CCQM-P58.1: Immunoassay Quantitation of Human Cardiac Troponin I.

Co-ordinating Laboratories: NIST, NPL

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Introduction

Immunoassay is a common method used to measure a wide variety of measurands for a wide range of applications. Because immunoassays typically utilize two or more antibodies, the measurands most amenable to immunoassay have sufficient molecule size to allow binding of one or more antibodies and for the production of antibodies which have high specificity to the measurand. As such, large molecules, such as proteins and glycans, are most often measured by immunoassay. The high measurement specificity of immunoassay, their robustness, speed, and low-cost, as well as the simplicity of instrumentation used in immunoassay all contribute to the widespread use in food analysis, forensics, and medical diagnostics. Due to the widespread use of immunoassay, NMIs (and/or their designated institutes) are often tasked to provide metrological support for routine use of immunoassay and may now, or in the future, employ immunoassay in their own measurement and calibration services.

The CCQM study P58.1 assessed the equivalence of immunoassay measurements between participating NMIs. The aim of P58.1 was to demonstrate the equivalence of immunoassay measurements to determine the mass concentration of the clinically-relevant protein human cardiac troponin I (cTnI) present at low concentration relative to the protein concentration of the sample matrix. The measurement equivalence will be assessed using traceability to a common certified reference material. To quantify cTnI, participants will use a homogeneous sandwich-based immunoassay with an enzymatic amplification step. The antibody format consists of a single capture and single detection antibody (referred to as 1 + 1), both were supplied to study participants.

The CCQM study P58.1 assessed the utility of a candidate immunoassay reference procedure to quantify a protein antigen based on the detection of two epitopes within the antigen. For this approach to succeed, a complete understanding and assessment of the uncertainty associated with the use of an immunoassay is required to assign mass values to a secondary certified reference material (CRM) from the concentration of a primary CRM. The P58.1 study determined the uncertainty associated with the use of the candidate reference immunoassay to assign values of the concentration of human cardiac troponin I (cTnI) in an aqueous protein solution.

In the previous P58 study, ELISA measurement results were compared between laboratories which all used common ELISA reagents (including 96-well plates), samples, a standard for the production of calibrants, and a detailed ELISA protocol, which were supplied by a single laboratory. The P58.1 study only utilized common samples, a standard of the production of calibrants, and a set of monoclonal antibodies (mAbs). Because much of the experimental procedure for the P58 study was essentially standardized across participating labs, the study primarily highlighted between-laboratory differences in plate sampling designs and in plate reader response. As the participants in the P58.1 study had to produce most of their own analytical reagents and develop their own measurement procedure, the study provides a better evaluation of the equivalence of ELISA measurements between the participating laboratories.

Description of the Measurand

The immunoassay used in P58.1 targets human cardiac troponin I through the use of two mouse monoclonal antibodies. The epitope of the monoclonal antibody (mAb 560) used for antigen capture is the region between residue 83 and residue 93 of human cTnI. For detection of the bound protein and signal generation, another monoclonal antibody was used (HyTest mAb 19C7), its epitope being the region between residue 41 and residue 49.

Participants

All participants (Table 1) have agreed to have their respective results identified throughout this report.

Table 1. Participants in the P58.1 Study

Laboratory	Country
BAM	Germany
CENAM	Mexico
INMETRO	Brazil
IRMM	Belgium (E.U.)
KRISS	South Korea
NIMC	China
NIMT	Thailand
NIST	United States
NMIJ	Japan
NPL	United Kingdom
PTB	Germany
UME	Turkey
VNIIM	Russia

Preparation of Samples

The samples for P58.1 were prepared at NIST through gravimetric dilution of NIST SRM 2921 (Human Cardiac Troponin Complex). The dilution buffer was 20 mmol/L Tris (Sigma-Aldrich), 150 mmol/L sodium chloride (Fluka), 1 mmol/L calcium chloride (Sigma-Aldrich), pH 7.4 containing 7.5 g/L bovine serum albumin (BSA) (Sigma-Aldrich). The final level of cTnI in the three study samples were in the range 0.5 µg/L to 10 µg/L. Approximately 200 aliquots of each of the three samples were prepared in 500 µL polypropylene screw-cap vials (Sarstedt), each with a sample fill volume of approximately 300 µL. After preparation and aliquoting, all samples were stored at ≤ -70 °C.

The immunoassay capture anti-human cTnI mAb clone 560 and detection anti-human cTnI mAb clone 19C7 were purchased from HyTest Ltd., Finland. The choice of these monoclonal antibodies was based on the recommended guideline and research finding (1, 2).

Homogeneity testing of Samples

The homogeneity analysis of unknown sample 2 was performed on 8 vials from each sample batch; 4 vials were chosen randomly from early in the fill order (i.e., vial numbers 1 through 50) and 4 vials were chosen randomly from late in the fill (vial numbers 150 through 200). From each of the 8 samples, three aliquots were analyzed using the candidate reference measurement procedure (RMP), described in Appendix2, on a single plate. Through comparison of the ELISA fluorescence intensity readouts from all 8 samples shown in Figure 1, no evidence of sample inhomogeneity of unknown sample 2 was observed. As all three samples are similar in nearly every aspect, it is assumed that the homogeneity observed for sample 2 also reflects the homogeneity of sample 1 and sample 3. Both pre-study test runs by us and actual study runs performed by participants validated the assumption.

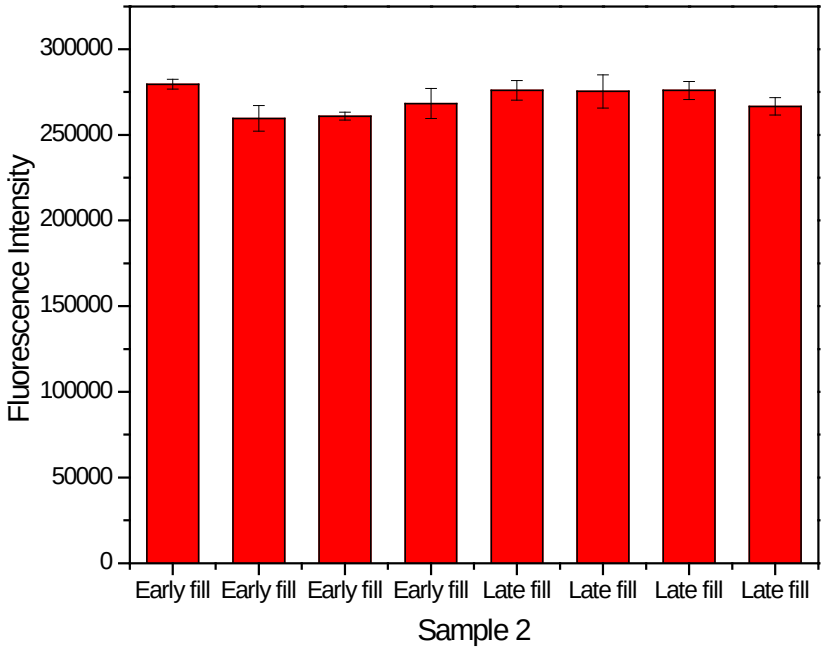

Figure 1: ELISA fluorescence intensity readouts of 8 individual sample vials for homogeneity assessment of unknown sample 2.

Stability testing

Stability testing of unknown sample 1 was performed with four sets of two vials of the samples stored at approximately 22 °C, 5 °C, and -20 °C, (with remaining sample aliquots stored at -80 °C) for approximately 3 days, 14 days, 28 days, and 42 days. Using an isochronous study design at the end of the 42 day study period, all aliquots were analyzed by the same ELISA method described below on a single plate. Through comparison of the ELISA response from all 26 vials as shown in Figure 2, there was a statistically significant decrease in the measured cTnI responses of samples stored at -20 °C relative to those stored at 5 °C ($p < 0.0001$). T-test showed no difference between cTnI responses of samples stored at 5 °C and at 22 °C ($p = 0.242$). Significant difference in cTnI responses was also observed between the control samples stored at ≤ -70 °C and samples stored at approximately -20 °C ($p < 0.0001$). Therefore, unknown samples were shipped to participants with dry ice and specified to be stored at ≤ -70 °C prior to the measurement. As all three samples are similar in nearly every aspect, it is assumed that the stability observed for sample 1 also reflects the stability of sample 2 and sample 3. Both pre-study test runs by NPL and actual study runs performed by participants validated the assumption.

Distribution of samples and report instructions

The P58.1 study description that was send to participants is in Appendix 1. Each participating institution was sent three vials each of Samples 1 through 3 and was requested to store these samples at ≤ -70 °C and thawed completely before sub-samples were taken for measurement. Participants were requested to analyse three separate sub-samples from each of the three P58.1 samples provided. The analysis of all three samples was repeated on a separate day, again with the analysis of each sample with triplicate aliquots.

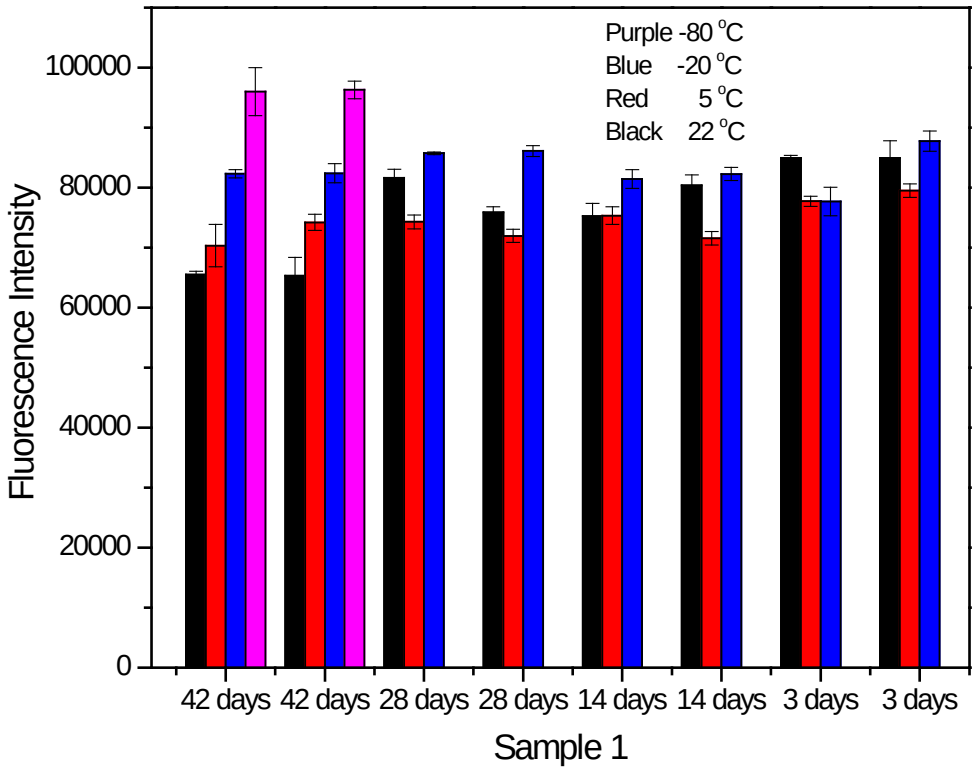

Figure 2 ELISA fluorescence intensity readouts of 26 individual sample vials for stability assessment of unknown sample 1. Two vials of sample 1 stored at ≤ -70 °C (purple columns) serve as controls for evaluating sample stability at three other different storage temperatures.

Participants additionally received three vials of the human cardiac troponin I standard (NIST SRM 2921) and were asked to store at ≤ -70 ºC until used to prepare calibration solutions. Participants also received stock solutions of the capture and detection monoclonal antibodies to be used in the 1+1 ELISA. Approximately 19 µL of a 5.3 mg/mL solution of capture mAb clone 560 and approximately 95 µL of a 3.6 mg/mL solution of detection mAb clone 19C7 were sent to participants. Participants were requested to store both mAb stock solutions at approximately 4 °C until used.

A detailed ELISA protocol (developed at NPL, see Appendix 2) was supplied to all participants as an example of a methodology that could be used in this study.

Participants were requested to report:

- The measured mass concentration of the cTnI in Samples 1 through 3 expressed in µg/L.
- A full uncertainty budget for the mass concentration of cTnI in Samples 1 through 3.
- An outline of the methodology, a full measurement equation and a breakdown of the uncertainty estimation submitted.

Participant method details

Although participants were provided with an example protocol, they were free to use any method, reagents (excluding antibodies and primary calibrant), or modify the protocol to fit in-house procedures. Twelve out of 15 participants returned data sets within the designated study period. NIMT supplied data after the deadline for data submission. Two participants (BAM and IRMM) performed the assay and measured on two instruments (Table 2). The mean response from these two instrument data sets was used in all subsequent data analysis unless indicated. CENAM provided data run on

two independent assays: RT-PCR and a commercial clinical analyser. The clinical analyser used by CENAM did not employ the monoclonal antibody pair supplied to the participants, a requirement of this study. Therefore, for statistical analysis, 13 unique data sets were used in the P58.1 study, excluding the data from NIMT and one of the sets submitted by CENAM.

Where indicated, all participants used a diluent containing 7.5 g/L BSA except NPL who used a diluent containing 75 g/L BSA to prepare the cTnI calibration samples. The use of fluorescent standards to compare/normalize plates and understand the uncertainty associated with the instrument (plate reader) was not performed by all participants. Table 2. Experimental factors used by the P58.1 study participants. Table 2 highlights which participants used this approach and the fluorescent standard employed.

Participant Data and Uncertainty Estimates

Table 3 details the reported measured cTnI concentrations for samples 1, 2 and 3, the given uncertainty estimate and coverage factor used.

