Transient Transfection of HEK293 Cells in Suspension: Process Characterization and Optimization by Applying Invasive Nucleotide and Non-invasive Electronic Nose Technology

Doctoral Thesis

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Transient Transfection of HEK293 Cells in Suspension: Process Characterization and Optimization by Applying Invasive Nucleotide and Non-invasive Electronic Nose Technology

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To my husband and my little son

Ignacio A. Favale and Juan Ignacio Favale Bassani

To my parents

Humberto A. Bassani and Blanca E. Molinas de Bassani

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Table of contents

Abreviations

AA	amino acids
ADP	adenosine diphosphate
AEC	adenylate energy charge
Ala	alanine
AMP	adenosine monophosphate
Arg	arginine
Asn	asparagine
Asp	aspartate
ATCC	american type culture collection
ATP	adenosine triphosphate
BHK	Baby hamster kidney
bp	base pair
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CMV	cytomegalovirus
Conc.	concentration
CTP	cytidine triphosphate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylen diamine tetra acetatic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked imnunosorbent assay
EPO	erythropoietin
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
Fig.	figure
FSC	forward scatter
GalNAc	N-acetyl-galactosamine
GDP	guanosine diphosphate
GFP	green fluorescent protein
GlcNAc	N-acetyl-glucosamine
Gln	glutamine
Glu	glutamate
Gly	glycine
GMP	guanosine monophosphate
GNAc	GlcNAc + GalNAc
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney
His	histidine
HPLC	high performance liquid chromatography
hpt	hours post transfection
IRES	internal ribosome entry site
Iso	isoleucine

LB	Luria Bertoni
Leu	leucine
Lys	lysine
MCS	multiple clone size
Met	methionine
NAD	nicotine adenine dinucleotide
NADP	nicotine adenine dinucleotide phosphate
No.	number
NTP	nucleotide triphosphate
ori	origin of replication
Orn	ornithine
PBS	phosphate buffered saline
PEI	polyethylenimine
Phe	phenylalanine
Pl.	pluronic F68
rhuEPO	recombinant human erythropoietin
RT	room temperature
SD	standard deviation
Ser	serine
SMIF	Scharfenberg's modification
SSC	side scatter
Thr	Threotnine
TRIS	tris-(hydroxymethyl)-aminomethane
Trp	tryptophan
TTran.	transient transfection
Tyr	tyrosine
U	uridine
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate
Val	valine
Vit.	vitamin solution

Units

g	gram
mg	milligram
µg	microgram
ng	nanogram
L	liter
mL	milliliter
µL	microliter
mol	mole
mmol	millimole
µmol	micromole
pmol	picomole
% W V^{-1}	gram of solute in 100 mL of solution
% V V^{-1}	volume of solute in 100 mL of solution
m	meter
mm	millimeter
µm	micrometer
nm	nanometer
h	hour
min	minute
s	second
V	volt
Ω	ohm
µF	microfaraday
kW	kilowatt
rpm	revolutions per minute
× g	relative centrifugal forces
µkat	microkatal (µmol s^{-1})
kDa	kilodalton

Summary

The increasing demand for milligrams of recombinant proteins (r-proteins) to be used for therapeutics and structural studies justifies the need for a rapid and scalable expression system. Transient transfection of mammalian cells is a powerful technology for the fast production of large amounts of these r-proteins. This system depends on a multitude of different factors including the cell line, its physiological state, the type of expression vector and the medium formulation. Moreover, monitoring bioprocess parameters, and control of the bioprocess at its optimal state, enable the reduction of production costs, increase the yield and maintain the quality of the desired product. Off-line and on-line monitoring methods have been used to characterize and to optimize the transient transfection process using HEK293 cells and polyethylenimine (PEI) as transfection mediator. As an off-line method the intracellular nucleotide analysis was used, which has been considered as a reliable tool reflecting the metabolic status, the growth potential, and the overall physiological condition of the cell. As an on-line and non-invasive method a completely new technology, the electronic nose, was applied for bioprocess monitoring and the identification of characteristic process states.

HEK293 cells were cultured in suspension (HEK293s) using a completely new medium, totally free of proteins or animal-origin components, which facilitates the transfection process by allowing growth and transfection of the cells without medium exchange. This is a central prerequisite for scaling the technology up to large volume cultivation processes. A special bicistronic plasmid was constructed under control of the cytomegalovirus promoter. The plasmid DNA was combined with PEI using an efficient minimum amount of DNA at a DNA:PEI ratio of 0.50:1.50 (µg:µg) showing 70-80 % of GFP positive cells under serum-containing as well as serum-free culture conditions. Specific cells taken from an early (from 40 to 77) and a late (from 89 to 150) passage number were compared as hosts for transient transfection.

The combined NucleotideTriPhosphate ratio expressed as NTP/U = [ATP+GTP] [UDP-GNAc] / [UTP+CTP] [UTP] was calculated in parallel during cultivation and transient transfection under serum-containing and serum-free culture conditions for process monitoring and characterization. The results showed that nucleotide ratios can be used to distinguish between:

- HEK293s cells from early and late passage numbers.
- Cells cultured under serum-containing conditions from those under protein/serum-free medium conditions.
- Transfected and untransfected cells in culture.
- DNA:PEI complex uptake (transfection procedure) from PEI uptake alone.

The BioNose was connected to the off-gas line of the bioreactor cultivation. The data were collected using the NST Senstool software and evaluated applying multivariate methods such as Principal Component Analysis (PCA). The results showed, that the BioNose can generate

characteristic pattern specific for a particular process being an easily accessible tool to monitor cell cultivations as well as to distinguish between different culture conditions.

In conclusion, a transient transfection procedure easy to perform, cost-effective, for a large-scale application using the mammalian cell line HEK293s cultivated in a medium totally free of proteins or components of animal origin was developed. In addition, the characterization of the transfection procedure using an off-line method such as intracellular nucleotide patterns, and an on-line method such as the electronic nose, has shown the applicability of both methods as appropriate and even excellent tools for process optimization.

1. Introduction

1.1 Animal cell culture: historical outline

Nearly 100 years of progress and drawbacks have elapsed from the beginning of animal cell "cultivation" to the mass production of cells and products. Animal cell cultivation began in the last decade of the nineteenth century with the first tentative experiments to maintain pieces of tissue in plasma or ascites fluid over several days or even weeks. The success of those experiments was limited by the quality of the nutrient fluid and the sterility of the experimental set up. In 1907, Ross Harrison was the first to record the maintenance and growth of nerve cells in a hanging drop over a period of up to 30 days. Harrison and his successors observed that strict aseptic conditions were crucial for the success of such experiments. These experiments (Harrison 1907) showed that normal cell functions can continue *in vitro.* Therefore the year 1907 is commonly regarded as marking the beginning of cell cultivation (Kretzmer 2002).

During the next four decades, the progress of cell cultivation was limited due to the stringent sterility controls necessary. The development of antibiotics in the late 1940s was another milestone. The addition of antibiotics eased the handling of complex undefined culture media. At the same time, the development of sterility techniques took place. During the following decade, great progress was made towards mass cultivation of animal cells and production processes. Maitland and Maitland (1928) had developed a simple method for virus propagation in tissue culture, but it took the findings of Enders and his co-workers, who showed that poliomyelitis virus could be cultured in HeLa cells and used as a vaccine, to indicate a great milestone towards production processes (Enders et al. 1949).

The most important breakthrough towards large-scale cultivation was achieved by Earle and Eagle, who made an extensive analysis of the requirements of cells *in vitro*. In 1955, Eagle reported a chemically defined medium known as EMEM (Eagle´s Minimum Essential Medium), which could replace the biological fluids used thus far (Eagle 1955). However, the cellular requirements for growth were not found to be satisfied until a 5 % to 10 % addition of blood serum was made (Butler 1986).

Serum from young or unborn animals was found to be particularly efficacious. It contains undefined materials essential for cell proliferation. However, blood serum is of undefined composition and varies between batches. This leads to many difficulties in reproducing experiments on a small-scale and in maintaining consistent yields of cell or their products on the large-scale. Consequently, numerous attempts have been made to

develop media suitable for the growth of cells in vitro in the absence of serum (Higuchi 1973).

The first permanent animal cell lines were established in the fifties and sixties (Capstick et al. 1962; Stoker and Macpherson 1964). Furthermore, in the beginning of the eighties the development of the hybridoma technique and expression of recombinant proteins in cultured animal cells contributed to their technological and commercial exploitation.

The development of a chemically defined medium, together with the development of permanent cell lines that can be sub-cultured indefinitely and which have the possibility to grow in suspension, had an enormous impact on large-scale cultivation of animal cells.

1.2 Animal cell culture: importance in protein production

The progress in gene manipulation and cell fusion has made it possible to produce large amounts of physiologically active proteins through cell cultivation. In the production of pharmaceuticals, microorganisms such as *Escherichia coli* have been chosen first as host cells due to their relative simple cultivation. The production of proteins by these microorganisms, however, revealed that some products fail to manifest their biological properties sufficiently. One particular reason for this was a deficiency of the products in their tertiary or quaternary structure. Yeasts also have the capacity to perform post-translational modifications in their synthesized proteins, but these were found to differ from their natural form (Andersen and Krummen 2002). Consequently, the production of recombinant proteins (r-proteins) has been briskly investigated using animal cells as hosts because these cells can perform the post-translational modification in an authentic manner.

Recombinant proteins can also be produced using transgenic plants and animals, as well as plant tissue cultures (Doran 2000; Giddings et al. 2000; Larrick and Thomas 2001). For example, more than 50 recombinant proteins have already been obtained from the milk of transgenic animals (Houdebine 1997) but the recombinant proteins obtained in this milk do not always have their normal biochemical structure. They may be underglycosylated, not fully γ-carboxylated or cleaved. In addition, limits in glycosylation and the problem of proteolysis have been cited as one challenge for recombinant production in plant culture.

Insect cells have been used in a variety of protein expression applications (Jäger 1996), particularly related to high-throughput expression of sequences for functional screening. However, some limitations had appeared to generate corrected glycosylated proteins (Andersen and Krummen 2002).

Due to the ability to carry out post-translational modifications as well as protein folding in an authentic manner (Hauser 1997), mammalian cells are the most common choice of production host for recently approved protein therapeutics (Chu and Robinson 2001). Over the past seven to eight years there has been considerable progress in fine-tuning mammalian expression systems for high-level recombinant gene expression (Andersen and Krummen 2002).

Some properties of host cell lines for recombinant protein production are listed below (Hauser 1997):

- Efficient expression of foreign genes.
- Post-translational modifications.
- Stability of transgenic expression.
- Cultivation requirements.
- Optimal production under controlled fermentation conditions.

Mammalian cells are classified on the basis of their proliferation mode into anchorage-dependent cells and anchorage-independent cells. Anchorage-dependent cells attach to solid surfaces and proliferate, while the others proliferate in suspension and do not need attaching surfaces (Tokashiki and Yokoyama 1997). The development of bioreactors has been made mainly on the basis of the techniques utilizing these properties. Bioreactors for suspension cells are classified by two types: Stirred tank bioreactor (Griffiths 1988) and Airlift or bubble column (Birch et al. 1985; Griffiths 1988). Nowadays, a large number of cultivation processes is existing to cover the cellular demand such as batch and fed-batch culture, and continuous culture, that includes chemostat and perfusion culture (Tokashiki and Yokoyama 1997). The process type and the scale chosen for the considered product depend on the aim of the study, the amount required, the market price and the feasibility of the process.

In the present, the ability to adapt many cell types to suspension culture and the use of polymeric additives to reduce shear effects led to widespread applications of suspension cell cultures (Chu and Robinson 2001).

1.3 Gene expression in mammalian cells

Developments in molecular biology over the past two decades have led to the cloning of numerous mammalian genes. The study of mammalian gene structure and function has been greatly enhanced by the use of eukaryotic expression vector systems. These vector systems facilitate the introduction of specific genes into the mammalian cells, the transcription of these genes and the subsequent production of proteins that are appropriately modified to reflect native molecular structure and biological function.
These mammalian expression vector systems have advantages that include but are not limited to the ability to (Colosimo et al. 2000):

- Express constitutive and inducible proteins.
- Produce a large quantity of protein that is post-translationally modified and appropriately folded.
- Characterize the impact of specific mutations on cell metabolism.

Nowadays, a wide variety of techniques and reagents are used to deliver macromolecules into eukaryotic cells. Currently, the following two approaches are used to deliver specifically nucleic acid into these cells (Guide to eukaryotic transfections with cationic lipid reagents, Life Technologies, 1999):

- *Transfection*: process in which the gene of interest is introduced into eukaryotic cells by biochemical or physical methods.

- *Infection*: viral mediated process where target cells are infected with a virus carrying the cloned sequence of interest in its genome.

DNA delivery by infection is more complicated than transfection. Generally, infection requires more steps and more time than does transfection, in addition biosafety issues also may arise, depending on the virus being used. While viral infection efficiencies are high in permissible cell lines, efficiencies are low or non-existent in cell lines lacking the receptor for the virus. DNA delivery by transfection, however, is faster and requires only a few reagents including plasmid DNA containing the gene of interest under the control of a strong promoter. Its simplicity and the numerous advances that have increased its level of efficiency have made transfection a far more popular method for nucleic acid delivery (Guide to eukaryotic transfections with cationic lipid reagents, Life Technology, 1999).

When the cells take up DNA, they express it transiently over a period of several days to several weeks and eventually the DNA is lost from the population.The ability to express this DNA over a short period of time is called “transient expression” (Kaufman

1997). The DNA is transferred into the nucleus of the cell but does not integrate into the chromosomes. Whilst when the transfected DNA is either integrated into the chromosomal DNA or maintained as an episome, the transfection is called “stable transfection” (The QIAGEN transfection resource book, QIAGEN, 1999) (See below under 1.3.1).

Many transfection techniques have been developed. Desirable features include high efficiency transfer of DNA to the nucleus, minimal interference with normal cell physiology, low toxicity, ease of use, reproducibility, successful generation of stable transfectans, and *in vivo* efficacy (Transfection guide, Promega, 1999). The techniques developed for gene transfer can be broadly classified as either chemical reagents (calcium phosphate, cationic polymers, liposomes, molecular conjugates) or physical methods (electroporation, biolistic, microinjection) (Colosimo et al. 2000).

The selection of DNA transfer method depends on numerous factors including the efficiency required, whether the transfection is *in vitro* or *in vivo* and whether the expression is stable or transient. The use of any strategy depends on the goals of the experiment and study. Once the endpoints are defined, it is possible to design the most effective strategy to achieve a desired outcome.

1.3.1 Transient transfection versus stable transfection

Transient transfection

Following DNA transfection, expression of the transgene may be detected transiently for 1 to 4 days. The optimal time interval depends upon the cell type, the doubling time of the cells and the specific characteristics of expression for the transferred gene (Colosimo et al. 2000). The DNA delivered to the cells is transported to the nucleus for transcription without integration into the chromosomes. This means, that many copies of the gene of interest are present, leading to high levels of expressed protein. Transient transfection is most efficient when supercoiled plasmid DNA is used (The QIAGEN transfection resource book, QIAGEN, 1999). Within a few days most of the foreign DNA is degraded by nucleases or diluted by cell division; and after a week, it can no longer be detected. Transient expression assays detect gene expression from unrearranged plasmid DNA. Thus, the level of expression is position independent and not influenced by surrounding chromosomal elements. Less time and labor are required to assay transient transfection than stable expression but as the efficiency of DNA uptake and level of expression can variable from one assay to the next, experiments must be carefully controlled.

Transient expression systems are extremely useful for studying elements that regulate gene expression or when it is important to have experimental results within a

short time frame (Colosimo et al. 2000). This system offers a convenient means to compare different vectors and ensures that an expression plasmid is functional before using the expression plasmid to establish a stable transfected expression cell line (Kaufman 1997). More recently, this method has demonstrated its ability to produce large amounts of recombinant proteins in large-scale (see under 1.3.2) (Durocher et al. 2002; Girard et al. 2002; Jordan et al. 1996; Meissner et al. 2001; Pham et al. 2003; Pick et al. 2002; Schlaeger and Christensen 1999; Wurm and Bernard 1999).

Transient expression system can be evaluated in terms of the protein products synthesized in the transfected cell, such as the activity of a reporter gene that is not endogenously expressed in the cell type used. The most common reporter gene systems include bacterial chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), firefly luciferase (Luc), human placental alkaline phosphatase (AP), β-glucuronidase (GUS) and green fluorescent protein (GFP) (Transfection guide, Promega, 1999; Guide to eukaryotic transfections with cationic lipid reagents, Life Technologies, 1999; The QIAGEN transfection resource book, QIAGEN, 1999; Alam and Cook 1990). Presently, GFP is widely used to estimate DNA uptake efficiencies in mammalian cells because it can be detected with high sensitivity and minimal invasive treatment in live cells (Pick et al. 2002). Moreover, the GFP variant, the enhanced GFP (EGFP), includes chromophore mutations that make it 30 – 35 times brighter than wild-type GFP. It can be detected by a variety of methods such as spectrofluorimetry, microscopy and flow cytometry (www.bdbiosciences.com/clontech).

Stable transfection

In order to achieve stable transfection, the transgene must be able to replicate in synchrony with the cell. This occurs as a result of spontaneous integration of the transfected plasmid into the host genome. In a small fraction of transfected cells, the added DNA is incorporated into the cell's genome by recombination. Although linear DNA results in lower DNA uptake, it yields optimal integration of DNA into the host genome (The QIAGEN transfection resource book, QIAGEN, 1999). Cells containing integrated DNA are rare and must be amplified by selection for drug resistance or identified as a result of phenotypic alteration. Stable gene expression experiments require several weeks to perform and longer if it is necessary to verify protein yield. However, the resulting cell line could be a stable source for protein production or used to develop transgenic animals (Guide to eukaryotic transfections with cationic lipid reagents, Life Technologies, 1999).

These systems are particularly useful when large quantities of protein are required. Stable transfection technologies have delivered kilograms of complex proteins from mammalian cells (Colosimo et al. 2000; Wurm 1990).

Typically, cells are maintained in non-selective medium for 1 – 2 days post-transfection, then cultivated in selective medium containing the drug. The use of the selective medium is continued for 2 – 3 weeks, with frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized. Individual colonies are sub-cloned to multi-well plates for further propagation in the presence of selective medium.

A typical vector used to enrich successfully transfected cells will carry a gene essential for the survival of a given cell line that is either defective in the gene or void of the gene. Classic selectable markers such as thymidine kinase (tk), dihydrofolate reductase (dhfr), hypoxanthine guanine phosphoribosyl transferase (hprt) and adenyl phosphoribosyl transferase (aprt) genes can only be used in cells deficient in TK, DHFR, HPRT and APRT, respectively. Alternatively, genes that confer resistance to cytotoxic drugs are quite effective in situations when the cell line is not defective in one of the endogenous genes mentioned earlier. For example, the most common selection marker is the aminoglycoside phosphotransferase (aph) gene that confers resistance to antibiotics such as kanamycin, neomycin and geneticin (Transfection guide, Promega, 1999; Guide to eukaryotic transfections with cationic lipid reagents, Life Technologies, 1999; The QIAGEN transfection resource book, QIAGEN, 1999).

An additional approach to select or enrich transfected cells relies on the fluorescence-activated cell sorting (FACS) analysis. Cell selection is based on expression of cell surface proteins that bind specific antibodies, (for example, human and murine CD24), that will facilitate FACS identification of cells carrying the expression vector (Colosimo et al. 2000).

1.3.2 Large-scale transient transfection

The great advances made in genomics and proteomics during the past decade have positioned r-proteins as major therapeutic biomolecules and drug targets (Chu and Robinson 2001; Kelley 2001). The increasing demand for milligrams of r-proteins to be used in pre-clinical, biochemical, and biophysical studies justifies the need for a rapid and scalable expression system. As was discussed above, mammalian expression systems are more appropriate for providing soluble and fully processed r-proteins. Widely used stable expression systems require considerable investment in time, human resource and bioreactor equipment, therefore this technology is not readily amenable to high-throughput mode, while transient transfection technology has demonstrated to produce large amounts of r-proteins within a few days (Bernard 2000; Durocher et al. 2002; Girard et al. 2002; Jordan et al. 1998; Meissner et al. 2001; Pham et al. 2003; Pick et al. 2002; Schlaeger and Christensen 1999; Wright et al. 2003; Wurm and Bernard 1999; Wurm and Bernard 2001).

Transient gene expression in mammalian cell lines was reviewed by Wurm and Bernard (1999) and they described the key features of most transient expression systems, of which some have been mentioned above (1.3.1):

- Simplicity, particularly in the construction of expression vectors.
- Extremely short time frame for the generation of product (days).
- Intrinsic genetic stability and consistency due to extremely short time frame between generation of a vector and the product recovered.
- Applicability to a wide range of host cell lines.
- Suitability for multiple processing, allowing studies of many genes or mutants at the same time.

For an optimal large-scale transient transfection and r-protein expression in mammalian cells, some key aspects need to be taken into account such as the cell line, the expression vector, the transfection vehicle, and the culture medium.

Cell line

Three cell types dominate the field: human embryonic kidney 293 (HEK293), COS and baby hamster kidney (BHK) (Wurm and Bernard 1999). Recently, Chinese hamster ovary (CHO) cell line was used by Genentech (Wurm and Bernard 2001).

HEK293, is widely used for r-protein production as it offers many advantages such as high transfection yields with most gene transfer vehicles, simple adaptation to growth in suspension culture, and adaptation to serum free-media (Garnier et al. 1994; Graham 1987). Moreover, the cells can be efficiently transfected in suspension using low-cost vehicles such as polyethylenimine (Durocher et al. 2002; Pham et al. 2003; Schlaeger and Christensen 1999; Schlaeger et al. 1998a; Schlaeger et al. 1998b) or calcium-phosphate (Girard et al. 2002; Jordan et al. 1998; Jordan et al. 1996; Jordan and Wurm 2004; Meissner et al. 2001; Pick et al. 2002; Wright et al. 2003).

Several different cell lines have been derived from the HEK293 parent. The two most popular variants for transient expression are HEK293T, expressing the SV-40 large T antigen (Kim et al. 1997) and HEK293-EBNA, expressing the nuclear antigen from Epstein-Barr virus EBNA (Cachianes et al. 1993), that allow episomal amplification of plasmids containing the viral SV40 (293T) or EBV (293E) origins of replication (Van Craenenbroeck et al. 2000).

HEK293 cells are also used for virus production, cell cycle research, gene expression, metabolism, receptor binding, and other studies. This epithelial cell line was derived from primary human embryonic kidney cells that were transformed using shared DNA from adenovirus type 5 (Graham et al. 1977).

The expression vector

For high level r-protein expression it is important to use vectors with promoters that are highly active in the host cell line, such as the human cytomegalovirus (CMV) or SV40 promoters. One non-viral promoter, the elongation factor (EF)-1 promoter, has also been used because it appears to be as strong as some of the viral promoters (Wurm and Bernard 1999). The CMV promoter is particularly powerful in HEK293 cells, where it has been shown to be strongly transactivated by the constitutively expressed adenovirus E1a protein (Gorman et al. 1989).

The transfection vehicle

Even though many highly effective gene transfer reagents are commercially available, only a few are relatively cheap and are considered for large-scale transfections, namely calcium phosphate, polyethylenimine (PEI) and electroporation (Wurm and Bernard 1999).

Especially the cationic polymer, PEI, exhibits several properties which are extremely important for high efficient gene delivery (Boussif et al. 1995; Boussif et al. 1996). For example, PEI-mediated transfection methods are highly efficient in a broad range of different cell lines and the cationic agent shows a low level of cell toxicity, it is simple to use and is effective in suspension cultures. Moreover, it is cheap and therefore useful for scale up experiments (Durocher et al. 2002; Pham et al. 2003; Schlaeger and Christensen 1999; Wurm and Bernard 1999). This polymer is available in both, linear and branched isoforms with a wide range of molecular weights and polydispersities (Godbey et al. 1999). Boussif et al, (1995 and 1996) described PEI with a high cationic-charged-density potential. This cationic charge density is due to the presence of a potentially protonable amino nitrogen at every third atom which may participate in DNA condensation. These protonable amino groups may also play a protective role in preventing DNA degradation in cytoplasmic endosomes as it has a high pH-buffer capacity. The so-called proton sponge effect was postulated to cause an early escape of DNA:PEI complexes from lysosomes, thus avoiding lysosomal degradation (Fig. 1.3.2a).

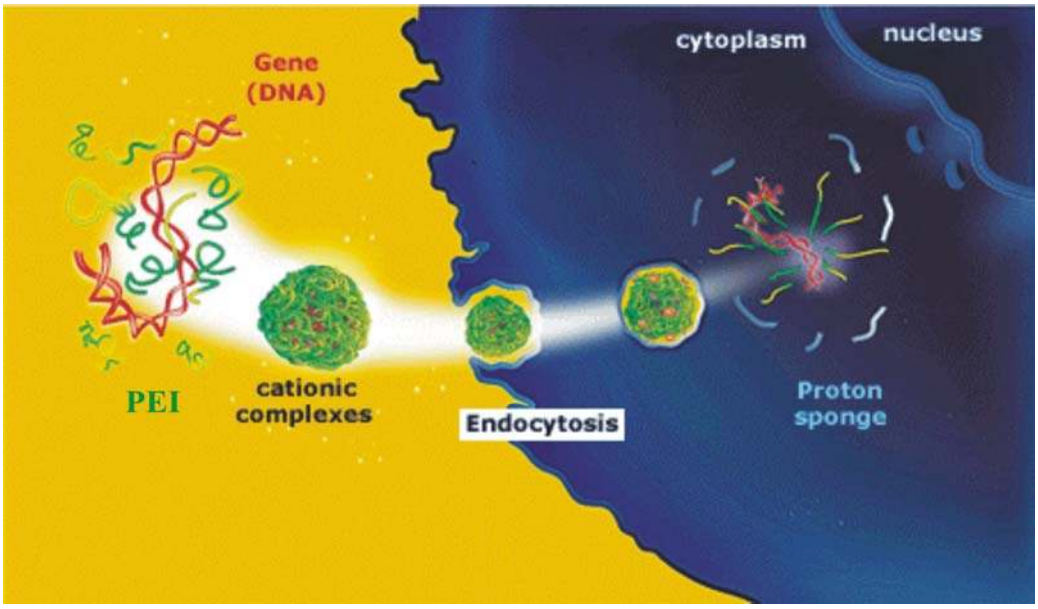


Fig. 1.3.2a: Mechanism of gene transfer into eukaryotic cells by PEI:DNA complexes (taken from: www.qbiogen.com, modified).

The culture medium

Some gene transfer reagents work only in serum-free medium whereas others are insensitive to the presence of serum, moreover, serum significantly increases the expression of r-proteins (Durocher et al. 2002). Also, the presence of cellular by-products in conditioned medium is associated with poor transfection yield. Hence, it is often necessary to perform a complete medium exchange prior to transfection (Schlaeger and Christensen 1999). However, this step does not satisfy the need for a robust large-scale transient transfection process. Additionally, in the production of secreted proteins, serum greatly interferes with their subsequent purification.

Moreover, numerous problems are associated with the presence of serum in culture. These are possible contaminations by infectious microorganisms which may be present in the serum, or by serum proteins, as well as the high cost and the batch-to-batch variability (Broad et al. 1991; Butler 1986).

Many commercially available serum-free media have been developed for HEK293 cells; however many of them do not support good transfection when using PEI and their cost-effectiveness is rather questionable (Pham et al. 2003). In addition, serum-free media offer the advantage of lowering the burden on purification of the r-proteins.

1.4 Monitoring of cultivation process

Monitoring and control of the cellular physiology is one of the great challenges in bioprocess engineering today. Unexpected variations in cell behavior often accompany fermentation processes due to unknown reasons. These variations strongly influence

process efficiency and therefore require adequate monitoring and control. Controlling the bioprocess at its optimal state enables the reduction of production costs, increases the yield and at the same time maintains the quality of the desired product. In general, bioprocesses are difficult to control. The main problem results from the inability to properly influence the cells internal environment by manipulation of their external environment. Several other factors further contribute to the control problem, such as the non-linear behavior of the cell, the highly complex metabolic network and that reliable methods for process state characterization are rarely available.

The particular control concept has been referred to as physiological state control, which was first proposed by Konstantinov and Yoshida (1989). Here, the physiological state of the cell culture is quantified by process variables that provide information about the status of the cells. Normally, these variables are specific metabolic rates or metabolic rate ratios. Despite the fact that today many of those variables can be measured on-line with commercially available sensors, this control strategy has not yet been implemented as a standard in industrial bioprocesses.

There are many aspects which contribute to the term “cell physiology”, as was defined by Grammatikos et al (1999). This term should mean the balance of all intracellular metabolic, growth-promoting and growth-inhibiting events, which result in cell growth and viability (or in growth arrest and/or cell death). Without cells in good physiological state, rapid growth without lags cannot be achieved, resulting in excessively long and, hence, costly pre-production scale-up steps. Cells in poor physiological state may not respond favorably to stress. Therefore, cell physiology should play the central role in a successful process development. Intracellular nucleotides were found to present one of the most valuable targets as they reflect the exact physiological state of a culture (Ryll and Wagner 1992). Therefore, nucleotides as a tool of monitoring bioprocess off-line will be discussed in detail later (see under 1.4. 1).

On the other hand, on-line monitoring of the physiological state of a cell culture is the method of choice but difficult to realize. For resolving the physiological state of a cell culture, one would have to incorporate several different sensors in the monitoring set up. However, a typical production scale set up often includes dissolved oxygen, pH and temperature sensors, and eventually a mass spectrometer (Heinzle 1992). Sensors for the determination of nutrient, metabolite and product concentrations, like biosensors, gas chromatography, HPLC, flow-injection and others, require sophisticated sterile liquid sampling systems and are therefore difficult to integrate into an existing reactor set up (Bachinger 1999). In the last years, the use of an electronic nose device, a combination of gas sensor arrays and pattern recognition techniques to simulate the human olfactory sense, has been used for bioprocess monitoring (Mandenius et al.

1997) solving some of these problems discussed above. The use of an electronic nose in bioprocesses monitoring will be discussed in detail under section 1.4.2.

1.4.1 Nucleotides and bioprocess control

Although only a small number of different cell lines are used regularly by the biotechnology industry, it is extremely important to realize that every recombinant cell is different. Every cell line has specific demands on the nutrient composition and the process control has to involve the measurement of several parameters with different uptake and secretion rates (Ryll and Wagner 1992). Moreover, the expression vectors, the transfection, cloning, and selection process, and the recombinant product of interest itself lead to as many different phenotypes as there are recombinant proteins. Because of this, universal culture conditions and universal production processes does not exist. For every new cell and product, often extensive process development has to be done for achieving maximum productivity and minimum costs (Grammatikos et al. 1999). The development and optimization of bioprocesses and the control of cellular growth is generally performed by the determination of specific nutrients such as glucose, glutamine, and sometimes other essential amino acids as well as secreted waste products, especially lactate and ammonia. The information provided is useful but often incomplete because it gives rare information about the state of the cells at the time of sampling and allows no reliable prediction. Cell counting allows only a post factor assessment of the cell state because the extent of cell proliferation measured at any given time point reflects the outcome of the respective pre-existing physiological condition. Due of these limitations, an exact reliable prediction of the growth behavior of a culture based on cell concentration measurement is not yet possible. The cellular viability, the most valuable parameter at present, is only an end result of intracellular physiological processes; a viability of x % does not reveal anything about the state of the remaining viable cells (Grammatikos et al. 1999).

Nucleotides have shown to be one of the most important substances for cell metabolism. They are involved in a number of cellular processes and have widespread regulatory potential. They participate as substrates, products, effectors, or energy donators in many cellular reactions. Additionally, fluctuations of pool size could affect alterations in transport processes, macromolecular synthesis, and cell growth (Hauschka 1973). Some evidence had been found supporting the idea that the pool size of ATP, the adenylate energy charge (AEC) or the pool size of UTP are, either influenced by or depended on the cell cycle (Rapaport et al. 1979), the stimulation of cells by serum or hypoxanthine (Grummt et al. 1977) or colchicine (Chou et al. 1984). Additionally, in plant cells a specific variation of the intracellular concentrations of nucleotides has been shown to be dependent on the growth cycle (Wylegalla et al. 1985).

Ryll and Wagner (1992) described the sensitivity of certain ratios of nucleotide triphosphates in responding to physiological changes of BHK and hybridoma cells and showed these parameters being useful in monitoring and control of production processes. In particularly, three combinations gave characteristic responses: the ratio of the sum of purine trinucleotides and pyrimidine trinucleotides (PurTP/PyrTP = NTP = [ATP + GTP]/[UTP + CTP]), the U ratio expressed as the quotient of UTP and UDP-GNAc (UDP-GNAc = sum of N-acetylated UDP-activated sugars) (UDP-GlcNAc + UDP-GalNAc) and the combination of both parameters NTP ratio/U ratio. The NTP ratio can be thought of as a measure of metabolic energy (represented by ATP + GTP) versus growth potential (represented by UTP + CTP) and is found to increase as a culture progresses, mainly due to notable decreases in UTP and CTP as cells enter the reduced exponential and eventually the stationary phase (Grammatikos et al. 1999; Ryll and Wagner 1992). The U ratio can be regarded as an argument of the growth energy versus the accumulation of ammonia which contributes UTP to the formation of UDP-GNAc (Ryll et al. 1994). Furthermore, when the U ratio decreases culture conditions get worse, mainly due to decreasing UTP concentrations and further due to an increase of the UDP-GNAc pool (due to accumulation of ammonia). Finally, the NTP/U ratio amplifies the NTP and U ratios and is, therefore, a very sensitive parameter which increases dramatically, compared to the increase of the NTP and the decrease of the U value. Increase in the NTP/U ratio can be taken to identify a worsening in cellular physiology and growth potential. These intracellular parameters have many applications, not only in the monitoring and the control of production processes but also in the development of efficient cell culture processes (Grammatikos et al. 1999).

1.4.2 Electronic nose and bioprocess control

Monitoring bioprocess parameters on-line is a method of choice. Apart from continuously being able to sample data by automatically programmed procedures, it is not necessary to take a sample manually which is time-consuming on the one hand and brings the necessary information about the process with time-shifting on the other hand.

The concept of the electronic nose, the combination of gas sensor arrays and pattern recognition techniques to model the human olfactory sense, was first proposed by Persaud and Dodd in 1982. Since then, this concept has been applied to a variety of applications with the main focus on the classification of the flavor of beverages or foodstuffs (Gardner and Bartlett 1994). Other examples of its applications are the analysis of medication off-odors (Schiffman et al. 1997), diabetes diagnosis (Ping et al. 1997) and the identification of microorganisms (Gardner et al. 1998; Gibson et al. 1997).

The use of the electronic nose for bioprocess monitoring was first evaluated by Mandenius et al., 1997 and Namdev et al., 1998. Mandenius et al. used a metal oxide sensor based electronic nose to explore the possibility of monitoring ethanol and biomass on-line in production scale baker's yeast fermentations. Namdev et al., applied a polymer sensor based instrument to detect abnormalities like variability in media composition in bioprocess. The potential of non-invasive chemical sensor arrays has been also shown in key process variables such as biomass and specific growth rate in *Escherichia coli*, as well as ethanol and glucose concentrations in *Saccharomyces cerevisiae* batch cultivations, which were estimated on-line with high accuracy (Bachinger et al. 1998b; Bachinger et al. 1998a). This technology had been applied for on-line monitoring of state transitions in perfusion culture of a CHO cell line producing recombinant human blood coagulation factor VIII in which it was possible to identify characteristic process states and visualize transitions related to product and lactate formation (Bachinger et al. 2000a; Bachinger et al. 2000c) and identify early contamination (Bachinger et al. 2002).

One limitation to on-line monitoring is often the need of instruments that endure heat sterilization still maintaining their sensor stability, reliability and sensitivity. For this reason it is most commonly found that parameters important for describing the cellular state are related to off-line sampling and off-line analysis and hence information is only obtained with a low sampling frequency (Bachinger and Mandenius 2000b).

An electronic nose prototype called BioNose (EU BioNose Project QLK3-CT-1999-00435) has been built according to a similar principle as the human nose. The nose uses an array of non-specific sensors that give a different pattern when exposed to different odors or gaseous emissions. The broad and overlapping sensitivities of the sensors in the array produce a flow of signals very rich in information. The signal pattern is the unique "fingerprint" of the emission produced by the measured object that is analyzed by the pattern recognition system. The system is trained to recognize a given emission with the help of a pattern recognition algorithm or other types of advanced mathematical algorithms. These methods together with unique combinations of sensors constitute the strength of the electronic nose technique.

An array of chemical gas sensors – including typically conducting polymers, metal oxide semiconductors (MOS), metal oxide semiconductor field efficient transistors (MOSFET) or quartz microbalance sensors – are combined (Dickinson et al. 1998; Mandenius 1999). Every sensor is by itself more or less unspecific to the measured gas mixture and the information is instead revealed by studying the complex total response pattern from the whole array.

MOS sensors: The device is based on the properties of semiconducting metal oxides. Thin films of materials, like tin oxide and copper oxide, change their conductivity in the presence of reducing or oxidizing gases.The selectivity and sensitivity of the surface are modulated by oxide composition, amounts of trace elements, e.g. palladium, platinum, gold or rhodium, and by operating temperatures, usually in the range between 100 and 400 °C. Oxygen in the air is adsorbed onto the sensor surface, removing electrons from the conduction band of the semiconductor, thereby increasing its electrical resistance and leading to a decreased conductivity of the device. The interaction of reducing gases with the surface-adsorbed oxygen decreases this electron trapping, leading to characteristic increases in electrical conductance of the sensor (Fig. 1.4.2a).

The MOS sensors exhibit varying sensitivity towards organic compounds and the analyte affinity to the surface is more or less affected by its chemical properties. Examples of typical analytes to which selectivity can be attained are hydrogen, carbon monoxide, ammonia, hydrogen sulfide, nitrogen oxide, sulfurous compounds, alcohols, and hydrocarbons (Bachinger 1999; Dickinson et al. 1998; Mandenius 1999; Persaud and Dodd 1982).

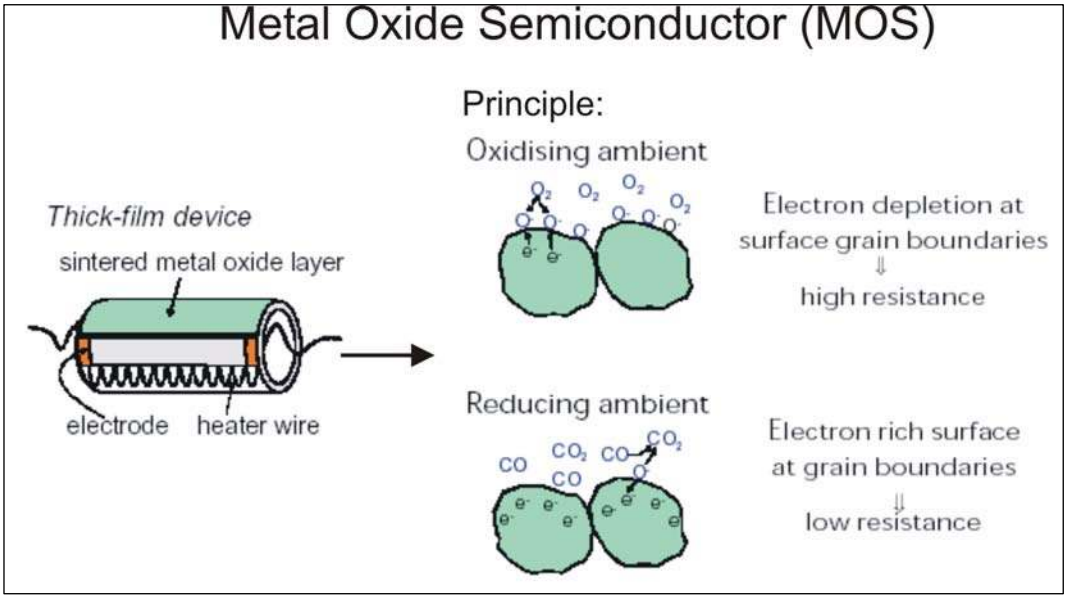


Fig. 1.4.2a: Design and principle of a MOS-sensor (from Nordic Sensor Technology (NST) Senstool manual, 2000).

MOSFET sensor: These metal oxide semiconductor field effect transistor devices can be built as capacitors and transistors. The semiconductor is normally silicon and the insulator silicon dioxide. A number of organic and inorganic compounds can be catalytically decomposed on such a surface resulting in dipoles that cause shifts in the capacitance of the semiconductor device. The working principle relies on the fact that the charge distribution at the insulator-semiconductor interface can be controlled by a

potential on the metal gate. As metal gates, continuous layers of palladium, or thin discontinuous layers of catalytic metals like platinum, iridium, rhodium and palladium are used. A schematic illustration of a MOSFET sensor is shown in figure 1.4.2b.

The principle of operation is that hydrogen atoms from dissociated hydrogen molecules on the catalytic metal surface, diffuse through the gate and adsorb at the metal-insulator interface where they give rise to a dipole layer or electrical polarization. Devices with thick continuous metal gates are merely sensitive to hydrogen atoms, whereas devices with discontinuous gates are sensitive to e.g. ammonia, ethanol, hydrogen, sulfurous hydrogen and unsaturated hydrocarbons.

The selectivity of the MOSFET sensors is modulated by using different catalytic metals, e.g. palladium, iridium, platinum and by the operating temperature (< 250 °C). Different compositions of the metals, different thickness and different working temperatures result in a large number of different selectivity patterns towards given gas mixtures. This sensor type is therefore of great interest in sensor arrays used to analyze complex gas mixture or odors (Bachinger 1999; Dickinson et al. 1998; Mandenius 1999; Winquist et al. 1993).

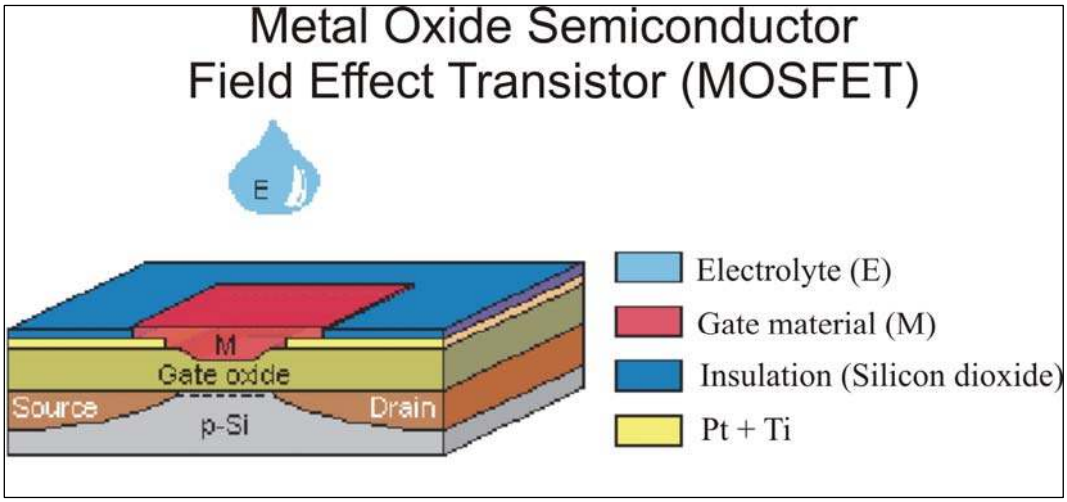


Fig. 1.4.2b: Structure of a MOSFET-sensor.

Typical sizes of a sensor array for electronic noses are 20 to 40 sensors. A set of different signal types (response, on/off derivative, on/off integral) can be obtained with these arrays. Using multivariate methods such as principal component analysis (PCA), partial least squares (PLS) and/or artificial neural networks (ANN) it is possible to analyze the signal pattern and identify important sensor responses. Furthermore, these methods can be used to build predictive models for various substances (Mandenius 1999).

The principle of monitoring a bioprocess using an electronic nose is illustrated in figure 1.4.2c. The gaseous emissions from the culture in the bioreactor are sampled continuously (on-line) from the gas exit of the bioreactor and are exposed to the sensor array of the electronic nose without violating the sterile barrier. The signal response generated by the array is analyzed by pattern recognition and correlations are sought that have significance for the bioactivity of the culture. These correlations include metabolite concentrations, cell viability, contaminating species and other relevant process variables. The pattern-recognition methods used have a key role in electronic-nose technology. For example, in the main static pattern-analysis techniques, PCA (1.4.3.1), PLS, cluster analysis and ANN are used today (Bachinger and Mandenius 2000b).

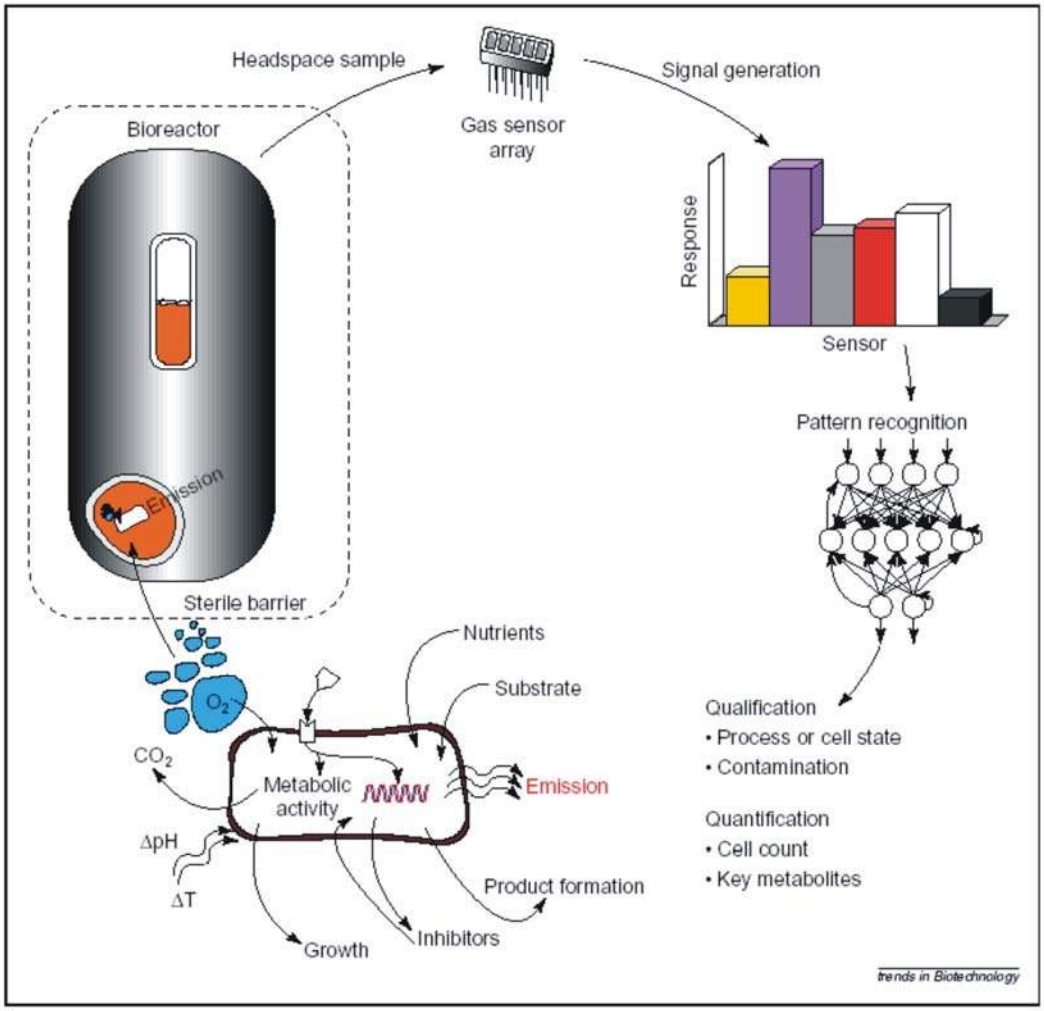


Fig. 1.4.2c: Process monitoring the electronic nose. Sample-containing emissions from cells or the medium in the bioreactor are withdrawn from the headspace volume of the bioreactor and transferred to the gas-sensor array of the electronic nose. Signals are generated in the array, which are analysed by pattern recognition using, for example, an artificial neural network (ANN) model. An ANN can quantify or qualify important culture variables, such as metabolic activity, biomass and nutrient concentrations (Bachinger and Mandenius 2000b).

1.4.3 Multivariate analysis

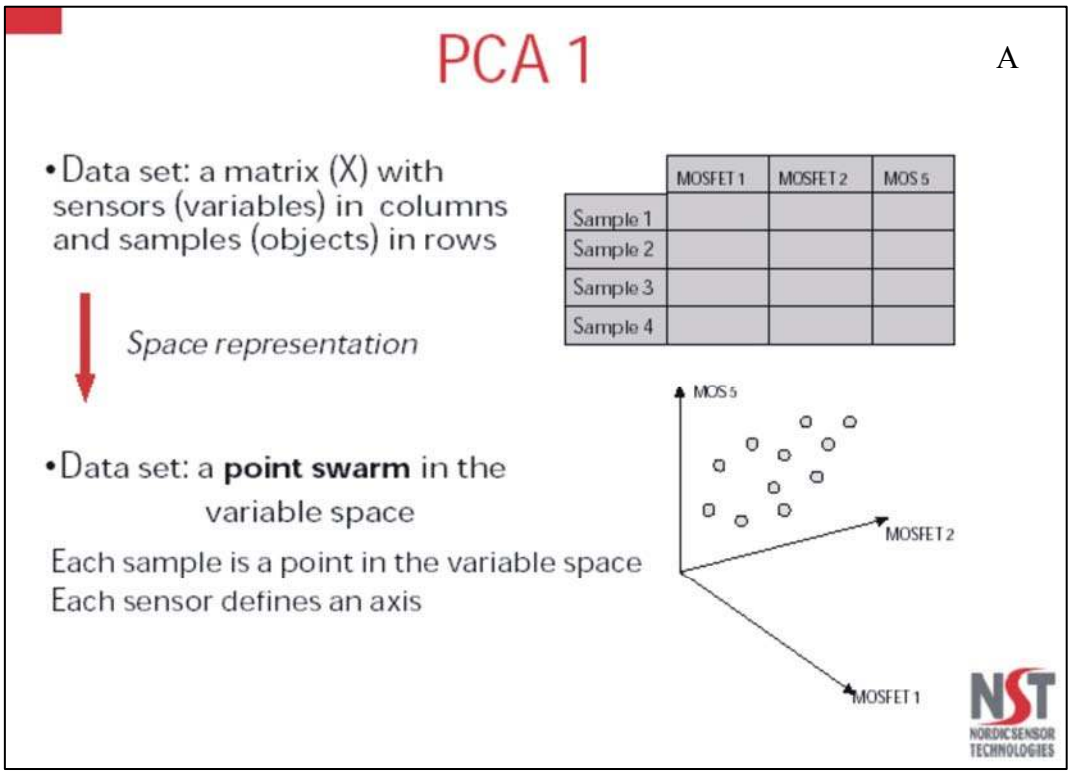
When the human brain is confronted with large data sets, for example more than 20 variables from various numbers of measurements, it has at first great difficulty just getting an overview of all the presented information followed by problems in filtering out what parts are really essential and informative (Unscrambler user Manual, 1998). Multivariate analysis simplifies the problem such that data can be viewed in a better way (NST Senstool manual, 2000).

1.4.3.1 Principal component analysis (PCA)

PCA can be seen as the basis of multivariate analysis. It is a method for approximating or reorganizing large data sets in a way that the most important information is compressed and can be presented in few very informative plots without loosing important information on the way. Mathematically PCA can be seen as a method for reducing the dimensions of the original problem. PCA operates by finding directions in the sensor space where a data set has maximal variation. These directions are called principal components (PCs). The PCs are calculated in order of importance. The first PC explains as much of the variance in the data set as possible. The second PC is orthogonal to the first and explains as much as possible of the variance that is not explained by the first PC. This continues until no variance remains in the data set (NST Senstool manual, 2000).

In other words, having K measured variables and N measured samples, this can be seen as a K-dimensional room with N data points. The PCA will rearrange this K-dimensional space into a new vector room build p of new variables called principal components (PCs). These PCs are linear combinations of the original variables and chosen in such a way that they create favorable mathematical properties, which in turn have shown to be very informative as well. The strength of the method is that most of the original information can be captured using only a few PCs and the remaining variation described by higher PCs will originate from non-systemic variations or noise (Wold 1987).

Figure 1.4.3.1a; A, B and C, illustrate the computation principles for a principal component analysis with two PCs (PC1 and PC2) based on three sensors signals (from NST Senstool manual, 2000).



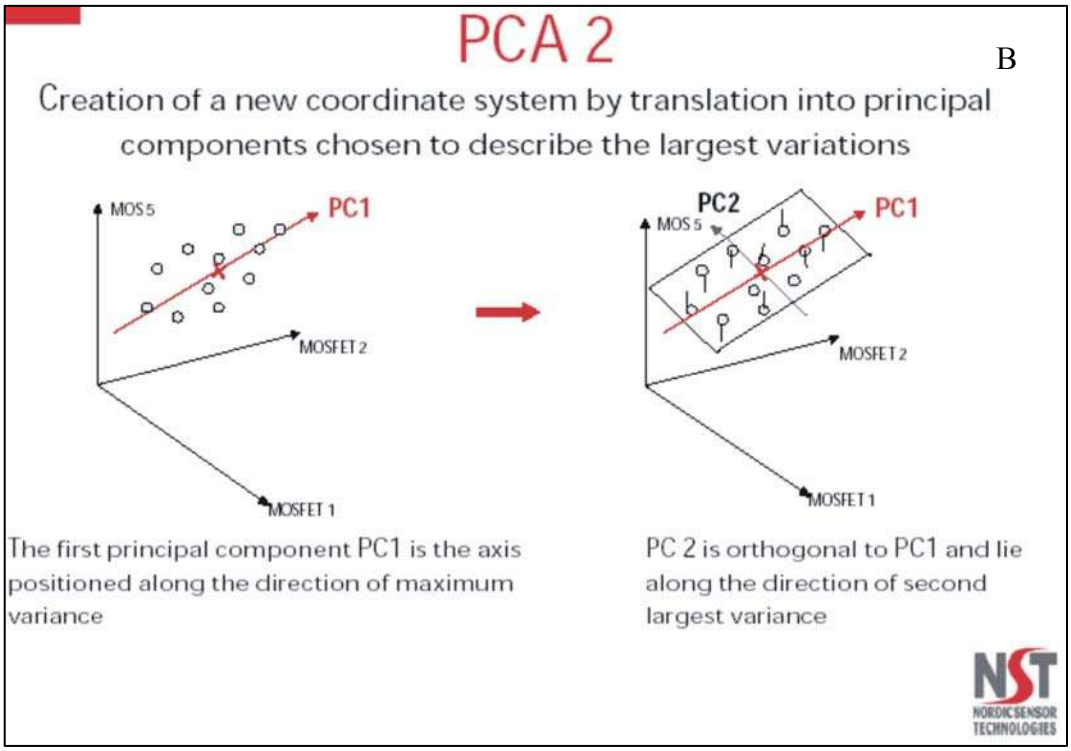


Fig. 1.4.3.1a; A, B and C: Illustration of the computation principles for a principal component analysis with two principal components (PC1 and PC2) based on three sensors signals. The first PC explains as much of the variance in the data set as possible. The second PC is orthogonal to the first and explains as much as possible of the variance that is not explained by PC1 (from NST Senstool manual, 2000).

