

**United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol**

SAM 617

**Supplemental Assay Method for Potency Assay *Leptospira kirschneri*
serogroup *grippotyphosa* Bacterins**

Date: **July 7, 2025**

Number: SAM 617.08

Supersedes: SAM 617.07, May 13, 2025

Standard Requirement: 9 CFR 113.104

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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Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippotyphosa* 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 Preparation of equipment/instrumentation**
 - 3.3 Preparation of reagents**
 - 3.4 Preparation of the sample**
- 4. Performance of the Test**
 - 4.1 Vaccination of hamsters**
 - 4.2 Challenge procedure**
 - 4.3 Challenge of test hamsters**
 - 4.4 Observation of hamsters after challenge**
- 5. Interpretation of the Test Results**
- 6. Report of Test Results**
- 7. References**
- 8. Summary of Revisions**

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Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippityphosa* Bacterins

1. Introduction

This Supplemental Assay Method (SAM) describes the hamster vaccination-challenge method used to determine potency of *Leptospira grippityphosa* bacterins as prescribed by title 9, *Code of Federal Regulations* (9 CFR), part 113.104.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Microscope with darkfield capability

2.1.2 Forceps, 5 1/2-inch, rat-tooth

2.1.3 Dissecting pins, 1-to 1 1/2-inch

2.1.4 Necropsy board

2.1.5 Stomacher[®] blender and sterile bags (alternatively, tissue grinders, 15 mL, TenBroeck may be used)

2.1.6 Balance, analytical

2.2 Reagents/supplies

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

2.2.1 Syringes, 1-mL tuberculin

2.2.2 Needles, appropriate size

2.2.3 Scalpels

2.2.4 Glass screw-top tubes, 20 x 150-mm (or equivalent container)

2.2.5 Serum bottles with rubber stoppers (or equivalent container for preparing dilutions of liver homogenate)

2.2.6 70% (v/v) ethanol

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippotyphosa* Bacterins

2.2.7 Microscope slides and coverslips

2.2.8 Pipettes, assorted sizes, cotton-plugged

2.2.9 1% Bovine Serum Albumin Diluent (BSAD)

2.2.10 P80-BA semi-solid medium

2.2.11 0.85% NaCl solution (saline)

2.2.12 *L. grippotyphosa* challenge culture, hamster-virulent

2.3 Animals

2.3.1 Hamsters, adult, 50-90 g. Ten hamsters are required for each bacterin tested. Twenty additional hamsters are required per test session for nonvaccinated controls and LD₅₀ titration.

2.3.2 The hamsters must be obtained from the same source and colony. Use either all male or all female hamsters for any one test.

2.3.3 House and feed all hamsters in an identical manner.

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 must have specific training and experience in the safe handling of live *Leptospira* spp. Personnel need specific training in the care and handling of laboratory hamsters and in the performance of this assay.

3.2 Preparation of equipment/instrumentation

3.2.1 Operate and maintain all equipment according to manufacturers' recommendations and applicable in-house standard operating procedures.

3.2.2 Sterilize all glassware before use.

3.2.3 Use only sterile supplies (pipettes, syringes, needles, rubber stoppers, etc.).

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Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippityphosa* Bacterins

3.3 Preparation of reagents

3.3.1 1% Bovine Serum Albumin Diluent – National Centers for Animal Health (NCAH) Media #20133

Sodium phosphate, dibasic	0.664 g
Potassium phosphate, monobasic	0.087 g
Bovine serum albumin, fraction V	10 g
Deionized water	q.s. 1.0 L

Mix until dissolved. If necessary, adjust pH to 7.5 ± 0.1 . Sterilize by filtration, using a 0.22- μm filter. Store 20°- 25°C for no longer than 1 year.

3.3.2 0.85% saline – NCAH Media #30201

Sodium chloride	8.5 g
Deionized water	q.s. 1.0 L

Autoclave at 121°- 125°C for 15 to 20 minutes. Store 20°- 25°C for no longer than 1 year.

3.3.3 P80-BA semi-solid medium – NCAH Media #10117

Sodium phosphate, dibasic	0.664 g
Potassium phosphate, monobasic	0.087 g
Sodium chloride	1.925 g
Ammonium chloride	0.268 g
Magnesium chloride	0.191 g
Deionized water	790 mL

Stir to dissolve. Add:

Cupric sulfate solution (300 mg/L, pH 5.8)	1 mL
Zinc sulfate solution (0.4g/L, pH 6.3)	10 mL
Ferrous sulfate solution (2.5 g/L)	20 mL
L-cystine	0.2 g

Stir. Do not attempt to dissolve L-cystine completely. Do not heat. Filter through triple thickness #1 Whatman paper. If filtrate is not clear, filter again.

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippityphosa* Bacterins

Combine filtered media with:

Vitamin B12 solution (10 mg/L)	20 mL
Thiamine HCl solution (2 g/L, pH 3.8)	0.1 mL
Tween 80	1.2 mL
Deionized water	q.s. 1 L

Place 800 mL of this mixture in a large container and add 1.3 g purified agar. Autoclave at 121°- 125°C for 20 to 25 minutes. Cool to 56°± 1°C.

