Immunoassay Kits:

Immunoassay kits are among our most useful and popular products. When used as directed, they can provide sensitive and specific quantification of specific molecules in complex biological fluids. For large molecules with multiple epitopes, we provide sandwich ELISA kits, of which employs one antibody to capture the target molecule and another to detect it. Examples are immunoassays for tPA and myeloperoxidase. Our assay kits for transcription factor activation also employ a sandwich approach, although the capture of the transcription factor is mediated by a bait oligonucleotide.

Competitive ELISAs are employed for small molecules, such as eicosanoids and steroids, that have only a single epitope. Although these assays have a smaller useful dynamic range and the results are inversely related to the analyte concentration, they can provide very accurate results if used properly. The article reproduced below details analytical constraints of competitive ELISA assays with an eye to maximizing their use for quantitative analysis. These assays typically provide high sensitivity, a wide dynamic range, and results that are directly proportional to the concentration of the target molecule in the sample.

Importance of the enzyme conjugate: Our ELISA kits for small molecules are based on the competition of the analyte and a synthetic standard conjugated to horseradish peroxidase. Horseradish peroxidase (HRP) is the enzyme that has been most widely used for immunochemical and immunoassay protocols because of several factors, including stability, resilience to protocols in which it is conjugated to small haptens, and a range of substrates for colorimetric or chemiluminescent detection. Some other enzymes, including catalase and acetyl cholinesterase (ACE), have higher turnover numbers and thus can theoretically provide greater sensitivity. However, they do not provide as robust an assay. ACE, for example, is less stable and can be inactivated by trace metal ions in many buffers or samples.

Validation: Immunoassays are typically characterized using pure standards in buffer. Real biological samples are much more complex. Hence, as possible, we validate results obtained using ELISA to those obtained by other methods such as GC/MS. In order to eliminate interference by other molecules, especially in serum, one must first partially purify the analtye in question. For urine and tissue culture samples, this step is usually unnecessary. Requirements and limitations of individual kits vary and are detailed here and on our web site.

How to Obtain Reproducible Quantitative ELISA Results

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ELISA assays have become one of the most popular biomedical methods for the quantifi-cation of analytes in samples because, in addition to their sensitivity and specificity, they are simpler and less costly than most other analyses. However, the ease with which ELISAs can be performed may mask some complex features of analyzing biological samples by ELISA. This article will review the basic steps used to obtain reliable ELISA data from biological samples using both major types of ELISA assay, the sandwich ELISA and the competitive ELISA. While these two formats share several components, sandwich ELISA and competitive ELISA differ fundamentally with respect to the standard curves obtained and the methods used for data analysis.

Sandwich ELISA: A sandwich ELISA is used for the detection of antigen-analytes containing at least two epitopes. Typically a microplate is coated with an IgG specific for one epitope and any remaining binding sites blocked. Next, the free analyte standard or analyte-containing sample is incubated on the coated plate,

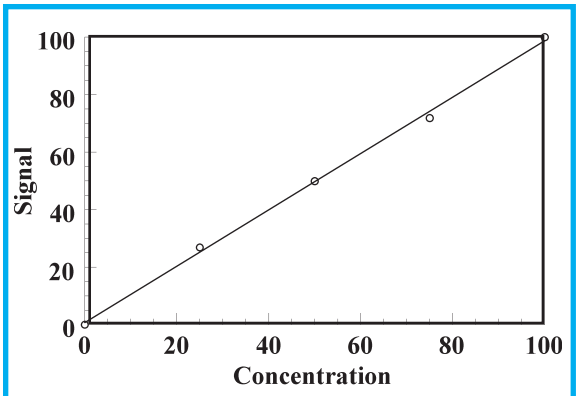

Figure 1. Standard curve for a typical sandwich ELISA assay showing a linear relation between the signal and the analyte concentration. y = mx + b, where y = signal, x = concentration, m = slope, b = y-intercept.

followed by incubation with an enzyme-conjugated antibody specific for a second epitope. The presence of the amount of enzyme conjugate bound to the plate is then detected following incubation with an appropriate substrate and measuring the resulting signal with a microplate reader.

