

# **Implementation of targeted proteomics methods for the characterization of peptides and proteins in liquid biopsies**

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## 1. ABSTRACT

Targeted proteomics methods emerged to detect and quantify well-defined sets of peptides with a high degree of specificity and sensitivity. Internal standard triggered-PRM (IS-PRM) was designed to increase the number of peptides monitored in one analysis without affecting the quality of the data obtained by using stable isotopically labeled (SIL) peptides as internal standards. We aimed to investigate the technical reproducibility of a type of IS-PRM method called SureQuant and to quantify the added SIL peptides and their corresponding endogenous forms.

Three aliquots of commercially available human serum samples were digested with trypsin and a set of 802 isotopically-labelled standard (SIL) peptides were spiked-in to later be analyzed by LC-MS using SureQuant acquisition method.

Instrument variability showed a coefficient of variation (CV) of 9.3%, whereas sample preparation variability increases up to 34.7% and 39.1%. The technique enabled to detect, on average, 772 SIL peptides and 738 endogenous forms.

Data obtained shows very good instrument reproducibility, whereas sample preparation procedure presents greater variability. Thus, this study proves the need for an improvement to achieve reproducibility and obtain reliable quantitative results.

## 2. INTRODUCTION

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) approaches are one of the most effective methods to study complex proteomes. On the one hand, discovery proteomics strategy is based on protein identification by sampling a randomly fraction of the proteome in each measurement (Fig. 1). In this way however, the sample is biased towards abundant peptides leading to a lack of reproducibility and quantitative limitations (1, 2). On the other hand, targeted proteomics methods emerged to detect and quantify well-defined sets of peptides with a high degree of specificity and sensitivity. Since targeted approaches are hypothesis-driven, only subset proteins of interest are under study (Fig. 1) (3). The increased use of targeted proteomics plays a key role in understanding the molecular mechanisms by studying post translational modifications, protein conformation and signalling pathways, among others. Also, its clinical applications, mainly include the verification of protein biomarkers in different diseases (4).

Although there is a wide variety of targeted proteomics methods, selected reaction monitoring (SRM) is one of the main techniques used. It is generally performed in a triple quadrupole (QqQ) mass spectrometer in which a predefined precursor ion of interest is selected in the first quadrupole (Q1), fragmented in the second quadrupole (Q2) and filtered in the third (Q3) to finally reach the detector (Fig. 2A) (5). SRM is considered to be a highly sensitive and specific technique, becoming a useful method to acquire precise quantification over a broad dynamic range (6, 7, 3). This allows the detection of low-abundance proteins in complex samples from any body fluids. One drawback is the low power resolution of both Q1 and Q3 mass analyzers, that can lead to interfering signals when analyzing the samples, obtaining biased results. Another disadvantage is that targeted peptides are limited in the experiment to guarantee the specificity of the measurements (3).

An efficient alternative that appeared with the implementation of fast scanning high resolution/accurate mass (HRAM) instruments is parallel reaction monitoring (PRM) (3). The principle of this targeted proteomics strategy is comparable to SRM, but it enables a simultaneous measurement of all product ions resulting from the fragmentation of a single targeted peptide, instead of recording a few transitions for a given precursor (3, 8). This is due to the fact that PRM experiments are typically performed on high-resolution hybrid quadrupole-orbitrap or time-of-flight instruments (Fig. 2B) (8). The PRM technique presents numerous advantages in comparison to the traditional SRM approach. First, it has increased sensitivity, since all potential product ions of a peptide are available to confirm its identity (6). Second, as fragmented ions are acquired with HRAM instruments and product ions are less likely to be affected by interfering ions, selectivity and specificity are also greatly improved. Finally, this strategy does not need a previous selection of fragmented ions and requires less method development than SRM (8, 7).

In targeted methods, either SRM or PRM, a cycle corresponds to the measurement of all the analytes of interest, which is repeated all along the chromatographic gradient. The time that it takes to complete a cycle (i.e. between one data point and the next one) is called the cycle time. The cycle time needs to be short enough to guarantee enough data points collected per analyte along its elution profile. Therefore, the number of analytes targeted within a cycle is limited. Despite this limitation on the number of analytes that can be included in one cycle, the development of scheduled PRM methods enables to increase the number of peptides monitored in one analysis by monitoring the peptides only during a time window around their actual elution time (Fig. 2C) (3). However, mass spectrometry acquisition parameters need to be adjusted when PRM is used for the analysis of large numbers of peptides, affecting the

quality of the data obtained. To overcome this issue, a new method called internal standard triggered-parallel reaction monitoring (IS-PRM) was designed. It is based on the use of stable isotopically labelled (SIL) peptides as internal standards to adjust acquisition parameters in real-time to measure endogenous peptides (Fig. 2D) (1). Therefore, taking everything into account, we can consider the PRM approach as an efficient method for quantitative analyses in complex biological samples, such as human tissue samples and liquid biopsies, for the study of biomarkers.

Liquid biopsies, such as blood, are indeed a source of many biomarkers useful for non-invasive diagnosis and disease monitoring (9). Human blood plasma is probably the most informative proteome from a medical point of view, since plasma interacts with each organ in the body (10). However, the presence of highly abundant proteins, like albumin, increases the difficulty to analyze low abundant but biologically relevant proteins (11, 12). Accordingly, synthetic peptides can be used to detect and quantify lower abundant molecules.

The aim of this project is to analyze human serum samples with the reference peptide kit PQ500 (containing 802 isotopically labelled reference peptides) and the SureQuant method (IS-PRM commercially available version) to assess the technical reproducibility of the method. The use of mass spectrometry-based approaches to identify and quantify plasma proteins is a valuable opportunity to contribute to human health through early diagnosis or by developing a personalized medicine/health. The data obtained in this work will be used as a basis for future projects to monitor the health condition of healthy and diseased individuals.

### **3. RESULTS**

In this work, we investigated the technical reproducibility of the targeted proteomics method known as SureQuant, which is a type of internal standard triggered-PRM acquisition method. This reproducibility was divided between technical variation of the instrument and variation among sample preparation, specifically in the digestion step. Also, we aimed to detect and quantify the added internal standard peptides and their corresponding endogenous forms. To do so, a total of 9 serum samples were used (3 samples with their corresponding triplicates) (Fig. 3).

All samples were prepared as indicated (Fig. 3, Methods section). Briefly, commercial serum samples were digested with trypsin and a set of 802 isotopically-labelled standard (SIL) peptides were spiked-in. Samples were then analyzed by LC-MS using SureQuant acquisition

method in which data for the selected endogenous peptides and their isotopically-labelled counterparts were recorded (see list of targeted peptides in Supplementary table).

In order to process the data obtained during the PRM assays, the list of targeted proteins, peptides and fragments was first specified in the open-source software Skyline (Fig. 4). Then, the acquired raw files were loaded and a two-step process was used to process the data. First, those peaks showing zero or poor signal were removed in each of the triplicates. Second, it was verified that the peak automatically chosen by Skyline was correct and, if not, the new peak was integrated manually. The identity of the peptides was corroborated by following two criteria. On one hand, co-elution with internal standards allowed identity confirmation of the corresponding endogenous forms. On the other hand, it was ensured that the ranking of transitions was the same between SIL and endogenous peptides, meaning that the most intense transition of the SIL peptide has to be the most intense transition of the endogenous peptide (Fig. 4).

The SureQuant method was applied to analyze a set of 802 stable isotopically labelled peptides (corresponding to 500 human proteins). The technique enabled to successfully identify a subset of 775 of the 802 internal standard peptides targeted in sample 1; 770 in sample 2 and 770 in sample 3. On the other hand, the technique allowed the identification of 741 endogenous peptides in sample 1; 737 in sample 2 and 735 in sample 3 (Fig. 5).

