



Cardiac-Specific Troponin-I ELISA Kit Protocol

INTENDED USE

The cTnI ELISA is intended for the quantitative determination of cardiac troponin I in human serum. Measurement of troponin I values are useful in the evaluation of acute myocardial infarction (AMI). **FOR RESEARCH ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES**

WARNINGS AND PRECAUTIONS FOR USERS

1. **CAUTION:** This kit contains human material. The source material used for manufacture of this component tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples, should be considered potentially infectious. It is recommended that the reagents and patient samples be handled according to the OSHA Standard on Bloodborne Pathogens³² or other appropriate national biohazard safety guidelines or regulations.³³⁻³⁴
2. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. Replace caps on reagents immediately. Do not switch caps.
5. Do not pipette reagents by mouth.

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. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. Patient samples may contain human anti-mouse antibodies (HAMA) which are capable of giving falsely elevated or depressed results with assays that utilize mouse monoclonal antibodies. The vendor's cTnI ELISA assay has been designed to minimize interference from HAMA-containing specimens; nevertheless complete elimination of this interference from all patient specimens cannot be guaranteed.
5. Test results that are inconsistent with the clinical picture and patient history should be interpreted with caution.

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SUMMARY AND EXPLANATION OF TEST

Troponin is the inhibitory or contractile regulating protein complex of striated muscle. It is located periodically along the thin filament of the muscle and consists of three distinct proteins: troponin I, troponin C, and troponin T.1-5 Likewise, the troponin I subunit exists in three separate isoforms; two in fast-twitch and slow-twitch skeletal muscle fibers, and one in cardiac muscle.(6-8) The cardiac isoform (cTnI) is about 40% dissimilar, has a molecular weight of 22,500 daltons, and has 31 additional amino acid residues that are not present on the skeletal isoforms. (3-4,8-12) Antibodies made against this cardiac isoform are immunologically different from antibodies made against the other two skeletal isoforms, (10,13) and the unique isoform and tissue specificity of cardiac troponin I is the basis for its use as an aid in the diagnosis of acute myocardial infarction (AMI). (2-4,8-9,13-17)

Cardiac troponin I (cTnI) has been useful in the differential diagnosis of patients presenting to Emergency Departments (ED) with chest pain. 18-20 Myocardial infarction is diagnosed when blood levels of sensitive and specific biomarkers, such as cardiac troponin, the MB isoenzyme of creatine kinase (CK-MB), and myoglobin, are increased in a clinical setting of acute ischemia.(21-22,23)

The most recently described and preferred biomarker for myocardial damage is cardiac troponin (I or T) (23). The cardiac troponins exhibit myocardial tissue specificity and high sensitivity. Likewise, cardiac TnI and CK-MB have similar release patterns (4-6 hours after the onset of pain), but the level of cTnI remains elevated for a much longer period of time (6-10 days), thus providing for a longer window of detection of cardiac injury. (23-24)

Normal levels of cTn I in the blood are very low. After the onset of an AMI, cTnI levels increase substantially and are measurable in serum within 4 to 6 hours, with peak concentrations reached in approximately 12 to 24 hours after infarction. (24-28) The fact that cTnI remains elevated in serum for a much longer period of time, added to its enhanced diagnostic

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sensitivity and cardiac specificity, allows for the detection of AMI much earlier after the onset of ischemia (4 hours),^{1,25} as well as the diagnosis of peri-operative infarction in situations where a high serum level of skeletal muscle proteins are expected. (17)

Additionally, recent data have identified a measurable relationship between cardiac troponin levels and long-term outcome after an episode of chest discomfort. (24,29) The studies suggest that the use of the cTnI demonstrates high predictive value in delineating the high risk group of unstable angina patients,⁽³⁰⁾ and that these tests may be particularly useful in evaluating patient condition prior to discharge from the ED. (25,29,31)

The cTnI Enzyme Immunoassay provides a rapid, sensitive, and reliable assay for the quantitative measurement of cardiac-specific troponin I. The antibodies developed for the test will determine a minimal concentration of 1.0 ng/ml, and there is no cross-reactivity with human cardiac troponin T or skeletal troponin T or I.

PRINCIPLE OF THE TEST

The cTnI ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes four unique monoclonal antibodies directed against distinct antigenic determinants on the molecule. Three mouse monoclonal anti-troponin I antibodies are used for solid phase immobilization (on the microtiter wells). The fourth antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the four antibodies, resulting in the troponin I molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 90-minute incubation at room temperature, the wells are washed with water to remove unbound-labeled antibodies. A solution of tetramethylbenzidine (TMB) Reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N hydrochloric acid (HCl) changing the color to yellow. The concentration of troponin I is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

INSTRUMENTATION

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

STORAGE

1. Store the 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. **DO NOT FREEZE**
2. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

LIST OF COMPONENTS

Materials Provided with the Kit:

- Antibody-Coated Wells (1 plate, 96 wells)
Microtiter wells coated with mouse monoclonal anti-TnI.
- Reference Standard Set (1 set, 1.0 ml/vial)
Contains 0, 2.0, 7.5, 30, and 75 ng/ml TnI, lyophilized.
- cTnI Enzyme Conjugate Reagent (13 ml/vial)
Contains mouse monoclonal anti-TnI conjugated to horseradish peroxidase in Tris Buffer-BSA solution with preservatives.
- TMB Reagent (11 ml/bottle)
Contains one-step TMB solution.
- Stop Solution (11 ml/bottle)
Contains diluted hydrochloric acid (1N HCl).

