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AccuBind
ELISA Microwells

Troponin-I (cTnI)
Product Code: 3825-300

Intended Use: The Quantitative Determination of Circulating Troponin-I concentrations in Human Serum by a Microplate Immunoenzymometric assay

SUMMARY AND EXPLANATION OF THE TEST

The cardiac-specific isoform of Troponin I (cTnI) has been known as a marker of heart damage and myocardial cell death, due to myocardial infarction, for just over 10 years. Troponin-I (cTnI, 24 kDa) is the inhibitory subunit of the Troponin complex of striated muscle. Most of the Troponin (I & T) proteins are located within the contractile apparatus of the striated muscle. The concentration of these subunits is increased in circulation for many days after AMI, because release from the structural elements requires degradation of myofibril itself. This location and release from the specific myocardial tissue and their sustained appearance in circulation make Troponin subunits reliable bio-markers of AMI. Unlike CK-MB and Myoglobin, Troponin does not suffer from non-specificity issues. The immunological determination of cTnI does have some issues surrounding it but those are mainly due to lack of mass standardization, the presence of post-translationally modified cTnI in the circulation and variations in the antibody cross-reactivities to the various detectable forms of cTnI. However, cTnI, unlike Myoglobin, has not been found in circulation in marathon runners and other cases of skeletal injury or trauma. Careful selection of antibodies and diligent preparation of stable cTnI for calibration permits cTnI to be an excellent biochemical marker of myocardial damage.

Troponin I (cTnI) is the inhibitory subunit of the Troponin complex, which regulates the calcium modulated interaction of actin and myosin in striated muscle. The complex is a heterodimer consisting of troponins C, I and T, which are tightly bound to the contractile apparatus; hence, circulating concentrations are low. Even though Troponin C and T are both considered to be equally good markers for AMI, the NH_2 terminus of cTnI has 31 additional amino acids not present in the skeletal isoforms and has generated an interest in easily creating cTnI-specific MoAb's, by researchers. Troponin I, however, has its own problems that researchers are dealing with. Troponin I is not very stable. A major portion of it is bound with tropinin C (cTnC) and the remaining small part is free in circulation. Again, the post translational modifications, including selective degradation, covalent complex formation and phosphorylation of cTnI in the post-ischemic myocardium complicate the matters further more. Also, the cTnI proteolysis is even more extensive in human myocardium. Some researchers attribute it in part to the heterogeneity of disease states present in a given patient population. The key to overcoming these problems is a very careful selection of antibodies, the matrix for stabilizing the cTnI and the cTnI protein used as the standard itself. Monobind has been able to successfully put together an assay that promises to have overcome those issues.

In this method, Troponin-I 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 Troponin-I* are added and the reactants mixed. Reaction between the various *Troponin-I* antibodies and native *Troponin-I* forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme- Troponin-I antibody bound conjugate is separated from the unbound enzyme-Troponin-I 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 color.

The employment of several serum references of known Troponin-I 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 Troponin-I concentration.

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:

$$Enz_{Ab_{(m)}} + Ag_{cTnI} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} Enz_{Ab_{(m)}}\text{-}Ag_{(cTnI)}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$Ag_{cTnI.}$ = Native Antigen (Variable Quantity)

$Enz_{Ab_{(m)}}$= Enzyme labeled MoAb (Excess Quantity)

$Enz_{Ab_{(m)}}\text{-}Ag_{cTnI.}\text{-}{}^{Btn}Ab_{(m)}$ = 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:

$$Enz_{Ab_{(m)}}\text{-}Ag_{cTnI.}\text{-}{}^{Btn}Ab_{(m)} + Strept_{CW} \Rightarrow \text{immobilized complex}$$

$Strep_{C.W.}$ = 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.

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.

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. 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.

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 repetitive freezing and thawing. When assayed in duplicate, 0.050 ml of the specimen is required.

REAGENTS AND MATERIALS PROVIDED:

A. Troponin-I Calibrators – 1.0 ml/vial (Lyophilized) [A – F]
Six (6) vials of references for *Troponin-I* antigen at levels of 0(**A**), 1.0(**B**), 3.0(**C**), 6.0(**D**), 15(**E**), and 30(**F**) ng/ml. ***Reconstitute each vial with 1.0ml of distilled or deionized water.***
The reconstituted calibrators are stable for 24 hours at 2-8°C. In order to store for a longer period of time aliquot the reconstituted calibrators in cryo vials and store at -20°C. ***DO NOT FREEZE THAW MORE THAN ONCE.*** A preservative has been added.
Note: The calibrators, human serum based, were calibrated using NIST standard for cTnI # 2921.

B. Troponin-I Enzyme Conjugate – 13 ml/vial [Icon]
One (1) vial containing enzyme labeled affinity purified antibody and biotin labeled monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin Microplate – 96 wells – [Icon]
One 96-well 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 [Icon]
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. Substrate A – 7.0ml/vial – [Icon S^A]
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

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

G. Stop Solution – 8.0ml/vial - Icon STOP
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

H. Product Instructions.

Required But Not Provided:

1. Pipette(s) capable of delivering 25µl and 100µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5% (optional).
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Storage container for storage of wash buffer.
10. Distilled or deionized water.
11. Quality Control Materials.

