June 20, 2002

VETERINARY SERVICES MEMORANDUM NO. 800.73

Subject: General Requirements for Immunodiagnostic Test Kits for the Detection of Antibody or Antigen

To: Biologics Licensees, Permittees, and Applicants
Directors, Center for Veterinary Biologics

I. PURPOSE

This memorandum provides guidance to licensees, permittees, and applicants concerning the requirements to support an application for a U.S. Veterinary Biological Product License or a U.S. Veterinary Biological Product Permit for antigen/antibody-based immunodiagnostic test kits for the diagnosis of animal disease and/or immunological status, as authorized by Title 9 Code of Federal Regulations, Part 114.9(f).

II. CANCELLATION

This memorandum cancels Veterinary Services Memorandum 800.73, dated August 19, 1997.

III. DEFINITION OF TERMS USED IN THIS MEMORANDUM

A. **Accuracy**: Closeness of agreement between the value produced by the assay and the correct value. Accuracy may be evaluated by comparing assay values with values accepted as correct by convention, such as an accepted gold standard, an accepted validated reference value, or the nominal value of a preparation formulated and validated for that purpose.

B. **Precision**: Degree of scatter among a series of measurements obtained from multiple observations of the same homogeneous sample under specified conditions. Precision is considered at several levels:

1. *Within-assay*: Precision among replicated samples in the same assay. For immunoassay plate-based kits, this refers to samples on the same plate and may also be called *within-plate* when assays consist of more than one plate run at the same time. This is evaluated by residual error.

2. *Between-plate*: Precision among assays run independently, either concurrently or within a short time interval, under the same operating conditions. For immunoassay plate-based kits, this refers to samples on different plates when more than one plate is processed concurrently. This is also called **repeatability**.

3. *Between-assay*: Precision among assays run independently under similar, but not necessarily identical, operating conditions. This refers to assays run at different times on the same day. This is one component of **intermediate precision**.

4. *Within-laboratory*: Precision among assays run within the same laboratory under different conditions, such as on different days or by different operators. This, also, is one component of **intermediate precision**.

5. *Between-laboratory*: Precision of the same assay procedure run on the same sample by different laboratories. This is also called **reproducibility**.

C. **Ruggedness**: Measure of the capacity of the assay to remain unaffected by deliberate small variations in method parameters. Sometimes called **robustness**, it provides an indication of assay reliability under normal use.

D. **Sensitivity**: Ability of a test to correctly identify samples from positive animals. This is expressed as a percentage, based on the following calculation:

true positive/(true positive + false negative) x 100%

Animals are classified as positive based on a gold standard or the best possible evaluation if more than one criterion is available. "True positives" are positive animals that test positive in the assay under consideration. "False negatives" are positive animals that test negative in the assay.

E. **Specificity**: Ability of a test to correctly identify samples from negative animals. Expressed as a percentage, based on the following calculation:

true negative/(true negative + false positive) x 100%

Animals are classified as negative based on a gold standard or the best possible evaluation if more than one criterion is available. "True negatives" are negative animals that test negative in the assay under consideration. "False positives" are negative animals that test positive in the assay.

F. **Dynamic Range**: Interval between the upper and lower amounts of analyte for which the assay has a suitable level of precision and accuracy.

G. **Gold Standard**: As a working definition for this document, Gold Standard is defined as an accepted reference standard or diagnostic test for a particular disease (the method that provides the true status of an animal as best as can be determined). In some cases, the Gold 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.)

IV. GENERAL REQUIREMENTS

Manufacturing practices and production standards for immunodiagnostic test kits shall be characterized in accordance with the requirements prescribed in the applicable Standard Requirements specified in Title 9, Code of Federal Regulations (9 CFR), and as specified by the manufacturer in the filed Outline of Production.

A. Manufacturing Standards

The preparation of test kit components and reagents shall be described using the Outline Guide for Diagnostic Test Kits, specified in 9 CFR, Part 114.9(f).

B. Requirements for Master Seeds and Ingredients of Animal Origin

1. Master Seeds that are whole cultures of a virus that are used in immunodiagnostic test kits shall be tested for extraneous viable bacteria and fungi, as specified in 9 CFR Part 113.27(c), and extraneous viruses, as prescribed in 9 CFR Part 113.55. They also shall be tested for appropriate identity characteristics, as specified in a filed Outline of Production.

