

Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Feline
Calicivirus in Cell Culture

Date: March 6, 2001
Supersedes: December 16, 1998
Number: MVSAM0306.02
Standard Requirement: 9 CFR, Part 113.314
Contact Person: Sally A. Hanna, (515) 663-7337
Marsha J. Hegland, (515) 663-7659

Approvals:

_____ Date: _____
Linn A. Wilbur, Head/Team Leader
Mammalian Virology Section

_____ Date: _____
Ann L. Wieggers, Quality Assurance Manager

/s/ Randall Levings _____ Date: 3/23/01__
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell
Culture

Table of Contents

1. Introduction
 - 1.1 Background
 - 1.2 Keywords
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

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* titration method for assaying modified-live feline calicivirus (FCV) vaccines for viral content. The method uses plaque forming units (PFU) in a cell culture system for titration of FCV.

1.2 Keywords

Feline calicivirus, FCV, potency test, titration, PFU, titration, *in vitro*, plaque

2. Materials

2.1 Equipment/instrumentation

2.1.1 Syringe,¹ self-refilling, repetitive, 2 ml

2.1.2 Micropipettor,² 200 µl, and tips³

2.1.3 Blender⁴

2.1.4 Media bottle,⁵ borosilicate glass with screw-top lid, 1000 ml

2.1.5 Incubator,⁶ 36° ± 2°C, high humidity, 5% ± 1% CO₂

2.1.6 Water bath⁷

2.1.7 Inverted light microscope⁸

¹ Wheaton® 13-689-50C, Fisher Scientific Corp., 2000 Park Ln., Pittsburg, PA 15275 or equivalent

² Pipetman® Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

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

⁴ Waring blender, Cat. No. 14-509-35, Fisher Scientific Corp. or equivalent

⁵ Wheaton® 219760, Fisher Scientific Corp. or equivalent

⁶ Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

⁷ Cat. No. 15-461-10, Fisher Scientific Corp. or equivalent

⁸ Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11717-3157 or equivalent

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

2.1.8 Vortex mixer⁹

2.1.9 Pipette-aid¹⁰

2.2 Reagents/supplies

2.2.1 FCV Reference,¹¹ C-14 strain

2.2.2 Crandell feline kidney (CRFK) cell culture,¹² free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Minimum Essential Medium (MEM)

2.2.3.1 9.61 g MEM with Earle's salts without bicarbonate¹³

2.2.3.2 2.2 g sodium bicarbonate (NaHCO₃)¹⁴

2.2.3.3 Dissolve with 900 ml deionized water (DW).

2.2.3.4 Add 5.0 g lactalbumin hydrolysate or edamin¹⁵ to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.3.3** with constant mixing.

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).¹⁶

2.2.3.6 Sterilize through a 0.22-µm filter.¹⁷

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

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

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

¹² CCL-94, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

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

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

¹⁵ Edamin, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

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

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

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

2.2.3.7 Aseptically add:

1. 25 units/ml penicillin¹⁸
2. 50 µg/ml gentamicin sulfate¹⁹
3. 100 µg/ml streptomycin²⁰

2.2.3.8 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

2.2.4.2 Aseptically add:

1. 100 ml gamma-irradiated fetal bovine serum (FBS)
2. 10 ml L-glutamine²¹

2.2.4.3 Store at 4° ± 2°C.

2.2.5 2X Medium

2.2.5.1 19.22 g MEM with Earle's salts without bicarbonate

2.2.5.2 2.2 g NaHCO₃

2.2.5.3 Dissolve with 900 ml DW.

2.2.5.4 Add 5.0 g lactalbumin hydrolysate or edamin to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.5.3** with constant mixing.

2.2.5.5 Q.S. to 1000 ml with DW and adjust pH to 6.8-6.9 with 2N HCl.

2.2.5.6 Sterilize through a 0.22-µm filter.

¹⁸Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth,

NJ 07033 or equivalent

¹⁹Cat. No. 0061-0464-04, Schering Laboratories or equivalent

²⁰Cat. No. S-9137, Sigma Chemical Co. or equivalent

²¹L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

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

2.2.5.8 Prior to preparing the Overlay Medium, aseptically add:

1. 25 units/ml penicillin
2. 50 $\mu\text{g/ml}$ gentamicin sulfate
3. 100 $\mu\text{g/ml}$ streptomycin

2.2.6 2% Tragacanth Gum (Trag)