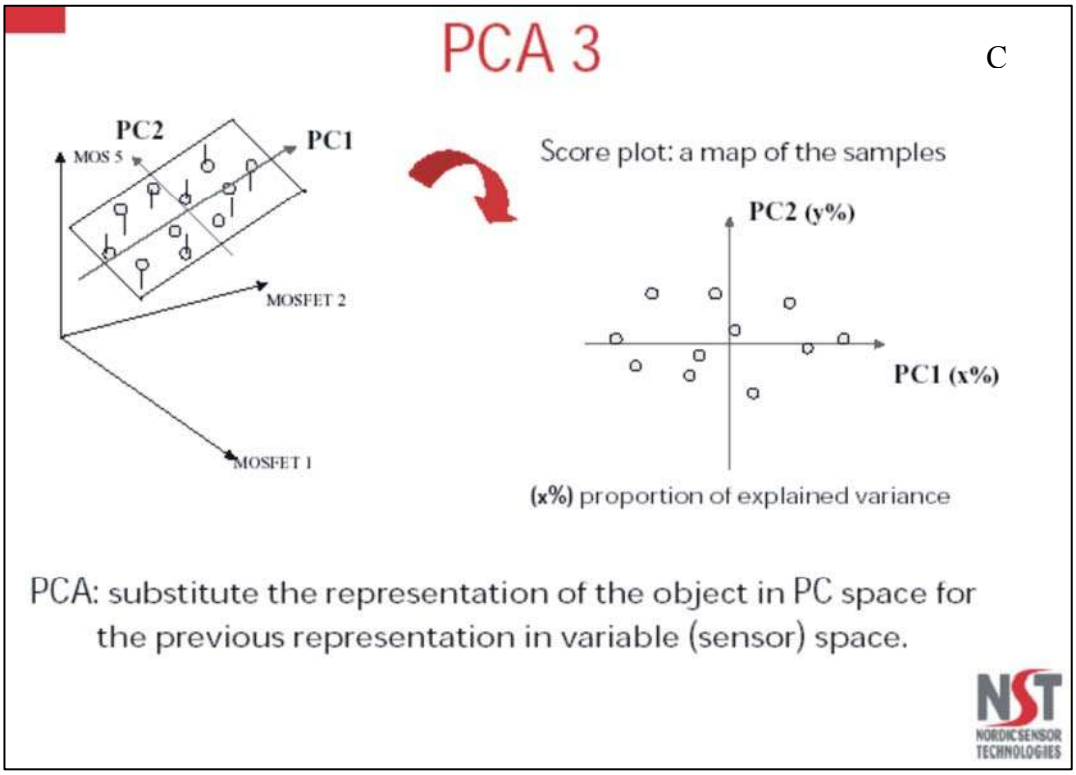


Fig. 1.4.3.1a. (Continued)

The results obtained from PCA are usually visualized in so called score and loading plots. The scores are the original data points, the measuring points, projected onto the new PCs while the loading plots describe the contribution the original variable has to the new PC. These PCs are obtained by projection of these original variables onto the new PC coordinate system. A score plot shows a condensed view of all measurement points in a data set, which is much easier to interpret than a whole collection of raw data bar charts. Since the scores contain information on the measurements, the score plot can be used to explore the relationships between different measurements, such as grouping/spreading of measurements on samples with similar/ different properties, outliers, and systematic errors including drifts (Fig. 1.4.3.1b; A). The loading plot contains information about the influence of the original variables on each PC, thus it can be used to explore the relationships between different variables. One important property is that variables arranged along the same direction in a loading plot give similar information. Another is that variables close to the origin give very little information (Fig. 1.4.3.1b; B) (NST Senstool manual, 2000).

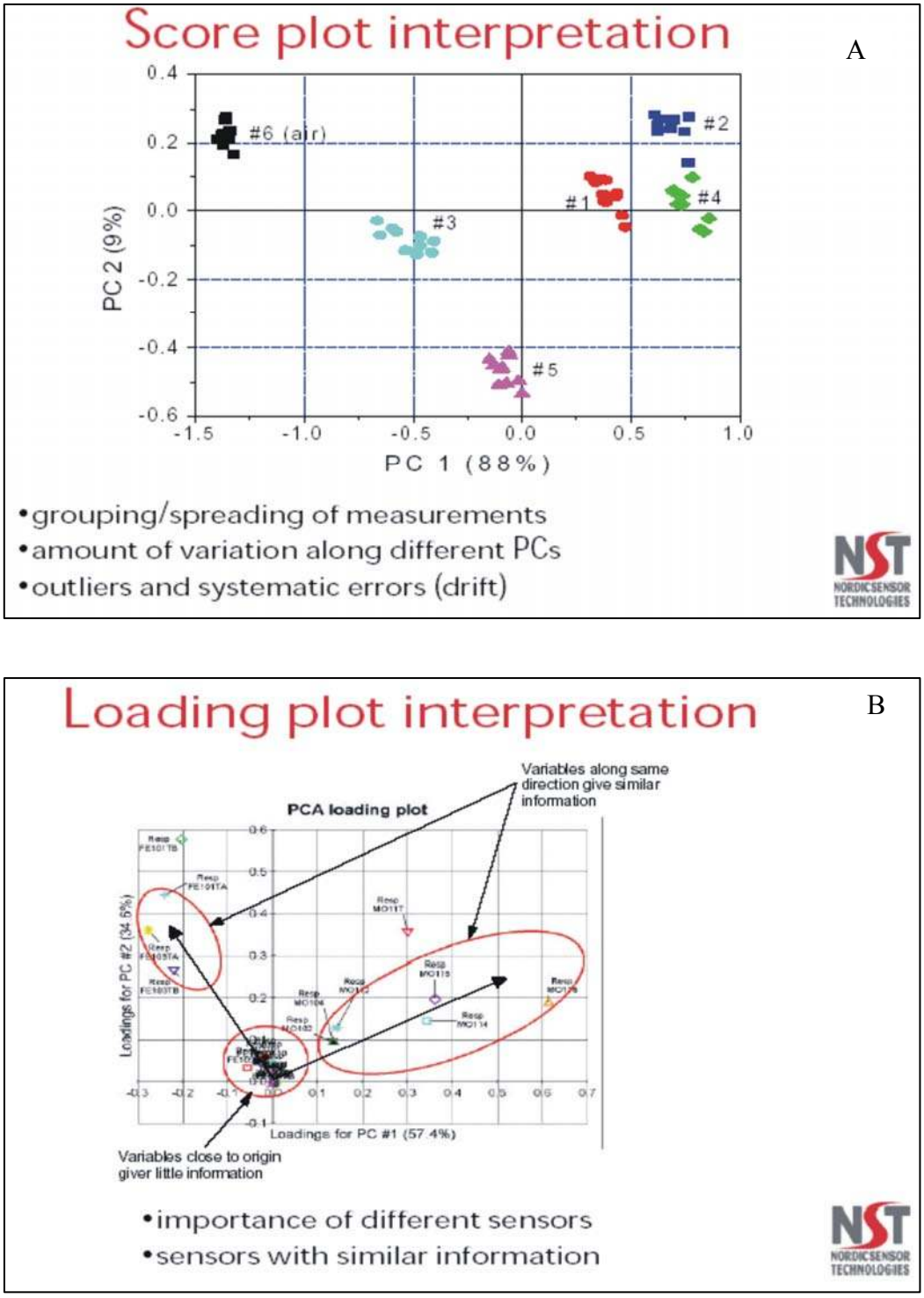


Fig. 1.4.3.1b: Illustration of a score plot (**A**) and loading plot interpretation (**B**) (from NST Senstool manual, 2000).

1.5 Problems in production of large amounts of r-proteins- Aims of the work

The development of industrial cell culture processes for the production of commercially relevant recombinant proteins is governed by constraints that pertain to issues such as cost, human resources, competitiveness, and the meeting of project timelines. Usually, the time allowed for proper process development is rather short, and in this time culture conditions and scale-up protocols have to be defined in such a way to maximize cell productivity and to minimize process length and overall cost. Due to high demand, the need for faster and cheaper approaches for the production of recombinant products is evident (Grammatikos et al. 1999; Wurm and Bernard 1999). The consideration of transient system for production of milligram to gram quantities of protein is rather new and it is now generating great interest because of its demonstrated ability to produce large amounts of r-proteins within a few days (Durocher et al. 2002; Girard et al. 2002; Jordan et al. 1998; Meissner et al. 2001; Pham et al. 2003; Schlaeger and Christensen 1999). However, there remains a high demand for simplification or reduction of handling steps, and reduction in the amount of DNA especially for large-scale transfection and the complete elimination of serum, proteins and undefined components from the production process.

Monitoring and control of the cellular physiology, and through this, control of bioprocess also is important to have success in a proper process development and it is a great challenge in bioprocess engineering. As was mentioned under section 1.4, bioprocesses are generally difficult to control due different and several factors.

Considering the problems mentioned above, this work was focused in the following aims:

- **First, development of a transient transfection process easy to perform, cost-effective, for large-scale application using the mammalian cells, HEK293 in suspension (HEK293s), comprising the development and optimization of a chemically defined medium to produce cultures totally free of serum and animal proteins or animal-origin components.**

- **Second, characterization and optimization of this transfection process using two different methods:**

  a) **Determination of the intracellular nucleotide ratios, which reflect the physiological state of mammalian cells, prerequisite for a successful**

production process, and therefore, evaluate the HEK293s cells metabolic energy and growth potential with respect of increasing productivity.

b) The use of the electronic nose, BioNose, for on-line monitoring to characterize and optimize transfection process.

To achieve the first aim, the initial experiments of this work show results towards the adaptation of HEK293s cells either to serum-free or protein-free media formulations to guarantee chemically defined culture conditions for further transient transfection processes. This has implicated the development of a serum/protein-free medium that facilitates the transient transfection procedure, avoiding washing of cells prior to the addition of the transfection complex and without changing the medium after the procedure.

Once that cells were adapted to the developed medium, further experiments were performed to optimize the DNA:PEI ratio in order to obtain highest transfection efficiencies using less amount of DNA.

To achieve the second aim of this thesis work, analysis of the intracellular nucleotide ratios was applied to the characterization of the HEK293s cells from an early and a late passage number, transfected and untransfected, either cultivated in serum-containing and serum-free media. This method gives information about the cell metabolic energy under different culture conditions, guiding the optimization of the transfection process towards an increase in the cell productivity.

On the other hand, on-line monitoring using a prototype electronic nose, BioNose, has been applied to investigate its use as a reliable tool for process characterization, monitoring the cell cultivation and distinguishing between different process conditions.

2. Materials and Methods

All standard chemicals used were of reagent grade, and purchased from Sigma (Deisenhofen, Germany), Difco Laboratories (Detroit, MI, USA), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Riedel-de-Haen (Seelze, Germany), Fluka (Buchs, Switzerland), J.T. Baker (Griesheim, Germany) if not specified.

All the solutions were prepared in ultra pure water obtained from the water system Super-Q™ (Millipore GmbH, Eschborn, Germany).

2.1 Equipments used

The equipments used in this work are described in the table 2.1a.

Table 2.1a: Equipments used in this work.

Equipment	Manufacturer	Model	Others
Autoclave	Souter Moller GmbH, Köln, Germany.	Sterilizer	
Autoclave (barrel)	Webeco GmbH, Bad Schwartau, Germany.	83343 - Sterilizer	
Cell count chamber	Assistent, Germany.	Neubauer Improved	0,0025 mm² 0,1 mm depth
Cell counter CASY 1	Schärfe System GmbH, Reutlingen, Germany	Model TTC	
Centrifuge (Eppendorf)	Heraeus, Kendro Laboratory products, Hanau, Germany	Omnilab Biofuge pico	
Centrifuge (conic tubes)	Heraeus, Kendro Laboratory products, Hanau, Germany	Digifuge GL	
Electroporator	Bio-Rad Laboratories, Life Science Research, Hercules, CA, USA	1-Gene pulser II 2- Pulse controller plus 3-Capacitance extender plus	
ELISA reader	SLT Lab instruments, Crailsheim, Germany	340 ATTC	Software: Easy WIN basic. Ver.1.0b
Flow cytometry	Becton Dickinson, Bedford, MA, USA	FACSCalibur	CellQuest Pro software

Equipment	Manufacturer	Model	Others
Fine balance	Sartorius AG, Göttingen, Germany	Analytic A 200 S	
Gas mix station	MKS Instrument Deutschland GmbH, München, Germany	BS 931 A	
Gel-Analysis	Bio-Rad Laboratories, Life Science Research, Hercules, CA, USA	Gel Doc 2000	Quantity One software
Glucose-Lactate-Analyzer	YSI 2700, Yellow springs instruments, Ohio, USA	Select biochemistry analyzer	Glucose and Lactate membranes
Incubator	Forma Scientific, Labotec GmbH, Göttingen, Germany	CO_2 incubator Model 3028	
	Heraeus, Kendro Laboratory products, Hanau, Germany	CO_2 incubator, Cytoperm 8080	
Ion pair reversed-phase HPLC	Kontrom Instruments, Milan, Italy		
Balance	Sartorius AG, Göttingen, Germany	LC 2201 P	
Microscope	Nikon, Düsseldorf, Germany	Inverse microscope Diaphot	
Microtiter plate reader	Labsystems Multiskan, Finland	MCC/340	
Mini gel chamber	GIBCO BRL, Life Technologies, Gaithersburg, MD, USA	HORIZON®58	
Osmometer	KNAUER, Berlin, Germany	Semi-micro osmometer type Dig.L	
pH-Meter	Knick, Berlin, Germany	761 Calimatic	
Power source	Bio-Rad Laboratories, Life Science Research, Hercules, CA, USA	Power-PAC 300	

Equipment	Manufacturer	Model	Others
Refrigerated centrifuge	Heraeus, Kendro Laboratory products, Hanau, Germany	Multifuge 3 S-R	
Shaker	B.Braun AG, Melsungen, Germany	Certomat	
Spectrophotometer	Amersham Pharmacia Biotech, Upsala Sweden	Ultraspec 3100 pro UV/Visible Spectrophotometer	
Spinner tableau (biological stirrer)	Wihl O. Schmidt GmbH, Braunschweig, Germany	Techne, MCS-1045	
Sterile clean-bench	Heraeus, Kendro Laboratory products, Hanau, Germany	LaminAir HLB 2472	
Tubes pump	Millipore Corporation, Bedford, MA, USA	Masterflex L/S easy load	
Ultracentrifuge	Sorvall Instruments, Kendro Laboratory products, Hanau, Germany	RC5C	Rotor: GSA and SS-34
UV-light (equipment)	Herolab, Molekulare Trenntechnik, Wiesloch, Germany	RH-5	
Video copy processor	Herolab, Molekulare Trenntechnik, Wiesloch, Germany		
Vortex	Scientific Industries, INC Bohemia, NY, USA	Vortex Genie 2	
Water bath	Kottermann, Uetze-Hänigsen, Germany	Type 3042	
Water bath: circulators	LAUDA Dr. Wobser GmbH & CO.KG Königshofen, Germany	Type RC 6 CP	

2.2 Materials used

The materials used in this work are described in the table 2.2a.

Table 2.2a: Materials used in this work.

Article	Manufacturer	Specification
Cell culture flask	BD Biosciences, Bedford, MA, USA	Tissue culture flask 0.2 µm vented blue plug seal cap
Conic tubes	Becton Dickinson Labware, Franklin Lakes, NJ, USA	50.0 mL polystyrene conical tube
	Becton Dickinson Labware, Franklin Lakes, NJ, USA	15.0 mL polystyrene conical tube
Cryogenic tubes	Nalge Company, Rochester, NY, USA	Nalgene Cryoware, Cryogenic vials
Eppendorf tubes	Eppendorf AG, Hamburg, Germany	Eppendorf tubes: 1.5 mL 2.0 mL
FACS-tubes	Becton Dickinson Labware, Franklin Lakes, NJ, USA	5.0 mL polystyrene round-bottom tube
Filter for tube pump	Pall Gelman Laboratory, Ann Arbor, MI, USA	AcroCap 0.1 µm
Freezing container cells, NALGENE "Mr. Frosty"	Nalge Company, Rochester, NY, USA	
Glass flasks	Schott Glass, Mainz, Germany	40.0 mL, 500.0 mL, 1000.0 mL, 2000.0 mL, 5000.0 mL, 10000.0 mL
Micro pipettes	Gilson, Inc., Middleten, WI, USA	P2, P10, P20, P100, P200, P1000
Pipette	Becton Dickinson Labware, Franklin Lakes, NJ, USA	Serological pipette Polystyrene
Pipettes tips	Sarstedt, Nümbrecht, Germany	Pipette-tips 200 µL
	Greiner GmbH, Esslingen, Germany	Bio-one pipette-tips 1000 µL
Plasmid extractions kits	Qiagen GmbH, Hilden, Germany	QIAprep spin miniprep kit
		QIAGEN plasmid maxi kit
		QIAGEN plasmid giga kit
		QIAquick gel extraction kit

Article	Manufacturer	Specification
Poly-propylene membrane tubing	Akzo Nobel, Wuppertal	
Reactor	ISOTHERM, Karlsruher Glastechisches Werk (KGW), Germany	1.6 L
Spinner flask	Techne, Cambridge, UK	150.0 mL flask
		1000.0 mL flask
Syringes	Omnifix-F, B.Braun, Melsungen, Germany	1.0 mL 50.0 mL
Syringe filters	Pall Gelman Laboratory, Ann Arbor, MI, USA	Acrodisc syringe filter 0.1 µm Supor membrane
		Acrodisc syringe filter 0.2 µm Nylon membrane, DMSO safe
	Millex®-HV, Millipore Corporation, Bedford, MA, USA	0.22 µm filter, PVDF (Durapore)
		0.45 µm filter – 4 mm
Well-plates	Becton Dickinson Labware, Franklin Lakes, NJ, USA	12-well polystyrene non-tissue culture treated plate
		96 well tissue culture plate, flat bottom with low evaporation lid
	Nalge Nunc International, Rochester, NY, USA	Nunc- Immuno™96 well™ plates

2.3 Cell line and bacterial strains

2.3.1 Cell line

- Human embryonic kidney 293 cells:

All the experiments were performed using human embryonic kidney cell line 293 (HEK293). A cell line derived from ATCC no. CRL 1573 adapted to grow in suspension (HEK293s) (Coté et al. 1998; Garnier et al. 1994).

These cells were kindly provided by Dr. Amine Kamen (Animal Cell Technology and Downstream Processing Group, Biotechnology Research Institute, National Research Council Canada, Montreal, Canada).

2.3.2 Bacterial strains

- *Escherichia coli* JM109 competent cells, high efficiency (Promega, USA).
- *Escherichia coli* XL 1 Blue, competent cells (Stratagene, USA).

2.4 Culture media

When the medium was prepared in the laboratory, sterilization was done by filtration through a 0.1 μm pore diameter filter. The additives were added to the different media following manufacturer's instructions. Supplements were first sterilized. In all cases, culture media were tested for sterility by incubating at 37 °C for three days and stored at 4 °C.

FCS (from Sigma or Biochrom, KG, Berlin, Germany) was heated by incubation in a water bath at 56 °C for 1 h for complement inactivation and further stored at 4 °C.

2.4.1 For mammalian cells:

- *DMEM -Ca^{2+}* (GBF). Homemade calcium-free DMEM was used as basic medium. When necessary, DMEM was supplemented either with FCS, vitamin solution (Vit.; MEM Vitamin solution 100 ×; Sigma, Catalogue no. M6895) or Pluronic F-68 (Pl.; Sigma). The medium chemical composition is described in Table A1 in the Appendix section.

- *Gene Therapy Medium-2. Serum-free* (Sigma, Germany; Catalogue no. G0791). Medium containing small amounts of recombinant proteins and polypeptides derived from plant protein hydrolysates. It contains no animal-derived proteins.

- *Pro 293s-CDM* (BioWhittaker, UK; Catalogue no. 12-765Q). Chemically defined medium, containing only one protein, r-Insulin. It is optimized for high-density production cultures.

- *CD 293* (Gibco, Invitrogen™, life technologies, Germany; Catalogue no. 11913-019). Chemically defined serum- and protein-free medium. It has been formulated without components of human or animal origin.

- *293 SFM II* (Gibco, Invitrogen™, life technologies, Germany; Catalogue no. 11686-029). A defined, serum-free medium formulated without human or animal origin components. It has a low protein content (10 mg L^{-1}). For specific experiments, this medium was supplemented with 1 mmol L^{-1} Asp, 150 μmol L^{-1} His, 500μmol L^{-1} Thr and 100 μmol L^{-1} Trp. (see results section 3.1.1)

- *FreeStyle™ 293 Expression Medium* (Gibco, Invitrogen™, life technologies, Germany; Catalogue no. 12338-026). Serum- and protein-free medium specifically developed to support the growth and transfection of FreeStyle 293-F cells grown under a suspension-type culture conditions. The L-Glutamine substitute, Glutamax-1, was present in the medium.

- *Opti-MEM® I* (Gibco, Invitrogen™, life technologies, Germany) (Catalogue no. 31985-047). Medium contains low serum concentration and low protein content.

- *Hybridoma-SFM (H-SFM)* (Gibco, Invitrogen™, life technologies, Germany; Catalogue no. 12045-076). Serum-free and low protein medium for hybridoma growth and monoclonal antibody production. For specific experiments, this medium was supplemented either with Pl., lipids (Lip.; Sigma) or EDTA as is described in the Table A2 in the Appendix.

- *SMIF 7* (GBF, Braunschweig, Germany). Chemically defined and protein-free medium developed for BHK-21 cell culture. Details about its composition are in http://www.gbf.de/eu/index.htmL.

- *Ex-Cell 293™* (JRH Biosciences, KS, USA; Catalogue no. 14570). Serum-free and animal-protein free medium developed for the long-term growth of HEK293 and related cells.

- *ExCell 293™ modified* (JRH Biosciences, KS, USA; Catalogue no. 81079). This medium has the same characteristic as *Ex-Cell 293™* (JRH Biosciences, KS, USA; Catalogue no. 14570) but it does not contain dextran sulfate.

- *FS:DMEM* (GBF, Braunschweig, Germany). This medium was developed during this thesis work (see details under 3.5.3 and 3.6.1). Briefly, it was a mixture of FreeStyle™ 293 Expression Medium (Gibco, Invitrogen™, life technologies, Germany; Catalogue no. 12338-026) and DMEM -Ca^{2+} + 4 % Vit. (MEM Vitamin solution 100 ×; Sigma, Catalogue No. M6895). When amino acids (600 µmol L^{-1} Asp and 100 µmol L^{-1} Glu) were added it was called *FS:DMEM + AA*.

During this work, many different combinations of media and additives were used. Detailed information about each medium will be given for every experiment in the respective section.

2.4.2 For bacterial culture:

- *LB (Luria Bertani) medium*: 10 g L^{-1} Bacto-tryptone, 5 g L^{-1} Bacto-yeast extract, 10 g L^{-1} NaCl. The components were diluted in Milli Q water and the pH was adjusted to 7.0. This medium was autoclaved for 20 min at 121 °C.

- *LB-Ampicillin medium*: Sterile LB medium was supplemented with ampicillin (Sigma) stock solution (50 mg mL^{-1}) to a final concentration of 50 µg mL^{-1}.

- *LB-Ampicillin agar*: To prepare LB solid medium, 15 g L^{-1} Bacto-agar was added. After sterilizing and cooling, ampicillin was added. The final concentration was the same as used for the liquid medium. The medium was mixed, filled into petri dishes under the sterile hood and stored at 4 °C.

- *LB-Kanamycin medium*: Sterile LB medium was supplemented with kanamycin to a final concentration of 50 µg mL^{-1} (stock solution of 50 mg mL^{-1}).

- *LB-Kanamycin agar:* Same agar as the LB-Ampicillin agar but ampicillin was replaced by kanamycin to a final concentration of 50 µg mL^{-1} (stock solution of 50 mg mL^{-1}).

- *SOB-medium*: 20 g L^{-1} Bacto-tryptone, 5 g L^{-1} Bacto-yeast extract, 0.5 g L^{-1} NaCl. The components were dissolved in Milli Q water and the pH was adjusted to 7.0. This medium was autoclaved 20 min at 121 °C.

- *SOC-medium*: 50 µL of 2 mmol L^{-1} Glucose (sterile) and 50 µL of 1 mmol L^{-1} Mg-solution ($MgSO_4 \times 7\ H_2O$) were added, just before use, to 5 mL SOB-medium.

2.5 Culture conditions of HEK293s cells

2.5.1 Cultivation under serum-containing medium

The cells were cultured in suspension using spinner flasks in humidified incubators at 37 °C and 12 % of CO_2.

The spinner experiments were performed either in small spinner flask (150 mL) with a maximum working volume of 70 mL, or in big spinner flasks (1000 mL) with a maximum working volume of 400 mL. Cells were stirred at 40 rpm in the first experiments and further at 80 rpm to avoid aggregates (see result section 3.1.1), on a

spinner tableau. Small or big spinners were inoculated at cell concentrations between 2.0×10^5 mL^{-1} and 3.5×10^5 mL^{-1}.

Bioreactor cultivations are explained in section 2.5.4.

All cultivation were performed in batch mode (Tokashiki and Yokoyama 1997).

2.5.2 Cultivation under serum-free medium

The culture conditions were the same as in 2.5.1. However, the initial cell concentration was in the range of 3.5×10^5 mL^{-1} and 5.0×10^5 mL^{-1}.
Bioreactor cultivations are explained in section 2.5.4.

2.5.3 Cell clusters disintegration

To avoid the formation of cells clusters, the cells were vigorously vortexed before being inoculated into the appropriate culture flask. The cell inoculum was transferred into a 50 mL conic flask with 10 mL of the culture and vortexed for 15 to 20 s. The same procedure was used for the sub-culturing until the cultures grew as homogenous single cells (1 to 3 – 4 passages). Cells were stirred at 80 rpm.

For cells that were already in culture, the spinner speed was increased from 40 to 80 rpm and the same procedure as was explained above was followed.

2.5.4 Bioreactor cultivation

HEK293s cells were grown in bioreactors using serum-containing and serum-free media.
The cultures were carried out in a stirred tank bioreactor at 37 °C and 40 rpm. A 1.8 L bioreactor was used with a working volume between 1.4 L and 1.6 L.

The bioreactor was equipped with 7 m of polypropylene membrane tubing to enable bubble-free aeration of the culture (Lehmann et al. 1987). The total gas flow was adjusted to 100 mL per minute (12.5 % CO_2, regulated O_2 and N_2). Dissolved oxygen was measured on-line and maintained at 40 % of air saturation. The electronic nose system was connected to the off-gas line of the reactor. For details see section 2.15. All bioreactors were inoculated with a starting cell concentration between 2.5×10^5 mL^{-1} and 3.5×10^5 mL^{-1}. Batch culture processes were performed.

Cultures using serum-free medium were carried out under the same conditions as in serum-containing medium but using a higher stirring rate (50 to 70 rpm), and a higher cell inoculum concentration ($3.5 - 5.0 \times 10^5$ mL^{-1}).

2.6 Adaptation of cells to serum-free culture medium

The cells from the mid-exponential growth phase with a viability above 90 % (2.14.3) were used for adaptation to serum-free culture. Different serum-free media were used as explained in detail in the results sections 3.1.1 and 3.5.

All procedures were performed in small spinner flasks using a working volume of 50 mL.

During the adaptation procedure a sample was taken daily to measure the total cell number, viability, glucose and lactate, LDH and amino acids concentration (see section 2.10 and 2.11).

The cells were considered fully adapted to the new serum-free medium when they grew as single cells (or small clumps), with a viability of more than 90 % and with growth rate similar to those in serum-containing medium. The adaptation time depended upon the medium and the protocol used, and it varied from 5 to 10 cell passages (sub-culture at an initial cell concentration of about 5.0×10^5 mL^{-1} every time that they have reached a cell concentration of 1.5×10^6 mL^{-1}). Different adaptation protocols were used:

2.6.1 Sequential adaptation using cell density effect

The initial cell concentration was 5.0×10^5 mL^{-1}. Every third day a medium exchange was performed by progressive replacing the complete cell volume. This helped to achieve a successful conditioning during the adaptation of the cells.

At the beginning of the culture, the amount of serum was 5 % and the successive passage were done in order to have 1 %, 0.2 %, 0.04 % and 0 % serum content, respectively. In this last step the cells were washed with the new serum-free medium and grew until 1.5×10^6 mL^{-1}. An aliquot of the cells was taken for cryopreservation(see section 2.7.1) and the rest of the cells was sub-cultured at a cell concentration of 5.0×10^5 mL^{-1}.

For every medium change, the complete volume was taken from the spinner flasks and transferred to conical tubes. A volume of 10 mL was taken from the tube and refilled into the spinner flask. The rest of the cell culture was centrifuged (1200 rpm, 3 min), the supernatant was discarded, and 40 mL of new serum-free medium was added.

2.6.2 Sequential adaptation with successive cell dilutions

In this case a progressive medium exchange was performed when the cell concentration reached 1.5×10^6 mL^{-1}. Subsequently, the cells were sub-cultured at a cell concentration

of 5.0×10^5 mL^{-1} in a medium containing half of the serum content compared to the previous culture.

Serum reduction was started from a portion of 5 % in the initial cultures to 2.5 %, 1.25 %, 0.62 %, 0.31 % and 0 %. At this point, cells were washed once with the new serum-free medium. As it was described before, when the cells reached a concentration of 1.5×10^6 mL^{-1}, a cell bank was deposited, and the cell culture was continued with different sub-cultures at an initial cell concentration of 5.0×10^5 mL^{-1} to 1.5×10^6 mL^{-1} until the cells were fully adapted.

2.6.3 Direct adaptation

Cells at a concentration of 5.0×10^5 mL^{-1} growing in serum-containing medium were washed one time with the serum-free medium and directly sub-cultured under the new conditions. Every time, that the culture reached a cell concentration between 1.5×10^6 mL^{-1} and 2.0×10^6 mL^{-1}, the cells were sub-cultured at the same cell concentration as the initial inoculum. This sub-culture was repeated between 3 to 6 times until the cells were fully adapted.

2.7 Cryopreservation of cell lines and bacterial strains

2.7.1 Cryopreservation of cell lines

Cells were frozen at a rate of 1 °C to 3 °C per minute using cryoprotective additives or chemicals to minimizing the detrimental effects of increasing solute concentrations and ice crystal formation. A special freezing container “Mr. Frosty” (see table 2.2a) filled with isopropanol was used. The cells should have a high viability above 95 %, being in the exponential growth phase. The container and the cryoprotectant medium were pre-incubated at 4 °C. 1.0×10^7 cells were frozen on each vial. Cells were centrifuged (3 min, 1200 rpm, RT), the pellet was dissolved in the freezing medium and 1.8 mL of this suspension were transferred to the cryogenic vials. The vials were immediately transferred to Mr. Frosty, and stored at –70 °C for at least one day. Subsequently, they were stored in the gas phase of liquid nitrogen at a temperature below –135 °C.

The different cryoprotector media used were the following:

- Cells growing in DMEM -Ca^{2+} + 5 % FCS + 0.1 % Pluronic F68 + Vitamin solution: 10 % cryoprotective agents, dimethylsufoxide (DMSO; Riedel-de-Haen, Seelze, Germany) + 90 % fresh medium.

- Cells growing in serum free media: 10 % DMSO + 10 % methylcellulose + 80 % fresh medium.

- Cells growing in FreeStyle and (FreeStyle-DMEM) media: 10 % DMSO + 90 % of a mixture of conditioned and fresh media (1:1).

2.7.2 Cryopreservation of bacteria

The recombinant bacteria were cultivated in LB liquid medium at 37 °C overnight. 500 µL of the culture was added into a sterile vial with 500 µL of glycerol as cryopreservative.It was homogenized and frozen at –70 °C.

2.8 Revitalization of cell lines and bacterial strains

2.8.1 Revitalization of cell lines

To revitalize the cell lines in all different media, the same procedure was applied. The cryogenic vial was taken from the liquid nitrogen storage tank and quickly thawed by incubating in a water bath at 37 °C for 3 min. The cells were transferred to a conic tube with 10 mL of fresh medium, warmed at 37 °C, following centrifugation at 1200 rpm for 3 min at RT. The supernatant was discarded and the pellet was resuspended in 10 mL of warm fresh medium, left for 3 min at RT to allow the diffusion of the DMSO from the cells to the media, and centrifuged again in the same conditions as the first time. This pellet was resuspended in a definite fresh warm medium volume and counted to determine the cell viability and the percentage of cell recovery.

2.8.2 Revitalization of bacteria

To recover bacteria, a small inoculum was taken with a loop from the tubes with the frozen bacteria. The inoculum was streaked in a LB-agar plate with the corresponding selective antibiotic. The tube was again stored at –70 °C and the plate was incubated at 37 °C overnight. After that, one colony was taken from the plate and inoculated into the LB liquid culture medium with the appropriate antibiotic.

2.9 Master cell bank and work cell bank

Once the cells were fully adapted to a new medium, a master cell bank was generated. Cell viability, growth rate, duplication time (see section 2.14), fungal and bacterial contaminations were tightly controlled. When all of these parameters were satisfied, cells were expanded. When the cells reached the middle of the exponential growth phase, 5 to 10 cryogenic tubes with 1.0×10^7 cells were frozen (see section 2.7.1) and this constitutes the master cell bank. One to three tubes from this bank were thawed (see section 2.8.1) for quality control, assessing viability, growth rate, population doubling time and contamination. Once these tests were satisfactory, cells were cultured as mentioned before, and 50 cryogenic tubes containing 1.0×10^7 cells of each one were

frozen. This constitutes the working cell bank. The cells to perform all the experiments were taken from this cell bank, which guaranteed to work always with cultures in the same range of passage number and quality.

2.10 Cell analysis

2.10.1 Total cell determination

Total cell number was assessed using different methods.

- Nuclei fixation and staining: 0.5 – 1.0 mL of cell suspension were taken, centrifuged (3 min, 1200 rpm, RT) and 80 % of the supernatant were replaced with crystal violet solution (see below). This solution was homogenized using a vortex for at least 1 min, and nuclei were counted with a hemocytometer (Sandford et al. 1951).

Crystal violet solution: 0.1 mol L^{-1} citric acid, 0.1 mg mL^{-1} crystal violet and 1 % V V^{-1} Triton X-100.

- Nuclei concentration was assessed by using an automatic cell counter (CASY 1): between 0.5 mL and 1.0 mL of cell suspension was taken, centrifuged (3 min, 1200 rpm, RT) and 80 % of supernatant was replaced with CASY cell extraction buffer (see below). The components were dissolved, the solution was heated at 40 °C, sterilized by filtration and stored at RT. The pellet was resuspended by vortexing for 30 s. An aliquot of this suspension (100 µL) was transferred to 5.0 mL CASY-ton buffer (see below) and counted (capillary: 150 µm, span with: 0 – 20 µm).

CASY cell extraction buffer: 21.00 g L^{-1} citric acid. H_2O, 10.01 g L^{-1} cetyltrimethylammoniumbromide.

CASY-ton buffer: 7.93 g L^{-1} NaCl, 0.35 g L^{-1} Na_2.EDTA, 0.4 g L^{-1} KCl, 0.22 g L^{-1} $NaH_2PO_4 \times H_2O$, 2.45 g L^{-1} $Na_2HPO_4 \times 2H_2O$, 0.2 g L^{-1} NaN_3 (0.02 %). The buffer was filtrated and stored at 4 °C.

2.10.2 Determination of viable cells

Viable cells were measured using the Trypan blue exclusion method.

Briefly, cells were diluted 1:1 or 1:2 with 0.5 % trypan blue solution (Roche Diagnostics, Mannheim, Germany), incubated for al least 1 min at RT and viable cells were counted by using the Neubauer hemocytometer chamber.

2.11 Metabolism analysis

2.11.1 Glucose and lactate determination

Glucose and lactate concentration were determined daily in duplicates in every cell culture supernatant using a glucose/lactate analyzer (Table 2.1a). The method is based on enzymatic oxidation (immobilized glucose oxidase and L-lactate oxidase, respectively). The formed hydrogen peroxide is oxidized at a platinum electrode and the respective electricity is measured.

2.11.2 Determination of lactate dehydrogenate (LDH) activity

LDH activity was measured as the reduction of pyruvate to lactate. The reduction is coupled to the oxidation of NADH to NAD^+, which was followed spectrophotometically at 340 nm (Wrobleswski and LaDue 1955).

60 µL of supernatant (store at 4 °C not more than 3 days) were mixed with 420 µL of 30 mmol L^{-1} NaH_2PO_4 buffer and 60 µL of 10 mmol L^{-1} pyruvate, preheated to 37 °C. 60 µL of 2.7 mmol L^{-1} NADH solution was quickly added, obtaining a total volume of 600 µL and the enzyme kinetics were immediately measured at 340 nm during 60 s using an Ultraspec 3100Pro. A regression coefficient was calculated expressing the activity as catalytic units per minute. The NADH solution was prepared freshly every two to three days and kept at 4 °C together with the other two solutions.

2.11.3 Amino acid analysis

Amino acids were quantified using a reversed phase high performance liquid chromatography (RP-HPLC) and an internal Norvalin or Citrulin standard (Larsen and West 1981).

In this work, 500 µL of supernatant were taken from every cell culture sample and stored at –20 °C until they were measured.

2.12 Intracellular nucleotide analysis

The method was used as previously reported (Ryll and Wagner 1991; Ryll and Wagner 1992) with few modifications.

Briefly, samples taken from spinner flasks were determined 1 h after standard analysis (see above). For bioreactor experiments, one common sample was taken and the nucleotides were analyzed 0.5 h before cell concentration and other parameters tested.

A sample of 1.0 – 5.0 mL containing about 2.0×10^6 cells was taken and immediately stored on ice. The sample was centrifuged for 3 min at 190 × g at 0 °C followed by the

removal of the supernatant. The cell pellet was resuspended in 300 – 500 µL chilled 0.5 mmol L^{-1} perchloric acid (PCA), stored on ice for 3 min, and centrifuged at 2000 × g for 2 min at 0 °C. The supernatant was collected and stored on ice, and the pellet was extracted once again, stored on ice and centrifuged as previously described. The second supernatant was pooled with the first one and the pH was adjusted to 6.5 using a microelectrode and a chilled solution of 2.5 mmol L^{-1} KOH and 1.5 mmol L^{-1} KH_2PO_4. After mixing the sample, the sample was place on ice for 2 min and centrifuged at 2000 × g for 2 min at 0 °C to precipitate $KClO_4$. The volume of the clear supernatant was determined, filtered through a 0.45 µm filter, frozen in N_2 liquid and stored at –70 °C until analysis on an ion pair reversed-phase HPLC (IP-RP-HPLC).

The HPLC separation of nucleotides was principally carried out as described by Ryll and Wagner (1991), using a gradient system of two buffers containing KH_2PO_4/ K_2HPO_4 and KH_2PO_4/K_2HPO_4/CH_3OH. The buffer's pH was set to 5.6 and 5.9 respectively using KOH (modification of the standard method). Peak identification was performed by comparing retention times with nucleotide standard runs between every 10 – 12 samples. Quantification of the peaks was done by manual integration using a MT-450 Kromasystem 2000 and relating the integrated peak area to standard curves in the range of 100 – 10 000 pmol. In this range, the authors found a linear correlation between peak area and concentration.

2.12.1 Special calculation using the intracellular nucleotide pools

- *Adenylate energy charge (AEC)*: AEC was defined by Atkinson (Atkinson 1969) as an indicator of the metabolic state of the cells, giving the information of the intracellular energy charge.

$$AEC = \frac{(ATP + 0{,}5ADP)}{(ATP + ADP + AMP)}$$

The following ratios were defined by Ryll and Wagner (Ryll and Wagner 1992):

- *NTP ratio*: it is the ratio of the sum of purines triphosphates and pyrimidines triphosphates.

$$NTP = \frac{(ATP + GTP)}{(UTP + CTP)} = \frac{Purines}{Pyrimidines} = \frac{"metabolic \cdot energy"}{"growth \cdot potential"}$$

- *U ratio*: it is the ratio of UTP and both UDP N-acetyl-hexosamines, UDP-GalNAc and UDP-GlcNAc.

$$U = \frac{UTP}{UDP-GNAc} = \frac{"growth \cdot energy"}{"accumulation \cdot of \cdot NH_4"}$$

$$UDP-GNAc = UDP-GalNAc + UDP\text{-}GlcNAc$$

- *NTP/U ratio*: it is the ratio of the NTP ratio and the U ratio.

$$NTP/U = \frac{(ATP+GTP)\cdot UDP-GNAc}{(UTP+CTP)\cdot UTP}$$
$$= \uparrow = "worsening\ in\ cellular\ physiologic\ state\ and\ growth\ potential"$$

- *Percentage determination*: indicate the relative amount of one component to the total nucleotide pools. For example, the following is the percentage of UTP.

$$\%UTP = \frac{UTP}{\sum Nucleotide} \cdot 100$$

$$\sum Nucleotide = ATP + GTP + UTP + CTP + UDP\text{-}GNAc + ADP + GDP + AMP + NAD + NADP$$

2.13 Protein analysis

The protein content of the supernatant was measured using the Coomasie Plus™ Protein Assay Reagent (Pierce, USA). This is a quick and ready-to-use coomassie-binding, colorimetric method for total protein quantification. It is a modification of the well-known Bradford method (Bradford 1976) but with an improved linearity for a defined range of protein concentration. The assay was performed in test tubes. For high protein concentration, 50 µL of the sample was transferred to 1.5 mL of the reagent (macro test). For the samples with lower protein concentration, 1 mL of the sample was taken and transferred to 1 mL of the reagent (micro test). After 10 min, the color formed was measured at 595 nm (Instructions Coomassie Plus ™ Protein Assay Kit, Pierce, Rockford, IL, USA).

The protein concentration was estimated using the absorbance obtained from serial dilutions of a protein (BSA; Gibco, Invitrogen™, life technologies, Germany) at a standard concentration as a reference. The standard curve was performed for both

methods (micro and macro test) following the procedure recommended (Stoscheck 1990).

2.14 Mathematical analysis

2.14.1 Growth rate (μ)

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1}$$

Where x_1 and x_2 are the concentration of the living cells per mL in the time point t_1 and t_2.

The growth rate (μ) is possible to calculate manually or directly from the slope value from the regression curve in the exponential phase of the cell culture. In this work, the logarithmic of the viable cells was always plotted as a function of the cultivation time and normally, μ was directly obtained from the slope curve.

2.14.2 Population doubling time (t_d)

$$td = \frac{\ln 2}{\mu}$$

Where μ is the growth rate.

2.14.3 Cellular viability (V %)

$$V = \frac{Cv}{Ct} \times 100$$

Where *Cv* is the concentration of the viable cells (2.10.2) and *Ct* is the total cell concentration (2.10.1).

2.15 BioNose

As explained in the Introduction section (1.4.2), an electronic nose, BioNose (EU BioNose Project QLK3-CT-1999-00435; Applied Sensors, Sweden), is build up according to the same principle as the human nose. An array of chemical gas sensors - typically conducting polymers, metal oxide semiconductors (MOS), metal oxide semiconductor field efficient transistors (MOSFET) are combined (Mandenius et al. 1999). Every sensor is more or less unspecific to the measured gas mixture and the information is instead revealed by studying the complex total response pattern from the whole array. All measurements are performed in cycles during which the sensors are alternatively exposed to reference air, to the sample headspace, and then again to

reference air to allow the sensors recover. A cycle is divided into three phases: Baseline, Sample and Recovery. The duration of these phases should be optimized for each application. For each sensor and each cycle, several characteristic signal parameters are then calculated: baseline, on/off derivative, on/off integral and response - describing the sensor-substance dynamics (Fig. 2.15a).

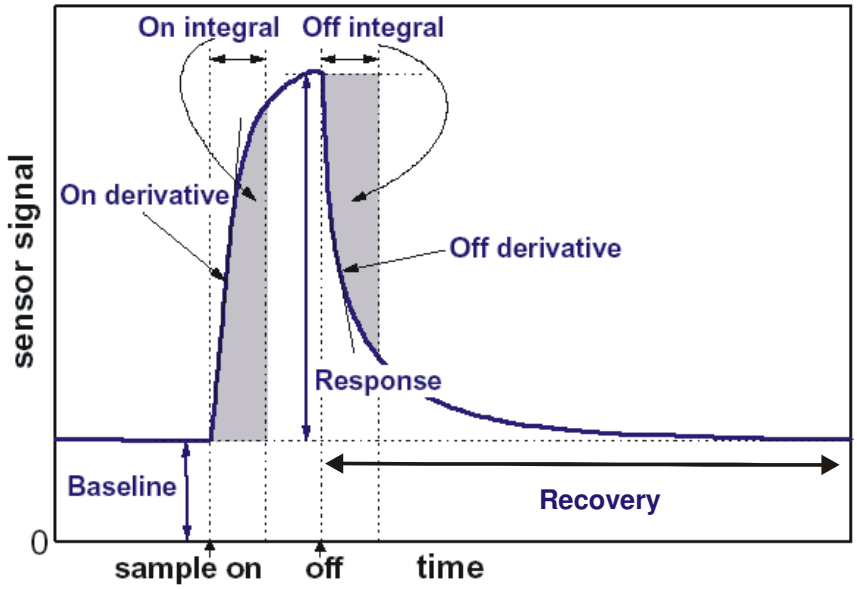


Fig. 2.15a: The measurement cycle of the electronic nose. The on integral and off integral represent chosen times over which the rise of the signal is integrated, and on derivative and off derivative chosen times where the signal rise and fall are derivated. Diagram from NST Senstool manual, 2000.

Using multivariate analysis (see section 2.16) and/or artificial neural networks it has been shown the possibility to select the important sensor responses in the array pattern and, based on them, build prediction models for various substances (Mandenius et al. 1999).

Connected to the off-line of the bioreactor, the sensors are presented to gas mixtures coming from the culture off gas for short time periods and a response is measured. Before and after exposure, the sensors are ”washed” with reference gas. To obtain measurements unaffected by the prior measurement, an important aspect in setting up the BioNose, is to ensure that the time the sensor is cleaned with reference gas is long enough (Bachinger and Mandenius 2000b).

As long as a constant flow is present in the direction from the bioreactor, it is possible for the BioNose to be connected at any time without violating the sterile barrier. For this reason the BioNose does not need to withstand sterilization in an autoclave. This enables the use of normal sensors, which do not need to withstand heat sterilization.

Different Bionose experiments were performed. HEK293s cells were used growing in serum-containing medium and in serum-free medium. Both cultures were also evaluated during transfection procedure mediated by PEI (see section 2.18.1).

For details see section result no. 3.7.2.

2.15.1 BioNose setup

The BioNose used, was equipped with 10 MOSFET sensors and 6 MOS sensors operated at 140 – 170 °C and 300 °C respectively. The electronic nose also contained a CO_2 sensor, a temperature sensor block and temperature controlled reference air humidification. Data was collected using NST Senstool software. Reference gas was measured for 100 s to obtain a base line level, followed by a 30 s sampling from the reactor off gas and 1070 s recovery time applying reference gas. Each sample cycle took 20 min. Reference gas flow was 160 mL min^{-1}, gas flow 62 mL min^{-1} and sensor block temperature 55 °C. Reference gas was cooled to 12 °C, the reactor off gas was pre-treated by cooling at 20 °C in order to reduce humidity. The exhaust gas line temperature was 34.5 °C.

The total bioreactor and BioNose set up can be seen in figure 2.15.1a.

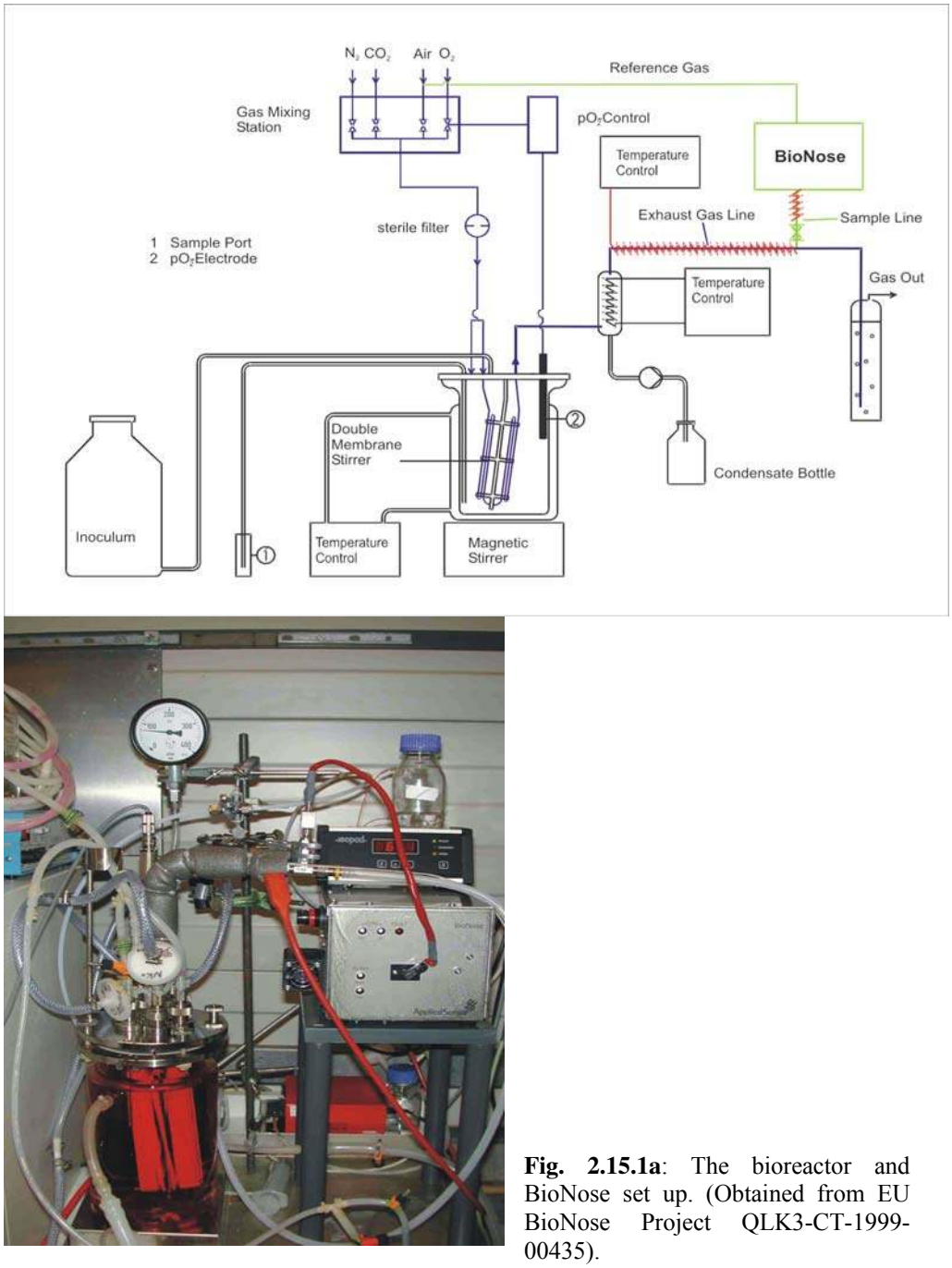


Fig. 2.15.1a: The bioreactor and BioNose set up. (Obtained from EU BioNose Project QLK3-CT-1999-00435).

2.16 Multivariate analysis

In practice, the main use of multivariate methods is to extract the relevant information from a huge data set.

2.16.1 Principal component analysis (PCA)

The principle of the PCA has been already explained in the Introduction section under 1.4.3.1. PCA can be seen as the basis of multivariate analysis and was used to evaluate the pattern response. Different PCA models were built using the NST Senstool

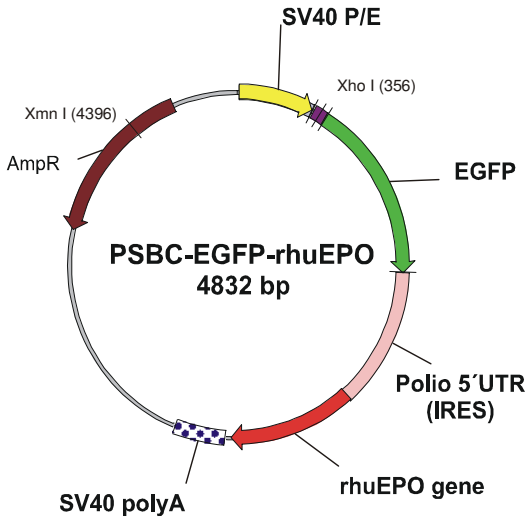
software. Sensors, which gave a noisy response, were avoided. The outlier's measurements (points that deviates significantly from the rest of the population) were deleted. The most relevant sensors were selected using the loading plot and the most relevant principal components were found. Data analyses were performed either with or without scaling method looking which of them gives best result. Normalization scaling was done such that the all measurement vectors obtain a total length of one, giving all measurements equal importance. The idea behind normalized measurement scaling is to extract information from the signal patterns rather than from the magnitudes of the signal.

2.17 Molecular cloning

2.17.1 Expression vectors

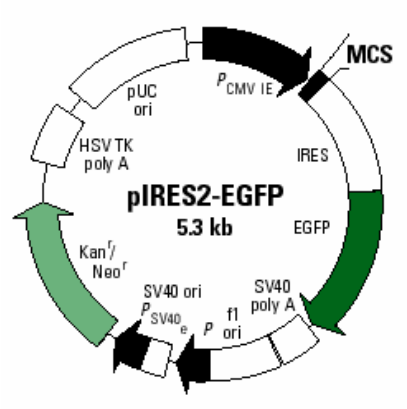
The expression vectors used in this work were the following:

- pSBC-EGFP-rhuEPO (4832 bp)



This expression vector was constructed in our Cell Culture Technology Department, (GBF, Braunschweig, Germany) by Noushin Irani (Irani 2000; Irani et al. 2002). It is a bicistronic plasmid, which contains the internal ribosome entry sites (IRES) from the poliovirus between the enhanced green fluorescent protein (EGFP) and the recombinant human erythropoietin gene (rhuEPO). These genes are under control of the SV40 virus promoter/enhancer (SV40 P/E). This vector also bears the ampicillin resistance gene.

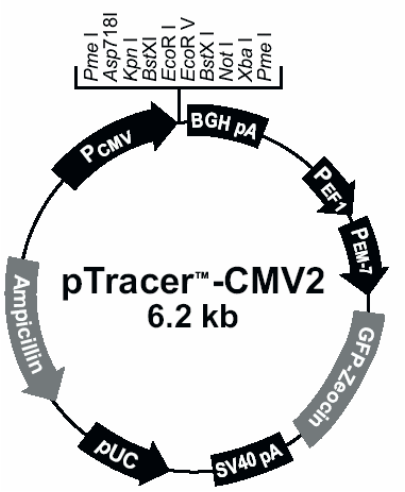
- pIRES2-EGFP (5300 bp) (BD Biosciences, Clontech, CA, USA)



(From: http://www.bdbiosciences.com/clontech/techinfo/vectors/vectorsF-I/pIRES2-EGFP.shtmL)

This plasmid contains the IRES element of the encephalomyocarditis virus (ECMV) between the multiple clone side (MCS) and the EGFP gene to be translated from a single bicistronic mRNA. It was designed for the efficient selection (by flow cytometry or other methods) of transiently transfected mammalian cells expressing EGFP and the protein of interest. Genes are under control of the immediate early Cytomegalovirus promoter (pCMV IE, 589 bp). This vector bears the kanamycin and neomycin resistance genes.

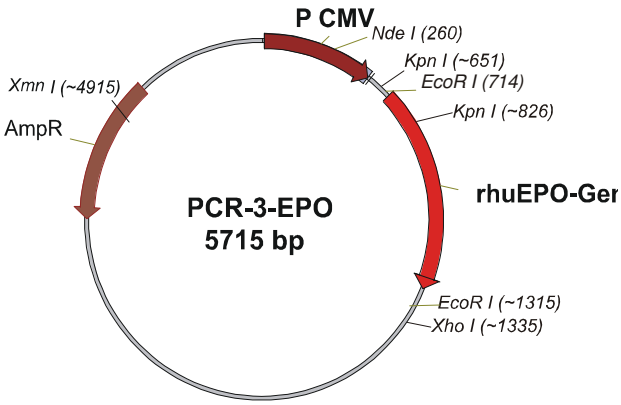
- pTracer™ -CMV2 (6210 bp) (Invitrogen, Life Technologies, CA, USA)



This vector was kindly provided by Dr. Manfred Wirth, Regulation and Differentiation Department GBF, Braunschweig, Germany. It contains the immediate-

early CMV promoter (654 bp) and the ampicillin and zeocin resistance genes (http://www.invitrogen.com/content/sfs/manuals/ptracercmv2_man.pdf).

