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## The ELISA Standard Save: Calculation of sample concentrations in assays with a failed standard curve

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### Abstract

The enzyme-linked immunosorbant assay (ELISA) is often used to measure protein levels in plasma and other solutions. In order for the assay to be quantitative, a standard curve must be prepared for each assay. Technical blunders in preparing the standard curve can render otherwise representative sample values useless. In order to recover these data, a protocol has been developed whereby a new standard curve is generated using the  $\Delta OD$  values of the samples. This new standard is applied to the original plate in order to determine sample concentrations. To validate this method, sample concentrations obtained using an acceptable standard curve were plotted against those obtained using the new standard curve. Linear regression analysis showed a 1:1 correlation between concentrations, with  $r^2$  values ranging from 0.98–0.99 and slopes ranging from 0.97–1.10. In this manner, data for these samples are preserved, saving the investigator the time and money involved in repeating experiments.

### Keywords

ELISA; standard curve; cytokine; sample concentration; recovery

### Introduction

Given its ease of performance, high throughput capabilities and the increasing availability of recombinant standards and antibodies for various proteins, the ELISA has become the assay of choice for measuring proteins in various biological matrices (Salonen and Vaheri, 1981; Boscato and Stuart, 1988). The ELISA technique has recently been optimized to measure protein concentrations from as little as 10 $\mu$ L of sample, with proper dilution. This greatly benefits many in vivo applications in which only small volumes of sample can be collected in order to monitor disease progression over time (O'Connor et al., 2004; Osuchowski et al., 2006). Unfortunately, human error and other unidentifiable factors sometimes result in unsuccessful assays in which a reliable standard curve can not be generated, leaving otherwise representative sample readings useless. This can prove detrimental when only small sample volumes can be collected, requiring both time and resources to repeat experiments.

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Here, we outline a method by which the concentrations of samples may be calculated by deriving a separate standard curve. This method is not recommended as a primary means of data collection, but is a rescue protocol for instances in which there is a need for salvage. This method is applicable when it is determined that the defect is with the standard only. Standard curves are deemed failed if they meet the following criteria: five or more samples have  $\Delta OD$  0.5 higher than the most concentrated standard; a 4-parameter regression can not be calculated ( $\Delta OD$  does not strictly decrease as standard concentration decreases); and  $r^2 < 0.97$ . In our experience, technical blunders involving the capture antibody result in little to no color development, while problems with detection antibody results in uniform faint color in all wells, including blanks. As such, experienced ELISA users can determine whether or not sample  $\Delta OD$  values are usable as they will show a wide range of values. Specific examples of possible mistakes where only the standard has failed would be using mouse IL-6 standard in an ELISA for human IL-6, or selecting the IL-1 standard when the assay was meant to measure IL-10.

In this method, a new standard curve is generated using the  $\Delta OD$  values of the samples themselves from the original assay. 8–10 samples with maximal range of  $\Delta OD$  values are chosen and re-run with a verified working standard protein. The concentrations of these samples are then used to generate a new standard curve, which is applied to the original plate in order to determine sample concentrations. Linear regression analysis of these curves showed a 1:1 correlation between concentrations, with  $r^2$  values ranging from 0.98–0.99 and slopes ranging from 0.97–1.10 for each cytokine assayed.

## Methods

1. Initial Sample Run
  - a. Samples are run according to previously optimized standard ELISA protocol (DeForge and Remick, 1991; Nemzek et al., 2001).
  - b. Immediately prior to stopping the color reaction, the  $OD_{590}$  is measured.
  - c. The reaction is stopped with sulfuric acid and  $\Delta OD$  values are used for data analysis. At this point, if a working standard curve can be generated, the data may be analyzed normally. If not, the following steps can be taken for preservation of sample data.
2. Troubleshoot Standard Curve
  - a. Prepare fresh dilution buffer and rerun standard curve. In order to proceed with the assay, troubleshooting must be successful so that standard curves can be reliably generated.
3. New Standard Run
  - a. Choose 8–10 samples to rerun to generate the new standard curve.
    - i. Samples with the maximum and minimum  $\Delta OD$  values are always chosen, along with others with  $\Delta OD$  values that are evenly distributed within this range.
  - b. These samples are rerun by standard ELISA protocol.
  - c. Prior to addition of sulfuric acid solution,  $OD_{590}$  is monitored. The reaction should not be stopped until the  $OD_{590}$  values are equal to that read in the initial sample run. The plates can be left in a covered plate reader and reread until the desired  $OD_{590}$  is obtained. This is to ensure that differences in extent of color development do not affect sample concentrations calculated using the new standard.

