

### Recovery of spiked troponin I in four routine assays

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#### Abstract

**Introduction:** This study aimed to examine the recovery of spiked human cardiac troponin I (cTnI) results measured by four routine assays, and investigate possible interference from microclots.

**Materials and methods:** 457 consecutive samples with cTnI concentration below limit of quantitation (12 ng/L), declared by the Vitros TnI ES assay (reference assay), were measured on Beckman Coulter Accu TnI+3, Siemens TnI-Ultra and Roche TnI STAT assays. These samples were enriched with native full-length cTnI to a concentration of 100 ng/L and retested. A post-spiking result that exceeded the critical difference at a predefined probability of 0.0005 of the target concentration (the median post-spiking result for each individual assay) was considered as outlier. To determine whether microclots were a significant cause of critically discrepant outlier results, a separate 50 samples were centrifuged twice between two post-spiking measurements using the Vitros TnI ES assay.

**Results:** The median recovery of the enriched cTnI was highest with the Roche assay (271 ng/L) and lowest with the Vitros assay (29 ng/L). The Vitros assay had the highest percentage of results that exceeded the critical difference (49%), followed by the Siemens (38%), Roche (18%) and Beckman Coulter (7%) assays. None of the 50 additional samples produced a critically lower cTnI result after re-centrifugation.

**Conclusions:** Our findings underscored the variability of cTnI assays in measuring native cTnI. The lack of cTnI results that became significantly lower after re-centrifugation suggested that microclots are unlikely to be a major cause of the outlier results.

**Key words:** cardiovascular diseases; troponin; immunoassay; clinical chemistry

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#### Introduction

The diagnosis of acute myocardial infarction (AMI) requires the biochemical evidence of a change (rise or fall) in serial plasma concentration of cardiac troponin (cTn) I or T, with at least one concentration above the 99th percentile upper reference limit (1). It also requires at least one additional supporting feature involving clinical assessment, electrophysiology, imaging, angiography or autopsy. Because of the high diagnostic weight given to cTn, a spurious result can have significant clinical consequences (2,3). Moreover, the difference in limit of quantification, limit of detection and upper reference limit among routine cTn assays may result in differing diagnostic performance and confuse physicians.

The increasingly sensitive cTn assays can amplify minor variations / errors in pre-analytical and analytical processes, increasing the likelihood of spurious results. Although what constitutes a significant change between serial cTn results is still debated (4), an accurate and precise assay is clearly necessary. Yet, the measurement of cTn is confounded by many factors related to patient sample such as heterophile antibodies (5,6), rheumatoid factors (7), troponin autoantibodies (8,9), and microclots or micro-particles (10). Despite improved assay design, laboratory interference causing inconsistent cTn measurement remains a concern (11,12).

The presence of interfering factors in patient samples can cause spuriously high or low cTn measurements (5-12). We hypothesized that spiking of native cTn protein into patient samples and measuring their recovery using routine cTn assay may provide information about their potential susceptibility to these patient-specific factors. Here, we aimed to examine the recovery of spiked native human cTnI in patient samples using four routine cTnI assays.

## Materials and methods

### Materials

The study was performed at the National University Hospital, Singapore, between November 2014 and January 2015. It was performed as part of a pre-implementation laboratory evaluation of the performance of four cTnI assays and was exempted from local ethics review. The principles of the Declaration of Helsinki were observed throughout the study.

A total of 457 consecutive leftover patient samples, collected in lithium heparinized plasma separation tubes (catalog no. 367962, BD Vacutainer, BD Biosciences, Manchester, United Kingdom), with cTnI concentrations below the lower limit of quantification of the Vitros TnI ES assay ( $< 12$  ng/L), were included in this study. There was no specific restriction on the patient population. These samples were stored at  $4^{\circ}\text{C}$  and retrieved within twelve hours of the initial testing for re-analysis. They were not subjected to re-centrifugation prior to analysis. One mL of each anonymized plasma sample was used to measure cTnI on the other three assays. These results served as the baseline (pre-spiking) concentrations. None of these samples had elevated hemolysis, icteric or lipemic indices, as determined by the Vitros 5600 platform using spectrophotometry principle, which may interfere with the cTnI measurements.

