

**Center for Veterinary Biologics  
and  
National Veterinary Services Laboratories  
Testing Protocol**

**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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## 1. Introduction

### 1.1 Background

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

### 1.2 Keywords

Canine adenovirus; CAV; CPE; TCID<sub>50</sub>; potency test; titration; *in vitro*

## 2. Materials

### 2.1 Equipment/instrumentation

2.1.1 Incubator,<sup>1</sup> 36° ± 2°C, high humidity, 5% ± 1% CO<sub>2</sub>, meeting the requirements of the current version of GDOCSOP004

2.1.2 Water bath,<sup>2</sup> 36° ± 2°C

2.1.3 Microscope,<sup>3</sup> inverted bright light

2.1.4 Vortex mixer<sup>4</sup>

2.1.5 Syringe,<sup>5</sup> self-refilling, repetitive, 2 ml

2.1.6 Pipettor<sup>6</sup> with tips<sup>7</sup> and/or motorized microliter pipette<sup>8</sup>

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<sup>1</sup> Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750 or equivalent

<sup>2</sup> Cat. No. 66648, Precision Scientific, 3737 West Cortland St., Chicago, IL 60647 or equivalent

<sup>3</sup> Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

<sup>4</sup> Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

<sup>5</sup> Wheaton, Cat. No. 13-689-50C, Fisher Scientific Co., 2000 Park Ln, Pittsburgh, PA 15275 or equivalent

<sup>6</sup> Cat. No. P-200, Rainin Instrument Co., P.O. Box 4026, Mack Rd., Woburn, MA 01801-4628 or equivalent

<sup>7</sup> Cat. No. RT-200, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent

<sup>8</sup> Cat. No. E2-250, Rainin Instrument Co. or equivalent

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2.1.7 Micropipettor, 300  $\mu$ l x 12 channel<sup>9</sup>

2.1.8 Pipette-aid<sup>10</sup>

## 2.2 Reagents/supplies

2.2.1 CAV Reference: Mirandola strain of CAV Type 1 or Manhattan strain of CAV Type 2<sup>11</sup>

2.2.2 Monospecific antisera,<sup>11</sup> 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.

2.2.3 MDCK cell line,<sup>12</sup> free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.4 Minimum essential medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts<sup>13</sup>

2.2.4.2 2.2 g sodium bicarbonate ( $\text{NaHCO}_3$ )<sup>14</sup>

2.2.4.3 Dissolve with 900 ml deionized water (DW).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamin<sup>15</sup> to 10 ml DW, heat to  $60^\circ\text{C} \pm 2^\circ\text{C}$  until dissolved, and add to the solution in **Section 2.2.4.3** with constant mixing.

2.2.4.5 Q.S. to 1000 ml with DW and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).<sup>16</sup>

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<sup>9</sup> Finnpiettes, Cat. No. NV204662D, A. Daigger Co., 199 Carpenter Ave., Wheeling IL 60090 or equivalent

<sup>10</sup> Cat. No. 183, Drummond Scientific Co., 500 Pkwy., Broomall, PA 19008 or equivalent

<sup>11</sup> Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

<sup>12</sup> ATCC CCL 81, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852

<sup>13</sup> Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

<sup>14</sup> Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

<sup>15</sup> Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

<sup>16</sup> Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

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2.2.4.6 Sterilize through a 0.22- $\mu$ m filter.<sup>17</sup>

2.2.4.7 Aseptically add:

1. 25 units/ml penicillin<sup>18</sup>
2. 50  $\mu$ g/ml gentamicin sulfate<sup>19</sup>
3. 100  $\mu$ g/ml streptomycin<sup>20</sup>

2.2.4.8 Store at 4°  $\pm$  2°C.

2.2.5 Growth Medium

2.2.5.1 940 ml MEM

2.2.5.2 Aseptically add:

1. 50 ml gamma-irradiated fetal bovine serum (FBS)
2. 10 ml L-glutamine<sup>21</sup>

