PIG CK-MM ELISA KIT
Life Diagnostics, Inc., Catalog Number: CKMM-9

ELISA for the Determination of Pig Creatine Kinase MM (CK-MM) in Serum or Plasma

INTRODUCTION

Creatine kinase (CK) is a key metabolic enzyme. It is a dimer of two subunits, each with molecular weights of approximately 43 kDa. Two different subunits occur, M and B. The CK holoenzyme therefore exists as MM and BB homodimers and an MB heterodimer. The MM and BB isoforms are expressed primarily in skeletal muscle and brain respectively while both MB and MM are expressed in heart. The relatively high expression levels of the MB isoform in heart explains its use as an established biomarker for heart disease. Likewise, CK-MM can be used as a specific biomarker for skeletal muscle injury. Studies in pigs have demonstrated that serum CK-MM levels can increase 50-fold or more as a result of skeletal muscle injury.[1]

PRINCIPLE OF THE TEST

The pig CK-MM test kit is a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified CK-MM antibodies for solid phase immobilization and a horseradish peroxidase (HRP) conjugated CK-MM monoclonal antibody for detection. Standards and diluted test samples are incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed, and HRP conjugate is added and incubated for 45 minutes. As a result, CK-MM molecules are sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies, and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of CK-MM is proportional to the optical density of the test sample and actual concentrations are determined by reference to a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-Pig CK-MM Antibody Coated Microtiter Plate with 96 wells (provided as 12 detachable strips of 8)
- 2x HRP Conjugate, 6 ml
- Reference Pig CK-MM Stock (lyophilized)
- 20x Wash Buffer: TBS50-20, 50 ml
- Diluent: YD50-1, 50 ml
- TMB Reagent: TMB11-1, 11 ml
- Stop Solution (1N HCl): SS11-1, 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-plate incubator/shaker with mixing speed of 150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional)

STORAGE

The kit should be stored at 4°C, and the microtiter strips should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described.

GENERAL INSTRUCTIONS

1. Please read the entire kit insert before starting the ELISA.
2. All reagents should be allowed to reach room temperature (25°C) before use.

WASH SOLUTION PREPARATION

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.

HRP CONJUGATE PREPARATION

The HRP conjugate is provided as a 2x stock. Prior to use, estimate the final volume of HRP conjugate required for your assay and dilute one (1) volume of the 2x stock with one (1) volume of diluent. Typically, we prepare 1 ml of conjugate for each 8-well strip used in the assay. This is prepared by mixing 0.5 ml of 2x HRP conjugate with 0.5 ml of diluent. Mix gently prior to use. Prepare the working conjugate no more than one hour in advance.

STANDARD PREPARATION

1. The reference CK-MM stock is provided in lyophilized form. Reconstitute with diluent as directed on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable for 1 day at 4°C)***.
2. Label 5 polypropylene or glass tubes as 50, 25, 12.5, 6.25 and 3.13 ng/ml.
3. Prepare the 50 ng/ml standard as described on the reference stock vial label.
4. Dispense 250 µl of diluent into the tubes labeled 25, 12.5, 6.25 and 3.13 ng/ml.
5. Pipette 250 µl of the 50 ng/ml CK-MM standard into the tube labeled 25 ng/ml and mix. This provides the working 25 ng/ml CK-MM standard.
6. Similarly prepare the 12.5, 6.25 and 3.13 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Samples must be diluted at least 10-fold with the provided diluent before use. Sample dilutions $\geq$ 10-fold eliminate interference caused by serum/plasma components. In validation studies we observed baseline plasma CK-MM levels of approximately 200 ng/ml. However, CK-MM levels may vary depending on experimental conditions, and optimum sample dilutions should be determined empirically. Assuming that a dilution of 10-fold is used, samples should be treated as follows:

1. Pipette and mix 25 µl of the serum/plasma sample with 225 µl of diluent in a polypropylene micro centrifuge tube. This provides a 10-fold diluted sample in sufficient volume for duplicate determinations.
2. Repeat this procedure for each sample to be tested.

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ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (25°C) for 45 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This should be performed using a plate washer (400 µl/well). If a plate washer is not available, use a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 µl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (25°C) for 45 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 µl of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (25°C) for 20 minutes.
13. Stop the reaction by adding 100 µl of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 5 minutes*.

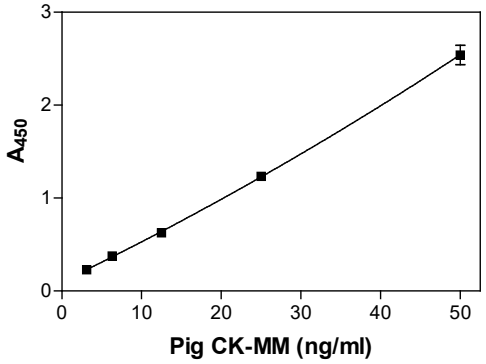
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y axis and concentrations on the horizontal or X axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of CK-MM in ng/ml from the standard curve.
4. Multiply the derived concentration by the dilution factor to determine the actual concentration of CK-MM in the serum/plasma sample.
5. PC graphing software may be used for the above steps. A linear fit of the data usually works well. However, if there is a slight curvature of the standard curve, fitting to a polynomial second order equation is advised.
6. If the A_{450} values of samples exceed that of the high standard the samples should be further diluted and re-tested. Samples with absorbance values below the lowest standard should be assigned a value of < 3.13 x A ng/ml, where A represents the dilution factor used for the sample.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against CK-MM concentrations on the X axis is shown below. A standard curve should be run for each experiment.

CK-MM (ng/ml)	Absorbance (450 nm)
50	2.540
25	1.234
12.5	0.626
6.25	0.375
3.13	0.229



LIMITATIONS OF THE PROCEDURE

1. Do not use samples at a dilution less than 10-fold (i.e., do not use a dilution of 5-fold). High serum content of the samples can interfere with the ELISA.
2. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

SPECIFICITY

Pig CK-BB and pig CK-MB were not available when this kit was developed, and specificity was therefore investigated using purified human CK-MM, CK-MB, and CK-BB. Human CK-MM was strongly recognized, but CK-BB was not recognized at concentrations up to 10 micrograms/ml. The ELISA was approximately 400-fold more selective for CK-MM compared to CK-MB.

REFERENCES

1. K Thoren-Tolling and L Jonsson. Creatine Kinase Isoenzymes in Serum of Pigs Having Myocardial and Skeletal Muscle Necrosis. Can J Comp Med. 47:207-216 (1983)

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For technical assistance please email us at
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