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Review

Targeted mass spectrometry approaches for protein biomarker verification

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ABSTRACT

The search for protein biomarkers has been a highly pursued topic in the proteomics community in the last decade. This relentless search is due to the constant need for validated biomarkers that could facilitate disease risk stratification, disease diagnosis, prognosis, monitoring as well as drug development, which ultimately would improve our quality of life. The recent development of proteomic technologies including the advancement of mass spectrometers with high sensitivity and speed has greatly advanced the discovery of potential biomarkers. One of the bottlenecks lies in the development of well-established verification assays to screen the biomarker candidates identified in the discovery stage. Recently, absolute quantitation using multiple-reaction monitoring mass spectrometry (MRM-MS) in combination with isotope-labeled internal standards has been extensively investigated as a tool for high-throughput protein biomarker verification. In this review, we describe and discuss recent developments and applications of MRM-MS methods for biomarker verification.

Contents

Abbreviations: MRM-MS, multiple-reaction monitoring mass spectrometry; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assays; SRM, selected-reaction monitoring; Q, quadrupole; PSA, prostate specific antigen; CRP, C-reactive protein; CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantitation; LC, liquid chromatography; SISCAPA, Stable Isotope-Labeled Standards with Capture on Anti-Peptide Antibodies; QconCat, quantification concatamer; PSAQ, protein standard absolute quantification

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1. Introduction

A biomarker is a measurable indicator that provides status of biological state of the patient. Validated biomarkers and/or biomarker panels could be applied for targeted medicine where they can be used to measure disease progression and treatment efficacy. Therefore, biomarker discovery has always been of great interest of clinical research community. In the year 2010 alone, there were over 26,000 publications related to the term "biomarker" in PubMed. Arguably the protein biomarker discovery process has been greatly improved due to technology advancement in the last decade, leading to the exponential increase in potential biomarkers presented in the scientific literature. But as noted in a recent review by Anderson [1], even with great interest and plenty of funding, there is only on average about 1 protein per year in recent years that could eventually ended up as a well-characterized assay that is used by the biomedical community.

Once potential biomarkers are discovered, validation has to be done to make sure the biomarkers are specifically associated with a defined biological state and can be reproducibly performed. Most potential biomarkers are discovered through extensive sample fractionation followed by mass spectrometry (MS) analysis. In most cases, only a limited number of clinical samples of various sample types including body fluids and tissues are analyzed due to the low throughput of the discovery phase. Before these biomarkers can be developed into useful clinical assays, they have to be validated as a true biomarker for the intended condition and their sensitivity and specificity must be established. Biomarker validation requires a very large number of clinical samples (e.g. >1000) compare to discovery stage (e.g. <100) [2]. Biomarker validation requires a higher threshold of certainty than either discovery or verification. It is important to establish sensitivity and specificity of the biomarker that its levels must be measured against many thousands of samples in which its variation within the targeted population is precisely reflected. This cohort must include not only healthy individuals but also those with similar conditions to that being studied. Between the discovery and validation phases, resides verification [2]. The goal of the verification stage is to select those potential biomarkers that have the necessary specificity and sensitivity to potentially pass a final validation phase. This phase requires analyzing several hundred samples, but unlike the discovery phase is focused on a smaller number of analytes (i.e. tens) and for MS experiments, measures the absolute quantity of each not the relative abundance as is measured in the discovery phase. Changing from relative to absolute measurement is challenging as it requires validation of the analytic method used in the biomarker verification phase. Optimizing the measurement of the selected targets requires a variety of different sample preparation methods and a large number of different internal standards need to be synthesized. Essentially the verification phase requires development of a number of assays equal to the number of targets selected based on the discovery phase.

Verification studies need to be very carefully designed and cannot simply be an analysis of more of the same samples used in the discovery phase. Similar to validation, the cohorts of samples should be increased to not only include samples obtained from disease-affected and healthy donors, but also those that have similar diseases and a broad range of individuals within the population. For example, males and females, broad age range, different races and ethnic backgrounds, pre- and post-menopausal women, etc. should be included to get a statistically empowered measurement of the concentration range of the potential biomarkers being targeted.

The types of sample that are used are almost always serum or plasma, owing to their availability as well as the hypothesis that their molecular content represents the physiological and pathological state of the whole human body. Other biological samples such as urine and CSF are also utilized. While the discovery phase continues to generate many potential biomarkers, the verification phase has become one of the major bottlenecks in producing clinically useful biomarkers.

Mass spectrometry has played a pivotal role in the biomarker discovery stage. For MS-based biomarker discovery, extensive sample procedure and data dependent acquisition are generally utilized to facilitate the most thorough and unbiased sample analysis. The potential biomarker candidates identified at discovery stage have a high false positive rate due to the limited number of samples analyzed and analytical variation caused by extensive sample preparation. For biomarker verification, the data acquisition strategy targets specific potential biomarkers instead of measuring as many species as possible. Targeting specific biomarkers enables absolute quantitation measurements of each molecule to be recorded with greater accuracy and precision than afforded in the discovery phase. High throughput multiplexed assays have to be developed to screen and verify a large number biomarker candidates identified within the discovery stage. Once the biomarkers are validated, the effort can be devoted to develop assays that can actually be used in clinical setting. While enzyme-linked immunosorbent assays (ELISA) are widely used for biomarker verification and validation, developing individual ELISA assays for a large number of potential biomarkers (if one is not presently available) is very time consuming and expensive [3]. If an ELISA is available for a potential biomarker, it is an excellent choice since this type of assay can be easily automated leading to very high throughput.

