Application Note 624

Practical, Broadly Applicable Workflow for Rapid Development of MS-based SRM Methods for Translational Research

Bryan Krastins and Mary F. Lopez; Thermo Fisher Scientific BRIMS, Cambridge, MA

Key Words

LTQ Orbitrap XL, TSQ Vantage, Pinpoint, MSIA, mass spectrometric immunoassay, SRM, selected-reaction monitoring, biomarker, apolipoprotein, ceruloplasmin, vitamin D binding protein, beta-2 microglobulin, C-reactive protein, procalcitonin, parathyroid hormone, insulin-like growth factor, prostate-specific antigen, erythropoietin, proprotein convertase subtilisin kexin, amyloid beta, PTH, PSA, translational research

Goal

Present a practical, broadly applicable workflow for rapid development of MS-based SRM methods that enable detection of proteins relevant to clinical research within established ranges in donor samples. The workflow allows analytically specific quantification of individual protein isoforms, thus addressing the challenge of detecting protein heterogeneity.

Introduction

Many disease-related proteins are truncated, modified by post-translational modifications (PTMs), or single nucleotide polymorphisms (SNP)[1] and thus are present in many active and inactive isoforms. It is important to be able to measure relative concentrations of these isoforms in order to gain the necessary analytical specificity for clinical research methods.[2-8] For example, protein heterogeneity has frustrated efforts to develop a prostate-specific antigen (PSA) method that delivers easily interpreted and consistent results.[9,10] Because PSA is typically present in numerous truncated and modified isoforms, the collective quantification of all forms may not provide enough analytical specificity for an accurate disease-related prognosis and has likely resulted in a high false-positive rate (FPR), limiting its application.[11] Because MS can resolve proteins at the sequence level, it can provide the selectivity needed to distinguish among variants and PTM heterogeneity in clinical research.

Though routine measurement of proteins by MS in biological fluids such as plasma and serum has not yet been widely adopted, advances in instrumentation, sample preparation, and enrichment, as well data processing, have made MS-based targeted protein methods attractive.

Despite these advances, the large dynamic range of proteins in serum and plasma challenges the ability of MS methods to detect low-abundance analytes. Accurate quantification of markers important to clinical research often requires enrichment of less-abundant species[12,13] prior to MS detection. Numerous approaches have been used including fractionation using multiple LC columns, prior depletion of abundant proteins, enrichment using solid phase extraction (SPE) or nanoparticles, or immunoaffinity enrichment by various techniques including magnetic beads.[14-19] Most of these approaches have not demonstrated the necessary combination of sensitivity across the appropriate concentration range, high precision (CVs ≤20%), and speed (less than 24 hour sample turnaround times) when running donor samples, and none adequately address protein heterogeneity and isoforms.[20]

To address the challenges of peptide variant detection and quantification in biological matrices, a workflow that combines highly selective affinity capture with MS detection and quantification of targeted surrogate peptides, including truncated variants, was employed. Thermo Scientific™ MSIA™ (Mass Spectrometric Immunoassay) is a highly selective workflow solution that provides affinity purification prior to detection by MS. Thermo Scientific™ MSIA™ D.A.R.T.'S (Disposable Automation Research Tips) consist of a porous monolithic microcolumn functionalized with an anti-protein antibody that is fixed within a pipette tip housing. MSIA overcomes many of the problems encountered with other workflows, resulting in methods with improved reproducibility, sensitivity, dynamic range, speed, and ease of use.[21] Additionally, the relative lack of analytical specificity of most antibodies to minor changes in protein sequence or the presence of PTMs presents an opportunity to collectively enrich an entire range of isoforms of a particular disease-related protein.

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Method development begins with the use of high-resolution LC-MS/MS to produce a map of the protein isoforms present in a collection of samples likely to contain disease-related variants. Once the variants are identified, targeted selected-reaction monitoring (SRM)-based MS methods are constructed for the variant-specific peptides. Thermo Scientific™ Pinpoint™ software facilitates SRM method development and targeted protein quantification.

Sixteen proteins spanning seven areas important to clinical research were analyzed using the MSIA-SRM workflow. Analyses were performed on donor plasma or serum samples. The panel of proteins included:

- Members of the apolipoprotein family (ApoE, ApoA1, ApoCI, ApoCIII, and ApoJ [clusterin])
- Medium-to-high abundance proteins (ceruloplasmin, vitamin D binding protein, beta-2 microglobulin, and C-reactive protein)
- Low-abundance proteins (procalcitonin, parathyroid hormone, insulin-like growth factor 1, prostate-specific antigen, erythropoietin, proprotein convertase subtilisin/kexin type 9, and amyloid beta)
- Numerous variant and isoform-specific peptides for other proteins not previously detected using SRM methods.