There is a direct linear relationship between the signal variable (y, in units of absorbance, fluorescence, or chemiluminescence) and the analyte concentration variable (x) related by a slope constant, m, and a y-intercept constant, b. The greater the signal, the greater the concentration of free analyte throughout the range of the standard curve, Figure 1. To obtain reliable results it is recommended that the sample dilutions provide signals that fall in the vicinity of 50% B/B_0 wherein $B = y - b$, $B_0 = y_0 - b$, and y_0 = the maximum signal. Also, to report values that have statistical significance, biological samples require 3 samplings or dilutions (n=3) regardless of the number of replicate wells. Typically coefficients of variance (CV, standard deviation/mean) will be less than 15%. If statistical significance or precision is not a major concern (e.g. for well established assay procedures designed to determine the presence or absence of an analyte), single dilutions of a sample can be used to obtain reproducible results as long as the values fall within the linear range of the standard curve.

Competitive ELISA: This type of ELISA is frequently used for the detection of small analyte antigens containing a single epitope. Typically the plate is coated with antibody specific for the single epitope on the analyte. Next, free analyte and analyte ligated to a detection enzyme are incubated on the coated plate. The quantity of the enzyme-ligand conjugate bound to the plate is detected after incubation with an appropriate substrate and the resulting signal measured with a microplate reader. In competitive ELISA, there is an inverse relationship between the concentration of the analyte in the sample and the signal obtained, due to the competition between the free analyte and the ligand-enzyme conjugate for the antibody coating the microplate, i.e. the more analyte the lower the signal. See Figure 2.

For competitive ELISAs, the rate of change of signal vs. concentration value varies throughout the standard curve concentration range, from steep to shallow. The relatively narrow standard curve range (x ~ 0.05 - 2.0 concentration units for 20% to 80% B/B_0, signal/maximum signal, adjusted to full scale range) defines

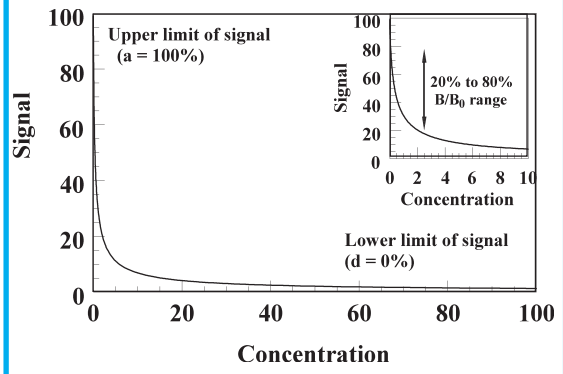

Figure 2. Model standard curve for a typical competitive ELISA assay. The raw data are initially fitted using the four parameter equation below:

$$y = \frac{}{1 + \left(\frac{}{}\right)^{b}} + d$$

The signal intensities are then scaled to set $B_0 = a = 100$ and $d = 0$, in order to express intensities on a 0 to 100% scale. The midpoint signal intensity (50%) occurs at c (here 0.335 units of concentration) and serves to define the 20% to 80% range generally used for the evaluation of unknown samples (see inset). These data are more conveniently graphed on a semilog plot as shown in Figure 3.

the portion of the standard curve that has the most reliable concentration dependence. The vertical portion of the curve exhibits large signal changes for small concentration changes, whereas, the horizontal portion of the curve exhibits small signal changes for large concentration changes.

In contrast to typical sandwich ELISAs, the concentration dependence of competitive ELISAs requires that samples, or dilutions of initial samples, fall within the relatively narrow concentration range of the most reliable portion of the standard curve (20% to 80% B/B_0). If only a rough concentration estimate is required (i.e. a coefficient of variance of 100-200% is acceptable) then signals that fall within 20% to 80% B/B_0 range are adequate. However, if CVs less than 25% are required, it may be necessary to use values obtained for only the 40%-60% B/B_0 portion of the standard curve.

