Validation of Serum Cardiac Troponin I Reference Materials Using an Immunoprecipitation Method Coupled with Fluorescent Western Blot Analysis

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List of Abbreviations: cTnI, cardiac troponin I; cTnT, cardiac troponin T; AACC, American Association for Clinical Chemistry; SRM, standard reference material; NIST, National Institute of Standards and Technology; IFCC, International Federation of Clinical Chemistry.

Abstract

Cardiac troponin I (cTnI) is adopted as the 'gold standard' cardiac biomarker following the universal definition of myocardial infarction (MI). Presently, the performance of commercial cTnI immunoassays varies significantly despite the recalibration using a troponin standard reference material, NIST SRM 2921. To standardize the cTnI immunoassays, a secondary reference material consisting of a panel of cTnI-positive human serum pools, is proposed by IFCC Working Group for Standardization of Troponin I (IFCC WG-TNI) for assay manufacturers to reference their assay calibrations and for improving intra-assay comparability and harmonization. The establishment of a reference measurement procedure for cTnI is required for the value assignment of the cTnI concentration of the proposed secondary reference material.

We developed an immunoprecipitation method coupled with fluorescent western blot analysis, which is designed to reduce the interference from cTnI heterogeneity and cTn autoantibodies and determine the cTnI concentration. We used magnetic beads coupled with 6 different anti-cTnI monoclonal antibodies that bind specifically to different amino acid sequence regions of the cTnI molecule to immunoprecipitate the cTnI proteins from a cTnI positive serum pool sample followed by sensitive detection using a fluorescent western blot. The cTnI degradation in the positive pool sample was confirmed and a total concentration of cTnI was determined. Moreover, cTnI and cTn autoantibodies were found in the same pool sample by a slightly modified method. We demonstrated the utility of this method for supporting the development of the secondary cTnI-positive serum based reference material for the standardization of clinical cTnI immunoassays.

Key Words: troponin; serum-based reference material; immunoprecipitation; western blot; validation.

Introduction

Since the first report of the use of cardiac troponin I (cTnI) for diagnosis of myocardial infarction (MI) in 1987, cTnI has currently been adopted as the 'gold standard' cardiac biomarker following the universal definition of myocardial infarction (1, 2). It is therefore important that cTnI is measured with highly reliable and standardized methods to achieve comparability of results and independence of test reagents, platforms, and the laboratories where the procedure is carried out (3). Though only one cTnT assay is available from Roche Diagnostics due to patent licensing, more than twenty different cTnI immunoassays are currently marketed by various vendors. All of these assays are 'sandwich' type immunoassays where either an immobilized antibody or two different kinds of immobilized antibodies specifically bind the cTn (I or T) present in human serum or plasma. The captured cTn is then reacted with one detection antibody or in some cases, with two different types of detection antibodies that are further coupled to an indicator molecule. Several of these assays utilize monoclonal, anti-human cTnI antibody pairs with high binding specificities to the invariant part of the cTnI molecule (amino acid residues 30 to 110) (4, 5).

Due to the use of various commercially available diagnostic cTnI immunoassay kits used in clinics, problems arise from their performance differences in selectivity, specificity and sensitivity (6). The measured cTnI levels showed approximately 20- to 40-fold differences between these assays. Even after recalibration with a standard reference material, NIST SRM 2921, which was developed by AACC cTnI Standardization Committee in collaboration with NIST, harmonization was not improved (7). The large discrepancies are results of various forms of cTnI measurand defined by the binding epitopes of antibodies used in the

assays (complexation and post-translational modifications, e.g. degradation, amino acid phosphorylation and oxidation) (10, 11) and the lack of a human serum pool reference material for method harmonization (7). The differences may also be generated from the different procedures applied to minimize potential interferences associated with different assays platform (8, 9). It is still unknown at the moment the clinical relevance of the individual form of cTnI measurand, i.e. disease specific and/or stage specific. In addition, the effects of the modified products on assay performance are not fully understood (12, 13). All these bring enormous challenges for attaining harmonization and standardization of current commercial assay performance. Understanding of the various cTnI forms will likely affect the design of future clinic immunoassays.

