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Master thesis

Evaluation of lgfIB and Sec10 Effects on Growth and Productivity in CHO cells

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„Der Zweifel ist der Beginn der Wissenschaft. Wer nichts anzweifelt, prüft nichts. Wer nichts prüft, entdeckt nichts. Wer nichts entdeckt, ist blind und bleibt blind.“ Teilhard de Chardin

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Abstract

Although Chinese Hamster Ovary cells are widely used in therapeutic protein production, their growth and productivity can still be improved. The objective of this thesis was to improve their performance through *IgfIB* and *Sec10* gene overexpression and knockdown of the IgfIB inhibiting *IgfBp4*. Furthermore it was examined, whether the effect of the *IgfIB* expression or the effect of the Igf-1 or LongR^{3}Igf-1 (Igf-1 analogue) as media supplement causes a greater improvement of the cells performance. In addition the effect of the LongR^{3}Igf-1 and the Igf-1 media supplement was tried to be improved through the knockdown of *IgfBp4*. The findings demonstrated that the overexpression of the *IgfIB* in combination with the knockdown of the *IgfBp4* leads to a significantly better growth. Furthermore the shBp4 transfection in combination with the Igf-1 supplement improved the specific productivity and growth. Also the effect of the LongR^{3}Igf-1 supplement could be improved through the shBp4 transfection of the cells. So far the *Sec10* overexpression did not improve the performance of the cell line. From those findings it can be concluded, that the combination of the Igf-1 supplement with the shBp4 transfection is a possible tool to improve the therapeutic protein production in Chinese Hamster Ovary cells.

Zusammenfassung

Chinese Hamster Ovary Zellen werden sehr häufig zur Herstellung von rekombinanten Proteinen verwendet. Die Wachstumsrate und ebenso die spezifische Produktivität dieser Zellen müssen aber noch immer verbessert werden. Ziel dieser Arbeit war es die Leistung der Zellen durch die *IgfIB* und *Sec10* Überexpression und den Knockdown des IgfIB inhibierenden *IgfBp4* zu verbessern. Darüber hinaus wurde getestet, ob die Überexpression des *IgfIB* oder die Verwendung eines Igf-1 oder LongR^{3}Igf-1 (Igf-1 Analogon) Medienzusatzes einen stärkeren Effekt in den Zellen hervorruft. Zusätzlich wurde versucht den Effekt des LongR^{3}Igf-1 und des Igf-1 Medienzusatzes mit Hilfe des *IgfBp4* Knockdowns zu verbessern. Die Ergebnisse zeigten, dass eine Kombination aus *IgfIB* Überexpression und *IgfBp4* Knockdown eine signifikant höhere Wachstumsrate verursacht. Weiters führte die shBp4 Transfektion in Kombination mit dem Igf-1 Medienzusatz zu einer höheren Wachstumsrate und einer besseren spezifischen Produktivität. Der Effekt des LongR^{3}Igf-1 Medienzusatzes konnte durch die shBp4 Transfektion auch verstärkt werden. Bislang führte die Überexpression von *Sec10* zu keiner Verbesserung der Leistung der Zellen. Diese Ergebnisse lassen den Schluss zu, dass die therapeutische Proteinproduktion in Chinese Hamster Ovary Zellen mit Hilfe einer Kombination aus shBp4 Transfektion und Igf-1 Medienzusatz verbessert werden kann.

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1 Introduction

Although many different cell factories like microorganisms, insect cell lines, plant cell lines and mammalian cell lines are available for the production of recombinant therapeutic proteins, the most popular ones for complex and glycosylated proteins are the mammalian cell lines (Grillberger et al., 2009).

Among the mammalian cell lines the Chinese hamster ovary (CHO) cells are the most important for recombinant therapeutic protein production (Kim et al., 2012). CHO cells were originally isolated from the ovaries of the Chinese hamster (Cricetulus griseus). In 1957 Theodore T. Puck isolated the first CHO cells from a female Chinese hamster (Tjio and Puck, 1958).

The first authorized therapeutic protein, which was produced with the help of recombinant mammalian cells, was the human tissue plasminogen activator, which became approved in 1986 (Wurm, 2004). From that time on the recombinant protein production in mammalian cells gained more and more importance. Nowadays 60 – 70 % of recombinant produced proteins are produced with the help of mammalian cells (Wurm, 2004) and CHO cell lines are known as the best workhorse for the production of recombinant therapeutic proteins, since they produce glycosylation patterns (Krampe and Al-Rubeai, 2010) and other post-translational modifications, which are comparable to human structures. Another reason for the popularity of CHO cells is that they have been used as safe hosts for more than two decades and are therefore accepted as safe production hosts by several regulatory agencies (Kim et al., 2012).

The main disadvantage of mammalian cell factories is the low volumetric yield. Mammalian hosts have a 10 – 100 fold lower volumetric yield than microbial production systems. The slower growth and the lower specific production rate are the key factors influencing the low volumetric yield of mammalian production systems (Krampe and Al-Rubeai, 2010). In order to eliminate the main drawback of the low volumetric productivity and the low product titer there are several main approaches. One opportunity is process optimization by improving media compositions and developing sophisticated feeding strategies, thus significantly increasing the integral of viable cells during a bioprocess (Krampe and Al-Rubeai, 2010).

The second opportunity is to optimize the process by process parameter shifting in a biphasic cultivation system. This means, that the process consists of a cell proliferation phase for generating a high amount of viable cells and a subsequent production phase,

where the cell proliferation is almost stopped and the product yields as well as the viable cell days are increased. The process parameters have to be pre-optimized according to the produced protein. An optimal result was obtained with shifting the temperature and pH values in the production phase (reduced temperatures and decreased pH values) to slow down the cell metabolism. These shifts lead to a significantly improved volumetric productivity without affecting the quality of the secreted product (Trummer et al., 2006).

An alternative strategy and nowadays a standard procedure in industrial processes is the use of methotrexate (MTX) as selective agent against the selection marker dihydrofolate reductase (dhfr). The production cell lines, which are *dhfr* $^-$, will be transfected with a plasmid encoding for the gene of interest (GOI) and the *dhfr* gene, by subsequently adding MTX, which acts as an inhibitor of the *dhfr* gene, the cells react with amplifying the *dhfr* gene. If the GOI and the *dhfr* gene are integrated at the same locus, the GOI is also amplified with the *dhfr* gene. Another, very similar acting system is the glutamine synthetase (GS) as selection marker and methionine sulphoximide (MSX) as selective marker (Mattia Matasci, 2008). These gene amplification strategies are combined with screenings for high producers.

Another opportunity is to improve cell line properties by applying genetic engineering strategies. One very promising cell engineering strategy is to overcome the disadvantages of mammalian cell lines by overexpression or introduction of genes regulating apoptosis, cell cycle, glycosylation, metabolism or protein expression. Anti-apoptotic genes are for example *Bcl-xL, Bcl-2* (Mastrangelo et al., 2000) and *c-Myc* (Ifandi and Al-Rubeai, 2005). These genes can be used alone or in combination (Meents et al., 2002).

RNA interference (RNAi) or Zinc-finger nucleases (ZFN) are also novel tools for cell engineering (Wu, 2009) (Porteus and Carroll, 2005). With the help of the RNAi technology a knockdown of gene translation can be achieved with high efficiency. The ZFNs enable a complete knockout of the desired gene. ZFN or short interfering RNAs (siRNAs) have been designed for example against pro-apoptotic sequences like Bak or Bax (Lim et al., 2006). The RNAi technology consists of two main methods, the siRNA technology and the microRNA (miRNA) technology (Siomi and Siomi, 2009). siRNAs are short (approx. 21 Bp), double stranded RNAs, which are processed out of long dsRNAs (shRNAs) (Elbashir et al., 2001) by Dicer, a ribonuclease III enzyme (Bernstein et al., 2001). The siRNA duplex is then bound by the RISC complex (RNA-induced silencing complex) and the passenger strand is degraded. After degradation of the passenger strand the RISC complex is guided by the incorporated siRNA to the target mRNA and RNA silencing takes place (Schwarz et al., 2003). Silencing occurs in two different ways, either translational

repression or mRNA degradation (Siomi and Siomi, 2009). miRNAs are small, endogenous non-coding RNAs, which are similar to siRNAs, but they differ in their silencing mechanism (miRNAs aren´t designed artificially against one mRNA, they can regulate a whole gene network) and biogenesis (Siomi and Siomi, 2009).

During this thesis two different genes, *IgfIB* and *Sec10*, were tested for their effects on volumetric productivity of a recombinant DUKXB-11 CHO cell line expressing an Erythropoietin-Fc fusion protein. IgfIB is one of the three most popular isoforms produced through alternative splicing of the *Igf–1* gene. Igf–1 belongs to the insulin-like growth factor family, which consists of the Igf-1, Igf-2 and insulin (Baxter, 1986). These growth factor family members, mainly the Igf-1 interact with the Igf1R and play an important role in the regulation of growth, development and survival of normal cells (Barres et al., 1993; Baserga et al., 1994; DeAngelis et al., 1995; LeRoith et al., 1995; Sell et al., 1995). Through interactions with the Igf-1 receptor Igf-1 has mitogenic effects and anti-apoptotic effects, which are mediated through the MAPK and PI3K signaling cascades (Mulligan et al., 2002; Sachdev and Yee, 2001). The Igf1R is a receptor with tyrosine kinase activity (Hwa et al., 1999). The complex network of the Igf signalling system consists out of receptors [Igf1R, Igf2R and the insulin receptor], ligands [Igf-1 and Igf-2] and six Igf binding proteins [IgfBp1-6] (Vorwerk et al., 2002). The IgfBps regulate the interactions between the Igf-1 and the Igf1R (Camacho-Hubner et al., 1992). High-affinity binding of Igfs by IgfBps influences their bioavailability through reduced binding of Igfs to the Igf1R (Noelle-Anne Sunstorm et al., 1998).

IgfBp4 is one of the six known IgfBps, which helps to protect the cells from Igf overstimulation through binding the Igf and therefore inhibiting Igf induced cell growth (Mohan et al., 1989). Depending on the cell type different isoforms of IgfBps are expressed and secreted. In CHO cells IgfBp4 and IgfBp3 are expressed constitutively in response to Igf-1 (Sunstrom et al., 1998). The IgfBp4 mRNA level increases with the amount of IgfIB (Conover et al., 1995). With the help of a specific protease, which cleaves the IgfBp into two protein fragments, the inhibitory effect of the IgfBp4 can be suppressed (Conover et al., 1993). Another option for suppressing the inhibitory IgfBp4 effect is the use of the RNAi technology and inhibiting the *IgfBp4* with the help of an in silico designed mix of 3 short-hairpin RNA molecules (shBp4). In this project the IgfBp4 inhibiting effect was blocked with a shBp4 mix. It was hypothesized, that cells transfected with shBp4 have an increased Igf-1 bioactivity and consequently exhibit a better growth performance than negative control transfected cells.

Apart from the *IgfIB* gene introduction and the silencing of the *IgfBp4*, the LongR^{3}Igf-1 supplement (Sigma-Aldrich, MO, USA) and an Igf-1 media supplement (Sigma-Aldrich, MO, USA) were used in order to obtain a higher volumetric productivity and a higher production titer. Using LongR^{3}Igf-1 media leads to an increased productivity, increased cell survival and boosts cell proliferation (Morris and Schmid, 2000). The LongR^{3}Igf-1 is an analogue of the Igf-1, but due to its marginal structure difference to Igf-1 it reveals an advantage over the native Igf-1, because it has a lower affinity for IgfBps. An extension on the N-terminus of the native Igf-1 with 13 amino acids and a substitution of the glutamine acid with an arginine on position three distinguishes the native Igf-1 from the LongR^{3}Igf-1 (Francis et al., 1992).

The protein encoded by the *Sec10* gene, is one component of the exocyst complex, which is highly conserved in higher eukaryotes (TerBush et al., 1996). It is also called *Exoc5* (exocyst complex component 5). The exocyst complex is composed out of eight proteins Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Hsu et al., 1996; Novick et al., 1980). This multiple protein complex is involved in tethering and spatial targeting of vesicles to their precise fusion sites. Beside this function the exocyst complex has many other functions (Finger and Novick, 1998; Guo et al., 1999; TerBush et al., 1996). Sec10 is necessary for primary ciliogenesis, regulates cytogenesis and is a crucial component of the exocyst complex (Zuo et al., 2009).

2 Materials and Methods

2.1 Cell Culture

The Chinese hamster ovary cells were cultivated in 125 ml shaker flasks (Corning® Life Sciences, NY, USA) at 37°C, 140 rpm and in a humidified atmosphere containing 7 % CO_2.

- CHO DUKX-B11 EpoFc 14F2
  The CHO DUKX-B11 EpoFc 14F2 cells grew in Gibco CD CHO Medium (Life Technologies, CA), glutamine free, supplemented with 1 ml Anti Clumbing Agent and 0,19 µM MTX per 500 ml of medium.

For the 4th Igf batch additional media supplements were used. After optimizing the concentration of LongR^{3}Igf-1 (Sigma-Aldrich, MO, USA) media supplement, 50 ng/ml were used as media supplement. Igf-1 (Sigma-Aldrich, MO, USA) was also used as media supplement. Following the optimization of the optimal concentration, 10 ng/ml of Igf-1 were used as media supplement.

2.1.1 Media Optimisation 4th Igf Batch

In order to determine the optimal concentration of the Igf-1 media supplement and the LongR^{3}Igf-1 media supplement for the 4th Igf batch some media supplement experiments were made.

In case of the Igf-1 media supplement batches with 10 ng/ml, 1 ng/ml and 0,1 ng/ml of Igf-1 were run. Before getting started 50 µg of the lyophilized Igf-1 were mixed with 50 µl of dH_2O in order to get a concentration of 1mg/ml Igf-1 media supplement. In the next step the Igf-1 supplement was 1:100 diluted and mixed to the final media optimization concentrations with the CHO DUKX-B11 EpoFc 14F2 medium and 1,5 x 10^5 cells/ml. The final volume of the batch was 25 ml. The batch was run in 2 technical replicates for 8 days and under the same conditions as described in point 2.1 cell culture.

The optimal media concentration for the LongR^{3}Igf-1 media supplement was determined with the help of 100 ng/ml, 50 ng/ml, 25 ng/ml and 10 ng/ml LongR^{3}Igf-1 media supplement. The LongR^{3}Igf-1 media supplement was mixed with 50 µl of 100 mM HAc according to manufactures protocol. The 20 µg/ml LongR^{3}Igf-1 media supplement was mixed to the final media optimization concentrations with the CHO DUKX-B11 EpoFc 14F2 medium and 1,5 x 10^5 cells/ml. Batch conditions and final volume were the same as for the Igf-1 media optimization.

2.2 Cell Counting

The total cell number was determined with the help of the Electrical Sensing Zone Method of the Multisizer™ 3 Coulter Counter (Beckman Coulter, CA). The ViCell analyser (Beckman Coulter, CA) was used to determine the viability, viable cell number and total cell number. This machine determines the viable and dead cell number with the help of trypan blue. Trypan blue stains the dead cells blue because it can pass through the broken cell membrane.

In some batches also the hemocytometer method was used to determine the number of viable cells, dead cells and viability. This method works with the same principle as the ViCell, as both methods determine the number of viable and dead cells with the help of trypan blue staining. Dead cells are stained blue whereas viable cells stay colourless. After counting the number of viable and dead cells in four out of the nine big squares some was able to determine the number of cells per ml and the total cell number with the following formulas.
The formula for calculating the viability can be found under the point 2.7. Igf Batch.

$$cells/ml = (average\ number\ of\ cells/square) \times dilution\ factor \times 10^4$$

Formula 1: Number of viable or dead cells/ml

$$total\ cell\ number = cells\ per\ ml \times initial\ volume\ of\ the\ cell\ suspension$$

Formula 2: Total cell number

2.3 Bacterial Transformation

2.3.1 Electroporation

Electrical impulses are used to make the membrane temporary permeable so that the vector with the gene of interest can pass through. Due to this method it is possible to get great porosities without destroying the intracellular membranes.

Before getting started it was important to obtain the right vector concentration (100 ng/µl). After diluting the vector, it had to be mixed with electrocompetent DH5α E.coli and transferred into the electroporation cell. Electroporation took place at voltage 2500 V, resistor 200 Ω and capacitor 25 µF.

Right after electroporation, 140 µl SOC Medium were added and subsequently transferred into an Eppendorf tube with 860 µl of SOC Medium. Then the mixture was incubated on a thermoblock for 1 hour at 37°C with 650 rpm. After incubation the bacterial solution was

streaked out on LB^{amp} plates (undiluted and 1:10 diluted) and cultured over night at 37°C. The LB^{amp} plates contained 100 µg/ml ampicillin.

2.3.1.1 IgfIB-pCIneo, phIgf2-pCIneo and pCIneo

These vectors were obtained from the AKH (Martin Schreiber et al., Department of Internal Medicine I, Medical University of Vienna, Austria). The empty pCIneo mammalian expression plasmid was 5472 bp long and contained a neomycin and ampicillin resistance. The IgfIB cDNA and the phIgf2 cDNA were derived from humans. The plasmid map can be found in the appendix.

pCIneo	2460 V	5,7 ms
IgfIB - pCIneo	2460 V	5,6 ms
phIgf2 – pCIneo	2460 V	5,6 ms

Table 1: Electroporation's conditions (voltage and time constant)

2.3.2 Chemical Transformation

2.3.2.1 IgfBp4 shRNA

Cloning of the IgfBp4 shRNA sequence was done according to the Invitrogen Block-iT™ Pol II miRNAi expression vector Kit (Life Technologies, Carlsbad, CA, USA). The shBp4 RNA was inserted into a pcDNA6.2-GW/EmGFP-miR vector according to manufacturer´s protocol. Three different *IgfBp4* Oligos were used for insertion into the pcDNA6.2-GW/EmGFP-miR vector.

Transformation was done with the help of chemical competent DH5α E. coli cells. 25 ng of DNA were added to the chemical competent DH5α E. coli cells, and after 5 minutes of incubation on ice, the cells were heat-shocked for 45 seconds at 42°C and incubated for another ten minutes on ice. Following this incubation step 250 µl of SOC-medium were added and the transformed cells were incubated for 45 minutes at 37°C and 300 rpm. After 45 minutes of incubation, the cell suspension was plated on LB-Agar plates containing 50 µg/ml spectinomycin and incubated overnight at 37°C.

2.3.2.2 Colony PCR

Following overnight incubation, colony PCR of eight chosen colonies from the three different clones was performed with the help of EmGFP Fwd Primer and miRNA Rev Primer. Colonies were picked from the LB-Agar plates with the help of sterile pipette tips. One part of the picked colonies was mixed with the PCR reaction mix (PCR conditions and reaction mix

reagents can be found in the appendix) and the second part was used to prepare a starter culture with 2 ml of LB-Medium containing 50 µg/ml spectinomycin.

After running an 1 % agarose gelelectrophoresis with the colony PCR products (45 min, 130 V), five clones with a "proper" insert in the vector were chosen and 800 µl of the culture were mixed with 200 µl of 85 % glycerol in order to prepare a cryostock of the selected colonies. The cryostock was stored at -80°C. The rest of the selected cultures was incubated overnight in 10 ml of LB-Medium with 50 µg/ml spectinomycin at 37°C and 180 rpm. The next day plasmid minipreps of the five chosen overnight cultures were performed using the PureYieldTM Plasmid Miniprep (Promega, WI). The purified plasmids were sent for sequencing to Eurofins MWG operon (Ebersberg, Germany).

After sequence confirmation three clones were used for preparing an overnight culture with the help of the cryostock. Plasmid maxipreps were performed using EndoFree Plasmid Maxi Kit (Qiagen, Germany). The plasmid pellet was resuspended in 150 µl of TE Puffer. The eluted plasmids were pooled and used as IgfBp4 shRNA mix after determining the RNA concentration and ratio.

2.3.2.3 Sec10

The *Sec10* gene including pCMV6 Kan/ Neo plasmid (OriGene) was ordered. Transformation was done as described under the point 2.3.2.1. IgfBp4 shRNA, except of the amount of the used plasmid (2µl of the 100 ng/µl Sec10 including plasmid) and the antibiotics containing LB Agar plates, because the *Sec10* including plasmid had a Kanamycin resistance.

2.4 Plasmid Amplification and Purification

2.4.1 IgfIB-pCIneo, phIgf2-pCIneo and pCIneo

One colony of each plasmid overnight culture was picked with a sterile pipette tip and submerged into 4,5 ml LB^{amp} medium (100 µg/ml). It was incubated for 8 hours at 37°C and 180 rpm.

Plasmid minipreps of the starter cultures were done with the help of the PureYield^{TM} Plasmid Miniprep (Promega, WI). Parts of the minipreps were sent for sequencing to Eurofins MWG operon (Ebersberg, Germany), in order to determine whether the right construct was amplified. A part of the purified plasmid was used to construct a cryostock (120 µl suspension and 180 µl 50 % glycerol).

Maxiprep was done with the help of EndoFree Plasmid Maxi Kit (Qiagen, Germany) and overnight culture. Therefore 200 µl of the starter culture were added to 200 ml of selective LB^{amp} medium and cultured over night at 37°C and 180 rpm. During the whole process LB^{amp} was used as selection media as the pCIneo vector carries an ampicillin resistance gene.

2.4.2 IgfBp4 shRNA

Plasmid amplification and purification of IgfBp4 shRNA were done as described under point 1.3.2.2. Colony PCR.

2.4.3 Sec10

Plasmid amplification and purification of the *Sec10* including plasmids was done as described under point 2.4.1. IgfIB-pCIneo, phIgf2-pCIneo and pCIneo except that three colonies were picked and LB^{Kan} medium was used instead of LB^{Amp} medium.

2.5 DNA/RNA Concentration and Quality

2.5.1 IgfIB-pCIneo, phIgf2-pCIneo and pCIneo

The concentration and quality of DNA was determined by measuring the absorption at 260 nm. Measurements were carried out with the help of the UV/VIS spectrophotometry (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific Inc., Waltham, WA, USA). The concentration of the DNA was calculated with the help of the following formula and under the assumption that the concentration of pure double-stranded DNA with an A_{260} of 1,0 is 50 µg/ml.

$$c(DNA)\ [\mu g/ml] = Abs_{260} \times 50\ \mu g/ml$$

Formula 3: DNA concentration

DNA quality was assessed by additional measurements of absorbances at 280 nm and 230 nm. High quality DNA should have an A260/280 ratio between 1,8 – 2,0 and an A260/230 ratio greater than 1,5.

2.5.2 IgfBp4 shRNA

This was done according to point 1.5.1.

2.5.3 Sec10

This was done according to point 1.5.1.

2.6 Transfection

2.6.1 IgfIB-pCIneo, phIgf2-pCIneo, pCIneo and IgfBp4 shRNA

10^7 CHO EpoFc 14F2 cells in the exponential growth phase and with viability greater than 95 % were used for transfection. First of all, the cells were centrifuged at 200 x g for 10 minutes. In the meantime, the required bulk of each plasmid sample (10 µg or 20 µg depending on the experiment) was prepared. After centrifugation, the supernatant was carefully removed from the cell pellet. Right after removing the supernatant, the pellet was resuspended in 100 µl Nucleofector mixture (82 µl cell Nucleofector Solution V and 18 µl supplement I) and 20 µg of the respective plasmid. After resuspending the pellet, the cell suspension was transferred into an Amaxa certified cuvette and inserted into the cuvette holder for electroporation. In this experiment, transfection took place after the program H-14 was selected. Right after transfection 2 ml of pre-warmed medium were added with the help of a special pipette. This mixture was carefully transferred into a conical flask containing 58 ml of pre-warmed medium. For generating technical replicates the suspension was quickly split into two flasks. The cells were incubated for 2 hours at 37°C in a humidified 5 % CO_2 incubator without shaking. After 2 hours, they were transferred into a humidified 37°C/5 % CO_2 incubator with shaking. Over the next 8 days, cells were incubated under these conditions.

All transfections were carried out with the Amaxa Nucleofector System (Amaxa cell line Nucleofector Ltd., 4002 Basel, Switzerland).

Transfections were done with:

- 10 µg of IgfIB pCIneo vector and 10 µg of NCemGFP plasmid (IgfIB)
- 10 µg of IgfIB pCIneo vector and 10 µg of IgfBp4 shRNA Mix (Mix)
- 10 µg of pCIneo plasmid and 10 µg of NCemGFP plasmid (negative control - NC)
- 10 µg of IgfBp4 shRNA Mix and 10 µg of pCIneo plasmid (shBp4)

The transfections for the Igf-1 and LongR^{3}Igf-1 supplemented batches were done with:

- 10 µg of NCemGFP plasmid (negative control, without Igf-1 and LongR^{3}Igf-1 media supplement - NC)
- 10 µg of NCemGFP plasmid (negative control, with Igf-1 media supplement – NC Igf)
- 10 µg of NCemGFP plasmid (negative control, with LongR^{3}Igf-1 media supplement – NC Long)
- 10 µg of IgfBp4 shRNA Mix (shBp4, without Igf-1 and LongR^{3}Igf-1 media supplement)
- 10 µg of IgfBp4 shRNA Mix (Mix, with Igf-1 media supplement – Mix Igf)
- 10 µg of IgfBp4 shRNA Mix (Mix, with LongR^{3}Igf-1 media supplement – Mix Long)

More information about the respective plasmids (plasmid map) can be found in the appendix. Later on, the batches done with these transfections were named Igf batch.

2.6.2 Sec10

Transfection of the CHO DUKX-B11 EpoFc 14F2 cells with the *Sec10* including plasmid took place as described under point 2.6.1 IgfIB-pCIneo, phIgf2-pCIneo, pCIneo and IgfBp4 shRNA.

Transfections were done with:

- 10 µg of pCMV6 with *Sec10* insert
- 10 µg of pCMV6 without *Sec10* insert
- Without any plasmid (Mock transfection)

More information about the plasmid (plasmid map) can be found in the appendix.

2.7 Igf Batch

Each batch was monitored for 8 days. Viable cell density and viability were determined with the help of the viable cell analyser (Beckman Coulter, USA) or with the hemocytometer method on day 1, 2, 3, 4, 6 and 8. Total cell number was determined with Coulter Counter measurements on day 1, 2, 3, 4, 6 and 8.

Using the Coulter Counter method it was possible to obtain more exact results of the total cell density. With the help of the ViCell or hemocytometer information about the viability, some was able to determine the viable cell density out of the Coulter Counter measurements.

$$viability = \frac{viable\ cells}{total\ cells} * 100$$

Formula 4: Viability

The growth rate can be calculated with the help of the viable cell density (VCD).

$$growth\ rate\ \left[\frac{1}{d}\right] = \frac{ln\left(\frac{x_1}{x_0}\right)}{d}$$

Formula 5: Growth rate

The supernatant was taken on day 1, 2, 3, 4, 6 and 8. It was used for determining the specific productivity with the help of the Enzyme Linked Immunosorbent Assay (ELISA) and

for determining the glucose and lactate concentration on day 2, 4 and 8 with the help of a bioanalyser.

