Animal and Plant Health Inspection Service

Veterinary Services

1400 Independence Ave, SW

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VETERINARY SERVICES MEMORANDUM NO. 800.112

TO: Veterinary Services Leadership Team
Directors, Center for Veterinary Biologics
Biologics Licensees, Permittees, and Applicants

FROM: John R. Clifford
Deputy Administrator

JOHN CLIFFORD

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SUBJECT: Guidelines for Validation of In Vitro Potency Assays

I. PURPOSE

This memorandum provides guidance concerning the information a firm should provide when submitting a new potency assay for consideration by the Center for Veterinary Biologics (CVB). This memorandum further clarifies information found in title 9, *Code of Federal Regulations* (9 CFR), sections 102.3(b)(2)(ii) and 113.8(a)(3)(ii), and in Veterinary Services (VS) Memorandum No. 800.50.

II. REPLACEMENT

This memorandum replaces VS Memorandum No. 800.112 dated August 29, 2011, in order to correct an error in part IV, Scope, and to remove reference to an obsolete VS Memorandum in appendix 1, section 2.6

III. BACKGROUND

Assay validation provides evidence that an assay does what it is intended to do. All assays, regardless of format or function, must be relevant, reliable, reproducible, and scientifically sound. The formal process for evaluating these characteristics is commonly known as validation. This document provides guidance for validating veterinary biologics potency assays.

IV. SCOPE

These guidelines apply to *in vitro* assays used to determine the potency of veterinary biological products. They provide a framework for designing *in vitro* potency assays and the studies needed to validate those assays.

V. GUIDELINES

The following documents containing guidance for the validation of *in vitro* potency assays are attached as appendices to this memorandum.

Appendices
Appendix I, Guidelines for Validation of In Vitro Potency Assays
Appendix II, Suggested Validation Report Topics
Appendix III, Guidance for Validating ELISA Relative Potency Assays

Appendix I

Guidelines for Validation of In Vitro Potency Assays

1. *Introduction.*

   1.1. *Aim*. These guidelines include general principles intended to apply to in vitro assays. While specific methods or criteria may vary due to the nature of particular assay types, a common set of concepts underlies the idea of assay validation. This document is intended to outline only a general approach to validation; not every recommendation may be applicable to every assay in every circumstance.

   1.2. *Validation phases*. The validation of a potency assay begins when the assay is proposed and its relationship to efficacy in the target species is first investigated. Validation continues through the development of the assay as it is first optimized and various aspects of its precision and accuracy are characterized. Subsequent validation activities involve the transition to implementation and monitoring the behavior of the assay over time when it is in routine use.

   1.3. *Validation vs. use*. Before implementation, the assay is optimized and its essential characteristics are demonstrated. When that is done, the assay is considered validated for its intended use; and, so long as the test is conducted according to the optimized procedure, the results of the assay are considered to be valid. Consequently, procedures required to be performed during assay optimization may not be necessary after adoption for routine use. For example, the range of dilutions used during validation of the assay may be greater than the range necessary for routine use. Consequently, the validation process must include an evaluation of the performance of the assay under routine use conditions.

2. *Outline of the Validation Process*. Validating a test procedure occurs in steps that include conceptualization, development, optimization, and verification that the test does what it is supposed to do. Reports may be submitted to Center for Veterinary Biologics (CVB) after completion of any step in the process. The final report typically includes data from the verification steps, and may also refer to earlier work on the conceptualization, development, and optimization of the assay.

   2.1. *Conceptualization:* Issues that should be addressed early in assay development:

   - The relationship between the response measured in the potency test and efficacy in the target species.
   - The composition of the reference or standard.
   - The availability of reagents.
   - The ability of the assay to measure the analyte or parameter of interest.

- The ability of the assay to measure the analyte in the range of concentrations expected in test and control preparations.
- The type of sample processing required.
- The potential effect of interfering or cross-reacting materials in the test preparation.

