Exploring Enzyme-Linked Immunosorbent Assay (ELISA) Data with the SAS® Analyst Application

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Abstract

ELISA methods are fundamental tools in the pharmaceutical industry with applications in drug discovery, animal studies, and clinical trials. ELISAs are readily automated and thus capable of high throughput quantification of analyte concentrations. Like other categories of bioassay, ELISAs are often performed on a 96-well microplate in a standard 8-row by 12-column format. Response measures from microplates often exhibit reproducible row and column patterns and several levels of variability. The SAS Analyst Application provides a graphical user interface for basic statistical techniques that can be used by scientists to explore and assess the magnitude of patterns and variability sources in assay execution. The Analyst Application provides results in a project format that is useful as a template for scientists to communicate with statisticians about basic statistical analyses.

Key words: Biological Assay Optimization, Variance Component Estimation, 96-well Microplate, ELISA, Data Visualization

Introduction

Bioassay

Enzyme-Linked Immunosorbent Assay (ELISA) methods are immunoassay techniques used for detection or quantification of a substance based on an immunological reaction (Kemeny 1991). ELISA methods were initially developed to detect serum antibodies, and they prove to be useful in many fields. For example, they have been used in environmental studies for the direct analysis of the thiocarbamate herbicide molinate (Harrison et al. 1989), and for quantitating cross reacting herbicides in environmental samples (Wortgerg et al. 1996). The food science industry uses ELISA in the analysis of food toxins (Chu et al. 1987; Dixon-Holland and Katz 1988). Agriculture and medical industries employ ELISA methods in the quantification of hormones (Rajkowski et al. 1989) and drugs (Laurie et al. 1989).

ELISA has become a fundamental tool for drug discovery, animal studies, and clinical trials in the pharmaceutical industry because of the ability to assess large quantities of samples. In the pharmaceutical industry, over 100,000 compounds are routinely tested using several different ELISA assays in early drug screens to identify promising compounds. The methods must be capable of high throughput performance, with runs quickly and reliably performed in high volume.

ELISA methods are routinely conducted in a standard 8-row by 12-column 96-well microplate format (Figure 1) that is readily automated and capable of high throughput quantification of analyte concentrations . This general format is currently being extended to plates having 16 or more rows and 24 or more columns, producing microplates with 384 or more wells.

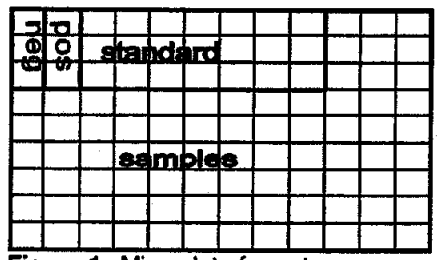

Figure 1. Microplate format

Each well of the microplate is treated in a specific manner to produce a response. For many ELISA methods, the typical response is a color change of the solution in the well, which is measured by a spectrophotometer (a device that measures the amount of light that passes through a well of the microplate). As the color of the solution in the well changes from a clear liquid (no reaction) to a dark color (for example, a 100 % reaction results in a dark blue), the optical density correspondingly increases. The optical density is then the response recorded by the spectrophotometer and used for subsequent analyses.

A common objective of a bioassay is to estimate the concentration of a test preparation by comparing its response to that of a standard preparation of known concentration. In general, the bioassay is performed by progressively diluting a standard preparation and one or more test preparations of unknown concentration. The dilution is typically carried out in an n-fold serial manner (for example, 2-fold: 0, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048). A subset of dilutions is transferred to the microplate and undergoes several processes including binding, incubating, and washing. In the final step, the spectrophotometer measures the optical density of each well. Depending on the assay, the optical density either increases or decreases as the test and standard materials are diluted, and this produces a dose-response

curve. Models are fit to estimate analyte concentration, and underlying assumptions must be tested to ensure proper estimation.

For most assay methods, the lower bound on the optical density converges to zero. In addition, for most ELISA methods there is also an upper bound that the biological response tends to achieve and/or the spectrophotometer can reasonably measure. The upper asymptote generally contains more variability than the lower asymptote. There exists a range of dilution levels between the asymptotes that produces an approximately linear response.

