Die approbierte Originalversion dieser Diplom-/Masterarbeit ist in der Hauptbibliothek der Technischen Universität Wien aufgestellt und zugänglich.
http://www.ub.tuwien.ac.at

The approved original version of this diploma or master thesis is available at the main library of the Vienna University of Technology.
http://www.ub.tuwien.ac.at/eng

Martina Marchetti-Deschmann

TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Masterarbeit

Proteomics characterisation of the secretome of mesenchymal stem cells

ausgeführt am

Institut für Chemische Technologien und Analytik

der Technischen Universität Wien

unter der Anleitung von

Assoc.-Prof. Mag.rer.nat. Dr.rer.nat. Martina Marchetti-Deschmann

durch

Anna-Katharina Koch, Bakk.rer.nat.

Habsburgerstraße 77, 2500 Baden

21. September 2015

Anna-Katharina Koch

Table of Content

Danksagung

An dieser Stelle möchte ich mich gerne bei folgenden Personen bedanken:

Danke *Univ.Prof. Mag.pharm. Dr.rer.nat. Günter Allmaier* für die Aufnahme in die Arbeitsgruppe.

Mein großer Dank gilt vor allem *Associate Prof. Mag. rer.nat. Dr.rer. nat. Martina Marchetti-Deschmann.* Sie hat es mir ermöglicht an diesem tollen Thema zu arbeiten. Ich bedanke mich bei Ihr für Ihr großes Vertrauen, die aufbauenden Worte und Ihre Geduld, wenn ich sie dringend brauchte. Danke für die tolle Unterstützung, aber auch für die Nachsicht die Sie mit mir hatte.

Außerdem möchte ich mich gerne bei *Univ. Prof. Dipl.-Chem. Dr. Cornelia Kasper* und *B.Sc. Dipl.-Ing.(FH) Dr. Verena Charwat* bedanken für die nette Zusammenarbeit während dieser Arbeit.

Mein herzlicher Dank gilt der gesamten *Arbeitsgruppe*. Besonders hervorheben möchte ich dabei *Nicole Engel* die ich als gute Freundin gewinnen konnte. Sie hat mich immer wieder aufgebaut und ermutigt; vielen Dank für die lustigen Momente die wir hatten. Mein Dank gilt auch *Benedikt Putz* für die ganzen Späße die wir hatten und die nette Unterstützung. *Victoria Dorrer*, danke für die Einführung im Labor und die lustige Zeit die wir hatten. *Elisabeth Soher*, danke für dein ansteckendes Lachen und die tolle Zeit in Berlin. *Victor Weiss*, danke für deine offene Art und deine Hilfe, wenn immer ich Fragen hatte.

Mein größter Dank gebührt meiner Familie. Danke *Mama und Papa* für eure tolle Unterstützung und euren Glauben an mich auch in schwierigen Zeiten. Ohne euch wäre ich nie so weit gekommen, DANKE ich hab euch lieb.

Bedanke will ich mich auch bei meiner *Schwester* für die lustigen SMS und Telefonate die wir hatten, gerade auch dann, wenn die Situation nicht einfach war.

Christoph Müller, dir gebührt ebenso mein größter Dank. Ohne dich wäre das alles hier nicht möglich gewesen. Ich danke dir für deine Geduld und dein Verständnis das du mit mir hattest. Ich danke dir für deine Versuche mich aufzuheitern und deine Unterstützung. Du bist der wichtigste Mensch in meinem Leben, ich liebe dich.

Ebenso bedanken möchte ich mich bei all meinen *Freunden* und meiner restlichen *Familie*.

Krise ist ein produktiver Zustand. Man

muss ihr nur den Beigeschmack der

Katastrophe nehmen.

Max Frisch

1 Introduction

1.1 Abstract

Depending on a distinct induction of mesenchymal stem cells, e.g. normoxie or hypoxie, protein expression levels can be influenced, potentially leading to a variation in their secretome. Normoxie and hypoxie are tools to control cell differentiation, tools of special interest in biotechnology. However, if cell differentiation is studied in a serum containing medium, the detection and characterization of changes in secretome expression levels is very difficult because of the presence of high-abundance proteins in the surrounding medium superimposing the rather low concentrated secretome. In the present study we are focussing on the development of a sample pre-fractionation method to characterize and detect changes in the secretome of mesenchymal stem cells cultivated in serum containing medium.

As a first step protein precipitation from the serum was studied. Precipitation with trichloroacetic acid (TCA) solution and ice-cold acetone (v:v/1:8) turned out to give reproducible results which were checked by SDS-PAGE. In a second step two dimensional gel electrophoresis (2D GE) had to be established for a good and reproducible separation of the rather high number of proteins in the serum containing medium.

As the focus of this study was the assessment of secretome changes, a dedicated sample preparation had to be established to have access to these rather low concentrated proteins. Therefore, high-abundance serum proteins from the medium had to be significantly removed.

Two completely different depletion methods were compared with respect to the efficiency of high-abundance serum protein removal. The first method is based on a one-step removal of the twelve most abundant protein species using antibody-based affinity columns. The second approach is relying in the enrichment of the secretome on beads carrying specific, covalently bound ligands (combinatorial peptide library). Finally the secretome of mesenchymal stem cells after normoxic and hypoxic cultivation was studied in a difference gel electrophoresis (DIGE) approach.

By using Top12 depletion spin columns, an efficient reduction of high-abundance proteins was observed, resulting in a reduced number of protein bands in 1D and a lower number of spots in a two dimensional polyacrylamide gel electrophoresis (2D PAGE). However, co-depletion of low abundant proteins was considered to be very likely because potential carrier proteins e.g. serum albumin were almost completely removed. The combinatorial peptide library showed a lower efficiency for serum protein removal. Serum albumin was still observed, however less concentrated. We assumed that co-depletion was reduced under these conditions and a more complete coverage of the secretome was achieved. We evaluated the latter approach to show reproducibility and robustness of sample preparation for 2D GE and 2D DIGE analysis. DIGE analysis showed variable intensities for some protein spots, indicating different expression levels of certain proteins.

1.2 Zusammenfassung

Eine Induktion wie beispielsweise Normoxie oder Hypoxie kann die Differenzierung von mesenchymalen Stammzellen beeinflussen, wodurch es zu Veränderungen in ihrem Sekretom kommen kann. Im Allgemeinen werden Normoxie und Hypoxie als biotechnologische „Werkzeuge“ angesehen, die die Zelldifferenzierung kontrollieren. Allerdings ist die Charakterisierung solcher Veränderungen des Sekretoms, aufgrund der Anwesenheit von hochabundanten Proteinen des umgebenden Mediums, sehr schwierig. Daher wurde der Fokus in der vorliegenden Arbeit auf die Entwicklung einer Proben-Vorfraktionierungsmethode gelegt, um mögliche Variationen im Sekretom zu detektieren. Für eine robuste Methodenevaluierung wurde als erster Schritt die Proteinfällung untersucht. Die Präzipitation, mit Trichloressigsäure und eisgekühltem Aceton (v:v/1:8), erzielte reproduzierbare Ergebnisse, welche durch SDS PAGE verifiziert wurden. Als zweiter Schritt wurde ein reproduzierbarer zwei dimensionaler Gel Elektrophorese (2D GE) Ansatz etabliert, um eine reproduzierbare Proteinseparation zu erzielen. Aufgrund der Kultivierung in humanserumhaltigem Medium, war der Hauptfokus dieser Arbeit die hochabundanten Serumproteine abzureichern, um niedermolekulare Sekretomproteine detektieren zu können. Dazu wurden zwei unterschiedliche Vorfraktionierungsmethoden, auf deren Effektivität, in der Entfernung dieser hochabundanten Serumproteine verglichen. Mit der ersten Methode wurden die 12 am häufigsten vorkommenden Serumproteine, mittels Antikörper basierten Affinitätssäulchen (Top 12 depletion spin columns), entfernt. Die zweite Methode basiert auf der Anreicherung von Sekretomproteinen, welche von spezifischen Liganden gebunden werden, die kovalent an Beads gebunden sind (Combinatorial peptide ligand library). Für die Detektion möglicher Unterschiede, aufgrund verschiedener Kultivierungsbedingungen (Normoxie und Hypoxie), wurde eine Differenz Gelelektrophorese (DIGE) durchgeführt. Die Verwendung von Antikörper basierten Affinitätssäulchen resultierte in einer effektiven Reduktion der hochabundanten Serumproteine, was sich in einer reduzierten Anzahl von Proteinbanden im eindimensionalen (1D), sowie in einer geringeren Anzahl von Proteinspots im zweidimensionalen Gel (2D) wiederspiegelt. Die Co-Abreicherung, von in geringen Mengen vorhandenen Sekretomproteinen, schien sehr wahrscheinlich, da potentielle Trägerproteine, wie das Serumalbumin nahezu komplett entfernt wurden. Die zweitgenannte Methode, war zwar in der Entfernung der Serumproteine weniger effizient, Serumalbumin war weiterhin vorhanden, jedoch weniger konzentriert. Wir nehmen daher an, dass die zuvor genannte Co-Abreicherung unter diesen Bedingungen reduziert wird, und eine vollständige Erfassung des Sekretom erreicht wird. Für weitere Versuche wurde der zweite Ansatz herangezogen, um die Reproduzierbarkeit und Robustheit der Probenvorbereitung für 2D GE und DIGE Analysen zu zeigen. Die DIGE Analyse zeigte für einige Proteine variable Spot Intensitäten, was auf einen unterschiedlichen Expressionsgrad von gewissen Proteinen hinweist.

1.3 Motivation

The aim of this work was to establish a reproducible sample pre-fractionation protocol that gives access to the less abundant secretome of mesenchymal stem cells grown in cell medium containing 10 % human serum. Yet these media contain a number of highly abundant serum proteins hindering the detection of minor protein species. Therefore an efficient reduction of high-abundance proteins had to be established. Final Goal – not followed in detail in this diploma thesis – is to detect statistically significant variations in the secretome of human mesenchymal stem cells cultivated in dependence of different oxygen supply levels by 2D DIGE.

1.4 Stem cells

1.4.1 General overview

The human organism consists of about 200 different cell types. The differentiation of these cells is depending on the surrounding milieu, which in turn is influenced by external factors like growth factors and by the cell itself. This influenceability of the cell differentiation is fundamental for the regeneration capacity. Explorations of these mechanisms provide fundamentals for the targeted therapy of tissue damage, also known as regenerative medicine [4].

Stem cells are known as specialized cells, which possess the ability of self-renewal through cell division, are able to differentiate into multi-lineages and possess varied potency depending on a distinct induction [4-6]. Due to their biological importance, as well as their clinical application, stem cells are popular subjects for many research areas, especially for tissue engineering. According to the basis of their origin and potency they are categorized as embryonic stem cells (ESCs) and adult stem cells (ASCs) [6]. Both classes of stem cells present its own benefits, limitations and challenges in bioprocess development, as well as common features, like the ability to proliferate and vary in their differentiation potential as shown in Figure 1. ESCs are initially isolated from the inner cell mass of blastocysts. They are characterized to be pluripotent and are able to give rise to all lineages. According to those facts ESCs are promising cells for immune-, and cellular therapy, as well as for regenerative medicine [4]. ACSs are localized in specific cell niches in different organs in regenerative tissues of adult organisms. Like ESCs, ASCs have the ability for self-renewal and to differentiate in various cell types under certain conditions. However, unlike ESCs, they are only multi-, oligo- or unipotent. ASCs require a defined environment in the organism, in which their stem cell character is sustained by the influence of various intrinsic and extrinsic signals. This particular environment is called stem cell niche. This hypothesis was introduced by Ray Schofield in 1978 [7]. According to his hypothesis, several niches are located in defined areas in the organism, in which stem cells renew themselves and various procedures are regulated like the cell’s survival, proliferation, or maintaining of character [5].

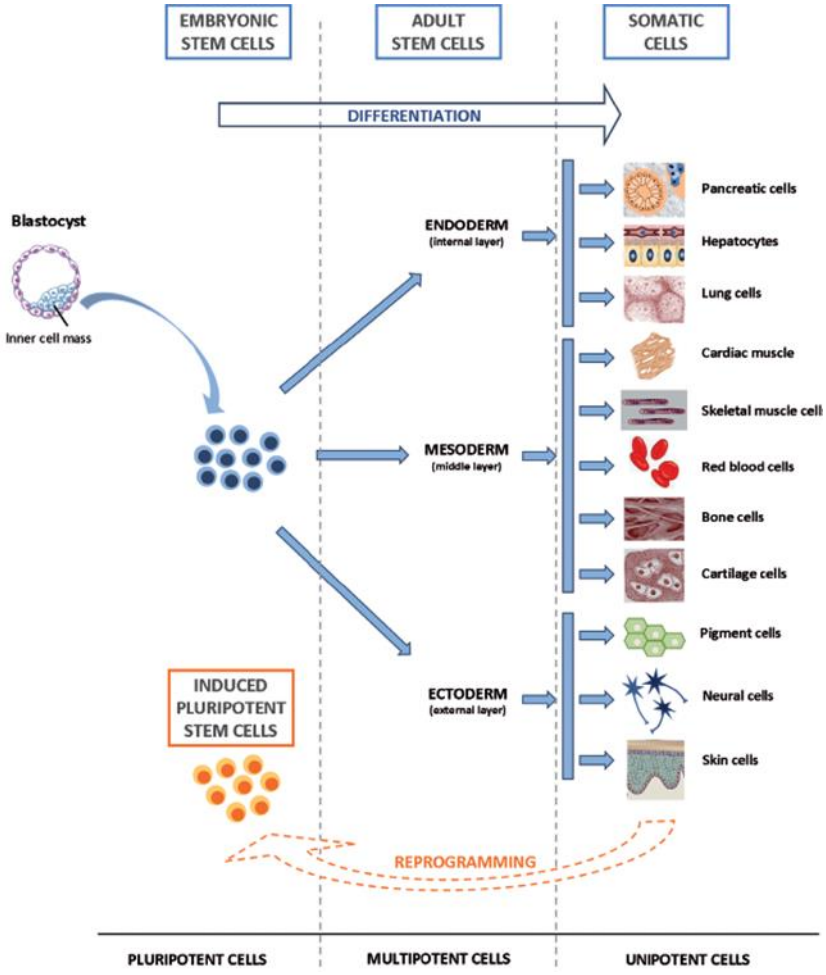


Figure 1: Stem cell characteristics. Picture adapted from Margarida Sera, "Bioengineering strategies for stem cell expansion and differentiation", URL: http://canalbq.spb.pt/docs/canalBQ_0007-30-37.pdf (retrieved at 24.07.2015)

1.4.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are dedicated to be ASCs and are also known as multipotent stromal cells [8]. In this master thesis MSCs were obtained from human adipose tissue and they are able to differentiate into various cell types including osteogenic, chondrogenic and adipogenic lineages, as shown in Figure 2 [9].

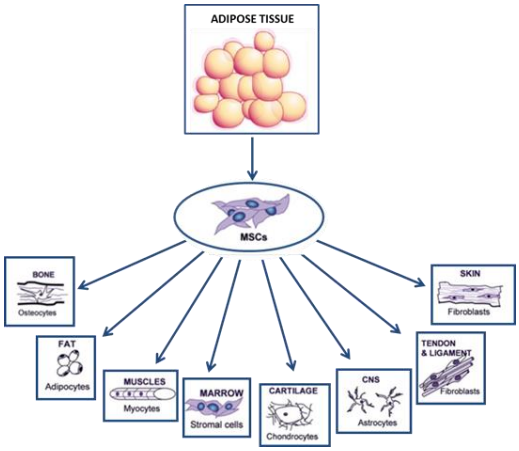


Figure 2: Differentiation potency of MSCs derived from adipose tissue

Those cell populations possess a high capacity of self-renewal which allows generating a large number of cells and they can derive from various tissues without ethical concern. MSCs are classified according to their origin independent of their differential potential. MSCs were first reported in the bone marrow 1970 [10], and further found and isolated from almost all tissues, see Table 1.

Source	Differential potential
Bone marrow	*Adipogenic, Chondrogenic, Osteogenic, Myogenic, Neuronal*
Adipose tissue	*Adipogenic, Chondrogenic, Osteogenic, Myogenic*
Cartilage	*Adipogenic, Chondrogenic, Osteogenic*
Dermis	*Adipogenic, Chondrogenic, Osteogenic, Myogenic*
Dental pulp	*Adipogenic, Chondrogenic, Osteogenic, Myogenic, Neuronal*
Breast milk	*Adipogenic, Chondrogenic, Osteogenic*
Blood	*Adipogenic, Osteogenic, Osteoclastic, Fibroblastic*
Umbilical cord blood	*Adipogenic, Chondrogenic, Osteogenic, Neuronal, Epithelial, Hepatogenic, Myogenic*
Urine	*Urothelial, Myogenic*
Wharton's jelly	*Adipogenic, Chondrogenic, Osteogenic, Myogenic, Neuronal, Endothelial, Hepatogenic, Pancreagenic*
Placental/Chorion	*Chondrogenic, Osteogenic, Myogenic, Neuronal*
Placental/Amnion	*Adipogenic, Chondrogenic, Osteogenic, Myogenic, Endothelial*

Table 1: Differentiation potential and source of MSCs. Table adapted from [11]

Nowadays the application of living cells for medical treatment has gained increasing relevance. Therefore MSCs are an effective and promising tool for therapeutic purposes, such as tissue engineering or cell therapy. Because of the fact that they possess multi lineage potential, as well as cytokine and anti-inflammatory molecule secretion, they are highly suited for chronic diseases treatment, tissue repair or regenerative medicine in general [12]. The International Society for Cellular Therapy (ISCT) established minimal criteria which have to be fulfilled by MSCs: they have to (a) show plastic adherence, (b) positively express a specific set of surface antigen markers (CD73, D90, CD105) and (c) lack the expression of CD14, CD34, CD45 and HLA-DR [13].

2 Theoretical Background

2.1 Cell cultivation

2.1.1 General overview

Cell cultures are defined as certain cells that have been isolated from an organism or the surrounding tissue and are cultivated *in vitro* under controlled conditions. These *in vitro* conditions have a specific influence on cell behaviour. Optimizations enable isolated cells to grow, proliferate, differentiate and fulfil specific tasks. Thus, it is important to adjust and simulate *in vivo* requirements and needs in an artificial environment. For each cell type cultivation varies and that is why environmental conditions like temperature, pH-value, osmolarity, the adequate supply of essential nutrients and oxygen as well as the removal of toxic substrates and provision for contaminations, have to be well defined [14].

In general cell cultures are divided into two categories: adherent cells and suspension cells. Adherent cells attach to the surface of the culture vessel during proliferation as for example epithelial cells, cartilage cells and fibroblasts do. Reason for this is the overall surface charge of the cells, which is defined by the glycocalyx, bivalent cations, especially magnesium and calcium, and several proteins. Suspension cells are able to float and grow in a suspension, lymphocytes and stem cells are typical examples [15].

Cell cultivation can be performed in different types of materials. Initially it was done in glass vessels which benefit from reuse and eco-friendliness. However, it is important to purify and pre-treat glass to eliminate unwanted ions, which are released from the glass itself and to remove contaminations attached to the material during transport or manufacturing. Soon plastic replaced glass as cultivation material because it can be produced in each shape in which it is needed. Besides glass and plastic, there also exist the less commonly used stainless steel, titanium or palladium vessels. Concerning the shape of the cultivation vessel bottles, made of glass or plastic, petri dishes or in microliter plates, both plastic, exist. For

industrial cell cultivation multi-trays, hollow fibre modules or spinner flasks are used. Latter are especially used for suspension cultures [15].

In general a culture medium is defined to support the growth and development of the cells. For a successful cultivation certain standards have to be fulfilled for the used media, which depend on the cell line itself. Therefore the development of well-defined and designed media was an important step for the cell cultivation. There exist different types of media for cultivation [14, 15].

2.1.2 General cultivation for the expansion of MSCs

For clinical application of MSCs, it is important that their expansion becomes effective, safe and robust. Therefore several techniques and approaches were invented to generate a necessary and relevant cell number in the recent years.

2.1.2.1 Static conventional cultivation

This technique is also known as a two-dimensional cell culture where cells are cultured in flat plastic flasks as shown in Figure 3. Due to the fact that only a moderate number of cells can be obtained by this technique and a scale-up makes the cultivation time consuming and prone to contamination, cell factories were introduced. Cell factories facilitate a specific type of cell culture flask where more than five, ten or even more cultivation chambers are combined in multi-layer stacks. That technique provides more growth surface and reduces the risk of contamination. However it is a static method and therefore no online monitoring and control of the cell growth is possible [14, 15].

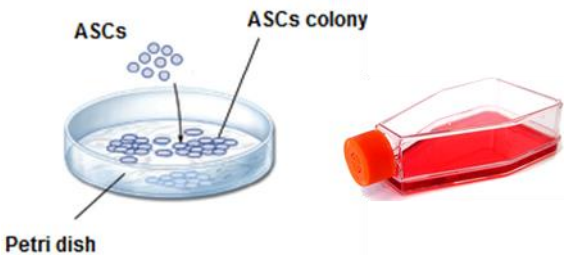


Figure 3: Stem cells cultivation with Petri dish or tissue culture flasks.

2.1.2.2 Static and dynamic cultivation on microcarriers

The cultivation and expansion of MSCs can also be carried out on microcarriers, as shown in Figure 4. They provide a supporting matrix, which allows cultivating cells on small solid particles suspended in the growth medium. Today macroporous and smooth microcarriers are in use. The difference between both is that in case of the smooth microcarriers the cells grow on the surface of the particles and do not enter the core, whereas in case of the macroporous microcarrier cells can grow inside the supporting matrix. Both types are available in several different materials. Smooth microcarriers are available in gelatine,

polystyrene or dextran. Macroporous microcarriers are obtainable in glass, cellulose, polyethylene or collagen [16]. Smooth microcarriers possess a high potential for up-scaling, simplify the monitoring of several cultivation parameters and can be used in bioreactors.

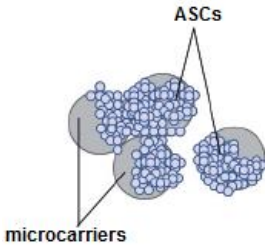


Figure 4: Stem cell cultivation on microcarriers.

2.1.2.3 Dynamic cultivation in bioreactor

The expansion of cells in a bioreactor is a dynamic system that is a promising tool for future application (Figure 5). Due to integrated sensors information about nutrient consumption and cell growth can be obtained. Furthermore this technique provides an automated controlling system and monitors online several cultivation parameters like pH value, temperature, oxygen or shear forces, and controls and maintains constant nutrient and gas supply. Controlling those facts make them important to mimic and recreate the physiological microenvironment of the tissue niche, which supports the optimal growing conditions, leading to a relevant number of cells and making the cultivation a promising tool for the clinical application of those cell lineages.

Figure 5: Cultivation of stem cells in a Z®RP 2000 H bioreactor and GMP-breeder from Zellwerk.
Picture take from http://zellwerk.biz/MSC/Flyer_BM_MSCs.pdf (retrieved at 24.07.2015)

2.1.3 Mesenchymal stem cell cultivation under normoxic and hypoxic conditions

The proliferation capacity and differentiation potential of MSCs can be influenced by several *in vitro* cell culture parameters. Due to different cell culture media compositions, types of sera or addition of supplements, an effective cell expansion can be achieved. One of the most important parameters is the oxygen (O_2) concentration. Variation in concentration leads to changes in gene expression. This makes O_2 concentration an important factor, which is supposed to have an influence on MSCs biology, as well as on their efficiency and biosafety for therapeutic application. Most of the cell cultivation protocols are applying ambient O_2 concentration (21% in the gas phase), which is approximately 4 to 10 times higher than the O_2 concentration in the MSCs niches [17]. Due to the fact that the natural microenvironment MSCs is characterized by low O_2 supply, a high resistance to O_2 limitation can be assumed. Higher O_2 levels may cause oxidative stress for *in vitro* cultured cells and moreover can lead to early senescence, longer population doubling times, DNA damages [18] or cell death by apoptosis and necrosis [19]. In recent years several studies have shown that the cultivation from MSCs under reduced O_2 concentrations lead to enhanced cell proliferation and can maintain the cells' differentiation properties [20].

2.1.4 Addition of serum to the culture medium of MSCs

In this work an alpha minimum essential medium (αMEM) was used for the cultivation of MSCs. This medium was developed by Harry Eagle in 1971, and is an advancement of the Minimum Essential Medium (MEM) which is also an advancement of the Basal Medium Eagle (BME) but contains a higher amount of amino acids, salts, glucose and vitamins [21, 22]. The αMEM differs from the MEM standard formula by the following additional components: vitamin B12, ascorbic acid, non- essential amino acids, pyruvate, fatty acid and D-biotin but also nucleosides [21, 22]

The addition of serum in amount of 3-25 % to the medium, depending on the requirements of each cell line is seen as a routine technique for cell cultures [21, 22]. Different serum types can be categorized according to the development stage of the animal (fetal, new-born or adult) or to the species of the animal (bovine, pig, horse) [15]. Nowadays also human sera are used for different tissue engineering or tissue culture approaches. Sera supply the cultures with hormones, growth and attachment factors, binding and transport proteins (*e.g.*: fibronectin and transferrin), with plenty of amino acids, inorganic salts, trace elements as well as buffer and neutralization systems such as albumin, immunoglobulins or protease inhibitors [14, 15]. Beside higher molecular weight materials such as polypeptides, growth factors and hormones, also cholesterol, fatty acids and lipids mostly in form of lipoproteins are introduced into the cell culture by the serum. Furthermore they can increase the viscosity of the medium,

provide mechanical protection by the reduction of shear forces and they inactivate toxic metabolic end products [15]. There are also some disadvantages for the application of serum containing media. Serum ingredients vary qualitatively and quantitatively from batch to batch, or they contain inhibitors [15].

2.2 Gel based Proteome Analysis

Proteomics was first introduced by Marc R. Wilkins in 1996 [23]. The term “Proteome” describes the entire protein complement expressed by a genome [23]. In thus, proteomics is defined as the analysis of all proteins which are expressed at a given time under defined conditions in a cell, tissue or in an organism. Even if there exists only one genome it codes for a full repository of proteins and their variants, the proteome at a given biological states. A proteome is the result of a combination of genomic transcription and translation, protein turnover and posttranslational modification. Today proteomics has developed from single protein profiling to high throughput identification and quantification of multiple biological samples. In general quantitative proteomic approaches can be classified as either “gel-free” or “gel-based” methods but also as “label-free” and “label-based” approaches, which can further be subdivided into chemical and metabolic labeling [24, 25]. During this master thesis a gel based approach in combination with label based or label free detection of proteins was carried out, as shown in Table 2.

	Gel-based
Label based	Fluorescence labelling (G-Dyes)
Label free	Non-covalently bound dye (e.g. Coomassie brilliant blue, Silver staining, fluorescence dyes)

Table 2: An overview of used quantitative proteomic approaches during the master thesis.

The gel-based proteome approach consists of the following major steps [25], shown in Figure 6 and discussed in more details below:

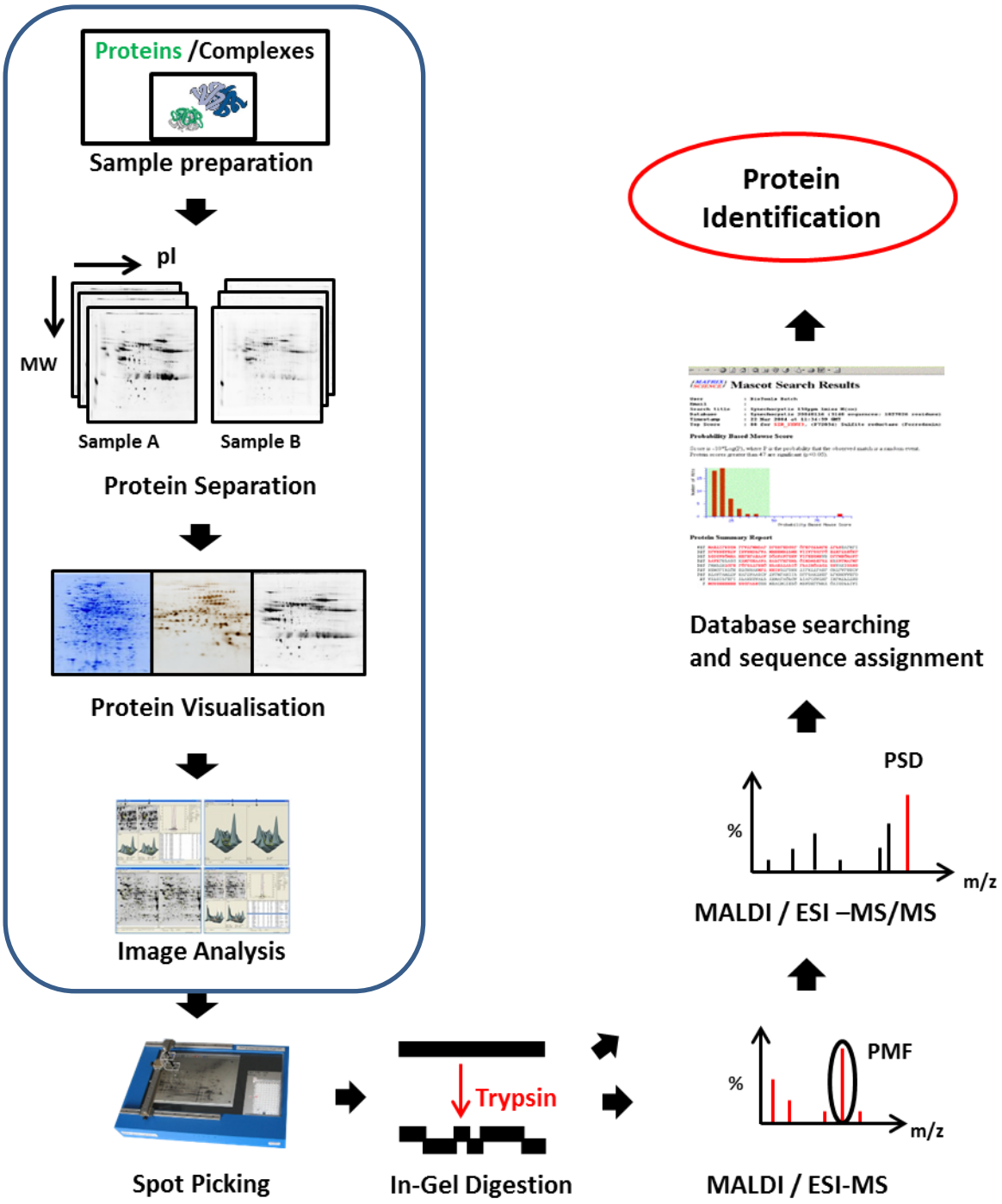


Figure 6: The main steps of a gel-based proteome approach. Steps highlighted in blue were performed during this work. To complete the gel-based proteome approach further steps including spot picking, in-gel digestion, MS-analysis and protein identification have to be done. These steps were not done during this work.

2.2.1 Sample preparation

2.2.1.1 Protein precipitation and solubilization

In general cells or tissues were disrupted and lysed by different techniques such as enzymatic digestion with cellulases or proteases, changing the osmotic pressure by introducing higher salt concentrations or with detergents. Mechanic disruption is performed

through homogenization with beads, sonication or grinding the cell material in liquid nitrogen. After this proteins have to be solubilized in an appropriate solvent, a buffer, and interfering substances like proteases, nucleic acids, polysaccharides, lipids and salt ions have to be removed by centrifugation, precipitation, organic solvent extraction or inhibitors in the case of enzymes.

2.2.1.2 Depletion of high-abundant proteins

For clinical researches, in particular for regenerative medicine and the search of potential biomarkers, the analysis of the human proteome becomes more and more interesting. Especially biomarkers are supposed to be found in the "deep" proteome, *i.e.* very low concentrated proteins. Proteome analysis is very challenging due to the extremely large dynamic concentration range, which spans over 12 or more orders of magnitude [26], however instruments reliably cover only 4 to 5 orders of magnitudes. Furthermore only a few high-abundance proteins represent a large majority of the protein mass, as shown in Table 3, and therefore the detection of other species is hindered.

High-abundance proteins	Source	Prevalence [%]
Albumin	Serum, CSF	50-60
IgG	Serum, CSF	10-15
Hemoglobin	Blood cell lysate	85-95
Ovalbumin	Egg white	35-45
Lactalbumin	Milk	30-40
β-Lactoglobulin	Milk	15-20
RuBisCO	Plant leaf extract	40-60
Actin	Cell extracts	15-20

Table 3: Overview of high-abundance proteins in various biological extracts. Table adapted from [26]

As shown in Table 3, albumin comprises 50–65% of the serum protein content [27] and in total about 90% consist of immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), Apolipoprotein A-II, Transferrin, alpha2-Macroglobulin, alpha1-acid glycoprotein, haptoglobin, apolipoprotein A-1 and fibrinogen besides the already mentioned serum albumin. Indeed, 1% of the entire serum proteome is considered to be of low-abundance (e.g. cytokines, chemokines, peptide hormones and proteolytic fragments of larger protein) but of great interest in the search of potential biomarkers [28]. So, depletion of the twelve mentioned high abundance proteins is necessary to detect these low abundance proteins. However, simple removal of albumin could go along with the elimination of important species because albumin acts as a carrier and transport protein within the blood for lipophilic

substances such as cytokines, lipoproteins or hormones. Considering this, protein depletion is a critical step to detect minor protein species. Nevertheless protein fractionation is an important step to get access to low concentrated peptides and proteins, which are currently masked by high-abundance proteins. So it is necessary to reduce the complexity and the wide dynamic concentration range.
For this purpose two different depletion methods were tested in this thesis: (a) a combinatorial hexapeptide library (CPLL) [29] and (b) a depletion column that removes the 12 most abundant serum proteins.

2.2.1.2.1 Combinatorial hexapeptide library

In the early 1990s combinatorial chemistry was initiated allowing an accelerated way to synthesize a large number of compounds differing by one substituent. By the work of Merrified (who first performed peptide synthesis on solid phase) [30, 31], Furka, (who invented the so-called split, combine, and pool method which reduced the number of synthesize steps and number of reaction vessels) [32] and Lam (who introduced the one-bead one-structure concept) [33] a novel screening approach was launched for studying the nature of intermolecular communication, as well as for separating proteins from complex mixture.

The combinatorial peptide ligand library corresponds to an assemblage of peptides received from an amino acid selection that compose peptides in all possible combinations. It was first introduced by Thulasiraman in 2005 [29] and aims for the reduction of the dynamic concentration range of proteins, especially in biological samples, to enrich very low-abundance proteins. Consequently high-abundance species are depleted. This ProteoMiner (Bio-Rad) or Equalizer beads technology features a combinatorial library of hexameric peptide ligands on their surface. Each bead has millions of copies of a single, unique peptide and each bead potentially has a different peptide ligand. The diversity of ligands is defined by two parameters: the number of amino acid building blocks and the length of the peptide. By placing a peptide library in contact with a biological sample the reduction of high-abundance proteins and enrichment of low-abundance ones is achieved (principle is shown in Figure 7). Through the restricted binding capacity of the beads, a saturation effect is rapidly reached for the most concentrated proteins. Proteins in excess cannot bind any further and are then discarded in the flow-through. In contrast, low-abundance species are concentrated and enriched on their specific affinity ligands, as long as the sample is loaded. Proteins which are retained by the beads are eluted from the affinity binder showing a nearly similar composition as the initial ones, however with significantly lower concentrations. The relative concentration

of each retained protein species and the presence of their specific affinity ligands define the composition of the eluted proteins.

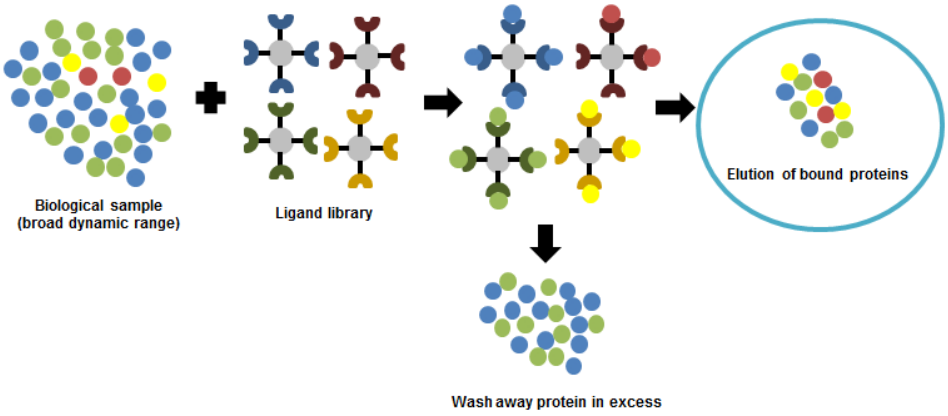


Figure 7: Working principle of CPLL.

Due to the work of Simó et al. [34] combinatorial peptides of different lengths from a single amino acid up to a hexapeptide were evaluated for a better understanding of the behaviour of solid-phase combinatorial peptide ligands. The obtained result shows that a non-insignificant portion of the proteome can be shown with the use of mixed beads with a single amino acid attached. It was further demonstrated that an increase of the captured proteins was achieved by increasing the length of the peptide bait [34]. It seems that the best results are obtained by using hexamer baits, resulting in a larger protein population and a better specify [35].

However, after this treatment absolute protein quantification is no longer possible since the dynamic range is reduced. Though, the proportionality is maintained whereby a relative quantification is still possible [36]. Literature states that combinatorial hexapeptide libraries allow the exploration of the entire proteome [37]. Yet, adsorption and elution conditions can significantly influence the final results and are crucial steps for a successful analysis [38]. The ratio between the given protein amount and the volume of beads influences the reduction of the dynamic range and analytical conditions, like pH, temperature or the ionic strength of the buffer, can significantly affect the affinity of the proteins for their specific peptide ligand.

2.2.1.2.2 Depletion columns

Depletion approaches mainly use, physicochemical features such a charge and size (ion exchange chromatography and gel filtration), or ligands and biochemical properties (affinity chromatography) [39].

Affinity Chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatographic matric. This working principle enables purification, concentration and enrichment of low-abundance protein species, removes contaminations and reduces the broad dynamic concentration range by depleting high-abundance serum or plasma proteins. A method that utilizes antibodies or antigens as ligands is also called immune-affinity chromatography. They are used to create a highly selective media for the depletion of the most high-abundant plasma and serum proteins. The antibodies are immobilized to a column. For a successful depletion a biospecific ligand, covalently attached to a chromatography matrix, is required. Some typical used ligands and their specific interaction partners are listed below:

Ligand	Biological interaction
Enzyme	substrate analogue, inhibitor, cofactor
Antibody	antigen, virus, cell
Lectin	polysaccharide, glycoprotein, cell surface receptor, cell
Nucleic acid	complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein
Hormone, vitamin	receptor, carrier protein.
Glutathione	glutathione-S-transferase or GST fusion proteins
Metal ions	poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces, enrichment of phosphorylated proteins

Table 4: Used biospecific ligands and their corresponding interaction molecule. Table adapted from GE Handbook "Affinity Chromatography, Principles and Methods", URL: http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/Affinity_chromatography_handbook.pdf (retrieved at 24.7.2015)

Some requirements for the used ligand are the reversible binding of the target molecule in an active form and that the specific binding affinity must be maintained for the target molecules during washing away unbound material. Electrostatic or hydrophobic interactions, van der Waals' forces or hydrogen bonding are responsible for the interaction between the ligand and target molecule. For example albumin is bound via electrostatic or hydrophobic interactions to Cibacron™ Blue F3G-A, a synthetic polycyclic dye linked to a Sepharose matric [40]. Fibronectin binds specifically to gelatin which is also linked to speharose by physiological pH

and ionic strength. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity of the elution solution.

For the removal of the high abundance proteins, protein depletion spin columns were tested. The used columns contain immobilized antibodies to remove twelve of the most abundant serum proteins in a single removal step, see Table 5 [41]. The working principle of those depletion columns is shown in Figure 8.

α1-Acid Glycoprotein	Fibrinogen
α1-Antitrypsin	Haptoglobin
α2-Macroglubulin	IgA
Albumin	IgG
Apolipoprotein A-I	IgM
Apolipoprotein A-II	Transferrin

Table 5: Top 12 highly abundant serum proteins which are depleted by the depletion columns according to manufacturer

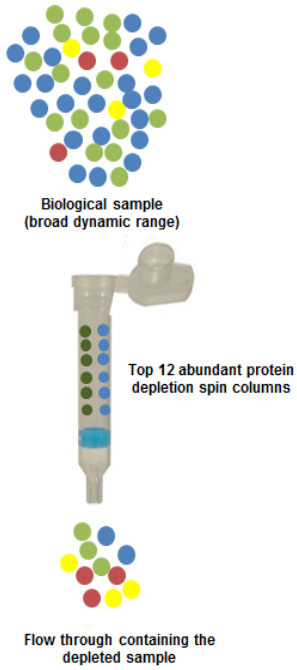


Figure 8: Working principle of depletion columns.

Chromy et al.[42] compared two groups of depletion approaches, each using two different kits: an ion exchange-based technique versus an antibody-based affinity method. Both kits of the former approach were albumin depletion kits. One of the latter approach was a six high abundance protein depletion kit and the other an albumin only depletion kit. The best results were shown for the removal of the six high abundance proteins increasing the protein spots from 866 (in crude serum) to more than 1500.

The two major limitations of depletion columns are the appearance of co-depletion of low molecular weight target proteins or the dilution of collected depleted sample [43]. Furthermore high clearance levels of low abundance proteins and the cost intensive materials are further disadvantages.

2.2.1.3 Denaturating / Non-denaturating conditions

Sample preparation can be done under denaturating and non denaturating conditions. Disrupting the naturally occurring secondary, tertiary and quarterly structure of the analyte, leads to a separation solely based on the primary structure of the protein. This basically means that the mobility of the analyte depends on its MW. The most common used denaturing method is the use of sodium dodecyl sulphate (SDS) which is often used during sample preparation for polyacrylamide gel electrophoresis (PAGE) a standard method for protein separation [44]. During the sample preparation procedure for SDS-PAGE, the analyte is boiled in an SDS containing buffer to disrupt hydrogen bonds. Those denaturating conditions are used in denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and temporal temperature gradient electrophoresis (TTGE) [2, 45, 46].

For some clinical applications or detection of antibodies, non-denaturated samples are applied in order to remain their primary, secondary, tertiary and ideally quaternary structure. Sample preparation is done without boiling the proteins in any detergent, so disulphide bridges are still present and therefore MWs cannot be determined.

2.2.2 Protein Separation

2.2.2.1 Electrophoresis

Electrophoresis is a separation technique that was invented by the Swedish chemist Arne Tiselius in 1937 [47, 48]. Today electrophoresis is a standard method in biochemical and biomedical research to isolate and separate nucleic acids, amino acids, carbohydrates, peptides and proteins. In principal, electrophoresis is separating anionic or cationic, low molecular (bio-)substances as well as larger (bio-)polymers, like high molecular weight proteins, cells and other charged particles. An electrophoresis apparatus contains an anode and a cathode on which constant voltage is applied. These electrodes are tipped into a solution containing the charged particles intended to be separated. Through the impact of the applied voltage, charged sample molecules migrate in the applied electric field towards the electrode of opposite charge inducing a current. The induced migration of the sample molecules is based on their different charges and molecular weights, and the speed of migrations is generally termed as electrophoretic mobility. Due to different electrophoretic mobilities the sample molecules are separated into individual zones. Furthermore the choice

of the buffer system is crucial. Proteins, which have a low to medium pI, are negatively charged and will migrate towards the anode, when basic buffer systems like Tris-chloride or Tris-glycine are used. Acidic buffer systems, like Glycine-acetate, are needed for basic proteins where they become positively charged and will migrate toward the cathode. Native gel electrophoresis is of interest if the natural state of a molecule, protein or DNA, is of interest.

Electrophoretic separations can be carried out in a free solution as in a capillary and free flow systems, or in stabilizing media such as gels which are polymerized in a thin-layer onto glass plates or a supporting films [2].

A) Carrier-free electrophoresis: The separation principle of a free-flow electrophoresis is described as following: the separation chamber is traversed by continues buffer flow and the electric field is adjusted vertical to the flow direction. Thus, sample components are differentially deflected and impinge on defined parts at the end of the separation chamber. This technique is normally used to separate large particles based on their different charges.
B) Carrier electrophoresis: This method separates molecules based on their different size and charge using a gel or a membrane as support material. The method is described in more detail in chapter 2.2.2.3 (page 28).
Nowadays three different electrophoretic separation methods are used as shown in Figure 9:

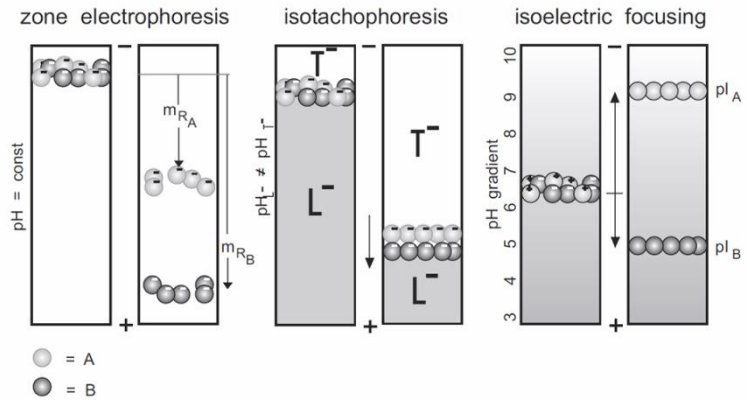


Figure 9: The three electrophoretic separation principles. A, B are sample components. Figure adapted from [2]

Zone electrophoresis (with a carrier or carrier free)

The separation principle of zone electrophorese is based on the different migration velocities of charged particles in an electric field. The electrophoretic mobility is dependent on the physic-chemical properties of the proteins and its charge is influenced again by pH, ionic strength and temperature in the buffer. In most cases basic buffers are used to lead mostly negatively charged proteins to migrate towards the anode. With ionic dyes, in principle very small charged molecules such as bromphenolblue, the relative electrophoretic mobility can

be determined [45]. During the whole separation time a homologues buffer system is used and adjusted to guarantee constant pH-value. According to diffusion, blurred zones can occur during the separation, which is responsible for the reduced resolution and detection limits [2].

Isotachophoresis

Isotachophoresis is also called constant velocity electrophoresis [49]. The electrophoretic separation of either cations or anions takes place in a discontinuous buffer system [49]. The sample of interest migrates between the leading and the terminating ion, which possess the highest and the lowest electrophoretic mobility, however all analytes in one separation zone migrate with the same speed. Thus, the separation is based on different electrophoretic mobilites and on the MWs. Compared with zone electrophoresis, a concentration regulating effect exists which should prevent for diffusion [2].

