

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

SAM 308

**Supplemental Assay Method for Potency Testing of Inactivated Rabies
Vaccine in Mice Using the National Institutes of Health Test**

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Contact: Alethea M. Fry, (515) 337-7200
Peg A. Patterson

Approvals: /s/Geetha B. Srinivas Date: 22Jun15
Geetha B. Srinivas, Section Leader
Virology

/s/Byron E. Rippke Date: 22Jul15
Byron E. Rippke, Acting Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 28Jul15
Rebecca L.W. Hyde, Section Leader
Quality Management
Center for Veterinary Biologics

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 Testing of Inactivated Rabies Vaccine
in Mice Using the National Institutes of Health Test**

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**Supplemental Assay Method for Potency Testing of Inactivated Rabies Vaccine
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1. Introduction

This Supplemental Assay Method (SAM) describes testing inactivated rabies vaccines for relative potency (RP). This method is in support of title 9, *Code of Federal Regulations* (9 CFR), section 113.209(b)(1). The method uses immunized mice to measure protection following a challenge with challenge virus standard (CVS). The RP is determined by comparing the Test Vaccine against a standardized reference vaccine. This SAM is based on the standard National Institutes of Health (NIH) test method as described in *Laboratory Techniques in Rabies*, fourth edition, edited by F. X. Meslin, M. M. Kaplan, and H. Koprowski, 1996, published by the World Health Organization (WHO), Geneva, Switzerland.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.2 Water bath

2.1.3 Strongly encouraged (Center for Veterinary Biologics (CVB) Notice No. 12-12)

2.1.3.1 Downdraft anesthesia table

2.1.3.2 Anesthesia machine

2.2 Reagents/supplies

All reagents and supplies must be sterile.

2.2.1 CF-1 female mice weighing 13-20 g.

2.2.2 Challenge Virus Standard (CVS) supplied by CVB

2.2.3 Veterinary rabies reference vaccine (VRRV) supplied by the CVB

2.2.4 Syringe, 0.5-1.0 mL and needle, 26-28-gauge x 3/8 - 1/2 inch (challenge)

2.2.5 Syringe, 1-3-mL and needle, 23-26 gauge x 1/2 - 5/8-inch (vaccination)

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2.2.6 NIH Phosphate buffered saline (PBS) (National Centers for Animal Health (NCAH) Media #30087)

1. 0.804 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
2. 0.136 g potassium phosphate, monobasic, monohydrate (KH_2PO_4)
3. 8.5 g sodium chloride (NaCl)
4. Q.S. to 1000 mL with deionized water (DI).
5. Adjust pH to 7.6 ± 0.1 with 0.1 N sodium hydroxide (NaOH).
6. Sterilize through a 0.22- μm filter.
7. Store at $2^\circ - 7^\circ\text{C}$.

2.2.7 7.5% Sodium Bicarbonate (NCAH Media #41009)

1. 7.5 g sodium bicarbonate (NaHCO_3)
2. Q.S. to 100 mL with DI.
3. Store at room temperature.

2.2.8 CVS Diluent (NCAH Media #30086)

1. 20 mL heat-inactivated, rabies antibody-free horse serum
2. 500,000 units penicillin
3. 1 g streptomycin
4. Q.S. to 1000 mL with DI.
5. Adjust pH to 7.6 with 7.5% Sodium Bicarbonate.
6. Sterilize through a 0.22- μm filter.
7. Store at $2^\circ - 7^\circ\text{C}$.

2.2.9 Pipettes: 2-mL, 5-mL, 10-mL and 25-mL

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2.2.10 Strongly encouraged (CVB Notice No.12-12)

2.2.10.1 Isoflurane

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in laboratory dilution techniques, the handling and discarding of human pathogens, and the handling and inoculation of mice.

3.2 Preparation of CF-1 female test mice

3.2.1 Minimally, mice are housed in a BL-2 animal rooms.

3.2.2 On the day of first vaccination, mice are weighed in groups of 16 (VRRV, test vaccine) and 10 (back titration) and must weigh between 13 and 20 grams.

3.2.3 For each Test Vaccine, 5 groups of 16 mice are recommended. Mice are housed in cages labeled with the Test Vaccine identification and dilution.

