

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 613

**Supplemental Assay Method for *In vitro* Potency Testing of
Erysipelothrix rhusiopathiae Bacterins**

Date: **June 17, 2022** Approved, pending Standard Requirement

Number: SAM 613.05

Supersedes: SAM 613.04, May 18, 2016

Standard Requirement:

Contact: Email: Methodsrequest.notification@usda.gov
Phone: Center for Veterinary Biologics, 515-337-6100

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

UNCONTROLLED COPY

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* 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**
 - 3.4 Preparation of the sample**
- 4. Performance of Capture ELISA**
- 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 serial**
- 6. Report of Test Results**
- 7. Summary of Revisions**

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

1. Introduction

This Supplemental Assay Method (SAM) for *in vitro* potency testing of *Erysipelothrix rhusiopathiae* bacterins employs a capture enzyme-linked immunosorbent assay (ELISA) specific for the 65-kD protein of *E. rhusiopathiae*. Relative potency is determined by comparing the 65-kD protein content of the test serial to the 65-kD protein content present in a suitably qualified reference bacterin.

2. Materials

2.1 Equipment/instrumentation

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

- 2.1.1 Microplate reader with dual wavelengths (450 and 650 nm)
- 2.1.2 Micropipettors, to cover the range of 5.0- μ L to 1000- μ L
- 2.1.3 8- or 12-channel micropipettor
- 2.1.4 Orbital shaker
- 2.1.5 Balance, to measure 150 mg to 15 gram
- 2.1.6 A computer software program capable of implementing the calculations described in **CVBSOP0102**, *Using Software to Estimate Relative Potency*
- 2.1.7 High-intensity ultrasonic processor, 20 kHz

2.2 Reagents/supplies

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

- 2.2.1 Carbonate coating buffer (pH 9.6)
- 2.2.2 Phosphate-buffered saline (PBS) (pH 7.2)
- 2.2.3 Phosphate-buffered saline with 0.05% Tween 20 (PBST)
- 2.2.4 Blocking diluent (2% nonfat dry milk in PBS)
- 2.2.5 Reagent diluent (2% nonfat dry milk in PBST)

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

- 2.2.6** Phosphate buffer for antigen elution (optional)
- 2.2.7** 0.5% sodium deoxycholate (desoxycholate) buffer for antigen elution (optional)
- 2.2.8** Sodium citrate, reagent grade, for antigen elution (optional)
- 2.2.9** 3,3',5,5'-tetramethylbenzine (TMB) substrate (Kirkegaard-Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879, #50-76-00)
- 2.2.10** Stop solution (2.5 M H₂SO₄)
- 2.2.11** 96-well flat-bottom microtitration plates (Immulon 2; Dynex Laboratories, Inc.)
- 2.2.12** 96-well U-bottom, non-binding microtitration plates (transfer plate)
- 2.2.13** Plate sealers or Parafilm[®]
- 2.2.14** Monoclonal antibody (MAb) against the 65-kD protein of *E. rhusiopathiae*, MAb ERHU1-B60-91. This reagent is available from the Center for Veterinary Biologics (CVB). Refer to the current reagent data sheet for additional information.
- 2.2.15** Rabbit origin polyclonal antiserum against 65-kD protein of *E. rhusiopathiae* (monospecific). This reagent is available from the CVB. Refer to the current reagent data sheet for additional information.
- 2.2.16** Horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.)
- 2.2.17** Qualified unexpired reference bacterin containing *E. rhusiopathiae*

CRITICAL CONTROL POINT: The reference and test serials should be adjuvanted with the same adjuvant. Ideally, the reference and test serials should be produced by the same Outline of Production.

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel should have working knowledge of the use of general laboratory chemicals, equipment, and glassware; performance of ELISA assays; and knowledge and experience in the operation of an automated microplate reader and data recording and evaluation software.

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

Caution: Concentrated solutions of acids and bases are used to prepare some of the following reagents. Both are hazardous and should be handled properly. Consult Material Safety Data Sheets (MSDS) (current version) for proper safety procedures.

3.3.1 Carbonate coating buffer (0.05 M, pH 9.6)

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 no longer than 1 week.

3.3.2 Phosphate buffered saline (PBS) (pH 7.2)

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. 1 L

Adjust pH to 7.2 ± 0.1 with 0.1 M NaOH or 0.1 M HCl. Store at 20°- 25°C for no longer than 6 months. If the longer-term storage is desired, autoclave to sterilize before storage.

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

3.3.3 PBS-Tween 20 (PBST)

PBS (see Section 3.3.2)	1 L
Tween 20	0.50 mL

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

3.3.4 Blocking diluent (2% nonfat dry milk in PBS)

Nonfat dry milk	4.0 g
PBS (see Section 3.3.2)	200 mL

Make fresh daily.

3.3.5 Reagent diluent (2% nonfat dry milk in PBST)

Nonfat dry milk	4.0 g
PBST (see Section 3.3.3)	200 mL

Make fresh daily.

3.3.6 Phosphate buffer for antigen elution

KH ₂ PO ₄	8.2 g
Deionized water	q.s. to 100 mL

Adjust pH to 6.5 ± 0.1. Store at 20°- 25°C for up to 30 days.

3.3.7 0.5% sodium deoxycholate (desoxycholate) for antigen elution

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

Store at 2°- 7°C for no longer than 1 month. Warm to 20°- 25°C prior to use.
Warning: This solution gels at 2°- 7°C.

