

Essentials in Bioassay Design and Relative Potency Determination

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2/29/2016

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This paper describes common components of a relative potency bioassay and provides a framework for assay development, calculation, and control.

In defining bioassays and relative potency, the *United States Pharmacopeia (USP)* states (1):

“Because of the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically similar, statistical similarity (a consequence of the Test and Standard similarity) should be present, and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unitless measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations.”

The purpose of this article is to provide a presentation of the most common components of a relative potency bioassay and to provide a framework or platform for assay development, calculation, and control. It is not meant to be mutually exclusive or mutually exhaustive of all the issues associated with a bioassay, but it covers the essential components in relative potency calculation and reporting.

Bioassay Purpose and Fitness for Use

Bioassays can be *in vitro*, *in vivo*, or *ex vivo* and can be used for a variety of purposes. Those purposes include the following:

- Process development, process characterization, and product development
- Product release testing for drug substance or drug product
- In-process control and intermediates testing
- Stability and product Integrity testing.

Bioassay dose response

Getting the bioassay dose response dilution scheme correct is one of the most important aspects of a bioassay. There are many dose response strategies that may be used, such as the following:

- Single dose (not recommended)
- Two dose (not recommended)
- Three dose (not recommended)
- Four dose (good but difficult to mask for saturated doses)
- Five dose (good for parallel line analysis [PLA] and dose masking)
- Nine dose + (see Figure 1) or more (good for four parameter [4PL] fitting and dose masking).

Generally, one-, two-, and three-dose schemes should be avoided as they are difficult to control variation and will be problematic during relative potency calculation. The ideal dose response curve is a five-point curve for parallel line analysis (linear) and a nine-point dose scheme or more for sigmoidal fits. For a sigmoidal curve, ideally it should be three points in the inactive or low response section of the curve, three points in the linear part of the curve and three points in the saturated area of the curve on the log or log₁₀ scale from a serial dilution. In a parallel line analysis (non-sigmoidal), a five-dose scheme is ideal in the linear part of the curve.

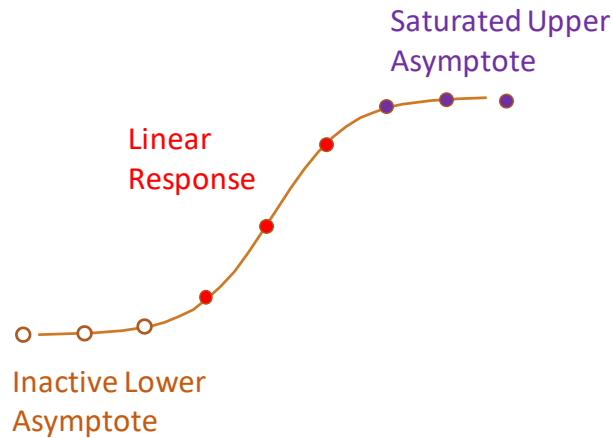


Figure 1: Nine-dose sigmoidal curve.

4PL versus 5PL fitting

If fitting a logistics sigmoidal curve, there are two common curve-fitting techniques: four parameter (4PL) and five parameter (5PL). Four-parameter curves assume symmetry in the upper and lower region of the curve fit, and five-parameter curves assume the curve shapes of the upper and lower asymptote are different. An evaluation of the residuals from the 4PL fit will indicate if the curve fitting technique is appropriate or if a 5PL fit is warranted. Residuals should be random and normal and show no systematic pattern across the dose response if the 4PL fit is good.

Outlier analysis

In discussing outliers (see Figure 2),

USP 1032 states, "The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample."

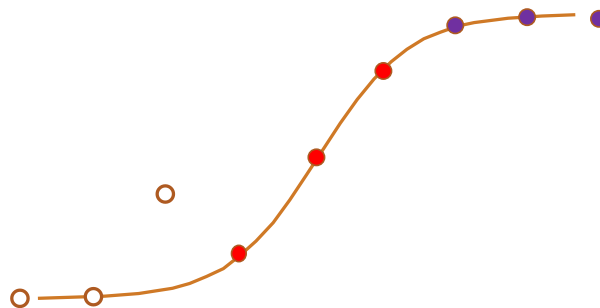


Figure 2: Nine-dose 4PL with outlier.

