

**United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol**

SAM 321

**Supplemental Assay Method for Quantitating the GP70 Antigen of Feline
Leukemia Virus in Veterinary Vaccines**

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Supplemental Assay Method for Quantitating the GP70 Antigen of Feline Leukemia
in Virus Veterinary Vaccines

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**Supplemental Assay Method for Quantitating the GP70 Antigen of Feline Leukemia
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1. Introduction

This Supplemental Assay Method (SAM) describes the enzyme-linked immunosorbent assay (ELISA) titration method for quantitation of the 70,000 Dalton glycoprotein (gp70) antigen of feline leukemia virus (FeLV) vaccines by the relative potency (RP) method. The RP of a Test Vaccine is determined by comparing the amount of gp70 antigen of a Test Vaccine to the gp70 antigen content of a Reference Preparation that has been directly or indirectly shown to be protective in a host animal immunogenicity trial.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Incubator $36^{\circ}\pm 2^{\circ}\text{C}$, $5\% \pm 1\% \text{CO}_2$, high humidity (Model 3158, Forma Scientific Inc.)

2.1.2 Microplate reader (Model MRX, Dynex Technologies Inc.)

2.1.3 Microplate washer (Model EL404, Bio-Tek Instruments Inc.)

2.1.4 Single and 12-channel micropipettors and tips of appropriate size.

2.1.5 Current version of the U.S. Department of Agriculture, Center for Veterinary Biologics' (CVB's) *Relative Potency Calculation Software (RelPot)*

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 0.01 M Phosphate buffered saline (PBS)

1. 1.33 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2. 0.22 g sodium phosphate, monobasic, monohydrate ($\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$)

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3. 8.5 g sodium chloride (NaCl)
4. Q.S. to 100 mL with distilled water (DW)
5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl).
6. Sterilize by autoclaving at 15 psi, $121^{\circ}\pm 2^{\circ}\text{C}$ for 35 ± 5 minutes.
7. Store at 2° - 7°C .

2.2.2 0.05 M Carbonate/Bicarbonate Coating Buffer, pH 9.6

1. 0.159 g sodium carbonate (Na_2CO_3)
2. 0.293 g sodium bicarbonate (NaHCO_3)
3. Q.S. to 100 mL with DW.
4. Adjust pH to 9.6 with 1.0 N HCl.
5. Store at 2° - 7°C ; use within 1 week.

2.2.3 Blotto

1. 1.5 g nonfat dry milk powder (*Flavorite* Instant Nonfat Dry Milk, extra grade, Preferred Products Inc.) (Brands and lots may vary in blocking ability. Determine appropriate percentage of dry milk for each batch.)
2. 100 mL PBS
3. Add 2 μL of Antifoam A.
4. Store at 2° - 7°C ; use within 1 week.

2.2.4 Diluent Buffer

1. 100 mL Blotto
2. 1.0 mL Triton X-100
3. Store at 2° - 7°C ; use within 1 week.

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2.2.5 Washing Buffer

1. 1000 mL of 0.01 M PBS
2. 1.0 mL Tween-20
3. Store at 2°- 7°C.

2.2.6 Goat Anti-FeLV gp70 Polyclonal Antibody

2.2.7 Anti-FeLV gp70 monoclonal antibody (MAb)

2.2.8 Goat anti-mouse horseradish peroxidase conjugate (Goat Anti-mouse Conjugate)

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

1. Solution A, ABTS
2. Solution B, Hydrogen Peroxide

2.2.10 FeLV gp70 Positive Control (available from the CVB)

2.2.11 Reference preparation: Each manufacturer provides a Reference Vaccine 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 the manufacturer must have an RP equal to or greater than the RP value contained in the APHIS filed Outline of Production.

2.2.12 Immulon II[®], flat bottom, 96-well plate

2.2.13 Flat bottom, 96-well plate (transfer plate)

2.2.14 Plate Sealer

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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

On the day of the reading, the ELISA reader must be turned on at least 30 minutes prior to determination of the OD. Dual wavelength settings, 405-nm test wavelength measured against a 490-nm reference wavelength, are used. The ELISA reader is zeroed on air prior to use.

3.3 Preparation of reagents/control procedures

3.3.1 Test plate preparation. One to five days prior to test initiation, dilute the Goat Anti-gp70 Polyclonal Antibody, per the CVB Reagent Data Sheet, in Carbonate/Bicarbonate Coating Buffer.

