United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

SAM 631

Supplemental Assay Method for Potency Testing of *Salmonella typhimurium* Bacterins

Date:	**June 19, 2006**
Number:	SAM 631.02
Supersedes:	BBSAM0631.01, June 14, 1999
Standard Requirement:	9 CFR, Part 113.120
Contact:	Janet M. Wilson, (515) 663-7245 JMW 21 JUN 06

Approvals: Paul Hauer — Date: 22 JUN 06
Paul J. Hauer, Section Leader
Bacteriology

Date: 23 JUN 06
Byron E. Ripplke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

Date: 26 Jun 06
Rebecca L.W. Hyde, Section Leader
Quality Assurance
Center for Veterinary Biologics

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 Potency Testing *Salmonella typhimurium* Bacterins

Table of Contents

1. Introduction

2. Materials

   2.1 Equipment/instrumentation
   2.2 Reagents/supplies
   2.3 Animals

3. Preparation for the test

   3.1 Personnel qualifications/training
   3.2 Selection and handling of test mice
   3.3 Preparation of supplies/equipment
   3.4 Preparation of reagents

4. Performance of the test

   4.1 Vaccination of test animals
   4.2 Preparation of challenge in biological safety cabinet
   4.3 Timing and administration of challenge
   4.4 Post-inoculation plate count in biological safety cabinet
   4.5 Observation of mice after challenge

5. Interpretation of the test results

6. Report of test results

7. References

8. Summary of revisions

UNCONTROLLED COPY

JMW 21Jun06

Supplemental Assay Method for Potency Testing *Salmonella typhimurium* Bacterins

1. Introduction

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing *Salmonella typhimurium*, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.120. Mice are vaccinated twice, 14 days apart, and challenged with a standard dose of virulent *S. typhimurium* 7 to 10 days after the second vaccination.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Spectrophotometer, Spectronic 20D+, (Spectronics Instruments)

2.1.2 Sterile inoculating loop

2.1.3 Bunsen burner (if non-sterile wire inoculating loop is used)

2.1.4 Incubator, 35°- 37°C

2.1.5 Micropipettors, 20- to 1000-µL

2.1.6 Test tube mixer, vortex-type

2.1.7 Crimper for aluminum rings on serum vials

2.1.8 Rotary shaker

2.1.9 Biological safety cabinet

2.2 Reagents/supplies

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

2.2.1 *S. typhimurium* challenge culture, available from the Center for Veterinary Biologics (CVB). Refer to the current reagent data sheet for additional information.

2.2.2 Test bacterin(s) containing *S. typhimurium*

UNCONTROLLED COPY

JMW 21 Jun 06

2.2.3 *S. typhimurium* reference bacterin, available from the CVB. Refer to the current reagent data sheet for additional information.

2.2.4 Syringes, 1-mL Tuberculin

2.2.5 Needles, 26-gauge, 3/8-inch

2.2.6 Glass serum bottle, 10- to 100-mL,

2.2.7 Rubber stopper, 13 x 20-mm, and aluminum cap for serum bottle

2.2.8 Screw-cap glass tubes, 13 x 100-mm and 15 x 125-mm

2.2.9 Pipettes, 5-mL, 10-mL, and 25-mL

2.2.10 Micropipette tips, up to 1000-µL capacity

2.2.11 Glass rod, bent into L-shape

2.2.12 Tryptose agar plates or Bovine Blood Agar Plates

2.2.13 Tryptose broth

2.2.14 Phosphate-buffered saline (PBS)

2.2.15 Brain-heart infusion broth

2.2.16 Screw cap flask, 1-L

2.3 Animals

2.3.1 Mice, 16-22 g. Although the 9 CFR does not specify a mouse source, some colonies of mice may be relatively resistant to salmonellosis and therefore less suitable for this assay.

2.3.2 Sixty mice are required for each serial to be tested (20 mice/dilution; 3 dilutions/serial). Sixty additional mice are required for the reference bacterin. Thirty mice are required to determine the LD_{50} of the challenge inoculum. All mice must be from the same source colony and of similar weight and/or age.

UNCONTROLLED COPY

21 June 06

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 and have specific training and experience in sterile technique, the handling of live bacterial cultures, and the handling of mice.

