# Proteomic analysis of hypothermically cultivated CHO cells

**Diploma thesis**

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# *Table of contents*

# Abstract

Within this thesis the expression of proteins in CHO cell lines, used for the production of recombinant proteins, during hypothermic cultivation is studied. Cultivation at hypothermic conditions results in positive changes in growth behaviour, metabolism and the quantity of recombinant proteins expressed by the cells.

A suspension batch cultivation of two host and two recombinant cell lines was performed under mild (33 ℃) hypothermic conditions and at 37 ℃ as reference. Data about cell concentration, viability, glucose and glutamine uptake as well as lactate production were obtained. Furthermore samples were taken for proteomic analysis, which enabled the identification of significantly higher or lower expressed proteins by using the 2D DIGE (differential in gel electrophoresis) method. Proteins of interest were picked and characterised by mass spectroscopy.

It was not possible to identify all picked spots. However, those identified included the down-regulated proteins: dihydrolipoyl dehydrogenase, ERp57 protein disulfide isomerase, protein disulfide-isomerase A6 precursor and stress 70 protein of the comparison of the 33 ℃ and the 37 ℃ cultures.
These results indicate that hypothermic cultivation significantly influences protein folding and processing.

# Abbreviations

2D DIGE – two dimensional differential in gel electrophoresis

APS – ammonium persulfate

ATP – adenosine triphosphate

CCB – Coomassie brilliant blue

CHO – Chinese Hamster Ovary cell line

CSPs – cold-shock proteins

Dhfr – dihydrofolate reductase

DMF – dimethylformamide

DTT – dithiothreitol

Epo – erythropoietin

ER – endoplasmatic reticulum

HGPRT – hypoxanthine-guaninephosphoribosyltransferase

IEF – isoelectric focusing

IRESs – internal ribosome entry segments

MTX – Methotrexate

PDIs – protein disulfide isomerases

pI – isoelectric point

(m)RNA – (messenger) ribonucleic  acid

rP – recombinant proteins

RT – room temperature

SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

sf – serum free

TEMED – N,N,N’,N’-tetramethylethylenediamine

# Chapter 1 - Introduction

## 1.1 CHO cell lines for the production of recombinant proteins

Cultivated mammalian cells have become the dominant system for the production of recombinant proteins for clinical applications because of their capacity for proper protein folding, assembly and post-translational modification [1]. Thus, the quality and efficacy of a protein can be superior when expressed in mammalian cells versus other hosts such as bacteria, plants and yeast.

In the mid- 1980s the Chinese Hamster Ovary cell line (CHO) was one of the first mammalian cell lines successfully developed for use in the production of therapeutically valuable proteins [1]. Today, variations of this cell line are widely used for the production of therapeutical proteins, above all glycosylation or multimer proteins, as the glycosylation of the proteins has a strong influence on the structure, the biological activity in vivo and the immunogenicity.

To ensure maximal productivity of recombinant proteins (rP) during production it is typical to encourage an initial phase of rapid cell proliferation to achieve high biomass followed by a stationary phase where cellular energies are directed towards production of rP [2]. During many such biphasic cultures, the initial phase of rapid cell growth at 37℃ is followed by a growt h arrest phase induced through reduction of the culture temperature [2]. Low temperature induced growth arrest is associated with many positive phenotypes including increased productivity, sustained viability and an extended production phase, although the mechanisms regulating these phenotypes during mild hypothermia are poorly understood [2].

## 1.2 The hypothermic cultivation of cells

Notably, all organisms [4] – [7] from prokaryotes to plants and higher eukaryotes respond to cold shock in a comparatively similar manner. The general response of cells to cold stress is the rapid over expression of a small group of proteins, the so-called cold-shock proteins (CSPs). The majority of these CSPs are regulated sequentially in response to temperature downshift and have fundamental functions that determine cell fate such as DNA replication, transcription, translation, RNA stabilization and ribosome assembly [8].

The use of cold-shock [4] or decreased-temperature cultivation for the improvement of recombinant protein yields from in vitro cultured mammalian cells, and the mechanisms at work, have been reviewed recently [3]. A number of reports have shown that exposing yeast or mammalian cells to sub-physiological temperatures (<30 or <37 ℃ respectively) invokes a co-ordinated cellular response involving a profound reduction of metabolism, reduction in glucose and glutamine consumption, inhibition of ATP expenditure, a decrease in free radical oxygen species, inhibition of metabolic waste release, a reduction in protease activity, arrest of the cell cycle (mainly at G1 phase), increased viability, disassembly of the cell cytoskeleton, inhibition of translation (at the levels of both initiation and elongation), transcription attenuation, delayed apoptosis and increased resistance against shear stress [3], [9] – [28].

Furthermore cold shock results in changes in membrane permeability, which are leading to increases in cytosolic $Na^+$ and $H^+$ (albeit with a decrease in intracellular $K^+$). Cold stress can also produce alterations in the properties of the lipid bilayer, some of which (such as phase transitions) are simply due to the reduction in temperature, whereas others (such as changes in the fatty acid composition of the membrane) likely reflect a cellular physiological response to cold stress [29].

Previous reports [3] have suggested, that there are five mechanisms by which cold-shock-induced changes occur in gene expression in mammalian cells. The five proposed mechanisms are: (i) a general reduction in transcription and translation, (ii) inhibition of RNA degradation, (iii) increased transcription of specific target genes via elements in the promoter region of such genes, (iv) alternative

pre-mRNA splicing, and (v) via the presence of cold-shock specific IRESs (internal ribosome entry segments) in mRNAs that result in the preferential and enhanced translation of such mRNAs upon cold shock [29].

Furthermore, decreased temperature not only results in temperature stress, but also potentially in oxygen stress, which is also due to the fact that oxygen dissolves at higher concentrations as the temperature is decreased [30]. It is thought that the hypoxic signal transduction pathway and the cold-shock response share common components [31]. Further support for this lies in the fact that expression of the two major mammalian CSPs, Rbm3 and Cirp is regulated in response to hypoxic conditions [32].

During mild hypothermic cultivation [3], as it is the case in this study, the cytoskeleton does not disassemble like during temperatures lower than 30 ℃ and translation is most likely to be limited via the well characterized de-phosphorylation of key initiation and elongation factors, a process which is known to limit global translation initiation under conditions of environmental stress [33], [34]. Increased mRNA stability [3] and the formation of stable secondary structures are also likely to contribute to compromise global translation efficiency at mild-hypothermic temperatures.

The following table presents the effect of hypothermic incubation on the productivity of recombinant proteins in in-vitro cultured mammalian cells.

*Table 1.1 - The effect of sub-physiological temperature (< 37 ℃) in vitro culturing of mammalian cells on recombinant protein productivity. [3]*

| Cell line | Recombinant protein | Cold-shock cultivation temperature (°C) | Change in cell-specific productivity | Change in volumetric productivity | Source |
|---|---|---|---|---|---|
| BHK-21 | Antithrombin III | Various | — | No change | Weidemann et al., 1994 |
| CHO | Erythropoietin | 33 | Fourfold ↑ | 2.5-fold ↑ | Yoon et al., 2003b |
| | | 30 | 5.6-fold ↑ | | |
| CHO | Anti-4-1BB antibody | 33 | 1.2-fold ↑ | No change | Yoon et al., 2003a |
| | | 30 | No change | 3.9-fold ↓ | |
| CHO | Human granulocyte macrophage colony stimulating factor | 33 | 2.1-fold ↑ | 2.3-fold ↑ | Fogolin et al., 2004 |
| CHO | Secreted alkaline phosphatase (SEAP) | 30 | 1.7-fold ↑ | 3.4-fold ↑ | Kaufmann et al., 1999 |
| CHO | Chimeric Fab | 28 | — | 14-fold ↑[a]<br>38-fold ↑[b] | Schatz et al., 2003 |
| CHO | Interferon-γ | 32 | Twofold ↑ | 40–90% ↑ | Fox et al., 2004a |
| CHO | C-terminal amidating enzyme | 35 | — | 1.8-fold ↑ | Furukawa and Ohsuye, 1998 |
| CHO | Not disclosed | 32 | Sixfold ↑ | — | Ducommun et al., 2002 |
| CHO | Tissular plasminogen activator | 32 | — | 1.7-fold ↑ | Hendrick et al., 2001 |
| Hybridoma | Anti-interleukin-2 | 34 | No change | — | Bloemkolk et al., 1992 |
| Murine hybridoma | Monoclonal antibody | 33 | 21% ↓ | — | Barnabe and Butler, 1994 |
| Mouse hybridoma | Cytotoxic monoclonal antibody | 29 | Not determined | 95% ↓ | Sureshkumar and Mutharasan, 1991 |
| | | 33 | 42% ↓ | 20% ↓ | |
| | | 35 | No change | 10% ↑ | |
| Hybridoma | Monoclonal antibody | Various | Decrease or no change | | Reuveny et al., 1986 |

[a]Serum-containing medium ↑increase.
[b]Serum-free medium ↓decrease.

## 1.3 The proteomic analyses

To find out more about the difference in the expression of intracellular proteins during the hypothermic cultivation of the CHO cells, the proteomic analysis method of choice was the 2D DIGE (differential in gel electrophoresis). This technique [35] has been developed for the separation of proteins by two-dimensional polyacrylamide gel electrophoresis. Due to its resolution and sensitivity, this technique is a powerful tool for the analysis and detection of proteins from complex biological sources. Proteins are separated according to their isoelectric point by isoelectric focusing in the first dimension, and according to their molecular weight by sodium dodecyl sulfate electrophoresis in the second dimension.

Since these two parameters are unrelated, it is possible to obtain an almost uniform distribution of protein spots across a two-dimensional gel. Furthermore the Ettan DIGE method from Amersham Bioscience enables the production of qualitative and quantitative data and the comparison of different gels by means of fluorescent markers and an internal pooled standard. With this technique a wide

range of statistical analyses of the resulting images is possible and thus valuable biological information can be obtained.

In the scope of this work the level of change in protein expression between the hypothermic cultures incubated at 33 ℃ and the reference cultures incubated at 37 ℃ cultures was determined by means of the average ratio value and the students t-test value. The identified proteins were defined as proteins of interest and six of them could be characterised by mass spectrometry.

# Chapter 2 - Objectives

We were interested in the behaviour of recombinant CHO cells under hypothermic conditions. For that purpose information was acquired about the differences in growth, viability and metabolic parameters during a batch cultivation of CHO cells at 33 ℃ for the hypothermic cultures and 37 ℃ as reference cultures by analysis of cell concentration, viability, the glucose and glutamine uptake and the lactate production. Two host cell lines and two recombinant cell lines were chosen to obtain information about differences betweens these two types of cells.

In addition, differences in expression of intracellular proteins were analysed by proteomic analysis. The objective here was the identification of significantly higher or lower expressed proteins. Several levels of comparison were evaluated, for example between all cultures at 33 ℃ vs. 37 ℃ or between host cell lines and production cell lines.

# Chapter 3 - Materials

## 3.1 Cells

For all experiments four different cell lines were used.

*Host cell lines:*

CHO **K1PD**

CHO **dhfr$^-$**

*Recombinant cell lines:*

CHO EpoFc / III-HI5b / **3F8** / serum-free (sf)

CHO EpoFc / III-HI5b / **14F2** / sf

**CHO K1 cells** are sub-clones of the parental CHO cell line that was initiated from an ovary biopsy of an adult Chinese hamster in 1957 (DSMZ no.: ACC110) [41] – [43]. They require proline, because of the absence of the appropriate synthesis apparatus.

**CHO dhfr$^-$ cells** are dihydrofolate reductase (dhfr) negative. The dihydrofolate reductase, reduces folate to tetrahydrofolate. Glycine, thymidine and purine synthesis needs the dhfr gene and therefore deficient cells cannot grow without nucleotide precursors such as thymidine and hypoxanthine [44].

The cells express hypoxanthine-guaninephosphoribosyltransferase (HGPRT) and therefore can synthesize purines from hypoxanthine and survive in the absence of dhfr by using the salvage pathway. Methotrexate [45] is a dihydrofolate analogue that binds DHFR, thereby inhibiting its enzymatic activity and preventing cell growth by inhibiting the folic acid metabolism. Cells without the salvage pathway cannot synthesise purines anymore.

Dhfr$^-$ cells show excellent growth behaviour and when this host cell line is used for transfection and expression of recombinant proteins, a high protein yield can be achieved. Hereby the desired gene and the dhfr gene are co-transfected into the dhfr$^-$ cells. During the following cultivation, where the HT-addition is removed, only those cells survive, which contain the dhfr gene. Upon increasing methotrexate concentration, the dhfr gene and frequently the gene of interest are co-amplified [46].

Chronic MTX treatment [45] (MTX inhibits the dhfr gene) can lead to MTX resistance, which enables amplification of the DHFR gene, which results in increased DHFR mRNA and (recombinant) protein levels. Several laboratories have reported that DHFR enzyme levels are elevated by treatment of cells with MTX [47] – [50] or hydroxyurea [50] and that the stimulation by hydroxyurea is due to elevated mRNA levels [50]. The drug-induced increase in DHFR mRNA levels may result from increased transcription, decreased mRNA degradation, or accumulation of mRNA due to blockage of cells at the G1/S boundary of the cell cycle, when DHFR gene transcription is maximal [51] [52]. The stimulation of DHFR by MTX and hydroxyurea may also be the result of inhibition of DNA synthesis, resulting in re-replication of the gene (i.e., gene amplification) and subsequent elevated expression from the increased number of transcription templates [53].

The recombinant **CHO EpoFc cell lines** [36] express EpoFc, a fusion protein consisting of an erythropoietin (Epo) part and an Fc part, which is derived from a human IgG1 antibody. Erythropoietin is a glycoprotein hormone, a cytokine responsible for inducing red blood cell production in the bone marrow.
The glycosylation of proteins is influenced by the cultivation conditions, such as the concentration of ammonium inside the cell and the lactate concentration outside the cell, which both influence the intracellular pH value [37] – [39]. Because of the glycosylation variants of Epo and the many tools available for targeting the Fc part, this fusion protein is a good model to study the production of complex glycoproteins.

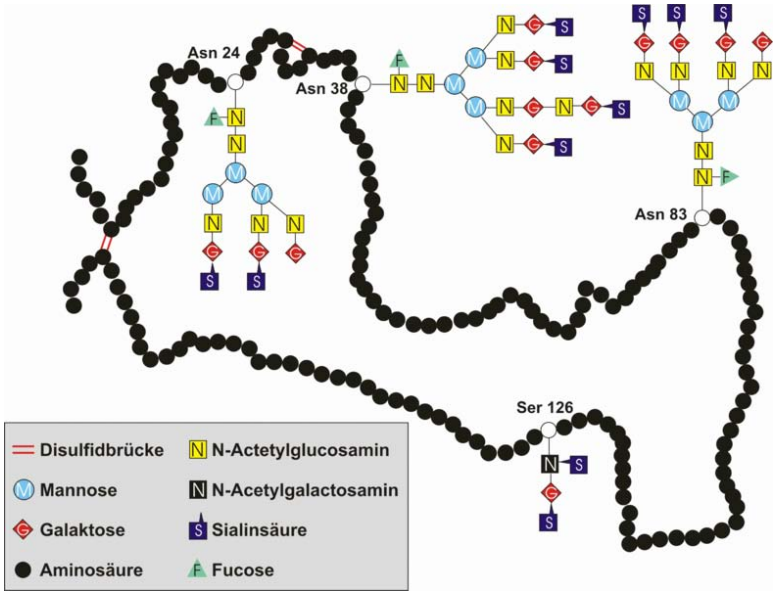


*Fig. 3.1 [40] Schematic representation of the erythropoietin molecule, showing the glycosylation sites*

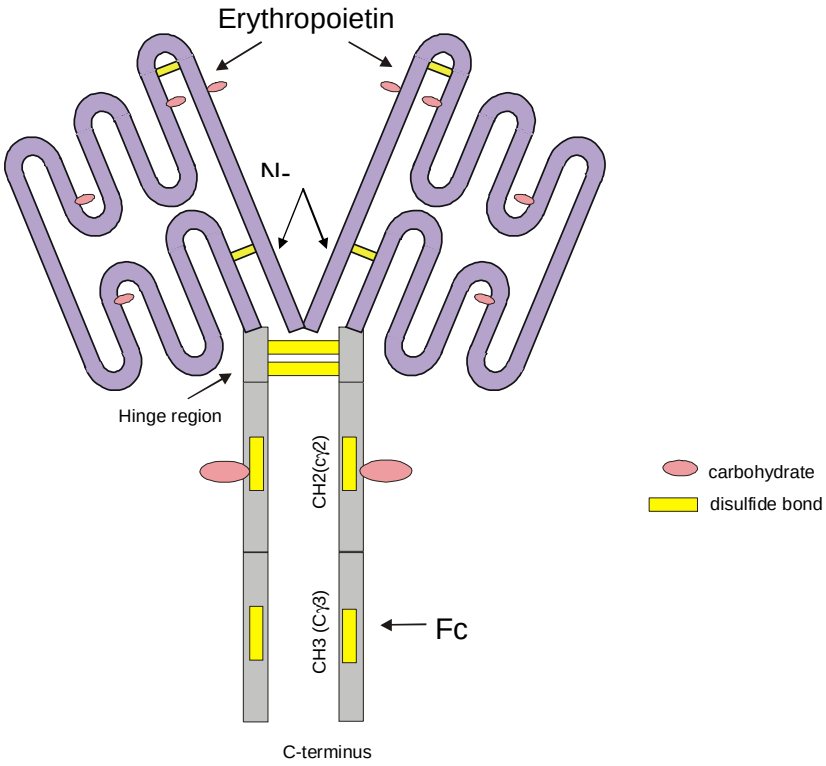


*Fig. 3.2 Schematic representation of the EpoFc recombinant protein. The approximate molecular weight of the erythropoietin amounts to 34 kDa and the approximate molecular weight of the Fc-terminus amounts to 60 kDa.*

## 3.2 Medium for the cell cultures

The quality of all reagents used during the cell cultivation was either cell culture tested or pro analysis.

### *3.2.1 Medium for K1PD cells - SMD M0012*

- DM122P3 (Sandoz) without MTX 250 ml
- 8 mM L-glutamine 8 ml of a stock solution, stored at –20 ℃

### *3.2.2 Medium for dhfr⁻ cells*

- DMEM / HAMs F12 250 ml / 250 ml (1:1)
- 0.25 % soy Peptone 10 ml
- HT addition 10 ml
- 4 mM L-glutamine 8 ml
- PF addition 1:100 L0018 5 ml
- 0.1 % Pluronic F68 5 ml

### *3.2.3 Medium for the 3F8 & 14F2 cells*

- DMEM / HAMs F12 — 250 ml / 250 ml (1:1)
- 4 mM L-glutamine in HQ water — 8 ml of a ready solution, stored at –20 ℃
- 0.25 % soy peptone — 10 ml
- 0.1 % Pluronic F68 — 5 ml
- PF addition 1:100 L0018 — 5 ml
- 0.19 µM methotrexate — 98.9 µl (pipetting of 99 µl)

### *3.2.4 About the substances*

**DMEM medium** - Dulbecco's Modified Eagle Medium is a basic nutrition solution for cell cultures. It consists of an energy and carbon source in the form of glucose, amino acids, inorganic salts, vitamins, sodium pyruvate and phenol red (pH indicator).
Sodium pyruvate functions as scavenger. It was found that sodium pyruvate was most effective, by a wide margin, in eliminating hydrogen peroxide and its toxic effects [54].

**HAM's medium** includes the 20 amino acids needed for protein synthesis in CHO, purine (hypoxanthine) and pyrimidine (thymidine) (synthesis of nucleotides), vitamins (8 of them are used as coenzymes), inorganic salts, the coenzyme lipoic acid, precursors needed for phospholipid synthesis (cholin and inositol), sodium pyruvate (as described above) and energy and carbon source in the form of glucose.

**L-glutamine** is an unstable essential amino acid at the pH of the medium and is therefore added before use.

**Soy Peptone** is added, because it contains many nutrients such as peptides and carbohydrates.

**HT addition** contains hypoxanthine and thymidine

**PF addition** – protein free (proprietary recipe) – is added to help the cells grow during the serum free cultivation.

**Pluronic** is a detergent and therefore increases the viscosity of the solution. This protects the cells from shear stress during the cultivation.