Recently, quantitation assays based on multiple-reaction monitoring (MRM) MS in combination with stable-isotope labeled internal standards have been extensively investigated as an alternative to ELISAs for protein biomarker verification purposes [4–6]. Fortunately, many of the techniques required to develop biomarker verification studies have been well established and widely utilized for small molecule analysis were developed over a decade ago [7,8]. The capability of this targeted MS-based approach to monitor multiple peptide transitions in parallel offers both the high specificity and the throughput needed for verification of the large numbers of biomarker candidates proposed in the biomarker discovery stage. Therefore, this review focuses on the recent progress of the application of this targeted MS approach for biomarker verification including sample preparation strategies proposed to improve sensitivity when dealing with commonly used sample types such as plasma, software solutions developed to

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facilitate the automation of both the experimental design and data processing as well as issues encountered applying the approach for peptide analysis compared to small molecules.

2. Multiple reaction monitoring-mass spectrometry

Multiple reaction monitoring-mass spectrometry is generally done using triple quadrupole or triple quadrupole ion trap mass spectrometers because of their short duty cycle as well as their linear quantitation range. The targeted MS-based approach to be applied to biomarker verification relies on quantification of peptides derived from the protein biomarker candidates. Normally 3 to 5 peptides per protein that fit certain criteria (peptides are generally between 8 and 25 amino acids long, do not contain easily modified amino acids or known glycosylation sites, and have well defined termini) are used to quantify the protein biomarker. While these are useful guidelines, it is obvious that in some cases these "rules" need to be broken to target a specific site of interest. These peptides could be those identified during the discovery stage after further filtering using the above mentioned criteria and/or selected from predicted high-responding peptides after an in silico proteolytic digestion [9].

A schematic of stable isotope dilution MRM-MS showing steps required for method development and optimization is shown in Fig. 1. MRM-MS is done by isolating the precursor ion in the first quadrupole (Q1), fragmenting it within Q2, and monitoring the optimum fragment ions using Q3. The selectivity and specificity is achieved through a combination of Q1 only isolating precursor peptide ions within a narrow mass window and monitoring fragment ion masses corresponding to the specific precursor ions in Q3. The selectivity typically increases when multiple transitions are monitored for the same precursor ion, with 3 to 5 transitions typically being used for selectivity purpose. As shown in Fig. 2, absolute quantitation is done by comparing the peak area ratios between the endogenous peptides (best performing transition peak area or peak area from combined transitions) and those obtained from the spiked stable isotope-labeled internal standard. Peptides and subsequent transitions suitable for MRM-MS assays have to be evaluated and optimized to achieve best performance with least interference. In fact, peptides seen in discovery stages are not necessarily the best candidates to give best performance in MRM-MS assays. Additional optimization MRM-MS experiments using both best performing peptides seen at discovery stage as well as predicted high-responding peptides of the same protein are still required to narrow down the best peptides and/or the best transitions to monitor in the interested sample matrix to achieve the best sensitivity and reproducibility.

To be used for quantitative biomarker verification, the assay performance of the targeted MRM-MS assay including sensitivity, specificity, reproducibility, linear dynamic range as well as potential throughput has to be evaluated and established in a matrix similar to that being used in the clinical study. As plasma is one of the most attractive sample types for biomarker studies, a recent publication by Addona et al. [2] reported a multi-site (eight sites) systematic assessment of the performance of MRM-MS targeted assays applied to quantification of peptides and proteins spiked into a human plasma matrix. Seven proteins encompassed by 11 peptides were targeted in a human plasma matrix and analyzed without any sample fractionation. Five out of the seven target proteins were non-human in origin, thereby not interfering with the endogenous proteins within the sample matrix. The two human target proteins were prostate-specific antigen (PSA) and C-reactive protein (CRP). PSA is a well-known prostate cancer biomarker present at a concentration of less than 4 ng/ml in healthy men [10]. CRP is an acute phase protein present at sub mg/ml concentration in healthy population, which has shown implications in both cardiovascular diseases and cancers [11,12]. Phase I evaluated the analytical performance of MRM-MS assay using previously digested human plasma spiked with synthetic peptides and demonstrated excellent inter- and intra-laboratory reproducibility and precision. In phase II, the targeted 11 peptides were generated via tryptic digestion of the 7 intact recombinant proteins instead of using the synthetic light peptides. Peptide recoveries calculated from heavy isotope labeled internal peptide standards were generally lower in phase II compared to phase I, which were expected considering both the enzyme digestion efficiency and the sample loss due to extra clean up step could result in less light peptides. However, as targeted light peptides were generated by one digestion and diluted accordingly to generate the samples, the impact of the digestion as well as the clean up procedure has minimum impact on CVs. The majorities of interlaboratory CVs are less than 15% in both phases I and II. In phase III, all the sample

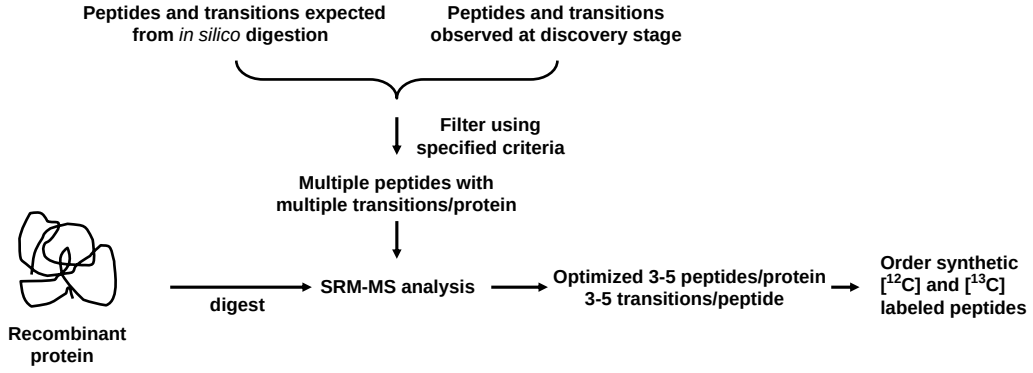

Fig. 1 – Stable isotope dilution MRM-MS method development and optimization workflow.

