DakoCytomation

Accessory Reagents for PMT Serology

Code No. K 0038

3rd edition

For the determination of antibodies to *Pasteurella multocida* toxin (PMT) in serum or colostrum from swine infected with toxigenic *P. multocida* or vaccinated with vaccines containing PMT or PMT analogs.

Accessory Reagents for PMT Serology (K 0038) must be used together with the DakoCytomation Pasteurella Multocida Toxin ELISA Kit (K 0009). The product insert for K 0038 describes the modifications required for making K 0009 suitable for antibody determination.

For veterinary use only.

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Intended Use

The present manual describes a modification of the DakoCytomation Pasteurella Multocida Toxin ELISA Kit (code No. K 0009), which can be used in order to detect antibodies to the *Pasteurella multocida* toxin (PMT) in serum or colostrum specimens from swine infected with toxigenic *P. multocida* or vaccinated with vaccines containing PMT or PMT analogs. The serological analysis can be performed either as a qualitative test in screening or as a quantitative test in titre determination.

This manual is a supplement to the printed Working Procedure enclosed with the DakoCytomation Pasteurella Multocida Toxin ELISA Kit and therefore must be read with the Working Procedure alongside.

Summary and Explanation

The primary purpose of the DakoCytomation Pasteurella Multocida Toxin ELISA Kit is to demonstrate toxigenic strains of *P multocida* in bacterial specimens obtained from swine since this is the essential criterion for the laboratory diagnosis of progressive atrophic rhinitis (see refs. 1 and 2). In contrast to this direct demonstration of the etiological agent, some laboratory situations require a method for determination of antibodies to PMT (see ref. 3). This is especially the case in health surveillance programs based on screening of serum or colostrum specimens for antibodies to PMT. Furthermore, serological analysis is important when monitoring efficacy of vaccines for progressive atrophic rhinitis by determination of anti-PMT titres.

Similar to the simple one-step ELISA procedure for detection of PMT described in the Working Procedure of the DakoCytomation Pasteurella Multocida Toxin ELISA Kit, the procedure for detection of anti-PMT antibodies is very simple and fast. The procedure is independent of the source of antibodies and therefore can be used for detection of anti-PMT antibodies in serum or colostrum specimens from any animal species. The present manual describes how to perform this modified assay.

Principle of the Assay

The detection of anti-PMT antibodies is performed by using reagents supplied with the DakoCytomation Pasteurella Multocida Toxin ELISA Kit. However, some surplus reagents are needed. The serological assay depends on addition of a fixed amount of PMT to all microwells of the ELISA plate and therefore more PMT than supplied as Positive Control Antigen (vial 2) in the DakoCytomation Pasteurella Multocida Toxin ELISA Kit is required. DakoCytomation Accessory Reagents for PMT Serology (code No. K 0038) includes the “extra” lyophilized PMT, reconstitution buffer, as well as positive and negative control serum needed for utilizing an entire DakoCytomation Pasteurella Multocida Toxin ELISA Kit (96 test wells) for serology.

Each serum or colostrum specimen to be analyzed is preincubated with a fixed amount of PMT. After this preincubation, the mixture is transferred to microwells precoated with mouse monoclonal antibody to PMT where it is incubated simultaneously with peroxidase-conjugated Fab’ fragment of rabbit antibody to PMT. After the ELISA incubation step the wells are washed, and chromogenic substrate is added. After stopping the enzymatic reaction, the color intensity is read photometrically, and the results for specimens and controls are determined by comparison to the absorbance (OD) obtained for buffer controls.

Reagents

For a description of the materials provided in the DakoCytomation Pasteurella Multocida Toxin ELISA Kit and their preparation and storage, please see items A and B, respectively, of the printed Working Procedure of the DakoCytomation Pasteurella Multocida Toxin ELISA Kit.

The DakoCytomation Accessory Reagents for PMT Serology are:

- **"Extra" PMT**
  Vials 5 (2 vials of each 500 ng PMT)
  Affinity-isolated, native, lyophilized PMT. Contains carrier protein and 20 µg merthiolate as preservative.
- **"Extra" Reconstitution Buffer**
  Vials 5A (2 vials of each 3 mL)
  For reconstitution of "Extra" PMT. Buffer-containing protein, detergent, and 0.05% merthiolate as preservative. Colored blue for identification.
- **Positive Control Serum**
  Vial 6 (0.4 mL)
  A positive anti-PMT reference serum. Contains 15 mmol/L sodium azide as preservative.
- **Negative Control Serum**
  Vial 7 (0.4 mL)
  A negative reference serum. Contains 15 mmol/L sodium azide as preservative.

