United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol

**SAM 312**

**Supplemental Assay Method for the Detection of Extraneous Cytopathogenic Agents in Master Seed Virus**

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Standard Requirement: **9 CFR 113.46 and 113.55**

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1. Introduction

This Supplemental Assay Method (SAM) detects extraneous cytopathogenic agents in master seed viruses (MSV) used in the production of veterinary vaccines. Cytopathic effect (CPE) of extraneous agents is determined by microscopic examination and May-Grünwald-Giemsa staining of tissue culture monolayers.

2. Materials

2.1 Equipment/instrumentation

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

- Inverted light microscope

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Cell culture flask, 25-cm², of cellular monolayers meeting the requirements of title 9, Code of Federal Regulations (9 CFR), parts 113.51 and 113.52

1. Monolayers at least 7 days from last subculture.

2. One flask for each cell type to be inoculated with MSV (MSV Flasks).

3. One flask for each cell type to be used as uninoculated negative control (NC Flasks).

2.2.2 Deionized water (DI)

2.2.3 0.01 M phosphate buffered saline (PBS)

1. 1.33 g sodium phosphate, dibasic, anhydrous (Na₂HPO₄)

2. 0.22 g sodium phosphate, monobasic, monohydrate (NaH₂PO₄•H₂O)

3. 8.5 g sodium chloride (NaCl)

4. Q.S. to 1000 mL with DI.
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5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl).

2.2.4 Absolute Methanol

2.2.5 May-Grünwald Stain

2.2.6 Whatman® No. 2 filter paper

2.2.7 Giemsa Stain

2.2.8 Pipettes: 1-mL, 5-mL, 10-mL, and 25-mL

2.2.9 Graduated cylinder, 500-mL

2.2.10 Mineral oil

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture, MSV extraneous agent testing as described in the 9 CFR 113.55 and 113.46, cell staining techniques, and use of inverted light microscopes.

3.2 Preparation of reagents/control procedures

3.2.1 May-Grünwald Stain, working dilution

1. Using a graduated cylinder, measure 500 mL of Absolute Methanol. The Absolute Methanol shall be at room temperature (23°C ± 2°C), prior to use.

2. Dissolve 0.5 g May-Grünwald Stain in Step 1.

3. Filter Step 2 using a No. 2 filter.

4. Store at room temperature.
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3.2.2 Giemsa Stain, working dilution

1. Using a 25-mL pipette, measure 24 mL DI into a suitable container. Using a 1-mL pipette, add 1 mL of Giemsa Stain, or dilute as specified by the manufacturer.

2. Store at room temperature.

3.3 Preparation of the sample

MSV shall be tested as described in the 9 CFR 113.55. The last subculture shall meet the minimum requirements specified in the 9 CFR 113.46. Monolayers shall be microscopically examined regularly for evidence of CPE during the 14-day maintenance period. Monolayers at least 7 days from the last subculture shall be tested for extraneous CPE agents by the May-Grünwald-Giemsa staining procedure, using at least 1 MSV Flask and 1 NC Flask for each cell type. Just prior to staining, all flasks are examined for CPE.

4. Performance of the Test

CAUTION: Precautionary measures for using methanol and Giemsa Stain: Keep away from heat, spark, or flames; keep container closed; avoid breathing vapor; use with adequate ventilation; wear appropriate protective clothing including gloves and safety goggles (no contact lenses); wash thoroughly after handling. Store in a flammable liquid storage cabinet. Place used chemicals in appropriate containers for disposal in a manner consistent with safe laboratory practices.

May-Grünwald-Giemsa staining:

4.1 Decant tissue culture media from the MSV Flasks and NC Flasks.

4.2 Using a 10-mL pipette, rinse monolayers with 10 mL of room temperature PBS.

4.3 Decant the PBS rinse.

4.4 Using a 5-mL pipette, add 4 ± 1 mL of the working dilution May-Grünwald Stain into each flask.

4.5 Incubate at room temperature for 10 ± 5 minutes.

4.6 Decant the stain and dispose of it properly.
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4.7 Using a 5-mL pipette, add 3 mL of the working dilution Giemsa Stain into each flask.

4.8 Incubate at room temperature for 15 ± 5 minutes.

4.9 Decant the stain and dispose of it properly.

4.10 Using a 10-mL pipette, rinse monolayers twice with 10 mL of DI.

4.11 Decant the DI.

4.12 While monolayers are still moist, using an inverted microscope at 100-200X, compare the MSV Flasks to the NC Flasks of the corresponding cell line tested. Mineral oil, 2 mL, may be placed in each flask to allow examination at a later time.

4.12.1 Examine each flask for the presence of extraneous intracellular agents. The May-Grünwald-Giemsa differentially stains DNA and RNA nucleoproteins. DNA nucleoprotein stains red-purple while RNA nucleoprotein stains blue.

4.12.2 Examine for the presence of CPE, such as inclusion bodies, giant cell formation, syncytia, or other cell abnormalities which may be attributable to an extraneous agent.

5. Interpretation of the Test Results

5.1 Criteria for a valid test

5.1.1 The NC Flask is free of extraneous intracellular agents, CPE, or cellular abnormalities.

5.1.2 The NC Flask and the MSV Flask are free of bacterial or fungal contamination.

5.1.3 The MSV Flask is free of CPE attributable to the MSV agent.

5.1.4 If any of the validity criteria in Sections 5.1.1 through 5.1.3 are not met, the test is a NO TEST and is repeated without prejudice.

5.2 Satisfactory tests

If the test is valid and the MSV Flask is found free of extraneous intracellular agents, CPE, or cellular abnormalities, the MSV is SATISFACTORY.
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5.3 Retests

5.3.1 If the initial test is valid and extraneous intracellular agents, CPE, or cellular abnormalities are found, the test is repeated (1st retest), using only the cell type found positive. The test is repeated using a new vial of MSV.

5.3.2 If the second valid test (1st retest) confirms the initial result, the MSV is UNSATISFACTORY.

5.3.3 If the second valid test (1st retest) fails to confirm the initial result, the MSV is tested a third time (2nd retest). The test is repeated using a new vial of MSV.

1. If the second and third valid tests (1st and 2nd retests) result in the MSV Flasks being free of extraneous intracellular agents, CPE, or cellular abnormalities, the MSV is SATISFACTORY.

2. If the third valid test (2nd retest) confirms the initial result, the MSV is UNSATISFACTORY.

6. Report of Test Results

Record all test results on the test record.

7. References


7.2 Gurr, E., Staining Practical and Theoretical, Williams and Wilkins Co., Baltimore, Maryland, 1962, p. 220.

8. Summary of Revisions

Version .03

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for 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:

- The document number has been changed from MVSAM0312 to SAM 312.
- The Contact information has been updated.

Version .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 SAM 312, dated June 1, 1985.

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