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Research Article

Development of a candidate secondary reference procedure (immunoassay based measurement procedure of higher metrological order) for cardiac troponin I: I. Antibody characterization and preliminary validation

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Abstract

In this study, the first steps in the development of a secondary reference measurement procedure (RMP) 'higher metrological order measurement procedure' to support the cardiac troponin I (cTnI) standardization initiative is described. The RMP should be used to assign values to serum-based secondary reference materials (RMs) without analytical artifacts causing bias. A multiplexed bead-based assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to identify the optimum monoclonal antibody pair (clones 560 and 19C7) for the RMP. Using these antibodies, an ELISA-based procedure was developed to accurately measure the main cTnI forms present in blood. The proposed RMP appears to show no bias when tested on samples containing various troponin complexes, phosphorylated and dephosphorylated forms, and heparin. The candidate assay displayed suitable linearity and sensitivity (limit of detection, 0.052 μg/L) for the measurement of the proposed cTnI secondary RMs. Preliminary comparison data on patient samples with a commercial cTnI assay are also provided to support the suitability of RMP for value assignment to RMs. Full validation and final assessment of the RMP will be performed through transferability and intercomparison studies.
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Keywords: antibody specificity; antigen-antibody reactions; candidate reference procedure; ELISA; reference standards; troponin I.

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Nonstandard abbreviations: MI, myocardial infarction; cTnI, cardiac troponin I; WG-TNI, Working Group on Standardization of Troponin I; NPL, National Physical Laboratory; IRMM, Institute for Reference Materials and Measurements; RMP, reference measurement procedure; CRM, certified reference material; SRM, standard reference material; SA-PE, streptavidin-phycoerythrin; AP, alkaline phosphatase; and 4-MUP, 4-methylumbelliferyl phosphate.

Introduction

Increased cardiac troponin concentrations in blood have been reiterated recently by the Global Task Force on the Universal Definition of Myocardial Infarction as the main criterion for the diagnosis of the acute myocardial infarction (MI) (1). It is, therefore, critical that this clinically relevant biomarker, on which important medical decisions are made, is measured with highly reliable and standardized methods as a way to achieve comparable results, independent of the assay used or the laboratory where the test is performed.

At present, cardiac troponin I (cTnI) measurement results are not standardized due to the lack of a complete reference

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measurement system (2). This can lead to a disagreement between results from different commercial assays. Not only is a higher-order level of reference for cTnI required to achieve calibration traceability of cTnI measurement results globally, but also the establishment of a reference measurement system for cTnI would enable calibration without bias of manufacturer's assays. There is now international consensus that the standardization of quantitative measurements in laboratory medicine requires application of a reference measurement system for calibration and validation of routine methods (3–5). Two critical components of the reference measurement system for cTnI, a secondary reference measurement procedure (RMP) and a secondary serum-based certified reference material (CRM) do not currently exist. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group on Standardization of Troponin I (WG-TNI) has been formed to undertake these two issues and fill the gaps in the metrological traceability chain for cTnI measurements.

The diagnostic companies are now producing more analytically sensitive versions of their 'sandwich-type' immunoassays for the measurement of cTnI concentrations in human serum or plasma. These assays utilize either an immobilized antibody or two different kinds of immobilized antibodies to specifically bind the cTnI in the test samples. The captured cTnI is then reacted with one or more detection antibodies that are coupled to an indicator molecule. In the development of a cTnI RMP, we have used two different measurement techniques to characterize the equilibrium binding between six monoclonal antibodies (mAb) and the cTnI molecule. One technique is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and another is the multiplexed bead array that resembles the commercial bead- or particle-based immunoassays. The capture mAb molecules are covalently immobilized on different bead populations to capture cTnI. The bound cTnI is recognized by a biotinylated detection antibody followed by binding of a fluorescent reporter, streptavidin-phycoerythrin (SA-PE). The ease of assay multiplexing and low cost of the instrumentation makes the bead array platform advantageous for the antibody pair optimization for cTnI detection.

The measurement issues associated with immunoassay detection of cTnI have been reviewed (6). In this study, an optimal anti-human cTnI mAb pair resulting from the mAb characterization with high specificities to the invariant part of the cTnI molecule (amino acid residues 30–110) was used to develop the '1 + 1' format candidate RMP in agreement with the IFCC recommendations (7). Details of the progress on the development and assessment of this candidate RMP are provided, with discussion on the specific RMP requirements to certify serum-based cTnI secondary RM.