Complementary research published in *Clinical Biochemistry* provides a detailed description of the experimental procedure, workflow, and results for the proteins measured.[22]

Experimental

Samples

All donor samples were procured in accordance with the approval of institutional IRB protocols. The disease states represented by the donor samples included Alzheimer's/neurological, cardiovascular/cerebrovascular, renal failure/endocrine function/bone metabolism/low vitamin D, growth disorders, and cancers.

Antibodies and Recombinant Proteins

Supplementary Table S1 in the complementary research paper[22] lists the sources for antibodies and recombinant proteins used in the development of the methods. Epitopes for the PTH, insulin-like growth factor (IGF1), and procalcitonin (PCT) antibodies were mapped using phage display technology (Differential Proteomics, Research Triangle, NC) and were determined to be aa 72–79, 87–92, and 99–106, respectively. The best performing antibodies were selected after testing several commercially available sources with standard commercially available recombinant proteins.

Sample Enrichment and Preparation

Custom MSIA D.A.R.T.'S were activated with relevant antibodies and subjected to binding and elution using a Thermo Scientific™ Versette™ automated liquid handler or Thermo Scientific™ Matrix™ Platemate™ robotic workstation. Plasma or serum sample volumes ranged from 1 to 1000 µL, depending on the abundance of target proteins. After sample extraction with the activated MSIA D.A.R.T.'S, the proteins were eluted using an acidic buffer into 96-well microtiter plates and then pH neutralized, reduced, alkylated, and digested with trypsin at 50 °C. In some cases, other proteolytic enzymes such as staphylococcus aureas V-8 protease (V8) were used to generate peptides with the desired target sequences.

High-Resolution LC-MS/MS Data Analysis and Protein Identification

High-resolution LC-MS/MS analysis was carried out on a Thermo Scientific™ LTQ Orbitrap XL™ hybrid ion trap-Orbitrap mass spectrometer coupled to a Thermo Scientific™ EASY-nLC liquid chromatography system at a flow rate of 375 nL/min. To identify proteins and peptides, data were analyzed using Thermo Scientific™ Proteome Discoverer™ software version 1.3 prior to SRM method development.

Targeted SRM Method Development

SRM methods were developed using a Thermo Scientific™ TSQ Vantage™ triple quadrupole mass spectrometer equipped with a Thermo Scientific™ Ion Max source with a high-flow metal needle coupled to a Thermo Scientific™ Transcend™ LX-1 system. A Thermo Scientific™ Transcend™ LX-4 system was tested to evaluate multiplexing. Depending on the experiment, flow rates of 240–800 µL/min were used. Thermo Scientific™ Accucore™ aQ 2.1 x 50 mm columns (P/N 17126-052130) were used for all analyses, except for the amyloid beta (A beta) analysis, where a Thermo Scientific™ ProSwift™ C18 1.0 x 250 mm column (P/N 164922) was used.

Calibration Curve Generation

Recombinant proteins were added to human blood or plasma or serum controls as calibration standards (typically N=9). Serial dilutions were used for all targeted proteins. The spiked calibration samples were processed with MSIA D.A.R.T.'S, eluted, and subsequently reduced, alkylated, and digested with trypsin or other proteolytic enzyme prior to MS. Therefore, every point on the calibration curve reflected the entire MSIA workflow.

Every sample analyzed included 100 fmol of heavy-isotope-labeled peptides as internal standards. The internal standards were added after digestion of the recombinant protein and before LC-MS analysis.

Quantitative analysis was performed using Pinpoint software. Calibration curve replicate points were run adjacently. Heavy peptides were also spiked into every sample and were used to calculate run-to-run variance to add confidence to the analysis.

Choice of Proteins, Peptides, and Transitions

Pinpoint software was used to facilitate MS method development and targeted protein quantification by combining four approaches:

1. Spectral libraries produced during high-resolution LC-MS/MS discovery analyses of the recombinant protein standards were imported into Pinpoint software for SRM method development and optimization.
2. Algorithmic predictions of optimal peptides and transitions were carried out *in silico* using Pinpoint software. These were added to the list of peptides and transitions already identified in the spectral libraries.
3. Manual sequence analysis identified peptides that would theoretically result from tryptic and other proteolytic digestion of natural variants such as apolipoprotein AI (Apo AI) peptides YTKKLNTQ and YTKKLNT. These sequences were added to the methods.
4. Intact sequences of naturally occurring variants (such as A beta peptides aa 1–38, 1–40 and 1–42) were added to the methods.

Peptide identities were confirmed by chromatographic co-elution of light- (endogenous) and heavy-isotope-labeled transitions. For additional verification and elimination of interferences, the SRM transition ratios were confirmed using discovery spectra. Time alignment and relative quantification of the transitions were performed using Pinpoint software.

Peptide sequences, transitions, collision energies and all other relevant parameters are provided in Table S2 of the complementary research paper.[22] All samples were analyzed in triplicate.