- d. A non-linear 4-parameter regression is used to generate a standard curve. Note that any computer program capable of generating a 4-parameter regression can be used. The concentrations of the samples are then used to generate a new standard curve as follows:
  - i. Each well corresponding to a sample in the new standard run is re-labeled as a standard and its concentration is entered manually.
  - ii. Once all samples are renamed and concentrations entered, those wells are used to generate the new standard curve and 4-parameter equation.
  - iii. The new standard curve is then applied to samples from the initial sample run to interpolate concentrations of all the samples.

## Results and Discussion

The OD<sub>590</sub> is monitored in order to ensure consistent color development in each ELISA run. Plotting of OD<sub>590</sub> versus  $\Delta$ OD resulted in an  $r^2$  value of 0.98, indicating that OD<sub>590</sub> is an effective method to monitor color development (Figure 1). Once concentrations are determined for the samples in the new standard run, those samples must be re-labeled as standards so they may be applied back to the initial run. In order to illustrate this, a sample 96-well plate layout for the new standard run is shown in Figure 2. The chosen samples are rerun by the same method as the initial sample run. Once these concentrations are determined, these same wells are re-labeled as standards and the measured concentrations are entered manually. This new standard is applied back to the initial sample run to interpolate sample concentrations.

In order to validate this method, 40 samples were prepared by spiking normal mouse plasma with a range of concentrations of the mouse recombinant cytokines TNF $\alpha$ , IL-4 and IL-6. Concentrations of the samples determined in the initial sample run plotted against those determined by applying the newly generated standard results in a linear correlation between the two methods with an  $r^2 \geq 0.98$  for all cytokines (Figure 3). Upon performing linear regression analysis, a slope of 1 indicates perfect agreement between the initial run and the application of the new standard. The slopes of the regression lines for all cytokines used were between 0.92 and 1.10, indicating that the new standard curve is a highly effective means by which to determine sample concentration.

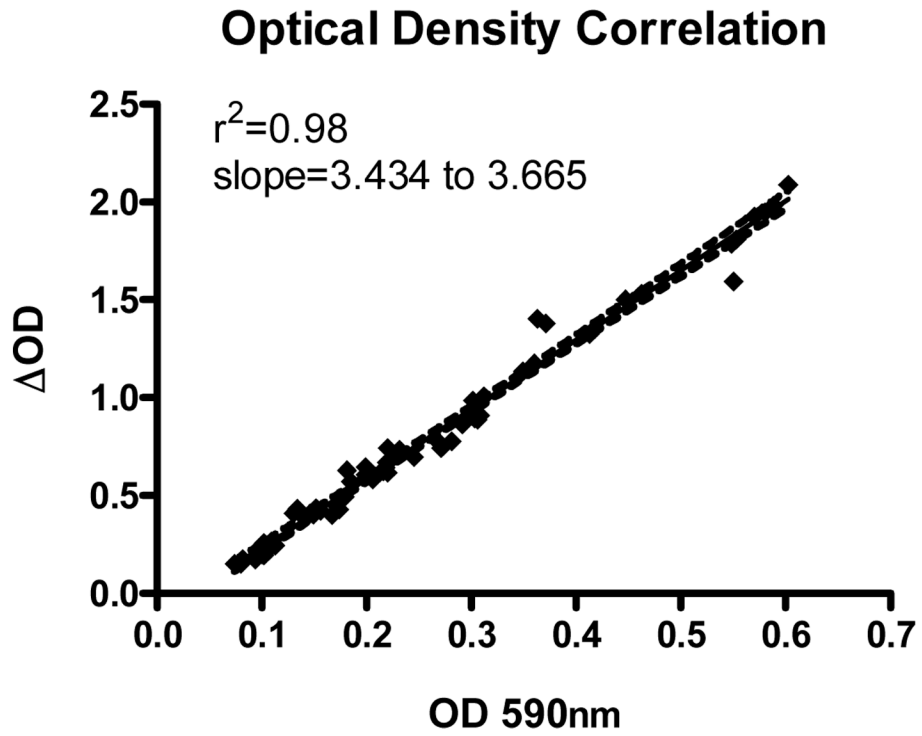
This method is based upon the concept that an unsuccessful standard curve does not negate the usefulness of the entire ELISA assay. Additionally, this method is most effective with samples that contain varying concentrations of the protein to be assayed. Samples with a wide range of concentrations will maximize the dynamic range of the new standard curve, making it more robust. We must emphasize the importance of measuring the OD<sub>590</sub> for all ELISA assays having greater than 20 samples, as it provides the researcher the option to use this method if the standard curve is not satisfactory. Significant resources may be saved as this method requires only the time needed to rerun 10 or fewer samples, saving both the time and money otherwise required for repeating experiments to collect more sample.

## Acknowledgments

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**Figure 1.** Correlation of  $OD_{590}$  and  $\Delta OD$ - Samples were read before and after addition of acid to stop color development.  $OD_{590nm}$  was plotted against  $\Delta OD$  to reveal a linear relationship.

