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Bacterial Artificial Chromosome (BAC) Based vs. Conventional Plasmid Transfection

A Comparison Study in CHO Cells

Master Thesis

Submitted by
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All truths are easy to understand once they are discovered;
the point is to discover them.
(Galileo Galilei)

Abstract

The market and fields of application for therapeutic proteins, such as monoclonal antibodies, are constantly growing. Therefore, stable production systems and high yields become increasingly important. CHO cells are widely used for expressing recombinant proteins and there are several techniques available for generating a producing cell line. Conventional plasmid transfection is a common method, but for establishing a high producer cell line, gene amplification and time-consuming screenings are necessary. An alternative method is the application of BACs as transfection vectors, which generate high producing cell lines without the need for gene amplification. The BAC itself mediates this as it provides a highly transcribed open chromatin region.

For this work, two scFc antibodies, 3D6scFc and 2F5scFc, were expressed in CHO cells. Cell lines were established using conventional plasmid transfection and compared to BAC transfectants. Therefore, growth and productivity were continuously monitored. Additionally, qPCR was performed to compare the cell lines regarding gene copy numbers and transcript level. Additionally, intracellular product concentration was determined by flow cytometry.

Specific productivities were three to four times higher for BAC transfectants compared to plasmid transfection without amplification. Gene copy numbers and intracellular product concentration were partly higher but transcript levels were significantly increased for BAC transfectants.

Thus, BAC transfection demonstrated to be a superior method for establishing stable, high producing CHO cell lines.

Der Markt und die Anwendungsgebiete für therapeutische Proteine, wie unter anderem monoklonale Antikörper, sind durch starkes Wachstum geprägt. Deshalb gewinnen stabile Produktionssysteme und hohe Ausbeuten an Bedeutung. CHO Zellen sind für die Expression von rekombinanten Proteinen weit verbreitet und es gibt eine Vielzahl an Techniken, um eine Produktionszelllinie zu generieren. Weit verbreitet ist die konventionelle Plasmidtransfektion, doch um hiermit einen guten Produzenten zu etablieren, benötigt man Genamplifikation und zeitintensive Screening-Runden. Eine alternative Methode ist die Verwendung von BACs als Transfektionsvektoren welche hohe Zellproduktivitäten auch ohne Genamplifikation erzielen können. Dies liegt daran, dass BACs höher transkribierte, so genannte offene Chromatinregionen mitbringen.

Für diese Arbeit wurden zwei scFc Antikörper, 3D6scFc und 2F5scFc, in CHO Zellen exprimiert. Hierzu wurden Zelllinien mit konventioneller Plasmidtransfektion generiert und mit BAC-transfizierten Zellen verglichen. Dabei wurde kontinuierlich das Wachstum und die Produktivität eruiert. Zusätzlich wurden qPCR Analysen durchgeführt um die Zelllinien bezüglich Gen-Kopienzahlen und Transkriptmengen beurteilen zu können. Die intrazelluläre Produktkonzentration wurde mittels Durchflusszytometrie bestimmt.

Die spezifischen Produktivitäten waren in BAC-Transfektanten drei- bis vierfach höher als in den Plasmidtransfektanten ohne Genamplifikation. Die Gen-Kopienzahlen und intrazellulären Produktkonzentrationen waren zum Teil höher, doch besonders deutlich war ein Anstieg der Transkriptmenge in BAC-Transfektanten festzustellen.

Insgesamt hat sich die Transfektion mittels BAC-Vektoren als eine vergleichsmäßig gute Methode zur Generierung von stabilen, hoch produzierenden CHO Zelllinien herausgestellt.

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BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
dH_2O	Deionised water
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleoside triphosphates
ELISA	Enzyme-linked Immunosorbent Assay
EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence Activated Cell Sorting
FIU	Fluorescence intensity unit
GCN	Gene copy number
gDNA	Genomic DNA
GOI	Gene of interest
HT	Hypoxanthine and thymidine
LSD	Least significant difference
μ	Specific growth rate
MFI	Median fluorescence intensity
MTX	Methotrexat
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qp	Specific productivity
RT	Room Temperature
scFc	Single chain variable fragment - crystallizable fragment
Vs.	Versus

1 Introduction

1.1 General Information

Recombinant antibodies are widely used for therapy, research and diagnostic purposes (Schirrmann et al., 2008). Thus, they became the fastest growing class among therapeutic proteins. A variety of production systems is available, but cultivated mammalian cells have taken a prevalent role due to their abilities regarding protein folding and assembly, as well as post-translational modifications (Wurm, 2004). Within the group of mammalian expression systems, immortalized Chinese hamster ovary (CHO) cells are most commonly used for the large-scale expression of recombinant protein pharmaceuticals (Kim et al., 2012).

For large-scale production, it is of crucial importance to have a stable cell line that is reaching high product titers. If the cells are transfected with a conventional plasmid, it is not possible to influence the locus and number of integration sites in the genome.

Therefore, powerful selection and gene amplification methods were developed. A very commonly used system is CHO/DHFR (Chusainow et al., 2009). It is based on the isolation of dihydrofolate reductase (DHFR) deficient CHO cells (Urlaub and Chasin, 1980), which are not able to convert dihydrofolate to tetrahydrofolate. Thus, cells grow only in a medium supplemented with hypoxanthine and thymidine (HT). By co-transfection of the genes of interest and the DHFR gene, CHO cells can be selected by cultivation in a medium lacking HT. Additionally, these introduced genes can be amplified by methotrexate (MTX). MTX inhibits the DHFR enzyme and the cells amplify the DHFR gene together with the genes of interest to overcome the inhibition (Chusainow et al., 2009). With this process, the productivity can be efficiently improved. However, gene amplification is labor-intensive and time-consuming (Kim et al., 2012). Therefore, other systems for improving product yields are continuously investigated.

Apart from number of genomic integrations, also the site of chromosomal integration directly effects the transcription rate (Wilson et al., 1990). This fact is referred to as position effect and can positively influence the productivity. If transgenes are inserted into tightly packed DNA regions, the heterochromatin, product expression can

be limited or completely inhibited. On the contrary, integration into euchromatin, the lightly packed form of DNA, frequently enables expression.

One way of achieving integration into highly transcribed regions of the genome is the application of bacterial artificial chromosomes (BACs) as transfection vectors. BACs have a large cloning capacity of up to 300 kbp and can contain genes which are considered as euchromatin, such as the *Rosa26* locus (Blaas et al., 2009). For the generation of transgenic mice, BACs have already been widely used (Giraldo and Montoliu, 2001), but the application for recombinant protein production is comparably new.

In 2009, Blaas et al. expressed an Fc fragment in HEK 293 cells and obtained a more than ten times higher specific productivity with BAC transfected clones compared to cell clones transfected with a conventional plasmid (Blaas et al., 2009). Furthermore, they found a correlation between specific productivity and transgene copy numbers and analyzed the protein production over a longer cultivation period. After 30 passages, the product yield was not significantly decreased.

1.2 Project proposal

Purpose of this work is a comparison of BAC and plasmid based transfection methods in CHO cells. Therefore, in total six cell lines are established, two of them with BAC transfection. The other four cell lines are co-transfected with a conventional plasmid containing the genes of interest and a plasmid containing the DHFR gene. Further, two of the four generated cell clones are subjected to amplification by MTX treatment.

For better comparability, the model protein used in this study is not a complete antibody. A full immunoglobulin G (IgG) molecule contains a heavy and a light chain, which would need the integration of two genes into the host cell genome. Therefore, simpler versions of the antibodies are engineered. Single chain variable fragment - crystallizable fragment (scFv-Fc, also denoted as scFc) antibodies are used as model in this study. The structure of a scFc antibody is presented in Fig. 1.1. It consists of the variable regions of a human IgG which are connected by a peptide linker, with an additional Fc part. For better comparison two different scFc antibodies, 3D6scFc and 2F5scFc, are used.

3D6scFc is a non-neutralizing monoclonal antibody and 2F5scFc is a broadly neutralizing monoclonal antibody, both targeting the envelope protein gp41 of HIV-1 (Kunert et al., 1998).

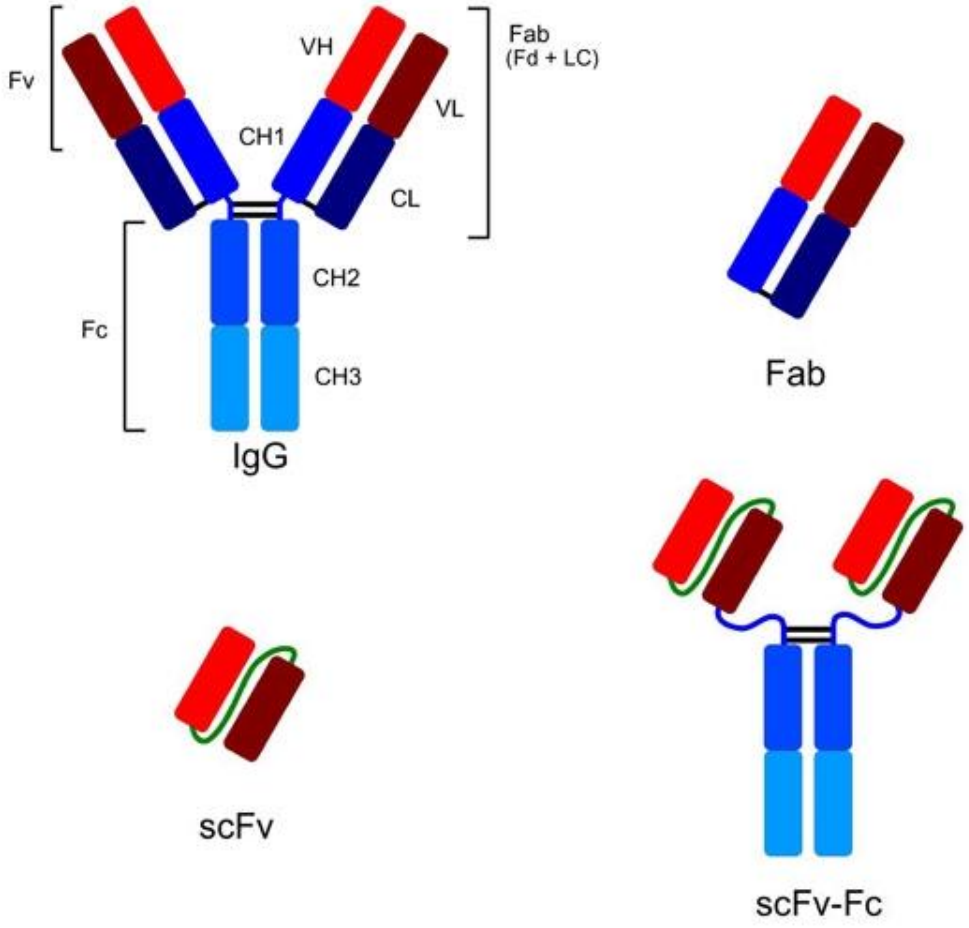

Fig. 1.1 Antibody structures: From IgG to scFv-Fc

The BAC transfectants were established by Martina Hofbauer (Polymun Scientific Inc.) and Willibald Steinfellner (BOKU Vienna, working group of Prof. Kunert). Plasmid transfections and cell line establishments are done within this project.

All six final cell lines are monitored for growth and productivity. Because of the time needed for MTX amplification, the amplified cell lines are not used for further comparisons within this work.

For BAC and plasmid transfectants without amplification (Fig. 1.2), also the gene copy numbers (GCN), transcript amounts and intracellular product concentrations are analysed. The GCN describes the prevalence of genes of interest in the host cell's genome and transcript amount equals the mRNA present in the cells. Both are analysed by Real-time qPCR. The intracellular product is analysed by flow cytometry.

sms involved

recombinant

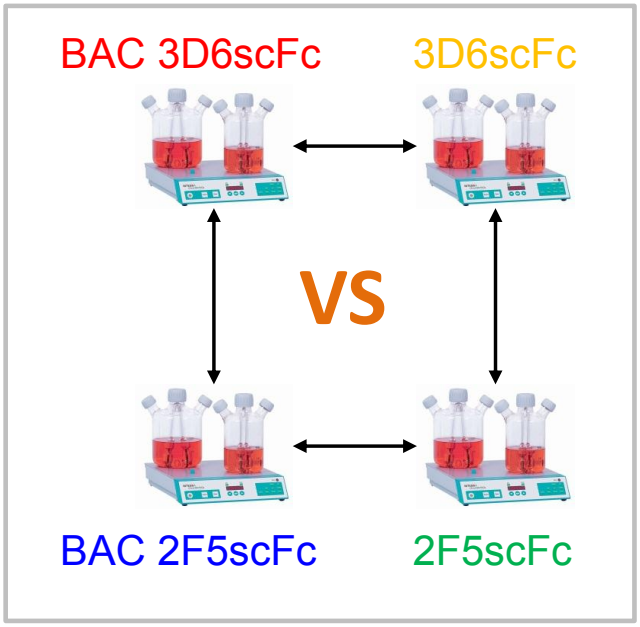

Fig. 1.2 Cell lines used for comparison of GCN, transcript amount and intracellular product concentration

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2 Material and Methods

2.1 Amplification of Plasmids

2.1.1 Material

Tab. 2.1: Material used for plasmid amplification in E.coli

Component	Definition
SOC Medium	Yeast extract 0.5 % (w/v) (5 g/l) Tryptone 2 % (w/v) (20 g/l) Sodium chloride 10 mM (0.6 g/l) Potassium chloride 2.5 mM (0.2 g/l) Magnesium chloride 10 mM Magnesium sulfate 10 mM D-Glucose 20 mM
LB medium	Tryptone 10 g Yeast extract 5 g Sodium chloride 10 g Added up to 1 L with dH_2O
LB agar medium	LB medium 1.5% Agar-Agar
Ampicillin Stock Solution	1000x Ampicillin Stock Solution (100 mg/mL)
Competent *E.coli* TOP10 cells	Invitrogen, One Shot ® TOP10 Electro-comp™ *E.coli* Cat. N°: C4040-52
Maxi Prep Kit	Macherey-Nagel REF 740424.10 LOT 1108/001

Electroporator	BioRad, Gene Pulser Xcell™ Electroporation System Cat. N°: 165-2660
Nanophotometer	Implen NanoPhotometer P-300

2.1.2 Transformation and cultivation of E.coli

For the transformation, 1 µL of plasmid DNA solution was added to 40 µL of competent *E.coli* TOP10 cells. This mixture was used for electroporation with 1.8 kV, 25 µF and 200 Ω in a 2 mm self-made electroporation cuvette (aluminium foil sticked to the inside of a disposable plastic cuvette). After the electroporation, 250 µL SOC medium were added and the mixture was incubated for 1 hour at 37 °C and 500 rpm.

50 µL and 100 µL aliquots were plated on petri dishes with LB agar medium containing 100 µg/mL ampicillin. The plates were incubated at 37 °C over night. On the next day the plates were sealed with parafilm and stored at 4 °C for further usage.

For the suspension culture, 500 mL of LB-Medium supplemented with ampicillin (100 µg/mL) was prepared. For the starter culture, 5 mL of this medium was inoculated with 1 single colony from the 50 µL aliquot - plate. This culture was incubated at 37 °C for 6 hours while shaking. The flask lid was slightly opened for air exchange. After incubation, two 500 mL flasks were prepared with 100 mL of the LB-Amp-Medium each. Afterwards, 100 µL of the starter culture were added to each of the 2 flasks. The incubation was at 37 °C, over night while shaking.

2.1.3 Purification of plasmid DNA

Plasmid preparation was done with a Maxi Prep Kit according to manufacturer's instructions. The purified plasmid was dissolved in 500 µL of endotoxin-free H_20. Afterwards, the DNA concentration was determined photometrically with the Nanophotometer.