1. Pipette 100 μ L/well of the Diluted Goat Anti-FeLV gp70 Polyclonal Antibody to all wells of a flat bottom, 96-well Immulon II[®] plate. This becomes the Test Plate.

2. Cover the Test Plate with a Plate Sealer and incubate at 3 ± 2 days, at 2° - 7° C.

3.3.2 Diluted gp70 Positive Control preparation. On the day of testing, dilute the gp70 Positive Control, per the CVB Reagent Data Sheet, in Diluent Buffer.

3.3.3 Diluted Anti-gp70 MAb preparation. On the day of testing, dilute the Anti-gp70 MAb, per the CVB Reagent Data Sheet, in Blotto.

3.3.4 Diluted Goat Anti-mouse Conjugate. On the day of testing, dilute the Goat Anti-mouse Conjugate, per previously determined optimal dilution, in PBS. Optimal dilution results in an OD reading between 0.400 and 0.700 when incubated at $36^{\circ} \pm 2^{\circ}$ C for 20 ± 10 minutes.

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3.3.5 Substrate Solution preparation. On the day of testing, just prior to substrate addition, mix equal volumes of ABTS Solution A and Hydrogen Peroxide Substrate Solution B per the manufacturer's instructions. The resulting Substrate Solution should remain clear and should be at room temperature at time of use.

3.4 Preparation of the sample

3.4.1 Antigen Extraction (optional). If the Test Vaccine contains 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. The CVB 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 without extraction. If the Reference Preparation is a product reference, both the Reference Preparation and Test Vaccine must be treated identically. If the Reference Preparation is a purified preparation, the extraction procedure is not required.

3.4.2 All samples must be at room temperature before testing. The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). Make twofold dilutions of the Reference Preparation and the Test Vaccine in Diluent Buffer in a flat bottom, 96-well plate which becomes the Transfer Plate.

3.4.3 An initial dilution of the Reference Vaccine and/or Test Vaccine may be made. This dilution is determined by the firm to assure the 6 dilutions tested encompass the linear portion of the regression curve. The starting dilution for the Reference Vaccine and/or the Test Vaccine 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.

- 1.** Pipette 150 μ L of Diluent Buffer to wells in rows C-G of the Transfer Plate with a 12-channel micropipettor (see **Appendix**).
- 2.** Pipette 300 μ L of the starting dilution of the Reference Preparation to 3 wells in row B of the Transfer Plate (see **Appendix**).
- 3.** Pipette 300 μ L of the starting dilution of the first Test Vaccine to 3 adjacent wells in row B of the Transfer Plate. An additional Test Vaccine may be tested into the next 3 adjacent wells.

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4. With a 12-channel micropipettor and the appropriate number of tips to correspond to the number of wells used in a row, mix row B (7 ± 2 fills) and transfer 150 μL to row C of the Transfer Plate. Replace tips, mix row C, and transfer 150 μL to row D.
5. Continue as in **Step 4** for the remaining rows D-G of the Transfer Plate, transferring 150 μL from the previous to the next row.

4. Performance of the Test

- 4.1 On the day of testing, remove the Plate Sealer and pipette 200 μL of thoroughly mixed Blotto to each well of the Test Plate already containing Diluted Goat Anti-gp70 Polyclonal Antibody. Do not remove the Diluted Goat Anti-gp70 Polyclonal Antibody prior to adding the Blotto. Reseal plate and incubate at $2^\circ - 7^\circ\text{C}$ for 90 ± 30 minutes.
- 4.2 Decant Blotto from the Test Plate. Pipette 200-300 μL of Washing Buffer into each well. Immediately decant Washing Buffer from the plate. Repeat for a total of 3 washes. At no time should wells dry between washes or incubations. An automatic microplate washer may be used.
- 4.3 Transfer 100 μL /well of each dilution in the Transfer Plate (starting in row G, most dilute, and ending in row B, least dilute) to appropriate rows of the Test Plate (see **Appendix**). Tips need not be changed between rows when proceeding from most dilute to successively more concentrated dilutions.
- 4.4 Pipette 100 μL /well of Diluted gp70 Positive Control to wells 11-B, 11-C, and 11-D of the Test Plate.
- 4.5 Pipette 100 μL /well of Diluent Buffer to wells 11-E, 11-F, and 11-G of the Test Plate to serve as blanks.
- 4.6 Seal the Test Plate with a Plate Sealer; incubate at $36^\circ \pm 2^\circ\text{C}$ for 60 ± 10 minutes.
- 4.7 Wash the Test Plate as in **Section 4.2**.
- 4.8 Pipette 100 μL /well of Diluted Anti-gp70 MAb to all wells of the Test Plate.
- 4.9 Seal the Test Plate with a Plate Sealer; incubate at $36^\circ \pm 2^\circ\text{C}$ for 60 ± 10 minutes.
- 4.10 Wash the Test Plate as in **Section 4.2**.

