MCP Papers in Press. Published on November 11, 2011 as Manuscript M111.008235

Accurate quantification of cardiovascular biomarkers in serum using Protein Standard Absolute Quantification (PSAQ™) and Selected Reaction Monitoring

Céline Huillet[1,2,3], Annie Adrait[1,2,3], Dorothée Lebert[1,2,3,4], Guillaume Picard[1,2,3], Mathieu Trauchessec[1,2,3], Mathilde Louwagie[1,2,3], Alain Dupuis[1,2,3], Luc Hittinger[6], Bijan Ghaleh[5], Philippe Le Corvoisier[5], Michel Jaquinod[1,2,3], Jérôme Garin[1,2,3], Christophe Bruley[1,2,3] and Virginie Brun[1,2,3,4]

[1] CEA, IRTSV, Biologie à Grande Echelle, F-38054 Grenoble, France.

[2] INSERM, U1038, F-38054 Grenoble, France.

[3] Université Joseph Fourier, Grenoble 1, F-38000, France.

[4] Promise Advanced Proteomics, Grenoble, F-38040, France.

[5] Plateforme de Ressources Biologiques, INSERM, Centre d'Investigation Clinique 006 et U 955 Equipe 03, Groupe Hospitalier Henri Mondor, Créteil, F-94000, France.

[6] Fédération de Cardiologie, Groupe Hospitalier Henri Mondor, Créteil, F-94000, France.

Corresponding author: Dr Virginie BRUN

Tel: 334-38789657

Fax: 334-38785051

E-mail: virginie.brun@cea.fr

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RUNNING TITLE: PSAQ™ for accurate biomarker quantification

ABBREVIATIONS

CKB: Creatine kinase B chain

CKM: Creatine kinase M chain

CKBB: Creatine kinase BB homodimer

CKMB: Creatine kinase MB heterodimer

CKMM: Creatine kinase MM homodimer

LDH-B: Lactate dehydrogenase B

PCI: Primary percutaneous coronary intervention

SID: Stable isotope dilution

SRM: Selected reaction monitoring

PSAQ: Protein standard absolute quantification

SUMMARY

Development of new biomarkers needs to be significantly accelerated to improve diagnostic, prognostic, and toxicity monitoring as well as therapeutic follow-up. Biomarker evaluation is the main bottleneck in this development process. Selected Reaction Monitoring (SRM) combined with stable isotope dilution has emerged as a promising option to speed this step, particularly because of its multiplexing capacities. However, analytical variabilities due to upstream sample handling or incomplete trypsin digestion still need to be resolved. In 2007, we developed the PSAQ™ method (Protein Standard Absolute Quantification) which uses full-length isotope-labelled protein standards to quantify target proteins. In the present study we used clinically validated cardiovascular biomarkers (LDH-B, CKMB, myoglobin and troponin I) to demonstrate that the combination of PSAQ and SRM (PSAQ-SRM) allows highly accurate biomarker quantification in serum samples. A multiplex PSAQ-SRM assay was used to quantify these biomarkers in clinical samples from myocardial infarction patients. Good correlation between PSAQ-SRM and ELISA assay results was found and demonstrated the consistency between these analytical approaches. Thus, PSAQ-SRM has the capacity to improve both accuracy and reproducibility in protein analysis. This will be a major contribution to efficient biomarker development strategies.

INTRODUCTION

Introduction of new diagnostic assays in the clinical setting requires an operating pipeline to efficiently translate putative biomarkers into validated biomarkers. Despite the discovery platforms' capacity to generate well populated lists of candidate biomarkers, very few proteins reach the patient bedside as fully fledged "FDA-approved" biomarkers. This is largely because of divergences between analytical needs and performances of the techniques available for candidate biomarker evaluation (1, 2).

Candidate biomarker evaluation is a major process of the biomarker pipeline, positioned downstream of the biomarker discovery phase and necessary before clinical validation. Candidate evaluation aims to select, among hundreds of putative biomarkers, those of clinical relevance. Evaluation phase combines 2 steps which respectively consist in: (i) confirming a difference between physiological and pathological concentrations in biofluids (the so-called "qualification phase") and (ii) assessing the specificity of candidate biomarkers (the so-called "verification phase") (1). Currently, due to its high throughput and high sensitivity, quantitative ELISA is the preferred assay format for studies evaluating biomarkers. However, as most candidates are likely to fail as relevant biomarkers, developing ELISA tests (with high quality antibodies) for all candidates is a financial burden for the diagnostics industry (3).

Thus, there exists an urgent need to develop analytical methods capable of reliable candidate evaluation, at high throughput and reasonable cost. Selected Reaction Monitoring (SRM) mass spectrometry combined with stable isotope dilution (SID-SRM) has shown promise as a solution to this technological hurdle (4, 5). MS analysis in SRM mode offers the unique possibility to specifically and simultaneously monitor the signatures of hundreds of

target peptides generated by trypsin digestion of proteins. Combined with isotope-labelled quantification standards (6), SRM can provide quantitative data for each protein targeted (5).

Recently, in an effort to demonstrate the potential of SID-SRM for candidate biomarker evaluation, a multi-laboratory study was set up to assess its analytical performances and potential transferability (7). Exogenous proteins, 7 in all, were added to unfractionated plasma samples. The spiked samples were analyzed by eight independent laboratories using SRM and isotope-labelled peptides as standards. The results obtained clearly demonstrated the capacity of SID-SRM to specifically and precisely quantify protein biomarkers in plasma. However, the results also revealed that the protein digestion rate was highly variable between laboratories. This variability had a significant effect on peptide recovery and on the accuracy of protein quantification. As suggested by the authors, this type of bias could be avoided if properly folded isotope-labelled protein standards were used as quantification standards (7, 8).

In 2007, we developed the PSAQ™ (Protein Standard Absolute Quantification) method which uses full-length isotope-labelled proteins as internal standards for absolute quantitative MS analysis. We demonstrated that, in contrast with peptide standards, adding isotope-labelled proteins before sample digestion enables accurate protein quantification, even for proteins resistant to trypsin digestion (9, 10). In addition, we, and others, have shown that this type of protein standard ("PSAQ standard") also corrects for protein losses that may occur during sample handling prior to trypsin digestion and LC-MS analysis (11-17). This latter feature is a particular advantage for MS analysis of blood biomarkers. Indeed, as plasma/serum are highly complex matrices and display a huge dynamic range, sample prefractionation must be performed to detect low-abundance protein biomarkers (4).

In this study, we have tested a combination of the PSAQ strategy with SRM (PSAQ-SRM) for quantification of cardiovascular biomarkers in serum samples. Selected biomarkers

include LDH-B, CKMB, myoglobin and troponin I. For some of these validated biomarkers, a comparison of PSAQ-SRM data and ELISA results was performed on samples from patients having suffered myocardial infarction.

EXPERIMENTAL PROCEDURES

Biomarkers and clinical samples

Human LDH-B, creatine kinase MB heterodimer (CKMB) and myoglobin were purchased from Applichem (Darmstadt, Germany). Human troponin I and healthy human serum were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Serum samples from 5 patients were provided by the Plateforme de Ressources Biologiques (Groupe Hospitalier Henri Mondor, Créteil, France). Five patients undergoing primary percutaneous coronary intervention (PCI) for ST-elevation myocardial infarction were included in the study. The protocol was approved by the hospital's institutional review board and all patients provided written informed consent for participation. Blood samples were taken at hospital admission and at regular intervals following PCI. Samples were collected in non-treated tubes (BD Biosciences, le Pont de Claix, France). All blood samples were centrifuged at 2200 g for 10 min to obtain serum supernatants. These were immediately aliquoted and frozen at -80°C. Additional blood samples were analyzed at the clinical chemistry laboratory for standard cardiac biomarker evaluation (Total CK activity, troponin I and myoglobin) as described below. In this study, only serum samples collected at hospital admission and at 3 or 8 days after PCI were analyzed using the PSAQ-SRM approach.

