

KAMIYA BIOMEDICAL COMPANY

Rat High-Sensitive CRP ELISA

**For the quantitative determination of
C-reactive protein in rat serum or plasma**

Cat. No. KT-099

For Research Use Only.

PRODUCT INFORMATION**Rat High-Sensitive CRP ELISA**
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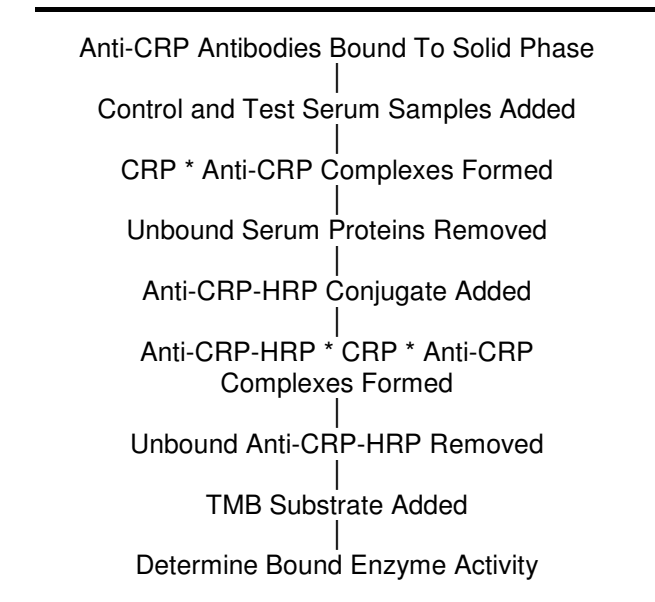
The Rat High-Sensitive CRP ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of C-reactive protein (CRP) in rat serum or plasma. For research use only.

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 pneumoniae*. 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 level in serum rises quickly following acute tissue damage and can reach a level 1,000-fold higher within 24 to 48 hours. 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 serum sample reacts with the anti-CRP antibodies that have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound serum proteins by washing, anti-CRP antibodies conjugated with horseradish peroxidase (HRP) are added. This HRP-conjugated antibody forms a complex with the previously bound serum 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 serum dilution.

Figure 1.

COMPONENTS

1. Diluent Concentrate
One bottle containing 50 mL of a 5X concentrated phosphate buffered saline (PBS) solution with 0.25% Tween, protein stabilizer and 0.25% Proclin 300 as a preservative.
2. Wash Solution Concentrate
One bottle containing 50 mL of a 20X concentrated PBS solution with 1% Tween.
3. Enzyme-Antibody Conjugate Concentrate
One vial containing 200 μ L of a 100X concentrated affinity-purified anti-rat CRP antibody conjugated with HRP in stabilizing buffer.
4. TMB Substrate Solution
One vial containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.
5. Stop Solution
One vial containing 12 mL of 0.3 M sulfuric acid. **WARNING:** Avoid contact with skin.
6. Microtiter Plate
Twelve removable eight-well micro strips in well holder frame. Wells are coated with affinity-purified anti-rat CRP.
7. Rat CRP Calibrator
One vial containing a lyophilized Rat CRP Calibrator.
8. Positive Control
One vial containing 50 μ L of serum with 0.1% sodium azide. See the Control Certificate for the concentration.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes (2 μ L to 200 μ L)
- Test tubes
- Microplate washer/aspirator
- Distilled or de-ionized H₂O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Vortex mixer

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
 - Diluent contains 0.25% Proclin 300 as a preservative. Positive Control contains 0.1% sodium azide.
5. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.
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 eyes and skin is possible. Flush with water after contact.

REAGENT PREPARATION

1. Diluent Concentrate

The Diluent solution supplied is a 5X concentrate and must be diluted 1:5 with distilled or de-ionized water.

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

The Enzyme-Antibody Conjugate supplied is a 100X concentrate and must be diluted 1:100. The required amount of working conjugate solution for each microtiter plate is prepared by adding 100 µL Enzyme-Antibody Conjugate to 9.9 mL of 1X Diluent. Mix uniformly, but gently. Avoid foaming.

