

shall be considered suspicious and shall be retested in accordance with paragraph (a)(6) of this section.

(10) On the retest if HI titers of 1:80 and/or SPD titers of 1:10 or higher are found, the flock shall be considered infected: *Provided*, That, at the discretion of the Official State Agency, additional tests may be conducted in accordance with paragraph (a)(6) of this section before final determination of the flock status is made.

(11) If HI titers of 1:80 and/or SPD titers of 1:10 or higher are found on the second retest, the flock shall be considered infected for the Mycoplasma for which it was tested.

(12) If the tube agglutination or serum plate tests are found on the second retest to be positive at the same or higher rate and the HI and/or SPD tests are negative, the flock should be considered infected: *Provided*, That if the status of the flock is considered to be equivocal, the Official State Agency may examine reactors by the in vivo bio-assay, PCR-based procedures, and/or culture procedures before final determination of the flock status is made.

(13) If the in vivo bio-assay, PCR-based procedures, and culture procedures are negative, the Official State Agency may qualify the flock for the classification for which it was tested.

(14) If the in vivo bio-assay, PCR-based procedures, or culture procedures are positive, the flock will be considered infected. However, the following considerations may apply:

(i) In PCR-positive flocks for which there are other negative mycoplasma test results, the flock's mycoplasma status should be confirmed through either seroconversion or culture isolation of the organism, or through both methods, before final determination of the flock's status is made.

(ii) In flocks for which only the bio-assay is positive, additional in vivo bio-assay, PCR-based procedures, or cultural examinations may be conducted by the Official State Agency before final determination of the flock's status is made.

(15) If the in vivo bio-assay, PCR-based procedures, or cultures are positive on retest, the flock shall be con-

sidered infected for the mycoplasma for which it was tested.

(b) [Reserved]

[40 FR 1504, Jan. 8, 1975, as amended at 41 FR 48726, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 47 FR 21993, May 20, 1982; 50 FR 19900, May 13, 1985; 54 FR 23957, June 5, 1989; 59 FR 12799, Mar. 18, 1994; 61 FR 11521, Mar. 21, 1996; 62 FR 44070, Aug. 19, 1997; 63 FR 3, Jan. 2, 1998; 65 FR 8019, Feb. 17, 2000]

§ 147.7 Standard test procedures for mycoplasma.⁵

The serum plate agglutination test, the tube agglutination test, and the enzyme-linked immunosorbent assay (ELISA) test should be considered basic screening tests for mycoplasma antibodies. The test selected will depend on preference, laboratory facilities, and availability of antigen. These three tests, though quite accurate, determine flock status rather than individual bird status, since occasional reactions are nonspecific. Under normal circumstances, the rate of such nonspecific reactions is low. Nonspecific reactions may occasionally be high, particularly after the use of erysipelas bacterin in turkeys and where mycoplasma antibodies are present for closely related mycoplasma other than for the species being tested. The hemagglutination inhibition (HI) test is too cumbersome for routine screening use. Positive reactions are extremely accurate however, and are useful in evaluating serum samples that react with the ELISA, plate, and/or tube antigens. The test should be conducted with 4 HA units. Titers of 1:80 or greater for both chicken and turkey sera are considered positive, while a 1:40 or 1:20 titer would be strongly suspicious and additional tests should be required.

(a) *Serum plate agglutination test.* (1) The serum plate agglutination test for mycoplasma is conducted by contacting and mixing 0.02 ml of test

⁵For additional information on mycoplasma test procedures, refer to the following references: Proc. 77th Annual Meeting, U.S. Animal Health Association, 1973; Isolation and Identification of Avian Pathogens, 3rd Edition; Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens, 1991.

serum with 0.03 ml of serum plate antigen on a glass at room temperature. The standard procedure is:

(i) Allow antigen and test serums to warm up to room temperature before use.

(ii) Dispense test serums in 0.02 ml amounts with a pipette or standardized loop (rinsed between samples) to 1½ inch squares on a ruled glass plate. Limit the number of samples (no more than 25) to be set up at one time according to the speed of the operator. Serum should not dry out before being mixed with antigen.

