Myoglobin is an enzyme, found primarily in cardiac and skeletal muscle. It is an oxygen binding protein and exists as a monomeric tetramer—myoglobin has one fourth the molecular weight (18 kD) of hemoglobin. Since Myoglobin, like FABP (fatty acid binding protein) is a low molecular mass cytoplasmic protein present not only in heart but also in other tissues, it offers a specific, sensitive, and stable plasma marker for muscle cell viability to discriminate between heart or skeletal muscle injury. The Myoglobin content of human heart, however, is lower than that of skeletal muscle.

Serial measurement of biochemical markers is now accepted universally as an important determinant in ruling in or ruling out acute myocardial infarction. Myoglobin is one of the most important markers in ruling out acute myocardial infarction (AMI) within 2h of admission because of chest pains. AMI disrupts cardiac cell membranes, releasing intracellular cardiac proteins into the vascular system, and these proteins including myoglobin, creatine kinase-MB (CK-MB), lactate dehydrogenase Type 1 (LDH) and cardiac troponin subunits I and T (TnI and TnT) have proven useful in diagnosing AMI. The optimal clinical utility of each marker depends on specific protein characteristics. Myoglobin, being the smallest of these markers, diffuses rapidly throughout the vascular system and provides the earliest indication of AMI. Myoglobin levels rise between 0.5 – 2.0 h after presentation, peak within 5-12 hours and the kidneys rapidly eliminate myoglobin from the system, restoring normal circulating concentrations within 16-36 hours. If allowed to persist, the protein rapidly clears from the system, myoglobin concentrations can reliably indicate reinfarction. Also, myoglobin measurements can preclude AMI. According to the Heart Emergency Room (ER) Program model two consecutive low measurements, the first upon admission of the patient and the second within 2 hours of the first, can exclude AMI in nearly 99% of the cases. Myoglobin participates in aerobic metabolism in cardiac and skeletal muscle cells thus showing high concentrations in these tissues. Renal failures and other kidney problems exhibit high levels of myoglobin levels as well. Most complications are accompanied by distinctive clinical symptoms that make the differential diagnosis possible. Serial testing for myoglobin and CK-MB isoenzyme mass on presentation and 3, 6 and 9 h later in patients with symptoms suggestive of acute ischemic coronary syndrome presenting with a non-diagnostic or equivocal electrocardiogram has been shown to be more effective than continuous intracoronary catheter balloon Doppler, echocardiography, and graded exercise testing. (Monobind has an excellent Chemi test system – Cat# 2975-300 for the determination of circulating levels of CK-MB in human serum or plasma.)

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

After the completion of the required incubation period, the enzyme- Myoglobin antibody bond conjugate is separated from the unbound enzyme-Myoglobin 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 (Myoglobin) levels together with the production 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 Myoglobin concentrations.

### 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 mononal anti-myoglobin antibody.

Upon mixing biontin 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:

\[
A^* + A + E + B = \text{immobilized complex}
\]

**4.0 REAGENTS AND MATERIALS PROVIDED:**

- **Materials Provided:**
  - Myoglobin Calibrators – 2.0 ml (Dried) Icons A – F
  - Streptavidin Conjugate reference standards are stable for 30 days at 2-8°C. In order to store for a longer period of time aliquot the recombinant calibrators in cryo vials and store at -10°C. **DO NOT FREEZE THE ABOVE**. A preservative has been added.

- **Note:** The calibrators, human serum based, were calibrated using ultracentrifuged myoglobin antigen at levels of 0(0A), 10(B), 25(C), 50(D), 150(E), and 400(F) ng/ml.

- **Reconstitute each vial with 2.0 ml of distilled or deionized water**

- **Note:** All Myoglobin antigen standards and streptavidin conjugates are stable for 30 days at 2-8°C. In order to store for a longer period of time aliquot the recombinant calibrators in cryo vials and store at -10°C. **DO NOT FREEZE**. A preservative has been added.

- **Note:** The calibrators, human serum based, were calibrated using ultracentrifuged myoglobin antigen at levels of 0(0A), 10(B), 25(C), 50(D), 150(E), and 400(F) ng/ml.

- **Reconstitute each vial with 20 ml – Icon**

- **Note:** One that vials contain a surfactant dissolved in buffered saline. A preservative has been added. Store at 2-30°C.

- **Note:** If the specimen(s) cannot be assayed within this time, the calibrators may be stored at temperatures of 2-8°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the 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. Performance of quality control materials 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 deviations 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

- **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.

- **Working Wash Buffer**
  - To Working Concentrate, add 200 ml of distilled water and mix well. Store at 2-30°C.

- **Wash Buffer**
  - To Working Concentrate, add 200 ml of distilled water and mix well. Store at 2-30°C.

