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VS

NATIONAL POULTRY IMPROVEMENT PLAN PROGRAM STANDARDS

August 2014

NPIP

NATIONAL POULTRY IMPROVEMENT PLAN

PROGRAM STANDARDS OF THE NATIONAL POULTRY IMPROVEMENT PLAN

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Introduction

These program standards describe specific tests and sanitation procedures. Formerly, these tests and procedures were outlined in our regulations at title 9, *Code of Federal Regulations* (9 CFR) part 147. We have amended our regulations to require that tests to be performed and sanitation to be maintained in a manner approved by the Administrator. The recommended tests and sanitation procedures are contained in this document. These changes streamline the provisions of the Plan, keep those provisions current with changes in the poultry industry, and provide for the use of new sampling and testing procedures.

Changes to this document are voted on and approved by the voting delegates at the Plan's Biennial National Plan Conference.

The National Poultry Improvement Plan (NPIP, also referred to as "the Plan") is a cooperative Federal-State-industry mechanism for controlling certain poultry diseases. The Plan consists of a variety of programs intended to prevent and control poultry diseases. Participation in all Plan programs is voluntary, but breeding flocks, hatcheries, and dealers must first qualify as "U.S. Pullorum-Typhoid Clean" as a condition for participating in the other Plan programs.

The Plan identifies States, flocks, hatcheries, dealers, and slaughter plants that meet certain disease control standards specified in the Plan's various programs. As a result, customers can buy poultry that has tested clean of certain diseases or that has been produced under disease prevention conditions.

The NPIP regulations in 9 CFR parts 145 and 146 contain requirements that must be observed by flocks that participate in the Plan. These requirements include requirements to test poultry for the specific disease addressed by each classification in which the flock participates. The procedures by which that testing is conducted have historically been contained in 9 CFR part 147, subparts A, B, and D. Subpart A set out blood testing procedures, subpart B set out bacteriological examination procedures, and subpart D set out molecular examination procedures, which currently include polymerase chain reaction (PCR) tests. In addition, subpart C of part 147 contained sanitation procedures. These test and sanitation procedures are now incorporated into this Program Standards document.

We have prepared this program standards document for several reasons. First, there are constant changes in the science and technology that go into developing effective, efficient tests. To have a successful voluntary program to reduce the incidence of disease in poultry, we need to be able to update the NPIP testing procedures when new scientific evidence indicates that different procedures can increase the reliability of a test, or when new technology is developed to make a test more efficient or accurate.

In addition, new tests are also continually developed that can provide valuable alternatives to existing approved tests. For example, there has been a great deal of progress in developing PCR tests in recent years. Adding such tests allows NPIP participants to take advantage of the latest testing technology.

Similarly, the sanitation procedures used as best practices to prevent the introduction or spread of disease in poultry flock are constantly changing, as more information becomes available about possible sources of infection and about the effectiveness of various means of preventing infection.

In the past, we have updated the regulations once every 2 years, following the biennial Plan Conference. However, with the continual changes in diagnostic science and testing technology, and in best practices for maintaining sanitation, the biennial update schedule has resulted in the regulations becoming out of date between updates. When this happens, sometimes the Plan's General Conference Committee (GCC) approves interim changes to the tests or sanitation procedures in accordance with the process outlined in 9 CFR147.43(d)(5)(iii). We hope to make the program more effective by making all participants aware of the new tests and sanitation procedures immediately by updating this document which we intend to be recognized in the regulations as a resource for tests and sanitation procedures.

Finally, tests can be difficult to render in the regulations. The current regulations in 9 CFR 147.11 and 9 CFR 147.12, for example, contain diagrams and flow charts that are part of larger processes, all of which require several pages to describe in narrative format. These program standards allow us to lay out our tests in an easier-to-understand format.

Definitions

Administrator	The Administrator, Animal and Plant Health Inspection Service, or any other employee of the Animal and Plant Health Inspection Service delegated to act in the Administrator's stead.
Affiliated flock	A meat-type flock that is owned by or has an agreement to participate in the Plan with a slaughter plant and that participates in the Plan through that slaughter plant.
Affiliated flock owner	A flock owner who is participating in the Plan through an agreement with a participating hatchery.
Animal and Plant Health Inspection Service (APHIS, the Service)	The Animal and Plant Health Inspection Service of the U.S. Department of Agriculture.
Authorized agent	Any person designated under title 9, *Code of Federal Regulations* (9 CFR) 145.11(a) to collect official samples for submission to an authorized laboratory as described in Program Standards Supbart A Section (1) and Subpart B Section (3). An authorized agent is also a person designated to perform functions under 9 CFR 146.10(a).
Authorized laboratory	An authorized laboratory is a laboratory that meets the requirements of9 CFR 147.52 and is thus qualified to perform the assays described in Program Standards Subpart D Section (7).
Authorized testing agent	Any person designated under 9 CFR 145.11(a) to collect official samples for submission to an authorized laboratory as described in Program Standards Supbart A Section (1) and Subpart B Section (3) and to perform the stained antigen, rapid whole blood test for pullorum typhoid.
Avian influenza	An infection or disease of poultry caused by viruses in the family *Orthomyxoviridae*, genus Influenzavirus A.
Baby poultry	Newly hatched poultry (chicks, poults, ducklings, goslings, keets, etc.).
Classification	A designation earned by participation in a Plan program.
Colon bacilli	For the purpose of this chapter, those organisms which are gram negative, non-spore-forming bacilli, which ferment lactose with gas formation, and serve as an index of fecal contamination.
Commercial meat-type flock	All of the meat-type chickens, spent fowl, meat-type turkeys, commercial upland game birds, or commercial waterfowl on one farm. However, at the discretion of the Official State Agency, any group of poultry which is segregated from another group in a manner sufficient to prevent the transmission of H5/H7 LPAI and has been so segregated for a period of at least 21 days may be considered as a separate flock.
Commercial table-egg layer flock	All table-egg layers of common age or pullet source on one premises.

Commercial table-egg layer premises	A farm containing contiguous flocks of commercial table-egg layers under common ownership.
Commercial table-egg layer pullet flock	A table-egg layer flock prior to the onset of egg production.
Cooperating State Agency	Any State authority recognized by the Department to cooperate in the administration of the provisions of 9 CFR part 56. This may include the State animal health authority or the Official State Agency.
Dealer	An individual or business that deals in commerce in hatching eggs, newly hatched poultry, and started poultry obtained from breeding flocks and hatcheries. This does not include an individual or business that deals in commerce in buying and selling poultry for slaughter only.
Department	The U.S. Department of Agriculture.
Domesticated	Propagated and maintained under the control of a person.
Equivalent or equivalent requirements	Requirements which are equal to or exceed the program, conditions, criteria, or classifications with which they are compared, as determined by the Official State Agency and with the concurrence of the Service.
Exposed (Exposure)	Contact with birds, equipment, personnel, supplies, or any article infected with, or contaminated by, communicable poultry disease organisms.
Flock:	(1) As applied to breeding. All poultry of one kind of mating (breed and variety or combination of stocks) and of one classification on one farm. (2) As applied to disease control. All of the poultry on one farm except that, at the discretion of the Official State Agency, any group of poultry which is segregated from another group and has been so segregated for a period of at least 21 days may be considered as a separate flock.
Fluff sample	Feathers, shell membrane, and other debris resulting from the hatching of poultry.
Fowl typhoid or typhoid	A disease of poultry caused by *Salmonella gallinarum*.
Franchise breeder	A breeder who normally sells products under a specific strain or trade name and who authorizes other hatcheries to produce and sell products under this same strain or trade name.
Franchise hatchery	A hatchery which has been authorized by a franchise breeder to produce and sell products under the breeder's strain or trade name.
H5/H7 low pathogenic avian influenza (LPAI)	An infection of poultry caused by an influenza A virus of H5 or H7 subtype that has an intravenous pathogenicity index in 6-week-old chickens of less than 1.2 or less than 75 percent mortality in 4- to 8-week-old chickens infected intravenously, or an infection with influenza A viruses of H5 or H7 subtype with a cleavage site that is not consistent with a previously identified highly pathogenic avian influenza virus.

H5/H7 LPAI virus infection (infected)

(1) Poultry will be considered to be infected with H5/H7 LPAI for the purposes of this part if:
   (i) H5/H7 LPAI virus has been isolated and identified as such from poultry;
   or
   (ii) Viral antigen or viral RNA specific to the H5 or H7 subtype of AI virus has been detected in poultry;
   or
   (iii) Antibodies to the H5 or H7 subtype of the AI virus that are not a consequence of vaccination have been detected in poultry. If vaccine is used, methods should be used to distinguish vaccinated birds from birds that are both vaccinated and infected. In the case of isolated serological positive results, H5/H7 LPAI infection may be ruled out on the basis of a thorough epidemiological investigation that does not demonstrate further evidence of H5/H7 LPAI infection, as determined by APHIS.

(2) The official determination that H5/H7 LPAI virus has been isolated and identified, viral antigen or viral RNA specific to the H5 or H7 subtype of AI virus has been detected, or antibodies to the H5 or H7 subtype of AI virus have been detected may only be made by the National Veterinary Services Laboratories.

Hatchery

Hatchery equipment on one premises operated or controlled by any person for the production of baby poultry.

Independent flock

A flock that produces hatching eggs and that has no ownership affiliation with a specific hatchery.

Infected flock

A flock in which an authorized laboratory has discovered one or more birds infected with a communicable poultry disease for which a program has been established under the Plan.

Midlay

Approximately 2–3 months after a flock begins to lay or after a molted flock is put back into production.

Multiplier breeding flock

A flock that is intended for the production of hatching eggs used for the purpose of producing progeny for commercial egg or meat production or for other nonbreeding purposes.

NPIP

The National Poultry Improvement Plan.

NPIP Program Standards

A document that contains tests and sanitation procedures approved by the Administrator under 9 CFR 147.53. This document may be obtained from the NPIP Web site at http://www.poultryimprovemet.org or by writing to the Service at National Poultry Improvement Plan, APHIS, USDA, 1506 Klondike Road, Suite 101, Conyers, GA 30094.

NPIP Technical Committee

A committee made up of technical experts on poultry health, biosecurity, surveillance, and diagnostics. The committee consists of representatives from the poultry and egg industries, universities, and State and Federal governments and is appointed by the Senior Coordinator and approved by the General Conference Committee.

Official State Agency	The State authority recognized by the Department to cooperate in the administration of the Plan.
Official supervision:	(1) As applied to Plan programs. The direction, inspection, and critical evaluation by the Official State Agency of compliance with the provisions of the Plan. (2) As applied to non-Plan but equivalent State poultry improvement programs. The direction, inspection, and critical evaluation by an officer or agency of a State government, of compliance with a publicly announced State poultry improvement program.
Person	A natural person, firm, or corporation.
Plan	The provisions of the National Poultry Improvement Plan.
Poultry	Domesticated fowl, including chickens, turkeys, ostriches, emus, rheas, cassowaries, waterfowl, and game birds, except doves and pigeons, which are bred for the primary purpose of producing eggs or meat.
Primary breeding flock	A flock composed of one or more generations that is maintained for the purpose of establishing, continuing, or improving parent lines.
Products	Poultry breeding stock and hatching eggs, baby poultry, and started poultry.
Program	Management, sanitation, testing, and monitoring procedures which, if complied with, will qualify, and maintain qualification for, designation of a flock, products produced from the flock, or a state by an official Plan classification and illustrative design, as described in 9 CFR 145.10.
Public exhibition	A public show of poultry.
Pullorum disease or pullorum	A disease of poultry caused by *Salmonella pullorum*.
Reactor	A bird that has a positive reaction to a test required or recommended in 9 CFR part 145 or these Program Standards for any poultry disease for which a program has been established under the Plan.
Salmonella	Any bacteria belonging to the genus *Salmonella*, including the arizona group.
Sanitize	To treat with a product which is registered by the Environmental Protection Agency as germicidal, fungicidal, pseudomonocidal, or tuberculocidal, in accordance with the specifications for use as shown on the label of each product. The Official State Agency, with the concurrence of the Service, shall approve each product or procedure according to its specified usage.

Senior Coordinator An employee of the Service whose duties may include but will not necessarily be limited to:

(1) Serving as executive secretary of the General Conference Committee.
(2) Serving as chairperson of the Plan Conference described in 9 CFR 147.47.
(3) Planning, organizing, and conducting the Plan Conference.
(4) Reviewing NPIP authorized laboratories as described in 9 CFR 147.51.
(5) Coordinating the State administration of the NPIP through periodic reviews of the administrative procedures of the Official State Agencies, according to the applicable provisions of the Plan and the Memorandum of Understanding.
(6) Coordinating rulemaking to incorporate the proposed changes of the provisions approved at the Plan conference into the regulations in 9 CFR parts 145, 146, and 147.
(7) Directing the production of official NPIP publications.
(8) Proposing an annual budget for plan activities and the General Conference Committee.
(9) Providing overall administration of the NPIP.

Service The Animal and Plant Health Inspection Service of the U.S. Department of Agriculture.

Serial The total quantity of completed product which has been thoroughly mixed in a single container and identified by a serial number.

Sexual maturity The average age at which a species of poultry is biologically capable of reproduction.

Started poultry Young poultry (chicks, pullets, cockerels, capons, poults, ducklings, goslings, keets, etc.) that have been fed and watered and are less than 6 months of age.

State Any of the States, the District of Columbia, the Commonwealth of Puerto Rico, Guam, the Commonwealth of the Northern Mariana Islands, the Virgin Islands of the United States, or any territory or possession of the United States.

State Inspector Any person employed or authorized to perform functions under 9 CFR145.11(b) or 9 CFR 146.10(b).

Stock A term used to identify the progeny of a specific breeding combination within a species of poultry. These breeding combinations may include pure strains, strain crosses, breed crosses, or combinations thereof.

Strain Poultry breeding stock bearing a given name produced by a breeder through at least five generations of closed flock breeding.

Succeeding flock A flock brought onto a premises during the 12 months following removal of an infected flock.

Suspect flock A flock shall be considered, for the purposes of the Plan, to be a suspect flock if any evidence exists that it has been exposed to a communicable poultry disease.

Trade name or number	A name or number compatible with State and Federal laws and regulations applied to a specified stock or product thereof.
United States	All of the States.

Subpart A—Blood Testing Procedures

(1) The standard tube agglutination test[1]

(a) The blood samples should be collected and delivered as follows:

(1) The blood samples should be taken by properly qualified and authorized persons only, and in containers provided by the laboratory. The containers should be stout-walled test tubes, preferably3/8 by 3 inches, without lip, or small well-selected medicine vials, which have been thoroughly cleaned and dried in a hot-air drying oven. If stoppers are used, they should be thoroughly cleaned and dried.

