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Center for Veterinary Biologics
Testing Protocol

SAM 624

Supplemental Assay Method for *In vitro* Potency Testing of *Leptospira interrogans* serogroup *pomona* Bacterins

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Entered into CVB Quality Management System by: <u>/s/Linda S. Snavelly</u> <u>09May17</u> Linda S. Snavelly Date Quality Management Program Assistant
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Supplemental Assay Method for *In vitro* Potency Testing of *Leptospira interrogans* serogroup *pomona*
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1. Introduction

This is a Supplemental Assay Method (SAM) using a sandwich enzyme-linked immunosorbent assay (ELISA) system to measure the relative potency of bacterins containing *Leptospira interrogans* serogroup pomona compared to a suitably qualified, nonexpired reference.

2. Materials

2.1 Equipment/instrumentation

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

- 2.1.1 Micropipettor to cover the range of 5- μ L to 1000- μ L
- 2.1.2 8- or 12-channel micropipettor, to cover the range of 50- μ L to 200- μ L
- 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

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

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- 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 *L. pomona* monoclonal antibody (LP MAb) produced from clone 2D7. LP MAb is available from the Center for Veterinary Biologics (CVB). Refer to the current reagent data sheet for additional information.
- 2.2.12 Polyclonal *L. pomona* antiserum (LP PAb) of rabbit origin. LP PAb is available from the CVB. Refer to the current reagent data sheet for additional information.
- 2.2.13 Goat anti-mouse IgA (alpha) horseradish peroxidase-labeled antibody, Human Serum Adsorbed (Kirkegaard and Perry Laboratories, Inc.)
- 2.2.14 Test bacterin(s) containing *L. pomona*
- 2.2.15 Reference bacterin (nonexpired) containing *L. pomona* (must be approved by the Animal and Plant Health Inspection Service (APHIS))

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.

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3.3 Preparation of reagents/control procedures

**3.3.1 Carbonate coating buffer – National Centers for Animal Health (NCAH)
Media #20034**

Na ₂ CO 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) – NCAH Media #10559

NaCl	8.00 g
KCl	0.20 g
Na ₂ HPO ₄	1.15 g
KH ₂ PO ₄	0.20 g
Deionized water	q.s. to 1 L

Adjust pH to 7.2 ± 0.1. Autoclave for 20 minutes at ≥ 121°C. 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. Sterile filter and store at 20°- 25°C for no longer than 3 months.

**3.3.4 Phosphate buffered saline with 0.05% Tween 20 (PBS-Tween 20) –
NCAH 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.

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

3.3.6 Phosphate buffer for antigen elution

KH₂PO₄ (Mallinckrodt, Inc., 7100, or equivalent) 8.2 g
Deionized water 94 mL

Adjust pH to 9.3 \pm 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 for up to 30 days. Warm to 20°- 25°C prior to use (buffer gels at 2°- 7°C).

3.3.8 LP MAb from clone 2D7

Obtain LP MAb from the CVB. Store at 2°- 7°C for several weeks or store long-term at -70°C or colder. Refer to the current reagent data sheet for additional details.

3.3.9 LP PAb of rabbit origin

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

3.3.10 Bacterins containing *L. pomona* 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.

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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. pomona* 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

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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 ELISA plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.6 Dilute LP MAb to the current use dilution (refer to 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 ELISA plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.8 Dilute the goat anti-mouse IgA horseradish peroxidase conjugate 1:1000 in antibody diluent. Alternatively, the appropriate use dilution may be specified by the 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 ELISA 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.

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5. Interpretation of the Test Results

5.1 Relative potency calculation method

5.1.1 Calculate the relative potency of the test bacterin as compared to that of the reference bacterin as described by standard operating procedures, **CVBSOP0102**, *Using Software to Estimate Relative Potency*.

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 less than 0.10 in minimum absolute value for relative potency calculations.

5.2 Requirements for a valid assay

5.2.1 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 (\geq) 0.95.

5.2.2 The reference regression line and the test bacterin regression line must show parallelism (slope ratio 0.80 to 1.25).

5.2.3 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 a 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.

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7. Summary of Revisions

Version .07

- Updated minimum slope expectations for consistency with PEL Reviewer Manual Work Instruction 4.6.2.

Version .06

- The Bacteriology Section Leader and PEL Director were updated.
- References to Relative Potency Calculation Software (RelPot) and associated SAM 318 were removed.

Version .05

- The contact person was changed to Angela M. Walker.
- Minor changes were made to clarify practices currently in use at CVB.

Version .04

- Additional conjugate information has been added throughout the document 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 changed to deoxycholate to reflect Sigma catalog listing (the two chemicals are equivalent).
- **4.2** Test well replicates changed from 3 to 2.

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- References to the current reagent data sheet were added throughout the document as appropriate.
- The contact person has been changed to Mary C. Rasmusson.

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