2.2 PCR

2.2.1 Material

Tab. 2.2 Material used for the PCR of plasmid DNA

Component	Definition
dNTPs	New England Bilolabs deoxynucleotide solution mix 10 mM each dNTP N0447L, stored at -20 °C
ThermoPol Buffer	New England BioLabs, 10x ThermoPol Reaction Buffer #B9004S, 10x Concentrate Lot: 0010908, Amt.: 1.5 mL
dH2O	deionized, autoclaved H2O
Taq Polymerase	New England BioLabs, Taq DNA Polymerase w/ ThermoPol Buffer #M0267L, 5.000 U/mL Lot: 8000908, Amt.: 0.4 mL
Primers	3D6scFc_Std_sense, 3D6scFc_Std_as, 13H5A5_LC306_se , according to chapter 2.11. 10 µM (1:10 dilution of 100 µM stock solution)
Thermocycler	BioRad C1000 thermal cycler
pCAGGS 3D6scFc	pCAGGS 3D6scFc plasmid as template, 2.86 µg/µL
pCIneo 3D6scFc	pCIneo 3D6scFc plasmid as template, 0.5 µg/µL
pCAGGS 2F5scFc	pCAGGS 2F5scFc plasmid as template, 0.5 µg/µL
pCIneo 2F5scFc	pCIneo 2F5scFc plasmid as template, 102 ng/µL

2.2.2 Procedure

Primers were diluted to a working stock concentration of 10 µM. The master mix was made according to Tab. 2.3. Four aliquots of 29.5 µL were taken for the PCR and 0.3 µL (3 pmol) of each Primer and 0.2 µL template were added.

Tab. 2.3 Reaction mixes used for PCR of plasmids

solution	pCAGGS 3D6scFc	pCIneo 3D6scFc	pCAGGS 2F5scFc	pCIneo 2F5scFc	Master Mix 5x
dNTPs	0.6 µL	0.6 µL	0.6 µL	0.6 µL	3 µL
ThermoPol Buffer	3 µL	3 µL	3 µL	3 µL	15 µL
dH2O	25.48 µL	25.48 µL	25.48 µL	25.48 µL	127.4 µL
Taq Polymerase	0.12 µL	0.12 µL	0.12 µL	0.12 µL	0.6 µL
3D6scFc_Std_sense	0.3 µL	0.3 µL			
3D6scFc_Std_as	0.3 µL	0.3 µL	0.3 µL	0.3 µL	1.5 µL
13H5A5_LC306_se			0.3 µL	0.3 µL	
pCAGGS 3D6scFc	0.2 µL				
pCIneo 3D6scFc		0.2 µL			
pCAGGS 2F5scFc			0.2 µL		
pCIneo 2F5scFc				0.2 µL	
SUM	**30 µL**	**30 µL**	**30 µL**	**30 µL**	**147.5 µL**

The PCR was performed according to Tab. 2.4. Afterwards the PCR mixes were stored at -20°C and analyzed by agerose gel electrophoresis (chapter 2.3).

Tab. 2.4 Cycles used for PCR of plasmids

Temperature	Time	Number of cycles
95 °C	2 minutes	1 x
94 °C	30 seconds	30 x
51 °C	30 seconds	
72 °C	1 minute	
72 °C	5 minutes	1 x

2.3 Agarose gel electrophoresis

2.3.1 Material

Tab. 2.5 Material used for agarose gel electrophoresis

Component	Definition
50x TAE buffer	242 g Tris base (121.1 g/mol) 57.1 mL glacial acetic acid 100 mL EDTA (0.5 M) Added up to 1 L with dH_2O
Agarose	peqlab peqGOLD Universal-Agarose, Article N°: 35-1020
EtBr	Ethidium bromide solution, 10 mg/mL in H_2O
Electrophoresis system	BioRad Sub-cell system for horizontal nucleic acid electrophoresis
Power supply	BioRad Power Pac Basic, Cat N°: 164-5050
Molecular Imager	BioRad Gel Doc XR System Cat N°: 170-8170

2.3.2 Preparation of gels

A 1% agarose solution in 1x TAE buffer was prepared and heated in a microwave oven until the agarose melted. To guarantee good separation and clear pictures, no turbidities should be present in the solution. After cooling it down to approximately 65 °C, 3 µL EtBr were added per 100 mL of agarose solution. The still liquid gel was gently mixed and poured into gel casting trays. Combs were used to obtain the pockets for pipetting the samples into the gel.

The gels polymerized on a static place in about 30 min to 1 hour. Afterwards they were stored at 4 °C submerged in 1x TAE-buffer.

2.3.3 Electrophoresis run

In an electrophoresis chamber, one gel was submerged in 1x TAE buffer. The samples were added to the pockets and the separation was performed at 110 V for approximately one hour. After this time, the gel was carefully taken out of the electrophoresis chamber and put into the molecular imager to photograph the bands.

2.4 Restriction Digestion

2.4.1 Material

Tab. 2.6 Material used for restriction digestion of plasmids

Component	Definition
Buffer 3	New England BioLabs NEBuffer 3 #B7003S, 10x Concentrate Lot: 0010903, Amt.: 1.5 mL
BSA	New England BioLabs Purified BSA 100x #B9001S, 10 mg/mL Lot: 0460804, Amt.: 0.25 mL
EcoRV	New England BioLabs EcoRV-HF #R3195S, 20.000 U/mL Lot: 0010811, Amt.: 0.2 mL, Assay: 11/08
dH2O	deionized, autoclaved H2O

pCAGGS 3D6scFc	pCAGGS 3D6scFc plasmid, 2.86 µg/µL
pCIneo 3D6scFc	pCIneo 3D6scFc plasmid, 0.5 µg/µL

2.4.2 Procedure

A master mix was prepared according to Tab. 2.7. Subsequently, two aliquots of 2.7 µL were used for the restriction mixes. For each sample, 0.5 µg of template DNA was added and dH_20 to reach a total volume of 20 µL. The applied volumes were calculated from the plasmid concentrations.

The restricition digestion mixes were incubated at 37°C for one hour. Afterwards, they were stored at -20 °C for further analysis and analyzed using agarose gel electrophoresis (chapter 2.3).

Tab. 2.7 Reaction mixes for restriction digestion

solution	pCAGGS 3D6scFc	pCIneo 3D6scFc	Master Mix 5x
Buffer 3	2 µL	2 µL	5 µL
BSA	0.2 µL	0.2 µL	0.5 µL
EcoRV	0.5 µL	0.5 µL	1.25 µL
dH2O	17.1 µL	16.3 µL	
pCAGGS 3D6scFc	0.2 µL		
pCIneo 3D6scFc		1 µL	
pCAGGS 2F5scFc			
pCIneo 2F5scFc			
SUM	20 µL	20 µL	6.75 µL

2.5 Cell culture

Experimental work was conducted in an animal cell culture laboratory. Sterile handling was done in laminar flow hoods to avoid microbial contamination, as no antibiotics were used for cultivation of animal cells.

2.5.1 Material used for cell cultivation

Tab. 2.8: Media components, media and cell culture material

Media components	
DMEM/Ham's F-12	Biochrom AG, DMEM/HAM's F12 (1:1) w/o L-Glutamine, Cat. N°: F4815
ProCHO5	Lonza, ProCHO5 w/ Pluronic F68 w/o L-Glutamine or Nucleosides (HT), Cat. N°: BE12-766Q
L-Glutamine	PAA, Stable Glutamine (200 mM), Cat. N°: M11-006
Protein free supplement	Polymun Scientific, Protein free supplement, 100x, L0018, Lot N°: 200711
Soy peptone	Polymun Scientific, Soy peptone 125 g/kg, Ultrafiltrated, 50x, L0021
Pluronic F-68	Polymun Scientific, Pluronic F-68 10%, 100x, L0024, Lot N°: 060711
HT Supplement	GIBCO, HT Supplement 50x, REF 41065-012
Phenol red	Sigma-Aldrich, Phenol red solution 0.5 % in DPBS, Cat. N°: P0290
G418	PAA, G-418 Sulphate Solution (50 mg/mL), Cat. N°: P11-012
MTX	Polymun Scientific, Methotrexat, 960 µM, L0002
CryoMaxx II	PAA, CryoMaxx II Cryopreservation Medium, Cat. N°: J05-012

Media	
CHO DHFR- Medium	DMEM/Ham's F-12 + L-Glutamine 4 mM + Protein free supplement 1x + Soy peptone 1x + Pluronic F68 1x + HT Supplement 1x
Transfection Medium	ProCHO5 + L-Glutamine 4 mM + HT Supplement 1x + Phenol red 15 mg/L
Selection Medium	ProCHO5 + L-Glutamine 4 mM + G418 0.5 mg/mL + Phenol red 15 mg/L
Amplification Medium (either 0.048 µM or 0.096 µM MTX)	ProCHO5 + L-Glutamine 4 mM + G418 0.5 mg/mL + Phenol red 15 mg/L + 0.048 µM MTX or 0.096 µM MTX
Material and Reagents	
Culture vessels	Nunc Nunclon Δ surface 96 well plates, Cat. N°: 167008 Nunc Nunclon Δ surface T25 flasks, Cat. N°: 163371 Nunc Nunclon Δ surface T80 flasks, Cat. N°: 153732 Techne complete culture vessel 125 mL spinner flask, PART N°: F7987
CryoTubes	Nunc CryoTubes™, 1.8 mL, Cat. N°: 377267

Trypan blue	Sigma-Aldrich, Trypan blue solution 0.4 %, Cat. N°: T8154
PEI	Polysciences Inc., Polyethylenimine 1 mg/mL, CatN°: 23966-2
Particle counter	Beckmann Coulter, Z2 Coulter counter
Neubauer-chamber	Optik Labor, Neubauer-chamber, 0.100 mm depth Profondeur 0.0025 mm²
Cell lysis buffer	0.1 M citric acid 2 % Triton X-100
Isotone	Beckmann Coulter isotone II diluent, REF N°: 8448011

2.5.2 Cell count with haemocytometer

For determination of cell concentration and viability, 500 µL of cell suspension was homogenously taken from the culture vessel. After addition of 100 µL Trypan blue solution and subsequent mixing, an aliquot was transferred to the Neubauer-chamber. Viable and dead cells (stained blue due to loss of membrane integrity) were counted optically using a microscope at ten-fold magnification.

Viable cell concentration and viability was calculated using Equ. 2.1 and Equ. 2.2.

$$VCC\ [cells/mL] = MVC\ x\ \frac{6}{5}\ x\ 10000 \qquad \textbf{Equ. 2.1}$$

$$viability\ [\%] = \frac{VC}{TC}\ x\ 100 \qquad \textbf{Equ. 2.2}$$

VCC: viable cell concentration
MVC: mean viable cells
6/5: dilution factor from addition of Trypan blue
10000: expansion factor from volume of the chamber to volume of 1 mL
VC: viable cells, TC: total cells

2.5.3 Cell count with particle counter

A 2 mL sample of cell suspension was harvested and centrifuged for 10 min at 190 x g. The supernatant was discarded and the cell pellet was resuspended in 1 mL of cell lysis buffer. After two hours of incubation at RT, an aliquot of 25 to 200 µL of the prepared sample was diluted in 9 mL of isotone solution and measured with the particle counter. As the cell lysis buffer lysed the cell membranes but not the cell nuclei, every detected particle represented a nucleus. The volume of the aliquot was chosen to result in a particle count within the optimal range of 6000 to 20000 counts. Each sample was measured in duplicates and the particle diameter was adjusted to a size of > 2.8 µm. Fig. 2.1 shows a typical peak obtained from the particle count of CHO cell nuclei. Afterwards, the cell concentration was calculated according to Equ. 2.3.

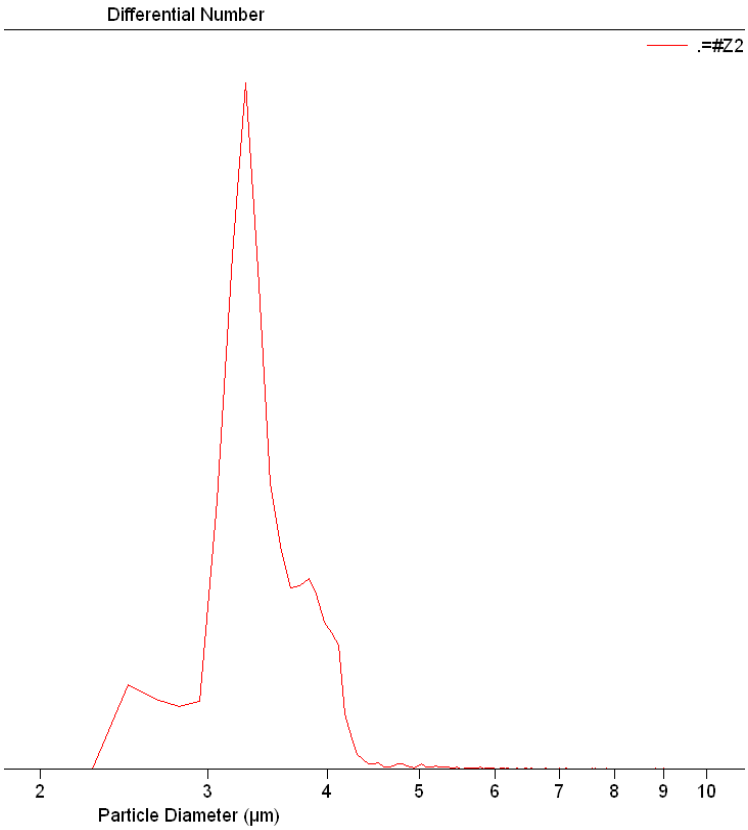

Fig. 2.1: Example graph derived from a measurement with the particle counter
The particle diameter is depicted on the horizontal axes and the particle count on the vertical axes.

$$CC\ [cells/mL] = \frac{count_m\ x\ V_{tot}\ x\ 2}{V_{aliq}\ x\ V_{sample}} \qquad \textbf{Equ. 2.3}$$

CC: cell concentration

$count_m$: mean particle count

V_{tot}: total volume used for measurement (9 mL isotone + volume of aliquot)

V_{aliq}: volume of aliquot

2: correction factor due to volume used by the particle counter

V_{sample}: volume of sample taken from cell suspension, usually 2 mL

2.5.4 Subculturing of cells

CHO cells are immortal cells, which will grow until they reach a critical cell density. To maintain the cells in a viable and exponential growth phase the culture needs to be split regularly to prevent them entering the stationary phase, which would result in growth arrest and further death of cells.

The CHO cell lines used in this work grew in suspension, thus no pretreatment such as trypsinization was needed. Host cell line was CHO DUKX-B11, which is deficient in dihydrofolate reductase (dhfr) (Urlaub and Chasin, 1980).

After determination of the cell concentration by particle count (chapter 2.5.3), the split ratio was calculated to result in an initial seeding density of 2×10^5 to 3×10^5 cells/mL. Cell lines with a higher specific growth rate were seeded with a lower cell concentration (approx. 2×10^5 cells/mL) to maintain their state in the exponential growth phase. Other cell lines, particularly fresh transfectants, had to be subcultured at a lower split ratio with higher seeding densities.

For subculturing, the cells were homogenously taken and transferred to a new culture vessel, except for the spinner flasks, where the cells were transferred back to the same spinner. Afterwards, fresh medium was added to reach a final volume of 10 mL for T25 flasks, 30 - 40 mL for T80 flasks or 50 mL for spinner flasks. The static cultures were incubated in an incubator at 37 °C, 7% CO_2 and 95% relative humidity. Spinner flasks were preconditioned with 60 ml CO_2 and cultivated at 37 °C on a magnetic stirrer platform at 50 rpm (Fig. 2.2). All established cell lines were splitted twice a week.