3.2.4 For the VRRV, 4 or 5 groups of 16 mice are recommended. Mice are housed in cages labeled with the VRRV and dilution.

3.2.5 For the CVS Back Titration, 4 groups of 10 mice are recommended. Mice are housed in cages labeled with the CVS Back Titration and dilution. These mice are reserved until the day of CVS challenge.

3.3 Preparation of reagents/control procedures

3.3.1 On the day of initial vaccination, reconstitute the VRRV with sterile DI as described on the CVB Reagent Data Sheet.

1. Prepare test dilutions of the VRRV in NIH PBS as described on the CVB Reagent Data Sheet.

a. Maintain the VRRV dilutions on ice until mice are inoculated. The diluted VRRV is used within 3 hours of preparation.

b. Freeze the remaining reconstituted VRRV in the original container at $-70^{\circ}\pm 5^{\circ}\text{C}$ for use in preparing the *second vaccination* series.

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2. Preparation of VRRV for the *second vaccination* (7 ± 1 days post inoculation)

a. Rapidly thaw the frozen, reconstituted VRRV in a $36^{\circ} \pm 2^{\circ}\text{C}$ water bath or at room temperature.

b. Prepare test dilutions of VRRV as described in the Reagent Data Sheet and once prepared, maintain the dilutions on ice until mice are inoculated. The diluted VRRV is used within 3 hours of preparation.

3.3.2 Preparation of the sample

The initial dilution of a Test Vaccine is based on the dilution determined in 5 replicate NIH tests at the time of the initiation of the host animal efficacy trial. The starting dilution should optimally protect 85-100% of vaccinates and this dilution **shall be stated in Part V of the Animal and Plant Health Inspection Service (APHIS) approved Outline of Production**. Starting with the initial dilution, prepare a set of five 5-fold serial dilutions for each Test Vaccine.

3.3.3 Preparation of Working CVS on the day of challenge

The **stock CVS** is a concentrated biological reagent that is diluted to a lower concentration for use. Stock reagents are used to save preparation time, conserve materials, reduce storage space, and improve the accuracy and stability.

Working CVS is a dilution of the stock reagent prepared and administered to test mice by the intracranial (IC) route.

1. Rapidly thaw stock CVS in a $36^{\circ} \pm 2^{\circ}\text{C}$ water bath.

2. Prepare test dilutions of the stock CVS in CVS diluent as described on CVB Reagent Data Sheet. The working CVS will contain ≥ 12 50% lethal dose (LD_{50})/0.03 mL.

3. The Working CVS is maintained on ice until inoculation into mice. The Working CVS is administered within 3 hours of preparation.

4. Preparation of CVS Back Titration on the day of challenge

a. Prepare three 10-fold serial dilutions from the working CVS.

b. The CVS Back Titration is maintained on ice until mice are inoculated. The CVS Back Titration is administered within 3 hours of preparation.

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4. Performance of the Test

4.1 Fill a 1-3 mL syringe with a 23-26-gauge x ½ to 5/8-inch needle with diluted Test Vaccine or VRRV.

4.2 Beginning with the most dilute preparation, inoculate 0.5 mL of assigned vaccine dilution into each of 16 mice/dilution by the intraperitoneal (IP) route. Repeat for each dilution of a Test Vaccine and the VRRV using a new group of 16 mice caged according to dilution. (After all animals have been vaccinated, dispose of residual dilutions of the Test Vaccines and VRRV.)

4.3 At 7 ± 1 days post inoculation, revaccinate animals as described above.

4.4 At 14 ± 1 days post-vaccination, all vaccinated mice (vaccine and VRRV) are administered 0.03 mL of working CVS intracerebrally (IC), using a 0.5 - 1.0-mL syringe with a 26-28 gauge x 3/8 – ½ inch needle. The working dilution of CVS is inoculated through the frontal bone, midway between the right eye and the midline. (Optional: mice may be anesthetized using a rodent anesthesia machine containing isoflurane and an induction chamber to scavenge residual anesthesia prior to challenge.)

