

PASTEURELLA
MULTOCIDA
ANTIBODY
TEST KIT

GENERAL INFORMATION AND
INTENDED USES

Pasteurella multocida (PM) is the causative agent of fowl cholera, a contagious septicemic disease of domestic poultry and wild birds that is often associated with high morbidity and mortality¹.

The ProFLOK® PM ELISA Kit is a rapid serologic test for the detection of PM antibody in chicken serum samples. The ProFLOK® PM ELISA test was developed primarily to aid in the serodiagnosis of Fowl Cholera and the detection of pre and post-vaccination PM antibody levels in chickens.

The assay is designed to measure PM antibody bound to PM antigen coated plates. The principle of the test is as follows: Serum obtained from chickens exposed to *Pasteurella multocida* contains specific anti-PM antibodies. Serum, diluted in Dilution Buffer, is added to a PM antigen coated plate. Specific PM antibody in the serum forms an antibody-antigen complex with the PM antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step reacts with the conjugate. After a brief incubation period, the unreacted conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromagen color change (from clear to green-blue) occurs in the presence of the peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of PM antibody in the serum. After the substrate has incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm.

REAGENTS REQUIRED TO PERFORM 90
TESTS

- a) 1 PM antigen coated plate
- b) 10 µl PM Positive Control Serum
- c) 10 µl Normal Control Serum (NCS)
- d) 100 µl Goat anti-Chicken IgG (H+L) Peroxidase Conjugate Solution
- e) 40 ml Dilution Buffer
- f) 10 ml ABTS-Hydrogen Peroxide Substrate Solution
- g) 2.5 ml 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
- h) 20 ml 20X Wash Solution (dilute [1:20] with laboratory grade water)

NOTE: Store all reagents provided in the kit at 2-7°C. Reagents should not be frozen.

EQUIPMENT AND MATERIALS
REQUIRED BUT NOT PROVIDED

- a) High precision pipette (i.e. 1-20 microliter pipette)
- b) 0.2 ml, 1.0 ml and 5.0 ml pipettes
- c) 8 or 12 channel pipette (or transplating device) and pipette tips
- d) 2 graduated cylinders (50 ml)
- e) 1 ml or 5 ml borosilicate glass test tubes
- f) Uncoated low binding 96 well plates (i.e. Nunc catalog #269620)
- g) Laboratory grade (Distilled or R.O.) water
- h) 96 well plate reading spectrophotometer with 405-410 nm filter
- i) Plate washing apparatus
- j) Waste container with bleach or other oxidizing agent

WARNINGS TO THE USERS OF REAGENTS
AND PM ANTIGEN COATED PLATES

- a) Handle all reagents and samples as biohazardous material.
- b) Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- c) Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontaminated with bleach or other strong oxidizing agent before disposal.
- d) Take special care not to contaminate any of the test reagents with serum or bacterial agents.
- e) Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
- f) The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
- g) Do not use this kit after the expiration date.
- h) NEVER PIPETTE BY MOUTH.

ALLOW ALL REAGENTS TO COME TO
ROOM TEMPERATURE BEFORE STARTING!

SAMPLE COLLECTION

For routine serologic flock monitoring, it is suggested that at least 30 or more sera per flock be randomly collected at standard time intervals (i.e. every four weeks). Proper sample collection procedures, serum harvest and serum sample storage (4°C for up to four days or -20°C for longer periods) are needed to provide reliable test results.

SAMPLE DILUTION PROCEDURE

Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Frozen serum samples should be completely thawed and thoroughly mixed before diluting. Set up samples and controls as shown in Figure 1.

PREPARATION OF THE SERUM
DILUTION PLATE

- a) Add 300 µl Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.
- b) Add 6 µl unknown serum per well as per Figure 1 (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). For example, wells 1 through 30 contain the diluted sera of flock 1, wells 31-60 contain the diluted sera of flock 2, etc.
- c) Add 6 µl of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10 and H12.
- d) Aspirate and remove any liquid in dilution plate wells A1, A3 and H11.
- e) Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to a PM antigen coated ELISA plate.
- f) Diluted serum should be tested within 24 hours.

This dilution format provides adequate quantities of diluted serum samples to conduct four additional ProFLOK® ELISA tests (i.e. IBD, REO, IBV or NDV) using the same serum dilution plate.

Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	(+)	(-)	(+)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
B	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)
C	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)	(31)	(32)	(33)
D	(34)	(35)	(36)	(37)	(38)	(39)	(40)	(41)	(42)	(43)	(44)	(45)
E	(46)	(47)	(48)	(49)	(50)	(51)	(52)	(53)	(54)	(55)	(56)	(57)
F	(58)	(59)	(60)	(61)	(62)	(63)	(64)	(65)	(66)	(67)	(68)	(69)
G	(70)	(71)	(72)	(73)	(74)	(75)	(76)	(77)	(78)	(79)	(80)	(81)
H	(82)	(83)	(84)	(85)	(86)	(87)	(88)	(89)	(90)	(-)	(+)	(-)

Preparation of PM Positive Control

A PM Positive Control Serum has been provided with this kit. Dilute the appropriate volume of PM Positive Control Serum with Dilution Buffer (1:50) in a clean, glass test tube. For example, dilute 6µl of positive control serum in 300 µl Dilution Buffer. Mix well. 150 µl of diluted PM Positive Control is needed per ELISA plate.

Preparation of Conjugate Solution

The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP Stabilizer. Dilute 100 µl stock conjugate in 10 ml Dilution Buffer (1:100 dilution). Mix well. This 10 ml preparation will supply sufficient conjugate for one 96 well ELISA plate.

Preparation of 1X Wash Solution

Dilute 20 ml concentrated Wash Solution in 380 ml laboratory grade (distilled or R.O.) water (1:20). Mix well. Approximately 400 ml Wash Solution is needed for each 96 well ELISA plate.

Preparation of the Substrate Solution

The Substrate Solution is ready to use. Each plate will require approximately 10 ml substrate solution. For example, 10 plates requires 100 ml substrate. For best results, the substrate solution must be equilibrated to room temperature before use.

Preparation of 1X Stop Solution

Dilute 2.5 ml concentrated Stop Solution in 10 ml laboratory grade (distilled or R.O.) water (1:5). Mix well. Approximately 12.5 ml Stop Solution is needed for each 96 well ELISA plate.

NOTE: Storage of 5X Stop Solution at refrigerated temperatures may cause the formation of a white solid. This does not affect product performance. Warm at room temperature or 37°C to dissolve before use.

ELISA TEST PROCEDURE

PREPARING THE TEST PLATE

- Remove a PM antigen test plate from the protective bag and label according to serum dilution plate identification.
- Add 50 μ l Dilution Buffer to all wells on the test plate.
- Add 50 μ l diluted Positive Control Serum to wells A1, A3 and H11. Discard pipette tip.
- Using an 8 or 12 channel pipette transfer 50 μ l/well of each of the diluted serum samples and Normal Control Serum samples from the serum dilution plate to the corresponding wells of the PM coated test plate (yields a 1:100 dilution). Discard pipette tips after each row of sample is transferred. Transfer of samples to the ELISA plate should be done as quickly as possible.
- Incubate plate for 30 minutes at room temperature.

WASH PROCEDURE

- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using an 8 or 12 channel pipette (or comparable automatic washing device), fill each well with approximately 300 μ l Wash Solution. **Allow to soak in wells for 3 minutes;** then discard contents into an appropriate waste container (waste container should contain bleach solution). Tap inverted plate to ensure that all residual liquid is removed. **Repeat wash procedure 2 more times.**

NOTE: The wash procedure is a very critical step in any ELISA procedure. Please follow the above steps as directed.

ADDITION OF ANTI-CHICKEN IgG PEROXIDASE CONJUGATE, SUBSTRATE AND STOP SOLUTION

- Using an 8 or 12 channel pipette (or transplating device) dispense 100 μ l diluted conjugate (prepared as described above) into each assay well. Discard pipette tips.
- Incubate for 30 minutes at room temperature.
- WASH** as in steps f and g above.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 μ l Substrate Solution into each test well. Discard pipette tips.
- Incubate 15 minutes at room temperature.
- Using an 8 or 12 channel pipette (or transplating device) add 100 μ l diluted Stop Solution (prepared as described above) to each test well.
- Allow bubbles to dissipate before reading plate.

MANUAL PROCESSING OF DATA

- Read the plate using an ELISA plate reader set at 405-410 nm. Be sure to blank the reader as directed.