Model

For any single preparation, the relationship between the observed response and dilution levels often results in a sigmoidal curve. This curve can be fit using the 4-parameter logistic equation:

$$y = \frac{a-d}{1+(x/c)^b} + d + \varepsilon$$

where

y = optical density
x = dose or dilution
a = maximum response
d = minimum response
c = concentration that results in 50% response
b = slope-like parameter
ε = random error

The model parameters of this equation have an intuitive biological interpretation. The parameters *a* and *d* relate to the maximum and minimum observed optical density, and they are influenced by the amount of bound antibody and absorptive properties of the antibody to the plate. Jones et al. (1995) demonstrated significant differences in *a* and *d* among microplates, different incubation times, days, and laboratories. The parameters *c* and *b* reflect properties of the binding reaction itself. Significant changes in *c* and *b* suggest a fundamental assay change such as a change in specificity. Analyte concentration estimates are significantly influenced by the steepness of the linear portion of the curve (Karpinski 1990).

Using a variation of this model, you can use optical density readings to estimate test preparation concentrations. Within this context, the standard is often used to generate a calibration curve (Bunch, Rocke, and Harrison 1990), or in a parallel line assay approach (Rodbard and Frazier 1975; Finney 1976).

Variability and Bias

Assay methods typically exhibit experimental variability at intra- and inter-assay levels. Both types of variation can arise from several sources. Because dilutions of material are often made serially, there can be considerable correlation among a dilution series, which results in serial dilution error, a source of intra-assay variation (Racine-Poon, Weihs, and Smith 1991). Research continues on the effects of serial dilution error and methods of dealing with it (Giltinan and Davidian 1994). The microplates themselves can exhibit reproducible row and column patterns. These patterns result in considerable intra-assay variability when not identified and dealt with appropriately (Jones et al. 1995).

The microplate can also be a source of inter-assay variability when different plate types, manufacturers, and lots result in variation in the observed optical densities. Additional sources of inter-assay variation include experimental conditions under which the assay is conducted, such as various environmental conditions (for example, incubation temperature and incubation time), analysts, and biological reactants. These factors can enter the process as fixed and/or random effects, increasing the variability of the optical density in either a systematic or random fashion, respectively. These fixed and random patterns in optical density affect model fitting by increasing the intra- and/or inter-assay variability or bias in the model parameter estimates.

Increases in the variability or bias of parameter estimates produces greater variability in the estimated analyte concentration. This variability and bias negatively impact drug discovery, pharmacokenetic studies, drug stability, and clinical trials. For example, increased variability could increase the sample size necessary to obtain acceptable precision or lead to the wrong conclusions because of a significant undetected bias.

Therefore, variability and bias effects impact the system at several different levels: (1) the raw optical density readings, (2) the estimation of model parameters, and (3) the estimation of analyte concentration. The identification and elimination of these effects are difficult because they arise from numerous sources and impact the bioassay process at many levels. Several methods have been suggested to detect, control, or eliminate these effects (Bunch, Rooke, and Harrison 1990; Buonaccorsi 1986; Lansky 1997; Larholt and Sampson 1995; Plikaytis et al. 1994; Rodbard 1974; Rocke and Jones 1997; Sittampalam, et al. 1996).

Testing for Common Slopes

Parallel line bioassay techniques are often used to estimate the unknown analyte concentration of a test preparation relative to the known concentration of a standard, and they are significantly affected by variability patterns. The use of a parallel line bioassay model requires a common slope between the standard and test preparation. Therefore, differences in the slope of the standard and test preparation curves (Finney 1978) must be identified. If the test for parallel lines fails, then another method for estimating the concentration must be used.

There are two fundamental aspects of this test. First, the degree of difference between the standard and test preparation slopes should be biologically important. A priori experimentation needs to be conducted to determine the maximum amount of deviation between the two slopes that can be tolerated and still provide a biologically useful endpoint. Second, the correct error term must be used to appropriately conduct this hypothesis test. Because this class of bioassay methods are run on microplates, rows and columns can be arranged as whole and subplots (Milliken and Johnson 1984) to form split-plot and strip-plot experimental designs from which the correct error terms can be constructed for statistical tests of interest.