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.

7. Rat CRP Calibrator

Add 1.0 mL of distilled or de-ionized water to the lyophilized Rat CRP Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 2.59 µg/mL (the reconstituted calibrator should be aliquoted and frozen if future use is intended). Rat CRP Calibrators need to be prepared immediately prior to use (see the following chart). Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent	Volume of 1X Diluent
1	200	50 µL of Rat CRP Calibrator	598 µL
2	100	300 µL of Calibrator 1	300 µL
3	50	300 µL of Calibrator 2	300 µL
4	25	300 µL of Calibrator 3	300 µL
5	12.5	300 µL of Calibrator 4	300 µL
6	6.25	300 µL of Calibrator 5	300 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 Rat CRP Calibrator and Positive Control.**

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 can be stored at room temperature (RT, 16-25°C) or at 4°C.

4. Enzyme-Antibody Conjugate

Undiluted anti-CRP-HRP conjugate should be stored at 4°C and diluted immediately prior to use. The working conjugate solution is stable for one day at 4°C.

5. TMB Substrate Solution

The TMB Substrate Solution should be stored at 4°C 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-rat CRP coated wells should be stored at 4°C in the sealed foil pouch with desiccant pack and are stable until the expiration date.

8. Rat CRP Calibrator

The lyophilized Rat 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 and are stable for one day.

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.

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. Specimens may be shipped at RT and then stored refrigerated at 4°C if testing is to take place within one week after collection. If testing is to take place later than one week, specimens should be stored at -20°C. Avoid repeated freezing/thawing.

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

ASSAY PROTOCOL

Dilution of Serum Samples

Due to the high-sensitive nature of the assay each serum sample should be diluted before use for a normal assay. A 1:12,000 dilution is appropriate for most samples. For absolute quantification of samples that yield results outside the range of the calibration curve, a lesser or greater dilution might be required.

To prepare a 1:12,000 dilution of sample, transfer 5 µL of sample to 495 µL of 1X Diluent. This gives you a 1:100 dilution. Mix thoroughly. Then dilute this by transferring 5 µL to 595 µL of 1X Diluent. You now have a 1:12,000 dilution of your sample. Make sure to mix thoroughly at each stage.

Procedure

Bring all reagents to RT before use.

1. Add 100 µL of 1X Diluent to each of the wells in A1 & A2. These will serve for an evaluation of the background associated with the assay.
2. Pipette 100 µL of
 - Calibrator 1 (200 ng/mL) into wells B1 & B2
 - Calibrator 2 (100 ng/mL) into wells C1 & C2
 - Calibrator 3 (50 ng/mL) into wells D1 & D2
 - Calibrator 4 (25 ng/mL) into wells E1 & E2
 - Calibrator 5 (12.5 ng/mL) into wells F1 & F2
 - Calibrator 6 (6.25 ng/mL) into wells G1 & G2
3. Pipette 100 µL of diluted Positive Control into wells H1 & H2.
4. Pipette 100 µL of diluted serum sample (test sample 1) into wells A3 & A4. The next sample goes in wells B3 & B4, the next in C3 & C4 and so on.
5. Incubate the Microtiter Plate at 22°C (RT) for ten (10 ± 2) minutes. Keep plate 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 μ L of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at RT in the dark for ten (10 ± 2) minutes.
9. Wash and blot the wells as described in Steps 6 and 7.
10. Pipette 100 μ L of TMB Substrate Solution into each well.
11. Incubate in the dark at RT for precisely five (5) minutes.
12. After five minutes, add 100 μ L of Stop Solution to each well.
13. Determine the absorbance at 450 nm of the contents of each well. Zero the plate reader to air.

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

Expected Values

The CRP concentration in normal rat serum has not yet been firmly established.

QUALITY CONTROL

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 pipetting.

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY

12779 Gateway Drive, Seattle, WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: techsupport@kamiyabiomedical.com
www.kamiyabiomedical.com