Materials and methods

Materials

Six purified anti-human cTnI mAbs (clones 3C7, 267, 560, MF4, 19C7 and M18) and the cTnI Diversity Kit were purchased from HyTest (Turku, Finland). The reported location of epitopes recognized by these six different mAbs, obtained from the HyTest data sheet (8), is shown in Supplemental Figure 1. Two of the mAbs have reported epitopes located in the stable and invariant part of cTnI, 19C7 [amino acid (a.a.) residues 41–49] and 560 (a.a. residues 83–93), two of the mAbs have N-terminal epitopes, M18 (a.a. residues 18–28) and 3C7 (a.a. residues 25–40), and two have C-terminal epitopes, 267 (a.a. residues 169–178) and MF4 (a.a. residues 190–196). Human cardiac troponin complex, SRM 2921 was provided by the National Institute of Standards and Tehnology (NIST). A patient cTnI-positive serum pool (PS) (cTnI concentration, 15 μg/L by Dimension RxL assay) was obtained at the University of Maryland School of Medicine with Institutional Review Board approval, and in accordance with Clinical and Laboratory Standards Institute (CLSI) document C37a for the current investigation (9). The normal (undetectable cTnI < 0.02 μg/L, Centaur cTnI-Ultra assay, Siemens, UK) serum pool from healthy patients (NS) was obtained from Bioreclamation (Liverpool, NY, USA). For preliminary comparison studies, anonymous leftover serum samples were provided by Dr. David Gaze from Clinical Blood Sciences, St. George's Hospital and Medical School (London, UK). Reagents for development of the candidate RMP were obtained from Sigma (Poole, UK), unless indicated.

SDS-PAGE

Screening of immunoprecipitation efficiency of mAbs against cTnI was performed by SDS-PAGE. Six micrograms of SRM 2921 were 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 (Burgess Hill, UK) and incubated with 5 μg of one of the six purified anti-human cTnI mAbs for 16 h at 4°C, followed by the addition of 20 μL protein G-agarose (Upstate Biotechnologies, Lake Placid, NY, USA) for 90 min at 4°C. The immunocomplexes on agarose beads were recovered by centrifugation and then washed twice with the immune precipitation buffer, two times with buffer A (25 mmol/L HEPES, pH 7.6, 1 mL/L Triton X-100) supplemented with 0.15 mol/L of NaCl, and finally twice with buffer A. The immunoprecipitated complexes were then separated by 14% SDS-PAGE under reducing conditions. The gels were stained using EZBlue Gel staining reagent (Sigma, St. Louis, MI, USA) and imaged using a 12-bit CCD camera. Band intensity was quantified using Gel-Pro Analyzer from Media Cybernetics (Bethesda, MD, USA) according to the manufacturer's instructions. The immunoprecipitation efficiency of antibodies was calculated as a ratio of the band intensity of cardiac troponin T (cTnT) to the band intensity of the heavy chain of the antibody used (see Results).

Multiplexed bead arrays

Antibody characterization was performed by multiplexed bead arrays. Antibody coupling to the carboxylated beads (-COOH Microspheres, Luminex Corp, Austin, TX, USA) was performed according to the manufacturer's protocol with minor modifications. The coupled beads were stored in a blocking buffer [PBS, pH 7.4, 0.1% (mass fraction) bovine serum albumin, 0.02% (mass fraction) Tween-20, 0.05% (mass fraction) NaN_3] at 4°C in the dark. A FluroReporter Mini-Biotin-XX-Protein Labeling kit from Invitrogen (Carlsbad, CA, USA) was used for the biotinylation of the detection antibody. A commercial Luminex 100 instrument (Luminex Corp) was used to perform the sandwich-type immunoassays detecting

cTnI. The optimized bead array assay protocol was as follows: 12.5 μL of 15 mmol/L EDTA in 0.1 mol/L acetate buffer, pH 5.2, was added, followed by addition of 10 μL NS, 7.5 μL of SRM 2921 spiked in PBS blocking buffer [PBS, pH 7.4, 1% BSA (mass fraction), 0.05% NaN_3 (mass fraction)], or 5–10 μL PS with 12.5–7.5 μL PBS blocking buffer to make up 17.5 μL sample volume, and then 10 μL blocking buffer. After beads (14 μL) were added and incubated for 30 min, a 10-μL biotinylated mAb (8.1×10^4 μg/L) was added. After 30 min, a 10-μL SA-PE (1.0×10^5 μg/L) was added as a fluorescent reporter and incubated for 10 min prior to the fluorescence readout (10).

Development of candidate RMP

The candidate RMP was developed in an ELISA format that can be transferred to clinical chemistry laboratories using reagents obtainable from multiple sources. The candidate RMP was developed in a '1+1' non-competitive, sandwich format (clones 560 and 19C7 were employed as the capture and detection antibodies, respectively) based on the screening data from the SDS-PAGE and bead-based assays described previously. The candidate RMP used an alkaline phosphatase (AP) enzymatic amplification step. The detection antibody '19C7' was labeled with AP from calf intestine using activated-AP (enzyme pre-labeled with chemical agent for coupling to an antibody) from Roche (Cat # 11464752001). The 19C7-AP conjugate was prepared according to the manufacturer's protocol and the resulting conjugates were stored at −20°C.

High protein binding 96-well plates (Greiner Bio One, Stonehouse, UK), either black or white plastic for fluorescent or chemiluminescent measurements were incubated with 100 μL of 2.5 mg/L of clone 560 mAb prepared in PBS, and stored overnight at 4°C. The plate was then washed with 300 μL washing buffer 'WB' [20 mmol Tris-HCl, 150 mmol NaCl and 0.05% Tween-20 (mass fraction)] and repeated for three cycles using a plate washer. Each well was blocked with 250 μL of T20 Superblock (Pierce, Rockford, IL, USA) and incubated on a plate shaker (30 rpm) for 90 min at ambient temperature.

