RABBIT CRP

Immunoperoxidase Assay for Determination of C - REACTIVE PROTEIN in Rabbit Sera

DIRECTIONS FOR USE

INTENDED USE

The CRP test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring CRP in serum or plasma of rabbits.

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[1]. This C-reactive protein (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 levels less that 0.3 mG/dL. The levels in serum rise quickly following acute tissue damage and can reach levels 1000-fold within 24 to 48 hours and also falls 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 these in plasma or serum can provide valuable diagnostic information in the detection, prognosis, and monitoring of disease not only in humans, but in companion animals and farm herds as well[2].

PRINCIPLE OF THE ASSAY

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the CRP present in serum sample reacts with the anti-CRP antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound serum proteins by washing, anti-CRP antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes 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 varies directly with 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 standard curve constructed from the standards, and corrected for serum dilution.

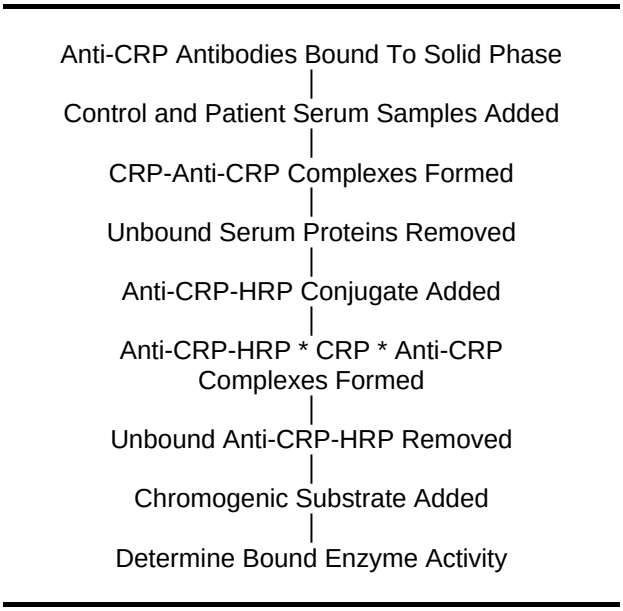

Figure 1.

REAGENTS (Quantities sufficient for 96 determinations)

1. DILUENT
One bottle containing 50 ml of phosphate buffered saline (PBS) solution containing bovine serum albumin, 0.05% Tween, and 0.02% thimerosal as a preservative.

2. WASH SOLUTION CONCENTRATE
One bottle containing 50 ml of a 10X concentrated phosphate buffered saline (PBS) solution containing 0.05% Tween.

3. ENZYME-ANTIBODY CONJUGATE
One vial containing 120 µL of affinity purified chicken anti-rabbit CRP antibody conjugated with horseradish peroxidase in a phosphate buffered saline solution with 50% glycerol.

4. CHROMOGEN-SUBSTRATE SOLUTION
One vial containing 12 mL of 3,3',5,5'-tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. STOP SOLUTION
One vial containing 12 ml 0.3 M sulfuric acid.

WARNING: Avoid contact with skin.

6. Anti-Rabbit CRP COATED WELLS
Eight removable twelve (12) well micro well strips in well holder frame. Each well is coated with affinity purified chicken anti-rabbit CRP.

7. Rabbit CRP STANDARDS
Five vials (0.5 mL/vial) containing pre-diluted reference Standard solutions. Standard 1 is adjusted to contain 25 nG/mL of rabbit CRP; Standard 2, 3, 4, and 5 are serial two-fold dilutions of Standard 1.

8. POSITIVE CONTROL
One vial containing 50 µL of serum with HIGH levels of CRP.

FOR IN VITRO USE ONLY

REAGENT PREPARATION

1. DILUENT
Ready to use. Mix gently before use. Avoid foaming.

2. WASH SOLUTION CONCENTRATE
The Wash Solution supplied is a 10X Concentrate and must be diluted 1:10 with distilled or deionized 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
The required amount of working conjugate solution for each microtitre plate is prepared by adding 100 µL Enzyme-Antibody Conjugate to 10 mL of Diluent. Mix uniformly, but gently. Avoid foaming.

4. CHROMOGEN-SUBSTRATE SOLUTION
Ready to use as supplied.

5. STOP SOLUTION
Ready to use as supplied.

6. CRP ELISA Micro plate
Ready to use as supplied.

7. CRP STANDARDS
Ready to use. Mix gently before use. Avoid foaming.

8. POSITIVE CONTROL (0.022 mg/ml)
The Control Sera should be diluted in a manner identical to the test serum samples. Varying dilution of the control sera will yield results that span the detection range of the assay.