REAGENT PREPARATION:

1. **Wash Buffer**
   Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.
2. **Working Substrate Solution**
   Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.
   Note: Do not use the working substrate if it looks blue.

TEST PROCEDURE

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

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 2-8ºC.**
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 Troponin-I Enzyme Reagent to each well. **It is very important to dispense all reagents close to the bottom of the microwell.**
   Note: Use a multichannel pipet to quickly dispense the 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 (20-27°C).
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 300µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) 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 two (2) additional times.**
8. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section).

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9. Incubate at room temperature for fifteen (15) minutes.
10. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

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

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 trends should be maintained to monitor batch to batch consistency.

CALCULATION OF RESULTS

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

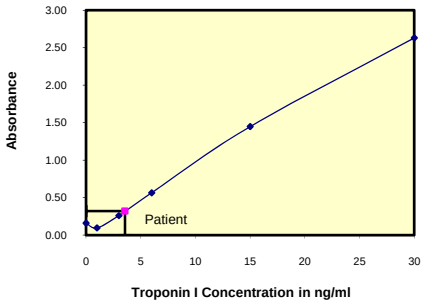
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1
2. Plot the absorbance for each duplicate serum reference versus the corresponding cTnI concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of cTnI 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 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 absorbance (0.322)

intersects the dose response curve at (3.55ng/ml) cTnI concentration (See Figure 1).

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	0.017	0.016	0
	B1	0.016		
Cal B	C1	0.094	0.096	1
	D1	0.097		
Cal C	E1	0.276	0.260	3
	F1	0.244		
Cal D	G1	0.574	0.564	6
	H1	0.554		
Cal E	A2	1.480	1.448	15
	B2	1.416		
Cal F	C2	2.660	2.630	30
	D2	2.599		
Ctrl 1	E2	0.050	0.051	0.43
	F2	0.051		
Ctrl 2	G2	1.256	1.245	12.75
	H2	1.233		
Patient	A3	0.320	0.322	3.61
	B3	0.324		

Figure 1



*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.

Q.C. PARAMETERS

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

1. The absorbance (OD) of calibrator 'A' should be ≤ 0.07.
2. The absorbance (OD) of calibrators 'F' should be ≥ 1.3.
3. Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

A. Assay Performance\

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Highly lipemeic, hemolysed or grossly contaminated specimen(s) should not be used.
8. Patient samples with Troponin-I concentrations above 30 ng/ml may be diluted with the zero calibrator or Troponin-I free pooled human serum or urine and re-assayed. Multiply the value obtained by the dilution factor to obtain the corrected value.
9. Use components from the same lot. No intermixing of reagents from different batches.

B. Interpretation

1. 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.
2. The reagents for AccuBind™ Troponin-I AccuBind™ Elisa procedure 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 used in combination with clinical examination, patient's history and, all other clinical findings.

EXPECTED VALUES

Troponin-I values are different in plasma and serum. In addition, plasma sample may be influenced by the additives used. For example, heparin minimally affects the results but oxalate and EDTA have significant effect. A serum sample that has been quickly separated from red cells is preferred.

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:**

Adult (Normal) ≤ 1.3 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.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the Troponin-I AccuBind™ Elisa 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	C.V.
Pool 1	20	0.44	0.014	3.3%
Pool 2	20	3.55	0.072	2.0%
Pool 3	20	12.75	0.311	2.4%

TABLE 3

Between Assay Precision* (Values in ng/ml)

SAMPLE	N	X	σ	C.V.
Pool 1	10	0.48	0.038	7.9%
Pool 2	10	3.68	0.242	6.6%
Pool 3	10	13.56	0.745	5.5%

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

B. Accuracy

The Troponin-I AccuBind™ Elisa was compared with a predicate radioimmunoassay assay. Biological specimens from population (symptomatic and asymptomatic) were used. (The values ranged from N/D – 18.1 ng/ml). The total number of such specimens was 151. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (y)	3.04	y = 0.3500+0.9266(X)	0.950
Reference (x)	*2.92*		

Only slight amounts of bias between this method 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.

C. Sensitivity

The sensitivity (detection limit) was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose. The assay sensitivity was found to be 0.05 ng/ml.

D. Specificity

The cross-reactivity of the Troponin-I Elisa method to selected substances was evaluated by adding the interfering compounds to a serum matrix at the following concentration(s). The antibody system used did not detect any hemoglobin, CK-MB, TnT 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.

E. Hook Effect

Human serum samples spiked with concentrations up to 10,000 ng/ml of cTnI did not show any hook effect with cTni AccuBind™ Elisa assay system.

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Cat# 3825-300

Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml 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)
	G)	1 (8ml)	2 (8ml)

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