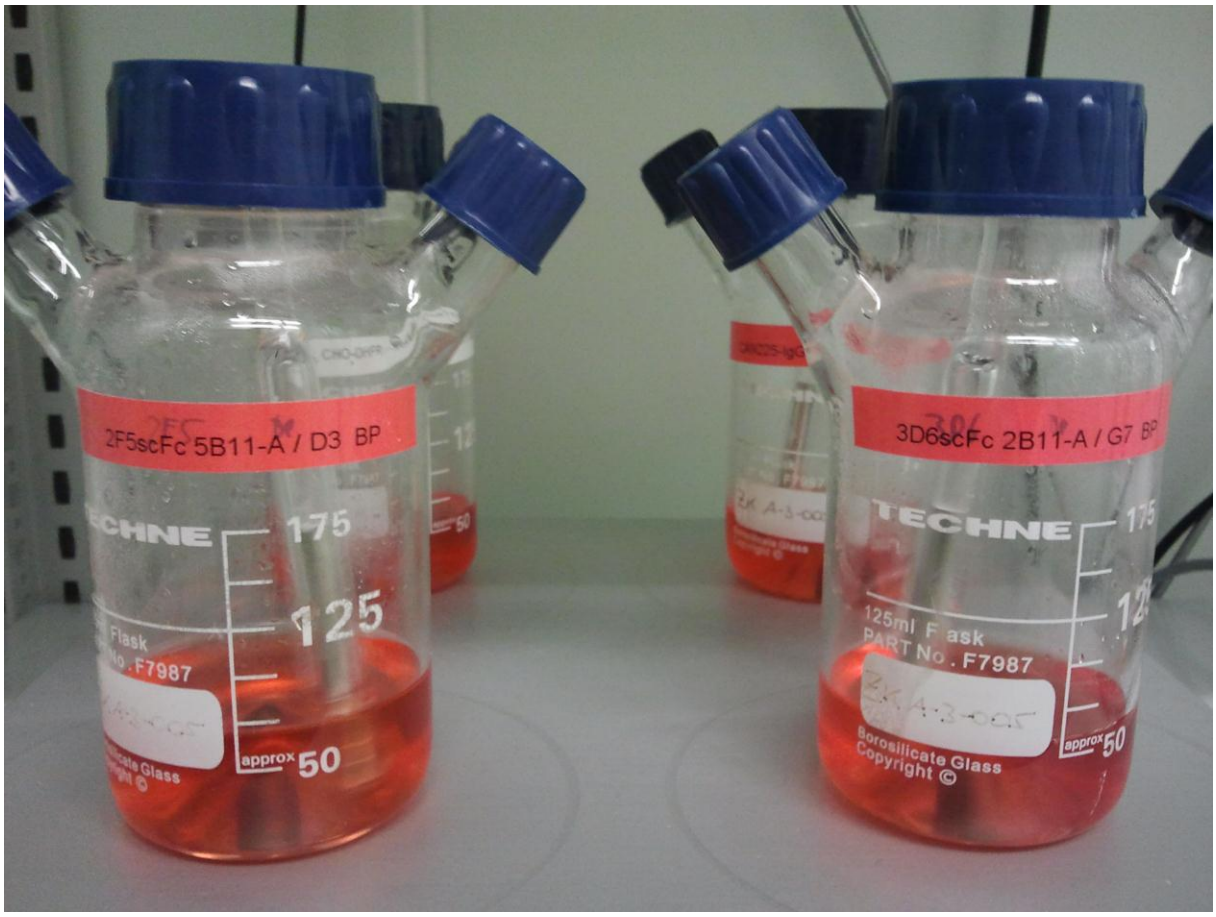

Fig. 2.2 Cultivation of CHO cells in spinner flasks

2.5.5 Transfection of CHO cells

The introduction of foreign genes into the genome of CHO cells was done by PEI mediated transfection. Cells were split in a ratio between 1:2 and 1:3 the day before transfection to keep the cells at a high viability and in exponential growth phase.

On the day of transfection, the cell concentration and viability was measured as described in chapter 2.5.2. Afterwards, 5 x 10^6 cells for the reaction mix and 1 x 10^6 cells for the negative control were taken and centrifuged at 190 x g for 10 min. The supernatant was discarded and the cell pellets were resuspended in transfection medium (5 x 10^6 cells in 10 mL and 1 x 10^6 cells in 2 mL of medium). The transfection mixes were prepared according to Tab. 2.9.

Tab. 2.9: Solutions used for the transfection of CHO cells

Solution	Definition
Negative control solution	350 µL HBS 50 µL PEI
Plasmid solution	20 µg pCAGGS plasmid with scFc antibody 5 µg P2_DHFR plasmid Added up to 1000 µL with HBS
PEI solution	750 µL HBS 250 µL PEI

The negative control solution was incubated for 20 min at RT. The plasmid solution and the PEI solution were incubated separately for 10 min at RT, mixed together and incubated for another 10 min at RT.

Subsequently the negative control solution was added to the prepared 1 x 10^6 cells in 2 mL and the transfection solution was added to the 5 x 10^6 cells in 10 mL. After 4 hours of incubation at 37 °C and 7 % CO_2 medium was exchanged by centrifugation at 190 x g for 10 min, and the cells were resuspended in fresh transfection medium. The cells were incubated overnight in T25 flasks at 37 °C and 7 % CO_2.

On the next day, the cells were centrifuged at 190 x g for 10 min and the supernatant was discarded. Afterwards, the cells were resuspended in 10 mL (for the negative control) and 50 mL (for the transfectants) of selection medium. The cells of the negative control as well as the transfected cells were plated in 96 well microtiter plates with 100 µL of cell suspension per well. This resulted in a seeding density of approximately 1 x 10^4 cells/well.

One week after plating, the wells were fed with 100 µL fresh selection medium and the growth was continuously monitored by microscopy. After two weeks, the wells were fed again with 100 µl fresh selection medium which resulted in a maximum volume of 300 µL per well.

2.5.6 Cell line establishment

Initially, volumetric titers in the transfection plates were screened by ELISA (chapter 2.6). Afterwards, the 12 best clones for each antibody were chosen for further cultivation. Therefore, they were transferred to an expansion plate and the volumes and cell numbers were increased from one well to eight wells for each clone. In the end of this process every clone was cultivated in a total volume of 2.4 mL (8 x 300 µL). From this expansion plate the clones were transferred into T25 flasks by splitting them 1:2 with fresh medium. According to their cell growth, the T25 flasks were added up to the total volume of 10 mL after cultivation for about 3-4 days. After this step, the clones were subcultured according to chapter 2.5.4 and the supernatants were collected and stored at -20 °C for achieving a continuous monitoring of the volumetric titers and specific productivities.

From the point on, where the cell numbers were increased in the expansion plates, there was also a division to cultivation in selection medium (without MTX) and with amplification medium (0.048 µM MTX). Additionally, for the clones in amplification medium, the MTX concentration was increased to 0.096 µM and they were subcloned (chapter 2.5.7) in order to have a homogenous population. These amplified clones were cryopreserved (chapter 2.5.8) for further experiments.

The clones that were best in regard to specific productivity and specific growth rate, were increased in biomass, transferred to spinner flasks and further cultivated in 50 mL medium.

2.5.7 Subcloning of cells

Subcloning medium was prepared by centrifuging cells from the last passages of a certain clone at 2000 rpm for 7 minutes. The supernatant was collected, mixed with fresh medium at a ratio of 1:1 and filtered through a sterile 0.22 µm Millipore ExpressTMPLUS filter. This step resulted in a subcloning medium that was able to supply all components needed or cell growth and mimic an environment with other cells being present.

For every clone, three microtiter plates were seeded and the cells were diluted to densities of 27, 9 and 3 cells/well. As 150 µL of suspension was used per well, the applied cell concentrations were 180, 60 and 20 cells/mL. The plates were incubated at 37 °C in a 7% CO_2 incubator with over 95% relative humidity.

2.5.8 Cryopreservation of animal cells

For long time storage of newly developed recombinant CHO cell clones, cryostocks were prepared. Therefore, CryoMaxx II medium was precooled at 4 °C and CryoTubes were precooled at -20 °C. First, 2.5 x 10^7 cells were centrifuged with 190 x g for 10 min and the supernatant (cultivation medium) was discarded. After addition of 5 mL CryoMaxx II and gentle resuspending, aliquots of 1 mL (5 x 10^6 cells respectively) were filled into CryoTubes. As long incubation with the preservation medium would harm the cells, the tubes were immediately put in a slightly open polystyrene box at -80 °C. This step aimed for a linear freezing gradient of approximately 1 °C/min which is optimal for cells to survive the freezing process.

On the next day, the cells were put in a liquid nitrogen tank at -196 °C and registered in a database to ensure high traceability.

2.5.9 Calculation of cell culture parameters

To describe the cell line characteristics concerning growth and productivity, the specific growth rate (μ) and the specific productivity (qp) were calculated according to Equ. 2.4 and Equ. 2.5.

$$\mu\,[1/d] = \frac{\ln(\frac{X_1}{X_0})}{\Delta t} \qquad \textbf{Equ. 2.4}$$

$$qp\left[\frac{\mu g}{d\ x\ 10^6 cells}\right] = \frac{P_1 - P_0}{X_1 - X_0}\ x\ \mu\ x\ 10^6 \qquad \textbf{Equ. 2.5}$$

μ: specific growth rate,
X_0: initial cell density, X_1: final cell density,
Δt: time [d],
qp: specific productivity,
P_0: initial product titer, P_1: final product titer

2.6 ELISA

2.6.1 Material

Tab. 2.10: Material and equipment used for ELISA

Component	Definition
10x PBS stock solution, 5 L	57.5 g Na_2HPO_4 * 2 H_2O 10 g KH_2PO_4 10 g KCl 400 g NaCl filled up to a total volume of 5 L with dH_2O
Washing buffer (1x TPBS), 1 L	100 mL of 10x PBS stock solution 1 mL Tween filled up to a total volume of 1 L with dH_2O
Dilution buffer, 100 mL	100 mL Washing buffer 1 g BSA
Coating buffer, 1 L	8.4 g $NaHCO_3$ 4.2 g Na2CO_3 filled up to a total volume of 1 L with dH_2O pH 9.5 - 9.8 Storage: 1 week at RT
ELISA Standards	3D6scFc, affinity purified standard stock solution, 200ng/ml.
Colouring buffer, 1 L	7.3 g Citric Acid * H_2O 11.86 g Na_2HPO_4 * 2 H_2O pH 4.8 - 5.0 filled up to a total volume of 1 L with dH_2O Storage: 1 month at 4°C

OPD	ortho-Phenylenediamine 100 mg/mL
Capture antibody	Sigma-Aldrich I3382-1MG 030M6049 SL10191 Anti-Human IgG (gamma-chain specific), Developed in Goat, Affinity isolated antigen specific antibody 1 mg/mL
Detection antibody	Invitrogen HRP-Goat Anti-Human IgG REF 628420 LOT 876293A Contains 0.1 % Proclin Exp.: 02/2013 Storage: 4°C 1 mg/mL
H_2SO_4	25 % H2SO4
Coating plates	Nunc, Immuno 96 MicroWell™ Solid Plates, MaxiSorp™ Cat. N°: 456537
Dilution plates	Nunc, 96 MicroWell™ plate Cat. N°: 269620
Plate washer	Tecan, Microplate Washer, 96 Plate Washer™ (96 PW)
Plate reader	Tecan, Infinite® M1000 PRO

2.6.2 Double antibody sandwich ELISA

Coating:

The capture antibody was diluted 1:2000 in coating buffer. Afterwards, 100 µL of the solution was added to each well of a coating plate. The plate was incubated for two hours at RT or over night at 4°C.

Afterwards, the plate was washed three times with 300 µL of washing buffer per well. This was achieved automatically with a programmed routine on the plate washer.

Addition of the samples:

a) Screening of transfection plates:

For first screenings, column 1 of the plate was loaded with blanks (dilution buffer) and the second column with the ELISA standards (from 100 ng/mL in well H2 in 1:2 dilution to 0.78 ng/mL in well A2). Rows 3 to 12 were loaded with the samples, with one sample per well (see Fig. 2.3). The samples were diluted to be in the range of the highest standards.

Each well was loaded with 50 µL and afterwards incubated for one hour at RT. Subsequently, the plate was washed with 300 µL per well for three times with the plate washer.

b) Determination of product concentration during cultivation:

As shown in Fig. 2.4, column 1 of the plate was loaded with blanks (dilution buffer) and the second and third column with the ELISA standards (from 100 ng/mL in wells H2 and H3 in 1:2 dilution to 0.78 ng/mL in wells A2 and A3). Columns 4 to 12 were filled with one sample per column (presented as the blue box). Thereby, the samples were diluted to approximately 100 ng/mL in well H. Afterwards, a 1:2 dilution series was made from row to row (From H to A), resulting in eight measurements for each sample. Therefore, the main differences to ELISA for screening of transfection plates, as mentioned before, were use of two columns for the standards and a dilution series with multiple measurements for the samples.

Each well was loaded with 50 µL and afterwards incubated for one hour at RT. Subsequently, the plate was washed with 300 µL per well for three times with the plate washer.

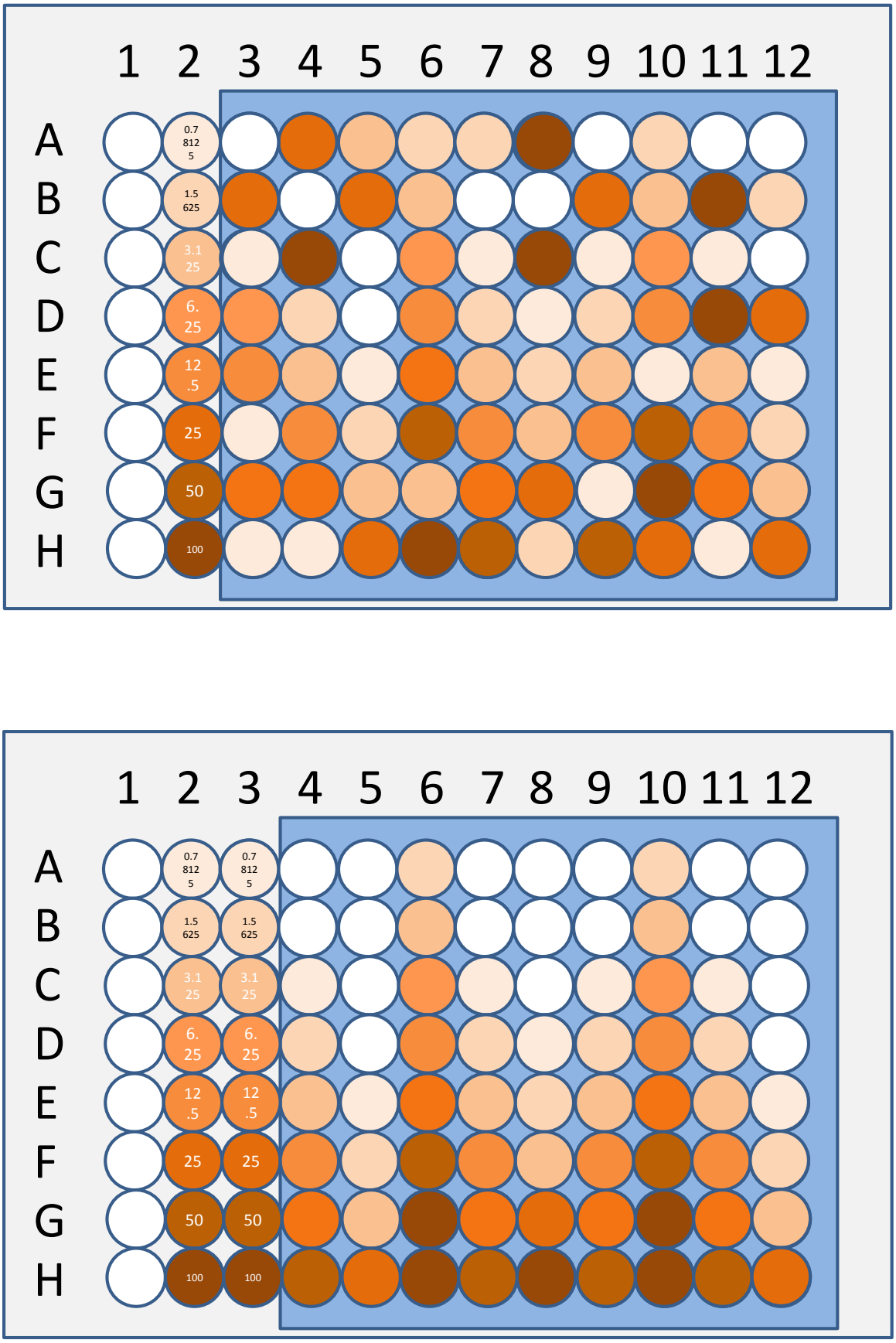

Fig. 2.4 ELISA plate used for determination of product concentrations
Blanks are in column 1, standards in columns 2 and 3, concentrations are given in ng/mL. columns 4 to 12 are used for the samples (blue box).

Addition of detecting antibody:

The HRP-Goat Anti-Human IgG antibody was diluted 1:3000 in dilution buffer. Afterwards, 50 µL of the solution was added to each well and incubated for one hour at RT. The plate was washed three times with 300 µL of washing buffer per well.

Addition of the substrate and enzyme activation:

For one plate, 100 µL of OPD and 6 µL of H_2O_2 were added to 10 mL of colouring buffer. Horseradish peroxidase, which is linked to the second antibody, catalyses the conversion of o-phenylenediamine (OPD) to 2.3-diaminophenazine (DAP) (Hamilton et al., 1999). DAP has an orange-brown colour and can be detected by photometric measurement at 492 nm.

