

United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Detection of Hemagglutinating Viruses

Date: **January 9, 2018**

Number: VIRPRO0096.06

Supersedes: VIRPRO0096.05, July 17, 2014

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Detection of Hemagglutinating Viruses

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Detection of Hemagglutinating Viruses

1. Introduction

This Testing Protocol (PRO) describes the procedures used to detect and quantitate the presence of extraneous Newcastle disease virus (NDV) or other hemagglutinating (HA) viruses that may be present in veterinary vaccines or master seeds (MS) other than live NDV MS or vaccines. Testing for extraneous HA viruses is required by title 9, *Code of Federal Regulations* (9 CFR), section 113.34, for MS testing, special request testing, or serial release testing of all products of chicken embryo origin. Note that HA is used interchangeably in this protocol as hemagglutination or hemagglutinating.

2. Materials

2.1 Equipment/instrumentation

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

- 2.1.1 Humidified, rotating egg incubator (Midwest Incubators, Model 252)
- 2.1.2 Laminar Flow Class II Biological Safety Cabinet (NuAire Inc., Labgard)
- 2.1.3 Freezers ($-20^{\circ}\pm 5^{\circ}\text{C}$, -65°C or colder)
- 2.1.4 Refrigerator ($4^{\circ}\pm 2^{\circ}\text{C}$)
- 2.1.5 Low speed, refrigerated centrifuge (Beckman J6-MI centrifuge with JS 4.2 rotor)
- 2.1.6 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)
- 2.1.7 Electric engraver (Acme Burgess Inc., Model 74)
- 2.1.8 Digital timer
- 2.1.9 (Optional) Vacuum source for aspirating liquids (vacuum pump with sidearm flask or Chapman-Type filter pump attached to a water line)
- 2.1.10 Single and multi-channel micropipettes (25- μL to 250- μL)
- 2.1.11 Various sizes of test tube racks
- 2.1.12 Pipette Aid or pipette filler (bulb type)

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2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents, glassware, and expendables should be sterile.

2.2.1 Specific-Pathogen Free (SPF) embryonated chicken eggs, 9- to 11-day-old

2.2.2 Phosphate-buffered saline (PBS), 0.1 M, pH 7.2 ± 0.1 (National Centers for Animal Health (NCAH) Media #30054)

1. 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
2. 0.22 g sodium phosphate, monobasic, monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)
3. 8.5 g sodium chloride (NaCl)
4. Q.S. to 1000 mL with distilled water (DW).
5. Adjust pH to 7.2 with 0.1 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl).
6. Sterilize by autoclaving at 15 psi, $121^\circ \pm 2^\circ\text{C}$ for 35 ± 5 minutes.
7. Store at 4° to $25^\circ \pm 2^\circ\text{C}$.

2.2.3 Alsever's solution (NCAH Media #20031)

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. 0.55 g citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)
3. 4.2 g NaCl
4. 20.5 g dextrose
5. Q.S. to 1000 mL with DW.
6. Filter sterilize with 0.22- μm filter.
7. Store at $4^\circ \pm 2^\circ\text{C}$.

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2.2.4 Chicken blood from SPF roosters in an equal volume of Alsever's Solution. Store for up to 2 weeks at $4^{\circ}\pm 2^{\circ}\text{C}$.

Note: Turkey blood may be substituted for chicken if swine influenza virus is suspected as a contaminant. Washing of turkey blood would be the same as for chicken blood in Section 3.3.

2.2.5 Known positive NDV control antigen for hemagglutination (HA) and hemagglutination-inhibition (HI) tests. Optional: Known positive control antigen for other type A influenza viruses, such as: avian, swine, equine, and canine influenza.

2.2.6 Known positive and negative NDV control antiserums for HI test. Optional: Known positive control antiserums for other type A influenza viruses, such as: avian, swine, equine, and canine influenza

2.2.7 U-bottom microtiter plates (96-well) and plate covers

2.2.8 Microtiter plate sealing tape

2.2.9 Reagent reservoirs

2.2.10 Glassware/plastic ware

- Pipettes (serologic, 1-mL, 5-mL, 10-mL, 25-mL)
- Micro pipette tips filtered and non-filtered (200- μL)
- Graduated cylinder (100-mL)
- 13 x 75-mm snap-cap tubes
- 50-mL conical centrifuge tubes
- Erlenmeyer flasks

