National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2921

Human Cardiac Troponin Complex

This Standard Reference Material (SRM) is primarily intended for use in evaluating the accuracy of clinical procedures for the determination of cardiac troponin I (cTnI) in human serum. It can be used to validate working or secondary reference materials. A unit of SRM 2921 consists of five vials, each containing approximately 115 µL of a dilute solution of human cardiac troponin complex.

The troponin complex was purified from human heart tissue and consists of three subunits: troponin T (cTnT), troponin I (cTnI), and troponin C (cTnC). The choice of a human cardiac troponin complex was made with the assistance of the Cardiac Troponin I Standardization Subcommittee of the American Association for Clinical Chemistry (AACC). Through two interlaboratory comparison studies, various troponin preparations, including recombinant and native troponin complexes of the troponin T, troponin I, and troponin C subunits, recombinant troponin I/troponin C complex, and recombinant and native cTnI, were evaluated using fifteen commercial cTnI assays [1]. Based on the results of these interlaboratory comparison studies, the human cardiac troponin complex was chosen for its ability to harmonize clinical assays and for its commutability.

Certified Human Cardiac Troponin Concentration: The certified concentration value of human cTnI was determined through a combination of amino acid analysis and liquid chromatography (LC). The amino acid analysis used SRM 2389 Amino Acids in 0.1 mol/L Hydrochloric Acid as an external standard.

Certified cTnI Concentration: 31.2 mg/L ± 1.4 mg/L

The certified value is the weighted mean of results obtained from two methods. The uncertainty in the certified concentration is calculated as $U = ku_c$. The quantity u_c is the combined standard uncertainty calculated according to the ISO/NIST Guides [2], where u_c is intended to represent, at the level of one standard deviation, the combined effect of within-method variation for both methods and between method variation for the two methods. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and a 95 % level of confidence.

Reference Concentration Values: Reference concentration values for cTnT and cTnC are provided in Table 1. The reference values are noncertified values that are the best estimates of the true values; however, these values do not meet NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Certification: The certification of this SRM is valid until **30 January 2010**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate. However, the certification is invalid if the SRM is contaminated or modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

Coordination of the technical measurements leading to certification of this SRM was performed by D.M. Bunk and M.J. Welch of the NIST Analytical Chemistry Division.

Willie E. May, Chief
Analytical Chemistry Division

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Gaithersburg, MD 20899
Certificate Issue Date: 30 April 2004

Preparation of the material and analytical measurements were performed by D.M. Bunk of the NIST Analytical Chemistry Division.

Consultation of the statistical design of the experimental work and evaluation of the data were provided by C.R. Hagwood of the NIST Statistical Engineering Division.

The support aspects involved in the preparation, certification, and issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by B.S. MacDonald of the NIST Measurement Services Division.

NOTICE AND WARNING TO USERS

Warning: SRM 2921 IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of this human protein has reported that the donor tissue, from which this protein was extracted, has been tested and found non-reactive for Hepatitis B surface antigen (HbsAg), human immunodeficiency virus (HIV), and human immunodeficiency virus antigen (HIV-1Ag). However, no known test method can offer complete assurance that hepatitis B virus, HIV, or other infectious agents are absent from this material. Accordingly, this human tissue product should be handled at the Biosafety Level 2 or higher as recommended for any potential human material in the Centers for Disease Control/National Institutes of Health Manual [3].

Storage: The SRM is shipped frozen on dry ice and, upon receipt, should be stored frozen until ready for use. A freezer temperature of -20 °C is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at or below -50 °C. The SRM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room or refrigerator temperature may result in changes in the analyte concentration.

INSTRUCTIONS FOR USE

Vials of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature (20 °C to 25 °C) until thawed. After the material is thawed, it should be used immediately. The material should be mixed before aliquots are removed. Any unused solution should be discarded.

