USDA
United States Department of Agriculture

Animal and Plant Health Inspection Service

Veterinary Services

1400 Independence Ave, SW

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VETERINARY SERVICES MEMORANDUM NO. 800.73

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

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SUBJECT: General Guidance for Test Kits Intended for the Diagnosis of Animal Diseases

I. PURPOSE

This memorandum provides guidance to licensees, permittees, and applicants to support an application for a U.S. Veterinary Biological Product License or U.S. Veterinary Biological Product Permit for test kits intended for the detection of animal disease or immunological status, as authorized by title 9, *Code of Federal Regulations* (9 CFR), part 101.2.

II. REPLACEMENT

This memorandum replaces Veterinary Services (VS) Memorandum No. 800.73 dated June 20, 2002.

III. BACKGROUND

Diagnostic test kits are intended to detect the disease or immunological status of an animal. Kits must be validated to demonstrate the fitness for intended use. The kit, regardless of format or function, must be reliable, reproducible, and scientifically sound. The formal process for evaluating these, and the diagnostic performance characteristics, is known as validation.

IV. SCOPE

This document outlines an approach for validating a diagnostic test, writing an Outline of Production, and confirming the expiration dating.

V. DEFINITIONS

A. *Gold Standard.* Ideally, a gold standard is an error-free method which gives an individual's true disease status. The accuracy and precision of a diagnostic method is measured against the gold standard. Because a true gold standard may be unavailable or impractical, new diagnostic tests are often compared to a reference method which has been accepted as a standard. In some cases, the

standard may be a composite of multiple evaluations (e.g., Johne's Disease classification may be based on cumulative results from histopathology, clinical signs, and culture, taking into consideration the herd status and age of the animal). A standard may be fallible, but may be deemed acceptable to use for designating the disease status of an animal when assessing the accuracy of a new diagnostic test kit. When estimating diagnostic sensitivity and specificity using the results of an imperfect reference standard, the estimates will likely be biased. There may be situations in which the ultimate goal is to mimic the results of a true gold standard. Studies may also be designed to use methods which have been developed to estimate sensitivity and specificity when a gold standard test is unavailable.

B. *Diagnostic Sensitivity*. Diagnostic sensitivity is the probability of obtaining a positive test result for a truly positive sample. When the results of the gold standard are accepted as correctly classifying true disease status, diagnostic sensitivity can be expressed as a percentage using the following calculation:

$$(\text{true positive}/(\text{true positive} + \text{false negative})) \times 100\%$$

False negative samples are truly positive samples classified as negative by the test kit.

C. *Diagnostic Specificity*. Diagnostic specificity is the probability of obtaining a negative test result for a truly negative sample. When the results of the gold standard are accepted as correctly classifying true disease status, diagnostic specificity can be expressed as a percentage using the following calculation:

$$(\text{true negative}/(\text{true negative} + \text{false positive})) \times 100\%$$

False positive samples are truly negative samples classified as positive by the test kit.

D. *Ruggedness*. Ruggedness is the measure of the capacity of the assay to remain unaffected by deliberate small variations in method parameters. It provides an indication of assay reliability under normal use.

E. *Receiver Operating Characteristic (ROC) Curve*. The ROC curve is a plot of a test's sensitivity versus its false positive rate (1-specificity) where each point on the empirical ROC curve is generated by a different potential cutoff value. A ROC curve is useful in visualizing the compromise between sensitivity and specificity for different cutoff values and for ultimately selecting a cutoff value.

F. *Repeatability*. Repeatability can be described as the variation observed in the measurements taken on a single sample by a single operator. In the context of a diagnostic test kit where a sample will be classified as positive or negative, interest lies in the ability of an operator to consistently classify a set of samples.

G. *Reproducibility*. Reproducibility can be described as the variation observed between measurements made by different operators. In the context of a diagnostic test kit where a sample will be classified as positive or negative, interest lies in the ability of the test system to produce consistent results for a set of samples when testing is conducted by different individuals.

H. *Sample*. In this document 'sample' usually refers to a diagnostic specimen rather than a statistical sample of units from a population. The meaning should be clear from the context.

