HORSE C-REACTIVE PROTEIN

Immunoperoxidase Assay for Determination of C-Reactive Protein in Horse Samples

DIRECTIONS FOR USE

Version2 1.0 -- 1

For Research Use Only, NOT for Diagnostic Purposes

Please Read this Package Insert Completely Before Using This Product

INTENDED USE

The CRP test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring CRP in Horse biological samples.

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 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 samples reacts with the anti-CRP antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-CRP antibodies conjugated with horseradish peroxidase (HRP), are 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 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 sample dilution.

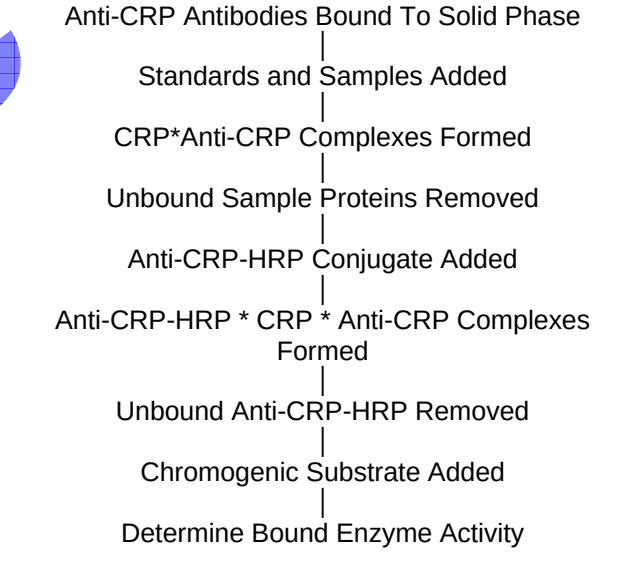

Figure 1.

REAGENTS (Quantities sufficient for 96 determinations)

1. DILUENT CONCENTRATE (Running Buffer)
One bottle containing 50 ml of a 5X concentrated diluent running buffer.

2. WASH SOLUTION CONCENTRATE
One bottle containing 50 ml of a 20X concentrated wash solution.

3. ENZYME-ANTIBODY CONJUGATE 100X
One vial containing 150 µL of affinity purified anti-Horse CRP antibody conjugated with horseradish peroxidase in a stabilizing buffer.

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-HORSE CRP ELISA MICRO PLATE
Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-Horse CRP.

7. HORSE CRP CALIBRATOR
One vial containing Horse CRP calibrator.

FOR IN VITRO USE ONLY

REAGENT PREPARATION

1. DILUENT CONCENTRATE
The Diluent Solution supplied is a 5X Concentrate and must be diluted 1/5 with distilled or deionized water (1 part buffer concentrate, 4 parts dH2O).

2. 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 dH2O). 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
Calculate the required amount of working conjugate solution for each microtitre 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.

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

5. STOP SOLUTION
Ready to use as supplied.

6. ANTI-HORSE CRP ELISA MICRO PLATE
Ready to use as supplied. Unseal Microtiter Pouch and remove plate from pouch. Remove all strips and wells that <u>will not</u> be used in the assay and place back in pouch and re-seal.

7. HORSE CRP CALIBRATOR
The Horse CRP Calibrator should be aliquoted and stored frozen. It is at a concentration of 4.837ug/ml and needs to be diluted in 1X diluent according to the chart below for each run. **Horse CRP standards need to be prepared immediately prior to use (see chart below).** Mix well between each step. Avoid foaming

Standard	**ng/ml**	**Volume added to 1x Diluent** →	**Volume of 1x Diluent**
6	320	60 µl Horse CRP Calibrator	847 µl
5	160	300 µl standard 6	300 µl
4	80	300 µl standard 5	300 µl
3	40	300 µl standard 4	300 µl
2	20	300 µl standard 3	300 µl
1	10	300 µl standard 2	300 µl
0	0		500 µl

STORAGE AND STABILITY

The expiration date for the package is stated on the box label.

1. 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 4-8°C.

2. 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 (16-25°C) or at 4-8°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 up to 8 hours.

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

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

6. ANTI-HORSE CRP ELISA MICRO PLATE
Anti-Horse CRP coated wells are stable until the expiration date, and should be stored at 4-8°C in sealed foil pouch with desiccant pack.

7. HORSE CRP CALIBRATOR
Long Term Storage: Upon receipt, aliquot the calibrator and store them frozen. They will be stable until expiration date. Short Term Storage: the calibrator is stable for up to 14 days at 4ºC. The working standard solutions should be prepared immediately prior to use and are stable for up to 8 hours.

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. The serum should be 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 freeze-thaw cycles.

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 SAMPLES

The assay for quantification of CRP in samples requires that each test sample be diluted before use. For a single step determination a dilution of 1/1,000 is appropriate for most serum/plasma samples. For absolute quantification, samples that yield results outside the range of the standard 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.**

1. To prepare a 1/1,000 dilution of sample, transfer 2 µL of sample to 198µL of 1X diluent. This gives you a 1/100 dilution. Next, dilute the 1/100 samples by transferring 30 µL, to 270 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 before use.**

2. Pipette 100 µL of
   Standard 0 (0.0 ng/ml) in duplicate
   Standard 1 (10 ng/ml) in duplicate
   Standard 2 (20 ng/ml) in duplicate
   Standard 3 450 ng/ml) in duplicate
   Standard 4 (80 ng/ml) in duplicate
   Standard 5 (160 ng/ml) in duplicate
   Standard 6 (320ng/ml) in duplicate

3. Pipette 100 µL of sample (in duplicate) into pre designated wells.

4. Incubate the micro titer plate at room temperature for thirty (30 ± 2) minutes. Keep plate covered and level during incubation.

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

6. 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. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100 µL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at room temperature 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 5/6.

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

10. Incubate in the dark at room temperature for precisely ten (10) minutes.

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

12. Determine the absorbance (450 nm) of the contents of each well. Calibrate 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 four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.

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

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 deionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

3. Do not mix or substitute reagents with those from other lots or sources.

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.

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the proprietary information.