2.2.11 Latex or nitrile surgical gloves

2.2.12 Syringes, 1-cc and 3-cc single use

2.2.13 Needles, 25-gauge x 5/8-inch 18-gauge x 1-inch, 16- to 22-gauge x 1 1/2-inch (Becton Dickinson & Co.)

2.2.14 Iodine 2% in alcohol, NCAH Media #30013

2.2.15 70% alcohol (70 mL ethyl alcohol + 30 mL water)

2.2.16 Cotton balls/cotton swabs

2.2.17 Duco cement

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2.2.18 Water, distilled or deionized, or water of equivalent purity

2.2.19 Clean, sterile glassware of appropriate size and type

2.2.20 Mirrored microtiter plate reader

3. Preparation for the Test

3.1 Personnel qualifications/training

The laboratory personnel must have experience with or training in this protocol. Personnel must be familiar with aseptic biological laboratory techniques and proper handling, diluting, pipetting, storing, and disposal of test reagents and biological materials. Personnel must have knowledge of safe operating procedures and policies.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer's instructions and monitor for compliance with current corresponding standard operating policies/procedures (SOPs).

3.3 Preparation of reagents/control procedures

Note: Washing and preparing the chicken red blood cell suspension is not actually done until just prior to or after the amnio-allantoic fluid (AAF) has been collected from the eggs in Section 4.3. Turkey blood is washed and standardized in the same manner as the chicken blood.

3.3.1 Washing chicken red blood cells (RBCs)

1. Dispense 10-20 mL rooster blood preserved in Alsever's solution into a 50-mL conical centrifuge tube.
2. Fill the tube with PBS to the 45-50 mL mark and gently invert the tube several times to wash the RBCs.
3. Centrifuge at 800 x g (1,800 rpm in a Beckman J6-MI centrifuge with JS 4.2 rotor) for 10 minutes at $4^{\circ} \pm 2^{\circ}\text{C}$.
4. Aspirate PBS and buffy coat (bone-colored layer of cells on top of red blood cells) from the tube using a pipette or vacuum-type aspirator.
5. Repeat **Steps 2 to 4** for a total of 3 washes. Washed RBCs can be stored at $4^{\circ} \pm 2^{\circ}\text{C}$ for up to 1 week.

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3.3.2 Preparation of 0.5% RBC suspension

1. Dispense 199 mL PBS into an appropriately labeled 250-mL Erlenmeyer flask.
2. Using a 1- or 2-mL pipette, add 1 mL washed packed RBCs to the PBS, rinsing the pipette thoroughly to remove all RBCs.
3. The 0.5% RBC suspension can be stored for up to 1 week at $4^{\circ} \pm 2^{\circ}\text{C}$. Discard if hemolysis is observed.

Note: The amount of standardized RBC suspension can be adjusted depending on quantity needed per test. For example 99.5 mL PBS and 0.5 mL RBCs, etc.

3.3.3 Preparation of NDV positive control antigen for HA/HI

Use a standardized NDV positive reference/control antigen (virus) for a positive control for the HA test. To conserve antigen, dilute NDV antigen with PBS to a concentration of 8 HA units (HAU) per 50 μL or 4 HAU per 25 μL and store at 4°C . See **Section 4.4** for HA test procedures and substitute antigen in place of AAF.

3.3.4 Preparation of known positive NDV control antiserum for HI

Use an NDV positive reference/control antiserum for the HI test. Dilute positive control serum to a titer between 1:16 and 1:64 with PBS. The titer is determined by HI test with 4 HAU/25 μL of homologous antigen (see **Sections 4.4 and 4.5** for standardization of antigen and HI testing).

3.4 Preparation of eggs

3.4.1 Use a minimum of ten 9- to 11-day-old SPF embryonated eggs per serial of vaccine or MSV. Prepare the eggs in accordance with the current version of **VIRSOP0022**, *Chicken Egg Preparation and Inoculation: Allantoic Route*. Make an additional line on the egg shell opposite from the inoculation site at the level of the air cell. This will be the drill site at the time of AAF collection.

3.4.2 Fill out an egg test worksheet (see the current version of **VIRTWS0007**, *Hemagglutination (HA) Testing of Vaccine, Egg Inoculation*). Label each egg on the side of the egg with the sample identification using a permanent marker. The label markings must correspond with the ones on the worksheet. Label a minimum of 5 eggs as normal controls.

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3.5 Preparation of the sample

3.5.1 Obtain and store samples for testing as described in the current version of **VIRWI2040**, *Sample Tracking in the CVB Virology Section*.