The DakoCytomation Accessory Reagents for PMT Serology should be stored at 2-8 °C. Expiry of reagents is stated on the label on the outside of the K 0038 box.

Preparation of "Extra" PMT: Add 2.5 mL of Reconstitution Buffer (vial 5A) to the lyophilized "Extra" PMT (vial 5). Mix the contents by gentle swirling. The "Extra" PMT should be reconstituted at least 30 minutes prior to use. Reconstituted PMT should be stored at 2-8 °C and used within three months. One vial of reconstituted "Extra" PMT will be sufficient for serological testing in 48 microwells (corresponding to 3 Clear Immunostrips of the DakoCytomation Pasteurella Multocida Toxin ELISA Kit). Thus the 2 vials "Extra" PMT (vial 5) and the 2 vials "Extra" Reconstitution Buffer (vial 5A) are sufficient for one DakoCytomation Pasteurella Multocida Toxin ELISA Kit.

Precautions

Please see the printed Working Procedure of the DakoCytomation Pasteurella Multocida Toxin ELISA Kit. Especially, please note that the DakoCytomation Accessory Reagents for PMT Serology contains a total of 1 µg of native PMT.

Specimen Preparation

Serum and colostrum specimens do not need any special treatment before being analyzed by the DakoCytomation Pasteurella Multocida Toxin ELISA Kit.

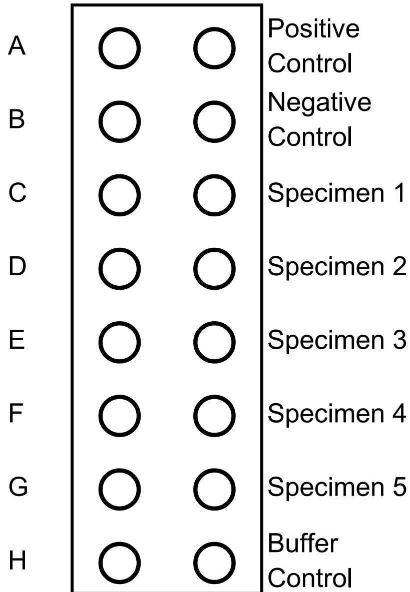
For screening of sera (or colostra), testing in only one dilution is recommended. For determination of titres, testing of several dilutions is required.

For Screening:

The microwells of the White Strips are used for preincubation of serum sample and PMT. Determine the number (N) of White Strips needed:

Number of sera (or colostra)	1-5	6-12	13-19	20-26	27-30	34-40
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.



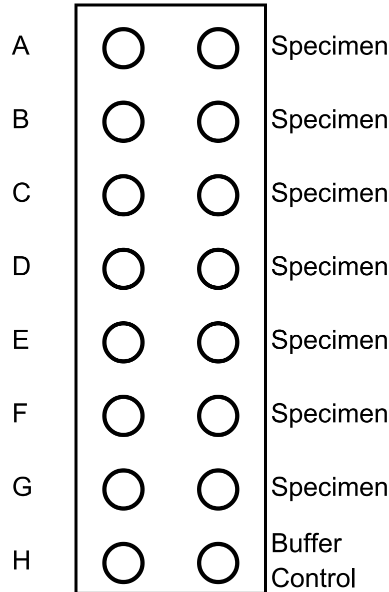


Figure 1. Transfer 30 µL of H_2O to all microwells in rows A to G of the White Strip(s). Transfer 60 µL of H_2O to the microwells in row H of the White Strip(s). To each of the two microwells A1 and A2 is added 30 µL of the Positive Control Serum (vial 6). To each of the two microwells B1 and B2 is added 30 µL of the Negative Control Serum (vial 7). For each of the specimens to be tested 30 µL is transferred to each of the two wells as indicated on the figure. Do not add specimens in row H. All wells of the White Strip now contain 60 µL of sample. Finally, by use of an 8-channel pipette add 40 µL of the reconstituted "Extra" PMT to all microwells of the White Strip(s). Beware of contaminations from one well to another during this step. Incubate the specimen mixtures (100 µL) for 30 minutes at 37 °C in the microwells of the White Strips covered by Sealing Tape.

The set-up in Figure 1 is just an example. However, the set-up must assure that the sample volume in all wells before addition of PMT is 60 µL and that the volume of PMT added to all wells is 40 µL/well. Furthermore, Buffer Control wells must always be included in the analysis.

