United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 625

Supplemental Assay Method for *In vitro* Potency Testing of *Leptospira interrogans* Serovar *canicola* Bacterins

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Supplemental Assay Method for *In vitro* Potency Testing of *Leptospira interrogans* Serovar *canicola* Bacterins

Table of Contents

1. Introduction

2. Materials

   2.1 Equipment/instrumentation
   2.2 Reagents/supplies

3. Preparation for the Test

   3.1 Personnel qualifications/training
   3.2 Preparation of equipment/instrumentation
   3.3 Preparation of reagents/control procedures
   3.4 Preparation of the sample

4. Performance of the Test

5. Interpretation of the Test Results

   5.1 Relative potency calculation method
   5.2 Requirements for a valid assay
   5.3 Requirements for a satisfactory test bacterin

6. Report of Test Results

7. Summary of Revisions

Supplemental Assay Method for *In vitro* Potency Testing of *Leptospira interrogans* Serovar *canicola* Bacterins

1. Introduction

This Supplemental Assay Method (SAM) uses a sandwich enzyme-linked immunosorbent assay (ELISA) to measure the relative potency of bacterins containing *Leptospira interrogans* serovar *canicola* compared to a suitably qualified, nonexpired reference bacterin.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Micropipettors, to cover the range of 5.0-µL to 1000-µL

2.1.2 8- or 12-channel micropipettor, to cover the range of 50-µL to 200-µL

2.1.3 Orbital shaker

2.1.4 Automatic microplate washer (optional)

2.1.5 Microplate reader with dual wavelengths (405 nm and 490 nm)

2.1.6 Balance, validated from 150 mg to 15 g

2.1.7 Relative Potency Calculation Software (United States Department of Agriculture [USDA], Veterinary Services, Center for Veterinary Biologics [CVB]), current version of **SAM 318**

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 96-well flat-bottom microtitration plates (Immulon 2, Dynatech Laboratories, Inc.)

2.2.2 96-well non-binding microtitration plates suitable for making serial dilutions (transfer plate)

2.2.3 Plate sealers

2.2.4 Carbonate coating buffer

2.2.5 Phosphate buffer for elution (optional)

2.2.6 Sodium citrate for elution (optional)

2.2.7 Sodium deoxycholate (desoxycholate) elution buffer (optional)

2.2.8 Phosphate-buffered saline with 0.05% Tween 20 (PBS-Tween 20)

2.2.9 Antibody diluent with Normal rabbit serum (negative for *Leptospira* spp.)

2.2.10 ABTS (2,2'-azino-di-3-ethylbenziazoline sulfonate) substrate, 1- or 2-component (Kirkegaard and Perry Laboratories, Inc.)

2.2.11 *Leptospira canicola*-specific (clone 4DB) monoclonal antibody (MAb). *L. canicola* 4DB MAb is available from the CVB. Refer to the current reagent data sheet for additional information.

2.2.12 Polyclonal *Leptospira canicola* antiserum (PAb), rabbit origin. *L. canicola* PAb is available from the CVB. Refer to the current reagent data sheet for additional information.

2.2.13 Goat anti-mouse IgM (Mμ) horseradish peroxidase-labeled antibody, Human Serum Adsorbed (Kirkegaard and Perry Laboratories, Inc.)

2.2.14 Test bacterin(s) containing *L. canicola*

2.2.15 Reference bacterin (nonexpired) containing *L. canicola* (must be approved by the Animal and Plant Health Inspection Service)

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware; automated microplate washer and microplate reader; and data analysis software. They need specific training in the performance of this assay.

3.2 Preparation of equipment/instrumentation

Operate and maintain all equipment according to manufacturers' recommendations and applicable in-house standard operating procedures.

3.3 Preparation of reagents/control procedures

3.3.1 Carbonate coating buffer--National Veterinary Services Laboratories (NVSL) Media #20034

Na_2CO_3	0.159 g
$NaHCO_3$	0.293 g
Deionized water	q.s. to 100 mL

Adjust pH to 9.6 ± 0.1. Store at 2°- 7°C for up to 1 week.

3.3.2 Phosphate buffered saline (PBS)--NVSL Media #10559

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Deionized water	q.s. to 1 L

Adjust pH to 7.2 ± 0.1. Autoclave for 20 minutes at ≥ 121ºC Store at 20°- 25°C for no longer than 6 months.