Patient serum samples were thawed following storage at −80°C and allowed to equilibrate to room temperature before centrifugation at 5000 *g* for 10 min to sediment any precipitated material. Calibration standards using SRM 2921 were prepared by gravimetric dilution of the standard stock into either a serum substitute 'SS' [WB substituted with 1 mmol $CaCl_2$ and 7.5% (mass fraction) BSA-essentially protease free], or pooled serum from male donors (cTnI <0.02 μg/L, Centaur cTnI-Ultra). Calibration standards and diluents were prepared fresh immediately before assaying the test samples. Fifty microlitre of test samples and standards were mixed separately with an equal volume of detection antibody prepared at 0.5 mg/L in WB supplemented with 10 mg/L mouse IgG. This mixture was applied to the washed test plate and incubated for 60 min at 31°C. The plate was washed for five-cycles and the AP substrate was added as described. The fluorogenic substrate 4-methylumbelliferyl phosphate (4-MUP) was prepared at 30 mg/L in 20 mmol Tris-HCl, 1 mmol $MgCl_2$ pH 8.0; 100 μL of this dilution was added to each well and incubated at 31°C for 30 min. The reaction was quenched by the addition of 100 μL of 200 mmol EDTA, pH 8.5, and the fluorescence signal was recorded using excitation and emission filters set to 355 nm and 460 nm, respectively. When the chemiluminescence detection method was implemented, the chemiluminescent substrate APS-5 from Lumigen (Southfield, MI, USA) was added to the washed plate and the signal readout was completed within 2–60 min using standard chemiluminescent settings.

The 2+1 and 2+2 assays described in the text refer to different combinations of mAbs used in the ELISA. The 2+1 format employed clones 560 and MF4 as capture antibodies and 19C7 for detection, whereas the 2+2 format employed 560 and MF4 for capture and 19C7 and 267 for detection. The 2+1 and 2+2 assays were performed as described for the 1+1 assay using a mixture of equal amounts of each mAb. Thus, the total amount of antibody applied to all tests was equivalent.

Specificity of RMP for various cTnI forms

The specificity of the RMP to the different forms of cTnI seen in MI patient samples was assessed using the cTnI Diversity Kit from HyTest that encompasses various purified cTnI forms. The samples were prepared according to the manufacturer's instructions and analyzed using the described cTnI candidate RMP. The proteolysis sample was prepared by the addition of one-part troponin complex (ITC) solution (0.24 g/L) with four-parts human cardiac tissue extract solution. This was then incubated at 37°C for 120 h. Extract solution was prepared by homogenizing human cardiac tissue (100% mass fraction) in 20 mmol Tris-HCl, pH 7.8, 150 mmol KCl and 5 mmol $CaCl_2$, the mixture was then centrifuged at 10000 *g* for 40 min and the supernatant stored at −70°C.

Results

Screening immunoprecipitation efficiency of anti-cTnI antibodies

Immunoprecipitation was performed with the incubation of mAb and troponin complex SRM 2921 in solution. Because in some cases the cTnI band is very close to the band of the antibody light chain (shown in Figure 1), 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. The mean results (±SD), calculated from at least three measurement replicates, were: MF4 mAb (1.08±0.11) >560 mAb

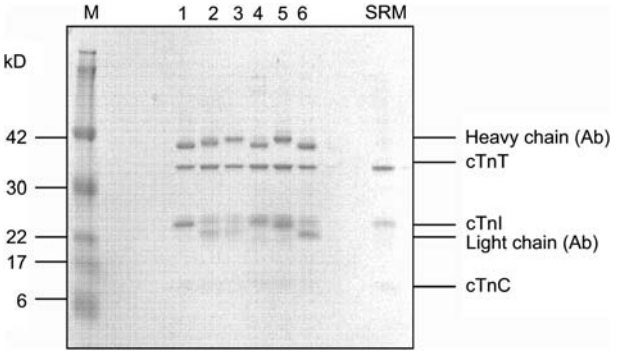

Figure 1 Immunoprecipitation efficiency screening for the evaluated monoclonal antibodies (mAb) against cardiac troponin I (cTnI).
SRM 2921 (6 μg) was diluted in the immune precipitation buffer and incubated with 5 μg of purified anti-human cTnI mAbs followed by SDS-PAGE and staining. The mAbs used for screening were as follows: 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.

(0.92 ± 0.06) > 267 mAb = 19C7 mAb (0.89 ± 0.06) > 3C7 mAb (0.58 ± 0.05) > M18 mAb (0.56 ± 0.03).

Antibody characterization by multiplexed bead arrays

Multiplexed bead array measurements were performed over multiple capture-detection antibody pairs. Four different capture mAbs (MF4, 560, M18 and 3C7) and three different detection mAbs (19C7, 267 and MF4) were used to make a total of eleven '1 + 1' capture and detection antibody pairs. Fluorescence intensity as a function of cTnI SRM 2921 concentration generated for the five best performing pairs in PBS blocking buffer only is shown in Figure 2. With MF4 serving as the capture mAb and 19C7 as the detection mAb, optimum assay sensitivity was obtained. The fluorescence signal increased monotonically with increasing cTnI SRM concentrations. When 560 was used as the capture mAb and 19C7 as the detection mAb, or 3C7 as the capture mAb and 19C7 as the detection mAb, the cTnI detection sensitivities were similar. Assay performance with M18 as capture mAb was not as good as with the other capture mAbs employed. However, the assay performed better with 19C7 than with MF4 as detection mAb.

