Application Note: 443

Quantitative Proteomic Workflow for Discovery of Early Rejection Kidney Transplant Peptide Biomarkers and Subsequent Development of SRM Assays in Urine

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Key Words

- LTQ Orbitrap XL
- SIEVE Software
- SRM Analysis
- TSQ Quantum Ultra
- Endogenous Peptides

Introduction

The accurate diagnosis of renal allograft rejection currently depends on a biopsy. Transplant medicine would benefit greatly from the availability of non-invasive tests for early detection of rejection as well as immunosuppressive drug therapeutic monitoring. Only a limited number of studies have been published to date on specific proteins associated with allograft rejection. Typically, renal dysfunction due to humoral transplant rejection or other pathologies results in the increase of protein excreted in urine.[1-5] In blood, endogenous peptides (not generated by trypsin digestion *ex vivo*) are likely candidate biomarkers for many diseases and pathologies as they are secreted from tissues and enter the bloodstream.[6-7] By a similar mechanism, the analysis of endogenous protein and peptide fragments in urine may also provide a non-invasive, early indication of kidney transplant rejection or disease.

Endogenous peptide recovery from body fluids poses numerous hurdles. First, proper collection and storage must minimize the generation of artifactual peptides that may be generated *ex vivo*. Second, the dynamic range of molecular size and abundance requires separation of proteins from peptides and metabolites.

Recently, there has been an increased focus in the clinical community on the development of targeted SRM-based assays as an alternative to traditional but less specific ELISA. Mass spectrometry-based assays offer a number of alternatives over antibody-based assays including higher specificity, robust quantification based on heavy labeled standards and the ability to monitor a panel of diagnostic markers in a multiplexed assay format. Unfortunately, designing effective targeted assays still remains a challenge, and may require large amounts of samples and multiple iterations to empirically determine the optimal proteotypic peptides and transitions. Fortunately, in many cases, SRM experiments are preceded by discovery-based experiments to develop a list of target proteins and peptide biomarkers. We have found that high resolution accurate mass MS/MS spectra generated in discovery experiments can be directly used to increase the efficiency and accuracy of SRM assay development by guiding the selection of optimal transitions (Figure 1).

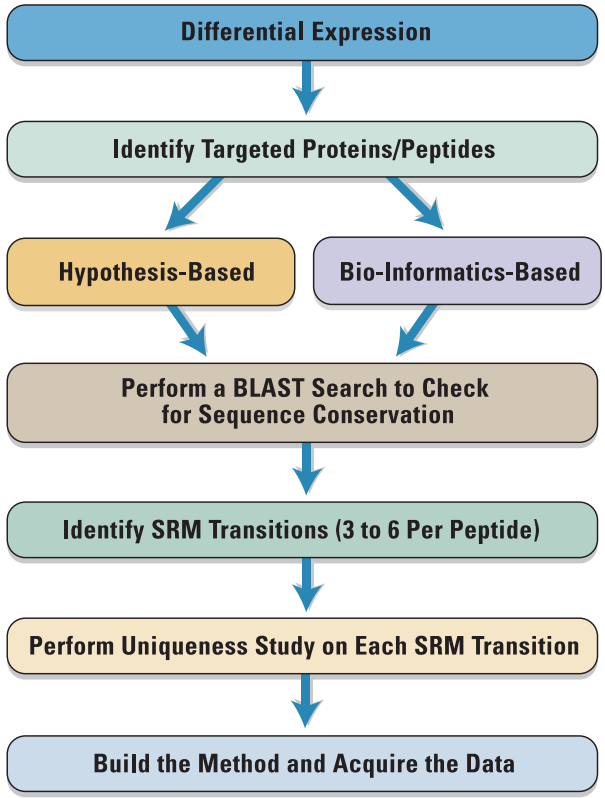

Figure 1: Workflow for integrating differential expression analysis with targeted protein quantitation

Goal

In this study, we describe the development of a workflow utilizing off-line size fractionation coupled with on-line liquid chromatography and high resolution tandem MS on an LTQ Orbitrap XL™ hybrid mass spectrometer specifically geared toward the identification of differentially expressed proteins and endogenous peptides in urine. Selected protein or peptide biomarkers identified in the high-resolution discovery workflow were subsequently quantified in targeted selective reaction monitoring (SRM) assays on a TSQ Quantum Ultra™ triple quadrupole mass spectrometer. The described quantitative workflows were used to analyze urine samples from normal, early rejection and acute humoral rejection transplant patients.

