DakoCytomation

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PMT ELISA Kit

Code No. K 0009

3rd edition

One-step enzyme immunoassay for the determination of *Pasteurella multocida* toxin (PMT).

For veterinary use only.

The kit contains 96 test wells and has been designed for the assay of up to 46 bacterial specimens (duplicate tests) plus controls. The kit allows up to 6 individual runs; the number of specimens depends on the number of individual runs as shown in the table:

Number of runs	1	2	3	4	5	6
Number of specimens	46	44	42	40	38	36

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Intended Use

The PMT ELISA Kit is designed for the detection of *Pasteurella multocida* toxin: The causal factor of progressive atrophic rhinitis in pigs (1).

Summary and Explanation

Progressive atrophic rhinitis is a disease of pigs, caused by infection with toxigenic *P. multocida* (1). The pathogenesis of the disease is linked to a single, well-characterized component: The *Pasteurella multocida* toxin (PMT) (other names for PMT are: dermonecrotic toxin (DNT), heat-labile toxin (HLT), turbinate atrophy toxin (TAT) and osteolytic toxin). The clinical manifestations of the disease are shortening or distortion of the snout, sneezing, nasal discharge and epistaxis. Reduced growth rate is seen in severe cases. Subclinical forms of the disease also occur.

Host factors as well as other determinants - infectious or non-infectious - may influence the course of infection with toxigenic *P. multocida*. An important predisposing factor is infection with *Bordetella bronchiseptica*. Factors such as climate, housing and environment may influence the severity of infection as well as the persistence of toxigenic *P. multocida*, and thus, the clinical and economic burdens of the disease.

Health monitoring programmes should be based on laboratory tests for demonstration of toxigenic strains of *P. multocida* combined with clinical inspections of herds and snout sections at slaughter. The traditional laboratory tests are based on demonstration of the biological activity of toxin obtained from pure cultures of *P. multocida*. One of the main advantages of the PMT ELISA Kit is that it assures a specific and sensitive demonstration of PMT in any type of sample, including extracts of primary mixed cultures obtained from e.g. nasal swabs or washings (2).

Principle of the Assay

The kit contains microwells coated with mouse monoclonal antibody to PMT. Extracts of bacterial cultures and peroxidase-conjugated Fab' fragment of rabbit antibody to PMT are incubated simultaneously in the microwells. After the incubation step, the microwells are washed and chromogenic substrate added. Presence of PMT in the extracts of the bacterial cultures is indicated by a golden-brown colour in the microwells. The intensity of the colour is measured at 490 nm and compared with the absorbance obtained for the positive and negative controls.

Reagents

A. Materials provided

White Strips
Six white microwell strips (2 x 8 wells each). Supplied in a plastic frame.
The White Strips are used for preparing extracts of bacterial specimens harvested from agar plates.

Clear Immunostrips
Six optically clear microwell strips (2 x 8 wells each). Supplied in a plastic frame.
The Clear Immunostrips are precoated with mouse monoclonal antibody to PMT.

Conjugate, Anti-PMT
Vial No. 1 (7 mL)
Peroxidase-conjugated Fab' fragment of rabbit antibody to PMT. Ready-to-use. In buffer with protein and 0.05% merthiolate as preservative. Coloured blue for identification.

Positive Control Antigen
Vial No. 2 (200 ng PMT)
Affinity-isolated, native, lyophilized PMT. Contains carrier protein and 20 µg merthiolate as preservative.

Reconstitution Buffer
Vial No. 2a (1 mL)
Buffered saline for reconstitution of Positive Control Antigen. Contains detergent and 0.01% merthiolate as preservative.

Substrate Buffer
Vial No. 3 (25 mL)
Buffer containing hydrogen peroxide.

OPD Tablets
Six tablets of 3.52 mg 1,2-phenylenediamine dihydrochloride corresponding to 2 mg OPD. The concentration of 1,2-phenylenediamine dihydrochloride in each tablet is 2.9%. The tablets are effervescent when dissolving.

Stop Solution
Vial No. 4 (25 mL)
0.5 mol/L H_2SO_4.

Sealing Tape
Two sheets of sealing tape.