**Methotrexate** – abbreviation MTX; Replaces the more toxic aminopterin and inhibits the enzyme dihydrofolate reductase. → see CHO dhfr$^-$ cells

## 3.3 Other reagents used in the cell culture

- T/C buffer for cell count determination (Coulter Counter)
  - citrate × $H_2O$ 21 g/kg
  - Triton X 100 20 g/kg
- 70 % ethanol (disinfection)

## 3.4 Kits for the protein sample preparation

The following two Kits were purchased from Amersham Bioscience.

- 2D Clean-Up Kit; Prod. No. 80-6484-51
- 2D Quant Kit; Prod. No. 80-6483-56

## 3.5 Mass spectrometric analysis

- LC-chromatograph "Ultimate 3000"; Dionex
- ESI-QToF MS - ElectroSprayIonization - Quadrupol - Time-Of-Flight Mass-spectrometer "QToF Global Ultima"; Waters

Software used for the analysis:

- MassLynx version 4.1; Waters
- ProteinLynx Global Server version 2.1; Waters

DI Johannes Stadlmann from the group of Prof. Altmann group (BOKU, department of biochemistry) performed the mass spectrometric analysis.

## 3.6 Reagents for the gels

The quality of all reagents was pro analysis. If not stated otherwise the item in question was purchased from Sigma-Aldrich.

- CyDye DIGE Fluor minimal labelling kit – includes dye Cy2, Cy3 & Cy5 each with an amount of 5 nmol; source Amersham Bioscience
- IPG Buffer pH 4-7 L (linear); source Amersham Bioscience
- IPG Buffer pH 3-11 NL (not-linear); source Amersham Bioscience
- Immobiline Drystrip 3-7 NL (24 cm); source Amersham Bioscience
- Immobiline Drystrip 3-11 NL (24 cm); source Amersham Bioscience
- N,N dimethylformamide (DMF)
- Plus One TEMED (N,N,N',N'-tetramethylethylenediamine)
- iodoacetamide
- butanol
- glycine
- SDS
- magnesium acetate
- tris
- CHAPS
- urea
- thiourea
- acrylamide + bisacrylamide 40 %
- 70% ethanol
- undenaturated ethanol
- HCl
- ammonium persulfate (APS)
- dithiothreitol (DTT)
- distillate water

## 3.7 Equipment used

During the experiment usual lab equipment was used like centrifuges, micro centrifuges, microscopes, photometer and so on. Furthermore the following special equipment was used:

- Multisizer 3 Coulter Counter; Beckmann
- YSI 7100 Multiparameter Bioanalytical System; YSI Life Sciences
- Ettan IPGphor – Isoelectric Focusing System; Amersham Bioscience
- Ettan Dalt six – Electrophoresis System; Amersham Bioscience
- Typhoon 9400 scanner; GE Healthcare Life Sciences

- personal computer - software
    - DeCyder; Version 5.0
    - ImageQuant; Version 5.0
    - Typhoon Scanner Control software; Version 4.0
    - MS Word 2000 or higher
    - MS Excel 2000 or higher

## 3.8 Prepared solutions

### 3.8.1 Solutions for cell wash and lysis

Preparation of *Cell Wash Buffer*

| substance | add | end concentration |
|---|---|---|
| 100 mM tris / HCl | 5.0 ml | 10 mM |
| 1 M MgAc[1)] | 0.25 ml | 5 mM |

add diluted HCl to set pH = 8.0

add distilled water to get 50 ml

[1)]MgAc … magnesium acetate

Preparation of the *Standard Cell Lysis buffer – 50 ml*

| substance | add | end concentration |
|---|---|---|
| 1 M tris / HCl | 1.5 ml | 30 mM |
| thiourea | 7.61 g | 2 M |
| urea | 2.1 g | 7 M |
| CHAPS | 2 g | 4 % |

add diluted HCl to set the pH = 8.5

fill up to 50 ml with distilled water

Thiourea improved the solubilization of proteins, urea solubilized and denatured the proteins, while CHAPS was used as a detergent to solubilize hydrophobic proteins and minimize protein aggregation.

### 3.8.2 Solutions for the IEF

Preparation of the *Rehydration / Sample Buffer Stock* (stored at −20 ℃)

| | |
|---|---|
| 7 M | urea |
| 2 M | thiourea |
| 2 % | CHAPS |

Preparation of the *Rehydration / Sample Buffer Solution* (stored at −20 ℃)

| | |
|---|---|
| 1 ml | *Rehydration / Sample Buffer Stock* |
| 5 µl | IPG buffer (pH 3 - 11) / *Equilibrium buffer* |
| trace | DTT (in protocol 4 mM) - tip of a spatula |
| 10 µl | 0.1 % bromophenol blue |

### 3.8.3 Solutions for the SDS-PAGE

*Preparation of the 10x Running Buffer*

| | |
|---|---|
| 30.25 g | tris |
| 144.1 g | glycine |
| 10 g | SDS |
| add 1 l | AD |

*Preparation of the 1.5 M tris/HCl; pH = 8.8*

| | |
|---|---|
| 63.6 g | tris |
| add 400 ml | AD |

The pH was adjusted by adding diluted HCl to get the desired pH of 8.8.

This solution was sterile filtered with the Millipore Express Plus 0.22 µm filter system and stored at room temperature.

*Preparation of the 12.5 % Polyacrylamide Gel Solution*

| | |
|---|---|
| 150 ml | 1.5 M tris/HCl pH = 8.8 |
| 187.5 ml | acrylamide 40 % / bisacrylamide 1.04 % mixture |
| 260.5 ml | AD |
| 85 µl | TEMED (>99 %) |
| 1 ml | 40 % APS |

APS and TEMED were added to this solution just previous to pouring the gels. An amount of 600 ml was sufficient for 6×1.5 mm gels.

*Re-equilibration Solution (stock)* – stored at −20 ℃ (25 ml aliquot)

| | |
|---|---|
| 10 ml | 1.5 M tris/HCl ; pH = 8.8 |
| 36 g | urea |
| 34.5 ml | glycerol |
| 2 g | SDS |
| add 100 ml | AD |

3 ml of that stock solution was needed for each strip.

*Agarose Sealing Solution* (30 ml were sufficient for 6 gels.)

| | |
|---|---|
| 30 ml | 1x Running buffer |
| 0.3 g | (1 %) agarose |
| ~10 µl | bromophenol blue |

A heating stirrer or a microwave oven was used to completely dissolve the agarose.

### 3.8.4 Colloidal Coomassie staining solution

*Solution A*

| | |
|---|---|
| 16 ml | ortho-phosphoric acid |
| 768 ml | distilled water |
| 80 g | ammonium sulfate |

*Solution B*

| | |
|---|---|
| 16 ml | 5 % Coomassie brilliant blue (CCB) G250 |
| 200ml | methanol |

*Solution A* and *Solution B* were mixed immediately before use. Just before the gels were put into the container with this staining solution, 200 ml methanol were added by pouring it slowly into the container.

The final concentrations were 0.08 % CBB 250, 1.6 % o-phosphoric acid, 8 % ammonium sulfate and 20 % methanol.

# Chapter 4 - Methods

## 4.1 Cell revitalisation

3F8, 14F2 and K1PD cells were revitalised prior to cultivation. The following procedures were performed.

The cells in the glass ampoules were thawed by hand warmth after their recovery from the nitrogen storage tanks. The cell suspension was pipetted into 8 ml cold medium and centrifuged at 180 to 200×g for 10 minutes. The supernatant was removed and the pellet was resuspended in 10 ml medium, mixed and transferred into the prepared T-flask. This procedure removed the cryo agents, which would be harmful to the cells under cultivation conditions. The cells were then incubated at 37 ℃ as described below.

## 4.2 Cell cultivation process

During all incubations of T-flasks the caps were opened a half turn immediately before they were put into the incubator. By doing so the atmosphere inside the T-flask could equilibrate with the incubator atmosphere. There was no pre-incubation during the passaging of the cells previous to the batch start.

*100 ml spinners were* used for all batch cultures. There was one incubator with a set temperature of 33 ℃ and an incubation chamber at 37 ℃. Both were equipped with spinner platforms. $CO_2$ was transferred into the spinner after the addition of the medium. This was done inside a laminar flow hood by injecting the $CO_2$ through an autoclaved Millipore MT GR 05000 0.2 µm syringe filter with a 60 ml syringe. The spinners were incubated under cultivation conditions for at least 1 hour, to reach the appropriate temperature before the cells were added and the batch cultivation could start.

*3F8 and 14F2:*

The cultivation of the cells was started with 20 ml of cell suspension (undefined cell concentration). Three days later 10 ml of medium was added to the cell suspensions.

On the fifth day the cell suspensions were transferred into 125 ml spinner flasks using the following procedure. 40 ml medium were propounded in a 125 ml spinner and $CO_2$ was injected by a 60 ml syringe through a filter as described above. The spinners were placed onto a spinner platform at 37 ℃ and incubated for a few hours. 20 ml of the cell suspension was pipetted into the respective spinner, with a resulting 1:3 dilution. These two spinners were incubated on spinner platforms at 37 ℃.

For security measure, backup cultures were cultivated parallel to the spinner cultures in T-flasks with a volume of 10 ml at 37 °C and were always passaged 1:5.

Four days later cell count and viability of the spinner cultures were determined for the first time.

| | total cell concentration | viability |
|---|---|---|
| • 3F8 | $1.57\times10^6$ cells/ml | 94.6 % |
| • 14F2 | $1.21\times10^6$ cells/ml | 95.5 % |

With this information the cell cultures were passaged to a starting cell concentration of $2\times10^5$ cell/ml. → see passaging of a spinner (4.2.1).

*K1PD*

K1PD cells were revitalised as described above and directly cultivated in spinner flasks. A pre-dilution was performed to allow the cells to adapt to the new conditions. The starting volume of medium amounted to 35 ml plus 5 ml of the cell suspension.

Three days later the total cell concentration and the viability of all three cultures were determined.

| | total cell concentration | viability |
|---|---|---|
| • 3F8 | $3.9\times10^5$ cells/ml | 97.3 % |
| • 14F2 | $4.7\times10^5$ cells/ml | 98.9 % |
| • K1PD | $5.8\times10^5$ cells/ml | 96.1 % |

The new passage was calculated as described below to obtain a resulting cell concentration of $1.5\times10^5$ cells/ml and a volume of 80 ml.

$c = V_{pip} \bullet CC \bullet via / V_{ges}$

| | | |
|---|---|---|
| $V_{pip}$ | … | pipetting volume [ml] |
| CC | … | determined cell concentration [cells/ml] |
| via | … | current viability [] |
| $V_{ges}$ | … | whole volume [ml] |

Three days later the cold shock experiment started. This time point was designated as day one of the experiment. On that day the cell suspension from every cell line was split into two spinners each with a starting volume of 100 ml and a cell concentration of $1 \times 10^5$ cells/ml. One culture was incubated at 33 °C, the other at 37 °C, respectively.

*dhfr* $^-$

The dhfr $^-$ cell line was included in the experiment upon receiving the cells from a culture derived from another experiment in the lab.

*Sampling*

Samples were taken daily from each culture, beginning at the day of inoculation. For the purpose of sampling all spinners were transferred from one of the incubation temperature ranges into a laminar flow hood simultaneously. Thus the cultures would not be stressed too much, but in the same way (e.g. time), while standing out at room temperature.

4 ml of each cell suspension were taken and transferred into a separate 10 ml tube for further analyses.

- 1 ml of that sample was used for the determination of the approximate cell count and the viability by the haemocytometer method. → see 4.2.2

- The remaining 3 ml of the cell suspension were centrifuged at 381×g for 10 minutes at room temperature (RT).

- 1 ml supernatant of every sample was transferred into a micro-centrifuge tube and stored at −20 ℃ for later analysis of the glucose-, lactate-, glutamine- and glutamate concentration by the YSI. → see 4.2.4
- The pellet was used for cell count determination using the Coulter Counter. → see 4.2.3

*Samples for proteomic analyses*

Two time points of interest were defined by consulting the growth curve. Because of the limited volume of the batch cultures, the samples for the proteomic analyses were taken only at these time points.

The first time point was determined to be well in the exponential growth phase of the cultures. This was achieved by evaluating the results of the specific growth rate of the cells and defining the cell concentration of $5\times10^5$ cells/ml as the appropriate value. This was the case for

- K1PD at day three,
- 3F8 and 14F2 at day four and
- $dhfr^-$ at day five.

On that time point $1\times10^7$ cells were taken from the spinners.

The second time point of the cell cultivation for each cell line was at the end of the batch, when the viability of one of the two cultures was around 80 %. Here all of the remaining cell suspension was used for the proteomic analysis. → see 5. Results

- 3F8 - total cell concentration -
  - 33 ℃ $1.38\times10^6$ cells/ml volume of 21 ml
  - 37 ℃ $0.92\times10^6$ cells/ml volume of 33 ml
- 14F2
  - 33 ℃ $2.12\times10^6$ cells/ml volume of 35 ml
  - 37 ℃ $1.52\times10^6$ cells/ml volume of 41 ml

- K1PD
  - 33 ℃ $2.20\times10^{6}$ cells/ml volume of 40 ml
  - 37 ℃ $1.75\times10^{6}$ cells/ml volume of 40 ml

- $dhfr^{-}$
  - 33 ℃ $1.02\times10^{6}$ cells/ml volume of 38 ml
  - 37 ℃ $0.92\times10^{6}$ cells/ml volume of 47 ml

### *4.2.1 Passaging of a cell suspension cultivated in a spinner:*

An empty T-flask was prepared and 80 ml were marked, when passaging a 100 ml spinner. Then the pipetting volume was calculated to get a set starting cell concentration.

$$V_{pip} = V_t \bullet c_t / ( CC \bullet via )$$

| | | |
|---|---|---|
| $V_{pip}$ | … | pipetting volume [ml] |
| $V_t$ | …. | target volume [ml] |
| $c_t$ | …. | target concentration [cells/ml] |
| CC | … | determined cell concentration [cells/ml] |
| via | … | current viability [] |

$V_{pip}$ was pipetted into the prepared T-flask and the pre-incubated medium was added to this cell suspension up to the marked 80 ml of volume. The remaining suspension inside the spinner was discarded. Afterwards, the cell suspension was transferred from the T-flask back into the spinner and the incubation upon a spinner platform (50 U/min) at 37 ℃ was continued.

### *4.2.2 Haemocytometer*

The haemocytometer method was performed by means of a microscopic counting chamber like Neubauer or Türcker-Bürck. 70 % ethanol was used for cleaning the carrier, to moisten the two bridges and to press and rub the cover slip upon these till the Newton rings could be seen. Previous to counting with a microscope, the 1 ml samples were vital stained by adding 200 µl trypan blue (mixed by pipetting).

This should be done just before the counting was started, because trypan blue is toxic for the cells and therefore could alter the result of the viability determination after a certain time.
The determination of the total cell count was the first step. Two of the four large squares of each chamber were counted.

$$c = CC / q \bullet d$$

| | | |
|---|---|---|
| c | … | cell concentration [cell/ml] |
| CC | … | Σ of counted cells [cells] |
| q | … | number of large squares that were counted |
| d | … | dilution – volume under the cover slip [$ml^{-1}$] |

The second step was the determination of the viability by counting the Trypan blue stained cells performed in the same ways as in the first step. These blue stained cells were assumed to be dead, because the dye can only pass through damaged cell membranes.

$$viab = CC / (CC + r) \bullet 100$$

| | | |
|---|---|---|
| viab | … | viability |
| CC | … | Σ of counted cells [cells] (step one) |
| r | … | Σ of dead cells |

### *4.2.3 Determination of the cell concentration*

For that purpose the Multisizer III Coulter Counter from Beckmann was used. It detects and counts the nuclei of lysed cells, which pass with the nuclei suspension and buffer between two electrodes and thereby change the conductivity. The lysis of cells was achieved by adding 1 ml T/C buffer to the pellet of 3 ml cell suspension (3 → 1) and incubation for at least 2 hours to over night at room temperature.
The procedure was as follows: After the sample was taken and 1 ml removed for the haemocytometer, the remaining 3 ml of the cell suspension were centrifuged at

381×g for 10 minutes at RT. 1 ml of the supernatant was transferred into an Eppendorf tube and stored at –20 ℃ for later analy sis → see 4.2.4
1 ml of the T/C buffer (Citrate×$H_2O$/Triton X 100) was added to the pellet (the cells) and incubated for 2 to 4 hours. Over night incubation was also possible at room temperature. The cell suspension was vortexed, 50 µl of the cell suspension was transferred into a coulter measure beaker and 9 ml coulter isotonic (physiologic solution) was added and measured.

The result consisted of a size distribution of the nuclei showing two connected peaks. The counts in front of these two peaks represented cell debris and had to be removed from the result. The left big peak represented the nuclei, which were in the $G_0$ and $G_1$ phase, while the right peak represented the nuclei in the $G_2$ phase. Additional small peaks to the right of the two main ones were assumed to be unlysed cells and were counted as well. The two markers, the first at the beginning of the first large peak and the second at the end to the right, were set to remove the cell debris from the result.

Calculation of the cell concentration:

$$c = x \bullet 2^{1)} \bullet (V_{phy} + V_s) / V_s / V_p$$

[1)] 0.5 ml were measured by the Coulter Counter during one analysis. The factor of 2 was multiplied to get the resulting concentration per 1 ml.

| | | |
|---|---|---|
| c | … | cell concentration [cells/ml] |
| x | … | average value of the two counts |
| $V_{phy}$ | … | volume of the physiologic solution [ml] |
| $V_s$ | … | volume of the sample used for the Coulter [ml] |
| $V_p$ | … | volume of the cell suspension analysed [ml] |

### *4.2.4 Determination of substrates and metabolites in the supernatant*

The YSI 7100 MBS, a multiparameter bioanalytic system, was used for the analysis of the concentrations of *glucose, glutamate*, *glutamine* and *lactate* in the supernatant samples stored at −20 ℃. This instrume nt consists of the biosensors, which are linked to a transductor (signal converter) and an electrical amplifier.

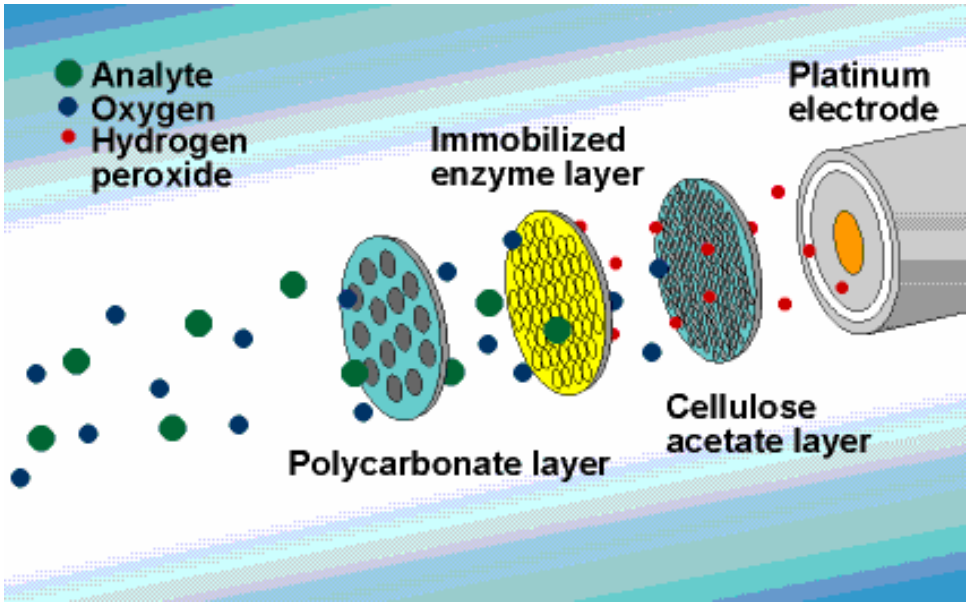


*Fig. 4.1 [55] Composition of the biosensor – The YSI membranes consists of three layers. The first layer is composed of porous polycarbonate, which restricts the diffusion of the substrate into the second layer, which consists of the immobilised enzymes. That prevents the reaction from becoming enzyme limited. The third layer consists of cellulose acetate and enables only small molecules, such as hydrogen peroxide, to reach the electrode. That layer blocks out many electrochemically-active compounds that would interfere with the measurement.*

The biosensor [55] consists of enzymes, which are specific for the substrate of interest, immobilised between two other membrane layers (Fig. 4.1). Indifferent of the specific substrate of interest, if the analyte binds to this enzyme, it will always be oxidised and one of the reaction products is always hydrogen peroxide. $H_2O_2$ transits the third layer and comes into contact with a platinum electrode, where it is oxidised. The resulting current is proportional to the concentration of the hydrogen peroxide and further on to the concentration of the specific substrate, as described later on.