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Experimental Conditions/Methods

Sample Preparation

Urine samples were collected from study participants with full consent and approval under IRB protocols. The samples were transported frozen and thawed before sample processing. Urine samples were concentrated using 2 mL single well devices (Vivaspin 2 3 kDa MWCO, Vivascience, MA). Concentrated urine samples were diluted 4:1 with 8 M guanidine HCl, 10 mM dithiothreitol (DTT), 150 mM tris HCl pH 8.5 (adjusted @ 20 °C) and mixed before incubation at 37 °C for 1h. The denatured samples were then alkylated with 45 mM iodoacetic acid (500 mM stock concentration in 1 M ammonium bicarbonate) in the dark for 1 h at room temperature. The residual alkylation agent was then reacted with 15 mM DTT followed by adding trifluoroacetic acid (TFA) to a final concentration of 2% (v/v) to acidify the sample.

Sample Desalting Employing C18 Reverse Phase Resin

HyperSep-96™ C18 solid phase extraction media (Thermo Fisher Scientific) was used to desalt the denatured, reduced and alkylated samples prepared in the above step. A 25 mg packing volume SPE device (P/N 60300-422) was adapted for single use by centrifugation (500 g for 3 – 5 min) or in a 96 well format using a plate adaptor (P/N 60300-302) and a HyperSep universal vacuum manifold (P/N 60104-230). In both cases the centrifugation and vacuum conditions were adjusted to achieve a processing time of 0.5 – 1 min. residence time in the media bed. Long centrifugation times/high speeds or prolonged vacuum processing should be avoided to ensure that the reverse phase resin does not dry out, requiring re-wetting with organic solvent. The HyperSep C18 resin was first conditioned before use; a) Fill the SPE device with n-propanol and pass though C18 media slowly (1–2 min). Repeat 2x. b) Follow with 0.25% (v/v in water) TFA to rinse the whole SPE device. Repeat 2x. Load 100 – 125 µL of sample (equivalent to 20 – 25 µL of original serum sample) prepared as described above and process by centrifugation or vacuum to flow through the SPE media for a residence time of 0.5 – 1 min. Prepare a wash buffer of 8M guanidine HCl, 10 mM dithiothreitol (DTT), 150mM tris HCl pH 8.5 (adjusted @ 20 °C) diluted 4:1 with TFA to a final concentration of 0.25% (v/v). Fill the SPE device and pass through the resin bed as described above. Note: this step removes the non-adsorbed sample in the interstitial fluid space within the SPE packed bed. Wash 3x with 0.25% (v/v) TFA as described above.

Elute the desalted sample with 500 µL of 50% (v/v) n-propanol in 0.1% (v/v) formic acid by centrifugation (single devices) or vacuum filtration.

Sample Clean-up Using Ultrafiltration Membranes

Sample clean up by ultrafiltration was carried out in single well devices (Microcon 50 kDA MWCO membrane, Millipore Corporation, Billerica, MA) using a microcentrifuge. Multiple single well UF devices can also be loaded into a carrier plate (Millipore Corporation) and processed by vacuum. Multiwell plates fitted with a 30 kDA MWCO membrane (Vivascience MA, P/N CMR 07211-1, Sartorius Stedim Biotech SA) were also used with larger numbers of samples on a vacuum manifold. Before use all UF membranes and devices were washed with 50% (v/v) n-propanol, 0.1% (v/v) Formic acid.