Isoelectric focusing

Isoelectric focusing is defined as an electrophoretic technique, which is used for the separation of amphoteric molecules with different isoelectric points, which is performed in a pH gradient [49, 50]. Amphoteric substances are nucleic acids, glycoproteins or proteins, which possess acidic and basic buffering groups. Depending on the pH value of the surrounding medium those groups become protonated or deprotonated [46]. In an acidic environment the basic groups become positively charged and contrary in a basic environment the acidic groups become negatively charged. IEF is employed for many different purposes: first dimension in 2D GE or pre-fractionation of complex protein mixtures according to charge. In general two different variants of IEF are available and applied for proteome analysis: (a) carrier ampholine (CA)-based IEF and (b) immobilized pH gradient (IPG)-based IEF. In the first mentioned method a pH gradient is generated during the focusing process by amphoteric, oligoamino-oligocarbonic acids with high buffer capacities at their pIs [51, 52]. However some problem can appear by using CA-based IEF: limited reproducibility because of the use of synthetic CAs which become instable over time, cathodic drift or batch to batch variations of CAs [53, 54]. To overcome these problems immobilized pH gradients were invented [55] which are based on an immobilized pH-gradient (IPG) directly polymerized into the gel [53, 55]. Nowadays different IPG-strips with variations in pH range (narrow or broad; linear or not linear) and length (7-24cm) are available. Both types of IEFs can be combined with a second dimension, usually SDS-PAGE. An IPG gel strip consists of a polyacrylamide matrix into which bifunctional immobiline reagents are copolymerized. Immobilines are acrylamide derivatives with functional groups for weak acids or bases, with a defined pK value. Due to the copolymerization of the acrylamide matrix with

the reactive ends of those acidic and basic immobilines, an extreme stable pH gradient is formed [54, 56]. Thereby an increased reproducibility, loading capacity and resolution as well as a separation of alkaline proteins can be obtained [54, 57].

2.2.2.2 Theory of electrophoresis

On the basis of the different charges and molecular masses of the analysed sample molecules they migrate at different speed when an electric field is applied. This so called electrophoretic mobility (u) is substance specific and determined by the drift velocity of this substance in an electric field [45, 49]. Different forces such as friction force (F_{fr}) and accelerating force (F_e) are interacting on the particles. In the following equation E is the electric field force, e is the elementary charge and z is the charge number [45, 49]:

$$F_e = q * E \qquad q = z * e \qquad \text{(Equation 1)}$$

F_{fr} acts against this motion which results in a certain deceleration of the analytes. This effect arises when large molecules as proteins are moving in a solution and is even more pronounced when a gel matrix is present (for more details see chapter 2.2.2.3 (page 28)). Thereby, joule heat occurs which has to be dissipated, usually by cooling the system (details see chapter 2.2.2.3.2.4 (page 32). The friction force (F_{fr}) is depending on the frictional coefficient (f_c) and on the velocity of migration (v). Equation 2 describes the dependency between F_{fr} and the frictional coefficient (f_c), which again depends on the viscosity of the used medium and the pore size of the matrices [2, 45, 49]:

$$F_{fr} = f_c * v \qquad \text{(Equation 2)}$$

The balance of these two forces causes that the particles are moving with a constant speed in the electric field. This leads to Equation 3, where u is defined as mobility and also as a proportionality factor between the present migration rate and the electric field strength. The electrophoretic mobility is furthermore depending on the pK values of the charged groups and the size of the sample molecules. Besides, the used buffer, its concentration and pH value, as well as the temperature, the field strength and the nature of the support material can have an impact on u [2, 45]:

$$F_e = F_{fr} \qquad q * E = f_c * v \rightarrow v = \frac{q*E}{f_c} = u * E \qquad \text{(Equation 3)}$$

For small spherical analytes and for the calculation of the friction force (F_{fr}) the Stoke's law can be applied, leading to the following equitation for the mobility (u): [46, 49]

$$F_{fr} = 6 * \pi * r * \eta * v \qquad \text{(Equation 4)}$$

$$u = \frac{q}{f_c} = \frac{z \times e}{6 \times \pi \times r \times \eta} \qquad \text{(Equation 5)}$$

Here, η is the viscosity of the liquid medium and r is the radius of the hydrated ion also called Stokes radius. For analytes such as proteins that do not have a spherical shape, an empirical relationship between mobility and the molecular weight (M) can be stated [45, 49]:

$$u = \frac{q}{M^{2/3}} \qquad \text{(Equation 6)}$$

2.2.2.3 Gel Electrophoresis

Gel Electrophoresis (GE) is a method where stable matrices are used to separate and analyse macromolecules according to their charge and size. The separation principle is based on the different migration velocities through the gel matrix according to the different sizes of the sample particles. The charged molecules are pushed by an electric field through the gel that contains small pores. Those molecules will travel through these pores at a speed that is revers related to their size. Thus, small molecules migrate a greater distance than larger molecules. According to their charge they will migrate toward either the positive or negative pole. During the electrophoretic separation Joule heat occurs which has to be dissipated. Therefore it is important to maintain a constant temperature and a cooled chamber during separation to guarantee reproducible results. The gels are cooled directly over water cooling or indirectly through anode and cathode buffer.

Usually a supporting gel matrix is applied for electrophoretic separations. In general two different gel types are used, granulated or compact gels [46]. For GE usually compact gels are used, e.g.: agarose or polyacrylamide gels.

2.2.2.3.1 Agarose gel electrophoresis

Agarose gels are applied when large pore sizes are needed for analysing molecules over 10 nm in diameter. In particular therefore pore sizes from 150 nm (with 1% agarose) to 500 nm (with 0.16% agarose) are used [2]. The pore size is defined by the concentration of agarose, in particular the weight of agarose in relation to the volume of water. For the analysis of serum proteins 0.7% to 1% agarose gels with 1 to 2 nm thicknesses are utilized [46]. Agarose is a polysaccharide which is gained from red seawater by removing the amylopectin. Agarose is characterized according to its melting point and degree of electroendosmosis [2]. The advantages of agarose gels are that the components are not toxic, gels are simply prepared and show a good separation for proteins with MWs over 500 kDa [45]. Disadvantages are that the gels are never free of electroendosmosis effects and a low sieving action is achieved for proteins under 100 kDa [45].

2.2.2.3.2 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel are received through copolymerisation of acrylamide monomers with a cross linking reagent, N, N'-methylenebisacryamide which leads to clear and transparent gels not exhibiting electroendosmosis. Normally Tetramethylethylenediamine (TEMED) is used as radical starter for polymerization and ammonium persulfate as catalyst [45].

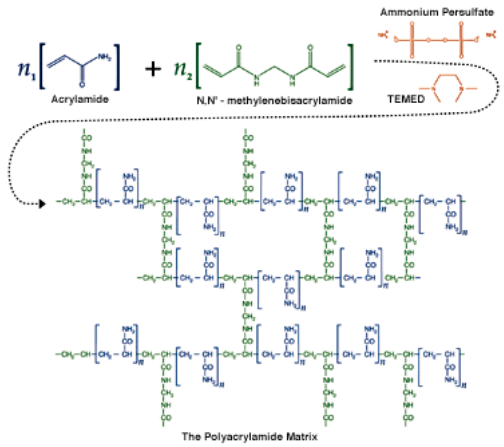


Figure 10: Structure of a polyacrylamide gel. Picture adapted from National diagnostics: Fundamental of Electrophoresis – The Polyacrylamide Matrix by: https://www.nationaldiagnostics.com/electrophoresis/article/polyacrylamide-matrix (retrieved 24.07.2015)

The pore size can be adjusted by the total acrylamide concentration, T, and the degree of cross linking, C [46]:

$$T = \frac{(a+b)\times 100}{V}\ [\%], \quad C = \frac{b\times 100}{a+b}\ [\%] \qquad \text{(Equation 7)}$$

In equation 7, a is the mass of acrylamide (g), b is the mass of methylenbisacrylamide (g) and V is the used volume (mL). If C stays constant and T increases the pore size will decrease. When C increases and T remains constant, the pore size follows a parabolic function leading to, large pore sizes, at high and low C values. Usually T values ranging from 4% to 16% are utilized for the separation of proteins and peptides according to their molecular weight. Higher values very likely lead to stuck proteins. For isoelectric focusing, protein separation according to pI, C values between 2.5% and 3% are used. In general for polymerisation O_2 has to be removed because it leads to chain breakup and interrupt the chain formation. To reduce O_2 uptake gels are polymerised in closed, vertical cassettes or for horizontal systems onto a carrier foil [46]. The effectivity of the polymerisation depends on different parameters like temperature, the pH value of the buffer, and the total acrylamide and catalysts concentrations. The advantages of polyacrylamide gels are that they are chemically inert and mechanically stable. Furthermore they possess a good sieving characteristic over a wide separation range and faster separation times still maintain well defined protein bands. Furthermore acrylamide gels are compatible with several staining techniques. The disadvantages of polyacrylamide gels are the toxicity of the monomers, the pore size is limited to proteins smaller than 200 kDa and the basic pH reduces the shelf life of the gels [45].

2.2.2.3.2.1 Gradient Gels

By changing the acrylamide concentration in the polymerisation solution during pouring the gel a pore gradient gel is obtained. For the preparation of gels with linear or exponential gradients two polymerisation solutions with different monomer concentrations are continuously mixed in a mixing chamber during casting, so that T decreases from bottom to top in the cassette shown in Figure 11. The solution of higher PA concentration is also mixed with saccharose or glycerols to avoid mixing of different layers in the gel casting cassette. To achieve a linear gradient both liquid levels have to be at equal height throughout pouring the gradient. For exponential gradients a stopper is placed in the mixing chamber, so the volume is fixed [2, 45]. Gradient gels are applied as immobilized pH gradient for isoelectric focusing, as porosity gradients in blue native electrophoresis and partly in SDS gel electrophoresis.

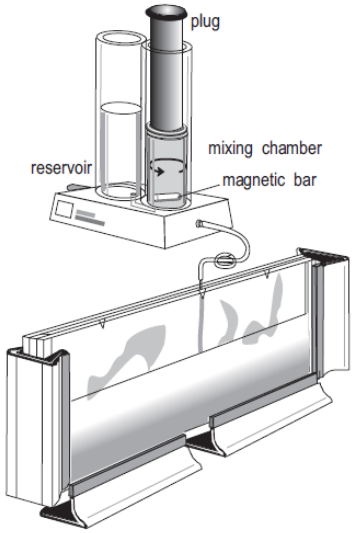


Figure 11: Preparation of a gradient gel. Picture adapted from [2]

2.2.2.3.2.2 SDS-PAGE

SDS-PAGE is considered to be the most applied electrophoretic technique for proteome analysis and characterisations. It was introduced by Ulrich K. Laemmli who invented this discontinues electrophoretic system to separate proteins with molecular weights between 5 and 250 kDa [58]. It is employed for many different purposes: as second dimension in 2D GE and Blue native electrophoresis or as 1D protein separation prior to tryptic digestion and LC-MS. Sample preparation is an essential step for the success of the SDS-PAGE. Because unlike in IEF (see chapter 2.2.2.1 (page 7), the intrinsic charge of the proteins should not have influence on the separation. Therefore SDS, a strong anionic detergent which solubilizes mostly all proteins and masks their charges by forming anionic micelles with a constant net charge per masse unit (1.4 g SDS / g protein), is added in excess to the samples and heated [45]. SDS disrupts hydrogen bonds and electrostatic interactions and therefore unfolds the secondary and tertiary structure of a protein. Furthermore to prevent back folding and aggregating of subunits, reducing agents as ß-mercaptoethanol or dithiothreitol, are added to cleave covalent disulfide bonds, which stabilize the tertiary and secondary structure. Due to the high resolution, which can be achieved with the discontinuous electrophoresis, a SDS containing discontinuous Tris-chloride/Tris-glycine buffer system is used as standard for protein separation [58]. A stacking gel (Tris-glycine buffer, pH 6.8; 3-4% acrylamide) overlays usually a separation gel (Tris-glycine buffer, pH 8.8; 5-20% acrylamide). The longer the separation gel is, the better is the resulting separation and the thinner the gel is, the sharper and clearer are the receiving bands [50]. Schägger and Jagow [59] introduced a method where they increased the molarity of the buffer and replaced glycine by tricine. Due to that, a better resolution of low molecular weight proteins and peptides, especially in the range between 5 and 20 kDa, was achieved at lower acrylamide concentrations, than in Tris-glycine-SDS PAGE systems.

2.2.2.3.2.3 Discontinues Gel Electrophoresis

With the help of discontinuous electrophoresis high resolution is achieved, sharp protein bands are obtained and aggregation and precipitation are prevented. The term “discontinuity” refers to the application of different gel structures (small or large pore) and various pH values, ionic strengths and buffer species [49]. In general, discontinuous gels are prepared by pouring a resolving gel into a glass cassette and then a stacking gel is polymerized on top of that gel as shown in Figure 12. Both gels differ in buffer composition, strength and pH, and pore size. The resolving gel shows a smaller pore size compare to the stacking gel, which is normally applied for one dimensional sodium dodecyl sulphate.

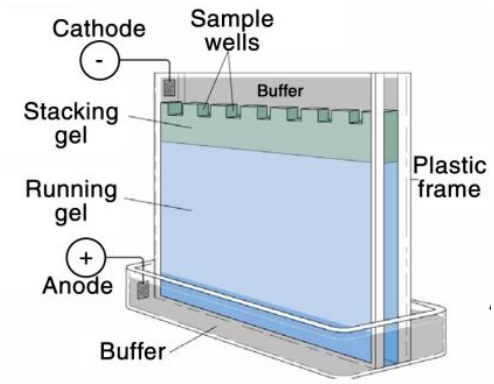


Figure 12: A depiction of a discontinuous gel. Picture adapted from http://www.sapd.es/revista/article.php?file=vol33_n3/03 (retrieved 24.07.2015)

2.2.2.3.2.4 Modern gel electrophoretic systems

Nowadays electrophoretic separation can be carried out either in a vertical or a horizontal system. Almost 40 years ago O’Farrell introduced the first approach for proteome analysis via high-resolution 2D GE. He combines IEF as first dimension and discontinuous SDS-PAGE in vertical glass cassettes as second dimension for the separation of proteins completely under denaturing conditions [52]. Difficulties concerning reproducibly were solved for the first dimension by the introduction of immobilised pH gradients, polymerized onto a precast film-backed gel [44].

Traditional SDS-PAGE is performed in vertical systems. The gels are completely covered in glass cassettes containing buffer. The samples are applied into wells (Figure 13), which are formed during the polymerization step with a comb. Glycerol is added to sample buffer to keep the samples in the well [45]. Vertical systems have a higher protein amount loading capacity, because thicker gels can be used. However blotting of those gels is disadvantageous and could lead to protein loss. Further disadvantages of this system are that it is limited in technical possibilities, it consists of many pieces which have to be cleaned

and set up and thinner gels cannot be used because it's complicated for sample application and the IPG strip would not fit between the glass plates. Furthermore the conventional way of running SDS-PAGE gels employs a high volume of buffers to guarantee effective cooling during the separation and to prevent blurred zones and U-shaped forming separation front ("smiling effect") [60].

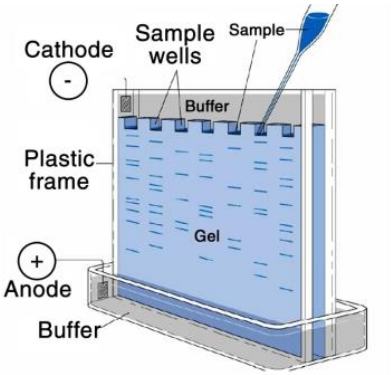


Figure 13: Setup for a vertical electrophoresis system. Picture adapted from http://www.sapd.es/revista/article.php?file=vol33_n3/03 (retrieved 24.07.2015)

In contrast the second dimension of the 2D GE can also be performed in a horizontal system. Through the introduction of a high performance electrophoresis (HPE) setup for the second dimension and precast polyacrylamide gels on film-backing, a higher resolution, sharper protein spots and better reproducibly results were obtained [60]. Furthermore those gels can be run on a multilevel flatbed electrophoresis apparatus. The rehydrated IPG strip, containing the analysed sample, can be applied directly into a narrow trench on the gel surface. Thus, a complete transfer of all proteins from the IPG-strip into the SDS gel is offered. Furthermore through the possibility that the gel layers can be thinner an effective cooling can be obtained which supports separation and reproducibility leading to faster separation times and sharper spots. Those facts are especially beneficial for two dimensional gel electrophoresis [2, 45, 46]. Furthermore low molecular weight proteins were able to with the horizontal setup. This is due the following facts: To maintain a long-term stability of the HPE gels, they were polymerized with a buffer with pH below 7, in order to prevent alkaline hydrolysis. Therefore, it is necessary to replace the glycine in the running buffer of traditional SDS-PAGE gels by tricine in case of HPE gels, in order to achieve a good separation quality. Through tricine the resolution in a HPE gel is extended down to 6 kDa, in contrast to glycine containing buffer system. For this master thesis a high performance electrophoresis (HPE) setup for the second dimension and HPE large gels, NF, of 12.5% T and 2% C in the resolving gel were used [60]. Figure 14 shows the used HPE Tower.

Figure 14: HPE tower from Serva used for the second dimension.

2.2.2.4 *Two-dimensional polyacrylamide gel electrophoresis*

The method with the highest resolving power for the analysis of complex protein mixtures is the two-dimensional gel electrophoresis (2D GE). The 2D GE was performed under native conditions and combined two orthogonal separation techniques, leading to a higher separation efficiency as well as higher numbers of analysed proteins and peptides. In the first dimension the sample was separated according to the isoelectric points of the constituents, (details see 2.2.2.1 (page 24). In the second dimension the analyte was separated according to the MW using SDS-PAGE and was carried out either in a horizontal flat-bed apparatus or in a vertical gel chamber (details see chapter 2.2.2.3.2.4 (page 32) [61]. Through the introduction of completely denaturing conditions by O'Farrell [52] whole cell contents were successfully separated leading to thousands of protein spots. These conditions are achieved by the presence of non-charged chaotropes (e.g. urea and thiourea), reducing agents and non-ionic or zwitterionic detergent during sample preparation and in the focused gel. Furthermore it is possible to visualize over 10.000 spots on one single gel. Figure 16 shows the working principle for 2D GE.

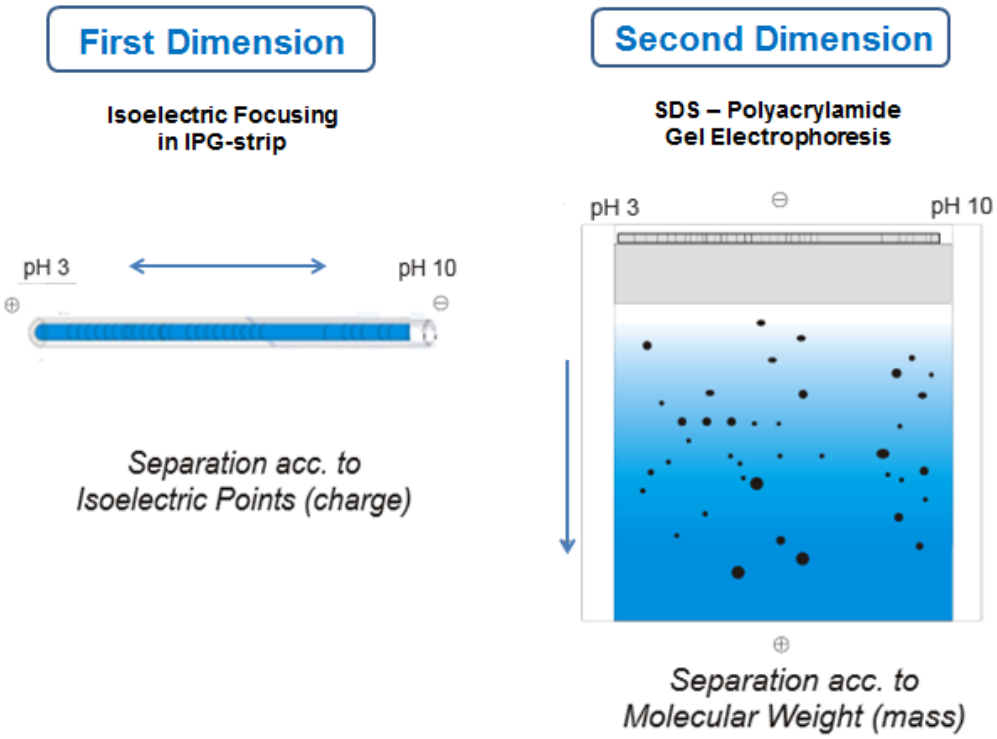


Figure 15: Principle of 2D GE.

2.2.3 Protein Visualisation

Proteins which are separated in a polyacrylamide gel are colourless. Therefore protein staining is used to enable visualisation of separated proteins. In general an ideal dye should bind to the proteins and the binding should be proportional to the protein concentration as well as it should not show any saturation effects to enable the quantitative evaluation. Furthermore the staining process should have a broad dynamic range, be very sensitive, visualize low abundant proteins and ideally be compatible with mass spectrometry for further analyses. Table 6 lists different protein visualisation methods.

Coomassie brilliant blue (CBB) is a triphenylmethane dye binding to basic side chains of amino acids, is easy to use and costs are low. Yet it is not very sensitive and the linear dynamic range covers only two orders of magnitude. CBB staining is preferred to determine relative amounts of proteins because it binds stoichiometrically to the protein.

Silver staining is more sensitive compared with CBB. The limit of detection is 1 ng of protein (more details see chapter 2.2.3.1.1 (page 37)).

Fluorescence staining can be used as pre or post electrophoretic stain and provides an alternative to prior mentioned methods. The labelling process is much more sensitive and shows a wider linear dynamic range, however it is very expensive (more details see chapter 2.2.3.2 (page 38) [25, 62].

Staining method	Detection Method	Limit of Detection (ng)	Linear range (orders of magnitude)
Post-staining			
CBB-R	Colorimetry	8-10	1-1.3
CBB-G (colloidal)	Colorimetry	8-10	1-1.3
Silver nitrate	Colorimetry	1	2
Serva Purple	Fluorescence	<1	4
Pre-staining			
CyDyes® saturation labeling	Fluorescence	0.1-0.2	3-5
CyDyes® minimal labeling	Fluorescence	0.005-0.01	3-5

Table 6: General protein stains and their sensitivity ranges. Table adapted from [63]

2.2.3.1 Post-electrophoretic stains

2.2.3.1.1 Silver nitrate staining

Silver nitrate used under acidic or basic conditions is a staining method that resembles to the process of developing photos. It was introduced 1979 to detect proteins after electrophoretic separation [64]. It has a high sensitivity, which is 10 times higher than colloidal Coomassie blue staining and 100 times higher than classical Coomassie brilliant blue [3, 65]. The staining method is used in Proteomics because it combines high sensitivity with cheap equipment and chemicals. However former protocols suffer from high background and silver mirrors. Two more facts have to be considered: (a) the compatibility with mass spectrometry (MS) for subsequent protein identification, due to some chemicals used for staining, like glutaraldehyde, can interfere with downstream analysis and the limited quantitative response, because different proteins tend to interact differently with silver ions, and (b) saturation effects or negative staining ("doughnut effect") of highly abundant spots are very likely. During the years over 100 different silver staining protocols were published and improved, however all based on the same principle.

First step is the fixation. In this step disturbing compounds are removed like carrier ampholytes, SDS or Tris and proteins are insolubilized in the gel. For high sensitivity proteins are usually crosslinked with Glutaraldehyde in the gel. However this is not compatible with MS and fixation is usually limited to alcohol treatment to precipitate the proteins in the gel.

The second step is sensitization; it should support image formation.

The third step is silver impregnation with ammoniacal silver or acidic silver nitrate. Ammoniacal silver staining is said to be more sensitive for basic proteins [66]. However, the ammonia concentration (storage) is a critical point for the reproducibility [63] and it has also been shown that ammoniacal silver staining is not compatible with mass spectrometry. On the other hand acidic silver nitrate staining can be used for gels after IEF and SDS-PAGE and is compatible with mass spectrometry. Therefore glutaraldehyde should be avoided in the sensitizing solution and formaldehyde in the silver nitrate solution.

The fourth step is gel development. Because of the impregnation step, silver ions are bound to proteins, in particular to negatively charged side chains (glutamic acid, cysteine and aspartic acid). The image development can in general be done with dilute carbonate, for acid staining, or citric acid for alkaline staining. In both cases a minor amount of formaldehyde is added. Through the reduction, silver ions are reduced to elementary silver, which consequently stain proteins in gels black, yellowish brown and gold with respect to their nature. Development is stopped when the desired image level is obtained by putting the gel into the stopping solution. Normally it contains acetic acid, in case of ammoniacal silver, or an amide, in case of silver nitrate, to reach a pH of seven. With this staining method it is possible to detect between 100 pg and 1 ng protein per band. However silver staining shows

a limited dynamic (refer to Table 6) range and as a consequence of that is not reliable for quantification. Also highly abundant spots can be saturated or negatively stained which leads to poor linearity [3].

2.2.3.1.2 Fluorescence staining

Several fluorescent dyes are available for post staining processes. They are appropriated for the quantification of proteins, because they provide high reliable sensitivity down to < 50 pg and they cover a wide dynamic range, about four orders of magnitude. Well established and sensitive dyes are Flamingo Pink™, Sypro Ruby and RuBPS, which are based on heavy metal ions and are chemically synthesize. Other fluorescent dye, like Serva purple, are naturally occurring fluorophors, which are compatible with mass spectrometry and DIGE approaches. Serva Purple binds reversible to lysine, arginine and histidine residues in proteins [67, 68] and is produced by the fungus *Epicoccum nigrus*. Serva purple is moreover sustainable as it is biodegradable, possesses very low background fluorescence and produces gels that are more stable as no highly concentrated organic solvents is required. However for the visual detection a fluorescence imager is needed in case of fluorescence dyes [67, 68].

2.2.3.2 Pre-electrophoretic labeling – the basis for difference gel electrophoresis (DIGE)

Difference gel electrophoresis (DIGE) is an method which allows multiplexed detection of co-migrated proteins due to the fact that all protein samples are labeled with different, spectrally distinct fluorescent dyes prior to electrophoretic separation as shown in Figure 16 [45, 46]. It was first described by Ünlü in 1997 [69]. After separate labeling all samples are pooled and run on the same 2D gel. This enables the same condition for all analysed protein samples, which eliminates gel-to-gel variations. Applying an internal standard, a pooled sample of all single samples within one experiment, is used for the normalization of the data between the gels allowing easy gel-to-gel comparison by increasing the confidence of the match and [45, 46] minimizing experimental variation [70].

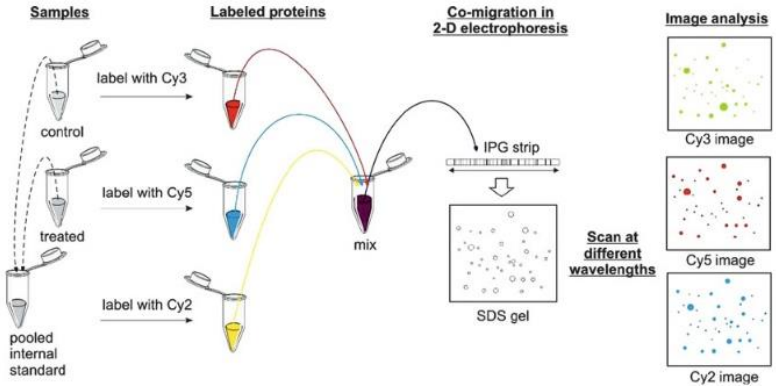


Figure 16: Principle of a difference gel electrophoresis (DIGE) approach, showing how to create an internal standard, which is labeled with Cy2. The samples are labeled with Cy5 and Cy3. Figure adapted from [1]

There are two labeling types available: minimal and saturation labeling.

2.2.3.2.1 Minimal Labeling (Lysine Labeling)

Minimal labeling means that the dye/protein ratio is kept low (at 3% or lower) to ensure that only a single lysine in each protein is labeled. This should prevent the appearance of multiple labels which could lead to multiple vertical spots per protein and reduction sample solubility [63]. Today, many different labeling kits are commercially available. One of them contains three different cyanine-based dyes (CyDyes®, Cy2, Cy3, and Cy5). Those dyes are coupled with an N-hydroxy succinimidyl ester, which reacts covalently and irreversibly with the Ɛ-amino groups of lysine side chains via an amide linkage, giving a sensitive and stable fluorescent signal. For this method it is very important that the dyes are size and charge matched. The used dyes are pK_a matched with the Ɛ-amino group of lysine to avoid pI shifts from protein of interest during labelling, by adding a basic buffering group to each dye [71] which compensates the loss of the positive charge. Further, the increase in molecular mass between labeled (1-3%) and unlabeled (97-99%) proteins differ from 434 to 464 Da depending on the attached dye [71, 72]. This little mass increase is not possible to resolve by SDS-PAGE and have an insignificant impact on the electrophoretic migration (spot position) in the 2D gel [71]. Furthermore it is important that the pH value of the sample is above 8.0, because the optimum of this labeling reaction lies at pH 8.5. Minimal labeling was used during this master thesis because an unknown sample was analysed and lysine is one of the common occurring amino acids in proteins [1, 45].

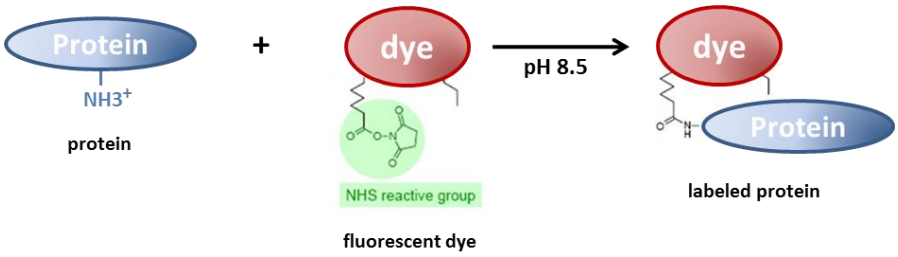


Figure 17: Principle of minimal labeling (lysine labeling).

2.2.3.2.2 Saturation Labeling (Cysteine Labeling)

The second technique, the saturation or cysteine labeling, tags every cysteine within a protein. The dye contains a maleimide group which covalently binds to cysteine via thioether linkage, shown in Figure 18, to avoid pI shifts [63]. Indeed the obtained 2D gel pattern could not be compared with those obtained from minimal labeled or non-labeled; because all accessible cysteine within a protein are labeled leading to slower migration of those proteins during SDS-PAGE and a stronger light emission signal. Therefore it is important that the

dyes are size matched. The mass shift is about 650 Da per label [46]. Prior to the labeling a reducing agent is used to cleave disulfide bridges. In contrast to lysine labeling the dye is coupled with a reactive maleimide group, the temperature is higher and reaction time is longer. It may seem that saturation labeling is more sensitive than minimal labeling; however cysteine is less present in proteins compared to lysine [1, 45]. Another important point is that the quantitative amount of cysteines in the sample cannot be predicted which demands high efforts for sample optimization. Often a pI shift of labelled proteins towards the acidic end can be observed, because lysine is non-specifically labeled due to dye excess. On the other hand, if not enough dye is applied for cysteine labeling, some proteins will migrate faster due to lower molecular sizes resulting in vertical streaks or elongated spots [63]. For saturation labeling only two cyanine-based dyes (Cy3 and Cy5) are available.

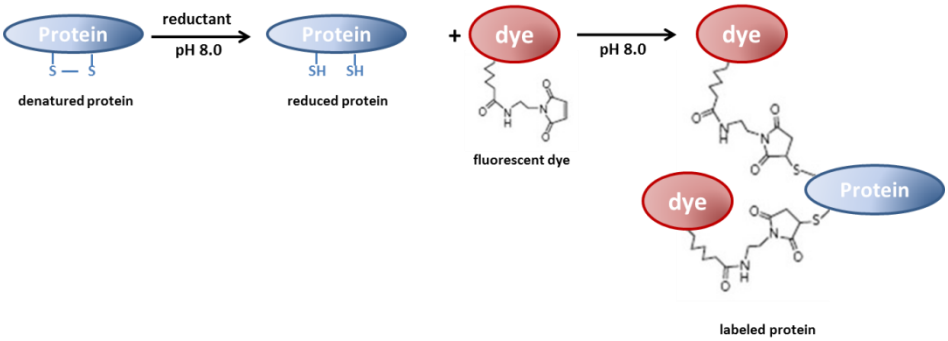


Figure 18: Principle of saturation labeling (cysteine labeling).

Beside the CyDyes® from GE Healthcare further developed technologies for multiplex-fluorescence 2D GE are available like the Refraction-2D™ Labeling Kit (G-Dyes) from NHDyeAGNOSTICS. This labeling kit contains four fluorescent dyes and those are photostable compare to CyDyes®. Both technologies are compatible with other staining protocols and do not interfere protein identification by mass spectrometry.

2.2.3.3 Image Analysis

For DIGE analysis the scanning of the gels at different wavelengths corresponding to the used dyes with a fluorescent imager is necessary. The signal measure with the imager is proportional to the amount of labeled protein because the dyes are excited with monochromatic light and therefore they emit light which is in proportion with the labeled protein in the sample. Through the visual comparison of the gels the sample preparation and labeling as well as the separation quality can be controlled. Following this the generated image has to be analysed (Figure 19). Therefore image analysis software of different kinds, from various companies, are available for the final evaluation of the achieved results.

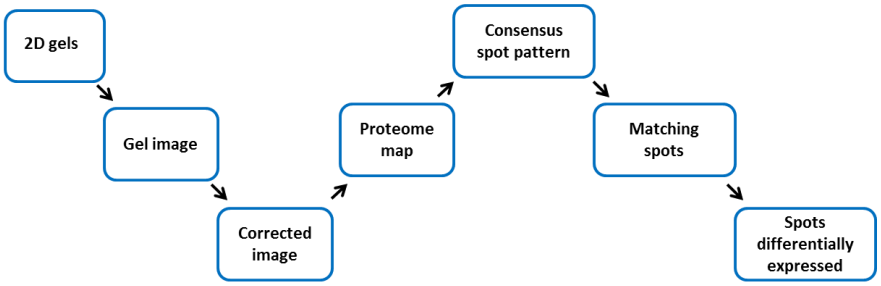


Figure 19: Strategy of the evaluation of a 2D gel, starting by getting a gel image and leading to identify different expressed spots.

For a quantitative analysis of separated proteins, alignment of the gel spots over multiple gels is essential and to quantify the intensities of the gel spots on the different gels, image analysis software are necessary. The gels are digitalized to detect differentially expressed proteins by a robust statistical analysis. The digitalization is done with either a special scanner allowing standardized scanning conditions or in case of fluorescence staining, with a fluorescent scanner [73-75].

In case of DIGE, image acquisition is crucial. Good quality of the raw data is very important and influences the final results. After receiving the images, an image warping step is done. It removes variations in the same spot position on the gel replicates and contributes to a good spot matching. After this a fused image is compiled which represents the summary of all proteins detected on all gels within an experiment. This compiled image is also called proteome map. Based on that, spot positions, their boundaries and their quantities are determined. The next critical step is the normalization of the spot quantities. The aim behind that step is to mitigate systemic differences between the gel images. A subsequent spot matching step is carried out to compare spot intensities, where each spot on a gel is mapped to its corresponding spot on others. Through application of consensus patterns, spot boundaries from the new created proteome map are transferred and compared to the original image and demodulated. The intensities of all pixels within those boundaries are summed up and so spot quantities are received. Further statistical tests need to be done to indicate and confirm the changes [73-75].

3 Materials and Methods

3.1 Stem cell cultivation

Stem cell culture supernatant preparation was performed by the working group of Cornelia Kasper at the Vienna University of Natural Resources and Life Sciences according to the following protocol:

3.1.1 Chemicals

Product	Product number	Company
Adipose derived MSCs; adMSC101013		AKH Vienna
αMEM	12000-63	
Human serum		From blood bank in Linz
Gentamycin sulphate	882067	Biozym

3.1.2 Requirements

- medium with 10% (human) serum
- control medium and supernatants prepared and treated identically (except for the absence/presence of cells)
- supernatant should be as concentrated as possible (low liquid volume per cell)
- large amount of supernatant and cell-free control medium (ca 100 mL each) but no replicas

3.1.3 Materials

- Cells: adipose derived human MSCs; adMSC101013
    - Cells isolated from adipose tissue (provided by plastic surgeons at AKH Wien) on 10.10.2013 by VC at AG Kasper according to group internal protocol.
    - Characterization:
        - tested negative for mycoplasma in Nov 2013 (P1), GK
        - Cells correctly present MSC surface marker (analysis of CD expression using a flow cytometry kit; 24.10.2013, P1, VC)
        - successful differentiation (application of Miltenyi Differentiation media for 11 days) into osteogenic, adipogenic and chondrogenic lineage (analysis: ECM specific staining's); Nov 2013, VC
        - Aliquot used for generation of supernatants: 4 cryovials (stored in N2 tank), P3, 0.5Mio cells/vial, normoxie, frozen on 31.01.2014, VC

- Medium: alphaMEM with 10% human serum and 0.5% gentamicin
    - αMEM: Gibco 12000-063 powder for 10 L / Lot: 1391544X 2 L prepared on 03.03.2014, VC; 2.2 g/L NaHCO3 added; sterile tested before use
    - human serum: from blood bank Linz; stored at -20°C in 40 mL aliquots; tube used labeled as “huS F&E (2) 11.11.13 DT 9/44”
    - gentamycin: Gentamycin sulphate 10mg/ml Lonza 882067; BE02-012E; Lot: 2MB257; stored at ambient temperature
- Centrifuge: Eppendorf 5702 (with holders for 4x 50 mL tubes), sing out rotor

3.1.4 Experimental Protocol

- **Cell thawing**: cells adMSC101013 P3 were thawed (4 cryovials)
    - thawing according to AG Kasper group internal protocol
    - viability after thawing: 74%; total (viable) cell count: 1,28Mio
    - cells cultivated in 1T175 (seeding = 8143 cells/cm²) P4, static cultivation
    - cultivation in cell culture (normoxie) incubator

- **Cell splitting**: adMSC101013 1T175 P4 were split into 6xT175
    - splitting according to AG Kasper group internal protocol
    - P4: cells look good, ca 50-60% confluent; 3.55Mio viable cells (=20000cells/cm²)
    - P5: seeding of 3380cells/cm² (split ratio ca 1:6); 20 mL medium/flask
    - further cultivation in normoxie incubator
- **Medium preparation**

  400 mL medium were prepared (for supernatants and control medium)
    - medium preparation according to AG Kasper group internal protocol
    - 358 mL alphaMEM
    - 40 mL human serum: frozen tube was thawed in fridge overnight (13.-14.03.2014); directly before use the serum was centrifuged 10min at 3000 xg, only the supernatant was used;
    - 2 mL gentamycin
    - medium was stored in fridge (4°C) in glass Schott flask until use
- **Application of Medium for secretome enrichment**

  Medium in the adMSC101013 P5 flasks was changed to the medium prepared on 14.03.2014
    - adMSC101013 P5, 6xT175; cells looked very good, ca 90-95% confluent
    - the old medium was discarded (vacuum suction pump) from each flask without disturbing the cell layer

- 20 mL of fresh preheated (37°C water bath) medium (prepared on 14.03.2014) was gently added to each flask
- additionally - for the cell-free control – 120 mL of the medium prepared on 14.03.2014 were transferred to one T175 flask and also put in the normoxie incubator (lying position)

- **19.03.2014: Replenishment of Medium**

  additional medium was added to the cell culture flasks to ensure sufficient nutrient supply and avoid cell stress/death
  - adMSC101013 look good in all 6 flasks; very confluent (>100%); medium starts turning orange
  - 5 mL of the medium prepared on 14.03.2014 were added to each flask
  - accordingly 6x5=30 mL of the medium were also added to the cell-free control flask
  - all flasks were put back into the normoxie incubator
- **21.03.2014 Collection of Supernatants**

  Cell supernatant and cell-free control medium were harvested and transferred to TU Wien.
  - adMSC101013 cells look good in all 6 flasks; even more cells than on 19.03.2013; however low number of rounded (dividing) cells (contact inhibition)
  - Cell-free medium:
    - 4x50 mL centrifuge tubes were filled with 37.5 mL of the cell-free sample each
    - 10min centrifugation at 300xg --> no pellet visible
    - Supernatants (ca 36 mL) transferred to fresh 50 mL tubes (last mL medium discarded)
    - 10min centrifugation at 3000xg (=max speed) --> no pellet visible
    - Supernatants (ca 35 mL) transferred to sterile glass Schott bottle (total ca 140 mL)
    - put immediately on ice and transferred to TU Wien on ice
  - Cell culture supernatant
    - Supernatant from 6 T175 collected (ca 25 mL/flask) and transferred to 4x50 mL tubes
    - 10min centrifugation at 300xg --> small pellet (ring) visible (dead/detached cells and large cell debris)
    - Supernatants (ca 36.5 mL/tube) transferred to fresh 50 mL tubes (last bit of medium and pellets discarded)
    - 10min centrifugation at 3000xg (=max speed) --> no pellet visible

        - Supernatants (ca 35 mL) transferred to sterile glass Schott bottle (total ca 140 mL)
        - put immediately on ice and transferred to TU Wien on ice
    - Cell harvest and counting
        - to define the final cell number cells from all 6 T175 were harvested (standard AG Kasper group internal protocol); long (ca 7 min) incubation times were allowed for detachment
        - cells detached as complete layers (not as single cells or small cell aggregates)
            - counting in hemocytometer not possible
            - instead: counting of nuclei in coulter counter (according to AG Kasper group internal protocol)
        - all cells were harvested into one 50 mL tube (total 36 mL)
        - the tube was centrifuged 5 min at 300xg
            - cell layers sedimented, but failed to form a nice, dense cell pellet
        - supernatant was discarded as well as possible (ca 5,5 mL remain to avoid losing cells)
        - The cell pellet (an remaining supernatant) were mixed with 7 mL coulter counter lysis buffer
            - Lysis Buffer: prepared by WS (Kunert group) in Jan 2014, non-sterile, stored at 4°C
        - Tube was vortexed and put in fridge for ca 15 min.
        - Quite a lot of aggregated cell layer remains --> 18.5 mL more lysis buffer was added --> total 30 mL
        - Sample was again vortexed and put in fridge for 1.5 h
        - A few structures were still not dissolved
        - However, coulter counter analysis was performed (with help of WS)
        - A total of 1,04x10^8 cells +/-1Mio cells was counted. This corresponds to ~100 000 cells/cm² in the 6xT175 flasks and an average doubling time of 34.5 h.

- **21.03.2014 Protein concentration and aliquot storage**

  Protein content in both samples was determined and samples were frozen in aliquots.

3.2 Determination of Protein concentration (Bradford Assay)

3.2.1 Chemical

Product	Product number	Company
Bovine serum albumin (BSA)	A8022-10G	Sigma Aldrich
Coomassie Brilliant Blue G250 (CBB)	27815	Fluka
Phosphoric acid	4380815	Sigma Aldrich
Ethanol	1.00983.2500	Merck
Urea	0.568-1KG	Amresco
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
CHAPS	C9426-5G	Sigma Aldrich
Ultra-high quality water (UHQ) from a Simplicity …. **(18.2 MΩ cm resistivity at 25 °C)**		Milipore Molsheim

3.2.2 Equipment

Product	Company
Nano Photometer	Implen
Cuvette	
Centrifuge mini star, silver line	VWR
Thermomixer comfort	Eppendorf

3.2.3 Working solution

Bradford reagent (in total 400 mL)	
0.01% Coomassie Brilliant Blue G250 (CBB)	40 mg
1.6M Phosphoric acid	40 mL
0.8M Ethanol	18.8 mL
UHQ	adjust to 400 mL

BSA – stock solution	
Stock solution BSA	1 mg / mL in IPG – buffer

IPG – buffer (in total 50 mL)	
7M Urea	21.02 g
2M Thiourea	7.61 g
2% CHAPS	1 g
UHQ	adjust to 50 mL

3.2.4 Protocol

- Bradford reagent: 40 mg CBB, 40 mL phosphoric acid and 18.8 mL ethanol were mixed and stirred for three hours or overnight in the hood
- Then, the volume was filled up to 400 mL with UHQ
- It is stored at 4°C protected from light and the reagent was filtrated each time before use

- Calibration: BSA stock solution was diluted to concentrations of 1 µL/mL, 2 µL/mL, 4 µL/mL, 7 µL/mL and 10 µL/mL with IPG-buffer
- Two blanks were prepared (2 x 10 µL IPG-buffer)
- Precipitated samples (preparation shown in chapter 3.3.4 (page 48)) were diluted (1:1000) with IPG-buffer
- 10 µL of each BSA standard, samples and blanks were mixed with 90 µL fresh filtrated Bradford reagent
- The determination of the protein concentration was performed at 595 nm with the Nano Photometer

3.3 Protein precipitation

3.3.1 Chemicals

Product	Product number	Company
Trichloroacetic acid (TCA)	1.00807.0250	Merck
Acetone	1.00014.2500	Merck
Urea	0.568-1KG	Amresco
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
CHAPS	C9426-5G	Sigma Aldrich

3.3.2 Equipment

Product	Company
Refrigerated centrifuge 3-30K	Sigma Aldrich
Centrifuge tubes, 15 ml	VWR
Centrifuge mini star, silver line	VWR
Rotor 12171	VWR
Thermomixer comfort	Eppendorf

3.3.3 Working solution

TCA - solution (in total 13 mL)	
TCA	10 g
UHQ	7 mL

Acetone – solution (80%)	
Acetone	40 mL
UHQ	10 mL

IPG – buffer (in total 50 mL)	
7M Urea	21.02 g
2M Thiourea	7.61 g
2% CHAPS	1 g
UHQ	adjust to 50 mL

3.3.4 Protocol

- The supernatant collected at Vienna University of Natural Resources and Life Sciences (details see chapter 3.1.4.) was used for further analysis
- Protein precipitation: 1 mL sample was mixed with 1 mL TCA and 8 mL ice-cold acetone, mixture was kept at –20°C for one hour
- Samples were centrifuged at 14000 x g for 15 minutes at 4°C
- Supernatant was discard, pellets were washed first with 2 mL ice-cold 80% acetone and afterwards twice with 2 mL ice-cold 100% acetone
- During washing steps samples were centrifuged at 14000 x g for 15 minutes at 4°C
- Final pellets were dried and dissolved in 60 µL of IPG buffer at 4°C overnight

3.4 Depletion of High-abundance proteins

3.4.1 Combinatorial hexapeptide library

3.4.1.1 Chemicals

Product	Product number	Company
Proteominer ™ Protein Enrichment Kit	163-3006	BioRad
Washing buffer	163-3006	BioRad
Elution reagent	163-3006	BioRad
Rehydration reagent	163-3006	BioRad
Trichloroacetic acid	1.00807.0250	Merck
Acetone	1.00014.2500	Merck
Urea	0.568-1KG	Amresco
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
CHAPS	C9426-5G	Sigma Aldrich
UHQ		

3.4.1.2 Equipment

Product	Company
Analog Vortex Mixer	VWR
Thermomixer comfort	Eppendorf
Refrigerated centrifuge 3-30K	Sigma Aldrich
Centrifuge mini star, silver line	VWR

3.4.1.3 Working solution

Proteominer ™ Washing buffer (in total 50 mL)
Phosphate-buffered saline buffer (PBS) (150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4)

Proteominer ™ Elution reagent (2X vials)
Lyophilized Urea (8 M) and CHAPS (2 %)

Proteominer ™ Rehydration reagent (in total 5 mL)
5% Acetic Acid

TCA - solution (in total 13mL)	
TCA	10 g
UHQ	7 mL

Acetone – solution (80%)	
Acetone	40 mL
UHQ	10 mL

IPG – buffer (in total 50 mL)	
7M Urea	21.02 g
2M Thiourea	7.61 g
2% CHAPS	1 g
UHQ	adjust to 50 mL

3.4.1.4 Protocol

- First columns were centrifuged at 1000 x g for 30-60 seconds for removing storage solution (20% beads, v/v aqueous EtoH, 0.5% v/v ACN)
- Beads were washed with 200 µL washing buffer and shaken on the thermomixer for 5 minutes
- Columns were again centrifuged at 1000 x g for 30-60 seconds, the flow through was discarded
- Washing step was repeated two times
- Columns contain 20 µL of settled beads
- 200 µL of precipitated sample were applied on the column and shaken on a thermomixer at room temperature for two hours and vortexed every 5 minutes
- To remove unbound proteins, columns were centrifuged at 1000 x g for 30-60 seconds
- Washing step was performed twice with 200 µL washing buffer and once with 200 µL UHQ
- Between the washing steps the columns were shaken on the thermomixer at room temperature for 5 minutes and then centrifuged at 1000 x g for 30-60 seconds
- 610 µL rehydration reagent were added to the lyophilized elution reagent to prepare the elution reagent
- 20 µL of elution reagent were added to each column and vortexed several times over a period of 15 minutes and then centrifuged at 1000 x g for 30-60 seconds always in the same collection tube
- The entire elution step was repeated four times

- Eluted proteins were again precipitated (as described in chapter 3.3.4.) to remove the acetic acid, which disturbs 2D GE.