Subsequently, the remaining aliquots were enriched with native full-length (non-complexed) cTnI protein purified from human cardiac tissue (catalog no. ab9936, Abcam, Massachusetts, USA), which has been pre-diluted with 1X-phosphate buffered saline, to a final concentration of 100

ng/L. The purity of this preparation is  $> 95\%$  by visual estimation of the SDS PAGE gel, according to the manufacturer. This commercially acquired preparation is not traceable to any international reference standard. After the initial round of testing,  $33\ \mu\text{L}$  of the pre-diluted native cTnI protein was spiked into  $690\ \mu\text{L}$  of the remaining aliquot of sample. The enriched samples were allowed to incubate at room temperature ( $\sim 23^{\circ}\text{C}$ ) for two hours before repeating measurement of cTnI. The measurement was repeated within four hours of the initial assay.

Two trained laboratory technicians, with more than five years of laboratory experience each, carefully performed all manual laboratory procedures. The variability of the manual pipetting procedures was assessed by pipetting and dispensing  $33\ \mu\text{L}$  and  $1\ \text{mL}$  of water into a laboratory precision balance (Cubis<sup>®</sup> Analytical Balance, Sartorius GmbH, Goettingen, Germany). The weight of these volumes of water was recorded. This was performed in 20 replicates for each pipetting volume. The coefficient of variation of the recorded weight of these two pipetting volumes were calculated, and considered the coefficient of variation of pipetting procedure. They were used to calculate the critical difference (see below).

To determine whether outlier results were caused by microclots, an additional 50 aliquots of leftover samples were enriched with cTnI and measured on the Vitros TnI ES assay. Following this, these samples were immediately protected with paraffin film, centrifuged twice consecutively ( $3000 \times g$ , for a total of 10 minutes, at  $5^{\circ}\text{C}$ ) using the PrO-Hospital centrifuge (Centurion Scientific, West Sussex, United Kingdom), and retested. The additional centrifugation step aimed at eliminating any free-floating microclots that may interfere with the cTnI measurement. A fall in cTnI measurement after re-centrifugation that exceeds the critical difference (as defined below) indicates that microclots have likely caused a spuriously elevated pre-centrifugation result.

### Methods

The four assays under evaluation included: a) the Accu TnI+3 (adopted on the Beckman Dxl plat-

form, both Beckman Coulter Diagnostics, California, USA); b) Vitros TnI ES (adopted on the Vitros 5600 platform, both Ortho Clinical Diagnostics, New York, USA); c) TnI-Ultra (adopted on the Immulite 2000 XPi platform, both Siemens Medical Solutions Diagnostics, Munich, Germany) and d) TnI STAT (adopted on the Cobas e411, both Roche Diagnostics, Mannheim, Germany). The technical specifications of these assays, as reported in the product inserts by the manufacturers, are summarized in Table 1.

In our laboratory, cTnI is routinely measured by the Vitros TnI ES assay within an hour after sample collection. The Vitros TnI ES assay was considered as reference assay in this study.

### Statistical analysis

The baseline cTnI results of all four assays were summarized using simple descriptive statistics. The mean concentration of the post-spiking samples of each assay was considered the target concentration. The recovery of each sample was calculated as  $[(\text{measured post-spiking cTnI concentration}) / (100 \text{ ng/L, the spiked cTnI concentration}) \times 100\%]$ . The percentage difference between the post-spiking cTnI concentration in each individual sample and the target concentration was calculated as  $[(\text{measured cTnI concentration after spiking a particular sample}) - (\text{assay-specific target cTnI concentration}) / (\text{assay-specific target cTnI concentration}) \times 100\%]$ . A percentage difference between

the post-spiking results and the target concentration of more than  $[z\text{-score} \times 2^{0.5} \times (\text{analytical coefficient of variation near the target concentration}^2 + \text{coefficient of variation of pipetting procedure}^2)^{0.5}]$  was considered critically different. A z-score of 3.5, corresponding to a predefined probability of 0.0005, was selected in this study. A result is considered an outlier if the recovery exceeds the critical difference of the target concentration. Two-tailed Fisher's exact test was performed to determine whether the difference in the proportion of outlier results between any two of the four routine assays were statistically significant (defined as  $P < 0.05$ ).