(iii) Dispense 0.03 ml of antigen beside the test serum on each square. Hold antigen dispensing bottle vertically.

(iv) Mix the serum and antigen, using a multimixing device if large numbers are to be run at one time.

(v) Rotate the plate for 5 seconds. At the end of the first minute, rotate the plate again for 5 seconds and read 55 seconds later.

(2) A positive reaction is characterized by the formation of definite clumps, usually starting at the periphery of the mixture. Most samples that are highly positive will react well within the 2-minute test period. Reactions thereafter should be considered negative, although partial agglutination at 3 and 5 minutes may warrant further retesting. High-quality antigen contacted with negative serum will usually dry up on the plate without visible clumping. Whenever samples are run, the antigen should be tested against known positive and negative control serums. Standard reference antigens and negative and positive titered sera are available from the National Veterinary Services Laboratories (NVSL), P.O. Box 884, Ames, Iowa 50010.

(3) Since it is difficult to measure uniform amounts of serum with a calibrated loop, this technique should not be used in conducting an official test.

(b) *Serum plate dilution test.* (1) The serum plate dilution (SPD) test may be used to evaluate possible nonspecific reactions, gain additional information to evaluate positive plate tests occurring in an unexpected manner, and/or to evaluate the level of mycoplasma antibodies present in the serum sam-

ple. If sufficient serum is available, the following method would provide the dilutions required to conduct the test.

(i) Rack three tubes and put 0.8 ml of phosphate-buffered saline (PBS) in tube 1 and 0.5 ml of PBS in tubes 2 and 3.

(ii) Pipette 0.2 ml of the test serum into tube 1 and discard the pipette.

(iii) With a pipette, mix the serum and PBS in tube 1 and withdraw 0.5 ml and add to tube 2.

(iv) Repeat the process in step (iii), mixing the contents of tube 2 and transferring 0.5 ml to tube 3.

(v) Conduct the test, as described for the serum plate test in paragraph (a), on the undiluted sample and on samples in tubes 1, 2, and 3 after proper mixing of each dilution.

(vi) To assist in the evaluation of the test, conduct concurrent SPD tests using both positive 1:80 and positive 1:160 HI sera for the mycoplasma being tested. The antigen should be pretested for reactivity with standard serum at the 1:5 and 1:10 dilution.

(vii) Interpretation of the SPD test results should be based on the criteria in § 147.6.

(c) *Tube agglutination test.* (1) The mycoplasma tube agglutination test is conducted by mixing 0.08 ml of test serum with 1.0 ml of diluted (1:20) antigen in a tube and allowing the mixture to react for 18-24 hours at 37° C. The diluent will be the standard phosphate-buffered saline with phenol. This solution is made up as follows:

	Grams
Sodium hydroxide (C.P.)	0.15
Sodium chloride (C.P.)	8.5
Potassium dihydrogen phosphate (KH ₂ PO ₄) (C.P.)	0.68
Phenol (Crystal) (C.P.)	2.5
Distilled water to make 1,000 ml	

The pH of the buffered phenolized saline will be 7.1-7.2 if all reagents are accurately measured. The stock tube antigen is diluted 1:20 with buffered phenolized saline. The procedures for the tube test are as follows:

(i) Rack 12x75 mm clean tubes and identify the tubes according to the sample to be tested.

(ii) Add 0.08 ml of the individual test serum to each tube. This will create

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approximately a 1:12.5 screening dilution of test serum when 1.0 ml of diluted antigen is added. The use of a pipetting device will insure proper mixing of serum and antigen.

(iii) To interpret positive reactions to the 1:12.5 dilution, two additional dilutions may be made by adding 0.04 ml of serum for 1:25 dilution and 0.02 ml of serum for 1:50 dilution, with the addition of 1.0 ml of diluted antigen as indicated in paragraph (c)(1)(ii) of this section.

(iv) Shake racks and incubate test systems for 18-24 hours at 37° C.

