

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

SAM 304

**Supplemental Assay Method for Titration of Infectious Canine Hepatitis
Virus in Primary Canine Kidney Cell Culture**

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**Supplemental Assay Method for Titration of Infectious Canine Hepatitis Virus
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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live infectious canine hepatitis (ICH) or canine adenovirus (CAV) type 1 virus vaccines for viral content. ICH endpoint is determined by cytopathic effect (CPE) in primary dog kidney (DKp) cell culture.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Incubator, $36^{\circ} \pm 2^{\circ}\text{C}$, high humidity, $5\% \pm 1\%$ CO_2 (Model 3336, Forma Scientific Inc.)

2.1.2 Water bath, $36^{\circ} \pm 2^{\circ}\text{C}$

2.1.3 Microscope, inverted light (Model CK, Olympus America Inc.)

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

2.1.5 Syringe, 2-mL self-refilling, repetitive

2.1.6 Pipettor with tips and/or motorized microliter pipette

2.1.7 Micropipettor, 300- μL x 12-channel

2.1.8 Pipette-aid

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 ICH Positive Control, Mirandola strain of ICH virus, type 1

2.2.2 Monospecific antisera, free of ICH antibody, that neutralize the non-ICH fractions present in multifraction vaccines, e.g. canine parainfluenza virus (CPI), canine parvovirus (CPV), canine distemper virus (CDV), etc.

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2.2.3 Primary dog kidney (DKp) cell culture, free of extraneous agents as tested by title 9, *Code of Federal Regulations* (9 CFR)

2.2.4 Minimum essential medium (MEM)

1. 9.61 g MEM with Earles salts without bicarbonate
2. 1.1 g sodium bicarbonate (NaHCO_3)
3. Dissolve with 900 mL deionized water (DI).
4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DI, heat to $60^\circ \pm 2^\circ\text{C}$ until dissolved, then add to the solution in **Step 3** with constant mixing.
5. Q.S. to 1000 mL with DI, and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).
6. Sterilize through a 0.22- μm filter.
7. Aseptically add 50 $\mu\text{g/mL}$ gentamicin sulfate
8. Store at 2° - 7°C .

2.2.5 Growth Medium

1. 940 mL MEM
2. Aseptically add:
 - a. 50 mL gamma-irradiated fetal bovine serum (FBS)
 - b. 10 mL L-glutamine (200 mM)
 - c. Store at 2° - 7°C .

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

1. 8.0 g sodium chloride (NaCl)
2. 0.2 g potassium chloride (KCl)
3. 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)

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4. 0.1 g magnesium chloride, hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)
5. Dissolve reagents in **Steps 1 through 4** with 900 mL DI.
6. Add 1.03 g sodium phosphate, dibasic anhydrous (Na_2HPO_4) to 10 mL DI, heat to $60^\circ \pm 2^\circ\text{C}$ until dissolved. Add to the solution in **Step 5** with constant mixing.
7. Dissolve 0.1 g calcium chloride, anhydrous (CaCl_2) with 10 mL DI and add slowly to the solution in **Step 6** to avoid precipitation.
8. Q.S. to 1000 mL with DI; adjust pH to 7.0-7.3 with 2N HCl.
9. Sterilize through a 0.22- μm filter.
10. Store at 2° - 7°C .

2.2.7 Cell culture plates, 96-well

2.2.8 Polystyrene tubes, 12 x 75-mm

2.2.9 Pipettes, 10-mL

2.2.10 Reagent reservoir

2.2.11 Syringe, 1-mL tuberculin

2.2.12 Needles, 18-gauge x 1 1/2-inch

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantitation of virus infectivity by CPE.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at $36^\circ \pm 2^\circ\text{C}$.