100 µL of the solution were transferred to each well and the enzymatic reaction was stopped with 100 µL of H_2SO_4 per well at the point of time when the most diluted standards had a visible colour.

Subsequently, the plate was measured photometrically on a TECAN infinite M1000 at 492 nm with a reference wavelength of 620 nm.

2.7 Intracellular determination of product formation by flow cytometry

2.7.1 Material

Tab. 2.11: Material used for FACS analysis

Component	Definition
Flow cytometry buffer	100 mM Tris/HCl 2 mM $MgCl_2$ 0.1 % Triton X-100 pH 7.4
FCS	d.FCS (PG0004) ID-Nr.: BF003.05-PA Lot: 01-C0026/150904/240904 Exp.date: 12/2005 (as medium supplement)
γ-chain Antibody	Sigma-Aldrich Anti-Human IgG (γ-chain specific)-R-Phycoerythrin, antibody produced in goat P9170-.5ML, 079K6032, Affinity isolated antibody
Flow cytometer	Beckman Coulter, Gallios™ 10/3 flow cytometer
Analysis software	Beckman Coulter, Kaluza® Analysis Software

2.7.2 Ethanol Fixation of Cells

1 x 10^6 cells were centrifuged for 10 min at 190 x g and the supernatant was discarded. The cells were fixed with 1 mL of 70% ethanol at -20°C and afterwards stored at 4 °C. The ethanol was added drop-wise under gentle vortexing to prevent cells from clumping.

2.7.3 Incubation with γ-chain Antibody

- The fixed cells were centrifuged for 10 min at 190 x g and washed with 1 mL flow cytometry buffer. The buffer was added drop-wise under gentle vortexing.
- Afterwards the cells were washed with 1 mL DNA-buffer containing 10% FCS.
- Incubation with 100 µL DNA-buffer containing 10% FCS at 37 °C for 30 min.
- Addition of 100 µL γ-chain antibody (diluted 1:50 in DNA-buffer containing 10% FCS) and incubation at 37 °C for 1 hour. After addition of γ-chain antibody, the samples were protected from light.
- 1 mL of DNA-buffer was added and cells are centrifuged for 10 min at 190 x g.
- The pellet was resuspended in 200 µL DNA-buffer and transferred to FACS tubes. The negative control was resuspended in 400 µL DNA-buffer in order to have more volume for adjusting the measurement parameters.
- The samples were analysed with the flow cytometer and evaluated with the analysis software.

2.8 Genomic DNA (gDNA) Isolation

2.8.1 Material

Tab. 2.12: Material used for gDNA isolation

Component	Definition
DNA purification kit	QIAGEN QIAamp DNA Blood Mini Kit, Cat N°: 51106
PBS	1.15 g Na_2HPO_4 * 2 H_2O 0.2 g KH_2PO_4 0.2 g KCl 8 g NaCl Added up to 1 L with dH_2O
Nanophotometer	Implen NanoPhotometer P-300

2.8.2 Isolation of gDNA

For the isolation, 2 x 10^6 cells were centrifuged at 190 x g for 10 min and the pellet was immediately frozen in liquid nitrogen and stored at -80 °C. The pellets of different samples at various time points were collected and the genomic DNA of all samples was isolated together.

The gDNA was isolated using the DNA purification kit according to the manufacturer's protocol. Briefly, after proteinase K and RNAase A treatment, a lysis buffer was added, the DNA was precipitated with ethanol and centrifuged through a provided column. After washing steps, gDNA was eluted from the column with dH_2O.

Concentration of gDNA was determined at 260 nm using a nanophotometer and calculated according to Equ. 2.6. Purity of the DNA was verified by the ratio of absorbance at 260 nm to 280 nm.

Afterwards, the samples were diluted to a DNA concentration of 1 ng/µL and heated to 99 °C for 10 min in order to assure a proper denaturation before storing aliquots for real-time PCR analysis (chapter 2.12). These aliquots were stored a 4 °C, to prevent long DNA strands from breaking while being frozen.

$$[DNA]\left[\frac{ng}{\mu L}\right] = Abs_{260nm}\ x50\ ng/\mu L \qquad \textbf{Equ. 2.6}$$

[DNA]: DNA concentration

Abs_{260nm}: Absorbance at 260 nm

2.9 RNA Isolation

2.9.1 Material

Tab. 2.13: Material used for RNA isolation

Component	Definition
Trizol Reagent	Ambion Tri reagent solution AM9738, stored at 4 °C
UPW	Ultra Purified Water, stored at -20 °C
Chemicals	Isopropanol 99.9%, Ethanol 70%, Chloroform ≥99.8%
RNAse free DNAse I reaction buffer	QIAGEN RNAse free DNAse set 79254, stored at 4 °C
DNAse I	QIAGEN RNAse free DNAse set 79254, stored at -20 °C 1500 Kunitz units / 550 µL
RNAse inhibitor	Invitrogen RNAse out ribonuclease inhibitor 40 U / µL, stored at -20 °C 10777-019
Nanophotometer	Implen NanoPhotometer P-300

2.9.2 Isolation of RNA

Analogous to the sampling for gDNA (chapter 2.8), 2 x 10^6 cells were centrifuged at 190 x g for 10 min and the pellets were shock frozen in liquid nitrogen. After storage at -80 °C, the RNA of the samples was isolated together.

- The cells were lysed by addition of 1 mL Trizol reagent and 200 µL chloroform. After mixing, the samples were incubated for 10 min on ice.
- The samples were centrifuged in a pre-cooled centrifuge at 4 °C and 16100 x g for 15 min.

- The colourless upper phase was transferred into a centrifugation tube and after addition of 500 µL isopropanol, the samples were incubated at -20 °C for 15 min.
- Afterwards, the samples were centrifuged at 4 °C and 16100 x g for 15 min. and the supernatant was discarded.
- Each pellet was washed with 1 mL of 70 % ethanol and centrifuged at 16100 x g for 5 min. at RT. After discarding the supernatant, the pellets were dried at RT until no alcohol was left.
- 70 µL of RNAse free DNAse I reaction buffer, 10 µL DNAse I and 4 µL RNAse Inhibitor were added to each pellet. After incubation at RT for 30 minutes, DNAse I was inactivated by heating the samples to 75 °C for 10 min.
- For precipitation of the RNA, 250 µL isopropanol was added and incubated at -20 °C for 15 min.
- The supernatant was discarded and the pellets were washed with 1 mL of 70 % ethanol and centrifuged at 16100 x g for 5 min at RT. After discarding the supernatant, the pellets were dried at RT until only the dry pellets were left.
- The derived RNA was dissolved in 23.5 µL UPW and 1.5 µL RNAse inhibitor by incubation at RT for 10 min and afterwards stored at -20 °C.

The RNA concentration was determined by photometric measurement at 260 nm and calculated according to Equ. 2.7. The purity was checked by the ratio of absorbance at 260 nm to absorbance at 280 nm.

$$[RNA]\ [ng/\mu L] = Abs_{260nm}\ x\ 40\ ng/\mu L \qquad \textbf{Equ. 2.7}$$

[RNA]: RNA concentration

Abs_{260nm}: Absorbance at 260 nm

2.10 Reverse Transcription

2.10.1 **Material**

Tab. 2.14: Material used for reverse transcription of RNA to cDNA

Component	Definition
dNTPs	New England Bilolabs deoxynucleotide solution mix 10 mM each dNTP N0447L, stored at -20 °C
RNAse inhibitor	Invitrogen RNAse out ribonuclease inhibitor 40 U / µL 10777-019, stored at -20 °C
Reverse transcriptase buffer	Promega M-MLV RT 5x buffer M531A, stored at -20 °C
Reverse transcriptase	Promega M-MLV reverse transcriptase 200 U/µL M170A, stored at -20 °C
Random primers	Promega Cat. # C1181 System Lot # 319110 C118A Lot # 31195905 500 µg/mL
UPW	Ultra Purified Water, stored at -20 °C

2.10.2 **Procedure**

Tab. 2.15: Reaction mix used for reverse transcription

mRNA	1 µg / 250 ng / 100 ng
Random Primers	0.5 µg Primers / 1 µg of RNA
dNTPs	1 µL
UPW	to a total volume of 14 µL

- For annealing the primers to the mRNA, the samples were heated to 70 °C for 5 min and cooled to RT in PCR reaction tubes.
- Afterwards 1 µL RNAse Inhibitor, 4 µL Reverse transcriptase buffer and 1 µL Reverse Transcriptase were added to each sample.
- As “No Reverse Transcription (NRT)” controls, 1 µL RNAse Inhibitor, 4 µL Reverse transcriptase buffer and 1 µL UPW were added to the same samples.
- The samples were incubated at 37 °C for 60 min, at 42 °C for 30 min and afterwards heated to 95 °C for 5 min to inactivate the enzymes.

2.11 Primers used for PCR and qPCR

Binding regions of the scFc and plasmid primers are displayed on the detailed plasmid maps in black colour (chapter 6.2).

Tab. 2.16: List of primers and hydrolysis probes for PCR and qPCR reactions

Amplicon	Sequences	Amplicon size
scFc	Primer s: ACG AGG ACC CTG AAG TGA AG Primer as: CGG TAG GTG GAG TTG TAC TGT TC Probe: AAG TGC ACA ACG CCA AGA CCA AGC	100 bp
ß-actin	Primer s: TGA GCG CAA GTA CTC TGT G Primer as: TTG CTG ATC CAC ATC TCC TG Probe: CCA TCC TGG CCT CAC TGT CCA CCT	78 bp
3D6scFc Plasmid	3D6scFc_Std_sense: AAC GCC AAG AAC TCC CTG TA 3D6scFc_Std_as: ATC ACG GAG CAG GAG AAC AC	1156 bp
2F5scFc Plasmid	13H5A5_LC306_se: CGC CAC CTA CTA CTG CCA G 3D6scFc_Std_as: ATC ACG GAG CAG GAG AAC AC	710 bp

Primer s: sense Primer, Primer as: antisense Primer, Probe: FAM/TAM conjugated hydrolysis probe, Synthesizing by Sigma-Aldrich

2.12 Real-time quantitative PCR (qPCR)

2.12.1 Material and equipment

Tab. 2.17: Material applied for the real time quantification of PCR product

Component	Definition
iQ Supermix	BioRad iQ™ supermix, 2X Supermix for Real-Time PCR, Cat. N°: 170-8862, Control 730001045
PCR plates	BioRad Multiplate PCR plates low 48-well white, Cat. N°: MLL4851
PCR sealers	BioRad PCR Sealers, Microseal B film, Cat. N°: MSB1001
Primers	Primer s, primer as and hydrolysis probe for scFc and ß-actin according to chapter 2.11
MiniOpticon	BioRad MiniOpticon™ real-time PCR detection system (for FSD), Cat. N°: 359-1592
Software	BioRad CFX Manager (provided with MiniOpticon), LinRegPCR: analysis of qPCR data V. 12.17 (Ruijter et al., 2009), (Tuomi et al., 2010), STATGRAPHICS Centurion XV

2.12.2 Procedure

For each sample of gDNA (prepared according to chapter 2.8), 3 ng DNA were used as a template for the reaction. As the samples were pre-diluted to a concentration of 1 ng/µL, for each reaction mix 3 µL of the samples were added. The scheme of a reaction mix with a total volume of 20 µL is depicted in Tab. 2.18.

.

Tab. 2.18: qPCR reaction mix for one sample

Component	Amount	
iQ Supermix 2x	10 µL	
Template	3 µL	= 3 ng for gDNA = unknown amount for cDNA
Primer s	0.6 µL	= 6 pmol DNA
Primer as	0.6 µL	= 6 pmol DNA
Probe	0.4 µL	= 4 pmol Probe
dH_2O	5.4 µL	
SUM	20 µL	

The plates with the reaction mixes were analysed with the MiniOpticon using the parameters described in Tab. 2.19.

Tab. 2.19 Cycles used for qPCR

Temperature	Time	Number of cycles
95 °C	5 minutes	1 x
55 °C	60 seconds	45 x
Fluorescence signal detection		
95 °C	15 seconds	
72 °C	5 minutes	1 x

2.12.3 Quantification of data

The DNA content of one cell was calculated according to Equ. 2.8.

Equ. 2.9 to Equ. 2.12 were used for determining the parameters needed for comparison of the different qPCR samples.

$$\frac{DNA}{cell}[pg] = \frac{2.45x\ 10^{9}\ bp\ x\ M_{bp}\ g\ mol^{-1}\ x\ 10^{12}\ pg\ g^{-1}}{N_A\ bp\ mol^{-1}} = 2.7\ pg \qquad \textbf{Equ. 2.8}$$

M_{bp}: average molecular weight of base pairs, 660 g mol^{-1}
N_A: Avogadro constant, 6.022 x 10^{23} mol^{-1}

$$\Delta Cq = Cq_{scFc} - Cq_{ß-actin} \qquad \textbf{Equ. 2.9}$$

$$-\Delta Cq = Cq_{ß-actin} - Cq_{scFc} \qquad \textbf{Equ. 2.10}$$

$$ratio_{scFc/ß-actin} = 2^{-\Delta Cq} \qquad \textbf{Equ. 2.11}$$

$$ratio_{sample1/sample2} = 2^{-\Delta\Delta Cq} \qquad \textbf{Equ. 2.12}$$

2.12.4 Statistical analysis

For statistical evaluation of Cq values, the software package STATGRAPHICS Centurion XV was used. Thereby, a one-way analysis of variance was performed with –ΔCq values.

- Scatterplots and box-and-whisker plots were displayed to visualize the distribution of the values and check for outliers.
- A multiple range test was made to check for homogenous groups.

3 Results

3.1 Plasmids

3.1.1 Plasmid maps

Two pCAGGS plasmids were used as presented in Fig. 3.1. Besides the product sequences (chapter 6.1), the plasmids contained an ampicillin resistance for selection in *E.coli*, a neo/kan resistance, which can be used for additional selection with the aminoglycoside antibiotic Geneticin (G418) during transfection and attb-sites for homologous recombination into the BACs. This recombination was catalysed by a ΦC31 integrase system (Groth et al., 2000) and is schematically represented for 3D6scFc in Fig. 3.2.

The DHFR gene used for selection and amplification was co-transfected with an additional plasmid (Fig. 3.3).