*Measuring principles of the particular analytes* [55]

1) The *D-glucose* is oxidised in the presence of the immobilised *glucose oxidase*, which produces hydrogen peroxide and glucono-lactone.

*glucose oxidase*

$$\text{D-glucose} + O_2 \rightarrow \text{D-glucono-ð-lactone} + H_2O_2$$

2) *L-glutamate and L-glutamine* are co-detected by one membrane. Two enzymes are immobilised on the glutamine membrane, which are the *glutaminase* and *L-glutamate oxidase*. L-glutamine is oxidised in the presence of glutaminase and the reaction produces $NH_3$ and L-glutamate.

*glutaminase*

$$\text{L-glutamine} + O_2 \rightarrow \text{L- glutamate} + NH_3$$

After that, the total *L-glutamate*, which is the glutamate generated by the enzymatic reaction and from the solution, are oxidised, catalysed by *L-glutamate oxidase*, which results into a produced hydrogen peroxide and the combined result of *L-glutamate* - and *L-glutamine* concentration.

*L-glutamate oxidase*

$$\text{L-glutamate} + O_2 \rightarrow H_2O_2 + \alpha\text{-ketoglutarate} + NH_3$$

Parallel to that, *L-glutamate* is detected with the immobilised *L-glutamate oxidase* by another electrode. The difference of this resulting *L-glutamate* concentration and the combined result of the co-detected *L-glutamate and L-glutamine* gives the *L-glutamine* concentration.

3) *L-lactate* is oxidised in the presence of *lactate oxidase*, which results in the production of hydrogen peroxide and pyruvate.

*L-lactate oxidase*

$$\text{L-lactate} + O_2 \rightarrow H_2O_2 + \text{pyruvate}$$

4) All the resulting signals detected are derived from hydrogen peroxide. $H_2O_2$ is oxidised at the platinum anode and triggers the release of electrons. The electron flow is proportional to the $H_2O_2$ concentration and, therefore, to the concentration of the substrate.

*platinum anode*

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

### *4.2.5 Calculations with the cell cultivation analytic results*

The following formulas were used in the calculations with the analytic results of the cell cultivation to create the diagrams in chapter 5.
The index ‘n’ represents the value from the time of sampling, while ‘n-1’ means that the value is from the day before day ‘n’.

c … cell concentration [cells / ml]
t … time [d]
$v_i$ … viable cell integral
index ‘v’ … viable cells

$\mu_{viable}$ [$d^{-1}$] … specific growth rate of the viable cells

$$\mu_{viable} = (\ln c_{v\,n} - \ln c_{v\,n-1}) / \Delta t$$

viable cell integral [cells d / ml]

$$\text{viable cell integral} = (c_{v\,n} - c_{v\,n-1}) / \mu_{viable}$$

total cell integral [cells d / ml]

$$\text{total cell integral} = (c_{total\,n} - c_{total\,n-1}) / \mu_{total}$$

$\mu_{total}$ [$d^{-1}$] … total specific growth rate

$$\mu_{total} = (\ln c_{total\,n} - \ln c_{total\,n-1}) / \Delta t$$

$q_{glucose}$ [ng / cell d] … uptake rate of glucose

$$q_{glucose} = (c_{glucose\ n-1} - c_{glucose\ n}) / v_{i\ n} \times 1000$$

$q_{lactate}$ [ng / cell d] … production rate of lactate

$$q_{lactate} = (c_{lactate\ n} - c_{lactate\ n-1}) / v_{i\ n} \times 1000$$

$q_{glutamine}$ [ng / cell d] … uptake rate of glutamine

$$q_{glutamine} = (c_{glutamine\ n-1} - c_{glutamine\ n}) / v_{i\ n} \times 1000$$

## 4.3 Cell wash and lysis

The cell suspension was centrifuged at 190×g for 10 minutes at room temperature. The pellet was washed with a volume of the *Cell Wash Buffer,* that equalled half of the centrifuged suspension volume. That was repeated three times. The cell suspension was transferred into 1.5 ml microcentrifuge tubes (Eppendorf) and centrifuged at 190×g in an Eppendorf Minispin (Microcentrifuge). The pellet (cells) was resuspended in *Standard Cell Lysis buffer* with a volume ratio of 1 : 2 (pellet : buffer). This suspension was vortexed and incubated for 15 minutes at room temperature. Afterwards it was centrifuged at > 12000×g for 30 minutes at room temperature. The supernatant containing the proteins was transferred into new 1.5 microcentrifuge tubes and stored at −80 ℃.

## 4.4 Protein Clean-up

The 2D Clean-up Kit from Amersham Bioscience was used for cleaning of the protein samples. This product is especially designed to prepare samples for 2D gel electrophoreses. This clean-up kit removes lipids, nucleic acids, phenols and charged detergents, which would interfere with the separation of the proteins by electrophoresis.

Procedure A of the product manual was used, because the volume of the samples per microcentrifuge tube was < 100 µl. For the detailed step description the manual can be acquired from the manufacturer homepage of Amersham

Bioscience [56]. During the whole process the samples remained on ice. The microcentrifuge steps were performed with an Eppendorf Centrifuge 5415R with a set temperature of 4 ℃. The protein samples were r esuspended in *Standard Cell Lysis buffer* and stored at −80 ℃.

## 4.5 Protein quantification

For the determination of the concentration of proteins in the samples, the 2D Quant Kit form Amersham Bioscience was chosen.

This assay works with copper ions, which bind to the proteins in the sample. During the procedure, the proteins are precipitated and resolved in a copper solution. The unbound copper ions which remain in the solution are measured through absorbance at 480 nm. The intensity of the colour is inversely proportional to the protein concentration.

Results of this protocol are linear for protein concentrations in a range of 0 to 50 µg. To quantify the proteins a standard curve has to be created.

The procedure for Ettan DIGE was performed. For the detailed step description the manual can be acquired from the manufacturer's homepage [56].

## 4.6 Principle of 2D DIGE

Ettan DIGE [35] (difference gel electrophoresis) is a method for pre-labelled protein samples. During the process the samples are separated in two dimensions, followed by an analysis of differences in the resulting gel images with the DeCyder software.

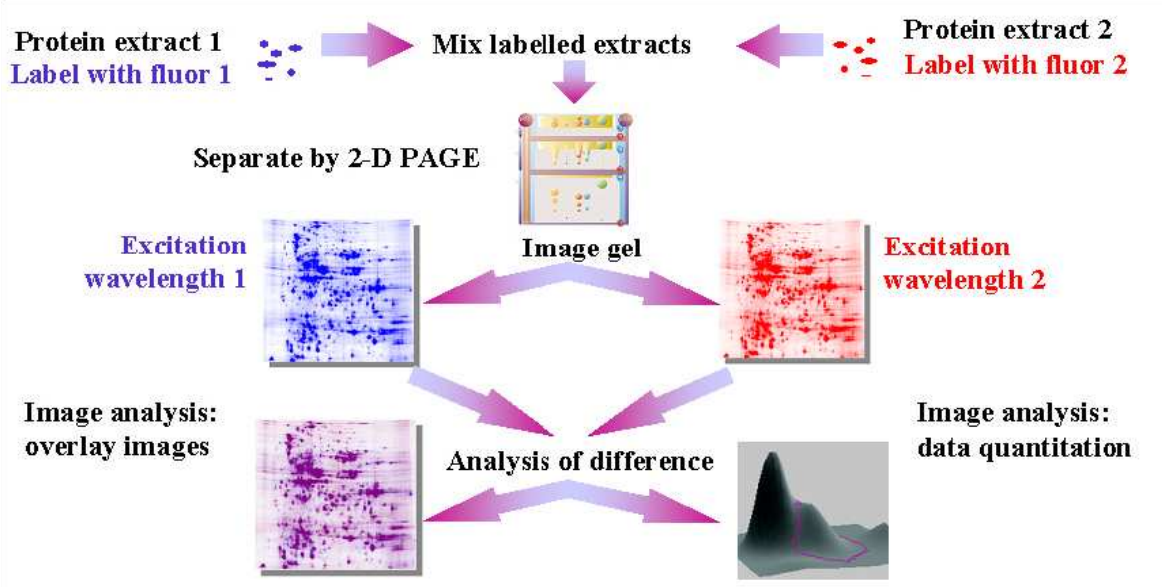


*Fig. 4.2 [56] Schematic structure of the Ettan DIGE method, starting with the extraction of the proteins, their labelling and the two dimensional separation. The applied CyDyes had a different excitation wavelength and the resulting images were then analysed by the DeCyder software.*

The protein samples were labelled by CyDyes, which had different excitation wavelengths. For the accurate wavelengths consult 4.10.

In the experiment an internal pooled standard was used, which consisted of equal parts from each sample. It was included in all gels to make it possible to compare them with each other and ensuring that all spots could be matched as the standard contained all proteins.

The labelled samples were applied onto an Immobiline Dry strip for the first dimension of separation, the isoelectric focusing (IEF). By that method the proteins are separated according to their isoelectric point (pI). At a pH, which represents its pI, the protein has no net charge and therefore stops migrating within an electric field. → *see 4.8*

The second dimension of separation was performed applying SDS PAGE. SDS PAGE means sodium dodecyl sulfate – polyacrylamide gel electrophoresis. During this separation the polypeptides are distributed according to their molecular weight. → *see 4.9*

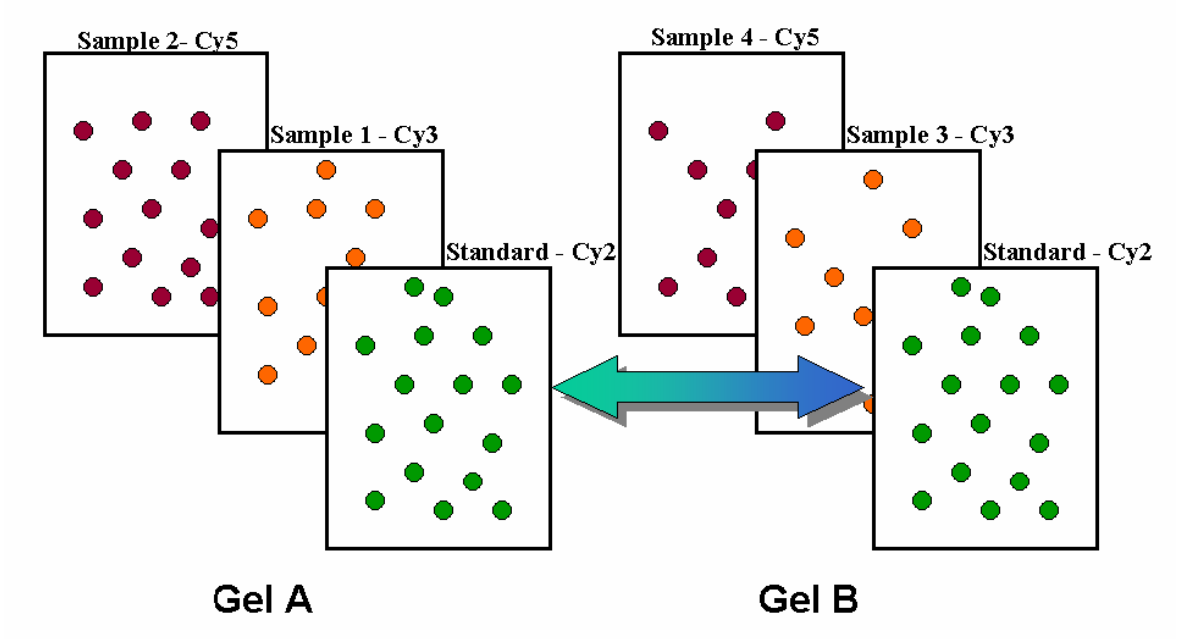


*Fig. 4.3 [56] Principle of gel matching by internal standard.*

When the gel runs were completed and the gels were scanned, the master gel was defined (gel with the best internal standard image) and the internal standards of all other gels were matched to the internal standard of the master gel. The intensities of the spots, which were equivalent to the amount of protein, were analysed and compared to the spots of the internal standard and the statistical difference was determined.
For detailed information see 4.10 and 4.11.

## 4.7 Protein minimal labelling by CyDye

The CyDye DIGE Fluor minimal labelling kit from Amersham Bioscience was used to label the protein samples. It included Cy2, Cy3 and Cy5 with an amount of 5 nmol each.
These three fluorochromes are so designed as to have the same diffusion abilities on the gel. The reactive group of the CyDyes are NHS ester groups, which are bound by an amide linker to the fluorochrome. This reactive group could bind covalently to the lysine residues of proteins (amide linkage). Minimal labelling means that only a small part of the total proteins of the sample was labelled. Approximately 1 to 2 % of the lysine residues were bound by the fluorescent markers.

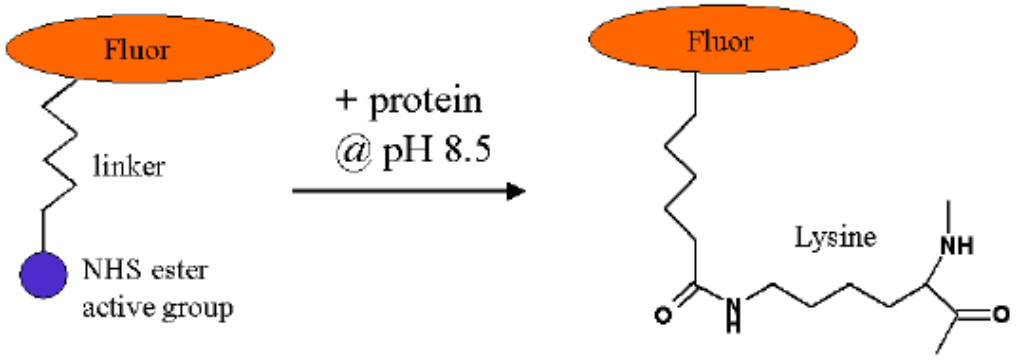


*Fig. 4.4 [56] Binding of CyDye fluorochromes to the lysine residue of a protein with the NHS ester active group.*

Important for the binding efficiency of the CyDyes to the proteins is the pH value of the sample buffer (*Standard Cell Lysis buffer*). It has to be higher than 8.0, whereby the optimum is a pH of 8.5. If the pH is lower than that, it can be compensated by adding a sample buffer with a higher pH value. A spot check was conducted with the samples to confirm that the pH was within range.

Before the delivered CyDyes can be used, they have to be reconstituted with dimethylformamide (DMF). It is important that the DMF is anhydrous and therefore as little exposed to the air (humidity) as possible. 5 µl of DMF were added to achieve a *1 mM CyDye Stock Solution*. The CyDyes, before and after reconstitution, had to be stored at −20 °C.

400 pmol of CyDye were used to label 50 µg of protein. The labelling could be done either by preparing a working solution first, for better handling, or by pipetting 400 pmol of the CyDye stock solutions directly to the samples.

For each sample and the internal pooled standard a microcentrifuge tube, containing the amount of 50 µg protein, was prepared. The internal pooled standard consisted of an equivalent part from all the samples and had an amount of 50 µg protein per sample and gel.

The labelling reaction was performed after adding the appropriate CyDye to the sample and incubation in the dark for 30 minutes. The reaction was stopped by adding 1 µl of a *10 mM Lysine* solution. After labelling the samples could be stored for up to three months at −80 °C.

## 4.8 IEF – 1st Dimension

After labelling, the samples were split into 3 groups, which where finished sequentially.

- 1 × the samples of time point (1)
- 2 × the samples of time point (2)

The Ettan IPGphor and the Immobiline Dry strips, with an immobilized pH gradient of 3 – 11, are products from Amersham Bioscience and were used during this procedure.

### 4.8.1 Rehydration of the IEF gel strips - procedure

A closable rehydration strip holder from Amersham Bioscience was used for this procedure. The spirit level of the strip holder was centred to ensure a homogeneous rehydration of the IPG Dry Strip. The *Rehydration Buffer Solution* (→ see 3.8.2 for the preparation) was prepared from the stock. The Dry strips were transferred into the strip holder with the gel side *facing downward*. So as not to harm the Dry strip gel surface, the protection foil was left for the time being. About 500 µl of that solution was pipetted onto each strip and spread by levelling the case. The Dry strips were heaved on one side by a pincer to get a good spread without bubbles. A moistened paper was put over the strip holder between cover and body. After 3 to 4 hours the *Rehydration Buffer Solution* was spread again. After a minimum of 8 hours, but not longer than over night incubation, a homogeneous rehydration of the Dry strip was ensured and the removal of the foil from the gel surface was done while transferring it to the ceramic tray of the IPGphor.

### 4.8.2 IEF – first dimension run

The ceramic tray from the IPGphor was used to place the Dry strips into it. The tray had to be clean and dry. A special detergent from Amersham Bioscience was used for that purpose so as not to damage the sensitive surface. The ceramic tray was positioned onto the Ettan IPGphor platform. The pointed end had contact to the anodic electrode area and the blunt end to the cathode electrode area of the IPGphor platform. The Dry strips were recovered from the rehydration strip holder, the foil was removed and the strips were transferred to the ceramic tray, with the

gel side *facing up*. The anodic (+, pointed) end of the Dry strip was directed toward the pointed end of the strip holder. The strip was centred inside the tray with a pincer. Two pre-cut papers were moistened with 150 µl distilled water per pad or soaked with distilled water and dabbed on a paper towel. These moistened paper strips were put halfway onto the Dry strips. The 2 electrodes were assembled with their copper wire onto the paper strips for contact. The contact of the metal needles to the surface of the copper conduct was checked.

The *Cup loading method* was used, which means that the samples were applied by a cup holder onto the Dry strips. The sample cups were positioned over the Dry strip as close to the *anodic electrode (+ pole)* as possible and pressed down, not too firm as the cup would cut through the gel of the Dry strip. The 4 feet of the cup were pressed into the tray line by a pincer or the plug (standard accessory of the IPGphor).

The whole ceramic tray and the sample cups were layered over with paraffin as cover fluid. 50 µg (100 µl - 400 µl) of CyDye labelled protein samples were pipetted into the cups as deeply as possible. The strip number was noted, the IPGphor cover was put over the device to protect the CyDyes from light during the run and the IEF was started with the following settings.

*IEF settings*

The current was set to 20 µA per strip (less than 50 µA per strip) and the whole runs had total volt-hours of 55 – 68 kVh. Total volt-hours were calculated according to the selected modus.

| time | voltage | modus |
|---|---|---|
| 6 h | 500 V | step 'n hold |
| 3 h | 1000 V | Gradient |
| 8 h | 8000 V | Gradient |
| → | ~ 68 kVh | resulting total volt-hours |

The low initial voltage minimized the sample aggregation and allowed the parallel separation of samples with different salt concentrations. When the voltage was lower than the set voltage during the steps of the run, the run was prolonged with 8000 V, step ‘n hold, till the intended total volt-hours were reached.

After the run the strips were either further processed or stored at −80 ℃.

## 4.9 SDS PAGE – 2nd Dimension run

### 4.9.1 Preparations

Preparation of the 10x Running Buffer, the 1.5 M tris/HCl; pH = 8.8 and the 12.5 % Polyacrylamide Gel Solution was conducted. → see 3.8.3 for details

*Preparation of the gels*

A gel caster for six gels was prepared. To get better results, always all six gels were poured and the four best, the gels with the most straight gel top, were chosen for the SDS run. All glasses and plastic inserts had to be cleaned with undenaturated ethanol, because denaturated ethanol would fluorescence during scanning.

The backside glass was positioned at an edge of the caster and the front glass was put onto the plastic isolation of the backside glass. Onto that followed a plastic insert and so on. Electrode paper could be put between the glass plates for labelling.