RNA samples were taken on day 2, 4 and 8 for determining gene expression using qPCR. On day 2, approximately 48 h after transfection, 1 ml of cell suspension was spun down and resuspended in PBS. After resuspending the cell pellet, the sample was analysed with the help of a flow cytometer with FACSCalibur (BD Biosciences). The aim of this analysis was to determine the amount of GFP expressing cells. Cells that were successfully expressed have a strong GFP signal, whereas cells that have no pCIneo plasmid have a weak GFP signal. Non transfected CHO DUKX-B11 EpoFc 14F2 cells were used as negative control.

2.8 RNA Isolation

Every second, fourth and eighth day 0,5 x 10^6 – 1,2 x 10^6 cells were harvested and resuspended in 500 µl of TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Right after adding TRIzol reagent, the cells were lysed with the help of a vortex mixer, incubated for 5 minutes on room temperature, vortexed again, and frozen at -80°C until RNA extraction was performed.

After thawing the samples 0,1 ml of Chloroform were added, mixed and incubated at room temperature for 3 minutes. Right after this step the solution was centrifuged at 4°C, 12000 x g and for 15 minutes. Chloroform was added for separating the homogenate into an aqueous, RNA containing phase, a DNA containing interphase and a protein containing organic phase. Continuing with the aqueous, upper RNA containing phase, 0,25 ml of isopropanol were added in order to precipitate the RNA. This solution was mixed, incubated for another 10 minutes and centrifuged again for 10 minutes at 4°C and 12000 x g.

Continuing with discarding the supernatant, the pellet was washed with 0,5 ml of 70 % Ethanol, mixed, and spun down for 5 minutes at 7500 x g and 4°C. The supernatant was carefully removed as well as possible. After drying the pellet it was dissolved in 40 µl of NF water and incubated at 60°C for 10 minutes. RNA quality and concentration was determined by measuring the absorption at 260 nm, the A260/280 and the A260/230 ratio using NanoDrop (Thermo Scientific, Wilmington, USA). The A260/280 ratio for pure RNA should lie between 1,8 and 2,0 and the A260/230 ratio should exert 2,0. After that procedure the RNA was stored at -80°C.

2.9 Reverse Transcription (Igf Experiment)

Max. 1 µg of RNA were reverse transcribed with the help of the DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA synthesis mixture was prepared according to the DyNAmo cDNA Synthesis Kit. The Kit includes oligo dT primers and random hexamer primers. The reverse transcription of the Igf experimental RNA was done with the help of random hexamer primers, which transcribe all RNA so that the transcribed cDNA covers the whole transcript. The cDNA synthesis step was extended up to 60 minutes in order to gain long or rare transcripts.

2.10 Quantitative PCR (Igf Experiment)

The use of qPCR to amplify cDNA products is a routine tool for studying gene expression with reliable and rapid quantification results. The method is very sensitive and even allows quantification of small changes in gene expression. A table of the used primers and the associated sequence can be found in the appendix.

2.10.1 qPCR Amplification (Igf Experiment)

Amplification of the *IgfIB*, *Igf1R*, *IgfBp4* and the housekeeping gene *actb*, was done using the same primers as for standard preparation and the SensiMix SYBR Hi-ROX Kit (Bioline, London, UK). qPCR was performed with the help of the Rotor-Gene Q machine (QIAGEN, Hilden, Germany) and a reaction volume of 10 µl. The reaction components and their amounts in the qPCR mix as well as the cycling conditions can be found in the appendix. With the help of a relative quantification model the quantity of the target gene was first normalized against a reference gene like *actb* for the amount of RNA added to the reverse transcription reactions (formula 6). The amplification efficiency of the target gene and the reference gene should be the same. If it is not the same the results have to be corrected for the efficiency.

$$\Delta C_{T,\ sample} = C_{T,\ target} - C_{T,\ reference}$$

Formula 6: Difference of the threshold cycles between target and reference

If the efficiency of the target gene and the reference gene is the same the comparative $2^{-\Delta\Delta cT}$ can be used for relative quantification. In this model the data is presented as fold change in gene expression, normalized to an endogenous reference gene and relative to the untreated control.

$$2^{-\Delta\Delta C_T} = 2^{-\Delta C_{T,sample} - \Delta C_{T,untreated\ control}}$$

Formula 7: Fold change in gene expression between sample and untreated control

The gene expression was only analysed with help of the $2^{-\Delta\Delta cT}$ method because the melting curves of the standards didn´t fit together.

2.11 ELISA

Enzyme Linked Immunosorbent Assay (ELISA) was used to determine the EpoFc titer and the specific productivity. The 96 well plates (Nunc-Immuno 96 Micro Well Solid Plates Maxisorp™, Thermo Fisher Inc., Waltham, MA, USA) were coated with a 1:1000 diluted capture antibody (1 mg/ml Goat Anti-Human IgG gamma-Chain Specific, Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4°C. The capture antibody was diluted with coating buffer. The next day, the plates were washed four times with washing buffer to get rid of the non-bound capture antibody and coating buffer.

After the washing step, 50 µl of the pre-diluted culture samples and standards, which were serially diluted with dilution buffer, were added to the coated 96 well plates and incubated for 1 hour at room temperature. In the next step, the plates were washed again four times with washing buffer and 50 µl of horse radish peroxidase conjugated antibody (0,5 – 4,0 mg/ml Goat Anti Human IgG-HRP, Life Technologies, Carlsbad, CA, USA) were added. The HRP-GAH antibody was pre-diluted 1:1000 with dilution buffer. The incubation of the conjugated antibody took place for another hour at room temperature. After one hour the plates were washed again four times and 100 µl of staining solution were added per well. The reaction was stopped by adding 100 µl of 2,5 M H_2SO_4. The absorbance was determined with the help of a 96 well plate reader (Sunrise, TECAN Group Ltd.) at 492 nm and 620 nm as a reference. Analysis of the absorbance was done with the help of the Magellan™ software (TECAN Group Ltd.). The specific productivity (formula 8) was determined with the growth data from the batches and the titers, which were calculated under the use of the standard curve.

$$qP\left[pg * cells^{-1} * day^{-1}\right] = \frac{p_n - p_1}{\sum_{i=1}^{n} \frac{\left(x_{i+1} - x_i\right) * \left(t_{i+1} - t_i\right)}{\left(ln\left(x_{i+1}\right) - ln\left(x_1\right)\right)}}$$

Formula 8: Specific productivity. p_n and p_1 are the titers in $pg*ml^{-1}$ at the time points t_{i+1} and t_i. X_{i+1} and x_i are viable cell densities at the time points t_{i+1} and t_i.

The recipes for the coating buffer, wash buffer, dilution buffer, staining buffer and staining solution can be found in the appendix.

2.12 ANOVA (Analysis of Variance)

Analysis of variance (ANOVA) was used to test whether the transfections showed significant differences. ANOVA is a statistical method to perform a comparison between more than two

groups. Depending on the number of factors a one – way or multiple – way analysis of variance are made. The "factor" is defined as an interesting characteristic (e.g. different transfections) measured on a nominal scale. With the help of the p – value the probability of obtaining a result at least as extreme as the observed one assuming the null hypothesis can be described. A typical null hypothesis expects that all treatments have the same effect (e.g. all different transfections show nearly the same average VCD). The null hypothesis is rejected when the p – value is below 0,05. A p – value below 0,05 indicates a significant difference, whereas p – values greater than 0,05 indicate no significant difference.

For performing a correct ANOVA two main assumptions need to be achieved:

- The distribution of the residuals should be normal
- The variance between data in groups should be the same (homogeneity of variances)

2.12.1 Post – hoc significance tests

The Bonferroni test and the Tukey test belong to the group of the post-hoc significance tests. The ANOVA only determines if there is a significant difference between a group of mean values. For determining which mean value is significant to each other the Bonferroni test or Tukey test have to be made. Analysis of variance and the Bonferroni test were computed with GenStat. To determine the exact p – values the Bonferroni test and Tukey test were made with Minitab.

2.13 Sec10 Batch

The transfected cells were incubated for 8 days under batch conditions. On day 1, 2, 3, 4 and 8 the viable cell density and viability were determined using the ViCell analyser (Beckman Coulter, USA).The growth rate was calculated with the help of the viable cell density as described under point 1.7. Supernatant samples for determining specific productivity with the help of the ELISA method were taken on day 1, 2, 3, 4 and 8. On day 2, 4 and 8 RNA samples were taken.

2.13.1 Specific Productivity

Specific productivity was determined with the help of the ELISA method described in point 2.11.

3 Results

3.1 Igf Batches – Comparison between the Different Transfections

The aim of this study was to test the effect of the transient overexpression of *IgfIB* and *Sec10* and the transient knockdown of *IgfBp4* on viability, viable cell density, specific productivity and protein titer of a recombinant EpoFc expressing CHO cell line.

For this purpose four transient transfection experiments were performed, with the effect of knockdown and overexpression being monitored during batch cultivation with two technical replicates. Batches 1 and 2 were biological replicates. Each of the four different batches was run with cells transfected with the empty plasmid as negative control, the IgfIB expression vector and cells transfected with a mix of the shBp4 and IgfIB expression vectors to see whether knockdown of *IgfBp4* enhances the effect of Igf-1 on cell growth. In addition, during the third batch a transfection of shBp4 alone was tested. The fourth batch was performed in media supplemented either with LongR^{3}Igf-1 or Igf-1 instead of the IgfIB expression. In order to test the effect of *IgfBp4* knockdown under these conditions, the cells were additionally transfected with shBp4.

3.1.1 Igf Batch 1 and 2 – GFP Measurement

In this experiment the cells were transfected with the empty plasmid as negative control, the IgfIB expression vector and a mix of the shBp4 and IgfIB expression vectors (Mix). All the different expression vectors included an emGFP sequence, which was used to determine the transfection efficiency. The same constructs were used in all four Igf batches. The emGFP expression from the shRNA vectors, the IgfIB expression vectors and the negative control vectors was monitored 48 hours after transfection using flow cytometry.

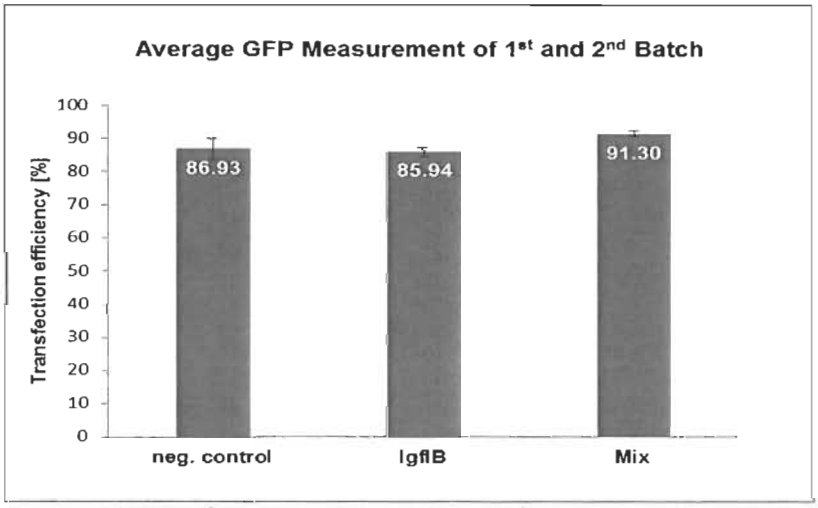

Figure 1: Flow cytometry analysis of the samples 48 h after transfection. The average transfection efficiency of Igf 1st and 2nd batch is displayed. The original data is displayed in the appendix.

Untransfected CHO EpoFc 14F2 cells were used to determine the amount of autofluorescence. Figure 1 indicates that more than 86 % of the negative control transfected cells, which were selected by gate G1 contained GFP. The cells transfected with the *IgfIB* construct showed more than 85 % of cells expressing GFP. Around 91 % of the Mix transfected cells contained GFP. Overall, GFP expression is consistent, indicating a high and reproducible transfection efficiency.

3.1.2 Quantification of IgfBp4 by quantitative RT-PCR (qPCR)

As described in point 2.8, RNA samples from the different batches were collected. After reverse transcription of the different isolated RNA samples, the expression level of the *IgfBp4* gene was measured by qPCR in all transfections. *Actb* was used as housekeeping gene for normalization according to the deltadelta-Ct method. The results indicate a successful knockdown of *IgfBp4* expression after 48 h for both the individual shBp4 and mixed shBp4 transfection, as shown in Figure 2 in the case of batch 3.

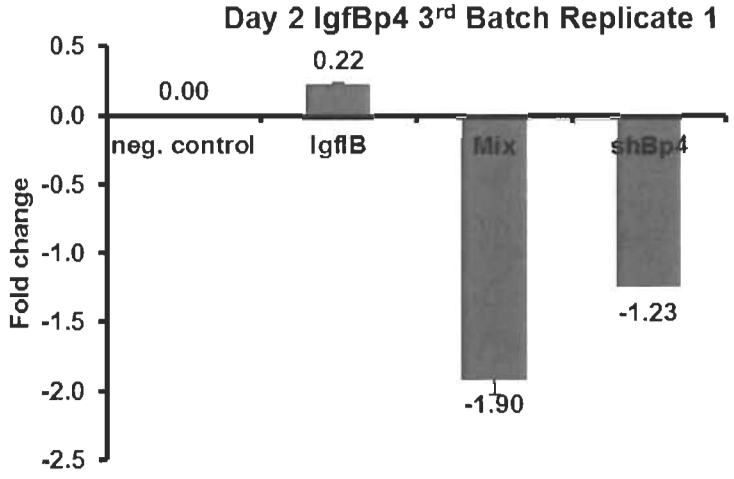

Figure 2: Fold change of *IgfBp4* gene expression normalized to the negative control. *IgfBp4* gene expression was analysed with the help of generated cDNA from the four different transfection samples of the 3rd batch on day 2 of batch culture. In this case the examined cDNA was taken from replicate 1.

3.1.3 Igf Batch 1 and 2 – Viable Cell Density (VCD) and Growth

The viable cell density and growth of the different batches were determined with the help of the ViCell analyser and Multisizer™ 3 Coulter Counter (see section 2.2 and section 3.1.5.1).

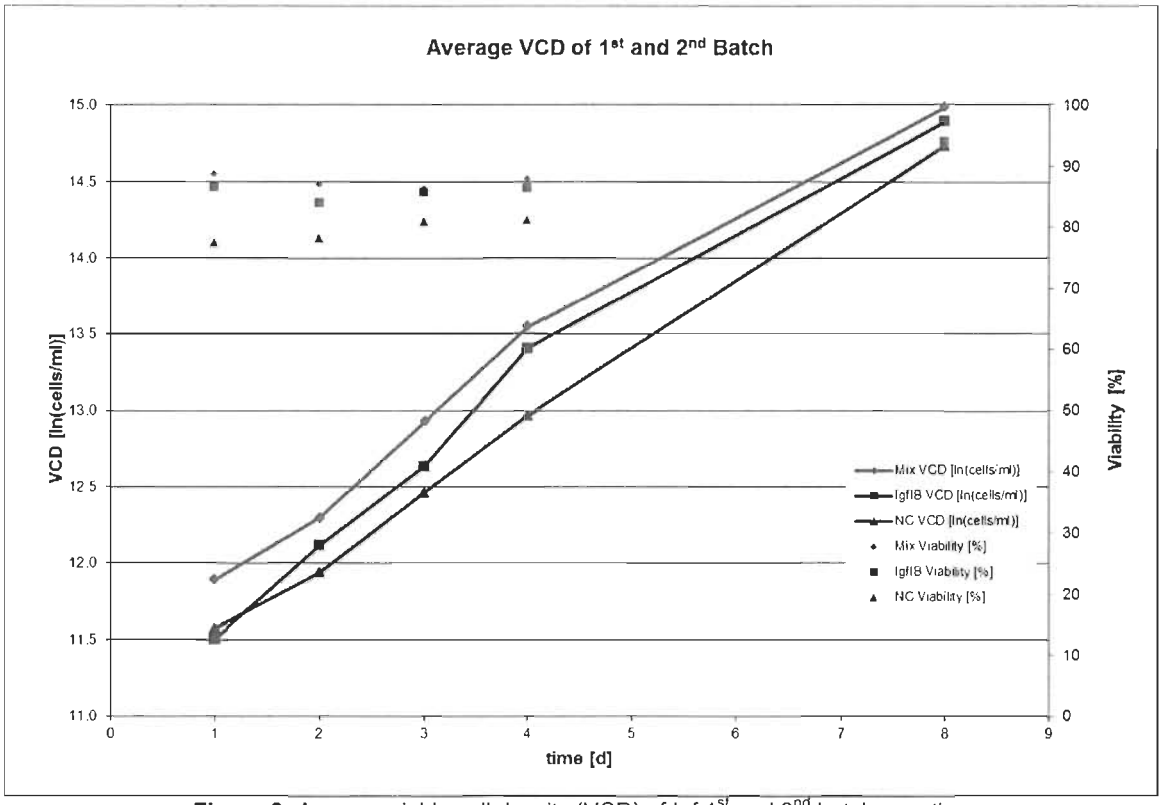

Figure 3: Average viable cell density (VCD) of Igf 1st and 2nd batch over time

Figure 3 indicates that starting with day 2 all samples had a higher VCD than the negative control. Over all eight days of batch culture the cells transfected with *IgfIB* and shBp4 (Mix) revealed the highest VCD. On day 1 of the batch culture the *IgfIB* transfected cells had the lowest VCD, probably because these cells were stressed more than the others through transfection. Observing the viability of the different samples reveals, that the negative control cells had the lowest viability until day 8 of batch culture and the Mix transfected cells had the highest viability. On day 8 of batch culture all samples had 94 % viability.

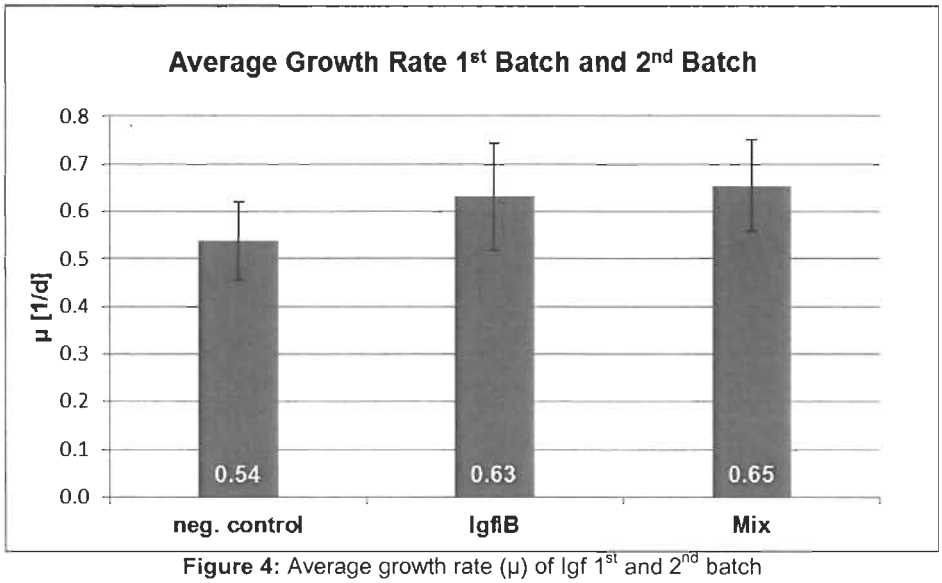

Figure 4: Average growth rate (μ) of Igf 1st and 2nd batch

The average growth rates shown in figure 4 were calculated with the help of the viable cell densities between day 1 and 4 of batch 1 and 2 and the equation shown in formula 5. The Mix transfected cells (*IgfIB* and shBp4 transfected cells) and *IgfIB* transfected cells had a 1,2 times higher growth rate than the negative control cells, but both transfections did not reveal a significant better result than the negative control.

3.1.3.1 Comparison between the ViCell analyser and Multisizer™ 3 Coulter Counter

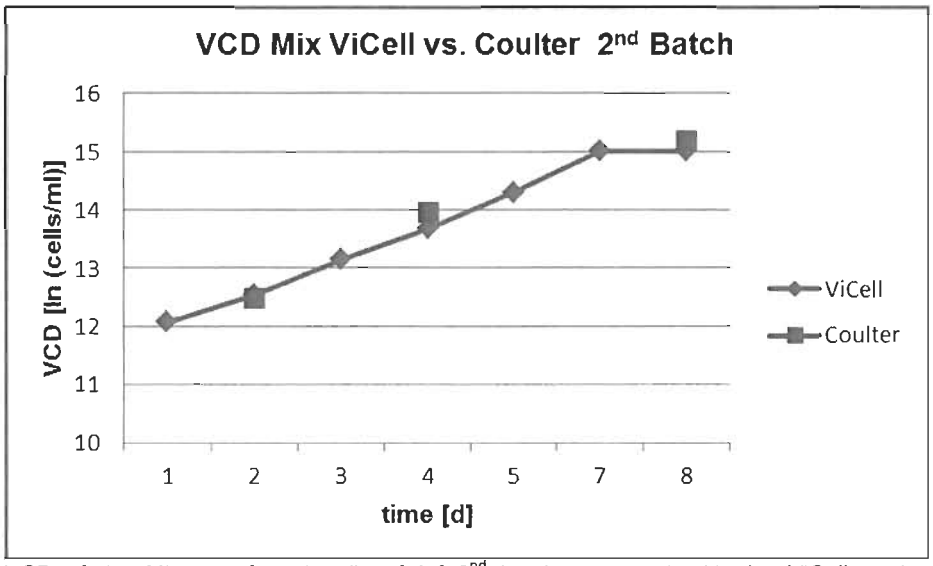

Figure 5: VCD of the Mix transfected cells of Igf 2nd batch measured with the ViCell analyser and the Multisizer™ 3 Coulter Counter.

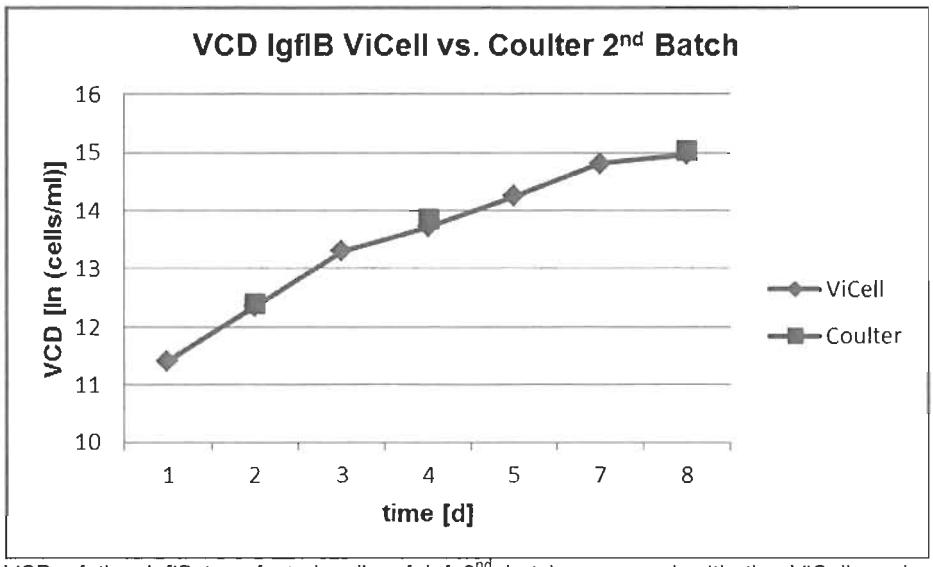

Figure 6: VCD of the *IgfIB* transfected cells of Igf 2nd batch measured with the ViCell analyser and the Multisizer™ 3 Coulter Counter.

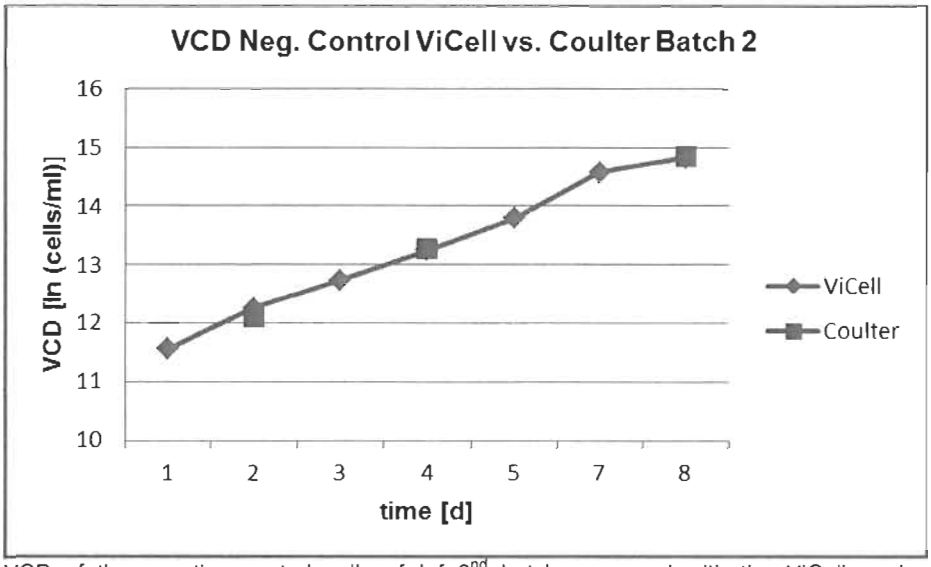

Figure 7: VCD of the negative control cells of Igf 2nd batch measured with the ViCell analyser and the Multisizer™ 3 Coulter Counter.

Figure 5, 6 and 7 indicate, that there is no big difference between the two measurements of the VCD. As a result of this test the VCD`s of all batches were calculated with the viability measured with the help of the ViCell analyser and the total cell density measured with the Multisizer™ 3 Coulter Counter according to formula 4.

3.1.3.2 Comparison between Igf Batch 1 and Igf Batch 2

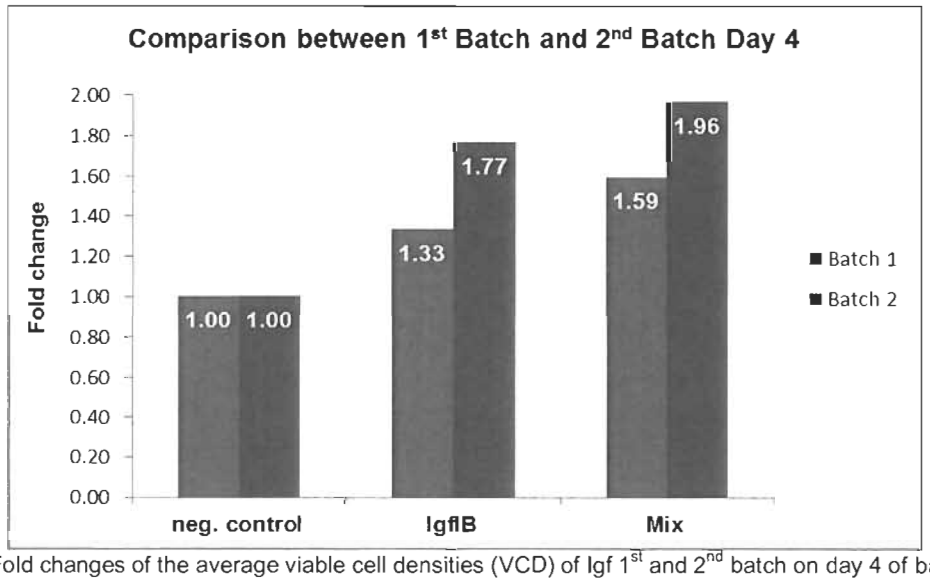

Figure 8: Fold changes of the average viable cell densities (VCD) of Igf 1st and 2nd batch on day 4 of batch culture

This figure indicates the general trend, that the cells transfected with *IgfIB* and shBp4 (Mix) had the highest average viable cell density, followed by the VCD of the *IgfIB* transfected cells. Figure 8 also reveals that the average VCD of batch 2 is higher than the average viable cell density of batch 1.