Once the list of identified endogenous peptides was established, we proceeded to the quantification of the analytes. For this purpose, we integrated the area under the curve of each transition, and used them to compare the endogenous peptide amounts (light peptides) with the reference amounts given by the SIL peptides (heavy peptides).

As a first step, we used the quantification of the peptides to refine the list of peptides identified with confidence. We considered a threshold value of 0.01 in the ratio light to heavy (light area/heavy area) to say that a peptide was confidently identified. With this criteria, the number of endogenous peptides detected decreased drastically. However, such threshold is important as the signal of endogenous peptides with such low ratios, might be due to the presence of impurities in the SIL peptide and not due to the presence of the peptide itself. Accordingly, 285, 331 and 310 endogenous peptides were detected in sample 1, 2 and 3, respectively.

To evaluate the reproducibility of the quantitation of the SureQuant method, coefficients of variation (CV) were calculated using the areas extracted by the Skyline software. Samples coming from the same digestion showed very good reproducibility, since a coefficient of

variation of 10.67% was observed in triplicates from sample 1; 10.1% in triplicates from sample 2 and 8.03% in triplicates from sample 3. Therefore, the CV mean is 9.6% (<10%) which is considered very favorable (Fig. 6A).

Nevertheless, when the coefficient of variation was calculated taking into account all 9 samples together, regardless of the digestion where they come from, the mean value increased up to 34.7%, considered unacceptable to ensure technical reproducibility (Fig. 6B). The same happens when the coefficient of variation was calculated taking into account the first triplicate of each sample, the second and the third, separately. The resulting CV value was 39.1%, which is even higher than the previous one (Fig. 6C). This is due to the fact that, in this last case, the variability is calculated taking into account triplicates from different samples.

#### **4. DISCUSSION**

As previously mentioned, the aim of this study was to assess the technical reproducibility of the SureQuant method and to quantify the number of peptides detected in the three different samples.

To assess instrument variability, the coefficient of variation was calculated taking into account triplicates coming from the same digestion. The mean value obtained was 9.6%, which implies very good instrument reproducibility. However, regarding the number of SIL peptides detected in each sample, it can be observed that it is lower than the total number of SIL peptides targeted. We would expect the same amount detected in both cases (802 peptides), but the number of SIL peptides detected was 775 in sample 1 and 770 in sample 2 and 3. Almost all of those 30 peptides (approximately) that could not be detected in any of the three samples correspond to peptides with a retention time between 0 and 12 minutes. In these cases, ion chromatograms in Skyline showed no signal or very low signal. Thereby, this fact leads us to think that there are instrument irregularities, such as a poor calibration of the chromatographic gradient. The no detection of other peptides that do not belong to the group of peptides co-eluting between 0 and 12 minutes, could be due to precipitation, oxidation or stability reasons.

Following with the number of peptides detected, the amount of endogenous forms identified is even lower. We do not consider this decrease as an instrument detection problem, but rather the absence of these peptides in the sample or, as in the previous case, due to precipitation, oxidation or stability reasons.

On the other hand, the two other coefficients of variation obtained show unacceptable values (34.7% and 39.1%) indicating that sample preparation variability is too high to ensure good reproducibility. Previous studies highlight the importance of sample preparation in order to obtain reproducible and high quality data (15). Sample preparation implies different steps, including reduction, alkylation and tryptic digestion. In the digestion step, proteins from the sample are converted into peptides by enzymatic proteolysis using, typically, the proteolytic enzyme trypsin. It is known that digestion is a source of variability and it can be due to several reasons. First, every protein is affected in a different way during the digestion procedure due to structural (i.e. disulfide bridges and solubility issues) and biological reasons (i.e. resistance to proteolysis) (16). Second, incomplete enzymatic digestion could lead to the production of miss cleavage and non tryptic peptides, decreasing reproducibility (16). Third, it has been reported that different types of commercially available trypsin show different efficiency, specificity and reproducibility depending on its the quality (16, 17).

Taking into account the previous information we can conclude that instrument variability is low, although it needs to be improved by re-calibration of the chromatographic system in order to detect peptides with short retention times (between 0-12 minutes). Furthermore, the high variability observed in sample preparation, forces us to repeat the experiment. There are several protocols for tryptic digestion in literature but, as digestion conditions depend on each protein, there is no protocol that is best for all proteins (16). Therefore, it would be important to monitor the tryptic digest at defined time points to assess digestion procedure and ensure reproducibility among different samples.

These improvements will increase the number of SIL peptides detected and will decrease procedure variability. Reproducibility achievement is essential to obtain reliable quantitative results and to be able to apply the SureQuant method in future projects to monitor the health condition of healthy and diseased individuals.

## 5. MATERIALS AND METHODS

### Sample preparation

Three aliquots of commercial human serum samples (50 µg, P/N H4522, Sigma-Aldrich) were reduced with dithiothreitol (30 nmol, 37 °C, 60 min) and alkylated in the dark with iodoacetamide (60 nmol, 25 °C, 30 min). The resulting protein extract was first diluted to 2M urea with 200 mM ammonium bicarbonate for digestion with endoproteinase LysC (1:10 w:w, 37°C, o/n, Wako, cat # 129-02541), and then diluted 2-fold with 200 mM ammonium bicarbonate for trypsin digestion (1:10 w:w, 37°C, 8h, Promega cat # V5113).

After digestion, the peptide mix was acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc) prior to LC-MS/MS analysis. Finally, the reference peptide kit PQ500 (Biognosys) was added at a ratio 1µg sample:10 fmol PQ500 and total protein abundances were adjusted to 1 µg/µL.

### Chromatographic and mass spectrometric analysis

Data was acquired using a Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an EASY-nLC 1200 (Thermo Fisher Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column and were separated by reversed-phase chromatography using a 50-cm column with an inner diameter of 75 µm, packed with 2 µm C18 particles spectrometer (Thermo Scientific, San Jose, CA, USA).

Chromatographic gradients started at 95% buffer A and 5% buffer B with a flow rate of 300 nl/min for 5 minutes and gradually increased to 25% buffer B and 75% A in 105 min and then to 40% buffer B and 60% A in 15 min. After each analysis, the column was washed for 10 min with 10% buffer A and 90% buffer B. Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile.

The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.4 kV and source temperature at 275°C. Ultramark 1621 for the was used for external calibration of the FT mass analyzer prior the analyses, and an internal calibration was performed using the background polysiloxane ion signal at m/z 445.1200.

A pool of the three samples was used to determine the intensity of the heavy standards using the PQ500 Survey template available in the instrument control software. Briefly, a full MS scan at resolution 120000 was used over a mass range of m/z 300-1500 with detection in the Orbitrap mass analyzer followed by a targeted mass filter that includes the precursor mass of the PQ500 heavy standards and an intensity threshold of 1e5. Those precursors with intensity

greater than the threshold were fragmented via high-energy collision dissociation (HCD) at normalized collision energy of 32% and they were acquired in the Orbitrap at 7500 resolution. Individual samples were analyzed using the PQ500 SureQuant template available in the instrument control software. Briefly, a full MS scan at resolution 120000 was used over a mass range of m/z 300-1500 with detection in the Orbitrap mass analyzer followed by a targeted mass filter that includes the precursor mass of the PQ500 heavy standards and an intensity threshold of 1% of the signal obtained in the survey run. Those precursors with intensity greater than the threshold were fragmented via HCD at normalized collision energy of 32% and they were acquired in the Orbitrap at 7500 resolution. When at least 5 of the 6 predefined fragments were detected, a fragmentation of the endogenous peptide was triggered via HCD at normalized collision energy of 32% and they were acquired in the Orbitrap at 60000 resolution. All data were acquired with Xcalibur software v4.3.73.10.

Digested bovine serum albumin (New england biolabs cat # P8108S) was analyzed between each sample to avoid sample carryover and to assure stability of the instrument and QCloud (13) has been used to control instrument longitudinal performance during the project.

#### Data Analysis

Acquired spectra were analyzed using Skyline daily (v 20.1.1.134) software (14).