SOURCE, PREPARATION, AND ANALYSIS[1]

Source of Material: The human cardiac troponin complex was prepared by HyTest Ltd., Turku, Finland. The human cardiac troponin complex was supplied by HyTest in a buffer composed of 150 mM sodium chloride, 5 mM calcium chloride, and 20 mM tris buffer (pH 7.5) and was diluted at NIST with the same buffer to the certified concentration given on this certificate.

Certification Analysis: All analyses for the certified and reference concentration values were performed at NIST. Two different methods were used for the determination of the concentration of cTnI in the SRM. The first method used reversed-phase liquid chromatography with UV detection (220 nm) to separate the three troponin subunits. A Zorbax SB300-C8 LC column (Agilent, Palo Alto, CA), 2.1 mm × 150 mm, held at 35 °C was used for the analysis. The separation utilized gradient elution that started at 70 % solvent A (0.1 % (v/v) trifluoroacetic acid in water) and 30 % solvent B (0.08 % (v/v) trifluoroacetic acid in acetonitrile) and finished at 20 % solvent A and 80 % solvent B after 25 min. The quantification of cTnI in the SRM was based on the peak height of the cTnI peak, using a calibration curve of peak height vs. cTnI concentration derived from external calibration standards prepared from purified human cTnI. The concentration of the cTnI calibration standards was determined by amino acid analysis using SRM 2389 as the external standard. Analysis of SRM 2921 was performed in quadruplicate each day, on four separate days. A typical reversed-phase chromatogram of SRM 2921 is shown in Figure 1.

The second method to determine the cTnI concentration in the SRM used amino acid analysis of the cTnI subunit purified from the troponin complex using reversed-phase liquid chromatography, as described above. The reversed-phase LC cTnI peak fractions were lyophilized and then hydrolyzed using HCl vapor at 110 °C for approximately 24 h. After hydrolysis, the hydrolyzed amino acids were quantitated using a modified version of the amino acid analysis procedure developed by Agilent (Palo Alto, CA) [4], which uses the amine-specific derivatization agent *o*-phthalaldehyde (OPA). The Agilent procedure utilizes the autosampler to mix the samples and OPA reagent

[1]Certain commercial products are identified in this certificate to adequately describe the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for this purpose.

and then inject them for gradient LC analysis with UV detection. The liquid chromatographic system used consisted of a binary pump, thermostated autosampler, column oven, and variable wavelength UV detector. The column used for the separation of amino acids was a Zorbax Eclipse AAA (Agilent, Palo Alto, CA; 4.6 × 150 mm, 3.5 μM particle size). SRM 2389 was used as an external standard for the amino acid analysis. The analysis was carried out in duplicate each day, on four separate days. 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 molecule of cTnI.

The concentrations of the cTnT and cTnC subunits in SRM 2921 were also measured using this combination of reversed-phase LC and amino acid analysis. These amino acid analysis results of the determination of cTnT and cTnC in the SRM are listed in Table 1.

Table 1. Reference Concentrations of cTnT and cTnC from Amino Acid Analysis

cTnT Concentration	36.9 mg/L ± 3.8 mg/L
cTnC Concentration	24.2 mg/L ± 1.3 mg/L

The uncertainties in the reference concentration values are calculated as $U = ku_c$. The quantity u_c is the combined standard uncertainty calculated according to the ISO/NIST Guides [2], where u_c is intended to represent the measurement error at the level of one standard deviation. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and a 95 % level of confidence. The reference concentration values are noncertified values that are the best estimates of the true values; however, these values do not meet NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

In addition to the quantitative analysis, structural characterization of the three troponin subunits in the SRM were also carried out by liquid chromatography coupled to mass spectrometry (LC/MS) and tryptic digestion followed by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Molecular mass determinations of the troponin subunits were performed by capillary LC/MS using a single quadrupole mass spectrometer. The proteins were separated on a Vydac C4 reversed-phase LC column (Grace Vydac, Hesperia, CA; 50 mm × 0.5 mm) using gradient elution with 0.05 % (v/v) trifluoroacetic acid in water, and 0.0425 % (v/v) trifluoroacetic acid in acetonitrile as mobile phases. The flow rate used was 25 μL/min. Full scan mass spectra were obtained of the column effluent by scanning from m/z 600 to m/z 1500 in 2 sec. LC/MS analysis of SRM 2921 was performed in duplicate. A typical total ion chromatogram from the LC/MS analysis is shown in Figure 2.

