United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 313

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

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

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1. Introduction

This Supplemental Assay Method (SAM) describes a method for detection of extraneous hemadsorbing (HAd) agents in master seeds (MS) used in the production of veterinary vaccines. Not all viruses cause a noticeable change in the cellular appearance, but can be detected by HAd of red blood cells (RBC) to virus-coded glycoproteins incorporated in the cell membrane. To perform the test, cell monolayers are washed and covered with a suspension of guinea pig and chicken RBC. Following incubation, uninfected cells will appear normal, while RBCs will adhere to cell monolayers that contain HAd surface viral antigens. Detection is accomplished by both macroscopic and microscopic reading methods.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below. Equipment is calibrated and certified according to current standard operating procedures.

2.1.1 Microscope, inverted bright light

2.1.2 Illumination box

2.1.3 Low speed, refrigerated centrifuge (Beckman J-6B centrifuge with JS 4.0 rotor or equivalent).

2.1.4 Refrigerator, 2°-7°C

2.1.5 Micropipettors and appropriate tips

2.1.6 Vacuum source for aspirating liquids (vacuum pump with side arm flask of Chapman-type filter pump attached to a water line)

2.1.7 Laminar Flow Biological Safety cabinet (NuAire, Inc. Labgard or equivalent)

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 Appropriate vessels at least 7 days from the last subculture containing:

- MS-inoculated
- negative control cell monolayers.

The preferred vessel is 25-cm^2 flasks , but any suitable alternative may be used if the surface area is ≥ 6 cm^2 each.

2.2.2 A vessel containing a monolayer of Vero cells, ≥ 6 cm^2 to be used in preparation of positive control (PC).

2.2.3 PC virus (current approved lot of Bovine Parainfluenza type 3)

2.2.4 Alsever's solution

1. 8.0 g sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)

2. 0.55 g citric acid ($C_6H_8O_7 \cdot H_2O$)

3. 4.2 g sodium chloride (NaCl)

4. 20.5 g glucose ($C_6H_{12}O_6$)

5. Q.S. to 1000 mL with deionized water (DI).

6. Filter sterilize with a 0.22-µm filter.

7. Store at 2°- 7°C.

2.2.5 Guinea pig and chicken RBC in equal volumes of Alsever's Solution. Store at 2°- 7°C.

2.2.6 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 ($NaH_2PO_4 \cdot H_2O$)

3. 8.5 g NaCl

4. Q.S. to 1000 mL with DI.

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°± 2°C for 35 ± 5 minutes.

7. Store at 2°- 7°C.

2.2.7 Pipettes: 1-mL, 5-mL, and 25-mL

2.2.8 Conical tubes: 50-mL

2.2.9 Cell culture flasks: 25-cm^2 (or appropriate vessel)

2.2.10 Graduated cylinder

3. Preparation for the Test

3.1 Personnel qualifications/training

Laboratory personnel must be trained in the operation of instruments and equipment used prior to performing this test. Personnel must be familiar with proper use of test reagents and biological materials. Personnel must have knowledge of safe operating procedures and policies.

3.2 Preparation of RBC

3.2.1 Wash guinea pig and/or chicken RBC

1. Dispense 10-20 mL of guinea pig and chicken blood preserved in Alsever's solution into separate 50-mL conical centrifuge tubes.

2. Fill the tube with Alsever's/PBS and gently invert tube several times to wash RBC.

3. Centrifuge RBC for 15 ± 5 minutes at 400-800 x *g* (e.g. 1500-1800 rpm, J6-B centrifuge with a JS-4.0 or JS-4.2 rotor) at 4°± 2°C.

4. Aspirate PBS and buffy coat (bone colored layer of cells on top of RBC) using a pipette or vacuum type aspirator**.**

5. Repeat **Steps 2 through 4** for a total of 3 or more washes (until supernatant is clear). Store the packed guinea pig and chicken RBC at 2°-7°C for up to 1 week. RBC should be discarded if hemolysis is observed.

3.2.2 Preparation of 0.2% RBC on day of test.

1. Dispense 49.8 mL of PBS into a suitable container.

2. Using 1-mL pipettes, add 100 µL of washed guinea pig RBC and 100 µL of chicken RBC to PBS. Rinse pipette(s) thoroughly to dispense all RBC. Gently swirl to mix.