The Analyst Application

Detecting and understanding variability is paramount to making bioassay a useful analytical method because inflated variability results in unstable parameter estimates and/or biased responses that are biologically important. The SAS Analyst Application provides scientists access to powerful statistical and graphical capabilities in a graphical user interface so that they can investigate these basic assay questions. Designed for the desktop environment of PCs and workstations, it is a useful tool for students and researchers as well as for experienced statisticians who desire a convenient interface to widely used statistical techniques. Additionally, the Analyst Application provides a unique opportunity for statisticians working with scientists to easily discuss the impact of variability and bias through illustration and advanced statistical models.

The Analyst Application provides a wide range of analytical and graphical tasks. You can compute descriptive statistics, create crosstabulations, and analyze distributions. Basic modeling tasks enable you to fit one-way analysis of variance and simple regression models. You can fit complex linear models with interaction and nested terms, and you can fit models incorporating both fixed and random effects using the mixed models task. Other statistical tasks include linear and logistic regression, survival analysis, multivariate statistics, and repeated measures analysis. In addition, you can produce several types of graphs, including histograms, box plots, and scatter plots.

The Analyst Application organizes results in a project format for analyses performed on one or more related data sets, such as within a clinical trial. A project is displayed graphically in the Project Tree within the main window of the application. Each time you run an analysis or create a graph, the project tree expands to include nodes for analysis results, graphs, and output data sets. You can customize the Project Tree to include HTML formatted results as well as access to the SAS code submitted to perform the task.

You can invoke the Analyst Application by either typing **analyst** at the command line or selecting Solutions > Analysis > Analyst from the Display Manager System.

Two examples are used to demonstrate some of the features and capabilities of the Analyst Application. The first example is from a plate uniformity trial. The second example is from a parallel line bioassay used to examine the concentration of a drug over several trials.

Example 1. Plate Data

Introduction

A plate uniformity trial was conducted to assess the consistency of observed responses within and between plates. In the trial, the same known quantity of material was aliquotted to each of the 96 wells over an entire plate. Two microplates were run in this manner to investigate differences within and between plates.

Histogram

Histograms enable you to explore your data by displaying the distribution of a particular variable. Using histograms, you can gain an indication of the shape of the distribution and compare distributions of a variable based on other classification variables.

To open the ASSAY data set, select **File > Open by SAS Name** and double-click the ASSAY data set.

Select **Graphs > Histogram**. Specify CPM as the analysis variable and PLATEID as the class variable. In the Fit window, click on normal distribution to produce a fitted curve. Click Ok to run the analysis.

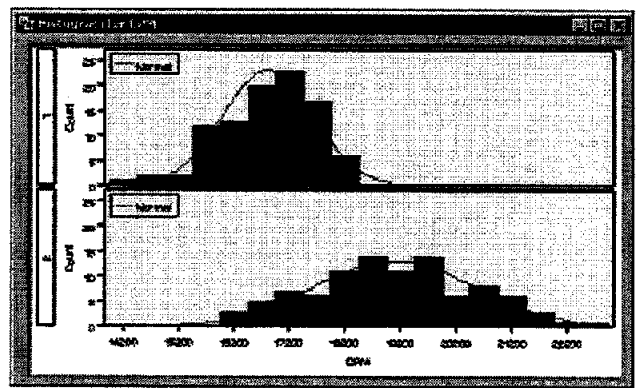

Figure 2. Comparative histogram of CPM by PLATEID with fitted Normal distribution

CPM appears to be approximately normally distributed for both the first and second plates (Figure 2). The first plate

has a smaller mean CPM than the second plate, since the distribution is shifted slightly to the left. In addition, the first plate's distribution is more concentrated about its mean, indicating that the first plate exhibited less variability than the second plate.

Although not clearly evident here, CPM is generally considered to be distributed as Poisson. Investigation into a reasonable and necessary transformation of the response can also be explored via probability plots (Figure 3). Exploratory analyses such as these often lead to further discussion regarding the impact of transformations on the error structure, the appropriateness of such transformations, and useful alternatives when transformations are not desired or appropriate.