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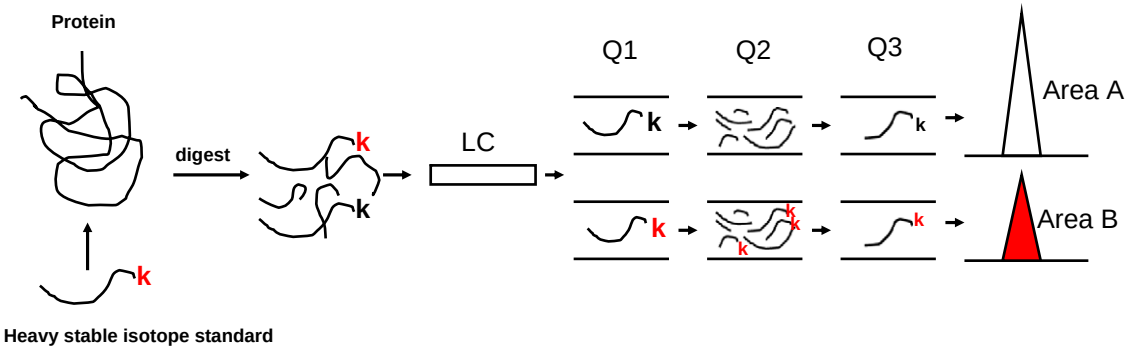

Fig. 2 – Absolute quantitation using MRM-MS in combination with isotope-labeled internal standard.

handling and preparation steps were performed on-site as would be expected if the approach is applied to verify large number of biomarker candidates. The median percent sample recovery of the peptides at the mid-concentration point (46 fmol/μl) was 48.9% for phase III (compared to 119.8% for phase I and 79.6% for phase II). The median interlaboratory CVs of eight out of eleven peptides measured, however, were still less than 25%. This invaluable study helps to establish some baseline parameters when applying targeted MRM-MS assay with isotope-labeled internal standards to verify biomarkers in a complex sample matrix. Overall, this study systematically demonstrated that the MRM-MS assay could be highly reproducible in a highly complex sample matrix such as plasma even across labs on different instrument platforms. For future large scale biomarker verification study, quantitation accuracy could depend on how to minimize variation caused by sample preparation and optimization of target peptides release. The low fmol/μl limit of quantitation (LOQ) achievable in phase I also mean only biomarkers in plasma with sub to low μg/ml or above concentrations can be reliably quantitated if the current MRM-MS approach is applied without any sample enrichment using current technology.

Kim et al. applied MRM-MS to verify biomarkers for diabetic retinopathy using clinical vitreous and plasma samples from 49 patients at 3 different stages [5]. This study utilized a typical potential biomarker verification work flow when MRM-MS is applied. Biomarker candidates were determined from previously generated proteome profiles and the monitored MRM transitions were selected from both earlier discovery effort and *in-silico* predictions. Targeted proteins quantified in both vitreous and plasma samples were reported to show different expression patterns and more experiments are probably needed for further biomarker verification. Kuzyk et al. [6] demonstrated quantitation of 45 endogenous proteins of moderate to high abundance in plasma without sample enrichment or fractionation in a single LC–MS run using MRM-MS in combination with 45 concentration-balanced stable-isotope labeled peptide standards according to their endogenous protein level. This study demonstrated the high throughput and multiplexing capability potential of this approach for protein biomarkers verification in a highly complex sample matrix. The concentration-balanced internal standard mixture not only facilitated better quantitation accuracy of analytes with $>10^4$ dynamic range but also improved the analyses CVs compared using equimolar concentration internal standard mixture. Therefore, the concentration of internal standards used needs to be carefully considered when developing multiplexed MRM-MS assays for biomarker verification.

Although the recent studies demonstrated the excellent precision and reproducibility of MRM-based approaches for biomarker verification, it also made it clear that the needed pg/ml–ng/ml sensitivity required to measure many biomarkers in plasma is not achievable with the current technology without any sample enrichment. Therefore, sample strategies developed previously for the low abundance biomarker discovery in plasma are being explored for biomarker verification purpose with emphasis being placed on their reproducibility and throughput.

3. Sample enrichment strategies applied to improve sensitivity

To improve detection sensitivity in plasma samples, the first sample preparation strategy that generally comes to mind is immuno-depletion of highly abundant proteins in serum and plasma; a technique that has been widely applied to biomarker discovery studies [13,14]. The reproducibility and the efficiency of immuno-depletion strategies for depletion of the intended abundant proteins have been thoroughly investigated and demonstrated [13–15]. Thus the depletion strategy in combination with further fractionation using techniques such as strong cation exchange chromatography have been applied to plasma samples prior to their analysis by MRM-MS. This combination has successfully achieved the ng/ml LOQs for clinical relevant biomarkers including PSA and cardiovascular biomarkers in patient samples [16–18]. While immunodepletion columns can deplete the targeted abundant plasma proteins efficiently and reproducibly, there are caveats that need to be considered when using this sample preparation strategy. In a recent study by Tu et al., only 23 proteins at less than ng/ml level were identified after immuno-depletion was performed [15]. In addition, the depleted high abundance proteins could potentially carry clinical relevant biomarkers either due to direct interaction or due to the so called “sponge” effect. Gundry et al. reported that nine of the 26 albumin-associated proteins that are considered potential biomarkers were only identified in the albumin-enriched fraction [19]. The non-targeted sample preparation strategy of immunodepletion in combination with further

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fractionation also has low multiplexing capabilities and low throughput.