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3.3 Preparation of reagents/control procedures

3.3.1 Preparation of DKp cell culture plates (Test Plates)

Cells are prepared from healthy, confluent DKp cell cultures at second or third passage. On the day of test initiation, using the 12-channel micropipettor, add 200 μL /well of approximately $10^{4.7}$ to $10^{5.2}$ cells/mL suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 Test Plate for the controls and 1 Test Vaccine. Each additional Test Plate allows testing of 3 additional Test Vaccines. Incubate at $36^{\circ}\pm 2^{\circ}\text{C}$ in a CO_2 incubator and use within 4 hours.

3.3.2 Preparation of ICH Virus Positive Control

1. On the day of test initiation, rapidly thaw a vial of ICH Virus Positive Control in a water bath.
2. Dispense 1.8 mL MEM into enough 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB Reagent Data Sheet, and label (e.g., 8 tubes labeled 10^{-1} through 10^{-8} , respectively).
3. With a 200- μL pipettor, transfer 200 μL of the ICH Virus Positive Control to the first tube labeled 10^{-1} ; mix by vortexing.
4. Using a new pipette tip, transfer 200 μL from the 10^{-1} -labeled tube (**Step 3**) to the 10^{-2} tube; mix by vortexing.
5. Repeat **Step 4** for each of the subsequent dilutions, transferring 200 μL from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 Prepare dilutions of each neutralizing non-ICH antiserum according to the CVB Reagent Data Sheet or manufacturer's instructions.

3.4 Preparation of the Test Vaccine

3.4.1 Conduct the initial test of a Test Vaccine with a single vial (a single sample from 1 vial). On the day of inoculation, using a sterile 1.0-mL syringe and an 18-gauge x 1 1/2-inch needle, rehydrate a vial of the Test Vaccine with the provided diluent by transferring 1.0 mL for a 1.0-mL-dose vaccine, 0.5 mL for a 1/2-mL-dose vaccine, etc., into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

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3.4.2 For multifraction ICH vaccines, neutralize the non-ICH fractions with antiserum specific to each virus fraction.

1. Dispense 200 μ L of each of the required neutralizing antiserum into a 12 x 75-mm polystyrene tube labeled 10^{-1} and q.s. with MEM to 1.8 mL. For example, to neutralize the 3 viral non-ICH Virus components of a CDV/ICH/CPI/CPV vaccine, dispense 200 μ L of each of the diluted CDV, CPI, and CPV antisera into the tube labeled 10^{-1} ; add 1.2 mL of MEM to obtain a final volume of 1.8 mL.
2. Pipette 200 μ L of the reconstituted Test Vaccine to the labeled tube to yield a 10^{-1} dilution; mix by vortexing.
3. Incubate at room temperature for 30 ± 5 minutes.

3.4.3 For vaccines containing only the ICH fraction, the 10^{-1} dilution is prepared by adding 200 μ L of the Test Vaccine to 1.8 mL of MEM in a 12 x 75-mm polystyrene tube, labeled 10^{-1} ; mix by vortexing.

3.4.4 Serial tenfold dilutions

1. Dispense 1.8 mL MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB Reagent Data Sheet; appropriately label (e.g., 8 tubes, labeled 10^{-1} through 10^{-8} , respectively).
2. Using a new pipette tip, transfer 200 μ L from the tube labeled 10^{-1} to the next dilution tube labeled 10^{-2} ; mix by vortexing.
3. Repeat **Step 2** to the remaining tubes, transferring 200 μ L from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

4. Performance of the Test

4.1 On the day of test initiation, label the Test Plates and inoculate each of 8 wells/dilution with 25 μ L of the Test Vaccine, starting with the highest dilution (most dilute). In a similar manner, inoculate 8 wells of the ICH Virus Positive Control (dilutions 10^{-8} through 10^{-5} for the example in **Section 3.3.2[2]**). Change tips between each unique sample (i.e., each Test Vaccine and the ICH Virus Positive Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-8} through 10^{-5}).

