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United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 407

Supplemental Assay Method for Titration of Marek's Serotype 3 (Herpesvirus of Turkeys Strain FC-126), Lyophilized

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1. Introduction

This Supplemental Assay Method (SAM) describes a procedure using chick embryo fibroblast (CEF) cell cultures, for titrating cell-free Marek's Serotype 3 (herpesvirus of turkeys [HVT] Strain FC-126) used as a vaccine against Marek's disease. The vaccine is composed of lyophilized cell-free preparation of the virus and a suitable diluent.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)

2.1.3 Water-jacketed incubator with a humidified 5 ± 1% CO_2 atmosphere and temperature set at 37º± 2°C (Forma Scientific, Model No. 3158)

2.1.4 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)

2.1.5 Magnetic stir plate

2.1.6 Scissors, sterile (Roboz RS-6800)

2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)

2.1.8 Pipette (Rainin Pipetman, P1000, or equivalent)

2.1.9 250-mL trypsinizing flask with stir bar, sterile

2.1.10 Erlenmeyer flask with a stirring bar, sterile

2.1.11 Hemocytometer

2.1.12 Bunsen burner

2.1.13 Blunt thumb forceps, sterile

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 Cotton swab

2.2.2 Tissue culture dish, 150 x 10-mm

2.2.3 Tissue culture dish, 100 x 10-mm

2.2.4 Plastic funnel covered with 4 layers of fine gauze

2.2.5 Polypropylene conical tube, 29 x 114-mm, sterile, 50-mL

2.2.6 Polypropylene centrifuge tubes, 250-mL

2.2.7 Roller bottles, 1000-mL (plastic)

2.2.8 Serological pipettes

2.2.9 60-mm gridded cell culture dish, tissue culture treated

2.2.10 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.11 Fetal Bovine Serum (FBS)

2.2.12 L-glutamine

2.2.13 Trypsin, 2.5%

2.2.14 Pipette tips

2.2.15 Solutions

All solutions are filter sterilized.

1. Trypsin Solution (0.25%)

Rapidly thaw frozen 2.5% (10X) trypsin in warm water. Immediately make a working solution (0.25%) by aseptically diluting the 10X solution 1:10 with Dulbecco's PBS, $CaCl_2$ and Mg free.

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2. Growth Medium

Medium 199 (with Earles salts) (powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry powder)	2.95 g
$NaHCO_3$	2.5 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
HEPES	11.97 g
Fetal Bovine Serum* (gamma-irradiated)	60 mL
q.s. with distilled or deionized water (DW)	2185 mL

Adjust pH to 7.35 to 7.4 by adding $NaHCO_3$ solution. Before use, add 1.0 mL of a 200 mM concentration of L-glutamine per 100 mL medium.

3. Maintenance Medium

Medium 199 (with Earles salts) (powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry powder)	2.95 g
$NaHCO_3$	2.75 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
HEPES	11.97 g
Fetal Bovine Serum* (gamma-irradiated)	10-20 mL
q.s. with DW	2142 mL

Adjust pH to 7.5 by adding $NaHCO_3$ solution. Before use, add 1.0 mL of a 200-mM concentration of L-glutamine per 100 mL medium.

*Previously tested for freedom from extraneous agents

4. SPGA Diluent

Sucrose	74.62 g
KH_2PO_4	0.45 g
K_2HP0_4	1.35 g
Mono Sodium Glutamate	0.80 g
1% Bovine Albumin (Fraction V)	10.0 g
q.s. with DW	1000 mL

2.2.16 Primary CEF preparation

Primary CEF cultures can be prepared from 9- to 11-day-old genetically susceptible (C/E) embryos from an Avian Leukosis Virus negative chicken flock in the following manner, or alternate methods of preparation are acceptable:

1. Disinfect the air cell end of the egg with 70% ethanol and break open the shell with sterile forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable 150 x 10-mm tissue culture or petri dish. Remove and discard the heads and viscera of the embryos with sterile forceps. Wash the embryo carcass several times with growth media (without FBS or L-glutamine) to remove excess blood. Place the washed embryos in a sterile dry 100 x 10-mm tissue culture or petri dish and mince them thoroughly using sharp sterile scissors.

2. To further wash, put the minced tissue into a trypsinizing flask containing 50 mL growth medium (without FBS or L-glutamine) and a magnetic stir bar. Place the flask on a stir plate and stir with a moderate vortex for 5 minutes. Allow the cells to settle and decant (and discard) the supernatant and repeat.

3. To trypsinize the tissues, first rinse the residual media from the cells by adding 10 ml of the 0.25% trypsin solution, and then immediately decant the trypsin solution. Next add 40 mL of the 0.25% trypsin solution to the flask and mix on a magnetic stir plate for 15 minutes. (Set stir plate to produce a moderate vortex.)