Heavy-Isotope-Labeled Peptides

Heavy-isotope-labeled versions (purity ≥97%) of each target peptide were synthesized. Heavy-isotope-labeled peptides had sequences identical to their respective endogenous peptides, but the C-terminal lysine or arginine residues were fully labeled (≥98.5%) with 13C or 15N. See Table S2 for peptide sequences.[22]

Results and Discussion

SRM Method Development and Optimization

Figure 1 shows the automated workflow for SRM method development. The workflow utilizes Pinpoint software to combine spectral library prediction with empirically collected high-resolution LC-MS/MS data to determine the optimal peptides and their transitions. Specifically, Pinpoint software integrates empirically observed transitions from the spectral library data with algorithmically predicted transitions. Figure 2 shows a workbook in the Pinpoint software method for Apo AI listing all peptides detected in the spectral libraries and *in silico* predicted peptides. Hydrophobicity factors are provided to assist in method creation by facilitating peptide scheduling along the LC gradient. This approach creates an initial SRM method containing a complete list of all possible target peptides and transitions. Once the initial method is built, and if recombinant protein standards (digested or intact) are available for testing, the list can be rapidly shortened by automated iterative optimization using Pinpoint software. If recombinant proteins are not available, synthetic peptides can be used to build the optimized method.

During iterative optimization, parameters such as collision energy, LC gradient and SRM scheduling windows are varied and the highest intensity transitions are retained for the next iteration. After three to four iterations, the method is optimized and ready for sample analysis. With the help of Pinpoint software, the entire MS method development process typically can be completed in two to three hours.