For Determination of Titres:

The row of dilutions for each specimen is prepared in the microwells of the White Strips enclosed with the DakoCytomation Pasteurella Multocida Toxin ELISA Kit. The number of dilutions tested for each specimen and the factor of dilution may vary for different situations. In the following example, the specimen preparation is shown for testing of 6 different specimens in five 3-fold dilutions. When testing each dilution in a single determination, the number (N) of White Strips needed is 3 corresponding to 48 microwells:

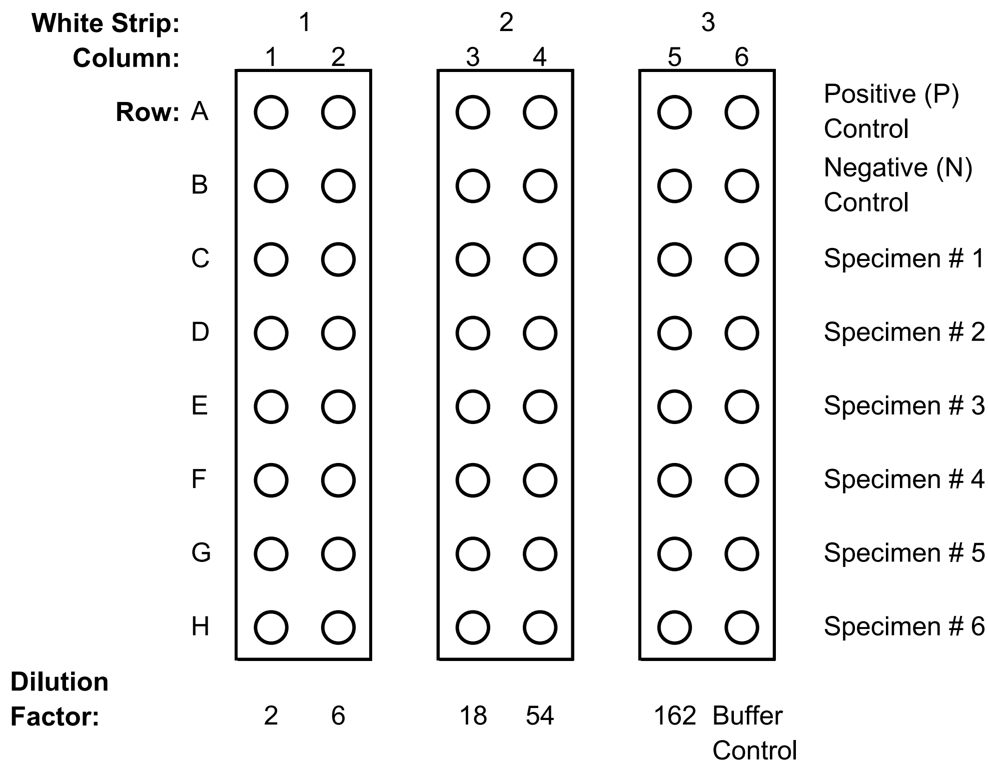


Figure 2. Set-up: 45 µL of H_2O is added to the 8 White Strip wells in column 1 (A1 to H1) and 60 µL of H_2O is added to the remaining 40 White Strip wells in column 2 to 6. To microwell A1 is added 45 µL of the Positive Control Serum (vial 6) and to the microwell B1 is added 45 µL of the Negative Control Serum (vial 7). For each of the Specimens #1 to #6, 45 µL is transferred to wells C1 to H1, respectively. By using an 8-channel pipette, the 3-fold dilution rows are prepared simultaneously for the 2 controls and the 6 specimens in rows A to H by transferring 30 µL from column 1 to column 2 and so on until column 5. For each step, the tips must be carefully emptied and the solution in the wells homogenized by use of the pipette before transfer of 30 µL to the next column. Remember to remove 30 µL from each microwell in column 5. These 8 x 30 µL are waste, do not transfer them to column 6. All wells of the White Strip now contain 60 µL of sample. Finally, by use of the 8-channel pipette add 40 µL of the reconstituted “Extra” PMT to all 48 microwells of the White Strips. Beware of contaminations from one well to another during this step. Incubate the specimen mixtures (100 µL) for 30 minutes at 37 °C in the microwells of the White Strips covered by Sealing Tape.