3.4.2 Top 12 High-abundance proteins depletion columns

3.4.2.1 Chemicals

Product	Product number	Company
Pierce ™ Top 12 Abundant Protein Depletion Spin Columns	85164	Thermofisher
TCA	1.00807.0250	Merck
Acetone	1.00014.2500	Merck
Urea	0.568-1KG	Amresco
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
CHAPS	C9426-5G	Sigma Aldrich
UHQ		

3.4.2.2 Equipment

Product	Company
Analog Vortex Mixer	VWR
Thermomixer comfort	Eppendorf
Refrigerated centrifuge 3-30K	Sigma Aldrich
Centrifuge mini star, silver line	VWR

3.4.2.3 Working solution

Pierce ™ Top 12 Abundant Protein Depletion Spin Columns
6 columns; 62% slurry in 10 mM PBS, 0.15 M NaCl, 0.02% sodium azide, pH 7.4

TCA - solution (in total 13 mL)	
TCA	10 g
UHQ	7 mL

Acetone – solution (80%)	
Acetone	40 mL
UHQ	10 mL

IPG – buffer (in total 50 mL)	
7M Urea	21.02 g
2M Thiourea	7.61 g
2% CHAPS	1 g
UHQ	adjust to 50 mL

3.4.2.4 Protocol

- Columns were equilibrated at room temperature for several minutes
- 10 µL samples (always over 200 µg in total) were applied directly to the resin slurry, columns were then inverted several times
- Columns were shaken on a thermomixer for one hour and vortexed every 5 minutes
- Columns were placed in fresh collection tubes and were centrifuged at 1000 x g for 2 minutes
- Eluted samples were then dissolved in PBS buffer
- For further 2D GE experiments, samples were again precipitated (as described in chapter 3.3.4.)

3.5 1D GE

3.5.1 Chemicals

Product	Product number	Company
Nupage 4-12% Bis-Tris gel , 1.0mm x 15 well	NP0323BOX	Invitrogen
Nupage LDS sample buffer (4x)	NP0007	Invitrogen
Nupage Mes SDS running buffer (20x)	NP0002	Invitrogen
DL-Dithiothreitol	43815-5G	Sigma Aldrich
See Blue Plus 2 prestained Standard		Invitrogen
UHQ		

3.5.2 Equipment

Product	Company
Thermomixer comfort	Eppendorf
Electrophoresis Power Supply Powerease™ 500	Amersham Bioscience
XcellSurelock® Mini Cell	Invitrogen

3.5.3 Working solution

Running buffer (in total 1 L)	
Nupage Mes SDS running buffer (20x)	50 mL
UHQ	950 mL

0.1 M DTT (in total 1 mL)	
DTT	0.15 g
UHQ	1 mL

3.5.4 Protocol

- Samples were diluted to achieve a protein amount of 1 and 2 µg per sample well
- Diluted samples were mixed with 5 µL LDS sample buffer and 1 µL 1 M DTT; the volume was filled up to 20 µL with UHQ

- Samples were heated on the thermomixer at 95°C for 5 minutes and 950 rpm
- The gel chamber was filled with 800 mL running buffer, precasted gel was inserted
- 1 µg/lane (5 µL) and 2 µg/lane (10 µL) samples were loaded onto the gel, 3 µL ladder was loaded as molecular weight marker
- The electrophoretic separation was initiated under the following start conditions, which show maximal values

Nupage Gel Program			
Voltage [V]	**Milliampere [mA]**	**Watt [W]**	**Time [h]**
120	60	25	1:35

3.6 2D GE

3.6.1 Pre-electrophoresis fluorescence labeling (DIGE)

3.6.1.1 Chemicals

Product	Product number	Company
Refraction-2D™ Labeling Kit	PR09	NHDyeAGNOSTICS
G-Dye100	PR09	NHDyeAGNOSTICS
G-Dye200	PR09	NHDyeAGNOSTICS
G-Dye300	PR09	NHDyeAGNOSTICS
G-Dye solvent	PR09	NHDyeAGNOSTICS
G-Dye labeling stop solution	PR09	NHDyeAGNOSTICS
Urea	0.568-1KG	Amresco
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
CHAPS	C9426-5G	Sigma Aldrich
UHQ		

3.6.1.2 Equipment

Product	Company
Centrifuge mini star, silver line	VWR
Thermomixer comfort	Eppendorf
Ice	

3.6.1.3 Working solution

Refraction-2D™ Labeling Kit for 12 gels	
G-Dye100	add 12.5 µL G-Dye solvent
G-Dye200	add 12.5 µL G-Dye solvent
G-Dye300	add 12.5 µL G-Dye solvent
G-Dye solvent	contains DMF, $HCON(CH_3)$
G-Dye labeling stop solution	

IPG – buffer (in total 50 mL)	
7M Urea	21.02 g
2M Thiourea	7.61 g
2% CHAPS	1 g
UHQ	adjust to 50 mL

3.6.1.4 Protocol

- 12.5 µL of G-Dye solvent were added to each G-Dye, vortexed and spun down
- The internal standard (IS) was labeled with 1 µL G-Dye100; the samples were labeled with 1 µL G-Dye200; both were incubated for 30 minutes on ice
- 1 µL of labeling stop solution was added to quench the labeling reaction
- IS and samples were vortexed, spun down and again incubated for 10 minutes on ice

Gel number	IS (labeled with G-Dye100)	Samples (labeled with G-Dye200)
Gel 1	12 µg of normoxic secretome + 12 µg of hypoxic secretome	24 µg of normoxic secretome
Gel 2	12 µg of normoxic secretome + 12 µg of hypoxic secretome	24 µg of normoxic secretome
Gel 3	12 µg of normoxic secretome + 12 µg of hypoxic secretome	24 µg of normoxic secretome
Gel 4	12 µg of normoxic secretome + 12 µg of hypoxic secretome	24 µg of hypoxic secretome
Gel 5	12 µg of normoxic secretome + 12 µg of hypoxic secretome	24 µg of hypoxic secretome
Gel 6	12 µg of normoxic secretome + 12 µg of hypoxic secretome	24 µg of hypoxic secretome

3.6.2 IEF - in-gel rehydration

3.6.2.1 Chemicals

Product	Product number	Company
Urea	0.568-1KG	Amresco
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
CHAPS	C9426-5G	Sigma Aldrich
Bromphenol Blue	B8026	Sigma Aldrich
DL-Dithiothreitol	43815-5G	Sigma Aldrich
Servalyte , 3-10 analytical grade	42940	Serva
Serva IPG blue strip, 3-10 NL 24cm	43022.01	Serva
Paraffin, highly liquid	1.07174.2500	Merck
UHQ		

3.6.2.2 Equipment

Product	Company
Reswelling tray	Serva
Multiphor II electrophoresis system	GE Healthcare
Power supply EPS 3501 XL	Amersham Bioscience
Thermomixer comfort	Eppendorf
IEF Electrode strips	GE Healthcare

3.6.2.3 Working solution

Rehydration buffer (in total 50 mL)	
7M Urea	21.02 g
2M Thiourea	7.61 g
2% CHAPS	1 g
0.002 % Bromphenol blue	
1M DDT	9 µL
Ampholytes, 3-10, NL	9 µL
UHQ	adjust to 50 mL

3.6.2.4 Protocol

- Samples were mixed with 9 µL 1M DTT, 9 µL Ampholytes and the volume was filled to 450 µL with rehydration buffer
- The IPG strip was placed in IPG buffer containing reswelling tray with gel side facing down
- The strip was covered with paraffin oil to prevent from drying and rehydrated overnight
- Next day IEF unit was cooled to 20°C, the cooling plate was covered with paraffin oil and the Drystrip Aligner was placed onto the cooling plate
- The rehydrated IPG-Strip was placed in the alignment tray with gel side up
- Two electrode wicks were placed onto both ends of the strip, once soaked with UHQ and the other with 200 µL 0.1 M DTT
- Electrodes were applied on the wicks
- The entire system was covered with paraffin oil
- The focussing steps are carried out according to the following IEF-program below

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	150	1	5	3:00
2	300	1	5	3:00
3	1000	1	5	6:00
4	3500	1	5	5:00
5	3500	1	5	14:00

3.6.3 IEF – cup loading

3.6.3.1 Chemicals

Product	Product number	Company
Urea	0.568-1KG	Amresco
CHAPS	C9426-5G	Sigma Aldrich
Bromphenol Blue	B8026	Sigma Aldrich
DL-Dithiothreitol	43815-5G	Sigma Aldrich
Servalyte , 3-10 analytical grade	42940	Serva
Serva IPG blue strip, 3-10 NL 24cm	43022.01	Serva
Paraffin, highly liquid	1.07174.2500	Merck
UHQ		

3.6.3.2 Equipment

Product	Company
Reswelling tray	Serva
Hoefer IEF 100	Serva
Clipin Cups	Serva
Thermomixer comfort	Eppendorf
IEF Electrode strips	GE Healthcare

3.6.3.3 Working solution

Rehydration solution (in total 10 mL)	
8M Urea	4.8 g
0.5% CHAPS (w/v)	50 mg
0.28% DTT (w/V)	28 mg
0.5% Servalyte 3-10 (v/v)	50 µL
0.002% Bromphenolblue	10 µL
UHQ	adjust to 10 mL

3.6.3.4 Protocol

- The IPG-strip was pre-rehydrated without the samples overnight covered with paraffin oil
- Next day the IEF unit was cooled to 20°C, the cooling plate was covered with paraffin oil and the Drystrip Aligner was placed onto the cooling plate
- The samples were labeled with G-Dyes prior to the application of the sample to the IPG-strip
- The rehydrated IPG-Strip was placed in the alignment tray with gel side up

- The samples were applied into a loading cup either on the anodal or cathodal end (depending on the analysed sample properties)
- Two electrode wicks were placed onto both ends of the strip, once soaked with UHQ and the other with 200 µL 0.1 M DTT
- Electrodes were applied on the wicks
- The entire system was covered with paraffin oil
- The focussing steps are carried out according to the following IEF-program below

Step		Voltage [V]	Time [h]
1	**step & hold**	250	3:00
2	**step & hold**	500	3:00
3	**gradient**	1000	6:00
4	**gradient**	10.000	1:00
5	**step & hold**	10.000	3:00 (24 kVh)

3.6.4 SDS-PAGE – horizontal system

3.6.4.1 Chemicals

Product	Product number	Company
See Blue Plus 2 prestained Standard		Invitrogen
Serva cooling fluid	43371.07	Serva
Serva SDS Anode buffer	43801.07	Serva
Serva SDS Cathode buffer	43802.07	Serva
2D HPE Large Gel NF 12.5%, 255 x 200 x 0.65mm	43857-00	Serva
Serva equilibration buffer	43805.07	Serva
DL-Dithiothreitol	43815-5G	Sigma Aldrich
Iodoacetamide	I1149-25G	Sigma Aldrich

3.6.4.2 Equipment

Product	Company
Reswelling tray	Serva
HPE tower	Serva
Power supply EPS 3501 XL	Amersham Bioscience
Electrode Wicks	Serva

3.6.4.3 Working solution

Equilibration solution - reduction (in total 6 mL)	
Urea	1.8 g
DDT	50 mg
Serva equilibration buffer	5 mL

Equilibration solution - alkylation (in total 6 mL)

Urea	1.8 g
Iodoacetamide	125 mg
Serva equilibration buffer	5 mL

3.6.4.4 Protocol

- Before the second dimension the IPG strip was washed with UHQ and placed with gel side facing down in the reduction solution for 15 minutes
- The reduction solution was exchanged with the alkylation solution and the IPS strip was placed again in the new solution for 15 minutes
- The HPE tower was prepared by setting the thermostatic circulator to 15°C and by setting the valve to “bypass” to avoid water condensation
- Two wicks were soaked with 45 mL from each electrode buffer
- The cooling plate was cleaned with UHQ water and 4 mL of the cooling fluid was applied
- The gel was bent into a “U-shape” and with the film supporting side the cooling fluid was distributed over the cooling plates
- The gel was placed onto the cooling plate, air bubbles were smoothed out; the unnecessary cooling fluid was removed with lint-free paper
- The edges of both wicks should overlap the gel by only ~3 mm on each side
- Then the IPG-strip was placed with gel side down into the slot of the gel
- 5 µL ladder were applied to the well next to the slot for the IPG-strip
- The lid was closed and the electrodes were placed on the wicks
- The valve was turned to “cooling” and the required program was selected
- After 1 hour 10 minutes the program was paused to remove the IPG-strip, the run was continued

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

3.6.5 SDS-PAGE – vertical system

3.6.5.1 Chemicals

Product	Product number	Company
Tris	741883	Sigma Aldrich
Glycin	1041691000	Merck
SDS	L3771-25G	Sigma Aldrich
Urea	0.568-1KG	Amresco
Glycerol	G5516-1L	Sigma Aldrich
Bromphenolblue	B8026	Sigma Aldrich
DL-Dithiothreitol	43815-5G	Sigma Aldrich
Iodoacetamide	I1149-25G	Sigma Aldrich
Acrylamide	A3553-500G	Sigma Aldrich
Bisacryamide	146072-500G	Sigma Aldrich
Ammonium Persulfate	US12300	Merck
TEMED	110732	Merck
UHQ		

3.6.5.2 Equipment

Product	Company
Vertical Gel Electrophoresis Unit (SE260)	
Reswelling tray	Serva
Power supply EPS 3501 XL	Amersham Bioscience

3.6.5.3 Working solution

Running Buffer for 2nd dimension (in total 6 L)	
250 mM Tris	15.14 g
0.192 M Glycin	77.07 g
SDS	5 g
UHQ	adjust to 5 L

Equilibration solution (in total 500 mL)	
6M Urea	180.18 g
50mM Tris-HCl (pH=8.8)	166.67 g
30% Glycerol (v/v)	150 mL
2% SDS	10 g
0.002% Bromphenolblue	

Equilibration solution – reduction (in total 10 mL)	
Equilibration solution	10 mL
DL-Dithiothreitol	100 mg

Equilibration solution – alkylation (in total 10 mL)	
Equilibration solution	10 mL
Iodoacetamide	250 mg

Monomer solution (in total 100 mL)	
30% Acrylamide	30 g
0.8% Bisacryamide	0.8 g
UHQ	adjust to 100 mL

4x Resolving Gel buffer	
1.5M Tris (pH=8.8)	18.17 g
UHQ	adjust to 100 mL

Gel recipe T= 12.5% (in total 60ml)	
Monomer Solution	20 mL
4x Resolving Gel Buffer	15 mL
10% SDS	0.6 mL
UHQ	24.8 mL
10% Ammonium Persulfate	0.3 mL
TEMED	20.3 µL

3.6.5.4 Protocol

- The gel was prepared by mixing all components together, ammonium persulfate and TEMED at last
- The gel solution was filled between glass plates without trapping air bubbles and was covered with water saturated butanol solution
- The equilibrated IPG strips was removed from the Incubation Tray with tweezers and was rinsed carefully with UHQ
- The strip was placed on a glass plate with gel side facing up
- The IPG strips was cut to proper size by removing gel and plastic layer with a spatula and scalpel at the acidic and basic end (IPG Strip length: 24cm, pocket size in SDS-PAGE gel: 13 cm)
- The IPG strip was then placed on top of the SDS-PAGE gel – basic end should point towards molecular weight marker
- A filter paper (ca 5 x 7 mm) is placed on the gel soaked with 5 µL of molecular weight marker
- The gel sandwich/IPG strip was sealed on top of the IPG strip to hold it in place during separation with agarose sealing solution
- The lower buffer chamber was filled 50% of the volume with Running Buffer, and a magnetic stirrer was placed on the bottom
- The gel sandwich and a dummy were connected to the upper buffer chamber
- The gel sandwiches was placed in the Lower Buffer chamber and the rest of the running buffer was added, then the water cooling was turned on
- The chosen Power/Voltage program was started (300 V, 50 mA, 20 W approx. 3-4 h)

Separation was finished when the molecular weight marker had a distance of approx. 1 cm to the lower boarder of the gel

Voltage [V]	Current [mA]	Watt [W]	Time [h]	Voltage [V]
300	50	20	3-4	300

3.7 Post-electrophoresis staining and detection

3.7.1 Fluorescence Staining

3.7.1.1 Chemicals

Product	Product number	Company
Serva Purple	43386	Serva
Citric Acid > 99.5%	251275-100G	Sigma Aldrich
Boric Acid	15660	Fluka
Sodium hydroxide	1.06482.5000	Merck
Ethanol	1.00983.2500	Merck
Ammonium bicarbonate	09830-500G	Fluka
Methanol	1.06035.2500	Merck
UHQ		

3.7.1.2 Equipment

Product	Company
Typhoon ™ FLA 9000	GE Healthcare
Centrifuge mini star, silver line	VWR
Thermomixer comfort	Eppendorf

3.7.1.3 Working solution

Fixing solution (in total 1 L)	
UHQ	850 mL
Citric acid	10 g
Ethanol	150 mL

Staining buffer (in total 1 L)	
UHQ	1 L
Boric acid	6.2 g
Sodium hydroxid	3.85 g

Washing solution (in total 1 L)	
UHQ	850 mL
Ethanol	150 mL

Acidification (in total 1 L)	
UHQ	850 mL
Citric acid	10 g
Ethanol	150 mL

Destaining solution (in total 400 mL)	
Ammonium carbonate	1.58 g
Methanol	200 mL
UHQ	200 mL

3.7.1.4 Protocol

- After the second dimension the gel was fixed in fixing solution for one hour
- Meanwhile Serva purple reagent was brought to room temperature
- 1 mL Serva purple was mixed with 250 mL staining solution
- The fixing solution was removed
- The gel was staining in the staining solution for one hour while gentle shaking
- The staining solution was removed and the gel was washed with washing solution for 30 minutes
- For the acidification the gel was incubated for 30 minutes in fixing solution again
- The gels were then scanned with the fluoresce imager
- For destaining the gel was incubated overnight in the destaining solution

Gel type		MiniGel	Standard Flatbed	Large Flatbed	Large gel (1 mm)	
Process	***Solution***			***Volume per gel***		***Time***
Fixing	Solution 1	100 mL	200 mL	300 mL	400 mL	1 h
Staining	Solution 2	0.4 mL dye in 100 mL	0.8 mL dye in 200 mL	1 mL dye in 250 mL	1.6 mL dye in 400 mL	1 h
Washing	Solution 3	100 mL	200 mL	300 mL	400 mL	30 min
Acidification	Solution 1	100 mL	200 mL	300 mL	400 mL	30 min

3.7.2 Silver Staining

3.7.2.1 Chemicals

Product	Product number	Company
Ethanol	1.00983.2500	Merck
Acetic Acid >99.8%	33209-2.5L	Sigma Aldrich
Sodium thiosulfate-5-hydrate	31459	Riedel den Haen
Silver nitrate	1.01512.0025	Merck
Sodium carbonate >99.8%	31432-1KG-R	Sigma Aldrich
Formaldehyde solution	F8775-25ml	Sigma Aldrich
Potassium hexacyanoferrate(III)	1049711000	Merck
Sodium thiosulfate	1065122500	Merck
UHQ		

3.7.2.2 Equipment

Product	Company
Office desk scanner	
Centrifuge mini star, silver line	VWR
Thermomixer comfort	Eppendorf

3.7.2.3 Working solution

Fixing solution (in total 100 mL)	
50% Ethanol	50 mL
5% Acetic Acid	5 mL
45% UHQ	45 mL

Washing solution (in total 50 mL)	
50% Ethanol	25 mL
50% UHQ	25 mL

Sensitization solution (in total 100 mL)	
Sodium thiosulfate-5-hydrate	0.02 g
UHQ	100 mL

Incubation solution (in total 100mL)	
Silver nitrate	0.1 g
UHQ	100 mL

Developing solution (in total 300 ml)	
2% Sodium carbonate	5 g
0.04% Formaldehyde	100 µL
UHQ	250 mL

Stopping solution (in total 300 mL)

5% Acetic Acid	15 mL
95% UHQ	285 mL

Storage solution (in total 100 mL)	
1% Acetic Acid	1 mL
99% UHQ	99 mL

Destaining solution (in total 300 mL)	
100 mM Sodium thiosulfate	150 mL
30 mM Potassium hexacyanoferrate(III)	150 mL

3.7.2.4 Protocol

- The staining process was performed according to Shevchenko [3] as shown in the table below
- SDS-PAGE gels and 2D gels were stained with this method and were compared with fluoresce staining

Solution	Incubation duration	Volume	Components
Fixing	20 min	100 mL	50% ethanol 5% acetic acid 45% UHQ
Wash	10 min	50 mL	50% ethanol 50% UHQ
Wash	2 hours or overnight after changing solution	50 mL	UHQ
Sensitization	1 min	100 mL	0.02g $Na_2S_2O_3$* 5 H_2O 100 mL UHQ
Wash	2 x 1 min	2 x 100 mL	2 x UHQ
Incubation	20 min (4°C)	100 mL	0.1 g silver nitrate 100 mL UHQ
Wash	2 x 1 min	2 x 100 mL	2 x UHQ
Developing	develop until protein bands are visible change solution several times	3 x 100 mL	5 g sodium carbonate (= 2%) 100 µL formalin (= 0.04 %) 250 mL UHQ
Stop	3 x 5 min	300 mL	5% acetic acid 95% UHQ
Storage	4°C	100 mL	1% acetic acid 99% UHQ
Destaining	unitl no silver protein spots are visible change solution several times	300 mL	50% sodium thiosulfate (100 mM) 50% potassium hexacyanoferrate(III) (30 mM)

- For destaining: the stained gel was incubated in the destaining solution as long as silver stained spots were visible
- The destaining solution was changed several times during the process
- The gel was flushed with UHQ water regularly

4 Results and Discussion

4.1 Technical Aspects for 2D GE / DIGE

Technical aspects for the 2D GE involve the following points: Protein precipitation and reproducibility, sample introduction into the first dimension of 2D GE, comparison of vertical and horizontal 2D GE, reproducibility and gel staining. The different aspects were tested and processed at different time points during this work, and different samples were used for these comparisons in the following chapters: (A) Synovial fluid (B) αMEM (C) αMEM/Norm for normoxic cultivation and αMEM/Hypo for hypoxic cultivation. As a starting point to setup proper 2D Ge conditions synovial fluid was used and equally treated like the analysed αMEM/Norm samples because analytical similarity was expected with respect to protein patterns and content.

4.1.1 Protein precipitation and reproducibility

To test the reproducibility of protein precipitation Synovia, αMEM and αMEM/Norm samples were precipitated in triplicates with 61mM trichloroacetic acid solution and ice-cold acetone according to chapter 3.3.4 (page 48). The protein concentrations of the samples were determined via Bradford assay according to chapter 3.2.4 (page 46). A calibration curve of the BSA standards was established to be able to get a linear regression. The samples were diluted according to their determined protein concentration, see chapter 3.2.4 (page 46), to achieve an average protein amount of 1 and 2 µg per lane. Afterwards the samples were separated using 1D PAGE see 3.5.4 (page 51), stained with Serva purple and silver.

<u>First replicate of precipitation</u>

The linear regression curve for the BSA standards is shown in Figure 56. The obtained results for the analysed samples are shown in Table 41. The composition of the sample containing loading buffer is described in Table 42. After 1D PAGE separation the gels were stained and scanned shown in Figure 20.

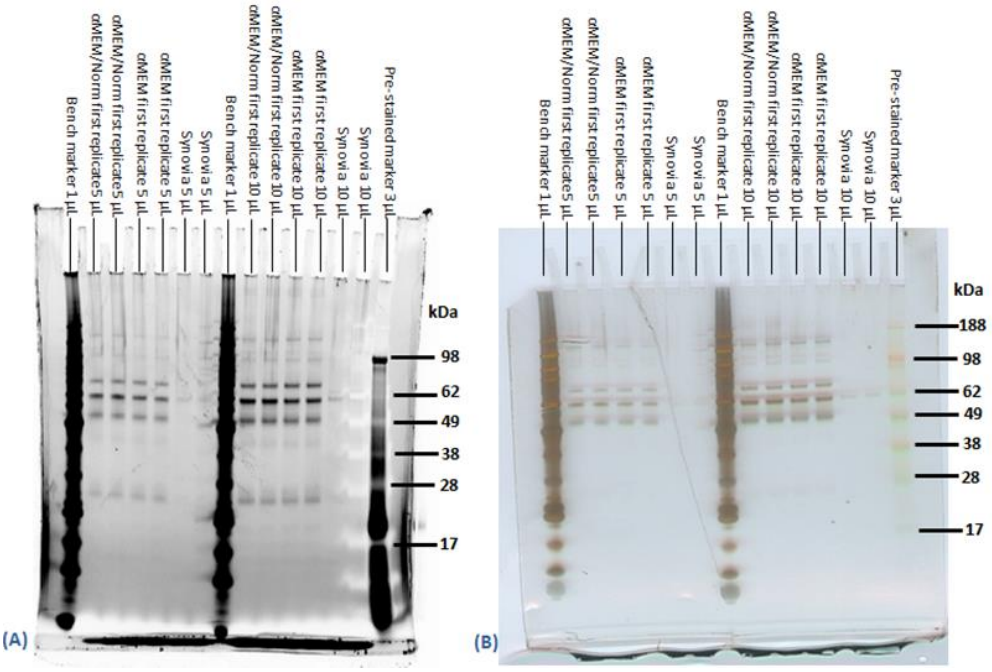


Figure 20: SDS PAGE of precipitated samples from Synovia, αMEM/Norm and αMEM. 1D gel after (A) post- fluorescent staining and (B) silver staining. Sample separation was performed on a 4-12% Bis-Tris gel. A pre-stained molecular weight marker was used.

Second replicate of precipitation

The obtained linear regression for the BSA standards and determined protein concentration for the analysed samples which were 1:500 diluted can be seen in Figure 21 and Table 7. Composition of sample containing loading buffer is shown in Table 8 and Table 9. After separation by 1D PAGE the gel was fluorescence and subsequent silver stained, as shown in Figure 22.

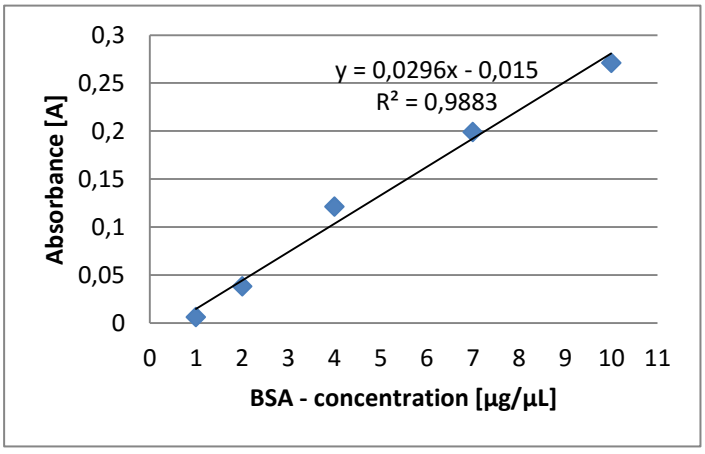


Figure 21: Linear regression for BSA calibrants.

Sample		Absorbance		Concentration	
		Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm (1:500)	measurement 1 0.112 measurement 2 0.106 measurement 3 0.108 measurement 4 0.111	0.109	4.386 4.188 4.254 4.353	4.295	**21.48**
αMEM (1:500)	measurement 1 0.105 measurement 2 0.099 measurement 3 0.096 measurement 4 0.075	0.094	4.156 3.958 3.860 3.169	3.785	**18.93**

Table 7: Overview on calculations for protein concentration of the used samples.

Component	Amount
DL-Dithiothreitol (1M)	1 µL
LDS sample buffer (4x)	5 µL
αMEM/Norm (1:20)	**3.72 µL (79.9 µg)**
UHQ water	10.28 µL
In total	20 µL

Table 8: Composition of 1D PAGE loading buffer including the analysed αMEM/Norm sample.

Component	Amount
DL-Dithiothreitol (1M)	1 µL
LDS sample buffer (4x)	5 µL
αMEM (1:20)	**4.23 µL (79.9 µg)**
UHQ water	9.77 µL
In total	20 µL

Table 9: Composition of 1D PAGE loading buffer including the analysed αMEM sample.

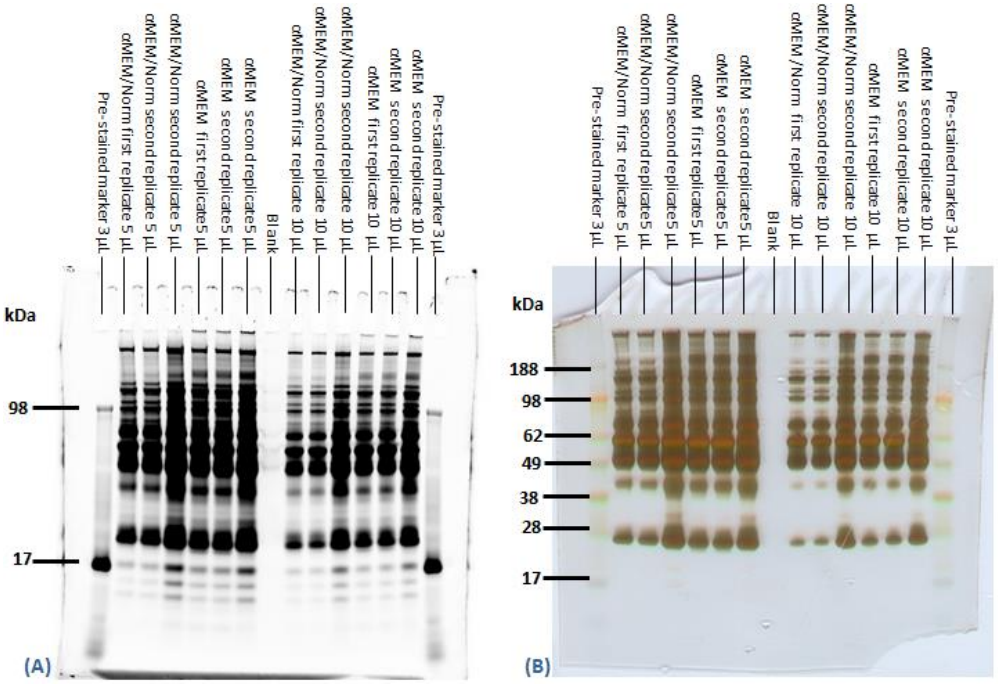


Figure 22: SDS PAGE separation of two precipitation replicates from αMEM/norm and αMEM. 1D gel after (A) post- fluorescent staining and (B) silver staining. Sample separation was performed on / by a 4-12% Bis-Tris gel. A pre-stained molecular weight marker was used.

Third replicate of precipitation

Figure 23 and Table 10 shows the linear regression and obtained results for the analysed samples. Table 11 and Table 12 point out the composition of the sample containing loading buffer. Separation was performed by 1D PAGE and the resulting gels were stained as shown in Figure 24.

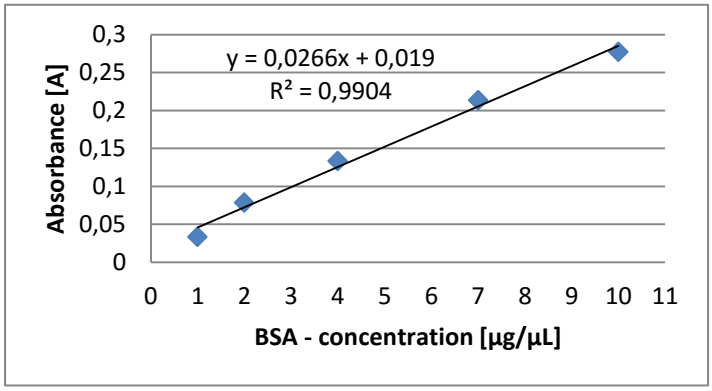


Figure 23: Linear regression for BSA calibrants.

Sample		Absorbance		Concentration		
			Average		*Average*	*µg/µL*
αMEM/Norm (1:1000)	measurement 1	0.139	0.135	4.447	4.326	**43.26**
	measurement 2	0.132		4.195		
	measurement 3	0.145		4.663		
	measurement 4	0.126		3.998		
αMEM (1:500)	measurement 1	0.110	0.106	3.402	3.267	**16.34**
	measurement 2	0.115		3.582		
	measurement 3	0.101		3.078		
	measurement 4	0.099		3.006		

Table 10: Protein concentrations determined for samples used to test protein precipitation repeatability.

Component	Amount
DL-Dithiothreitol (1M)	1 µL
LDS sample buffer (4x)	5 µL
αMEM/Norm (1:50)	**4.62 µL (199.8 µg)**
UHQ water	9.38 µL
In total	20 µL

Table 12: Composition of 1D PAGE buffer including the analysed αMEM/Norm sample.

Component	Amount
DL-Dithiothreitol (1M)	1 µL
LDS sample buffer (4x)	5 µL
αMEM (1:20)	**4.90 µL (µg)**
UHQ water	9.10 µL
In total	20 µL

Table 11: Composition of 1D PAGE buffer including the analysed αMEM sample.

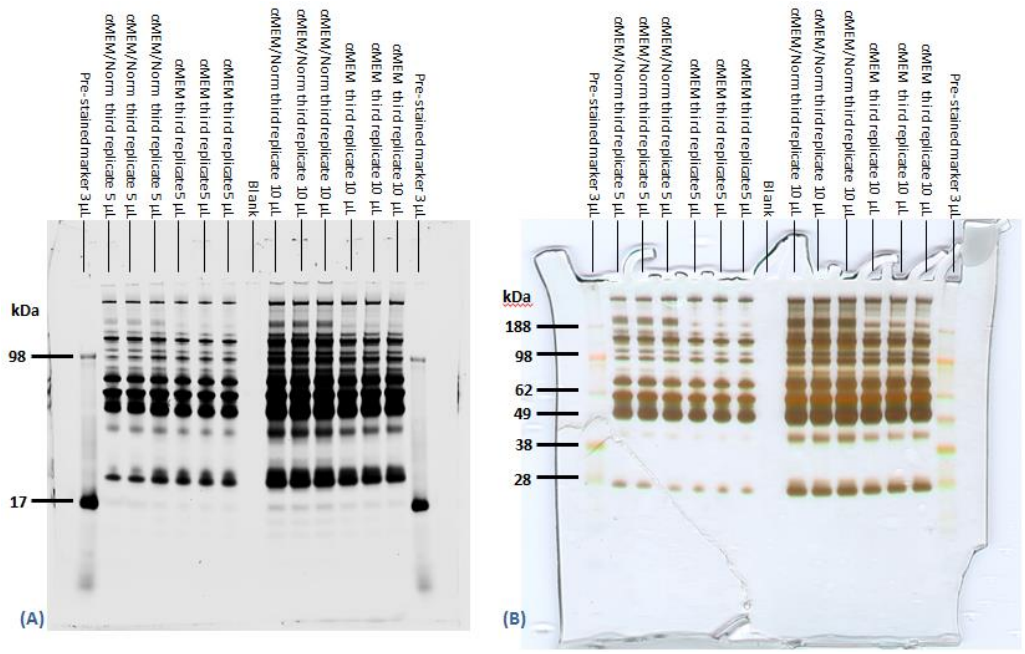


Figure 24: SDS PAGE of third precipitated replicate from αMEM and αMEM/NormMSC. 1D gel after (A) post-fluorescent staining and (B) silver staining. Sample separation was performed on / by a 4-12% Bis-Tris gel. A pre-stained molecular weight marker was used.

Comparison of all three precipitation replicates

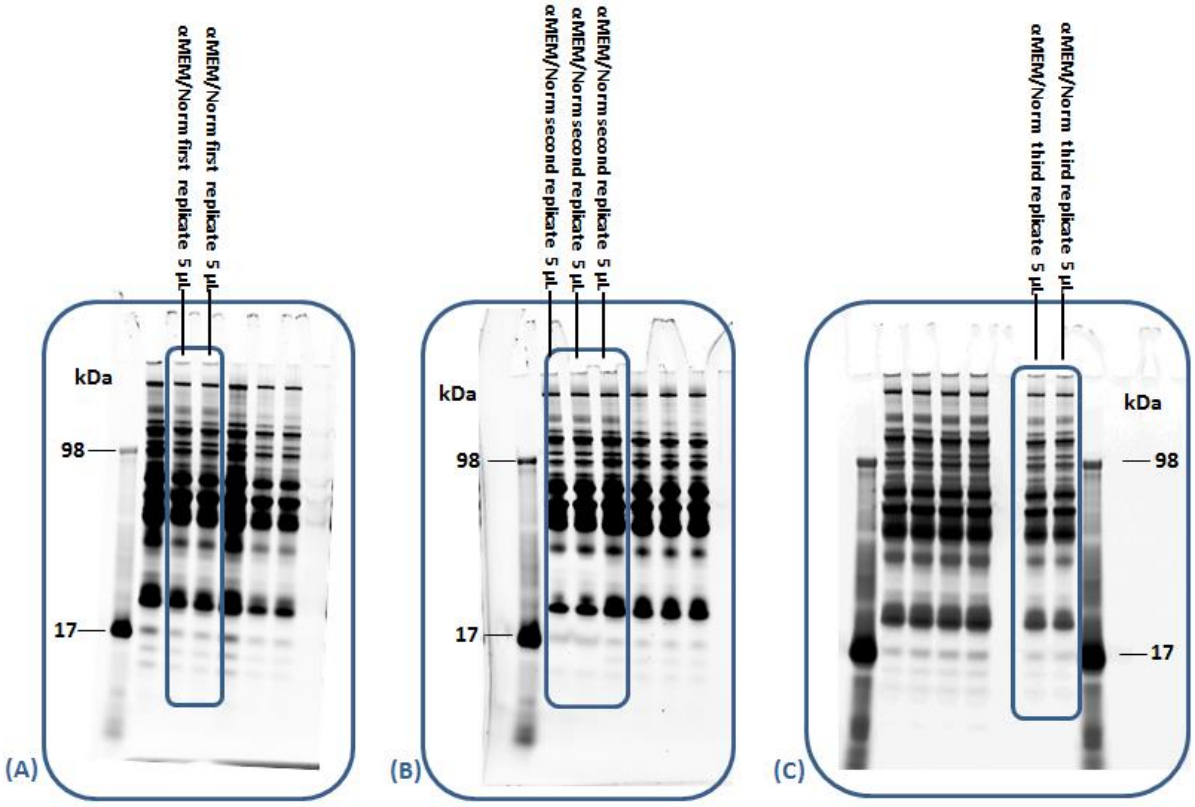


Figure 25: Three technical replicates of protein precipitation in direct comparison. (A) First, (B) second and (C) third replicate of TCA precipitation of proteins from three αMEM/Norm sample aliquots.

Figure 25 (A-C) shows all three technical replicates for the precipitated αMEM/Norm samples. The lanes highlighted in blue contain 1 µg total protein from each corresponding αMEM/Norm precipitation replicate. By visual comparison of the marked lanes it could be seen that all three replicates show almost similar protein band patterns, especially between MW 90 kDa to 120 kDa and 17 kDa to 30 kDa. The obtained results showed that this type of precipitation with TCA and ice-cold acetone was successful and reproducible for this type of sample.

4.1.2 Sample application for the first dimension of 2D GE (comparison of cup loading and in-gel rehydration)

Although the setup for the 2D GE approach was already established with the available equipment, another technique for sample application into the first dimension was tested. Available instrumentation in the lab allowed the introduction of proteins into the first dimension via effective in gel rehydration. However, during a Workshop held at the Vienna University of Technology (April 22–25, 2014; Institute of Chemical Technologies and Analytics) it was possible to test cup loading as an alternative for the same sample. For this purpose the same αMEM/Norm and αMEM/Hypo samples were used for in-gel rehydration as well as for cup-loading. The samples were precipitated with TCA/acetone according to the protocol given in chapter 3.3.4 (page 48). The samples were diluted 1:1000 with IPG buffer before measurement. Afterwards the protein concentration was determined via Bradford Assay (see chapter 3.2.4 (page 46)). To remove high-abundance proteins the samples were treated with CPLL as described in chapter 3.4.1.4 (page 49). After the elution step the samples had to be precipitated again to exchange the acetic acid from the elution reagents. The samples were again diluted (αMEM/Norm 1:10) (αMEM/Hypo 1:20) with IPG buffer prior measurement. The protein concentration was again determined and results are shown in Table 13.

Sample	Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm before depletion	measurement 1	0.079	0.076	3.847	3.709	**37.09**
	measurement 2	0.077		3.738		
	measurement 3	0.075		3.630		
	measurement 4	0.074		3.621		
αMEM/Hypo before depletion	measurement 1	0.064	0.063	3.034	2.993	**29.93**
	measurement 2	0.061		2.871		
	measurement 3	0.063		2.980		
	measurement 4	0.065		3.088		

αMEM/Norm after depletion	measurement 1	0.188	0.188	9.751	9.751	**0.97**
	measurement 2	0.189		9.805		
	measurement 3	0.188		9.751		
	measurement 4	0.187		9.697		
αMEM/Hypo after depletion	measurement 1	0.176	0.174	9.101	8.966	**1.79**
	measurement 2	0.175		9.074		
	measurement 3	0.172		8.885		
	measurement 4	0.171		8.830		

Table 13: Protein concentrations determined for samples used to compare cup-loading and in gel rehydration as sample preparations steps in IEF.

Table 13 shows the final results for the αMEM/Norm sample. The calculated concentration values include the dilution factor (1:1000 and 1:10), the volume used for protein determination (10 µL) and the conversion factor (1000) from µg/mL to µg/µL. Further a high reduction of the protein concentration through the treatment with CPLL was obtained and it can be seen that the protein concentration from the αMEM/Hypo sample after depletion is two-fold higher than those of the αMEM/Norm sample after depletion. Afterwards 12.67 µg (from αMEM/Norm sample) and 13.45 µg (from αMEM/Hypo sample) were pooled and labeled with 1 µL of G-dye 100, to prepare the internal standard (IS). 26 µg of the respectively analysed sample (in this case αMEM/Hypo sample) was labeled with G-dye 200 accoridng to the protocol, see chapter 3.6.1.4 (page 53).

Further experiments were done for the same sample (αMEM/Hypo) with different sample application methods for the first dimension of 2D GE.

4.1.2.1 In-gel rehydration

For the first dimension, immobilized pH gradient gel strips (24cm, pH 3-10, non-linear) were used. The isoelectric focussing step was again performed according chapter 3.6.1.4 (page 53). Both labeled extracts were mixed and introduced via overnight in gel rehydration. Table 14 shows the composition of the IEF buffer and the used sample volume. After the rehydration step, the IPG strip was focussed according to the set program and the actual progress of current and voltage over 24 hours of focussing is given in Figure 26. The sample was focussed in total for 52.9 kVh, 11 mAh and 25:01 h.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Hypo + IS	**36.5 µL**
Rehydration-buffer	429 µL

In total	450 µL

Table 14: Composition of the IEF buffer containing the analysed sample.

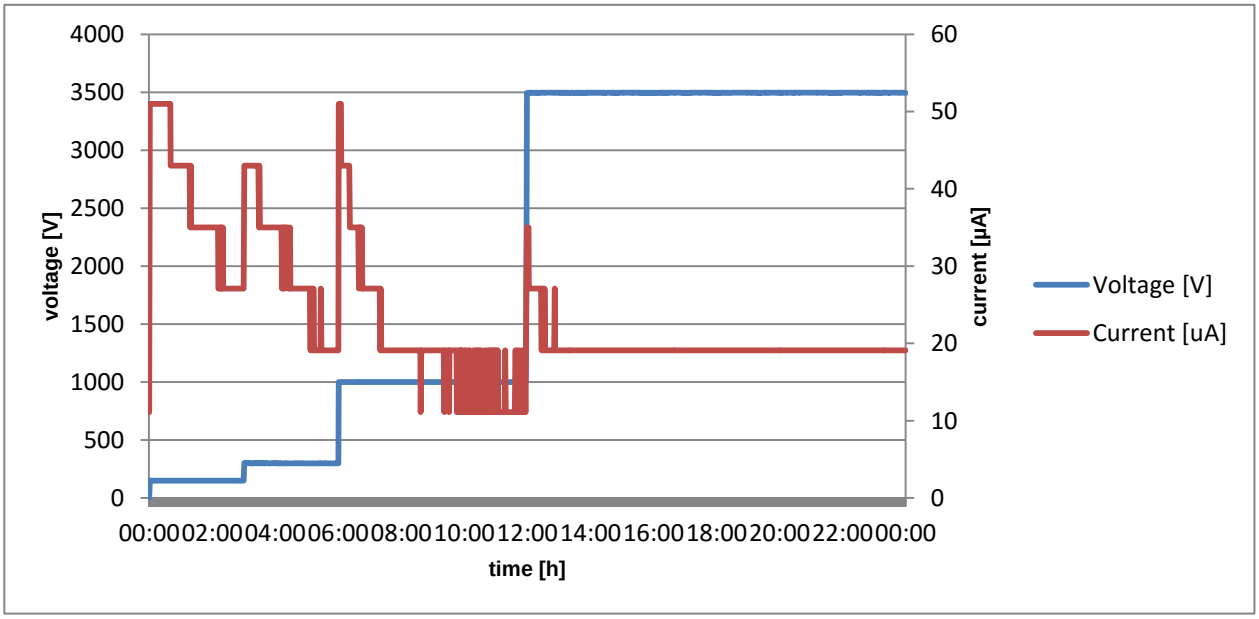


Figure 26: Voltage and current over the course of time for the analysed sample.