Production of full-length stable-isotope-labelled proteins (PSAQ standards)

PSAQ standards were synthesized as previously described (9). Briefly, LDH-B, creatine kinase B chain (CKB), creatine kinase M chain (CKM), myoglobin and troponin I genes were amplified by PCR using a cardiac cDNA library as template (Biochain Institute, Hayward, CA, USA) (see supplemental Table 1 for primer sequences and PCR reaction conditions). Genes were cloned into the pIVEX 2.4d expression vector (5 Prime, Hamburg,

Germany) using the In-Fusion™ PCR cloning system (Clontech, Saint Germain en Laye, France). The pIVEX 2.4d vector provides a N-terminal hexahistidine purification tag. Plasmids were cloned into XL1-Blue cells (Agilent Technologies, Massy, France), purified and sequenced (Cogenics, Meylan, France). Cell-free protein expression and isotope-labelling was performed using the RTS 500 Proteomaster *E. coli* HY kit (5 Prime) in the presence of [$^{13}C_6$, $^{15}N_2$] L-lysine and [$^{13}C_6$, $^{15}N_4$] L-arginine (Eurisotop, Saint-Aubin, France). PSAQ standards were purified on a nickel affinity column (Ni Sepharose 6 Fast Flow resin, GE Healthcare, Orsay, France) using an imidazole gradient. PSAQ standards were checked for purity on SDS-PAGE using Coomassie staining (>95% purity). N-terminal hexahistidine purification tags were not removed as they are not expected to significantly modify PSAQ biochemical properties. Isotope-labelled proteins were quantified by amino acid analysis (MScan SA, Plan les Ouates, Switzerland). Isotope incorporation was verified by LC-MS and LC-SRM analysis and was found to be greater than 99% (see supplemental Figure 1).

Depletion of serum samples

Serum samples (14 µL) from healthy donors and patients were spiked with defined amounts of PSAQ standards. To set up a titration experiment, samples from healthy donors were spiked with defined amounts of exogenous, unlabelled LDH-B, CKMB, myoglobin or troponin I (see supplemental LC-SRM data). Serum samples were depleted of the 6 most abundant proteins using the human Multiple Affinity Removal Spin cartridge (MARS 6) (Agilent Technologies) according to the manufacturer's instructions. The flow-through was concentrated to 15 µL using a 5000 Da cut-off ultrafiltration device (Vivascience, Hannover, Germany). Laemmli buffer (10 µL) was added before SDS-PAGE analysis.

Troponin I antibody biotinylation

Troponin I antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was dialyzed against PBS buffer. The antibody was then incubated with NHS-PEG$_4$-Biotin reagent (Pierce/Thermo Fischer Scientific, Brebières, France) for 3 hours at room temperature (NHS-PEG$_4$-Biotin/antibody ratio: 20/1). After biotinylation, the antibody was once again dialyzed in PBS buffer. Biotinylation was checked by dot-blot analysis with neutravidin-HRP on a PVDF membrane.

Immunoenrichment of serum samples

Before immunoenrichment, serum samples (1 mL) were spiked with defined quantities of troponin I PSAQ standard. To generate the titration curve, a range of concentrations of exogenous unlabelled troponin I was added to healthy donor serum samples (see supplemental LC-SRM data). Biotinylated antibody (1 µg) was added to the samples before overnight incubation at 4°C on a rotating wheel. Dynabeads M-280 Streptavidin (Invitrogen, Cergy Pontoise, France) were added (15 µL Dynabeads per sample) and samples were incubated for a further 4h at 4°C with rotation. Supernatants were eliminated and beads were washed 3 times with PBS/0.1%Tween, then with PBS and finally with water. Captured proteins were eluted from the beads with 0.5% formic acid. Eluates were dried by vacuum centrifugation and resuspended in Laemmli buffer before SDS-PAGE analysis.

SDS-PAGE analysis and trypsin digestion

After protein depletion or immunocapture prefractionation, samples were resuspended in Laemmli buffer and loaded onto precast NuPAGE Novex Bis-Tris 4-12% acrylamide gradient gels (Invitrogen). After migration, gels were fixed and stained with Coomassie staining solution (Biorad, Marnes la Coquette, France). Gel regions containing targeted

biomarkers were excised and cut into small pieces (see supplemental Figures 2A, 3A and 4A). For multiplex detection of biomarkers in depleted serum samples, gel pieces obtained from 2 excised regions (15 to 30 kDa and 35 to 45 kDa regions) were pooled before in-gel digestion (see Figure 1A and supplemental Figure 6A). For immunoenriched samples, SDS-PAGE was performed in the "stacking" mode and samples were entirely excised from the gel (see Figure 1A, supplemental Figures 5A and 6B). Gel pieces were destained by alternating washing cycles in NH_4HCO_3 25 mM and NH_4HCO_3 25 mM (50%)/ACN (50%) for 15 min. After destaining, gel pieces were dried by vacuum centrifugation and oxidized with a 7% H_2O_2 solution for 15 min (18). Gel pieces were washed with pure water and dehydrated in 100% ACN before overnight trypsin (Promega, Madison, WI, USA) digestion at a protein/enzyme ratio of 1:20 (w/w) in 25 mM NH_4HCO_3 at 37°C. Tryptic peptides were extracted from the gel in three successive steps (15 min each), using 50% ACN, 5% formic acid and 100% ACN extraction solutions. After drying by vacuum centrifugation, tryptic peptides were resuspended in 2% ACN and 0.2% formic acid before LC-SRM analysis. Most samples were reconstituted in 10 µL to allow a 6 µL pick-up injection on the nanoLC-chromatography system. However, samples containing higher amounts of biomarkers (highest points of titration curves) were resuspended in a larger volume (20 to 50 µL).

LC-SRM analysis

SRM transitions were selected based on proteotypic peptide LC-MS/MS spectra (see supplemental LC-MS/MS data) and were experimentally validated by LC-SRM analysis of trypsin-digests containing each biomarker and its corresponding PSAQ standard. LC-SRM analyses were performed on a 5500 Q-Trap hybrid triple quadrupole/linear ion trap mass spectrometer (400 to 1250 m/z range) equipped with a Nano III electrospray ion source and operating with Analyst software (version 1.5, Applied Biosystems/MDS Sciex, Les Ulis,

France). The instrument was coupled to an Ultimate 3000 nanoLC-chromatography system (Dionex, Voisins Le Bretonneux, France). Chromatography was performed using a two-solvent system combining solvent A (2%ACN/0.1%formic acid) and solvent B (80%ACN/0.08%formic acid). First, samples (6 µL pick-up injections) were concentrated for 3 min on a 300-µm x 5-mm PepMap C_{18} precolumn (Dionex) at 20 µL/min flow rate. Peptide digests were then separated onto a 75-µm x 150-mm C_{18} column (Dionex) at a flow rate of 300 nl/min and using a 60-min gradient from 10% B to 40% B in 30 min and from 40% to 90% in 5 min. Data were acquired in a positive ion mode with an ion spray voltage of 2200 Volts, curtain gas at 20 p.s.i, a nebulizer gas at 12 p.s.i and an interface heater temperature of 150°C. Collision exit, declustering and entrance potentials were set to 15, 75 and 12 Volts, respectively. Collision energy values were calculated using linear equations based on the unlabelled peptide precursor *m/z* ratios:

For doubly charged precursors: $CE = 0.05\ m/z + 5$ (Volts)

For triply-charge precursors: $CE = 0.05\ m/z + 4$ (Volts)

Collision energy was identical for both labelled and unlabelled versions of each peptide (Table 1). At least 2 SRM transitions per peptide were monitored. These were acquired at unit resolution both in Q1 and Q3 quadrupoles with a dwell time set at 50 ms and a cycle time set at 4.9 s (multiplex detection in depleted serum samples) or 2.1 s (troponin I detection in immunoenriched serum samples).