3.5.2 Vaccines to be tested may be either lyophilized, frozen, or liquid. The lyophilized products are reconstituted with the firm's diluent as recommended on the label or with sterile distilled water. Dilute vaccines with 30 mL of sterile distilled water per 1,000 doses and use as inoculum. (See **Section 3.5.4** for treatment of MS.)

Some vial doses are larger than 1000 and require additional dilutions to reach the proper use dilution for inoculation. Dilute as in Table 1.

Table 1

Number of doses/vial	Reconstituted volume of vaccine	Final Dilution
500	15 mL	None
1,000	30 mL	None
2,000	60 mL	None
2,500	75 mL	None
5,000	150 mL	None
10,000	300 mL	None
15,000	100 mL	2.0 mL vaccine + 7.0 mL sterile water
20,000	100 mL	1.5 mL vaccine + 7.5 mL sterile water
25,000	100 mL	1.0 mL vaccine + 6.5 mL sterile water

Mix these dilutions well by vortexing and keep reconstituted and diluted vaccines on ice for at least 15 minutes before egg inoculation.

3.5.3 All oil-based killed virus vaccines are to be used as is with no further dilution.

3.5.4 Master seeds may be diluted 1:10 in tryptose phosphate broth and may be mixed with equal parts of homologous antiserum and incubated at $37^{\circ}\pm 2^{\circ}\text{C}$ for 60 minutes in order to neutralize the MS prior to testing in eggs.

Note: Neutralization is necessary for testing hemagglutinating MS viruses such as avian and mammalian type A influenza as per the current version of VIRSOP2007, Master Seed Testing in the Virology Section.

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4. Performance of the Test

4.1 Egg inoculation

Note: Eggs should be incubated for 4 to 5 days after inoculation. It is best to inoculate eggs so that 24-hour deaths can be removed and discarded and live embryos can be refrigerated at 4 to 5 days postinoculation. Egg inoculation should be performed in a biological safety cabinet.

4.1.1 Retrieve the labeled eggs from the egg incubator. Refer to the current version of **VIRSOP0022** for proper inoculation technique for this test. Disinfect the egg shell over the drilling mark with 2% tincture of iodine and drill a small hole into the shell with an electric engraving tool.

4.1.2 Use a 5/8-inch, 25-gauge needle with a 1-cc tuberculin syringe and inoculate 0.2 mL into each of the eggs marked for that vaccine or MSV. When inoculating an oil-based killed virus vaccine, use an 1-inch, 18-gauge needle with the 1-cc tuberculin syringe and inoculate 0.2 mL into each egg.

4.1.3 Seal the eggs with Duco cement and be careful not to let the cement run down the side of the egg because it will cause the egg shell to stick to the paper egg flat.

4.2 Egg incubation

4.2.1 Place the egg flat in the $37^{\circ}\pm 2^{\circ}\text{C}$ humidified egg incubator basket for 24 hours postinoculation. At the end of the 24-hour postinoculation period, candle the eggs, record and discard all the dead eggs. The 24-hour deaths are considered to be due to trauma. After the candling, the eggs may be placed into an incubator rotating tray for the remaining time of the test.

Note: At the completion of the incubation period, there must be at least 7 viable eggs to complete a valid test. If there are less than 7 viable eggs left, then the test is considered a no test and should be repeated.

4.2.2 Candle all eggs daily (inoculated and negative controls). It is not necessary to candle eggs on weekends or holidays. Continue to record any deaths occurring after 24 hours and place these embryos in the walk-in cooler or refrigerator at $4^{\circ}\pm 2^{\circ}\text{C}$.

4.2.3 On the 4th or 5th day postinoculation, place all remaining viable embryos (both inoculated and negative controls) in a refrigerated unit and hold at $4^{\circ}\pm 2^{\circ}\text{C}$ for a minimum of 2 hours. Cooling will increase the transparency of the chorio-allantoic membrane to facilitate proper allantoic fluid collection and reduce hemorrhage into fluids.

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4.3 Collection of AAF from eggs

Note: The chicken RBCs can be washed and a 0.5% suspension made, as per Sections 3.3.1 and 3.3.2, at this time or just prior to HA testing.

4.3.1 Prior to collecting AAF, fill out a microtiter HA/HI test worksheet, in ink (see the current version of **VIRTWS0019, HA/HI Test Worksheet**). Label a microtiter plate(s) to correspond with the worksheet. One additional row of wells should be included for a known positive HA control antigen (usually Newcastle disease). Also include 3 or more wells for an RBC cell control (CC). See **Appendix I** for an example of plate set up.

