Short- and Long-Term Cardiac Troponin I Analyte Stability in Plasma and Serum from Healthy Volunteers by Use of an Ultrasensitive, Single-Molecule Counting Assay

To the Editor:

Cardiac troponin I (cTnI) is the gold standard biomarker for diagnosing patients with acute coronary syndromes. Expert panels have defined the decision limit at >99th percentile of a reference population and assay imprecision of ≤10%. Prototype ultrasensitive cTnI assays allow accurate detection of low concentrations of cTnI in healthy individuals. However, these novel assays require analytical and biological validation before they can be put into clinical practice.

We previously reported the 99th percentile cutoff of 7 ng/L for the Erenna cTnI immunoassay (Singulex), with the requisite precision. Using a single-molecule counting technology, the limit of detection (0.2 ng/L) surpassed many other commercial assays. The improved limit of detection is not an artifact of nonspecific-binding events (1). We also showed that the increased analytical sensitivity of the assay enabled measurement of the short- and long-term biological variation of cTnI from healthy individuals (2). However, to what extent minute levels of analyte instability may have contributed to this variation was not established.

We determined the in vitro stability of cTnI in whole blood and serum over short- and long-term intervals in both healthy subjects and in a small subset of patients who presented to the San Francisco General Hospital Emergency Department. A protocol for the use of leftover blood from routine collections and for collecting blood of healthy subjects was reviewed and approved by the University of California San Francisco Committee on Human Health. Previous reports demonstrated cTnI stability at high concentrations in patients with cardiac injury (3–5), despite the fact that these samples may contain proteolytic enzymes that are coreleased following cardiac injury. None of these studies used high-sensitivity assays to demonstrate analyte stability at the low concentrations observed in healthy individuals. Because the Erenna cTnI immunoassay has high analytical sensitivity, our study will answer whether differences in clinical study design, such as timing between blood draws, removal of serum or plasma from cells, and subsequent specimen testing procedures, will contribute variability to cTnI measurements.

Four milliliters of blood from apparently healthy volunteers who self-reported no cardiac symptoms was drawn into EDTA-plasma and serum separator tubes (SSTs). To assess stability over a short-term period of 6 h, 4 EDTA and 4 SST samples were collected from each of 15 volunteers. The first sample (t = 0) for each specimen type was centrifuged and immediately frozen at −80 °C. The remaining samples were left at room temperature for 2, 4, and 6 h before centrifugation and −80 °C storage. Analyte stability after 1- and 6-h room temperature storage were also assessed in 10 healthy subjects and 12 emergency department patients who presented with chest pain and had low cTnI concentrations. To assess stability over a long-term period of 48 h, we collected 3 EDTA and 3 SSTs from each of 15 volunteers. EDTA plasma samples were either centrifuged and frozen immediately or left at room temperature for 24 or 48 h before spinning and −80 °C storage. SST samples were spun first, then either frozen immediately or left at room temperature for 24 or 48 h before −80 °C storage. Resulting samples were thawed and batch tested using the Erenna cTnI immunoassay.

The mean CV was 11% (range 4%–24%) for cTnI measurements over 6 h, and 14% (range 2%–34%) over 48 h (Table 1). The variation was similar when compared to other conventional assays. The percent changes over 1 h for healthy subjects and 6 h for chest pain patients were <1% and <3%, respectively. Compared to other conventional assays, analyte variability was not different in this study. A previous experiment performed using the iSTAT assay noted <4% change in cTnI concentration between samples stored refrigerated or at room temperature over 14 days (3). A study using the Immulite system found <15% differences in samples stored with different protocols (4). However, variability among plasma and serum samples determined with the Vidas system at different temperatures was statistically significant (P < 0.0001) (5). In our analysis, the change in analyte concentration per hour during the 6-h interval was minimal [mean 0.001 (SD 0.031) ng/L]. For the long-term, 48-h interval, we noted a small positive increase in the change in analyte concentration per hour [0.015 (0.033) ng/L, or 0.339 (0.793) ng/L per day]. Variation in cTnI above the analytical precision cutoff (≥20% CV) was observed in 17% of short-term and 33% of long-term samples, suggesting that measurements are most precise when freshly prepared samples are used. However, these limits did not exceed the reference change values of −32% and −45% calculated from the biologic variation of cTnI (2), suggesting that minor analyte in-

1 Nonstandard abbreviations: cTnI, cardiac troponin I; SST, serum separator tube.
stability will not lead to results that are clinically relevant. Analyte stability was not affected by sample type in our study, with both whole blood–EDTA plasma and serum samples providing similar measurements.