Combine the following:

Bovine serum albumin, fraction V	20 g
Sodium phosphate, dibasic	0.133 g
Potassium phosphate, monobasic	0.017 g
Deionized water	q.s. 200 mL

Adjust pH to 7.4 to 7.6 and sterilize by filtration (0.2 µm).

Add filtered albumin solution to the cooled (56°C) solution prepared previously.

Adjust to pH 7.2 to 7.8 with sterile 10% NaOH. Dispense in 9-mL aliquots into screw-capped tubes. Store, tightly capped at 20°- 25°C for no longer than 1 year.

3.4 Preparation of the sample

3.4.1 Shake each bacterin to mix contents thoroughly.

3.4.2 Disinfect the top of the bacterin container with 70% ethanol.

3.4.3 Dilute each bacterin with saline so that 1 hamster dose (0.25 mL) is equivalent to 1/800 of the recommended host-animal dose.

1. For 2-mL-dose products, dilute the bacterin 1:100 in saline.

2. For 5-mL-dose products, dilute the bacterin 1:40 with saline.

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippotyphosa* Bacterins

4. Performance of the Test

4.1 Vaccination of hamsters

4.1.1 For each bacterin to be tested, vaccinate 10 hamsters with 0.25 mL of appropriately diluted bacterin (see **Section 3.4.3**) using the route recommended by the manufacturer. If the recommended vaccination route is intramuscular, or if the product is labeled for either intramuscular or subcutaneous use, vaccinate the hamsters intramuscularly in the hind leg. If the label limits administration of that product to the subcutaneous route, vaccinate the hamsters subcutaneously in the abdominal area. For all vaccinations, use a 1.0-mL syringe fitted with an appropriate size needle.

4.1.2 Retain 10 nonvaccinated hamsters as controls.

4.1.3 Retain 20 nonvaccinated hamsters to determine the LD₅₀ of the challenge inoculum if required

4.1.4 Cryopreserved challenge culture should be inoculated into the first hamsters for serial passage on the day of vaccination or within sufficient time to perform at least three serial passages prior to use in a regulatory test. *Leptospira* passage through hamsters is conducted according to the current version of **CVB-WI-0253, *Leptospira* Passage through Hamsters**.

4.1.5. Challenge all hamsters with a virulent suspension of *L. grippotyphosa* 14 to 18 days after vaccination.

4.2 Challenge procedure

The challenge inoculum is a liver homogenate from a clinically ill hamster. The Center for Veterinary Biologics (CVB) maintains virulent, cryopreserved challenge organisms.

4.2.1 Select a clinically ill (preferably moribund) hamster from a group of hamsters that were infected 3 to 4 days previously with *L. grippotyphosa*.

4.2.2 Euthanize the hamster with CO₂. Rapid administration from a compressed gas cylinder is recommended because the inflow to the chamber can be regulated precisely. Follow the euthanasia procedure approved by the Animal Care and Use Committee.

4.2.3 Pin the dead hamster to a posting board (ventral aspect up) and disinfect the skin with 70% ethanol.

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Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippotyphosa* Bacterins

4.2.4 Using aseptic technique, reflect the abdominal skin. Discard the instruments used to expose the abdominal musculature. Using fresh instruments, reflect the abdominal musculature to expose the abdominal viscera. Discard the instruments used to open the abdominal cavity.

4.2.5 Using fresh instruments, aseptically remove approximately 1 gram of liver tissue and place in a sterile previously weighed container. Weigh the container again with liver tissue and add or subtract additional tissue to obtain a mass as close to 1.0 gram as possible ± 0.1 gram. Aseptically place liver in a sterile blender bag and add 9 mL of sterile BSAD to the bag. Thoroughly homogenize the liver, taking care to avoid foam formation. This suspension is considered the 1:10 dilution.

4.2.6 Prepare 5 additional serial tenfold dilutions (10^{-2} through 10^{-6}) of the tissue suspension in BSAD (1.0 mL suspension + 9.0 mL diluent). Hold the dilutions at room temperature (20° - 25° C) and complete challenge inoculations within 1 hour after preparation of dilutions.

4.2.7 Place 2 drops of the 10^{-4} dilution on a microscope slide from an 18g needle, cover with a 22x40mm coverslip, and examine under a 200X magnification with a darkfield microscope. The 10^{-4} dilution should have approximately 4 to 20 organisms per field.

4.2.8 If the 10^{-4} dilution has 4 to 20 organisms per field, use the 10^{-5} or 10^{-6} dilution for the challenge inoculum. (This usually is sufficient to deliver the required challenge of 10-10,000 LD₅₀.) The challenge inoculum should be prepared in an appropriate sterile container.