After IEF the IPG strip was reduced and alkylated according to chapter 3.6.4.4 (page 57) before separating the proteins according to their molecular weight on 255 x 200 x 0.65 mm 2D HPE Large Gel NF T% 12.5 using the HPE tower. Figure 27 shows the corresponding voltage and current run over time. The second dimension took 5:50 h, leading to 3479 kVh and 197 mAh.

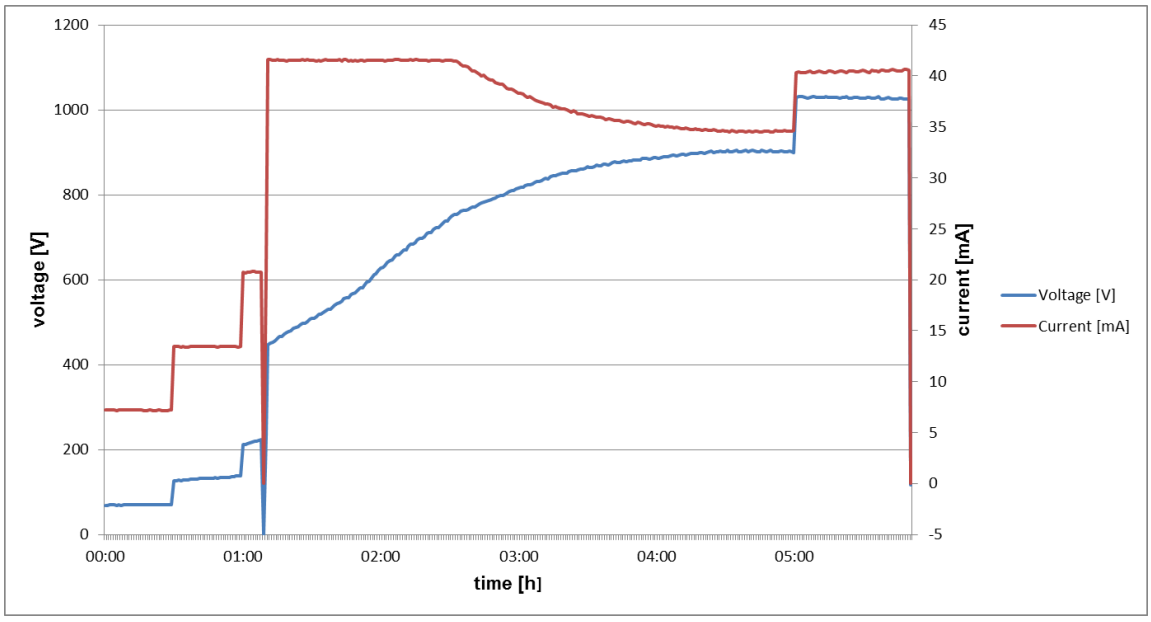


Figure 27: Voltage and current over the course of time for the analysed sample.

Afterwards the gels were scanned at the wavelength specific for each G-dye (Figure 29).

4.1.2.2 Cup loading

The same αMEM/Norm and αMEM/Hypo samples were used as for in-gel rehydration. The IPG strip was pre-rehydrated with rehydration buffer (composition see chapter 3.6.2.3 (page 54) but without containing the sample (composition see chapter 3.6.2.3 (page 54)). The αMEM/Hypo sample (26.12 µg) and IS (13.45 µg αMEM/Hypo and 12.67 µg αMEM/Norm) were labeled and applied into a loading cup on the anodal end of the IPG strip. It was hoped that the sample was transported and separated more quickly into the gel. Furthermore, all proteins were positively charged and did not aggregate, therefore protein interaction could be reduced and all the proteins approached to their pIs from the same side. The isoelectric focussing was carried out in the Hoefer IEF 100 during the 2D gel workshop according to the following program:

Step		Voltage [V]	Time [h]
1	**step & hold**	250	3:00
2	**step & hold**	500	3:00
3	**gradient**	1000	6:00
4	**gradient**	10.000	1:00
5	**step & hold**	10.000	3:00 (24 kVh)

Table 15: Running conditions for the first dimension with cup loading for the αMEM/Hypo sample.

Due to the fact that the isoelectric focusing was carried out with equipment during the 2D gel workshop no actual progress of current and voltage over time was obtained. After the reduction and alkylation step, the second dimension was performed according to chapter 3.6.2.4 (page 54). For the performance the HPE tower was used. The actual progress of voltage and current can be seen in Figure 28.

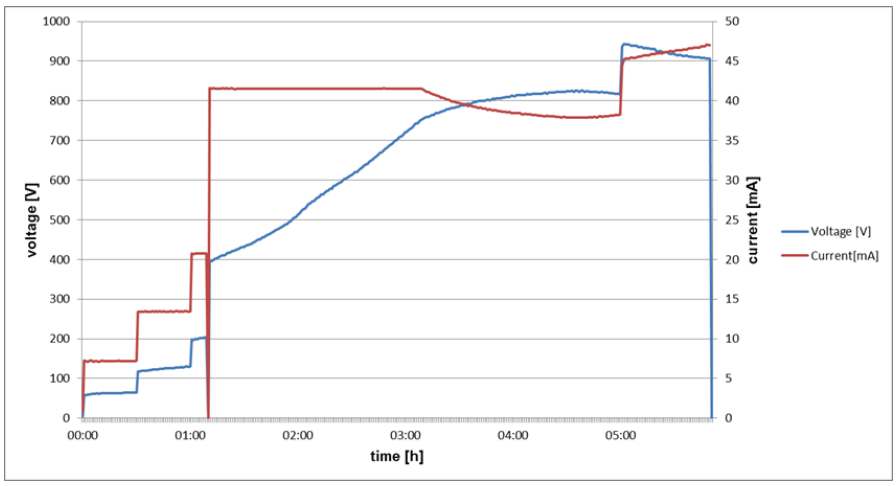


Figure 28: Voltage and current over the course of time for the analysed sample.

The second dimension took 5:50 h, leading to 3470 kVh and 193 mAh. Afterwards the gels were scanned at the wavelength specific for each G-dye, as shown in Figure 29 and Figure 30.

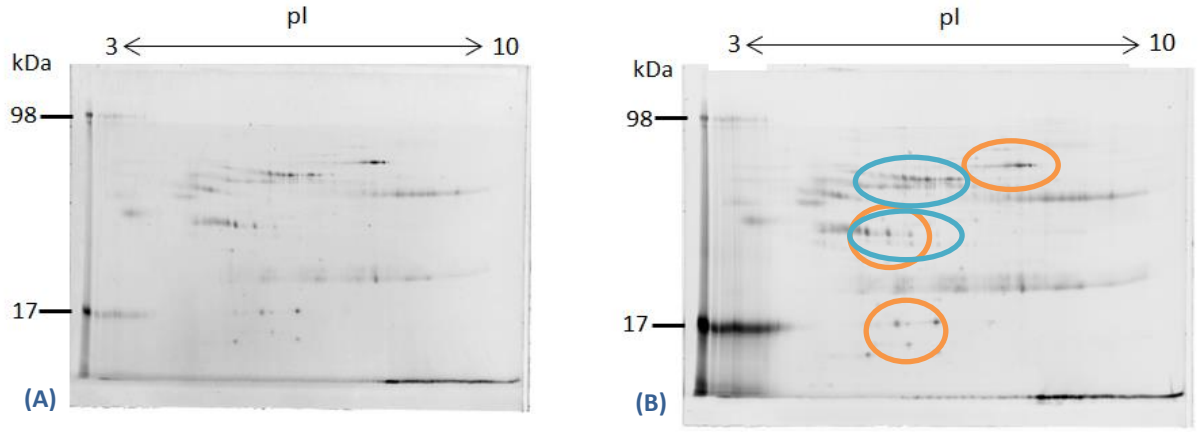


Figure 29: 2D gels of the analysed αMEM/Hypo sample and IS. Samples were applied by in-gel rehydration overnight on a 24cm IPG strip (pH 3-10, not linear). (A) 26µg IS were labeled with G-dye 100 and (B) 26 µg αMEM/Hypo were labeled with G-dye 200. A pre-stained molecular weight marker was used.

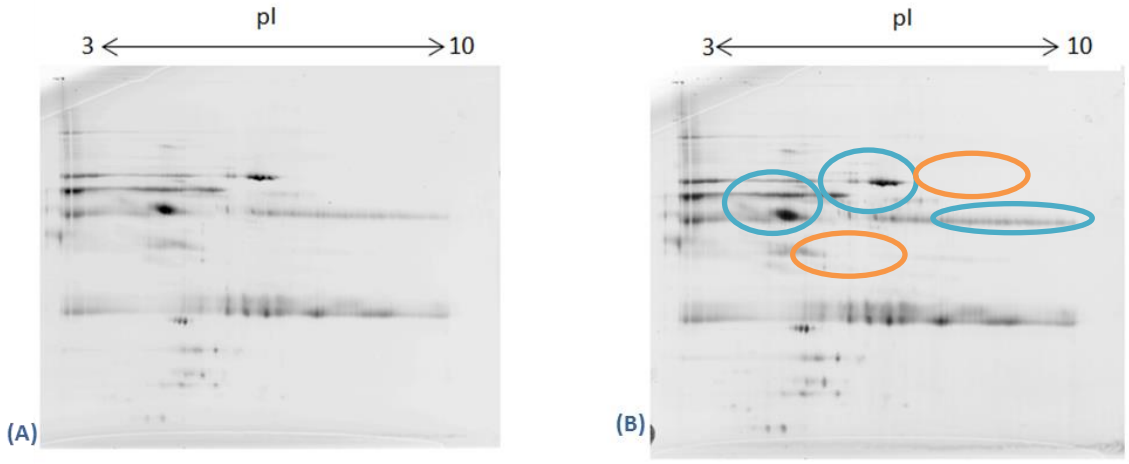


Figure 30: 2D gels of the analysed αMEM/Hypo sample and IS. Samples were applied by cup loading on a 24cm IPG strip (pH 3-10, not linear) at the acidic area. (A) 26µg IS were labeled with G-dye 100 and (B) 26 µg αMEM/Hypo were labeled with G-dye 200. No pre-stained molecular weight marker was used.

The results supported the decision to do further experiments with in-gel rehydration rather than with cup loading. The same protein amount was applied in both cases. As Figure 30 B illustrates the obtained protein spots were not clearly focused (highlighted in blue) and especially in the neutral and basic area less protein spots were detected compared with the results obtained by in-gel rehydration (highlighted in orange) see Figure 29B . Proteins with a pI (pI 3 till 5) near the application site of the cups were not well introduced into the gel and therefore they did not migrate and remained near the cups because they were more prone to precipitate. Reasons for the obtained results could be: Cup loading was performed during the

2D gel workshop where the experiment was done by different persons leading to mistakes during sample preparation (labeling or rehydration) or handling (sample application by cups). Furthermore sample application on the cathodal end should also be tested to check if better results were obtained. To verify the observed results further repetitions of the experiment had to be done, yet time constraints during the master thesis did not allow for further experiments. Therefore in-gel rehydration was chosen as the sample application method for future experiments.

4.1.3 Comparison of vertical and horizontal 2D GE and reproducibility

For the second dimension a vertical electrophoresis system and a horizontal flatbed system were tested and compared. For this, synovia fluid sample were used and the separation was tested first by the horizontal HPE system using HPE large 12.5% gels and second by a vertical system using self-made gels. Synovia samples were used without protein precipitation but diluted 1:500 with IPG buffer before measurement. The protein concentration was determined via Bradford assay as described in chapter 3.2.4 (page 46). The obtained concentration value is shown in Table 16.

Sample		Absorbance			Concentration	
			Average	*µg/mL*	*Average*	*µg/µL*
Synovia sample	measurement 1	0.292	0.290	6.710	6.395	33.3
	measurement 2	0.288		6.609		

Table 16: Overview on the calculation of the protein concentration of the synovia sample.

Table 16 shows the final concentration value for the synovia sample. The value includes the dilution factor (1:500), the volume used for protein determination (10 µL) and the conversion factor (1000) from µg/mL to µg/µL. Afterwards the sample was dived into two aliquots, one was used for the separation on a horizontal system, the other one for the separation on a vertical system. After the determination of the protein concentration, both synovia sample aliquots were analysed using 2D gel electrophoresis. For the first dimension the samples were treated according to the chapter 3.6.1.4 (page 53). Prior to the IEF step, the 24 cm pH 3-10 NL IPG strip was rehydrated overnight in IEF buffer, which also contained the synovia sample. Table 17 shows the composition of the rehydration buffer and the used sample volume. After rehydration the IPG strip was focussed according to the program given in Table 18 and the actual voltage and current over 23 hours of focussing is given in Figure 31. The sample was focussed in total for 70.8 kVh, 18.8 mAh and 23:13 h.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
Synovia	**3 µL** (99.9 µg)
Rehydration-buffer	429 µL
In total	450 µL

Table 17: Composition of the IEF buffer containing the analysed sample.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	300	1	5	1:00
2	300	1	5	1:00
3	1000	1	5	3:00
4	3500	1	5	4:00
5	3500	1	5	15:00

Table 18: Isoelectric focusing program used for Synovia.

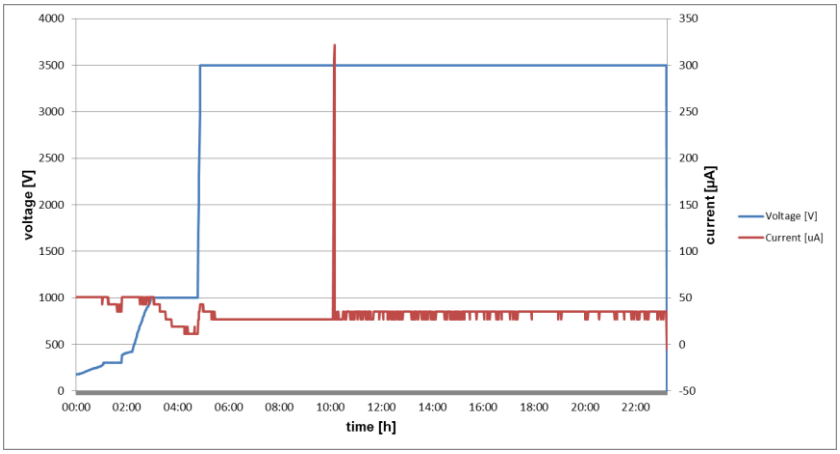


Figure 31: Voltage and current over the course of time for Synovia sample.

Figure 31 shows a normal current flow during the first dimension. At 10:00 h a non-sample caused noise was detected. This was explained by inherent detector fluctuations, instrumental errors or impurities in the sample as fluff or salt.

After IEF and prior to the second dimension, the IPG strip was reduced and alkylated according to chapter 3.6.4.4 (page 57).

4.1.3.1 Horizontal electrophoresis system as second dimension

The separation of the synovial sample according to its molecular weight was performed on a 255 x 200 x 0.65 mm 2D HPE Large Gel NF T% 12.5 using the HPE tower. Table 19 shows the used program for this step and Figure 32 the corresponding voltage and current run over time. The second dimension takes 5:50 h, leading to 3588 kVh and 193 mAh.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 19: HPE tower program for the second dimension for the synovia sample.

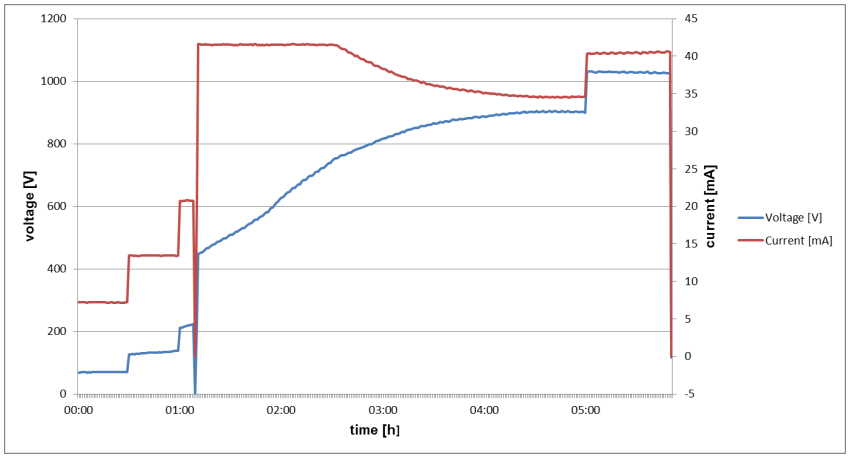


Figure 32: Voltage and current over the course of time for the analysed synovia sample.

The obvious current decrease after 1:10 h is caused by a pause of the separation to remove the IPG strip. After separation, the 2D gel was silver stained according to the protocol given in chapter 3.7.2.4 (page 63) [3].

4.1.3.2 Vertical electrophoresis system as second dimension

In this case the separation in the second dimension was done in a self-made polyacrylamide gel of 12.5% T and 2.6% C (preparation see chapter (page)) on a vertical gel electrophoresis unit (SE260). The used program can be seen in Table 20.

Voltage [V]	Current [mA]	Watt [W]	Time [h]
300	50	20	3-4

Table 20: Applied program for the second dimension.

After the setup was reassembled, the chamber was filled with the corresponding running buffer (composition see chapter 3.6.5.3 (page 58)). The IPG strip prepared as described before was placed on top of the SDS-PAGE gel and an agarose sealing solution was added on top of the IPG strip to keep it in place during the separation. The electrophoretic separation was stopped when the molecular weight marker had a distance of approximately 1 cm to the lower boarder of the gel. After separation the 2D gel was silver stained according to the protocol given in chapter 3.7.2.4 (page 63) [3].

4.1.3.3 *Results from the horizontal system in comparison with the results from vertical system*

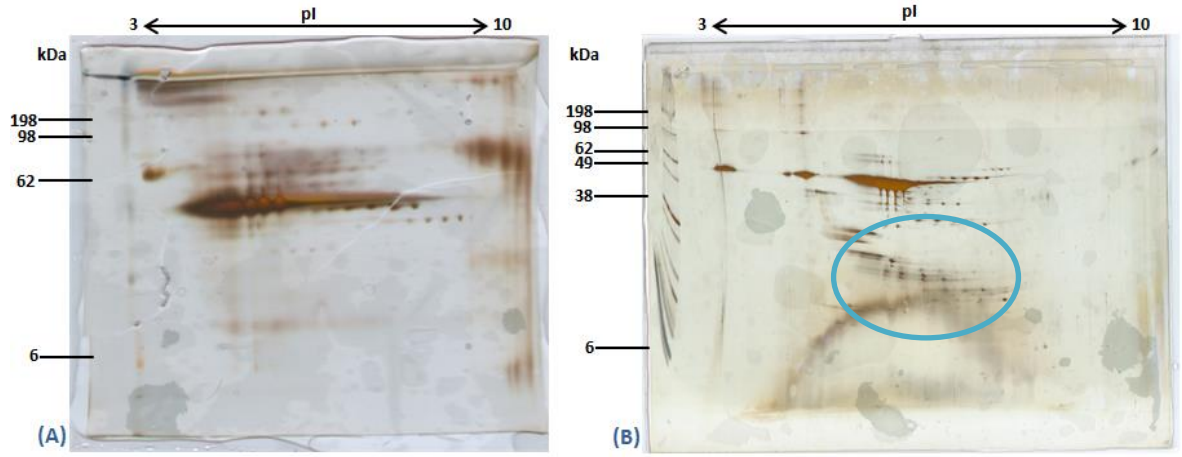


Figure 33: Comparison of the separation (A) on a self-made SDS-PAGE T 12.5% gel , performed on a vertical system and (B) on a precast 2D HPE large T 12.5% gel, performed on a horizontal system.

According to Figure 33 further experiments were done with precast 2D gels on the horizontal system. First of all a higher resolution and sensitivity was achieved with the precast gels compared to the self-made gels. More focused spots were obtained with the precast gels compared to self-made gels. Furthermore low molecular weight proteins were detected (highlighted in blue) with the horizontal setup. This is due the following fact: To maintain a long-term stability of the HPE gels, they were polymerized with a buffer with a pH below 7, in order to prevent alkaline hydrolysis. For this glycine is replaced by tricine in case of HPE gels, therefore a good separation quality is simultaneously achieved. Through tricine the resolution in a HPE gel is extended down to 6 kDa, in contrast to glycine containing buffer system [60]. Additionally the horizontal flatbed system with its ready-made gels has a higher reproducibility in comparison to self-made gels.

Furthermore up to four gels can be run at the same time in the multilevel flatbed apparatus (HPE tower) leading to a higher reproducibility because same separation conditions are maintained.

The HPE gels used in this master thesis are 650 µm thick. They have a trench for the application of the IPG-strip which is located in the stacking gel area (6% T and 3% C). The resolving gel contained 12.5% T and 2% C. In contrast the self-made gel is 1 mm thick and the resolving gel contained 12.5% T and 2.6% C. Even if the amount of crosslinker is slightly higher in the self-made gels, their performance is lower. Therefore an influence of acrylamide composition and thickness of the gel is improbable [60].

4.1.3.4 Reproducibility of 2D GE

The better separation on the horizontal gel system advised for further use throughout this thesis. However the reproducibility of the 2D gels had to be evaluated. Therefore αMEM/Norm samples were precipitated with TCA/acetone and the protein concentration was determined. Samples were aliquoted and separated by 2D GE in triplicate on 24 cm IPG strips (non-linear pH gradient from 3 to 10) and T% 12.5 SDS-PAGE. Specifics of single 2D GE experiments were as follows:

Table 21 gives an overview about the mixture of the used IEF buffer, containing the corresponding αMEM/Norm samples for all three replicates.

	Replicate 1	Replicate 2	Replicate 3
DL-Dithiothreitol (1M)	9 µL	9 µL	9 µL
Servalyte, 3-10	9 µL	9 µL	9 µL
αMEM/Norm	**100 µL (904 µg)**	**50 µL (450 µg)**	**50 µL (450 µg)**
Rehydration-buffer	332 µL	382 µL	382 µL
In Total	450 µL	450 µL	450 µL

Table 21: Composition of the analysed sample containing IEF-buffer for all three replicates.

Table 22 and Table 23 show the applied program for both dimensions. An overview of all three progresses of currents and voltages for both dimensions is given in Figure 34 and Figure 35.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [µA]**	**Watt [W]**	**Time [h]**
1	150	1	5	3:00
2	300	1	5	3:00
3	1000	1	5	6:00
4	3500	1	5	5:00
5	3500	1	5	15:00

Table 22: Isoelectric focussing program for the first dimension for all three replicates.

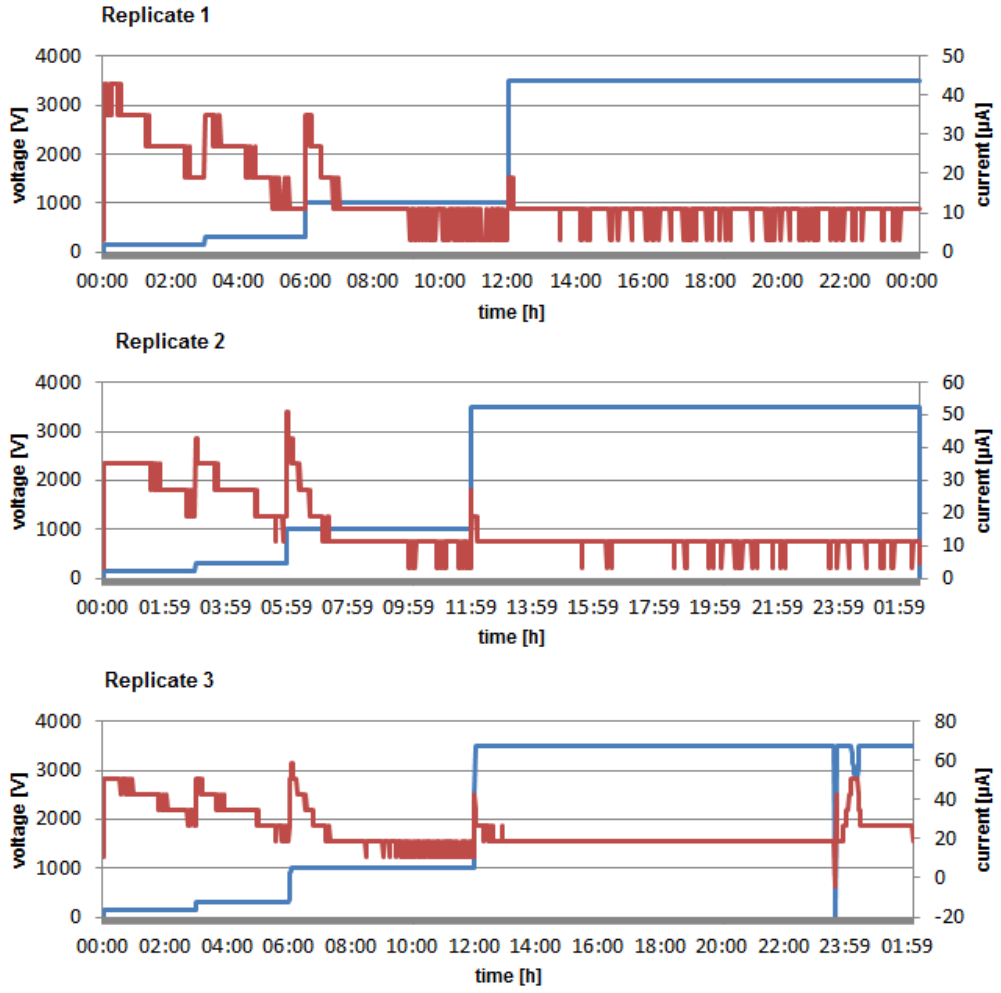


Figure 34: Voltage and current over the course of time for all three αMEM/Norm sample replicates.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 23: HPE tower program for the second dimension for all three replicates.

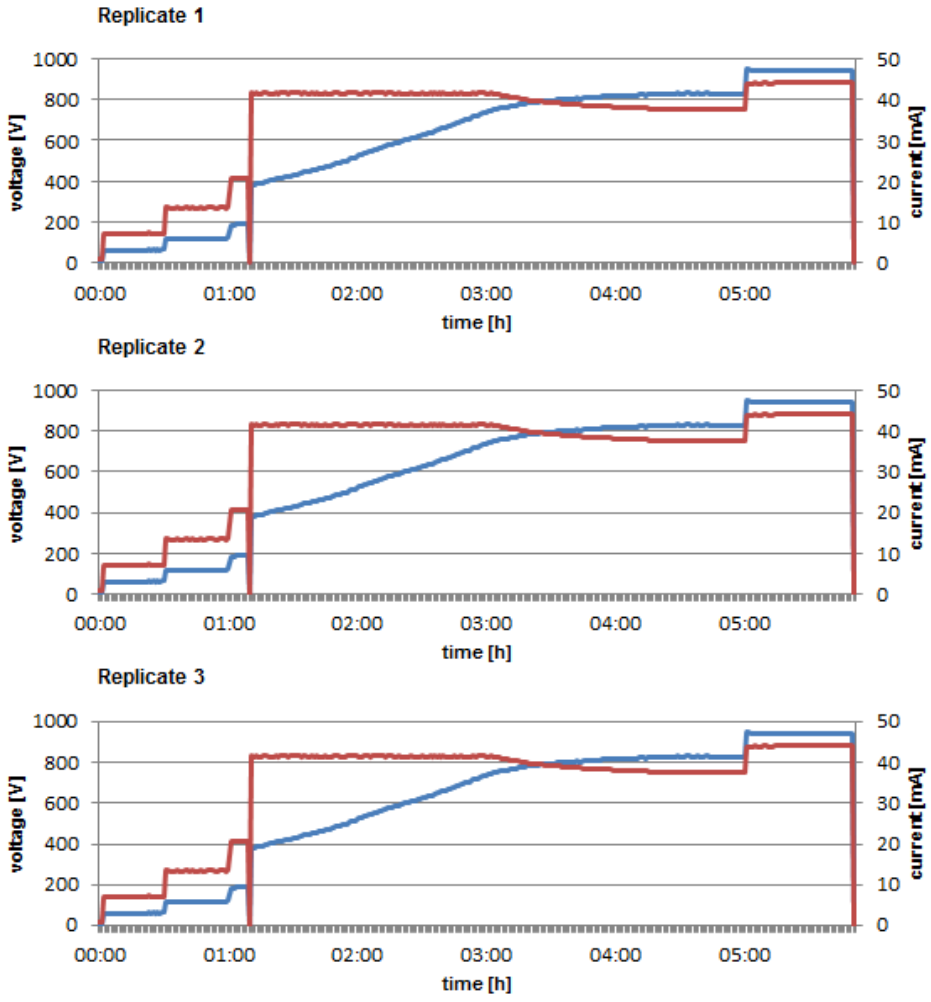


Figure 35: Voltage and current over the course of time for all three αMEM/Norm sample replicates.

Voltage and current did not show uncommon behavior over time for the first and second dimension as shown in Figure 34 and Figure 35.

Sample replicates were focused by IEF (on average 58.9 kVh, 10 mAh, 26.6 h) and separated by SDS-PAGE (on average 3.535 kVh, 195.6 mAh, 5:50 h) (Details can be seen below in Table 24).

	Replicate 1	Replicate 2	Replicate 3
Protein load	**100 µl (904 µg)**	**50 µL (450 µg)**	**50 µL (450 µg)**
IEF	61.6 kVh	58.6 kVh	56.7 kVh
	9 mAh	9 mAh	12 mAh
	27:30 h	26:38 h	26:07 h
SDS PAGE	3517 kVh	3517 kVh	3572 kVh
	196 mAh	196 mAh	195 mAh
	5:50 h	5:50 h	5:50 h
Gel stain	fluorescence/silver	fluorescence/silver	fluorescence/silver

Table 24: Overview of the obtained parameters for the first and second dimension for all three αMEM/Norm samples replicates.

The obtained 2D gels were stained with Serva purple and subsequent with silver staining as shown in Figure 36.

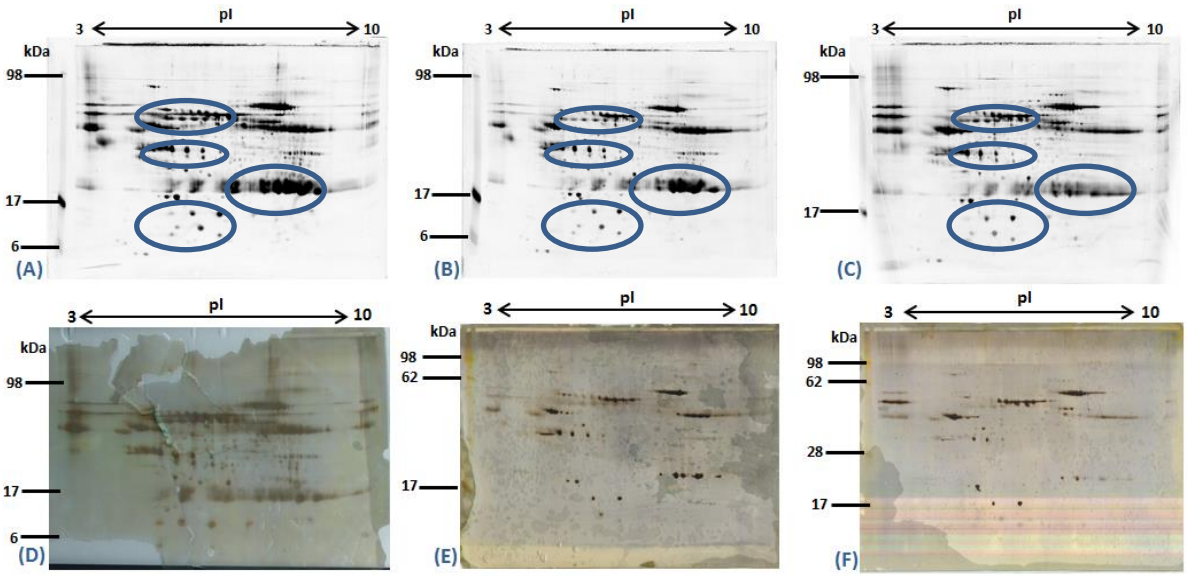


Figure 36: 2D GE of proteins depleted from one αMEM/Norm sample. (A+D) 900µg, (B+E) and (C+F) are replicates of 450 µg protein load.

For method evaluation 2D GE has to be reproducible. According to Figure 36 a similar 2D gel spot pattern for all 2D gel triplicates was received. The gels were only analysed qualitative due to less time for the evaluation, limited possibility from the software and limited number of focussed spots. Therefore the intensity of only few spots, highlighted in blue, was visually

compared. Normally gel image analysis software is applied for a quantitative evaluation of separated protein spots. Nevertheless it can be said that similar protein spot pattern were received (areas are marked in blue) and sample preparation appears to be reproducible. Based on the presences of highly abundant proteins it was difficult to detect and visualize low-abundance proteins which were assumed to be highly relevant for secretome analysis. So good quality of the raw data is very important and influences the final results. Sample pre-fractionation strategies were expected to allow better access to the secretome, maybe even concentrating proteins of interest.

4.1.4 Comparison of silver and fluorescence stained 2D gels of αMEM/Norm samples

During this work two different post-electrophoresis staining methods (silver and Serva purple a fluorescent dye) were tested for their sensitivity and ability to give full protein spot detection. Therefore αMEM/Norm and αMEM samples were precipitated, diluted 1:500 and protein concentration was determined. Figure 38 show the linear regression curve for BSA standards and Table 25 lists the results for the αMEM/Norm and αMEM samples.

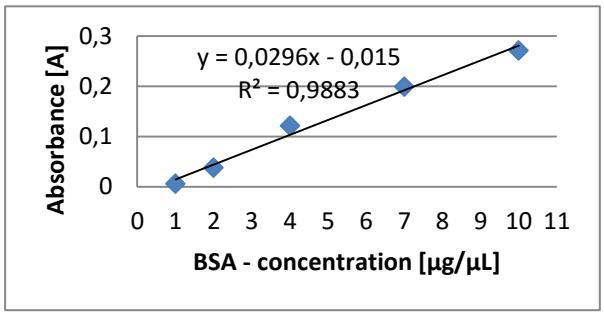


Figure 38: Linear regression for BSA calibrants.

Sample	Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm (1:500)	measurement 1	0.112	0.109	4.386	4.295	**21.48**
	measurement 2	0.106		4.188		
	measurement 3	0.108		4.254		
	measurement 4	0.111		4.353		
αMEM (1:500)	measurement 1	0.105	0.094	4.156	3.785	**18.93**
	measurement 2	0.099		3.958		
	measurement 3	0.096		3.860		
	measurement 4	0.075		3.169		

Table 25: Overview on calculations for protein concentration of the used αMEM/Norm and αMEM samples.

For the separation with 2D GE only the αMEM/Norm sample was used. Table 26 shows the composition of the IEF buffer and the used sample. The IPG strip was focussed according to the program as shown in Table 27 and the actual progress of current and voltage over 26 hours of focussing is given in Figure 39. The sample was focussed in total for 58.6 kVh, 9.0 mAh and 26:39 h.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Norm	**50 µL** (450 µg)
Rehydration-buffer	382 µL
In total	450 µL

Table 26: Composition of the IEF buffer containing the analysed sample.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [µA]**	**Watt [W]**	**Time [h]**
1	150	1	5	3:00
2	300	1	5	3:00
3	1000	1	5	6:00
4	3500	1	5	5:00
5	3500	1	5	15:00

Table 27: Isoelectric focusing program for the αMEM/Norm sample.

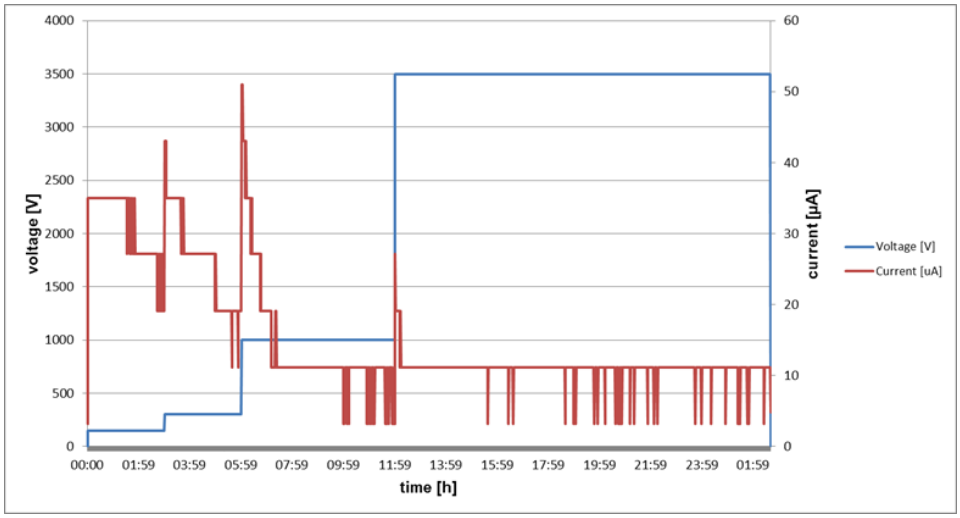


Figure 39: Voltage and current over the course of time for the analysed αMEM/Norm sample.

For the second dimension the set program is shown in Table 28 and the corresponding progress of voltage and current could be seen in Figure 40.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 28: HPE tower program for the second dimension for the αMEM/Norm sample.

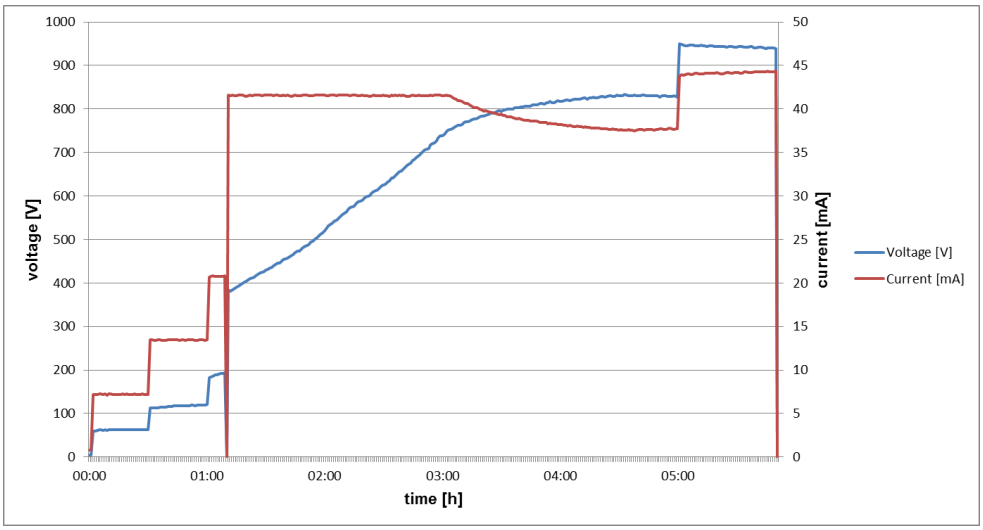


Figure 40: Voltage and current over the course of time for the αMEM/Norm sample.

The second dimension took 5:50 h and 3519 kVh as well as 196 mAh were obtained. For the first and second dimension no abnormalities for the current over time were observed as shown in Figure 39 and Figure 40.

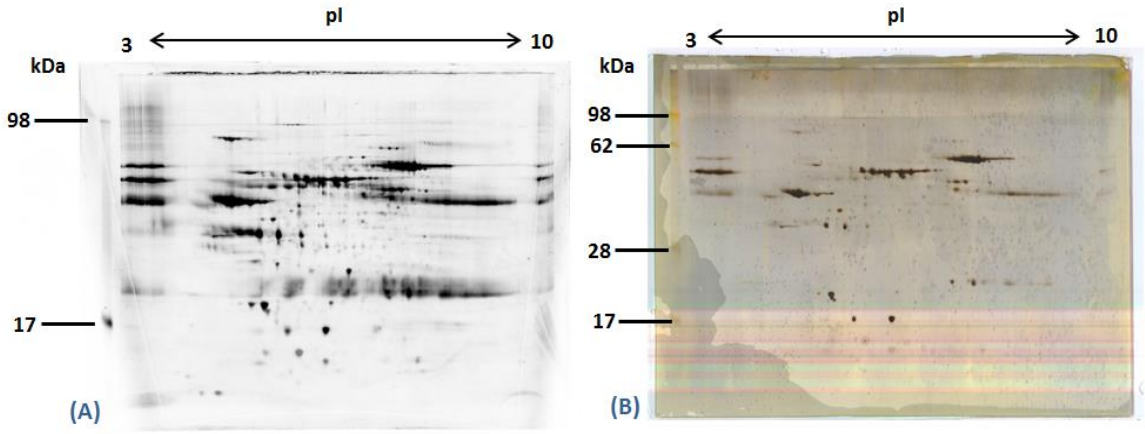


Figure 41: 2D gel of αMEM/Norm sample after (A) fluorescence staining using Serva purple and (B) silver staining according to Shevchenk [3]. In both cases 450 µg protein were loaded.

Due to the fact that we detected numerous protein spots, partly showing a higher intensity, with the fluorescence staining compared to silver staining it was decided to take a closer look at the observed differences. In general it can be said that fluorescence staining with Serva purple is more sensitive (down to 50 pg) compared to silver staining (down to 0.1 - 1 ng) and it is also compatible with MS analysis, a very important point for a complete gel-based proteome approach. Figure 41 shows the 2D gels of αMEM/Norm sample stained with Serva purple in comparison to silver staining. Based on a higher sensitivity of the fluorescent dye an increased protein spot recovery compared to silver was received. Both techniques are reversible and compatible with MS analysis however Serva purple showed lower background fluorescence compared to silver. Furthermore Figure 41 indicates that the protein pattern was distorted and horizontal streaks in the second dimension were observed at MW 30 kDa to 65 kDa. Moreover some spot are only partially resolved, especially in the basic (pI 8 to 9) and acidic (pI 3 to 5) area, generated by the still too high protein load and insufficient protein separation caused by probably protein precipitation or non-protein impurities in the sample. Indeed, Serva purple provided an improve yield of protein spots leading to use it for further experiments.

4.2 Synovia – A biological fluid to evaluate 2D GE conditions

4.2.1 Determined Protein Concentration via Bradford Assay

To get introduced to the topic and the applied techniques, first experiments were conducted with synovial fluid. Synovia is a viscous, non-Newtonian fluid found in the cavities of synovial joints. The principal role of synovial fluid is to reduce friction between the articular cartilage of synovial joints during movement [76]. The fluid contains numerous plasma proteins and enzymes, which makes it a suitable sample to get an insight into 2D GE.

Synovia samples were used without protein precipitation but diluted 1:500 with IPG buffer before measurement. The samples were diluted and divided into two aliquots. The protein concentration for both technical replicates was determined according to chapter 3.2.4 (page 46) and the measurement was carried out on the photometer by pressing the measurement-button twice. A calibration curve using BSA in IPG buffer (1 – 10 µg/µL) was established, as shown in Figure 42.

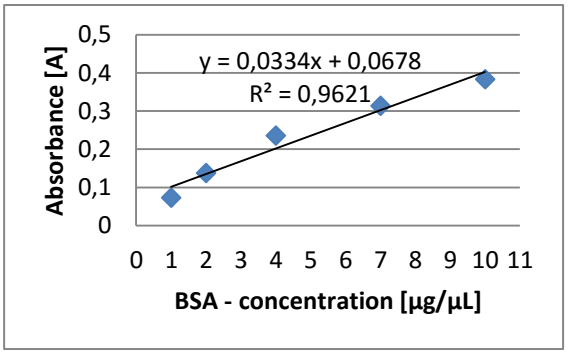


Figure 42: Linear regression for BSA calibrants.

Table 29 shows the final results for the synovia sample. The calculated concentration values include the dilution factor (1:500), the volume used for protein determination (10 µL) and the conversion factor (1000) from µg/mL to µg/µL. Every absorbance measurement was carried out in duplicate.

Sample		Absorbance			Concentration	
			Average (SD)	µg/mL	Average (SD)	µg/µL
Synovia	measurement 1	0.292	0.290 (+/- 0.002)	6.710	6.395 (+/- 0.05)	**33.3**
	measurement 2	0.288		6.609		
Synovia	measurement 1	0.246	0.243 (+/- 0.002)	5.337	5.256 (+/- 0.08)	**26.28**
	measurement 2	0.241		5.176		

Table 29: Overview on calculations for protein concentration of the used synovia sample.

Table 29 shows a broad variation (24%) between the obtained concentration values from both technical replicates. Here it has to be mentioned that this was the first experiment of this master thesis, in which errors during sample preparation are very likely. Further reasons for the result can be that the Bradford reagent was not enough filtered, the sample was not enough mixed during sample preparation or the pipettes were not calibrated.

4.2.2 Analysing the Synovia Samples via 2D GE

4.2.2.1 First dimension - Isoelectric focusing

After the determination of the protein concentration via Bradford assay, the synovia sample was analysed using 2D gel electrophoresis. Therefore for the first dimension of the 2D GE the samples were treated according to the chapter 3.6.1.4 (page 53). Prior to the IEF step, the 24 cm pH 3-10 NL IPG strip was rehydrated overnight with IEF buffer, which also contained the synovia sample. Table 30 shows the composition of the rehydration buffer and the used sample volume. After rehydration the IPG strip was focussed according to the program given in Table 31 and the actual voltage and current over 23 hours of focussing is given in Figure 43. The sample was focussed in total for 70.8 kVh, 18.8 mAh and 23:13 h.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
Synovia	**3 µL** (99.9 µg)
Rehydration-buffer	429 µL
In total	450 µL

Table 30: Composition of the IEF buffer containing the analysed sample.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	300	1	5	1:00
2	300	1	5	1:00
3	1000	1	5	3:00
4	3500	1	5	4:00
5	3500	1	5	15:00

Table 31: Isoelectric focusing program used for Synovia.

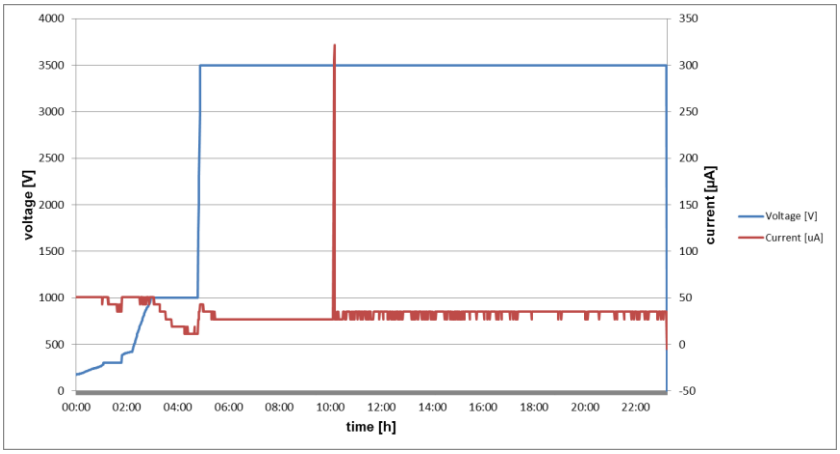


Figure 43: Voltage and current over the course of time for Synovia sample.

