

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

SAM 309

**Supplemental Assay Method for Titration of Canine Parainfluenza Virus in
Vero Cell Culture**

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Supplemental Assay Method for Titration of Canine Parainfluenza Virus in Vero Cell Culture

1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live canine parainfluenza (CPI) virus vaccines for viral content. Presence or absence of CPI virus is determined by hemadsorption (HAd) of guinea pig red blood cells (GPRBC).

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

2.1.3 Centrifuge and rotor (Beckman Coulter)

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

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

2.1.6 Syringe, self-refilling repetitive, 2-mL

2.1.7 Pipettor with tips and/or motorized microliter pipette and tips

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 CPI Positive Control Virus, D008 strain

2.2.2 Monospecific antisera, free of CPI antibody, which neutralize the non-CPI virus fractions present in multifraction vaccines (e.g., canine distemper virus (CDV) and canine adenovirus (CAV))

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2.2.3 African green monkey kidney (Vero) cell line, 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 reagents in **Steps 1 and 2** with 900 mL deionized water (DI).
4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DI, and heat to $60^\circ \pm 2^\circ\text{C}$ until dissolved. 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 $2^\circ - 7^\circ\text{C}$.

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)
3. Store at $2^\circ - 7^\circ\text{C}$.

2.2.6 Alsevers Solution

1. 8.0 g sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$)
2. 0.55 g citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)

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3. 4.2 g sodium chloride (NaCl)
4. 20.5 g glucose (C₆H₁₂O₆)
5. Q.S. to 1000 mL with DI and dissolve.
6. Sterilize through a 0.22-μm filter.
7. Store at 2°- 7°C.

2.2.7 Dulbecco's phosphate buffered saline free of calcium (Ca⁺⁺) and magnesium (Mg⁺⁺) ions.

1. 8.0 g NaCl
2. 0.2 g potassium chloride (KCl)
3. 0.2 g potassium phosphate, monobasic, anhydrous (KH₂PO₄)
4. Dissolve reagents in **Steps 1 through 3** with 900 mL DI.
5. Add 1.03 g sodium phosphate, dibasic, anhydrous (Na₂HPO₄) to 10 mL DI, and heat to 60°± 2°C until dissolved. Add to **Step 4** with constant mixing.
6. Q.S. to 1000 mL with DI; adjust pH to 7.0-7.3 with 2N HCl.
7. Sterilize through a 0.22-μm filter.
8. Store at 2°- 7°C.

2.2.8 Dulbecco's phosphate buffered saline (DPBS)

1. 8.0 g NaCl
2. 0.2 g KCl
3. 0.2 g KH₂PO₄
4. 0.1 g magnesium chloride, hexahydrate (MgCl₂•6H₂O)
5. Dissolve reagents in **Steps 1 through 4** with 900 mL DI.

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6. Add 1.03 g Na_2HPO_4 to 10 mL DI, and heat to $60^\circ \pm 2^\circ\text{C}$ until dissolved. Add to **Step 5** with constant mixing.
7. Dissolve 0.1 g calcium chloride, anhydrous (CaCl_2) with 10 mL DI, and add slowly to **Step 5** 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 $2^\circ - 7^\circ\text{C}$.

2.2.9 Guinea pig blood in an equal volume of Alsevers Solution

2.2.10 Cell culture plates, 24-well

2.2.11 Polystyrene tubes, 12 x 75-mm

2.2.12 Pipettes, 10-mL and 25-mL

2.2.13 Conical tubes, 50-mL

2.2.14 Syringe, 1-mL tuberculin, and needle, 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 HAd.

3.2 Preparation of equipment/instrumentation

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

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of Vero cell culture plates (Test Plates)

Cells are prepared from healthy, confluent Vero cells that are maintained by passing every 3 to 4 days. One day prior to test initiation, add 1.0 mL/well of $10^{4.7}$ to $10^{5.2}$ cells/mL cells suspended in Growth Medium into all wells of a 24-well cell culture plate. Prepare 1 Vero plate for the controls and 1 for each Test

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Vaccine. These become the Test Plates. Incubate at $36^{\circ}\pm 2^{\circ}\text{C}$ in a CO_2 incubator for one day.

