Agar Gel Immunodiffusion (AGID)
Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Avian Influenza Agar Gel Immunodiffusion Test to Detect Serum Antibodies to Type A Influenza Viruses

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1. Introduction

1.1 Background

The avian influenza (AI) agar gel immunodiffusion (AGID) test is used to detect circulating antibodies to Type A influenza group-specific antigens, namely the ribonucleoprotein (RNP) and matrix (M) proteins. Therefore, this test will detect antibodies to all influenza A virus subtypes. The AGID test can also be used as a group-specific test to identify isolates as Type A influenza viruses. The method used is similar to that described by Beard.

The basis for the AGID test is the concurrent migration of antigen and antibodies toward each other through an agar gel matrix. When the antigen and specific antibodies come in contact, they combine to form a precipitate which is trapped in the gel matrix and produces a visible line. The precipitin line forms where the concentration of antigen and antibodies is optimum. An extreme variation in the concentration of antigen or antibodies will alter the location of the line or cause it to be dissolved. Electrolyte concentration, buffer, pH and temperature also affect precipitate formation.

1.2 Key Words

avian influenza, AI, agar gel, AGID

2. Materials

2.1 Equipment/instrumentation

2.1.1 Refrigerator (4 C)

2.1.2 Freezer (-20 C)

2.1.3 Incubator or closed plastic container for room temperature (25 C) incubations.

2.1.4 Autoclave

2.1.5 Hot plate (optional)

2.1.6 Vacuum pump

2.1.7 Microscope illuminator
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2.1.8 Template cutter, 7-well pattern--a center well surrounded by 6 evenly spaced wells. Wells are 5.3 mm in diameter and 2.4 mm apart.

2.1.9 Top loading balance (capable of measuring 0.1 gm)

2.1.10 Micropipettor

2.2 Reagents/supplies

Note: All chemicals should be reagent grade unless specified.

2.2.1 Sodium phosphate monobasic (NaH$_2$PO$_4$) and dibasic (Na$_2$HPO$_4$)

2.2.2 Agarose (Type II Medium grade) agar (Sigma Chemical Co. Cat. number A-6877)

2.2.3 Sodium Chloride (NaCl)

2.2.4 Avian influenza AGID antigen (current version of AVRPP0100) and antiserum (current version of AVRPP0101)

2.2.5 Strong positive, weak positive and negative reference sera (optional)

2.2.6 Water--distilled or deionized water or water of equivalent purity. Heat sterilized.

2.2.7 Common laboratory supplies and glassware--Erlenmeyer flasks, graduated cylinders, pipettes, 100 x 15-mm and/or 60 x 15-mm disposable plastic petri plates, flexible silicone or rubber tubing, side-arm flask (500 ml or larger), and a 12- to 14-gauge blunt-ended cannula.

Note: All glassware and disposable labware should be sterile unless otherwise stated.
3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must be familiar with:

3.1.1 Preparation and proper handling of test reagents and biological materials.

3.1.2 Calibration, maintenance, and use of instruments listed in section 2.1.

3.2 Preparation of equipment/instrumentation

Equipment is calibrated and certified according to respective National Veterinary Services Laboratories (NVSL) Standard Operating Procedures (SOPs).

3.3 Preparation of reagents/control procedures

3.3.1 Phosphate buffered saline (PBS), 0.01 M, pH 7.2 (NVSL media number 30054, see appendix 8.1)

3.3.2 Preparation of AI AGID agar:

3.3.2.1 Weigh 9.0 gm of Agarose (see 2.2.2) and 80 gm of NaCl and add to 1 liter of PBS (0.01 M, pH 7.2) in a 2 liter Erlenmeyer flask.

Note: Larger or smaller volumes of agar can be prepared by multiplying or dividing each ingredient by the same factor. The size of flask used should be at least twice the volume of the contents so that when heated, the contents will not boil over.

3.3.2.2 Dissolve the mixture by bringing to a boil on a hot plate using a magnetic stir bar to mix the contents in the flask while heating.

OR
Autoclave the mixture for 10 min and mix the contents by swirling after removing from the autoclave to ensure a homogeneous mixture of ingredients.

3.3.2.3 After boiling, allow the agar to cool at room temperature (25 C) for 10 to 15 min before dispensing into petri plate(s).

3.3.2.4 Agar can be stored in the flask at 4 C for several months and melted and dispensed into plate(s) as needed.

OR

The liquid agar solution can be kept in a 45 C waterbath for several weeks and used as needed.

Note: Do not use agar if mold or precipitate is observed.

3.3.3 AI AGID antigen is prepared according to the current version of protocol AVRPP0100.

3.3.4 AI AGID control antiserum is prepared according to the current version of protocol AVRPP0101.

4. Performance of the test

4.1 Detection of serum antibodies

4.1.1 Dispense 15 to 17 ml of melted agar into a 100 x 15-mm petri plate or 5 to 6 ml agar into a 60 x 15-mm petri plate using a 25-ml pipette. The agar thickness should be approximately 2.8 mm.

4.1.2 Allow plates to cool in a relatively dust-free environment with the lids off to permit escape of water vapor. The lids should be left off for at least 15 min, but not longer than 30 min, as electrolyte concentration of the agar may change due to evaporation and adversely affect formation of precipitin lines.
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Note: Plates should be used the same day they are poured.

4.1.3 Fill out an AGID test worksheet in ink with sample identification, reagent lot numbers, test date, initials of the person(s) performing and reading the test, as well as any other pertinent information.

4.1.4 Using a template, cut the agar after it has hardened. Up to 7 template patterns can be cut in a 100 x 15-mm plate and 2 patterns can be cut in a 60 x 15-mm plate.

4.1.5 The agar plugs are removed by aspiration with a 12- to 14-gauge cannula connected to a side arm flask with a piece of silicone or rubber tubing which is connected to a vacuum pump with tubing. Adjust the vacuum so that the agar surrounding the wells is not disturbed when removing the plugs.

4.1.6 Place approximately 50 µl of each unknown sera in the appropriate well (the well should be filled as near level as possible without overflowing), using a micropipette with an attached pipette tip. A clean tip must be used for each new serum sample tested. Place approximately 50 µl AI AGID positive control antiserum (current version of protocol AVRPP0101) in each of three alternate peripheral wells. Place approximately 50 µl of AI AGID antigen (current version of protocol AVRPP0100) in the center well (see Figure 1). This arrangement provides a positive control line on each side of the test serum, thus facilitating accurate determination of lines of identity.