These five sets of assay data were fitted by using Eq. [3] described in the Appendix for the determination of the equilibrium constant K per bead between the capture mAb and cTnI SRM. We assume that all bound cTnI complexes are detected by the detection mAb because the molar concentration of the detection mAb with respect to that of cTnI complexes is in large excess. The equilibrium constant K $(\mu g/L)^{-1}$ was as follows: MF4 (0.98, ○) > 560 (0.71, ▼) > 3C7 (0.62, ●) > M18 (0.34 with MF4 as the detection mAb, ■; 0.30 with 19C7 as the detection mAb, △). The similar binding constants obtained for M18 with two different detection mAbs (MF4 and 19C7) further validate our assumption.

It is well-known that a human serum matrix can introduce inhibitory effects, causing lower concentrations of cTnI to be reported by immunoassays (11, 12). When cTnI SRM was spiked into NS, a severe inhibitory effect was observed in a similar fashion for all five mAb pairs shown in Figure 2. By incorporating both EDTA and acetate buffer, pH 5.2, to facilitate the binding between the capture mAb immobilized on beads and cTnI complexes as described in the 'Methods' section, the assay performance was considerably improved with all three pairs of mAbs tested (MF4/19C7, 560/19C7 and M18/19C7) (10). The assay performance characteristic for MF4/19C7 was still the best, with a trend similar to those shown in Figure 2.

Figure 3 shows fluorescence intensity results obtained from multiplexed bead arrays employing different mAb pairs for NS (7.5 μL) serving as the background controls and PS with different volumes (5 μL, 7.5 μL or 10 μL) added to make up a total of 17.5 μL sample volume as described in the 'Methods' section. Of the four best performing mAb pairs for PS, MF4 as the capture mAb and 19C7 as the detection mAb, 560 as the capture mAb and 19C7 as the detection

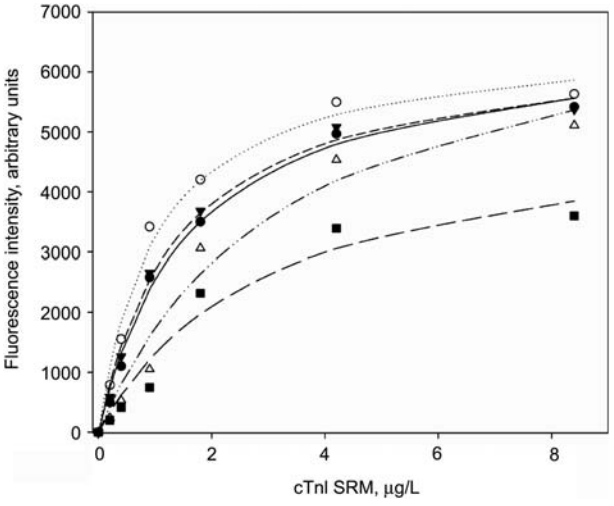

Figure 2 Fluorescence intensity as a function of cTnI SRM 2921 concentrations generated using multiplexed bead arrays in PBS blocking buffer using various capture-detection antibody pairs: MF4 as capture and 19C7 as detection (○); 560 as capture and 19C7 as detection (▼); 3C7 as capture and 19C7 as detection (●); M18 as capture and 19C7 as detection (△); M18 as capture and MF4 as detection (■), respectively.
The CVs of fluorescence intensity values were < 7.2% from replicates in the same experiment and from different experiments. Fitting curves for the determination of the equilibrium constant K were obtained using Eq. [3] described in the Appendix.

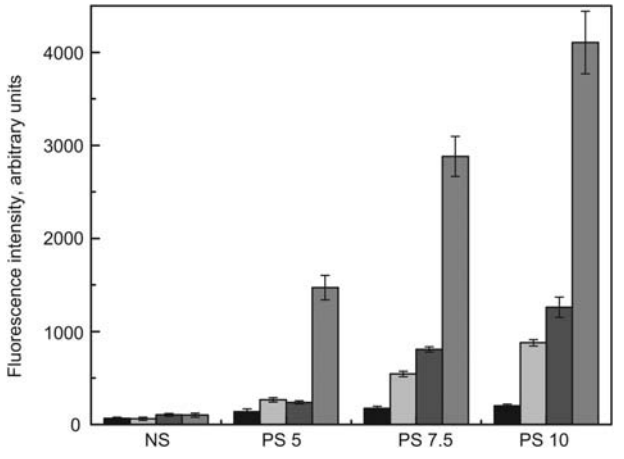

Figure 3 Fluorescence intensity results obtained from multiplexed bead arrays using different mAb pairs for normal serum (NS, 7.5 μL) serving as the background controls and cTnI-positive serum with different volumes (PS, 5 μL, 7.5 μL or 10 μL) added to produce a 17.5 μL sample volume as described in the 'Methods' section.
3C7 as the capture mAb and 19C7 as the detection mAb (black bar); 560 as the capture mAb and 19C7 as the detection mAb (light gray bar); MF4 as the capture mAb and 267 as the detection mAb (dark gray bar); MF4 as the capture mAb and 19C7 as the detection mAb (gray bar). The error bar refers to the SD of each value averaged from replicates in the same experiment and from different experiments.

mAb, and 3C7 as the capture mAb and 19C7 as the detection mAb unexpectedly had the same performance sequence as for cTnI SRM in PBS blocking buffer (Figure 2). When MF4 served as the capture mAb and 267 as the detection mAb, the assay performed slightly better than with the 560/19C7 pair.