(2) Tests are read against a dark background under indirect fluorescent light. Regarded as a positive reaction is a clearing of the supernatant fluid, with visible sediment in the bottom of the tube. Incomplete reactions are suspect. Positive and negative control serums should be incorporated into each day's run of tests. Reactions at 1:25 or greater are considered positive. They should be confirmed by the HI test. Incubation for periods greater than 24 hours may be helpful in evaluating suspicious reactions and need for possible retesting or other diagnostic tests.

(d) *Hemagglutination Inhibition (HI) test.* The mycoplasma HI test is conducted by the constant-antigen, decreasing-serum method. This method requires using a 4-hemagglutination (HA) unit of diluted antigen. Differences in the number of HA units used will change the titers of positive sera markedly. Standard HA antigens for *Mycoplasma gallisepticum*, *M. synoviae*, and *M. meleagridis* are available from NVSL. The antigen has been titrated and diluted to approximately 1:640. The HA titration of each sample should be checked as described in paragraph (d)(2) on initial use or after long storage. To maintain HA activity, the undiluted HA antigen should be stored at -60° to -70° C. The test procedures are illustrated in Tables 2 and 3 of this paragraph.

(1) *Preparation of materials.* (i) Prepare phosphate-buffered saline (PBS) as follows:

	Grams
Sodium hydroxide (C.P.)	0.15
Sodium chloride (C.P.)	8.5
Potassium dihydrogen phosphate (KH ₂ PO ₄) (C.P.)	0.68

	Grams
Distilled water to make 1,000 ml	

The pH of the PBS will be 7.1-7.2 if all reagents are accurately measured.

(ii) Collect the turkey or chicken red blood cells (RBC's) in Alsever's solution which has been prepared as follows:

	Grams
Sodium citrate	8.0
Sodium chloride	4.2
Dextrose	20.5
Distilled water to make 1,000 ml.	

The sodium citrate and sodium chloride are dissolved in 800 ml distilled water and sterilized at 15 lbs. pressure for 15 minutes. Dissolve the dextrose in 200 ml distilled water, sterilize by Seitz or other type of filtration and then add aseptically to the sterile sodium citrate and sodium chloride solution.

(iii) From a turkey(s) or chicken(s) known to be free of the mycoplasma being tested, withdraw sufficient blood with a syringe containing Alsever's solution to give a ratio of 1 part blood to 5 parts Alsever's solution (e.g., 8 ml blood in 40 ml of Alsever's solution). Centrifuge the blood suspension at 1,000 rpm for 10 minutes and remove the Alsever's solution or supernatant with a pipette.

(iv) Wash the RBC's two times in 10 or more parts of Alsever's solution, centrifuging after each washing. Centrifugation is at 1,000 rpm for 10 minutes. The supernatant fluid is removed and the RBC deposit resuspended to give a 25 percent suspension of packed RBC's in Alsever's solution. (In testing either chicken or turkey sera, the homologous RBC system must be used; i.e., use chicken cells when testing chicken serum and turkey cells when testing turkey serum.) If this suspension is kept refrigerated, it should keep for 7 or 8 days after the blood has been collected.

(v) For the test, 1 ml of the 25 percent RBC's is added to 99 ml of buffered saline to make a 0.25 percent RBC suspension.

(2) *Hemagglutination (HA) antigen titration.* The HA stock antigen is stored at -70 ° C in PBS buffer containing 25

percent glycerin (vol/vol) in a concentrated suspension (*i.e.*, 320-640 HA units/ml) in screwtype vials. Under such conditions, potency will be retained for years. There will be a rapid

loss of titer if improperly stored. The titer of HA antigen is determined as illustrated in Table 1 and described in paragraphs (d)(2)(i) through (x) of this section.

TABLE 1 Titration of Hemagglutination (HA) Antigen

	Tube No.							
Reagents (ml)	1	2	3	8	9	10	11 ^a
PBS	0.8	0.5	0.5	0.5	0.5	0.5	0.5
Antigen	0.2							
Transfer	0.5→	0.5→	0.5→→	0.5→	0.5→	0.5→	0.5→ ^c
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ant. dilution	1:5	1:10	1:20	1:640	1:1280	1:2560	
Results ^b	+	+	+	+	-	-	

^a Tube 11, PBS/RBC control.

