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Review

Standardization of troponin I measurements: an update[1)]

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Abstract

Standardization of cardiac troponin I (cTnI) measurement is important because of the central role for diagnosis of myocardial infarction. In blood, cTnI is present as a heterogeneous mixture of different molecular species. The analytical problem caused by this heterogeneity may be circumvented by recognition of a unique, invariant part of the molecule that is common to all components of the mixture. Antibodies used for the development of cTnI assays should selectively recognize epitopes within this invariant part, leading to a consequential increase in the homogeneity of immunoassay reactivity. This should be associated with the use of a reference material that represents the natural and major antigen in blood after tissue release, i.e., the troponin complex. Although a primary reference material for cTnI is available, studies indicate that cTnI assays remain without harmony after recalibration using this material. To achieve closer comparability of cTnI values between assays, the use of a secondary reference material, consisting of a panel of human serum pools, is proposed for use by manufacturers to calibrate their assays. To assign true cTnI concentration values to this secondary reference material, establishment of a reference measurement procedure for cTnI is required. A practical approach to the development of a reference procedure could be to design an immunochemical assay with well-characterized specificity to the invariant part of the cTnI molecule and calibrated using the primary reference material.
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Introduction

The recently released document by the Global Task Force on the universal definition of myocardial infarction (MI) has strengthened the role of the measured increase of the blood concentration of cardiac troponin as the main criterion for MI definition (1). It is therefore pivotal that this clinically relevant biomarker, on which important critical decisions will rest, is measured with highly reliable and standardized methods to achieve comparability of results, independent of the measurement test reagents and platforms, as well as the laboratory where the procedure is carried out. Standardization of troponin measurements would ensure the interchangeability of results over time and space and significantly contribute to further improvements in healthcare by allowing results of clinical studies undertaken in different locations or times to be universally applied.

Because of an international patent and a monopoly position of the vendor, cardiac troponin T assays are commercially available from only a single manufacturer, so that comparability or harmonization of results for this marker is not a problem, even if troponin T measurements are not definitively standardized to higher order certified materials. Conversely, a variety of companies currently market assays for cardiac troponin I (cTnI) measurement. Very often, these assays use different standard materials and antibodies with different epitope specificities. As a consequence, analytical systems may give results that are unique to a certain method or instrument, so that different results from different cTnI assays and platforms may be obtained. Cooperative studies and external quality assessment schemes that measure cTnI concentrations in common samples have often shown quite large scatter. For instance, an Interna-

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tional Federation of Clinical Chemistry and Laboratory Medicine (IFCC)-coordinated study involving 14 commercial platforms showed more than 20-fold differences in cTnI values among methods (2). It is evident that the consequential inability to define common decision limits for cTnI may create a substantial problem for the clinical and laboratory communities when marker results are interpreted.

The concept of the reference measurement system

There is now an international agreement that the standardization of quantitative measurements in Laboratory Medicine requires the consistent definition and application of a reference measurement system for calibration and validation of routine methods (3–6). Such a structure is based on the concepts of metrological traceability and of a hierarchy of analytical measurement procedures (7). Key elements of the system are the reference measurement procedure and different types of reference materials. The reference procedure, which is calibrated using a primary reference material, is used to assign a certified value to a secondary reference material, which typically consists of the analyte present in a complex matrix comparable to that of a routine sample. Once the appropriate reference material is certified, this material and the manufacturer's testing procedure can be used in industry to assign values to commercial calibrators. Clinical laboratories use routine procedures with validated calibrators, both from commercial sources, to measure human samples. In this way, the value obtained will be traceable to the reference procedure and materials, and measurement standardization – the process of realizing measurement traceability and achieving trueness of measured values – will be reached. However, because no reference measurement procedure or secondary reference materials for cTnI are available at present, manufacturers usually prepare their own calibrators and assign values through different approaches, thus contributing to a lack of harmonization among assays.

