universität wien

MASTERARBEIT

„Immunological and mass spectrometry based methods for the sensitive and accurate determination of selected interleukins and growth factors, including IL-6, IL-8 and CXCL3“

Verfasser

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, Juli, 2012

Studienkennzahl lt. Studienblatt:	A 066 863
Studienrichtung lt. Studienblatt:	Masterstudium Biologische Chemie
Betreuerin / Betreuer:	ao. Univ.-Prof. tit. Univ.-Prof. Mag. Dr. Andreas Rizzi

Abstract

The use of standard methods for the identification or quantification of proteins using Western blot (WB) analysis or ELISA may lead to unexpected results with respect to sensitivity, specificity, reliability and robustness, because of matrix effects. We have performed a systematic comparison of different antibody-based and mass spectrometry-based methods in order to assess their advantages and disadvantages, as well asspecific limits of each of them

By the combination of the advantages of both types of methods, high sensitivity of antibody-based methods and high reliability and robustness of mass spectrometry-based methods, the best results were obtained particularly when dealing with samples containing complex.

Zusammenfassung

Bei der Anwendung von Standard Methoden für die Identifizierung und Quantifizierung von Proteinen, wie z.B. Western Blot (WB) Analyse oder ELISA, kann es auf Grund von unerwünschten Matrixeffekten zu verfälschten Ergebnissen kommen. Diese Effekte können die Robustheit, Sensitivität, Spezifität und Verlässlichkeit der Ergebnisse beeinträchtigen.

Wir haben einen systematischen Vergleich von verschiedenen Methoden, welcher auf die Verwendung von Antikörpern und auf Massenspektrometrie basierenden Methoden durchgeführt, um die Vor- und Nachteile der jeweiligen Methoden und deren analytischen Grenzen festzustellen.

Durch das Ausnützen der Vorteile beider Methoden, z.B. die hohe Sensitivität der Antikörper basierenden Methoden und die hohe Robustheit und Verlässlichkeit der MS-basierende Methoden, konnten bei deren Kombination die besten Ergebnisse erzielt. Diese Vorteile kommen besonders bei der Analyse von Proben mit komplexer Matrix zum Tragen.

Acknowledgments

I am thankful to Univ. Prof. Andreas Rizzi for enabling and supervising my master thesis in a very challenging and dynamic field such as mass spectrometry. Additionally, I want to thank him for the given possibility to cooperate with Univ. Prof. Christopher Gerner, who supported me in all steps of this project, It was a big benefit for me being part of this very productive and complementary cooperation.

I want to thank Dr. Astrid Slany for her encouragement and for introducing me to the ``Gerner lab``. The mass results would not be that impressive without the support of Dr. Michael Grössel, for which he has my honest acknowledgment. I would further like to thank Editha Bayer, Philip Klepeisz as well as all member of the research group for a good working atmosphere.

Especially I am thankful to the family of Ismet Spahija , Nesim Duja and lot of friends for helping me at the beginning of my live in Vienna.

I want to express my gratefulness to my family for being with me all this time.

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Part I: Methodology of low-abundant protein quantification

1. Quantification methods using specific antibodies

1.1 Luminex™ Assay - principles and methodology

For a long time specific antibody-based Enzyme-Linked-Immuno-Sorbent-Assays (ELISAs) have been the standard method for quantitative analysis of low-abundant protein biomarkers like cytokines or others. Studying and monitoring the course of most diseases require rapid measurements of a set of such proteins in serum or plasma samples, in both clinical as well as research laboratories. Such circumstances make simultaneous (multiplexed) measurements of these analytes irreplaceable, requiring an ability that ELISA does not have. Nowadays, several Multiplex Bead Array Assays (MBAAs) are available, the description thereof can be found in the literature as far back as 1977 (Horan& Wheeless, 1977). One of the most widely used multiplexing systems nowadays is the Luminex™ System released from Luminex Corporation in the late 1990´s(Elshal& McCoy, 2006).
The Luminex™ System uses the xMAP (Multi-Analyte-Profiling) technology which combines a flow cytometer, fluorescent-dyed microspheres (beads), laser light sources and digital signal processing to effectively allow multiplexing of up to 100 unique assays within a single sample. The main part of the Luminex xMAP technology consists of the polystyrene microspheres (beads) with a size of 5.6 micron. The beads are internally dyed with red and infrared fluorophores. By using different ratios of these dyes a set of 100 different beads with unique spectral signature is created. This fluorescence is used as reporter system that allows distinguishing each bead type according to its unique fluorescence intensities. These intensities are recorded after excitation with red laser in flow cytometry when the beads pass through a detection chamber (Chowdhury et al., 2009).

The beads' surface allows coupling of biomolecules such as antibodies, oligonucleotides, peptides etc. giving the coupled beads the properties to capture (targeting) particular molecules in the sample. The Luminex assay (Figure 1) is based on the principle of a sandwich immunoassay - the same Principe as in ELISAs - using a biotin-streptavidin system in which the streptavidin carries phycoerythrin as fluorescent reporter group. The antibody directed against a targeted analyte is coupled on a specific and known bead type. After incubation with the sample, the beads with the captured analyte will be incubated one more time with the biotinylated primary antibody, and finally the biotin-streptavidin binding will take place when incubating the sample with streptavidin coupled to phycoerythrin. Eventually, the phycoerythrin emission, stimulated by excitation with a secondary laser, is correlated with the amount of analyte molecules bound on the coated beads (Leng et al., 2008).

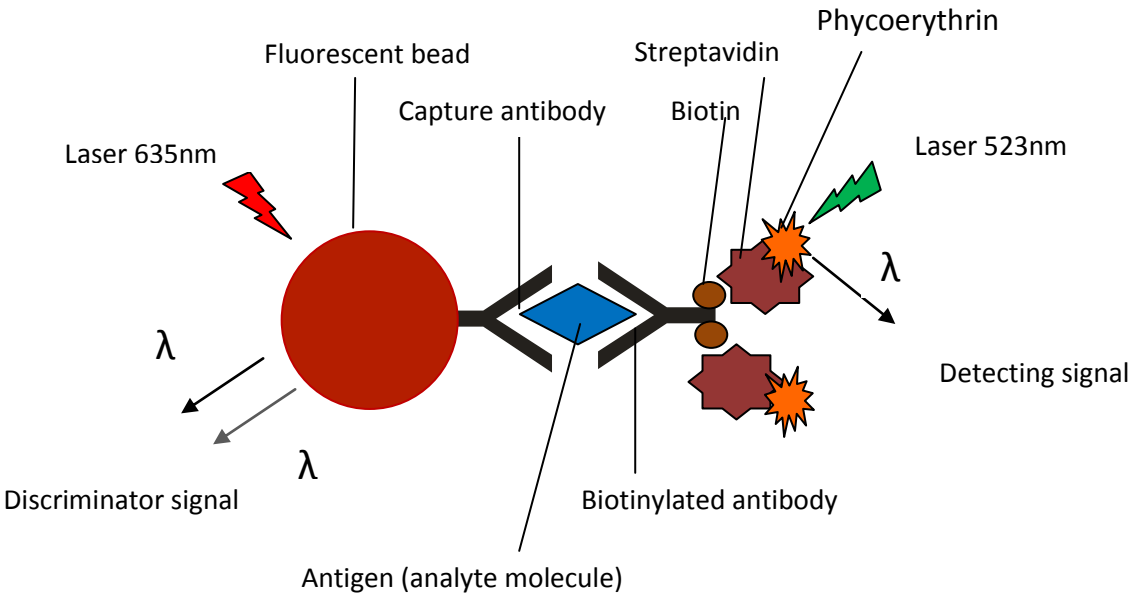


Figure 1. *Schematic drawing of a Luminex assay.*

The next part of the Luminex System (Figure 2) is the Luminex 100™ analyzer which utilizes principles of flow cytometry combined with advanced fluidics and high-speed digital signal processing. Two lasers are installed in the detection chamber which excite the fluorochromes embedded within the beads as well as the phycoerytrin-dye bound to the analyte molecule. By passing through the detector chamber via the fluid stream, the dyes will be excited and the intensities of the corresponding fluorescence signals will be measured by the detectors, one as detecting signal (analyte molecule), the other one as discriminating signal (identifying beads' type) (Dunbar et al., 2003).

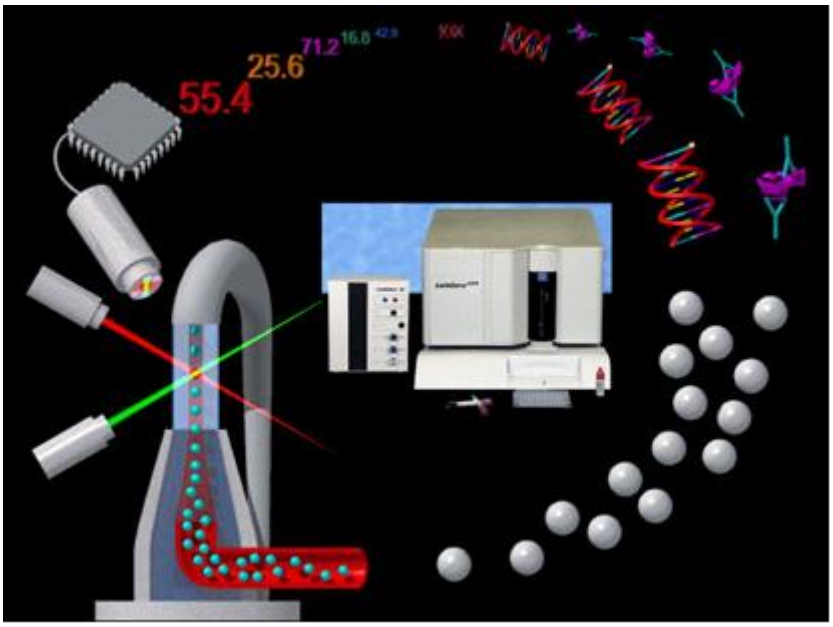


Figure 2. *Main parts of Luminex™ System: biomolecules; microspheres (beads); flow analyzer and high-speed signal processing, (taken from (Dunbar& Vander Zee& Oliver& Karem& Jacobson, 2003)).*

The possibility to distinguish about 100 different bead types in such a rapid way makes it possible to analyze many analytes from the same sample at the same time. For this purpose, different beads will be coated separately with antibodies directed against several different antigens, each known bead type coated with a certain antibody. Coated beads are first mixed and then incubated with the sample. As the Luminex assay needs small sample volumes only, the reactions can take place in 96-well plates. Finally, the measured fluorescence intensities will be processed and depicted in a diagram as Median Fluorescent Intensity (MFI). This value takes into account the carryover of the beads from one well to the next within the measurements by comparing with standard concentration using the following curve fitting function (eqn. 1):

$$MFI = a + \frac{b}{1 + \left(\frac{x}{c}\right)^{d}}$$

Equation 1. *Curve fitting equation using 4 fitting parameters: a - estimated response at zero concentration; b - estimated response at infinite concentration; c - mid-range concentration (EC50); d - slope factor (Hill Slope)*

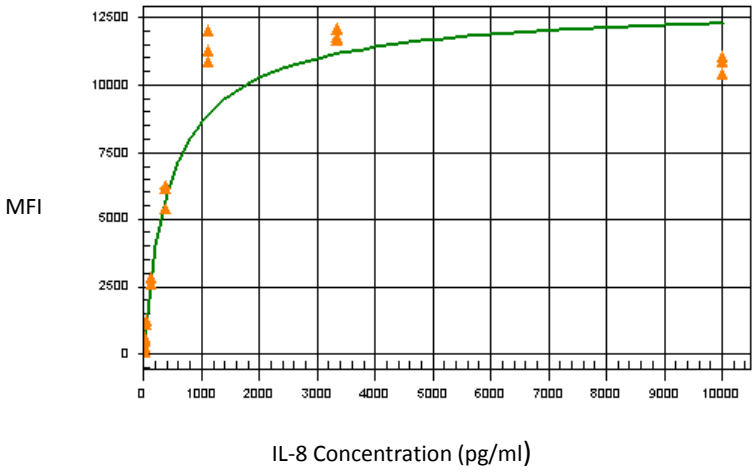


Figure 3. *Example of MFI response in dependence on analyte concentration: Experimental data are given by orange triangles; fitted curve based on eqn. 1 is given as green line. This curve is a typical output of the instrument's "data interpretation report".*

Using fluorescence intensities for analyte quantification offers many advantages including the very low detection limit (LOD) in the pg/ml range, and high selectivity. On the other hand, fluorescence-based calibration lines deviate from linearity at even not so high concentrations as a result of self-quenching and self-absorption. The evident advantages of

multiplexing arrays are the high throughput attainable, the low sample volume needed and the efficiency in time and costs.

1.2 Enzyme-Linked Immuno-Sorbent Assay - principles and methodology

Enzyme-Linked immuno-Sorbent Assays (ELISAs) were developed during the 1970s as alternative to Radio-Immuno Assays (RIAs). Potential health problems associated with the use of radioactive isotopes could be avoided when using enzyme-catalyzed color reactions instead of radioactivity as reporter principle. ELISAs belong nowadays to the most widely used methods in clinical and research laboratories, enabling accurate as well as sensitive detection and quantification of targeted protein (Lequin, 2005).

Commonly, the ELISA consists of two antibodies, the first one interacts with the antigen (analyte) determining in this way the assay's specificity, while the second one interacts with a constant region of the first (primary) antibody enabling detection by means of the reporter enzyme linked to this secondary Ab. With "competitive assays", the antigen is linked with a reporter enzyme. In order to amplify the signal, the biotin-streptavidin interaction is used: antibodies are biotinylated and streptavidin is linked to the reporter enzyme.

Three main formats of ELISAs can be distinguished: (i) indirect assays, (ii) sandwich assays and (iii) competitive assays. Sandwich assays are usually more sensitive compared to the other ones. However, a requirement for this type of assay is that the antigen has two epitopes which interact with the two antibodies at the same time. One of these antibodies is linked with the reporter enzyme.

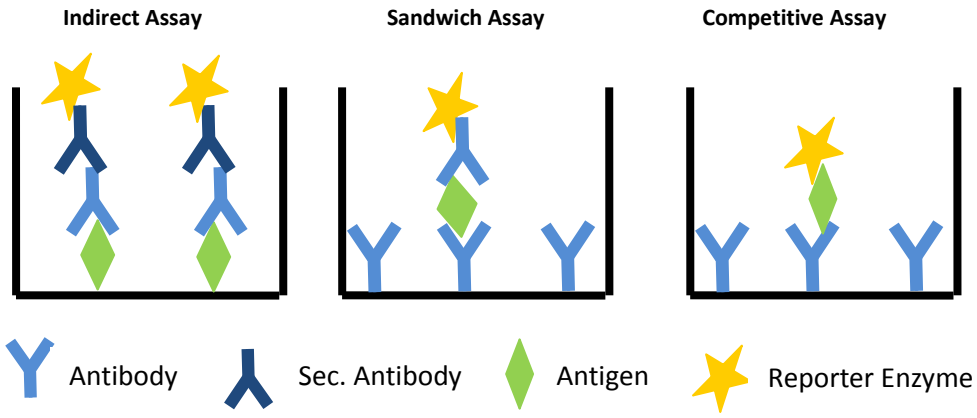


Figure 4. *The three ELISA formats.*

Two enzymes are widely used as reporter: horseradish peroxidase (HRP) and alkaline phosphatase. HRP reduces H_2O_2 to H_2O by using the tertramethylbenzidin (TMB) as substrate reagent. Its reduction leads to the colored solution.

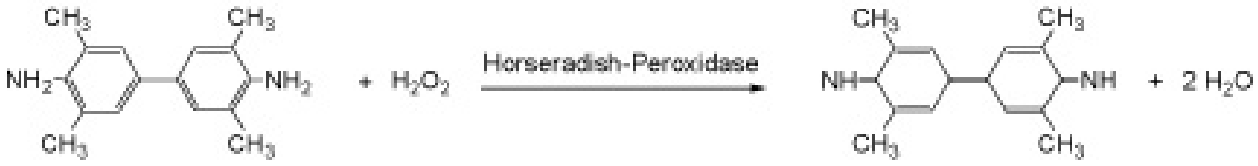


Figure 5. *Scheme of the Horseradish Peroxidase reaction.*

Usually, ELISAs are provided as a kit with all reagents included. Often, they are performed in a 96-well plate format, and a sandwich assay includes the following steps: Into a 96-well plate, onto which antibodies directed against a specific protein are coated, the sample containing the antigen is added and incubated for two hours ore overnight depending on the performance of the antibody used. The next steps include the incubation with the biotinylated (as well antigen binding) antibody, addition of streptavidin linked with reporter enzyme; the addition of the subtract reagent, TMB, and finally stopping the reaction with sulfuric acid and measuring the absorbance at 450nm. A calibration curve is constructed by blotting the concentrations of the calibration standards *vs*. the corresponding absorbance values measured. From the measured analyte absorbance analyte concentration is calculated *via* this curve.

ELISAs based on antibody-antigen interaction offer sensitivities in the pg/ml range and exhibit a high specificity allowing the quantification of the targeted proteins. It is frequently considered as the best validated method as it is usually well reproducible and robust. However, development of ELISAs is very expensive, and the assays usually have a limited dynamic range, their selectivity is strong depended on the quality of antibodies used, they have no multiplexing ability, and the influence of unspecific binding to matrix components can be considerable (Leng& McElhaney& Walston& Xie& Fedarko& Kuchel, 2008,Whiteaker et al., 2007).

1.3 Western-Blotting Assays: Principles and methodology

The procedure of Western blotting (WB) consists of three mean steps: (i) electrophoretic separation of the proteins, (ii) protein transfer (blotting) from the electrophoresis gel onto a blotting membrane, and (iii) the “development” of the membrane.

The electrophoretic separation is usually performed as a one dimensional SDS-PAGE by which proteins are separated according to their molecular weight. The poly-acrylamide

(PAA) gels employed are made by polymerizing a mixture of acrylamide- and bisacrylamide-monomers; the resulting “pore” size depends on the total monomer concentration (%T) and the content of the cross-linker in the mixture (%C). Usually PAA gels 12%T are used. Ammonium-persulfate is used to initiate the polymerization reaction, TEMED to catalyze it. For allowing a higher protein loading and concentrating of the analytes, two gel sections exhibiting different pore sizes are used (concentration gel and separation gel). In these zones different buffers with deferent pH-values are used: in the concentration zone (stacking gel) a pH value of 6,8 is commonly used, whereas in the separation zone the pH is 8,8 (Laemmli, 1970). Just before loading onto the gel the sample is mixed with loading buffer. This buffer contains mercaptoethanol as reducing agent for cleaving the disulfide bridges in the proteins, causing protein ´´linearization´´. In this way the interaction with SDS (present in loading buffer) is facilitated. By “complete” SDS loading all proteins become net negatively charged and reach a status where they have a more or less uniform charge to size ratio which effects that their mobility in free solution would be identical and independent on the original charges. In the polymer network of the gel the effective mobilities of the proteins is then only determined by the size (usually substituted by the logarithm of the molecular weight) of the proteins.

In a next step the proteins are transferred from the gel onto the membrane (Towbin et al., 1979), which commonly consists of nitrocellulose or PVDF (see Figure 5). This so called blotting process is based on an electrophoretic migration orthogonal to the gel and is based on the fact that all proteins are still negatively charged. This blotting process is carried out in the transfer cell. In order to check the completeness of the transfer process, all transferred proteins can be visualized by staining with adsorptive dyes, e.g. Ponceau Red (cf. Figure 7a).

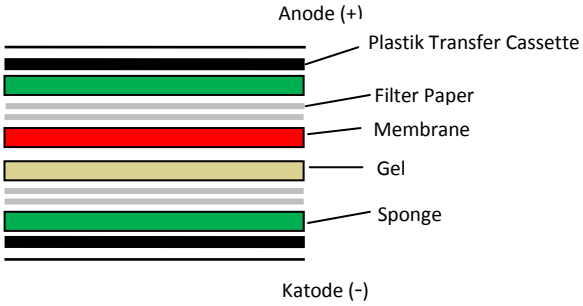


Figure 6. *Scheme of the various layers used during the blotting process in WBAs.*

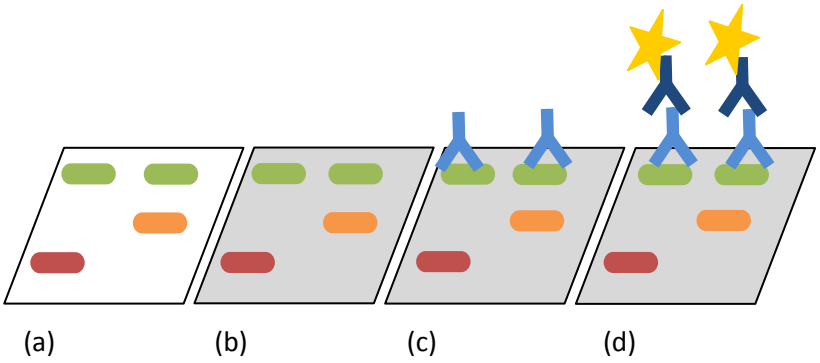
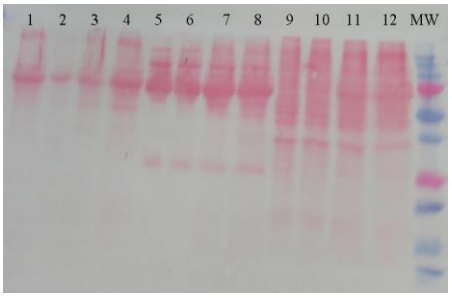
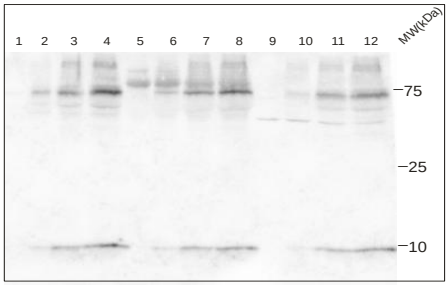


Figure 7. *Consecutive steps in membrane "development": (a) membrane after protein transfer; (b) blocking the membrane on the sites not occupied by transferred proteins; (c) incubation with primary antibodies; (d) incubation with enzyme-linked secondary antibody and the color-reagent (chemiluminescence) solution.*

The development of the membrane is the last step of WBA. The transferred proteins are detected by using specific antibodies. The membrane is electrified during transfer, meaning that on the membrane sites free of transferred proteins any protein can bind unspecifically, including the primary Abs. This would cause false positive spots. To avoid such an error, the protein free places on the membrane have to be blocked by adsorbing a protein which does not interact with the primary antibody, usually powdered milk (step (b) in Fig. 6). Afterward, the membrane is incubated with the primary antibody directed against the targeted protein, and finally, the secondary antibody directed against a constant region in the first antibody is added. As this secondary antibody is coupled with a reporter enzyme the targeted protein is visualized upon adding a chemiluminescence solution and luminescence intensities can be measured (Figure 6 and 7b).



(a)



(b)

Figure 8*. Western blot membranes: (a) after protein transfer and staining all proteins by an adsorptive dye (Ponceau Red); and (b) after development with primary and secondary Abs using a HRP reaction. Visible are bands of proteins recognized by the primary antibody.*

Verification of the identity of a protein is based on the Mw assessed via the migration distance. The quantification is based on the measurement of the optical density of the bands (intensity of reflected light) and its relation to intensity values gained from samples containing spiked a target protein standard in known amounts.

2. Mass Spectrometry based quantification methods

2.1 Shot-gun Proteomics – ways of comprehensive, non-targeted quantitative proteomics with and without labelling

Protein quantification in complex biological sample is not only important for finding differences between physiological states in a biological system. It is also a most challenging task in comprehensive proteomics because of the enormous number of proteins simultaneously under consideration and their extensive dynamic rang in biological samples like serum, plasma or cell culture supernatants.

Mass spectrometry (MS) based methods provide a powerful tool which allows to overcome the problems in terms of sample complexity and extended dynamic range. One approach established for this purpose is named “shot-gun” proteomics and consists of following steps: (i) cell lysis, some kind of pre-separation of intact proteins followed by tryptic cleavage (or cleavage by other specific proteolytic enzymes) of the intact proteins yielding a mixture of peptides; (ii) separation of this most complex mixture of peptides by multi-dimensional separation methods, particularly high-performance liquid chromatography (HPLC) in miniaturized systems; (iii) peptide analysis by multi-stage MS involving various mechanisms of ion fragmentation like collision induced dissociation (CID) and/or electron-transfer-dissociation (ETD). Based on these fragmentation patterns the final computer assisted data analysis assesses partial protein sequences by using informatics tools, and from these partial sequences protein identification can commonly be attained cases when dealing with organisms with completely sequences genome.

There are some particularly critical steps is sample preparation when addressing cell compartment-specific proteomics which needs for instance fractionation in cytoplasm, nucleus, various membranes, etc. When dealing with proteins secreted from cells growing in cell cultures, the situation is much easier as only the supernatant has to be abstracted by centrifugation. The first pre-separation of the intact proteins can be done by 1D-GE which is followed by silver staining, excision of the stained protein bands and tryptic digestion yielding complex mixtures of peptides.

This shot-gun proteomics approach is widely used for protein identification. When dealing with protein quantification the situation is more delicate as the registered signal intensities depend on the ionization yield attained for each peptide in the ion source, and this yield is influenced by the physicochemical properties of peptides. Therefore, for accurate quantification it is required to widely separate the peptides for avoiding ionization suppression effects and it is generally preferable to compare identical individual peptides originating from two different experiments under comparison.

An effective approach for accurately comparing signal intensities of corresponding (i.e., identical) peptides originating from different samples is their derivatization with stable-isotope coded labels. In addition, the mass difference caused by the different label masses might be useful for identification of corresponding peptides. Isotope labels can be introduced into peptides in several ways: chemically, enzymatically or metabolically, and synthetic peptides can be spiked as standards. Recently, different label-free relative quantification strategies have emerged: They are based either on comparing absolute peak intensities of corresponding peptides in different runs (corresponding to different samples), or introduce the concept that the number of peptides that can be determined by MS/MS-based partial sequencing (or the number of acquired MS/MS spectra) reflects to some extent the abundance of the peptide (Cox& Mann, 2011).