2.2. *Development and optimization:* During development and optimization, the firm should:

- Evaluate assay reactivity against placebo material.
- Determine the optimum extraction and/or antigen elution conditions.
- Assess the effect of long-term contact of the entity of interest with adjuvant, if appropriate.
- Assess the effect of adjuvant saturation (a critical concern when comparing a monovalent reference to multivalent products).
- Determine the final assay conditions and reagent concentrations.
- Determine the criteria for acceptance for reagents, references, and controls.
- Determine assay performance with actual unknown.
- Incorporate the use of controls and methods of monitoring assay and reagent performance.

2.3. *Verification.* The firm should determine accuracy, precision, selectivity, sensitivity, and ruggedness with the test method and provide that data to CVB. The guidance in this section should be taken as suggestions that are not necessarily applicable to every assay in every circumstance.

2.3.1. *Specificity/selectivity.* Evaluate the ability of the assay to selectively detect the analyte without being significantly affected by cross-reactive substances. This may be done by assessing the response curve for placebo vaccines, vaccines spiked with potentially interfering substances, or vaccines containing similar but nonidentical analytes. Ideally, such preparations would show no evident dose-response in the assay, and any detectable signal would be trivial compared with the signal of the analyte. Test methods that are used for potency testing multivalent vaccine formulations must show that the dose-response curve of each formulation is similar to the reference or standard dose-response curve. More than one serial or serial prototype of each formulation should be evaluated. CVB may ask for additional data if

the data for specificity/selectivity suggest multivalent formulations have different dose-response curves.

2.3.2. *Analytical sensitivity*.

- *Limit of detection (LOD)*. Determine the least amount of analyte that can be distinguished from background, but not necessarily quantified.

- *Limit of quantitation (LOQ).* Determine the lowest and highest concentrations of an analyte that can be quantified with an acceptable level of accuracy and precision.

- *Signal to background (S/B).* Evaluate the ratio of the signals of analyte and reagent blank.

2.3.3. *Accuracy*. To evaluate accuracy, compare measurements produced by the assay to values that are assumed to be correct, such as those associated with an accepted standard, or the nominal values of preparations formulated for that purpose. The concentrations of the analyte should span a range appropriate for the assay.

2.3.4. *Precision*. Design a study to evaluate precision appropriate to the application, which may include within-assay, between-run, and between-day variance components. Depending on the analyte level, interest may be focused on the precision of a raw measurement (such as optical density) or the final value (for example, titer). A nested design with adequate replication for each variance is often appropriate (such as several runs on each of several days by each of several operators).

2.3.5. *Discrimination*. The ability to discriminate between close values is a function of both accuracy and precision. Formulate preparations with a range of potencies (analyte concentrations) suitable for determining the ability of the assay to discriminate between satisfactory levels of analyte and marginally unsatisfactory levels. The discrimination of a potency assay reflects its diagnostic sensitivity, in the sense of reliably detecting an unsatisfactory serial.

2.3.6. *Ruggedness*. Evaluate ruggedness by observing the effect of changes in incubation time, incubation temperature, operators, reagent lot, or other test conditions on the test result. Also, consider the possibility that systematic features of the assay's structure and design may have an effect on the test results. An example is enzyme-linked immunosorbent assays (ELISA) plate location effects, which may be evaluated with a uniformity plate.

2.3.7. *Other*. Verification of particular types of assays may need to include the evaluation of other critical elements specific to the assay type. Critical elements for specific assay types are outside the scope of this document.

2.4. *Serial release*. Determine the format for routine use of the assay in serial release testing. Describe the method of calculating the potency estimate and proposed validity specifications for an individual test. Requests to consider an assay that does not entirely meet ideal assay assumptions for use in serial release testing should be accompanied by:

- An explanation of the mechanism producing the departure from the ideal;
- Experimental evidence supporting the explanation; and
- A quantitative assessment of the size of the departure and its impact on potency estimation.