^b + = HA; - = no HA (sample titer 1:640).

^c Discard 0.5 ml.

(i) Rack a series of 11 chemically clean 12x75 mm test tubes. Label the tubes 1-11 left to right.

(ii) Put 0.8 ml of PBS in tube 1 and 0.5 ml of PBS in each of tubes 2-11.

(iii) Add 0.2 ml of antigen to tube 1. This will make a 1:5 dilution of antigen. Discard pipette.

(iv) Mix contents of tube 1 thoroughly with a clean pipette, and transfer 0.5 ml to tube 2. This will make a 1:10 dilution of antigen in tube 2. Discard pipette.

(v) Continue making serial twofold dilutions of antigen, changing pipettes after each transfer, through tube 10. This will result in a series of twofold dilutions ranging from 1:5 to 1:2560. Discard 0.5 ml of antigen dilution from tube 10.

(vi) Add 0.5 ml of 0.25 percent RBC's to tubes 1-11. Tube 11 will serve as PBS/RBC control.

(vii) Shake the rack and incubate at room temperature until the cells in the

PBS/RBC control tube have settled into a compact button at the bottom of the tube.

(viii) If turkey sera is also to be tested for HI titer, repeat steps outlined in paragraphs (d)(2)(i) through (vii) of this section, using 0.25 percent turkey RBC's.

(ix) The end point of the titration is the highest dilution of antigen that produces complete agglutination of the RBC's, as evidenced by the formation of a thin sheet of cells covering the concave bottom of the tube. For example, if complete agglutination is produced through tube 8 (a dilution of 1:640 of antigen), the antigen would be said to titer 640, the reciprocal of the dilution.

(x) Specificity of HA antigen should be determined by conducting HI tests with specific chicken sera of variable HI titers. Specific turkey sera of varying HI titers should be used if turkey sera is also to be tested.

TABLE 2 Hemagglutination Inhibition (HI) Test:

Reagents (ml)	Tube No.						
	1 ^a	2	3 8	9	10	11 ^b
PBS	0.8	0	0		0	0	0.5
8-unit antigen	0	0.5	0		0	0	0
4-unit antigen	0	0	0.5	0.5	0.5	0.5	0
Test serum	0.2	0	0	0	0	0	0
Transfer	0.5→	0.5→	0.5→...→	0.5→	0.5→	0.5→	0.5↓ ^c
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Serum dilution	1:5	1:10	1:20 ...	1:640	1:1280	1:2560	

^a Tube 1. Serum control.

^b Tube 11. PBS/RBC control.

^c Discard 0.5 ml.

TABLE 3 Antigen Control:

Reagents (ml)	Tube No.				
	1	2	3	4	5
4-unit antigen	1.0	0	0	0	0
PBS	0	0.5	0.5	0.5	0.5
Transfer	0.5→	0.5→	0.5→	0.5→	0.5 ^b ↓
0.25% RBC	0.5	0.5	0.5	0.5	0.5
Unit Antigen/tube	4	2	1	1/2	1/4
Results ^a	+	+	+	-	-

^a + = HA; - = no HA.

^b Discard 0.5 ml.

(3) *Reagents for mycoplasma HI test.* (i) Eight-unit antigen (Dilution factor for stock antigen is established by dividing titer by 8; *i.e.*, 640 antigen is diluted 1:80 in PBS to make 8-unit antigen.)

(ii) Four-unit antigen (made by diluting surplus 8-unit antigen 1:2 with PBS).

(iii) PBS at pH 7.0.

(iv) Unknown test serums.

(v) Positive control serum of known titer (should be from the same species as the unknown).

(vi) Negative control serum (should be from the same species as the unknown).

(vii) Solution of 0.25 percent washed RBC's.

(4) *Test outline.* (i) Rack 10 chemically clean 12x75 mm tubes for each serum,

including controls, to be tested. Identify each row of tubes, and label tubes in each row 1-10, left to right. In row 1, add tube 11 for a PBS/RBC control.