Note: A pattern should be included with positive, weak positive and negative reference serum in the test sera wells to aid in the interpretation of results.

4.1.7 Cover each plate after filling all wells and allow plate(s) to set for a few minutes before moving. This will reduce the possibility of spillage.

4.1.8 Incubate the plate(s) for 24 hr at room temperature (25 C) in a closed chamber to prevent evaporation. Humidity can be provided, if necessary,
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by placing a damp paper towel at the bottom of the incubation chamber.

Note: Temperature changes during migration may lead to artifacts.

4.2 Alternate procedure for identifying isolates as type A influenza viruses

4.2.1 Dispense agar and cut plates as in steps 4.1.1 to 4.1.5.

4.2.2 Place approximately 50 µl of AI AGID positive control antiserum (current version of protocol AVRPP0100) in the center well using a micropipette.

4.2.3 Dispense AI AGID antigen (current version of protocol AVRPP0100) in alternate wells around the center well. Test antigen consisting of amnionic-allantoic fluid or a crude chorio-allantoic membrane suspension is placed in the remaining alternate peripheral wells.

4.2.4 Plates are covered and incubated as in steps 4.1.7 and 4.1.8.

5. Interpretation of test results

5.1 Serum antibody detection

5.1.1 Remove the lid and read plate(s) over an intense narrow beam of light against a dark background. A microscope illuminator works well and allows for varying intensities of light and positions.

5.1.2 The type of reaction will vary with the concentration of antibody in the sample being tested. The positive control serum line is the basis for reading the test, and if the line is not distinct, the test is not valid and must be repeated. The following types of reactions are observed:

5.1.2.1 Negative reaction--the control lines continue into the test sample well without bending or with a slight bend away
from the antigen well and toward the positive control serum well (see Figure 1).

5.1.2.2 Positive reaction--control lines join with and form a continuous line (line of identity) with the line between the test serum and antigen. The location of the line will depend on the concentration of antibodies in the unknown sample. Weakly positive samples may not produce a complete line between the antigen and test serum but may only cause the tip or end of the control line to bend inward toward the test well (see Figure 1).

Figure 1. Immunodiffusion test pattern with AI antigen in the center well; AI positive control serum in wells A, C, and E; negative test serum in well B; positive test serum in well D; and weak positive test serum in well F.

5.1.2.3 Non-specific lines--these lines occasionally are observed between the antigen and test serum well. The control lines will pass through the non-specific line and continue on into the test serum well. The non-specific line does not form a continuous line (line of identity) with the positive control lines.
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Figure 2. Immunodiffusion test pattern with examples of nonspecific line formation (wells B and F). These reactions are not specific for AI and should be disregarded.

5.2 Antigen detection (alternate procedure)

5.2.1 Plates are read as in step 5.1. The unknown virus is identified as type A influenza virus if a line of identity forms with the positive control antigen.

6. Report of test results

6.1 Record test results, in ink, on the AGID worksheet using the following notations: “+” = positive reaction, “-” = negative reaction, “I” = inconclusive results, and “NSL” designates a nonspecific line. Positive results may also be recorded in degrees to denote strength of the reaction, ie., “+4” = strong positive, “+3” = positive, “+2” = weak positive, and “+1” = very weak positive.

6.2 Record test results from AGID worksheet to summary worksheet.

OR

If AI AGID results of all samples are negative, and no additional test is performed, results are recorded on original submission sheet.
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Note: If new reagents are being evaluated, then record results on the appropriate reagent production worksheets, then go to step 6.6.

6.3 Enter the number of serums tested in the specimens block of program 1 (log new cases) in the Avian Isolation Menu of Reflections (HP3000) so serology summaries can be electronically generated.

6.4 Enter status of the case and summary of results in program 7 (update case status) on the Avian Isolation Menu.

6.5 Give case report (APHIS form 10-4 or equivalent) and summary worksheet to Head of Avian Viruses Section for reporting.

6.6 File worksheet(s) and copies of summary sheet(s).

7. References


7.2 Version .02, April 13, 1998, was a revision superseding the February 28, 1997 version. Some wording changes were made.

7.3 Version .03, June 15, 1999, was a revision superseding the April 13, 1998 version. Additions were made in part 5.1.2 to include figures 1 and 2.

7.4 Version .04, May 10, 2001, was a revision superseding the June 15, 1999 version. Some minor wording changes were made.

7.5 Version .05, April 1, 2003, was a revision superseding the May 10, 2001 version. Additions were made in part 4.1.3, part 6.2, and part 6.3. Changes were made in part 4.1.6 and 4.2.2.
8. Appendices

8.1 Phosphate buffered saline:

Sodium phosphate dibasic, 11.9 gm
Sodium phosphate monobasic, 2.2 gm
Sodium chloride, 85.0 gm
Distilled water, QS to 10L

Adjust final pH to 7.2. Autoclave on slow exhaust.

9. Quick reference

_____ Prepare buffers and reagents
_____ Prepare AI AGID agar
_____ Pour AGID plates
_____ Fill out worksheet
_____ Cut and remove agar plugs from agar gel plates
_____ Fill plates with reagents and samples
_____ Read plates after 24 hr incubation
_____ Record test results
_____ Enter test results into the computer (Avian Isolation Menu number 1 and number 7)
_____ Give results to Section Head for reporting
_____ File worksheet(s) and testing results
Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Reagent Production Protocol

Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

Date: May 10, 2001
Supersedes: April 2, 1999
Number: AVRPP0100.04
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Approvals:
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Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

1. Introduction

Avian influenza (AI) agar gel immunodiffusion (AGID) antigen is used to detect group specific antibodies to Type A influenza virus in serum. The procedure for antigen preparation is based on the work of Dr. C. W. Beard, *A Laboratory Manual for the Isolation and Identification of Avian Pathogens*, published by the American Association of Avian Pathologists (Reference 7.1).