2. Master Seeds that are whole cultures of a bacterium that are used in immunodiagnostic test kits shall be tested for viable extraneous bacteria and fungi, as specified in 9 CFR Part 113.27(d), and for appropriate biochemical and cultural characteristics, as specified in the filed Outline of Production.

3. Master Seeds that are genetically modified (gene-deleted or recombinant) organisms shall be tested according to the requirements in Section IV.B.1-2, as appropriate and applicable. If alternative purity, identity, or expression assays are necessary, genetically-modified Master Seeds shall be tested by laboratory procedures that are acceptable to the Animal and Plant Health Inspection Service (APHIS) and described in the filed Outline of Production.

4. Master Seeds of other microbial classes (e.g., fungi, rickettsiae) must be adequately identified and tested for purity by laboratory procedures acceptable to APHIS and described in the filed Outline of Production.

5. When synthetic antigens are used in immunodiagnostic test kits, the Master Seed shall be a Master Sequence. The amino acid, nucleotide, or carbohydrate sequence of the antigen, along with any other critical structural specifications and criteria necessary to ensure antigen quality, shall be described in the filed Outline of Production in a manner acceptable to APHIS.

6. Ingredients of animal origin, whether produced in the U.S. or imported, and other reagents obtained from foreign sources shall be subject to the requirements and restrictions specified in 9 CFR Parts 104, 113.50, 113.53, and 122. Ingredients of animal origin must be sourced from countries whose BSE status is either no risk or low risk as defined by the National Center for Import-Export and 9CFR 94.18.

7. Cell cultures that are used in the preparation or propagation of Master Seeds or in the preparation of other kit components shall meet the applicable requirements prescribed in 9 CFR Parts 113.51 (primary cells) and 113.52 (cell lines).

V. COMPONENTS OF IMMUNODIAGNOSTIC KITS

The source and/or formula for all components of immunodiagnostic test kits must be described in the filed Outline of Production. All components must be lot controlled. Prior to changing the specified source or formula of any component, validation data, demonstrating that the performance level of the kit is retained (or improved), must be submitted to, and accepted by, APHIS.

A. Agent Antigen or Antibody

1. Defined as any reagent(s) that participates in, or competes with, the specific agent antigen-antibody reaction being measured by the kit, including control sera.

2. Should be prepared from an APHIS-approved Master Seed or Master Cell stock on licensed premises. Use of reagents prepared in a different manner must be justified and requires specific APHIS approval.

3. Must be prepared and stored in a clean manner and shown to be stable at recommended storage temperatures.

4. Bulk lots must be tested for purity, identity, and sterility by methods described in the filed Outline of Production and acceptable to APHIS.

5. If the agent antigen or antibody is not prepared by the firm or is derived from a source other than an approved Master Seed or Master Cell, additional quality testing, acceptable to APHIS, may be required, and each lot must be approved by APHIS prior to use. Confirmatory testing by the Center for Veterinary Biologics-Laboratory (CVB-L) may be required on each lot.

6. Significant changes in microbial strain, line, or passage level may require confirmation of kit sensitivity and specificity and/or additional suitability testing.

7. All kits in a single serial must be prepared using a single lot of agent antigen or antibody.

B. Anti-Species Antibody or Conjugate

1. Defined as any non-antigen-specific reagent(s) used to coat a solid phase or to amplify/report an antigen-antibody reaction. Includes anti-species antibody; protein-A, -G, or -L; biotin; or enzyme-labeled versions of any of these.

2. Does not need to be prepared on licensed premises, but each lot must be validated, either by certificate of analysis from the manufacturing source or by in-house testing, prior to use in a manner acceptable to APHIS. Acceptance criteria must be specified in the filed Outline of Production.

3. All kits in a single serial must be prepared using a single lot of anti-species antibody or conjugate.

C. Coated Solid-Phase Components

1. When coated solid-phase components (e.g., immunoassay plates 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.

2. Solid-phase components should be coated on licensed premises. Exemptions require specific approval by APHIS.

3. Each coated component piece in a lot must be prepared with a single lot of coating reagent (or multiple lots that were well mixed (homogeneous) prior to application) and a single lot of solid-phase substrate.