The following are recommended approaches for outlier detection and removal (4):

- Studentized residuals from the curve fit is greater than 2.576 (99% risk)
- Jackknife z score of the removed point of 2.576 or more (99% risk).

The influence of the removed point on the curve can be evaluated by examination of the change in R^2 and Root Mean Squared Error (RMSE) of the residual error. Generally, no more than one point or one dose on the dose curve should be removed.

Transformation and Curve Weighting

Data transformation may be done in both the dilution and the measured response if it can be shown to improve the linearity of the assay. *USP 1034 (2)* states,

“Fit the statistical model for detection of potential outliers, as chosen during development, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.”

Weighting reduces the variation in relative potency determination if the variation along the dose response lacks homogeneity. Typically, $1/\text{variance}$ of the residuals at each dose is used when the variation is statistically different. An unequal variances test using Bartlett's or Levene's can be used to determine if the variances are statistically different. Fixed $1/\text{variance}$ weighting is generally the recommended approach. Weighting is applied to both reference and test.

Masking for Parallel Line Analysis

Masking or removal of the saturated measurement (see Figure 3) in a linear parallel line analysis is recommended. Only the high or low (saturated) dose of the response curve is removed, and sequential three or four doses are used for relative potency determination depending on the dosing scheme. Masking will remove the saturation (hockey stick effect) in the parallel line analysis.

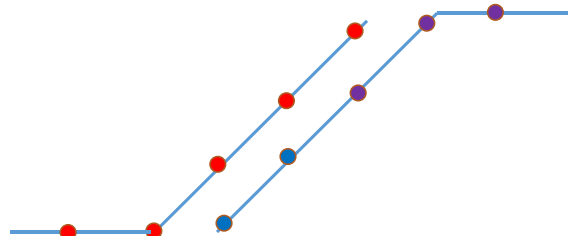


Figure 3: Hockey sticking (saturated) dose response for a linear fit.

Systems Suitability and Evaluating Parallelism

There are several techniques for the evaluation of parallelism. Table I provides a summary of methods for the evaluation of parallelism. Other systems suitability criteria include the measurement of a known control, relative variation (%CV) of repeated measures, and curve depth for signal strength.

Evaluation Method	Recommendation	Limit	Rationale
F Test	Not Recommended	Report Only	Too sensitive
Chi-Square Test	Not Recommended	Report Only	Too sensitive
Upper Asymptote Ratio (Test/Reference)	Recommended	Ratio Limit or Equivalence Test	Measures relative parallelism
Lower Asymptote Ratio (Test/Reference)	Not Recommended	Ratio Limit or Equivalence Test	Scaled by low values, not reliable
Slope Ratio (Test/Reference)	Recommended	Ratio Limit or Equivalence Test	Measures relative parallelism
Constrained-Unconstrained RP Difference	Recommended	Limit	Measures relative parallelism
PLA Interaction p-value	Recommended	Alpha = 0.05 or 0.01	Measures relative parallelism
Curve Depth Ratio (Test/Reference)	Recommended	Ratio Limit or Equivalence Test	Measures relative signal

Table I: Evaluation of parallelism and systems suitability.

Calculation of Relative Potency

The calculation of relative potency is shown in Equation 1 and may change depending on the assay. Reference/Test or depending on the assay Test/Reference.

$$\frac{EC_{50} \text{ Reference}}{EC_{50} \text{ Test}} * 100 + CF_{\text{Reference}} + CF_{\text{Reference Stability}}$$

[Eq. 1]

EC50 is calculated from the 4PL constrained inflection point and back transformed (antilog) using the following equations depending if it is log 10 or log transformed in the dilution factor (see Equation 2).

$$\begin{aligned} \text{Log 10} \quad EC_{50} &= 10^{\text{Inflection Point}} \\ \text{Log} \quad EC_{50} &= \text{Exp}(\text{Inflection Point}) \end{aligned}$$

[Eq. 2]

Controlling the Influence of the Standard in Relative Potency

Notice in Equation 1 that there is a correction factor (CF) applied to the relative potency determination. CF reference is a correction factor for qualification of a new reference to correct for shifts in potency due to a change in reference. CF reference stability is a correction for the loss of potency of the reference over time. Failure to include these correction factors in the potency calculator will cause the assay to become hyper or hypo potent overtime.

