

HOLD-OVER INFORMATION FROM VETERINARY SERVICES MEMORANDUM NO. 800.90

Industry has requested that some portions of obsoleted VSM 800.90 be preserved until the required information can be put into a rewrite of VSM 800.211. Therefore, the information below is included in the Reviewers' Manual on an interim basis.

I. INTRODUCTION

These guidelines provide parameters and procedures for conducting an enzyme-linked immunosorbent assay (EIA or ELISA) for the quantitation of antigen in a veterinary biological product. The guidelines also establish uniformity for such *in vitro* testing. In the ELISA or EIA system described, the concentration of a test serial is determined by comparing the absorbance values (optical density or OD) of that serial to the OD values of a reference preparation (Master or Working Reference). The relative potency of the test serial is determined from the relationship of the OD's. These guidelines address the following: definitions, immunogen test specificity, *in vitro* test design, reference qualification, reference requalification, Qualifying Serials, reference storage, reference dating, testing adjuvanted product, and statistical methods.

II. DEFINITIONS

A. Applicable 9 CFR definitions include:

1. Master Reference, 9 CFR 101.5 (o). A Master Reference is a reference whose potency is correlated, directly or indirectly, to host animal immunogenicity. The Master Reference may be used as the working reference in *in vitro* tests for relative potency. The Master Reference may also be used to establish the relative potency of a serial of product used in requalification studies and to establish the relative potency of working references. The Master Reference as described in a filed Outline of Production may be:
 - a. A product reference that is a completed serial of vaccine or bacterin prepared in accordance with a filed Outline of Production. Product references may be:
 - i. Monovalent references composed of a single fraction or agent, or
 - ii. Polyvalent references composed of two or more fractions or agents
 - b. A purified preparation of a protective immunogen or antigen, or
 - c. A nonadjuvanted harvested culture of microorganisms.
2. Working Reference, 9 CFR 101.5 (p). A Working Reference is the reference preparation that is used in the *in vitro* test for the release of serials of product.

Working References may be:

- a. Master References, or
 - b. Serials of product that have been prepared and qualified, in a manner acceptable to the Animal and Plant Health Inspection Service (APHIS), for use as reference preparations.
3. Qualifying Serial, 9 CFR 101.5 (q). A Qualifying Serial is a serial of biological product used to test for immunogenicity when the Master or Working Reference is a purified antigen or nonadjuvanted harvest material. Qualifying Serials shall be produced in accordance with the filed Outline of Production, tested for immunogenicity in accordance with methods deemed appropriate by APHIS, and have a geometric mean relative potency, when compared to the Master Reference, of not greater than 1.0 as established by independent parallel line assays with five or more replicates or by other valid assay methods for determining the relative antigen content which demonstrate linearity, specificity, and reproducibility at least equivalent to the parallel line assay and are acceptable to APHIS. Qualifying Serials used to requalify or extend the dating period of a Master Reference shall be determined to be immunogenic in accordance with methods deemed appropriate by APHIS and, in addition, shall be within their permitted dating period and have been prepared in accordance with the production method described in the currently filed Outline of Production.
 4. Immunogenicity, 9 CFR 101.5 (r). The ability of a biological product to elicit an immune response in animals as determined by test methods or procedures acceptable to APHIS.

III. DETERMINING THE TARGET OF THE TEST SYSTEM

- A. Prior to the establishment of a testing method in the filed Outline of Production or Special Outline, the following should be supplied to and approved by APHIS:
 1. Evidence that the *in vitro* relative potency test measures a protective immunogen as demonstrated by one of the following methods:
 - a. Host animal studies using a purified subunit (e.g. glycoprotein or bacterial outer membrane antigen extract) that elicits protection against the specific animal disease,
 - b. Passive protection by the monospecific antibody component(s) of the *in vitro* test system,
 - c. Data published in peer reviewed scientific journals generally recognized by the scientific community and acceptable to APHIS,
 - d. Demonstration of *in vitro* neutralization of viable organisms by the detecting reagent, or
 - e. Other methods acceptable to APHIS.

2. Demonstration of the specificity of the reaction between a protective immunogen and the detection antibody by polyacrylamide gel electrophoresis/immunoblotting or other similarly specific techniques.

B. In general, the preferred ELISA or EIA potency test system uses a monoclonal antibody or mono-specific polyclonal antibody for a protective immunogen.

IV. REQUIREMENTS FOR A REFERENCE

A. The antigen content of a Master Reference should directly or indirectly correlate to protection of the host animal or support label claims.

1. A direct correlation is established when the Master Reference is used in the host animal protection study.
2. An indirect correlation is established when a Qualifying Serial is tested in host animals or when a Master Reference or Qualifying Serial is administered to animals other than host animals.

B. The reference used in the *in vitro* test for serial release is by definition a Working Reference by can, in addition, be the Master Reference.