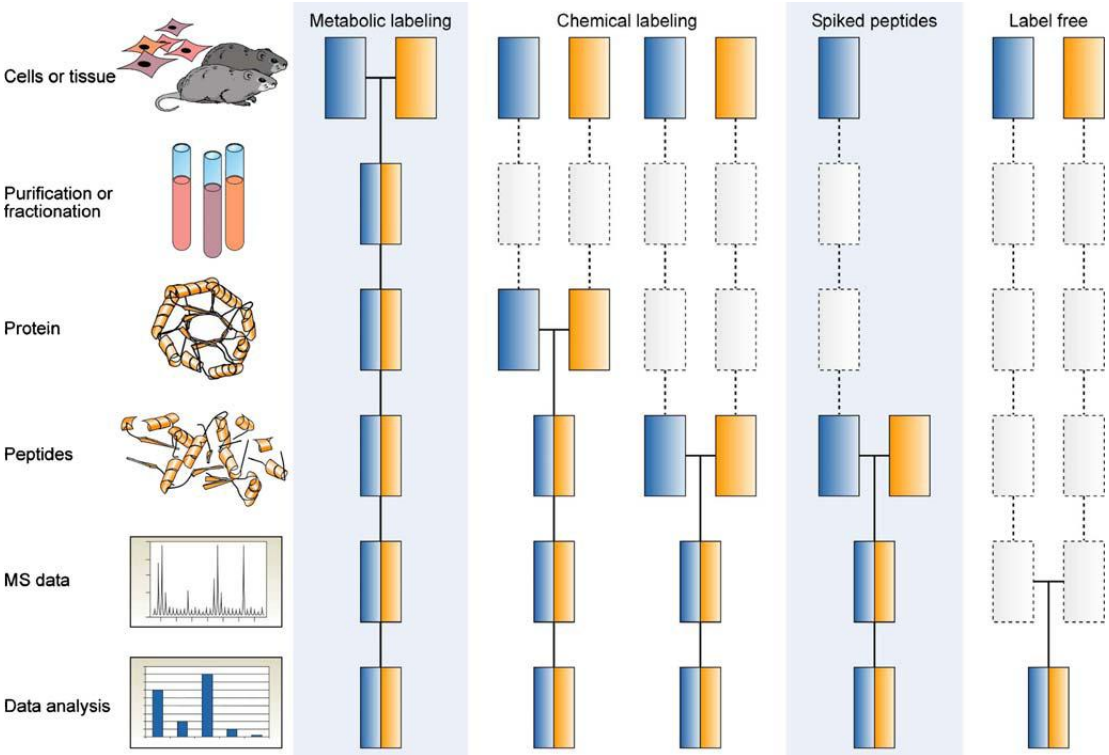


Figure 9. *Quantitative mass spectrometry workflows introducing heavy/light isotope-codes at different stages of the sample pretreatment. Boxes in blue and yellow represent two experimental conditions. Horizontal lines indicate when samples are combined. Dashed lines indicate points at which experimental variation and thus quantification errors can occur, (taken from (Bantscheff et al., 2007)) .*

2.1.1 Quantification with stable-isotope coded labeling

This concept is based on derivatizing peptides by heavy/light-isotope labeling that leads to mass differences between these differently labeled forms of corresponding peptides. The

relative abundance of these corresponding peptides is determined according to their peak intensities. This labeling can be performed at intact protein level in a metabolic (SILICA) or chemical (particularly *via* the reactive amino acids Cys and Lys) way; or on peptide level in an chemical or enzymatic (introducing ^{18}O by hydrolysis in $H_2{}^{18}O$) way (Bantscheff& Schirle& Sweetman& Rick& Kuster, 2007).

Metabolic labeling:

With this technique protein labeling occurs during cell growth when isotope-labeled amino acids are incorporated into the cell from the cell culture medium. Whereas a ^{15}N- enriched cell culture medium was initially used for total labeling, nowadays the most popular form of metabolic labeling is the addition of $^{13}C_6$ –Arg and $^{13}C_6$ –Lys or deuterium coded amino acids. The method is known under the name Stable Isotope Labeling by Amino acid in Cell culture (SILAC). When using ^{13}C –coded Arg and Lys, after tryptic digestion every peptide should contain at least one labeled amino acid. The protein concentration ratio is calculated by comparing the peak intensities of corresponding unlabeled (light) and labeled (heavy) peptides.
With metabolic labeling the two samples that should be compared (light and heavy labeled, respectively) are mixed together right at the beginning of the sample preparation procedure minimizing error rates introduced during sample preparation steps. This fact is a main advantage of the SILAC method in comparison to the other methods with which the mixing of samples occurs later during the sample preparation work flow (cf. the scheme in Figure 8). The impossibility to compare more than three distinguishable conditions in a sample (i.e., no heavy isotopes, $^{13}C_6$, $^{13}C_6{}^{15}N$), as well as the time required for protein labeling over 6 to 8 passages are main disadvantages of SILICA.

Chemical labeling:

There are several approaches for chemical labeling of proteins and peptides consisting on the incorporation of an isotope-coded tag (label) on specific, reactive amino acid side chains. Again, the relative quantification is based on comparing peak intensities of corresponding peptides labeled with a light and heavy tag, respectively. In principle, every reactive amino acid side chain could be used for tag incorporation; however, the most used sites of labeling are the ε-amino group in Lys and the sulfhydryl group in Cys.
With the Isotope-Coded Affinity Tag (ICAT) method (Gygi et al., 1999), for instance, cysteine residues are derivatized by a reagent containing iodoacetamide as reactive group (interacting with the Cys), a linker chain carrying the isotope coding, and finally a biotin moiety for affinity purification (using streptavidin sorbents) (Figure 9). The light variant of the linker chain contains only hydrogen atoms, whereas in the heavy variant 8 hydrogen atoms are substituted by deuterium atoms giving a mass difference of 8.04928 Da between the light and heavy variants. The proteins in the two samples to be compared (e.g. control vs. stressed; or two different cell states) are separately labeled with light or heavy reagent variants. Afterward, the two samples are combined and digested. Isolation of the labeled Cys-containing peptides can be done by affinity separation using the biotin-avidin/streptavidin system. As only Cys-containing peptides are labeled by this method the

entire peptide mixture is significantly simplified which facilitates the subsequent relative quantification via HPLC/MS (Figure 10) However, cystein is a rather rare amino acid, and proteins in which cysteins are under-represented are on risk not to be detected. Further, the chance of finding posttranslational modifications in proteins is restricted to those present in peptides containing cysteins.

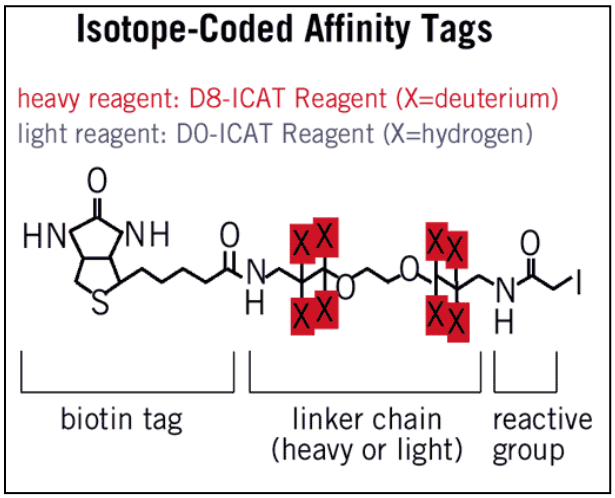


Figure 10. *Scheme of the ICAT reagent,(taken from(Gygi& Rist& Gerber& Turecek& Gelb& Aebersold, 1999)).*

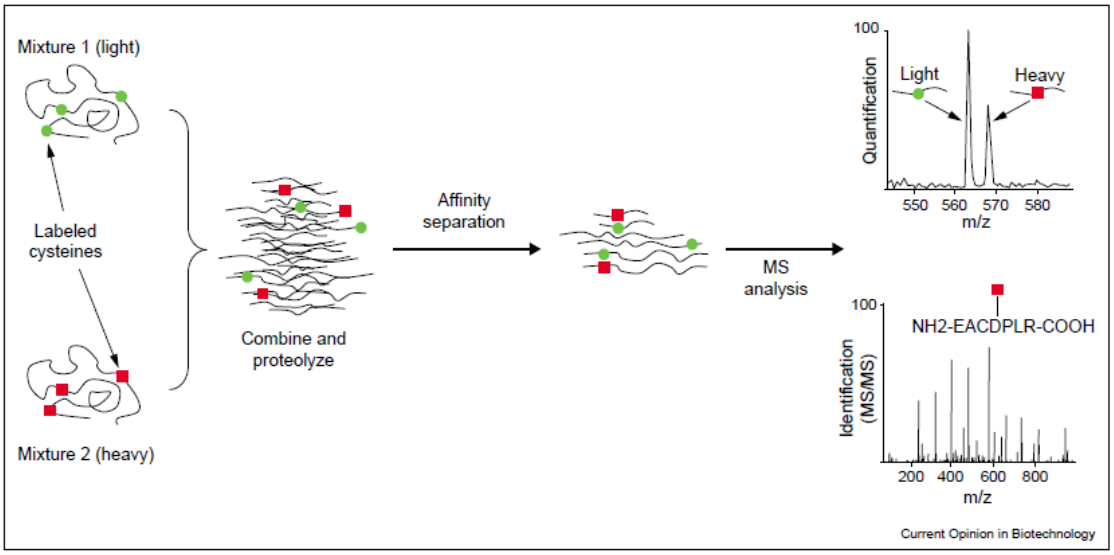
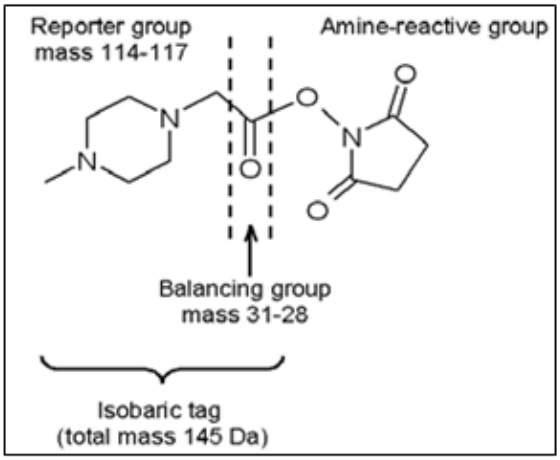


Figure 11. Scheme exemplifying the ICAT strategy for differential quantifying proteins (taken from(2003)

Other labeling reagents use *N*-hydroxysuccinimide (NHS) for coupling with amino groups, e.g. the ε-amino group in Lys or the *N*-terminus of the peptides. A major advantage of this labeling is that all tryptic peptides can be covered. An example is the Isotope Coded Protein Label (ICPL) which exists of *N*-nicotinolyloxy-succinimide in two variants, as light (d0) and

heavy (d4) reagent. Sample preparation steps and MS analysis are the same as for the ICAT method, except that the affinity isolation is not included.

A further of chemical labeling method for protein quantification, called Isotope Tag for Relative and Absolute Quantification (iTRAQ), uses isobaric tags that can be distinguished by the masses of the fragment ions resulting from of the tags after CID in the MS/MS mode. Again amino groups are labeled by means of an *N*-hydroxy succinimide moiety in the reagent. The main part of the isobaric tags consists of a reporter group (with molecular masses of 114, 115, 116 and 117, respectively) and a balance group (with molecular masses of 31, 30, 29 and 28, respectively) according to the number of heavy isotopes of C, N, O (cf Figure 11). The quantification is based on comparison of peak intensities of the reporter groups, not of the peptides. The low masses of the reporter groups and the availability of four variants of the label allow multiplexing of the analyses with 4 samples in one run. Since recently, an advanced iTRAQ® Reagent – 8plex is commercially available by using a modified balance group). With iTRAQ the labeling commonly occurs at peptide level. Whereas the other methods mentioned are based on HPLC-MS quantification, where the chromatographic retention time is correlated with MS spectra, the iTRAQ quantification is based on MS/MS spectra, where the reporter and balance group are separated.



Mass	Reporter	Balance	Mass
114	1 x ^{13}C	1 x ^{13}C, 1x ^{18}O	31
115	2 x ^{13}C	1x ^{18}O	30
116	2 x ^{13}C, 1x ^{15}N	1 x ^{13}C	29
117	3 x ^{13}C, 1x ^{15}N		28

MReporter +MBalance = Constant

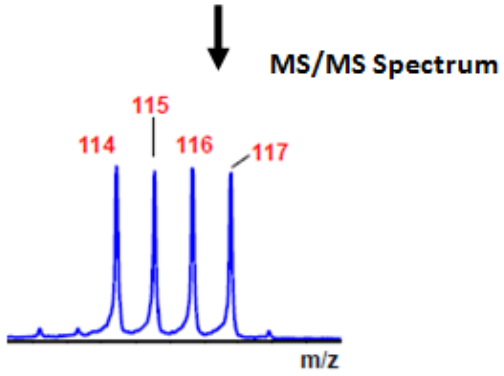


Figure 12. Left side: i*TRAQ label consisting of isobaric tag and reactive group; right side: Table giving the isotope composition of four isobaric tags; and typical MS/MS spectrum showing the reporter-group section, (taken from(Ross et al., 2004)).*

Enzymatic labeling:

This method is based on introducing an ^{18}O atom on the *C*-terminus of peptides during trypsin or Glu-C digestion in $H_2{}^{18}O$. The labeling occurs at peptide level. Both enzymes

catalyze the incorporation of two ^{18}O atoms, leading to a mass shift of 4 Da when compared to unlabeled peptides. The main practical problem of this method that complicates data analysis is the fact that full labeling is rarely achieved.

Spiking with isotope-labeled synthetic peptides:

Spiking with isotope-labeled synthetic peptides is a method known as Absolute Quantification of Proteins (AQUA). The absolute quantification can be performed by adding of known quantity of synthetic peptide in the sample, before (to be sure that sample and standard are treated equally) or after digestion and after HPLC/MS analysis and comparing peak intensities between sample and standard. With the method of synthetic peptides we can investigate just one peptide/protein, but when using gene technology (synthetic genes) the producing of a peptide library including several standards is easier and enable quantification of many peptides in the same sample.

2.1.2 Label-free quantification:

Most labeling methods suffer from certain disadvantages including time requirements, additional sample preparation steps which might influence quantification accuracy and precision, incomplete and non-uniform labeling, additional effort to get rid of the reagent components, and high costs of the reagents. Most labeling methods do not allow comparing more than two samples in one run, except iTRAQ allowing a comparison of up to 8 samples. The complexity of biological samples and particularly the need for high-throughput procedures require faster and simpler methods for protein quantification. Therefore, the development of label-free quantification methods came again in the focus of scientific research.

The main steps in label-free quantification are: (i) sample preparation, protein fractionation/ separation, and digestion to peptides; (ii) peptide separation by one- or two-dimensional HPLC hyphenated to multi-stage MS; and finally (iii) data analysis. When working with label-free methods, the samples to be compared are not mixed together but are treated separately.

For relative quantification two types of data can be used as basis: first, the peak intensity data for selected peptides or, alternately, the number of peptides detected per protein (number of assigned MS/MS spectra: “spectral counts”). Additionally, absolute quantification methods are also available (Zhu et al., 2010).

Quantification by peak intensity:

Protein quantification is based on the peak intensity of LC-MS spectra of precursor peptides. Peak intensities of peptides are in linear correlation with the protein concentration. For data analyses the following algorithms are required for (-) distinguishing peptides peak from noise and neighboring peaks; (-) deconvolution of charge state distribution; (-) adjusting the LC-MS retention time values for correct peak matching between multiple LC-MS runs (-) and statistical analysis.

Quantification by spectral count:

This method is based on counting the number of MS/MS spectra leading to identified peptides (spectral count). Studies have shown a good linear correlation of protein abundance in the samples and the number of MS/MS spectra leading to peptide identification (Liu et al., 2004). The larger a protein is the more spectral counts will be obtained. For avoid this discrepancy, a normalization factor was introduced in the statistical analysis, called normalized spectral abundance factor (NSAF). NSAF is calculated as the number of spectral counts (SpC) identifying a protein, divided by the length of the protein (L), divided by the sum of SpC/L for all proteins in the experiment (Chen& Yates, 2007,Florens et al., 2006). In comparison with the quantification based on peak intensities, the spectral count method is much easier and - except of the calculation of the normalization factor - do not require any specific algorithms.

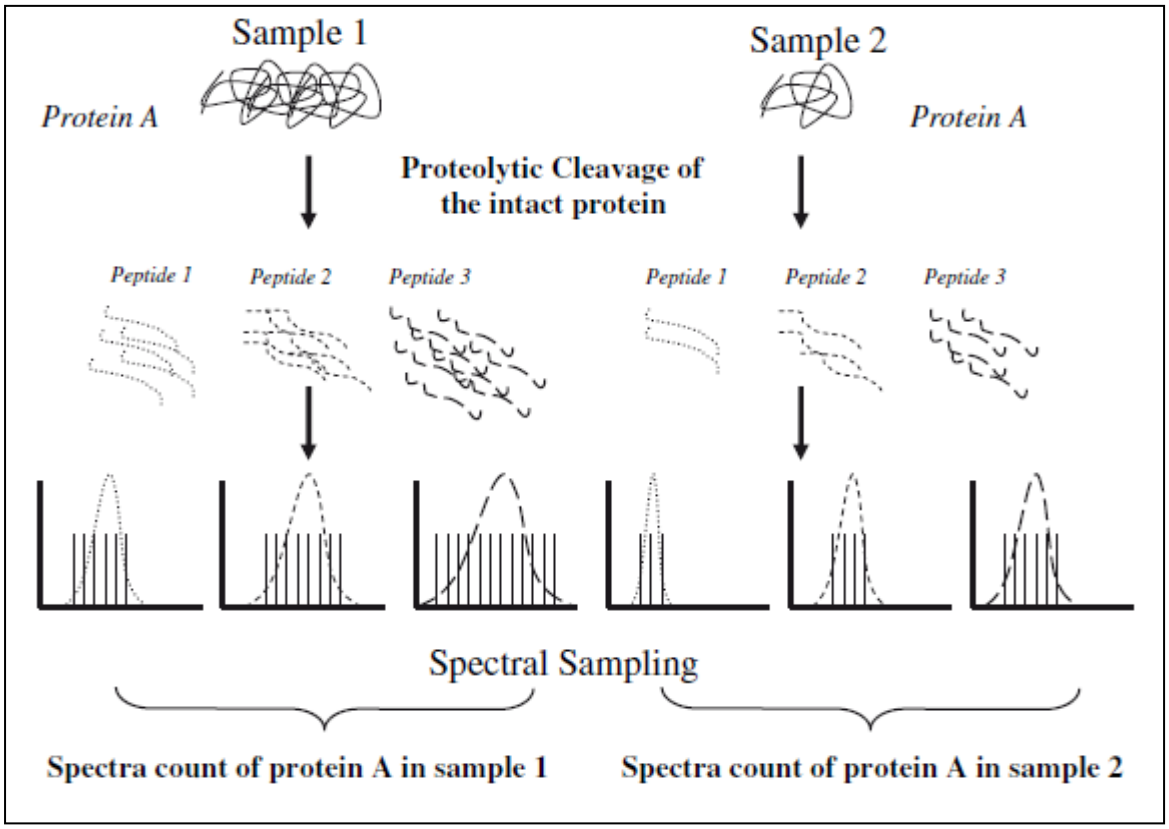


***Figure 13**. Schematic representation of the concept of protein quantification by the method of "spectral counts", (taken from (Chen& Yates, 2007)).*

Absolute quantification:

There exist additional methods for absolute protein quantification based on a label-free approach. The assessment of the Protein Abundance Index (PAI) is one of those. The PAI is defined as the number of identified peptides divided by the number of theoretically observable tryptic peptides for each protein. It was found that the exponential form of the PAI, i.e., the emPAI which equals to 10^{PAI}-1, is linearly correlated with the protein concentration on the sample.

$$\textbf{Protein content}\ (mol\ \%) = \frac{\textbf{emPAI}}{\Sigma\ \textbf{(emPAI)}}\ \textbf{x 100}\ ;$$

$$\textbf{Protein content}\ (weight\ \textbf{\%}) = \frac{\textbf{emPAI x Mr}}{\Sigma\ \textbf{(emPAI x Mr)}}\ \textbf{x 100;}$$

where Mr is the molecular mass of the protein; and Σ (emPAI x Mr) is the sum of emPAI values multiplied by the mass of the proteins for all identified proteins.

The emPAI method turned out as being useful in comprehensive approaches for the determination of protein concentrations, as for instance in shot-gun proteomics. However, the reliability of the method may be compromised by sample complexity, by the predominance of highly abundant proteins which can influence peptide identification, and by saturation effects. Such problems can be minimized by a pre-separation of proteins e.g. by 1D electrophoresis which is commonly applied within a shot-gun proteomics approach. Using MS instruments with short MS/MS cycle times might improve the linearity between emPAI values and protein concentration. Besides absolute quantification, also relative quantification is possible by comparing the emPAI values between two samples (Ishihama et al., 2005).

Absolute Protein EXpression (APEX) is the name of another method for absolute protein quantification per cell. The method is based on the proportionality between protein abundance and the number of peptides originating from this protein that can be observed in a experiment. The APEX method introduces an correction factor that make the fraction of expected number of peptides proportional to the fraction of observed number of peptides (Lu et al., 2007) .

2.2 Quantitative targeted proteomics

Unlike the non-targeted proteomics, which do not require information about the proteins to be quantified, targeted proteomics needs such information for selection of peptides specific for the targeted protein. There are two approaches widely used in this field: Multiple-Reaction-Monitoring (MRM) and Selected-Ion-Monitoring (SIM). The needed information for appropriate MRM can be received from MS/MS spectra, for SIM by simple MS spectra.

2.2.1 Multiple-Reaction-Monitoring (MRM):

Multiple-Reaction-Monitoring (MRM) is a tandem mass spectrometric method for rapid, sensitive and selective quantification of proteins which has been widely used in recent years. The method comprises as a first step the isolation of a specific peptide of the targeted protein (precursor peptide ion) from all other peptides in the ion beam, followed by a fragmentation step (e.g.by CID) and the analyzing of the fragment ions. Usually, a relative quantification is performed by comparing the peaks intensities of different samples, or an absolute quantification by spiking with synthetic labelled peptides as calibration standards (Kuzyk et al., 2009).

When fragmenting the precursor peptide ion several ions are created and each of them represents a “transition” or a “reaction”. Single Reaction Monitoring (SRM) would mean

quantification by analyzing only one of these transitions. For attaining better accuracy and reliability the quantification is usually based on analysing several transitions for a given precursor peptide ion, e.g. three. In these instances, the name Multiple Reaction Monitoring (MRM) is chosen.

MRM experiments are usually performed on triple-quadrupole instruments. In the first quadrupole (Q1) the precursor peptide ion is isolated from the ion beam, in the second quadrupole (q2) the isolated peptide is fragmented, and the third quadrupole (Q3) is used with a limited number of open (narrow) m/z windows for selecting the fragment ions with chosen m/z values and for measuring the ion counts of these fragments (peaks intensities) (Figure 1). The peptides to be selected for the quantification of the "parent"-protein, have to be "proteotypic" peptides (PTP). This means that such a peptide has to have a unique amino acid sequence allowing to identify the associated protein. Secondly, the chosen PTP should be easily ionisable and thus easily detectable by MS (Huttenhain et al., 2009). MRM is a highly sensitive and selective method. The high selectivity is achieved by the narrow mass filtering in the quadrupoles Q1 and Q3. This filtering leads to a significant reduction of the chemical noise, which, together with the non-scanning mode of operation in the two quadrupols Q1 and Q3, results in high sensitivity and a low limit of detection.

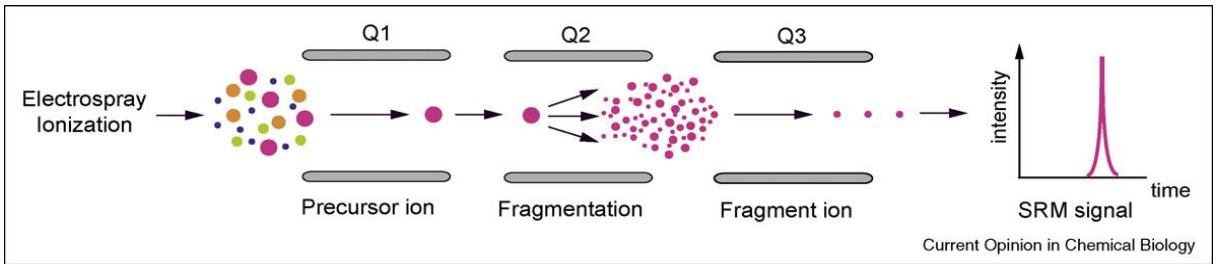


Figure 14. *Schematic illustration of the principle of Selected Reaction Monitoring (SRM): Q1- precursor ion isolation; Q2- precursor ion fragmentation; Q3- mass analysis and quantification of a specific precursor ion fragment (transition), (taken from (Huttenhain& Malmstrom& Picotti& Aebersold, 2009)).*

For establishing an appropriate MRM assay the following steps should be considered: (-) selection of candidate PTPs: These PTPs can be found by using MS spectra of shotgun-type experiments or by using computational tools to predict the most likely MS observable peptides; (-) determination of the best transition for a given PTP and of optimal instrument parameters. These parameters are usually determined by performing MS/MS analysis with the selected PTP.

2.2.2 Selected-Ion-Monitoring (SIM):

Selected-Ion-Monitoring (SIM) is a further approach used in targeted proteomics. Unlike to MRM, SIM is not based on tandem mass spectrometry by quantifying selected fragment ions but is based on single-stage MS quantifying selected peptide ions, which in case of MRM would be the precursor ions.

SIM comprises the isolation of selected peptide ions from the ion beam followed by the analysis of these isolated peptide-ions. Unlike MRM, SIM requires only the information on peptide-ions to be expected from a targeted protein and which will be quantified from the full MS spectrum. For attaining high sensitivity, SIM is best done with a continuous ion-beam from which the selected peptide-ions can be separated ´in space´. Such a procedure is done when using a quadrupole filter/analyzer for ion separation. For attaining high resolution and high accuracy in mass determination, the use of an Orbitrap analyzer allows to reach resolution values above 60.000 and mass accuracy values below 3 ppm. Combining a quadrupol filter for ion separation with multiplexed selected ion monitoring in a way in which the selected ions of several SIMs were stored (in the C-trap) and simultaneously injected into an Orbitrap analyzer, as it is done with the “Q Exactive” mass spectrometer, makes this type of instrument particularly well suited for the SIM approach (Michalski et al., 2011).

When monitoring MRMs and SIMs of different peptides in a so-called “scheduled” approach the different peptides are measured only across a specified time window of their expected retention time during the LC gradient. This procedure reduces the number of spectra (peptides) to be measured and allows maintaining dwell times and cycle times at optimal values for accurate and sensitive quantification (Lange et al., 2008).

Part II: The Investigation Program:

1. Introduction:

Cytokines are small proteins secreted by the cells of immune system as response to diverse cellular stresses. They are produced by phagocytes, macrophages, T lymphocytes, bone marrow stromal cells etc. They regulate the growth, differentiation and activation of immune cells, including stimulation the growth and differentiation of lymphocytes, activation and proliferation of effector cells of immune system and also stimulate the development of hematopoietic cells. Additionally, the induction of synthesis of other cytokines is also known. They perform their function at the produced site and in the distance by enter the circulation.

Being secreted by the cells of immune system cytokines participate on this system by stimulating of inflammatory reaction (e.g. acting on endothelial cells), recruiting, activation, and regulation of effectors cells (e.g. phagocytes, neutrophils etc.).

Hence, participation of cytokines on a complex network of immune system and inducing the immune reactions make their role on whole functions of an organism very important. Research revealed the association of cytokines at pathogenesis of many inflammatory diseases. Further, the released cytokines in response to inflammation or infection can inhibit tumor development and progressing. On the other hand host cytokines can also promote cancer progression by inducing the growth of cancer cells. Pro-inflammatory cytokines are important player on communication between the immune system and the central nervous system, indicating the key role of cytokines on diseases correlated with nervous system (Dranoff, 2004,Skaper et al., 2012).

Interleukin 6 (IL-6) is known as multifunctional cytokine and belongs to IL-6 superfamily and to pro–inflammatory cytokines. It is involved in regulation of immune response, hematopoiesis, inflammatory and pro-inflammatory effects with main function as antibody inducer (early identified as B cells differentiation factor). Additionally, IL-6 acts on T cells, hepatocytes, hematopoetic progenitor cells etc., induces nerve cell differentiation and myeloma and plasmacytoma growth. IL-6 maybe produced by B cells, macrophages, dendritic cells, fibroblasts, epithelial cells etc. (Maccio& Madeddu, 2012).

Higher levels of IL-6 are obtained at numerous infectious, inflammatory and cancer diseases. Recent studies show implication of IL-6 on development and progression of epithelial ovarian cancer and higher serum IL-6 levels are obtained at patients with Schizophrenia (Pedrini et al., 2012).