LTQ Orbitrap XL Mass Spectrometry Analysis

Samples in 5% (v/v) Acetonitrile 0.1% (v/v) Formic acid were injected with a Thermo Scientific Micro Autosampler onto a 75 µm x 25 cm fused silica capillary column packed with Hypersil GOLD™ aQ™ 5 µm media, a Surveyor MS pump running with a flow splitter (1:1000), in a 250 µL/min gradient of 5%(v/v) Acetonitrile, 0.1% (v/v) Formic acid to 30% (v/v) Acetonitrile, 0.1% (v/v) Formic acid over the course of 180 minutes with a total run length of 240 minutes. The LTQ Orbitrap XL was run with a 60 k resolution full scan with 1e6 target values, and 1000 ms inject times with up to five precursor ions selected for MS/MS. HCD MS/MS at 15 K resolution were acquired with data dependent 8e4 trigger thresholds, 2e5 target values, 2000 ms injection times. A normalized collision energy of 45 was used. CID MS/MS were acquired with 3e4 target values, 1e4 data dependent trigger thresholds. In both experiments, monoisotopic precursor selection is enabled, with +1 and unassigned charge states rejected. Label-free differential analysis was performed using the SIEVE algorithm.

TSQ Quantum Ultra and Development of SRM Assay Methodology

The Quantum Ultra Mass spectrometer was equipped with a Thermo Scientific Surveyor™ MS pump, and Micro Autosampler (flow split pre-Autosampler as used in the discovery instrument format). The source was the IonMax Source equipped with a low flow metal needle, with a 1 mm x 50 mm Hypersil GOLD AQ column (part number 25302-051030). Solvent A was Fisher Optima LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was Fisher Optima LC-MS grade 30% (v/v) Acetonitrile with 0.2% (v/v) formic acid. The peptides found to be differentially present in the discovery data were imported directly into SRM Workflow software. Transitions were based on predominant fragments found in the discovery data (> 5 transitions per peptide), overlapped with the predominant ion fragments SRM builder. Alignment and relative quantification of the transitions were performed with SRM builder.

Results and Discussion

Analysis of High Resolution MS/MS Data by SIEVE

Although lower resolution trap based CID scans can be triggered on lower abundance peptides, when searching a database "no enzyme", the large number of potential misidentifications, even with high parent mass accuracy, makes for a difficult high confidence sequence assignment. This, coupled with high charges and uneven charge distributions (unlike the case when peptides digested with trypsin), makes for a difficult assignment and an even more difficult prediction of a useful SRM transition. Using the HCD cell to perform the fragmentation of these difficult to analyze peptides, yields an accurate mass product ion with an easily assignable charge state.

Typical re-constructed ion chromatograms from non-differentially expressed (Figure 2a) and highly over-expressed (Figure 2b) putative protein markers are shown in Figure 2. A summary of the top 31 peptides/proteins increased in stable vs. pre-rejection (putative early markers) and stable vs. rejecting (putative late markers) are shown in Table 1. The majority of frames, *i.e.*, peptides were up-regulated in the disease state. Few peptides were down-regulated or equivalent (ratio close to 1). This result is consistent with the hypothesis that endogenous peptide fragments represent a "metabolic snapshot" of proteolytic activities induced by disease or inflammation. Presumably, proteolytic fragments are excreted into the urine and may be a rich source of disease biomarkers. In this case, only an increase and not a decrease in endogenous peptides would be expected in disease samples.