4.3.2 After eggs are chilled (see **Section 4.2.3**), remove from refrigeration and place in a biological safety cabinet. Disinfect the top of the egg shell with 70% alcohol. Using the electric engraver, etch a small hole in the shell above the previously marked air cell line.

4.3.3 Using a 16- to 22-gauge x 1 1/2-inch needle attached to a 3-cc syringe, insert the needle horizontally into the hole then tip the needle down at a 45° angle. This should place the tip of the needle in the allantoic cavity. Draw up to 3 mL of the clear fluid. The amount collected depends on whether it is desired to save some of the sample in a tube or if the sample is to be added directly into a collection well of a microtiter test plate.

Note: If yolk or blood gets drawn up in the syringe, discard it and try again in another area of the egg. If AAF cannot be collected with a needle and syringe, it may be necessary to remove part of the egg shell over the air cell with a sterile forceps and use a pipette to collect a sample.

4.3.4 If AAF sample is to be collected directly into a 96-well microtiter plate for screening then add PBS to the plate first. Using a multi-channel micropipette, dispense 50 µL of PBS per well into all wells, except for the AAF collection wells. After adding PBS, collect and dispense AAF samples into collection wells of a 96-well microtiter plate or into an appropriately labeled tube if the sample is to be saved. For AAF samples collected in tubes, PBS is added to plates prior to testing.

Important: If collecting samples into a 96-well microtiter plate, save the eggs. If the sample is HA positive, additional AAF is collected and a hemagglutination-inhibition (HI) test is performed.

4.3.5 Repeat **Sections 4.3.3 and 4.3.4** for each sample. Be sure to use a new syringe and needle for each egg. If samples are collected in tubes, store them at 4°C until ready to test by HA.

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4.4 Hemagglutination (HA) test

4.4.1 If AAF was collected directly into the plate, go to **Section 4.4.4**.
If the samples were collected in tubes, then number the tubes to match the HA test worksheet and proceed to the next **Section 4.4.2**.

4.4.2 Using a multi-channel micropipette, add 50 μ L of PBS per well into all wells of the 96-well plate, including the CC wells.

4.4.3 Using a single channel micropipette and filtered tips, add 50 μ L of each sample to the first well in each row that corresponds to that sample (either test or normal control sample). Also include a positive control antigen (see **Section 3.3.3**).

4.4.4. Add 50 μ L of positive control antigen to the first well of the row that was marked as a positive control.

4.4.5 Using filtered tips and a multi-channel micropipette set to transfer 50 μ L, make serial twofold dilutions of each sample from well 1 through the last well of each row. If the first well(s) were used as a collection well, then use the multi-channel micropipette to transfer 50 μ L to well 2 in each row and then make serial twofold dilutions. Discard 50 μ L from the last well after mixing and discard tips. Repeat for each test plate. The resulting dilutions will range from 1:2 in the first well to 1:256 if samples were collected in tubes, or 1:2 in the second well, to 1:128 in the 8th well, if the first well in each row was used as a collection well. Typically AAF samples are carried out 8 wells. However, the samples can be carried out 12 wells (1:2 to 1:2,048 or 1:4,096 dilutions) to capture endpoints.

4.4.6 Using a multi-channel micropipette, add 50 μ L of 0.5% RBC suspension to each well, including the CC wells, and gently shake/agitate the plate on a flat surface to thoroughly mix.

Important: Keep RBCs thoroughly suspended during the dispensing process.

4.4.7 Cover the plate with microtiter plate sealing tape and incubate at room temperature until distinct buttons have formed in the CC wells (which usually takes 20 to 30 minutes).

4.4.8 Record results on the worksheet as follows:

+ = (positive HA) wells with complete hemagglutination

- = (negative HA) wells with a distinct button formation

I = (incomplete HA) wells with partial button formation of fuzzy margins, donut-like appearance

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4.4.9 If the sample is HA positive, the last well (highest dilution of antigen) with **complete** hemagglutination is the endpoint of the titration. See **Appendix II** for an example of an HA test.

4.4.10 The endpoint dilution of an HA positive sample is considered to be 1 HA unit (HAU); 8 HA units in 50 μ L or 4 HAU in 25 μ L are used in the HI test. The dilution containing 8 HA units in 50 μ L is determined by dividing the endpoint by 8 (the desired number of HAUs in 50 μ L), e.g., if the HA endpoint titer was 1:128, the antigen dilution which would contain 8 HA units in 50 μ L should be 1:16 ($128 \div 8$).

