Myoglobin (Human)
ELISA Kit Protocol

(Cat. No.:EK-311-17)

PHOENIX PHARMACEUTICALS, INC.

INTENDED USE

For the quantitative determination of myoglobin concentration in human serum. **FOR RESEARCH ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES**

LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. Samples may contain human anti-mouse antibodies (HAMA) which are capable of giving falsely elevated results with assays that utilize mouse monoclonal antibodies. The Myoglobin ELISA assay has been designed to minimize interference from HAMA-containing specimens; nevertheless complete elimination of this intereference from all speicmens cannot be guarenteed.

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INTRODUCTION AND PROTOCOL OVERVIEW

Myoglobin, a heme protein with a molecular weight of approximately 17,500 Daltons is found in both cardiac and skeletal muscle. Dmage to either tyoe of muscle following conditions such as trauma, ischemia, and diseases that cause myopathy, is associated with the release of myoglobin into serum.[1,2] Specifically, following cardiac necrosis associated with myocardial infarction (MI), myoglobin is onw of the fist markers to rise avobe normal levels. Myoglobin levels increase measureably above baseline within 2-4 hours post-infarct, peaking at 9-12 hours, and returning to baseline within 24-36 hours.[1,2,3,4]

In the absence of skeletal muscle trauma or other factors associated with a non-cardiac realted increase in circulating myoglobin, its levels have been used as an early marker for myocardial infarct.[4,6,7] A number of reports suggest using the measurement of myoglobin as a diagnostic aid in ruling out myocardial infarction[5,8]with negative predictive values of up to 100% reported at certain time periods after the onset of symptoms.[9-15] Unlike the other cardiac enzymes such as creetine kinase and the amb isoform (i.e. CK and CK/MB) which do not reach serum levels until serveral hours post-infarction (approx. 19 hours), myoglobin levels can be expeacted to peack within 6 to 9 hours.[16]

The Myoglobin Enzyme Immunoassay provides a rapid, sensitive, and reliable assay for the quantitative measreument of myoglobin in serum. The antibodies developed for the test will determine a minimal concentration of 5.0 ng/ml, and there is no cross-reactivity with related cardiac or skeletal enzymes.

CAUTION: Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use and establish optimum sample concentrations.

PRINCIPLE OF THE TEST

The Myoglobiin ELISA test is based on the priciple of a solid phase enzyme-linked immunsorbent assay.[17,18] The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the myoglobin molecule. Mouse monoclonal anti-myoglobin antibody is use for the solid phase immobilization (on the microtiter wells). A goat anti-myoglobin antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the myoglobin molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 45 minute incubation at room temeprature, the wells are washed with water to remove unbound labeled antibodies. A TMB (Tetramethryl-benzidine) Reagenet is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop Solution changing the color to yellow. The concentration of myoglobin is directly porportional to the colot intesisty of the test sample. Absorbance is measure spectrophotmoetrically at 450nm.

LIST OF COMPONENTS

Materials Provided with the Kit:

- **Antibody-Coated Wells (1 plate, 96 wells)**
  Microtiter wells coated with murin monoclonal anti-myoglobin.
- **Reference Standard Set (0.5ml/vial, 1 set/kit)**
  Contains 0, 25, 100, 250, 500, and 1000 ng/ml myoglobin, liquid, ready-to-use. These standards have been pre-diluted 10-fold. Please do not dilute them again.
- **Sample Diluent (25 ml/bottle)**
  Contains phosphate buffer and 1.0% (w/v) Pro-Clin as preservative.
- **Enzyme Conjugate Reagent (22 ml/vial)**
  Contains anto-myoglobin conjugated to horseraddish perosidase in Tris buffer-BSA solution with preservatives.
- **TMB Reagent (11 ml/bottle)**
  Contains one-step TMB solution
- **Stop Solution (1 bottle, 11 ml/bottle)**
  Contains diluted hydrochloric acid (1N HCl)

Materials required but not provided:

- Precision pipettes: 20µl, 50µl, 100µl, 200µl, and 1.0 ml.
- Disposable pipette tips.
- Distilled water.
- Vortex mixer or equivalent.
- Absorbent paper or paper towel.
- Graph paper.
- Microtiter plate reader.