3.2 Selection and handling of test mice

3.2.1 Mice of either sex may be used, but females are recommended.

3.2.2 All mice must be housed and fed in a similar manner.

3.2.3 Identify each cage of mice by treatment group.

3.2.4 If any mice die after vaccination, but prior to challenge with live *S. typhimurium*, necropsy these mice to determine cause of death if the cause of death is not outwardly apparent. If the cause of death is unrelated to vaccination, file the necropsy report with the test records, and no additional action is needed. If death is attributable to the test bacterin, report the death immediately to the CVB-Inspection and Compliance, which may request further safety testing of the bacterin.

3.2.5 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the mice and to sanitize the contaminated rooms.

3.3 Preparation of supplies/equipment

3.3.1 Use only sterile bacteriological supplies.

3.3.2 Operate and maintain all equipment according to manufacturers' recommendations and applicable standard operating procedures.

3.4 Preparation of reagents

3.4.1 *S. typhimurium* bacterin, available from the CVB. Refer to current reagent data sheet for details.

3.4.2 *S. typhimurium* challenge culture, available from the CVB. Refer to current reagent data sheet for details.

UNCONTROLLED COPY

JMW 21 Jun 06

3.4.3 Phosphate-buffered saline--National Veterinary Services Laboratories (NVSL) Media #10559

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Sodium phosphate, dibasic	1.15 g
Potassium phosphate, monobasic	0.2 g
Deionized water	q.s. 1 L

Adjust pH to 7.2 ± 0.1. Autoclave 20 minutes at ≥121°C. Store at 20°- 25°C for no longer than 6 months.

3.4.4 Tryptose broth--NVSL Media #10404

Tryptose broth powder (BBL or equivalent)	26 g
Deionized water	q.s. 1000 mL

Autoclave 15 minutes at ≥121°C. Cool before using. Store at 20°- 25° no longer than 6 months.

3.4.5 Tryptose agar--NVSL Media #10093

Tryptose agar powder (BBL or equivalent)	41 g
Deionized water	q.s. 1000 mL

Autoclave 25 minutes at ≥121°C. Cool in a 56°- 60°C water bath. Pour into sterile petri dishes. Allow to cool to 20°- 25°C. Store at 2°- 7°C for no longer than 6 months.

3.4.6 Brain-heart infusion broth--NVSL Media #10009

Brain-heart infusion (BBL or equivalent)	37 g
Deionized water	q.s. 1000 mL

Autoclave 20 minutes at ≥121° C. Store at 20°- 25°C for no longer than 6 months.

3.4.7 Bovine blood agar--NVSL Media #10006

Blood agar base powder	40 g
Deionized water	950 mL

Autoclave for 20 minutes at ≥121° C. Cool to 45°- 47°C.

UNCONTROLLED COPY

JMW 21JUN06

Add:
Defibrinated blood 50 mL

Pour into sterile petri dishes. Allow to cool to 20°- 25°C. Store at 2°- 7°C for no longer than 6 months.

4. Performance of the test

4.1 Vaccination of test animals

4.1.1 Check the label on each product and Section VI of the current Outline of Production to confirm identity and dose volume.

4.1.2 Test each test bacterin and the reference bacterin at 3 tenfold dilutions. Typically, test the bacterins at 1:10, 1:100, and 1:1000 dilutions. Refer to the current reagent data sheet for any starting dilutions of the reference bacterin. It is permissible to make tenfold dilutions other than those described as long as the reference and test bacterins are tested at the same dilutions. For viscous bacterins, it is advisable to start at 1:2 or 1:3, and make tenfold dilutions from this starting point, to increase injectability of the product at the low dilution.

4.1.3 Thoroughly mix product by inverting end-to-end at least 10 times. Make the appropriate tenfold dilutions of the reference bacterin in PBS. Make identical tenfold dilutions of the test bacterin(s) in the diluent approved in the specific Outline of Production for that product. (Some oil-adjuvanted products require oil-based diluents.) Place each dilution in a separate sterile injection vial. Prepare dilutions immediately prior to use; do not store in diluted form.