Participants were asked to develop an uncertainty equation for the use of immunoassay technique to quantitate cTnI using SRM2921. The uncertainty equations where provided by participants have been listed in Appendix 4, with the majority of the participants identifying two sources of uncertainty that they considered to be significant, that associated with repeated measurement and the uncertainty associated with the certified concentration of cTnI in the common standard used for calibration (i.e., SRM 2921).

Many of the uncertainty components estimated by participants were influenced by the cTnI sample tested. Table 4, Table 5 and Table 6 express the uncertainty components as a percentage of the total uncertainty. Based on participants prior knowledge and measurements performed during the study to assess the contribution of certain assay steps the magnitude of uncertainty components varies considerably from participant to participant. Uncertainty associated with repeated measurement and that associated with the certified concentration of the calibration material SRM 2921 are common as significant contributor to the overall uncertainty across the majority of participants.

Table 2. Experimental factors used by the P58.1 study participants

Laboratory	Method	Plate Reader	[BSA] in diluent, g/L	Fluorescent Standard
BAM	ELISA	Molecular Devices M5 multilabel reader	7.5	None – not compatible with protocol
		Edinburgh Instruments FS 920		
CENAM	RT-PCR	Taq-man Protein Analysis Oligo probe	7.5	
	Commercial Clinical analyser	Immunometric Vitros		
INMETRO	ELISA	BioTek Synergy2 SFA	7.5	SRM 1932 - not compatible with protocol
IRMM	ELISA	Victor 3 multilabel counter	7.5	BAM F003
		Fluostar Galaxy		
KRISS	ELISA	Victor 3 multilabel counter	7.5	Quinine sulphate
NMIC	ELISA	Tecan T200	7.5	
NIST	ELISA	Fluorolog microwell plate reader	7.5	Quinine sulphate
NMIJ	ELISA	Appliskan (Thermofisher Scientific)	7.5	50 μL of 5 μM 4-methylumbelliferone
NPL	ELISA	Victor 2 multilabel counter	75.0	SRM 1932 (100 nM, 100 μL)
PTB	ELISA	Biotek Synergy H4 double monochromators	7.5	
UME	ELISA	DTX 880 Beckman Coulter	7.5	
VNIIM	DELFIA	FLUOROFOT (Nanospectr, RF)	7.5	
NIMT	ELISA	Biotek SynergyHT	7.5	SRM 2921

Table 3. Participants reported results and uncertainty statements for Samples 1-3. Note: the significant figures listed are those reported by participants.

	Sample 1			Sample 2			Sample 3		
Laboratory	[cTnI], µg/L	U, µg/L	k	[cTnI], µg/L	U, µg/L	k	[cTnI], µg/L	U, µg/L	k
BAM_1 (reader 1)	0.604	0.336	2	1.12	0.582	2	4.21	2.06	2
BAM_2 (reader 2)	0.565	0.327	2	1.14	0.483	2	4.41	2.14	2
CENAM_1 (RT-PCR)	1.3	0.6	2	3.7	2.4	2	-	-	
INMETRO	0.957	0.166	2	3.17	0.548	2	13.15468	2.244595	2
IRMM_1 (reader 1)	0.61	0.07	2	1.85	0.42	2	7.47	0.53*	2
IRMM_2 (reader 2)	0.60	0.08	2	1.82	0.42	2	7.39	0.80	2
KRISS	0.94	0.16	2	2.28	0.17	2	8.18	1.02	2
NIMC	0.82	0.16	2	2.19	0.34	2	9.6	1.4	2
NIST	0.85	0.04	2	2.472	0.0897	2	8.212	0.551	2
NMIJ	0.598	0.130	2	1.950	0.172	2	7.144	0.527	2
NPL	0.852	0.11	2	2.554	0.361	2	7.186	0.96	2
PTB	1.14	0.48	2	3.32	2.14	2	**	**	**
UME	0.733	0.119	2	2.097	0.677	2	9.157	2.702	2
VNIIM	0.671	0.432	2	2.159	0.4867	2	10.179	3.124	2
CENAM_2 (Vitros)	1.7	0.13	2	4.7	0.13	2	17.4	1.22	2
NIMT	0.34	0.06	2	1.58	0.18	2	6.26	1.08	2

* For the calculation of the expanded uncertainty for Sample 3, the s_{ip} uncertainty component (Appendix 4) from Sample 1 was used.

** Sample 3 was not received by PTB.

Table 4. Breakdown of participants' reported uncertainty components for Sample 1. The uncertainty components represent the mean % across either the two or three plates reported. NR; uncertainty components not reported. Uncertainty components less than 1 % are not listed.

Laboratory	Overall		% of total Uncertainty				
	μg/L	U μg/L	uSTAND	uMEAS	uLIQ_H	uCAL	uPLATE
BAM_1	0.604	0.336	28.1	17.9	54.1		
BAM_2	0.565	0.327					
CENAM_1	1.300	0.600					
INMETRO	0.957	0.166	22.6	75.2	2.2		
IRMM	0.61	0.07					
KRISS	0.945	0.165	15.7	58.0		5.6	10.1
NIMC	0.820	0.160	15.9	17.4		66.7	15.9
NIST	0.850	0.0475			15.5	22.4	62.1
NMIJ	0.598	0.130	13.0	61.4	17.5		9.4
NPL	0.852	0.110	22.4	57.9	2.6	13.0	4.1
PTB	1.140	0.480		40.0	28.5		9.4
UME	0.733	0.119	14.9	66.9	2.0	39.6	
VNIIM	0.671	0.432	41.8	39.3	6.0		13.0
NIMT	0.34	0.06	18.7	21.5	20.0		
CENAM_2	1.700	0.130					

Table 5. Breakdown of participants' reported uncertainty components for Sample 2. The uncertainty components represent the mean % across either the two or three plates reported. Uncertainty components less than 1 % are not listed.

Laboratory	Overall		% of total Uncertainty				
	μg/L	U μg/L	uSTAND	uMEAS	uLIQ_H	uCAL	uPLATE
BAM_1	1.12	0.582	30.2	17.3	52.5		
BAM_2	1.14	0.483					
CENAM_1	3.700	2.400					
INMETRO	3.171	0.548	22.6	75.2	2.2		
IRMM	1.85	0.42					
KRISS	2.283	0.167	35.6	38.4		10.7	23.0
NIMC	2.190	0.340	19.0	18.2		62.9	19.0
NIST	2.472	0.0897			21.9	48.0	30.1
NMIJ	1.950	0.172	21.4	36.3	28.9		15.6
NPL	2.554	0.361	21.0	60.5	2.4	12.2	3.9
PTB	3.320	2.140		68.6	25.8		8.503
UME	2.097		0.7	9.9	77.7	1.3	67.0
VNIIM	2.159		0.5	39.9	8.5	16.3	35.4
NIMT	1.58	0.18	20.9	29.1	20.0		
CENAM_2	4.700	0.130					

Table 6. Breakdown of participants' reported uncertainty components for Sample 3. The uncertainty components represent the mean % across either the two or three plates reported. Uncertainty components less than 1 % are not listed.

Laboratory	Overall		% of total Uncertainty				
	µg/L	U µg/L	uSTAND	uMEAS	uLIQ_H	uCAL	uPLATE
BAM_1	4.21	2.06	36.8	15.7	47.5		
BAM_2	4.41	2.14					
CENAM_1							
INMETRO	13.155	2.245	22.6	75.2	2.2		
IRMM	7.47	0.53					
KRISS	8.177	1.019	24.9	62.1		7.2	16.1
NIMC	9.570	1.400	20.0	16.4		63.6	20.0
NIST	8.212	0.551			26.2	63.0	10.9
NMIJ	7.144	0.527	23.4	30.5	31.5		17.0
NPL	7.186	0.960	22.0	58.8	2.5	12.6	4.0
PTB							
UME	9.157	2.702	10.3	71.3	1.3	62.3	
VNIIM	10.179	3.214	37.9	27.1	11.1		24.0
NIMT	6.26	1.08	22.6	31.8	20.0		
CENAM_2	17.400	1.220					

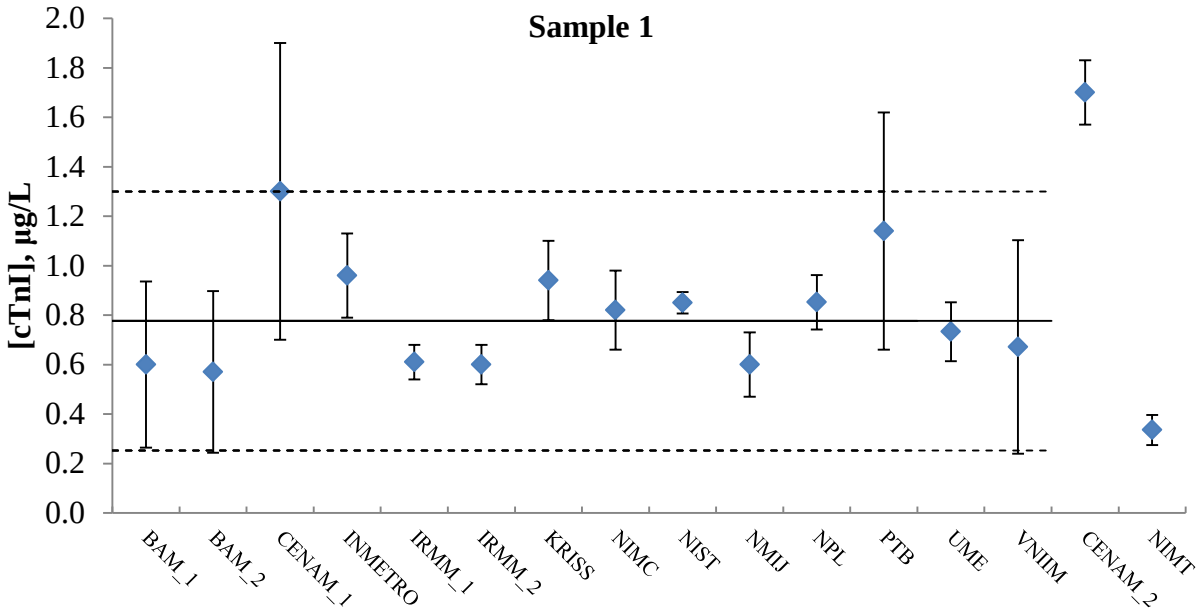

Figure 3: Comparison plot of participants' results for the measurement of the mass concentration of cTnI in Sample 1. The solid line indicates the median value of all measurement results (excluding NIMT and CENAM_2) while the dotted lines approximates the 95 % confidence interval of the median (± 1 median absolute deviation (MADe)). The uncertainty associated with each laboratory's data is the expanded uncertainty (U_{95}) as reported by the laboratory.

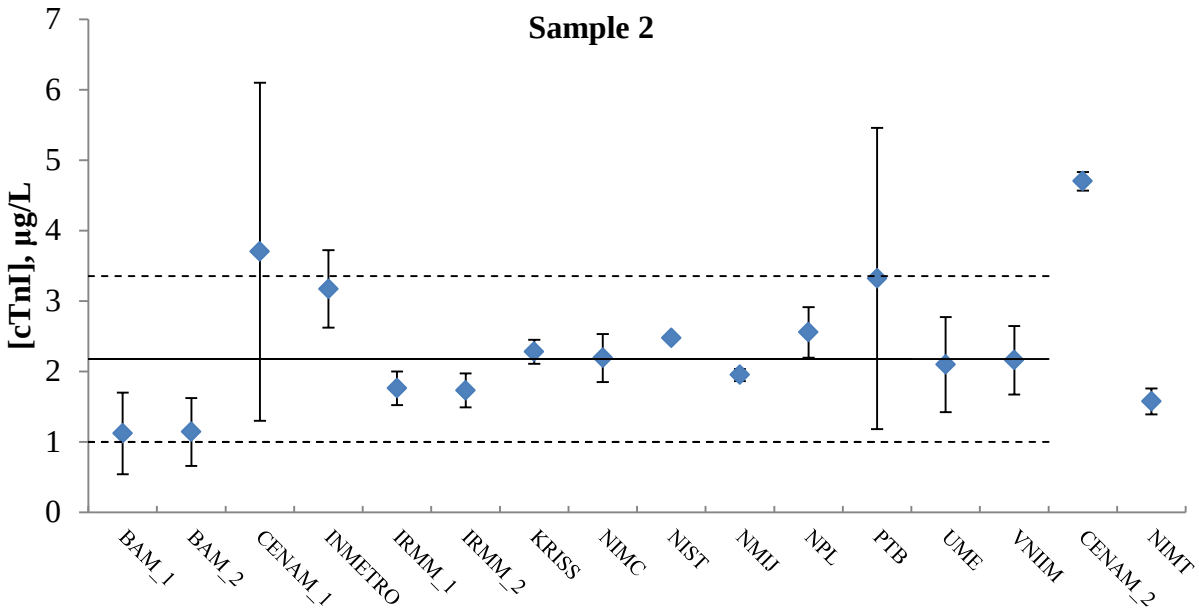

Figure 4: Comparison plot of participant's results for the measurement of the mass concentration of cTnI in Sample 2. The solid line indicates the median value of all measurement results (excluding NIMT and CENAM_2) while the dotted lines approximates the 95 % confidence interval of the median (± 1 MADe). The uncertainty associated with each laboratory's data is the expanded uncertainty (U_{95}) as reported by the laboratory.