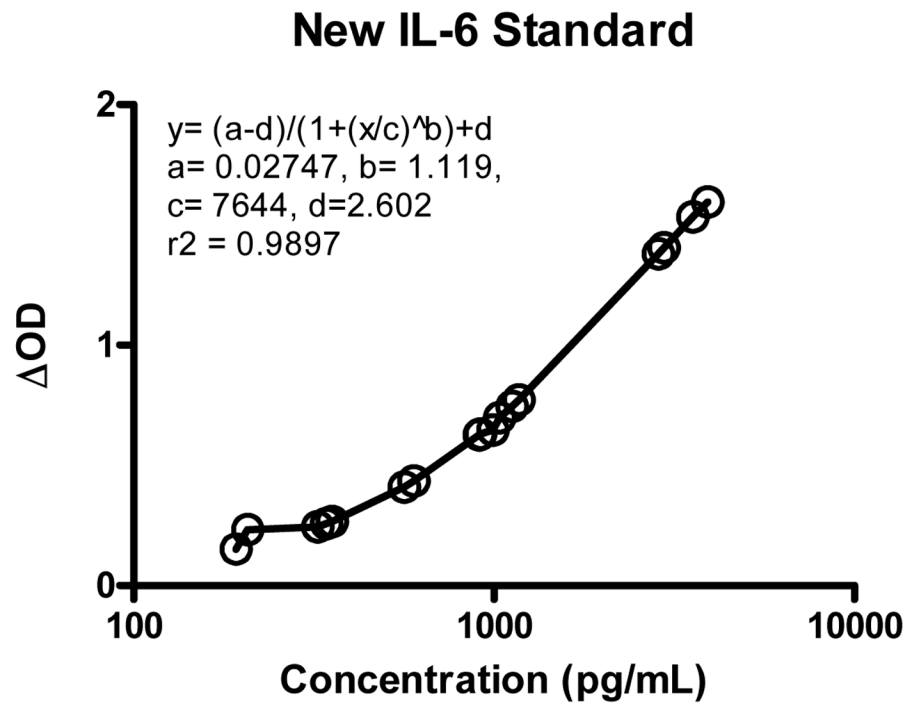
A)

	1	2	3	4	5	6	7	8
<b>A</b>	Standard 1 10,000 ng/ml	Standard 1 10,000 ng/ml	Sample 1 $\Delta$ OD: 1.38 2858.3 ng/ml	Sample 1 $\Delta$ OD: 1.4050 2961.9 ng/ml	Sample 9 $\Delta$ OD: 0.6310 916.79 ng/ml	Sample 9 $\Delta$ OD: 0.6270 909.89 ng/ml		
<b>B</b>	Standard 2 3330.0 ng/ml	Standard 2 3330.0 ng/ml	Sample 2 $\Delta$ OD: 1.5320 3562.5 ng/ml	Sample 2 $\Delta$ OD: 1.5940 3911.7 ng/ml				
<b>C</b>	Standard 3 1108.9 ng/ml	Standard 3 1108.9 ng/ml	Sample 3 $\Delta$ OD: 0.7450 1122.7 ng/ml	Sample 3 $\Delta$ OD: 0.7720 1174.5 ng/ml				
<b>D</b>	Standard 4 369.26 ng/ml	Standard 4 369.26 ng/ml	Sample 4 $\Delta$ OD: 0.1530 192.7 ng/ml	Sample 4 $\Delta$ OD: 0.1530 192.7 ng/ml				
<b>E</b>	Standard 5 122.96 ng/ml	Standard 5 122.96 ng/ml	Sample 5 $\Delta$ OD: 0.2340 307.79 ng/ml	Sample 5 $\Delta$ OD: 0.2460 324.80 ng/ml				
<b>F</b>	Standard 6 40.496 ng/ml	Standard 6 40.496 ng/ml	Sample 6 $\Delta$ OD: 0.6470 944.57 ng/ml	Sample 6 $\Delta$ OD: 0.6990 1037.3 ng/ml				
<b>G</b>	Standard 7 13.635 ng/ml	Standard 7 13.635 ng/ml	Sample 7 $\Delta$ OD: 0.2590 343.25 ng/ml	Sample 7 $\Delta$ OD: 0.2680 356.05 ng/ml				
<b>H</b>	Standard 8 0.00001 ng/ml	Standard 8 0.00001 ng/ml	Sample 8 $\Delta$ OD: 0.4110 564.24 ng/ml	Sample 8 $\Delta$ OD: 0.4350 600.45 ng/ml				

B)