3.1.3.3 Correlation Transfection Efficiency – Viable Cell Density (VCD) Igf Batch 1 and 2

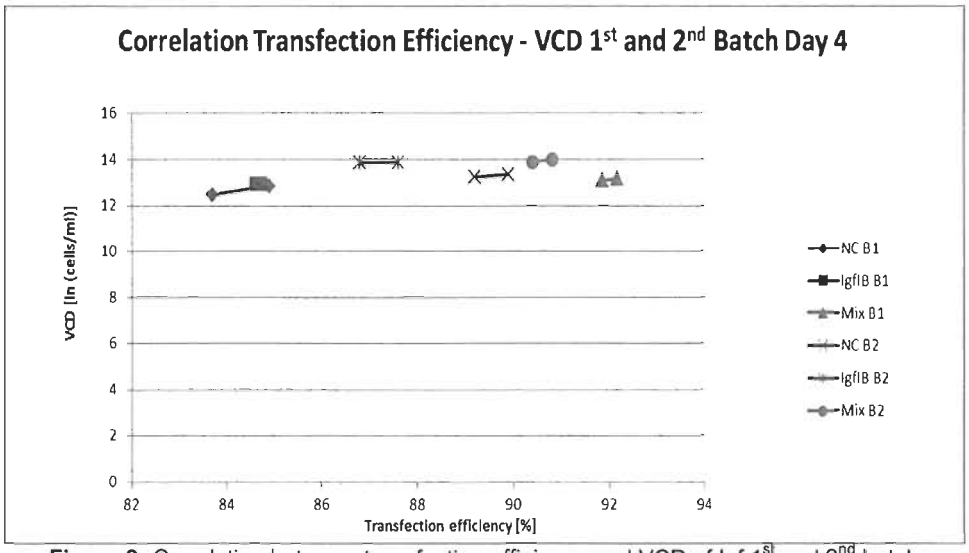

Figure 9: Correlation between transfection efficiency and VCD of Igf 1st and 2nd batch

Figure 9 reveals that the transfection efficiency does not influence the viable cell density of the different transfected samples, because the slope of the straight lines is nearly zero.

3.1.4 Igf Batch 1 and 2 – Specific Productivity and Titer

The ELISA method was used to determine the impact of the different transfections on the specific productivity and the EpoFc titer.

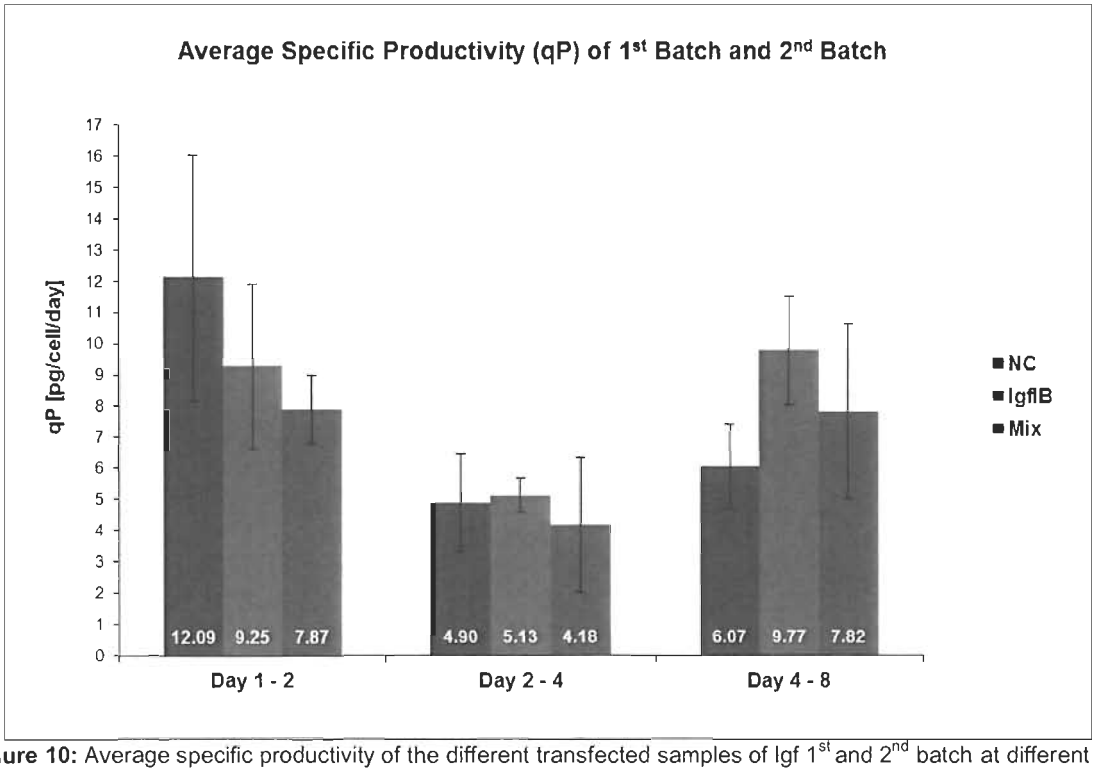

Figure 10: Average specific productivity of the different transfected samples of Igf 1st and 2nd batch at different days

Figure 10 describes the average specific productivity of the different samples on different days out of the 1st and 2nd batch. The highest specific productivity was found on day 1 – 2 (lag phase). The lowest specific productivity was found on day 2 – 4 (exponential phase). Comparing the highest productivities on the different days indicated that the negative control cells had the highest productivity on day 1 – 2, whereas on day 2 – 4 and on day 4 – 8 the *IgfIB* transfected cells showed the highest productivity of all different samples.

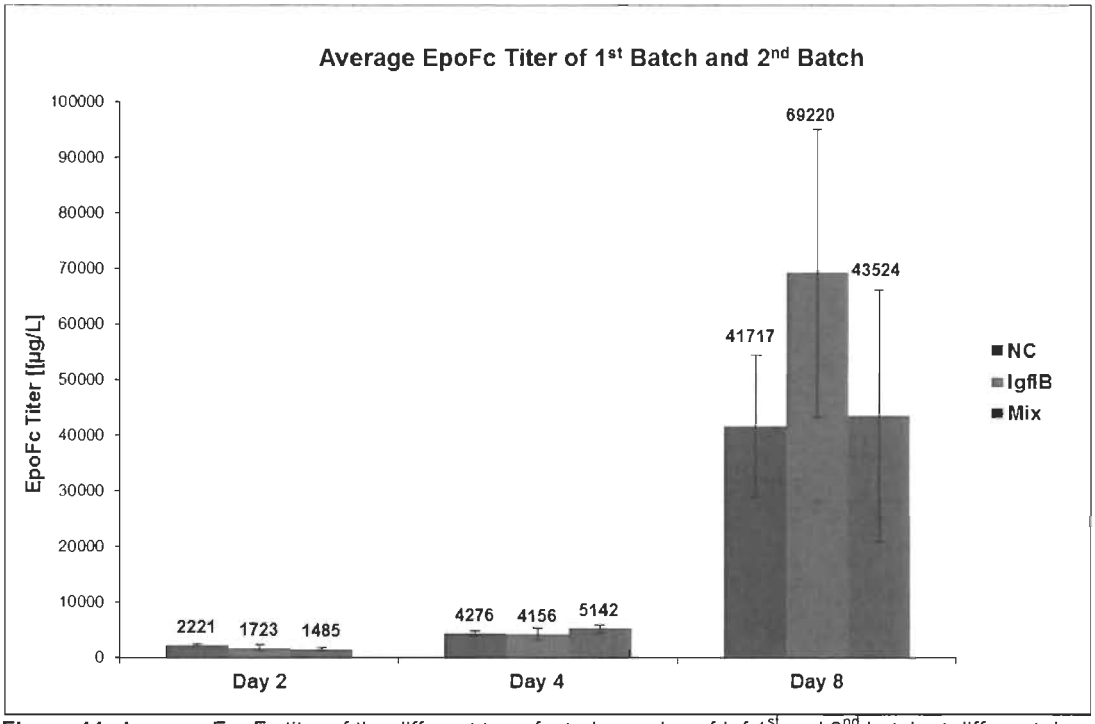

Figure 11: Average EpoFc titer of the different transfected samples of Igf 1st and 2nd batch at different days

Figure 11 shows the average EpoFc titer of the different samples out of the 1st and 2nd batch. Despite the high standard deviation of all samples on day 8 of batch culture, the *IgfIB* transfected sample revealed the highest amount of product formation, followed by the negative control and the Mix transfected sample, which showed the lowest amount of product formation.

3.1.5 Igf Batch 3 – GFP Measurement

In order to test the effect of the shBp4 alone, an additional transfection was performed in Igf batch 3. The rest of this experiment was identical to Igf batch 1 and Igf batch 2. With the help of flow cytometry 10 000 events were analysed 48 hours after transfection to determine the amount of viable transfected, GFP expressing cells.

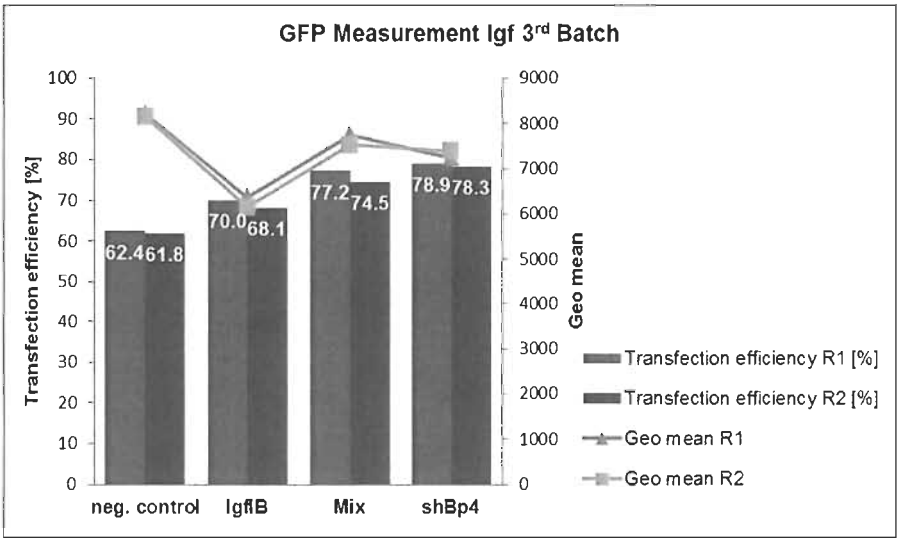

Figure 12: Flow cytometry analysis of the samples 48 h after transfection. The transfection efficiency and geo mean are displayed. The original data is displayed in the appendix.

Untransfected CHO EpoFc 14F2 cells were used to determine the amount of autofluorescence. Figure 12 indicates, that more than 61 % of the negative control transfected cells, which were selected by gate P1 contained GFP. Around 68 % of the *IgfIB* transfected cells expressed GFP. More than 74 % of the Mix transfected cells expressed GFP. Among the shBp4 transfected cells 78 % contained GFP.

3.1.6 Igf Batch 3 – Viable Cell Density (VCD) and Growth

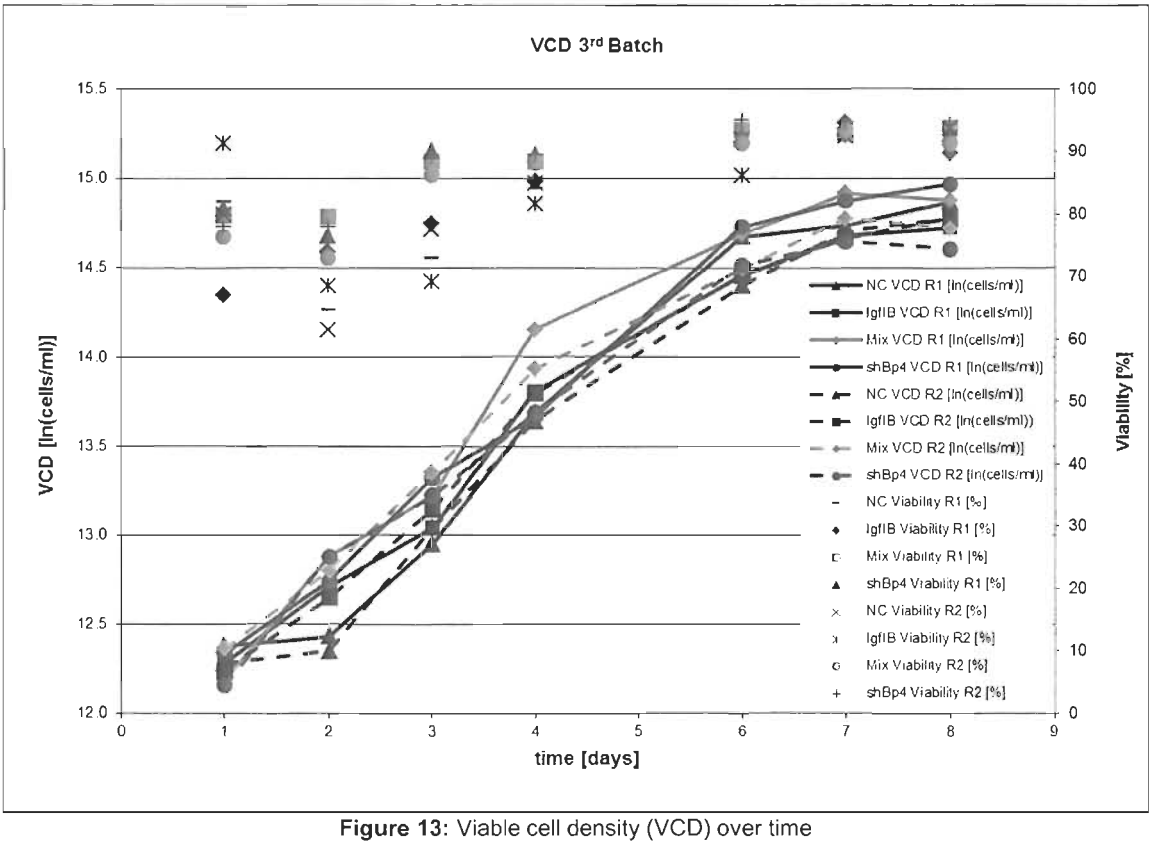

Figure 13: Viable cell density (VCD) over time

Figure 13 reveals that the negative control replicate 2 had the lowest viable cell density until day 7 of batch culture. The Mix transfected replicates (shBp4 and *IgfIB* transfected cells) showed the highest VCD until day 7 of batch culture. Between day 7 and 8 the Mix transfected samples were already in the decline phase whereas the other samples were in the stationary phase or still in the late exponential phase. Interestingly between the first and the second day of batch culture it looks like the negative control samples are still stress exposed, in contrast to the other transfected samples, which do not seem to be stressed anymore. Considering the viability of the different samples, from day 4 on all samples had a viability above 80 %. The *IgfIB* transfected replicate 1 had the lowest viability on day 8 of batch culture (89,8 % viability). The viability of the samples between day 1 and 2 was always above 60 %. Interestingly all samples revealed a decrease in viability on day 2 of batch culture, on day 3 of batch culture the viability of all samples was again elevated and above 70 %.

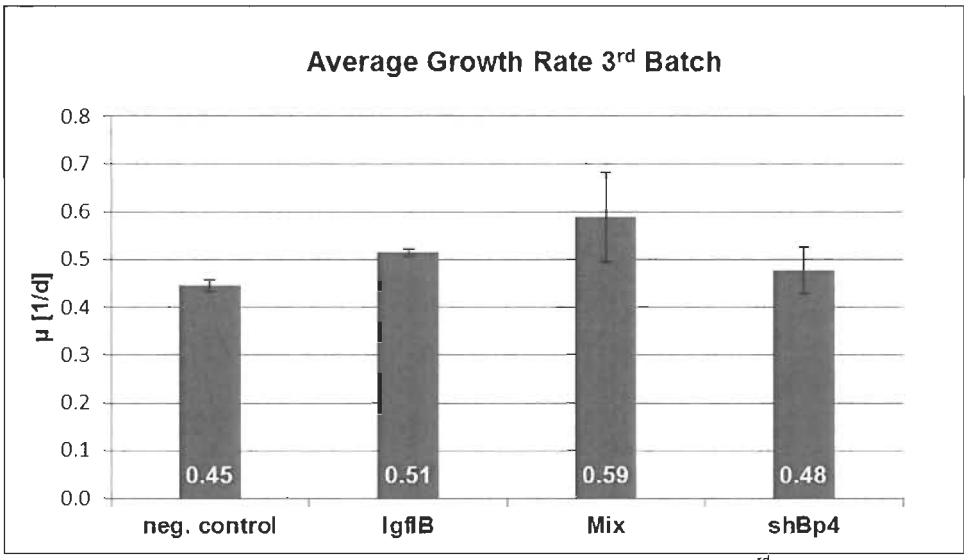

Figure 14: Average growth rate (μ) of the 2 replicates of Igf 3rd batch

Figure 14 displays the average growth rates, which were calculated with the help of the viable cell densities between day 1 and day 4 of batch 3 and the equation shown in formula 5. Comparing the average growth rate of the Mix transfected cells (*IgfIB* and shBp4 transfected cells) with the negative control cells indicates, that the Mix transfected cells had a 1,3 times higher average growth rate. The *IgfIB* transfected cells, the shBp4 transfected cells and the negative control cells showed no big difference in growth characteristics.

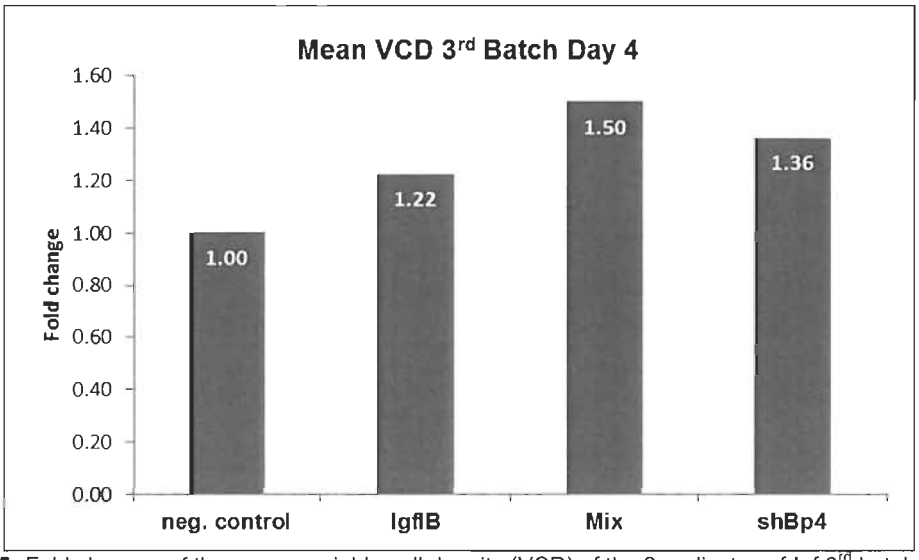

Figure 15: Fold changes of the average viable cell density (VCD) of the 2 replicates of Igf 3rd batch on day 4

The fold changes of the VCD in figure 15 reveal that the Mix transfected sample had the highest average VCD, followed by the shBp4 transfected sample and the *IgfIB* transfected sample.

3.1.6.1 Correlation Transfection Efficiency – Viable Cell Density (VCD) Igf Batch 3

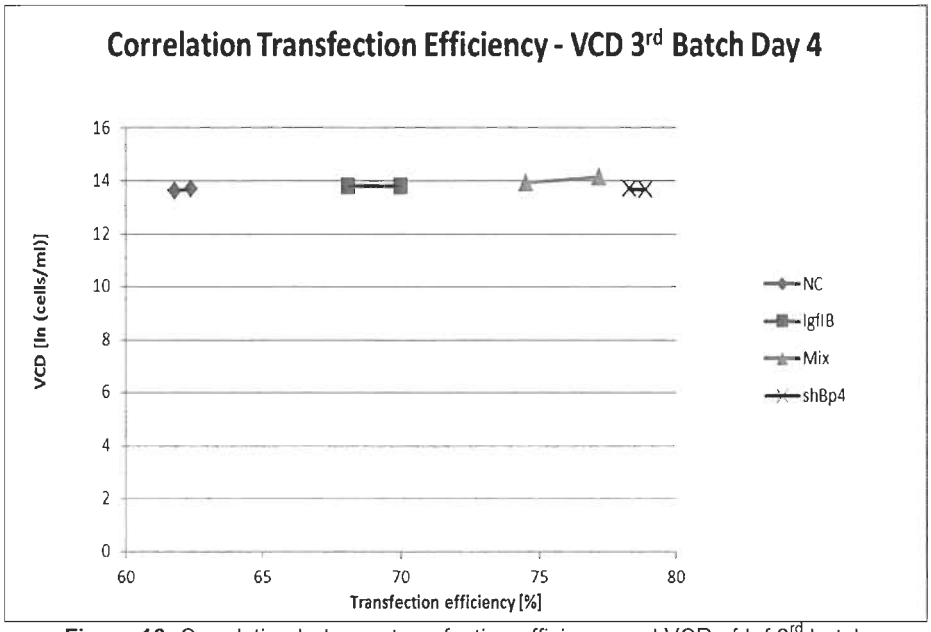

Figure 16: Correlation between transfection efficiency and VCD of Igf 3rd batch

The slope of the straight lines in figure 16 indicates that the transfection efficiency does not influence the viable cell density of the different transfected samples in batch 3.

3.1.6.2 Statistical Analysis of the Viable Cell Density (Igf Batch 1, 2 and 3)

In order to determine whether the transfections showed significant differences an ANOVA of the log10 of the viable cell densities was made. ANOVA was made with the log10 of the VCD, because the non-transformed data showed too high differences between the different batches. In total three batches were analysed. Each transfection had two technical replicates of which the mean values of the VCD per batch were calculated. The ANOVA method is sensitive to outliers and skew distributions. Before starting with the ANOVA analysis the data has to be visualized. In this study a scatterplot was made to verify, if there are outliers or skew distributions within the groups. The scatterplot in Figure 17 illustrates a comparison of the log10 of the mean VCD values per batch. Figure 17 indicates that the assumptions of ANOVA are valid.

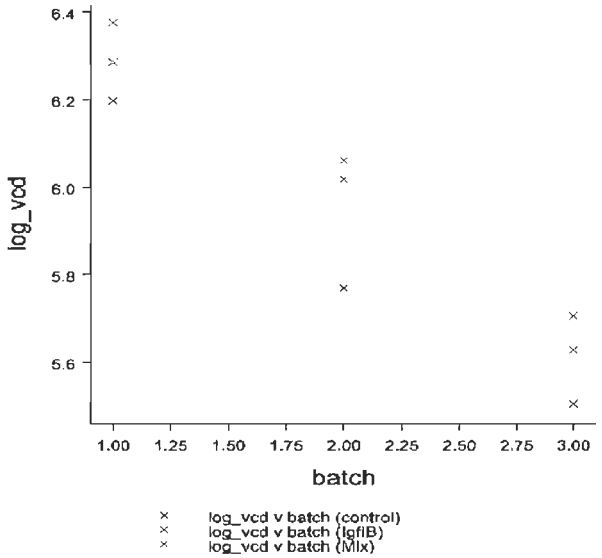

Figure 17: Scatterplot of the log10 of the mean VCD´s of batch 1, batch 2 and batch 3

A two - way ANOVA was chosen to test the effects of the transfection (negative control, IgfIB and Mix) and of batch (batch 1, 2 and 3) on log10 VCD.

Analysis of variance

Variate: log_VCD

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
transfection	2	0.078327	0.039163	20.61	**0.008**
batch	2	0.680687	0.340343	179.12	<.001
Residual	4	0.007600	0.001900		
Total	8	0.766614			

Table 2: Analysis of variance results; d. f.: degree of freedom; s.s.: sum of squares; m.s.: mean squares; v.r.: variance ratio, also labeled F - value; F pr.: probability of obtaining the observed v.r. or a larger one; also labeled P – value.

The red labeled value in table 2 reveals that there is a significant difference between the different transfections. Differences between the batches are also significant.

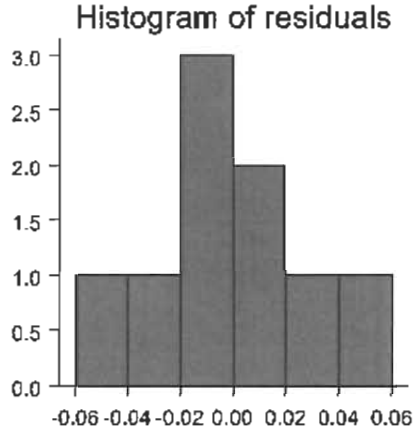

Figure 18: Histogram of the residuals. The x – axis equals the residuals and the y – axis equals the frequency of the values.

The histogram of residuals (figure 18) indicates that the distribution of the residuals approximately corresponds to a normal distribution. Hence the presence of outliers and skew distributions can be excluded.

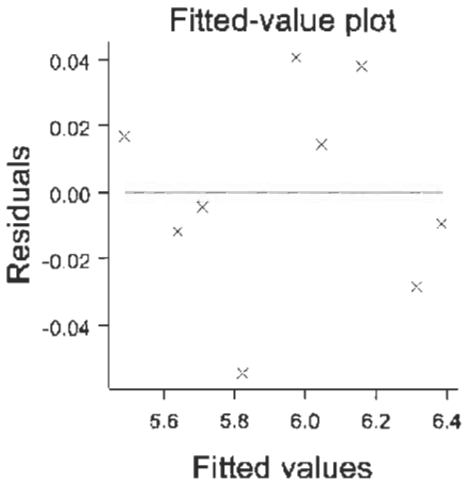

Figure 19: Fitted-value plot. The x – axis displays the log10 VCD of the three batches and the y-axis displays the residuals.

The fitted-value plot in figure 19 displays, that there are not any extremely conspicuous features. As it is already demonstrated in the histogram, no extremely outlying residuals can be found. To determine, which of the different transfections show a significant difference, a Bonferroni test was made.