2.5. *Monitoring.* Include a plan in the final validation report for monitoring the performance of the assay in routine use to show it continues to behave as expected. A monitoring plan typically includes control preparations, statistical and graphical tools for assessing the performance and stability of the assay, standards, and references.

2.6. *Report*. Validation reports may be submitted in stages. Upon completion of validation and compilation of preliminary monitoring data, the firm should submit a validation report containing information on the work that has not been previously submitted. A suggested list of topics for the report can be found in Appendix II. The report must include:

- Raw data in an electronic file as outlined in the veterinary biologics Electronic Submissions web page;
- Complete dose-response data graphically plotted; and
- Relevant estimates of potency, variance components of potency, and variances or coefficients of variation of other quantities (e.g., optical densities) where appropriate.

3. *Definitions*.

3.1. *Accuracy.* The closeness of agreement between the value produced by the assay and the correct value.

3.2. *Analyte.* The component of an unknown that is measured by the test system.

3.3. *Internal control.* The internal control (IC) is a preparation included in an assay to serve as an independent measure of the assay's performance. ICs may be crude

preparations, semi-purified or purified fractions of the unknown, or other materials that respond similarly to the reference and analyte in the assay. An IC is stored under conditions that preserve stability and maintain consistent performance. An independent measure would be another test method or panel of test methods that rely on a different principle than the test system being monitored. These independent test methods must address qualitative and quantitative features of the IC.

3.4. *Precision.* Degree of scatter among a series of measurements obtained from multiple observations of the same homogeneous sample under specified conditions. Precision may be considered at several levels. To illustrate the idea of variance components, the following levels of precision may be considered for plate-based assays. The list is neither prescriptive nor exhaustive.

3.4.1. *Within-plate.* The precision among replicated specimens on the same plate. This is evaluated by residual error.

3.4.2. *Between-plate.* The precision of tests run under the same operating conditions concurrently or within a short time interval. This is also called repeatability.

3.4.3. *Intermediate precision.* The precision among the results of tests run under varying conditions within the same laboratory. Elements of intermediate precision may include:

3.4.3.1. *Between assay.* Precision among assays run independently under similar, but not necessarily identical, operating conditions such as assays run at different times on the same day.

3.4.3.2. *Within-laboratory*. Precision among assays run within the same laboratory under different conditions, such as on different days or by different operators.

3.5. *Reagent blank.* The reagent blank (RB) consists of all components of the test sample except the analyte being measured. The RB is processed the same as the unknown.

3.6. *Reference.* A reference is a preparation that has had its clinical or immunological activity or analyte concentration established in a valid, well-controlled study, series of studies, or assays.

3.7. *Reproducibility.* The precision of the assay run on the same specimen by different kinds of laboratories. (e.g., research and development vs. quality control).

3.8. *Ruggedness.* The capacity of an assay (method) to remain unaffected when small changes in environment or operating conditions are made.

3.9. *Sensitivity*.

3.9.1. *LOD*. The limit of detection is the lowest concentration of analyte in a sample that can be distinguished from background, but not necessarily quantified.

3.9.2. *LOQ*. The limits of quantitation are the lowest and highest concentrations of an analyte in a specimen that can be quantified with an acceptable level of accuracy and precision. The lower LOQ is greater than the LOD.

3.10. *Specificity/selectivity.* Specificity is the ability of an assay to measure the analyte of interest to the exclusion of other relevant components; that is, it detects only one analyte. Selectivity is the extent to which an assay can measure a particular analyte in a complex mixture without interference from other components in the mixture. During optimization, the goal is to enhance selectivity by carefully choosing conditions, pretreatments, and controls.

3.11. *Standard.* A standard is a preparation with known analyte concentration.