Two additional pCIneo plasmids (provided by Andreas Maccani, working group of

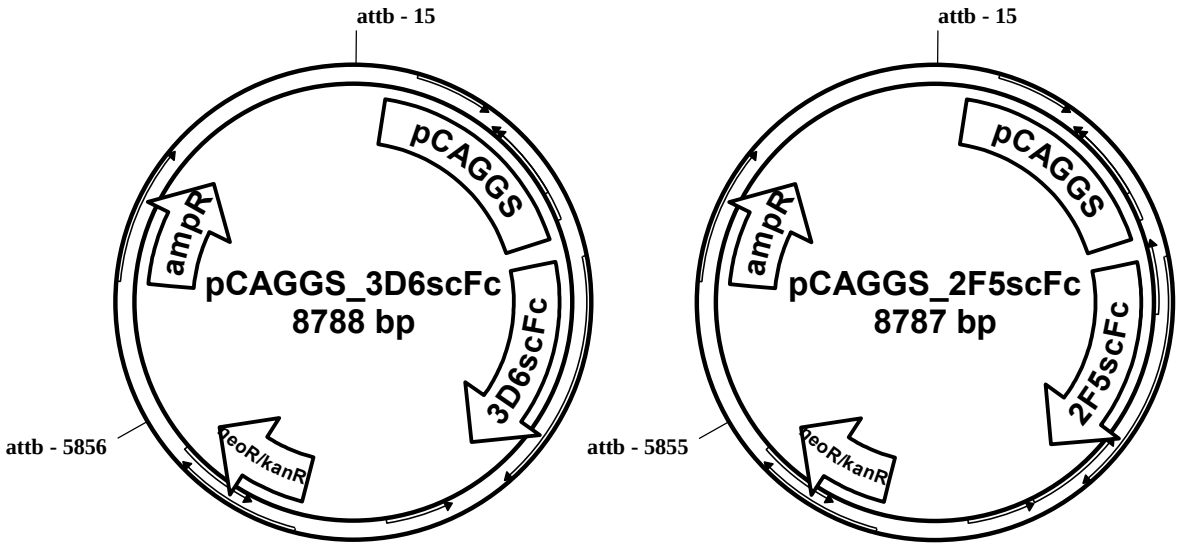

Fig. 3.1 Product plasmids used for transfection

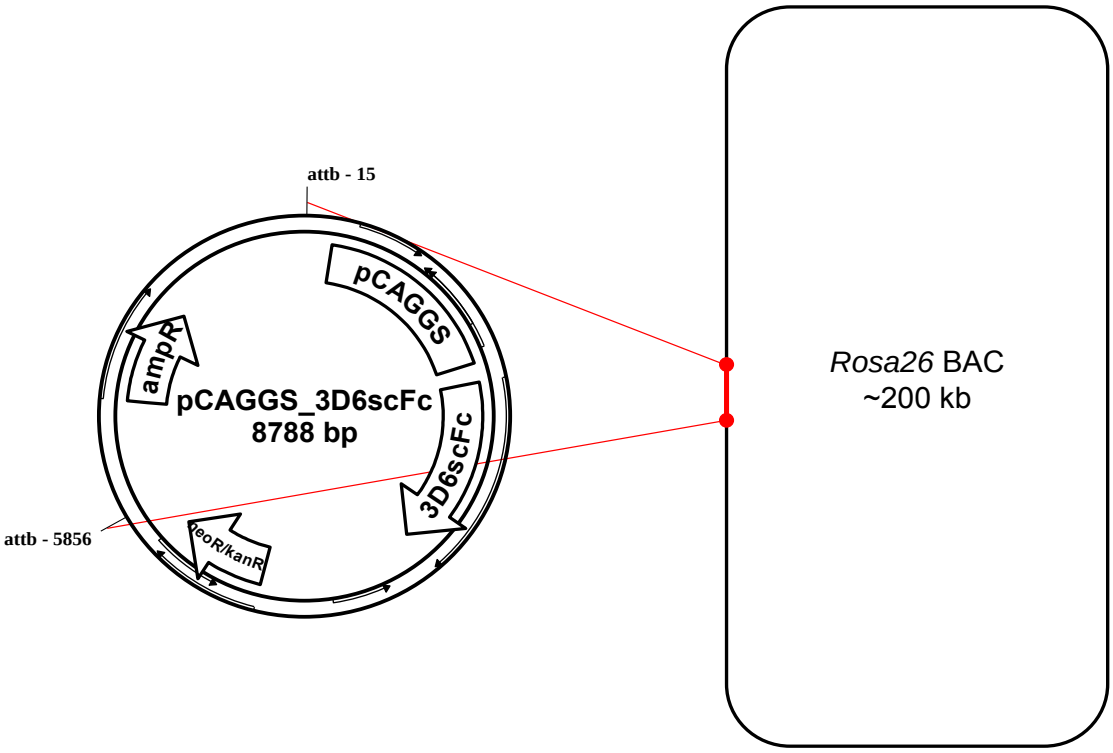

Fig. 3.2 Recombination sites of plasmid and BAC

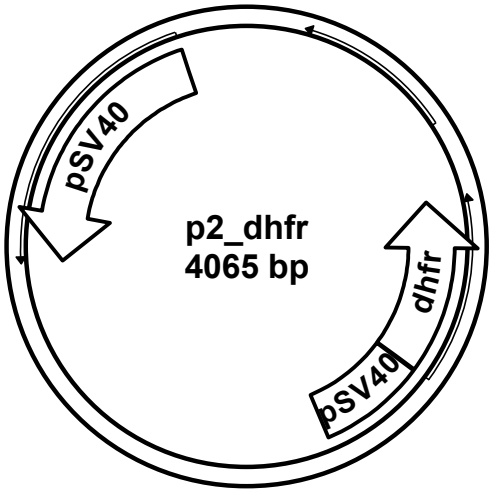

Fig. 3.3 DHFR plasmid used for transfection

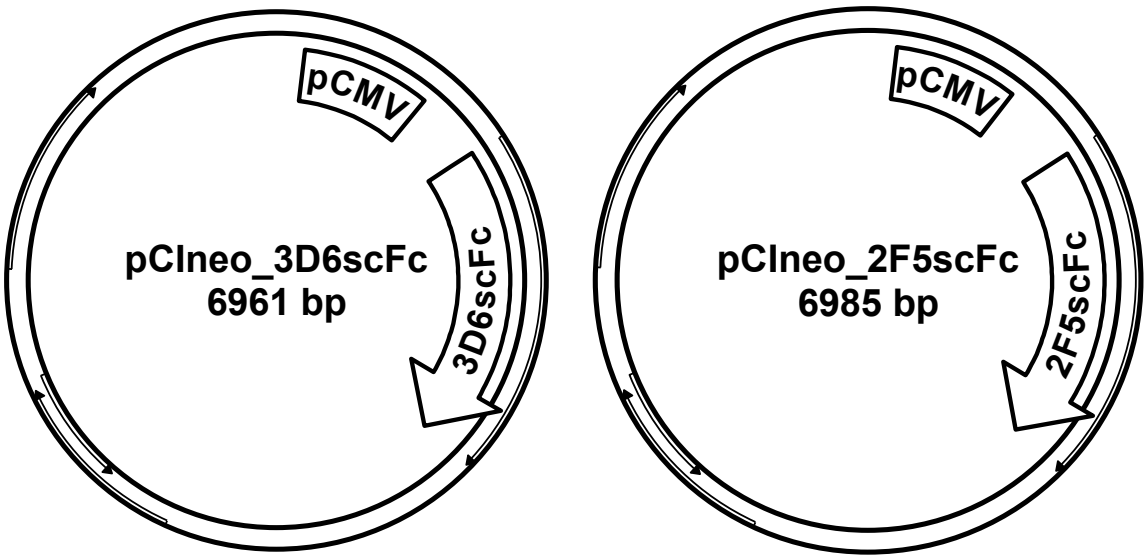

Fig. 3.4 Control plasmids for characterisation

3.1.2 Plasmid amplification

The plasmid pCAGGS 2F5scFc (Fig. 3.1) was already available in sufficient amounts. The second plasmid, pCAGGS 3D6scFc had to be prepared by transformation and amplification in E.coli.

For electroporation of the plasmid pCAGGS 3D6scFc, the time constant was 5 ms and the effective voltage 1.786 kV. The whole cell suspension of 200 mL was used for the Maxi prep kit. After purification, it yielded a DNA concentration of 2.86 µg/µL and a purity coefficient (A260/A280) of 1.84, which was in the optimal range of 1.7 to 1.9.

3.1.3 Characterisation by PCR products

The PCR was performed to check the plasmids for integrated, correct product genes. As the two constructs were quite similar, except for the variable domains, the primer 3D6scFc_Std_as was chosen to be on a homologous part between the two sequences. Therefore, the same antisense primer could be used for 3D6scFc and 2F5scFc and only the sense primers had to be switched (chapter 2.11). Two pCIneo plasmids containing the sequences of 3D6scFc and 2F5scFc were used as controls (Fig. 3.4).

The amplicon size for the PCR with 3D6scFc was 1156 bp. A band around 1200 bp was identified for both plasmids (Fig. 3.5 [A]).

For 2F5scFc, the amplicon was 710 bp long and for both plasmids a band over 700 bp was visible (Fig. 3.5 [B]). For pCAGGS, two additional bands with a lower intensity were present around 10000 bp and 5500 bp. These bands were probably from the plasmid due to high template concentration.

Therefore, for both products, the antibody genes were confirmed on the plasmids used for transfection.

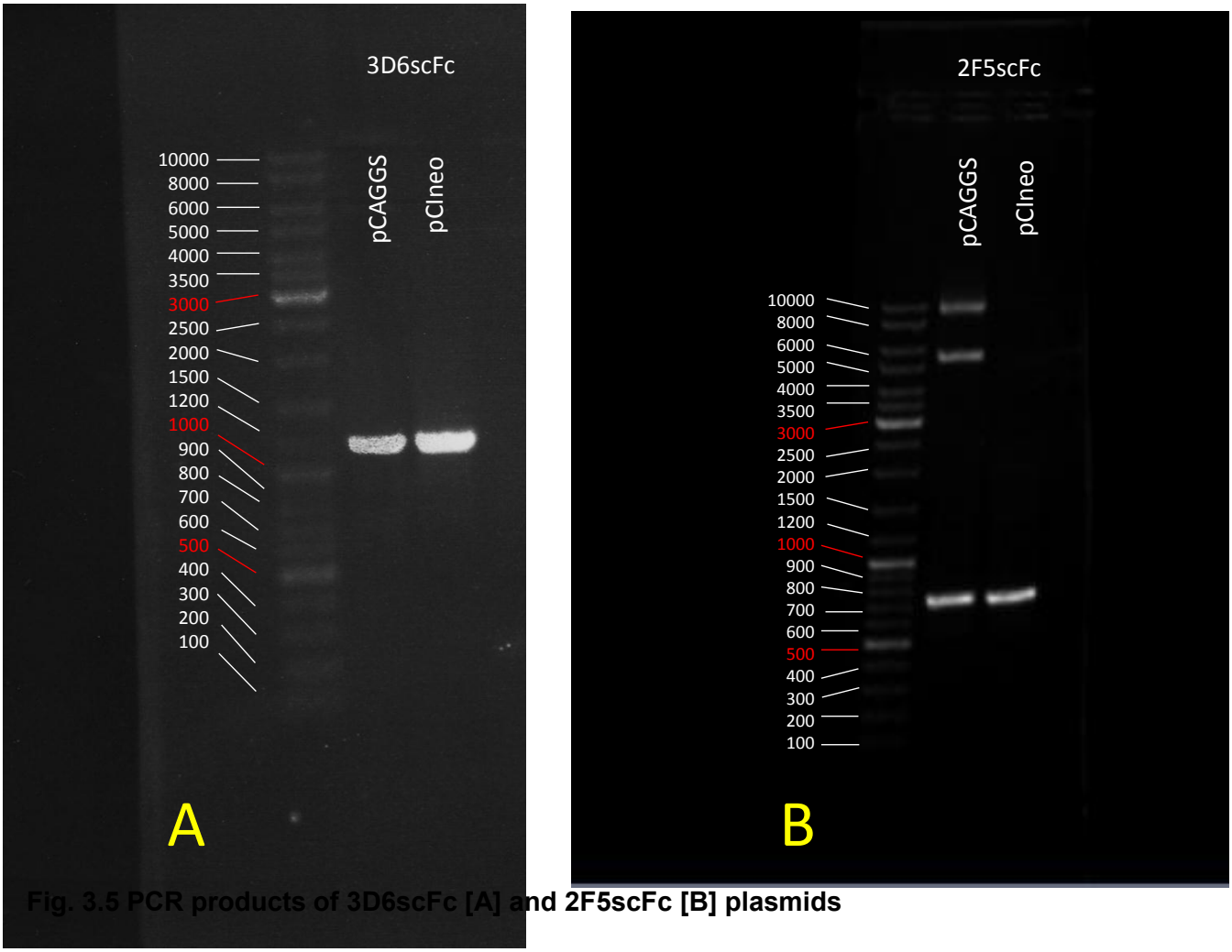

Fig. 3.5 PCR products of 3D6scFc [A] and 2F5scFc [B] plasmids

3.1.4 Characterisation by restriction digestion

For pCAGGS 3D6scFc (the amplified plasmid), an additional restriction digestion was performed to test for length of the plasmid. Control plasmid was pCIneo 3D6scFc. EcoRV cuts both plasmid at one site, pCAGGS 3D6scFc at position 3197 and pCIneo 3D6scFc at position 2383.

For pCAGGS a band around 9000 bp and for pCIneo a band around 7000 bp was found (Fig. 3.6, considering the movement direction of the standard). The difference between the two linearized plasmid bands is around 2000 bp. These values correlate with the actual lengths of 8788 bp for pCAGGS (Fig. 3.1) and 6961 bp for pCIneo (Fig. 3.4).

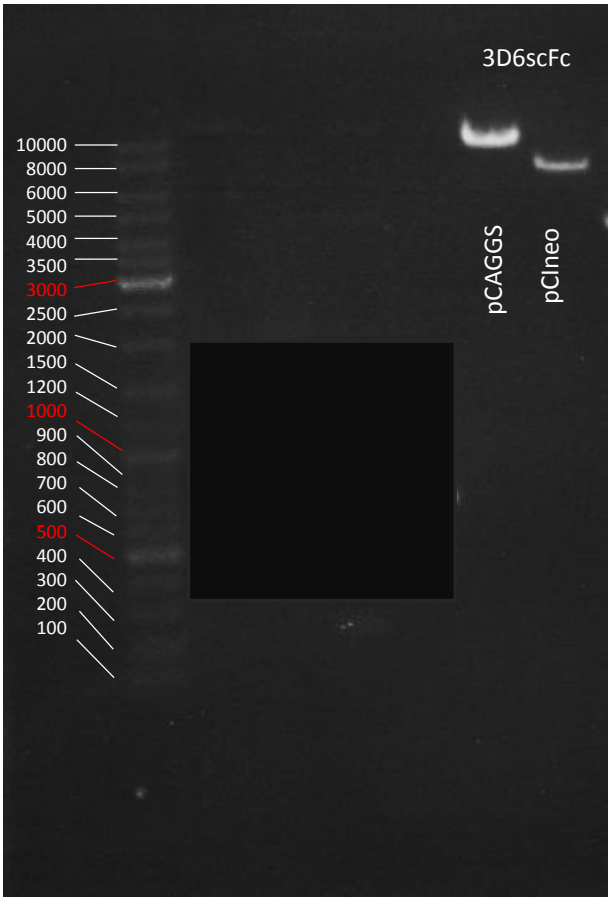

Fig. 3.6 Restriction digestion of 3D6scFc plasmids

3.2 Cell Culture

3.2.1 Transfection

Preliminary to transfection, the viability and cell concentration of the CHO host cell line was determined by haemocytometer (chapter 2.5.2). The results are summarized in Tab. 3.1. The transfections were made on two different days with the same host cell line and therefore different passage numbers are given. For both introduced plasmids, the CHO host cell line had a high viability and good growth characteristics, which made it suitable for transfection.

Tab. 3.1: Characteristics of host cell line before transfection

Transfection of	Passage number	Viable cell concentration	Viability
3D6scFc	27	5.5 x 10^5 cells/mL	98 %
2F5scFc	29	6.6 x 10^5 cells/mL	99 %

The evaluation of growth in microtiter plates approximately four weeks after transfection is shown in Tab. 3.2. For both cell lines, the transfection was successful and no cells grew in the negative control plates. Therefore, the selection pressure worked and the cells that grew in the positive wells had integrated the plasmid DNA into their genome. The fact that for 3D6scFc transfection efficacy was three times higher compared to 2F5scFc, already depicts the tendency that CHO cells might have less problems with the production of 3D6scFc.

Tab. 3.2: Growth evaluation of microtiter plates after transfection

Plates	Plated wells	Grown wells	Transfected wells [%]	Wells screened with ELISA
3D6scFc	480	146	30.4	204
3D6scFc neg. control	96	0	0	0
2F5scFc	480	55	11.5	53
2F5scFc neg. control	96	0	0	0

An example of applied microtiter plates for transfection is presented in Fig. 3.7. Certain wells were marked according to macroscopic (e.g. colour) and microscopic (e.g. cell densities, deriving of cell population from one single cell) attributes and according to their product formations. The colour change in wells is due to a pH change, visualized by the indicator phenol red. When CHO cells grow, they lower the pH of the medium by production of lactic acid and the colour shifts from red to yellow.