2.2.5.3 Store at 4°  $\pm$  2°C.

2.2.6 Dulbecco's phosphate buffered saline (DPBS)

2.2.6.1 8.0 g sodium chloride (NaCl)<sup>22</sup>

2.2.6.2 0.2 g potassium chloride (KCl)<sup>23</sup>

2.2.6.3 0.2 g potassium phosphate, monobasic, anhydrous (KH<sub>2</sub>PO<sub>4</sub>)<sup>24</sup>

2.2.6.4 0.1 g magnesium chloride, hexahydrate (MgCl<sub>2</sub>•6H<sub>2</sub>O)<sup>25</sup>

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<sup>17</sup> Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

<sup>18</sup> Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

<sup>19</sup> Cat. No. 0061-0464-04, Schering Laboratories or equivalent

<sup>20</sup> Cat. No. S-9137, Sigma Chemical Co. or equivalent

<sup>21</sup> 200 mM (100X) liquid, Cat. No. G-7513, Sigma Chemical Co. or equivalent

<sup>22</sup> Cat. No. 3624-01, J.T. Baker, Inc. or equivalent

<sup>23</sup> Cat. No. P217-500, Fisher Scientific Co. or equivalent

<sup>24</sup> Cat. No. 3246-01, J.T. Baker, Inc. or equivalent

<sup>25</sup> Cat. No. M33-500, Fisher Scientific Co. or equivalent

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**2.2.6.5** Dissolve reagents in **Section 2.2.6.1** through **Section 2.2.6.4** with 900 ml DW.

**2.2.6.6** Add 1.03 g sodium phosphate, dibasic anhydrous ( $\text{Na}_2\text{HPO}_4$ )<sup>26</sup> to 10 ml DW, heat to 60° ± 2°C until dissolved. Add to **Section 2.2.6.5** with constant mixing.

**2.2.6.7** Dissolve 0.1 g calcium chloride, anhydrous ( $\text{CaCl}_2$ )<sup>27</sup> with 10 ml DW and add slowly to **Section 2.2.6.5** to avoid precipitation.

**2.2.6.8** Q.S. to 1000 ml with DW; adjust pH to 7.0-7.3 with 2N HCl.

**2.2.6.9** Sterilize through a 0.22-µm filter.

**2.2.6.10** Store at 4° ± 2°C.

**2.2.7** Cell culture plates,<sup>28</sup> 96 well

**2.2.8** Polystyrene tubes,<sup>29</sup> 12 x 75 mm

**2.2.9** Pipettes,<sup>30</sup> 10 ml

**2.2.10** Reagent reservoir<sup>31</sup>

**2.2.11** Syringe,<sup>32</sup> 1 ml tuberculin

**2.2.12** Needles,<sup>33</sup> 18 ga x 1½ in

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<sup>26</sup> Cat. No. 3828-01, J.T. Baker, Inc. or equivalent

<sup>27</sup> Cat. No. 4225-05, J.T. Baker, Inc. or equivalent

<sup>28</sup> Cat. No. 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent

<sup>29</sup> Falcon® 2058, Becton Dickinson Labware, 2 Oak Park, Bedford, MA 01730 or equivalent

<sup>30</sup> Falcon® 7530, Becton Dickinson Labware or equivalent

<sup>31</sup> Cat. No. 4870, Costar Corp. or equivalent

<sup>32</sup> Cat. No. 309602, Becton Dickinson & Co., 1 Becton Dr., Franklin Lakes, NJ 07414-1884 or equivalent

<sup>33</sup> Cat. No. 305196, Becton Dickinson & Co. or equivalent

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### **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  $36^{\circ} \pm 2^{\circ}\text{C}$ .

#### **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 Serial. Each additional MDCK Plate allows testing of 3 additional Test Serials. Incubate at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator and use within 4 hr.

##### **3.3.2 Preparation of CAV Reference Control**

**3.3.2.1** On the day of test initiation, rapidly thaw a vial of CAV Reference in the water bath.

**3.3.2.2** Using the 2-ml self-refilling repetitive syringe, dispense 1.8 ml MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent Sheet; label (for example: 8 tubes labeled  $10^{-1}$  through  $10^{-8}$ , respectively).

