United States Department of Agriculture
Center for Veterinary Biologics
Standard Operating Policy/Procedure

Using Software to Estimate Relative Potency

Date: **September 10, 2015**

Number: CVBSOP0102.03

Supersedes: CVBSOP0102.02, February 9, 2015

Contact: Christopher Tong, (Christopher.H.Tong@aphis.usda.gov)

Approvals:

/s/Steven A. Karli Date: 21Sep15
Steven A. Karli, Director
Inspection and Compliance
Center for Veterinary Biologics

/s/Byron E. Rippke Date: 11Sep15
Byron E. Rippke, Acting Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Byron E. Rippke Date: 11Sep15
Byron E. Rippke, Director
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

INTERNAL USE ONLY

Entered into CVB Quality Management System
by: /s/Linda S. Snavely 25Sep15
Linda S. Snavely Date
Quality Management Program Assistant

Table of Contents

1. Introduction

1.1 Relative potency

Relative potency (RP) assays compare two preparations of a particular analyte that behave in the assay as if they are identical but for their respective concentrations. This document describes methods used by the Center for Veterinary Biologics (CVB) Laboratories for estimating relative potency in enzyme immunoassays (e.g., ELISA) during testing activities similar to the serial release testing of veterinary vaccines. The methods described are not appropriate for evaluating parallelism in assay validation studies. For general information about relative potency estimation and assay validation of relative potency assays, see Veterinary Services Memorandum (VSM) 800.112, Appendix III.

1.2 Preparations

Relative potency immunoassays of veterinary vaccines typically compare a reference vaccine (reference) and the vaccine lot being tested (serial). The reference shall be a serial of product produced per an approved *Outline of Production* or a purified reference consisting of only the agent, a subunit of the agent, or a non-adjuvanted harvested culture of microorganisms. The reference shall be an Animal and Plant Health Inspection Service (APHIS) approved reference that has been directly or indirectly correlated to a host animal efficacy trial in which sufficient protection was elicited to pass the minimum requirements set by APHIS for the agent being tested.

1.3 Methods

Two methods are described. Select the one that follows the validated method in the approved Outline of Production.

The first method is loosely based on the parallel line assay concept, and its data model is a straight line (simple linear regression). This method is appropriate for assays designed, optimized and validated for parallel line analysis, which has the response restricted to the approximately linear portion of the usually nonlinear response curve. Data from such assays are termed ‘fully linear’ in this document. Data that include the pseudo-linear region plus part(s) of the curved regions of the dose response will require *masking* some dilutions prior to analysis; such data are here termed ‘partially linear.’ (Masking refers to removing the dilutions that are not part of the pseudo-linear region for analysis.)

The second method is based on the full dose response concept, and its data model is the 3-parameter logistic (3PL) sigmoid curve. Data of this type are termed ‘full dose response.’ This method assumes a constant variance function and no asymmetry in the response curve.

2. Materials

2.1 *In vitro* enzyme immunoassay test results

2.2 Equipment/instrumentation

A computer software program capable of implementing the calculations described below.

Presently, the CVB Laboratories use SoftMax Pro (SMP) versions 6.2.1 or 6.3 on the Windows 7 platform; later versions of an SMP/operating system combination may be used if their behavior is not substantially different. The CVB does not endorse any particular software program and the calculations described below can be readily implemented in several other software programs. Use of the same software package by the firm and the CVB is not required if both software packages implement the same method of statistical calculation.

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel should be familiar with enzyme-linked immunoassays. A minimal knowledge of personal computers and the use of software described in **Section 2.2** is also required.

3.2 Preparation of equipment/instrumentation

If using SMP, CVB users should employ the current versions of an SMP protocol file ("partiallylinear.spr" or "fulldose.spr") prepared by the CVB Statistics Section. These protocol files are tested for use in SMP versions 6.2.1 and 6.3 only and need validation before use in alternate versions of SMP. **Section 4** of this document describes the methods provided in the SMP protocols. Example files illustrating these calculations have been posted on the Molecular Devices Community website for SoftMax Pro.