4. All kits in a single serial must be prepared using the same lot of coated solid-phase component.

D. Substrates

1. Defined as substances that undergo a color change or other detectable reaction when catalyzed by an enzyme-labeled kit component.

2. May be purchased; the filed Outline of Production must specify the source and the criteria for acceptance of each lot. Changes in source must be approved by APHIS.

3. All kits in a single serial must be prepared using a single lot of substrate.

E. Inert Buffers and Diluents, Stop Solutions

1. Defined as inert liquids used to dilute test samples/other kit components, wash solid-phase components, or stop substrate reactions.

2. Permissible to use more than one lot of buffer, diluent, or stop solution in the manufacture of a single kit serial

3. Must be manufactured from clean ingredients and be sterile or stable (i.e., microbiostatic) in the final container.

4. Methods used to sterilize or stabilize the liquids, as well as a validated, maximum acceptable time interval between manufacture and sterilization or stabilization, should be described in the filed Outline of Production to ensure lack of contamination with by-products of bacterial growth.

VI. PRELICENSING REQUIREMENTS

A. Data Considerations

The primary data considerations for diagnostic test kits are listed below:

1. Sensitivity

The sensitivity of the kit should be estimated by testing known positive samples, as determined by a recognized gold standard acceptable to APHIS. Samples covering a range of reactivities (strong positive, weak positive) should be evaluated; data from these samples are also used to define the dynamic range of the kit. APHIS may request evaluation of samples obtained at different time intervals after infection/vaccination to determine the period during which the kit has acceptable sensitivity. If certain conditions or interfering substances are known to affect the performance of the kit, appropriate samples should be included so that the magnitude and clinical significance of the effect(s) can be evaluated.

2. Specificity

The specificity of the kit should be estimated by testing known negative samples, as determined by a recognized gold standard acceptable to APHIS. Samples should be obtained from uninfected animals from several sources, to provide a representative sampling of the general population. Kits for the detection of antibody should be specifically tested against immune sera to related antigens and sera obtained from animals vaccinated with biological products commonly used in that animal species. Cross-reacting and/or interfering substances should be identified.

The number of samples necessary to estimate sensitivity and specificity is dependent on the species of animal to be tested, the proposed use of the kit, proposed kit claims, the acceptability of the proposed gold standard, and the availability of adequately validated samples. If the kit is to be labeled for more than one animal species, animals from each species must be included. An adequate number of each sample type (e.g., whole blood, serum, plasma) from each of the animal species for which the kit is to be labeled should be included.

3. Ruggedness, Repeatability, and Suitability

Experimental kits must be evaluated in typical laboratory settings. The data generated from these field trials will be used to assess assay repeatability, ruggedness, suitability; to evaluate the adequacy of test instructions for general use; and to confirm sensitivity, specificity, and precision estimates generated by the kit manufacturer.

At least two prelicense serials should be evaluated in at least three geographically distinct cooperating laboratories. The kit should be provided in its final form and include the instructions for use that will be supplied to consumers after licensure. Cooperating laboratories should perform the assay exactly as stated in the supplied instructions. Each laboratory should test a panel of at least 20 reference samples supplied by the manufacturer of the test kit. In addition, each laboratory is asked to test well-characterized samples taken from its own inventory.

The reference panel supplied by the kit manufacturer may be the same panel used for serial release potency testing (see Section VII.C.) or may be an alternative panel of samples meeting the same criteria. The identity of the samples must be coded so that the cooperating laboratories are blinded to sample identity and classification. Each sample should be provided in duplicate or triplicate, with each replicate uniquely identified, so that error and repeatability data may be generated.

Cooperating laboratories must submit to the kit manufacturer all raw data regarding the assay response of the coded samples. For each laboratory inventory sample that is tested, the official classification (positive/negative) and the test

method (gold standard) used to classify the sample should be submitted in addition to the assay response value.

Diagnostic test kits for diseases with U.S. State and/or Federal eradication/control programs will be provided to the National Veterinary Services Laboratories (NVSL) by the Center for Veterinary Biologics (CVB) for evaluation for program use. In some cases, APHIS may provide the core samples for a reference serum panel. The license and/or permit for such kits may restrict distribution to APHIS-approved laboratories.