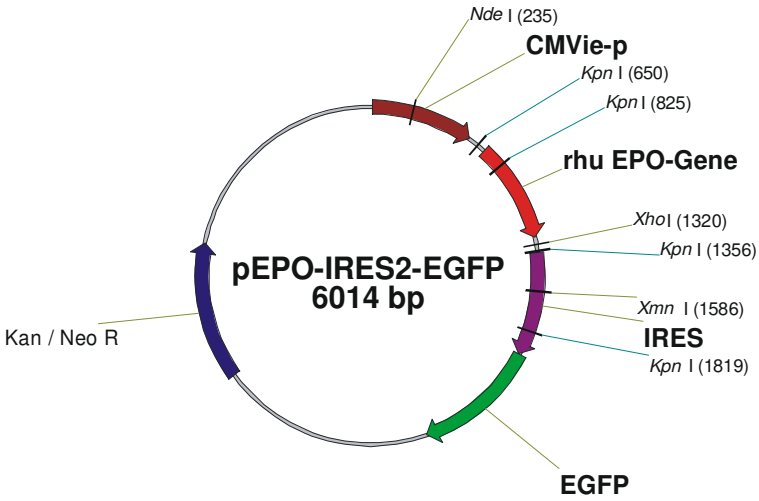
- pCR-3-EPO (5715 bp)



This plasmid was kindly provided by Dr. Eckart Grabenhorst, Proteinglycosylation Department, GBF, Braunschweig, Germany.

It was constructed by using the vector pCR™ 3 (Invitrogen, Life Technologies, CA, USA) (http://invitrogen.com/content/sfs/vectors/pcr3.pdf) and a cloned human erythropoietin gene (Powell et al. 1986). The rhuEPO gene is under the CMV promoter control. The vector also contains the ampicillin resistance gene.

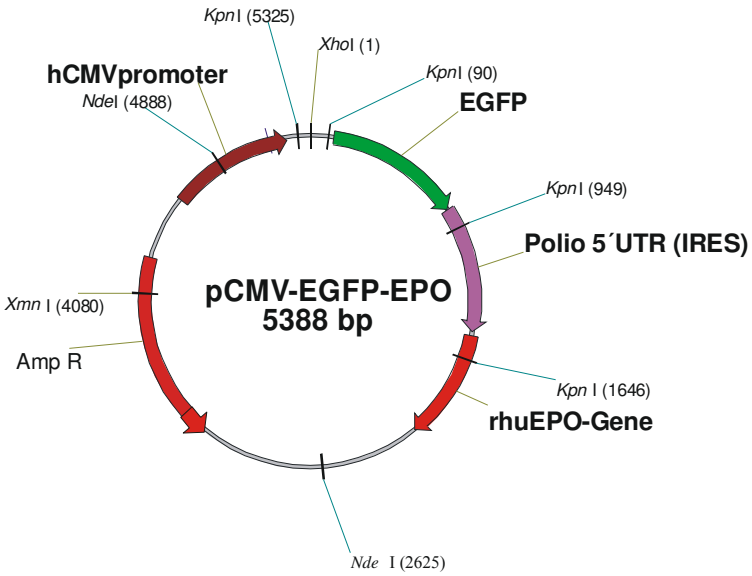
- pEPO-IRES2-EGFP (6014 bp)



This bicistronic vector was generated during this thesis work. It has the rhuEPO and the EGFP genes under the CMV promoter control. It was constructed using the plasmids pIRES2-EGFP and pCR-3-EPO (Fig. 2.17.2a). This vector contains the

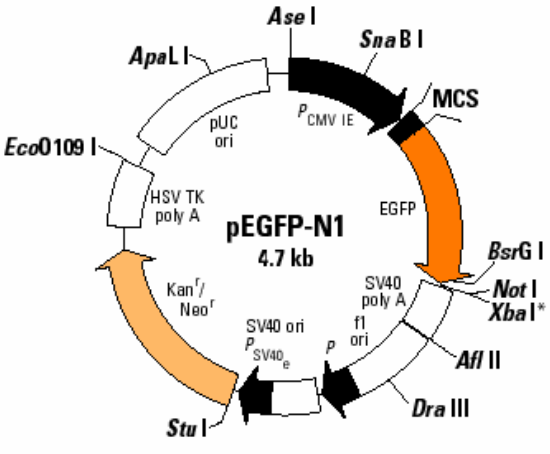
kanamycin and neomycin resistance genes. For details see the result section number 3.3.1.

- pCMV-EGFP-EPO (5388 bp)



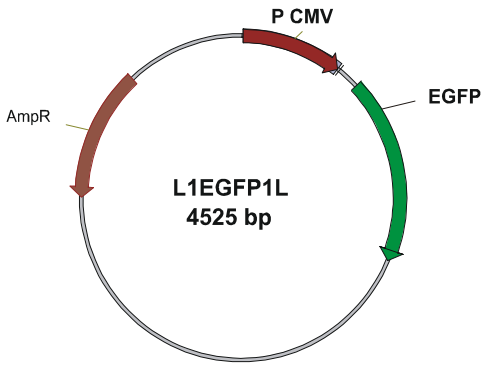
This bicistronic vector was generated during this thesis work using the plasmids pTracer™ -CMV2 and pSBC-EGFP-rhuEPO. It contains an upstream EGFP gene and a downstream rhuEPO gene. These genes are under the CMV promoter. It contains the gene, which confers resistant to ampicillin (see Fig. 2.17.2b). For details see the section 3.3.2.

- pEGFP-N1 (4700 pb) (BD Biosciences, Clontech, CA, USA)



(From:http://www.bdbiosciences.com/clontech/techinfo/vectors/vectorsE/pEGFP-N1.shtmL). This vector contains the EGFP gene under control of the immediate early Cytomegalovirus promoter and carries the kanamycin and neomycin resistance genes.

- pL1EGFP1L (4525 bp)



This vector contains the EGFP gene under control of the immediate early Cytomegalovirus promoter. It carries the ampicillin resistance gene.

These last two plasmids were kindly provided by Dr. Christiane Beer, Regulation and Differentiation Department GBF, Braunschweig, Germany.

2.17.2 Construction of bicistronic vectors

Both plasmids were constructed with the aim to have a bicistronic plasmid with a strong promoter (CMV) for expression in mammalian cells, a model-protein to evaluate the productivity of transient transfected mammalian cells (rhuEPO), and reporter gene to evaluate the transfection efficiency (EGFP) (see 2.19).

For the construction of bicistronic vectors, standard protocols were followed. These protocols are described below (2.17.2.1 to 2.17.2.8) (Sambrook et al. 1989).

Figures 2.17.2a and 2.17.2b show a scheme of the strategy performed for the construction of the plasmid pEPO-IRES2-EGFP and pCMV-EGFP-EPO, respectively.

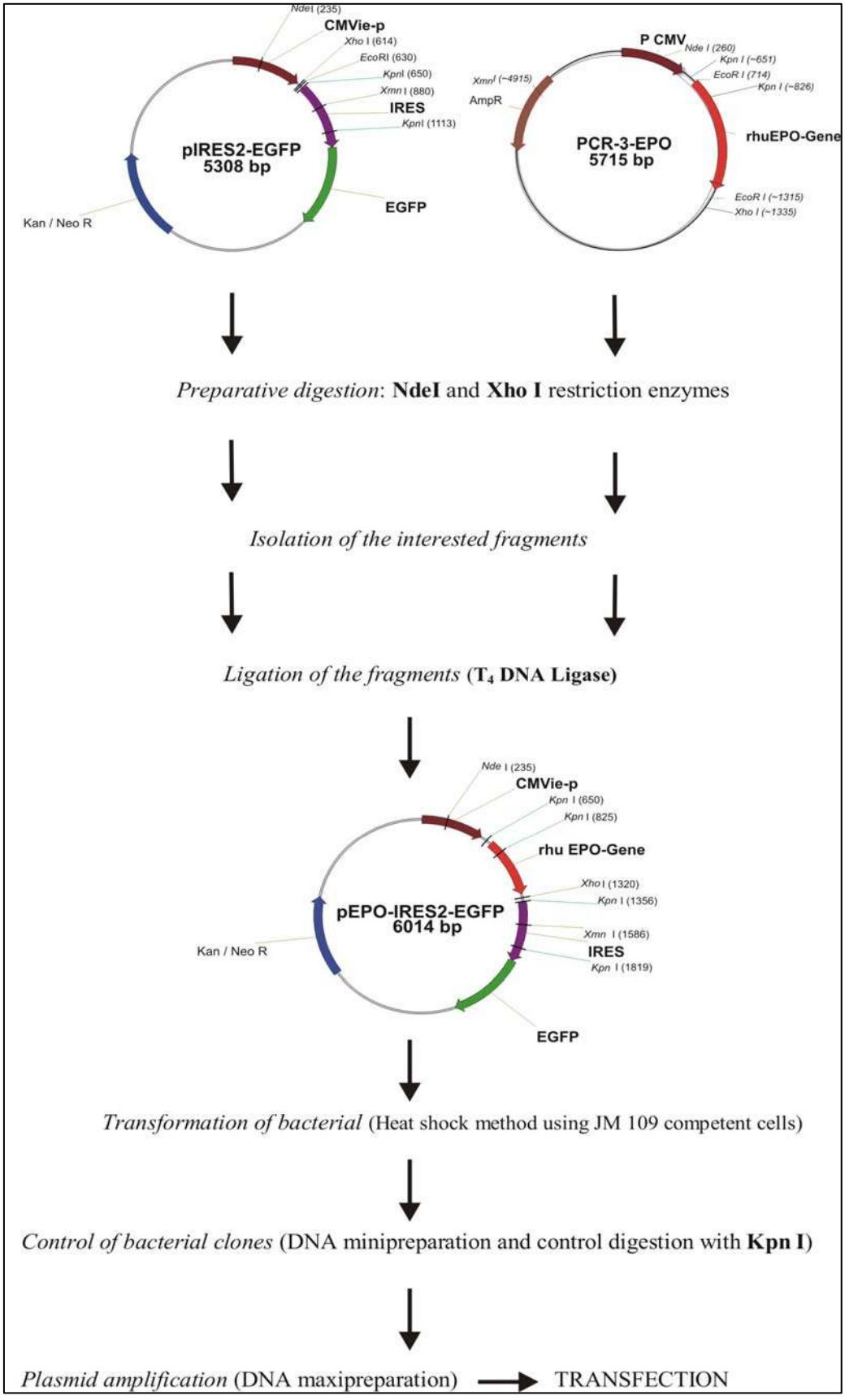


Fig. 2.17.2a: Strategy for construction the bicistronic vector pEPO-IRES2-EGFP.

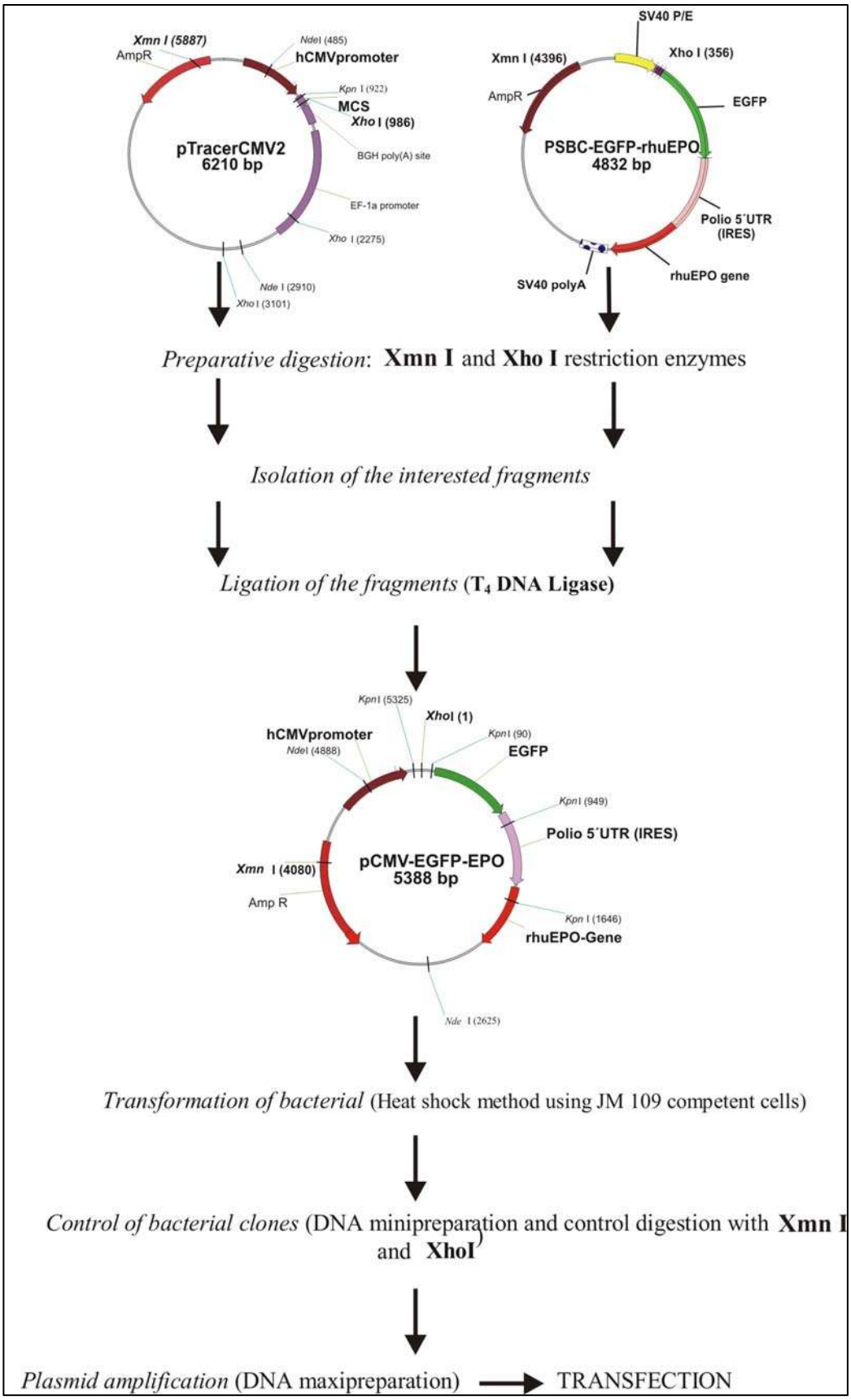


Fig. 2.17.2b: Strategy for construction the bicistronic vector pCMV-EGFP-EPO.

2.17.2.1 *E. coli* transformation

Two different protocols were used to transform the different *E. coli* strains (manufacturers' recommendations):

- *Electroporation*: it was the recommended method to transform the XL 1 Blue competent cells, which were used to host all plasmids. Electroporation was performed using the following settings on the electroporator: capacitance extender: 500 μF; capacitance: 25 μF; pulse controller: 200 Ω; gene pulser (volt-set): 2.5 kW (max.).

In an eppendorf tube, 1 μL of plasmid of a concentration of 0.1 μg μL^{-1}, was added. The competent *E. coli* cells were thawed very quickly on ice, 50 μL of the competent cells were added to the plasmid solutions, resuspended once, and incubated for approximately 30 s on ice. During this time, 500 μL of SOC medium was prepared. The solution (plasmid and *E. coli*) was transferred to a transformation cuvette and put into the gene pulser. The two buttons of the pulser were pushed until signal and the 500 μL of prepared medium were given to the cuvette. The solution was transferred to a new reaction tube, which was immediately placed on ice. 50 μL were used to prepare a LB agar plate with the corresponding antibiotics. The plates were incubated upside down, overnight at 37 °C.

- *Heat shock*: this standard method was recommended to transform the JM 109 competent cells (see Technical Bulletin No. 095, Promega, Madison, WI, USA). Briefly, sterile polypropylene tubes were chilled on ice and the competent cells were thawed on ice for approximately 5 min. The thawed competent cells were mixed gently and 50 μL were transferred into each of the chilled tubes. 1 to 25 ng of DNA (in a volume not higher than 5 μL) were added per 50 μL of competent cells and the tube were quickly flicked several times. The tubes were immediately placed on ice for 10 min. After that, the cells were heat-shocked for 45 – 50 seconds in a water bath at exactly 42 °C and then, placed on ice for 2 min more. After that, 450 μL of cold (4 °C) SOC medium were added to each transformation reaction. The mix was incubated for 60 min at 37 °C with shaking (180 rpm). After that, the tubes were centrifuged (2000 rpm, 5 min) to concentrate the transformed bacterial, and aliquots were evenly spread onto LB plates with the appropriate antibiotics. They were incubated overnight at 37 °C.

2.17.2.2 DNA mini-preparation

Successful transformations were checked by means of plasmid mini-preparations using the "QIAprep spin miniprep kit protocol" (see table 2.2a).

Selected colonies were taken with a sterile toothpick. A small sample was taken in order to have a backup. The bacteria were transferred into LB-antibiotic medium

reagent cups (2 mL of prepared medium in 10 mL glass cup). These cups were incubated either 6 h or overnight at 37 °C in a shaker at 180 rpm. The following day, the cell suspension was transferred into a 2.5 mL Eppendorf tube and centrifuged (2 min, 7000 rpm, at RT). After this step, the pellet can be frozen at –20 °C, if necessary; otherwise the Qiagen protocol was followed. The DNA concentration should be between 25 µg and 50 µg in 50 µL of the elution buffer.

2.17.2.3 Digestion of DNA with restriction enzymes

The purpose of endonuclease digestion is to cleave the DNA in specific sites either for analytical or for preparative purposes. The amount of employed DNA for analytical purposes depends on the expected DNA bands in the gel and is normally between 0.5 µg and 1.0 µg. For preparative purposes, the DNA content varies between 10 to 20 µg.

The amount of the restriction enzyme depends also on the DNA content. With increasing DNA content either the applied enzyme or the incubation time of digestion must be increased. However, the maximum amount of enzyme must be 10 % of the total cocktail volume, otherwise the elevated glycerin concentration, in which the enzymes are stored, would disturb the enzymatic activity. For each enzyme, its own 10× buffer or/and 100× BSA was used, as indicated by the manufacturer. The final volume of enzyme digestion was completed with water. In the case of a double digestion, a buffer in which both enzymes were active was used.

In this work, all enzymes were purchased from New England $Biolabs_{Inc.}$, Hitchin, Hertfordshire, UK in the concentrations indicated below:

- *EcoR*I (Catalogue no.: R0101S): 20,000 U mL^{-1}
- *Nde*I (Catalogue no.: R0111S): 20,000 U mL^{-1}
- *Xho*I (Catalogue no.: R0146S): 5,000 U mL^{-1}
- *Kpn*I (Catalogue no.: R0142S): 10,000 U mL^{-1}
- *Xmn*I (Catalogue no.: R0194S): 20,000 U mL^{-1}

"Control digestion" was performed to check whether the DNA isolated from the bacterial clones has the correct fragment of interest in correct orientation and position.

For a final reaction volume of 10 µL, the following master-mix was prepared:

- 1 µL 10× enzyme buffer
- max. 1 µL enzyme
- × µL DNA (2.5 to 5.0 µL)
- 0.1 µL 100× BSA
- × µL water (complete to 10 µL of total volume)

"Preparative digestion" was performed with the aim to have more desired DNA fragments and for isolating it from the agarose gel.

For example for a final reaction volume of 40 µL:

- 4 µL 10× enzyme buffer
- 1.5 or 2.0 µL enzyme 1
- 2.5 or 2.0 µL enzyme 2
- × µL DNA (6.0 to 10.0 µL)
- 0.5 µL 100× BSA
- × µL water (complete to 40 µL of total volume)

The digestion started when the enzyme/s and the sample/s were incubated 1 – 2 h at 37 °C. For preparative digestion, a minimum incubation time of 2 h and maximum of overnight were used. Samples were prepared for electrophoresis.

2.17.2.4 Gel preparation and electrophoresis

For analytical and preparative purpose a 1 % agarose (Agarose NA, Pharmacia, Uppsala, Sweden) gel were prepared as follows: agarose was dissolved in 30 mL running buffer (TAE-buffer, see below), shortly heated in a microwave oven, cooled (≈ 85 °C) and ethidium bromide was added to a final concentration of 0.5 µg mL^{-1}. The liquid agarose gel was mixed carefully, poured in a mini gel chamber avoiding bubble formation, and it was left to solidify. The running buffer contained the same ethidium bromide concentration as the gel.

5× Loading buffer was added to the DNA samples (1/5 of cocktail volume). Samples were loaded into the gel wells, and were run at 100 V. The process was stopped when the bromophenol blue band was closed to the end of the gel. A DNA marker, λ Hind III (prepared in the Regulation and Differentiation Department GBF, Braunschweig, Germany) was loaded in parallel with the samples. The DNA bands were detected under UV-light and photographed.

50x TAE-buffer: 242 g L^{-1} Trisma-Base, 57.1 mL L^{-1} acetic acid, 100 mL L^{-1} 0.5 mol L^{-1} EDTA, pH 8.0.

50x TAE buffer was diluted 1:50 before use.

5x Loading buffer: 0.25 % (V V^{-1}) Bromophenol blue, 40 % (W V^{-1}) sucrose in water.

2.17.2.5 Isolation of the plasmid

To isolate the DNA fragment of interest from the plasmid, a restriction enzyme digestion was carried out for two hours at a 37 °C. The DNA fragments were separated in an agarose preparative gel. The gel was run as it was explained in the previous section (2.17.2.4). The DNA fragments were excised from the gel using a scalpel visualized under the UV-light. The DNA was eluted from the gel using the QIAquick gel extraction kit (see table 2.2a) according to the manufacturer's description. After the DNA extraction, an analytical gel was prepared to estimate the amount of DNA recovered from the gel.

2.17.2.6 Ligation

The ligation cocktails used generally contained three times the molar amount of the insert respect to the vector. The amount of DNA was calculated from the analytical gel (2.17.2.5). The T_4 DNA Ligase (Catalogue No.: M0202S; 400,000 U mL^{-1}, New England $Biolabs_{Inc.}$, Hitchin, Hertfordshire, UK) was used for all the experiments.

1 µL of the T_4 DNA Ligase in a 20 µL of total reaction was used, according to the manufacturer's instructions:

- 2 µL 10× ligase buffer
- 1 µL T_4 DNA Ligase
- × µL of fragment 1 (vector)
- × µL of fragment 2 (insert, must be two or three times more concentrated than the vector)
- × µL water (complete to 20 µL of total volume)

Incubation time was between 2 to 3 hours at RT.

2.17.2.7 DNA maxi-preparation – DNA giga-preparation

Maxi-preparation or giga-preparation is necessary if a large amount of plasmid-DNA is needed. In order to confirm whether the selected colony contained the right insert, a mini preparation was done. Once, it was confirmed, the bacteria were cultivated in large-scale culture and the plasmid was isolated.

For this purpose, the selected colony was incubated in 10 mL LB-antibiotic medium for approximately 5 h at 37 °C to produce the pre-culture. Two 2-L Erlenmeyer flasks were filled, each with 200 mL with the same medium, inoculated with 5 mL pre-culture and incubated at 37 °C overnight in a shaker (180 rpm). The next day, either the Qiagen plasmid maxi kit or Qiagen plasmid giga kit (see table 2.2a) was used to isolate the plasmid DNA, according to manufacturer's instructions.

The concentration of DNA was measured (see below, 2.17.2.8) and the presence of the expected plasmids was checked by restriction pattern analysis (2.17.2.3).

2.17.2.8 Determination of DNA concentration

The DNA concentration and the quality of the DNA solution were determined by measuring the absorption at two wavelengths. The DNA solution was diluted 1:100 in TE-buffer (see below) and 100 µL of this solution was transferred into a quartz cuvette (Pharmacia, Uppsala, Sweden). The absorption was measured spectrophotometrically at 260 nm and 280 nm. The ratio between OD_{260} and OD_{280} should be between 1.75 and 2.0; otherwise, if the ratio is lower, the DNA is contaminated with proteins. An OD_{260} = 1 corresponds to a DNA concentration (double stranded) of 50 µg mL^{-1}.

DNA concentration (µg mL^{-1})= OD_{260} × 50 × dilution factor

TE-buffer: 10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1}, pH: 8.0.

2.18 Transient transfection methods

One of the aims of this work was to develop and optimize a scale-up transient transfection method in mammalian cells using serum-free medium. One of the DNA delivery methods for plasmid, polyethylenimine (PEI), has been shown to be suitable for scale-up operations. It has the advantage of being cost-effective (see Introduction section 1.3.2). For this reason, in this work, different experiments using PEI were performed working with cell culture under serum-containing and serum-free conditions.

2.18.1 Transient transfection mediated by PEI

All the experiments were performed using the HEK293s cells, growing in different media which are described in each experiment (see details in results sections 3.2, 3.4, 3.6 and 3.7). During the first phase of the exponential growth, the cells were in very good conditions showing a viability above 90 % and grew as single cells or small clusters at a concentration between 5.0×10^5 mL^{-1} and 1.0×10^6 mL^{-1}. Cells were incubated at 37 °C and 12 % of CO_2.

Stock solutions of PEI were prepared using a 25 kDa linear PEI (Polysciences, Warrington, PA, USA) in a concentration of 1 mg mL^{-1}. They were prepared in water, neutralized with HCl, sterilized by filtration (0.22 µm) and aliquoted and stored at –70 °C.

In all the experiments a plasmid concentration between 0.02 µg $µL^{-1}$ and 2.0 µg $µL^{-1}$ with a A260/A280 ratio between 1.75 and 2.00 was used. pEPO-IRES2-EGFP and pCMV-EGFP-EPO plasmids were utilized in these experiments.

2.18.1.1 Small-scale transient transfection

Two protocols were used. In both cases, the transfection procedures were performed in serum-containing or/and in serum-free medium. (See details in sections 3.2, 3.4 and 3.6). All experiments were done in duplicate in a 12-well plate.

For the complex formation, different ratios between DNA and PEI were used to determine the lowest amount of DNA with the highest transfectability. This was prepared at RT under a laminar flow sterile hood as it is explained below.

- Protocol A: Cell wash step before the addition of transfection complex

Three hours before transfection, cells cultured under the conditions explained in 2.18.1, were harvested in the middle of the exponential growth phase. They were centrifuged (3 min at 1200 rpm), washed one time with fresh new medium and finally, resuspended in this medium at a concentration of 1.0×10^6 mL^{-1} in a conic tube. In order to enrich single cells, the tube was vortexed for 20 s and 500 µL of the cell suspension were distributed per well. The plate was incubated at 37 °C, 12 % CO_2 for three hours, until the addition of the transfection complex.

For the preparation of the transfection complex, DNA was diluted in fresh serum-free medium in a volume equivalent to one-tenth of the culture volume to be used in the transfection. Two minutes later, PEI was added, and the mixture was immediately vortex-homogenized and incubated for 10 min at RT prior to the addition to the cells.

Once the DNA:PEI complex was added to the cells, the plate was additionally incubated for three hours under the same conditions. Then, the volume was completed to 1 mL by the addition of fresh medium, gently shaken and further incubated until flow cytometry analysis (see 2.19.1) (normally between 48 h to 72 h later). The supernatants were stored at –20 °C for later analysis of EPO (see 2.19.2).

- Protocol B: Transfection without cell wash step before the addition of transfection complex

Cells cultured under the conditions explained in the section before and collected in the middle of the exponential growth phase, were counted 24 h before to perform the transfection procedure. They were diluted to a concentration of about 3.0×10^5 mL^{-1} and 5.0×10^6 mL^{-1} and were further incubated until next day. Cells were counted again and when they nearly doubled their concentration (between 5.0×10^5 mL^{-1} and 1.0×10^6 mL^{-1}, 24 h conditioned medium), they were transfected as explained below.

900 µL of cell culture were seeded per well, previous vortex-homogenization for approximately 20 s. The plate/s was/were incubated for the time during the complex formation was prepared in the same condition as before. The complex formation was

done as explained in the protocol A, section 2.18.1.1. 100 µL of this was added to the cells, gently shaken and incubated further until its analysis using flow cytometry (see 2.19.1). The supernatants were stored at –20 °C for later analysis of EPO (see 2.19.2).

2.18.1.2 Transfection in spinner flask

The transfections were performed without cells washing step before the addition of the transfection complex, using serum-containing and serum-free media. In all cases the preparation of transfection complexes were made following the protocol A explained under 2.18.1.1.

Cells were inoculated in a big spinner flask (1000 mL) using a working volume of 200 mL, with an initial concentration between 3.0×10^5 mL^{-1} and 5.0×10^5 mL^{-1}. Cells were transfected 24/48 hours later, when the cell concentration was reached 6.0×10^5 mL^{-1} and 1.0×10^6 mL^{-1}, respectively.

The complex was prepared by diluting the DNA in 20 mL of fresh serum-free medium (1/10 from the total volume to be transfected) and by adding PEI two minutes later. This mixture was vortexed and incubated for 10 min at RT until the addition to the culture.

Then the culture was further incubated under the same conditions as before and a sample was taken daily to determine the cell concentration, the viability, glucose and lactate, LDH, amino acids (named as standard determinations, section 2.10 and 2.11) and nucleotide concentrations (2.12). A fraction of the cells was taken to analyze the GFP expression using flow cytometry (see 2.19.1). Culture supernatants were stored at –20 °C for later analysis of EPO (2.19.2).

2.18.1.3 Transfection in bioreactor

A 1.8 L bioreactor with a working volume between 1.4 L and 1.6 L, was inoculated at a cell concentration between 3.0×10^5 mL^{-1} and 5.0×10^5 mL^{-1}. When the cell concentration was nearly duplicated, 24/48 hours later, the cells were transfected. For the complex formation, the DNA was diluted in 50 mL of fresh serum-free medium and two minutes later PEI was added. After 10 min of incubation at RT, the complex was added to the bioreactor through the inoculum tube, following by a wash with 20 mL of serum-free medium. The conditions of the culture were the same as those described in 2.5.4, with the difference that the stirrer speed was increased to 60 and 70 rpm. A sample was taken daily to perform the standard determinations (section 2.10 and 2.11) and nucleotide concentrations (2.12). A fraction of the cells was taken to analyze the GFP expression using flow cytometry (see 2.19.1). Culture supernatants were stored at –20 °C for later analysis of EPO (2.19.2).

2.19 Evaluation of transfection

In this work, the model proteins, EGFP and rhuEPO, were used to evaluate transfection procedures.

2.19.1 Green Fluorescent Protein determination

The EGFP, the reporter protein, was measured by flow cytometry. This parameter was used to evaluate the transfection efficiency.

Cells were counted and 5.0×10^5 to 1.0×10^6 of them were added into a sterile Eppendorf tube and centrifuged 3 min at 2000 rpm. The supernatants were stored at –20 °C for later EPO analysis. Cells were washed twice with PBS (without Ca^{2+} and Mg^{2+}) and resuspended in a volume of 300 µL to 400 µL of the same buffer for immediate flow cytometry analysis. The cells were transferred to the FACS-tube, homogenized using a vortex for guaranteeing single cells measurement, and analyzed.

The percentage of GFP-positive cells was assessed using the CellQuest Pro software. Only viable cells were determined for the expression of GFP, considering 3000 events per measurement. A control of untransfected cells (negative control) was measured to eliminate fluorescent background from the cells.

2.19.2 Erythropoietin quantification

Erythropoietin quantification was performed by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) to evaluate the productivity of the transient transfected mammalian cells.

ELISA microplates were coated with 400 ng of affinity-purified monoclonal antibody mAb 2B2 (Laboratorio de Cultivos Celulares, Santa Fe, Argentina) diluted in coating buffer (50 mmol L^{-1} carbonate buffer, pH: 9.6) and incubated either 1 h at 37 °C or overnight at 4 °C. The plates were washed with 0.05 % Tween 20 in PBS (T-PBS) and then blocked with PBS-1 % BSA to prevent non-specific binding (1 h at 37 °C). After washing with T-PBS, the plates were loaded with the samples. Standard curves were achieved by 1:2 serial dilutions of purified rhuEPO (Laboratorio de Cultivos Celulares, Santa Fe, Argentina) from 4 µg mL^{-1} to 7.8 ng mL^{-1} dissolved in cell culture medium (the same medium in which the transfected cells were cultivated). This control was performed in triplicates. Samples were diluted 1:2 and serial dilutions were performed with the same culture medium. Plates were incubated for 1 h at 37 °C, washed with T-PBS and incubated with rabbit anti-rhuEPO polyclonal antibodies (pAb, Laboratorio de Cultivos Celulares, Santa Fe, Argentina), diluted 1:1,000 with 0.1 % BSA – 0.05 % Tween 20 in PBS (BSA-T-PBS). After 1 h incubation at 37°C, the plates were washed and incubated with peroxidase-labelled goat anti-rabbit antibody (DAKO,

DakoCytomation, CA, USA; Cat. no. P0448) diluted 1:1,000 in BSA-T-PBS. After the final wash, the plates were incubated in the dark with 50 mmol L^{-1} citric acid-phosphate buffer, pH: 3, containing 3.0 mg mL^{-1} o-phenylenediamine and 0.12 % (V V^{-1}) hydrogen peroxide (substrate solution). The absorbance was measured at 450 nm with a micro-titer plate reader.

EPO concentration was calculated by interpolating the absorbance from the plot in logarithmic scale of standard absorbance vs. standard concentration.

3. Results

3.1 Adaptation to serum-free medium

First, HEK293s cells were either adapted to a serum-free medium or a protein-free medium formulation to guarantee chemically defined culture conditions for further transient transfection processes.

3.1.1 Sequential adaptation using cell density effect

The following experiments were performed by a progressive medium exchange, using high cell concentrations in order to facilitate the cell adaptation process due to a better conditioning of the culture medium. The following serum-free media were used: (1) Gene Therapy Medium-2 (GTM-2), (2) Pro 293s-CDM, (3) CD 293 and (4) 293 SFM II. The adaptation was done according to the protocol described in 2.6.1. DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium was used as control.

Samples were taken from the four spinners daily. For each culture, cell concentration, viability, glucose and lactate concentration, as well as LDH activity and amino acids concentration were routinely assessed (data not shown). These measurements were called standard determination (2.10 and 2.11).

During the adaptation process and the first passage in the new serum-free medium, cells growth was characterized by forming large aggregates (more than 50 cells) making a homogenous sampling difficult. Despite this, a backup of cell samples from all four cultures was performed and 10 to 15 cryogenic vials per spinner flask were stored in the gas phase of liquid nitrogen using the cryopreservation protocol (see under 2.7.1).

One cryogenic vial of each different condition (1), (2), (3) or (4) was thawed. Cells were cultured in the respective serum-free medium and sub-cultured when they reached a concentration between 1.5×10^6 mL^{-1} to 2.0×10^6 mL^{-1}. Generally, four sub-cultures were performed. Medium (1) was excluded from further experiments because the cells did not grow during 12 days of cultivation.

During cell cultivation in the three remaining media, large cell aggregates of more than 50 cells occurred. Therefore, the following experimental steps were carried out in order to break down the cell clumps (2.5.3). Figure 3.1a show an example of the different steps during cluster disintegration.

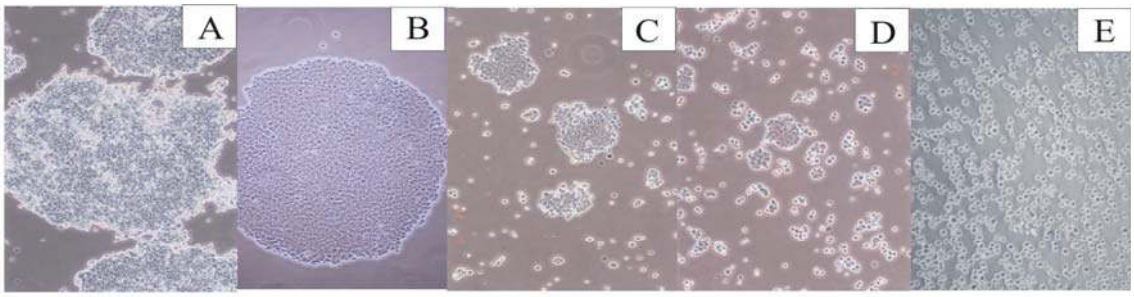


Fig. 3.1a: Different development of aggregates during cell cluster disintegration of HEK293s cells cultured in 293 SFM II. (from **A** to **E**): Cell culture stirred at 40 rpm (**A**) and in the next four sub-cultures at 80 rpm (**B** to **E**).

After four sub-cultivations, cells in medium (3) and (4) grew as a single cell culture and those in medium (2) grew in clusters (more than 20 – 30 cells).

Taken into account that a homogeneous cell suspension is an important prerequisite for successful and efficient transfection and due to the fact, that cells only cultured in medium (3) and (4) grew as single cells, medium (2) was excluded from the further test phase.

Batch cultures were performed for eight days in small spinner flasks using CD 293 (3) and 293 SFM II (4) media. Standard parameters were determined. At the end of the batch cultivation, four amino acids were found to be limited (data not shown). Therefore, both media were suplemented with 1 mmol L^{-1} Asp, 150 µmol L^{-1} His, 500 µmol L^{-1} Thr, and 100 µmol L^{-1} Trp.

Succesive sub-cultivations were performed using both suplemented media. The standard parameters were measured. Similar results were found for both media (data not shown).

Population doubling time (t_d) of HEK293s cells growing in CD 293 + AA was comparably high and between 60 h and 70 h, whilst t_d of the respective cells growing in 293 SFM II + AA was lower and between 30 h and 35 h. Notice, that the control in serum-containing DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium was between 20 and 30 h. Cells growing in 293 SFM II + AA showed a better disintegration of clusters and single cell-type growth than cells growing in CD 293 + AA. Therefore, HEK293s cells growing in 293 SFM II + AA were considered to be fully adapted to the new serum-free medium. Therefore, this medium was selected for further experiments. Finally, a batch culture, was performed to better characterize HEK293s in this 293 SFM II + AA medium (Fig. 3.1b). The maximum cell concentration was about 3.5×10^6 mL^{-1} with a viability above 95 % during the first 96 h of culture. After this period the viability dropped dramatically when the cells entered the stationary phase (A), and correlated to a drastic increase of the LDH (C). The lactate concentration in the supernatant was below 0.6 g L^{-1} and the glucose concentration was maintained above 1.5 g L^{-1} (C).

These sets of experiments showed that the adaptation protocol using progressive medium exchange under high cell concentrations allowed the HEK293s cells to fully adapt to the serum-free 293 SFM II + AA medium. Therefore, these cells growing in this medium were chosen to perform the preliminary transfection experiments (see section 3.2 and section 3.4.1).

3.2 Preliminary transient transfection experiments using PEI and the pSBC-EGFP-rhuEPO vector plasmid

The pSBC-EGFP-rhuEPO vector plasmid was used for stable expression in BHK-21 cells transfected using the calcium-phosphate method as previously reported (Irani 2000; Irani et al. 2002). The preliminary transient transfection experiments with HEK293s cells were also performed using this vector, but PEI as mediator according to Protocol A (cell wash step before the addition of transfection complex), section 2.18.1.1.

HEK293s cells cultured in serum-free 293 SFM II + AA medium and in DMEM-Ca^{2+} + Vit. + Pl. + 5 % FCS medium were transfected using different ratios of DNA:PEI. Results from flow cytometry analysis showed no significant transfection, evaluated by GFP expression, for all DNA:PEI ratios adjusted after 48 h or 72 h post transfection (hpt) (data not shown).

Two expression vectors, pEGFP-N1 and L1eGFP 1L (see details under 2.17.1) bearing the EGFP gene under control of the CMV promoter were tested. First, small-scale transient transfections (Protocol A, section 2.18.1.1) using HEK293s cultured in serum-containing medium (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) were performed.

A ratio of 1.0:1.5 of DNA:PEI (µg:µg), having the highest transfection efficiency in previous experiment (Durocher et al. 2002) was used. For the complex formation the following media were used:

1- Serum-free medium (w/o s): DMEM -Ca^{2+} + Vit. + Pl.
2- Serum-containing medium (with s): DMEM -Ca^{2+} + Vit. + Pl.+ 5 % FCS

Transfections were performed using both vectors containing the CMV promoter, and were compared to the reference vector pSBC-EGFP-rhuEPO containing the SV40 promoter.

Cells were analyzed by flow cytometry at 72 hpt. The respective results are shown in figure 3.2.a. Transfection efficiencies were higher than 95 % with pEGFP-N1 and L1eGFP 1L plasmids for complexes formed in serum-containing and serum-free medium. A transfection efficiency of nearly 20 % was reached with the pSBC-EGFP-rhuEPO plasmid only for complex formed under serum-free medium conditions.

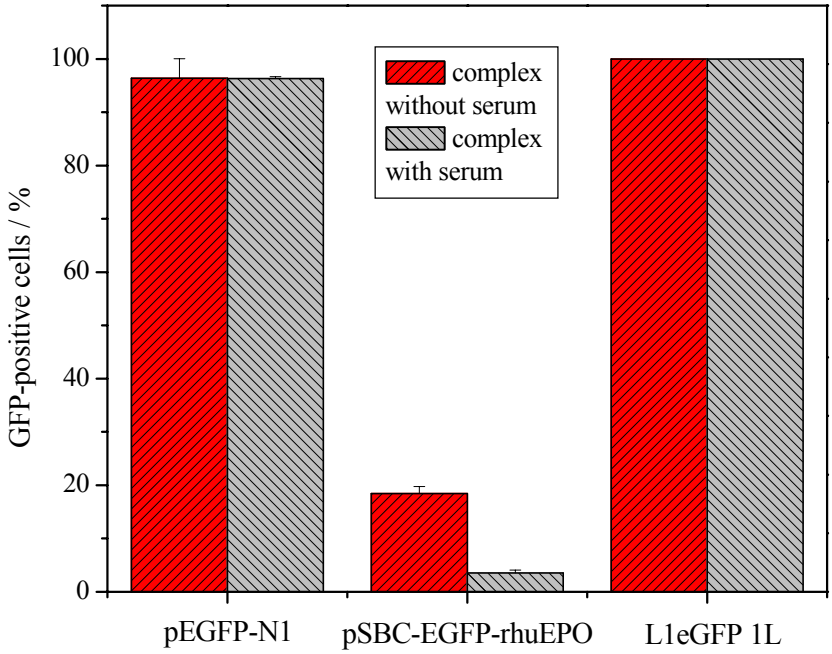


Fig. 3.2a: HEK293s cells cultured in serum-containing medium (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) were transfected with 1 µg DNA from different plasmid and 1.5 µg PEI. The percentage of GFP-positive cells was measured 72 hpt. The DNA:PEI complex for transfection was prepared in serum-free medium (DMEM -Ca^{2+} + Vit. + Pl.) (hatched red columns: complex without serum) and in serum-containing medium (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) (hatched gray columns: complex with serum). Errors bars represent + standard deviation (SD) of one experiment done in duplicate.

These results showed, that the highest transfection efficiency was reached under PEI-mediated transient transfection using vectors bearing the CMV promoter. Therefore, for the constructions of the new bicistronic vectors containing the EGFP reporter gene and the EPO model gene, the CMV promoter was chosen.

3.3 Construction of bicistronic plasmids

Two different bicistronic plasmids were constructed, both containing the EGFP and the EPO genes under control of the CMV promoter, thus generating different model plasmids as explained below.

3.3.1 Construction of pEPO-IRES2-EGFP vector

The construction of the pEPO-IRES2-EGFP vector followed the protocol indicated in 2.17.2, figure 2.17.2a. Part of the CMV promoter and the rhuEPO gene were removed from the pCR-3-EPO vector using the *Nde*I and *Xho*I restriction enzymes (2.17.2.3) and ligated with the *Nde*I / *Xho*I digested pIRES2-EGFP vector (2.17.2.6). The CMV

promoter was regenerated and the rhuEPO gene was inserted upstream of the IRES site of the encephalomyocarditis virus (ECMV) and the EGFP gene (Fig. 3.3.1a).

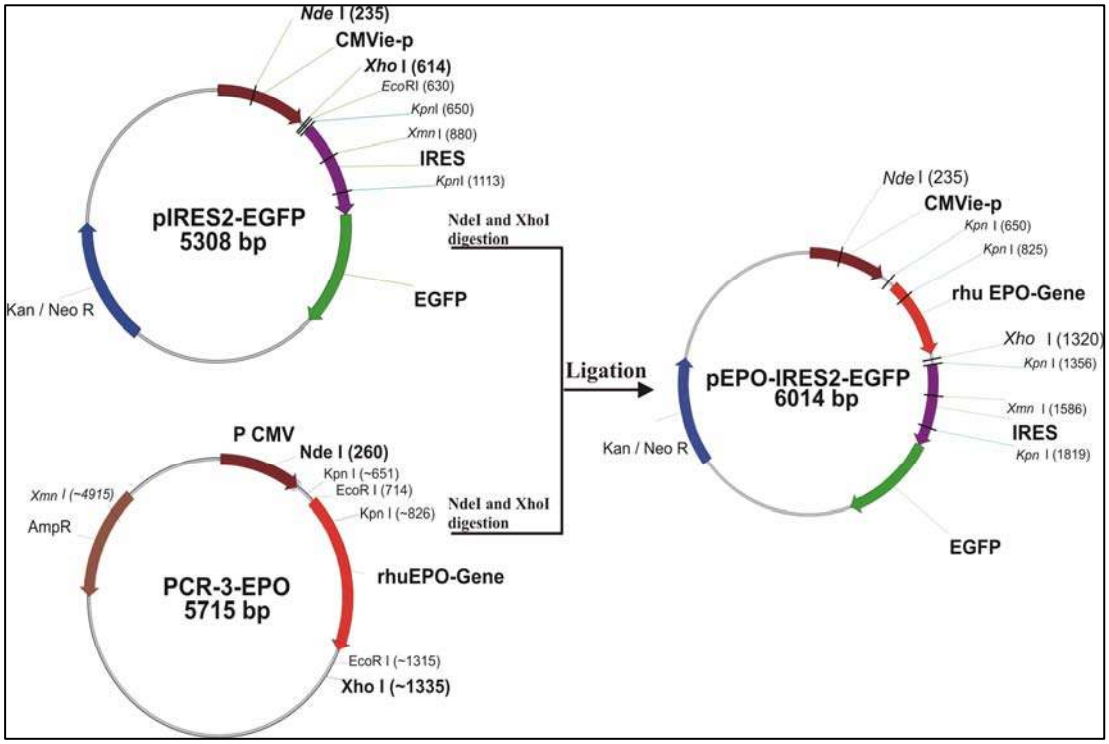


Fig. 3.3.1a: Construction of the bicistronic vector pEPO-IRES2-EGFP. pIRES2-EGFP and the pCR-3-EPO vector were digested with *Nde*I and *Xho*I. The revealing rhuEPO gene fragment plus part of the CMV promoter were ligated with the pIRES2-EGFP fragment. The CMV promoter was regenerated and the rhuEPO gene was inserted in the MCS, upstream of the IRES site and the EGFP gene resulting in the new bicistronic vector, pEPO-IRES2-EGFP.

pIRES2-EGFP and pCR-3-EPO vectors were digested (2.17.2.3) as described below and separated in the agarose gel under preparative conditions. Fragments were cut out from the gel under UV light (2.17.2.5; Fig. 3.3.1b).

Digestion was carried out using *Nde*I and *Xho*I restriction enzymes at the same time for a total volume of 40 µL according to the protocol described in section 2.17.2.3. The digestion was incubated 2h at 37 °C.

The *Nde*I enzyme cuts both plasmids within the CMV promoter. The *Xho*I restriction sites are in the MCS of the pIRES2-EGFP vector and downstream of the rhuEPO gene in the pCR-3-EPO vector. As a result, part of the CMV promoter plus the rhuEPO gene fragment were cut out from the pCR-3-EPO vector obtaining one insert of about 1075 bp. This fragment was used for subsequent ligation with the fragment obtained from the pIRES2-EGFP vector (4929 bp) (Fig. 3.3.1b).

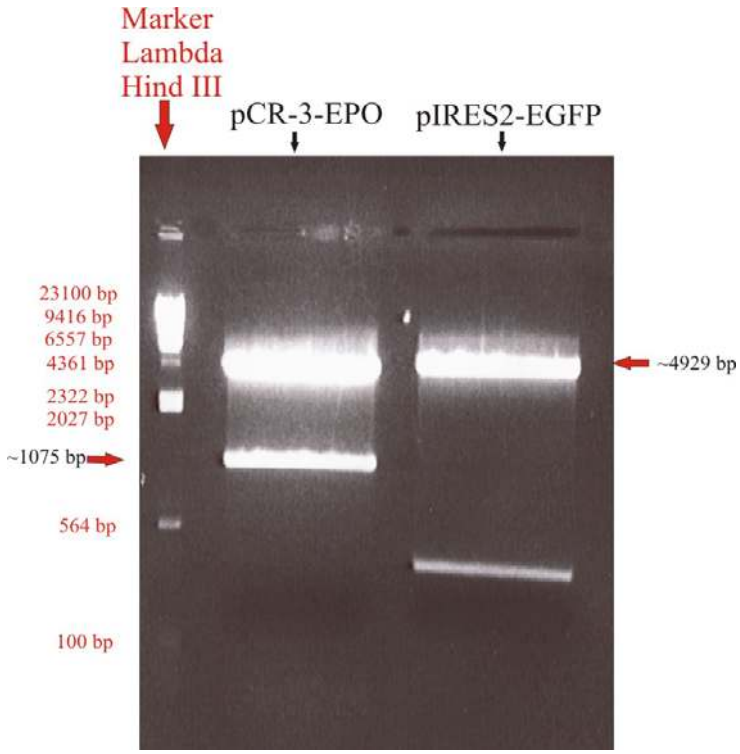


Fig. 3.3.1b: Preparative digestion with the *Nde*I and *Xho*I restriction enzymes of the plasmids pCR-3-EPO and pIRES2-EGFP. The digested plasmids were separated on 1 % agarose gel. Fragment of ~ 1075 bp (from pCR-3-EPO vector) contains part of the CMV promoter plus the rhuEPO gene. For pIRES2-EGFP, the interested fragment was the one of ~ 4929 bp. In this plasmid, part of the CMV promoter and the MCS were cut out.

Both fragments were purified using the QIAquick gel extraction kit protocol and the purified yield was verified in a 1 % agarose gel (data not shown). Fragments were ligated following the protocol as indicated in 2.17.2.6 for a total volume of 20.0 µL.

The generated plasmid was amplified in bacteria JM 109, using the heat-shock method for the transformation (2.17.2.1).

For the screening, ten clones were selected and cultured in LB-Kanamycin medium in order to be used for DNA mini-preparation (2.17.2.2). Accuracy of the constructed vector was controlled with a restriction analysis using the *Kpn*I enzyme.

Four *Kpn*I restriction sites exist within the new bicistronic vector pEPO-IRES2-EGFP located at 650 bp, 825 bp, 1356 bp and 1819 bp. Hence, four bands were expected: the smallest at 175 bp, two bands around 531 bp and 436 bp and the biggest band at about 4845 bp (Fig. 3.3.1c). The results showed that nearly all clones were correct.

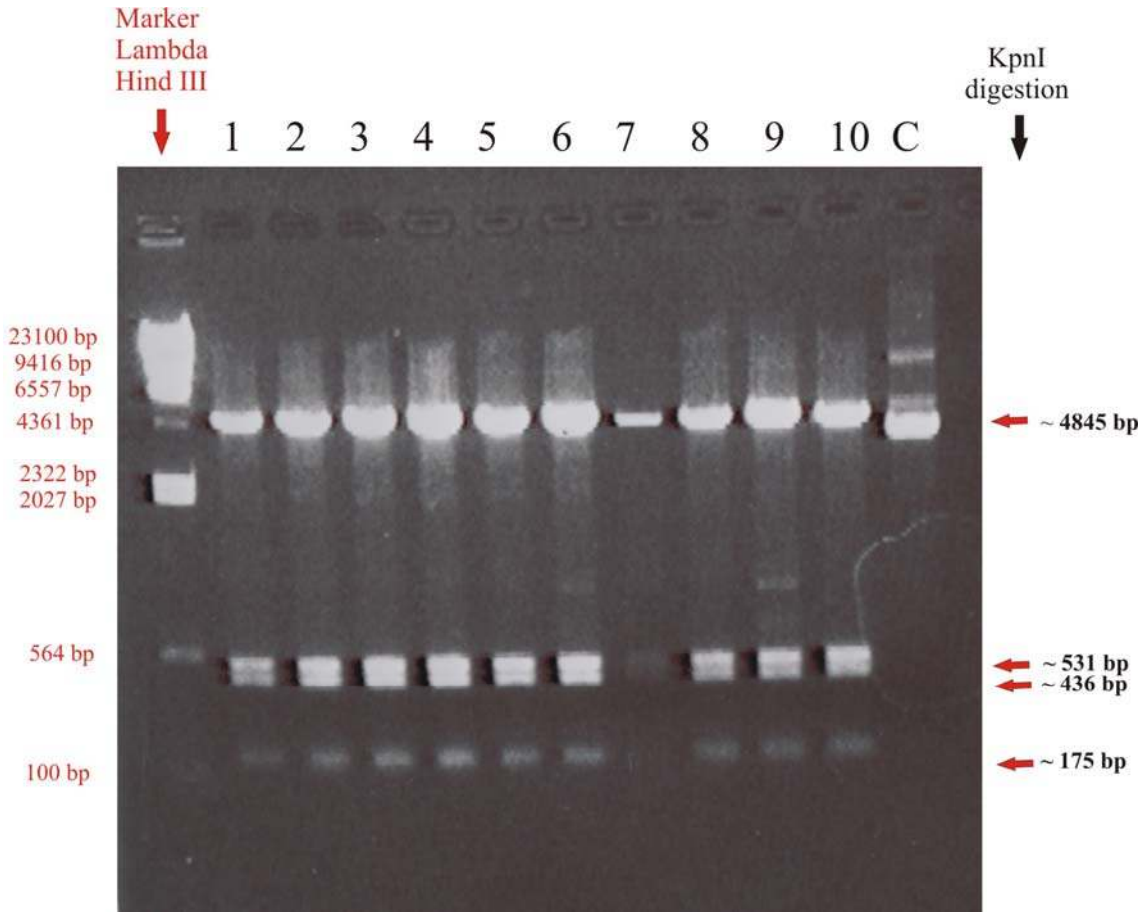


Fig. 3.3.1c: Electrophoresis of 1 % agarose gel showing different clones (1 to 10) from the pEPO-IRES2-EGFP vector digested with the *Kpn*I. The expression vector has four restriction sites for *Kpn*I generating four bands in the gel, aproximately at 4845 bp, 531 bp, 436 bp and 175 bp. C: control of the vector without digestion.

One clone was selected to perform the DNA maxi-preparation (2.17.2.7) to prepare sufficient DNA for further transfection processes. DNA concentration and purity were measured as indicated in 2.17.2.8. The 260/280 ratio was higher than 1.75, indicating a DNA purity above 95 %. See table 3.3.1a.

Table 3.3.1a: pEPO-IRES2-EGFP purity and concentration obtained from the DNA maxi-preparation.

Plasmid	Absorbance		Ratio: 260/280	DNA-concentration
	260 nm	280 nm		
pEPO-IRES2-EGFP	0.438	0.246	1.78	2.19 mg mL^{-1}

Finally, a control digestion with the *Kpn*I enzyme was performed using the DNA obtained from the DNA maxi-preparation. The results were the same as in figure 3.3.1c, confirming the correct ligation of the new bicistronic vector (data not shown).

This new bicistronic vector, pEPO-IRES2-EGFP, was used for the optimization of the transient transfection process.

3.3.2 Construction of pCMV-EGFP-EPO vector

For the construction of the pCMV-EGFP-EPO vector (section 2.17.2, Fig. 2.17.2.b), parts of the ampicillin resistant gene and the CMV promoter from the pTracerCMV2 vector (2.17.1) were removed by *Xmn*I and *Xho*I digestion. The pSBC-EGFP-rhuEPO vector (2.17.1) was cleaved with *Xmn*I and *Xho*I restriction enzymes and an ampicillin resistant gene fragment plus the SV40 promoter were removed. After ligation, the ampicillin resistant gene was regenerated in the new construct and the CMV promoter was placed upstream of the EGFP and rhuEPO genes having the polio 5´UTR IRES site located between both genes (Fig. 3.3.2a).