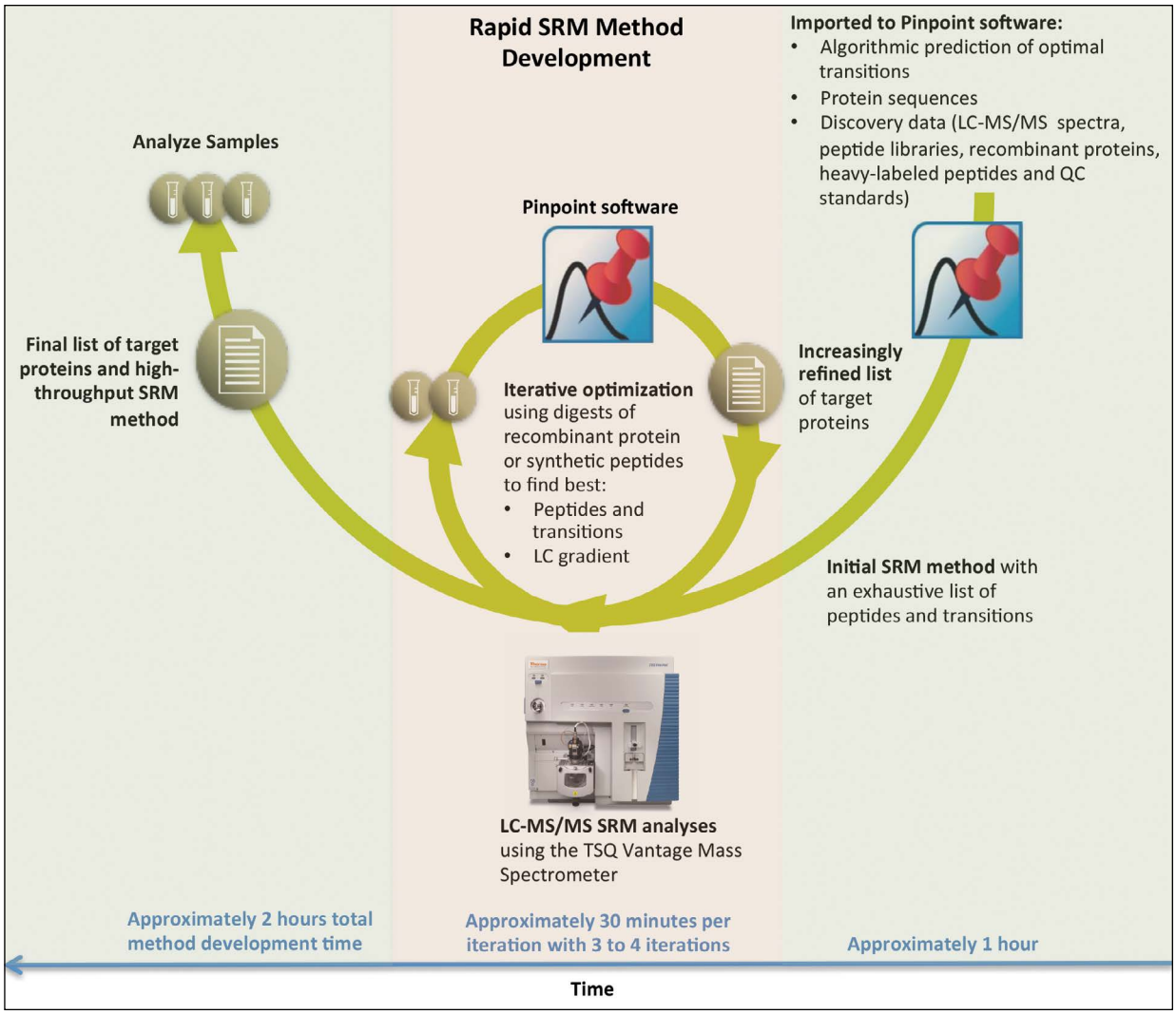
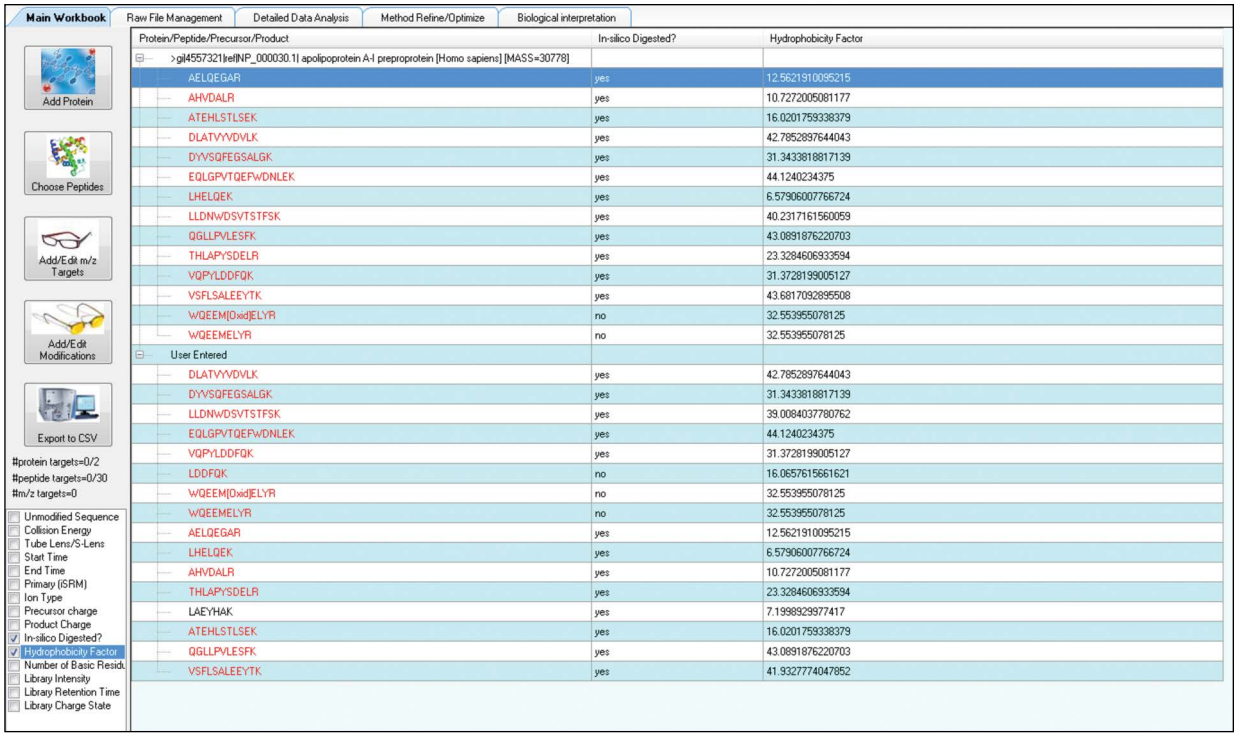


Figure 1. Automated development and optimization of SRM methods using Pinpoint software.



Protein/Peptide/Precursor/Product	In-silico Digested?	Hydrophobicity Factor
>gi|4557321|ref|NP_000030.1| apolipoprotein A-I preproprotein [Homo sapiens] [MASS=30778]		
AELQEGAR	yes	12.5621910095215
AHVDALR	yes	10.7272005081177
ATEHLSTLSEK	yes	16.0201759338379
DLATVYVDVLK	yes	42.7852897644043
DYVSQFEGSALGK	yes	31.3433818817139
EQLGPVTQEFWDNLEK	yes	44.1240234375
LHELQEK	yes	6.57906007766724
LLDNWDSVTSTFSK	yes	40.2317161560059
QGLLPVLESFK	yes	43.0891876220703
THLAPYSDELR	yes	23.3284606933594
VQPYLDDFQK	yes	31.3728199005127
VSFLSALEEYTK	yes	43.6817092895508
WQEEM[Oxid]ELYR	no	32.5539550781250
WQEEMELYR	no	32.5539550781250
User Entered		
DLATVYVDVLK	yes	42.7852897644043
DYVSQFEGSALGK	yes	31.3433818817139
LLDNWDSVTSTFSK	yes	39.0084037780762
EQLGPVTQEFWDNLEK	yes	44.1240234375
VQPYLDDFQK	yes	31.3728199005127
LDDFQK	no	16.0657615661621
WQEEM[Oxid]ELYR	no	32.5539550781250
WQEEMELYR	no	32.5539550781250
AELQEGAR	yes	12.5621910095215
LHELQEK	yes	6.57906007766724
AHVDALR	yes	10.7272005081177
THLAPYSDELR	yes	23.3284606933594
LAEYHAK	yes	7.1998929977417
ATEHLSTLSEK	yes	16.0201759338379
QGLLPVLESFK	yes	43.0891876220703
VSFLSALEEYTK	yes	41.9327774047852