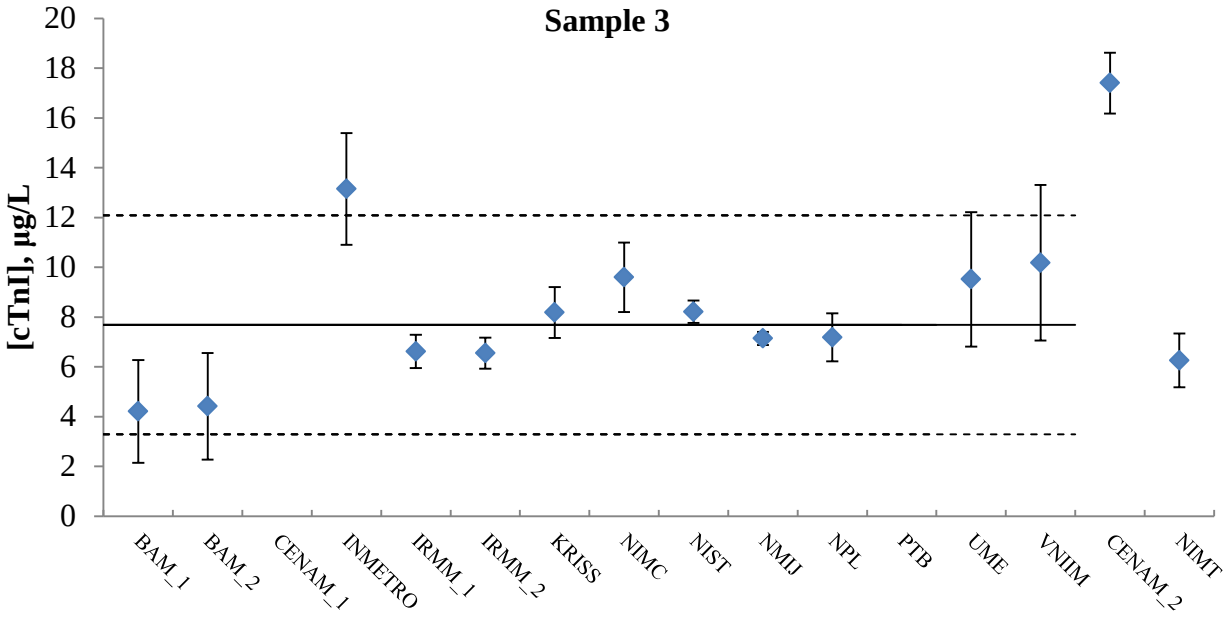

Figure 5: Comparison plot of participants' results for the measurement of the mass concentration of cTnI in Sample 3. The solid line indicates the median value of all measurement results (excluding NIMT and CENAM_2) while the dotted lines approximates the 95 % confidence interval of the median (± 1 MADe). The uncertainty associated with each laboratory's data is the expanded uncertainty (U_{95}) as reported by the laboratory.

Analysis of Results

Figure 3 through Figure 5 compare the measured concentration of cTnI in Sample 1 through Sample 3, respectively. In these figures, the solid line represents the median value of participant data and the dotted lines are ± 1 MADe (the standard estimate from the median absolute deviation), approximately the 95 %

confidence interval of the median. Robust statistics were used to analyze the P58.1 study data to reduce the impact of inconsistent results. Because the results from NIMT were submitted after the study deadline and after other study participant's data had been made available, the NIMT results were not used in any data analysis. The commercial clinical assay results submitted by CENAM (CENAM_2) did not use the anti-cTnI clones 560/19C7 in a similar format as requested in the P58.1 study protocol. Therefore, the measurand defined in the P58.1 study as epitopes specific to clones 560 and 19C7 was not specifically measured by the commercial clinical assay. As such, the CENAM_2 results were not included in the analysis of the P58.1 study data. However, the NIMT and CENAM_2 results are included in Figure 3 through Figure 5 to facilitate comparisons.

Coherent Statistical Analysis

In the P58.1 study, each participant was free to use any statistical method to analyze their immunoassay measurement data and arrive at cTnI concentration estimates and uncertainties. Discrepancies between participants' result in Figures 3-5 and Figures A1-A3 could thus be caused, in part, by applying different statistical methods or by applying methods which are not fit for purpose. Reanalysis of the P58 study (4) showed that discrepancies between immunoassay concentration estimates due to statistical analyses can be substantial.

Eight participants provided raw data (i.e. intensity measurements and calibrant concentrations) for each measured immunoassay plate. We reanalyze each of these data sets by the same statistical method (4). This method consists of a non-linear regression with heteroscedastic Gaussian errors for the calibration and subsequent prediction of the unknown concentration – both tasks are solved applying Bayesian inference. Previously, this approach was shown to be applicable and to give reliable estimates and uncertainties.

Bayesian analysis requires prior distributions. These priors are newly developed for the current study, because the priors used previously for analysis of the P58 study data (4), relied on additional measurements of control samples which are unavailable in P58.1. On the other hand, an assumption of no prior information on any of the calibration parameters (requiring uninformative prior distributions) leads to identifiability issues for at least three immunoassay data sets in this study. Instead, information (i.e. expertise), gained during the precursor P58 study, can be used to derive prior distributions for the current study. Because the precursor study P58 covered a wide range of experimental settings (different international participants, different laboratory equipment, personnel and procedures, etc.), we can assume that the calibration curves estimated for P58 (and their uncertainties) cover the plausible range of future calibration curves if

- the intensity measurements and calibrant concentrations are suitably scaled, and if
- the measurements can be assumed to follow the same statistical model (the 4-parametric logistic function and Gaussian errors whose variance is linear in the concentrations).

Approximating (or smoothing) the posterior distribution of each calibration parameter from all P58-participants with a parametric distribution from a heavy-tailed family (the *t*-distribution in particular) may thus represent the

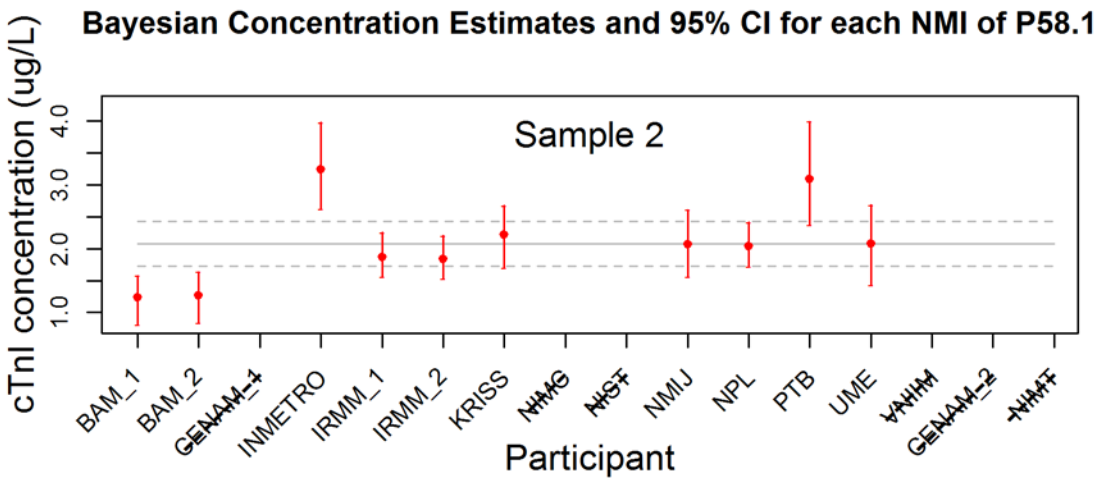

Figure 7: Bayesian concentration estimates and 95% credible intervals (CI) based on each participants measurement of the mass concentration of cTnI in sample 1. NMIs whose names are struck out did not submit raw data to perform a Bayesian analysis.

knowledge which was available prior to performing the current study and is displayed in Figure of Appendix 7.

The robustness of estimates and uncertainties with respect to the employed prior distributions were tested. For those data sets of the current study where application of uninformative prior distributions on all calibration parameters is possible, the estimates and uncertainties are almost identical to the ones displayed below. For the remaining data sets, the employed prior distributions solve identifiability issues and thus enable an analysis.

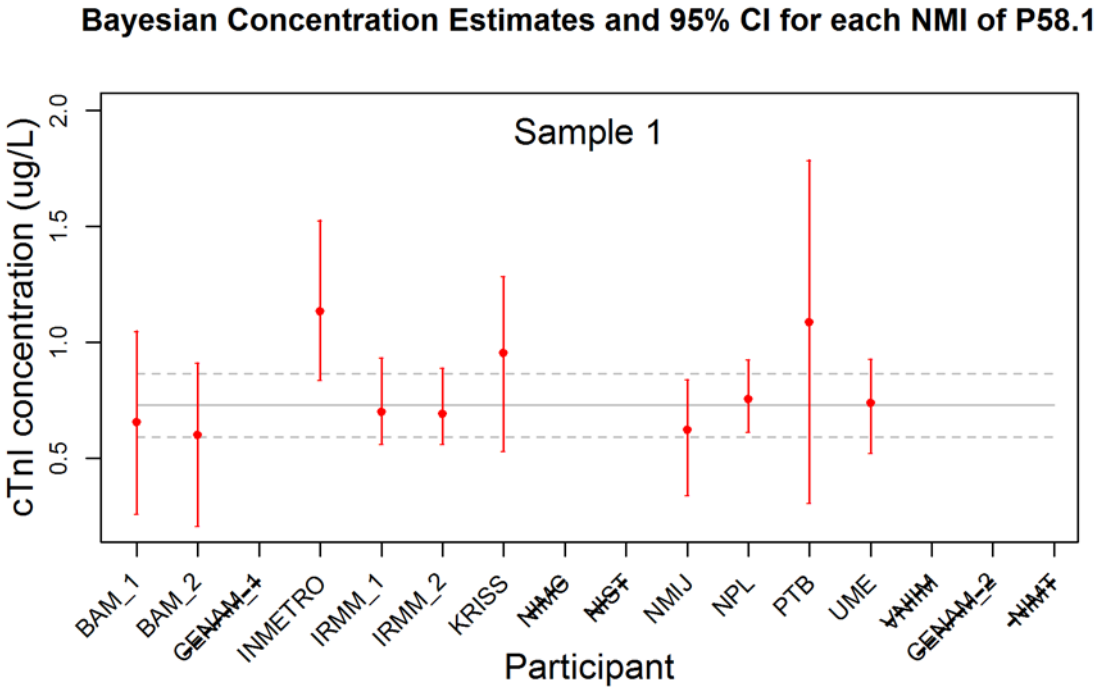

Figure 6: Bayesian concentration estimates and 95% credible intervals based on each participants measurement of the mass concentration of cTnI in sample 2. NMIs whose names are struck out did not submit raw data to perform a Bayesian analysis.

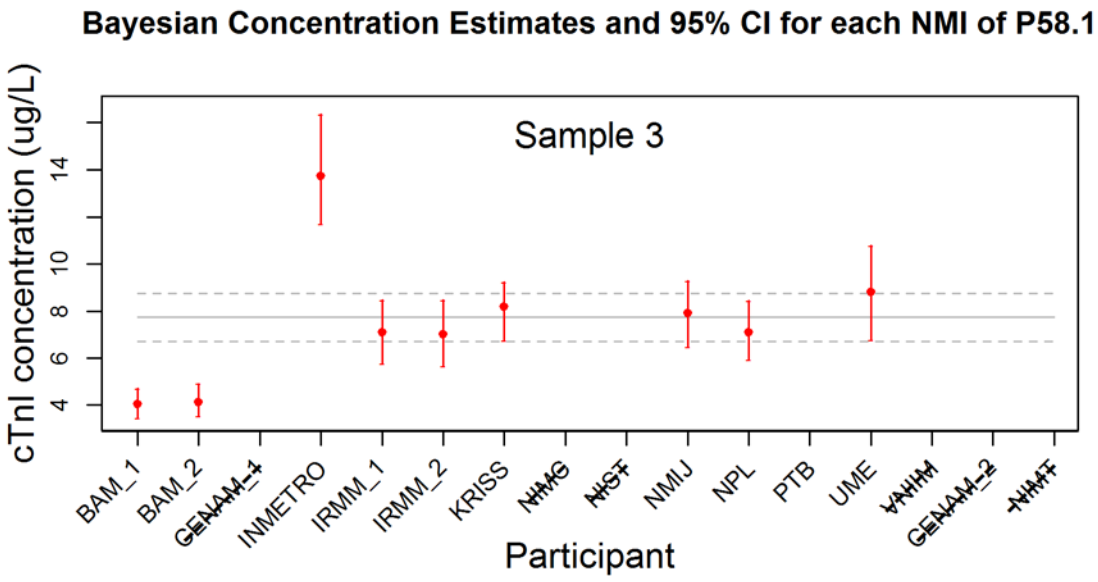

Figure 8: Bayesian concentration estimates and 95% credible intervals based on each participants measurement of the mass concentration of cTnI in sample 3. NMIs whose names are struck out did not submit raw data to perform a Bayesian analysis.

Estimates and Uncertainties from the Coherent Statistical Analysis

Application of the Bayesian approach leads to cTnI concentration estimates for each immunoassay plate. Subsequent linear pooling (5) of these two or three plate estimates (i.e. adding their posterior distributions), gives the P58.1 cTnI concentration estimates for Samples 1 through 3 displayed in Figure 6 through Figure 8, respectively.

None of the cTnI concentration estimates attained by each participant's approach contradict the ones from the Bayesian approach (i.e. the intervals overlap). More remarkable, however, is that all the uncertainties evaluated by the Bayesian approach are larger for sample 1 and most are larger for samples 2 and 3, compared to each participant's approach. In other words, especially at lower concentrations, part of the immunoassay variability is not well-explained by simpler statistical methods (such as the linear fit suggested in the data analysis section of Appendix 2).