2. Materials

2.1 Equipment/instrumentation

2.1.1 Refrigerated (4 C) low-speed centrifuge (Beckman model J-6B)

2.1.2 Sorvall Omni-mixer or equivalent model of tissue homogenizer

2.1.3 Sonicator

2.1.4 Horizontal platform rotator

2.1.5 Multi-wrist action shaker

2.1.6 Automatic dispensing pump or Cornwall syringe

2.1.7 Ultra-low freezer (-70 C)

2.1.8 Lyophilizer

2.1.9 Autoclave

2.1.10 Incubator

2.1.11 Refrigerator (4 C)

2.1.12 General purpose microscope lamp (AO Scientific Instruments or equivalent) for candling eggs
Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

2.1.13 Biological safety cabinet (class II)

2.1.14 Common laboratory apparatus and glassware--top loading balance (capable of measuring 0.01 g), pH meter, magnetic stir plate, magnetic stir bar, vortex mixer, beakers, Erlenmeyer flasks, graduated cylinders, pipettes, centrifuge tubes, 2-ml glass serum vials, 13-mm slotted stoppers and aluminum seals

Note: All glassware and disposable labware should be sterile unless otherwise stated.

2.2 Reagents/supplies

Note: All chemicals should be reagent grade unless specified.

2.2.1 Phosphate buffered saline (PBS), 0.01 M, pH 7.2 (National Veterinary Services Laboratories [NVSL] media number 30054, see appendix 8.1)

2.2.2 10T antibiotic medium (NVSL media number 10411, see appendix 8.2)

2.2.3 Sodium N-lauroylsarcosine (Sarkosyl Reagent)--C_{15}H_{28}NO_{3}Na

2.2.4 Glycine--(C_{2}H_{5}NO_{2})

2.2.5 Sodium Azide--(NaN_{3})

2.2.6 Sulfuric Acid--(H_{2}SO_{4})

2.2.7 Sodium Hydroxide--(NaOH)

2.2.8 β-propiolactone (Sigma catalog number p5648)

2.2.9 Water-distilled or deionized water or water of equivalent purity. Autoclave sterilize.

2.3 Biological materials

2.3.1 Specific-pathogen-free (SPF) embryonating chicken eggs, 10 to 11 days old.
3. Preparation for production

3.1 Personnel qualifications/training

Personnel must be familiar with:

3.1.1 Preparation and proper handling of test reagents and biological materials.

3.1.2 Calibration, maintenance, and use of instruments listed in section 2.1.

3.2 Preparation of equipment/instrumentation

Equipment is calibrated and certified according to respective NVSL Standard Operating Procedures.

3.3 Preparation of reagents/control procedures

Note: All reagents should be stored at 4 C for up to 3 mo unless otherwise indicated.

3.3.1 10% Sodium N-lauroylsarcosine (Sarkosyl Reagent)

Dispense 70 ml water into a 250-ml beaker placed on a magnetic stir plate. Add, slowly, a little at a time, 10 g of Sarkosyl and mix with a magnetic stir bar until dissolved. Adjust the pH to 7.5-8.0 with 10% H$_2$SO$_4$ or 10% NaOH. Add water and QS to 100 ml.

3.3.2 Glycine-Sodium N-lauroylsarcosine (Glycine Sarkosyl Buffer)

Add 9.25 g glycine to 200 ml water in an Erlenmeyer flask. Mix until dissolved and adjust pH to 9.0 ± 0.05. If the pH is below 8.95, adjust by adding a few drops of 10 N NaOH. Add 25 ml of 10% Sarkosyl Reagent to the pH-adjusted glycine solution. Add water and QS to 250 ml and thoroughly mix.

3.3.3 10% Sodium Azide (NaN$_3$)
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Place 10 g sodium azide in an Erlenmeyer flask and add water and QS to 100 ml. Mix well.

**Caution:** Sodium Azide (NaN₃) is highly toxic. Refer to Materials Safety Data Sheets (MSDS) for information regarding safe handling and disposal of materials containing this chemical.

### 3.3.4 10% Sodium Hydroxide (NaOH) solution

Add 10 g of NaOH to 80 ml water in an Erlenmeyer flask and mix until dissolved. QS to 100 ml with water and mix well.

### 3.3.5 10 N NaOH solution

Add 40 g of NaOH to 80 ml water in an Erlenmeyer flask and mix until dissolved. QS to 100 ml with water and mix well.

### 3.3.6 10% Sulfuric Acid (H₂SO₄) solution

Add 10 ml of concentrated H₂SO₄, slowly, to 90 ml water in an Erlenmeyer flask. Mix well.

### 3.3.7 1% β-propiolactone (BPL)

Add 1 ml BPL to 99 ml of cold (4°C) sterile PBS and mix well.

**Critical Control Point:** Do not dilute the BPL until it is needed as it hydrolyzes rapidly in aqueous solutions. Once diluted, the BPL solution must be used within 15 min. BPL is a carcinogen. Use caution when handling. Refer to Materials Safety Data Sheets (MSDS) for information regarding safe handling of this material.

### 3.4 Preparation/harvest of virus/product

**Critical control point:** All work with live virus should be performed in a biological safety cabinet.
3.4.1 Select a low pathogenic strain of influenza virus type A which replicates to a high titer in chicken embryos, e.g., A/Turkey/MN/81 (H5N2).

3.4.2 Inoculate 10- to 11-day-old SPF embryonating chicken eggs with 0.1 ml of a $10^{-3}$ dilution of virus in 10T antibiotic medium via the allantoic sac route.

3.4.3 Incubate eggs at 37 C for 5 days and candle daily. Discard all embryos dying within 24 hr of inoculation. Refrigerate all embryos dying on the second through the fifth days.

Note: Most of the embryos should die on the second and third days post-inoculation.

3.4.4 Harvest the amnionic-allantoic fluid (AAF) and the chorioallantoic membranes (CAM) from the refrigerated embryos. Collect the AAF and CAM material into separate tubes. Freeze the CAM material at -70 C. Clarify the AAF by centrifugation at 1,500 x g (2,500 rpm in a Beckman J-6B centrifuge) for 20 min and freeze the supernatant at -70 C. AAF will be used later for antiserum production.

4. Preparation of the product

Critical control point: Parts 4.2, 4.4 and 3.4.2 should be performed in a biological safety cabinet.

4.1 Remove tubes with CAM material from the freezer and thaw at room temperature (25 C).

4.2 Homogenize the CAMs (no diluent should be added) using a Sorvall Omnimixer at a speed setting of 7 for 2, 2-min cycles in an ice bath.

4.3 Freeze-thaw the homogenate 3 times.

4.4 Homogenize again as in step 4.2.

4.5 Centrifuge the homogenate at 1,500 x g (2,500 rpm in a Beckman J-6B) for 20 min at 4 C and save the supernatant and the pellet. Add 10% sodium azide to the supernatant to give a final concentration of 0.1% sodium azide and store at 4 C.
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4.6 Extract additional antigen from the pellet by treating the pellet with glycine-sarkosyl buffer (approximately 1 ml per 10 ml sediment).