3. RBC suspension should be discarded following completion of test.

3.3 Preparation of the sample

For each cell line, seed at least one 25-cm^2 flask with MS inoculated cells and at least one 25-cm^2 flask with un-inoculated cells to be tested sequentially at 4°C and 25°C. In addition, seed two 25-cm^2 flasks of un-inoculated Vero cells to serve as positive controls, one flask for each temperature. The positive control flasks are seed-inoculated with bovine parainfluenza virus 3 at the time of the last subculture.

4. Performance of the Test

The HAd test is conducted at 2 temperatures: 4°± 2°C and room temperature (23°± 2°C) for MS and NC flasks of each cell type. Two PC flasks (Vero cell monolayers) are tested (one at 4° and one at room temperature). Alternatively, MS, NC and PC flasks of each cell type may be incubated simultaneously at each temperature (**Section 4.2**).

4.1 Vessels incubated at each temperature separately

4.1.1 Decant medium from MS, NC and PC flasks.

4.1.2 Add 5 mL of PBS to each of the MS, NC and PC flasks, swirl gently and decant. Repeat for a total of 3 washes.

4.1.3 Gently swirl the 0.2% RBC mixture and add 5 mL into each MS, NC and PC flasks (**Section 3.2.2**).

4.1.4 Incubate all flasks except one PC flask at 4°± 2°C for 25 ± 5 minutes.

4.1.5 Decant the 0.2% RBC mixture.

4.1.6 Wash vessels 3 times as described in **Section 4.1.2.**

4.1.7 Using an illumination box, compare each MS flask to the corresponding NC and PC control flasks. Look for areas of HAd on the cell monolayer (see **Appendix I**).

4.1.8 Using an inverted microscope at 100X, compare each MS flask to the corresponding NC and PC flask(s). Look for HAd of individual RBC to cell membranes (see **Appendix II**).

4.1.9 If HAd is not detected, add 0.2% RBC mixture to MS, NC cell monolayers and remaining PC flask as described in **Section 4.1.3.**

4.1.10 Incubate all vessels at room temperature for 25 ± 5 minutes.

4.1.11 Repeat **Sections 4.1.6 through 4.1.9**.

4.2 Separate vessels incubated simultaneously at each temperature

4.2.1 Repeat **Sections 4.1.1 through 4.1.3** for duplicate sets of vessels.

4.2.2 Incubate MS and NC flasks for each cell line, as well as a PC flask (Vero) at 4°± 2°C for 25 ± 5 minutes.

4.2.3 Incubate separate MS and NC flasks for each cell line, as well as a PC flask (Vero) at room temperature for 25 ± 5 minutes.

4.2.4 Repeat **Sections 4.1.6 through 4.1.9** for each set of vessels.

5. Interpretation of the Test Results

For a valid test, the PC cell culture must demonstrate HAd while the NC cell culture must be free of HAd activity.

HAd activity in the MS inoculated cell monolayers other than the non-neutralized MS is indicative of infection by extraneous viruses, and the MS is determined unsatisfactory. The MS may be retested to confirm original results if warranted. Additional investigative work may be warranted to determine cause of HAd.

6. Report of Test Results

All records are kept in accordance with current recordkeeping practices

7. References

Title 9, *Code of Federal Regulations,* parts 113.46, 113.51, 113.52 and 113.55, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .05

- **The coversheet and contact information has been updated.**

Version .04

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

Version .03

- The Contact information has been updated.

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:

- **1:** An overview of the test method has been added.
- **2.1:** More current and appropriate equipment has been added.
- **2.2:** Surface area criteria to test and a Positive Control have been added to the test.
- **3.1:** Personnel qualifications and training have been clarified.
- **3.3:** Additional detail to washing and preparation of 0.2% RBC has been added.
- **3.4:** A Positive Control has been added to the test system.
- **4.2:** The Positive Control flask has been added to the incubation step.
- **5.1:** This section has been condensed and the roles of the Positive and Negative Control vessels have been clarified.