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- 4.2** Eight uninoculated wells serve as negative cell controls.
- 4.3** Incubate the inoculated Test Plates in the CO₂ incubator for 11 days ± 1 day.
- 4.4** After incubation, read at 100X or 200X magnification on an inverted light microscope and examine cells for CPE characterized by cell rounding and lysis.
- 4.4.1** Wells displaying one or more CPE foci are considered to be positive for ICH Virus.
- 4.4.2** Record results as the number of CPE-positive wells versus total number of wells examined for each dilution of the Test Vaccine and the ICH Virus Positive Control.
- 4.5** Calculate the ICH endpoints of the Test Vaccine and the ICH Virus Positive Control using the Spearman-Kärber method as commonly modified by Finney. The titers are expressed as log₁₀, 50% tissue culture infective doses (TCID₅₀).

Example:

10⁻² dilution of Test Vaccine = 8/8 wells CPE positive
10⁻³ dilution of Test Vaccine = 5/8 wells CPE positive
10⁻⁴ dilution of Test Vaccine = 1/8 wells CPE positive
10⁻⁵ dilution of Test Vaccine = 0/8 wells CPE positive

Spearman-Kärber formula:

Test Vaccine Titer = (X - d/2 + [d • S]) where:

X = log₁₀ of highest dilution with all wells CPE positive (2)

d = log₁₀ of tenfold dilution factor (1)

S = sum of proportion of wells CPE positive for all dilutions tested:

$$\frac{8}{8} + \frac{5}{8} + \frac{1}{8} + \frac{0}{8} = \frac{14}{8} = 1.75$$

$$\text{Test Vaccine titer} = (2 - 1/2) + (1 \cdot 1.75) = 3.25$$

Adjust the titer to the Test Vaccine dose as follows:

A. divide the **Test Vaccine Dose** by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this ICH test vaccine, the recommended dose is 2 mL)

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Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this ICH test vaccine, the inoculation dose is 0.025 mL)

$$\frac{2 \text{ mL dose}}{0.025 \text{ mL inoculum}} = 80$$

B. calculate log₁₀ of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

$$\text{Log of } 80 = 1.9$$

$$\text{Test Vaccine titer} = 3.25 + 1.9 = 5.15$$

Therefore the titer of the **ICH Test Vaccine** is 10^{5.15} TCID₅₀/2mL.

5. Interpretation of the Test Results

5.1 Valid assay

5.1.1 The calculated titer of the ICH Virus Positive Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The lowest inoculated dilution of the ICH Virus Positive Control must exhibit a 100% positive CPE reaction (8/8). If an endpoint is not reached (1 or more wells are CPE positive wells at the highest dilution), the titer is expressed as “greater than or equal to” the calculated titer. If an endpoint is critical to testing, the highest (most dilute) must exhibit no positive CPE reaction (0/8).

5.1.3 The uninoculated cell controls must not exhibit any CPE, degradation, or cloudy media that would indicate contamination.

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

5.3 In a valid test, if the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Vaccine is considered **SATISFACTORY**.

5.4 In a valid test, if the titer of the Test Vaccine is lower than the titer contained in the APHIS filed Outline of Production for the product under test, the Test Vaccine may be retested in accordance with 9 CFR, Part 113.8.b.

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6. Report of Test Results

Results are reported as TCID₅₀ per dose of Test Vaccine.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.305, U.S. Government Printing Office, Washington, DC.

7.2 Cottral, GE (Ed.), *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca and London, 1978, pg. 731.

7.3 Finney, DJ, *Statistical method in biological assay*. Griffin, London, 3rd ed., 1978, pp. 394-401.

8. Summary of Revisions

Version .06

- **Coversheet and contact information have been updated.**

Version .05

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

Version .04

- The Contact information has been updated.

Version .03

- The term “Reference” has been changed to “Positive Control” throughout the document.

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:

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- **2.2.4.2** The amount of sodium bicarbonate (NaHCO_3) has been changed from 2.2 g to 1.1 g.
- **2.2.4.7** Penicillin and streptomycin have been deleted.
- **4.5** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- **5.1.2** Recording the rate of positive reaction for validity requirements.
- 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.

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