SPECIMEN COLLECTION AND PREPARATION

- The use of SERUM samples is required for this test.
- Specimens should be collected using standard venipuncture techniques. Remove serum from the coagulated or packed cells within 60 minutes after collection.
- Specimens which cannot be assayed within 24 hours of collection should be frozen at -20°C or lower, and will be stable for up to six months.
- Specimens should not be repeatedly frozen and thawed prior to testing. DO NOT store in "frost free" freezers, which may cause occasional thawing.
- Specimens which have been frozen, and those which are turbid and/or contain particulate matter, must be centrifuged prior to use.

STORAGE

1. Store unopened kit at 2-8°C upon receipt and when it is not in use, until the expiration shown on the kit label. Refer to the package label for the expiration date.
2. The opened and used reagents are stable until the expiration date if stored properly at 2-8°C.
3. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

ASSAY PROCEDURE

1. All reagents should be brought to room temperature (18-25°C) before use.
2. Serum and control serum should be diluted 10 fold before use. Prepare a series of small tubes (such as 1.5 ml microcentrifuge tubes) and mix 20 µl serum or plasma with 180 µl (0.180 ml) Sample Diluent. **PLEASE DO NOT DILUTE THE STANDARDS - THEY HAVE ALREADY BEEN PRE-DILUTED 10-FOLD.**
3. Secure the desired number of coated wells in the holder.
4. Dispense 20 µl of myoglobin standards, diluted specimens and diluted controls into the appropriate wells.
5. Dispense 200 µl of Enzyme Conjugate Reagent into each well.
6. Thoroughly mix for 30 seconds. It is very important to mix completely.
7. Incubate at room temperature (18-25°C) for 45 minutes.
8. Remove the incubation mixture by flicking plate contents into a waste container.
9. Rinse and flick the microwells 5 times with distilled or deionized water . (Please do not use tap water.)
10. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water drops.
11. Dispense 100 µl of TMB Reagent solution into each well. Gently mix for 5 seconds.

12. Incubate at room temperature for 20 minutes.
13. Stope the reaction by adding 100 µl of Stop Solution to each well
14. Gently mix 30 seconds. ***It is important to make sure that all the blue color changes to yellow color completely.***
15. Read absorbance at 450 nm with a microtiter well reader **within 15 minutes.**

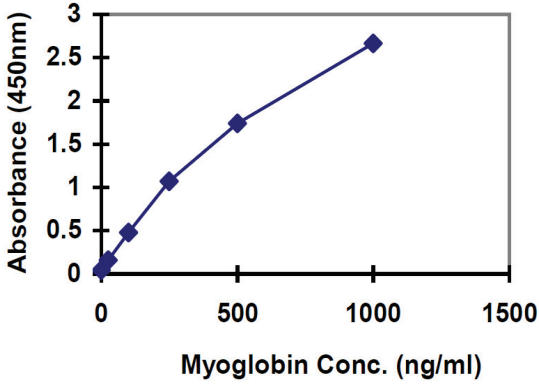
CALCULATION OF RESULTS

1. Calculate the mean absorbance value (A450) for each set of reference standards, controls and patient samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in µIU/ml on graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.
3. Use the mean absorbance values for each specimen to determine the corresponding concentration of TSH in µIU/ml from the standard curve.
4. Since the reference standards have already been pre-diluted 10-fold, there is no need for the samples or control sera observed values to be multiplied by the dilution factor of 10. However, if the samples are diluted to 100-fold, the observed values should be multiplied by 10.

EXAMPLE OF STANDARD CURVE

Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against TSH concentrations shown in the X axis. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.

Myoglobin(ng/ml)	Absorbance (450 nm)
0	0.046
25	0.158
100	0.476
250	1.070
500	1.741
1000	2.664



EXPECTED VALUES

- Normal serum myoglobin levels range for 12 to 100 ng/ml. Values increase slightly with age.22
- Each facility should establish its own reference intervals for myoglobin.
- Serial sampling may be required to detect elevated levels.