(2) Sufficient blood should be procured by making a small incision in the large median wing vein with a small sharp lancet and allowing the blood to run into the tube, or by the use of a small syringe (with 20 or 21 gage needle) which is properly cleansed between bleedings with physiological saline solution. To facilitate the separation of the serum, the tubes should be placed in a slanted position until the blood has solidified. After the blood has completely clotted, they should be packed and shipped by mail (special delivery), rapid express, or by messenger, to the laboratory. All labeling must be clear and permanent, and may be done with a suitable pencil on etched portions of the tube, or by means of fast-gum labels.

(3) The blood samples must reach the laboratory in a fresh and unhemolyzed condition. Hemolyzed samples should be rejected. It is imperative, therefore, to cool the tubes immediately after slanting and clotting, and unless they reach the laboratory within a few hours, to pack them with ice in special containers, or use some other cooling system which will insure their preservation during transportation. In severe cold seasons, extreme precautions must be exercised to prevent freezing and consequent laking. The samples must be placed in cold (5° to 10°C.) storage, immediately on arrival at the laboratory.

(b) The antigen shall consist of representative strains of S. pullorum which are of known antigenic composition, high agglutinability, but are not sensitive to negative and nonspecific sera. The stock cultures may be maintained satisfactorily by transferring to new sloped agar at least once a month and keeping at 18° to 25°C. (average room temperature) in a dark closet or chest, following incubation for from 24 to 36 hours at 37°C. The antigenic composition and purity of the stock cultures should be checked consistently.

[1] The procedure described is a modification of the method reported in the Proceedings of the U.S. Live Stock Sanitary Association, November 30 to December 2, 1932, pp. 487 to 491.

(c) A medium which has been used satisfactorily has the following composition:

Water	1,000 cc.
Difco beef extract	4 gm. (0.4 percent).
Difco Bacto-peptone	10 gm. (1.0 percent).
Difco dry-granular agar.	20 gm. (2.0 percent).
Reaction—pH 6.8 to 7.2.	

(d) Large 1–inch test tubes, Kolle flasks, or Blake bottles should be streaked liberally over the entire agar surface with inoculum from 48-hour slant agar cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated 48 hours at 37°C., and the surface growth washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5°to 10°C.) in tightly stoppered bottles.

(e) Thiosulfate-Glycerin (TG) medium may be used as an alternate medium for the preparation of tube agglutination antigen. The TG medium, formerly used for the preparation of stained, whole-blood antigen, is described in more detail in the article by A. D. MacDonald, Recent Developments in Pullorum Antigen for the Rapid, Whole-Blood Test, Report of the Conference of the National Poultry Improvement Plan, pages 122–127, 1941. This medium provides a tube antigen of excellent specificity and greatly increases the yield of antigen from a given amount of medium. The TG medium has the following composition:

Beef infusion	1,000 cc.
Difco Bacto-peptone	20 gm. (2.0 percent).
Sodium thiosulfate	5 gm. (0.5 percent).
Ammonium chloride	5 gm. (0.5 percent).
Glycerin, U.S.P. (95 percent)	20 cc. (2.0 percent).
Difco dry-granular agar.	30 gm. (3.0 percent).
Reaction—pH 6.8 to 7.2.	

Large 1–inch test tubes, Kolle flasks, Blake bottles, or Erlenmeyer flasks should be seeded over the entire agar surface with inoculum from 24–hour beef infusion broth cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated 96 hours at 37°C., and the surface growth washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigen then should be

centrifuged. The mass of bacteria should be removed from the centrifuge tubes or bowl and resuspended in saline (0.85 percent) solution containing 0.5 percent phenol. After the bacterial mass has been uniformly suspended in the diluent, it should be again passed through a cotton pad in a Buchner funnel without the aid of suction. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5° to 10°C.) in tightly stoppered bottles.

(f) The diluted antigen to be used in the routine testing should be prepared from the stock antigen by dilution of the latter with physiological (0.85 percent) saline solution containing 0.25 percent of phenol to a turbidity corresponding to 0.75–1.00 on the McFarland nephelometer scale. The hydrogen-ion concentration of the diluted antigen should be corrected to pH 8.2 to 8.5 by the addition of dilute sodium hydroxide. New diluted antigen should be prepared each day and kept cold. The diluted antigen may be employed in 2 cc. quantities in 4 by1/2–inch test tubes, or 1 cc. quantities in smaller tubes, in which the final serum-antigen mixtures are made and incubated. The distribution of the antigen in the tubes may be accomplished by the use of long burettes, or special filling devices made for the purpose.

(g) The maximum serum dilution employed must not exceed 1:50 for chickens, nor 1:25 for turkeys. The available data indicate that 1:25 dilution is the most efficient. In all official reports on the blood test, the serum dilutions shall be indicated. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum-delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37°C.

(h) The results shall be recorded as:

N, or − (negative), when the serum-antigen mixture remains uniformly turbid.

P, or + (positive), when there is a distinct clumping of the antigen, and the liquid between the agglutinated particles is clear.

S, or ? (suspicious), when the agglutination is only partial or incomplete.

M, or missing, when samples listed on the original record sheet are missing.

H, or hemolyzed, when blood samples are hemolyzed and cannot be tested.

B, or broken, when sample tubes are broken and no serum can be obtained.

(Some allowance must always be made for the difference in sensitiveness of different antigens and different set-ups, and therefore, a certain amount of independent, intelligent judgment must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of S. pullorum.)

(2) The rapid serum test[2]

(a) The procedure for the collection and delivery of blood samples in the rapid serum test is the same as that described in Section A(1)(a) of these standards.

(b) The selection and maintenance of suitable strains of S. pullorum and the composition of a satisfactory medium are described in Section 1(b) and 1(c).

(c) Large 1–inch test tubes, Kolle flasks, or Blake bottles are streaked liberally from 48–hour slant-agar cultures prepared from stock cultures of the selected strains.

(d) The antigen-growing tubes or bottles should be incubated 48 hours at 37°C., and the surface growth washed off with a very slight amount of 12 percent solution of sodium chloride containing 0.25 to 0.5 percent phenol, filtered through lightly packed sterile absorbent cotton placed in the apex of a sterile funnel.

(e) The washings should be adjusted (using 12 percent sodium chloride containing 0.25 to 0.5 percent phenol) so that the turbidity is 50 times greater than tube 0.75 of McFarland's nephelometer, or to a reading of 7 mm. by the Gates nephelometer.

(f) The individual strain antigens should be tested with negative sera for their insensitivity and with positive sera for high agglutinability in comparison with known satisfactory antigen. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5° to 10°C.) in tightly stoppered bottles.

(g) The tests should be conducted on a suitable, smooth plate. The serum-antigen dilution should be made so that the dilution will not exceed 1:50 when compared to the standard tube agglutination test. When testing turkey blood samples, it is desirable to use a serum-antigen dilution equivalent to the 1:25 in the tube method. The serum should be added to the antigen and mixed thoroughly by use of the tip of the serum pipette. Most strong positive reactions will be plainly evident within 15 to 20 seconds. The final reading should be made at the end of 2 or 3 minutes. Heating the plate at approximately 37°C. will hasten agglutination. Before reading, the plate should be rotated several times.

(h) The results shall be recorded as described in Section 1(h).

(3) The stained-antigen, rapid, whole-blood test[3]

(a) The description of the preparation of antigen is not herein included because the antigen is a proprietary product produced only under license from the Secretary of Agriculture.

(b) A loop for measuring the correct quantity of blood can usually be obtained from the manufacturer of the antigen. A satisfactory loop may be made from a

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[2] The procedure described is a modification of the method reported by Runnels, Coon, Farley, and Thorpe, Amer. Vet. Med. Assoc. Jour. 70 (N.S. 23): 660–662 (1927).

[3] The procedure described is a modification of the method reported by Schaffer, MacDonald, Hall, and Bunyea, Jour. Amer. Vet. Med. Assoc. 79 (N. S. 32): 236–240 (1931).

piece of No. 20 gage nichrome wire, 21/2inches long, at the end of which is fashioned a loop three-sixteenths of an inch in diameter. Such a loop, when filled with blood so that the blood appears to bulge, delivers 0.02 cc. A medicine dropper whose tip is adjusted to deliver 0.05 cc. is used to measure the antigen. A glass plate about 15 inches square, providing space for 48 tests, has proved satisfactory for this work. The use of such a plate enables the tester to have a number of successive test mixtures under observation without holding up the work to wait for results before proceeding to the next bird.

(c) A drop of antigen should be placed on the testing plate. A loopful of blood should be taken up from the wing vein. When submerged in the blood and then carefully withdrawn, the loop becomes properly filled. On looking down edgewise at the filled loop, one observes that the blood appears to bulge. The loopful of blood then should be stirred into the drop of antigen, and the mixture spread to a diameter of about 1 inch. The loop then should be rinsed in clean water and dried by touching it to a piece of clean blotting paper, if necessary. The test plate should be rocked from side to side a few times to mix the antigen and blood thoroughly, and to facilitate agglutination. The antigen should be used according to the directions of the producer.

(d) Various degrees of reaction are observed in this as in other agglutination tests. The greater the agglutinating ability of the blood, the more rapid the clumping and the larger the clumps. A positive reaction consists of a definite clumping of the antigen surrounded by clear spaces. Such reaction is easily distinguished against a white background. A somewhat weaker reaction consists of small but still clearly visible clumps of antigen surrounded by spaces only partially clear. Between this point and a negative or homogeneous smear, there sometimes occurs a very fine granulation barely visible to the naked eye; this should be disregarded in making a diagnosis. The very fine marginal clumping which may occur just before drying up is also regarded as negative. In a nonreactor, the smear remains homogeneous. (Allowance should be made for differences in the sensitiveness of different antigens and different set-ups, and therefore, a certain amount of independent, intelligent judgment must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of S. pullorum.)

(4) The microagglutination test for pullorum-typhoid

Routinely, the microagglutination test is applied as a single-dilution test and only a single 18–24 hour reading is made.

(a) The procedure for the collection and delivery of blood samples in the microagglutination test is the same as that described in Section A(1)(a). A method that has proven advantageous is to transfer the serum samples from the blood clot to a microplate as described in "Applied Microbiology," volume 24, No. 4, October 1972, pages 671–672. The dilutions are then performed according to paragraphs (d) or (e) of this section.

(b) Stained microtest antigen for pullorum-typhoid is supplied as concentrated stock suspension and must be approved by the Department.[4] Directions for diluting will be provided with the antigen. The stock as well as the diluted antigen prepared each day should be kept sealed in the dark at 5° to 10°C. when not in use.

(c) Available data indicate that a 1:40 dilution for the microagglutination test is most efficient for the detection of pullorum-typhoid agglutinins in both chickens and turkeys. In all official reports on the blood test, the serum dilutions shall be indicated.

(d) The recommended procedure for the 1:40 dilution in the microagglutination test is as follows:

(1) Add 100 microliters (0.10 cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) Using a microdiluter or a multimicrodiluter handle fitted with twelve 10 microliter microdiluters, transfer 5 microliters (0.005 cc.) of the serum sample from the collected specimen to the corresponding well of the microplate. This is accomplished by touching the surface of the serum sample with the microdiluter and then transferring and mixing with the diluent in the microplate well. The microdiluter is removed, blotted, touched to the surface of the distilled water wash, and again blotted. Other acceptable methods of serum delivery are described in Applied Microbiology, volume 21, No. 3, March 1971, pages 394–399.

(3) Dilute the microtest antigens with 0.50 percent phenolized saline and add 100 microliters (0.1 cc.) to each microplate well.

(4) Seal each plate with a plastic sealer or place unsealed in a tight incubation box as described in Applied Microbiology, volume 23, No. 5, May 1972, pages 931–937. Incubate at 37°C. for 18–24 hours.

(5) Read the test results as described in paragraph (f) of this section.

(e) The recommended procedure for a microagglutination test titration is as follows:

(1) Add 50 microliters (0.05cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) To the wells representative of the lowest dilution in the titration, add an additional 50 microliters (0.05 cc.) of 0.85 percent physiological saline making a total of 100 microliters in these wells.

(3) Transfer each serum sample as described in (4)(d)(2) of these Standards to the first well containing 100 microliters (0.10cc.) in the titration, which represents the lowest dilution.

(4) Make twofold serial dilutions of each serum by transferring 50 microliters (0.05cc.) of diluted serum from one well to the next using twelve 50-microliter microdiluters fitted in a multimicrodiluter handle. When transfers have been made to all of the wells of the desired series, the 50 microliters remaining in the microdiluters are removed by blotting, touching the microdiluters to the surface of the distilled water wash, and blotting again.

(5) Dilute the desired microtest antigen with 0.50 percent phenolized saline and add 50 microliters (0.05 cc.) to each microplate well.

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[4] Information as to criteria and procedures for approval of concentrated stock suspension of stained microtest antigens may be obtained from the National Poultry Improvement Plan, Veterinary Services, APHIS, USDA, 1506 Klondike Road, Suite 101, Conyers, GA 30094.

(6) Seal each plate with a plastic sealer or place the unsealed microplates in a tight incubation box and incubate at 37 °C. for 18 to 24 hours.

(7) Read the test results as described in paragraph (f) of this section.

(f) Read the test results with the aid of a reading mirror. Results are interpreted as follows:

(1) N, or − (negative), when the microplate well has a large, distinct button of stained cells; **or**

(2) P, or + (positive), when the microplate well reveals no antigen button; **or**

(3) S, or ? (suspicious), when the microplate well has a small button. Suspicious reactions may tend to be more positive than negative [±] **or** vice versa [±] and can be so noted if desired.

(5) Procedure for determining the status of flocks reacting to tests for Mycoplasma gallisepticum, Mycoplasma synoviae, and Mycoplasma meleagridis.

Procedures for isolation and identification of Mycoplasma may be found in A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens, published by the American Association of Avian Pathologists; *Avian Mycoplasmosis* (*Mycoplasma gallisepticum*), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Fifth Ed., Office International des Epizooties, pp 842–855, 2004; and Program Standards, Subpart B sections (6) and (7).

(a)Determining flock Mycoplasma status

(a) The status of a flock for Mycoplasma shall be determined according to the following criteria:

(1) If the enzyme-labeled immunosorbent assay (ELISA), official molecular examination procedure, or serum plate test is negative, the flock qualifies for the classification for which it was tested.

(2) If the ELISA or serum plate test is positive, the hemagglutination inhibition (HI) test or a molecular examination procedure shall be conducted: *Provided,* for the HI test, that if more than 50 percent of the samples are positive for *M. gallisepticum, M. meleagridis,* or *M. synoviae,* the HI test shall be conducted on 10 percent of the positive samples or 25 positive samples, whichever is greater. HI titers of 1:40 or more may be interpreted as suspicious and appropriate antigen detection samples should be taken promptly (within 7 days of the original sampling) from 30 clinically affected birds and examined by an approved cultural technique individually, or pooled (up to 5 swabs per test) and used in a molecular examination procedure or in vivo bioassay.