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- 4.11 Pipette 100 μ L/well of Diluted Goat Anti-mouse. Conjugate to all wells of the Test Plate.
- 4.12 Seal the Test Plate with a Plate Sealer; incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ for 60 ± 10 minutes.
- 4.13 Wash the Test Plate as in **Section 4.2**, then wash 2 times with PBS instead of Washing Buffer.
- 4.14 Pipette 100 μ L/well of Substrate Solution to all wells of the Test Plate.
- 4.15 Incubate the Test Plate at room temperature for 20 ± 10 minutes.
- 4.16 Read the Test Plate at 405-nm test wavelength against a 490-nm reference wavelength, when the Positive Control wells (11-B, C, D) read between 0.400 and 0.700 OD after the average blank reading is subtracted.
- 4.17 The arithmetic mean of the blank wells is determined; this becomes the average blank reading. The average blank reading is subtracted from all readings before analysis of the data by *RelPot*.
- 4.18 Evaluate results using the current version of *RelPot*.

5. Interpretation of the Test Results

- 5.1 If the corrected OD of the Positive Control is not within the 0.400-0.700 OD range, the test is considered a **NO-TEST** and can be repeated without prejudice.
- 5.2 All validity criteria in the current version of *RelPot* 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).
- 5.3 For a given Test Vaccine 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 contained in an APHIS filed Outline of Production.
- 5.4 For a Test Vaccine result less than the RP contained in an APHIS filed Outline of Production, the test may be repeated if the test meets 9 CFR 113.8(c)(5) criteria.

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6. Report of Test Results

Results are reported as the RP for the Test Vaccine. The highest RP of the top scores shall be considered the RP value for reporting purposes.

7. References

7.1 Title 9, *Code of Federal Regulations*, section 113.8, U.S. Government Printing Office, Washington, DC.

7.2 Shibley GP, Tanner JE, Hanna SA, United States Department of Agriculture licensing requirements for feline leukemia virus vaccines. *JAVMA*, (1991), Vol. 199, No. 10, 1402-1406.

8. Summary of Revisions

Version .06

- **The coversheet and contact information has 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.
- Reference to SAM 318 has been removed throughout the document.

Version .04

- The Contact information has been updated.

Version .03

- **2.1.4:** Changes were made to the type and sizes of micropipettors to allow for variation as appropriate.

Version .02

This document was rewritten 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:

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- 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.
- “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.

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Appendix
Transfer and Test Plate Format

	1	2	3	4	5	6	7	8	9	10	11	12									
A	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>									
B	<input type="radio"/>	R E F E R E N C E	R E F E R E N C E	R E F E R E N C E	S E R I A L 1	S E R I A L 1	S E R I A L 1	S E R I A L 2	S E R I A L 2	S E R I A L 2	+	C N T R	<input type="radio"/>								
C	<input type="radio"/>												<input type="radio"/>								
D	<input type="radio"/>										<input type="radio"/>										
E	<input type="radio"/>										<input type="radio"/>	B L A N K	<input type="radio"/>								
F	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>										
G	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>										
H	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>									

Locations for dilution plate:

Row B, columns 2, 3, and 4: lowest dilution of Reference Vaccine
Row B, columns 5, 6, and 7: lowest dilution of Test Serial 1
Row B, columns 8, 9, and 10: lowest dilution of Test Serial 2

Locations for test plate:

Rows B through G, columns 2, 3, and 4: increasing dilution of the Reference Vaccine
Rows B through G, columns 5, 6, and 7: increasing dilution of Test Serial 1
Rows B through G, columns 8, 9, and 10: increasing dilution of Test Serial 2
B-11, C-11, and D-11: gp70 Positive Control
E-11, F-11, and G-11: Blank