To investigate the feasibility of a comparable and stable cTnI-positive serum pool reference material and to establish a candidate immunoassay reference measurement procedure for cTnI concentration assignment, the IFCC has created a new Working Group for Standardization of Troponin I (IFCC WG-TNI) (7, 14). As part of the effort, we have developed various measurement techniques to investigate binding affinity between six monoclonal antibodies (mAb) each serving as either the capture or the detection antibody and cTnI in the form of either a reference material (NIST SRM 2921) or a cTnI-positive serum pool (PS) to support the development of the reference immunoassay procedure. The objective of the present study is to develop measurement methods that facilitate the characterization of a stable cTnI-positive serum pool reference material that could be used to ensure comparable results between the different assays. The methods developed are based on immune-precipitation and fluorescence western blot analysis enabling us to evaluate the extent of degradation and total concentration of cTnI. These methods will allow the

investigation of cTnI stability over temperature and other storage conditions and characterization of different serum pool samples.

Materials and methods

Antibodies and serum samples

Six purified monoclonal anti-human cTnI antibodies, clone 3C7, 267, 560, MF4, 19C7 and M18 were purchased from HyTest, Turku, Finland. A patient cTnI-positive serum pool was provided by Dr. Robert Christenson at University of Maryland School of Medicine with IRB approval and in accordance with CLSI document C37a for the current investigation. The normal serum pool was obtained from NCI, NIH (Frederick, MD).

Western blot analysis

The electrophoresis gels containing proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (describe apparatus- time and current); the membrane was blocked for 1 hr at room temperature in PBS with 5 g/L BSA (Sigma) and 1 mL/L Tween 20. The troponin I protein was subsequently analyzed by enhanced chemiluminescence (ECL) or fluorescence based western blot analysis.

The ECL based western blot was performed using the anti-cTnI primary antibody, followed by the horseradish peroxidase (HRP) - labeled secondary antibody for chemiluminescence detection. Briefly, blocked membranes were incubated for 16 h at 4 °C with primary antibodies at a concentration of 1 mg/L each. Membranes were then washed and incubated with 1:10,000 dilution of anti-mouse IgG conjugated to HRP for 60 min at room temperature. After wash, bound antibodies were detected with an ECL Western blot analysis detection system (GE).

The modified fluorescence based western blot analysis was performed with the use of biotinylated primary antibodies (at a concentration of 1 mg/L), followed by incubation with the phycoerythrin-labeled streptavidin (SA-PE, at a concentration of 0.1 mg/L). The time course was kept the same as for the ECL based western blot. Final fluorescence detection was carried out with a HITACHI FMBIO III multiview image scanner (MiraiBio Inc. Alameda, CA). The biotinylated primary antibodies were prepared according to the manufacturer-recommended procedure of FluoReporter Mini-Biotin-XX-Protein Labeling Kit from Invitrogen (Carlsbad, CA). The fluorescent band intensity was quantified using FMBIO Image Analysis 3.0 software according to the instructions. The cTnI analyte concentration was calculated from troponin I band intensity in accordance with the calibration curve of SRM 2921.

Detection of serum cTnI by western blot – direct serum assay (WB-DSA)

The assay was performed under denaturing and reducing conditions according to the procedures of Madsen et al (15, 16). Briefly, positive or normal serum was diluted in a sample buffer containing 16.5 g/L sodium dodecyl sulfate, 16.5 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 16.5 mL/L Nonidet P-40, 0.5 mol/L dithiothreitol, 1 mol/L urea, 0.25 mol/L Tris-HCl (pH 6.8), 0.01 gm/L bromophenol blue, and 500 ml/L mL glycerol. The serum was resolved by 14% (mass fraction of acrylamide) SDS- polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Gel-resolved proteins were then transferred onto a PVDF membrane in the presence of 10 mmol/L CHAPS, pH 11. The western blot procedure was then performed as described in the method of western blot analysis using biotinylated antibody clone M18, 560 and MF4 against cTnI as primary antibodies with the fluorescence detection method instead of the ECL method used by Madsen and coworkers (15, 16).