Interleukin-8 (IL-8) belongs to a chemokine family of cytokines and is also known as C-X-C chemokine 8. It is known for their chemotactic properties on leukocytes, which caused the chemokine name. Various cells produce IL-8 as response to growth factors, inflammatory

cytokines and pathophysiologic conditions, including endothelial cells, epithelial cells, macrophages, monocytes, fibroblasts etc.

It is involved on requirements of neutrophils, basophils and T lymphocytes on site of injury and inflammation. Further, IL-8 is involved on neutrophil activation, possess tumorigenic and proangiogenic properties and generally is implicated in autoimmune, inflammatory and infectious diseases (Xing et al., 2012,Brat et al., 2005).

Involving of IL-8 in many processes on organisms and spatially on different diseases have led to correlation of changes on IL-8 levels with several diseased states, making IL-8 useful as biomarker and potentially interesting as target or effector for new therapies. Promising examples of using IL-8 as a biomarker are: urinary bladder cancer, prostatitis, acute pyelonephritis, chorioamnionitis etc. (Shahzad et al., 2010).

C-X-C motif ligand 3 (CXCL3) known also as growth regulated oncogene (GRO3) or GRO protein gamma (GROg) belong to the same family of cytokines as IL-8 , namely those containing the CXC sequence (the first two cysteine residue are separated by an amino acid (X))and ELR motif. In contrast to IL-8, CXCL3 has lover binding affinity to their receptor (CXCR2). CXCL3 can be produced by leukocytes, endothelial cells, fibroblasts and epithelial cells. Beside its important role within the immune system, CXCL3 is involved also in tumorigenesis and metastasis (Hannelien et al., 2012).

Cytokines show to be important players involved in development, progression and inhibition of many diseases. Further, being part of a complex protein interaction network that leads to a diseased state, different therapeutic approaches include targeting of cytokines. Additionally, several diseases are direct correlated with changes on cytokines levels in comparison to healthy state. In these entire situations the accurate and sensitive determination of cytokines in clinical samples is crucial for investigation of their implication.

In plasma, the top 10 abundant proteins constitute approximately 90% of the total protein content and the following 10 abundant proteins constitute another 9% of the total protein weight. The remaining proteins, including cytokines, cover only 1% of the total protein weight. These leads to a wide dynamic range, covering more than 10 orders of magnitude in terms of concentration, additionally the high variety of distinct proteins increase enormously the sample complexity. By dealing with biological samples, quantification of small changes on proteins amount (concentration range under 10ng/ml), in a such complex environment is very challenging. Systematic comparison of most used antibody and mass spectrometric based methods is performed in order assess their performances in respect to sample complexity.

2. Materials, Instrumentation and Methods

2.1 Samples, antibodies and recombinant proteins

Samples:

- Cultured human umbilical vein endothelial cells (HUVEC) grown in endothelial basal medium (EBM-2) supplemented (or not supplemented) with 10% fetal calf serum (FCS) (CC-3156; Lonza, Basel, Switzerland), were treated with IL-1β for 48h. After 24h the medium was changed and then cells are grown without IL-1 β for the residual 24h. Controls were cultured in the same way without adding IL-1β.

- Skin fibroblasts grown in fibroblasts basal medium (FBM) supplemented with 10% FCS (CC3131, provided by Lonza)., untreated and treated with IL-1b (medium changed after 24h and cells are grown without IL-1b).

  For preparation of the supernatant (SN), the cells' supernatant was transferred in a 15ml falcon tube and centrifuged for 5 min at 1200 rpm at RT. The supernatant was then transferred in a new falcon tube and stored in aliquots at -20°C.

- Human serum used for studying of matrix effects was pooled from blood taken from 8 healthy donors. For the preparation of serum, blood was kept for 1h at RT after sampling and centrifuged in a Vacuette serum tube at 1200g for 20 min at RT. The serum collected from the upper part of the tube was stored in 150 µl aliquots at -80°C.

Antibodies:

- *Anti-Interleukin 8 (CXCL8/IL-8)* –A monoclonal mouse antibody (IgG_1) directed against *E. Coli*-derived recombinant human CXCL8/ IL-8 as immunogen, Protein A or G purified from hybridoma culture supernatant and supplied in lyophilized state was purchased from R&D Systems (MAB208). It was reconstituted with sterile PBS to a concentration of 500 µg/ml and stored at -20°C in aliquots of 20µl.

- *Anti-Interleukin 6 (IL-6)* - A monoclonal mouse antibody (IgG_1), directed against *E. Coli*-derived recombinant human IL-6 as immunogen, Protein A or G purified from hybridoma culture supernatant, supplied in lyophilized state was purchased from R&D Systems (MAB206). It was reconstituted with sterile PBS to a concentration of 500 µg/ml and stored at -20°C in aliquots of 20µl.

- *Anti- GRO gamma (C-X-C motif chemokine 3; CXCL3)* - A polyclonal rabbit antibody (IgG) directed against human CXCL3 purified by affinity chromatography, supplied as a liquid in sterile PBS (1mg/ml) was purchased from USBio (G8975-26). It was stored at -20°C in aliquots of 10µl.

- *Anti-Lumican* - rabbit polyclonal antibody (IgG) directed against a synthetic peptide corresponding to the amino acid sequence 205-235 from the center region of human LUM (KLH) as immunogen, purified by ammonium sulphate precipitation, supplied as a liquid in sterile PBS (1mg/ml) was purchased from USBio (L6025). It was stored at 4°C in aliquots of 10µl.

- *Anti-Tumor necrosis factor-inducible gene 6 protein (TSG6, TNFAIP6)* – A polyclonal rabbit antibody directed against a synthetic peptide corresponding to the amino acid sequence surrounding Valine 37 of human TSG6 as immunogen, affinity purified and supplied as a liquid in sterile PBS (1mg/ml) was purchased from Novus (NBP1-01046). It was stored at -20°C in aliquots of 10µl.

- The enzyme conjugated secondary antibodies
  Anti mouse IgG (H+L)-HRP conjugated were obtained from Bio-Rad (1706516) and *Anti rabbit IgG H&L Chain specific (Goat) peroxidase conjugated* from Calbiochem (401353), respectively.

Recombinant proteins:

- *Recombinant human Interleukin 8 (1-77) (CXCL8/IL-8)* - produced in *E. Coli* was purchased from PROSPEC (#CHM-327). This interleukin consists of a single, non-glycosylated polypeptide chain of 77 amino acids with a molecular mass of 8904 Dalton. It was obtained as sterile filtered, white, lyophilized (freeze-dried) powder and was reconstituted over night at 4°C in sterile water (18MΩ-cm) to yield a concentration of 100µg/ml. It was stored at -20°C.

- *Reconstitution of serial dilutions of recombinant IL*-8:
  Recombinant human interleukin 8 spiked in three matrices, i.e. PBS; PBS supplemented with 10% human serum; and supernatant of untreated HUVECs supplemented with 10% FCS. The rIL-8 stock solution in water (100µg/ml) was diluted 1:10 (v/v) with water (giving a concentration of 10µg/ml) and, beginning from this solution, a serial dilution was performed in each matrix (PBS; PBS/10% human serum; untreated HUVEC SN /10% FCS). The final solutions had concentrations of: 1µg/ml; 100ng/ml; 10ng/ml, 1ng/ml, 100pg/ml, 10pg/ml, 3pg/ml and 1pg/ml.
  Upon reconstitution the samples were aliquoted corresponding to each method and stored at -20°C. In order to avoid sample degradation and to hold all preparation steps equal each aliquots were used just one time. Before starting with an experiment the samples were incubated at 37°C for 10 min and centrifuged at 13,000 rpm for 5min.

2.2. Immuno-Precipitation (IP)

Chemicals

The immunoprecipitation Kit - Dynabeads® Protein G was purchased from Invitrogen (100.07D) . This kit contained:

- Dynabeads Protein G (100.04D),
- Binding & Washing Buffer,
- Washing Buffer (citrate-phosphate buffer, pH 5.0),
- Elution Buffer (0,1 M citrate, pH 2-3).

The following buffer solutions were self-prepared

- PBS buffers supplemented with 0,01%; 0,05%; 0,1% Tween-20, resp., were used as Binding & Washing Buffer;
- 0,1M Glycine-HCl Buffer, pH 2.7, was used as Elution Buffer.

Other solutions:

- Bovine serum albumin (BSA) (obtained from Ruth, Karlsruhe, Germany) 2% (w/v) in water;
- Tween-20 (Bio-Rad, Ref.Nr. 170-6531) 10% (w/w) in water,
- Triton X-100 (Sigma –Aldrich; 9002-93-1) 10% (w/w) in water;
- NaCl 5M, and NaOH 5M in water
- 5x SDS Loading Buffer (5x SDS-LB)

Instrumentation

- Rotator (horizontal rotation)
- Magnets for 0,5 ml and 1,5 ml tubes
- 0,5 ml and 1,5 ml Eppendorf tubes
- A set of pipettes (0,5-10; 2-20; 10-100; 20-200; 100-1000µl) (Socorex, Lausanne, Switzerland)

Immuno-Precipitation procedure

Our work with Immunoprecipitation was started using the protocol from Invitrogen (provided with kit) which was improved later on step by step to account for the specific requirements of the proteins targeted and the antibodies used. All these steps are described in the `Results and Discussion´ section. Here, the basic protocol is described which provided the starting point.

The IP-protocol is divided in three parts consisting of (i) antibody binding (beads coating), (ii) the immunoprecipitation process of the antigen and (iii) the elution of the target protein. All these steps are performed at room temperature (RT).

Antibody binding (beads coating): Before use, Dynabeads were rotated for 5 min., 100 µl of this homogeneous bead solution were then transferred to a tube (500 µl Eppendorf tube). The supernatant was removed after placing the tube on the magnet. To the prepared beads 2 µg (4 µl) antibody diluted in 200 µl Binding &Washing Buffer was added and incubated for 30 min at RT under rotation. After antibody binding, the tube was placed on the magnet again and the clear supernatant solution is transferred to a clean tube (for investigating the completeness of Ab-immobilization). Supernatant after coating is denominated here a **SN1**.

Immunoprecipitation of antigen: The coated beads were re-suspended in 200 µl Binding & Washing Buffer by pipetting, and 5µl of this suspension were transferred to a new tube for immunoprecipitation of the targeted protein. (The remaining coated beads (195 µl), after removing the supernatant, were again re-suspended in PBS/0,05% Tween-20 and stored at 4°C and can be used later.) To the mentioned 5 µl of coated beads suspension 400 µl of sample solution were added and incubated for 30 min at RT under rotation. Afterwards the beads were separated by the magnet and the supernatant was transferred to a clean tube (for investigating the completeness of IP). This supernatant after IP is denominated here a **SN2**.
The beads-antigen complex was washed three times with 200 µl Washing Buffer, in between the tube was placed on the magnet and the supernatant was removed. At the end the beads-antigen complex was re-suspended by pipetting in 100 µl Washing Buffer and transferred to a new tube. A new tube was chosen to avoid any co-elution of proteins bound to the tube wall.

Elution: After removing the supernatant (Washing Buffer), 20 µl Elution Buffer were added to beads-antigen complex and incubated for 10 min at RT under rotation to dissociate the complex. Thereafter the beads were placed on the magnet and the supernatant was transferred to a clean tube. This solution is denominated here as eluate **(EL)**.

For a re-use of the beads, the pellet after transfer of the supernatant (elution) was neutralized with 100 µl phosphate buffer pH 8 under 10 min of rotating and re-suspended in PBS/0,05% Tween-20 and stored at 4°C.

Control of IP efficiency by WB analysis: All IP experiments were controlled by Western blot analysis. Just before loading, 20 µl of the acidic eluate were neutralized with 1 µl 5M NaOH and mixed with 5 µl 5xSDS-LB (see “Solutions and buffers composition”). For the 1-DE, a mixture of 20 µl Elution Buffer, 1 µl 5 M NaOH and 5 µl 5xSDS-PP was loaded onto the gel between two samples. When investigating SN1 and SN2, 40 µl of SN1 and SN2 were mixed with 5 µl water and 5 µl 5xSDS-LB shortly before loading.

2.3 Luminex™ Assay

Chemicals

- xMAP®Reagents – MicroPlexTM Microspheres from Luminex; Product Number L100-C136-01
- Anti-Interleukin 8(CXCL8/IL-8) - monoclonal mouse antibody; the identical antibody as for IP was used
- Biotinylated anti-human CXCL8/IL-8 antibody from R&D Systems; (Minneapolis, USA, ref. Nr. BAF208)
- Streptavidin-R-phycoerythrin (PE) from Fluka/Sigma (Schnelldorf, Germany; ref. Nr. 42250)
- Recombinant human CXCL8/IL-8, used for the standard calibration curve, from R&D Systems; 208-IL
- Sulfo-NHS (*N*-hydroxysulfosuccinimide)(50mg/ml), from Pierce , (**France)**; Ref.Nr. 24510
- EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimid hydrochloride)(50mg/ml) from Pierce 77149 22980
- Samples: standard calibration solutions using recombinant IL-8 spiked into three matrix solutions: (i) HUVEC SN /10% FCS treated with IL-1ß; (ii) HUVEC SN /10% FCS untreated (negative control); (iii) PBS/10% human serum
- Activation Buffer (see "Solutions and buffers composition")
- Coupling Buffer (see "Solutions and buffers composition")
- Luminex Buffer (see "Solutions and buffers composition")
- Aqua bidest.
- Isotonic saline solution (0.9 % NaCl)
- Sterile PBS (see "Solutions and buffers composition")

Instrumentation

- Luminex100 Integrated System™ (Luminex corporation, Austin, USA)
- Filterplate – Multi-Screen®HTS – 1.2µm Hydrophilic Low Protein Binding Durapore®Membrane
- Coulter Counter Z2 (Beckman coulter INC, USA)
- Labinco L46 vortexer (Labinco, Breda, Netherlands)
- Ultra-sonication –water bath
- Stuart rotator SB3
- Eppendorf LowBind Tube 1.5ml
- Multi channel pipette
- Vacuum Manifold
- Shaker-BioDancer (New Braunswick Scientific, Enfield, USA)
- A set of pipettes (Socorex)
- Paper towels, Parafilm

Luminex™ Assay

The bead stock "solution" with a concentration of 1,25x10^{7}beads/ml was vortexed for 30 sec followed by sonication for further 30sec to prevent bead aggregation. Afterward, 200 µl of this suspension was transferred to a LowBind tube (used to avoid beads attaching on the tube wall.)

Beads activation: Beads have to be activated before coating with antibody. For this purpose, beads in the LoBind tube were pelleted by centrifugation over 2 min at 800 rpm, then the SN was removed and beads were washed two times with 100 µl bidestilled water (with 30 sec vortexing, 30 sec sonication and 2 min centrifugation by 800 rpm in between). Washed beads were resuspended in 80 µl activation buffer (by 30 sec vortexing and sonication), and 10 µl of Sulfo-NHS (50 mg/ml) and 10 µl EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimid hydrochloride; 50 mg/ml) were added with gently vortexing in between. The beads were protected from light by using an aluminum foil and incubated for 20 min at RT under 30 rpm rotation. After removing the SN-solution, the activated beads were resuspended in 200 µl Coupling Buffer by vortexing over 30 sec and sonication and were pelleted by centrifugation with 800 rpm over 2 min. The resuspension in Coupling Buffer was repeated leading to a second resuspension in 100 µl Coupling Buffer. After this step the beads are ready for coating.

Coating with antibody: To the suspension of activated beads (in 100 µl Coupling Buffer) 100 µg IL-8 antibody (i.e., 200 µl antibody solution in PBS) were added and diluted with Coupling Buffer to a final volume of 500 µl. This mixture was gently vortexed for 2h under rotation (16 rpm) and light protection. Afterward, the supernatant (gained by centrifugation at 800 rpm over 2 min) was removed, and the beads pellet was resuspended in 500 µl Luminex Buffer by vortexing and sonication. If coated beads were used at the same day, they were incubated for 30 min at RT under gentle (30rpm) rotation. Beads were washed again two times with 400 µl Luminex Buffer (with vortexing, sonication and removing the supernatant after 2 min of centrifugation at 800 rpm in between) and at the end resuspended in 500 µl Luminex Buffer. Then beads were counted and stored at 2-8°C.

Beads Counting: For Sandwich-Immunoassays, commonly 30 µl of coated beads (containing 1500 beads) were used per well. This corresponds to a solution with a concentration of 5x10^4 beads/ml. To prepare such a concentration, the coated beads have to be counted using a Coulter Counter. For this purpose, 20 µl of the coated beads suspension were diluted in 10 ml of isotonic saline solution. The blank value measured was 64000 Counts (should be below 200,000 counts) and for the beads suspension a concentration of 2,7x10^6 beads/ml was measured. To attain the wanted concentration of 5x10^4 beads/ml a dilution step by a factor of 63 was necessary.

Sandwich Immunoassay: A solution of recombinant IL-8 with a concentration of 50 µg/ml was diluted in two steps to a concentration of 10.000pg/ml and then, a serial dilution always by a factor of 1/3 using Luminex Buffer was undertaken to generate a set of 9 standard solutions for calibration covering a concentration range from 10.000pg/ml to 1,5pg/ml. The Luminex Buffer alone was used as a blank. The coated beads suspension was vortexed, sonicated and 1:63 diluted with Luminex Buffer to yield a solution with $5x10^4$ beads/ml. This solution was rotated until the well plate was ready for pipetting.
The assay was performed in a 96-well filter bottom plate and the wells to be used were pre-wetted with 50 µl sterile PBS for 10-20 min and this volume was then aspirated using a vacuum manifold and finally blotted with the bottom of the plate on paper towels to remove excess fluid. By using a multi-channel Pipette, 30µl standard and samples were added to each well followed by the addition of 30µl beads suspension. All samples and standards are loaded in triplicate. Before incubation over night at 4°C in the dark under shaking, the loaded well plate was vortexed very shortly. After incubation, the fluid was removed from the wells using a vacuum manifold, afterwards the beads were washed three times with 110 µl sterile PBS, each time the fluid was removed using a vacuum manifold and at last the plate was blotted on the paper towels to remove excess fluid. Afterward, 25µl biotinylated antibody (1:100 diluted with Luminex Buffer) was added to each well and the plate was gently vortexed and incubated for 1h at RT and light protected on the shaker. After aspiration of the biotinylated antibody solution, the wells were washed another three times with 110 µl sterile PBS. Then, 30 µl Streptaviidin-R-phycoerythrin (PE) solution (diluted with Luminex Buffer at a concentration of 2 µg/ml) was added, and the plates were vortexed and incubated in the dark for a further 30 min by shaking. Finally, the wells were washed two times with 110 µl sterile PBS (at the last time the excess fluid was removed by blotting on paper towels) and at the end the beads were resuspended in 110 µl sterile PBS, gently vortexed and measured on Luminex 100ISTM. If measurements could not be performed on the same day, the well-plate was stored at 4°C over night. For longer storage, fresh PE has to be added before the measurements.

The parameters in the analysis software were set for acquiring data using 50 beads per well for 200 sec. The raw data were measured as mean fluorescence intensity (MFI). The analytes' concentrations were calculated by means of the calibration curve created without the first two highest standards, covering a concentration range from 1.5 to 1,111 pg/ml.

2.4. ELISA (Enzyme-Linked Immunosorbent Assay)

Chemicals

- Human IL-8 ELISA kit , from RayBio (Cat#: ELH-IL8-001)
- Samples: standard calibration solutions using recombinant IL-8 spiked into three matrix solutions: (i) HUVEC SN /10% FCS treated with IL-1ß; (ii) HUVEC SN /10% FCS untreated (negative control); (iii) PBS/10% human serum

Instrumentation

- A set of pipettes (Socorex)
- Shaker, MS1 minishaker, IKA
- Paper towels, Parafilm

ELISA Assays

The ELISAs were performed using the human IL-8 ELISA kit from RayBio (Cat#: ELH-IL8-001), containing a 96-well plate coated with IL-8 specific antibody. All reagents were provided with the kit and were prepared according to the manufacturers´ protocol booklet provided. Standard solutions of recombinant IL-8 were prepared by using Assay Diluent A to yield a concentration of 50 ng/ml, followed by another dilution step to prepare a stock solution of 600pg/ml, from which then a serial dilution by factors of 1/3 was done to yield a series of seven concentrations ranging from 600 pg/ml to 0.8 pg/ml. The Diluent was used as blank. ELISAs were performed with “undiluted” sample and diluted sample (IL-8 in PBS/10% human serum 1:10 diluted with Assay Diluent A and HUVEC SN with 10% FCS 1:100 diluted with 1x Assay Diluent B). All standards and samples were measured in triplicate.

For measuring, 100 µl standard and sample, resp., were added to each well, and the plate was incubated over night at 4°C under shaking. The supernatant solution was then discarded and the wells were washed four times with 300 µl 1X Wash Solution. At the last wash the plate was inverted and blotted against clean paper towels to completely remove all fluid. The well plate was incubated with 100 µl/well of prepared biotinylated anti-IL-8 antibody for 1h at RT under gentle shaking. After discarding the supernatant solution wells were washed four times with 300 µl 1x Washing Buffer, followed by incubation with 100µl/well HRP-Streptavidin solution at RT for 45 min under gentle shaking. After removing the solution from wells and four times washing with 1x Washing Buffer, 100 µl of TMB were added to each well and the plates were incubated for 30 min at RT in the dark under gentle shaking. After adding 50 µl/well of Stop Solution the plate was immediately measured at 450nm.

2.5. Western–Blotting

Chemicals

- Primary antibody - mouse anti-IL-8 antibody- (1:500 diluted in 5%PM/TBST/ NaN_3)
- Secondary antibody - anti mouse IgG (H+L)-HRP conjugated (1:10.000 diluted in 5%PM/TBS)
- Samples: standard calibration solutions using recombinant IL-8 spiked into three matrix solutions: (i) HUVEC SN /10% FCS treated with IL-1ß; (ii) HUVEC SN /10% FCS untreated (negative control); (iii) PBS/10% human serum
- Non-fat dry milk (powdered milk, PM)
- Blotting Buffer (see "Solutions and buffers composition")
- 1x Tris-Buffered-Saline (TBS)
- 1x Tris-Bufered-Saline-Tween-20 (TBST)
- Electrophoresis Buffer (see "Solutions and buffers composition")
- Marker - Precision Plus Protein™ Standards-Dual Color von BIO-RAD
- 30% polyacrylamid (PAA) solution
- 2M Tris - HCl pH 8.8 Buffer
- 1M Tris - Glycine pH6.8 Buffer
- 5x SDS Loading Buffer (5x SDS-LB)
- Sample Buffer (SB)
- Ponceau S Staining (0.2% Ponceau S in 3% trichloroacetic acid)
- ECL™ Prime Western Blotting Detection Reagent (GE Healthcare)
- TEMED
- 10% Ammonium Persulfate (APS) solution
- 20% (w/w) Sodium Dodecyl Sulfate (SDS) solution

Instrumentation

- peQLab Biotechnology Chemilumineszenz-Imaging (peQ Lab Biotecnologie GmbH, Erlangen, Germany
- Electrophoresis equipment: Mini-ProteanTM- Cell Tetra System from BIO-RAD, (Vienna, Austria) (1mm Spacer)
- Blotting equipment: Mini Trans-Blot Electrophoretic Transfer Cell from BIO-RAD
- Nitrocellulose Membrane (PORTMAN) (Whatman GmbH, Dassel, Germany)
- Magnetic Mixer with magnetic stir bar
- Desiccator
- Filter paper, sponges

1-DE-WB-Procedure

SDS-PAGE: Western blot analysis is preceded by protein separation using one-dimensional SDS-PAGE. For this purpose, samples were loaded onto a 12% PAA gel in amounts according to the aim of application. E.g. in the case of antibody validation samples with different amounts of total protein were loaded (see corresponding figures in the antibody validation

section). If the sample volume was lower than 10 µl, the samples were diluted to a total volume of 10 µl by adding water, if a sample volume was higher than 10 µl they were diluted to 20 µl. In both cases 5xSDS-loading buffer were added, 4 µl of buffer to 10 µl of sample and 5 µl of buffer to 20µl sample-water solution. By the comparison of various methods to 10µl sample was added 4µl 5xSDS-loading buffer and 20µl of immunoprecipitated samples were mixed with 5 µl 5xSDS-loading buffer**.** Just before loading the samples are vortexed and briefly spinned. The application wells in the gel were loaded with sample solutions, in between the samples solutions of Sample Buffer (mixed 2:1 with 5x SDS-LB) and on the lane at the right pre-stained markers (Precision Plus Protein^{TM} Standards-Dual Color from BIO-RAD #161--0374). Electrophoresis was carried out for 45 min at RT by 20mA and 200V, when the sample arrived at the separating gel current and voltage were increased to 40 mA per gel and 300V.

Transfer of Proteins: The separated proteins were transferred from the gel onto a nitrocellulose membrane by electroblotting for 2h at RT applying 80 V with 400 mA current, or over night at RT with 30 V and 400 mA current.

Membrane development: After blotting, the membrane was washed briefly with water and all proteins were visualized by Ponceau staining (3 min incubation in the staining solution at RT under shaking followed by washing for a short time with water). The next steps in membrane developing included: blocking with 5 %MP/TBST for 1h under shaking, three times washing with TBST Buffer, 2h incubation with primary antibody (1:500 in 5% MP/TBST/NaN_3 diluted), again three times washing with TBS Buffer followed by 1h incubation with secondary antibody (1:10000 in 5% MP/TBS diluted), and finally, washing three times with TBS. The final measurement was carried out after addition of 4 ml luminescence solution and 3 min of incubation.

2.6. Shot-gun proteomics

Chemicals

- 1D-SDS-PAGE: see Western-Blotting part (2.5)
- Molecular mass marker: Precision Plus ProteinTM Standards-Dual Color from BIO-RAD, Austria, ref. Nr. 161—0374
- Methanol, technical from BDH Prolab (VWR ref.Nr. 20903.368)
- Methanol for analysis Merck (Darmstadt, Germany) (VWR ref. Nr. *1.06009.2511)*
- Acetic acid for analysis Merck (Darmstadt, Germany) (VWR ref. Nr. *1.00063.2511)*
- Sodium thiosulfate for analysis ($Na_2S_2O_3$ $5H_2O$) Merck (Darmstadt, Germany) (VWR ref. Nr 1.06516.0500)
- Silver nitrate $AgNO_3$ from Sigma-Aldrich (Schnelldorf, Germany, ref. Nr. S6506)
- Sodium carbonate (Na_2CO_3) for analysis from Merck (Darmstadt, Germany) (VWR ref. Nr 1.06392.1000)

- Sodium hydroxide from BDH Prolabo (VWR ref. Nr. 1.06498.1000
- Formaldehyde 37% Merck (Darmstadt, Germany) (VWR ref. Nr. 1.04003.1000)
- Potassium ferricyanide (III) ($K_3Fe(CN)_6$) from Sigma-Aldrich (Schnelldorf, Germany, ref. Nr. 702587)
- Ammonium hydrogen carbonate (NH4HCO3) from Sigma-Aldrich (ref. Nr. A6141)
- Dithiothreitol (DTT) from Gerbu (Wieblingen, Germany, ref. Nr.1008.0025)
- Iodacetamide (IAA) from Sigma-Aldrich (Schnelldorf, Germany, ref. Nr. I-6125)
- Acetonitrile (ACN) hypergrade for LC/MS Merck (Darmstadt, Germany) (VWR ref. Nr. 1.00029.2500)
- Formic acid (FA) for analysis Merck (Darmstadt, Germany) (VWR ref. Nr. 1.00253.1000)
- Trypsin sequencing grade from Roche (Mannheim, Germany, ref. Nr. 11418475001)

Instrumentation

- Electrophoresis equipment: Mini-ProteanTM- Cell Tetra System from BIO-RAD, Austria
- Desiccator from Kartell (VWR ref. Nr. 467-2120)
- Scalper from SWANN-MORTON (VWR ref. Nr. 233-5482)
- Silicon coated tubes
- Thermomixer Comfort from Eppendorf(VWR ref. Nr. 460-1112)
- Vortex-Genie from Scientific Industries (VWR ref. Nr. 444-5900)
- Ultrasonic bath SONOREX DIGITAL from Bartelt (Gtraz, Austria, ref. Nr. 9877059)
- Vacuum centrifuge GeneVac miVac from Bartelt (ref. Nr. DNA23050B00)
- Q Exactive mass spectrometer from Thermo Scientific (Massachusetts, USA)
- UltiMate 3000 RSLCnano System form Dionex (California, USA)

Analytical procedure

Shotgun Analysis requires several steps of sample preparations before mass spectrometry analysis can be performed. In our experiments protein separation was done by electrophoresis (1D-PAGE); silver staining and tryptic digestion. Only samples with forth higher concentration (from 1µg/ml to 1ng/ml) were analyzed per shotgun approach.

