Monobind Inc.
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AccuLite
CLIA Microwells

Myoglobin Test System
Product Code: 3275-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Circulating Myoglobin concentrations in Human Serum, or Urine by an Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Myoglobin is an enzyme, found primarily in cardiac and skeletal muscle. It is an oxygen biding protein and exists as a monomeric form of hemoglobin. Being a monomer of hemoglobin – which is a 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 ctyoplasmic protein present not only in heart but also in the skeletal muscle it is difficult to use it as a 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 myocardial markers used 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. Some of the proteins including *myoglobin,* creatine kinase-MB (CK-MB), lactate dehydrogenase type-1 (LD1) and cardiac troponin subunits I and T (TnI & 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 hours after the onset of chest pains and peak within 5-12 hours. The kidneys rapidly eliminate myoglobin from the system, restoring normal circulating concentrations within 16-36 hours. Since 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 two hours later, negatively predict AMI in nearly 100% of the cases. Myoglobin participates in aerobic metabolism in cardiac and skeletal muscle cells thus showing high concentrations in muscle traumas. Renal failures and other kidney problems exhibit high levels of myoglobin levels as well. Most complications are accompanied by distinct 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 equivalent electrocardiogram has been shown to be more effective than continuous serial electrocardiograms, echocardiography, and graded exercise testing.
(Monobind has an excellent Chemi test system Product Code: 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 monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of Myoglobin are added and the reactants mixed. 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 bound 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 permits the 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 Myoglobin 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-myoglobin 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:

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

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)
Ag_{Myo} = Native Antigen (Variable Quantity)
$^{Enz}Ab_{(m)}$ = Enzyme labeled MoAb (Excess Quantity)
$^{Enz}Ab_{(m)} - Ag_{Myo} - {}^{Btn}Ab_{(m)}$ = Antigen-Antibodies 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:
$^{Enz}Ab_{(m)} - Ag_{Myo} - {}^{Btn}Ab_{(m)} + Strept_{CW} \Rightarrow$ immobilized complex
$Strept_{CW}$ = Streptavidin immobilized on well
Immobilized complex = sandwich complex bound to the solid surface

After sufficient time for reaction, 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 AND MATERIALS PROVIDED:

Materials Provided:

A. Myoglobin Calibrators – 2.0 ml/vial (Dried) - Icons A-F
Six (6) vials of references for Myoglobin antigen at levels of 0(**A**), 10(**B**), 25(**C**), 50(**D**), 150(**E**), and 400(**F**) ng/ml. ***Reconstitute each vial with 2.0ml of distilled or deionized water.*** The reconstituted calibrators are stable for 30 days at 2-8°C. In order to store for a longer period of time aliquot the reconstituted calibrators in cryo vials and store at -10°C. ***DO NOT FREEZE THAW MORE THAN ONCE.*** A preservative has been added.
Note: The calibrators, human serum based, were calibrated using gravimetric protein weight from a >99% purified preparation as measured by PAGE.

B. Myoglobin Tracer Reagent - 13 ml/vial - Icon Ⓔ
One (1) vial containing enzyme labeled affinity purified antibody and biotin labeled monoclonal mouse anti-myoglobin IgG 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 2-30°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. 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.

4.1 Required 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 in type, and the usual precautions in the collection of venipuncture samples should be observed; Urine samples may also be used when treated appropriately. The blood should be collected in a plain red-top venipuncture tube without additives. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells. Centrifuging serum samples before a complete clot forms may result in the presence of fibrin. To prevent erroneous results due to the presence of fibrin make sure that complete clot formation has taken place prior to the centrifugation of the samples.

Urine samples may be collected without preservatives and stored at 2-8°C for up to 8 hours. For longer storage the samples should be immediately aliquoted and stored at -20°C. (*Please contact Monobind for special Urinary Myoglobin procedure*).

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 two (2) 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 repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) 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. 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.

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 reference calibrators and controls to room temperature (20-27°C). *****Test procedure should be performed bya 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 (25µl) of the appropriate calibrators, controls and samples into the assigned 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.*
4. Swirl the microplate gently for 20-30 seconds to mix. Cover with a plastic wrap.
5. Incubate for 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 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 (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 five (5) minutes in the dark.
10. Read the Relative Light Units (RLU) in each well using a 96 well 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 myoglobin in unknown specimens.

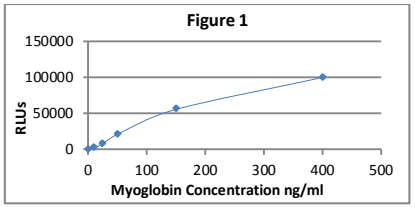
1. Record the RLUs obtained from the printout of the microplate luminometer as outlined in Example 1.
2. Plot the light intensity for each duplicate serum reference versus the corresponding myoglobin concentration in ng/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of myoglobin for an unknown, locate the average RLUs of the unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLUs (60424) of control 2 intersects the calibration curve at (165.2ng/ml) myoglobin concentration (See Figure 1).