4.7 Sonicate the pellet/buffer mixture in an ice bath for 2, 1-min cycles at a 2 sec pulse cycle setting. Store pellets at -70 C overnight.

Note: Tune the sonicator probe prior to use to ensure optimum operation. Refer to the manufacturers instruction manual.

4.8 Thaw and dilute the treated, sonicated sediment with PBS at a ratio of 10-20 ml PBS per 10 ml of sediment. Shake each tube vigorously and use a vortex mixer to resuspend the pellet in the PBS. Then shake vigorously for 15 to 20 min using a multi-wrist action shaker.

4.9 Repeat steps 4.5 through 4.7. After the second antigen extraction from the pellet, centrifuge and add sodium azide as in step 4.5. Discard the pellet.

4.10 Keep antigen pools from steps 4.5, 4.6, and 4.9 separate.

4.11 Inactivate the virus in each antigen pool by adding 1% \( \beta \)-propiolactone to a final concentration of 0.1% (1:10 dilution). Incubate for 2 hr on a horizontal rotator at room temperature, then store at 4 C overnight.

Note: The antigen should be allowed to equilibrate to room temperature (25 C) before adding the BPL. Gently agitate the antigen fractions while adding the BPL to insure even dispersal of the chemical.

4.12 After quality control testing of the antigen (see 5.1 and 5.2), store the antigen in bulk in an ultra-low freezer (-70 C) until needed.

5. Quality control testing

5.1 Sensitivity testing

5.1.1 Testing by Viral Reagents Unit

Test each antigen fraction individually and pooled by the AI AGID test (current version of AVPRO00100) and
5.1.1.1 Each antigen fraction is evaluated by AI AGID against AI negative reference and positive control sera. Use one pattern to evaluate each fraction, placing antigen fractions in the center wells and alternating the negative and positive control serums in the outside wells. Include a control pattern on the plate using a matched lot of antigen and control antiserum.

5.1.1.2 Pool all fractions that produce a single bold precipitin line (the stronger the antigen, the closer the precipitin line will be to the antiserum well).

5.1.1.3 Retest the antigen pool by AI AGID against a minimum of 6 different positive control serums.

5.1.1.4 At this point, if the antigen pool appears satisfactory, go to part 5.2 and test the antigen to ensure inactivation of the virus by the β-propiolactone.

5.1.1.5 After the antigen has been found to be free of viable virus, the antigen may be evaluated with AI antiserum (go to step 5.1.1.6) or stored in bulk at -70 C for later use. Before freezing, aliquot 2 ml into each of 3 to 5 vials for the freezer repository. (The repository samples can be used later for evaluating with AI antiserum pools.)

5.1.1.6 Evaluate dilutions of the antigen pool (may use repository samples if bulk antigen is frozen) against dilutions of AI antiserum in the AI AGID test to determine which dilutions produce a single, sharp precipitin line midway between the antigen and antiserum wells.

5.1.1.7 Evaluate the optimal dilutions of antigen and matching antiserum against a panel of 4 each of weakly positive, negative, and strongly positive AI reference serums. Also evaluate the serum panel with an approved
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(control) lot of matching antigen and antiserum. Test results must concur.

5.1.1.8 The optimally diluted antigen and antiserum must also be reevaluated against the panel of reference serums after lyophilization.

5.2 Testing to ensure the inactivation of live virus by

5.2.1 Dilute the BPL-inactivated antigen pools 1:10 in 10 T antibiotic medium and inoculate 5, 9- to 11-day-old SPF embryonating chicken eggs via the allantoic sac route.

5.2.2 Incubate eggs at 37 C for 5 days and candle daily. Discard all embryos dying within 24 hr of inoculation. Refrigerate all embryos dying on the second through the fifth days as well as the live embryos on the fifth day.

5.2.3 Harvest the amnionic-allantoic fluid (AAF) from the dead and live embryos. Keep the AAF from each dead embryo separate, but the AAF from the live embryos can be pooled.

5.2.4 Clarify the AAFs by centrifugation at 1,500 x g (2,500 rpm in a Beckman J-6B) for 10 min.

5.2.5 Test the AAFs for hemagglutinating (HA) activity (see part 4 of the current version of AVPRO0805).

5.2.6 If no HA activity is observed, make a second passage of the pooled AAF material by following steps 5.2.1 through 5.2.5.

5.2.7 If HA activity is not observed in the second passage, the antigen is considered to be free of viable virus. If there is HA activity after the first or second passage, add 1% BPL as in step 4.11, and repeat steps 5.2.1 through 5.2.7.

5.3 Testing by Avian Diagnostic Section

Send the matching antigen and antiserum (final lyophilized products) to the Avian Diagnostic Section of the Diagnostic Virology Laboratory for evaluation by the AI AGID test against a panel of reference sera.
Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

6. Post preparatory steps

6.1 Bottling

6.1.1 If bulk antigen is frozen, thaw at room temperature (25 C), then dilute (if necessary) to optimally match a positive control serum (see parts 5.1.1.5 and 5.1.1.7)

6.1.2 Dispense 2 ml aliquots of antigen into sterile 2-ml glass vials with a sterile dispensing pump or Cornwall syringe. Cap each vial loosely with a sterile 7 x 13-mm slotted stopper.

Note: At this point, the vials can be capped, sealed, labeled, and stored at -70 C if product is not going to be lyophilized. Although lyophilization is the ideal method for product storage, it is optional.

6.1.3 Freeze the antigen at -70 C for at least 24 hr and then lyophilize for 24 to 48 hr.

6.1.4 Vacuum seal the vials of lyophilized antigen before removing from the lyophilizer and cap with 13-mm aluminum seals.

6.2 Assign expiration date

Not available.

6.3 Labeling instructions

Label bottles with a yellow label with the product name, serial number, storage temperature, and amount of sterile water required to reconstitute the antigen to the original volume.

6.4 Place product on inventory

6.4.1 The antigen must pass quality control testing (see 5.1.1.8) before being added to the inventory.

6.4.2 A Reagent Data Sheet (see current version of NVSLSOP0008) should be created for each new lot of product.
Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

6.4.3 Save back 3 vials of lyophilized antigen for the repository before adding to the inventory.

6.5 Product storage

Store lyophilized antigen at 4 C.

6.6 Directions for use

The antigen is used as specified in the AI AGID test protocol (current version of AVPRO0100).