3.12. *Signal to background (S/B) ratio.* S/B is the ratio of the signal of the analyte to the RB signal. It is important for the RB to be identical to the test sample except for the analyte being measured unless otherwise justified during the validation process. Buffer or air blanks are usually not satisfactory indicators of background because they may not account for all extraneous signals. In assay validation studies, the term signal-to-noise ratio is often used, and it is understood that noise refers to background rather than random scatter.

3.13. *Unknown*. The unknown is the test preparation that is assayed to determine the content of the analyte.

3.14. *Validation.* A process that provides evidence that an assay method does what it is intended to do.

3.15. *Verification*. The term used in this document to describe a subset of the validation process where specificity/selectivity, accuracy, precision, discrimination, and ruggedness of the test method are evaluated.

Appendix II

Suggested Validation Report Topics

1. Title of method
2. Principle of method and its relationship to efficacy
3. Development and optimization
   - 3.1. Developmental work description
   - 3.2. Optimization work
     - 3.2.1. Description of approach
     - 3.2.2. Materials and methods
     - 3.2.3. Results
     - 3.2.4. Analysis
     - 3.2.5. Discussion and conclusion. (Include critical specifications of reagents, equipment, and the test procedure)
4. Description of procedures for assessing
   - 4.1. Accuracy
   - 4.2. Precision
   - 4.3. Ruggedness
   - 4.4. Sensitivity
   - 4.5. Specificity
5. Results (for each of the items in 4)
   - 5.1. Data summary
   - 5.2. Graphs
   - 5.3. Analysis
   - 5.4. Discussion and conclusions
6. Finalized procedure
7. Description of monitoring plan
   - 7.1. References
   - 7.2. Controls
   - 7.3. Standards
   - 7.4. Preliminary data
   - 7.5. Proposed action plan: May include control charts, statistical methods, and specifications

Appendix III

Guidance for Validating ELISA Relative Potency Assays

1. *Introduction.* This appendix presents details specific to the validation of enzyme-linked immunosorbent assay (ELISA) designed as relative potency assays (RPAs). Appendix III is intended to supplement Appendix I, which outlines general principles applicable to the validation of all types of in vitro assays. The principles in Appendix I should be thoroughly understood before proceeding to the details in Appendix III, as they are not repeated here.

2. *Steps in the Validation of ELISA RPAs*

   2.1. *Conceptualization*

      2.1.1. Assays are sometimes categorized as analytical or comparative. Analytical assays are those designed to measure the concentration of a specific analyte; they may therefore be termed analyte-based. Comparative assays are those designed to compare preparations based on their response in the assay; they may therefore be termed response-based.

      A completely analyte-based assay would be a quantitative, rather than relative, potency assay, and test preparations would be compared to a standard, rather than a reference, preparation. Relative potency assays were initially conceived as nearly analyte-based, but have since spanned the continuum to purely response-based.

      2.1.2. Firms should design ELISA RPAs that are analyte-based rather than response-based. Every effort should be made to identify immunogens and epitopes that are critical to efficacy. Doing so may allow new references to be qualified by in vitro methods alone. Response-based ELISA RPAs typically rely on animal challenge studies to qualify references because the active component has not been identified or the reference preparation is poorly characterized. The Center for Veterinary Biologics (CVB) encourages the development of in vitro assays that do not require animal challenge studies to qualify references.

      The focus of this appendix is on ELISA RPAs that may not be completely analyte-based but have references that have been characterized and monitored well enough for in vitro qualification of new references. Much of the guidance in this appendix may be applied to completely analyte-based assays as well. Further guidance for specific assays is available from CVB.

2.2. *Development and Optimization.*

2.2.1. *Optical density range.* CVB recommends that the optical density (OD) of the saturation portion of the ELISA curve not be greater than 2.0 even though the instrument can measure higher OD values. Since higher ODs correspond to lower measured signal (transmitted light) in the spectrophotometer, small variations in the execution of the assay may have a greater impact at high ODs than at lower ODs.

2.2.2. *Reagent blank.* The reagent blank should produce ODs of 0.15 or less.

