

**Center for Veterinary Biologics  
and  
National Veterinary Services Laboratories  
Testing Protocol**

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

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## 1. Introduction

### 1.1 Background

This Supplemental Assay Method (SAM) describes a method for detection of extraneous hemadsorbing (HAd) agents in master seed viruses (MSV) used in the production of veterinary vaccines. Extraneous HAd agents are detected in tissue culture monolayers using a mixture of 0.2% guinea pig and chicken red blood cells (RBCs). Detection of HAd RBCs is accomplished by both macroscopic and microscopic methods.

### 1.2 Keywords

Master seed virus; hemadsorption; HAd; blood; RBCs; tissue culture; *in vitro*

## 2. Materials

### 2.1 Equipment/instrumentation

2.1.1 Microscope,<sup>1</sup> inverted light

2.1.2 Illumination box<sup>2</sup>

2.1.3 Centrifuge<sup>3</sup> and rotor<sup>4</sup>

2.1.4 Refrigerator,<sup>5</sup> 4° ± 2°C

### 2.2 Reagents/supplies

2.2.1 Tissue culture monolayers meeting the requirements in the Code of Federal Regulations, Title 9 (9 CFR), Parts 113.51 & 113.52.

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<sup>1</sup> Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

<sup>2</sup> Model #11-8C Glow-Box, Instruments for Research and Industry, 100 Franklin Ave., Cheltenham, PA 19012-2222 or equivalent

<sup>3</sup> Model J6-B, Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92834-3100 or equivalent

<sup>4</sup> Model JS-4.0, Beckman Instruments, Inc. or equivalent

<sup>5</sup> Model 3766, Barnstead/Thermolyne, 2555 Kerper Blvd., Dubuque, IA 52001 or equivalent

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2.2.1.1 Monolayers at least 7 days from last subculture

2.2.1.2 1 or 2 flasks for each cell type to be inoculated with MSV (MSV Flasks)

2.2.1.3 1 or 2 flasks for each cell type to be used as uninoculated negative control flasks (NC Flasks)

2.2.2 Alsever's Solution

2.2.2.1 8.0 g sodium citrate ( $C_6H_5Na_3O_7 \cdot 2H_2O$ )<sup>6</sup>

2.2.2.2 0.55 g citric acid ( $C_6H_8O_7 \cdot H_2O$ )<sup>7</sup>

2.2.2.3 4.2 g sodium chloride (NaCl)<sup>8</sup>

2.2.2.4 20.5 g glucose ( $C_6H_{12}O_6$ )<sup>9</sup>

2.2.2.5 Q.S. to 1000 ml with deionized water (DW).

2.2.2.6 Sterilize with a 0.22- $\mu$ m filter.<sup>10</sup>

2.2.2.7 Store at  $4^\circ \pm 2^\circ C$ .

2.2.3 Guinea pig and chicken RBCs in equal volumes of Alsever's Solution. Store at  $4^\circ \pm 2^\circ C$ .

2.2.4 0.01 M Phosphate buffered saline (PBS)

2.2.4.1 1.33 g sodium phosphate, dibasic, anhydrous ( $Na_2HPO_4$ )<sup>11</sup>

2.2.4.2 0.22 g sodium phosphate, monobasic, monohydrate ( $NaH_2PO_4 \cdot H_2O$ )<sup>12</sup>

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<sup>6</sup> Cat. No. S 4641, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

<sup>7</sup> Cat. No. C 7129, Sigma Chemical Co. or equivalent

<sup>8</sup> Cat. No. S 9625, Sigma Chemical Co. or equivalent

<sup>9</sup> Cat. No. G 8270, Sigma Chemical Co. or equivalent

<sup>10</sup> Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

<sup>11</sup> Cat. No. S 0876, Sigma Chemical Co. or equivalent

<sup>12</sup> Cat. No. S 9638, Sigma Chemical Co. or equivalent

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**2.2.4.3** 8.5 g NaCl

**2.2.4.4** Q.S. to 1000 ml with DW.

**2.2.4.5** Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)<sup>13</sup> or 1.0 N hydrochloric acid (HCl).<sup>14</sup>

**2.2.4.6** Sterilize by autoclaving at 15 psi, 121° ± 2°C for 35 ± 5 min.

**2.2.4.7** Store at 4° ± 2°C.

**2.2.5** Pipettes: 1 ml, 5 ml, and 25 ml<sup>15</sup>

**2.2.6** Conical tube,<sup>16</sup> 50 ml

**2.2.7** Cell culture flask,<sup>17</sup> 25 cm<sup>2</sup>

**3. Preparation for the test**

**3.1 Personnel qualifications/training**

Personnel shall have experience in the preparation and maintenance of cell culture and MSV extraneous agent testing as described in 9 CFR, Parts 113.46 and 113.55.

**3.2 Preparation of reagents/control procedures**

**3.2.1** Upon receipt of the RBCs, prepare Washed RBCs as follows:

**3.2.1.1** Transfer 20 ml of RBCs from each species into separate 50-ml conical tubes.

**3.2.1.2** Q.S. to 50 ml with Alsever's Solution.

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<sup>13</sup> Cat. No. 925-30, Sigma Chemical Co. or equivalent

<sup>14</sup> Cat. No. 920-1, Sigma Chemical Co. or equivalent

<sup>15</sup> Falcon® 7521, 7543, and 7525 respectively, Fisher Scientific Corp., 2000 Park Ln., Pittsburg, PA 15275 or equivalent

<sup>16</sup> Cat. No. 62.547, Sarstedt, Inc., P.O. Box 468, Newton, NC 28658-0468 or equivalent

<sup>17</sup> Cat. No. 430168, Corning Costar Corp., 45 Nagog Park, Acton, MA 01720 or equivalent

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**3.2.1.3** Mix by inverting several times.