Note: Computer data reduction software designed for chemiluminescence assays may 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 Number	RLU(A)	Mean RLU (B)	Value (ng/ml)
Cal A	A1	82	83	**0**
	B1	84		
Cal B	C1	2028	2099	**10**
	D1	2169		
Cal C	E1	8559	8530	**25**
	F1	8501		
Cal D	G1	21088	21289	**50**
	H1	21289		
Cal E	A2	55695	55845	**150**
	B2	55995		
Cal F	C2	100457	100000	**400**
	D2	99543		
Ctrl 1	E2	24418	24845	**58.1**
	F2	25273		
Ctrl 2	G2	59709	60424	**165.2**
	H2	61138		
Patient 1	A3	17353	17564	**42.3**
	B3	17775		

* 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 RLUs of the calibrators have been normalized to 100,000 RLUs 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.



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 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. 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.
9. 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.
10. 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.
11. 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 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 gathered by Monobind in concordance with the published literature the following ranges have been assigned. **These ranges should be used as guidelines only:**

TABLE 1

Adult (Normal)	**≤ 96 ng/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 Myoglobin AccuLite® CLIA Test System 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 ng/ml)

SAMPLE	N	X	σ	CV
Low	12	42.3	2.5	5.9%
Medium	12	127.5	6.7	5.3%
High	12	235.4	8.5	3.6%

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

SAMPLE	N	X	σ	CV
Low	10	41.5	2.7	6.5%
Medium	10	131.2	7.2	5.5%
High	10	238.9	10.1	4.2%

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

14.2 Sensitivity

The sensitivity (detection limit) was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2**σ** (95% certainty) statistic to calculate the minimum dose. The assay sensitivity was found to be 0.03 ng/ml.

14.3 Specificity

The cross reactivity of the Myoglobin AccuLite® CLIA test system to selected substances was evaluated by adding the interfering substance(s) to a serum matrix at the following concentration(s). The antibody system used did not detect any hemoglobin, CK-MB, TnI or FABP when tested at very high concentrations. The presence of lipemia (25 mg/ml); hemoglobin (4.0 mg/ml) and bilirubin (2.5 mg/ml) did not affect the assay precision.

14.4 Accuracy

The Myoglobin AccuLite® CLIA test system was compared with a predicate ELISA assay. Biological specimens from population (symptomatic and asymptomatic) were used. (The values ranged from N/D – 185 ng/ml). The total number of such specimens was 85. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Coefficient Coefficient
Monobind (y)	23.42	y= 1.02(x) + -1.04	0.983
Reference (x)	24.23		

Only slight amounts of bias between the Myoglobin AccuLite® CLIA test system and the reference method are indicated by the closeness of the mean values. 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 in serum. However, samples expected to be over 400ng/ml should be diluted 1:10 and 1:100 in normal pooled human serum and the normal pool assayed along side to obtain a 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

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2. Gibler WB, Lewis LM, Erb RE, et al, "Early detection of acute myocardial infarction in patients presenting with chest pain and nondiagnostic ECGs; serial Troponin-I sampling in the emergency department", *Ann Emerg Med*, 19, 1359-66 (1990).
3. Kallner A, Sylven C, Brodin U, et al, "Early diagnosis of acute Myocardial infarction; a comparison between chemical predictors", *Scand J Clin Lab Invest*, 49, 633-9 (1989).
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5. Lee TH, Rouan GW, Weisberg MC, et al, "Sensitivity of routine clinical criteria for diagnosing Myocardial infarction within 24 hours of hospitalization", *Ann Intern Med*, 106, 181-6 (1987).
6. Gibbler WB, Runyon JP, Levy RC, Sayre MR, Kacich R, Hattemar CR, et al, "A rapid diagnostic and treatment center for patients with chest pain in the emergency department", *Ann Emerg Med,* 25, 1-8 (1995).

Revision: 4 **Date: 2019-Jul-16** **DCO: 1353**
MP3275 **Product Code: 3275-300**

Size		96(A)	192(B)
Reagent (fill)	A)	2ml set	2ml set
	B)	1 (13ml)	2 (13ml)
	C)	1 plate	2 plates
	D)	1 (20ml)	1 (20ml)
	E)	1 (7ml)	2 (7ml)
	F)	1 (7ml)	2 (7ml)

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Glossary of Symbols
(EN 980/ISO 15223)