

## Chapter 4.1.4 Validating Alternate Techniques for Detection of Mycoplasmas

### Subject

Guidance for validating alternative techniques for the detection of mycoplasma.

### Purpose

This document provides guidance concerning the information a firm should provide when submitting a new mycoplasma detection method for consideration by the Center for Veterinary Biologics (CVB). Recently alternative methods have been developed for mycoplasma testing of veterinary biologics. Most of the methods are nucleic acid amplification technique (NAT)-based assays which are more rapid than the direct culture method cited in title 9, Code of Federal Regulations (9 CFR), Part 113.28. This document serves to outline expectations for validating a new alternative method.

### Background

Assay validation provides evidence that an assay meets all of the requirements of its intended use. NAT methods for mycoplasma detection can be qualitative, semi-quantitative, or quantitative. Endpoint PCR assays are considered limit tests designed to give a positive or negative result regarding the presence of an analyte. Ideally NAT tests have a high degree of specificity and a low limit of detection. The current 9 CFR method for mycoplasma detection is a qualitative method; therefore, it is acceptable that the alternative method is qualitative. This diminishes the validation work to be done since fewer validation parameters have to be addressed.

### Method

- The sensitivity of the method should be similar to that of 9 CFR 113.28, which will serve as the “gold standard”.
- The method should include a positive control for the extraction process.
- The method should include positive and negative controls for the amplification process.
- Additional controls may be required, depending on the assay. [REDACTED]

### Validation

- Sensitivity
  - Direct and concurrent comparison with the 9 CFR method using the same samples (with a defined titer, CFU/mL or CCU) and dilutions. The dilution series should not be more than 10-fold. The proposed alternative method should be as sensitive or more so than the 9 CFR method.

- [REDACTED]
- Firms validating a different assay: It is recommended that firms follow the guidelines of the European Pharmacopoeia 6.1, Section 2.6.7 [REDACTED]
- Use of the 5 VICH strains for the validation is recommended, but the CVB will entertain the use of alternative strains.
  - *Acholeplasma laidlawii*
  - *Mycoplasma fermentans*
  - *Mycoplasma hyorhinis*
  - *Mycoplasma orale*
  - *Mycoplasma synoviae*
- In addition to the VICH strains, firm should include all Mycoplasma strains that are used by the firm, as these strains are potential contaminants for the firm.
- Specificity
  - One important feature of NAT-based assays is the ability to amplify a wide range of mycoplasma species; however, mycoplasma has a close phylogenetic relationship to Bacillus, Lactobacillus, Streptococcus, and Clostridium, therefore assay specificity must be evaluated. Specificity is validated by demonstrating that the assay can differentiate between closely related organisms. For NAT assays, primers and probes should amplify the target sequence but fail to amplify closely related targets. If a method does show cross-amplification of related targets, or a high rate of false positives, the firm should present a method for discrimination.
- Precision
  - With appropriate replication as discussed above, the studies used to evaluate sensitivity also provide relevant data on within and between assay/day repeatability.
  - To further evaluate intermediate precision, when applicable, additional replication of the dilution series should be done using multiple technicians and equipment.
- Reproducibility and Lab Transfer
  - If more than one laboratory will be running the assay, each laboratory should evaluate the above criteria in its own hands. Replicates from multiple labs within a firm (but **not** from outside the firm, such as contract testing labs) may be used to achieve the minimum 24 replicates/dilution (see Sensitivity above).
- Ruggedness/Robustness

- Provide evidence that deliberate small variations in method parameters, including different kit & reagent lots, have negligible effects on the outcome of the test. The performance of the assay should also be analyzed at the extreme values of any allowed assay parameter ranges, such as incubation time and temperatures, if applicable.
- Miscellaneous (System suitability)
  - Test the assay against product matrices or sample types to ensure that the product does not interfere with or inhibit the assay causing false negative results. [REDACTED]
  - [REDACTED] . When grouping products, the following should be considered:
    - Product formulation, including all reagents and adjuvant components that are added at batching and the percentage of each component; tested at the final concentration.
    - Composition and volume of diluent used to rehydrate lyophilized product (i.e. diluted product for mass vaccination).
    - Source of antigen (i.e., cell culture, allantoic fluid, ground tissue, etc.).
    - Method and degree of concentration.
    - Method of inactivation.
- Protocol
  - The particular design of a validation study for a given test method depends on the unique characteristics of that method. Firms are strongly advised to submit a protocol for review prior to initiating a test method validation study.

### Outline of Production

- The Outline of Production or Special Outline should describe the assay in sufficient detail that the CVB would be able to reproduce the assay without requiring additional information. The primer or probe sequences and source of all reagents should be included.
- Controls should be clearly described. The concentration of the positive control should be specified.
- For NAT methods, the Outline should state the type of thermocycler and other critical equipment that is used, and the instruments should be calibrated on a regular basis. Validation data (demonstration of fitness for purpose) will be required for each PCR machine.
- The Outline should describe the detection system in detail. [REDACTED]
- If the proposed method involves the use of equipment and/or reagents that the CVB does not have and is not able to obtain, the CVB will test product according to 9 CFR 113.28.

Section V.A of the Outline of Production should clearly state that the CVB will test according to 9 CFR 113.28.

- Validity criteria should be clearly stated.
- Acceptance criteria should be clearly stated.
- The Outline of Production should clearly state how positive results will be handled and should clearly specify the retest provisions. Sequencing of a PCR fragment is NOT an acceptable means to demonstrate that results are “false positive”. If the retest provision is the NAT test, the entire assay must be performed, starting with a new vial(s). The 9 CFR 113.28 test may be used as the definitive test.