Supplemental Assay Method for Potency Testing Enterotoxigenic (987P Pilus) *Escherichia coli* Bacterins

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 622

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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

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1. Introduction

This Supplemental Assay Method (SAM) for potency testing inactivated *Escherichia coli* bacterins employs a capture enzyme-linked immunosorbent assay (ELISA) for 987P pilus antigen. Relative potency is determined by comparing the 987P antigen content of the test bacterin to the 987P antigen content present in an unexpired, 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 (490 nm and 650 nm)

2.1.2 Automatic microplate washer (optional)

2.1.3 Micropipettors, to cover the range of 5-µL to 1000-µL

2.1.4 8- or 12-channel micropipettor, to cover the range of 50-µL to 200-µL

2.1.5 Orbital shaker

2.1.6 Balance, to measure 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 high-binding microtitration plates (Immulon 2; Dynatech Laboratories, Inc.)

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

2.2.3 Plate sealers

2.2.4 Carbonate coating buffer

2.2.5 Phosphate-buffered saline (PBS), pH 7.2

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

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2.2.9 Phosphate elution buffer (optional)

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2.2.12 Citrate buffer (substrate diluent)

2.2.13 o-Phenylenediamine dihydrochloride (OPD)

2.2.14 Hydrogen peroxide, 30%, stabilized

2.2.15 2.5M H_2SO_4 Stop solution

2.2.16 987P-specific antigen-capture polyclonal antibody (987P PAb), rabbit origin (available from the Center for Veterinary Biologics (CVB)). Refer to the current reagent data sheet for details.

2.2.17 987P-specific antigen-detection monoclonal antibody (987P MAb), mouse origin (available from the CVB). Refer to the current reagent data sheet for details.

2.2.18 Horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) obtained from a commercial source (Jackson ImmunoResearch Laboratories, Inc., 115-035-062)

2.2.19 Test bacterin(s) containing 987P antigen

2.2.20 Reference bacterin containing 987P antigen (must be approved by the Animal and Plant Health Inspection Service and within dating)

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 reader; and data recording and evaluation 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 standard operating procedures.

3.3 Preparation of reagents/control procedures

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

3.3.1 Carbonate coating buffer – National Centers for Animal Health (NCAH) 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 no longer than 1 week.

3.3.2 Phosphate buffered saline (PBS) – NCAH 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. Store at 20°- 25°C. If long term storage (up to 1 year) is desired, autoclave for 20-30 minutes at ≥ 121°C to sterilize following manufacturer's recommendations.

3.3.3 Phosphate Buffered Saline with 0.05% Tween 20 (PBS-Tween) – NCAH Media #30179

PBS (see **Section 3.3.2**)	1 L
Tween 20	0.5 mL

Store at 20°- 25°C for no longer than 1 year.

3.3.4 PBS-Tween with 2.0% bovine albumin fraction V (MAb diluent)

PBS-Tween (see **Section 3.3.3**)	25 mL
Bovine albumin fraction V (Scientific Protein Laboratories, Viobin Corp., Waunakee, Wisconsin 40-6197-2-1160 or equivalent)	0.5 g

Prepare immediately prior to use. Swirl gently to dissolve the powder.

3.3.5 PBS-Tween 20 with 1.0% normal rabbit serum (conjugate diluent)

PBS-Tween 20 (see **Section 3.3.3**)	24.75 mL
Normal rabbit serum (negative for *E. coli* antibodies)	0.25 mL

Prepare immediately prior to use. Swirl gently to mix.

3.3.6 Phosphate elution buffer

KH_2PO_4 (reagent grade)	8.2 g
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 desoxycholate (reagent grade)	0.50 g
PBS (see **Section 3.3.2**)	100 mL

Store at 2°- 7°C for no longer than 1 month. Warm to 20°- 25°C prior to use. (The buffer gels at 2°- 7°C.)

3.3.8 Citrate buffer (pH 5.0) – NCAH Media #20033

Citric acid monohydrate (reagent grade)	5.26 g
$Na_2HPO_4 \cdot 7H_2O$	6.739 g
Water	q.s. to 1 L

Adjust pH to 5.0 ± 0.1 and filter sterilize. Store at 2°- 7°C for no longer than 2 months. Use to prepare substrate solution (**Section 3.3.9**).

3.3.9 Substrate solution (quantities for 1 plate)

Citrate buffer	12 mL
o-Phenylenediamine dihydrochloride (OPD) (Sigma P8787 or equivalent)	4 mg
30% H_2O_2 (stabilized)	5 µL

Prepare within 15 minutes of use.

Caution: o-Phenylenediamine dihydrochloride is a carcinogen. See appropriate MSDS for precautions when handling this product.