3.4 Preparation of the sample

Some bacterins do not require antigen-elution treatment prior to being tested in this assay. Representative serials should be tested with and without antigen-elution treatment to determine if the treatment specifically enhances the capture of 65-kD protein. If no enhancement of antigen capture can be demonstrated, the bacterins should be tested without antigen-elution treatment. The reference bacterin and the test serials for each product must be treated by the same procedure. The following section discusses some of the more common antigen elution treatments. However, alternative elution procedures

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

may be more appropriate for some bacterins. Antigen-elution treatments should be optimized for each manufacturer's bacterin(s). Treat bacterins prior to making serial twofold dilutions in reagent diluent.

3.4.1 Aluminum-adjuvanted bacterins

1. Sodium citrate elution

Add 1.0 g sodium citrate to 10.0 mL bacterin (10% w/v). Place on an orbital shaker (100-130 rpm) 16 to 24 hours at 35°- 38°C. Treated bacterin is considered undiluted.

2. Phosphate buffer elution

Add 1.0 mL of phosphate elution buffer to 1.0 mL of bacterin. Place on an orbital shaker (100-130 rpm) 16 to 24 hours at 35°- 38°C. Treated bacterin is considered diluted 1:2.

3.4.2 Oil-adjuvanted bacterins

Mix 1.0 mL 0.5% sodium deoxycholate with 1.0 mL bacterin. Place on an orbital shaker (100-130 rpm) 16 to 24 hours at 35°- 38°C. Treated bacterin is considered diluted 1:2.

4. Performance of the Capture ELISA

4.1 Dilute MAb ERHU1-B60-91 to the current use dilution (refer to current reagent data sheet) in cold carbonate coating buffer. Place 100 µL diluted MAb into each well of a 96-well flat-bottom microtitration plate. Seal plates with adhesive plate sealers or Parafilm®. Incubate overnight at 2°- 7°C for at least 16 hours. Coated plates may be held at 2°- 7°C up to 5 days.

4.2 Wash the plate 3 times with PBST. 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.

4.3 Place 100 µL blocking buffer in each well of the plate. Incubate at 35°- 38°C for 60 to 75 minutes.

4.4 Wash plate as described in **Section 4.2**.

4.5 Make twofold dilutions of reference and test bacterins in a transfer plate. Place 125 µL of reagent diluent into each well of a 96-well U-bottom microtitration plate. Add 125 µL of bacterin to the first well of each of a minimum of 2 columns or rows per

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

bacterin. Use a multichannel pipetting device to make serial twofold dilutions of each bacterin across the plate. At least 2 wells must be reserved without bacterin for use as a blank.

The use of at least 7 serial twofold dilutions 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 and bacterin may differ.

4.6 Transfer 100 μ L diluted bacterin from each well of the transfer plate to the corresponding well on the test plate. Seal plate and incubate for 60 to 75 minutes at 35°- 38°C.

4.7 Wash plate as described in **Section 4.2**.

4.8 Dilute polyclonal rabbit antiserum to current use dilution (refer to the current reagent data sheet) in reagent diluent. Place 100 μ L diluted antibody in each well of the test plate. Seal plate, and incubate for 60 to 75 minutes at 35°- 38°C.

4.9 Wash plate as described in **Section 4.2**.

4.10 Dilute anti-rabbit horseradish peroxidase conjugate in reagent diluent to appropriate use dilution as determined by checkerboard titration for that lot. Place 100 μ L diluted conjugate in each well of the test plate. Seal plate and incubate for 60 to 75 minutes at 35°- 38°C.

4.11 Measure out the necessary volume (10 mL/plate) of TMB substrate. Store in the dark during the conjugate incubation step to allow substrate to come to 20°- 25°C.

4.12 Wash plate as described in **Section 4.2**.

4.13 Place 100 μ L TMB substrate solution into each well of the test plate. Incubate at 20°- 25°C for 7 to 15 minutes, or until adequate color change has occurred.

4.14 Add 100 μ L stop solution (2.5M H₂SO₄) to each well of the plate.

4.15 Read the ELISA plate(s) at 450/650 nm. Calculate the mean absorbance for the blank wells. Subtract the mean absorbance of the blank wells from each bacterin well absorbance value prior to data analysis.

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

5. Interpretation of the Test Results

5.1 Relative potency calculation method

Refer to the current version of **CVBSOP0102** for estimating the relative potency.

5.2 Requirements for a valid assay

5.2.1 An assay must meet the validity requirements of **CVBSOP0102** to be considered valid.

5.2.2 Lines determined by first-order linear regression must have a correlation coefficient (r) of ≥ 0.95 .

5.2.3 The reference dose-response line and the test serial dose-response line must show parallelism.

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 4 independent assays, the serial shall be deemed unsatisfactory.

5.3 Requirements for a satisfactory serial

To be considered satisfactory, a test serial must have an RP value of ≥ 1.0 in a valid assay. Serials 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 the reported RP values of both of the retests are ≥ 1.0 , the serial is satisfactory. If the test serial is not retested, the serial is unsatisfactory.

6. Report of Test Results

Results of the test(s) are reported as described by standard operating procedures.

7. Summary of Revisions

Version .05

- Updated coversheet and contact information.

Version .04

- Section Leader and Director information updated.

Supplemental Assay Method for *In vitro* Potency Testing of *Erysipelothrix rhusiopathiae* Bacterins

- Relative Potency reference document changed from SAM 318 to CVBSOP0102.

Version .03

- The Contact information has been updated.

Version .02

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

- **2.2** The use of 2,2'-azino-di-3-ethylbenzothiazoline sulfonate (ABTS) substrate has been discontinued and this has been removed from the document.
- Desoxycholate has been changed to deoxycholate (the two chemicals are equivalent).
- Reference to the current reagent data sheet has been added throughout the document as appropriate.
- The title of this document has been revised to clarify that this SAM describes an in-vitro assay, not the codified mouse assay (described in **SAM 611**).
- The contact person has been changed to Janet Wilson.