LC-SRM data analysis

Data analysis was performed using MultiQuant software (version 1.1 Applied Biosystems/MDS Sciex). Unlabelled/labelled peak area ratios were calculated for each SRM transition after careful verification of coelution profiles. Ratios obtained from the different SRM transitions were used to calculate the corresponding average peptide ratio. Then, ratios

obtained for the different proteotypic peptides were combined to calculate the protein ratio and determine biomarker concentration in serum (see supplemental LC-SRM data and supplemental Figures 2 to 5). From the 787 calculated peak area ratios, 11 (1.4%) outlier values were excluded from analysis (see supplemental LC-SRM data). Most of these outliers were related to matrix interferences impairing labelled or unlabelled peptide transition signals.

LLOQ determination

LLOQ corresponds to the lowest concentration of an analyte that can be determined with acceptable precision and accuracy. Various approaches for determining LLOQ can be used, including signal-to-noise analysis, statistical analysis based on blank sample variance determination (19) and least-squares linear regression analysis (20). In this study, blank samples were not available (non-spiked serum samples contained endogenous levels of biomarkers). Consequently, LLOQ was determined according to the FDA criteria described in the guidelines for bioanalytical method validation (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances). LLOQ was established as the lowest concentration of the titration curve that was measured with precision (CV) inferior to 20 % and accuracy comprised between 80 and 120 %.

ELISA and enzymatic assays

Myoglobin concentration in patient serum samples was determined using the Human Myoglobin ELISA Kit (Alpco Diagnostics, Salem, NH, USA) according to the manufacturer's instructions. To investigate troponin I concentration in patients' samples, the Access AccuTnI Troponin I Assay (Beckman Coulter, Roissy, France), which is not a highly sensitive troponin I assay, was used as described by the manufacturer. Total CK activity was measured by spectrophotometry using a COBAS system (Hoffman La Roche, Basel,

Switzerland) at the local clinical chemistry laboratory. All assay parameters are presented in supplemental Table 2.

RESULTS

Selection of proteotypic peptides and SRM transitions.

Once expressed and purified, each PSAQ standard was submitted to SDS-PAGE, in-gel digestion with trypsin and LC-MS/MS analysis (see supplemental LC-MS/MS data). This allowed us to experimentally determine the signature peptides (so-called "proteotypic" peptides) to be monitored for each cardiovascular biomarker targeted. Proteotypic peptides were chosen for their sequence uniqueness, verified by BLAST search, and ease of detection with LC-MS/MS. Peptides containing methionine and cysteine residues were not excluded from the proteotypic peptide panel. Indeed, our group has recently demonstrated that sulphur-containing peptides can be used for quantification if the redox status of the target protein and its PSAQ analog are "equalized" by H_2O_2 treatment before trypsin digestion (article under preparation, see also supplemental Figures 2 and 5 which show titration curves obtained with such modified peptides). Based on LC-MS/MS data analysis, a list of putative SRM transitions was established, which included 3 to 6 SRM transitions per proteotypic peptide. Then, using prefractionated serum matrix spiked with PSAQ standards and digested with trypsin, we experimentally selected the "best" SRM transitions, *i.e.* those effectively detected in the matrix and presenting no signs of interference. In the final SRM methods used for titration curves and patient samples, only the "best" SRM transitions (2 to 4 per proteotypic peptide) were retained (Table 1). Importantly, for each selected SRM transition, 2 precursor/fragment ions pairs were actually monitored: one for the labelled form of the peptide and one for its unlabelled form. Hence, LDH-B was characterized by 4 proteotypic peptides. We also monitored peptide VIGSGCNLDSAR, which is shared between the three LDH isoforms (LDH-A, LDH-B and LDH-C). This additional peptide allowed us to measure total LDH content in serum samples. Quantification of myoglobin, which is a small protein

(17 kDa), was based on only one proteotypic peptide. Troponin I levels were evaluated using 3 proteotypic peptides. For CKMB, heterodimer concentrations could not be specifically investigated. However, we could examine the levels of CKB chains (using 2 proteotypic peptides) and CKM chains (using 3 proteotypic peptides) independently. Notably, in serum samples, CKB and CKM chains, in addition to making up the CKMB heterodimer, may also form CKMM and CKBB homodimers.

Quantification of cardiovascular biomarkers in depleted serum samples.

A rapid sample preparation workflow, combining depletion of the 6 most abundant serum proteins with short SDS-PAGE migration, was developed (Figure 1A). For each cardiovascular biomarker, a 4-point titration experiment was performed by spiking 14 µL serum samples with defined amounts of both unlabelled protein (LDH-B, CKMB, myoglobin or troponin I) and corresponding PSAQ standard. Zero samples were also constituted. The spiked LDH-B, CKMB, myoglobin and troponin I quantities were calculated to generate titration curves covering physiological levels to highest pathological concentrations. All titration points were performed in full-process triplicates (see supplemental LC-SRM data). For CKMB heterodimer, we investigated the levels of CKM chains and CKB chains by simultaneously spiking CKM and CKB PSAQ standards. After prefractionation and trypsin digestion, samples were analyzed by LC-SRM. LDH-B, CKM chains and myoglobin could be detected at their physiological levels (*i.e.* in non-spiked healthy serum samples) estimated at 510 ng/mL, 260 ng/mL and 40 ng/mL, respectively (Figure 2). For CKB, endogenous levels could not be detected. In healthy donor serum, normal levels of CKB are < 3 ng/mL. Titration curves for LDH-B, CKM and myoglobin were linear for concentrations ranging from endogenous levels to the highest concentrations detected in serum samples. In contrast, troponin I was undetectable in these samples (concentration range: 0.1 ng/mL to 272.5

ng/mL). Based on these titration curves, we determined the analytical performances of the PSAQ-SRM assay for each biomarker targeted (Table 2). Ideally, blank serum should have been used to determine LLOQ. However, for CKMB heterodimer, it was difficult to co-deplete CKMB, CKBB and CKMM homodimers, which also contain CKB and CKM chains. Thus, we decided to determine LLOQ in healthy donor serum. For each biomarker, LLOQ was deduced from the average titration curve obtained from all proteotypic peptides (Figure 2) and according to the FDA recommendations (see the "Experimental procedures" section). Despite extensive sample prefractionation and trypsin digestion, excellent quantification accuracy, as indicated by the slope of the average titration curve, was found for both LDH-B and myoglobin (slope value of 1.18 and 1.14, respectively) (Figures 2A and 2B). Titration of CKMB heterodimer gave unexpected results (Figure 2C), with slopes of 0.2 (CKB) and 0.45 (CKM), rather than the expected 0.5 for both proteins. This indicates that: (1) commercial CKMB added into serum samples was underestimated and (2) CKM and CKB chains were not added in the same stoeichiometry. To test the latter hypothesis, we investigated "pure" CKMB heterodimer by mixing 900 ng of commercial CKMB with 450 ng of CKB PSAQ standard and 450 ng of CKM PSAQ standard. After in-gel trypsin digestion, the mix was analyzed by LC-SRM which revealed a 2/1 CKM/CKB stoichiometry. This indicates that the commercial CKMB heterodimer contained excess CKM chains, possibly originating from a contaminating CKMM homodimeric form. Commercial CKMB is purified from myocardium containing excess CKMM (75%), thus contamination of CKMB preparations would not be surprising. The underestimation of CKMB in serum may stem from CKB and CKM PSAQ standards and CKMB heterodimer behaving differently during prefractionation and/or digestion. This hypothesis was supported by native-PAGE analysis of CKB and CKM PSAQ standards. CKB PSAQ standard was found to be monomeric while CKM PSAQ standard was structured as a CKMM homodimeric (data not shown).

