Monobind Inc.
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AccuLite
CLIA Microwells

High Sensitivity C-Reactive Protein (hs-CRP) Test System

Product Code: 3175-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of CRP C-Reactive Protein concentration in Human Serum, Plasma or Whole Blood by a Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

C-Reactive Protein has traditionally been used to diagnose and monitor acute inflammation. It was named as such for its ability to bind and precipitate the C-polysaccharide of pneumococcus. It is an alpha globulin (MW 110-140 kD). CRP is synthesized in the liver and is normally present as a trace constituent of serum or plasma at levels less than 0.3 mg/dl. It has numerous physiological functions similar to those of immunoglobulins and acts as a host defense mechanism.

CRP is one of the acute phase proteins, the circulatory levels of which rise during general, non-specific response to a wide variety of diseases. These include infections by bacteria, acute phase of rheumatoid arthritis, abdominal abscesses and inflammation of the bile duct. High levels of CRP may also be found in patients with some viral infections, tuberculosis, acute infectious hepatitis, many other necrotic and inflammatory disease, burn and surgical trauma victims. Although the elevated levels of CRP are not indicative of any particular disease, the sudden rise of CRP does indicate an inflammatory process. CRP levels rise in circulation within 24-48 hours following acute tissue damage, reach a peak (upto 1000 times the constitutive level) and decrease with the resolution of trauma or inflammation. The elevated levels of CRP may last for several days before reaching back to normal levels.

Since, elevated levels of CRP are always associated with pathological changes; the CRP assays provide useful information for the diagnosis and therapeutic monitoring of inflammatory processes and associated diseases. Measurement of CRP by high sensitivity CRP assays adds to the predictive value of other cardiac markers like Myoglobin, CK-MB, cTnI and cTnT to assess the risk of cardiovascular and peripheral vascular disease. Rifai and Ridker – in a study for CDC – have proposed that medical decision points established by prospective epidemiological studies be used to interpret individual patient CRP results in risk assessment for cardiovascular disease. This is similar to the approach used by the National Cholesterol Education Program for blood lipids that requires that assays for CRP be standardized to provide comparable results. With the advent of sensitive methodologies, the use of high sensitivity CRP assays is becoming more routine to aid in the determination of inflammation due to cardiovascular trauma. Since CRP is not specific for anything in particular, Monobind hs-CRP assay results should be used in conjunction with other historical, physiological and pathological findings.

In this method, CRP calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of CRP) are added and the reactants mixed. Reaction between the various CRP antibodies and native CRP forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-CRP antibody bound conjugate is separated from the unbound enzyme-CRP conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce light.

The employment of several serum references of known CRP levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with CRP concentration.

3.0 PRINCIPLE

Immunoenzymometric assay (TYPE 3):
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, **in excess,** and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-Troponin-I antibody.

Upon mixing biotin labeled monoclonal antibody, the enzyme-labeled antibody and a serum containing the native antigen reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(m)} + Ag_{CRP} + {}^{Btn}Ab_{(m)} \underset{k_a}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(m)} - Ag_{CRP} - {}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)
Ag_{CRP} = Native Antigen (Variable Quantity)
$^{Enz}Ab_{(m)}$ = Enzyme labeled Antibody (Excess Quantity)
$^{Enz}Ab_{(m)} - Ag_{CRP} - {}^{Btn}Ab_{(m)}$ = Antigen-Antibodies Sandwich Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:
$^{Enz}Ab_{(m)} - Ag_{CRP} - {}^{Btn}Ab_{(m)} + \underline{Streptavidin}_{CW} \Rightarrow$ Immobilized complex
$\underline{Streptavidin}_{CW}$ = Streptavidin immobolized on well
Immobilized complex = sandwich complex bound to the solid surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided

A. CRP Calibrators – 1ml/vial - Icons A-F
Six (6) vials of references CRP antigen manufactured at levels of 0(**A**), 0.5(**B**), 2.0(**C**), 5.0(**D**), 15(**E**) and 30(**F**) µg/ml. Store at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the international reference material CRM 470.