Gene Name	Protein Name	Presence: Early or Early and Late	Fold Change Early	P-value Early	Number of Peptides Detected	Fold Change Late	P-value Late	Number of Peptides Detected, Late
TAF1C	TATA box binding protein (TBP)-associated factor, RNA polymerase	E	3032.132	0.041	1			
EIF4G2	eukaryotic translation initiation factor factor 4 gamma	E	343.174	0.072	1			
MYH14	myosin, heavy chain 14	E	343.174	0.072	1			
ADAM20	ADAM metallopeptidase domain 20	E	59.244	0.339	1			
PTGDS	prostaglandin D2 synthase 21kDa (brain)	E	31.801	0.505	1			
ERP29	endoplasmic reticulum protein 29	E	27.143	0.407	1			
MTUS1	mitochondrial tumor suppressor 1	E	27.143	0.407	1			
SIGLEC5	sialic acid binding Ig-like lectin 5	E	27.143	0.407	1			
STIL	SCL/TAL1 interrupting locus	E	10.919	0.051	1			
PPM1G	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	E	9.256	0.485	1			
MBD4	methyl-CpG binding domain protein 4	E	7.206	0.327	1			
TIAL1	TIA1 cytotoxic granule-associated RNA binding protein-like	E	5.151	0.333	1			
AZGP1	alpha-2-glycoprotein 1, zinc-binding	E	4.835	0.638	2			
IBTK	inhibitor of Bruton agammaglobulinemia tyrosine kinase	E	4.759	0.042	1			
TF	transferrin	E	4.356	0.523	6			
OTOR	otoraplin	E	2.936	0.115	1			
ASPN	asporin	E	2.538	0.428	1			
CCT7	chaperonin containing TCP1, subunit 7 (eta)	E	2.08	0.526	1			
PTPRS	protein tyrosine phosphatase, receptor type, S	E	1.74	0.522	1			
PCTK3	PCTAIRE protein kinase 3	E	1.738	0.888	1			
TP53RK	TP53 regulating kinase	EL	3032.132	0.041	1	66.092	0.334	1
UMOD	uromodulin (uromucoid, Tamm-Horsfall glycoprotein)	EL	760.173	0.142	4	42.433	0.324	4
ALB	albumin	EL	462.544	0.434	28	7.228	0.532	7
TTR	transthyretin (prealbumin, amyloidosis type I)	EL	133.893	0.199	1	1043.262	0.089	7
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin)	EL	51.572	0.451	17	1940.058	0.068	24
LMAN2	lectin, mannose-binding 2	EL	25.029	0.398	1	81.286	0.62	1
DNAH5	dynein, axonemal, heavy chain 5	EL	11.505	0.041	1	2.45	0.417	1
WWTR1	WW domain containing transcription regulator	EL	6.063	0.612	1	36.653	0.319	1
INCENP	inner centromere protein antigens 135/155kDa	EL	2.538	0.428	1	2.134	0.494	1
B2M	beta-2-microglobulin	EL	1.439	0.407	3	10.869	0.533	4

Table 1: Summary of label-free differential analysis results.