VI. ASSAY DEVELOPMENT

Validating a diagnostic test kit occurs in steps which include conceptualization, development, and verification that the test kit will perform consistently. The final report typically includes data from the verification steps, but may also refer to earlier work on the conceptualization and development of the assay.

A. *Conceptualization.* Several issues should be addressed early in the development of the diagnostic test kit:

1. The ability of the kit to detect the analyte of interest. Diagnostic analytes are typically antigens, antibodies, or genetic sequences.

2. The ability of the kit to measure the analyte in the range of concentrations expected in diagnostic test samples.

3. The type of sample and the sample processing required.

4. The potential effect of cross-reacting materials in the test preparation.

B. *Development.* During the development phase, the firm should:

1. Determine the final test kit conditions and reagent concentrations.

2. Incorporate the use of controls and methods of monitoring the performance of the test kit.

3. Determine the criteria for acceptance for reagents and controls, including serial release panel members.

4. For plate based assays, determine if there are location effects on the plate.

C. *Validation.* The firm should determine the performance characteristics, namely diagnostic sensitivity and diagnostic specificity, as well as demonstrate the ruggedness of the kit and provide all raw data to the Center for Veterinary Biologics (CVB) for review. It is strongly recommended that the firm submit a

detailed protocol to the CVB describing the proposed validation prior to initiation of the study. Recommended formats for raw data may be accessed at the CVB website under the Biologics Regulations and Guidance section, Electronic Submissions subsection.

1. *Diagnostic Sensitivity and Diagnostic Specificity.* The protocol should address the analysis method planned to estimate diagnostic sensitivity and diagnostic specificity. The protocol should specify the gold standard test and proposed statistical methods, particularly for those utilizing imperfect, composite, or no gold standards. For kits in which the response is determined by visual inspection, but a more objective measurement has been made, such as densitometry, the sensitivity will be estimated based on the visual classification. The visual classification should be made in the absence of knowledge of the objective measurement in these instances.

2. *Samples for Estimating Diagnostic Performance Characteristics.* Determining the true status of the sample requires testing by one or more additional tests. A reference test may exist which is the currently accepted method of designating the status of a sample (gold standard). In some cases, a composite of multiple evaluations may be used in determining the true disease status of an animal. The reference test or tests (in the event of a composite of multiple evaluations) should be applied uniformly to all animals to determine their diagnostic status. Do not do selective retesting based on the results of the experimental kit. The selection of test specimens is instrumental for accurately estimating the diagnostic performance characteristics of the test kit. The samples used should be the same specimen type intended for use in the kit. If the label claim for the kit is for more than one specimen type (e.g., whole blood, serum, plasma) and/or more than one species, diagnostic sensitivity and specificity should be estimated for each specimen type/species combination. An adequate number of positive and negative samples of each type should be tested. The positive samples should cover the range of activity from weak positive to strong positive. Generally, diagnostic samples should be obtained from U.S. sources to account for disease agent isolates unique to the U.S. The protocol should address the species, sample types, proposed number of samples, and the acquisition of samples, which could include any pertinent information such as geographic location, sample treatment, storage, shipping conditions, etc. The protocol should provide a justification for the intended sample size(s). Describe how the sample set represents the target population.

3. *Determining the Cutoff Value.* Some kits may produce a semi-quantitative test result used in conjunction with a cutoff value for determining the status (positive/negative) of the sample. The results of testing the diagnostic samples may aid in specifying the cutoff value. The ROC curve may be useful in guiding a decision regarding the most appropriate cutoff value for the intended use of the test. The final report should describe how the cutoff value

was selected and include estimates of diagnostic sensitivity and diagnostic specificity for the proposed cutoff value.

4. *Ruggedness.* Evaluate ruggedness by observing the effect of changes in incubation time, incubation temperature, operators, reagent lots, or other test conditions on the final results.