C. It is recommended that the Master Reference and/or Working Reference be a product reference.

1. A product reference will have all the components in the same relative proportions as are found in the serial of product being tested.
2. A product reference can be either a monovalent reference or a polyvalent reference.
 - a. If a monovalent reference is used to evaluate a polyvalent product, it is unacceptable to compensate mathematically for *in vitro* interference occurring between fractions in the test serial.
 - b. If using a polyvalent reference to test product with fewer fractions than the reference, it is necessary to demonstrate that *in vitro* interference between fractions is not occurring. If *in vitro* interference occurs, then a monovalent reference or reference with similar antigen content is required.

D. Purified references are acceptable, provided that:

1. The test system is not influence by the other components present in the normal product,
2. Linearity and parallelism between the purified Master or Working References and the product being tested are maintained,

3. A Qualifying Serial is used to establish the purified Master Reference, and
 4. There is no selective addition of components or compounds to a purified reference to compensate for nonlinearity or lack of parallelism.
- E. Master References should be established to have a relative potency (RP) of one (1.0). This may be accomplished as follows:
1. For nonfrozen product references the RP of 1.0 is based on the concentration of antigen in the immunogenicity serial which demonstrated a protective response or supports label claims in a host animal immunogenicity trial.
 2. For purified concentrated Master or Working References (frozen or non-frozen), the dilution of the reference equivalent to an RP of 1.0 is established at the time of reference qualification using a Qualifying Serial which demonstrates a protective response or supports label claims in a host animal immunogenicity trial.
 - a. Dilution of the purified Master or purified Working Reference prior to use in the test system should not exceed 1:100 and should be done with an inert-ingredient diluent (phosphate buffered saline, Dulbecco's phosphate buffered saline, physiological saline, water, etc.).
 3. Frozen product Master Reference should be established to have an RP of 1.0 and validated during qualification by the use of a qualifying product serial demonstrating a protective response or supporting label claims in a host animal immunogenicity trial.
 - a. Dilution of a product Master Reference with an inert ingredient diluent (phosphate buffered saline, Dulbecco's phosphate buffered saline, physiological saline, water, etc.) to compensate for a difference in dose is allowed.
- F. It is recommended that a new vial of reference be used for each test. Vials of frozen references should not be refrozen or used for more than one (1) day unless the additional time or freeze-thaw cycles are supported by data submitted and approved by APHIS and the criteria specified in the Outline of Production or Special Outline.
- G. The Master and/or Working Reference should be uniquely identified by lot number and expiration date in the filed Outline of Production or Special Outline.
1. The Outline of Production or Special Outline should specify the reference (including lot number) used for testing.
 2. The Master Reference should be available in sufficient quantities to allow the manufacturer and the CVB-L to conduct testing throughout the dating of the reference and for an additional period if requalification is anticipated.

V. QUALIFICATION OF A REFERENCE

- A. Qualification of a Master Reference for use in *in vitro* relative potency assays is either the initial establishment of a Master Reference or the establishment of a new Master Reference to replace an existing reference. The dating of the Master Reference is for a defined time period as supported by data and approved by APHIS.
- B. Master Reference that are unfrozen product references stored similarly to product can be directly qualified in animals.
- C. Master References that cannot be directly qualified in animals, but require a Qualifying Serial for qualification, include:
 1. Master References that are frozen product references,
 2. Master References that are stabilized and frozen, and
 3. Purified Master References.
- D. The immunogenicity of a new and replacement Master Reference should be established as prescribed in the applicable 9 CFR Standard Requirement or, for agents without codified requirements, in accordance with protocols acceptable to APHIS. Alternative immunogenicity proposals for qualifying Master References may be considered by APHIS, but should:
 1. Include a statistically significant number of animals as would be required for initial licensure of a product,
 2. Include an assessment of immunogenicity and/or efficacy of a product in the host species,
 3. Be directly correlated to protection or support label claims, and
 4. Be submitted to the firm's assigned reviewer in the Center for Veterinary Biologics-Licensing and Policy Development (CVB-LPD) soon enough to allow for comment and approval prior to test initiation.

VI. REQUALIFICATION OF A REFERENCE

- A. Requalification of a Master Reference requires demonstration of the immunologic stability of a previously qualified Master Reference.
 1. The dating of a Master Reference may be extended beyond its expiration date by confirming its immunogenicity in a manner acceptable to APHIS.
 2. A Master Reference can be requalified multiple times, provided that the stability of the Master Reference is shown to be maintained.
- B. Requalification of a Master Reference should be done prior to the end of dating.

1. Products tested with expired Master References are not eligible for release.
- C. Demonstrating the efficacy of a reference either directly or indirectly is one method of confirming immunologic stability.
- D. Immunologic methods not requiring vaccination and challenge may demonstrate the stability of a reference, provided that the immunologic response was initially correlated to protection during a host animal efficacy study.
- E. An unfrozen product reference stored similarly to product can be directly requalified in animals.
- F. Frozen product references, stabilized and frozen references, or purified references cannot be directly requalified in animals. The difference in composition and/or freeze-thaw cycle precludes direct requalification, as the continued correlation to product efficacy cannot be ensured.
- G. For references that cannot be directly requalified in animals or where the manufacturer wishes to establish a Working Reference different from the Master Reference, a Qualifying Serial shall be used in requalification trials.