Screening immunoprecipitation efficiency of antibodies against troponin I

Six μg of human cardiac troponin complex SRM 2921 was diluted in an immune precipitation buffer (PBS, pH 7.4, supplemented with 0.1 g/L BSA, 1 mL/L Triton X-100, 0.1 g/L sodium azide, and the protease inhibitor cocktail set I from Roche) and incubated with 5 μg of one of the 6 purified monoclonal anti-human cTnI antibodies (clones 3C7, 267, 560, MF4, 19C7 or M18) for 16 h at 4 °C, followed by addition of 20 μL protein G-agarose (Upstate Biotechnologies, Inc.) for 90 min at 4 °C. The immunocomplexes on agarose beads were recovered by centrifugation at 5,000g for 5 min followed by resuspension in buffer to wash them. The immunocomplexes were washed twice with the immune precipitation buffer, two times with 25 mmol/L of HEPES, pH 7.6, 1 mL/L Triton X-100, 0.15 mol/L of NaCl, and finally twice with 25 mmol/L HEPES, pH 7.6, 1 mL/L Triton X-100. The immunoprecipitated complexes were freed from the agarose beads by heating in SDS sample buffer and then separated by 14% (mass fraction of acrylamide) SDS- PAGE under reducing conditions. The gels were stained using EZBlue Gel Staining reagent (Sigma, St. Louis, MI). The gels were imaged using a 12-bit CCD camera and the band intensity was quantified using Gel-Pro Analyzer from Media Cybernetics (Bethesda, MD) according to the manual instructions. The immunoprecipitation efficiency of antibodies was calculated as a ratio of the band intensity of cTnT to the band intensity of the heavy chain of the antibody used.

Detection of cTn autoantibody

Troponin SRM 2921 or cTnI protein (Abcam Inc, Cambridge, MA) was buffer exchanged and immobilized on the MyOne™ Tosylactivated Dynabeads® from Invitrogen according to the manufacturer's protocol except for the absence of BSA in all of the buffers. Capture of the autoantibodies in the positive serum pool was done

using, Dynabeads (coupled with either 10 μg of cTnI protein or 10 μg of troponin complex SRM 2921). The beads were incubated with 200 μL positive serum pool in 800 μL buffer containing 25 mmol/L of HEPES, pH 7.6 supplemented with 1 mL/L Triton X-100 at 4 °C overnight. Dynabeads without protein coupling served as the negative control. The Dynabeads were collected with a magnet and washed twice with 25 mmol/L of HEPES, pH 7.6, 1 ml/L Triton X-100 supplemented with 0.15 mol/L of NaCl, followed by washing twice with 25 mmol/L of HEPES, pH 7.6, 1 mL/L Triton X-100. The immunoprecipitated complexes were freed from the beads by boiling in SDS sample buffer for 5 min eluted and resolved by 4~12% SDS-PAGE under the reducing condition. Gel-resolved proteins were then transferred onto a PVDF membrane and probed with biotinylated goat anti-human IgG (Invitrogen) at a concentration of 0.5 mg/L, followed by incubation with the phycoerythrin-labeled streptavidin (SA-PE) and subsequent fluorescence detection.

Immunoprecipitation with Dynabeads coupled with anti-human cTnI antibodies

Immunoprecipitation were performed using the Dynabeads coupled with the antibodies against human cTnI in serum sample solutions (positive serum or troponin SRM 2921 spike in normal serum). The coupling of the antibodies to the Dynabeads was according to the manufacture's protocol (Invitrogen) and a ratio of 40 μg antibody per 1 mg MyOne™ Tosylactivated Dynabeads.

