United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 610

Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serogroup Icterohaemorrhagiae Bacterins

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Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serogroup Icterohaemorrhagiae Bacterins

Table of Contents

1. Introduction

This Supplemental Assay Method (SAM) describes the hamster vaccination-challenge method used to determine potency of *Leptospira icterohaemorrhagiae* bacterins as prescribed by title 9, *Code of Federal Regulations* (9 CFR), part 113.102.

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

2.1.7 Calibrated weight set

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted as necessary.

2.2.1 Syringes, 1-mL tuberculin for animal injections and 3-mL for preparing challenge dilutions in 1% BSA glass serum vials.

2.2.2 Needles of appropriate gauge (e.g. 23g or 25g for hamster injections and larger 18g for preparing challenge dilutions in 1% BSA glass serum vials)

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

2.2.7 Microscope slides and coverslips

2.2.8 Pipettes, assorted sizes, cotton-plugged

2.2.9 1% Bovine Serum Albumin Diluent (BSAD) National Centers for Animal Health (NCAH) Media #20133 in 10 mL glass serum vials and also in 20mm x 125mm sc test tubes.

2.2.10 Sterile Petri plates 15mm X 100mm

2.2.11 0.85% NaCl solution (saline) NCAH Media #10191

2.2.12 *L. icterohaemorrhagiae* challenge culture IRP 655, frozen, hamster-virulent

2.3 Animals

2.3.1 Hamsters, adult, 50-90 g. Ten hamsters are required for each bacterin tested. Ten hamsters are always required per test session for nonvaccinated controls, and an additional twenty hamsters are required for LD_{50} titration if the back-titration exemption is not used (see **CVB Public Notice 15-13**). Sufficient hamsters should always be available to perform at least three *in vivo* serial passages of the *Leptospira* prior to challenge.

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, etc.).

3.3 Preparation of reagents

3.3.1 Phosphate Buffer 25X Solution – NCAH Media #40105

Sodium Phosphate Dibasic Anhydrous	16.6 g
Potassium Phosphate Monobasic	2.172 g
Deionized water	q.s. 1000 mL

Mix until dissolved. Filter sterilize (0.2-µm filter). Store refrigerated up to 6 months.

3.3.2 1% Bovine Serum Albumin Diluent – NCAH Media #20133

Phosphate Buffer 25X Solution (NCAH) Media #40105	40mL
Bovine serum albumin	10 g
Deionized water	q.s. 960mL

Mix until dissolved. If necessary, adjust pH to 7.5 ± 0.1. Filter sterilize (0.22-µm filter). Store at 20°- 25°C for no longer than 1 year.

3.3.3 0.85% saline – NCAH Media #10191

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

Autoclave at 121°C for 20 minutes. Store 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 1/80 of the recommended host-animal dose.

Examples:

1. For 1-mL- host dose products, dilute the bacterin 1:20 in saline.
2. For 2-mL-dose products, dilute the bacterin 1:10 in saline.
3. For 3-mL-host dose products, dilute the bacterin 1:6.7 (6.67) in saline.
4. For 4-mL-host dose products, dilute the bacterin 1:5 in saline.
5. For 5-mL-dose products, dilute the bacterin 1:4 in saline.

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 LD_{50} 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. icterohaemorrhagiae* 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. icterohaemorrhagiae*.

4.2.2 Euthanize the hamster with CO_2. 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.

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. Measure the liver on an analytical scale in a tared sterile container in order to obtain 1.0 ± 0.1 gram of infected tissue. Aseptically place liver in a sterile blender bag. Add 9 mL of sterile BSAD to the bag. Thoroughly homogenize the liver with a Stomacher or tissue grinder, 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, cover with a 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, then the 10^{-6} dilution will be the challenge inoculum. (The challenge inoculum is the dose sufficient to deliver the required challenge of 10-10,000 LD_{50}.) The challenge inoculum should be prepared in an appropriate sterile container.

Note: If the 10^{-4} dilution does not contain the appropriate number of spirochetes per field, determine which dilution contains between 4–20 spirochetes/microscopic field. Then utilize the chart below to determine appropriate dilution to use as the challenge inoculum.

Dilution Containing 4 - 20 spirochetes/field	Challenge Inoculum Dilution
10^{-4}	10^{-6}
10^{-5}	10^{-7}
10^{-6}	10^{-8}
10^{-7}	10^{-9}

If the 10^{-4} dilution contains < 4 organisms per field, select another clinically ill hamster (Section 4.2.1) and prepare another challenge inoculum.

4.2.9 If the LD50 is required, prepare 4 additional tenfold dilutions beyond the dilution selected for the challenge inoculum. Retain these dilutions to determine the LD_{50} of the challenge inoculum.

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

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

4.3 Challenge of test hamsters

4.3.1 Within 1 hour after preparation, inject intraperitoneally (IP) 0.2 mL of the challenge inoculum 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 needle.

4.3.2 Inject 5 hamsters (0.2 mL, IP) with each of the dilutions prepared in **Section 4.2.9** if a back-titration is required. These 4 groups of hamsters will be used to calculate the LD_{50} of the challenge inoculum.

4.3.3 The $10^{-2 \text{ or } -3}$ 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.

4.4.3 Calculate the LD_{50} of the challenge inoculum using the Reed-Muench, Dragstedt-Behrens, or Spearman-Kärber method of calculation. The CVB typically uses the Dragstedt-Behrens method.

5. Interpretation of the Test Results

5.1 Interpret the results as described in 9 CFR 113.102.

5.2 The test is valid if 8 or more of the nonvaccinated hamsters that received the challenge inoculum are counted as deaths. An LD_{50} of 10-10,000 is an additional validity requirement without a backtitration exemption.

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 113.102, U.S. Government Printing Office, Washington, DC.

CVB-WI-0253 Leptospira Passage through Hamsters.

CVB Public Notice 15-13 Option to Remove Back-titration Hamsters from In Vivo Potency Tests for Leptospira Serogroups Canicola and Icterohaemorrhagiae

8. Summary of Revisions

Version .07

- Formatting updated, updated coversheet, minor process updates
- **2.1.7** Added calibrated weight set
- **2.2.1, 2.2.2, 2.2.9, 2.2.11** Items were revised and updated
- **2.2** Updated to allow equivalent reagents/supplies to be used as necessary

- **2.2.10** Removed P80-BA semi-solid medium and added Sterile Petri plates 15mm X 100mm

- **2.2.12** Revised to *L. icterohaemorrhagiae* challenge culture IRP-655, frozen, hamster-virulent

- **2.3** Added reference to **CVB Public Notice 15-13**

- **3.3.1, 3.3.2, 3.3.3** Items were revised and updated. P80-BA semi-solid medium removed Phosphate Buffer 25X Solution was added

- **3.4.3** Revised with more dilution examples added

- **4.3.3** Changed recommended dilution from 10^{-4} to $10^{-2 \text{ or } -3}$

- **4.4.1** Updated humane endpoints description for consistency

Version .06

- Formatting has been updated according to current practices.

- The LD50 calculation was clarified.

- The use of back-titration hamsters has been updated in agreement with current public guidance.

- Serogroup specificity testing and retention of shipping cultures has been updated based on current practices.

Version .05

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

Version .04

- The Contact information has been updated.

- The title has been changed to be more in agreement with current nomenclature practices.

- References to NVSL have been changed to NCAH throughout the document.

- Minor revisions have been made to clarify practices currently in use at the Center for Veterinary Biologics.

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.