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 reactors mixed. The interaction between the various CRP antibodies and native CRP forms a sandwich complex which binds with the streptavidin coated to the well. After the completion of the required incubation period, the enzyme-CRP antibody bond conjugate is separated from the unbound enzyme-CRP conjugate by aspiration or decantation. The activity of the two antibodies used for ascertainment is quantitated by reaction with a suitable substrate to produce color.

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 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-CRP antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reactive reagents in excess form an insoluble sandwich complex without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

\[
\text{CRP} + \text{EnzAb} + \text{AbCRP} \rightarrow \text{CRP-EnzAb-AbCRP}
\]

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reactive reagents in excess form an insoluble sandwich complex without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

\[
\text{CRP} + \text{EnzAb} + \text{AbCRP} \rightarrow \text{CRP-EnzAb-AbCRP}
\]

4.1 Required But Not Provided:

1. Pipette capable of delivering 25µl & 50 µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 ml and 0.300 ml volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Pad for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.

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 negative 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 handling, unpacking and reading back to normal levels of CRP may last for up to 48 hours.

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 methods.

Materials Provided:

A. CRP Calibrators – 1ml/vial - Icons A-F
B. CRP Enzyme Reagent – 13ml/vial.
C. Streptavidin Coated Plate – 96 wells - Icon
D. Serum Diluent – 20ml
E. Wash Solution Concentrate – 20 ml - Icon
F. Substrate A – 7ml/vial - Icon
G. Substrate B – 7ml/vial - Icon
H. Stop Solution – 8ml/vial - Icon
I. Product Instructions.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain non-venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

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, 0.050ml of the diluted specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in each test run. Qualitative control charts should be maintained to follow the performance of the supplied reagents. Trending of results should be used to ascertain trends. 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. Serum Diluent

Dilute the serum to 200ml in a suitable container with distilled or deionized water. Store at 2-8°C.

2. Wash Buffer

Dilute contents of wash solution to 1000 ml with distilled or deionized water in a suitable storage container. Store at 2-8°C.

3. Working Substrate Solution

Pour the contents of the amber vial labeled Solution ‘A’ into the amber vial labeled B. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. 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 inverting. Store at 2-8°C for up to forty-eight (48) hours.

Note 1: Do not use the working substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

Note: THE CALIBRATORS ARE READY TO USE.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references 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, control, 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, dilution control or specimen (see Patient Sample Preparation above) into the assigned wells.
3. Add 0.010ml (100µl) of the CRP Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.
4. Note: Use the black pipette tip to quickly dispense the Enzyme Reagent to avoid drift if the dispensing is to take many minutes.
5. Swirl the microplate gently for 20-30 seconds to mix and cover.
6. Incubate 15 minutes at room temperature.
7. Add 0.375µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times. 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 two (2) additional times.
8. Add 0.010 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section).

10. Incubate at room temperature for fifteen (15) minutes.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

11. Do not store reagents that are contaminated or have bacteria growth.

Note: THE CALIBRATORS ARE READY TO USE.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references 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, control, 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, dilution control or specimen (see Patient Sample Preparation above) into the assigned wells.
3. Add 0.010ml (100µl) of the CRP Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.
4. Note: Use the black pipette tip to quickly dispense the Enzyme Reagent to avoid drift if the dispensing is to take many minutes.
5. Swirl the microplate gently for 20-30 seconds to mix and cover.
6. Incubate 15 minutes at room temperature.
7. Add 0.375µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times. 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 two (2) additional times.
8. Add 0.010 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section).

**Do not shake the plate after substrate addition.**

11. Incubate at room temperature for fifteen (15) minutes.
Add 0.050ml (50µl) of stop solution to each well and mix gently.

Read the absorbance in each well at 450nm (using a reference graph paper) after 30 minutes of adding the stop solution.

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 C-reactive protein in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as described in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding CRP concentration in µg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best fit curve through the plotted points.

4. To determine the concentration of CRP for an unknown, locate the average absorbance of the duplicates for each 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 absorbance (0.959) intersects the dose response curve at (0.65µg/ml) CRP concentration (See Figure 1).

