Determining the Accuracy of an ELISA using Spike-and-Recovery and Linearity-of-Dilution 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 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. Two of the most common and essential pre-measurement experiments are called, 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 analyte 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. Observing high assay background OD readings is often a result of non-specific interference that will limit the quantification range of the assay.

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" 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, milk, 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 are likely to 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. ***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). 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 1). Recoveries outside this range indicate interference from sample components possibly due to incorrect choice of sample diluent.

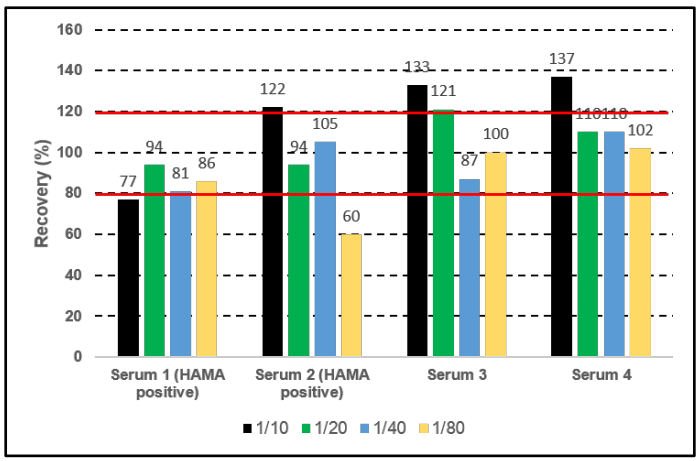


Figure 1: Recovery of spiked human proNGF in four serum samples at four dilutions (BEK-2226). This data demonstrates that under the given assay conditions with the chosen sample diluent a minimum sample dilution of 1:20 (green bars) is required to achieve accurate results for these four human serum samples, 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.

2. ***Linearity-of-dilution assays*** also measure the accuracy of the ELISA assay and its compatibility with a sample matrix. This experiment involves serially diluting a sample, preferably with a high endogenous concentration of analyte. If no sample with high endogenous concentration is available, a known concentration of analyte can be added as a spike to the sample and then diluted. The antigen concentration is then measured against the standard curve. 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 starts to be linear or constant and is between 80-120% of expected sample recovery becomes the Minimum Required Dilution (MRD) for *that* particular sample (Table 1). A serially diluted sample that is not affected by matrix effects should be parallel with the expected calibration curve.

Calculation of Linearity-of-dilution:

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

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

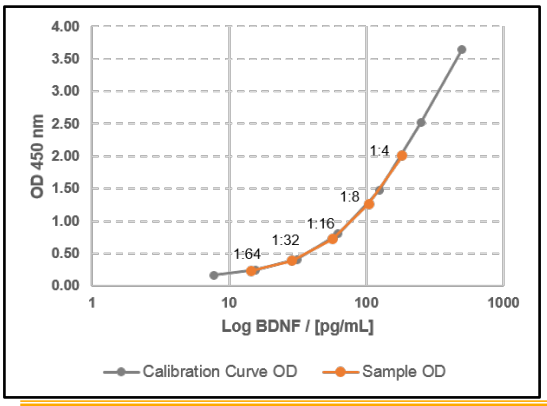
$$\% \text{ change in concentration from previous dilution (1:16)} = \frac{c\ (analyte\ at\ 1{:}16\ dilution)}{c\ (analyte\ at\ 1{:}8\ dilution)}\ x\ 100\%$$

etc. for all other subsequent dilutions.

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

Table 1 & Figure 2: Dilutional linearity, recovery and parallelism for BDNF 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. Superimposing calibration OD readings with sample OD readings (right graph) demonstrates parallelism, and thus validates the use of this sample diluent for accurate quantification of BDNF in human milk with a Minimum Required Dilution (MRD) of at least 1:4. 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



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

Poor assay performance as demonstrated by 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. This will indicate the ELISA requires further assay optimization.

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. For instance, using cell culture media rather than PBS as diluent for analyte in culture supernatants.

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

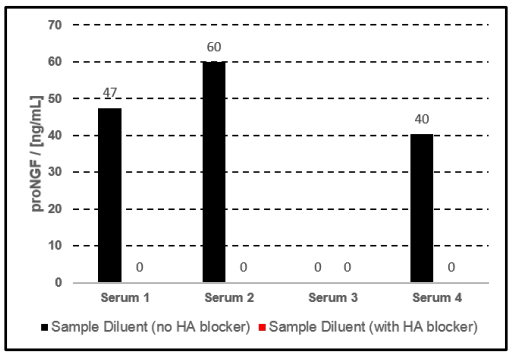


Figure 3: Evaluation of proNGF levels ((BEK-2226)) in four human serum samples at 1:20 sample dilution. Initial experiments demonstrated lack of dilutional linearity for serum samples 1, 2 and 4 (data not shown). Comparison of sample diluents with and without heterophilic antibody (HA) blocker reveals 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, which may include changing out antibody components that are not reactive

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