The observed relative molecular masses for the troponin subunits in SRM 2921 are listed in Table 2. The molecular weight distributions for the cTnT, cTnI, and cTnC subunits in SRM 2921, obtained from LC/MS, are shown in Figure 3. The tentative identification of the molecular species listed in Table 2 and seen in Figure 2 are based on the observed relative molecular masses of each species, the known post-translational modifications of each troponin subunit, and, when observed, the peptides in the tryptic digest of each troponin subunit. The uncertainties in the relative molecular mass values in Table 2 are calculated as $U = ku_c$. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and a 95 % level of confidence.

The observed molecular mass distribution of the cTnI in SRM 2921 shows a substantial degree of heterogeneity. The pattern of components and specifically the mass differences between components indicate post-translational modifications such as phosphorlyation and truncation of the C-terminus. Both types of post-translational modifications have been reported previously for human cardiac troponin I [5]. The types of cTnI species observed in SRM 2921 are nearly identical to those found in the two candidate reference materials that were obtained previously from HyTest. However, SRM 2921 seems to differ in the relative distribution of these species; SRM 2921 appears to contain more C-terminally truncated materials than what was observed in previous lots of the troponin complex. Additionally, a species with a molecular mass of 23 918 (tentatively assigned as non-phosphorylated, intact cTnI) was not previously observed.

In addition to the three troponin subunits, additional components were observed in SRM 2921 through the LC/MS analysis. The peak labeled **A** in Figure 2, eluting before the cTnT peak, produced a noisy mass spectrum which could be deconvoluted to indicate a component with a molecular mass of 26 780 ± 40. Because this species elutes so close to cTnT, it could be a degraded form of cTnT. There were two components co-eluting under the peak labeled with **B** in Figure 2 at approximately 25 min. The first component has a polymeric distribution, with a mass difference between peaks of 44 u, the unified atomic mass unit, extending up to approximately m/z 900. A mass

difference of 44 u is usually associated with a polyethylene glycol contamination. The second component, eluting after cTnI, had an observed molecular mass of 32 755 ± 20. The peak labeled **C** in Figure 2 was consistently observed with a strong signal. However, the averaged mass spectrum associated with this peak did not show a clear polymeric or multiply-charged distribution. This could indicate either a very high molecular mass protein contaminant or a protein contaminant with substantial structural heterogeneity.

Table 2. Relative Molecular Masses from the LC/MS Analysis of SRM 2921

Component	Calculated Relative Molecular Mass[a]	Observed Relative Molecular Mass[b] (± 95 % CI)	Tentative Identification
CTnT	34 459.0	**34 503 ± 6**	N-acetylation of the N-terminus
		33 775 ± 8	cTnT[Ac1-281] = 33 773
		34 374 ± 8	cTnT[Ac1-286] = 34 373
		34 524 ± 8	
CtnI	23 918.4	24 079 ± 8	Bis-phosporylated
		23 999 ± 6	Mono phosphorylated
		23 918 ± 5	Expected molecular mass with N-terminal acetylation
		23 785 ± 8	Mono phosphorylated + C-terminal truncation of SE
		23 704 ± 8	C-terminal truncation of SE
		23 637 ± 8	Mono phosphorlyation + C-terminal truncation of SEF
		23 556 ± 6	C-terminal truncation of SEF
		23 427 ± 8	C-terminal truncation of SEFK
CTnC	18 416.6	**18 445 ± 4**	
		18 426 ± 6	
		18 463 ± 8	

[a] Mass is calculated from the amino acid sequence obtained from the Swiss-Prot database (http://us.expasy.org/sprot/sprot-top.html) using entry names TRT2_HUMAN for cTnT, TRIC_HUMAN for cTnI, and TPCC_HUMAN for cTnC.
[b] The major component observed is indicated in bold.