- Calculate the average Positive Control Serum absorbance (Optical Density [O.D.]) using the absorbance values of wells A1, A3 and H11. Calculate the average Normal Control Serum (NCS) absorbance values obtained from wells A2, H10 and H12. Record both averages.
- Subtract the average negative (NCS) absorbance from the average positive absorbance. The difference is the Corrected Positive Control.
- Calculate a sample to positive (Sp) ratio by subtracting the average negative control absorbance from each sample absorbance. The difference is divided by the corrected positive control. Use the following equation format:

$$SP = \frac{(\text{SAMPLE ABSORBANCE}) - (\text{AVERAGE NORMAL CONTROL ABSORBANCE})}{\text{CORRECTED POSITIVE CONTROL ABSORBANCE}}$$

- A PM ELISA titer can be calculated by the following suggested equation:

$$\text{LOG}_{10} \text{ TITER} = (1.464 \times \text{LOG}_{10} \text{ Sp}) + 3.197$$
$$\text{TITER} = \text{ANTILOG OF LOG}_{10} \text{ TITER}$$

Example:

- Example Positive Control Absorbance:
0.585, 0.610, 0.590
Average = $(0.585 + 0.610 + 0.590) / 3 = 0.595$
- Example Normal Controls:
0.078, 0.067, 0.057
Average = $(0.078 + 0.067 + 0.057) / 3 = 0.067$
- Corrected Positive Control:
 $(0.595) - (0.067) = 0.528$
- Example Sp value calculation:
Absorbance of sample = 0.560
 $(0.560) - (0.067) / 0.528 = 0.934$
- Example of Calculation of titer using the Sp from above:
 $\text{Log}_{10} \text{ Titer} = 1.464 \times (\text{Log}_{10} 0.934) + 3.197$
Titer = ANTILOG 3.15
Titer = 1413

RESULTS

Assay Control Values:

Valid PM ELISA results are obtained when the average optical density (O.D.) value of the Normal Control Serum is less than 0.200 and the Corrected Positive Control value range is between 0.250 and 0.900. If either of these values are out of range, the PM test results should be considered invalid and the samples should be retested. Samples testing with an Sp value of less than or equal to

0.199 will receive a 0 titer value and are considered negative for PM antibody.

Under optimal conditions* the suggested O.D. value ranges of **0.060 to 0.08 for PM Normal Control Serum** and **0.40 to 0.750 for PM Positive Control Serum** should be strived for to ensure the most consistent laboratory test results. Please note that tests with O.D. values which do not fall within the suggested O.D. ranges above does not constitute an invalid test.

*Optimal conditions are at room temperature [70 to 75°F (21 to 24°C)]. Higher room temperatures may result in slightly higher O.D. values.

Interpretation of Results

The PM ELISA titer values obtained represent a comparison of the PM antibody level within each field chicken serum tested and the PM ELISA kit positive and normal control sera. Therefore, it is important to first determine that the PM ELISA positive and normal control sera values obtained are valid as detailed above in the "Assay Control Values" section of this pamphlet before PM ELISA results are interpreted.

A "0" PM ELISA titer represents a chicken serum sample that contains an extremely low to insignificant PM antibody level compared to the PM ELISA kit positive and normal control sera.

A PM ELISA titer value above "0" indicates only that a chicken serum sample contains a significant and ELISA-detectable PM antibody level compared to the PM ELISA kit positive and normal control sera. However, these titers do **not** imply or ensure "protection" nor provide serologic differentiation between a PM vaccine response or PM field infection.

Optimal PM vaccine administration practices and "protective" flock PM titer target values must be determined by each PM ELISA kit user by comparing flock pre- and post-vaccination PM ELISA results [i.e. coefficient of variation (%CV) and geometric titer (GMT) values] with flock performance parameters (i.e. morbidity, mortality, flock body weight gain or uniformity) over time.

BIBLIOGRAPHY

- Rhoades, K.R. and R.B. Rinler. Pasteurellosis. In: *Diseases of Poultry*, 9th ed. American Association of Avian Pathologists. Iowa State University Press, Ames, Iowa. pp. 145-171. 1991.

Please contact Synbiotics Technical Service at
800-228-4305 or 816-464-3500 with questions and comments.

03-3056-0410

PASTEURELLA MULTOCIDA ANTIBODY TEST KIT

ITEM NO. 96-6527

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