INTRODUCTION

Pig Major Acute-Phase Protein (MAP), serum levels increase significantly during inflammatory conditions (ref 1). In studies at Life Diagnostics, we found levels ranging from approximately 1 mg/ml in healthy pigs to >10 mg/ml in pigs with bacterial infections.

PRINCIPLE OF THE ASSAY

The assay uses two peptide-specific MAP antibodies developed at Life Diagnostics. One is used as coating antibody. The other is conjugated to HRP and used for detection. Standards and diluted samples (100 µl) are incubated in anti-MAP coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If MAP molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If MAP is present, a blue color develops. Color development is stopped after 20-minutes by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of MAP is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-MAP coated plate (12 x 8-well strips)
- Anti-MAP HRP stock
- MAP stock, 1 vial. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the standard stock vial at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl aliquots to the wells of the antibody-coated plate.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

WASH SOLUTION

The Wash Solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DILUENT

The Diluent (YD50-1) is formulated for measurement of MAP in serum. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of pig MAP in a stabilizing matrix. Reconstitute it with deionized water as described on the vial label and gently mix. Prepare the 500 ng/ml standard as described on the label.
2. Label seven polypropylene tubes as 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.91 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 500 ng/ml MAP standard into the tube labeled 250 ng/ml and mix. This provides the 250 ng/ml MAP standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

IMPORTANT – If future use of the stock is intended, the sealed vial must be frozen at or below -20°C within 30 minutes of reconstitution.

HRP CONJUGATE

The HRP conjugate is provided as a concentrated stock. Dilute the stock with YD50-1 diluent as described on the vial label right before use.

[1] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

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SAMPLES

The assay is intended for measurement of MAP in pig serum or plasma. In serum from sick pigs, we found MAP levels ranging from 0.5 to 7 mg/ml. A dilution of 20,000-fold in YD50-1 worked well for most samples, but optimal dilutions should be determined by the end user. Diluent YD50-1 must be used for sample dilution. A 20,000-fold dilution can be obtained as follows.

1. Dispense 0.198 ml and 0.398 ml of YD50-1 diluent into separate microcentrifuge tubes.
2. Dilute 2.0 µl of serum or plasma into the tube containing 0.198 ml of diluent and mix. This provides a 100-fold dilution.
3. Dilute 2.0 µl of the 100-fold diluted sample into the tube containing 0.398 ml of diluent and mix. This provides a 20,000-fold dilution.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 µl of standards and samples into appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
5. Dispense 100 µl of diluted HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
8. If necessary, strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 µl of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20 minutes stop the reaction by adding 100 µl of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

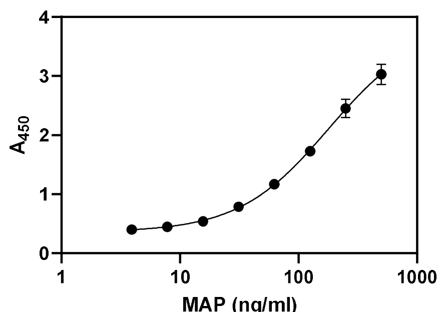
RESULTS

1. Using curve fitting software, graph the absorbance values of the standards on the Y-axis versus $\log_{10}$ of MAP concentration on the X-axis.
2. Fit the curve to a sigmoidal four parameter logistic equation and derive the concentration of MAP in the diluted samples.
3. Multiply derived values by the dilution factor(s) to determine MAP concentration in the original sample.
4. If the absorbance values of diluted samples fall outside the standard curve, samples should be further diluted and re-tested.

TYPICAL STANDARD CURVE

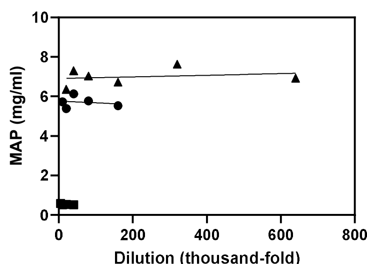
A typical standard curve is shown below. This curve is for illustration only.

MAP (ng/ml)	A_{450}
500	3.027
250	2.453
125	1.729
62.5	1.167
31.25	0.789
15.63	0.542
7.81	0.447
3.91	0.403



PERFORMANCE

Linearity: To assess the linearity of the assay, serum samples with MAP concentrations of 0.53, 5.7 and 7.0 mg/ml diluted with YD50-1 to give values within range of the assay.



REFERENCES

1. Heegard PMH. et al. Optimal combinations of acute phase proteins for detecting infectious disease in pigs. Veterinary Research 2011, 42:50

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For technical assistance please email us: techsupport@lifediagnostics.com

[2] If absorbance of the high standard is ≥4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.