For pilot studies to determine the appropriate dilution ranges necessary to obtain more precise values, competitive ELISAs will require a minimum of three sample dilutions to obtain concentration estimates that fall within the most reliable range of the standard curve. Even with established competitive ELISAs, single dilutions of sample are not recommended due to the relatively narrow target range of the standard curve and routine variations among assays that may affect the range.

Know the characteristics of the biological sample to be analyzed. Obtaining reproducible results with either ELISA method requires minimizing factors that may affect the quality of the sample. Obtaining reproducible results in a competitive ELISAs requires more vigilance due to the relatively narrow target range of the assay.

What is the reported range of analyte for a given type of sample? This information is needed to determine the quantity of sample needed, the appropriate size for aliquots, and the sample dilutions to use. One or two dilutions may be adequate for a sandwich ELISAs but three or more are preferable for a competitive ELISA. Concentration values obtained for a minimum of two dilutions should be comparable, and additional values may be needed for statistical analysis (because n = 1 for one dilution regardless of the number of replicates).

Decide upon the most useful way to express the concentration of the analyte and the best method for normalizing values. For example, the concentration of an analyte in urine should be normalized to the creatinine concentration; values for tissue homogenates or cell lysates to the protein concentration.

Identify proper storage conditions, including containers, temperature,and preservation methods. Store frozen samples in aliquots so that they will not undergo multiple freeze-thaw cycles.

Optional but highly recommended: Obtain a large volume of an internal reference sample. Aliquots of such a sample can be used in pilot experiments and included as an internal reference standard with established assays.

Use an appropriate method for data analysis. For the sandwich ELISAs, the standard curve is a straight line with a positive slope. A linear regression curve can be used to obtain sample concentration estimates. Small numbers of

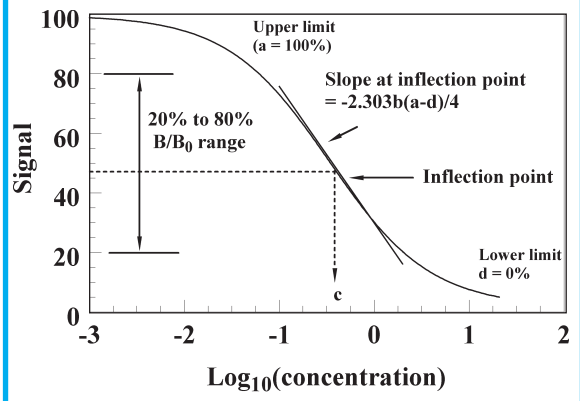

Figure 3. Data from Fig. 2 are replotted using $\log_{10}$(concentration) as the x-axis, which reveals the two plateau regions and the point of inflection (at concentration = c) more clearly. a = the maximum signal, d = the minimum signal, b = a slope-related term at the inflection point, and c = the concentration at the inflection point (50% B/B_0). B_0 = a - d and B = y – d. Values for 20%, 50% and 80% B/B_0 are then determined. Note: although the concentration extremes cannot be used for analyte determinations, they are absolutely required for the estimation of parameters a and d.

biological samples can be easily evaluated with a hand calculator or graph paper. Larger numbers of samples are conveniently evaluated using simple spreadsheet software.

For competitive ELISAs, the standard curve can be mathematically represented as a 4-parameter logistic fit (see Fig. 3) and subjected to nonlinear regression analysis. Most modern microplate readers include software that perform nonlinear regression analyses using the 4-parameter logistic model, which is the regression equation of choice (Diamandis and Christopoulos, 1996; Maciel, 1985).