3.3.10 Stop solution (2.5 M H_2SO_4)--NCAH Media #30171

Concentrated (98%) H_2SO_4	13.6 mL
Water	86.4 mL

Add acid to water. Solution may be stored no longer than 1 year at 20°- 25°C.

3.3.11 Bacterins containing 987P antigen

1. Reference bacterin

2. Test bacterin

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

Antigen-elution treatments: 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 the 987P antigen capture. If no

enhancement of the 987P 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-adjuvanted bacterins

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

- Sodium citrate elution

Add 1.0 g sodium citrate, dihydrate to 10.0 mL of bacterin (10% w/v). Place on an orbital shaker (100- 120 rpm) overnight at 35°- 37°C. Consider treated bacterin to be undiluted.

- Phosphate buffer elution

Add 1.0 mL of phosphate elution buffer to 1.0 mL of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 35°- 37°C. Consider treated bacterin to be diluted 1:2.

3.4.2 Oil-adjuvanted bacterins

Mix 1.0 mL of the sodium desoxycholate elution buffer with 1.0 mL of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 35°- 37°C. Consider treated bacterin to be diluted 1:2.

4. Performance of the Test

4.1 Dilute the 987P PAb in cold carbonate coating buffer (refer to the current reagent data sheet for dilution). Place 100 µL in each well of 96-well flat-bottom high-binding microtitration plates. Seal coated plates with plate sealers. Incubate coated plates overnight at 2°- 7°C. Store sealed plates at 2°- 7°C for no longer than 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 micropipettor 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 and the test bacterin may differ.

4.3 Wash the coated ELISA plates 3 times with PBS-Tween 20. An automatic plate washer (200-300 µL/well, 10- to 40-second soak cycle) may be used. Alternatively, plates may be washed by hand. Tap the plates upside down on absorbent material to remove residual fluid.

4.4 Use a multichannel micropipettor to transfer the bacterin dilutions from the transfer plates to the coated ELISA plates (100 µL/well). Seal the ELISA plates and incubate them on an orbital shaker (100-120 rpm) for 30 minutes (± 5 minutes) at 20°-25°C.

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

4.6 Dilute the 987P MAb in MAb diluent to the appropriate use dilution (refer to the current reagent data sheet for dilution) and add 100 µL to each well. Seal the ELISA plates and incubate on an orbital shaker (100-120 rpm) for 30 minutes (± 5 minutes) at 20°- 25°C.

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

4.8 Dilute the peroxidase-conjugated goat anti-mouse IgG in conjugate diluent to the appropriate use dilution and add 100 µL to each well. Seal the ELISA plates and incubate on an orbital shaker (100-120 rpm) for 30 minutes (± 5 minutes) at 20°- 25°C.

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

4.10 Add 100 µL substrate solution to each well. Incubate the ELISA plates on an orbital shaker (100-120 rpm) for 10 minutes (± 5 minutes) or until sufficient color develops at 20°- 25°C.

4.11 Stop the substrate color development by adding 100 µL stop solution to each well.

Note: The OPD substrate buffer undergoes a color shift from yellow to orange when stop solution is added.

4.12 Read the ELISA plates using an ELISA reader with dual wavelengths (490 nm test, 650 nm reference). 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 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 (<) 0.05 (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 are determined by first-order linear regression of at least 3 contiguous points and must have a correlation coefficient (r) of greater than or equal to (≥) 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).

5.3 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. Reporting of Test Results

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

7. Summary of Revisions

Version .07

- Updated coversheet and contact information.

Version .06

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

Version .05

- The Bacteriology Section Leader has been updated.
- Clarified media expiration dates.
- Removed references to Relative Potency Calculation Software (RelPot) and associated SAM 318.

Version .04

- The Contact information has been updated.
- All references to National Veterinary Services Laboratories media have been changed to National Centers for Animal Health media.

Version .03

- Clarification that sodium citrate, dihydrate should be used (rather than sodium citrate monobasic, anhydrous) has been added throughout the document.

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:

- **2.2.1** The specification of the assay plate as high-binding has been added.
- **2.2.2** The specifications of the transfer plate as non-binding have been added.
- **2.2.15 and 3.3.10** The stop solution has been further defined and storage information updated.
- **3.3.1** The sodium carbonate molecular formula has been corrected.
- **3.3.5** The formula has been updated including the specification that the serum not contain *E. coli* antibodies.
- **3.3.8** Filter sterilization of the solution has been added.
- **4.10** Additional details for stopping the reaction have been added.
- References to the current reagent data sheet have been added throughout the document.
- References to internal CVB documents have been replaced with summary information.
- The contact person has been changed to Janet Wilson.