### Results

The number of pre-spiking samples and their concentrations above the lower limit of quantification of the other three assays are summarized in Table 2. Two of the seven results that were above the upper reference limit belonged to the same sample and were high on Siemens cTnI-Ultra and Beckman Coulter Accu TnI+3 assays, respectively.

The post-spiking cTnI concentrations measured by the four assays are summarized in Figure 1. The within-assay and between-assays recoveries of the enriched cTnI were highly variable. The Siemens TnI-Ultra assay had the widest range of enriched cTnI concentration recovered.

The coefficient of variation of the manual pipetting procedures was 1.8%. The critical differences

**TABLE 1.** Technical specifications reported by the manufacturers' product inserts of the assays used

Assay	Lower limit of detection, ng/L	Lower limit of quantification, ng/L	Upper reference limit, ng/L	Concentration at 10% CV, ng/L	Total analytical imprecision (CV) at selected cTnI concentration	Critical difference, %
Vitros TnI ES	12	12	40	27	4.3% at 70 ng/L	23.1
Beckman Coulter Accu TnI+3	8	40	40	40	8.0% at 50 ng/L	40.6
Siemens cTnI-Ultra	6	30	40	30	5.3% at 80 ng/L	27.7
Roche TnI STAT	160	300	160	234	4.8% at 323 ng/L	25.4

CV - coefficient of variation.

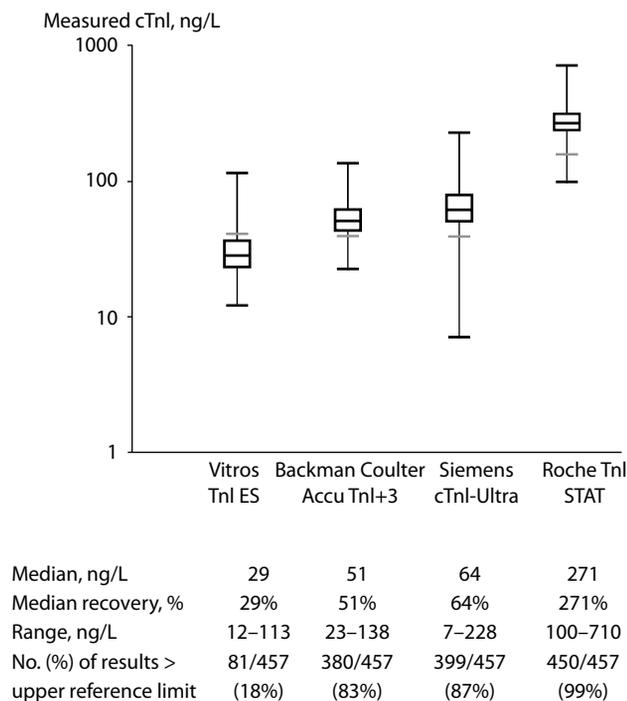
The critical difference was calculated as  $[4.95 \times (\text{analytical coefficient of variation near the target concentration}^2 + \text{coefficient of variation of pipetting procedure}^2)^{0.5}]$ , at a predefined probability of 0.0005.

**TABLE 2.** Pre-spiking samples above the limit of quantification of the three assays investigated and their concentrations.