Note: For this protocol, dilution terminology of 1:16, 1:24, 1:32, etc., specifies 1 part plus 15 parts (liquid), 1 part plus 23 parts, etc.

4.4.11 For HI testing of HA positive AAF samples, prepare a small quantity (> .4 mL) of each sample diluted to 8 HAU in 50 μ L using PBS as diluent. This will become the standardized AAF sample after confirmation of virus concentration. (If samples were collected directly into a microtiter plate for HA testing, then AAF will need to be collected into a snap cap tube from each egg that that was HA positive.)

4.4.12 The AAF sample dilutions are then retested by HA as per **Sections 4.4.2 to 4.4.8** to confirm the correct concentration of virus. This is also known as a back titration and should be done the same day that the HI test is performed.

Note: If the sample dilution contains 8 HAU in 50 μ L, there should be complete hemagglutination in the first 3 wells (1:2, 1:4, and 1:8 dilutions) and there can be partial (incomplete) or no hemagglutination in the fourth and fifth wells.

4.4.13 If the diluted sample(s) contains 8 HAU in 50 μ L, it is ready to be tested by HI. If the sample dilution does not exhibit the correct number of HAU (too strong or weak), make adjustments in the dilution and run another HA (back titration).

4.5 Hemagglutination-inhibition (HI) test

If an AAF sample is HA positive it should be further tested by HI to identify the agent causing hemagglutination. Typically the HI test is performed using NDV antiserum to confirm the presence of NDV. If the AAF is NDV HI test negative, the AAF may be further tested by HI using specific antisera for other hemagglutinating viruses such as avian, swine, equine, or canine, type A influenza viruses.

4.5.1 Fill out a microtiter HA/HI test worksheet, in ink (see the current version of **VIRTWS0019**). Label a microtiter plate(s) to correspond with the worksheet.

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At least one additional row of wells should be included for a known positive HI control (usually Newcastle disease). Also include 3 or more wells for CC. See **Appendix III** for example of HI plate set up.

4.5.2 Leaving the first well empty in each row, dispense 25 μ L of PBS into all test wells of each HI plate including the positive control serum wells. Add 50 μ L of PBS to the CC wells.

4.5.3 Add 50 μ L of NDV positive reference serum (see **Section 3.3.4**) to the first well in each row.

4.5.4 Using a multi-channel micropipette set to transfer 25 μ L, make twofold serial dilutions of the serum beginning with the first well through the last well of each row.

4.5.5 Add 25 μ L of the standardized AAF sample to each well of the corresponding row for that sample. See **Sections 4.4.11 through 4.4.13** for standardization of HA positive (AAF) samples to 8 HAU/50 μ L. Continue to do this for each standardized AAF sample and also for the standardized NDV positive reference antigen.

4.5.6 Gently shake/agitate the plate on a flat surface to thoroughly mix. Cover each plate (plates can be stacked) and incubate for 30 minutes at room temperature.

4.5.7 After incubation, add 50 μ L of the 0.5% RBC suspension to each well and gently shake/agitate the plate to thoroughly mix.

Note: Keep RBCs thoroughly suspended during the dispensing process.

4.5.8 Cover the plate with microtiter plate sealing tape and incubate at room temperature until distinct buttons have formed in the CC wells (usually takes 20 to 30 minutes).

4.5.9 Record results on the worksheet as follows:

+ = wells with complete hemagglutination (negative for inhibition of HA)

- = wells with a distinct button formation (positive for inhibition of HA)

I = (incomplete HA) wells with partial button formation of fuzzy margins, or donut-like appearance

When interpretation between complete and incomplete inhibition is doubtful, tilt the microtiter plate at about a 45° angle for 20 to 30 seconds and look for a “tear

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drop” appearance of RBCs in the wells with complete inhibition. Wells with partial inhibition (incomplete HA) will not “tear drop”.

4.5.10 Samples are considered HI positive if complete **inhibition** of hemagglutination is observed. The button (inhibition of hemagglutination) is recorded using a minus sign (-) just as for the HA test, but it means that the sample is HI positive. Complete hemagglutination is recorded with a plus (+) sign (the same as for the HA test), but is negative on the HI test. Endpoints are reported as the highest serum dilution resulting in **complete inhibition** of hemagglutination. See **Appendix IV** for an example of an HI test

5. Interpretation of HA/HI Test Results

5.1 An HA test is considered valid if:

- The HA titer for the NDV positive reference/control antigen is within the acceptable range as determined by previous titrations;
- There is no hemagglutination with the AAF samples from the negative control eggs; and
- The CC wells exhibit well-defined buttons.