Quantification of troponin I in immunoenriched serum samples

Generally, LC-SRM detection of serum proteins present below 100 ng/mL is difficult when using depletion as unique prefractionation method (4). However, we hypothesized that the combination of depletion and SDS-PAGE might allow troponin I detection, particularly for samples with concentrations in the range of 100 ng/mL. Indeed, myoglobin could be detected at just 40 ng/mL using this procedure. However, troponin I could not be detected, even at the highest concentration tested (272.5 ng/mL). Most likely, this is because of its interaction with abundant proteins retained on the depletion cartridge (19). Therefore, a prefractionation method based on immunocapture coupled to SDS-PAGE was developed to validate the PSAQ-SRM approach for this specific biomarker (Figure 1A). To perform titration experiments, healthy serum samples (1 mL) were spiked with defined amounts of troponin I and its corresponding PSAQ standard. An immunoenrichment protocol was optimized using a biotinylated anti-troponin I antibody and streptavidin coated beads. Because of the strong biotin-streptavidin interaction, anti-troponin I antibody was retained on streptavidin beads during elution, further improving sample decomplexification. However, as Laemmli buffer was used to elute troponin I, direct trypsin digestion was not possible. Therefore, samples were submitted to “stacking” SDS-PAGE before in-gel digestion (supplemental Figure 5A). After trypsin digestion and peptide extraction samples were analyzed using LC-SRM analysis. With this serum prefractionation method, troponin I could be detected at 500 pg/mL of serum, which is slightly higher than physiological concentrations (≈ 350 pg/mL). However, sensitive and accurate troponin I quantification was possible over the pathological concentration range (5.5 to 272.5 ng/mL) (Figure 2D, supplemental Figure 5). PSAQ-SRM assay analytical performances after troponin I immunoenrichment are presented in Table 2.

Interestingly, the troponin I proteotypic peptide ISADAMMQALLAGR includes serine 149 which was previously reported to be phophorylatable (21). In this study, all quantification results obtained from this peptide were consistent with those obtained from the 2 other proteotypic peptides (see suplemental Figure 5 and supplemental LC-SRM data). Therefore, we can hypothesize that: (i) serine 149 was primarily not phosphorylated or (ii) serine 149 phosphorylation was removed as troponin I was released in blood flow.

Multiplexed PSAQ-SRM analysis of clinical samples and correlation with ELISA/enzymatic assay results

Once the prefractionation and quantification processes had been characterized for each biomarker, multiplexed LC-SRM detection was assessed on healthy donor serum samples. After spiking the samples with defined quantities of PSAQ standards, prefractionation and SDS-PAGE, gel bands were cut, pooled and proteins were digested with trypsin (Figure 1A). LDH-B, myoglobin, CKM and CKB could be simultaneously detected in depleted serum samples without difficulty and without time-scheduled acquisition (Figure 1B). We then applied this multiplex PSAQ-SRM method to the analysis of serum samples from patients with myocardial infarction. Serum samples from five patients were collected at two time-points: (i) immediately after hospital admission and (ii) 3 to 8 days after PCI. All samples were analyzed using the PSAQ-SRM method after depletion (14 µL) or troponin I immunoenrichment (1 mL). LDH-B, myoglobin, CKM and CKB chains and troponin I were all detected in these samples (Table 3, supplemental Figures 6 and 7). For each patient, biomarker concentration changes between the two collection time-points were consistent with blood-release kinetics *i.e.* an early increase in troponin I, myoglobin and CKMB (day 0) and a delayed LDH-B augmentation (days 3 or 8). As myoglobin, CKMB and troponin I are routinely used clinically to confirm myocardial injury, correlation between ELISA or

enzymatic assays and the PSAQ-SRM approach could be assessed for these patient samples (Table 3 and Figure 3). Total CK enzyme activity and CKB and CKM concentrations estimated by PSAQ-SRM correlated well, with a R^2 value of 0.89 (Figure 3A). Excellent correlation between ELISA and PSAQ-SRM results (R^2 = 0.95) were observed for myoglobin. Surprisingly however, the slope of the correlation curve was equal to 5.29, suggesting that PSAQ-SRM systematically quantifies 5 times more myoglobin than ELISA (Figure 3B). Following this result, we compared the commercial myoglobin ELISA standard directly to our PSAQ standard by mixing them in equal amounts. After trypsin digestion and LC-SRM analysis of the 1/1 mix, the ELISA standard was found to be 5.39 times less concentrated than the PSAQ standard (see supplemental Figure 8). From these results, we can surmise that either the concentration of the myoglobin ELISA standard was overestimated by the supplier or that it had been degraded during storage. For troponin I quantification, a correlation coefficient (R^2) of 0.79 was found between ELISA and PSAQ-SRM with a correlation curve slope of 1.41 (Figure 3C). Possibly, this lesser correlation originates from troponin I interactions with serum proteins. By masking epitopes, such interactions could differentially influence ELISA detection (based on 2 monoclonal antibodies) and PSAQ-SRM immunocapture (based on 1 monoclonal antibody).

DISCUSSION

In 2006, Rifai and co-workers described the biomarker pipeline and the promise MS held for biomarker research (1). In particular, they highlighted how SID-SRM analysis might solve the technological hurdle of biomarker evaluation. However, application of SID-SRM as part of biomarker development requires key analytical performances to be attained, including specificity, sensitivity and confident quantification. In addition, to surpass ELISA it is crucial to offer multiplexing capabilities and antibody-free prefractionation. Recently, an inter-laboratory study assessed the analytical features of a multiplexed SID-SRM assay and demonstrated the accelerated throughput and transferability of this type of analysis. This study, however, highlighted quantification accuracy as a limitation of the method, particularly when sample prefractionation was necessary (7, 8). The goal of our study was to demonstrate that the use of full-length isotopically-labelled proteins used as quantification standards (PSAQ standards) could significantly advance the performances of a SRM-based biomarker evaluation platform.

LDH-B, CKMB, myoglobin and troponin I were chosen as model biomarkers to assess the performances of the PSAQ-SRM method. Troponin I, CKMB and myoglobin are currently used in hospital laboratories to rapidly confirm myocardial injury. Measurement of LDH-B levels was abandoned because its release in the blood stream is delayed, reaching its maximal concentration 72h after myocardial infarction. However, to evaluate the PSAQ-SRM method, the combination of these 4 biomarkers was of particular interest. Firstly because they belong to different concentration classes, with LDH-B being the most abundant (µg/mL of serum) and troponin I requiring very sensitive assays (below 1 ng/mL in serum). Secondly, ELISA or enzymatic tests are available for troponin I, myoglobin and CKMB, making a comparison between ELISA and PSAQ-SRM assays possible.

Samples were fractionated using a decomplexification method based on the depletion of abundant proteins combined with SDS-PAGE. Using this straightforward sampling, only 14 µL of serum were necessary to simultaneously quantify 3 biomarkers at their physiological levels. Likely due to the high ion-current potential of its proteotypic peptide (22), myoglobin could be quantified down to 40 ng/mL (equivalent to 33 femtomoles in 14 µL). For troponin I quantification, however, this type of fractionation, applied to tiny volumes of serum, was not sufficient. Not only is troponin I a low-abundance biomarker, but it has also been shown to interact with abundant proteins retained by serum depletion devices (19). Therefore, we developed an immunocapture approach to quantify troponin I below ng/mL concentrations in serum. Measurement of this protein might also have been possible using depletion on larger serum volumes, combined with mild-detergents to improve elution from the depletion cartridge and by replacing SDS-PAGE with chromatographic peptide separation. Indeed, Keshishian and co-workers have recently described an antibody-free sampling approach involving protein depletion, trypsin digestion and SCX peptide separation before LC-SRM analysis (19). With this methodology, they were able to detect troponin T down to 5 ng/mL in 100 µL plasma samples. Another recent study detected 4 ng/mL of PSA in 100 µl serum samples using albumin depletion, trypsin digestion, SPE peptide separation and LC-SRM analysis (23).