VII. QUALIFYING SERIAL

- A. Qualifying Serials shall be produced in accordance with a filed Outline of Production.
- B. Dilution of a production serial to produce a Qualifying Serial is allowed, provided that after dilution the relative concentration of all the components other than the agent being requalified is at release levels per the filed Outline of Production.
- C. Qualifying Serials should be produced within six (6) months prior to the immunogenicity trial, but a serial is acceptable if it was produced within the dating period of the product and as specified in the currently filed Outline of Production. The use of a recently produced Qualifying Serial ensures that the relationship of the reference to the product has not been altered by gradual changes in the production method or materials over time.
- D. A serial prepared by a method which is different from the method specified in the currently filed Outline of Production is not acceptable for use as a Qualifying Serial.
- E. Qualifying Serials used to requalify a Master Reference or Working Reference used in serial release testing shall have a relative potency less than or equal to 1.0 ([1.0) when compared to the Master Reference or Working Reference. A minimum of 5 independent replicate assays shall be used to confirm the relative potency value of the Qualifying Serial.
- F. Qualifying Serials may be used as Working References, provided that:
 1. The Qualifying Serial has a relative potency of 1.0 when compared to the

Master Reference,

2. The Qualifying Serial is designated as the Working Reference in the filed Outline of Production or Special Outline.

G. The dating of Qualifying Serials used as Working Reference shall be:

1. Equal to the dating of the product, or
2. Equal to the dating allowed the Master Reference, if:
 - a. The relative potency of either the Qualifying Serial or Working Reference when compared with the Master Reference is equivalent to 1.0,
 - b. The equivalence of the Qualifying Serial/Master Reference relationship is monitored at intervals not less than at mid-dating of the Master Reference using the *in vitro* assay specified in the filed Outline of Production or Special Outline, and
 - c. The monitoring plan is specified in the filed Outline of Product or Special Outline.

H. If the Qualifying Serial/Master Reference relationship is shown to be nonequivalent, the Qualifying Serial is no longer eligible for use in the test. In that event, the Master Reference may be designated as the Working Reference in the Outline of Production or Special Outline; or a new Working Reference may be qualified.

VIII. STORAGE OF A REFERENCE

- A. The storage conditions for the Master or Working Reference used in an *in vitro* test for serial release should be stated in the filed Outline of Production or Special Outline.
- B. Master References that are product references and stored in a manner similar to product should not require further dilution or bench-level manipulation prior to being used as Working References to test for serial release.
- C. Master Reference that are purified antigens and stored in a manner similar to product may be diluted up to 1:100 prior to being used as Working References to test for serial release.
- D. If stabilizers are used to extend the storage period of a frozen reference that is used as a Working Reference in an *in vitro* test for serial release, the following apply:
 1. The stabilized frozen reference can be used in the *in vitro* potency test without further sample treatment if:
 - a. The stabilizer is added prior to freezing the reference,

- b. The stabilizer is added to the reference prior to initiating the immunogenicity study,
 - c. A Qualifying Serial is used in the immunogenicity study, and
 - d. The relative potency of the stabilized and frozen Master Reference and the Qualifying Serial are equivalent when the immunogenicity study is initiated. A minimum of five (5) independent replicate assays in the *in vitro* test system shall be used to establish equivalence.
2. If stabilizer is added to the Master Reference after the initiation of the immunogenicity study, the *in vitro* test protocol should provide for similar treatment of the test sample. Treatment of the sample should be specified in the filed Outline of Production or Special Outline.
 3. Mathematical corrections cannot be used to compensate for added stabilizer.

IX. REFERENCE REQUALIFICATION

The purpose of requalifying a Master Reference is to demonstrate its immunogenic stability and thereby extend the dating period for use for potency testing. Methods for demonstrating immunogenic stability include:

A. Host animal vaccination-challenge studies

1. Host animal protection studies in which a single dilution of the Master Reference or Qualifying Serial is administered by the least immunogenic of the recommended routes of vaccine administration may be used to extend the dating of the Master Reference for a period equal to that allowed for product dating.

B. Nonhost animal vaccination-challenge studies

1. Nonhost animal vaccination-challenge studies may be used to demonstrate the stability of the Master Reference if the protective immune response in nonhost animals is correlated to the protective response in host animals.

C. Host animal serology studies

1. Serological response in the host animal may be used to requalify a Master Reference when a relationship between titer and protection has been established.

X. TESING ADJUVANTED PRODUCT

- A. When both the production serial and the Working Reference contain adjuvant, treatment to release bound adjuvant is allowed, provided that:
 1. The test serial and the Working Reference receive similar treatment,

2. The treatment method is specified in the filed Outline of Production or Special Outline, and
 3. The treatment method was demonstrated to be applicable at the time of an immunogenicity or Master Reference requalification study.
- B. When adjuvant is present in the test serial but not in the Working Reference, treating the test serial to release bound antigen without having to similarly treat the Working Reference is allowed if:
1. The serial used in the immunogenicity test was treated, and
 2. The treatment method is specified in the filed Outline of Production or Special Outline.