3.3.2 Preparation of CPI Positive Control Virus

1. On the day of test initiation, rapidly thaw a vial of CPI Positive Control Virus in a water bath.
2. 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., 9 tubes, labeled 10^{-1} through 10^{-9} , respectively).
3. With a 200- μL pipettor, transfer 200 μL of the CPI Positive Control Virus 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 of the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 Prepare a dilution of each neutralizing non-CPI antiserum in DPBS according to the supplied CVB Reagent Data Sheet or as determined for that specific antiserum.

3.3.4 Preparation of washed guinea pig red blood cells (GPRBC)

1. Transfer 20 mL of the guinea pig blood into a 50-mL conical tube.
2. Q.S. to 50 mL with Alsevers Solution, and mix by inverting several times.
3. Centrifuge for 15 ± 5 minutes at $400 \times g$ (1500 rpm in a Beckman J6B centrifuge with JS-4.0 rotor) at $4^{\circ}\pm 2^{\circ}\text{C}$.
4. Remove supernatant and buffy coat by pipetting.
5. Repeat steps in **Steps 2 through 4** for a total of 3 washes.

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6. Add an equal volume of Alsevers Solution to the washed GPRBC. Store at 2°- 7°C; use within 1 week of collection.

3.3.5 0.5% GPRBC Suspension for the HAd assay

1. On the day of conducting the HAd test, pipette 500 µL of washed, packed GPRBC into 100 mL of Ca⁺⁺, Mg⁺⁺-Free DPBS, rinsing the pipette thoroughly to remove the GPRBC.
2. Mix by inverting, store at 2°- 7°C, and use within 1 week of collection.

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 1-mL-dose vaccines, 0.5 mL for 0.5-mL-dose vaccines, etc., into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 For multifraction CPI vaccines, neutralize the non-CPI viral fractions with antiserum specific to each non-CPI virus fraction. It is not necessary to neutralize canine parvovirus (CPV) since CPV is not expected to replicate in confluent Vero cells.

1. Prepare dilutions of each neutralizing non-CPI antiserum according to the CVB Reagent Data Sheet or manufacturer's instructions.
2. Dispense 200 µL 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 2 non-CPI viral fractions in a 3-fraction CDV/CPI/CAV vaccine, dispense 200 µL of each of the diluted CDV and CAV antisera into the tube labeled 10⁻¹; add 1.4 mL of MEM to obtain a final volume of 1.8 mL.
3. Pipette 200 µL of the reconstituted Test Vaccine to the 10⁻¹ labeled tube containing the neutralizing antisera; mix by vortexing.
4. Incubate at room temperature for 60 ± 10 minutes.

3.4.3 For Test Vaccines containing only the CPI viral fraction, prepare the 10⁻¹ dilution by mixing 200 µL of the reconstituted Test Vaccine with 1.8 mL of MEM in a labeled 12 x 75-mm polystyrene tube.

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3.4.4 Serial tenfold dilutions

1. Dispense 1.8 mL MEM into each of 5, 12 x 75-mm polystyrene tubes labeled 10^{-2} through 10^{-6} (or more if the expected CPI endpoint of the Test Vaccine is higher than 10^{-6}).
2. Using a new pipette tip, transfer 200 μ L from the tube labeled 10^{-1} to the next dilution tube; 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 inoculation, label the Test Plates and inoculate each of 5 wells/dilution with 200 μ L of dilutions 10^{-6} through 10^{-3} of each Test Vaccine. In a similar manner, identify and inoculate 5 wells/dilution of the CPI Positive Control Virus (with dilutions 10^{-9} through 10^{-6} for the example in **Section 3.3.2**). Change tips between each unique sample (i.e., each Test Vaccine and the CPI Positive Control Virus); tip changes are not necessary between dilutions in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-9} through 10^{-6}).