7. References


7.2 Version .03, April 2, 1999, was a revision superseding the March 27, 1998 version. Additions and changes were made to parts 3.3.3, 3.4.1, 3.4.3, 3.4.4, 4.7, 5.1.1, and 5.1.5. Step 3.4.5 was combined with step 3.4.4. Step 6.4.3 was added. Some minor wording changes were also made.

7.3 Version .04, May 10, 2001, was a revision superseding the April 2, 1999 version. Additions and changes were made to parts 2.1, 3.4, 4, 4.8, and 6.1.2. Parts 5.1 and 5.2 were switched and an additional step was added to part 5.1.1. Step 5.3 was added. Some minor wording changes were also made.

8. Appendices

8.1 Phosphate buffered saline:

Sodium phosphate dibasic, 11.9 gm
Sodium phosphate monobasic, 2.2 gm
Sodium chloride, 85.0 gm
Water, QS to 10 L.

Adjust final pH to 7.2. Autoclave.

8.2 10T antibiotic medium

8.2.1 Basal (make in advance and store in desired quantities in cooler)
Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

Trizma base, 1.21 gm
Tryptose broth, 26 gm
Water, 1,000 ml

Autoclave 20 min on slow exhaust.

8.2.2 Antibiotics for 10T

Penicillin (1,586 U/mg), 6.3 gm
Streptomycin (747 U/mg), 2.68 gm

Place 15 ml sterile Dulbecco’s PBS into a sterile 250-ml cylinder. Add penicillin and streptomycin and stir briefly to dissolve. Aseptically add:

13.0 ml kanamycin sulfate (50 mg/ml)
20.0 ml gentocin (50 mg/ml)
0.04 ml mycostatin (5,000,000)—to make new stock, rehydrate with 10 ml sterile PBS

Adjust the pH antibiotic solution to 6.6 with 1 N NaOH. QS to 50 ml in a measuring cylinder with sterile PBS and add to 950 ml basal. Aseptically dispense in desired quantities in sterile 12 x 75-mm snap-cap tubes. Freeze at -20 C.

9. Quick reference

_____ Prepare buffers and reagents
_____ Prepare virus dilution and inoculate eggs
_____ Harvest AAF and CAM’s from dead embryos
_____ Freeze and thaw CAM’s and homogenize
_____ Freeze and thaw homogenate 3 times
_____ Homogenize, centrifuge, and collect supernatant
_____ Add preservative to supernatant
_____ Add glycine-sarkosyl buffer to pellet and sonicate
Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test

- Add diluent to pellet, process and collect supernatant
- Add preservative
- Repeat antigen extraction from pellet
- Add preservative
- Sensitivity tests
- Bottle antigen and freeze
- Lyophilize antigen
- Label antigen
- Store antigen
- File worksheet and testing results
- Place antigen on inventory
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

Date: May 10, 2001
Supersedes: September 14, 1999
Number: AVRPP0101.04
Contact Person: Amy Shafer, (515) 663-7551

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Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

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Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

1. Introduction

1.1 Background

Avian influenza (AI) agar gel immunodiffusion (AGID) antiserum is used as a positive control serum with AI AGID antigen to enhance the detection of group specific antibodies to type A influenza virus in serum. The method for antiserum preparation is based on procedures outlined by J. E. Pearson and D. A. Senne in Proceedings of the Third International Symposium on Avian Influenza. (Reference 7.1).

1.2 Key words

Avian influenza, AI, antiserum, agar-gel immunodiffusion, AGID

2. Materials

2.1 Equipment/instrumentation

2.1.1 Refrigerated (4 C) low speed centrifuge (Beckman model J-6B or equivalent)

2.1.2 Refrigerated (4 C) ultracentrifuge (Beckman model L8-80 or equivalent)

2.1.3 Fixed angle ultracentrifuge rotor (Beckman model Ti-60 or equivalent)

2.1.4 Refrigerator (4 C)

2.1.5 Incubator (37 C)

2.1.6 Automatic dispensing pump or Cornwall syringe capable of dispensing 10-ml quantities

2.1.7 Ultra-low freezer (-70 C)

2.1.8 Freezer (-20 C)

2.1.9 Lyophilizer

2.1.10 Autoclave

2.1.11 Biological safety cabinet (class II)
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

2.1.12 Common laboratory apparatus, supplies and glassware--top loading balance (capable of measuring 0.01 g), magnetic stir plate, magnetic stir bar, vortex mixer, beakers, Erlenmeyer flasks, graduated cylinders, pipettes, 50-ml conical centrifuge tubes, 15 × 75-mm snap cap tubes, 30- and 60-cc disposable syringes, 3- and 5-cc syringes, 25-gauge × 5/8-inch and 18-gauge × 1½-inch needles, 12-gauge × 2½-inch stainless steel needles, disposable latex gloves, 8-10-mm thick glass rod, 25 × 89-mm thick-walled polycarbonate ultracentrifuge tubes, 10-ml glass serum bottles, and 20-mm slotted stoppers and aluminum seals.

Note: All glassware and disposable labware should be sterile unless otherwise stated.

2.2 Reagents/supplies

Note: All chemicals should be reagent grade unless specified.

2.2.1 Phosphate buffered saline (PBS), 0.01 M, pH 7.2 (National Veterinary Services Laboratories [NVSL] media number 30054, see appendix 8.1)

2.2.2 10T antibiotic medium (NVSL media number 10411, see appendix 8.2)

2.2.3 Sodium N-lauroylsarcosine--C_{15}H_{28}NO_{3}Na

2.2.4 Glycine--C_{2}H_{5}NO_{2}

2.2.5 Sodium Azide--NaN_3

2.2.6 Ketamine hydrochloride (100 mg/ml)

2.2.7 Iodine-based disinfectant

2.2.8 Water--distilled or deionized water or water of equivalent purity. Heat sterilized.

2.3 Biological materials

2.3.1 Specific pathogen free (SPF) embryonating chicken eggs, 10- to 11-day-old.

2.3.2 SPF mature white Leghorn chickens
Preparation for production

3.1 Personnel qualifications/training

Personnel must be familiar with:

3.1.1 Preparation and proper handling of test reagents and biological materials.

3.1.2 Calibration, maintenance, and use of instruments listed in section 2.1.

3.1.3 Proper care and handling of laboratory animals as outlined in the NVSL Animal Users Manual.

3.2 Preparation of equipment/instrumentation

Equipment is calibrated and certified according to respective NVSL Standard Operating Procedures (SOPs).