Select **Graphs > Probability Plot**. Specify CPM as the analysis variable, click on Variables, and select PLATEID as the BY-group variable. Click Ok to run the analysis.

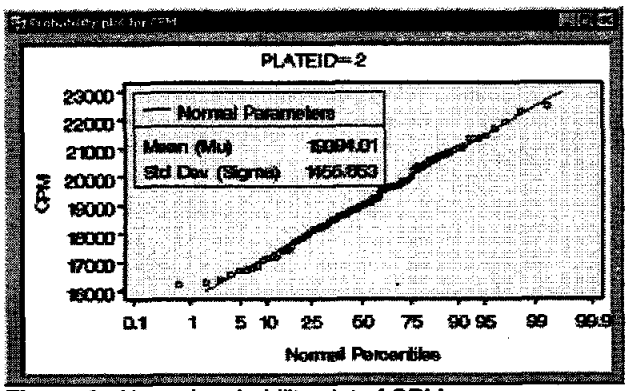

Figure 3. Normal probability plot of CPM

Boxplots

Box plots are a useful technique for displaying one-dimensional data, and they provide a concise picture of multiple distributions by placing them side by side for easy comparison.

Select **Graphs > Boxplot**. Select CPM as the analysis variable and ROW as the classification variable. In the Variables window, specify PLATEID as the BY-group variable. Click on Ok to run the analysis.

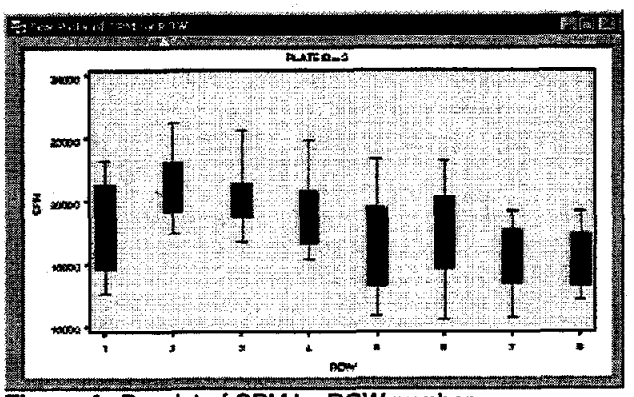

Figure 4. Boxplot of CPM by ROW number

The boxplot (Figure 4) reveals that there is a systematic relationship between plate row and CPM. There appears to be a decrease in CPM as row number increases, and a depressed response on the first row that could be attributable to edge effects. "Edge effects" are a phenomenon commonly encountered that result in biased observed responses in the wells along the outside rows and columns of a plate (that is, the 36 wells along the outer edge of a 96-well microplate).

As an example of how these edge effects might negatively impact a clinical study, consider a bioassay used to estimate blood plasma levels of a toxic metabolite resulting from a treatment. Assume that a parallel line bioassay was used to estimate the concentration of the toxic metabolite in each patient. If edge wells were always systematically assigned to blood plasma samples from low dose patients, then significantly biased concentration estimates could occur. The biased concentration estimates could be the result of the test and standard curves having different slopes.

Upwardly biased toxic metabolite concentration estimates could result in incorrectly concluding that the therapeutic window for the drug is unacceptably narrow based on the levels of the toxic metabolite relative to drug concentration. Under these conditions, the drug could be considered too dangerous when in fact it is perfectly acceptable. The results could prompt further unnecessary and expensive research into identifying an alternative delivery system or formulation, or even keep the drug from being developed and brought to market.

In this brief example, differences in slopes are biologically important and an appropriate statistical test should be used to detect when differences occur. Proper randomization and design of the assay should be implemented to avoid and detect when blood plasma concentration estimates are at significant risk of being biased due to a failure of the data to meet the necessary underlying model assumptions. As stated previously, some of these underlying model assumptions are parallel slopes, common upper and lower asymptotes (when a logistic model is fit), and homogeneous variance.