Figure 43 indicates an almost normal current flow during the first dimension. However at 10:00 h a non-sample conditional noise was detected. This is explained by inherent detector fluctuations, instrumental errors or impurities in the sample as fluff or salt.

4.2.2.2 Second dimension – SDS PAGE

After IEF the IPG strip was reduced and alkylated according to chapter 3.6.4.4 (page 57) before separating the proteins according to their molecular weight on 255 x 200 x 0.65 mm 2D HPE Large Gel NF T% 12.5 using the HPE tower. Table 32 shows the used program for

this step and Figure 44 the corresponding voltage and current run over time. The second dimension took 5:50 h, leading to 3588 kVh and 193 mAh.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 32: HPE tower program for the second dimension used for Synovia

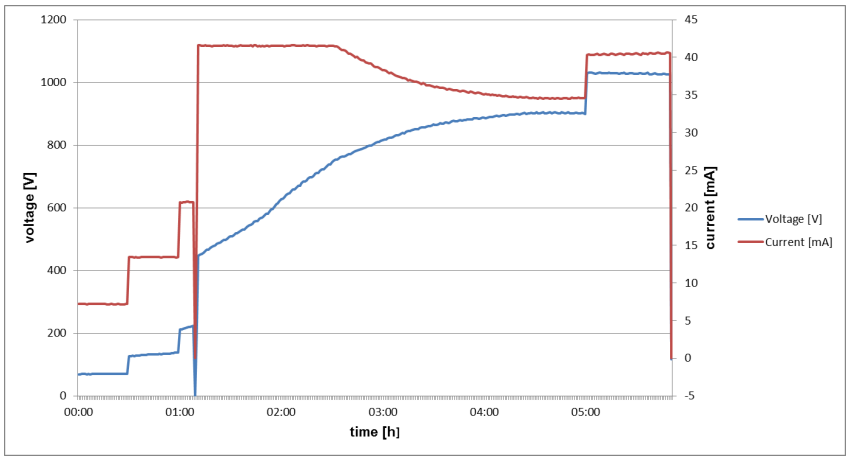


Figure 44: Voltage and current over the course of time for the Synovia sample.

The fall at 1:10 h is caused by a pause to remove the IPG strip. After separation the 2D gel was silver stained according to the protocol given in chapter 3.7.2.4 (page 63) [3], the obtained result is shown in Figure 45.

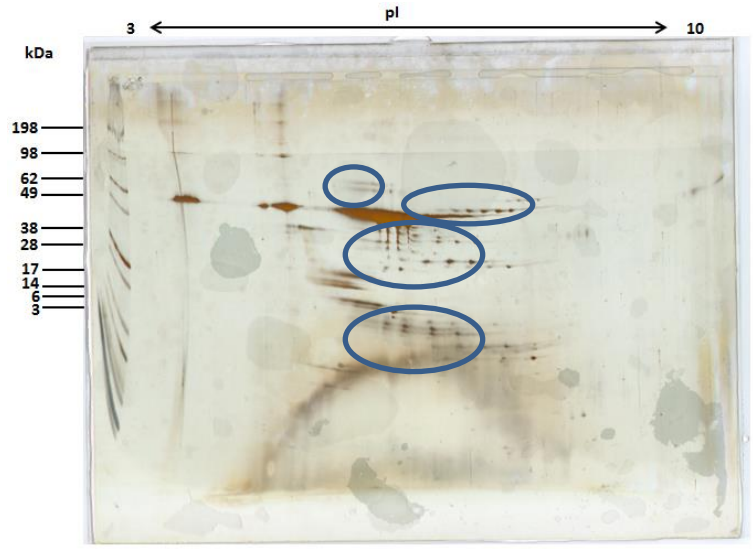


Figure 45: 2D gel of synovia, separated on a 24 cm IPG strip containing a non-linear pH-gradient 3-10, followed by SDS PAGE using T% 12.5 gels. The gel was silver stained. A pre-stained molecular weight marker was used.

Figure 45 displays the spot pattern of a silver stained 2D gel, showing the separated synovia sample. Only a few defined protein spots, highlighted in blue, with acidic pIs and MWs from 30 kDa to 60 kDa were detected. Furthermore a cloudy background, a blurred molecular weight marker and horizontal streaks were observed. Assumptions for that could be that micelles between SDS and the zwitterionic and non-ionic detergents have formed, impurities in the sample or sample buffer have occurred or a too long focusing time which leads to protein precipitation and so hampers the protein separation. Also mistakes during sample preparation and handling or the quality of the gel contribute to this result.

4.3 2D GE of the MSC secretome without sample pre-fractionation

In vitro cell cultivation of MSCs is carried out under ambient O_2 concentration (21%) which is also defined as “normoxic” condition. In contrast, *in vivo* MSCs usually are not exposed to such O_2 concentrations [20]. To establish a first protein map of the MSC secretome, MSCs cultivated under different O_2 conditions (21% or 5% O_2) in a bioreactor with αMEM medium containing 10% human serum and 0.5% gentamicin were used (details see chapter 3.1.4 page 43). The supernatant, which contains the secretome, was collected and analysed. For better comparison, sole αMEM was also analysed. These first experiments were supposed to give a first insight into sample behaviour during 2D GE. Furthermore these experiments were expected to give first information on protein quantity and pattern, of the αMEM medium containing the MSC secretome respectively.

Protein concentration determination by Bradford assay showed that the concentration was too low to be measured by VIS spectroscopy and therefore also too low for 2D GE. Protein precipitation using 61 mM trichloroacetic acid and ice-cold acetone was performed (details see 3.3.4 page 48) for the secretome and alphaMEM sample. Afterwards the protein concentration of the αMEM and αMEM/Norm sample was measured. A calibration curve was established (See Figure 46 for details).

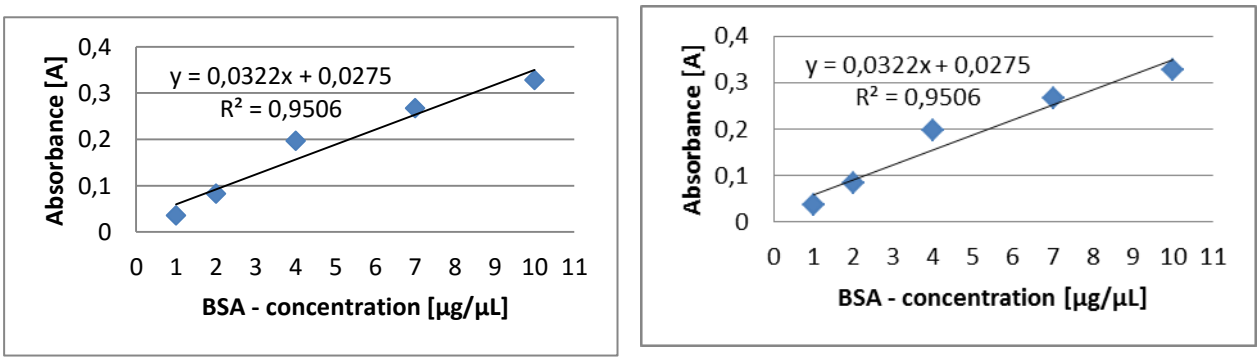


Figure 46: Linear regression for BSA calibrants.

Table 33 shows the obtained results for the measured αMEM and αMEM/Norm sample. The calculated concentration values include the dilution factor (1:1000), the volume (10 µL) and the conversion factor (1000) from µg/mL to µg/µL. Absorbances were measured in duplicates.

Sample	Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm	measurement 1	0.204	0.216	6.252	6.64	**66.4**
	measurement 2	0.229		7.028		
αMEM	measurement 1	0.209	0.197	6.407	6.050	**60.5**
	measurement 2	0.186		5.694		

Table 33: Overview on calculations for protein concentration of the used αMEM and αMEM/Norm samples.

After the determination of the protein concentration via Bradford assay, the samples were analysed using 2D GE. For the first dimension immobilized pH gradient gel strips (24cm, pH 3-10, non-linear) were used to get an overview of protein distribution. The isoelectric focussing step was again performed according chapter 3.6.1.4 (page 53). The sample was introduced via overnight in gel rehydration. Table 34 shows the composition of the IEF buffer and the used sample volume. After the rehydration step, the IPG strip was focussed according to the set program as shown in Table 35 and the actual progress of current and voltage over 24 hours of focussing is given in Figure 47. The sample was focussed in total for 69.8 kVh, 19.8 mAh and 24:13 h.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Norm	**2 µL** (121 µg)
Rehydration-buffer	430 µL
In total	450 µL

Table 34: Composition of the IEF buffer containing the analysed sample.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	300	1	5	1:00
2	300	1	5	1:00
3	1000	1	5	3:00
4	3500	1	5	4:00
5	3500	1	5	15:00

Table 35: Isoelectric focusing program for the αMEM/Norm sample.

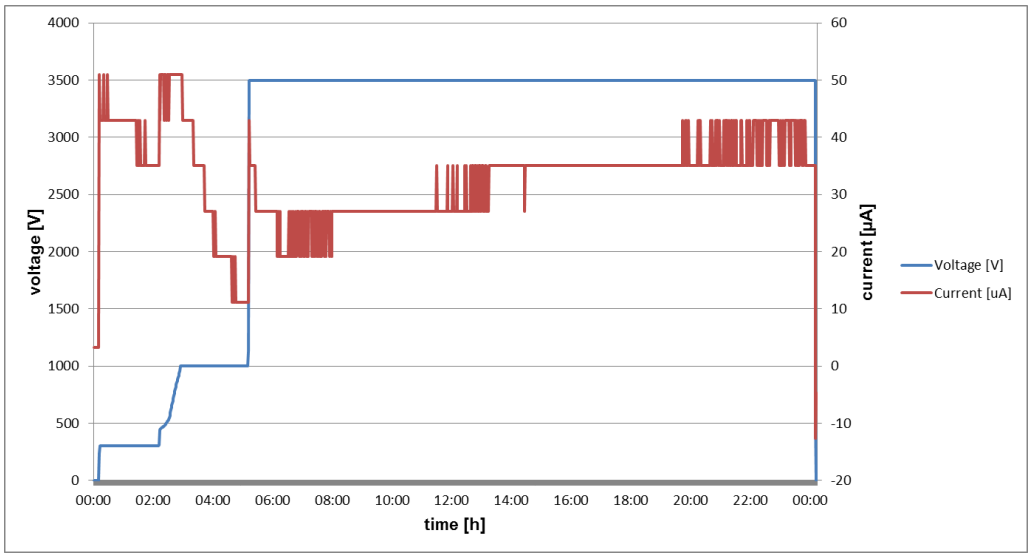


Figure 47: Voltage and current over the course of time for the αMEM/Norm sample.

A reduction and alkylation steps was performed prior to the second dimension according to chapter 3.6.2.4 (page 54). For the performance the HPE tower was used. The set program is shown in Table 36 and the corresponding progress of voltage and current could be seen in Figure 48.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 36: HPE tower program for the second dimension for the αMEM/Norm sample.

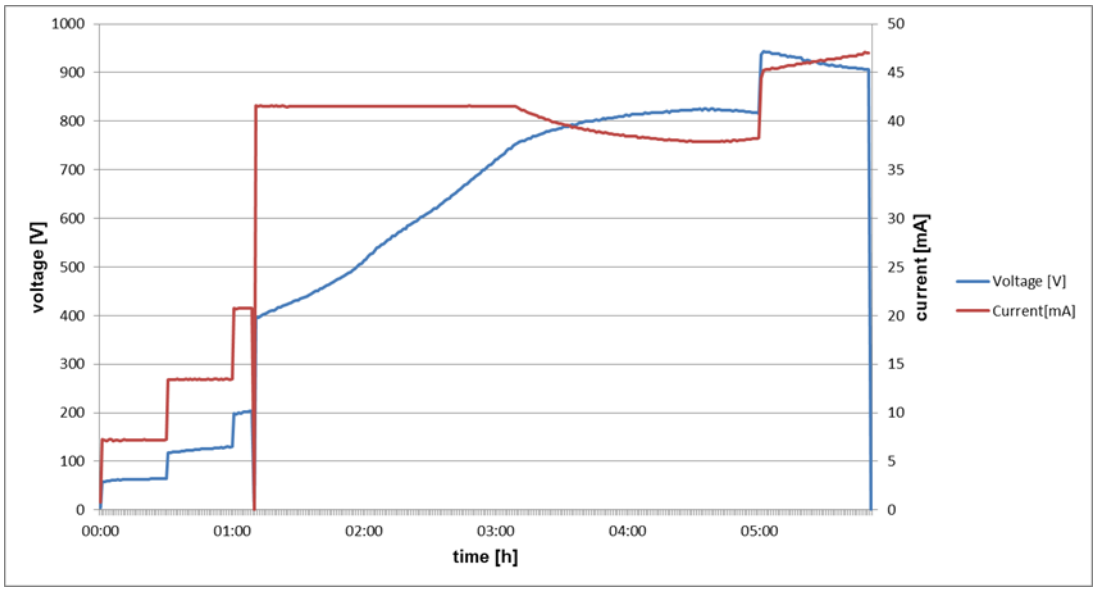


Figure 48: Voltage and current over the course of time for the αMEM/Norm sample.

The drop at 1:10 h is the result of a pause during the program duo to remove the IPG strip. The second dimension took 5:50 h and 3604 kVh and 195 mAh were obtained.

After these steps the 2D gel was stained silver according to the protocol in chapter 3.7.2.4 (page 63) [3], the obtained result is shown in Figure 49.

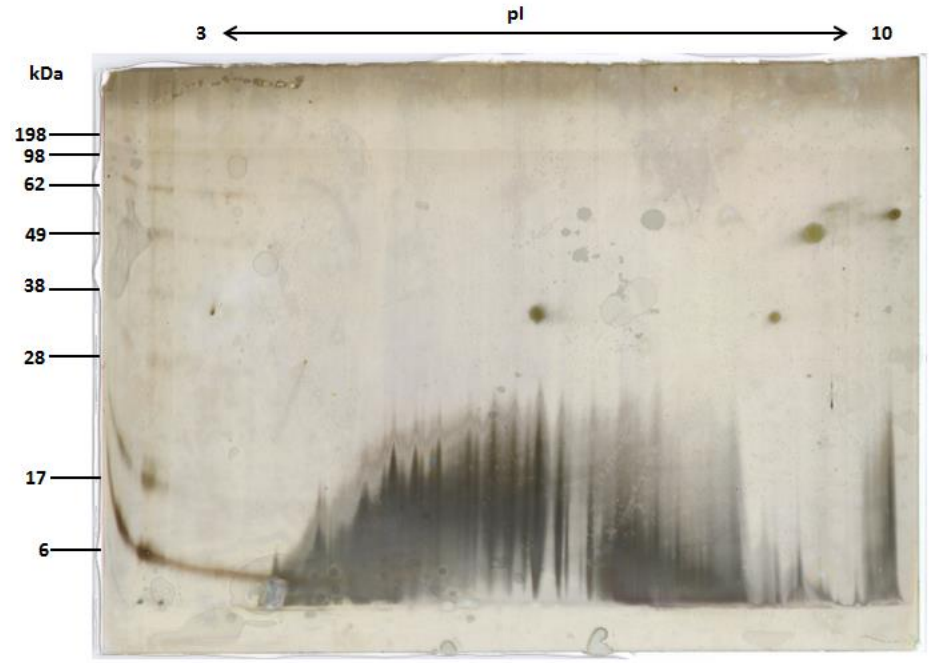


Figure 49: 2D gel of αMEM/Norm sample, separated on a 24 cm IPG strip containing a non-linear pH-gradient 3-10, followed by SDS PAGE using T% 12.5. gels. The gel was silver stained. A pre-stained molecular weight marker was used.

2D GE did not perform well. Due to low protein content or maybe formed protein aggregates or complexes the sample was either not focussed well during IEF or did not migrate into the gel in the second dimension.

For better results sample preparation parameters were changed. New Serva ampholytes (3-10) were used, the sample volume was increased during the IPG strip rehydration and the 2D gel was stained with Serva purple, a more sensitive fluorescence dye.

Proteins were again precipitated in duplicates (αMEM/Norm (1) and (2)) from the same αMEM/Norm sample and αMEM. Protein concentration was determined and Figure 50 shows the linear regression curve. Table 37 lists the results for the αMEM/Norm (1) and (2) samples.

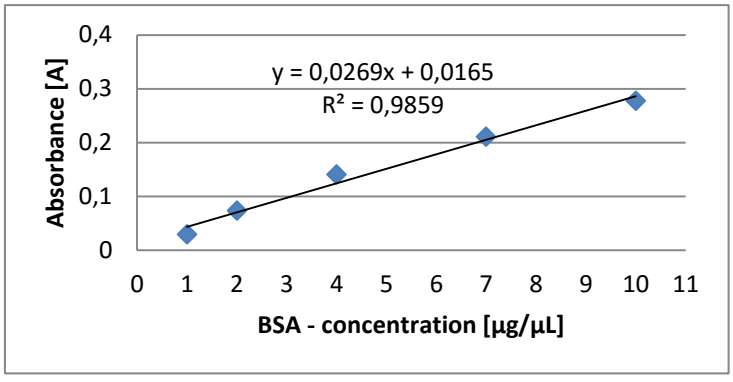


Figure 50: Linear regression for BSA calibrants.

Sample		Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm (1)	measurement 1 measurement 2	0.227 0.228	0.2275	7.815 7.852	7.834	**78.3**
αMEM/Norm (2)	measurement 1 measurement 2	0.218 0.217	0.2175	7.481 7.444	7.463	**74.63**
αMEM (1)	measurement 1 measurement 2	0.206 0.209	0.208	7.035 7.147	7.091	**70.91**
αMEM (2)	measurement 1 measurement 2	0.177 0.191	0.184	5.959 6.478	6.219	**62.19**

Table 37: Overview on calculations for protein concentration of the used αMEM/Norm samples and the αMEM medium.

Table 38 shows the composition of the IEF buffer and the used sample volume. For the focussing the IEF program was adapted. The second focusing step was raised from 300 V to 500 V as shown in Table 39. The actual progress of voltage and current over 23 hours of focussing is given in Figure 51. The sample was focussed in total for 66.8 kVh, 18.0 mAh and 23:33 h.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Norm	**6 µL** (447.8 µg)
Rehydration-buffer	426 µL
In total	450 µL

Table 38: Composition of the IEF buffer containing the analysed sample.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [µA]**	**Watt [W]**	**Time [h]**
1	300	1	5	1:00
2	500	1	5	1:00
3	1000	1	5	3:00
4	3500	1	5	4:00
5	3500	1	5	15:00

Table 39: Isoelectric focusing program for the αMEM/Norm sample.

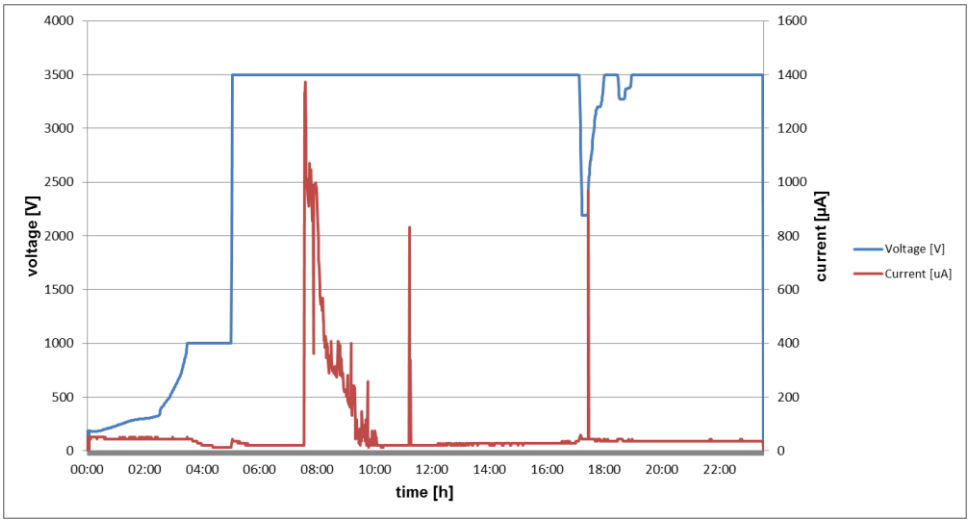


Figure 51: Voltage and current over the course of time for the αMEM/Norm sample.

Figure 51 shows the voltage and current progress during IEF for the second attempt of separating the αMEM/Norm sample with 2D GE. The abrupt increase of the current flow at 8:00 h is probably caused by the presence of buffer ions or a high salt concentration. Again at 18:00 h the maximum current of 50 µA was exceeded and separation was current restricted (max. values). This leads to a decrease of the voltage flow, hampering the protein separation.

After reduction and alkylation given in chapter 3.6.2.4 (page 54), the second dimension was conducted at the HPE tower. The set program is shown in Table 40 and the corresponding progress of voltage and current could be seen in Figure 52.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 40: HPE Tower program for the second dimension for the αMEM/Norm sample.

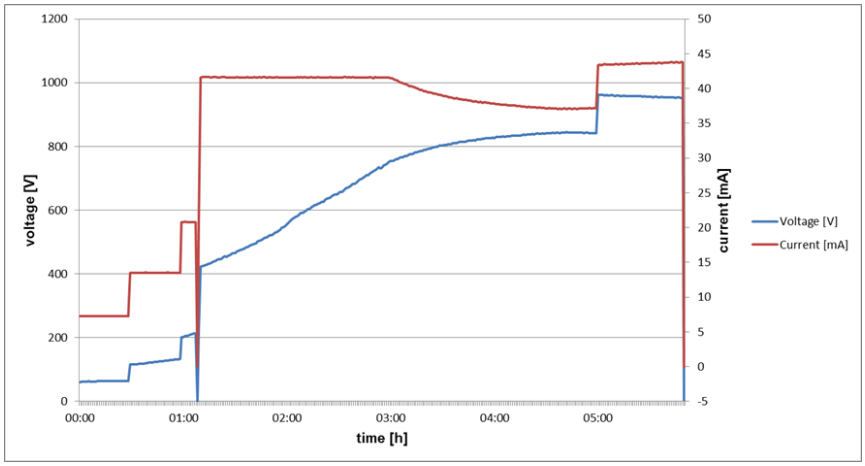


Figure 52: Voltage and current over the course of time for the αMEM/Norm sample.

The fall at 1:10 h is caused by a pause to remove the IPG strip. For the second dimension following parameters were obtained: 3603 kVh, 192 mAh and 5:50 h.

The 2D gel was post-stained with Serva purple according to chapter 3.7.1.4 (page 61). Figure 53 shows the fluorescence stained gel after 2D separation. It can be seen that the staining procedure was not successful, no protein spots and no marker were visualized. To rule out staining artifacts, the gel was again stained with Serva purple. For this the gel was incubated in solution 1 (composition see chapter 3.7.1.3 (page 60)) overnight and then staining was finished according to the protocol. The obtained gel scan can be seen in Figure 55.

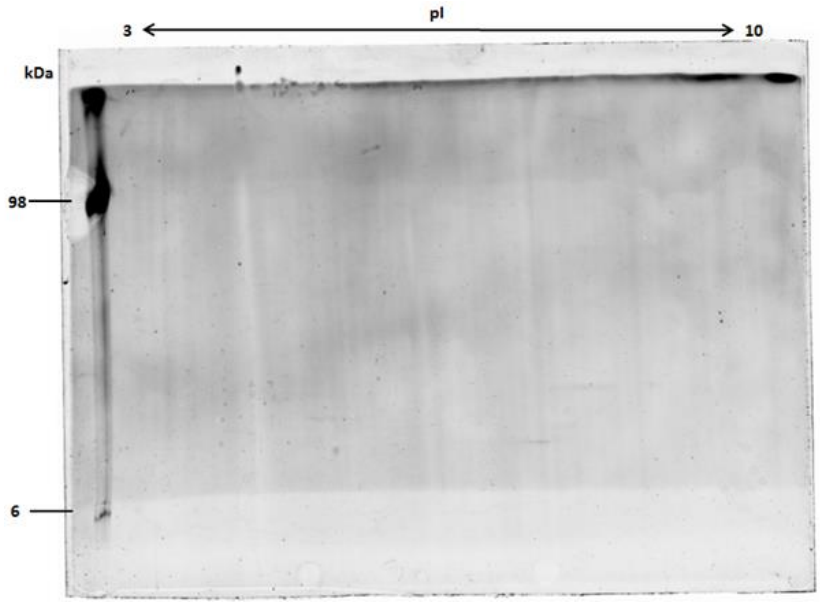


Figure 53: 2D gel of secretome sample, separated on a 24 cm IPG strip containing a non-linear pH-gradient 3-10, followed by SDS PAGE using T% 12.5. gels. The gel was fluorescence stained using Serva purple. A pre-stained molecular weight marker was used.

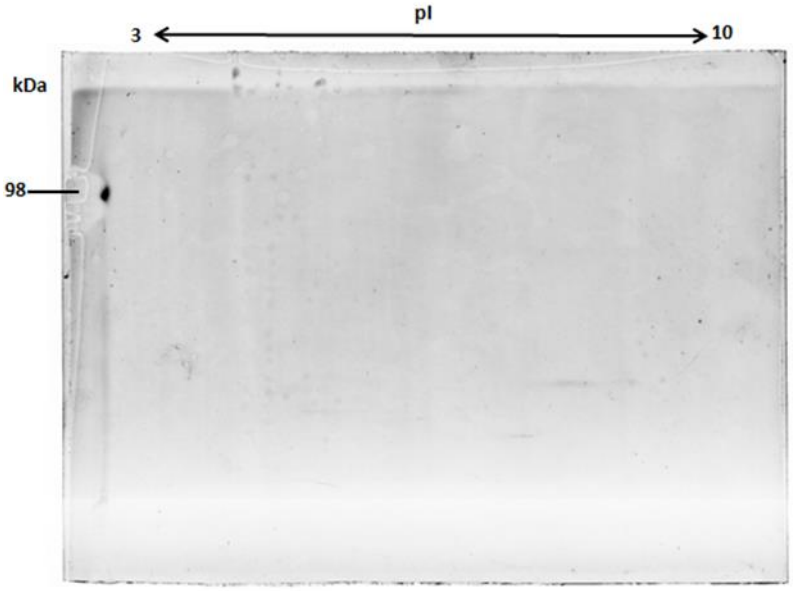


Figure 54: Second fluorescence stain with Serva purple on the same 2D gel as in Figure 53.

The repeated staining process did not improve the results. No protein spots were visible again. As a further attempt subsequent silver staining of the very same gel was carried out. For this, the gel was destained with 50mM ammonium carbonate solution in 50% methanol and UHQ water overnight and silver stained according to chapter 3.7.2.4 (page 63) [20].

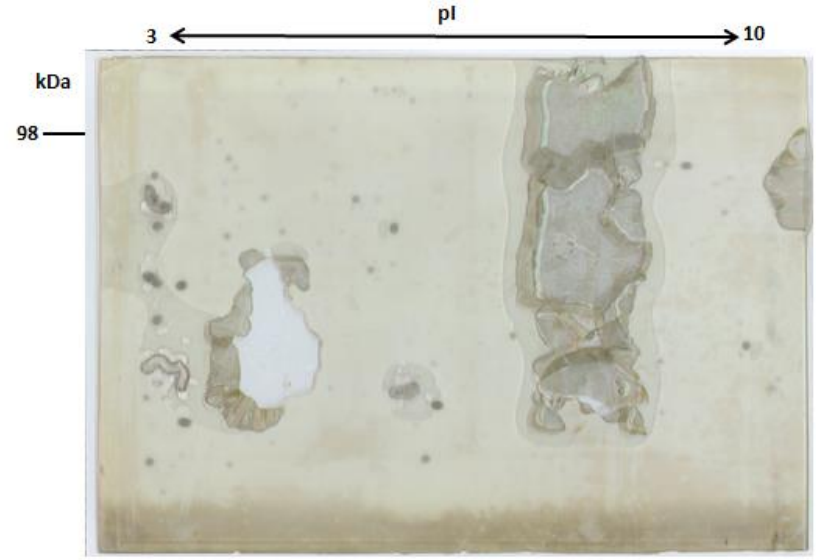


Figure 55: Same 2D gel as in Figure 53 and Figure 54 after silver staining.

Figure 55 shows that even silver staining did not improve the result; again no protein spots were able to detect. Furthermore the gel was detached from the film and was partly destroyed, probably according to too long incubation times in methanol containing solutions.

To proof whether the selected precipitation strategy with TCA/ice-cold Acetone (details see chapter 3.3.4 (page 48)) works for the analysed αMEM/Norm sample and to check if the sample contains a detectable protein amount, a 1D PAGE was performed. This time 1D GE was done directly after protein precipitation. Only if protein bands were detected and of reasonable intensity, further 2D GE was performed. Again proteins were precipitated and protein concentration was determined by Bradford assay before 2D GE. A synovia fluid sample was equal treated like the analysed αMEM/Norm samples.

Figure 56 displays the obtained calibration curve for the BSA standards. Table 41 shows the obtained results from the measured αMEM, αMEM/Norm and synovia fluid sample. The calculated concentration values include the dilution factor (αMEM and αMEM/Norm 1:100; Synovia fluid 1:500), the volume (10µL) and the conversion factor (1000) from µg/mL to µg/µL. The absorbances from the analysed samples were measured in duplicates.

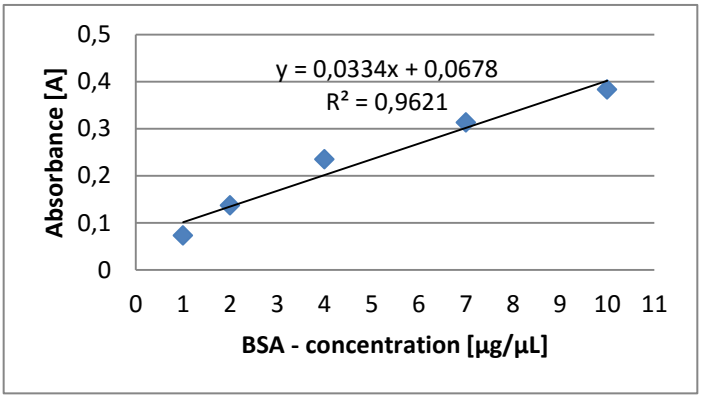


Figure 56: Linear regression for BSA calibrants.

Sample	Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm (1:100)	measurement 1 measurement 2	0.387 0.380	0.384	9.552 9.343	9.448	**9.45**
αMEM (1:100)	measurement 1 measurement 2	0.235 0.261	0.248	5.003 5.782	5.392	**5.39**
Synovia fluid (1:500)	measurement 1 measurement 2	0.186 0.170	0.178	3.537 3.058	3.297	**16.49**

Table 41: Overview on calculations for protein concentration of the used αMEM, the αMEM/Norm and synovia fluid sample.

As shown in Table 41, the analysed αMEM/Norm sample contains 9.5 µg/µL protein, which is two-fold more than the αMEM contains.

The samples were diluted (see Table 42) to achieve an average protein amount of 1 µg (5 µL) and 2 µg (10 µL) per lane.

	αMEM/Norm (1:100)	αMEM (1:100)	Synovia fluid (1:500)
DL-Dithiothreitol (1M)	9 µL	9 µL	9 µL
LDS sample buffer (4x)	9 µL	9 µL	9 µL
Sample	**4.23 µL (40.1 µg)**	**7.42 µL (40 µg)**	**2.43 µL (40 µg)**
UHQ water	9.77 µL	6.58 µL	11.53 µL
In total	20 µL	20 µL	20 µL

Table 42: Composition of 1D PAGE buffer containing the analysed samples.

The samples were separated using a 4-12% Bis-Tris gel according to chapter 3.5.4 (page 51), and a MES buffer was used as running buffer. The electrophoretic separation was initiated with starting conditions as presented in Table 43 giving the maximal values. The gel was stained with Serva purple, left overnight in UHQ water and silver stained on the next day. The obtained scans of the stained gels can be seen in Figure 57.

Nupage Gel Program			
Voltage [V]	**Current [mA]**	**Watt [W]**	**Time [h]**
120	60	25	1:35

Table 43: Initial starting conditions for SDS PAGE.

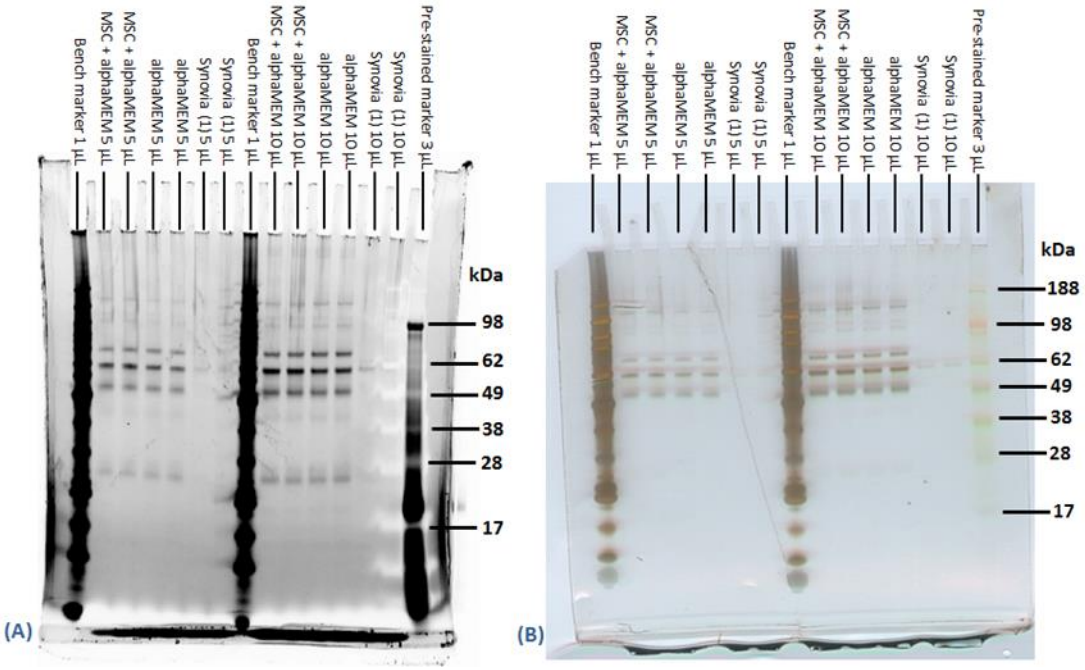


Figure 57: SDS PAGE of synovia, αMEM and αMEM/Norm samples. 1D gel after (A) post- fluorescent staining and (B) silver staining. A pre-stained molecular weight marker was used.

According to Figure 57 protein bands were detected with 1D PAGE. It can be seen that Serva purple (Figure 57A) is more sensitive compared to silver staining (Figure 57B) leading to more visible low abundant protein bands. The obtained result proved that the analysed αMEM/Norm samples contained proteins, however less concentrated to become visible in the second dimension, and the precipitation method worked and was not responsible for bad protein spot detection in the first experiment. The fact that gels that passed their expiring dates were used, was expected to be the reason for the bad results. Furthermore too high salt concentrations or problems during sample preparation can be possible reasons for the obtained results. Moreover samples or samples buffers from cell cultivation and harvesting can contain non-protein impurities. This leads to protein precipitation and further that those proteins did not enter the second dimension resulting in no protein spots in the 2D gel.

After these results αMEM/Norm samples were further used for 2D GE in hope to detect sample spots. To ensure that enough protein material is used for 2D GE the protein amount loaded onto the IPG strip was significantly increased. The possibility of poor sample separation in the first dimension was taken into account in favour of protein visualization on the 2D gel and testing of the 2D GE setup. With this we wanted to prove that proteins are actually separated via IEF and they were actually entered the separation gel in the second dimension. Table 44 shows the composition of the sample buffer now containing 904 µg of total protein, which is two-fold more than before.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Norm	**100 µL** (904 µg)
Rehydration-buffer	332 µL
In total	450 µL

Table 44: Composition of the IEF buffer containing the analysed sample.

After rehydration of the IPG strip the IEF program was again adjusted. The first focusing step was decreased from 300 V to 150 V and the second step from 500 V to 300 V. Furthermore the focusing time for step 1 till 4 was extended this time as shown in Table 45. The actual voltage and current over 27 hours of focussing is given in Figure 58. The sample was focussed in total for 61.6 kVh, 9 mAh and 27:30 h.

IEF-program for first dimension				
Step	**Voltage [V]**	**Current [µA]**	**Watt [W]**	**Time [h]**
1	150	1	5	3:00
2	300	1	5	3:00
3	1000	1	5	6:00
4	3500	1	5	5:00
5	3500	1	5	15:00

Table 45: Isoelectric focusing program for the αMEM/Norm sample.

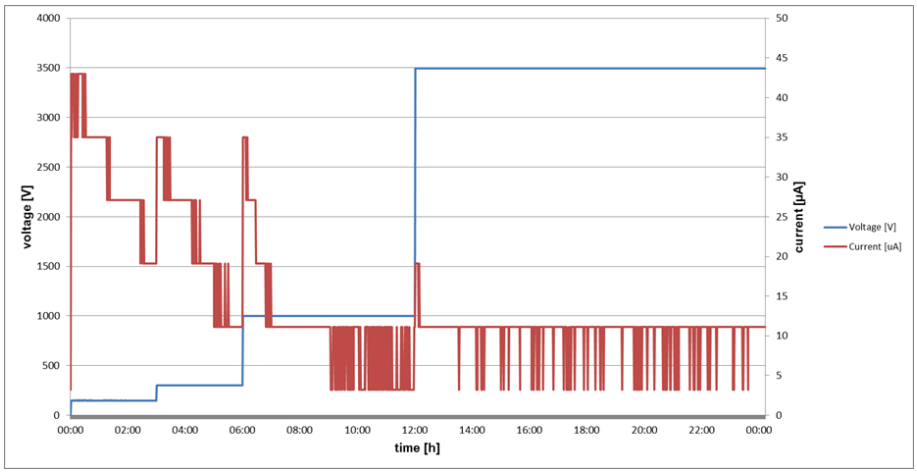


Figure 58: Voltage and current over the course of time for the αMEM/Norm sample.

HPE tower program for second dimension				
Step	**Voltage [V]**	**Current [mA]**	**Watt [W]**	**Time [h]**
1	100	7	1	0:30
2	200	13	3	0:30
3	300	20	5	0:10
4	1500	40	30	3:50
5	1500	45	40	0:50

Table 46: HPE tower program for the second dimension for the αMEM/Norm sample.

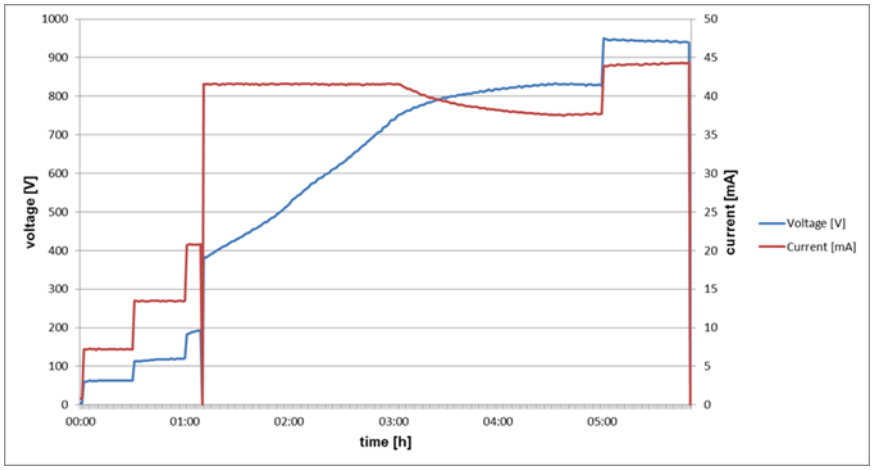


Figure 59: Voltage and current over the course of time for the αMEM/Norm sample.

Table 46 and Figure 59 shows the used program as well as the actual progress of current and voltage for the second dimension leading to the following parameters: 3517 kVh, 196 mAh and 5:50h. No uncommon current over time was observed during the first or the second dimension.

Figure 60 shows the gel obtained after Serva purple and subsequent silver staining.

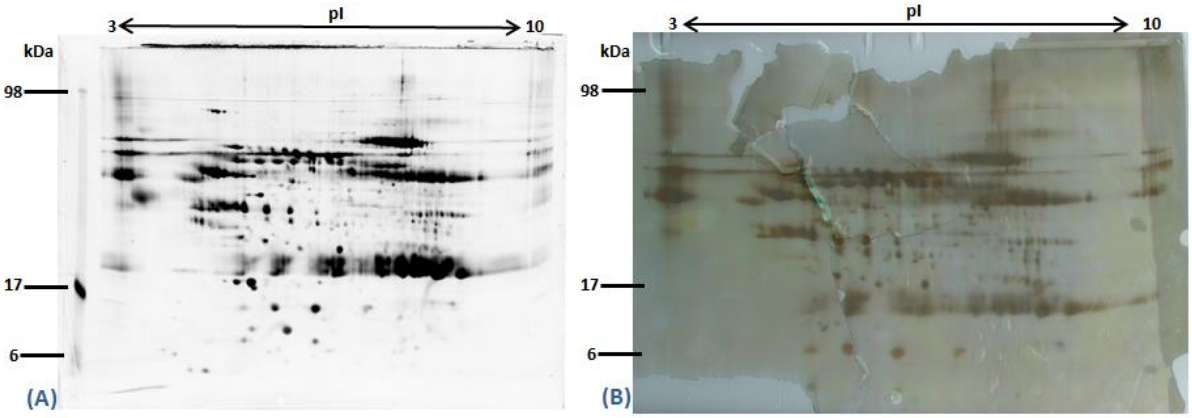


Figure 60: 2D gel of 900 µg αMEM/Norm sample, separated on a 24 cm IPG strip containing a non-linear pH-gradient 3-10, followed by SDS PAGE using T% 12.5. gels. The gel was (A) fluorescence stained using Serva purple and (B) silver stained. A pre-stained molecular weight marker was used.

It can see that this time a high number of protein spots were detected. As a rather high protein amount was loaded proteins were not well focused in the first dimension. Furthermore it can be seen that protein load is especially high in some basic (pI 7 to 8) and some acidic (pI 3.3 to 5.0) areas. As mentioned before we took this possibility into account to at least test our 2D setup, however it has also to be mentioned at this point that high protein amounts can lead to protein aggregation during IEF which becomes visible as horizontal streaks in the second dimension. This phenomenon is especially observed at MW 30 kDa till 60 kDa in Figure 60. Again a detaching of the gel from the plastic support film was observed due to long incubation times in organic solvents.

4.4 Comparison of antibody-based affinity columns and a combinatorial hexapeptide library for serum fractionation

To be able to perform secretome analysis high-abundance serum proteins had to be depleted without losing valuable secreted proteins. Therefore two different depletion methods were used and compared. Both reduce the high dynamic concentration range of proteins, however in two different ways. The first method utilizes a combinatorial hexapeptide library, which enriches very low-abundance proteins and at the same time reduces highly concentrated species (further details see chapter 2.2.1.2.1 (page 20)). The second technique, the top 12 highly abundant protein depletion columns, is a method that removes twelve of the most abundant serum proteins in a single removal step by columns that contain immobilized antibodies (more details see chapter 2.2.1.2.2 (page 22)). Hence, we have here a depletion strategy compared to the combinatorial hexapeptide ligand library (CPLL) where we have an enrichment strategy. An overview of both working principle is shown in Figure 61.

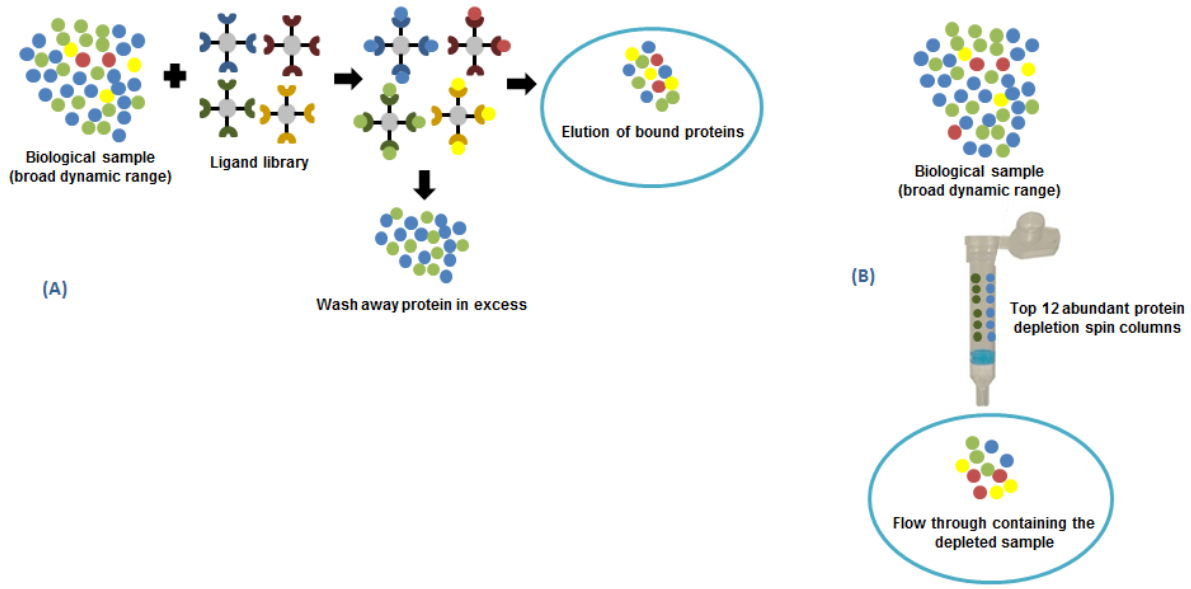


Figure 61: Comparison of the working principle of (A) CPLL and (b) Top 12 depletion columns.

Both methods differ in the binding principle to reduce the dynamic concentration range. High-abundance proteins were eliminated either by binding to antibodies (Figure 61B) or by not binding the hexamer peptide ligands (Figure 61A). In case of CPLL, proteins which were retained by the beads, were eluted from the affinity binder (highlighted in blue) and further used for analysis. In contrast, with the Top 12 depletion columns the flow though (marked in blue) containing the depleted sample was used. Pre-fractionated samples from both methods were analysed by 1D PAGE and 2D GE.