## **6. ACKNOWLEDGEMENTS**

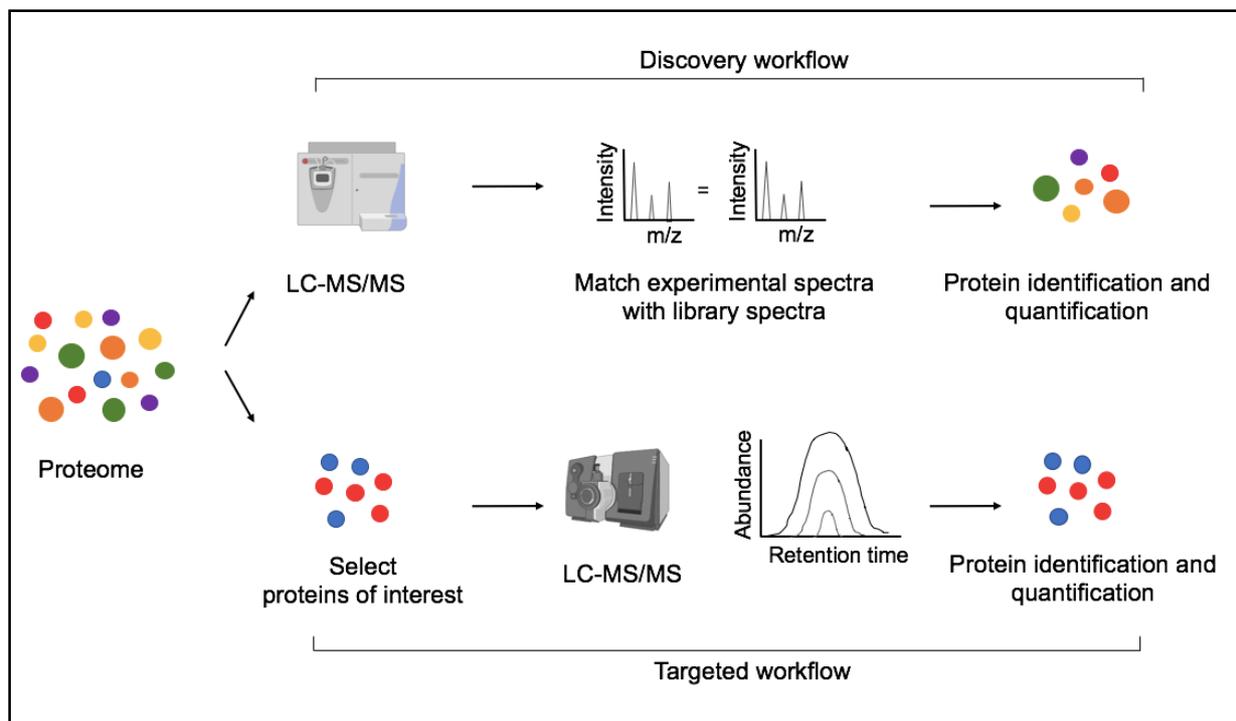
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## 7. BIBLIOGRAPHY

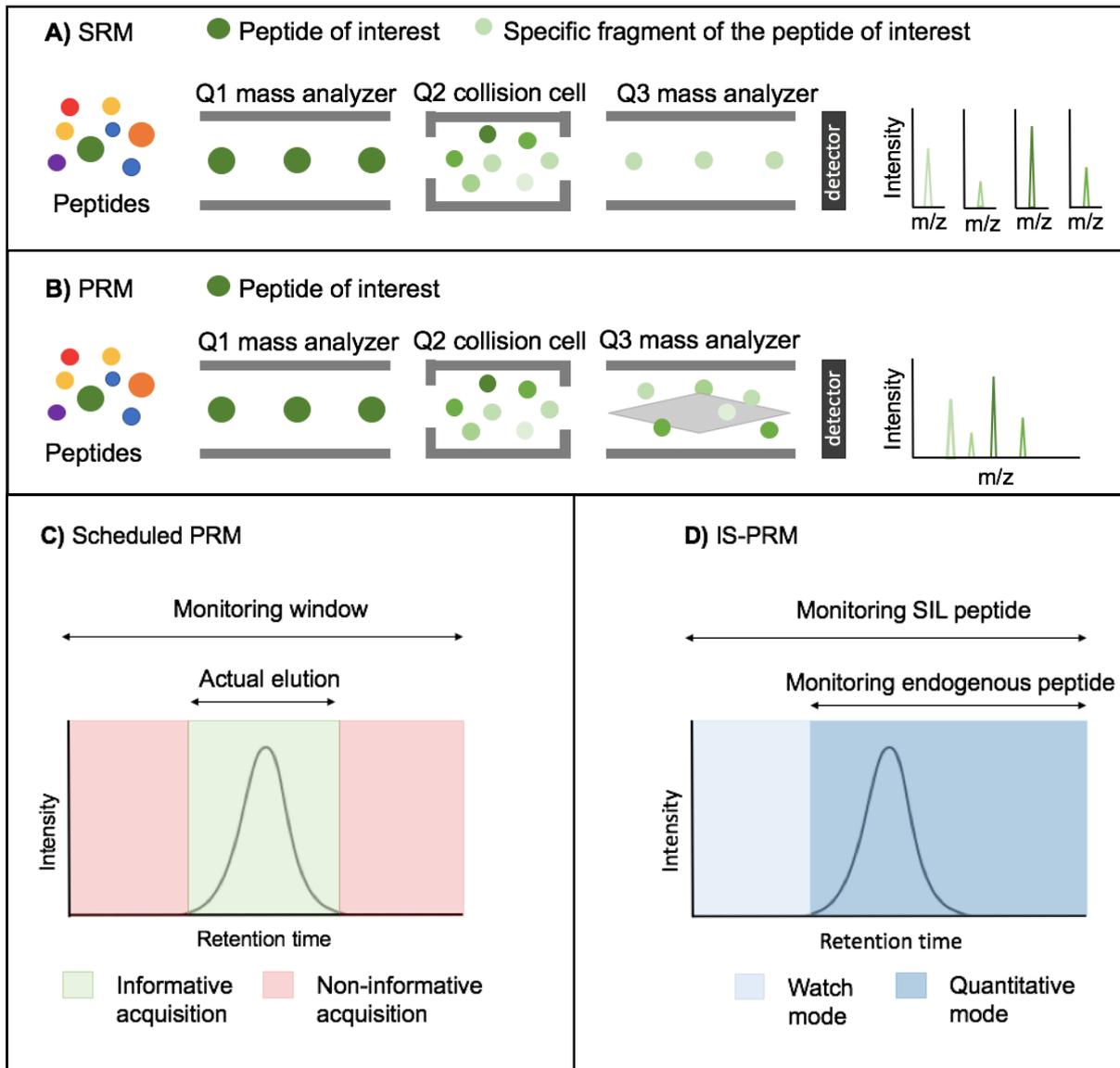
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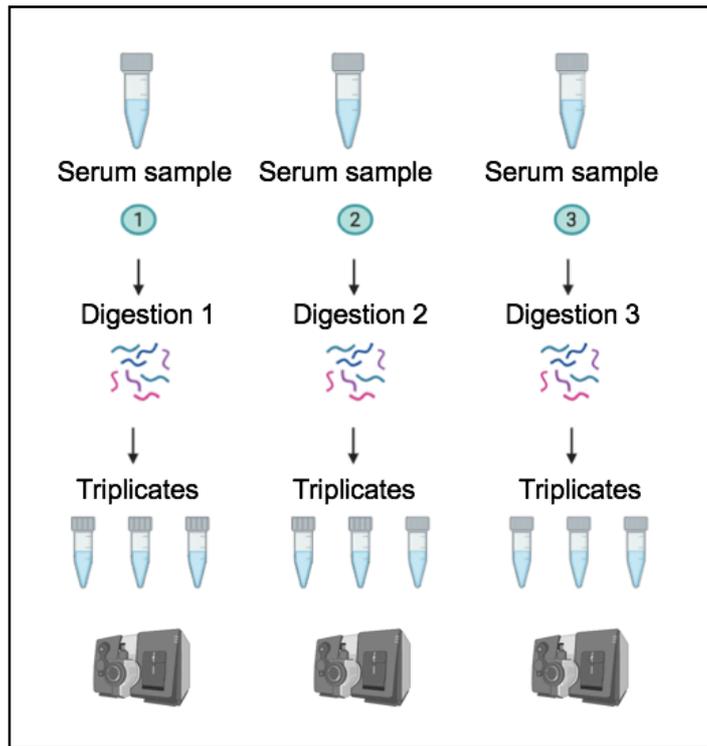
## 8. FIGURES