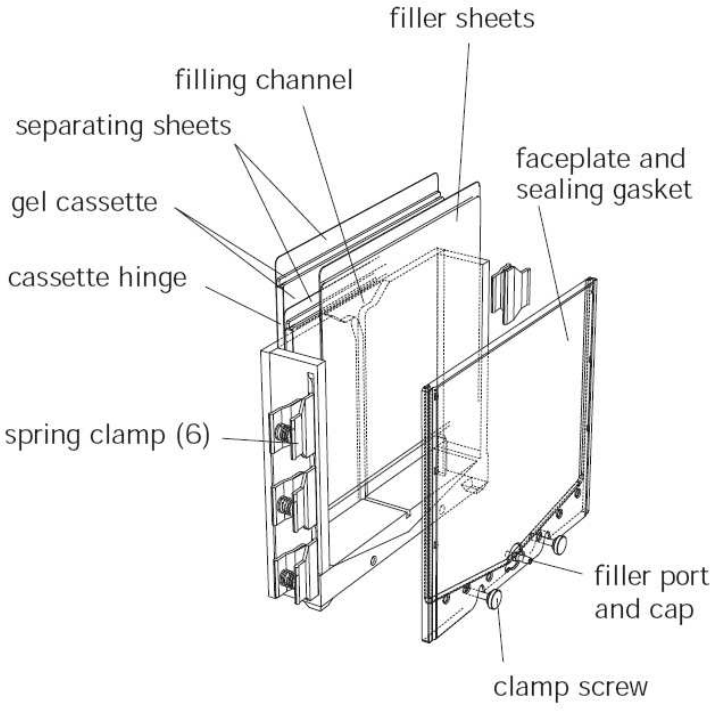


*Fig. 4.5 [56] Gel caster with the capacity for six gels.*

The *12.5 % Polyacrylamide Gel Solution* was poured into the gel caster as fast as possible, before it could polymerise. A layer of butanol was poured onto the gel tops to get them even. Around 8 hours later distilled water was poured over the

gels top, so that there was no butanol left on the gels. The gels were left for further polymerisation inside the gel caster over night. The following day the gels were removed from the gel caster for immediate use or stored at 4 ℃.

### 4.9.2 Re-equilibration of the Dry strips

Before the SDS separation could be started, the Immobiline Dry strips had to be re-equilibrated and the *Re-equilibration Solution* and the *Agarose Sealing Solution* were prepared (→ see 3.8.3 for details).

For re-equilibration the Dry strips were put into a strip holder with the gel side facing up. For the first part of the re-equilibration a *Re-equilibration solution* with *0.5 % DTT* (reductant) was prepared and 3 ml were pipetted onto every strip. The solution was distributed by tipping the strip. After 15 minutes of incubation, inside a fume hood, the solution was removed and 3 ml of the second solution, the *Re-equilibration solution with 4.5 % iodoacetamide*, was pipetted onto each strip. After another 15 minutes of incubation the solution was removed and the strips were placed onto the SDS gels by using a plastic pusher or a spatula, avoiding bubble inclusion.

The first re-equilibration step with DTT saturated the IPG strip with the SDS buffer system required for the 2D separation. The second step replaced the reductant with iodoacetamide, which alkalised thiol groups on proteins and prevented their re-oxidation during electrophoresis.

### 4.9.3 The second dimension

The anode of the Dry strip was on the left side and the gel side was facing to the front glass. The setup was sealed with the *Agarose Sealing Solution.* If a protein marker was needed, it was positioned at the left or the right side of the gel. The marker was applied onto the gel by creating a pocket with a space holder put onto the strip before the set-up was sealed with the *Agarose Sealing Solution*. The protein marker was pipetted into the so formed pocket.
The gels were placed into the chamber in between the blanks, when there were less than 6 gels. Blank cassette inserts were used for the unoccupied slots.

4 l of *1x Running Buffer (anode buffer)*, diluted from the *10x Running Buffer,* were filled into the chamber till the fluid level was at the first mark signed onto the Ettan Dalt six. The plastic sealing of the separation insert of the cassette carrier had been moistened by SDS electrophoresis buffer previously, so that it sealed off properly. The separation insert was transferred into the upper part of the chamber and filled with 1 l to 1.5 l of *2x Running Buffer (cathode buffer)* till the fluid level was within the marks on the apparatus. *1x Running Buffer* was filled into the outer chamber up to the same height as the *2x Running Buffer* in the separation insert.

In the beginning the second dimension separation settings were 2.5 W/gel for 30 minutes. After that the performance was set to 100 W overall for approximately 4 - 5 hours. The separation was ended just before the blue front (bromophenol blue) reached the end of the gel.

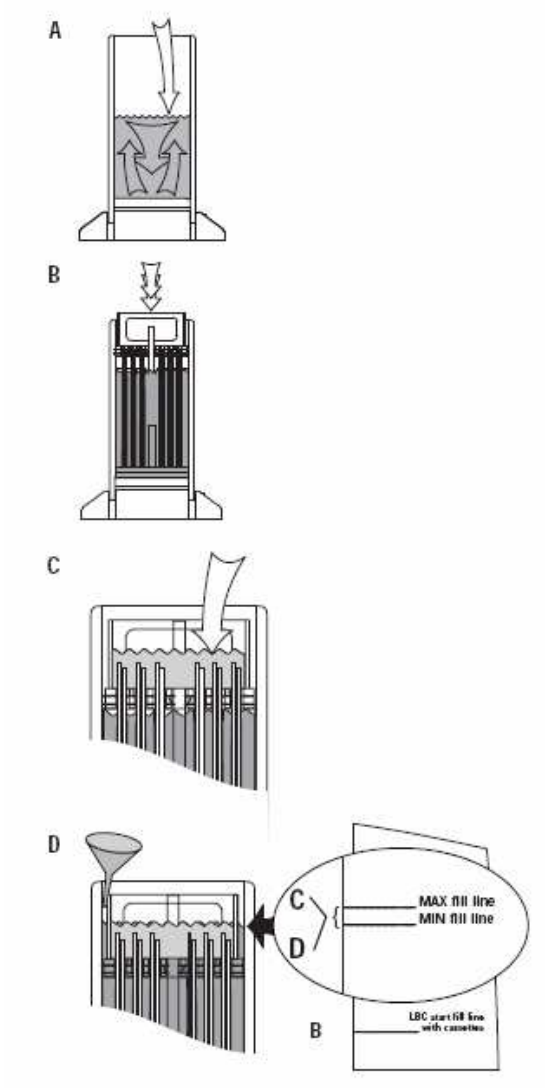


*Fig. 4.6 [56] Preparation of the Ettan Dalt six apparatus.* ***A*** *The 1x Running Buffer / anode buffer was poured into the container up to the first fill line (B in picture D).* ***B*** *The gels in their holder and the separation insert were introduced into the apparatus.* ***C*** *The 2x Running Buffer / cathode buffer was poured into the separation insert.* ***D*** *Both, the 1x Running Buffer and the 2x Running Buffer, were filled up to the marked fill lines, between Max and Min (C and D marked).*

## 4.10 Gel scanning and storing

After the SDS separation the *Typhoon 9400 scanner* was pre-heated for 30 minutes. The glass plates of the gels were cleaned by undenaturated ethanol and scanned. A pre-scan was performed with a resolution of 1000 µm (microns), which means that one pixel had the edge length of 1 mm, to see if the intensity had to be regulated. Red areas indicated that the intensity of the laser was too high and had to be lowered. When the intensity was ok, a scan at a resolution of 100 µm was performed.

The emission filter settings were as follows.

| | | | excitation wavelength |
|---|---|---|---|
| • Cy2 | filter 520 | blue2 | λ = 488 nm |
| • Cy3 | filter 580 | green | λ = 532 nm |
| • Cy5 | filter 670 | red | λ = 633 nm |

The starting PMT (intensity of the laser) value amounted to 600 V, and was lowered when red areas were present in the pre-scan.

To prevent further diffusion of the proteins after scanning, the gels were transferred from the glass plates into a container and fixed using a *Storing Solution*, which consisted of 10 % acetic acid, 40 % methanol in distilled water. The gels were incubated on a shaker for at least 1 hour. Afterwards the container was transferred into a 4 ℃ storage room till the s oftware analysis was finished and the spots of interest had to be picked.

## 4.11 Gel analysis by DeCyder

Before the analysis of the gel files (*.gel) were started with the DeCyder software, the image files were prepared by the *Image Quant software*. The areas of interest were determined and new image files were created, which were analysed by the DeCyder software later.

The *DeCyder software* is a differential analysis software. With that software the following processes were performed: spot detection, background subtraction, in-

gel normalization (internal standard), gel artefact removal, gel to gel matching and statistical analysis.

Three modules of the DeCyder were used:

- Batch Processor
- BVA – biological variation analysis
- DIA – differential in-gel analysis

The *Batch Processor* was used for the automated image spot detection and matching of all the gels in the experiment. All gel images were integrated into the batch list. The gel image with the best quality, which was from the gel D2G2, the image of CHO EpoFc 14F2(1), was chosen as the *master gel*. The exclusions filter option was not used, because no adequate settings could be found for these gels, which would remove the dust particles and not the protein spots as well. Two groups were determined. E.g. In some analyses “Group 1” consisted of the images of the 33 ℃ samples and “Group 2” of the 37 ℃ sam ple images. The batch process was started and a *.bva file was generated, which was further worked on by the *BVA* module.

The *BVA* was used to correct the spot matching of the batch processors *.bva file and to perform statistical analysis of the spots. At first a visual spot exclusion was performed in the match table. Hereby all spots on the master gel were analysed and the spots were either defined as proteins or else removed. Afterwards big spots were marked in the landmark mode on the internal standard image of the master gel and on all other internal standards of the gels in the experiment. That enables the software to link the positions of these spots in the internal standard of all gels to the position of the same spot on the master gel. By doing so a better matching rate of the spots was obtained during the matching of all gels. By using the protein table, the spots were sorted by the average ratio of abundance and the search for proteins of interest. The proteins of interest were defined by a students t-test value of $< 0.05$, which means, that there is a 5 % possibility that the resulting value is random, and an average ratio of the compared data of $< -2$ or $> 2$, as to be of biological significance. These values of the two parameters were used in the

manufacturer's manual. The so defined spots of interest were located in the gel picture and a print of that image was used for comparison during spot picking.

The *DIA* module was for protein spot detection and quantification of up to three images (Cy2, Cy3 and Cy5) in one gel. That module was applied to import spot maps of gels, which were not properly computed by the batch processor.

## 4.12 Staining and picking of the gels

The gels were *Colloidal Coomassie* stained [57], which is a protocol for a working detection range of 50 µg to 500 µg of protein mass, where 150 µg of protein mass were on the gels.

The gels were put into the staining solution (see 3.8.4 for the preparation of the solution), and the container was transferred onto a rotary shaker and the gels were incubated over night. On the following day the gels were de-stained by removing the staining solution and washing of the gels with distilled water several times till the background was transparent. During the de-staining of the gels with distilled water, the container remained on the rotary shaker for approximately 15 minutes per step.

The picking of the spots of interest was carried out by using new sterile (not autoclaved) pipette tips, which were cut into the desired diameter, and a picking platform, which trans-illuminated the gel during the procedure. Distilled water was pipetted to the spots in the microcentrifuge tubes to protect the picked gel spots from dehydration. The spots were stored at 4 ℃ unt il mass spectrometric analysis.

# Chapter 5 – Results and discussion

## 5.1 Description of the experimental setup

A suspension batch cultivation of two host and two recombinant cell lines was performed to obtain information about hypothermic cultivation. In our case we cultivated the CHO cells under mild (33 ℃) hypothe rmic conditions and at 37 ℃ as reference.

The objectives were to compare the effect of the two incubation temperatures, host and recombinant cell lines and the differences between the two host cell lines and between the two recombinant cell lines.
The cell lines were chosen because of their difference in characteristics. The two host cell lines differ in their maximum growth rate, while the two recombinant cell lines differ in their metabolism.

Samples for the proteomic analyses were acquired at the exponential phase and in the stationary phase of the process.

## 5.2 Batch cultures

Results of the batch cultivation are presented in three figures for each cell line.

- The first graph –1– gives information about the growth and the viability during the cultivation. The two green arrows indicate the sampling points for the proteomic analysis.
- The second graph –2– gives information about the glucose, glutamine and lactate concentration alterations over the time.
- The third graph –3– gives information about the glucose and glutamine uptake rate and the lactate production rate.

### 5.2.1 CHO K1PD

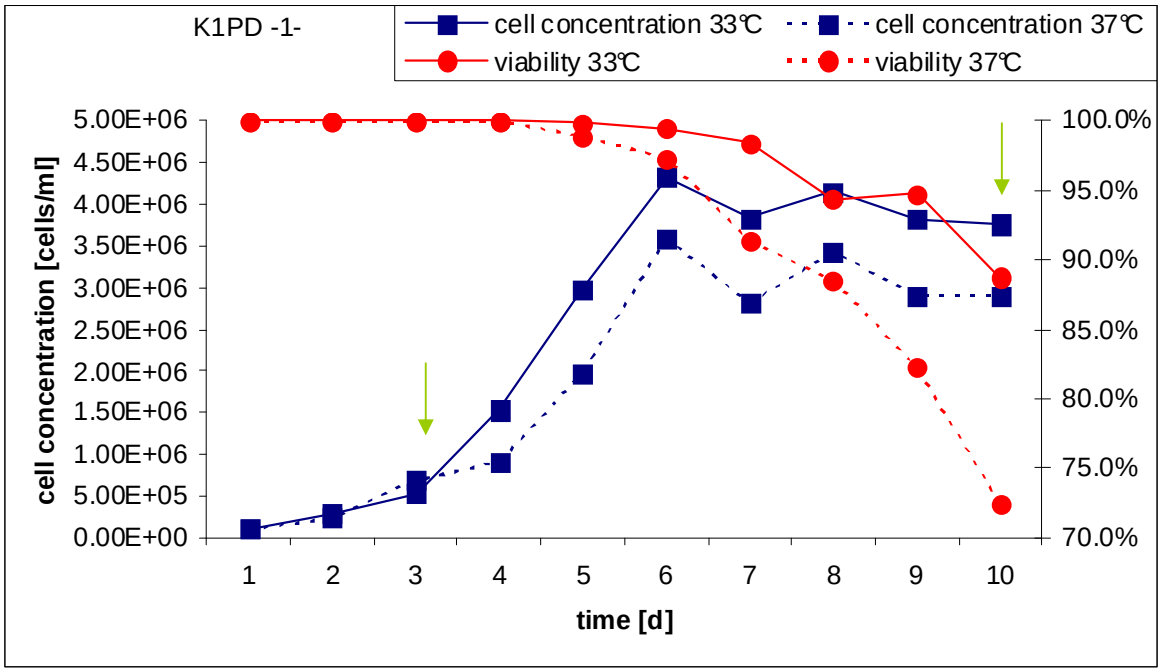


*Fig. 5. 1 The growth curve and the viability of the CHO K1PD during the 33 ℃ and the 37 ℃ cultivation. The arrows indicate the samp ling points for the proteomic analyses.*

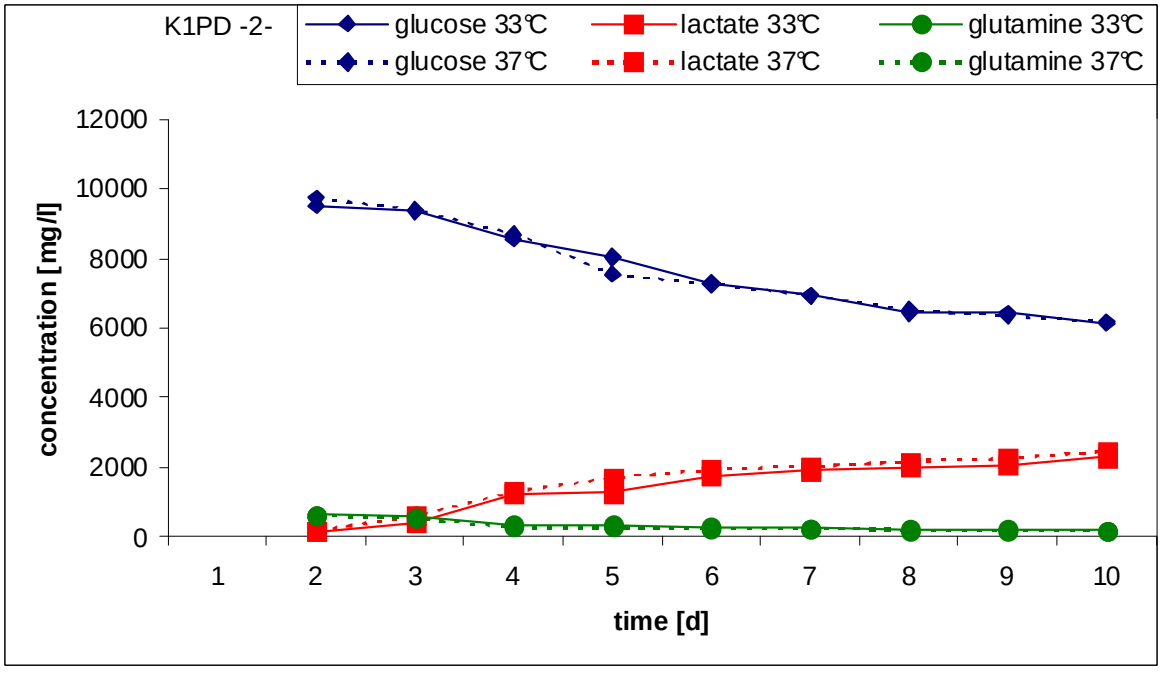


*Fig. 5. 2 The concentration of glucose, lactate and glutamine of the CHO K1PD during the 33 ℃ and the 37 ℃ cultivation. No samp les were taken on day 1 and by that no data acquired.*

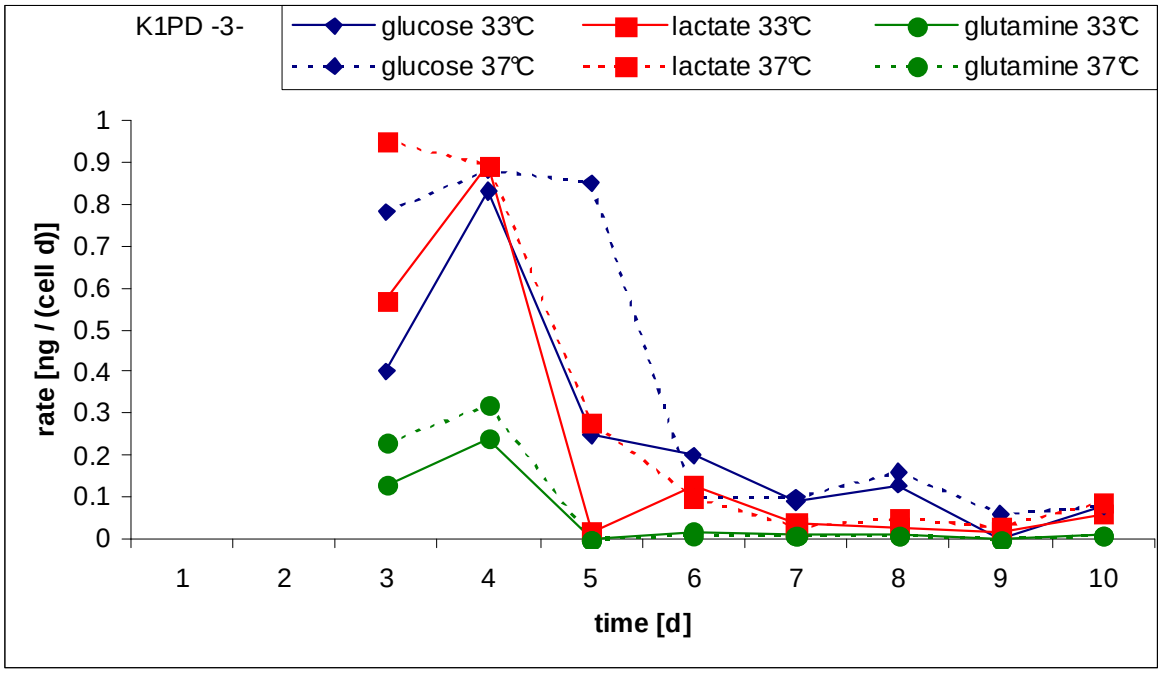


*Fig. 5. 3 The glucose and glutamine uptake rate and the lactate production rate of the CHO K1PD during the 33 ℃ and the 37 ℃ cultiva tion. Because of the missing values of the concentrations from day 1, the rates of day 1 and 2 could not be calculated.*

*Table 5. 1 Specific growth rates $\mu_{viable}$ [$d^{-1}$] of the K1PD cultures during the cultivation.*

| day | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| 33 ℃ | 1.24 | 0.60 | 1.07 | 0.67 | 0.36 | -0.13 | 0.04 | -0.08 | -0.08 |
| 37 ℃ | 1.00 | 1.07 | 0.28 | 0.76 | 0.58 | -0.31 | 0.17 | -0.24 | -0.12 |

*Differences between the 33 ℃ and the 37 ℃ culture*

The CHO K1PD had a remarkable growth rate at 33 ℃ and also reached a higher cell concentration in the same period of cultivation time than the 37 ℃ culture. Even though the cell concentration of the 33 ℃ cul ture was higher than that of the 37 ℃ culture, the viability at 33 ℃ was better. O n day 4 of the cultivation the 33 ℃ culture reached its highest growth rate and by t hen the glucose and glutamine uptake rates and the lactate production rate had their highest values during this cultivation. After 10 days the viability of the 37 ℃ culture was so low, that the K1PD cultures reached the termination condition. The glucose and the glutamine uptake and the lactate production were very similar in their course, as they were all high in the first four days. After that the rates dropped to zero. On day 3 and 5

the glucose uptake rates of the 37 ℃ culture was higher than the uptake rate of the 33 ℃ culture, while on day 4 they were the same.