(3) If the in vivo bioassay, molecular examination procedure, or culture procedure is negative, the Official State Agency may qualify the flock for the classification for which it was tested. In the event of contaminated cultures, the molecular examination technique must be used to make a final determination.

(4) If the in vivo bioassay, molecular examination procedure, or culture procedure is positive, the flock will be considered infected.

(b) [Reserved]

(6) Standard test procedures for mycoplasma.[5]

The serum plate 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 two 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 and/or plate 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 titer would be suspicious and additional tests should be required.

(a) Serum plate agglutination test

The serum plate agglutination test for mycoplasma is conducted as per NPIP approved manufactures' directions, whenever samples are run, the antigen shall be tested against known positive and negative control serums. Negative and positive sera are available from the National Veterinary Services Laboratories (NVSL), P.O. Box 884, Ames, Iowa 50010. Positive and negative control sera are also available commercially.

(b) 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 paragraphs (b)(2)(ii) or (b)(3)(ii) on initial use or after long storage. To maintain HA activity, the undiluted HA antigen should be stored at -60° to -70°C.

(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_2PO_4) (C.P.)	0.68
Distilled water to make 1,000 ml	

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

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 heparin (1,000 units per mL) or 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 turkeys or chickens known to be free of the mycoplasma being tested, withdraw sufficient blood with a syringe containing heparin (approximately 0.2 mL heparin (1,000 units per mL) per 10 mL of blood) or 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 or supernatant with a pipette.

(iv) Wash the RBCs two times in 10 or more parts of Alsever's solution or buffered saline, 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 or buffered saline. (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, 2 ml of the 25 percent RBCs is added to 98 ml of buffered saline to make a 0.25 percent RBC suspension.

(2) Procedure No. 1.

(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 (RBCs) suspension 0.5 percent (2 ml of 25 percent RBCs 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 RBCs.)

(ii) 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.

Table 1—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

(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÷8=80, or a dilution of 1:80 containing 8 HA units. Or 640÷4=160, a dilution of 1:160 containing 4 HA units per 0.05 ml.

(iii) 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 contains1/2unit; and well 6 contains1/4unit.

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

(*7*) After 20 to 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 RBCs 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 to 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 is suspicious.

(L) Sample test results are illustrated in Table 1 in this paragraph.

(iv) 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.

(3) 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 µL pipettor (Pipetman P200).
(G) Pipette tips.
(H) 0.5 percent homologous red blood cells (RBCs) in PBS (use RBCs from the same species being tested).
(I) Plate-sealing tape.
(J) Mirrored plate reader.

(ii) *Hemagglutination antigen (HA) titration.*

(A) Perform standard hemagglutination test (HA) on mycoplasma antigen to determine titer of antigen.
(1) Dispense 50 µL of PBS into each well of 3 rows of a 96-well microtiter plate.
(2) Dispense 50 µL of stock antigen into the wells of 2 rows.
(3) Perform serial twofold dilutions (50 µL) using a 12-channel pipettor. The dilution series will be from 1:2 to 1:4096.
(4) Add 50 µL of 0.5 percent homologous RBCs 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 RBCs 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 HA units ÷ 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 µL of PBS to the top row of wells (row A) of the plate.
(C) Add 25 µL of PBS to all remaining wells of the plate.
(D) Add 10 µL of each test sera to well A of each column (making a 1:5 sera dilution).
(E) Serially dilute 25 µL from well A through H using a 12-channel pipettor. Discard the final 25 µL. Row A = 1:5...row H = 1:640.
(F) With an Oxford doser, add 25 µL of 4 HA unit antigen to wells B through H. Well A serves as sera control.
(G) Prepare an antigen backtitration by adding 25 µL of PBS to each well of one column. Add 25 µL of diluted antigen to well A and serially dilute 25 µL 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 µL of 0.5 percent RBCs to all wells. Note: Do not agitate after RBCs have been added (agitation may result in false

positive reactions by causing the RBCs 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 RBCs. 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 RBCs (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 RBCs by adding equal volumes of packed RBCs 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 RBCs.

(*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.

(7) 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 9 CFR 145.23(c)(1)(ii)(C) and 9 CFR 145.33(c)(1)(ii)(C), for retaining the classification U.S. M. Synoviae Clean under 9 CFR 145.23(e)(1)(ii)(B) and 9 CFR 145.33(e)(1)(ii)(B), and for retaining the classification U.S. H5/H7 Avian Influenza Monitored under 9 CFR 146.23(a), 9 CFR 146.33(a), and 9 CFR 146.44(a).

(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 Section A(6) (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 Section A(6).

(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 9 CFR 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.

(8) Standard test procedures for avian influenza

(a) agar gel immunodiffusion (AGID) test

(a) The agar gel immunodiffusion (AGID) test should be considered the basic screening test for antibodies to Type A influenza viruses. The 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 viruses, regardless of subtype. 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.[6] 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 that is trapped in the gel matrix and produces a visible line. The precipitin line forms where the concentration of antigen and antibodies is optimum. Differences in the relative concentration of the antigen or antibodies will shift the location of the line towards the well with the lowest concentration or result in the absence of a precipitin line. Electrolyte concentration, pH, temperature, and other variables also affect precipitate formation.

(1) *Materials needed.*

(i) Refrigerator (4 °C).

(ii) Freezer (−20 °C).

(iii) Incubator or airtight container for room temperature (approximately 25 °C) incubations.

(iv) Autoclave.

(v) Hot plate/stirrer and magnetic stir bar (optional).

(vi) Vacuum pump.

(vii) Microscope illuminator or other appropriate light source for viewing results.

(viii) Immunodiffusion template cutter, seven-well pattern (a center well surrounded by six evenly spaced wells). Wells are 5.3 mm in diameter and 2.4 mm apart.

(ix) Top loading balance (capable of measuring 0.1 gm differences).

(x) Pipetting device capable of delivering 50µl portions.

(xi) Common laboratory supplies and glassware—Erlenmeyer flasks, graduated cylinders, pipettes, 100 × 15 mm or 60 × 15 mm petri dishes, flexible vacuum tubing, side-arm flask (500 mL or larger), and a 12- or 14-gauge blunt-ended cannula.

(2) *Reagents needed.*

(i) Phosphate buffered saline (PBS), 0.01M, pH 7.2 (NVSL media #30054 or equivalent).

(ii) Agarose (Type II Medium grade, Sigma Chemical Co. Cat.# A–6877 or equivalent).

(iii) Avian influenza AGID antigen and positive control antiserum approved by the Department and the Official State Agency.

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[6] Beard, C.W. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bull. Wld. Hlth. Org. 42:779–785. 1970.

(iv) Strong positive, weak positive, and negative control antisera approved by the Department and the Official State Agency (negative control antisera optional).

(3) *Preparing the avian influenza AGID agar.*

(i) Weigh 9 gm of agarose and 80 gm of NaCl and add to 1 liter of PBS (0.01 M, pH 7.2) in a 2 liter Erlenmeyer flask.

(ii) To mix the agar, either:

(A) Autoclave the mixture for 10 minutes and mix the contents by swirling after removing from the autoclave to ensure a homogeneous mixture of ingredients; or

(B) 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. After boiling, allow the agar to cool at room temperature (approximately 25°C) for 10 to 15 minutes before dispensing into petri plates.

(iii) Agar can be dispensed into small quantities (daily working volumes) and stored in airtight containers at 4°C for several weeks, and melted and dispensed into plates as needed.

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

(4) *Performing the AGID —*

(i) *Detection of serum antibodies.*

(A) Dispense 15 to 17 mL of melted agar into a 100 × 15 mm petri plate or 5 to 6 mL agar into a 60 × 15 mm petri plate using a 25 mL pipette. The agar thickness should be approximately 2.8 mm.

(B) Allow plates to cool in a relatively dust-free environment with the lids off to permit the escape of water vapor. The lids should be left off for at least 15 minutes, but not longer than 30 minutes, as electrolyte concentration of the agar may change due to evaporation and adversely affect formation of precipitin lines.

Note: Plates should be used within 24 hours after they are poured.

(C) Record the sample identification, reagent lot numbers, test date, and identification of personnel performing and reading the test.

(D) Using the template, cut the agar after it has hardened. Up to seven template patterns can be cut in a 100×15 mm plate and two patterns can be cut in a 60×15 mm plate.

(E) Remove the agar plugs by aspiration with a 12- to 14-gauge cannula connected to a side arm flask with a piece of silicone or rubber tubing that 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.

(F) To prepare the wells, place 50 µl of avian influenza AGID antigen in the center well using a micropipette with an attached pipette tip. Place 50 µl AI AGID positive control antiserum in each of three alternate peripheral wells, and add 50 µl per well of test sera in the three remaining wells. This arrangement provides a positive control line on each

side of the test serum, thus providing for the development of lines of identity on both sides of each test serum (see figure 1).

Note: A pattern can be included with positive, weak positive, and negative reference serum in the test sera wells to aid in the interpretation of results (*see* figure 2).

(G) Cover each plate after filling all wells and allow the plates to incubate for 24 hours at room temperature (approximately 25 °C) in a closed chamber to prevent evaporation. Humidity should be provided by placing a damp paper towel in the incubation chamber.

Note: Temperature changes during migration may lead to artifacts.

(ii) *Interpretation of test results.*

(A) Remove the lid and examine reactions from above by placing the plate(s) over a black background, and illuminate the plate with a light source directed at an angle from below. A microscope illuminator works well and allows for varying intensities of light and positions.

(B) 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. If the line is not distinct, the test is not valid and must be repeated. The following types of reactions are observed (see figure 3):

(*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.

(*2*) *Positive reaction.* The 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 test serum. 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.

(*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 with positive control lines.

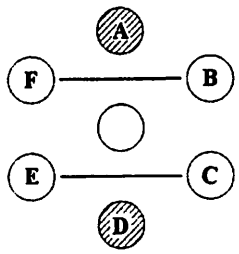


FIGURE 1.—*Immunodiffusion test that uses AI AGID antigen in the center well; AI-positive control serum in wells A and D; and AI-negative test serum in wells B, C, E, and F.*

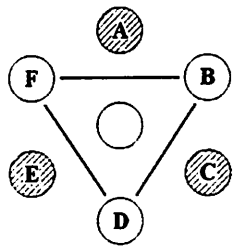


FIGURE 2.—*Immunodiffusion test that has AI AGID antigen in the center well; AI-positive control serum in wells A, C, and E; and AI-negative test serum in wells B, D, and F.*

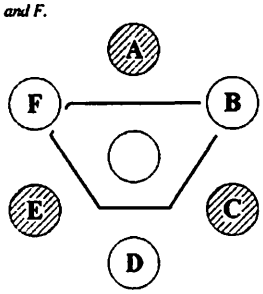


FIGURE 3.—*Immunodiffusion test that has AI AGID antigen in the center well; AI-positive control serum in wells A, C, and E; AI-negative test serum in well B; AI-positive test serum in well D; and weak positive test serum in well F.*

(b) enzyme-linked immunosorbent assay (ELISA)

(b) The enzyme-linked immunosorbent assay (ELISA) may be used as a screening test for avian influenza. Use only federally licensed ELISA kits and follow the manufacturer's instructions. All ELISA-positive serum samples must be confirmed with the AGID test conducted in accordance with paragraph (a) of this section.

Subpart B—Bacteriological Examination Procedure

(1) Laboratory procedure recommended for the bacteriological examination of egg-type and meat-type breeding flocks with salmonella enteritidis positive environments.

Birds selected for bacteriological examination from egg-type and meat-type breeding flocks positive for *Salmonella enteritidis* after environmental monitoring should be examined as described in Section B(2)(a) of these Program Standards, with the following exceptions and modifications allowed due to the high number of birds required for examination:

(a) Except when visibly pathological tissues are present, direct culture, Section B(2)(a)(1) of these standards, may be omitted.

(2) Laboratory procedure recommended for the bacteriological examination of salmonella from birds

(a) For egg- and meat-type chickens, turkeys, waterfowl, exhibition poultry, and game birds

All reactors to the pullorum-typhoid tests, up to 25 birds, and birds from *Salmonella enteritidis* (SE) positive environments should be cultured in accordance with both the direct enrichment (paragraph (a)(1)) and selective enrichment (paragraph (a)(2)) procedures described in this section: *Provided,* if there are more than four reactors to the pullorum-typhoid tests in the flock, a minimum of four reactors as provided for in 9 CFR 145.14(a)(6)(ii) shall be submitted to the authorized laboratory for bacteriological examination. Careful aseptic technique should be used when collecting all tissue samples.

(1) Direct culture (refer to illustration 1). Grossly normal or diseased liver, heart, pericardial sac, spleen, lung, kidney, peritoneum, gallbladder, oviduct, misshapen ova or testes, inflamed or unabsorbed yolk sac, and other visibly pathological tissues where purulent, necrotic, or proliferative lesions are seen (including cysts, abscesses, hypopyon, and inflamed serosal surfaces) should be sampled for direct culture using either flamed wire loops or sterile swabs. Since some strains may not dependably survive and grow in certain selective media, inoculate non-selective plates (such as blood or nutrient agar) and selective plates (such as MacConkey [MAC] and brilliant green novobiocin [BGN] for suspect *S. pullorum* or *S gallinarum* and MAC, BGN, and xylose-lysine-tergitol 4 [XLT 4] for SE). Refer to illustration 1 for recommended bacteriological recovery and identification procedures.[7] Proceed immediately with collection of organs and tissues for selective enrichment culture.

(2) Selective enrichment culture (refer to illustration 1). Collect and culture organ samples separately from intestinal samples, with intestinal tissues collected last to prevent cross-contamination. Samples from the

[7] Biochemical identification charts may be obtained from "A Laboratory Manual for the Isolation and Identification of Avian Pathogens," chapter 2, Salmonellosis. Fourth edition, 1998, American Association of Avian Pathologists, Inc., Kennett Square, PA 19348.

following organs or sites should be collected for culture in selective enrichment broth:

(i) Heart (apex, pericardial sac, and contents if present);
(ii) Liver (portions exhibiting lesions or, in grossly normal organs, the drained gallbladder and adjacent liver tissues);
(iii) Ovary-Testes (entire inactive ovary or testes, but if ovary is active, include any atypical ova);
(iv) Oviduct (if active, include any debris and dehydrated ova);
(v) Kidneys and spleen; and
(vi) Other visibly pathological sites where purulent, necrotic, or proliferative lesions are seen.