Unless edge effects are resolved, the 36 wells along the edge of the plate are unusable. Edge effects are difficult to identify with a quick glance at the data as it is read directly from the spectrophotometer. However, the scientist can quickly visualize edge effects by producing a surface plot with the Analyst Application.

Surface Plot

The Surface Plot task can be used to produce three-dimensional surface plots. For surface plots, the x- and y-values form an evenly spaced grid of horizontal values, and the z-values are predicted using an estimation method such as linear interpolation or smoothing splines.

> Select **Graphs > Surface Plot**. Specify ROW as the x-axis variable, COLUMN for the y-axis, and CPM as the z-axis variable to be predicted. Open the Display window to specify a rotation angle of 125. In the Variables window, specify PLATEID as the BY-group variable. Click Ok to produce the plot.

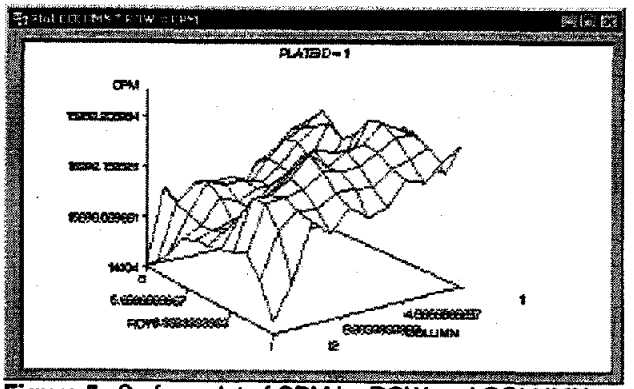

Figure 5. *Surface plot of CPM by ROW and COLUMN*

The three-dimensional surface plot (Figure 5) clearly illustrates the presence of row and column effects in this 96-well plate bioassay. Additionally, very depressed CPM readings are observed in some corners of the plate; "corner effects" are another commonly observed problem in assay methods using microplates.

Linear Model

The Linear Models task fits general linear models using the method of least squares. This analysis technique is used for experimental data in which there is a continuous response variable and one or more independent classification variables and/or one or more independent quantitative variables. The total variation in the response variable is explained as the sum of the variation due to the effects of the model and the variation due to random error.

> Select **Statistics > ANOVA > Linear Models**. Specify CPM as the dependent variable and ROW, COLUMN, and PLATEID as class variables. Click Ok to run the analysis.

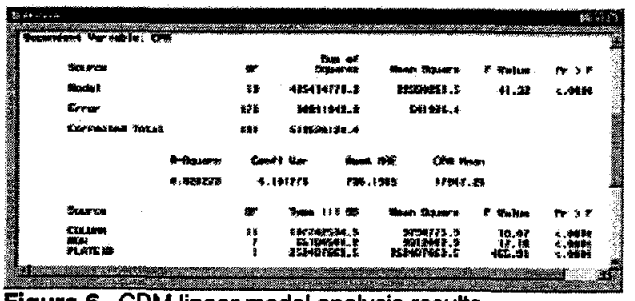

Figure 6. CPM linear model analysis results

A simple linear model confirmed that microplates (PLATEID), rows, and columns (Figure 6) were significant model effects. All three effects were significant with *p*-values less than 0.0001. The patterns observed in this example can lead to inter- and intra-assay run variability and biases that need to be identified and dealt with before the assay is used to make critical decisions. Bioassay variability components need to be appropriately identified and estimated so that comparisons of interest can be made correctly (for example, estimating differences in the concentration of a toxic metabolite relative to the drug concentration delivered to patients across treatments).

Example 2. Assay Runs for Trials

Introduction

Consider several trials conducted to investigate the concentration of a drug, where the expected concentration was 80 units. Samples of the drug were taken at the same 14 time points for each trial, but some sampling times could not be evaluated for every trial and therefore each sampling time did not have the same sample size. The objectives were to determine if there were significant differences in drug concentration among trials and sampling times.

A bioassay was designed to estimate the concentration of a drug at a target concentration of 70 $\pm$ 35 units. The throughput of the bioassay was such that a high volume of samples could be evaluated quickly. Significant inter- and intra-assay run variability was known to exist for this bioassay.