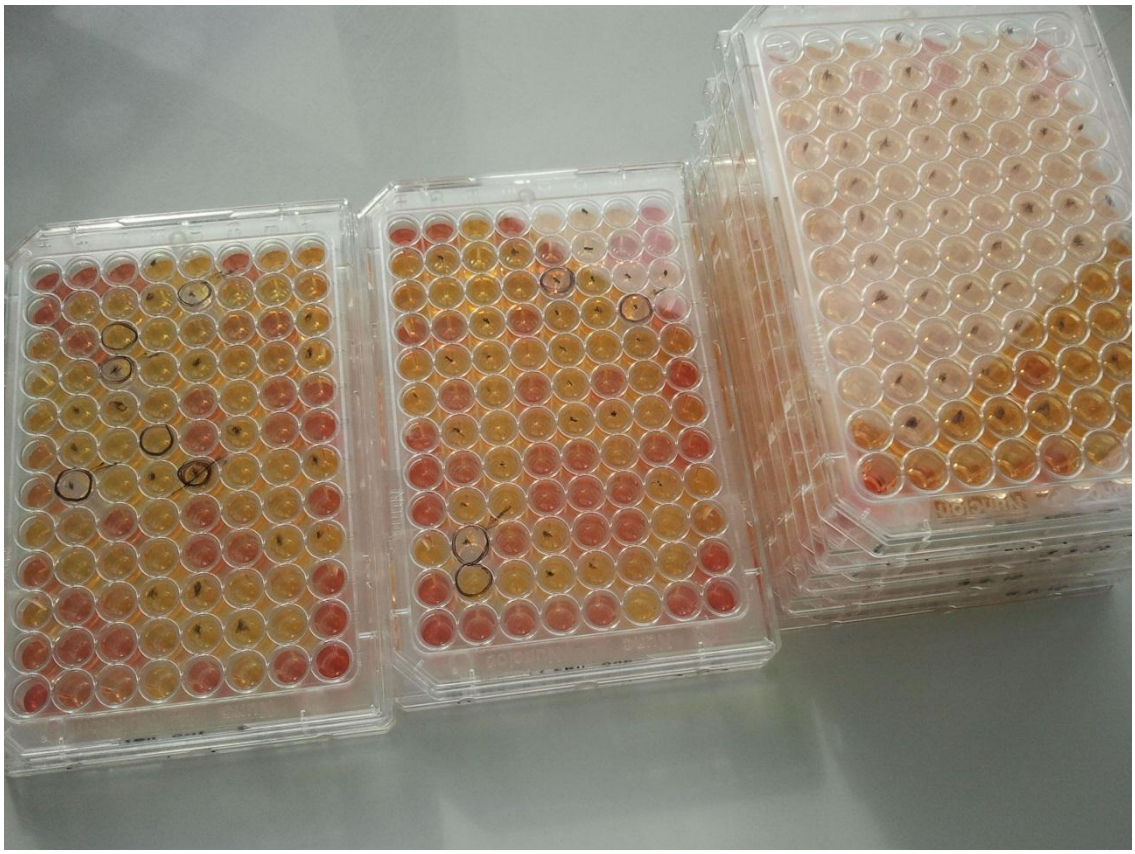

Fig. 3.7 Microtiter plates with transfected CHO cells

3.2.2 Cell line establishment

An overview of the major steps in establishing the cell lines is depicted in Fig. 3.8. After transfection and first screenings of product formation, as mentioned in chapter 2.5.6, the biomass of the selected clones was increased and they were transferred into T25 flasks. The best two clones for each antibody and medium were furthermore cultivated in spinner flasks with 50 mL each.

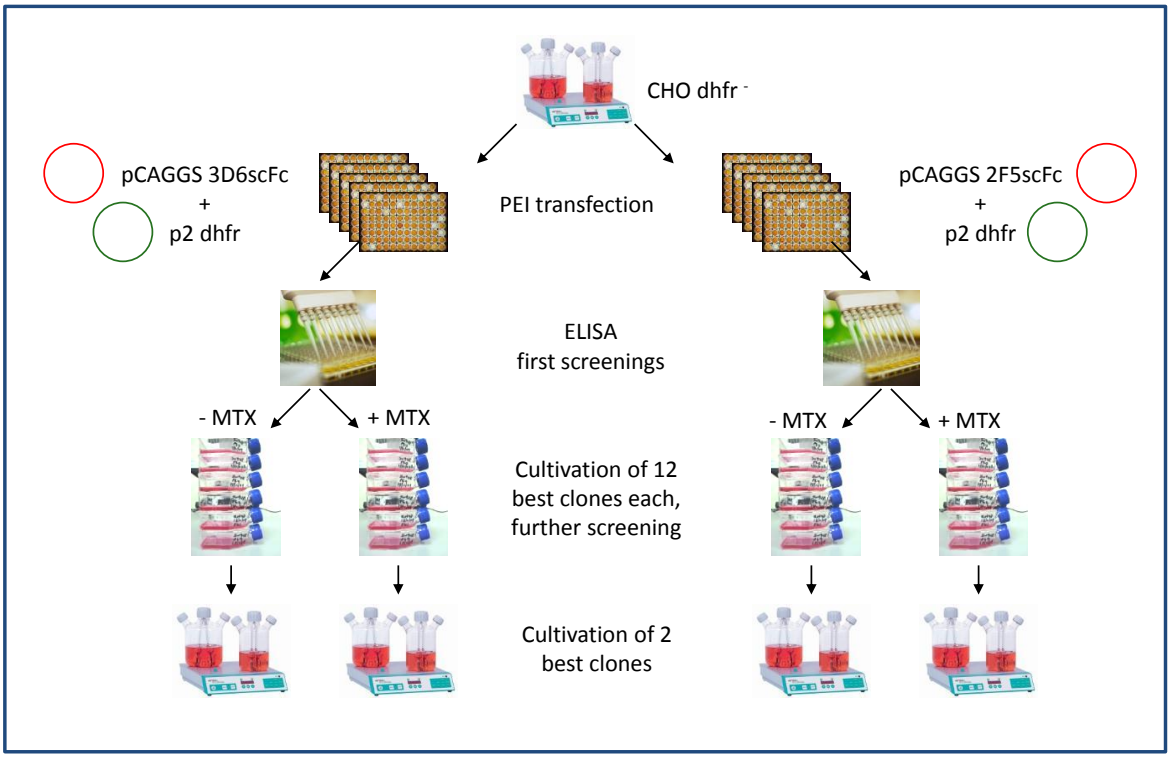

Fig. 3.8 Overview of the important steps in establishing the final cell lines

According to first ELISA screenings on productivity, the 12 best clones were chosen for each recombinant cell line and further cultivated in T25 roux flasks with selection medium (without MTX) and amplification medium (with 0.048 µM MTX). The cells cultivated in selection medium were used for the direct comparison to the BAC transfected cells. One ELISA screening plate is depicted in Tab. 3.3. Column 3 to column 8 represent 80 wells of the second transfection plate of 3D6scFc. For determination of the best clones, the surrounding wells (row A, row H, column 1, column 12) were generally discarded because the volume of these wells was less than in the middle wells. The reason was unequal evaporation from the microtiter plates because of long time incubation at 37 °C. Therefore, the surrounding wells had generally higher concentrations and direct comparison with the inner wells was not possible. Since the clone 2A5 in Tab. 3.3 was the only clone with a product concentration above the measurement threshold, it was also further cultivated to get accurate data on its productivity.

After discarding the outer wells, the inner wells were marked according to their product concentrations. In the example, all wells with absorbance over value 3 were marked green. Clone 2B8 was also chosen to be further cultivated in T25 roux flasks.

In Fig. 3.9, the cultivation of the clones in roux flasks with approximately 10 mL medium is shown.

Besides product concentration, also macroscopic (colour of the well, as mentioned in chapter 3.2.1) and microscopic (form of colony in the well, monoclonal colonies) observations had an impact on the choice of the best clones.

Tab. 3.3 ELISA screening of 3D6scFc transfectants - plate 2 excerpt

Plate 2	Blank	Std	Col. 3	Col. 4	Col. 5	Col. 6	Col. 7	Col. 8
A	0.024	0.100	1.345	2.665	OVER	1.348	3.713	3.481
B	0.024	0.172	0.890	1.226	2.099	2.561	2.954	3.427
C	0.023	0.318	1.064	2.831	3.283	1.698	3.140	3.000
D	0.023	0.552	1.246	0.998	3.318	2.606	2.999	3.142
E	0.022	0.907	0.870	0.725	2.543	1.645	2.245	2.984
F	0.027	1.273	0.876	0.833	2.891	2.382	2.791	2.865
G	0.023	2.038	0.836	1.443	0.622	1.278	1.972	0.807
H	0.029	2.457	0.701	0.559	0.682	0.644	0.988	0.817

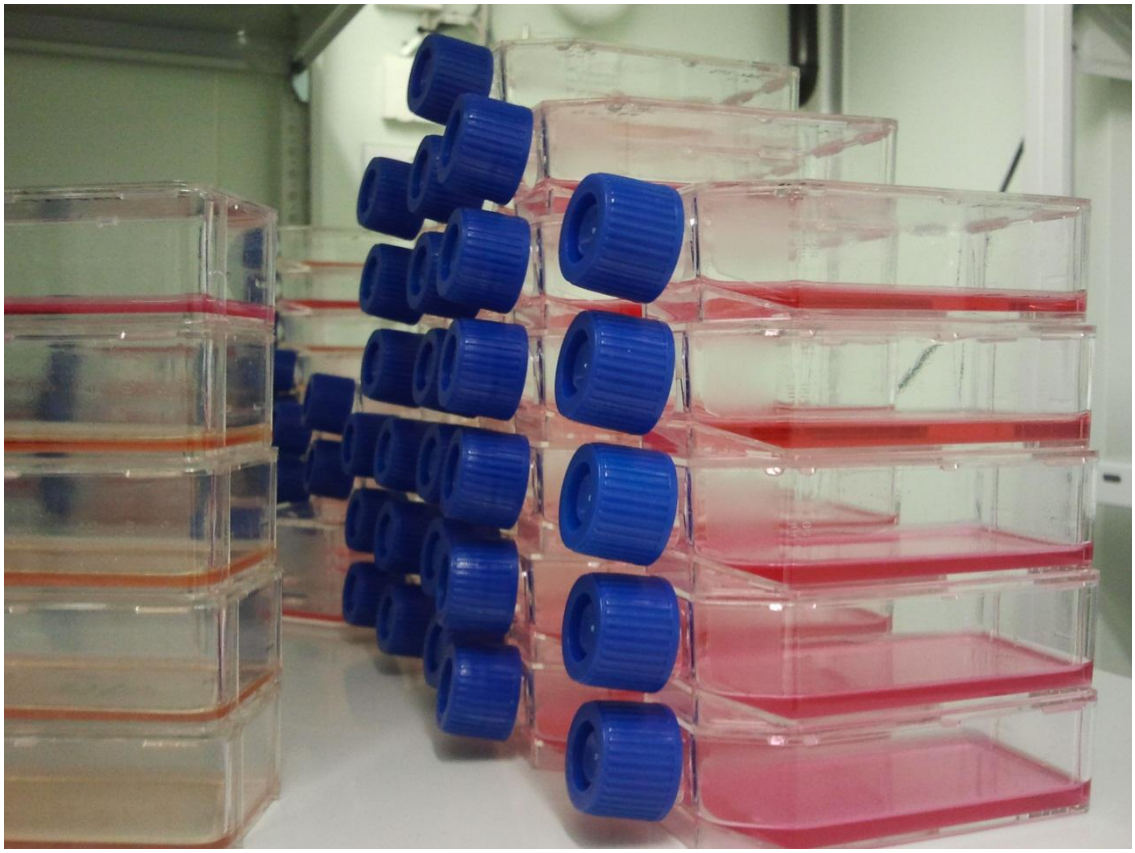

Fig. 3.9 Cultivation of transfected clones in T25 flasks

Product concentrations and specific productivities of the chosen clones are depicted in Fig. 3.10 to Fig. 3.17. Each bar represents the value for a certain time point and the dots and numbers characterize the mean values over all measurements. The values were measured twice a week, every time they were also subcultured according to chapter 2.5.4. According to their growth, for each clone the cell and product concentration was determined between five to seven times. For some clones, the adjustment to T25 roux flasks and increase in cell mass was faster and therefore they had to be subcultured earlier than other clones. As a result, there were more time points for measuring the mentioned characteristics. Few clones did not survive the adaption to roux flasks and therefore insufficient or no data is present for these clones in Fig. 3.10 to Fig. 3.17. For example, according to Fig. 3.14 it was just possible to determine the product concentration for clone 4E4 one time and therefore no value for specific productivity could be calculated (Fig. 3.15). After the first passage, the clone stopped growing and could not be further cultivated.

In detail, for 3D6scFc the three best producing clones were 2A5, 2B11 and 1D2 (Fig. 3.10 to Fig. 3.13). This result was obtained for cultivation in selection medium, as well as for cultivation in amplification medium with 0.048 µM MTX. When the cell mass for these clones in selection medium was increased for cultivation in spinner flasks, clone 2A5 stopped growing and would not recover after several medium changes. Therefore, the clones 2B11 and 1D2 were transferred to spinner flasks and further cultivated. Also for amplification medium with 0.048 µM MTX, clone 2A5 suddenly stopped growing, which depicts the instability of this clone.