2.2.3. *Signal to background (S/B) ratio.* The S/B ratio is a key consideration in determining the optimum reagent working dilutions. An S/B ratio of 10 or greater when measured near the saturation portion of the ELISA curve is usually adequate.

2.2.4. *Plate uniformity.* Plate edge effects are common and, when they occur, the involved wells should not be used for reference or unknown preparations. Gradient or other location effects may preclude the use of specific plate types or indicate additional assay development is necessary.

- Firms should check for location effects with uniformity plates, which have all wells filled with a single preparation of the reference at the same volume and dilution. Use at least three uniformity plates.

- Firms should select a dilution of the reference that produces an OD that is approximately three-fourths of the OD at saturation.

- If edge or gradient effects are suspected, it may be useful to assess additional plates to confirm the effects.

2.2.5. *Parallelism.* A valid RPA depends on a comparison of parallel curves. This means that the ELISA curves for the reference and test preparations have the same shape and differ only by a horizontal shift. The amount of shift indicates the log relative potency (RP) of the unknown preparation in comparison to the reference. Quantitative analyte-based assays also require that the standard and test preparations have parallel dose response curves, although the analysis is usually interpolation on the standard curve and the results are reported as the concentration of the analyte.

2.2.5.1. A dilution series that extends from the saturation through extinction portions of the ELISA curves for both the reference and unknown preparations is necessary. This should be taken into account when determining the dilution factor and dilution series placement on the

plate. Use blank-corrected ODs (ODs with the mean of the reagent blank subtracted).

2.2.5.2. Fit a nonlinear regression function to the data for each preparation and estimate the parameters determining the curve's shape. A three-parameter logistic function (3PL) is usually used for the types of curves most commonly seen in ELISAs of veterinary biologics. The 3PL curve has parameters for asymptote, scale, and location, which correspond to the immunological reaction's saturation, relative rate of change, and dilution at its midpoint. Alternative curve functions may be considered if demonstrated to be appropriate and necessary.

2.2.5.3. Firms should compare the scale and asymptote parameters of the two preparations by taking the ratio of the two preparations' corresponding parameter estimates. They should also construct individual 90 percent confidence intervals for the ratios. If the confidence intervals fall between 0.9 and 1.1, the parameters may be considered equivalent, and the curves may be considered parallel.

2.2.5.4. Usually 5 to 10 independent replicate plates are used to demonstrate parallelism, but more may be necessary. CVB suggests testing across multiple days. Only one replicate can come from each plate. The entire dilution series for each preparation should be on an individual plate.

2.2.5.5. When a separate working reference is proposed, parallelism between the master and working reference should also be demonstrated.

2.2.5.6. Generally, the test preparations are pre-license or production serials manufactured according to an Outline of Production.

- Unless otherwise approved by CVB, a serial from every product code that will be tested with the proposed reference should be evaluated in the assay. For a line of combination products, only the largest and smallest combinations need to be tested if the composition of the intermediate combinations is identical to them for all other components.

- Firms should evaluate at least two serials. It is sometimes useful to assess replicate dilution sequences of the reference to isolate sources of assay variability.

2.2.6. *Frozen references*. For references that will be frozen, an assessment of the effect of freezing on the reference should be conducted by evaluating parallelism and relative potency between frozen and refrigerated reference preparations.

- The reference and test serial must be treated the same unless it can be demonstrated that the freeze-thaw process has no effect.

- CVB recommends a minimum of five replicates. Additional replicates may be necessary when a conclusion is not obvious.

- If the test method proposes multiple freeze/thaws of a vial of reference during its use, the assessment of freezing should be made at the maximum number of proposed freeze/thaw cycles.

2.2.7. *Assay specifications*. Firms should determine the procedures, plate layout, and potency estimation method for serial release testing by:

- Specifying the dilution series for each preparation.

- Specifying the complete plate layout.