- Analyzing partially linear data for serial release testing: http://www.softmaxpro.org/protocol/show/58
- Analyzing full dose response data for serial release testing: http://www.softmaxpro.org/protocol/show/60

The following information is specific to the test and must be garnered from relevant technical documents, such as Special Outlines, Standard Operating Procedures, or Supplemental Assay Methods:

- Serials and controls, their dilution sequences, and their placement on the plate (plate layout);
- Optical reader wavelength(s);

- Calculations for optical density (OD) corrections, such as subtracting background ODs from different wavelengths or subtracting an average blank OD from all well ODs;
- Assay validity criteria, such as minimum optical density (OD), minimum slope, and parallelism criteria, as well as valid ranges for controls.

Once the above information is acquired, they should be entered into the appropriate fields in the SMP protocol, when applicable. Regions of the plate that are empty or not part of the study should be labeled 'NPS' to indicate 'Not Part of Study.' In SMP, optical reader wavelength(s) are specified in the Settings menu, and simple OD corrections can be specified under the Data Reduction menu.

4. Instructions for Methods

4.1 General approach

4.1.1 Data from a given plate should be analyzed together and not combined with data from other plates.

4.1.2 Evaluate plate validity criteria, such as those for the blank and control wells. If the validity criteria are satisfied, proceed.

4.1.3 Graph the optical density (OD) data of the reference and test serial on the same plot. The OD is displayed on the vertical axis, and the log-transformed dilution is displayed on the horizontal axis. At this stage, the OD data from each well (not their average for a given dilution and preparation) should be displayed. Ensure that the appropriate correction (e.g., by the mean plate blank OD) is applied to the data. Visually evaluate the OD data for suitability. If the data has the expected dose response, proceed. If there is insufficient or unexpected dose response (e.g., approximate flat line response), no RP estimate should be reported, as the data are not suitable for relative potency estimation. If the Special Outline, Outline of Production, or Standard Operating Procedure specifies rules for handling extreme or unusual values (outliers), implement these prior to curve fitting.

4.1.4 Fit and plot appropriate data models to the data from each serial *individually*. Visually evaluate the quality of fits (e.g., how closely the fitted curves pass through the data). If the latter is acceptable, evaluate the validity criterion for parallelism corresponding to the appropriate data model. If the criterion is satisfied, proceed; if not, no RP estimate should be reported. **Sections 4.2 and 4.3** provide details for specific data models, respectively, for fully or partially linear data, and full dose response data.

4.1.5 Fit and plot a *simultaneous* data model constrained to have parallelism between the reference and test serial. (If there are multiple test serials on the plate,

fit a *separate* simultaneous data model for *each* test serial with the reference.) Visually evaluate the quality of fit. If the latter is acceptable, report the RP estimate. Ensure that the reported RP is qualitatively reasonable, e.g., if the test serial curve is to the right of the reference, the RP estimate should be greater than one, in most conventional ELISAs. Again, details for specific data models may be found in **Sections 4.2 and 4.3**.

4.2 Fully or partially linear data

4.2.1 Decide if the data from all dilutions may be used (fully linear case) or whether a subset of the dilutions, i.e., a pseudo-linear region, should be identified (partially linear case).

4.2.2 If a pseudo-linear region should be identified, use the guidance in the relevant Outline of Production, Special Outline, or Standard Operating Procedure to identify the dilutions covering the region. That document should specify the suitable regions identified during assay validation. When no such guidance is available, an official test is not possible, but testing may be done for unofficial reasons. In that case, choose the pseudo-linear regions using the following criteria, in order of decreasing importance.

a. Choose *at least three,* preferably as many as possible, *consecutive* dilutions for a given serial. Do not select non-consecutive regions of the curve.
b. Choose sections of the curve with the least amount of curvature (relatively straight).
c. Choose sections of the curve corresponding to the steepest part of the curve, avoiding flatter, more horizontal regions.
d. Choose sections of the curve most parallel between the reference and the test serial. (The subset of dilutions need *not* be the same for the reference and test serial.)

4.2.3 The data model is simple linear regression of the (corrected) OD vs. the log dilution. This data model has two parameters, the slope m and the intercept b. The equation is $y = m \times x + b + \varepsilon$, where y is the optical density, x is the log dilution, and ε is the error term.