### 4.1 Required But Not Provided:

1. Pipette(s) capable of delivering 25µl and 100µl volumes with a precision of better than 1.5%.
2. Dispensers (for repetitive deliveries of 0.100 ml and 0.300 ml volumes with a precision of better than 1.5% (optional)).
3. Microplate washer or a squeeze bottle (optional).
4. Micropette Luminometer
5. Container(s) for mixing of reagents (see below).
6. Ethanol (96%) for cleaning 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.

**Note:** Do not use reagents that are contaminated or have bacteria growth.

### 9.0 TEST PROCEDURE

**Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).**

- **Test procedures should be performed by a skilled individual or trained professional**

1. Format the microplates’ wells for 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 (25µg) of the appropriate controls, controls, standards and patient specimens into the wells.
3. Add 0.100 ml (100µl) of the Myoglobin Tracer Reagent to each well. It is very important to dispense all reagents close to the bottom of the microwell.

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

1. Swirl the microplate gently for 20-30 seconds to mix. Cover with a plastic wrap.
2. Incubate for 15 minutes at room temperature.

6. **SPECIMEN COLLECTION AND PREPARATION**

The specimens shall be blood, serum in type, and the usual precautions in the collection of venipuncture samples should be observed. Reagents will be prepared by a skilled individual or trained professional.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7. Add 350 μL of wash buffer (see Reagent Preparation Section), decant, and then 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 instructions. For all washes, if a squeeze bottle is used, fill each well to the top by squeezing the container. Avoiding air bubbles. Decant the wash and repeat four (4) additional times.

8. Add 0.100 ml (100 μL) of Working Signal Reagent to all wells (see Reagent Preparation Section).

9. Incubate at room temperature for five (5) minutes in the dark.

10. Read the Relative Light Units (RLU) in each well using a 96 well microplate luminoimeter.

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 myoglobin in unknown specimens.

1. Record the RLU’s obtained from the printout of the microplate luminoimeter as outlined in Example 1.

2. Plot the light intensity for each duplicate serum sample versus the corresponding myoglobin 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. The concentration in ng/ml is read from the horizontal axis of the graph. (The duplicates of the unknown may be averaged as indicated).

5. To determine the average RLU’s (60424) of control 2 intersects the calibration curve at (165.2ng/ml) myoglobin concentration. (See Figure 1).

Note: Computer data reduction software designed for chemiluminescent assay testing can 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. 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 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) minutes to avoid assay drift.

3. Highly lipemic, hemolyzed or grossly contaminated specimens should not be used.

4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.

The addition of a Signal Reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-difference during reaction.

Failure to achieve complete solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

Use components from the same lot. Use reagents from the same lot and preparation when appropriate.

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 the patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinations.

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 could 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 VALUES

Myoglobin values are consistently the same in plasma and serum. However, a serum sample that has been quickly separated from the red cells is preferred. Myoglobin levels are higher in trained athletes or people who are used to a daily regimen of strenuous exercise.

Based on the clinical data measured with Monobind in concordance with the expected values established by the Manufacturer only until an agreement. The least square regression equation and correlation coefficient indicates excellent method agreement.

14.5 High Dose Hook-Effect:

The test will not be affected by Myoglobin concentrations up to 10,000ng/ml. Samples expected to be over 400ng/ml should be diluted 1:10 and 1:100 in normal pooled serum and the normal pooled serum should be added to the base value. The base value and dilution factor should be taken into account to get the corrected concentration of Myoglobin in the sample.

15.0 REFERENCES


Revision: 3 Date: 032012 Cslt 3275-300 DCO: 0641..CA 3275-300

For Orders and Inquiries, please contact

Tel: 1-940.091.2685 Email: info@monobind.com
Fax: 1-940.920.3830 Web: www.monobind.com

Please visit our website to learn more about our other interesting products and services.

Table 4

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (ng/ml)</th>
<th>Least Square Regression Analysis</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>This Method (y)</td>
<td>23.42</td>
<td>y = 1.02x + -1.04</td>
<td>0.983</td>
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<tr>
<td>Reference (x)</td>
<td>24.23</td>
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Only slight amounts of bias between the Myoglobin AccuLite™ CLIA test system and the reference method are indicated by the closeness of the linear regression equation and correlation coefficient indicates excellent method agreement.

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<tr>
<td>Low</td>
<td>12</td>
<td>42.3</td>
<td>5.9%</td>
</tr>
<tr>
<td>Medium</td>
<td>12</td>
<td>127.5</td>
<td>6.7%</td>
</tr>
<tr>
<td>High</td>
<td>12</td>
<td>208.4</td>
<td>8.5%</td>
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*As measured in ten experiments in duplicate over ten days.

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<td>10</td>
<td>14.5</td>
<td>2.6%</td>
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<tr>
<td>Medium</td>
<td>10</td>
<td>131.2</td>
<td>7.2%</td>
</tr>
<tr>
<td>High</td>
<td>10</td>
<td>138.9</td>
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