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Testing Protocol

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Supplemental Assay Method for Conducting the
Hemagglutination Inhibition Assay for Equine Influenza
Antibody

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method for determining the hemagglutination inhibition (HI) antibody titer of sera from guinea pigs vaccinated with type A equine influenza viruses as part of a potency test for veterinary vaccines.

Note: For this SAM, the dilution terminology of 1:10, 1:20, etc., specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Micropipettors: 200- μ l and 1000- μ l single channel, 5- to 50- μ l x 12-channel, and tips

2.1.2 Centrifuge and rotor (Model J6-B centrifuge and Model JS-4.0 rotor, Beckman Instruments Inc.)

2.1.3 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

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 Round-bottom plate, 96-well

2.2.2 Polystyrene tube, 12 x 75-mm

2.2.3 Conical tube, 50-ml

2.2.4 Pipettes, 2-ml and 25-ml

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2.2.5 Reagent reservoir

2.2.6 0.01 M Phosphate buffered saline (PBS) Media
30054

2.2.6.1 1.19 g sodium phosphate, dibasic,
anhydrous (Na_2HPO_4)

2.2.6.2 0.22 g sodium phosphate, monobasic,
monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

2.2.6.3 8.5 g sodium chloride (NaCl)

2.2.6.4 Q.S. to 1000 ml with distilled water
(DI).

2.2.6.5 Adjust pH to 7.2-7.6 with 0.1 N sodium
hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl).

2.2.6.6 Sterilize by autoclaving at 15 psi,
 $121^\circ \pm 2^\circ\text{C}$ for 35 ± 5 minutes.

2.2.6.7 Store at $2^\circ - 7^\circ\text{C}$.

2.2.7 10% Kaolin Suspension

2.2.7.1 10 g kaolin in 100 ml PBS

2.2.7.2 Store at $2^\circ - 7^\circ\text{C}$.

2.2.8 Alsever's Solution

2.2.8.1 8.0 g sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$)

2.2.8.2 0.55 g citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)

2.2.8.3 4.2 g NaCl

2.2.8.4 20.5 g glucose

2.2.8.5 Q.S. to 1000 ml with DI.

2.2.8.6 Sterilize with a $0.22\text{-}\mu\text{m}$ filter.

2.2.8.7 Store at $2^\circ - 7^\circ\text{C}$.

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2.2.9 Chicken red blood cells (RBC) from specific-pathogen-free chickens in an equal volume of Alsever's Solution. Store at 2°- 7°C.

2.2.10 Test Virus. Each manufacturer provides each strain of type A equine influenza virus present in the Test Serial that has been correlated to protection in a host animal immunogenicity trial. Each Test Virus is identified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production.

2.2.11 Equine influenza virus reference antiserum (Reference Serum) to serve as a positive control [available from the Center for Veterinary Biologics (CVB)]

3. Preparation for the assay

3.1 Personnel qualifications/training

Personnel shall have training in hemagglutination (HA) and HI techniques and in standard laboratory procedures.

3.2 Preparation of reagents/control procedures

3.2.1 Upon receipt of the RBC, prepare Washed RBC as follows:

3.2.1.1 Transfer 20 ml of RBC into a 50-ml conical tube.

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

3.2.1.3 Mix by inverting several times.

3.2.1.4 Centrifuge for 10 minutes at 400 X g (1500 rpm in the J6-B centrifuge with a JS-4.0 rotor).

3.2.1.5 Remove supernatant and white blood cell layer 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.

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3.2.1.7 Store the packed RBC in Alsever's Solution at 2°- 7°C; use within 1 week of collection of RBC.

3.2.2 0.5% RBC Suspension for the HA or HI assays. Pipette 500 µl of packed Washed RBC into 100 ml of PBS. Store at 2°- 7°C; use within 1 week of collection of RBC.

3.2.3 5% RBC Suspension for removal of nonspecific agglutinins from guinea pig sera (GPS). Pipette 100 µl of packed Washed RBC into 1.9 ml of PBS. Store at 2°- 7°C; use within 1 week of collection of RBC.

3.2.4 Test Virus Working Dilution. Each Test Virus should contain 4-8 HA units (HAU) per 25 µl. The Test Virus Working Dilution is determined by an HA assay and verified by back titration for each HI test performed.

3.2.4.1 On the day of test initiation, prepare a series of twofold dilutions of each Test Virus in duplicate from undiluted through 1:2048 in a 96-well, round-bottom plate (See Template, **Appendix I**). The same pipette tips may be used throughout the dilution scheme.

1. Pipette 50 µl/well of PBS into columns 2-12 with a 12-channel micropipettor.
2. Thaw frozen Test Virus at room temperature. Add 100 µl of undiluted Test Virus to wells A1 and B1. Additional Test Viruses are tested in duplicate in rows C through F.
3. Transfer 50 µl from column 1 to column 2 with a 12-channel micropipettor; mix (7 ± 2 fills by aspiration and expulsion of the 12-channel micropipettor).
4. Continue **Section 3.2.4.1(3)** for columns 3-12, transferring 50 µl from the previous well to next well in the column until the dilution sequence is completed. Remove and discard 50 µl from the last column.