Note: If the 10^{-4} dilution contains > 20 organisms per field, dilute the challenge with BSAD until numbers of organisms are between 4 and 20 per field. If < 4 organisms are present per field in the 10^{-4} dilution examine the 10^{-3} dilution; if the 10^{-3} dilution contains the required number of organisms, challenge with the 10^{-4} or 10^{-5} dilution. If the 10^{-3} dilution contains < 4 organisms per field, select another clinically ill hamster (Section 4.2.1) and prepare another challenge inoculum that more closely matches the desired organism density. Prepare an inoculum from a liver homogenate preparation that contains highly motile organisms.

4.2.9 Prepare 4 additional tenfold dilutions beyond the dilution selected for the challenge inoculum. Retain these dilutions to determine the LD₅₀ of the challenge inoculum.

4.2.10 The remaining liver homogenate from the challenge preparation may be used to infect additional hamsters to serve as a source of inoculum for future

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippityphosa* Bacterins

potency tests. The dilution and dose volume should be tailored to suit the frequency with which serial passage is performed.

4.2.11 Transfer 1 mL of the challenge inoculum into 9 mL of P80-BA semisolid medium. Incubate semisolid cultures at 25°- 30° C for 2 to 4 weeks. Cultures may be maintained at 20°- 25°C for up to 6 months. These cultures serve as an archival source of the challenge inoculum if additional analysis is warranted. The CVB prepares additional cultures (i.e., duplicate cultures of inoculum from each of the 10⁻⁴ through 10⁻¹⁰ dilutions) for internal use.

4.3 Challenge of test hamsters

4.3.1 Within 1 hour after preparation, inject intraperitoneally (IP) 0.2 mL of the dilution selected in **Section 4.2.8** into each of the vaccinated hamsters and 10 nonvaccinated control hamsters. Use a 1.0-mL syringe fitted with an appropriate size needle.

4.3.2 Inject 5 hamsters (0.2 mL, IP) with each of the dilutions prepared in **Section 4.2.9**. These 4 groups of hamsters will be used to calculate the LD₅₀ of the challenge.

4.3.3 The 10⁻² or ⁻³ dilution should be retained and later tested for serogroup specificity through the current version of **CVB-TWS-0048**, *Leptospira ELISA Screen*.

4.3.4 Disinfect all work surfaces with 70% ethanol. Sterilize all contaminated equipment and supplies in the autoclave.

4.4 Observation of hamsters after challenge

4.4.1 Observe all hamsters daily for 14 days following challenge. Record deaths.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

4.4.2 At the end of the 14-day observation period, count the remaining hamsters and record results.

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippityphosa* Bacterins

4.4.3 Calculate the LD₅₀ of the challenge inoculum using the Reed-Muench or Spearman-Kärber method of calculation.

5. Interpretation of the Test Results

5.1 Interpret the results as described in 9 CFR 113.104.

5.2 If 8 or more nonvaccinated controls die the test is valid. A LD₅₀ of 10-10,000 is an additional validity requirement.

5.3 If 3 or 4 vaccinates die in the first stage test, conduct a second stage test in a manner identical to the first stage. If the second stage is used, evaluate each serial according to the second part of the table. Serials pass or fail on the basis of cumulative results. Evaluate the results according to the following table:

Stage	Number of Vaccinates	Cumulative Number of Vaccinates	Cumulative Total Dead Hamsters for Satisfactory Serial	Cumulative Total Dead Hamsters for Unsatisfactory Serial
1	10	10	2 or less	5 or more
2	10	20	5 or less	6 or more

6. Report of Test Results

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

7. References

Title 9, *Code of Federal Regulations*, part 113.104, U.S. Government Printing Office, Washington, DC.

CVB-WI-0253 *Leptospira* Passage through Hamsters.

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippotyphosa* Bacterins

8. Summary of Revisions

Version .08

- Added details to microscopy and left policy about LD50 groups vague with "if required" language.
- Added in cryopreservation language.

Version .07

- Range for acceptable inoculation dilution updated.
- Details on microscopy added.

Version .06

- The coversheet has been updated

Version .05

- The Contact information has been updated, and minor edits to accurately reflect current practices.

Version .04

- *Leptospira interrogans* Serovar *grippotyphosa* has been changed to *Leptospira kirschneri* serogroup *grippotyphosa* to reflect the most current molecular identification results.
- The Contact information has been updated.
- **3.3.1:** The pH for the BSA diluent has been altered to allow ± 0.1 deviation from 7.5.
- References to NVSL have been changed to NCAH throughout the document.

Supplemental Assay Method for Potency Assay *Leptospira kirschneri* serogroup *grippityphosa* Bacterins

Version .03

- Minor changes have been made to update the document to current formatting practices.

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:

- **2.1** A Stomacher[®] blender and analytical balance have been added to the list of equipment and instrumentation.
- **2.2** The list of reagents and supplies has been updated to more accurately reflect what is used for this assay method.
- **3.3** The formulas have been updated to reflect that the solution may be stored at room temperature for up to one year.
- **3.3.3** P80-BA semi-solid medium has been added to the Preparation of Reagents section.
- References to internal CVB documents have been replaced with summary information.
- The contact person has been changed to Mary C. Rasmusson.