(3) From each bird, aseptically collect up to10 to 15 grams of each organ or site listed in paragraph (a)(2) of this section. Mince, grind, or blend and place in a sterile plastic bag. All the organs or sites listed in paragraph (a)(2) of this section from the same bird may be pooled into one bag. Do not pool samples from more than one bird. Add sufficient tetrathionate enrichment broth to give a 1:10 (sample to enrichment) ratio. Incubate the sample at 37°C or 42.0° C for 20 to 24 hours. Follow the procedure outlined in illustration 1 for the isolation and identification of *Salmonella.*

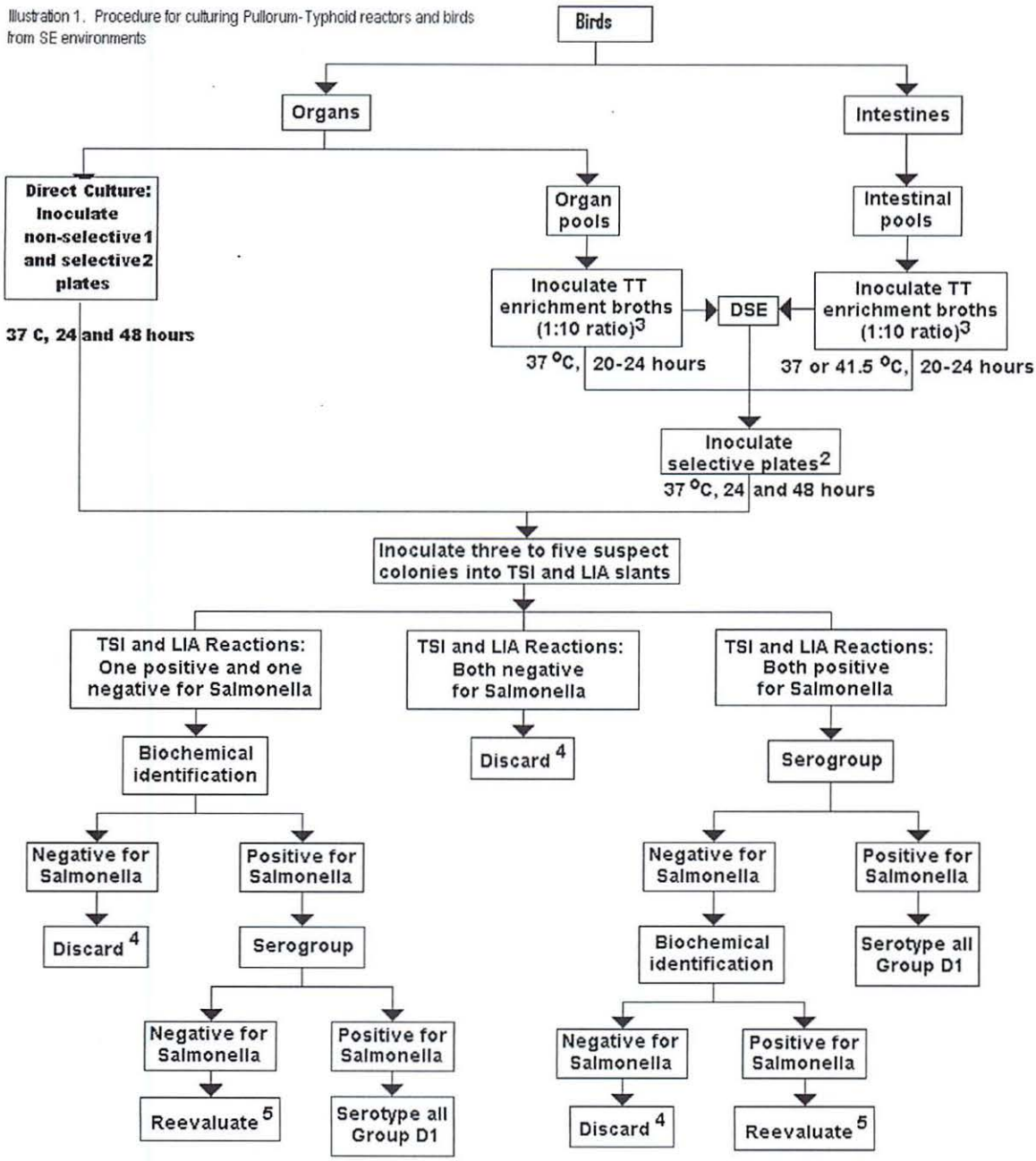
(4) From each bird, aseptically collect 10 to 15 grams of each of the following parts of the digestive tract: Crop wall, duodenum, jejunum (including remnant of yolk sac), both ceca, cecal tonsils, and rectum-cloaca. Mince, grind, or blend tissues and pool them into a sterile plastic bag. Do not pool tissues from different birds into the same sample. Add sufficient tetrathionate enrichment broth to give a 1:10 (sample to enrichment) ratio. Follow the procedure outlined in illustration 1 for the isolation and identification of *Salmonella.*

(5) After selective enrichment, inoculate selective plates (such as MAC and BGN for *S. pullorum* or *S gallinarum* and MAC, BGN, and XLT 4) for SE. Incubate the plate at 37° C for 20 to 24 hours. Inoculate three to five *Salmonella*-suspect colonies from plates into triple sugar iron (TSI) and lysine iron agar (LIA) slants. If there are no suspect colonies after 24 hours of incubation, incubate the plates an additional 24 hours before considering negative. Screen colonies by serological (*i.e.*, serogroup) and biochemical procedures (e.g., the Analytical Profile Index for Enterobacteriaceae [API]) as shown in illustration 1.

(6) If the initial selective enrichment is negative for *Salmonella,* a delayed secondary enrichment (DSE) procedure is used. Leave the tetrathionate-enriched sample at room temperature for 5 to 7 days. Transfer 1 mL of the culture into a tube containing 10 mL of fresh tetrathionate enrichment broth, incubate at 37 C for 20 to 24 hours, and plate as in Section B(2)(5).

(7) Serogroup all isolates identified as salmonellae and serotype all serogroup D1 isolates. Phage-type all SE isolates.

Illustration 1. Procedure for culturing Pullorum-Typhoid reactors and birds from SE environments



1. Non-selective plates such as blood or nutrient agar.
2. Selective plates such as MacConkey, Brilliant Green Novobiocin (BGN) for pullorum-typhoid reactors and MacConkey, BGN, and xylose-lysine tergitol 4 (XLT 4) for SE.
3. Tetrathionate enrichment broth.
4. Reevaluate if epidemiologic, necropsy, or other information indicates the presence of an unusual strain of Salmonella.
5. If biochemical identification and serogroup procedures are inconclusive, restreak original colony onto non-selective plating media to check for purity. Repeat biochemical and serology tests.

Illustration 1

(3) Procedures for collection, isolation, and identification of Salmonella from house environmental samples, cloacal swabs, and hatchery samples.

Information concerning the pen arrangement and number of birds per pen should be obtained from the owner so that the required number of samples per pen and per flock can be determined. A means of identifying each sample by pen of origin should be provided. The vehicle transporting the personnel taking the samples should be left as far as practical from the poultry pens. Biosecurity precautions, including using disinfected sampling equipment, sterile sampling supplies and personal cleanliness, should be followed. The hands should be carefully washed with a sanitizing soap before the sampling. Outer clothing, including gloves, should be changed between visits to different premises so that clean clothing is worn on entering each premise. A minimum size of 3 inches by 3 inches should be used for the sterile gauze pads.

The used and clean apparel and sampling materials should be kept separate. Boots or footwear should be cleaned and disinfected between visits to different premises. Disposable caps or hair nets should be provided and discarded after use on each premises. After collection, the samples should be protected from drying, light, and excessive temperatures and delivered to the laboratory within one day. If delivery is delayed, samples should be refrigerated.

(a) For egg- and meat-type chickens, turkeys, waterfowl, exhibition poultry, and game birds

All samples and swabs described in this paragraph should be cultured in accordance with illustration 2. All salmonellae recovered shall be serogrouped or serotyped.

(1) *Poultry house environmental samples.*

(i) Fecal material, litter or dust. With a clean gloved hand or sterile collection device, collect fecal material, litter, or dust from several locations representing all areas of the pen or house into a sterile bag or container. A suggested number of samples would be 5 samples from pens or houses with less than 500 birds; 10 samples from pens or houses with 500 to 2,500 birds; and 15 samples from pens or houses with more than 2,500 birds.

(ii) Drag swabs (DS). DS, which consist of gauze pads or commercially available sponges, enable the sampling of large areas of the pen or house.

(A) Preparation. DS may be purchased commercially or be user prepared. One suggested method of making the DS assemblies is as follows: A sterile gauze pad is folded in half and a 2-foot long (60cm) piece of twine is securely attached to the folded pad using a paper clip, staple, or similar device. A second sterile gauze pad is similarly fastened to a 5-foot (150 cm) long piece of twine. The shorter piece of twine is then tied to the longer piece producing a DS sample set of two swabs arranged in a Y-shaped configuration. Alternatively, two separate DS samplers may be prepared. The twine is wrapped around the swabs, and the swabs moistened with double-strength skim milk (DSSM). The moistened swabs are placed in an instrument package. The sterilized swabs contained in the instrument pack may be frozen (to prevent drying) until use.

(B) Procedure. At the farm the thawed DS assemblies are unraveled and the ends of the twine held in gloved hands. The swabs are dragged across the environmental surfaces of the house for 15 minutes or the length of the house (down and back). One set of swabs (two individual pads) is dragged across the center of the house floor and another set of swabs (two individual pads) is dragged across the inside perimeter of the house floor. The four pads are individually placed in labeled, sterile bags. If necessary to prevent drying out, additional DSSM (evaporated skim milk) may be added to the bags. The bags should be protected from excessive heat and submitted as soon as possible to the authorized laboratory for testing. If the samples cannot be submitted to the laboratory the same day, they should be stored 2°- 4 °C or placed in a cooler with ice or ice packs for no more than 5 days before culturing.

(iii) Shoe cover swabs.

Absorbable fabric shoe covers involve the exposure of the bottom surface of shoe covers to the surface of floor litter and slat areas. Wearing clean gloves, place the shoe covers over footwear that is only worn inside the poultry house. This can be footwear dedicated to the facility or disposable overshoes. Each pair of shoe covers should be worn while walking at a normal pace over a distance of 1,000 feet (305 meters). For flocks with fewer than 500 breeders, at least 1 pair of shoe covers should be worn to sample the floor of the bird area. For flocks with 500 or more breeders, at least 2 pairs of shoe covers should be worn to sample the floor of the bird area. After sampling, place each shoe cover in a sterile container with 30 ml of double strength skim milk, unless pre-moistened swabs are used. Seal the sterile containers and promptly refrigerate them at 2° to 4 °C or place in a cooler with ice or ice packs. Do not freeze. Samples should be stored at refrigerator temperatures of 2° to 4 °C no more than 5 days before culturing.

(iv) Nest box or egg belt swabs as alternative sampling source

(A) Two sterile pre-moistened (ex. DSSM) gauze pads or sponges are swabbed inside approximately 10 percent of the nest boxes. Each swab or sponge is placed into a separate sterile bag and submitted to the authorized laboratory.

(B) Two sterile pre-moistened (ex. DSSM) gauze pads or sponges are used to swab the egg belts. At least 30 feet of belt material is swabbed with each swab. Each swab is placed into a separate sterile bag and submitted to the authorized laboratory.

(2) *Cloacal swabs.* Cloacal swabs for bacteriological examination shall be taken from each bird in the flock or from a minimum of 500 birds in accordance with the procedure described this section. A sterile cotton-tipped applicator or swab is inserted into the cloaca and rectum of the bird in such a manner to ensure the collection of fecal material. The applicator may be broken off in to a sterile tube. The cloacal swabs may be combined in multiples of five or in combinations specified by the authorized laboratory.

(3) *Hatchery samples*. Hatchery-related samples, such as chick box papers, meconium, and fluff, may be examined for the presence of Salmonella to indicate the transfer of Salmonella from parent to offspring.

(i) Chick box papers. Chick box paper samples may be collected by an authorized agent according to paragraph (a)(3)(i)(A) of this section or

may be submitted directly to an authorized laboratory for testing according to paragraph (a)(3)(ii)(B) of this section. It is important to remove the paper from the chick box before the box is placed in the brooding house.

(A) Instructions for sampling chick box papers. One chick box paper is collected for every 10 boxes of chicks placed in a house. With sanitized and gloved hands lay out the papers on a clean, disinfected surface. Saturate a sterile gauze pad or sponge with DSSM and swab the surface of 5 chick box papers. The pad should be rubbed over approximately 75 percent of each paper with sufficient pressure to remove any dried meconium. Addition of more DSSM may facilitate sampling. The process is repeated with a second swab and the other five chick box papers. Both swabs may be added to a single sterile, labeled plastic bag and submitted to the authorized laboratory. Promptly refrigerate the Whirl-Pak bags containing the samples and transport them, on ice or otherwise refrigerated, to a laboratory to be cultured within 5 days of collection.

(ii) The Plan participant may send chick box papers directly to a laboratory, where samples may be collected as described in paragraph (a)(3)(i)(A) of this section. To send chick box papers directly to a laboratory:

(A) Collect 1 chick box paper for each 10 boxes of chicks placed in a house and place the chick papers immediately into large plastic bags and label and seal the bags.

(B) Place the plastic bags containing the chick box papers in a clean box and transport them within 48 hours to a laboratory. The plastic bags do not require refrigeration.

(iii) Chick meconium. After collection, the container of meconium is mixed to obtain a uniform consistency. In the laboratory a 25-gram sample will be removed for bacteriological examination.

(iv) Fluff. Fluff samples may be collected from the floor of the hatchery or from the tray following hatching. The fluff sample may be collected by either swabbing the floor or tray with a pre-moistened gauze pad or sponge or by placing fluff material directly into a sterile bag.

(b) Isolation and identification of Salmonella

There are two enrichment procedures approved for the isolation of *Salmonella* as described in this section (See Illustration 2). Alternatively, approved rapid methods may be used to detect the presence of *Salmonella*, positive samples which must then be isolated.

(1) Direct tetrathionate enrichment followed by MSRV enrichment (Illustration 2).

(i) Fresh Tetrathionate enrichment broth is added to the sample to give a 1:10 (sample to enrichment) ratio. Incubate the samples at 37° C or 42° C for 20 to 24 hours.

(ii) After incubation transfer approximately 100 microliters (3 drops) of the enriched culture into (subsurface) an MSRV plate. Incubate the plate right side up at 42° C for 24 hours.

(iii) Observe the MSRV plate for growth migrating from the point of inoculation. If present, insert a sterile loop into the outer edge of the zone of growth and inoculate selective agar plates, such as BGN and XLT4.

(iv) If no zone of growth is present, incubate the MSRV plate at 42° C for another 24 hours. Observe the MSRV plate for growth migrating from the point of inoculation. If growth is present, insert a sterile loop into the outer edge of the zone of growth and inoculate selective agar plates, such as BGN and XLT4. If still no zone, insert the loop into the point of inoculation and inoculate selective agar plates. This ensures that weakly or non-motile strains of Salmonella will not be missed.