5. *Inter-laboratory comparison.* Firms are required to conduct an inter-laboratory comparison to evaluate the suitability of the test kit when used by cooperating laboratories.

    a. *Test Panel*. A test panel, created by the manufacturer for use in the field trial, should consist of at least 20 reference samples spanning the expected range of reactivity. The panel should not contain more than five negative samples. The negative samples could be positive for another analyte which might cross-react with the kit. The report should discuss how the reactivity of the remaining samples was determined. Every effort should be made to use samples from natural infection/exposure rather than spiked samples. It is recommended that one to two reference samples be duplicated within the panel. The CVB recognizes there may be instances in which this is not possible. If the test panel cannot be created from diagnostic samples from unique, naturally infected animals, the protocol should discuss the rationale and provide details regarding the samples to be used in the panel.

    b. *Field Study.* The panel will be sent to each of three participating laboratories, shipped in accordance with 9 CFR 103.3. Each laboratory will test the panel members in each of two prelicense serials. The panel members should be randomized and the protocol should discuss the method of randomization used. Further, the individual(s) within each participating laboratory conducting the testing should be blinded to the sample status (positive/negative) as well as the number of positive and negative samples within the panel.

    c. Retesting samples with discrepant results is unnecessary and should be avoided. The report must include a table displaying all test results for each sample. All raw data should be supplied to the CVB in electronic format.

    d. Participating laboratories should be encouraged to determine the suitability of the test kit by testing samples submitted to their laboratory. This is especially critical for fresh samples such as whole blood and fecal samples.

D. *Program Diseases.* The National Veterinary Services Laboratories (NVSL) will evaluate diagnostic test kits intended for U.S. State and/or Federal eradication/control programs. The CVB will provide prelicense serials to the

NVSL for evaluation. The license and/or permit for such kits may restrict distribution to Animal and Plant Health Inspection Service (APHIS)-approved laboratories. A U.S. Veterinary Biologics License or Permit does not guarantee the test kit will be used in an official eradication program.

Significant changes in disease prevalence may affect the diagnostic implication of a test result and, hence, the role of the kit in disease eradication/control programs.

VII. OUTLINE OF PRODUCTION

Manufacturing practices and production standards for test kits shall be characterized in accordance with the requirements prescribed in the applicable Standard Requirements specified in 9 CFR, and as specified by the manufacturer in the filed Outline of Production. The preparation of test kit components and reagents shall be described using the Outline Guide for Diagnostic Test Kits, specified in 9 CFR 114.9(f). The subsequent items in this section provide additional guidance but are not all-inclusive.

A. *Introduction Section*. The kit description, list of components, and test interpretations and limitations contained in this section should be included in the product circular. The description should avoid stating or implying the test kit is a quantifying assay. Statements such as "proportional to the OD value" should be avoided. Quantification statements will only be allowed for poultry antibody test kits.

B. *Section I. Antibody Production*

1. Agent-specific antibody is defined as any reagent(s) which participates in, or competes with, the antigen-antibody reaction being measured by the kit.

2. May be purchased or prepared on licensed premises.

    a. The filed Outline of Production must specify the identity, source (including country of origin), the acceptance criteria, and additional quality testing the firm performs on each lot. The statement, "accepted under a Certificate of Analysis" is not an acceptance criterion.

    b. Purchased monoclonal antibodies must be fully characterized and specify the clone designation or reacting epitope. Changes in source must be approved by the CVB. If antibodies are obtained from another licensed establishment, they do not require a "For Further Manufacture" product license. Firms are encouraged to obtain monoclonal antibodies from sources where the hybridoma meets the requirements prescribed in 9 CFR 113.52.

c. Master Cell Hybridomas used in the preparation of monoclonal antibodies prepared on licensed premises shall meet the applicable requirements prescribed in 9 CFR 113.52. The filed Outline of Production must specify the identity, source, the acceptance criteria, and additional quality testing the firm performs on each lot used on production.

3. A statement indicating ingredients of animal origin must be sourced from countries whose Bovine Spongiform Encephalopathy status is either no risk or minimal risk as defined by the National Center for Import-Export and 9 CFR 94.18 shall be included in the filed Outline of Production in either sections I, II, or III. Ingredients of animal origin, used in the preparation of any components for a diagnostic test kit, whether produced in the U.S. or imported from foreign sources, may be exempted from the requirements and restrictions specified in 9 CFR 113.50, 113.53, and 122 if a risk assessment has been submitted to the CVB and found to have negligible risk to U.S. animal health. If requesting an exemption, an assessment may be performed defining the risk associated with not conducting the specified 9 CFR testing in a manner acceptable to APHIS.