To deal with the sample complexity issue and improve sensitivity for plasma samples, a better strategy would include targeted sample preparation in which low abundant targets can be enriched while the sample matrix can be simultaneously simplified. Targeted sample preparation strategies can either enrich for target proteins or enrich for peptides that act as surrogates for protein quantitation. Common enrichment strategies performed at the protein level include the use of antibodies, gel electrophoresis or off-gel fractionation. Unfortunately these techniques are generally time consuming and difficult to multiplex with low throughput. A recent approach termed Stable Isotope-Labeled Standards with Capture on Anti-Peptide Antibodies (SISCAPA) has shown great potential for low abundant biomarker verification when applied to enrich for targeted peptides. The peptides of interest are enriched from digested plasma samples that are spiked with known amounts of their stable isotope labeled internal standard counterparts. The enrichment uses immobilized antibodies generated against specific peptides. The peptides are released from the antibody and then quantitated using MRM-MS [20]. Whiteaker et al. [21] presented an improved SISCAPA approach through automating the process using a magnetic-bead-based platform capable of targeting 9 peptides in the same assay. The positive or negative impact of the multiplexing format on peptide target recovery seems to depend on the individual peptide but the overall performance of the 9-plex panel was not adversely affected. While using 10 μl plasma sample, the multiplexed SISCAPA process provided enrichment of 100–1000 fold with overall quantitation median CVs of 12.6%. This automated and multiplexed sample enrichment approach allows for quantifications of proteins in plasma at ng/ml level without any depletion and fractionation steps. What's more, as an enrichment strategy, the SISCAPA approach can be easily applied to larger sample volumes to further improve its sensitivity. As demonstrated in the same study, applying the method to 1 ml of plasma resulted in LODs and LOQs that were further decreased to pg/ml protein level for most targets while still maintaining assay performance. A potential drawback for the SISCAPA process is that not all peptides are antigenic as only 9 out of the original 15 peptides used in the study generated working antibodies. Multiple peptides per protein could potentially assist in generating useful antibodies, although the cost and overall lead time will also increase.

Alternatively, another affinity enrichment strategy based on combinatorial hexapeptide ligand library coupled to beads shows potential to achieve enrichment of low abundant proteins while minimizing sample dynamic range in a non-targeted manner compared to SISCAPA [22]. The non-targeted manner is in the sense that it is not targeting at any specific protein or proteins, but bind proteins up to its capacity. The combinatorial peptide ligand library reduces sample dynamic range by equalizing the concentrations of all proteins by capturing them up to its binding capacity for all proteins, resulting in the overall enrichment of low abundance proteins. It has been widely used for various sample types including human sera and urine [22]. Although it has not been exploited in combination with MRM-MS for plasma and serum samples, it has recently been applied in combination with an MRM-MS assay for quantitation of low abundance proteins in patient ovarian cancer ascites digest [23]. A multiplexed MRM-MS assay of five proteins including kallikrein 6 (in ng/ml level) were developed and the quantitation values obtained for this protein after combinatory peptide library treatment correlated well with those from earlier ELISA results ($R^2 = 0.988$). However, the reproducibility, efficiency and overall applicability of this sample enrichment strategy for multiple targets in a highly complex matrix obviously need to be further investigated and validated before it can be applied for protein biomarker verification purpose.

4. Mass spectrometry-based strategies to improve sensitivity

Besides all the sample preparation efforts being developed to improve the sensitivity of targeted MRM-MS approaches, Fortin et al. [24] reported a MS-based strategy (termed MRM^3) to improve limit of quantitation by taking advantage of the ability of a hybrid triple quadrupole/linear ion trap mass spectrometer to further fragment the product ions monitored in Q3. In MRM^3, the ion chromatogram is reconstructed from fragments of the product ion in the linear ion trap as shown in Fig. 3 instead of from the product ion as in the MRM mode. The MRM^3 mode further reduces background interference, which leads to a lower LOQ. It was demonstrated that MRM^3 generally resulted in 3- to 5-fold improvements in LOD and LOQ using 5 model proteins with only a slight reduction of accuracy and precision compare to a conventional MRM assay. The MRM^3 method demonstrated its potential for biomarker verification when it was applied to quantitate PSA in plasma samples obtained from three cancer patients using only a simple solid phase extraction sample preparation step targeted at PSA peptides. The low ng/ml results obtained using MRM^3 correlated very well with the ELISA tests. Besides the above strategy, Hossain et al. [25] reported that modification of a triple quadrupole mass spectrometer's ion source by coupling a multi-capillary inlet/dual electrodynamic ion funnel interface improved ion transmission efficiency that increased MRM peak intensities from 20- to 150-fold depending on the individual peptides. Further testing of the interface demonstrated a 10-fold improvement of LOD to 40–80 ng/ml range for the proteins spiked in non-depleted mouse plasma with much better reproducibility. Further advancement of mass spectrometer technology both from ion transmission efficiency and background noise reduction show great potential for reliable low abundant (ng/ml) biomarker quantitation and could be applied later to achieve the sensitivities needed for protein biomarker verifications either by itself or in combination with sample enrichment strategies.

5. Stable isotope labeled internal standards used

Stable isotope labeled peptides of the target proteins synthesized by chemical methods is the most commonly used internal standards for MRM-MS assays. They are normally