Information about the stoichiometry of the troponin subunits in the SRM can be obtained by comparing the molar concentrations of each subunit, as determined by amino acid analysis. Table 3 shows the ratios of the molar concentrations of the subunits observed for SRM 2921. The uncertainties in the values in Table 3 are calculated as $U = ku_c$. The coverage factor, k, is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and a 95 % level of confidence.

Table 3. Reference Ratios of the Molar Concentration of Troponin Subunits in SRM 2921

[cTnT]/[cTnI]	0.84 ± 0.05
[cTnC]/[cTnI]	1.03 ± 0.04

The results shown in Table 3 indicate that a 1:1 stoichiometry exists between the cTnI and cTnC subunits. However, the amount of cTnT appears to be present at less than unit stoichiometry with respect to the cTnI and cTnC subunits.

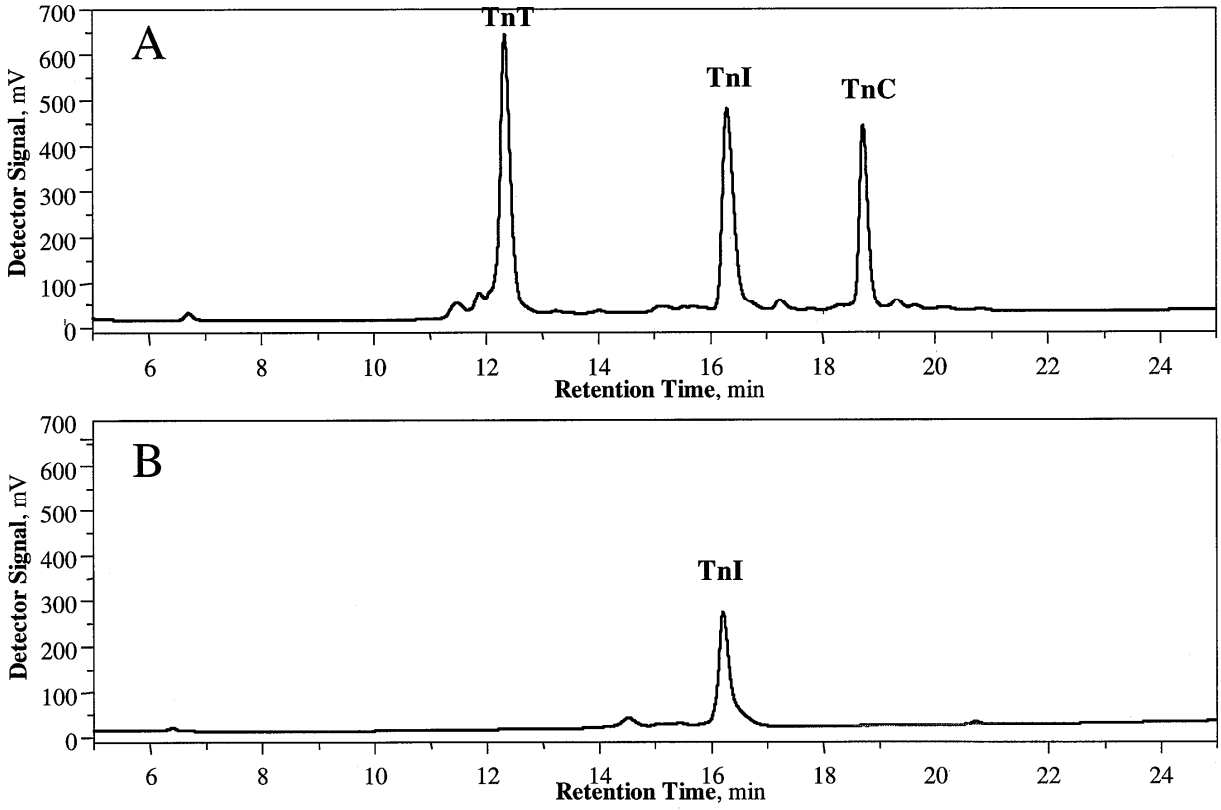

Figure 1. Chromatograms from the reversed-phase LC analysis of **A**) SRM 2921 and **B**) a cTnI calibration standard. Chromatographic conditions are described in the text.