Materials Required but not provided:

- Distilled or deionized water
- Precision pipettes: 5 µl, 10 µl, 50 µl, 100 µl and 1.0 ml
- Disposable pipette tips
- Microtiter well reader capable of reading absorbance at 450 nm.
- Vortex mixer, or equivalent
- Absorbent paper
- Graph paper
- Cardiac Marker Tri-level Control; Cat. No. 685 (Bio-Rad Laboratories Diagnostics Group, Hercules, CA 94547)

SPECIMEN COLLECTION AND PREPARATION

1. The use of SERUM samples is required for this test.
2. Specimens should be collected using standard venipuncture techniques. Remove serum from the coagulated or packed cells within 60 minutes after collection.
3. 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.

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4. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples (after centrifugation).
5. 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.

REAGENT PREPARATION

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Reconstitute each lyophilized standard with 1.0 ml distilled water. Allow the reconstituted material to stand for at least 20 minutes and mix gently. **The Reconstituted standards will be stable for up to 21 days when stored sealed at 2-8°C.** Discard the reconstituted Standards after 21 days. **To assure long term (more than 21 days) maximum stability of the reconstituted Standards, they should be aliquoted and frozen (-20°C or below) immediately after reconstitution has been achieved. Each aliquoted Standard should be frozen and thawed only once.**
3. Samples with expected Troponin I concentrations over 100 ng/ml may be quantitated by dilution with diluent available from vender.

PROCEDURAL NOTES

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

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards, specimens, and controls into appropriate wells.
3. Gently mix for 10 seconds.
4. Dispense 100 µl of Enzyme Conjugate Reagent into each well.
5. Thoroughly mix for 30 seconds. It is very important to mix completely.
6. Incubate at room temperature (18-25°C) for 90 minutes.

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7. Remove the incubation mixture by flicking plate contents into a waste container.
8. Rinse and flick the microtiter wells 5 times with distilled or deionized water. (Please do not use tap water.)
9. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
10. Dispense 100 μ l of TMB Reagent into each well. Gently mix for 10 seconds.
11. Incubate at room temperature for 20 minutes.
12. Stop the reaction by adding 100 μ l of Stop Solution to each well.
13. Gently mix for 30 seconds. **It is important to make sure that all the blue color changes to yellow color completely.**
14. Read absorbance at 450nm with a microtiter well reader **within 15 minutes.**

CALCULATION OF RESULTS

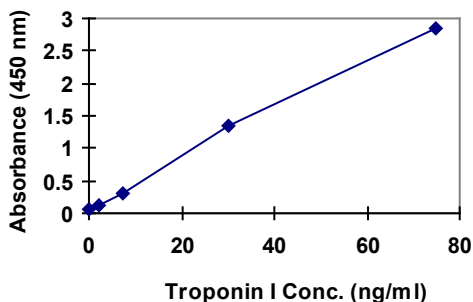
1. Calculate the mean absorbance value (OD450) for each set of reference standards, controls and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of troponin I (ng/ml) from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
4. Patient samples with cTnI concentrations greater than 75 ng/ml should be diluted 10-fold with vender's Troponin I Sample Diluent. The final cTnI values should be multiplied by 10 to obtain cTnI results in ng/ml.

STANDARD CURVE

Quality control specimens (controls) must be run with each calibration curve to verify assay performance. To ensure proper performance, control material should be assayed repeatedly to establish mean values and acceptable ranges. *Results of a typical standard run with absorbency readings at 450nm shown on the Y axis against troponin I concentrations shown on the X axis. NOTE: This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve in each experiment.*

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cTnI (ng/ml)	Absorbance (450 nm)
0	0.048
2.0	0.110
7.5	0.307
30	1.357
75	2.853



EXPECTED VALUES AND SENSITIVITY

An evaluation of the clinical data was conducted to determine the normal expected value, as well as the clinical sensitivity and clinical specificity of the BioCheck cTnI assay (see below). Two-hundred and twenty-five (225) apparently healthy adults were assayed using the BioCheck test to establish the normal expected value, which was determined to be ≤ 0.5 ng/ml cTnI. All values from the normal population tested were below the sensitivity level of the assay (1.0 ng/ml).