(v) Incubate the selective agar plates at 37° C for 20 to 24 hours. Observe the plates for Salmonella suspect colonies. Screen three to five colonies by inoculating them individually into triple sugar iron agar (TSI) and lysine iron agar (LIA) slants or equivalent method. Incubate the slants at 37° C for 20 to 24 hours. Screen the colonies by serological (i.e., serogroup) or biochemical (e.g. API) procedures as shown in Illustration 2.

(vi) Serogroup all isolates identified as *Salmonella* and serotype all serogroup D isolates. Phage type one SE isolate per flock per submission.

(2) Pre-enrichment followed by selective enrichment. (Illustration 2.)

(i) Pre-enrichment broth (e.g. buffered peptone water, BPW) is added to the sample to give a 1:10 (sample to enrichment) ratio. Incubate the sample at 37° C for 20 to 24 hours.

(ii) Transfer 1 ml of the pre-enriched sample into a tube containing 10 ml of tetrathionate enrichment broth and transfer 0.1 ml into either a tube containing 10 ml of Rappaport-Vassiliadis enrichment broth or into a MSRV plate. Incubate at 42° C for 20 to 24 hours.

(iii) After incubation inoculate the tetrathionate and RV enrichments onto separate selective agar plates, such as BGN and XLT4. If the MSRV media was inoculated then follow the steps in (1)(iii) and (1) (iv).

(iv) Screen the selective agar plates for *Salmonella* as described in (1)(v) and (1)(vi).

(3) Approved rapid methods for the detection of Salmonella.

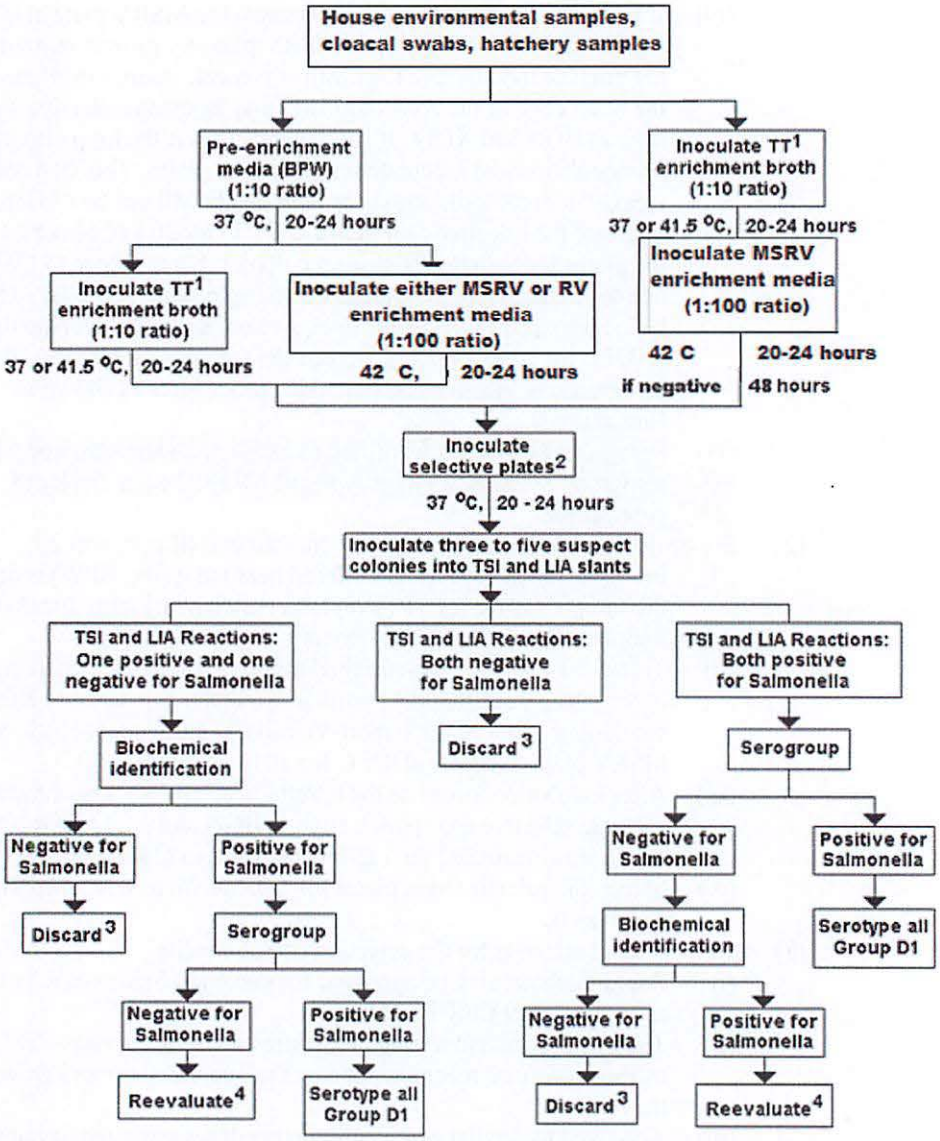
(i) Rapid methods may be approved for detecting Salmonella by the NPIP as set forth in 9 CFR 147.54.

(ii) The enrichment and testing procedures used for the respective rapid method are those recommended by the manufacturer and approved by the NPIP.

(iii) Approved molecular examination procedures are listed in Subpart D.

(iv) Samples that are positive with the rapid method must be confirmed as *Salmonella* by inoculation of selective plating media, such as BGN and XLT4, from the enrichment broths used in the detection process.

(v) Follow the isolation procedures in (b)(1)(v) of this section.



1. Tetrathionate enrichment broth, e.g., Rappaport-Vassiliades (RV) or modifed semisolid RV (MSRV).
2. Selective plates such as Brilliant Green Novobiocin (BGN) or xylose-lysine tergitol 4 (XLT 4).
3. Reevaluate if epidemiologic, necropsy, or other information indicates the presence of an unusual strain of Salmonella.
4. If biochemical identification and serogroup procedures are inconclusive, restreak original colony onto non-selective plating media to check for purity. Repeat biochemical and serology tests.

Illustration 2. Culture procedures for house environmental samples, cloacal swabs, and hatchery samples

(4) Procedure for bacteriological culturing of eggshells for colon bacilli organisms

Proper precautions to avoid environmental contamination of the samples during the collection and laboratory process, and proper handling of the samples following collection are essential. Each State Inspector involved in eggshell culture activities must receive instruction in the necessary sanitation procedures, sampling procedures, and sample handling by the authorized laboratory involved. The Official State Agency will maintain a record showing that the required instruction was given to each State Inspector.

(a) Sample selection

Forty eggs in the top flats of each of three randomly selected cases of sanitized eggs from each flock will be used for each sampling.

(b) Swab procedure

A 2.5 centimeter diameter circular area of the large end of each of the eggs will be rubbed with a sterile swab previously moistened with sterile lactose broth or other suitable liquid media provided by the authorized laboratory. One swab will be used for five eggs, and four swabs will be pooled to each sterile, capped tube provided by the authorized laboratory.

(1) From the tube containing four swabs and lactose broth or other suitable media, 1 ml. will be transferred to 10 ml. lactose in a fermentation tube.

(2) Incubate at 37 °C for 48 hours. The presence of acid, and gas in the amount of 10 percent or more after 24 and 48 hours of incubation, provides a presumptive conclusion of the presence of colon bacilli organisms.

(5) Procedures to determine status and effectiveness of sanitation monitored program

The following monitoring procedures[8] may be applied at the discretion of the Official State Agency:

(a) Monitor effectiveness of sanitation program

(1) Culture the surface of cased eggs periodically for fecal contaminating organisms as described in Section B(4).

(2) Culture a sample of dead-in-shell eggs periodically from each breeding flock for coliforms. Such eggs should also be cultured for the dependable recovery of *salmonellae.* Culturing for the dependable recovery of *salmonellae* should include the use of:

   (i) Preenrichment broths supplemented with 35 mg ferrous sulfate per 1,000 ml preenrichment to block iron-binding, *Salmonella* -inhibiting effects of egg conalbumin; and

   (ii) Tetrathionate selective enrichment broths, competitor-controlling plating media (XLT4, BGN, etc.), delayed secondary enrichment procedures, and colony lift assays detailed in paragraph (a)(5) and illustration 2 of these Program Standards.

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[8] Laboratory procedures for monitoring operations proposed here are described in the following two publications: Isolation and Identification of Avian Pathogens, American Association of Avian Pathologists, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348–1692, 1980, and Culture Methods for the Detection of Animal Salmonellosis and Arizonosis, Iowa State University Press, Ames, Iowa 50010, 1976.

(6) Laboratory procedure recommended for the bacteriological examination of mycoplasma reactors[9]

(a) Turbinates, trachea, air sacs, sinuses, nasal passages, respiratory exudates, synovial fluid, eggs (including yolk, yolk sacs, membranes and allantoic fluid), should sterile swabs.

Aseptic techniques are very important as some organisms may not be suppressed by the antimicrobial agents used in this procedure. Tissue suspensions from large volumes are sometimes desirable from the sites listed above and occasionally from the oviduct and cloaca. Tissues should be ground or blended completely in 10 times their volume of Mycoplasma Broth Medium (MBM). (See paragraph (f) of this section.) Specimens submitted to referral laboratories in order of preference for recovery of the mycoplasma organisms are: live birds, refrigerated fresh tissues, and tissue specimens packed with dry ice.

(b) Inoculate 5–10 ml of MBM with a swab, wire loop or 0.1 ml of the tissue suspension.

When evidence of growth is observed (lowered pH or turbidity of broth) transfer each broth culture as needed to maintain the original isolates. Incubate tubes at 37 °C for at least 21 days before discarding as negative. When growth is first observed or if no growth occurs by the *4th or 5th* day of incubation, inoculate broth culture onto a plate of Mycoplasma Agar Medium (MAM). (See paragraph (g) of this section.) Several cultures may be inoculated on one plate by using a wire loop or a cotton swab. Incubate plates 3–5 days at 37 °C in a high humidity chamber. If preferred, 5 percent CO_2 may be added or a candle jar may be used. Tiny circular and translucent colonies with elevated centers are very suggestive of mycoplasma. Indirect lighting and a low power or dissecting microscope are recommended for observation of the colonies as they are rarely more than 0.2–0.3 mm in diameter.

(c) Isolates must be typed.

(1) Isolates may be shipped in MBM with ice packs if shipment will be in transit less than 2 to 3 days. Longer shipments require freezing of the MBM with dry ice, or shipping MAM slants at room temperature. Isolates must have indications of growth before shipment is made.

(2) Isolates may be stored in MBM at -20 °C for 2 to 3 weeks, or they may be stored at -68 °C for several years.

(d) Alternate method of culture: An overlay enrichment culture for fastidious and sensitive mycoplasma, especially for M. meleagridis should be included.

(1) Pour 2–3 ml of MAM into a test tube and tilt the tube until a slant (approximately 45 degrees) is obtained. Other containers are acceptable.

(2) Overlay the slant with sufficient MBM, so that the media (including inoculum) covers the agar slope.

(3) Inoculate the culture as indicated in paragraph (b) of this section.

(4) Incubate and examine the overlay as indicated in paragraph (b) of this section.

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[9] Yoder, H. W., Jr., "Mycoplasmosis." In: Isolation and Identification of Avian Pathogens. (Stephen B. Hitchner, Chairman, Charles H. Domermuth, H. Graham Purchase, James E. Williams.) 1980, pp. 40–42, Creative Printing Company, Inc., Endwell, NY 13760.

(e) Preparation of media components:[10]

(1) Deionized distilled water suitable for cell culture fluids should be used.

(2) All glassware should be carefully washed with a nonresidue detergent such as Alcojet and rinsed three times in tap water and twice in deionized distilled water.[11]

(3) Thallium acetate in a 10 percent solution is added to an approximate final concentration of 1:4000; however, highly contaminated specimens may require a final concentration of 1:2000.[12] Thallium acetate is added to deionized distilled water first, except as noted in paragraph (e)(4) of this section, to prevent the precipitation of proteins.

(4) Mycoplasma Broth Base, dextrose, phenol red, and cysteine hydrochloride are added to deionized distilled water first if autoclave sterilization is used.[13] Thallium acetate and then the remaining components are added aseptically after cooling the autoclaved media to 45 °C or less.

(5) Use sterile deionized distilled water to reconstitute penicillin.

(6) Sterile serum should be inactivated by heating at 56 °C for 30 minutes. Swine serum may be used for *M. gallisepticum, M. synoviae, M. gallinarum,* and *M. meleagridis* isolation; however, horse serum is usually recommended for *M. meleagridis* isolation.

(7) Phenol red should be prepared as a 1 percent solution.

(8) NAD (beta nicotinamide adenine dinucleotide or coenzyme I) should be prepared as a 1 percent solution.[14]

(9) Cysteine hydrochloride, prepared as a 1 percent solution, is used to reduce the NAD for *M. synoviae* growth.

(10) A purified agar product such as Noble (Special agar) is used in the MAM.[15]

(11) Adjust the pH with 0.1 M NaOH.

(12) Sterilization may be accomplished by two methods:

- (i) Filtration sterilization through a 0.20 micron filter is the recommended method. Aseptic techniques must be used.
- (ii) Autoclave sterilization at 120 °C, 15 pounds pressure (103 kPa), for 15 minutes may be used, if preferred, when following the procedure described in paragraph (e)(4) of this section.

(13) Phenol red, dextrose, and NAD may be omitted when culturing for *M. meleagridis* and *M. gallinarum.*

(14) When culturing for *M. meleagridis* from contaminated samples include 100 units/ml of Polymyxin B in MBM.

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[10] Trade names are used in these procedures solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.

[11] Alcojet is available from: Alconox Inc., White Plains, NY 10603.

[12] Thallium acetate may be obtained from Fischer Scientific Company.

[13] Mycoplasma Broth Base may be obtained from: (a) Product #CM0403, from Therom Scientific.

[14] NAD Grade III may be obtained from: Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178.

[15] Noble Agar may be obtained from: BD (Becton, Dickinson, and Company), Cat #214220.

(f) Mycoplasma Broth Medium (Frey) is prepared as follows:

To 850 to 880 ml of deionized distilled water;
Add:
Thallium acetate (ml)—2.5 (1:4000)
Potentially contaminated samples (ml)—5.0 (1:2000)
Mycoplasma Broth Base (g)—22.5
Aqueous penicillin (units)—500,000
Sterile serum (ml)—120 to 150.0
Phenol red plus (ml)—2.5
NAD (ml)—12.5
Cysteine hydrochloride (ml)—12.5
Dextrose (g)—1.0–1.5
Adjust pH to 7.8
Filter sterilize

(1) Broth may be stored at 4 °C for at least 2 weeks or at -40 °C for longer periods.