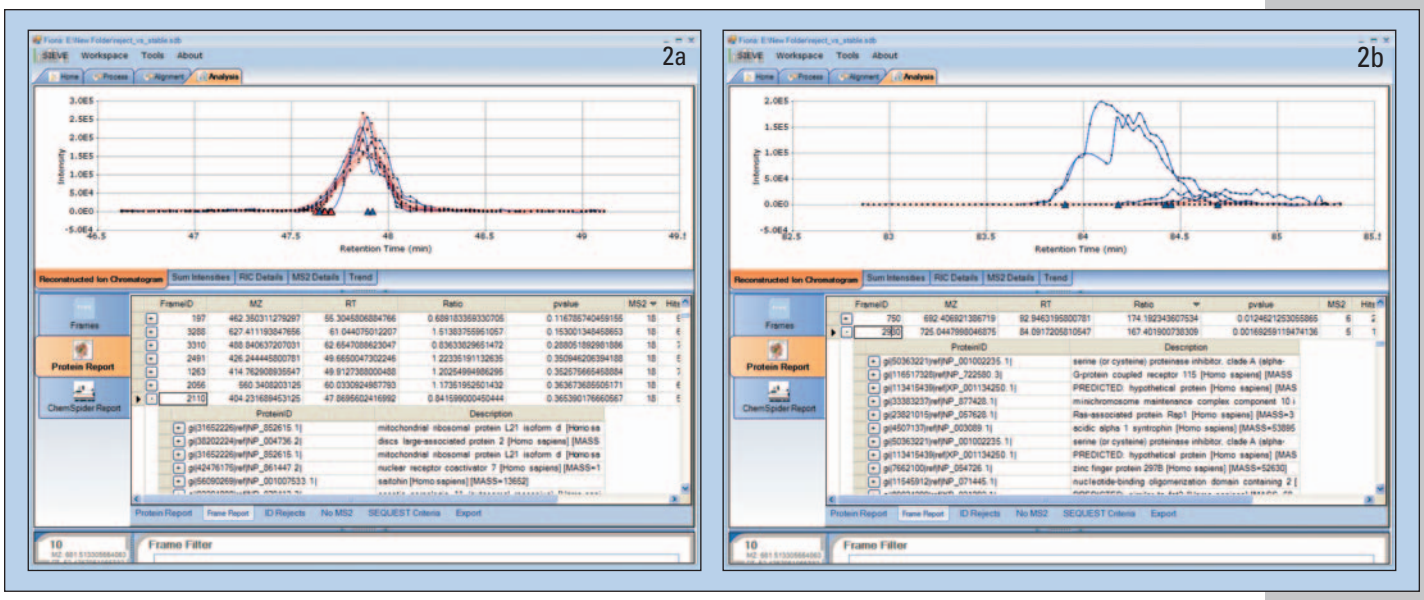

Figure 2: Re-constituted Ion Chromatograph data analysis of high resolution MS/MS data by SIEVE. 2a) Non-differentially expressed putative biomarker (ratio 0.8, mitochondrial ribosomal protein) and 2b) Increased expression (ratio 167.4) of putative biomarker.