2.2.6.1 20 g Trag²²

2.2.6.2 1000 ml DW

2.2.6.3 Mix small amounts of Trag at a time vigorously, with a blender set on high.

2.2.6.4 Pour 500 ml each into 1000-ml media bottles.

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

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

2.2.7 7.5% Sodium Bicarbonate

2.2.7.1 7.5 g NaHCO_3

2.2.7.2 Q.S. to 100 ml with DW.

2.2.7.3 Sterilize by autoclaving at 15 psi, $121^{\circ} \pm 2^{\circ}\text{C}$ for 30 ± 10 min.

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

2.2.8 70% Ethyl Alcohol

2.2.8.1 73 ml ethyl alcohol²³

2.2.8.2 27 ml DW

²²Acros AC42138-5000, Fisher Scientific Corp. or equivalent

²³Denatured, 190 proof, Cat. No. 7018, J.T. Baker, Inc. or equivalent

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

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

2.2.9 Crystal Violet Stain

2.2.9.1 7.5 g crystal violet²⁴

2.2.9.2 50 ml 70% Ethyl Alcohol

2.2.9.3 Dissolve crystal violet in alcohol; add remaining ingredients.

2.2.9.4 250 ml formaldehyde²⁵

2.2.9.5 Q.S. to 1000 ml with DW.

2.2.9.6 Filter through Whatman® #1 filter paper.²⁶

2.2.9.7 Store at RT.

2.2.10 Tissue culture plates,²⁷ 4 well

2.2.11 Polystyrene tubes,²⁸ 12 x 75 mm

2.2.12 Pipettes,²⁹ 25 ml

2.2.13 Needles,³⁰ 18 ga x 1½ in

2.2.14 Syringes (tuberculin),³¹ 1 ml

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

²⁴Cat. No. C 0775, Sigma Chemical Co. or equivalent

²⁵37% by weight, Cat. No. F79, Fisher Scientific Corp. or equivalent

²⁶Cat. No. 1001, Fisher Scientific Corp. or equivalent

²⁷Cat. No. 7603705, ICN Biochemicals, Inc., 3300 Hyland Ave., Costa Mesa, CA 92626 or equivalent

²⁸Falcon® 2058, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent

²⁹Cat. No. 13-675-30, Fisher Scientific Corp. or equivalent

³⁰Cat. No. 305196, Becton Dickinson Labware or equivalent

³¹Cat. No. 309602, Becton Dickinson Labware or equivalent

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

and maintenance of animal viruses and the quantitation of virus infectivity by PFU.

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 CRFK tissue culture plates (Test Plates)

Cells are prepared from healthy, confluent CRFK cell cultures that are maintained by passing every 3 to 4 days. Two days prior to test initiation, add 8.0 ml/well of approximately $10^{5.0}$ to $10^{5.3}$ cells/ml suspended in Growth Medium into all wells of a 4-well tissue culture plate. Prepare 1 Test Plate for the FCV Reference Control and 1 for each Test Serial. Incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator.

3.3.2 Preparation of FCV Reference Control

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

3.3.2.2 Using a self-refilling, repetitive syringe, dispense 1.8 ml of MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB-L Reference and Reagent sheet; appropriately label (e.g., 9 tubes, labeled 10^{-1} through 10^{-9} , respectively).

3.3.2.3 Transfer 200 μl of the FCV Reference to the tube labeled 10^{-1} ; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 μl from the 10^{-1} -labeled tube 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 μl of the previous dilution to the next dilution tube until the ten-fold dilution series is completed.

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

3.3.3 On the day of test initiation, prepare Overlay Medium. Approximately 35 ml per plate is required.

3.3.3.1 Aseptically add to a volume of 2X Medium:

1. 10% gamma-irradiated FBS
2. 2% 7.5% Sodium Bicarbonate
3. 1 volume of 2% Trag

3.3.3.2 Mix and warm the Overlay Medium in the water bath for 60 ± 10 min prior to performing the procedure in **Section 4.5**.

3.4 Preparation of the sample

3.4.1 The initial test will be with a single vial (a single sample from 1 vial). On the day of test initiation, using a 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 a ½-ml-dose vaccine, etc., into the vial containing the lyophilized Test Serial; mix by vortexing. Incubate for 15 ± 5 min at RT.

3.4.2 Dispense 1.8 ml MEM into each of 6, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6} , using a 2-ml self-refilling, repetitive syringe.

3.4.3 Transfer 200 µl of the Test Serial to the tube labeled 10^{-1} ; mix by vortexing.

3.4.4 Using a new pipette tip, transfer 200 µl from the tube labeled 10^{-1} to the 10^{-2} tube; mix by vortexing.

3.4.5 Repeat **Section 3.4.4** 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.

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

4. Performance of the test

4.1 On the day of test initiation, label the Test Plates and remove the Growth Medium.

4.2 Inoculate 1 well/dilution with 200 μ l from dilutions 10^{-6} through 10^{-3} of the Test Serial, starting with the highest dilution (most dilute). Inoculate 1 well/dilution from dilutions 10^{-9} through 10^{-7} of the FCV Reference Control. Change tips between each unique sample (i.e., each Test Serial), but tip changes are not necessary between each dilution in a series when pipetting from the most dilute to the most concentrated (e.g., 10^{-9} through 10^{-7}). Gently rotate the plates to evenly disperse the inoculum.