- Identifying test validity criteria for the plate controls. Minimally, a reagent blank and positive control are necessary. Criteria may be adjusted after complete verification if the additional testing suggests minor changes are appropriate.

2.2.8. *Analysis*. Firms should describe the statistical method for estimating relative potency. They should provide enough detail so that a statistician can reproduce all aspects of the procedure without using specific software. Include the criteria that will be used to validate individual runs, such as a criterion for parallelism or precision.

2.2.9. *Software*. Firms should identify the software to be used for serial release and provide all necessary details about how the serial release analysis will be run with the software.

2.2.10. *Dynamic range*. It may be useful at this stage to consider the range of potencies for which the assay can produce accurate and precise estimates. A strategy for prediluting high potency serials may be necessary.

2.3. *Verification*.

2.3.1. *Assay format*. For the verification phase of validation, all testing must use the test format proposed in the Special Outline or Outline of Production by the laboratory that will be conducting serial release testing.

2.3.2. *Assay reagents*. For accuracy and precision, all testing should be conducted with the same lots of critical reagents (e.g., conjugate, capture, and detection

antibody). Ideally, these lots would also have been used in the later stages of optimization. CVB encourages using multiple lots when testing ruggedness.

2.3.3. *Accuracy and precision.* Firms should evaluate accuracy and precision over the expected range of potencies, between operators, and across days. The release RP should be determined based on the precision and accuracy characteristics of the assay, incorporating information on precision and accuracy to set a release value that minimizes the likelihood of subpotent serials being released.

2.3.3.1.*Design*. This testing should be done by at least two operators on at least 3 days. The test preparations should be prepared from the same bulk antigen lot used to formulate the Master Reference and include subpotent and potent preparations.

2.3.3.2.*Preparations*.

- *Subpotent preparations*. Firms should formulate test preparations at RPs of 0.8 and 0.9 to demonstrate the ability of the assay to discriminate between potent and subpotent serials. These preparations may be serials formulated to the target RP based on antigen input. It is also acceptable to use preparations made by diluting the reference or a serial with a reagent blank. For assays with a coefficient of variation of 10 percent or more, additional preparations with potency of 0.7 and 0.6 should also be tested.

- *Potent preparations*. Firms should test preparations with analyte concentrations spanning the range expected in production serials of the products. Firms should test at least one preparation with 20 percent more antigen then the reference and one at the maximum expected production serial antigen content. Prelicense serials may also be assessed.

- *Reference only*. It is sometimes useful to include replicate dilution sequences of the reference to isolate sources of assay variability.

2.3.4. *Ruggedness*. Firms should estimate the RP of at least two representative serials on 3 separate days under the allowed range of assay conditions. For example, firms should run the tests at the minimum and maximum temperatures and minimum and maximum incubation times. Firms should use the potent preparations described in the previous section.

2.4. *Monitoring*.

2.4.1. *Monitoring plan*. Firms should prepare a plan to monitor the reference as well as the assay system as a whole. The plan should include a description of the validated independent quantitative and qualitative test methods used to

monitor reference stability, frequency of testing, reference storage conditions and monitoring, and the trend analysis tools used to evaluate the monitoring parameters.

2.4.2. *Reference lifetime*. References will be assigned renewable 15-year dating. CVB will permit continuous use of a reference (frozen or unfrozen) if the potency of the reference has not declined as determined by an acceptable stability monitoring program.

2.4.3. *Reference stability testing*. Monitoring the stability of a reference means evaluating its potency and determining when the potency starts to decline (9 CFR 113.8). ELISA RPAs cannot self-monitor because the assay response is not a measure of the intrinsic properties of the reference. Stability monitoring of the reference thus requires independent measures of quantitative and qualitative parameters relevant to the potency of the reference using validated test methods

2.4.3.1.*Number of assay methods*. Firms need at least one quantitative and one qualitative test method. More than one of each category of testing methods evaluating the reference may be of value.