(ii) Put 0.8 ml of PBS in tube 1 of each test row; put 0.5 ml of 8-unit antigen in tube 2 of each test row; put 0.5 ml of 4-unit antigen in each of tubes 3-10 in each test row; and put 0.5 ml of PBS in tube 11.

(iii) Add 0.2 ml of test serum to tube 1. This tube will be the serum control in the test system.

(iv) Mix and make 0.5 ml transfers from tube 1 through tube 10. This will result in serial twofold dilutions of serum starting with 1:5 and ending with 1:2560. Discard 0.5 ml from tube 10.

(v) Rack five tubes in which to set up an antigen control.

(vi) In tube 1, put 1.0 ml of 4-unit antigen; put 0.5 ml of PBS in tubes 2-5.

(vii) Make 0.5 ml serial transfers from tube 1 through tube 5, changing pipettes after each transfer. Discard 0.5 ml from tube 5. This will result in a series of tubes respectively containing 4, 2, 1, 1/2, and 1/4 units of antigen.

(viii) After 20-30 minutes at room temperature to permit antibody-anti-

gen reaction, add 0.5 ml of 0.25 percent washed RBC's to each tube. Shake racks and incubate as for HA titration.

(ix) In this test system, positive serum should inhibit the HA activity of the antigen, while negative serum should have no effect. Inhibition will be evidenced by the formation of a free-flowing bottom of cells in the bottom of the tube. The titer of the serum can be calculated as the reciprocal of the highest dilution of serum that produces complete HI. Controls should read as follows:

(A) Serum control (tube 1). Cells should settle out.

(B) PBS/RBC control (tube 11). Cells should settle out.

(C) Antigen control. HA in tubes 1-3. Cells should settle out in tubes 4-5.

(D) Positive and negative serum control. Positive control should inhibit to its known titer; negative control should have no inhibitory effect.

(x) With this test system and 4 units of antigen, HI titers of 80 or above are considered positive and titers of 40 are strongly suspicious. However, titers of 10 or 20 are usually negative. Sample test results are illustrated in Table 4 in this paragraph.

TABLE 4—SAMPLE RESULTS OF HI TESTS
[Tube and Serum Dilution]

	1	2	3	4	5	6	7	8	9	10
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Serum A (HI neg.)	-	+	+	+	+	+	+	+	+	+
Serum B (HI 1:40)	-	-	-	-	+	+	+	+	+	+
Serum C (HI 1:160)	-	-	-	-	-	-	+	+	+	+
Serum D (HI 1:20)	-	-	-	+	+	+	+	+	+	+

+, HA.
-, no HA or HI.

(xi) If serological results from agglutination tests complemented by the HI test are inconclusive, cultural examination, bio-assay, or retesting of samples after an interval of at least 21 days may be indicated.

(e) *Procedure for mycoplasma hemagglutination inhibition tests using microtiter technique—(1) Procedure No. 1.* The microtiter mycoplasma HI test was developed from the tube HI test described in §147.7(d). Refer to these procedures for preparation of materials not listed below.

(i) *Materials needed.* (A) Microtiter equipment (minimal); *i.e.*, microplates, microdiluters, micropipettes, go-no-go diluter delivery tester, (0.05 ml).

(B) Phosphate-buffered saline (PBS).

(C) Reagents from NVSL; *i.e.*, HA antigen and negative and positive titered sera for the mycoplasma to be tested.

(D) Homologous red blood cells (RBC's) suspension 0.5 percent (2 ml of 25 percent RBC's to 98 ml of PBS) obtained from birds free of the mycoplasma to be tested. (See paragraphs

(d)(1)(ii) through (v) of this section for preparation of RBC's.)

(ii) *Microtiter hemagglutination (HA) antigen titration.* (A) Mark off two rows of 10 wells each for antigen titer (HA is done in duplicate).

(B) Mark last well in each row for cell controls.

(C) Prepare in small test tube (12×75 mm) a starting dilution of antigen by combining 0.1 ml antigen with 0.9 ml PBS. This is a 1:10 dilution.

(D) Add 0.05 ml PBS to all wells, including cell controls.

