Development of SRM methods for the detection and quantification of chromosome 16 proteins

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1. Summary

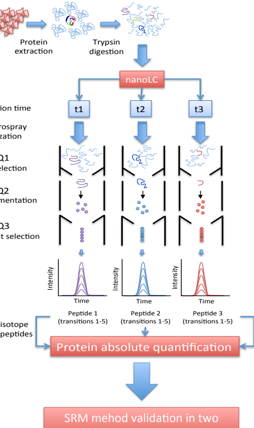
Detection and quantification of chromosome 16 proteins in biological matrices by SRM procedures is one of the major goals of the SpHPP chr16 Consortium. Two strategies have been undertaken to tackle chr16 proteome: one focused on proteins with experimental evidences and the second to the search for the missing proteins, which is based on the expression of recombinant proteins in a cell free translation system to gather MS information to define the SRM methods to be used in real biological samples. A group of chr16 known proteins were selected according to their score in GPMDB and their tryptic peptides were assayed in biological matrices to select the best transitions. Upon optimisation, methods were developed for 50 proteins allowing detection in total cell lysates of MCF7, Ramos and CCD18 cell lines. Cross validation was performed in three independent labs combining data from ABSciex 4000 and 5500 QTrap instruments. Among all peptides assayed, those observed in parallel shotgun proteomic analysis of the same cell lines were preferentially observed and confirmed with synthetic peptides, supporting the strategy of collecting shotgun MSMS data to facilitate the design of SRM methods. Moreover, an SRM method for detection and quantification cardiotrophin 1, an important hepatoprotective factor, in complete plasma samples has been developed. A database collecting all SRM data is under construction.

2. SRM analysis

On the basis of both the MS/MS spectra observed in the LC-MS analysis of the samples, predictions derived from the sequences, and data available from databases, several peptide precursor and fragment ion masses were selected per each protein and assayed for SRM analysis. Analyses were performed on AB Sciex 4000 and 5500 QTRAP instruments. After precolumn desalting, tryptic digests (1-2 µg) were separated on C18 nanocolumns (75 µm id, 15 cm, 3 µm particle size) (LC Packings, Netherlands; Eksigent, USA; Thermo Scientific, USA) at a flow rate of 300 nl/min, with a 90 min lineal gradient from 5 to 40% ACN in 0.1% formic acid. The mass spectrometer was interfaced with nanospray sources equipped with uncoated fused silica emitter tips (20 µm inner diameter, 10 µm tip, NewObjective, Woburn, MA) and was operated in the positive ion mode. MS Source parameters were as follows: capillary voltage 2800 V, source temperature 150 ºC, declustering potential (DP) 135 V, curtain and ion source gas (Nitrogen) 20 psi, and collision gas (Nitrogen) medium. The dwell time for each transition was 20 ms. Collision energies for each peptide were automatically computed using the embedded rolling collision energy equations of the MRM Pilot software.

In order to confirm the identity of the peptides a MRM-initiated Detection and Sequencing (MIDAS) experiment was performed for each peptide. The mass spectrometer was instructed to switch from MRM to enhanced product ion (EPI) scanning mode when an individual MRM signal exceeded 500 counts. Each precursor was fragmented a maximum of twice before being excluded for 30 s. Data were analyzed by submitting the MS/MS data to the ProteinPilot software (ABSciex, version 4.0). All searches were performed against the HPP chromosome 16 database.

SRM data analysis was done using the quantitation module of the Analyst 1.5.1 software (ABSciex), using the IntelliQuant algorithm for peak integration.