Definition of the measurand

The traceability model emphasizes in particular the importance of a detailed definition of the analyte to be measured (8, 9). In certain cases, e.g., for analytes that are well-defined chemical entities, such as cholesterol or creatinine, the definition of the analyte is straightforward. However, when considering much more complex substances, such as most protein biomarkers, including cTnI, the definition may not be as clear because of potential intrinsic or acquired heterogeneity. In blood, cTnI is present as a heterogeneous mixture of different molecular species. Intact cTnI and a spectrum of modified products have been detected in sera from patients with MI (10). In turn, for the definition of the analyte ''cardiac troponin I'', it must be decided whether the term refers to a) a mixture of different forms, i.e., free and complexed with troponin C and troponin T, or to only one prevalent form, b) composition classes (e.g., oxidation or phosphorylation), and c) content classes (e.g., percentage of phosphorylation) (11).

As a matter of fact, the heterogeneity of cTnI may be circumvented by the definition of a unique, invariant part of the molecule that is common to all components of the mixture present in blood, e.g., the epitopes that are located in the central part of the cTnI molecule and are not affected by troponin IC or ITC complex formation and other 'in vivo' modifications (12). Antibodies used for the development of cTnI assays should selectively recognize these epitopes with a consequent increase in the homogeneity of immunoassay reactivity (13). Standardization of cTnI assays is in fact not possible if differences in antibody specificities among the assays are not minimized.

Reference materials

The major prerequisite for guaranteeing comparability of results among different assays remains the availability of suitable reference materials for calibration. Several studies have shown that the quantitative differences in cTnI test values among currently available commercial assays are largely attributable to the lack of a common calibrator for use by the manufacturers of these assays. In their landmark study, Katrukha et al. (14) showed that use as common calibrator of a material containing equimolar concentrations of human cardiac troponins I, T, and C significantly reduced the interassay variability of cTnI values for a troponin-positive serum sample.

On the basis of this experimental evidence, an international agreement was reached recommending the selection as reference material for cTnI of a compound representing the natural and major form of the antigen in blood after tissue release, i.e., the troponin complex form (13). The American Association for Clinical Chemistry (AACC) committee for cTnI standardization, working in cooperation with the National Institute of Standards and Technology (NIST), the IFCC Committee on Standardization of Markers of Cardiac Damage (C-SMCD), and diagnostic companies, was involved in the development of this type of reference material which eventually became NIST standard reference material (SRM) 2921. The first phase of the process involved the evaluation of a number of different candidate materials, consisting of human native and recombinant proteins, in liquid-frozen and lyophilized forms (15). Preliminary characterization studies using mass spectrometry (MS) to verify the material composition were conducted at NIST (16). Through comparison studies conducted with the manufacturers of cTnI assays, the various preparations were evaluated and the best material was chosen.

An extensive structural characterization of the selected material, a troponin ITC ternary complex

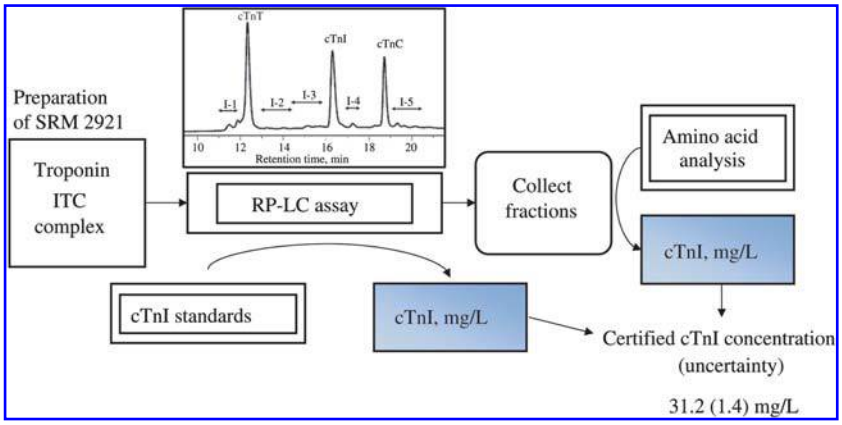

Figure 1 Steps of the certification analysis of the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2921 – human cardiac troponin complex.
RP-LC, reversed-phase liquid chromatography; cTnI, cardiac troponin I.