(g) Mycoplasma Agar Medium (Frey) is prepared as follows:

To 850 to 880 ml of deionized distilled water;
Add:
Mycoplasma Broth Base (g)—22.5
Adjust pH to 7.8
Purified agar (g)—12.0
Autoclave and cool in 45 °C water bath
Thallium acetate (ml)—2.0; (1:4000)
Sterile serum at 45 °C (ml)—150.0
Aqueous penicillin (units)—400,000
NAD (ml)—12.5
Cysteine hydrochloride (ml)—12.5

(1) Rotate flask gently and pour about 15 ml of media into each petri dish.
(2) Stack petri dishes only 2 to 3 high in a 37 °C incubator up to 2 hours to remove excess moisture.
(3) Wrap inverted plates in sealed bundles and store at 4 °C for not more than 15 days.

(h) New component or media batches should be monitored to compensate for changes in formulation due to alterations of purity concentration, preparation, etc.

A known series of titrations from a single culture should be made on both new and old media. The media should be compared on the basis of growth, colony size, and numbers of colonies which develop.[16]

[16] "Laboratory Procedures and Medium For The Isolation Of Mycoplasma From Clinical Materials." *Laboratory Diagnosis of Mycoplasma in Food Animals,* Proceedings of Nineteenth Annual Meeting, The American Association of Veterinary Laboratory Diagnosticians, 1976, pp. 106–115, AAVLD, 6101 Mineral Point Road, Madison, Wisconsin 53705.

(7) Procedure for the evaluation of mycoplasma reactors by in vivo bio-assay (enrichment)

This procedure has been shown to be sensitive enough to detect less than 100 mycoplasma organisms under proper conditions.[17] Proper conditions are defined in this section.

(a) Obtain chickens or turkeys (test birds) which are at least 3 weeks of age and are free of *M. gallisepticum, M. synoviae,* and *M. meleagridis* and transport them in a manner to prevent their being contaminated by any infectious avian disease.
   (1) Maintain test birds in an area that has been effectively cleaned and disinfected.
   (2) The area should be isolated from other birds or animals.
   (3) Personnel caring for the test birds should take the necessary precautions (Section C(6)(b)) to prevent the mechanical transfer of infectious avian diseases from other sources.

(b) Test birds to be used for inoculation with contaminated tissues should be serologically negative by the serum plate agglutination test.
   (1) Inoculated test birds should be isolated from non-inoculated control birds for the length of any experiment.

(c) Aseptically obtain tracheal, turbinate, and sinus mucosa, lung and sinus exudates, cervical, thoracic, and abdominal airsac tissues (including lesions), and portions of oviduct and synovial fluid from at least four suspect donor birds. In a sterile device, blend the tissues completely in four times their volume of Mycoplasma Broth Medium (Frey), (see Section B(6)(f)). Suspensions may be made from tissue pools. Inoculate test birds within 30 minutes for preparation of suspensions.
   (1) Inoculate at least four test birds for each suspension pool via the abdominal air sac and infraorbital sinus, with up to1/2 ml of inoculum per site.
   (2) Test birds should be bled every 7 days for 35 days to identify sero-converters.
   (3) At 35 days, test birds should be sacrificed and bacteriologic isolation and identification of mycoplasma attempted (see Section B(6)). Note especially the sites of inoculation for typical gross or microscopic mycoplasma lesions.

(d) Donor birds are considered infected when:
   (1) Test birds have serum plate antibodies for the mycoplasma for which the donor birds were tested, regardless of HI test results, *and* control birds stay serologically negative;
   or
   (2) Mycoplasma organisms are isolated from the test birds and serotyped positive for the mycoplasma for which the donor birds were tested, *and* control birds stay serologically and culturally negative.

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[17] Research results are described in the following two publications: (a) Bigland, C. H. and A. J. DaMassa, "A Bio-Assay for Mycoplasma Gallisepticum." In: United States Livestock Sanitary Association Proceedings, 67th, 1963, pp. 541–549. (b) McMartin, D. A., "Mycoplasma Gallisepticum in the Respiratory Tract of the Fowl." In: The Veterinary Record, September 23, 1967, pp. 317–320.

(e) Laboratory findings may be verified by direct cultures of material from sick birds or by inoculating seronegative birds from the suspect flock and comparing serological findings with those from the test birds.

(8) Laboratory procedure recommended for the bacteriological examination of cull chicks and poults for salmonella.

The laboratory procedure described in this section is recommended for the bacteriological examination of cull chicks from egg-type and meat-type chicken flocks and waterfowl, exhibition poultry, and game bird flocks and poults from turkey flocks for salmonella.

(a) For cull chicks, from 25 randomly selected 1- to 5-day-old chicks that have not been placed in a brooding house, prepare 5 organ pools, 5 yolk pools, and 5 intestinal tissue pools as follows. For poults, from a sample of 10 poults that died within 10 days after hatching, prepare organ pools, yolk pools, and intestinal pools as follows:

(1) *Organ pool:* From each of five chicks or two poults, composite and mince 1- to 2-gram samples of heart, lung, liver, and spleen tissues. Include the proximal wall of the bursa of Fabricius for chicks only.

(2) *Yolk pool:* From each of five chicks or two poults, composite and mince 1- to 2-gram samples of the unabsorbed yolk sac or, if the yolk sac is essentially absent, the entire yolk stalk remnant.

(3) *Intestinal pool:* From each of five chicks or two poults, composite and mince approximately 0.5 cm^2 sections of the crop wall and 5-mm-long sections of the duodenum, cecum, and ileocecal junction.

(b) Transfer each pool to tetrathionate selective enrichment broth (Hajna or Mueller-Kauffmann) at a ratio of 1 part tissue pool to 10 parts broth.

(c) For cull chicks, repeat the steps in paragraphs (a) and (b) of this section for each 5-chick group until all 25 chicks have been examined, producing a total of 15 pools (5 organ, 5 yolk, and 5 intestinal). For poults, repeat the steps in paragraphs (a) and (b) of this section for each two-poult group until all the poults in the sample have been examined.

(d) Culture the tetrathionate pools as outlined for selective enrichment in illustration 2 of these Standards. Incubate the organ and yolk pools for 24 hours at 37 °C and the intestinal pools at 41.5 °C. Plate as described in illustration 2 of these Standards and examine after both 24 and 48 hours of incubation. Confirm suspect colonies as described. Further culture all salmonella-negative tetrathionate broths by delayed secondary enrichment procedures described for environmental, organ, and intestinal samples in illustration 2. A colony lift assay may also be used as a supplement to TSI and LI agar picks of suspect colonies.

Subpart C—Sanitation Procedures

(1) Flock sanitation.

To aid in the maintenance of healthy flocks, the following procedures should be practiced:

(a) Baby poultry should be started in a clean brooder house and maintained in constant isolation from older birds and other animals. Personnel that are in contact with older birds and other animals should take precautions, including disinfection of footwear and change of outer clothing, to prevent the introduction of infection through droppings that may adhere to the shoes, clothing, or hands. (See Section C(4)(a).)

(b) Range used for growing young stock should not have been used for poultry the preceding year. Where broods of different ages must be kept on the same farm, there should be complete depopulation of brooder houses and other premises following infection of such premises by any contagious disease.

(c) Poultry houses should be screened and proofed against free-flying birds. An active rodent eradication campaign is an essential part of the general sanitation program. The area adjacent to the poultry house should be kept free from accumulated manure, rubbish, and unnecessary equipment. Dogs, cats, sheep, cattle, horses, and swine should never have access to poultry operations. Visitors should not be admitted to poultry areas, and authorized personnel should take the necessary precautions to prevent the introduction of disease.

(d) Poultry houses and equipment should be thoroughly cleaned and disinfected prior to use for a new lot of birds. (See Section C(4)(a).) Feed and water containers should be situated where they cannot be contaminated by droppings and should be frequently cleaned and disinfected. Dropping boards or pits should be constructed so birds do not have access to the droppings.

(e) Replacement breeders shall be housed at the proper density consistent with the type of building and locality and which will allow the litter to be maintained in a dry condition. Frequent stirring of the litter may be necessary to reduce excess moisture and prevent surface accumulation of droppings. Slat or wire floors should be constructed so as to permit free passage of droppings and to prevent the birds from coming in contact with the droppings. Nesting areas should be kept clean and, where appropriate, filled with clean nesting material.

(f) When an outbreak of disease occurs in a flock, dead or sick birds should be taken, by private carrier, to a diagnostic laboratory for complete examination. All Salmonella cultures isolated should be typed serologically, and complete records maintained by the laboratory as to types recovered from each flock within an area. Records on isolations and serological types should be made available to Official State Agencies or other animal disease control regulatory agencies in the respective States for followup of foci of infection. Such information is necessary for the development of an effective Salmonella control program.

(g) Introduction of started or mature birds should be avoided to reduce the possible hazard of introducing infectious diseases. If birds are to be introduced, the health status of both the flock and introduced birds should be evaluated.

(h) In rearing broiler or replacement stock, a sound and adequate immunization program should be adopted. Since different geographic areas may require certain specific recommendations, the program recommended by the State experiment station or other State agencies should be followed.

(i) Feed, pelleted by heat process, should be fed to all age groups. Proper feed pelleting procedures can destroy many disease-producing organisms contaminating feedstuffs.

(2) Hatching egg sanitation.

Hatching eggs should be collected from the nests at frequent intervals and, to aid in the prevention of contamination with disease-causing organisms, the following practices should be observed:

(a) Cleaned and disinfected containers, such as egg flats, should be used in collecting the nest eggs for hatching. Egg handlers should thoroughly wash their hands with soap and water before and after egg collection. Clean outer garments should be worn.

(b) Dirty eggs should not be used for hatching purposes and should be collected in a separate container from the nest eggs. Slightly soiled nest eggs may be gently dry cleaned by hand.

(c) Hatching eggs should be stored in a designated egg room under conditions that will minimize egg sweating. The egg room walls, ceiling, floor, door, heater, and humidifier should be cleaned and disinfected after every egg pickup. Cleaning and disinfection procedures should be as outlined in Section C(4) of these Standards.

(d) The egg processing area should be cleaned and disinfected daily.

(e) Effective rodent and insect control programs should be implemented.

(f) The egg processing building or area should be designed, located, and constructed of such materials as to ensure that proper egg sanitation procedures can be carried out, and that the building itself can be easily, effectively, and routinely sanitized.

(g) All vehicles used for transporting eggs or chicks or poults should be cleaned and disinfected after use. Cleaning and disinfection procedures should be as outlined in Section C(4).

(3) Hatchery sanitation.

An effective program for the prevention and control of *Salmonella* and other infections should include the following measures:

(a) An effective hatchery sanitation program should be designed and implemented.

(b) The hatchery building should be arranged so that separate rooms are provided for each of the four operations: Egg receiving, incubation and hatching, chick/poult processing, and egg tray and hatching basket washing. Traffic and airflow patterns in the hatchery should be from clean areas to dirty areas (*i.e.*, from egg room to chick/poult processing rooms) and should avoid tracking from dirty areas back into clean areas.

(c) The hatchery rooms, and tables, racks, and other equipment in them should be thoroughly cleaned and disinfected frequently. All hatchery wastes and offal

should be burned or otherwise properly disposed of, and the containers used to remove such materials should be cleaned and sanitized after each use.

(d) The hatching compartments of incubators, including the hatching trays, should be thoroughly cleaned and disinfected after each hatch.

(e) Only clean eggs should be used for hatching purposes.

(f) Only new or cleaned and disinfected egg cases should be used for transportation of hatching eggs. Soiled egg case fillers should be destroyed.

(g) Day-old chicks, poults, or other newly hatched poultry should be distributed in clean, new boxes and new chick papers. All crates and vehicles used for transporting birds should be cleaned and disinfected after each use.

(4) Cleaning and disinfecting

The following procedures are recommended:

(a)In the poultry houses:

(1) Remove all live "escaped" and dead birds from the building. Blow dust from equipment and other exposed surfaces. Empty the residual feed from the feed system and feed pans and remove it from the building. Disassemble feeding equipment and dump and scrape as needed to remove any and all feed cake and residue. Clean up spilled feed around the tank and clean out the tank. Rinse down and wash out the inside of the feed tank to decontaminate the surfaces and allow to dry.

(2) Remove all litter and droppings to an isolated area where there is no opportunity for dissemination of any infectious disease organisms that may be present. Housing where poultry infected with a mycoplasmal disease were kept should remain closed for 7 days before removal of the litter.

(3) Wash down the entire inside surfaces of the building and all the installed equipment such as curtains, ventilation ducts and openings, fans, fan housings and shutters, feeding equipment, watering equipment, etc. Use high pressure and high volume water spray (for example 200 pounds per square inch and 10 gallons per minute or more) to soak into and remove the dirt to decontaminate the building. Scrub the walls, floors, and equipment with a hot soapy water solution. Rinse to remove soap.

(4) Spray with a disinfectant which is registered by the Environmental Protection Agency as germicidal, fungicidal, pseudomonocidal, and tuberculocidal, in accordance with the specifications for use, as shown on the label of such disinfectant.

(b) In the hatchers and hatchery rooms:

(1) Use cleaning agents and sanitizers that are registered by the U.S. Environmental Protection Agency as germicidal, fungicidal, pseudomonocidal, and tuberculocidal. Use manufacturer's recommended dilution. Remove loose organic debris by sweeping, scraping, vacuuming, brushing, or scrubbing, or by hosing surface with high pressure water (for example 200 pounds per square inch and 10 gallons per minute or more). Remove trays and all controls and fans for separate cleaning. Use hot water (minimum water temperature of 140°F) for cleaning hatching trays and chick separator equipment. Thoroughly wet the ceiling, walls, and floors with a stream of water, then scrub with a hard bristle brush. Use a cleaner/sanitizer that can penetrate protein and fatty deposits. Allow the chemical to cling to treated surfaces at least 10 minutes before rinsing off. Manually scrub any remaining deposits of organic material until they are removed. Rinse until there is no longer any deposit on the walls, particularly near the fan opening,

and apply disinfectant. Use a clean and sanitized squeegee to remove excess water, working down from ceilings to walls to floors and being careful not to recontaminate cleaned areas.

(2) Replace the cleaned fans and controls. Replace the trays, preferably still wet from cleaning, and bring the incubator to normal operating temperature.

(3) The hatcher should be fumigated (see Section C(5)) or otherwise disinfected before transferring the eggs.

(4) If the same machine is used for incubating and hatching, the entire machine should be cleaned after each hatch. A vacuum cleaner should be used to remove dust and down from the egg trays; then the entire machine should be vacuumed, mopped, and fumigated (see Section C(5)) or otherwise sanitized.