4.1.4 Vaccinate separate groups of 20 mice with each of the test bacterin dilutions and reference bacterin dilutions. For reference bacterin groups, inject each mouse with 0.25 mL intraperitoneally. Inject test bacterins intraperitoneally at a dose volume that corresponds to 1/20 of the lowest dose recommended on the product label or Section VI of the current Outline of Production. This volume must not be <0.1 mL.

Note: It is permissible to vaccinate a few extra mice in each group to compensate for any potential deaths that may occur prior to challenge and are not related to vaccination. However, if extra mice are vaccinated, all surviving at the time of challenge must be challenged with live *S. typhimurium* and included in data calculations.

4.1.5 Revaccinate the mice in a similar manner 14 days after the first vaccination.

UNCONTROLLED COPY

JMW 22 Jun 06

4.1.6 Retain 30 non-vaccinated mice to determine LD_{50} of the challenge.

4.2 Preparation of challenge in biological safety cabinet

4.2.1 Reconstitute a vial of challenge in 1 mL tryptose broth.

4.2.2 Inoculate 3 tubes containing 10 mL of brain-heart infusion broth with 500 µL of reconstituted culture.

4.2.3 Incubate the inoculated tubes at 35°- 37°C for 16 to 20 hours.

4.2.4 Perform a Gram stain on the overnight culture, using standard methods. If the bacteria in the Gram stain are short, Gram negative rods (evidence of pure culture), proceed to the next step. If the challenge appears contaminated, discard affected tubes.

4.2.5 Adjust the density of the overnight culture to 57-61% T at 620 nm using a Spectronic 20D+ spectrophotometer. Dilute the culture, as needed, with tryptose broth. Place the culture in a 13 x 100-mm screw-cap tube for spectrophotometric determination. Use sterile tryptose broth in a 13 x 100-mm tube as a blank for the spectrophotometer.

4.2.6 Prepare a 10^{-1} dilution of the standardized culture in tryptose broth. **This inoculum is used to challenge the mice.** Dispense challenge liquid into a serum vial and seal with a rubber stopper and aluminum ring.

4.2.7 Prepare additional tenfold dilutions of the standardized culture for LD_{50} determination (10^{-3} to 10^{-5}) and post-inoculation plate count (10^{-5} to 10^{-7}) Dispense an aliquot of each LD_{50} dilution in a separate serum vial and seal. Alternatively, save an aliquot of the challenge inoculum or 10^{-5} dilution to prepare the additional plate count dilutions later (see **Section 4.4.1**).

4.2.8 Place the vial(s) of challenge inoculum and additional dilution tubes on ice. Keep on ice through challenge procedure and until culture is added to plates for post-inoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge all vaccinates 7 to 10 days after the second vaccination.

4.3.2 Challenge non-vaccinated LD_{50} controls at the same time as the vaccinates.

UNCONTROLLED COPY

jmw
22Jun06

4.3.3 Inoculate each vaccinated mouse with 0.25 mL of challenge inoculum intraperitoneally, using a 1 mL tuberculin syringe and 26-gauge, 3/8-inch needle.

4.3.4 Inoculate separate groups of 10 non-vaccinated control mice intraperitoneally with 0.25 mL of each of the LD_{50} dilutions.

4.4 Post-inoculation plate count in biological safety cabinet

4.4.1 After mice are challenged, prepare 10^{-5} to 10^{-7} tenfold dilutions using tryptose broth diluent (if not previously prepared in **Section 4.2.7**).

4.4.2 All bacterial suspensions must be mixed well prior to placing an aliquot on an agar plate. Plate each dilution in triplicate using 0.1 mL on bovine blood agar or tryptose agar. Inoculum must be spread evenly on the surface of the agar plates and not allowed to pool around the edges. Complete all plate inoculations within 1 hour of challenge.

4.4.3 Incubate the plates aerobically at 35°- 37°C for 18 to 30 hours.

4.4.4 Using the dilution yielding 30-300 colonies per plate, calculate the colony forming units (CFU)/challenge dose according to the following formula:

$$\frac{\text{Colony count sum}}{\text{Number of plates}} \times \frac{1}{\text{Dilution factor plated}} \times \frac{1}{\text{Plated volume (ml)}} \times \frac{\text{Challenge dilution}}{1} \times \frac{\text{Challenge vol. (ml)}}{\text{Dose}} = \frac{\text{CFU}}{\text{Dose}}$$

4.4.5 Record the plate count (CFU/dose) of the challenge on the test result form. This information is for informational purposes to track trends and to troubleshoot problem tests. The 9 CFR does not specify a minimum or maximum CFU/dose for this test.

