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MASTERARBEIT

“Purification of the Cytokine CXCL5 via Immuno- precipitation and Quantification from Different Matrices Applying NanoUHPLC-ESI-Orbitrap”

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Abstract

For the quantification of biomarker molecules from complex biological samples ELISA and other immunological methods are currently the golden standard in clinical practice. The advantages of ELISA in comparison to other non-immunological quantification methods are its high sensitivity and the ease of implementation without the need of expensive and maintenance-intensive instrumentation. On the other hand, many ELISAs may be quite sensitive to matrix effects and exhibit only limited linear range and accuracy in complex samples. Therefore other detection methods with less matrix sensitivity like mass spectrometry-based methods are of increasing interest for biomarker quantification. In spite of the tremendous technological progress mass spectrometry has experienced in the last few decades, the dynamic range of potential analytes in clinically relevant samples like blood serum are still overpowering the capabilities of even high-end mass spectrometers. This implicates the need for efficient sample prefractionation in combination with high performance liquid chromatography in order to reduce sample complexity. On the basis of cytoplasm samples from Jurkat cells, an efficient separation workflow combining protein separation by electrophoresis with peptide separation by chromatography was optimized. In order to assess the clinically relevant pg/mL range, an additional step of immunoprecipitation was applied for a small low abundant protein. As an example for such a protein the biologically interesting cytokine CXCL5 was chosen as analyte. Although significantly higher signal intensities were achieved for CXCL5 using our separation strategies in combination with the immunoprecipitation step, the lower limit of detection could not be improved below 1 ng/mL.

Zusammenfassung

Für die Quantifizierung von Biomarkermolekülen aus komplexen biologischen Proben stellen ELISA und andere immunologische Methoden zurzeit die Methode der Wahl in der klinischen Anwendung dar. Die Vorteile von ELISA gegenüber anderen nicht Antikörper-basierenden Methoden sind sowohl die hohe Sensitivität der Antikörper als auch die einfache Anwendung der Methode ohne die Notwendigkeit teurer oder wartungsintensiver Instrumente. Andererseits zeigen viele dieser Methoden eine große Anfälligkeit gegenüber Matrixeffekten, wodurch es zu verminderter Genauigkeit und eingeschränktem dynamischem Bereich der Analyse kommen kann. Hieraus ergibt sich ein verstärktes Interesse an anderen Methoden zur Biomarkerbestimmung, welche stabiler gegenüber Matrixeinflüssen wie beispielsweise die Massenspektrometrie. Trotz der außerordentlichen technologischen Fortschritte, die auf dem Gebiet der Massenspektrometrie in den letzten Jahrzehnten erzielt werden konnten, überfordert der riesige dynamische Bereich potentieller Analyten, der in klinisch relevanten Proben, wie Blutserum, zu finden ist, nach wie vor selbst high-end MS Instrumente. Angesichts dessen muss die Probenkomplexität durch effiziente Fraktionierungsschritte oder Auftrennungsschritte bereits vor der Messung im Massenspektrometer reduziert werden. Am Beispiel von Cytoplasmaproben von Jurkatzellen wurde eine effiziente Trennmethodik, bestehend aus einem Proteinfractionierungsschritt mittels Elektrophorese und einem Peptidauftrennungsschritt mittels Chromatographie, optimiert. Um den klinisch interessanten pg/mL Konzentrationsbereich analytisch zu erfassen, wurde zusätzlich ein Immunpräzipitationsschritt zur spezifischen Anreicherung eines ausgewählten kleinen, niedrige-abundanten Proteins durchgeführt. Als Beispiel für ein solches Protein wurde das Cytokin CXCL5 ausgewählt. Obwohl deutlich höherer Signalintensitäten für CXCL5 erzielt werden konnten, wenn die obig beschriebene Trennmethodik um die selektive Immunpräzipitation erweitert wurde konnte eine untere Nachweisgrenze von 1 ng/mL nicht unterschritten werden.

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List of Abbreviations

<i>UHPLC</i>	<i>Ultra High Performance Liquid Chromatography</i>
<i>SN</i>	<i>Supernatant</i>
<i>con</i>	<i>Control</i>
<i>CYT</i>	<i>Cytosol</i>
<i>NE</i>	<i>Nuclear extract</i>
<i>MS</i>	<i>Mass spectrometry</i>
<i>QQQ</i>	<i>Triple Quadrupole</i>
<i>HUVEC</i>	<i>human umbilical vein endothelial cells</i>
<i>ELISA</i>	<i>Enzyme-linked immunosorbent assay</i>
<i>HRP</i>	<i>Horse radish peroxidase</i>
<i>DDA</i>	<i>Data-dependent acquisition</i>
<i>ESI</i>	<i>Electrospray ionization</i>
<i>HCD</i>	<i>Higher-energy collision-induced dissociation</i>
<i>HPLC</i>	<i>High-pressure liquid chromatography</i>
<i>iTRAQ</i>	<i>Isobaric tags for absolute and relative quantification</i>
<i>LC</i>	<i>Liquid chromatography</i>
<i>MRM</i>	<i>Multiple reaction monitoring</i>
<i>MS</i>	<i>Mass spectrometry</i>
<i>MS/MS</i>	<i>Tandem mass spectrometry</i>
<i>PAI</i>	<i>Protein abundance index</i>
<i>PSAQ</i>	<i>Protein standard absolute quantification</i>
<i>FDR</i>	<i>False discovery rate</i>
<i>PSM</i>	<i>Peptide-to-spectrum match</i>
<i>PTM</i>	<i>Post-translational modification</i>
<i>QTOF</i>	<i>Quadrupole time of flight</i>
<i>SILAC</i>	<i>Stable isotope labeling with amino acids in cell culture</i>
<i>SRM</i>	<i>Selected reaction monitoring</i>
<i>TMT</i>	<i>Tandem mass tag</i>
<i>UPLC</i>	<i>Ultra-high-pressure liquid chromatography</i>
<i>TIC</i>	<i>Total ion chromatogram</i>
<i>XIC</i>	<i>Extracted ion chromatogram</i>
<i>RT</i>	<i>Room temperature</i>
<i>IDs</i>	<i>Identifications</i>
<i>PAA</i>	<i>Poly acrylamide</i>
<i>SDS-PAGE</i>	<i>Sodium dodecyl sulfate poly acrylamide gel electrophoresis</i>

Introduction

Motivation

For thousands of years mankind suffered greatest from those diseases, that emerged relatively early in people's lives as the life expectancy was relatively short. In the United States the leading causes of death in 1900 were infectious diseases ranging from pneumonia over influenza and tuberculosis to gastrointestinal infections.[1] These types of infections alone caused more than 5 million deaths in the year 1900 in the U.S. while heart diseases and cancer, which are the major reasons for death in the present, made up a significantly less part of the total death toll. Along with the improvements in hygiene standards, nutrition and medicinal treatment the overall life expectancy increased significantly, replacing the infectious diseases as major death causes and facilitating the role of cancer and heart diseases.

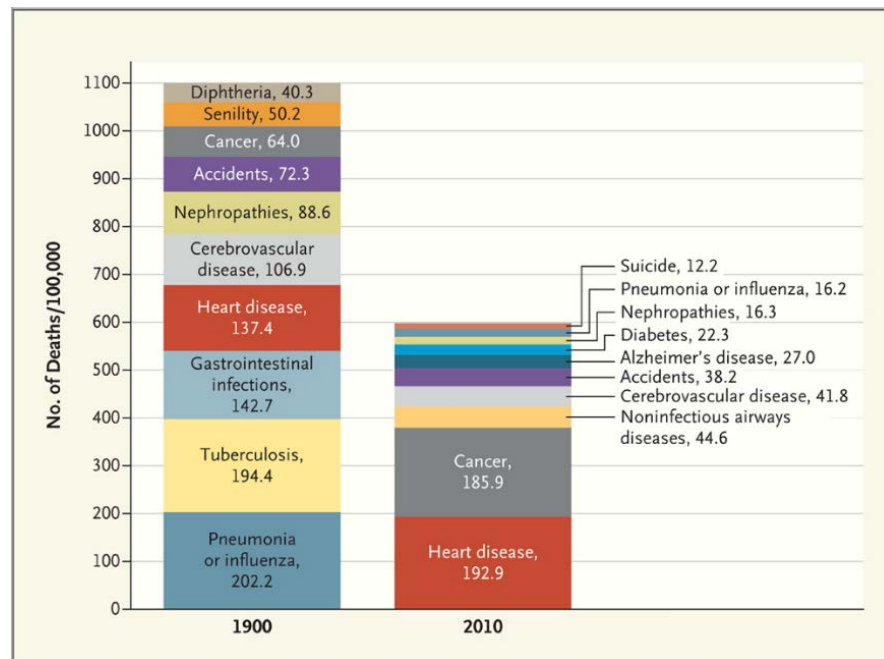


Figure 1: Main death causes in the US in 1900 and 2010 [1]

These two diseases usually do not emerge from one day to the other but mostly require at least some years to develop and outmaneuver the bodies' own defense mechanisms. Hence, the early detection and reliable diagnosis of cancer and heart diseases is of utter importance as the success rates in curing or treating these diseases dramatically increase with early diagnosis. All diseases can be traced back to changes in cell state of the patient's cellular endowment. These changes are often associated with

changes in the protein expression pattern of a specific cellular subpopulation commonly localized in one specific organ or region in the organism.

This observation led to the discovery of a range of so-called “biomarkers”, which represent proteins or other molecules that are known to be up- or down-regulated as a consequence of a certain disease or functional disorder. These biomarkers could not only be utilized for the early diagnosis of certain diseases but also to monitor therapy success, to determine individual responsiveness of patients for specific therapies and for many more applications. For a reliable detection of changes in biomarker levels robust, accurate and selective quantification methods are essential. As human organisms consist of billions of cells belonging to a wide variety of different cell types, finding and reliably quantifying good biomarkers is far from trivial. The huge number of different proteins expressed by the entity of all human cells creates an extremely complex sample. Human blood serum, for example, contains a concentration range of different proteins of about ten orders of magnitude and many hundreds of different types of proteins. Unfortunately many biologically interesting proteins and biomarkers occur at very low concentrations and are therefore hard to detect and quantify in a robust manner. One group of these biologically interesting low-abundant proteins are the so called cytokines.

Cytokines as markers for disease and cancer

Cytokines are mostly small signaling proteins which facilitate and modulate the immunological answer to an inflammatory stimulus.[2] While the major functional role of all cytokines is to manipulate the immune response within an organism, the structural diversity within this class is rather high.[3]

This group of immunomodulating agents can be subclassified either into structural similar groups or functionally related clusters, with the functional subclassification proving to be significantly more useful in terms of clinical relevance. Cytokines can be grouped into three families namely the four- α -helix bundle family, the IL-1 family and the IL-17 family with the four- α -helix bundle family further dividing into the IL-2, the interferon and the IL-10 subfamily. [4]

Functionally all members of the cytokine family can be classified as type 1 or type 2 cytokines. While type 1 cytokines like IFN- γ and TGF- β tend to facilitate cellular immune response, those of type 2 (like IL-4 and IL-10) favor antibody responses. [4]

Beside the already mentioned crucial role for the immune response, cytokines also play an important role in cancer development and the onset and progression of many other diseases. According to Hanahan

and Weinberg the transformation of an ordinary, normal cell to a cancer cell happens in six steps: Self-sufficiency for growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis.[5] Many of these steps can occur due to accumulation of mutations and are mainly controlled by the cell itself. Other important processes for tumor survival and proliferation on the other hand depend on the communication of the tumor cells with the nearest neighboring non-tumor cells the so called stromal cells. One example of this interaction is the initiation of blood vessel remodeling around the tumor to ensure a sufficient supply with oxygen and nutrients for the highly proliferative tumor. Angiogenesis, the creation and remodeling of blood vessels, usually happens during embryogenesis and in the course of wound healing but is otherwise suppressed in adults. By secreting angiogenic growth factors like VEGF to its surrounding the tumor can induce angiogenesis and ensure adequate growth conditions for the tumor. Another important hallmark in tumorigenesis, namely metastasis and tissue invasion, also requires the cooperation of tumor and stromal cells often involving enzymes like matrix metalloproteases for the degradation of the extracellular matrix.[6]

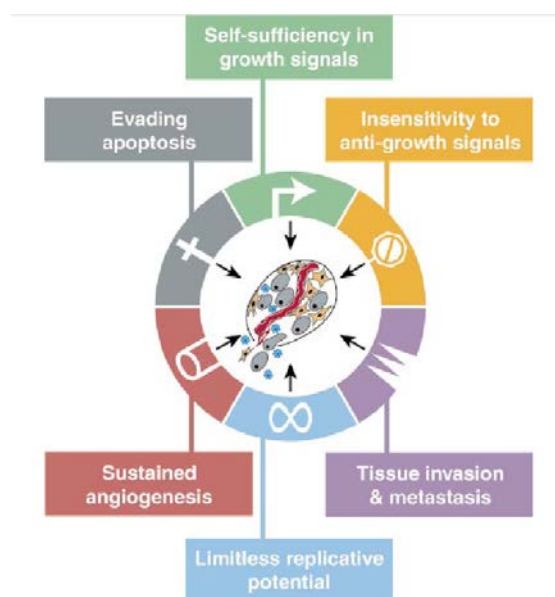


Figure 2: Successively acquired functional disorders en route to cancer [5]

Aside from the previously mentioned hallmarks during cancer promotion Hanahan and Weinberg concluded in 2011 that avoiding the defense mechanisms of the innate immune system and deregulation of the cells own energy metabolism are two more important mechanisms for tumor cell survival and promotion. Furthermore the potential role of chronic inflammation and genomic instability as important prerequi-

sites for cancerogenesis were highlighted.[7] Particularly the tumor promoting effect of chronic inflammation and the evasion of the immunological defense involve cytokine signaling and are therefore likely to be assessed by the determination of blood plasma cytokine levels. One example for a cytokine up-regulated on inflammatory stimulation is CXCL5.

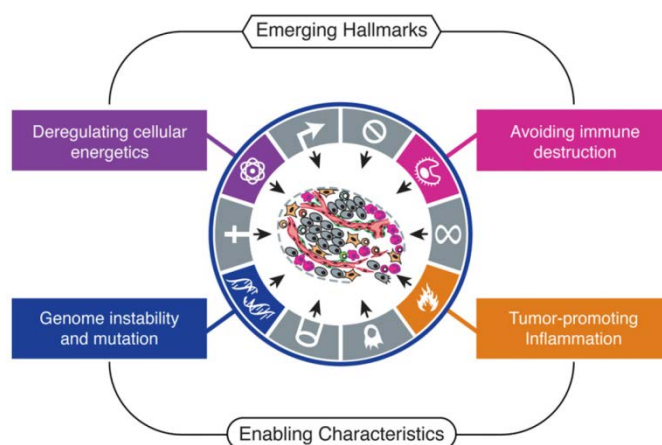


Figure 3: Tumor promoting characteristics and emerging hallmarks for cancer [7]

CXCL5 – the C-X-C motif chemokine 5

The C-X-C motif chemokine 5 (CXCL5) is a small signaling protein with a molecular mass of about 12 kDa. It is also referred to as Epithelial-derived neutrophil-activating protein 78, neutrophil-activating peptide ENA-78 or small-inducible cytokine B5.[8] Furthermore CXCL5 belongs to the C-X-C motif chemokine family and is involved in the activation of neutrophils during inflammation. Post-translational N-terminal proteolytic cleavage into ENA-78(8-78) or ENA-78(9-79-8) occurs after the segregation of CXCL5 from peripheral blood monocytes.[8] CXCL5 is not only produced and secreted by immune cells like monocytes and neutrophils but can also be segregated by non-immune cells like epithelial cells, fibroblasts and endothelial cells.[9] As CXCL5 is expressed and secreted by a broad range of cell types it can be used as a general marker for inflammation, which was also the reason for choosing it as analyte in the course of this work.

Quantification of low abundant marker proteins

As discussed earlier cytokines and other biomarker proteins can serve as valuable indicators in human blood plasma for various diseases. In recent years many biomarker candidates were discovered by mass spectrometric methods but only a very small number could actually be evaluated and utilized in clinical routine diagnostics. This phenomenon can be at least partly related to the conceptual oversimplification of the biomarker model that many scientists used as starting point for their research. Accessing one single

specific cellular state with only one biomarker in such a complex organism as the human body seems a bit optimistic. More realistically a panel of at least ten or more biomarkers could provide the specificity required for a reliable and specific detection of diseased cell states in the human body.

Another potential reason for the failure of mass spectrometry to generate more biomarkers for the clinic is most likely the complex matrix of blood plasma, which greatly hampers sensitive, accurate and reproducible quantification. Most biomarker quantification methods used in clinical diagnostics are based on immunological detection principles like ELISA (enzyme-linked immunosorbent assay) and therefore strongly depend on highly specific antibodies.

While these immunological quantification methods can detect proteins at very small concentrations in the pg/ml range, matrix effects can strongly reduce accuracy and reproducibility. Furthermore, the development and evaluation of specific antibodies against new biomarkers can be very costly and time consuming. Mass spectrometry based methods could overcome these downsides of immunological methods and provide a cost efficient, highly reproducible and fast alternative for routine diagnostic applications. In the following sections the most important immunological and MS-based methods will be described and the advantages and disadvantages of each single method described in more detail.

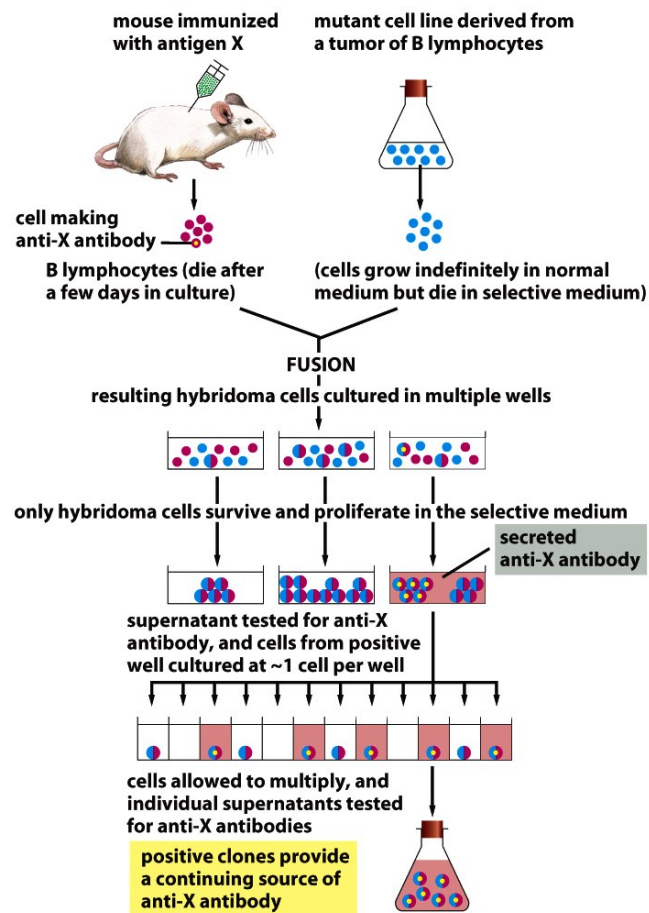


Figure 8-8 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 4: The long and costly path to the production of antibodies [10]

Western Blot

One of the most simple and frequently used immunological quantification methods for proteins is western blot. This technique can be broken down to essentially three different steps. These steps are firstly the separation of sample proteins according to molecular mass by means of a SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), secondly the transfer of the separated proteins from the gel onto the surface of a specific membrane (blotting) and finally the detection of the protein of interest by incubation with a protein specific primary antibody and an enzyme conjugated secondary antibody.

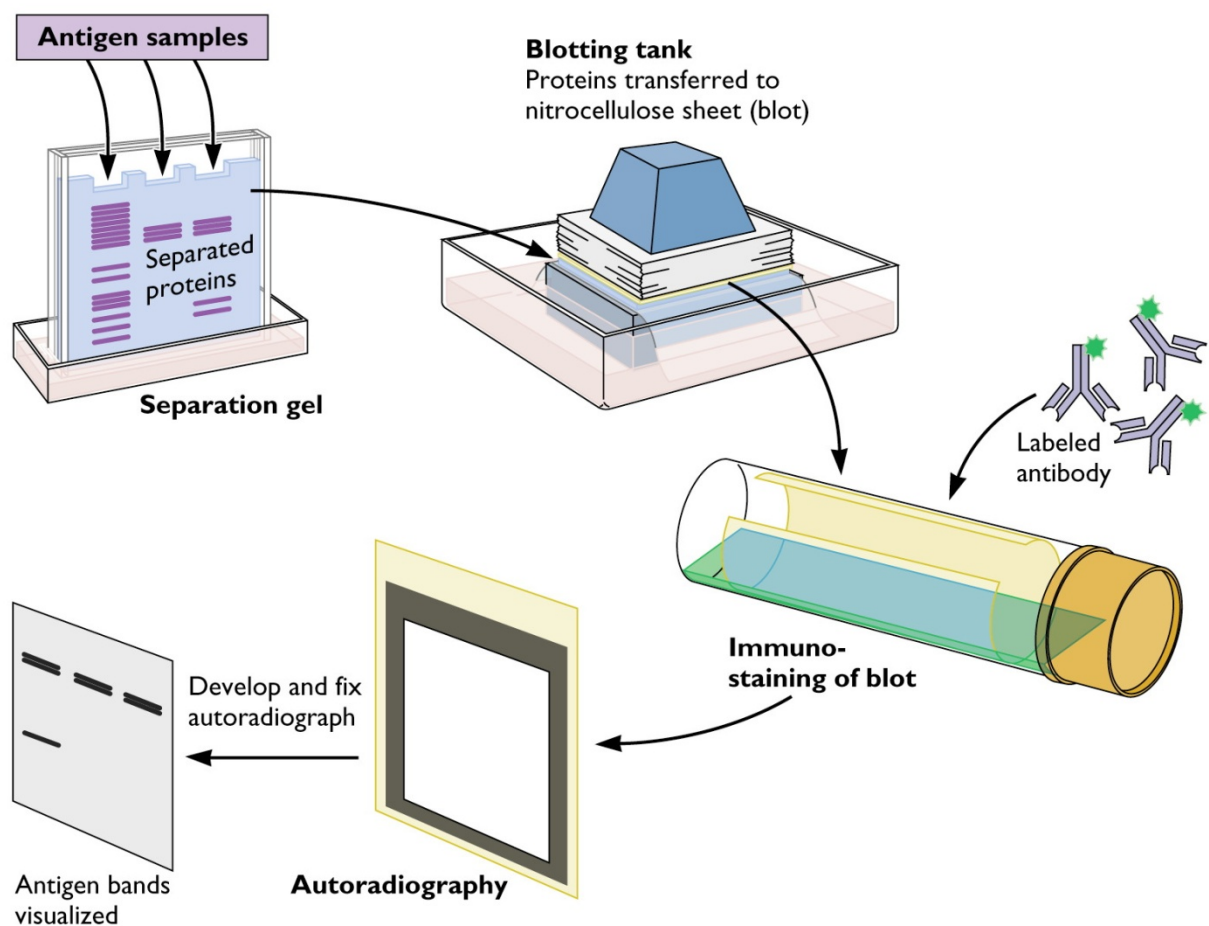


Figure 5: General steps in western blotting [11]

SDS-PAGE

The first step in the course of a western blot analysis is the so called SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein sample, which usually contains many different protein species, is mixed with laemmli buffer that mainly contains bromphenol blue, glycerol, mercaptoethanol and SDS. Mercaptoethanol is required to destroy inter- and intramolecular protein disulfide bonds. SDS denatures the proteins, opens up the complex tertiary and secondary structure of the proteins and covers the surface of all proteins with a uniform negative charge, so that all proteins will migrate to the positive electrode during electrophoresis. Glycerol is added to the sample to increase the density so that the sample will sink down to the bottom of the sample slots in the gel after loading of the samples onto the gel. The bromphenol blue stains the sample intensively blue so that the migration front of the samples during the electrophoresis can be monitored visually.

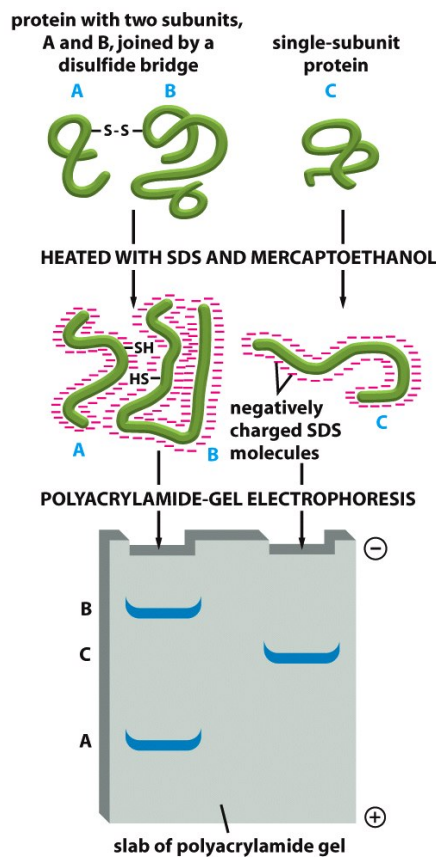


Figure 8-18b Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 6: Main task of SDS and mercaptoethanol for SDS PAGE [10]

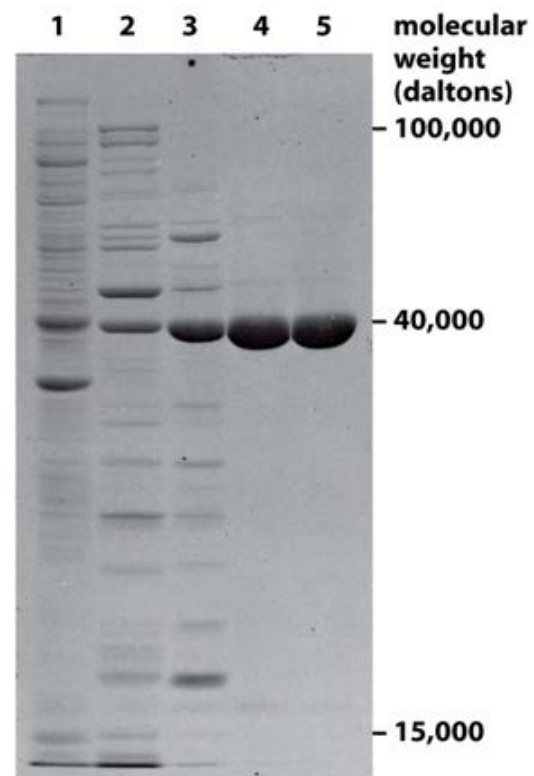


Figure 8-19 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 7: Visualized protein bands separated by SDS PAGE according to molecular weight [10]

Before the sample loading can be performed the acrylamide gel is transferred to an electrophoresis apparatus and buffer, usually Tris-glycine with SDS, added so that the gel is in contact with the buffer. After the addition of Laemmli buffer, the samples are loaded into slots of the vertically arranged polyacrylamide gel. Most polyacrylamide gels consist of two different regions. The upper part of the gel consists of the so called “stacking gel” which has wider pores than the separation gel and consequently does not hinder the migration of the proteins but focusses the sample. The lower part of the gel is called the “separation gel” or “resolving gel” and has smaller pores than the stacking gel and also a higher pH, which both facilitate separation of the proteins. Along with the protein samples a marker is usually loaded onto the gel which consists of several proteins of known different molecular masses. The marker proteins are usually pre-stained and can be used to for the correlation of migration distance to molecular weight. To start the electrophoresis a fixed voltage or current is applied on the gel and kept constant until the blue front has reached the bottom of the gel. The larger molecules are retarded stronger by the gel matrix and stay behind while the smaller molecules migrate faster.