(E) Add 0.05 ml antigen (1:10 dilution) with diluters to the first well in both rows, mix thoroughly, transfer diluter to second well of each row and mix, continuing through the 10th well of each row. With mixture in diluter from last well, check diluter on go-no-go card, then place diluter in distilled water. If diluter checks out, antigen dilution will be 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120.

(F) Add 0.05 ml of 0.5 percent RBC suspension to all wells using a 0.05 dropper.

(G) Seal plate (if plate is to be held over 2 hours); shake and allow to stand at room temperature until cells in cell control gather in compact button. The titer is the highest dilution in which agglutination is complete. The dilution contains 1 HA unit in 0.05 ml.

(H) Prepare a dilution of antigen which contains 8 HA units in 0.05 ml. Example: if the antigen titer is 1:640, then that dilution contains 1 HA unit per 0.05 ml. Then $640 \div 8 = 80$, or a dilution of 1:80 containing 8 HA units. Or $640 \div 4 = 160$, a dilution of 1:160 containing 4 HA units per 0.05 ml.

(iii) *Microtiter HI test.* (A) Prepare two dilutions of antigen, one containing 8 HA units per 0.05 ml and one containing 4 HA units per 0.05 ml. The 4-unit antigen can be prepared from the 8-unit antigen by mixing with equal parts of PBS.

(B) Mark off one row of 8 wells for each test.

(C) Prepare a 1:5 dilution of each sera to be tested in a small test tube (12×75 mm): 0.1 ml serum plus 0.4 ml PBS or 0.05 ml serum plus 0.20 ml PBS.

(D) Add 0.05 ml PBS with the 0.05 ml dropper to the first well in each row.

(E) Add 0.05 ml of 8-unit antigen to well 2 in each row.

(F) Add 0.05 ml of 4-unit antigen to well 3 through 8 for each row.

(G) For each serum to be tested, load 0.05 ml diluter with 1:5 dilution as prepared in paragraph (iii) above and place in first well of row.

(H) Mix well and transfer loaded diluter to well 2. Continue serial twofold dilutions through well number 8.

(I) Well 1 (serum dilution of 1:10) is serum control. Well 2=1:20 dilution; well 3=1:40 dilution; well 4=1:80 dilution; well 5=1:160 dilution; well 6=1:320 dilution; well 7=1:640 dilution; and well 8=1:1280 dilution.

(J) *Antigen control.* (1) Mark off 6 wells for antigen controls.

(2) Add 0.05 ml PBS to wells 2, 3, 4, 5, and 6.

(3) Add 0.05 ml 8-unit antigen to wells 1 and 2.

(4) With empty diluter, mix contents of well 2. Continue serial twofold dilutions through well 6.

(5) Well 1 contains 8 units; well 2 contains 4 units; well 3 contains 2 units; well 4 contains 1 unit; well 5 contains $\frac{1}{2}$ unit; and well 6 contains $\frac{1}{4}$ unit.

(6) Mark off two wells for cell controls and add 0.05 ml PBS to each.

(7) After 20-30 minutes at average room temperature (20°-23°C) to permit antibody-antigen reaction, add 0.05 ml of a 0.5 percent suspension of RBC's to all wells.

(8) Seal all wells (if wells are to be held over 2 hours). Shake the plate thoroughly.

(9) Incubate at room temperature for 30-45 minutes.

(K) *Interpretation:* The HI titer is the highest serum dilution exhibiting complete inhibition of hemagglutination as indicated by flowing of cells when the plate is tilted. Serum having a titer of 1:80 or greater is considered positive. A titer of 1:40 or 1:20 is suspicious.

(2) *Procedure No. 2.* Purpose: To test for antibodies to avian mycoplasma by hemagglutination inhibition (HI). The test uses the constant antigen, titered-sera method for measuring antibodies to *M. gallisepticum*, *M. synoviae*, or *M. meleagridis*.

(i) *Materials needed.* (A) *M. gallisepticum*, *M. synoviae*, and/or *M. meleagridis* HI antigens.

(B) Positive and negative control sera.

(C) Phosphate buffered saline (PBS).