3.3 Preparation of reagents/control procedures

Note: All reagents can be stored at 4 C for up to 3 mo unless otherwise indicated.

3.3.1 10% Sodium N-lauroylsarcosine (Sarkosyl Reagent)

Dispense 70 ml water into a 250-ml beaker and place on a magnetic stir plate. Add, slowly, a little at a time, 10 g of Sodium N-lauroylsarcosine and mix with a magnetic stir bar until dissolved. Adjust pH to 7.50-8.00 with 10% H₂SO₄ or 10% NaOH. Add water QS to 100 ml.

3.3.2 Glycine-Sodium N-lauroylsarcosine (Glycine Sarkosyl Buffer)

Add 9.25 g glycine to 200 ml water in an Erlenmeyer flask. Mix until dissolved and adjust pH to 9.00 ± 0.05. If pH is below 8.95, adjust by adding a few drops of 10 N NaOH. Add 25 ml 10% Sarkosyl Reagent to the pH-adjusted glycine solution. Add water QS to 250 ml and mix thoroughly.
3.4 Propagation/Preparation of virus

Critical control point: Parts 3.4.1 and 3.4.2 should be performed in a biological safety cabinet.

3.4.1 Preparation of the type A influenza ribonucleoprotein (RNP) for chicken inoculation.

3.4.1.1 Propagate virus in SPF embryonating chicken eggs as outlined in current version of AVRPP0100, “Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test”, section 3.4.

3.4.1.2 Remove amnionic allantoic fluid (AAF) from freezer and thaw at room temperature (25 C).

3.4.1.3 Centrifuge AAF at 1500 × g (2500 rpm) for 20 min at 4 C to clarify. Pour supernatant through a funnel containing 2 layers of sterile gauze into an Erlenmeyer flask(s).

3.4.1.4 Evaluate the AAF (supernatant) for viral hemagglutinating (HA) activity (see current version of AVPRO0805). If no HA activity is present, then the AAF is not suitable for RNP production.

3.4.1.5 If HA activity is satisfactory, pipette supernatant into 8 thick-walled ultracentrifuge tubes (20 ml per tube). Place tubes in a fixed angle rotor (Ti-60) and ultracentrifuge at 251,720 × g (50,000 rpm) for 90 min to pellet virus.

3.4.1.6 Decant supernatant into a container with an iodine-based disinfectant. Gently rinse pellet with approximately 5 ml PBS then decant PBS into the container with the disinfectant.

3.4.1.7 Add 0.1 ml glycine-sarkosyl buffer to each tube and break up pellet with a glass rod. Incubate at room temperature (25 C) for 60 min.
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

3.4.1.8 Add 1.0 ml PBS to each tube, pool RNP and freeze at -70 C in a closed container until needed.

3.4.1.9 Disinfect thick-walled ultracentrifuge tubes with an iodine-based or other suitable disinfectant. Scrub out the tubes with a soft-bristled test tube brush and rinse tubes thoroughly with distilled water and dry. Autoclave sterilize. Tubes can be reused several times but should be discarded if any cracks or signs of wear are noted.

3.4.2 Preparation of live virus chicken inoculum.

3.4.2.1 Select a type A influenza virus that grows to a high titer in embryonating chicken eggs and has a hemagglutinin (H) and neuraminidase (N) surface antigen different from the virus used to produce the AAF in section 3.4.1, e.g., A/Eq1-Bel (H7N1) subtype.

3.4.2.2 Follow the same procedure for virus propagation and collection of AAF as in step 3.4.1.1. Store AAF at -70 C until ready to use.

4. Production of the product

4.1 Remove RNP inoculum from the freezer, thawing only what is needed for the number of birds being inoculated.

4.2 Inoculate mature white Leghorn chickens via the brachial vein (main wing vein) with 0.5 ml of RNP solution.

Note: All intravenous injections are made using a 3- or 5-cc syringe with a 25-gauge × 5/8-inch needle. Latex gloves should be worn for protection.

4.3 Twenty-seven to 29 days after the first injection, thaw at room temperature (25 C) the AI virus prepared in part 3.4.2 and centrifuge at 1500 × g (2500 rpm) for 20 min to clarify. Save the supernatant and refrigerate at 4 C until ready to use. Inoculate each chicken with 5.0 ml of live virus inoculum via the brachial vein.
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

**Note:** Virus inoculum may be loaded into syringes and stored at 4°C one day prior to bird inoculation.

4.4 Thirteen to 15 days after the second inoculation, thaw RNP and prepare a water-in-oil emulsion of equal parts of the antigen in Freund's complete adjuvant (FCA). Inject 0.8 ml RNP-adjuvant mixture per chicken into 3 or 4 sites subcutaneously at the base of the neck. Inoculations are performed with a 5-cc syringe and 18-gauge × 1½-inch needle.

**Note:** Follow NVSL guidelines for proper use of FCA in animals.

4.5 Exsanguinate birds 13 to 15 days after the last inoculation, as follows:

4.5.1 Inject each chicken intramuscularly with 7 to 10 mg/lb Ketamine hydrochloride.

4.5.2 When birds appear sedated, exsanguinate by cardiac puncture through the thoracic inlet using a 12-gage × 2½-inch stainless steel needle attached to a 60-cc syringe.

4.5.3 Fill syringe no more than 2/3 full as sufficient air space is needed in the syringe along the length of the clot for good separation of the serum from the clot.

4.5.4 It may be necessary to use more than 1 syringe to fully exsanguinate each bird. After filling the first syringe 2/3 full, quickly detach the syringe from the needle without removing needle from the thoracic inlet and attach a new 60- or 30-cc syringe. Use care not to move the needle during the exchange of syringes. Continue to exchange syringes until the bird is fully exsanguinated.

4.5.5 Label all syringes from each bird with the same identification (I.D.) number. Make sure the cap is placed back on the tip of each syringe.
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

4.6 Position the syringes horizontally with the capped end slightly raised to allow an air space to form along the entire length of the syringe barrel and incubate at room temperature (25°C) for 18 to 24 hr. Serum separation may be enhanced by incubating syringes at 37°C for ≤ 2 hr before incubation at room temperature.

Critical control point: Serum from each bird should be kept separate and tested individually before pooling.

4.7 Remove plunger from syringe barrel and decant serum into a 50-ml centrifuge tube. Serum from each bird should be pooled and the centrifuge tube labeled with the corresponding I.D. number. Syringes and 50-ml tubes can be refrigerated overnight at 4°C so that additional serum can be allowed to separate from the clots. Any additional serum can be decanted into the same 50-ml centrifuge tube as before. Centrifuge tubes at 1200 × g (2000 rpm) for 15 min.