4. Changes in propagation method, growth media, hybridoma line, or passage level may require confirmation of kit sensitivity and specificity and/or additional testing and a change to the Outline of Production.

5. All kits within a serial must be prepared from a same lot of antibody.

C. *Section II. Antigen Preparation, including PCR primers*

1. May be purchased or prepared on licensed premises

a. For purchased antigen, the filed Outline of Production must specify the identity, source, the acceptance criteria, and additional quality testing of each lot. The statement “accepted under a Certificate of Analysis” is not an acceptance criterion. Changes in source must be approved by the CVB. Firms are encouraged to obtain antigen preparations from sources where the antigen meets the requirements prescribed in 9 CFR 113.27(c) and 113.55.

b. Master Seed Viruses (MSV) specified in the Outline of Production shall be tested for extraneous viable bacteria and fungi, as specified in 9 CFR 113.27(c), and extraneous viruses, as prescribed in 9 CFR 113.55. The MSV shall be tested for appropriate identity characteristics, as specified in a filed Outline of Production. Master Cell Stock (MCS) cultures used in the preparation or propagation of

Master Seeds on licensed premises shall meet the applicable requirements prescribed in 9 CFR 113.51 and 113.52. The filed Outline of Production must specify the identity, the acceptance criteria, and additional quality testing of each lot used on production.

2. Master Seed Bacteria (MSB) prepared on licensed premises specified in the Outline of Production shall be tested for viable extraneous bacteria and fungi, as specified in 9 CFR 113.27(b), and for appropriate biochemical and cultural characteristics, as specified in the filed Outline of Production. The filed Outline of Production must specify the identity, the acceptance criteria, and additional quality testing of each lot used on production.

3. Genetically modified (gene-deleted or recombinant) Master Seed (MS) organisms prepared on licensed premises shall be tested according to the requirements in section VII.C.2 or VII.C.3, as appropriate and applicable. If alternative purity, identity, or expression assays are necessary, genetically modified MS shall be tested by laboratory procedures acceptable to the CVB. The filed Outline of Production must specify the identity, source, the acceptance criteria, and additional quality testing of each lot used on production.

4. Master Seeds of other microbial classes (e.g., fungi, rickettsiae, parasites) must be adequately identified and tested for purity by laboratory procedures acceptable to the CVB. The filed Outline of Production must specify the identity, source, the acceptance criteria, and additional quality testing of each lot used on production.

5. When synthetic antigens or oligonucleotides are used in test kits, the amino acid, nucleotide sequence, or carbohydrate composition, along with any other critical structural specifications and criteria necessary to ensure quality, shall be described in the filed Outline of Production in a manner acceptable to the CVB. Synthetic antigens or oligonucleotides may be purchased; the filed Outline of Production must specify the identity, source, the acceptance criteria, and additional quality testing of each lot.

6. All kits in a serial must be prepared from the same lot of agent-specific antigen, synthetic antigen, or oligonucleotide.

D. *Section III. Preparation of Standard Reagents*

Test kit components are subject to the requirements and restrictions indicated in the following chart:

Component	Produced in Licensed Establishment	Same Lot for Entire Serial	Source Identified and/or Formula in Outline	Submit Data Before Changing	Dating of Serial
Anti-species Antibody or Conjugate	No	Yes	Yes	Yes	Yes
Agent Antigen or Antibody	No	Yes	Yes	Yes	Yes
PCR Master Mix	Yes	Yes	Yes	Yes	Yes
Sample Diluent	No	No	Yes	Yes	Yes
Controls	No	Yes	Yes	Yes	Yes
Stop Solution	No	No	Yes	Yes	No
Prepared Solid Surface	Yes	Yes	Yes	N/A	Yes

1. *Section III.A.* Describe the manufacture of the positive and negative controls used in the kit. The controls may be purchased; the filed Outline of Production must specify the identity, source, acceptance criteria, and additional quality testing performed on each lot. The statement "accepted under a Certificate of Analysis" is not an acceptance criterion. All kits in a single serial must be prepared from the same lot of the control.