1D-PAGE for subsequent Shotgun Analysis: With all samples, 10 µl of them were mixed with 2 µl H_2O and 3 µl of 5xSDS-sample buffer and after vortexing and spinning the samples were loaded onto a 12% PAA gel. Electrophoresis was carried out (at 200V, 40mA for two gels) until the complete separation of a set of pre-stained molecular mass markers was visible (about 1.5cm total migration distance). Afterwards, the gels were fixed with 50% methanol/10% acetic acid solution and subsequently silver stained as described below. Gel lanes were cut out of the gel and were digested using trypsin as described below.

MS-Compatible Silver Staining: The gels were fixed and washed with 50% methanol solution, and sensitized with 0.02% $Na_2S_2O_3$ solution. Then the gels were stained with 0.1% $AgNO_3$ solution for 10 min, rinsed with bidistilled water and subsequently developed with 3% Na_2CO_3/0.05% formaldehyde solution as previously described in (Mortz et al., 2001)

Tryptic Digestion: Protein lanes under 25 kDa were divided into slices (*i.e.*, into two slices with the PBS/0,1% BSA samples, and three slices with the PBS/10% Serum and the HUVEC SN samples) and were cut out of the gel. The gel-pieces were destained with 15 mM $K_3Fe(CN)_6$/50 mM $Na_2S_2O_3$ solution and intensively washed with 50% methanol/10% acetic acid. Afterward, the pH was adjusted by adding 200 µl 50 mM NH_4HCO_3 solution and the gel pieces were incubated in a shaker (Thermomixer) for 5min at 800 rpm. After removal of the supernatant the proteins in the gel pieces were reduced by adding 200 µL of a 10 mM DTT/ 50 mM NH_4HCO_3 solution for 30 min at 56 ^oC. After removal of the supernatant the gel pieces were again washed with the 50 mM NH_4HCO_3 solution and spinned for buffer removal. Afterwards the proteins were alkylated by adding 200µL 50 mM iodacetamide/50 mM NH_4HCO_3 solution over 20 min in the dark. After removal of the supernatant and washing by the NH_4HCO_3 buffer, the gel-pieces were treated with acetonitrile (ACN) and dried in a vacuum centrifuge. Dry gel-slices were treated with a 0.1 mg/mL trypsin solution in 50 mM NH_4HCO_3 for 10 min on ice. Afterward, the slices were covered with 50 mM NH_4HCO_3 and were subsequently stored overnight at 37^oC. The peptides obtained by digestion were eluted by adding 50 mM NH_4HCO_3 solution, transferring the supernatant into silicon coated tubes and repeating this procedure two times with 5% formic acid/ 50% acetonitrile. Between each elution step the gel-spots were ultra-sonicated for 10 min. Finally, the peptide solution was concentrated in a vacuum centrifuge to 20µl volume. Just before performing LC-MS analysis, the samples are diluted to 60µl by a solution containing 98% H2O /2% ACN/0.2% FA, then 5µl of standard peptides solution (containing *in toto* 20 fmol/µl of each peptide) was added in order to check the instrument performance during LC-MS analysis. After vortexing and centrifugation at 1000rpm for 3min 60µl of this solution was pipetted into a well plate.

HPLC-Mass Spectrometric Analysis: First, the peptides were separated by nano-HPLC using the UltiMate 3000 RSLC nano System (Pre-column: Acclaim PepMap 100, C18 100 µmx2 cm; Analytical column: Acclaim PepMap RSLC C18 75 µm x 15cm; Dionex, California, USA) operating at a flow rate of 500 nL/min and using a solvent gradient from 99% A; 1% % B to 68% A; 32%B over the time window of 15 min. Solvent composition A: 98% water, 2% ACN, 0,2% FA; Solvent composition B: 20% water, 80% ACN; For each run 15 µl of sample solution were injected (corresponding to a quarter of the entire sample solution gained from the gel). (When starting from 1µg/ml protein, a maximum of 250ng/ml can be expected being injected into the column.) All shot-gun-type measurements were performed in duplicate and, additionally, a single SIM measurement was done.

Peptide identification was done via MS/MS fragmentation analysis using a Q Exactive mass spectrometer from Thermo Scientific (Massachusetts, USA) equipped with a nanospray ion source. The MS data were acquired using a data-dependent "top 6 method" which dynamically chooses the six most abundant precursor ions from full scan MS (400-1500 m/z), followed by precursor ions isolation performed with an isolation window of 2 Th and "High

energy collision induced decay" (HCD) fragmentation. The full scan MS was acquired at a resolution of 70.000 and the MS/MS scan at 17.500, both at m/z 200. Protein identification was achieved using Proteome Discoverer 1.3 (Thermo) employing Mascot and Sequest search algorithms and searching against Uniprot human database (04/2012) using following searching parameters: maximum two missed cleavages and maximum allowed mass deviation of 5ppm for peptide ions and 20ppm for fragment ions. Further, carbamidomethylation of cysteines was set as fixed and protein *N*-terminal acetylation methionine oxidation as variable modification. The false discovery rate was adjusted to 0,01. Quantification of IL-8 from raw MS data was performed using the "Perseus" tools available within the "MaxQuant" software 1.3 taking into account all IL-8 identified peptides in all gel slices.

2.7. Selected Ion Monitoring (SIM)

Sample preparation steps prior to SIM analysis are identical to those for the shotgun approach described above (i.e., protein separation by 1D-PAGE, silver staining and tryptic digestion of proteins).

The proteins captured on the beads by immunoprecipitation from 200 µl of sample were eluted from the beads with 40 µl elution buffer (pH 2.73). One half of this volume was used for SDS-PAGE with SIM analysis (giving that only 100 µl of sample were used for SIM analysis), the other half for a WB-analysis for comparison. Just before loading the sample solution onto the 12 % PAA gel, the 20 µL sample was neutralized with 1µl 5M NaOH (Merck/VWR 1.06498.1000), mixed with 5µl 5xSDS-loading buffer, vortexed and shortly centrifuged. When loading the samples onto the gel, elution buffer instead of SB was added in the lanes between the samples in order to hold the protein separation in narrow bands. Unlike to non-precipitated samples, the precipitated ones were separated in the separating gel over a migration distance of just 0.5 cm because of the much lower sample complexity after IP.

After digestion of the proteins and elution from the gel, the end volume was 20 µl and this volume was diluted to 60 µl with a solution containing 98% water/2% ACN/0.2% FA. For each HPLC-SIM run 15 µl of them were used. All SIM measurements were performed in triplicate.

HPLC-Mass Spectrometric Analysis.

SIM was done by using the same HPLC-MS equipment as described for the shotgun approach. The MS analysis was run in the positive ion mode and for the quantification of IL-8 the following peptide ions were isolated with a window of *in toto* 3 *m/z*: 562.94727 (z=3); 739.03644(z=3); 883.95868 (z=2); 437.72073 (z=2); 589.64172 (z=3); 589.64172 (z=2); 701.8534 (z=2). A cycle consists of: one full scan MS with an accumulation time of 30 ms for

5E5 ions and resolution of 70.000; seven SIM scans for chosen peptides, each SIM with an accumulation time of 120 ms for 5E4 ions and 70.000 resolution; finally a MS/MS scan, triggered if the targeted peptide ions were found in SIM mode, with accumulation times of 250 ms for 5E4 ions and resolution of 17.500. All this steps gives a total cycle time of ca. 2.5 s. The resolution in the Q Exactive is specified as FWHM at m/z 200. Peptide identification from MS/MS spectra was done as described previously at shot-gun proteomics part.

The quantification of IL-8 from the SIM data of all identified peptides (depending on the protein concentration) was performed by using the "Sieve" software 2.0 (Producer,Thermo).

As described above from the SDS/PAGE gels two pieces were cut out covering the Mw range between 25 kDa to 10 kDa, and 10 to 5 kDa, respectively, when dealing with samples where an IP pre-treatment was included. When no IP was done before, three gel slices were taken, corresponding to a Mw range from 25 kDa to 5 kDa divided in three equally width gel pieces. For the SIM based quantitation of IL-8, only the slice (containing the predominant amount of IL-8 was taken (when an IP treatment was done before or only standard und reference samples were run); in the case of analysis without IP pretreatment, the middle slice (containing the predominant amount of IL-8) was used.

2.8. Solutions and buffer composition

30% polyacrylamide (PAA) solution:
292 g Acrylamide from Gerbu 1001
8 g Piperazine di-acrylamide(PDA) (1,4-Bis(acrylol) piperazine) from Sigma-Aldrich 14470
add to 1 L water

12 % separating gel:
4,8 ml 30% PAA
2,25 ml 2M Tris-HCl buffer pH 8,8
4,83 ml water

4% stacking gel:
1,066 ml 30% PAA
1 ml 1M Tris-HCl buffer pH 6,8
5,86 ml water

Phosphate buffered saline (PBS), pH 7,3
80 g NaCl from Merck/VWR 1.06404.1000
2 g KCl from Merck/VWR 1.04936.0500
2 g KH2PO4 from Merck/VWR 1.04873.0250
14,4 g Na2HPO4 x 2H2O from Merck/VWR 1.06580.1000
add to 1 L water

5X SDS - loading buffer (5x SDS-LB):
5 ml 1M Tris-HCl buffer pH 6,8
2 g SDS (sodium dodecyl sulfate) from Gerbu 1012
10 ml Glycine from Gerbu 1023
0,05 g Bromphenol blue from Merck/VWR 1.11746.0005
add to 17,5 ml water
before use to 437,5 µl above mixture 62,5 µl 2-mercaptoethanol was added (Sigma-Aldrich M3148)

Sample buffer (SB):
4,5 g Urea from Merck/VWR 1.08488.1000
1,14 g Thiourea from Sigma-Aldrich T8656
0,4 g CHAPS from Gerbu 1083.0025
25 µl 20% SDS (w/w)
1 ml 1M DTT
add to 10 ml water

Electrophoresis buffer:
100 ml 10X Tris-Glycine
5 ml 20% SDS (w/w)
895 ml water

10x Tris-Glycine:
30 g Tris X from Gerbu 1044.1000
144 g Glycine from Gerbu 1023
add to 1L water

10x Tris buffered saline Tween (TBST):
100 ml 1M Tris-HCl buffer pH 8
300 ml 5M NaCl
10 g Tween 20
add to 1 L water

Tris buffered saline (TBS):
5 ml 1M HEPES/NaOH buffer pH 7,4 ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) from Gerbu 1009.0250)
15 ml 5M NaCl
add to 500ml water

Blotting buffer:
100 ml 10x Tris –Glycine
150 ml Methanol technical from BDH Prolab/VWR (20903.368)
1 ml 20% SDS (w/w)
750 ml water

Activation buffer:
100 mM sodium phosphate, monobasic anhydrous (NaH_2PO_4 x H_2O from Sigma, S3193) pH adjusted to 6,2 by 5M NaOH from Fisher, SS256-500

Coupling buffer:
50 mM MES (2 [N-Morpholino] ethanesulfonic acid from Sigma, M2933)
pH adjusted to 5.0 by 5M NaOH

Luminex buffer:
0,250 ml Tween-20
0,25 g sodium azide (NaN_3 from Sigma-Aldrich S8032)
5 g BSA (bovine serum albumin from Ruth-Germany)
500 ml PBS

3. Results and Discussion

3.1. Specific immunochemical enrichment of the interleukins IL-6, IL-8 and CXCL3

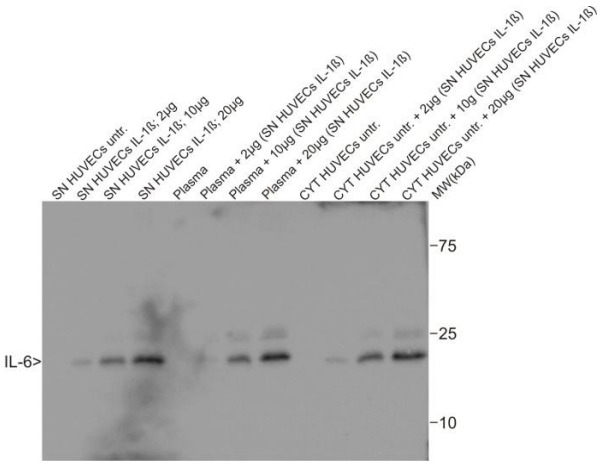
3.1.1 Characterization of antibodies regarding unspecific interactions and sensitivity

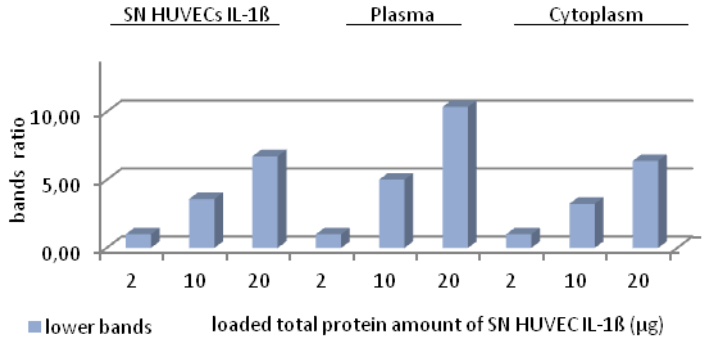
In a very first step those antibodies used for immunoprecipitation (IP) of the various cytokines were characterized regarding sensitivity and selectivity by means of Western Blot (WB) analysis. HUVEC and skin fibroblast cell cultures were used and samples were taken from cell culture supernatant (monitoring proteins secreted), from human plasma (monitoring predominantly secreted proteins as well), and from cytoplasm of the cultured cells. Antibody sensitivity was determined according to the band intensities when loading three different amounts of total protein on PAA gels, namely 2µg, 10µg and 20µg, respectively.

The experimental setting was such that identical amounts of total protein (2µg, 10µg and 20µg, resp.) gained from the supernatant of HUVECs or skin fibroblasts treated with IL-1ß were spiked in human plasma and cell cytoplasm of untreated cells. Plasma from healthy persons, cytoplasm of untreated HUVECs and skin fibroblasts, and the supernatant of untreated HUVECs were used as negative controls. The commercially available antibodies for the cytokines IL-6, IL-8 und CXCL3 specified in the Materials and Methods section were investigated within this work.

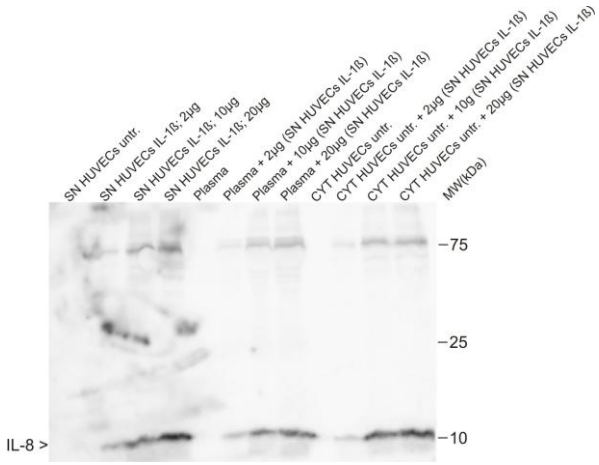
The WBs and the corresponding band intensity data for IL-6, IL-8 und CXCL3 are shown in the Figs. 15 (a), (b) and (c), respectively. The bands originating from the targeted cytokines (expected molecular masses of 23,718, 11,098, and 11,342 Da, respectively) are seen with increasing intensities when higher total protein amount was loaded. Such a response is a positive indication of antibodies' specificity and sensitivity. However, unexpected bands are present in addition: a faint band at about 30kDa in the WB for IL-6, and rather strong bands at 75kDa in the WBs for IL-8 and CXCL3. In a complex matrix of cell supernatants, cell cytoplasm or plasma, unspecific interactions of antibodies with other proteins are not unlikely, and binding of the targeted antigen to high abundant proteins building antigen-protein complexes (which are not dissociated by SDS) cannot be excluded as well. The intensities of these additional bands increase in all samples with increasing amount of total protein (corresponding to increasing volumes of the supernatant from IL-1ß treated HUVECs added). (Figure 15 right-side). The supernatant of untreated HUVECs doesn't show any bands. From these data we conclude that binding of the interleukins to other abundant proteins is more likely the source of additional bands than it is an unspecific interaction of the antibody. However, cross-reactivity of antibodies with other proteins that are up regulated by treating HUVECs with IL-1ß cannot be excluded as well.

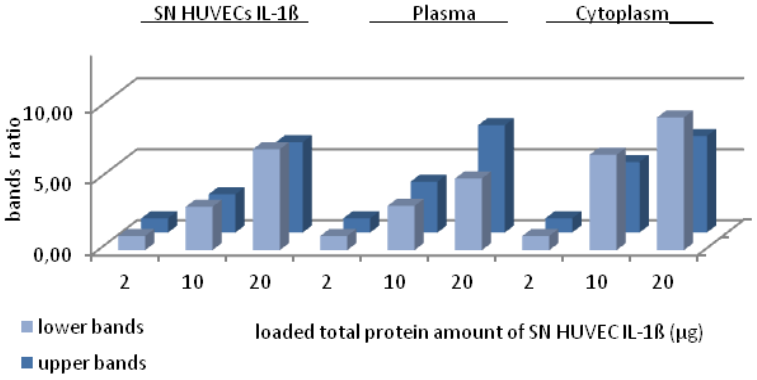
(a)


SN HUVECs untr.
SN HUVECs IL-1ß; 2µg
SN HUVECs IL-1ß; 10µg
SN HUVECs IL-1ß; 20µg
Plasma
Plasma + 2µg (SN HUVECs IL-1ß)
Plasma + 10µg (SN HUVECs IL-1ß)
Plasma + 20µg (SN HUVECs IL-1ß)
CYT HUVECs untr.
CYT HUVECs untr. + 2µg (SN HUVECs IL-1ß)
CYT HUVECs untr. + 10g (SN HUVECs IL-1ß)
CYT HUVECs untr. + 20µg (SN HUVECs IL-1ß)
MW(kDa)
75
25
10
IL-6>

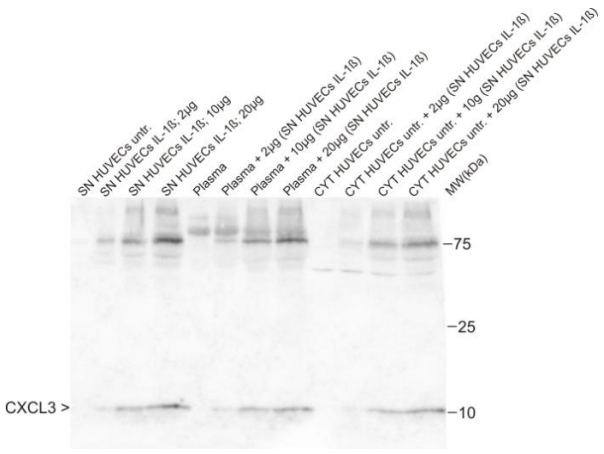

SN HUVECs IL-1ß
Plasma
Cytoplasm
bands ratio
10,00
5,00
0,00
2
10
20
lower bands
loaded total protein amount of SN HUVEC IL-1ß (µg)

(b)


SN HUVECs untr.
SN HUVECs IL-1ß; 2µg
SN HUVECs IL-1ß; 10µg
SN HUVECs IL-1ß; 20µg
Plasma
Plasma + 2µg (SN HUVECs IL-1ß)
Plasma + 10µg (SN HUVECs IL-1ß)
Plasma + 20µg (SN HUVECs IL-1ß)
CYT HUVECs untr.
CYT HUVECs untr. + 2µg (SN HUVECs IL-1ß)
CYT HUVECs untr. + 10g (SN HUVECs IL-1ß)
CYT HUVECs untr. + 20µg (SN HUVECs IL-1ß)
MW(kDa)
75
25
10
IL-8 >


SN HUVECs IL-1ß
Plasma
Cytoplasm
bands ratio
10,00
5,00
0,00
2
10
20
lower bands
upper bands
loaded total protein amount of SN HUVEC IL-1ß (µg)

(c)


SN HUVECs untr.
SN HUVECs IL-1ß; 2µg
SN HUVECs IL-1ß; 10µg
SN HUVECs IL-1ß; 20µg
Plasma
Plasma + 2µg (SN HUVECs IL-1ß)
Plasma + 10µg (SN HUVECs IL-1ß)
Plasma + 20µg (SN HUVECs IL-1ß)
CYT HUVECs untr.
CYT HUVECs untr. + 2µg (SN HUVECs IL-1ß)
CYT HUVECs untr. + 10g (SN HUVECs IL-1ß)
CYT HUVECs untr. + 20µg (SN HUVECs IL-1ß)
MW(kDa)
75
25
10
CXCL3 >

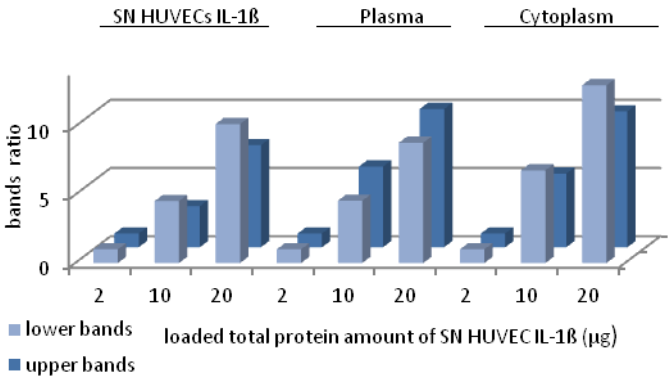

SN HUVECs IL-1ß
Plasma
Cytoplasm
bands ratio
10
5
0
2
10
20
lower bands
upper bands
loaded total protein amount of SN HUVEC IL-1ß (µg)

Figure 15. ***Left side****: WB analysis of cytokines using primary Abs targeting (a) IL-6; (b) IL-8; (c) CXCL3. Cytokines were determined in cell supernatant (SN), plasma, and cell cytoplasm (CYT) as described below:*
Lanes: 1: supernatant (SN) of untreated HUVECs (negative control; total protein amount 20µg); lanes 2-4: SN of HUVECs after treatment of cells with IL-1ß; volumes applied to WB correspond to total protein amounts of 2µg, 10µg and 20µg respectively; lane 5: human plasma (negative control; total protein amount 20µg); lanes 6-8: human plasma to which SN from HUVECs treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.; lane 9: cytoplasm of untreated HUVECs (negative control; total protein amount 20µg); lanes 10-12: CYT of untreated HUVECs to which SN from HUVECs treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.
Right side*: Bands intensity ratios measured by using ImageQuant 5.0. A value of 1 was assigned to the band with the lowest intensity (2µg total protein amount of SN-HUVEC-IL-1ß).*

In addition to the three anti-interleukin antibodies (against IL-6, IL-8 and CXCL3), an antibody targeting Tumor Necrosis Factor-inducible Gene 6 protein (TSG6) was tested as well. This protein has a calculated molecular mass of 31,203Da. The WB shown in Fig. 16 exhibits 4 bands, the strongest thereof at about 18-20 kDa. As these bands appear in cytoplasm only and there even in the negative control, the data indicate unspecific interactions of the TSG6 antibody. This Ab was not used for further investigations.

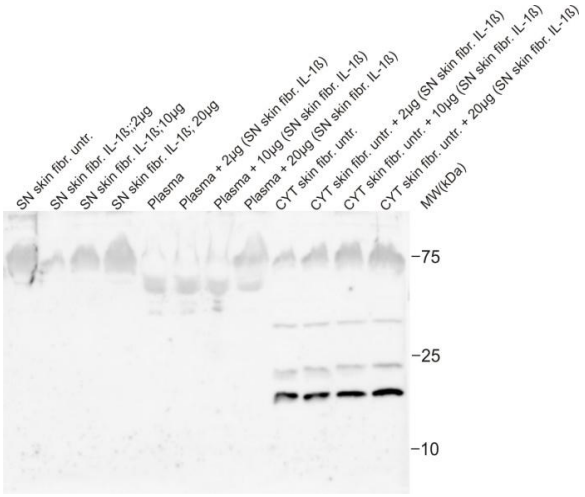


Figure 16. *WB analysis targeting Tumor Necrosis factor-inducible Gene 6 protein (TSG6) in cell supernatant of skin fibroblasts, in human plasma, and in skin fibroblast cell cytoplasm after addition of cell SN.*
Lanes: 1: supernatant (SN) of untreated skin fibroblasts (negative control; total protein amount 20µg); lanes 2-4: SN of skin fibroblasts after treatment of cells with IL-1ß; volumes applied to WB correspond to total protein amounts of 2µg, 10µg and 20µg respectively; lane 5: human plasma (negative control; total protein amount 20µg); lanes 6-8: human plasma to which SN from skin fibroblasts treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.; lane 9: cytoplasm of untreated skin fibroblasts (negative control; total protein amount 20µg); lanes 10-12: CYT of untreated skin fibroblasts to which SN from skin fibroblasts treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.

3.1.2 Determination of appropriate IP conditions for the targeted proteins (following the Invitrogen protocoll)

- ***Selection of beads***

Immunoprecipitation was performed using Dynabeads Protein G. Dynabeads consist of a polymer encapsulated shell with a magnetic pigment inside (usually iron oxide) giving beads magnetic properties. The used Dynabeads from Invitrogen carry reactive sulfonyl esters (R-SO_2-OR´) that can react with proteins e.g. by covalent binding to the thiol groups of the amino acid cysteine or to the primary amino group of lysines. Dynabeads used in this work were pre-coupled with recombinant protein G. Protein G binds IgGs on their Fc region with strong affinity at neutral pH by a non-immune mechanism but does not contain albumin binding sites. As antibodies from mouse and rabbit (like those used here) exhibit higher affinity to protein G than to protein A, Dynabeads coated with protein G were chosen for our IP experiments.

The entire IP procedure consists in three steps: (i) Antibodies directed against a specific cytokine (primary Abs) were diluted in "Binding and Washing Buffer" and loaded (coated) onto the Dynabeads *via* interaction with Protein G. For testing the completeness of the antibody coating reaction under given coating conditions, the supernatant remaining after the coating step (i.e., "SN1" which contains still some primary antibodies) is transferred in a clean tube and analyzed by WB using a secondary ("reporter") antibody. (ii) In the second step, the coated beads are then incubated with the sample to attain antigen precipitation ("capture"). At the end of IP, the remaining supernatant ("SN2") is collected in a new tube and is again analyzed by WB testing for completeness of IP of the antigen. (iii) In the last step the immunoprecipitated antigen is eluted from the beads by using "Elution Buffer" and the antigen concentration is determined by WB analysis.