Given that most proteins of clinical interest are present in the ng/mL range in serum or plasma, matrix decomplexification is generally mandatory to successfully detect biomarker candidates (4). In this context, the use of isotope-labelled peptide standards, added at late stages of the analytical workflow, may not provide optimal quantification accuracy and reproducibility (7-9). Ideally, quantification standards should correct for losses occurring during sample decomplexification and compensate for digestion variability. Our results demonstrate that accurate and precise biomarker quantification can be achieved by spiking

isotopically labelled proteins (PSAQ standards) into serum samples early in the sample preparation process. Provided that PSAQ standards behave exactly like the target proteins, quantification is accurate, even in cases where recovery may have been affected by extensive fractionation (depletion or immunocapture, and SDS-PAGE). Similarly, PSAQ standards also compensate for variable digestion yields (17). In this study, we also tried to quantify a protein heterodimer, CKMB, using both CKB and CKM standards. Possibly because the standards did not exactly mimic the biochemical behaviour of the CKMB heterodimer, quantification was less accurate for this protein complex than for the other proteins studied. Nevertheless, in patient serum samples, increases in CKM and CKB chains measured by PSAQ-SRM were highly consistent with total CK activity results.

Accuracy is not essential for biomarker verification as this step is mainly focused on determining the specificity of biomarker candidates. Because of this, the use of isotope-labelled peptides as quantification standards is relevant and straightforward in this phase (19). However, during the qualification phase, the difference between healthy and pathological samples has to be established. For this, a method that can accurately and precisely quantify biomarkers over both physiological and pathological concentration ranges would be of particular value. In addition to meeting this criterion, as PSAQ standards correct for variable recovery due to differences in sample handling and digestion efficiency, their use should significantly improve inter-assay and inter-laboratory reproducibility.

Due to its selectivity, SRM analysis provides a high detection specificity (5). However, when working with highly complex samples such as serum, this selectivity might not be sufficient to avoid matrix interferences (24). In this context, the use of isotope dilution standards, such as labelled peptides or proteins, which co-elute with the target improve the specificity of the analysis (12, 25). Finally, we have shown that PSAQ standards offer the

largest coverage for quantification even making it possible to take cystein- and methionin-containing peptides into account after protein oxidation (18).

In our study, multiplexing was limited to the detection of 4 biomarkers in depleted serum samples. However, technological advances, including scheduled SRM (26, 27) and iSRM (28), have made it possible to monitor hundreds of proteins in a single experiment. Recently, Kuzyk and co-workers used isotope-labelled peptide standards and LC-SRM to quantify 45 proteins in plasma. 31 of these proteins were potential biomarkers of cardiovascular diseases (25). The most frequent question about implementation of the PSAQ method concerns the availability of PSAQ standards. In this study, the cell-free expression and isotope labelling of LDH-B, CKB, CKM, myoglobin and troponin I PSAQ standards were optimized in less than 2 months. These biomarkers are not post-translationally modified, which facilitates their expression in bacterial lysates. However, phosphorylated or glycosylated PSAQ standards can be produced using specific production systems (11, 13). Certainly, the throughput of PSAQ standard production could be further enhanced by availing of cDNA libraries specifically developed for protein expression (29, 30).

In conclusion, this study clearly demonstrates the relevance of using isotope-labelled protein standards for multiplex and reliable quantification of biomarkers in prefractionated clinical samples. We are currently concentrating our efforts on the generation of isotope-labelled protein libraries to increase the availability of PSAQ standards and widen the use of the PSAQ-SRM analytical strategy. Applying PSAQ-SRM as part of the biomarker development pipeline should help bridge the gap between biomarker discovery and clinical applications.

REFERENCES

1. Rifai, N., Gillette, M. A., and Carr, S. A. (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* **24**, 971-983

2. Makawita, S., and Diamandis, E. P. (2009) The Bottleneck in the Cancer Biomarker Pipeline and Protein Quantification through Mass Spectrometry-Based Approaches: Current Strategies for Candidate Verification. *Clin. Chem*. **56**, 212-222

3. Paulovich, A., Whiteaker, J., Hoofnagle, A., and Wang, P. (2008) The interface between biomarker discovery and clinical validation: The tar pit of the protein biomarker pipeline. *PROTEOM. CLIN. APPL.* **2**, 1386-1402

4. Huttenhain, R., Malmstrom, J., Picotti, P., and Aebersold, R. (2009) Perspectives of targeted mass spectrometry for protein biomarker verification. *Curr. Opin. Chem. Biol.* **13**, 518-525

5. Lange, V., Picotti, P., Domon, B., and Aebersold, R. (2008) Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* **4**, 222

6. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U S A* **100**, 6940-6945

7. Addona, T. A., Abbatiello, S. E., Schilling, B., Skates, S. J., Mani, D. R., Bunk, D. M., Spiegelman, C. H., Zimmerman, L. J., Ham, A. J., Keshishian, H., Hall, S. C., Allen, S., Blackman, R. K., Borchers, C. H., Buck, C., Cardasis, H. L., Cusack, M. P., Dodder, N. G., Gibson, B. W., Held, J. M., Hiltke, T., Jackson, A., Johansen, E. B., Kinsinger, C. R., Li, J., Mesri, M., Neubert, T. A., Niles, R. K., Pulsipher, T. C., Ransohoff, D., Rodriguez, H., Rudnick, P. A., Smith, D., Tabb, D. L., Tegeler, T. J., Variyath, A. M., Vega-Montoto, L. J., Wahlander, A., Waldemarson, S., Wang, M., Whiteaker, J. R., Zhao, L., Anderson, N. L.,

Fisher, S. J., Liebler, D. C., Paulovich, A. G., Regnier, F. E., Tempst, P., and Carr, S. A. (2009) Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* **27**, 633-641

8. Hoofnagle, A. N. (2010) Quantitative Clinical Proteomics by Liquid Chromatography-Tandem Mass Spectrometry: Assessing the Platform. *Clin. Chem.* **56**, 161-164

9. Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., Vandenesch, F., and Garin, J. (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. *Mol. Cell. Proteomics* **6,** 2139-2149

10. Brun, V., Masselon, C., Garin, J., and Dupuis, A. (2009) Isotope dilution strategies for absolute quantitative proteomics. *J. Proteomics* **72**, 740-749

11. Ciccimaro, E., Hanks, S. K., Yu, K. H., and Blair, I. A. (2009) Absolute quantification of phosphorylation on the kinase activation loop of cellular focal adhesion kinase by stable isotope dilution liquid chromatography/mass spectrometry. *Anal. Chem.* **81**, 3304-3313

12. Hanke, S., Besir, H., Oesterhelt, D., and Mann, M. (2008) Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the attomole level. *J. Proteome Res.* **7**, 1118-1130

13. Heudi, O., Barteau, S., Zimmer, D., Schmidt, J., Bill, K., Lehmann, N., Bauer, C., and Kretz, O. (2008) Towards absolute quantification of therapeutic monoclonal antibody in serum by LC-MS/MS using isotope-labeled antibody standard and protein cleavage isotope dilution mass spectrometry. *Anal. Chem.* **80**, 4200-4207

14. Izrael-Tomasevic, A., Phu, L., Phung, Q. T., Lill, J. R., and Arnott, D. (2009) Targeting interferon alpha subtypes in serum: a comparison of analytical approaches to the detection and quantitation of proteins in complex biological matrices. *J. Proteome Res.* **8**, 3132-3140

15. Janecki, D. J., Bemis, K. G., Tegeler, T. J., Sanghani, P. C., Zhai, L., Hurley, T. D., Bosron, W. F., and Wang, M. (2007) A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. *Anal. Biochem.* **369**, 18-26

16. Kumar, V., Barnidge, D. R., Chen, L. S., Twentyman, J. M., Cradic, K. W., Grebe, S. K., and Singh, R. J. (2010) Quantification of serum 1-84 parathyroid hormone in patients with hyperparathyroidism by immunocapture in situ digestion liquid chromatography-tandem mass spectrometry. *Clin. Chem.* **56**, 306-313

17. Lesur, A., Varesio, E., and Hopfgartner, G. (2009) Accelerated tryptic digestion for the analysis of biopharmaceutical monoclonal antibodies in plasma by liquid chromatography with tandem mass spectrometric detection. *J. Chromatogr. A*. **1217**, 57-64