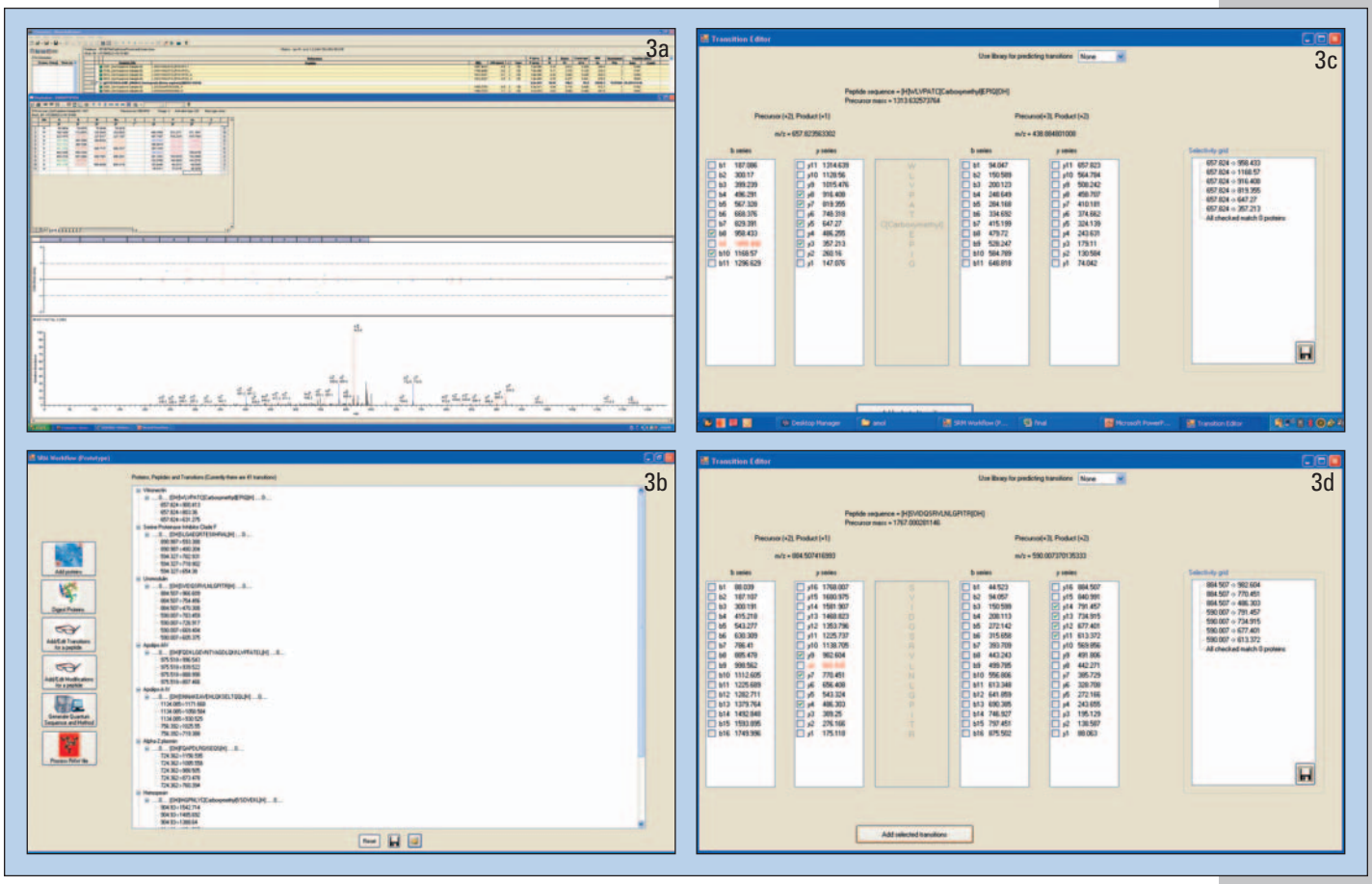

Figure 3: Discovery driven design of SRM assay method. 3a) High resolution MS/MS spectra of target proteins/peptides; 3b) Building a method for the TSQ-Quantum Ultra with SRM Builder software; 3c) Transitions for a vitronectin peptide; and 4d) Transitions for a uromodulin peptide.