- ***Influence of sample pre-cleaning***

Protein G pre-coated on Dynabeads can bind IgGs present in the samples and this undesired enrichment of "wrong" antibodies often leads to co-immunoprecipitation of other proteins present in the sample. In order to minimize unwanted binding during IP and to avoid interactions of other compounds in the sample with the beads or the beads' ligands, the sample was pre-incubated for 40 min at RT with 20µl Dynabeads. The effect of this pre-cleaning procedure was demonstrated by comparing pre-cleaned with non pre-cleaned samples after IP and detecting the remaining interleukin (IL-6) in the sample by WB analysis (Fig. 17). As there was no significant change in the IL-6 band intensities comparing the samples without and with pre-cleaning step, we conclude that the influence of sample pre-cleaning on the IP efficiency is marginal. The bands seen in the WB at about 100kDa are assumed being dimmers of the heavy chains (HC) of the IGgs . They could be removed by

boiling the sample. Based on the conclusion that IP is not significantly influenced by sample pre-cleaning, this step was not applied in all further future IP experiments.

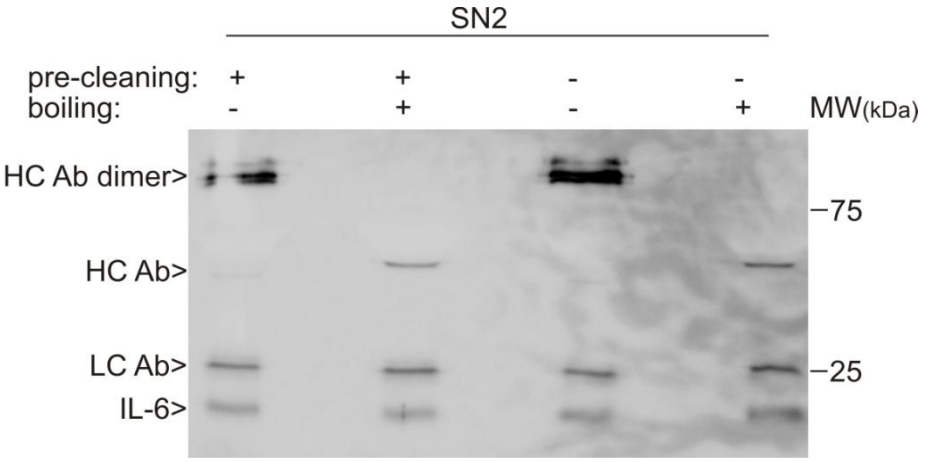


Figure 17. *WB analysis targeting IL-6 in HUVEC-SN2 (i.e., supernatant remaining after IP of IL-6). IP was performed with and without sample pre-cleaning aimed for removing IgGs present in the sample using Dynabeads Protein G (100µl beads coated with 2µg antibody; 50µl of them were used for IP). Dissociation of the protein complex (HC dimmers) with Mw of approx. 100 kDa was attained by boiling the sample for 5min at 95°C after mixing with loading buffer.*

- ***Influence of the amount of coated beads employed***

To start the optimization, IP was performed following the manufacturers' protocol booklet provided and using buffer solutions taken from the pre-prepared kits. In this procedure 100µl beads were coated with 2µg antibody and then IP was performed with different amount of coated beads, namely 2,5µl; 50µl ;100µl, respectively. According to the protocol, coating of beads with antibody and subsequent IP was each carried out by incubation over 30min at RT under horizontal rotation; the following elution was done with 20µl Elution Buffer and rotation over 10min. When the band of the light chain of the antibody (LC) appeared in the WB of the supernatant sample after IP (SN1), we had an indication that not all the amount of antibody used for coating (2µg) has successfully bound to the beads.

In Fig. 18, the band of IL-6 is seen in the supernatant sample remaining after IP (SN2) in all three experiments using different amounts of beads. However, the band intensity of IL-6 becomes much lower in SN2 samples when using higher amounts of coated beads (50µl and 100µl) indicating a more complete IP when higher amounts of antibody (theoretically 1µg and 2µg) are offered. (For comparison, with 2,5µl beads only 50ng of Abs are theoretically available.)

As the optimum conditions for IP are strongly dependent on the antibody used, IP with anti-IL-8 antibodies were tested under analogous condition as described for anti-IL-6 Abs using 50µl and 100µl of coated beads, respectively (Fig. 19). Complete IP of IL-8 was not yet

achieved as can be seen by the IL-8 band still appearing in the WB of the SN2 sample. However the intensity of this band is lower when using 100µl coated beads.

The IL-8 bands in eluate samples (EL) indicate that IP could be successfully performed. As different volumes of elution buffer were used in Fig. 19a and b, the comparison of band intensities would be misleading (discussion on `elution optimization` in the Supplemental Material).

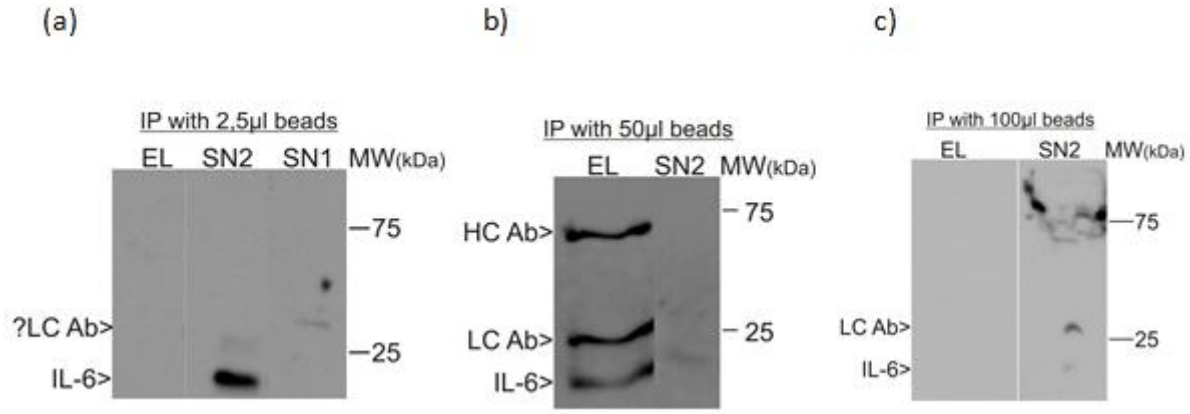


Figure 18. *WB analysis targeting IL-6 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using different amounts of coated beads: (a) 2.5µl; (b) 50µl, (c) 100µl. Coating conditions: 100µl beads were incubated with 2µg antibodies for 30min at RT under rotation; IP conditions: incubation for 30min at RT under rotation.*

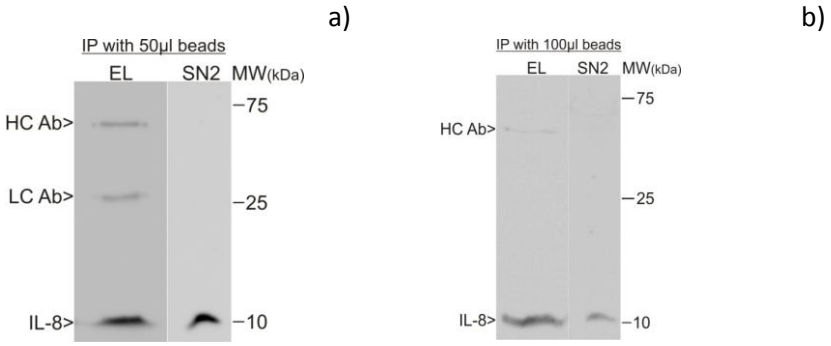


Figure 19. *WB analysis targeting IL-8 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using different amounts of coated beads: (a) 50µl, (b) 100µl. Coating and IP conditions as described in Fig. 18.*

- ***Influence of amount of antibodies immobilized on the beads***

From the experiments described above we concluded that a rather high amount of antibody is needed for attaining nearly complete IP of interleukins. In these former experiments 100µl beads were reacted with 2µg of antibody. In order to further enhance the completeness of

IP, the same amount of beads as previously were coated now with higher amounts of antibodies under the same conditions as described before: *i.e.*, 100µl beads were coated now with 10µg of antibody, and IP of IL6 was performed using 2,5µl and 50µl coated beads: This means that 250ng and 5µg antibody, respectively, were offered for the precipitation reaction. For IP of IL-8, 50µl beads were coated with 2,5µg antibody and the entire volume of 50µl was used.

The data in Fig. 6a indicate that 250ng of Ab available in 2.5 µl of beads is not yet sufficient for attaining IP of IL-6 to a reasonable extent: no IL-6 band is obtained in the eluate (EL) from the beads, whereas most of the IL-6 is recognized in the supernatant after IP (SN2). By using 50µl of beads containing a total amount of 5µg of Ab gives satisfactory results for both interleukins. After IP of IL-6 (Fig. 20b) no IL-6 band is seen in SN2, (The lack of the IL-6 band in EL is caused by too extensive washing after IP.), and with IL-8 (Fig. 21), the bands in the WB of both, SN2 and EL, indicate that more or less complete IP could be attained under these conditions.

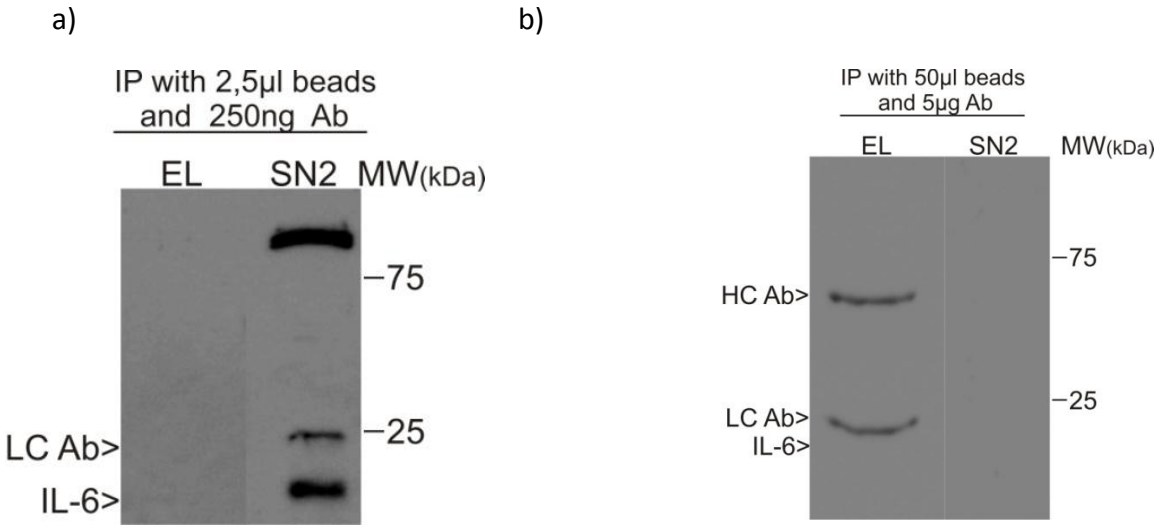


Figure 20. *WB analysis targeting IL-6 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using enhanced amounts of Ab coated onto the beads: (a) 2.5µl of beads containing 0.25 µg of Ab and (b) 50µl of beads coated with 5µg of Ab.; (b) 50µl l of beads coated with 5µg of Ab. Other conditions as described above.*

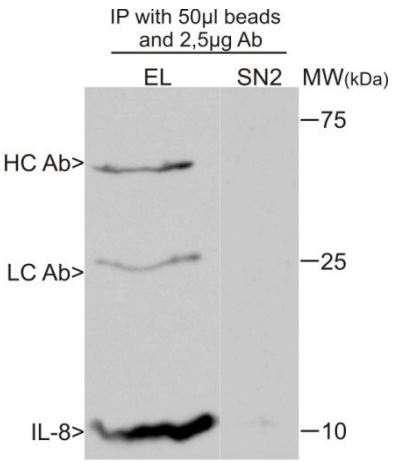


Figure 21. *WB analysis targeting IL-8 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using enhanced amounts of Ab coated onto the beads: 50µl of beads coated with 2.5µg of Ab. Used samples: HUVEC-SN*

3.1.3 Optimization of IP when using low amounts of coated beads with high amounts of antibodies coated under a modified IP protocoll

In the first part we tried to optimize IP regarding to the amounts of beads and antibody following the Invitrogen protocol. In this way we achieved complete IP of IL-6 and IL-8 using 50µl beads coated with 5µg and 2.5µg antibody, respectively.

In order to keep the consumption of beads small, we now change to a strategy using lower amounts of coated beads for IP and improving some steps in the Invitrogen protocol, *i.e.*, particularly including more washing steps, using larger buffer volumes, and increasing the IP incubation time from 30min to 2h.

For preparing the beads for the coating reaction and then later for IP, the beads were washed three times with Binding & Washing Buffer (in volume ratio 1:5 beads to washing buffer) over 5min under rotation. After IP the beads were washed three times with Washing Buffer (ratio 1:5 as previously) in order to dissociate unspecific binding. For the final elution, twice the volume of Elution Buffer was used as µl of coated beads were taken for IP.

For both interleukins, IL-6 and IL-8, 100µl beads were coated with 5µg of corresponding antibody and IP was performed using 10µl and 20µl of coated beads (corresponding to 0.5µg and 1µg antibodies). The WB results are shown in Fig. 22. From the absence of bands in SN1 one can conclude that the entire amount of antibody added was bound onto the beads (which were probably facilitated by the additionally applied washing steps.). However, the presence of an interleukin specific band in SN2 indicated, that the IP of IL-8 was not yet complete.

In a further attempt to improve the effectiveness of IP, the sample was supplemented prior to IP with Tween-20 (1% final concentration) in order to reduce non-specific binding, to prevent aggregation of beads and to facilitate bead mixing, and NaCl was added to the sample to a final concentration of 150mM. Other conditions and the amount of coated beads (20µl) used for IP were identical as in the last experiments. The presence of the IL-8 band in SN2 shown in Fig. 23 still indicates insufficient IP.

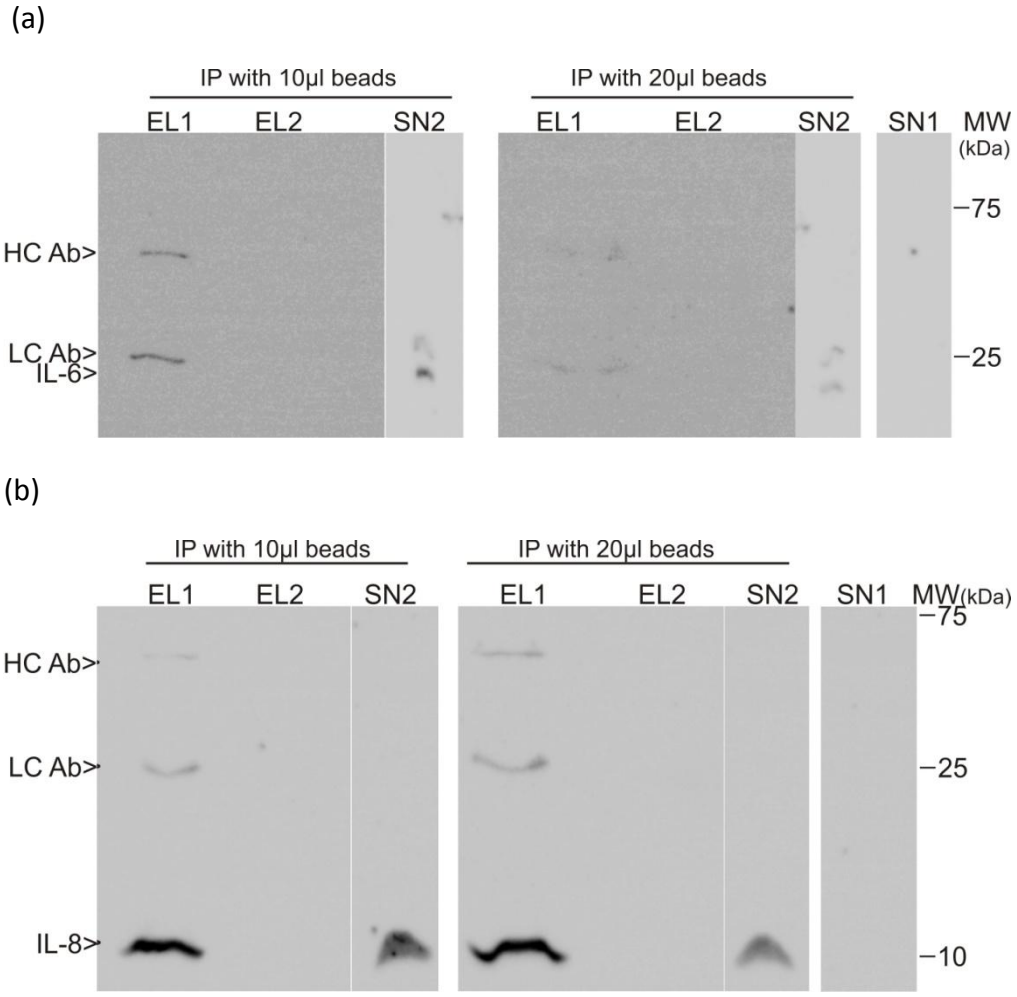


Figure 22. *Western blot analysis targeting (a) IL-6 and (b) IL-8 in the supernatant after IP. Higher amounts of antibody IP was performed using enhanced amounts of Ab coated and lower volumes of beads. 100µl beads were coated with 5µg of corresponding antibody and IP was performed using 10µl and 20 µl of coated beads. Other IP conditions: incubation over 2h at RT; (SN1- supernatant after coating; SN2 – supernatant after IP).*

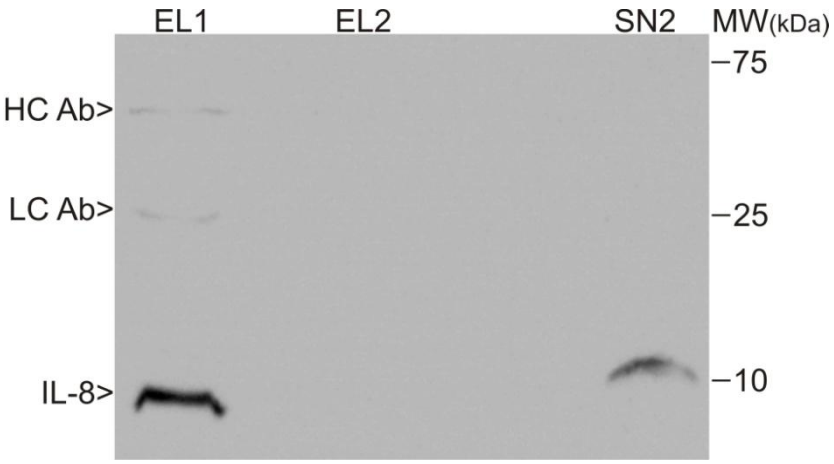


Figure 23. *WB analysis targeting IL-8 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed after adding 1% Tween-20 and NaCl at 150mM final concentration to the sample. 100µl beads were coated with 5µg antibody and 20µl of them were used for IP.*

In a next step, the beads were coated to their maximal capacity, i.e., 50µl beads were coated with 10µg antibody, and 20µl of coated beads were used for IP. In this case the coating procedure lasted over 60 instead for 30min, and the sample volume was reduced to 300µl. The other experimental conditions were kept constant, except Triton X-100 was used instead of Tween-20).

Recognition of the IL-8 antibody in SN1 and of IL-8 in SN2 demonstrates (Fig. 24a) that neither the antibody coating onto the beads nor the IP of IL-8 was carried out completely. However, IL-8 was completely eluted from the beads in the first elution step (no bands in EL2).

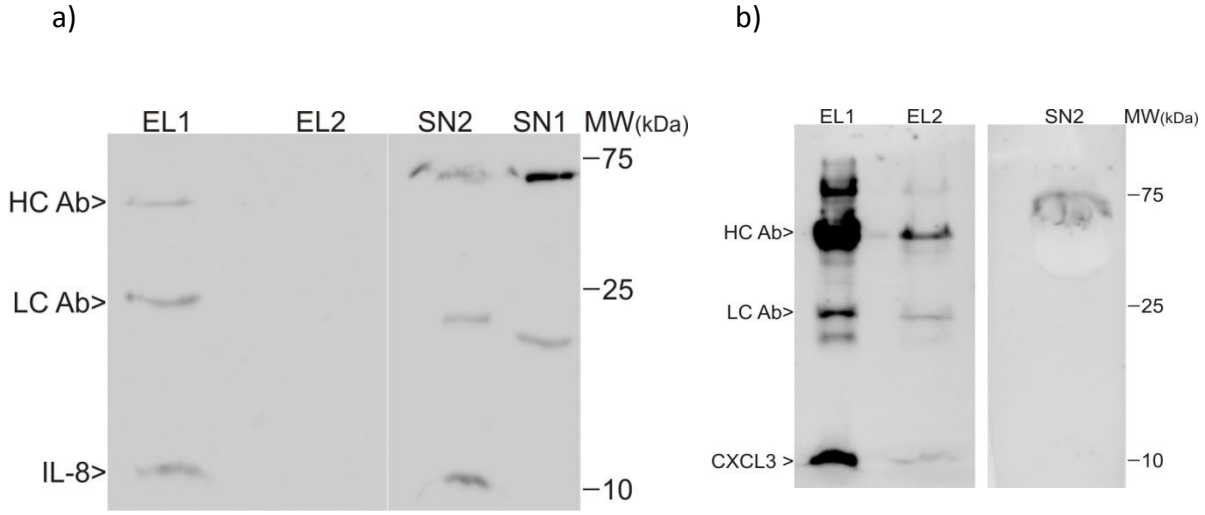


Figure 24. *Western blot analysis targeting IL-8 and CXCL3 8 in the supernatant after IP.*

IP was performed using 1% Triton X-100 and 150mM NaCl; Other conditions: 50µl beads coated with 10µg antibody (maximum capacity), 20µl of coated beads used for IP.

With CXCL3 the outcome was even better, as the entire amount of CXCL3 could be immunoprecipitated, as indicated by the absence of a CXCL3 specific band (11kDa) in SN2. In the case of CXCL3 the antigen – antibody interaction seems to be strong. Under the chosen elution conditions, not all CXCL3 was eluted by the first elution step (EL1) as indicated by the presence of a fainty CXCL3 band in EL2. In EL1 a number of other bands can be seen in addition to CXCL3, probably originating from antibodys' heavy and light chains. The band at 75kDa was also found before in a non immunoprecipitated sample and might originate from a complex of CXCL3 with an abundant protein present in the samples which is then co-immunoprecipitated or from non-specific interactions of the anti-CXCL3-Ab. However, it does not negatively affect the completeness of the IP of CXCL3.

Triton X-100 is known as detergent that counteracts weak protein–protein interaction better than other nonionic detergents. It seems that in the case of CXCL3 the strong interaction of CXCL3 with the Ab is not interrupted by Triton X-100 whereas unspecific interactions of the Ab with other proteins are minimized.

In an effort to coat 5 µg of anti-cytokine antibody to 50 µl of beads, Tween-20 was added to the antibody solution in the Binding & Washing Buffer (to a final concentration of 0.1%). This was aimed to facilitate beads mixing, to preserve non-covalent protein-protein interactions and to reduce non-specific interactions. Additionally, 30µl of a 2% BSA solution was added for quenching non specific binding onto the coated beads. Fig. 25 shows the corresponding WB results. The fact that no band is detected in SN1 (with both targeted proteins IL-8 and CXCL3) indicates that in both cases the entire amount of antibodies was bound onto the beads. However, even though IL-8 as well as CXCL3 specific bands were detected in SN2, indicating incomplete IP. This outcome can be a consequence of too low antibody amounts immobilized, or of the use of an inappropriate detergent (Twenn-20) and/or omitting NaCl. The absence of any CXCL3 band in EL2 indicates the (for the first time) complete elution which was achieved by using three times more µl of Elution Buffer (60µl) than µl of coated beads used for IP.

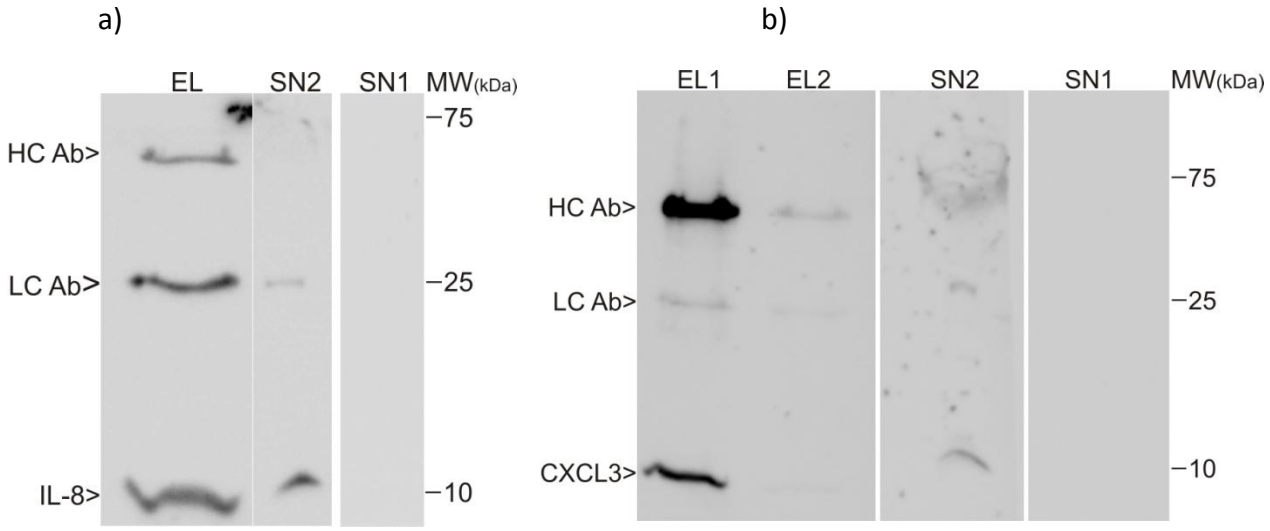
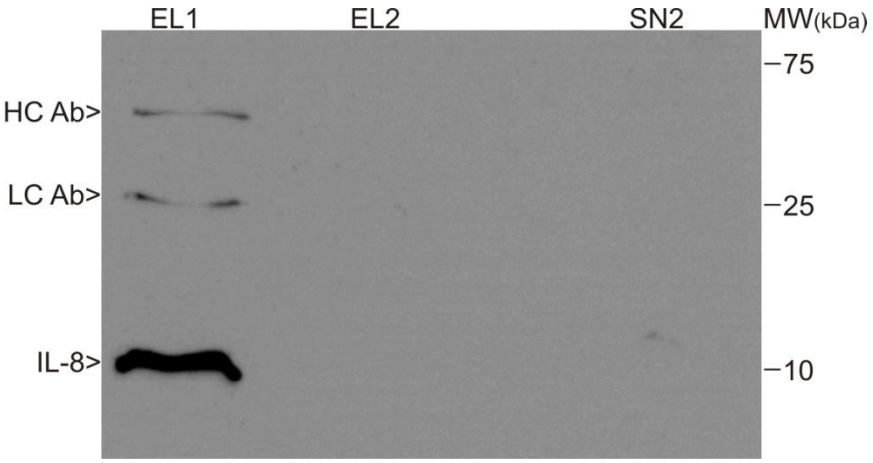


Figure 25.*Western blot analysis targeting (a) IL-8 and (b) CXCL3.*
Coating was made under addition of 0.1% Tween-20 in Binding & Washing buffer and samples were supplemented with Tween-20 (0,1% final concentration) and 30µl of 2% BSA . Other conditions: 50µl beads coated with 5µg antibody, 20µl of beads (containing 2µg antibody) used.