Development of candidate RMP

Based on the data from SDS-PAGE and bead array analysis, the antibody clones 560 and 19C7 were selected for the development of the cTnI reference immunoassay. The candidate RMP was optimised to obtain suitable precision over the working concentration range (tentatively designated as 0.1–10 μg/L), and linear and equimolar response (i.e., same reactivity to various cTnI forms). A fluorescent enzymatic readout assay with a substrate employed in many diagnostic instruments and laboratories was first used for immunoassay development. However, through the development of the RMP, data generated using the chemiluminescent substrate APS-5 provided superior detection limits and showed extended linearity. Using the chemiluminescent substrate, a linear response on a log-log plot from 0.05 to 50 μg/L (r^2 0.9968) was obtained. The fluorescent substrate 4-MUP generated a similar dynamic range. However, there was significant loss in linearity at both high and low cTnI concentrations leading to greater uncertainty in results measured in these concentration ranges.

The limit of detection (LOD) and the limit of the blank (LOB) for the chemiluminescent assay were determined as described in the CLSI document EP17-A (13). Negative (cTnI < 0.020 μg/L) and low cTnI positive sample populations (< 4 LOB) were assigned using the Centaur cTnI-Ultra assay and quantitated using the candidate RMP. The LOB and the LOD were estimated to be 0.030 μg/L and 0.052 μg/L, respectively, using data from 11 negative and 14 positive cTnI serum samples, each analysed four times.

A preliminary comparison of cTnI results from 30 patient samples obtained using the Centaur assay and the candidate RMP showed generally lower values by cRMP (Passing-Bablok regression slope 0.729 [CI 0.563, 0.935], y-intercept 0.032 μg/L [CI –0.087, 0.015], Figure 4A; mean % difference –48.5 [CI –72.2, –24.8], Figure 4B). While methods were correlated ($r^2 = 0.801$), there was some discordance between the methods. One sample was grossly discordant with 9.1 μg/L measured using the Centaur compared with 0.05 μg/L by cRMP, and was excluded from the analysis (not shown).

RMP specificity for cTnI forms

cTnI is thought to be released into the blood in free and complexed forms (14, 15). Modification and complexation of cTnI can affect the epitopes available to interact with the selected antibodies. With the use in the proposed RMP of mAbs that bind in the 'stable' region of cTnI, i.e., clones 560 (designated epitope a.a. 83–93) and 19C7 (designated epitope a.a. 41–49), it was anticipated that the antibodies will be able to bind intact and nicked cTnI forms present in the blood. Purified cTnI forms were used to assess the concept of an isoform insensitive assay (Figure 5) (16). For comparison of the responses to free cTnI and binary complex with cTnC, the obtained RMP results were normalized to the response to the ternary complex SRM 2921 diluted in pooled cTnI negative sera. The candidate RMP appeared to show equimolarity when free cTnI response was compared with those of binary complex (Figure 5A). Phosphorylated and dephosphorylated cTnI and the presence of heparin do not significantly change the reactivity of the assay. A similar pattern of responses with various cTnI forms was also

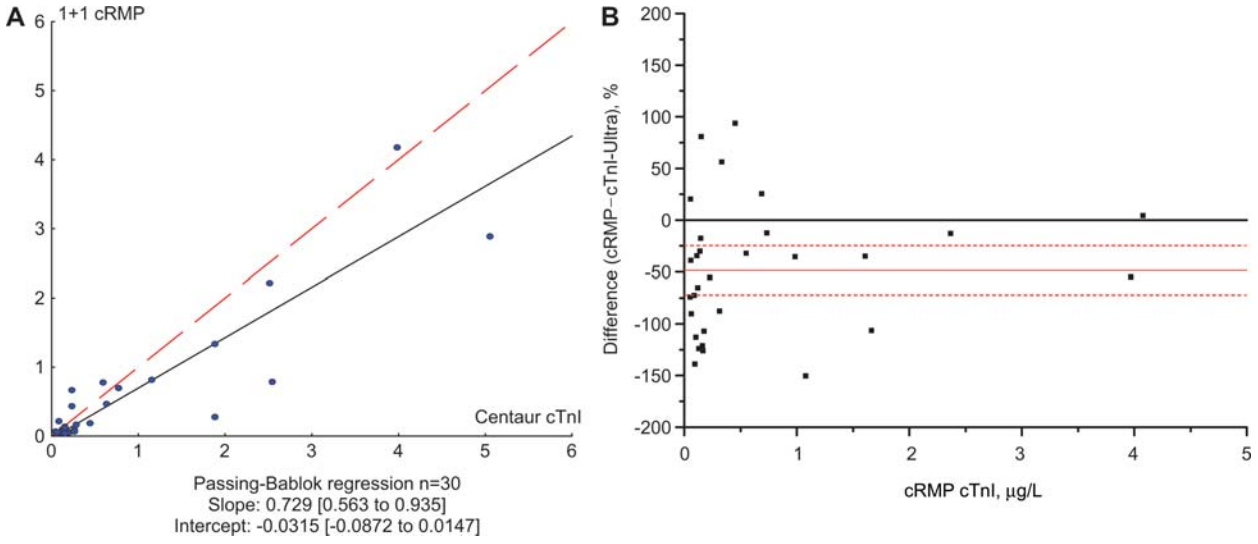

Figure 4 Comparison of the candidate reference measurement procedure (cRMP) with a commercial cTnI assay (Centaur cTnI-Ultra). (A) Passing-Bablok regression and (B) bias plot of the differences.