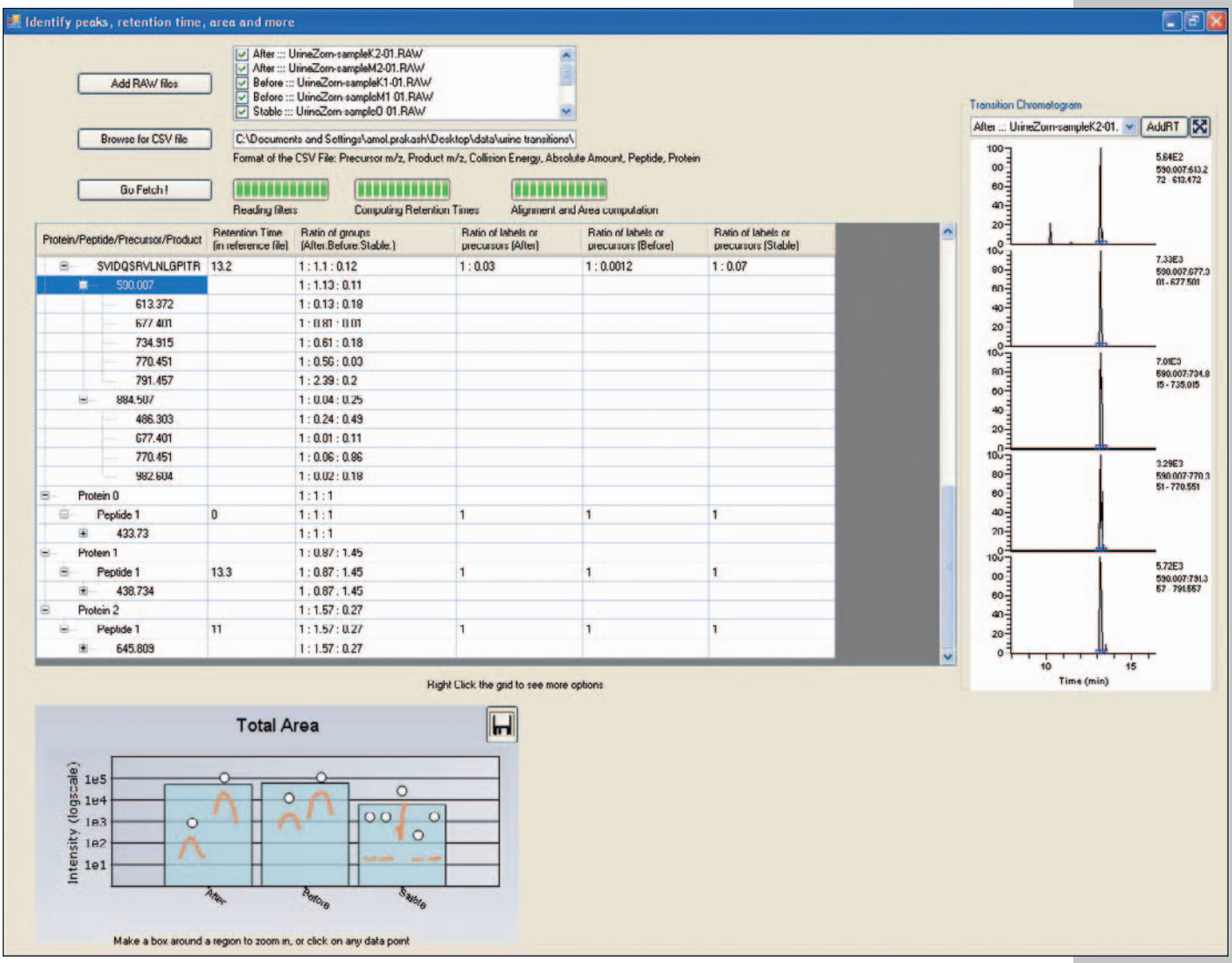

Figure 4: SRM assay results for uromodulin peptide transitions in pre-rejection and rejecting clinical samples.

Development of a Discovery Driven Single Reaction Monitoring (SRM) Assay on a TSQ Quantum Ultra Triple Quadrupole Mass Spectrometer

High resolution MS/MS spectra of target proteins/peptides illustrated in Figure 3a were analyzed to determine the most sensitive or intense transitions. The selected peptides and transitions were used to build an SRM method for TSQ Quantum Ultra with SRM Workflow software (Figure 3b). Individual transitions for a vitronectin peptide (Figure 3c) and a uromodulin peptide (Figure 3d) are illustrated. Preliminary results are summarized in Figure 4 for the putative biomarkers vitronectin and uromodulin in pre-rejection and rejecting patient samples. The 5 transitions for the uromodulin peptide, charge state +3, 590.007 (but not charge state +2, 884.507) were observed in the pre-rejection and rejecting patient samples but not in the stable patient samples. This demonstrates the presence of a specific endogenous peptide in a patient sample under early and full rejection conditions. The absence of the same endogenous peptide in the stable patient samples suggests it may be a useful marker for early rejection, although the sample set is very small and much further verification is, of course, needed. The confidence level for this result is high because all 5 transitions for the peptide were observed. The probability of this result due to random chance is very low.

Conclusion

- A robust workflow was created for the recovery of intact endogenous peptides (not generated by proteolytic cleavage *ex vivo*) from urine.
- Quantitative, label-free differential analysis was applied to high-resolution MS spectra to drive the selection of putative peptide markers for early transplant rejection.
- The high-resolution accurate mass MS/MS fragmentation data facilitated design of a targeted SRM assay, by yielding highly accurate fragment assignments on difficult to analyze endogenous peptides.
- A targeted SRM assay for one of the peptides in the panel was tested on the patient sample set and quantitative data were obtained.
- The described workflows can serve as a model to link biomarker discovery and validation.

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