Bonferroni test

Experimentwise error rate = 0.0500
Comparisonwise error rate = 0.0167

Mean	vs Mean	t	significant
Neg.control	IgfIB	-4.307	**Yes**
Neg. control	Mix	-6.277	**Yes**
IgfIB	Mix	-1.970	**No**

Table 3: Bonferroni test results; t: t – value.

The Bonferroni test (table 3) shows, that the difference between the VCD of the negative control and the *IgfIB* transfected cells is significant (p = 0,0377). The same is valid for the difference between the mean VCD of the negative control and the mean VCD of Mix transfected cells (p = 0,0099). The difference between the mean VCD of the *IgfIB* transfected cells and the mean VCD of the Mix transfected cells is not significant (p = 0,3606). The significance observations are based on p – values < 0,05.

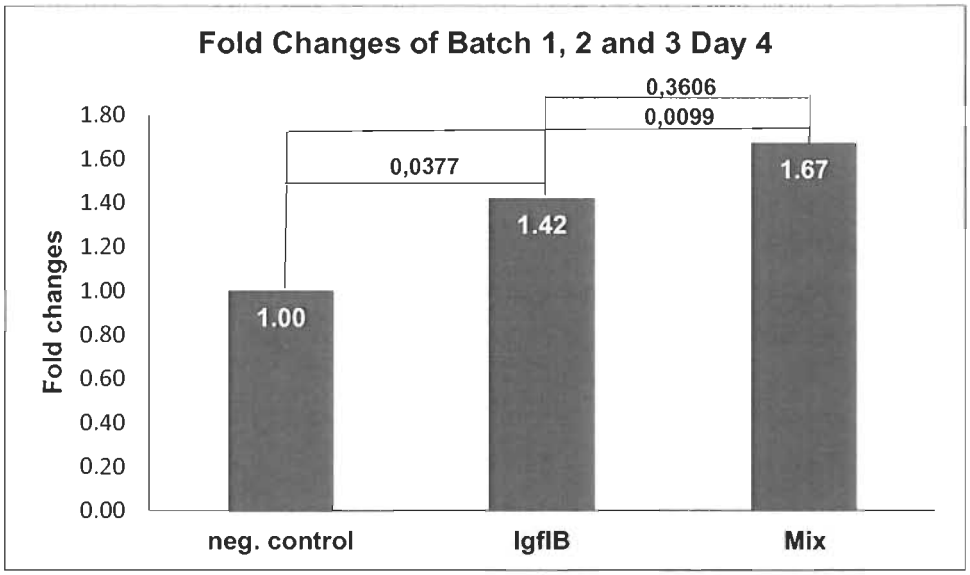

Figure 20: Fold changes of the original mean VCD values of batch 1, 2 and 3. The p – values for the different samples are displayed between the respective samples.

Figure 20 shows, that the mean VCD of the *IgfIB* transfected cells was 1,42 times higher than the mean VCD of the negative control. Also the Mix transfected cells revealed a 1,67 times higher mean VCD as the negative control. These values are also reflected in the p – values between the samples. As described in figure 20 the mean VCD of the Mix transfected cells is only 1,18 times higher than the mean VCD of the *IgfIB* transfected cells, also the p – value indicates a non-significant difference between the two samples.

3.1.6.3 Statistical Analysis of the Growth Rate (Igf Batch 1, 2 and 3)

The two – way ANOVA was also used to test the effects of the different batches (batch 1, 2 and 3) and the different transfections (neg. control, IgfIB and Mix) on the growth rate.

Analysis of Variance for μ

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Batch	2	0,051308	0,051308	0,025654	27,74	0,005
Clone	2	0,024603	0,024603	0,012302	13,30	0,017
Error	4	0,003700	0,003700	0,000925		

Table 4: Analysis of variance results; DF: degree of freedom; Seq SS: sequential sum of squares; Adj SS: adjusted sum of squares; Adj MS: adjusted mean squares; F: F – value; P: P – value.

The red labeled value in table 4 indicates that there is a significant difference between the different transfections and also the differences between the batches are significant. In order to determine, which transfections show significant differences, a Tukey test was made.

Tukey Simultaneous Tests
Response Variable μ
All Pairwise Comparisons among Levels of clone

clone = IgfIB subtracted from:

Clone	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
Mix	0,04003	0,02483	1,612	0,3407
Neg	-0,08534	0,02483	-3,437	0,0559

clone = Mix subtracted from:

Clone	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
neg	-0,1254	0,02483	-5,049	0,0158

Table 5: Tukey test; SE of Difference: standard error of difference.

Table 5 reveals that the differences between the mean growth rate of the *IgfIB* transfected cells and the Mix transfected cells (*IgfIB* and shBp4 transfected cells) as well as differences between the mean growth rate of the negative control cells and the *IgfIB* transfected cells were not significant. By comparing the growth rate of the Mix transfected cells with the growth rate of the negative control, a significant difference was observed (p = 0,0158).

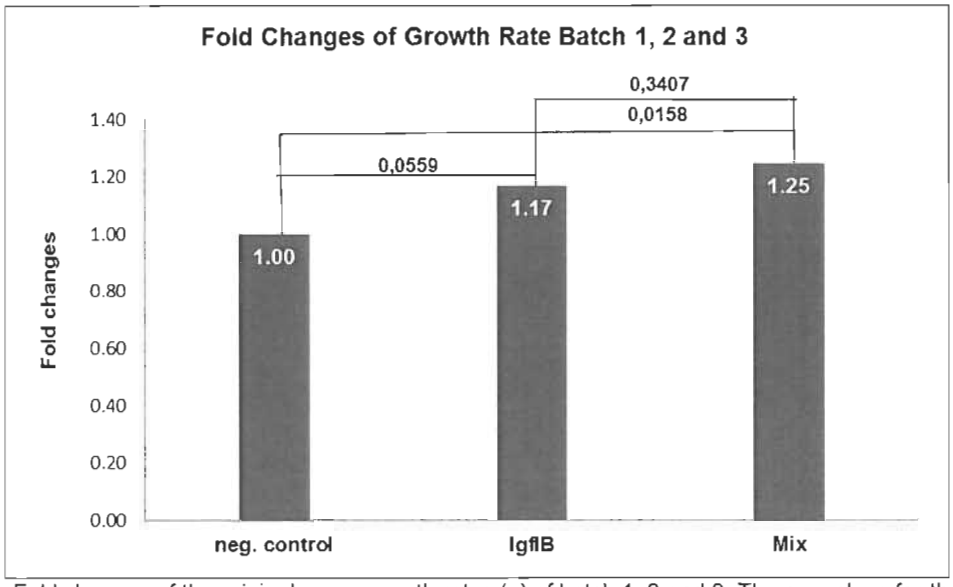

Figure 21: Fold changes of the original mean growth rates (μ) of batch 1, 2 and 3. The p – values for the different samples are displayed between the respective samples.

The fold changes in figure 21 reveal, that the Mix transfected cells (*IgfIB* and shBp4 transfected cells) had a 1,25 times higher mean growth rate than the negative control cells. This observation is also confirmed by the p – value between the samples. In contrast, the mean growth rate of the *IgfIB* transfected cells was only 1,17 times higher than the mean growth rate of the negative control and indicated no significant difference. Regarding the mean growth rate of the *IgfIB* transfected samples and the Mix transfected samples shows, that there is no significant difference between the samples, the two samples revealed nearly the same mean growth rate.

3.1.7 Igf Batch 3 – Specific Productivity and Titer

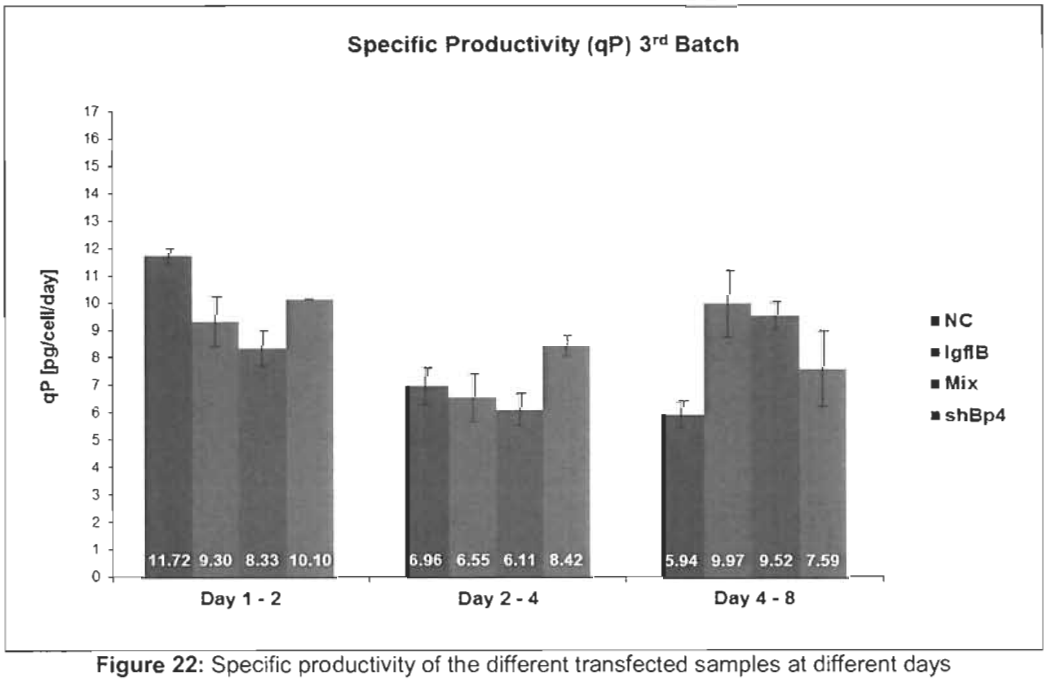

Figure 22: Specific productivity of the different transfected samples at different days

Figure 22 compares the specific productivity of the different samples on different days of batch culture. The highest specific productivity was found in the lag phase (day 1 – 2). The lowest specific productivity was found in the exponential phase on day 2 – 4.The *IgfIB* transfected cells and the Mix transfected cells (*IgfIB* and shBp4 transfected cells) had their respective highest productivity between day 4 and 8. Comparing the specific productivities on the different days indicated that the negative control cells had the highest specific productivity on day 1 – 2 and the shBp4 transfected cells had the highest productivity on day 2 – 4. Considering the high standard deviation of the *IgfIB* transfected samples on day 4 – 8, the *IgfIB* and shBp4 transfected cells (Mix) revealed the highest specific productivity on day 4 – 8.

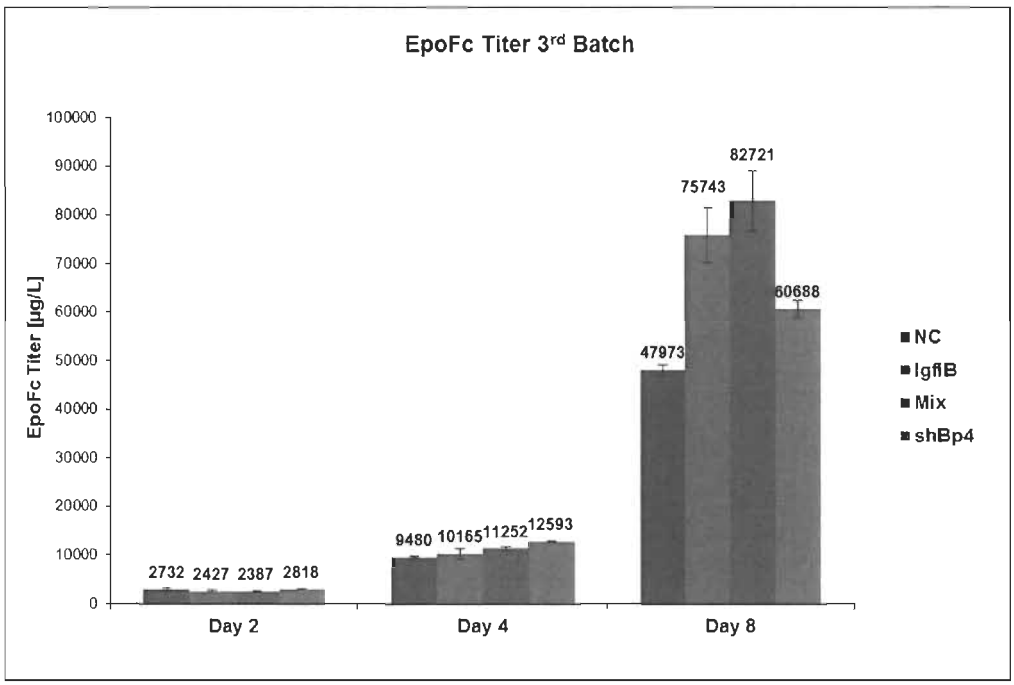

Figure 23: EpoFc titer of the different transfected samples at different days

Figure 23 displays the EpoFc titer of the different samples. On day 8 of batch culture the Mix transfected cells showed the highest amount of product formation, they were followed by the *IgfIB* transfected cells, which showed the second highest amount of product formation. The product formation of the *IgfIB* transfected cells was followed by the shBp4 transfected cells.

3.1.8 Igf Batch 4 – Media Optimisation

Before starting with Igf batch 4 media optimisation experiments had to be done in order to determine the optimal concentration of the Igf-1 and the LongR^{3}Igf-1 media supplement.

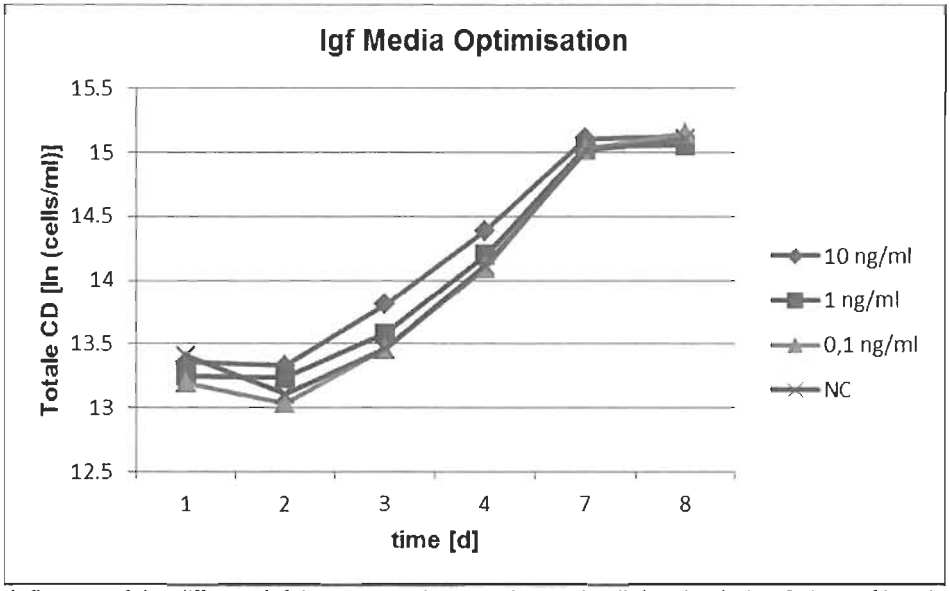

Figure 24: Influence of the different Igf-1 concentrations on the total cell density during 8 days of batch culture

Figure 24 reveals that 10 ng/ml Igf-1 media supplement had the best effect on the total cell density over all 8 days of batch culture. Hence 10 ng/ml Igf-1 media supplement were used for the Igf-1 including samples in Igf batch 4.

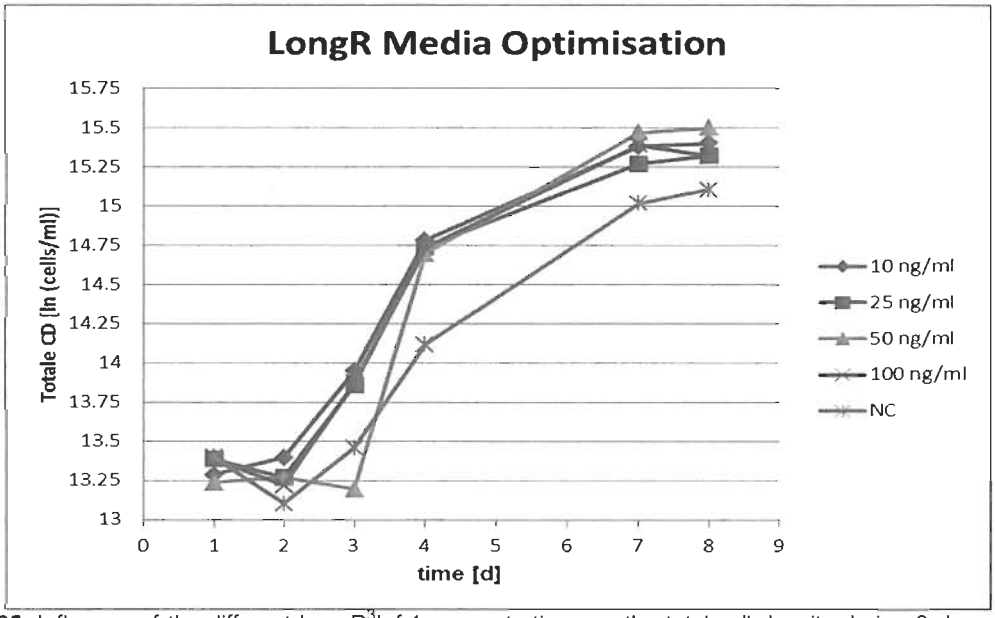

Figure 25: Influence of the different LongR^{3}Igf-1 concentrations on the total cell density during 8 days of batch culture

The LongR^{3}Igf-1 media optimisation diagram (figure 25) indicates, that 50 ng/ml of the LongR^{3}Igf-1 media supplement is the optimal concentration to obtain the highest total cell density in batch culture. Thus 50 ng/ml LongR^{3}Igf-1 media supplement were used for the LongR^{3}Igf-1 including samples in Igf batch 4.

3.1.9 Igf Batch 4 – GFP Measurement

In order to determine the amount of viable transfected GFP expressing cells a presentable amount of each sample was analysed 48 hours after transfection with the help of flow cytometry.

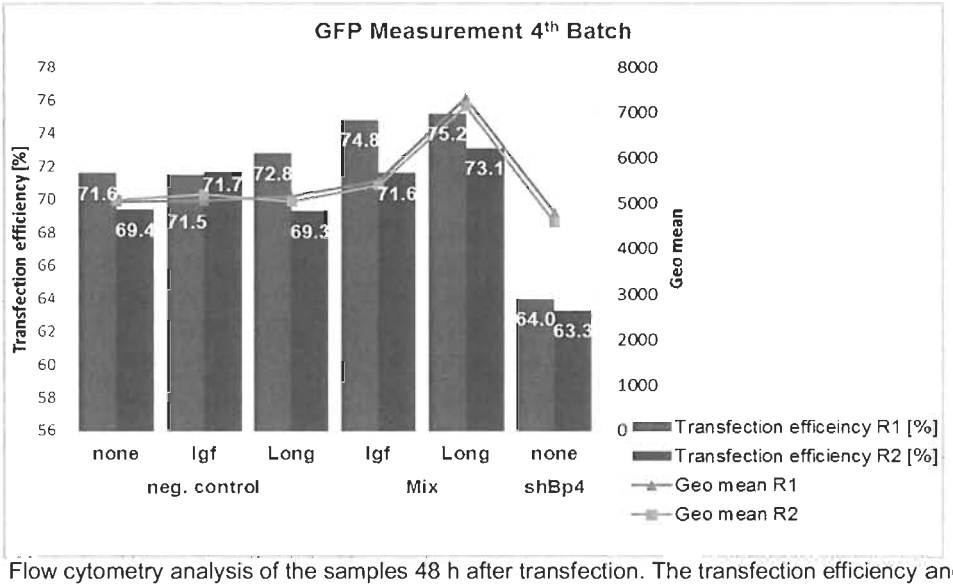

Figure 26: Flow cytometry analysis of the samples 48 h after transfection. The transfection efficiency and geo mean are displayed. The original data is displayed in the appendix.

Untransfected CHO EpoFc 14F2 cells were used as control to determine the amount of GFP positive cells 48 hours after transient transfection. Figure 26 displays that more than 69 % of the negative control transfected cells without any additional media supplement expressed GFP. 71 % of the negative control cells in the Igf-1 medium were GFP positive. More than 69 % of the negative control cells in the LongR^{3}Igf-1 supplemented medium expressed GFP. Around 71 % of the shBp4 transfected cells in the Igf-1 medium (Mix Igf) contained GFP. Among the shBp4 transfected cells in the LongR^{3}Igf-1 supplemented medium (Mix Long) 73 % and more showed GFP expression. Around 63 % of the shBp4 transfected cells contained GFP.

3.1.10 Igf Batch 4 – Viable Cell Density and Growth

Based on the observations in the 3rd batch a 4th different batch was made. During the first three batches it was observed, that the Mix transfected cells (shBp4 and *IgfIB* transfection) revealed the highest VCD and growth rate. The *IgfBp4* knockdown seems to have an important effect on the binding of the IgfIB to the Igf1R and the resulting starting of the anti-apoptotic and mitogenic cascade. The 4th batch consisted out of six different samples, the negative control cells without any additional media supplement, the negative control cells with Igf-1 as additional media supplement, the negative control cells with LongR^{3}Igf-1 as additional supplement, the shBp4 transfected cells with Igf-1 as additional media supplement, the shBp4 transfected cells with LongR^{3}Igf-1 as additional media supplement

and the shBp4 transfected cells without any additional media supplement. The LongR^{3}Igf-1 has a lower affinity to the IgfBps than the native Igf-1 and therefore the cells, which are grown in this media, should reveal a higher VCD than the cells grown in the Igf-1 media. The higher VCD is caused through uninhibited binding of the LongR^{3}Igf-1 to the Igf1R and the resulting starting of the signal cascade. The aim of this experiment was to address the question, if the shBp4 transfection in combination with the Igf-1 media supplementation reveals a higher VCD than the LongR^{3}Igf-1 media supplementation with either shBp4 transfected cells or with negative control cells, which were only transfected with the empty plasmid. Another interesting question was, if the shBp4 transfection could improve the effect of the LongR^{3}Igf-1 media or the Igf-1 media. Furthermore based on the knowledge, that the expression of an additional protein like e.g. the IgfIB elicits additional cell stress, it was tested, if the Igf-1 media supplement instead of the *IgfIB* transfection reveals a better performance of the cells considering the specific productivity and VCD.

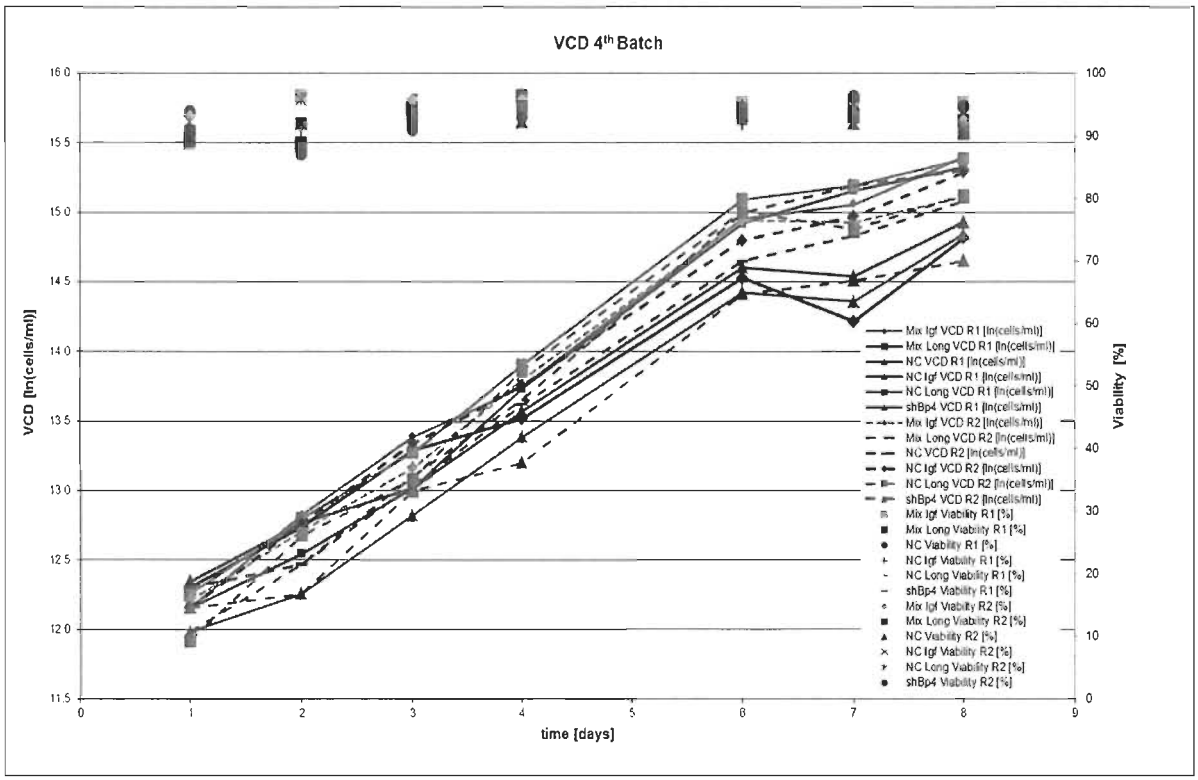

Figure 27: Viable cell density (VCD) over time; NC cells were transfected with the empty plasmid and had no additional media supplement; NC Igf cells were transfected with the empty plasmid and grown in Igf-1 supplemented media; NC Long cells were transfected with the empty plasmid and grown in LongR^{3}Igf-1 media; Mix Igf cells were transfected with the plasmid including the shBp4 insert and were grown in Igf-1 supplemented media; Mix Long cells were transfected with the plasmid including the shBp4 insert and were grown in LongR^{3}Igf-1 media; shBp4 cells were transfected with the plasmid including the shBp4 insert and had no additional media supplement.

During all days of batch culture, except on day 7, the shBp4 transfected replicates had the lowest VCD. The negative control replicate 1 in the LongR^{3}Igf-1 supplemented media revealed the highest VCD of all samples between day 4 and 8. Considering the viability, all different samples revealed a viability higher than 80 % during the whole batch culture. Replicate 2 of the Mix transfected cells in the LongR^{3}Igf-1 supplemented media revealed the lowest viability on day 8 of batch culture (90,5 % viability).