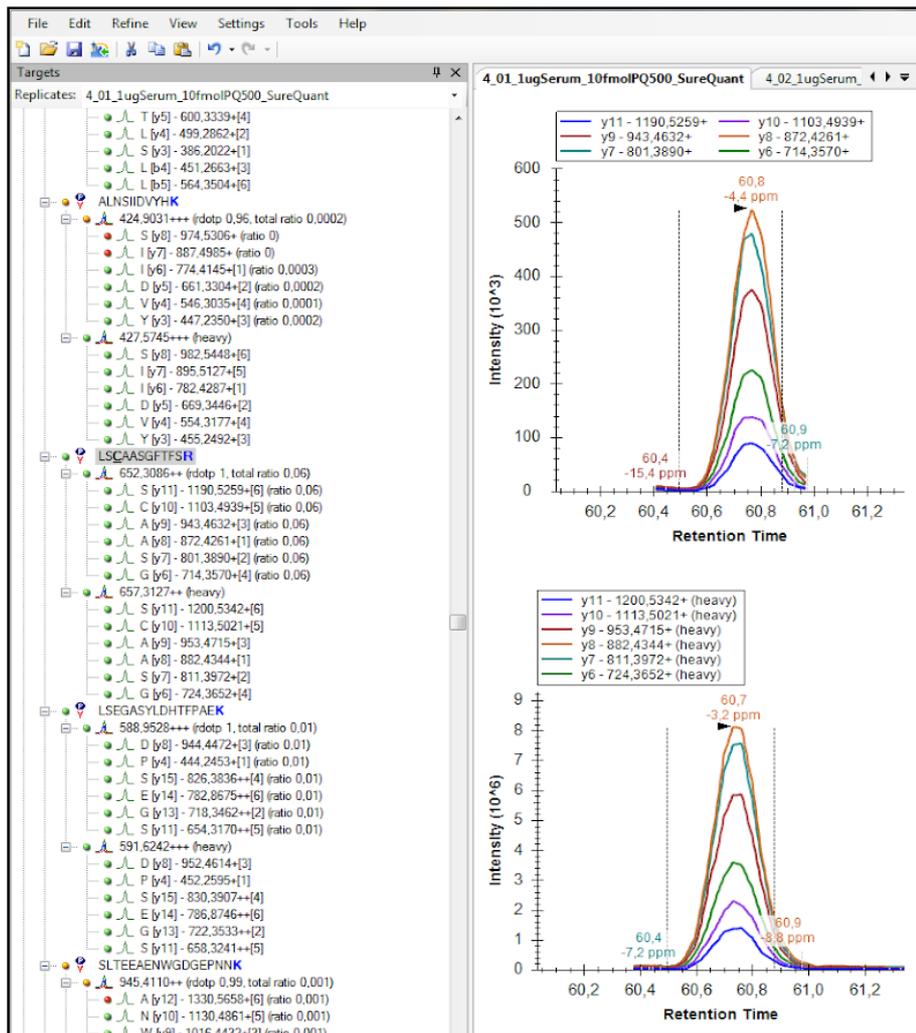
**Figure 1. Schematic representation of liquid chromatography coupled to tandem mass spectrometry approaches.** Discovery proteomics experiments analyse samples containing proteins over a broad dynamic range using LC-MS/MS. Once the spectra of the experimental sample is obtained, it is matched with a library spectra of reference in order to quantify, but mainly identify as many proteins as possible. In contrast, targeted proteomics typically analyse specific proteins of interest (less than 100), also using LC-MS/MS to identify and quantify them with high precision.



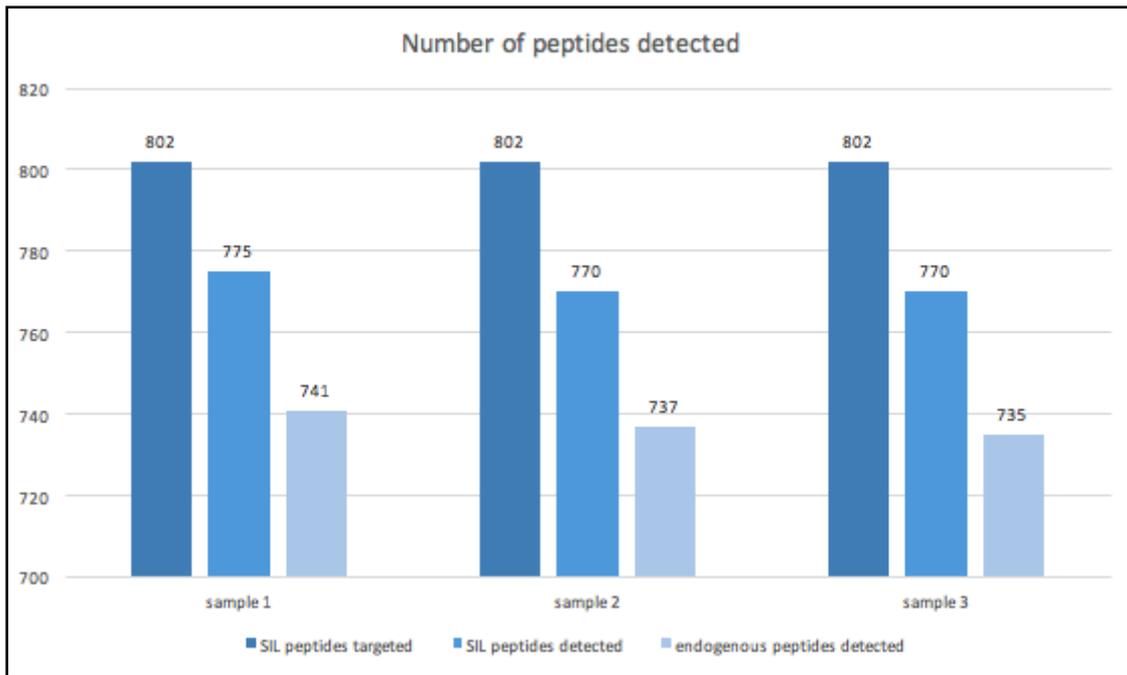
**Figure 2. Schematic representation of targeted proteomic approaches.** (A) In SRM, a predefined peptide of interest of a particular mass-to-charge ( $m/z$ ) ratio is selected in the first quadrupole (Q1). Then, in the second quadrupole (Q2), the previously isolated precursor is fragmented. Lastly, one of the resulting fragmented ions is filtered in the third quadrupole (Q3) to finally reach the detector, where the detected signals are recorded as an ion chromatogram. (B) In contrast, in PRM all product ions of the peptide of interest are measured simultaneously. (C) In scheduled PRM, peptides of interest are only monitored during a time window around their actual elution time. (D) IS-PRM operates alternating two different modes. In the “watch mode” (lower resolution), only SIL peptides are monitored. When a SIL peptide is detected above a threshold value of intensity, the “watch mode” is switched to the “quantitative mode”, enabling the measurement of the SIL and the endogenous peptide concurrently.