If these conditions are not met the test is not valid and should be repeated.

5.2 An HI test is considered valid if:

- The HA positive diluted sample and NDV reference/control antigen contains the correct number of HAUs (8 in 50 μ L or 4 in 25 μ L) as determined by the back titration;
- The expected HI titer for the NDV positive reference serum is within the acceptable range as determined by previous titrations; and
- The CC wells exhibit well-defined buttons.

If these conditions are not met the test is not valid and should be repeated.

5.3 If RBCs in the CC wells do not exhibit a well-defined button, check the following as possible causes:

- Incorrect formulation of PBS
- Excessive moisture evaporation from plates during the test
- RBCs are too old
- Incorrect concentration of RBCs
- RBC suspension isn't thoroughly mixed
- Incorrect formulation of Alsever's solution

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6. Report of HA/HI Test Results

6.1 Results are reported out as either negative or positive for the detection of HA viruses.

6.2 A satisfactory test result occurs when no HA activity is detected in any of the vaccine or MS inoculated eggs.

6.3 If the sample is HA positive, it is unsatisfactory and is further tested by HI to identify the extraneous agent causing hemagglutination.

Report results as negative or positive, as described in the current version of **VIRSOP0027**, *Testing Roles, Responsibilities, and Procedures for Reporting Test Results in the Virology Section*.

7. References

Title 9, *Code of Federal Regulations*, Subchapter E., section 113.34, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .06

- The Contact information has been updated.
- **3.5.1:** Replaced VIRSOP2041 (inactivated) with VIRWI2040.
- Updated dilution table and nomenclature.

Version .05

- The Contact information has been updated.

Version .04

- The Contact information has been updated.
- **3.5.1:** The reference document information has been updated.
- **6.0:** The reference document information has been updated.

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Version .03

- The Contact information has been updated.
- **2.1.5:** This section has been updated with the current model of Beckman centrifuge.
- **3.3:** Turkey blood, in addition to chicken blood, has been added.
- **3.5.4:** Changed the incubation temperature and time to reflect current Virology Section policies.
- Minor changes have been made throughout the remainder of the document to add clarification and detail to test procedures.
- Reference documents have been updated through the document.

Version .02

- The document number has been changed from PYSOP0096 to VIRSOP0096.
- Amy Shafer has been added as a Contact for this document.
- **2:** Significant changes have been made to include a more detailed list of reagents and supplies required for new test procedures.
- **3.3:** Significant changes have been made to collection and preparation of chicken RBC suspension.
- **3.3 and 3.4:** These sections have been changed in sequence and include changes in preparation and standardization of test control reagents.
- **3.5.4:** This section concerning MS testing has been added.
- **4.4:** Major changes have been made in the procedure used for HA testing. This procedure changed from a rapid plate test (Minnesota testing box) to a microtiter HA test.
- **4.5:** A microtiter HI test has been added.
- **5:** This section has been changed to add more detail to test validity requirements
- **6.3:** This section has been added to include HI testing to report of results

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- Minor changes have been made to the remainder of the document to add more detail and clarification to test procedures.
- The worksheet which had been included as an Appendix in the previous version has been removed and is now an independent document (**VIRTWS0096**).
- References to **VIRTWS0019** have been added to the document.
- Appendices have been added to include HA and HI plate and test examples.

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Appendix I

Example of test plate for HA test (1st well in each row used for sample collection)

