United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 623

Supplemental Assay Method for Potency Testing Enterotoxigenic (F41 Pilus) *Escherichia coli* Bacterins

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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 F41 pilus antigen. Relative potency is determined by comparing the F41 antigen content of the test bacterin to the F41 antigen content present in a 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 (United States Department of Agriculture, Veterinary Services, Center for Veterinary Biologics [CVB]), current version

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)

2.2.7 PBS-Tween with 2.0% bovine albumin fraction V (monoclonal antibody [MAb] diluent)

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

2.2.9 Phosphate elution buffer (optional)

2.2.10 Sodium citrate for antigen elution (optional)

2.2.11 Sodium desoxycholate elution buffer (optional)

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 F41-specific antigen-capture polyclonal antibody (F41 PAb), rabbit origin (available from the CVB). Refer to the current reagent data sheet for details.

2.2.17 F41-specific antigen-indicator MAb, SDSU 56/85 (F41 MAb) (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 F41 antigen

2.2.20 Reference bacterin containing F41 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 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 no longer than 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. 1 L

Adjust pH to 7.2 ± 0.1. Store at 20°- 25°C. If long-term storage (up to 6 months) is desired, autoclave 20 minutes at ≥121°C to sterilize.

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

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

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

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, WI, 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
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 (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)--NVSL Media #20033

Citric acid monohydrate (reagent grade)	5.26 g
$Na_2HPO_4 \cdot 7H_2O$	6.74 g
Deionized 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)--NVSL Media #30171

Concentrated (98%) H_2SO_4	13.6 mL
Deionized 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 F41 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

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 F41 antigen capture. If no enhancement of F41 antigen capture can be demonstrated, test the bacterins without antigen-elution treatment. Treat the reference bacterin and the test bacterins for each product by the same 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 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 deoxycholate 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 F41 PAb in cold carbonate coating buffer (refer to the current reagent data sheet for the current use dilution). Place 100 µL in each well of a 96-well flat-bottom, high-binding microtitration plate. Seal coated plates with plate sealers. Incubate the 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 multichannel 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 hand washed. 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 F41 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 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 optical density (O.D.) values of <0.05 (after subtraction of the mean absorbance of the blank) in the relative potency calculations.

5.1.3 Do not use first-order linear regression lines with slopes > -0.150 in the relative potency calculations. Enter a minimum slope assay parameter of 0.150 in the RelPot spreadsheet in place of the 0.000 default.

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

5.1.5 Report the highest relative potency value (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 of 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 ≥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, report the test bacterin as 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. Reporting of test results

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

7. Summary of revisions

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 assay plate has been further described as high-binding.
- **2.2.2** The transfer plate has been further described as non-binding.
- **2.2.15** and **3.3.10** The stop solution has been further defined and storage information has been updated.
- **3.3.5** The formula has been updated, including the specification that the serum not contain *E. coli* antibodies.
- **3.3.7** The alternate chemical name has been included.
- **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 M. Wilson.