4.2 Five uninoculated wells serve as Negative Cell Controls.

4.3 Incubate the Test Plates in a $36^{\circ}\pm 2^{\circ}\text{C}$ CO_2 incubator for $8 \text{ days} \pm 1 \text{ day}$.

4.4 After incubation, perform the HAd assay on the cell monolayers with a 0.5% GPRBC suspension.

4.4.1 Decant the media from the plates into an autoclavable container.

4.4.2 Submerge each Test Plate in a reservoir of Ca^{++} , Mg^{++} -Free DPBS and decant.

4.4.3 Mix the 0.5% GPRBC suspension by gentle inversion, and add 1.0 mL/well.

4.4.4 Incubate at 2° - 7°C for 25 ± 5 minutes.

4.4.5 Wash Test Plates by repeating **Section 4.4.2** twice.

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4.4.6 Read the wet Test Plate at 100X magnification on an inverted light microscope, and examine CPI-infected vero cells for HAd.

1. Wells displaying GPRBC adsorbed onto the cell monolayer are considered to be positive for CPI.
2. Record the results as the number of HAd- positive wells versus total number of wells examined for each dilution of the Test Vaccine and the CPI Positive Control Virus.

4.5 Calculate the CPI Virus endpoints of the Test Vaccine and the CPI Positive Control Virus using the Spearman-Kärber method as modified by Finney. The titers are expressed as \log_{10} 50% HAd infective doses (HAdID₅₀).

Example:

10^{-3} dilution of Test Vaccine = 5/5 wells HAd positive
 10^{-4} dilution of Test Vaccine = 4/5 wells HAd positive
 10^{-5} dilution of Test Vaccine = 1/5 wells HAd positive
 10^{-6} dilution of Test Vaccine = 0/5 wells HAd positive

Spearman-Kärber formula:

Test Vaccine titer = $(X - d/2 + [d \cdot S])$ where:

X = \log_{10} of highest dilution with all wells HAd positive (3)

d = \log_{10} of tenfold dilution factor (1)

S = sum of proportions of wells HAd positive for all dilutions tested:

$$\frac{5}{5} + \frac{4}{5} + \frac{1}{5} + \frac{0}{5} = \frac{10}{5} = 2.0$$

CPI Test Vaccine titer = $(3 - 1/2) + (1 \cdot 2.0) = 4.5$

Adjust the titer to the recommended Test Vaccine dose as follows:

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

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this CPI vaccine, the recommended dose is 1 mL)

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Inoculation Dose = amount of diluted Test vaccine added to each well of the Test Plate (for this test CPI vaccine, the inoculation dose is 0.2 mL)

$$\frac{1 \text{ mL dose}}{0.2 \text{ mL inoculum}} = 5$$

B. calculate \log_{10} of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

$$\text{Log of } 5 = 0.7$$

$$\text{CPI Test Vaccine titer} = 4.5 + 0.7 = 5.2$$

Therefore, the titer of the CPI Test Vaccine is $10^{5.2}$ HAID₅₀/mL.

5. Interpretation of the Test Results

5.1 Validity requirements

5.1.1 The calculated titer of the CPI Positive Control Virus 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 CPI Positive Control Virus must exhibit a 100% positive HAd reaction (5/5). If an endpoint is not reached (1 or more wells are HAd 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 HAd reaction (0/5).

5.1.3 The Negative Cell Controls shall not exhibit degradation, HAd, or cloudy media indicative of contamination.

5.2 If the validity requirements are not met, 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 less 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 113.8.

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6. Report of Test Results

Results are reported as HAdID₅₀ per dose of Test Vaccine.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.316, U.S. Government Printing Office, Washington, DC.

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

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

8. Summary of Revisions

Version .06

- **The 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:

- **2.2.4.2** The amount of sodium bicarbonate (NaHCO₃) has been changed from 2.2 g to 1.1 g.

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- **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 the 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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