- *Influence of incubation time for IP*

Carrying out the IP with rotation at 4°C overnight (under otherwise identical conditions) and an almost complete IP of IL-8 could be attained (Fig. 26).
IP lasting overnight was performed also with other antibodies tested here, i.e., TSG6 (Fig. 26b) and Lumican (data not shown). In both cases the antibodies were coated successfully onto the beads (indicated by the low HC/LC band intensity in SN1), and complete IP was achieved (as indicated by the absence of target protein bands in SN2). The absence of Lumican and TSG6 was corroborated by MS data using a “shot-gun proteomics” related methodology.

(a)



(b)

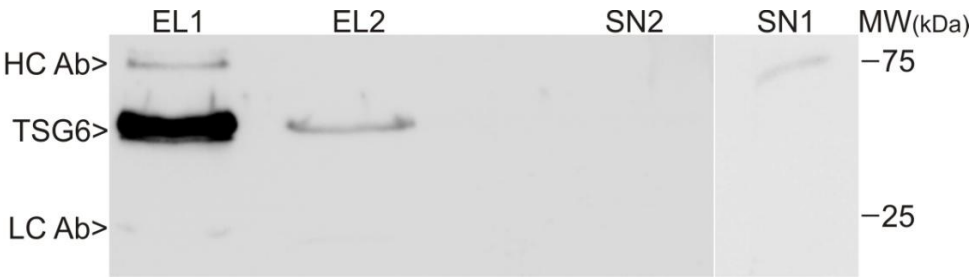


Figure 26. *Western blot analysis of (a) IL-8 and (b) TSG6.*
IP was carried out over night at 4°C; other conditions identical to those described in Figure 15

- *Sources of loosing analytes during IP*

The entire IP procedure includes several steps: (-) coating of beads with the selected antibody targeting the protein, (-) incubation with sample for antigen capture, and finally (-) elution of the captured antigen. In between there are included many washing and pipetting steps which together with inappropriate incubation conditions could lead to various losses: loss of beads (i.e., before and after coating; before and after IP); loss of antibodies (i.e., by preparing the antibody solution; by sub-optimal coating conditions); and loss of targeted protein (i.e., during washing steps; not optimum IP and elution conditions).

Until now, our investigations were oriented primarily on bringing the IP to completeness. The information received from the absence of target protein bands in SN2 is sufficient for evaluating the completeness as no sample treatment steps other than incubating the sample with coated beads and transferring the SN2 in e new vial are included. In this section we consider the loss of target protein after capture on beads and it can happen by washing steps and by incomplete elution of the captured antigen. That the elution conditions were already optimized was demonstrated by the absence of bands after second elution in several experiments. The loss of the target protein by washing steps (*i.e.,* after IP and before elution) is discussed here.

The usual procedure includes washing of the beads after IP for three times with 4 fold higher volume of Washing Buffer than µl of coated beads are used for IP. Fig. 27 (right-site) shows that IL-6 is actually present in the Washing Buffer used for the first washing step already when loading only 20% (40µl) of the used Washing Buffer volume onto the WB gel.

When considering potential loss of target protein during sample pre-cleaning steps, we pre-incubated 300µl of sample with 20µl of beads. Then, the beads were eluted with 40µl Elution Buffer and this volume was loaded to WB analysis. Fig. 27 (left site) shows an IL-6 band with low intensity indicating some loss of antigen during pre-cleaning. The low intensity of this band, however, implicate that more antigen is lost by washing steps, particularly if these steps will be carried out repeatedly.

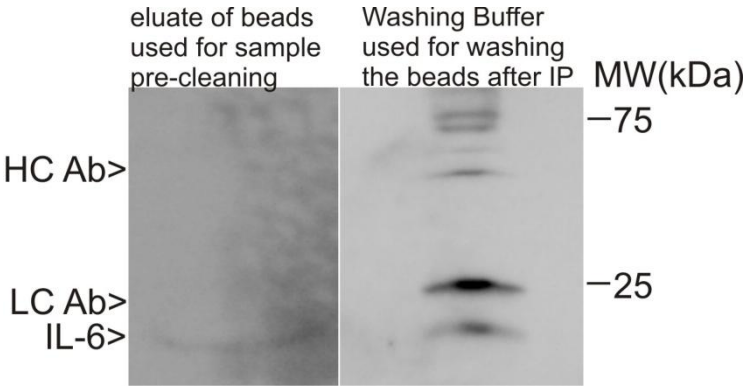


Figure 27. *Western blot analysis of IL-6 in bead eluate and in washing buffer after IP.*
Left: Eluate from beads used for sample pre-cleaning ;
Right: Washing Buffer eluted from the beads after IP (single washing step).

- *Influence of the composition of the binding buffer*

The exact composition of the Binding & Washing Buffer obtained from Invitrogen was not defined within the protocol provided with the kit, the only given information was that it is PBS. In an effort to optimize the buffer solution for coating the antibodies onto the beads, we used a procedure where the Binding & Washing Buffer PBS was supplemented with 0.01%; 0.05% and 0.1% Tween-20 whereas all the other IP conditions were not changed (*i.e.,* the sample was supplemented with 0.1% Tween-20 and 30µl BSA2%, all incubations times and washing steps remained the same.) The WB data in Fig. 28 indicate that a higher amount of antibody could be coated onto the beads when using PBS/0.05% Tween-20 buffer. No IL-8 band is recognized in SN2, in contrast to IP using PBS/0.01% and 0.1% Tween-20 Binding Buffer, where the IL-8 specific band can be detected with low intensity.

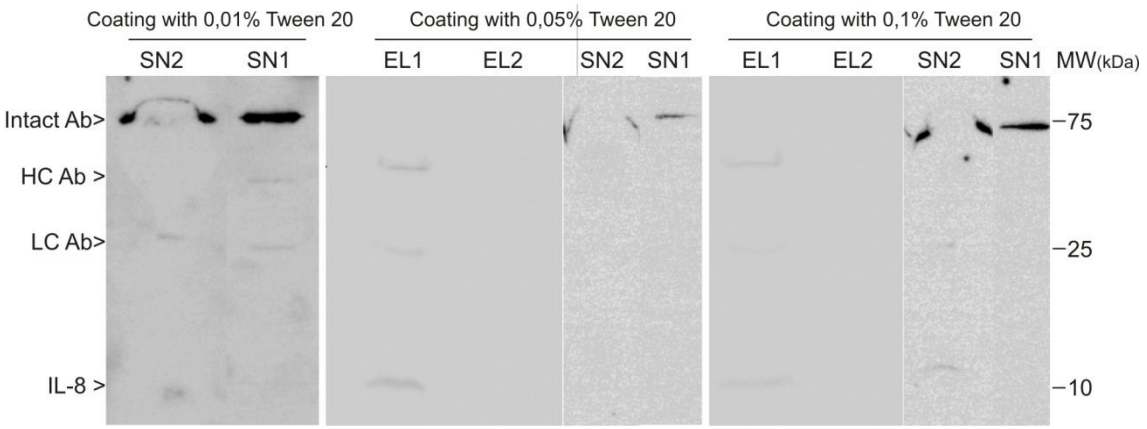


Figure 28. *Western blot analysis IP targeting IL-8 under different buffer compositions during coating. Coating was made using a Binding & Washing Buffer consisting of PBS supplemented with different concentrations of Tween 20 (final concentration 0,01%, 0,05 and 0,1%).*
Other conditions: After pre-cleaning, Tween 20 (0.1% final concentration) and 30µl BSA (2% final concentration) were added to the sample. 50µl beads were coated with 5µg antibody and 20µl of those were used for IP.

3.1.4 Conclusion regarding IP

IP was performed using different antibodies directed against various proteins. The IP conditions you should apply for a selected target protein are determined by the binding properties of the used antibodies. The optimum coating conditions are influenced by the species the antibody belongs to. During this study we used anti-IL-6 and anti-IL-8 antibodies from mice and anti-CXCL3-, anti-TSG6- and anti-Lumican antibodies from rabbits. Both types bind equally strong to protein G, thus the coating conditions were more or less the same for all antibodies with a maximum achieved binding capacity of 5µg antibodies coated onto 50µl beads (antibody solution supplemented with 0.1% Tween-20; Invitrogen buffer or PBS supplemented with 0.5% Tween-20; and incubation over 1h at RT under rotation.

It became obvious that the IP results are depending on the antibodies-antigen binding properties. We could show that with the anti-IL-6 antibody used complete IP could be achieved by using 50µl beads coated with 5µg antibody. With the anti-IL-8 antibody (exhibiting stronger antigen-antibody binding) just 2.5µg of the antibody was needed for attaining complete IP (using the same amount of beads and identical experimental conditions).

In addition, we could demonstrate that complete IP can be achieved with the smaller amount of antibody (2µg) coated onto 20µl beads only when incubation is carried out overnight and when adding Tween-20 and 2% BSA (facilitating bead mixing, preserving non covalent protein–protein interactions and quenching non-specific binding to the coated beads). The longer incubation time and the addition of detergent turned out as being

necessary when using the smaller volume of beads (20µl) and the lower amounts of antibodies (2 µg).

With antibodies exhibiting very good binding properties (i.e. CXCL3) complete IP of the antigen could be attained already after 2h incubation. In this case a higher amount of antibody was used and a stronger detergent could be added to the sample for avoiding non-specific binding to the coated beads. With antibodies exhibiting inappropriate binding to the target protein (e.g. TSG6;) IP will remain incomplete and non-selective.

3.2 Comparison of various methods for the quantitative determination of IL-8 in different matrices

In this chapter various protein analysis methods are tested for specific quantification of IL-8 in different matrixes and are evaluated with respect to specificity and reliability: the methods considered are (-) Western blot analysis (WB), (-) Luminex^{TM} assay and ELISA (Enzyme-Linked ImmunoSorbent) assay as three antibody based methods, and (-) shotgun proteomics as well as SIM as mass spectrometry based methods. SIM analysis were carried out without and with preceding immunoprecipitaton (IP). In this case, half of IP eluates were used for WB analysis, the other half for MRM. (In order to avoid losses of target protein the beads were not washed after IP.)

Eight different concentrations of recombinant IL-8 covering six orders of magnitude were spiked into three different matrices: 1) phosphate buffered saline (PBS); 2) PBS supplemented with 10% human serum and 3) supernatant of untreated human umbelical vein endothelial cells (HUVECs) grown in endothelial basal medium (EBM-2) supplemented with 10% fetal calf serum (FCS). Sample volumes and protein amounts used in the various test runs are listed in Table 1.

Table 1. IL-8 concentrations, sample volumes and amounts of IL-8 applied within the different test runs and methods mentioned.

Nr.	rec.IL-8 conc.	**WB (10µl)**	**IP (200µl)**	**Shotgun (10µl)**	**Luminex^{TM} (30µl)**	**ELISA (100µl)**
1	1 µg/ml	10ng	200ng	10ng	30ng	100ng
2	100 ng/ml	1ng	20	1ng	3ng	10ng
3	10 ng/ml	100pg	2ng	100pg	300pg	1ng
4	1 ng/ml	10pg	200pg	10pg	30pg	100pg
5	100 pg/ml	1pg	20pg	1pg	3pg	10pg
6	10 pg/ml	0,1pg	2pg	0,1pg	0,3pg	1pg
7	3.3 pg/ml	0,03pg	0,66pg	0,03pg	0,09	0,3pg
8	1.1 pg/ml	0,01pg	0,22pg	0,01pg	0,03	0,1pg

3.2.1 Western blot analysis and immunoprecipitation

WB samples were performed parallel for each tested matrix including the final chemiluminescence imaging. IP for the samples with different IL-8 concentrations within the same matrix were performed parallel as well and the beads coated in four coating experiments (50µl beads with 5µg antibodies) were pooled before use in IP. Experiments were performed with all IL-8 concentrations given in Table 1, though Fig. 29 shows only those samples in which the targeted IL-8 was detectable by both methods.

The WB analyses gave comparable results (band intensities) for the equally concentrated samples independent from the respective matrix, probably due to the electrophoretic separation process inherent to WB analysis. Interestingly, the results show comparable band intensities of non-IP-treated and IP-treated samples within the same matrix, and between IP-treated samples in different matrices.

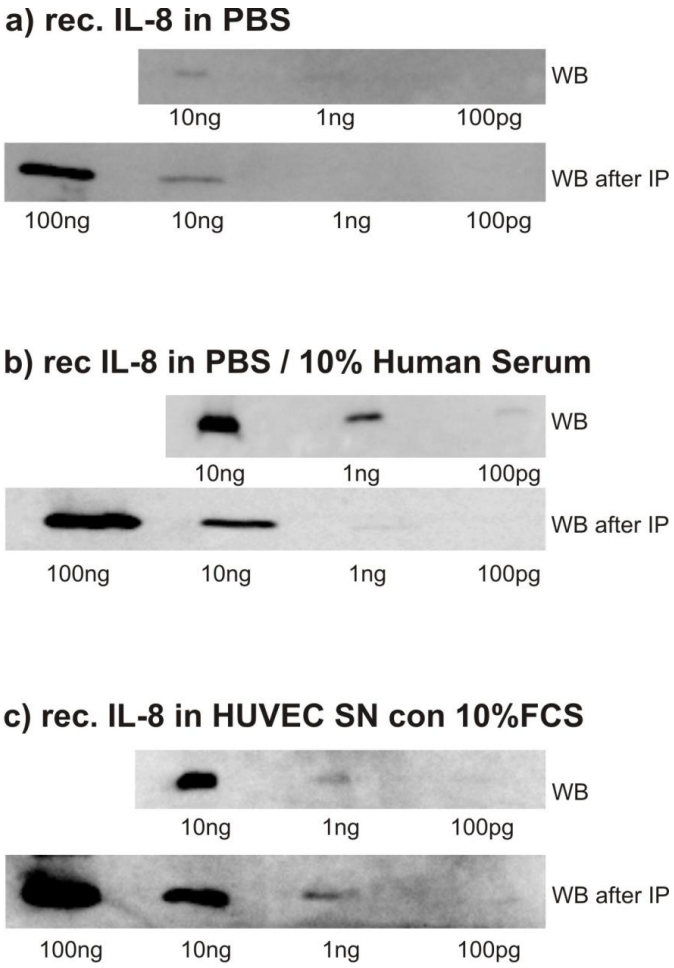


Figure 29. *WB analysis of rec. IL-8 spiked into different matrices for attaining concentrations ranging from 100pg to 100ng. Matrices: (a) PBS, (b) PBS with 10% human serum, (c) supernatant of untreated HUVECs with 10%FCS. WB data without IP and WB with preceding IP are given*.

The bands intensities as determined by the ImageQuant5.0 software are shown in Fig. 30. The data obtained without IP are given in the first row, those with preceding IP in the second one. The band ratios are normalized by to the theoretical (expected) amount of IL-8. The agreement of the results obtained without and with IP is excellent, especially at measurements in HUVEC supernatant. Only the IP-WB sample in PBS shows much higher band intensity than non-IP one. With some samples, LLODs of 100pg could be obtained by both, the WB and the IP-WB methods.

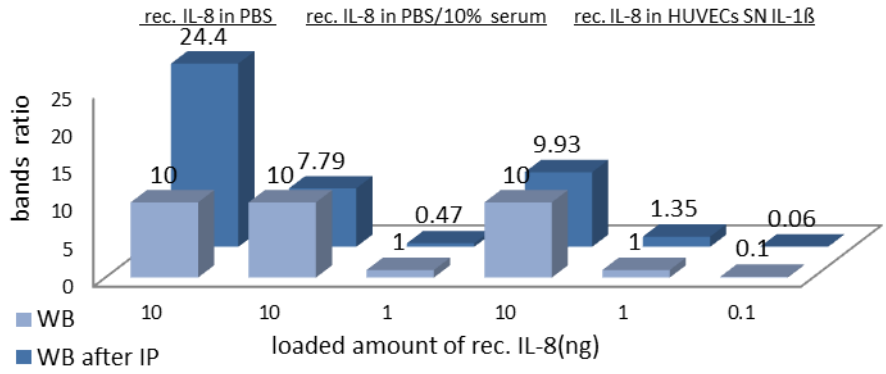


Figure 30. *WB data of IL-8 samples without preceding IP (first row) and without IP (second row) as determined by the ImageQuant 5.0 software. The band ratio was normalized to the band intensities obtained by direct Western blotting.*

When comparing the band intensities of samples containing a lower and a higher amount of IL-8 as determined by ImageQuant 5.0, a saturation effect is found for the 100ng band at all tested matrices with the IP samples within the same matrix (Fig. 31). This means that there is a maximum capability for the IP of a targeted protein under certain IP conditions. Using the HUVECs supernatant samples as an example, the maximal amount of IL-8 that can be immunoprecipitated under the chosen conditions is about 17ng.

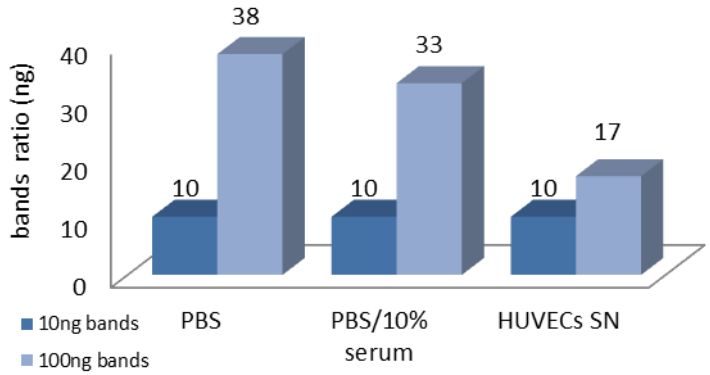


Figure 31*. Band ratios of IP samples containing IL-8 amounts of 10ng and 100ng, respectively, within different matrices. A matrix dependent saturation effect of IP is demonstrated.*

3.2.2 Luminex™ -Assay

All measurements by the Luminex™ assay were performed in triplicates for each concentration and each matrix.

- *calibration curve in Luminex buffer*

Standards were reconstituted by diluting recombinant IL-8 with Luminex buffer, the standard calibration curve (Fig. 32) shows approximate linearity only up to 100 - 200 pg/ml, above 2000 pg/ml the curve approaches the saturation level.

Tabel 2. Standard curve within the concentration range 0 - 1111pg/ml as measured with the Luminex assay.

Expected Concentration (pg/ml)	MFI (1)	MFI (2)	MFI (3)	Average of determined concentrations (pg/ml)	%RSD
1111	10887,5	11271	12008	1115,51	9,42
370	5383.5	6119.5	6300	379,76	12,13
123	2625.5	2617.5	2846	123,22	6,63
41,5	1246.5	1237.5	1105.5	40,87	8,93
13,7	574	655	512	14,88	17,95
4,5	290.5	257	245.5	4,24	16,09
1,5	178.5	187.5	152.5	1,77	24,88
0	83.5	140.5	101.5	0,62	88,96

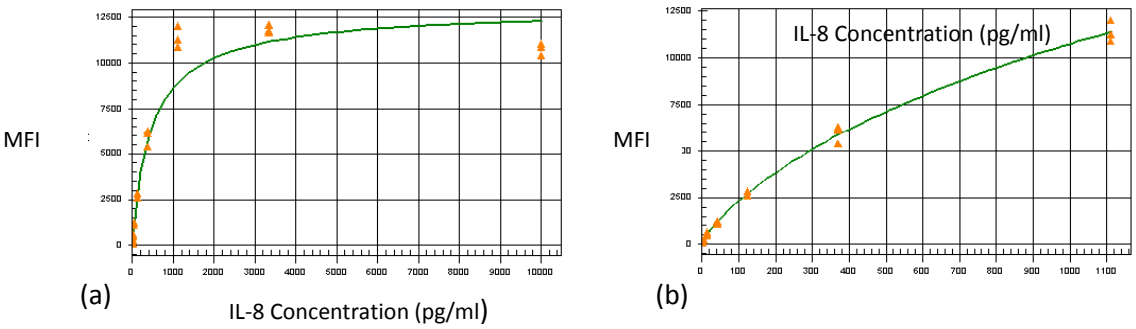


Figure 32. *Standard calibration curves of Luminex™: (a) curve covering the entire concentration range of IL-8 standards; (b) standard curve in the concentration range < 1111pg/ml. The series of standard concentrations were made using Luminex buffer.*

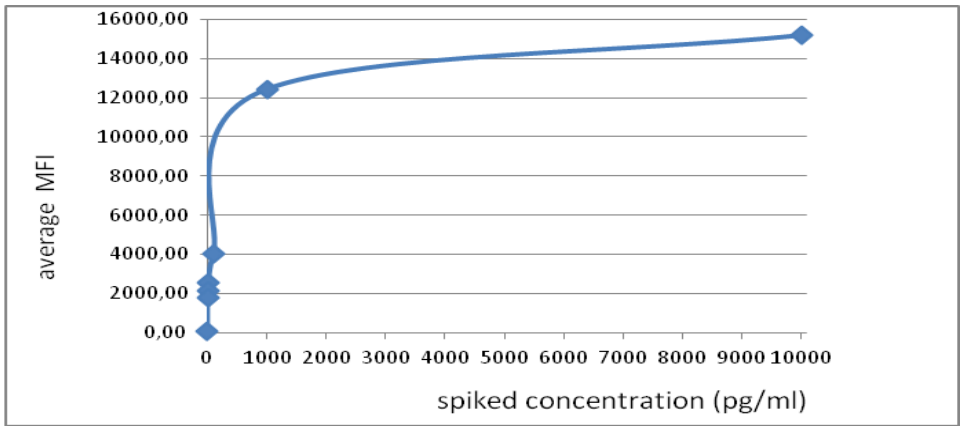
- *sample data in PBS/10% human serum matrix*

When measuring IL-8 in PBS/10% human serum matrix (Figs. 33, 34 and Table 3) far too high IL-8 concentrations were assessed, particularly at concentrations lower than 100pg/ml, and saturation appears already above a concentration of 100pg/ml.

Table 3. Luminex assay data for recombinant IL-8 measured in PBS/10% human serum.

Expected Concentration (pg/ml)	MFI (1)	MFI (2)	MFI (3)	Average of determined concentrations (pg/ml)	%RSD
$1*10^4$	15706	15758.5	14063.5	>1111	
$1*10^3$	12980	12249	12036	>1111	
100	3907.5	4025.5	4109	178,9	4,28
10	2401	2731.5	2531	114,4	8,93
3	2117	2187	2128	90	2,39
1	1804	1850	1740	70,9	4,15
0	40,5	46	47,5	<0	

(a)



(b)

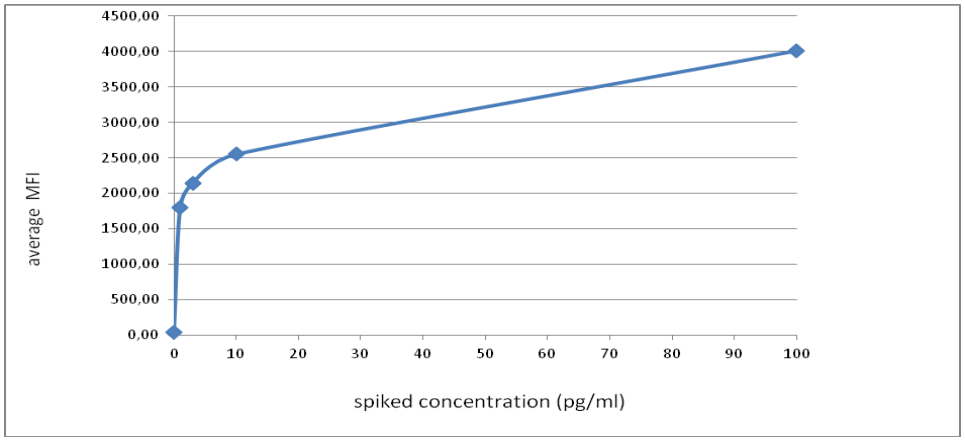


Figure 33. *MFI curve of rec. IL-8 spiked to PBS/ 10% human serum covering the concentration ranges of (a) 0 – 10.000pg/ml; and (b) 0 – 100pg/ml.*

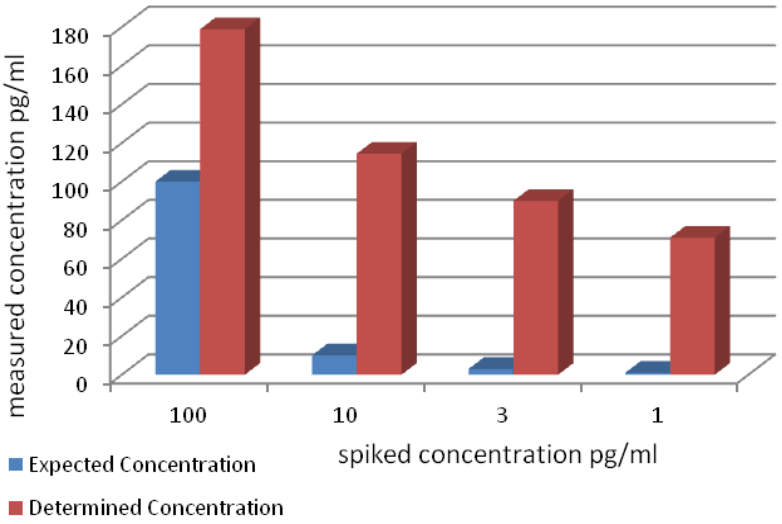


Figure 34. *Comparison of "true" spiked amounts of IL-8 with the values assessed by means of the Luminex™ assay when measured in PBS supplemented with 10% human serum.*

- sample data in HUVECs supernatant / 10% FCS

Table 4. Luminex assay data for recombinant IL-8 measured in HUVEC SN/10% FCS.

Expected Concentration (pg/ml)	MFI (1)	MFI (2)	MFI (3)	Average of determined concentrations (pg/ml)
$1*10^4$	15108.5	16075.5	16003.5	>1111
$1*10^3$	15003.5	15302.5	14727	>1111
100	14854.5	14992	14960	>1111
10	14604.5	16172.5	15180	>1111
3	15183	16298	14793	>1111
1	15230.5	15139.5	14909	>1111
0	16537.5	14651	13326.5	>1111

When carrying out the measurements in HUVECs supernatant / 10% FCS (Table 4) saturation is found over the entire range of tested concentrations (1pg/ml to 10ng/ml).
In toto, the results obtained for IL-8 in PBS/10% human serum and in HUVECs supernatant gave indications for significant cross-reactivity of the Luminex assay leading to false positive results and to saturation effects at concentration lower as expected.
However, even the determination of IL-8 in neat PBS (data not show) did not correspond with the expected values. Obviously, this observed bias is caused by conditions inappropriate for the Luminex assay. One can speculate whether the presence of carrier proteins in PBS might be benefitial for avoiding unspecific interaction of the IL-8 with parts of the equipment.

3.2.3 ELISA

ELISA tests were performed using the ELISA kit from RayBio. According to the kit protocol, this kit should be appropriate for measurements of serum and cell supernatant samples. All standards and samples were incubated overnight at 4°C and measured in triplicate.

The series of calibration standards was prepared using Assay Diluent A, and the corresponding calibration curve showed linearity up to a concentration of 100pg/ml (Fig. 35 and Table 5). According to the kit protocol the upper limit of linearity should be at 600pg/ml. ELISA tests were performed with undiluted and diluted samples.