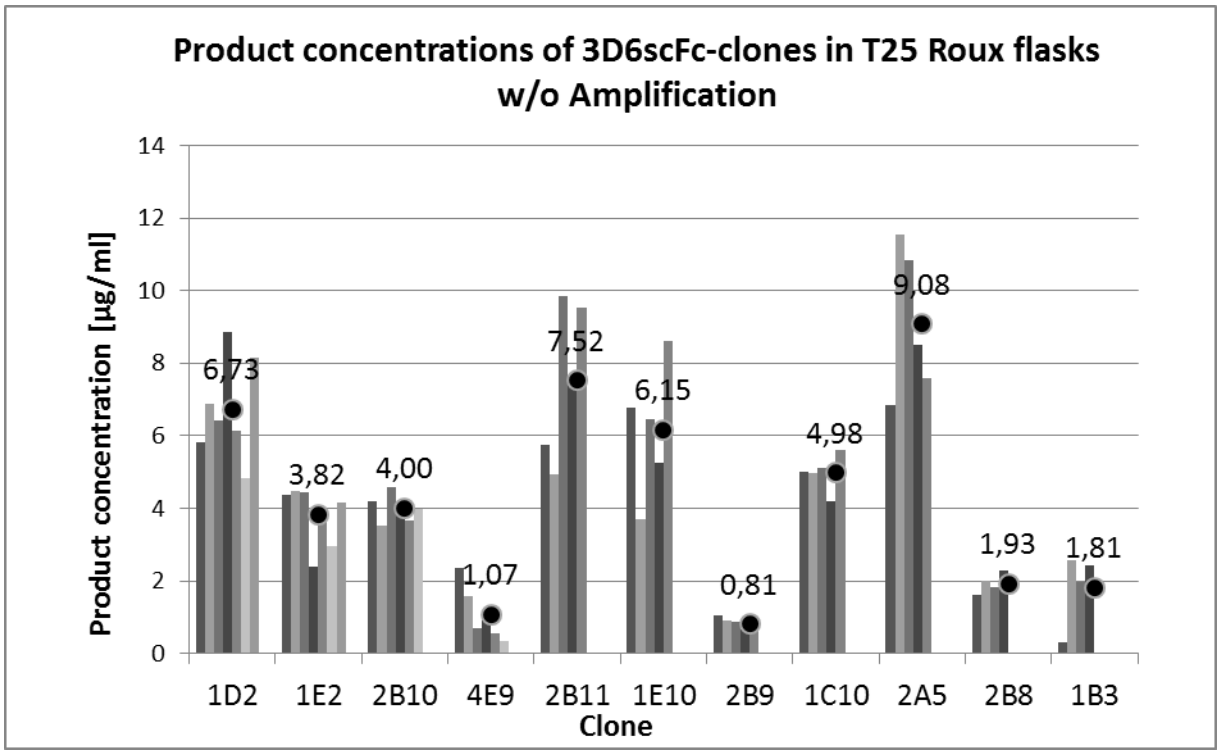

Fig. 3.10 Product concentrations of 3D6scFc in selection medium

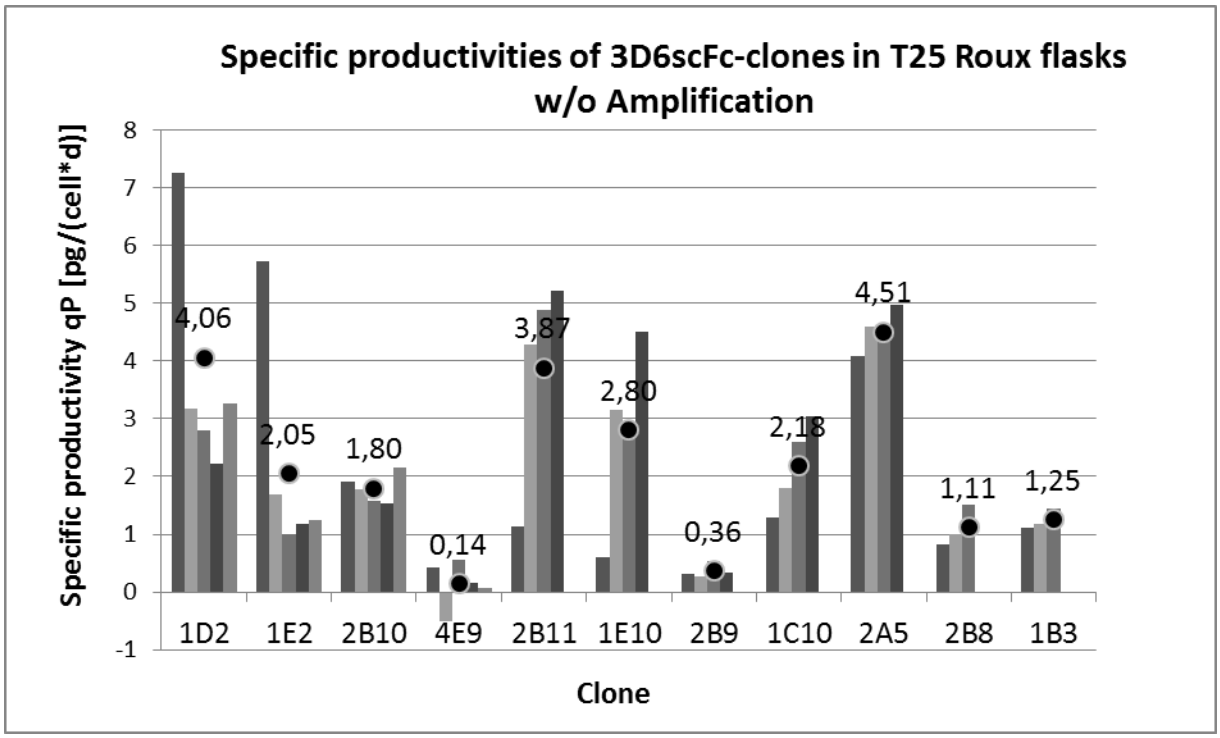

Fig. 3.11 Specific productivities of 3D6scFc in selection medium

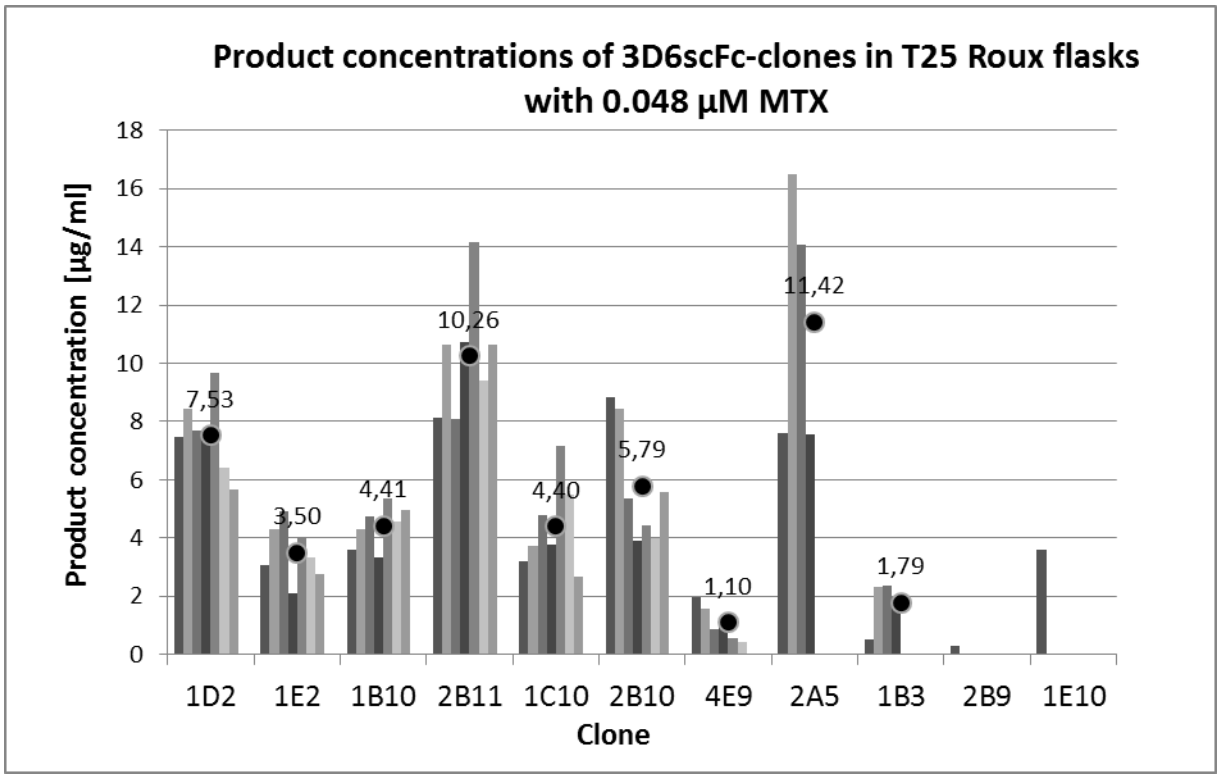

Fig. 3.12 Product concentrations of 3D6scFc in amplification medium (0.048 µM MTX)

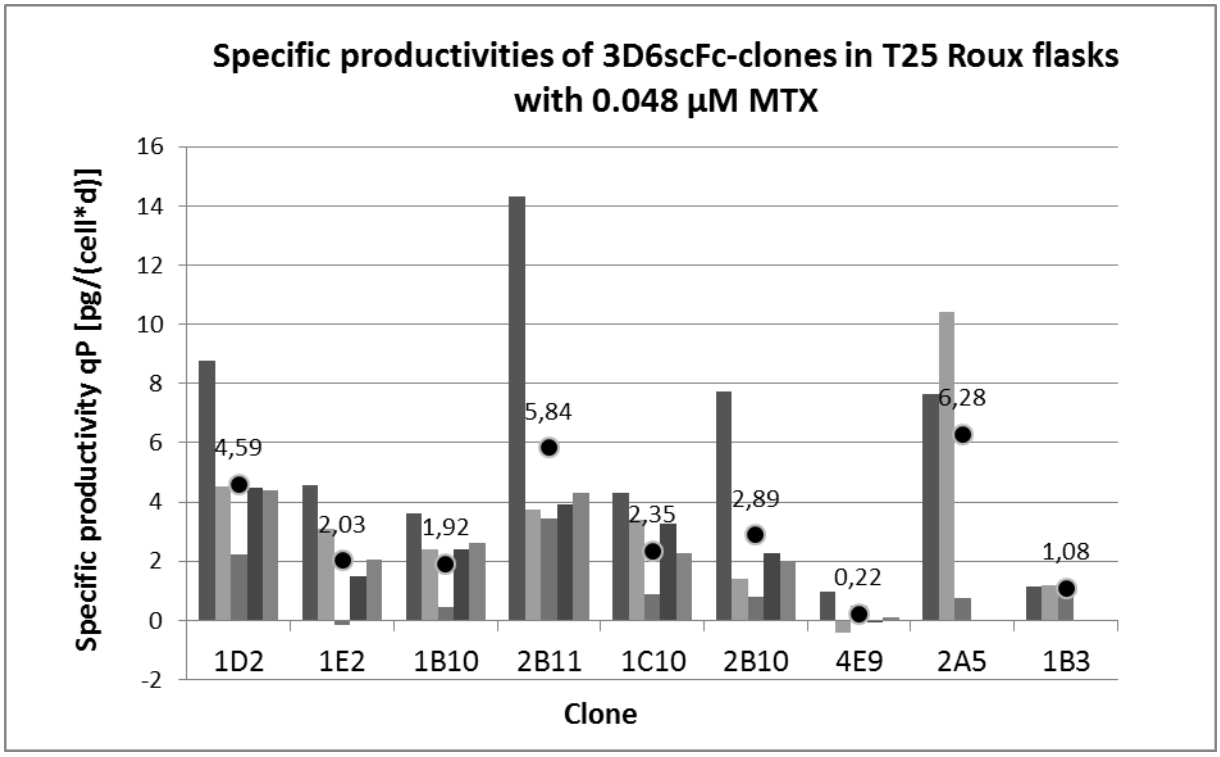

Fig. 3.13 Specific productivities of 3D6scFc in amplification medium (0.048 µM MTX)

For 2F5scFc transfectants, the two best producing clones, which were used for further cultivation in spinner flasks, were 5B11 and 4E9. Analogous to 3D6scFc transfectants, the best clones in selection medium were also the best clones in amplification medium with 0.048 µM MTX. At this time point, the clones had just started to adapt to the amplification medium after a few passages and the MTX concentration was still comparably low.

In Fig. 3.17 some clones have negative bars at the third point of determination. These are probably negative due to inaccuracies at measurements with the particle counter at one day. The determined cell concentrations were lower than expected and therefore specific growth rates and subsequently specific productivities resulted in negative values.

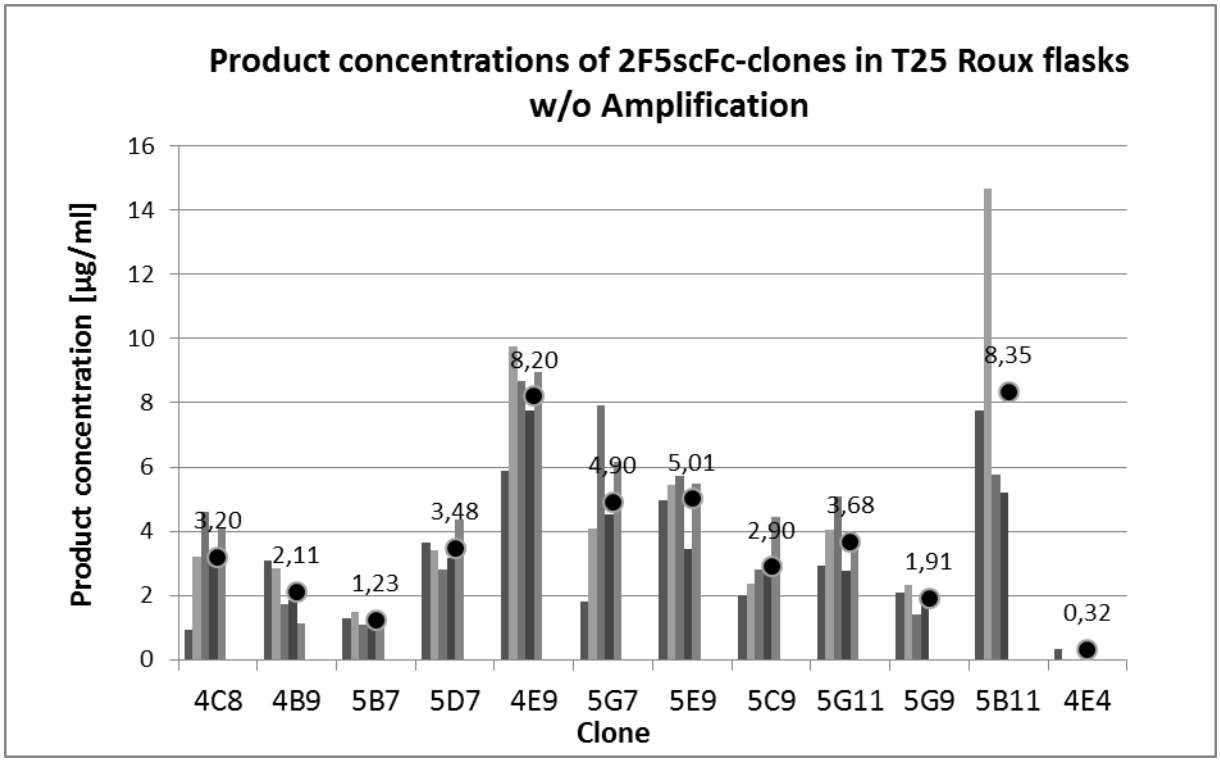

Fig. 3.14 Product concentrations of 2F5scFc in selection medium

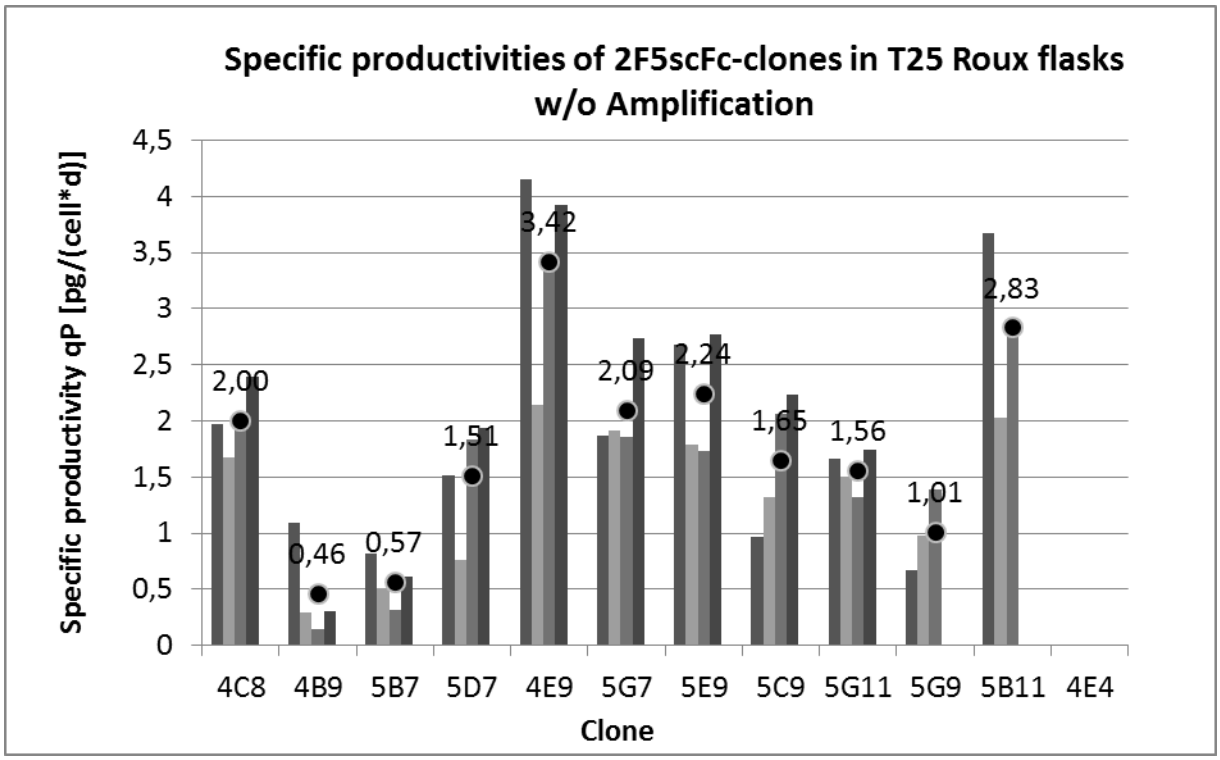

Fig. 3.15 Specific productivities of 2F5scFc in selection medium

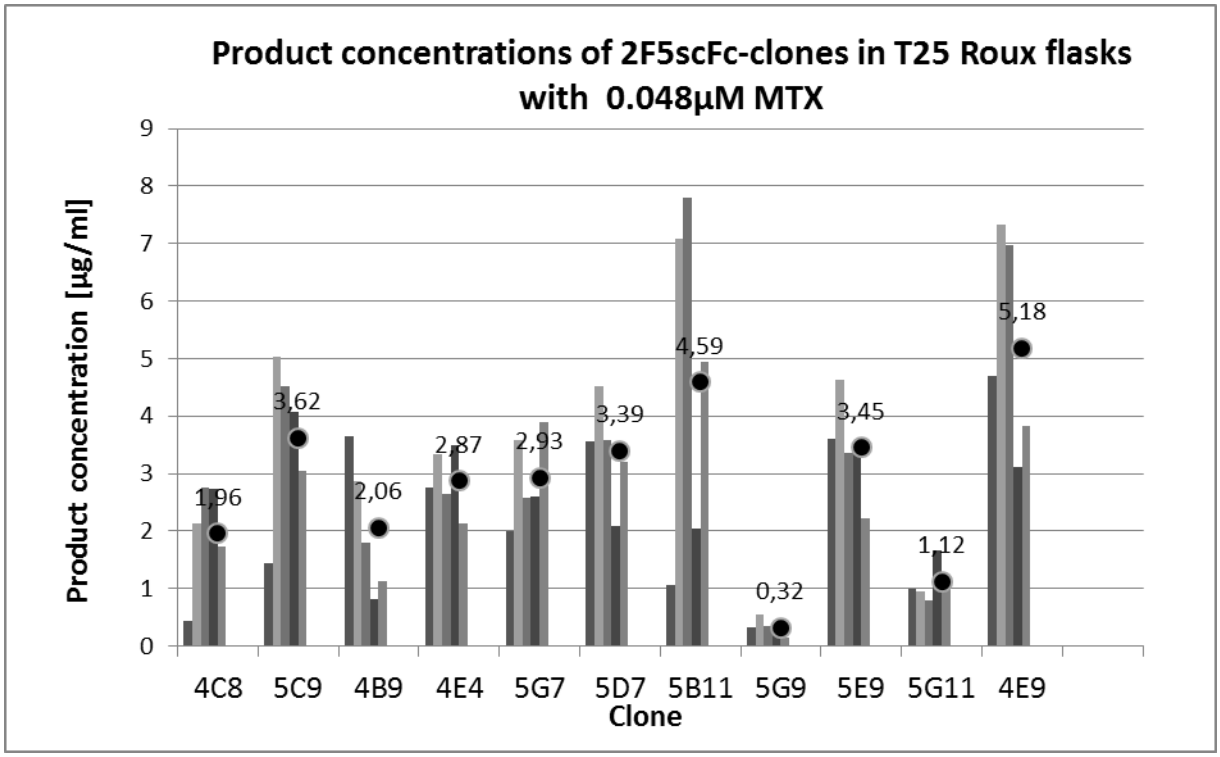

Fig. 3.16 Product concentrations of 2F5scFc in amplification medium (0.048 µM MTX)

