

United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol

SAM 621

Supplemental Assay Method for Potency Testing Enterotoxigenic (K88 Pilus)  
*Escherichia coli* Bacterins

Date: **May 10, 2017** Approved, pending Standard Requirement

Number: SAM 621.06

Supersedes: SAM 621.05, April 3, 2014

Standard Requirement:

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Supplemental Assay Method for Potency Testing Enterotoxigenic (K88 Pilus) *Escherichia coli* Bacterins

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**Supplemental Assay Method for Potency Testing Enterotoxigenic (K88 Pilus) *Escherichia coli* Bacterins**

**1. Introduction**

This Supplemental Assay Method (SAM) for potency testing inactivated *Escherichia coli* bacterins employs a capture enzyme-linked immunosorbent assay (ELISA) for K88 pilus antigen. Relative potency is determined by comparing the K88 antigen content of the test bacterin to the K88 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 Micropipettors, to cover the range of 5- $\mu$ L to 1000- $\mu$ L
- 2.1.2 8- or 12-channel micropipettor, to cover the range of 50- $\mu$ L to 200- $\mu$ L
- 2.1.3 Orbital shaker
- 2.1.4 Automatic microplate washer (optional)
- 2.1.5 Microplate reader with dual wavelengths (490 nm and 650 nm)
- 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 plate)
- 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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- 2.2.7** PBS-Tween with 1.0% ovalbumin (conjugate diluent)
- 2.2.8** Phosphate elution buffer (optional)
- 2.2.9** Sodium citrate, dihydrate for antigen elution (optional)
- 2.2.10** Sodium desoxycholate elution buffer (optional)
- 2.2.11** Citrate buffer (substrate diluent)
- 2.2.12** o-Phenylenediamine dihydrochloride (OPD)
- 2.2.13** Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 30%, stabilized
- 2.2.14** 2.5 M H<sub>2</sub>SO<sub>4</sub> Stop solution
- 2.2.15** K88-specific antigen-capture monoclonal antibody (available from the Center for Veterinary Biologics (CVB)). Refer to the current reagent data sheet for details.
- 2.2.16** Horseradish peroxidase-conjugated K88-specific monoclonal antibody (available from the CVB). Refer to the current reagent data sheet for details.
- 2.2.17** Test bacterin(s) containing K88 antigen
- 2.2.18** Reference bacterin containing K88 antigen (approved by the Animal and Plant 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 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 <sub>2</sub> CO <sub>3</sub>	0.159 g
NaHCO <sub>3</sub>	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 <sub>2</sub> HPO <sub>4</sub>	1.15 g
KH <sub>2</sub> PO <sub>4</sub>	0.20 g
Deionized water	q.s. to 1 L

Adjust pH to 7.2 ± 0.1. Store at 20°- 25°C for no longer than 6 months. 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 <b>Section 3.3.2</b> )	1 L
Tween 20	0.5 mL

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

**3.3.4 PBS-Tween with 1.0% ovalbumin (conjugate diluent)**

PBS-Tween (see <b>Section 3.3.3</b> )	20 mL
Ovalbumin (Calbiochem 32467 or equivalent)	0.2 g

Add ovalbumin to the PBS-Tween 20 within 10 minutes of use. Swirl gently to dissolve the crystals.

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**3.3.5** Phosphate elution buffer

KH <sub>2</sub> PO <sub>4</sub> (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.

**3.3.6** Sodium desoxycholate (desoxycholate) elution buffer

Sodium desoxycholate (Sigma Chemical D6750 or equivalent)	0.5 g
PBS (see <b>Section 3.3.2</b> )	100 mL

Store at 2°- 7°C for no longer than 1 month. Warm to room temperature prior to use; (the buffer gels at 2°- 7°C).

**3.3.7** Citrate buffer (pH 5.0) – NCAH Media #20033

Citric acid monohydrate (reagent grade) (Fisher Scientific A104-500 or equivalent)	5.26 g
Na <sub>2</sub> HPO <sub>4</sub> •7H <sub>2</sub> O	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 (see **Section 3.3.8**).

