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

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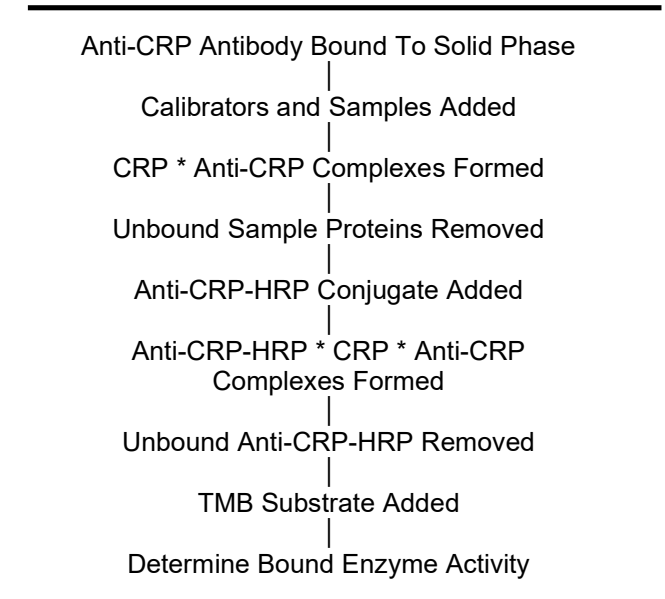
INTENDED USE
The Pig High-Sensitive CRP ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of C-reactive protein (CRP) in pig biological samples. For research use only.

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 CRP present in the sample reacts with the anti-CRP antibody, which has been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-CRP antibody conjugated with horseradish peroxidase (HRP) is added. This HRP-conjugated antibody forms a complex 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.



COMPONENTS
1. Diluent
   One bottle containing 60 mL of a 1X diluent running 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-pig CRP antibody conjugated with HRP in 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 strips in well holder frame. Wells are coated with affinity-purified anti-pig CRP.

7. Pig CRP Calibrator
   One vial containing a lyophilized Pig CRP Calibrator.

8. Positive Control
   One vial containing 50 µL of 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 de-ionized H_2O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Centrifuge for sample collection
- Anticoagulant for plasma collection

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 eyes and skin is possible. Flush with water after contact.

REAGENT PREPARATION

1. Diluent
   Ready to use as supplied.

2. Wash Solution Concentrate
   The Wash Solution supplied is a 20X concentrate and must be diluted 1:20 with distilled or de-ionized water. 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.

3. 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. Avoid foaming. Dilute immediately before use and protect from light.

4. TMB Substrate Solution
   Ready to use as supplied.

5. Stop Solution
   Ready to use as supplied.

6. Microtiter Plate
   Ready to use as supplied. Unseal microtiter pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.

7. Pig CRP Calibrator
   Add 1.0 mL of distilled or de-ionized water to the lyophilized Pig CRP Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 6.40 µg/mL **(the reconstituted calibrator should be aliquoted and frozen if future use is intended)**. Pig CRP Calibrators need to be prepared immediately prior to use (see chart below). Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent	Volume of 1X Diluent
6	200	30 µL Pig CRP Calibrator	930 µL
5	100	300 µL Calibrator 6	300 µL
4	50	300 µL Calibrator 5	300 µL
3	25	300 µL Calibrator 4	300 µL
2	12.5	300 µL Calibrator 3	300 µL
1	6.25	300 µL Calibrator 2	300 µL
0	0		600 µL

8. 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.

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. **Note: See long term storage recommendations below for the Pig CRP Calibrator and Positive Control.**

2. Diluent
   The diluent is stable until the expiration date and should be stored at 4°C.

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 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 and is stable until the expiration date. Protect from light.

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 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 less than 7 days can be at 4°C. Avoid multiple freeze/thaw cycles.

INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the calibrator solutions should be within 20% of the expected values.

SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freezing/thawing.

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds. EDTA may affect CRP binding, therefore it is not recommended for use as an anti-coagulant.

ASSAY PROTOCOL

Dilution of Samples

Due to the high sensitive nature of the assay each sample should be diluted before use for a normal assay. A dilution of 1:2,000 is appropriate for most serum/plasma samples. For absolute quantification of samples that yield results outside the range of the calibration curve, a lesser or greater dilution might be required. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**

To prepare a 1:2,000 dilution of sample, transfer 5 µL of sample to 495 µL of 1X Diluent. This gives you a 1:100 dilution. Mix thoroughly. Next, dilute the 1:100 samples by transferring 20 µL to 380 µL of 1X Diluent. You now have a 1:2,000 dilution of your sample. Mix thoroughly at each stage.

Procedure

1. **Bring all reagents to RT before use.**

2. The Calibrators and the test sample(s) should be loaded into the ELISA wells as quickly as possible to avoid a shift in OD readings. Using a multichannel pipette would reduce this occurrence.

3. Pipette 100 µL of
   Calibrator 0 (0.0 ng/mL) in duplicate
   Calibrator 1 (6.25 ng/mL) in duplicate
   Calibrator 2 (12.5 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

4. Pipette 100 µL of diluted Positive Control (in duplicate) into pre-designated wells.

5. Pipette 100 µL of diluted sample (in duplicate) into pre-designated wells.

6. Incubate the Microtiter Plate at 22°C (RT) for thirty (30 ± 2) minutes. Keep plate covered and level during incubation.

7. Following incubation, aspirate the contents of the wells.

8. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with wash buffer, invert the plate then pour/shake out the contents in a waste container. Following this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

9. Pipette 100 µL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (RT) for thirty (30 ± 2) minutes. Keep plate covered in the dark and level during incubation.

10. Wash and blot the wells as described in Steps 7 and 8.

11. Pipette 100 µL of TMB Substrate Solution into each well.

12. Incubate in the dark at RT for precisely ten (10) minutes.

13. After ten minutes, add 100 µL of Stop Solution to each well.

14. Determine the absorbance at 450 nm of the contents of each well within 30 minutes. Calibrate the plate reader to manufacturer's specifications.

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

RESULTS

1. Subtract the average background value from the test values for each sample.

2. Using the results observed for the calibrators construct a calibration curve. The appropriate curve fit is that of a four-parameter logistics curve, although a second order polynomial (quadratic) or other curve fits may also be used.

3. Interpolate test sample values from calibration curve. Correct for sample dilution factor to arrive at CRP concentration in original sample.

PERFORMANCE CHARACTERISTICS

In accord with good laboratory practice, the assays for specific CRP require meticulous quality control. Each laboratory should use routine quality control procedures to establish inter- and intra-assay precision and performance characteristics.

LIMITATION OF THE PROCEDURE

1. 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.
2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or de-ionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

FOR RESEARCH USE ONLY

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