4.8 Carefully pipette or decant serum off red blood cell (RBC) pellet into another labeled 50-ml centrifuge tube. Refrigerate tubes of serum at 4°C. Discard the tube with the RBC pellet. An average yield of serum from an adult leghorn chicken is 15 to 20 ml.

5. Quality control testing

5.1 Evaluation of sera

5.1.1 Test an aliquot of serum from each bird by the AI AGID test (current version of protocol AVPRO0100). Test undiluted serum as well as dilutions of 1/2, 1/4, 1/6, 1/8, and 1/10. Prepare serum dilutions with PBS. Sera exhibiting strong precipitin lines at the 1/10 dilution are further diluted and tested by AI AGID to determine the optimum dilution for use. Discard sera which exhibit lines of nonidentity (i.e., non-specific or double lines) at all dilutions. Save all sera exhibiting clear, sharp precipitin lines, including those which may have non-specific lines at lower dilutions but not at the higher dilutions.
5.1.2 Pool undiluted sera according to optimum dilution range, e.g., pool sera with sharpest precipitin lines at lower dilutions and make a separate pool of the higher titered sera. Centrifuge each serum pool at 1500 x g (2500 rpm) for 20 min and save supernatant. Each pool can be used as a separate lot of AI AGID antiserum or pooled with other lots of serum of similar titers.

Note: At this point, undiluted serum pools can be frozen at -20 C and used later or proceed to the next step.

5.1.3 Thaw antiserum pool(s), if frozen, and evaluate small aliquots of each antiserum pool against several lots of AI AGID antigen (current version of protocol AVRPP0100) by the following method:

5.1.3.1 Make dilutions of antiserum in a range which is appropriate for each serum pool made in step 5.1.2.

5.1.3.2 Make dilutions of antigen to test against the antiserum dilutions (see current version of protocol AVRPP0100 step 5.2).

5.1.3.3 Place AI negative control serum in alternate wells of the test patterns. A control pattern consisting of a known matched set of antigen and antiserum with AI negative control serum in alternate wells should also be included on each plate as a reference.

5.1.3.4 The optimal dilution(s) of antigen and antiserum should produce a single, sharp precipitin line that forms midway between the antigen and antiserum wells. Complete precipitin lines should extend to but do not bend into the negative serum wells.

5.1.3.5 Usually, more than 1 pattern, i.e. antigen and antiserum dilution appears to be optimal. A final evaluation of the optimal dilutions by AI AGID should include a weak positive control serum in at least 1 well of each pattern. This will aid in determining the best dilution of antigen and antiserum to use for a matched set.
5.2 Sensitivity testing

5.2.1 Testing by Viral Reagents Unit

Evaluate optimally diluted antiserum and matching antigen against a panel of a minimum of 4 each of weak positive, negative, and strong positive reference serums. Compare results to one or more previous lots of antiserum and antigen. Results obtained with panel serums must concur with previous test results. Reevaluate the antiserum and matching antigen with the reference sera panel after final bottling and lyophilization.

5.2.2 Testing by Avian Viruses Section

Send the matching antiserum and antigen (final lyophilized product) to the Avian Viruses Section of the Diagnostic Virology Laboratory for evaluation by the AI AGID test with a panel of reference sera.

6. Post preparatory steps

6.1 Bottling

6.1.1 Centrifuge the bulk antiserum pool (thaw first if frozen) at 1500 x g (2500 rpm) for 20 min to clarify.

6.1.2 Pipette or pour the antiserum off the pellet into a measuring cylinder then into a large Ehrlenmeyer screw-cap flask. Discard the pellet.

6.1.3 Dilute the antiserum with sterile PBS (if necessary) to obtain the optimal dilution for use as determined in section 5.1.3.

6.1.4 Add sodium azide to the diluted antiserum to achieve a final concentration of 0.1% (w/v). Thoroughly mix on a stir plate with a magnetic stir bar at 4 C for a minimum of 30 min.

6.1.5 Dispense 6 ml aliquots of antiserum into sterile 10-ml glass serum vials with a sterile dispensing pump or Cornwall syringe. Cap each vial loosely with a sterile 13 x 20-mm slotted stopper.
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

Note: At this point the vials can be capped, sealed, labeled, and stored at -70 C if product is not going to be lyophilized. Although lyophilization is the ideal method for product storage, it is optional.

6.1.6 Freeze the antiserum at -70 C for at least 24 hr, then lyophilize for ≥36 hr.

6.1.7 Vacuum seal vials of lyophilized antiserum before removing from lyophilizer and cap with 20-mm aluminum closures.

6.2 Assign expiration date

Not available.

6.3 Labeling instructions

Label bottles with a green label containing the following information: product name, serial number, amount of sterile water required to reconstitute the antiserum to the original volume, and laboratory name and location.

6.4 Place product on inventory

6.4.1 Reevaluate as in step 5.2.1 before adding to the inventory.

Note: This step is not necessary if the product has been evaluated within 6 mo prior to placing on inventory.

6.4.2 A Reagent Data Sheet (see current version of NVSLSOP0008) should be created for each new lot of product.

6.4.3 A minimum of 3 vials of each lot or serial should be placed into the repository before placing product on inventory.

6.5 Product storage

Store lyophilized antigen at 4 C.
6.6 Directions for use

Use antiserum as specified in current version of protocol AVPRO0100.

7. References


7.2 Version .02, was a revision superseding the June 3, 1998, February 24, 1997, version. Section 6.4.2 was added and there were some minor changes in wording.

7.3 Version .03, September 14, 1999, was a revision superseding the June 3, 1998 version. Section 1 was updated to include format changes (key words). Changes and/or more details were added to steps 3.4.1.3, 3.4.1.5, 3.4.1.6, and 3.4.1.9. An additional step 3.4.1.4 was added to section 3.4.1 and the amount of PBS added in step 3.4.1.8 was changed from 2 ml to 1 ml. A range of days for injections or bleeding was added to steps 4.3 through 4.5. Procedural changes were added to steps 4.3, 4.6, 4.7 and 5.1.1. Section 5.1.3 was changed and divided into steps. An additional step 5.1.3.5 was added to section 5.1.3. Additions/changes were made to steps 6.1.4, 6.1.6, 6.3, and 6.4.1. Step 6.4.3 was added. There were some minor wording changes made to add clarity.