4.5 Observation of mice after challenge

4.5.1 Observe the mice daily for 14 days after challenge. Record deaths.

4.5.2 If deaths occurring after challenge are suspected to be due to causes other than salmonellosis, necropsy such mice to determine the cause of death. If cause of death is unrelated to vaccination and/or challenge, do not include the deaths in the total deaths for the test.

UNCONTROLLED COPY

JMW
22 Jun 06

5. Interpretation of the test results

Interpret the test as prescribed in 9 CFR, Part 113.120.

5.1 Calculate the LD_{50} (theoretical dose/dilution at which the challenge would be lethal to 50% of the control mice) of the challenge inoculum using the Reed-Muench or Spearman Kärber method of estimation. A valid test must have an LD_{50} between 100 and 10,000.

5.2 Calculate the PD_{50} of the reference bacterin and each test bacterin (theoretical dose/dilution at which the bacterin would protect 50% of the mice) using the Reed-Muench or Spearman Kärber method of estimation.

5.3 At least 2 dilutions of the reference shall protect >0% and <100% of the mice for a valid test. The lowest dilution of the reference shall protect >50% of the mice and the highest dilution of the reference shall protect <50% of the mice.

5.4 If the PD_{50} of the reference cannot be calculated because the lowest dilution tested protects <50% of the mice, the serial may be retested, ***provided the following:***

1. If the serial is not retested, it is unsatisfactory.

2. If the protection provided by the lowest dilution of the standard exceeds that provided by the lowest dilution of the test serial by at least 6 mice, the test serial is unsatisfactory without additional testing.

3. If the total number of mice protected by the reference (sum of survivors in all dilution groups) exceeds the total number protected by the test serial by 8 mice or more, the test serial is unsatisfactory without additional testing.

5.5 If the PD_{50} of the test serial in a valid test cannot be calculated because the highest dilution protected more than 50% of the mice, the serial is satisfactory without further testing.

5.6 Divide the PD_{50} of each test serial by the PD_{50} of the reference to calculate the relative potency (RP) for each serial.

5.7 If the RP of the test serial(s) is ≥0.3, the serial is satisfactory.

5.8 If the RP of the test serial(s) is <0.3, the test serial is unsatisfactory.

UNCONTROLLED COPY

JMW 22 June 06

5.9 A test serial with an RP <0.3 may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. Calculate the results of the retests in the following manner:

1. Average the RP values of the retests.

2. If the average RP of the retests is <0.3, the serial is unsatisfactory.

3. If the average RP of the retests is ≥0.3 **AND** the RP obtained in the original test is <1/3 than the average (RP) of the retests, the test bacterin is satisfactory. Consider the initial test to be the result of test system error.

4. If the average of the retests is ≥0.3 **BUT** the RP of the original test is >1/3 of the average RP of the retests, calculate a new average RP using the RP values obtained in all tests (original plus retests). If the new average RP is ≥0.3, the test bacterin is satisfactory. If the new average RP is <0.3, the test bacterin is unsatisfactory.

6. Report of test results

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

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.120, U.S. Government Printing Office, Washington, DC, 2006.

7.2 Reed LJ, Muench H, 1938. A simple method of estimating 50% endpoints. *Am J Hygiene*, 27: 493-497.

8. 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:

- **2.1** A sterile inoculating loop and biological safety cabinet have been added.
- **2.2.8** Screw-cap tubes, 15 x 125-mm, have been added.

UNCONTROLLED COPY

- **2.2.12** Bovine blood agar has been added as an additional media option for the plate counts.
- **2.2.16** A 1-L flask has been added
- **2.3.2** The number of mice currently used for the test has been updated.
- **3.4.7** The recipe for 5% bovine blood agar plates has been added.
- The use of a biological safety cabinet has been added throughout the document.
- References to the current reagent data sheet have been added throughout the document.
- The currently used spectrophotometer parameters have been updated.
- References to internal documents have been replaced with summarized information.
- The contact has been changed to Janet Wilson.

UNCONTROLLED COPY

JMW
22Jun06