STORAGE AND STABILITY

The expiry date for the package is stated on the outer label. However, each component is stable until the date stated on each bottle label. The recommended storage temperature for the complete package is 4-8°C.

1. DILUENT
The Diluent should be stored at 4-8°C and is stable until the expiration date stated on the reagent bottle label.

2. WASH SOLUTION
The Wash Solution Concentrate is stable until the expiration date stated on the reagent bottle label. The working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C).

3. ENZYME-ANTIBODY CONJUGATE
Undiluted horseradish peroxidase anti-CRP conjugate should be stored at 4-8°C and diluted immediately prior to use. The working conjugate solution is stable for one day.

4. CHROMOGEN-SUBSTRATE SOLUTION
The Substrate Solution should be stored at 4-8°C and is stable until the expiration date stated on the reagent bottle label.

5. STOP SOLUTION
The Stop Solution should be stored at 4-8°C and is stable until the expiration date stated on the reagent bottle label.

6. CRP ELISA Micro plate
Anti-rabbit CRP coated wells are stable until the expiration date listed on the package label, and should be stored at 4-8°C.

7. CRP STANDARDS
All standards should be stored at 4-8°C and are

stable until the expiration date indicated on the reagent vial label.

8. POSITIVE CONTROL
Control sera should be stored at 4-8°C and are stable until the expiration date indicated on the reagent vial label.

INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the standard 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 room temperature and then stored refrigerated at 2-8°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 freeze/thawing.

1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

MATERIAL PROVIDED

See "REAGENTS"
MATERIALS REQUIRED
BUT NOT PROVIDED

- Precision pipette (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H_2O
- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Vortex mixer

ASSAY PROTOCOL

DILUTION OF SERUM SAMPLES

The assay for quantification of CRP in serum requires that each test sample be diluted before use. For a single step determination a dilution of serum at 1:3000 is appropriate for most samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required.

1. Prepare an appropriate dilution of control reagent and specimen to be tested. In two consecutive steps prepare a 1:3000 serum dilution. Transfer 10 µL of sample to 490 µL (1:50) of diluent, then dilute this by transferring 10 µL to 590 µL (1:60) of diluent. Mix thoroughly at each stage.

PROCEDURE

Bring all reagents to room temperature before use.

1. Add 100 µL of 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
Standard 1 into wells A3 & A4
Standard 2 into wells A5 & A6
Standard 3 into wells A7 & A8
Standard 4 into wells A9 & A10
Standard 5 into wells A11 & A12

3. Pipette 100 µL of Diluted Control into wells B1 & B2

4. Pipette 100 µL of serum sample (test sample 1) into wells B3 & B4. The next sample goes in wells B5 & B6, the next in B7 & B8 and so on.

5. Incubate the micro titer plate at 22°C (room temperature) for thirty (30 ± 2) minutes. Keep plate level during incubation.

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

7. Fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. Finally, invert the plate on absorbent

paper (paper towel) and blot the excess fluid from the wells.

8. Pipette 100 µL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (room temperature) for thirty (30 ± 2) minutes.

9. Wash and blot the wells as described in Steps 6/7.

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

11. Incubate in dark, at room temperature for precisely fifteen (15) minutes.

12. After fifteen minutes, add 100 µL of Stop Solution to each well.

13. Determine the absorbance (450 nm) of the contents of each well. Zero the plate reader to air.

Stability of the final reaction mixture

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 standards construct a Standard Curve. The appropriate curve fit is that of a second order polynomial (quadratic).

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at CRP concentration is original sample.

PERFORMANCE CHARACTERISTICS

The precision of the assay for quantification of rabbit CRP was evaluated on five separate occasions. The average intraassay coefficient of variation was calculated to be 5.29% (range 0.77-5.66%); whereas, the interassay coefficient of variation was 9.14%.

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 redistilled or deionized water, and accuracy of reagent and sample pipettings.

REFERENCES

1. Tillett, W.S. and T. Francis. 1930. Serological reactions in pneumonia with non-protein somatic fraction of pneumococcus. J. Exp Med. 52:561-571.

2. Eckersal, P.D. 2000. Recent advances and future prospects for the use of acute phase proteins and markers of disease in animals. Revue Med. Vet. 151(7): 577-584.

Manufactured by:

Immunology Consultants Laboratory, Inc.