18. Jaquinod, M., Villiers, F., Kieffer-Jaquinod, S., Hugouvieux, V., Bruley, C., Garin, J., and Bourguignon, J. (2007) A proteomics dissection of Arabidopsis thaliana vacuoles isolated from cell culture. *Mol. Cell. Proteomics* **6**, 394-412

19. Keshishian, H., Addona, T., Burgess, M., Mani, D. R., Shi, X., Kuhn, E., Sabatine, M. S., Gerszten, R. E., and Carr, S. A. (2009) Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* **8**, 2339-2349

20. Sanagi, M. M., Ling, S. L., Nasir, Z., Hermawan, D., Ibrahim, W. A., and Abu Naim, A. (2009) Comparison of signal-to-noise, blank determination, and linear regression methods for the estimation of detection and quantification limits for volatile organic compounds by gas chromatography. *J. AOAC Int.* **92**, 1833-1838

21. Buscemi, N., Foster, D. B., Neverova, I., and Van Eyk, J. E. (2002) p21-activated kinase increases the calcium sensitivity of rat triton-skinned cardiac muscle fiber bundles via

a mechanism potentially involving novel phosphorylation of troponin I. *Circ. Res.* **91**, 509-516

22. Fusaro, V. A., Mani, D. R., Mesirov, J. P., and Carr, S. A. (2009) Prediction of high-responding peptides for targeted protein assays by mass spectrometry. *Nat. Biotechnol.* **27**, 190-198.

23. Fortin, T., Salvador, A., Charrier, J. P., Lenz, C., Lacoux, X., Morla, A., Choquet-Kastylevsky, G., and Lemoine, J. (2009) Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests. *Mol. Cell. Proteomics* **8**, 1006-1015

24. Sherman, J., McKay, M. J., Ashman, K., and Molloy, M. P. (2009) How specific is my SRM? The issue of precursor and product ion redundancy. *Proteomics* **9**, 1120-1123

25. Kuzyk, M. A., Smith, D., Yang, J., Cross, T. J., Jackson, A. M., Hardie, D. B., Anderson, N. L., and Borchers, C. H. (2009) Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* **8**, 1860-1877.

26. Stahl-Zeng, J., Lange, V., Ossola, R., Eckhardt, K., Krek, W., Aebersold, R., and Domon, B. (2007) High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. *Mol. Cell. Proteomics* **6**, 1809-1817

27. Picotti, P., Rinner, O., Stallmach, R., Dautel, F., Farrah, T., Domon, B., Wenschuh, H., and Aebersold, R. High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. *Nat. Methods* **7**, 43-46

28. Kiyonami, R., Schoen, A., Prakash, A., Peterman, S., Zabrouskov, V., Picotti, P., Aebersold, R., Huhmer, A., and Domon, B. Increased selectivity, analytical precision, and throughput in targeted proteomics. *Mol. Cell. Proteomics*, in press

29. Goshima, N., Kawamura, Y., Fukumoto, A., Miura, A., Honma, R., Satoh, R., Wakamatsu, A., Yamamoto, J., Kimura, K., Nishikawa, T., Andoh, T., Iida, Y., Ishikawa, K., Ito, E., Kagawa, N., Kaminaga, C., Kanehori, K., Kawakami, B., Kenmochi, K., Kimura, R., Kobayashi, M., Kuroita, T., Kuwayama, H., Maruyama, Y., Matsuo, K., Minami, K., Mitsubori, M., Mori, M., Morishita, R., Murase, A., Nishikawa, A., Nishikawa, S., Okamoto, T., Sakagami, N., Sakamoto, Y., Sasaki, Y., Seki, T., Sono, S., Sugiyama, A., Sumiya, T., Takayama, T., Takayama, Y., Takeda, H., Togashi, T., Yahata, K., Yamada, H., Yanagisawa, Y., Endo, Y., Imamoto, F., Kisu, Y., Tanaka, S., Isogai, T., Imai, J., Watanabe, S., and Nomura, N. (2008) Human protein factory for converting the transcriptome into an in vitro-expressed proteome. *Nat. Methods* **5**, 1011-1017

30. Holz, C., Lueking, A., Bovekamp, L., Gutjahr, C., Bolotina, N., Lehrach, H., and Cahill, D. J. (2001) A human cDNA expression library in yeast enriched for open reading frames. *Genome Res.* **11**, 1730-1735

ACKNOWLEDGMENTS

We thank Christel Cabon for preparing patient samples, Marie-Laure Bourhis for logistical help, EDyP Service team for LC-MS/MS analyses, Dr Christophe Masselon for scientific discussions and Dr Maighread Gallagher-Gambarelli for editorial assistance. This project was supported by grants from the GRAVIT consortium, the French National Agency for Research (Project ANR-08-EBIO-008) and the 7th Framework Programme of the European Union (Contract no. 262067- PRIME-XS).

FIGURE LEGENDS

Figure 1. Sample preparation workflows for the quantification of cardiovascular biomarkers in serum.

A) Sample preparation workflow using either depletion of abundant proteins or troponin I immunoenrichment before SDS-PAGE, in-gel trypsin digestion and LC-SRM analysis. **B**) Multiplex LC-SRM detection of LDH-B, CKB chain, CKM chain and myoglobin in a healthy donor serum sample after depletion of abundant proteins. For better clarity, a single SRM transition from one proteotypic peptide (labelled and unlabelled versions) is shown for each biomarker.

Figure 2. Titration of cardiovascular biomarkers in serum samples

A) LDH-B titration curve. This curve was generated using 4 proteotypic peptides. Titration curves for each proteotypic peptide and related SRM transitions are presented in supplemental Figure 2. **B**) Myoglobin titration curve. These results were obtained using one proteotypic peptide. Two SRM transitions were monitored and the corresponding data are presented in supplemental Figure 3. **C**) CKM chain, CKB chain and CKMB titration curves. CKMB dimer was artificially spiked into samples. CKB and CKM chain concentrations were estimated independently (from 2 and 3 proteotypic peptides, respectively). The CKM+CKB titration curve represents a combination of the CKB and CKM titration curves. Further details on the different proteotypic peptides monitored and SRM transitions considered are available in supplemental Figure 4. **D**) Troponin I titration curve based on 3 proteotypic peptides. Titration curves for each proteotypic peptide and related SRM transitions are presented in supplemental Figure 5. For all titration curves, error bars represent standard deviations obtained from 3 full-process replicates.

Figure 3. PSAQ-SRM quantification of cardiovascular biomarkers in patient samples and correlation with enzymatic or ELISA assays

A) Correlation between total CK enzymatic activity and CKMB concentration, as determined by PSAQ-SRM, in clinical serum samples after depletion of abundant proteins (5 patients with myocardial infarction, 2 collection time-points). **B**) Correlation between ELISA and PSAQ-SRM results for quantification of myoglobin in clinical serum samples after depletion of abundant proteins (5 patients with myocardial infarction, 2 collection time-points). **C**) Correlation between results for quantification of troponin I in clinical serum samples by ELISA or in immunoenriched samples by PSAQ-SRM (5 patients with myocardial infarction, 2 collection time-points).