purified from human heart tissue, was carried out at NIST by liquid chromatography (LC) coupled to MS and tryptic digestion followed by matrix-assisted laser desorption/ionization (MALDI)-MS (17). The concentration of cTnI in the material was determined through a combination of LC and amino acid analysis (Figure 1). The first method used reversed-phase (RP)-LC to separate the three troponin subunits present in the reference material. cTnI quantification was based on the height of the cTnI peak and was interpolated from a calibration curve of peak height vs. cTnI concentration derived from external calibrators prepared from purified human cTnI. The second method to determine the cTnI concentration in the reference material used amino acid analysis of the cTnI subunit purified from the troponin complex by RP-LC, as described above. A certified reference material for amino acids (NIST SRM 2389) was used as external standard for the amino acid analysis. The measured concentrations of the amino acids alanine, valine, and leucine were used to calculate the concentrations of cTnI, accounting for the number of each amino acid present in one cTnI molecule. The measured concentrations of cTnI in SRM 2921, determined by both the RP-LC and amino acid methods, were combined to give the certified concentration (17).

The next phase of the program included an experimental evaluation of the ability of this reference material, used for direct calibration of commercially available methods, to achieve a significant improvement in comparability of results (18). The use of SRM 2921 as common calibrator in commercial systems did not, however, improve result comparability of cTnI measurements of human sera, indicating that the mere availability of a primary reference material and its use for calibration is not sufficient by itself for standardizing cTnI results. There may be several underlying reasons to explain these results (19). First consider that, although the content of the SRM 2921 material attempted to mimic the major form of cTnI found in biological specimens, the analytes in the reference material and in biological fluids are definitely non-identical. Primary reference materials for heterogeneous proteins can serve only as surrogates for the analytes to be measured in patient samples. Although such materials resemble to some extent the analyte present in the human fluids, they may, however, represent only an ''average'' condition, and this may invalidate the basic rule of immunoprocedures, which is to compare ''like with like''. Furthermore, besides the removal of the matrix background the purification procedures used can lead to partial modification of the structure of the troponin molecule, which in turn might affect the immunologic reaction, at least for some assays. Bunk et al. (16) have shown that some posttranslational modifications in the cTnI extracted from human heart may be attributable to the purification process. Altogether, these facts raise a potential commutability issue of this reference material with native clinical samples (18). Commutability is defined as the ability of a reference or control material to show interassay properties similar to those of human samples. It is a fact that only commutable materials can be used by industry for direct value assignment of manufacturers' calibrators, having great importance to ensure metrological traceability (20).

An appropriate solution to these problems can be the identification of preparations with a composition and matrix similar to that in clinical samples and their

Table 1 Hierarchy of reference materials for immunoassays [modified from ref. (21)].

Primary reference material: pure analyte (recombinant or human purified protein), with values assigned by mass determination/calculation;
Secondary reference material: matrix-based, with values assigned by a reference procedure against the primary material. Prepared with:
a) pool of human sera spiked with the corresponding purified antigen,
b) pool of human sera containing the corresponding native antigen in detectable concentrations.

use as secondary reference materials to calibrate commercial assays (Table 1) (21). The cTnI concentrations of these samples could be assigned against the previously described primary reference material, thus assuring the traceability of patient results directly to the SI unit. Producing a secondary reference material with appropriate concentrations of cTnI can theoretically be accomplished by spiking of the purified analyte into normal serum. Studies performed using human pooled serum spiked with NIST SRM 2921 have, however, shown that this spiked material is highly unstable (22). The selection of pools of human sera collected from individuals with elevated cTnI concentrations is therefore the preferred option (9).

