

Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Determination of the
Specific Viral Antigen Content in Inactivated Canine
Coronavirus Vaccines

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Supplemental Assay Method for Determination of the Specific Viral Antigen Content
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1. Introduction

This is an enzyme-linked immunosorbent assay (ELISA) method for the quantitation of the viral antigen content of inactivated canine coronavirus (CCV) vaccines. The relative potency (RP) of CCV vaccines is determined by comparing the amount of CCV in a Test Serial to the CCV content of a Reference Preparation that has been shown, directly or indirectly, to be protective in a host animal immunogenicity study.

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator, $36^{\circ} \pm 2^{\circ}\text{C}$, $5 \pm 1\%$ CO_2 , high humidity,¹ meeting the requirements of the current version of GDOCSOP0004

2.1.2 Microplate reader²

2.1.3 Microplate washer³

2.1.4 Micropipettors: 200 μl and 500 μl single channel,⁴ 50-200 μl x 12 channel,⁵ and tips⁶

2.1.5 Microtiter plate shaker⁷

2.1.6 RP Calculation Method: Current version of *Supplemental Assay Method for Evaluation by the Relative Potency Method of In Vitro Enzyme Immunoassays Used In Testing of Veterinary Vaccines* (MVSAM0318).⁸ Current version of the U.S. Department of Agriculture, Veterinary Biologics Program's *Relative Potency Calculation Software (RelPot)*.⁸

¹ Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

² Model MRX, Dynex Technologies, Inc., 14340 Sullyfield Circle, Chantilly, VA 20151 or equivalent

³ Model EL404, Bio-Tek Instruments, Inc., Highland Park, Box 998, Winooski, VT 05404-0998 or equivalent

⁴ Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

⁵ Cat. No. 77-705-00, Flow Laboratories, 7655 Old Springhouse Road, McLean, VA 22102 or equivalent

⁶ Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent

⁷ Model 4625, Labline Instruments, Inc., 15 & Bloomingdale Ave., Melrose Park, IL 60160 or equivalent

⁸ Available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010

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2.1.7 Vortex mixer⁹

2.2 Reagents/supplies

2.2.1 0.01 M Phosphate buffered saline (PBS)

2.2.1.1 1.9 g sodium phosphate, dibasic,
anhydrous (Na_2HPO_4)¹⁰

2.2.1.2 0.22 g sodium phosphate, monobasic,
monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)¹¹

2.2.1.3 8.5 g sodium chloride (NaCl)¹²

2.2.1.4 Q.S. to 100 ml with distilled water (DW).

2.2.1.5 Adjust pH to 7.2-7.6 with 0.1 N sodium
hydroxide (NaOH)¹³ or 1.0 N hydrochloric acid
(HCl).¹⁴

2.2.1.6 Sterilize by autoclaving at $121^\circ \pm 2^\circ\text{C}$,
15 psi for 35 ± 5 min.

2.2.1.7 Store at $4^\circ \pm 2^\circ\text{C}$.

2.2.2 0.05 M Carbonate Coating Buffer, pH 9.6

2.2.2.1 0.159 g sodium carbonate (Na_2CO_3)¹⁵

2.2.2.2 0.293 g sodium bicarbonate (NaHCO_3)¹⁶

2.2.2.3 Q.S. to 100 ml with DW.

2.2.2.4 Adjust pH to 9.6 with 2 N HCl .¹⁷

2.2.2.5 Store at $4^\circ \pm 2^\circ\text{C}$; use within 1 wk.

⁹ Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716
or equivalent

¹⁰ Cat. No. S 0876, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

¹¹ Cat. No. S 9638, Sigma Chemical Co. or equivalent

¹² Cat. No. S 9625, Sigma Chemical Co. or equivalent

¹³ Cat. No. S 925-30, Sigma Chemical Co. or equivalent

¹⁴ Cat. No. 920-1, Sigma Chemical Co. or equivalent

¹⁵ Cat. No. S 1641, Sigma Chemical Co. or equivalent

¹⁶ Cat. No. S 6014, Sigma Chemical Co. or equivalent

¹⁷ Cat. No. 251-2, Sigma Chemical Co. or equivalent

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2.2.3 Blocking Solution

2.2.3.1 1 g casein¹⁸ in 100 ml Carbonate Coating Buffer

2.2.3.2 Store at 4° ± 2°C; use within 1 wk.

2.2.4 Wash Buffer

2.2.4.1 500 µl Tween-20¹⁹ in 1000 ml 0.01 M PBS

2.2.4.2 Store at room temperature (RT),
23° ± 2°C.

2.2.5 Diluent Buffer

2.2.5.1 1 g casein²⁰ in 100 ml Wash Buffer

2.2.5.2 Store at 4° ± 2°C; use within 1 wk.

2.2.6 Feline infectious peritonitis virus antibody²¹
(FIPV Ab), ammonium sulfate-precipitated

2.2.7 CCV monoclonal antibody (CCV MAb)²²

2.2.8 Rabbit anti-mouse horseradish peroxidase
conjugate²³ (Rabbit Anti-mouse Conjugate)

2.2.9 (2,2'-azino-di-{3 ethyl-benzthiazaline sulfonate
6}) (ABTS) peroxidase substrate solution²⁴ (Substrate
Solution)