7.4 Version .04, May 10, 2001, was a revision superseding the September 14, 1999, version. Changes or additions were made to steps 4.4 and 5.1.2. There were some minor wording changes.

8. Appendices

8.1 Phosphate buffered saline:

Sodium phosphate dibasic, 11.9 gm
Sodium phosphate monobasic, 2.2 gm
Sodium chloride, 85.0 gm
Distilled water, QS to 10 L.

Adjust final pH to 7.2. Autoclave on slow exhaust.
8.2 10T antibiotic medium

8.2.1 Basal (make in advance and store in desired quantities in refrigerator)

- Trizma base, 1.21 gm
- Tryptose broth, 26 gm
- Distilled water, 1,000 ml

Autoclave 20 min on slow exhaust.

8.2.2 Antibiotics for 10T

- Penicillin (1,586 U/mg), 6.3 gm
- Streptomycin (747 U/mg), 2.68 gm

Place 15 ml of sterile Dulbecco’s PBS into a sterile 250-ml cylinder. Add penicillin and streptomycin and stir briefly to dissolve. Aseptically add:

- 13.0 ml kanamycin sulfate (50 mg/ml)
- 20.0 ml gentocin (50 mg/ml)
- 0.04 ml mycostatin (5,000,000) -- to make new stock, rehydrate with 10 ml sterile PBS

Adjust the pH of the antibiotic solution to 6.6 with 1 N NaOH. QS to 50 ml in a measuring cylinder with sterile PBS and add to 950 ml basal. Aseptically dispense in desired quantities in sterile 12 x 75-mm snap-cap tubes. Freeze at -20°C.
Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

9. Quick reference

_____ Prepare buffers and reagents
_____ Prepare RNP inoculum
_____ Prepare live virus inoculum
_____ Inoculate chickens with RNP
_____ Inoculate chickens with live virus
_____ Inoculate chickens with RNP-adjuvant
_____ Bleed out chickens
_____ Evaluate antiserum from individual birds by AI AGID
_____ Sensitivity tests
_____ Make optimal dilution of antiserum
_____ Add preservative
_____ Bottle antiserum and freeze
_____ Lyophilize antiserum (optional)
_____ Label antiserum
_____ Store antiserum
_____ File worksheet and testing results
_____ Place antiserum in inventory
Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Standard Operating Procedure

Filter Paper Method of Blood Collection
for Serologic Test(s)

Date: July 27, 1998

Supersedes: New

Number: AVSOP0800.01

Contact Person: Dennis Senne, (515) 239-8551

Approvals:

____________________________  Date:_________
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Avian Viruses Section

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of other products that may be suitable.
Filter Paper Method of Blood Collection for Serologic Test(s)

1. **Purpose:**

Avian blood samples can be collected and preserved in filter paper. Blood collected in this manner can be used in place of serum and/or plasma for serologic tests such as hemagglutination-inhibition (HI) test. The technique is especially useful in field situations where aseptic collection and adequate preservation of serum and/or plasma may be a problem.

2. **Materials:**

   2.1 Filter paper (903, Schlicher and Schuell Inc., Keene, New Hampshire 03431)
   
   2.2 Flat-bottom microtiter plates (96-well) with covers
   
   2.3 Microtiter plate shaker
   
   2.4 Micropipettes (single and multichannel)
   
   2.5 Micropipette tips (200 µl)
   
   2.6 Refrigerator (4 °C ± 2 °C)
   
   2.7 Paper punch (5 mm diameter hole)
   
   2.8 Ink pen (indelible)
   
   2.9 Paper stapler
   
   2.10 Forceps (fine tip)
   
   2.11 Phosphate buffered saline (PBS). See appendix.

3. **Preparation and assembly of filter paper strip clusters:**

   3.1 Cut filter paper into 1.2 x 10 cm strips.
   
   3.2 Arrange 3 strips, each overlapping the other in the middle, to form a 6-spoke wheel configuration. Staple strips together at the center of the wheel. Each spoke is used to collect blood from one bird.

4. **Collection of blood on filter paper strips:**

   4.1 Puncture/nick the brachial wing vein or cut a toe nail of the bird to be sampled.
Filter Paper Method of Blood Collection for Serologic Test(s)

4.2 Briefly allow blood to pool on the skin at the puncture site. The blood should not be allowed to clot before soaking the filter paper strip.

4.3 Saturate the distal 1.2 to 2.5 cm of a filter paper strip with blood by soaking the flat side (not the edge) in the blood that has pooled at the puncture site on the wing or from a toe. Collect sufficient blood so that both surfaces of the strip become saturated.

4.4 Write the animal identification on the unused portion of the strip with an ink pen.

4.5 Bend the tip of each strip slightly upward so the cluster of 6 "spokes" will have a concave shape and can be placed on any flat surface so that the blood-containing portion of the strip will not be in contact with the storage surface.

4.6 Allow the strips to air-dry.

4.7 Place the dry strips in a plastic bag and seal the bag.

4.8 Ship/deliver samples to the laboratory without refrigeration.

4. Processing dried blood in filter paper:

4.1 Store the dried blood filter paper strips at 4 C until processed.

4.2 Label a flat-bottom microtiter plate to match the identification of samples on filter paper strips. One well per sample will be needed.

4.3 Punch 3, 5 mm discs from the saturated portion of a filter paper strip. Transfer the discs with a pair of forceps to the appropriate well of the microtiter plate.

4.4 Dispense 200 µl PBS to each well containing dried blood discs.

4.5 Place microtiter plate on a Micro plate shaker and mix for 1 hr (± 10 min) at a setting that will adequately mix the samples but not displace the liquid from the wells.
Filter Paper Method of Blood Collection for Serologic Test(s)

4.6 Incubate plates at 4 C overnight to allow for maximum elution of blood. The eluted sample is equivalent to a 1:10 dilution of serum.

4.7 Perform HI test according to the standard protocol (current version of AVPRO800, Hemagglutination-Inhibition Test to Detect Serum Antibodies to Avian Paramyxoviruses).

Note: The initial serum dilution will be 1:20 when tested by the standard protocol.

5. References:


6. Appendix:

Phosphate buffered saline (PBS), 0.1 M, pH 7.2.

Combine the following reagents: Sodium chloride 8.5 g, sodium phosphate dibasic 1.33 g, sodium phosphate monobasic 0.22 g, distilled water q.s. to 1 liter.