The set-up in Figure 2 is meant as an example. Other combinations of dilution factor, length of row of dilutions, and number of specimens may be used. However, the set-up must assure that the sample volume in all wells before addition of PMT is 60 µL, and the volume of PMT added to all wells is 40 µL/well. Furthermore, Buffer Control wells must always be included in the analysis.

Assay Procedure

The number of Clear Immunostrips needed is identical to the number (N) of White Strips used for specimen preparation. 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 specimen mixtures

- Pipette N x 1 mL of the Conjugate, Anti-PMT (vial 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.

- Transfer 50 µL of specimen mixture from each microwell of the White Strips to the corresponding microwell of the Clear Immunostrips. The transfer should be done within 30 minutes after completion of the pre-incubation of specimen mixtures at 37 °C.

  For the set-up described in Figure 1: Change tips N times (once for each strip). For the set-up described in Figure 2, the transfer can be done in a very simple way. An 8-channel pipette is used for simultaneous transfer of 50 µL of each sample in Column 6 of the White Strip to the corresponding wells in Column 6 of the Clear Immunostrip. Without changing the 8 tips of the pipette, Column 5 subsequently can be transferred from the White Strip to the Clear Immunostrip and so on until Column 1 has been transferred.

  Beware of contaminations from one well to another if retaining the tips of the pipette when using another set-up.

- Cover the Clear Immunostrips by Sealing Tape and incubate for <u>45</u> minutes at room temperature (20-25 °C) on an orbital shaker/shaking table.

2. Washing

- Wash the Clear Immunostrips 4 times with deionized or distilled water using a hand-operated or an 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 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 colors 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 <u>15</u> minutes at room temperature (20-25 °C).
- Transfer N x 4 mL of Stop Solution (vial 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 addition of Stop Solution.

Calculation of Screening Results

1. Positive control

- Calculate $OD_{positive\ control}$, the mean OD-value for the positive control wells. (For the set-up in Figure 1: Microwells A1 and A2).

2. Negative control

- Calculate $OD_{negative\ control}$, the mean OD-value for the negative control wells. (For the set-up in Figure 1: Microwells B1 and B2).

3. Buffer control

- Calculate $OD_{buffer\ control}$, the mean OD-value for the negative control wells. (For the set-up in Figure 1: All microwells of the White Strip(s) in row H). The ODcut-off = 0.5 x ODbuffer control.

4. Specimens

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

5. Quality control

- Check that $OD_{buffer\ control}$ is higher than 0.600. The expected value is approx. 1.400.
- Check that $OD_{positive\ control}$ is less than 25% of $OD_{buffer\ control}$. The expected value is approx. 5% of $OD_{buffer\ control}$.
- Check that $OD_{negative\ control}$ is higher than 65% of $OD_{buffer\ control}$. The expected value is approx. 90% of $OD_{buffer\ control}$.

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

Interpretation of Screening Results

All serum and colostrum specimens resulting in a mean OD-value below the $OD_{cut-off}$ are positive.

Specimens resulting in a mean OD-value above 65% of the $OD_{buffer\ control}$ are negative.

Specimens resulting in a mean OD-value between the $OD_{cut-off}$ and 65% of the $OD_{buffer\ control}$ are inconclusive. Re-testing in several dilutions is recommended.

Calculation of Results of Titre Determination

1. Buffer Control

- Calculate $OD_{buffer\ control}$, the mean OD-value for the negative control wells. (For the set-up in Figure 2: All microwells of the White Strip(s) in column 6). $OD_{cut-off} = 0.5 \times OD_{buffer\ control}$.

2. Quality control

- Check that $OD_{buffer\ control}$ is higher than 0.600. The expected value is approx. 1.400.
- Check that OD of the Positive Control Serum diluted 1:2 (for the set-up in Figure 2: Microwell A1) is less than 25% of $OD_{buffer\ control}$. The expected value is approx. 5% of $OD_{buffer\ control}$.
- Check that OD of the Negative Control Serum diluted 1:2 (for the set-up in Figure 2: microwell B1) is higher than 65% of $OD_{buffer\ control}$. The expected value is approx. 90% of $OD_{buffer\ control}$.

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

Interpretation of Results of Titre Determination

There are several methods which can be used in order to estimate the titre of serum and colostrum specimens. This manual describes an estimation method which can be performed on a relatively simple calculator and is based on only two selected OD-values from the row of dilutions. Other methods performed on computers or by graphic presentation may be based on more than two OD-values from the row of dilutions, and an estimate might also be based on just a single OD-value.