3.3.3 Polyvinyl alcohol, 1%, in PBS

PBS (see **Section 3.3.2**)	70 mL
Polyvinyl alcohol, 88% hydrolyzed, MW 13,000-23,000 (Aldrich Chemical, Cat. No. 36,317-0, or equivalent)	0.7 g

Stir to dissolve using low heat if needed. Store at 20°- 25°C for no longer than 1 week.

3.3.4 Phosphate buffered saline with 0.05% Tween 20 (PBS-Tween 20)--NVSL Media #30179

PBS (see **Section 3.3.2**)	1000 mL
Tween 20	0.50 mL

Store at 20°- 25°C for no longer than 6 months.

3.3.5 Antibody diluent with normal rabbit serum (negative for *Leptospira* spp.)

Polyvinyl alcohol, 1%, in PBS (see **Section 3.3.3**)	43.6 mL
Normal rabbit serum	400 µL

Mix immediately prior to use. Store at 2°- 7°C for up to 1 week.

3.3.6 Phosphate buffer for antigen elution

KH_2PO_4 (Mallinckrodt, Inc., 7100, or equivalent)	8.2 g
Deionized water	94 mL

Adjust pH to 9.3 ± 0.1, or other appropriate pH as optimized for use with a specific bacterin. Store at 20°- 25°C for no longer than 1 month.

3.3.7 Sodium deoxycholate (desoxycholate) elution buffer

Sodium deoxycholate (Sigma Chemical D6750, or equivalent)	0.50 g
PBS (see **Section 3.3.2**)	100 mL

Store at 2°- 7°C up to 30 days. Warm to 20°- 25°C prior to use (buffer gels at 2°- 7°C).

3.3.8 MAb specific for *Leptospira canicola*, clone 4DB

Obtain MAb 4DB from the CVB. Store long-term at -70°C. The MAb may be stored at 2°- 7°C for several weeks. Refer to the current reagent data sheet for additional details.

3.3.9 Polyclonal *Leptospira canicola* antiserum, rabbit origin

Obtain the rabbit antiserum from the CVB. Store long-term at -70°C. The antiserum may be stored at 2°- 7°C for several weeks. Refer to the current reagent data sheet for additional details.

3.3.10 Bacterins containing *Leptospira canicola* antigen

1. Reference bacterin

2. Test bacterin(s)

CRITICAL CONTROL POINT: Ideally, the reference and test bacterins should be produced by the same Outline of Production. If reference formulation

differs from that of the test bacterin, the assay must be validated to show that this does not adversely affect assay performance or accuracy of results.

3.4 Preparation of the sample

Many bacterins do not require antigen-elution treatment prior to being serially diluted in twofold increments with PBS-Tween 20. Test representative batches of each adjuvanted product with and without each antigen-elution treatment to determine if the treatment specifically enhances antigen capture. If no enhancement of antigen capture can be demonstrated, test the bacterins without antigen-elution treatment. Treat the reference bacterin and the test bacterins by the same elution procedure. Alternative elution procedures, other than those described here, may be more appropriate for some bacterins.

3.4.1 Aluminum hydroxide-adjuvanted bacterins

Bacterins adjuvanted with aluminum hydroxide may be treated with either sodium citrate or phosphate buffer prior to making serial twofold dilutions in PBS-Tween 20.

1. Sodium citrate elution

Mix 1 g sodium citrate with 10 mL of bacterin (10% w/v). Place on an orbital shaker (100-120 rpm) overnight at 36°- 38°C. Consider treated bacterin to be undiluted.

2. Phosphate buffer elution

Mix 1 mL of phosphate elution buffer with 1 mL of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 36°- 38°C. Consider treated bacterin to be diluted 1:2.

3.4.2 Oil-adjuvanted bacterins

Mix 1 mL of sodium deoxycholate (desoxycholate) elution buffer with 1 mL of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 20°- 25°C. Consider treated bacterin to be diluted 1:2.

4. Performance of the Test

4.1 Dilute *L. canicola* rabbit antiserum to the current use dilution (refer to current reagent data sheet) in cold carbonate coating buffer. Dispense 100 µL into each well of a 96-well microtitration plate (test plate). Seal plate and incubate at 2°- 7°C for 16 to 20 hours. Coated plates may be stored at 2°- 7°C for up to 5 days.