4.4.1 Combinatorial hexapeptide library

To achieve a reduction of the dynamic concentration range of the analysed αMEM/Norm samples, they were processed with the CPLL approach according to the set up from the working group of Klaus Kratochwill. Therefore two αMEM/Norm sample aliquots from the same origin sample were used. Protein concentration was determined by Bradford assay showing that the concentration was too low to be measured and therefore sample were precipitated using TCA and ice-cold acetone (details see 3.3.4 page 48). Afterwards the protein concentration of both αMEM/Norm sample aliquots (αMEM/Norm before depletion (1)+(2)) was measured again. Precipitated samples were further prepared with the ProteoMiner Small-Capacity kit (Bio-Rad). Both samples were applied to the calibrated columns, containing the bead bound hexapeptide ligands. After the incubation time, proteins which had not bound to the beads were washed away in the flow through during the washing step. Proteins which had bound were eluted from the attached beads and further analysed. Due to the containing acetic acid in the elution buffer the samples had to be precipitated again to exchange it. At first the protein concentration was determined for both depleted samples (αMEM/Norm after depletion (1)+(2)). Those were then pooled (αMEM/Norm pooled after depletion (1)+(2)) to increase the available sample volume for further analysis. The linear regression curve and the obtained calculated concentration values were shown in Figure 62 and Table 47

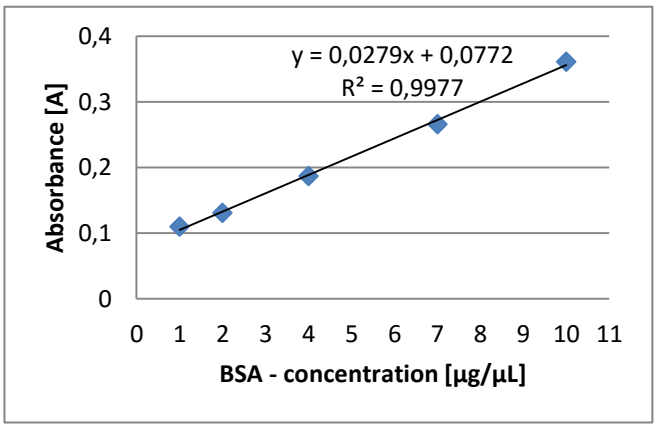


Figure 62: Linear regression for BSA calibrants.

Sample		Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm before depletion (1)	measurement 1 measurement 2 measurement 3 measurement 4	0.112 0.106 0.108 0.111	0.109	4.386 4.188 4.254 4.353	4.295	**21.48**
αMEM/Norm after depletion (1)	measurement 1 measurement 2	0.170 0.171	0.1705	3.309 3.345	3.327	**0.33**
αMEM/Norm before depletion (2)	measurement 1 measurement 2 measurement 3 measurement 4	0.139 0.132 0.145 0.126	0.135	4.447 4.195 4.663 3.998	4.326	**43.26**
αMEM/Norm after depletion (2)	measurement 1 measurement 2	0.116 0.113	0.115	1.345 1.236	1.291	**0.13**
αMEM/Norm pooled after depletion (1)+(2)	measurement 1 measurement 2	0.171 0.169	0.170	3.345 3.236	3.291	**0.33**

Table 47: Overview on calculations for protein concentration of the used αMEM/Norm samples before and after using CPLL approach.

Table 47 shows the obtained results for the measured αMEM/Norm samples. The calculated concentration values include the dilution factor before depletion (αMEM/Norm (1) 1:1000, αMEM/Norm (2) 1:500) and after depletion (αMEM/Norm (1) + (2) 1:10), the volume (10 µL) and the conversion factor (1000) from µg/mL to µg/µL. Absorbance was measured in quadruplicates (before depletion) and duplicates (after depletion). It could be seen that protein concentration of CPLL-treated samples decreases clearly (0.33 µg/µL and 0.13 µg/µL) compared with untreated samples (21.48 µg/µL and 43.26 µg/µL) before loading onto the columns. Here it has to be mentioned that all samples were handled with the set up for CPLL according to the protocol kindly provided by the working group of Klaus Kratochwill (Medical University of Vienna). No buffer or pH value adjustment, which could improve the elution efficiency of the sample, was performed due to less time for further experiments leading to possible less protein recovery after depletion. However, in a comparative study Candiano et al. [43] showed that a subsequent elution of captured proteins from CPLL with eluting agents was not fully effective concerning the protein recovery. Therefore they tested different elution ways to try to release all those proteins which were adsorbed by the CPLL beads by dominant ionic interactions. The best result was shown with the elution in boiling 10% SDS added with 3% DTE. By the hydrophobic interaction from SDS with the captured

proteins, those where released from the hydrophobic interaction with peptides attached to the beads. However SDS has to be removed prior 2D GE analysis.

The pooled and treated αMEM/Norm samples were analysed using 2D GE. For the first dimension a gel strip with an immobilized pH gradient from 3 to 10 was used. The isoelectric focussing step was performed according to the IEF program from chapter 3.6.2.4 (page 54). On 24 cm strips, after overnight sample introduction Table 48 gives details ion sample preparation and Figure 63 on voltage and current changes. Details on the second dimension, which was again run according to chapter 3.6.4.4 (page 57), are given in Figure 64. The gel was stained with Serva purple (see chapter 3.7.1.4 (page 61)) and silver (see chapter 3.7.2.4 (page 63)) see Figure 65.

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Norm pooled after depletion (1)+(2)	**170 µL** (56 µg)
Rehydration-buffer	262 µL
In total	450 µL

Table 48: Composition of the IEF buffer containing the analysed sample.

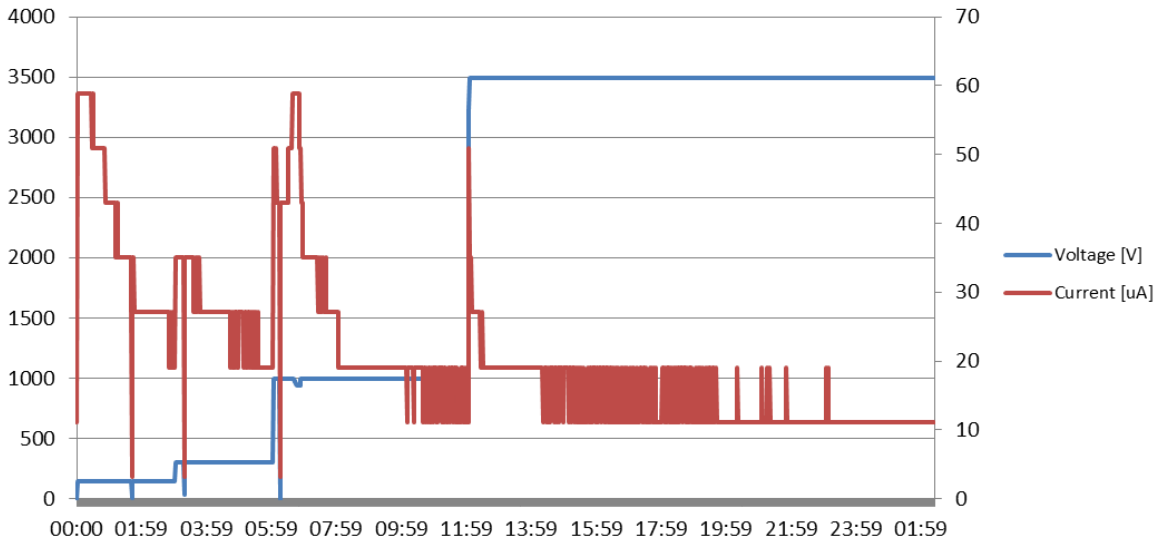


Figure 63: Voltage and current over the course of time for the pooled and depleted αMEM/Norm samples.

As Figure 63 shows the sample was focussed in total for 57.5 kVh, 10 mAh and 26:19 h.

A reduction and alkylation step was performed prior to the second dimension according to chapter 3.6.2.4 (page 54).

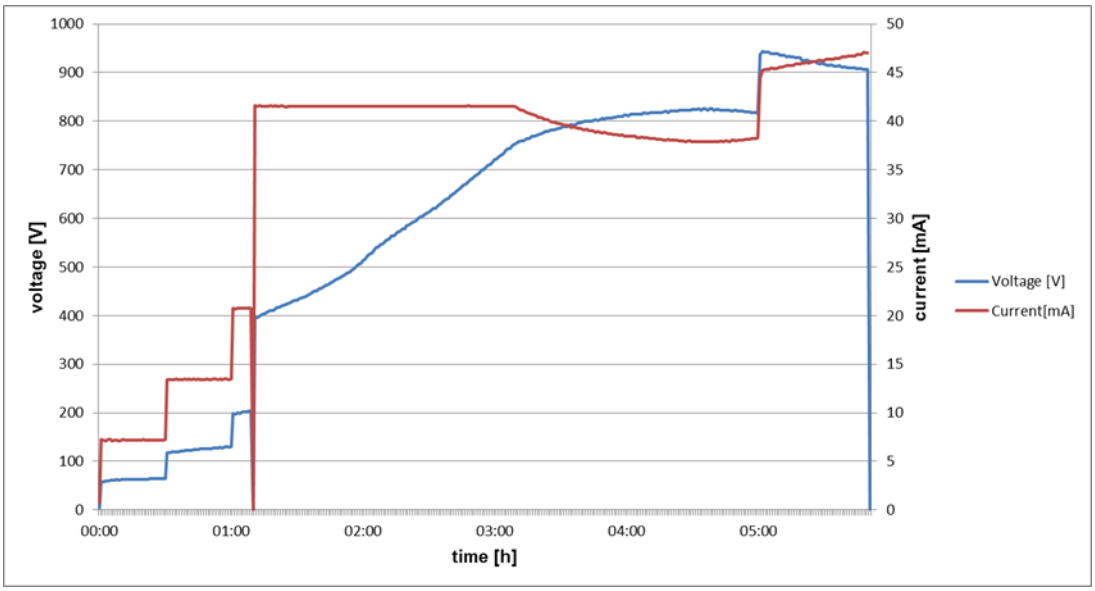


Figure 64: Voltage and current over the course of time for the pooled and depleted αMEM/Norm samples.

The second dimension took 5:50 h and 3460 kVh and 197 mAh were obtained. No uncommon current over time was observed during the first or the second dimension.

The 2D gel was post-stained with Serva purple according to chapter 3.7.1.4 (page 61) and subsequent silver stained according to chapter 3.7.2.4 (page 63). The obtained results are shown in Figure 65.

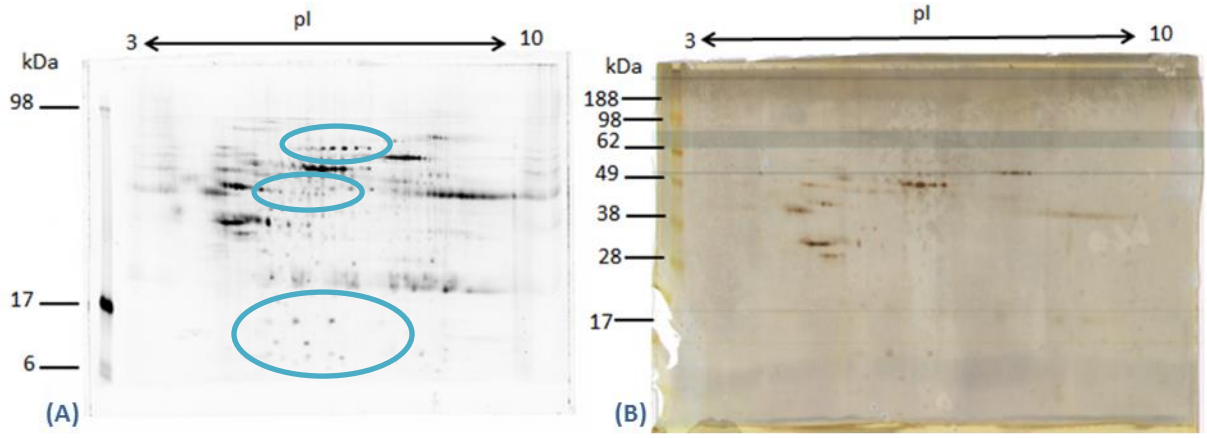


Figure 65: 2D GE of MSCs cultivated under normoxic conditions in αMEM after removing high-abundance proteins by a combinatorial peptide ligand library. 56 µg proteins were loaded on the first dimension after the depletion, followed by SDS PAGE using T% 12.5 gels. The gel was (A) fluorescence stained using Serva purple and (B) silver stained. A pre-stained molecular weight marker was used.

Figure 65 shows the 2D gel from the CPLL treated αMEM/Norm sample. A comparison with the untreated samples (as shown in Figure Figure 60 (page 103)) showed a lower efficiency for serum protein removal. The major high-abundance proteins *e.g*. serum albumin (66.5 kDa) or heavy (50 kDa) and light (23 kDa) chains of immunoglobulin were still observed, however less concentrated and intensive. It was assumed that co-depletion is reduced under these conditions and a more complete coverage of the secretome will be achieved. However, some spots (highlighted in blue) were now more focussed and able to be detected. Normally the flow-through fraction form CPLL-treated samples (it should show a similar protein pattern as the native sample related to the presents of high-abundance proteins) has to be analysed to verify efficient sample enrichment. Due to time constraints this was not done during this master thesis. Indeed in a comparative study from Bandow [77] this attempt was conducted leading to the following results: Plasma protein fractions from CPLL elution and flow-through were compared with each other and with untreated native plasma samples by 1D PAGE and 2D DIGE. The 1D gel shows that the CPLL flow-through fraction shared the same major protein bands as the native plasma sample, which confirmed that high-abundance proteins were present in this fraction. In contrast the CPLL elution fraction shows different band patterns. For the DIGE approach the CPLL elution and flow-through fractions were compared. The resulting pattern looked very different also compared with those from the untreated plasma sample. However it has to be mentioned that after CPLL treatment major proteins could not be compared and quantified. Furthermore the reduction of high-/medium-abundance proteins depends on several parameters e.g. environmental conditions, temperature or diffusion time, which were also not adjusted.

Extending the time period for the precipitation step will enhance protein recovery because especially in case of TCA precipitation, protein loss was probably due to incomplete solubilisation of the pellets and the acetone wash step. Further dissolving the obtained pellet will again increase protein concertation. Therefore the same samples as previous described were again used for a new peptide library approach. According to a mistake during determination of the protein concentration, the pooled sample for further experiments was contaminated with Bradford reagent. So the experiment was repeated and the new sample details are given in Table 49. Please mark that now only 50 µL were used to dissolve the sample after CPLL treatment and following precipitation, leading to an estimated increase of protein concentration.

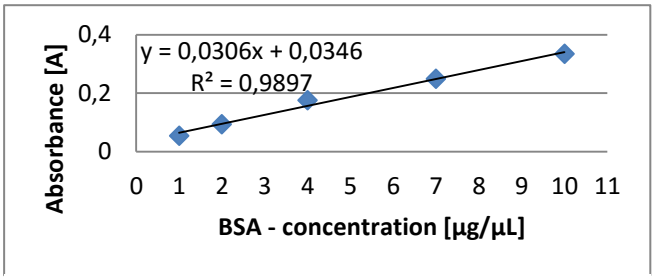


Figure 66: Linear regression for BSA calibrants.

Sample	Absorbance			Concentration		
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm before depletion (1)	measurement 1	0.112	0.109	4.386	4.295	**21.48**
	measurement 2	0.106		4.188		
	measurement 3	0.108		4.254		
	measurement 4	0.111		4.353		
αMEM/Norm after depletion 1-1	measurement 1	0.286	0.292	8.221	8.409	**0.08**
	measurement 2	0.293		8.450		
	measurement 3	0.294		8.483		
	measurement 4	0.295		8.484		
αMEM/Norm before depletion (2)	measurement 1	0.139	0.135	4.447	4.326	**43.26**
	measurement 2	0.132		4.195		
	measurement 3	0.145		4.663		
	measurement 4	0.126		3.998		
αMEM/Norm after depletion 2-1	measurement 1	0.116	0.113	2.660	2.570	**0.03**
	measurement 2	0.113		2.562		
	measurement 3	0.113		2.562		
	measurement 4	0.111		2.496		
αMEM/Norm pooled after depletion (1-1)+(2-1)	measurement 1	0.211	0.216	5.768	5.951	**0.06**
	measurement 2	0.214		5.866		
	measurement 3	0.217		5.964		
	measurement 4	0.221		6.095		

Table 49: Overview on calculations for protein concentration of the new αMEM/Norm samples before and after using CPLL approach.

Table 41 shows that again a decrease of the protein concentration after using CPLL. Suggestions for that could be that again the proteins of interest were again captured and not eluted from the beads due to no adjustment of the pH value or buffer for the elution. Furthermore it could be that the used hexapeptide ligands were not specific for the wanted secretome proteins resulting in a probable loss of proteins of interest. To increase protein concentration both samples were again pooled followed by analysing them with 1D PAGE, composition of the loading buffer is shown in Table 50.

Component	Amount
DL-Dithiothreitol (1M)	1 µL
LDS sample buffer (4x)	5 µL
αMEM/Norm pooled after depletion (1-1)+(2-1)	**10 µL (0.6 µg)**
UHQ water	4 µL
In total	20 µL

Table 50: Composition of 1D PAGE loading buffer including the analysed αMEM/Norm sample.

Figure 67 shows the 1D gel first stained with Serva purple and subsequent with silver.

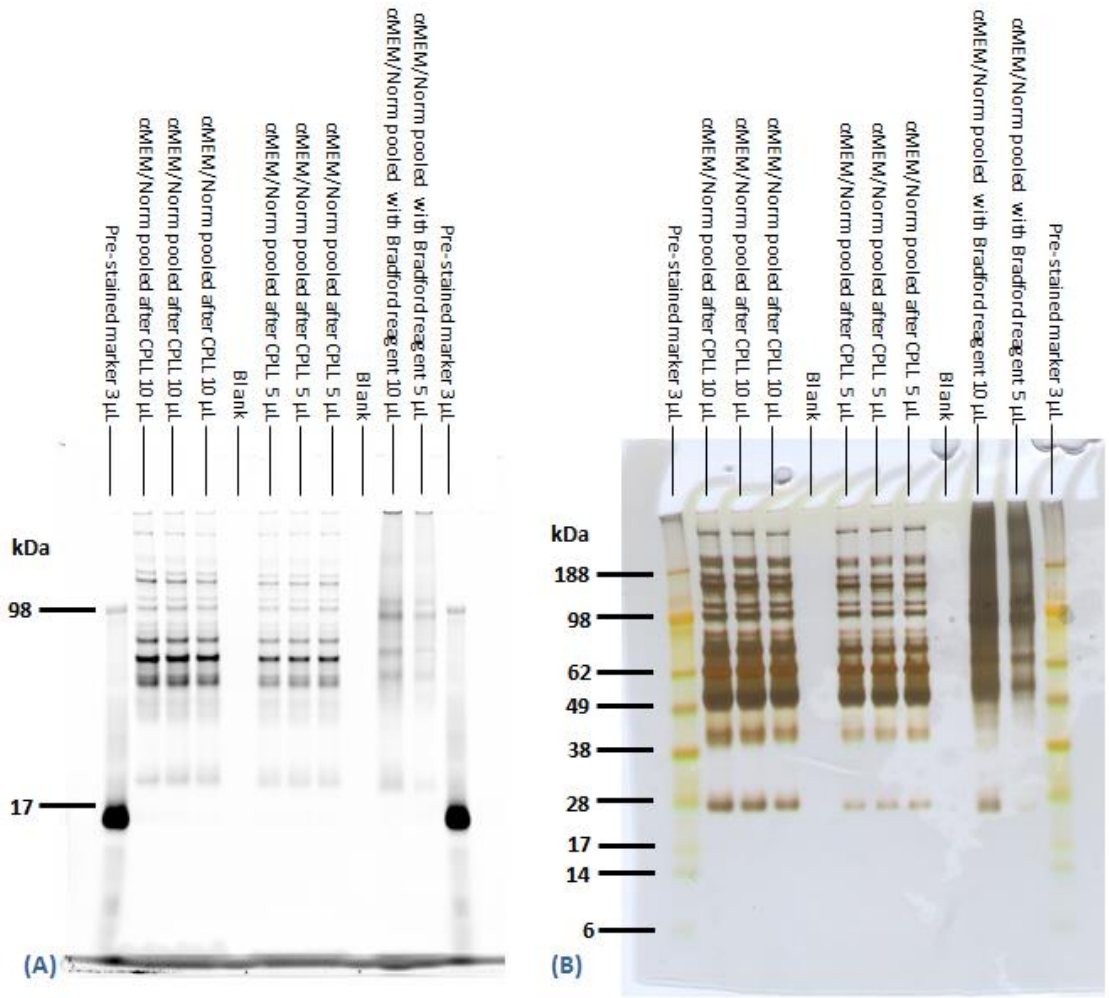


Figure 67: First experiment for analyzing αMEM/Norm samples where low abundant proteins were enriched by CPLL approach. 1D gel after (A) post-fluorescent staining and (B) silver staining. Sample separation was performed on a 4-12% Bis-Tris gel. A pre-stained molecular weight marker was used.

As shown in Figure 67 the protein band patterns from the CPLL- treated and untreated αMEM/Norm sample (see Figure 60 (page 103)) look similar, however less intense and concentrated leading to a reduction of major proteins. To improve the results further experiments need to be done to test different buffer or elution systems as well as the influence of different parameter e.g. pH or temperature concerning the obtained results. Results will be further discussed and compared in chapter 4.4.3 (page 119).

4.4.2 Antibody-based affinity columns

4.4.2.1 First experiment

For comparison, the same samples, namely αMEM/Norm (1) and (2), used in the previous experiment were used for better comparison. Here it has to be mentioned again that the analysed sample was applied onto the depletion columns and high-abundance serum proteins were eliminated by binding to antibodies. The flow-through fraction containing less concentrated major serum proteins was analysed. The samples were prepared according to the protocol see chapter 3.4.2.4 (page 51). The protein concentration was determined before and after reduction of the twelve major serum proteins via Bradford assay (see chapter 3.2.4 (page 46)) and calculated values are shown in Table 51.

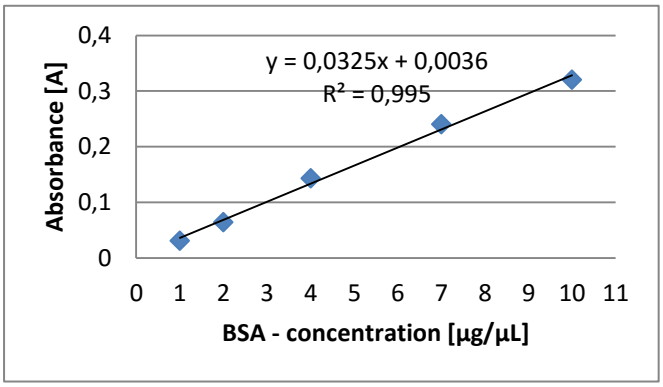


Figure 68: Linear regression for BSA calibrants.

Sample	Absorbance		Concentration			
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm before depletion (1)	measurement 1 measurement 2 measurement 3 measurement 4	0.112 0.106 0.108 0.111	0.109	4.386 4.188 4.254 4.353	4.295	**21.48**
αMEM/Norm after depletion (1)	measurement 1 measurement 2	0.018 0.016	0.017	<LOD	< LOD	**< LOD**
αMEM/Norm before depletion (2)	measurement 1 measurement 2 measurement 3 measurement 4	0.139 0.132 0.145 0.126	0.135	4.447 4.195 4.663 3.998	4.325	**43.26**
αMEM/Norm after depletion (2)	measurement 1 measurement 2	0.039 0.042	0.041	1.088 1.181	1.135	**0.0113**

Table 51: Overview on calculations for protein concentration of the used αMEM/Norm samples before and after using the antibody-based affinity columns approach.

Due to the fact that the determination of protein concentration was not possible in the first sample a 1D gel was prepared to determine whether protein bands can be detected. The samples were separated using 1D PAGE using 4-12% BisTris gels and a MES running buffer. Due to probably less protein content the gel pockets were loaded with the maximum allowable sample volume (10 µL). The composition of 1D PAGE buffer including the analysed sample could be seen in Table 52. Afterwards the gel was stained with Serva purple and silver, results are shown in Figure 69.

Component	Amount
DL-Dithiothreitol (1M)	1 µL
LDS sample buffer (4x)	5 µL
αMEM/Norm after depletion (2)	**10 µL (0.11 µg)**
UHQ water	4 µL
In total	20 µL

Table 52: Composition of 1D PAGE loading buffer including the analysed αMEM/Norm sample.

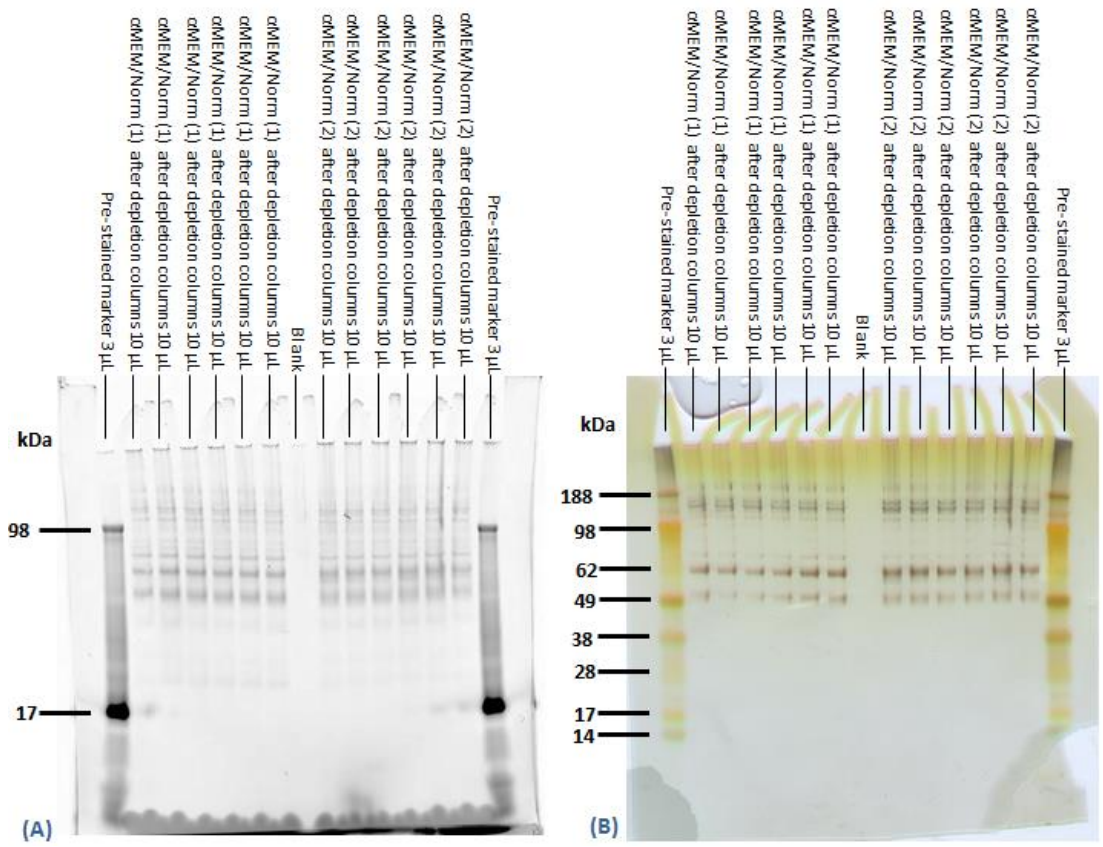


Figure 69: 1D PAGE analysis of αMEM/Norm (1) and (2) samples after using the Top 12 depletion columns. (A) Serva purple stainging (B) silver staining.

An efficient reduction of high-abundance proteins was observed resulting in a reduced number of protein bands in 1D as shown in Figure 62. The obtained result confirms literature named method limitation (co-depletion of low abundance proteins). The high possibility of co-depletion of low abundant proteins was considered to be very likely.

4.4.2.2 Second experiment

Because of too low protein content the pre-fractionation with the top 12 columns was repeated. Therefore the same samples were used as before; however this time in order to increase the protein concentration, the pellet from the newly depleted and precipitated samples was dissolved in the total volume of 50 µL αMEM/Norm after depletion (2) sample from experiment 1 (details see chapter 4.4.2.1 (page 112)). Again the total protein concentration was measured (Figure 70 and Table 53).

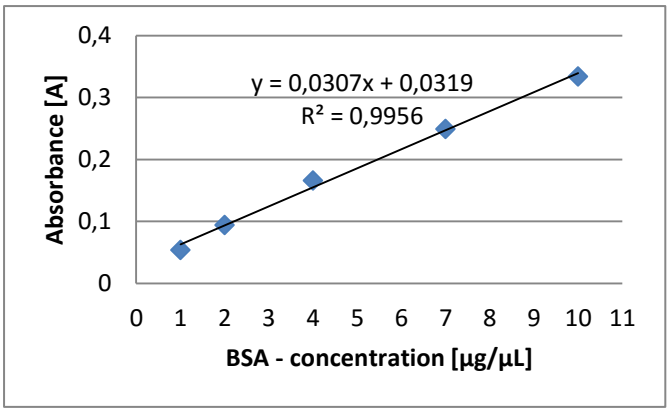


Figure 70: Linear regression for BSA calibrants.

After measuring the protein concentration of both new depleted and precipitated samples (αMEM/Norm after depletion (1-1) and (2-1)) via Bradford assay, both samples were further pooled. All results are shown in Table 53.

Sample	Absorbance			Concentration		
			Average	*Average*	*µg/mL*	*µg/µL*
αMEM/Norm after depletion (1-1)	measurement 1	0.174	0.174	4.558	4.558	**0.045**
	measurement 2	0.175		4.591		
	measurement 3	0.173		4.525		
αMEM/Norm after depletion (2-1)	measurement 1	0.084	0.089	1.614	1.777	**0.017**
	measurement 2	0.090		1.810		
	measurement 3	0.093		1.909		
αMEM/Norm pooled after depletion (1-1) + (2-1)	measurement 1	0.142	0.145	3.511	3.620	**0.036**
	measurement 2	0.145		3.609		
	measurement 3	0.149		3.740		

Table 53: Overview on calculations for protein concentration of the used αMEM/Norm samples before and after using the antibody-based affinity columns approach.

Because of the still too low protein concentration, the processing of the sample with the top 12 depletion columns was again repeated.

4.4.2.3 Third experiment

Again, the pellet of the new third-depleted and precipitated sample was dissolved in the prior already pooled sample (αMEM/Norm pooled after depletion (1-1) + (2-1)); results are shown in Table 54.

Sample		Absorbance		Concentration	
			Average	*µg/mL*	*Average*	*µg/µL*
αMEM/Norm after depletion (3) pooled with pooled sample (1-1) + (2-1)	measurement 1	0.186	0.190	4.950	5.065	**0.051**
	measurement 2	0.188		5.016		
	measurement 3	0.190		5.081		
	measurement 4	0.194		5.211		

Table 54: Overview on calculations for protein concentration of the top 12 –treated and pooled αMEM/Norm samples.

Table 46 shows the final concentration of the final sample used for further analysis. The samples were separated using 1D PAGE. Afterwards the gel was stained with Serva purple and silver, results are shown in Figure 71.

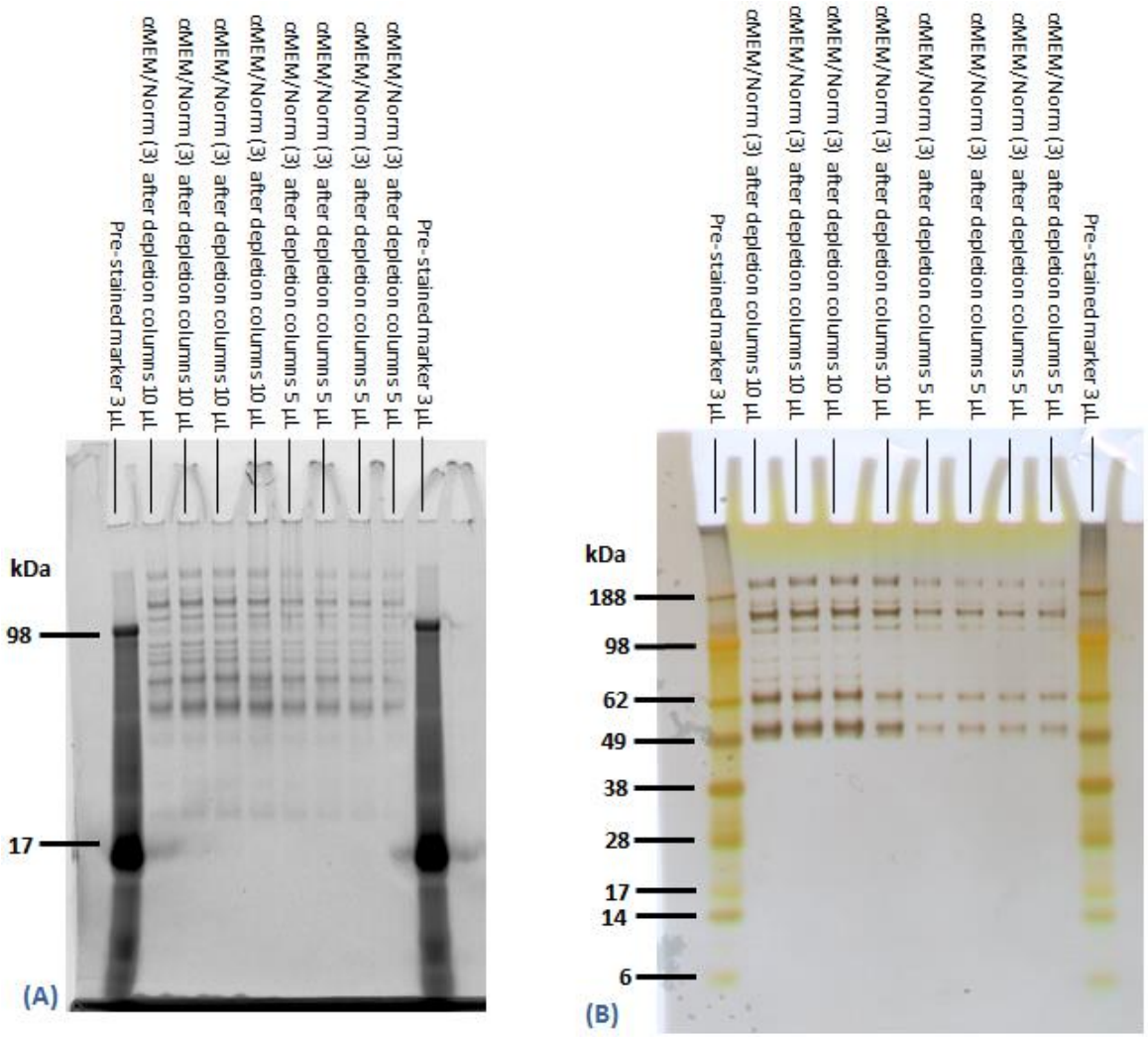


Figure 71: 1D PAGE analysis of the final αMEM/Norm (3) sample after using the Top 12 depletion columns. (A) Serva purple staining (B) silver staining.

As Figure 64 shows more intensive protein bands were obtained compared to Figure 62. Due to pooling of all Top12-treated samples a final protein concentration of 0.05 µg/µL was received. The final sample, namely αMEM/Norm (3), was further analysed by 2D GE. In this case 90 µL (4.5 µg) were loaded onto the IPG-strip (Table 55). The first dimension was performed according to chapter 3.6.1.4 (page 53) and the corresponding progress of current and voltage over 24 hours is given in Figure 72. The sample was focussed in total for 46.1 kVh, 14.0 mAh and 24:13 h. The corresponding second dimension took 5:50 h and 3438 kVh and 192 mAh were obtained (Figure 73). The 2D gel was stained with (A) Serva purple and (B) silver (see Figure 74).

Component	Amount
DL-Dithiothreitol (1M)	9 µL
Servalyte , 3-10	9 µL
αMEM/Norm (3)	**90 µL** (4.5 µg)
Rehydration-buffer	342 µL
In total	450 µL

Table 55: Composition of the IEF buffer containing the analysed sample.

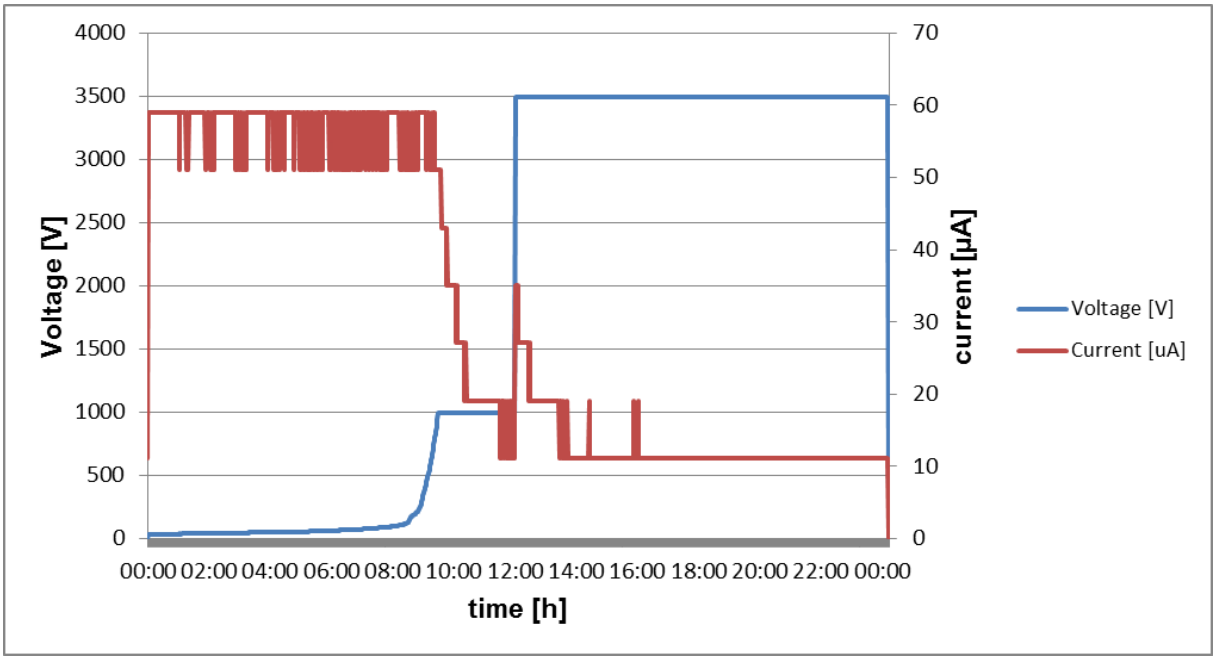


Figure 72: Voltage and current over the course of time for final top12-treated αMEM/Norm samples (3).

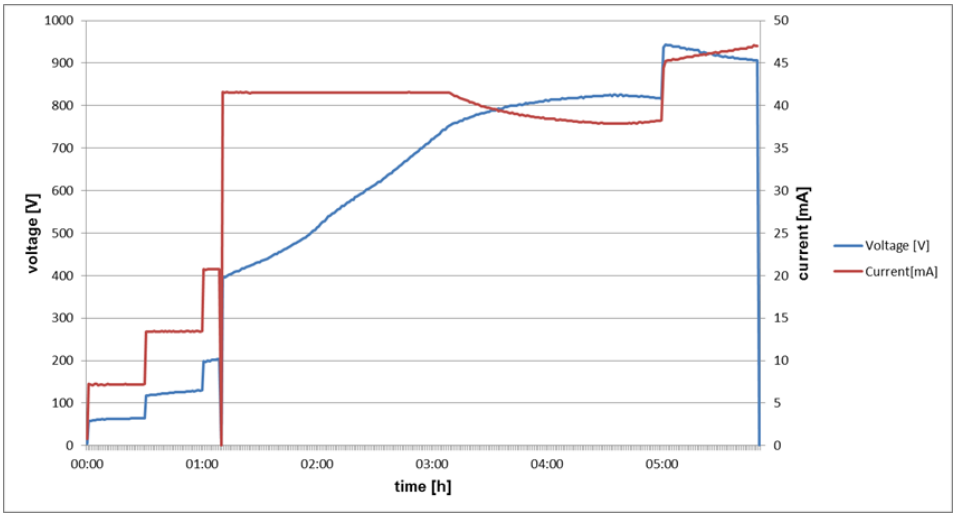


Figure 73: Voltage and current over the course of time for final top12-treated αMEM/Norm samples (3).

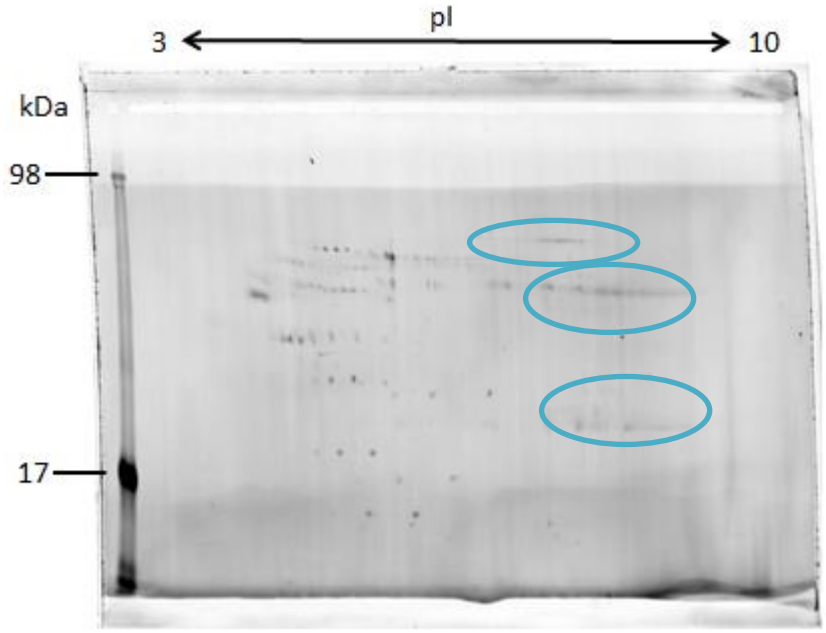
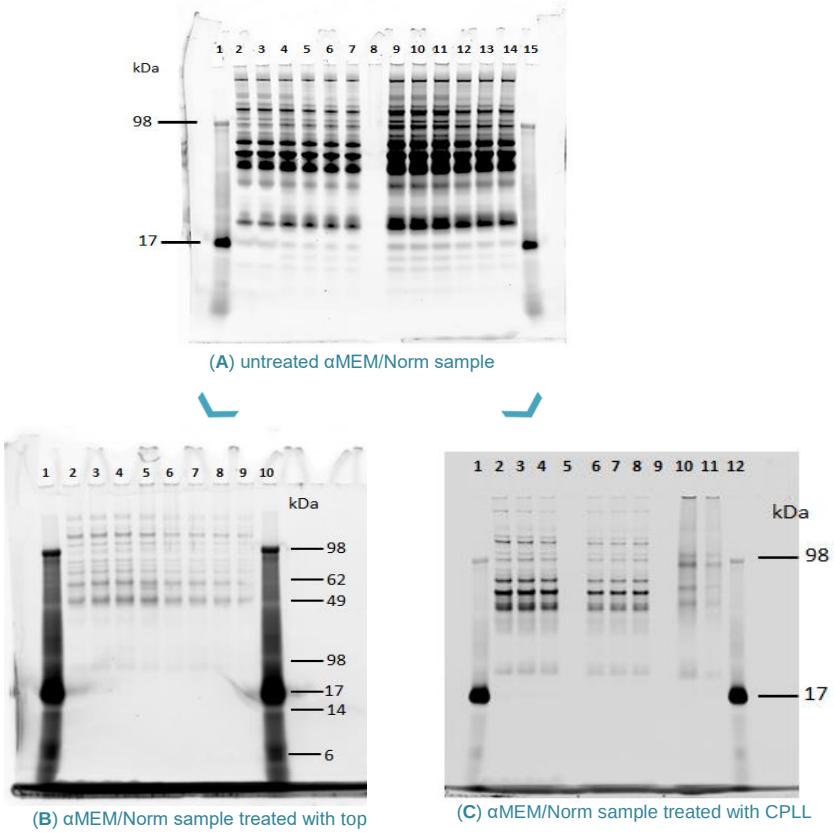


Figure 74: 2D GE of MSCs cultivated under normoxic conditions in αMEM after removing high-abundance proteins by top12 columns. 4.5 µg proteins were loaded on the first dimension, followed by SDS PAGE using T% 12.5 gels. The gel was fluorescence stained using Serva purple. A pre-stained molecular weight marker was used.

According to Figure 74 a highly effective reduction of high-abundance proteins was achieved. Possible carrier proteins (*e.g.* serum albumin or immunoglobulin marked in blue) for low-abundance proteins of interest were almost completly removed leading to the suggestion that co-depletion of minor species are very likely. However compared to the untreated αMEM/Norm sample clear focused spots were obtained. Due to long incubation times in organic solvents a detaching of the gel from the plastic support film was observed resulting in no possible scan of the silver stained 2D gel. Results will be further discussed and compared see chapter 4.4.3 (page 119).

4.4.3 CPLL results in comparison with antibody-based affinity columns results

A significant removal of high-abundant serum proteins, without losing valuable secreted proteins enables the access to the low abundant secretome. Therefore two depletion methods were compared. Protein fractions were first analysed by 1D PAGE, depicted in Figure 75.



(**A**) untreated αMEM/Norm sample

(**B**) αMEM/Norm sample treated with top 12 depletion columns

(**C**) αMEM/Norm sample treated with CPLL

Figure 75: 1D gel comparison of the two pre-fractionation methods: (A) untreated αMEM/Norm sample, (B) Fractionation with top 12 depletion columns (C) Fractionation with CPLL.

Top 12-treated samples show an efficient reduction of major protein species, however resulting in less intensive bands compared to the untreated sample and the CPLL approach as shown in Figure 75. Plausible reasons for that are that the removal of abundant carrier proteins like serum albumin can cause co-depletion of several low-abundance proteins, hindering their detection [39]. Further limitations of antibody-based affinity columns reported by Millioni et al. [78] are that column-bound antibodies do not recognize all isoforms and fragments of all high-abundance proteins and the quantity of them could saturate and exceed the binding capacity. Further many depleted fractions have to be pooled which leads to an extensive manipulation and introduces errors or contaminations [78]. It is recommended by the manufacture to shake the columns on an end over end tumbler for one hour at room temperature. Because the equipment was unavailable the columns were vortexed every 15 minutes which possibly influences the obtained results.