**3.2.1.4** Centrifuge for  $15 \pm 5$  min at  $400 \times g$  (1500 rpm in a J6-B centrifuge with a JS-4.0 rotor).

**3.2.1.5** Remove supernatant and buffy coat by aspirating with a 25-ml pipette.

**3.2.1.6** Repeat **Sections 3.2.1.2 through 3.2.1.5** for a total of 3 washes.

**3.2.1.7** Store the packed RBCs in Alsever's solution at  $4^\circ \pm 2^\circ\text{C}$ .

**3.2.2** Preparation of 0.2% RBCs Mixture on the day of test

**3.2.2.1** Using a 25-ml pipette, measure 49.9 ml of PBS into a suitable container.

**3.2.2.2** Using 1-ml pipettes, add both 100  $\mu\text{l}$  of washed packed guinea pig RBCs and 100  $\mu\text{l}$  of washed packed chicken RBCs to **Section 3.2.2.1**. Gently swirl to mix.

**3.2.2.3** Store at  $4^\circ \pm 2^\circ\text{C}$ ; use within 1 wk of collection of RBCs.

**3.3 Preparation of the sample**

Both MSV Flasks and NC Flasks shall be tested at least 7 days from the last subculture for extraneous HAd agents using 1 or 2 MSV Flasks of each cell type and 1 or 2 NC Flasks of each cell type.

**4. Performance of the test**

The HAd is conducted at 2 temperatures:  $4^\circ \pm 2^\circ\text{C}$  and room temperature (RT)( $23^\circ \pm 2^\circ\text{C}$ ). This may be accomplished by using the same MSV Flasks and NC Flasks of each cell type for both

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temperatures (**Section 4.1**) OR separate MSV Flasks and NC Flasks of each cell type may be incubated simultaneously at each temperature (**Section 4.2**).

**4.1** Same flask used at both temperatures

**4.1.1** Decant media from the MSV Flasks and NC Flasks.

**4.1.2** Add 5 ml of PBS to each MSV Flask and NC Flask, swirl gently and decant. Repeat for a total of 3 washes.

**4.1.3** Gently swirl the 0.2% RBCs Mixture and then add 5 ml into each MSV Flask and NC Flask (**Section 3.2**).

**4.1.4** Incubate at  $4^{\circ} \pm 2^{\circ}\text{C}$  for  $25 \pm 5$  min.

**4.1.5** Decant the 0.2% RBCs Mixture.

**4.1.6** Repeat **Section 4.1.2**.

**4.1.7** Using an illumination box, compare each cell line MSV Flask to the corresponding cell line NC Flask. Look for areas of HAd of RBCs to the cell sheet.

**4.1.8** Using an inverted microscope at 100X, compare each cell line MSV Flask to the corresponding cell line NC Flask. Look for HAd of the individual RBCs to cell membranes.

**4.1.9** If no HAd is detected, repeat **Section 4.1.3**.

**4.1.10** Incubate at RT for  $25 \pm 5$  min.

**4.1.11** Repeat **Sections 4.1.5 through 4.1.8**.

**4.2** Separate flasks simultaneously at 2 temperatures

**4.2.1** Repeat **Sections 4.1.1 through 4.1.3** for 2 sets of flasks.

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**4.2.2** Incubate an MSV Flask and an NC Flask for each cell line at RT for  $25 \pm 5$  min. Incubate a separate MSV Flask and a separate NC Flask for each cell line at  $4^\circ \pm 2^\circ\text{C}$  for  $25 \pm 5$  min.

**4.2.3** Repeat **Sections 4.1.5 through 4.1.8** for each set of flasks.

## 5. Interpretation of the test results

### 5.1 Criteria for a valid test

**5.1.1** The NC Flasks shall contain no evidence of HAd.

**5.1.2** The NC Flasks and the MSV Flasks shall contain no evidence of bacterial or fungal contamination.

**5.1.3** The MSV Flasks shall contain no evidence of HAd 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** If the MSV Flask contains no evidence of HAd and the test is valid, the MSV is **SATISFACTORY**.

### 5.3 Retests

**5.3.1** If the initial test is valid and evidence of HAd is found, the test is repeated (1st retest), using only the cell line or lines found positive for HAd. The test is repeated using new vials 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 new vials of MSV.

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**5.3.3.1** If there is no evidence in the second and third valid tests (1st and 2nd retests) of HAD in the MSV Flask, the MSV is **SATISFACTORY**.

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

**6. Report of test results**

The test results are appropriately recorded and reported on a data sheet.

**7. References**

Code of Federal Regulations, Title 9, Parts 113.46, 113.51, 113.52 and 113.55, U.S. Government Printing Office, Washington, DC, 1999.

**8. Summary of revisions**

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. The following is a list of the significant changes made from the previous protocol:

- 8.1** Addition of retesting criteria (**Section 5.3**)