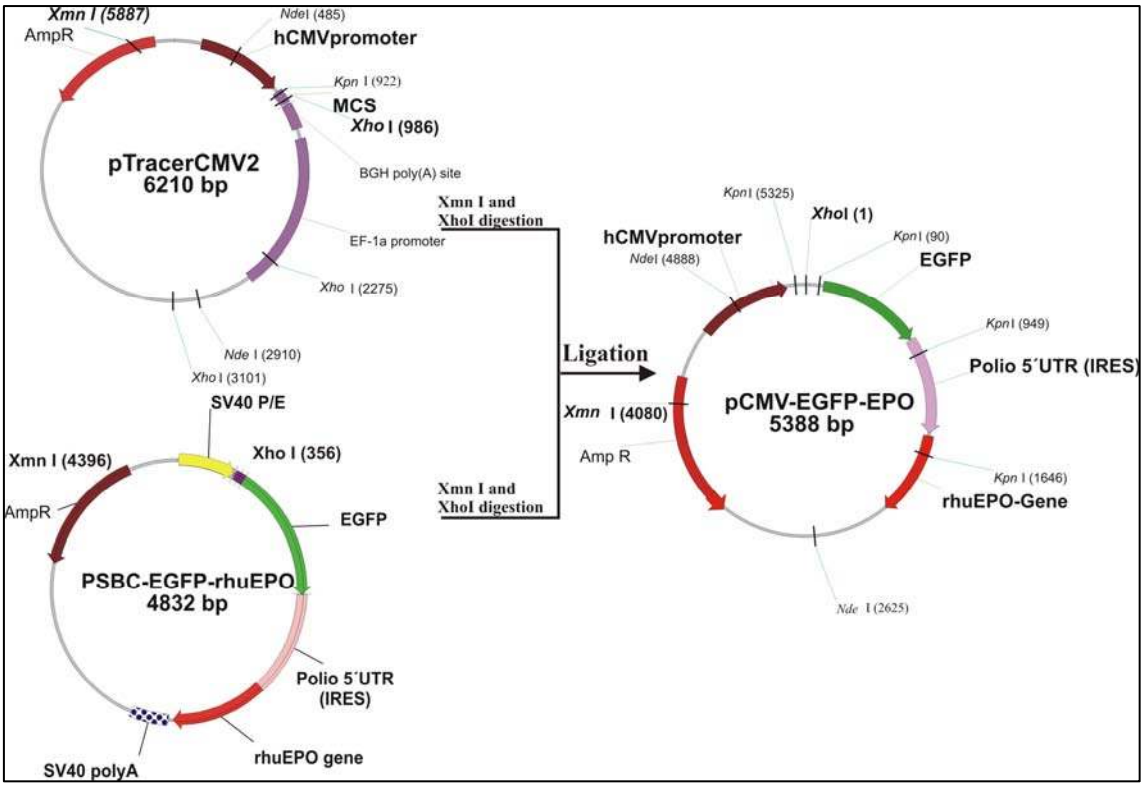


Fig. 3.3.2a: Construction of the bicistronic vector pCMV-EGFP-EPO starting from pTracerCMV2 and the pSBC-EGFP-rhuEPO vectors. After cleaving both plasmids by *Xmn*I and *Xho*I digestion, the CMV promoter plus a fragment of the ampicillin resistant gene were ligated into the fragment from the pSBC-EGFP-rhuEPO, from which a fragment of the ampicillin resistant gene plus the SV40 promoter were removed. The ampicillin resistant gene was regenerated and the EGFP and rhuEPO genes placed under control of the CMV promoter, resulting in new bicistronic vector, pCMV-EGFP-EPO.

pTracerCMV2 and pSBC-EGFP-rhuEPO vectors were digested using both enzymes simultaneously in a total volume of 40 µL according the protocol described in material and methods, section 2.17.2.3. They were incubated overnight at 37 °C.

Digested fragments were preparatively separated in an agarose gel (2.17.2.4) and cut out (2.17.2.5, Fig. 3.3.2b). The *Xmn*I enzyme has one restriction site in the ampicillin resistant gene of both plasmids and one additional site at 1609 bp in the pTracerCMV2 plasmid. The *Xho*I enzyme has three restriction sites in this plasmid, one in the MCS, at 986 bp, and the other two, at 2275 bp and at 3101 bp. The pSBC-EGFP-rhuEPO plasmid, has only one restriction site after the SV40 promoter and upstream of the EGFP-gene. Therefore, two bands were expected in the case of this vector, one of 4040 bp, the interested one, and the other of 792 bp. Five bands were expected for the pTracerCMV2 vector: 2786 bp, 1309 bp (interested fragment), 826 bp, 666 bp and 623 bp (Fig. 3.3.2b).

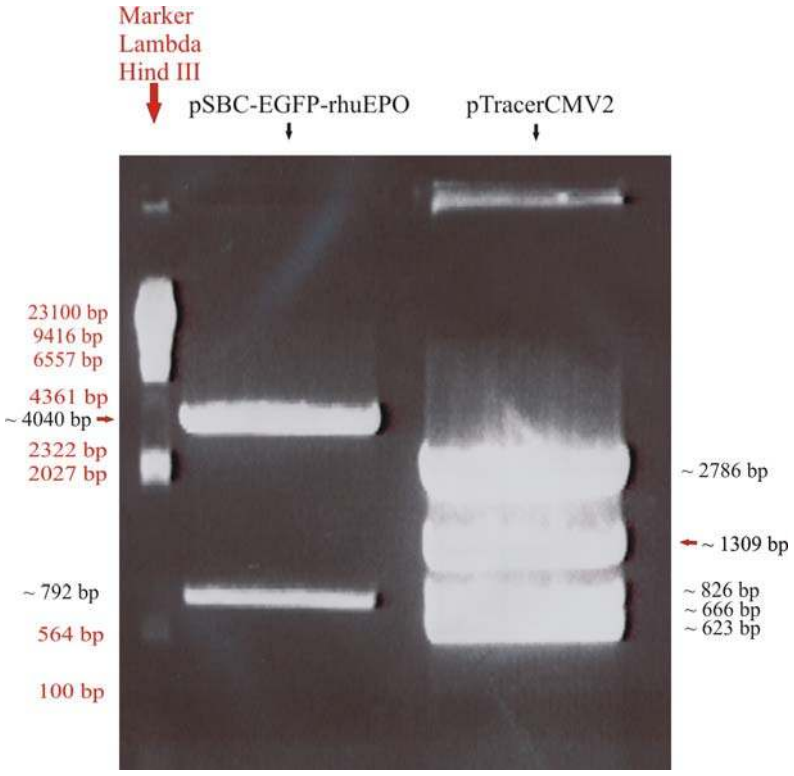


Fig. 3.3.2b: Preparative digestion with the *Xmn*I and *Xho*I restriction enzymes of the plasmids pSBC-EGFP-rhuEPO and pTracerCMV2. The digested plasmids were separated on 1.0 % agarose gel. Fragment of ~ 4040 bp (from pSBC-EGFP-rhuEPO), that contains part of the ampicillin resistant gene and the SV40 promoter, was removed. For pTracerCMV2, the desired fragment was the one of 1309 bp, which has parts of the ampicillin resistance gene and the CMV promoter.

As was explained for the construction of the pEPO-IRESs-EGFP vector, both fragments were purified using the QIAquick gel extraction kit protocol and the purified

yield was verified in a 1 % agarose gel (data not shown). Fragments were ligated following the protocol as indicated in 2.17.2.6.

The new plasmid was amplified in bacteria JM 109, using the heat-shock method for the transformation (2.17.2.1).

Ten clones were selected to continue with the liquid cultures in LB-Ampicillin medium and later, DNA mini-preparation was performed (2.17.2.2). The vector was verified by restriction analysis using the same restriction enzymes as for the preparative digestion (*Xmn*I and *Xho*I). Therefore two fragments were expected, one at approximately 4000 bp (from pSBC-EGFP-rhuEPO plasmid) and the other at about of 1300 bp (from pTracerCMV2 plasmid) (Fig. 3.3.2c).

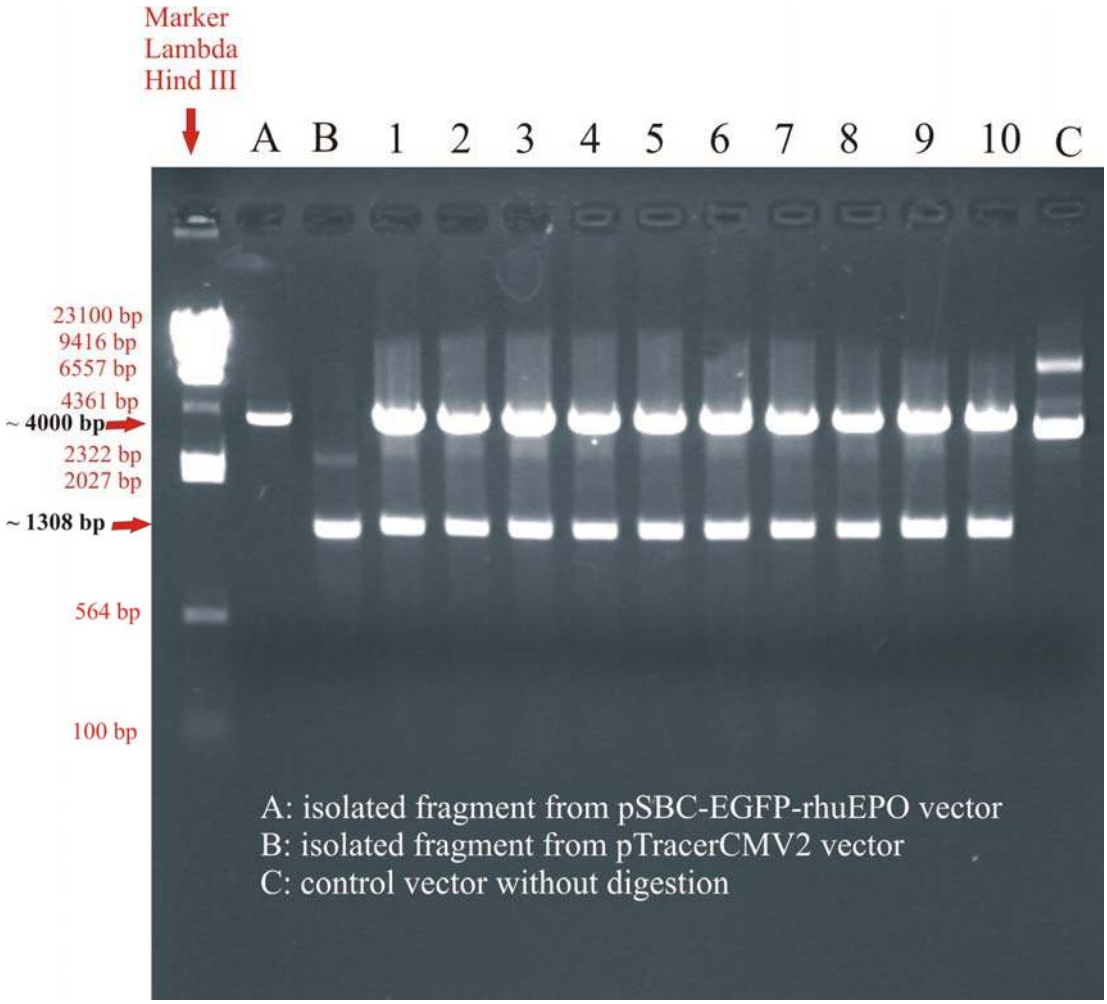


Fig. 3.3.2c: Electrophoresis of 1 % agarose gel showing different clones (1 to 10) from the pCMV-EGFP-EPO vector digested with *Xmn*I and *Xho*I restriction enzymes. This figure shows, that all clones were correct. **A**: isolated fragment from pSBC-EGFP-rhuEPO (~ 4000 bp); **B**: isolated fragment from TracerCMV2 (~ 1308 bp); **C**: control of the vector without digestion.

These results showed that all clones were correctly transformed. Clone number 9 was selected for the DNA maxi-preparation (2.17.2.7) to produce enough DNA for further transfection processes. DNA concentration and purity were measured as indicated under 2.17.2.8. The 260/280 ratio was higher than 1.70, indicating a DNA purity above 95 %. See table 3.3.2a.

Table 3.3.2a: pCMV-EGFP-EPO purity and concentration obtained from the DNA maxi-preparation.

Plasmid	Absorbance		Ratio: 260/280	DNA-concentration
	260 nm	280 nm		
pCMV-EGFP-EPO	0.417	0.242	1.73	2.09 mg mL^{-1}

Finally, a control digestion with the same restriction enzymes (*Xmn*I and *Xho*I) was performed using the DNA obtained from the maxi-preparation. The result was the same as shown in figure 3.3.2c, thus verifying the integrity of the new bicistronic vector (data not shown).

In this section two different bicistronic vectors were constructed to be used in different further transient transfection processes. Both vectors harbored the EGFP and the EPO genes under control of the CMV promoter (CMVp: 589 bp and 654 bp from pEPO-IRES2-EGFP and pCMV-EGFP-EPO vectors, respectively).
The pEPO-IRES2-EGFP plasmid contained the rhuEPO gene upstream of the IRES site of the encephalomyocarditis virus (ECMV) and the EGFP gene downstream of the IRES. In contrast, the pCMV-EGFP-EPO vector was characterized by the inverse orientation containing the EGFP first followed by the IRES site from the Polio 5´UTR, and the rhuEPO as second gene. pEPO-IRES2-EGFP was used as standard vector to optimize the transfection procedure.

3.4 Small-scale transient transfection mediated by PEI using bicistronic plasmid

3.4.1 Cell wash before the addition of the transfection complex

The following experiments were performed with cells cultured in small-scale using a 12-well plate. Cells were washed prior to transfection under PEI-mediated conditions. This transfection procedure followed Protocol A as indicated under section 2.18.1.1, using the bicistronic vector pEPO-IRES2-EGFP.

HEK293s cells cultured in serum-free 293 SFM II + AA medium and in serum-containing DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium were transfected using a ratio of 1.0:1.5 of DNA:PEI (µg:µg). The complex was formed in serum-free 293 SFM II + AA or DMEM -Ca^{2+} + Vit. medium according to the better results obtained in the preliminary experiments described in section 3.2. Cells were analyzed by flow cytometry 48 and 72 hpt. The results showed 72 % and 67 % GFP-positive cells after 48 hpt and 72 hpt, respectively for transfection in serum-containing medium. No significant transfection for the procedure in serum-free conditions could be observed (data not shown). The experiment was performed three times obtaining always the same results (data not shown).

In order to evaluate whether these results were due to an inhibition in the transfection procedure from the 293 SFM II + AA medium applied, HEK293s cells cultured in this medium were washed with DMEM -Ca^{2+} + Vit. + Pl. + 5 % medium 3 h before transfection, and sub-cultured in this same medium. The complex was prepared in DMEM -Ca^{2+} + Vit. and added to the cells. Six hours later, the cells were centrifuged (2000 rpm, 3 min) and washed one time with the 293 SFM II + AA medium. Cells were seeded again in 12-well plates, using the same serum-free medium as washed, for further incubation until flow cytometry analysis, 48 hpt. In parallel, HEK293s cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS and in 293 SFM II + AA media were transfected. The results showed, that similar transfection efficiency as in serum-containing medium was reached (75 % and 70 %) with cells cultured under serum-free conditions when one medium exchange for the transfection was done (293 SFM II + AA medium to DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium). In contrast, transfection under serum-free conditions without medium exchange showed no significant transfectability (less than 1.5 %) (Fig. 3.4.1a).

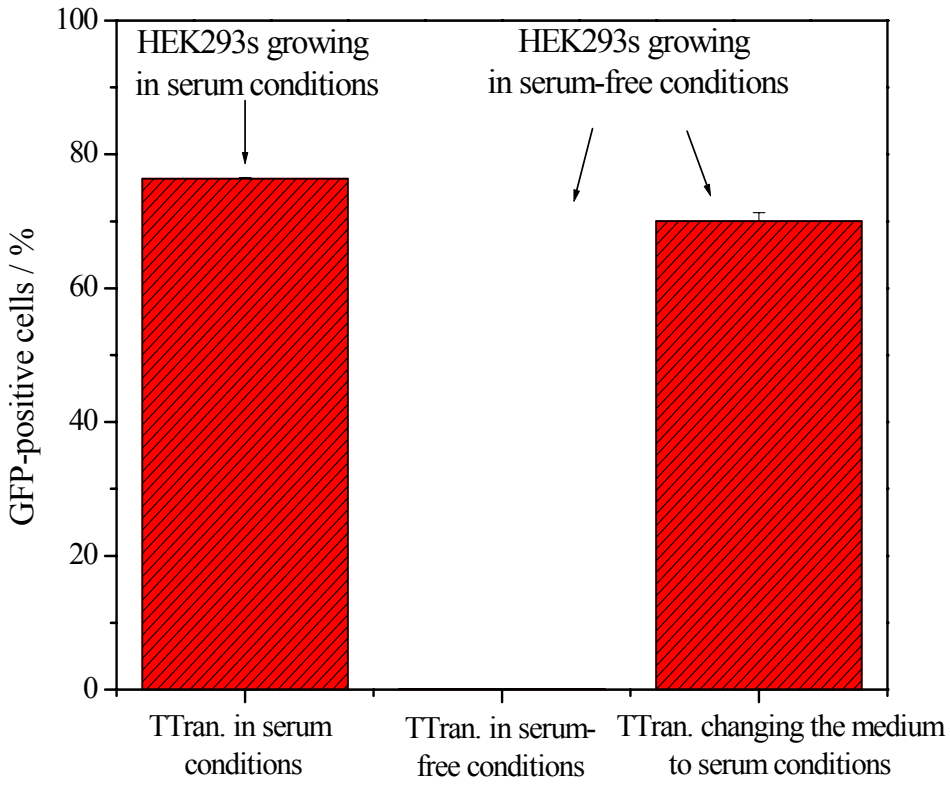


Fig. 3.4.1a: HEK293s cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS and in 293 SFM II + AA medium were transfected with 1.0 µg of DNA and 1.5 µg of PEI. The complex was prepared in serum-free medium (DMEM -Ca^{2+} + Vit.). The figure shows that cells growing under serum-free conditions with one medium exchange from 293 SFM II + AA medium to DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS for transfection reached a similar transfection efficiency as cells cultured under serum-containing conditions. In contrast, transfection under serum-free conditions alone showed no significant transfection. TTran.: Transient Transfection. Errors bars represent + SD of one experiment done in duplicate.

3.4.2 Transfection without cell wash before the addition of transfection complex

The first aim of this work was to define a simple and scalable transfection process. In order to reach this objective, two steps had to be simplified: the 3 h incubation period of the DNA:PEI complex with cells in reduced culture volume, and the medium change 3 h prior to transfection. We assumed, that a reduced culture medium could promote the interaction of the DNA:PEI complex with the cells and thus increasing transfection efficiency (Durocher et al. 2002). A medium change was reported to be necessary to avoid deleterious effects of conditioned medium on the transfection efficiency (Schlaeger and Christensen 1999). This medium exchange step is a comparably simple procedure in small-scale but represents a significant hurdle at scales in the range of liters. Therefore, transient transfection without the preceding cell wash step was

performed under PEI-mediating conditions, following the protocol B described under the section 2.18.1.1.

HEK293s cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium were used to perform this experiment. The complex formation was prepared as indicated in the previous section 3.4.1 (1.0 µg of pEPO-IRES2-EGFP and 1.5 µg of PEI) and added to the cells. Results from flow cytometry analysis showed transfection efficiency in the same range (70 %) 48 hpt as it was found for the transfection procedure with the wash step (data not shown).

Transfection was done with both plasmids (pEPO-IRES2-EGFP and pCMV-EGFP-EPO) obtained the results showed in figure 3.4.2a. Transfection efficiencies higher than 60 %, with the first plasmid, and 90 % with the second, were reached following the transfection procedure without cell wash before the addition of the transfection complex.

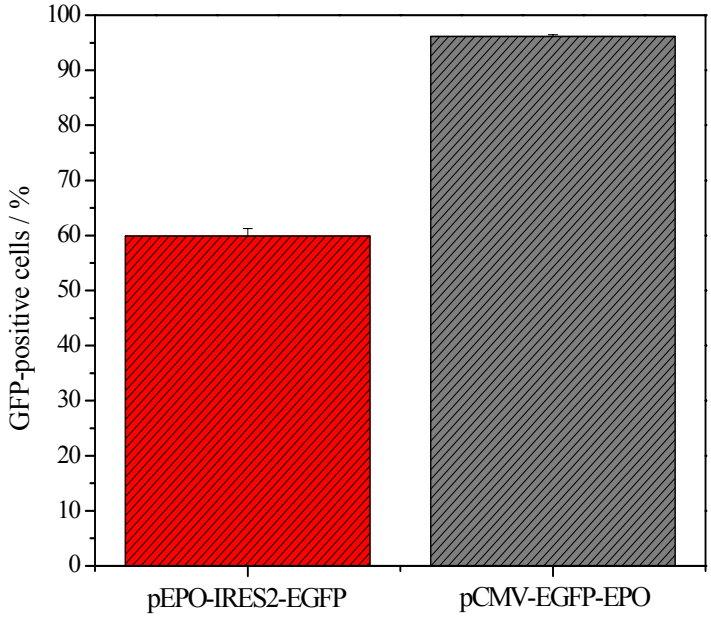


Fig. 3.4.2a: HEK293s growing in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS were transfected with DNA:PEI (1.0µg:1.5µg) without a preceding cell wash step before the addition of transfection complex. pEPO-IRES2-EGFP (in red) and pCMV-EGFP-EPO (in gray) were used. GFP-positive cells were quantified by flow cytometry, 48 hpt. Errors bars represent + SD of one experiment done in duplicate.

These results clearly showed that the transfection without a preceding wash step was possible in HEK293s cultures under serum-containing conditions.

3.5 Optimization of serum-free medium for transfection

The next experiments were directed to the development of a serum-free medium, in which a successful transfection without cell wash step of adapted HEK293s will be possible.

FreeStyle™ 293 expression medium (see 2.4.1) was especially developed for supporting growth and transfection (without cell wash step) of the special FreeStyle 293-F cell line, which is a subclone of the 293 cell line (FreeStyle™293 Expression Manual, www.invitrogen.com). Therefore, this serum-free medium was chosen to perform the next experiments.

HEK293s cells already adapted to the SFM 293 II + AA serum-free medium were thawed (revitalization protocol under 2.8.1) and cultured in the same medium. Three sub-cultures were performed under these media conditions. Subsequently, cells were washed one time with FreeStyle™ 293 expression medium and were directly cultured in this new formulation at a cell concentration of about 5.0×10^5 mL^{-1}. The cells were sub-cultured four times always when they reached a cell concentration of about 1.5×10^6 mL^{-1}. The same standard parameters as indicated in section 3.1.1 were determined. The viability was always above 90 % (data not shown). In the last sub-culture, cells entered a lag phase-like situation after 15 days, growth ceased and viability dropped down (data not shown).

Due to the fact, that the cells were not completely adapted to the new FreeStyle™ 293 expression medium, one direct experiment was performed in order to check whether it is already possible to transfect the cells without wash step. In parallel, FreeStyle™ 293 expression medium was compared to the standard serum-containing medium DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS. The same protocol as already explained under 3.4.2 was applied using the pEPO-IRES2-EGFP vector for complex formation. This complex was built in DMEM -Ca^{2+} + Vit., and in Opti-MEM® I medium according to manufacturer's protocol. This medium contained less than 1 % of FCS. The day before transfection, cells from sub-culture 2 after adaptation and growing in FreeStyle™ 293 expression medium, were taken and a new inoculate of a concentration between 3.5×10^5 mL^{-1} and 5.0×10^5 mL^{-1} was performed. Approximately 24 h later, a cell concentration of about 7.0×10^5 mL^{-1} and a viability of 96 % was adjusted. Transfection in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS was used as control. Results taken from the flow cytometry analysis (48 hpt) revealed nearly 25 % GFP-positive cells in FreeStyle™ 293 expression medium/Opti-MEM® I medium, and nearly 10 % when the complex was prepared in DMEM -Ca^{2+} +Vit. (Fig. 3.5a).

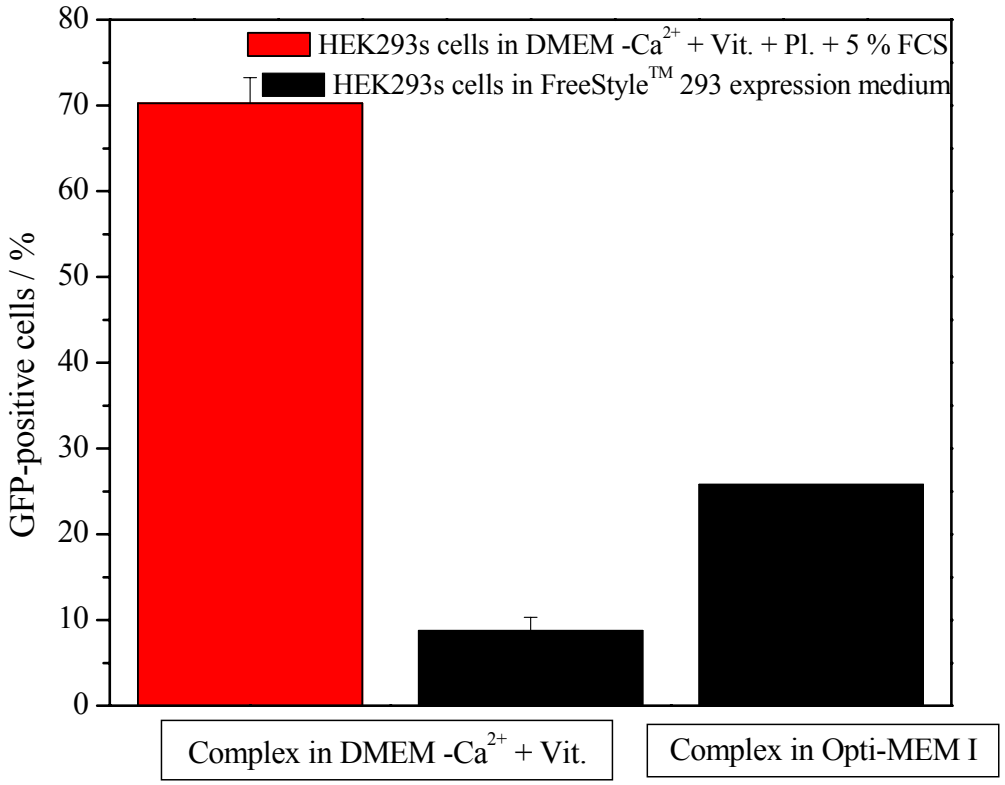


Fig. 3.5a: Transfection of HEK293s cells (without wash step) cultured in DMEM -Ca^{2+}+ Vit. + Pl. + 5 % FCS (in red) and in FreeStyle™ 293 expression medium (in black). Cells were transfected with 1 µg of DNA (pEPO-IRES2-EGFP) and 1.5 µg of PEI. GFP was measured by flow cytometry 48 hpt. The complex was prepared in DMEM -Ca^{2+} + Vit. for both cultures and either in Opti-MEM I medium, for cells cultured under serum-free condition. Errors bars represent + SD of one experiment done in duplicate.

The Hybridoma-SFM medium (H-SFM, section 2.4.1) was initially developed for growth of hybridoma cells. A custom-made low-calcium H-SFM medium supplemented with 1 % of FCS was used in several experiments previously reported (Coté et al. 1998; Durocher et al. 2002) to support cell growth of HEK293s cells and to perform transient transfection with these cells. Therefore, H-SFM + Pl. medium (2.4.1) was decided to use also in the next experiments.

The same adaptation protocol as taken for the FreeStyle™ 293 expression medium was applied. HEK293s cells grew to a cell concentration of 1.5×10^6 mL^{-1} within two days. Very large clusters (more than 50 cells) occurred which could not be disintegrated following the protocol described in section 2.5.3. Due to the fact, that a homogeneous cell suspension is considered to be an important prerequisite for successful transfection, this culture medium was excluded from further transfection test.

In order to find a suitable serum-free medium for growth and transfection of HEK293s cells as single cell suspension without applying a wash step before

transfection, different serum-free adaptation protocols to several media manufactures were tested in parallel as is explained below.

3.5.1 Sequential adaptation with successive cell dilution

The following experiments were characterized by a discontinuous reduction of the FCS content when HEK293s cells reached a cell concentrations of 1.5×10^6 mL^{-1}. The adaptation process to the different serum-free media H-SFM + Pl.and SMIF 7 followed the protocol described in material and methods under 2.6.2.

HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS was used as initial culture. Samples were taken daily and the standard parameters (2.10 and 2.11) were determined routinely (data not shown).

During adaptation to H-SFM + Pl., large clusters, of more than 50 cells, occurred which could not be disintegrated (see protocol under 2.5.3). Therefore, this medium was discarded from further test.

Very big aggregates also appeared during adaptation to SMIF 7 medium. When SMIF 7 with a reduced amount of Ca^{2+} (10 µmol L^{-1} $CaCl_2$) was used, smaller clumps appeared, but the HEK293s cells stop growing after the third sub-culture. They entered a lag phase of seven days resulting in the decision to stop further evaluation of this medium formulation (data not shown).

3.5.2 Direct adaptation

Some medium manufactures recommend a direct adaptation. Here, the cells cultured in serum-containing medium were washed with the respective new serum-free medium and directly sub-cultured under the new conditions. This direct adaptation process was used for H-SFM + Pl. and Ex-Cell 293™ media (2.4.1) following the protocol described under 2.6.3.

The direct adaptation to H-SFM + Pl. medium was performed without successful results. Large cell aggregates were occurred as also observed for the other two adaptation processes. H-SFM + Pl. was supplemented with EDTA to prevent formation of clumps, and additionally lipids were added according to Cote et al., 1998 (2.4.1). A new direct adaptation protocol with this supplemented medium (H-SFM + Pl. + Lip. + EDTA) was performed obtaining the same results as before (data not shown) such that the medium was excluded for further experiments, too.

The direct adaptation to the serum-free Ex-Cell 293™ medium gave better results. HEK293s cells grew as small clumps between 5 and 10 cells after the third sub-culture. Additional three sub-cultures were run and a batch cultivation was performed during eight days in a small spinner flask. The standard parameters (2.10 and 2.11) were determined. The viable cell concentration reached 8.0×10^6 mL^{-1} with viability higher

than 95 % and no glucose limitation was observed during the entire batch culture (data not shown). The population doubling time of HEK293s cells growing in ExCell 293™ medium was 31.5 h. Therefore, HEK293s cells growing in ExCell 293™ medium were considered fully adapted to this new serum-free medium.

A direct adaptation to a serum-free medium was successfully performed from Ex-Cell 293™ to the Ex-Cell 293™ modified medium (2.4.1). Cell clusters between 20 to 50 cells were formed at high cell concentration. To evaluate the medium as standard in future cultivations, a batch culture was performed for eight days. Standard parameters (2.10 and 2.11) were determined. The viable cell concentration reached nearly 8.0×10^6 mL^{-1} with a viability of more than 90 %. Subsequently, the cells rapidly entered the death phase (data not shown). No glucose limitation was observed as in batch culture using ExCell 293™ medium (data not shown). The population doubling time of these cells was 30.8 h.

3.5.3 Preliminary transient transfection experiments using different serum-free media

Transient transfection in small-well plates was performed using the different serum-free media in which HEK293s cells could be fully adapted (293 SFM II + AA, ExCell 293™ and Ex-Cell 293™ modified media).

1 % of FCS was added to the serum-free medium in parallel experiments as recommended by Durocher et al. (2002) to improve transient transfection (FreeStyle™ 293 Expression Manual, www.invitrogen.com, Invitrogen Technical Services).

The following mediums were used as specified below:

1- Ex-Cell 293™ medium
2- Ex-Cell 293™ medium + 1 % FCS
3- Ex-Cell 293™ modified medium
4- Ex-Cell 293™ modified medium + 1 % FCS
5- 293 SFM II + AA + 1 % FCS

For the HEK293s cells cultured in media 1 and 3, cells were counted and seeded in the plate at a concentration of 5.0×10^5 mL^{-1} per well (900 µL × well), three hours before transfection. Plates were further incubated.

For media 2, 4 and 5, cells growing in the respective medium without serum were washed one time with the corresponding medium with 1 % FCS and seeded in the plate at the same cell concentration mentioned above. They were further incubated for three hours until the transfection procedure was performed.

Transfection in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS was performed in parallel as control. HEK293s cells cultured in each different medium (1 to 5), without transfection, were used as negative control.

The DNA:PEI (1.0:1.5, µg:µg) complex was formed in DMEM -Ca^{2+} + Vit. for all transfections, using the pEPO-IRES2-EGFP vector.

Flow cytometry measurements were performed 48 hpt.

The following table (Table 3.5.3a) shows the results for each medium. Every measurement was calibrated with the corresponding control. These results showed no significant transfection for every medium tested. Every data represent the average from two different experiments.

Table 3.5.3a: Small-scale transient transfection of HEK293s cells in different serum-free media. Three hours before transfection, cells were washed with the respective medium and seeded in the plate for transfection. The DNA (pEPO-IRES2-EGFP):PEI (1.0:1.5, µg:µg) complex was prepared in DMEM -Ca^{2+} + Vit. for all cases, incubated at RT for 10 min and added to the cells. Flow cytometry measurements were performed 48 hpt. Transfection in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS was performed as positive control. Every data represents the average from two experiments.

Medium:	**Positive control (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS)**	**1 Ex-Cell 293™ medium**	**2 Ex-Cell 293™ medium + 1 % FCS**	**3 Ex-Cell 293™ modified medium**	**4 Ex-Cell 293™ modified medium + 1 % FCS**	**5 293 SFM II + AA + 1 % FCS**
GFP-positive cells (%)	74.59	0.24	0.10	3.02	1.50	0.10

Further experiments were performed, but preparing the transfection complex in each serum-free medium used in the previous experiment instead of DMEM -Ca^{2+} + Vit. medium. The results taken from flow cytometry analysis (48 hpt) showed no significant transfection for all media used. Untransfected (controls) and transfected cell cultures showed less than 0.5 % of GFP-positive cells (data not shown).

In order to investigate, whether the presence of serum improves the transfectability, experiments were done using Ex-Cell 293™ modified medium and the 293 SFM II + AA medium supplemented with different amounts of FCS (2.5 % and 5 %), but without any successful (increase transfectability) results.

At this point, only 10 % GFP-positive cells were reached after transfection in FreeStyle™ 293 expression medium and complex formation in serum-free DMEM -Ca^{2+} + Vit. medium (Fig. 3.5a) and only 3.02 % GFP-positive cells were found using the Ex-Cell 293™ modified medium (see table 3.5.3a). It was not clear whether the low transfectability was due to an inhibition by components of the serum-free medium and/or by the absence of unknown components important for transfection, which are

present in FCS. This assumption was concluded from results obtained when cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS were washed before transfection and transferred in the same medium without serum, resulting in no significant transfection (data not shown). In order to identify a possible inhibition from medium components, these two media were diluted with DMEM -Ca^{2+} + Vit., expecting to have a higher percentage of GFP-positive cells. Media were prepared in the following way:

I) [Ex-Cell 293™ modified:(DMEM -Ca^{2+} + Vit.)], [1:1]

II) [293 FreeStyle™ expression medium:(DMEM -Ca^{2+} + Vit.)], [1:1]

Cells already adapted to Ex-Cell 293™ modified serum-free medium were washed one time with each medium (I and II) in the mid-exponential growth phase and sub-cultured in these media at an initial cell concentration of 5.0×10^5 mL^{-1}. These two new cultures were continuously sub-cultured every second or third day to maintain the cell concentration between 1.5×10^6 mL^{-1} and 2.0×10^6 mL^{-1}. After the third passage, cells were prepared for small-scale transient transfection without cell wash. The complex (1.0:1.5, µg:µg; DNA (pEPO-IRES2-EGFP):PEI) was prepared in DMEM -Ca^{2+} + Vit. medium. GFP-positive cells were visualized by flow cytometry analysis 48 hpt. Transfection in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS was used as control. Figure 3.5.3a shows that the mixed medium II, [293 FreeStyle™ expression medium:(DMEM -Ca^{2+} + Vit.)], [1:1] revealed a transfection efficiency as obtained for the control.

Transfection under serum-free medium II conditions was performed three times in duplicate and the same result was obtained (data not shown).

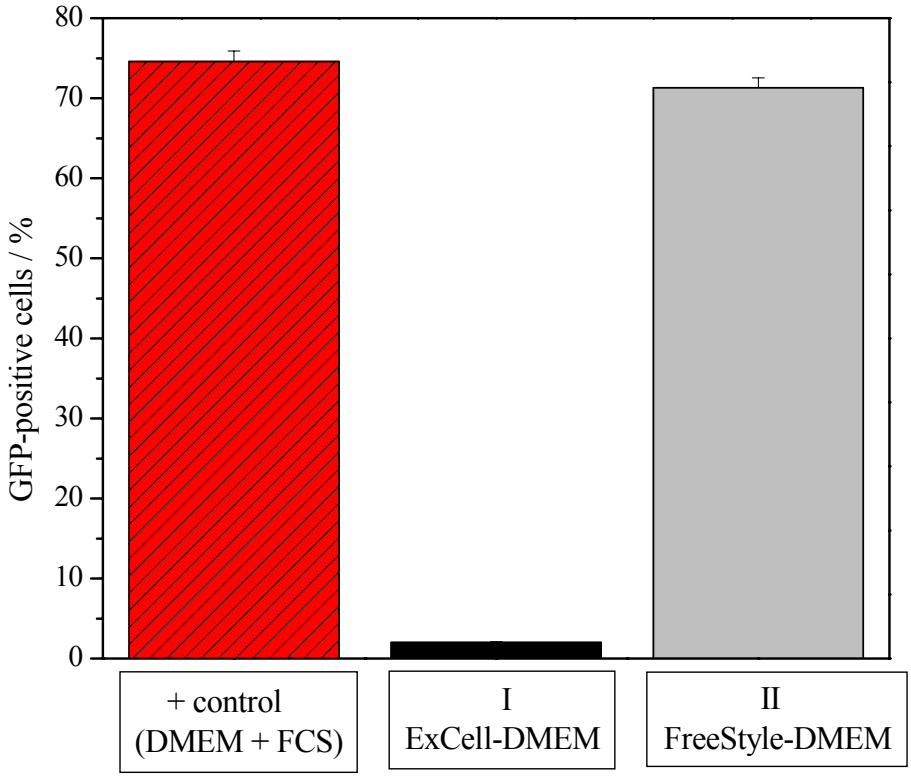


Fig. 3.5.3a: HEK293s cells growing in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS (in red), in [Ex-Cell 293™ modified:(DMEM -Ca^{2+} + Vit.)], [1:1] (in black) and in [293 FreeStyle™ expression medium:(DMEM -Ca^{2+} + Vit.)], [1:1] (in gray) were transfected with (DNA:PEI), (1.0:1.5, µg:µg) prepared in DMEM -Ca^{2+} + Vit.. GFP was measured by flow cytometry analysis 48 hpt. Errors bars represent + SD of one experiment done in duplicate.

With this result the first aim of the work was achieved, namely to find a serum-free and/or protein-free medium formulation to guarantee chemically defined culture conditions for further transient transfection processes without washing the cells prior to the addition of the complex and without changing the medium after the transfection procedure – a prerequisite for large-scale transfection.

3.6 Process optimization for transfection in serum-free medium

The next experimental step was driven to optimize and characterize the HEK293s cell in the new serum-free [FreeStyle™ 293 expression medium:(DMEM -Ca^{2+} + Vit.)], [1:1] medium (2.4.1).

3.6.1 Establishment of cells completely adapted to the new serum-free medium

Two different batch cultivations in small spinner flask (50 mL working volume) were performed for eight days in the new serum-free medium composition, [FreeStyle™ 293

expression medium:(DMEM -Ca^{2+} + Vit.)], [1:1], (FS:DMEM) in order to characterize the HEK293s cells under these new conditions.

Cell concentration, viability, glucose and lactate concentration, as well as the LDH activity were measured (Fig. 3.6.1a). The cell concentration reached was about 3.0×10^6 mL^{-1}. Viability was higher than 95 % during the cultivation period (Fig. 3.6.1a; A). Population doubling time was between 30 and 37 h. When glucose fell short of a concentration of 1.50 g L^{-1}, cells started to consume lactate (Fig. 3.6.1a; B). At the end of the cultivation, LDH was comparably high at a value of about 10 µkat L^{-1} (Fig. 3.6.1a; C).

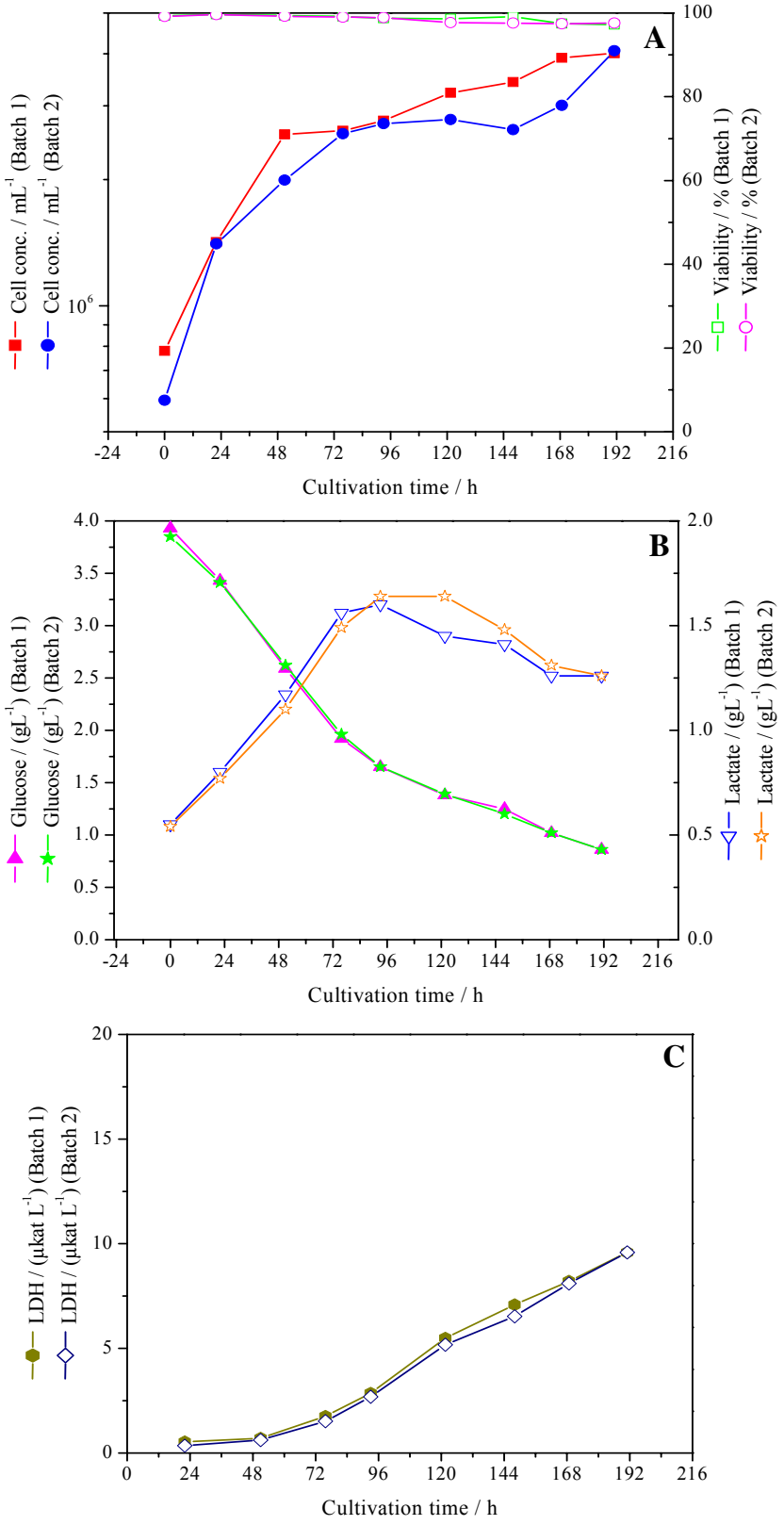


Fig. 3.6.1a: Two batch cultivations of HEK293s cells growing in FS:DMEM medium. Both cell cultures were performed in small spinner flasks (50 mL working volume) during eight days. Cell concentration and viability (**A**), glucose and lactate concentration (**B**), and LDH activity (**C**) were determined.

Amino acids were analyzed (2.11.3) to identify a possible nutrient limitation. The pattern of amino acids was similar for both batch cultures. Figure 3.6.1b shows the amino acid concentrations in the supernatant of HEK293s during batch culture in FS:DMEM medium. The arrows (↓) indicate a depletion in the amino acids aspartate (Asp) and glutamate (Glu) at the end of the batch culture (216 h). Therefore, this medium was supplemented with 600 µmol L^{-1} of Asp and 100 µmol L^{-1} of Glu in future and was called FS:DMEM + AA.

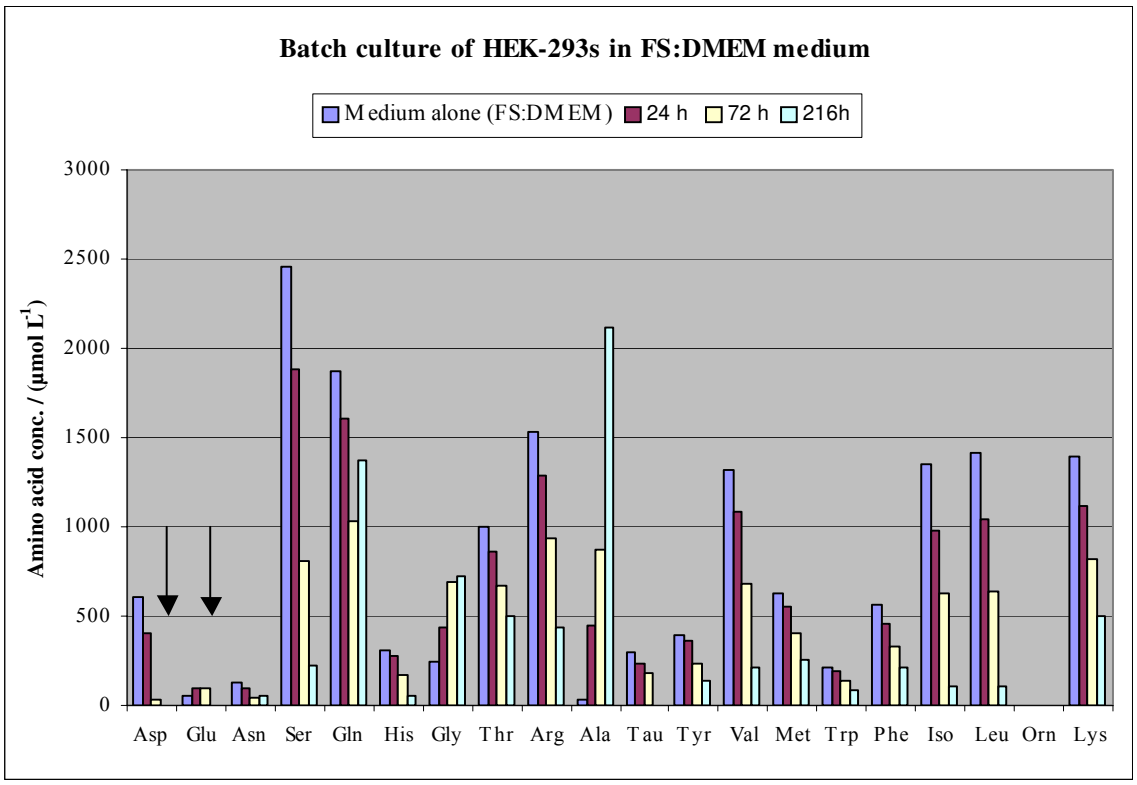


Fig. 3.6.1b: Amino acid concentrations in the culture supernatant of HEK293s cells growing in FS:DMEM medium. The ↓ indicate Asp and Glu limitation at the end of the culture batch (216 h).

HEK293s cells were cultured in this FS:DMEM + AA medium and, after a minimum of four sub-cultures, a batch cultivation was performed for ten days.

Standard parameters (Fig. 3.6.1c) were measured. The viable cell concentration was higher in medium with AA at the end of the exponential growth phase compared to the non-supplemented formulation (6.5×10^6 mL^{-1} compared to 3.0×10^6 mL^{-1}). Cell viability was higher than 95 % during the complete batch process (Fig. 3.6.1c; A). Lactate was consumed after 144 h of cultivation although the glucose concentration was still at 1.50 g L^{-1} at this cultivation time (Fig. 3.6.1c; B). At this point, LDH presented values of more than 6 µkat L^{-1} (Fig. 3.6.1c; C). At the beginning of the stationary growth phase (at 144 h) aggregates of about 20 to 30 cells occurred. The population double time of the HEK293s in this new serum-free medium was between 30 and 36 h.

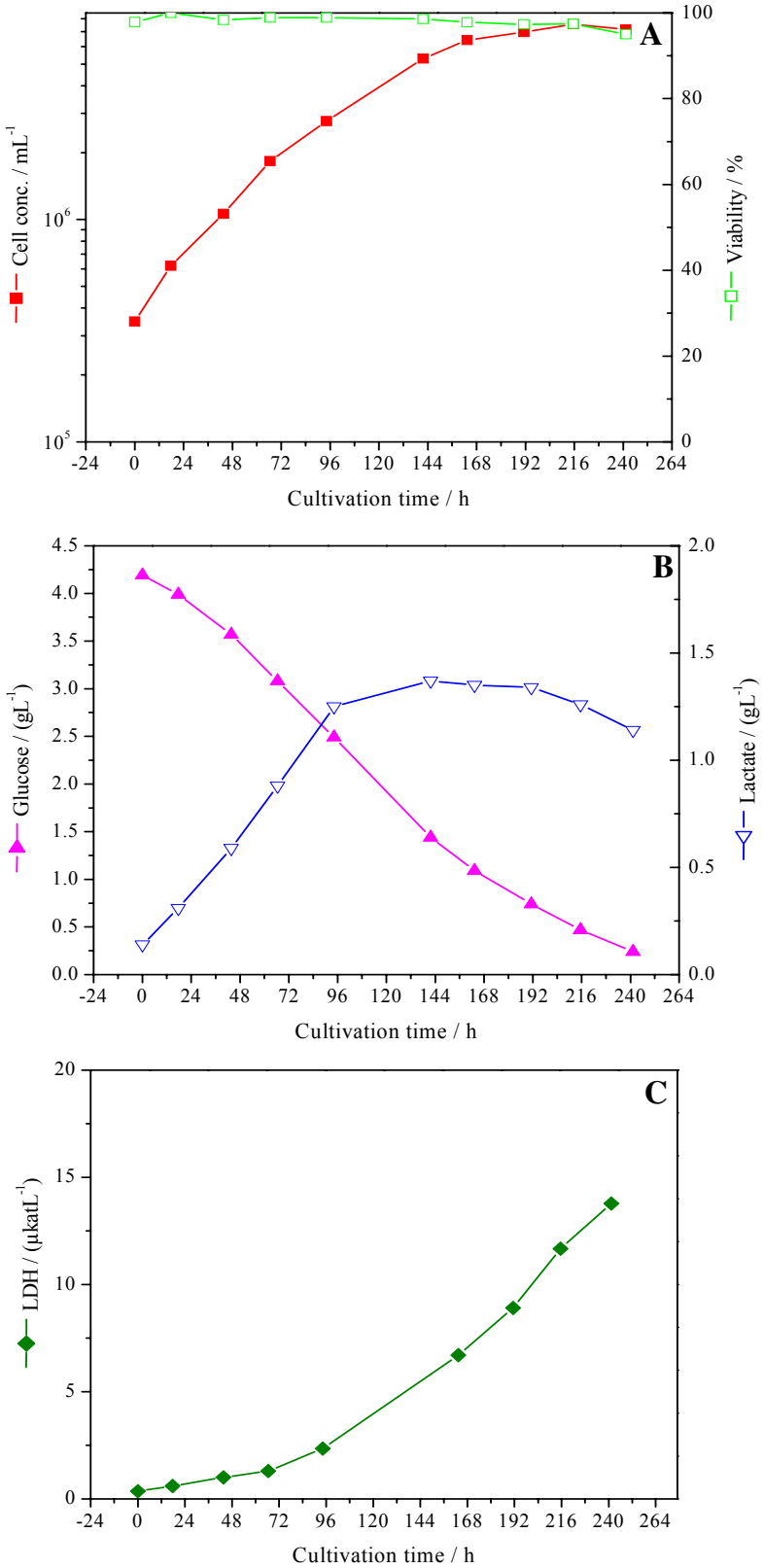


Fig. 3.6.1c: Batch culture of HEK293s cells in FS:DMEM + AA medium. Culture was performed in small spinner flasks (50 mL working volume) during ten days. Cell concentration and viability (**A**), glucose and lactate concentration (**B**), and LDH activity (**C**) were determined.

Amino acids were analyzed in this new batch culture and no limitation could be detected (data not shown).

HEK293s cells were considered completely adapted to the new serum-free and protein-free FS:DMEM + AA medium and a working cell bank was generated. These cells had the passage number 60.
Finally, this medium was chosen to perform all the following experiments under serum-free conditions.

3.6.2 DNA:PEI ratio optimization

Different DNA:PEI ratios were evaluated in order to decrease the amount of the DNA used obtaining highest transfectability at minimum DNA dosage. HEK293s cultured in FS:DMEN + AA medium were transfected using different ratios of DNA (pEPO-IRES2-EGFP):PEI (µg:µg). One small-scale experiment (12-well plate) was performed in duplicate. Flow cytometry analyses were performed 48 hpt. Figure 3.6.2a shows that the highest transfection efficiencies were obtained using a DNA:PEI (µg:µg) ratio of 0.50:1.50.

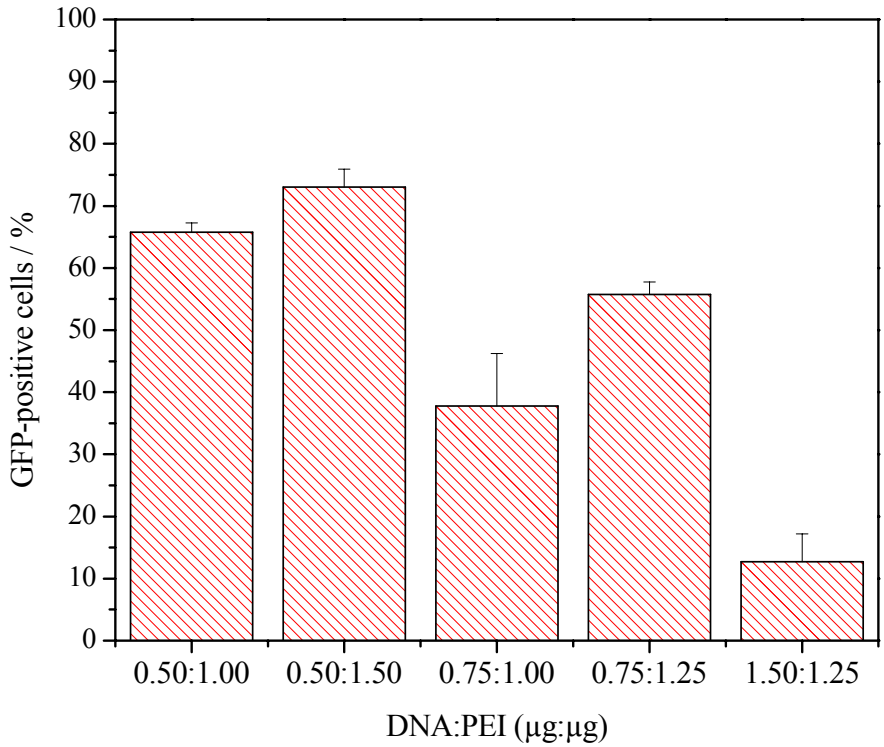


Fig. 3.6.2a: HEK293s cells cultured in FS:DMEM + AA medium were transfected using different ratios of DNA (pEPO-IRES2-EGFP vector) and PEI. One small-scale experiment (12-well plate) was performed in duplicate. GFP was measured by flow cytometry analysis 48 hpt. Errors bars represent + SD of one experiment done in duplicate.

This experiment, using the same DNA:PEI ratios, was repeated and gave the same results as presented above (data not shown). In order to further decrease the amount of the DNA used, another transfection experiment was performed for DNA:PEI (µg:µg) ratios: 0.25:1.00 and 0.25:1.50. The transfectability was below 30 % (data not shown).