PERFORMANCE CHARACTERISTICS

- **SENSITIVITY**
  The lowest detectable level ofmyoglobin by this assay is estimated to be 5 ng/ml.

- **HOOK EFFECT**
  No high-dopse hook effect is observed in this test with sample concentrations up to 10,000 ng/ml

- **PRECISION**
  a. ***Intra-Assay Precision***
  Within-run precision was determined by replicate determinations of five different serum samples in one assay. Within-assay variability is shown below:

Serum Sample	1	2	3	4	5
# Reps.	20	20	20	20	20
Mean Myo. (ng/ml)	55.6	214.3	294.9	505.9	1,437
S.D.	2.2	12.9	16.2	26.3	94.0
C.V. (%)	3.9	6.0	5.5	5.2	6.6

  b. ***Inter-Assay Precision***
  Between-run precision was determined by replicate measurements of five different serum samples over a series of individually calibrated assays. Between-assay variability is shown below:

Serum Sample	1	2	3	4	5
# Reps.	35	35	35	35	35
Mean Myo. (ng/ml)	59.2	244.4	330.5	568.3	1451.7
S.D.	4.6	12.8	38.9	52.7	104.7
C.V. (%)	7.8	5.2	11.8	9.3	7.2

- **RECOVERY**

Various serum samples of known myoglobin levels were combined and assayed in duplicate. The mean recovery was 102.8%.

Pair No.	Expected [Myoglobin] (ng/ml)	Observed [Myoglobin] (ng/ml)	% Recovery
1	280	250	89.3
2	451	495	109.8
3	255	241	94.5
4	269	300	111.5
5	39	41	105.1
6	240	231	96.0
7	90	88	95.9
8	209	214	102.0
9	340	328	96
10	214	213	100.0
11	551	655	118.8
12	431	436	101.2
13	757	824	108.8
14	747	768	102.8
15	780	894	114.6
16	575	569	98.9

- **LINEARITY**

Three samples were serially diluted to determine linearity. The mean recovery was 105.8%.

#	Dilution	Expected Conc. (ng/ml)	Observed Conc. (ng/ml)	% Expected
1	Undiluted	--	--	--
	1:2	540	542.6	100.5
	1:4	270	290.8	107.7
	1:8	135	153.3	113.6
	1:16	67.5	75.3	111.6
	1:32	33.8	38.7	114.5
	1:64	16.9	18.8	111.2
	1:128	8.5	8.6	101.2
	1:256	4.3	3.9	90.7
				Mean= 106.4%
2	Undiluted	--	--	--
	1:2	945	956	101.2
	1:4	472.5	500	105.8
	1:8	236.3	262.8	111.2
	1:16	118.1	131.7	111.5
	1:32	59.1	65.2	110.3
	1:64	29.5	31.1	105.4
	1:128	14.8	12.8	86.5
				Mean=104.6%
3	Undiluted	--	--	--
	1:2	--	--	--
	1:4	691.0	691.4	100.0
	1:8	362.3	345.7	104.8
	1:16	173.9	172.8	100.6
	1:32	95.7	86.4	110.8
	1:64	45.8	43.2	106.0
	1:128	21.2	21.6	98.0
	1:256	13.5	10.8	125.0
				Mean=106.5%

- **SPECIFITICITY**

The following materials were tested for cross-reactivity at concentrations up to the levels indicated below. No cross-reactivity was observed for any of the components.

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Material Tested	Test Concentration
Interfereing Substances	
Cardiac Tnl	1000ng/ml
Cardiac TnT	1000ng/ml
Cardiac TnC	1000ng/ml
Skeletal Tnl	1000ng/ml
CK-MB	1000ng/ml
CK-MB2	100ng/ml
CK-MM	5µg/ml
CK-BB	10µg/ml
Tropomyosin	1000ng/ml
Myosin Light Chain Kinase (MLCK)	1000ng/ml
Actin	1000ng/ml
Endogeneous Substances	
Bilirubin	20mg/dl
Cholesterol	500mg/dl
Triglyceride	1,500mg/dl
Total Protein	3g/dl
Total Protein	10g/dl
Therapeutic Substances	
Aspirin	0.3ng/ml
Coumadin	1000µg/ml
Digoxin	200ng/ml
Flurosemide(Lasix)	400µg/ml
Sodium Heparin	8U/ml