### 5.2.2 CHO dhfr⁻

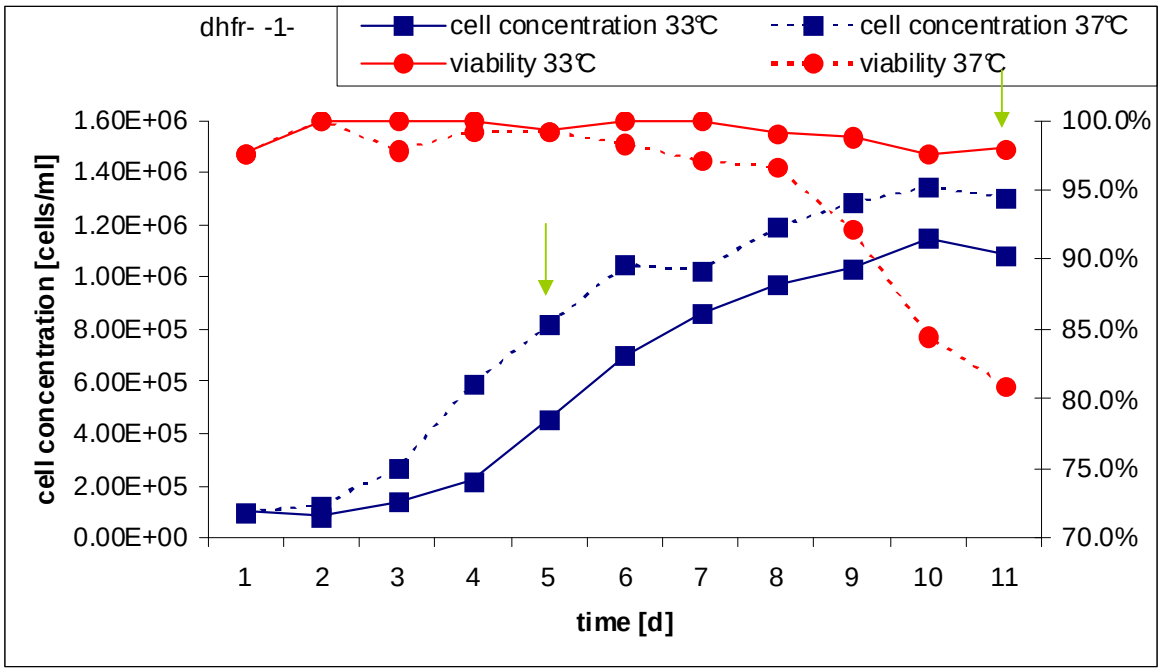


*Fig. 5. 4 The growth curve and the viability of the CHO dhfr⁻ during the 33 ℃ and the 37 ℃ cultivation. The arrows indicate the sampling points for the proteomic analyses.*

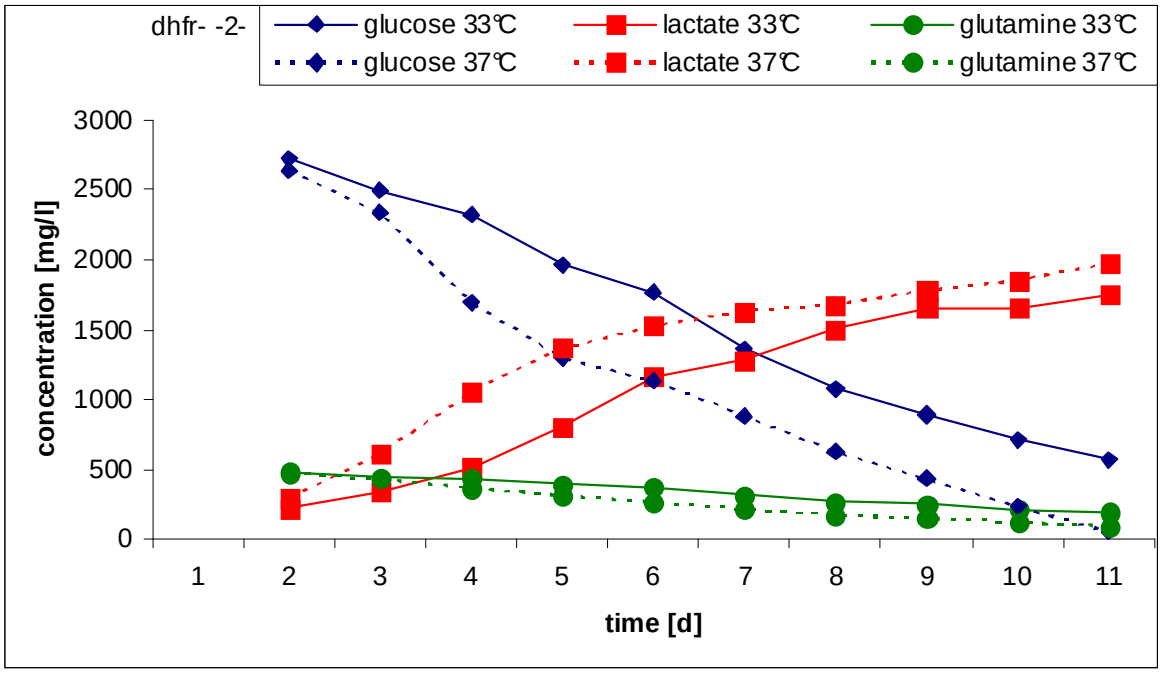


*Fig. 5. 5 The concentration of glucose, lactate and glutamine of the CHO dhfr⁻ during the 33 ℃ and the 37 ℃ cultivation. No samp les were taken on day 1 and by that no data acquired.*

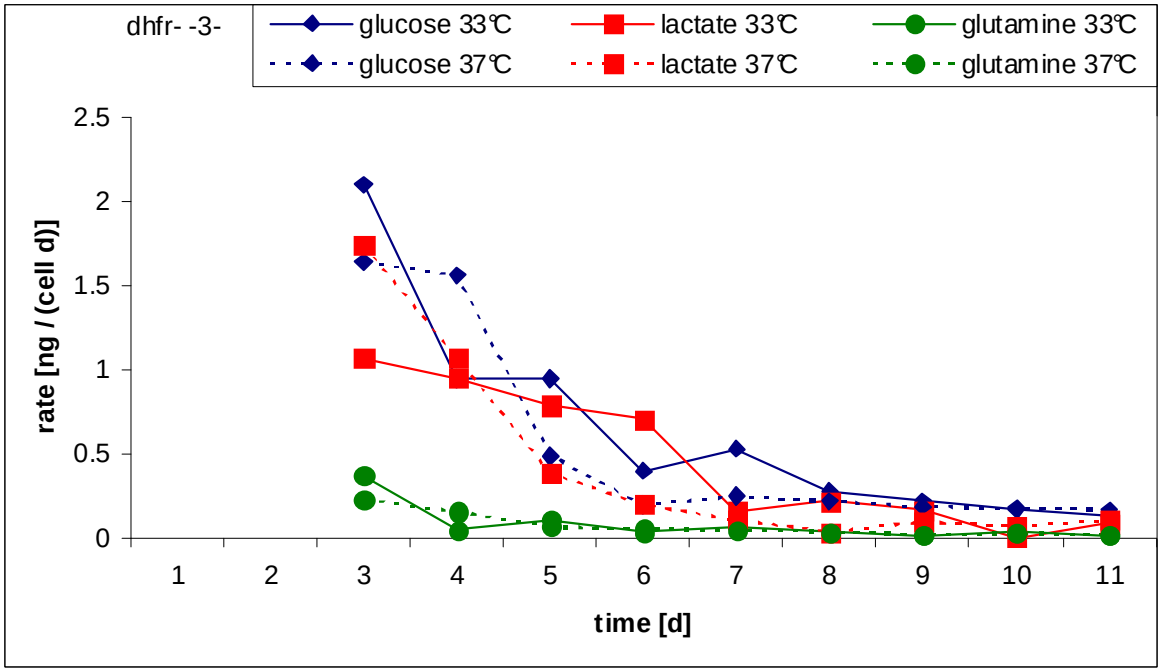


*Fig. 5. 6 The glucose and glutamine uptake rate and the lactate production rate of the CHO dhfr⁻ during the 33 ℃ and the 37 ℃ cultivation. Becaus e of the missing values of the concentrations from day 1, the rates of day 1 and 2 could not be calculated.*

*Table 5. 2 Specific growth rates $\mu_{viable}$ [$d^{-1}$] of the CHO dhfr$^-$ cultures during the cultivation.*

| day | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| 33 ℃ | -0.19 | 0.51 | 0.45 | 0.62 | 0.49 | 0.21 | 0.10 | 0.07 | 0.10 | -0.05 |
| 37 ℃ | 0.32 | 0.72 | 0.79 | 0.27 | 0.28 | -0.04 | 0.13 | 0.03 | -0.04 | -0.07 |

*Differences between the 33 ℃ and the 37 ℃ culture*

The CHO dhfr$^-$ cells of the 37 ℃ culture showed better growth th an the 33 ℃ culture. The viability of the 37 ℃ culture started to decrease on day 9 with a value of 92 % and falling, while the viability of the 33 ℃ culture was still constantly over 95 %. The glucose and the glutamine uptake rates and the lactate production rate were similar for both cultures.

### 5.2.3 CHO EpoFc 3F8

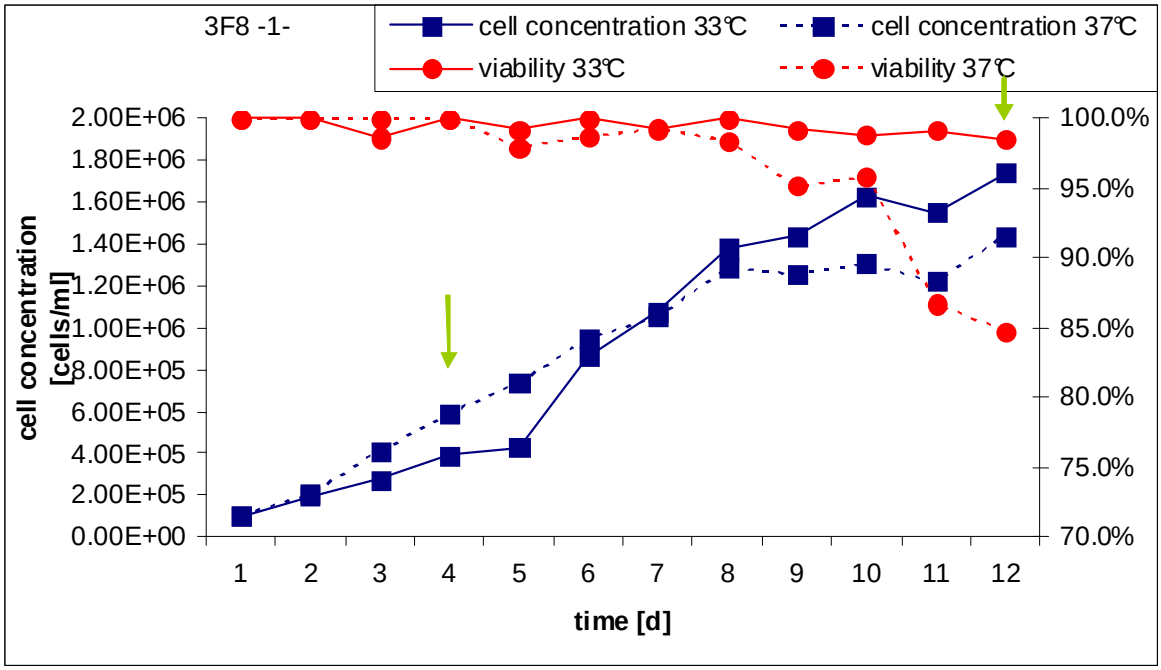


*Fig. 5. 7 The growth curve and the viability of the CHO EpoFc 3F8 during the 33 ℃ and the 37 ℃ cultivation. The arrows indicat e the sampling points for the proteomic analyses.*

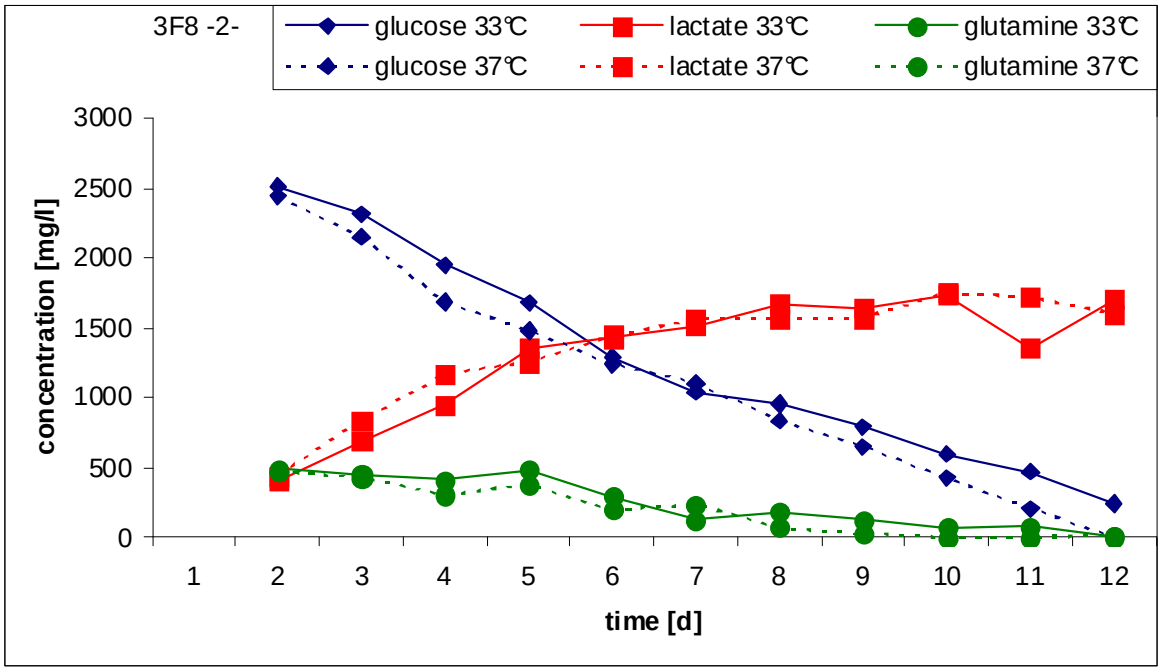


*Fig. 5. 8 The concentration of glucose, lactate and glutamine of the CHO EpoFc 3F8 during the 33 ℃ and the 37 ℃ cultivation. No samples were taken on day 1 and by that no data acquired.*

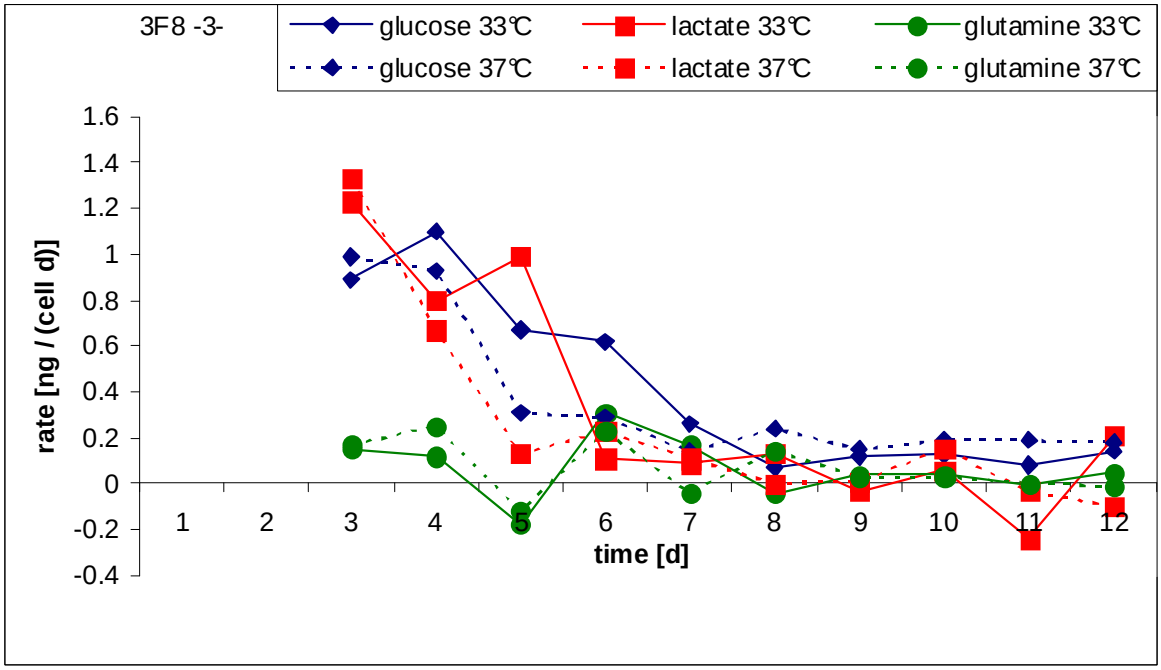


*Fig. 5. 9 The glucose and glutamine uptake rate and the lactate production rate of the CHO EpoFc 3F8 during the 33 ℃ and the 37 ℃ cu ltivation. Because of the missing values of the concentrations from day 1, the rates of day 1 and 2 could not be calculated.*

*Table 5. 3 Specific growth rates $\mu_{viable}$ [$d^{-1}$] of the CHO EpoFc 3F8 cultures during the cultivation.*

| day | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 ℃ | 0.78 | 0.32 | 0.38 | 0.08 | 0.71 | 0.21 | 0.25 | 0.04 | 0.12 | -0.05 | 0.011 |
| 37 ℃ | 0.84 | 0.70 | 0.36 | 0.20 | 0.26 | 0.11 | 0.19 | -0.06 | 0.05 | -0.17 | 0.14 |

*Differences between the 33 ℃ and the 37 ℃ culture*

In the beginning the growth rate of the recombinant cell line CHO EpoFc 3F8 cultivated at 33 ℃ was lower compared to the growt h rate at 37 ℃. From day 6 on the growth rate of the 33 ℃ culture was always hig her than the growth rate of the 37 ℃ culture. On day 8 the viability of the 37 ℃ cultures became lower than the viability of the 33 ℃ cultures. After that the gro wth rate of the 37 ℃ culture declined further. From day 8, the 33 ℃ culture had higher cell concentrations than the 37 ℃ culture. In the end the 33 ℃ culture ha d a higher cell concentration and a better viability. On day 9 the glutamine of the 37 ℃ culture was used up, while the 33 ℃ culture had around 130 mg/l left. The lac tate concentration was for both cultures approximately the same during the whole process. One exception was day 6 of the 33 ℃ culture, where the culture had a high growth rate increase and with that a high lactate production rate. The glucose uptake of the 37 ℃ culture was higher than that of the 33 ℃ culture and on da y 12 the 37 ℃ culture had no glucose left in the medium. On day 12 of the experiment the viability of the 33 ℃ cultures was 98.5%, while the 37 ℃ was around 84%. Overall both cultures had a slow increase of the cell concentration. In the beginning of the batch cultivations the glucose uptake rate and the lactate production rate were high at both incubation temperatures. From day 7 on both cultures had a constant uptake respectively production rate of glucose and lactate.

The glutamine uptake rate was relatively constant till day 9, where the glutamine was used up.

