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 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. Comparison of known dose response curve, an unknown specimen’s activity can be correlated with Troponin-I 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 microparticle well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[
\text{EnzAb(m)-Ag(cTnI)-BtnAb(m)}
\]

\[
\text{ka} = \text{Rate Constant of Association}
\]

\[
\text{kd} = \text{Rate Constant of Dissociation}
\]

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[
\text{Strepavidin immobilized on well}
\]

\[
\text{Streptavidin immobilized complex = sandwich complex bound to the solid surface}
\]

After sufficient time for reaction, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The endogenous enzyme activity is then determined. The enzyme concentration is directly proportional to the native antigen concentration. By utilizing several portion of reference standards 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

#### Materials Provided:

A. Troponin-I Calibrators – 1.0 ml/vial (Lyophilized) [A – F]  
(6) 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. This 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 Troponin-I.

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.  
Store at 2-8°C.

C. Streptavidin Microplate – 96 wells – Icon  
One 96-well microplate coated with streptavidin and packaged.

D. Wash Buffer (50 ml) – Icon  
One (1) vial containing a surfactant in buffered saline.  
A preservative has been added. Store at 2-8°C.

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

F. Substrate B – 7.0 ml/vial – Icon S  
One (1) bottle containing hydrogen peroxide (H2O2) in buffer.  
Store at 2-8°C.

G. Stop Solution – 8.0 ml/vial - Icon  
One (1) bottle containing a strong acid (1N HCl).  
Store at 2-8°C.

### 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 handling and disposal of infectious materials shall be as 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, “Biohazard in Microbiological and Biomedical Laboratories,” 2nd Edition, 1988, HTTPS.

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. The blood should be collected in a plain red top 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 sample(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, 0.050 ml 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. Any and all methods should be reviewed and aimed to ascertain trends. Significant deviation from established performance can indicate that there are problems with equipment or reagents and adjustment or re-calibration 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 solution to 100ml with distilled or deionized water in a suitable storage container. Store at 2-8°C.

2. Working Substrate Solution  
Pour the contents of the amber vial labeled Solution ‘A’ into the stock amber vial labeled Solution ‘B’. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.

### 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 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 calibrator, 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. If a single channel pipet is used, the fluid will drip 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 aspirations,allow the plate dry and blot with absorbent paper.

7. **Add the appropriate substrate (see Reagent Preparation Section), decant (tap and blot) and 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. Discard the wash and repeat two (2) additional times.

8. Add 0.100 ml (100µl) of working substrate solution to all wells. **DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION**.

9. Incubate for an additional 15 minutes at room temperature (20-27°C).

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 and 620nm wavelength. **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.
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 3.55 ng/ml cTnI.

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validity of the software should be ascertained.

1.3.0 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 calibrator ‘F’ should be ≥ 1.3. Four out of five quality control pools should be within the established ranges.

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 reagents should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated samples may result in a falsely elevated value.
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 decantation wash step(s) may result in poor replication and spuriously results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. 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.

10. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from the provided’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 before use and within the preventative maintenance.

13. Risk Analysis, as required by CE Mark (IV) Directive 93/42/EC, is 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 determinate factors.

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.

6. 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. Heterophile antibodies often cause such test interactions and have been known to be problems for all kinds of immunosassays. (Boscato LM Stewart MC. Heterophile antibodies: a problem for all immunosassays 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 other clinical findings.

13.0 EXPECTED VALUES

Troponin-I values are different in plasma and serum. In addition, plasma samples may be used 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.

| 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 in the laboratory 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 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

<table>
<thead>
<tr>
<th>Pool</th>
<th>Mean Value</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.44</td>
<td>0.014</td>
<td>3.3%</td>
</tr>
<tr>
<td>2</td>
<td>0.55</td>
<td>0.072</td>
<td>2.0%</td>
</tr>
<tr>
<td>3</td>
<td>1.275</td>
<td>0.311</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

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

### TABLE 3

<table>
<thead>
<tr>
<th>Pool</th>
<th>Mean Value</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.48</td>
<td>0.038</td>
<td>7.9%</td>
</tr>
<tr>
<td>2</td>
<td>3.68</td>
<td>0.242</td>
<td>6.6%</td>
</tr>
<tr>
<td>3</td>
<td>13.56</td>
<td>0.745</td>
<td>5.5%</td>
</tr>
</tbody>
</table>

14.2 Sensitivity

The sensitivity (detection limit) was ascertained by determining the variability of the blank serum, calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose. The assay sensitivity was found to be 0.030 ng/ml.

14.3 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

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (x)</th>
<th>Regression Analysis</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>This Method</td>
<td>3.04</td>
<td>y = 0.3500+0.0266(x)</td>
<td>0.950</td>
</tr>
</tbody>
</table>

Only slight amounts of bias between this method and the reference method were indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

14.4 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 very high concentration(s).

<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>ND</td>
</tr>
<tr>
<td>FABP</td>
<td>ND</td>
</tr>
<tr>
<td>TnT</td>
<td>ND</td>
</tr>
</tbody>
</table>

The presence of Lp(a) (25 mg/ml), hemoglobin (4.0 mg/ml) and bilirubin (2.5 mg/ml) did not affect the assay precision.