate results for these four human serum samples, because only dilutions > 1:20 give spike recovery values within the 80-120% range.

Calculation of Spike-and-recovery:

$$\% \text{ Recovery} = \frac{c\ (spiked\ sample) - c\ (unspiked\ sample)}{c\ (diluent\ spike)}\ x\ 100\%$$

Note: Recovery values are calculated before adjusting analyte concentrations (c) for dilution factors and are based on analyte concentrations and not OD values.

Correcting for Poor Parallelism, Spike-and-Recovery and Linearity-of-Dilution

Poor assay performance as demonstrated by absence of parallelism indicates the presence of matrix effects, or different nature of reference standard as compared to native protein, and requires assay optimization. This can include: choice of different reference protein, different assay antibodies, optimization of incubation times, or choice of different assay buffer.

Failed spike-and-recovery and Linearity-of-dilution experiments indicate incompatibility of the sample diluent with the sample matrix which could be either due to incorrect buffer choice, heterophilic antibody interference or a mixture of both. Further assay optimization is required. Potential solutions are:

(1) Higher sample dilutions to avoid matrix interferences, provided that endogenous analyte concentrations are high enough.

(2) Adjustment of the sample diluent that needs to reflect the complexity of the sample matrix. This involves creating calibration curves in the blank matrix of the sample if one is available. For instance, using cell culture media rather than PBS as diluent for culture supernatants and reference standard, if target is quantified in culture supernatants. In cases where matrix is not easily available in large quantities, surrogate matrix can be used. Such surrogate matrices are commercially available.

(3) If heterophilic antibody interference is observed, additional blockers may need to be evaluated and added to the sample diluent (Figure 3)

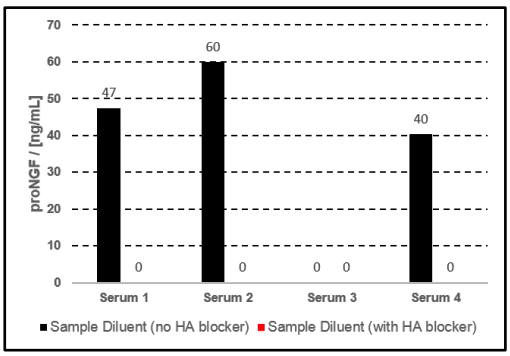


Figure 3: Evaluation of proNGF levels (BEK-2226) in four human serum samples at 1:20 sample dilution. Initial experiments demonstrated lack of dilutional linearity for serum samples 1, 2 and 4 (data not shown). Comparison of sample diluents with and without heterophilic antibody (HA) blocker reveals false-positive readings for serum 1, 2 and 4 due to HA interference when using sample diluent without the addition of a blocking agent (serum sample 3, no detection). This indicates that further experimental work will be necessary to overcome the HA interference,

References

Kragstrap, T.W. *et al.*. A simple set of validation steps identifies and removes false results in a sandwich enzyme-linked immunosorbent assay caused by anti-animal IgG antibodies in plasma from arthritis patients. *SpringerPlus*. 2013 June 2(1): 2-10

Tate, J. and Ward, G. Interferences in Immunoassay. *Clin. Biochem. Rev*. 2004 May 25(2): 105-120