TECH TIP #57

How to use a protein assay standard curve

Introduction

Typical protein assays are used to determine protein concentration by comparing the assay response of a sample to that of a standard whose concentration is known. Protein samples and protein standards are processed in the same manner by mixing them with assay reagent and using a spectrophotometer to measure the absorbances. While protein quantitation from absorbance values is straightforward, one common source of confusion is the assumption that dilution of the sample with assay reagent is a necessary consideration. This Tech Tip describes how to properly calculate sample protein concentrations using a standard curve. Thermo Scientific® Pierce Protein Assays are used as examples, but the principles apply to protein assay methods in general.

Fundamental Principles of Standard Curve Assays

A. Identically assayed samples are directly comparable ("Equal treatment means equal opportunity.")

Sample assay responses are directly comparable to each other if they are processed in exactly the same manner. Variance in protein quantity is the only possible cause for differences in final absorbance (color intensity) if samples are dissolved in the same buffer and the same stock solution of assay reagent is used for all samples. Of course, different proteins will generate different absorbance values even at the same concentration, an issue that is discussed more fully in Pierce® Protein Assay product instructions (see Related Products).

B. Units in equals units out ("What you put in is what you get out.")

The unit of measure used to express the standards is by definition the same unit of measure associated with the calculated value for the unknown sample (i.e., final results for unknown samples will be expressed in the same unit of measure as was used for the standards). For example, if the standards are expressed as micrograms per milliliter, then the value for the unknown sample, which is determined by comparison to the standard curve, is also expressed as micrograms per milliliter.

The Total Amount of Protein Per Well is Irrelevant

With these principles of standard curve assays in mind, one can easily understand why it is neither necessary nor even helpful to know the actual amount (e.g., micrograms) of protein applied to each well or cuvette of the assay. Consider a simple example in which the Coomassie Plus Protein Assay Kit (Product No. 23236) is used to assay two protein samples: a test sample whose concentration is not known, and a standard whose concentration is 1mg/mL (= 1000µg/mL). (Ordinarily, an entire set of standards is necessary to establish a response curve, but this is a simplified example.)

In the microplate protocol, one adds 10µL of sample (test or standard) and 300µL of assay reagent per well. Because 10µL of the standard sample is added to a well, there is $0.010\text{mL} \times 1000\text{µg/mL} = 10\text{µg}$ of protein in the well. If the assay results in the test sample having the same final absorbance as the standard sample, then the conclusion is that the test sample contains the same amount of protein as the standard sample. Because there was 10µg of standard/well, one could report the determined concentration of test sample as "10µg/well." However, the amount of protein per well is almost certainly not the value of interest; instead, one usually wants to know the protein concentration of the original test sample. Because the original standard was 1000µg/mL, the test sample that produced the same absorbance in the assay also must be 1000µg/mL.

The Protein Concentration in Assay Reagent is Irrelevant

Furthermore, it is neither necessary nor helpful to know the protein concentration as it exists when diluted in assay reagent. In the above example, because the 10µg standard was diluted to 310µL after adding of 300µL of assay reagent, the final concentration in the well is $10\text{µg}/310\text{µL} = 0.0323\text{µg/µL} = 32.3\text{µg/mL}$. Therefore, one could report the determined concentration of test sample as "32.3µg/mL." However, the protein concentration when diluted by assay reagent is almost certainly not the value of interest; instead, one wants to know the protein concentration of the original test sample. Because the original standard was 1000µg/mL, the test sample that produced the same assay absorbance also must be 1000µg/mL.