(D) Microtiter plates, 96-well, U-bottom.

(E) 12-channel pipettor (Titerek).

(F) 50 μ L pipettor (Pipetman P200).

(G) Pipette tips.

(H) 0.5 percent homologous red blood cells (RBC's) in PBS (use RBC's from the same species being tested).

(I) Plate-sealing tape.

(J) Mirrored plate reader.

(ii) *Microtiter hemagglutination antigen (HA) titration.* (A) Perform standard hemagglutination test (HA) on mycoplasma antigen to determine titer of antigen.

(1) Dispense 50 μ L of PBS into each well of 3 rows of a 96-well microtiter plate.

(2) Dispense 50 μ L of stock antigen into the wells of 2 rows.

(3) Perform serial two-fold dilutions (50 μ L) using a 12-channel pipettor. The dilution series will be from 1:2 to 1:4096.

(4) Add 50 μ L of 0.5 percent homologous RBC's to each well of all 3 rows. The row with no antigen serves as an RBC control.

(B) Incubate at room temperature (approximately 30 minutes) until the control RBC's give tight buttons. The HA titer is read as the last well to give a complete lawn (hemagglutination).

(C) Dilute stock antigen to 4 HA units for the HI test. The dilution required to give 4 HA units is calculated by dividing the stock antigen HA titer by 8. (Example: $1:320 \text{ HA units} \div 8 = 40$, dilute stock antigen 1:40.)

(iii) *Hemagglutination inhibition assay.* (A) Label one column (A to H) of a 96-well, U-bottom microtiter plate for each sample, each positive and negative control sera, antigen backtitration, and RBC control.

(B) Add 40 μ L of PBS to the top row of wells (row A) of the plate.

(C) Add 25 μ L of PBS to all remaining wells of the plate.

(D) Add 10 μ L of each test sera to well A of each column (making a 1:5 sera dilution).

(E) Serially dilute 25 μ L from well A through H using a 12-channel pipettor. Discard the final 25 μ L. Row A = 1:5...row H = 1:640.

(F) With an Oxford doser, add 25 μ L of 4 HA unit antigen to wells B through H. Well A serves as sera control.

(G) Prepare an antigen backtitration by adding 25 μ L of PBS to each well of one column. Add 25 μ L of diluted antigen to well A and serially dilute 25 μ L from wells A to D. This prepares 1:2, 1:4, 1:8, and 1:16 dilutions. (It is recommended that the antigen control backtitration be performed before the diluted antigen is used in the assay. Dilution problems could be detected and corrected before the inappropriately diluted antigen is used in the assay.)

(H) Leave a column of wells blank for an RBC control.

(I) Agitate gently and incubate for 30 minutes at room temperature.

(J) Add 50 μ L of 0.5 percent RBC's to all wells. Note: Do not agitate after RBC's have been added (agitation may result in false positive reactions by causing the RBC's to fall, resulting in "false" buttons).

(K) Cover the plate with sealing tape. Incubate at room temperature for 30 minutes or until control RBC's give a tight button.

(L) Read the reaction on a mirrored plate reader.

(iv) *Results.* (A) The titer is reported as the reciprocal of the last dilution to give a tight button of RBC's. The final dilution scheme includes the antigen in the dilution calculation and is as follows: B=1:20, C=1:40, D=1:80, E=1:160, F=1:320, G=1:640, H=1:1,280.

(B) For the assay to be valid:

(1) The positive control sera must give a result within one dilution of the previously determined titer.

(2) The negative control sera must be negative.

(3) The backtitration of the antigen must be 1:4 or 1:8.

(4) The RBC control must give tight, non-hemolyzed buttons.

(5) Sera controls (well A of each test sera) must not have non-specific agglutination or hemolysis. If negative, report as "negative with non-specific agglutination or non-specific hemolysis" or "unable to evaluate due to non-specific agglutination or hemolysis" or treat the serum to remove the non-specific agglutination and repeat the test. (See paragraph (e)(2)(v) of this section.)

(v) *Treatment to remove non-specific agglutination*—(A) *Purpose.* Treatment of serum to remove non-specific agglutination that is interfering with HI assays.

