K-ASSAY®

KAMIYA BIOMEDICAL COMPANY

Pig High-Sensitive CRP ELISA

For the quantitative determination of C-reactive protein in pig biological samples

Cat. No. KT-184

For research use only.

PRODUCT INFORMATION
Pig High-Sensitive CRP ELISA
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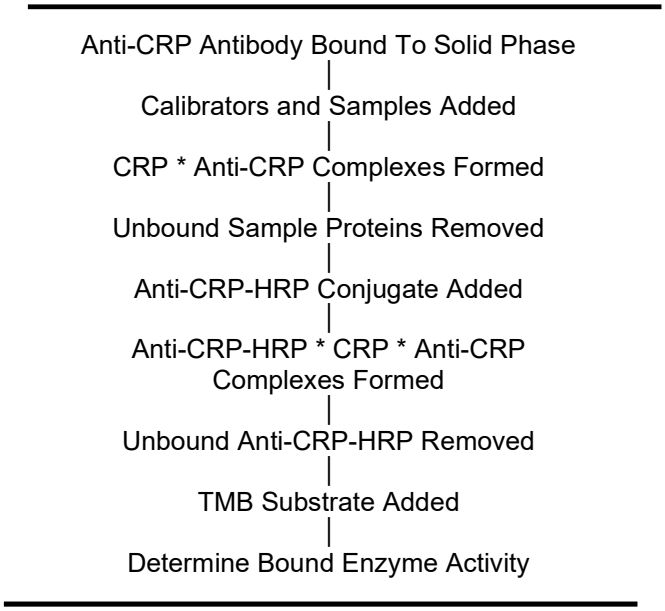
INTENDED USE
The Pig High-Sensitive CRP ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of C-reactive protein (CRP) in pig biological samples. For research use only.

INTRODUCTION
Acute phase proteins are plasma proteins which increase in concentration following infection, inflammation or trauma. The first acute phase protein to be recognized was discovered in humans by Tillet and Frances in 1930. This CRP is so named because it is able to effect precipitation of somatic C-polysaccharide of *Streptococcus pneumonia.* CRP is an alpha globulin protein with a mass of 110,000 to 140,000 daltons, and composed of five identical subunits, which are non-covalently assembled as a cyclic pentamer. It is synthesized in the liver and, in humans, is normally present as a trace constituent of serum at a level less than 0.3 mg/dL. The CRP levels in serum rise quickly following acute tissue damage and it also falls very rapidly once the stimulus is removed. It has been proposed that CRP aids in complement activation, influences phagocytic cell function, and augments cell-mediated cytotoxicity. Investigations over the past few years have shown that quantification of CRP in plasma or serum can provide valuable information in the detection, prognosis, and monitoring of disease not only in humans, but in companion animals and farm herds as well.

PRINCIPLE
The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the c-reactive protein (CRP) present in the sample reacts with the anti-CRP antibodies, which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-CRP antibodies conjugated with horseradish peroxidase (HRP) is added. These enzyme-labeled antibodies form complexes with the previously bound CRP. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme is proportional to the concentration of CRP in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of CRP in the test sample. The quantity of CRP in the test sample can be interpolated from the calibration curve constructed from the calibrators and corrected for sample dilution.

Figure 1.



LIMITATION OF THE PROCEDURE

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC PURPOSES. IN VITRO USE ONLY.

Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

COMPONENTS

1. **Diluent Concentrate**
   One bottle containing 60 mL of a 1X concentrated diluent buffer.
2. **Wash Solution Concentrate**
   One bottle containing 50 mL of a 20X concentrated wash solution.
3. **Enzyme-Antibody Conjugate Concentrate**
   One vial containing 150 µL of a 100X concentrated affinity-purified anti-CRP antibody conjugated with HRP in a stabilizing buffer.
4. **TMB Substrate Solution**
   One bottle containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.
5. **Stop Solution**
   One bottle containing 12 mL of 0.3 M sulfuric acid.
   WARNING: Avoid contact with skin.
6. **Microtiter Plate**
   Twelve removable eight-well micro strips in a well holder frame. Wells are coated with affinity-purified anti-CRP
7. **Pig CRP Calibrator**
   One vial containing a lyophilized Pig CRP Calibrator.
8. **Positive Control**
   One vial containing of 50 µL serum with 0.1% sodium azide. See the Control Certificate for the concentration.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Squirt bottle or Microplate washer/aspirator
- Distilled or deionized H_2O
- Microplate reader
- Assorted glassware for the preparation of reagents and solutions
- Timer
- Centrifuge for sample collection
- Anticoagulant for plasma collection

STORAGE AND STABILITY

1. **Complete Kit**
   The expiration date for the kit is stated on the outer label. The recommended storage temperature is 4°C. All components should be stable up to the expiration date if stored and used per this kit protocol insert. **Note: See long-term storage recommendations below for the Pig CRP Calibrator and Positive Control.**
2. **Diluent**
   The 1X Diluent is stable until the expiration date.
3. **Wash Solution**
   The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4°C.
4. **Enzyme-Antibody Conjugate**
   Undiluted anti-CRP-HRP conjugate should be stored at 4°C in the dark (protect from light) and diluted immediately prior to use. The undiluted conjugate solution is stable until the expiration date.
5. **TMB Substrate Solution**
   The TMB Substrate Solution should be stored at 4°C in the dark (protect from light) and is stable until the expiration date.
6. **Stop Solution**
   The Stop Solution should be stored at 4°C and is stable until the expiration date
7. **Microtiter Plate**
   Anti-pig CRP coated wells are stable until the expiration date and should be stored at 4°C in the sealed foil pouch with a desiccant pack.
8. **Pig CRP Calibrator**
   The **lyophilized** Pig CRP Calibrator should be stored at 4°C or frozen until reconstituted. The reconstituted calibrator should be aliquoted and stored frozen. Avoid multiple freeze/thaw cycles. **The working calibrator solutions should be prepared immediately prior to use.**
9. **Positive Control**
   For storage longer than 7 days, keep frozen until the expiration date. Storage for less than 7 days can be at 4°C. Avoid multiple freeze/thaw cycles.

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.
4. Preservatives
   - Positive Control contains 0.1% sodium azide.
5. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
6. Other precautions:
   - Do not interchange kit components from different lots.
   - Do not use kit components beyond the expiration date.
   - Protect reagents from direct sunlight.

- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
- Avoid all contact with the reagents by using gloves.
- Stop solution contains diluted sulfuric acid. Irritation to the eyes and skin is possible. Flush with water after contact.

SPECIMEN COLLECTION AND HANDLING

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing.

If blood samples are clotted, grossly hemolyzed, lipemic, or the integrity of the sample is of concern, make a note and interpret results with caution.

The sample collection and storage condition listed below are intended as general guidelines. Sample stability has not been evaluated.

- **Serum samples** - Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation. Remove serum and assay immediately or aliquot and store samples at -80˚C (preferably) or -20˚C. Avoid multiple freeze-thaw cycles
- **Plasma samples** - Blood should be collected into a container with an anticoagulant and then centrifuged. Assay immediately or aliquot and store samples at -80°C (preferably) or -20˚C. Avoid multiple freeze-thaw cycles.
- **Urine samples** - Collect mid-stream using a sterile or clean urine collector. Centrifuge to remove cell debris. Assay immediately or aliquot and store samples at -80°C (preferably) or -20˚C. Avoid multiple freeze-thaw cycles.
- **Known interfering substances** - Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.