How to Apply Dilution Factors

One situation in which the dilution factor is important to consider is when the original sample has been pre-diluted relative to the standard sample. Continuing with the same example, suppose that the original protein sample is actually known to be approximately 5mg/mL. This is too concentrated to be assayed by the Coomassie Plus Protein Assay Kit, whose assay range in the standard microplate protocol is 100-1500µg/mL. However, one could dilute it 5-fold in buffer (i.e., 1 part sample plus 4 parts buffer) and then use that diluted sample as the test sample in the protein assay. If the test sample produces the same absorbance as the 1000µg/mL standard sample, then one can conclude that the test (5-fold diluted) sample is 1000µg/mL, and therefore the original (undiluted) sample is $5 \times 1000µg/mL = 5000 µg/mL = 5mg/mL$.
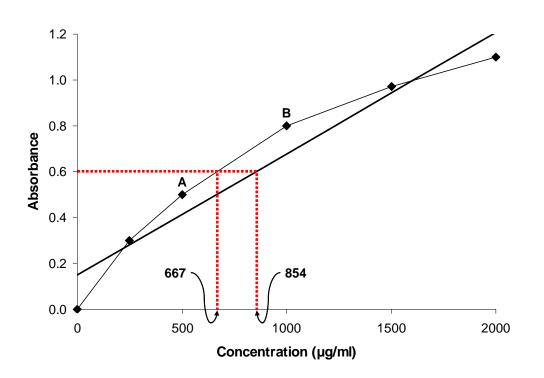
How to Interpolate on a Complete Standard Curve

Unlike the example described above, most assays use an entire set of protein standards whose concentrations span the effective assay range (e.g., 100-1500µg/mL for the Coomassie Plus Protein Assay Kit). Rarely, if ever, will the test sample produce an assay response that corresponds exactly to one of the specific standard samples. Therefore, a method is needed to calculate or interpolate between the Standard sample points. Most modern plate readers and spectrophotometers have associated software that automatically plots a linear or curvilinear regression line through the standard points, interpolates the test samples on that regression line, and reports the calculated value.

Consider the following example involving a set of six standard points (0, 250, 500, 1000, 1500 and 2000µg/mL). The same point-to-point relationship for this set of standards is plotted in Figures 1 and 2, but different methods were used to determine a mathematical equation that describes this absorbance-concentration relationship. If a test sample results in an absorbance of 0.6, then one must interpolate between values obtained for the 500 and 1000µg/mL standards to determine the test sample concentration.

On a merely graphical basis, one can see that the test sample must be ~ 650µg/mL.
- The line segment AB in the point-to-point graph is described by the equation $y = 0.0006(x + 333.33)$.
- Solving this equation for x gives $x = 1666.7y - 333.33$.
- If $y = 0.6$, then $x = 667µg/mL$.

Figure 1. Example standard curve involving six points. The thin line is a point-to-point graph through the plotted standards. The thick line is linear regression for the entire set of standard points. Dashed lines represent interpolations for a test sample having absorbance 0.6. See text for additional details.

Many researchers plot a linear regression for the entire set of standards, assuming that the overall relationship between concentration and absorbance is best described by a straight line. The thick, straight line in Figure 1 is the linear regression that best describes the entire set of standard points ($R^2 = 0.9355$).
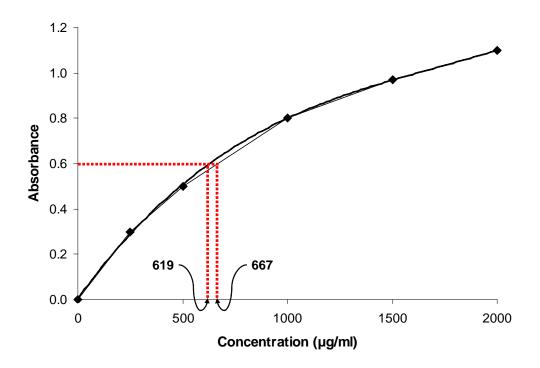
- The equation for this line is $y = 0.00056x + 0.1174$.
- Solving for x gives $x = 1770.4y - 207.91$.
- For $y = 0.6$, $x = 854\mu g/mL$.

As is obvious from the graph, this linear regression does not provide a good basis for interpolating test sample concentrations relative to assay results for the standards.

The best method for interpolation in this example is by reference to a curvilinear regression, in this case a 3-parameter polynomial equation that can be calculated by most plate reader software or standard spreadsheet programs (see box below).