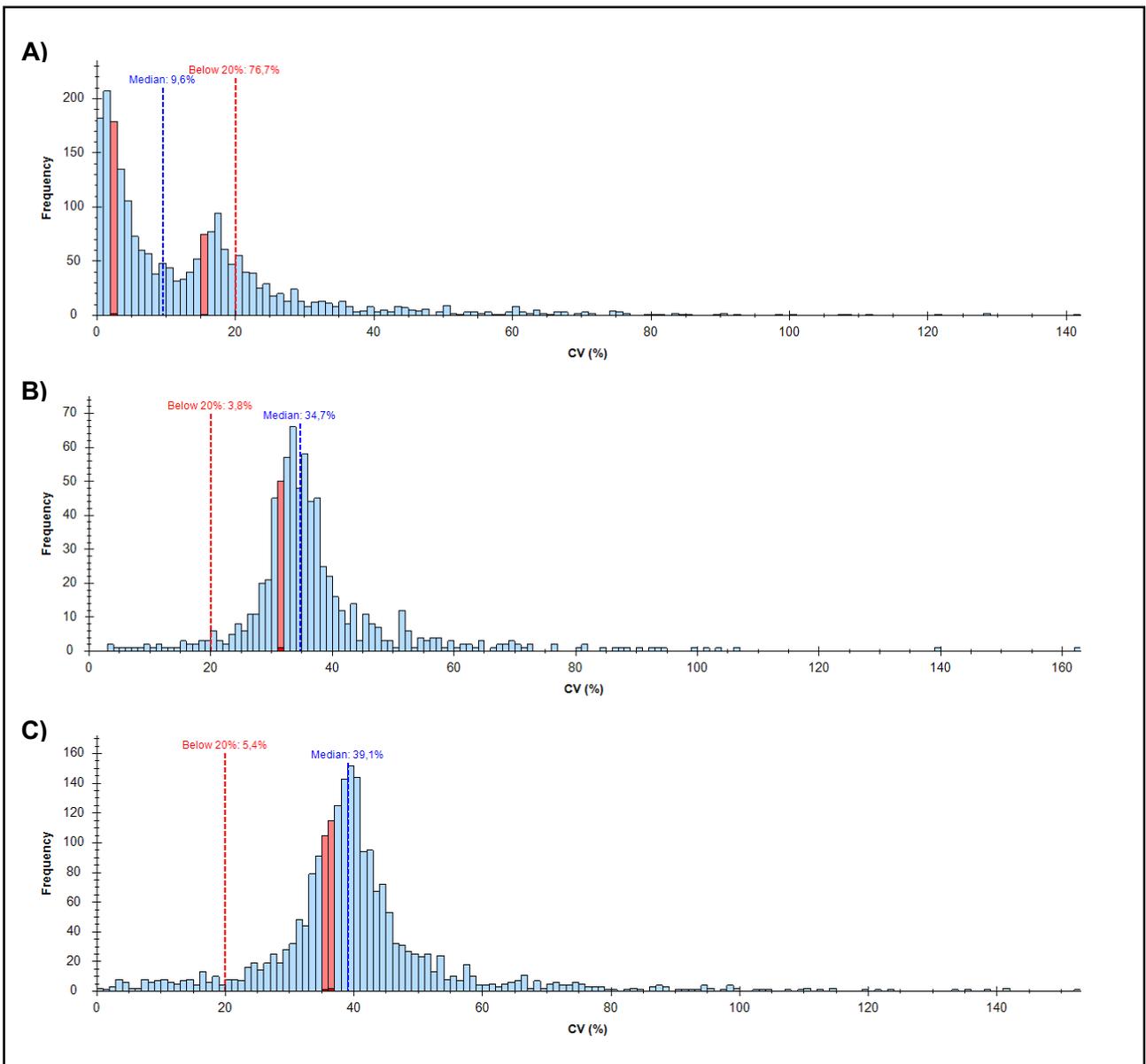
**Figure 3. Schematic representation of the samples used in the experiment.** Three aliquots of commercial human serum samples were digested independently. Once the peptide mix was obtained from each of the three aliquots, each sample was divided into triplicates, being a total of 9 samples that will later be injected to the LC-MS/MS instrument to perform the experiment.



**Figure 4. Analysis of the data obtained during PRM using Skyline software.** On the left, green, yellow and red dots indicate how well Skyline did at finding an acceptable group of co-eluting peaks for the transitions analyzed. Also on the left, the list of peptides and transitions is displayed. On the right, ion chromatograms of precursor and fragments are shown for SIL (above) and endogenous peptides (below). In this specific peptide (LSCAASGFTFSR), 6 transitions in both heavy and light peptides are seen, being y8 (orange) the most intense fragment in both cases, confirming the identity of the endogenous form. Retention time is indicated above the peak (60,7 in both heavy and light peptide), enhancing its identification.



**Figure 5. Graphical representation of the number of peptides detected.** The number of SIL peptides targeted is 802 in each of the three samples. In the first sample, 775 SIL peptides and 741 endogenous peptides were detected. Similar values are observed in the other two samples, where 770 SIL peptides were detected in both sample 2 and sample 3 and 737 and 735 endogenous peptides were detected, respectively.



**Figure 6. Technical reproducibility of the SureQuant method.** A) Samples coming from the same digestion show a coefficient of variation of 9,6% on average and 76,7% of peptides are below a coefficient value of 20%. B) The nine samples coming from the three different digestions show a coefficient of variation of 34,7% and only 3,8% of the peptides are below 20%. C) Finally, the coefficient of variation calculated taking into account the first, the second and the third triplicate of each sample separately shows a mean value of 39,1% and 3,8% of peptides are below the coefficient of variation of 20%.

## 9. SUPPLEMENTARY TABLE

**Table 1. List of the 802 targeted peptides.**

Peptide Sequence	Peptide Sequence	Peptide Sequence
VGVETTKPSK	KVAQELEEK	STPSLTTK
RPSDKYR	TSPVDEK	SPENSLDPK
HYEGSTVPEK	SHTALLR	APLGSPSPR
GCQGKPLPK	LQEGPTSCSG	TPLGDTTHTCPR
QESEQGPCR	KPAITYGTR	PVSLTGK
DNGGEVTDKPVK	TASHGDLIR	HLACLPR
AEPEKTEGAAEAK	ARELISR	VSAQQVQGVHAR
TTVSNVK	SGSGLVGR	AHVPSWK
SHKSYSCQVTHEGSTVEK	VLSPADK	YPNCAYR
GASQELK	VTESEIK	HTSALAFR
STISAEK	TAGQGPGQLQR	GTYSTTVTGR
IPSNPSHR	VGEFSGANK	VYSTSVTGSR
HCSQVDSVR	GHFDTLK	IEALNEK
RAVSPPAR	FAHTVVTSR	YSGSEGSTQTLTK
LGSYEHR	VTAHAEGYTPSAK	TAAQNLYEK
TGAVSGHSLK	ISSPTETER	AHVNSLGENLK
ATNYNAGDR	APLTKPLK	ANLQSVPHASASR
VTTHPLAK	NYAEVGR	LRPLSGSEAPR
NANAEPAVQR	VHENENIGTTEPGEHQEAK	SLPTEDCENEK
SDTSSNHAVLK	YGSPYTK	SSSISFK
ETGEHLVHVK	TPLFHSK	VTYDVSR
TLVVHEK	SAGSVESPSVSSTHR	YSQKEDKYEEEEIK
SSSHPIFHR	VNHVTLQPK	EEAPSLR
AQTTVTCTEK	ELDSTGTPTGK	HLVALSPK
KEEEAVEPQSSPAK	SPELQAEAK	ALYEAGER
TESIIHR	GNFHAVYR	SPDGDSSLAASER
SQVEPETR	SALDTAAR	HSIGYSIR
ANHEEVLAAGK	SSDANLYR	EDARPVPQGSCQSELHR
SSQSLLSK	LASAYGAR	VISVSTGK
TAVAHKPGAFK	ISVIRPSK	DVAEEAGVSK
AGPNLRL	SGELEQEEER	SGAEEQGPIDGSPK
TTSHTELVR	DGLAPEKTSPDR	GPPAPPEPR
LSELHCDK	TLYSSSPR	LSSPSIK
TVTISKPCGK	VVVHPDYR	SQSSVLADSETSK