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

- Appendices containing macro- and microscopic images depicting hemadsorbtion have been added.

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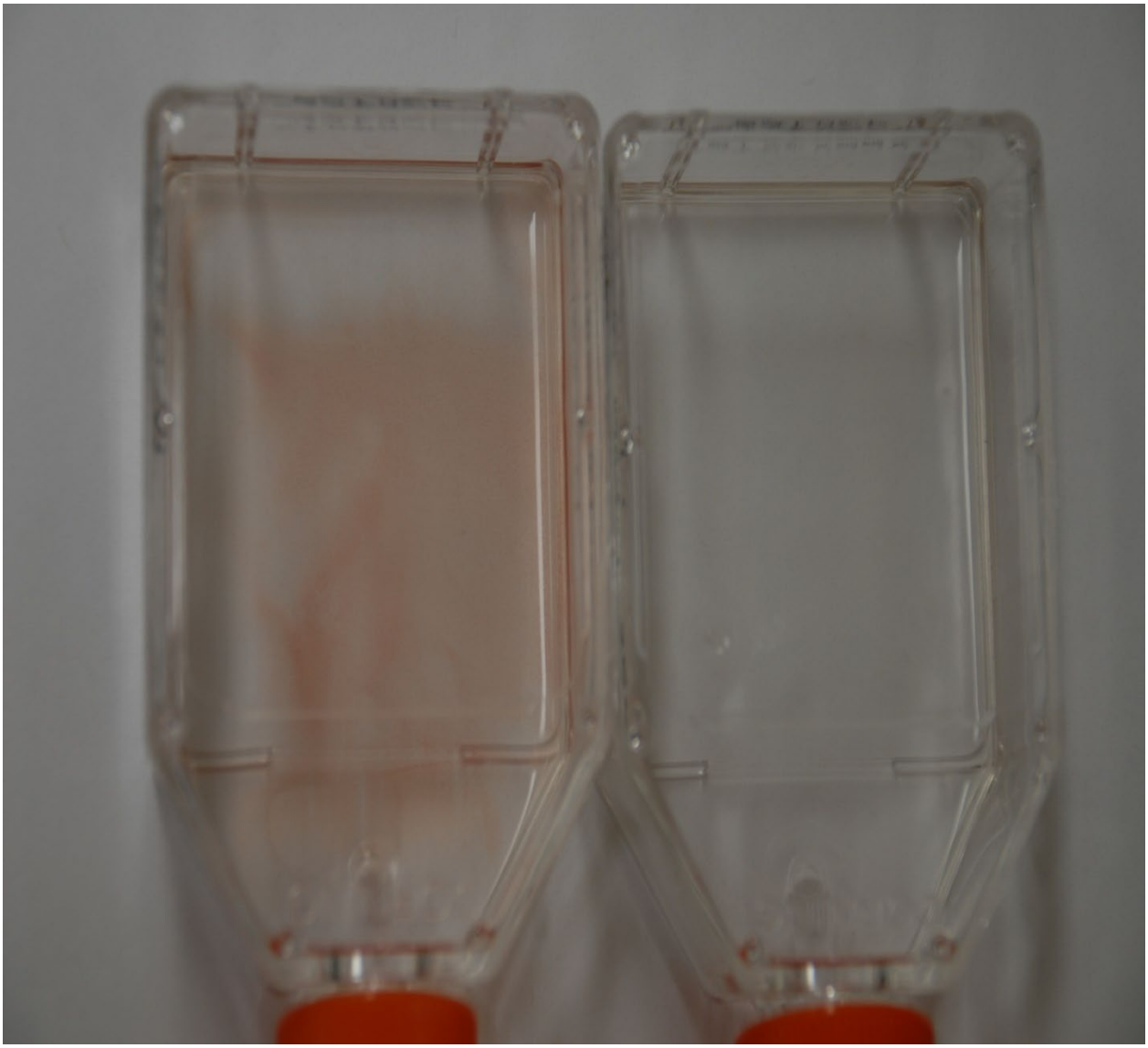
Appendix I

Macroscopic view of hemadsorption



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Appendix II

Microscopic view of hemadsorption