4. Predictive Values

Sharp 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. When prevalence estimates are available, the projected positive and negative predictive values of a test result may be considered in optimizing the kit for program use. APHIS may use these projections to suggest cutoff values (which affect the relative sensitivity and specificity of the kit) that are appropriate for the needs of the program at a given stage in the eradication/control effort. For example, early in eradication/control programs, cutoff values may be set to ensure high sensitivity; when eradication/control programs have progressed to the point that low prevalence occurs, cutoff values may be set to ensure high specificity.

B. Sources of Samples

In general, samples tested in support of licensure should be acquired from domestic sources. However, if antigenic variation is not considered to be a significant feature of the etiologic agent for which the kit is designed, exceptions may be made, upon prior approval by APHIS. Contact the CVB-Licensing and Policy Development (CVB-LPD) to obtain the appropriate permits prior to importing test samples or other potentially infectious materials from other countries.

If a kit is intended for the diagnosis of a disease that is exotic to the United States, testing of all potentially infectious samples will be performed in facilities that are appropriate for the disease and are specified and approved by APHIS, which may include the NVSL or a foreign laboratory.

C. Study Design

Studies to support licensure of antigen/antibody-based immunodiagnostic kits should be designed with the following points in mind. The CVB encourages firms to submit protocols for comment prior to initiating pivotal studies. See VS Memorandum 800.200 for additional guidance.

1. Identify the target population and state how it is emulated by the sampling frame. Describe how the subjects will be selected from the sampling frame. Subjects are selected from a subject pool; if that pool emulates a population in some way, it is termed a sampling frame, and, if properly sampled, inference may be drawn to the associated population. The choices made in sample selection will impact the allowed claims for kit performance.

2. Identify the gold standard or other reference method, and justify the comparison of the experimental product to that method. If the gold standard is a composite of more than one assay, describe the serial or parallel testing sequence. In any case, testing procedures (e.g., types of tests performed, number of replicate samples tested, number of retests) must be applied uniformly to all test samples. To avoid bias, do not apply the gold standard or reference method differently to samples based on the result obtained with the experimental product.

D. Study Reports

In addition to preparing summary reports, submit the raw data, in the format indicated in Appendix I, for each sample. If possible, submit one copy of the data on electronic disk; see VS Memorandum 800.96 (Guidelines for Submitting Electronic Data Files for Statistical Analysis) for additional guidance.

Do not pool estimates, such as those of sensitivity and specificity, unless warranted by clinical and statistical homogeneity. When estimates diverge widely between laboratories, state the range of observed values. For example, with sensitivity estimates of 48%, 92% and 71%, state that sensitivity at three laboratories ranged from 48% to 92% instead of stating that average sensitivity was 70%. It may be appropriate to present observed sensitivity and specificity estimates in table format on kit inserts.

E. Confirmatory pre-license testing by the CVB-L

Upon approval by the CVB-LPD, serials of prelicense product will be submitted to the CVB-L for confirmatory testing. The CVB-L will test the product using the criteria specified in Section V of the Outline of Production and may conduct additional testing to confirm kit claims.

VII. SERIAL RELEASE TESTING

A. Sterility and Purity

Plates, gels, and antigen/antibody on a solid substrate are exempt from the sterility and purity tests described in 9CFR Parts 113.26, 113.27, and 113.28.

Each lot of buffer, diluent, or other liquid of non-animal origin should be sterile or stable. Sterility or stability of stop solutions composed of strong acid (e.g., 1M H_2SO_4), or other chemicals generally accepted as not supporting microbial growth does not need to be demonstrated. Ingredients of animal origin must meet the requirements prescribed in 9 CFR Part 113.53.

B. Safety

Finished kits are exempt from animal tests for safety. All potentially infective material must be appropriately labeled. Chemical safety instructions must be included in labeling for all hazardous materials. Disposal instructions must be given in the Outline and on the final packaging label.

C. Potency

The manufacturer must perform a potency test on each serial of assembled test kits. The potency test must be performed in accordance with the instructions in the test kit insert and as specified in the filed Outline of Production, using reference samples that are acceptable to APHIS. The purpose of the potency test is to ensure that each serial of the assay is producing accurate test results when properly performed. Serial release testing provides confidence that each serial will perform to the specificity and sensitivity standards determined at the time of licensure.