The following method is used for each specimen (please also see Example 1 below):

- If possible, locate the two neighbor wells of the row of dilutions giving a lower and a higher OD-value than $OD_{cut\text{-}off}$, respectively.
- If this is not possible because the OD-value of the first well in the row of dilutions has an OD-value higher than the $OD_{cut\text{-}off}$, the corresponding specimen is either seronegative (> 0.65 x $OD_{buffer\ control}$) or inconclusive (between the $OD_{cut\text{-}off}$ and 0.65 x $OD_{buffer\ control}$). In both cases the titre is considered “not determinable” (ND).
- If this is not possible because the OD-value of the last well in the row of dilutions has an OD-value less than the $OD_{cut\text{-}off}$, the specimen must be further diluted before retesting in order to estimate its titre.

Example 1:

The OD-values were obtained by using the set-up described in Figure 2 for the determination of anti-PMT titres in 6 different specimens. Each specimen (#1 to #6) was tested by a row of dilution using five 3-fold dilutions (row C to H):

	1	2	3	4	5	6
A	0.042	0.057	0.376	1.042	1.359	1.465
B	1.291	1.370	1.416	1.440	1.438	1.487
C	0.032	0.030	0.033	0.049	0.084	1.478
D	1.422	1.533	1.502	1.499	1.506	1.502
E	0.034	0.091	*0.412*	*1.180*	1.337	1.514
F	0.035	0.032	0.035	0.111	*0.839*	1.526
G	0.074	*0.256*	*0.970*	1.302	1.476	1.482
H	*0.185*	*0.934*	1.378	1.435	1.487	1.453
Dilution Factor	2	6	18	54	162	Buffer Control

The $OD_{buffer\ control}$ is calculated as the mean OD-value of the 8 microwells in column 6: 1.488. The 50% cut-off value is then: $OD_{cut\text{-}off}$ = 0.5 x $OD_{buffer\ control}$ = 0.744.

For the positive control serum (row A) and each specimen (row C-H) the two neighbor wells of the row of dilutions giving a lower and a higher OD-value, respectively, than the $OD_{cut\text{-}off}$ (0.744) are located when possible. In the table above the values of OD_{low} (left) and OD_{high} (right) are shown in *italics*.

Specimen	OD_{low}/dilution factor	OD_{high}/dilution factor	Titre *)
Positive Control, #	0.376 (18)	1.042 (54)	33
1 (C)	Too high titre	Too high titre	Above 162
2 (D)	Sero-negative	Sero-negative	ND
3 (E)	0.412 (18)	1.180 (54)	29
4 (F)	0.111 (54)	0.839 (162)	140
5 (G)	0.256 (6)	0.970 (18)	13
6 (H)	0.185 (2)	0.934 (6)	4.5

*) The titre (T) of a specimen is the estimated dilution factor corresponding to an OD-value at the level of $OD_{cut\text{-}off}$. T is calculated by using the OD_{low} and the OD_{high} and their corresponding dilution factors (F_{low} and F_{high}, respectively) in this expression:

$$\log T = \frac{OD_{high} - OD_{cut-off}}{OD_{high} - OD_{low}} \text{ x } \log \frac{F_{low}}{F_{high}} + \log F_{high}$$

For Specimen #3 (row E):

$$\log T = \frac{1.180 - 0.744}{1.180 - 0.412} \text{ x } \log \frac{18}{54} + \log 54 \rightarrow T = 29$$

Procedural Notes and Limitations

- For optimal results, the simultaneous incubation of conjugate and specimen mixture should be done on an orbital shaker/shaking table.
- The range of titres which can be estimated in a set-up will depend on several parameters:
  - the number of dilutions tested for each specimen
  - the size of the fold of dilution

  By using a low fold of dilution (e.g. 2-fold dilutions) the precision of the determination is increased, but a higher number of dilutions must be tested in order to assure the determination of a wide range of titres. Due to lack of precision, it is not recommended to use more than 5-fold dilutions.
- If the specimens are expected to include a wide range of titres, it might be useful to pre-dilute specimens with very high concentrations of anti-PMT antibodies in order to reduce the number of dilutions needed for the test.

References

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2. Mattsson S, Foged NT, Soderlind O. Evaluation of the 1-step DAKO PMT ELISA for detection of toxin-producing *Pasteurella multocida*. Proceedings International Pig Veterinary Society Congress 1992:173.
3. Foged NT. *Pasteurella multocida* toxin. The characterisation of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. APMIS 1992;100 Suppl 25:1-56.

Technical Advice and Customer Service

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