Table 1. Peptide sequences and SRM transitions

Biomarker	UniProt Accession Number	Peptide sequence	SRM transitions		Collision energy (Volts)
			Q1 m/z	Q3 m/z	
LDH-B	P07195	IVVVTAGVR	457.3	503.3	27.9
			457.3	602.4	27.9
			457.3	701.4	27.9
		IVVVTAGV**R**	462.3	513.3	27.9
			462.3	612.4	27.9
			462.3	711.4	27.9
		GLTSVINQK	480.3	502.3	29.0
			480.3	601.4	29.0
			480.3	688.4	29.0
		GLTSVINQ**K**	484.3	510.3	29.0
			484.3	609.4	29.0
			484.3	696.4	29.0
		SLADELALVDVLEDK	815.4	504.3	45.8
			815.4	718.4	45.8
			815.4	1001.6	45.8
			815.4	1114.6	45.8
			544.0	504.3	31.2
			544.0	718.4	31.2
			544.0	817.4	31.2
		SLADELALVDVLED**K**	819.4	512.3	45.8
			819.4	726.4	45.8
			819.4	1009.6	45.8
			819.4	1122.6	45.8
			546.6	512.3	31.2
			546.6	726.4	31.2
			546.6	825.4	31.2
		LIAPVAEEEATVPNNK	848.0	472.3	47.4
			848.0	1201.6	47.4
		LIAPVAEEEATVPNN**K**	852.0	480.3	47.4
			852.0	1209.6	47.4
Total LDH: LDH-A, LDH-B and LDH-C	P00338 P07195 P07864	VIGSGC(ox^3)NLDSAR	620.3	333.2	36.0
			620.3	448.2	36.0
			620.3	675.3	36.0
		VIGSGC(ox^3)NLDSA**R**	625.3	343.2	36.0
			625.3	458.2	36.0
			625.3	685.3	36.0
Creatine kinase B	P12277	DLFDPIIEDR	616.8	742.4	35.8
			616.8	857.4	35.8
		DLFDPIIED**R**	621.8	752.4	35.8
			621.8	867.4	35.8
		VLTPELYAELR	652.4	990.5	37.6
			652.4	1091.6	37.6
		VLTPELYAEL**R**	657.4	1000.5	37.6
			657.4	1101.6	37.6

Table 1 (continued).

Biomarker	UniProt Accession Number	Peptide sequence	SRM transitions		Collision energy (Volts)
			Q1 m/z	Q3 m/z	
Creatine kinase M	P06732	FEEILTR	454.3	502.3	27.7
			454.3	631.4	27.7
		FEEILT**R**	459.3	512.3	27.7
			459.3	641.4	27.7
		ELFDPIISDR	602.8	700.4	35.1
			602.8	962.5	35.1
		ELFDPIISD**R**	607.8	710.4	35.1
			607.8	972.5	35.1
		GGDDLDPNYVLSSR	754.4	935.5	42.7
			754.4	1050.5	42.7
		GGDDLDPNYVLSS**R**	759.4	945.5	42.7
			759.4	1060.5	42.7
Myoglobin	P02144	VEADIPGHGQEVLIR	545.0	553.3	31.2
			545.0	702.9	31.2
		VEADIPGHGQEVLI**R**	548.3	558.3	31.2
			548.3	707.9	31.2
Troponin I	P19429	TLLLQIAK	450.3	685.5	27.5
			450.3	572.4	27.5
		TLLLQIA**K**	454.3	693.5	27.5
			454.3	580.4	27.5
		NITEIADLTQK	623.3	675.4	36.2
			623.3	1018.5	36.2
		NITEIADLTQ**K**	627.3	683.4	36.2
			627.3	1026.5	36.2
		ISADAM(ox^2)M(ox^2)QALLGAR	756.4	303.2	42.8
			756.4	416.3	42.8
			756.4	529.4	42.8
		ISADAM(ox^2)M(ox^2)QALLGA**R**	761.4	313.2	42.8
			761.4	426.3	42.8
			761.4	539.4	42.8

Peptides from PSAQ standards are mentioned with the C-terminal isotope-labelled amino acid in bold.
Methionine dioxidation (ox^2) or cystein trioxidation (ox^3) modification states are indicated.

Table 2. Analytical performances of PSAQ-SRM quantifying cardiovascular biomarkers in serum samples.

<table>
<tr><th>Biomarker</th><th>Sample prefractionation method</th><th>Serum volume</th><th>LLOQ* (ng/mL)</th><th>Range of tested concentrations (ng/mL)</th><th>Linearity (R^2)</th><th colspan="2">Accuracy (slope value)</th><th>Precision at LLOQ (CV in %)</th></tr>
<tr><td>LDH-B</td><td>Depletion + SDS-PAGE</td><td>14 µL</td><td>510</td><td>510 (endogenous) → 5500</td><td>0.99</td><td colspan="2">1.18</td><td>8</td></tr>
<tr><td>CKB chains</td><td>Depletion + SDS-PAGE</td><td>14 µL</td><td>ND</td><td>20 (endogenous) → 1125</td><td>0.99</td><td>0.20</td><td rowspan="2">0.65 (CKMB)</td><td>ND*</td></tr>
<tr><td>CKM chains</td><td>Depletion + SDS-PAGE</td><td>14 µL</td><td>ND</td><td>260 (endogenous) → 1125</td><td>1</td><td>0.45</td><td>ND*</td></tr>
<tr><td>Myoglobin</td><td>Depletion + SDS-PAGE</td><td>14 µL</td><td>500</td><td>40 (endogenous) → 1000</td><td>1</td><td colspan="2">1.14</td><td>8</td></tr>
<tr><td>Troponin I</td><td>Immunoenrichment + SDS-PAGE</td><td>0.5 to 1 mL</td><td>5.5</td><td>0.29 (endogenous) → 272.5</td><td>1</td><td colspan="2">0.95</td><td>3</td></tr>
</table>

LLOQ was established as the lowest concentration of the average titration curve that was measured with precision (CV) inferior to 20 % and accuracy comprised between 80 and 120 %.* CKMB LLOQ could not be determined as quantification accuracy was lower than 80% (65%).

Table 3. PSAQ-SRM and ELISA quantification of LDH-B, CKB, CKM, myoglobin and troponin I in patient serum.

Patient, day after hospital admission	PSAQ-SRM					ELISA or enzymatic assay		
	Depletion				Immuno -enrichment	Total CK activity (UI/L)	Myoglobin (ng/mL)	Troponin I (ng/mL)
	LDH-B (µg/mL)	CKB (ng/mL)	CKM (ng/mL)	Myoglobin (ng/mL)	Troponin I (ng/mL)			
Patient 1, day 0	3.21	80**	9160	8120	121	4983	1353	80
Patient 1, day 3	4.32	30*	730	60*	16	439	50	29
Patient 2, day 0	1.07	100*	2320	2530*	41	1269	496	13.3
Patient 2, day 3	2.70	50*	1280	170	12	188	28	6.6
Patient 3, day 0	1.93	150	5430	4975	51	1338	1149	14.6
Patient 3, day 8	2.10	120*	230	90	6	107	36	10.3
Patient 4, day 0	1.03	70*	1050	2735	16	626	719	9.3
Patient 4, day 3	2.18	120*	470	80	6	218	36	8.9
Patient 5, day 0	1.88	110	2750	2305	33	1219	500	29.4
Patient 5, day 3	4.19	20*	360	45	10	228	38	12.9