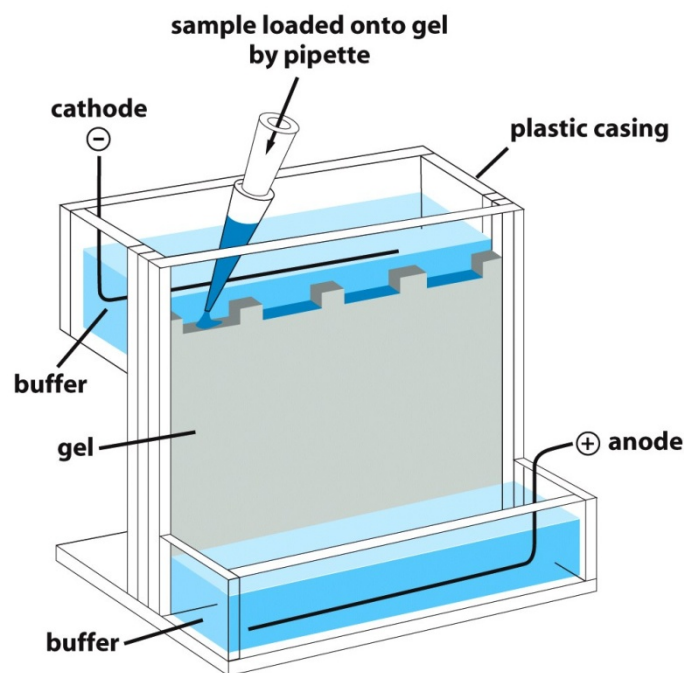


Figure 8-18a Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 8: Sample loading on SDS gel and gel electrophoresis apparatus [10]

Blotting

For the detection of the protein of interest the separated proteins need to be transferred onto the surface of a specific membrane (e.g. nitrocellulose). This can either be performed by tank blotting or semi-dry blotting. Both methods have in common that the still uniform negatively charged proteins are “pulled” out

of the gel towards a membrane that is in direct contact with the gel and very strongly bind all proteins. The build-up of the “sandwich” for both blotting methods is quite similar and shown in Figure 9. The difference between the two methods is in principal the volume of buffer required. While semi-dry blotting requires only small amounts, tank blotting requires typically more than 500ml of blotting buffer. Semi-dry blotting has become more common in recent years due to the fact that the buffer consumption is significantly less and it can also be performed faster when using specialized one-use electrodes.

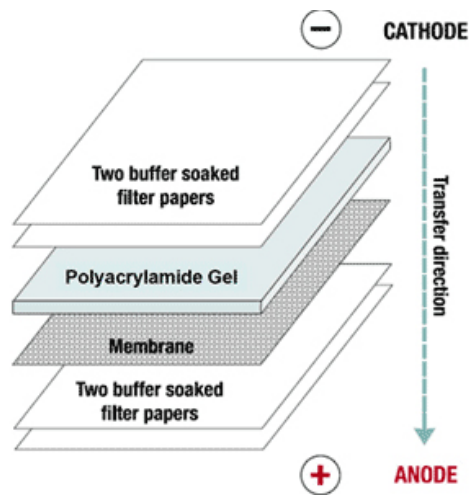


Figure 9: Build-up of a blotting sandwich [12]

Antibody Detection

After the blotting the protein-containing membrane is usually stained with PonceauS solution to confirm the successful transfer from the SDS gel to the membrane. The staining with PonceauS is only reversible and will be washed away during the following incubation steps. Blocking the protein-free surface of the membrane is the next essential step in line. This process is usually performed with milk powder or BSA and mainly serves the task of saturating the surface of the blotting membrane, so that the antibodies applied in later steps do not bind in an unspecific manner to the surface of the membrane but only selectively to the protein species of interest.

Following the blocking the membrane is washed several times and then incubated with the primary antibody which binds specifically to the targeted protein. The most important factor at this stage and probably for the whole western blot analysis is the specificity of the primary antibody. Low quality antibodies often tend to not only recognize the intended analyte but also other proteins, which can lead to false-positive results or an overestimation of the analyte of interest. The incubation with the primary antibody is again followed by several washing steps and the incubation with the secondary antibody. The secondary anti-

body is coupled to an enzyme mainly HRP that provides the signal for the final detection step. The secondary antibody is directed against the animal in which the primary antibody was produced (e.g. rabbit) therefore binding to the primary antibody.

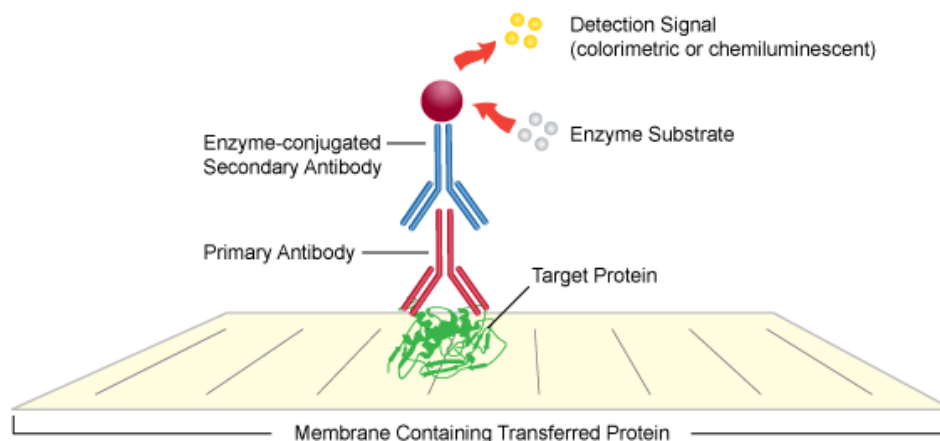


Figure 10: Detection principle of analytes in western blot [13]

The last step in the western blot analysis after some more washing steps is the addition of ECL solution to the membrane. This ECL solution contains mainly hydrogen peroxide and luminal. The luminal gets oxidized in the presence of the horse radish peroxidase by the hydrogen peroxide and emits light. This light blackens a photo-sensitive foil that is placed on top of the membrane. Dark spots on the photo-sensitive foil therefore represent the analyte and the intensity correlates well with the analyte concentration.

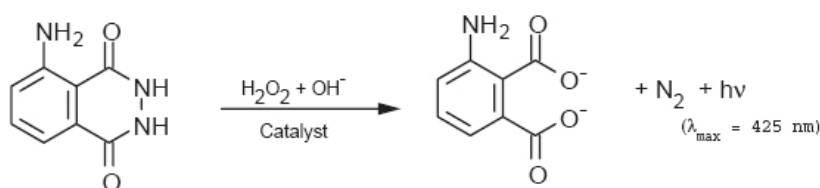


Figure 11: Signal generating reaction for detection via luminol and H_2O_2 [14]

ELISA – Enzyme-linked Immunosorbent Assay

The Enzyme-linked Immunosorbent Assay (ELISA) is currently the most frequently used method for the quantification of proteins from biological samples. Especially in clinical diagnostics and molecular biology ELISA is the standard technique of choice. Most ELISAs can be associated with one of the three kinds indicated in Figure 12. Direct assays require only one antibody and also only one epitope on the surface of the antigen to interact with the antibody. The antigen is therefore unspecifically bound on the surface of a 96-well plate and then directly detected with the use of a single antibody. Another positive factor of direct detection is the absence of potential cross reactivity arising from a secondary detection antibody. One of the downsides of direct detection are that every new antibody for a specific antigen needs to be labeled with an enzyme for detection, which is time consuming, expensive and can also hamper the effective antibody-antigen interaction.[15] Another negative factor is the minimal signal amplification compared to the indirect or the Sandwich assay. For indirect detection assays the antigen is also bound directly to the wellplate but instead of using only a single antibody two antibodies are applied. Firstly, a primary antibody that recognizes the antigen is utilized and followed by a secondary antibody which is coupled to an enzyme and directed against the primary antibody. The last step of the assay is the enzyme-substrate reaction, which is detected by means of a platereader photometer. One advantage of an indirect assay is that for all primary antibodies arising from one animal species the same secondary antibody can be used saving time and money and conserving the maximal antibody-antigen affinity for the primary antibody. Another positive effect is the flexibility in the choice of the enzyme-substrate visualization reaction.

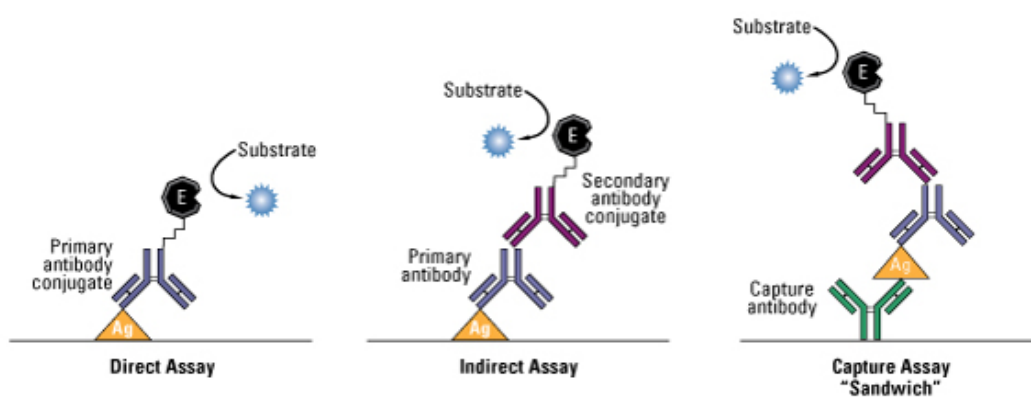


Figure 12: Different types of ELISA formats [15]

A third type of ELISA is the so called Sandwich ELISA. As indicated in Figure 13 the 96-well plate, in which the assay is performed, is coated with a capture antibody that immobilizes the target antigen when the sample is applied to the well plate. For the detection of the captured antigen a second antibody is used that recognizes another specific site of the antigen and binds to it. In most cases the second antibody is not directly coupled to the enzyme giving rise to the signal but coupled to biotin, which binds the enzyme via the streptavidin-biotin interaction, or another antibody which then carries the signalling enzyme. The signaling enzyme is usually not directly coupled to the antibody as the indirect coupling via the streptavidin-biotin complex causes improved signal intensity due to multiple signaling enzymes per detection antibody.

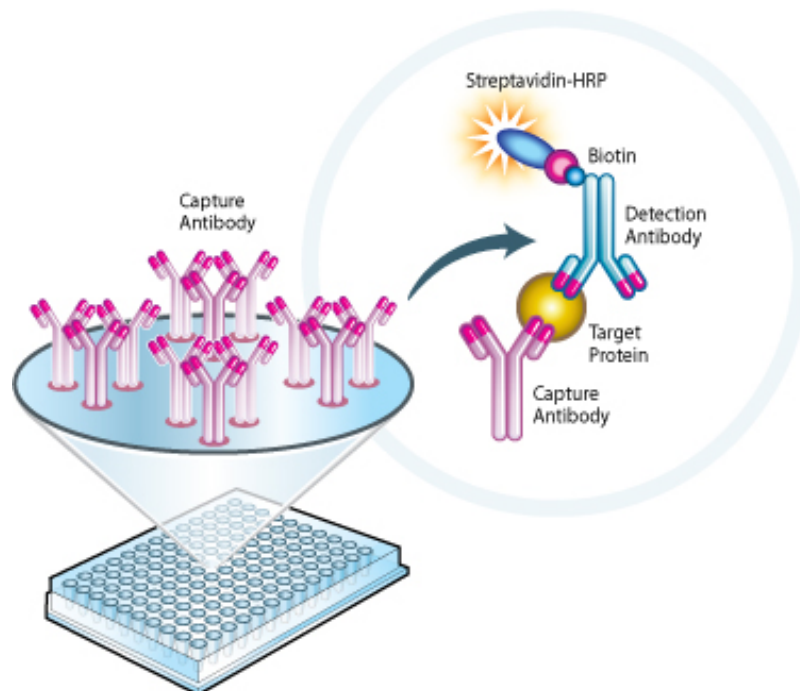


Figure 13: Detection principle of Sandwich ELISA [16]

Most ELISAs available on the market at the moment are Sandwich ELISAs. According to the guidelines of many of the commercially available ELISA kits the manufacturers state that a concentration range from the low ng/ml to the low pg/ml range can be quantified even in very complex matrices like plasma or serum.

Immunoprecipitation (IP)

Immunoprecipitation can be used for the enrichment and purification of specific proteins or cells from complex samples. This method also utilizes the highly specific antigen-antibody interaction between either celltype-specific marker proteins located on the cell surface or proteins in solution. A common way to immobilize the antibodies is given with the so called Dynabeads™. These are magnetic beads coupled with either protein A or protein G that can bind IgG or IgA antibodies specific for a certain antigen.[17] As indicated in Figure 14 the beads coated with protein G are incubated with the chosen IgG sample and bind to the protein G via the constant region of the antibody. The bead - protein G – IgG complex is then incubated with the sample, which contains the target protein, and binds the protein of interest. The beads are then washed and the target protein eluted from the antibody.

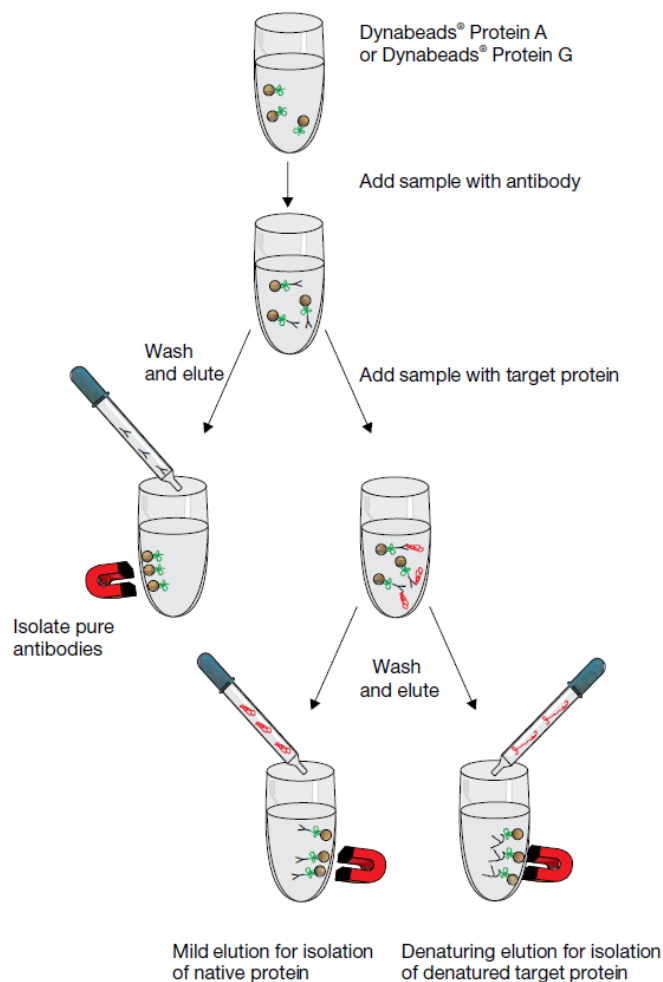


Figure 14: IP methodology using protein A or protein G coupled magnetic Dynabeads™ [17]

Quantification Methods based on Mass Spectrometry

Besides the immunological quantification methods also mass spectrometric techniques offer great potential for the reliable and accurate quantification of marker proteins in complex matrices. The big advantage of mass spectrometric techniques over immunologically based methods is the independency from antibody development and so the assay development could be achieved faster and cheaper. A downside of mass spectrometric methods is that the selective and accurate quantification in complex matrices is still problematic. Furthermore modern high performance mass spectrometers are costly and complex instruments that demand skilled and experienced operators as well as regular maintenance and performance evaluation. Several MS-based protein quantification methods have been developed especially in the last 15 years ranging from label-free methods over spiking methods applying peptides or proteins to labeling methods utilizing isotopic labels.

Labeling Methods

Isotope Coded Affinity Tags – ICATs

One approach for relative quantification in proteomics uses so called isotope-coded affinity tags (ICATs). For the comparison of two different samples (e.g. cell lysate of treated and untreated cells) both samples are labeled separately with two different types of labels, namely light and heavy reagent, prior to pooling, proteolytic digest and LC/MS analysis.

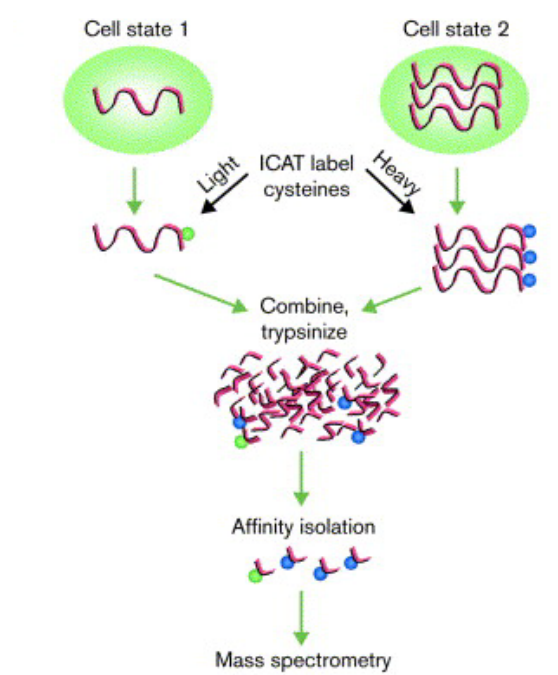


Figure 15: Scheme for ICAT labeling of protein samples [18]

These labels differ in the mass of their linker groups due to use of light and heavy isotopes (^{12}C and ^{13}C or ^1H and ^2H). Both, the heavy and the light reagent, bind to all cysteines in the sample and hence increase the mass of each protein by a certain value per cysteine incorporated in the protein. The separately labeled samples are then pooled, further processed and proteolytically digested. The resulting labeled peptides are then separated from non-labeled peptides by affinity chromatography with avidin. The analysis of the labeled peptides is afterwards carried out by LC – MS/MS. The relative peptide quantification is performed by determining the relative signal intensities of corresponding peptide ions, which show mass differences equal to the mass difference between light and heavy label. The peptide identification is carried out by MS/MS of the chosen peptides.[19],[20]

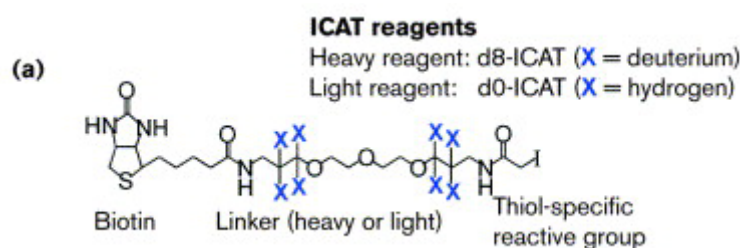
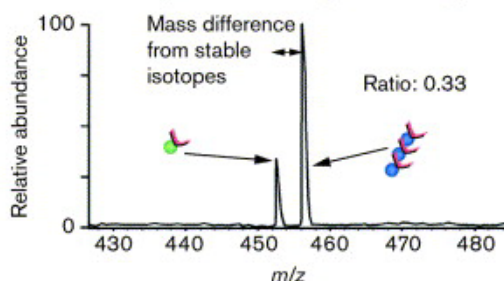
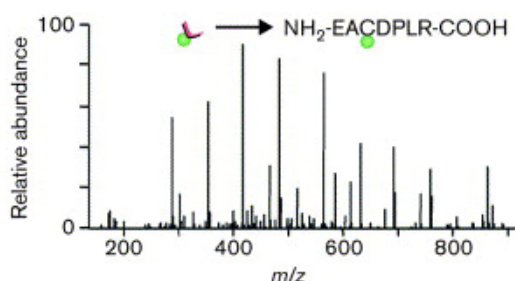


Figure 16: Structure of ICAT reagent [18]

Quantitate relative protein levels by measuring peak ratios



Identify peptide by sequence information (MS/MS scan)



Current Opinion in Chemical Biology

Figure 17: MS and MS/MS of labeled peptides [18]

Isobaric Tag for relative and absolute Quantitation – iTRAQ

Another way to quantitate peptides from complex mixtures are isobaric tags for relative and absolute quantitation (iTRAQs). In principle the general idea of ICAT and iTRAQ are similar in terms of labeling a set of different samples with distinguishable labels, pooling, digesting and analyzing the samples in the MS. While ICAT labels can be differentiated by mass initially, iTRAQ labels have the same overall mass and can only be differentiated upon fragmentation. Therefore relative quantification happens on the MS² level instead of the MS¹ level. In the MS² the iTRAQ labels are fragmented and the relative intensities of the different reporter group masses can be compared and associated with the corresponding sample. As all iTRAQ labels have the same overall mass but variable reporter group masses a balance group is incorporated in the label to generate a uniform total mass of the label. The third functional group is an amine specific peptide reactive group which binds to the N-terminus and sidechain amines of all peptides in the sample.

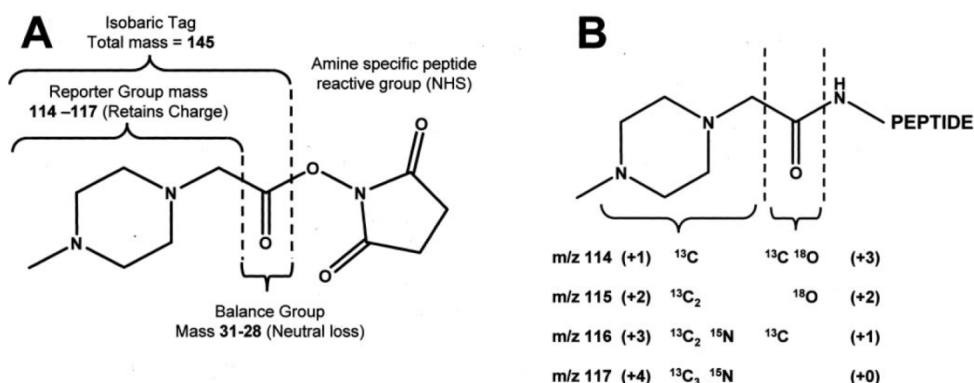


Figure 18: A - Structure of the iTRAQ label, B - Isotopic pattern for different iTRAQ labels [21]

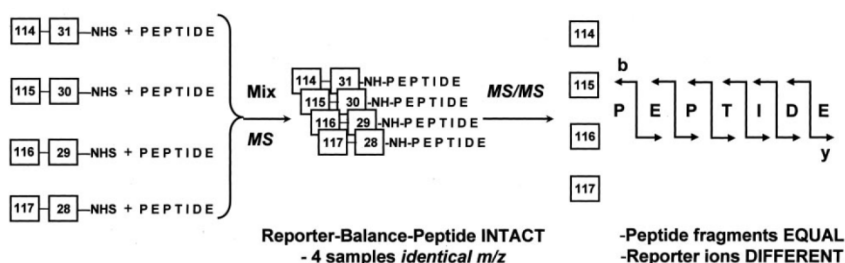


Figure 19: MS and MS/MS fragmentation pattern of labeled peptides [21]

Stable Isotope Labeling of Amino Acids in Cell Culture- SILAC

SILAC is a metabolic labeling method based on the need of mammalian cells for essential amino acids (e.g. histidine or leucine), which they can't synthesize themselves. Hence, mammalian cells in culture have to be supplied with essential amino acids for successful cultivation. For SILAC, one amino acid in cellculture medium (e.g. arginine or leucine) is replaced by a heavy variant using ^{13}C instead of ^{12}C . These heavy SILAC amino acids do not change cell morphology or cellular growth rates and allow to distinguish between peptides originating from cell state A (light normal amino acids) and cell state B (heavy SILAC amino acids).

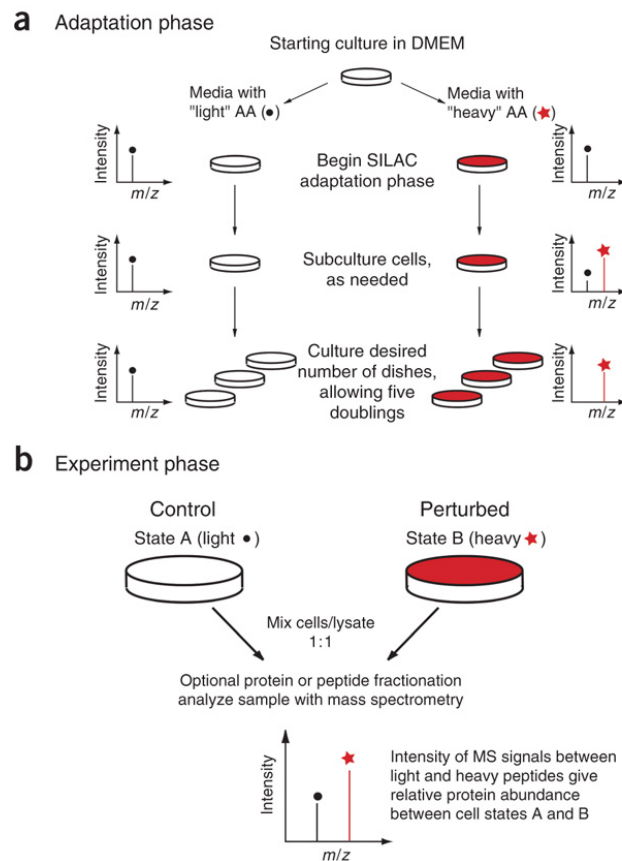


Figure 20: Classical SILAC - comparison of cell states [22]

For this enzymatic labeling technique the samples to be compared are digested in separate entities. One of the protein samples is digested in the presence of normal water while the other sample is digested in the presence of H_2^{18}O . The application of ^{18}O in combination with trypsin results in the introduction of two heavy oxygens at the C-terminal site of all proteolytic peptides. The mass of all proteolytic peptides is therefore shifted by +4. When another protease than trypsin is used for digestion this mass shift may differ. Endo-Lys-N, for instance, introduces only one heavy oxygen into each proteolytic peptide and therefore increases the mass only by +2. This method can only be used for the comparison of two different samples and does not account for sample treatment variations occurring prior to the digestion. [23]

Protein and Peptide Spiking Methods

Absolute Quantification of Proteins -Protein AQUA

Most of labeling methods described previously can only be applied for relative quantification and direct comparison of a few samples. AQUA on the other hand represents a technique that can be used for both relative and absolute quantification. It was developed by Gygi and colleagues and is based on the addition of a defined amount of stable isotope labeled peptides to the sample prior to digestion.