Work Flow in the Potency Assay

Figure 4 presents a good workflow for a bioassay.

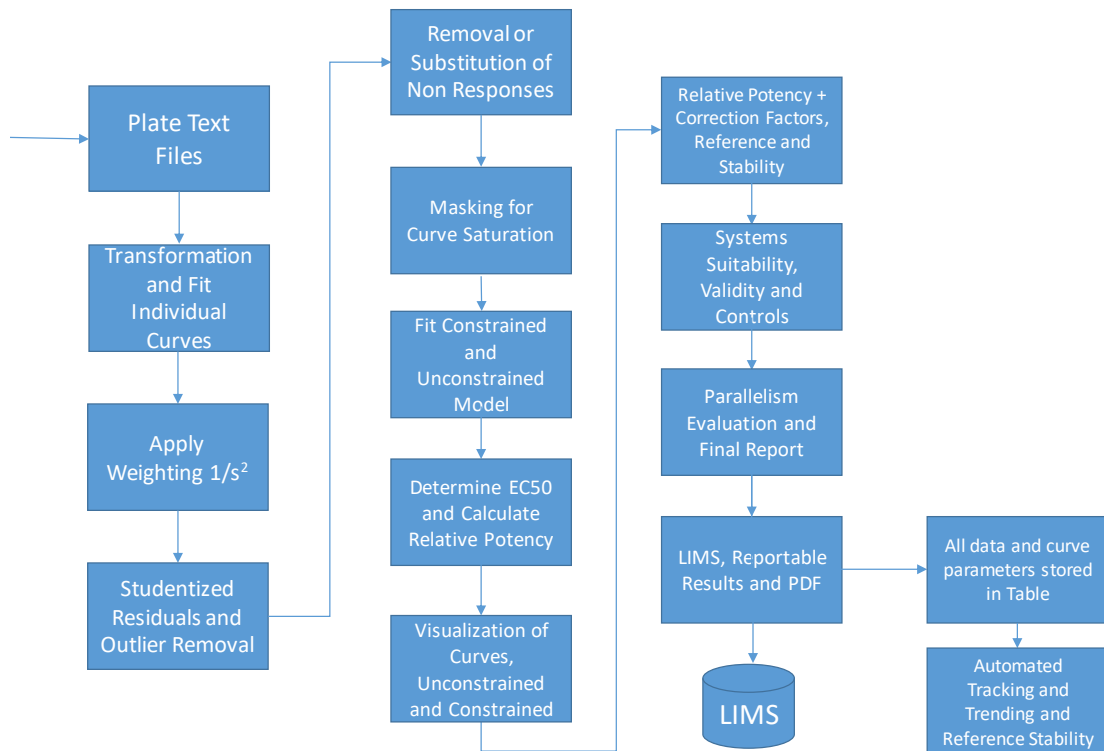


Figure 4: Bioassay workflow.

Data from the bioassay should be quality control checked, and all reportable results should be sent to a laboratory information management system (LIMS). Key parameters and reagents from the bioassay should be used for tracking and trending.

Monitoring and controlling relative potency

It is critical to monitor the performance of the bioassay over time. The unconstrained EC_{50} standard is generally considered the best measure of the stability and consistency of the assay. To detect if assay drift is a method issue or standard issue a comparison to the unconstrained EC_{50} should be made. If both the reference and the test are drifting, it is change in the assay over time but no impact to relative potency. If the test is stable and the reference is trending it will impact relative potency and should be corrected or a new reference selected.

Conclusions

Bioassays are powerful tests of relative potency and biological activity but need care in the design of the assay and the analysis to make sure they are a reliable indication of the change in potency. Validation of the assay (3) will demonstrate the accuracy, repeatability, and linearity of the assay and its fit for use in the measurement of relative potency. Care in the selection of the dose response, outlier detection and removal, masking, transformation, and weighting will make the assay more stable and repeatable.

References:

1. USP, <1032> Design and Development of Biological Assay, USP–NF [38], December 2015.
2. USP, <1034> Analysis of Biological Assays, USP–NF [38], December 2015.
3. USP, <1033> Biological Assay Validation, USP–NF [38], Dec. 2015.
4. T. Little, Out of Trend Identification and Removal, *BioPharm International* 29 (1) (January 2016).