4. Place a sterile gauze wrapped funnel into the opening of a 250-mL conical centrifuge tube. To stop the trypsinizing action on the cells, pour 2 mL of FBS through the gauze and then pour the trypsinized contents of the flask through the gauze funnel into the centrifuge tube. Bring the total volume to approximately 125 mL with growth media. Centrifuge for 10 minutes at 250 x *g* (1050 rpms using a Beckman J-6B centrifuge with a JS-4.2 rotor) with the temperature set at 10°C.

Resuspend the cells in growth medium to a concentration of approximately 750,000 cells per mL. Plant the cells in 60-mm plastic petri dishes (gridded or plain), 5 mL per dish (approximately 3.75 x 10^6 cells). Incubate at 37º± 1ºC in a high-humidity atmosphere containing approximately 5 ± 1% CO_2. In 24 hours the monolayers should be complete and ready for inoculation.

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies and training in the operation of the laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturers' instructions and monitor in compliance with current corresponding standard operating procedures.

3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 Preparation of vaccine for titration

Rehydrate the vaccine (HVT) to field strength (1 dose/0.2 mL) with the proper amount of the manufacturer's diluent. Mix thoroughly.

3.4.2 Holding period

Place the vaccine bottle in an ice bath for 2 hours prior to proceeding with the titration.

4. Performance of the Test - Preparing Dilutions and Inoculating Plates

Prepare dilution blanks containing 9 mL of the manufacturer's diluent or SPGA diluent. Remove the medium from the titration plates as follows: Set the plates on a slanted surface. Remove fluid using a pipette, then allow the plates to drain an additional 60 to 90 seconds. Next, remove the remaining fluid accumulated at the lower edge of the plates. Mix the virus suspension thoroughly by inverting several times. Put 1.0 mL of the vaccine into the 9-mL dilution blank. This makes a 1:10 dilution of the vaccine. (For vaccine expected to have a relatively high-virus content, a 1:20 dilution may be used.) Mix this dilution thoroughly, and inoculate 0.05 mL per plate onto the center of each of 5 plates (on a horizontal surface). Incubate the inoculated plates at 37º± 1°C in a humidified atmosphere for a 30 to 45 minute absorption period. Add 5 mL maintenance medium to each plate, and continue incubation at 37º± 1°C in a high-humidity atmosphere of

approximately 5 ± 1% CO_2 for 6 days. The maintenance medium may be replaced after 2 or 3 days if the pH of the culture fluids becomes too acidic.

Titrate a known positive reference virus with each group of titrations. Uninoculated negative control cells are maintained to monitor the integrity of the cell culture system.

5. Interpretation of the Test Results

5.1 Controls

The titer of the positive reference must be within the established range for the test results to be valid. Uninoculated negative control cells confirm the integrity of the cell culture system.

5.2 Making plaque counts and calculating titer

5.2.1 Counting

1. Well-developed plaques are usually observed by day 4 postinoculation. On day 6 postinoculation, make a plaque count using an inverted microscope (and a grid-adapted stage if plain plates have been used). By day 6, all primary plaques should have developed and secondary plaques should not be developed well enough to cause problems in counting using these testing methods.

2. Count all the plaques on each of the plates of the titration series. A plaque is counted as 1 regardless of size unless it has apparently arisen from 2 distinct centers. At least 4 plates should be counted for a valid test.

5.2.2 Calculating plaque-forming units (PFUs)

Calculate the number of plaque-forming units (PFUs) per plate and multiply this value by the final dilution factor (plated dilution divided by amount plated) and then multiply by the bird dose volume of 0.2 mL (assuming the volume of bird dose is 0.2 mL).

Example: (56+48+47+53)/4 = 51 average PFUs per plate

(51)(10÷0.05 mL = 200)(0.2)=2040 PFUs/bird dose

5.3 Retests

Conduct retests as required by title 9, *Code of Federal Regulations* (9 CFR), section 113.8(b) and requirements of minimum release in the firm's current Outline of Production, Part V.

5.4 Evaluation of test results

5.4.1 The 9 CFR 113.8(b) defines the criteria for a satisfactory/unsatisfactory serial.

5.4.2 The firm's requirements of minimum release/stability titers for each Marek's vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of Test Results

Titers are reported out as PFUs per bird dose.

7. 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 document number has been changed from VIRSAM0407 to SAM 407.
- The Contact information has been updated.

Version .02

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This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. No significant changes impacting the test were made from the previous protocol.

- The title change of SAM 407 is in accordance with the True Name nomenclature revision (Veterinary Biologics Notice dated June 12, 1996).
- **2.2.13** The trypsin has been changed from 0.25% to 2.5%.
- **2.2.15(1)** The Trypsin Solution has been revised for clarity.
- **2.2.15(2)** and **2.2.15(3)** HEPES has been added to the mediums and "heat-inactivated" has been changed to "gamma-irradiated" for the Fetal Bovine Serum.
- **2.2.16** The preparation of primary chick embryo fibroblasts (CEFs) has been added to the document.
- **4.1** Positive reference and uninoculated control procedures have been added for clarity.
- "Foci" or "focus" has been changed to "plaques" or "plaque" throughout the document.