United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 319

Supplemental Assay Method for Titration of *Chlamydophila felis* (formerly Feline *Chlamydia psittaci*) in Cell Culture

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Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) describes a titration method for assaying live cell culture-adapted *Chlamydophila felis* (formerly feline *Chlamydia psittaci*) vaccines for potency. The method uses a McCoy cell line as the test system. The presence or absence of the chlamydophila agent is determined by staining inoculated cell cultures by an indirect fluorescent antibody (IFA) method using a monoclonal antibody (MAb) specific for *C. felis*.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Cabinet, laboratory biosafety level-2

2.1.2 Incubator, 36°± 2°C, high humidity, 5% ± 1% CO_2 (Model 3336, Forma Scientific Inc.)

2.1.3 Incubator, 36°± 2°C, aerobic (FA Model 2, Precision Scientific)

2.1.4 Water bath

2.1.5 Centrifuge, rotor, and rotor/microplate carriers (Avanti J-E and JS–5.3 rotor, Beckman Coulter)

2.1.6 Microscope, ultraviolet (UV) light (Model BH2, Olympus America Inc.)

2.1.7 Pipettors

2.1.8 Multichannel pipettor, 50-300 µL x 12-channel

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

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 Chicken-embryo-adapted or cell-culture-adapted Chlamydophila positive control, Cello Strain

2.2.2 Monospecific Antisera free of neutralizing antibodies to nonchlamydophila virus fractions present in multifraction Test Vaccines, e.g., feline panleukopenia virus, feline calicivirus, feline viral rhinotracheitis virus.

2.2.3 "*C. psittaci"* MAb, FP 4E1FD7, feline specific

2.2.4 Anti-mouse fluorescein isothiocyanate conjugate (Anti-mouse FITC conjugate)

2.2.5 A McCoy cell line (McCoy B cell line, mouse fibroblast) free of extraneous agents as tested in accordance with title 9, *Code of Federal Regulations* (9 CFR)

2.2.6 Minimum essential medium (MEM)

1. 9.61 g MEM with Earles salts without bicarbonate

2. 5.0 g lactalbumin hydrolysate or edamine

3. 1.1 g sodium bicarbonate ($NaHCO_3$)

4. Q.S. to 1000 mL with deionized water (DI), and adjust the pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

5. Filter through a 0.22-µm filter.

6. Adjust the pH of the solution with 2N HCl to 7.1-7.2 following filtration.

7. Store at 2°- 7°C.

2.2.7 Growth medium for cell culture (Growth Medium)

Aseptically add to 900 mL MEM:

1. 100 mL gamma-irradiated fetal bovine serum (FBS)

2. Gentamicin sulfate, 50 µg/mL

3. Store at 2°- 7°C.

2.2.8 Chlamydophila Stock Solution

1. 13.37 g Dulbecco's MEM with high glucose

2. 3.7 g sodium bicarbonate

3. Q.S. to 1000 mL with DI; adjust the pH to 6.8-6.9 with 2N HCl.

4. Sterilize through a 0.22-µm filter.

5. Aseptically add 50 µg/mL gentamicin sulfate; adjust the final pH of the solution to 7.0-7.2 with 2N HCl.

6. Store at 2°- 7°C.

2.2.9 7.5% Sodium Bicarbonate

1. 7.5 g sodium bicarbonate

2. Q.S. to 100 mL with DI.

3. Sterilize by autoclaving at 121°± 2°C, 15 psi for 30 ± 10 minutes.

4. Store at 2°- 7°C**.**

2.2.10 Maintenance Medium

Aseptically add:

1. 10.0 mL sterile tryptose phosphate broth[24] (29.5 g/L in DI)

2. 2.0 mL 1M HEPES buffer

3. 4.0 mL gamma-irradiated FBS

4. 0.3 mL cycloheximide stock solution (1000 µg/mL in DI) for a final concentration of 3 µg/mL

5. Q.S. to 100 mL with Chlamydophila Stock Solution.

6. Adjust pH to 7.0-7.2 with 7.5% Sodium Bicarbonate.

7. Store at 2°- 7°C**.**

2.2.11 Chlamydophila Diluent

To prepare 100 mL Chlamydophila Diluent, add aseptically:

1. 10.0 mL tryptose phosphate broth

2. 2.0 mL 1M HEPES buffer

3. Q.S. to 100 mL with Chlamydophila Stock Solution.

4. Adjust pH to 7.0-7.2 with 7.5% Sodium Bicarbonate.

5. Store at 2°- 7°C**.**

2.2.12 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)

4. 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \bullet 6H_20$)

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°± 2°C until dissolved, then add to above mixture with constant mixing.