### 5.2.4 CHO EpoFc 14F2

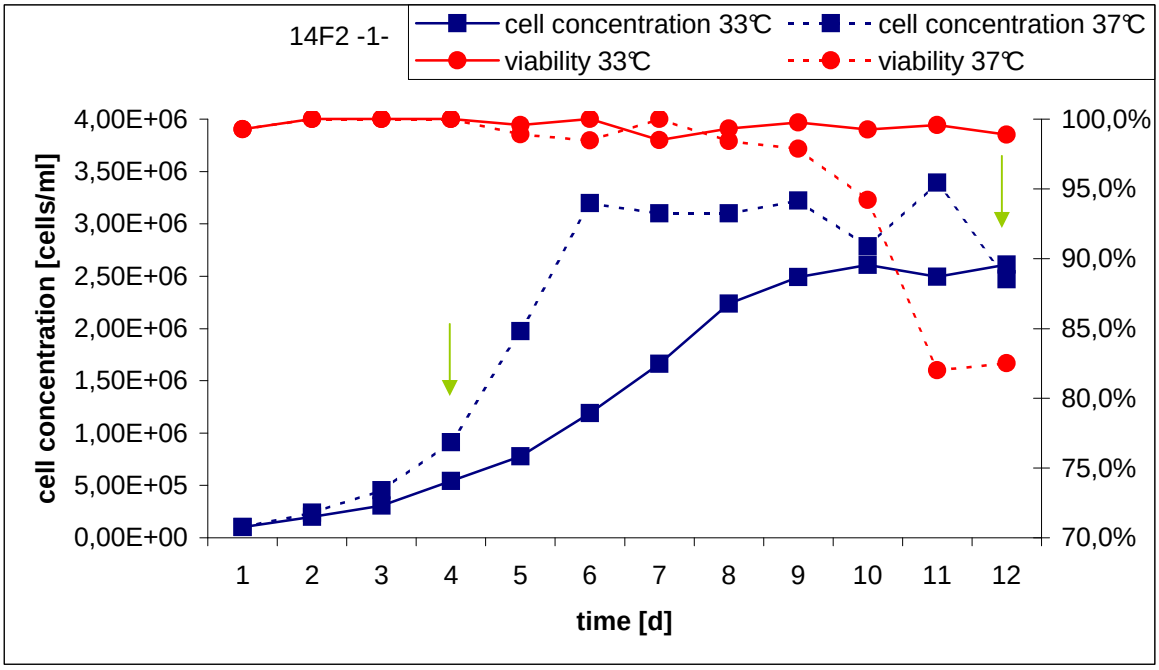


*Fig. 5. 10 The growth curve and the viability of the CHO EpoFc 14F2 during the 33 ℃ and the 37 ℃ cultivation. The value of the c ell concentration of the 33 ℃ culture of day 7 was an aberration and was corrected to acquire useable values. The arrows indicate the sampling points for the proteomic analyses.*

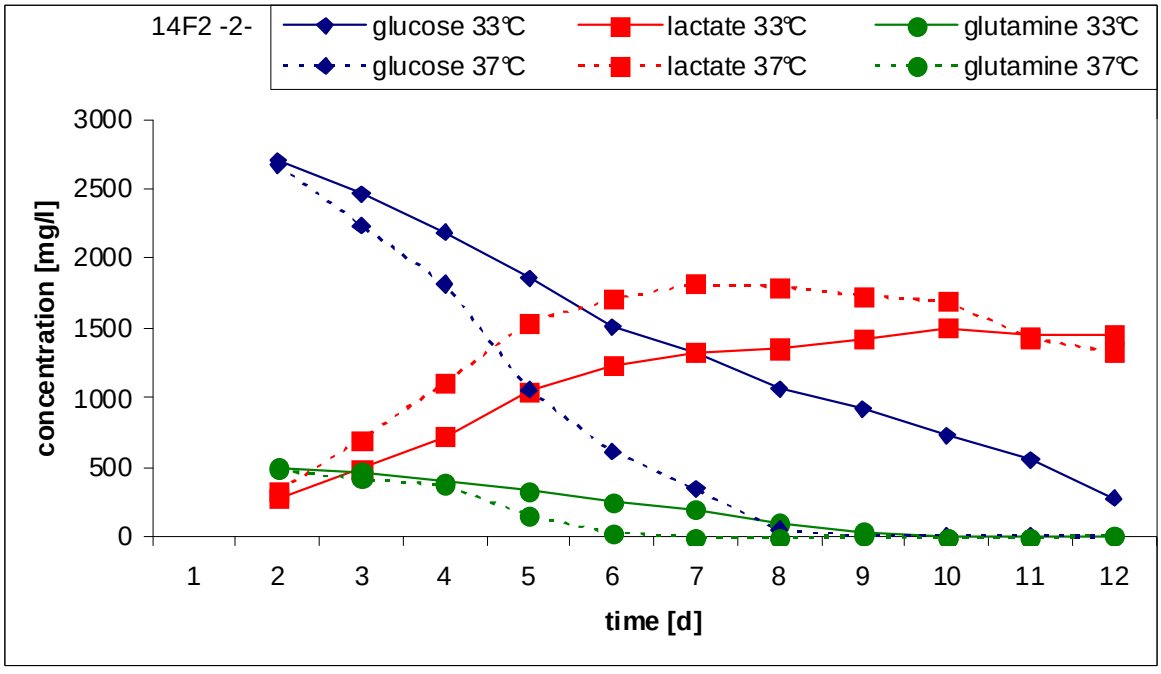


*Fig. 5. 11 The concentration of glucose, lactate and glutamine of the CHO EpoFc 14F2 during the 33 ℃ and the 37 ℃ cultivation. No samples were taken on day 1 and by that no data acquired.*

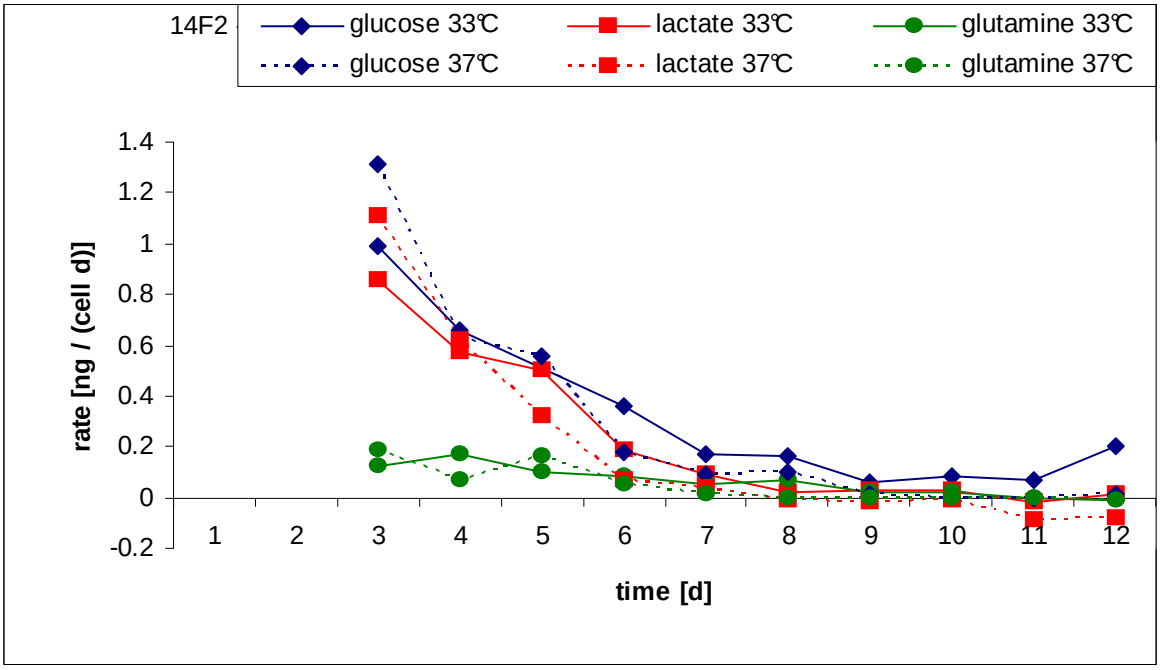


*Fig. 5. 12 The glucose and glutamine uptake rate and the lactate production rate of the CHO EpoFc 14F2 during the 33 ℃ and the 37 ° C cultivation. Because of the missing values of the concentrations from day 1, the rates of day 1 and 2 could not be calculated.*

*Table 5. 4 Specific growth rates $\mu_{viable}$ [$d^{-1}$] of the CHO EpoFc 14F2 cultures during the cultivation.*

| day | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 ℃ | 0.81 | 0.43 | 0.57 | 0.36 | 0.43 | 0.33 | 0.30 | 0.11 | 0.04 | -0.04 | 0.07 |
| 37 ℃ | 1.02 | 0.64 | 0.70 | 0.76 | 0.48 | -0.02 | -0.02 | 0.03 | -0.18 | 0.06 | -0.58 |

*Differences between the 33 ℃ and the 37 ℃ culture*

The growth rate of the CHO EpoFc 14F2 cells with the incubation temperature of 33 ℃ was lower than the one of the 37 ℃ cultivati on. The 37 ℃ culture showed a faster growth rate, but with the downside of an earlier drop of the viability at day 9. The 37 ℃ culture reached the stationary phase at d ay 7, while the 33 ℃ culture entered the stationary phase at day 10.

On day 12 of the experiment the 33 ℃ culture had t he same cell concentration as the 37 ℃ culture. The viability of the 33 ℃ cultu re was still constant (stationary phase), when the 37 ℃ culture was already in the d ying phase. In the end of the experiment the 33 ℃ culture did not reach the maxi mum cell concentration of the 37 ℃ culture.

The glucose and glutamine uptake rates and the lactate production rate of the 33 ℃ and the 37 ℃ culture were similar.

On day 9 the glucose was used up in the medium of the 37 ℃ culture, while the 33 ℃ culture had one third of the starting glucose concentration left. On day 6 the glutamine was used up in the medium of the 37 ℃ culture, where the 33 ℃ culture had glutamine left till day 10. Furthermore the glucose uptake rate of the 33 ℃ culture was increased without the presence of glutamine in the medium.

### 5.2.5 Comparisons and general conclusion

*Host cell lines*

By comparing the two host cell lines, CHO K1PD and CHO dhfr$^-$, the K1PD turned out to be the fast growing and shorter living cell line. On day 6 the 33 ℃ culture of the K1PD reached a cell concentration of $4.32\times10^6$ cell/ml. The 33 ℃ culture of the K1PD showed a better growth than the 37 ℃ culture, while for the dhfr$^-$ it was vice versa. The 33 ℃ culture of both cell lines maintained viability longer than its 37 ℃ counter part, but the 33 ℃ culture of the K1 PD was like its 37 ℃ culture already in the dying phase in the end of the experiment.

The glucose concentration in the medium of the K1PD cultures was far higher than the glucose concentration in the medium of the dhfr$^-$, which influenced the uptake rates. The total glucose consumed by the K1PD amounted to around 3500 mg/l, while the total glucose consumed by the dhfr$^-$ was 2200 mg/l for the 33 ℃ culture and 2600 mg/l for the 37 ℃ culture. The lactate production rates of the K1PD were lower than the lactate production rates of the dhfr$^-$. Around 70% of the glutamine in the medium were metabolised by K1PD during the whole cultivation, while the dhfr$^-$ cell line incubated at 33 ℃ consumed around 60 % and the 37 ℃ culture consumed around 80 %.

*Recombinant cell lines*

By comparing the two recombinant cell lines, CHO EpoFc 14F2 and CHO EpoFc 3F8 the 14F2 turned out to be the culture with the faster growth. Both 14F2 cultures reached a higher cell concentration than the 3F8 cultures. For both recombinant cell lines the 33 ℃ culture had a stable viability, while at 37 ℃ the culture viability rapidly decreased in the end, which was day 8 for the 3F8 37 ℃ culture and day 9 for the 14F2 37 ℃ culture.

The 33 ℃ cultures of both cell lines had a similar glucose uptake rate, which was > 0.50 till day 5. The 3F8 37 ℃ culture had a high glucose uptake rate of > 0.90 till day 4. The 14F2 37 ℃ culture had high glucose upta ke rates, > 0.50, till day 5, which resulted in combination with the higher cell concentration (compared to the other 3 cultures of recombinant cell lines) in an earlier drop of the glucose concentration.

The 33 ℃ culture of the 14F2 had the lowest lactat e production rate that resulted in less than 1500 mg/l of lactate in the medium. The glutamine uptake of the 37 ℃ culture of the 14F2 cell line was higher than the 33 ℃ culture and both 3F8 cultures. The main difference in the lactate production rate between the 33 ℃ cultures and the 37 ℃ cultures was, that the 33 ℃ cultures could maintain a higher lactate production rate over a longer period of time than the 37 ℃ cultures. That was the case, because of the lower cell concentrations and with that the higher total glucose concentration in the culture medium of the 33 ℃ cultures compared to the 37 ℃ cultures.

*Comparison of the host cell lines to the recombinant cell lines*

Each cell line group had one member with slow growth, which reached only a low total cell concentration and one member with fast growth, which reached a high total cell concentration. The slow growing cell lines were the CHO EpoFc 3F8 and the CHO dhfr$^-$ and the fast growing cell lines were the CHO EpoFc 14F2 and the CHO K1PD. The K1PD cell line grew fastest and died earlier than the other cell lines in the experiment. Both dhfr$^-$ cultures had the slowest growth rate in the first two days, while the K1PD 33 ℃ culture had the fast est growth rate. At day 3 and following the growth rates of all cultures were similar. The CHO EpoFc 14F2 reached a higher total cell concentration than the CHO EpoFc 3F8 and is therefore as a production cell line of more interest. CHO K1PD is a favourite host cell line for hypothermic cultivation, because of the high cell concentration reached during the process.

The main difference between the host cell lines and the recombinant cell lines is, that both recombinant cell line cultures had a longer life time during this experiment under hypothermic conditions.

*General conclusion*

The hypothermic cultivation at 33 °C, in contrast to the physiological incubation temperature of 37 °C [3], brings the benefit of a longer life time. Further advantages are the reduced lactate production and the glucose and glutamine uptake during the cultivation at 33 °C. These qualities make the hypothermic cultivation interesting for the production of recombinant proteins.

## 5.3 Experimental design of the 2D-gels

The following table contains the number of the run, the number of the gel in that particular run, the time point and the volume of protein suspension containing 50 µg of protein for the labelling with 400 pmol CyDye. Furthermore, the CyDye, with which the sample was labelled, and the file name of the gel, designated during the analysis with the DeCyder software, can be seen. The number of the Immobiline Dry strip used for the isoelectric focusation is also given. There was sufficient amount of sample available to perform the experiment as presented in the table below.

*Table 5. 5 The experimental design for the 2D DIGE*

| run | gel # | 1st sampling | [µl] sample (50µg protein) | CyDye | file name | Dry strip # |
|---|---|---|---|---|---|---|
| 2 | 1 | 3F8 33 | 108,70 | Cy3 | D2G1 | 34967 |
| 2 | 1 | 3F8 37 | 123,97 | Cy5 | D2G1 | 34967 |
| 2 | 2 | 14F2 33 | 118,11 | Cy3 | D2G2 | 34968 |
| 2 | 2 | 14F2 37 | 153,06 | Cy5 | D2G2 | 34968 |
| 2 | 3 | K1PD 33 | 99,60 | Cy3 | D2G3 | 34969 |
| 2 | 3 | K1PD 37 | 65,22 | Cy5 | D2G3 | 34969 |
| 2 | 4 | dhfr- 33 | 214,29 | Cy3 | D2G4 | 34971 |
| 2 | 4 | dhfr- 37 | 166,67 | Cy5 | D2G4 | 34971 |

| run | gel # | 2nd sampling | [µl] sample (50µg protein) | CyDye | file name | Dry strip # |
|---|---|---|---|---|---|---|
| 1 | 1 | 3F8 33 | 107,14 | Cy3 | D1G1 | 51275 |
| 1 | 1 | 3F8 37 | 72,46 | Cy5 | D1G1 | 51275 |
| 1 | 2 | 14F2 33 | 98,68 | Cy3 | D1G2 | 51276 |
| 1 | 2 | 14F2 37 | 61,48 | Cy5 | D1G2 | 51276 |
| 1 | 3 | K1PD 33 | 69,44 | Cy3 | D1G3 | 51277 |
| 1 | 3 | K1PD 37 | 115,38 | Cy5 | D1G3 | 51277 |
| 1 | 4 | dhfr- 33 | 170,45 | Cy3 | D1G4 | 51278 |
| 1 | 4 | dhfr- 37 | 93,75 | Cy5 | D1G4 | 51278 |
| 3 | 1 | 3F8 33 | 107,14 | Cy3 | D3G1 | 51279 |
| 3 | 1 | 3F8 37 | 72,46 | Cy5 | D3G1 | 51279 |
| 3 | 2 | 14F2 33 | 98,68 | Cy3 | D3G2 | 51280 |
| 3 | 2 | 14F2 37 | 61,48 | Cy5 | D3G2 | 51280 |
| 3 | 3 | K1PD 33 | 69,44 | Cy3 | D3G3 | 34974 |
| 3 | 3 | K1PD 37 | 115,38 | Cy5 | D3G3 | 34974 |
| 3 | 4 | dhfr- 33 | 170,45 | Cy3 | D3G4 | 51269 |
| 3 | 4 | dhfr- 37 | 93,75 | Cy5 | D3G4 | 51269 |

## 5.4 Results of the DeCyder analysis

The following image shows the master gel, D2G2, after the definition of the area of interest by the ImageQuant software.

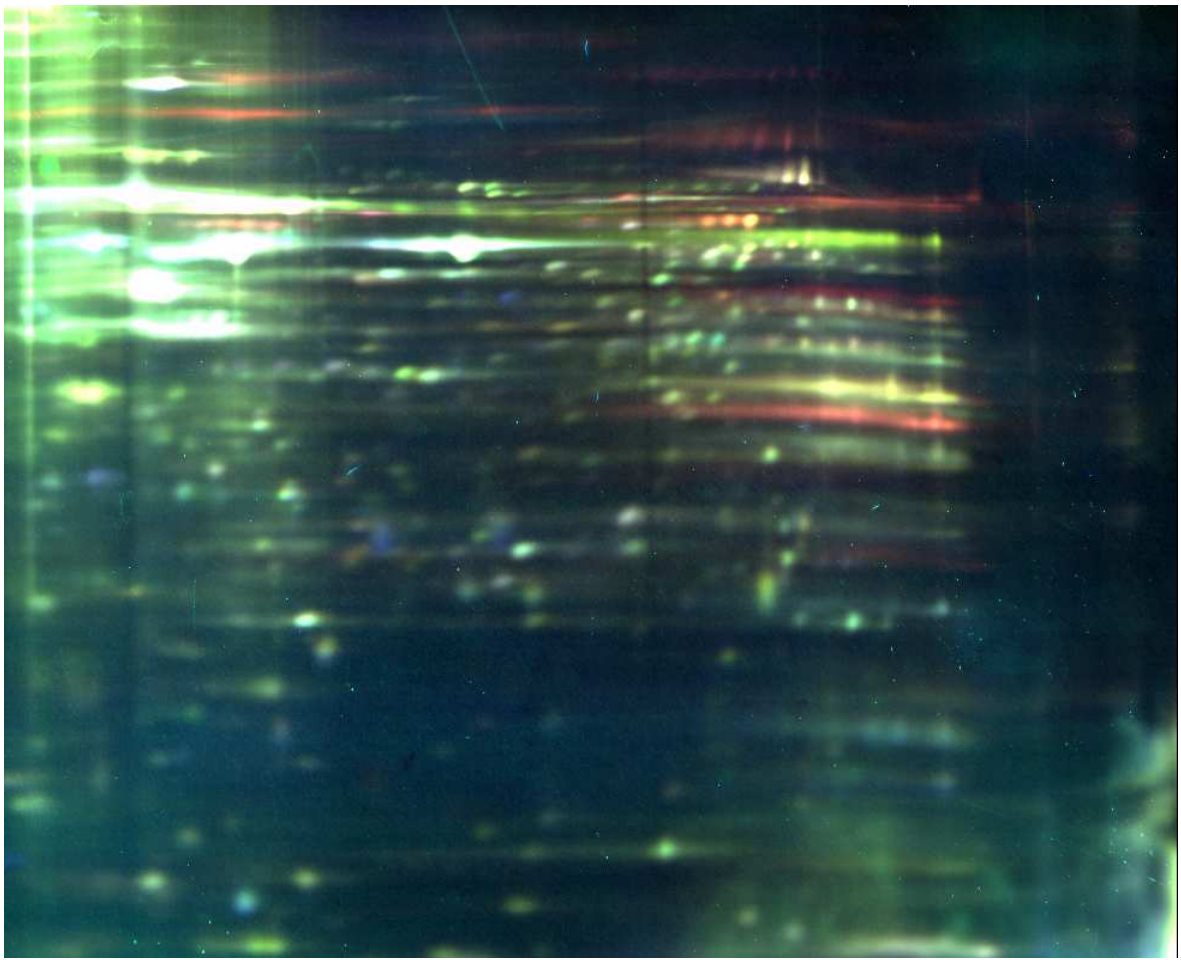

*Image 5. 1 – Master gel D2G2 with all CyDyes excited and overlaid to get an impression of the quantity and the distribution of the proteins on the gel.*

After the analysis with the batch processor and the BVA module of the DeCyder software were conducted, the master gel, D2G2, and a second gel, D2G1, were chosen to be *Colloid Coomassie stained* and the spots of interest were picked afterwards. The images of these two gels are presented here. The master gel, D2G2, was the gel of the samples of both incubation temperatures of the CHO EpoFc 14F2 from the first time point and the internal pooled standard. The second gel, D2G1, was the gel of the samples of both cultivation temperatures of CHO EpoFc 3F8 from the first time point and the internal pooled standard.