It is recommended that each laboratory establish its own normal range based on the patient population, geography, dietary and environmental factors; likewise current practice and clinical criteria for AMI diagnosis must be considered. However, based on published literature, the diagnostic cut-off for AMI patients is determined to be 1.5 ng/ml.⁽³⁵⁾ Any conditions resulting in myocardial cell damage can potentially increase cardiac troponin-I levels above the expected value. These conditions have been documented clinically to include unstable angina, myocarditis, congestive heart failure, and cardiac surgery or invasive testing. (3,29)

NOTE: Serial sampling may be required to detect elevated levels.

STANDARDIZATION

Human Troponin I-T-C Complex was obtained from a qualified vendor, and cTnI concentration was determined. The material was further diluted with the cTnI Sample Diluent and served as “Standard Stock Solution” for preparing cTnI reference Standard Sets. The target value of the “Standard Stock Solution” was confirmed by the Abbott AxSym Troponin I immunoassay.

PERFORMANCE CHARACTERISTICS

1. Sensitivity

The minimum detectable concentration of the cTnI ELISA assay as measured by 2SD from the mean of a zero standard is estimated to be 1.0 ng/ml. Additionally, the functional sensitivity was determined to be 0.75 ng/ml ((as determined with inter-assay %C.V. $\leq 10\%$). **Lower limit of cTnI ELISA \approx 0.48 ng/ml cTnI; upper limit = 1.0 ng/ml cTnI.**

2. Hook Effect

No hook effect was observed in this assay at cardiac troponin-I concentrations up to 10,000 ng/ml.

3. Precision

a. Intra-Assay Precision

Within-run precision was determined by replicate determinations of four different serum samples in one assay. Within-assay variability is shown below:

Samples	1	2	3	4
# Reps.	20	20	20	20
Mean cTnI (ng/ml)	5.93	24.3	44.9	89.8
S.D.	0.22	1.35	1.78	2.52
C.V. (%)	3.7	5.6	4.0	2.8

b. Inter-Assay Precision

Between-run precision was determined by replicate measurements of four different serum samples over a series of individually calibrated assays. Between-assay variability is shown below:

Samples	1	2	3	4
# Replicates	26	26	26	26
Mean cTnI (ng/ml)	5.88	24.56	48.91	85.81
S.D.	0.28	1.14	2.23	3.76

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C.V. (%)	4.8	4.7	4.6	4.4
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4. Specificity

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

Material Tested	Test Concentration
Rabbit skeletal muscle troponin C	2,500 ng/ml
Human cardiac troponin T	2,500 ng/ml
Human skeletal muscle troponin T	2,500 ng/ml
Human skeletal muscle troponin I	2,500 ng/ml
Hemoglobin	1.2 g/dl
Bilirubin	20 mg/dl
Cholesterol	500 mg/dl
Triglyceride	1,000 mg/dl
Total Protein	10 g/dl

5. Recovery Studies

Various patient serum samples of known Estradiol levels were combined and assayed in duplicate. The mean recovery was 101.3%.

PAIR NO.	EXPECTED [cTnI] (ng/ml)	OBSERVED [cTnI] (ng/ml)	% RECOVERY
1	4.25	3.95	92.9%
2	8.97	8.50	94.8%
3	11.43	10.49	91.8%
4	14.97	14.00	93.5%
5	32.34	29.62	91.6%
6	32.77	30.49	93.0%
7	81.00	77.21	95.3%

6. Linearity Studies

Four patient samples were serially diluted to determine linearity. The mean recovery was 101.7%.

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#	Dilution	Expected Conc. (ng/ml)	Observed Conc. (ng/ml)	% Expected
1.	Undiluted	----	----	----
	1:2	74.9	74.9	100%
	1:4	37.5	37.4	99.7%
	1:8	18.7	19.3	103.2%
	1:16	9.4	9.9	105.3%
	1:32	4.7	5.0	106.4%
	1:64	2.4	2.6	108.3%
	1:128	1.2	1.3	108.3%
				<i>Mean = 105.3%</i>
2.	Undiluted	----	----	----
	1:2	68.4	68.4	100.0%
	1:4	34.2	34.2	100.0%
	1:8	17.1	17.6	102.9%
	1:16	8.6	8.5	98.8%
	1:32	4.3	4.4	102.3%
	1:64	2.2	2.4	109.1%
	1:128	1.1	1.2	109.1%
				<i>Mean = 103.2%</i>
3.	Undiluted	----	----	----
	1:2	----	----	----
	1:4	62.5	64.0	102.4%
	1:8	31.3	31.3	100.0%
	1:16	15.6	14.5	92.9%
	1:32	7.8	7.2	92.3%
	1:64	3.9	3.7	94.9%
	1:128	1.9	2.0	105.3%
				<i>Mean = 98.0%</i>
4.	Undiluted	----	----	----
	1:2	----	----	----
	1:4	86.4	88.0	101.9%
	1:8	43.2	43.1	99.8%
	1:16	21.6	21.7	100.5%
	1:32	10.8	10.2	94.4%
	1:64	5.4	5.4	100.0%
	1:128	2.7	2.8	103.7%
				<i>Mean = 100.1%</i>

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ASSAY DIAGRAM