(c) The egg and chick/ poult delivery truck drivers and helpers should use the following good biosecurity practices while picking up eggs or delivering chicks or poults:

(1) Spray truck tires thoroughly with disinfectant before leaving the main road and entering the farm driveway.

(2) Put on sturdy, disposable plastic boots or clean rubber boots before getting out of the truck cab. Put on a clean smock or coveralls and a hairnet before entering the poultry house.

(3) After loading eggs or unloading chicks or poults, remove the dirty smock or coveralls and place into a plastic garbage bag before loading in the truck. Be sure to keep clean coveralls separate from dirty ones.

(4) Reenter the cab of the truck and remove boots before placing feet onto floorboards. Remove hairnet and leave with disposable boots on farm.

(5) Sanitize hands using appropriate hand sanitizer.

(6) Return to the hatchery or go to the next farm and repeat the process.

(5) Fumigation

Fumigation may be used for sanitizing eggs and hatchery equipment or rooms as a part of a sanitation program. APHIS disclaims any liability in the use of formaldehyde for failure on the part of the user to adhere to the Occupational Safety and Health Administration (OSHA) standards for formaldehyde fumigation, 29 CFR 1910.1048.

(6) Procedures for establishing isolation and maintaining sanitation and good management practices for the control of Salmonella and Mycoplasma infections

(a) The following procedures are required for participation under the U.S. Sanitation Monitored, U.S. M. Gallisepticum Clean, U.S. Synoviae Clean, U.S S. Enteritidis Monitored, and U.S. S. Enteritidis Clean classifications:

(1) Allow no visitors except under controlled conditions to minimize the introduction of *Salmonella* and *Mycoplasma.* Such conditions must be approved by the Official State Agency and the Service.

(2) Maintain breeder flocks on farms free from market birds and other domesticated fowl. Follow proper isolation procedures as approved by the Official State Agency.

(3) Dispose of all dead birds by locally approved methods.

(b) Recommended procedures:

(1) Avoid the introduction of *Salmonella, Mycoplasma gallisepticum*, or *Mycoplasma synoviae* infected poultry.
(2) Prevent indirect transmission from outside sources through contaminated equipment, footwear, clothing, vehicles, or other mechanical means.
(3) Provide adequate isolation of breeder flocks to avoid airborne transmission from infected flocks.
(4) Minimize contact of breeder flocks with free-flying birds.
(5) Establish a rodent control program to keep the rodent population and other pests under control.
(6) Tailor vaccination programs to needs of farm and area.
(7) Clean and disinfect equipment after each use.
(8) Provide clean footwear and provide an adequate security program.
(9) Clean and disinfect houses before introducing a new flock.
(10) Use clean, dry litter free of mold.
(11) Keep accurate records of death losses.
(12) Seek services of veterinary diagnostician if unaccountable mortality or signs of disease occur.
(13) Adopt and maintain a clean egg program.
(14) Use only crates and vehicles that have been cleaned and disinfected in accordance with Section C(4)(a) to haul live poultry to and from the premises.

(7) Procedures recommended to prevent the spread of disease by artificial insemination of turkeys

(a) The vehicle transporting the insemination crew should be left as far as practical from the turkey pens.
(b) The personnel of the insemination crew should observe personal cleanliness, including the following sanitary procedures:
    (1) Outer clothing should be changed between visits to different premises so that clean clothing is worn on entering each premises. The used apparel should be kept separate until laundered. This also applies to gloves worn while handling turkeys.
    (2) Boots or footwear should be cleaned and disinfected between visits to different premises.
    (3) Disposable caps should be provided and discarded after use on each premises.
(c) The use of individual straw or similar technique is highly recommended. Insemination equipment which is to be reused should be cleaned and disinfected before reusing. Equipment used for the convenience of the workers should not be moved from premises to premises.
(d) No obviously diseased flock should be inseminated. If evidence of active disease is noted after insemination is begun, operations should be stopped and the hatchery notified.
(e) Care should be taken during the collection of semen to prevent fecal contamination. If fecal material is present, it should be removed before the semen is collected. Likewise, care should be taken not to introduce fecal material into the oviduct of the hen.

(8) Hygiene and Biosecurity Procedures for Poultry Primary Breeding Flocks and Hatcheries

(a) Recommendations applicable to breeding establishments.

(1) The choice of a suitably isolated geographical location, taking into account the direction of the prevailing winds, facilitates hygiene and disease control. The establishment should be surrounded by a security fence and a gateway to control traffic and access to the site. A sign indicating restricted entry should be posted at the entrance. If a vehicle must enter the biosecure area, it must be cleaned and disinfected before entry.

(2) Poultry breeding establishments should be single-purpose. Single-species enterprises, and ideally an all-in, all-out single-age group principle, should be adopted whenever possible.

(3) Where several flocks are maintained on one premises, the individual flocks should be managed as separate entities.

(4) Buildings housing poultry or those used to store feed or eggs should be free of vermin and not accessible to wild birds.

(5) Poultry houses should be constructed so that all surfaces inside the buildings can be cleaned and disinfected.

(6) The area immediately surrounding the poultry houses should be free from debris and ideally this should consist of an area of concrete, gravel, or other similar material. Grass surrounding the poultry houses should be kept short.

(7) Domestic animals should not be permitted access to poultry houses.

(8) Appropriate disease security precautions should be adopted for all visitors to the premises and for all staff entering individual poultry houses.

(9) When a poultry house or premise is depopulated, all manure should be removed from the houses and effective cleaning and disinfection procedures applied. Bacteriological monitoring of the efficacy of disinfection procedures is recommended. When necessary, rodent and insect control procedures should also be carried out.

(10) Repopulation of poultry houses or premises should only be made from poultry flocks of known high health status and which are regularly monitored for Salmonella and other poultry pathogens.

(11) All feed used in poultry houses should be manufactured and stored in a manner that prevents exposure to wild birds, waterfowl, and rodents.

(12) The water supply to poultry houses should be of a satisfactory potable status.

(13) Sick and dead birds should be removed from poultry houses as soon as possible and effective and safe disposal procedures implemented.

(14) Full records relating to mortality, disease diagnosis, treatments, and vaccinations should be maintained on an individual flock basis. Such records should be readily available for inspection.

(b) Recommendations applicable to hatching egg hygiene and transport.

(1) The litter in the laying house should be kept dry and in good condition.

(2) Eggs should be collected at frequent intervals of not less than twice per day and placed in clean, disinfected containers.

(3) Dirty, broken, cracked, leaking, and dented eggs should be collected in a separate container and should not be used for hatching purposes.

(4) The clean eggs should be sanitized as soon as possible after collection. The methods of sanitation are described in Section C(8)(g).

(5) The sanitized eggs should be stored in a clean, dust free room used exclusively for this purpose.

(6) The eggs should be transported to the hatchery in new or clean containers that have been fumigated or sanitized with a liquid disinfectant. The cleaning and disinfection of vehicles must be a regular part of the hatchery routine

(c) Recommendations applicable to hatchery buildings.

(1) The design of the hatchery should be based on suitable work flow and air circulation principles. It should be constructed so that there is a one-way flow for the movement of eggs and chicks. The air flow should also follow this same one-way direction.

(2) The hatchery buildings should include physical separation of all work areas. If possible, separate ventilation should be provided for these work areas, namely, the rooms for:
- (i) Egg receiving and egg storage.
- (ii) Egg traying.
- (iii) Fumigation.
- (iv) Setting or initial incubation.
- (v) Hatching.
- (vi) Sorting, sexing, and placing chicks in boxes.
- (vii) Material storage, including egg and chick boxes, egg flats, box pads, chemicals, and other items.
- (viii) Facilities for washing equipment and disposal of waste.
- (ix) Employee meals.
- (x) Other office functions.

(3) Openable windows, ventilators, and other open areas should be screened against insects and vermin.

(d) Recommendations applicable to hatchery building hygiene.

(1) The area adjacent to the hatchery buildings should have limited access to traffic, and access into the building should be restricted.

(2) Wild birds and domestic and wild animals must be excluded from the hatchery area. When necessary, a specific program for fly control should be implemented.

(3) The hatchery area should be maintained free from all hatchery waste, garbage of all kinds, and discarded equipment.

(4) Approved disposal methods and adequate drainage must be available.

(5) All hatchery equipment, tables, and horizontal surfaces in rooms must be promptly and thoroughly cleaned of organic material, washed, scrubbed, rinsed with clean water, and finally disinfected with an approved disinfectant.

(e) Requirements applicable to personnel and visitors.

(1) Clean clothing and footwear must be provided for all personnel and visitors entering the premises or the hatchery.

(2) Clean and disinfected footwear or a means to clean and disinfect footwear should be available at each entrance. Washing the hands in disinfectant solution or with soap and water should be required.

(3) Personnel and visitors should have no direct contact with other poultry or poultry products.

(f) Hygiene measures during the handling of eggs and day-old birds.

(1) Day-old poultry must be delivered or distributed in new chick boxes; or in used boxes made of suitable material which have been thoroughly cleaned and disinfected or fumigated.

(2) The day-old poultry should be delivered directly from the hatchery by personnel wearing clean, disinfected outer clothing. Outer clothing should be changed or disinfected between each delivery.

(3) The delivery truck must be cleaned, and disinfected before loading each consignment of day-old poultry.

(g) Sanitation of hatching eggs and hatchery equipment.

Sanitation means:

(i) Fumigation with formaldehyde, or

(ii) Spraying with or immersion in an egg shell disinfectant in accordance with the manufacturer's instructions, or

(iii) made hygienic by another method approved by the veterinary authority.

(iv) Should notifiable avian influenza (NAI) be detected within a State where a primary breeder has production facilities, then the biosecurity procedures for breeding flocks and hatcheries will be implemented in accordance with the USDA-approved State response and containment plan for NAI.

Subpart D—Molecular Examination Procedures

(1) Laboratory procedure recommended for the polymerase chain reaction (PCR) test for Mycoplasma gallisepticum and M. synoviae.

(a) DNA isolation. Isolate DNA from 1 mL of eluate from tracheal swabs in PBS or 1 mL of broth culture by a non-phenolic procedure. Centrifuge samples at 14,000 x g for 5 to 10 minutes. Decant supernatant and wash the pellet with 1 mL of PBS. Centrifuge as above and re-suspend the pellet in 25 to 50 µl of 0.1 percent DEP (Diethyl Pyrocarbonate; Sigma) water. Boil at 100 °C for 10 minutes followed by 10 minutes incubation at 4 °C. Centrifuge as above and transfer the supernatant DNA to a nuclease-free tube. Estimate the DNA concentration and purity by spectrophotometric reading at 260 nm and 280 nm.

(b) Primer selection.

(1) *M. gallisepticum.* The primer for *M. gallisepticum* should consist of the following sequences:

MG-F	5' GAG CTA ATC TGT AAA GTT GGT C
MG-R	5' GCT TCC TTG CGG TTA GCA AC

(2) *M. synoviae.* The primer for *M. synoviae* should consist of the following sequences:

MS–F	5' GAG AAG CAA AAT AGT GAT ATC A
MS–R	5' CAG TCG TCT CCG AAG TTA ACA A

(c) Polymerase chain reaction.

(1) Treat each sample (100 to 2000 ng/5 µl) with one of the following 45 µl PCR cocktails:
   - (i) 5 µl 10x PCR buffer, 1 µl dNTP (10 mM), 1 µl of Reverse primer (50 µM), 1 µl of Forward primer (50 µM), 4 µl $MgCl_2$(25 mM), 1 µl taq-polymerase (5 U), 32 µl DEP water.
   - (ii) 18 µl water, 25 µl PCR mix (Promega), 1 µl Reverse primer (50 µM), 1 µl Forward primer (50 µM).

(2) Perform DNA amplification in a Perkin-Elmer 9600 thermocycler or in a Hybaid PCR Express thermocycler.[18] The optimized PCR program is:

Temperature (°C)	Duration	Cycles
94	30 seconds	30–40
55	30 seconds	30–40
72	1 minute	30–40
72	5 minutes	1 (final extension).

(d) Electrophoresis. Mix PCR products (5 to 10 µl) with 2 µl loading buffer (Sigma) and electrophorese on a 2 percent agarose gel containing 0.5 µg/mL ethidium bromide in TAE buffer (40 mM tris; 2 mM EDTA; pH 8.0 with glacial acetic acid) for 30 minutes at 80 V. *M. gallisepticum* (185 bp) and *M. synoviae* (214 bp) amplicons can be visualized under an ultraviolet transilluminator along with the PCR marker (50 to 2000 bp; Sigma).

(e) Alternative methods Alternative methods (equipment and reactions components) may be used by an approved laboratory as long as the listed MG and MS PCR primers in Subpart D section 1 are used and a proficiency test as outlined in Program Standards Subpart D section 3. has been carried out to the satisfaction of the Official State Agency and the Service, indicating the laboratory is performing equivalent or better detection levels with their desired PCR method. Quantified positive controls should be made to use with each run of this assay from the Hemagglutination antigens provided from the Service. Records shall be maintained over time to show the production of the controls and consistency of the reactions of said controls in this assay over time.

(2) Laboratory procedures recommended for the real-time polymerase chain reaction test for Mycoplasma gallisepticum (MGLP ReTi).

(a) DNA extraction. Use Qiagen Qiamp Mini Kit for DNA extraction or equivalent validated technique/procedure. This kit uses the following methods: 100 µl of swab suspension incubates with 10 µl of proteinase K and 400 µl of lysis buffer at 56 °C for 10 minutes. Following incubation, 100 µl of 100 percent ethanol is added to lysate. Wash and centrifuge following extraction kit recommendations.

(b) Primer selection. A forward primer mglpU26 (5'-CTA GAG GGT TGG ACA GTT ATG–3') located at nucleotide positions 765,566 to 765,586 of the *M. gallisepticum* R strain genome sequence; a reverse primer mglp164 (5'-GCT GCA CTA AAT GAT ACG TCA AA–3') located at nucleotide positions 765,448 to 765,470 of the *M. gallisepticum* R strain genome sequence; and a Taqman dual-labeled probe mglpprobe (5'-FAM–CAG TCA TTA ACA ACT TAC CAC CAG AAT CTG–BHQ1–3') located at

[18] Trade names are used in these procedures solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.

nucleotide positions 765,491 to 765,520 of the *M. gallisepticum* R strain genome should be used to amplify a 139-bp fragment of the lp gene.