Comparing the raw plate cTnI concentration estimates derived by the common Bayesian approach (displayed in Figure A4 through Figure in Appendix 6) to the ones derived by each participant (Figures A1-A3, in Appendix 3), supports the finding of a more reliable evaluation of uncertainties by the Bayesian method. Nevertheless, a few of the plate estimates differ significantly between the methods (plate 1 of IRMM_1 and IRMM_2, plate 2 [and plate 1 for sample 2] of NPL). Additionally, substantial discrepancies between plate estimates of the same participant remain (for INMETRO, IRMM, NMIJ, NPL and UME for the Bayesian approach) – especially for large concentrations. That is, some sources of immunoassay variability are (currently) not captured by an analysis of a single immunoassay plate. While two or three independent repeated measurements [1] display this variability in the current study, this was not the case in the previous P58 study (possibly due to the more restrictive P58 protocol).

[1] BAM and KRISS performed repetitions on the same day and show smaller inter-plate variability.

Table 7. Median and MADe values for the estimation of cTnI using all methods (although excluding the CENAM_2 and NIMT results). For participants that provided two values for each sample, where a common plate was measured on two independent instruments a mean value from the two data sets has been used in calculation of the overall study data.

	Median [cTnI], µg/L	MADe, µg/L	CV, %	Gravimetric [cTnI] ± U_{95}, µg/L
Sample 1	0.78	0.26	33.7	1.00 ± 0.05
Sample 2	2.17	0.59	27.1	2.43 ± 0.11
Sample 3	7.68	2.20	28.6	8.76 ± 0.39

Overall Results and Discussion

The combined data for all methods are presented in Table 7, and for ELISA only protocols in Table 8, i.e. excluding the clinical analyser submitted by CENAM. Because the results from NIM-T were submitted after the study deadline and after other study participant's data had been made available, the NIMT results were not used in any data analysis. The commercial clinical assay results submitted by CENAM (CENAM_2) did not use the anti-cTnI clones 560/19C7 in a similar format as requested in the P58.1 study protocol. Therefore, the measurand defined in the P58.1 study as epitopes to clones 560 and 19C7 was not specifically measured by the commercial clinical assay. As such, the CENAM_2 results were not included in the analysis of the P58.1 study data (Table 7 and Table 8).

In addition to the data obtained from the P58.1 study, Table 7 and Table 8 also list the "gravimetric" [cTnI] concentrations of all three samples. The "gravimetric" concentration is an estimate of the concentration of each samples based on the gravimetric dilution and the certified concentration of SRM 2921, from which the samples were prepared. As there is the possibility of cTnI losses during the more than 1000-fold dilution process used to prepare the study samples (due to, for example, cTnI adsorption onto pipette tips and other surfaces), the "gravimetric" concentration of the samples might not reflect the actual concentrations. The "gravimetric" concentrations should be considered to be the highest possible cTnI concentration of each sample, the expected concentration if there was no loss of cTnI during sample preparation.

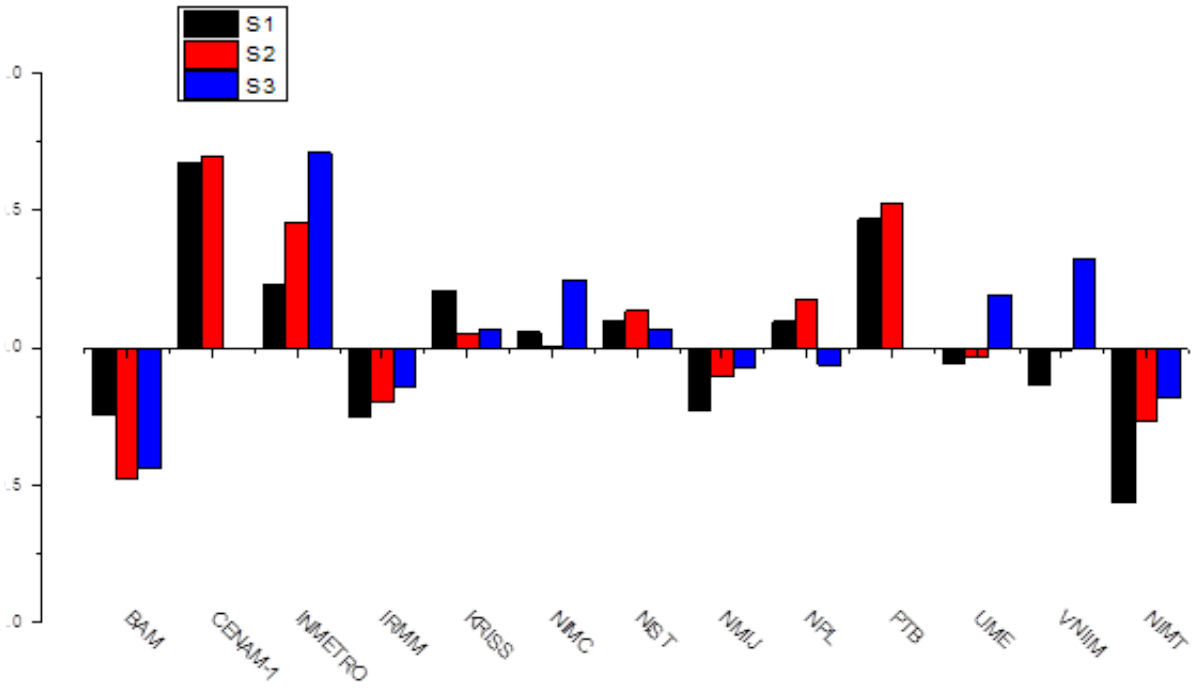

Figure 9: Bias plot – For each sample the difference from the overall median value, expressed relative to the overall median was compared.

Participants employed different approaches to test either the 2, or 3 batches of unknown cTnI standards provided, for example using common calibration samples between different plates (batches of cTnI samples). A plot comparing the uncertainty from each of the participant plates, highlighting intra-plate error is shown in Appendix 3, Figure A1, Figure A2, and Figure A3, respectively for Samples 1, 2 and 3. Comparison of uncertainty either within-plate, or plate-to-plate (including fresh preparation of the calibration samples) did not highlight an obvious trend. Where significant plate-to-plate variation occurred (compared to the intra-plate variation), this could indicate that either repeatability of the immunoassay technique or the preparation of the calibration response was resulting in significant uncertainty.

The bias plot in Figure 9 highlights any non-linearity associated with the quantitation of the cTnI samples using the immunoassay method. Data has been normalised relative to the median response from all participants (average response from the IRMM and BAM data sets used in the calculation of the mean). Assay non-linearity in determination of the unknowns will be highlighted by a wide distribution in bias values obtained

Table 8. Median and MADe values for the estimation of cTnI using ELISA methods only. For participants that provided two values for each sample, where a common plate was measured on two independent instruments a mean value from the two data sets has been used in calculation of the overall study data.

	Median [cTnI], µg/L	MADe, µg/L	CV, %	Gravimetric [cTnI] ± U_{95}, µg/L
Sample 1	0.73	0.20	26.9	1.00 ± 0.05
Sample 2	2.16	0.59	27.1	2.43 ± 0.11
Sample 3	7.68	2.20	28.6	8.76 ± 0.39

for each of the cTnI samples. A plot of the cTnI concentration as estimated using gravimetric dilution, and the median response from all methods (Figure 10), generates a good correlation with an adjusted r^2 of 0.994. The correlation observed suggests linearity of the ELISA approach for determination of the cTnI concentrations represented by the samples analysed within the P58.1 study.

Similar meta-analyses can be carried on the basis of the results from the coherent statistical analysis. On the level of laboratory concentration estimates the Bayesian cTnI concentration estimates appear largely consistent with each other. One possibility to investigate this for each sample in detail is an analysis based on a linear fixed effects model (6). Such an analysis is robust to inconsistent measurements, and under the assumption of at least 4 consistent measurements, it suggests the reference concentrations listed in Table 9 (and also depicted in Figure 6 through Figure 8 as the solid and dashed horizontal lines in gray) . Note that only eight laboratories (BAM (set 1), INMETRO, IRMM (set 1), KRISS, NMIJ, NPL, PTB, and UME) contributed to this reference value, because one of each of the double estimates for BAM and IRMM were excluded and five laboratories did not contribute raw data for the Bayesian analysis. Also in Table 9 are the median (and associated uncertainties) cTnI concentrations calculated from the values submitted by these participating labs.

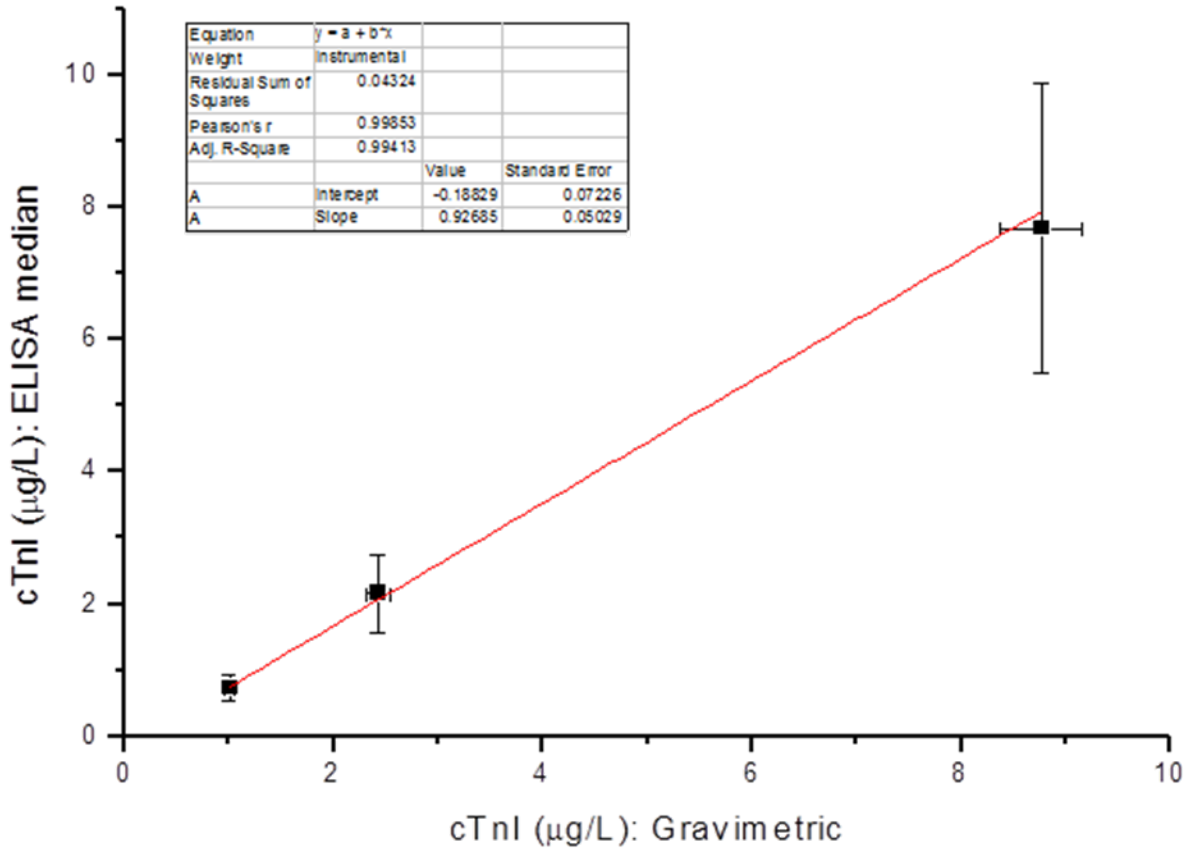

Figure 10: Comparison of participant Median and MADe values for the estimation of cTnI using all methods (although excluding the CENAM_2 and NIMT results) with those estimated from gravimetric dilution.

Table 9. Samples cTnI reference concentration estimated by applying a linear fixed effects model to the participants Bayesian concentration estimate. For comparison, the median, MADe, and CV of the data reported by included. Note that data from BAM (set 1), INMETRO, IRMM (set 1), KRISS, NMIJ, NPL, PTB, and UME are included in the comparison.

	Estimated reference [cTnI], µg/L	95 %CI, µg/L	CV, %	Median [cTnI], µg/L	MADe, µg/L	CV, %
Sample 1	0.73	(0.59, 0.86)	14.3	0.79	0.27	33.7
Sample 2	2.08	(1.72, 2.43)	5.6	2.19	0.59	26.9
Sample 3	7.73	(6.71, 8.76)	1.9	7.19	1.47	20.5

The fact that the estimated reference concentrations (calculated from the coherent statistical analysis of participant's raw data) in Table 9 have smaller uncertainties than the median of the values submitted by study participants is caused by the difference in methods to estimate this reference (and not necessarily by the difference in concentration estimates for each participant). Additionally, the analysis gives evidence that one participant has measured sample 2 with bias, and two participants have measured sample 3 with bias (not shown).