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5. Add 50 μ l of 0.5% RBC to each well containing Test Virus.
6. Place 50 μ l of the 0.5% RBC Suspension in each of 3 wells as an RBC Control. Add 50 μ l of PBS to each of the 3 RBC Control wells.
7. Mix by tapping the edge of the plate with fingers. Incubate the plate uncovered for 35 \pm 10 minutes at room temperature. The optimum time to evaluate results is when distinct buttons have formed in the RBC Control wells.
8. Read the assay as follows: Record "+" for complete agglutination (hazy appearance to the RBC) and "-" for no agglutination (distinct button) and \pm for incomplete agglutination. To confirm specificity of agglutination, tilt the plate at a 45° angle for 20-30 seconds. If the settled RBC's "run" or form a tear-drop appearance, that particular well is considered to be negative for agglutination.
9. The endpoint HA titer is considered to be the highest dilution of virus that completely agglutinates the RBCs. Wells with partial (incomplete) agglutination are not considered when determining endpoint values. The RBC diluent control wells should have distinct buttons. The endpoint HA titer is considered to be the dilution of Test Virus that contains 1 HA unit (HAU) per 50 μ l. A virus concentration of 4-8 HAU per 25 μ l is used for the HI assay. In order to determine a dilution factor of the Test Virus containing 4-8 HAU per 25 μ l, the endpoint dilution is divided by 12. For example, if the endpoint titer of the Test Virus is 256, the dilution containing 4-8 HAU per 25 μ l would be approximately 1:20 (256 divided by 12). The concentration of virus in this preparation (working dilution) should be confirmed by a virus back titration prior to performing the HI assay and is repeated when the HI portion of the assay is conducted.

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3.3 Preparation of the sample

3.3.1 Guinea pig sera from test animals may be stored frozen indefinitely at $-20^{\circ}\pm 2^{\circ}\text{C}$. Treated serum may be stored at 2° - 7°C for up to 48 hours, or frozen indefinitely at $-20^{\circ}\pm 2^{\circ}\text{C}$.

3.3.2 Treatment of GPS samples for the removal of nonspecific inhibitors

3.3.2.1 On the day of test initiation, pipette 200 μl of each GPS into individually labeled 12 x 75-mm polystyrene tubes, one tube for each serum to be tested.

3.3.2.2 Pipette 1.0 ml of 10% Kaolin Suspension (keep kaolin in suspension by periodic shaking) to each tube.

3.3.2.3 Vortex the kaolin/serum mixture tubes on high speed to resuspend the kaolin every 5 minutes for 20 ± 5 minutes.

3.3.2.4 Centrifuge at 800 X g (2000 rpm in the J6-B centrifuge with a JS-4.0 rotor) for 20 ± 5 minutes.

3.3.2.5 Pipette 100 μl 5% RBC to each tube.

3.3.2.6 Pipette 700 μl PBS to each tube.

3.3.2.7 Gently shake the rack of tubes every 5 minutes for 20 ± 5 minutes to keep the RBCs in suspension. The kaolin pellet should not be disturbed by this treatment.

3.3.2.8 Centrifuge at 400 X g (1500 rpm in the J6-B centrifuge with a JS-4.0 rotor) for 20 ± 5 minutes. The RBC pellet will pack down on top of the kaolin pellet.

3.3.2.9 Pour the supernatant from each treated GPS to a clean, labeled 12 x 75-mm polystyrene tube. This is considered to be a 1:10 dilution of Treated GPS.

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4. Performance of the HI assay

4.1 Pipette 50 μ l each of the Treated GPS into the first well of 2 adjacent columns on a 96-well, round-bottom plate for each Test Virus (See Template, **Appendix II**). This is the Sample Test Plate.

4.2 Pipette 25 μ l of PBS into the remaining wells of each column with the 12-channel micropipettor.

4.3 Twofold dilutions are made as follows:

4.3.1 Transfer 25 μ l of serum from row 1 to row 2; mix (7 \pm 2 fills by aspiration and expulsion of the 12-channel micropipettor).

4.3.2 Continue the 25- μ l transfer until all dilutions have been made through row 8. Remove and discard 25 μ l from the last row.

4.4 Add 25 μ l of the Test Virus Working Dilution (4-8 HAU/25 μ l) (**Section 3.2.4**) to each well.

4.5 Virus Back Titration is performed for each Test Virus (See Template, **Appendix II**).

4.5.1 Add 100 μ l of the Test Virus Working Dilution in the first wells of 2 adjacent columns of the Sample Test Plate (such as A11 and A12).

4.5.2 Add 50 μ l of PBS in each of the 5 wells in the rows below the first wells of the 2 adjacent columns (B11 and B12 through F11 and F12).