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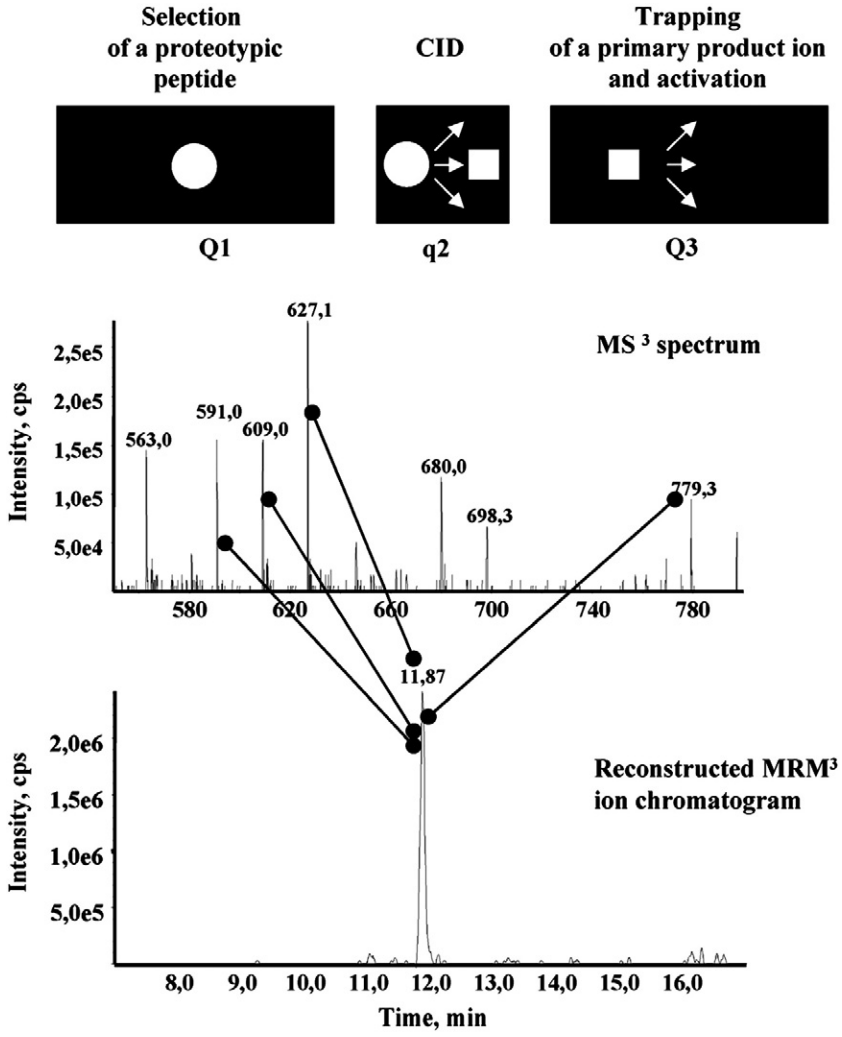

Fig. 3 – Schematic representation of MRM^3 on a hybrid triple quadrupole linear ion trap mass spectrometer with extracted ion chromatogram peak for quantitation. (reproduced with permission from [24]).

purified to high purity and quantified by amino acid analysis to assure quantification accuracy. The synthetic peptides are generally straightforward to synthesize and it is also applicable when modifications need to be quantified as long as the modification can be synthesized onto the labeled standard peptides. If a large number of peptides are to be monitored, purchasing synthetic peptides becomes costly. Therefore, an approach denoted quantification concatamer (QconCat), in which an artificial protein is expressed using gene encoding a concatamer of the target standard peptides in E coli system, was developed [26,27]. The QconCat proteins were reported to be highly amenable to proteolytic digestion due to their lack of higher order structure. The result is an equimolar mixture of the target peptides upon digestion. QconCat is a convenient and economic approach to obtain an equimolar ratio of large number of labeled internal standard peptides. It was evaluated by Mirzaei et al. [28] to be comparable but not necessarily superior to the chemical synthesis method to generate labeled internal standards. Even though QconCat does not completely compensate for target protein digestion efficiency due to their completely different structure, it could still partially compensate for the digestion procedure as they are typically added before the digestion step. Chemically synthesized peptides are usually added after the digestion step. However, QconCat peptides are not useful for quantitating modified peptides as the protein is expressed in *E. coli*. In addition, when multiplexing for biomarker verification, a variable amount of internal standards may need to be added for the different proteins to obtain the best overall quantitation result [6]; a procedure that is impossible using QconCat proteins. For targeted MRM-MS in combination with stable isotope internal standard approach, peptides are quantified as surrogates for protein targets. Therefore, the reproducibility and efficiency of the peptides release from proteins upon digestion are critical for their quantification and could be a major cause of data variation and discrepancies as noticed in various studies [4,29,30].

Efforts have to be made to optimize digestion procedure in plasma for biomarker quantitation [29,31]. It was noted that although an overall best performing digestion procedure can be achieved for all proteins in a sample, the optimum procedure for best digestion efficiency and reproducibility for any individual protein is in fact protein dependent [31]. Therefore, a solution could be using isotope labeled full length

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recombinant proteins as internal standards for MRM assays. The recombinant protein standards could be added at the beginning of any sample preparation and thus compensate for digestion efficiency as well as variation due to sample preparation throughout the entire procedure. Isotope labeled recombinant proteins have been explored as internal standards for improved absolute quantitation accuracy [32–35]. Brun et al. [32] denoted their approach as protein standard absolute quantification (PSAQ) and demonstrated that PSAQ outperforms both AQUA and QconCAT for quantifying the public health biomarker staphylococcus super-antigenic toxins in urine samples (Fig. 4). A comparison of the introduction points of these three type isotope labeled internal standards in sample procedure is shown in Fig. 5. Isotope labeled recombinant proteins are also the only internal standards compatible with any sample enrichment, fractionation and preparation strategies that are applicable for biomarker verification purpose. These recombinant proteins are generally expressed in either cell free or *E coli* systems, therefore they are not applicable for quantifying specific post-translational modifications. As one of the first application of isotope labeled recombinant protein used in clinical sample setting, quantification method of urinary albumin with a clinically relevant dynamic range of ~3 mg/l to 300 mg/l using