2. *Section III.B.* The Anti-Species Antibody or Conjugate is defined as any reagent(s) used to amplify/report an antigen-antibody reaction. It includes anti-species antibody; protein-A, -G, or -L; colloidal gold; biotin; or enzyme-labeled versions of any of these. It does not need to be prepared on licensed premises, but each lot must be validated in a manner acceptable to the CVB. Acceptance criteria must be specified in the filed Outline of Production. All kits in a serial must be prepared from the same lot of anti-species antibody or conjugate.

3. *Section III.C.* The Substrate is defined as a substance which undergoes a color change or other detectable reaction when catalyzed by an enzyme-labeled kit component. It may be purchased. The filed Outline of Production must specify the source and the acceptance criteria of each lot.

Changes in source must be approved by the CVB. It is permissible to use more than one lot in the manufacture of a serial.

4. *Section III.D.* List all buffers included in the kit. Buffers are defined as inert liquids used to dilute test samples/other kit components, perform washes, or stop substrate reactions. Describe the source and/or formula for all buffers contained in the test kit. Each lot of buffer, diluent, or other liquid of non-animal origin should be stable in the final container. Methods used to stabilize the liquids, as well as a validated, maximum acceptable time interval between manufacture and stabilization to ensure lack of contamination with bacterial by-products, should be described. Stability of stop solutions composed of strong acid (e.g., 1M H2SO4), or other chemicals generally accepted as not supporting microbial growth, does not need to be demonstrated.

5. Changes in any of the test kit reagents should be supported by data demonstrating that the changes have not altered the sensitivity and/or specificity of the test kit.

E. Section IV. Preparation of the Product

1. *Section IV.A.* List the preservative(s) and concentration for each component containing preservatives.

2. *Section IV.B.* When coated solid-phase components (e.g., immunoassay plates, beads, or membranes) are prepared, they must be assigned a lot identity separate from those of the coating reagent (antigen/antibody) and the uncoated solid-phase substrate. The solid-phase component type should be identified in the Outline of Production. Solid-phase components should be coated on licensed premises; exemptions require specific approval by the CVB. Each lot of coated solid-phase component must be prepared with the same lot of coating reagent and a single lot of solid-phase substrate. All kits in a serial must be prepared using the same lot of coated solid-phase component. The formulas for reagents necessary to prepare the solid-phase components should be included. Changes in the coating method should be supported by data demonstrating the changes have not altered the sensitivity and/or specificity of the test kit.

3. *Section IV.C.* List the minimum and maximum fill volumes for each final container to ensure there is enough of the component to adequately perform the test(s).

4. *Section IV.D.* Describe the method used to dispose of unsatisfactory material. The firm may refer to VS Memorandum No. 800.56.

F. Section V. Testing

1. *Section V.A.* Test kits are exempt from the sterility and purity tests described in 9 CFR 113.26, 113.27, and 113.28.

2. *Section V.B.* Test kits are exempt from animal safety tests.

3. *Section V.C.* The manufacturer must perform a potency test using reference samples (serial release panel) on each assembled test kit serial. Each panel member must have an objective value for the acceptance criterion. The potency test must be performed in accordance with the instructions in the test kit insert and specified in the filed Outline of Production. Serial release testing provides confidence each serial will perform to the specificity and sensitivity standards determined at the time of licensure.

    a. The serial release panel used for the potency test must be well characterized. The panel should include examples of the following:

        (1) Negative/uninfected animals.

        (2) Strongly positive animals.

        (3) Moderately positive animals.