B. hsCRP Tracer Reagent – 13ml/vial – Icon Ⓔ
One (1) vial containing Biotin labeled monoclonal mouse IgG and Anti-CRP HRP in buffer, dye, and preservative. Store at 2-8°C.

C. Light Reaction Wells – 96 wells – Icon ⇓
One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate – 20 ml/vial - Icon
One (1) vial containing a surfactant dissolved in buffered saline. A preservative has been added. Store at 2-8°C.

E. Signal Reagent A – 7.0ml/vial – Icon C[A]
One (1) vial containing luminol in buffer. Store at 2-8°C.

F. Signal Reagent B – 7.0ml/vial – Icon C[B]
One (1) vial containing hydrogen peroxide (H_2O_2) dissolved in buffer. Store at 2-8°C.

G. Serum Diluent – 20ml/vial
One (1) vial containing buffer salts and a dye. Store at 2-8°C.

H. Product Insert

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.
Note 3: Above reagents are for a single 96-well microplate.

4.1Required But Not Provided:
1. Pipette(s) capable of delivering 0.025ml (25µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5% (optional).
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Luminometer
5. Container(s) for mixing of reagents (see below).
6. Absorbent Paper for blotting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.
10. Storage container for storage of wash buffer.
11. Distilled or deionized water.
12. Quality Control Materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface antigen, HIV 1&2 and HCV antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS.

Safe disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum, plasma or whole blood in type, and the usual precautions in the collection of venipuncture samples should be observed. The blood should be collected in a plain redtop venipuncture tube without additives or gel barrier. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050 ml (50µl) of the diluted specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated ranges for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition,maximum absorbance should be consistent with past experience significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
   Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution -** Store at 2 - 8°C.
   Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.
3. **Serum Diluent**
   Dilute the serum diluent concentrate to 200ml in a suitable container with distilled or deionized water. Store at 2-8°C.
4. **Patient Sample Dilution (1/200)**
   Dispense 0.010ml (10µl) of each patient specimen into 2ml of serum diluent. Cover and vortex or mix thoroughly by inversion. Store at 2-8°C for up to forty-eight (48) hours.

Note 1: THE CALIBRATORS ARE READY TO USE.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).
*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.025 ml (25µl) of the appropriate serum reference calibrator, diluted specimen or control (***see Patient Sample Preparation above)*** into the assigned wells.
3. Add 0.100 ml (100µl) of the CRP Tracer Reagent to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**

Note: *Use a multichannel pipet to quickly dispense Tracer Reagent to avoid drift if the dispensing is to take more than a few minutes.*

4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 15 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.**
8. Add 0.100 ml (100µl) of Working Signal Reagent to all wells (see Reagent Preparation Section).
   DO NOT SHAKE THE PLATE AFTER SIGNAL ADDITION
9. Incubate at room temperature for 5 minutes in the dark.
10. Read the Relative Light Units (RLU) in each well using a microplate luminometer. **The results should be read within thirty (30) minutes of adding the Working Signal Reagent.**

Note: Always add reagents in the same order to minimize reaction time differences between wells.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of CRP in unknown specimens.

1. Record the RLUs obtained from the printout of the microplate luminometer as outlined in Example 1.
2. Plot the light intensity for each duplicate serum reference versus the corresponding troponin concentration in ng/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of myoglobin for an unknown, locate the average RLU's of the unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (31561) of control intersects the calibration curve at (6.25µg/ml) CRP concentration (See Figure 1).