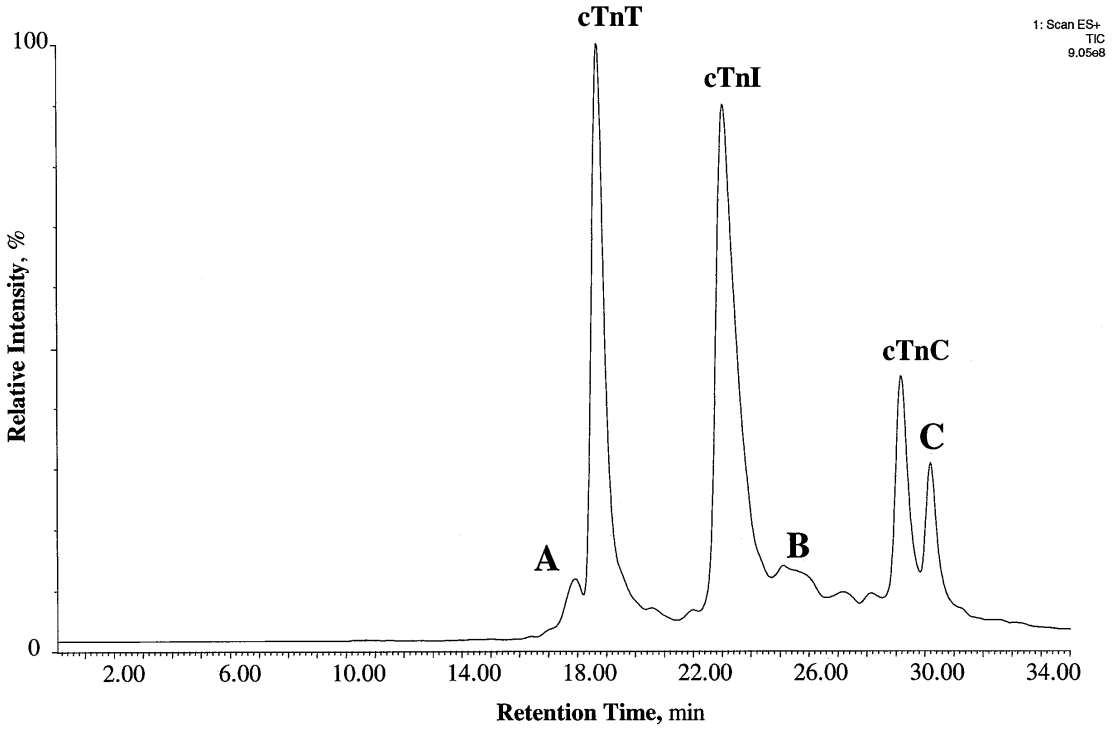

Figure 2. Total ion chromatograph from the LC/MS analysis of SRM 2921. Chromatographic conditions are described in the text.