WARNINGS AND PRECAUTIONS FOR USERS

1. **CAUTION:** This kit contains human material. The source material used for maufacture of this kit tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. Howeverm all human blood products, including serum samples, should be considered ptentially infectious. Handlling and disposal should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.[21]
2. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
3. Do not use the reagent when it becomes clody or contamination is suspected.
4. Do not use the reagent if vial is damaged.
5. Replace caps on reagents immediately. Do not switch caps.
6. Each well can be used only once.
7. Do not pipette reagents by mouth.
8. Solutions containing additives or preservatives, such as sodium axide, should not be used in the enzyme reaction.
9. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amonts of water and seek medical attention if irritation persists.

INSTRUMENTATION

A microtiter well reader with a bandwidth of 10nm or less and an optical density range of 0 to 3 OD or greater at 450 nm wavelength is accptable for absorbance measurement.

PROCEDURAL NOTES

- Pipetting Recommendations (single and multi0-channel): Pipetting of all standards, samples, and controls should be completed within 3 minutes.
- All standards, samples and controls should be run in duplicate concurrently so that all conditions of testing are the same.
- It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

QUALTIY CONTROL

- Good laboratory practice requires that quality contrl specimens (controls) be run with each calibration curve to verify assay performance.
- To ensure proper performance, control material should be assayed repeateadly to establish mean values and acceptable ranges.

STANDARDIZATION

Human myoglobin concentration Complex was obtained from a qualified vendor, and myoglobin concentration was determined. The material was further diluted with Sample Diluent and served as "Standard Stock Solution" for preparing myoglobin reference standard sets. The target value of the "Standard Stock Solution" was confirmed by the Abbott AxSym Myoglobin immunoassay.

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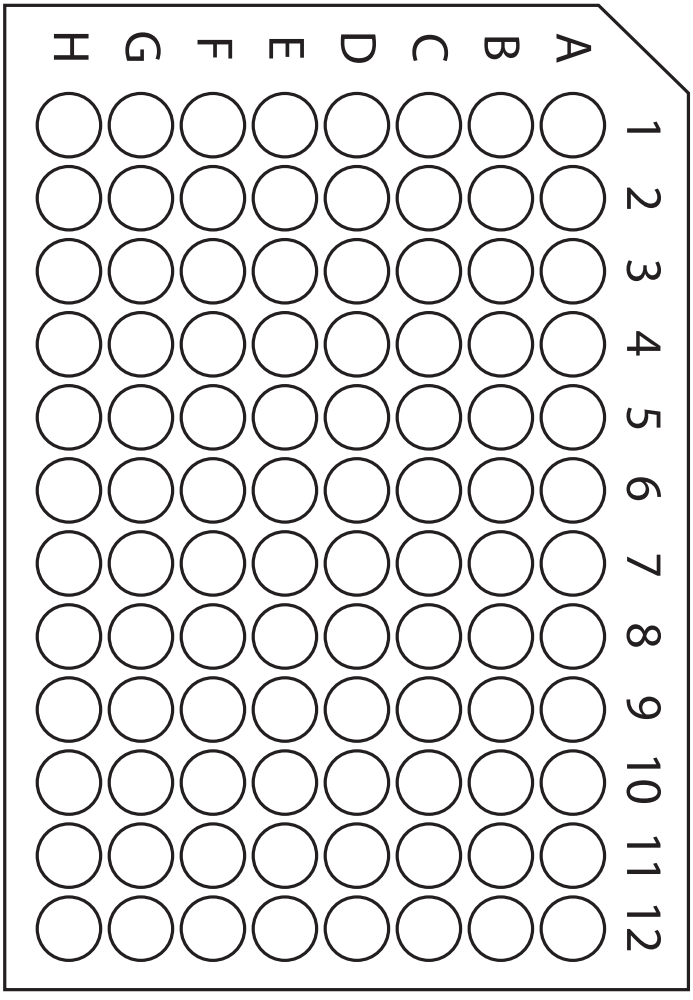
ASSAY DIAGRAM



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