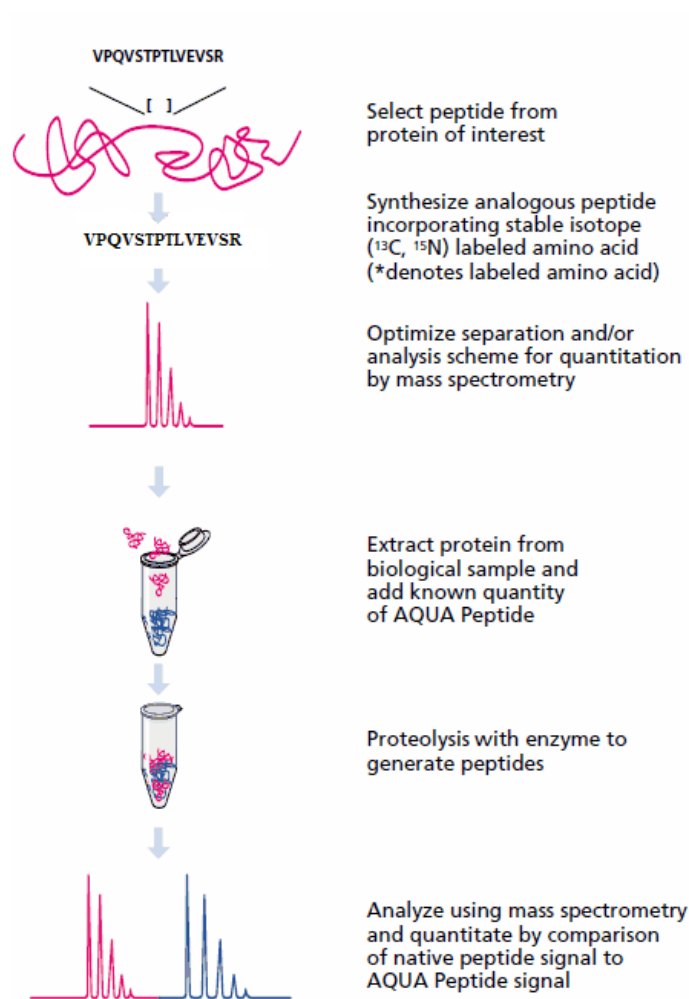


Figure 21: Workflow for absolute quantification of proteins using AQUA peptides [24]

Label-free Methods

Mainly two label-free approaches are commonly used in proteomic protein quantification currently. While one method is based on the measurement of the MS¹ signal intensities of peptides unique for one specific protein, spectrum counting, as the nomenclature of the approach already indicates, counts the number of identified peptides per protein and relates this information back to the quantity of the protein initially present.

Spectrum counting methods

Spectrum counting methods utilize the observation that proteins present at higher abundances give rise to a greater number of different peptides. The simplest spectrum counting method is probably peptide counting where only the number of peptides identified for a protein is used as an index for protein abundance. A method with better reproducibility and higher dynamic range than peptide counting is spectrum counting. Spectrum counting uses the number of peptide-to-spectrum matches as quantitative measure.[25] Another spectrum counting method for the quantitative analysis of proteins uses the average total intensity of all fragment ion spectra annotated to a protein. This measure provides improved accuracy and a wider dynamic range than spectrum counting.[26] The protein abundance index (PAI) is a measure frequently used for absolute protein quantification. Especially for the comparison of great numbers of samples the PAI is commonly used. Instead of only counting spectra or peptides it also takes into account the number of observable peptides per protein by dividing the observed peptides by the number of observable peptides.

MS¹-intensity based methods

In contrary to spectrum based approaches MS¹-based quantification methods refer to the intensity of intact peptide signals. The intensity assessment on the MS¹ level is usually performed by integrating the corresponding m/z value over its chromatographic elution window. Not surprisingly the signal intensities of the peptides originating from the same protein will not be equal due to different ionization properties and other factors. Hence these different intensities referring to one protein need to be combined to assess the protein concentration difference between the samples of interest. Different methods for the protein abundance assessment include adding up the peptide intensities corresponding to the same protein entity or averaging the individual peptide changes between two samples. [27]

Triple Quadrupole Mass Spectrometer(QqQ) and SRM/MRM

The instrument most commonly used and probably best suited for quantitative targeted proteomics is the triple quadrupole mass spectrometer. The heart of the instrument is an array of three quadrupoles (Q1, Q2 and Q3). Each of these quadrupoles consists of 4 conducting metal rods that can manipulate ions of specific m/z ratios by applying a distinct combination of constant and varying voltage. (<http://www.lcresources.com/resources/exchpost/quadrupole.pdf>) The three quadrupoles can be used in principle for a wide variety of different operation modes of which SRM/MRM is the most useful for targeted proteomics. SRM and MRM are commonly used terms referring to the same method in general. While SRM stands for “Selected Reaction Monitoring”, MRM is the short form of “Multi-reaction Monitoring”. As indicated in Figure 22 SRM/MRM is based on the selection of one peptide in the first quadrupole representative for the protein of interest, which is then fragmented in the second quadrupole and a further selection of the fragments performed in the third quadrupole providing additional specificity.

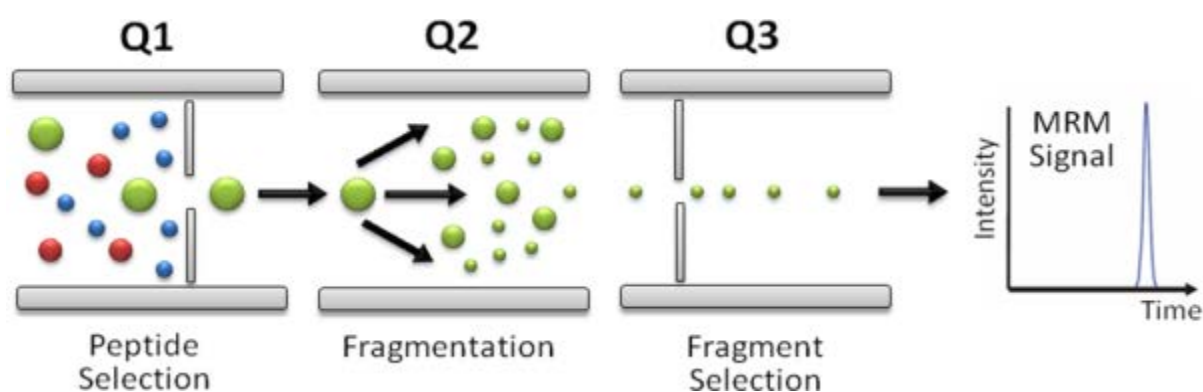


Figure 22: Scheme of SRM/MRM principle [28]

Selected Ion Monitoring (SIM)

This approach narrows the monitored m/z region down to a small specific m/z window (common width of one m/z) containing the analyte m/z and ignores all other species with different m/z values. Not surprisingly for analytes in complex matrices this approach is best performed with high-resolution mass spectrometers enabling the operator to distinguish and quantify peptides of very small mass differences on the MS^1 level. The quantification is hereby based on the intact peptide signal intensity from the MS^1 .

To observe another peptide than the desired one with the same exact mass may seem very unlikely but in fact it does happen. So to back up the identity of the observed peptide another parameter namely the retention time can be utilized. The retention time in combination with the exact mass as measure of identi-

ty is not just used in selected ion monitoring but also in normal shotgun analyses. The data analyses software MaxQuant implemented an option called “Match between runs”, which can identify peptides on the basis of their exact mass and retention time when this peptide was successfully identified in another sample of the same batch due to its MS² spectrum.

Experimental Approach for this Work

The quantification of biological relevant marker proteins from complex sample material like blood serum is most commonly performed with immunological detection methods like ELISA. These quantification methods often show high sensitivity also in complex biological matrices but are quite costly and suffer from poor reproducibility. Another down-side of immunological methods is the long lead time and the high costs for assay development. Other methods, like MS-based methods, could provide an interesting alternative to the currently established “golden standard” in the clinics. The aim of this work was to develop a sensitive and selective MS-based methodology for the quantitative detection of the cytokine CXCL5. The cytokine was spiked into matrices of different complexity at a concentration range from 1 pg/mL up to 1 µg/mL, which corresponds to six orders of magnitude, and analyzed via shotgun mass spectrometry. To reduce potential matrix effects and enrich CXCL5 prior to the analysis immunoprecipitation was applied. The initial immunoprecipitation protocol was optimized for the enrichment of CXCL5 and the efficiency of the precipitation assessed via Western Blot analysis. The immunoprecipitated sample and the non-immunoprecipitated sample were analyzed in parallel via shotgun mass spectrometry. In the course of this work also the LC-MS/MS methodology for the measurement of the samples was optimized.

Experimental

Antibodies, recombinant proteins and complex matrices as background

- Primary antibody – anti-CXCL5 antibody, Rabbit, polyclonal to CXCL5, 0,5 mg/mL (Abcam, no: ab9802) stored at -20°C
- Secondary antibody – anti-rabbit antibody HRP-conjugated stored at 4°C
- Recombinant CXCL5 protein, expressed in E.coli, >98%, 20 µg (Abcam, ab9803) reconstituted in 40 µL water bidest. and diluted to 400 µL with PBS 0,1% BSA stored at -80°C
- Supernatant of human umbilical vein endothelial cells supplemented with 10% FCS
- Human serum from a healthy person
- SN of A-Haut cells stimulated with IL-1b
- SN of HUVEC cells (untreated)

SDS-PAGE

Reagents

- H₂O bidest.
- PDA, 1,4-Bis(acryloyl)piperazine, BioXtra, suitable for electrophoresis, ≥99.0% (TLC) (Sigma)
- Acrylamide 4x, Recrystallized, >99.5%; pH 6-7; UV abs. (A1 cm, 10% in water) 300 nm: <0.1; conductivity (40% in water): <5 µS; low acrylic acid. MW 71.08 (Gerbü)
- TRIS x, Tris(hydroxymethyl)aminomethane, 2-Amino-2-(hydroxymethyl)-1,3-propanediol, THAM, Tris base, Trometamol, ≥99.9%; A 1 cm 10% in water 260nm: <0.02 / 40% in water 430nm: <0.02; MW 121.14 (Gerbü)
- HCl, Hydrochloric acid 32% for analysis EMSURE® 1 * 1 l (MERCK (MDA) INCL SCHUCHARDT)
- APS, Ammonium peroxodisulfate for analysis EMSURE® ACS, Reag. Ph Eur 1 * 500 g (MERCK (MDA) INCL SCHUCHARDT)
- TEMED, N,N,N',N'-Tetramethylethylenediamine, suitable for electrophoresis, ≥99% (Sigma)
- SDS 4x, Sodium dodecylsulfate, Na laurylsulfate; ≥99.0%; UV abs. (A 1cm 1% in water) 260nm:<0.2; powder; FW 288.38 (Gerbü)
- 2-Propanol LC-MS CHROMASOLV® (Sigma)
- 2-Mercaptoethanol for molecular biology, for electrophoresis, suitable for cell culture, BioReagent, 99% (GC/titration) (Sigma)
- Glycerol 85% for analysis EMSURE® Reag.Ph Eur 1 * 1 l (MERCK (MDA) INCL SCHUCHARDT)

- Bromophenol blue sodium salt water-soluble indicator ACS 1 * 5 g (MERCK (MDA) INCL SCHUCHARDT)
- TRIS x, Tris(hydroxymethyl)aminomethane, 2-Amino-2-(hydroxymethyl)-1,3-propanediol, THAM, Tris base, Trometamol, ≥99.9%; A 1 cm 10% in water 260nm: <0.02 / 40% in water 430nm: <0.02; MW 121.14 (Gerbu)
- SDS 4x, Sodium dodecylsulfate, Na laurylsulfate; ≥99.0%; UV abs. (A 1cm 1% in water) 260nm:<0.2; powder; FW 288.38 (Gerbu)
- Urea, Harnstoff, Ultrapure Reagent (Gerbu)
- Thiourea, ACS reagent, ≥99.0% (Sigma)
- CHAPS, Cholamidopropyltrimethylammonio-1-propanesulfonic acid; >98%; zwitterionic compound; MW 614.89 (Gerbu)
- Sodium thiosulfate pentahydrate for analysis EMSURE® ACS,ISO, REAG. PH EUR 1 * 500 g (MERCK (MDA) INCL SCHUCHARDT)
- SDS 4x, Sodium dodecylsulfate, Na laurylsulfate; ≥99.0%; UV abs. (A 1cm 1% in water) 260nm:<0.2; powder; FW 288.38 (Gerbu)
- Precision Plus Protein™ Dual Color Standards #161-0374 (Bio-Rad)
- TRIS x, Tris(hydroxymethyl)aminomethane, 2-Amino-2-(hydroxymethyl)-1,3-propanediol, THAM, Tris base, Trometamol, ≥99.9%; A 1 cm 10% in water 260nm: <0.02 / 40% in water 430nm: <0.02; MW 121.14 (Gerbu)
- Glycine Ultrapure min. 99,5 %, For molecular biology (Gerbu)
- Methanol TECHNICAL Plastic bottle 5l 1 * 5 l (VWR BDH Prolabo)
- Acetic acid (glacial) 100% anhydrous for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 2,5 l (MERCK (MDA) INCL SCHUCHARDT)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µl) (Socorex)
- Mini-PROTEAN® Tetra Cell Electrophoresis system including 1 mm spacer glass plate, short plates, casting frames, casting stand, tank and lid and 10 well comb and 15 well comb (Bio-Rad)
- Desiccator 150mm (VWR)
- Water-jet vacuum pump (VWR)
- Erlenmeyer flasks (VWR)

- Aluminium foil
- WHATMAN 3MM Filterpapierbögen 460 x 570mm (VWR)
- PowerPac Universal Power Supply (Bio-Rad)
- Gel loading tips 200 µL (Starlab)
- Plastic bowls
- Tube, Safe-Lock, PP, 1.5ml, clear (Eppendorf)
- Tube, Safe-Lock, PP, 0.5ml, clear (Eppendorf)

Solutions

SampleBuffer (SB)

22,5g Urea ad 7,5M
 5,7g Thiourea ad 1,5M
 2g CHAPS ad 4%
 125µL 20% SDS ad 0,05%
 5ml 1M DTT ad 100mM
 Filled to 50ml with H₂O bidest.
 1ml aliquots stored at -20°C; well mixed after thawing

30% Acrylamide/PDA (piperazine di-acrylamide) solution

292 g Acrylamide
 5 g PDA
 Filled to 1L with water bidest.
 Stored at 4°C

5x Laemmli Buffer

5ml 1M Tris-HCl pH=6,8 ad 250mM
 2g SDS ad 10%
 10ml Glycerin ad 50%
 0,05g Bromphenolblau ad 0,25%
 H₂O ad 17,5ml
 Aliquoted into 437,5 µL aliquots and stored at 4°C
 62,5 µL of mercaptoethanol added just before use to each aliquot (ad 12,5%)
 Complete 5x laemmli buffer stored at 4°C

2M Tris-HCl pH 8,8

242,28g Tris
 Adjusted to pH 8,8 with HCl
 Filled to 1l with H₂O bidest.
 Stored at 4°C

1M Tris-HCl 6,8

60,57g Tris
 Adjusted to pH 6,8 with HCl
 Filled to 0,5l with H₂O bidest.
 Stored at 4°C

Electrode buffer

100ml 10x Tris-Glycine buffer
5ml 20% SDS ad 0,1%
Filled to 1l with H₂O bidest.
Stored at RT

10x Tris-Glycine buffer

60g Tris ad 25mM
288g Glycine ad 192mM
Filled to 1l with H₂O bidest.
Stored at RT

Fixation solution

500 mL methanol (technical)
100 mL glacial acetic acid
400 mL water bidest.
Stored at RT

Practical Procedure for SDS PAGE for Western Blot

Preparing the Gel

For the separation gel for two 12% gels 4,8 mL of a 30% Acrylamide/PDA solution, 2,25 mL of a 2 M Tris-HCl (pH 8,8) and 4,83 mL of water are added to an Erlenmeyer flask and mixed. For the preparation of two 13% gels the separation gel is prepared with 5,2 mL acrylamide, 2,25 mL of 2 M TRIS-HCl buffer (pH 8,8) and 4,427 mL of water are added. The stacking gel is identical for 13% and 12% gels and 1,066 mL of 30% Acrylamide/PDA solution, 1 mL of 1 M Tris-HCl (pH 6,8) and 5,86 mL of water are transferred to an Erlenmeyer flask and mixed. 2 mL of the solution for the separation gel are transferred to a small beaker and the two Erlenmeyer flasks are covered with aluminium foil and degassed for 10 min. While the solutions are degassing the electrophoresis gel casting unit including the glass plates is assembled.

To the small beaker 5 µL of TEMED and 20 µL of 10% APS is added, mixed and quickly 500 µL pipetted between the assembled glass plates for the gels to plug the assembly and prevent from later leakage. The separation gel solution and the stacking gel solution are removed from the degasser after 10min. The border of the separation gel is marked on the glass plate with a permanent marker 2 cm(for Western Blot) and 2,5 cm (for Shotgun analysis) below the upper edge of the glass plate facing towards the operator. To the Erlenmeyer flask with the separation gel 50 µL of 20% SDS solution, 7,5 µL of TEMED and 45 µL of 10% APS are added, mixed and quickly pipetted between the glass plates up to the mark. On top of the separation gel 200 µL of 90% isopropanol are added to cover the gel. The gel was then allowed to

polymerize for at least 30 min. After this 30 min the isopropanol is removed by first pouring off and then drying the gel carefully with filter paper. To the stacking gel solution prepared previously 40 μ L of 20% SDS, 8 μ L of TEMED and 40 μ L of 10% APS are added, mixed and quickly pipetted on top of the separation gels up to the very edge of the lower glass plate. A 10-well or 15-well comb was then placed between the glass plates avoiding air bubbles and the gels are allowed to polymerize for at least 45 min. The gels are to be used within 24h.

Loading of the Gel

The polymerized gels are transferred from the gel casting stand to the electrode assembly and fixed with the higher glass plate facing outwards. The space between is filled with electrode buffer just between the edges of the higher and lower glass plates and the comb carefully removed. One of the loading schemes is provided below in Table 1. The samples, the marker and the sample buffer are then loaded according to the loading scheme using gel loading tips and the space between the two gels filled to the very top with electrode buffer.

Table 1: Example loading scheme for 1d-SDS-PAGE of JURKAT samples

1d-130117rm						
Gelposition	Sample number	Sample name	Protein concentration	Sample volume	H₂O volume	5x SDS-PP
left 1		EMPTY				
2		EMPTY				
3		SB 12 μ L + 3 μ L SDS-PP				3 μ L
4	1	jurkat_130116at_cyt	7.0 μ g/ μ L	2.9 μ L	9.1 μ L	3 μ L
5		SB 12 μ L + 3 μ L SDS-PP				3 μ L
6	2	jurkat_130116at_NE	2.0 μ g/ μ L	10.0 μ L	2.0 μ L	3 μ L
7		SB 12 μ L + 3 μ L SDS-PP				3 μ L
8	3	jurkat_130116rm_NE	2.0 μ g/ μ L	10.0 μ L	2.0 μ L	3 μ L
9		SB 12 μ L + 3 μ L SDS-PP				3 μ L
right 10	M	MW-Marker 5 μ L				

Running the Gel

The electrode assembly is placed in the tank filled also with electrode buffer and the lid placed on the top. The lid is connected to the power supply, the power supply set to 20 mA per gel and the voltage restricted to a maximum of 200V and the run started. The run is continued (a) until the front reaches the end of the gel (for Western Blot), (b) until the marker is separated for 1 cm (normal shotgun gel for LC-MS/MS optimization) or (c) until the marker is separated for 3-4 cm and is then stopped (quantification of CXCL5). The power supply is turned off, the gels removed from the tank and the gels freed from the glass plates and put into fixation solution.

Western Blot

Reagents

- H₂O bidest
- Methanol TECHNICAL Plastic bottle 5l 1 * 5 l (VWR BDH Prolabo)
- TRIS x, Tris(hydroxymethyl)aminomethane, 2-Amino-2-(hydroxymethyl)-1,3-propanediol, THAM, Tris base, Trometamol, ≥99.9%; A 1 cm 10% in water 260nm: <0.02 / 40% in water 430nm: <0.02; MW 121.14 (Gerbü)
- SDS 4x, Sodium dodecylsulfate, Na laurylsulfate; ≥99.0%; UV abs. (A 1cm 1% in water) 260nm:<0.2; powder; FW 288.38 (Gerbü)
- Glycine Ultrapure min. 99,5 %, For molecular biology (Gerbü)
- PonceauS
- Trichloroacetic acid
- TRIS x, Tris(hydroxymethyl)aminomethane, 2-Amino-2-(hydroxymethyl)-1,3-propanediol, THAM, Tris base, Trometamol, ≥99.9%; A 1 cm 10% in water 260nm: <0.02 / 40% in water 430nm: <0.02; MW 121.14 (Gerbü)
- HCl, Hydrochloric acid 32% for analysis EMSURE® 1 * 1 l (MERCK (MDA) INCL SCHUCHARDT)
- NaCl Sodium chloride for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Tween20 #170-6531, 100 ml, enzyme immunoassay grade polysorbate surfactant (detergent) (Bio-Rad)
- HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Gerbü)

- Sodium hydroxide pellets for analysis EMSURE® ISO 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- NaCl Sodium chloride for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Gerbü)
- Sodium hydroxide pellets for analysis EMSURE® ISO 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Non-fat dry milk (powdered milk, PM)
- Sodium azide, BioXtra (Sigma)
- Kit ECL prime western blot det. Reagent 1 * 1 KIT (GE HEALTHCARE)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µl) (Socorex)
- Glas bowl
- Mini-PROTEAN Tetra Cell plus Mini-Trans blot module, 10-well, 1,0mm (Bio-Rad)
- PowerPac Universal Power Supply (Bio-Rad)
- Blotting membrane PROTRAN BA83, 0,2µm, 300mm x 3m (VWR)
- WHATMAN 3MM filter paper 460 x 570mm (VWR)
- Magnetic stirring bar and magnetic stirrer
- Freezer pack
- Back & forth shaker, 450 x 450 mm, analog 1 * 1 ST (GFL (GESELLSCH. FÜR LABORT.))
- Camera
- Plastic bowls with lids
- Transparent film
- peQLab Biotechnology Chemilumineszenz-Imaging (peQ Lab Biotechnologie GmbH)

Solutions

Blotting buffer

100mL 10xTris-Glycine buffer (see section solutions in SDS-PAGE for Western Blotting)
 150mL Methanol
 Filled to 1l with H₂O bidest.
 1ml 20% SDS

Stored at 4°C

PonceauS Staining solution

0,2% PonceauS
in 3% trichloro acetic acid

1xTBST

Dilute 10xTBST 1:10 with H₂O bidest.
Stored at 4°C

10xTBST

100ml 1M Tris.HCl pH8,0
300ml 5M NaCl
10g Tween20 ad 1%
Filled up to 1l with H₂O bidest.
Stored at 4°C

1xTBS

10ml 1M Hepes/NaOH pH7,4
30ml 5M NaCl
Filled up to 1l with H₂O bidest.
Stored at 4°C

1M HEPES-NaOH pH7,4

23,831g HEPES (2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethane sulfonic acid)
Adjust to pH 7,4 with 5M NaOH
Filled up to 100 mL with H₂O bidest.
Stored at 4°C

Luminescence Solution

Amersham ECLTM Prime Western Blotting Detection Reagent (4°C): 1mL from brown and white flask,
prepared 5 min prior to use kept in the dark

5% milk powder in 1xTBST (= 5%MP/TBST)

5 g milk powder
Filled up to 100 mL with 1xTBST

5% milk powder in 1xTBS (= 5%MP/TBS)

5 g milk powder
Filled up to 100 mL with 1xTBS

Primary Antibody Solution

1:500 dilution of anti-CXCL5 antibody in 5%MP/TBST with 0,02% sodium azide
stored at 4°C

Secondary Antibody Solution

1:10 000 dilution of anti-rabbit antibody HRP-conjugated in 5%MP/TBS

Practical Procedure for Blotting

Protein transfer to Membrane

For cooling of the blotting buffer during the blotting process a freezer pack is stored at -20°C the day before the blotting was performed. For the assembly of the blotting equipment gloves are to be worn to avoid contamination of the blotting membrane. For the actual blotting assembly a blotting cassette is placed on some tissue with the white part down and the black part facing upwards. A sponge is then placed in a glass bowl filled with blotting buffer and placed on the white part of the blotting cassette. Next two pieces of filter paper are put into the bowl with blotting buffer and placed on top of the sponge. Then the blotting membrane is also wetted with blotting buffer and placed on top. A few mL of blotting buffer are then put on top of the membrane and the gel carefully placed in the centre of the membrane avoiding air bubbles between membrane and gel. The edges of the gel are then marked on the membrane for later cutting and two more wet filter papers placed on the gel.

Using a 50 mL Falcon tube any potential air bubbles are removed by rolling the tube with gentle pressure over the sandwich. A wet sponge is then placed on top of the sandwich and the cassette closed. The cassette is then placed in the holder with the white part facing the red side and the black side facing the black side. The holder with the cassette is then placed in the blotting tank filled to ~50% with blotting buffer. A magnetic stirring bar is placed in the tank and the frozen freezer pack is placed in the tank. The tank was filled to the top with blotting buffer and placed on a magnetic stirrer and the stirrer turned on. The lid is closed and the electrode cables plugged into the power supply (red to red and black to black). The power supply is set to 30 V with a limitation of maximal 400 mA, turned on and allowed to run over night.

PonceauS staining

After the blotting the array is disassembled and the membrane separated from the gel. The membrane is then cut with a clean pair of scissors (wash with water and ethanol beforehand) along the mark indicating the edges of the gel. The membrane is placed in a clean plastic bowl and briefly washed with water. The water is discarded and the membrane incubated und shaken for 5 min in PonceauS staining solution. The PonceauS staining solution is transferred back to the flask and the membrane two to three times briefly washed with water until the background staining is washed off and the sample bands are clearly visible and a photo is taken.