**Peptide Sequence**

ALLETSEK  
FGEGVSSPK  
FFESHVAR  
EAFAAVSK  
VLNQELR  
GSGDSSQVTQVSPQR  
TFHEASEDCISR  
EQPPSLTR  
CNPDSNSANCLEEK  
FGQTPVQER  
YSSLAEAASK  
LWEGSTSR  
NDPYHPDHFNCANCGK  
VEHSDLSFSK  
YYSALR  
WDAQIPHQHR  
TPITVVK  
GNGPHDNGIIVSTR  
GVTIPSQR  
DATQITQGPR  
IQNVLSEKPR  
YADAQLSCQGR  
HTSLGLEAK  
ALPTTYEK  
ASSFLGEK  
ESHVTLASPEETR  
VALTGVR  
EEFHEQSFR  
VALSAVRPGDR  
ASVSVTAEDEGTQR  
FRPDGLPK  
INENTGSVSVTR  
EGVVHGVATVAEK  
HYGNSYSVSNSEK

**Peptide Sequence**

FASTFDK  
GALALEEK  
TIDDLEEK  
DQCQVDSQCPGQMK  
SVVSDEGLK  
TGESAEFVCK  
GLPSSIEK  
YTHFLTQHYDAKPQGR  
ESSSHHPGIAEFPSR  
GAEDSLADQAANK  
VYKPSAGNNSLYR  
THYSNIEANESEEV  
HVLVTLGEK  
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VSLATVDK  
ISASAEELR  
IAFSATR  
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LNENHSGELWK  
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LYPTYSTK  
LSAAEAQNHCNQNGGNLATVK  
ATYIQNYR  
HGEYWLGNK  
VG DYGSLSGR  
GTFSTTVTGR  
RPSSQGWASPQVAGR  
CPAGFIDK  
IFYNQNHYDGSTGK

**Peptide Sequence**

HLFVQDPQTCK  
LENLEPEHEYK  
NLDGISHAPNAVK  
AQGYSGLSVK  
LVTDLTK  
YGHSLALYK  
FQASVATPR  
NPLPSKETIEQEK  
GKWERPFEVK  
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VTILADR  
GVFEVK  
HAEPEQNWEAVDGSQTETEEK  
SHLIIAQVAK  
EFGNTLEDK  
AAVYHHFISDGVR  
VIQTAFQR  
PAFSAIR  
SSEDPNEDIVER  
HLEDVFSK  
VQELEASVQEGK  
AVEPQLQEEER  
IDSVSEGNAGPYR  
VI AVNEVGR  
GTLSTTITGR  
IYFTDSSSK  
VTISVDTSR

**Peptide Sequence**

GLPAPIEK  
NANTFISPQQR  
CRPPVGCEELVR  
LFPAPSEK  
TSSDHTDHTYLSSTFTK  
SLGFCDTTNK  
VGAHAGEYGAEALER  
FEVYGCK  
AILGATEVK  
QYFYETK  
YEYLEGGDR  
LIANTLNSR  
FEHCNFDVTTTR  
APTAQVESFR  
TPLTATLSK  
AAISGENAGLVR  
VDSL VGAGPASR  
TYQGSYGFR  
FSTVAGESGSADTVR  
FGQSCQEQCPGISGCR  
GLTSVINQK  
FNSVPLTDTGHER  
GFVVAGPSR  
ITGAQVGTGCGTLNDGK  
ATAVVDGAFK  
IIELSQTAK  
LDGSVDFK  
TFAHYATFR  
TEGDGVYTLNNEK  
PALEDLR  
TPWHVTIK  
LSSPAVITDK  
IYFAHTALK  
LRPVAAEVYGTERR

**Peptide Sequence**

DHSAIPVINR  
PYTFHSHGITYYK  
SSPYYALR  
GFPGIQGR  
IQQNLDQLR  
TDAPDLPEENQAR  
ILSIDGGGTR  
TSTDLDVDVDQPKEEK  
GQTL LAVAK  
LQENLQAYR  
THPHFVIPYR  
ESDTSYVSLK  
ADLSGITGAR  
SIPQVSPVR  
DGETIELR  
LEGEACGVYTPR  
FPALTQE QK  
IYLVETK  
QVQLVQSGAEVR  
FSVVYAK  
YMQSSVQLR  
KPWLAYPHYKPPEK  
LIQGAPTIR  
EAQQYSEALASTR  
FYEA FSK  
ALAH LLEAER  
VTEPISAESGEQVER  
APGEGPQVACTGPPSAPR  
IVVVTAGVR  
LPATEKPVLLSK  
TLEAQLTPR  
DIVSGLK  
LLGPHVEGLK  
LTQLNLDR

**Peptide Sequence**

LPVAPLR  
EALQGVGDMGR  
ILAGPAGDSNVVK  
LYGGPGVPGGSSCGTQAR  
IRPHTFTGLSGLR  
YVVISQGLDKPR  
DDEEFIESNK  
SVGFHLPSR  
DHGETAFAVYDK  
LTVSIEAR  
LPTDSELAPR  
YFHHNSDFYIPK  
GDIGETGVPGAEGPR  
SGNCYLDIRPR  
GYILVGQAK  
AEIEYLEK  
VIIYEVNK  
DNCCILDER  
HQAQIDHYLGLANK  
TPDGSLLR  
TYLPAVDEK  
LAPLAEDVR  
SSGWHFVVK  
TVESITDIR  
SPAGPTVVSIGGGK  
STIYLVR  
ISVYYNEATGGK  
NKEDCVEIYIK  
TGIVSGFGR  
HESENLYSIACDKPQQVR  
SEAACLAAGPGIR  
VAAGAFQGLR  
GSTLTSPCQDFGK  
LVNIAVDER

**Peptide Sequence**

WEHGDGYPFDGK  
KPSLSVQGPVVPAGEK  
GIVEECCFR  
FVNPEDVAR  
DGAGDVAFVK  
FLQSFAR  
LTAFPSESVK  
SPGAPGLTLK  
NLVVIPK  
YEGSYALTSEEAEER  
ACIPTGPYPCGK  
SYDYQSVCEPGAAPK  
DVSYLRY  
SGNENGEFYLR  
VTIASLPR  
SLDSPAALAEER  
IDVHLVPCR  
VEQAVETEPEPELR  
GFYFNKPTGYGSSSR  
IAYGTQGSSGYSLR  
RKSSVTDSFSSLVNR  
TAAISGYSFK  
VVFDDTYDR  
LTFQCGSDAGYDR  
LVHCPIETQVLR  
KPDGYDYAFSK  
AESPEVCFNEESPK  
VDGSVDFYR  
DYWSTVK  
SQIHDIVLVGGSTR  
VHVGDDEFVHLR  
GILAADESTGSIK  
LLELTGPK  
TLLPVSKPEIR

**Peptide Sequence**

ILDDLSPR  
EALVPLVADHK  
GYTQQLAFR  
LHLDYIGPCK  
RDPSPVSGPVHLFR  
WYVDGVEVHNAK  
IESPGYLTPSGYPHSYHPSEK  
YTFELSR  
STVISYECCPGYEK  
LTFDEYR  
ATDFVVPGPCK  
LYEYIAR  
VSPVGETYIHEGLK  
SYLEITPSR  
AIGYLNTGYQR  
LGPGLVDAAQAR  
VNVDEVGGEALGR  
GNQWVGYYDDQESVK  
SIDVACHPGYALPK  
LFEELGK  
GPYHPSECCFTYTTYK  
EQLTPLIK  
LLDSLPSDTR  
LTPTLYVGK  
LFGPDLK  
LLPAQLPAEK  
YAATSQVLLPSK  
VGISAVALR  
FQNALLVR  
AATVGSLAGQPLQER  
YEVTVVSVR  
FLLYNR  
VYLGPGSDGHPYSTQSIQQGSAVSR  
DLELEVK