2.4.3.2.*Quantitative parameter*. The validated testing method must quantify the protective antigen with sufficient precision to detect a change of 20 percent.

2.4.3.3.*Qualitative parameter*. The validated testing method must verify the protective antigen is intact as compared to when the reference was first qualified. This testing may be in vivo, in vitro, or a combination of the two.

2.4.3.4.*Frequency of testing*. The Master Reference must be tested the date of first vaccination for the efficacy study and again at 3, 6, 12, and 30 months, and then at 2.5-year intervals along with a filed report. Each test used for this purpose must be approved by CVB. CVB recommends that a minimum of 5 vials of the reference be tested at each time interval except initially, when 20 vials should be analyzed.

2.4.3.5.*Trend analysis*. Trend analysis tools will facilitate detection of changes in the qualitative and quantitative parameters of the reference. More than one trending tool may be of benefit. Firms should start with validated test methods, determine the initial parameters, and perform the testing under ideal conditions.

2.4.4. *Quantitative and qualitative monitoring tools.* Physicochemical and immunochemical test methods for monitoring qualitative and quantitative

parameters of the reference and assay performance include, but are not limited to, the following:

Capillary electrophoresis
Peptide mapping
Isoelectric focusing (IEF)
IEF and SDS-PAGE (2-D)
Amino acid sequencing/analysis/N-terminal analysis
Mass spectrometry
HPLC-Ion exchange, hydrophobic interaction, reverse phase, affinity, size exclusion
Biosensor (SPR or equivalent)
Circular dichroism
Differential scanning calorimetry
Western blotting
PAGE (reduced, denatured, native)
Infrared spectroscopy
Nuclear magnetic resonance
Various types of immunoelectrophoresis (Laurell or rocket, 2-dimensional)

2.4.5. *Run-to-run monitoring*. In addition to regular testing by independent methods, firms should also monitor the ELISA serial release testing, which may give an early indication of a change in the assay or reference. This type of monitoring involves trend analysis of parameters estimated when the serial release test is run. Include summaries of this information with the reports noted in section 2.4.3.4 of this appendix.

2.4.6. *Replacement of critical reagents*. As an approved lot of capture antibody, detector antibody, or other critical reagent is depleted, a new lot will need to be procured and use dilution determined.

2.4.6.1. Firms should conduct preliminary testing to determine a suitable range of dilutions for the new lot. They should evaluate each dilution of the new lot and the current lot at its use dilution using the reference. Testing should be completed *using at least one plate on* each of 3 days. The reference response curve should extend from saturation through extinction. Firms should calculate the RP obtained with the new lot dilutions versus the current lot and select a dilution that results in an RP of 1.0 or greater.

2.4.6.2. Firms should confirm the selection by testing at least five serials of product with the approved lot and the proposed use dilution of the new lot. Firms should evaluate the RP estimates and make sure they do not suggest the new lot results in higher RPs.

2.4.7. *Working references*. CVB recommends that firms prepare working references at regular intervals as a precaution against declining reference.

3. *Report*. Firms should submit reports of validation studies as outlined in Appendices I and II. In addition, the report must include:

3.1. A summary of the optimized method describing the actual conditions of use with respect to the sample treatment, reagent concentrations, dilution series of the reference and unknowns (serials), and incubation conditions for coating, binding, washing, blocking, and reaction with substrate.

3.2. The full dose-response curves of the Master and Working Reference (M/WR) and representative serials for each product code affected. Provide graphs where appropriate.

3.3. Data evaluating plate position effects on the response of uniform samples.

3.4. A description of the software used for analyzing the data and determining relative potency with example output.

3.5. A description of the assay and reference monitoring plan, including the test method(s) used and the trending tools.

3.6. Submission of the complete data from all experiments in suitable electronic files will expedite CVB's assessment of the report. Questions about appropriate formats should be directed to the CVB Statistics Section Leader.