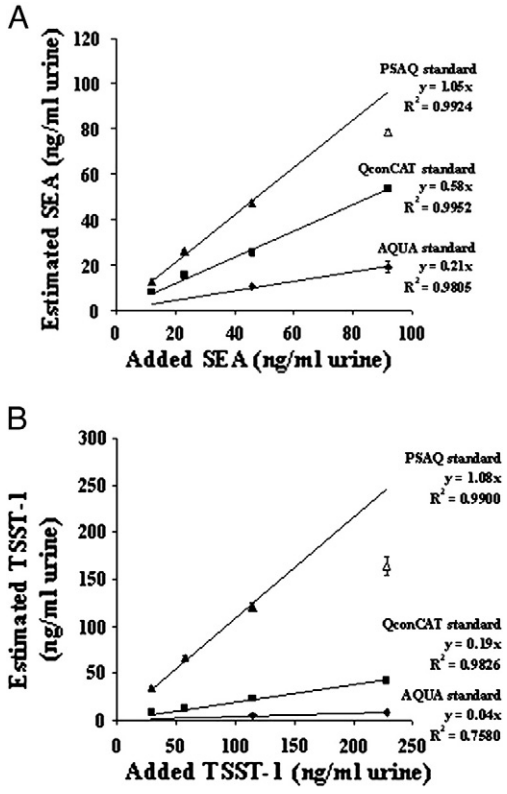

Fig. 4 – The comparison of concentration curves of peptides NVTVQELDLQAR (SEA) and LPTPIELPLK (TSST-1) in SEA (A) and TSST-1 (B) spiked urine samples obtained using different isotope labeled internal standards: synthetic peptides (AQUA) or generated from QconCAT or PSAQ. Each data point is the mean value ± S.E. of three analytical replicates. (reproduced with permission from [32]).

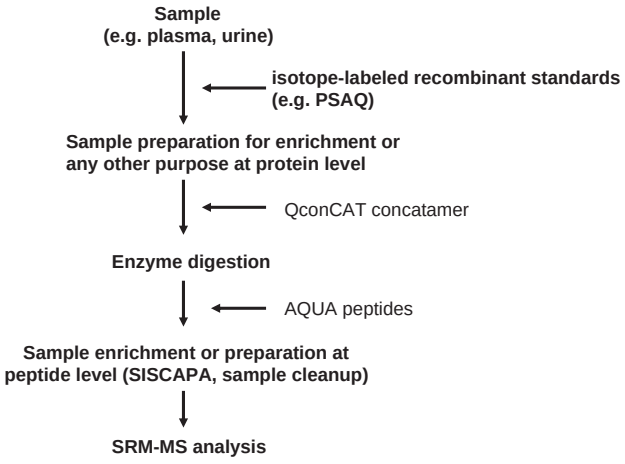

Fig. 5 – Introduction point of AQUA peptides, QconCAT concatemers, and isotope-labeled recombinant proteins as internal standards for quantification of protein biomarkers in sample processing procedure.

15^N-labeled recombinant human serum albumin (HSA) internal standard was developed and its clinical performance for 138 patent samples comparable to a commercially available immunoturbidometric assay was demonstrated [36]. However, isotope labeled recombinant proteins haven not been applied in any large scale biomarker verification studies as of yet, probably due to the time and expense required for their preparation. As the technology for recombinant protein expression and purification continues to develop, isotope labeled recombinant proteins could become a very useful tool for later targeted biomarker verification efforts especially when the efforts are performed across multiple sites where sample preparation procedure at different sites could cause significant data variation.

6. Bioinformatic software to facilitate application of MRM-MS for biomarker verification

With the large amount of potential biomarker candidates to be verified, setting up MRM-MS assay for each peptide with multiple transitions manually could become time consuming (and confusing) especially when time-scheduled transitions also need to be set up to increase both throughput and sensitivity. What's more, once high throughput multiplexed MRM-MS assay for parallel biomarkers verification are optimized, data processing has to be automated as well to improve throughput and minimize error. Bioinformatic tools to facilitate both the earlier stage surrogate peptides determination and the later stage data processing require continued development. Both commercial and open source software have been developed in recent years for these purposes and have greatly simplified the efforts needed to setup and optimize MRM-MS assays.

The commercial software include Pinpoint from Thermo Scientific and MultiQuant from AB Sciex, which is not surprising as their TSQ and QTRAP mass spectrometers are two of the most commonly used instruments for targeted peptide MRM-MS experiments. Both MultiQuant and Pinpoint

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have capability for MRM-MS method design, assay optimization and data processing for experiments and data generated by their specific instruments. The commonly used open source software includes Skyline and MRMer [37,38]. MRMer takes data in the platform-independent mzXML data format and allows data extraction, visualization as well as analysis. Among the software choices, Skyline takes data from all major instrument companies in their native form and could be used to design targeted MRM-MS assay directly exported in specific major instrument method forms. When deciding on peptides and transitions to monitor for biomarker verification, previous search result from biomarker discovery stage, public available spectral library results as well as in silico predictions could all be used in the decision making process. Skyline also fully supports quantitative data analysis using isotope-labeled internal standards as shown in Fig. 6. Collision energy is one of the instrument parameters that are often optimized for each peptide to achieve best signal intensity, which could be both time and resource consuming especially when large amount of peptides are involved. Skyline also provides instrument platform dependent collision energy predictions for major instrument companies that performs close to 92% on average to the performance of the empirically derived collision energy in MRM-MS assays as reported in a recent study [39]. Final result generated by skyline can be exported into tabular format that is compatible to Excel for easy statistic analysis. As Skyline seems to have the capabilities of method design for targeted MRM-MS in combination of isotope-labeled internal standards, support for assay optimization and support for automated data analysis for all the major instrument platforms, it would be a great tool later for biomarker verification studies when multi-site studies are being conducted.

Besides software tools developed to facilitate MRM-MS assay design and data processing automation, effort has also been spent to address inaccurate and imprecise quantification due to interferences or inconsistency in MRM-MS signals. An algorithm denoted automated detection of inaccurate and imprecise transitions (AuDIT) has been developed to automatically identify potential problematic data to be manually inspected and corrected. The program demonstrated better than 94% identification accuracy of errant data with high sensitivity and specificity [40]. Its implementation either as a post data-processing tool or further incorporated into the software packages mentioned above could greatly improve both the speed and the accuracy of future large scale biomarker verification effort using the targeted MRM-MS approach.