Several studies have shown that cTnI values are more comparable between routine measurement systems if one or more serum samples are used as the common calibrator. In a study by Tate et al. (23), the harmonization effect was assessed by determining the among-systems coefficient of variation (CV) after correction for calibration differences among seven assay systems by reference to two different materials used as common calibrator, a purified troponin ternary complex material and a serum sample collected from a patient with MI. Generally, test values were better harmonized among systems using the serum sample rather than the processed material as calibrator even if, in some samples, significant scatter among the different methods was still observed. In a similar study, performed by the AACC cTnI standardization committee, the alignment strategy with regression parameters using human serum pools produced a 5-fold improvement in intersystem variability. In particular, the variability of results among cTnI assays decreased from CVs of ~90% to CVs between 7% and 28% (18).

Taken together, this evidence suggests that a small number of human serum pools (e.g., three samples containing cTnI around the clinically relevant concentrations) could serve as a secondary reference material for cTnI measurement. These materials should be selected and prepared according to recommendations from the relevant ISO standards (24–26). Considering the substantial effort that goes into the selection, characterization, and distribution of these materials, a lot size large enough to meet the needs of the clinical community for at least 5 years should be prepared.

Reference measurement procedure

Use of human serum alone will, however, not provide value assignment; therefore, a reference measurement procedure that can be reproduced within defined specifications is essential for certification of cTnI values in the secondary reference materials. Unfortunately, for cTnI the search and the assessment of candidate reference materials have not been supported by the simultaneous development of a reference measurement procedure. A practical option is the development of a ''higher order'' immunochemical procedure using monoclonal antibodies with well-defined specificity against epitopes of the cTnI molecule that can be considered stable from the point of view of stereochemical inhibition of the binding (19). The main argument against this option is that an immunoassay procedure may be too dependent on a certain assay technology, because this technique is an indirect measurement approach (27); however, alternative non-immunochemical techniques, such as MS, that may possibly be preferred for standardization, currently lack the measurement sensitivity for direct measurement of cTnI, which occurs in blood at very low concentrations. A ''higher order'' immunoassay method is suitable for value assignment of a secondary reference material provided it has specificity to the invariant part of the cTnI molecule, acceptable assay performance, is calibrated against SRM 2921, and has diagnostic power comparable with that of validated commercial assays (Table 2).

Basic experiments for determination of the optimal combination of monoclonal antibodies by epitope mapping and affinity binding studies were recently started at NIST. Six monoclonal antibodies are under evaluation for use in the reference immunoassay based on literature evidence for appropriate capture and detection pairs for a sandwich cTnI immunoassay and the IFCC recommendations for the appropriate epitopes to target for serum cTnI measurement (12, 13, 28, 29). Analytical approaches used in the characterization of these antibodies are multiplexed bead arrays and hydrogen/deuterium exchange coupled to high resolution MS for epitope mapping, in conjunction with high resolution MS of the cTnI antigen captured by the immobilized antibodies. On the basis of results, the optimal capture and detection antibodies will be chosen to develop a robust and well-characterized immunoassay method for cTnI to be used as candidate reference measurement procedure (30).

Standardization vs. harmonization

Because of this complicated situation, it is clear that progress in the standardization of cTnI assays will be

Table 2 Requirements for a designated higher order reference measurement procedure for cardiac troponin I (cTnI).

Non-commercial sandwich-based non-competitive immunoassay:
• based on monoclonal antibodies directed against epitopes that can be considered pristine from the point of view of stereochemical inhibition of the binding,
• comparable epitope-specificity with the last-generation commercial assays,
• calibrated with NIST SRM 2921.
Thorough definition of assay characteristics including:
• antibody specificity,
• immunoreactivity to cTnI forms present in serum,
• detection limit and measurement uncertainty.