AAF dilutions →

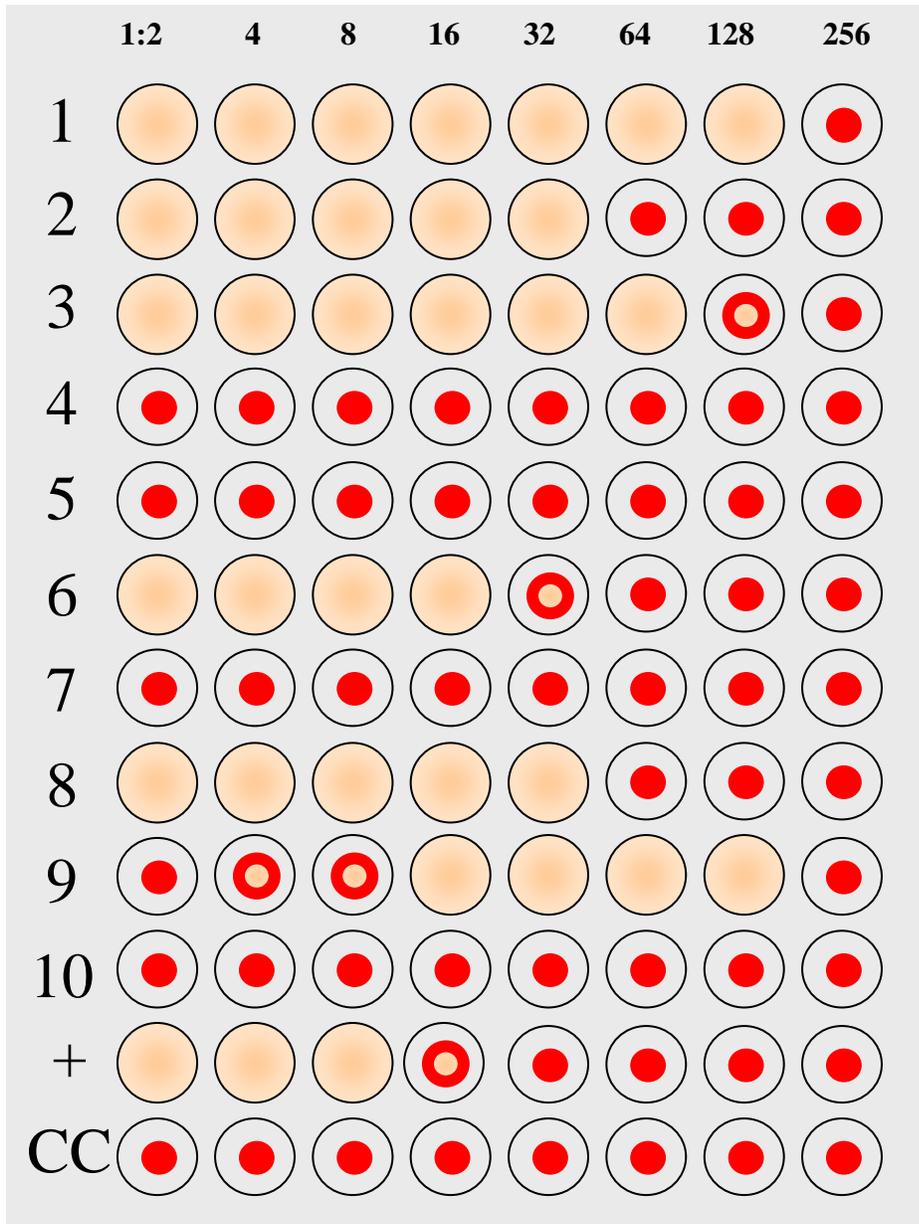
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
AAF 1							
AAF 2							
AAF 3							
AAF 4							
AAF 5							
AAF 6							
AAF 7							
AAF 8							
AAF 9							
AAF 10							
Pos +							
CC	CC	CC	CC	CC	CC	CC	CC

AAF = test samples (including negative control samples)
Pos + = positive control antigen
CC = RBC control

Detection of Hemagglutinating Viruses

Appendix II

Hemagglutination (HA) test with titers below (samples collected in tubes)



Sample 1 = 1:128
Sample 2 = 1:32
Sample 3 = 1:64
Sample 4 = Neg < 1:2
Sample 5 = Neg < 1:2
Sample 6 = 1:16

Sample 7 = Neg < 1:2
Sample 8 = 1:32
Sample 9 = 1:128 (prozone effect in 1st 3 wells)
Sample 10 = Neg < 1:2
+ control = 8 HAU / 50 μ L
Cell control = Negative (no hemagglutination)

Detection of Hemagglutinating Viruses

Appendix III

Example of test plate for HI test

Antiserum dilution*→ un. 1:2 1:4 1:8 1:16 1:32 1:64 1:128

AAF 1	AAF 1	AAF 1	AAF 1	AAF 1	AAF 1	AAF 1	AAF 1
AAF 2	AAF 2	AAF 2	AAF 2	AAF 2	AAF 2	AAF 2	AAF 2
AAF 3	AAF 3	→					
AAF 4	→						
AAF 5	→						
AAF 6	→						
AAF 7	→						
AAF 8	→						
AAF 9	→						
AAF 10	→						
Pos +	→						
CC	CC	CC	CC	CC	CC	CC	CC

*Antiserum added to 1st well of each row and diluted in PBS prior to addition of antigen (AAF)