**Peptide Sequence**

TYSVEYLDSSK  
LTGISDPVTVK  
ELLDTVTAPQK  
GHAHDGQALSTDLGVYTCEASNR  
PIIHFGSDYEDR  
EHVAHLLFLR  
SSGLVSNAPGVQIR  
GGYTLVSGYPK  
ALLAFQESK  
SDLVNEEATGQFR  
SDVVYTDWK  
LAILGIHNEVSK  
NIDALSGMEGR  
STDTSCVNPPTVQNAHILSR  
DALSSVQESQVAQQAR  
TALQHLHGVPQGALLEDNR  
IGESIELTCPK  
YALSNSIGPVR  
ILGPLSYSK  
VVCSSGAVGNYSGLLAVK  
NILTSNIDVK  
KDVLETFTVK  
NYPSELDK  
VLEGNEQFINAAK  
EPISVSSEQVLK  
LSGEAYGFVAR  
AYLEEECPATLR  
YGIDWASGR  
APQTGIVDECCFR  
TGQQLTSDQLPIK  
ILASTQFEPTAAR  
NSWGHNFGEEGYIR  
NCQTVLAPCSPNPCENAAVCK  
IVIEYVDR

**Peptide Sequence**

LCTPLLPK  
TVAACNLPIVR  
VEYQCQSYELQGSK  
GNLCVNLMR  
VPGTSTSATLTGLTR  
TEAPSATGQASSLLGGR  
IAWALSR  
YGFYTHVFR  
DAHSVLLSHIFHGR  
EVDQQLLSVQTR  
ELLETVVNR  
IQVLVEPDHFK  
AGLAASLAGPHSIVGR  
SWSVYVGAR  
NPNLPPETVDSLK  
DTDLDGFPDEK  
NIQSLEVIGK  
YLDWIHGHIR  
LSEPAELTDAVK  
FGSSLITVR  
VPLQQNFQDNQFQ GK  
IDYGVFAK  
LLIGTVFHK  
HLSLLTTLNLR  
ALNSIIDVYHK  
LSCAASGFTFSR  
LSEGASYLDHTFPAEK  
SLTEEAENWGDGEPNNK  
AALSIVSEIGK  
LALFPDK  
YLGVTLSR  
SAVTALWGK  
FITPEDLSK  
IWLNDNR

**Peptide Sequence**

LGPISADSTTAPLEK  
LFDEINPETK  
LTEPADTITDAVK  
VLFYVDSEK  
VLAIHDLNEDQLR  
LSLPTDLK  
VPTGGVEEGLLER  
HSQPWQVLVSR  
AGLLRPDYALLGHR  
FGSGYVSGWGR  
VGEYSLYIGR  
IILEALR  
LLLSSETPIEGK  
TALASGGVLDASGDYR  
LDIDSPPITAR  
HSLPDIQLLK  
ALSIGFETCR  
SHSSGSVLPLEGEGR  
IAQYYTFK  
FQSVFTVTR  
VTSIQDWVQK  
TGYFDGISR  
GVTFLR  
EGYYGYTGAFR  
YTFVVPEDTR  
AVGYLITGYQR  
QIQVSWLR  
HLQEYQDLLNVK  
LGPHAGDVEGHLSFLEK  
DTVENAIQITSGK  
YPLYVLK  
GLGEISAASEFK  
LVVQSSIDSSAFK  
VLASCYAVIEEHSWAHR

**Peptide Sequence**

GPSVFPLAPSSK  
AGALNSNDAFVLK  
LYIEYGIQR  
VSPGAFTPLVK  
ALEWLAR  
SYTITGLQPGTDYK  
ALQASALNAWR  
LTASAPGYLAITK  
AWFLESK  
SLLHTLALPSPK  
SELEEQLTPVAEETR  
NITEIADLTQK  
YGLVITYATYPK  
WIYHLTEGSTDLR  
VGALASLR  
ANVFVQLPR  
TVGSDTFYSFK  
NIPGDFECECEGYR  
DAVEDLESVKG  
TVAAGALASLSHLK  
GHGEDHCQFVDSFLDGR  
INGIPQQHTQVLFIAK  
ESLSSYWESAK  
FLNVLSR  
SASDLTWDNLK  
ALDFAVGEYNK  
AVASAAAALVLK  
DASGATFTWTPSSGK  
VQNATLAVANITNADSATR  
ILLDEQAQWK  
VGYVSGWGQSDNFK  
AVSPLPYLR  
VSTLPAITLK  
VDATEESDLAQQYGVR

**Peptide Sequence**

YGFIEGHVVIPR  
DSPSVWAAVPGK  
EENFYVDETTVVK  
SSNLIILEEHLK  
DSDLLSPSDFK  
ALFLETEQLK  
EDVYVVGTVLR  
FSGWYDADLSPAGHEEAK  
ETLLQDFR  
VYFAGFPR  
LLVQILQK  
LSFQHDPETSVLVLR  
FRPSEPHFTLDGNSFYK  
GSVQYLPDLDDK  
AADDTWEPFASGK  
TSFPEDTVITYK  
SFYTAYLQR  
DFEQPLAISR  
SLVELTPIAAVHGR  
WTLTAPPGYR  
NELESYAYSLK  
VTSVVTGFNNLPDR  
TNFDNDIALVR  
SVLGQLGITK  
GLETSLAECTFTK  
EIGELYLPK  
GKEESLSDLYAELR  
DLQNFLK  
FYNQVSTPLLR  
PSSLGQGAGEVWLR  
VILPQTS DAYQVSVAK  
SGFSFGFK  
GPESCSLGCAQATQCALCLR  
LLCGLLAER

**Peptide Sequence**

GNLVSIHNFNINYR  
FEDGVLPDYPR  
LLAENNEIISNIR  
TATSEYQTFNPR  
LLGLSLAGK  
VGLSDAFVVHR  
GYSIFSATK  
DLEEPINFR  
GFQVVVTLR  
STESYFIPEVR  
GAFFPLTER  
LQSGTHCLWTDQLLQGSEK  
DNENVVNEYSSELEK  
LGPNYLHIPVNCYPYR  
ESISVSSEQLAQFR  
GPGGVWAAEAISDAR  
LTSPLGALR  
ELGCGAASGTPSGILYEPPAEK  
VLEYLNDLK  
VGAGTGQIWL DNVQCR  
DFAEHLIPR  
AVGPHQFLGDQEAIQAAIK  
EVTVPVFYPTK  
NFPSPVDAAFR  
GPVTDVAYSHDGAFLAVCDASK  
TGGLDLPSPPTGASLK  
IANVFTNAFR  
FLASVSTVLTSK  
LNQALLDLHALGSAR  
ALQDQLVLVAAK  
AVGLLTVISK  
IQEVAGSLIFR  
LGTFEVEDQIEAAR  
FYYLIASETPGK

**Peptide Sequence**

RPDSLQHVLLPVLDLDR  
IHLISTQSAIPYALR  
GWSTPPICSFTK  
GQVPENEANVVITTLK  
HLYVLEFSDHPGIHEPLEPEVK  
LEYLLLSR  
YDPSLKPLSVSYDQATSLR  
GPGGAWAAEVISNAR  
NTGVISVVTGLDR  
DVDIDSYPDEELPCSAR  
TVTATFGYPFR  
FNALQYLR  
LSSGLVTAALYGR  
SALVLQYLR  
WDEELAAFAK  
LHEAFSPVSYQHDLALLR  
FVGQLDISIAR  
DFHINLFR  
ATISHVSPDSL YLFR  
LLQDFFNGR  
HPDLSIPELLR  
SLPTCLPVCGLPK  
GFTIPEAFR  
GWVTDGFSSLK  
YVYIAELLAHK  
LVVLPFPK  
AIPVAQDLNAPSDWDSR  
ELLENEFHQILK  
EEASDYLELDTIK  
IVLLDVPVR  
YSLTYIYTGLSK  
FPAIQNLALR  
LQDIVSALEDR  
DAGTIAGLNVLR