Comparison of P58 and P58.1 Study Results

The data obtained from the P58.1 can be compared to the previous P58 study that used ELISA for the quantitation of human interferon α2β (3). For the P58 study all the reagents were provided to the participant The P58 study design contrasts to P58.1, where only the analyte, reference sample and antibodies were provided. In the P58 study, the between-laboratory variability in the measured concentration of the single unknown sample used in the study was approximately 6 %. As indicated in Table 8, the between-laboratory variability for all three samples used in the P58.1 study was approximately 27 %. The higher level of between-laboratory variability in the P58.1 study can potentially be explained by a number of factors. Probably the most important factor is that the P58.1 study required participants to produce most of their ELISA reagents (most importantly, labelled and immobilized antibodies) while all reagents (including well-plates) were supplied to participants in the P58 study. Another difference between the P58 and P58.1 studies that may have contributed to higher between-laboratory variability in the P58.1 study is the concentration of the unknown sample. In P58, the concentration of the human interferon α2β in the study sample was approximately 1000-fold higher than any of the three cTnI samples used in the P58.1 study. One would anticipate higher between-laboratory variability for the measurement of very low concentration samples.

P58.1 Scope of Applicability

The P58.1 study supports a measurement claim for the determination by immunoassay of the mass concentration of a protein present in an aqueous protein solution for which the matrix protein concentration is significantly larger than the measurand concentration and the matrix protein composition is simple (one or a few matrix proteins).

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Appendix 1: Study Protocol

CCQM-P58.1

"Immunoassay Quantitation of cTnI Protein"

A pilot project organised by the Bioanalysis working group (BAWG) of the CCQM as a follow up to CCQM-P58 "Fluorescence measurement for the life sciences".

Introduction

The CCQM study P58.1 will assess the utility of using an immunoassay format to quantify a protein antigen based on the detection of two epitopes within the antigen. For this approach to succeed, a complete understanding and assessment of the uncertainty associated with the use of an immunoassay is required to assign mass values to a secondary certified reference material (CRM) from the concentration of a primary CRM. The P58.1 study will determine the uncertainty associated with the use of the candidate reference immunoassay to assign values of the concentration of human cardiac troponin I (cTnI) in an aqueous protein solution. Ultimately the P58 studies may lead to a key comparison based on the use of immunoassay for the value assignment of the mass concentration of a protein analyte in a complex-matrix CRM such as human serum.

The aim of P58.1 is to demonstrate the comparability of an immunoassay measurement to determine the mass concentration of a clinically-relevant protein (cardiac troponin I) present at low concentration relative to the protein concentration of the sample matrix. The measurement comparability will be assessed using traceability to a common certified reference material.

To quantify cTnI, participants will use a homogeneous sandwich-based immunoassay with enzymatic amplification step. The antibody format consists of a single capture and single detection antibody (referred to as 1 + 1). The detection antibody is coupled to an enzyme such as alkaline phosphatase, the relative concentration of which can be determined optically using a suitable fluorescent substrate. Participants will need access to a fluorescent plate reader, capable of measuring an enzymatic substrate, such as 4-MUP.

Description of the Measurand

The immunoassay used in P58.1 target human cardiac troponin I through the use of two mouse monoclonal antibodies. The epitope of the monoclonal antibody (MAb 560) used for antigen capture is the amino acids between residue 83 and residue 93 of human cTnI. For detection in the immunoassay, the epitope of the monoclonal antibody used (HyTest MAb 19C7) is the amino acids between residue 41 and residue 49.

Preparation of Samples

The final level of cTnI in the three study samples is expected to be in the range 0.5 µg/L to 25 µg/L. All three samples were prepared by gravimetric dilution of NIST SRM 2921 ("Human Cardiac Troponin Complex") with 7.5 g/L bovine serum albumin in 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 20 mmol/L Tris, pH 7.4. Approximately 200 aliquots of each of the three samples were prepared in 500 µL polypropylene screw-cap vials, each with a sample fill volume of approximately 300 µL. After preparation and aliquotting, all samples were stored at -80 °C.

Homogeneity testing of Samples

The homogeneity analysis of Sample 2 was performed on 8 vials from each

sample batch; 4 vials were chosen randomly from early in the fill order (i.e., vial numbers 1 through 50) and 4 vials were chosen randomly from late in the fill (vial numbers 150 through 200). From each of the 8 samples, three aliquots were analyzed by the ELISA method developed by NPL (see Appendix 2) on a single plate. Through comparison of the ELISA response from all 8 samples, no evidence of sample inhomogeneity of Sample 2 was observed. As all three samples are similar in nearly every aspect, it is assumed that the homogeneity observed for Sample 2 also reflect the homogeneity of Sample 1 and Sample 3.

Stability testing

Stability testing of Sample 1 was performed with four sets of two vials of the samples stored at 22 °C, 5 °C, and -20 °C, (with remaining sample aliquots stored at -80 °C) for approximately 3 day, 16 days, 28 days, and 45 days. Using an isochronous study design at the end of the 45 day study period, all aliquots will be analyzed by the ELISA method developed at NPL (see Appendix 2) on a single plate. Through comparison of the ELISA response from all 32 vials, Sample 1 demonstrated stability for all storage conditions for up to 28 days. For the Sample 1 aliquots stored at 22 °C and 5 °C for 48 days, there was a statistically significant decreased in the measured cTnI ELISA response relative to aliquots stored at -20 °C for 48 days and the control samples stored at -80 °C. As all three samples are similar in nearly every aspect, it is assumed that the stability observed for Sample 1 also reflect the stability of Sample 2 and Sample 3.

Instructions for use

Participants will receive three vials of the human cardiac troponin I standard (NIST SRM 2921). Each vial of SRM 2921 contains approximately 115 µL. Participants should use one vial of SRM 2921 to prepare calibration solutions on each of two days of analysis. Therefore, an additional vial of SRM 2921 is included which can be used in method development and test analysis. Participants should store SRM 2921 at -70 °C or below until used.

Participants will also receive stock solutions of the capture and detection monoclonal antibodies to be used in the 1+1 ELISA. Approximately 19 µL of a 5.3 mg/mL solution of capture MAb clone 560 and approximately 95 µL of a 3.6 mg/mL solution of detection MAb clone 19C7 will be sent to participant. Both MAb stock solutions should be stored at approximately 4 °C until used. There are not additional aliquots of the MAb stock solutions available; participants should use them sparingly. However, these MAb can be purchase through HyTest (www.hytest.fi) if a participant requires additional resources.

Participants will receive three vials each of Samples 1 through 3 (nine samples). The materials should be stored at -70 ºC and thawed completely before sub-samples are taken. One vial of each sample provided can be used for test analysis and method optimization.

Participants are requested to analyse three separate sub-samples from each of the three samples provided. The analysis of all three samples should be repeated on a separate day, again with the analysis of each sample with triplicate aliquots.

Methodology

Participants are requested to use their preferred 1+1 sandwich immunoassay methodology for the analysis of cTnI in each of the three samples. Because the antibodies used in an ELISA define the measurand, participants are also required to use the two supplied mouse

monoclonal antibodies in their 1+1 sandwich immunoassay. An example of an ELISA method for cTnI measurement that was developed at NPL can be found in Appendix 2.

Participants are required to use the supplied NIST SRM 2921 to prepare calibration solutions for their immunoassay measurement.

Reporting

Three results for the mass concentration of cTnI for each of the two vials of Samples 1 through 3 should be reported, as well as an overall combined result for both vials.

All results returned should include,

- The mass concentration of the cTnI in Samples 1 through 3 expressed in µg/L.
- A full uncertainty budget for the mass concentration of cTnI in Samples 1 through 3.
- An outline of the methodology, a full measurement equation and a breakdown of the uncertainty estimation submitted

Reports should be sent to Alex Knight (alex.knight@npl.co.uk)

Proposed Comparison Timetable

Deadline for signup to study: August 1, 2011

Distribution of sample materials: first two weeks of August 2011

Deadline for submission of results: November 30, 2011

Draft A report: April 2012

Appendix 2: Candidate Reference Measurement Procedure for cTnI[2]

This candidate reference measurement procedure (cRMP) is for the assignment of cTnI solution 'samples' relative calibrants prepared using a primary reference material (such as NIST SRM 2921). The procedure described is for the measurement of reference material prepared in a serum substitute for concentrations of cTnI from ranging from 100 – 10000 ng/L.

Reaction Principle

This measurement procedure uses a homogeneous sandwich-based immunoassay with enzymatic amplification step.

The antibody format consists of a single capture and single detection antibody (referred to as 1 + 1). The detection antibody is coupled to alkaline phosphatise, the relative concentration of which can be determined optically using a suitable substrate.

The measurement procedure described utilises a fluorescent substrate, 4-MUP for detection purposes.

Measurement Conditions

The measurement steps for the ELISA are:

Step (approximate duration, min)

Day 1: Antibody immobilisation (30)

Day 2: Blocking of the ELISA plate (90)

Day 2: Preparation of standards and samples (60)

Day 2: Immunoassay incubation (60)

Day 2: Measurement procedure (30)

Day 2: Data analysis (60)

Reagents

The following reagents have been used in this measurement procedure:

- SRM 2921 (one vial)
- Two vials of cTnI prepared in Solution 3 at undisclosed concentrations (within the range 330 – 50000 ng/L).
- Anti-human cTnI mAb: clone 560
- Anti-human cTnI mAb: clone 19C7
- Tris(hydroxymethyl)aminomethane (Tris) (C4H11NO3), Mr = 121.1
- Sodium chloride (NaCl), Mr = 65.01
- Potassium chloride (KCl), Mr = 74.55
- Polyoxyethylenesorbitan monolaurate (Tween 20)
- 4-Methylumbelliferyl Phosphate (4-MUP), Mr = 256.15
- Magnesium Chloride ($MgCl_2$), Mr = 125.84
- Bovine serum albumin (BSA), essentially protease free, purity . 98 %, Mr ≈ 68000
- Calcium chloride ($CaCl_2$), Mr = 110.98
- Sodium phosphate dibasic (Na2HPO4), Mr = 141.96
- Potassium phosphate monobasic (KH_2PO_4), Mr = 136.09
- Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), Mr = 372.24
- Sorbitol ($C_6H_{14}O_6$), Mr = 182.17
- Perchloric acid ($HClO_4$), Mr = 100.46, warning corrosive and oxidizing.
- SRM 936a, or other suitable liquid fluorescent standard.
- Activated alkaline phosphatase (AP, EC 3.1.3.1), from calf intestine (specific activity ~800 U/mg protein (25.C, pNPP at pH 9.8) (Roche Diagnostics)
- Sodium Carbonate (Na_2CO_3), Mr = 106
- Sodium hydrogen carbonate ($NaHCO_3$), Mr = 84.01
- Triethanolamine ($HOCH_2CH_2)_3N$), Mr = 149.19

[2] Certain commercial equipment, instruments, and materials are identified here to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

- Sodium borohydride ($NaBH_4$), Mr = 37.83 (warning: toxic. handle with appropriate safety equipment and procedures)
- Glycine (NH_2CH_2COOH), Mr = 75.07
- Zinc chloride ($ZnCl_2$), Mr = 136.3
- Sodium Azide (NaN_3), Mr = 65.01 (warning: toxic. handle with appropriate safety equipment and procedures)

Preparation of Solutions

Highly purified water should be used to prepare all solutions, comparable to bidistilled water (conductivity <0.055 µS/cm, TOC < 6 ppb, pH 6-7). Solutions should be filtered using a 0.2 µm (or similar pore size) filtration unit for prolonged storage.

Solution 1

137 mmol/L Sodium chloride
2.7 mmol/L Potassium chloride
10 mmol/L Sodium phosphate dibasic
1.76 mmol/L Potassium phosphate monobasic
-Adjust to pH 7.4 with 1 mol/L HCl
Stability at 4 ºC: 3 months

Solution 2

200 mmol/L Tris(hydroxymethyl) aminomethane
1.5 mol/L Sodium chloride
0.5 % (w/v) Polyoxyethylenesorbitan monolaurate (Tween 20)
-Adjust to pH 7.5 with 1 mol/L HCl
Stability at ambient temperature: 3 months
Dilute this solution 10 fold (100 mL Solution 2 into 900 mL water) to create the working stock.
Stability at ambient temperature: 3 months

Solution 3

20 mmol/L Tris (hydroxymethyl) aminomethane
150 mmol/L Sodium chloride
1 mmol/L Calcium chloride
7.5 % (w/v) Bovine serum albumin
-Adjust to pH 7.4 with 1 mol/L HCl
Stability at 4 ºC: 2 weeks

Solution 4

20 mmol/L Tris (hydroxymethyl) aminomethane
1.0 mmol/L Magnesium chloride
-Adjust to pH 8.0 with 1 mol/L HCl.
Stability at 4 ºC: 3 months

Solution 5

200 mmol/L Ethylenediaminetetraacetic acid disodium salt dihydrate
-
Add sodium hydroxide pellets and mix on electronic stirrer, until all the EDTA and NaOH pellets have dissolved
-Adjust to pH 8.5 with 1 mol/L NaOH.
Stability at ambient temperature: 3 months

Solution 6

100 mmol/L Tris
150 mmol/L Sodium chloride
2 % (w/v) Tween 20
5 % (w/v) Sorbitol
2.0 % (w/v) Bovine serum albumin
-Adjust to pH 7.5 with 1 mol/L HCl
Stability at 4 ºC: 3 months

Measurement Procedure

The assay is typically run over two days consisting of antibody immobilisation (Day 1) and subsequent running of the assay (Day 2). The assay time can be shortened by running the antibody immobilisation step at ambient or elevated temperature for two hours.