2.2.9.1 Solution A, ABTS

2.2.9.2 Solution B, Hydrogen Peroxide

¹⁸ Cat. No. C 0376, Sigma Chemical Co. or equivalent

¹⁹ Cat. No. 170-6531, BioRad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547 or equivalent

²⁰ Cat. No. C 0376, Sigma Chemical Co. or equivalent

²¹ Feline pleural ascites available upon request from the CVB-L

²² Mouse ascites available upon request from the CVB-L

²³ Cat. No. 61-6520, Zymed Laboratories, 52 S. Linden Ave., Suite 3, So. San Francisco, CA 94080 or equivalent

²⁴ Product Code 50-62-00, Kirkegaard & Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879-4174 or equivalent

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2.2.10 Flat bottom, 96-well ELISA plate²⁵ (ELISA Plate)

2.2.11 Plate sealer²⁶

2.2.12 Reference Preparation. Each manufacturer provides a Reference Preparation that has been directly or indirectly shown to be protective in a host animal immunogenicity trial. The Reference Preparation is the lot number identified in Part V of the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or special outline. All subsequent serials produced by a manufacturer must have an RP equal to or greater than the RP value contained in the APHIS filed Outline of Production.

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training and experience in the immunological basis of antigen capture ELISA assays, the principles of optical densitometry (OD), and computer software analysis.

3.2 Preparation of equipment/instrumentation

The microplate reader must be turned on at least 30 min prior to determination of OD readings. The microplate reader is zeroed on air prior to initial use.

3.3 Preparation of reagents/control procedures

3.3.1 Test Plate preparation. On the day of plate coating, dilute the FIPV Ab, per the Center for Veterinary Biologics-Laboratory (CVB-L) Reference and Reagent Sheet supplied with the reagent, in Carbonate Coating Buffer. Mix by vortexing and pipette 100 µl of diluted FIPV Ab to each well of an ELISA Plate, which becomes the Test Plate. Cover the Test Plate with a plate sealer and incubate 68 ± 52 hr, at $4^0 \pm 2^0$ C.

²⁵ Immulon II®, Cat. No. 011-010-3450, Dynex Technologies, Inc.

²⁶ Cat. No. 001-010-3501, Dynex Technologies, Inc. or equivalent

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3.3.2 Diluted CCV MAb preparation. On the day the Test Plate is read, dilute the CCV MAb, per the CVB-L Reference and Reagent Sheet supplied with the reagent, in Diluent Buffer; mix by vortexing.

3.3.3 Diluted Rabbit Anti-Mouse Conjugate preparation. On the day the Test Plate is read, dilute the Rabbit Anti-mouse Conjugate, per previously determined optimal dilution, in Diluent Buffer; mix by vortexing.

3.3.4 Substrate Solution preparation. On the day the Test Plate is read, just prior to substrate addition, mix equal volumes of ABTS Solution A and Hydrogen Peroxide Solution B, per the manufacturer's instructions. The resulting Substrate Solution must remain clear. The Substrate Solution must be at RT at time of use.

3.4 Preparation of the sample

3.4.1 Antigen Extraction (Optional). If the Test Serial contains an adjuvant which interferes with antigen detection, the firm may specify the procedure for extraction of the antigen from the adjuvant. If extraction is a necessary step, the extraction procedure will be included in Part V of the APHIS filed Outline of Production or special outline. If the Reference Preparation is a product reference, both the Reference Preparation and Test Serial must be treated identically. If the Reference Preparation is a purified reference, the extraction procedure is not required for the Reference Preparation. The CVB-L will extract antigen using the firm's protocol. If no protocol is stated in either the APHIS filed Outline of Production or special outline, the test will be conducted at the CVB-L without extraction.

3.4.2 All samples must be at RT before testing. The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). Twofold dilutions may be made in an additional ELISA Plate, which becomes the Transfer Plate, as follows (tips are changed between each dilution):

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3.4.2.1 Add 150 µl of Diluent Buffer to wells in rows B-H with a 12-channel micropipettor (see **Section 9.1**).

3.4.2.2 The starting dilution for the Reference Preparation and the Test Serial shall be stated in part V of the APHIS filed Outline of Production or special outline. Unless stated otherwise, the diluent will be Diluent Buffer for the initial dilution.

3.4.2.3 Add 300 µl of the starting dilution of the Reference Preparation to wells A1 and A2.

3.4.2.4 Add 300 µl of the starting dilution of the Test Serial to wells A3 and A4. Additional Test Serials may be tested in duplicate in columns 5-12.