Because of resource restrictions on the assay, it took several assay runs to evaluate all sampled material from any specific sampling time. Since the assay runs represent only a random sample of potential runs, assay run is considered a random effect and the mixed models task is appropriate for fitting this model using the Analyst Application.

From a philosophical perspective, sampling time could be considered random and it could enter the mixed model as a random effect. For example, consider a model where sampling time is a random effect. In this case, you could determine the impact on performing inference on the trials given a random sample from one or more sampling times, and run in R assay runs having N potency estimates per run. If the sampling time effect was considered random, it would add another level of random error in estimating the drug potency. However, interest lies in the differences among specific trials and sampling times. Thus, both trials and sampling time were designated as fixed effects.

Mixed Models

For this experiment, the mixed linear model is

$$y_{ijkl} = \mu + \alpha_i + \beta_j + r_{k(ij)} + \varepsilon_{ijkl}$$

where

y_{ijkl} is the observed potency in trial i at time j in run k

μ is the overall mean

α_i, β_j are the TRIAL and SAMPLING TIME effects, respectively

$r_{k(ij)}$ is the random effect for (inter-assay) run, distributed iid $N(0,\sigma_r^2)$

ε_{ijkl} is the (intra-assay) random error associated with the l^{th} observed potency, distributed iid $N(0,\sigma_\varepsilon^2)$

$r_{k(ij)}$ and ε_{ijkl} are assumed to be independent of each another.

The variance of each observed potency estimate is

$$\text{Var}(y_{ijkl}) = \text{Var}(r_{k(ij)} + \varepsilon_{ijkl}) = \sigma_r^2 + \sigma_\varepsilon^2$$

The Mixed Models task in the Analyst Application enables you to specify fixed and random effects and construct main effects, interactions, nested effects, and polynomial terms. You can select from one of six estimation methods, including maximum likelihood, residual maximum likelihood (REML), and MIVQUE. A number of graphics are integrated into the Mixed Models task, including means plots for fixed effects, predicted plots, and residual plots with and without random effects. In addition, you can compute mean comparisons, output predictions to a SAS data set, and perform weighted and BY-group analyses. You can access the mixed models task by selecting **Statistics > ANOVA > Mixed Models**.

Open the POTENCY data set by clicking **File > Open** and selecting the POTENCY data set. Select **Statistics > ANOVA > Mixed Models**. Specify CONC as the dependent variable and SAMPTIME, RUN, and TRIAL as class variables.

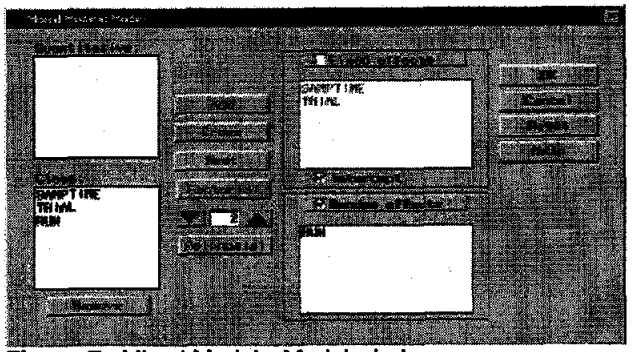

Figure 7. Mixed Models: Model window

In the Model window, select SAMPTIME and TRIAL simultaneously and click Add to specify both terms as fixed effects. Click on Random, select RUN, and click Add to specify assay run as a random effect. Click Ok.

In the Plots window, select both observed and predicted means for main effects, and click Ok.