1. Antibody immobilisation

a) Black high-binding polystyrene 96-well plates should be used for fluorescent assays to minimize reflection and scatter of light. The volumes given in

this protocol are for the preparation of a single plate and should be adjusted accordingly for multiple plates.

b) Prepare 10.5 mL of cTnI capture antibody (clone 560) by diluting to 2.5 mg/L in Solution 1. Note: the stock antibody concentration varies with batch but should be in the range of 3-10 g/L.
c) Calculate the volume of capture antibody required based on the number of plates to prepare and the batch concentration of capture antibody stock.
d) In a 15 mL plastic microfuge tube pipette the antibody stock, make up to 10.5 mL using Solution 1 and mix by inverting the capped tube.
e) Pipette 100 µl of the diluted capture antibody into each well, tap the plate gently to ensure the bottom of each well is fully covered with liquid.
f) Seal the plate using either a microplate seal, or cover with foil to avoid contamination and incubate overnight at 4 ºC
g) Stability at 4 ºC: 2 days.

2. Blocking of the ELISA plate

Various blocking buffers have been tested and can be substituted for Solution 6.

a) Wash the plate three times with 300 µL of Solution 2 per well. If using a plate washer, see section "Plate Washer Settings" below for recommended settings.
b) Tap the inverted plate onto a paper towel to remove excess washing solution.
c) Add 200 µL of blocking buffer to each well and incubate at ambient temperature for 90 minutes. If incubating on a plate shaker the following settings if applicable should be used: 30 rpm.
d) Note: While washing, plates should not be left dry for significant time periods.

Optional blocking buffer:
Superblock T20 (TBS) blocking buffer (Pierce, IL)

3. Preparation of calibration standards

The cRMP uses a 1-step ELISA format with sequential addition of the sample to the immobilised capture antibody, followed by the addition of the detection antibody conjugate. The cTnI SRM 2921 response curve is prepared from gravimetric dilution from a working stock solution. The preparation of the working stock solution '312 µg/L' from the SRM 2921 stock solution is performed as follows:

- Defrost the vial of SRM 2921 on ice. Unused standard stock should be aliquoted into fresh microfuge tubes, and stored at ≤ –70 ºC within 30 minutes of defrosting the SRM vial (these aliquots can be used for repeats of the assay). Any unused SRM 2921 stock and subsequent dilutions should be discarded after this time period.
- Gravimetric dilution can be performed in 1.5 ml microfuge tubes. Each prepared dilution should be store on ice until use. Diluted standards should be discarded after analysis. Stability of dilutions at 4 ºC: ≤ 2 hours.
- Gravimetric weighing for preparation of the cTnI standards should be performed on an electronic balance with a minimum resolution of 0.1 mg.
- Weigh an approximately 10 µL aliquot of SRM 2921 into microfuge tube.
- Dilute with approximately 990 µL of Solution 3.
- Gently and thoroughly mix.

Table A1 lists the steps for the preparation of the standard response plot, prepared from the working stock solution '312 µg/L'

Table A1. Preparation of cTnI calibration solutions.

cTnI concentration in calibrant, µg/L	Volume of calibrant used (Calibrant), µL	Volume of Solution 3, µL
50	160.3 (312 µg/L stock)	839.7
10	200 (50 µg/L)	800
3.33	6.6 (50 µg/L)	933.4
1	100 (10 µg/L)	900
0.33	100 (3.33 µg/L)	900
0.1	100 (1 µg/L)	900
0.033	100 (0.33 µg/L)	900
0	0	1000

detailed above. When prepared each dilution should be stored on ice until addition to the plate. Participants can prepare the plate in any format (an example template layout is given at the end of this Appendix)

Note: Table A1 gives instructions to prepare calibration solutions for a single plate, each repeated in triplicate. When more than one plate is run, the volumes may need to be adjusted accordingly.

4. Immunoassay Incubation

The samples are incubated in the plate in a 1-step reaction with the detection antibody. The preparation of the detection antibody is described section "Preparation of antibody clone 19C7-AP conjugate" below.

- Defrost the detection antibody on ice. Described is the preparation of 5.5 mL of detection antibody conjugate, sufficient for the analysis of a single plate, if more plates are to be assayed increase the volume of antibody prepared as appropriate. Dilute the detection antibody in a microfuge tube to 0.5 mg/L in Solution 2, approximately 5.5 ml will need to be prepared for a single plate. Mix the solution by inverting the tube. Stability at ambient temperature: 2 hours.
- Wash the plate three times with 300 µL of Solution 2 per well. Tap out any excess wash solution.
- Pipette 50 µL of sample, or standard into the 96-well plate, samples should be analysed in triplicate. A new pipette tip should be used for each sample.
- Add 50 µL of 19C7-AP conjugate to each well, taking care to avoid contamination between wells.
- Seal /cover the plate and incubate at 30 ^{o}C for 60 minutes (on an orbital shaker (30 rpm) if available).
- Note: If less than 96 samples are being analysed in a plate avoid the use of the edge wells 'A1, A12, H1 and H12', for the ELISA assay. The edge wells can be used for liquid fluorescent standard preparation where available.
- Note: The use of a multichannel pipette can improve throughput, however the dispensing volume from each channel should be checked during calibration to ensure no bias in sample liquid transfer.
- Wash the plate 5 times with 300 µL of Solution 2 per well. Tap out any excess wash solution.
- Prepare 11 mL/per plate of 4-MUP substrate by diluting to 40 µM in Solution 4. Protect this reagent from light. Add 100 µL of 4-MUP solution to each well. Seal and cover plate with

foil to protect from light. Incubate at 30 ºC (on an orbital shaker if available) for 30 minutes.

- Uncover plate and add 100 µL of Solution 5 to each well of the plate to stop the reaction, cover the plate with foil to protect from light.

5. Measurement Procedure

The conditions for measuring the fluorescence of the 4-MUP substrate are given in Table A2. The majority of commercial dedicated fluorescent plate reader instruments are either filter, or monochromatic-based. A single set of measurement settings cannot be applied to all instruments; therefore we recommend users to determine the dynamic range of their instrument where a doubling of the fluorophore concentration gives an equal increase in fluorescent intensity. Determine the fluorophore concentration where deviation from this relationship is observed, ideally the highest standard should generate a response ~ 50% less than this fluorophore concentration, ensuring all estimates are made within the measurement range of the instrument. The instrument setting should be adjusted (where appropriate) to maximise the signal generated by the 50000 ng/L standard and ensure it is in the linear measurement range of the instrument.

Where available a suitable fluorescent standard (examples can include SRM 1932 'quinine sulfate', or Fluorescence Spectral Standard C, certified (BAM-F003) sold by Sigma-Aldrich)) should be dispensed into spare wells to aid normalization.

Table A2. Initial conditions for measurement of 4-MUP.

Temperature	21.0 ºC ± 1 ºC
Lamp intensity	Low
Excitation wavelength (nm)	355
Reading (exposure) time	0.1 s
Emission wavelength (nm)	460
Detector sensitivity	Low

Note: Plate alignment and constant illumination across a plate should be checked regularly for plate readers using either:

- A physical plate standard in which solid phase fluorescent standard is present in all the wells (Matech, Westlake Village, CA).
- A liquid fluorescence standard that closely matches the excitation and emission peaks of 4-MUP, which can be pipetted into each well. Note that liquid transfer will generate uncertainty that will need to be assessed when using this approach.

Data Analysis

Plate reader instruments have different sensitivities therefore the quantitation range for the cTnI cRMP will vary between laboratories.

- Calculate the mean response from the blank (zero concentration standard) and subtract this from all the raw data values.
- Calculate the mean, standard deviation and % CV from all the data inputs. If sufficient replicates have been run outlier analysis techniques can be used to mark and exclude spurious data from single wells.
- From gravimetric data from the preparation of the samples for the standard response curve, calculate the corrected cTnI concentrations.

- Transform both the cTnI (ng/L) concentration and intensity values (arbitrary) into log values.
- Plot log concentration vs. log intensity for the cTnI standards and fit to a linear equation (y = ax + b). Determine quality and suitability of fit (see notes).
- Determine the concentration of the unknown samples using the equation derived above. Using anti-log convert to concentration estimates.

Note: The quality of the fit can be assessed by looking at the R^2 value, ideally this should be greater than 0.99. However the R^2 values should not solely be relied upon. The quality of the fit can be assessed by back-calculating the concentration of the standards using the y 'intensity' data and the equation derived. The % bias between the two values should be less than 20. If bias values of greater than 20 % are observed at either end of the response curve this indicates the limits of the quantitation range.

Data Reporting

Estimates for the three undisclosed cTnI samples and the associated uncertainty should be provided. We would also be grateful if participants could provide the following:

- The raw, untransformed fluorescent data.
- Details of any equations, methods used to transform the data, or remove any outliers (with justifications).
- Equations used to fit the data and associated performance metrics, i.e. goodness of fit.
- Optional: A breakdown of the components of the uncertainty and associated equation.

Sources of error

If significant bias is observed upon back calculation of the standard response when fitting to a linear equation, significant errors in estimating unknowns using this data set will be obtained. The following should be considered if significant bias is observed:

- Re-measure the standards to confirm these bias limits and adjust the assay measurement range accordingly.
- Analyse more standard concentrations in the areas of greater bias, allowing more complex data fitting models to be tested (i.e. logistic), or the use of multiple fitting procedures to model the data.

Example of a plate template format

Shown below is an example of a plate template that can be copied for use in the

	1	2	3	4	5	6	7	8	9	10	11	12
A	QS		50000 ng/L	50000 ng/L			S1	S1	S1			QS
B			10000 ng/L	10000 ng/L			S2	S2	S2			
C			3330 ng/L	3330 ng/L			S3	S3	S3			
D			1000 ng/L	1000 ng/L								
E			333 ng/L	333 ng/L			QS	QS	QS			
F			100 ng/L	100 ng/L								
G			33.3 ng/L	33.3 ng/L								
H	QS		0 ng/L	0 ng/L								QS

study.
Participants do not have to follow this plate layout, however when sending raw data we ask participants label the contents of each well in the spreadsheet. S1, S2 and S3 represent the undisclosed concentrations of cTnI in the study samples for the participants to estimate.

Plate washer settings

A wash step where the liquid is simultaneously dispensed and aspirated thereby creating a circular flow should be used where available. The following setting can be used where applicable to the plate washing instrument being used.
WASH (repeat for 3 cycles before final aspiration step):

- Z-position:Overflow
- Aspiration rate:High
- Aspiration time:1 s
- Wash rate:300 µl/s
- Head speed:10 mm/s

FINAL ASPIRATE:

- Aspiration rate:High
- Aspiration time:4 s
- Head speed:10 mm/s

Preparation of antibody clone 19C7-AP conjugate.

The antibody AP conjugate can be prepared using various methods, however the recommended one uses pre-activated AP available from Roche Diagnostics (Cat. #. 11464752001). For the conjugation to anti-cTnI clone 19C7:

Additional Reagents

- Mouse mAb anti cTnI clone 19C7 (Hytest, Finland)
- Activated alkaline phosphatase (AP, EC 3.1.3.1), from calf intestine (specific activity 800 U/mg protein (25 ^{o}C, pNPP at pH 9.8) (Roche Diagnostics)
- Sodium Carbonate (Na2CO3), Mr = 106
- Sodium hydrogen carbonate ($NaHCO_3$), M_r = 84.01
- Triethanolamine ($HOCH_2CH_2)_3N$), M_r = 149.19
- Sodium borohydride ($NaBH_4$), Mr = 37.83 (warning: toxic, handle with appropriate safety equipment and procedures)
- Glycine (NH_2CH_2COOH), M_r = 75.07
- Zinc chloride ($ZnCl_2$), M_r = 136.3
- Sodium Azide (NaN_3), M_r = 65.01 (warning: toxic, handle with appropriate safety equipment and procedures)

Solution 7

1 mol/L Sodium carbonate
1 mol/L Sodium hydrogen carbonate

- Adjust pH of the Sodium hydrogen carbonate solution to pH 9.4 using sodium carbonate solution.
- Add 10 ml of the sodium carbonate/hydrogen carbonate solution in an 100 ml volumetric flask, fill water (20 ^{o}C) up to the calibration mark of the volumetric flask
- Stability at 4 ºC: 1 week

Solution 8

50 mmol/L Triethanolamine,
150 mmol/L Sodium chloride
1 mmol/L MgCl2
0.1 mmol/L ZnCl2
10 mmol/L Glycine
0.1 % w/v Sodium Azide
5 % w/v BSA

- Adjust to pH 7.6 with 1 mol/L HCl
- Stability at 4 ºC: 3 months

Antibody conjugation

- Buffer exchange antibody solution (0.35 mg ~ 50 µL at ~ 6-8 g/L) into Solution 7 using centrifugation cartridges with a suitable cut-off (50 kDa).

- This step reduces the amount of contaminating sodium azide in the conjugation reaction.
- Exchange the buffer in the cartridge three times with Solution 7.
- Add the 19C7 sample, centrifuge and collect the liquid removed from the resin, estimate the volume of the buffer exchanged 19C7.
- Reconstitute the activated alkaline phosphatase in 0.5 mL of water, store unused reagent in 100 µL aliquots at −20 ºC after a snap freeze at −60 ^{o}C.
- In a 1.5 ml microfuge tube add 50 µL of the 19C7 in Solution 7 and add 100 µL of activated alkaline phosphatase.
- Incubate this vial at 21 ^{o}C ± 2 ^{o}C for three hours, with the occasional mixing of the tube by vortex, or inversion.
- Stop the reaction by adding 20 µL of 2 mol/L triethanolamine pH 8.0 and mix.
- Add 40 µL of cold (0-4.C) freshly prepared 200 mM sodium borohydride solution, mix and incubate at 4 ºC ± 2 ºC for 30 minutes.
- Add 5 µL of 2 mol/L triethanolamine pH 8.0, mix and incubate at 4 ºC ± 2 ºC for 2 hours.
- Add 10 µL of 1 mol/L glycine pH 7.0.
- Add 450 µL of Solution 8. Divide into 20 µL aliquots and store at -20 ºC for up to 1 year.
- Alternatives to using direct conjugation of AP to 19C7 antibody clone include; biotinylated antibody and streptavidin-alkaline phosphatase conjugate.