        (4) Weakly positive animals.

    b. It is acceptable for the serial release panel to be samples artificially created from antigens or antibodies, or dilutions of a single sample. However, these samples should be pre-diluted; to avoid dilution errors, a sample should not be diluted to produce multiple samples with different reactivity at the time the assay is performed. Serial release panels should be prepared in sufficient quantities and single-use aliquots to last for years.

    c. The serial release panel must be identified in the Outline of Production by lot number, recommended storage temperature, and acceptable assay ranges. The firm should submit data to show how the ranges are calculated. For example, for an ELISA test kit where results are expressed as a ratio of the optical densities of the sample to a positive control (S/P), the filed Outline of Production must specify an acceptable S/P range (including appropriate upper and lower limits) for each reference sample. For a test to be considered satisfactory, each serial release panel member must test within the specified range. For products containing positive and/or negative controls, each positive and negative control must test within the

specified range. If appropriate methods to obtain quantitative measurements are available, such as densitometry, objective criteria will be required for serial release of kits interpreted subjectively in the field.

d. The manufacturer of the kit must supply aliquots of each panel member to the CVB laboratory to be used for confirmatory serial release testing. However, samples are not required for inclusion in kits marketed to the consumer.

e. All potency testing of individual serials, whether by the firm or at the CVB, should be done using the same panel members. For serial release, the panel identification shall be recorded on the APHIS Form 2008 for the serial.

f. It will be necessary to replace members of the serial release panel as the stock depletes. The replacement sample should serve the same purpose as the sample it is replacing. Its performance should demonstrate its ability to fulfill the same role, but it need not have precisely the same reactivity as the previously approved panel member.

(1) Sufficient data should be obtained to demonstrate the replacement sample's performance in the assay. Validation data must be accepted by the CVB prior to use of the replacement sample in serial release testing.

(2) The replacement sample (e.g., positive samples not near the saturation or critical decision point of the assay) will be acceptable if the response distributions of the replacement and current member are similar. The replacement sample may also be deemed acceptable if the distributions are not similar, but it serves a similar role as the current panel member. In this case, serial release specifications may require adjustment.

(3) Weak positive replacement samples should have response values (in the assay) near the critical decision point. The acceptable result range should not cross the cutoff value between assay runs. Reactivity ranges should be established with an appropriate response range above the cutoff value.

(4) For samples at the extremes of the dynamic range, such as negative panel members, the reactivity of the replacement samples should be within the response range (e.g., all negative replacement samples should test within the negative range).

G. *Section VI. Post-preparatory Steps*

1. *Section VI.A.* List the number of final component containers in each kit box. Multiple variations of the kit components are acceptable.

2. *Section VI.B.* Describe the collection of submission samples and retention samples as specified in 9 CFR 113.3(b)(7) and 113.3(e)(1). A sufficient quantity should be submitted to the CVB in order to conduct the potency testing. Storage and shipment of the kits should be in accordance with 9 CFR 114.11.

3. *Section VI.C.* Each lot of each component in the kit shall be assigned an expiration date based on the stability of the individual component. The expiration date may be indicated on the component label. The expiration date of the serial shall be calculated from the date of initiation of the first potency test but shall not exceed the expiration date of any of the components. The dating for test kits shall not exceed 12 months unless real-time stability data to justify a longer interval have been approved by the CVB.

4. *Section VI.D.* Include the recommendations, qualifications, limitations, and test interpretations for use of the kit. Information in this section is the same as in the introduction section and what is included on the insert for use for the kit. The kit should define the acceptable qualities and the potential impact for a test sample to be suitable for analysis in the test kit. All potentially infective material must be appropriately labeled. Chemical safety instructions must be included in the labeling for all hazardous materials. Disposal instructions must be given in the Outline of Production and on the package insert.

VIII. CONFIRMATION OF DATING (COD)

Product dating must be confirmed.

A. *Number of Serials.* Three serials should be used in the COD study.

B. *Frequency of Testing.* Each serial should be tested at release and at the end of the proposed dating period using the serial release panel and criteria specified in the filed Outline of Production. Ideally, interim testing is also performed. The same serial release panel should be used for all tests performed on a given Serial.

IX. SHIPPING TEST KITS

If a manufacturer wishes to ship a test kit outside the recommended storage conditions specified on the label, data should be submitted to the CVB demonstrating that the test kit performs as expected for the temperatures outside the recommended storage conditions.

Manufacturers requesting an exemption to 9 CFR 114.11 during shipment should submit a protocol to the CVB prior to initiation of the studies. If granted, the date the exemption was granted and the specific shipping conditions should be included in section VI.B of the Outline of Production.