Detection using Antibodies

The membrane is then blocked with milk powder by adding 5% milk powder in TBST and incubated for one h. The membrane is then washed three times for 5 min with TBST at room temperature. The primary antibody solution (1:500 diluted in 5% milk powder in TBST with 0,02% sodium azide) is added to the blot and incubated under shaking for 2 h at room temperature. The primary antibody solution is transferred back into the storage tube and stored at 4°C for further use. The blot is washed three times with TBS for 5 min per washing step. 10 mL of the secondary antibody (1:10 000, anti-rabbit in 5% milk powder in TBS) are added to the blot and shaken for 1 h at room temperature. The blot is then washed three times for 5 min with TBS. The luminescence solution is prepared during the 5 min prior to the intended use and kept in the dark. The blot is incubated with the luminescence solution for 3 min by pipetting the 2 mL manually over the membrane repeatedly. The membrane is then placed in a transparent film, any air bubbles removed and the chemiluminescence measured with a blot reader using the Chemi Capt software.

Silver Staining

Reagents

- H₂O bidest.
- Methanol TECHNICAL Plastic bottle 5l 1 * 5 l (VWR BDH Prolabo)
- Acetic acid (glacial) 100% anhydrous for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 2,5 l (MERCK (MDA) INCL SCHUCHARDT)
- Sodium thiosulfate pentahydrate for analysis emsure# ACS,ISO, REAG. PH EUR 1 * 500 g (MERCK (MDA) INCL SCHUCHARDT)
- Silver nitrate, ReagentPlus®, ≥99.0% (titration) (Sigma)
- Sodium carbonate anhydrous for analysis emsure# ISO 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Formaldehyde solution min. 37% gr for analysis stabilized with about 10% methanol, ACS,REAG. PH EUR 1 * 1 l (MERCK (MDA) INCL SCHUCHARDT)
- Acetic acid (glacial) 100% anhydrous for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 2,5 l (MERCK (MDA) INCL SCHUCHARDT)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µL) (Socorex)
- Back & forth shaker, 450 x 450 mm, analog 1 * 1 ST (GFL (GESELLSCH. FÜR LABORT.))
- Plastic bowls with lids
- Illumination plate, glass plate, camera stand and camera
- 100 mL volumetric cylinder

Solutions

Fixation solution

500 mL methanol (technical)
100 mL glacial acetic acid
400 mL water bidest.
Stored at RT

50% Methanol

500 mL methanol (technical)
500 mL water bidest.
Stored at RT

1% Acetic acid

10 mL glacial acetic acid
990 mL water bidest.
Stored at RT

0,02% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ solution

1 mL of 2% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ solution
99 mL water bidest.
Prepared just before use

2% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ solution

2 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$
98 mL water bidest.
Stored at 4°C

Developing solution

3 g Na_2CO_3
100 mL water bidest.
Shortly before use add 130 µL formaldehyde

0,1% Silver nitrate solution

100 mg AgNO_3
100 mL water bidest.
Made just before use

Practical Procedure for Silver Staining

After a minimum time of 20 min. in the fixation solution, the solution is discarded, 50% methanol added and the gels shaken for 10 min. Afterwards the methanol is discarded and the gels are shaken two times for 5 min with water. The gels are then incubated for 1 min. with 0,02% $\text{Na}_2\text{S}_2\text{O}_3$, quickly washed two times with water and then incubated for 10 min in freshly prepared 0,1% silver nitrate solution (4°C). The silver solution is afterwards discarded to a distinct container for silver waste. The gels are quickly rinsed with water once and developing solution added to the gels. As soon as the gels are yellow to brownish colored the solution is poured off and the staining process stopped by adding 1% acetic acid. After 5 min in the 1% acetic acid the gels are transferred to the illumination plate and a picture is taken of the gels

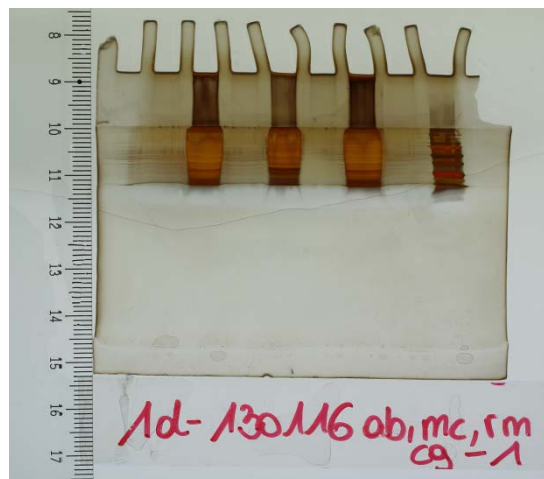


Figure 23: Silver staining of a 1d SDS gel

Proteolytic Digest

Reagents

- H₂O bidest
- Sodium thiosulfate pentahydrate for analysis emsure# ACS,ISO, REAG. PH EUR 1 * 500 g (MERCK (MDA) INCL SCHUCHARDT)
- Potassium hexacyanoferrate(III), ACS reagent, ≥99.0% (Sigma)
- Methanol zur Analyse EMSURE® ACS,ISO,Reag. Ph Eur 1 * 2,5 l (MERCK (MDA) INCL SCHUCHARDT)
- Acetic acid (glacial) 100% anhydrous for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 2,5 l (MERCK (MDA) INCL SCHUCHARDT)
- Ammonium bicarbonate, ReagentPlus®, ≥99.0% (Sigma)
- Dithiothreitol, for SH Groups Analysis, SP-Grade (Gerbü)
- Ammonium bicarbonate, ReagentPlus®, ≥99.0% (Sigma)
- Iodoacetamide, ≥99% (HPLC), crystalline (Sigma)
- Ammonium bicarbonate, ReagentPlus®, ≥99.0% (Sigma)
- Trypsin Sequencing Grade from bovine pancreas (Roche)
- Ammonium bicarbonate, ReagentPlus®, ≥99.0% (Sigma)
- Ice
- Acetonitrile hypergrade for liquid chromatography (LC/MS) LiChrosolv® (Merck Millipore)
- Formic acid, for mass spectrometry, ~98% (Sigma)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µl) (Socorex)
- Illumination plate, scalpel and glass plate
- Tube, Safe-Lock, PP, 1.5ml, clear (Eppendorf)
- 15 mL and 50 mL BD Falcon tubes (VWR)
- Vortex-Genie 2 230V European plug 1 * 1 item (SCIENTIFIC INDUSTRIES (ELMIS))
- Thermomixer Comfort basic device without thermoblock EU-plug 1 * 1 items (EPPENDORF)
- Exchangeable Thermoblock For Micro Test Tubes, 24 x 1.5ml, Thermomixer Comfort, Complete With IsoTherm Rack And IsoTherm Cool Pack 0C, Eppendorf 1 * 1 items (EPPENDORF)
- Bandelin Ultrasonic bath DK 102 P SONOREX DIGITAL, 3,0 ltr. (Bartelt)

- Speedvac Duo Plus Package, comprising, miVac Duo (without rotor), miVac Duo Pump, miVac SpeedTrap, tubing kit
- 48 x 1.5ml/2ml Eppendorf tube open aluminium rotor (supplied as std with DNA system)
- Clear-view Snap-Cap microtubes, 0,6ml, siliconized (Sigma)

Solutions

150mM $K_3Fe(CN)_6$ (10x):

0,4938g $K_3Fe(CN)_6$
 Filled up to 10ml with H_2O bidest.
 Stored at 4°C

500mM $Na_2S_2O_3$ (10x):

1,2409g $Na_2S_2O_3$
 Filled up to 10 mL with H_2O bidest.
 Stored at 4°C

Washing solution

50ml Methanol ad 50%
 40ml H_2O bidest.
 10ml Glacial acetic acid ad 10%
 Stored at 4°C

Destaining solution

1ml 10x $K_3Fe(CN)_6$ ad 15mM
 1ml 10x $Na_2S_2O_3$ ad 50mM
 8ml H_2O bidest.
 Freshly prepared prior to use

NH_4HCO_3 buffer solution

0,1977g NH_4HCO_3 ad 50mM
 Filled up to 50 mL with H_2O bidest.

1M DTT (Dithiothreitol) (100x)

1,5425g DTT
 Filled up to 10ml with H_2O bidest.
 Stored at -20°C in 500 μ L aliquots

500mM IAA (Iodacetamide) (10x)

0,9248g IAA
 Filled up to 10ml with NH_4HCO_3 buffer solution
 Stored at -20°C in 1,5 mL aliquots

Excision and Destaining

For the digestion of the samples each lane is cut into 4 slices. These 4 slices are then cut into smaller cubes of about 1 mm³ and the cubes of each slice transferred to separate 1,5 mL Eppendorf vials. 300 µL of destaining solution is added and vortexed until the cubes are colorless. The destaining solution is discarded and 300 µL of washing solution (50% methanol, 10% acetic acid) added to each slice followed by short vortexing and 5 min shaking at room temperature. The washing solution is then discarded and 200 µL of fresh washing solution are added and shaken for 10 min. This washing step is repeated one more time followed by a fourth washing step with 20 min shaking time.

Reduction

The washing solution is then removed and 200 µL of 50mM NH₄HCO₃ added to each slice followed by 5 min shaking. The solution is discarded and 200 µL of 10mM DTT in 50 mM NH₄HCO₃ solution added. The slices with the DTT are then shaken for 30 min at 56°C.

Alkylation

The gel pieces are then washed with 200 µL of 50mM NH₄HCO₃ for another 5 min. 200 µL of 50 mM IAA in 50 mM NH₄HCO₃ solution are added and the gel pieces incubated without shaking in the dark for 20 min.

Drying of the Gel Pieces

The gel slices are then washed one more time with NH₄HCO₃ and the washing solution discarded. 200 µL of acetonitrile (LC-MS grade) are added to each vial and shaken for 5 min. The vials are then centrifuged for only 5 s and the acetonitrile discarded. The vials are transferred into the SpeedVac and dried for 2 min at 37°C.

Addition of Trypsin

The dry gel pieces are retrieved from the SpeedVac and placed on ice. Trypsin (0,1875 µg/µL) in 50 mM NH₄HCO₃ is placed on ice as well and 15 µL directly added onto the gel pieces changing pipette tip after each sample. The gel pieces are then allowed to swell for 15 min on ice taking up the trypsin into the gel pieces. 25 µL of bicarbonate buffer is added after the swelling and the gel pieces placed in the incubator at 37°C over night.

Elution & Concentration

The next day 40 µL of the bicarbonate buffer is added to each vial, shortly vortexed and the vials placed in the ultrasonic bath for 15 min at 30% power. The vials are centrifuged for a few seconds and the supernatant containing the tryptic peptides transferred to siliconized 600 µL tubes. To the gel slices 40 µL of 50% acetonitrile/5% formic acid is added, vortexed and again placed in the ultrasonic bath for 10 min at 30% power. The supernatant is again taken off and pooled with the corresponding supernatant from the first extraction step. Another 40 µL of 50% acetonitrile/5% formic acid is added to each slice and for 10 min placed in the ultrasonic bath at 30% power. The supernatant is again pooled with the last two corresponding ones and the siliconized tubes placed in the SpeedVac. The samples are vacuum-concentrated at 37°C to a final volume of ~20 µL, removed from the SpeedVac and placed in a labeled plastic bag and stored at -20°C.

Immunoprecipitation

Reagents

- H₂O bidest.
- Dynabeads® Protein G for Immunoprecipitation (Fisher Scientific)
- Tween20 #170-6531, 100 ml, enzyme immunoassay grade polysorbate surfactant (detergent) (Bio-Rad)
- di-Sodium hydrogen phosphate dihydrate for analysis EMSURE® 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Sodium chloride for analysis EMSURE® ACS,ISO,Reag. Ph Eur 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Potassium dihydrogen phosphate for analysis emsure # ISO 1 * 250 g (MERCK (MDA) INCL SCHUCHARDT)
- Albumine fraction V, >98%, powder, for molecular biology (BSA) (Roth)
- Glycine Ultrapure min. 99,5 %, For molecular biology (Gerbü)
- HCl, Hydrochloric acid 32% for analysis emsure# 1 * 1 l (MERCK (MDA) INCL SCHUCHARDT)
- Sodium hydroxide pellets for analysis emsure# ISO 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µL) (Socorex)
- Programmable Rotator Mixer RM Multi-1 (Starlab)
- Magnets for 500 µL Eppendorf tubes (Eppendorf)
- Reaction vessel 0,5 ml, safe-lock, transparent (Eppendorf)

Solutions

10% Tween20 Solution

10 g Tween20
90 mL of water bidest.
Thoroughly mixed until completely dissolved
Stored at 4°C

PBS/0,05%Tween20

500 µL of 10% Tween20 solution
100 mL of PBS
Stored at 4°C

Phosphate buffered saline (PBS), pH 7,3

80 g NaCl
2 g KCl
2 g KH₂PO₄
14,4 g Na₂HPO₄ × 2H₂O
Dissolved in water bidest. and filled up to 1 L

Elution Buffer (0,1M Glycine-HCl, pH 2,7)

750,7 mg Glycine
Added 90 mL of H₂O bidest.
Adjusted pH to 2,7 with HCl
Filled up to 100 mL with H₂O bidest.

2% BSA Solution

1 g Bovine serum albumin
50 mL of H₂O bidest.

Practical Procedure for Immunoprecipitation

The initial immunoprecipitation method used as starting point for further optimization was kindly provided by Besnik Muqaku and is described in detail below. The optimization process as well as the description of the optimized method is listed in the Results and Discussion chapter.

Equilibration of Dynabeads

The dynabeads are resuspended by pipetting up and down or by vortexing for 5 to 10 min. 50 µL of the resuspended beads are transferred into a 500 µL Eppendorf vial. The vial is placed in a magnet suitable for IP with dynabeads and the clear supernatant removed after the magnetic beads were attached to the wall. The beads are resuspended in 200 µL of PBS/0,05% Tween20, rotated for 5 min, the clear supernatant removed by applying the magnet and the previous steps are repeated another two times.

Binding of the Antibody

5 µg of IgG antibody are diluted in 200 µL of PBS/0,05% Tween20. The diluted antibody solution is added to the equilibrated beads and the beads resuspended. The beads with the antibody solution are rotated at RT for 1h. The beads are precipitated using the magnet and the supernatant (SN1) stored in a separate vial at 4°C for later verification of completeness of protein G – IgG binding. The beads with the bound antibody are resuspended in 200 µL of PBS/0,05% Tween20.

Binding of Antigen from Sample

80 µL of the resuspended beads with bound antibody are transferred to a 500 µL Eppendorf vial, precipitated by use of the magnet and the supernatant removed. The beads are resuspended in 300 µL of sample with 30 µL of 2% BSA and 3 µL of 10% Tween20. The beads are rotated over night with the sample at 4°C. The beads are precipitated with the magnet and the supernatant transferred to a separate Eppendorf vial for later analysis of completeness of antigen binding (**SN2**). The beads are washed three times with PBS/0,05% Tween20. After the washing steps the beads are resuspended in 100 µL of PBS/0,05% Tween20 and transferred to a new Eppendorf vial.

Elution of Antigen from Beads

The beads are precipitated with the magnet, the supernatant discarded and resuspended in 100 µL of 0,1 M glycine/HCl-buffer (pH = 2,7). The beads are rotated for 10 min at RT, again precipitated and the supernatant transferred to a new Eppendorf vial (**eluate1**) for later analysis of completeness of elution.

The beads are resuspended again in 100 µL of glycine/HCl-buffer and rotated for another 10 min. The beads are precipitated again and the supernatant (**eluate2**) stored separately at 4°C for analysis of completeness of elution.

LC-MS/MS

Reagents

- H₂O (MilliQ grade)
- Acetonitrile hypergrade for liquid chromatography (LC/MS) LiChrosolv® (Merck Millipore)
- Formic acid, for mass spectrometry, ~98% (Sigma)
- 2-Propanol LC-MS CHROMASOLV (Sigma)
- Standard peptides
 - o Glu1-Fibrinopeptide B, Sequence: EGVNDNNEEGFFSAR, $[M+2H]^{2+} = 785,84206$ g/mol, from Sigma (F3261)
 - o PeptideM28, Sequence: TTPAVLSDGSYFLYSK, $[M+2H]^{2+} = 932,45418$ g/mol, from PSL
 - o Peptide HK0, Sequence: VLETKSLYVR, $[M+2H]^{2+} = 604,356435$ g/mol, from PSL
 - o HK1, Sequence: VLETK(e-AC)SLYVR, $[M+2H]^{2+} = 625,361717$ g/mol, from PSL
- Nitrogen gas(99%)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µL) (Socorex)
- Thermo Scientific Q Exactive Benchtop LC-MS/MS (Thermo Scientific)
- UltiMate® 3000 RSLCnano (former Dionex, now Thermo Scientific)
- Acclaim* PepMap* RSLC Column, 75µm*50cm and 15cm, nanoViper, C18, 2µm, 100Å (Thermo Scientific)
- Acclaim* PepMap* RSLC Nano Trap Column, 100µm*2cm, nanoViper, C18, 5µm, 100Å (Thermo Scientific)
- 20 µL (Thermo Scientific)

Solutions

Mobile phase A (2% ACN, 0,2% FA)

20 mL Acetonitrile (LC-MS grade)
980 mL H₂O (MilliQ quality)
2 mL Formic acid (~98%)

Mobile phase B (80% ACN, 0,2% FA)

800 mL Acetonitrile (LC-MS grade)
200 mL H₂O (MilliQ quality)
2 mL Formic acid (~98%)

10% Isopropanol

900 mL H₂O (MilliQ quality)
100 mL Isopropanol (LC-MS grade)

Standard peptide stock solution (10 pmol/μL)

79 μL of 0,2 mg/mL Glu1-Fibrinopeptide in 30% formic acid (c_{final} : 10 pmol/μL)
38 μL of 1,0 mg/mL Peptide M28 in 30% formic acid (c_{final} : 10 pmol/μL)
12 μL of 1,0 mg/mL Peptide HK0 in 30% formic acid (c_{final} : 20 pmol/μL)
13 μL of 1,0 mg/mL Peptide HK1 in 30% formic acid (c_{final} : 10 pmol/μL)
858 μL of 30% formic acid

Standard peptide solution (10 fmol/μL)

1:1000 dilution of standard peptide stock solution (10 pmol/μL)

Programs required for Data Acquisition and Analysis

- Proteome Discoverer 1.3.0.339
- RawMeat (version 2.1)
- Chromeleon 6.80 SR10 Build 2914 (180531)
- Xcalibur 2.2 (SP1.48)
- MaxQuant 1.3.0.5
- Q Exactive Tune Application for Benchtop Orbitrap MS 2.2 SP1 Build 1646

MS Settings

Table 2: Settings for 170 min MS method (=190 min total gradient time)

MS Acquisition Mode	Full MS/dd-MS ² (TopN)
Global Settings	Use lock mass: best Chromatographic peak width (FWHM): 15 s
Time	170 min
General	User role: Standard Runtime: 170 min Polarity: positive

	Default charge state: 2 Inclusion: - Exclusion: - Tags: -
Full MS	Resolution: 70 000 AGC target: 1e6 Maximum IT: 120 ms Scan range: 400 to 1400 m/z
dd-MS ² /dd-SIM	Resolution: 17 500 AGC target: 5e4 Maximum IT: 250 ms Loop count: 6 TopN: 6 Isolation window: 2 m/z Fixed first mass: 100 m/z NCE: 30
dd Settings	Underfill ratio: 10.0% Intensity threshold: 2.0e4 Apex trigger: 3 to 30 s Charge exclusion: unassigned, 1 Peptide match: - Exclude isotopes: on Dynamic exclusion: 30 s

Practical Procedure for LC-MS/MS Sample Measurement

Pipetting of the samples

The sample vials with 20 µL liquid sample are taken from the freezer (-20°C), thawed and centrifuged for 5 min. 15 µL of the sample are then transferred to a 96-well plate. The sample was then diluted with a certain volume of mobile phase A (volume depended on the sample). For the assessment of the LC performance a mix of four standard peptides dissolved in 30% formic acid is added to the diluted sample. The volume of the standard peptides that was added was 10% of the diluted sample. After the transfer of all samples to the well plate, dilution and addition of the standard peptides the well plate is covered with an adhesive plastic sheet. The plate is then placed in the autosampler of the UltiMate® 3000 RSLCnano LC system which is cooled down to 4°C.

Starting the measurement

Prior to the start of the measurement the sample sequence is written for the LC as well as for the mass spectrometer and saved. The MS sequence is started first followed by the LC sample sequence.

Data analysis

Every set of four raw files corresponding to the 4 bands of each sample obtained after the LC-MS/MS analysis is searched as MudPIT via Proteome Discoverer (Version 1.3.0.339) from Thermo. The search workflow is illustrated in Figure 24 and briefly described in Table 3 and the settings for the applied search protocol are listed in, Table 4, Table 5 and Table 6.

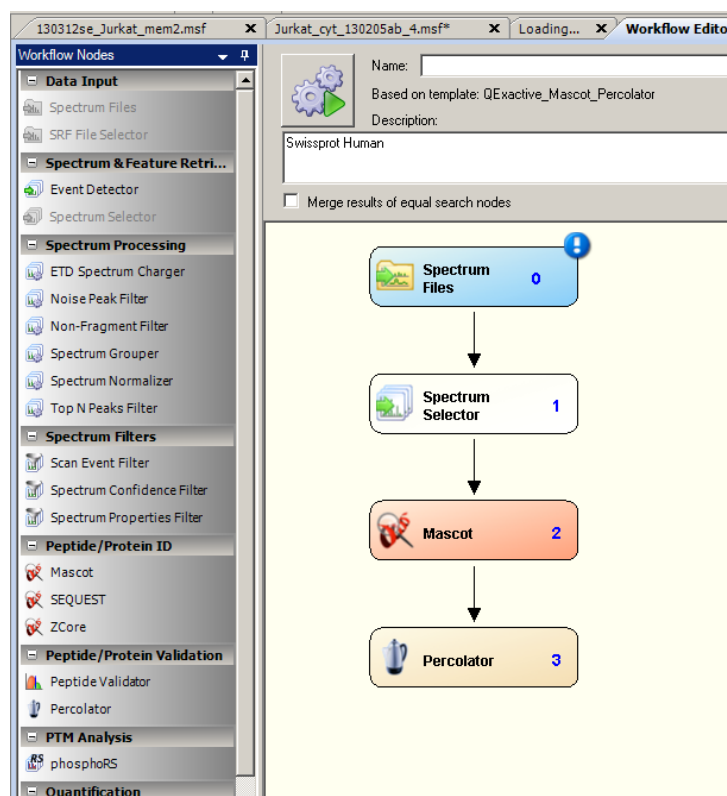


Figure 24: Proteome Discoverer search workflow overview

Table 3: Proteome Discoverer Search Workflow

DATA INPUT	Spectrum Files	Selection of the spectrum raw-files obtained from the analysis
SPECTRUM AND FEATURE RETRIEVAL	Spectrum Selector	Decision which spectra are to be submitted to the search engine, filters spectra based on mass and charge state and replaces unrecognized properties
PEPTIDE/PROTEIN IDENTIFICATION	Mascot	Identifies peptides on the basis of their MS ² spectra and associates the peptides to proteins
PEPTIDE/PROTEIN VALIDATION	Percolator	Validates the quality of the peptide identification

Table 4: Spectrum Selector Settings

General Settings	Use: MS1 Precursor
Spectrum Properties Filter	Lowest charge state: 2 Highest charge state: 8 Minimum precursor mass: 800 Da Maximum precursor mass: 10000 Da Total intensity threshold: 1 Minimum peak count: 3
Scan Event Filter	Mass analyzer: Any MS order: Is MS2 Activation type: Any Scan type: Is Full Ionization source: Any Polarity mode: Any
Peak Filters	S/N Threshold (FT-only): 3
Replacement for Unrecognized Properties	Unrecognized charge replacements: Automatic Unrecognized mass analyzer replacements: FTMS Unrecognized MS order replacements: MS2 Unrecognized activation type replacement: HCD Unrecognized polarity replacement: +

Table 5: Mascot Settings

Input Data	Protein database: swissprot_human Enzyme name: trypsin Maximum missed cleavage sites: 2 Instrument: ESI-TRAP Taxonomy: All entries
Tolerances	Precursor mass tolerance: 5 ppm Fragment mass tolerance: 20 mmu Use average precursor mass: False
Dynamic Modifications	1. Dynamic modification: Acetylation (Protein N-term) 2. Dynamic modification: Oxidation (M)
Static Modifications	1. Static modification: Carbamidomethyl (C)

Table 6: Percolator Settings

Input Data	Maximum Delta Cn: 0,05
Decoy Database Search	Target FDR (strict): 0,01 Target FDR (relaxed): 0,05 Validation based on: q-value

Cell culture

Reagents

- H₂O bidest.
- RPMI-1640 Medium from ATCC
- Fetal bovine serum (FCS) from ATCC
- Penicillin-Streptomycin-Amphotericin B Solution from ATCC
- Urea, Harnstoff, Ultrapure Reagent (Gerbü)
- Thiourea, ACS reagent, ≥99.0% (Sigma)
- CHAPS, Cholamidopropyltrimethylammonio-1-propanesulfonic acid; >98%; zwitterionic compound; MW 614.89 (Gerbü)
- SDS 4x, Sodium dodecylsulfate, Na laurylsulfate; ≥99.0%; UV abs. (A 1cm 1% in water) 260nm:<0.2; powder; FW 288.38 (Gerbü)
- Dithiothreitol, for SH Groups Analysis, SP-Grade (Gerbü)
- NaCl, Sodium chloride for analysis EMSURE® ACS, ISO, Reag. Ph Eur 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Gerbü)
- Magnesium chloride hexahydrate for analysis EMSURE® ACS, ISO, Reag. Ph Eur 1 * 250 g (MERCK (MDA) INCL SCHUCHARDT)
- Sucrose, Saccharose for biochem. applications Reag. PhEur 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, ≥97.0% (Sigma)
- Triton X-100, Octylphenolpoly-(ethyleneglycolether), especially purified for membrane research (Roche)
- Sodium hydroxide pellets for analysis EMSURE® ISO 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Ice
- Leupeptin hemisulfate salt (Sigma)
- Pepstatin A, microbial, ≥90% (HPLC), microbial, ≥75% (HPLC) (Sigma)
- Aprotinin from bovine lung, lyophilized powder, 3-8 TIU/mg solid (Sigma)
- PMSF Phenylmethanesulfonyl fluoride, ≥98.5% (GC) (Sigma)
- 2-Propanol LC-MS CHROMASOLV® (Sigma)

- TRIS x, Tris(hydroxymethyl)aminomethane, 2-Amino-2-(hydroxymethyl)-1,3-propanediol, THAM, Tris base, Trometamol, ≥99.9%; A 1 cm 10% in water 260nm: <0.02 / 40% in water 430nm: <0.02; MW 121.14 (Gerbü)
- HCl, Hydrochloric acid 32% for analysis EMSURE® 1 * 1 l (MERCK (MDA) INCL SCHUCHARDT)
- EDTA Ethylenediaminetetraacetic acid, BioUltra, anhydrous, ≥99% (titration) (Sigma)
- NaCl, Sodium chloride for analysis EMSURE® ACS, ISO, Reag. Ph Eur 1 * 1 kg (MERCK (MDA) INCL SCHUCHARDT)
- Nonidet™ P 40 Substitute, BioXtra, mixture of 15 homologues (Sigma)
- Ethanol absolute for analysis EMSURE® ACS, ISO, Reag. Ph Eur 1 * 2,5 l (MERCK (MDA) INCL SCHUCHARDT)
- Bio-Rad Protein Assay Dye Reagent Concentrate #500-0006 (Bio-Rad)

Materials and Instrumentation

- Set of pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µl) (Socorex)
- Sterile pipettes 5ml, 10ml, 25ml (Sterilin)
- Sterile working bench - HERASAFE KS 12 (Thermo Scientific)
- Pipetboy acu classic (VWR)
- Incubator - Doppel Heracell 150l CO2 Inkubator VA 230 (Thermo Scientific)
- Water bath - Ed-7a/b badthermostat 1 * 1 ST (JULABO)
- Zeiss Mikroskop Primo Vert mit binokularem Fototubus (Zeiss)
- Camera ERC 5S (Zeiss)
- Centrifuge - MEGAFUGE 16R (Thermo Scientific)
- 25 cm² and 75 cm² Cell Culture Flask, with filter cap (PAA)
- Desiccator 150mm (VWR)
- 15 mL and 50 mL BD Falcon tubes (VWR)

Cell lines

Cell line J45.01, T lymphocytes from acute T cell leukemia, human (CD45 deficient variant of Jurkat clone E6-1) from homo sapiens, male; from ATCC (catalogue no.: CRL-1990)

Solutions

Full medium

500 mL RPMI-1640 Medium
50 mL FCS
0,5 mL Penicillin-Streptomycin-Amphotericin B Solution

Serum-free medium

500 mL RPMI-1640 Medium
0,5 mL Penicillin-Streptomycin-Amphotericin B Solution

Sample Buffer (SB)

22,5g Urea ad 7,5M
5,7g Thiourea ad 1,5M
2g CHAPS ad 4%
125µL 20% SDS ad 0,05%
5ml 1M DTT ad 100mM
Filled to 50ml with H₂O bidest.
1ml aliquots stored at -20°C; well mixed after thawing

Protease inhibitor cocktail (PIC)

100 µL Pepstatin A
10 µL Leupeptin
10 µL Aprotinin
880 µL water bidest.