The rhuEPO concentration was quantified by the specific ELISA (2.19.2) in the cell culture supernatant stored at –20 °C, 48 hpt. Figure 3.6.2b shows the results in which the highest expression level of rhuEPO was obtained using the DNA:PEI (µg:µg) ratio of 0.50:1.50, which correlated well with the highest transfectability determined by the GFP-positive cells.

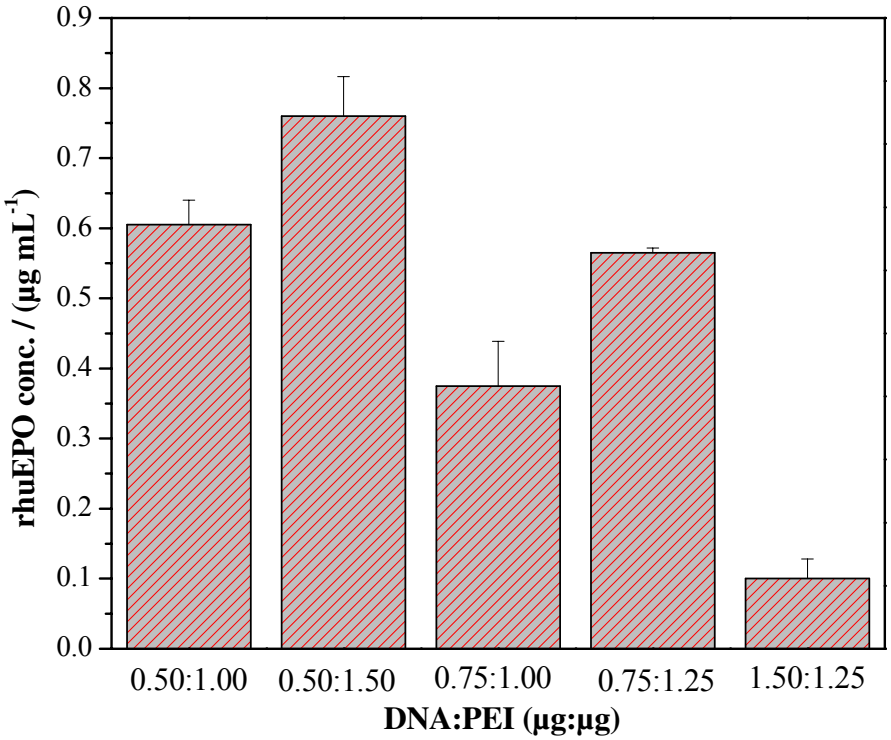


Fig. 3.6.2b: HEK293s cultured in FS:DMEM + AA medium were transfected with different amounts of DNA (pEPO-IRES2-EGFP vector) and PEI. rhuEPO from the cell culture supernatant at 48 hpt was quantified by ELISA. Errors bars represent + SD of one small-scale experiment done in duplicate.

Summarizing, these results showed that a DNA:PEI (µg:µg) ratio of 0.5:1.5 guaranteed on the one hand highest transfection efficiency and on the other hand the highest expression level for the model protein. Therefore, this ratio was chosen to perform future transient transfection processes.

3.7 *Scale-up and characterization of transient transfection*

The next step was to scale-up the transient transfection procedure to spinner flasks with 200 mL working volume. HEK293s cells growing in FS:DMEM + AA medium were initially cultured at a cell concentration of about 3.0×10^5 mL^{-1} to 5.0×10^5 mL^{-1}. Transfection procedure was performed, when the cell concentration reached a value between 6.0×10^5 mL^{-1} and 1.0×10^6 mL^{-1} and the viability maintained above 98 % (approximately 24h or 48 h after the seeding). The complex formation was induced in 20.0 mL of DMEM -Ca^{2+}+ Vit. (1/10 of the total volume to be transfected), as it was already explained under 2.18.1.2). The plasmid pEPO-IRES2-EGFP was used in the DNA:PEI ratio of 0.5:1.5, µg:µg. Briefly, first DNA was added and, two minutes later, PEI. The complex was homogenized (by vortex) and incubated for 10 minutes at RT before the addition to the cells. Every 12 hpt samples were taken for flow cytometry analysis and the respective cell supernatants were stored at –20 °C for the quantification of rhuEPO. Cell concentration, viability, substrates and metabolites were determined as described before (section 2.10 and 2.11). Figure 3.7a shows that the maximum transfectability, expressed as GFP-positive cells, was reached between 60 and 72 hpt with a value of 70 %. The highest rhuEPO productivity was reached 72 hpt with a concentration of 1.58 µg mL^{-1}.

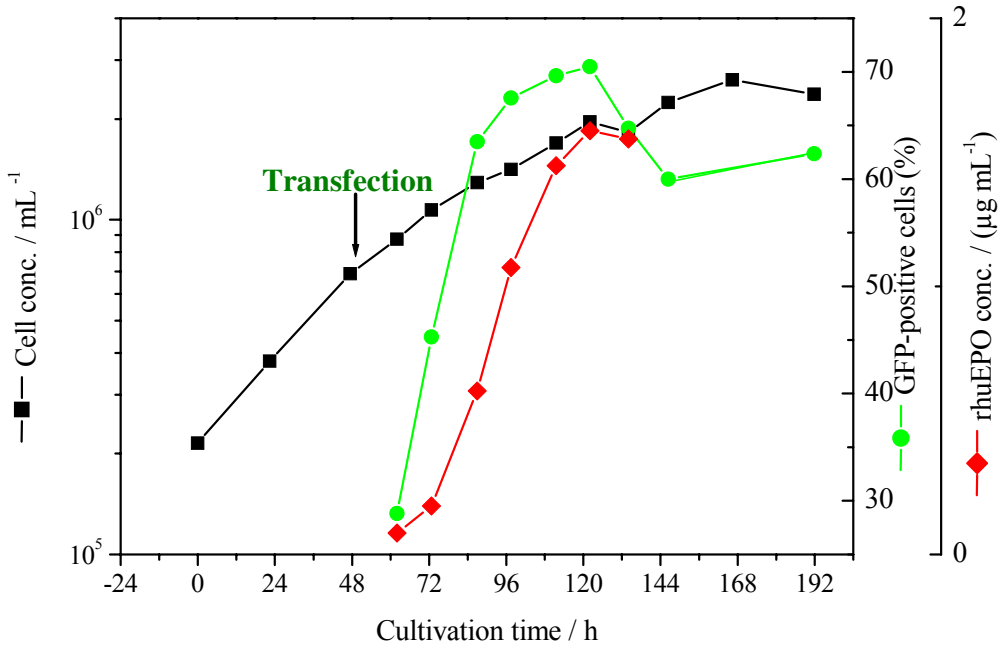


Fig. 3.7a: HEK293s cells growing in FS:DMEM + AA medium (200 mL working volume, in spinner flask) were transfected (↓) using a DNA:PEI (µg:µg) ratio of 0.5:1.5, without washing the cells. GFP-positive cells were quantified by flow cytometry (●) and rhuEPO (♦) was measured by ELISA every 12 hpt.

Different transfection experiments were performed in big spinner flasks (200 mL working volume) using HEK293s cells growing under serum-free (FS:DMEM + AA) and serum-containing (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) medium conditions following the same protocol as described before (section 2.18.1.2). The cells used were from an early (between 40 and 77) and late (between 89 and 150) passage numbers. Cell concentration, viability, substrates and metabolites as well as intracellular nucleotides were determined as described in material and methods section (2.10, 2.11 and 2.12).

Table 3.7a summarizes different transient transfections performed in serum-free medium with HEK293s cells from an early and a late passage number using pEPO-IRES2-EGFP and pCMV-EGFP-EPO plasmids. The GFP-positive cells were identified between 60 and 72 hpt as well as the rhuEPO and the total protein concentration of the cell supernatant stored at –20 °C.

These results showed, that cells from an early passage number (70 and 76), transfected with the plasmid pEPO-IRES2-EGFP, presented highest transfection efficiencies (73.22 % and 70.49 %, respectively) compared to their counterparts from a late passage number (89 and 101: 51.02 % and 55.62 %, respectively). The same results were also observed for cells transfected with the plasmid pCMV-EGFP-EPO with 95.97 % compared to 69.48 %. However, the absolute values reached were higher than for cells transfected with the first plasmid. Moreover, the expression of the rhuEPO showed the same tendency as the transfection efficiency expressed by the GFP-positive cells, but no difference between transfection with the different plasmids could be observed. EPO represented between 2.5 % and 10 % of the total protein concentration.

Table 3.7a: The different transfection experiments performed under serum-free conditions at a glance.

Transfection of HEK293s cells in serum-free medium (DNA:PEI = 0.5 µg:1.5 µg)				
Cell passage number:	Plasmid used:	**GFP-positive cells (%)** (60 – 72 hpt)	**rhuEPO conc. (µg mL^{-1})** (60 – 72 hpt)	**Total protein conc. (µg mL^{-1})** (60 – 72 hpt)
70 *(early)*	***pEPO-IRES2-EGFP***	73.22	1.54	14.80
76 *(early)*		70.49	1.58	17.12
89 *(late)*		51.02	1.17	34.51
101 *(late)*		55.62	1.29	51.18
70 *(early)*	***pCMV-EGFP-EPO***	95.97	1.59	19.94
101 *(late)*		69.48	n.d.	57.08

n.d.= not determined

Table 3.7b shows the same experiments as in Table 3.7a but the transfection procedure was done under serum-containing conditions. HEK293s cells from an early and a late passage number and the plasmids pEPO-IRES2-EGFP and pCMV-EGFP-EPO were used (same conditions as in serum-free medium). Identification of GFP-positive cells as well as rhuEPO and protein concentrations in the cell culture supernatant were determined between 60 and 72 hpt.

In this case, the highest transfection efficiencies (80.15 % – 86.39 % compared with 43.97 % to 71.3 %) were reached when cells from a late passage number (116 and 150) were transfected either with pEPO-IRES2-EGFP and pCMV-EGFP-EPO. Considering the results from transfection of cells from the same early passage no. 42, highest values of 58.37 % for the second plasmid, which contains EGFP as first gene, were found compared to 43.97 % for GFP-positive cells for the first plasmid. This has confirmed the results from transfections under serum-free conditions.

For the transfection procedure using the pEPO-IRES2-EGFP plasmid, highest productivity of the rhuEPO between 2.07 and 2.60 µg mL^{-1} were obtained in the supernatant of cells cultured under serum-containing conditions compared to 1.17 to 1.58 µg mL^{-1} in serum-free medium. This difference was not observed with the pCMV-EGFP-EPO plasmid.

EPO represented approximately 0.02 % of the total protein concentration.

Table 3.7b: The different transfection experiments performed under serum-containing conditions at a glance.

Transfection of HEK293s cells in serum-containing medium (DNA:PEI = 0.5 µg:1.5 µg)				
Cell passage number:	Plasmid used:	**GFP-positive cells (%)** (60 – 72 hpt)	**rhuEPO conc. (µg mL^{-1})** (60 – 72 hpt)	**Total protein conc. (µg mL^{-1})** (60 – 72 hpt)
40 *(early)*	***pEPO-IRES2-EGFP***	71.3	2.60	1381.96
42 *(early)*		43.97	2.07	1364.24
116 *(late)*		80.15	2.34	1369.50
42 *(early)*	***pCMV-EGFP-EPO***	58.37	n.d.	1367.46
150 *(late)*		86.39	1.41	1380.88

n.d.= not determined

3.7.1 Intracellular nucleotide pools

Intracellular nucleotide pools have been reported to reflect the physiological state of BHK, hybridomas and CHO cells culture (Grammatikos et al. 1999; Ryll et al. 1994; Ryll and Wagner 1992). They represent the driving forces for cell growth and metabolism. The knowledge of the correct cell physiology is a prerequisite for a successful production process.

In order to evaluate the HEK293s cells metabolic energy and growth potential with respect of increasing productivity, and therefore to characterize and optimize the transient transfection procedure, the intracellular nucleotide pattern of these cells was analyzed under different culture conditions.

Intracellular nucleotides were quantified as explained under materials and methods in section 2.12. AEC, NTP ratio, U ratio and NTP/U ratio were calculated as described before (2.12.1).

The absolute value of the NTP/U ratio for cells in good physiological state varies with cell type, medium and culture mode. Every cell line reveals a specific range which indicates a good physiological state (Grammatikos et al. 1999; Ryll and Wagner 1992). Therefore, the characteristic range of HEK293s cells under different conditions was studied.

HEK293s cells from early and late passage numbers were cultivated in 200 mL spinner flasks in serum-containing medium, DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS, and in serum-free medium, FS:DMEM + AA. In parallel, batch cultures without (w/o) transfection/with (w.) transfection were performed to characterize the cells. Cell concentration, viability, substrates and metabolites as well as intracellular nucleotides were determined every day (2.10, 2.11 and 2.12).

3.7.1.1 Early vs. late passage number

Serum-containing medium

First, HEK293s cells from early (77) and late (101) passage numbers growing under serum-containing conditions were evaluated. Diagram A from figure 3.7.1.1a; A shows that cells from the exponential growth phase proliferated to about 3.5×10^6 mL^{-1} within 72 h of cultivation before they entered the stationary phase. During the first hours of culture (cultivation time: 0 and 24 h) the NTP/U ratio maintained below 3.3 (horizontal black dash line). This period indicated best physiological conditions. Then (↓), this ratio increased more than two-fold in cultures with cells from an early passage number (from 3.28 to 9.77) and from 3.14 to 4.92 in cultures with cells from a late passage number. This increase indicated a worsening of the cellular conditions and a poor growth potential, and therefore, the cells entered the phase of reduced exponential growth. This situation was confirmed 1 day later by counting the cell number in both cultivations (vertical black dash line). Usually, a 50 – 100 % increase in NTP/U value from the basal level is enough to consider this phase as sub-optimal cell state (Grammatikos et al. 1999).

This increment in the NTP/U ratio showed different characteristics between cells from early and late passage numbers. In the first case, the NTP/U ratio reached its maximum after 96 h of culture, but in the second case 48 h later (blue dot line for early passage number, and red dot line for late passage number) indicating a further worsening of cell state and the beginning of the starvation phase (Fig. 3.7.1.1a; A). These results indicated a change in the cell physiological character during aging of this cell type.

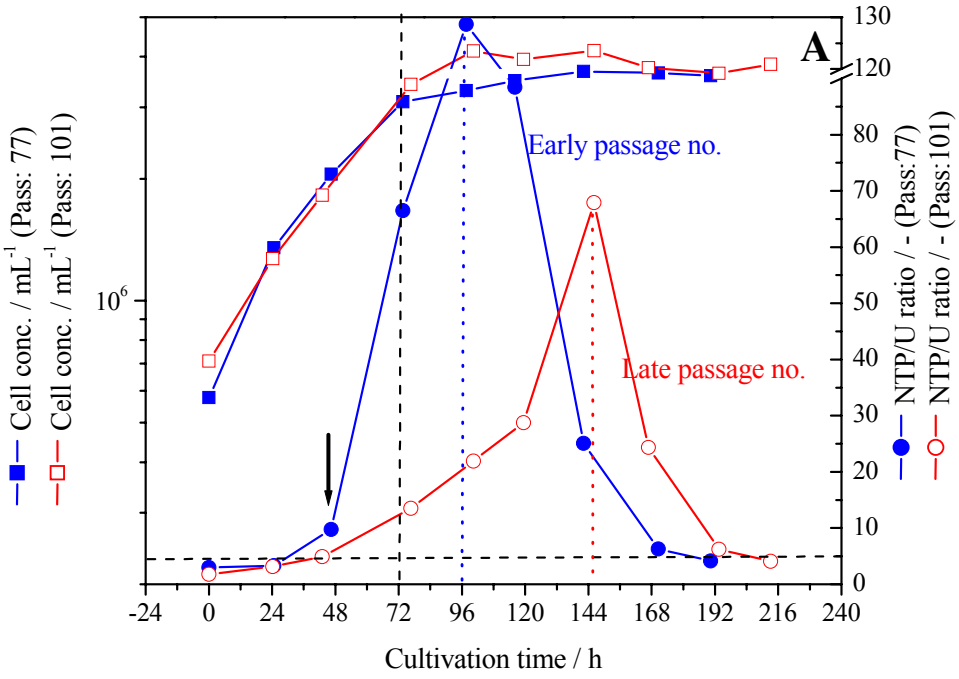


Fig. 3.7.1.1a; A: Two batch cultures of HEK293s from an early (blue filled symbol) and a late (red open symbol) passage number cultivated under serum-contaning conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS). The cell concentration (symbol: square) and NTP/U (symbol: circle) ratio were determined. The horizontal black dash line shows the low values of the NTP/U ratio, indicating best physiological state. The arrow indicates the point at which an increase in the NTP/U ratio was observed indicating a worsening of the cell conditions. The vertical blue dot line and the vertical red dot line indicate the maximum increase of the NTP/U ratio for cultures with cells from the early passage number and the late passage number, respectively. The vertical black dash line indicated the end of the exponential phase.

The adenylate energy charge (AEC) was determined and presented values of about 0.96 in both cell types throughout the batch culture.

Viability, glucose, lactate and LDH were compared between both batch cultures (Fig. 3.7.1.1a; B, C and D). The vertical black dash line indicates the end of the exponential phase. The behavior of the cultures with cells from different ages was the same in nearly all parameters, characterized by a viability of more than 90 % for the total culture time (Fig. 3.7.1.1a; B). Except lactate production, which was higher (1 g L^{-1} more) in cultures with cells from the early passage number (Fig. 3.7.1.1a; C).

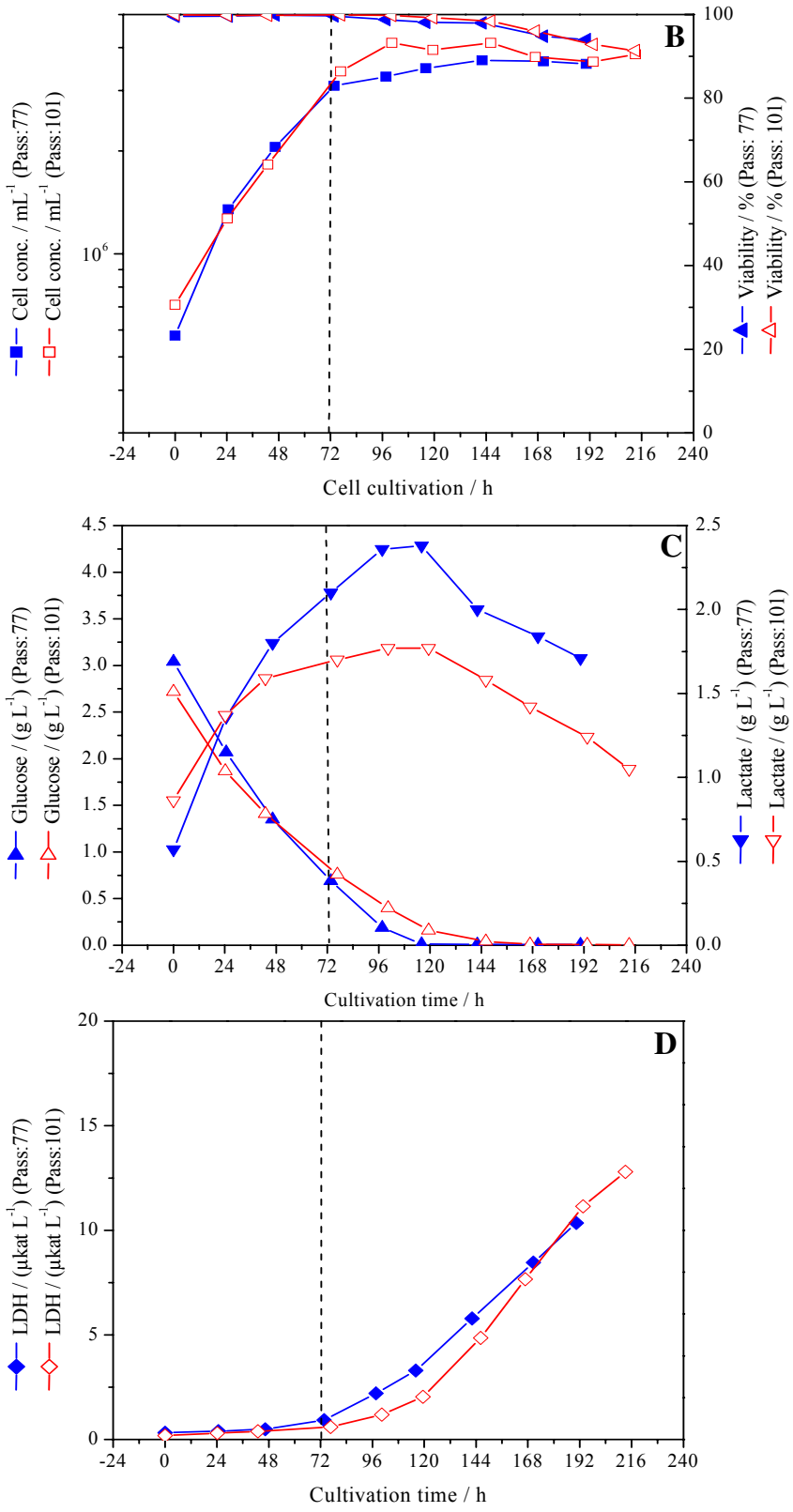


Fig. 3.7.1.1a; B, C and D: Two batch cultures of HEK293s from an early (blue filled symbol) and a late (red open symbol) passage number under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS). Viability (**B**), glucose and lactate (**C**) and LDH (**D**) were evaluated and compared between both cells ages. The vertical black dash line indicated the end of the exponential phase.

Serum-free medium conditions

The same procedure was done for cells growing under serum-free conditions. HEK293s from the early passage number 70 and from the late passage number 101 were cultivated in FS:DMEM + AA medium.

Diagram A from figure 3.7.1.1b shows that cells of a late passage number (red open square) grew exponentially to about 4.0×10^6 mL^{-1} within 72 h before entering the stationary phase. Cells of an early passage number (blue filled square) continuously grew until a cell concentration of 5.5×10^6 mL^{-1} within 120 h of culture. However, they showed a lower growth rate (μ) of about 19.4×10^{-3} h^{-1} (compared to a μ of 27.4×10^{-3} h^{-1} for cells of the late passage no.). The reduced growth rate could be visualized by the NTP/U ratio, which amounted to less than 2.3 and maintained more or less during the first hours (0 and 24; horizontal black dash line). Then it (↓) increased until the end of the batch culture without reaching a maximum. Cultures of cells from a late passage number also showed a NTP/U ratio of less than 2.3 and maintained during the first hours of the culture. Then (↓) the NTP/U ratio increased and reached a maximum at 144 h of the culture.

In these cells, an NTP/U ratio of less than 2.3 indicated cells could be found indicating good physiological conditions.

The NTP/U ratio of cultured cells from a late passage number ranged between 1.54 and 1.83 in the first two days, then it increased to 2.86 in the following 24 h indicating a worsening of the cell conditions and the end of the exponential growth phase (vertical red dash line). This could be confirmed 48 h later by counting the cells. For cultures with cells from an early passage number, the NTP/U ratio was between 1.57 and 1.91 at the beginning of the culture (0 and 24 h), followed by an increase of 50 % 48 h later. This indicated the end of the exponential growth phase which was also confirmed 48 h later by counting the stationary cell number (vertical blue dash line, 110 h of cultivation time). The further dramatically increase of this ratio indicated the starvation phase, when the cell viability dropped down (Fig. 3.7.1.1b; B).

An AEC of 0.98 to 0.95 throughout the batch culture was found for cells from the early passage number and of 0.97 to 0.93 for the respective cells from the late passage number.

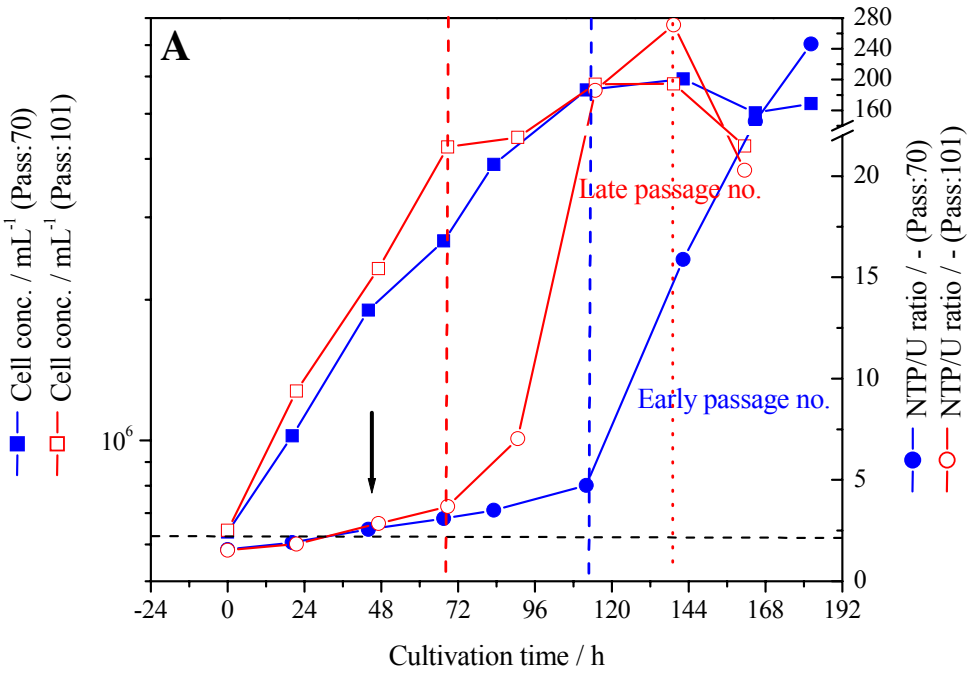


Fig. 3.7.1.1b; A: Cell concentration and NTP/U ratio were evaluated from two batch cultures of HEK293s from an early (blue filled symbol) and a late (red open symbol) passage number cultured under serum-free conditions (FS:DMEM + AA). The horizontal black dash line showsd low values of NTP/U ratio, indicating a good physiological state. The arrow indicates the point at which an increase in the NTP/U ratio was observed. The vertical red dot line indicated the maximum increase of the NTP/U ratio for cells from the late passage number. The vertical red dash line and the vertical blue dash line indicate the end of the exponential growth phase for cells from the late and the early passage number, respectively.

Viability, glucose, lactate and LDH from both cultures were compared (Fig. 3.7.1.1b; B, C and D, respectively). The vertical dash lines indicate the end of the exponential phase for cells from a late passage number (red) and for cells from an early passage number (blue). After this point, in both cases, the viability started to decline (B) and the LDH (D) increased abruptly. For cells from a late passage no., the glucose concentration was already depleted from the medium (conc.: 0.42 g L^{-1}) after 114 h of cultivation with a maximum lactate concentration of nearly 2.0 g L^{-1}. In contrast, glucose was still present at a concentration of 1.8 g L^{-1} for cells from an early passage number and lactate concentration reached 1.3 g L^{-1} (C). Hence, cells coming from a late passage number reached the stationary and starvation phase earlier.

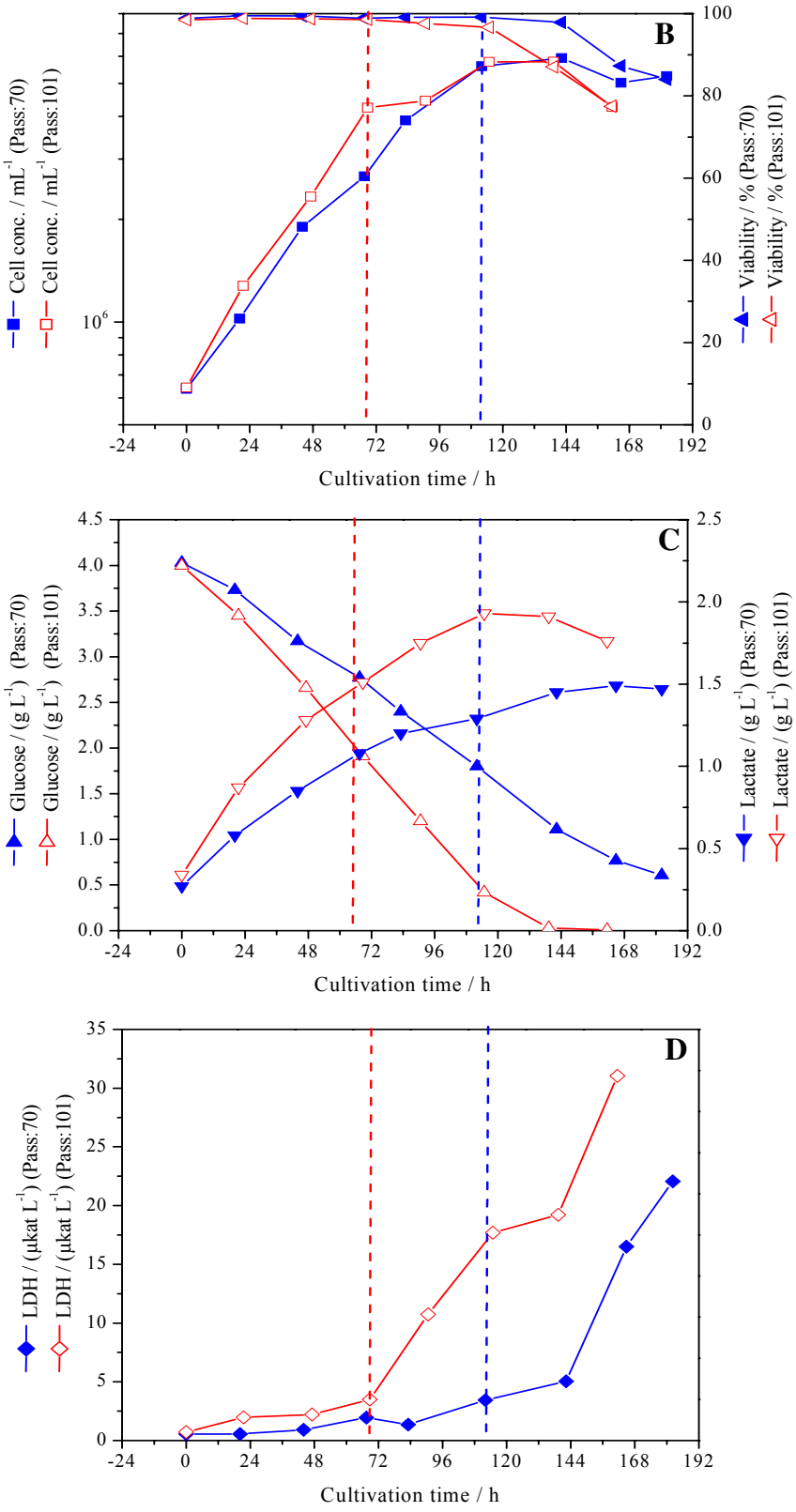


Fig. 3.7.1.1b; B, C and D: Batch cultures of HEK293s from an early (blue filled symbol) and a late (red open symbol) passage number cultivated under serum-free conditions (FS:DMEM + AA). Viability (**B**), glucose and lactate (**C**) and LDH (**D**) were evaluated and compared between cells from both passage numbers. The vertical dash line indicated the end of the exponential phase (blue for early passage no. and red for late passage no.).

This experiment, which compared cells taken from early and late passages and cultivated under serum-free and serum-containing conditions, was repeated and gave the same results as presented above (data not shown).

3.7.1.2 Early vs. late passage number – Transfected cells

Serum-containing medium

HEK293s cells from an early (40) and a late (116) passage number were cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium in a spinner flask (200 mL working volume). They were transfected following the standard protocol (see under 2.18.1.2), using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg). GFP was evaluated by flow cytometry 60 hpt.

The transfectability of cultured cells from the early passage was 71.30 % GFP-positive cells compared to 82.07 % in cultures with cells from a late passage (Table 3.7.1.2a).

Table 3.7.1.2a: Transfectability obtained in serum-containing medium.

Transfection of HEK293s in serum-containing medium (DNA:PEI = 0.50µg:1.50µg)	**Transfectability expressed in GFP-positive cells (%) (60 hpt)**
Early passage number (40)	**71.30**
Late passage number (116)	**82.07**

Diagram A of figure 3.7.1.2a shows the transfection of HEK293s cells at a concentration of 1.0×10^6 mL^{-1} (↓).Subsequently, they continued growing with a lower growth rate (see table 3.7.1.2b), as indicated by the NTP/U ratio and the population doubling time. During the first hours before transfection this ratio amounted to less than 3.3 and maintained in both cases (horizontal black dash line). Then (↓), the ratio started to increase slowly indicating a worsening of the cell conditions and a poor growth potential, thus reaching a maximum at 144 h of the cultivation time for cultures with cells from an early passage (blue dot line), whilst this maximum could not be determined for cultures with cells from a late passage. For cultures with youngest cells, the ratio increased more than 50 % 24 h after the transfection procedure, whilst with the oldest cells showed this increase after 48 h. The dramatic further increase indicated a further worsening of the cell state, which was noticed first in transfected cells from an early passage number.

This increment of the NTP/U ratio has shown different characteristics between transfected cells from an early and late passage number under serum-containing medium. This was also found for the same cells, but without transfection and cultivated

under identical conditions (3.7.1.1). These results confirmed a change in the cell character during aging of this cell line.

The AEC revealed values of approximately 0.97 during all the process for both batch cultures.

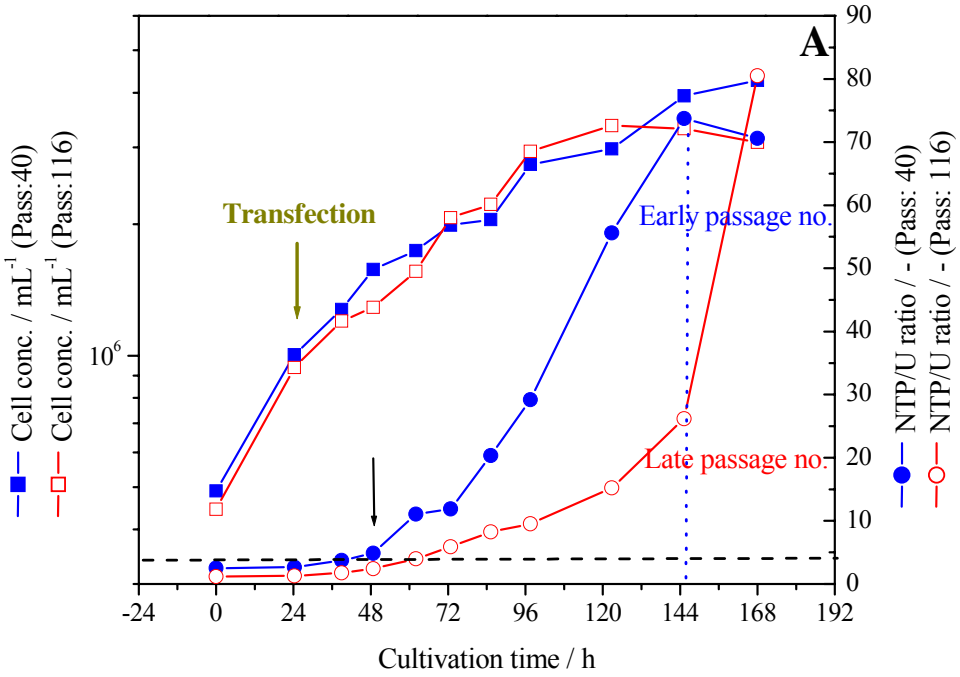


Fig. 3.7.1.2a; A: HEK293s cells from an early (blue filled symbol) and a late (red open symbol) passage number cultured under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg). The cell concentration (square symbol) and the NTP/U ratio (circle symbol) were estimated. The horizontal black dash line shows the low values of the NTP/U ratio, indicating best physiological state. The black arrow indicates the point at which a slow increase in the NTP/U ratio was observed indicating a worsening of the cell conditions and a poor growth potential. The vertical blue point line indicates the maximum increase of the NTP/U ratio of cells from the early passage.

Table 3.7.1.2b: Growth rate (µ) and population doubling time (t_d) of HEK293s cells from an early and a late passage number cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium before and after transient transfection (TTran.) procedure.

	Growth rate µ [h^{-1}]		**Population doubling time t_d [h]**	
	Before TTran.	After TTran.	Before TTran.	After TTran.
Early passage no. (40)	**29.6×10^{-3}**	**13.1×10^{-3}**	**23.4**	**52.9**
Late passage no. (116)	**30.8×10^{-3}**	**15.3×10^{-3}**	**22.5**	**45.3**

Viability of cells from both cultures was higher than 90 % (Fig. 3.7.1.2a; B). Determination of glucose and lactate concentration (Fig. 3.7.1.2a; C) as well as LDH activity (Fig. 3.7.1.2a; D) revealed no difference between transfected cells from an early and late passage number.

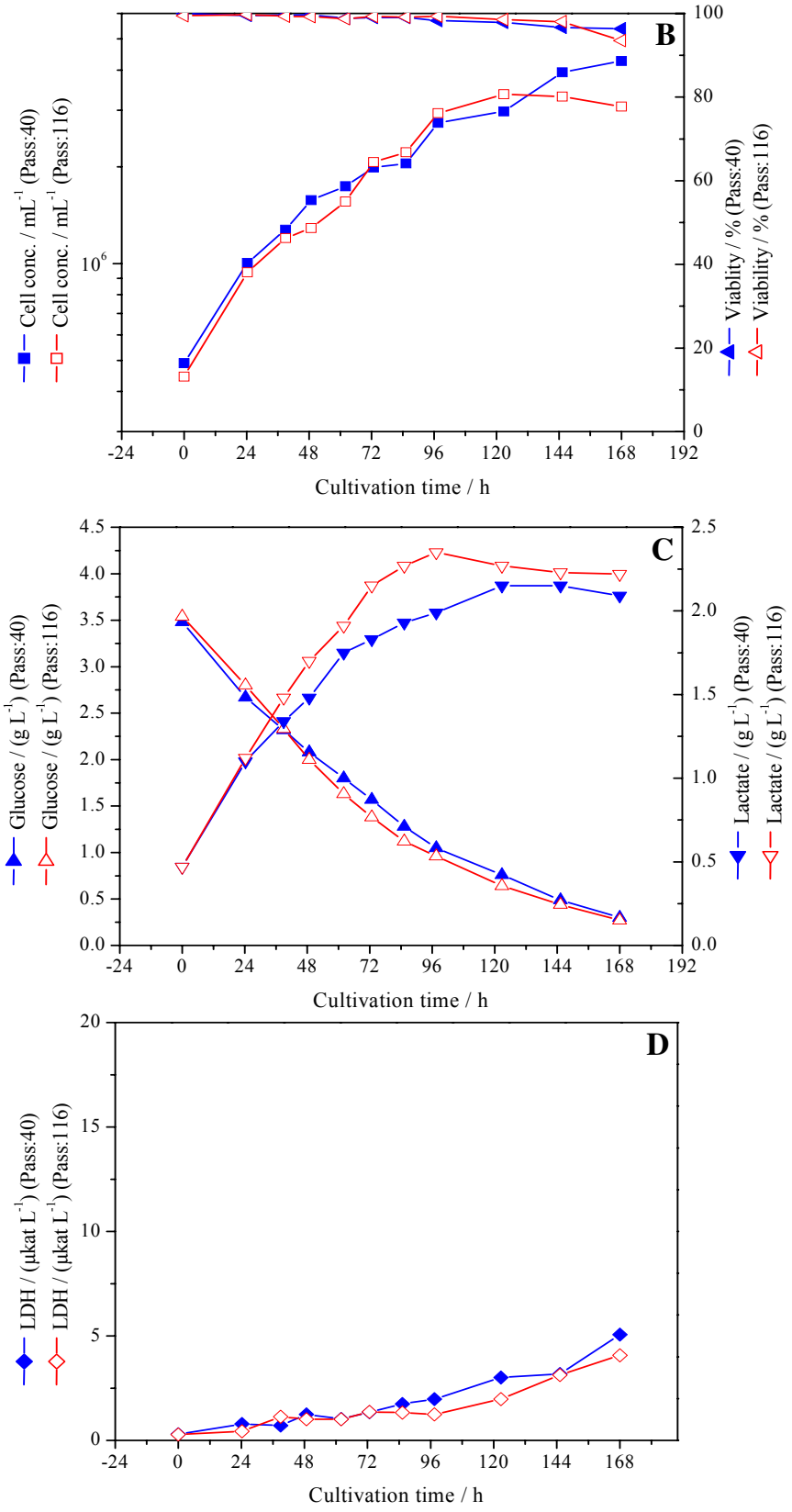


Fig. 3.7.1.2a; B, C and D: HEK293s cells from an early (filled blue symbol) and a late (open red symbol) passage number cultured under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) were transfected using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg). Viability (**B**), glucose and lactate (**C**) and LDH (**D**) were estimated and compared.

Serum-free medium

HEK293s cells from the early passage number 70 and from the late passage number 101 were cultivated in FS:DMEM + AA medium in 200 mL spinner flasks. They were transfected following the standard protocol (2.18.1.2), using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg) as explained above. GFP was evaluated using flow cytometry 60 hpt.

Higher transfectability was reached in cultures with cells from the early passage no. (73.22 %) compared to cultures with cells from the late passage no. (55.62 %) (Table 3.7.1.2c).

Table 3.7.1.2c: Transfectability obtained in serum-free medium.

Transfection of HEK293s in serum-free medium (DNA:PEI = 0.50µg:1.50µg)	**Transfectability expressed in GFP-positive cells (%) (60 hpt)**
Early passage number (70)	**73.22**
Late passage number (101)	**55.62**

The transfection procedure was performed at a cell concentration of 1.0×10^6 mL^{-1} (↓) as shown in diagram A of figure 3.7.1.2b. Subsequently, the growth rate of both cultures decreased (see table 3.7.1.2d) as in transfected cells under serum-containing conditions (Table 3.7.1.2b).

The NTP/U ratio was lower than 2.3 and more or less constant during the first hours, before transfection of both cell cultures (horizontal black dash line; diagram A of figure 3.7.1.2b). Then (↓), the NTP/U ratio started to increase slowly up to 5 during the following 24 h post transfection, which again indicated the reduced growth already observed for transfected cells under serum-containing medium (Fig. 3.7.1.2a; A). However, the NTP/U ratio of cells from the late passage number dramatically increased under serum-free conditions after 90 hpt and reached a maximum of more than 250 at 144 h of the cultivation time (red dot line) indicating that the cells entered the stationary and starvation phase. In contrast, the NTP/U ratio of the respective cells of the early passage number did not reach a maximum and only increased to a comparably low value below 10. In conclusion, the maximum value of the NTP/U ratio for the cells from the late passage was more than 20-fold higher compared to the respective value obtained from cells of the early passage.

The AEC maintained at 0.98 during both batch cultures and decreased to 0.90 at the end of the culture (161.50 h) for transfected cells from the late passage number.

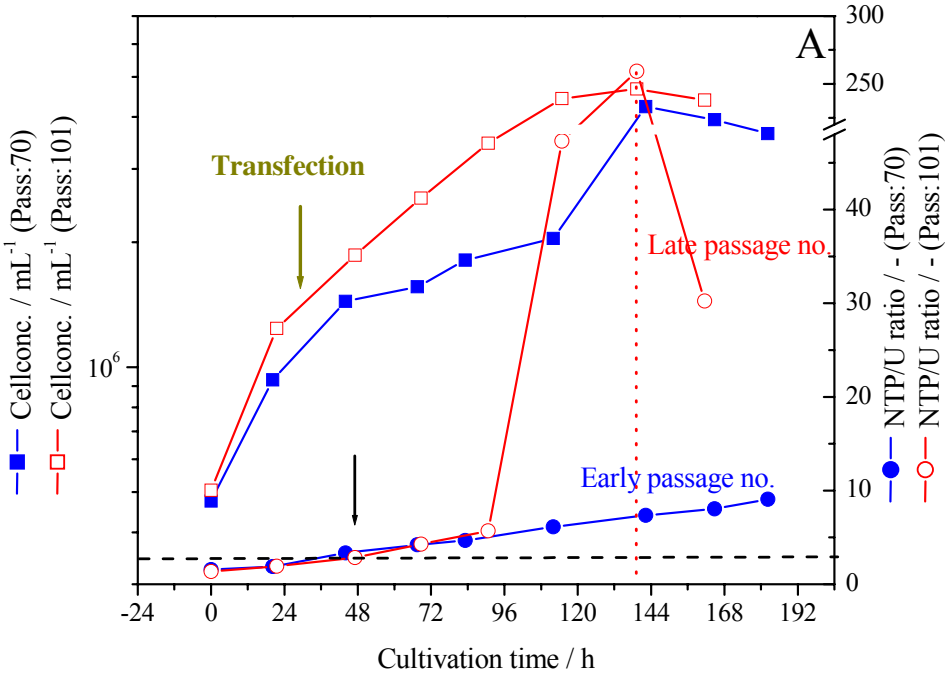


Fig. 3.7.1.2b; A: HEK293s cells from an early (blue filled symbol) and a late (red open symbol) passage number cultured under serum-free conditions (FS:DMEM + AA) were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg). The cell concentration (square symbol) and the NTP/U ratio (circle symbol) were estimated. The horizontal black dash line shows the low values of the NTP/U ratio, indicating best physiological state. The black arrow indicates the point at which a slow increase in NTP/U ratio was observed indicating a worsening of the cell conditions and a poor growth potential. The vertical red dot line indicates the maximum increase of the NTP/U ratio of cells from a late passage number.

Table 3.7.1.2d: Growth rate (µ) and population doubling time (t_d) of HEK293s cells from an early and a late passage number cultivated in FS:DMEM + AA medium before and after transient transfection (TTran.) procedure.

	Growth rate µ [h^{-1}]		**Population doubling time t_d [h]**	
	Before TTran.	After TTran.	Before TTran.	After TTran.
Early passage no. (70)	**33.3×10^{-3}**	**10.9×10^{-3}**	**20.8**	**63.6**
Late passage no. (101)	**41.8×10^{-3}**	**12.8×10^{-3}**	**16.6**	**54.1**

Standard parameters showed a viability of more than 90 % during the complete process with a decrease to 87 % at the end of the batch culture of transfected cells from a late passage no. (Fig. 3.7.1.2b; B). This last result correlated with the drastical increase of the LDH (Fig. 3.7.1.2b; D). As has been observed for cultures with cells from a late passage no. under the same medium conditions but without transfection (Fig. 3.7.1.1b; C), the glucose concentration was depleted at the end of the batch culture (140 h of the cultivation time; glucose conc.: 0.18 g L^{-1}) and the lactate concentration

reached a maximum of 2.18 g L^{-1}. In the respective culture, with cells from an early passage no., however, at the same time of cultivation, glucose was still present (conc.: 1.9 g L^{-1}) and the lactate concentration was 1.42 g L^{-1} (Fig. 3.7.1.2b; C). As was observed before (3.7.1.1), cells from a late passage reached the stationary and starvation phase earlier than cells from an early passage.

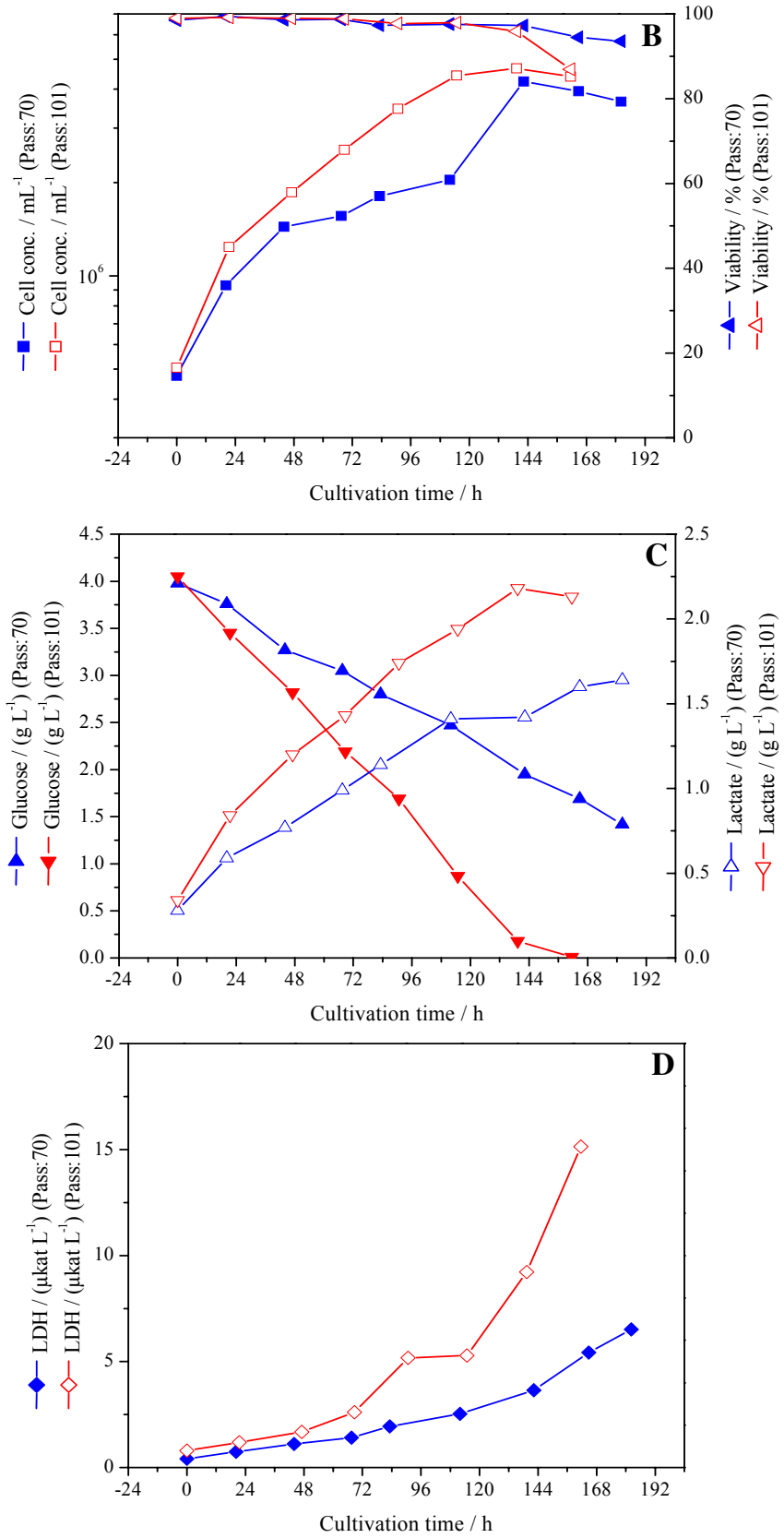


Fig. 3.7.1.2b; B, C and D: HEK293s cells from an early (blue filled symbol) and a late (red open symbol) passage number cultured under serum-free conditions (FS:DMEM + AA) were transfected using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg). Viability (**B**), glucose and lactate (**C**) and LDH (**D**) were evaluated and compared.

3.7.1.3 Transfected vs. untransfected cells

Serum-containing medium

HEK293s cells (passage no.: 40) were cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium in two spinner flasks (200 mL working volume) at a cell concentration of 5.0×10^5 mL^{-1}. Transfection was performed in one spinner flask as explained before (3.7.1.2) and samples were taken every 12 hpt for flow cytometry analysis. The cell culture supernatant was stored at –20 °C for its later quantification of the rhuEPO by the respective ELISA (section 2.19.2).

To the control, 20.0 mL of DMEM -Ca^{2+} + Vit. medium, without the transfection complex, were added to the second spinner flask.

The maximum transfection efficiency expressed as GFP-positive cells was approximately 70 % between 72 and 96 hpt (see Fig. 3.7.1.3a; A).

Cells were transfected at a concentration of 1.0×10^6 mL^{-1} (↓) and subsequently, the growth rate decreased (see table 3.7.1.3a). This correlated with the increase of the NTP/U ratio (red open circle, Fig. 3.7.1.3a; A), which showed a value of 3.3 before transfection (0 and 24 h of cultivation time) (horizontal black dash line), indicating good physiological conditions and cells in the exponential growth phase with a high growth potential. After transfection, the NTP/U ratio increased to a value above 5 within 24 h (↓) indicating a slight worsening of the culture conditions and a poorer growth potential. The NTP/U ratio further increased reaching a maximum value 96 hpt, indicating a very low quality of the culture conditions. Only by controlling the cell concentration and viability at 144 h of the culture (96 hpt) alone, one cannot foresee this worsening of the physiology of the cells (Fig. 3.7.1.3a; B).

For the control spinner, the first low increase of the NTP/U ratio was observed at 72 h of the cultivation time, indicating the end of the exponential growth phase. This could be confirmed one day later by counting the cells. The addition of the 20 mL DNA-free medium, did not substantially affect the growth rate (see table 3.7.1.3a). The drastical increased of the NTP/U ratio, reaching its maximum at 120 h, indicated the starvation phase, which could be verified one to two days later by a drop in the cell viability (81.63 %) and higher values of the LDH activity (between 8.99 and 11.61 μkat L^{-1}) (Fig. 3.7.1.3a; B and D). The maximum NTP/U value was reached 24 h before that of the transfected cells. Moreover, the absolute value of the NTP/ ratio for untransfected cells was two-fold higher (151.08) compared to 73.74 for transfected cells indicating the different cell characteristics between transfected and untransfected cells.

Despite the fact, that the transfected cells showed a growth rate, which was more than half of that of the untransfected ones (13.1×10^{-3} compared to 32.0×10^{-3}**;** table

3.7.1.3a), the glucose and lactate concentration showed no difference between both cell types throughout the total batch culture (Fig. 3.7.1.3a; C).

As expected, the AEC was higher than 0.90 for both cell cultures.

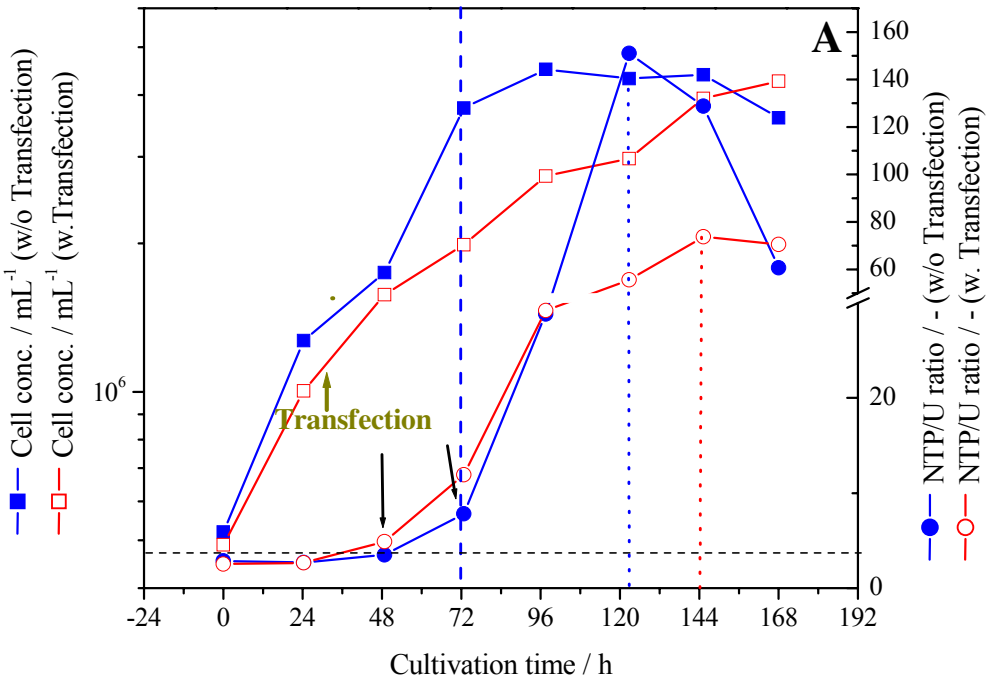


Fig. 3.7.1.3a; A: HEK293s cells (Pass: 40) were cultivated under serum-containing conditions (DMEM -Ca^{2+} +Vit. +Pl. + 5 % FCS) in two different spinner flasks. In one of them, cells were transfected (↑) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (red open symbol). To the control (blue filled symbol), 20.0 mL of DMEM -Ca^{2+} + Vit. medium without the transfection complex were added. Cell concentration (square) and NTP/U ratio (circle) were evaluated. The horizontal black dash line shows the low values of the NTP/U ratio, indicating best physiological state. The black arrows (↓) indicate the points at which an increase of 50 % in the NTP/U ratio could be observed (24 h for transfected cells, 72 h for untransfected cells). The vertical blue dot line and the red dot line indicate the maximum increase of the NTP/U ratio for untransfected cells and transfected cells, respectively. The vertical dash blue line indicates the end of the exponential phase for the control cells.