If nonlinear regression analysis software is not available, competitive ELISA data can be linearized and analyzed using a log-logit transformation, Figure 4 (Diamandis and Christopoulos, 1996). The resulting straight line can be used to evaluate samples by linear regression. However, only the linear portion of the standard curve can be used. If this method of analysis is chosen, prepare more standard dilutions in the linear portion of the curve but include high and low standard concentrations to establish values for a and d (read from the signal vs. log concentration plot). Given the base line-corrected maximum signal value, B_0 and the signal value, B, the 80%, 50% and 20% B/B_0 can be evaluated.

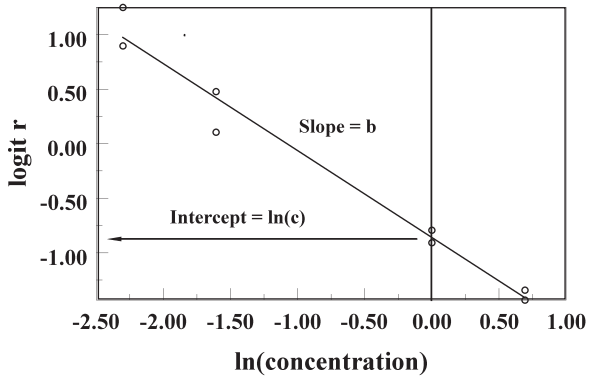

Fig. 4 Log-Logit transformation. In this linear transformation the data are fitted to the equation: Logit r = ln(c) - b ln(x), wherin r = B/B_0 and logit r = ln[r/(1-r)]This equation is derived from the four parameter fit based on the assumption that two of the parameters (a and d) are accurately known, e.g., by inspection of the y vs log(x) plot. This transformation linearizes the data in the region of interest and permits analysis of the data using a scientific hand calculator.In the above case, the estimates of c (0.42) and b (-0.80) agree well with those obtained using the four parameter fit (0.35 and -0.81, respectively).

Know the range within which your instrument provides accurate readings. Reliable ELISA values also depend on the sensitivity of the instrument within the useful range of the standard curve. This is especially important for competitive ELISAs, for which the reliable range is narrow relative to the maximum and minimum signals. For competitive ELISAs, absorbance readings have the smallest dynamic range and, thus, require more standard values to determine the reliable range. A typical microplate reader provides a linear response within the 0.050 to 1.000 absorbance range, equivalent to 1/10 decade of dynamic range and typically picomole/well sensitivity.

Evaluating the raw data and expressing ELISA results. Routine variations among ELISA standard curves and sample values include "intraassay" (within the plate, replicate values), "interassay" (between plates) or "day to day" variations, and are usually in the 5-20% range. The narrow standard curve range of the competitive assay is more susceptible than sandwich ELISAs to routine variations. Inclusion of an internal reference standard aids in the assessment of these variations. Validation of the exact concentration of the analyte in this internal reference standard by a "gold standard" assay procedure such as gas chromatography-mass spectroscopy (GC/MS) is ideal.

To obtain precise concentration estimates using competitive ELISA, values obtained for at least two dilutions of a sample should agree. The most precise values are those in the vicinity of 50% B/B_0, although the concentration estimate is subject to the routine variations described above If there is no sample value that falls in the vicinity of 50% B/B_0, repeat the experiment with adjusted sample dilutions. Give these values in the vicinity of 50% B/B_0 the highest priority and gauge the accuracy of other replicates with respect to these most reliable values. Do not use concentration estimates that deviate markedly (greater than or less than 50%) from those in the central portion of the curve. If only one sample dilution (n=1) yields a value

within the most reliable portion of the standard curve, the assay should be repeated until values obtained for at least two sample dilutions (n=2) are in agreement.

Finally, determine the concentration of a normalizing component (e.g. protein for tissue samples, creatinine for urine samples) and express the concentrations of analyte measured by ELISA relative to the normalizing parameter.

Conclusion: Unique demands of competitive ELISAs include: obtaining reliable values for two dilutions within the narrow target range of its standard curve, and using the preferred 4-parameter logistical fit method for analysis of a standard curve that requires non-linear regression analysis.Using these procedures, major sources of sample variability can be minimized, and reproducible quantitative values obtained.

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