4.5 The CVS Back Titration is administered IC to the remaining four groups of 10 back-titration mice using a new syringe and needle. Each of the four dilutions are administered to 10 mice per dilution; beginning with the most dilute concentration of the CVS through the most concentrated dilution of the CVS. The same syringe and needle may be used if starting with the most dilute followed by successively more concentrated dilutions. The CVS Back Titration is not administered until all the vaccinated mice have been challenged.

4.6 Animal Observations:

4.6.1 Animals are observed daily for 14 days post challenge. Deaths or animals humanely euthanized are recorded.

4.6.2 Mice dying prior to or on day 5 post-challenge are considered nonspecific deaths. Nonspecific deaths are not used in calculating the 50% effective dose (ED₅₀) of the Test Vaccine or VRRV.

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4.6.3 The following five disease stages can be recognized in mice infected with rabies virus (Bruckner et al. 2003):

Stage 1: *Ruffled fur and hunched back* indicate the first signs of clinical disease. These are general signs of illness in mice and can be observed for many other diseases. Therefore, these clinical signs in Stage 1 are not specific indicators for rabies, but do reflect that the animal is unwell and that its welfare is compromised.

Stage 2: *Slow or circular movements.* During Stage 2, animals lose their alertness. They walk more slowly than usual; and if observed carefully, show slow circling movements, mainly in one direction. These are the first clinical indicators of neurological disorder.

Stage 3: *Shaky movements, trembling and convulsions.* In Stage 3 the neurological signs become increasingly obvious, with trembling and shaky movements and convulsions. By this time there is marked loss of body weight. This stage with severe and unequivocal clinical signs, clearly indicates rabies infection.

Stage 4: *Signs of paralysis.* Lameness and paresis (partial paralysis), usually of the hind legs, are clear indicators of progressive infection with rabies and is soon followed by complete paralysis. The animals become dehydrated.

Stage 5: *Moribund animals.* In Stage 5, animals become moribund. They can be seen to be prostrate and recumbent, and they obviously do not eat or drink; yet they may survive for 1 or 2 more days.

4.6.4 Animals exhibiting Stage 3 or greater disease signs must be humanely euthanized. Deaths occurring 6 or more days post-challenge and euthanized animals are considered as deaths caused by the CVS challenge (9 CFR 117.4 and CVB Notice No. 12-12). Mice surviving the 14-day post challenge observation period are euthanized at the conclusion of the test.

4.6.5 Similar definitions should be incorporated into non-codified potency and safety tests and efficacy study protocols. Proposed endpoints will be reviewed on a case-by-case basis.

4.7 The ED₅₀ of each Test Vaccine, VRRV, and the LD₅₀ of the CVS are determined by the method of Spearman-Kärber as referenced in the fourth edition of the WHO, *Laboratory Techniques in Rabies*.

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4.8 The relative potency (RP) of the Test Vaccine (TV) is determined by the formula:

$$\text{RP} = \frac{\text{reciprocal ED}_{50} \text{ of TV}}{\text{reciprocal ED}_{50} \text{ of VRRV}} \times \frac{\text{dose of TV}}{\text{dose of VRRV}}$$

$$\text{ED}_{50} \text{ of TV} = 1:90$$

$$\text{ED}_{50} \text{ of VRRV} = 1:70$$

$$90/70 = 1.29 \text{ RP/mL}$$

4.9 The RP value may be expressed in international units (IU) by multiplying the RP by the IU/ml of the VRRV. For a VRRV with a known value of 1.0 IU/mL, in the above example:

$$1.29 \times 1.0 \text{ IU/mL} = 1.29 \text{ IU/mL}$$

5. Interpretation of the Test Results

5.1 For a valid test:

5.1.1 At least 70% of the mice receiving the most concentrated dilutions of VRRV and the Test Vaccine must survive (e.g., 11 out of 16 mice).

5.1.2 At least 70% of the mice receiving the least concentrated dilution of VRRV and the Test Vaccine must die (e.g., 11 out of 16 mice).

5.1.3 For a valid challenge, the CVS back titration must be $\geq 12 \text{ LD}_{50}/0.03 \text{ mL}$.

5.1.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.2 The minimum RP value for a satisfactory Test Vaccine is determined by the results of the host animal immunogenicity serial as required by the 9 CFR 113.209 and contained in an APHIS filed Outline of Production. If the validity requirements are met and the Test Vaccine meets or exceeds the minimum release RP value the Test Vaccine is considered **SATISFACTORY**.