Figure 2. Pinpoint software provides in silico predicted digested peptides along with peptides found by matching discovery data with the spectral library. Hydrophobicity factors help method creation by allowing scheduling of peptides along the LC gradient.

MSIA-SRM Workflow

Figure 3 shows the high-throughput MSIA-SRM workflow. Sample extraction and elution were typically completed in two hours or less, depending on the number of binding cycles. The optimal number of repetitive binding cycles (200–1500) was determined empirically and depended on (i) the affinity of the antibody for the analyte and (ii) the analyte abundance in the samples.

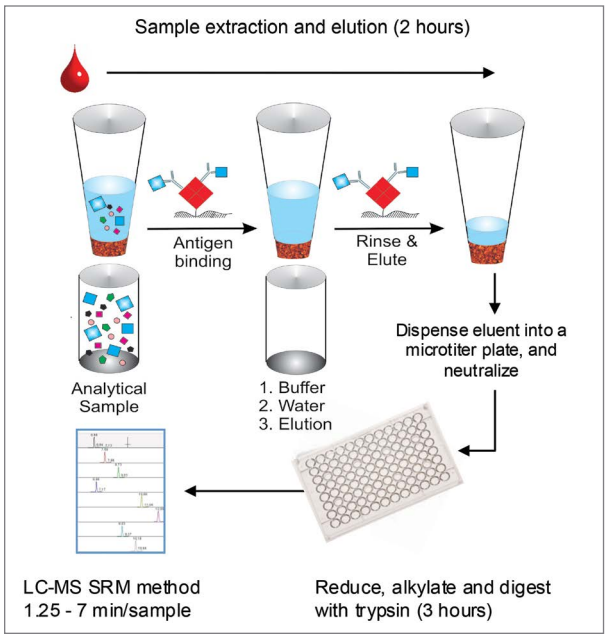


Figure 3. MSIA-SRM workflow.

Reduction, alkylation and digestion were completed in three hours, after which the sample was ready for injection into the LC–MS/MS system. Typical chromatographic separation times using fast-flow LC (240–800 µL/min) ranged from 1.25 to 7 min per sample, depending on the level of LC multiplexing, for example if a Transcend LX-4 system was used.

Some low-molecular-weight proteins or protein fragments such as A beta aa 1–38, 1–40, 1–42 were measured in their intact forms, i.e. without enzymatic digestion. In those cases, samples were introduced into the MS immediately following elution from the MSIA D.A.R.T'S. Intact protein analyses typically require lower LC flow rates or nanoflow LC in order to achieve the signal intensity needed to properly analyze the isotopic envelope produced by high molecular weight, highly charged species. Large intact proteins (>10 kDa) are best resolved on high-resolution ion trap-Orbitrap hybrid instruments rather than "beam" instruments such as triple quadrupole mass spectrometers.

For samples not requiring enzymatic digestion, the complete MSIA-SRM workflow (fast-flow) required just a few minutes more than two hours per 96-well plate. For analytes requiring enzymatic digestion to achieve sensitive detection, up to three more hours of sample preparation time may be needed. LC-MS analysis per sample was ten to twelve minutes in single-plex mode. Delivering multiple LC channels to a single MS instrument (multiplexing) increased the throughput by two to four times.

Method Sensitivity and Precision

Representative extracted SRM chromatograms of surrogate peptides from digested human plasma and serum samples and recombinant proteins are shown in Figure 4. Peak shapes were typically symmetrical and relatively free of interferences. SRM transition ion ratios were within ±15% of internal standard reference ratios.

In order to ensure selectivity, even for enriched samples, several SRM transitions were used for quantification of each peptide. Representative calibration curves for each set of analyses are shown in Figure 5. Calibration curves for all peptides demonstrated linear behavior (correlation coefficients ranged from 0.89 to 0.99 for triplicate analyses). The precision (% CV) of replicate test samples for all peptides within the linear range of the curve was ≤20%.