PMSF solution

1,742 g PMSF
100 mL 2-propanol

Isotonic fractionation buffer

1 mL 1 M HEPES/NaOH (pH 7,4)
200 µL 5 M NaCl
350 µL 1 M MgCl₂
500 µL 2 M EGTA
12,5 mL Sucrose
2,5 mL 20% Triton X-100

Practical Procedure for Culturing and Fractionation of Jurkat Cells

Culturing of Jurkat cells

Jurkat cells (T lymphocytes from acute T cell leukemia) are purchased from ATCC, thawed and cultured in a 25 cm² culture flask according to the product information sheet for the cell line CRL-1990. The cells are cultured in RPMI-1640 medium supplemented with 10% FCS and antibiotics at 37°C and 5% CO₂.

When the cells reach a density of $\sim 1 \times 10^6$ cells/mL, they are transferred to a 75 cm² flask. The cells are cultured and split until six 75 cm² flasks with a cell density of 1×10^6 are achieved. The cells are then transferred to protein-free RPMI medium only supplemented with antibiotics and cultivated for 4h.

Fractionation

The whole fractionation procedure is carried out on ice and the centrifuge set to 4°C. After the 4h of cultivation in serum-free medium the cell suspension within each flask is transferred to a 15 mL Falcon tube and centrifuged at 1100 rpm for 5 min. The supernatant is discarded and the cells resuspended in PBS and centrifuged at 1200 rpm for 5 min. the supernatant discarded again and the cells resuspended again in PBS and centrifuged again at 1200 rpm for 5 min. The supernatant is one more time discarded and 3 mL of fractionation buffer added. The cells are now lysed by applying shear stress by pipetting up and down with a 1000 μ L Gilson pipette about 5 times. The success of the lysis is monitored by transferring 20 μ L of cell suspension to a glass slide and visually inspecting the cells under the microscope. Only very small dark particles (the nuclei) should remain. If not pipetting needs to be continued until most cells are lysed.

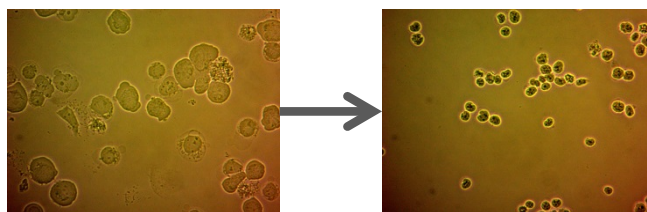


Figure 25: Jurkat cells before and after lysis

After complete lysis the cells are centrifuge at 3500 rpm for 5 min. The supernatant (cytoplasm) is transferred to a fresh 15 mL Falcon tube and filled up to 14 mL with cold absolute ethanol and placed at -20°C over night for protein precipitation.

The pellet gained from the last centrifugation step contains the nuclei and is further processed by drying the pellet with a paper tissue and estimating the volume of the nuclei pellet. 100 μ L of TE_{NaCl} including PIC and PMSF to inhibit proteases are added. The pellet is resuspended and then allowed to stand on ice for 10 min. Following 900 μ L of TE_{NP-40} are added and placed on ice for 15 min. The fraction is then centrifuged at 3500 rpm for 5 min at 4°C and the supernatant, which contains the nuclear extract, transferred

to a fresh 15 mL Falcon tube. The nuclear extract is filled up to 5 mL with cold absolute ethanol and stored at -20°C over night for protein precipitation.

The next day the ethanol precipitated samples are centrifuged at 5000 rpm and 4°C for 20 min. The supernatant of the Falcon tubes is discarded by decanting off and the pellets allowed to dry at room temperature for 10 min facing upside down. The Falcon tubes are then degassed for 10 min and urea added to each tube until saturation is achieved. To dissolve the protein pellets SB buffer according to the pellet size was added. 30 µL of SB buffer are added to the nuclear extract proteins and 50 µL to the cytoplasmic proteins and thoroughly mixed before placing the tubes on 4°C over night.

The next day the samples are completely dissolved by pipetting up and down with a 200 µL Gilson pipette and adding some more SB buffer if required. After the pellets are completely dissolved and the sample are clear and transparent they are transferred to pre-labelled 1,5 mL Eppendorf vials and then the total protein concentrations are assessed via Bradford assay.

Bradford assay

In an Eppendorf tube 199 µL of water and 1 µL of sample are pipetted and 50 µL of Bio-Rad Protein Assay Dye Reagent Concentrate added. The resulting blue color is then compared with a standard series and the matching concentration noted for later entry in the database as well as on the Eppendorf vial of the sample for storage.



Figure 26: Standard series with BSA for Bradford assay

Results and Discussion

Antibody Validation

A polyclonal antibody from rabbit against CXCL5 was purchased from Abcam and their cross reactivity assessed by Western Blot analysis. Samples that were analyzed earlier via shotgun proteomics were screened in the GPDE database for CXCL5 and the supernatant of A-Haut cells stimulated with IL-1b was found to contain CXCL5 and was therefore chosen as positive control. The supernatant of untreated HUVECs did not show CXCL5 and was therefore chosen as negative control. The cross-reactivity of the purchased antibody was determined by spiking the positive controls into three different matrices (water, blood plasma and HUVEC con CYT). The purchased antibody detected CXCL5 at the correct mass of 12 kDa and also the relative intensities of the CXCL5 bands showed good correlation with the applied amounts of positive control. The antibody also detected bands at higher molecular weights which might be explained by cross-reactivity of the antibody or incomplete separation of the CXCL5 protein from other proteins in the sample. The performance of the antibody was accepted as sufficient for further immunoprecipitation (IP) experiments as the main task of the IP was defined to enrich the analyte and strongly reduce sample complexity for MS quantification.

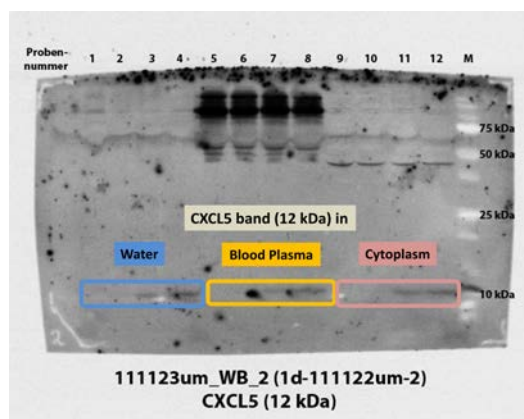


Figure 27: Antibody validation of CXCL5 via Western Blot

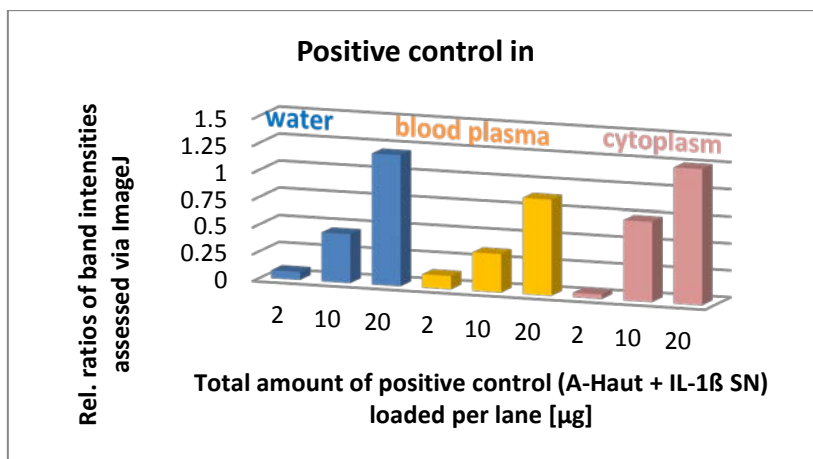


Figure 28: Comparison of relative intensities of CXCL5 bands for antibody validation

Immunoprecipitation

The basic immunoprecipitation protocol that was used as starting point for further optimization was kindly provided by Besnik Muqaku and is provided here:

Table 7: Immunoprecipitation with magnetic dynabeads coated with proteinG - initial procedure

Equilibration of Dynabeads	1. Resuspend the dynabeads by pipetting up and down or by vortexing for 5 to 10 min 2. Transfer 50 μL of the resuspended beads into a 500 μL Eppendorf vial 3. Place the Eppendorf vial in a magnet suitable for IP with dynabeads and remove the clear supernatant after the magnetic beads are attached to the wall 4. Resuspend the beads in 200 μL of PBS/0,05% Tween20, rotate for 5 min, remove clear supernatant by applying the magnet and repeat the previous steps another two times
Binding of the Antibody	5. 5 μg of IgG antibody is diluted in 200 μL of PBS/0,05% Tween20 6. The diluted antibody solution is added to the equilibrated beads and the beads resuspended 7. The beads with the antibody solution is rotated at RT for 1h 8. The beads are precipitated using the magnet and the supernatant (SN1) stored in a separate vial at 4°C for later verification of completeness of protein G – IgG binding 9. The beads with the bound antibody is resuspended in 200 μL of PBS/0,05% Tween20
Binding of Antigen from Sample	10. 80 μL of the resuspended beads with bound antibody are transferred to a 500 μL Eppendorf vial, precipitated by use of the magnet and the supernatant removed 11. The beads are resuspended in 300 μL of sample + 30 μL of 2% BSA + 3 μL of 10% Tween20 are added to the beads 12. Rotation over night at 4°C 13. Precipitation of beads with magnet and transfer of supernatant to separate Eppendorf vial for later analysis of completeness of antigen binding (SN2) 14. The beads are washed 3 times with PBS/0,05% Tween20 15. After the washing the beads are resuspended in 100 μL of PBS/0,05% Tween20 and transferred to a new Eppendorf vial
Elution of Antigen from Beads	16. The beads are precipitated with the magnet, the supernatant discarded and resuspended in 100 μL of 0,1 M glycine/HCl-buffer (pH = 2,7) 17. The beads are rotated for 10 min at RT 18. The beads are again precipitated and the supernatant transferred to a new Eppendorf vial (eluate1) for later analysis of completeness of

	<i>elution</i> 19. The beads are resuspended again in 100 μL of glycine/HCl-buffer and rotated for another 10 min 20. The beads are precipitated again and the supernatant (eluate2) stored separately at 4°C for analysis of completeness of elution
--	--

As sample for the evaluation of the initial immunoprecipitation method cytoplasm of A-Haut treated with IL-1b was used. The supernatants of the different steps of the IP were loaded onto a 12% PAA-gel (20 μ L + 5 μ L 5x laemmli buffer) and the CXCL5 as well as the antibody eluted from the beads detected via Western Blotting.

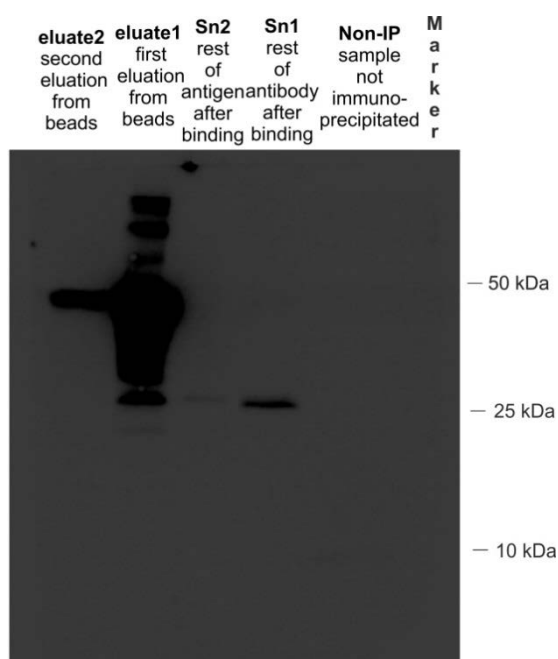


Figure 29: Western Blot of supernatants of initial IP method

Due to the fact that the antibodies eluting from the beads were highly abundant in the eluate1 compared to the CXCL5 antigen, the low molecular weight region (<20kDa) of the membrane was separately incubated with primary and secondary antibody and the signal intensity for the CXCL5 antigen could be drastically improved (Figure 30). It could be concluded from the comparison of the eluate1 and the positive control, which was not subjected to IP, that antigen was lost during the immunoprecipitation procedure and further optimization was necessary.

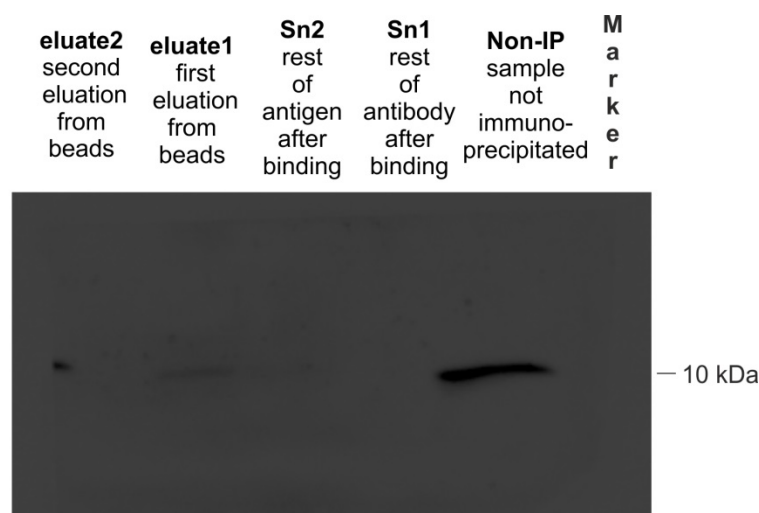


Figure 30: Western Blot of supernatants from Initial IP method for CXCL5 with the region below 20 kDa incubated a second time with antibodies for signal improvement

One potential step where antigen could have been lost was identified as the three washing steps after the binding of the antigen to the beads. Also the high volume of elution buffer and the short elution time were parameters suspected to reduce the concentration of the antigen in the final eluate. Therefore the 3 washing steps after the binding of the antigen to the antibodies were not performed in the next experiment, the buffer volume for the elution was reduced from 100 μ L to 40 μ L and the elution time was increased to 30 min.

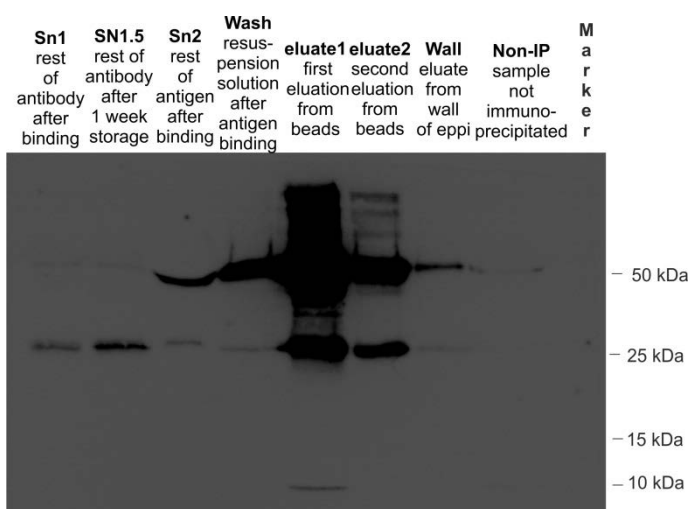


Figure 31: Western Blot of supernatants from modified IP method for CXCL5

As the signal intensity of the CXCL5 band at ~10 kDa was very weak again, the blot was cut in two and the lower part (<20 kDa) incubated with antibodies again.

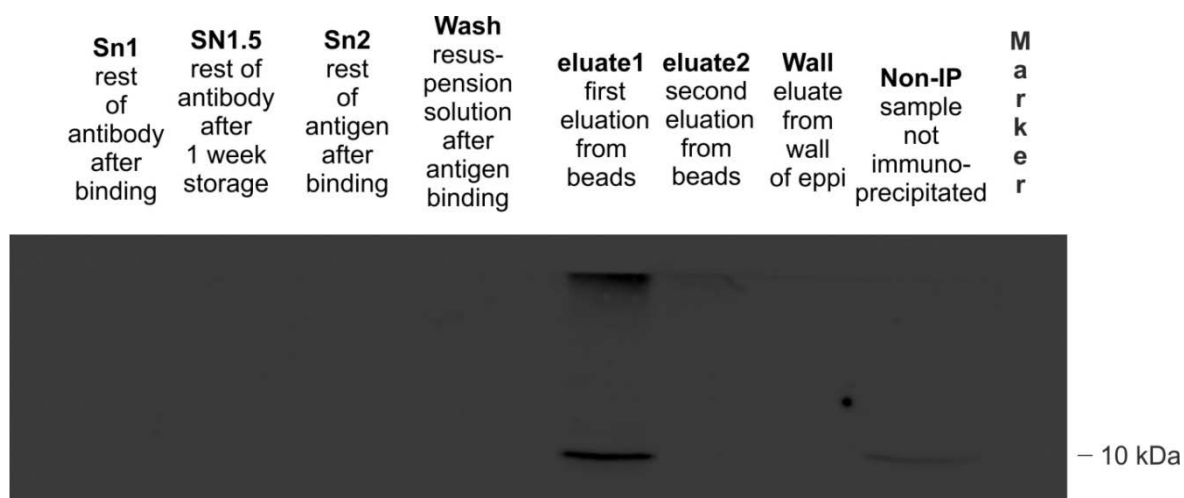


Figure 32: Western Blot of supernatants from modified IP method for CXCL5 with the region below 20 kDa incubated a second time with antibodies for signal improvement

After the second incubation with primary and secondary antibodies the signal intensity was drastically increased and the signal intensity for CXCL5 for eluate1 from the beads was significantly higher than the signal intensity for the non-IP positive control sample.

Optimisation of the LC and MS/MS conditions

Motivation of LC-MS/MS optimisation

The aim of this work was to develop a sensitive and selective MS-based methodology for the quantitative detection of the cytokine CXCL5. For this purpose the LC-MS/MS methodology was optimized in terms of reducing sample complexity reaching the mass spectrometer at any given time point to enable the detection of peptides of low abundant proteins and avoid ion suppression of high abundant coeluting peptides. Therefore Jurkat cytoplasm digests were used as complex samples and the number of successfully identified protein groups utilized as a measure for the ability to identify and quantify analytes from complex matrices such as blood plasma.

Overview on Experimental Realization of Optimization

Six 75 cm² flasks with Jurkat cells with a cell density of $\sim 1 \times 10^6$ cells/mL were fractionated into cytoplasmic and nuclear fractions, a Bradford assay performed to determine the absolute protein concentration of the fractions and the fractions stored at -20°C. 20 µg of each fraction was then loaded onto a 12%PAA gel, electrophoresis performed and each fraction cut into 4 bands. These bands were destained, the disulfide bonds broken and alkylated using DTT and IAA and then digested with trypsin over night at 37°C. The tryptic peptides were then eluted from the gel slices using an ultrasonic bath and the volume reduced in the SpeedVac to ~20µL and the samples stored at -20°C. The samples were then transferred to a 96-well plate and analysed with different LC-MS/MS settings.

Comparison of Protein and Peptide Identifications using Proteome Discoverer

The Thermo raw files obtained for the samples to be compared were searched via Proteome Discoverer using the workflow described in detail in the *Data analysis* section. The obtained search files to be compared were loaded via Proteome Discoverer into the same report file creating a multi report illustrated in *Figure 33*.

Protein	Accession	Description	iScore	iProteins	iUniquePeptides	iPeptides	iPSMs	Score A2	Coverage A2	iPeptides A2	#PSM A2	Score B2	Coverage B2	iPeptides B2	#PSM B2
4196	P49454	Centromere protein F OS=Homo sapiens GN=CENPF PE=...	0.50 %	1	1	2	2	41.11	0.50 %	2	2		0.00 %		
4197	Q49453	Poly (ADP-ribose) polymerase 14 OS=Homo sapiens GN=...	0.50 %	1	1	1	1	49.10	0.50 %	1	1		0.00 %		
4198	Q15015	Zinc finger protein 645 OS=Homo sapiens GN=ZNF645 PE=...	0.49 %	1	1	1	1	41.96	0.49 %	1	1		0.00 %		
4199	Q15058	Kinesin-like protein KIF14 OS=Homo sapiens GN=KIF14 PE=...	0.49 %	1	1	1	1	27.88	0.49 %	1	1		0.00 %		
4200	Q12765	Res- responsive element-binding protein 1 OS=Homo sapi...	0.41 %	1	1	1	1	24.33	0.41 %	1	2		0.00 %		
4201	A288P1	Neuroblastoma-amplified sequence OS=Homo sapiens GN=...	0.38 %	1	1	1	1	61.37	0.38 %	1	1		0.00 %		
4202	Q9NPA0	Ubiquitin carboxyl-terminal hydrolase 32 OS=Homo sapi...	0.37 %	1	1	1	1	28.73	0.37 %	1	1		0.00 %		
4203	Q15714	E3 ubiquitin-protein ligase HERC2 OS=Homo sapiens GN=...	0.24 %	1	1	1	1	25.85	0.24 %	1	1		0.00 %		
4204	Q15024	Thon OS=Homo sapiens GN=TTN PE=1 SV=1- [TTN]_L...	0.24 %	1	1	9	15	21.49	0.23 %	5	7	20.72	0.19 %	7	8
4205	P12042	Hemoglobin subunit delta OS=Homo sapiens GN=HBDEL PE=...	0.20 %	5	1	1	2		0.00 %			103.83	0.00 %	1	2
4206	P12765	Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=...	0.27 %	1	1	1	1		0.00 %			38.39	0.27 %	1	1
4207	Q10481	Amyloid-like protein 2 OS=Homo sapiens GN=ALPL2 PE=...	0.54 %	1	1	2	2		0.00 %			45.23	0.54 %	2	2
4208	P55378	6.8 kDa mitochondrial proteolipid OS=Homo sapiens GN=...	12.07 %	1	1	1	1		0.00 %			28.45	12.07 %	1	1
4209	Q10379	Galgin subfamily A member 2 OS=Homo sapiens GN=GO...	1.00 %	1	1	1	1		0.00 %			48.32	1.00 %	1	1
4210	Q10441	Proteolipid protein 2 OS=Homo sapiens GN=PLP2 PE=1 S...	8.55 %	1	1	1	2		0.00 %			27.66	8.55 %	1	2
4211	Q1UW68	Transmembrane protein 205 OS=Homo sapiens GN=TM2...	8.89 %	1	1	1	1		0.00 %			23.06	8.89 %	1	1
4212	Q15149	Snurportin-1 OS=Homo sapiens GN=SNUPN PE=1 SV=1...	8.06 %	1	2	2	2		0.00 %			55.02	8.06 %	2	2
4213	Q185V73	Centrosome-associated protein CEP250 OS=Homo sapien...	0.49 %	1	1	1	1		0.00 %			41.11	0.49 %	1	1
4214	P19719	Cytochrome b-c1 complex subunit 6, mitochondrial OS=H...	19.67 %	1	2	2	4		0.00 %			122.50	19.67 %	2	4
4215	Q188K19	Oxidoreductase-like domain-containing protein 1 OS=Ho...	14.29 %	1	2	2	2		0.00 %			26.18	14.29 %	2	2
4216	P12749	V-type proton ATPase 16 kDa proteolipid subunit OS=Ho...	11.81 %	1	1	1	2		0.00 %			39.40	11.81 %	1	2
4217	Q1H124	DnaJ homolog subfamily C member 5 OS=Homo sapiens...	7.58 %	1	1	1	1		0.00 %			51.45	7.58 %	1	1
4218	P13125	Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1- [L...	6.28 %	1	1	1	1		0.00 %			45.52	6.28 %	1	1
4219	Q17C78	Signal peptide peptidase-like 2A OS=Homo sapiens GN=SP...	2.61 %	1	1	1	1		0.00 %			32.53	2.61 %	1	1
4220	P49194	RNA-binding protein 25 OS=Homo sapiens GN=RBX2 PE=...	2.14 %	1	1	1	1		0.00 %			24.43	2.14 %	1	1
4221	Q187P01	Protein kish A OS=Homo sapiens GN=THM157A PE=2 S...	12.50 %	1	1	1	1		0.00 %			39.01	12.50 %	1	1
4222	Q191Q23	Transmembrane emp24 domain-containing protein 3 OS=...	20.74 %	1	3	3	3		0.00 %			85.65	20.74 %	3	3
4223	Q103305	Transmembrane protein 118 OS=Homo sapiens GN=TM1...	6.53 %	1	1	1	5		0.00 %			92.67	6.53 %	1	5
4224	Q100203	AP-3 complex subunit beta-1 OS=Homo sapiens GN=AP3...	5.39 %	1	3	3	3		0.00 %			83.56	5.39 %	3	3
4225	Q1H0A0	N-acetyltransferase 10 OS=Homo sapiens GN=NAAT10 PE=...	2.05 %	1	1	1	1		0.00 %			23.63	2.05 %	1	1
4226	Q157H19	RRP12-like protein OS=Homo sapiens GN=RRP12 PE=1 S...	2.85 %	1	2	2	2		0.00 %			44.95	2.85 %	2	2
4227	Q1U0V2	Tight junction protein 20-2 OS=Homo sapiens GN=TNFR...	0.92 %	1	1	1	1		0.00 %			49.25	0.92 %	1	1
4228	Q19938	Legumin OS=Homo sapiens GN=LGN PE=1 SV=1- [L...	7.16 %	1	2	2	2		0.00 %			49.13	7.16 %	2	2
4229	Q17955	Plastin-1 OS=Homo sapiens GN=PLT1 PE=1 SV=3- [PL...	1.87 %	1	1	1	1		0.00 %			31.03	1.87 %	1	1
4230	Q1H139	Neuroplastin OS=Homo sapiens GN=NPPLN PE=1 SV=2- [...	10.05 %	1	2	2	2		0.00 %			45.60	10.05 %	2	2
4231	Q13357	DnaJ homolog subfamily C member 3 OS=Homo sapiens...	3.20 %	1	1	1	1		0.00 %			42.56	3.20 %	1	1
4232	P13161	40S ribosomal protein S20 OS=Homo sapiens GN=PSL1 PE=...	20.34 %	1	3	3	5		0.00 %			123.16	20.34 %	3	5
4233	Q19919	Polytopic kidney disease 3-like 1 protein OS=Homo sape...	1.86 %	1	1	2	2		0.00 %			21.12	1.86 %	2	2
4234	Q10533	Alpha-2-macroglobulin receptor-associated protein OS=H...	3.08 %	1	1	1	1		0.00 %			45.28	3.08 %	1	1
4235	Q186WH2	Fufl14 domain-containing protein 2 OS=Homo sapiens G...	11.11 %	1	2	2	2		0.00 %			45.05	11.11 %	2	2
4236	Q11978	Nucleolar and coiled-body phosphoprotein 1 OS=Homo sa...	3.72 %	1	2	2	4		0.00 %			56.55	3.72 %	2	4

Figure 33: Proteome Discoverer multi report for two files

For the comparison of the searches of the two best methods (190 min gradient & 270 min gradient, Top10 and 50 cm separation column length) 5 465 merged proteins, 45 847 peptides, 134 247 peptide-to-spectrum matches were found for 325 719 search inputs without applying any filters or false discovery rates. Interestingly no protein groups could be assigned before applying the false discovery rates, which seems to be a bug in the software.