7. Dissolve 0.1 g calcium chloride, anhydrous ($CaCl_2$) with 10 mL DI and add slowly to **Step 5** to avoid precipitation.

8. Q.S. to 1000 mL with DI; adjust the pH to 7.0-7.3 with 2N HCl.

9. Sterilize through a 0.22-µm filter.

10. Store at 2°- 7°C.

2.2.13 Cell culture plates, 96-well

2.2.14 Polystyrene tubes, 12 x 75-mm

2.2.15 Syringe, 1-mL tuberculin

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

2.2.17 Acetone, 80%

1. 80 mL acetone

2. 20 mL DI

3. Store at room temperature.

2.2.18 Gauze. Cut into 20- x 15-cm, 4-layer pads; wrap in foil paper; sterilize by autoclaving at 121°± 2°C, 15 psi for 50 ± 10 minutes.

2.2.19 Labeling tape, 1/2-inch

3. Preparation for the Test

3.1 Personnel qualifications/training

3.1.1 Personnel shall have experience in the preparation and maintenance of cell culture, as well as in the propagation and maintenance of chlamydophila agents. Personnel shall be proficient with the quantitation techniques for chlamydophila infectivity including the IFA staining method.

This method requires the propagation of *C. felis* (formerly *C. psittaci*) agents which may be infectious for humans. All cultures and discarded materials should be considered potentially infective and handled in a manner consistent with safe laboratory practices and in accordance with the recommendations of the Centers for Disease Control and Prevention and the National Institutes of Health. All cultures, spent medium, and contaminated materials should be disinfected or autoclaved before discarding. The use of appropriate Laboratory Biosafety Level-2 cabinets is recommended for these procedures.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of cell seeding and then on the day of cell inoculation, set a water bath at 36°± 2°C and the centrifuge temperature at 36°± 2°C.

3.2.2 On the day of the IFA staining procedure, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DI.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of McCoy cell culture plates (McCoy Plates)

1. Prepare cells from healthy, confluent McCoy cells that are maintained by passing every 3 to 4 days. Using a multichannel pipettor, seed all wells of a 96-well cell culture plate 2 days prior to test initiation with 200 µL/well of cells suspended in growth media. The cell suspension should contain approximately $10^{5.39}$ to $10^{5.54}$ cells per mL. These become the McCoy Plates.

2. Incubate the McCoy Plates in a CO_2 incubator at 36°± 2°C for 2 days prior to inoculation. Only confluent cell monolayers should be used for the assay. (As illustrated in **Appendix I.** Avoid the use of outside wells if cell monolayers in those wells are not confluent.)

3.3.2 Preparation of the *Chlamydophila felis* Positive Control

1. On the day of test initiation, rapidly thaw a vial of Chlamydophila Positive Control in a 36°± 2°C water bath.

2. Dispense 1.8 mL Chlamydophila Diluent into an appropriate number of 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the Center for Veterinary Biologics (CVB) Reagent Data Sheet and label (e.g., 7 tubes, labeled 10^{-1} through 10^{-7}, respectively).

3. With a 200-µL pipettor, transfer 200 µL of the Chlamydophila Positive Control to the 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 from the previous dilution tube to the next dilution tube until the dilution sequence is completed.

3.3.3 Preparation of the Working *Chlamydophila felis* MAb

On the day the IFA staining procedure is conducted, dilute the Feline-specific "*C. psittaci"* MAb (*Chlamydophila felis* MAb) with DPBS as stated on the CVB Reagent Data Sheet.

3.3.4 Preparation of the Working Anti-mouse FITC Conjugate

On the day the IFA procedure is conducted, dilute the Anti-mouse FITC Conjugate with DPBS to its previously determined optimal working concentration.

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 by transferring 1.0 mL for 1-mL-dose vaccines, 0.5 mL for 1/2-mL-dose vaccines, etc., of the provided diluent into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 For multifraction feline "*C. psittaci"* vaccines (*C. felis* vaccines), neutralize the nonchlamydophila fractions with antiserum specific to each virus fraction.

1. Prepare dilutions of each neutralizing Monospecific Antiserum (nonchlamydophila) according to the CVB Reagent Data Sheet or the manufacturer's instructions.

2. With a 200-µL pipettor and a different tip, dispense 200 µL of each Monospecific Antiserum into a 12 x 75-mm polystyrene tube, labeled 10^{-1}. For example, to neutralize the 3 viral fractions of a feline *Chlamydophila felis*/FVR/FCV/FPV vaccine, dispense 200 µL of each of the FVR, FCV, and FPV working dilution antisera into the tube labeled 10^{-1}; add 1.2 mL of Chlamydophila Diluent 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 36°± 2°C for 30 ± 5 minutes.