Note 1: If the sample values need to be represented in mg/dl, 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 (see below) would be 2.19/10=0.219 mg/dl)

Note 2: Controls and standards designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

11.0 Q.C. PARAMETERS

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

1. Maximum Absorbance (Calibrator 'F') > 1.3
2. Maximum Absorbance (Calibrator 'A') < 0.1

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product is 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) divisions of the well to avoid pipette carryover.

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 substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.

6. Plate readers measure vertically. Do not touch the bottom of the wells.

7. Failure to remove adhering solution adequately in the aspiration or decalibration each step(s) may result in poor replication and spurious results.

8. Use components from the same lot. No intermixing of reagents from different batches is allowed.

9. Patient specimens with CRP concentrations above 30µg/ml may be further diluted (for example 1/50) with serum diluent and re-assayed. The serum dilution concentration is obtained by multiplying the result by the dilution factor (50).

10. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.

11. 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.

12. 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 preventive maintenance.

13. 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. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.

4. If test kits are altered, such as by mixing parts of different kits, which may produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.

5. 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 references a normal range for hsCRP AcuBind™ ELISA Microplate Test System was established.

**TABLE 1**

<table>
<thead>
<tr>
<th>CRP Value</th>
<th>Low Absorbance</th>
<th>Normal Absorbance</th>
<th>High Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1.0 µg/ml</td>
<td>1.000 - 1.500</td>
<td>1.501 - 2.000</td>
<td>&gt; 2.000</td>
</tr>
<tr>
<td>1.0 - 3.0 µg/ml</td>
<td>2.000 - 2.500</td>
<td>2.500 - 3.000</td>
<td>&gt; 3.000</td>
</tr>
<tr>
<td>&gt; 3.0 µg/ml</td>
<td>&gt; 3.000</td>
<td>&gt; 3.000</td>
<td>&gt; 3.000</td>
</tr>
</tbody>
</table>

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 "normal-range" depends on multiple 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 Microplate Elisa Procedure were determined by analyses on three different levels of control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 3 and Table 4.

**TABLE 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>CV%</th>
</tr>
</thead>
</table>
| Level 1 | 10 | 1.16 | 0.13 | 11.2%
| Level 2 | 20 | 5.9 | 0.52 | 8.8%
| Level 3 | 20 | 13.06 | 2.06 | 16.0%

**TABLE 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>CV%</th>
</tr>
</thead>
</table>
| Level 1 | 10 | 1.16 | 0.13 | 11.2%
| Level 2 | 20 | 5.9 | 0.52 | 8.8%
| Level 3 | 20 | 13.06 | 2.06 | 16.0%

14.2 Sensitivity

The hsCRP Microplate Elisa Procedure has a sensitivity of 0.014µg/ml. The sensitivity (S) was ascertained by determining the variability of the '0µg/ml' calibrator and using 20 (95% certainty) to calculate the minimum dose.

14.3 Accuracy

The MonoBind AcuBind™ hsCRP Elisa was compared against a predicate automated hsCRP method. Biological specimens (n=167) from population (symptomatic and asymptomatic) were used. The values ranged from 0 – 22 µg/ml. The correlation is presented in Table 4.

**TABLE 4**

<table>
<thead>
<tr>
<th>Method</th>
<th>Leass Square Regression Analysis</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>This Method</strong></td>
<td>0.976</td>
<td>y = 0.540 + 0.050x</td>
</tr>
<tr>
<td><strong>Reference</strong></td>
<td>3.94</td>
<td>---</td>
</tr>
</tbody>
</table>

14.4 Specificity

The cross-reactivity of the hsCRP Microplate Elisa Procedure to selected substances was evaluated by adding the interfering substances 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.

**TABLE 5**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>ND</td>
</tr>
<tr>
<td>Lipids</td>
<td>ND</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>ND</td>
</tr>
<tr>
<td>Human IgG</td>
<td>ND</td>
</tr>
</tbody>
</table>