

Early markers of kidney transplant rejection: Quantitative proteomic workflows for discovery and the development of non-invasive, targeted assays

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Abstract and 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). In blood, endogenous peptides (not generated by the kidney) are likely candidate biomarkers for many diseases and pathologies as they are secreted from tissues and enter the bloodstream (2,3). 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 artefactual peptides that may be generated *in 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, targeting 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 MS2 spectra generated during the discovery process can be directly used to increase the efficiency and accuracy of SRM assay development by guiding the selection of optimal transitions (Figure 1).

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. A panel of the protein or peptide biomarkers identified in the high-resolution discovery workflow were subsequently quantified in targeted selection reaction monitoring (SRM) assays on a TSO 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.

Materials & Methods

Urine samples were collected from study participants with full consent and approval under IRB protocols. Details of experimental design and sample preparation are given in Fig 2, 3. Prepared samples were run on an LTQ Orbitrap XL instrument (Thermo Scientific, Bremen, Germany) with CID and HCD as the fragmentation modes.

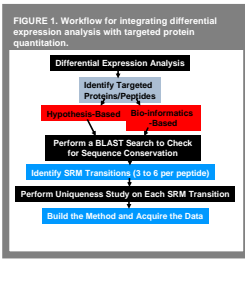


FIGURE 2. Schematic of the overall workflow for recovery and analysis of endogenous peptides from urine. Urine samples are collected and processed to eliminate small molecule metabolites and intact proteins before analysis by high resolution LC-MS/MS. A panel of differentially expressed proteins/peptides are selected for targeted quantification. High resolution MS2 fragmentation data from the targeted peptides are used to create an optimized SRM method.

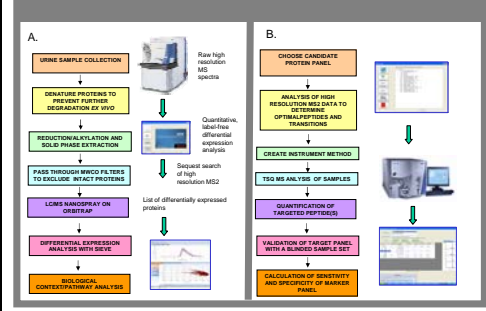


TABLE 1. Summary of label-free differential analysis results. The top 31 peptides/proteins increased in stable vs pre-rejection (putative early markers) and stable vs rejecting (putative late markers) are shown in Table 1. Several of these proteins (in yellow) have been previously identified as associated with transplant rejection or implantation failure (table 2).

Gene	Protein Name	Protein ID	Protein	Fold Change Early/Late	Fold Change Early/Late	Number of peptides detected, Late	Fold Change Early/Late	p-value	Number of peptides detected, Late
PTPRC	CD140a (transmembrane protein TSH) (inositol 1,4,5-bisphosphate 3-kinase)	Q99753	143,174	0.072	1				
EPHA2	ephrin-A2; transmembrane tyrosine kinase 2	Q99753	143,174	0.072	1				
MTOR	serine/threonine kinase 1	Q99753	143,174	0.072	1				
ADAM10	ADAM10 metalloprotease domain 20	Q99753	158,244	0.339	1				
PRKRI	protein kinase R, isozyme 1 (RimK) (RimK)	Q99753	191,801	0.459	1				
EPOR	erythropoietin receptor 2	Q99753	217,163	0.457	1				
MTUS1	mitochondrial tumor suppressor 1	Q99753	227,143	0.497	1				
MTUS2	mitochondrial tumor suppressor 2	Q99753	227,143	0.497	1				
SLIT1	SLIT1, 1 (semaphorin 3)	Q99753	10,939	0.051	1				
PTPST1	protein tyrosine phosphatase ST1 (Shc-PTP)	Q99753	4,226	0.456	1				
MRPS4	mitochondrial ribosomal protein 4	Q99753	7,206	0.327	1				
PTPST2	protein tyrosine phosphatase ST2 (Shc-PTP)	Q99753	4,133	0.433	1				
ANUP1	ankyrin 2; cytoplasmic 1 (zpc-binding)	Q99753	4,835	0.618	2				
PTPST3	protein tyrosine phosphatase ST3 (Shc-PTP)	Q99753	4,226	0.456	1				
TFE	transferrin	Q99753	4,356	0.523	6				
PTPST4	protein tyrosine phosphatase ST4 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST5	protein tyrosine phosphatase ST5 (Shc-PTP)	Q99753	4,226	0.456	1				
CTCF	chromatin insulator 1 (CTCF, subunit 2) (ctcf)	Q99753	2,098	0.526	1				
PTPST6	protein tyrosine phosphatase ST6 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST7	protein tyrosine phosphatase ST7 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST8	protein tyrosine phosphatase ST8 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST9	protein tyrosine phosphatase ST9 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST10	protein tyrosine phosphatase ST10 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST11	protein tyrosine phosphatase ST11 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST12	protein tyrosine phosphatase ST12 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST13	protein tyrosine phosphatase ST13 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST14	protein tyrosine phosphatase ST14 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST15	protein tyrosine phosphatase ST15 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST16	protein tyrosine phosphatase ST16 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST17	protein tyrosine phosphatase ST17 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST18	protein tyrosine phosphatase ST18 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST19	protein tyrosine phosphatase ST19 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST20	protein tyrosine phosphatase ST20 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST21	protein tyrosine phosphatase ST21 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST22	protein tyrosine phosphatase ST22 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST23	protein tyrosine phosphatase ST23 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST24	protein tyrosine phosphatase ST24 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST25	protein tyrosine phosphatase ST25 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST26	protein tyrosine phosphatase ST26 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST27	protein tyrosine phosphatase ST27 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST28	protein tyrosine phosphatase ST28 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST29	protein tyrosine phosphatase ST29 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST30	protein tyrosine phosphatase ST30 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST31	protein tyrosine phosphatase ST31 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST32	protein tyrosine phosphatase ST32 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST33	protein tyrosine phosphatase ST33 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST34	protein tyrosine phosphatase ST34 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST35	protein tyrosine phosphatase ST35 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST36	protein tyrosine phosphatase ST36 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST37	protein tyrosine phosphatase ST37 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST38	protein tyrosine phosphatase ST38 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST39	protein tyrosine phosphatase ST39 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST40	protein tyrosine phosphatase ST40 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST41	protein tyrosine phosphatase ST41 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST42	protein tyrosine phosphatase ST42 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST43	protein tyrosine phosphatase ST43 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST44	protein tyrosine phosphatase ST44 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST45	protein tyrosine phosphatase ST45 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST46	protein tyrosine phosphatase ST46 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST47	protein tyrosine phosphatase ST47 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST48	protein tyrosine phosphatase ST48 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST49	protein tyrosine phosphatase ST49 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST50	protein tyrosine phosphatase ST50 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST51	protein tyrosine phosphatase ST51 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST52	protein tyrosine phosphatase ST52 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST53	protein tyrosine phosphatase ST53 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST54	protein tyrosine phosphatase ST54 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST55	protein tyrosine phosphatase ST55 (Shc-PTP)	Q99753	4,226	0.456	1				
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PTPST70	protein tyrosine phosphatase ST70 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST71	protein tyrosine phosphatase ST71 (Shc-PTP)	Q99753	4,226	0.456	1				
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PTPST90	protein tyrosine phosphatase ST90 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST91	protein tyrosine phosphatase ST91 (Shc-PTP)	Q99753	4,226	0.456	1				
PTPST92	protein tyrosine phosphatase ST92 (Shc-PTP)	Q99753	4,226	0.456	1			</	