3.4.3 For Test Vaccines containing only a feline chlamydophila fraction, prepare the 10^{-1} dilution by adding 200 µL of the Test Vaccine to 1.8 mL of Chlamydophila Diluent in a 12 x 75-mm polystyrene tube, labeled 10^{-1}; mix by vortexing.

3.4.4 Vaccine tenfold dilutions

Using the same method described for diluting the Chlamydophila Positive Control in **Section 3.3.2** prepare an appropriate number of tenfold serial dilutions of the reconstituted Test Vaccine to bracket its expected endpoint specified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production.

4. Performance of the Test

4.1 On the day of test initiation, label the McCoy Plates, aseptically decant the Growth Medium, and blot the plate on a sterile gauze pad.

4.2 Inoculate 8 wells/dilution with 100 µL/well of the Test Vaccine dilutions and the Chlamydophila Positive Control dilutions (dilutions 10^{-3} through 10^{-7} for the example in **Section 3.3.2(2)**). Change tips between each unique sample (e.g., each Test Vaccine and the Chlamydophila 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^{-7} through 10^{-3}) (**Appendices I and II**).

4.3 Dispense 100 µL of Chlamydophila Diluent to each of 8 wells which serve as uninoculated cell culture controls (Cell Culture Controls) (**Appendices I and II**).

4.4 Seal each McCoy Plate by placing around the upper side of each plate sufficient 1/2-inch labeling tape to maintain the plate's lid in place.

4.5 Centrifuge the McCoy Plates at 466 x g (1500 rpm in an Avanti J-E and JS–5.3 Rotor, and Micro-Test Plate Carriers) at 36°± 2°C for 55 ± 5 minutes to aid adsorption and penetration of chlamydophila into the McCoy cells.

4.6 After centrifugation, pipette 100 µL/well of Maintenance Medium into the McCoy Plates including the Test Vaccine, Positive Control, and Cell Culture Controls.

4.7 Incubate the McCoy Plates at 36°± 2°C in a CO_2 incubator for 5 ± 1 days.

4.8 After incubation, decant the medium into an autoclavable waste container and pipette 200 µL of DPBS into each well of the McCoy Plates.

4.9 Decant the DPBS in the same autoclavable waste container as in **Section 4.8,** and pipette 200 µL of 80% acetone into each well of the McCoy Plates; incubate at room temperature for 15 ± 5 minutes.

4.10 Decant the 80% acetone in a suitable waste container, and gently blot the plate on paper towels. Allow the plates to air dry.

4.11 Dispense 90 ± 10 µL of the Working "Chlamydia" MAb into each well of the McCoy Plates.

4.12 Incubate the McCoy Plates at 36°± 2°C in a high humidity aerobic incubator for 30 ± 5 minutes.

4.13 Decant the MAb; wash the cells twice by pipetting and decanting 200 µL/well of DPBS. The first wash is decanted immediately after delivering the DPBS, whereas the second wash is allowed to remain for 10 ± 5 minutes before decanting. Decant and blot the plate.

4.14 Pipette 90 ± 10 µL of the Working Anti-mouse FITC Conjugate into each well of the McCoy Plates.

4.15 Incubate the McCoy Plates in a high humidity aerobic incubator at 36°± 2°C for 30 ± 5 minutes.

4.16 Repeat **Section 4.13**.

4.17 Fill wells completely with DI, decant, and air dry.

4.18 Examine the plates at 200X using a UV-light microscope for typical intracytoplasmic, apple-green fluorescent inclusions which are characteristic of *C. felis* infection.

4.18.1 Wells containing 1 or more cells exhibiting *C. felis* fluorescent inclusions are considered positive for feline *C. felis* infection.

4.18.2 Results are recorded as the number of IFA-positive wells versus the total number of wells examined for each dilution of a Test Vaccine and the Chlamydophila Positive Control.

4.19 Calculate the chlamydophila endpoints of the Test Vaccine and the Chlamydophila Positive Control using the Spearman-Kärber method as commonly modified by Finney. The titers are expressed as $\log_{10}$ 50% fluorescent antibody infective dose ($FAID_{50}$) per mL or per dose.

Example:

10^{-2} dilution of Test Vaccine = 8/8 wells IFA positive
10^{-3} dilution of Test Vaccine = 5/8 wells IFA positive
10^{-4} dilution of Test Vaccine = 1/8 wells IFA positive
10^{-5} dilution of Test Vaccine = 0/8 wells IFA positive

Test Vaccine titer = (X - d/2 + [d • S]) where:

X = reciprocal $\log_{10}$ of highest dilution with all wells IFA positive(2)
d = $\log_{10}$ of tenfold dilution factor (1)
S = sum of proportions of wells IFA positive for all dilutions tested starting at X:

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

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

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

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

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this *C. felis* Test Vaccine, the recommended dose is 1 mL)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this *C. felis* 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:

Log of 10 = 1.0

Test Vaccine Titer = 3.25 + 1.0 = 4.25

Therefore the titer of the ***C. felis* Test Vaccine** is $10^{4.3}$ $FAID_{50}$/mL.