B. Reagent preparation and storage

Store kit at 2-8 °C. Expiry of kit and reagents is stated on the label. The Clear Immunostrips should be stored in the tightly-closed foil bag with desiccant until use.

Preparation of Positive Control Antigen: Add 800 µL of Reconstitution Buffer (vial No. 2a) to the lyophilized Positive Control Antigen (vial No. 2). Mix the contents by gentle swirling. The Positive Control Antigen should be reconstituted at least 30 minutes prior to use. Reconstituted Positive Control Antigen should be stored at 2-8 °C and used within three months. The Positive Control Antigen will be sufficient for at least 6 duplicate tests.

C. Materials required but not provided

Deionized or distilled water.

Pipettes to deliver 50 µL, 200 µL and 800 µL.

An adjustable pipette to deliver 1-5 mL.

8-channel pipette(s) to deliver 50 µL and 100 µL.

Reagent reservoirs for 8-channel pipette.

Orbital shaker/shaking table for ELISA plates (optional).

ELISA microwell washer, hand-operated or automatic.

ELISA microwell reader equipped with a 490 ± 2 nm filter. (An additional reference filter between 600 nm and 650 nm is optional).

Generally available laboratory glassware and test tubes.

Precautions

All reagents, test tubes, pipette tips and microwell strips should be handled and disposed of as if they were infectious.

Do not pipette by mouth. The preservative used in the reagents is toxic if ingested. Avoid contact with eyes, respiratory system and skin. The Positive Control Antigen contains 200 ng of native PMT. The LD_{50} of PMT in laboratory animals is approximately 1000 ng/kg body weight when administered parenterally.

Do not use kit components beyond the expiry date stated on the label. Avoid any cross-contamination of samples and kit components as this may give erroneous results. Do not interchange kit components from different lots.

Specimen Collection and Preparation

Suitable porcine microflora can be obtained from several sources, including nasal swabs, nasal washings, tonsillar swabs or from lung or tonsil tissues. Nasal swabs should preferably be obtained from the posterior part of the nasal cavity in live pigs by the use of commercially available swabs, such as cotton or alginate swabs. The swabs should immediately be transferred to a transport medium e.g. saline. The transport time to the laboratory should be minimized as much as practically possible, and the swabs should preferably be kept at low temperatures (approx. 4 °C) during the transport (3).

Primary cultures of nasal swabs are prepared in Petri dishes (diameter 9 cm) on selective blood agar medium containing neomycin sulphate 2 µg/mL and bacitracin 3.5 µg/mL (4). In order to obtain an agar surface covered with numerous colonies, the inoculation can be done by distributing a few drops (50 to 100 µL) of the transport medium over the entire agar surface or by streaking the swab over the entire agar surface.

All colonies on the agar plate should be harvested after overnight incubation at 37 °C. Add 2 mL of deionized or distilled water to each plate and collect bacterial specimens using a Drigalski ("triangular"/"hockey stick") spatula and a pipette. A smaller volume of water should not be used for the specimen preparation, since this could cause antigen overloading.

The microwells of the White Strips are used for preparation of extracts of the harvested bacterial specimens. Determine the number (N) of White Strips needed:

Number of harvested bacterial specimens	1-6	7-14	15-22	23-30	31-38	39-46
Number (N) of White Strips needed	1	2	3	4	5	6

Store surplus of White Strips in the kit box for later use. Place N White Strips in the plastic frame.

Transfer 200 µL of the harvested bacterial specimen to each of two microwells of a White Strip as indicated in Figure 1:

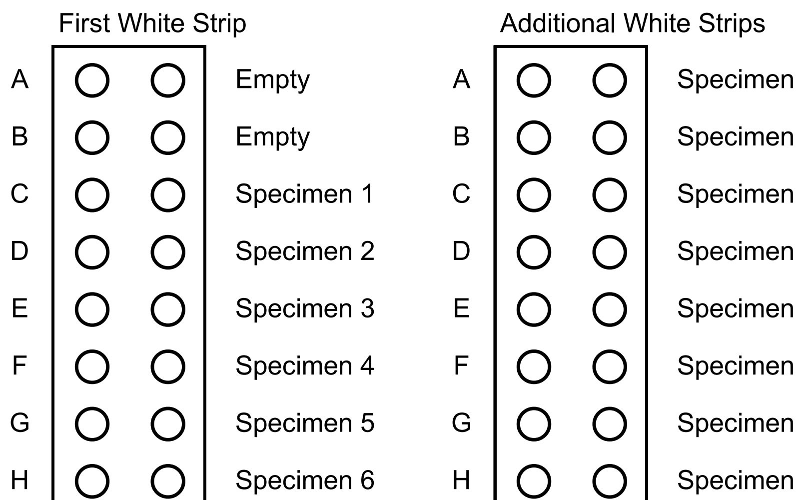

Figure 1. The first White Strip of the run allows extraction of six harvested bacterial specimens in duplicate (the four microwells A1, A2, B1 and B2 are left empty). Each additional White Strip of the run allows extraction of eight harvested bacterial specimens in duplicate.

Cover the microwells of the White Strips in the plastic frame by a lid or Sealing Tape and leave the harvested bacterial specimens for extraction overnight at 37 °C.

Assay Procedure

The number of Clear Immunostrips needed is also N. Place N Clear Immunostrips in the plastic frame. For use in later runs, store surplus Clear Immunostrips in the foil bag with silica-gel capsules. Carefully reseal the bag with adhesive tape and store it at 2-8 °C.

1. Addition of conjugate and specimens

- Pipette N x 1 mL of the Conjugate, Anti-PMT (vial No. 1) into a reagent reservoir. By use of an 8-channel pipette, transfer 50 µL of the conjugate from the reservoir to all microwells of the Clear Immunostrips.
- Add 50 µL of deionized or distilled water to each of the two negative control wells (A1 and A2 of the first Clear Immunostrip). Change tip after each pipetting.
- Add 50 µL of reconstituted Positive Control Antigen to each of the two positive control wells (B1 and B2 of the first Clear Immunostrip). Change tip after each pipetting.
- By use of an 8-channel pipette, transfer 50 µL of bacterial extract from each microwell of the White Strips to the corresponding microwell of the Clear Immunostrips. Change tips N times (once for each strip).
- Cover the Clear Immunostrips by Sealing Tape, and incubate for 1 hour at room temperature (20-25 °C) on an orbital shaker/shaking table. Alternatively, use static incubation for 2 hours.

2. Washing

- Wash the Clear Immunostrips 4 times with deionized or distilled water using a hand-operated or automatic ELISA washer. Make sure that the microwells are filled and emptied completely during each wash-cycle.

3. Preparation of Chromogenic Substrate

Transfer N x 4 mL of Substrate Buffer (vial No. 3) to a test tube and add N x 1 OPD Tablets.

Chromogenic Substrate must be protected from direct sunlight. When dissolving OPD Tablets (approximately 3 minutes) do not seal the container tightly as the tablets are effervescent.

The substrate should remain pale. If it colours increasingly it has been contaminated with peroxidase and it must be discarded.

4. Incubation with Chromogenic Substrate, reading of results

- Transfer the Chromogenic Substrate prepared in step 3 to a reagent reservoir. By use of an 8-channel pipette, transfer 100 µL of Chromogenic Substrate from the reservoir to each microwell at timed intervals. Only N x 1.6 mL of the Chromogenic Substrate will be consumed.
- Incubate in the dark for 10 minutes at room temperature (20-25 °C).
- Transfer N x 4 mL of Stop Solution (vial No. 4) to a reagent reservoir. By use of an 8-channel pipette, add 100 µL of Stop Solution to each microwell using the same, timed intervals as for the Chromogenic Substrate. Only N x 1.6 mL of the Stop Solution will be consumed.
- Read the absorbance (OD) of each microwell at 490 nm. For dual wavelength readers use a reference filter between 600 and 650 nm. Blank on air. Read results within 1 hour after the addition of Stop Solution.

Calculation of Results

1. Negative control

- Calculate $OD_{negative\ control}$, the mean OD-value for the two negative control wells (microwells A1 and A2).

2. Positive control

- Calculate the mean OD-value for the two positive control wells (microwells B1 and B2).