7. Conclusions and perspectives

With the backlog of biomarkers identified within the discovery phases continues to increase in size, it is becoming imperative

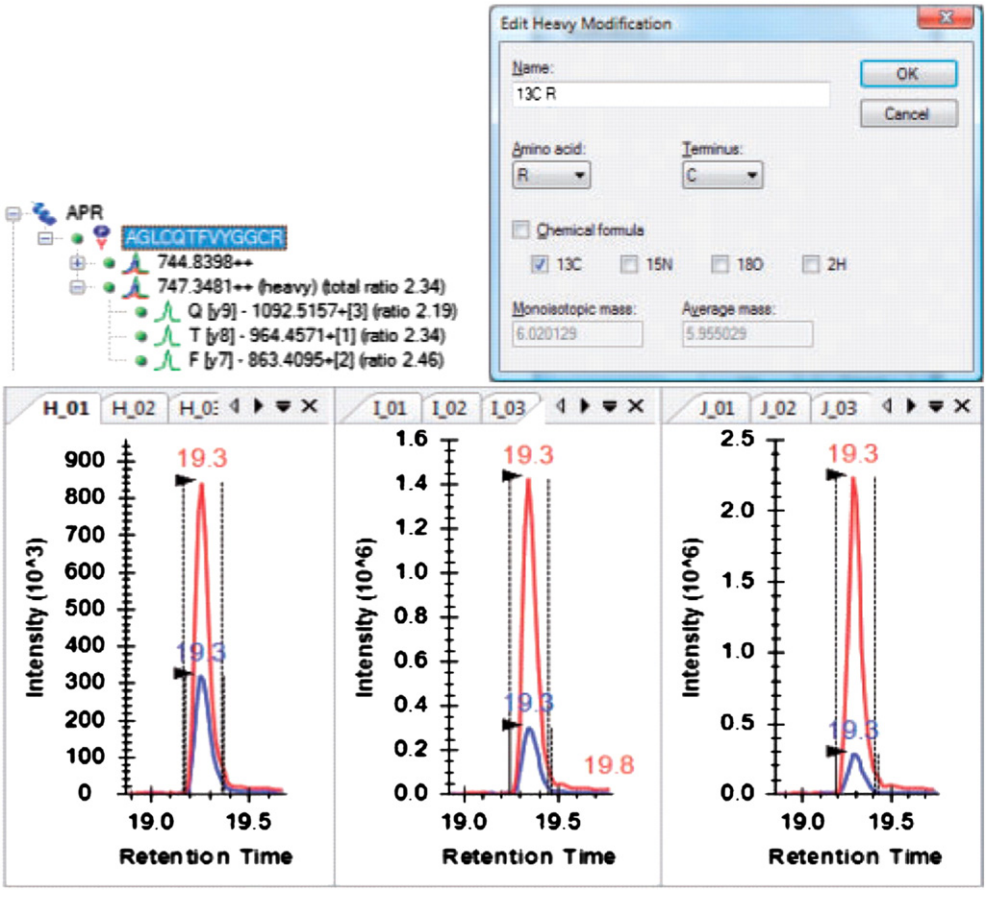

Fig. 6 – Skyline support for quantitative analysis of isotope-labeled internal standards and endogenous peptides. (reproduced with permission from [37]).

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that high-throughput tools for verifying the utility of any of these proteins soon become available. While MRM-MS approaches for verifying these potential biomarkers hold promise, each step in the process requires careful optimization. In needs to be kept in mind that the final quantitative results provided by MRM-MS must be accurate, precise, and highly reproducible. These requirements are not trivial as there is not a single "one size fits all" method to assay every candidate biomarker. The peptide selection, sample preparation method, LC conditions, and MS parameters must be optimized for each potential biomarker. Fortunately, the proteomics community can learn a lot from the small molecule community who have been developing quantitative assays for specific molecules for decades. While the specific steps involved in quantitating proteins versus small molecules are different, the fundamentals of extraction/enrichment, LC, MS, and data analysis are very similar.

To alleviate the backlog of biomarkers identified in the discovery phase, developing MRM-MS assays will need to become routine. Presently most verification studies are still conducted using antibody-based platforms since there are a large number of these assays already available. If an investigator has a choice between MRM-MS and an antibody-based assay, the decision is typically driven by availability. It makes more sense to utilize an already available antibody-based test rather than going through the expense of developing and MRM-MS assay. The best use of MRM-MS assays is for proteins where no antibody-based test is available or the present assay performs poorly. Another area where MRM-MS provides significant advantages is in multiplexing. Considering the speed and resolution afforded by modern mass spectrometers, it is easy to envision the day where up to a hundred proteins can be specifically quantitated using MRM-MS from a single patient sample. If this goal is achieved, it will represent a major step in making proteomics a contributor to personalized medicine.