After applying a relaxed FDR of 0,05 and a strict FDR of 0,01 the number of protein groups for both samples combined was 4 454 while the number of merged proteins, peptides, PSMs and search inputs remained unchanged. The actual relaxed false discovery rate was only 0,0119 and the actual strict false discovery rate 0,01 as indicated in *Figure 34*.



Figure 34: FDR settings applied in Proteome Discoverer

After applying the a “Mascot Significance Threshold” of 0,05 as peptide filter and changing the protein grouping option “Consider only PSMs with confidence at least” to “Low” as indicated in *Figure 35* the number of protein groups for both samples combined decreased from 4454 to 4420, the merged proteins from 5398 to 5465, the peptides from 45 847 to 40 036 and the PSMs from 134 247 to 116 617.

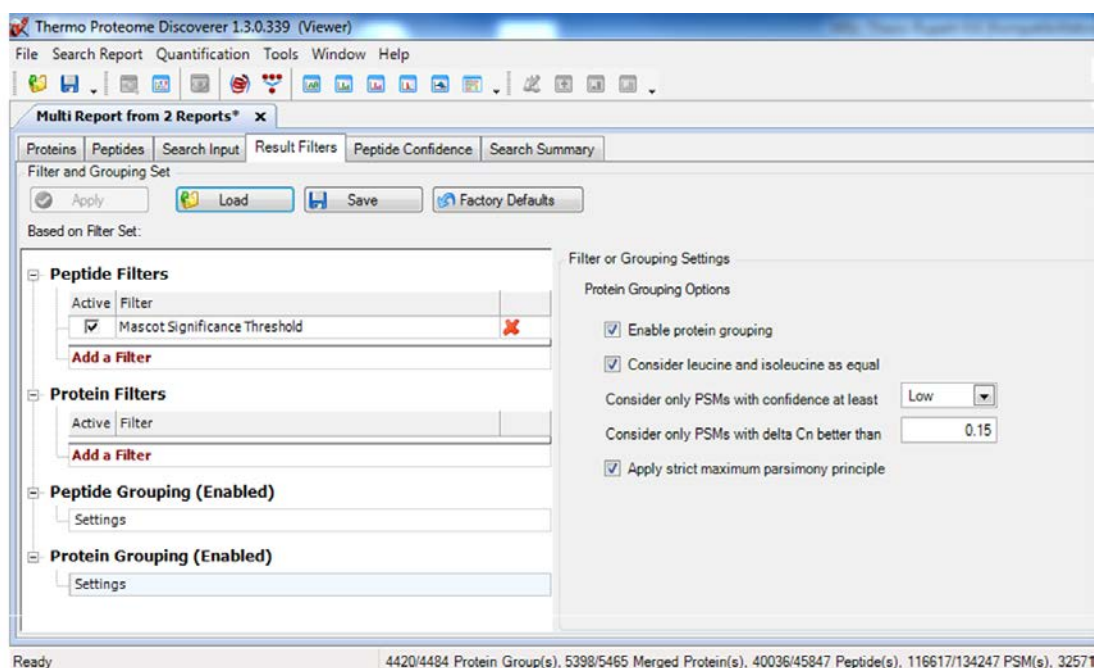


Figure 35: Filter setting in Proteome Discoverer

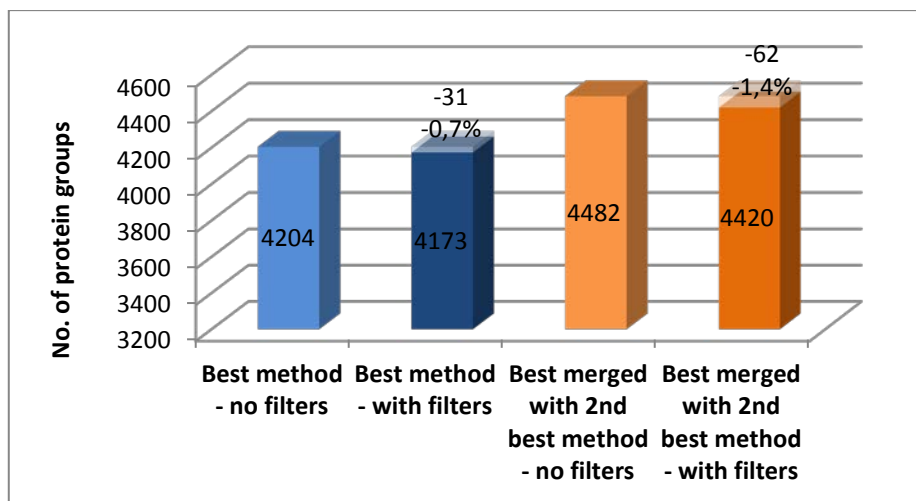


Figure 36: Comparison of protein group number for best method with/without filters and best+2nd best method with/without filters

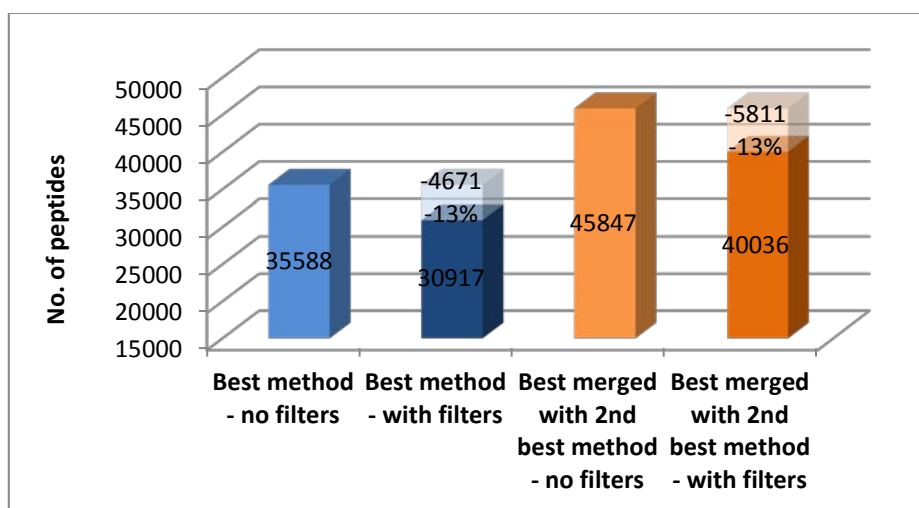


Figure 37: Comparison of peptide number for best method with/without filters and best+2nd method with/without filters

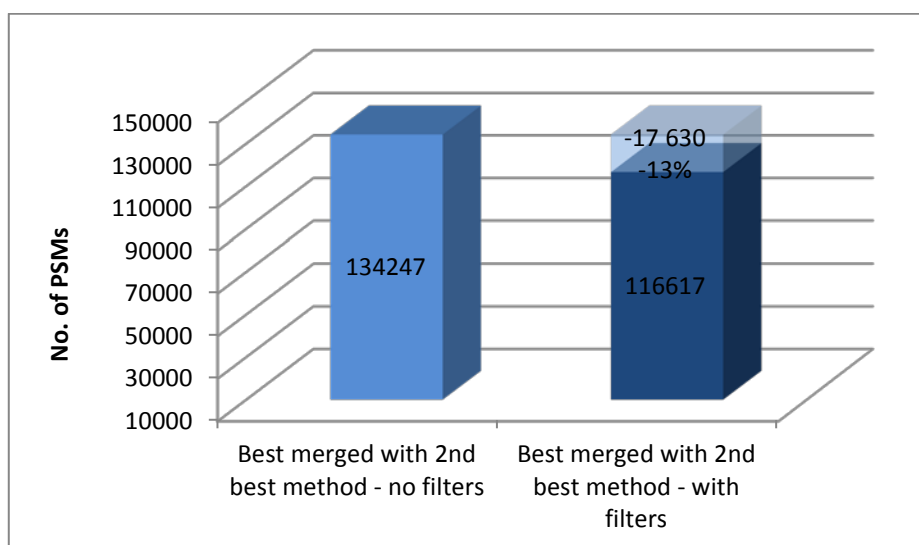


Figure 38: Comparison of PSMs for best method with/without filters and best+2nd best method with/without filters

Initial LC-MS/MS Method

The initial LC method utilized a 108 min gradient including washing steps and a 15 cm Acclaim* PepMap* RSLC separation column from Thermo. The mass spectrometric method was based on a Top6 method. The number of protein groups identified with this initial method for the Jurkat cytoplasm was 2721 and the number of identified peptides was 17530.

Increase of gradient length from 108 min to 190 min

The gradient time was then increased to 190 min keeping all other settings constant and the number of protein groups and peptides were compared in Figure 39 and Figure 40. The increase in the number of identified peptides was 49% and the increase for the number of identified protein groups was 26%.

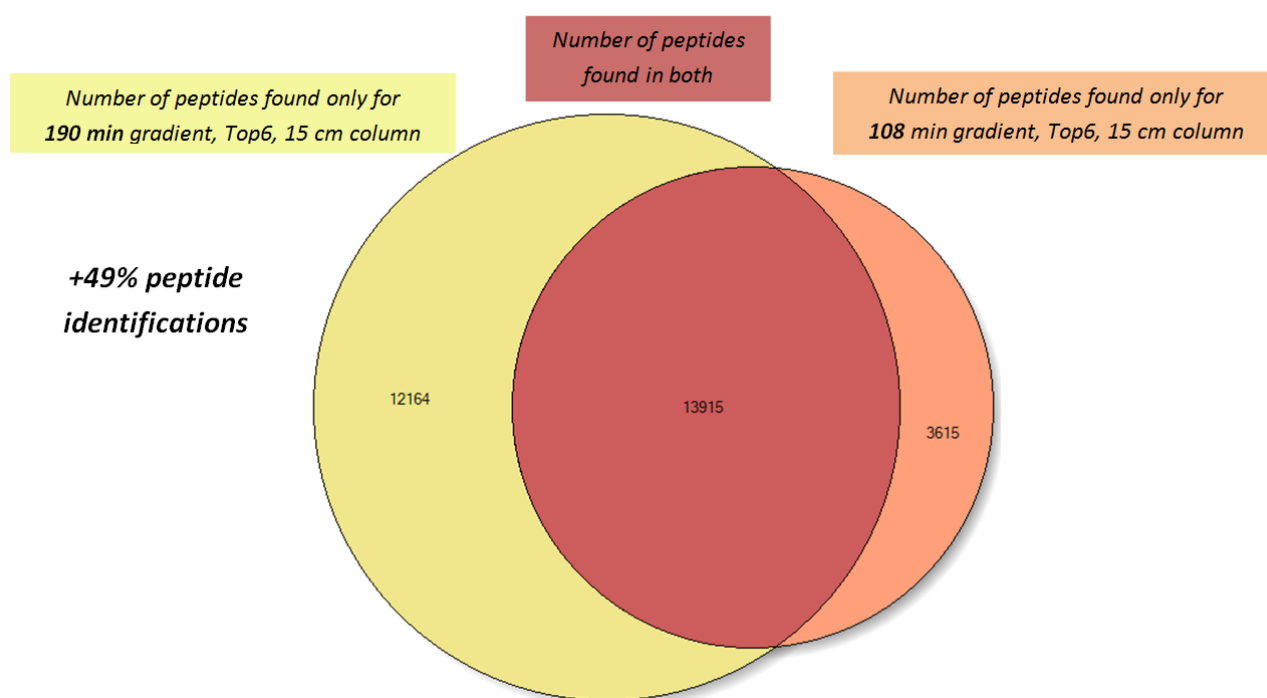


Figure 39: Comparison of peptide IDs for 108 min and 190 min gradient with Top6 and 15 cm column for Jurkat cytoplasm in-gel digest

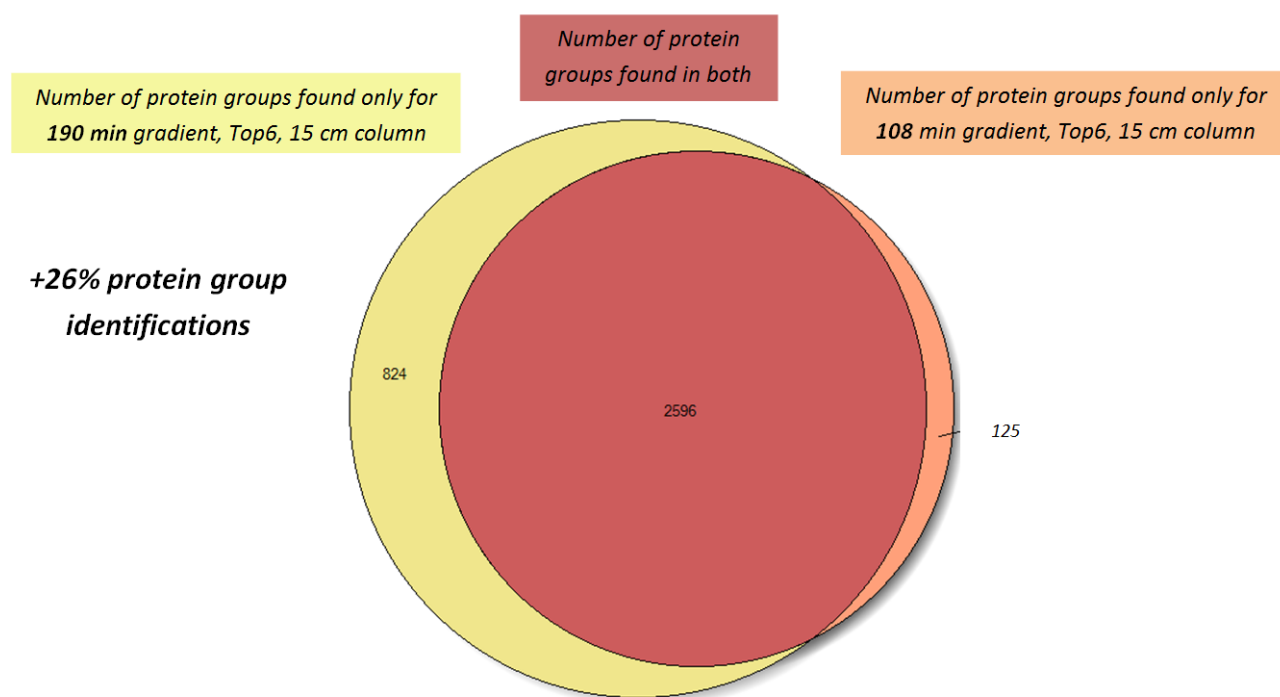


Figure 40: Comparison of protein group IDs for 108 min and 190 min gradient with Top6 and 15 cm column for Jurkat cytoplasm in-gel digest

When the raw data from the runs with 108 min and 190 min gradient time were assessed with the software “RawMeat” (Figure 41), the 108 min method showed a significantly higher ratio of Top6 than the 190 min method. Nevertheless, even for the 190 min method the number of Top6 performed was greater than any other TopN. This observation led to the conclusion that the sample was too complex for the applied method and most of the analysis time the six most abundant intact peptides were chosen for fragmentation potentially missing out on slightly lower abundant peptides. Therefore several other methods were tested with longer gradient time, Top10 instead of Top6 and a 50 cm column for better separation.

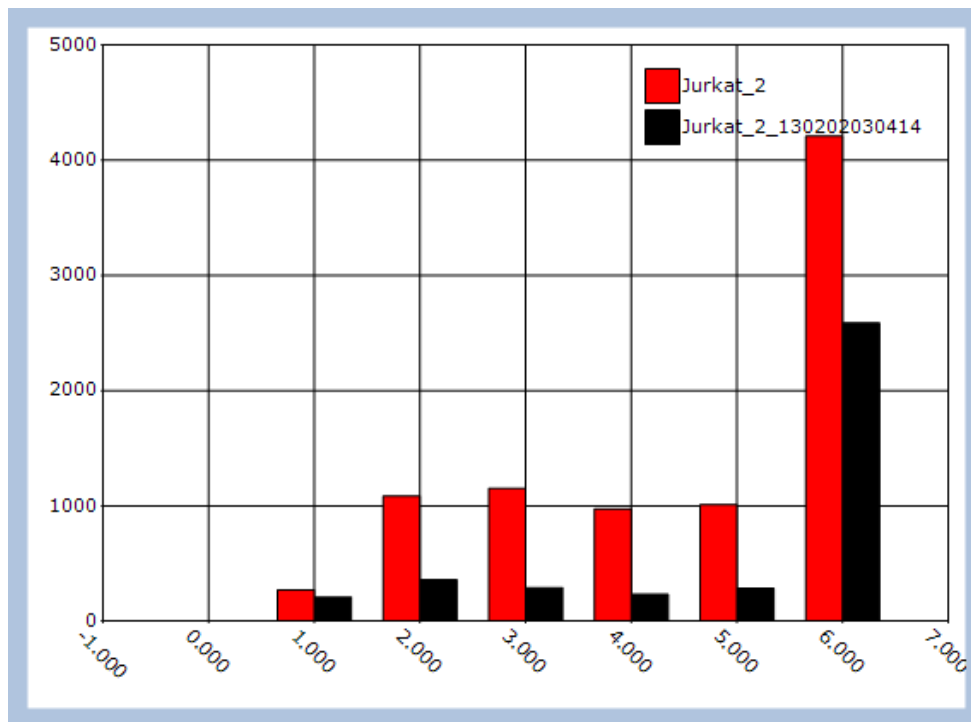


Figure 41: TopN spacing of band 2 for 108 min (black bars) and 190 min method (red bars)

Increase of Top6 to Top10 MS/MS method

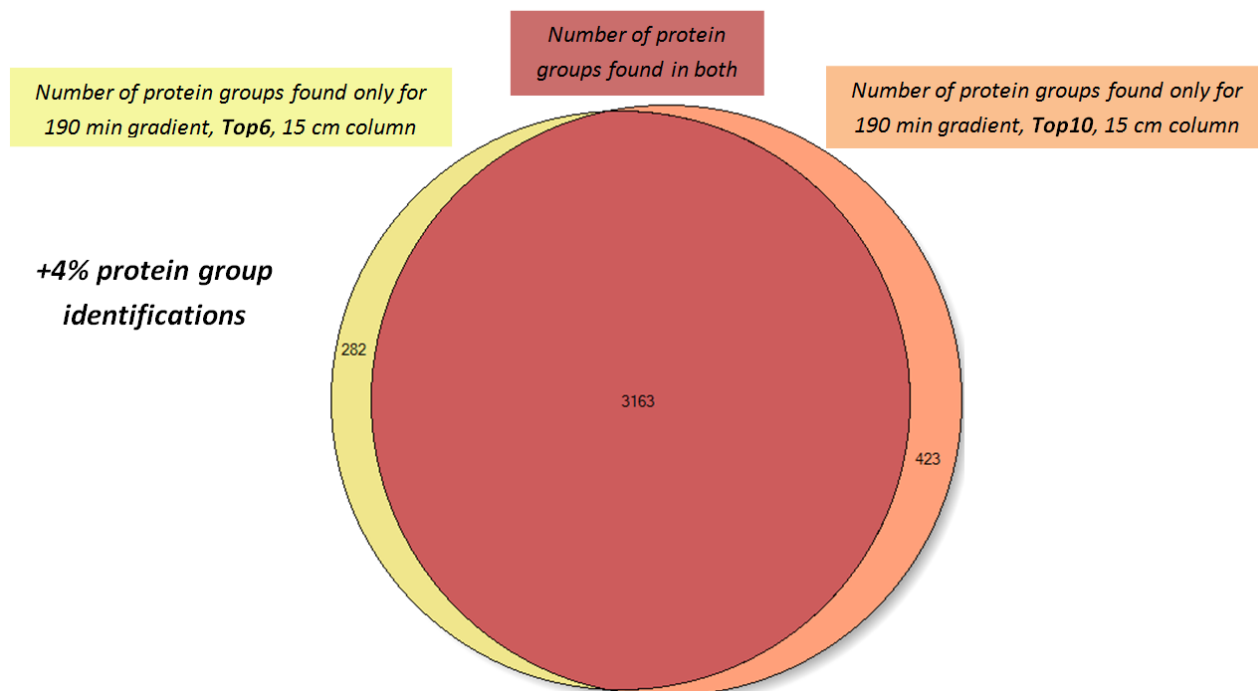


Figure 42: Comparison of protein group IDs for Top6 and Top10 with 190 min gradient and 15 cm column for Jurkat cytoplasm in-gel digest

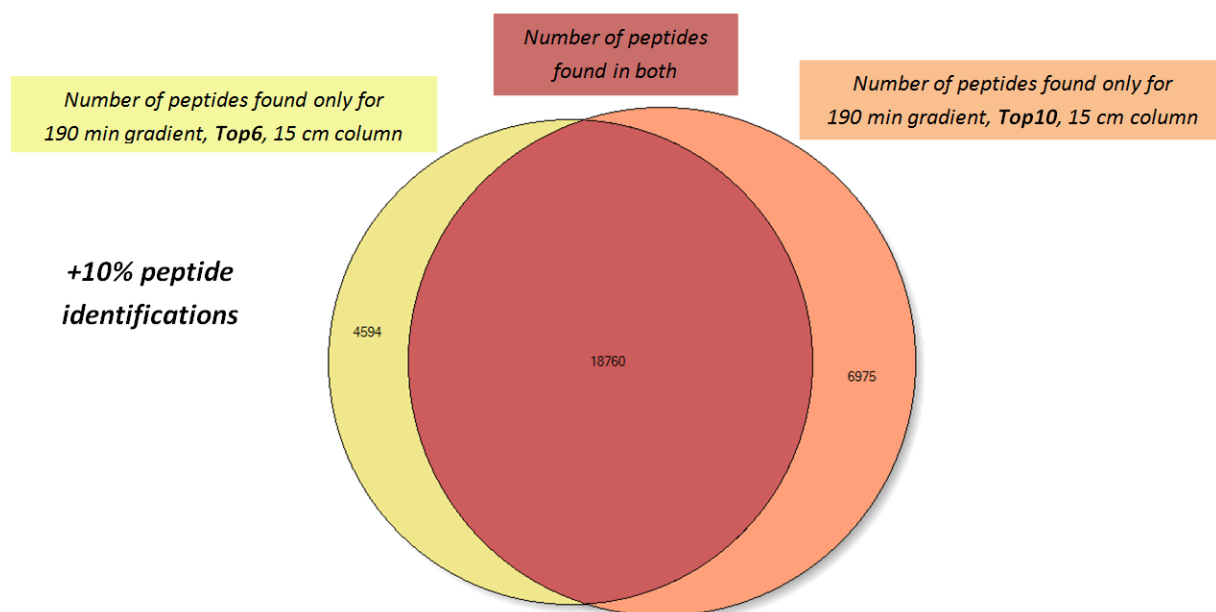


Figure 43: Comparison of peptide IDs for Top6 and Top10 with 190 min gradient and 15 cm column for Jurkat cytoplasm in-gel digest

The new Top10 method achieved 4% more protein group identifications and 10% more peptide identifications. This represents only a slight improvement but as no negative effects. This Top10 method was therefore chosen as starting point for further optimisation. In Figure 44 the distribution of the TopNs is shown. The black bars indicate the Top10 method and the red bars the Top6 method. While the Top6 method performed about 4000 Top6, only 1800 Top10s were performed for the Top10 method. This means that for the Top6 method the instrument might have missed peptides for fragmentation in 4000 cases, while for the Top10 method this happened only 1800 times. Hence, the complexity of the sample seems to be still too high even for the Top10 method and further reduction of sample complexity on the LC side has to be achieved.