Note 1: If the sample values need to be represented in mg% divide the value obtained (in Step#4) by 10 to convert the values in mg/dl (or mg %). (For Example the value for Patient #2 in example #2 would be 21.9/10=2.19 mg/dl)

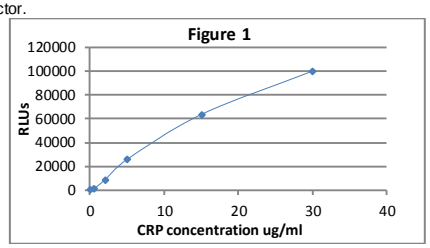
Note 2: Computer data reduction software designed for chemiluminescence assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Position	RLU (A)	Mean RLU (B)	Conc. (µg/ml)
Cal A	A1	57	56	0
	B1	55		
Cal B	C1	1119	1136	0.5
	D1	1154		
Cal C	E1	8404	8564	2.0
	F1	8724		
Cal D	G1	25534	25819	5.0
	H1	26103		
Cal E	A2	63497	63569	15
	B2	63641		
Cal F	C2	98557	100000	30
	D2	101443		
Control	E2	31214	31561	***6.52***
	F2	31907		
Patient 1	G2	416	434	***0.23***
	H2	453		
Patient 2	A3	62979	62782	***14.8***
	B3	62585		

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the F calibrator (greatest light output). This conversion eliminates differences cause by efficiency of the various instruments that can be used to measure light output. The conversion varies between instruments and should be established for each instrument before using it as a factor.

Figure 1



11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The Dose Response Curve should be within established parameters.
2. Four out of six quality control pools should be within the established ranges**.**

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Patient specimens with CRP concentrations above 30µg/ml may be diluted (for example an extra 1/10 dilution) with diluted serum diluent and re-assayed (final dilution = 1/2000). The sample concentration is obtained by multiplying the result by the additional dilution factor (10).
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
12. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato LM, Stuart MC. 'Heterophilic antibodies: a problem for all immunoassays' Clin. Chem. 1988:3427-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

13.0 EXPECTED RANGES OF VALUES

Based on a study of an apparent normal population and established literature references, an expected range for the hsCRP AccuLite® CLIA method was established (Table 1).

TABLE I
Expected Values for the hsCRP assay

Low Risk	=	< 1.0 µg/ml
Normal	=	1 – 3 µg/ml
High Risk	=	> 3.0 µg/ml

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precision of the hsCRP AccuLite® CLIA assay were determined by analyses on three different levels of pool control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in µg/ml)

SAMPLE	N	X	σ	CV
Low	12	1.5	0.08	5.3%
Medium	12	5.8	0.47	8.1%
High	12	13.1	0.98	7.5%

TABLE 3
Between Assay Precision (Values in µg/ml)

SAMPLE	N	X	σ	CV
Low	10	1.4	0.11	7.9%
Medium	10	6.0	0.52	8.6%
High	10	13.3	1.03	7.7%

*As measured in ten experiments in duplicate over ten days.

14.2 Sensitivity

The hsCRP AccuLite® CLIA Test System has a sensitivity of 0.007 µg/ml. The sensitivity was ascertained by determining the variability of the 0 µg/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum.

14.3 Accuracy

The hsCRP AccuLite® CLIA method was compared against a predicate automated CRP method. Biological specimens (n=74) from population (symptomatic and asymptomatic) were used. The values ranged from 0 – 22 µg/ml. The correlation is presented in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
Monobind (y)	3.50	y= 1.016(x) + -0.198	0.976
Reference (x)	3.89		

14.4 Specificity

The cross reactivity of the hsCRP AccuLite® CLIA test to selected substances was evaluated by adding the interfering substance to a pooled serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of CRP needed to produce the same absorbance.

Substance	Cross Reactivity
Bilirubin	ND
Lipids	ND
Triglycerides	ND
Human IgG	ND

14.5 High Dose Hook Effect

The test will not be affected by CRP concentrations up to 5000 µg/ml in serum or plasma. However, samples expected to be over 30 µg/ml should be further diluted in working serum diluent.

15.0 REFERENCES

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Glossary of Symbols
(EN 980/ISO 15223)

IVD — In Vitro - Diagnostic Medical Device
2° 8° — Temperature Limitation Storage Condition (2-8° C)
Consult Instructions for Use
REF — Catalogue Number
Σ — Contains Sufficient Test for Σ
LOT — Batch Code
Used By (Expiration Day)
Date of Manufacturer
Manufacturer
EC REP — Authorized Rep in European Country
CE — European Conformity