Appendix 3: Raw Data

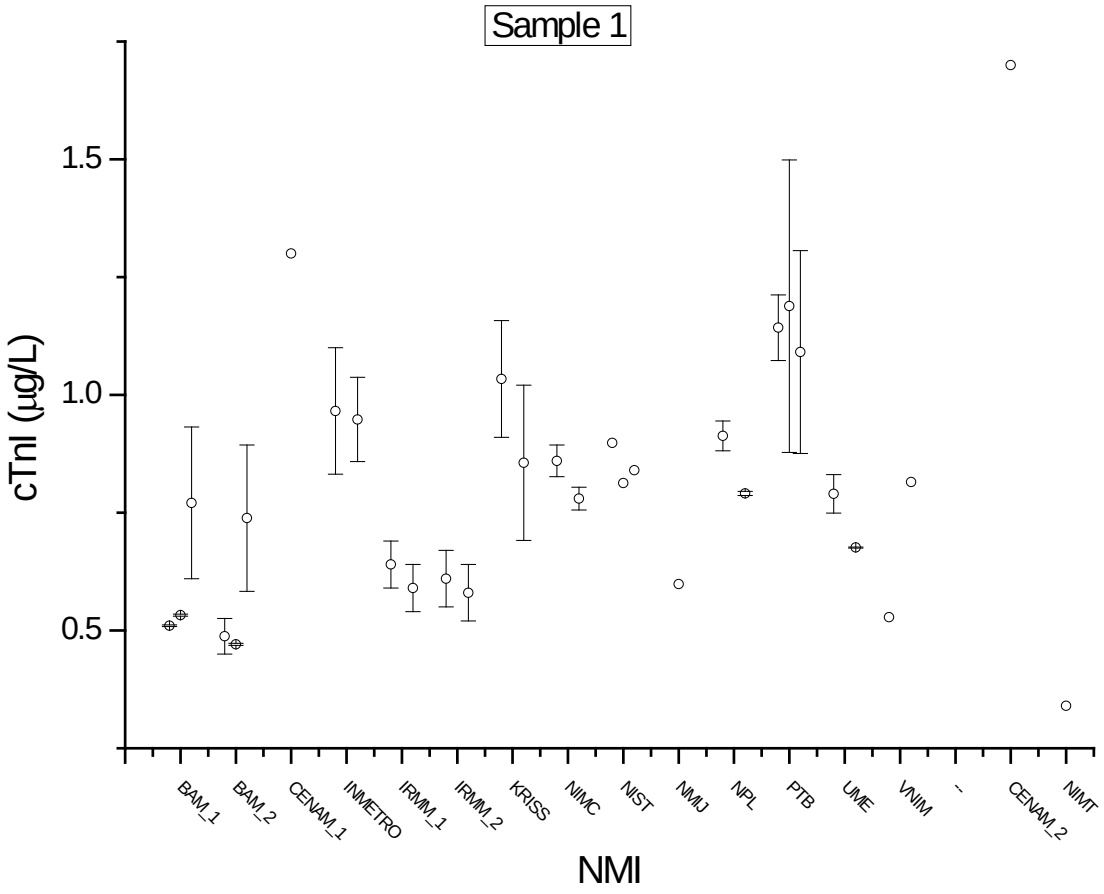

Figure A1: Raw data Sample 1. Mean response derived from individual assay 'plates' and the associated %CV.

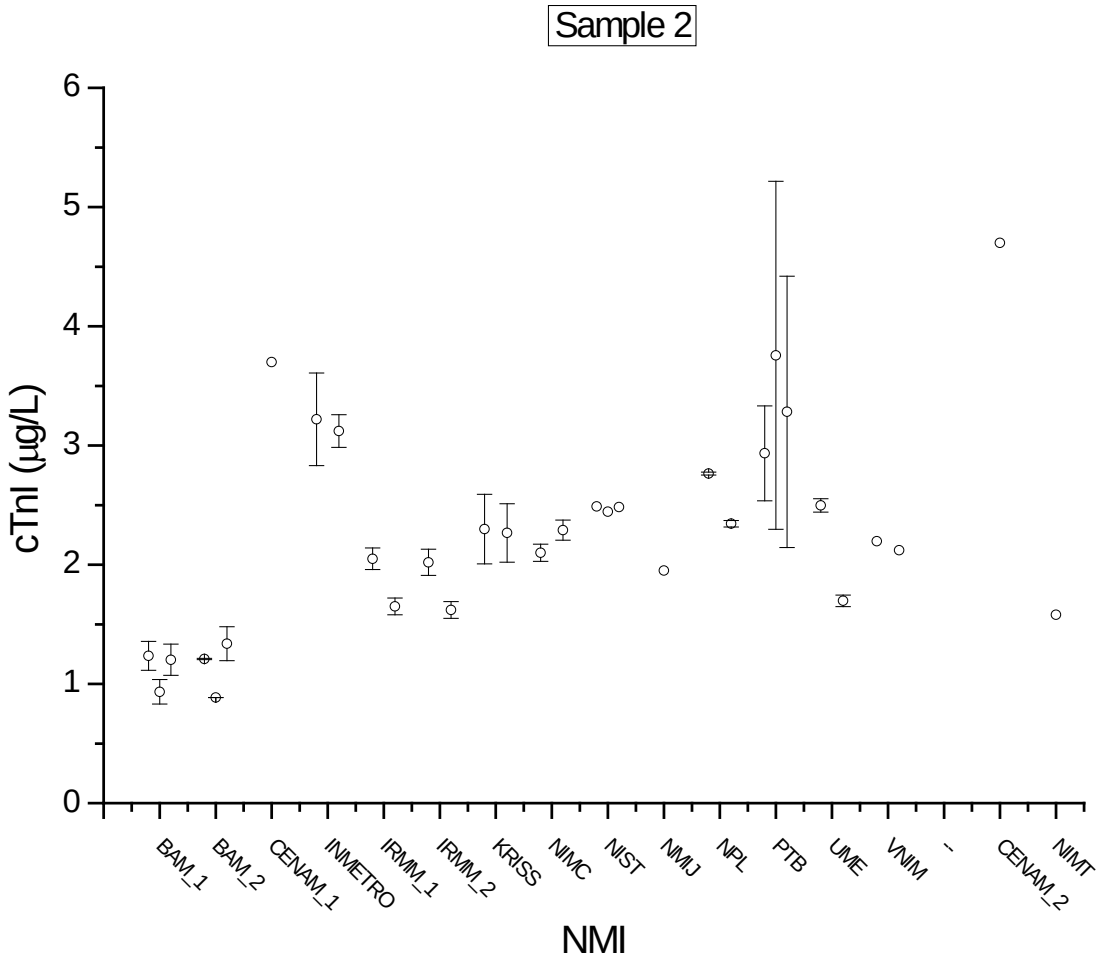

Figure A2: Raw data Sample 2. Mean response derived from individual assay 'plates' and the associated % CV.

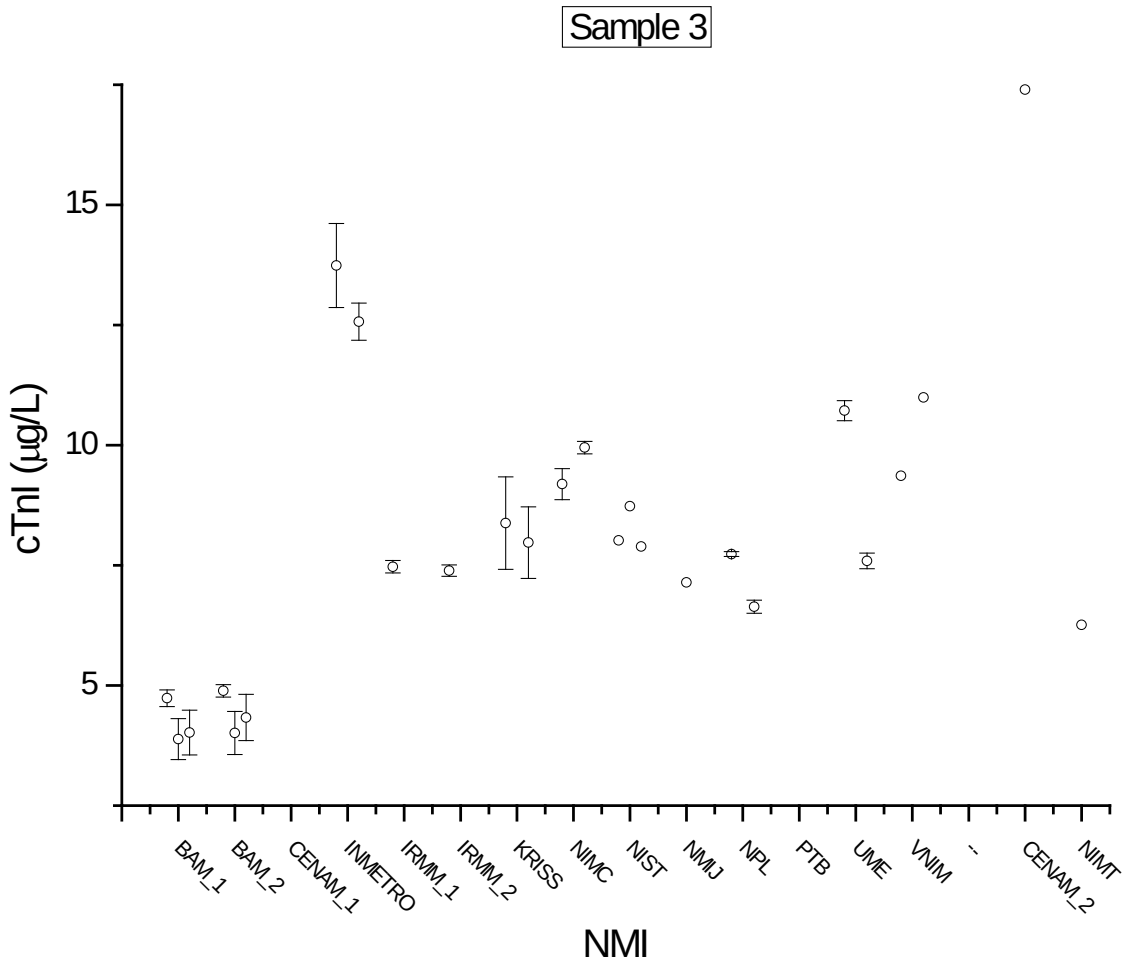

Figure A3: Raw data Sample 3. Mean response derived from individual assay 'plates' and the associated % CV.

Appendix 4: Participants' Uncertainty Equations

Laboratory	Uncertainty Equation
BAM	$\sqrt{sd^2 + s(meas)^2 + s(liqu.handling)^2 + s(calibration)^2}$
CENAM	Not provided
INMETRO	Not provided
IRMM	$U_{rel} = k \times \sqrt{u_{calibrant}^2 + \left(\frac{s_{ip}}{\sqrt{d}}\right)^2 + \left(\frac{s_r}{\sqrt{n}}\right)^2}$
KRISS	$u_{combined} = \sqrt{(u_{mean})^2 + (u_{calibrant})^2 + \left(u_{plate}\right)^2 + (u_{calibration})^2}$
NIMC	$u_{U1,c,rel} = \sqrt{u_{U1,A,rel}^2 + u_{U1,Cal,rel}^2 + u_{STD,rel}^2}$
NIST	$\sqrt{s(mean)^2 + s(pipetting, plate\ and\ instrument\ biases, and\ calibration\ at\ 95\%\ confidence)^2}$
NMIJ	$u(c) = \sqrt{\left(\frac{\partial \mathrm{C}}{\partial y}u(y)\right)^2 + \left(\frac{\partial \mathrm{C}}{\partial A_1}u(A_1)\right)^2 + \left(\frac{\partial \mathrm{C}}{\partial A_2}u(A_2)\right)^2 + \left(\frac{\partial \mathrm{C}}{\partial B}u(B)\right)^2 + \left(\frac{\partial \mathrm{C}}{\partial \mathrm{C}_0}u(\mathrm{C}_0)\right)^2}$
NPL	$\sqrt{s(mean)^2 + s(calibrant)^2 + s(plate)^2 + s(pipette)^2 + s(calibration)^2}$
PTB	$U_{c = \sqrt{(calibration)^2 + (measurement)^2 + (liquid\ handling)^2}}$
UME	$u_{combined} = \sqrt{\left(\frac{u_{repeatibility}}{C_{sample}}\right)^2 + \left(\frac{u_{reproducubility}}{C_{sample}}\right)^2 + \left(\frac{u_{calibration\ curve}}{C_{sample}}\right)^2 + \left(u_{prep}\right)^2 + (u_{312\ stock\ sln})^2}$
VNIIM	Not provided
NIMT	Not provided