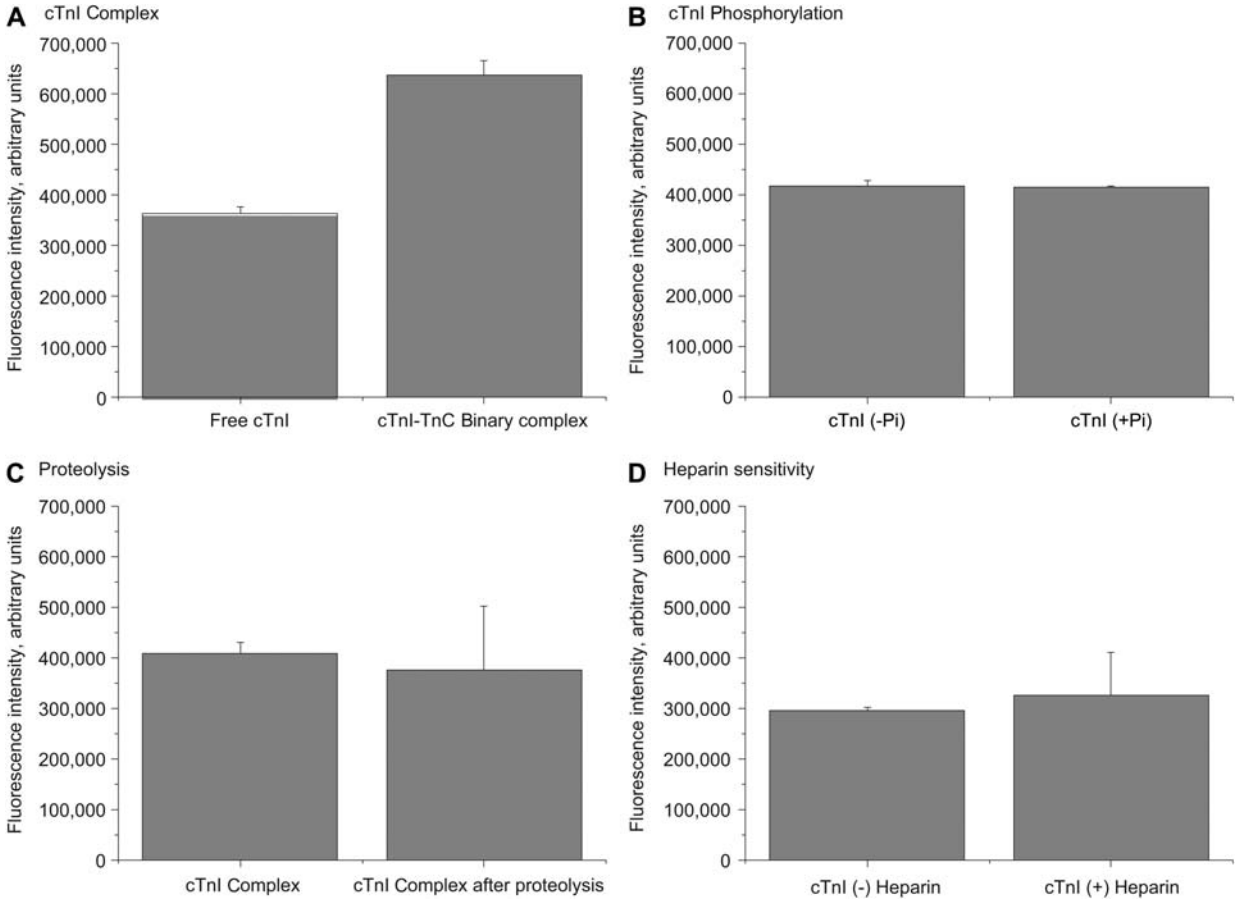

Figure 5 cTnI form specificity of the candidate reference measurement procedure (cRMP).
The error bars represent the SD from duplicate measurements. (A) Compares the responses of RMP to free cTnI and cTnI-cTnC binary complex [designated at 10.74 and 19.94 ng, respectively] after normalization to the SRM 2921 ternary complex response. (B) Shows the response of the RMP against phosphorylated and dephosphorylated cTnI forms [designated (+) Pi and (–) Pi, respectively] at 6.95 and 6.32 ng. (C) Compares the response of the troponin complex (ITC) before and after proteolysis [designated at 6.25 and 6.87 ng, respectively]. (D) Shows the effect of heparin, known to bind to cTnI and sterically interfere with epitope recognition by some antibodies [designated cTnI concentrations in the samples: 11.20 μg/L in (–) heparin and 12.21 ng in (+) heparin, respectively]. All samples were prepared in 0.465 mL of double deionized water as directed by the Diversity Kit supplier.

observed with both the 2+1 and 2+2 assay formats (data not shown).