The immunoprecipitation procedure was done by adding 1 μL of each antibody coupled Dynabeads (~ 7 μg beads/μL at a concentration of approximately 2 pmol antibody/μL beads) to a serum sample (50 to 400 μL) in 800 μL buffer solution containing 25 mmol/L of HEPES, pH 7.6, supplemented with 1 mL/L Triton X-100 followed by incubation overnight at 4 °C. The Dynabeads were collected (magnet) and washed twice with 25 mmol/L of HEPES, pH 7.6, 1 mL/L Triton X-100

supplemented with 0.15 mol/L of NaCl, followed by twice with 25 mmol/L of HEPES, pH 7.6, 1 mL/L Triton X-100. The immunoprecipitated complexes were freed from the Dynabeads by heating with SDS-PAGE sample buffer and ready for further analysis.

Results

Anti-cTnI antibody selection

The location of epitopes recognized by 6 different mAbs used in the present study is shown in the supplemental Fig. 1. Two of the mAbs have epitopes located in the stable and invariant part (amino acid residues 30-110) of cTnI, residues 41-49 for 19C7 and residues 83-93 for 560, respectively. Two of the mAbs have N-terminal epitopes, residues 18–28 for M18 and residues 25-40 for 3C7, respectively. Two of the mAbs have C-terminal epitopes, residues 169-178 for 267 and 190-196 for MF4, respectively. All six antibodies recognize both free cTnI and cTnI in complex forms.

Western blot analysis with ECL or fluorescence detection

ECL is commonly used method for the routine protein detection in western blots. The emitted light is captured on film or by a CCD camera making quantitation difficult and limiting sensitivity. Fluorescence is a very sensitive, photo-induced spectral analysis method where the molecules of the analyte are excited by irradiation at a certain wavelength and emit at a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis. Fluorescence detection method provides a high sensitivity and low limits of detection.

We compared the two detection methods for cTnI measurement. Using our conditions the fluorescence detection was 5 to 10 fold more sensitive compared to the ECL method because of the lower background of the fluorescence method

(Supplemental Fig. 2). Hence, we used fluorescence detection method for western blot analysis in the following measurements.

Recognition of proteolytic fragments of cTnI in SRM 2921 by different mAbs

cTnI and its proteolytic fragments in troponin complex SRM 2921 were visualized by mAbs with different epitope specificities (Fig. 1). We were able to differentiate four immunoreactive cTnI bands with molecular masses ranging from 14 kDa up to 27–28 kDa. Antibodies with epitopes located in the N-terminal (MAb M18 and 3C7) and C-terminal (267 and MF4) regions of the molecule recognized only limited numbers of cTnI bands. M18 and 3C7 show only the intact cTnI band, and mAb 267 and MF4 react with the intact cTnI and a lower molecular mass degradation product (arrows in Fig. 1). Moreover, mAb 19C7 and 560, which recognize residues 41–49 and 83-93 of cTnI, respectively, are able to detect up to 4 different cTnI bands (Fig. 1) even though the detection sensitivity of mAb 19C7 is much lower than that of mAb 560. The results demonstrate that the N- and C-terminal peptides of cTnI are easily removed from cTnI during proteolysis even if cTnI is in the complex forms.

Detection of serum cTnI by western blot – direct serum assay (WB-DSA)

Van Eyk et al had successfully applied WB-DSA detecting troponin modification from patients with acute myocardial infarction and performing the time course of cTnI degradation in patients with acute ST-elevation myocardial infarction (STEMI) (15, 16). However, we did not observe any significant difference between positive serum and normal serum (Supplemental Fig. 3). One explanation is that the different antibody clones we used may have different specificities and lower sensitivity for the cTnI detection.