4.3 One uninoculated well on a Test Plate serves as a negative cell control.

4.4 Incubate the inoculated Test Plates at $36^{\circ} \pm 2^{\circ}\text{C}$ for 75 ± 15 min in a CO_2 incubator. To evenly disperse the inoculum, gently rotate the plates at intervals of 25 ± 5 min.

4.5 Add 8 ml/well of the Overlay Medium to the plates with a 25-ml pipette. Discard any unused, warmed Overlay Medium.

4.6 Incubate the Test Plates undisturbed at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator for 96 ± 12 hr.

4.7 After incubation, without removing the Overlay Medium, pipette 5 ml of the Crystal Violet Stain (**Section 2.2.9**) into each well of the Test Plates with a 25-ml pipette; mix by gentle rotation.

4.8 Allow Test Plates to incubate at RT 25 ± 5 min.

4.9 Wash the Overlay Medium and the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container overflowing with running water from the cold water tap until the water is clear. Allow to air dry.

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

4.10 Count the number of FCV plaques for each well. If FRV and FCV plaques are counted together in a combination vaccine, the FCV plaques will contrast markedly from FRV. The FRV plaques are visible as small, clear plaques approximately 1 mm in diameter with distinct edges. The FCV plaques are large, clear circular areas (averaging 3 to 4 mm in diameter) with fuzzy edges. If the plaques are not distinguishable by size, use an inverted light microscope with 100X magnification to differentiate the plaque edges.

4.11 Record results as the number of FCV plaques for each dilution of a Test Serial and the FCV Reference Control.

4.12 Calculate the FCV titers of the Test Serial and the FCV Reference Control from the dilutions with plaque counts containing 10-100 plaques. The titer may be expressed as PFU per dose of vaccine.

Example:

$$\begin{aligned} \text{Log}_{10} \text{ of PFU (65)} &= 1.8 \\ \text{Log}_{10} \text{ of reciprocal of dilution counted (10}^{-3}\text{)} &= 3.0 \\ \text{Log}_{10} \text{ of reciprocal of dose factor} &= 3.0 \\ \frac{200 \text{ } \mu\text{l inoculum}}{1 \text{ ml-dose}} = \frac{1}{5} &= 0.7 \end{aligned}$$

$$\text{Total} = 5.5$$

Titer of the Test Serial is $10^{5.5}$ PFU.

4.13 One plaque represents a single infective unit (IU), whereas 1, 50% tissue culture infective dose (TCID₅₀) is statistically equivalent to a theoretical 0.69 IU. A TCID₅₀ endpoint will be 1.44 times those expressed as PFU per unit of inoculation. Therefore, to express PFU titer as TCID₅₀ titer, multiply the PFU titer by 1.44 or add 0.16 (the log of 1.44) to the log₁₀ value of the PFU titer. From the above example, the titer of the Test Serial is $10^{5.66}$ TCID₅₀.

5. Interpretation of the test results

5.1 Validity requirements

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

5.1.1 The calculated titer of the FRV Reference 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.

5.1.2 The uninoculated cell control cannot exhibit any plaques, cytopathic effects (CPE), or cloudy media that would indicate any contamination.

5.1.3 The Test Serial and the FCV Reference Control must have dilutions in which 10 to 100 PFU are counted.

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 If the validity requirements are met and 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 (OP) 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 titer contained in the APHIS filed Outline of Production for the product under test, the Test Serial may be retested in accordance with the Code of Federal Regulations, Title 9, Part 113.8.b.

6. Report of test results

Report results as PFU or TCID₅₀ per dose of Test Serial.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.314, U.S. Government Printing Office, Washington, DC, 2000.

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

Supplemental Assay Method for the Titration of Feline Calicivirus in Cell Culture

7.3 Davis, Dulbecco, Eisen, Ginsberg, ed. *Microbiology including Immunology and Molecular Genetics*, 3rd ed. Harper and Row, Hagerstown, 1980, pg. 880.

8. Summary of revisions

8.1 MVSAM0306.01:

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. No significant changes were made from the previous protocol.

8.2 MVSAM0306.02:

The following is a list of the changes made from the previous protocol:

- 1.** Changes have been made to the 2X Medium and Overlay Medium formulations.
- 2.** 7.5% Sodium Bicarbonate has been included for preparation of the Overlay Medium.
- 3.** A method was added to convert data from PFU to TCID₅₀.
- 4.** Minor changes in terminology have been made and additional details have been added for clarification and consistency with other SAMs.