Results and Discussion

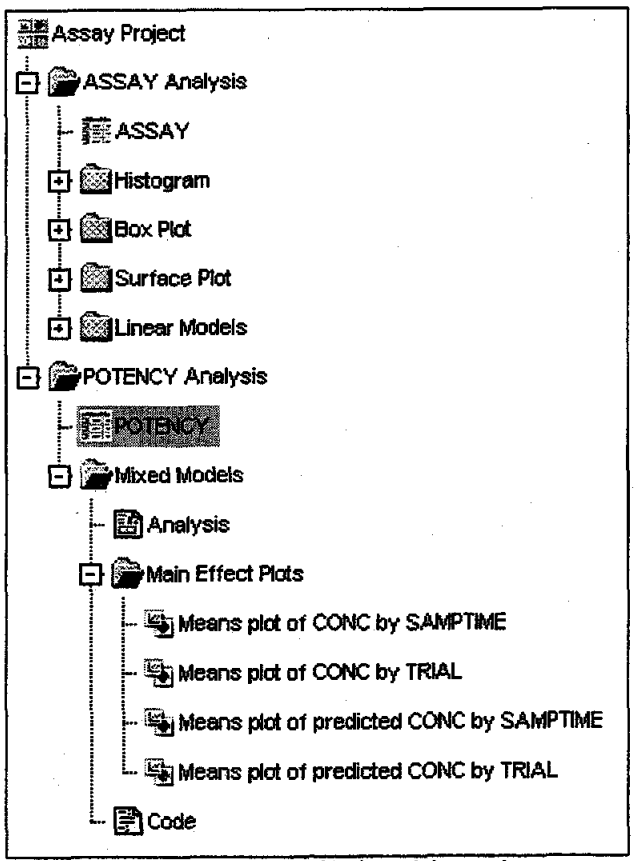

Figure 8. Project tree with mixed models results

The Analyst Application adds a folder to the Project Tree for each task you perform. In this case, the Mixed Models task produced a folder that contains analysis results, multiple main effects plots, and the SAS code that produced the results (see Figure 8). Within the project tree you can open and close a folder by clicking the plus (+) or minus (-) sign next to it, and you can open a node within a folder by double clicking on the node. In addition, you can view the same node in two or more windows, which is useful for visually comparing graphical results for different groups.

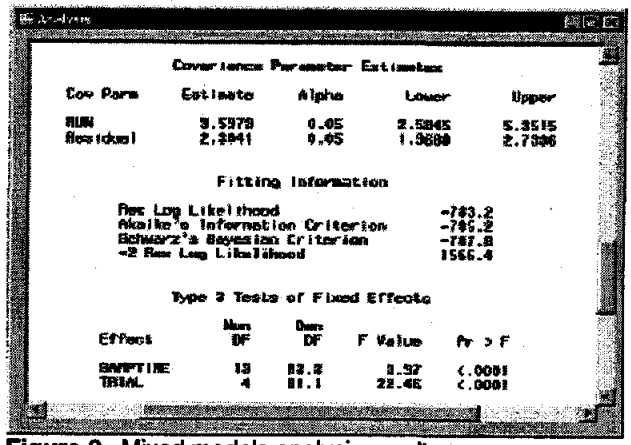

Figure 9. Mixed models analysis results

Trial and sampling time effects were both statistically significant ($p < 0.0001$, Figure 9). The means plot of concentration versus sampling time illustrates a large drop in the potency at sampling time 3 (Figure 10). After this drop, the potency proceeds to increase and stabilizes at sampling time 6.

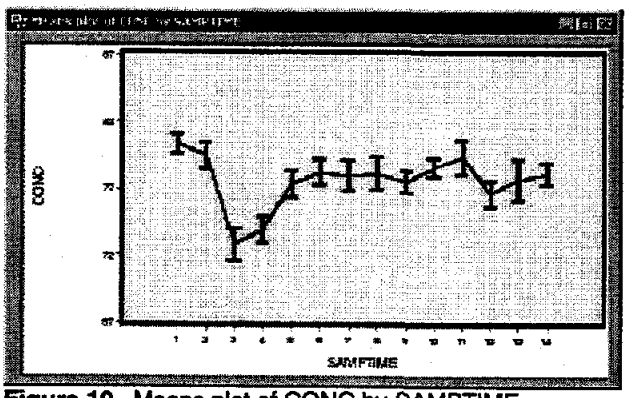

Figure 10. Means plot of CONC by SAMPTIME

Scientists interested in differences among sampling times and trials wanted to predict the response for both current and future sampling times and trials based on the formulated model of these data. Figures 10 and 11 provide a graphical framework within which a statistician can discuss the computational and philosophical differences between *confidence and prediction intervals* with scientists and management. Issues often revolve around

- Why are these intervals different?
- What is their interpretation?
- How do these philosophical differences in intervals impact the ability to make decisions about differences among trials and sampling times?