**3.3.2.3** With a 200- $\mu\text{l}$  pipettor, transfer 200  $\mu\text{l}$  of the CAV Reference to the first tube labeled  $10^{-1}$ ; mix by vortexing.

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**3.3.2.4** Using a new pipette tip, transfer 200  $\mu$ l from the  $10^{-1}$  labeled tube (**Section 3.3.2.3**) to the  $10^{-2}$  tube; mix by vortexing.

**3.3.2.5** Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200  $\mu$ 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 Serial 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-ga x 1½-in needle, rehydrate a vial of the Test Serial 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 Serial; mix by vortexing. Incubate for  $15 \pm 5$  min at room temperature (RT) ( $23^\circ \pm 2^\circ\text{C}$ ).

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

**3.4.2.1** Prepare a dilution of each neutralizing non-CAV antiserum in DPBS according to the CVB-L Reference and Reagent Sheet or as determined for that specific antiserum.

**3.4.2.2** Dispense 200  $\mu$ 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  $\mu$ 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.

**3.4.2.3** Pipette 200  $\mu$ l of the reconstituted Test Serial to the labeled tube to yield a  $10^{-1}$  dilution; mix by vortexing.

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**3.4.2.4** Incubate at RT for  $30 \pm 5$  min.

**3.4.3** For vaccines containing only the CAV fraction, the  $10^{-1}$  dilution is prepared by adding 200  $\mu$ l of the Test Serial 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

**3.4.4.1** 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 Serial is higher than  $10^{-5}$ ).

**3.4.4.2** Using a new pipette tip, transfer 200  $\mu$ l from the tube labeled  $10^{-1}$  to the next dilution tube labeled  $10^{-2}$ ; mix by vortexing.

**3.4.4.3** Repeat **Section 3.4.4.2** to the remaining tubes, transferring 200  $\mu$ 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  $\mu$ l of the Test Serial, starting with the highest dilution (most dilute). In a similar manner, inoculate 8 wells/dilution of the CAV Reference 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 Serial and the CAV Reference 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 Serials may be inoculated onto other MDCK Plates in a similar manner, 3 Test Serials per Test Plate.

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

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**4.3** Incubate the Test Plate in a  $36^{\circ} \pm 2^{\circ}\text{C}$   $\text{CO}_2$  incubator for  $11 \pm 1$  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 Serial and the CAV Reference Control.

**4.5** Calculate the CAV endpoints of the Test Serial and the CAV Reference 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 Serial = 8/8 wells CPE positive  
 $10^{-3}$  dilution of Test Serial = 5/8 wells CPE positive  
 $10^{-4}$  dilution of Test Serial = 1/8 wells CPE positive  
 $10^{-5}$  dilution of Test Serial = 0/8 wells CPE positive

Spearman-Kärber calculation of total CPE positive wells (14), using 8 wells per dilution = 1.3 log

$\log_{10}$  of reciprocal dilution ( $10^{-2}$ ) = 2.0 log

$\log_{10}$  of reciprocal of dose factor:

$\frac{0.025 \text{ ml inoculum}}{1 \text{ ml dose}} = \frac{1}{40} = 1.6 \text{ log}$

Total = 4.9 log

Titer of the Test Serial is  $10^{4.9}$   $\text{TCID}_{50}$ .

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## 5. Interpretation of the test results

### 5.1 Valid Assay

5.1.1 The calculated titer of the CAV Reference 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 Reference Control must have a 100% positive CPE reaction (8/8), and the highest (most dilute) must exhibit no positive CPE reaction (0/8).

5.1.3 The Negative 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.

5.3 In a valid test, if the titer of the Test Serial 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 Serial is considered **SATISFACTORY**.

5.4 In a valid test, if the titer of the Test Serial is less than the required minimum contained in an APHIS filed Outline of Production for the product under test, the Test Serial may be 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 Serial.

## 7. References

7.1 Code of Federal Regulations, Title 9, Part 113.305, U.S. Government Printing Office, Washington, DC, 1999.

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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, pg. 508.

## 8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.