(B) *Specimen.* Serum.

(C) *Materials.* Homologous RBC's (chicken or turkey), 50 percent solution PBS, centrifuge, incubator, 4C (refrigerator).

(D) *Procedure.* (1) Prepare a 1:5 dilution of test serum by adding 50 µL of serum to 200 µL of PBS.

(2) Prepare a 50 percent solution of RBC's by adding equal volumes of packed RBC's to PBS. Mix well.

(3) Add 25 µL of 50 percent RBC solution to the serum dilutions.

(4) Vortex gently to mix.

(5) Incubate at 4 °C for 1 hour.

(6) Centrifuge to pellet the RBC's.

(7) Use the supernatant to perform the HI assay. Modify the dilution scheme in the assay to consider the initial 1:5 dilution prepared in the treatment. For the 1:5 dilution scheme, do not add PBS to row A. Add 50 µL of the 1:5 treated supernatant to row A. Serially dilute 25 µL from rows A through H. This prepares a serum dilution of 1:10 through 1:640 in rows B through H.

[49 FR 19803, May 10, 1984, as amended at 57 FR 57342, Dec. 4, 1992; 59 FR 12799, Mar. 18, 1994; 63 FR 3, Jan. 2, 1998; 67 FR 8469, Feb. 25, 2002]

§ 147.8 Procedures for preparing egg yolk samples for diagnostic tests.

The following testing provisions may be used for retaining the classification U.S. M. Gallisepticum Clean under § 145.23(c)(1)(ii)(C) and § 145.33(c)(1)(ii)(C), for retaining the classification U.S. M. Synoviae Clean under § 145.23(e)(1)(ii)(b) and § 145.33(e)(1)(ii)(b), and for retaining the classification U.S. H5/H7 Avian Influenza Monitored under § 146.23(a), § 146.33(a), and § 146.44(a) of this chapter.

(a) Under the supervision of an Authorized Agent or State Inspector, the eggs which are used in egg yolk testing must be selected from the premises where the breeding flock is located, must include a representative sample of 30 eggs collected from a single day's production from the flock, must be identified as to flock of origin and pen,

and must be delivered to an authorized laboratory for preparation for diagnostic testing.

(b) The authorized laboratory must identify each egg as to the breeding flock and pen from which it originated, and maintain this identity through each of the following:

(1) Crack the egg on the round end with a blunt instrument.

(2) Place the contents of the egg in an open dish (or a receptacle to expose the yolk) and prick the yolk with a needle.

(3) Using a 1 ml syringe without a needle, aspirate 0.5 ml of egg yolk from the opening in the yolk.

(4) Dispense the yolk material in a tube. Aspirate and dispense 0.5 ml of PBS (phosphate-buffered saline) into the same tube, and place in a rack.

(5) After all the eggs are sampled, place the rack of tubes on a vortex shaker for 30 seconds.

(6) Centrifuge the samples at 2500 RPM (1000×g) for 30 minutes.

(7)(i) For egg yolk samples being tested to retain the U.S. M. Gallisepticum Clean and U.S. M. Synoviae Clean classifications, test the resultant supernatant for *M. gallisepticum* and *M. synoviae* by using test procedures specified for detecting IgG antibodies set forth for testing serum in § 147.7 (for these tests the resultant supernatant would be substituted for serum); except that a single 1:20 dilution hemagglutination inhibition (HI) test may be used as a screening test in accordance with the procedures set forth in § 147.7.

(ii) For egg yolk samples being tested to retain the U.S. H5/H7 Avian Influenza Monitored classification, test the resultant supernatant in accordance with the requirements in § 146.13(b).

NOTE: For evaluating the test results of any egg yolk test, it should be remembered that a 1:2 dilution of the yolk in saline was made of the original specimen.

[50 FR 19900, May 13, 1985; 63 FR 3, Jan. 2, 1998, as amended at 71 FR 56333, Sept. 26, 2006]

§ 147.9 Standard test procedures for avian influenza.

(a) The agar gel immunodiffusion (AGID) test should be considered the basic screening test for antibodies to