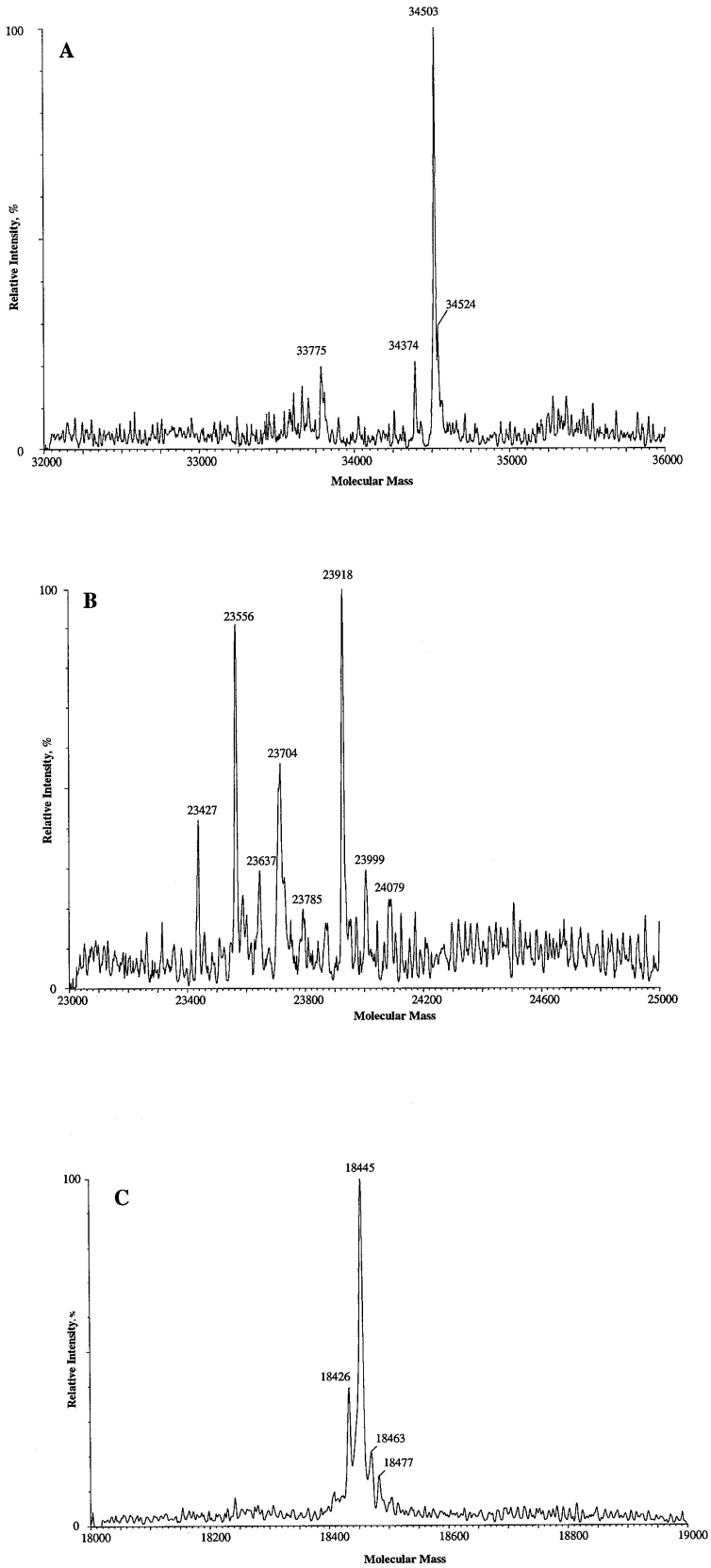

Figure 3. Deconvoluted molecular mass distributions of A) cTnT, B) cTnI, and C) cTnC obtained from the LC/MS analysis of SRM 2921.

REFERENCES

[1] Christenson, R.H.; Duh, S.H.; Apple, F.S.; Bodor, G.S.; Bunk, D.M.; Dalluge, J.; Panteghini, M.; Potter, J.D.; Welch, M.J.; Wu, A.H.B.; Kahn, S.E.; *Standardization of Cardiac Troponin I Assays: Round Robin of Ten Candidate Reference Materials*; Clin. Chem., Vol. 47, pp. 431-437 (2001).

[2] *Guide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9 1st ed. ISO, Geneva, Switzerland (1993); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing Uncertainty of NIST Measurement Results*; NIST Technical Note 1297, U.S. Government Printing Office: Washington, DC (1994); (available at http://physics.nist.gov/Pubs).

[3] U.S. Department of Health and Human Services; *Biosafety in Microbiological and Biomedical Laboratories*; U.S. Government Printing Office: Washington, DC (1988).

[4] Agilent Technical Publication No. 5980-1193E.

[5] Bunk, D.M.; Welch, M.J.; Anal. Biochem., Vol. 284, pp. 191-200 (2000).

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet http://www.nist.gov/srm.