ASSAY PROTOCOL

Dilution of Samples

The assay for quantification of CRP in samples requires that each test sample be diluted before use. All samples should be assayed in **duplicate** each time the assay is performed. The recommended dilutions are only suggestions. Dilutions should be based on the expected concentration of the unknown samples such that the diluted sample falls within the dynamic range of the calibration curve. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**

- **Serum and Plasma samples** – The recommended starting dilution is 1:2,000. To prepare a 1:2,000 dilution of a sample, transfer 5 µL of sample to 495 µL of 1X diluent. This gives you a 1:100 dilution. Next, dilute the 1:100 by transferring 20 µL into 380 µL of 1X diluent. You now have a 1:2,000 dilution of your sample. Mix thoroughly at each stage.

Reagent Preparation

1. **Bring all reagents to room temperature (RT) before use.**
2. **Diluent Concentrate**
   Ready to use as supplied.

3. **Wash Solution Concentrate**
   The Wash Solution supplied is a 20X concentrate and must be diluted 1:20 with distilled or deionized water. (1 part buffer concentrate, 19 parts dH_2O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.
4. **Enzyme-Antibody Conjugate Concentrate**
   Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 µL Enzyme-Antibody Conjugate to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently to avoid foaming. Dilute immediately before use and protect from light.
5. **TMB Substrate Solution**
   Ready to use as supplied.
6. **Stop Solution**
   Ready to use as supplied.
7. **Microtiter Plate**
   Ready to use as supplied. Unseal the Microtiter pouch and remove plate from pouch. Remove all of the strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.
8. **Pig CRP Calibrator**
   Prepare according to the lot specific **Certificate of Analysis**
9. **Positive Control**
   The concentration and recommended dilution are provided on the **Control Certificate**. Before use, briefly centrifuge the Positive Control to allow all of the liquid to collect in the bottom of the vial.

Procedure

1. **All Samples and calibrators should be assayed in duplicate.**
2. The Calibrators and the test sample(s) should be loaded into the ELISA wells as quickly as possible to avoid a shift in optical density (OD) readings. Using a multichannel pipette would reduce this occurrence.

   Pipette 100 µL of

Calibrator 0	(0.0 ng/mL) in duplicate
Calibrator 1	(6.25 ng/mL) in duplicate
Calibrator 2	(12.50 ng/mL) in duplicate
Calibrator 3	(25 ng/mL) in duplicate
Calibrator 4	(50 ng/mL) in duplicate
Calibrator 5	(100 ng/mL) in duplicate
Calibrator 6	(200 ng/mL) in duplicate

3. Pipette 100 µL of sample (in duplicate) into predesignated wells.
4. Incubate the **Microtiter Plate** at **RT** for **thirty** (30 ± 2) minutes. Keep the plate covered and level during incubation.
5. Following incubation, aspirate the contents of the wells.
6. Completely fill each well with the appropriately diluted **Wash Solution** and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill the wells with diluted **Wash Solution**, invert the plate and pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual Wash Solution. Repeat three times for a total of four washes.

7. Pipette 100 µL of appropriately diluted **Enzyme-Antibody Conjugate** to each well. Incubate at **RT** for **thirty** (30 ± 2) minutes. Keep plate covered in the dark and level during incubation.
8. Wash and blot the wells as described in Steps 5 and 6.
9. Pipette 100 µL of **TMB Substrate Solution** into each well.
10. Incubate in **the dark** at **RT** for precisely **ten** (10) minutes.
11. After **five** (5) minutes, add 100 µL of **Stop Solution** to each well.
12. Determine the absorbance at 450 nm of the contents of each well within **thirty** (30) minutes. Calibrate the plate reader to manufacturer's specifications.

RESULTS

1. Subtract the average background value (average absorbance reading of Calibrator zero) from the test values for each sample.
2. Average the duplicate readings for each standard and use the results to construct a Calibration Curve. Construct the calibration curve by reducing the data using computer software capable of generating a four-parameter logistic curve fit. A second order polynomial (quadratic) or other curve fits may al-so be used; however, they will be less precise fit of the data.
3. Interpolate test sample values from calibration curve. Correct for sera dilution factor to arrive at the CRP concentration in original sample.

FOR RESEARCH USE ONLY

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