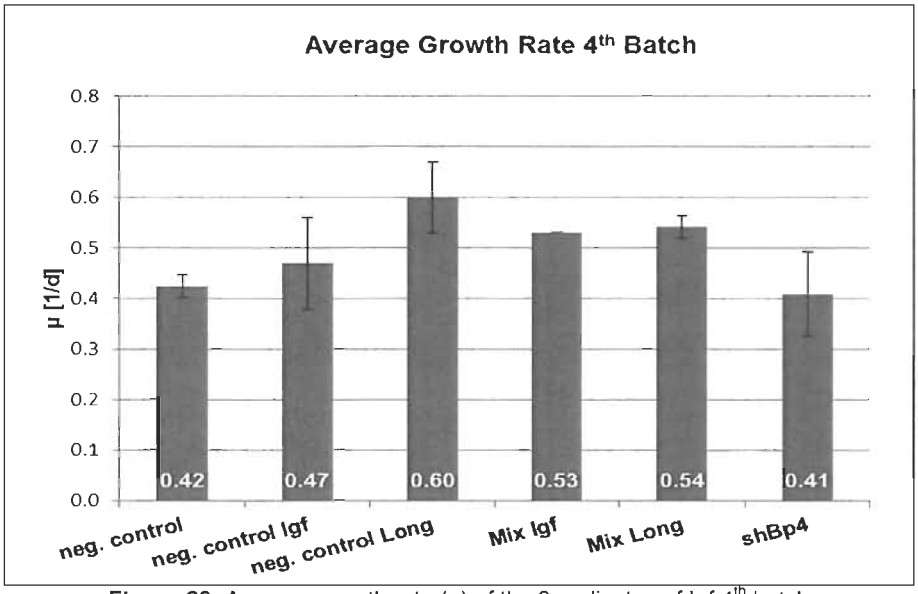

Figure 28: Average growth rate (μ) of the 2 replicates of Igf 4th batch

The results shown in figure 28 were calculated with the help of the viable cell densities between day 1 and day 4, using formula 5. All samples, which are comparable with the first three batches, revealed a lower growth rate than the ones from the remaining experiments. The negative control cells in the LongR^{3}Igf-1 media supplement (neg. control Long) had the highest growth rate of all different samples; it was 1,4 times higher than the average growth rate of the negative control cells. Interestingly the shBp4 transfected cells in the Igf-1 media supplement (Mix Igf) and the shBp4 transfected cells in the LongR^{3}Igf-1 media supplement (Mix Long) had nearly the same average growth rate. The negative control, shBp4 sample and the negative control sample in the Igf-1 supplemented media revealed nearly the same average growth rate.

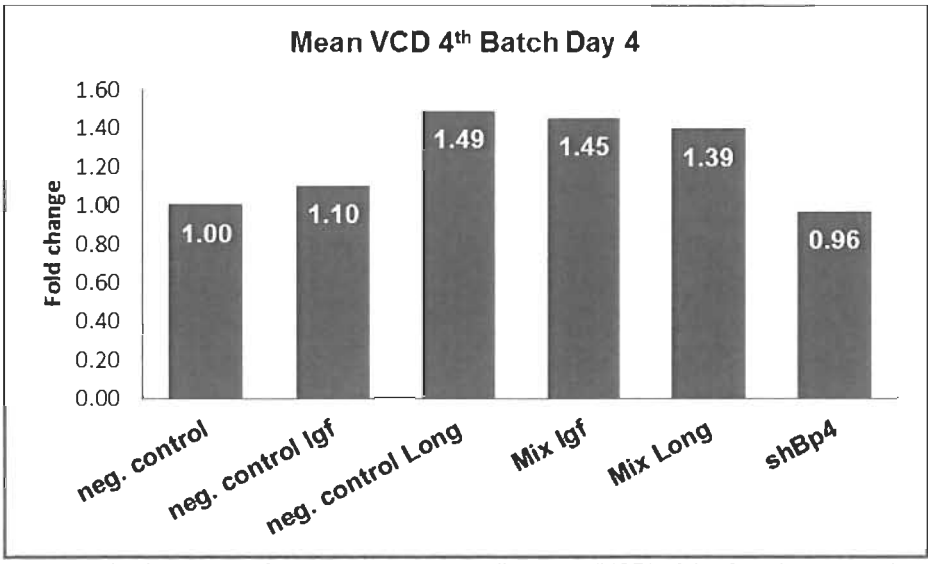

Figure 29: Fold changes of the average viable cell density (VCD) of the 2 replicates on day 4

Figure 29 indicates that the negative control cells in the LongR^{3}Igf-1 supplemented media had the highest mean VCD on day 4. The Mix transfected cells in the Igf-1 media show the second highest mean VCD value, followed by the Mix transfected cells in the LongR^{3}Igf-1 supplemented media. The lowest mean VCD is represented by the shBp4 transfected cells, which reveal an even lower mean VCD than the negative control cells.

3.1.10.1 Correlation Transfection Efficiency – Viable Cell Density (VCD) Igf 4th Batch

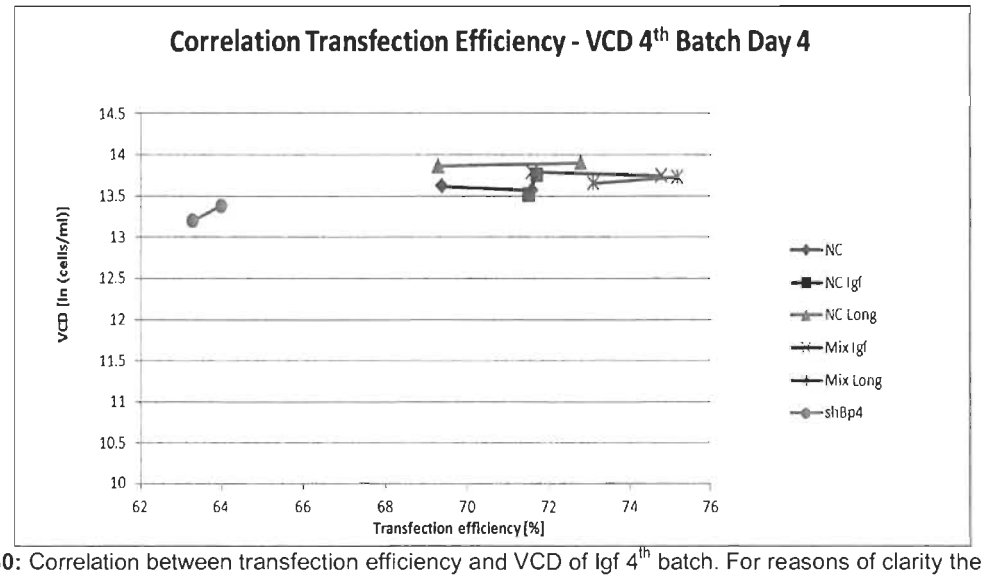

Figure 30: Correlation between transfection efficiency and VCD of Igf 4th batch. For reasons of clarity the y - axis does not start at zero.

The correlation between transfection efficiency and VCD, displayed in figure 30, indicates that the transfection efficiency does not influence the viable cell density of the different transfected samples.

3.1.11 Igf Batch 4 – Specific Productivity and Titer

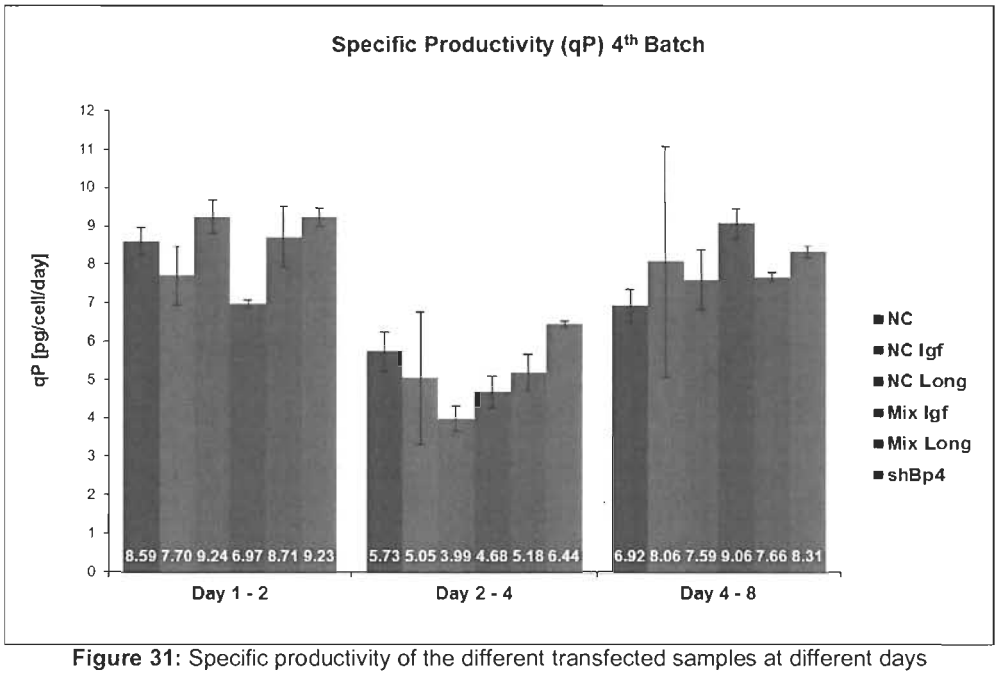

Figure 31: Specific productivity of the different transfected samples at different days

Figure 31 compares the specific productivity of the different samples on different days of batch culture. All samples which were grown in the LongR^{3}Igf-1 media supplement revealed a higher qP than their equal sample, which was grown in the Igf-1 media supplement on day 1 – 2 and day 2 – 4. On day 4 – 8 the shBp4 transfected cells in the Igf-1 media supplement (Mix Igf) had the highest qP. The highest specific productivity was found in the lag phase of the batch culture (samples between day 1 – 2). The lowest specific productivity was found between day 2 – 4 (exponential phase of batch culture).

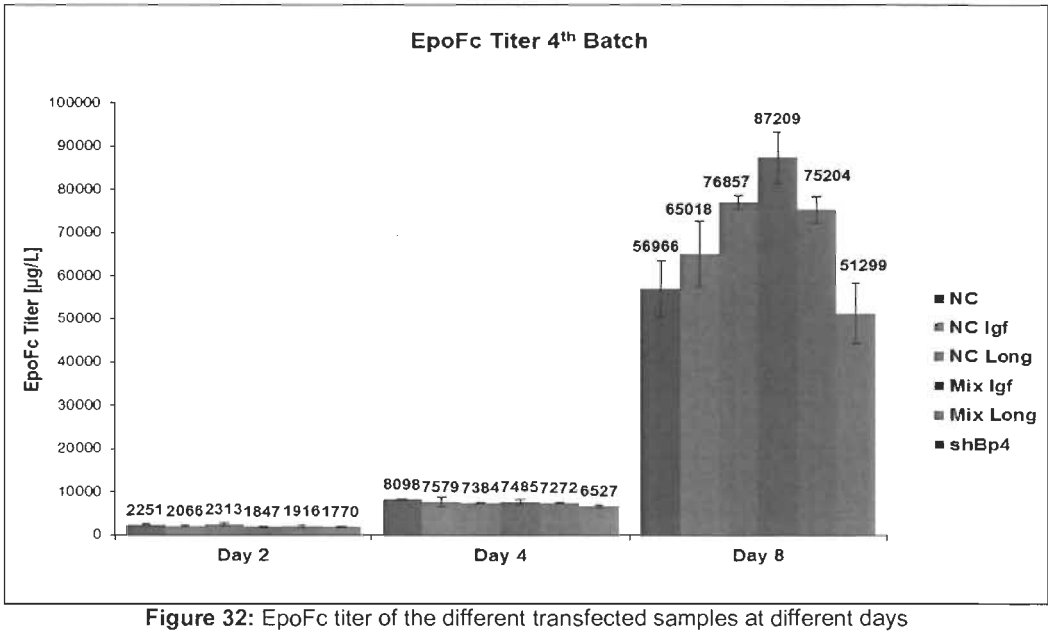

Figure 32: EpoFc titer of the different transfected samples at different days

Figure 32 displays the EpoFc titer of the different samples. The different transfections show the desired effect in the amount of product formation on day 8. Considering whether the Igf-1 (NC Igf) or the LongR^{3}Igf-1 media supplementation (NC Long) causes a positive effect on the amount of product formation compared to the negative control on day 8 of batch culture reveals, that the LongR^{3}Igf-1 media supplementation had a stronger effect. The Igf-1 media supplementation in combination with the shBp4 transfection (Mix Igf) indicates the strongest positive effect on the amount of product formation of all different transfections on day 8 of the batch culture. Combining the shBp4 transfection with the LongR^{3}Igf-1 media supplementation (Mix Long) exhibits nearly the same effect on the amount of product formation as the LongR^{3}Igf-1 media supplementation alone (NC Long).

3.2 Sec10 Batch

One Sec10 batch was run with two technical replicates. The batch was run with cells transfected with the empty plasmid, cells transfected with the vector including the *Sec10* insert and cells transfected only with the transfection reagent without any plasmid (Mock transfection). The Mock transfection was done in order to determine the potential effect of the transfection reagent on the cells.

3.2.1 Sec10 Batch – Viable Cell Density and Growth

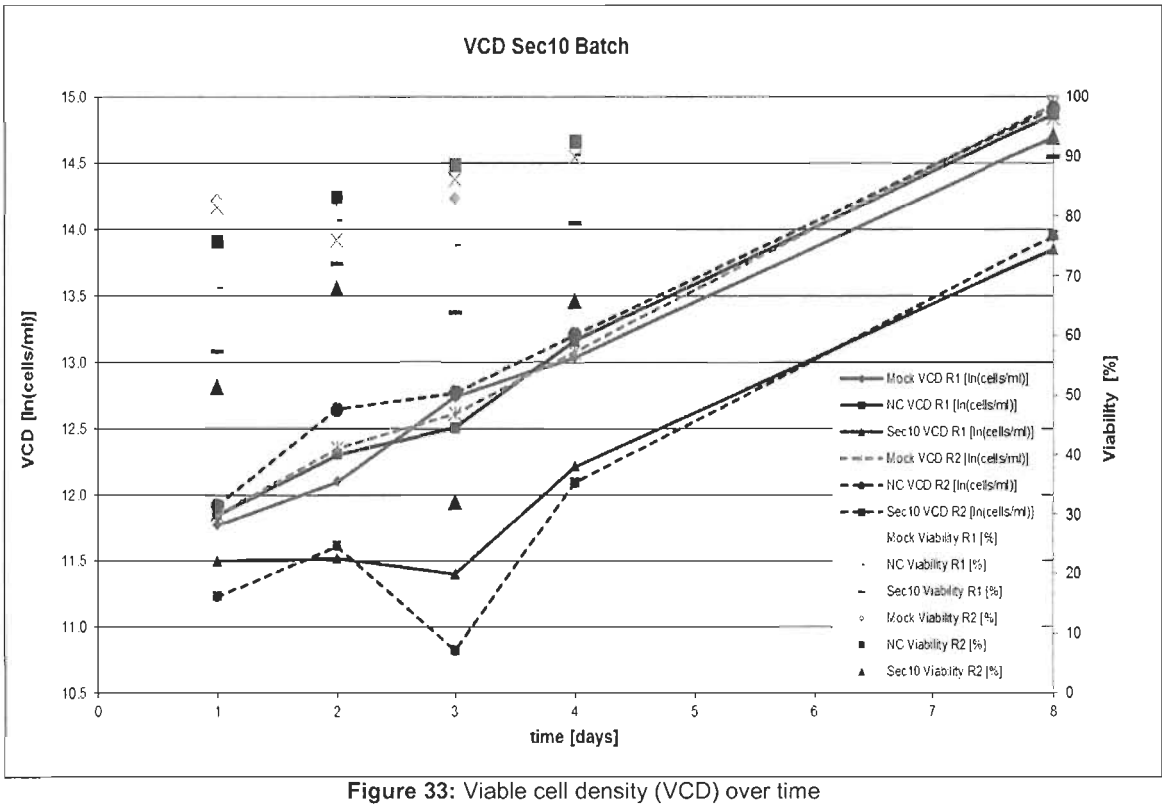

Figure 33: Viable cell density (VCD) over time

Figure 33 reveals that the *Sec10* transfected cells had the lowest VCD and viability during the entire batch culture. Between day 1 and day 4 in figure 33 the negative control replicate 2 showed the highest viable cell density and the *Sec10* transfected replicates showed the lowest VCD. The Sec10 sample with the lowest viability was found on day 3 of batch culture; only 32,1 % of the cells were viable. The viability of the Mock transfected cells and the negative control cells was always between 65 % and 90 % until day 4 of batch culture. On day eight all samples revealed a viability of 90 % or more. Probably the different cells were stressed too much during transfection and recovered slowly after this stress.

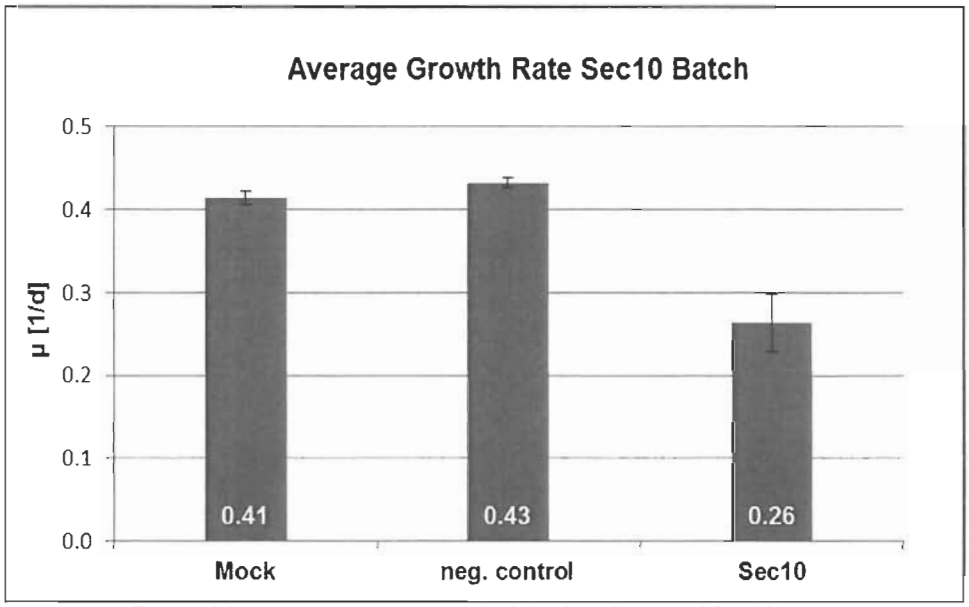

Figure 34: Average growth rate (μ) of the 2 replicates of Sec10 batch

The average growth rates (figure 34) were calculated with the help of the viable cell densities between day 1 and day 4, using formula 5. The *Sec10* transfection did not show any growth promoting effects, on the contrary, growth rate was reduced by more than 30%.

3.2.2 Sec 10 Batch - Specific Productivity and Titer

The ELISA method was used to determine the impact of the different transfections on the specific productivity and the EpoFc titer.

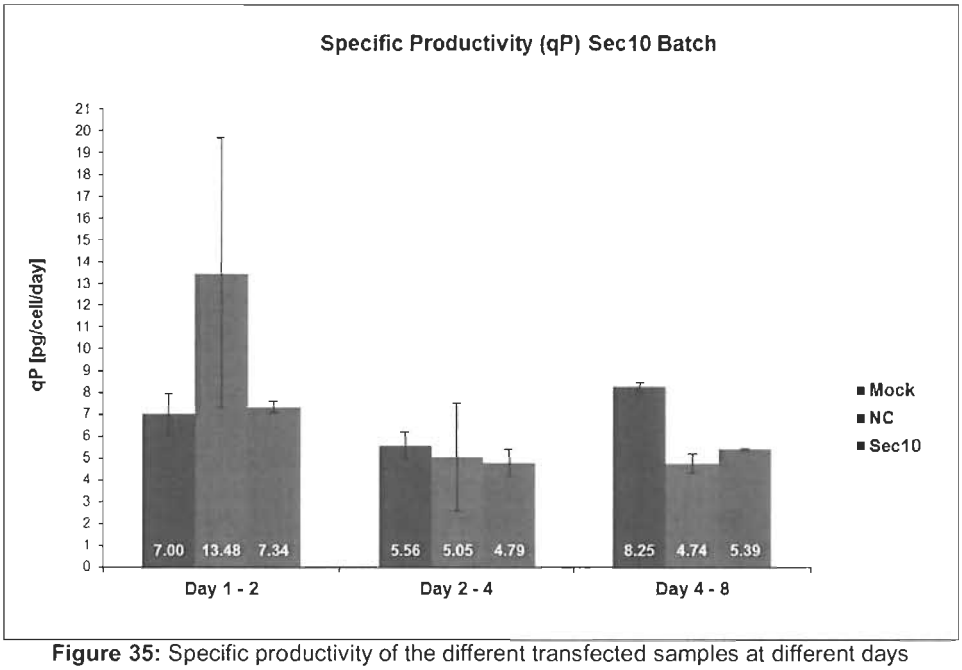

Figure 35: Specific productivity of the different transfected samples at different days

Figure 35 describes the specific productivity of the different samples on different days of the batch culture. The highest specific productivity was found in the lag phase of the samples on day 1 – 2. The lowest specific productivity was found in the exponential phase on day 2 – 4. Comparing the highest productivities on the different days indicated, that considering the high standard deviation of the negative control cells on day 1 – 2, all samples on day 1 – 2 had nearly the same specific productivity, whereas the Mock transfected cells indicated the highest productivity on day 2 – 4 and day 4 – 8.

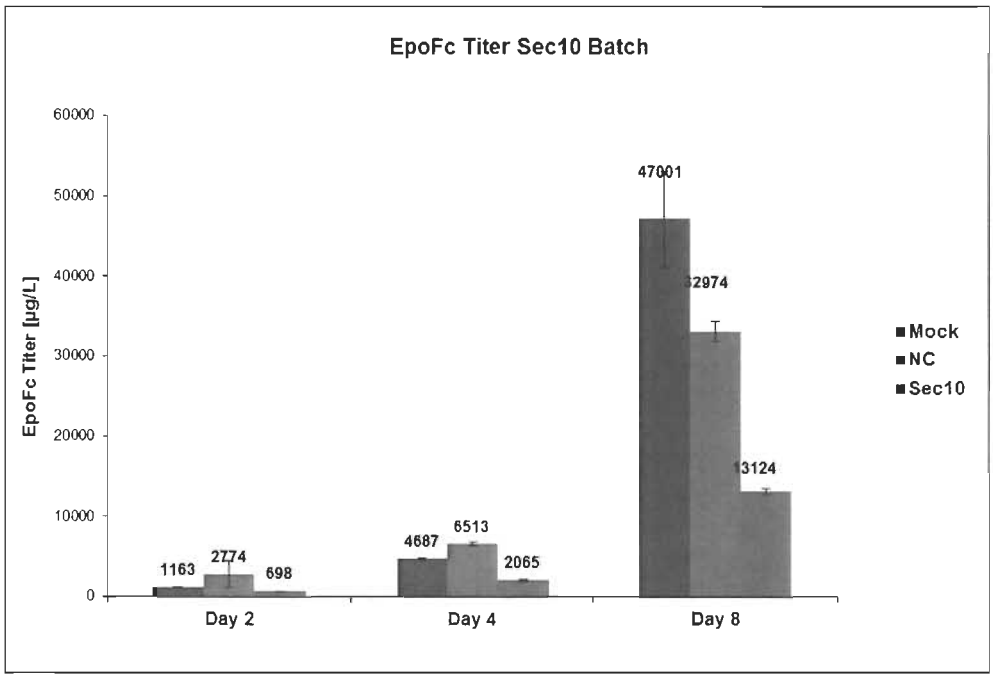

Figure 36: EpoFc titer of the different transfected samples at different days

Figure 36 shows the EpoFc titer of the different samples. On day 8 of batch culture the Mock transfected sample revealed the highest amount of product formation, whereas the negative control sample showed a lower amount of product formation and the *Sec10* transfected sample revealed the lowest amount.

4 Discussion

The aim of this thesis was to optimize the expression of recombinant proteins in CHO by targeted cell engineering. Out of many possibilities we tried to optimize the recombinant protein expression with the help of the *IgfIB* and *Sec10* gene overexpression.

4.1 Igf Batches

As assumed from the literature, the cells which were transfected with a combination of the *IgfIB* including plasmid and the shBp4 including plasmid (so called Mix transfected cells), revealed the highest VCD over the entire 8 days of batch culture in the first three batches. The *IgfIB* transfected cells and the shBp4 transfected cells also revealed a higher VCD than the negative control, but it was lower than the VCD of the Mix transfected cells. These results indicate that neither the IgfIB transfection alone nor the shBp4 transfection alone affects the cells as much as the combination out of them. The cells need the knockdown of the IgfBp4 via the shBp4, so that the produced IgfIB can bind without any inhibition by IgfBp4 to the Igf1R and thus start the anti-apoptotic and mitogenic signal transduction cascade. This is also confirmed by several papers, in which it is concluded that the IgfBp4 plays a great role in the Igf pathway by binding the IgfIB with high affinity and therefore blocking the pathway, because the Igf1R cannot bind the IgfIB. The very interesting observation that the shBp4 transfected cells showed a higher VCD than the IgfIB transfected cells during the 3rd batch could be associated with the fact that the CHO cells are predicted to produce Igf-1 also by themselves and this binds directly to the Igf1R because the IgfBp4 expression is silenced with the help of the shBp4 RNA. The viability of all different transfections indicated nearly the same effect or a slightly advantageous effect over the negative control sample.

These VCD observations are also confirmed by the ANOVA analysis of the first three batches and the negative control, IgfIB and Mix samples. The shBp4 transfected samples weren´t taken into account because this transfection was only performed in one out of three batches.

In the ANOVA analysis the negative control shows a significant lower VCD than IgfIB and Mix transfected cells. As expected, Mix transfected cells show a higher VCD than IgfIB transfected cells but this difference was not significant. Due to the low sample size, there were only 4 residual degrees of freedom in the ANOVA. This requires relatively high differences for detection of significance. Therefore the power to detect moderate differences between Mix and IgfIB transfected cells as significant is probably too low.

Focusing on the average growth rate of the different transfections also the Mix transfected samples (*IgfIB* and shBp4 transfected cells) revealed the expected result. They had a significantly higher growth rate than the negative control cells. The slightly higher average growth rates of the IgfIB transfected cells and the shBp4 transfected cells in comparison to the negative control confirm the hypothesis that the combination of the shBp4 transfection and the *IgfIB* transfection is the reason for the significantly better performance of the Mix transfected cells.

These very promising effects were observed after transient transfections; maybe the effects would be even better after stable transfections. Furthermore a stable transfection would be useful to observe the long term effects of the different transfections which may differ from the short time increase of expression of stimulatory genes.

Focusing on the specific productivity of the three different batches it is difficult to make a founded conclusion. With caution it can be said that the different transfections revealed no positive effect on the specific productivity in the beginning of the batch cultures. It can be mentioned, that the assumed positive effect of the different transfections requires time, because the overexpression of these genes might not be high enough to cause a difference in productivity within the first 2 days of batch culture. Another possible reason could be that the effect of the IgfIB and shBp4 needs time and isn´t developed enough between day 1 and day 2 of batch culture.

After the first two days of batch culture all different transfections showed an advantageous effect over the negative control samples. The reason for this interesting fact, that the shBp4 transfected cells revealed the highest productivity between day 2 and day 4, could be that the CHO cells also produce Igf-1 on their own. The cells seem to proliferate slowly, because they only express low amounts of Igf-1. This means, that not the whole energy of the cells goes into proliferation and there is a lot of energy left for production. It seems that, the not-so fast proliferating cells are producing more than the fast proliferating cells. This fact is probably also the reason for the lower specific productivity of the *IgfIB* and the Mix transfected cells in comparison to the shBp4 transfected cells between day 2 and day 4 of the batches.