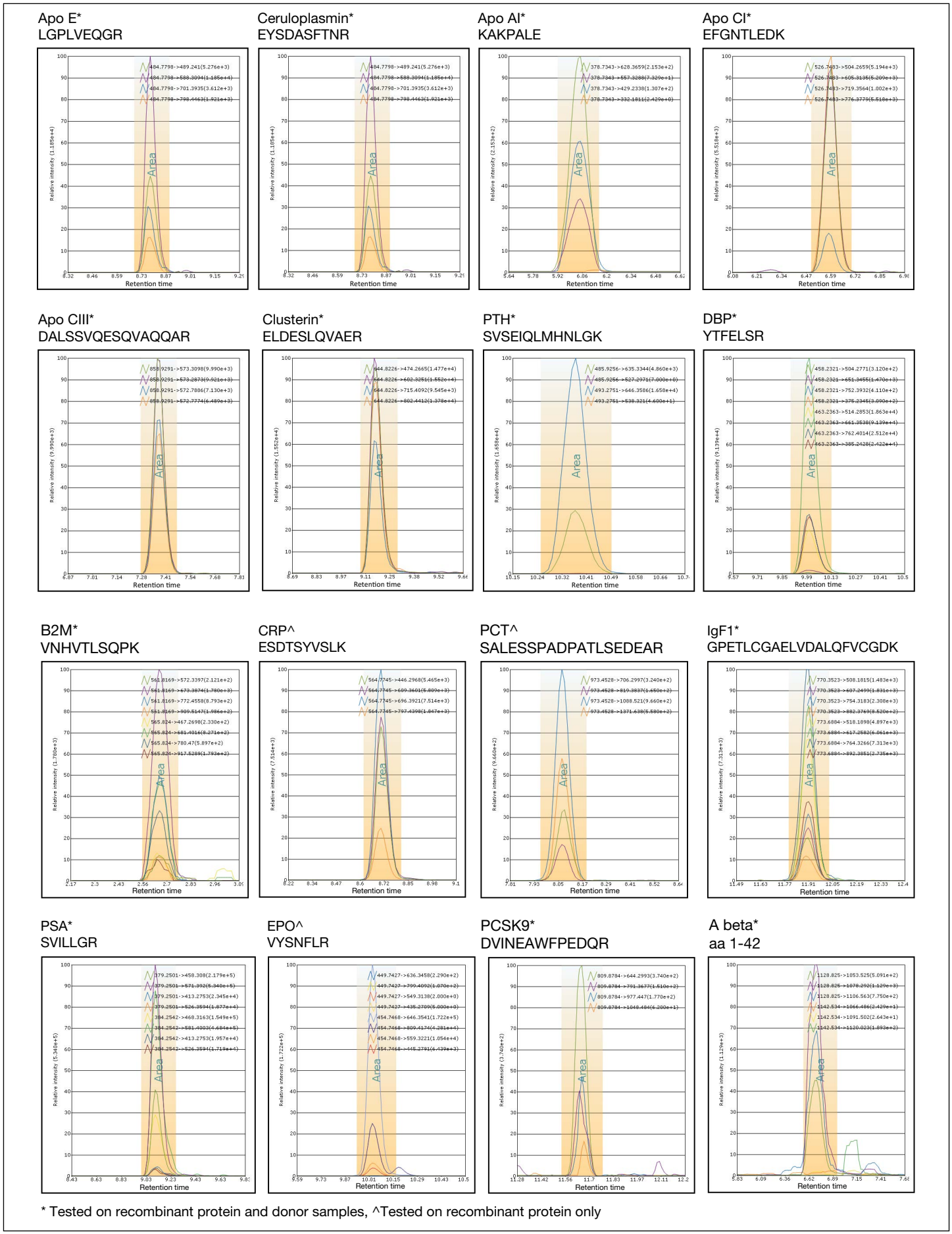


Figure 4. Extracted ion chromatograms of MSIA-SRM analyses of donor samples and recombinant proteins.