Table 3.7.1.3a: Growth rate (µ) and population doubling time (t_d) of two HEK293s cells batch cultures cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS medium, before and after the addition (add.) of medium alone (untransfected cells) or the transfection complex (transfected cells).

	Growth rate µ [h^{-1}]		**Population doubling time t_d [h]**	
	Before add.	After add.	Before add.	After add.
Untransfected cells	**36.8×10^{-3}**	**32.0×10^{-3}**	**18.8**	**21.6**
Transfected cells	**29.6×10^{-3}**	**13.1×10^{-3}**	**23.4**	**52.9**

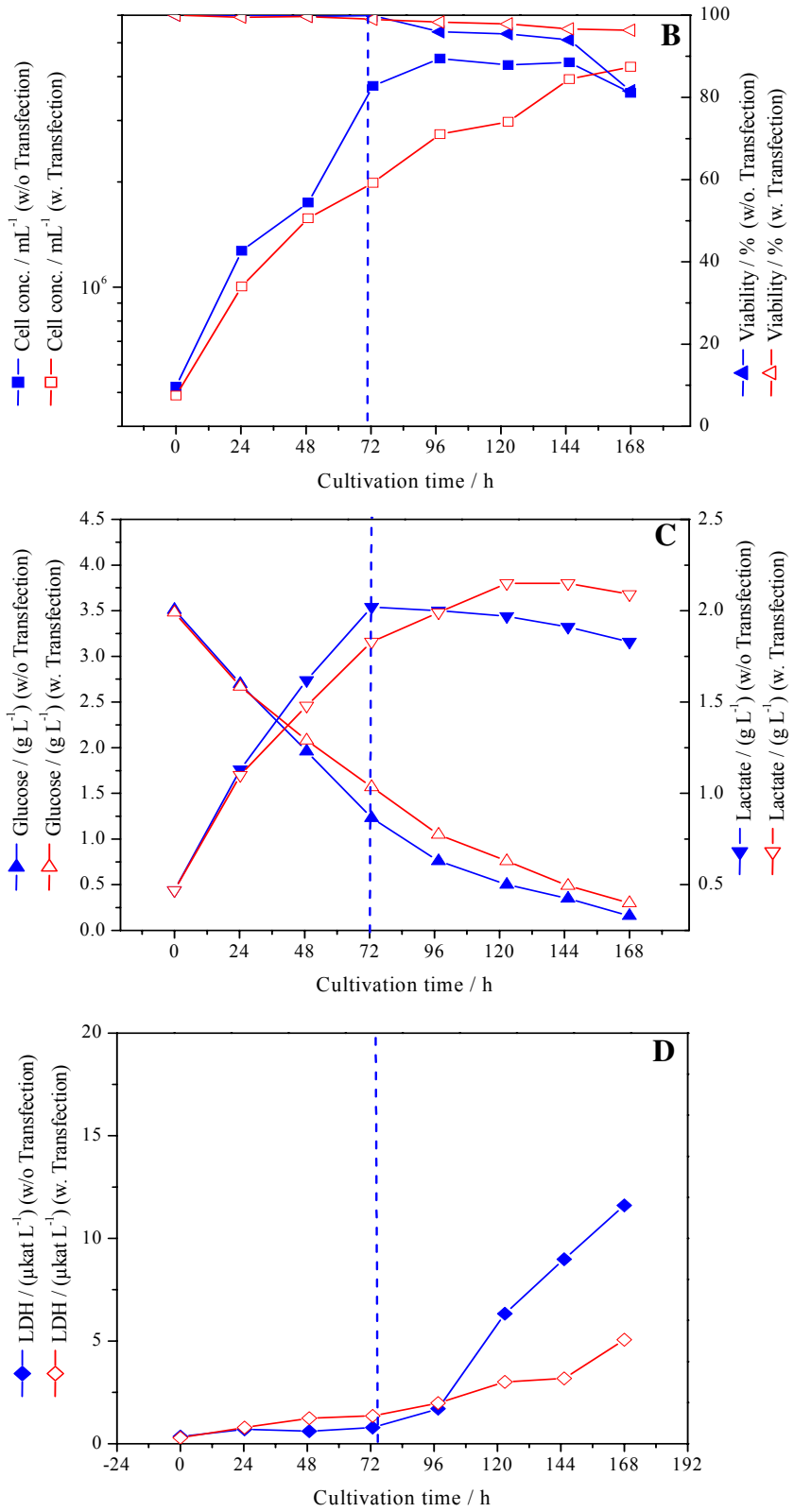


Fig. 3.7.1.3a; B, C and D: HEK293s cells (Pass: 40) were cultivated under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS) in two different spinner flasks. In one of them, cells were transfected using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (red open symbol). For the control (blue filled symbol), 20.0 mL of DMEM -Ca^{2+} + Vit. medium without the transfection complex were added. Viability (**B**), glucose and lactate (**C**) and LDH (**D**) were evaluated and compared. The vertical blue dash line indicates the end of the exponential phase for the control cells.

Production of rhuEPO as well as expression of GFP were analyzed during the batch culture and compared to the cell physiological state expressed as NTP/U ratio. Different works have shown that high productivity and good cell physiology may not always go hand in hand (Barnabé and Butler 1998; Grammatikos et al. 1999; Hayter et al. 1992; Milller et al. 1988; Ryll and Wagner 1992). This could be confirmed in this work. As shown in figure 3.7.1.3b, where the highest productivity of the rhuEPO (between 2.5 and 6.0 µg mL^{-1}) as well as the highest expression of the GFP-positive cells (approximately 70 %) were reached at the end of the batch culture. The respective increase of the NTP/U ratio showed a worsening of cell conditions and indicated a poor growth potential.

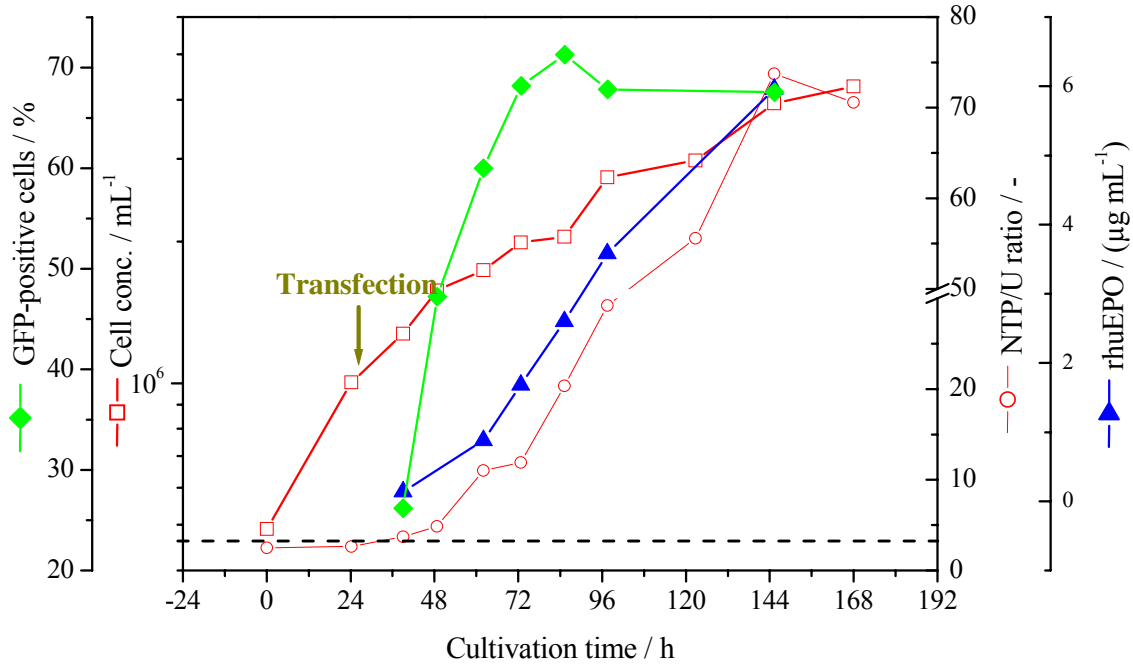


Fig. 3.7.1.3b: HEK293s cells (Pass: 40) cultivated under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS) were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium. NTP/U ratio (red open circle) was evaluated. The horizontal black dash line indicates the limit of the region of NTP/U values, where cells are in the exponential growth phase and have a good growth potential. GFP-positive cells (green filled diamonds) and rhuEPO (blue filled triangle) production were measured every 12 hpt.

Serum-free medium

HEK293s cells (passage no.: 76) were cultivated in FS:DMEM + AA in two spinner flasks (200 mL working volume) at a cell concentration of 3.0 × 10^5 mL^{-1}. The transfection procedure was performed in one spinner flask, whereas only medium was added to the other as control. The same protocol as for serum-containing medium was followed (see above under 3.7.1.3).

The maximum transfection efficiency expressed as GFP-positive cells was 70 % between 60 and 72 hpt (see Fig. 3.7.1.3c; A).

Cells were transfected at a concentration of 7.0×10^5 mL^{-1} (↓) and, as was previously shown (3.7.1.2), they continued growing with a lower rate (see table 3.7.1.3b). This was correlated with the increase of the NTP/U ratio (red open circles, Fig. 3.7.1.3c; A). Before transfection, the NTP/U ratio was less than 2.3 and more or less constant (horizontal black dash line). 24 hpt an increase of more than 50 % was observed (**1**↓), indicating worsening of the culture. The ratio further increased until the end of the culture reaching a value of 7.14, which was substantially lower compared to untransfected cells (see below). The viability throughout the culture was more than 90 % (Fig. 3.7.1.3c; B).

In control culture (w/o transfection, filled symbol, Fig. 3.7.1.3c; A) the NTP/U ratio maintained at low values of 1.45 – 2.08 for the first 72 hours of cultivation when cells grew exponentially. The addition of the 20 mL medium, did not substantially affect their growth rate (see table 3.7.1.3b) as was observed for cells under serum-containing medium. The end of the exponential phase (increase of the NTP/U ratio more than 50 %) was observed 48 h after the increase of the NTP/U ratio of the transfected cells (**2**↓; 96 h cultivation time). The ratio further increased and showed a dramatic rise of more than 6-fold at 144 h of the cultivation time (from 2.90 to 18.17), indicating the starvation phase. This was determined 24 h later with a drop of more than 10 % in the viability (Fig. 3.7.1.3c; B) and an increase of the LDH activity from 4.63 µkat L^{-1} to 13.02 µkat L^{-1} (Fig. 3.7.1.3c; D). At this cultivation time, the NTP/U ratio reached a value of 27.57 (Fig. 3.7.1.3c; A).

At the end of the batch cultures (168 h of cultivation), the glucose concentration of culture supernatants from untransfected cells was 0.85 g L^{-1} compared to 2.01 g L^{-1} for the respective supernatant from transfected cells. Lactate in both culture supernatants did not reach an inhibitory concentration throughout the complete process. Its maximum was 1.4 g L^{-1}, which was first achieved by the untransfected cells (120 h and 168 h of cultivation time, respectively) (Fig. 3.7.1.3b; C). Thus these cells, reached the stationary and the starvation phase earlier.

These results verified the different cell characteristics between transfected and unstransfected cells previously shown in the experiments mentioned above.

The AEC was more or less constant of about 0.98 for unstransfected and transfected cells throughout the batch cultures.

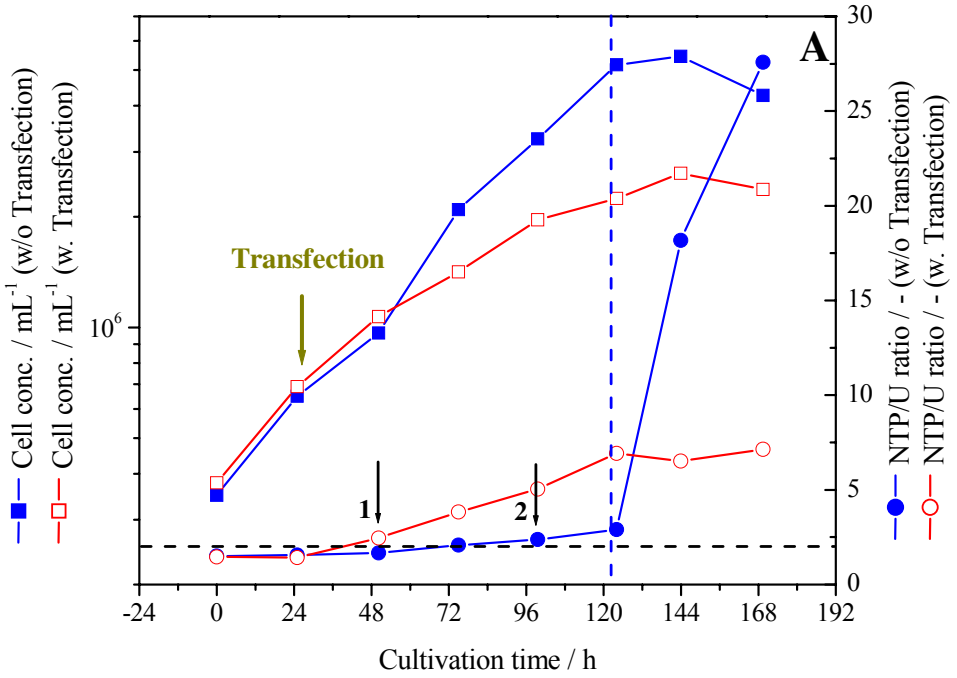


Fig. 3.7.1.3c, A: HEK293s cells (Pass: 76) were cultivated under serum-free conditions (FS:DMEM + AA) in two different spinner flasks. In one of them, cells were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (red open symbol). For the control (blue filled symbol), 20.0 mL of DMEM -Ca^{2+} + Vit. medium without the transfection complex were added. Cell concentration (square) and NTP/U ratio (circle) were evaluated. The horizontal black dash line shows the low values of the NTP/U ratio, indicating best physiological state. The black arrow 1 (**1**↓) indicates the point at which an increase of 50 % in the NTP/U ratio can be observed for transfected cells. For untransfected cells, the increase occured 48 h later (96 h cultivation time; **2**↓). The vertical dash lines indicate the end of the exponential phase for the control cells.

Table 3.7.1.3b: Growth rate (µ) and population doubling time (t_d) of two HEK293s cells batch cultures cultivated in FS:DMEM + AA medium, before and after the addition (add.) of medium alone (untransfected cells) and the transfection complex (transfected cells).

	Growth rate µ [h^{-1}]		**Population doubling time t_d [h]**	
	Before add.	After add.	Before add.	After add.
Untransfected cells	**24.8×10^{-3}**	**22.7×10^{-3}**	**27.9**	**30.5**
Transfected cells	**24.1×10^{-3}**	**11.1×10^{-3}**	**28.8**	**62.4**

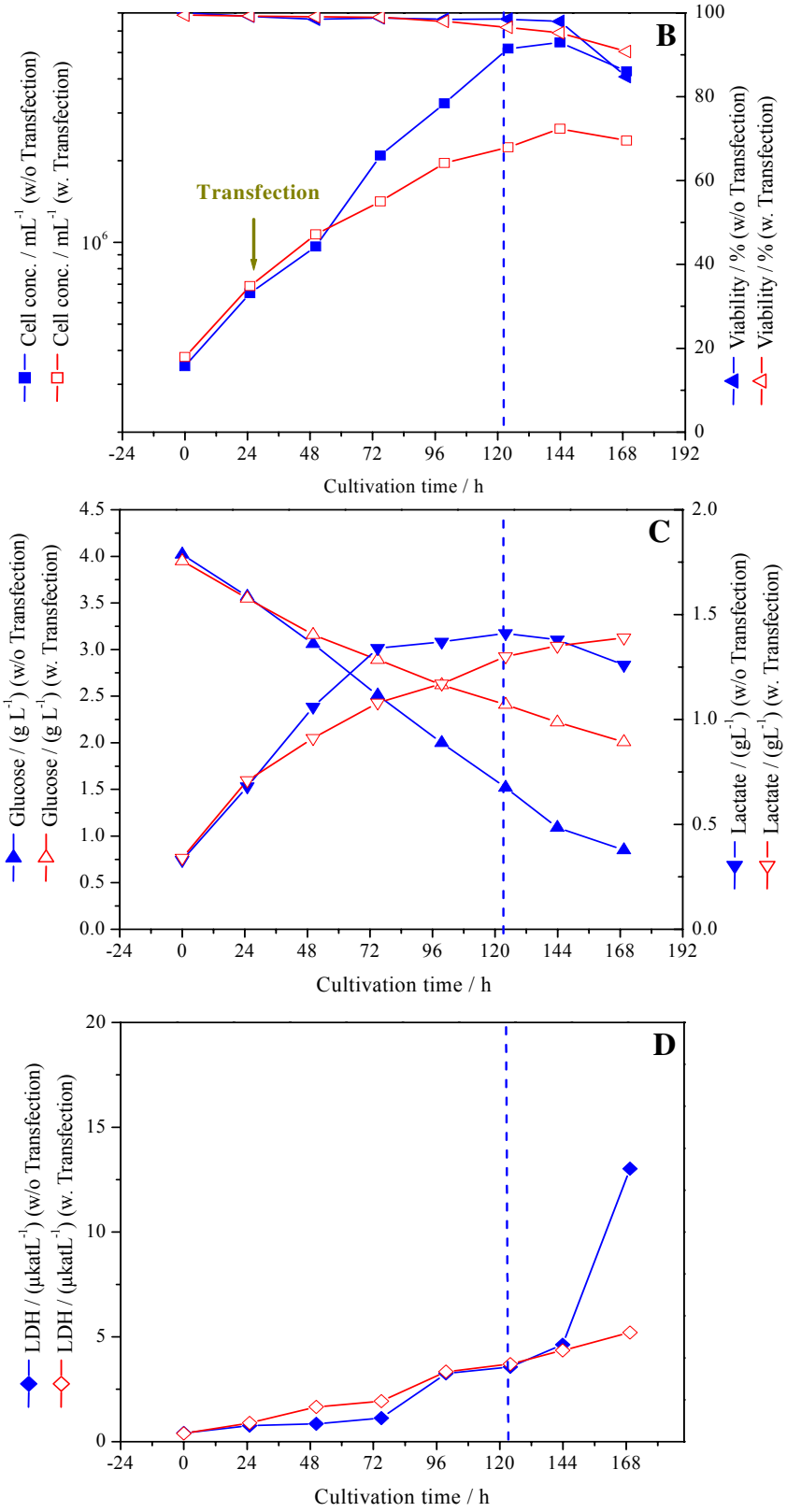


Fig. 3.7.1.3c; B, C and D: HEK293s cells (Pass: 76) were cultivated under serum-free conditions (FS:DMEM + AA) in two different spinner flasks. In one of them, cells were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (red open symbol). For the control (blue filled symbol), 20.0 mL of DMEM -Ca^{2+} + Vit. medium without the transfection complex were added. Viability (**B**), glucose and lactate (**C**) and LDH (**D**) were evaluated. The vertical dash lines indicate the end of the exponential phase for the control cells.

As was performed for transfected cells under serum-containing conditions, the production of the rhuEPO as well as the expression of GFP were analyzed during the batch culture. They were compared to the NTP/U ratio to characterize the cell physiological state during expression and production phase. It is shown in figure 3.7.1.3d that a high productivity and a good cell physiology do not simultaneously change. The highest concentration of the rhuEPO (approximately 2.0 µg mL^{-1}), as well as the highest percentage of the GFP expression (70 %) were reached between 60 and 72 hpt (84 to 108 h cultivation time), whereas the NTP/U ratio values showed a worsening of the cell conditions and a poor growth potential.

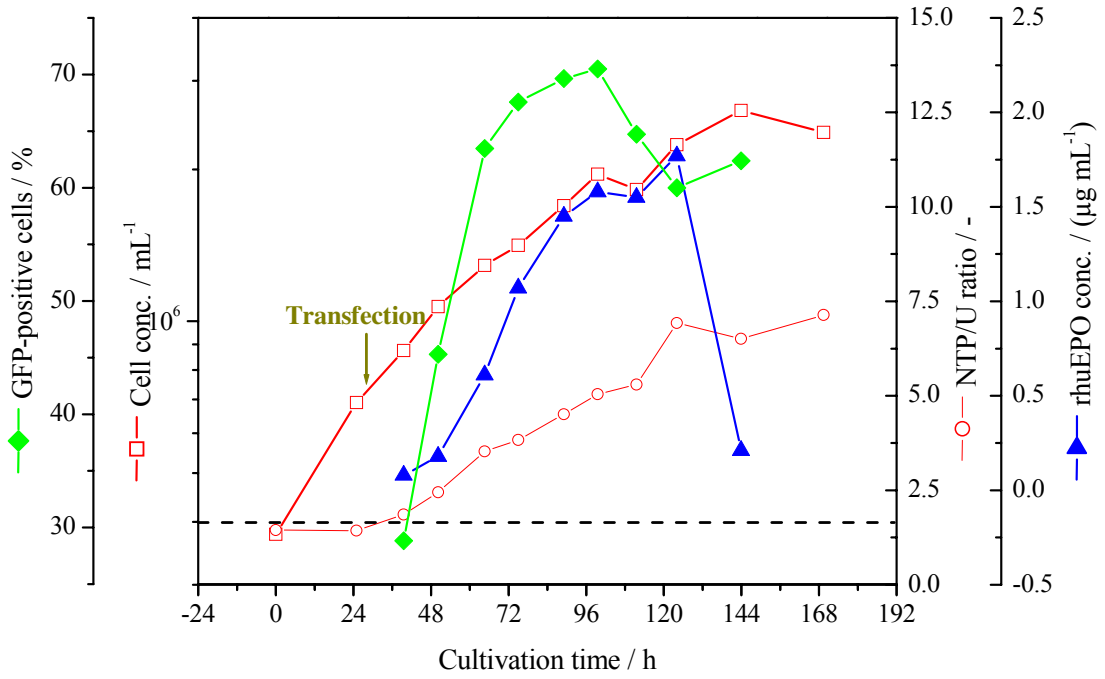


Fig. 3.7.1.3d: HEK293s cells (Pass: 76) were cultivated under serum-free conditions (FS:DMEM + AA) and transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (red open square). NTP/U ratio (red open circle) was evaluated. The horizontal black dash line indicates the limit, in which low ratio values means cells in the exponential phase and good growth potential. GFP-positive cells (green filled diamonds) and rhuEPO (blue filled triangle) production were measured every 12 hpt.

This experiment, which compared transfected and untransfected cells cultivated under serum-free conditions, was repeated one time and gave the same results as presented above (data not shown).

3.7.1.4 Transfected cells vs. addition of PEI alone

In order to investigate whether the different cell characteristics observed in transfected cells compared to untransfected cells was due to the physiological uptake of the PEI with DNA, PEI alone was added to the cell cultures under serum-containing and serum-

free medium conditions. These cell cultures were compared to the cultivated transfected cells previously performed under the same conditions.

The PEI was dissolved in 20.0 mL of DMEM -Ca^{2+} + Vit. medium. and added to the cell cultures when they reached a concentration between 7.0×10^5 mL^{-1} and 1.0×10^6 mL^{-1}.

Serum-containing medium

PEI was added to HEK293s cells (passage no.53) cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium when the cells reached a concentration of 8.75×10^5 mL^{-1} (↑). This culture was compared with one other in which the transfection procedure was performed when the cells (passage no. 40) reached a concentration of 1.0×10^6 mL^{-1} (↓). Diagram A of figure 3.7.1.4a shows an increase in the NTP/U ratio (↓) of more than 50 % after the addition of PEI (↑), indicating a worsening of the cell conditions. This increase was in the same range as observed for transfected cells. The ratio further increased reaching a maximum at 144 h of the cultivation time (vertical dot line) in both cultures. The difference between both cultures was in the absolute value of the ratio, which was more than 30 % higher in untransfected cells, indicating a worsening of the cell status at the end of the culture. This was verified 24 h later by counting the cells by identifying a drop in the viability (Fig. 3.7.1.4a; B) and an increase in the LDH activity (Fig. 3.7.1.4a; D). In addition, untransfected cells showed a higher growth rate of 15.0×10^{-3} h^{-1} compared to transfected HEK293s cells (13.1×10^{-3} h^{-1}), despite the fact that μ decreased to about 30 % after the addition of the PEI. Whilst in the transfected culture this decrease was clearer with 50 % after the addition of the transfection complex (see Table 3.7.1.4a).

In spite of the different growth rates of both cultures, the determination of the glucose concentration showed identical values throughout the complete cultivation. However, a higher concentration of lactate was detected in cultures of transfected cells, 2.15 g L^{-1} compared to 1.68 g L^{-1} for untrasnfected cells at the end of the batch culture (Fig. 3.7.1.4a; C).

The AEC maintained values of 0.97 throughout both batch cultures, indicating that the energy charge alone did not give favorable access to information about the physiological state of the cells.

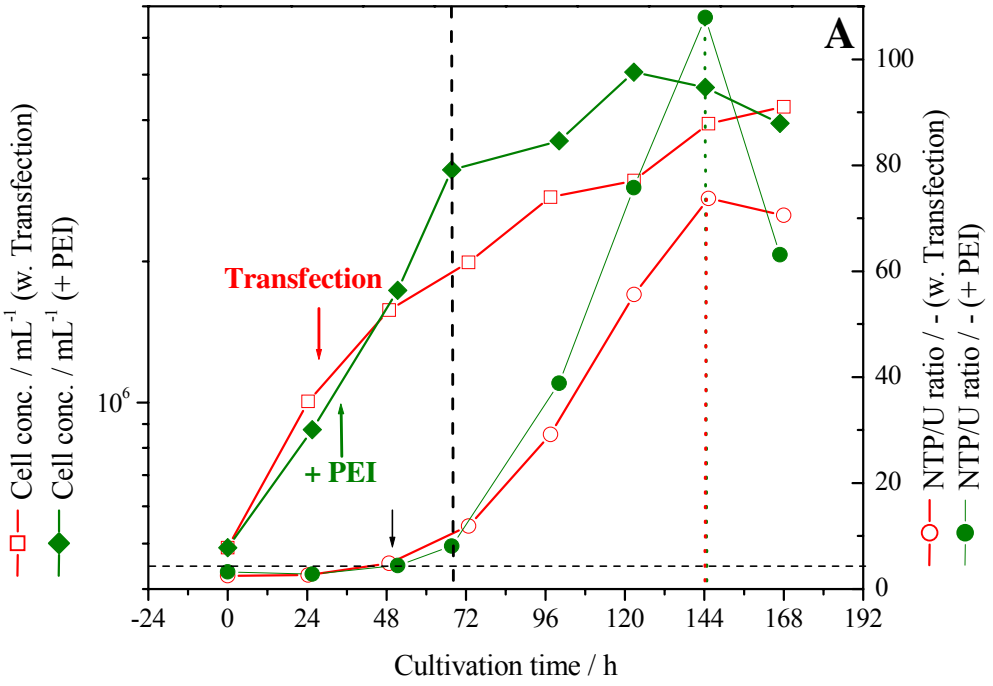


Fig. 3.7.1.4a; A: The addition of PEI alone (↑) to HEK293s cells cultured under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS) (green filled symbols) was compared to a PEI-mediated trasnfection of the same cells (red open symbols). Transfection was performed using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg) (↓). Cell concentration and NTP/U ratio were evaluated. The horizontal black dash line indicates the limit of the region, in which a low NTP/U ratio indicates cells in the exponential growth phase and in a good growth potential. The black arrow shows the point at which an increase of more than 50 % was observed for the NTP/U ratio, indicating a worsening in the cell status. The vertical black dash line indicates the end of the exponential phase for untransfected cells. The vertical green and red dot lines show the maximum increase of the NTP/U ratio for both batch cultures.

Table 3.7.1.4a: Growth rate (µ) and population doubling time (t_d) of two HEK293s cell batch cultures cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium before and after the addition (add.) of PEI alone (untransfected cells) and the transfection complex (transfected cells).

	Growth rate µ [h^{-1}]		**Population doubling time t_d [h]**	
	Before add.	After add.	Before add.	After add.
Untransfected cells (addition of PEI alone)	**22.6×10^{-3}**	**15.0×10^{-3}**	**30.6**	**46.2**
Transfected cells (addition of transfection complex)	**29.6×10^{-3}**	**13.1×10^{-3}**	**23.4**	**52.9**

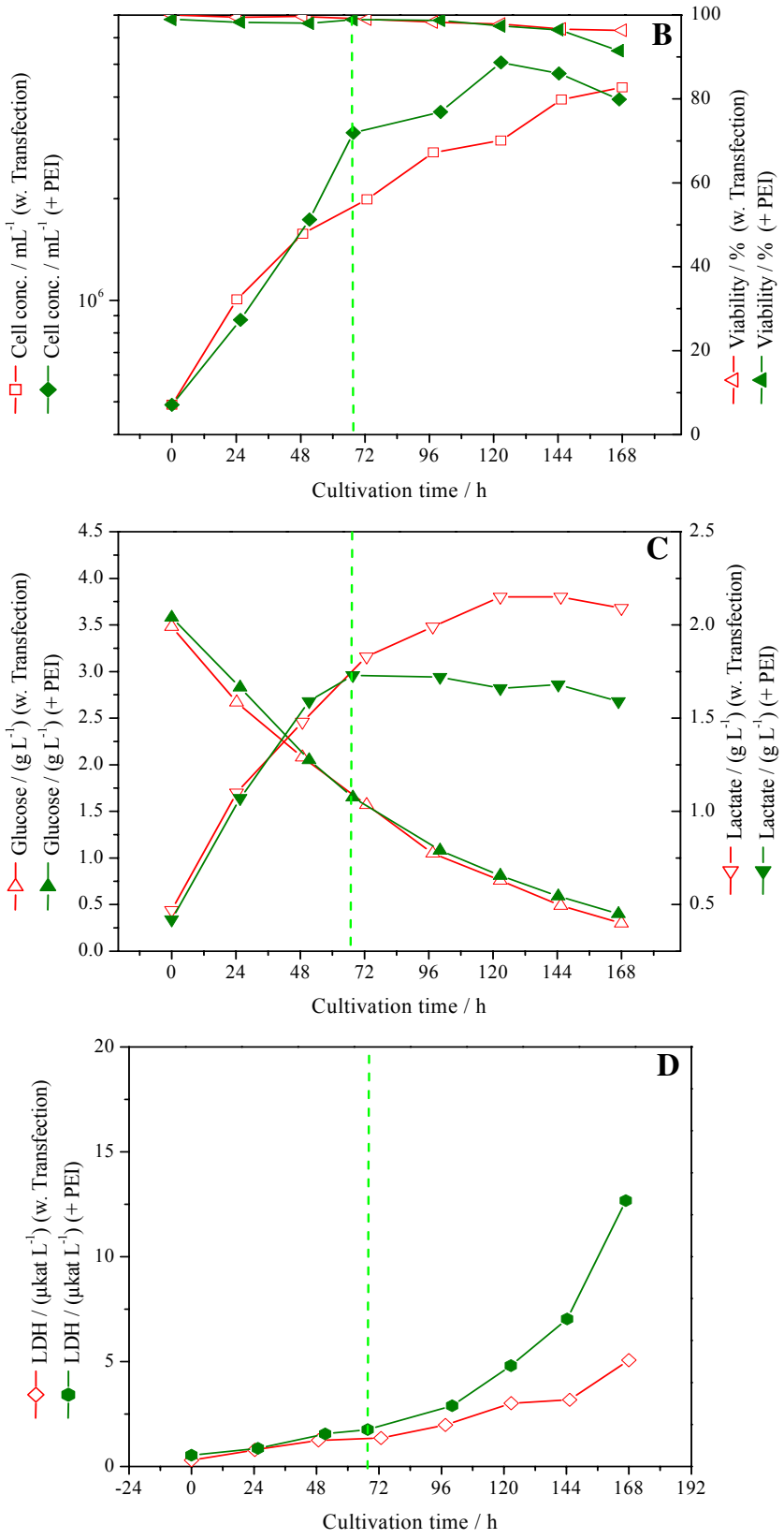


Fig. 3.7.1.4a; B, C and D: Viability (**B**), glucose, lactate (**C**) and LDH activity (**D**) were evaluated in transfected (red open symbols) and unstransfected (green filled symbols; addition of PEI alone) cells growing under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS). Cells were transfected using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg).

Serum-free medium

The same experiment about the addition of PEI alone (w/o DNA) as pointed above was performed under serum-free conditions (passage no. 73) and compared to a culture, in which a complete trasnfection (PEI + DNA) was done (transfected cells, passage no.70).

Diagram A of figure 3.7.1.4b shows, that after the addition of PEI (↓) the NTP/U (**2**↑) ratio increased more than 50 % compared to the basal level (2.3, horizontal black dash line). This was observed 48 h later compared to the transfected cells (**1**↓). The NTP/U ratio further increased up to high values of 60 – 63 for untransfected (+ PEI) cells at the end of the batch culture (216 h cultivation time), indicating the starvation phase. This was also verified by a drop in the viability (Fig. 3.7.1.4b; B) and an increase in the LDH activity (Fig. 3.7.1.4b; D). The maximum NTP/U ratios from both cultures have not been determined throughout the complete batch cultures.

As was already observed for untransfected cells growing under serum-containing conditions, a higher growth rate of 12.4×10^{-3} h^{-1} could also be detected compared to the transfected cells, despite the fact that the growth rate decreased after the addition of PEI more than 50 % (see Table 3.7.1.4b).

In addition, the determination of the glucose concentration showed identical values through the complete cultivation time for both cultures, despite the fact that different growth rates were measured. Meanwhile, a higher lactate concentration was measured at the end of the culture of transfected cells, as also was found in serum-containing medium. However, the values reached a maximum of approximately 2.0 g L^{-1}, double the amount of those found in untransfected cells under these conditions (Fig. 3.7.1.4b; C).

The AEC maintained constant showing a value of 0.98 throughout both batch cultures.

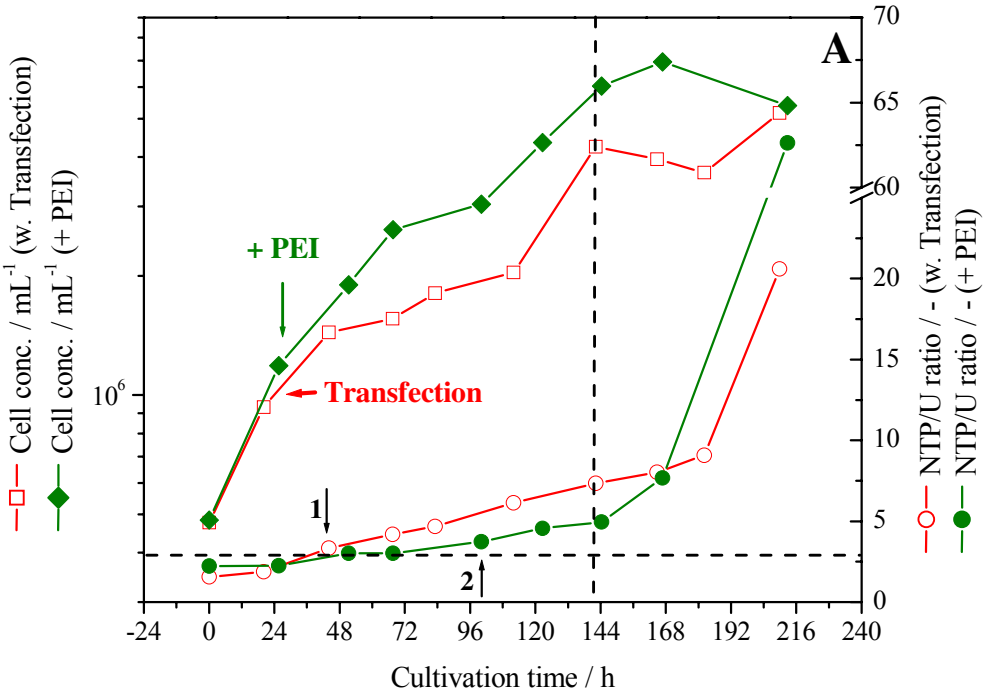


Fig. 3.7.1.4b; A: The addition of PEI alone (↓; **+ PEI**) to HEK293s cells cultured under serum-free conditions (FS:DMEM + AA) (green filled symbols) was evaluated and compared to a transfected culture (red open symbols), which was performed using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg) (←). Cell concentration and NTP/U ratio were evaluated. The horizontal black dash line indicates the limit of the region, in which a low NTP/U ratio indicates cells in the exponential growth phase and in a good growth potential. The black arrow shows the point at which an increase of more than 50 % was observed for the NTP/U ratio, indicating a worsening in the cell status, (**1↓:** for transfected cells; and **2↑:** addition of PEI alone). The vertical black dash line indicates the end of the exponential phase for untransfected cells.

Table 3.7.1.4b: Growth rate (µ) and population doubling time (t_d) of two HEK293s cell batch cultures cultivated in FS:DMEM + AA medium, before and after the addition (add.) of PEI alone (untransfected cells) and the transfection complex (transfected cells).

	Growth rate µ [h^{-1}]		**Population doubling time t_d [h]**	
	Before add.	After add.	Before add.	After add.
Untransfected cells (addition of PEI alone)	**35.1×10^{-3}**	**12.4×10^{-3}**	**19.7**	**55.7**
Transfected cells (addition of transfection complex)	**33.3×10^{-3}**	**10.9×10^{-3}**	**20.8**	**63.59**

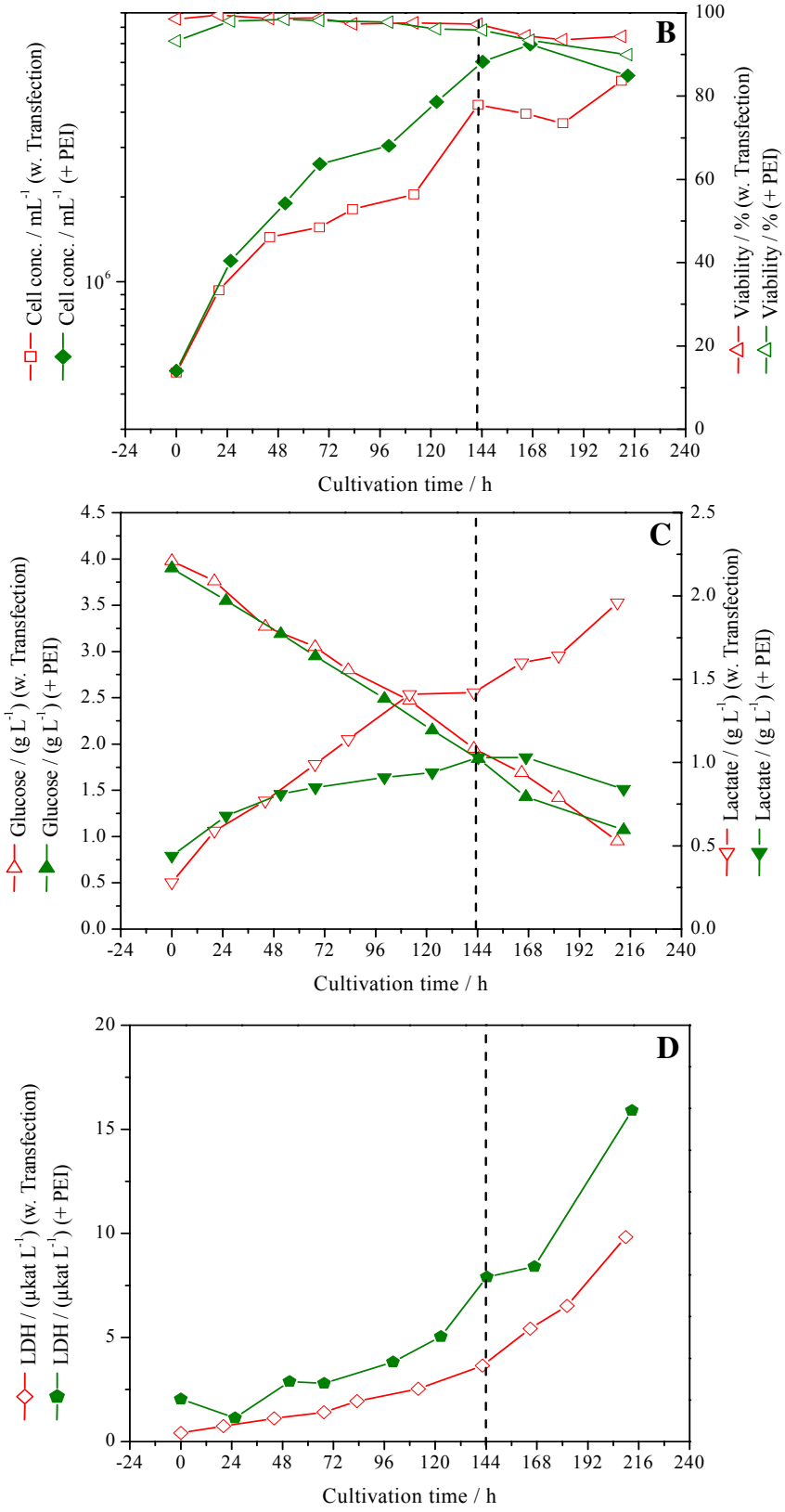


Fig. 3.7.1.4b; B, C and D: Viability (**B**), glucose, lactate (**C**) and LDH (**D**) were evaluated in cultures of transfected (red open symbols, conditions: DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg)) and unstranfected cells (green filled symbols; addition of PEI alone) growing under serum-free conditions (FS:DMEM + AA). The vertical black dash line indicates the end of the exponential phase for untransfected cells.

These results showed that nucleotide ratios can be used to distinguish between:

- **HEK293s cells from an early and a late passage number.**
- **Cells cultured under serum-containing conditions from those under protein/serum-free medium conditions.**
- **Transfected and untransfected cells in culture.**
- **Cells uptake of the DNA:PEI complex (transfection procedure) from the uptake of PEI alone.**

These results indicated a change in the cell physiology during aging of the cell line and in their behavior not only due to different media conditions, even more, due to different uptake and expression-production processes.

3.7.2 Scale-up to bioreactor scale. BioNose characterization

Before scale-up the inocula were prepared. Therefore, the HEK293s cells were cultivated in a big spinner flask (300 mL working volume) under serum-containing and/or serum-free conditions. The cells were in very good conditions showing a viability above 90 % and grew as single cells or small clusters. They were initially cultured at a cell concentration of about 2.0×10^5 mL^{-1} and 3.5×10^5 mL^{-1} (under serum-containing conditions; see 2.5.1) and between 3.0×10^5 mL^{-1} to 5.0×10^5 mL^{-1} (under serum-free conditions; see 2.5.2). Cells were incubated at 37 °C and 12 % of CO_2 and stirred at 80 rpm. When the culture reached a cell concentration between 1.5×10^6 mL^{-1} and 2.0×10^6 mL^{-1}, the cells were sub-cultured at the same cell concentration as the initial inoculum in new spinner flasks. This sub-culture was repeated until enough cells were prepared to inoculate the 1.8 L bioreactor (between 1.4 L and 1.6 L working volume). All bioreactors under serum-containing conditions, were inoculated with a starting cell concentration between 2.5×10^5 mL^{-1} and 3.5×10^5 mL^{-1}, in contrast to a cell concentration between 3.0×10^5 mL^{-1} to 5.0×10^5 mL^{-1} under serum-free conditions. The bioreactor cultures were performed using the conditions explained in section 2.5.4.

The BioNose was connected to the off-gas line of the bioreactor (section 2.15). The data were collected using the NST Senstool software. As explained in section 2.16.1, the response pattern was evaluated using multivariate methods, in which different PCA models were built using the response values. Sensors, which gave a noisy response, were avoided. The outlier's measurements were deleted. The most relevant sensors were selected using the loading plot and the most relevant principal components were found.

Data analyses were performed either with or without a scaling method in order to find which of them gave best results. Normalization scaling was done such that all the measurement vectors obtained a total length of one, giving all measurements equal importance. The idea behind the normalize measurement scaling is to extract information from the signal patterns rather than from the magnitudes of the signal.

The first experiments using the BioNose were performed under serum-containing conditions and the seconds, under serum-free conditions as specified below.

3.7.2.1 Transient transfection system in serum-containing medium

The pattern of the BioNose for different phases of the process have been characterized individually and in combination: Sterility test, addition of PEI to the culture medium, batch culture of HEK293s cells, addition of PEI to cultured HEK293s cells, transfection of cultured HEK293s cells by DNA:PEI complex, expression of the recombinant proteins by transfected HEK293s cells.

Sterile Test

The contributed sensors were evaluated with the loading plot (data not shown). Their response was constant during the entire sterile test, with values between 11 and 13 (arbitrary units) for MOSFET sensors (FE104 A and FE105A) and between 25 and 75 (arbitrary units) for MOS sensors (MO102, MO103, MO104, MO105 and MO106) (data not shown).

HEK293s batch culture

The bioreactor was inoculated with 3.0×10^5 mL^{-1} HEK293s cells cultured in DMEM - Ca^{2+} + Vit. + Pl. + 5 % FCS. The batch culture was stopped at a cell concentration of about 2.5×10^6 mL^{-1} with a viability above 98 % (Fig. 3.7.2.1a; A). At this point, glucose and lactate concentration in the culture supernatant were 1.78 g L^{-1} and 1.63 g L^{-1}, respectively (Fig. 3.7.2.1a; B).

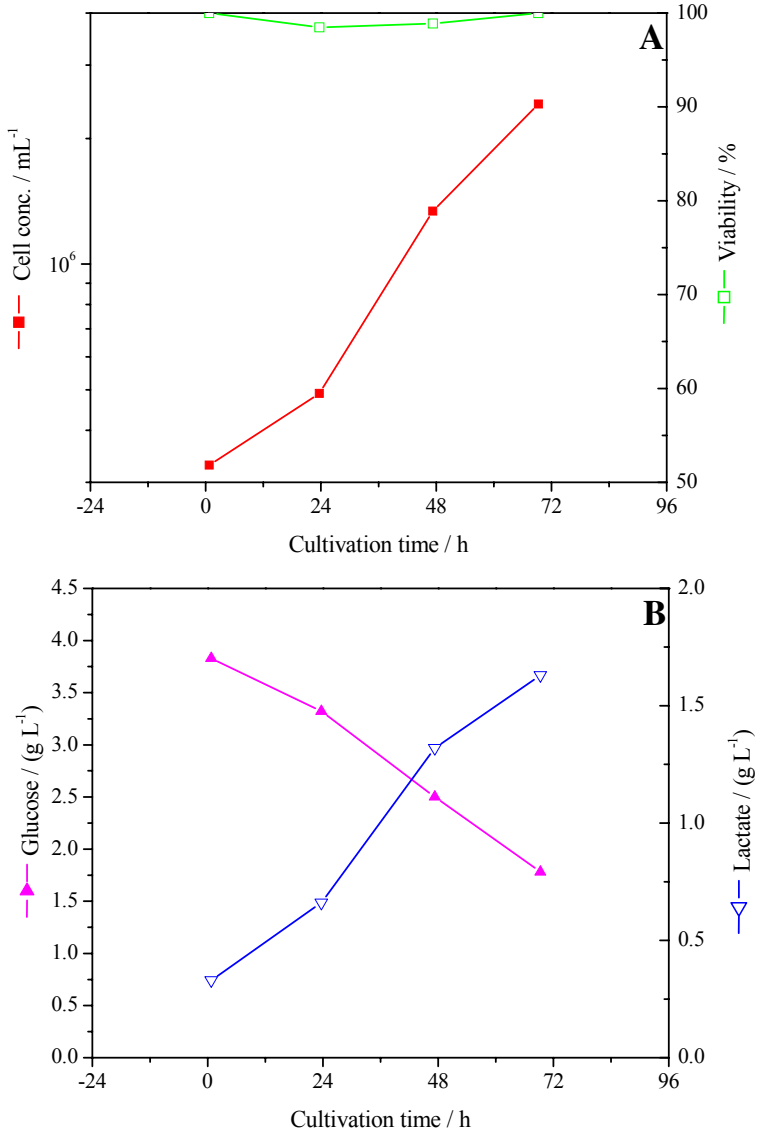


Fig. 3.7.2.1a: Batch culture of HEK293s cells in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium. The cell culture was performed in a bioreactor (1.4 L working volume) during 72 h. Cell concentration and viability (**A**), glucose and lactate concentration (**B**) were determined.

Analyzing the loading plot, three MOSFET sensor (FE 103 A, FE 103 B and FE 105 B) and the MOS sensors, MO 102, MO 104, MO 105 and MO 106 were contributing partly in the response of the cultivation of the batch culture (data not shown). A small decrease was observed during the growth phase of the cells whilst FE 104 A, FE 104 B and MO 103 presented a constant response during the culture period (data not shown). Figure 3.7.2.1b shows the normalized score plot characteristics from the batch culture. PC 1, PC 2, and PC 3 together explained 96.9 % of the signal variance (Fig. 3.7.2.1b).

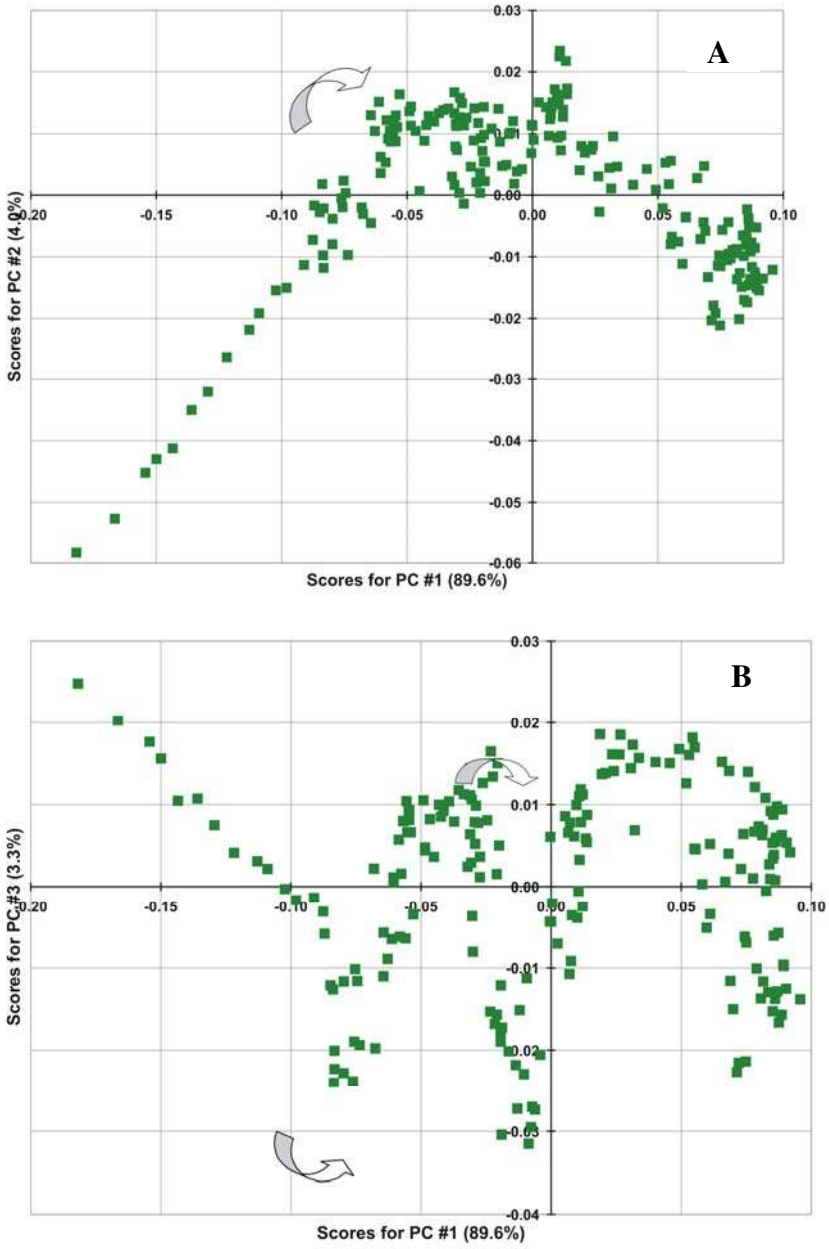


Fig. 3.7.2.1b: Score plot from the HEK293s culture batch in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS. PC1 versus PC2 (**A**) and PC1 versus PC3 (**B**). PC1, PC2, and PC3 together explained 96.9 % of the signal variance. The points in a plot represent the measurement of the electronic nose at a reduced interval of 20 min.

Addition of PEI to the cell culture

PEI dissolved in 50 mL of DMEM -Ca^{2+} + Vit. was added to the HEK293s cell culture when the cell concentration was between 5.0×10^5 $mL^{-1}$and 1.0×10^6 mL^{-1} with a viability of 100 % (Fig. 3.7.2.1c; A). The batch culture was performed during 125 h, showing at this time a glucose and lactate concentration of 1.13 g L^{-1} and 1.87 g L^{-1}, respectively (Fig. 3.7.2.1c; B).

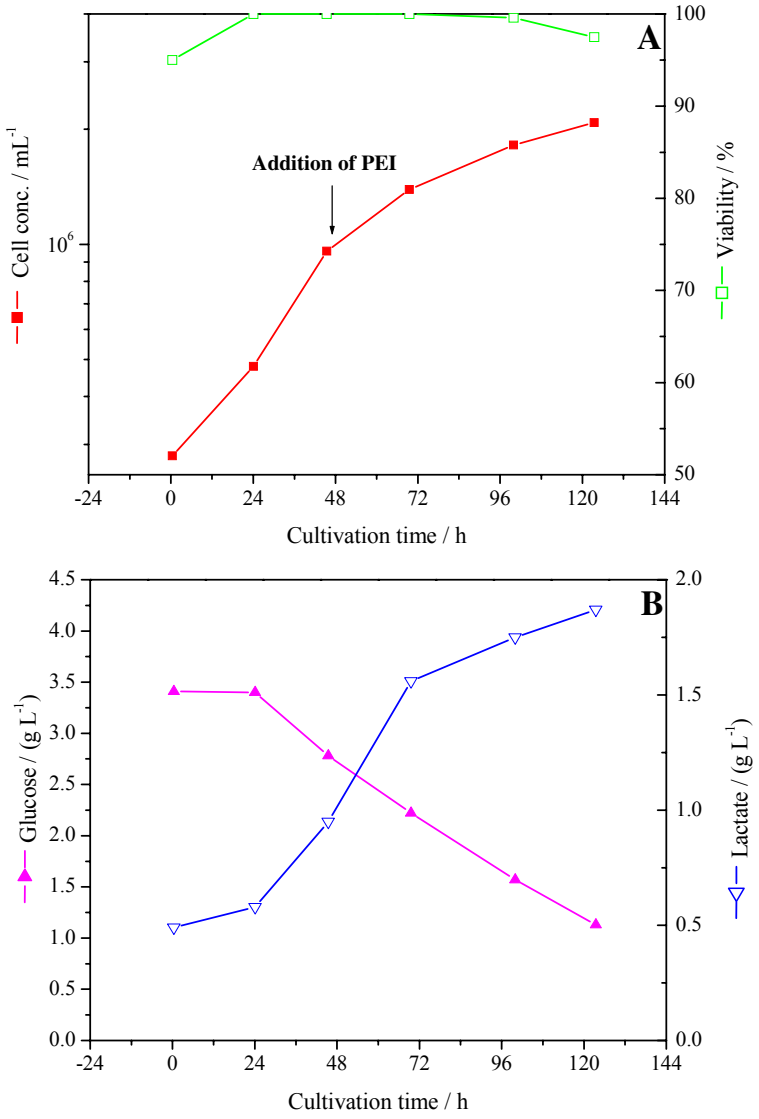


Fig. 3.7.2.1c: Addition of the PEI (↓) to the HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS (**A**). The cell culture was performed in a bioreactor (1.4 L working volume) during 125 h. Cell concentration and viability (**A**), glucose and lactate concentration (**B**) were determined.

