K-ASSAY®

KAMIYA BIOMEDICAL COMPANY

Dog High-Sensitive CRP ELISA

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

Cat. No. KT-093

For research use only

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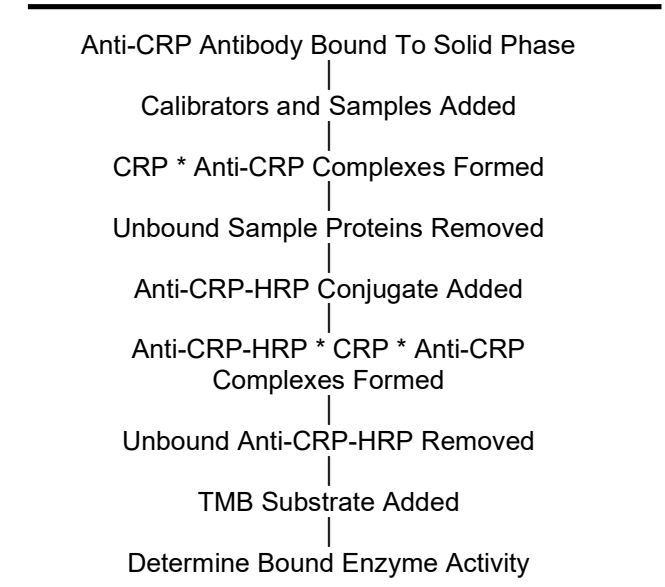
INTENDED USE
The Dog High-Sensitive CRP ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of C-reactive protein (CRP) in dog biological samples. For research use only.

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 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 levels in serum rise quickly following acute tissue damage and fall very rapidly once the stimulus is removed. It has been proposed that the function of CRP is to aid in complement activation, influence phagocytic cell function, and augment 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 antibodies that have 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 enzymes 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 de-ionized water, and accuracy of reagent and sample pipetting, washing technique, incubation time or temperature. Do not mix or substitute reagents with those from other lots or sources.

COMPONENTS

1. **Diluent Concentrate**
   One bottle containing 50 mL of a 5X 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-dog 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-dog CRP.
7. **Dog CRP Calibrator**
   One vial containing a lyophilized Dog 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

4. that in certain cases, unusual results may be obtained due to high levels of interfering factors.
5. Preservatives
   - Positive Control contains 0.1% sodium azide.
6. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
7. 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.

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. **See long term storage recommendations for the Dog CRP Calibrator and Positive Control below.**
2. **Diluent**
   The 5X Diluent 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.
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-dog 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. **Dog CRP Calibrator**
   The lyophilized Dog 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.

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.

REAGENT PREPARATION

1. **Bring all reagents to room temperature (RT) before use.**
2. **Diluent Concentrate**
   The Diluent solution supplied is a 5X concentrate and must be diluted 1:5 with distilled or de-ionized water. (1 part buffer concentrate, 4 parts dH_2O)
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. Dilute immediately before use and protect from light. Mix uniformly, but gently to avoid forming foam.
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 the plate from the 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.

8. **Dog CRP Calibrator**
   Add **1.0 mL** of distilled or de-ionized water to the lyophilized Dog CRP Calibrator and mix gently until dissolved. The calibrator is now at a concentration of **2.12 µg/mL (the reconstituted calibrator should be aliquoted and frozen if future use is intended)**. Dog 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
7	200	60 µL Dog CRP Calibrator	575 µL
6	100	300 µL Calibrator 7	300 µL
5	50	300 µL Calibrator 6	300 µL
4	25	300 µL Calibrator 5	300 µL
3	12.5	300 µL Calibrator 4	300 µL
2	6.25	300 µL Calibrator 3	300 µL
1	3.13	300 µL Calibrator 2	300 µL
0	0		600 µL

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.

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 1:1,000. To prepare a 1:1,000 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 sample by transferring 40 µL to 360 µL of 1X Diluent. You now have a 1:1,000 dilution of your sample. Mix thoroughly at each stage.

Procedure

1. **Bring all reagents to room temperature (16˚C to 25˚C) 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 optical density (OD) readings. Using a multi-channel pipette would reduce this occurrence.

   Pipette 100 µL of
   Calibrator 0 (0.0 ng/mL) in duplicate
   Calibrator 1 (3.13 ng/mL) in duplicate
   Calibrator 2 (6.25 ng/mL) in duplicate
   Calibrator 3 (12.5 ng/mL) in duplicate
   Calibrator 4 (25 ng/mL) in duplicate
   Calibrator 5 (50 ng/mL) in duplicate
   Calibrator 6 (100 ng/mL) in duplicate
   Calibrator 7 (200 ng/mL) in duplicate

3. Pipette 100 µL of diluted Positive Control (in duplicate) into predesignated wells.

4. Pipette 100 µL of diluted sample (in duplicate) into predesignated wells.
5. Incubate the Microtiter Plate at room temperature for ten (10 ± 2) minutes. Keep the plate covered and level during incubation.
6. Following incubation, aspirate the contents of the wells.
7. 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 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.
8. Pipette 100 µL of appropriately diluted **Enzyme-Antibody Conjugate** to each well. Incubate at 22°C room temperature for ten (10 ± 2) minutes. Keep plate covered in the dark and level during incubation.
9. Wash and blot the wells as described in Steps 6 and 7.
10. Pipette 100 µL of **TMB Substrate Solution** into each well.
11. Incubate in the dark at room temperature for precisely five (5) minutes.
12. After five minutes, add 100 µL of **Stop Solution** to each well.
13. Determine the absorbance at 450 nm of the contents of each well within 30 minutes. Calibrate the plate reader to manufacturer's specifications.

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. A second order polynomial (quadratic) or other curve fits may also be used.
3. Interpolate test sample values from the calibration curve. Correct for sample dilution factor to arrive at CRP concentration in original sample.

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