5. Interpretation of the Test Results

5.1 For a valid assay

5.1.1 The Cell Culture Controls shall not exhibit intracytoplasmic fluorescent inclusions, significant degradation of the cell sheet, CPE, or cloudy media that would indicate contamination.

5.1.2 The calculated $FAID_{50}$ titer of the Chlamydophila Positive Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established by a minimum of 10 previously determined chlamydophila titrations.

5.1.3 The lowest inoculated dilution of the Positive Control must induce specific IFA in 100% of the wells (8/8). If an endpoint is not reached (1 or more wells are IFA 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 IFA staining (0/8).

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

5.2 In a valid test, if the titer of the Test Vaccine is equal to or greater than the titer specified in the APHIS filed Outline of Production, the Test Vaccine is considered **SATISFACTORY**.

5.3 In a valid test, if the titer of the Test Vaccine is lower than the titer specified in the APHIS filed Outline of Production, the Test Vaccine shall be retested in accordance with the 9 CFR, Part 113.8(b).

6. Report of Test Results

Report results as $FAID_{50}$ per dose of the Test Vaccine.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.71, U.S.Government Printing Office, Washington, DC.

7.2 Cottral GE (Ed.). *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, New York, 1978, pg. 731.

7.3 Finney, DJ. *Statistical method in biological assay*, 3rd edition. Griffin, London, 1978, pp. 394-401.

7.4 Richmond JY, McKinney RW (Eds.). *Biosafety in microbiological and biomedical laboratories*. U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington, DC, 1993, pg. 177.

7.5 Smith TF. *Chlamydia*. In *Diagnostic Procedures for viral, rickettsial and chlamydial infections*, 6th edition, NJ Schmidt and RW Emmons (Eds.). American Public Health Assoc., Washington, DC. 1989, pg. 1165-1192.

7.6 Spears, P and Storz, J. *Biotyping of Chlamydia psittaci based on inclusion morphology and response to diethylaminoethyl-dextran and cycloheximide*. Infect. Immun. 24:224-232, 1979.

7.7 Spears, P and Storz, J. *Chlamydia psittaci*: *Growth characteristics and enumeration of serotypes 1 and 2 in cultured cells*. J. Infect. Dis. 140:959-967, 1979.

7.8 Storz, J. *Chlamydia and chlamydia-induced diseases*. Charles C. Thomas Publishing Company, Springfield, Illinois, 1971, pg.358.

8. Summary of Revisions

Version .06

- **The coversheet and contact information have 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.

Version .03

- The Contact has been changed from Ione Stoll to Sandra Conrad.

- Based on the present nomenclature, "Feline *Chlamydia psittaci*" has been changed to "*Chlamydophila felis*"; therefore, the Genus "Chlamydia" has been changed to "Chlamydophila" and the species "felis" has been introduced.

- 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:

- **2.2.6.3** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.

- **4.17** Additional steps have been added for clarification.

- **4.19** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.

- **5.1.3** Recording the rate of positive reaction for validity requirements.

- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°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.

Supplemental Assay Method for Titration of *Chlamydophila felis* (formerly Feline *Chlamydia psittaci*) in Cell Culture

Appendix I
Chlamydophila Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	10^{-1}*	→	→	→	→	→	→	→		CC	CC	
C	10^{-2}	→	→	→	→	→	→	→		CC	CC	
D	10^{-3}	→	→	→	→	→	→	→		CC	CC	
E	10^{-4}	→	→	→	→	→	→	→		CC	CC	
F	10^{-5}	→	→	→	→	→	→	→				
G	10^{-6}	→	→	→	→	→	→	→				
H												

CC = cell culture controls
* = Tenfold dilutions of *Chlamydophila felis* Test Vaccine

Appendix II
Chlamydophila Positive Control Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	10^{-3}#	→	→	→	→	→	→	→		CC	CC	
C	10^{-4}	→	→	→	→	→	→	→		CC	CC	
D	10^{-5}	→	→	→	→	→	→	→		CC	CC	
E	10^{-6}	→	→	→	→	→	→	→		CC	CC	
F	10^{-7}	→	→	→	→	→	→	→				
G												
H												

CC = cell culture controls
# = Tenfold dilutions of *Chlamydophila felis* Positive Control