The detection limit of the fluorescence method for cTnI is 0.05 ng/well or 25 μg/L cTnI (considering 2 μL net serum loaded in each well). Taking into account of

an undetectable cTnI level in the present normal serum sample by both immunoprecipitation and multiplexed bead arrays, the result suggests that the cTnI concentration in our positive serum pool sample may be lower than the detection limit of this method. It is therefore necessary to enrich the amount of cTnI in the positive serum sample. On the other hand, two bands at positions similar to molecular weight of antibody's heavy chain and light chain in Lane 1 to 6 were shown up for both normal and positive serum (Supplemental Fig. 3). These interference signals are not specific to cTnI and are likely generated from antibodies in serum samples. it is necessary to decrease such interference. To minimize this type of interference, we developed a method of immunoprecipitation coupled with western blot analysis (described in next section).

Screening immunoprecipitation efficiency of antibodies against troponin I

Immunoprecipitation was performed with the incubation of mAb and troponin SRM 2921 in solution to allow the immunocomplex to form and then followed by precipitation with protein-G agarose. The precipitated complexes were separated by SDS- PAGE and then subjected to protein staining. Because the cTnI band is very close to band of the light chain of antibodies in some cases as shown in Fig. 2, we evaluated the binding strength between the mAbs and troponin complex by the ratio of the band intensity of cTnT to the band intensity of the heavy chain of the antibody used. All 6 antibodies bind to the troponin complex and the binding strength in solution as measured by the stained intensity ratios of cTnT band to the antibody heavy chain (means are in the parenthesis with one standard deviation) are:, MF4 (1.08±0.11) > 560 (0.92±0.06) > 267 = 19C7 (0.89±0.06) > 3C7 (0.58±0.05) > M18 (0.56±0.03).

Detection of cTn autoantibody

In recent publications, Pettersson et al have shown that autoantibodies to cTn are commonly found in both healthy control subjects and hospitalized patients with or without a history of cardiac disease (17, 18). These autoantibodies were found to bind to the stable invariant part (amino acids 30 to 110) and even more so to the C-terminal part of cTnI (19). A recent publication also reveals autoantibodies that recognize troponin T (20). Although these reports have showed that autoantibodies inhibit the binding of cTnI-specific antibodies to the ternary cardiac troponin complexes, it was not ascertained if the autoantibodies are produced against specific epitopes of the cTnI molecule. In this study, we designed a method to detect the cTn autoantibodies using purified cTnI or troponin SRM 2921 coupled Dynabeads. The presence of the autoantibodies in the cTnI positive serum pool sample are shown by the bands of the heavy and light chains of the autoantibodies in Lane 2 and 3 (Fig. 3). It is worth noting that the stronger IgG bands for SRM 2921 coupled Dynabeads (lane 3) compared to than those for purified cTnI coupled beads (Lane 2), suggesting the presence of autoantibodies against troponin complexes and/or component of the complexes. The recent studies have shown that the prevalence of troponin autoantibodies that bind the stable region might interfere with the immunoassays designed for recognition of cTnI in the stable invariant region.

Validation of cTnI measurement using immunoprecipitation coupled with fluorescent western blot analysis

To overcome the complications resulted from troponin autoantibodies, troponin I modifications and association with other proteins, we established a validation method based on immunoprecipitation coupled with fluorescent western blot analysis. Two different experimental designs are shown in Fig.4,

immunoprecipitation with an increment of positive serum volume and with different amount of spike-in troponin complex in normal serum. We observed that the intensity of the lower molecular weight band increased with increasing the positive serum volume. This finding indicates the degradation of cTnI in positive serum pool with respect to SRM 2921 although we do not know if the degradation occurs because of a consequence of a disease specific modification or it is due proteolysis during the sample collection and/or storage process. The concentration of cTnI in the present positive serum pool sample is estimated to be 2 μg/L with an error of 10 to 20% according to the calibration curve generated by the intensity of the bands from SRM 2921 standard. This concentration value is comparable to the result estimated from the multiplex bead arrays (14).