3. SRM Results

Protein Accession	Gene_id	loge	# Laboratories	# Peptides	Ramos	MCF7	CCD18	TC28	Huh7	Jurkat	HUAECS
A5YKK6	CNOT1	-1186,1	2	1	x					x	
B5ME19	EIF3CL	-339,5	3	4	x	x	x		x		
O14983	ATP2A1	-392,6	3	1	x	x	x			x	
O43809	NUDT21	-206,8	3	4	x	x	x	x			
O60884	DNAJA2	-173,1	3	1	x	x	x	x	x		
O75150	RNF40	-187,3	2	2	x		x			x	
P00505	GOT2	-564,2	3	3	x	x	x			x	x
P00738	HP	-1400,2	3	4	x					x	
P04075	ALDOA	-1076,2	3	3	x	x	x		x	x	x
P15170	GSPT1	-284,5	3	3	x	x	x		x	x	
P15559	NQO1	-384,3	3	3		x		x			x
P15880	RPS2	-357,3	3	4	x	x	x		x		
P22695	UQCRC2	-361,5	3	4	x	x	x		x	x	
P31146	CORO1A	-598,1	3	5	x	x	x	x			
P35637	FUS	-365	3	4	x	x	x	x			
P49411	TUFM	-411,3	3	9	x	x	x			x	x
P49588	AARS	-543	3	3	x		x		x	x	x
P63279	UBE2I	-210,9	3	1	x	x	x		x	x	
P69849	NOMO3	-369,4	3	2	x	x	x	x		x	x
P80404	ABAT	-230,7	2	1		x			x		
Q08AM6	VAC14	-187,1	3	4	x	x	x	x			
Q12789	GTF3C1	-1039,8	3	1	x				x		
Q12931	TRAP1	-392,6	3	1	x	x	x	x		x	
Q13509	TUBB3	-901,2	3	7	x	x	x	x		x	
Q14019	COTL1	-236,4	3	2	x				x	x	
Q14694	USP10	-177,7	3	5	x	x	x	x			
Q14807	KIF22	-387,7	2	1			x		x	x	
Q15393	SF3B3	-702	3	4	x	x	x		x		
Q16775	HAGH	-196,9	3	2		x	x	x			
Q49A26	GLYR1	-271,3	3	2	x		x		x	x	
Q53FZ2	ACSM3	-198,8	3	1	x	x					
Q68EM7	ARHGAP17	-1107,8	2	1	x		x		x		x
Q6P2E9	EDC4	-435,1	3	3			x	x	x	x	
Q86W42	THOC6	-179,2	3	4	x	x	x	x		x	
Q8TBB5	KLHDC4	-285,4	3	1	x	x	x				
Q92793	CREBBP	-408,5	2	1	x				x		
Q93009	USP7	-312,2	3	5	x	x	x	x		x	x
Q96DA0	ZG16B	-265	2	1	x	x	x				
Q96QK1	VPS35	-335,9	3	3		x	x		x	x	x
Q9NUI1	DECR2	-185	2	2		x		x	x		
Q9NUU7	DDX19A	-203,5	3	7	x	x	x	x			
Q9UMR2	DDX19B	-219,6	2	1		x			x		
Q9UQ35	SRRM2	-1962,2	3	3	x	x	x		x	x	x

With the aim of establishing workflows and SOPs within the consortium, a set of Chr 16 proteins were selected and distributed to six different laboratories for SRM assay development. The initial set of 120 Chr-16 proteins were selected on the basis of their GPMDB scores, log e on the range -175 to -6000, belonging to the group defined as "known" Chr-16 proteins. Each laboratory explored the detectability by SRM of the assigned proteins in digests from at least three cell lines used in the chr16 SpHPP consortium: MCF7, CCD18, and Ramos, which are expected to provide a large coverage of expressed Chr16 proteins. Additional cell lines or plasma samples were also assayed in some laboratories. Initial selection of proteotypic peptides and transitions to monitor for each protein was done either from database available data, results or *in silico* prediction from the sequence.