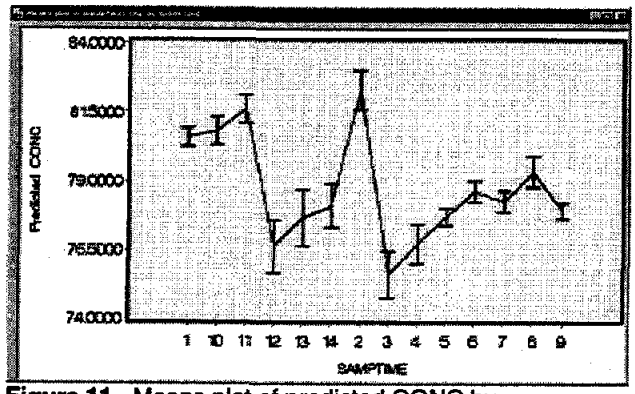

Figure 11. Means plot of predicted CONC by SAMPTIME

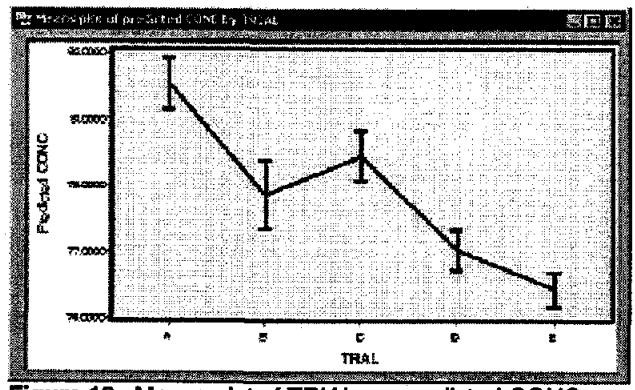

Figure 12. Means plot of TRIAL vs. predicted CONC

These graphical displays of confidence and prediction intervals are particularly useful when discussing how variability structures impact what has been observed versus what is to be predicted. The plot of predicted concentration with 95% prediction intervals versus trial illustrates that the concentration decreased with each successive trial. By comparing Figures 10, 11, and 12 with other plots generated utilizing different variance structures, scientists can see more clearly the impact of these structures on confidence and prediction intervals than if the plots were unavailable. Additionally, displaying the plots in a point-and-click interface stimulates a more interactive and dynamic discussion with scientists and management.

Because there is a significant amount of inter- and intra-assay run variability, estimating them ($\hat{\sigma}_r^2$ =3.5973 and $\hat{\sigma}_e^2$ =2.3041, Figure 9) may enable the scientist to more effectively implement the assay in the future. Based on these results, the scientist can determine the number of assay runs and potency determinations per run that will be required to achieve a desired total standard deviation (Table 1).

By inspection, the scientist can see that increasing the number of determinations across assay runs decreases the total assay standard deviation more quickly than does increasing the number of determinations within an assay run (Table 1). These results can be used to approximate the sample size necessary to achieve desired significance levels and powers.

Table 1. Table of standard deviations by assay run (R) and determinations per assay run (N)

R	N	SD
1	1	2.4293
1	2	2.1793
1	3	2.0893
1	4	2.0429
2	1	1.7178
2	2	1.5410
2	3	1.4774
2	4	1.4445
3	1	1.4025
3	2	1.2582
3	3	1.2063
3	4	1.1795
4	1	1.2146
4	2	1.0897
4	3	1.0447
4	4	1.0214

Summary

ELISA techniques are useful quantitative methods in many fields. The use of such equipment as microplates and automated systems for collecting data is coupled with a need for appropriately handling the data generated by these systems through proper experimental design and randomization. The Analyst Application is an extremely useful tool for the assessment by scientists and statisticians of these data throughout the lifetime of development and use of a bioassay. For more information on the Analyst Application, visit the SAS web site at http://www.sas.com/rnd/app/da/analyst.html.

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