Peptide Sequence

STVEELHEPIPSLFR  
DGSFVSVITGLR  
GTNYLADVFEK  
DYVSQFEGSALGK  
GTWTQPFDLASTR  
VPSLVGSFIR  
VTVFPIGIGDR  
LNCQVIGASVDSHFCHLAWVNTPK  
TEHPFTVEEFVLPK  
LLGPGPAADFSVSVER  
SCDLALLETYCATPAK  
LVLPLSISSR  
GLYDVVSVLR  
IGFFNQYAEQLR  
NEALIALLR  
HGATVLTALGGILK  
YFIDFVAR  
ITVVDALHEIPVK  
ICLDLQAPLYK  
GGEGTGYFVDFSVR  
GLAFTDVDVDSIK  
LSNNALSGLPQGVFGK  
LSPINLVVPVK  
RTVAAPSVFIFPPSDEQLK  
SIVVSPILIPENQR  
SGVQQLIQYYQDQK  
DILTIDIGR  
LPFPIIDDR  
NPQLCYQDTILWK  
SYSFDFYVPQR  
EGLDLQVLEDSGR  
QEPSQGTTTTFAVTSILR  
SFLVWVNEEDHLR  
PCFSALEVDETYVPK

Peptide Sequence

GSAADSEESPAIEAIHLLR  
LFDQAFGLPR  
SLDFTELDVAEEK  
TLAFPLTIR  
VWFELTQGSITK  
GHILELLTEVTR  
AVISPGFDVFAK  
LLIYDASSLESGVPSR  
LSELIQPLPLER  
DYIFGNYSIER  
SLPVSDSVLSGFQR  
LKPEDITQIQPQQLVLR  
YISPDQLADLYK  
VCPFAGILENGAVR  
HLDSVLQQLQTEVYR  
YPLVVFSHGLGAFR  
YEQAFLTSFVGLRPEK  
LSDLLAPISEQIK  
AFQVWSDVTPLR  
DFLGFYVVDShR  
APDLQDLPWQVK  
SLLGAFIPR  
TPEGLTLLFK  
LISWYDNEFGYSNR  
PFLVFIR  
DLLHVLAFSK  
IDPVNGQITTI AVLDR  
YAASSYLSLTPEQWR  
TSESGELHGLTTEEFVEGIYK  
VVSVLTVVHQDWLNGK  
ALESPPERPFLAILGGAK  
YWGVASFLQK  
DLHKPIQEVIIELTK  
ILHVFHGLLPGLFLVK

Peptide Sequence

NIETIINTFHQYSVK  
IFFPGVSEFGK  
LTQLGTFEDHFLSLQR  
VPLALFALNR  
SLGPALLLLQK  
YQFFVYLQEGK  
DFTFDLYR  
YPGAYYIFQIK  
LPLVPALDGCLR  
GPITSAAELNDPQSILLR  
LDTLAQEVALLK  
AQNDQLGWLWGQSR  
AQGFTEDTIVFLPQTDK  
PVAFSVVPTAAA AVSLK  
SDLAVPSELALLK  
FLVGPDGIPIMR  
LGFTDLFSK  
TVAGQDAVIVLLGTR  
LEPPWINVLQEDSVTLTCQGAR  
EIVDSYLPVILDIK  
TLYLADTFPTNFR  
GFFADYEIPNLQK  
GIISALLVPPETEEAK  
FYALSASFEPFSNK  
LQTVLEAVHDLLRPR  
PDDL PFSTVVPLK  
NALALFVLPK  
FVFGTTPEDILR  
NVHLFATPLAASLEEVAPGAR  
GFLLLASLR  
ELLALIQLER  
AALSPLADLHALVLR  
VSASPLLYTLIEK  
LLLSSLGIPVNHIEGSQK

Peptide Sequence

FTVDRPFLFLIYEHR  
VVLAYEPVWAIGTGK  
FIPLIPIPER  
DGTFFPLPIGESVTVTR  
LLGNVLCVLAHHFGK  
AANSLEAFIFETQDK  
GTVAAPSVFIFPPSDEQLK  
TTPPVLDSDGSFFLYSR  
DIEHLTSLDFFR  
GDELADSALEIFK  
SYDWSQITTVATFGK  
GLLQQIGDALSSQR  
LAEGFPLLLK  
EVPLNTIIFMGR  
STVLTIPLEIIK  
VKEEIIAEFVQELR  
GLPPVDFVPPIGVESR  
ELCVPLAVPYLDK  
GLPNVVTSAISLPNIR  
GTVSGTLIGLEFIR  
EILVGDVGQTVDDPYATFVK  
IIIPQDVGLTILK  
KIPNPdffEDLEPFR  
GDTYPAELYITGSILR  
LLGNVLCVRLAR  
SLETEILESLK  
ASSIIDELFQDR  
GSFALSPVESDVAPIAR  
IEIESFYEGEDFSETLTR  
LLSGPYFWSLPSR  
LDNLVAILDINR  
IYTSPTWSAFVTDSSWSAR  
LVFVHSAEGNEFWSALLEK  
TLAQLNPESLFIASK

Peptide Sequence

IVASTLSNPELFEEWTGNVK  
DFPLVDGHNDLPLVLR  
TTVILPLAPFVR  
SSFTVQDLKPFTEYVFR  
FSGTWYAMAK  
VSFLSALEEYTK  
IALGGLLPASNLR  
LASLFPALFSR  
FSISATYDLGATLLK  
LTVGAAQVPAQLLVGALR  
ISLSPEYVFSVSTFR  
SIPTCTDFEVIQFPLR  
LLTSFLPAQLLR  
LNIFDQSFYDLSLQTSEIKKKK  
VIPGPPALTLVPAELVR  
DITDTLVAVTISEGAHHLDR  
DIASGLIGPLIICK  
FSLVSGWGQLLDR  
SDVGFLLPPFPTLDPEEK  
VFVSVLDVNDNAPEFPFK  
ADEFNLNWHALFESIK  
TDTGFLQTLGHNLFGIYQK  
DVWGIIEGPIDAAFTR  
DLNELQALIEAHFENR  
RLDFTGNLIEDIEDGTFSK  
TLTLPSLPLNSADEIYELR  
YSVILLDTLLGR  
LEGSAPTDLVDSLTTIPELK  
AATFGLILDDVSLHLTFGK  
TAFDDAIAELDTLNEDSYK  
LLQDSVDFSLADAINTEFK  
ATLVCLISDFYPGAVK  
IGVAIGDQILDLSIIK  
DSPVLIDFFEDTER

Peptide Sequence

FSVPAGIVIPSFQALTAR  
EFEGEEYEILGITR  
GILSGTSDLLTFDEAEVR  
SANLVAATLGAILNR  
AAFDDAIAELDTLSEESYK  
DASIVGFFDDSFSEAHSEFLK  
SLADELALVDVLEDK  
FYPEDVSEELIQDITQR  
SSAGCEGIGDFVELLGGTGLDPSK  
FTTLVQDLANAFQQEAQTSGK  
HNVDDSLTTVGSLEDETYTVR  
FSTFLSLEAADLK  
NAPAIIFIDELDAIAPK  
DLADELALVDVIEDK  
LVSLDSGLLNSLALTELOFHR  
TAFDEAIAELDTLSEESYK  
FTPNWLSVLVDNLPGTK  
EPCVESLVSQYFQTVTDYGK  
LSVGLEDEEDLLEDLDQALK  
EFVEEFIWPAIQSSALYEDR