Towards to the end of the batch cultures, between day 4 and day 8, the *IgfIB* transfected cells had the highest productivity at least in batch 1 and batch 2. This fact could be attributed to the transient transfection and the fact that the percentage of plasmids that are diluted out increases with the proliferation rate of the cells. In case of the Mix transfected cells, the

proliferation rate is higher than in the *IgfIB* transfected cells, therefore the probability of plasmids that are diluted out and the resulting lower specific production rate is very high. The second possible reason could be, as already mentioned before during day 2 and day 4, that the Mix transfected cells proliferate very fast so that there is not enough energy left for a high productivity of these cells. Another possible reason why *IgfIB* transfected cells show the highest specific productivity could be the autocrine loop of the Igf pathway. In case of the Mix transfected cells, the cells are probably already saturated by IgfIB. IgfIB binds directly to the Igf1R and its activation of the Igf pathway is not blocked through the binding of the IgfIB with the IgfBp4. Cells, which have already enough IgfIB, stop the proliferation etc. through the autocrine loop.

Based on the very promising observations during the first batches a 4th batch with an Igf-1 media supplement instead of the recombinant IgfIB was conducted. This decision was made under the hypothesis, that the expression of the recombinant IgfIB stresses the cells more than the use of the Igf-1 media supplement. Additionally for a future production process it is more useful to use Igf-1 as a media supplement, because the IgfIB expression is a very high energy consuming process, that leads to a lower specific productivity in these cells. Furthermore another additional media supplement LongR^{3}Igf-1 was used in expectation, that the LongR^{3}Igf-1 media supplemented cells show a better performance in VCD, growth and specific productivity than the Igf-1 supplemented cells. LongR^{3}Igf-1 is more biological potent than Igf-1, because it has a lower affinity to the IgfBps. The 4th batch consisted out of 6 different samples, the negative control cells without any additional media supplement, the negative control cells with Igf-1 as additional media supplement, the negative control cells with LongR^{3}Igf-1 as additional media supplement, the shBp4 transfected cells with Igf-1 as additional media supplement (Mix Igf), the shBp4 transfected cells with LongR^{3}Igf-1 as additional media supplement (Mix Long) and the shBp4 transfected cells without any additional media supplement (shBp4). The shBp4 transfections were done, because we wanted to know whether the Igf-1 media supplementation with the shBp4 transfection may lead to even better growth performance than the LongR^{3}Igf-1 media supplement in combination with the shBp4 transfection or the LongR^{3}Igf-1 media supplementation alone. An additional question was whether the performance of the negative control cells in the LongR^{3}Igf-1 supplemented media and the performance of the negative control cells in the Igf-1 supplemented media could be improved through the shBp4 transfection.

Focusing on the first hypothesis, that the media supplementation instead of the expression of the IgfIB leads to lower cell stress and thereby to a higher VCD or growth rate, it is difficult to make a clear statement, because the VCD and also the growth rate of the comparable

samples in the 3rd and 4th batch varies a lot during all days. To make a clear, well stated conclusion about the first hypothesis, it would be useful to redo batch 3 and batch 4 with at least three replicates per batch.

As expected from the previous experiments, the shBp4 transfection improves the effect of the Igf-1 media supplementation. The VCD and also the growth rate of the shBp4 transfected cells in the Igf-1 supplemented media is higher than the VCD and growth rate of the negative control cells in the Igf-1 supplemented media. During the last days of batch culture the shBp4 transfected cells in the LongR^{3}Igf-1 supplemented media indicated a higher VCD than the negative control cells in the LongR^{3}Igf-1 supplemented media. Both samples showed no difference in the growth rate. A possible reason for that observation could be that the LongR^{3}Igf-1 media supplement still has a low affinity to the IgfBp4 and towards to the end of the batch culture there is only little LongR^{3}Igf-1 left. In the shBp4 transfected cells the IgfBp4 is inactivated, therefore the whole LongR^{3}Igf-1 media supplement can bind to the Igf1R without any inhibition.

Addressing the second hypothesis, that the LongR^{3}Igf-1 media supplemented cells show a better performance than the Igf-1 media supplemented cells, the average growth rate of the different samples reveals that the LongR^{3}Igf-1 media supplemented cells with/ without shBp4 transfection show the same performance as the Igf-1 supplemented cells in combination with the shBp4 transfection (Mix Igf). Towards the end of batch culture the shBp4 transfected cells in the LongR^{3}Igf-1 media supplement (Mix Long) had the highest VCD. It can be assumed, as already mentioned before, that the LongR^{3}Igf-1 media supplement still has a low affinity to the IgfBp4 and therefore the inhibitory effect of the shBp4 leads to a better bioavailability of the LongR^{3}Igf-1 supplement. During the first days of batch culture the shBp4 transfection in combination with the Igf-1 media supplement leads to a higher VCD than the negative control with the LongR^{3}Igf-1 media supplement, which is supposed to have a low affinity for IgfBps and therefore should lead to a better growth performance. Another very interesting observation is that the shBp4 transfected cells in the LongR^{3}Igf-1 supplemented media revealed a lower VCD than the shBp4 transfected cells in the Igf-1 supplemented media during the first four days of batch culture, but it seems that the shBp4 transfection improves the effect of the LongR^{3}Igf-1 towards to the end of batch culture. According to these observations it can be assumed that the promoting effect of the LongR^{3}Igf-1 in combination with the shBp4 transfection needs time.

Observing the specific productivity the shBp4 transfected cells showed the highest specific productivity during the first four days of batch culture, because of the own expressed Igf-1

and the blocked *IgfBp4.* A reason for the lower specific productivity of the other samples could be, as already assumed in the batches before, that the cells in the Igf-1 and LongR^{3}Igf-1 supplemented media with/ without shBp4 transfection are proliferating so fast, that there is not enough energy left for production of the EpoFc. Between day 4 and day 8 of the 4th batch the shBp4 transfected cells in the Igf-1 supplemented media (Mix Igf) showed the highest specific productivity. Probably the Mix Igf sample had the highest productivity because these cells did not reveal such a high VCD and therefore they have more energy left for producing EpoFc.

Summarized it can be said, that the knockdown of the IgfBp4 in combination with the Igf-1 media supplement leads to a high specific productivity and growth rate. The cells performed better or equal than in the LongR^{3}Igf-1 supplemented media. The LongR^{3}Igf-1 effect could slightly be improved through the knockdown of the *IgfBp4.*

4.2 Sec10 Batch

The VCD and the viability of the *Sec10* transfected cells was lower than the respective values of the negative control cells and the Mock transfected cells. As expected the *Sec10* gene expression did not lead to a higher VCD and viability, but it was surprising, that the VCD and viability was so low in comparison to the other cells. The Sec10 insert is more than 4,6 kBp long. Maybe the *Sec10* transfected cells are totally occupied with the *Sec10* transcription and translation, so that there is hardly any energy left for growing. The length of the Sec10 insert and the combined expression stress could also be the reason for the similar specific productivity of all samples. It was expected, that the cells show a higher specific productivity, with the help of the *Sec10* insert. Unfortunately this expectation wasn´t fulfilled.

5 Conclusion and Outlook

5.1 Igf Batches

Throughout all three batches, the cells, which were transfected with a combination of the shBp4 and the *IgfIB* including plasmids (so called Mix transfected cells), showed the highest VCD. In case of the specific productivity it is difficult to make a clear statement. In order to be able to make a well-founded statement I would recommend redoing the ELISA with at least three replicates. Another option to proof the overexpression of the *IgfIB* gene or the knockdown of the *IgfBp4* would be a qPCR with primers against these genes on day 2, 4 and 8 of batch culture. With the results out of this analysis it is possible to know how much of the plasmid including the insert can be found within the transformed cells and therefore it is possible to make a more precise conclusion about the effects of the different transfections.

Another very interesting analysis would be an ELISA with IgfIB specific antibodies, delivering information about how much of IgfIB is respectively expressed by the different transfected cells. Maybe these results led to a more accurate interpretation on the effects of the diverse transfections. Meaning that, if there is more expressed IgfIB, the anti-apoptotic and proliferative effects would be distinctively higher.

The most important thing for making a clear statement is a stable transfection. With the help of a stable transfection, the risk of discharged plasmids is minimized and it would be possible to also observe long term effects of overexpression.

Moreover an inducible promoter in front of the *EpoFc* gene would be useful to eliminate the probable effect of the lower specific production rate caused by the higher proliferation and growth rate of the respective cells. This is a very time consuming, expensive method and it has to be considered if it is worth to follow this idea.

Last but not least doing a Western Blot of each shBp4 transfected sample could be useful for detection if the *IgfBp4* silencing was successful.

Based on the observations of the first three batches the 4th batch was made with an Igf-1 media supplement and an analogue of the Igf-1 media supplement, the LongR^{3}Igf-1, which should lead to a better proliferation and anti-apoptotic effects than the Igf-1. The usage of these media supplements is very important because the expression of the *IgfIB* needs additional cell energy. During a production process as much energy as possible should be used for the production of the desired product. Additionally to the usage of the media

supplements it was tried to improve the effects of the supplements with the shBp4, based on the knowledge of the first three batches.

In general the effect of the Igf-1 could be improved through the shBp4 transfection. During the last days of batch culture also the effect of the LongR^{3}Igf-1 could be improved through the shBp4 transfection. With respect on the growth rate and the specific productivity I would recommend to use the shBp4 transfected cells in the Igf-1 media supplement for the production of recombinant proteins.

5.2 Sec10 Batch

Although the *Sec10* transfection seems to have a negative effect on the growth performance of the transfected cells, an inducible promoter in front of the *Sec10* gene could led to a higher specific production rate of the transfected cells. Using an inducible promoter would allow the cells to grow and express the *Sec10* gene in the stationary phase, when they already reached a high enough cell density. This method would probably lead to a higher EpoFc production in the late, stationary phase.

Furthermore a Western Blot analysis would verify, if there is a *Sec10* expression or not.

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7 Appendix

7.1 Primer List

Primer ID	Sequence (5´ - 3´)
>CGR_IGFBP4_807	AGGACCTAGTCATCATCCCTA
>CGR_IGFBP4_1665	TTTGGGTCTGGGTATTGGGTT
>CGR_IGFBP4_1853	CCTAATTGTGGTCTCTGGTCA
EmGFP forward primer	GGCATGGACGAGCTGTACAA
miRNA reverse primer	CTCTAGATCAACCACTTTGT
>igf1_hsa_primer1_fw	GCGCTGTGCCTGCTCACCTT
>igf1_hsa_primer1_rev	CAGGCTTGAGGGGTGCGCAA
>igf1r_cgr_primer1_fw	AGGCTGCACCGTGGTCAATGGC
>igf1r_cgr_primer1_rev	TGCCCTGAGTGATGGTGAGGTTGT
>IgfBp4_cgr_primer3_fw	TCAACCCCTGCAGTGCCCATGA
>IgfBp4_cgr_primer3_rev	GGCAGAGAGCCAGGACTCGGACC
>Sec10_cgr_primer4_fw	ACCTAGACAACGTGCAGTAGAAGCT
>Sec10_cgr_primer4_rev	TTCTGAATGACGTCTGCTGCTTCC
cho_actb_2_fw	TACGTGGGTGACGAGGCCCA
cho_actb_2_rev	AGCCAGGTCCAGACGCAGGA

7.2 shBp4 Mix Oligosequences

Oligo ID	Sequence (5´ - 3´)
CGR_IGFBP4_807	TGCTGTAGGGATGATGACTAGGTCCTGTTTTGGCCACTGACTGACA GGACCTACATCATCCCTA
CGR_IGFBP4_1665	TGCTGAACCCAATACCCAGACCCAAAGTTTTGGCCACTGACTGACTT TGGGTCGGTATTGGGTT
CGR_IGFBP4_1853	TGCTGTGACCAGAGACCACAATTAGGGTTTTGGCCACTGACTGACC CTAATTGGTCTCTGGTCA

7.3 Reagents

LB – agar (pH= 7,0)

	Amount
Bacto - Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
RO - water	to 1 l

LB – medium (pH = 7,0)

	Amount
Bacto - Tryptone	50 g
Yeast extract	25 g
NaCl	50 g
RO - water	to 5 l

1 % Agarose - Gel (TAE) 140 ml/ Gel

	Amount
Agarose	1,4 g
TAE (50x)	2,8 ml (= 2,88 g)
AD	Up to 140 ml

After melting cool down to 50° C and add 1 µl of Midori Green prior pouring the gel into the form.

Coating Buffer (pH = 9,6 – 9,8)

	Amount
$NaHCO_3$	8,4 g
Na_2CO_3	4,2 g
RO - water	to 1 l

Wash Buffer (pH =7,2 - 7,4)

	Amount
Na_2HPO_4 x 2 H_2O	1,15 g
KH_2PO_4	0,2 g
KCl	0,2 g
NaCl	8 g
Tween 20	1 ml
RO - water	to 1 l

Dilution Buffer

The dilution buffer consists of wash buffer with 1 % bovine serum albumin.

Staining Buffer (pH = 5,0)

	Amount
Citric acid x 2 H_2O	7,3 g
Na_2HPO_4 x 2 H_2O	11,86 g
RO - water	to 1 l

Staining Solution

	Volume
Staining buffer	10 ml
H_2O_2	6 µl
1,2-o-Phenyldiamindihydrochlorid (1 mg/ml)	100 µl

7.4 Pipetting Schemes and Temperature Profiles

Restriction Digestion

	Volume
DNA	x µl
Restriction enzymes	2,5 µl each
10 x Fast Digest Buffer	5 µl
NFW	y µl
Final Volume	50 µl

Time	Temperature
30 min	37° C
5 min	80° C

Klenow Restriction for Sticky Vector Ends

	Volume
dNTP's (10 µmol/l)	5 µl
DNA	x µl
10 x NEB Buffer 2	5 µl
DNA Polymerase I, Large (Klenow) Fragment	1 µl
NFW	y µl
Final Volume	50 µl

Incubation at 25° C for 15 minutes. After incubation purification takes place with the PCR Purifiction Kit and the purified DNA should be eluted in 20 µl of NFW.

Ligation

	Volume
T4 Ligationbuffer	1 µl
T4 Ligase	1 µl
DNA (20-30 ng)	x
NFW	y µl
Final Volume	10 µl

Incubation at room temperature for 3 hours.

Colony PCR (IgfBp4 shRNA)

	Volume
10 x Reaction Buffer Biotherm + 15 mM $MgCl_2$	2 µl
10 mM dNTP's	0,6 µl
50 mM $MgCl_2$	0,5 µl
EmGFP Forward Primer	0,2 µl
miRNA Reverse Primer	0,2 µl
Biotherm Polymerase	0,1 µl
NFW	16,4 µl
Final Volume	20 µl

Time	Temperature	Repeats
5 min	95° C	
30 sec	95° C	30 x
30 sec	80° C	
1 min	65° C	
10 min	72° C	

DyNAmo™ cDNA Synthesis

	Volume
2x RT buffer (includes dNTPs and $MgCl_2$)	10 µl
Random hexamer primer set (300 ng/µl)	1 µl
Template RNA (max. 1 µg)	x µl
M-MuLV RNase H^+ reverse transcriptase (includes RNase inhibitor)	2 µl
RNase – free H_2O (add water to fill up to the final reaction volume)	x µl
Final Volume	20 µl

Time	Temperature
10 min	25° C
60 min	37° C
5 min	85° C
Hold	4° C

qPCR of the mRNAs

	Volume
2 x Reaction Mix	5 µl
Forward Primer	0,25 µl
Reverse Primer	0,25 µl
Template	1 µl
NFW	3,5 µl
Final Volume	10 µl

Time	Temperature	Repeats
10 min	95° C	
15 s	95° C	40 x
15 s	60° C	
15 s	72° C	
0 s	80° C	

The fluorescence intensity was determined every cycle after 15 seconds at 72° C and again at 80° C. After the cycling, a melting curve was generated by increasing the temperature from 60° C to 99° C, 1° C per 5 seconds.

DyNAmo™ cDNA Synthesis

	Volume
2x RT buffer (includes dNTPs and $MgCl_2$)	10 µl
Random hexamer primer set (300 ng/ µl)	1 µl
Template RNA (max. 1 µg)	x µl
M-MuLV RNase H^+ reverse transcriptase (includes RNase inhibitor)	2 µl
RNase – free H_2O (add water to fill up to the final reaction volume)	x µl
Final Volume	20 µl

Time	Temperature
10 min	25° C
60 min	37° C
5 min	85° C
Hold	4° C

7.5 Vector Maps

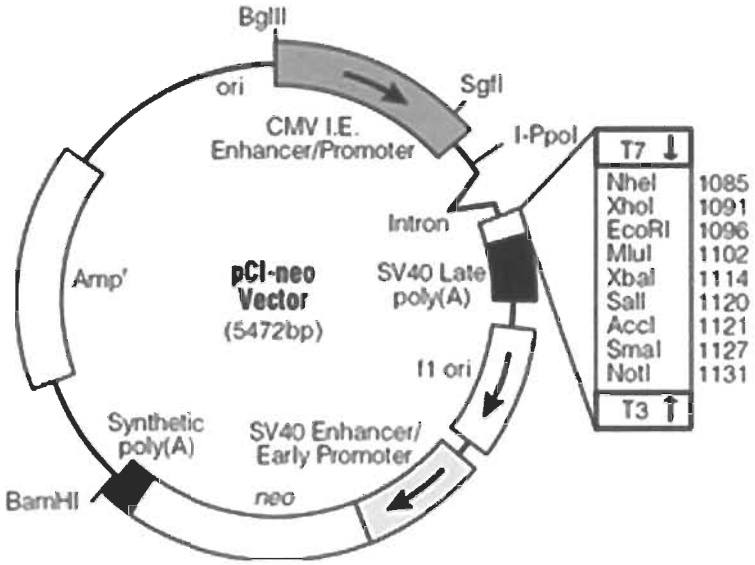


Figure 37: Vector map of pCI-neo, taken from http://promega.com

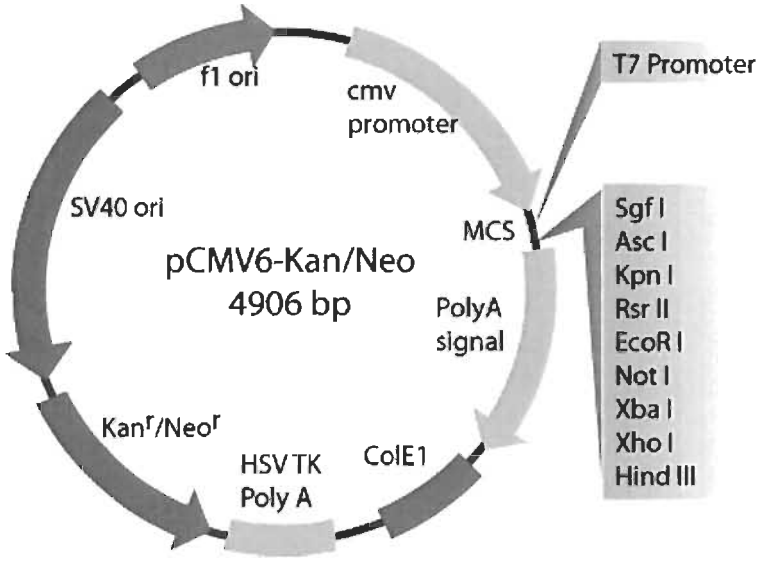
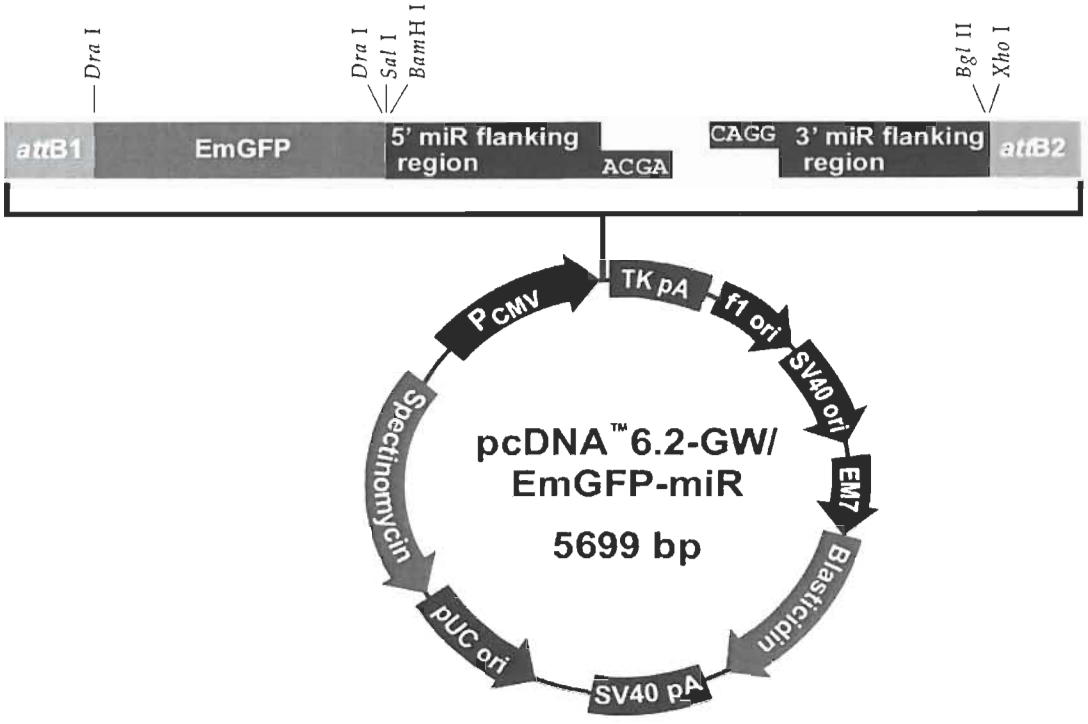


Figure 38: Vector map of pCMV6-Kan/Neo, taken from http://origene.com



Comments for pcDNA™ 6.2-GW/EmGFP-miR
5699 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680 - 704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
5' overhang (C): bases 1515-1518
5'overhang: bases 1519-1522
3' miR flanking region: bases 1519-1563
*att*B2 site (C): bases 1592-1616
miRNA reverse sequencing primer site (C): bases 1607-1626
TK polyadenylation signal: bases 1645-1916
f1 origin: bases 2028-2456
SV40 early promoter and origin: bases 2483-2791
EM7 promoter: bases 2846-2912
Blasticidin resistance gene: bases 2913-3311
SV40 polyadenylation signal: bases 3469-3599
pUC origin (C): bases 3737-4410
Spectinomycin resistance gene (C): bases 4480-5490
Spectinomycin promoter (C): bases 5491-5624

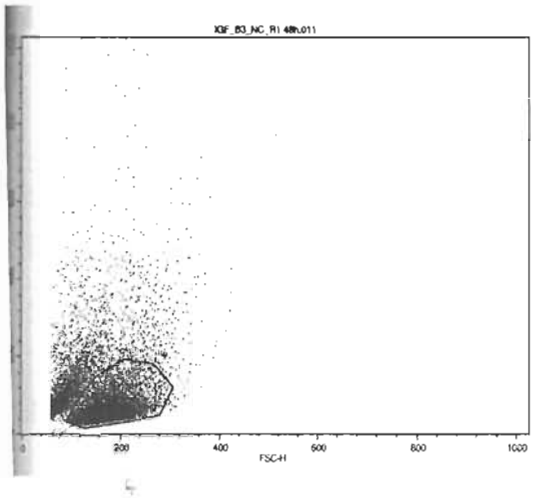
(C) = Complementary strand

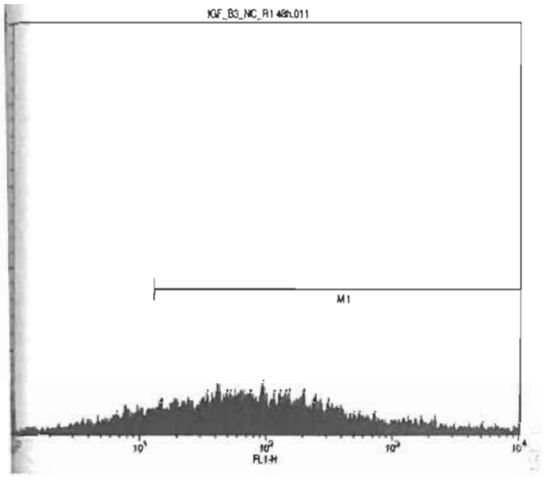
Figure 39: Vector map of pcDNA™6.2-GW/EmGFP-miR, taken from http://tools.invitrogen.com

7.6 GFP Measurements

7.6.1 Igf Batch 1

A)





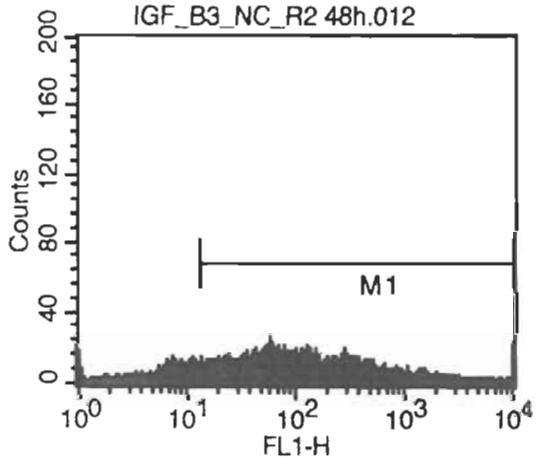
File: IGF_B3_NC_R1 48h.011

Gate	Events	% Total
G1	6602	66.02

File: IGF_B3_NC_R1 48h.011

Marker	Left, Right	% Gated	% Total	Geo Mean
All	1, 9910	100.00	66.02	76.92
M1	13, 9910	84.90	56.05	121.24

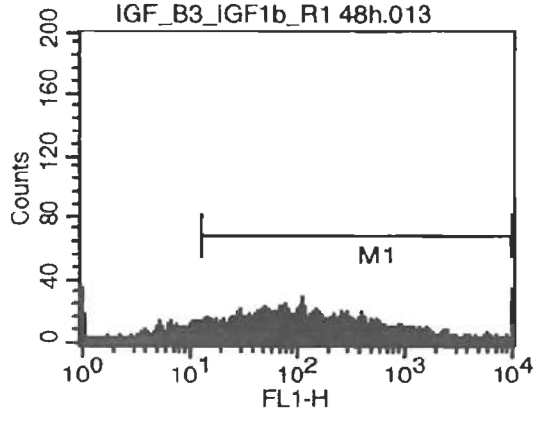
B)



File: IGF_B3_NC_R2 48h.012

Marker	Left, Right	% Gated	% Total	Geo Mean
All	1, 9910	100.00	64.23	74.12
M1	13, 9910	83.70	53.76	121.40