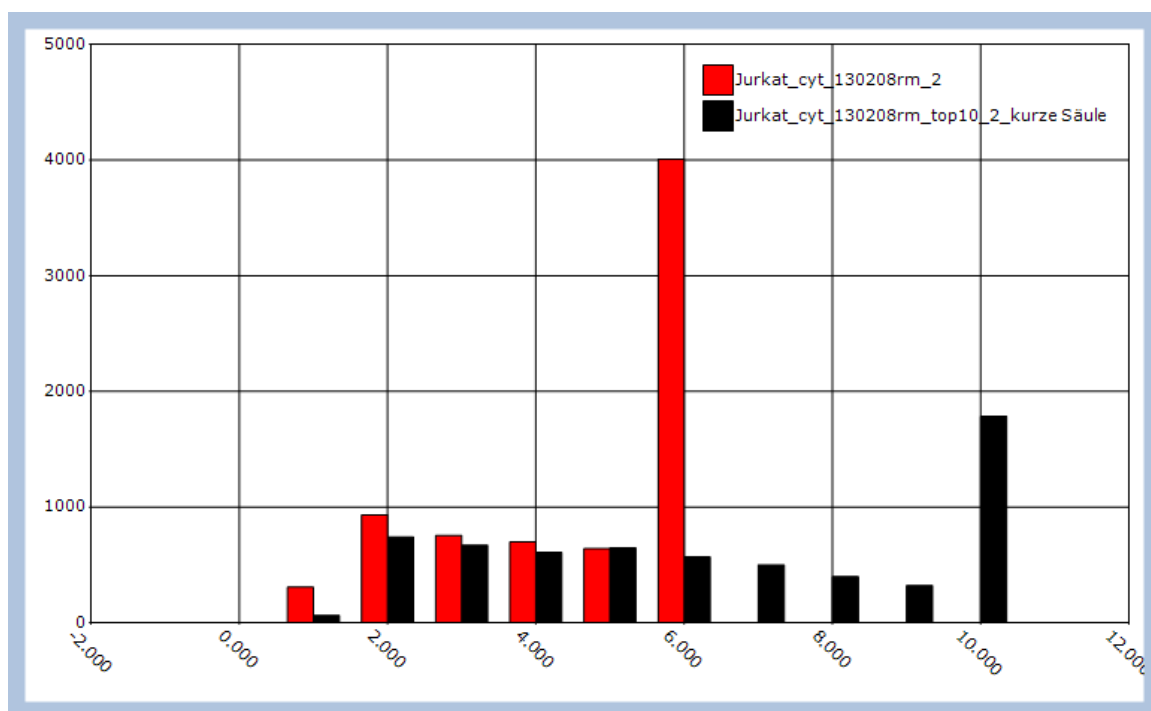


Figure 44: TopN distribution of band 2 for Top6 (red bars) and Top10 (black bars) method with 190 min gradient and 15 cm column

Increase of separation column length from 15 cm to 50 cm

The application of the longer separation column resulted in a 16% increase in protein group identifications and 17% increase in peptide identifications. The lower total number of protein groups and peptides is caused by the fact that only 3 of the 4 bands (band 2, 3 and 4) could be analyzed due to insufficient sample volume. The TopN distribution pattern did not change significantly when using the longer column as still somewhat less than 2000 Top10 were performed, which suggests that the sample complexity is still too high for the instrument to fragment all peptides present in abundances suitable for fragmentation.

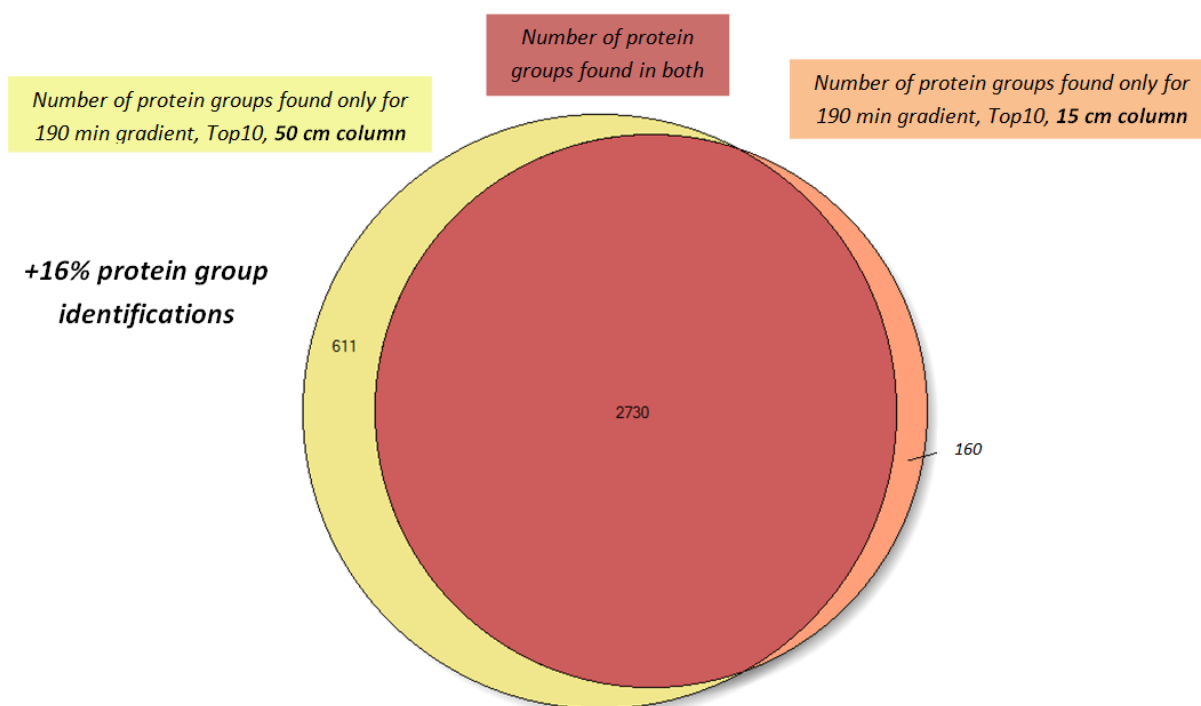


Figure 45: Comparison of protein group IDs for 15 cm and 50 cm column with Top10 and 190 min gradient time for Jurkat cytoplasm in-gel digest

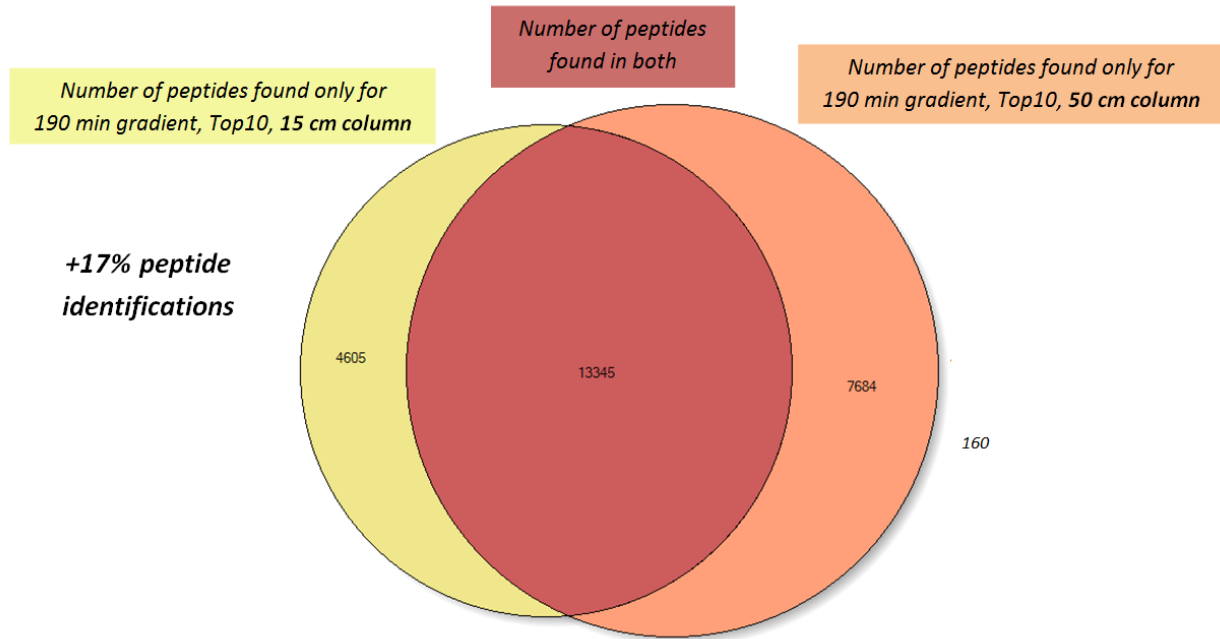


Figure 46: Comparison of peptide IDs for 15 cm and 50 cm column with Top10 and 190 min gradient time for Jurkat cytoplasm in-gel digest

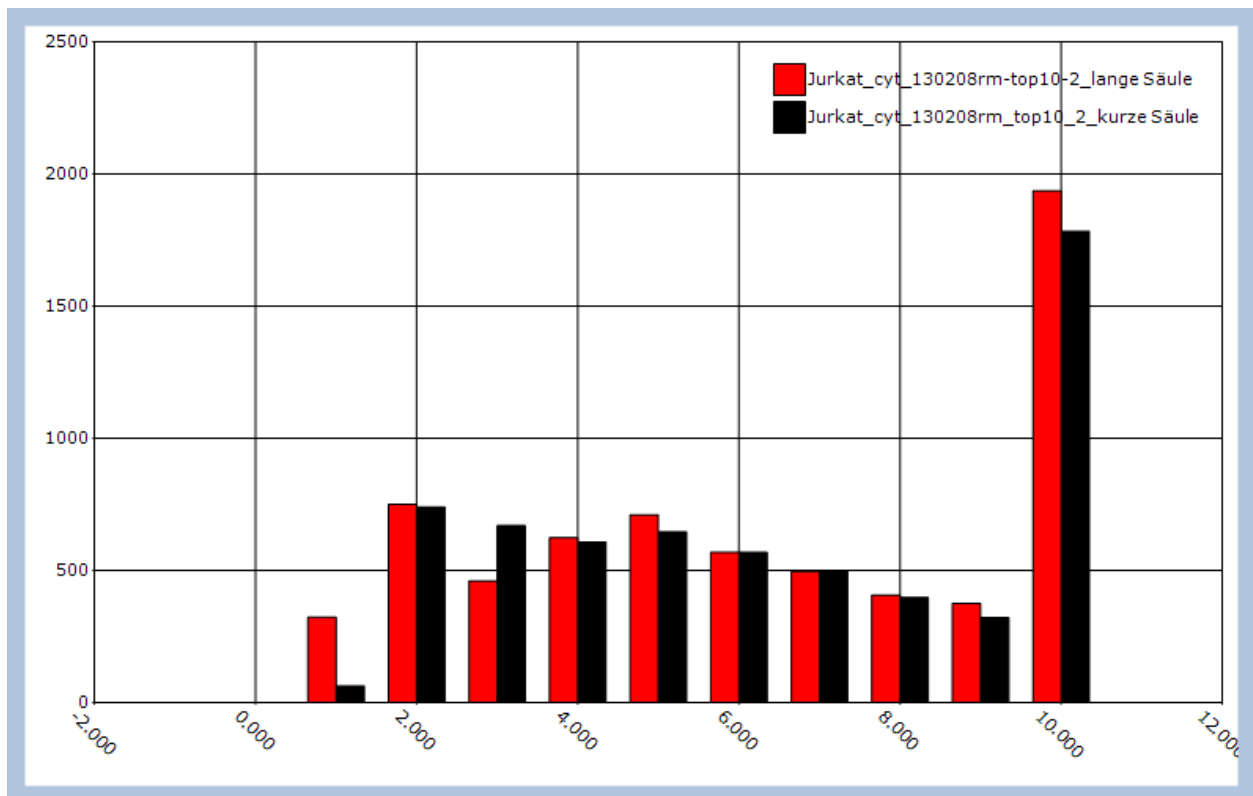


Figure 47: TopN distribution of band 2 of 15 cm (black bars) and 50 cm (red bars) column length methods

Increase of gradient length from 190 min to 270 min

To further reduce the sample complexity the MS instrument has to deal with at any given time of the gradient, the gradient length was increased further to 270 min. In Figure 48 it can be seen that prolonging the gradient time resulted in the desired complexity reduction. The TopN distribution clearly shows that for the 270 min method the number of Top10s is strongly reduced in comparison to the 190 min method, while the number of Top2 to Top7 is significantly increased. This indicates that the ionized peptides transferred to the instrument can now be isolated and fragmented in a more comprehensive way than with the shorter gradient. As a result of increased gradient time 15% more protein groups and 19% more peptides could be identified. In fact the gradient time could still be increased to further reduce sample complexity as there are still Top10s performed. A downside of a further gradient prolonging is the reduction in sample throughput and potential peak broadening, which would reduce the effective peptide abundance reaching the instrument at any given time point. This reduction in abundance could in turn reduce the number of identified peptides and protein groups.

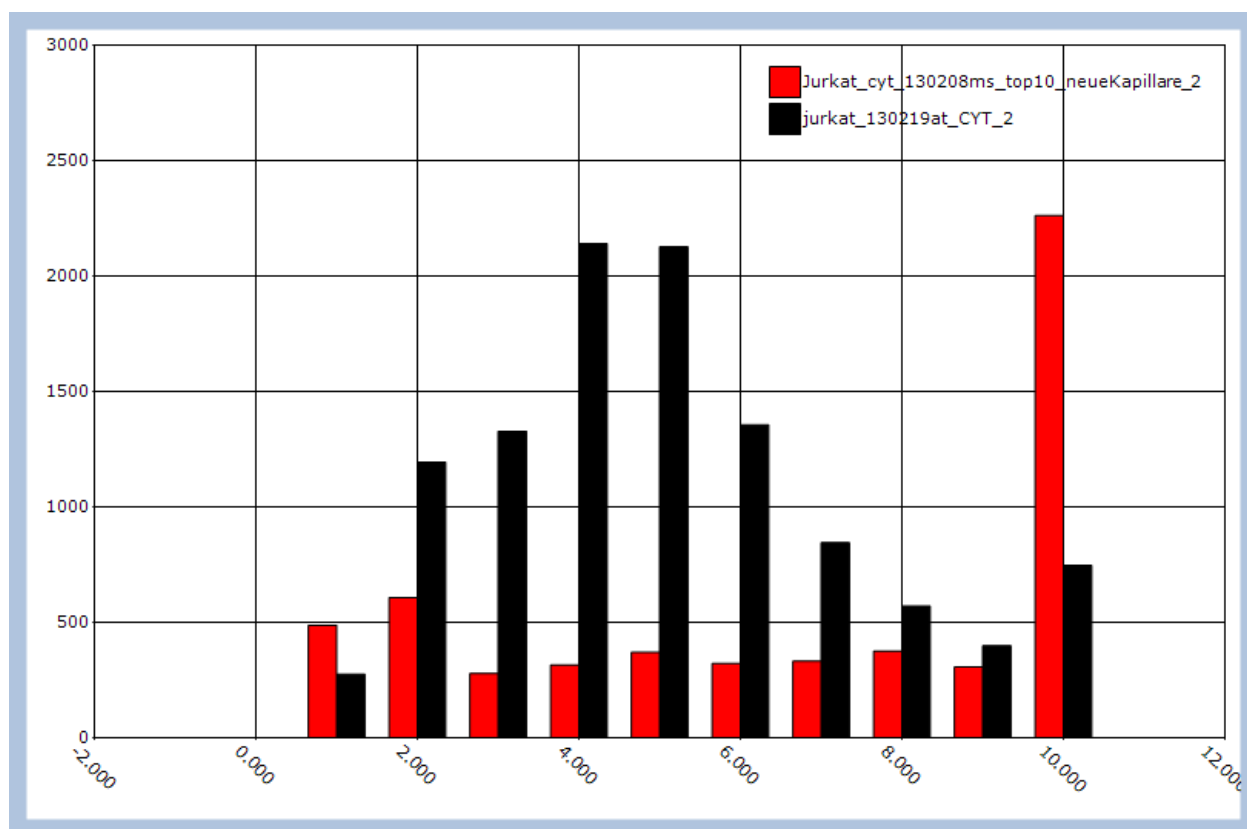


Figure 48: Comparison of 190 min (red bars) and 270 min (black bars) method for Jurkat cytoplasm in-gel digest

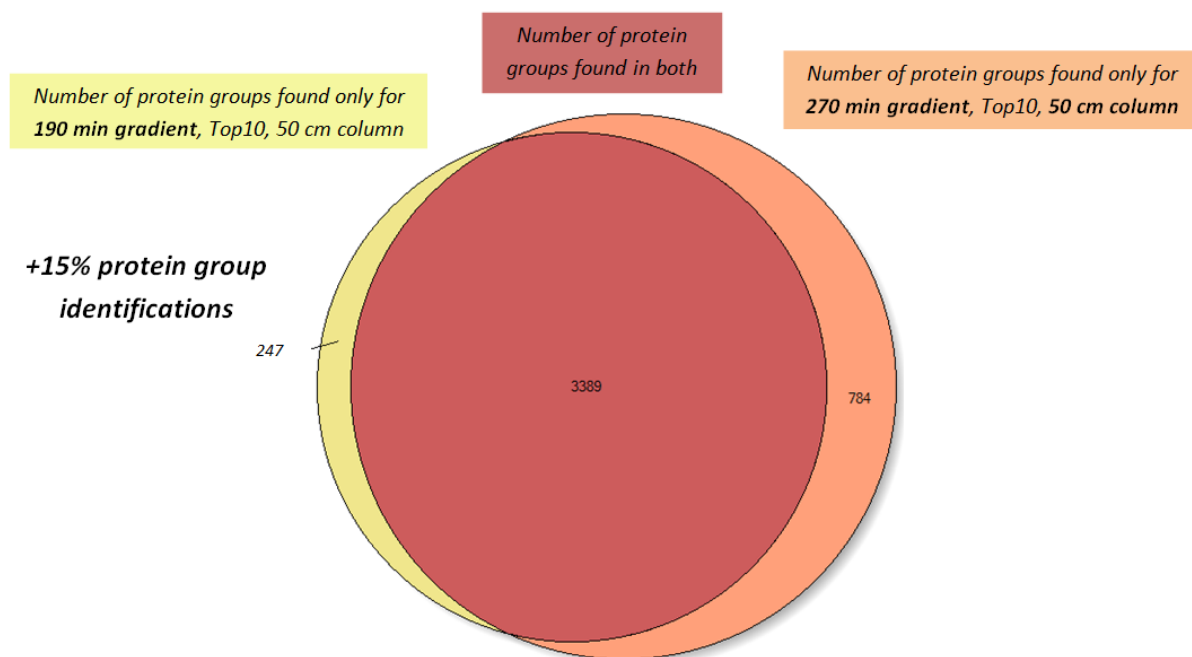


Figure 49: Comparison of protein group IDs for 270 min and 190 min gradient with Top10 and a 50 cm column for Jurkat cytoplasm in-gel digest

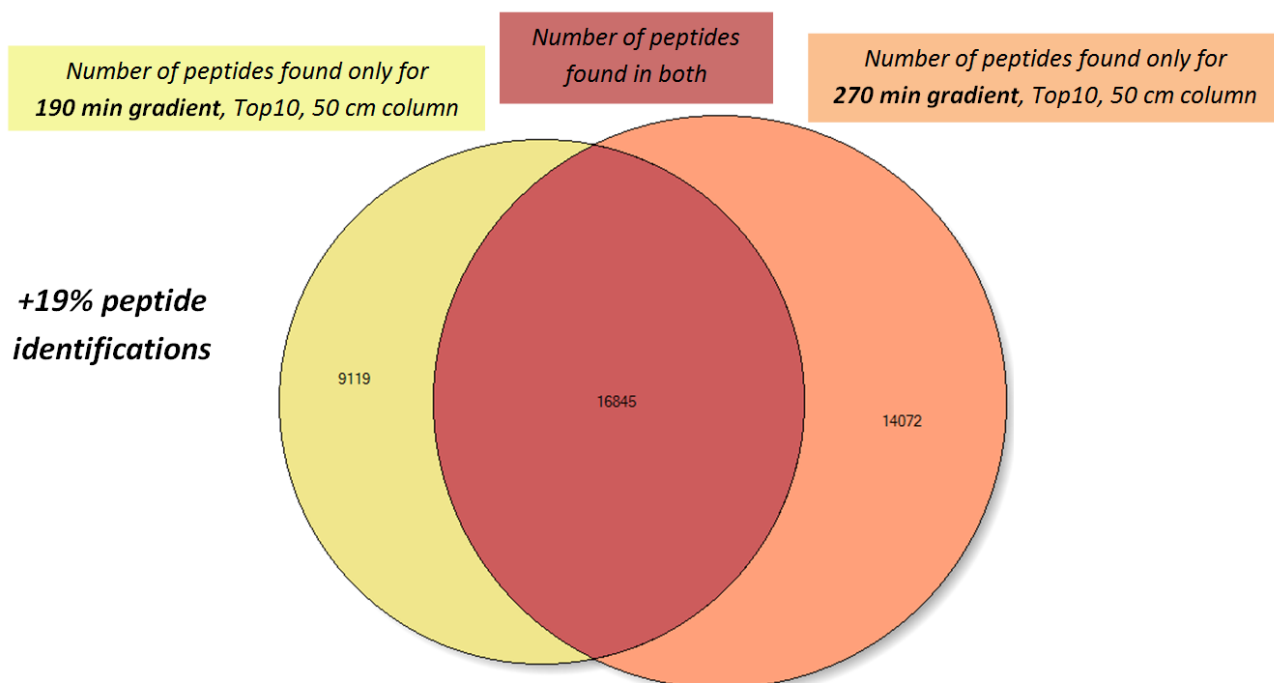


Figure 50: Comparison of peptide IDs for 270 min and 190 min gradient with Top10 and a 50 cm column for Jurkat cytoplasm in-gel digest

Conclusion of the LC-MS/MS Method Optimization

In the course of the optimization of the LC-MS/MS methods gradient time, separation column length and TopN method were assessed. Summing up all optimization steps an increase of about 60% for protein group identifications and about 95% for peptide identifications was achieved. This considerable improvement in identifications was on the other hand paid for with longer analysis time and subsequently lower throughput. Instead of the initial 108 min gradient the final gradient was 270 min long, which represents an increase of analysis time of 150%. Due to the necessity of this long gradient for identification and quantification of low abundant marker proteins like CXCL5 from complex matrices, this long analysis time was accepted as inevitable negative side effect.

These numbers are calculated from the method-to-method increases and differ from the numbers calculated from initial and final method only. The deviation on the protein level is rather small with 60% ID improvement for method-to-method calculation and 53% ID improvement for initial-final-only calculation. The difference on the peptide level on the other hand is quite high with 95% ID improvement for method-to-method calculation and only 76% ID improvement for initial-final-only calculation.

Table 8: Detailed data on LC-MS/MS optimization

Column length [cm]	Gradient time [min]	TopN method	No Proteins filtered	No peptides filtered	% of bands measured
15	108	6	2721	17530	100
15	190	6	3420	26079	100
15	190	6	3445	23354	100
15	190	10	3586	25735	100
15	190	10	2890	17950	75
50	190	10	3341	21029	75
50	190	10	3636	25964	100
50	270	10	4173	30917	100

Table 9: Overview of ID improvements based on methodology change

Change in Method	Peptide ID Increase	Protein Group ID Increase
108 min -> 190 min	49%	26%
Top6 -> Top10	10%	4%
15 cm -> 50 cm	17%	16%
190 min -> 270 min	19%	15%
TOTAL IMPROVEMENT	95%	60%

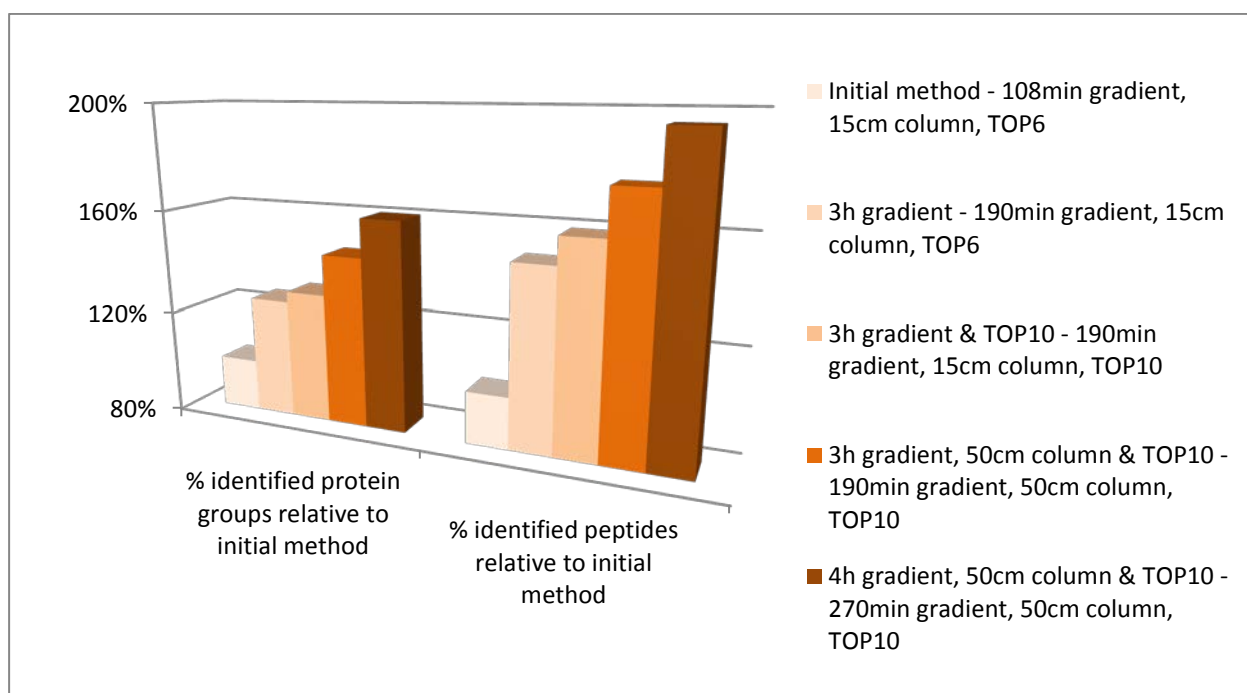


Figure 51: Overview of increase in peptide and protein group identifications with different methods applied

Quantification of CXCL5

Overview over experimental layout and file analysis

The recombinant protein CXCL5 was spiked into PBS/0,1%BSA as simple sample background and into PBS/10% serum as more complex sample background. The dilution series of CXCL5, which were prepared in the different matrices, ranged from 1 µg/mL to only 1 pg/mL and therefore covered six orders of magnitude.

20 µL of each sample concentration and background matrix +5 µL laemmli buffer were loaded onto a 13% PAA gel. Electrophoresis was performed until the marker was separated over a length of 3-4 cm and then stopped. After silver staining of the gels, one band per lane (below 15-20 kDa) was cut as indicated in Figure 52 and digested.