5.3 If the Test Vaccine does not meet the minimum RP value contained in an APHIS filed Outline of Production, as determined from the initial NIH test, the Test Vaccine may be retested. If the Test Vaccine is retested, 2 independent NIH tests shall be conducted. A geometric mean RP of all 3 retests is used to evaluate the Test Vaccine.

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If the Test Vaccine is not retested by or if the geometric mean RP value of the initial test and retests is below the minimum release RP value, the Test Vaccine is **UNSATISFACTORY**. If the geometric mean RP value of the initial test and retests meets or exceeds the minimum release, the serial is **SATISFACTORY**.

6. Report of Test Results

Test results are reported as the RP/mL.

7. References

- 7.1** Title 9, *Code of Federal Regulations*, section 113.209, U.S. Government Printing Office, Washington, DC.
- 7.2** Richmond JY, McKinney RW (eds.). Biosafety in microbiological and biomedical laboratories. U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington. 1993; p. 177.
- 7.3** Wilbur LA, Aubert MFA. The NIH test for potency. In *Laboratory Techniques in Rabies, 4th ed.*, Meslin, FX, MM Kaplan, and H Koprowski, eds. World Health Organization, Geneva. 1996, Chpt. 37.
- 7.4** Center for Veterinary Biologics Notice No. 13-10 (July 26, 2013)
- 7.5** Center for Veterinary Biologics Notice No. 12-12 (May 25, 2012)
- 7.6** Lukas Bruckner, Klaus Cussler, Marlies Halder, Jacques Barrat, Peter Castle, Karin Duchow, Donna M. Gatewood, Richard Gibert, Jan Groen, Bernhard Knapp, Robin Levis, Catherine Milne, Simon Parker, Karin Stünkel, Nico Visser, and Peter Volkers. Three Rs Approaches in the Quality Control of Inactivated Rabies Vaccines, The Report and Recommendations of ECVAM Workshop 48^{1,2} ATLA 31, 429–454, 2003.

8. Summary of Revisions

Version .06

- **5.3:** Rewritten to describe the provisions the Center for Veterinary Biologics will use if the serials do not meet the minimum RP value and associated retest provisions.

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Version .05

- Total revision of the document to reflect practices currently in use at the Center for Veterinary Biologics.

Version .04

- The Contact information has been updated.
- **1:** The challenge virus used in the test has been identified.
- **3.1:** “Potential” and “potentially” have been removed from sentences. Rabies is a human health hazard and should be treated accordingly.
- **3.3:** This section has been revised for clarification.
- **4.4:** The beginning dilution of the CVS has been added.
- **4.5:** The dilution series of the CVS has been changed to reflect the scheme used at the CVB.
- References to NVSL have been changed to NCAH throughout the document.

Version .03

- **2.2:** Media numbers have been added for reagents available from the National Veterinary Services Laboratories.
- **3.3.1:** The current dilution scheme of the veterinary rabies reference vaccine used at the CVB has been incorporated.
- **3.3.2(7):** This section has been revised to incorporate the current dilution scheme of the CVS back titration.
- **3.3.3(3):** An additional group of 16 test mice has been added to the VRRV.
- **4.5:** The administration of the CVS back titration has been clarified.

Version .02

This document was revised to clarify the 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:

- **1.2** “Key Words” has been deleted.

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- **3.3.1.2** The example of the fivefold dilution series starting at 1:10 was expanded.
- **3.4** has been reworded for clarification.
- **4.6.2** has been reworded for clarification.
- **5.2** has been reworded for clarification.
- The refrigeration temperatures have been changed from $4^{\circ} \pm 2^{\circ}\text{C}$ to $2^{\circ} - 7^{\circ}\text{C}$. This reflects the parameters established and monitored by the Rees system.
- “Test Serial” has been changed to “Test Vaccine” throughout the document.
- “Reference and Reagent Sheet” has been changed to “Reagent Data Sheet” throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.