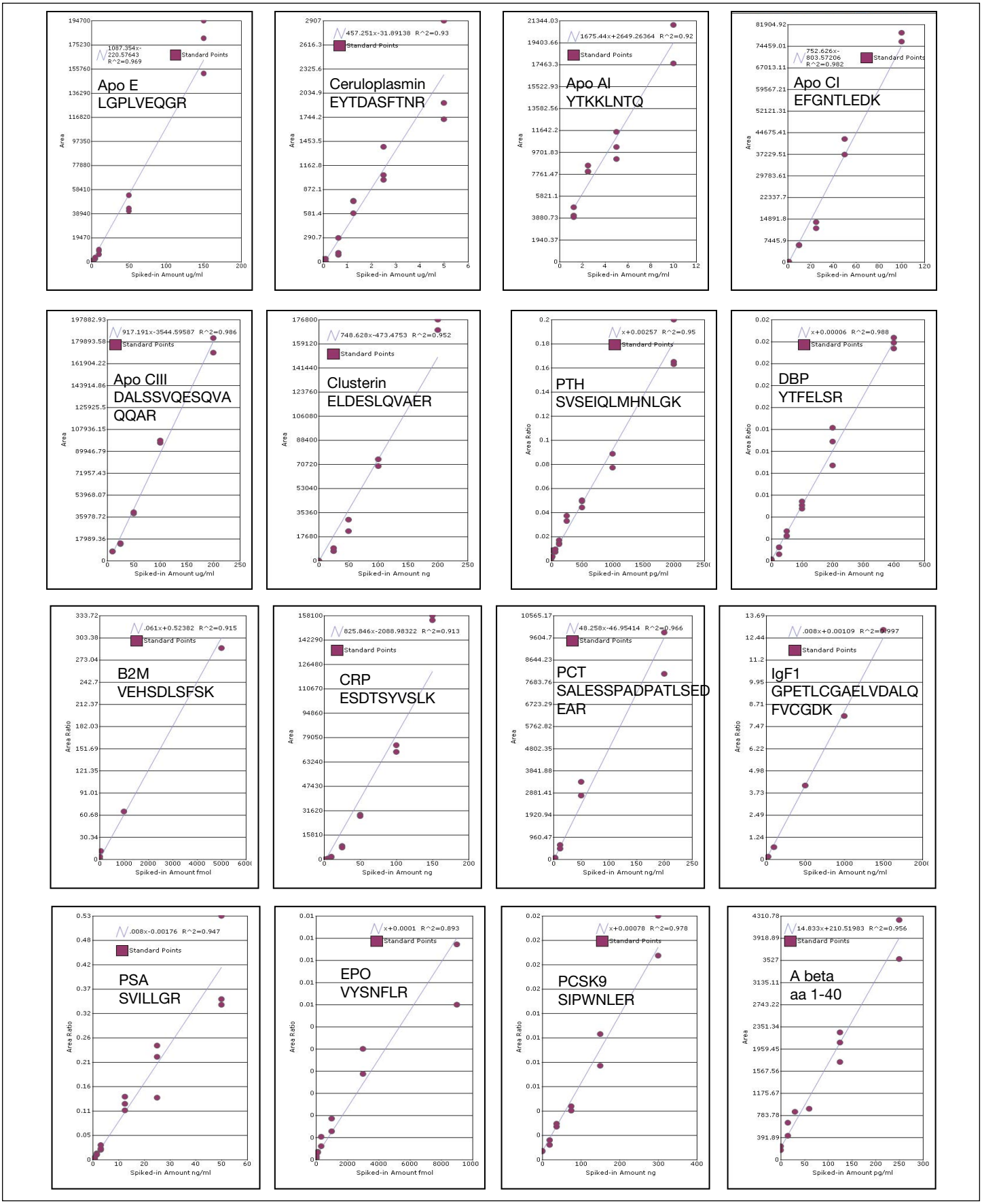


Figure 5. Calibration curves for targeted peptides. Linear regression curves were created using recombinant proteins subjected to the complete MSIA workflow. Because calibration data were extracted from several Pinpoint software workbooks covering experiments performed at different laboratories, the calibration curves are based either on area or area ratio, and either a spiked-in concentration or absolute amount of heavy-isotope-labeled peptide. R^2 values ranged from 0.89 to 0.98 and %CVs of full triplicates ranged from 0 to 13%.

Lower limit of quantification (LLOQ) values are given in Table 2 of the complementary research paper.[22] Detection limits and dynamic ranges for all targeted peptides were well within the useful research range for the selected analyses. The LLOQ values were close to or at the lower end of the calibration curve's linear range.

Analysis of Methods

The MSIA-SRM methods for the sixteen analytes important to clinical research were high-throughput, sensitive, and highly selective. The sixteen proteins represented a broad range of analytes that included members of the Apolipoprotein family (ApoE, ApoA1, ApoCI, ApoCIII, and ApoJ [clusterin]), some medium- to high-abundance proteins (ceruloplasmin, vitamin D binding protein, beta-2 microglobulin and C-reactive protein), and many other important low-abundance proteins (procalcitonin, parathyroid hormone, insulin-like growth factor 1, prostate-specific antigen, erythropoietin, proprotein convertase subtilisin/kexin type 9, and amyloid beta). SRM method development was rapid (on the order of hours), automated, and resulted in a method that can provide precise quantification within the required useful range for each analyte.

Analytical Specificity of MS

Because MS provides sequence information, the MSIA-SRM methods were highly selective. Traditional immunoassays may include intact and truncated protein species in quantitation results due to a lack of analytical specificity in the capture or detection antibodies (or both). This presents a problem for analytes where truncated species or other variants are relevant, such as for PTH.[23,24] For example, the PTH active site is aa 1–10 and the intact protein is rapidly cleaved *in vivo*. Therefore measurement of the N-terminal aa 1–13 tryptic peptide using SRM can provide a more accurate estimation of active protein. Although the primary antibody for the traditional immunoassay is directed at aa 1–34, it may not be absolutely analytically specific for only that protein because it is polyclonal. It may capture fragments from proteins that are missing the active site at the N-terminus, resulting in an overestimation of active protein. For clinical research purposes, detection with MS allows absolute quantification of different isoforms excluding any fragments that do not contain the active site at the N-terminal. Any analyte that exhibits fragmentation or cleavage *in vivo* would be subject to similar caveats when measured with traditional immunoassays.