3.4.2.5 Transfer 150 µl from row A to row B. Mix row B with the 12-channel micropipettor (7 ± 2 fills).

3.4.2.6 Continue as in **Section 3.4.2.5** for the remaining rows C-G, transferring 150 µl from the previous row to the next row. Note: Row H is not used and remains as Diluent Buffer for transfer to the blank wells.

4. Performance of the test

4.1 Remove the Test Plate prepared earlier from $4^{\circ} \pm 2^{\circ}\text{C}$ storage (see **Section 3.3.4**).

4.2 Decant the Test Plate contents in a suitable container. Fill each well with at least 200 µl of Wash Buffer. Immediately decant Wash Buffer from the Test Plate. Repeat for a total of 4 washes. At no time should wells dry between rinses or incubations. After the last wash, tap the Test Plate on paper towels to remove residual Wash Buffer. An automatic plate washer may be used.

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- 4.3** Add 200 μ l of Blocking Solution to each well of the Test Plate; seal the Test Plate with a plate sealer and incubate for 60 ± 10 min at $36^{\circ} \pm 2^{\circ}\text{C}$.
- 4.4** Wash the Test Plate as in **Section 4.2**.
- 4.5** Transfer 100 μ l of the Test Serial and the Reference Preparation diluted on the Transfer Plate (see **Section 3.4.2**) to corresponding wells of the blocked Test Plate. Tips need not be changed if proceeding from the most dilute to the most concentrated (Row H to Row A). Row H receives all assay reagents except antigen and serves as the blank wells.
- 4.6** Seal the Test Plate with a plate sealer and incubate on the microtiter plate shaker 120 ± 10 min, or overnight (14 ± 2 hr), at $36^{\circ} \pm 2^{\circ}\text{C}$ with sufficient agitation to keep the test samples in suspension. The incubation time shall be stated in Part V of the APHIS filed Outline of Production or special outline. If the incubation time is not specified, incubation will be overnight.
- 4.7** Wash the Test Plate as in **Section 4.2**.
- 4.8** Pipette 100 μ l/well of Diluted CCV MAb to each well of the Test Plate; seal the Test Plate with a plate sealer and incubate for 60 ± 10 min at $36^{\circ} \pm 2^{\circ}\text{C}$.
- 4.9** Wash Test Plate as in **Section 4.2**.
- 4.10** Pipette 100 μ l of Diluted Rabbit Anti-mouse Conjugate to each well of the Test Plate; seal the Test Plate with a plate sealer and incubate for 60 ± 10 min at $36^{\circ} \pm 2^{\circ}\text{C}$.
- 4.11** Wash the Test Plate as in **Section 4.2**.
- 4.12** Pipette 100 μ l of Substrate Solution to each well of the Test Plate; incubate the Test Plate at RT.
- 4.13** Read the Test Plate at 405-nm test wavelength against a 490-nm reference wavelength on the microplate reader when the color development gives a sufficient OD reading in at least the fourth dilution of the Reference Preparation ($\text{OD} \geq 0.05$ after the average blank reading is subtracted).

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4.14 Determine the arithmetic mean of at least 3 wells of the blank wells in Row H; this becomes the Average Blank Reading. The Average Blank Reading is subtracted from all readings before analysis of the data by *RelPot*.

4.15 Evaluate the data using *RelPot*.

5. Interpretation of the test results

5.1 All validity criteria in the current version of MVSAM0318 must be met for a valid test. An invalid test may be repeated. Testing may be repeated for equivocal tests as defined in 9 CFR 113.8(c)(4).

5.2 For a Test Serial to be satisfactory, the RP value of at least 1 valid RP from the group of the highest scoring valid RP values has to be greater than or equal to the RP stated in an APHIS filed Outline of Production.

5.3 For a Test Serial less than the RP stated in an APHIS filed Outline of Production, the test may be repeated when the test meets the criteria defined in 9 CFR 113.8(c)(5) (see MVSAM0318, Test Results and Interpretation).

6. Report of test results

Record RP results on the test record.

7. References

7.1 Horsburgh BC, Brown TDK. Sequence analysis of CCV and its relationship to FIPV, TGEV and PRCV. In: *Coronaviruses*, Laude H, Vautherot J, eds., pp 3-9. Plenum Press, New York, 1994.

7.2 Katz JB, Hanson SK, Patterson PA, Stoll IR. *In vitro* assessment of viral antigen content in inactivated aluminum hydroxide adjuvanted vaccines. *J Vir Meth* 1989, 25:101-108.

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8. Summary of revisions

MVSAM0322.01: 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. The following is a listing of the significant changes made from the superseded protocol:

8.1 Change in the Wash Buffer from 0.3% Tween-20 to 0.05% Tween-20.

8.2 Change in incubation time for the Test Serial to allow for flexibility in testing.

8.3 Minor changes in format and wording to add clarity.

9. Appendix

9.1 Transfer and Test Plate Format

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	REF	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B												
C												
D												
E												
F												
G												
H	BLK											

REF= Reference Preparation; TS= Test Serial; BLK= Blank