4.2 Make twofold dilutions of reference and test bacterins, using PBS-Tween 20 as a diluent. Add 125 µL PBS-Tween 20 to each well of a clean microtitration plate (transfer plate). Place 125 µL of bacterin in the first well of each row. Test each bacterin in at least 2 replicate rows. Test the reference bacterin and the test bacterin on the same plate.

Use a multichannel pipetting device to make serial twofold dilutions of each bacterin across the plate (125 µL transfer volume). Reserve at least 2 unused wells on each plate to serve as blanks.

The use of at least 7 serial twofold dilutions per bacterin is recommended. Ideally, the selected bacterin dilutions should delineate the sigmoid curve from antigen saturation to antigen extinction for each bacterin. The dilutions used for the reference bacterin and the test bacterin may differ.

4.3 Wash the test plate(s) once with PBS-Tween 20. An automatic plate washer (200-250 µL/well, 10- to 40-second soak cycle, 3 wash cycles) may be used, or the plates may be washed by hand. Tap the plates upside down on absorbent material to remove residual fluid.

4.4 Use a multichannel pipetting device to transfer the bacterin dilutions from the transfer plates to the coated ELISA plates (100 µL/well). Seal the ELISA plates and incubate the plates for 60 to 90 minutes at 36°- 38°C.

4.5 Wash the plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.6 Dilute MAb 4DB to the current use dilution (refer to the current reagent data sheet) in antibody diluent, and add 100 µL to each well of the test plate. Incubate the plates for 55 to 65 minutes at 36°- 38°C.

4.7 Wash the plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.8 Dilute the goat anti-mouse IgM horseradish peroxidase conjugate 1:4000 in antibody diluent; or to the appropriate use dilution as specified by manufacturer or determined by checkerboard titration for that lot. Add 100 µL to each well of the test plate. Incubate for 30 to 60 minutes at 36°- 38°C.

4.9 Wash the plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.10 Add ABTS substrate (100 µL) to all wells, and incubate plates for 15 to 30 minutes at 36°- 38°C.

4.11 Read plates at 405/490 nm. Calculate the mean absorbance for the blank wells. Subtract the mean absorbance of the blank wells from each bacterin test well absorbance value prior to data analysis.

5. Interpretation of the Test Results

5.1 Relative potency calculation method

5.1.1 Use the current version of the *Relative Potency Calculation Software* (RelPot) to calculate the relative potency of the test bacterin as compared to that of the reference bacterin.

5.1.2 Do not use bacterin dilutions with mean absorbance values less than (<) 0.050 (after subtraction of the mean absorbance of the blank) in the relative potency calculations.

5.1.3 Do not use regression lines with slopes greater than (>) -0.100 in relative potency calculations. Enter a minimum slope (Min Slope) assay parameter of 0.100 in the spreadsheet in place of the 0.000 default.

5.1.4 Enter the reference and test bacterin data, and execute the relative potency program as outlined in the current version of **SAM 318**.

5.1.5 Report the highest relative potency (RP) value included in the top scores from each test as the RP for the test bacterin.

5.2 Requirements for a valid assay

5.2.1 An assay must meet the validity requirements in the current version of **SAM 318** to be considered valid.

5.2.2 Lines determined by first-order linear regression of at least 3 contiguous points must have a correlation coefficient (r) of greater than or equal to (≥) 0.95.

5.2.3 The reference regression line and the test bacterin regression line must show parallelism (slope ratio ≥ 0.80).

5.2.4 Assays that are not valid may be repeated up to a maximum of 3 times. If a valid assay cannot be achieved with 3 independent assays, the test bacterin is unsatisfactory.

5.3 Requirements for a satisfactory test bacterin

To be considered satisfactory, a test bacterin must have an RP value of ≥ 1.0. Test bacterins with RP values <1.0 on a valid assay may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. If both retests are valid and the reported RP values of both of the retests are ≥ 1.0, the test bacterin is satisfactory.

6. Report of Test Results

Report the results of the test(s) as described by standard operating procedures**.**

7. Summary of Revisions

Version .04

- Additional conjugate information has been added to reflect current procedures.

Version .03

- Minor changes have been made to update the document to current formatting practices.

Version .02

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.7** Desoxycholate has been changed to deoxycholate to reflect Sigma catalog listing (the two chemicals are equivalent).

- **4.2** Test well replicates has been changed from 3 to 2.

- References to the current reagent data sheet have been added throughout the document as appropriate.

- The contact has been changed to Mary C. Rasmusson.