The data for IL-8 in neat PBS samples showed a rather high scatter similar to those observed with the Luminex™ assay (Table 6). Undiluted sample of IL-8 in PBS/10% human serum and HUVECs supernatant /10% FCS showed saturation at all concentrations within the given range (data not show). When diluting these samples (Fig. 36 and Table 7 for PBS/10% human serum; Fig. 37 and Table 8 for HUVECs supernatant /10%FCS) with a ratio of 1:10 and 1:100, an overestimation of the IL-8 concentration was observed and saturation effects took place at concentrations higher than 100pg/ml with both matrices.

Taken together, the ELISA shows cross reactivity with sample matrix components even in diluted sample, leading to an overestimation of concentrations and/or false positive results. Saturation affect appear earlier than expected. With all measurements excellent reproducibility values of lower than 10 %RSD (CV) were obtained (as expected according to the kit protocol), except the measurements in neat PBS.

Table 5. Standard calibration data with the used IL-8 specific ELISA

Standard concentration (pg/ml)	OD_{450nm} (1)	OD_{450nm} (2)	OD_{450nm} (3)	Average OD_{450nm}	Average (OD_{450nm}) – zero value (OD_{450nm})	%RSD
600	2,497	2,675	2,867	2,680	2,624	6,91
200	2,761	2,853	3,003	2,872	2,817	4,25
66,7	1,825	1,832	1,781	1,813	1,757	1,53
22,2	0,725	0,744	0,717	0,729	0,673	1,90
7,4	0,291	0,283	0,289	0,288	0,232	1,45
2,5	0,132	0,138	0,149	0,140	0,084	6,17
0,8	0,08	0,069	0,08	0,076	0,021	8,32
0	0,052	0,057	0,058	0,056	0,000	5,77

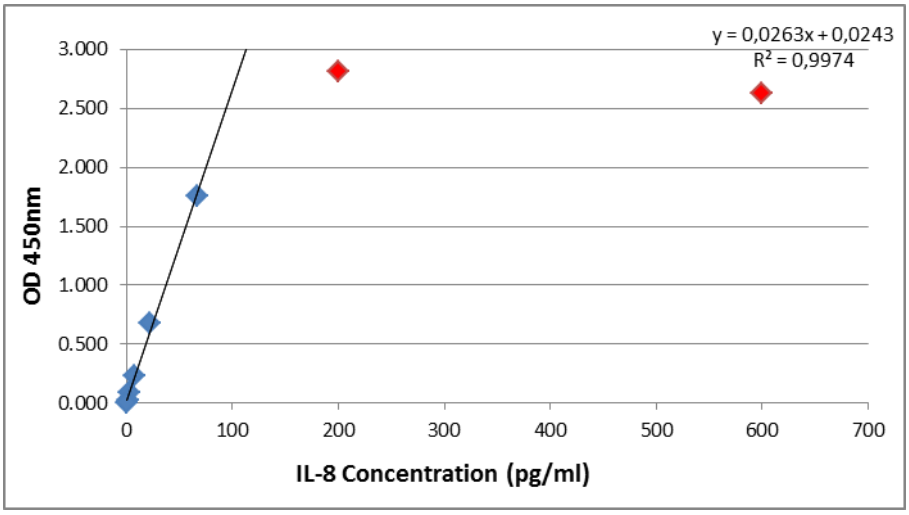


Figure 35. *Standard calibration curve with the used IL-8 specific ELISA.*

Table 6. ELISA data for recombinant IL-8 measured in PBS

Expected concentration (pg/ml)	OD_{450nm} (1)	OD_{450nm} (2)	OD_{450nm} (3)	Average OD_{450nm}	Average (OD_{450nm}) – zero value (OD_{450nm})	Average of determined concentrations (pg/ml)	%RSD
$1*10^6$	2,549	3,042	2,808	2,800	2,702	101,80	9,67
$1*10^5$	3,151	3,444	3,638	3,411	3,313	126,38	8,94
$1*10^4$	1,855	1,894	2,191	1,980	1,882	71,97	12,65
$1*10^3$	0,31	0,312	0,405	0,342	0,244	9,70	43,00
100	0,131	0,145	0,146	0,141	0,043	2,03	116,76
10	0,388	0,404	0,363	0,385	0,287	11,32	13,77
3	0,33	0,351	0,28	0,320	0,222	8,86	12,98
1	0,472	0,497	0,435	0,468	0,370	14,48	9,47
o	0,107	0,082	0,105	0,098	0,000		

Table 7 ELISA data for recombinant IL-8 measured in PBS/10% human serum, 1:10 diluted.

Expected concentration (pg/ml)	OD_{450nm} (1)	OD_{450nm} (2)	OD_{450nm} (3)	Average OD_{450nm}	Average (OD_{450nm}) – zero value (OD_{450nm})	Average of determined concentrations (pg/ml)	%RSD
$1*10^5$	3,327	3,394	3,498	3,406	3,301	124,58	3,63
$1*10^4$	3,762	3,655	3,654	3,690	3,585	135,38	1,63
$1*10^3$	3,47	3,479	3,473	3,474	3,368	127,15	0,54
100	3,326	3,342	3,388	3,352	3,246	122,51	0,93
10	1,596	1,608	1,688	1,631	1,525	57,06	2,99
1	0,961	0,962	1,002	0,975	0,869	32,13	2,26
o	0,112	0,09	0,115	0,106	0,000		

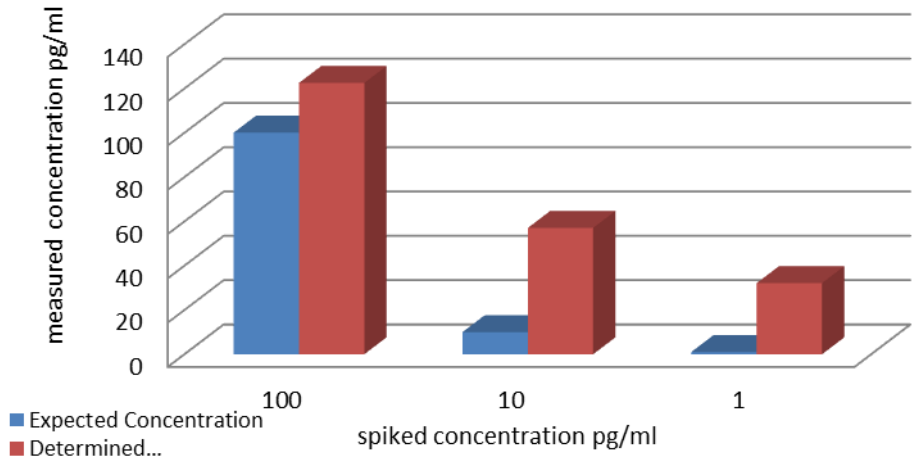


Figure 36. *Comparison of "true" spiked amounts of IL-8 with the values assessed by means of the IL-8 specific ELISA when measured in PBS supplemented with 10% human serum, 1:10 diluted.*

Table 8. ELISA data for recombinant IL-8 measured in HUVEC SN/10% human serum, 1:100 diluted

Expected concentration (pg/ml)	OD_{450nm} (1)	OD_{450nm} (2)	OD_{450nm} (3)	Average OD_{450nm}	Average (OD_{450nm}) – zero value (OD_{450nm})	Average of determined concentrations (pg/ml)	%RSD
$1*10^4$	3,435	3,448	3,561	3,481	1,426	53,28	7,18
$1*10^3$	3,68	3,755	3,782	3,739	1,683	63,08	3,40
100	3,469	3,49	3,52	3,493	1,437	53,73	5,25
10	2,371	2,508	2,365	2,415	0,359	12,73	14,00
1	1,934	1,994	2,023	1,984	-0,072	-3,66	-62,99
o	1,973	2,136	2,058	2,056	0,000		

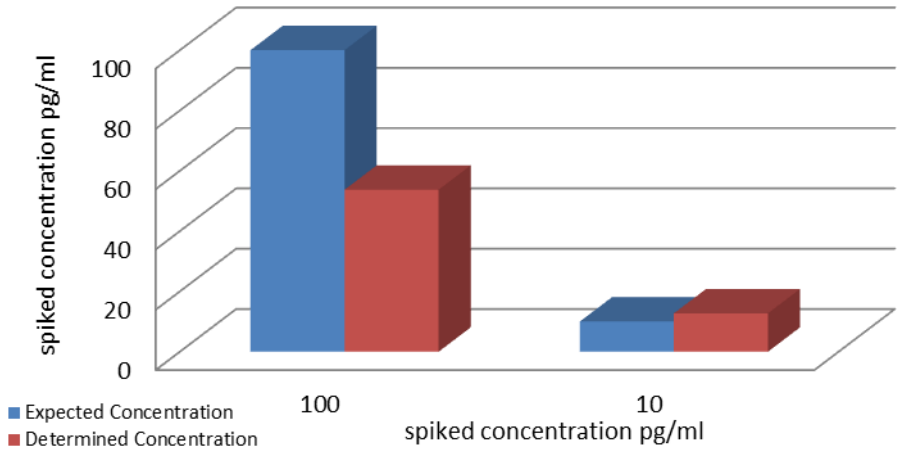


Figure 37. *Comparison of "true" spiked amounts of IL-8 with the values assessed by means of the IL-8 specific ELISA when measured in supernatant of HUVECs supplemented with 10% FCS, 1:100 diluted.*

3.2.4 Shot-gun proteomics

The methodology for protein quantification by shotgun proteomics is described in the introductory part, including an overview over the various other methods employed. This approach includes severel steps of sample preparation before the MS spectra can be obtained: i.e., protein separation by 1D SDS-PAGE and silver staining (Figure 38), cutting of the gel lanes into slices and in-gel tryptic digestion of the proteins present in these pieces, extraction of the peptides and, as final step, separation of peptides by means of nano-flow RP-HPLC followed by nano-ESI-MS analysis and assessment of the MS and the MS/MS fragmentation spectra for identification and quantification.

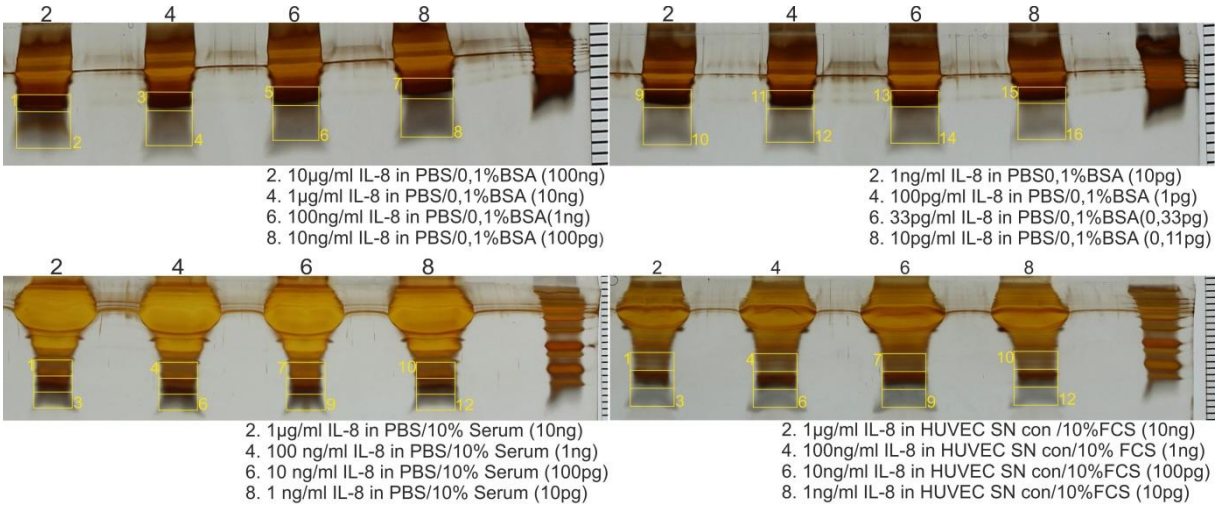


Figure 38. *SDS-PAGE gels photos of shotgun experiments after silver staining. For MS analysis are taken only the lanes part under 25 kDa: at PBS/0,1% BSA samples were divided in two and at serum and HUVEC samples in three slices, as it is shown with numbered yellow rectangles. 10µl of sample were loaded and theoretical corresponding IL-8 amount at each concentration is given in brackets.*

When handling with sample higher compleity, such as serum or plasma where thousands of proteins are presented at very different concentration ranges covering more than 10 orders of magnitude the sample pretreatment protocol has to be considered. Proteins with higher molecular weight gives higher numbers of peptides for mass spectrometric analysis after enzymatic digestion, which can lead to an underrepresentation of proteins with low molecular weight at MS-analysis. This has to be carefully considered by performing the shotgun approach as data dependent analysis, in order to achieve higher proteome coverage. Protein separation according to the molecular weight by 1D SDS-PAGE helps to reduce this problem. After protein digestion, the peptides are separated by RP-HPLC, an additional step for reducing sample complexity. However, sample complexity is still high as demonstrated in the mass-retention time plot of detected peptide ions in one HPLS-MS run of a single digested gel slice from a HUVEC SN sample (Figure 39). The larger number of different proteins in large concentration ranges make the identification and especially

quantification of proteins very challenging, as is demonstrated by the base peak chromatogram (Figure 40).

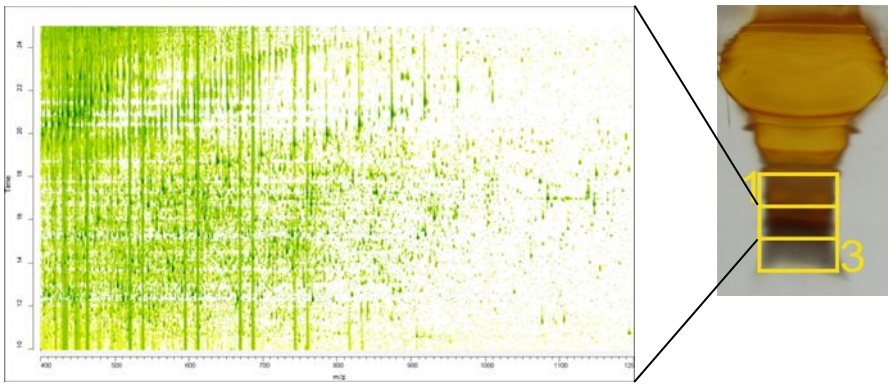


Figure 39. *Mass vs. retention time plot showing the detected peptiede ions in one HPLS-MS run of a singel gel slice from HUVEC SN sample analysed by MaxQuant software, demonstrating the sample coplexity and the recovering power of used MS instrument, Q Exactive.*

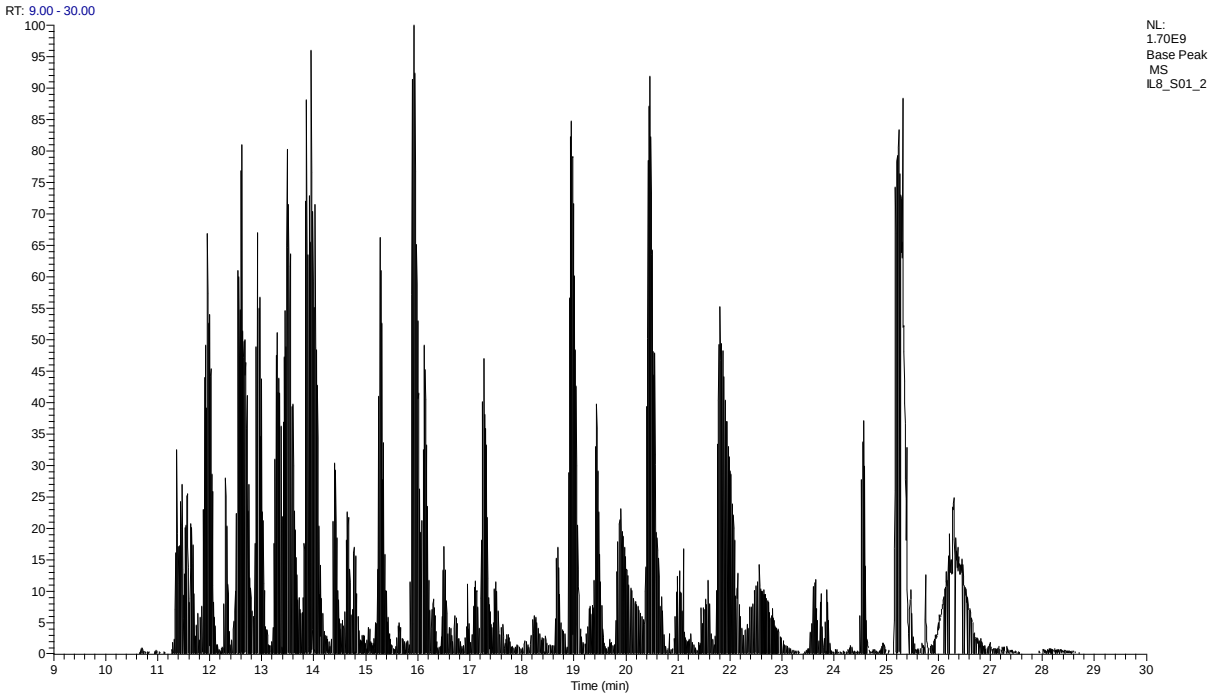


Figure 40. *A base peak chromatogram of a HPLC-MS run of a single gel slice from a HUVEC SN sample, presenting the relative abundance of peptides in the sample.*

The shotgun approach was performed using the top 6 method, a data dependent method, where the six relatively highest ions obtained from full scan MS were chosen for fragmentation and peptide identification (Figure 41). The MS/MS data analysis and protein

identification was done using Proteome Discoverer 1.3 (Thermo) employing Mascot and Sequest search algorithms and searching against the Uniprot human database (04/2012).

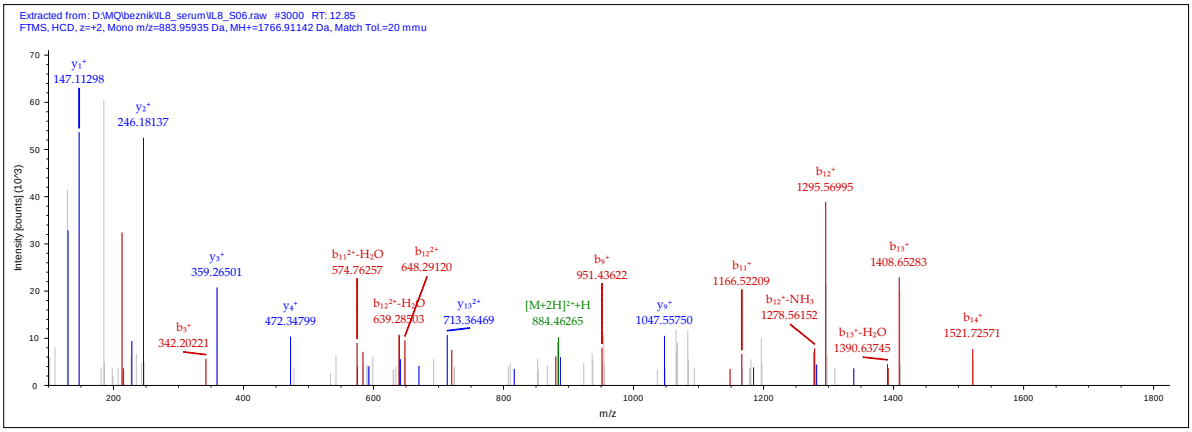


Figure 41. *The MS /MS spectrum identifying the peptide VIESGPHCANTEIIVK (883.95868 m/z), originating from IL-8 peptide. A high number of selected fragment ions (color coded) was classified to the IL-8 peptide, indicating identification with high reliability.*

The quantification of an identified protein relied on the MS data considering all identified different peptides per protein was based on the calculation of peak ares of the extracted ion chromatograms (Figure 42) of all identified peptides.

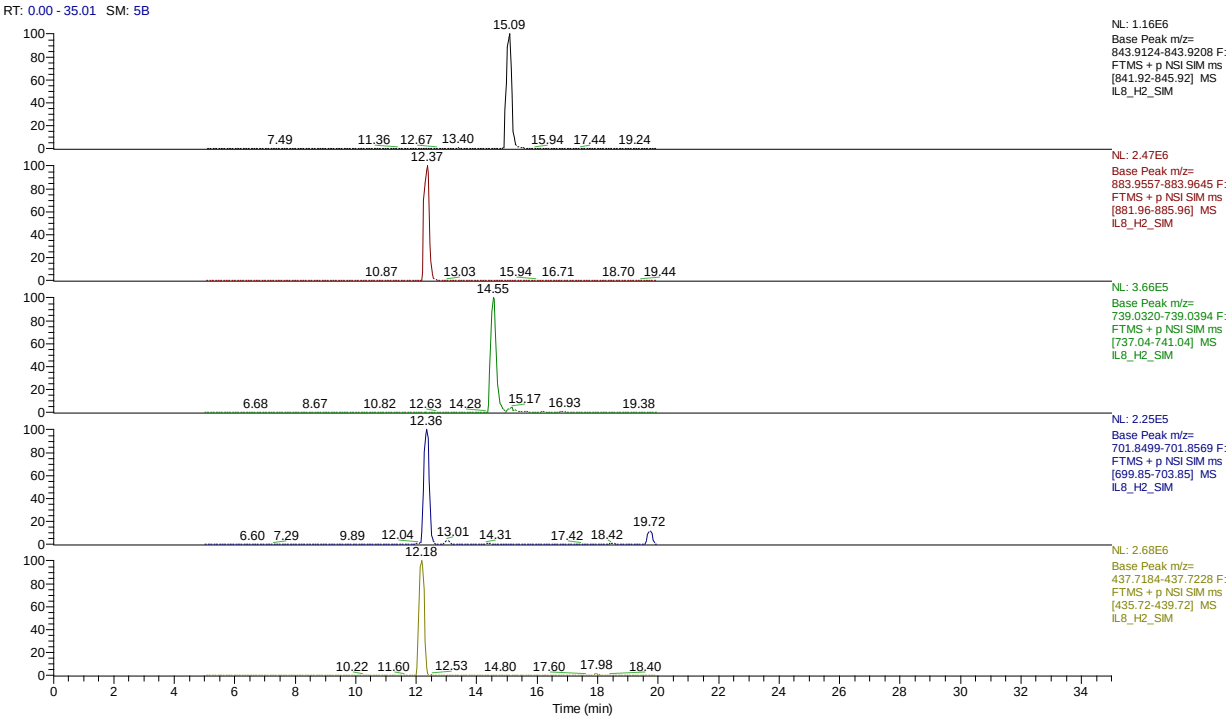


Figure 42. *Extracted ion chromatograms used for quantification of identified peptides based on calculation of peak area.*

The shotgun analysis was applied only for non-immunoprecipitated samples, using the ``top 6 `` data dependent method, and the MS analysis was done in duplicate by loading each time only a quarter of the available sample amount. The residual amount of sample was used for targeted proteomics (SIM), discussed in the next section.

For the quantification of IL-8 protein, the MS data from all gel slices(accumulating for all identified IL-8 peptides) were analyzed using MaxQuant software (Version 1.3), which allowed generation of quantitative data by consideration of three dimensions: peak intensity, m/z value and retention time (as visualized in Figure 39).

The experimental results are depicted in comparison to the expected ratio of IL-8 concentration within a matrix, (i.e., the expected value in the ideal case by calculation based on the values obtained with the highest IL-8 concentrations). In the y- axis the measured ratio are given, again in comparison to the values obtained with highest IL-8 concentration.

The results presented in Figure 43 show a very good agreement between measured and calculated values, espetially in case of serum samples, which are expected to present a much more complex matrix in comparison to HUVEC supernatant. Interestingly, LC identified a comparable number around 300 different proteins in both matrices. MaxQuant analysis calculated very low l p-values (< 0.01) for the reliability of quantitative data and revealed that the duplicates performed gave almost identical results. As a quite short (15min) gradient was used for the HPLC separation, the robustness and accuracy of data was better than otherwise expected for the shotgun approach. This indicates the robustness and selectivity of the presently employed MS analysis even when measuring in complex matrices.

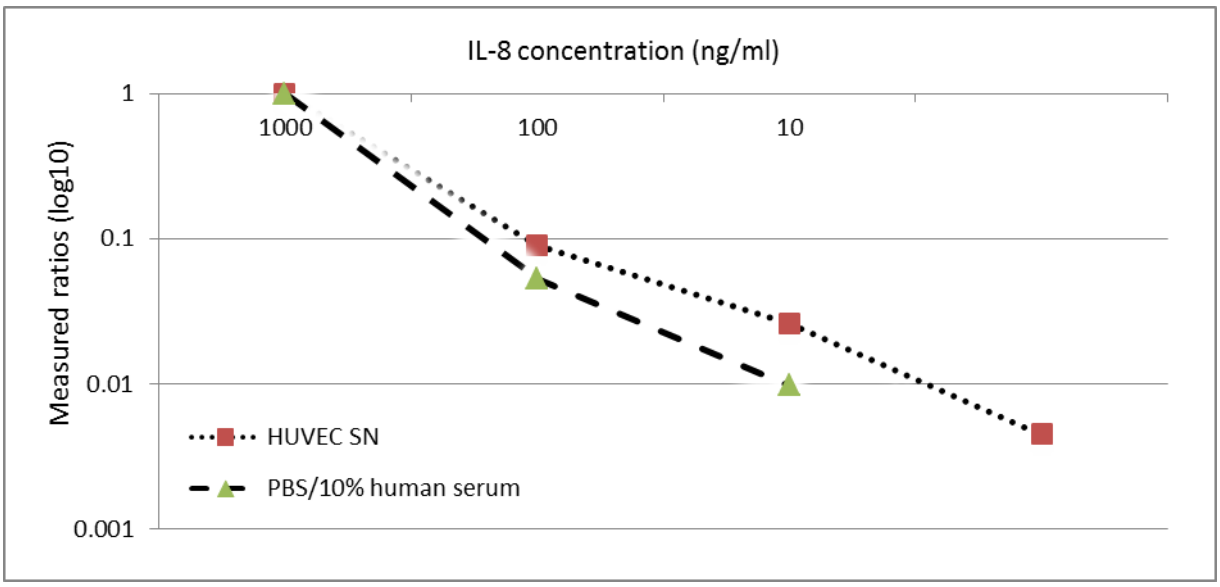


Figure 43. *The MS-shotgun results of IL-8 spiked into HUVEC SN and PBS/10% human serum. Calculated IL-8 concentrations were compared to the measured values normalized against the value obtained with 1µg/ml IL-8.*

3.2.5 Selected Ion Monitoring (SIM)

Sample preparation steps prior to SIM analysis were identical to those for the shotgun approach (i.e., protein separation by 1D-PAGE, silver staining and tryptic digestion of proteins).

With one half of the volume of immunoprecipitated samples we performed WB, whereas the residual sample volume was used for performing SIM analysis. In comparison to the non-immunoprecipitated samples, the sample IP were shortly separated on a gel by electrophoresis and was divided into two slices (Figure 44). Within these two slices subjected the gel part below Mw of 25 kDa to analysis, IL-8 was always identified in the upper one but not present any more in the lower one. Therefore, for quantification only the single IL-8 positive slice was used.