AAF = HA positive test samples standardized to 8 HAU in 50 µL (4 HAU per 25 µL)

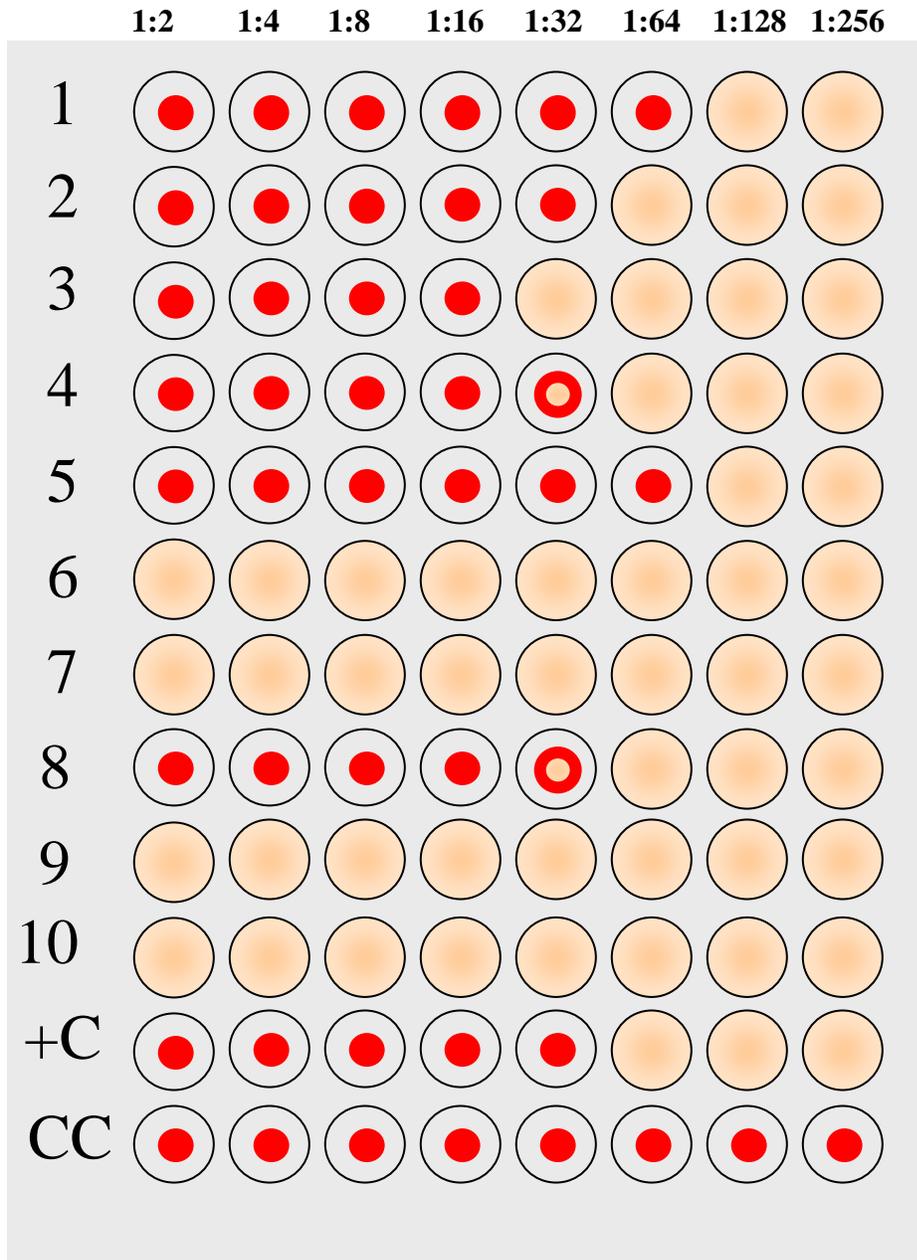
Pos + = positive control antigen diluted to 8 HAU in 50 µL

CC = RBC control

Detection of Hemagglutinating Viruses

Appendix IV

Hemagglutination-inhibition (HI) test with titers below



Sample 1 = 1:64
 Sample 2 = 1:32
 Sample 3 = 1:16
 Sample 4 = 1:16
 Sample 5 = 1:64
 Sample 6 = Neg < 1:2

Sample 7 = Neg < 1:2
 Sample 8 = 1:16
 Sample 9 = Neg < 1:2
 Sample 10 = Neg < 1:2
 + antiserum control = 1:32
 Cell control = Negative (no hemagglutination)