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REFERENCES

[1] Anderson NL. The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. Clin Chem 2010;56:177–85.
[2] Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 2006;24:971–83.
[3] Issaq HJ, Veenstra TD. Would you prefer multiple reaction monitoring or antibodies with your biomarker validation? Expert Rev Proteomics 2008;5:761–3.
[4] Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 2009;27:633–41.
[5] Kim K, Kim SJ, Yu HG, Yu J, Park KS, Jang IJ, et al. Verification of biomarkers for diabetic retinopathy by multiple reaction monitoring. J Proteome Res 2010;9:689–99.
[6] Kuzyk MA, Smith D, Yang J, Cross TJ, Jackson AM, Hardie DB, et al. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. Mol Cell Proteomics 2009;8:1860–77.
[7] Woolf E, Haddix HM, Matuszewski B. Determination of an in vivo metabolite of a human immunodeficiency virus protease-inhibitor in human plasma by high-performance liquid chromatography with tandem mass spectrometry. J Chromatogr A 1997;762:311–9.
[8] Schupke H, Hempel R, Eckardt R, Kronbach T. Identification of talinolol metabolites in urine of man, dog, rat and mouse after oral administration by high-performance liquid chromatography-thermospray tandem mass spectrometry. J Mass Spectrom 1996;31:1371–81.
[9] Fusaro VA, Mani JP, Mesirov SA. Carr. Prediction of high-responding peptides for targeted protein assays by mass spectrometry. Nat Biotechnol 2009;27:190–8.
[10] Catalona WJ, Smith DS, Ratliff TL, et al. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. N Engl J Med 1991;324:1156–61.
[11] Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, et al. Genetic loci associated with C-reactive protein levels and risk of coronary heart disease. JAMA 2009;302:37–48.
[12] Mahmoud FA, Rivera NI. The role of C-reactive protein as a prognostic indicator in advanced cancer. Curr Oncol Rep 2002;4:250–5.
[13] Darde VM, Barderas MG, Vivanco F. Depletion of high-abundance proteins in plasma by immunoaffinity subtraction for two-dimensional difference gel electrophoresis analysis. Methods Mol Biol 2007;357:351–64.
[14] Whiteaker JR, Zhang H, Eng JK, Fang R, et al. Head-to-head comparison of serum fractioination techniques. J Proteome Res 2007;6:828–36.
[15] Tu C, Rudnick PA, Martinez MY, Cheek KL, Stein SE, Slebos RJ, et al. Depletion of abundant plasma proteins and limitations of plasma proteomics. J Proteome Res 2010;9:4982–91.
[16] Fortin T, Salvador A, Charrier JP, Lenz C, Lacoux X, Morla A, et al. 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 2009;8:1006–15.
[17] Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 2007;6:2212–29.
[18] Keshishian H, Addona T, Burgess M, Mani DR, Shi X, Kuhn E, et al. Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 2009;8:2339–49.
[19] Gundry RL, Fu Q, Jelinek CA, Van Eyk JE, Cotter RJ. Investigation of an albumin-enriched fraction of human serum and its albuminome. Proteomics Clin Appl 2007;1:73–88.
[20] Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). J Proteome Res 2004;3:235–44.
[21] Whiteaker JR, Zhao L, Anderson L, Paulovich AG. An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction

monitoring mass spectrometry-based quantification of protein biomarkers. Mol Cell Proteomics 2010;9:184–96.
[22] Boschetti E, Righetti PG. The art of observing rare protein species in proteomes with peptide ligand libraries. Proteomics 2009;9:1492–510.
[23] Drabovich AP, Diamandis EP. Combinatorial peptide libraries facilitate development of multiple reaction monitoring assays for low-abundance proteins. J Proteome Res 2010;9: 1236–45.
[24] Fortin T, Salvador A, Charrier JP, Lenz C, Bettsworth F, Lacoux X, et al. Multiple reaction monitoring cubed for protein quantification at the low nanogram/milliliter level in nondepleted human serum. Anal Chem 2009;81:9343–52.
[25] Hossain M, Kaleta DT, Robinson EW, Liu T, Zhao R, Page JS, et al. Enhanced sensitivity for selected reaction monitoring-mass spectrometry-based targeted proteomics using a dual-stage electrodynamic ion funnel interface. Mol Cell Proteomics Apr 21 2010:20410378 [Epub ahead of print] PubMed PMID.
[26] Beynon RJ, Doherty MK, Pratt JM, Gaskell SJ. Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. Nat Methods 2005;2:587–9.
[27] Pratt JM, Simpson DM, Doherty MK, Rivers J, Gaskell SJ, Beynon RJ. Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. Nat Protoc 2006;1:1029–43.
[28] Mirzaei H, McBee JK, Watts J, Aebersold R. Comparative evaluation of current peptide production platforms used in absolute quantification in proteomics. Mol Cell Proteomics 2008;7:813–23.
[29] Proc JL, Kuzyk MA, Hardie DB, Yang J, Smith DS, Jackson AM, et al. A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. J Proteome Res 2010;9: 5422–37.
[30] Arsene CG, Ohlendorf R, Burkitt W, Pritchard C, Henrion A, O'Connor G, et al. Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. Anal Chem 2008;80:4154–60.
[31] Agger SA, Marney LC, Hoofnagle AN. Simultaneous quantification of apolipoprotein A-I and apolipoprotein B by liquid-chromatography–multiple-reaction-monitoring mass spectrometry. Clin Chem 2010;56:1804–13.
[32] Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M, et al. Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics 2007;6:2139–49.
[33] Janecki DJ, Bemis KG, Tegeler TJ, Sanghani PC, Zhai L, Hurley TD, et al. A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. Anal Biochem 2007;369:18–26.
[34] Hanke S, Besir H, Oesterhelt D, Mann M. Absolute SILAC for accurate quantitiation of proteins in complex mistures down to the attomole level. J Proteome Res 2008;7:1118–30.
[35] Singh S, Springer M, Steen J, Kirschner MW, Steen H. FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification potentially modified peptides. J Proteome Res 2009;8:2201–10.
[36] Seegmiller JC, Barnidge DR, Burns BE, Larson TS, Lieske JC, Kumar R. Quantification of urinary albumin by using protein cleavage and LC–MS/MS. Clin Chem 2009;55:1100–7.
[37] Maclean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 2010;26:966–8.
[38] Martin DB, Holzman T, May D, Peterson A, et al. MRMer, an interactive open source and cross-platform system for data extraction and visualization of multiple reaction monitoring experiments. Mol Cell Proteomics 2008;7:2270–8.
[39] Maclean B, Tomazela DM, Abbatiello SE, Zhang S, Whiteaker JR, Paulovich AG, et al. Effect of collision energy optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. Anal Chem 2010;82: 10116–24.
[40] Abbatiello SE, Mani DR, Keshishian H, Carr SA. Automated detection of inaccurate and imprecise transitions in peptide quantification by multiple reaction monitoring mass spectrometry. Clin Chem 2010;56:291–305.

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