3. Extracts of harvested bacterial specimens

- Calculate the mean OD-value for the two test wells for each bacterial specimen.

4. Quality control

- Check that $OD_{negative\ control}$ is less than 0.150, but greater than 0.000. If the value is above 0.150, inadequate washing or contamination of the Chromogenic Substrate may be the cause. If the value is less than 0.000, the ELISA reader should be reblanked on air and the wells re-read.
- Check that the mean OD-value of the positive control is greater than 0.500. If the value is below 0.500, a too vigorous washing procedure or too low temperature, particularly during incubation with the Chromogenic Substrate, may be the cause.

If the quality control requirements are not satisfied, test results may be invalid and the assay procedure should be repeated.

Interpretation of Results

PMT is present in the extract of a harvested bacterial specimen if both:

- the mean OD of the two test wells is $\geq 0.100 + OD_{negative\ control}$
- the mean OD of the two test wells is $\geq 5 \times OD_{negative\ control}$

If only one of the above criteria is fulfilled, re-test the extract of the harvested bacterial specimen or request a new specimen.

Procedural Notes and Limitations

The PMT ELISA Kit may also be used for the differentiation of toxigenic and non-toxigenic *P. multocida* in pure cultures. When testing pure cultures the extraction step used with extracts of primary mixed cultures can be omitted and the specimen can be tested immediately after being harvested from the agar plate.

By slight modifications of the assay procedure for the PMT ELISA Kit, it is possible to perform:

- very precise quantitative measurements of PMT
- detection of antibodies to PMT in serum or colostrum from animals infected with toxigenic *P. multocida* or vaccinated with vaccines containing PMT or PMT analogues.

Manuals describing these modified assays can be obtained on request from DakoCytomation Denmark A/S.

Performance Characteristics

The PMT ELISA Kit was compared with a conventional tissue culture method (5). The PMT ELISA Kit was used for direct testing of extracts of primary mixed cultures of porcine nasal swabs while specific detection of PMT by the tissue culture method required isolation of a pure subculture of *P. multocida* from the primary culture plate. As shown in Table 1, a much higher overall diagnostic sensitivity was observed when the procedure was based on the PMT ELISA Kit (118 positive swabs compared to 77). The reduced sensitivity of the tissue culture method was caused by the lower overall possibilities of isolating *P. multocida* from primary cultures with heavily microbial overgrowth (at least 17 of 97 cases) and the risk of isolating non-toxigenic *P. multocida* from primary cultures of nasal swabs containing both non-toxigenic and toxigenic *P. multocida* (24 of 76 cases). PMT was always detected by the PMT ELISA Kit when the tissue culture method was positive for toxigenic *P. multocida* (77 cases).

Table 1. Comparison of the PMT ELISA Kit and a tissue culture method.

PMT ELISA Kit	Tissue Culture Method			
	P. multocida not isolated	*P. multocida* isolated		Total
		Toxigenic	Non-toxigenic	
Presence of PMT	17	77	24	118
Absence of PMT	80	0	52	132
Total	97	77	76	250

It was concluded that abundance of non-toxigenic *P. multocida* colonies and/or heavy microbial overgrowth in the primary culture will significantly reduce the possibility of isolating toxigenic *P. multocida* colonies. With the PMT ELISA Kit where all colonies of a primary culture are harvested and tested the risk of overlooking toxigenic *P. multocida* colonies does not exist.

References

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3. Chanter N, Goodwin RFW, Rutter JM. Comparison of methods for the sampling and isolation of toxigenic *Pasteurella multocida* from the nasal cavity of pigs. Res Vet Sci 1989;47:355-8.

4. Barfod K, Pedersen KB. Influence of vaccination of sows with Bordetella-Pasteurella vaccines on the occurrence of atrophic rhinitis among their offspring after experimental infection with Bordetella bronchiseptica and toxigenic Pasteurella multocida. Nord Vet-Med 1984;36:337-45.

5. Rutter JM, Luther PD. Cell culture assay for toxigenic *Pasteurella multocida* from atrophic rhinitis in pigs. Vet Rec 1984;114:393-6.

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