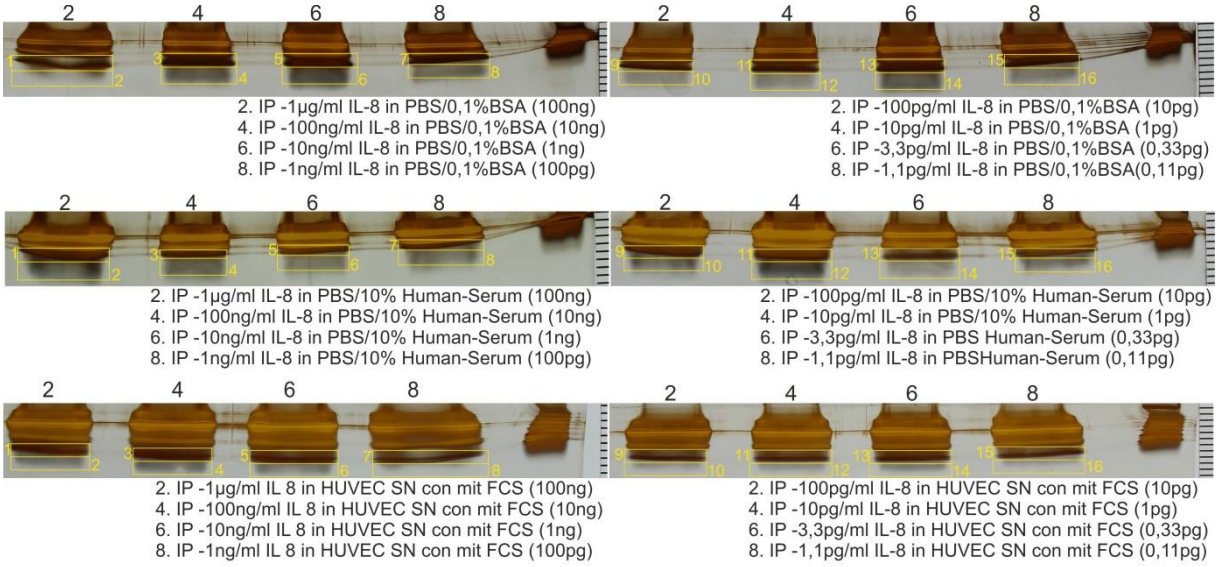


Figure 44. *SDS-PAGE gels of immunoprecipitated samples after silver staining. For SIM-MS analysis only the part of the lanes below 25 kDa was used. This part was divided in two slices as shown with numbered yellow rectangles. 100µl of the original sample were loaded and the theoretically corresponding IL-8 amount for each concentration is noted in brackets.*

The LS-MS analysis was performed under the identical gradient conditions as chosen before for the shot-gun experiments, while the mass spectrometer was operated in the SIM mode. Immunoprecipitated samples were measured in triplicate using each time only a quarter of the sample. The statistical significance of all SIM measurements was characterized by p-

values less than 0.01. Samples processed directly without IP were measured in duplicate and even in single measurements when dealing with PBS/0.1%BSA and PBS/10% human serum, HUVEC SN, respectively.

The ability of the IP protocol to attain complete immunoprecipitation of IL-8 independent from the matrices (at concentration higher then 10ng/ml) in all tested matrices is demonstrated in Figure 45 by showing a high agreement of theoretically expected ratios compared to the measured ones. Further, a good correlation even at higher concentrations (i.e., 1 000 and 100ng/ml) illustrates the power and the dynamic range (4-5 orders of magnitude) of the IP protocol for capturing even higher amounts of IL-8 (100ng at highest concentration) and of the SIM approach. A saturation effect within IP at high concentrations as obtained by WB analysis (see section 3.2.1) was not observed in case of SIM.This finding demonstrates the superior specificity and robustness of mass spectrometry compared to antibody based methods.

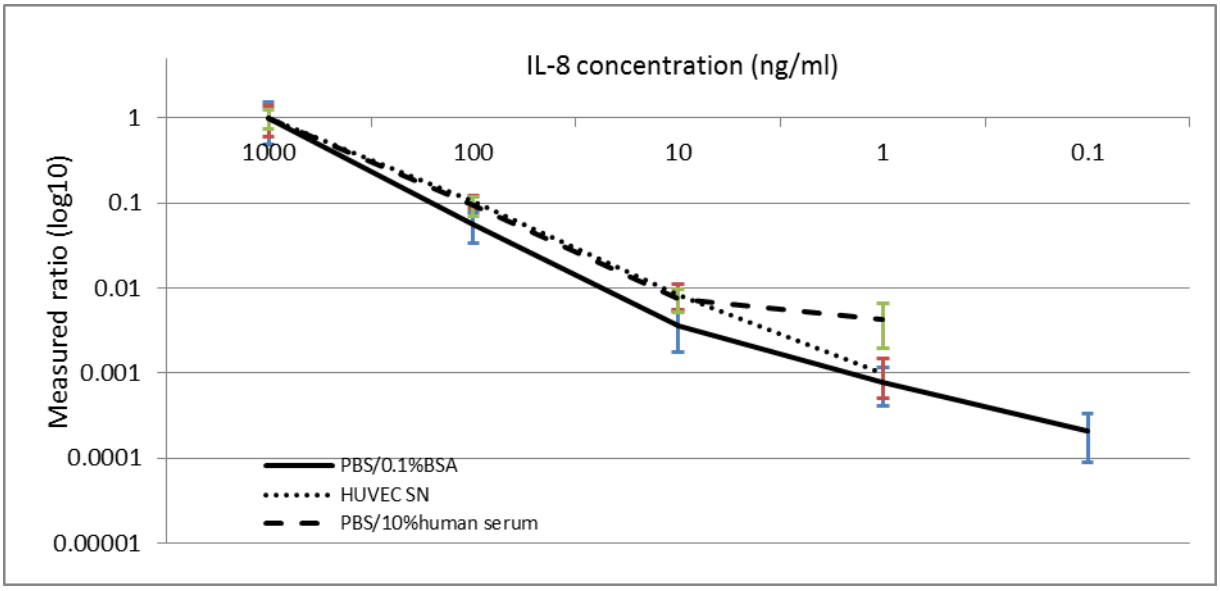


Figure 45. *The MS-SIM results of immunoprecipitated IL-8 which had been spiked into different matrices: PBS/0,1% BSA, HUVEC SN and PBS/10% human serum. Calculated IL-8 concentrations and measured values normalized against the highest IL-8 concentration are shown. Standard deviations within triplicate measurements for each peak are shown.*

A direct demonstration of the high performance of IP is shown by the comparison of samples analysed with and without IP (Figure 46). More or less identical correlations at the samples treated in the same way (except of IP enrichments steps) and performing all experiment in parallel, is another indication of extremly little sample loss during IP. Moreover, the detection signal obtained when by using only 2.5pg amount of IL-8 on column can be attributed to the effects of carrier protein (BSA) during IP, decgreasing the loss of analyte during the experimental procedure.

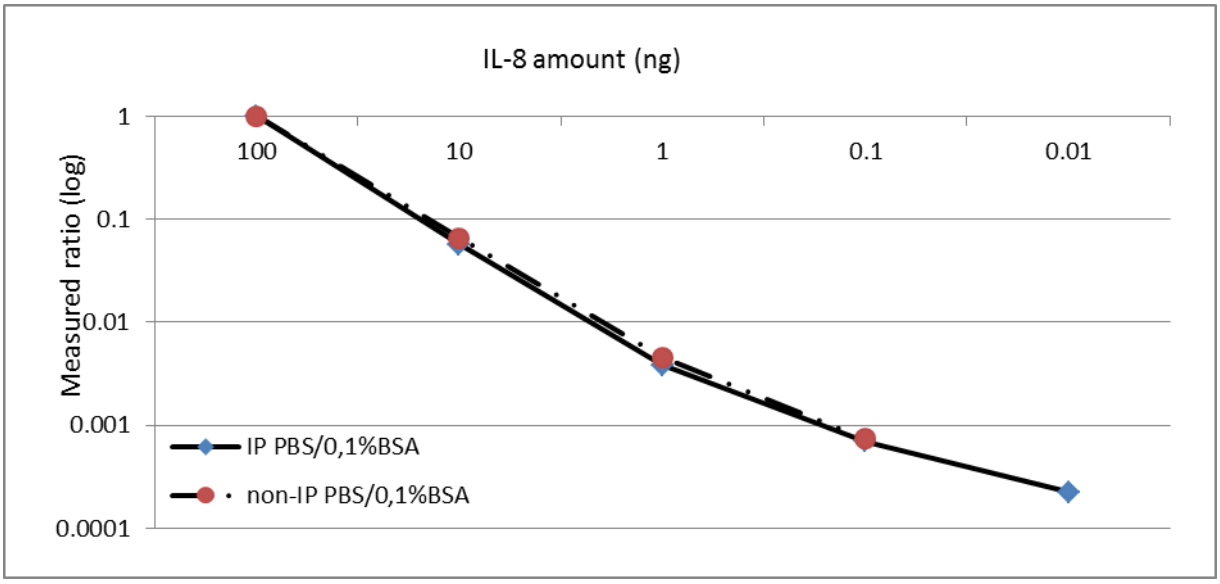


Figure 46. *The MS-SIM results for non-immunoprecipitated and immunoprecipitated IL-8 which had been spiked into PBS/0,1%BSA demonstrating the IP efficiency. Calculated IL-8 amount and the measured value normalized at highest IL-8 concentration are shown.*

The performance with respect to accuracy and the dynamic range covered is shown by a comparison of the results obtained by shotgun and the SIM experiment measuring the same samples (Figure 47).Experiment data presented on the diagram show a high agreement between two methods demonstrating the robustness and reliability of results obtained by mass spectrometric analysis

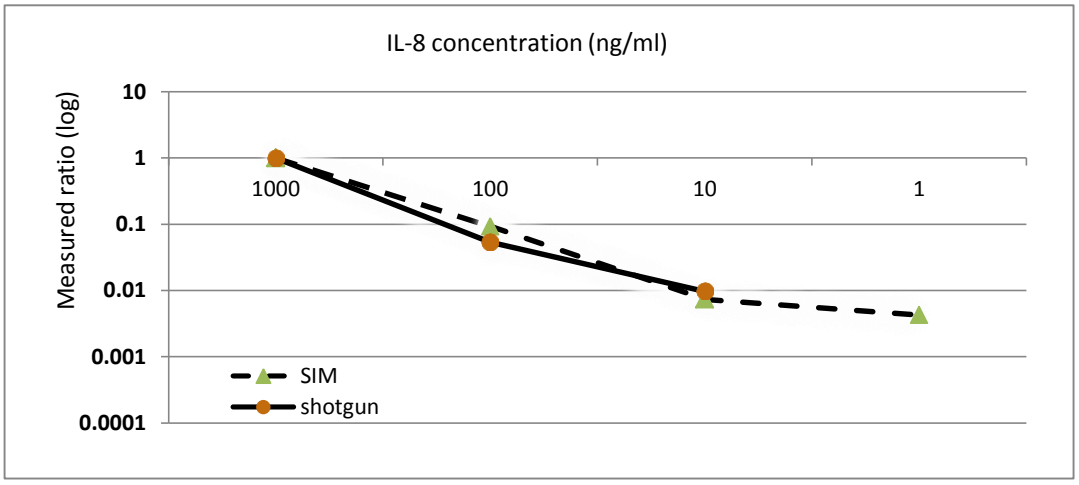


Figure 47*. Results presenting comparison with respect to accuracy and covered dynamic range of the shotgun and SIM obtained from with measurement with the same sample. IL-8 concentration and the measured value normalized against highest IL-8 concentration are shown.*

4. Conclusion

In this Master Thesis immunochemistry and mass spectrometry based methods for the quantification of Il-8 was tested, optimized and comparatively evaluated.

The anti-IL-8 antibody which was tested at the beginning in cell supernatant, plasma and cytoplasm was found not to recognize other proteins. Thus, there was a good chance that the Western Blot analyses could be performed without major matrix effects in all three tested matrices, particularly as the additional electrophoretic separation process would separate potential cross reactive proteins.

The Immunoprecipitation was optimized for three important cytokines, IL-6, IL-8 and CXCL3, using the supernatants of inflammatory stimulated cells grown in media supplemented with 10% FCS. Following such an optimized protocol which avoids washing steps after IP for reducing losses in target protein, has led to WB results for samples taken from HUVECs supernatant which were superior to other immunochemistry based methods. Hardly any loss of target protein during immunoprecipitation was found in comparison to non-IP-treated samples. Small losses observed in the case of the samples taken in PBS/10% human serum were not significant. With all tested matrices a saturation effect was found at 100ng, indicating that under the IP conditions applied the maximum amount of protein that can be immunoprecipitated is approximately 20ng in all matrices, the obsereved LLOD of Western blot was at 100pg.

When applying the Luminex^{TM} assay a significant cross-reactivity was found in case of the PBS/10% serum samples and saturation appeareds over the entire range of tested IL-8 concentrations with the HUVEC-SN samples. Similarly, with the IL-8 specific ELISA only diluted samples could be used in which, however, high cross-reactivity was observed.

Taken together, all antibody based methods tested here, with the exception of WB, were found as being significantly affected by matrix components. No significant matrix effect, however, was established with IP when using an optimized IP protocol. With the Luminex^{TM} assay the standard curve may achieve linearity from 10pg/ml to 1000pg/ml. In contrast, ELISA seems not to be the method of choice in terms of quantitative determination of low abundant proteins in complex matrices, as a false positive overestimation of analyte amounts could not be avoided even by the addition of detergent components to the buffer solutions.

The possibility of multiplexing Luminex^{TM} assays makes them attractive for many high-throughput applications. Inherent limitations of luminescence measurements like quenching and early saturation effects remain as certain limitations. Particularly, the cross reactivity to matrix components has to be considered (and excluded) carefully in each case. One of the major advantages of HPLC-MS based quantification methods is their robustness against matrix effects, even without preceding IP. IP as a tool for sensitive protein enrichment and

mass spectrometric methods aimed for highly selective and sensitive detection seem to give a promising combination for a sensitive, accurate and reliable quantitative determination of low abundant proteins in complex matrices.

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6. Figure Captions

Figure 1. Schematic drawing of a Luminex assay.

Figure2. Main parts of LuminexTM System: biomolecules; microspheres (beads); flow analyzer and high-speed signal processing, (taken from (Dunbar& Vander Zee& Oliver& Karem& Jacobson, 2003)).

Figure 3. Example of MFI response in dependence on analyte concentration: Experimental data are given by orange triangles; fitted curve based on eqn. 1 is given as green line. This curve is a typical output of the instrument's "data interpretation report".

Figure 4. The three ELISA formats.

Figure 5. Scheme of the Horseradish Peroxidase reaction.

Figure 6. Scheme of the various layers used during the blotting process in WBAs.

Figure 7. Consecutive steps in membrane "development": (a) membrane after protein transfer; (b) blocking the membrane on the sites not occupied by transferred proteins; (c) incubation with primary antibodies; (d) incubation with enzyme-linked secondary antibody and the color-reagent (chemiluminescence) solution.

Figure 8. Western blot membranes: (a) after protein transfer and staining all proteins by an adsorptive dye (Ponceau Red); and (b) after development with primary and secondary Abs using a HRP reaction. Visible are bands of proteins recognized by the primary antibody.

Figure 9. Quantitative mass spectrometry workflows introducing heavy/light isotope-codes at different stages of the sample pretreatment. Boxes in blue and yellow represent two experimental conditions. Horizontal lines indicate when samples are combined. Dashed lines indicate points at which experimental variation and thus quantification errors can occur, (taken from (Bantscheff et al., 2007)) .

Figure 10. Scheme of the ICAT reagent,(taken from(Gygi& Rist& Gerber& Turecek& Gelb& Aebersold, 1999)).

Figure 15. Scheme exemplifying the ICAT strategy for differential quantifying proteins (taken from Current Opinion in Biotechnology 2003, 14:110-118)

Figure 12. Left side: iTRAQ label consisting of isobaric tag and reactive group; right side: Table giving the isotope composition of four isobaric tags; and typical MS/MS spectrum showing the reporter-group section, (taken from(Ross et al., 2004)).

Figure 13. Schematic representation of the concept of protein quantification by the method of "spectral counts", (taken from (Chen& Yates, 2007)).

Figure 14. Schematic illustration of the principle of Selected Reaction Monitoring (SRM): Q1- precursor ion isolation; Q2- precursor ion fragmentation; Q3- mass analysis and quantification of a

specific precursor ion fragment (transition), (taken from (Huttenhain& Malmstrom& Picotti& Aebersold, 2009)).

Figure 15. Left side: WB analysis of cytokines using primary Abs targeting (a) IL-6; (b) IL-8; (c) CXCL3. Cytokines were determined in cell supernatant (SN), plasma, and cell cytoplasm (CYT) as described below:
Lanes: 1: supernatant (SN) of untreated HUVECs (negative control; total protein amount 20µg); lanes 2-4: SN of HUVECs after treatment of cells with IL-1ß; volumes applied to WB correspond to total protein amounts of 2µg, 10µg and 20µg respectively; lane 5: human plasma (negative control; total protein amount 20µg); lanes 6-8: human plasma to which SN from HUVECs treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.; lane 9: cytoplasm of untreated HUVECs (negative control; total protein amount 20µg); lanes 10-12: CYT of untreated HUVECs to which SN from HUVECs treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.
Right side: Bands intensity ratios measured by using ImageQuant 5.0. A value of 1 was assigned to the band with the lowest intensity (2µg total protein amount of SN-HUVEC-IL-1ß).

Figure 16. WB analysis targeting Tumor Necrosis factor-inducible Gene 6 protein (TSG6) in cell supernatant of skin fibroblasts, in human plasma, and in skin fibroblast cell cytoplasm after addition of cell SN.
Lanes: 1: supernatant (SN) of untreated skin fibroblasts (negative control; total protein amount 20µg); lanes 2-4: SN of skin fibroblasts after treatment of cells with IL-1ß; volumes applied to WB correspond to total protein amounts of 2µg, 10µg and 20µg respectively; lane 5: human plasma (negative control; total protein amount 20µg); lanes 6-8: human plasma to which SN from skin fibroblasts treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.; lane 9: cytoplasm of untreated skin fibroblasts (negative control; total protein amount 20µg); lanes 10-12: CYT of untreated skin fibroblasts to which SN from skin fibroblasts treated with IL-1ß were added in volumes corresponding to 2µg, 10µg or 20µg total protein, resp.

Figure 17. WB analysis targeting IL-6 in HUVEC-SN2 (i.e., supernatant remaining after IP of IL-6).
IP was performed with and without sample pre-cleaning aimed for removing IgGs present in the sample using Dynabeads Protein G (100µl beads coated with 2µg antibody; 50µl of them were used for IP). Dissociation of the protein complex (HC dimmers) with Mw of approx. 100 kDa was attained by boiling the sample for 5min at 95°C after mixing with loading buffer.

Figure 18. WB analysis targeting IL-6 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using different amounts of coated beads: (a) 2.5µl; (b) 50µl, (c) 100µl. Coating conditions: 100µl beads were incubated with 2µg antibodies for 30min at RT under rotation; IP conditions: incubation for 30min at RT under rotation.

Figure 19. WB analysis targeting IL-8 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using different amounts of coated beads: (a) 50µl, (b) 100µl. Coating and IP conditions as described in Fig. 4.

Figure 20. WB analysis targeting IL-6 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using enhanced amounts of Ab coated onto the beads: (a) 2.5µl of beads containing 0.25 µg of Ab and (b) 50µl of beads coated with 5µg of Ab.; (b) 50µl l of beads coated with 5µg of Ab. Other conditions as described above.

Figure 21. WB analysis targeting IL-8 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed using enhanced amounts of Ab coated onto the beads: 50µl of beads coated with 2.5µg of Ab. Used samples: HUVEC-SN

Figure 22. Western blot analysis targeting (a) IL-6 and (b) IL-8 in the supernatant after IP. Higher amounts of antibody IP was performed using enhanced amounts of Ab coated and lower volumes of beads. 100µl beads were coated with 5µg of corresponding antibody and IP was performed using 10µl and 20 µl of coated beads. Other IP conditions: incubation over 2h at RT; (SN1- supernatant after coating; SN2 – supernatant after IP).

Figure 23. WB analysis targeting IL-8 in the supernatant after IP (samples: SN of stimulated HUVECs). IP was performed after adding 1% Tween-20 and NaCl at 150mM final concentration to the sample. 100µl beads were coated with 5µg antibody and 20µl of them were used for IP.

Figure 24. Western blot analysis targeting IL-8 and CXCL3 8 in the supernatant after IP.
IP was performed using 1% Triton X-100 and 150mM NaCl; Other conditions: 50µl beads coated with 10µg antibody (maximum capacity), 20µl of coated beads used for IP.

Figure 25. Western blot analysis targeting (a) IL-8 and (b) CXCL3.
Coating was made under addition of 0.1% Tween-20 in Binding & Washing buffer and samples were supplemented with Tween-20 (0,1% final concentration) and 30µl of 2% BSA . Other conditions: 50µl beads coated with 5µg antibody, 20µl of beads (containing 2µg antibody) used.

Figure 26. Western blot analysis of (a) IL-8 and (b) TSG6.
IP was carried out over night at 4°C; other conditions identical to those described in Figure 11

Figure 27. Western blot analysis of IL-6 in bead eluate and in washing buffer after IP.
Left: Eluate from beads used for sample pre-cleaning ;
Right: Washing Buffer eluted from the beads after IP (single washing step).

Figure 28. Western blot analysis IP targeting IL-8 under different buffer compositions during coating. Coating was made using a Binding & Washing Buffer consisting of PBS supplemented with different concentrations of Tween 20 (final concentration 0,01%, 0,05 and 0,1%).
Other conditions: After pre-cleaning, Tween 20 (0.1% final concentration) and 30µl BSA (2% final concentration) were added to the sample. 50µl beads were coated with 5µg antibody and 20µl of those were used for IP.

Figure 29. WB analysis of rec. IL-8 spiked into different matrices for attaining concentrations ranging from 100pg to 100ng. Matrices: (a) PBS, (b) PBS with 10% human serum, (c) supernatant of untreated HUVECs with 10%FCS. WB data without IP and WB with preceding IP are given.

Figure 30. WB data of IL-8 samples without preceding IP (first row) and without IP (second row) as determined by the ImageQuant 5.0 software. The band ratio was normalized to the band intensities obtained by direct Western blotting.

Figure 31. Band ratios of IP samples containing IL-8 amounts of 10ng and 100ng, respectively, within different matrices. A matrix dependent saturation effect of IP is demonstrated.

Figure 32. Standard calibration curves of Luminex^{TM}: (a) curve covering the entire concentration range of IL-8 standards; (b) standard curve in the concentration range < 1111pg/ml. The series of standard concentrations were made using Luminex buffer.

Figure 33. MFI curve of rec. IL-8 spiked to PBS/ 10% human serum covering the concentration ranges of (a) 0 – 10.000pg/ml; and (b) 0 – 100pg/ml.

Figure 34. Comparison of "true" spiked amounts of IL-8 with the values assessed by means of the LuminexTM assay when measured in PBS supplemented with 10% human serum

Figure 35. Standard calibration curve with the used IL-8 specific ELISA.

Figure 36. Comparison of "true" spiked amounts of IL-8 with the values assessed by means of the IL-8 specific ELISA when measured in PBS supplemented with 10% human serum, 1:10 diluted.

Figure 37. Comparison of "true" spiked amounts of IL-8 with the values assessed by means of the IL-8 specific ELISA when measured in supernatant of HUVECs supplemented with 10% FCS, 1:100 diluted.

Figure 38. SDS-PAGE gels photos of shotgun experiments after silver staining. For MS analysis are taken only the lanes part under 25 kDa: at PBS/0,1% BSA samples were divided in two and at serum and HUVEC samples in three slices, as it is shown with numbered yellow rectangles. 10µl of sample were loaded and theoretical corresponding IL-8 amount at each concentration is given in brackets.

Figure 39. Mass-retentional time plot showing the detected peptiede ions in one LS-MS run of a singel gel slices from HUVEC SN sample analysed by MaxQuant software, demostrating the sample coplexity and power of mass spectrometric instrument, Q Exactive.

Figure 40. A base peak chromatogram of a LC-MS run of a single gel slice from a HUVEC SN sample, presenting the relative abundance of peptides in the sample.

Figure 41. The MS /MS spectrum identifying the VIESGPHCANTEIIVK (883.95868 m/z), an IL-8 peptide. High number of selected fragment ions (color coded) were classified to the IL-8 peptide, indicating identification with higher reliability.

Figure 42. LC-MS chromatogram used for quantification of identified peptides based on calculation of peak area.

Figure 43. The MS-shotgun results of IL-8 spiked into HUVEC SN and PBS/10% human serum. Calculated IL-8 concentrations were compared to the measured values normalized against the value obtained with 1µg/ml IL-8.

Figure 44. SDS-PAGE gels of immunoprecipitated samples after silver staining. For SIM-MS analysis only the lanes part under 25 kDa was used, divided in two slices as shown with numbered yellow rectangles. 100µl of the original sample were loaded and the theoretically corresponding IL-8 amount for each concentration is noted in brackets.

Figure 45. The MS-SIM results of immunoprecipitated IL-8 which had been spiked into different matrices: PBS/0,1% BSA, HUVEC SN and PBS/10% human serum. Calculated IL-8 concentration and the measured value normalized against the highest IL-8 concentration are shown. The standard deviations within triplicate measurements at each peak are shown.

Figure 46. The MS-SIM results for non-immunoprecipitated and immunoprecipitated IL-8 which had been spiked into PBS/0,1%BSA demonstrating the IP efficiency. Calculated IL-8 amount and the measured value normalized at highest IL-8 concentration are shown.

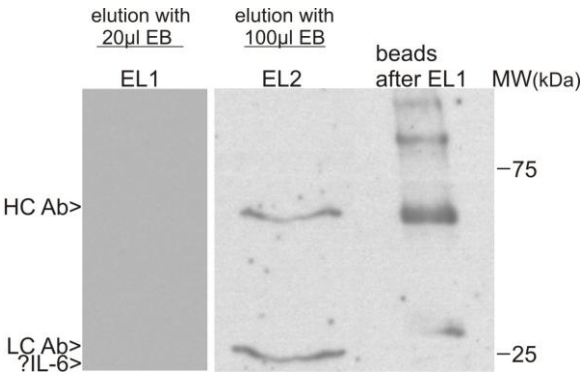
Figure 47. Results presenting comparison with respect to accuracy and covered dynamic range of the shotgun and SIM obtained from with measurement with the same sample. IL-8 concentration and the measured value normalized against highest IL-8 concentration are shown.

Figure (supplemental material). Elution optimization using high volumes of elution buffer (EB). Western blot analysis after IP: a) IP of IL-6; b) IP of IL-8.
EL1- first elution: beads were eluted with 20µl EB; EL2- second elution: after EL1 the same beads were eluted one more time with 100µl EB; beads after EL1- 20µl of beads after the first elution were dissolved in 20µl 5xSDS sample buffer and loaded.

7. Supplemental Material

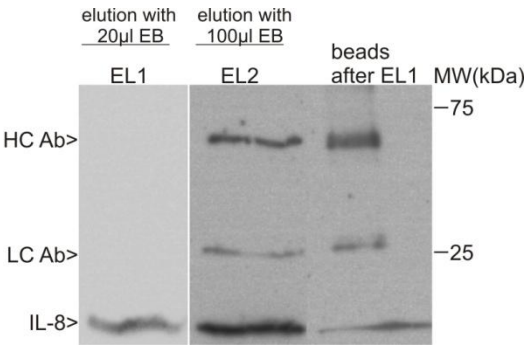
Elution optimization

a)



1d-110615-16bm_WB_IL6 (2+100>100) elution opt

b)



1d- 110615-16_Wb_IL8 (2+100>100) elution opt

Figure . Elution optimization using high volumes of elution buffer (EB). Western blot analysis after IP: a) IP of IL-6; b) IP of IL-8.
EL1- first elution: beads were eluted with 20µl EB; EL2- second elution: after EL1 the same beads were eluted one more time with 100µl EB; beads after EL1- 20µl of beads after the first elution were dissolved in 20µl 5xSDS sample buffer and loaded.

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