4.5.3 Transfer 50 μ l from duplicate wells of row 1 to row 2; mix (7 \pm 2 fills by aspirating and expulsion of the 12-channel micropipettor).

4.5.4 Continue **Section 4.5.3** for rows 3 through 6, transferring 50 μ l from the previous wells to the next wells in the column. Remove and discard 50 μ l from the last row used.

4.6 Place 25 μ l of each GPS in an individual well as an autoagglutination control (GPS Autoagglutination Control). Add 25 μ l of PBS to each of these GPS control wells.

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4.7 Mix by tapping the edge of the plate with fingers. Incubate the plate uncovered for 30 ± 5 minutes at room temperature.

4.8 Add 50 μ l of PBS to each of the 3 RBC Control wells and place 50 μ l of the 0.5% RBC Suspension in each of the 3 wells.

4.9 Add 50 μ l of 0.5% RBC to each well containing the Treated GPS/Test Virus mixture, the Virus Back Titration, or the GPS Autoagglutination Control.

4.10 Mix by tapping the edge of the plate with fingers. Incubate the plate uncovered for 35 ± 10 minutes at room temperature. The optimum time to evaluate results is when distinct buttons have formed in the RBC Control wells.

4.11 Read the agglutination results as follows:

"+" partial to complete agglutination (hazy appearance) indicates absence of antibody in the GPS.

"-" no agglutination (distinct button) indicates presence of antibody in the GPS.

4.12 The HI titer of the GPS is the reciprocal of the highest serum dilution with a distinct button of RBC in both test wells.

4.13 Group the test sera based on the vaccination serial. Determine the geometric mean titer (GMT) for each Test Virus from each group.

5. Interpretation of the HI results

5.1 Validity requirements

5.1.1 The Virus Back Titration must show complete agglutination in the first 3 dilutions, partial to full agglutination in the 4th dilution, and weak partial to no agglutination in the 5th and 6th dilution.

5.1.2 For a given fraction, the unvaccinated GPS must remain seronegative at the 1:10 dilution.

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5.1.3 None of the GPS Autoagglutination Control wells may exhibit autoagglutination.

5.1.4 The titer of the Reference Serum must fall within ± 1 dilution of its recorded titer, as determined by a minimum of 10 previous titrations.

5.1.5 If these validity requirements are not met, the test is **NO TEST** and may be repeated.

5.2 If the GMT of the GPS of a Test Serial for a given fraction in an initial valid test is equal to or greater than the requirements stated in the APHIS filed Outline of Production, the Test Serial is **SATISFACTORY**.

5.3 If the GMT of the GPS of a Test Serial in an initial valid test, for a given fraction, is less than the GMT specified in the APHIS filed Outline of Production, the Test Serial may be retested in an equivalent number of additional animals as the initial test tested.

5.3.1 If the GMT of the GPS from all vaccinated animals of a Test Serial in the initial valid test and the valid retest, for a given fraction, is equal to or greater than the requirements stated in the APHIS filed Outline of Production, the Test Serial is **SATISFACTORY**.

5.3.2 If the GMT of the GPS from all vaccinated animals of a Test Serial in the initial valid test and the valid retest, for a given fraction, is less than the GMT specified in the APHIS filed Outline of Production, the Test Serial is **UNSATISFACTORY**.

6. Report of the HI results

Record all test results on the test record.

7. References

7.1 Conrath TB. *Handbook of Microtiter Procedures*. Clinical and Research Applications Laboratory, Cooke Engineering Co., Alexandria, VA, 1972.

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7.2 Snedecor GW and Cochran WG. *Statistical Methods*, 6th ed., Iowa State University Press, Ames, IA, Chpt 11, 1967.

8. Summary of revisions

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:

- **3.2.4(8)** Clarification of agglutination specificity confirmation has been added.
- **3.2.4(9)** Clarification of endpoint HA titers has been added.
- 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.

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9. Appendices

Appendix I

Standardization of Test Viruses Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	A 1											
B	A 1											
C	A 2											
D	A 2											
E	A 3											
F	A 3											
G	UN	1:2	1:4	1:8	1:16	1:32	1:64	128	256	512	1024	2048
H	RBC	RBC	RBC									

A 1= Type A Influenza Isolate No. 1, etc. RBC= RBC Control

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Appendix II

Sample Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 1:10	GPS 1	GPS 1	GPS 2	GPS 2	GPS 3	GPS 3	GPS 4	GPS 4	GPS 5	GPS 5	BT	BT
B 1:20											1:2	1:2
C 1:40											1:4	1:4
D 1:80											1:8	1:8
E 1:160											1:16	1:16
F 1:320											1:32	1:32
G 1:640											RBC	RBC
H 1:1280											RBC	RBC

GPS= Treated Guinea Pig Serum

BT= Back Titration of the Test Virus Working Dilution

RBC= RBC Control