Reference samples must be well characterized and validated. The panel of samples should include examples of the following:

1. Negative/uninfected animals

2. Strongly positive animals

3. Weakly positive animals

4. Samples generating assay values just above, and just below, the cutoff value between positive and negative classification

5. Animals with reactivity to closely related (potentially cross-reactive) antigens and/or vaccinated animals

6. Animals reactive for only one, or a subset, of antigens for kits that detect reactivity to more than 1 antigen

The reference panel should include sera from at least 20 individual animals. Smaller panels may be considered for diseases where samples are difficult to obtain or validate. Pooled

sera should be avoided, when possible. A single serum sample may be used more than once in the panel to evaluate error among replicate samples, but a single serum sample must not be diluted with normal serum to produce multiple samples with different reactivities.

The reference samples must be identified in the Outline of Production by lot number, date of preparation, purpose, and acceptable assay range. The acceptable range should be expressed in the units used by the end-user to interpret results. For example, for an ELISA where results are expressed as a ratio of the absorbance of the sample and 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, the reference samples must test within the range(s) in a valid assay. If appropriate methods to obtain valid quantitative measures are available, objective criteria may be required to measure the potency of kits that are subjectively interpreted in the field; however, each serial also must pass potency testing by the subjective criteria used by consumers.

When the inventory of a reference sample is low, a replacement sample should be identified and validated. The replacement sample should serve the same purpose as the original sample (e.g., a strongly positive sample is replaced by another strongly positive sample). Validation data must be accepted by APHIS prior to use of the replacement sample in serial release testing; confirmatory testing at the CVB-L may be required.

The manufacturer of the kit must supply aliquots of each reference sample to the CVB-L for confirmatory serial release testing; however, samples are not required for inclusion in kits that are marketed to the consumer.

APHIS reserves the right to establish reference panels for certain disease agents and require their use in serial release potency tests. In such cases, panels are supplied to the licensees/permittees by the CVB-L. The CVB-L provides written notice to licensees and permittees when the CVB-L panels are replaced and gives the date after which all tests should be performed with the new panel. Diagnostic test kits must achieve a passing score on the CVB-L test panel prior to serial release. The CVB-L panel may contain current NVSL proficiency check-test samples and may be supplemented, as appropriate, by additional samples supplied by the CVB-L. The minimum criteria for acceptable potency (number of samples identified correctly) will be determined by the CVB but will not be lower than the requirements established by APHIS to certify laboratory proficiency. Firms may add additional samples, which will be tested per criteria established in Section V. of the Outline of Production, to the serial release panel.

Individual serials should be tested by the same panel(s) by the firm and the CVB-L, at serial release and throughout dating. The firm shall use current panel(s) to perform the first potency test on the serial, and the panel identification shall be recorded on the APHIS Form 2008 for the serial. The samples used for the first potency test shall comprise the panel that is used for all testing of that kit, regardless of whether panel members, or entire

panels, change prior to expiry of the serial. If it appears that samples used in the first potency test will be replaced prior to expiry of the serial, sufficient quantities of the original panel should be retained for any additional testing that may be performed on that serial throughout dating.

VIII. DATING OF IMMUNODIAGNOSTIC TEST KITS

Each lot of each component in the kit shall be assigned an expiration date based on the stability of the individual component, and the expiration date shall be indicated on the component label. The expiration date of the kit shall be calculated from the date that the first potency test is initiated but shall not exceed the expiration date of any of the components.

The dating for immunodiagnostic test kits shall not exceed 12 months unless real-time stability data are submitted to justify a longer interval. To confirm the initial dating period granted at licensure, potency testing shall be performed on the first three serials of kits on, or after, their expiration dates. The kits must have been stored within the recommended temperature ranges. Confirmatory data shall be submitted to, and approved by, APHIS.

/s/ W. Ron DeHaven

W. Ron DeHaven
Deputy Administrator
Veterinary Services

Appendix I

Sample format for data submission

Animal ID	Laboratory	Technician	Day	Run	Result (Signal: Pos Ratio)	Test Conclusion	Gold Std. Conclusion
32	CA	A	Wed	1	1.179	pos	pos
32	CA	A	Wed	2	1.402	pos	pos
34	NY	A	Mon	1	1.080	pos	neg
34	NY	A	Tues	1	0.900	neg	neg