**3.3.8** Substrate solution (quantities for 1 plate)

Citrate buffer	12 mL
o-Phenylenediamine dihydrochloride (OPD) (Sigma P8787 or equivalent)	4 mg
30% H <sub>2</sub> O <sub>2</sub> (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.**

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**3.3.9** Stop solution (2.5 M H<sub>2</sub>SO<sub>4</sub>) – NCAH Media #30171

Concentrated (98%) H <sub>2</sub> SO <sub>4</sub>	13.6 mL
Deionized water	86.4 mL

Add acid to water. Store at 20°- 25°C for no longer than 1 year.

**3.3.10** Monoclonal antibodies (MAb)

1. K88 antigen-capture monoclonal antibody (21BA1-1H1). Obtain MAb from the CVB. Refer to the current reagent data sheet for details on use and storage.

2. Horseradish peroxidase-labeled K88 antigen-indicator MAb (21BA1-1H1). Obtain conjugated MAb from the CVB. Refer to the current reagent data sheet for details on use and storage.

**3.3.11** Bacterins containing K88 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 the K88 antigen capture. If no enhancement of the K88 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. Alternate 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.

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- Sodium citrate elution

Mix 1.0 g sodium citrate, dihydrate with 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

Mix 1.0 mL of phosphate elution buffer with 1.0 mL 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 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 K88 antigen-capture MAb in cold carbonate coating buffer (refer to the current reagent data sheet for dilution) and 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. Coated plates stored at 2°- 7°C may be used 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 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, 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 micropipettor to transfer the bacterin dilutions from the transfer plates to the coated ELISA plates (100 µL/well). Seal the ELISA plates and



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incubate them on an orbital shaker (100-120 rpm) for 30 minutes ( $\pm$  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 horseradish peroxidase-labeled K88 antigen-indicator MAb in conjugate diluent to the current use dilution (refer to the current reagent data sheet for dilution) and add 100  $\mu$ L to each well. Seal the ELISA plates, and incubate on an orbital shaker (100-120 rpm) for 30 minutes ( $\pm$  5 minutes) at 20°- 25°C.

**Note: Ovalbumin will stick to plastic. Prepare the working dilution of the conjugate in a glass vial. Transfer the diluted conjugate into pipetting trays immediately prior to the addition of the conjugate to the ELISA plate.**

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

**4.8** Add 100  $\mu$ L freshly prepared substrate solution to each well. Incubate the ELISA plates on an orbital shaker (100-120 rpm) for 10 minutes ( $\pm$  5 minutes) or until sufficient color is observed at 20°- 25°C.

**4.9** Stop the substrate color development by adding 100  $\mu$ L stop solution to each well.

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

**4.10** 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 ( $<$ ) 0.050 (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.

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**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 ( $\geq$ ) 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).

**5.3 Requirements for a satisfactory test bacterin**

**5.3.1** To be considered satisfactory, a test bacterin must have an RP value of  $\geq 1.0$ . 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  $\geq 1.0$ , 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 .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.

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**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 assay plate has been further described as high-binding.
- **2.2.2** The transfer plate has been further described as non-binding.
- **2.2.14** The Stop Solution used has been further described.
- **2.2.18** Further description of the reference bacterin as unexpired has been added.
- **3.3.2** Additional information for long term storage has been added.
- **3.3.6** The alternate chemical name has been included.
- **3.3.7** Filter sterilization of the solution has been added.
- **3.3.9** The information on solution storage has been updated.
- **3.4.1.2** The incubation temperature has been altered from 20°- 25°C to 35°- 37°C.
- **4.8** Additional details for stopping the reaction have been added.
- References to internal CVB documents have been replaced with summary information.
- References to the current reagent data sheet have been added throughout this document.
- The contact person has been changed to Janet Wilson.