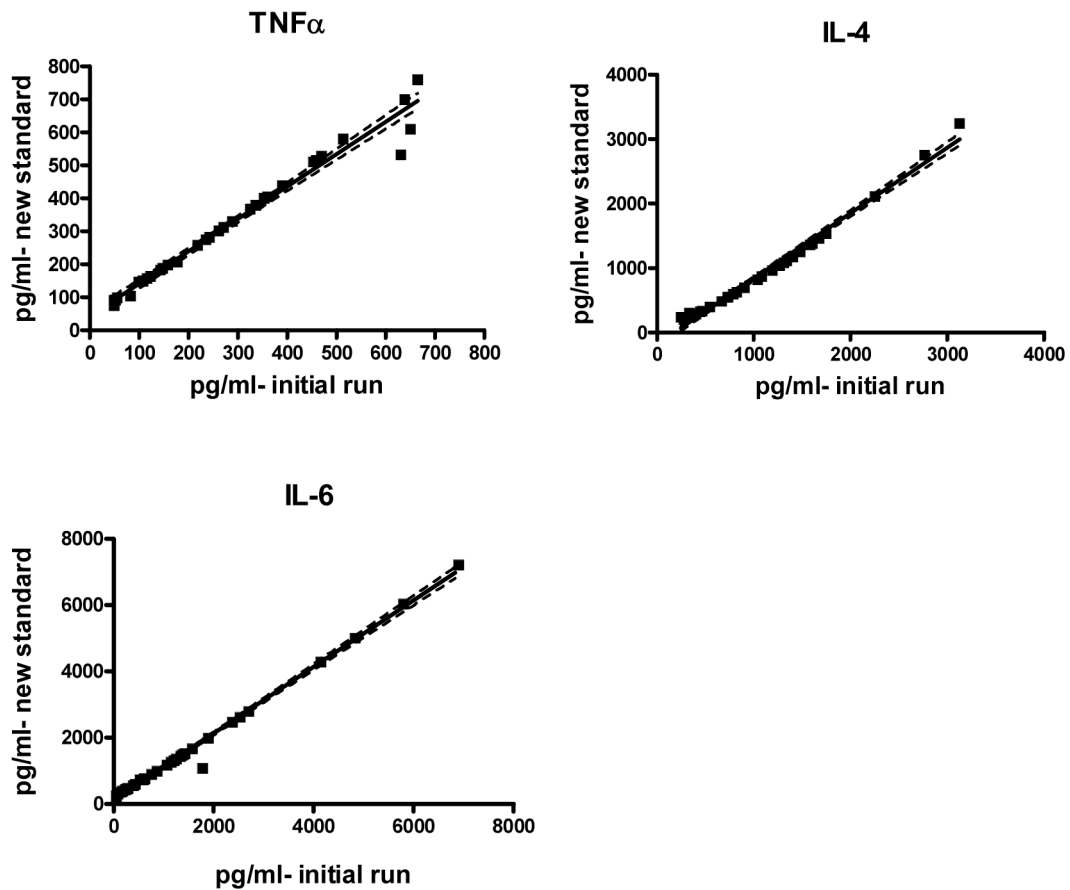
	1	2	3	4	5	6	7	8
<b>A</b>			Standard 2 2858.3 ng/ml	Standard 2 2961.9 ng/ml	Standard 5 916.79 ng/ml	Standard 5 909.89 ng/ml		
<b>B</b>			Standard 1 3562.5 ng/ml	Standard 1 3911.7 ng/ml				
<b>C</b>			Standard 3 1122.7 ng/ml	Standard 3 1174.5 ng/ml				
<b>D</b>			Standard 9 192.7 ng/ml	Standard 9 192.7 ng/ml				
<b>E</b>			Standard 8 307.79 ng/ml	Standard 8 324.80 ng/ml				
<b>F</b>			Standard 4 944.57 ng/ml	Standard 4 1037.3 ng/ml				
<b>G</b>			Standard 7 343.25 ng/ml	Standard 7 356.05 ng/ml				
<b>H</b>			Standard 6 564.24 ng/ml	Standard 6 600.45 ng/ml				

C)

**Figure 2.**

96-well plate layouts for generation of new standard curve. A) Plate layout with standard curve used to determine chosen sample concentrations. Standard concentrations are entered manually based on 1:3 dilutions of top standard. Sample concentrations are determined using standard curve. B) Re-labeling of sample wells as standards by manually inputting average concentrations of samples determined in the new standard run. C) Example of the new standard curve constructed for mouse IL-6.

A)



B)

	$r^2$ coefficient	slope
TNF $\alpha$	0.98	0.92-1.0
IL-4	0.99	0.97-1.10
IL-6	0.99	0.97-1.0

**Figure 3.**

Validation of the accuracy of the new standard curve. A) Sample concentrations determined by the initial sample run were plotted against those determined using the newly generated standard. The graphs show a nearly 1:1 correlation for all cytokines used, indicating the accuracy of this method. B)  $r^2$  values and slopes indicate the new standard correctly calculates the concentration.