Discussion

Discrepancies between cTnI results obtained using different commercial immunoassay methods may cloud the clinical interpretation of this biomarker (17). It is, therefore, important to standardize cTnI measurements to obtain comparable results in different locations or times.

One of the basic needs of the standardization project is the development of a RMP using a well-characterized mAb pair for value assignments of suitable cTnI RMs (2). The two methods utilized in this study, SDS-PAGE and multiplexed bead arrays, for the characterization of the relative binding strengths between mAbs and SRM 2921 unveil the same following binding sequence: MF4 > 560 > 3C7 > M18. Additional methods, such as mass spectrometry are currently under development for obtaining the absolute equilibrium binding constants between these mAbs and cTnI SRM. In accordance with recommendations for using mAbs with specificities to the invariant part of the cTnI molecule (7), the second optimal mAb pair 560/19C7 was chosen for the development of the candidate reference immunoassay procedure. It is also worth noting that when the 560/19C7 mAb pair was switched in bead array assays (19C7 serving as the capture mAb and 560 as the detection mAb), identical assay performance was observed with respect to the 560/19C7 pair. These results suggest that immobilization of the capture mAbs on bead surfaces has no significant effect on binding of the cTnI molecule.

Due to the lower sensitivity of the SDS-PAGE method, the bead array method was used to screen optimal mAb pairs for PS (Figure 3). Using this approach, similar results were obtained for PS when compared to cTnI SRM. Surprisingly,

MF4 mAb that targets the C-terminal epitope of cTnI showed the best performance as the capture mAb. As it has been demonstrated that cTnI in patients' blood is present as intact molecule and as degradation products of proteolytic processing of N- and C-terminal ends of the molecule (15), our results appear to be unexpected, at least when the PS was used as sample to screen mAbs.

The antibody pair 560/19C7 selected from bead array and SDS-PAGE analysis were used to develop an ELISA-based RMP. The need and requirements for a RMP to support the cTnI standardisation process has been highlighted (2). Using SRM 2921 as mass calibrator, the candidate RMP generated a linear response on a log-log plot from 0.05 to 50 μg/L, with a calculated LOD of 0.052 μg/L. This LOD is well below the lowest concentration in a panel of secondary RMs, tentatively designated to be 0.10 μg/L (2). However, this is above the cut-off values for MI used with many commercial assays (18). Further attempts will be made to improve the RMP sensitivity through, for example, further optimization of assay conditions.

A preliminary comparison of the RMP with a commerically available assay showed a significant bias. This observed difference could be due to differences in standard material composition and mass concentration assignment, antibody specificities and assay conditions. An extensive comparison with commercial assays will be performed through a round-robin study currently in preparation. The limited study with patient samples showed a low rate of discordant results by RMP when compared with a commercial assay, indicating possible analytical interferences in their measurements. If confirmed, this data may suggest the need of further optimisation of the candidate RMP. However, the pools collected to prepare candidate RMs will be tested for interferents and, if positive, eliminated before their characterization.

One of the requested characteristics of the RMP is equimolarity, i.e., the ability to measure all cTnI forms released into the blood of MI patients that contain the two designated epitopes within the stable region of the analyte. Using the proposed RMP, the variation in assay response to free and complexed cTnI, phosphorylated and dephosphorylated and heparin-treated cTnI was shown to be negligible. However, the cTnI forms employed in our experiments did not represent all cTnI forms present in blood of MI patients, nor other possible interferents, such as cTnI autoantibodies or heterophilic antibodies that are present sometimes in patient samples (11, 12).

In conclusion, this paper highlights the progress in the development of a RMP to support the implementation of a reference measurement system for a complex protein analyte, such as cTnI. Data are provided to support the approach of defining a complex analyte with multiple existing forms through the measurement of epitopes common to all of those. The ability of the candidate RMP to detect these epitopes and generate an equivalent response for the most important cTnI forms present in blood represents a first important step towards standardization of the measurements of this clinically important analyte.

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Conflict of interest statement

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Appendix

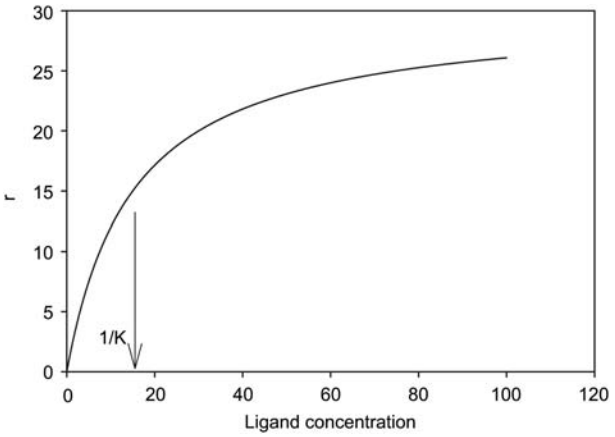
Start with the description of the reaction between the microspheres with attached antibodies and the antigen or ligand. We will view each microsphere as a large antibody with n sites for reacting with the ligand. From now on, when we speak of antibody we mean the whole microsphere. The fundamental reaction will be that between the site on the antibody (microsphere) and the ligand. The total concentration of free sites will be set to $[S]$ and the concentration of sites with attached ligand will be set to $[SL]$. The total number of sites will be distributed among the microspheres and will be written as $[S]+[SL]=n[Sp]$, where $[Sp]$ is the concentration of antibodies (microspheres) in the vial. At equilibrium, the equilibrium constant K is defined by