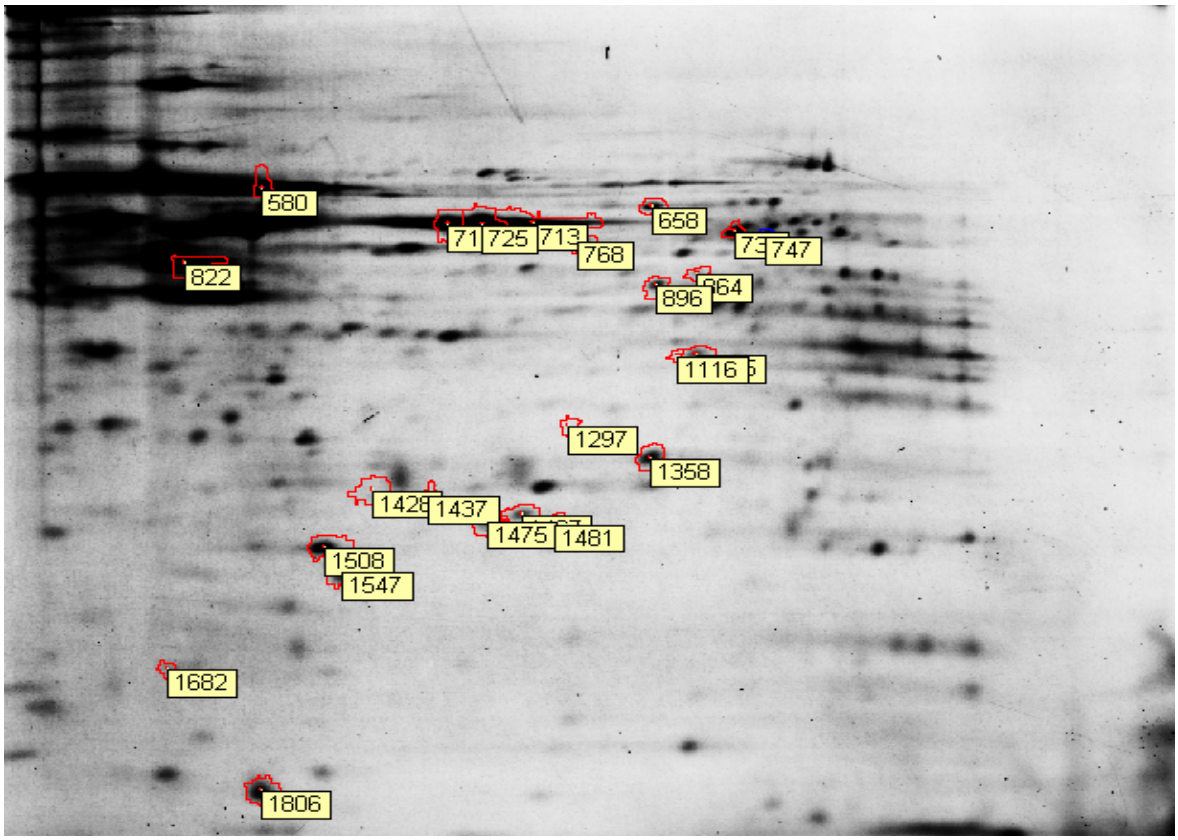


*Image 5. 2 – The master gel, D2G2, with the annotations of the protein spots of interest found during all analyses and picked for MS analysis.*

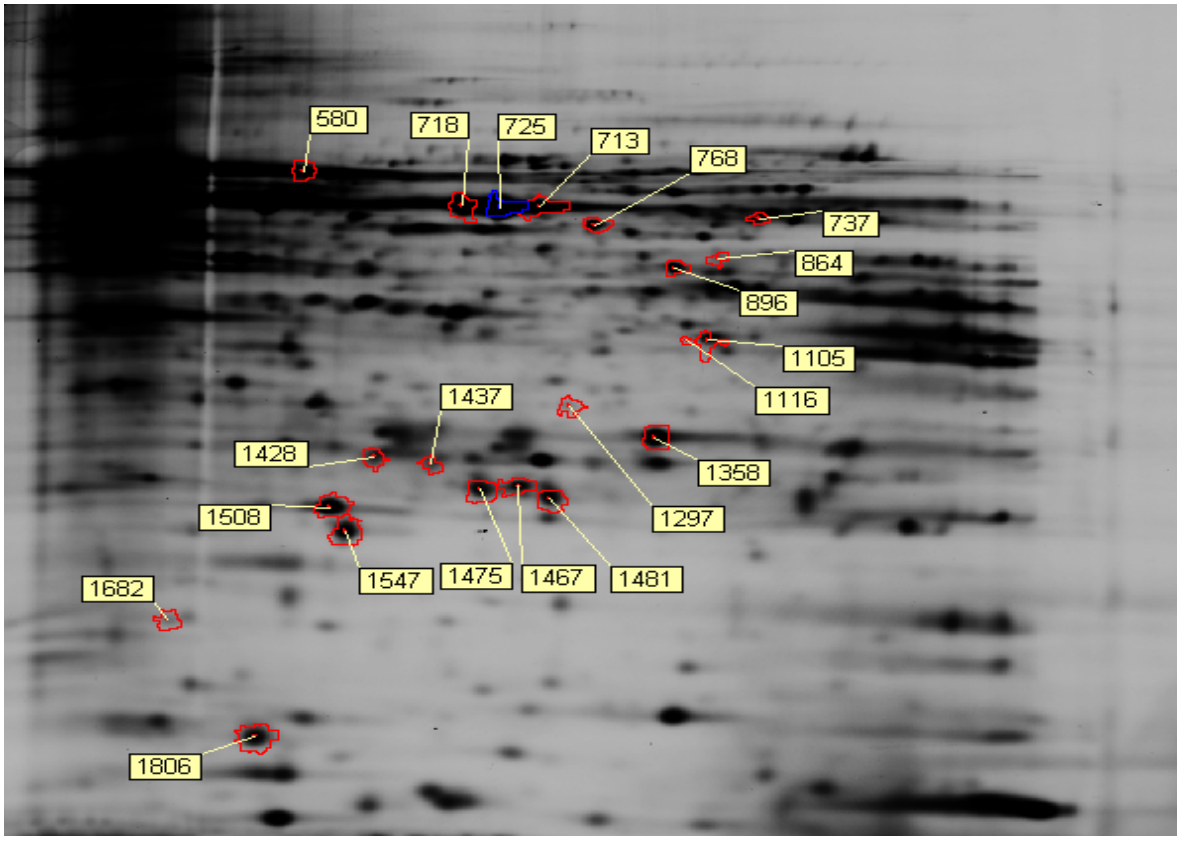


*Image 5. 3 – This gel image comprises from the second gel, D2G1, where the spots of interest, in this image annotated, were picked from and analysed by MS.*

During all analyses with the BVA module, proteins that were up- or down-regulated by the temperature shift from 37 ℃ to 33 ℃ were s earched. Therefore, up-regulated means, that the proteins are higher expressed at 33 ℃, while down-regulated means a higher expression of that protein during the 37 ℃ cultivation.

The usual boundaries defined in the method chapter were altered for analysis with 15 or more gels, to get a wider spread of proteins as result. In that cases proteins (spots) of interest were defined as proteins with an average abundance of < -1.3 or > 1.3, which defines a protein expression change of 30 %, and a Students T-test of 0.16, which represents a 16 % chance of the value to be random. For the analysis with an amount of gels < 15 the spots of interest were defined by an average abundance of < -1.5 or > 1.5 and a Students T-test of 0.07.

Seven different comparisons were conducted with the BVA module.

5.4.1 all gels, 33 ℃ $\rightarrow$ 37 ℃

5.4.2 host cell lines, 33 ℃ $\rightarrow$ 37 ℃

5.4.3 recombinant cell lines, 33 ℃ $\rightarrow$ 37 ℃

5.4.4 33 ℃ cultures, host cell lines $\rightarrow$ recombinant cell lines

5.4.5 37 ℃ cultures, host cell lines $\rightarrow$ recombinant cell lines

5.4.6 each cell lines is analysed by itself, 33 ℃ $\rightarrow$ 37 ℃

5.4.7 comparisons of the difference between the exponential phase and the stationary phase

*Description of the tables*

The tables include the following information. The master number of the chosen protein spot is assigned on the master gel, which is the same for every gel.

The appearance gives information about the number of gels, where the specific spot was found, while the value inside the brackets presents the number of gels in this specific analysis. The ‘A’ stands for analysis gel and the ‘M’ for master gel, which gives information about the gel type where the spot was present. The standard, Cy 2, of the master gel was included during all analyses. Therefore the value of the number of gels was in some specific analyses, where the CHO EpoFc 14F2 gel was not usually including, increased by one gel.

The student's t-test value and the average abundance value are also included in the tables. A positive average ratio was defined as a down-regulation of the protein, where a negative average ratio means that the proteins were higher expressed in the 33 ℃ cultures.

*colour code of the most significant spots*

| | |
|---|---|
| Spot showed up during most of the analyses. | |
| Spot found in the specific analysis. | |
| Spot characterised by MS | |

### 5.4.1 Analysis of all the samples

The data of all tables consist of a statistical analysis of two groups. The images of the gels derived from the 33 ℃ cultures of all cel l lines were defined as group 1, while the images of the gels derived from the 37 ℃ cultures of all cell lines were defined as group 2. The most significant spots of interest are indicated by a green background. During this statistical analysis all gels were included to find up- or down-regulated proteins that all cell lines had in common during the hypothermic cultivation.

The two picked columns (only in table 5.6 and 5.7) represent the spot picking efficiency, because some spots were too small to be properly stained and could not be found during the spot picking process. The re-discovery efficiency of the spots during picking was divided into 3 degrees.

1. The identity of the picked spot was indisputable the same as the spot in the gel image. – 'ok'
2. The identity of the picked spot was not indisputable, but with a high probability, the same as the spot in the gel image. – '~'
3. The spot could not be found during the picking process. – 'x'

*Table 5. 6 Down-regulated proteins of all cultures – The most significant spots were 580, 718, 725, 737, 822, 1358, 1428, 1437, 1467 and 1547.*

| position | master no. | pick #1 | pick #2 | appearance | students t-test | average ratio |
|---|---|---|---|---|---|---|
| 1 | 580 | ~ | ok | 24 (30) A, M | 0,013 | 1,61 |
| 2 | 713 | ok | ok | 30 (30) A, M | 0,05 | 1,44 |
| 3 | 718 | ok | ok | 24 (30) A, M | 0,08 | 1,67 |
| 4 | 725 | ok | ok | 30 (30) A, M | 0,012 | 1,55 |
| 5 | 737 | ok | ok | 24 (30) A, M | 3,30E-05 | 1,53 |
| 6 | 768 | ok | ok | 24 (30) A, M | 0,042 | 1,32 |
| 7 | 822 | ok | ok | 18 (30) A, M | 0,011 | 1,54 |
| 8 | 896 | ok | ok | 30 (30) A, M | 0,00027 | 1,45 |
| 9 | 1358 | ok | ok | 30 (30) A, M | 0,0027 | 1,9 |
| 10 | 1428 | x | x | 24 (30) A, M | 0,047 | 2,06 |
| 11 | 1437 | x | x | 24 (30) A, M | 0,013 | 1,85 |
| 12 | 1467 | ok | ~ | 30 (30) A, M | 0,052 | 1,72 |
| 13 | 1475 | ~ | ok | 30 (30) A, M | 0,16 | 1,59 |
| 14 | 1481 | ~ | ~ | 30 (30) A, M | 0,14 | 1,3 |
| 15 | 1508 | ~ | ok | 27 (30) A, M | 0,08 | 1,32 |
| 16 | 1547 | ok | x | 30 (30) A, M | 0,079 | 1,69 |

*Table 5. 7 Up-regulated proteins –*

*The most significant spot here were 864, 1116 and 1682.*

| position | master no. | pick #1 | pick #2 | appearance | students t-test | average ratio |
|---|---|---|---|---|---|---|
| 17 | 864 | x | x | 24 (30) A, M | 0,021 | -1,95 |
| 18 | 1105 | ok | ok | 27 (30) A, M | 0,075 | -1,68 |
| 19 | 1116 | x | x | 21 (30) A, M | 0,067 | -2,05 |
| 20 | 1297 | x | x | 21 (30) A, M | 0,026 | -1,54 |
| 21 | 1682 | x | x | 12 (30) A, M | 0,063 | -1,92 |
| 22 | 1806 | ok | ok | 18 (30) A, M | 0,62 | -1,14 |

*1806 was chosen, because it is assumed to be the protein S100 A6 [59] and was picked to verify its identity.*

During this analysis more proteins showed up, where the protein expression was down-regulated (higher expressed at the 37 ℃ cultu res), influenced by the hypothermic cultivation, than there were up-regulated proteins. The most significant down-regulated proteins were the protein spots with the master number 580, 718, 725, 737, 822, 1358, 1428, 1437, 1467 and 1547. Protein spot 1428 and 1437 could not be picked and by that not identified. The most significant up-regulated proteins were the proteins with the master number 864, 1116 and 1682.

### 5.4.2 Analysis of the host cell lines

This analysis was performed to obtain information about the different protein expression of the host cell lines. In this case only the gels of the host cell lines, CHO K1PD and CHO dhfr⁻, were analysed to find proteins that were down- or up-regulated during the hypothermic cultivation.

*Table 5. 8 Down-regulated proteins –*
*The most significant proteins were 580, 1358 and 1428. 1358 and 1428 were included despite their high students t-test value, because they also showed up as proteins of interest during the analysis including all gels.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 580 | 16 (16) A, M | 0,062 | 1,72 |
| 737 | 13 (16) A, M | 0,019 | 1,46 |
| 822 | 13 (16) A, M | 0,11 | 1,4 |
| 1358 | 16 (16) A, M | 0,26 | 1,72 |
| 1428 | 10 (16) A, M | 0,28 | 2,58 |
| 1481 | 16 (16) A, M | 0,12 | 1,4 |

*Table 5. 9 Up-regulated proteins –*
*The proteins with the best values were 1116 and 1682. 1116 was included despite its high students t-test value, because this protein also showed up as proteins of interest during the analysis including all gels.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1116 | 10 (16) A, M | 0,23 | -2,03 |
| 1682 | 7 (16) A, M | 0,026 | -1,62 |

The two most significant down-regulated proteins were 580, 1358 and 1428. The student's t-test values of 1358 and 1428 were rather high, but because of their significance during the other analysis the values seem to be adequate.
1116 and 1682 were the two most up-regulated proteins of interest.

### 5.4.3 Analysis of the recombinant cell lines

This analysis was performed to obtain information about the different protein expression of the two recombinant cell lines CHO EpoFc 3F8 and 14F2. Down- and up-regulated proteins were searched for.

*Table 5. 10 Down-regulated proteins – Analysis results of the down-regulated proteins of the recombinant cell lines, CHO EpoFc 3F8 and CHO EpoFc 14F2 only. The two orange indicated spots, 658 and 747, showed up only during this analysis and were also picked for MS analysis. 1358 (indicated in green) was, like during the 5.4.1 analysis, the most significant spot.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 658 | 9 (15) A, M | 0,032 | 1,59 |
| 725 | 15 (15) A, M | 0,05 | 1,6 |
| 737 | 12 (15) A, M | 0,0019 | 1,5 |
| 747 | 15 (15) A, M | 0,025 | 1,65 |
| 896 | 15 (15) A, M | 0,0033 | 1,5 |
| 1358 | 15 (15) A, M | 0,022 | 2,03 |
| 1428 | 15 (15) A, M | 0,14 | 1,78 |
| 1437 | 12 (15) A, M | 0,15 | 1,9 |
| 1467 | 15 (15) A, M | 0,042 | 1,61 |

*Table 5. 11 Up-regulated proteins –*

*The most significant spots were 864, 1116 and 1682. 1116 and 1682 were included despite their high students t-test values.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 864 | 9 (15) A, M | 0,00024 | -2,05 |
| 1116 | 12 (15) A, M | 0,22 | -2,06 |
| 1297 | 12 (15) A, M | 0,13 | -1,51 |
| 1682 | 6 (15) A, M | 0,22 | -1,98 |

The two spots 658 and 747 were added to the pick list. Spot 1358 was, like in the analysis which included all gels, the most significant down-regulated protein. Like in the analysis including all gels, 864, 1116 and 1682 were the most significant up-regulated proteins.

### 5.4.4 Analysis of the 33 ℃ cultures of the host cell lines compared to the 33 ℃ of the recombinant cell lines

This analysis was performed to obtain information about the different protein expression of the 33 ℃ cultures of the host cell lines as group 1 in the statistical analysis and the recombinant cell lines as group 2. A positive average ratio value indicates a higher expression in the recombinant cell lines and a negative value a higher expression in the host cell lines.

*Table 5. 12 Proteins higher expressed in the recombinant cell lines – The four most significant spots were 713, 1043, 1101, 1475 and 1481.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 713 | 20 (20) A, M | 0,022 | 1,56 |
| 1043 | 18 (20) A, M | 0,0077 | 2,42 |
| 1101 | 12 (20) A, M | 0,0015 | 2,64 |
| 1475 | 20 (20) A, M | 0,0042 | 2,19 |
| 1481 | 20 (20) A, M | 0,011 | 1,98 |

*Table 5. 13 Proteins higher expressed in the host cell lines – The most significant protein was spot 849.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 849 | 18 (20) A, M | 0,042 | -3,37 |
| 1467 | 20 (20) A, M | 0,12 | -1,57 |

The proteins with the spot number 713, 1043, 1101, 1475 and 1481 were the most significant higher expressed proteins of the recombinant cell lines.
849 was the highest expressed protein of the host cell lines at this comparison.

### 5.4.5 Analysis of the 37 ℃ cultures of the host cell lines compared to the 37 ℃ of the recombinant cell lines

This analysis was performed to obtain information about the different protein expression of the 37 ℃ cultures of the host cell lines as group 1 in the statistical analysis and the recombinant cell lines as group 2. A positive average ratio value indicates a higher expression in the recombinant cell lines and a negative value a higher expression in the host cell lines.

*Table 5. 14 Proteins higher expressed in the recombinant cell lines – The four most significant spots were 713, 1043, 1475 and 1481.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 713 | 20 (20) A, M | 0,013 | 1,86 |
| 1043 | 18 (20) A, M | 0,008 | 2,8 |
| 1475 | 20 (20) A, M | 0,018 | 2,46 |
| 1481 | 20 (20) A, M | 0,024 | 1,74 |

*Table 5. 15 Proteins higher expressed in the host cell lines – The most significant proteins were 962, 1280 and 1309.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 768 | 16 (20) A, M | 0,064 | -1,44 |
| 962 | 20 (20) A, M | 0,017 | -2,45 |
| 1280 | 18 (20) A, M | 0,053 | -2,05 |
| 1309 | 20 (20) A, M | 0,014 | -2,9 |
| 1806 | 12 (20) A, M | 0,11 | -1,83 |

The proteins with the spot number 713, 1043, 1475 and 1481 were the most significant higher expressed proteins of the recombinant cell lines. 962, 1280 and 1309 were the highest expressed proteins of the host cell lines.

### 5.4.6 Analysis of each cell line by comparing the protein expression of the 33 ℃ culture to the 37 ℃ culture

This analysis was performed to obtain information about the different protein expression of the 33 ℃ culture and the 37 ℃ cultu re for each cell line independently. Here the 33 ℃ cultures were assigne d group 1 and the 37 ℃ cultures were assigned group 2 during the statistical analysis.