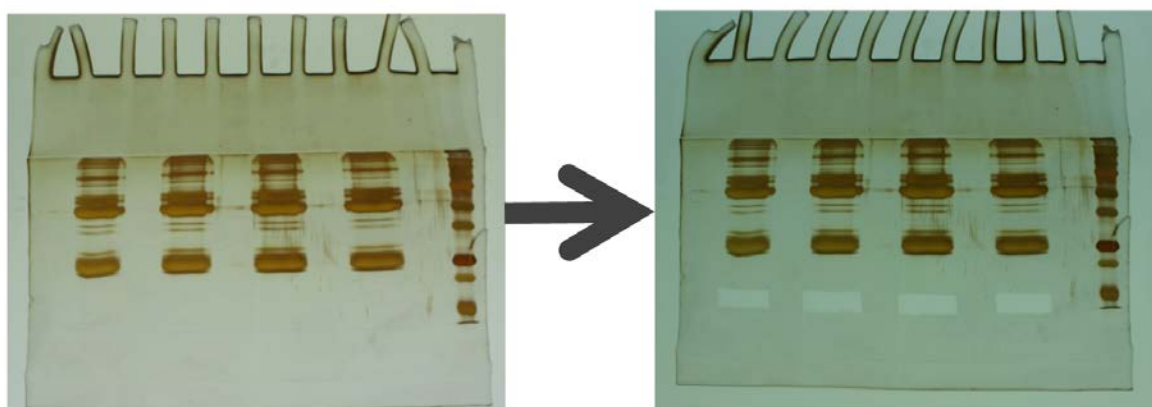


Figure 52: Excision of the gel bands for quantification of CXCL5

After the elution of the peptides from the gel pieces, the samples were concentrated down to 20 µL and stored at -20°C. For the LC-MS/MS analysis the samples were centrifuged and 15 µL transferred to a 96-well plate and diluted with 105 µL mobile phase A and 12 µL standard peptides (10 fmol/µL) added. 15 µL of the diluted sample was injected into the LC system and analyzed with a 85 min gradient with a Top10 method and a 50 cm separation column.

The raw files obtained from the sample run were manually controlled via Xcalibur for LC quality control. Therefore the position and the width of the peaks from the standard peptides, which were added just before the measurement, were assessed. An example for this is presented in Figure 53. The full peak width is about 30 s, the intensities within one order of magnitude and the elution order according to the hydrophobicity of the peptides. All these parameters are within specifications and therefore further analysis of the raw files via MaxQuant was performed.

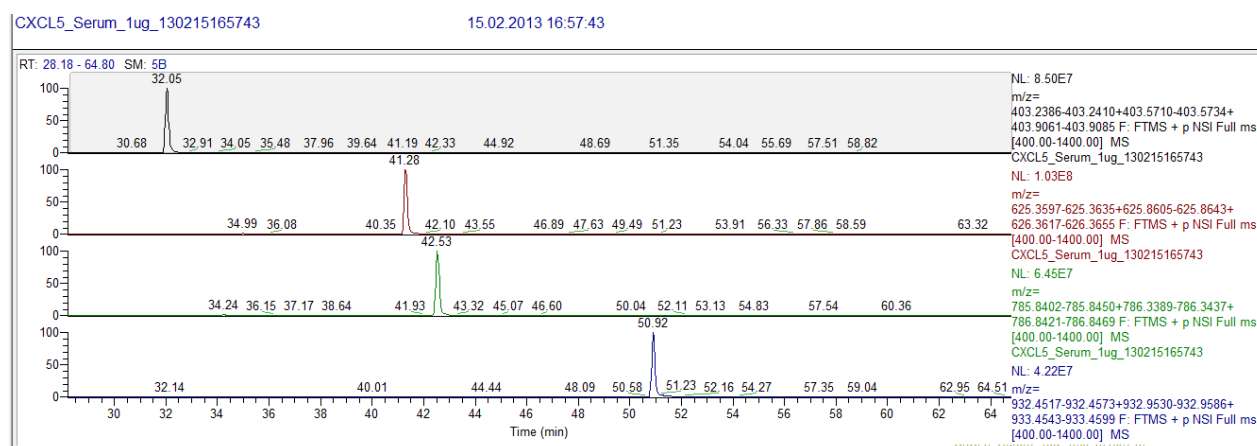
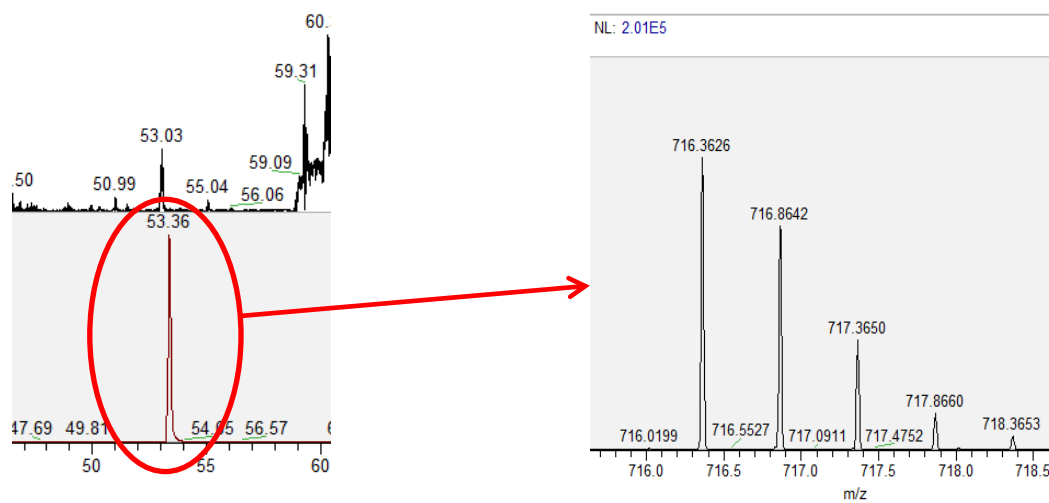


Figure 53: Extracted ion chromatograms of the four standard peptides for the assessment of the LC performance



**Figure 54: Left side - TIC of CXCL5 in PBS/0,1%BSA and XIC of one peptide specific (EICLDPEAPFLK) for CXCL5
Right side - Isotopic pattern for the CXCL5 specific peptide EICLDPEAPFLK**

General Information on MaxQuant Software

MaxQuant is a software package developed by the group of Matthias Mann for the analysis of quantitative MS-based proteomic experiments. This free software package was published in 2008 and was specifically designed for high resolution MS data obtained from large scale proteomic experiments. MaxQuant utilizes its own search engine called “Andromeda”. Beside labeled peptides it can also analyze label-free data and includes a very useful option named “match between runs”. This option enables the identification of peptides based on their exact mass and the retention time as long as the peptide was successfully identified in another sample that is co-analyzed in the same batch. For peptides of low abundant proteins this tool might be very valuable as the concentration could be high enough to obtain a decent MS¹ signal but might be too low to achieve a MS² spectrum of sufficient quality for identification. It could therefore be possible to build up a library of retention times and exact masses for the identification of peptides when keeping the retention times and therefore the LC method perfectly constant over time. A single cytoplasm sample of Jurkat cells analyzed in four runs resulted in the detection of more than 330 000 chromatographic features corresponding to peaks of potential peptides. Only 90 000 and therefore not even one third of these features were successfully identified. This relationship is graphically presented in Figure 55 which shows the MS¹ signals of one of the four runs of Jurkat cytoplasm with the identified peptides marked in pink and the unidentified features marked in blue. By building up the previously mentioned library of retention times in combination with exact masses of a large number of peptides the detection of a significant higher number of peptides per experiment seems quite feasible.

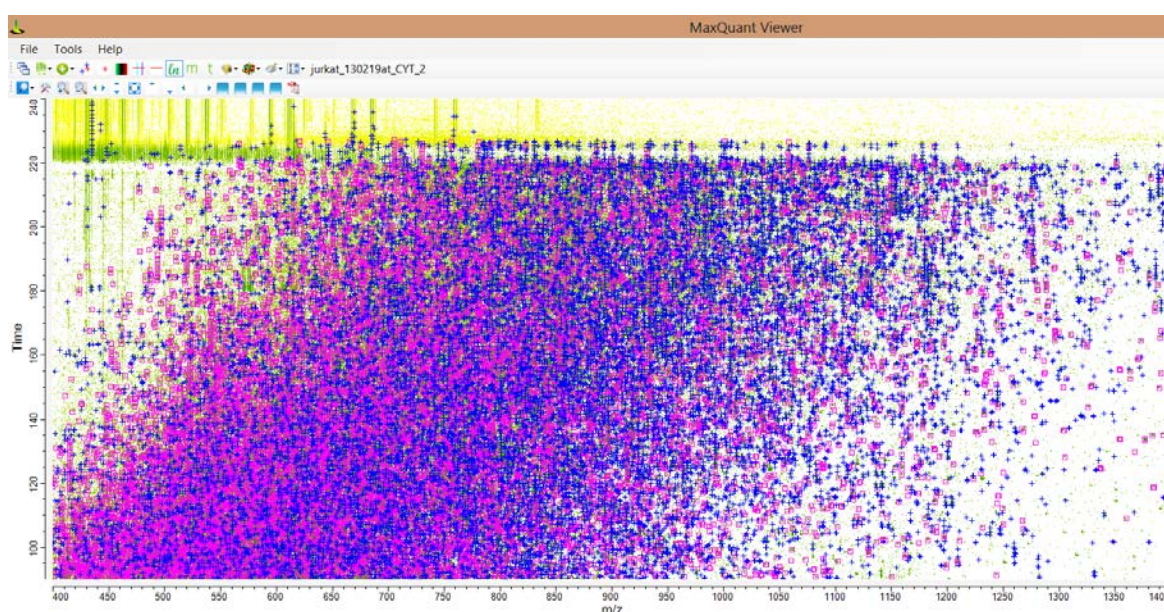


Figure 55: MaxQuant - plot of time against m/z with identified peptides represented by pink rectangles and not identified species represented by blue plus signs

The first step in data processing for MaxQuant is the detection of so called “features”, which essentially correspond to peaks arising from different species. These features are detected from each MS¹ spectrum as 2D peaks first and are then traced over time to obtain 3D peaks. In the course of the feature detection process these 3D peaks are then further processed as indicated in *Figure 57*. After these

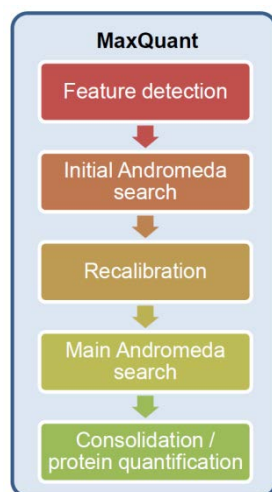


Figure 56: MaxQuant Data Processing Overview [29]

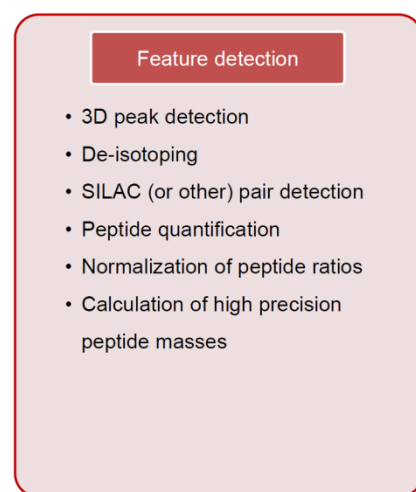


Figure 57: MaxQuant Feature Detection [29]

The obtained raw files were searched and analyzed via the MaxQuant software package (version 1.3.0.5). Therefore the files were loaded and an experimental design file created. The group specific parameters were changed to Oxidation (M) and Acetyl (Protein N-term) for variable modifications, the multiplicity was set to 1 (as no labeled experiment was performed), the maximum number of modifications per peptide was set to 2, the number of maximum missed cleavages to 2 the maximum charge to 7. Trypsin/P was chosen as enzyme and for the first search 10 ppm was entered. For the main search 5 ppm was chosen and the option "individual peptide mass tolerances" was activated. Type was set to standard and all other settings in the group-specific parameters were left on the default settings.

In the "MS/MS & sequences" tab the FASTA file for human, which was previously downloaded, was added (Uniprot_HUMAN_201207.fasta). As fixed modification Carbamidomethyl (C) was chosen and include contaminants turned on. All other settings were left at the default state.

In the "identification & quantification" menu the minimum number of unique peptides was set to 1 and the filter labeled amino acids option as well as the second peptides option unticked. The experimental design file was chosen and for protein quantification use only unmodified peptides and Oxidation (M) and Acetyl (Protein N-term) entered. The discard unmodified counterpart peptides option was unticked and 1 entered as minimum ratio count. All other features in the identification and quantification tab were left untouched.

In the miscellaneous part only match between runs were ticked and set to 2 min and label-free quantification. The LFQ min. ratio count was set to 1 and fast LFQ disabled. Re-quantify and iBAQ were both disabled. The number of threads to be used for the analysis was set to 3 and the analysis started. The results of the MaxQuant search were viewed with Perseus and the LFQ intensity of CXCL5 in the different samples assessed.

The detection limit and the linear range of CXCL5 were analyzed and compared for the matrices PBS with 0,1% BSA and PBS with 10% human blood serum. The desired detection limit was in the lower pg/mL range both for PBS/0,1% BSA and PBS/10% serum. It was expected that the detection limit for PBS/0,1% BSA might be slightly lower than for the more complex sample matrix with 10% serum.

Somewhat surprisingly the limit of detection for both sample matrices was identical. The lowest concentration that was detected in PBS/0,1% BSA and PBS/10% serum was 1000 pg/mL (= 1 ng/mL) (Figure 59). Unfortunately the low pg/mL concentration range could not be assessed without immunoprecipitation. The detection limit concerning the total amount of analyte injected for one run on the other hand was in the low pg range. As indicated in Figure 58 For both matrices 1,7 pg of CXCL5 was the lowest total amount loaded onto the trapping column that could still be detected. The linearity from 1 µg/mL down to 1 ng/mL was quite good for both matrices with R^2 values above 0,99 with slightly better linearity for PBS/0,1% BSA.

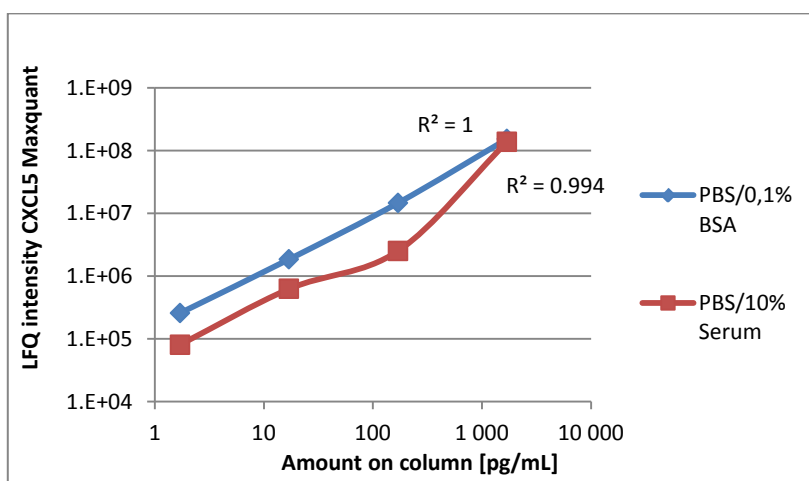


Figure 58: Comparison of non-IP samples of CXCL5 in PBS/0,1% BSA (blue) and PBS/10% serum (red)

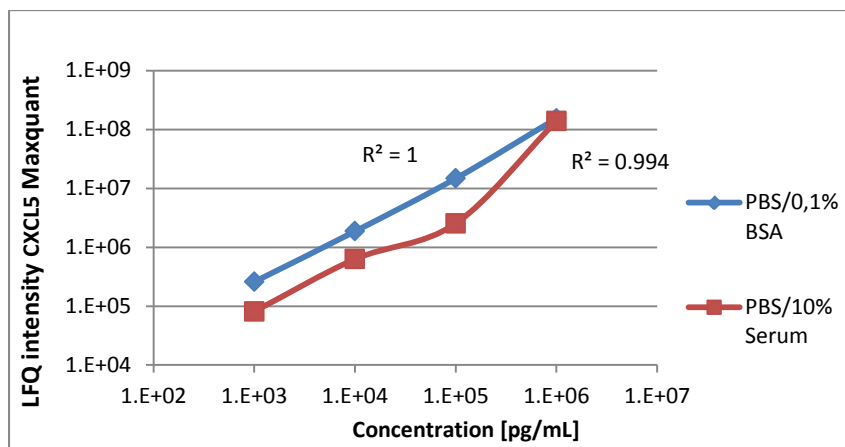


Figure 59: Comparison of non-IP samples of CXCL5 in PBS/0,1% BSA (blue) and PBS/10% serum (red)

Figure 60 and Figure 61 illustrate the signal intensity difference between the immunoprecipitated samples and the non-immunoprecipitated samples. The samples preconcentrated via immunoprecipitation show LFQ intensities about two orders of magnitude higher than the intensities of the non-IP samples. Despite this intensity increasing effect the lower limit of detection could not be decreased. Analogous to the non-IP samples no concentration that was smaller than 1 ng/mL could be detected. This may be caused by analyte losses during the IP, which are not evident at higher concentrations but could become more important at very low concentrations. In terms of linearity the immunoprecipitated samples suffered only minor losses in linearity with slightly better results again for PBS/0,1% BSA as background.

Compared to ELISA the sensitivity of the optimized MS-based detection method with a lower limit of detection of 1 ng/mL is rather poor as ELISAs typically detect the antigen of interest in the low pg/mL range. With a linear range over four orders of magnitude from 1 µg/mL down to 1 ng/mL and its stability against matrix effects the derived MS-based method seems to be ahead of the typical ELISA.

For the detection of smaller CXCL5 concentrations more sample volume than the 300 µL that were used for IP could be applied. (e.g. 10 mL) Besides higher sample volume for immunoprecipitation also more sensitive mass spectrometers like triple quadrupole instruments could also be applied to lower the limit of detection. Alternatively other precleaning strategies like depleting the plasma from the most abundant proteins via spin columns could be tested.

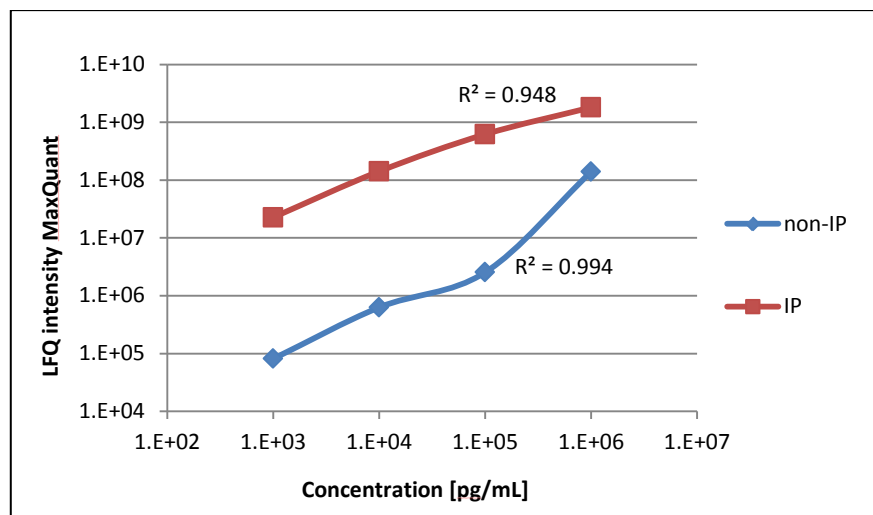


Figure 60: Comparison of non-IP (blue) and IP (red) samples of CXCL5 in PBS/10% serum

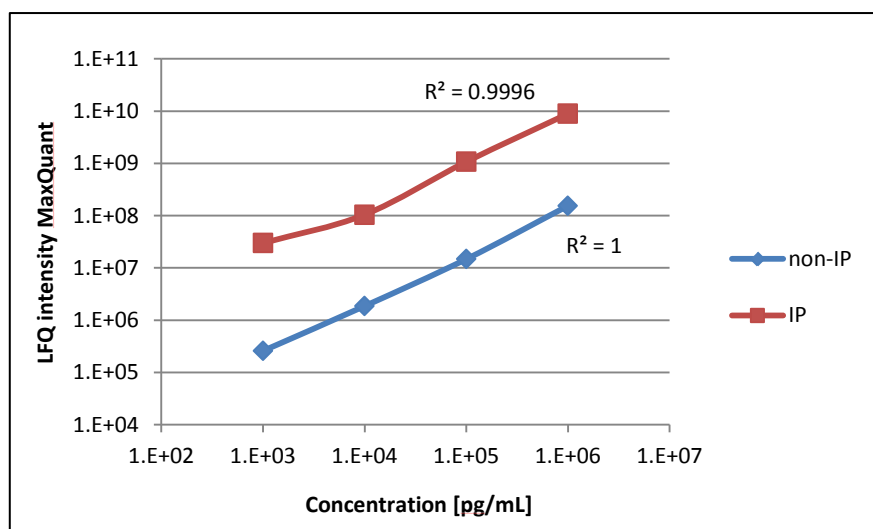


Figure 61: Comparison of non-IP (blue) and IP (red) samples of CXCL5 in PBS/0,1% BSA

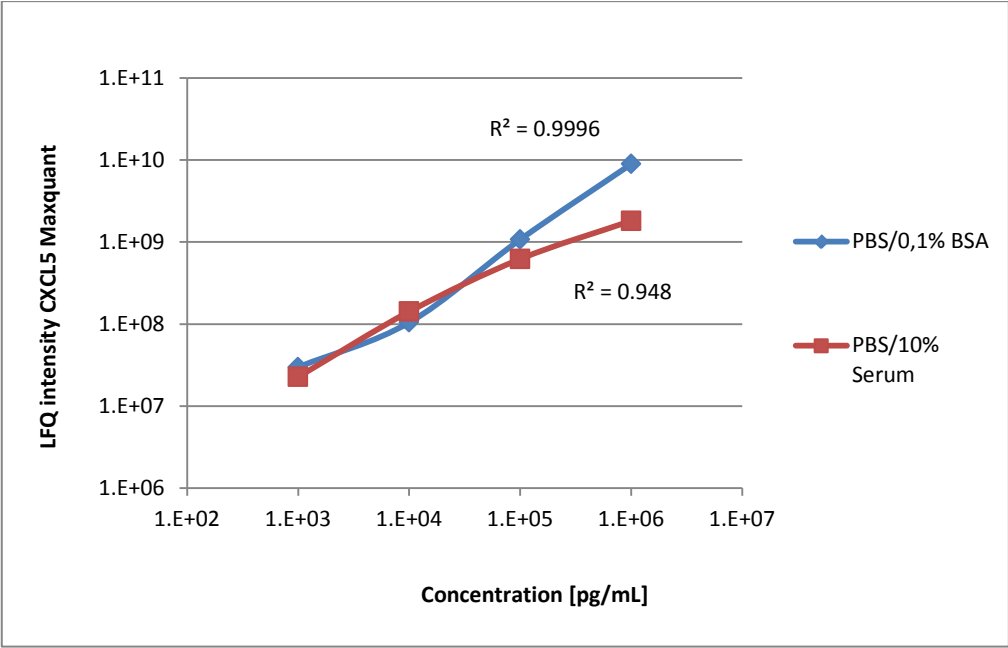


Figure 62: Comparison of IP CXCL5 samples in PBS/0,1% BSA (blue) and PBS/10% serum (red)

Conclusion

The aim of this work was to develop a sensitive and selective MS-based methodology for the quantitative detection of the cytokine CXCL5.

For this purpose the LC-MS/MS methodology was optimized using Jurkat cytoplasm digests. The number of successfully identified protein groups was taken as a measure for the ability to identify and quantify analytes from complex matrices such as blood plasma. The optimization of the LC-MS/MS method included the variation of gradient time length, separation column length and TopN method. The initial methodology consisted of a gradient that was 108 min long, utilized a 15 cm separation column and a Top6 method. With this method it was already possible to assign 2721 protein groups from 17530 peptides identified. In the course of the optimization the gradient was prolonged to 270 min, the column length increased to 50 cm and a Top10 method chosen. The same sample applying this method yielded 4173 protein groups with 30917 peptides. This corresponds to an increase in peptide identifications of 76% and an increase in protein group identifications of 53% but also an increase of analysis time of 150%.

CXCL5 was spiked into PBS/0,1% BSA and PBS/10% serum at a concentration range of 1 µg/mL to 1 pg/mL covering six orders of magnitude.

Immunoprecipitation was applied to reduce potential matrix effects and enrich CXCL5 prior to the MS analysis. The general immunoprecipitation protocol was optimized for CXCL5 and the efficient binding and enrichment were confirmed via Western Blot analysis. Both sample types, with and without immunoprecipitation, were analyzed via shotgun MS.

The lower limit of detection for all sample types and background matrices was found to be 1 ng/mL and could not be improved by applying immunoprecipitation. Although the signal intensity for the immunoprecipitated samples was significantly higher, lower concentrations could not be detected. The linearity in the concentration range of 1 µg/mL to 1 ng/mL was quite good for all samples but was slightly better for non-immunoprecipitated samples ($R^2 > 0,99$) than for immunoprecipitated samples ($R^2 > 0,94$).

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Born: 1.4.1988 in Vienna

Education

- 2009-now** **MSc “Biological Chemistry” with specialization in “Chemical Biology”**
- University of Vienna
- Modules: Bioanalytical Chemistry, Biophysical Chemistry, Bioinorganic Chemistry, Microbiology and Genetics, Cell Biology, Biotechnology
- MSc Project: Optimisation of LC and MS Conditions for the Separation of Complex Protein Samples on a nanoUHPLC-Orbitrap System and Quantitative Determination of Cytokines – CXCL5 as an Example
-
- 2008-2009** **BSc “Medicinal Chemistry and Pharmaceutical Sciences”**
- Dublin Institute of Technology, Ireland
- Graduation: Distinction
- Modules: Organic Chemistry, Inorganic Chemistry, Physical Chemistry, Separation Sciences, Quality Assurance, Computational Chemistry, Professional Skills, Pharmaceuticals, Medicinal Chemistry, Environmental Chemistry, Spectroscopy
- BSc Project: Preparation and Characterisation of N,F co-doped visible light active TiO₂ Photocatalyst
-
- 2007-2008** **Military Service – Education as paramedic**
-
- 2002-2007** **College of Chemistry Specialising in Biochemistry, Biotechnology and Genetic Engineering**
- 1170 Wien, Rosensteingasse 79
- Graduation: Distinction
- Diploma Thesis: Analysis of Mycotoxins and Glycoalkaloids in the Sugar and Starch Technology

Practical Experience

October 2012 – January 2013 – University of Vienna, Department for Analytical Chemistry

- ✓ Supervision of students in the “Analytical-chemical practical” and in the “Chemical basics practical II A”

Juli 2003 & 2005 – Analytical Laboratory “Errichtungsgesellschaft Marchfeldkanal”

- ✓ Sampling and analysis of ground and surface waters via UV/Vis spectroscopy, electrochemical, volumetric and gravimetric methods as well as microbiological tests.

Mai-Juli 2009 – Centre for Research in Engineering Surface Technology

- ✓ Synthesis and characterisation of inorganic materials via X-ray diffraction, FTIR, UV/Vis spectroscopy, BET surface analyzer and differential scanning calorimetry.

Other Qualifications

German (native language), English (advanced level)

Experienced use of Windows and MS Office

Hobbies

- ✓ Soccer – Local team - SCU Obersdorf/Pillichsdorf
- ✓ Chairman of the association “Katholische Jugend Obersdorf”
- ✓ Assistance on parental farm

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