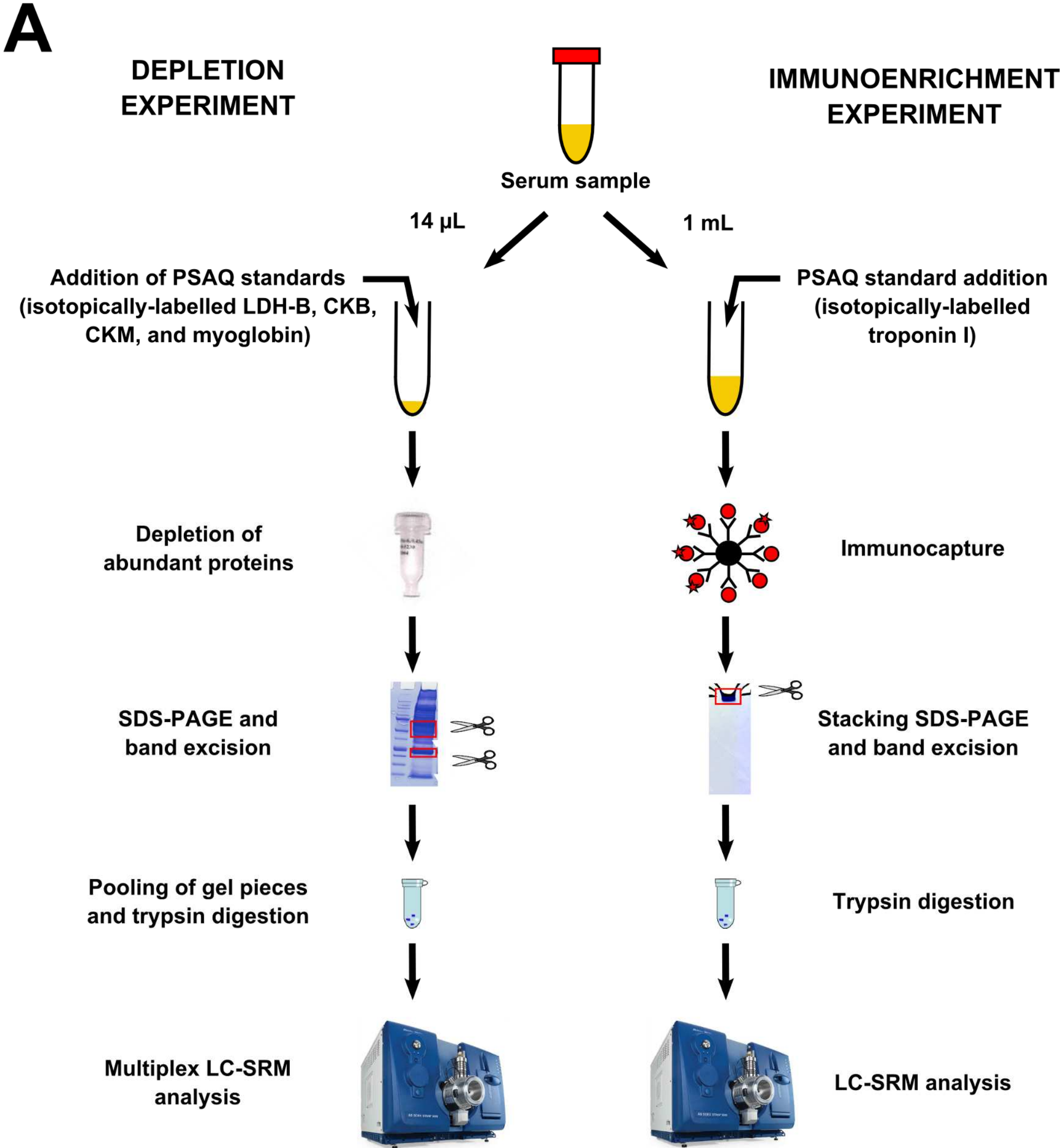
Biomarker concentrations were determined using at least 1 proteotypic peptide and 2 SRM transitions except for values indicated with:

* 1 proteotypic peptide detected with 1 SRM transition

** 2 proteotypic peptides, each detected with 1 SRM transition

See supplemental Figure 7 which shows the corresponding SRM transition chromatograms.

Figure 1.



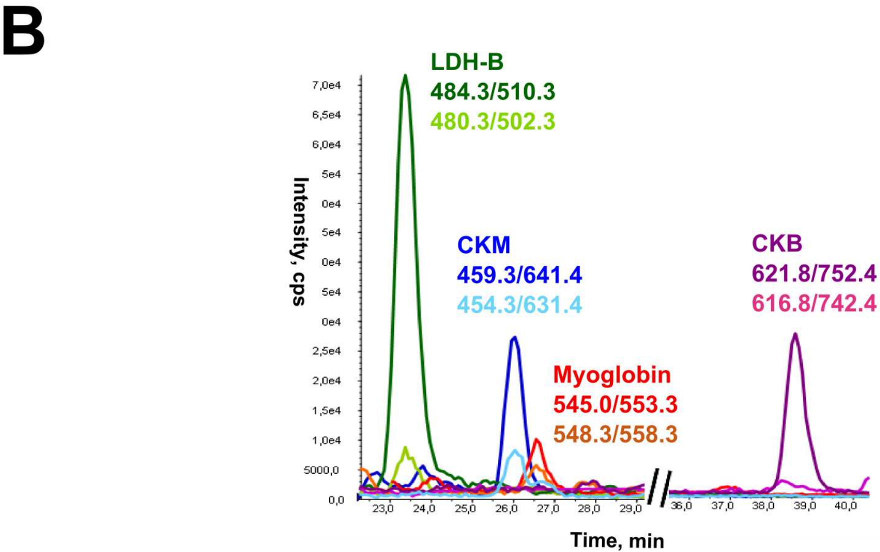


Figure 2.

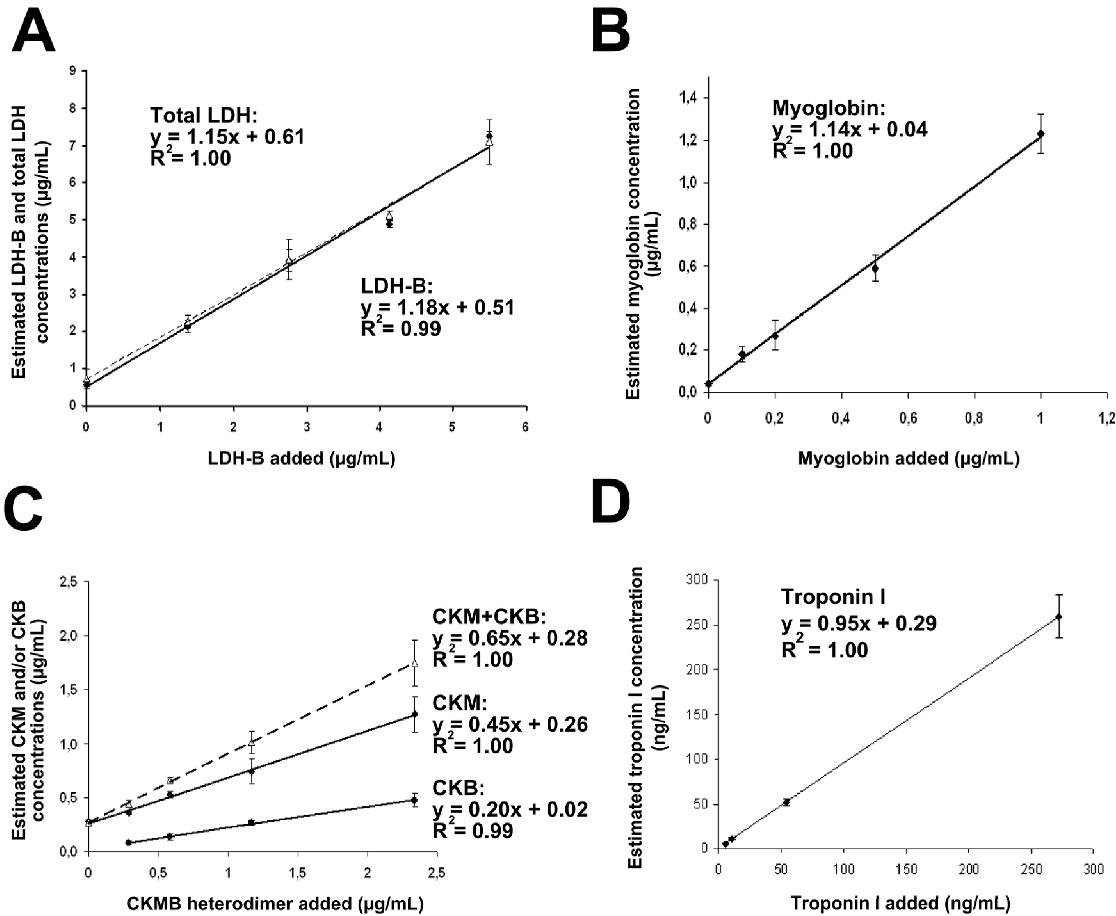


Figure 3.