- When solved for x, the 3-parameter equation ($R^2 = 0.9997$) is $x = 1372.2y^3 - 769.01y^2 + 1004.2y - 2.9373$.
- For $y = 0.6$, $x = 619\mu g/mL$.

On a merely graphical basis (Figure 2), this method can be seen as the most accurate for interpolating the test sample. If one had included a $750\mu g/mL$ standard, it surely would have occurred on this 3-parameter trend line rather than on the straight line segment AB or on the linear regression displayed in Figure 1.

Figure 2. Example standard curve involving six points. The thin line is a point-to-point graph through the plotted standards. The thick line is a 3-parameter regression for the entire set of standard points. Dashed lines represent interpolations for a test sample having absorbance 0.6. See text for additional details.

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3

Using Microsoft Excel to plot and apply standard curve

A protein assay, such the BCA Protein Assay, is an excellent tool for estimating the protein concentration of a sample. The intensity of the colored reaction product is a direct function of protein amount that can be determined by comparing its absorbance value to a standard curve. Plotting a graph with the absorbance value as the dependent variable (Y-axis) and concentration as the independent variable (X-axis), results in an equation formatted as follows: $y = ax^2 + bx + c$, where solving for x determines the protein concentration of the sample. By switching the axes and plotting protein concentration as the dependent variable on the Y-axis and absorbance as the independent variable on the X-axis, the protein concentration is represented by y and the equation is much easier to solve.

To use Excel for generating such an equation, enter the concentration values for the standards in Column A and their corresponding absorbance data in Column B. Highlight both columns and from the Insert menu select Chart and XY (Scatter). Click on the resulting graph and select Add Trendline from the Chart menu. While viewing the graph next to the open Format Trendline window, choose Polynomial and set the Order to 2, 3 or 4 until the best-fit appears. Check the box near the bottom called Display Equation on Chart; then close the Format Trendline window. Use the resulting equation to determine protein concentration (y) of an unknown sample by inserting the sample's absorbance value (x).

A Note About Interfering Substances

This Tech Tip has focused on proper calculation of test sample concentrations relative to the standard samples. The possible effects of interfering substances were not discussed because the assumption was that all protein samples were treated exactly the same, including the buffers in which the proteins were dissolved. In this situation, any interference caused by components of the buffers is exactly the same for both test and standard samples.

Nevertheless, interference by nonprotein substances in the samples that block or contribute to the assay color reaction is an important issue for any protein assay system. The instructions for the different Pierce Protein Assay Kits (see Related Products) describe the classes of compounds that are known to interfere, as well as several techniques for eliminating or minimizing their effects. The Reducing Agent Compatible BCA Protein Assay Kits (Product No. 23250 and 23252) contain a treatment reagent for specific eliminating interference by dithiothrietol (DTT) and other reducing agents that are commonly present in cell lysate and other protein samples.

Related Thermo Scientific Pierce Products

23209	**Bovine Serum Albumin Standard Ampules, 2 mg/ml**, 10×1mL ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide	
23208	**Bovine Serum Albumin Standard Pre-Diluted Set**, 7×3.5mL aliquots in the range of 125-2000 µg/ml	
23212	**Bovine Gamma Globulin Standard Ampules, 2 mg/ml**, 10 x 1mL ampules	
23213	**Bovine Gamma Globulin Standard Pre-Diluted Set**, 7×3.5mL aliquots in the range of 125-2000 µg/mL	
23225	**BCA Protein Assay Kit**, working range 20-2000µg/mL	
23235	**Micro BCA Protein Assay Kit**, working range of 0.5-20µg/mL	
23250	**BCA Protein Assay Kit – Reducing Agent Compatible**, working range 125-2000µg/mL	
23250	**Microplate BCA Protein Assay Kit – Reducing Agent Compatible**	
23236	**Coomassie Plus (Bradford) Assay Kit**, working range of 1-1500µg/mL	
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**, sufficient reagents to pre-treat 500 samples to remove interfering substances before total protein quantitation	

Current versions of product instructions are available at *www.thermoscientific.com/pierce*. Call 800-874-3723 or contact your local distributor.