In contrast CPLL-treated samples leads to a lower efficiency for serum protein removal resulting in partly similar intensive bands in the 1D gel compare to the untreated sample. As already mentioned previously all samples were processed with a fixed set up for CPLL form the working group of Klaus Kratochwill. No adjustment of buffer or pH value, which could improve the elution efficiency of the sample, was performed. In a comparative study Candiano et al. [43] showed that a subsequent elution of captured proteins from CPLL with eluting agents was not fully effective concerning the protein recovery. Therefore they tested different elution ways to try to release all those proteins which were adsorbed by the CPLL beads by dominant ionic interactions. The best result was shown with the elution in boiling 10% SDS added with 3% DTE. By the hydrophobic interaction from SDS with the captured proteins, those where released from the hydrophobic interaction with peptides attached to the beads. However SDS has to be removed prior 2D GE analysis. The columns were shaken on a thermomixer at room temperature for two hours and vortexed every 5 minutes; however it is recommended that columns are rotated on a rotational shaker for two hours at room temperature. This can influence the obtained results. To further characterize the efficiency of the depletion, the samples were separated by 2D GE afterwards and visualized by Serva purple and silver staining (see Figure 76)

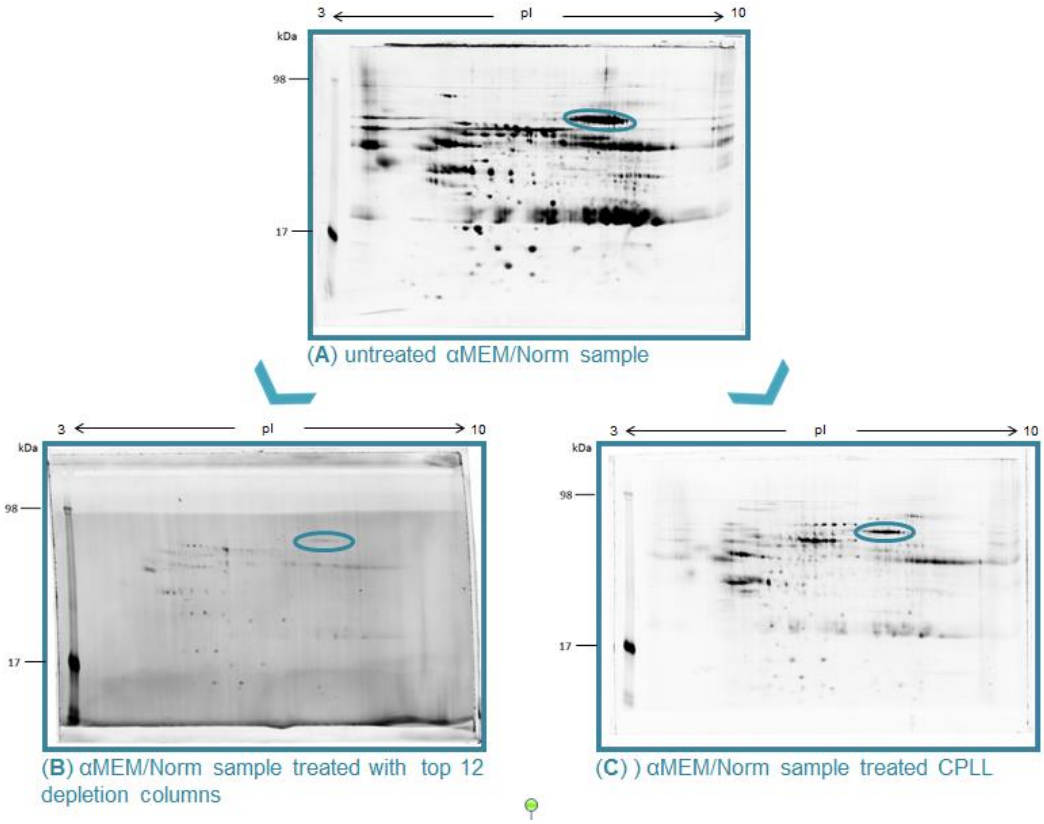


Figure 76: 2D gel comparison of the two pre-fractionation methods: (A) untreated αMEM/Norm sample, (B) Fractionation with top 12 depletion columns (C) Fractionation with CPLL. Serum album is highlighted in blue.

By using Top12 depletion spin columns an efficient reduction of high-abundance proteins was observed resulting in a lower number of spots in 2D PAGE (see Figure 76 B) compared to the CPLL approach (see Figure 70 C). As already mentioned co-depletion might remove minor protein species together with the affinity ligand [37]. Hence it has to be said that only 5 µg total protein was loaded in case of Top 12 columns which is only one-tenth of the total protein used for the CPLL approach (56 µg). So repetitions of the top 12 columns approach can lead to an increase of the total protein amount by pooling several fractions. Through the high possibility of co-depletion of low abundant proteins with the top 12 approach the combinatorial peptide library was also tested, but showed a lower efficiency for serum protein removal leading to more focused spots in the 2D gel (see Figure 76 C). Serum albumin was still observed, but less concentrated (highlighted in blue). It was assumed that co-depletion is reduced under these conditions.

A decrease of the protein concentration was observed after using CPLL. Suggestions for that could be that proteins of interest were captured and not eluted from the beads due to absent adjustment of the pH value or buffer for the elution. Furthermore it could be that the used hexapeptide ligands were not specific for the wanted secretome proteins resulting in a probably loss of proteins of interest in the flow though. Moreover the CPLL approach requires relatively high amounts of starting material to ensure efficient enrichment of low-abundance proteins; otherwise high- and medium-abundance proteins would not fully saturate their ligands and the elution would have the same profile as the initial sample [77]. A controversial study from Keidel et al. [37] point out that the interact from equalizer beads with a protein mixture is based on a hydrophobic binding mechanism where diversity in surface ligands is negligible. Data of Bandow [77] showed that the CPLL flow-through fraction had equal protein bands as the native plasma, which proved that this fraction contained high-abundance proteins. Different band patterns were shown in the CPLL elution fraction. Completely different patterns were also observed comparing the untreated plasma sample, the CPLL elution and flow-through fractions. Unfortunately adjustments for influence factors of the protein-reduction were not done within this master thesis. Conclusive there is not an ideal approach for the high abundance protein depletion because the information resulting of the different methods is not unique (45) and is therefore always a compromise between specify or completeness.

It was assumed that the CPLL approach would lead to a complete coverage of the secretome due to the fact that carrier proteins for minor species are still present. Further 2D DIGE experiments were conducted with CPLL.

4.5 DIGE to compare normoxic and hypoxic cultivation conditions after sample pre-fractionation using CPLL

Finally DIGE analysis was introduced to visualize proteome differences between the normoxic and hypoxic secretome of mesenchymal stem cells, following the removal of high abundant serum proteins with the peptide library approach. This technique was employed to alleviate the comparative difficulties between for more than one independent 2D gel. As described before DIGE allows multiplexing of samples reducing therefore significantly the technical variation making the observed variation more likely to be a biological one. For this approach from both untreated αMEM/Norm and αMEM/Hypo samples triplicates of each biological state *(*normoxie: *αMEM/Norm 1-1, αMEM/Norm 1-2 and αMEM/Norm 1-3*) (hypoxie: *αMEM/Hypo 1-1, αMEM/Hypo 1-2 and αMEM/Hypo 1-3*) were done and pooled in order to generate enough protein materials for 2D DIGE analysis. An overview for the workflow is outlined in Figure 77.

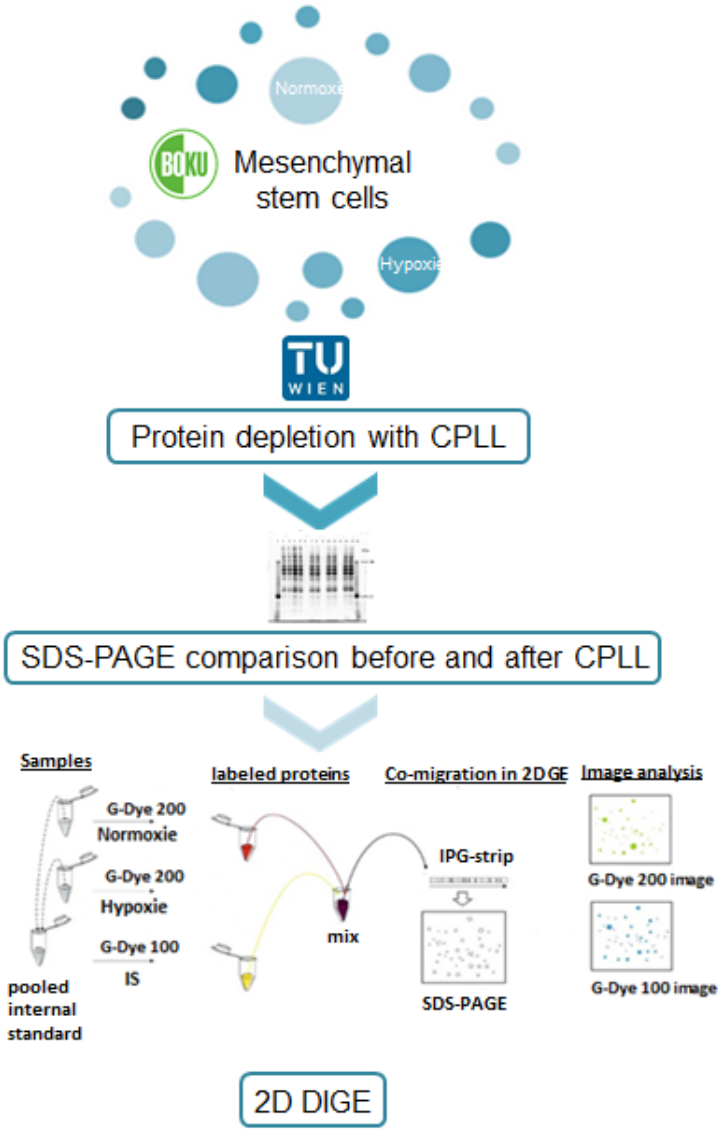


Figure 77: Workflow of the applied approach to deplete high abundant proteins and to analyze the secretome by 2D DIGE.

The untreated αMEM/Norm and αMEM/Hypo samples were processed with the CPLL approach in triplicates. For a preliminary screen the native αMEM/Norm and αMEM/Hypo samples as well as each replicate from both cultivation conditions were analysed by 1D PAGE (see Figure 78). The composition of the sample containing loading buffers is described in Table 56.

	αMEM/Norm (1)	αMEM/Norm 1-1	αMEM/Norm 1-2	αMEM/Norm 1-3	αMEM/Hypo (1)	αMEM/Hypo 1-1	αMEM/Hypo 1-2	αMEM/Hypo 1-3
DDT (1 M)	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
LDS buffer (4x)	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Sample	**5.35 µL (198.4 µg)**	**4.10 µL (3.9 µg)**	**6.94 µL (3.99 µg)**	**10.32 µL (3.99 µg)**	**5.84 µL (159.8 µg)**	**2.23 µL (4.0 µg)**	**5.18 µL (3.99 µg)**	**4.01 µL (3.98 µg)**
UHQ water	8.65 µL	9.89 µL	7.06 µL	3.67 µL	8.16 µL	11.76 µL	8.81 µL	9.98 µL
In total	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

Table 56: SDS PADE loading for all samples analysed by DIGE.

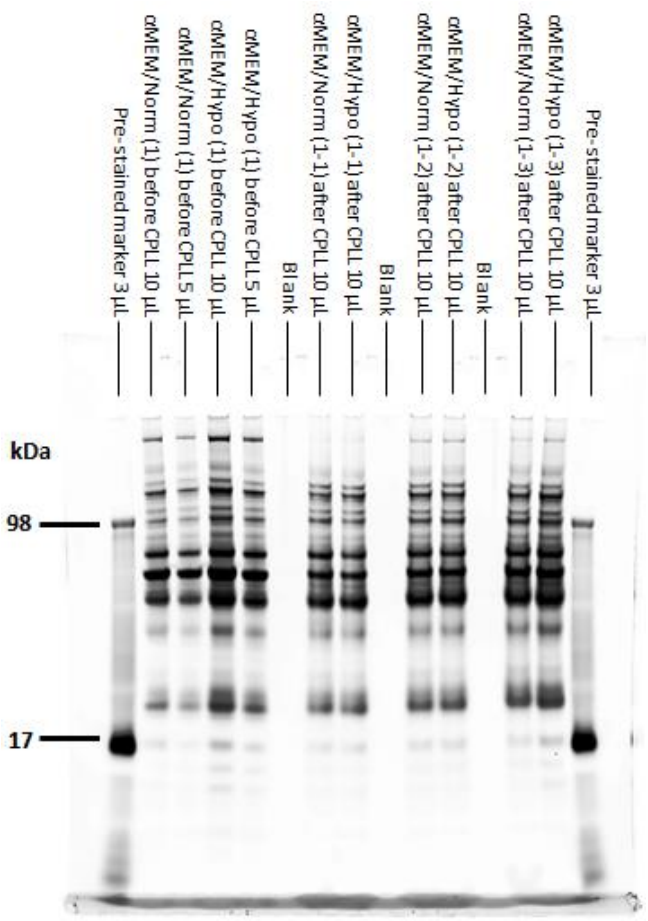


Figure 78: SDS-PAGE of αMEM/Norm and αMEM/Hypo samples before and after enrichment of low abundance proteins with CPLL. 1D gel was post-fluorescent staining with Serva purple.

Figure 78 shows the obtained 1D gel from the prior mentioned samples. It can be seen that partly higher concentrated protein bands were detected with 1D PAGE. It can futher be observed that the CPLL-treated αMEM/Hypo samples were more concentrated compared to the CPLL-treated αMEM/Norm samples. This result confirm that enough total protein for 2D DIGE analsyis is present.

Aliquots of 4 µg from all used samples in the experiment were pooled and labeled with 1 µL of G-dye 100, to prepare the internal standard (IS). 24 µg of the respectively analysed sample was labeled with G-dye 200 according to the protocol see chapter 3.6.1.4 (page 53). Both labeled extracts were mixed and separated by 2D GE (more details see Figure 79). Afterwards the gels were scanned at the wavelength specific for each G-dye generating images that can be overlayed directly by the DeCyder software, using the differential in-gel analysis (DIA) option for the identification of differentially expressed protein spots.

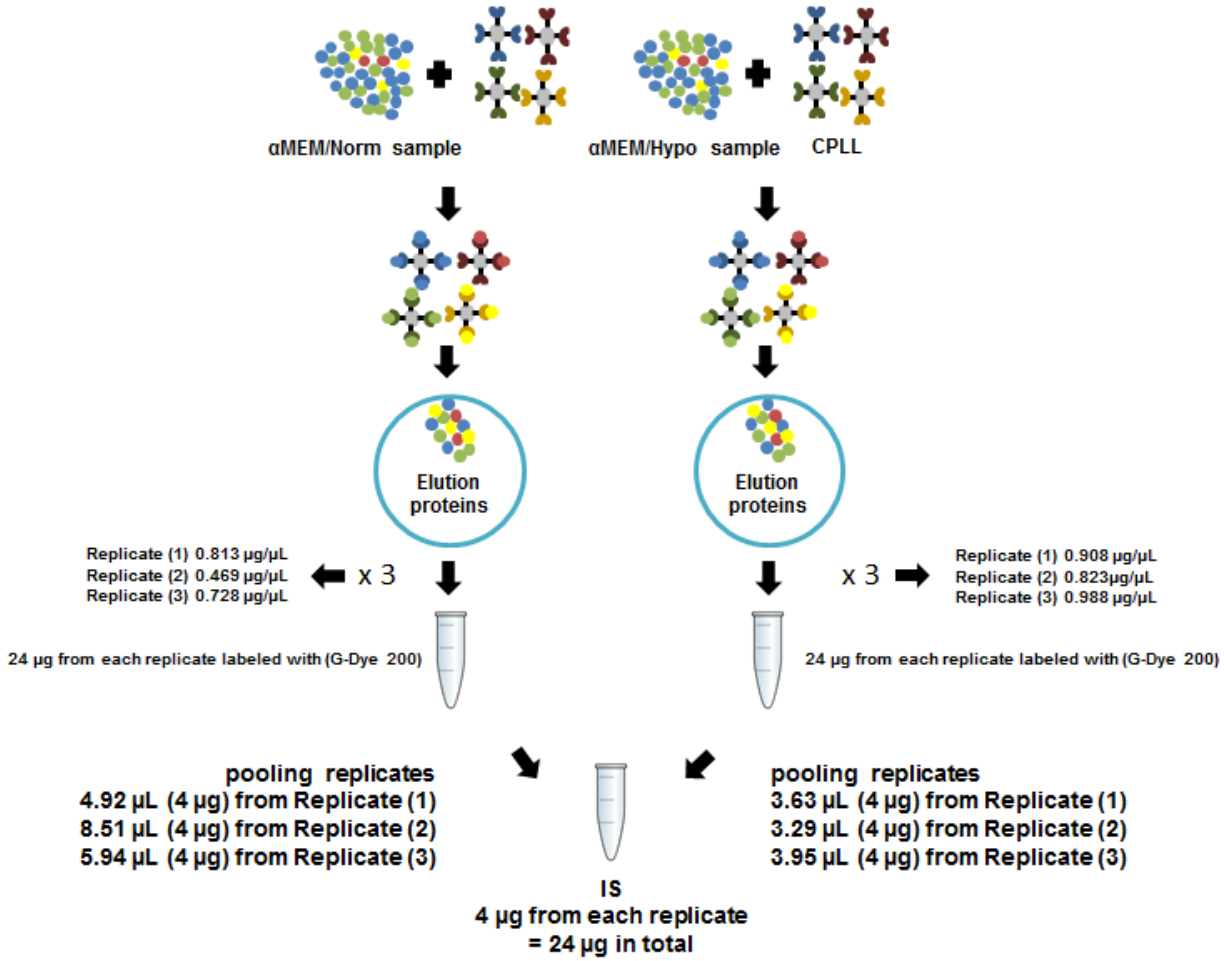


Figure 79: Workflow for generating the triplicates for both cultivation conditions as well as the internal standard.

4.5.1 Results for normoxic cultivation conditions

As previous mentioned for each cultivation condition three replicates were generated (see Figure 80) which were used for further 2D GE analysis. Table 57 should give an overview of the characteristics for each normoxic replicate, which will be discussed in more details in the following.

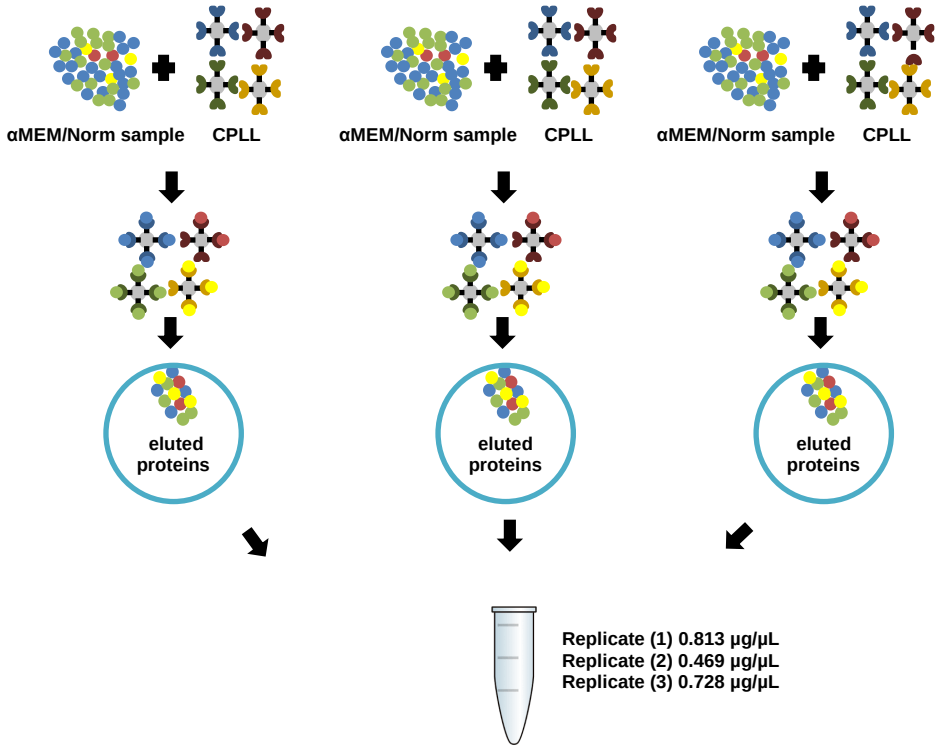


Figure 80: Overview of generating three replicates for the normoxic cultivation condition.

	Replicate (1)	Replicate (2)	Replicate (3)	Internal standard
Sample	αMEM/Norm 1-1	αMEM/Norm 1-2	αMEM/Norm 1-3	IS
Label	1 µL G-Dye 200	1 µL G-Dye 200	1 µL G-Dye 200	1 µL G-Dye 100
Protein load	**29.52 µL (24 µg)**	**51.09 µL (24 µg)**	**32.96 µL (24 µg)**	**4 µL from all samples (24 µg)**
IEF	49.5 kVh 10 mAh 24:02 h	55.3 kVh 10 mAh 25:42 h	52.8 kVh 12 mAh 24:59 h	
SDS-PAGE	3434 kVh 197 mAh 5:50 h	3658 kVh 125 mAh 5:50 h	3434 kVh 197 mAh 5:50 h	

Table 57: Characteristics for the three normoxic replicates.

2D DIGE analysis was performed after treatment with CPLL (see chapter 3.4.1.4 (page 49)). 24 µg of the analysed replicate and 24 µg of the IS (a pool of 4 µg equal aliquots of all replicates) were labeled and pooled with the corresponding G-dyes prior to electrophoresis (more details see chapter 3.6.1.4 (page 53)). The first and second dimension was performed according to the protocol (see chapter 3.6.2.4 (page 54) and 3.6.4.4 (page 57)). No uncommon current over time was observed during the first and second dimension for all three replicates (Figure 81 and Figure 82). Obtained parameters for both dimensions are shown in Table 57.

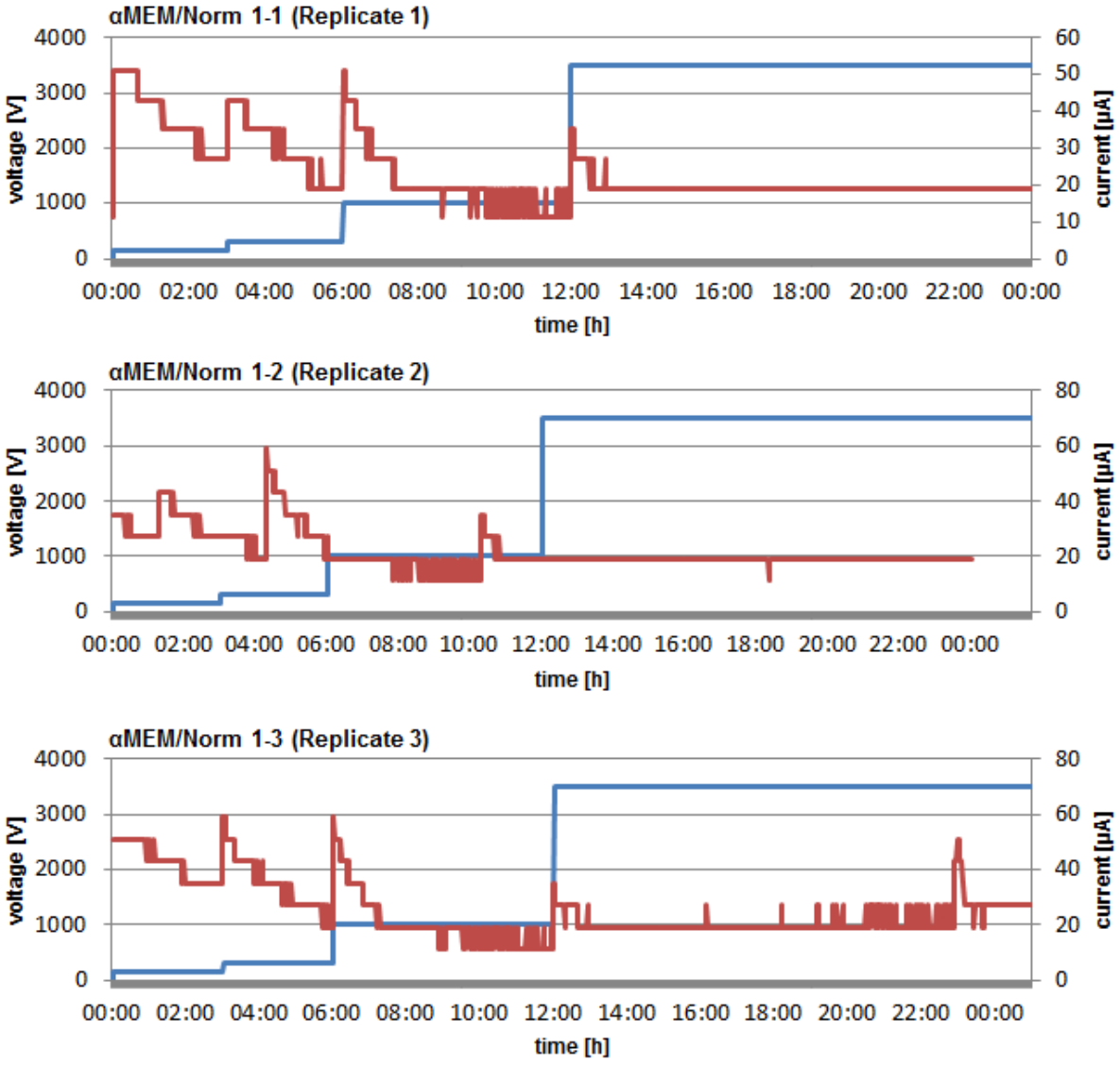


Figure 81: Voltage and current over the course of time for all three the αMEM/Norm replicates.

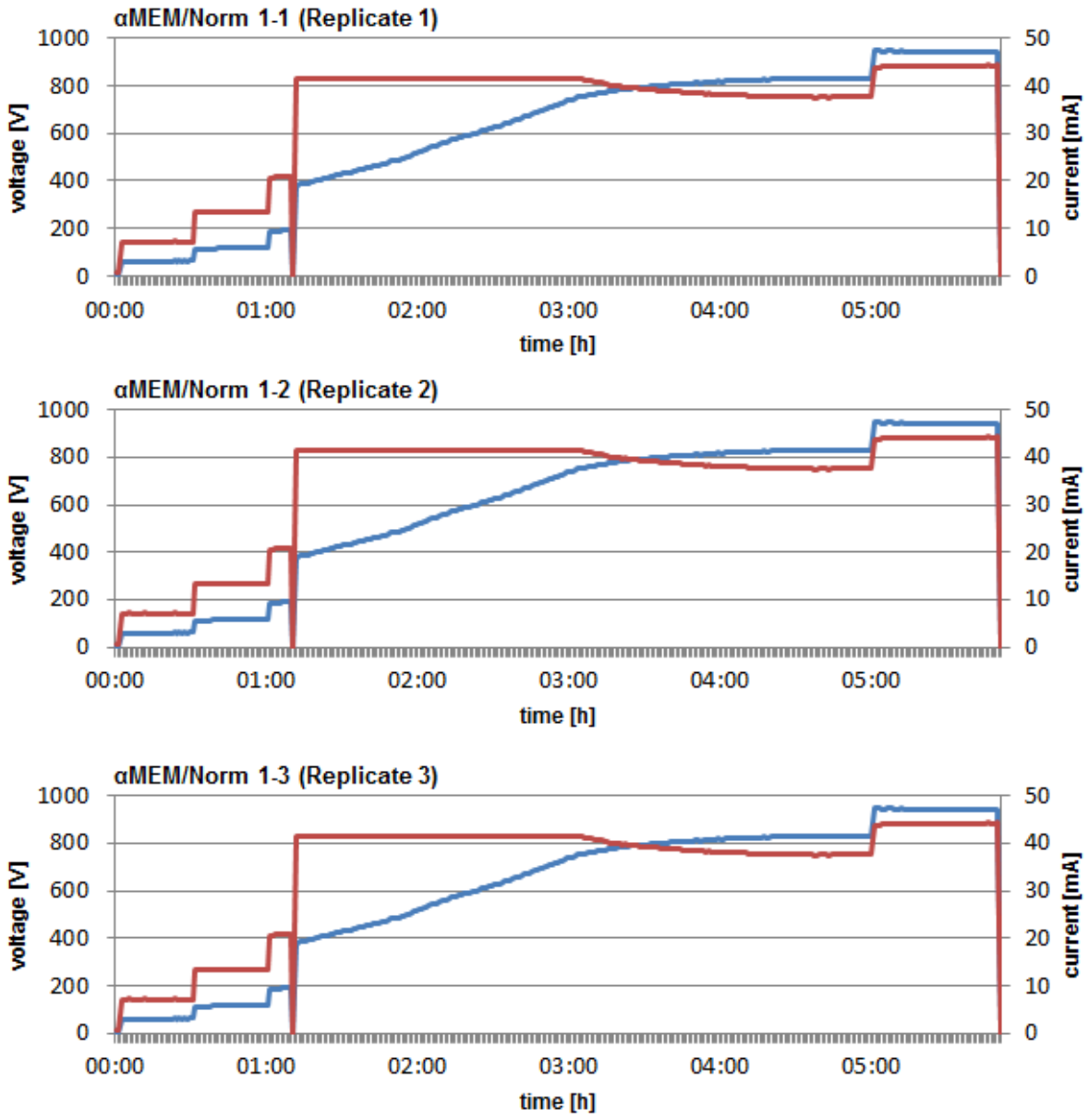


Figure 82: Voltage and current over the course of time for all three the αMEM/Norm replicates.

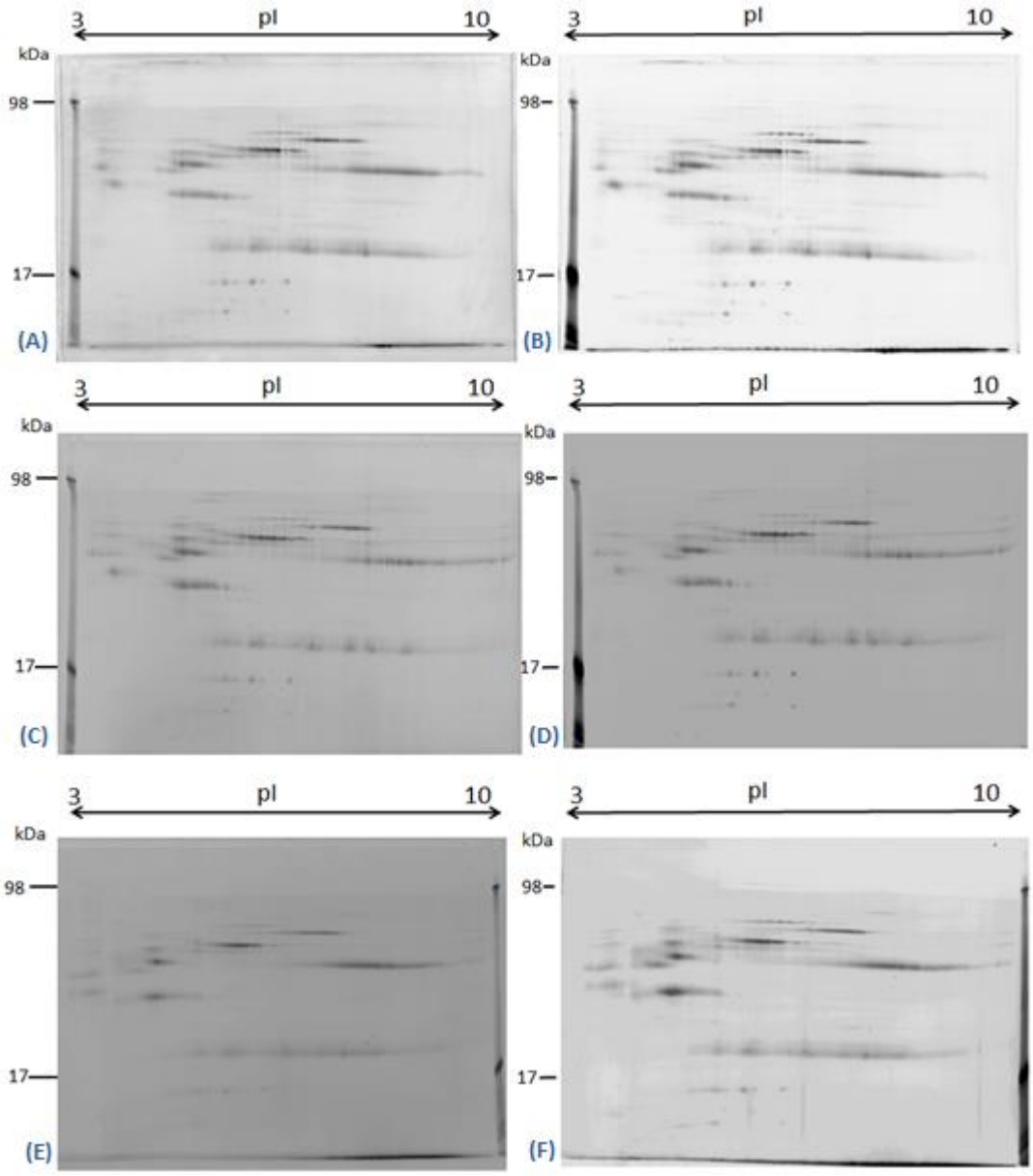


Figure 83: 2D gel of 24 µg (A) αMEM/Norm 1-1 replicate, (C) αMEM/Norm 1-2 replicate, (E) αMEM/Norm 1-3 replicate and 24 µg (B)+(D)+(F) IS, separated on a 24 cm IPG strip containing a non-linear pH-gradient 3-10, followed by SDS PAGE using T% 12.5 gels. A pre-stained molecular weight marker was used.

Figure 83 shows the obtained scans of the 2D gel for all three αMEM/Norm replicates and their corresponding IS. Compared to the prior 2D gel patterns, less protein spots which were not clearly focused were obtained. Assumptions for that could be impurities in the sample or sample buffer have occurred or a too long focusing time which leads to protein precipitation and so hampers the protein separation. Also mistakes during sample preparation and handling e.g. pooling of many samples, increase the risk of introduced errors and contaminations. However some spots between MW 6 kDa and 17 kDa good focussed spots were detected. Furthermore the gel was also post stained with Serva purple to enhance the

fluorescent stain (Figure 84). Figure 85 shows the overlay of the obtained images using DeCyder software.

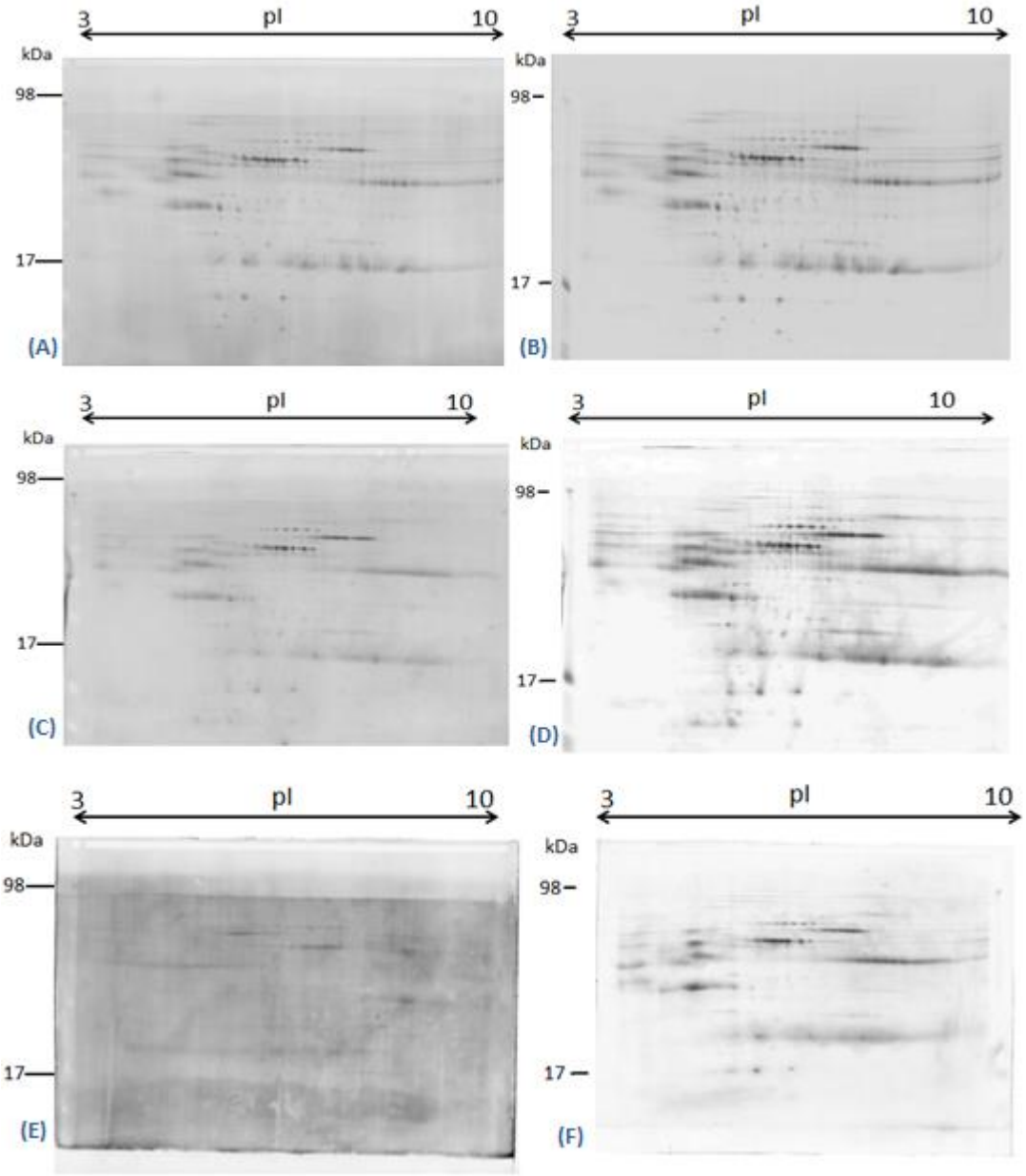


Figure 84: 2D gel of 24 µg (A) αMEM/Norm 1-1 replicate, (C) αMEM/Norm 1-2 replicate, (E) αMEM/Norm 1-3 replicate and 24 µg (B)+(D)+(F) IS, after post-staining with Serva purple to achieve an additive effect.

A further post-staining step with Serva purple was conducted to enhance the labeling reaction form the obtained results. Thus, more good focused spots become visible; especially at MW 55 kDa till 65 kDa (see Figure 84 (A), (B), (C) and (D)). An image overlay of the obtained gel images was done by using DeCyder software. In dual-channel images different gels are present in the colours red and green and placed on top of the other. Channel green illustrates the IS, red the analysed sample (normoxie or hypoxie) and yellow the occurrences of the spot in the IS as well as in the sample.

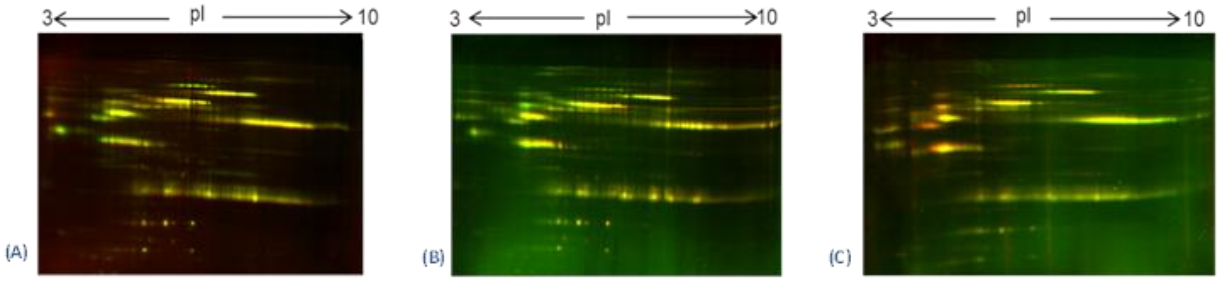


Figure 86: Image overlay of the generated gel scans to detect and quantify differentially expressed protein spots. (A) αMEM/Norm 1-1 replicate and IS overlay, (B) αMEM/Norm 1-2 replicate and IS overlay, (C) αMEM/Norm 1-3 replicate and IS overlay.

4.5.2 Results for hypoxic cultivation conditions

For the hypoxic cultivation condition also three replicates were generated (see Figure 87) prior to 2D GE analysis. An overview of the characteristics for each hypoxic replicate is given in Table 58 and will be discussed in more details in the following. After all three replicates were processed with CPLL a 2D DIGE analysis was performed (see chapter 3.4.1.4 (page 49)). Here, also 24 µg of the analysed replicate and 24 µg of the IS (a pool of 4 µg equal aliquots of all replicates) were labeled and pooled with the corresponding G-dyes prior to electrophoresis (more details see chapter 3.6.1.4 (page 53)). The first and second dimension showed no uncommon current over time for all three replicates (Figure 88 and Figure 89). Obtained parameters for both dimensions are shown in Table 58.

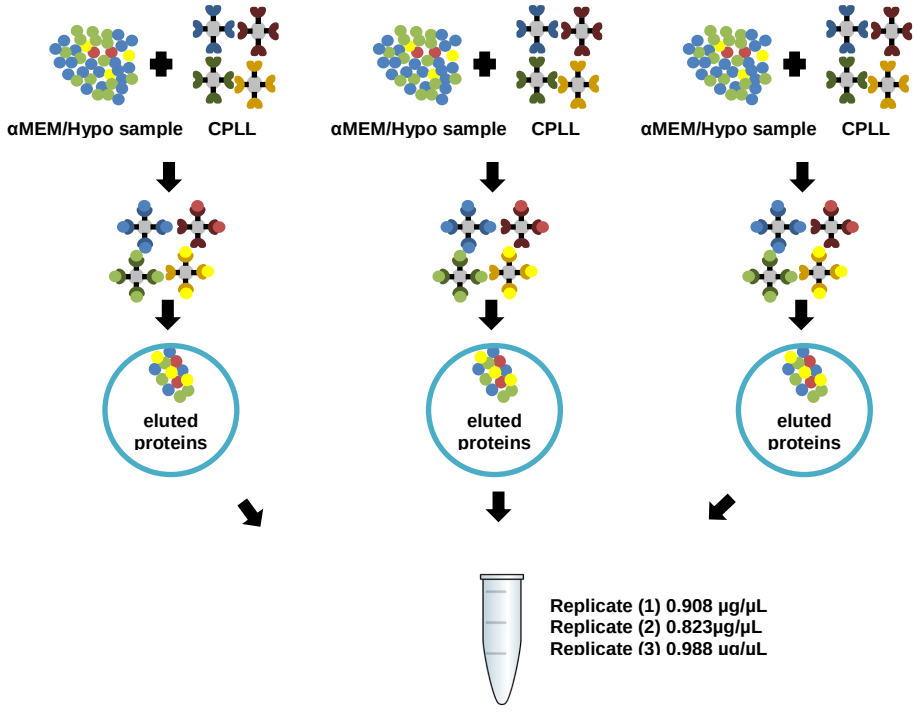


Figure 87: Overview of generating three replicates for the hypoxic cultivation condition.

	Replicate (1)	Replicate (2)	Replicate (3)	Internal standard
Sample	αMEM/Hypo 1-1	αMEM/Hypo 1-2	αMEM/Hypo 1-3	IS
Label	1 µL G-Dye 200	1 µL G-Dye 200	1 µL G-Dye 200	1 µL G-Dye 100
Protein load	**26.41 µL (24 µg)**	**29.16 µL (24 µg)**	**24.29 µL (24 µg)**	**4 µL from all samples (24 µg)**
IEF	54.2 kVh 10 mAh 25.23 h	55.5 kVh 11 mAh 25:47 h	48.6 kVh 10 mAh 23:47 h	
SDS-PAGE	3434 kVh 193 mAh 5:50 h	3710 kVh 191 mAh 5:50 h	3710 kVh 191 mAh 5:50 h	

Table 58: Characteristics for the three hypoxic replicates.

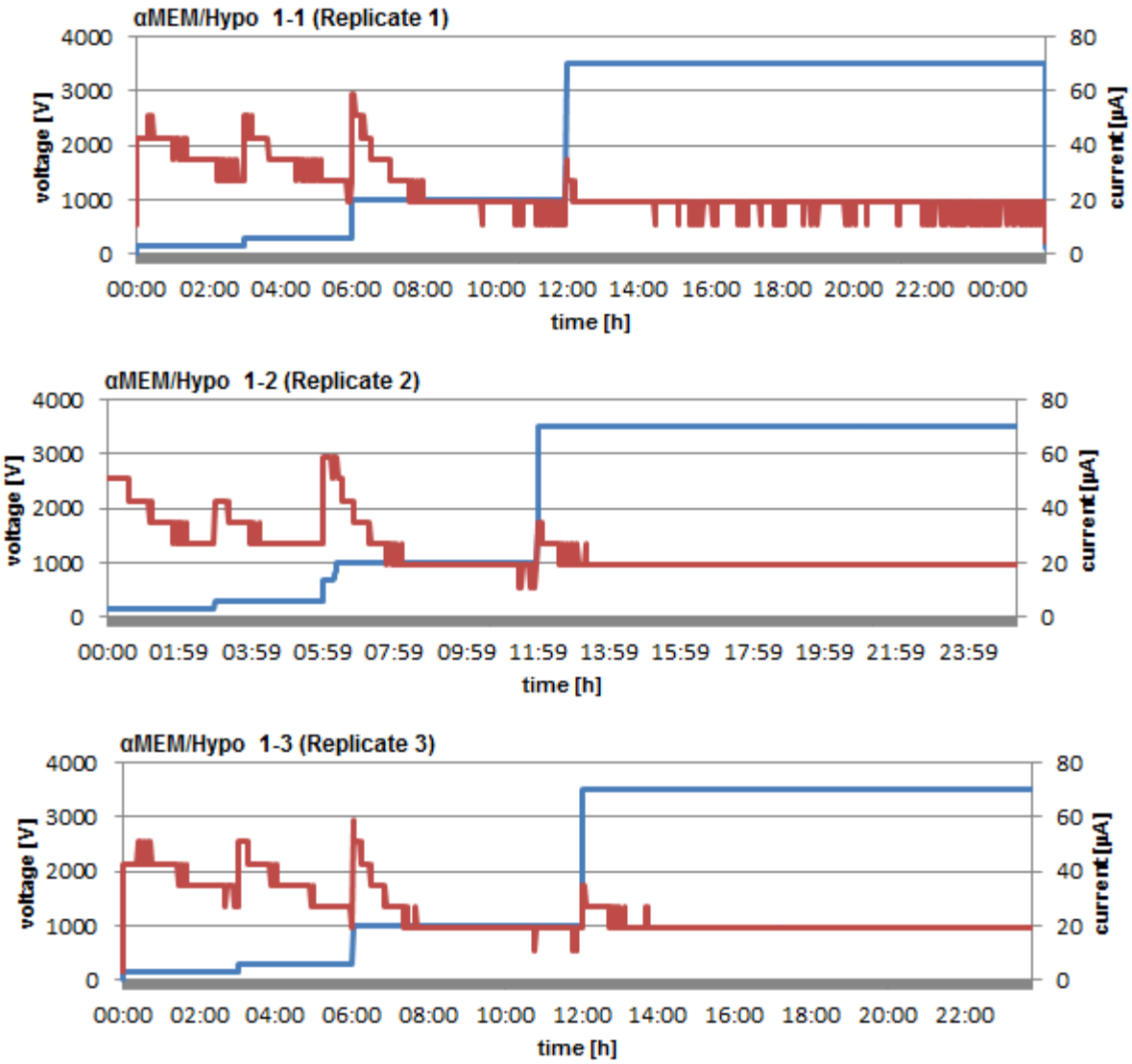


Figure 88: Voltage and current over the course of time for all three the αMEM/Hypo replicates.