Assay	Samples above lower limit of quantification				Samples above upper reference limit	
	Number, N	Mean cTnI, ng/L	Standard deviation, ng/L	Range, ng/L	Number, N	Range, ng/L
Beckman Coulter AccuTnI+3	2	60	25	42–77	2	42–77
Siemens cTnI-Ultra	13	39	11	33–68	2	51–68
Roche TnI STAT	3	410	80	330–480	3	330–480

**TABLE 3.** Samples exceeding the critical difference (at a predefined probability of 0.0005).

Assay	Critical difference, %	Negative difference, N (%)	Within critical difference, N (%)	Positive difference, N (%)
Vitros TnI ES	23.1	95 (21)	234 (51)	128 (28)
Beckman Coulter AccuTnI+3	40.6	23 (5)	426 (93)	8 (2)
Siemens cTnI-Ultra	27.7	115 (25)	283 (62)	59 (13)
Roche TnI STAT	25.4	37 (8)	375 (82)	45 (10)



**FIGURE 1.** Box and whisker plots showing the post-spiking cardiac troponin I result in the four assays. The y-axis is in logarithmic scale and the grey horizontal bar represents the upper reference limit of the respective assays. Values below the figure summarize the median and range of concentration of troponin I in the post-spiking samples, the median recovery (%) and results (%) above the upper reference limit.

of the four assays are provided in Table 1. The number of samples that exceeded the critical difference is summarized in Table 3. Using two-tailed Fisher exact test, all combinations of pair-wise between-assay comparison of the proportion of samples with recovery exceeding the critical difference were significantly different ( $P < 0.05$ ); except for samples with positive difference exceeding the critical difference measured by the Vitros TnI ES and the Siemens cTnI-Ultra assays.

The Vitros TnI ES assay had the highest percentage (49%) of results that are outliers, followed by the Siemens TnI-Ultra (38%), Roche TnI STAT (18%) and Beckman Coulter Accu TnI+3 (7%) assays. Fifty-five samples had post-spiking results that were above the target concentration in some assays, and below the target concentration in others (i.e. discordant recoveries between assays).

Of the 50 samples that were re-centrifuged twice between cTnI measurements, none produced a critically lower post-re-centrifugation cTnI result. Only one sample had critically discrepant cTnI result that was higher after re-centrifugation (pre-re-centrifugation: 22 ng/L vs. post-re-centrifugation:

**TABLE 4.** Pre- and post-re-centrifugation cardiac troponin I results.

Troponin I, ng/L	Pre-re-centrifugation	Post-re-centrifugation	% Difference
Mean	35	36	- 3
Median	32	33	- 3
Minimum	12	13	- 28
Maximum	76	77	13

Percent difference (%) was calculated as  $[(\text{pre-re-centrifugation result} - \text{post-centrifugation result}) / \text{pre-re-centrifugation result}] \times 100$ .

29 ng/L, translating into a 27% difference). The rest of the results are summarized in Table 4.

## Discussion

The baseline cTnI results were highly comparable across the four assays. There were only seven discordant results where the cTnI was elevated above the upper reference limit when measured by another assay. Interestingly, only one sample had discordant results in more than one laboratory method. This particularly sample had elevated cTnI measured by the Siemens cTnI-Ultra and Beckman Coulter Accu TnI+3 assays, and was negative on the others. This suggests that there may be patient-specific factors that infrequently affect the individual assays, causing discordant cTnI results.

As the cTnI concentration of these pre-spiking patient samples were consistently suppressed across multiple assays, we could assume that they were truly negative for cTnI. Hence, the clinical specificity  $[\text{true negative} / (\text{true negative} + \text{false positive}) \times 100\%]$  of these assays is  $> 99\%$ . This is generally in accordance with other clinical studies, which showed very high clinical specificity ( $> 90\%$ ) for cTnI assays in situations where the difference between serial measurements is small (4).

Many studies have looked at the prevalence of analytical interference in cTnI assays. Some of these studies have relied on finding discrepant results from duplicate testing of the same sample (12), while others directly evaluated the effect of a specific interfering factor on cTnI measurement (9,11), which includes heterophile antibodies (5,6), rheumatoid factors (7), troponin autoantibodies (8,9), and microclots or micro-particles (10).