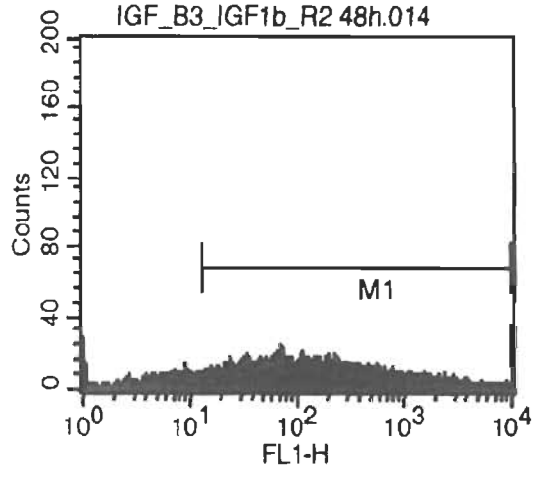
C)



File: IGF_B3_IGF1b_R1 48h.013

Marker	Left, Right	% Gated	% Total	Geo Mean
All	1, 9910	100.00	70.78	91.49
M1	13, 9910	84.74	59.98	152.22

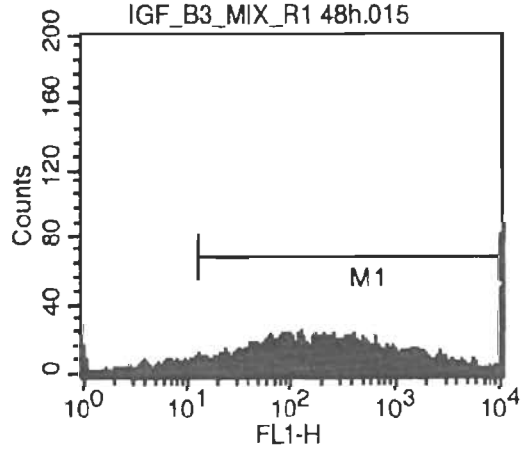
D)



File: IGF_B3_IGF1b_R2 48h.014

Marker	Left, Right	% Gated	% Total	Geo Mean
All	1, 9910	100.00	66.18	91.90
M1	13, 9910	84.62	56.00	153.34

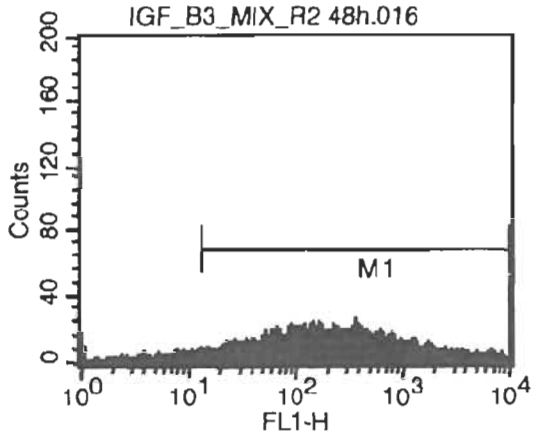
E)



File: IGF_B3_MIX_R1 48h.015

Marker	Left, Right	% Gated	% Total	Geo Mean
All	1, 9910	100.00	75.60	163.28
M1	13, 9910	91.85	69.44	219.20

F)



File: IGF_B3_MIX_R2 48h.016

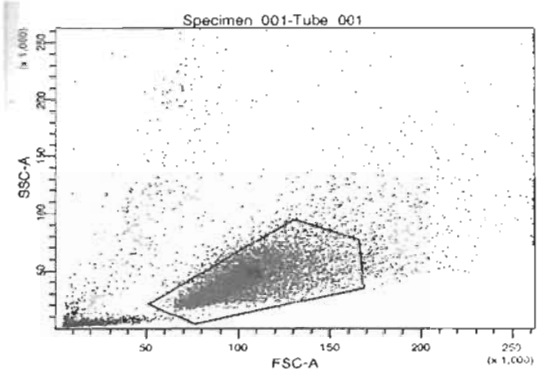
Marker	Left, Right	% Gated	% Total	Geo Mean
All	1, 9910	100.00	74.12	164.57
M1	13, 9910	92.16	68.31	219.24

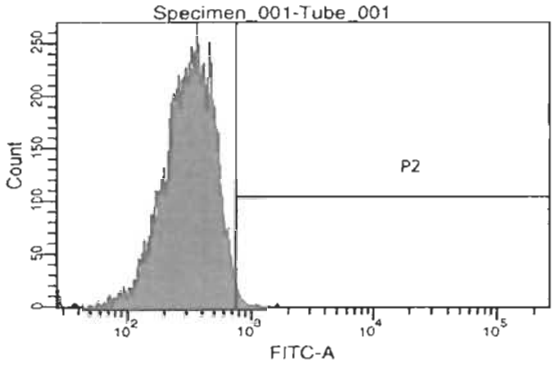
Figure 40: Flow cytometry analysis of the different transfection samples 48 hours after transfection. The number of viable cells was chosen with the help of gate G1 on the forward scatter / side scatter plot. The number of viable transfected cells was chosen by the M1 marker on the FL1 histogram.

A) NC R1
B) NC R2
C) IgfIB R1
D) IgfIB R2
E) Mix R1
F) Mix R2

7.6.2 Igf Batch 2

A)

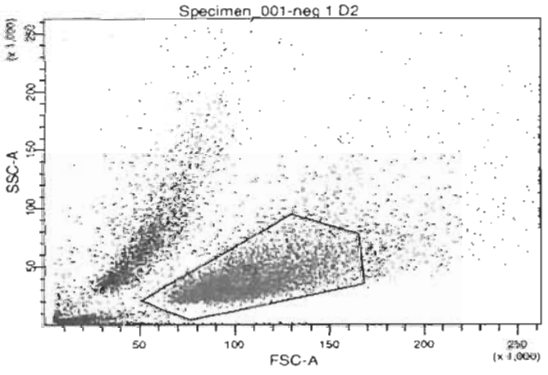


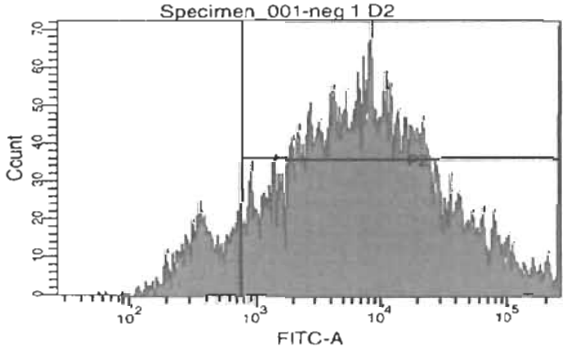


Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	Tube_001
Record Date:	Jun 22, 2011 10:13:23 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	437
P1	7,689	76.9	####	343
P2	103	1.3	974	1,018

B)

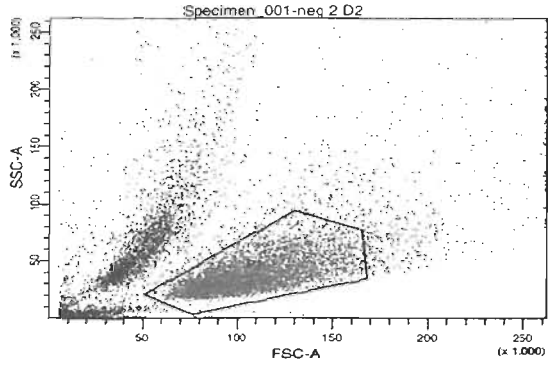


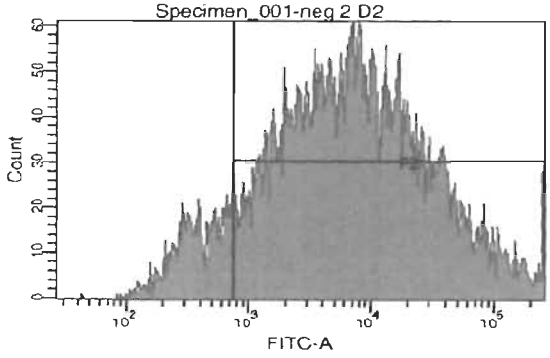


Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	neg 1 D2
Record Date:	Jun 22, 2011 10:21:05 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	17,800
P1	5,485	54.8	6,446	22,171
P2	4,903	89.4	9,030	24,753

C)

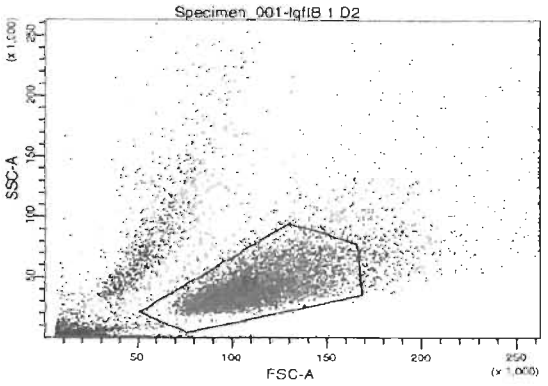


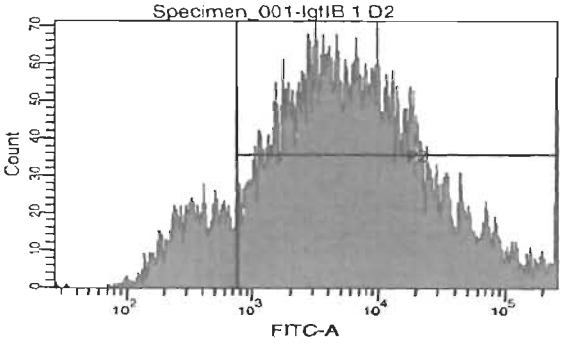


Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	neg 2 D2
Record Date:	Jun 22, 2011 10:22:24 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	17,257
P1	5,687	56.9	6,340	22,227
P2	5,073	89.2	8,925	24,866

D)

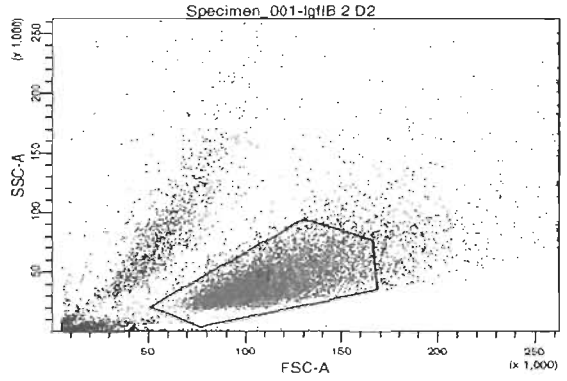


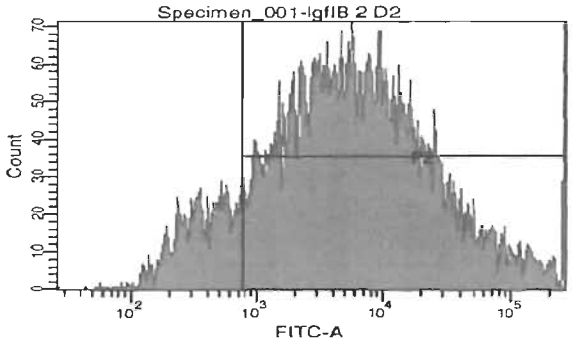


Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	IgfIB 1 D2
Record Date:	Jun 22, 2011 10:23:41 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	16,139
P1	6,392	63.9	5,116	18,537
P2	5,597	87.6	7,510	21,115

E)

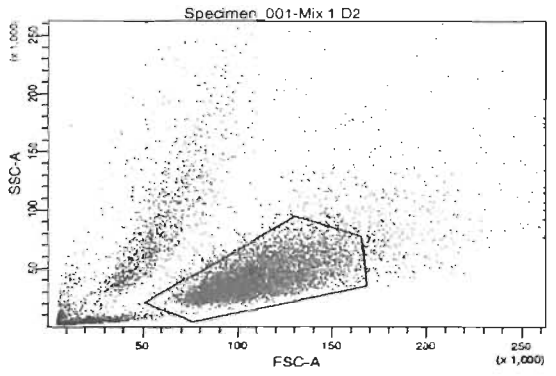


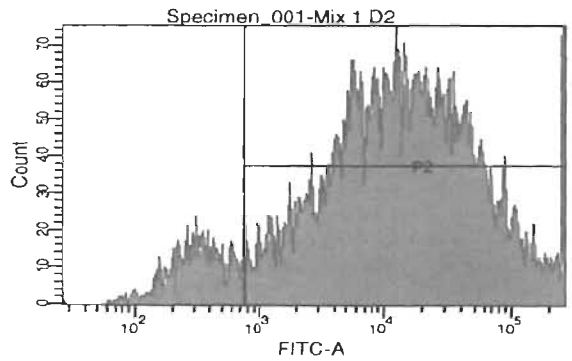


Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	IgfIB 2 D2
Record Date:	Jun 22, 2011 10:24:56 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	16,556
P1	6,513	65.1	5,060	18,958
P2	5,655	86.8	7,591	21,775

F)

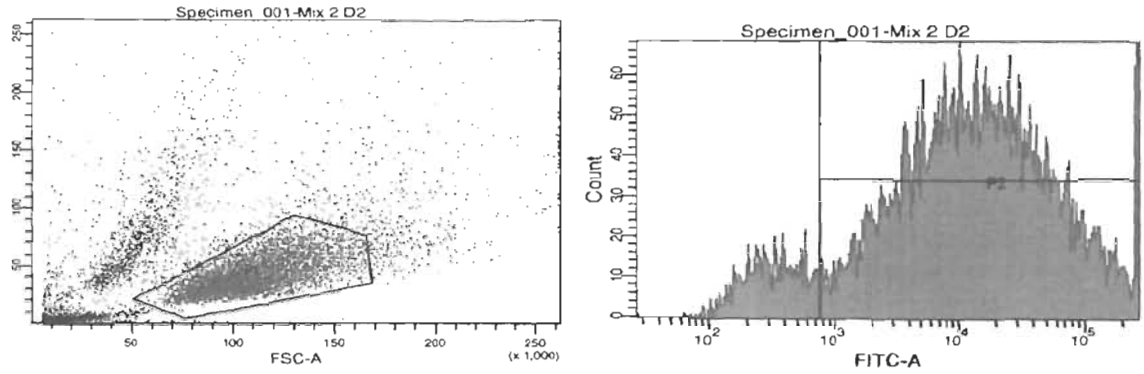




Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	Mix 1 D2
Record Date:	Jun 22, 2011 10:16:14 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	31,291
P1	6,717	67.2	10,885	35,834
P2	6,096	90.8	15,645	39,449

G)



Experiment Name:	Experiment_009
Specimen Name:	Specimen_001
Tube Name:	Mix 2 D2
Record Date:	Jun 22, 2011 10:19:44 AM

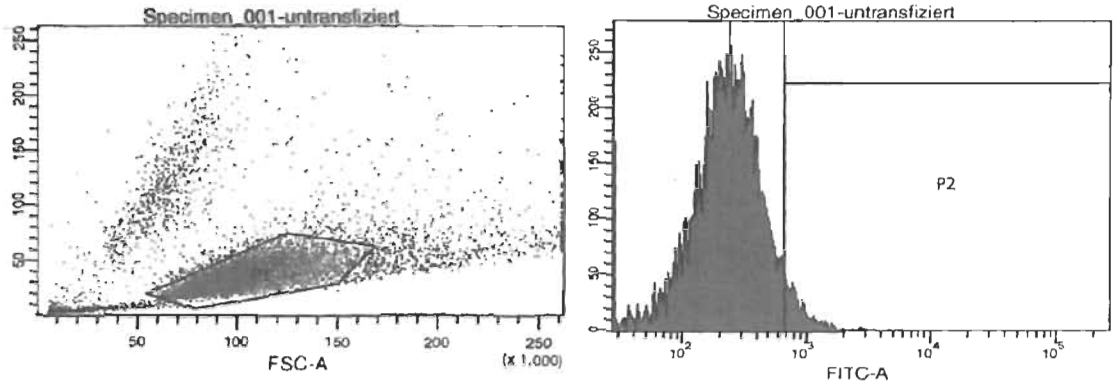
Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	29,844
P1	6,136	61.4	####	36,284
P2	5,544	90.4	15,702	40,121

Figure 41: Flow cytometry analysis of the different transfection samples 48 hours after transfection. The number of viable cells was chosen with the help of gate P1 on the forward scatter / side scatter plot. The number of viable transfected cells was chosen by the P2 population on the FITC histogram.

A) untransfected cells, blank GFP
B) NC R1
C) NC R2
D) IgfIB R1
E) IgfIB R2
F) Mix R1
G) Mix R2

7.6.3 Igf Batch 3

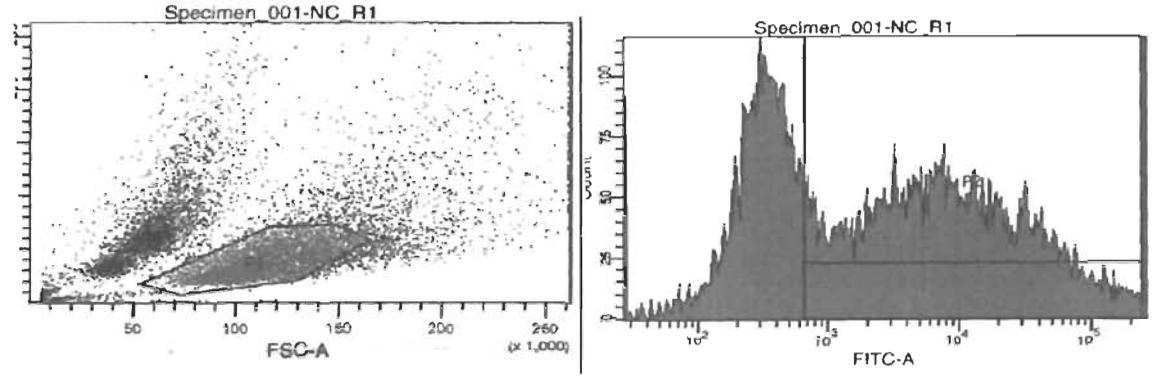
A)



Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	untransfiziert
Record Date:	Jul 27, 2011 11:23:2...

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	282
P1	7,457	74.6	####	227
P2	506	5.1	931	1,009

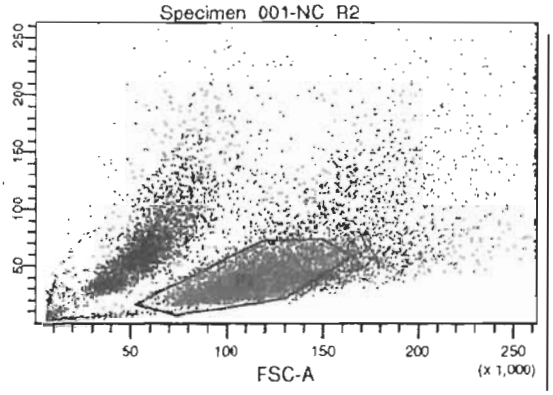
B)

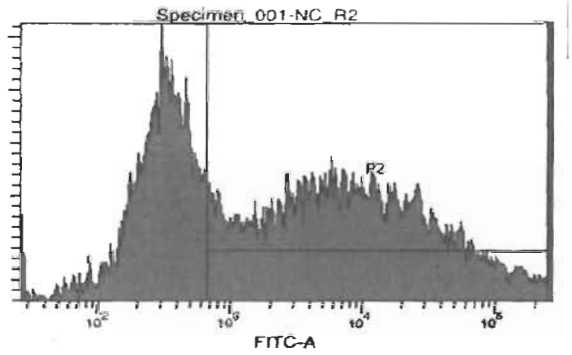


Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	NC_R1
Record Date:	Jul 27, 2011 11:30:41...

Population	#Eve...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	19,332
P1	4,576	45.8	####	23,471
P2	6,238	62.4	8,220	22,413

C)

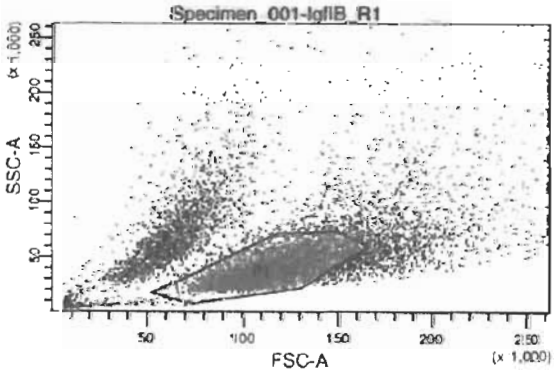


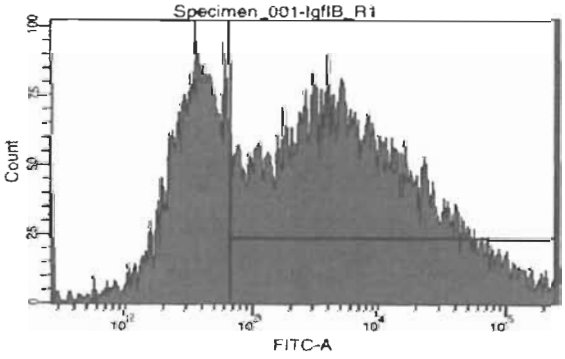


Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	NC_R2
Record Date:	Jul 27, 2011 11:34:04...

Population	#Eve...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	20,117
P1	4,430	44.3	6,531	24,982
P2	6,181	61.8	8,162	22,411

D)

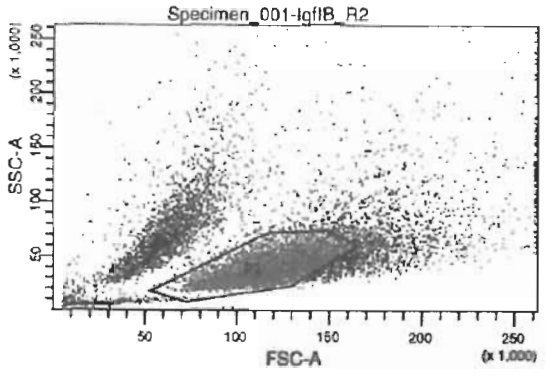


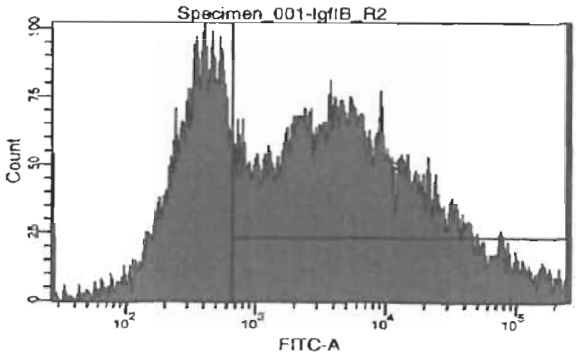


Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	IgfIB_R1
Record Date:	Jul 27, 2011 11:35:11 AM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	16,735
P1	5,323	53.2	4,487	19,182
P2	6,998	70.0	6,367	17,195

E)

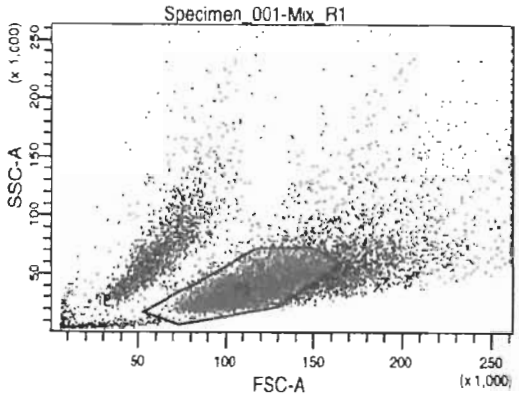


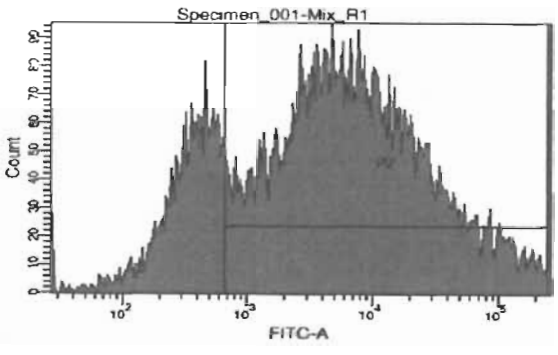


Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	IgfIB_R2
Record Date:	Jul 27, 2011 11:37:55 AM

Population	#Even...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	16,536
P1	5,135	51.3	4,425	19,106
P2	6,812	68.1	6,152	16,986

F)

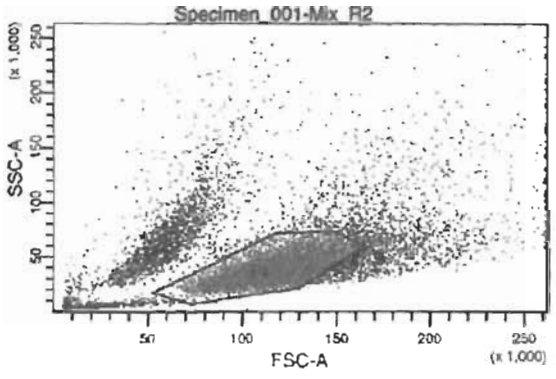


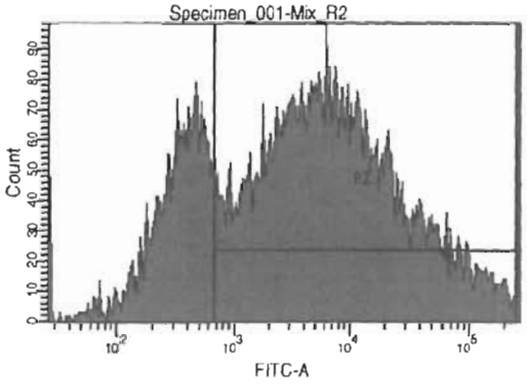


Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	Mix_R1
Record Date:	Jul 27, 2011 11:41:43 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	22,350
P1	5,565	55.6	6,003	21,796
P2	7,717	77.2	7,750	20,221

G)

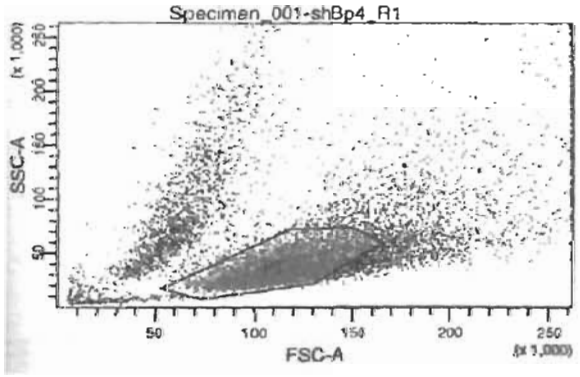


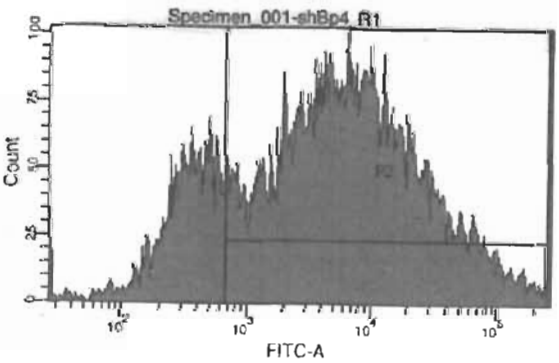


Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	Mix_R2
Record Date:	Jul 27, 2011 11:43:02...