Discussion

The clinical diagnostics of myocardial infarction is impeded by the discrepancies between cTnI values obtained using different commercially available immunoassay kits. The use of different antibodies (some of which recognize epitope regions prone to degradation) is likely to be one of the main reasons for the observed assay discrepancies. In the present study, using antibodies recognizing different regions of troponin I, we have confirmed that 3 different degradation products in addition to the intact cTnI are present in troponin SRM (Fig. 1). Substantial heterogeneity of molecular mass distribution of the cTnI has been observed for SRM 2921 by LC-MS analysis (21). The modified cTnI forms observed in SRM 2921 closely resemble those found by our western blot analysis; SRM 2921 appears to contain more C-terminally truncated products. Nevertheless, a N-terminal-truncated product band was observed only by our western blot analysis. Only with the use of the

immunoprecipitation method coupled with fluorescent western blot analysis, we observed 2 cTnI bands, intact cTnI band and N-terminal degradation product band, for the positive serum pool sample (data not shown). This observation could be due to the limited amount of cTnI present in positive serum pool sample and limited detection sensitivity of our assay. The mAb 560 (recognizes residues 83-93 of cTnI) is able to detect up to 4 product bands only at the cTnI loading quantity higher than 1 ng/well. Moreover, we observed a more intense cTnI degradation band than the intact band in the positive serum pool compared to the SRM 2921 (Fig. 4). This result is consistent with literature that some modifications of cTnI were observed in the myocardium of animal models and bypass patients, and up to 8 degradation fragments from both N- and C- terminal were presented along with intact cTnI in patients suffering from ST-elevation myocardial infarction (15, 16).

The dominant complex forms of cTnI are non-covalent ternary complex cTnT-I-C (TIC complex) and binary complex cTnI-C (IC complex) in serum as suggested by our recent study (14). The region of cTnI that interacts with TnC is presumably localized within amino acid residues 33–53, 129–150 or 161–181 of cTnI (22, 23) whereas the region within the residues 33–80 is likely to contain sites of interaction with cTnT (22). The human troponin autoantibodies, on the other hand, also interferes with the binding of assay antibodies to the central region of cTnI (19). In the present study, autoantibodies were found in the cTnI positive serum pool sample using either the tissue-derived ternary troponin complex SRM 2921 coupled or purified free cTnI coupled Dynabeads (Fig. 3). The more intense autoantibody bands in the case of SRM 2921 coupled beads suggest that these autoantibodies directly bind not only to cTnI protein but also to TnC or cTnT.

In addition to the N- and C-terminal truncations due to proteolysis and the presence of autoantibodies, the cTnI stable region also contains two cystine residues at positions 80 and 97 that could be subject to modification. Oxidation of the sulfhydryl groups of the cystine amino acids could affect the interaction of cTnI with other cTn proteins and binding with antibodies in commercially available cTnI assays (24). A mis-sense mutation (P82S) is also known in the cTnI stable region, possibly affecting the immunoassay performance (25).

Because of all these complications associated with cTnI protein described above, the current recommendation to use only cTnI antibodies directed to the stable, mid-fragment epitopes (26, 27) may be the most logical choice to lead to a substantial increase in the homogeneity of cTnI immunoassay results. However, there are still many challenges to be resolved such as production and characterization of antibodies with higher binding specificity for cTnC, cTnT, the reduction of interference by autoantibodies, and assay conditions to enhance the binding between antibodies and cTnI (14).

Considering that cTnI is heterogeneous and not well defined target, a practical approach for the standardization of clinical cTnI measurements is to build a complete reference measurement system (3). This measurement system includes a secondary cTnI-positive serum based commutable reference materials and a reference immunoassay measurement procedure for a value assignment of the comparable reference materials, along with the primary reference material, SRM 2921. The reference procedure will utilize a pair of well-characterized mAbs with high binding specificities to the stable invariant region of the cTnI molecule. The development of the positive serum based commutable reference material requires not only a set of measurement techniques for evaluating different pooling methods and sample stability

over time and storage conditions (with or without a protease inhibitor cocktail), but also the prudent coordination of the commercial cTnI assay method comparison in parallel with the reference immunoassay procedure.

For these reasons, we developed an immunoprecipitation procedure coupled with a fluorescent western blot procedure that has a reasonable sensitivity of detecting the cTnI in the positive serum pool from patients with diagnosed MI, a measurement that currently mass spectrometry methods are unable to do.