Using serum or EDTA-plasma, low concentrations of cTnI from blood of healthy subjects and emergency department patients appears to be as stable as the high concentrations released from patients with myocardial damage. Although the mechanism of protein release may be different between normal tissue turnover of healthy subjects and that from minor ischemic injury, once cardiac troponin is in the blood itself, there are no differences with respect to stability. These data will be useful for prospective and retrospective cardiovascular studies when measuring low cTnI concentrations using high-sensitivity assays.

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References


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To the Editor:

Update: Exon 15

RET Mutation Scanning

To the Editor:

Mutation scanning by high-resolution melting analysis has high diagnostic sensitivity and specificity (1, 2). Our 2006 Clinical Chemistry report demonstrated that mutation scanning of RET (ret protooncogene) detects mutations causative of multiple endocrine neoplasia type 2 (3). To illustrate mutation-scanning data for exon 15, we used cell lines that had the wild-type sequence or were heterozygous for a common, non-pathogenic exon 15 polymorphism (c.2712C>G, p.S904S), which occurs at an allele frequency of 11%–27% (4, 5), but samples with other exon 15 sequence variants were not available at that time. We have revisited this mutation-scanning assay for exon 15 because a sample with a novel exon 15 sequence variant (c.2673G>A, p.S891S) (5) became available for testing. Additionally, the published exon 15 forward primer had inadvertently been designed to anneal over a nonpathogenic polymorphism within intron 14, c.2608-24G>A (rs2472737). The variant allele for this polymorphism occurs at a frequency of 6%–28%, depending on the population (4, 5). Because this polymorphism is common and closer to the 5’ end of the exon 15 forward primer, we used samples of known genotypes to test the function of this published primer for amplification efficiency, allele dropout, and mutation-scanning results for RET exon 15.

The protocol for RET mutation scanning by high-resolution melting analysis was described previously (3). In brief, samples were amplified on the LightCycler® 1.5 system (Roche); the amplicons were then analyzed by high-resolution melting with the HR-1™ instrument (Idaho Technology). The exon 15 reverse and forward primers were 15R1 (5’-CTGG GAGCCCGGCTCAT-3’) and 15F1 (5’-GCCCTGAC(G)ACTCGT GCTATT-3’), respectively. The position of intron 14 polymorphism c.2608-24G>A in 15F1 is marked as (G). Another forward primer, 15F4 (5’-CACACACC ACCCTCTGCTG-3’), which does not anneal over the c.2608-24G>A polymorphism, was used with reverse primer 15R1 under the same experimental conditions. All de-identified samples were sequenced for RET exon 15, which includes the c.2608-24G>A polymorphism region (forward primer 15FSeq (5’-TGCTGGTACACC AGGCTG-3’) and reverse primer 15RSeq (5’-TGCCCCATGGTGCA CCTG-3’) were used for amplification and sequencing). Fig. 1 shows the primer positions and the locations of sequence variants. Of note, the 15F4 and 15FSeq primers are not recommended for mutation scanning, because their use would detect 2 common polymorphisms, complicating the assay results.

To test amplification efficiency, we compared quantification cycle (Cq) values for samples homozygous for intron 14 polymorphism c.2608-24G>A with samples that had the wild-type sequence at this polymorphism position. Because samples can have inherent differences, such as DNA concentration, that affect Cq values, we also amplified the samples with a different forward primer (15F4) that is unaffected by the c.2608-24G>A polymorphism. The samples homozygous for the c.2608-24G>A polymorphism amplified with primer 15F1 had a delay of approximately 1 cycle compared with the wild-type samples or when the 15F4 primer was used. Samples that were heterozygous for both polymorphisms (i.e., intron 14 c.2608-24G>A and exon 15 c.2712C>G) had similar Cqs with the two exon 15 forward primers 15F1 and 15F4.

Because the 15F1 primer has the wild-type G nucleotide at position c.2608-24, amplicons produced from any sample with that primer, including samples heterozygous or homozygous for the c.2608-24G>A polymorphism under the 15F1 primer, will also have the wild-type G nucleotide at that position (confirmed by sequencing; data not shown). Therefore, use of the 15F1 primer actually masks the presence of the c.2608-24G>A polymorphism, thereby avoiding this complication in analyzing melting-curve data. Samples homozygous wild type for