The addition of PEI (Fig. 3.7.2.1d; A) resulted in a transient increase of the contributing MOSFET sensors, particularly FE104A, FE101B, and FE104B for 16 hours. MOS sensors gave a weak response after the addition of PEI (Fig. 3.7.2.1d; B). The score plot revealed a characteristic pattern, which differentiated the response signal after the addition of the PEI with a displacement of these signals over a period of 14 - 16 h (Fig. 3.7.2.1e). PC1, PC2, PC3 and PC4 explained 96 % of the variance. In figure 3.7.2.1e PC1 is plotted versus PC2 (A) and PC1 versus PC3 (B). Normalization scaling was performed.

This experiment was repeated and gave the same results as reported here.

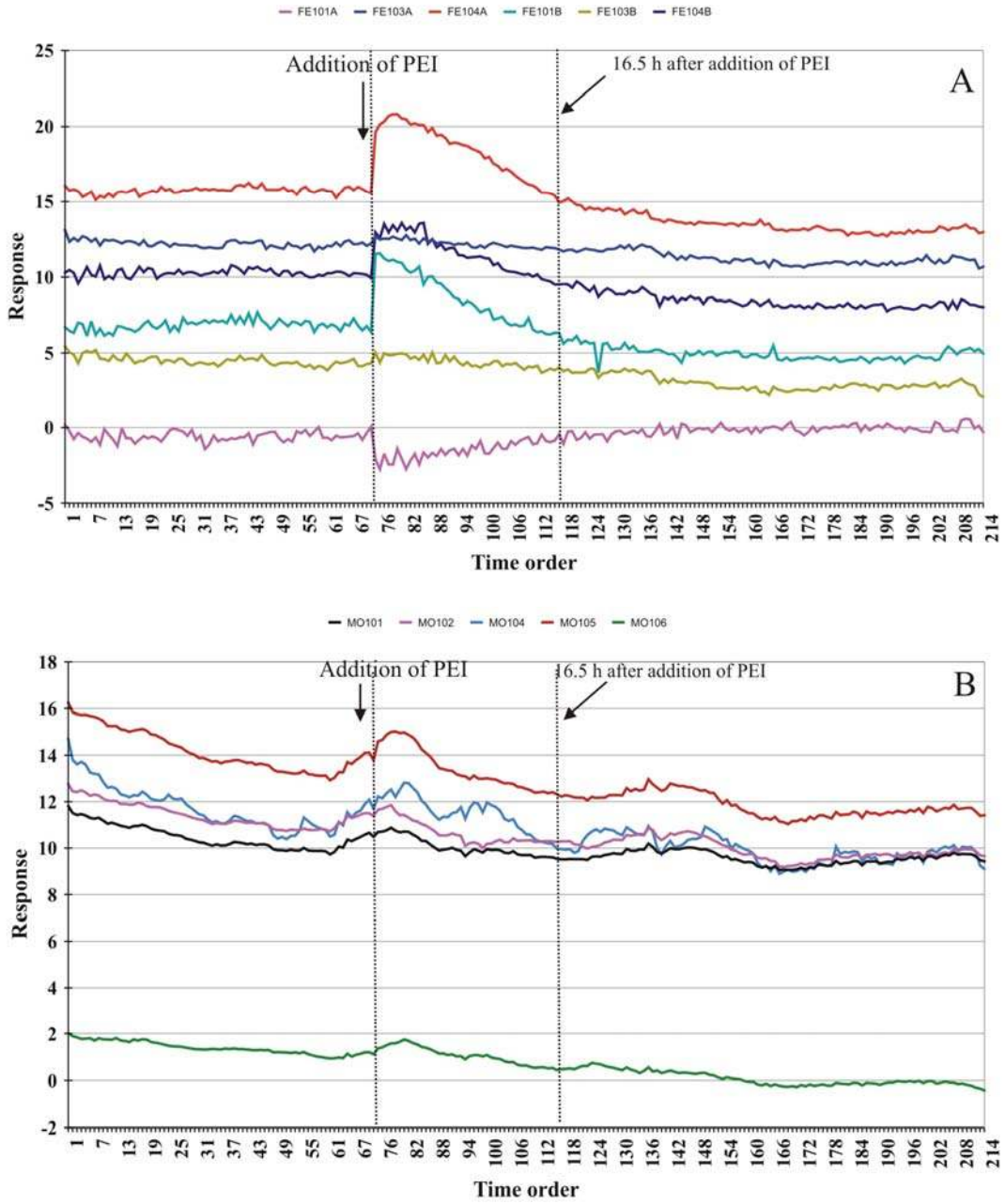


Fig. 3.7.2.1d: Addition of the PEI to the HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS. **A** shows the response of the contributed MOSFET sensors and in **B** of the MOS sensors. Sensor signals are given in arbitrary units. Time order represents every measurement of the electronic nose at a reduced interval of 20 min.

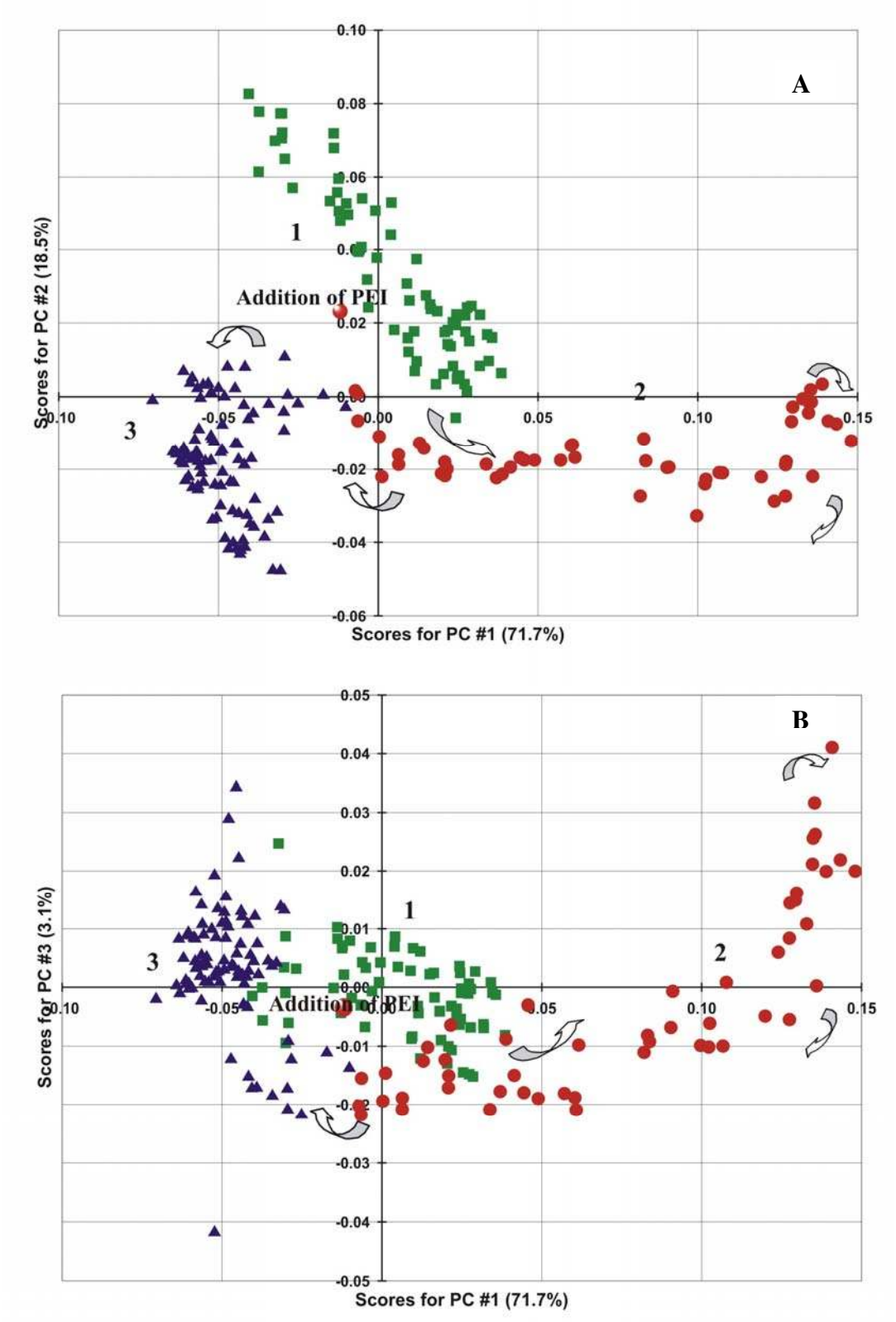


Fig. 3.7.2.1e: Score plot of the addition of the PEI to the HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS. PC1 versus PC2 (**A**) and PC1 versus PC3 (**B**). PC1, PC2, and PC3 together explained 93.3 % of the signal variance.(■): before addition of PEI, (●): uptake of PEI, (▲): end of batch culture. The points in the plot represent the measurements of the electronic nose at a reduced interval of 20 min. 1 to 3 show the trajectory of the signal response.

Transfection of HEK293 cells

HEK293s cells (passage no. 56) cultivated in DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS medium) were transfected following the protocol described under 2.18.1.3.

pEPO-IRES2-EGFP plasmid combined with PEI (1.0:1.5, µg:µg; respectively) were used for complex formation. The DNA:PEI complex was prepared in 50 mL of DMEM -Ca^{2+} + Vit. and added to the bioreactor through the inoculation tubing. This was washed with 20 mL of medium, immediately after inoculation. GFP was evaluated by flow cytometry every 12 hpt.

The cells were transfected at a concentration of 8.2×10^5 mL^{-1} with a viability above 99 % (Fig. 3.7.2.1f; A). The highest percentage of the GFP-positive cells (36.4 %) were reached at 56 hpt (111 h cultivation time), (Fig. 3.7.2.1f; A).

At 74 h of cultivation (20 hpt) cells aggregates (about 20 cells) occurred, which increased at the end of the batch culture (between 50 and 70 cells).

The glucose concentration was depleted at the end of the batch culture (210 h of the cultivation time; glucose conc.: 0.08 g L^{-1}) and the lactate concentration reached a maximum of 2.40 g L^{-1} (Fig. 3.7.2.1f; B).

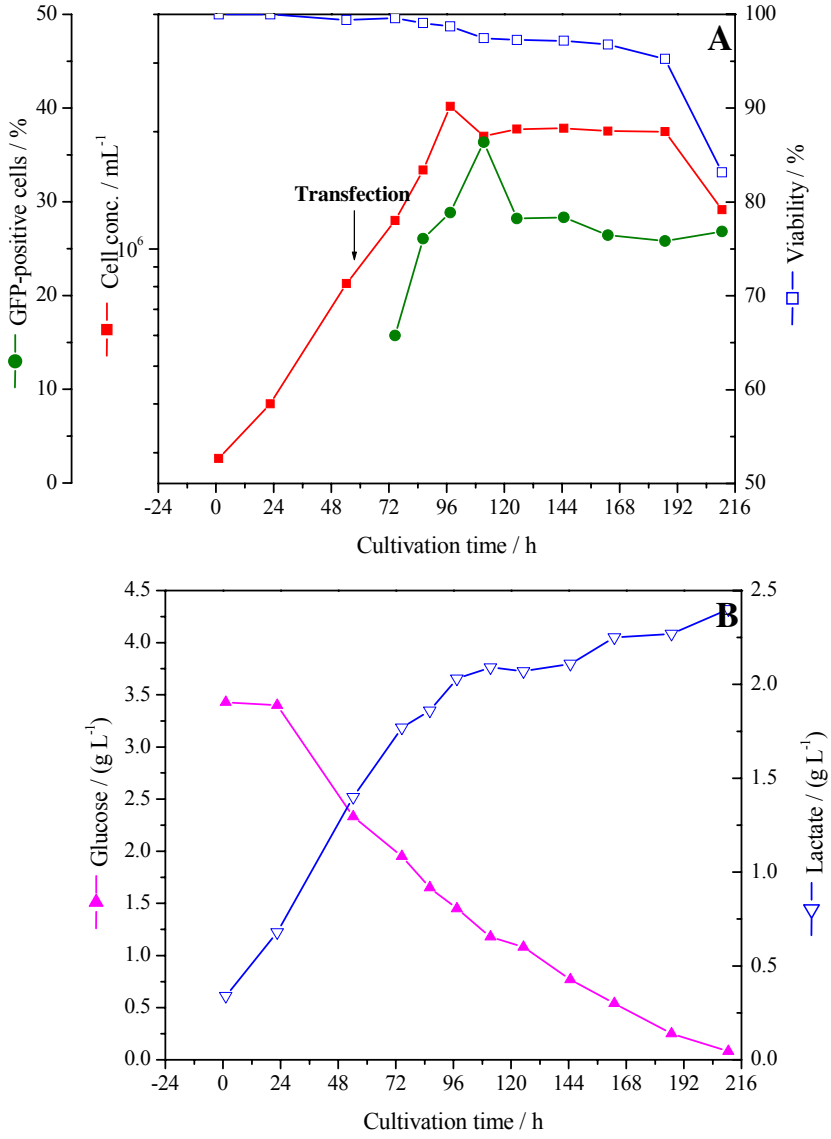


Fig. 3.7.2.1f: HEK293s cells cultivated under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS) were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 1.0:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium. GFP-positive cells was measured every 12 hpt (**A**). The cell culture was performed in a bioreactor (1.4 L working volume) during 216 h. Cell concentration and viability (**A**), glucose and lactate concentration (**B**) were determined.

The complete process was analyzed with the NST Senstool software. After transfecting, the MOSFET sensors (e.g. FE104A, FE104B) responded with a transient positive signal over a period of 25 - 27 h, which was longer, but similar, to the response of the sensors when only PEI was added (Fig. 3.7.2.1g; A). However, the MOSFET sensor, FE101B, which showed a suitable response pattern after the addition of PEI, did not show a signal, suggesting that it dropped out. In addition, MOS sensors also gave a characteristic weak signal after transfection of the cells (Fig. 3.7.2.1g; B). The normalized score plot revealed a characteristic pattern for the culture before and after transfection showing a clear shift of the data during the first 15 hpt, and from this time

until the end of the transfection culture (Fig. 3.7.2.1h). PC1, PC2, PC3, PC4, and PC5 contributed to 96.7 % of the variance (data not shown).

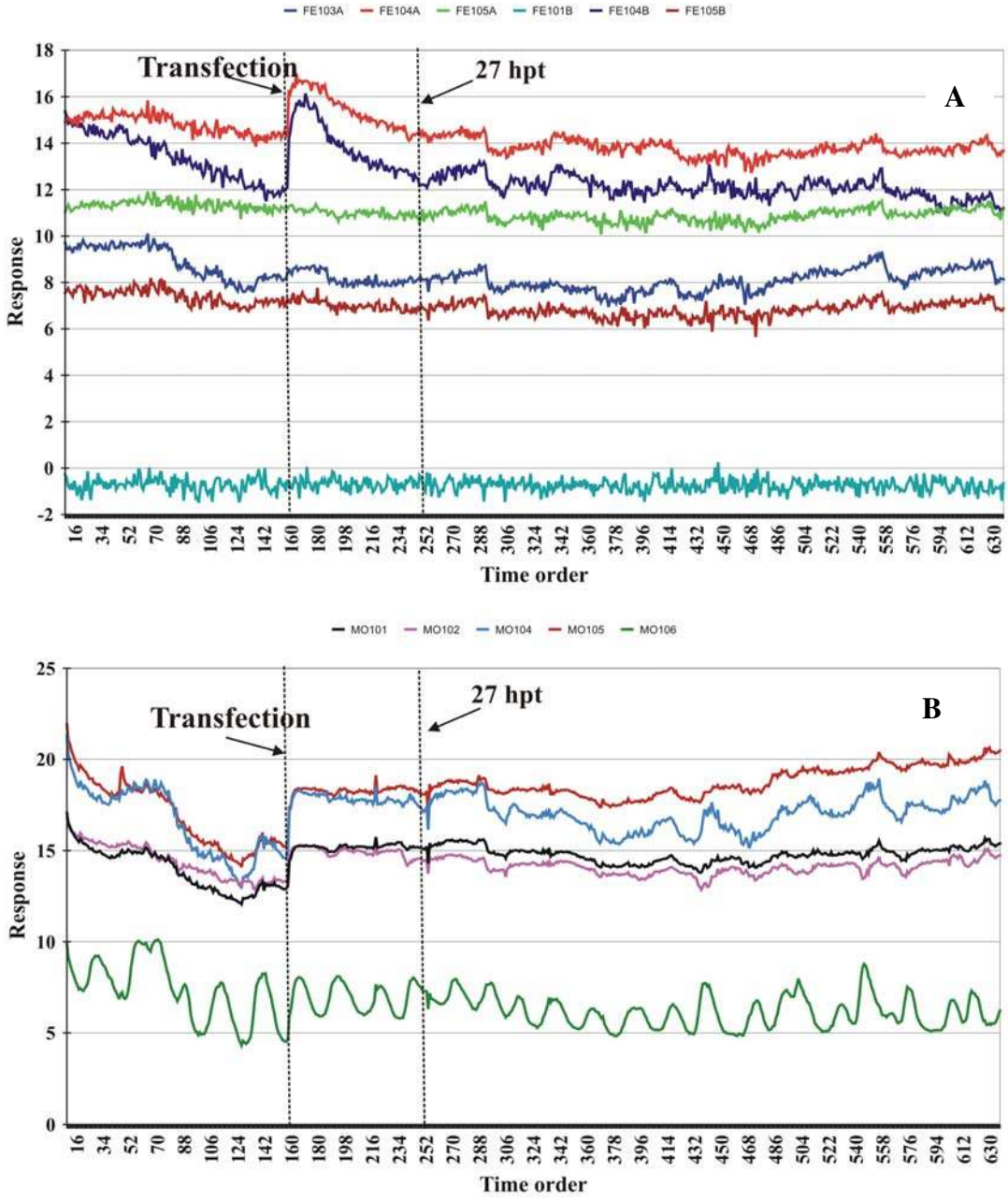


Fig. 3.7.2.1g: Transfection of the HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS. **A** shows the response of the contributing MOSFET sensors and in **B** of the respective MOS sensors. Sensor signals are given in arbitrary units. Time order represents every measurement of the electronic nose at a reduced interval of 20 min.

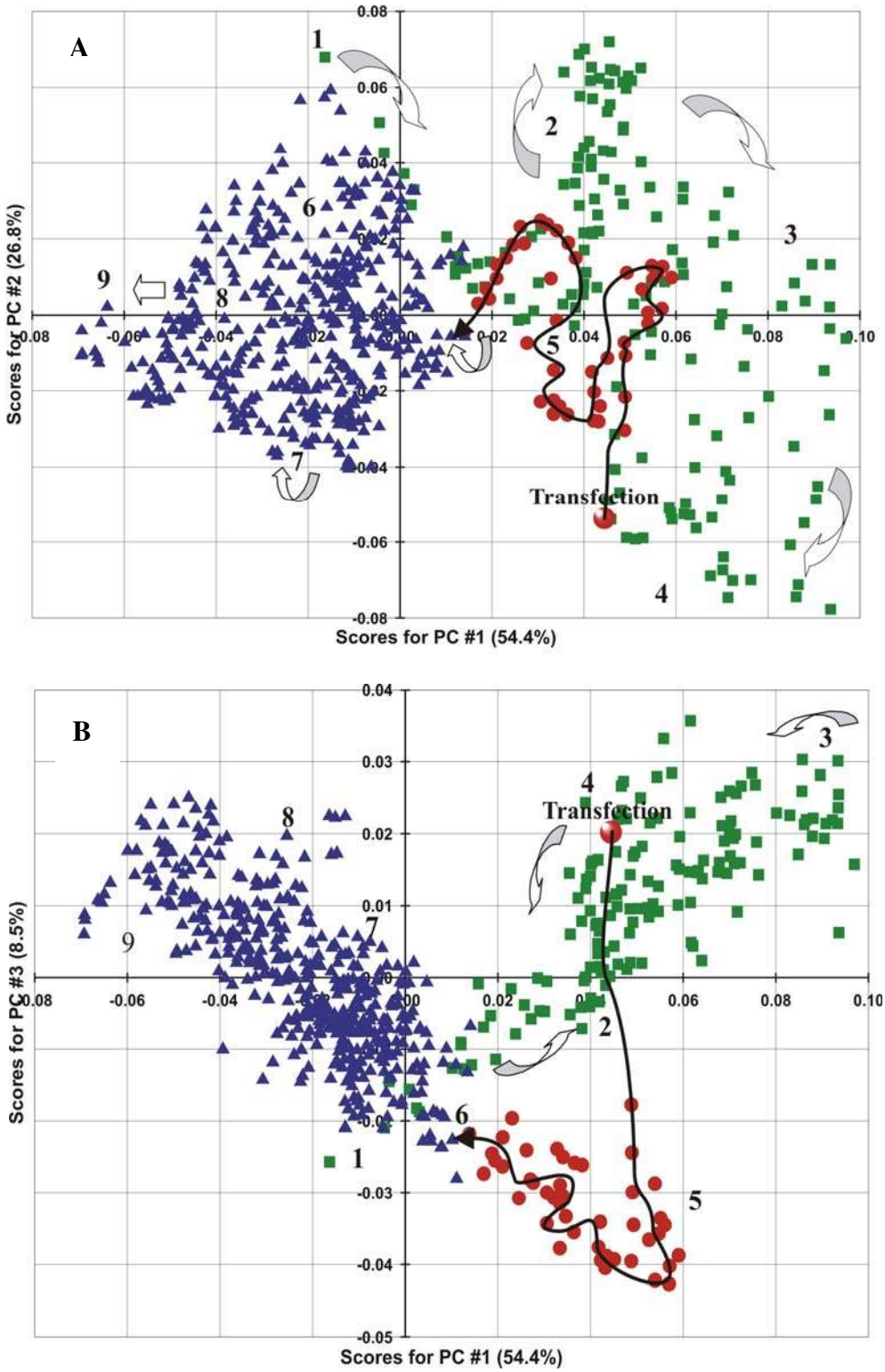


Fig. 3.7.2.1h: Score plot of the transfection process to the HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS. PC1 versus PC2 (**A**) and PC1 versus PC3 (**B**). PC1, PC2, and PC3 together contributed to 89.8 % of the signal variance. (■): before transfection, (●): 15 hours after transfection, (▲): 16 hpt until the end of the culture. The points in a plot represent the measurement of the electronic nose at a reduced interval of 20 min. 1 to 9 show the trajectory of the signal response from the beginning of the cultivation until the end of the procedure.

This experiment was repeated and gave the same results as presented above (data not shown).

A third transient transfection was performed under the conditions mentioned above with a small modification. Cells were stirred at 80 rpm instead of 40 rpm. GFP was measured at 24, 48 and 72 hpt.

The highest transfectability was 65 % between 48 and 72 hpt (Fig. 3.7.2.1i; A). The viability was above 95 % throughout the batch culture (Fig. 3.7.2.1i; A). The glucose concentration was depleted at the end of the culture (168 h of cultivation time) and the lactate concentration reached a concentration of 2.40 g L^{-1} (Fig. 3.7.2.1i; B).

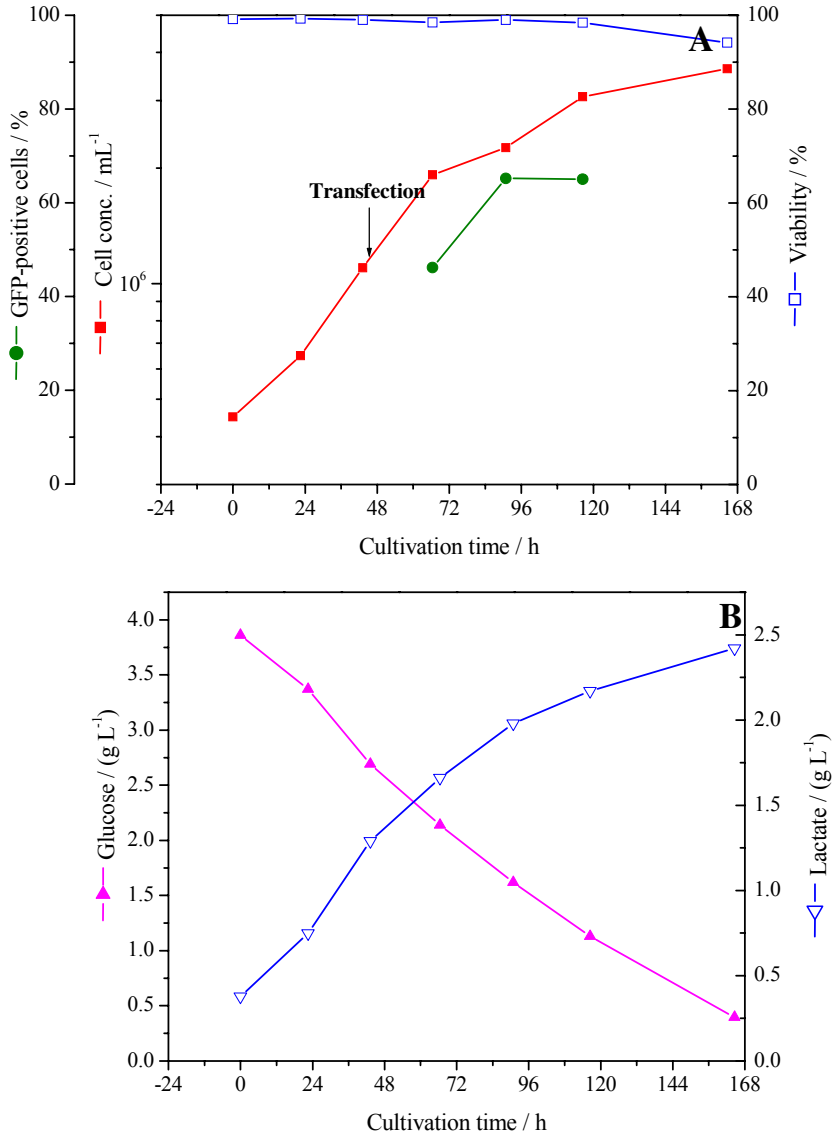


Fig. 3.7.2.1i: HEK293s cells cultivated under serum-containing conditions (DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS) were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 1.0:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium. GFP-positive cells was measured at 24, 48 and 72 hpt (**A**). The cell culture was performed in a bioreactor (1.4 L working volume) during 168 h at 80 rpm. Cell concentration and viability (**A**), glucose and lactate concentration (**B**) were determined.

Addition of PEI to the cell culture medium

In order to check, whether the BioNose response was actually due to the physiological uptake of the PEI with or without DNA or only an artificial signal caused by the presence of PEI in the medium, PEI alone was added to the cell culture medium without the presence of HEK293s cells in the bioreactor.

Figure 3.7.2.1j shows no response in the sensor signal when the PEI was added to the cell-free culture.

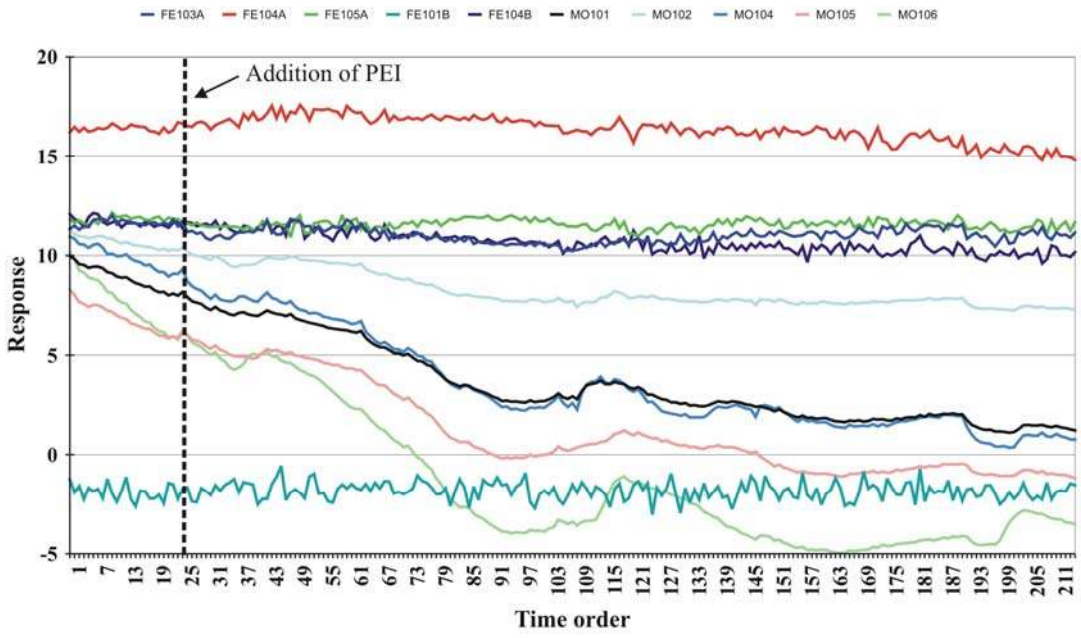


Fig. 3.7.2.1j: Response of MOSFET and MOS sensors to the addition of PEI to DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS medium. Sensor signals are given in arbitrary units. Time order represents every measurement of the electronic nose at a reduced interval of 20 min.

Figures 3.7.2.1k; A and 3.7.2.1k; B show the score plot after the addition of the PEI and the DNA:PEI, respectively to two different cultures. Both figures show the characteristic shift signal after the addition of either PEI or DNA:PEI.

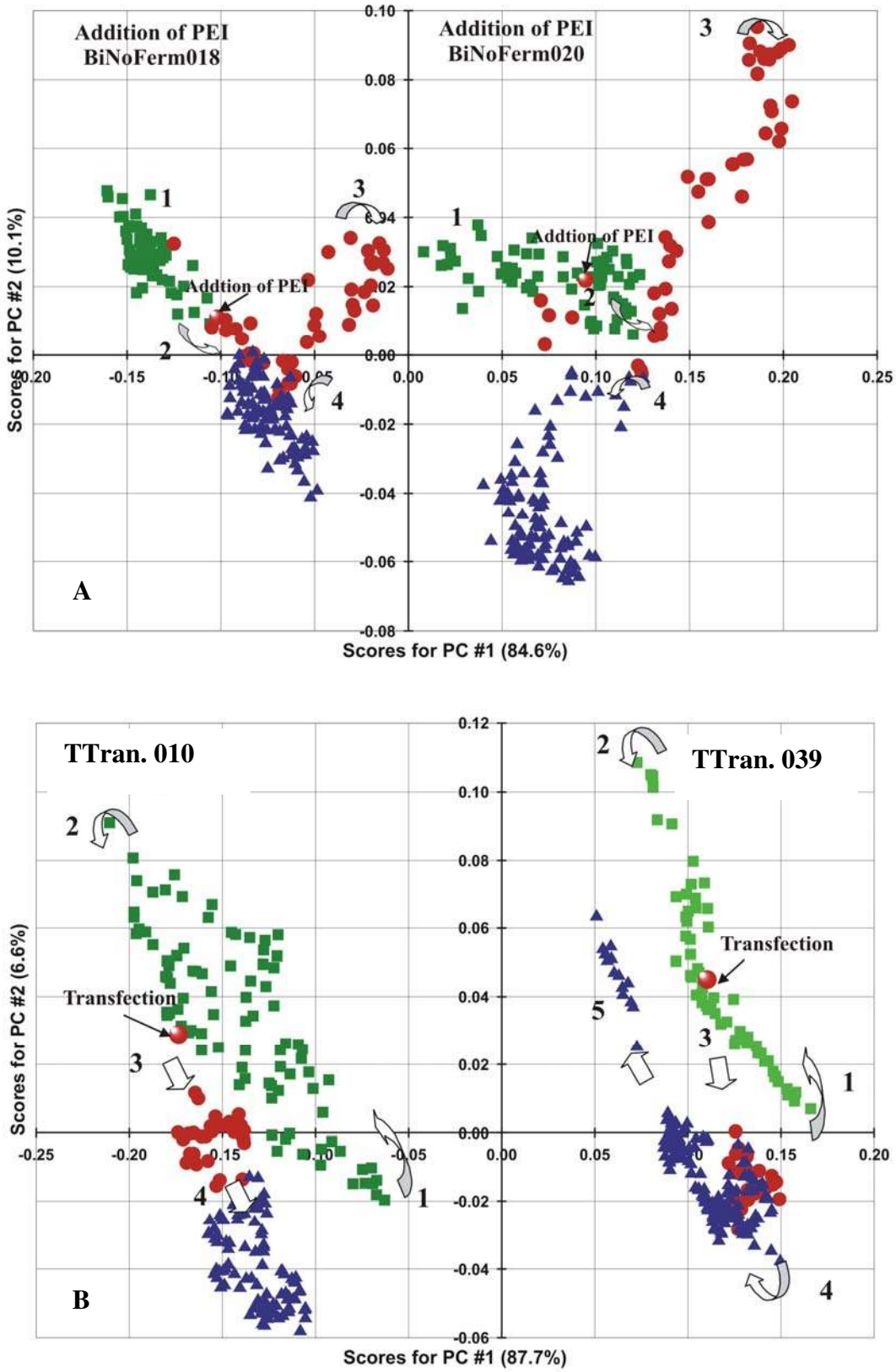


Fig. 3.7.2.1k: Score plot of the addition of the PEI (**A**) and DNA:PEI (**B**) to two different HEK293s cells cultured in DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS. (■): before addition of PEI or DNA:PEI, (●): between 10 to 15 hours after transfection, (▲): 11 to16 hpt until the end of the culture. 1 to 4/5 show the trajectory of the signal response. The points in a plot represent the measurement of the electronic nose at a reduced interval of 20 min.

3.7.2.2 Transient transfection under serum-free medium conditions

Two transient transfection experiments were performed in a 1.8 L bioreactor (1.6 L working volume) using the HEK293s cultivated in FS:DMEM + AA serum-free medium. These cultures were carried out under the same conditions as in serum-containing medium but using a higher stirring rate (70 rpm instead of 40 rpm) (2.5.4).

Sterile test

First, the sterile test with this serum-free medium was assessed with the BioNose during three days obtaining similar results as the sterile test in serum-containing medium. The response of the sensors was constant during the entire test although the sensor profile differed to the previous experiments. The contributing sensors were the MOSFET FE103 A, FE104 A and FE104B (FE104 A and FE105A, for serum-containing medium) with values between 13 and 17 (arbitrary units) and the MOS sensors MO101, MO102, MO104, MO105 and MO106 with values between 28 and 40 (arbitrary units) (MO102, MO103, MO104, MO105 and MO106 for serum-containing medium) (data not shown).

Transfection of HEK293s

After the sterile test, the bioreactor was inoculated with HEK293s cells at a concentration of 4.5×10^5 mL^{-1}. Once the cells reached a concentration between 7.0×10^5 mL^{-1} and 8.5×10^5 mL^{-1}, they were transfected using the DNA (pEPO-IRES2-EGFP):PEI ratio of 1.0:1.5 (µg:µg) prepared in 50 mL DMEM -Ca^{2+} + Vit. as explained for the transfection procedure under serum-containing conditions. Cell concentration, viability, glucose and lactate concentration, as well as intracellular nucleotides were determined as described in materials and methods section (2.10, 2.11 and 2.12). The GFP-positive cells were identified between 60 and 72 hpt.

The first experiment was performed with HEK293s cells from a passage number 70. They were transfected at a cell concentration of 7.55×10^5 mL^{-1} with a viability above 94 % (Fig. 3.7.2.2a; A). The transfectability was 55.5 % expressed as GFP-positive cells at 60 hpt.

The NTP/U ratio showed a value of 3.2 at the beginning of the culture, meaning cells in a poor physiological state. Before transfection, the ratio decreased to 2.7, but it was still higher than 2.3 (below to 2.3, indicated best physiological cell state). Then, the ratio further increased to a value of 11.75 without reaching a maximum throughout the cell culture (Fig. 3.7.2.2a; A).

The glucose concentration was maintained above 2.2 g L^{-1} at the end of the batch culture (240 h) and the lactate concentration reached a maximum of 1.5 g L^{-1} (Fig. 3.7.2.2a; B).

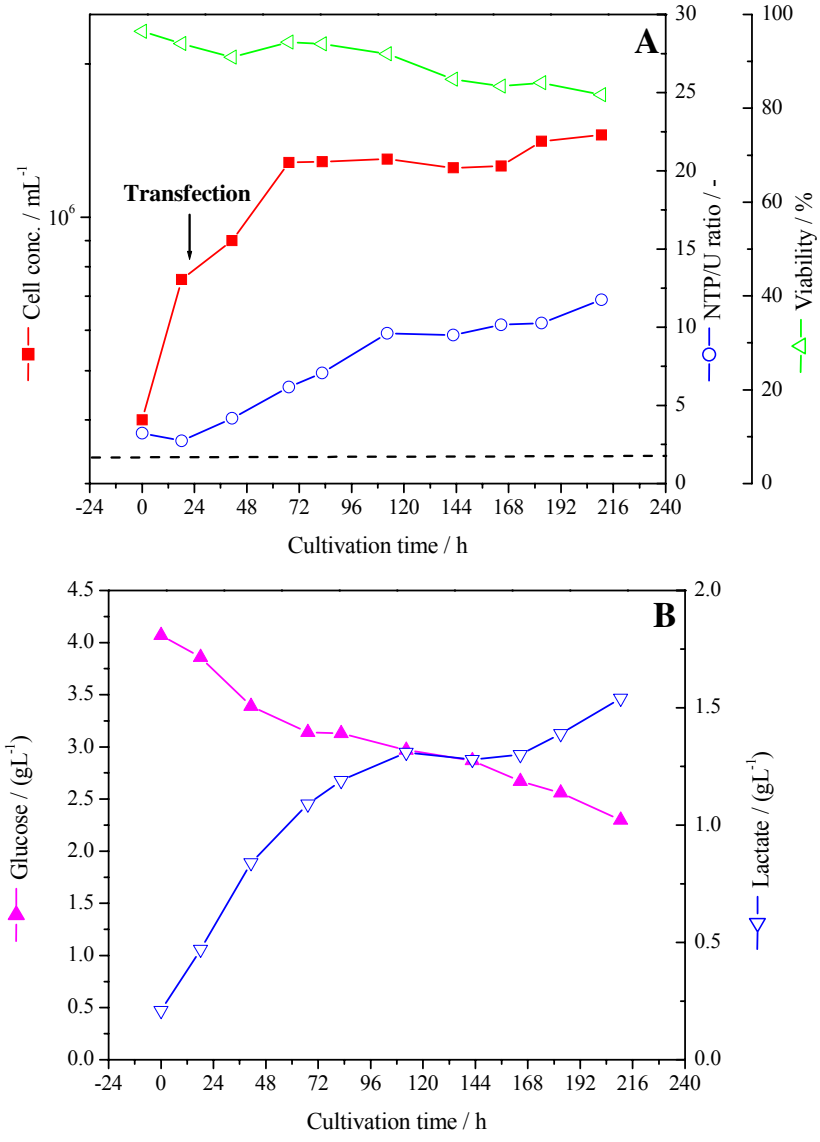


Fig. 3.7.2.2a: HEK293s cells were cultivated under serum-free conditions (FS:DMEM + AA) in a bioreactor (1.6 L working volume). The cells were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 1.0:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (**A**). Cell concentration, viability and NTPP/U ratio (**A**), as well as glucose and lactate concentration (**B**) were determined. The horizontal black dash line (**A**) shows the low values of the NTP/U ratio, indicating best physiological state.

The complete process was analyzed with the BioNose. Diagram A in figure 3.7.2.2b shows that mainly the MOSFET sensors FE104A and FE104B responded by a substantial increase when the bioreactor was inoculated. This response further increased when the transfection was performed over a period of 8 to 10 h. This result was similar

to that one obtained with FE104A and FE104B sensors under serum-containing conditions. MOS sensors presented a weak signal after the transfection procedure, especially MO104 and MO106 (Fig. 3.7.2.2b; B).

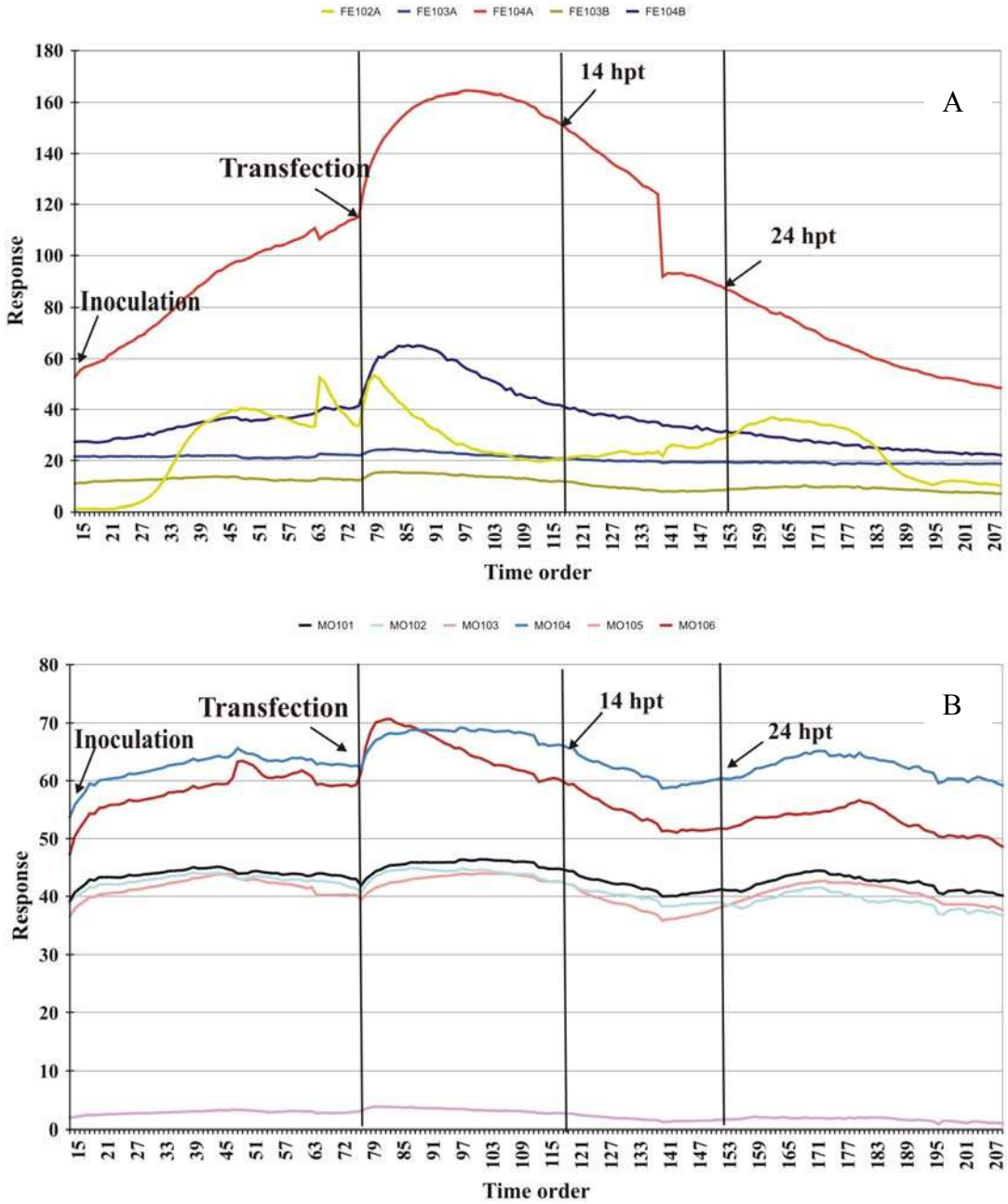


Fig. 3.7.2.2b: Transfection of the HEK293s cells cultured in FS:DMEM + AA serum-free medium. Diagram **A** shows the response of the contributing MOSFET sensors and diagram **B** of the respective MOS sensors. Sensor signals are given in arbitrary units. Time order represents every measurement of the electronic nose at a reduced interval of 20 min.

The normalized score plot of the transfection procedure (Fig. 3.7.2.2c) showed a clear shift of the data after the addition of the DNA:PEI complex. PC1 and PC2 contributed to 96.6 % of the variance response.

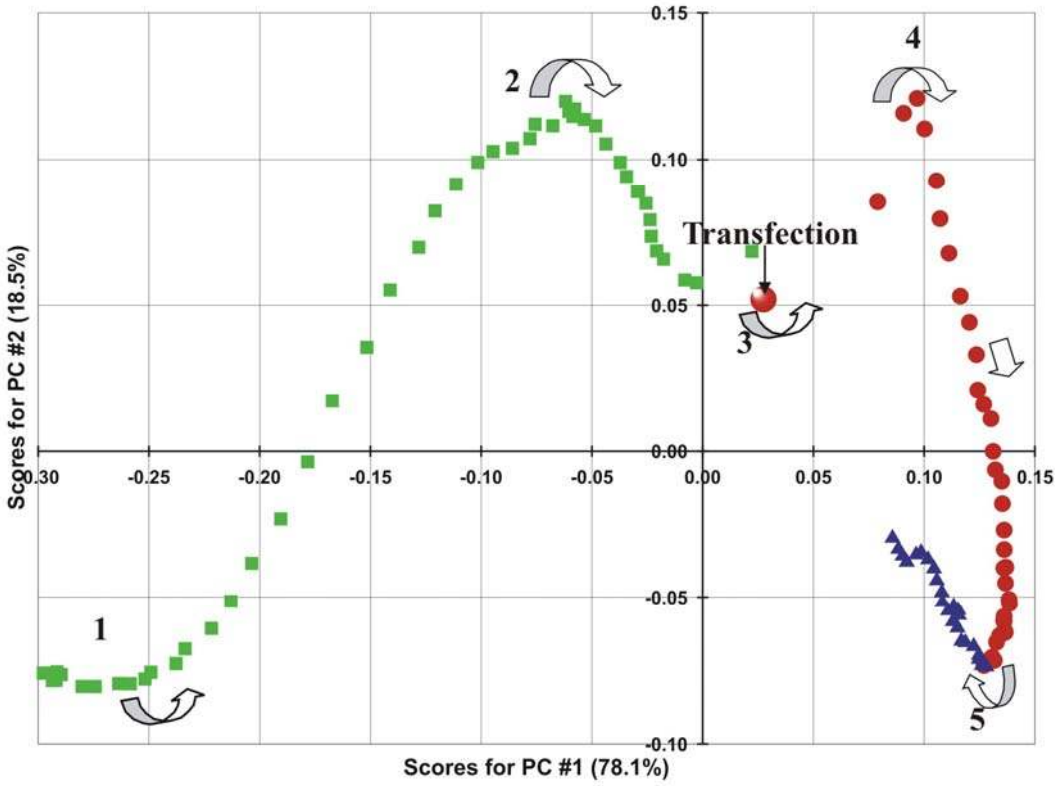


Fig. 3.7.2.2c: Score plot of the transfection process to the HEK293s cells cultured in FS:DMEM + AA serum-free medium.(■): before transfection, (●): 15 first hours after transfection, (▲): after 16 hpt. 1 to 5 show the trajectory of the signal response. PC1 versus PC2 contributed to 96.6 % of the signal variance. The points in a plot represent the measurement of the electronic nose at a reduced interval of 20 min.

A second transient transfection was performed using HEK293s cells (passage no. 89) in the 1.8 L bioreactor under the same conditions mentioned above. Cells were transfected at a cell concentration of 8.0 g L^{-1} with a viability above 92 % (Fig. 3.7.2.2d; A). The transfection efficiency was 44.5 % at 70 hpt.

The NTP/U ratio showed a value of 5.2 at the beginning of the culture, meaning cells in a poor physiological state. Before transfection, the ratio decreased to 2.3. After transfection, the ratio further increased showing a value of 250 at the end of the culture batch, indicating the starvation cell phase (Fig. 3.7.2.2d; A).

The glucose concentration was depleted at the end of the batch culture (216 h) and the lactate concentration reached a maximum of 2.4 g L^{-1} (Fig. 3.7.2.2d; B).

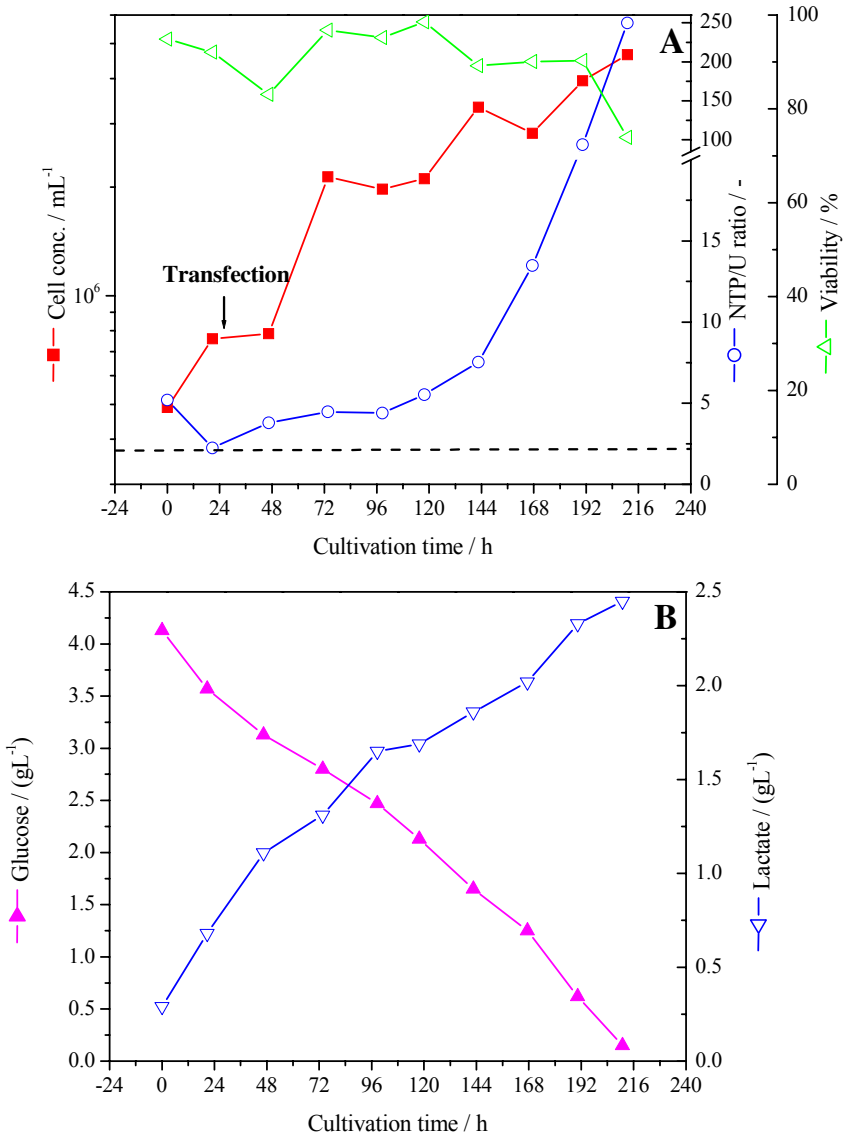


Fig. 3.7.2.2d: HEK293s cells were cultivated under serum-free conditions (FS:DMEM + AA) in a bioreactor (1.6 L working volume). The cells were transfected (↓) using a DNA (pEPO-IRES2-EGFP):PEI ratio of 0.5:1.5 (µg:µg), prepared in DMEM -Ca^{2+} + Vit. medium (**A**). Cell concentration, viability and NTPP/U ratio (**A**), as well as glucose and lactate concentration (**B**) were determined. The horizontal black dash line (**A**) shows the low values of the NTP/U ratio, indicating best physiological state.

Data from the BioNose of both transient transfection processes were compared. The BioNose analysis of the complete process showed similar patterns of the contributing sensors in both cultivations (data not shwon). MOSFET and MOS sensors showed the characteristic weak signal response after the transfection procedure (data not shown).

Diagrams A and B in figure 3.7.2.2e show the score plot of the complete transfection procedure from both cultivations (TTran. 040 and TTran. 044, respectively; TTran.: transient transfection). In the first one, the trajectory of the response data was clearer than in the second. It should be noticed, that during this last cultivation, the BioNose was shut down twice before adding the DNA:PEI complex and a third time 20

h after the transfection procedure, producing a distortion in the score plot pattern (Fig. 3.7.2.2e; B).

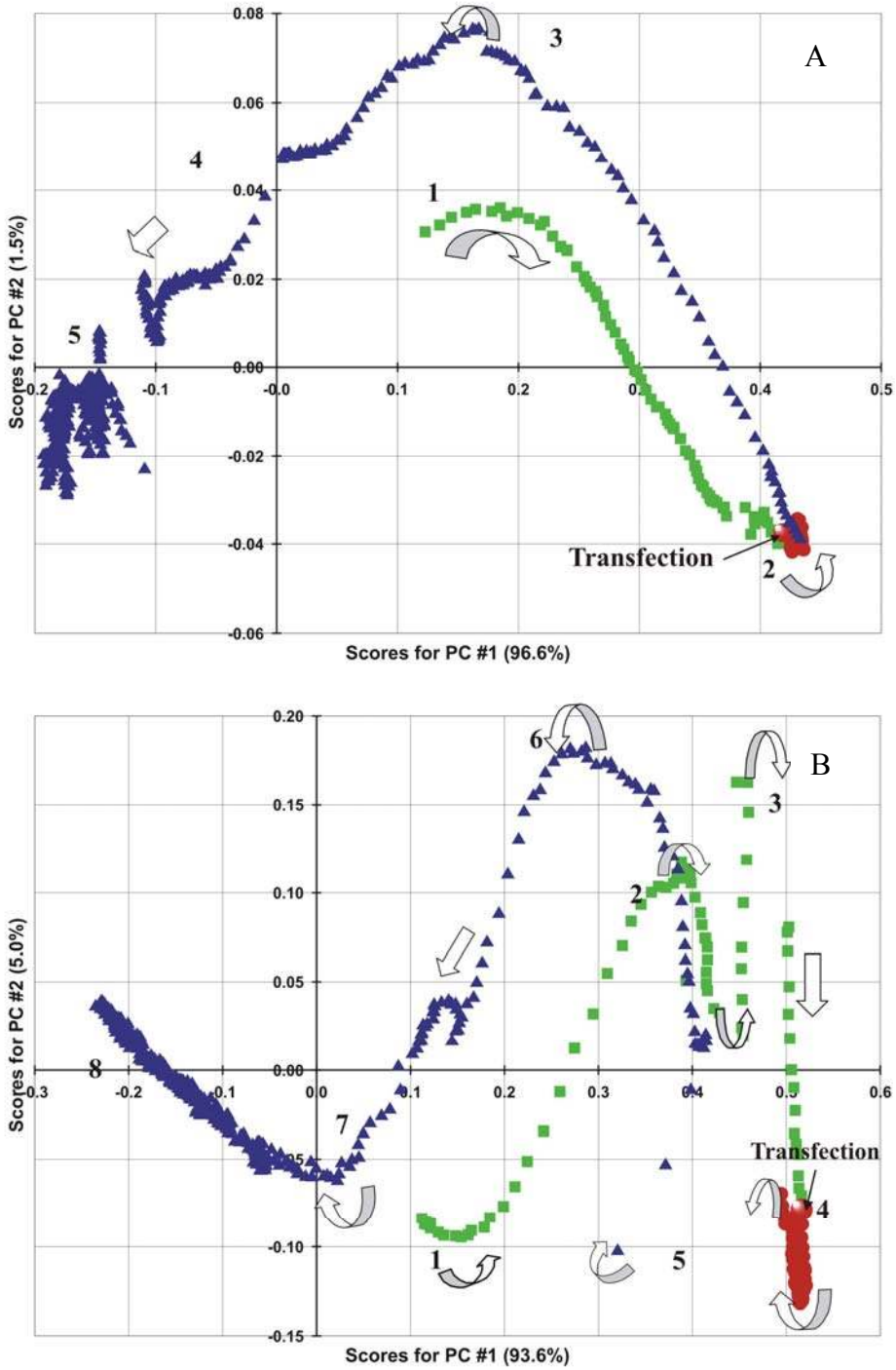


Fig. 3.7.2.2e: Score plot of the transient transfection procedure to two HEK293s cells bioreactor cultivations (TTran. 040 (**A**) and TTran. 044 (**B**); TTran.: transient transfection) using FS:DMEM + AA serum-free medium. (■): before addition of DNA:PEI, (●): between 10 to 15 hours after transfection, (▲): 11 - 16 hpt until the end of the culture. 1 to 5 in **A** and 1 to 8 in **B** show the trajectory of the signal response from the beginning of the cultivation until the end of the procedure. The points in a plot represent the measurement of the electronic nose at a reduced interval of 20 min.