The most abundant proteins in serum such as albumin and IgG are 100,000 times more concentrated than cTnI, and thus may lead to non- specific binding and interference in the assay. Our procedure overcomes the interference problem from the abundant proteins in serum that the WB-DSA in its current form did not.

The discrepancy between our results and published data is most likely due to the differences in the sensitivities of different antibodies from different sources. To capture as many forms of cTnI as possible, we combined the antibodies recognizing all 6 different epitopes of cTnI as capture antibodies in our assay system. We optimized this method so that only 100 μL serum was required for the reliable detection of cTnI. We found that a stronger degradation band was present in the positive serum with regard to SRM 2921. This finding further verified the complexity of cTnI in the positive patient serum pool and the necessity of using antibodies recognizing different epitopes of cTnI for the evaluation of the true cTnI concentration.

Our measurement procedure requires time and a number of steps; it is a robust method in that it provides information about the degradation of cTnI and total cTnI concentration. A protease inhibitor changes the results indicating that the proteolysis is present in the patient samples or occurred during storage and not during this

analytical procedure. This procedure will be a valuable tool for the selection and preparation of the secondary cTnI-positive serum reference material for the standardization of the cTnI assays.

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Figure legends

Figure 1. Recognition of proteolytic fragments of cTnI in troponin complex SRM 2921 by different mAbs. Various amount of SRM 2921 was diluted in PBS buffer, pH 7.4 supplemented with 1 g/L BSA, separated using 14 % gradient SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane. Different mAbs were used to visualize bands corresponding to cTnI proteolytic peptides. The apparent degradation products are marked by arrows. The epitope for each mAb is shown in parenthesis.

Figure 2. Immunoprecipitation efficiency screening for six different antibodies against troponin I. Human cardiac troponin complex SRM 2921 (6 μg) was diluted in the immune precipitation buffer and incubated with 5 μg of the purified monoclonal anti-human troponin I antibodies. The immunoprecipitated complexes were separated by 14% SDS- PAGE under reducing conditions and then subjected to staining with EZBlue Gel Staining. The mAb antibody used for screening: lane 1, 3C7; lane 2, 267; lane 3, 560; lane 4, MF4; lane 5, 19C7; lane 6, M18. SRM refers to troponin complex, NIST SRM 2921 and M is a protein molecular weight marker.

Figure 3. cTn autoantibody detection in positive serum pool. Dynabeads® coupled with either 10 μg of cTnI protein, 10 μg of troponin complex SRM 2921, or no-protein (control) were incubated with 200 μL positive serum pool in 800 μL buffer solution containing 25 mmol/L of HEPES, pH 7.6 supplemented with 1 mL/L Triton X-100. The captured antibody was visualized by goat anti-human IgG (H+L)-biotin conjugate, followed by incubation with the phycoerythrin-labeled streptavidin (SA-

PE). Loading sequence: Lane 1, control with Dynabeads alone; Lane 2, cTnI coupled Dynabeads; Lane 3, troponin SRM 2921 coupled Dynabeads.

Figure 4. Use of the immunoprecipitation-coupled fluorescent western blot for the determination of the cTnI concentration in the positive serum pool sample. Six different antibody coupled Dynabeads® (1 µL each bead population) at a concentration of ~2 pmol antibody/µL beads were added to a serum sample (50 to 400 µL) and incubated in 800 µL buffer solution containing 25 mmol/L of HEPES, pH 7.6, supplemented with 1 mL/L Triton X-100 overnight at 4 °C. The immunoprecipitated complexes were resolved by 14% SDS-PAGE, and detected with biotinylated primary antibody clone M18, 560 and MF4, followed by fluorescence western blot analysis. The gel for cTnI detection in positive serum and the gel for troponin SRM 2921 spike in negative serum for constructing a calibration curve were transferred to the same membrane to eliminate possible transfer and detection relevant variations.