

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

SAM 323

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

Date: August, 25, 2023

Number: SAM 323.06

Supersedes: SAM 323.05, November 20, 2014

Standard Requirement: 9 CFR, part 113.306

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

Table of Contents

- 1. Introduction**
- 2. Materials**
 - 2.1 Equipment/instrumentation**
 - 2.2 Reagents/supplies**
- 3. Preparation for the Test**
 - 3.1 Personnel qualifications/training**
 - 3.2 Preparation of equipment/instrumentation**
 - 3.3 Preparation of reagents/control procedures**
 - 3.4 Preparation of the sample**
- 4. Performance of the Test**
- 5. Interpretation of the Test Results**
- 6. Report of Test Results**
- 7. References**
- 8. Summary of Revisions**

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying the viral content of modified-live canine distemper virus (CDV) vaccines. This test is applicable to CDV vaccines when the master seed virus (MSV) has been adapted to African green monkey kidney (Vero) cells and the MSV produces cytopathic effect (CPE).

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 36°± 2°C, high humidity, 5% ± 1% CO₂ incubator (Model 3336, Forma Scientific Co.)

2.1.2 36°± 2°C water bath

2.1.3 Inverted light microscope (Model CK, Olympus America Inc.)

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

2.1.5 Self-refilling repetitive syringe, 2-mL

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

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 CDV Positive Control, Onderstepoort strain

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

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

2.2.3 Vero cells 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
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. Add to the solution in **Step 3** with constant mixing.
5. Q.S. to 1000 mL with DI; 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 mL)
3. 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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Supplemental Assay Method for Titration of Canine Distemper Virus in Vero Cell Culture

4. 0.1 g magnesium chloride, hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)
5. Dissolve reagents 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, then 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 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 $2^\circ - 7^\circ\text{C}$.

2.2.7 Polystyrene tubes, 12 x 75-mm

2.2.8 Pipettes, 10-mL

2.2.9 Reagent reservoir

2.2.10 Syringe (tuberculin slip tip), 1-mL

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

2.2.12 Cell culture plates, 96-well

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must 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 inoculation, set a water bath at $36^\circ \pm 2^\circ\text{C}$.

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

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of Vero cell culture plates (Vero Plates)

1. Cells are prepared from healthy, confluent Vero cell cultures. On the day of test initiation, using a 12-channel micropipettor and reagent reservoir, seed 100 μL /well in all wells of the 96-well cell culture plates with Vero cells suspended in Growth Medium at a density of approximately $10^{4.7}$ to $10^{5.0}$ cells/mL. Prepare 1 Vero Plate for the controls and 3 Test Vaccine. Each additional Vero Plate allows testing of 4 additional Test Vaccines.

2. Use the Vero Plates within 4 hours.

3.3.2 Preparation of CDV Positive Control

1. On the day of test initiation, rapidly thaw a vial of CDV Positive Control in a $36^{\circ}\pm 2^{\circ}\text{C}$ water bath.

2. Dispense 1.8 mL of 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., 5 tubes, labeled 10^{-1} through 10^{-5} , respectively).

3. With a 200- μL pipettor, transfer 200 μL of the CDV 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 of the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

3.4 Preparation of the sample

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

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

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

1. Prepare 1:2 dilutions of each neutralizing non-CDV antiserum by mixing equal volumes of antiserum and DPBS.
2. Dispense 200 μL of each of the required neutralizing antisera 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 CPI and CAV components in a 4-fraction CDV/CPV/CPI/CAV vaccine, dispense 200 μL of each of the diluted CPI 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 labeled tube to yield a 10^{-1} dilution; mix by vortexing.
4. Incubate at room temperature for 30 ± 5 minutes.

3.4.3 For Test Vaccines not containing CPI or CAV, the 10^{-1} dilution is prepared by adding 200 μL 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 of MEM into each of 4, 12 x 75-mm polystyrene tubes, labeled 10^{-2} through 10^{-5} .
2. Using a new pipette tip, transfer 200 μL from the tube labeled 10^{-1} to the 10^{-2} tube; mix by vortexing.
3. Repeat **Step 2** by transferring 200 μL from the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

4. Performance of the Test

4.1 Label the Vero Plates and inoculate each of 5 wells/dilution with 100 μL of dilutions 10^{-5} through 10^{-2} of the Test Vaccine. In a similar manner, inoculate 5 wells/dilution of the CDV Positive Control (with dilutions 10^{-5} through 10^{-2} from **Section 3.3.2**). Change tips between each unique sample (i.e., each Test Vaccine and the

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

CDV 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^{-5} through 10^{-2}).

4.2 Five uninoculated wells serve as negative cell controls.

4.3 Incubate Vero Plates in a $36^{\circ}\pm 2^{\circ}\text{C}$ CO_2 incubator for 7 days \pm 1 day.

4.4 After incubation, read the Vero Plate at 100X magnification on an inverted light microscope and examine cells for CDV CPE.

4.4.1 Wells displaying 1 or more CPE foci, characterized by cell fusion and lysis, are considered to be positive for CDV.

4.4.2 Results are recorded as the number of CPE positive wells versus total number of wells examined for each dilution of the Test Vaccine and the CDV Positive Control.

4.5 Calculate the 50% tissue culture infective dose (TCID_{50}) of the Test Vaccine and the CDV Working Positive Control using the method of Spearman-Kärber as modified by Finney.

Example:

10^{-3} dilution of Test Vaccine = 5/5 wells CPE positive

10^{-4} dilution of Test Vaccine = 4/5 wells CPE positive

10^{-5} dilution of Test Vaccine = 2/5 wells CPE positive

10^{-6} dilution of Test Vaccine = 0/5 wells CPE positive

Spearman-Kärber formula:

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

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

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

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

$$\frac{5}{5} + \frac{4}{5} + \frac{2}{5} + \frac{0}{5} = \frac{11}{5} = 2.2$$

Test Vaccine titer = $(3 - 1/2) + (1 \bullet 2.2) = 4.7$

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

Adjust the titer to the recommended 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 test CDV vaccine, the recommended dose is 1 mL)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this CDV test vaccine, the inoculation dose is 0.1 mL)

$$\frac{1 \text{ mL dose}}{0.1 \text{ mL}} = 10$$

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

$$\text{Log of } 10 = 1.0$$

$$\text{Test Vaccine titer} = 4.7 + 1.0 = 5.7$$

Therefore the titer of the **CDV Test Vaccine** is $10^{5.7}$ TCID₅₀/mL.

5. Interpretation of the Test Results

Valid Assay

5.1 The calculated titer of the CDV 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.

5.2 The lowest inoculated dilution of the CDV Positive Control must induce CPE in 100% of the wells (5/5). If an endpoint is not reached (1 or more wells are CPE positive 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 CPE (0/5).

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

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

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

5.5 If the validity requirements are met and 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**.

5.6 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Vaccine is retested according to 9 CFR 113.8.

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 113.306, 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 methods in biological assay*. Griffin, London. 3rd edition, 1978, pp. 394-401.

8. Summary of Revisions

Version .06

- The coversheet and contact information has 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.
- **3.3.2:** The temperature of the water bath has been added.

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

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:

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