(c) MGLP ReTi. Primers and probe should be used in a 25 µl reaction containing 12.5 µl of Quantitect Probe PCR 2X mix (Qiagen, Valencia, CA),[22] primers to a final concentration of 0.5 µmolar, and probe to a final concentration of 0.1 µmolar, 1µl of HK–UNG Thermolabile Uracil N-glycosylase (Epicentre, Madison, WI), 2 µl of water, and 5 µl of template. The reaction can be performed in a SmartCycler (Cepheid, Sunnyvale, CA) or other equivalent validated platform procedure for real-time thermocycler at 50°C for 2 minutes; 95°C for 15 minutes with optics OFF; and 40 cycles of 94°C for 15 seconds followed by 60°C for 60 seconds with optics ON.

(d) Determination of positive. For each MGLP ReTi assay reaction, the threshold cycle number (CT value) was determined to be the PCR cycle number at which the fluorescence of the reaction exceeded 30 units of fluorescence. For all samples tested, any MGLP reaction that has a recorded CT value was considered positive, while any MGLP reaction that had no recorded CT value was considered negative.

(e) Controls. Proper controls should be used when conducting the MGLP ReTi assay as an official test of the Plan. Positive, quantitative, extraction, and internal controls are commercially available from GTCAllison, LLC, Mocksville, NC

(f) Alternative methods Alternative methods (equipment and reactions components) may be used by an approved laboratory as long as the listed MG PCR primers in Program Standards Subpart D section 2 are used and a proficiency test as outlined in Program Standards Subpart D section 3 has been carried out to the satisfaction of Official State Agency and the Service, indicating the laboratory is performing equivalent or better detection levels with their desired PCR method.

(3) Laboratory procedures recommended for the conventional polymerase chain reaction test for Salmonella Enteritidis.

Allows the use of a second primer set suitable for a real-time polymerase chain reaction (PCR) application that targets a region already approved for conventional PCR detection of Salmonella Enteritidis.

(a) Sample enrichment. Samples (drag swabs, chick paper swabs, etc.) are enriched in Tetrathionate enrichment for 18 to 24 hours at 37° or 42.0°C (see Section B(3)) and subcultured onto Modified Semi-solid Rappaport-Vassiliadis (MSRV) Enrichment (see Section B(3)) in accordance with procedures set forth in these program standards.

(b) Quality control. A positive control, known Salmonella Enteritidis or ATCC strain from BHI broth should be inoculated onto MSRV media, incubated for 18 to 24 hours and 1 to 3 plugs harvested from the zone of white precipitate growth outside the initial inoculation site. A negative control is harvested plugs from an area of uninoculated MSRV plate.

(c) DNA extraction. DNA is extracted from 1 to 3 plugs (~100 µL) of MSRV agar in the zone of migration outside the initial inoculation site by boiling in 100µl PCR grade water for 10 minutes or by another DNA extraction method. Samples are cooled to room temperature before PCR use or stored at 2° to 8°C, if PCR is not performed

immediately. For the boiling method, the extracted samples are spun at 16,000 rcf for 3 minutes. The DNA is contained in the supernatant.

(d) Primer Selection.

The SE specific primers are:
sdf I (forward) – TGTGTTTTATCTGATGCAAGAGG
sdf I (reverse) – CGTTCTTCTGGTACTTACATGAC.

The internal control primers are:
rpl I (forward) – GGGTGATCAGGTTAACGTTAAAG
rpl I (reverse) – CTTCGTGTTCGCCAGTGGTACGC.

(e) Polymerase chain reaction.

The following multiplex-PCR reaction (2 sets of primersper reaction tube) should be set up in a 200µl PCR tube ora 25 µl PCR tube, in a clean environment.

Reaction Mix	*50 µL Volume*	*25 µL Volume*	*Final Concentration*
10X PCR Gold Buffer	5µl	2.5µl	1X
$MgCl_2$ (25mM)	5µl	2.5µl	2.5mM
10mM dNTP mix	3µl	1.5µl	150µM each
sdf I Forward Primer	4µl	2.0µl	0.4µM
sdf I Reverse Primer	4µl	2.0µl	0.4µM
rpl I Forward Primer	4µl	2.0µl	0.4µM
rpl I Reverse Primer	4µl	2.0µl	0.4µM
Amplitaq Gold Polymerase	0.5µl	0.25µl	2.5U
Sterile PCR Grade Water	15.5µl	5.25µl	
DNA Template*	5.0µl	5.0µl	

*The DNA template is 5.0µl of the boiled MSRV solution or DNA from another DNA extraction method.

(f) PCR amplification program:

Polymerase Activation Step	95°C for 10 minutes	1 cycle
Denaturation Step	95°C for 30 seconds	
Annealing Step	60°C for 30 seconds	35 cycles
Extension Step	72°C for 1 minute	
Final Extension	72°C for 7 minutes	1 cycle
4°C Hold	infinite hold until samples are refrigerated or frozen	

(g) Electrophoresis

After PCR is completed, samples should be analyzed by DNA electrophoresis. A 3 percent RAGE gel, or a 1 percent to 3 percent conventional gel with a sample volume of 3.5µl, in addition to 1.5µl of loading dye, is recommended. The *sdf I* primers will yield a 293bp band only in the presence of Salmonella Enteritidis DNA and the *rpl I* primers will yield a 343bp band in the presence of any bacterial DNA (the primers have worked well with every organism so far with the exception of *Proteus mirabilis*).

Reference:

1.Charlton BR, Walker RL, Kinde H, Bauer CR, Channing-Santiago SE, et al. (2005) Comparison of a Salmonella Enteritidis-Specific Polymerase Chain Reaction Assay to Delayed Secondary Enrichment Culture for the Detection of Salmonella Enteritidis in Environmental Drag Swab Samples. Avian Diseases: Vol. 49, No. 3 pp. 418–422.

Or alternatively in a real time SYBR based PCR assay the following primers may be utilized

F2 TTG ATG TGG TTG GTT CGT CAC T ,
R2 TCC CTG AAT CTG AGA AAG AAA AAC TC

(h) Alternative methods (equipment and reactions components)

Alternative methods may be utilized by an approved laboratory as long as appropriate PCR primers as listed in this subpart are utilized and a Group D Salmonella proficiency test provided by the service has been passed utilizing the method indicating the laboratory is performing equivalent or better detection levels with their desired PCR method. If using real time PCR assays Quantitative positive controls should be made to use with each run of this assay from a known Salmonella enteritidis Strain (as confirmed by NVSL), or ATCC Strain extracted using Qiagen DNeasy Tissue Kit Cat. No. 69506 or equivalent.) Records shall be maintained to show the production of the controls and consistency of the reactions of said controls in this assay over time to the Official State Agency upon request.

(4) Laboratory procedures recommended for the real-time polymerase chain reaction test for *Salmonella sp.* Group D

(a) Sample enrichment.

Samples (drag swabs, chick paper swabs, etc.) are enriched in Tetrathionate enrichment for 24 hours at 37° or 42° C (see Section B(3)) and subcultured onto Modified Semi-solid Rappaport-Vassiliadis (MSRV) Enrichment (see Section B(3)) .

(b) Quality control.

A positive control, known *Salmonella* Enteritidis or ATCC strain, from BHI broth should be inoculated onto MSRV media, incubated for 18 to 24 hours and 1 to 3 plugs harvested from the zone of white precipitate growth outside the initial inoculation site. A negative control is harvested plugs from an area of uninoculated MSRV plate.

(c) DNA extraction.

DNA is extracted from 1 to 3 plugs (~100 µL) of MSRV agar in the zone of migration outside the initial inoculation site by boiling in 100µl PCR grade water for 10 minutes or by another DNA extraction method. Samples are cooled to room temperature before PCR use or stored at 2° to 8°C, if PCR is not performed immediately. For the boiling method, the extracted samples are spun at 16,000 rcf for 3 minutes. The DNA is contained in the supernatant.

(d) Primer and probe selection.

sefAF (forward): 22 bp sequence 5'GGCTTCGGTATCTGGTGGTGTA3' (50 nM final concentration); (example: use volumes below if stock primer is 10 µM concentration).
sefAR (reverse): 24 bp sequence 5'GGTCATTAATATTGGCCCTGAATA3' (900 nM concentration); (example: use volumes below if stock primer is 10 µM concentration).
sefAPR (probe): 25 bp sequence 5'FAM/CCACTGTCCCGTTCGTTGATGGACA/TAMRA' or comparable quencher (250 nM concentration);
(FAM = 3' 5(6)-Carboxyfluorscein; TAMRA, 6-carboxytetramethylrhodamine (quencher dye)); (example: use volumes below if stock primer is 10 µM concentration).

(e) PCR reactions. The following real-time PCR reaction (or equivalent) should be set up in a clean environment. The reaction should be assembled using aerosol resistant pipette tips to decrease the chance of contamination. Total volume for reaction is 50µl (Opticon) or 25µl (SmartCycler).

Reaction Mix	50µl Volume	25µl Volume	Final Concentration
10X PCR Gold Buffer	5µl	2.5µl	1X
$MgCl_2$ (25mM)	5µl	2.5µl	2.5mM
10mM dNTP mix	4µl	2.0µl	200µM each
Forward Primer (*sefA*F)	0.2µl	0.1µl	0.05uM
Reverse Primer (*sefA*R)	4.6µl	2.3µl	0.9 uM
TaqMan probe (*sefA*PR)	0.6µl	0.3µl	0.25uM
Amplitaq Gold Polymerase	0.25µl	0.13µl	1.25U
Sterile PCR Grade Water	25.35µl	10.17µl	
DNA Template*	5.0µl	5.0µl	

*The DNA template is 5.0µl of the boiled MSRV solution or DNA from another DNA extraction method.

(f) PCR amplification program.

Pre-incubation	50°C for 2 minutes	1 cycle
Pre-incubation	50°C for 2 minutes	1 cycle
Taq activation	95°C for 10 minutes	1 cycle
Annealing Step	95°C for 15 seconds	
Extension Step	60°C for 60 seconds	40 cycles

(g) Analysis of results. A sample with a Ct value less than 35 is considered positive. A sample with a Ct value between 35 and 40 is considered to be indeterminate. Positive and indeterminate results are to be further tested by culture methods.

Reference:
1. Seto KH, Valentin-Bon IE, Brackett, RE, and Holt, PS. (2004) Rapid, Specific Detection of Salmonella Enteritidis in Pooled Eggs by Real-Time PCR. Journal Food Protection: Vol 67, No. 5, pp. 864-869.

(5) Laboratory procedure recommended to produce proficiency test sample sets for establishing inter-laboratory equivalence in molecular identification of plan diseases sampled in the poultry upper respiratory tract

A participant in any State wishing to work with the Official State Agency may use the following method to set up inter-laboratory proficiency sample sets to have a quantitative means to assure molecular based detection assays are detecting similar levels in laboratories providing service in a given region and to be linked to current antigens provided by the Service for use in the NPIP.

(a) A fresh batch of desired antigen shall be ordered from the NVSL in Ames, Iowa via http://www.aphis.usda.gov/animal_health/lab_info_services/downloads/AmesReagentManualCurrent.pdf (Mycoplasma gallisepticum hemagglutination (HA) Reagent code 100, Mycoplasma synoviae (HA) reagent code 120, and

Mycoplasma Meleagridis (HA) antigen reagent code 110). Mycoplasma HA antigens should be thawed when received and immediately repackaged into smaller aliquots of 250 microliter (µl) volume and refrozen in a -70 C° freezer with appropriate identification. Keep in mind when working with these HA antigens that they are live organisms and can transmit the disease as they are a huge concentration of target DNA. Use appropriate lab biosecurity.

(b) For each sample set to be produced go to an "NPIP CLEAN" flock and collect 75 swabs (normal type used by laboratory, but preference should be towards a dry-packed rayon-tipped swab with plastic shaft packaged in individual tubes or plastic pouches), from the birds choanal cleft region (palatine fissure), gently turning around collecting mucus and cells. *AVOID* getting the swabs bloody as this can cause false reactions with some assay types in the program (specifically the AI ACIA test). These will now be referred to as "CP" swabs. It is not necessary to use targeted animals for this; the objective is to collect background poultry cells and accompanying respiratory region microbes.

(c) Next, make test "pool" sets by adding one of the PBS or antigen-loaded swabs and four of the "poultry only" swabs to make a test pool set of five swabs. These will be kept in their individual packages but linked together in a small bag or with rubber bands. Keep track of the PBS (negative control) or antigen concentration identification of the one swab and the code applied to the pool if doing a proficiency test. Avoid leaving your temporary identification on the individual swabs with the added PBS or antigen.

(d) Pack up all the pool samples sets and save overnight in the refrigerator or ship via overnight mail for DNA extraction process and PCR analysis the next day.

(e) It is recommended to do a small test with your antigens and a minimized sample set before any big project to get the desired dilution range down. (You may need to readjust the recommended levels listed above if they do not hit the desired goal of finding the end point in your system and that of the other labs. This is also a great way to evaluate different swab types; it does make a difference which type one uses. Plain cotton swabs are really not desirable as you will see if you try this out.)

(f) Have the extraction and PCR/molecular method done as normal in the lab. (Find out if the lab desires different pool sizes or not to pool at all before setting up the proficiency test.)

(6) Use of rRt-PCR for AI testing in Waterfowl.

The NPIP supports the use of cloacal swabs from domestic ducks and poultry as an approved specimen for the rRT-PCR matrix test assay when performed with the Ambion MagMAX (catalog No. AM1835 from Life Technologies) magnetic bead procedure for the NPIP NAI US H5/H7 Avian Influenza Clean and the US H5/H7 Avian Influenza Monitored Programs. The rRT-PCR procedure will remain a screening test and all positive findings will need to be further tested as provided in 9 CFR 145.14(d) and 9 CFR 146.13(b).

(7) Approved tests

The following diagnostic test kits that are not licensed by the Service (e.g., bacteriological culturing kits) are approved for use in the NPIP:

1. Rapid Chek©Select TMSalmonella Test Kit, Romer Labs, Inc., Newark, DE 19713.

2. ADIAFOOD Rapid Pathogen Detection System for Salmonella spp., AES Chemunex Canada. Laval, QC (Canada) H7L4S3.

3. DuPont Qualicon BAX Polymerase Chain Reaction (PCR)-based assay for Salmonella 1 and 2 DuPont Qualicon, Wilmington, DE 19810.

4. Applied Biosystems TaqMan® Salmonella Enteritidis Real-Time PCR assay for the detection of Salmonella Enteritidis. Life Technologies Corporation. Foster City, CA 94404.

5. IDEXX MG/MS RT-PCR.

6. MicroSEQ Salmonella Species Detection Kit, Life Technologies Corporation, Austin, TX.

7. ANSR Salmonella Test, Neogen Corporation, Lansing, MI 48912.

8. Reveal 2.0 Group D1 Salmonella (Including SE) Kit, Neogen, Neogen Corporation, Lansing, MI 48912.

9. DNAble Salmonella Detection Kit, EnviroLogix, Inc., Portland, Maine 04103.

10. Bactotype MG/MS Kit, QIAGEN, Germantown, Maryland, 20874.