The use of recovery experiment is one of the routine methods for screening for laboratory interference (13,14). When the measured concentration (i.e. recovery) of a target analyte is significantly higher or lower than the spiked concentration, it indicates that the sample may contain substances that interfered with the laboratory measurement procedure. The spiking experiment design used in this study seeks to simulate an AMI event, where a patient with no measurable baseline cTnI is suddenly exposed (via *ex vivo* enrichment) to low concentrations of cTnI. The concentration of cTnI used in this study was deliberately kept low to enhance the sensitivity of the detection of potential assay interference. This study provides complementary data to the other experimental designs by looking at the how the different routine cTnI assays react with spiked native human cTnI protein in a large cohort of patient with no detectable cTnI at baseline. The native full-length (non-complexed) cTnI protein purified from human cardiac tissue is used as a standardized spiking material for this recovery experiment since the cTnI assays are designed to measure this molecule. Therefore, it provides a homogenous target molecule against which the performance of the assays can be assessed.

Under this experimental design, the prevalence of outlier cTnI results is highly variable among the four assays. The high number of outlier result seen in the Vitros TnI ES assay can be explained by the low cTnI recovery (low denominator), coupled with the high assay precision resulting in a small critical difference.

Several factors may contribute to highly variable recoveries of cTnI between the assays. Human cTnI is a heterogeneous group of molecules. They in-

clude different isoforms (15,16) and complexes (17,18), and are degraded into various lengths by proteases in blood (19,20). Significant systematic inter-method differences among cTnI assays are probably influenced by differences in standardisation, the use proprietary antibodies that have different cross-reactivities to different forms of cTnI, and different upper reference limits (17-20). Hence, the antibodies of the assay are likely to have different degree of recognition of the native human cTnI spiked into the sample, leading to the observed differences in the amount recovered.

On the other hand, the heterogeneity in cTnI recovery among patient samples when using the same assay probably reflects patient-specific interfering factors. This is particularly true since the same amount of native human cTnI was enriched in each sample. Recently, cTn autoantibodies have been gaining increasing attention as a common laboratory interferent. cTn auto-antibodies can affect the assay by inhibiting the interaction of the assay antibody and the cTn epitope (9,11). On the other hand, it may also form 'macro-cTn' and cause spuriously elevated measurement as a result of impaired clearance (21), which may be associated with chronically elevated cTnI (22). The prevalence of cTn autoantibodies has been reported to be present in 12.7% to 15.9% of the general population (23,24). It is conceivable that some of the samples with recoveries that exceeded the critical difference were due to the presence of cTnI autoantibodies.

There have been reports of irreproducible, falsely high cTnI results that are commonly referred to as "outliers" (or colloquially known as "fliers"). Typically, these results are detected upon repeat testing of the same sample that would return a difference that is significantly larger than the analytical variation of the instrument (12,25,26). The prevalence of these outlier results have been reported to be < 1% in several recent studies (25-27). They are more common in samples that have been stored (26) and are unrelated to the analyser, centrifugation speed or sample type (27). The cause of

such phenomenon is yet unclear, but fibrin strands have been suggested as a possibility (25).

Microlots / fibrin strands can cause spuriously elevated cTnI results. In this study, the double re-centrifugation of the 50 clinical samples did not produce any significantly lower result. This suggested that microclots / microparticles were unlikely to be a major interfering factor in this study.

Of note, the number of samples with post-spiking cTnI results above the upper reference limit was also highly variable. Clinically, this may indicate that different troponin assays have different ability to discriminate patients with raised cTnI, leading to variable clinical diagnostic performance.

There are several potential limitations in this study. The observed differences in measured concentrations between the assays may be in part related to between-method bias, which was not examined in this study, and should be interpreted with care. Additionally, the identity of the interfering factor in the critically discrepant samples was not examined in detail, as it was beyond the scope of this study. A detailed study of the prevalence of interfering factors affecting routine cTnI assays is an important area for further research. Finally, there was no specific restriction on the patient population included in this study. It is conceivable that some of these patients may have treatment such as heparin that may affect cTn measurement (28).

In conclusion, our findings underscored the variability of cTnI assays in measuring native cTnI. The lack of cTnI results that became significantly lower after re-centrifugation suggested that microclots are unlikely to be a major cause of the outlier results.

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### Potential conflict of interest

None declared.

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