Anti-peptide Versus Anti-protein Antibodies

A discussion of affinity purification coupled to MS detection should also include a discussion of anti-peptide versus anti-protein antibodies. Although there are specific cases where anti-peptide antibodies have application, for example with proteins where good antibodies are not available or where autoantibodies are present and already bound to the target analyte, affinity capture at the protein level provides significant advantages. Anti-protein antibodies provide global enrichment of multiple protein isoforms, simplifying distinguishing active from inactive forms. In addition, it is not necessary to develop highly analytically specific antibodies because capturing the collection of the protein isoforms is preferred. Using an anti-protein antibody raised toward an epitope common to all isoforms or variants is more economical because only one is needed to measure multiple forms.

If anti-peptide antibodies are used, they must be highly analytically specific such that a unique one is needed for each target peptide. For example, the PTH analysis described in this study would require seven unique anti-peptide antibodies. Not only is this more expensive, it does not allow addition of new isoforms to the method without delays to develop new anti-peptide antibodies. New isoforms can be added to an anti-protein based clinical research method in a matter of hours.

Anti-protein antibody capture provides significant advantages when trypsin digestion is used. Digestion in solution is highly dependent on the sample protein-to-trypsin ratio. Therefore, complex samples such as raw plasma or serum require relatively large amounts of trypsin and long digestion times (12–24 hours).[12] This requirement is further complicated by differences in the samples themselves. Samples from different states of health are expected to vary, the effects of which have never been studied to the degree necessary to demonstrate that digestion variability due to disease state does not exist. For these reasons, the use of anti-peptide antibodies that require digestion of the crude sample prior to capture, is more expensive, time-consuming and a potential source of error. Conversely, samples enriched at the protein level are digested after capture and are thus far less complex. Trypsin digestion of the enriched samples is typically fast in two to four hours, economical, and highly reproducible.[25-27]

Quantification of non-unique peptides when using anti-peptide antibodies can also be problematic. If the surrogate peptides are not carefully chosen or unique, digestion of a complex mixture followed by anti-peptide capture and quantification will result in incorrect measurements because one peptide sequence may be present in multiple proteins. This problem is not encountered when enrichment is done at the protein level.

Why Affinity Purification

Why is affinity purification necessary for medium or high abundance analytes? Enrichment of higher-abundance analytes serves several functions. As described above, digestion of complex mixtures is expensive and time-consuming. Although protein targets may be present in high abundance, variants important to clinical research are likely to be less so. Apo AI is one of the more abundant proteins in plasma and the major component of high-density lipoprotein (HDL). The ability of Apo AI to bind lipid effectively is highly dependent on the C terminal.[28] Truncated Apo AI differs from full length Apo AI by a single amino acid at the C-terminus (http://www.uniprot.org/uniprot/P02647). As demonstrated in this study, affinity purification and detection of Apo AI using the MSIA-SRM approach permits quantification of both isoforms, as well as the other C and N-terminally truncated variants that may be present in much lower amounts.

Injecting simple mixtures instead of raw plasma or serum digests into the LC-MS system reduces LC column and MS source fouling and thus maintenance frequency—a practical advantage. This also adds robustness and consistency to routine research measurements.

The most compelling reason to adopt MS in the clinical research lab is its ability to measure panels of several analytes at the same time. With current multiplexed ELISA and other immunoassay technologies, sensitivity is typically compromised as the number of analytes measured concurrently is increased. The MSIA-SRM approach allows multiplexing of analytes by serial extraction of the same sample using different MSIA D.A.R.T.'S. Serially extracted fractions are measured in the same SRM run. Thus the MSIA-SRM approach is fast, efficient, conservative of sample volume and avoids the need to accommodate several antibodies that have different binding and elution condition optima.

Conclusion

A practical, scalable method for rapid development of MS-based SRM methods for sixteen proteins of importance to clinical research in seven different disease groups was demonstrated. The MSIA workflow coupled high-throughput affinity purification with SRM analyses. Pinpoint software facilitated SRM method development and targeted protein quantification.

Extracted ion chromatograms of MSIA-SRM analysis of real biological (donor) samples were free of significant interferences. Calibration curves created using recombinant proteins demonstrated that the MSIA workflow could be used to detect and quantify target proteins at pg to mg/mL, within established useful ranges. The workflow allows analytically specific quantification of individual protein isoforms and thus addressed the challenges of protein heterogeneity found in clinical research applications.

For research use only. Not for use in diagnostic procedures.

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