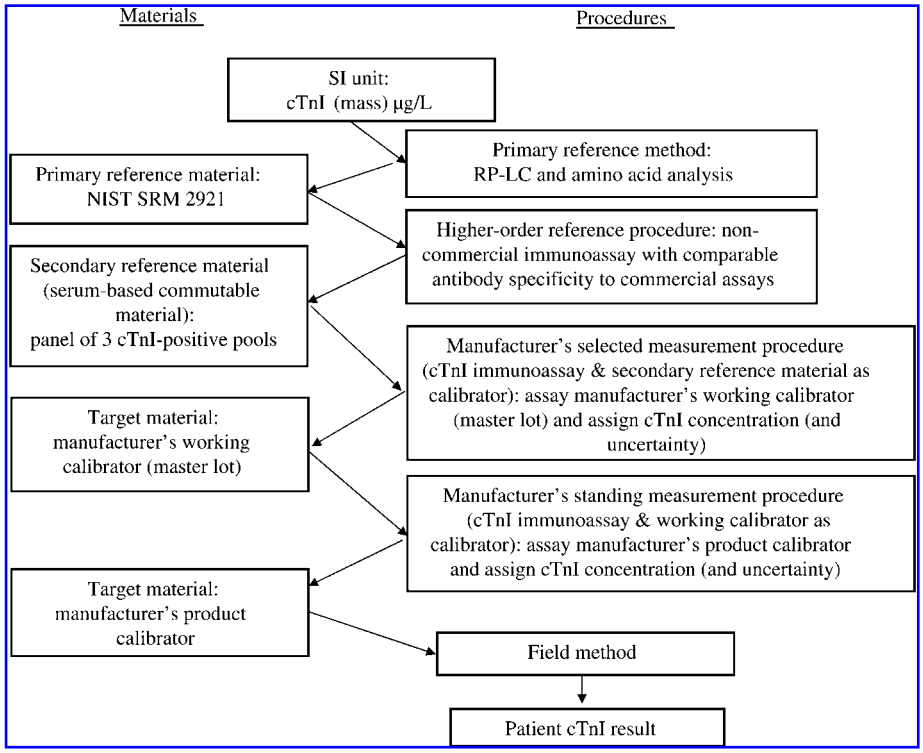

Figure 2 Suggested approach for the standardization of cardiac troponin I (cTnI) measurements through traceability implementation to the reference measurement system.
RP-LC, reversed-phase liquid chromatography.

relatively slow and that some of the problems in this field will not be solved soon. As an interim solution, an assay harmonization approach that incorporates recalibration of various assays to give the same results has been advocated (31). In this case, a ''designated'' common comparison method should provide the basis for harmonization. Historically, a form of harmonization already existed in which commercial companies, to fulfill US Food and Drug Administration demands, tried to adjust their assay results based on those of the first assay released in the market, which was the Baxter Stratus assay. Harmonization thus is possible only in a method-dependent manner. Furthermore, although methods can produce more or less similar results, these may be far from traceability and significantly biased in terms of trueness (19). Despite some practical problems, standardization rather than harmonization of cTnI measurements should, therefore, be the goal whenever possible.

In agreement with a metrologically correct approach, for the standardization and traceability of cTnI measurements, a reference measurement system is required (Figure 2). This is to be comprised of a primary reference material, a higher order reference procedure for the value assignment of matrix-based reference materials, and these secondary materials, represented by a panel of appropriately selected and certified human pooled serum samples. Once appropriate reference materials are available, these materials and the manufacturers' testing procedures can be used in industry to assign values to product calibrators. Clinical laboratories, using routine procedures with validated calibrators to measure patient samples, will finally obtain standardized cTnI values. Given that the diagnostic companies are now producing more analytically sensitive versions of their troponin assays, an opportunity exists for industry to be involved in cTnI standardization at the same time as these new ''high sensitivity'' assays are being developed (32).

With this proposal in mind, the IFCC has recently created a new Working Group for Standardization of Troponin I (WG-TNI) by involving professional and industry expertise and representatives of major national metrology institutes. The project phases are related: 1) to establish a candidate reference immunoassay procedure for cTnI based on an optimal combination of monoclonal antibodies; 2) to prepare a secondary multi-level (three) reference material for cTnI consisting of serum pools from MI subjects, and 3) to evaluate effectiveness of this reference measurement system to standardize cTnI measurement through a round robin study involving routine clinical assays after a value transfer using the secondary material as common calibrator. Although this is a research project and there is no guarantee of success, such experimental work is needed if there is to be progress in the standardization of cTnI and, in general, of heterogeneous clinical analytes measured by immunoassays.

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