*3F8*

*Table 5. 16 Down-regulated proteins – The two most significant spots were 1358 and 1428.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1358 | 7 (7) A, M | 0,07 | 2,28 |
| 1428 | 7 (7) A, M | 0,0093 | 2,21 |

*Table 5. 17 Up-regulated proteins – The only significant up-regulated spot found during this analysis was 1297.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1297 | 7 (7) A, M | 0,003 | -1,58 |

*14F2*

*Table 5. 18 Down-regulated proteins –*

1358 was the most significant protein.

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 737 | 9 (9) A, M | 0,014 | 1,53 |
| 896 | 9 (9) A, M | 0,019 | 1,53 |
| 1358 | 9 (9) A, M | 0,045 | 1,78 |

*Here were 9 gels instead of the 7 gels of the other comparisons, because during this analysis one more gel image was analysable.*

No *up-regulated* proteins of interest were found during this comparison.

*K1PD*

*Table 5. 19 Down-regulated proteins –*

*1376 was the most significant protein.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1376 | 7 (7) A, M | 0,042 | 2,07 |
| 1467 | 7 (7) A, M | 0,067 | 1,94 |

No *up-regulated* proteins of interest were found during this comparison.

*dhfr*$^{-}$

*Table 5. 20 Down-regulated proteins –*

*822 and 1481 were the most significant down-regulated proteins during this comparison.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 822 | 7 (7) A, M | 0.056 | 1.53 |
| 1481 | 7 (7) A, M | 0.037 | 1.6 |

*Table 5. 21 Up-regulated proteins –*

*864 was the most significant protein of interest during this analysis.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 864 | 7 (7) A, M | 0.013 | -2.23 |

This analysis showed that CHO EpoFc 3F8 had two significant down-regulated proteins, 1358 and 1428 and one significantly up-regulated protein, 1297.

The most significant down-regulated protein of CHO EpoFc 14F2 was the protein with the spot number 1358. No up-regulated proteins could be found for 14F2 during this analysis.

CHO K1PD had two significantly down-regulated proteins, 1376 and 1467. No up-regulated proteins could be found for K1PD during this analysis.
Dhfr$^{-}$ had two significantly down-regulated proteins, 822 and 1481, and one significantly up-regulated protein, 864 during this analysis.

### 5.4.7 Analysis of the differences in protein expression between the two time points of sampling

These analyses was performed to obtain information about the different protein expression of the cultures at the two time points - *tp* - of sampling, which represent the exponential phase and the stationary phase. These studies included the following comparisons:

- Comparison of tp 1 and 2 including all gels –
  Tp 1 was assigned to group 1 and tp 2 was assigned to group 2.
  Positive average abundance values represent a higher expression of the specific protein at tp 2.
- Comparison of tp 1 and 2 including only the host cell lines –
  Tp 1 was assigned to group 1 and tp 2 was assigned to group 2.
  Positive average abundance values represent a higher expression of the specific protein at tp 2.
- Comparison of tp 1 and 2 including only the recombinant cell lines –
  Tp 1 was assigned to group 1 and tp 2 was assigned to group 2.
  Positive average abundance values represent a higher expression of the specific protein at tp 2.
- Comparison of the protein expression of the host and the recombinant cell lines at tp 1 –
  The host cell lines were assigned to group 1 and recombinant cell lines were assigned to group 2.
  Positive average abundance values represent a higher expression of the specific protein in the recombinant cell lines.
- Comparison of the protein expression of the host and the recombinant cell lines at tp 2 –
  The host cell lines were assigned to group 1 and recombinant cell lines were assigned to group 2.

Positive average abundance values represent a higher expression of the specific protein in the recombinant cell lines.

- Comparison of the protein expression of the specific cell lines at tp 1 and tp 2 – Tp 1 was assigned to group 1 and tp 2 was assigned to group 2. Positive average abundance values represent a higher expression of the specific protein at tp 2.

All other comparisons were statistically not possible, because of the few gels at disposal.

*All gels*

This analysis was performed to obtain information about the differences in protein expression of the beginning of the experiment compared to the end. Here all cell lines of both incubation temperatures were included.

*Table 5. 22 Comparison of tp1 and tp2 including all gels – Protein 1103 was higher expressed at tp 1.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1103 | 27 (30) A, M | 0.0032 | -2.19 |

*Table 5. 23 Comparison of tp1 and tp2 including all gels – The proteins 551, 695 and 1271 were higher expressed at tp 2.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 551 | 18 (30) A, M | 0.048 | 2 |
| 695 | 24 (30) A, M | 0.00017 | 2.35 |
| 1271 | 27 (30) A, M | 0.00081 | 2 |

During this analysis the protein 1103 was found to be higher expressed at the samples taken at time point 1. The proteins 551, 695 and 1271 were higher expressed in samples of tp 2.

*Host cell lines*

This analysis was performed to obtain information about the differences in protein expression at the beginning of the experiment compared to the end. Here only the host cell lines of both incubation temperatures were included.

*Table 5. 24 Comparison of tp1 and tp2 including only the host cell lines – The proteins 717, 718 and 1169 were higher expressed at tp 2*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 717 | 10 (16) A, M | 0.0048 | 3.34 |
| 718 | 13 (16) A, M | 0.077 | 2.34 |
| 1169 | 10 (16) A, M | 0.03 | 2.05 |

No proteins were found, which were higher expressed at tp 1.

*Recombinant cell lines*

This analysis was performed to obtain information about the differences in protein expression of the beginning of the experiment compared to the end. Here only the recombinant cell lines at both incubation temperatures were included.

*Table 5. 25 Comparison of tp1 and tp2 including only the recombinant Cell lines – These proteins were higher expressed at tp 1*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 705 | 9 (13) A, M | 0.012 | -2.54 |
| 722 | 10 (13) A, M | 0.003 | -2.25 |
| 1208 | 12 (13) A, M | 0.013 | -2.18 |
| 1280 | 13 (13) A, M | 0.019 | -2.52 |
| 1470 | 10 (13) A, M | 0.013 | -2.47 |

No proteins were found, which were higher expressed at tp 2.

During the analysis of the host cell lines three proteins, 717, 718 and 1169, showed up, which were higher expressed at time point 2, while no protein was found, which were higher expressed at time point 1.

The results of the analysis of the recombinant cell lines were vice versa. Five proteins, 705, 722, 1208, 1280 and 1470, were found, which were higher expressed at time point 1 and no up-regulated proteins could be found at time point 2.

*Time point 1*

This analysis was performed to obtain information about the differences in protein expression of the host cell lines compared to the recombinant cell lines during exponential growth phase.

*Table 5. 26 Comparison of the protein expression of the host and the recombinant cell lines at tp 1 – These proteins were higher expressed in the host cell lines at tp 1.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 849 | 27 (30) A, M | 0.0052 | -5.44 |
| 1024 | 18 (30) A, M | 0.05 | -2.75 |
| 1255 | 18 (30) A, M | 0.007 | -3.83 |

*Table 5. 27 Comparison of the protein expression of the host and the recombinant cell lines at tp 1 – These proteins were higher expressed in the recombinant cell lines at tp 1.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 705 | 21 (30) A, M | 0.0081 | 3.52 |
| 713 | 30 (30) A, M | 0.0031 | 2.2 |
| 717 | 21 (30) A, M | 0.0019 | 2.09 |
| 719 | 24 (30) A, M | 0.0038 | 3.33 |
| 722 | 24 (30) A, M | 0.00027 | 3.42 |
| 1043 | 27 (30) A, M | 0.0025 | 3.15 |
| 1103 | 27 (30) A, M | 6.40E-05 | 2.31 |
| 1475 | 30 (30) A, M | 0.0063 | 2.94 |
| 1590 | 21 (30) A, M | 0.018 | 2.36 |

*Time point 2*

This analysis was performed to obtain information about the differences in protein expression of the host cell lines compared to the recombinant cell lines in stationary phase.

*Table 5. 28 Comparison of the protein expression of the host and the recombinant cell lines at tp 2 – These proteins were higher expressed in the host cell lines at tp 2.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 470 | 24 (30) A, M | 0.0077 | -2.81 |
| 962 | 30 (30) A, M | 0.00048 | -3.07 |
| 1280 | 27 (30) A, M | 0.014 | -2.5 |
| 1741 | 24 (30) A, M | 0.0028 | -2.71 |

*Table 5. 29 Comparison of the protein expression of the host and the recombinant cell lines at tp 2 – These proteins were higher expressed in the recombinant cell lines at tp 2.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1043 | 27 (30) A, M | 0.0052 | 2.51 |
| 1475 | 30 (30) A, M | 0.015 | 2.13 |
| 1481 | 30 (30) A, M | 0.0055 | 1.83 |

By comparing the results of the comparison of the host and the recombinant cell lines of the two time points it can be seen, that the protein expression in the host respectively recombinant cell lines changed during the experiment and the proteins higher expressed during the first time point were different from the proteins found during the second time point. Not one protein could be found as higher expressed at both time points.

*The cell lines*

This analysis was performed to obtain information about the differences in protein expression of the specific cell lines by comparing the two time points, tp 1 and tp 2.

*3F8*

*Table 5. 30 Comparison of the protein expression of CHO EpoFc 3F8 at tp 1 and tp 2 – These proteins were higher expressed at tp 1.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 722 | 8 (8) A, M | 0.052 | -2.13 |
| 1365 | 8 (8) A, M | 0.087 | -2.07 |
| 1753 | 8 (8) A, M | 0.055 | -2.1 |

No proteins were found, which were higher expressed at tp 2.

*14F2*

*Table 5. 31 Comparison of the protein expression of CHO EpoFc 14F2 at tp 1 and tp 2 – These proteins were higher at tp 1.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 859 | 9 (9) A, M | 0.00096 | -2.04 |
| 922 | 9 (9) A, M | 0.019 | -2.49 |

*Table 5. 32 Comparison of the protein expression of CHO EpoFc 14F2 at tp 1 and tp 2 – These proteins were higher expressed at tp 2.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 1501 | 9 (9) A, M | 0.017 | 2.08 |
| 1699 | 9 (9) A, M | 0.014 | 2.17 |

*K1PD*

*Table 5. 33 Comparison of the protein expression of CHO K1PD at tp 1 and tp 2 – These proteins were higher expressed at tp 1.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 747 | 7 (7) A, M | 0.016 | -3.32 |
| 970 | 7 (7) A, M | 0.001 | -4.11 |
| 1470 | 7 (7) A, M | 0.013 | -2.25 |

No proteins were found, which were higher expressed at tp 2.

*dhfr⁻*

*Table 5. 34 Comparison of the protein expression of CHO dhfr⁻ at tp 1 and tp 2 – These proteins were higher expressed at tp 2.*

| master no. | appearance | students t-test | average ratio |
|---|---|---|---|
| 200 | 10 (10) A, M | 0.016 | 4.19 |
| 725 | 10 (10) A, M | 0.013 | 2.61 |
| 1271 | 7 (10) A, M | 0.023 | 2.27 |
| 1358 | 10 (10) A, M | 0.018 | 3.19 |
| 1394 | 10 (10) A, M | 0.015 | 2.29 |

No proteins were found, which were higher expressed at tp 1.

At the CHO EpoFc 3F8 and CHO K1PD cultures only proteins were found, which were significantly higher expressed at time point 1, which represents the samples taken at the beginning of the experiment. For CHO dhfr⁻ only proteins were found, which were higher expressed at time point 2, which represents the samples taken at the end of the experiment. In the CHO EpoFc 14F2 cultures proteins at both time points were found to be significantly higher expressed.

## 5.5 Results of the MS analysis

Six spots could be identified. The peptide patterns from the other picked proteins, among them the prominent protein 1358, were acquired, but could not be identified based on the database. One of the problems was that the quantity of the samples was small. Also, peptide signal from trypsine, which was used during the sample preparation (tryptic digestion) resulted in a spectrum, that was over-layered by the trypsine spectrum.

*Table 5. 35 Identified proteins*

| spot number | expasy.org Acc. No. | protein name |
|---|---|---|
| 580 | O35501 | Stress 70 protein |
| 713, 718, 725 | Q91Z81 | ERp57 protein disulfide isomerase |
| 737 | Q8CIZ7 | *Dihydrolipoyl dehydrogenase* |
| 822 | P38660 | Protein disulfide-isomerase A6 precursor |

The spot *580* was identified as the *stress 70 protein*. This is a 75kDa glucose regulated protein from the heat shock protein 70 family [60]. It is encoded by the gene HSPA9 / GRp75 and is located in the inner membrane of the mitochondrion in the cell [61]. The protein is implicated in the control of the cell proliferation and cellular aging [60]. The Hsp70 family [62] of proteins constitutes one of the major molecular chaperone proteins in eukaryotic cells. These proteins perform an essential cellular role by binding to partially unfolded nascent polypeptides and assisting both in their proper folding and assembly into multi-protein complexes and in their transport across various intracellular membranes [63]. In eukaryotic cells distinct Hsp70 homologues encoded by separate genes are found in the cytosol, endoplasmatic reticulum, mitochondria and chloroplasts [63].

All members of the Hsp70 [63] chaperone class possess two distinct domains: a highly conserved N-terminal ATPase domain and a more divergent C-terminal domain, which binds short hydrophobic peptides of target substrates.

Hsp70 chaperone function requires the N-terminal ATPase domain, which, interestingly, is similar structurally to rabbit skeletal muscle actin despite little sequence similarity. Up-regulation of Hsps beyond a critical threshold may have deleterious cellular consequences [64]. Distinct functions between Hsp70 members were reported recently to exist in regions outside the peptide binding domain, suggesting additional levels of complexity to chaperone functions in vivo [65].

The three spots *713, 718 and 725* belonged to actually one protein, which was highly expressed and prominent on all gels. It was identified as *ERp57 protein disulfide isomerase*. Protein disulfide isomerases (PDIs) constitute a family of structurally related enzymes [66]. They are involved in the proper folding and in the formation and reshuffling of the disulfide bridges of the proteins synthesized in the rough ER, imported in the lumen of this structure and destined to be secreted or incorporated in the cell membrane. Each protein contains two or more active sites.

Their function, which is essential for cell viability, is that of chaperones and redox-catalysts. As chaperones they are part of a quality-control system for the correct folding of the proteins at the rough ER, while as redox catalysts, due to their thioredoxin-like active sites, they are important for the cell redox homeostasis. The ERp57 participates in the assembly of the major histocompatibility complex I (MHC I) [67].

In case of redox potential stress to the cells, after treating the cells with oxidants like $H_2O_2$ or NAC (N-acetyl-L-cysteine) the ERp57 was highly significantly up-regulated [68].

Spot *737* was identified as *dihydrolipoyl dehydrogenase* [69]. It has the synonym EC 1.8.1.4 *dihydrolipoamide dehydrogenase*. It is encoded by the gene Dld. One FAD per subunit binds to this protein as co-factor. This enzyme catalyses the oxidation of protein N(6)-(dihydrolipoyl)lysine to protein N(6)-(lipoyl)lysine.

Lipoamide dehydrogenase [70], a homodimeric FAD-dependent disulfide reductase, is usually described as the common component E3 of the mitochondrial 2-oxoacid dehydrogenase multi-enzyme complexes. These complexes catalyse the oxidative decarboxylation of 2-oxoacids (pyruvate, 2-oxoglutarate and the

three short branched-chain 2-oxoacids produced by transamination of the amino acids leucine, isoleucine and valine) to the corresponding acyl-CoA derivatives [71] – [73]. It is also the L-protein component of the glycine decarboxylase system [74] – [77]. The lipoamide dehydrogenase has a redox disulfide and a tightly, but noncovalently, bound FAD cofactor, both of which participate in the electron transfer from dihydrolipoamide to $NAD^+$ [78].

Spot 822 was identified as *protein disulfide-isomerase A6* (precursor) or EC 5.3.4.1 *protein disulfide isomerase P5* (synonym) [69]. It is encoded by the gene PDIA6 and member of the protein disulfide isomerase family as described above. It is located in the ER lumen and catalyses the rearrangement of –S–S– bonds in proteins. This enzyme contains two thioredoxin domains.

## 5.6 Description and discussion of the proteomic results

The goal was to identify proteins that were differently expressed during the cultivations. These proteins were significantly down-regulated or up-regulated on the gel images from the protein samples with the DeCyder software and identified by MS analysis. During the different statistical analysis conducted with the software, significantly regulated proteins were found. Most prominent down-regulated proteins, which showed up in the results of many analyses were 1358, 580 and 1428. 864 was the most frequent up-regulated protein at the analyses.

During the analysis, which included all gels, 16 down-regulated and 6 up-regulated proteins could be found. 10 of the down-regulated proteins had good values while 3 of the 6 up-regulated proteins were prominent. Among these most significant down-regulated spots were the 6 spots identified by the mass spectroscopic analysis. For the description of these proteins and their function in the cell see 5.5. By comparing the results from the analyses, which included either only the recombinant cell lines 5.4.2 or the host cell lines 5.4.3, one could see the following. At the proteins higher expressed in the recombinant cell lines of the 37 ℃ cultures, two proteins, 658 and 747, were found, which did not show up during the analysis of the host cell lines. 1358 had an average ratio value of 2.03, while at the host cells it had only an average ratio value of 1.72. That means that

this protein is more down-regulated respectively lower expressed at 33 ℃ in the recombinant cells, than it is in the host cells. Protein 1428 is with an average ratio value of 2.58 higher down-regulated in the host cell lines, than in the recombinant cell lines with an average ratio value of 1.78. Protein 580 was only significantly down-regulated in the host cells. By comparing the up-regulated proteins, the proteins 864 (with a difference of 50 % in the expression level) and 1682 (with a difference of 36 % in the expression level) were higher up-regulated respectively higher expressed at 33 ℃ in the recombinant cells, while protein 1116 was nearly identically up-regulated in both cell line groups.

By comparing the two analyses of the 33 ℃ cultures 5.4.4 and the 37 ℃ cultures 5.4.5, the following differences of the protein expressions showed up. The recombinant cells of the 37 ℃ cultures had a sligh tly higher protein expression of the proteins 713 (with a difference of 30 % in the expression level), 1043 (with a difference of 38 % in the expression level) and 1475 (with a difference of 27 % in the expression level) than the recombinant cells cultivated at 33 ℃. Protein 1481 was higher expressed by 24 % in the recombinant cells incubated at 33 ℃.
The higher expressed proteins in the host cells incubated at 37 ℃ were the proteins 962, 1280 and 1309. These proteins were not significantly altered in the host cells incubated at 33 ℃. Protein 849 was foun d in the analysis of the 33 ℃ cultures and had a very high relative expression rate (average ratio of -3.37) in the host cells compared to the recombinant cells.

When comparing protein expression in the cell lines at 33 ℃ and 37 ℃ incubation temperature (5.4.6), the following differences in protein expression were found. The two most significantly proteins higher expressed at 37 ℃, 1358 and 1428, will be discussed.

*1358*

CHO EpoFc 3F8 had the highest down-regulation effect respectively the highest reduction of the protein expression in the 33 ℃ cu lture with an average ratio value of 2.28 followed by the CHO K1PD with a value of 2, CHO EpoFc 14F2 with a value of 1.78 and CHO dhfr$^-$ with a value of 1.55.

*1428*

The CHO EpoFc 3F8 showed again the highest down-regulation with an average ratio value of 2.21, followed by CHO K1PD with a value of 2.08 and CHO EpoFc 14F2 with a not significant value of 1.32. $Dhfr^-$ had no calculable average ratio value.

The result of the analyses of the comparison of the protein expression in the exponential phase and the stationary phase showed, that all cell lines had different proteins expressed differentially.

## 5.7 Concluding remarks

DIGE, with DeCyder image analysis software, facilitates increased confidence of detection and quantification of protein differences between samples [58]. Many statistical analyses are possible by using an internal pooled standard, because of the many samples that can be correlated with the DIGE method.

The incorporation of a temperature reduction step is commonly employed during production cell culture in the biopharmaceutical industry. This temperature shift is used as a means of simultaneously inducing growth arrest and extending long term culture viability thus increasing recombinant protein productivity and yield [2].

The goals of this study were to obtain information about the cell behaviour and their metabolism and to identify proteins, whose expression was altered, under hypothermic conditions. The data obtained during this diploma thesis may help to optimise cultivation conditions for biopharmaceutical products [3].

# Chapter 6 – References

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