Figure 3.7.2.2f shows the score plot of both transfection procedures in details.

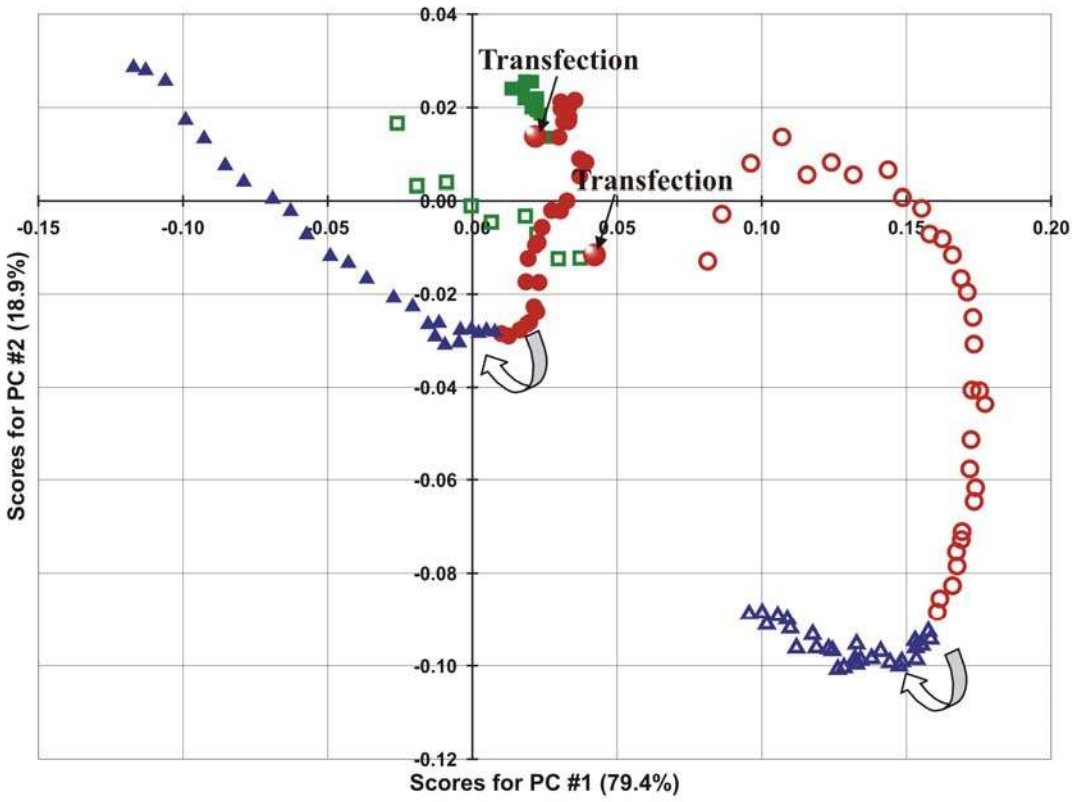


Fig. 3.7.2.2f: Score plot of the transfection procedure using the HEK293s cells cultured in FS:DMEM + AA serum-free medium. Green square symbols: 4 h before addition DNA:PEI, red circle symbols: between 10 to 15 hpt and blue triangle symbols: between 11-16 to 20-34 hpt. Filled symbols corresponded to the first transfection procedure (TTran. 040) and open symbols to the second one, TTran. 044. The points in a plot represent the measurement of the electronic nose at a reduced interval of 20 min.

These results showed, that the BioNose can generate characteristic pattern specific for a particular process. An electronic nose could be a tool to monitor cell cultivations as well as to distinguish between different culture conditions.

4. Discussion

As was mentioned in the introduction section (1.3.2), large transient transfection has numerous key features. In addition, with this procedure it has been demonstrated that large amounts of protein can be produced within a few days (Bernard 2000; Durocher et al. 2002; Girard et al. 2002; Jordan et al. 1998; Meissner et al. 2001; Pham et al. 2003; Pick et al. 2002; Schlaeger and Christensen 1999; Wright et al. 2003; Wurm and Bernard 1999; Wurm and Bernard 2001). This satisfies the increasing demand for milligrams of r-proteins to be used in pre-clinical, biochemical, and biophysical studies, showing the importance of this technology for biopharmaceutical manufacturing. In this context, the results presented here, contribute to science and technology development in this field.

4.1 Transient transfection

Different key aspects need to be taken into account for an optimal large-scale transient transfection and r-protein expression in mammalian cells (1.3.2), such as the cell line, the transfection vehicle, the expression vector and the culture medium. The cell line, HEK293 cells in suspension (HEK293s), and the transfection vehicle, PEI, had been chosen for their characteristics already described in the introduction section 1.3.2. The expression vector was evaluated and the culture medium was developed in this work, obtaining successful results, which will be discussed below.

The expression vector

The results presented in section 3.2 showed that the highest transfection efficiencies were reached using vectors bearing the CMV promoter. Therefore, new bicistronic vector were constructed using this promoter (section 3.3). These results elucidated, that CMV-vectors resulted in high-level expression of many different genes in a wide variety of cell types. They are considerably stronger than both, the SV40 promoter and the long terminal repeat of Rous sarcoma virus (Foecking and Hofstetter 1986). Although they marked out that the level of expression is cell-type dependent, it appears to form the ideal promoter for the expression in mammalian cells, with few, if any, known disadvantages (Brightwell et al. 1997). It was described (Boshart et al. 1985) that the strong activity of the CMV enhancer is due to the high concentration of binding sites of cellular transcription factors.

The culture medium

Numerous problems are associated with the presence of serum in culture, such as possible contamination by infectious microorganisms which may be present in the

serum, or by serum proteins, as well as the high cost, batch-to-batch variability and the difficulty in removing serum proteins when purifying the product of interest (Broad et al. 1991; Butler 1986). These concerns are heightened when the final product is to be used as an injectable therapeutic (Coté et al. 1998). Numerous studies have already dealt with serum-free medium development (Jayme et al. 1997) however, a universal cell adaptation protocol does not yet exist. Therefore, different adaptation protocols were performed with the HEK293s cells at the beginning of this thesis work. Furthermore, the literature often oversimplifies the development process of a serum-free medium. Although certain lines can be adapted spontaneously from serum-containing medium to serum-free medium, others need a sophisticated mixture of adaptation and screening steps to be weaned from serum-containing medium (Coté et al. 1998; Hesse and Wagner 2000; Sinacore et al. 2000).

Several commercial serum-free media were developed to support the growth of HEK293 cells. Normally, the manufactures recommend to perform a particular protocol for their adaptation to these specific media, but it is not always possible to have a successful outcome. As shown in results section 3.1 and 3.5, the growth of HEK293s cells as single cells or small clumps (less than 20 cells) with doubling times ranging from 30 to 36 h, was possible only with some of these commercial media (293 SFM II, Ex-Cell 293™ medium and Ex-Cell 293™ modified; see section 2.4.1 for media details) and in combinations of some of them (Ex-Cell 293™ modified plus DMEM -Ca^{2+} + Vit. and FreeStyle™ 293 expression medium plus DMEM -Ca^{2+} + Vit.). These results verified, that no universal method has been devised to address the serum-free development process.

Following the first aim of this work, further experiments were performed to find a medium totally free of animal proteins or animal-origin components, in which not only HEK293s cells were fully adapted, even more facilitate the transient transfection processes. This had implicated to avoid the washing step of cells prior to the addition of the transfection complex and the changing of the medium after the transfection procedure.

Different groups had reported a serum-free formulation to support growth of HEK293 cells (Berg et al. 1993; Coté et al. 1998; Durocher et al. 2002; Schlaeger and Christensen 1999). However, these media like the commercial ones did not satisfy all the prerequisites mentioned above. For example, Schlaeger and Christensen (1990) had developed a serum-free medium in which heparin was added to avoid cell aggregation. As heparin inhibits DNA uptake in transfection procedure, it has to be removed from the medium prior to performing this procedure. Consequently, cells have to be washed with a heparin-free medium previous to the addition of the transfection complex.

On the other hand, Invitrogen™, life technologies (CA, USA), had developed an expression system, using the FreeStyle™293-F cell line, derived from the HEK293 cell line (FreeStyle™ 293 Expression System; For large-scale transfection of suspension 293 cells in a defined, serum-free medium; Catalogue no. K9000-01). In this system, cells growing under serum-free conditions were transfected, but the transfection complex was prepared in a medium (Opti-MEM® I, see section 2.4.1), which contains less than 1 % of FCS. Hence, the system is not completely serum-free.

In addition, Durocher et al. (2002) had cultivated the HEK293s cells in a serum-free medium, but they marked out the requirement of 1 % bovine calf serum (BCS) medium supplementation to improve transient transfection mediated by PEI. These authors had shown that the presence of serum increases gene delivery and/or transgene expression through one mechanism that remains unclear. They used expression vectors containing the CMV promoter and they suggested that serum might contribute to augment transcriptional activity of this promoter as the CMV immediate early enhancer contains multiple binding sites for serum-activated transcription factors (Brightwell et al. 1997). However, they obtained only a partial recovery of transgene expression when serum was added to the cells 3 h after their transfection in serum-free medium. This suggested that, in addition to the potential serum-mediated CMV promoter transcriptional activation, other serum component(s) might increase transfection efficacy of DNA:PEI complexes (Durocher et al. 2002). However, the results presented here (Section 3.5.3, Table 3.5.3a) were contradictory with those showed by Durocher et al. (2002) as are discussed below.

In order to investigate whether the presence of serum improves the transfectability, 1 % FCS was added to the different serum-free cultures and transfection efficiencies lower than 1.5 % were obtained (Table 3.5.3a). These results suggested that the low transfectability was due to an inhibition by component(s) of the serum-free medium rather than a missing of unknown important component(s) of the FCS. This was verified when 60 % higher transfection efficiencies were obtained using the HEK293s cells cultivated in a mixture 1:1 of the commercially FreeStyle™ 293 expression medium with DMEM -Ca^{2+} + Vit. medium than using FreeStyle™ 293 expression medium alone (see Fig. 3.5.3a). Further investigations should be done to identify the possible inhibitory component(s) in order to better understand the serum mechanism in transfection mediated by PEI.

Moreover, transfection efficiencies of 60 – 70 % were reached using cultures with 24 h conditioned medium, showing that this does not affect the transfection procedure, as was postulated by Schlaeger and Christensen (1999). This therefore simplifies

process scale-up. These last results were in agreement with those presented by Durocher et al. (2002).

Further results in section 3.6 showed that HEK293s were successfully adapted to this new serum-free medium (3.6.1).

In order to obtain highest transfection efficiencies using lesser amount of DNA, an optimum DNA:PEI ratio of 0.5:1.5 (µg:µg) was found (3.6.2).

Under these conditions several transfections were performed with cells from an early and a late passage number using pEPO-IRES2-EGFP and pCMV-EGFP-EPO plasmids. GFP-positive cells and the rhuEPO concentration present in the cell supernatant were determined between 60 and 72 hpt (table 3.7a).

The results showed lowest transfection efficiencies with the first plasmid, which contains a downstream EGFP gene (section 3.3.1). Several authors had shown that, in a bicistronic vector, the IRES-dependent second gene expression is significantly lower than the cap-dependent first gene expression (Dirks et al. 1993; Hennecke et al. 2001; Mizuguchi et al. 2000). Hence, this could be one of the reasons for the differences in the GFP expression found between both plasmids. Moreover, the nature of the IRES element differs from one plasmid to the other. pEPO-IRES2-EGFP contains the IRES site of the ECMV and pCMV-EGFP-EPO that of the poliovirus (Fig. 3.3.1a and Fig. 3.3.2a). The strength of the internal translation initiation is defined by the nature of the IRES element and the cellular context (Hennecke et al. 2001).Therefore different levels of gene expression could be expected.

On the other hand, the results showed that cells from an early passage number presented higher transfection efficiencies expressed as GFP-positive cells compared to their older counterparts from a late passage number (Table 3.7a). Even more, the expression of the recombinant model gene rhuEPO showed the same tendency. Due to the complete protein-free medium conditions used, EPO represented between 8 to 10 % of the total protein concentration when cells from an early passage number were used, and between 2.5 and 3.5 % with cells from a late passage number. This is an additional advantage of the chemically defined process for the purification of the protein of interest. This can be explained when compared to the transfection performed under serum-containing conditions, in which EPO represented 0.02 % of the total protein concentration (Table 3.7b). Summarizing serum-free conditions have shown an efficient productivity, especially using HEK293s cells from an early passage number.

The concentration of the rhuEPO under serum-containing condition was higher than in serum-free medium. One reason for this difference could be the release of proteases into the medium. This process happens during the proliferation phase of the cells as well as after their death and following cell lysis. Medium supplemented with serum inhibits

proteases, as inhibitors make up 10 % of the total protein content of serum. Therefore, in cultures without serum the proteases can have a negative influence upon cell proliferation as well as in the desired synthesized product (Murakami 1989; Waldemar et al. 1991).

With the results presented here, a new approach in transient transfection system was developed, using HEK293s cells cultivated in a medium totally free of animal proteins or animal-origin components. Indeed, this is a unique method using this cell line in this medium condition (European patent application, Patent no. 03010206.5-2405). Recently, Pham et al. (2003) had published the development of a new serum-free medium, but with the addition of peptones, that sustains cell growth and PEI-mediated transfection of a HEK293/EBNA 1 cell line.

Following the second aim of this work, a process characterization towards the further optimization of the transient transfection procedure was performed. It was the first time that the use of an off-line and invasive method as intracellular nucleotide analysis, and the on-line and non-invasive method, the electronic nose, were used to characterize the transient transfection procedure using HEK293s cells. The results obtained have shown that both methods are suitable and even excellent tools for process optimization as is discussed below.

4.2 Intracellular nucleotide analysis

Ryll and Wagner (1991 and 1992) demonstrated the application and usefulness of the analysis of special intracellular nucleotides for the cultivation of animal cells. Nucleotides were found to be a good target as they play a central role in metabolism. Especially, the specific combination of these parameters resulted in growth specific functions, such as the NTP, U, and NTP/U ratio. Several works (Grammatikos et al. 1999; Grammatikos et al. 1998; Moore et al. 1997; Ryll et al. 1991a; Ryll et al. 1991b; Ryll et al. 1994; Schoenherr et al. 2000) verified the sensitivity of these ratios, and extended its application for monitoring, control, and regulation of bioprocess. Particularly, the sensitivity of the NTP/U ratio and its ability to report on the physiologic status and growth potential made a tool to predict the growth behavior of a culture, as was tested for BHK, hybridoma and CHO cells. In the present work, these results were further extended to HEK293s cells.

The NTP/U ratio

The NTP/U ratio amplifies the NTP and U ratio by combining their values. For all cultivations presented here (3.7.1) and for all batch cultures studied (> 25), this ratio has been shown to be a very sensitive parameter, which reported earlier in studies on the cell physiological status and the growth potential. Moreover, other investigators showed, that this parameter allowed the prediction of the behavior of cells in culture up to 2 days before any changes were noted by classic cell number and viability measurements (see 3.7.1) (Grammatikos et al. 1999; Ryll and Wagner 1992; Schoenherr et al. 2000). In this work it was demonstrated that nucleotide ratios could also be used to distinguish between HEK293s cells from early and late passage numbers (3.7.1.1 and 3.7.1.2) indicating a change in the cellular character during aging of these cells. Moreover, nucleotide ratios of transfected HEK293s clearly differed from those in untransfected cells and also differed in cells cultured under serum-free and protein-free medium conditions compared to the serum-containing media (3.7.1.3).

Grammatikos et al. (1999) had shown that the absolute value of the NTP/U ratio for cells in good physiological state varies with cell type, medium and culture mode. This could be verified with HEK293s cells cultivated under different medium conditions. 15 batch cultivations using serum-free medium and 11 cultures using serum-containing medium were performed in this work analyzing the NTP/U ratio. In the first studies, a value of 1.0 to 2.3 and in the second ones, 1.0 to 3.3, indicated a good physiological state of cells in the exponential growth phase and best growth potential. A 50 % to 100 % increase was considered as a worsening of the cells conditions and a poorer growth potential suggesting the beginning of the reduced exponential growth phase and later, the stationary phase.

Early vs. late passage number

As was shown in the results section (3.7.1.1), HEK293s cells cultivated either in serum-containing or in serum-free media showed a different behavior depending whether they were taken from an early (40 to 77) or a late (89 to 150) passage number.

Cells from an early passage number growing under serum-containing conditions, reached the maximum of the NTP/U ratio first (Fig. 3.7.1.1a; A). In contrast, under serum-free conditions, cells from a late passage number were the first to reach this ratio (Fig. 3.7.1.1b; A). These maximum NTP/U values were a result of the opposing values of the NTP and the U ratio, which have reached the maximum and minimum respectively (data not shown). As was demonstrated earlier (Grammatikos et al. 1999; Ryll and Wagner 1992) and confirmed for HEK293 cells here, the NTP ratio increased during progress of the culture, as a result of an appropriate decrease in the cellular UTP and the CTP pool when cells entered the reduced exponential phase. In parallel the U

ratio decreased when the culture conditions worsened mainly due to a decrease of the UTP pool and an increase of the respective UDP-GNAc pool (the explanation of this increasing can be found in a later paragraph of the discussion section).

These worse conditions and the growth reduction indicated that a limitation of one or more essential nutrient compounds had started. During progress of all batch cultures performed in this work an elevated NTP/U ratio could be measured (figures section 3.7.1). Under serum-free conditions, the maximum NTP/U ratio was reached later for cells from an early passage number compared to cells from a late passage number (Fig. 3.7.1.1b; A). This indicated that cells from late passages reached the stationary and/or the starvation phase earlier due to a better adaptation to the culture conditions.

Early vs. late passage number – Transfected cells

The highest transfection efficiencies, expressed as GFP-positive cells, were reached with cells that showed the maximum NTP/U ratio in a late phase of the culture (Table 3.7.1.2a and Fig. 3.7.1.2a; A for serum-containing conditions and, Table 3.7.1.2c and Fig. 3.7.1.2b; A for serum-free conditions). For cultures using serum-containing conditions, transfected HEK293 cells from late passage numbers reached higher transfection efficiencies than cells from early passage numbers (82.07 % compared with 71.30 %). The inverse behavior was found for the same cells cultured under serum-free conditions (cells from an early passage number 73.22 %, compared with 55.62 % from cells of late passage number). This tendency was observed in all the transfections performed (see Table 3.7a and Table 3.7b).

Highest transfection efficiencies were obtained when a low cellular UDP-GNAc content was measured during the complete batch culture (data not shown). In serum-free conditions, transfected cells from an early passage number showed a low percentage of UDP-GNAc (9 to 11 %) during the entire culture batch, whilst cells from a late passage number, presented values from 10 %, at the beginning of the culture, to 35 % at the end, at 168 h. In contrast, cells cultured under serum-containing medium, showed the lowest percentage of UDP-activated sugars for cells from a late passage number (from 11 % to 40 %, at the end of the batch culture), whilst transfected cells from an early passage number showed values from 14 % to 55 % at the same time.

Previous work considered the intracellular UDP-GNAc content as a key regulator of cell viability and productivity (Barnabé and Butler 1998; Grammatikos et al. 1998; Ryll et al. 1994). The increase of the UDP-GNAc pool can be caused by an elevated synthesis or reduced use. The activated N-acetylhexosamines are predominantly used for the formation of sugar side chains in glycoproteins and glycolipids (gangliosides). Glycoproteins are essential elements of the membranes or secreted molecules, which function as messengers within the macroorganims. Ryll et al. (1994) suggested that an

elevated intracellular UDP-GNAc pool is a mediator of the loss of cell viability induced in cultures by the accumulation or addition of ammonia. Others work groups identified ammonia as one of the responsible molecules increasing the UDP-GNAc pool (Gawlitzek et al. 1998; Grammatikos et al. 1998; Yang and Butler 2002). Indeed, the accumulation of ammonia is well known to inhibit cell growth and to affect protein production, thus perturb the glycosylation process (Butler and Spier 1984; Yang and Butler 2000; Yang and Butler 2002). Ammonia (as NH_3 or NH_4^+) is produced by the thermal degradation or intracellular deamination of glutamine, which is one of the major nutrient sources in cell culture medium (Doyle and Butler 1990; Tritsch and Moore 1962). Nevertheless, although ammonia has not been measured in this work, a high concentration at the end of the batch culture can be assumed.

Moreover, it was proposed that if the UDP-GNAc pool is kept at low levels during a production process, it is conceivable that cell growth is improved and uniform product quality is maintained (Grammatikos et al. 1998). In this context, it was shown here, that cultures under the influence of a low percentage of UDP-GNAc responded with higher transfection efficiencies.

Transfected vs. untransfected cells

HEK293s cells transfected in either serum-containing or serum-free media showed an increase of more than 50 % in the NTP/U ratio 24 h after the addition of the transfection complex (Fig. 3.7.1.3a; A and Fig. 3.7.1.3c; A). As well, they presented a decrease of more than 50 % in their growth rate (Table 3.7.1.3a and Table 3.7.1.3b). Reductions in growth rate have been associated in other studies with an increase in antibody production (Barnabé and Butler 1998; Hayter et al. 1992; Millller et al. 1988). Therefore this growth rate reduction of transfected HEK293s cells could be related to the production phase. The same but untransfected cells, to which only the complex medium was added, presented a slow and insignificant decrease in their growth rate of about 10 – 15 % (Table 3.7.1.3a and Table 3.7.1.3b). Moreover, the characteristic increase in the NTP/U ratio of more than 50 % of the basal levels appeared between 24 and 48 h later than in transfected cells.

The sensitivity and applicability of the NTP/U ratio was verified here again by the results of this work with HEK293s cells. Nucleotide ratios indicated that the physiological state of the cells has changed after the addition of the DNA:PEI complex for transfection. These changes in the NTP/U ratio were dominantly caused by a decrease in the U ratio (decrease of the UTP and increase of the UDP-GNAc pool; data not shown). As mentioned before, the increase of the UDP-GNAc pool can be caused by elevated synthesis or reduced use. The increase of the UDP-GNAc pools after growth rate reduction did not seem to be the result of a reduced turnover rate alone,

because the expression of secreted glycoproteins has been shown active during the phase of reduced growth (Al-Rubeai and Emery 1990; Barnabé and Butler 1998; Ryll and Wagner 1992). Therefore, in transfected cells this increase of the UDP-activated aminohexoses could be related to a high synthesis rate of glycoproteins or glycolipids.

Furthermore, many studies have shown the importance of the U ratio for earlier detection of changes in the cell physiology and the reduction of the growth rate (Grammatikos et al. 1999; Moore et al. 1997; Schoenherr et al. 2000). These authors discussed the importance of the UTP as key pool in cell growth. Moore et al. (1997) showed that a decrease of the UTP pool and an increase of the activated aminohexosamine pool correlated with a reduction of the temperature from 37 °C to 30 °C in arresting CHO cells. Reduction of the culture temperature may cause cell stress. In this context, the addition of the transfection complex could be considered as a stress situation and therefore could be one of the reasons for the decrease of the UTP and the increase of the UDP-GNAc pool in transfected cells in contrast to untransfected cells.

Additionally transfected cells, cultivated either in serum-containing or serum-free conditions, showed a less dramatical increase of the NTP/U ratio with a maximum value 50 % lower compared to untransfected cells. In general, a drastic increase in the UDP-GNAc pool is responsible for a dramatic decrease in the U ratio and an associated dramatic increase in the NTP/U ratio as discussed above. This was correlated with the result obtained in this work (section 3.7.1.3), where transfected cultures presented no substantial increase of the UDP-activatedhexosamine pool (data not shown) and therefore no substantial increase in the NTP/U ratio (Fig. 3.7.1.3a; A and Fig. 3.7.1.3c; A), especially in cultures under serum-free medium. One reason could be the use of the activated sugars for the formation of glycoproteins. Moreover, as was proposed by Grammatikos et al. (1998) (about keeping low levels of the UDP-GNAc pool during a production process), one can conclude that the conditions identified during optimization of the transfected cultures performed in this work were the most favorable to improve productivity.

Transfected cells vs. addition of PEI alone

When PEI was added to the cultures, a decrease in the cellular growth rate of approximately 30 % and 60 % was observed in serum-containing and serum-free media conditions, respectively. However, this decrease was not in the same magnitude as for DNA:PEI-transfected cells, which showed a decreasing of 55 % (for serum-containing medium) and 67 % (for serum-free medium) (Table 3.7.1.4a and 3.7.1.4b). Several studies have shown that the DNA:PEI complex added at a concentration for gene delivery, did not remarkably affect the cellular metabolism. However, higher amounts

of the complex or even free PEI did result in toxicity (Boussif et al. 1995; Godbey et al. 1999; Kichler et al. 2001). Godbey et al. (1999) postulated that a possible reason for the toxic effects of PEI on the cells is due to the membrane permeability, resulting in elevated endosomal concentrations of PEI.

The NTP/U ratio from these PEI-transfected cultures showed a different behavior in comparison to DNA:PEI-transfected cells (Fig. 3.7.1.4a; A and Fig. 3.7.1.4b; A). A 50 % increase of the NTP/U ratio was observed 24 h and 48 h after the addition of PEI in cells cultivated under serum-containing and serum-free media, respectively. This indicates that PEI has affected the cell physiology. As expected, the incorporation of PEI induced cell stress, which could be the reason for the increment of the NTP/U ratio (as discussed above). However, the maximum of the NTP/U ratio in PEI-transfected cells was 30 and 100 % higher compared to the DNA:PEI-transfected cells, showing clearly the difference between DNA:PEI and PEI uptake.

Summarizing, these data showed the intracellular nucleotide ratio as a reliable tool, describing the physiological conditions of HEK293s cells. Even more, the NTP/U ratio was useful to characterize the transfection procedure using different cellular ages and different medium conditions towards an optimal transfection process. In this context, it is important to emphasize, that in each example presented in the section 3.7.1 and discussed above, the NTP/U ratio was always below to 2.3 – 3.3 before the addition of the transfection complex in serum-free as well as in serum-containing media, respectively. These low NTP/U values indicated best physiological state for transfection.

Table 3.7b (section 3.7) shows that cells from the same early passage number 42, presented a lower percentage of GFP-positive cells as expected using either pEPO-IRES2-EGFP or pCMV-EGFP-EPO plasmids (44 % and 55 %, respectively) under serum-containing conditions. The analysis of the intracellular nucleotides revealed NTP/U ratios higher than 3.3 before transfections were performed (data not shown), indicating that the cells were in a poor physiological state. These results verified that it is important to have cells in best physiological conditions characterized by a high growth potential to perform a successful transfection procedure.

The adenylate energy charge (AEC) of cells in all cultivations maintained always at high values of more than 0.90 even if cells have entered the starvation phase. Such a high AEC seems to be characteristic for continuous mammalian cell lines (Ryll et al. 1991a; Ryll et al. 1991b) and it cannot be taken as a valid indicator of cell damage (Kristensen 1989).

The NTP/U plot

Ryll and Wagner (1992) proposed the plotting of the NTP value as a function of the U value, resulting in a curve that reflects the physiological progress of a cell culture during the growth cycle of a cell culture process. A particular area of this function, which is framed by a rectangle characterizing the upper and the lower limit of a permitted region, corresponds to the exponential growth phase and is called log-box. All the other values on the left and the right side of the exponential region belong to the lag phase, to the reduced exponential growth, or to the starvation phase. The authors postulated that the general behavior of the cells is as following: if the cells reduce their growth, the values move to the left upper region (increase NTP but decreasing U value). This movement can be reversed by a batchwise medium exchange, for example. Upon reaching the maximum point in the left upper side, the values move down again (reduced NTP value) which indicate a worsening of the cell conditions and a reduces viability. Ryll and Wagner (1992) proposed this function for monitoring, control and regulation of bioprocesses based on *in vitro* cell cultures. The advantage of this monitor and control system is based on the fact that direct changes of the cell physiology, but not only the cell viability, are detected. They suggested its importance for process validation and the maintenance of a constant product quality.

Considering these proposals, the NTP/U plot was applied to transfected and untransfected HEK293s cells cultured in serum-free and serum-containing media.

Diagram A and B of figure 4.2a show the NTP/U plot from transfected and untransfected HEK293s cells taken from an early and late passage number and cultured under serum-containing and serum-free conditions, respectively. The diagrams show a media-specific and cell-specific range in which all points are arranged. In both cases, when the physiological conditions of the cells alter, the values move from the log-box to the left upper side and reach the maximum of the NTP/U ratio (worse cell conditions). Subsequently, the points move down again (reduced NTP value) indicating a reduction in the cell viability. For untransfected cells cultivated under serum-containing medium the points move again to the right down side (increased U value) indicating the starvation phase (Fig. 4.2a; A). This increment on the U ratio was mainly due to the decrease of the UDP-GNAc pool. At this point, glucose, which is one of the components for the formation of the UDP-activated sugars, was completely depleted in the medium.

Comparing both plots, it is possible to identify the different behavior of the HEK293s cells cultivated under both media conditions. Even more, in every plot it is possible to see that neither transfected and untransfected cells nor cells taken from an early or late passage number, show different behavior.

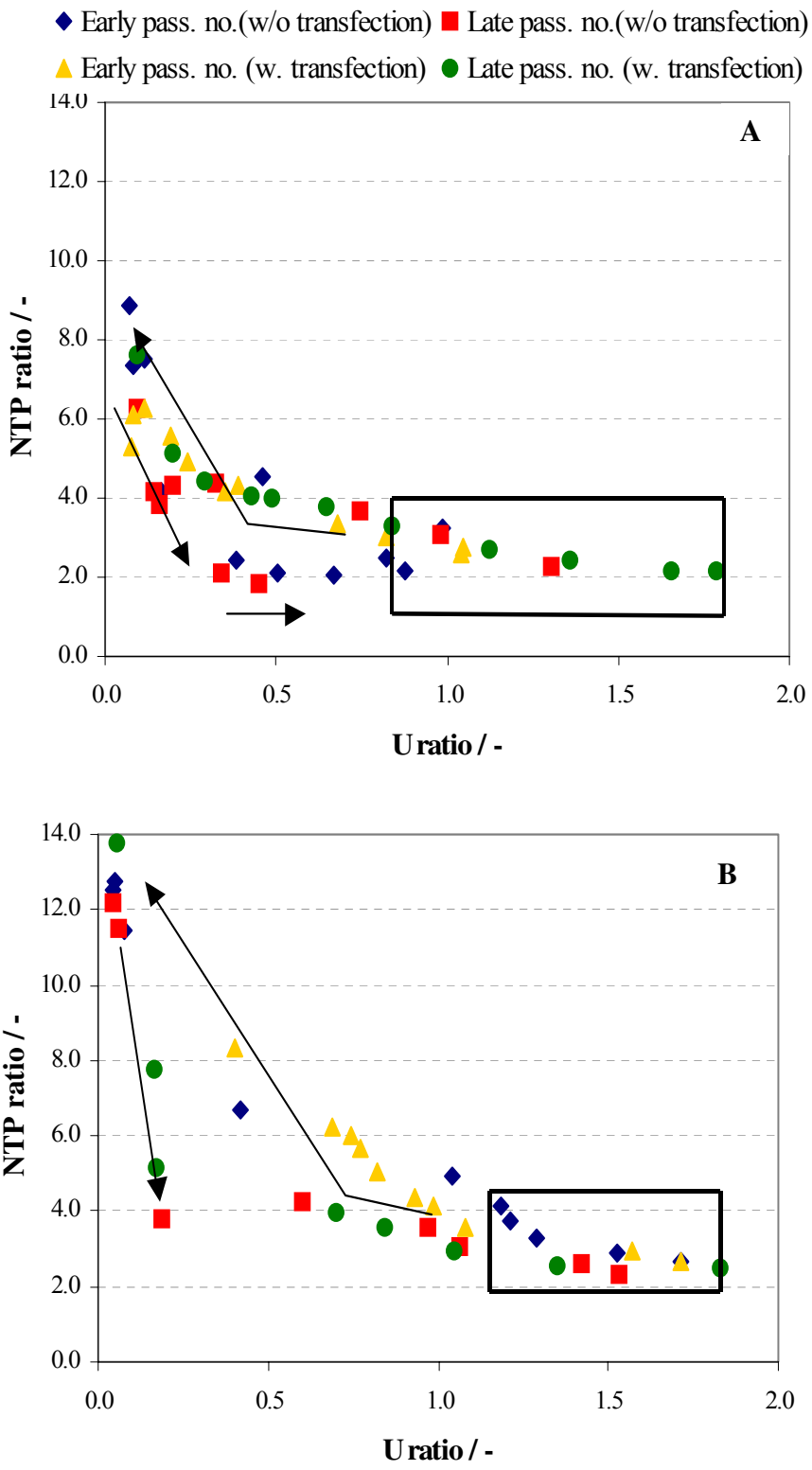


Fig. 4.2a: NTP/U-plot for the cultivation and transfection of HEK293s cells taken from an early and late passage number and cultured under serum-containing (**A**) and serum-free medium (**B**). The exponential growth phase is defined as a common region expressed by the rectangle. The arrow to the left upper part indicates the movement of the data (increase NTP ratio and decrease U ratio) when the cells change their physiology and reach the maximum of the NTP/U ratio (worse cell conditions). Upon this maximum, the points move down again (arrow to the down part) indicating the reduced cell viability. For untrasnfected cells cultivated under serum-containing medium (**A**), the points move again to the right down side (increase U value) indicating the starvation phase.

4.3 BioNose

In general, bioprocess parameters important for control of the cellular state, such as cell number and viability, glucose and lactate concentrations and the pool of intracellular nucleotides, are usually monitored off-line with long time intervals between sampling and analysis. Additionally, the process is expensive and time-consuming. This off-line analysis is mainly due to the fact that on-line sensors often do not fulfill the necessary requirements such like reliability, simplicity, sterility, and long-term stability. With these limitations, an accurate control of the cellular state in bioprocesses is difficult. However, the problem of controlling the status of a cultivation could also be solved by sensors that do not directly monitor the above-mentioned environmental parameters but that identify important transitions between different cultivation phases which gives access to optimal culture conditions (Bachinger and Mandenius 2000b). To assess the usefulness of the electronic nose for this purpose, different experiments were performed evaluating the BioNose sensor pattern for different phases of the transfection procedure using HEK293s cells of different cell age in serum-containing and serum-free media. As mentioned in the introduction, this work was part of an EU project. As already described by other authors for CHO cells (Bachinger et al. 2000a; Bachinger et al. 2000c; Bachinger et al. 2002; Mandenius 1999; Mandenius et al. 1998), the use of an electronic nose (BioNose) for monitoring mammalian cell cultivation was verified in this work for HEK293s cells, to distinguish between different process conditions on the one hand and as a tool for process characterization on the other hand. Although the sensors signal responses from the BioNose were much weaker than expected (100 times lower than expected due to different qualities of the sensors) the usefulness of the electronic nose could be demonstrated.

It is important to emphasize that the signal output is the result of the combination of many sensor responses without having specific knowledge about the individual sensors´ sensitivity. Generally, details on chemical surface reactions for specific analytes are not always known, but they are also not needed for accurate function of the electronic nose. Consequently, the identification of the different phase in a process by the BioNose is the indirect correlation of sensors responses to these analytes and not the direct detection of a certain analytes (Bachinger et al. 2000a).

The following phases of the process have been characterized individually and in combination: Sterility test, addition of PEI to culture medium, batch cultivation of HEK293s cells, addition of PEI to cultured HEK293s cells, transfection of cultured

HEK293s cells by the DNA:PEI complex, expression of the recombinant proteins in transfected HEK293s cells (3.7.2).

During the sterile test and the batch culture of HEK293s cells cultivated under serum-containing conditions were obtained constant sensor responses (3.7.2.1). The addition of PEI to the cell culture resulted in a transient increase of the contributing MOSFET sensors over a period of 14 – 16 hours (Fig. 3.7.2.1d; A). When the cells were transfected by the DNA:PEI complex, especially the same MOSFET sensors responded with a transient positive signal over a period of 25 – 27 h which was longer but similar to the response of the sensors when only PEI was added (Fig. 3.7.2.1g; A compared to Fig. 3.7.2.1d; A). In these cases the score plots revealed a characteristic pattern of the culture before and after the addition of PEI alone or DNA:PEI, respectively (Fig. 3.7.2.1e and Fig. 3.7.2.1h). This result was attributed to the cell physiological uptake of PEI / DNA:PEI, corroborated when the addition of the PEI alone to the cell-free culture medium showed no response in the sensor signal (Fig. 3.7.2.1j) presenting a characteristic process-specific BioNose response pattern for the transient transfection of HEK293s cells cultured in serum-containing medium.

The BioNose sensor responses for transient transfection of cells cultivated under serum-free conditions using chemically-defined protein-free medium showed the same characteristic increase as in serum-containing conditions (Fig. 3.7.2.2b compared to Fig. 3.7.2.1g). This increase in the sensor response was already observed when the bioreactor was inoculated, showing a different behavior of the cells growing under this serum-free conditions (Fig. 3.7.2.2b). Moreover, the respective score plots of the BioNose for transfection procedures in the different culture conditions even show the difference between the undefined (serum-containing medium) and the defined (serum-free medium) media (Fig. 3.7.2.1h and Fig. 3.7.2.2e; A – B) identifying different process conditions. The trajectory of the data signal was more define and clearer for transfection processes under serum-free medium compared to serum-containing medium. This difference can be assumed by the more complex mixture when serum is present.

Bachinger et al (2000a) identified characteristic states of recombinant CHO cells in culture and changes in the process operation by individual clusters in the score plot. Here, it was also possible to differentiate clusters corresponding to each culture state, especially for the procedure under serum-free conditions (Fig. 4.3a; A and B).

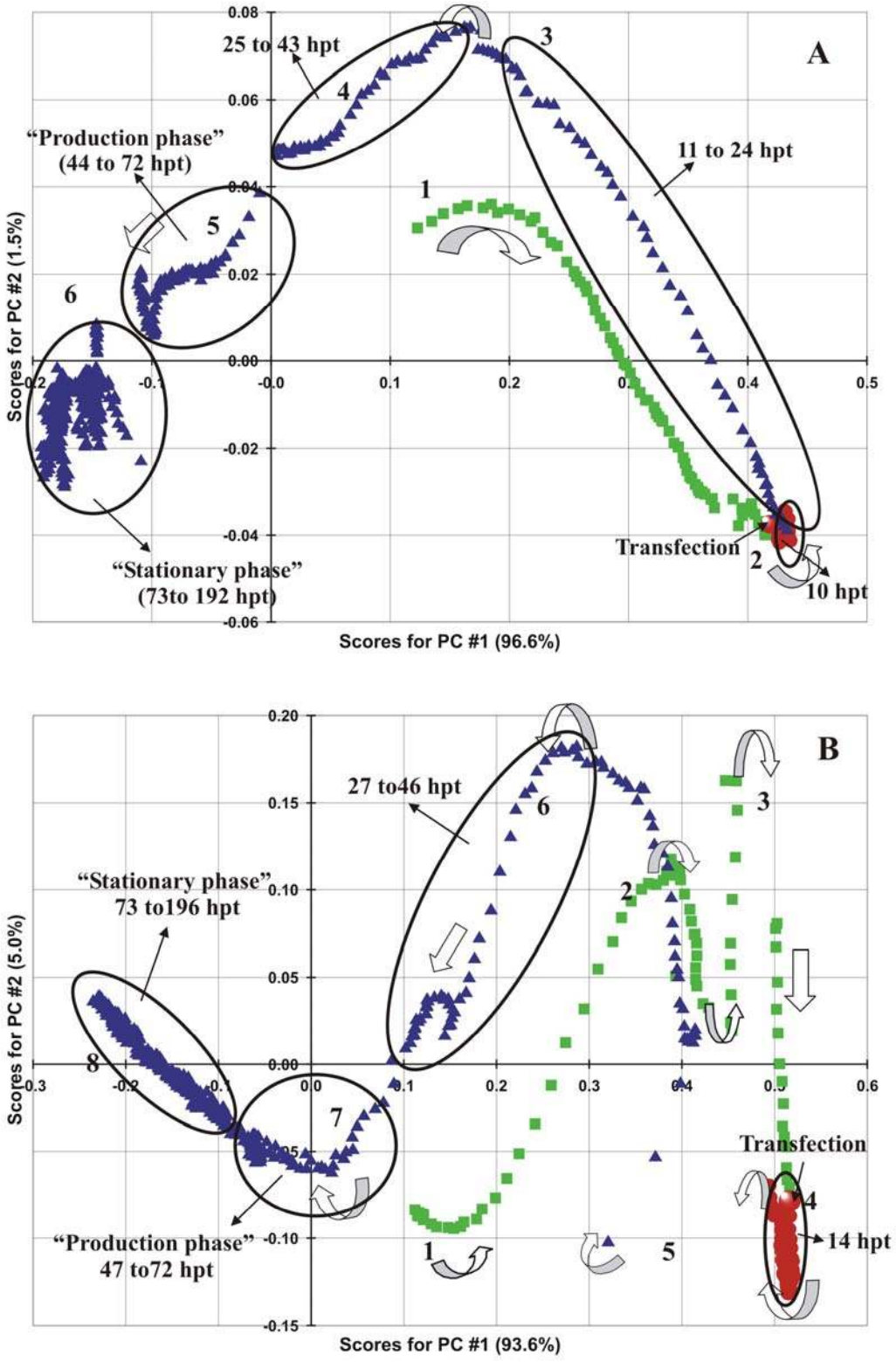


Fig. 4.3a: Score plot of the complete transfection procedure using the HEK293s cells cultured in FS:DMEM + AA serum-free medium. TTran. 040 (**A**) and TTran. 044 (**B**). (■): before addition of DNA:PEI, (●): between 10 to 15 hours after transfection, (▲): 11 -16 hpt until the end of the culture. 1 to 6 in **A** and 1 to 8 in **B** show the characteristic cluster representing different states of the culture process. Every point represents one measurement from the electronic nose at a 20 min interval.

During inoculation of the bioreactor and the first day of the batch culture until the transfection procedure, the data oscillate in the first cluster (see green squares in the figure 4.3a). Additionally, it must be considered that during the cultivation TTran. 044, (Fig. 4.3a; B), the BioNose was shut down two times before adding the DNA:PEI complex and one more time 17 hours later, producing a distortion in the score plot pattern. As described by Bachinger et al. (2000a) and it was corroborated here, the sensors are able to track faults related to reactor operation reflecting this in the score plot pattern.

During 10 to 15 hpt, the data move down in a cluster in the right part of the plot (red filled circle, cluster no. 2 in Fig. 4.3a; A and no. 4 in Fig. 4.3a; B), which coincided with the increase observed in the contributing sensor response (Fig. 3.7.2.2b). Then, the data start to shift to the left upper part reaching the top of the plot at 24 – 26 hpt. Subsequently, the data make a sharp turn down arranging a new cluster that corresponded to a time interval between 25 – 27 hpt and 43 – 46 hpt (blue filled triangle, cluster no. 4 in Fig. 4.3a; A and no. 6 in Fig. 4.3a; B). Following the score plot data down, another cluster can be identified, which can be attributed to the "production phase" and correlates with the determination of the GFP-positive cells measured by flow cytometry analysis at 60 hpt [represented by cluster 5 (44.5 % GFP-positive cells) and 7 (55.5 % GFP-positive cells) in Fig. 4.3a; A and B, respectively]. Finally (73 to 196 hpt), by the movement of the data into a new cluster, the stationary and the starvation phase was possible to identify in case of the first cultivation (cluster 6, Fig. 4.3a; A) and the stationary phase in the last cultivation (cluster 8, Fig. 4.3a; B); (see section 3.7.2.2 for cell cultivation parameters).

Summarizing, the data show that the BioNose can significantly improve the monitoring of a cell cultivation by topically following the progress of the complex process. Moreover, electronic noses can distinguish between different process conditions without measuring the concentration of specific species. The ideal cultivation process with respect to productivity can be found earlier and can be identified more reliably. Information generated can easily be implemented for the optimization of transient transfection processes. As was described by Bachinger et al. (2000a) to allow real-time identification of ideal process states, a mathematical model, like PCA, need to be applied onto the sensor signals. A PCA-model can be calculated with the sensor data from an ideal cultivation where the corresponding principal components represent ideal process states. In subsequent cultivations, the electronic nose signals can be projected in real-time onto this "base" PCA-model resulting in a new set of principal components. When comparing the plots one can notice if, for example, the production phase from the new cultivation is enclosed by boundaries for the region in the plot where the sensor

signals should ideally stay during this new cultivation. If, during a new cultivation, the real-time projection of the sensor signals onto the "base" PCA-model does not, a non-ideal state is indicated and control actions could be effectuated (Bachinger et al. 2000a). Even more, Mandenius et al. (1998) described the use of an electronic nose for process diagnosis, since they could identify bacterial infection in the course of an ongoing cultivation earlier than with alternative methods, such as dissolved oxygen and pH probes.

In addition, bioreactor cultivations under serum-free medium (section 3.7.2.2 and section 4.3, Fig. 4.3a; TTran. 040 (**A**) and TTran. 044 (**B**)) were analyzed using the NTP/U ratio. These results showed values higher than 2.3 (5.2 and 3.2) at the beginning of the culture (day 1), indicating cells in poor physiological conditions with a low growth potential (section 3.7.2.2, Fig. 3.7.2.2d; A and Fig. 3.7.2.2a; A). This was consistent with result of the transfection, which was performed at day 2, obtaining a lower percentage of GFP-positive cells, 44.5 % and 55.5 % respectively. Additionally the presence of cell aggregates before the addition of the transfection complex could affected the procedure; hence this is an important prerequisite for a good transfection efficiency.

It was interesting to observe that in the cell culture showing the lowest NTP/U ratio at day 1 (TTran. 044 with NTP/U of 3.2 that indicated better cell conditions compared to TTran. 040 with NTP/U ratio of 5.2) the highest transfectability was measured (55.5 % compared with 44.5 %). Moreover, cells used in both cultivations were derived from different passage numbers, 89 (late passage no.) for TTran. 040, and 70 (early passage no.) for TTran. 044 (section 3.7.2.2). These results agreed with the points already discussed in section 4.2. Cells cultivated under serum-free conditions, derived from an early passage no. showed a higher trasnfectability than cells derived from a late passage no., although the older cells were better adapted to this medium.

4.4 Intracellular nucleotide analysis vs. BioNose

This work showed that the intracellular nucleotide analysis and the electronic nose can be used to monitor a bioprocess and to identify different process conditions.

As already discussed (4.3), on-line monitoring of bioprocess parameters is the method of choice when possible. It has several advantages such as the possibility to continuously sample data using programmed procedures avoiding time-consuming manual sampling. Particularly, one of the dominant advantages of the electronic nose is the non-invasive sampling from the bioreactor off-gas line. Thereby the sterile barrier can be introduced simply via an off-gas filter, which is implemented in any typical industrial bioreactor system. Another advantage of the BioNose is the fact that the

knowledge about the molecular composition of the odor is not needed for identification of process parameters. However by combining the electronic nose with the principal component analysis it is possible to study a dynamic process. In one plot, a description of the process situation can be obtained without having direct information about the cellular and physiologic state.

Even though the intracellular nucleotide analysis is not an on-line method, nucleotide ratios give direct information about the cell status. Additionally, their combination amplifies small changes of the culture giving larger and more easily detectable variations. Moreover, they are quite robust and independent on the sampling method. Since the parameter is a ratio, it is also independent on the volume used for extraction.

Summarizing, the determination of intracellular nucleotides could even help to find an ideal cultivation and a suitable PCA-model for on-line process monitoring.
In addition, using nucleotide ratios pre-culture could be optimized. This is an important prerequisite for a successful bioreactor cultivation and thus for a successful large-scale transfection procedure.

5. Conclusions and future perspectives

In conclusion, a transient transfection procedure easy to perform, cost-effective, for a large-scale application using the mammalian cells HEK293s cultivated in a medium totally free of animal proteins or animal-origin components was developed.

The work performed in this thesis contributes to science and technology development in transient transfection. Important new goals were achieved in this field:

- Use of minimal amount of DNA.
- Develop of a new serum-free medium.
- Omission of the cell wash step prior and after transfection.

In addition, the characterization of the transfection procedure using an off-line method such as intracellular nucleotide patterns, and an on-line method such as the electronic nose, has showed the applicability of both methods as appropriate and even excellent tools for process optimization.

The advantage of obtaining early information on changes in cell physiology that will later translate to cell growth worsening, complete arrest or even decreasing viability finds many applications in process development. In the absence of NTP/U information, one cannot know whether cells are in a good physiological state with high growth potential or not. These are essential demands in process development. As was shown in this work, cells in sub-optimal physiological state will not respond favorably to stress. In this case, this was observed as a low transfection efficiency and as consequence low protein expression.

Moreover, information drawn by the NTP/U ratio give access to special cell properties e.g. growth retardation during scale-up. Knowledge of the exact time for transferring the cells to the next scale is of crucial importance, not only with regard to process time reduction but also with regard to efficiently design the respective scale-up protocol by avoiding a large number of time-consuming experiments.

In addition, the NTP/U ratio reflected different cell physiological patterns, characteristic for the respective process condition. In this context, as mentioned in the results section (3.7.1), this ratio had shown to be a useful and reliably tool to distinguish between:

- HEK293s cells from early and late passage numbers.
- Cells cultured under serum-containing conditions from those under protein/serum-free medium conditions.
- Transfected and untransfected cells in culture.
- DNA:PEI complex uptake (transfection procedure) from PEI uptake alone.

Even more, these results demonstrated that transfectability and productivity of transiently transfected HEK293s cells are highly dependent on the characteristic cell physiology under every process condition. In this context, the nucleotide ratio plays an essential role, more than the most common parameters such as cell number and viability, or the determination of glucose, lactate, LDH or amino acids in the culture supernatant or even the cell passage number.

NTP/U profiles could be used to find optimum process state in a series of production runs with regard to the best cell physiology status and the optimum time and conditions for transient transfection.

The use of the BioNose as a tool for process characterization, cell culture monitoring and optimization was demonstrated. Moreover, a characteristic process-specific BioNose pattern for the transient transfection of HEK293s was found. In addition, the data obtained here, can be used to generate a database for optimization of transfection processes based on cultured HEK293s cells. However, a further improvement of the sensors sensitivity is a prerequisite to provide a reliable robustness to this continues non-invasive real-time monitoring method.

Additional experiments are required to further improve the process:

- Further optimization of the transient transfection to larger scales.
- Extension of the results obtaining so far to all conceivable scales.
- Expansion of the results to different mammalian cell types.

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7. Appendix

7.1 Media composition

Table A1: DMEM -Ca^{2+} + Vit. + Pl. + 5% FCS and DMEM -Ca^{2+} + Vit. chemical composition media.

DMEM -Ca^{2+} + Vit. + Pl. + 5 % FCS	DMEM -Ca^{2+} + Vit.	Components	Concentration
x	**x**	$MgSO_4 \times 7H_2O$	200.0 mg L^{-1}
x	**x**	KCl	400.0 mg L^{-1}
x	**x**	$NaHCO_3$	3700.0 mg L^{-1}
x	**x**	NaCl	6400.0 mg L^{-1}
x	**x**	NaH_2PO_4	109.0 mg L^{-1}
x	**x**	L-Arginine × HCl	168.6 mg L^{-1}
x	**x**	L-Cystine × 2HCl	62.6 mg L^{-1}
x	**x**	Glycine	30.0 mg L^{-1}
x	**x**	L-Histidine × $HCl \cdot H_2O$	42.0 mg L^{-1}
x	**x**	L-Isoleucine	131.0 mg L^{-1}
x	**x**	L-Leucine	131.0 mg L^{-1}
x	**x**	L-Lysine × HCl	219.0 mg L^{-1}
x	**x**	L-Methionine	60.0 mg L^{-1}
x	**x**	L-Phenylalanine	66.0 mg L^{-1}
x	**x**	L-Serine	84.0 mg L^{-1}
x	**x**	L-Threonine	95.0 mg L^{-1}
x	**x**	L-Tryptophane	16.0 mg L^{-1}
x	**x**	L-Tyrosine	71.4 mg L^{-1}
x	**x**	L-Valine	94.0 mg L^{-1}
x	**x**	D-Glucose × H_2O	4950.0 mg L^{-1}
x	**x**	L-Glutamine	731.0 mg L^{-1}
x	**x**	$Fe(NO_3)_3 \times 9H_2O$	0.1 mg L^{-1}
x	**x**	Phenol red	1.5×10^{-3}% (V V^{-1})
x	**x**	MEM vitamins solution(100 ×)	4.0 %(V V^{-1})
x	**-**	Pluronic F-68, solution 10 %	0.1 % (V V^{-1})
x	**-**	FCS	5.0 % (V V^{-1})

Table A2: Description of H-SFM medium supplemented either with Pl., Lip. or EDTA.

H-SFM + Pl.	H-SFM + Pl. + Lip. + EDTA	**Components**	**Concentration**
x	**x**	Pluronic F-68, solution 10 %	0.1 % (V V^{-1})
-	**x**	Lipid medium supplement (0.1 %)	1.0 mL L^{-1}
-	**x**	EDTA (Stock sol. 1.6 g L^{-1})	1.4 mL L^{-1}

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PUBLICATIONS

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SCIENTIFIC COURSES, MEETINGS AND CONGRESS

1. "Research Projects (Art and Technique of its formulation). Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral. Santa Fe, Argentine. 14 th May, 1994.
2. "Animals laboratory: general and special aspects application in the research working andtechnology development in Biology Sciences". LETH (Laboratorio de Endocrinología y Tumores Hormonodependientes). Human Physiology Department. Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral. Santa Fe, Argentine. September - October 1995.
3. "High Performance Liquid Chromatography with application in food analysis" Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), Universidad Nacional del Litoral, Consejo Nacional de Investigaciones Científicas y Técnicas., Santa Fe, Argentine, 14 th March - 25 th April, 1997.
4. "First Workshop and Latinoamerican Conference of Autoimmunity". Buenos Aires, Argentine, 21 - 23 April, 1997.
5. "Practice and theory: Second course about recently advance in Immunogenetics and Histocompatibility". Sociedad Argentina de Inmunología (SAI); The International Union of Immunological Societies (IUIS);, The American Society for Histocompatibility and Immunogenetics (ASHI); Maestría en Biotecnología, Universidad de Buenos Aires (UBA); The American Board of Histocompatibility and Immunogenetics (ABHI); Buenos Aires, Argentine; 11 - 20 August, 1997.
6. "Immunology and Immunopathology". Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Argentine. (72 hs) May - October, 1997.
7. "Analysis of DNA in biological samples (Estudios de Filiación e Identidad)". Fundación Argentina de Investigaciones Biomoleculares (FIBIO). Buenos Aires, Argneine. 18 - 22 May, 1998.
8. "Actualization in Medical Molecular Biology". Instituto de Investigaciones Bioquímicas, Fundación Campomar. Buenos Aires, Argentine. 3 - 31 August, 1998.
9. "Molecular Neurobiology". Facultad de Farmacia y Bioquímica, Escuela de Graduados. Universidad de Buenos Aires, Argentine. May - June, 1998.
10. "Immune System Physiology". Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentine. October, 1998.

11. “Cytoquines and adhesion molecules in inflammation and autoimmunity”. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentine. May - June, 1999.
12. “Techniques in Biochemistry and Molecular Biology”, Instituto de Investigaciones Dr. JF Leloir (Fundación Campomar), Buenos Aires, Argentine. August - December, 1999.
13. “Research Methodology ”. Academia Nacional de Medicina de Buenos Aires. Instituto de Investigaciones Hematológicas “Mariano Castex”. Buenos Aires, Argentine. October, 2000.
14. I° LACTRIMS (Comité Latinoamericano para el Tratamiento y la Investigación en Esclerosis Múltiple) Congress . Buenos Aires, 9 -11 November, 2000.
15. 18th ESACT (European Society for Animal Cell Technology) MEETING. Granada, Spain, 11-14 May, 2003.