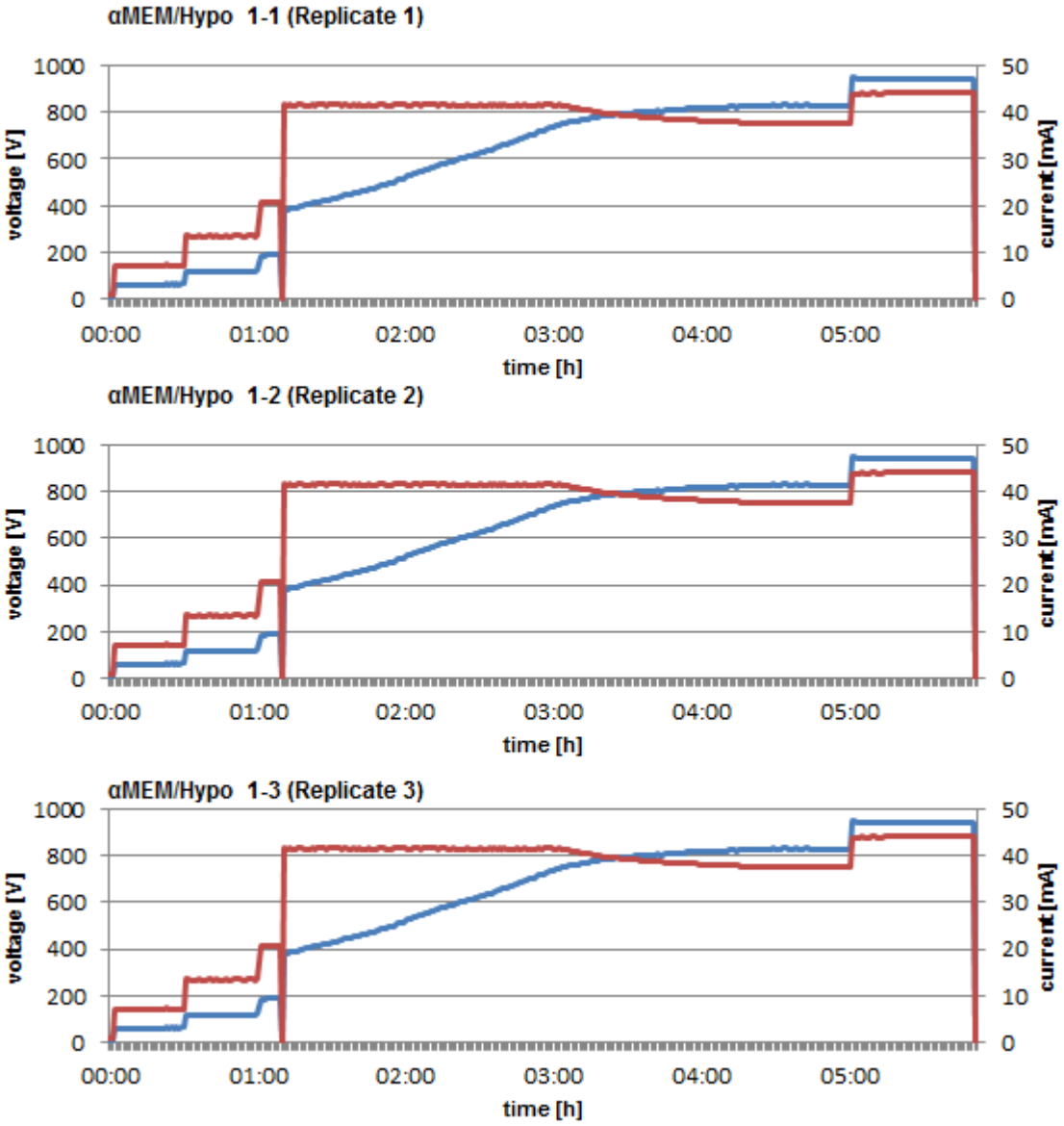


Figure 89: Voltage and current over the course of time for all three the αMEM/Hypo replicates.

The obtained scans of the 2D gel are shown in Figure 90. To enhance the fluorescent stain the gel was also post-stained with Serva purple (Figure 91). Figure 92 shows the overlay of the obtained images using DeCyder software.

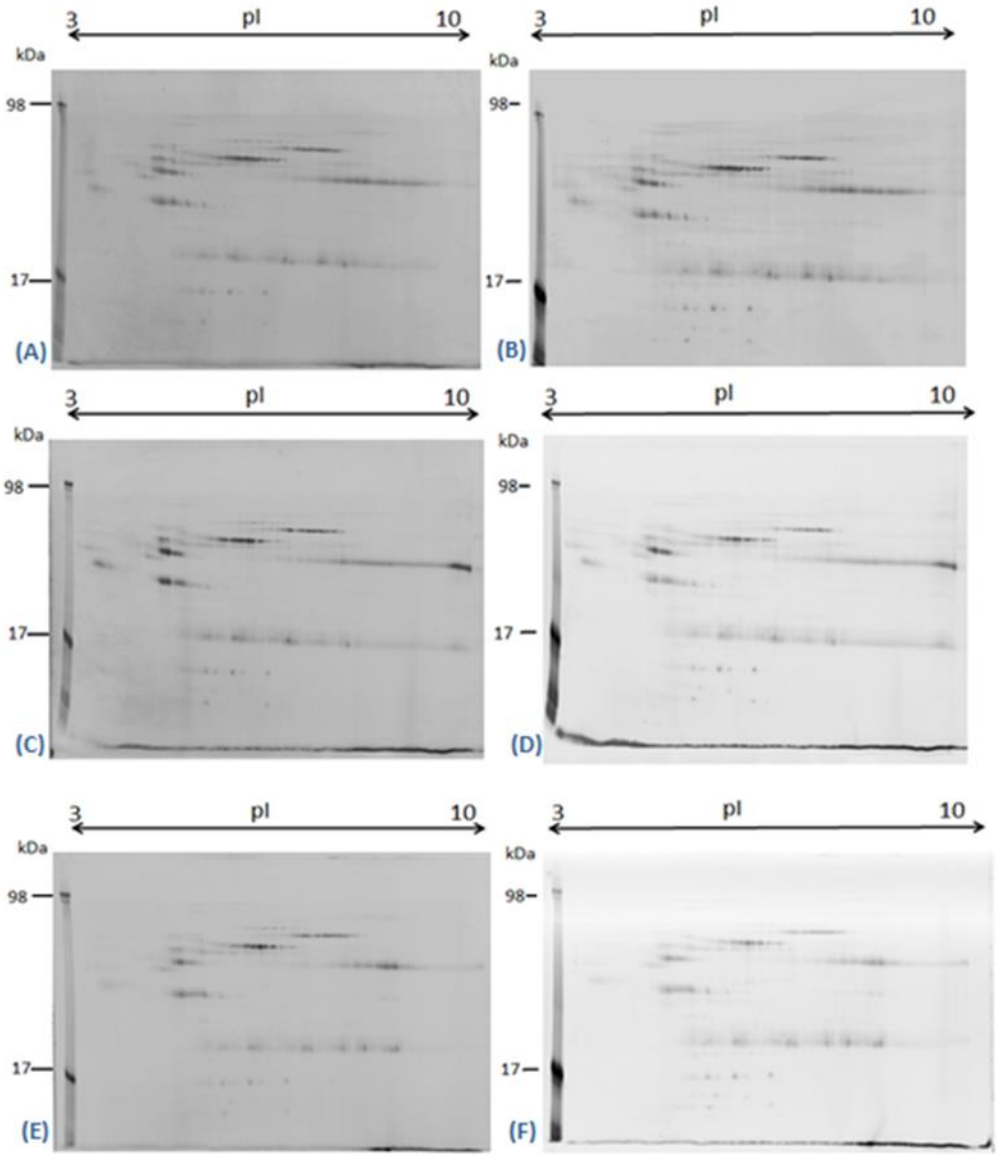


Figure 90: 2D gel of 24 µg (A) αMEM/Hypo 1-1 replicate, (C) αMEM/Hypo 1-2 replicate, (E) αMEM/Hypo 1-3 replicate and 24 µg (B)+(D)+(F) IS, separated on a 24 cm IPG strip containing a non-linear pH-gradient 3-10, followed by SDS PAGE using T% 12.5 gels. A pre-stained molecular weight marker was used.

For all three replicates less good focussed spots were obtained, as shown in Figure 90 (A), (C) and (E). Again horizontal streaks were observed as previous by the normoxic replicates (see Figure 90). Circumstances during sample preparation *e.g*. pooling of many samples could cause contaminations which hamper protein separation. The reduced number of protein spots can be caused by inefficient labeling due to disturbing amounts of salt despite precipitation, leading to a change of the pH. Moreover present ampholytes can competed with the protein for the dye during labeling reaction leading also to an inefficient labeling. Further possible reasons for the obtained results are that the IPG strips were frozen after the focusing to run all experiments parallel in one attempt for the second dimension on the HPE tower to exclude different separation conditions. Protein quality could be affected due to the freezing process; however this is a less likely reason.

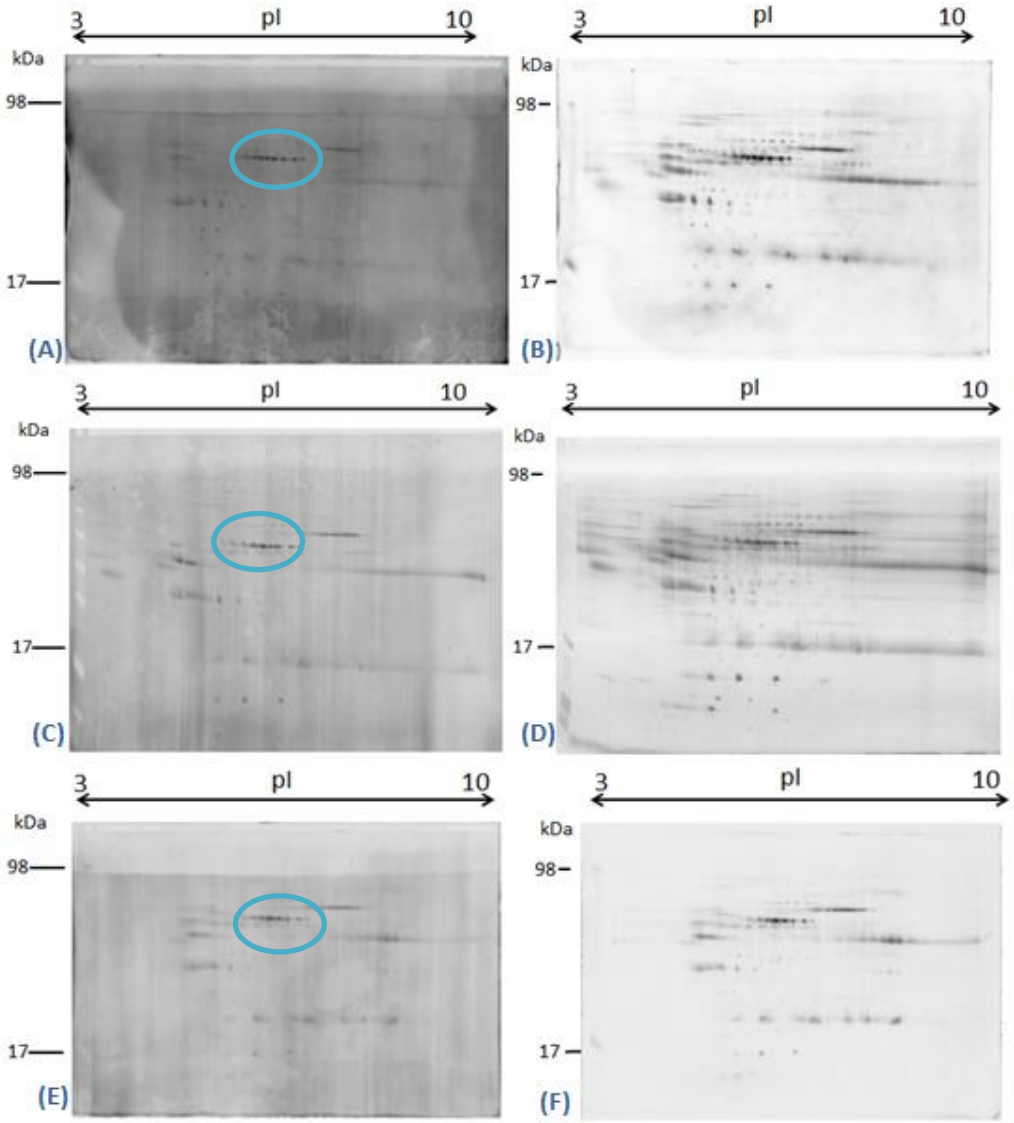


Figure 91: 2D gel of 24 µg (A) αMEM/Hypo 1-1 replicate, (C) αMEM/Hypo 1-2 replicate, (E) αMEM/Hypo 1-3 replicate and 24 µg (B)+(D)+(F) IS, after post-staining with Serva purple to achieve an additive effect.

To enhance the fluorescent labeling, a subsequent post-fluorescent staining was carried out with Serva purple. However, the resulting 2D gels showed no further spots as depicted in Figure 91 (A), (C) and (E). Only at MW 60 kDa some good focussed spots become better visible (highlighted in blue). The received spot patterns from the IS were improved by the additive staining, as shown in Figure 91 (B), (D) and (F).

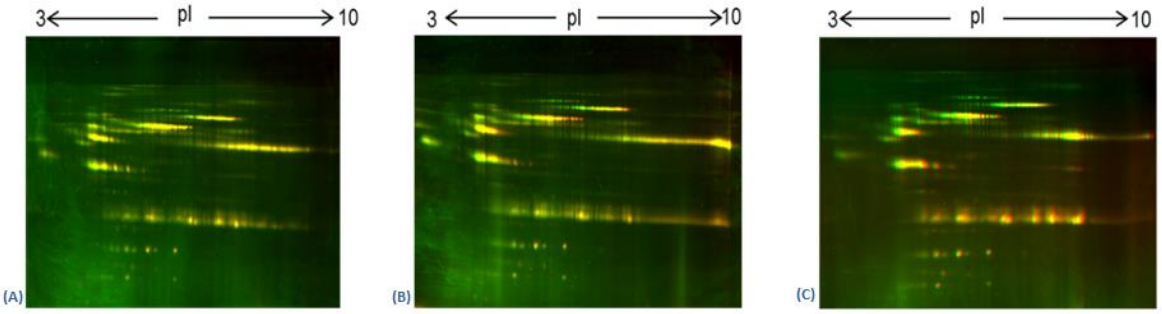


Figure 92: Image overlay of the generated gel scans to detect and quantify differentially expressed protein spots. (A) αMEM/Hypo 1-1 replicate and IS overlay, (B) αMEM/Hypo 1-2 replicate and IS overlay, (C) αMEM/Hypo 1-3 replicate and IS overlay.

4.5.3 Comparison of the results for both cultivation conditions

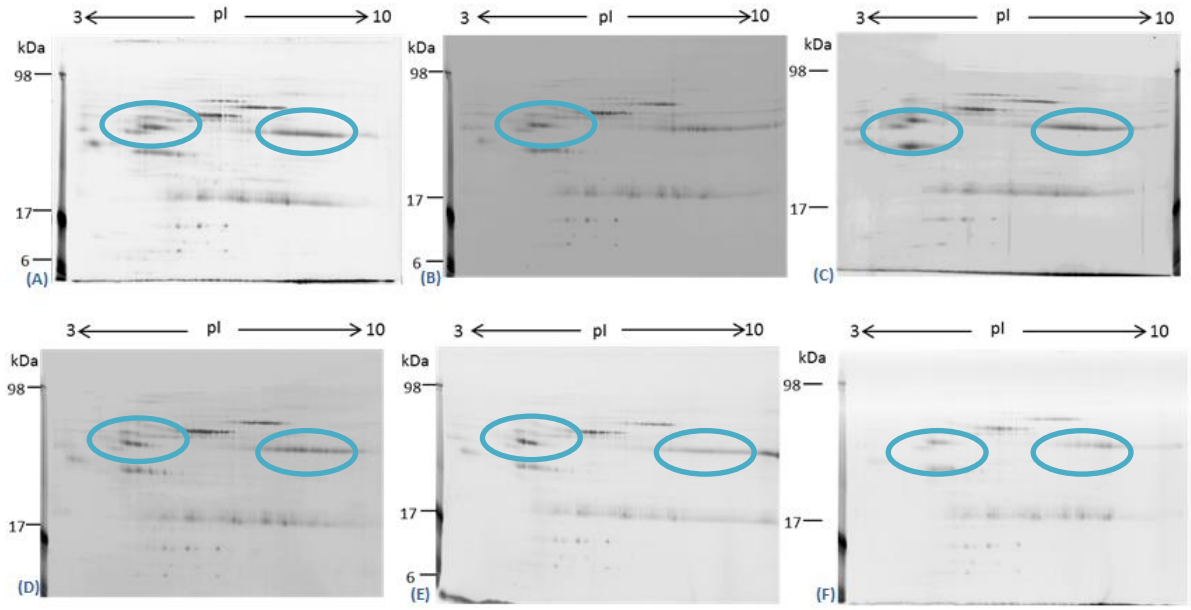


Figure 93: 2D DIGE comparison of different cultivation conditions. Three technical replicates for each cultivation condition were prepared (A) (B) and (C) normoxie and (D) (E) and (F) hypoxie. Images are acquired with a fluorescence scanner.

Figure 93 and Figure 94 show the comparison of 2D gels first labeled with fluorescence G-dyes and afterwards post stained with Serva purple to enhance the spot detection an to compare the sensitivity of both methods. As previous mentioned the obtained results were not satisfying. Less spots compared to prior experiments were obtained, which were further less intensive and not well focused (marked in blue). No uncommon current over time was observed during the first and second dimension for all replicates. Inefficient labeling due to changes of the pH caused by the presence of salts (despite protein precipitation) or presence of ampholytes that competed with the protein for the dye can be possible reasons for a less number of spots. Further the IPG strips were frozen after the first dimension to run all experiments in parallel to exclude different separation conditions. Because only one strip per day was able to be focussed and up to 3 strips were run in parallel on the HPE tower in the second dimension, the strips were frozen for one to three days at -20°C. Protein quality may be affected due to the freezing process. More likely sample preparation was inconsistent e.g. mistakes during handling or pooling of many samples can cause errors and contaminations, which hamper protein separation. To exclude some of the previous mentioned possibilities the gels were post-fluorescent stained with Serva purple, after 2D DIGE analysis to test if an improvement of the number of spots is possible shown in Figure 94.

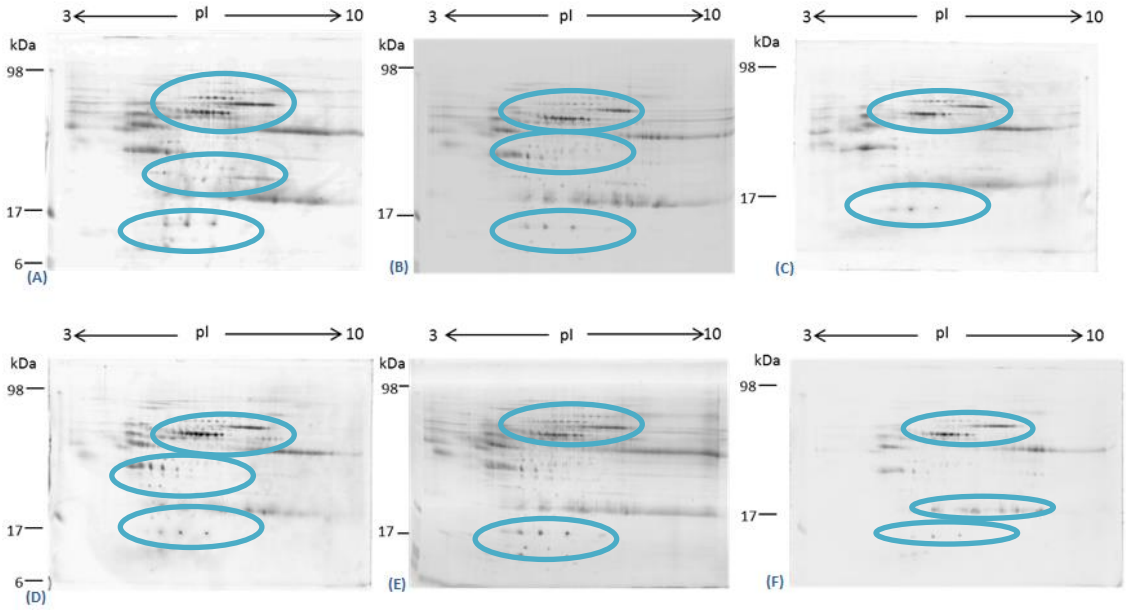


Figure 94: 2D DIGE gel comparison of both cultivation conditions after post-staining with Serva purple. (A) (B) and (C) normoxie and (D) (E) and (F) hypoxie replicates.

A 2D gel comparison of all replicates from both cultivation conditions after Serva purple staining is shown in Figure 94. Now more spots become visible for all replicates, highlighted in blue. Pre-fluorescent labeling is proven in literature to be much more sensitive compared to post-fluorescent staining. Nevertheless more spots were detected after the additional staining step. Possible causes for that could be that the minimal labeling is not fully optimized for our samples, because with minimal labeling only one lysine residue per protein will be labelled and only if lysine is freely available or present. If lysine is not available or not even present in the protein, it cannot be labeled leading to less protein spots which can be detected. The addition of a non-covalently binding fluorescence dye after separation allows unspecific attachment of dye molecules additionally increasing the fluorescence read-out, hence allowing the detection of more spots after post-staining compared to the minimal labeling strategy. More replicates of the experiment and testing of other labeling strategies (saturation labeling or other available dyes e.g. CyDyes) will be necessary to see if other approaches deliver better results. Due to less time further experiments were not performed.

Prior to the analysis the gel images were cropped to remove areas extraneous to the gel image by the program ImageQuant. Image overlay using DIGE technology for normoxie and hypoxie samples are shown in Figure 95.

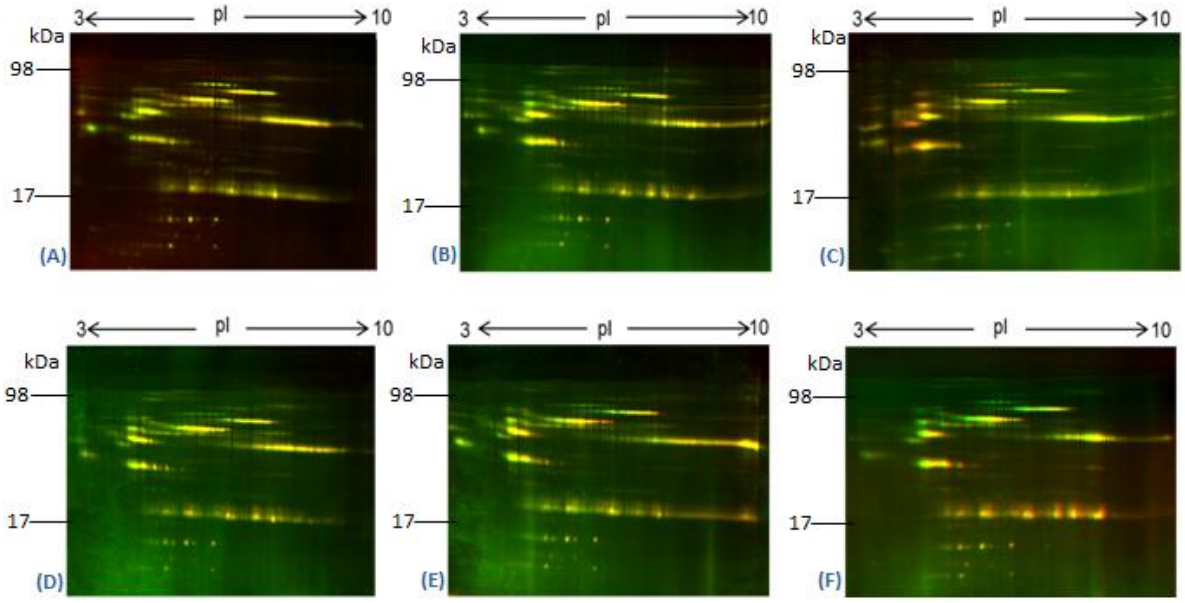


Figure 95: Comparison of image overlay of the 2D DIGE gels for both cultivation conditions. Three technical replicates for each cultivation condition were prepared. (A) (B) and (C) show the replicates for normoxic cultivation, (D) (E) and (F) the replicates for hypoxic cultivation.

The image overlays of the patterns visualize up- and down regulated proteins (Figure 95). Protein spots which are shown in green are present in the internal standard only (IS). Spots which are visible in red are only present in the samples (normoxie or hypoxie). If gel spots are yellow it indicates that this protein is present in both samples which moreover means that they have not changed between the conditions. Due to bad quality of the raw data a software based evaluation was not possible, only a visual comparison was done.

However slight changes cannot be determined without software based evaluation. Therefore one replicate of each cultivation condition was chosen. Only the left corner of the 2D gel (MW 6 kDa and 17 kDA, pI 3 and 6.2) provides well focussed spots, so an evaluation using DeCyder software was tried, depicted in Figure 96.

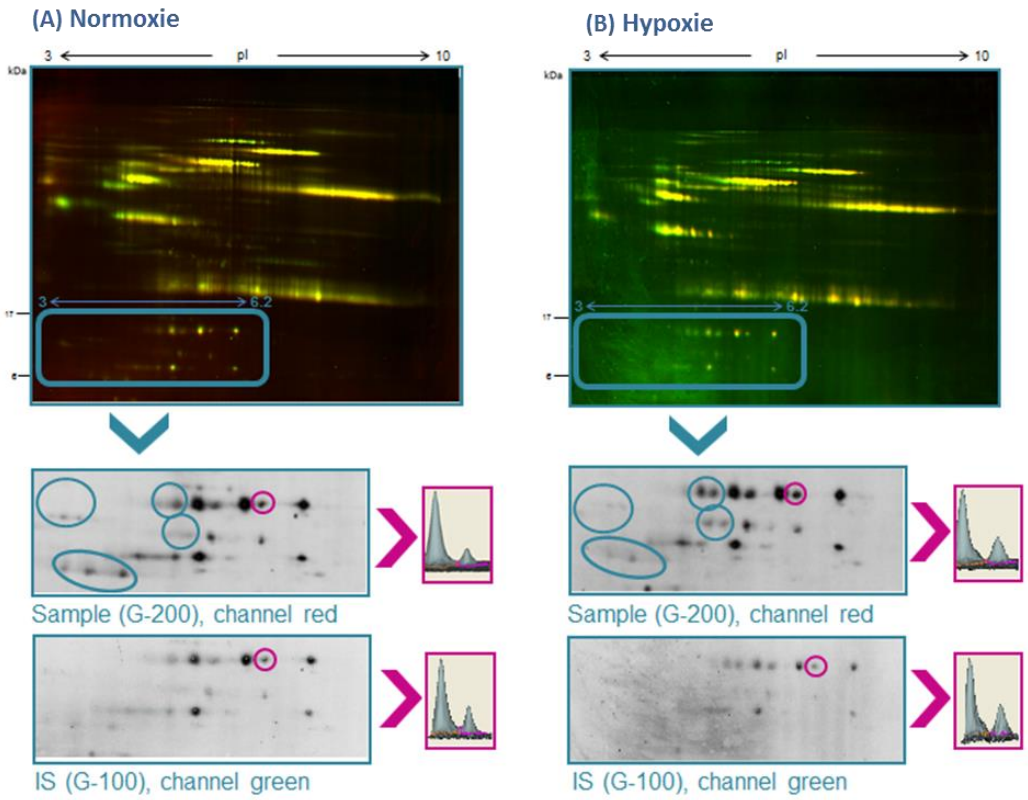


Figure 96: 2D DIGE comparison of different cultivation conditions (A) normoxie and (B) hypoxie

After background subtraction and in-gel normalization by the differential in-gel analysis (DIA) module from DeCyder gel analysis visually showed variable intensity for some protein spots, which could indicate differently expression levels of proteins for hypoxie and normoxie samples (highlighted in blue). However these statements are at the moment statistically not confirmed (see Figure 96) due to the limited number of analyses. However it can be used as preliminary result for further experiments.

Yet it was shown that differences in the secretome of mesenchymal stem cells treated under different oxygen levels are accessible. However more repetitions of the experiment are necessary to generated more data with better quality to be able to evaluate them with the corresponding software to statistically confirm the mentioned speculation. Furthermore mass spectrometric analysis has to be done to identify differentially expressed proteins and to confirm that those belong to the secretome of interest and not to the serum.

5 Conclusion

In the present work we were able to establish a good and reproducible strategy to get access to the secretome of mesenchymal stem cells. We could show that our protein precipitation strategy using TCA and ice-cold acetone was successful and reproducible, which was confirmed by 1D PAGE. To achieve reproducible 2D GE conditions different sample preparation strategies were evaluated. We tested two sample application methods in-gel rehydration and cup loading for their performance in the first dimension. The obtained results show that in-gel rehydration was the better choice for the work conducted in this master thesis. For the second dimension a horizontal and vertical gel electrophoresis system was compared leading to the decision that with the horizontal equipment a higher resolution, sensitivity and reproducibility was achieved. Finally different post-staining techniques were tested for their sensitivity. With Serva purple, a fluorescent dye, an increased number of protein spots was detected when compared to silver staining. To get access to low abundant proteins, high abundance serum proteins had to be depleted. Two depletion methods were tested and good results were achieved for both strategies. We assumed that the combinatorial peptide library approach reduces high abundant serum proteins to a lesser extent than Top12 depletion columns, however co-depletion of low abundance proteins is reduced and a more complete coverage of the secretome can be achieved. Yet, the combinatorial peptide ligand library approach will be further optimized to achieve even better reproducibility and therefore robustness of sample preparation.

By using DIGE technology we aimed to show differences for the secretome of MSCs cultivated under different oxygen supply levels (normoxie and hypoxie). DIGE gel analysis showed some variable intensities for some protein spots, which indicate different expression levels of proteins for both cultivation condition. However these statements are only based on visual comparison of spot intensities and are at the moment not statistically confirmed.

6 Outlook

For future experiments the following points need to be followed up:

- The CPLL approach needs to be optimized to further enhance results. For this buffer conditions in the sample pre-fractionation step and sample volumes, and maybe even protein precipitation has to be optimized.
- Due to not quit focused spots during the DIGE approach changing the labeling strategy should be considered.
- Saturation labeling might be used as an alternative because it has lower detection limits and therefore better results might be obtained.
- Furthermore the IEF program could be more adjusted to receive better results.
- Number of analyses for optimally prepared samples has to be increased to reach statistically significant results.
- As a final step protein identification by mass spectrometry is needed to characterize possible up- or down regulated proteins.

7 References

1. Cramer R, Westermeier R: **Difference Gel Electrophoresis (DIGE): Methods and Protocols**: Humana Press; 2012.
2. Westermeier R: **Electrophoresis in Practice**, 4th Edition edn: Wiley-VCH Verlag GmbH & Co. KGaA; 2001.
3. Shevchenko A, Wilm M, Vorm O, Mann M: **Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels**. *Anal Chem* 1996, **68**(5):850-858.
4. Ullah I Fau - Baregundi Subbarao R, Baregundi Subbarao R Fau - Rho G-J, Rho GJ: **Human Mesenchymal Stem Cells - Current trends and future prospective**. (1573-4935 (Electronic)).
5. Kühl S, Kühl M: **Stammzellbiologie**, 1. Auflage edn: UTB GmbH; 2012.
6. Eblenkamp M, Neuss-Stein S, Salber S, J. VR, Wintermantel E: **Stammzellen**. In: *Medizintechnik.* 5. Auflage edn: Springer-Verlag Berlin, Heidelberg; 2009: 443-471.
7. Schofield R: **The relationship between the spleen colony-forming cell and the haemopoietic stem cell**. *Blood Cells* 1978, **4**(1-2):7-25.
8. Prockop DJ: **Marrow stromal cells as stem cells for nonhematopoietic tissues**. *Science* 1997, **276**(5309):71-74.
9. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: **Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement**. *Cytotherapy* 2006, **8**(4):315-317.
10. Friedenstein AJ, Chailakhjan RK, Lalykina KS: **The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells**. *Cell Tissue Kinet* 1970, **3**(4):393-403.
11. Lavrentieva A: **Strategies in umbilical cord-derived mesenchymal stem cells expansion**. *Diss.* Hannover: Gottfried Wilhelm Leibniz Universität Hannover; 2012.
12. Ullah I, Baregundi Subbarao R, Rho GJ: **Human Mesenchymal Stem Cells - Current trends and future prospective**. *Biosci Rep* 2015.
13. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A: **Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement**. *Cytotherapy* 2005, **7**(5):393-395.
14. Schmitz S: **Der Experimentator Zellkulturen**, 3. Auflage edn: Spektrum Akademischer Verlag Heidelberg; 2011.
15. Gstraunthaler G, Lindl T: **Zell- und Gewebekultur**, 7. Auflage edn: Springer-Verlag Berlin, Heidelberg; 2013.
16. Martin Y, Eldardiri M, Lawrence-Watt DJ, Sharpe JR: **Microcarriers and their potential in tissue regeneration**. *Tissue Eng Part B Rev* 2011, **17**(1):71-80.
17. Haque N, Rahman MT, Abu Kasim NH, Alabsi AM: **Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy**. *ScientificWorldJournal* 2013, **2013**:632972.
18. Estrada JC, Albo C, Benguria A, Dopazo A, Lopez-Romero P, Carrera-Quintanar L, Roche E, Clemente EP, Enriquez JA, Bernad A *et al*: **Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis**. *Cell Death Differ* 2012, **19**(5):743-755.
19. Peterson KM, Aly A, Lerman A, Lerman LO, Rodriguez-Porcel M: **Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress**. *Life Sci* 2011, **88**(1-2):65-73.
20. Lavrentieva A, Majore I, Kasper C, Hass R: **Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells**. *Cell Commun Signal* 2010, **8**:18.
21. Eagle H: **The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure**. *J Exp Med* 1955, **102**(5):595-600.
22. Eagle H: **Nutrition needs of mammalian cells in tissue culture**. *Science* 1955, **122**(3168):501-514.

23. Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC, Yan JX, Gooley AA, Hughes G, Humphery-Smith I *et al*: **From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis**. *Biotechnology (N Y)* 1996, **14**(1):61-65.
24. Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K: **Gel-based and gel-free quantitative proteomics approaches at a glance**. *Int J Plant Genomics* 2012, **2012**:494572.
25. Monteoliva L, Albar JP: **Differential proteomics: an overview of gel and non-gel based approaches**. *Brief Funct Genomic Proteomic* 2004, **3**(3):220-239.
26. Righetti PG, Boschetti E: **Chapter 4 - Low-Abundance Protein Access by Combinatorial Peptide Libraries**. In: *Low-abundance Proteome Discovery.* Edited by Boschetti PGR. Boston: Elsevier; 2013: 79-157.
27. Anderson NL, Anderson NG: **The human plasma proteome: history, character, and diagnostic prospects**. *Mol Cell Proteomics* 2002, **1**(11):845-867.
28. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD: **Characterization of the low molecular weight human serum proteome**. *Mol Cell Proteomics* 2003, **2**(10):1096-1103.
29. Thulasiraman V: **Reduction of the concentration difference of proteins in biological liquids using a library of combinatorial ligands**. *Electrophoresis* 2005, **26**:3561-3571.
30. Merrifield RB: **Automated synthesis of peptides**. *Science* 1965, **150**(3693):178-185.
31. Merrifield B: **Solid phase synthesis**. *Science* 1986, **232**(4748):341-347.
32. Furka A, Sebestyen F, Asgedom M, Dibo G: **General method for rapid synthesis of multicomponent peptide mixtures**. *Int J Pept Protein Res* 1991, **37**(6):487-493.
33. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ: **A new type of synthetic peptide library for identifying ligand-binding activity**. *Nature* 1991, **354**(6348):82-84.
34. Simo C, Bachi A, Cattaneo A, Guerrier L, Fortis F, Boschetti E, Podtelejnikov A, Righetti PG: **Performance of combinatorial peptide libraries in capturing the low-abundance proteome of red blood cells. 1. Behavior of mono- to hexapeptides**. *Anal Chem* 2008, **80**(10):3547-3556.
35. Boschetti E, Righetti PG: **The ProteoMiner in the proteomic arena: a non-depleting tool for discovering low-abundance species**. *J Proteomics* 2008, **71**(3):255-264.
36. Righetti PG, Fasoli E, Boschetti E: **Combinatorial peptide ligand libraries: the conquest of the 'hidden proteome' advances at great strides**. *Electrophoresis* 2011, **32**(9):960-966.
37. Keidel EM, Ribitsch D, Lottspeich F: **Equalizer technology--Equal rights for disparate beads**. *Proteomics* 2010, **10**(11):2089-2098.
38. Boschetti E, Righetti PG: **Low-Abundance Protein Access by Combinatorial Peptide Libraries**. In: *Low-abundance Proteome Discovery: State of the Art and Protocols.* Elsevier Science Publishing Co Inc; 2013: 90-106.
39. Filip S, Vougas K, Zoidakis J, Latosinska A, Mullen W, Spasovski G, Mischak H, Vlahou A, Jankowski J: **Comparison of Depletion Strategies for the Enrichment of Low-Abundance Proteins in Urine**. *PLoS One* 2015, **10**(7):e0133773.
40. Bellei E, Bergamini S, Monari E, Fantoni LI, Cuoghi A, Ozben T, Tomasi A: **High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins**. *Amino Acids* 2011, **40**(1):145-156.
41. Beseme O, Fertin M, Drobecq H, Amouyel P, Pinet F: **Combinatorial peptide ligand library plasma treatment: Advantages for accessing low-abundance proteins**. *Electrophoresis* 2010, **31**(16):2697-2704.
42. Chromy BA, Gonzales AD, Perkins J, Choi MW, Corzett MH, Chang BC, Corzett CH, McCutchen-Maloney SL: **Proteomic analysis of human serum by two-dimensional differential gel electrophoresis after depletion of high-abundant proteins**. *J Proteome Res* 2004, **3**(6):1120-1127.
43. Candiano G, Dimuccio V, Bruschi M, Santucci L, Gusmano R, Boschetti E, Righetti PG, Ghiggeri GM: **Combinatorial peptide ligand libraries for urine proteome analysis: investigation of different elution systems**. *Electrophoresis* 2009, **30**(14):2405-2411.

44. Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W: **The current state of two-dimensional electrophoresis with immobilized pH gradients**. *Electrophoresis* 2000, **21**(6):1037-1053.
45. Lottspeich F, Engels J: **Bioanalytik**, 3. Auflage edn: Spektrum Akademischer Verlag, Heidelberg; 2012.
46. Westermeier R, Naven T, Höpker HR: **Proteomics in Practice. A Guide to Successful Experimental Design**, 2nd Edition edn: Wiley-VCH Verlag GmbH & Co. KGaA; 2008.
47. Tiselius A: **ELECTROPHORESIS OF PURIFIED ANTIBODY PREPARATIONS**. *J Exp Med* 1937, **65**(5):641-646.
48. Tiselius A: **Electrophoresis of serum globulin. I**. *Biochem J* 1937, **31**(2):313-317.
49. Gey M: **Instrumentelle Analytik und Bioanalytik: Biosubstanzen, Trennmethoden, Strukturanalytik, Applikationen**, 2. Auflage edn: Springer 2008.
50. Rehm H, Letzel T: **Der Experimentator: Proteinbiochemie / Proteomics**, 6. Auflage edn: Spektrum Akademischer Verlag, Heidelberg; 2009.
51. Klose J: **Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals**. (0018-7348 (Print)).
52. O'Farrell PH: **High resolution two-dimensional electrophoresis of proteins**. (0021-9258 (Print)).
53. Gorg A, Obermaier C Fau - Boguth G, Boguth G Fau - Harder A, Harder A Fau - Scheibe B, Scheibe B Fau - Wildgruber R, Wildgruber R Fau - Weiss W, Weiss W: **The current state of two-dimensional electrophoresis with immobilized pH gradients**. (0173-0835 (Print)).
54. Gorg A, Weiss W Fau - Dunn MJ, Dunn MJ: **Current two-dimensional electrophoresis technology for proteomics**. (1615-9853 (Print)).
55. Bjellqvist B, Ek K, Righetti PG, Gianazza E, Gorg A, Westermeier R, Postel W: **Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications**. *J Biochem Biophys Methods* 1982, **6**(4):317-339.
56. Westermeier R Fau - Postel W, Postel W Fau - Weser J, Weser J Fau - Gorg A, Gorg A: **High-resolution two-dimensional electrophoresis with isoelectric focusing in immobilized pH gradients**. (0165-022X (Print)).
57. Bjellqvist B, Sanchez Jc Fau - Pasquali C, Pasquali C Fau - Ravier F, Ravier F Fau - Paquet N, Paquet N Fau - Frutiger S, Frutiger S Fau - Hughes GJ, Hughes Gj Fau - Hochstrasser D, Hochstrasser D: **Micropreparative two-dimensional electrophoresis allowing the separation of samples containing milligram amounts of proteins**. (0173-0835 (Print)).
58. Laemmli UK: **Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4**. *Nature* 1970, **227**(5259):680-685.
59. Schagger H, von Jagow G: **Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa**. *Anal Biochem* 1987, **166**(2):368-379.
60. Moche M, Albrecht D, Maass S, Hecker M, Westermeier R, Buttner K: **The new horizon in 2D electrophoresis: new technology to increase resolution and sensitivity**. *Electrophoresis* 2013, **34**(11):1510-1518.
61. May C, Brosseron F, Pfeiffer K, Meyer HE, Marcus K: **Proteome Analysis with Classical 2D-PAGE**. In: *Quantitative Methods in Proteomics.* Edited by Marcus K, 1 edn: Humana Press; 2012: 37-46.
62. Patton WF: **Detection technologies in proteome analysis**. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002, **771**(1-2):3-31.
63. Miller I, Crawford J, Gianazza E: **Protein stains for proteomic applications: which, when, why?** *Proteomics* 2006, **6**(20):5385-5408.
64. Kerenyi L, Gallyas F: **[Errors in quantitative estimations on agar electrophoresis using silver stain]**. *Clin Chim Acta* 1973, **47**(3):425-436.
65. Drabik A, Bodzon-Kulakowska A, Silberring J: **Gel Electrophoresis**. In: *Proteomic Profiling and Analytical Chemistry.* Edited by Silberring J, Ciborowski P: Elsevier; 2013: 107-132.

66. Rabilloud T: **A comparison between low background silver diammine and silver nitrate protein stains**. *Electrophoresis* 1992, **13**(7):429-439.
67. Bell PJ, Karuso P: **Epicocconone, a novel fluorescent compound from the fungus epicoccumnigrum**. *J Am Chem Soc* 2003, **125**(31):9304-9305.
68. Coghlan DR, Mackintosh JA, Karuso P: **Mechanism of reversible fluorescent staining of protein with epicocconone**. *Org Lett* 2005, **7**(12):2401-2404.
69. Unlu M, Morgan ME, Minden JS: **Difference gel electrophoresis: a single gel method for detecting changes in protein extracts**. *Electrophoresis* 1997, **18**(11):2071-2077.
70. Gorg A, Drews O, Luck C, Weiland F, Weiss W: **2-DE with IPGs**. *Electrophoresis* 2009, **30 Suppl 1**:S122-132.
71. Hrebicek T, Durrschmid K, Auer N, Bayer K, Rizzi A: **Effect of CyDye minimum labeling in differential gel electrophoresis on the reliability of protein identification**. *Electrophoresis* 2007, **28**(7):1161-1169.
72. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M: **Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology**. *Proteomics* 2001, **1**(3):377-396.
73. Drabik A, Silberring J, Bodzon-Kulakowska A: **Quantitation of Protein Using 2D Gels**. In: *Proteomic Profiling and Analytical Chemistry.* 2013: 127-129.
74. Berth M, Moser FM, Kolbe M, Bernhardt J: **The state of the art in the analysis of two-dimensional gel electrophoresis images**. *Appl Microbiol Biotechnol* 2007, **76**(6):1223-1243.
75. Daszykowski M, Stanimirova I, Bodzon-Kulakowska A, Silberring J, Lubec G, Walczak B: **Start-to-end processing of two-dimensional gel electrophoretic images**. *J Chromatogr A* 2007, **1158**(1-2):306-317.
76. Feneis H: **Anatomisches Bildwörterbuch der internationalen Nomenklatur**, 7. Auflage edn: Thieme; 1993.
77. Bandow JE: **Comparison of protein enrichment strategies for proteome analysis of plasma**. *Proteomics* 2010, **10**(7):1416-1425.
78. Millioni R, Tolin S, Puricelli L, Sbrignadello S, Fadini GP, Tessari P, Arrigoni G: **High abundance proteins depletion vs low abundance proteins enrichment: comparison of methods to reduce the plasma proteome complexity**. *PLoS One* 2011, **6**(5):e19603.

8 Appendix

Product name	Product #	Company
2D HPE Large Gel NF 12.5%, 255 x 200 x 0.65mm	43857-00	Serva
Acetic Acid >99.8%	33209-2.5L	Sigma Aldrich
Acetone, for analytic	1.00014.2500	Merck
Albumin, from bovine serum	A8022-10G	Sigma Aldrich
Boric Acid	15660	Fluka
Bromphenol Blue	B8026	Sigma Aldrich
CHAPS suitable for electrophoresis > 98% (TLC)	C9426-5G	Sigma Aldrich
Citric Acid > 99.5%	251275-100G	Sigma Aldrich
Coomassie Brilliant Blue G250	27815	Fluka
DL-Dithiothreitol	43815-5G	Sigma Aldrich
Ethanol	1.00983.2500	Merck
Formaldehyde solution	F8775-25ml	Sigma Aldrich
Immobiline dry strip pH 3-10 NL, 24cm	17-6002-45	GE Healthcare
Iodoacetamide	I1149-25G	Sigma Aldrich
IPG-Buffer pH 3-10	17-6000-87	GE Healthcare
Methanol, lichrosolv gradient grade	1.06007.2500	Merck
Nupage 4-12% Bis-Tris gel , 1.0mm x 15 well	NP0323BOX	Invitrogen
Nupage LDS sample buffer (4x)	NP0007	Invitrogen
Nupage Mes SDS running buffer (20x)	NP0002	Invitrogen
Paraffin, highly liquid	1.07174.2500	Merck
Phosphoric acid	4380815	Sigma Aldrich
Pierce ™ Top 12 Abundant Protein Depletion Spin Columns	85164	Thermofisher
Refraction-2D™ Labeling Kit	PR09	NHDyeAGNOSTICS
Proteominer ™ Protein Enrichment Kit	163-3006	BioRad
SDS, molecular biology grade	A2263, 1000	AppliChem
See Blue Plus 2 prestained Standard		Invitrogen
Serva cooling fluid	43371.07	Serva
Serva equilibration buffer	43805.07	Serva
Serva IPG blue strip, 3-10 NL 24cm	43022.01	Serva
Serva Purple	43386.01	Serva
Serva SDS Anode buffer	43801.07	Serva
Serva SDS Cathode buffer	43802.07	Serva
Servalyte , 3-10 analytical grade	42940	Serva
Silver nitrate	1.01512.0025	Merck
Sodium carbonate >99.8%	31432-1KG-R	
Sodium hydroxide	1.06482.5000	Merck
Sodium thiosulfate-5-hydrate	31459	Riedel den Haen
Thiourea, minimum 99.0%	T7875-500G	Sigma Aldrich
Trichloroacetic acid	1.00807.0250	Merck
Urea	0.568-1KG	Amresco

Table 59: An overview about the used chemicals for the entire experiment.