Population	#Eve...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	21,054
P1	5,368	53.7	5,839	21,948
P2	7,451	74.5	7,525	20,037

H)

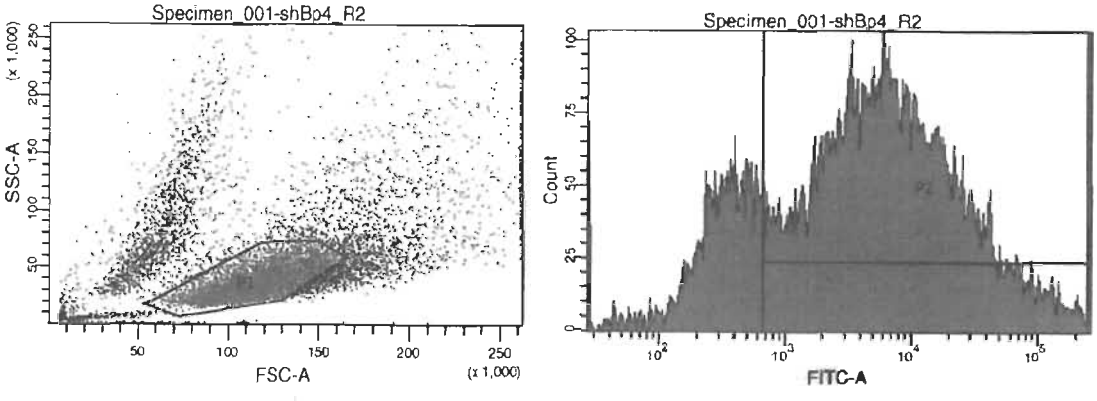




Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	shBp4_R1
Record Date:	Jul 27, 2011 11:44:11 AM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	18,304
P1	5,629	56.3	####	16,501
P2	7,894	78.9	7,242	17,429

I)



Experiment Name:	Experiment_012
Specimen Name:	Specimen_001
Tube Name:	shBp4_R2
Record Date:	Jul 27, 2011 11:45:40 ...

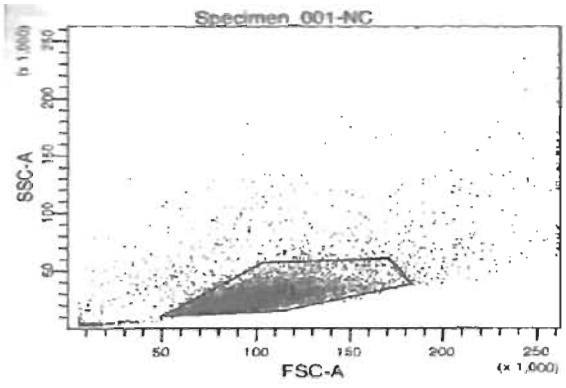
Population	#Even...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	17,597
P1	5,630	56.3	5,633	17,241
P2	7,827	78.3	7,286	17,549

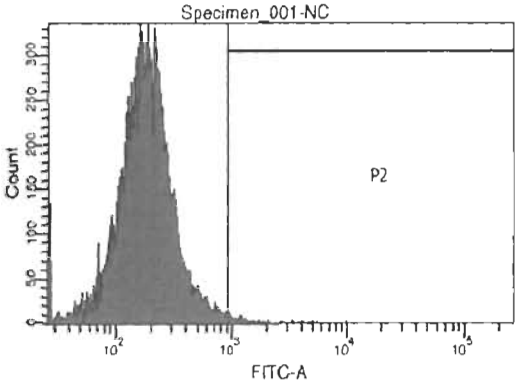
Figure 42: Flow cytometry analysis of the different transfection samples 48 hours after transfection. The number of viable cells was chosen with the help of gate P1 on the forward scatter / side scatter plot. The number of viable transfected cells was chosen by the P2 population on the FITC histogram.

A) untransfected cells, blank GFP
B) NC R1
C) NC R2
D) IgfIB R1
E) IgfIB R2
F) Mix R1
G) Mix R2
H) shBp4 R1
I) shBp4 R2

7.6.4 Igf Batch 4

A)

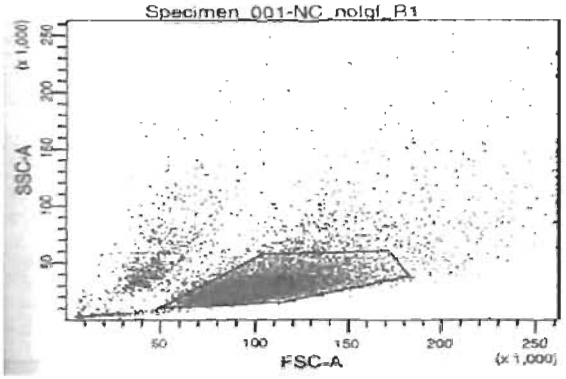


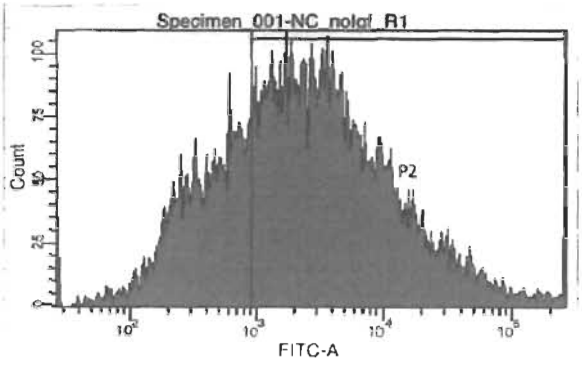


Experiment Name: Experiment_013
Specimen Name: Specimen_001
Tube Name: NC
Record Date: Aug 18, 2011 11:33:55 AM

Population	#Events	%Parent	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	221
P1	9,006	90.1	####	192
P2	115	1.2	1,391	1,588

B)

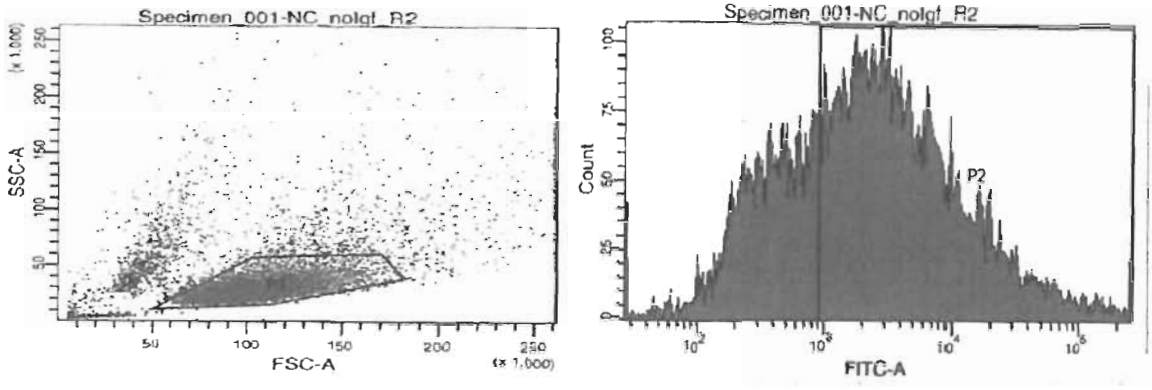




Experiment Name: Experiment_013
Specimen Name: Specimen_001
Tube Name: NC_nolgf_R1
Record Date: Aug 18, 2011 11:37:22 AM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	10,753
P1	8,095	81.0	####	9,832
P2	7,162	71.6	5,045	14,841

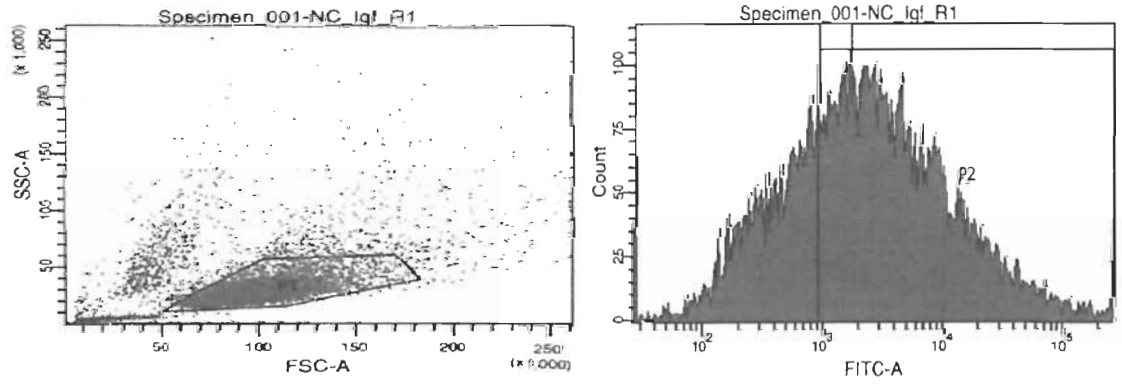
C)



Experiment Name:	Experiment_013
Specimen Name:	Specimen_001
Tube Name:	NC_nolgf_R2
Record Date:	Aug 18, 2011 11:45:44...

Population	#Eve...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	10,342
P1	7,714	77.1	2,614	9,465
P2	6,944	69.4	5,091	14,708

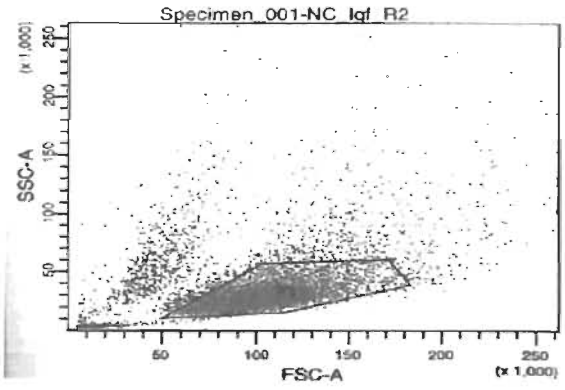
D)

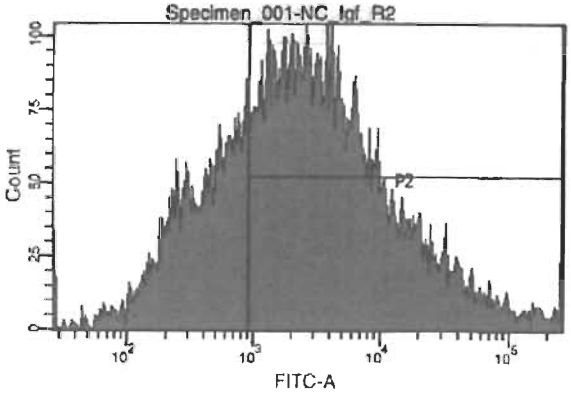


Experiment Name:	Experiment_013
Specimen Name:	Specimen_001
Tube Name:	NC_lgf_R1
Record Date:	Aug 18, 2011 11:46:58 AM

Population	#Events	%Par...	FITC-A GeoM...	FITC-A Mean
All Events	10,000	####	####	10,320
P1	8,119	81.2	####	9,918
P2	7,153	71.5	5,064	14,256

E)

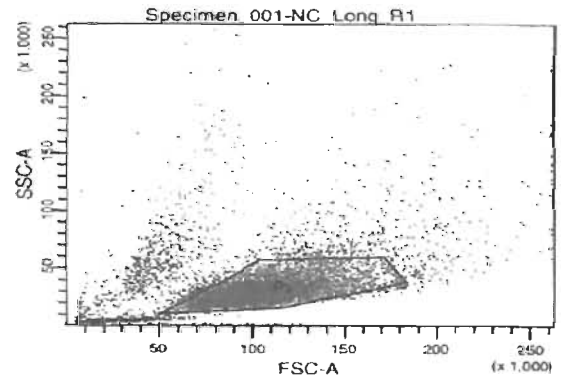


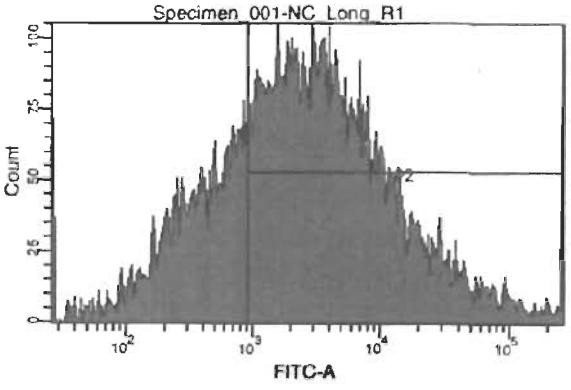


Experiment Name:	Experiment_013
Specimen Name:	Specimen_001
Tube Name:	NC_Igf_R2
Record Date:	Aug 18, 2011 11:48:23 ...

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	11,201
P1	8,155	81.6	####	10,824
P2	7,174	71.7	5,204	15,442

F)

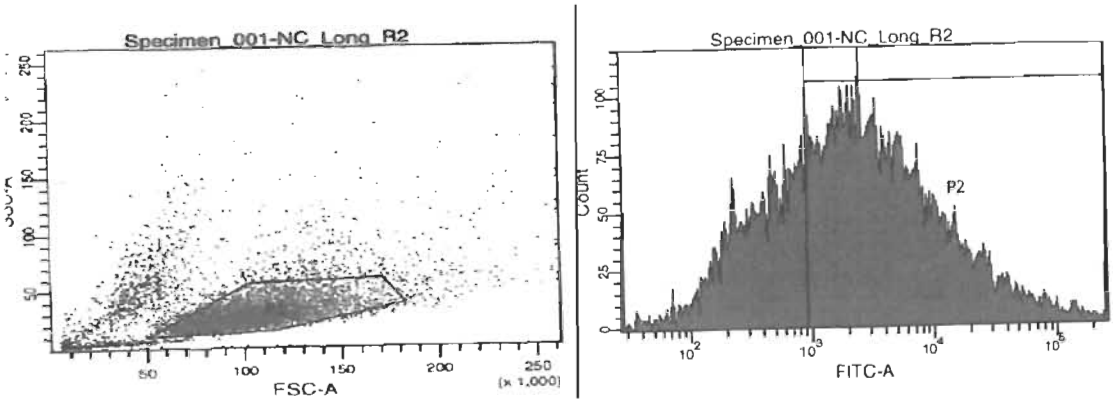




Experiment Name:	Experiment_013
Specimen Name:	Specimen_001
Tube Name:	NC_Long_R1
Record Date:	Aug 18, 2011 11:49:35 AM

Population	#Events	%Pare...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	11,025
P1	8,009	80.1	2,958	11,039
P2	7,279	72.8	5,179	14,990

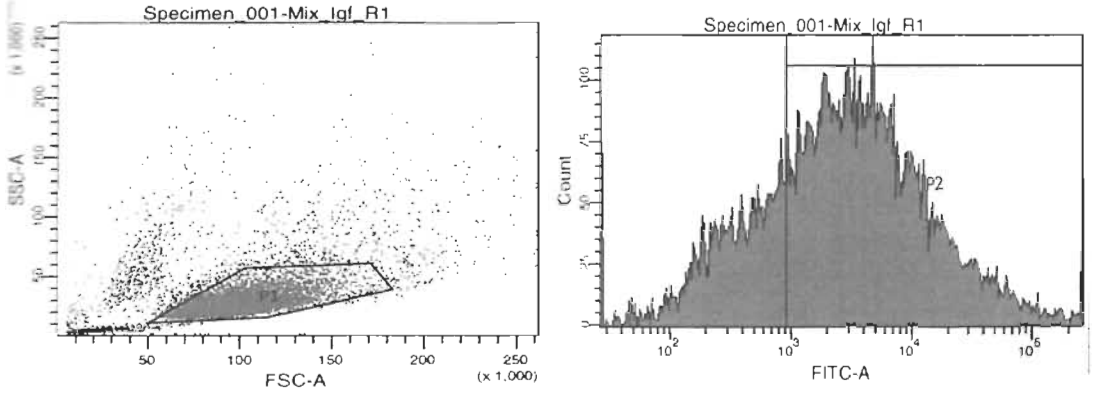
G)



Experiment Name: Experiment_013
Specimen Name: Specimen_001
Tube Name: NC_Long_R2
Record Date: Aug 18, 2011 11:51:09 AM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	10,314
P1	7,787	77.9	2,761	10,008
P2	6,928	69.3	5,051	14,706

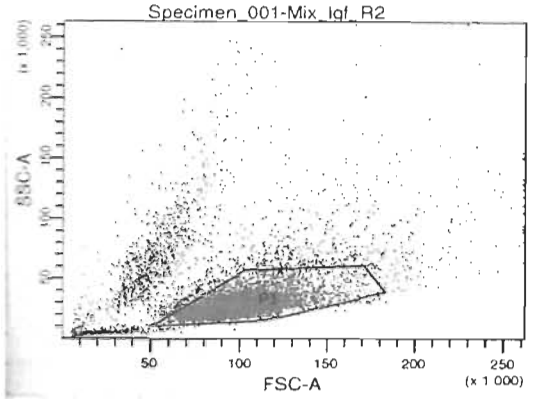
H)

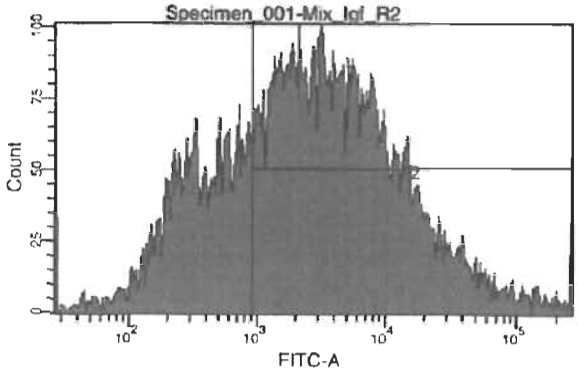


Experiment Name: Experiment_013
Specimen Name: Specimen_001
Tube Name: Mix_Igf_R1
Record Date: Aug 18, 2011 11:55:31 AM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	11,224
P1	8,105	81.0	####	10,537
P2	7,475	74.8	5,519	14,877

I)

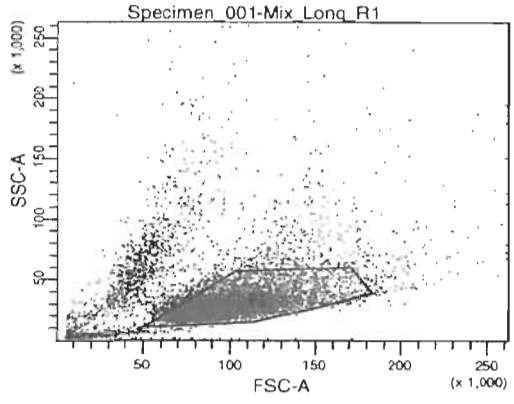


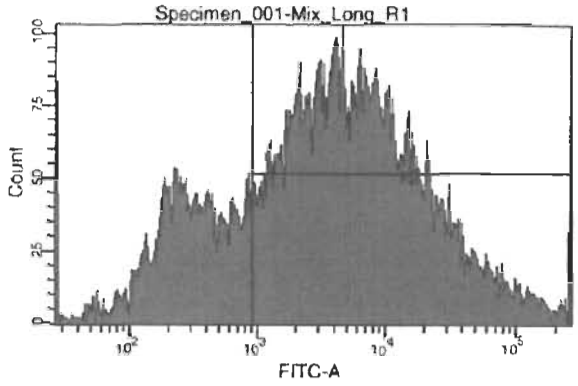


Experiment Name: Experiment_013
Specimen Name: Specimen_001
Tube Name: Mix_Igf_R2
Record Date: Aug 18, 2011 11:57:10 AM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	10,399
P1	7,926	79.3	####	10,054
P2	7,161	71.6	5,411	14,362

J)

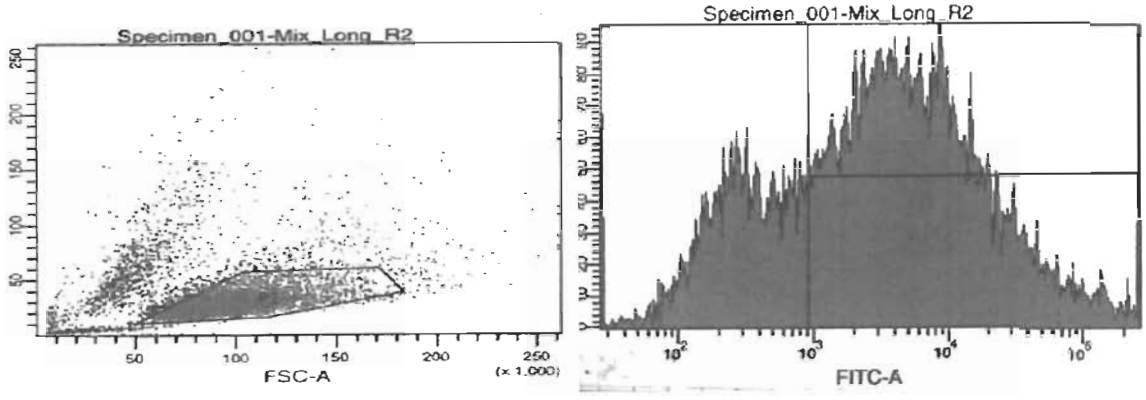




Experiment Name: Experiment_013
Specimen Name: Specimen_001
Tube Name: Mix_Long_R1
Record Date: Aug 18, 2011 11:58:20 ...

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	14,966
P1	7,624	76.2	####	15,588
P2	7,525	75.2	7,320	19,772

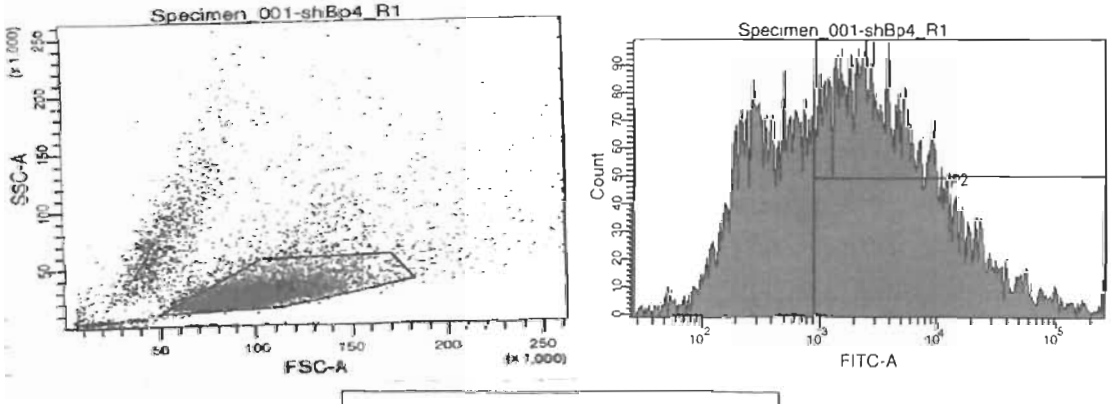
K)



Experiment Name:	Experiment_013
Specimen Name:	Specimen_001
Tube Name:	Mix_Long_R2
Record Date:	Aug 18, 2011 12:00:09 PM

Population	#Events	%Par...	FITC-A Geo M...	FITC-A Mean
All Events	10,000	####	####	14,813
P1	7,685	76.8	####	14,966
P2	7,314	73.1	7,153	20,121

L)



Experiment Name:	Experiment_013
Specimen Name:	Specimen_001
Tube Name:	shBp4_R1
Record Date:	Aug 18, 2011 11:52:32

Population	#Eve...	%Par...	FITC-A Geo ...	FITC-A Mean
All Events	10,000	####	####	8,268
P1	7,548	75.5	2,309	8,345
P2	6,400	64.0	4,816	12,697

M)

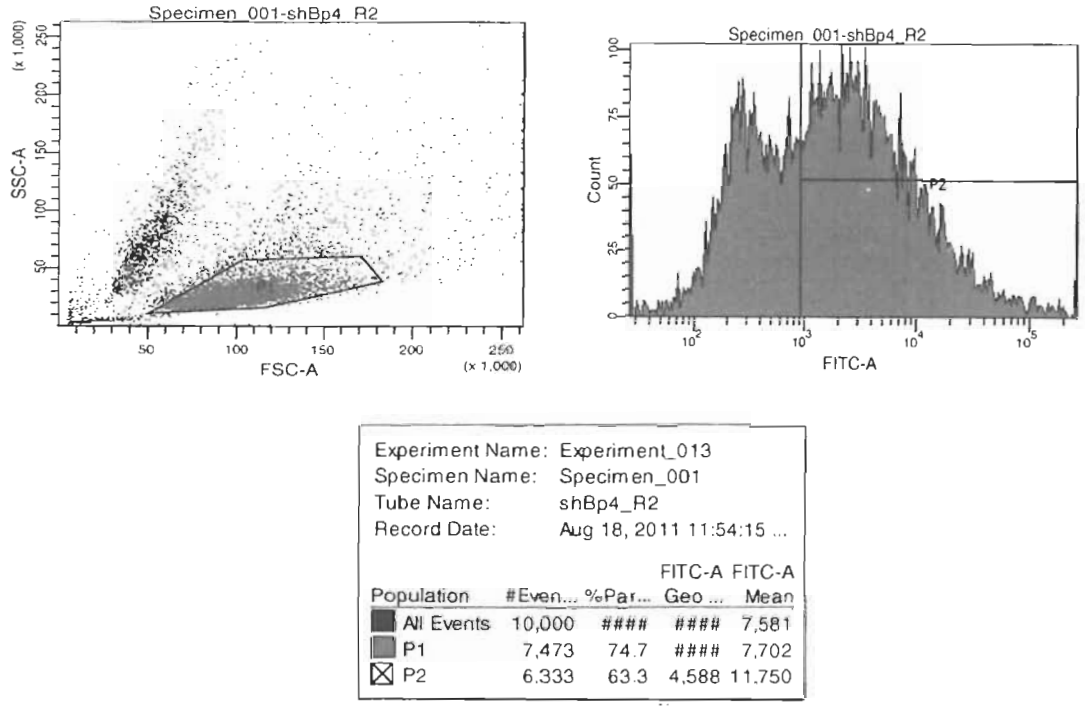


Figure 43: Flow cytometry analysis of the different transfection samples 48 hours after transfection. The number of viable cells was chosen with the help of gate P1 on the forward scatter / side scatter plot. The number of viable transfected cells was chosen by the P2 population on the FITC histogram.

A) untransfected cells, blank GFP
B) NC R1
C) NC R2
D) NC Igf R1
E) NC Igf R2
F) NC Long R1
G) NC Long R2
H) Mix Igf R1
I) Mix Igf R2
J) Mix Long R1
K) Mix Long R2
L) shBp4 R1
M) shBp4 R2