4.2.4 The parallelism validity criterion is that the ratio of slopes (between the test serial and reference) should fall within an acceptance window, to be specified by the Outline of Production, Special Outline, Standard Operating Procedure, or Supplemental Assay Method. In cases where no acceptance window is specified, such as a Special Outline that uses historical RelPot analysis software, use an interval of 0.80 to 1.25 inclusive.

4.2.5 If the parallelism criterion is met, the simultaneous data model should have the slope for both reference and test serial constrained to be equal. The

difference in intercepts is used to determine the relative potency using classical parallel line analysis (see e.g., Armitage et al., 2002; Finney, 1964; Redmond, 2005). Ensure that other validity criteria are met, e.g., the fitted slope exceeds the minimum slope. (We expect the Special Outline or Outline of Production to always specify a nonzero minimum absolute value of the slope for parallel line assays.)

4.3 Full dose response data

4.3.1 When enough of the full dose response, from saturation to extinction, is consistently exhibited for *both* test serial and reference, an *S*-shaped curve such as a 3PL (3-parameter logistic) curve may be fitted. The 3 parameters are often called the upper asymptote, inflection point, and scale factor. In many software programs, a 4-parameter logistic curve option is offered, but it can be converted to a 3PL by constraining the lower asymptote to be zero.

4.3.2 There are several parameterizations of the 4-parameter logistic curve. A commonly used parameterization, used by SMP software, is as follows.

$$y = D + \frac{A-D}{1+(x/C)^B} + \varepsilon,$$

where *y* is the optical density, *x* is the dilution, *A* and *D* are the asymptotes, *B* is the scale factor, and *C* is the inflection point, and ε is the error term. Depending on the type of assay and the sign of *B*, either *A* or *D* would correspond to the lower asymptote. In SMP, this data model is selected by "4-Parameter" under the Curve Fit dropdown. If not using the CVB protocol, please specify which parameter is being constrained.

4.3.3 The parallelism validity criteria are as follows. The upper asymptotes and scale factors of the reference and test serial should be compared. The ratio of the upper asymptote of the test serial to that of the reference should fall within a specified acceptance interval. Also, the ratio of the scale factor of the test serial to that of the reference should fall within a specified acceptance interval. *Both* of these criteria must be met to demonstrate acceptable parallelism. The Outline of Production, Special Outline, Standard Operating Procedure, or Supplemental Assay Method should specify the acceptance intervals; if not, consult the CVB Statistics Section.

4.3.4 When the parallelism validity criteria are met, the simultaneous data model should have the upper asymptotes for both test serial and reference constrained to be equal, and the scale factors for both test serial and reference constrained to be equal. The inflection points of the reference and test serial are used to estimate the relative potency, depending on the parameterization used. Ensure that other validity criteria are met.

4.3.5 The CVB Statistics Section can provide a technical document providing guidance on how to work with existing parameterizations of the 4-parameter logistic model, available in certain software programs, to implement the above calculations.

5. Interpretation of the Test Results

For a serial to be satisfactory, the relative potency estimate resulting from the above calculations must be greater than or equal to the required minimum contained in an approved APHIS Outline of Production for the product being tested. If any of the validity criteria are not satisfied, the result is considered a "no test".

6. References

6.1 P. Armitage, G. Berry, and J. N. S. Matthews, 2002: *Statistical Methods in Medical Research*, 4th edition. Blackwell Science (Malden, MA), Sec. 20.2.

6.2 D. J. Finney, 1964: *Statistical Method in Biological Assay*, 2d edition. Hafner (New York), Sec. 4.11.

6.3 C. K. Redmond, 2005: Parallel-line assay. In *Encyclopedia of Biostatistics*, 2d edition, edited by P. Armitage and T. Colton. Wiley (Chichester, U.K.), vol. 6, pp. 3936-3942.

6.4 D. Siev, 1997: Interpretation and estimation of relative potency in vaccines. *Journal of Immunological Methods*, 208: 131-139.

7. Summary of Revisions

Version .03

- Clarified last sentence of Section 4.2.5.

Version .02

- Revised the title of the document.
- Updated the Contact information.
- Added clarifying language at the end of Sec. 1.1 and beginning of Sec. 1.3.
- Improved readability of the equation in Sec. 4.3.2.