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Testing Protocol

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Supplemental Assay Method for Titration of Canine Adenovirus in Canine  
Kidney Cell Culture

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**Supplemental Assay Method for Titration of Canine Adenovirus in Canine Kidney Cell Culture**

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**Supplemental Assay Method for Titration of Canine Adenovirus in Canine Kidney Cell Culture**

**1. Introduction**

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live canine adenovirus (CAV) vaccines for viral content. CAV endpoint is determined by viral cytopathic effect (CPE) in a Madin-Darby canine kidney (MDCK) cell line.

**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,  $5\% \pm 1\%$   $\text{CO}_2$  (Model 3336, Forma Scientific Inc.)

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

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

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

**2.1.5** Syringe, self-refilling, repetitive, 2-mL

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

**2.1.7** Micropipettor, 300- $\mu\text{L}$  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** CAV Positive Control: Mirandola strain of CAV Type 1 or Manhattan strain of CAV Type 2

**2.2.2** Monospecific antisera, free of CAV antibody, that neutralize the non-CAV 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** MDCK 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<sub>3</sub>)
3. Dissolve with 900 mL deionized water (DI).
4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DI, heat to 60°± 2°C until dissolved, and 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 µg/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)
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<sub>2</sub>PO<sub>4</sub>)

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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 ( $\text{Na}_2\text{HPO}_4$ ) to 10 mL DI, 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 ( $\text{CaCl}_2$ ) 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- $\mu\text{m}$  filter.
10. Store at  $2^\circ - 7^\circ\text{C}$ .

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 the 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 MDCK cell culture plates (MDCK Plates)

Cells are prepared from healthy, confluent MDCK cells. On the day of test initiation, using a 12-channel micropipettor, add 200  $\mu\text{L}$ /well of  $10^{4.7}$  to  $10^{5.2}$  cells/mL suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 MDCK Plate for the controls and the first Test Vaccine. Each additional MDCK Plate allows testing of 3 additional Test Vaccines. Incubate at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator and use within 4 hours.

#### 3.3.2 Preparation of CAV Positive Control

1. On the day of test initiation, rapidly thaw a vial of CAV Positive Control in the 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, and label (for example: 8 tubes labeled  $10^{-1}$  through  $10^{-8}$ , respectively).
3. With a 200- $\mu\text{L}$  pipettor, transfer 200  $\mu\text{L}$  of the CAV Positive Control to the first tube labeled  $10^{-1}$ ; mix by vortexing.
4. Using a new pipette tip, transfer 200  $\mu\text{L}$  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  $\mu\text{L}$  from 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 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-mL-dose vaccine, 0.5 mL for 1/2-mL-dose vaccines, etc., into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for  $15 \pm 5$  minutes at room temperature.

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

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1. Prepare a dilution of each neutralizing non-CAV antiserum in DPBS according to the CVB Reagent Data Sheet or as determined for that specific antiserum.
2. Dispense 200  $\mu\text{L}$  of each of the required neutralizing antiserum into a 12 x 75-mm polystyrene tube labeled  $10^{-1}$  and q.s. to 1.8 mL with MEM. For example, to neutralize 3 non-CAV viral components of a CDV/CAV/CPI/CPV vaccine, dispense 200  $\mu\text{L}$  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.
3. Pipette 200  $\mu\text{L}$  of the reconstituted Test Vaccine to the labeled tube to yield a  $10^{-1}$  dilution; mix by vortexing.
4. Incubate at room temperature for  $30 \pm 5$  minutes.

**3.4.3** For vaccines containing only the CAV fraction, the  $10^{-1}$  dilution is prepared by adding 200  $\mu\text{L}$  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. Using a 2-mL self-refilling repetitive syringe, dispense 1.8 mL MEM into each of 4, 12 x 75-mm polystyrene tubes labeled  $10^{-2}$  through  $10^{-5}$  (or more if the expected CAV endpoint of the Test Vaccine is higher than  $10^{-5}$ ).
2. Using a new pipette tip, transfer 200  $\mu\text{L}$  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  $\mu\text{L}$  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 MDCK Plates and inoculate each of 8 wells/dilution with 25  $\mu\text{L}$  of the Test Vaccine, starting with the highest dilution (most dilute). In a similar manner, inoculate 8 wells/dilution of the CAV Positive Control (with 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 CAV 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  $10^{-5}$ ). This becomes the Test Plate. Additional Test Vaccines

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may be inoculated onto other MDCK Plates in a similar manner, 3 Test Vaccines per Test Plate.

**4.2** Eight uninoculated wells on the initial Test Plate serve as a Negative Cell Control.

**4.3** Incubate the Test Plates in a  $36^{\circ} \pm 2^{\circ}\text{C}$   $\text{CO}_2$  incubator for 11 days  $\pm$  1 day.

**4.4** After incubation, read the Test Plate at 100X or 200X magnification on an inverted light microscope and examine cells for CPE. CAV CPE is characterized by cell rounding and lysis.

**4.4.1** Wells displaying 1 or more areas of CPE are considered to be positive for CAV.

**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 CAV Positive Control.

**4.5** Calculate the CAV endpoints of the Test Vaccine and the CAV Positive Control using the method of Spearman-Kärber as modified by Finney. The titers are expressed as  $\log_{10}$ , 50% tissue culture infective doses ( $\text{TCID}_{50}$ ).

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 =  $(x - d/2 + [d \bullet S])$ , where:**

**X** =  $\log_{10}$  of highest dilution with all wells CPE positive (2)

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

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

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

**Test Vaccine titer =  $(2 - 1/2) + (1 \bullet 1.75) = 3.25$**

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

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

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

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

$$\frac{1 \text{ mL dose}}{0.025 \text{ mL}} = 40$$

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

$$\text{Log of } 40 = 1.6$$

$$\text{Test Vaccine Titer} = 3.25 + 1.6 = 4.85$$

Therefore the titer of the **CAV Test Vaccine** is  $10^{4.85}$  TCID<sub>50</sub>/mL.

**5. Interpretation of the Test Results**

**5.1 Valid assay**

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

**5.1.2** The lowest inoculated dilution of the CAV Positive Control must induce CPE in 100% of the wells (8/8). 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 (0/8).

**5.1.3** The Uninoculated Cell Control 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.

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**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 required minimum contained in an APHIS filed Outline of Production for the product under test, the Test Vaccine is retested in accordance with 9 CFR, Part 113.8.

## 6. Report of Test Results

Results are reported as TCID<sub>50</sub> 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, 1978. *Statistical method in biological assay*. Griffin, London. 3rd ed., 1978, pp. 394-401.

## 8. Summary of Revisions

### 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.

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**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 ( $\text{NaHCO}_3$ ) 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.
- “Reagent and Reference 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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