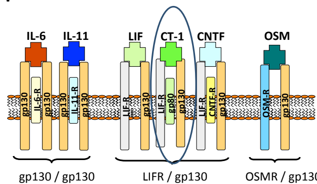
For 106 out of the 120 proteins selected (88.3%) a minimum of three co-eluting SRM transition signals were observed for a number of peptides ranging from 1-10 per protein, with an average of 4.16 peptides/protein. However, only those peptides for which an SRM-signal triggered MS2 spectra confirmed clearly the sequence were considered as bona-fide SRM assayable peptides. These amounted to 172 peptides from 51 proteins (42.5 % of the initially selected). It is worth mentioning that, as an additional validation, 47 peptides, belonging to 12 of the assayed proteins, were obtained by chemical synthesis and analyzed using the developed SRM assays. In all cases, the results confirmed the transitions selected, and the observed retention times closely matched those of the endogenous peptides.

After the initial round of SRM method development at the six laboratories, a second round of cross-validation was performed, assigning each of the final 51 proteins detected to two labs different from the one that initially developed the SRM method. After this second round of analysis validation, a total of 149 peptides from 49 proteins met the criteria of having been successfully detected, with at least three transitions per peptide, by at least two different laboratories. In most cases proteins were also detected in more than one different sample type.

Overall, we have been able to develop reliable SRM assays to monitor 49 chr16 proteins (40.8% of the initially selected, representing 5% of all chr16 proteins). All the relevant experimental conditions, peptide and transitions data from all laboratories has been gathered on a MySQL relational database constructed to keep track and query all the chr16 SRM information, and from which the curated data will be transferred to public repositories along the project.

One of our next goals is to build a library including all MIAPE compliant MS/MS spectra generated within the consortium as an attempt to increase the efficiency of the targeted experiments. To examine the suitability of this approach, we performed a simple test using Skyline software (MacCoss Lab. Department of Genome Sciences. University of Washington). All MS/MS spectra reported from one of the shotgun experiments (CNB_Jurkat_R1_HPLCRP) were used to create the library, and the SRM-detected proteins were added to map all peptides onto the available spectra. Matching was verified for all peptides pertaining to 81.3% of the SRM proteins further supporting the interest of using this approach to design targeted SRM methods.

4. Cardiotrophin 1 (CT1). A Chr16 protein involved in liver diseases?



- Cardiotrophin-1 (CT-1) is a member of the gp130 cytokine family.
- CT-1 is a natural defence against liver injury: apoptosis (Marques et al Hepatology 2007), Ischemia-reperfusion (Iñiguez et al JEM, 2006), lethal fulminant hepatitis (Tuñon, J. Virol. 2011).
- CT-1 plays a pivotal role in glucose and lipid metabolism (Moreno-Aliaga et al Cell metabolism 2011).
- CT-1 is located in 16p11.2 locus, which has been associated with obesity and cachexia (Walters et al, Nature 2010; Jacquemont et al Nature 2011).

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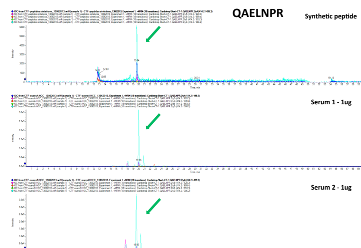
Three CT-1 peptides were initially selected to develop a quantitative assay and monitor potential differences of CT-1 levels in serum of patients with liver disorders.

The peptide QAELNPR has been positively detected in serum samples, with retention times and transitions resembling those measured for the synthetic standard



The support of ISCIII and MINECO is acknowledged

The linearity of the method was assessed with the standard peptide and spans 3 orders of magnitude from 2 to 2000 fmol.

The SRM method was used to compare circulating CT-1 levels between healthy individuals, and liver diseased patients suffering from liver cirrhosis and hepatocellular carcinoma.

Our preliminary analysis suggest a depletion of about 50% of CT-1 in the serum of patients and postulates this alteration as one of the factors contributing to the chronification and progression of liver injury.

Further analysis involving a larger number of patients as well as other less severe liver ailments are needed to extract reliable conclusions. However, in light of these observations and the reported hepatoprotective role of CT-1 it is tempting to speculate that CT-1 may emerge as a promising therapeutic candidate.