$$K=\frac{[SL]}{[S][L]}=\frac{[SL]}{\big([S]+[SL]-[SL]\big)[L]}=\frac{\dfrac{[SL]}{[Sp]}}{\left(\dfrac{[S]+[SL]}{[Sp]}-\dfrac{[SL]}{[Sp]}\right)[L]} \quad [1]$$

where $[L]$ is the concentration of ligand (antigen). From previous definitions, the quantity $r=[SL]/[Sp]$ is the number of occupied sites per microsphere (antibody). The quantity r is related to the measurement in the cytometer which measures the number of labeled sites on each microsphere. Eq. [1] can be rewritten as

$$K=\frac{r}{\left(n\dfrac{[Sp]}{[Sp]}-r\right)[L]}=\frac{r}{(n-r)[L]} \quad [2]$$

Eq. [2] can be rewritten to express the quantity r in terms of ligand concentration as



$$r = \frac{nK[L]}{1 + K[L]} \quad [3]$$

Therefore, the simple theory suggests that the number of sites with attached ligand will be proportional to the ligand concentration at low ligand concentrations, and saturate at high ligand concentrations. A typical response is shown in the figure above. The ligand concentration at which the response is half of the maximum response is the reciprocal of the equilibrium constant.

The response at large ligand concentrations is proportional to the number of sites on the microsphere.

References

1. Thygesen K, Alpert JS, White HD, Jaffe AS, Apple FS, Galvani M, et al. Universal definition of myocardial infarction. Circulation 2007;116:2634–53.
2. Panteghini M, Bunk DM, Christenson RH, Katrukha A, Porter RA, Schimmel H, et al. Standardization of troponin I measurements: an update. Clin Chem Lab Med 2008;46:1501–6.
3. Muller MM. Implementation of reference systems in laboratory medicine. Clin Chem 2000;46:1907–9.
4. Thienpont LM, Van Uytfanghe K, De Leenheer AP. Reference measurement systems in clinical chemistry. Clin Chim Acta 2002;323:73–87.
5. Panteghini M, Forest JC. Standardization in laboratory medicine: new challenges. Clin Chim Acta 2005;355:1–12.
6. Panteghini M. Assay-related issues in the measurement of cardiac troponins. Clin Chim Acta 2009;402:88–93.
7. Panteghini M, Gerhardt W, Apple FS, Dati F, Ravkilde J, Wu AH. Quality specifications for cardiac troponin assays. Clin Chem Lab Med 2001;39:175–9.
8. hsTnI MAbs™. Hytest News, 2007. Available at: http://www.hytest.fi/data_sheets/newsletters/hsTnI%20MAbs%20Newsletter.pdf. Accessed: 22 October 2010.
9. Clinical and Laboratory Standards Institute. Preparation and validation of commutable frozen human serum pools as secondary reference materials for cholesterol measurements procedures; approved guideline. CLSI Document C37-A. Clinical and Laboratory Standards Institute 940 West Valley Road, Suite 1400, Wayne, Pennsylvania, 1999.
10. Wang L, Bunk DM, He HJ, Cole KD. Removal of inhibitory effects in a serum cardiac troponin I immunoassay. Clin Chem 2009;55:2055–6.
11. Kim WJ, Laterza OF, Hock KG, Pierson-Perry JF, Kaminski DM, Mesguich M, et al. Performance of a revised cardiac troponin method that minimizes interferences from heterophilic antibodies. Clin Chem 2002;48:1028–34.
12. Eriksson S, Halenius H, Pulkki K, Hellman J, Pettersson K. Negative interference in cardiac troponin I immunoassays by circulating troponin autoantibodies. Clin Chem 2005;51:839–47.
13. Clinical and Laboratory Standards Institute. Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline. CLSI document EP17-A Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania, 2004;1–42.
14. Katrukha AG, Bereznikova AV, Esakova TV, Pettersson K, Lovgren T, Severina ME, et al. Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex. Clin Chem 1997;43:1379–85.
15. Katrukha AG, Bereznikova AV, Filatov VL, Esakova TV, Kolosova OV, Pettersson K, et al. Degradation of cardiac troponin I: implication for reliable immunodetection. Clin Chem 1998;44:2433–40.
16. Wu AH, Feng YJ, Moore R, Apple FS, McPherson PH, Buechler KF, et al. Characterization of cardiac troponin subunit release into serum after acute myocardial infarction and comparison of assays for troponin T and I. Clin Chem 1998;44:1198–208.
17. Christenson RH, Duh SH, Apple FS, Bodor GS, Bunk DM, Panteghini M, et al. Toward standardization of cardiac troponin I measurements part II: assessing commutability of candidate reference materials and harmonization of cardiac troponin I assays. Clin Chem 2006;52:1685–92.
18. Tate JR. Troponin revisited 2008: assay performance. Clin Chem Lab Med 2008;46:1489–500.