Appendix 5: Participants' Comments

Laboratory	Comments
BAM	We think that our results are consistently low due to degradation during transport of the protein samples to us. An important error source seems to us the difficult-to-perform synthesis of the antibody-enzyme conjugate. It would be better for future trials to provide a ready-made, stable conjugate for signal generation. The given protocol for the Sandwich ELISA should form an annex. Outliers have been eliminated by a Nalimov test. To us it seems safer to perform calibration over a larger range and use a sigmoidal calibration model then.
CENAM	
INMETRO	
IRMM	
KRISS	The signals for the 33.3 and 100 ng/L calibration standards were too low - Excluded from the standard curve. The signal for the 50000 ng/L standard was often out of the linear range - Excluded from the standard curve. The concentration of BSA in solution 3 caused slight difference in measurement The aliquots of cTnI SRM 2921 often caused inconsistent results protein attachment to microfuge tubes ?? protein degradation resulted from freeze-thaw actions ??
NIMC	
NIST	
NMIJ	
NPL	
PTB	
UME	We used DTX 880 Beckman Coulter fluorescent plate reader, with excitation filter of 360 nm, bandwidth 40 nm, emission filter of 460 nm, a bandwidth 40 nm and medium binding Greiner-Bio-One, 96 well Flat bottom plates, Cat no: 655076. We prepared the standards by gravimetric dilution. We added more dilution points to the calibration graphs: 30, 7.6, 1.7, 0.6, 0.5, 0.2 ng/L. For each set of samples, we used separate points of the calibration graph. Since the stock solution has very high concentration (32 000 µg/L), it was diluted 100 times, by taking 10 µl and diluting it to the 1000 µl, as it was suggested in the protocol. We believe that taking 10 µl might have increased the error in the preparation of the calibration graph because 10 ul might not be homogenous at the point where it was drawn from the bottle, instead, it could have been prepared in two steps each having 10 times dilutions, by taking 100 µl and diluting it to 1000 µl and repeating that step.
VNIIM	
NIMT	

Appendix 6: Bayesian Concentration Estimates for each ELISA

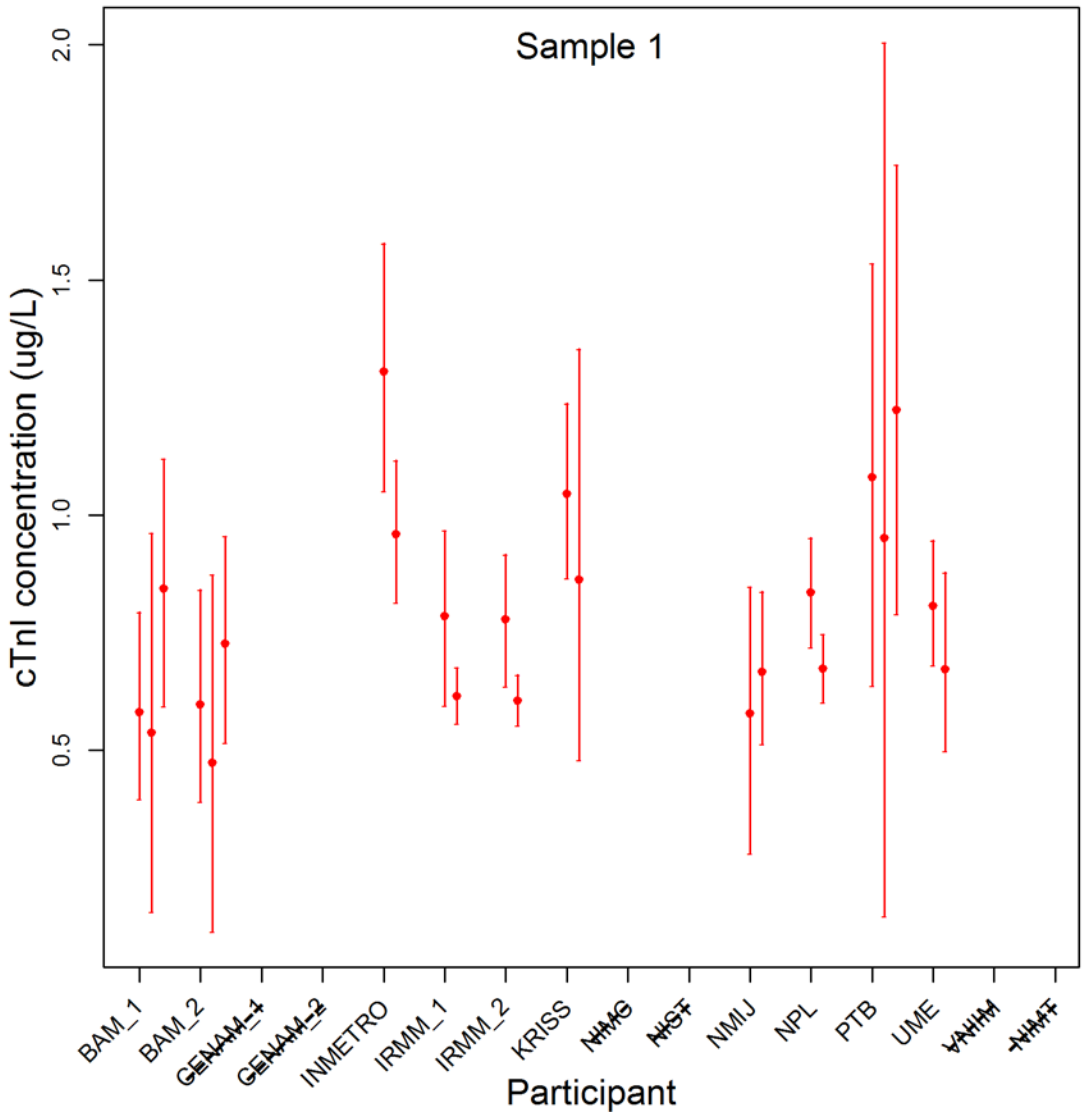

Figure A4: Bayesian concentration estimates and 95% credible intervals based on measurement of the mass concentration of cTnI for each ELISA and Sample 1. NMIs whose names are struck out did not submit raw data to perform a Bayesian analysis.

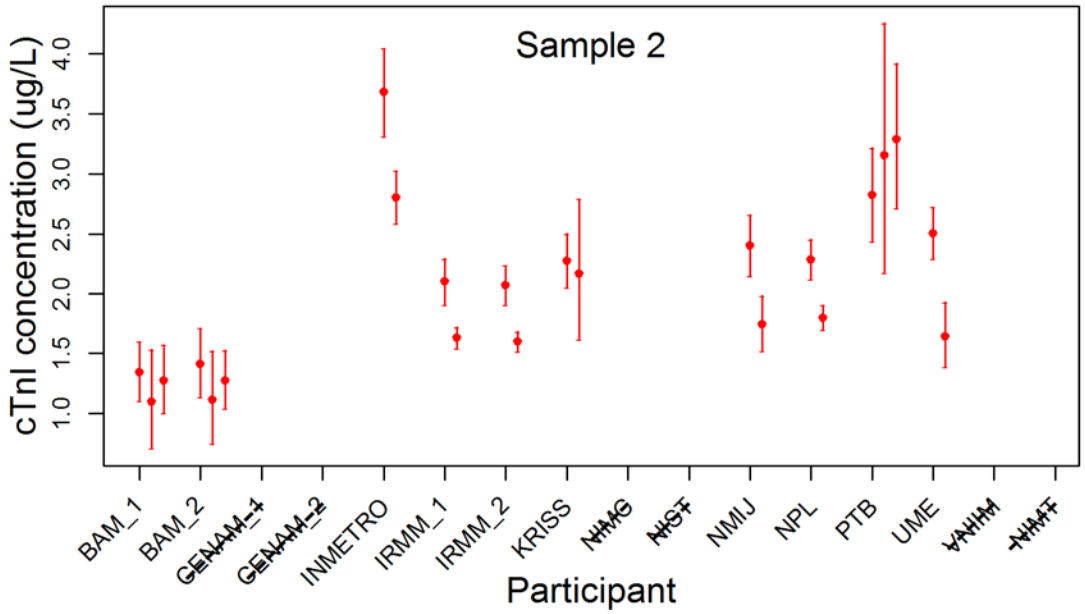

Figure A5: Bayesian concentration estimates and 95% credible intervals based on measurement of the mass concentration of cTnI for each ELISA and Sample 2. NMIs whose names are struck out did not submit raw data to perform a Bayesian analysis.

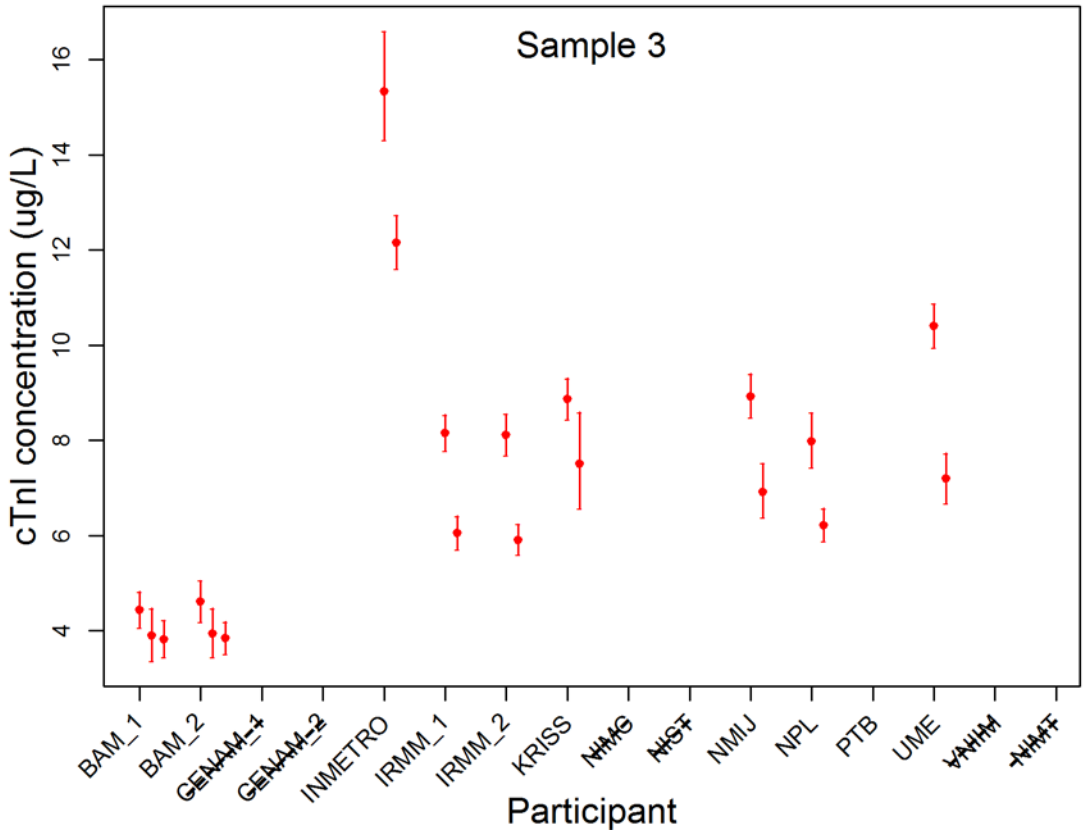

Figure A6: Bayesian concentration estimates and 95% credible intervals based on measurement of the mass concentration of cTnI for each ELISA and Sample 3. NMIs whose names are struck out did not submit raw data to perform a Bayesian analysis.

Appendix 7: Prior Knowledge

The range of a priori calibration curves is displayed in Figure A7. The high variability in a priori possible curves, that can be observed, affirms the claim that a wide range of experimental settings are covered.

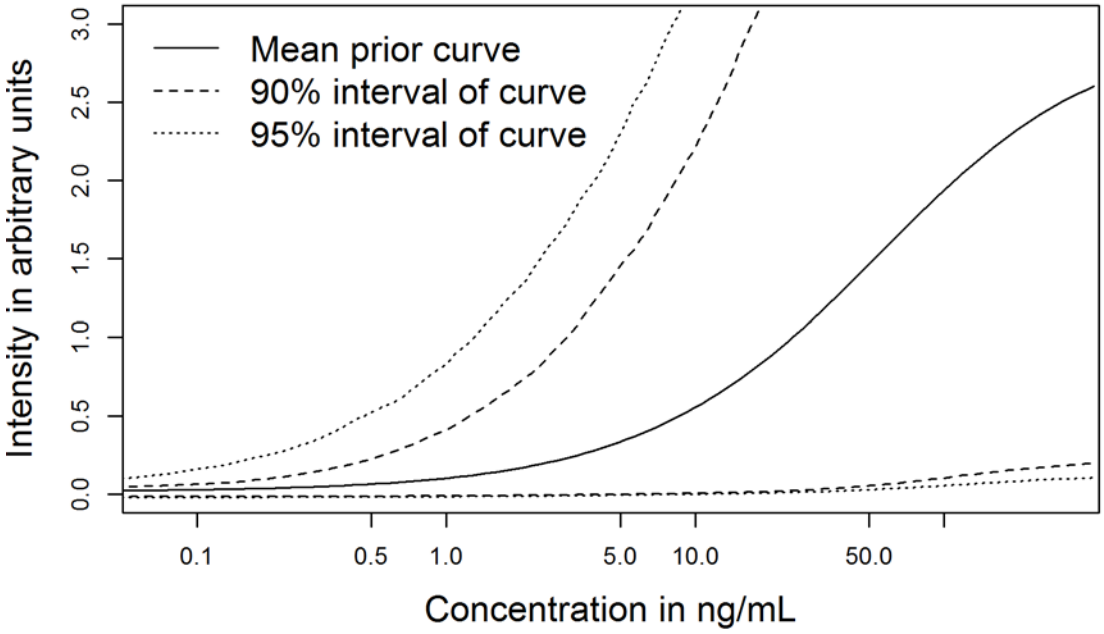

Figure A7: Prior information for immunoassay analyses is displayed by the mean calibration curve (solid lines) as well as the 90% and 95% interval curves (dashed and dotted lines, respectively). Please observe that this prior holds for scaled intensity measurements of at most 1.