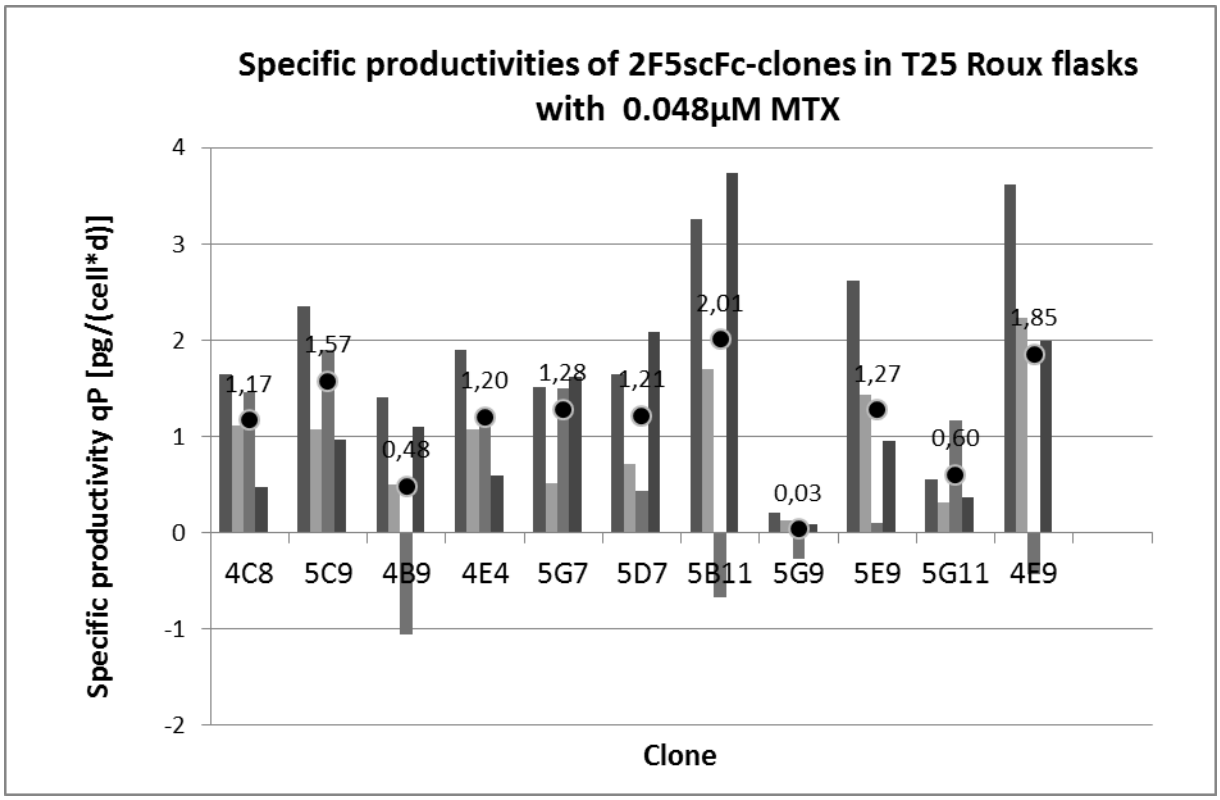

Fig. 3.17 Specific productivities of 2F5scFc in amplification medium (0.048 µM MTX)

3.2.3 Specific growth rates

The specific growth rates from cultivation of the clones in spinner flasks are displayed in Fig. 3.18 and Fig. 3.19. 3D6scFc BAC and 2F5scFc BAC were approximately growing at the same rate, with a mean µ below 0.4 d^{-1}. Plasmid transfected 3D6scFc clones were growing at higher rate, at an average of nearly 0.5 d^{-1}. The fastest growing cell line was plasmid transfected 2F5scFc clone 5B11, with a mean µ of over 0.55 d^{-1}. As the speed of plasmid transfectants was constantly increasing during the cultivation period, at the last three passages µ was already over 0.6 d^{-1}. Because of the limited timeframe for this work, the clones could not be monitored for more passages. According to Fig. 3.22, the qp of 2F5scFc clone 5B11 was not decreasing over time, indicating that increasing µ was not due to a loss of productivity. Probably, the reason for better growth was adaption to the cultivation in spinner flasks

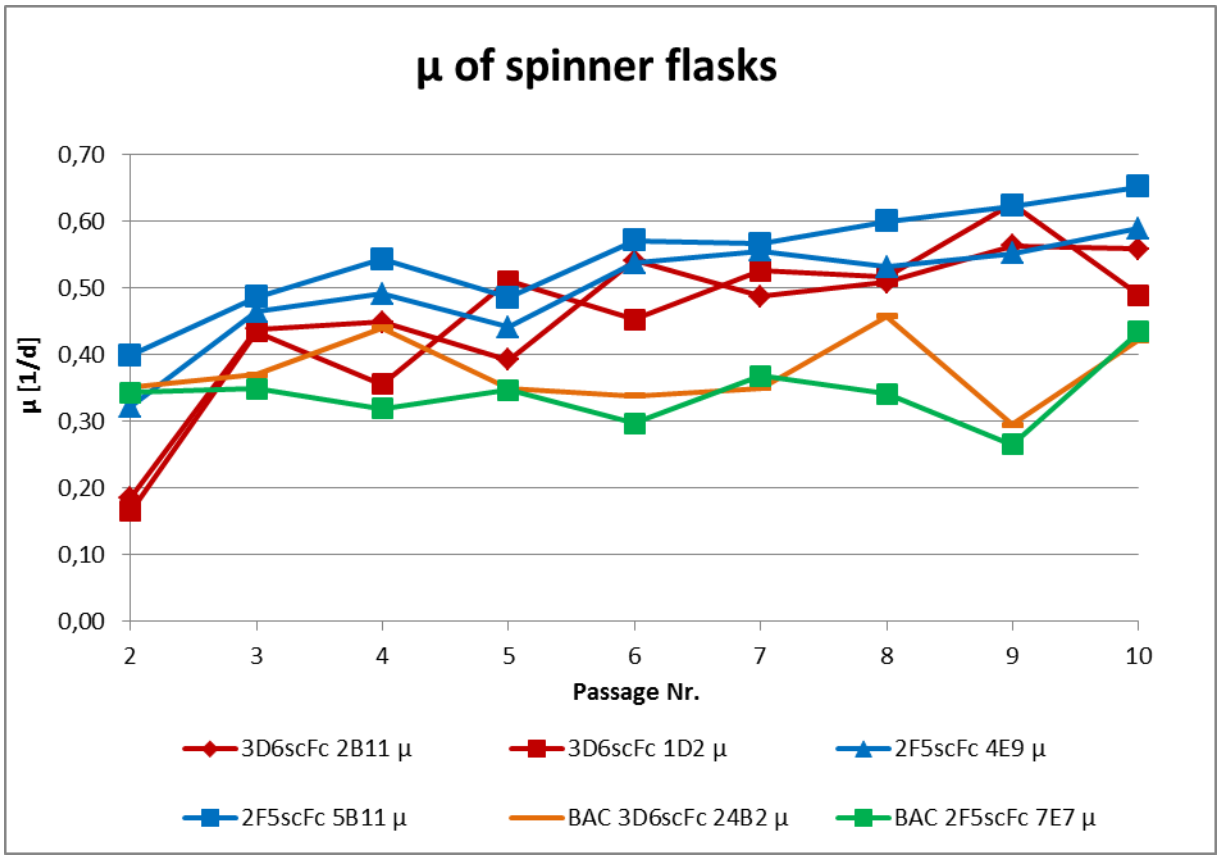

Fig. 3.18 Specific growth rates of clones in spinner flasks with 50 mL medium

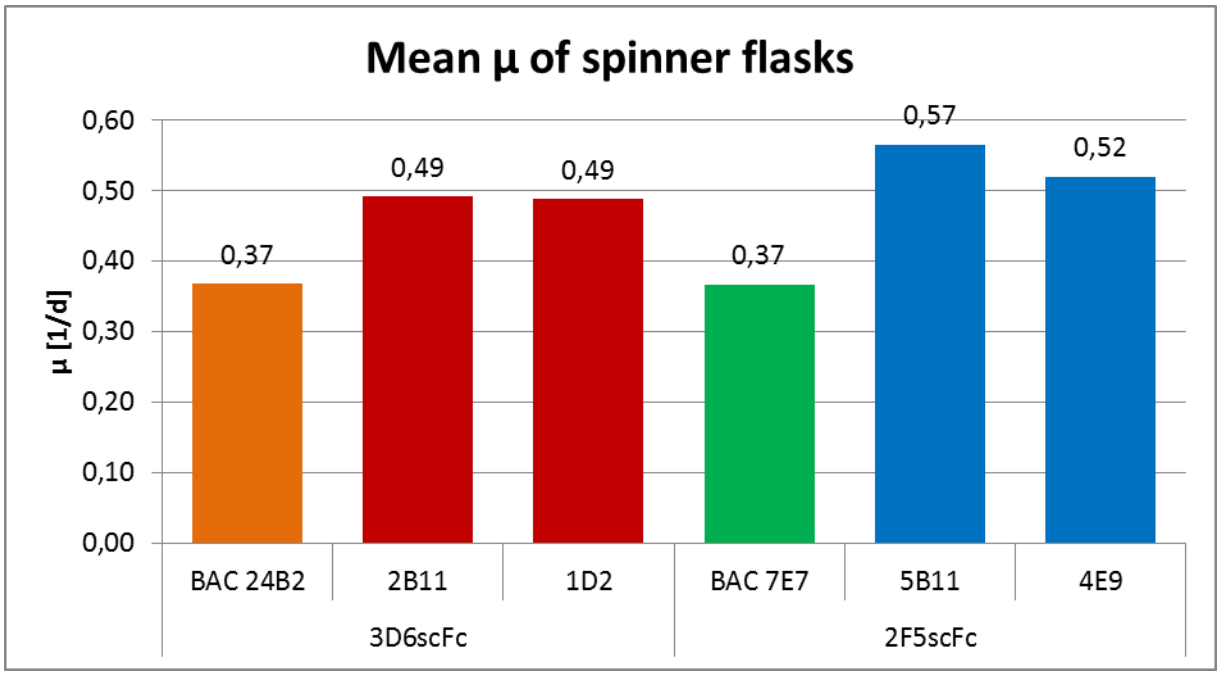

Fig. 3.19 Mean specific growth rates of clones in spinner flasks with 50 mL medium

3.2.1 Product concentrations

Concerning the product concentrations in the supernatant of the clones, 3D6scFc BAC was exclusively leading with an average of 46 µg/mL (Fig. 3.21). Second highest concentrations were obtained for plasmid transfected 3D6scFc clones, whereas 2B11 had approximately 16 µg/mL. 2F5scFc transfectants exhibited generally lower product concentrations. Around 11 µg/mL were found for 2F5scFc BAC and 5.8 µg/mL for the faster growing plasmid clone 5B11.

3D6scFc clone 1D2 had decreasing product concentrations (Fig. 3.20) and therefore the more stable clone 2B11, which was also superior in µ and qp, was selected for further comparisons with qPCR and FACS.

For 2F5scFc, the choice of the best clone was not as clear. Clone 5B11 was selected due to higher µ, higher product concentrations and overall better growth characteristics (clone 4E9 started to form aggregates after the first passages in spinner flasks). Nevertheless, because of faster growth, clone 5B11 had a slightly lower specific productivity (chapter 3.2.1), according to Equ. 2.5.

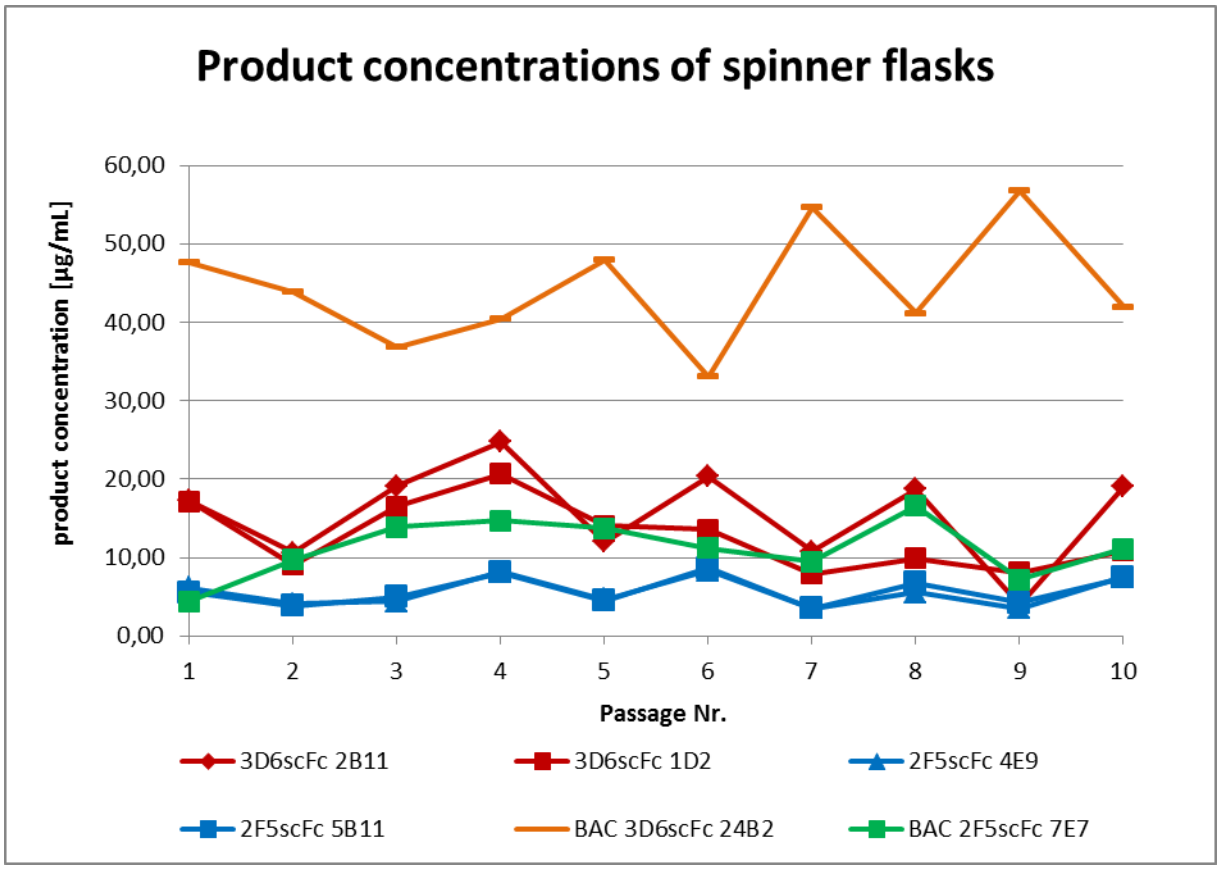

Fig. 3.20 Product concentrations of clones in spinner flasks with 50 mL medium

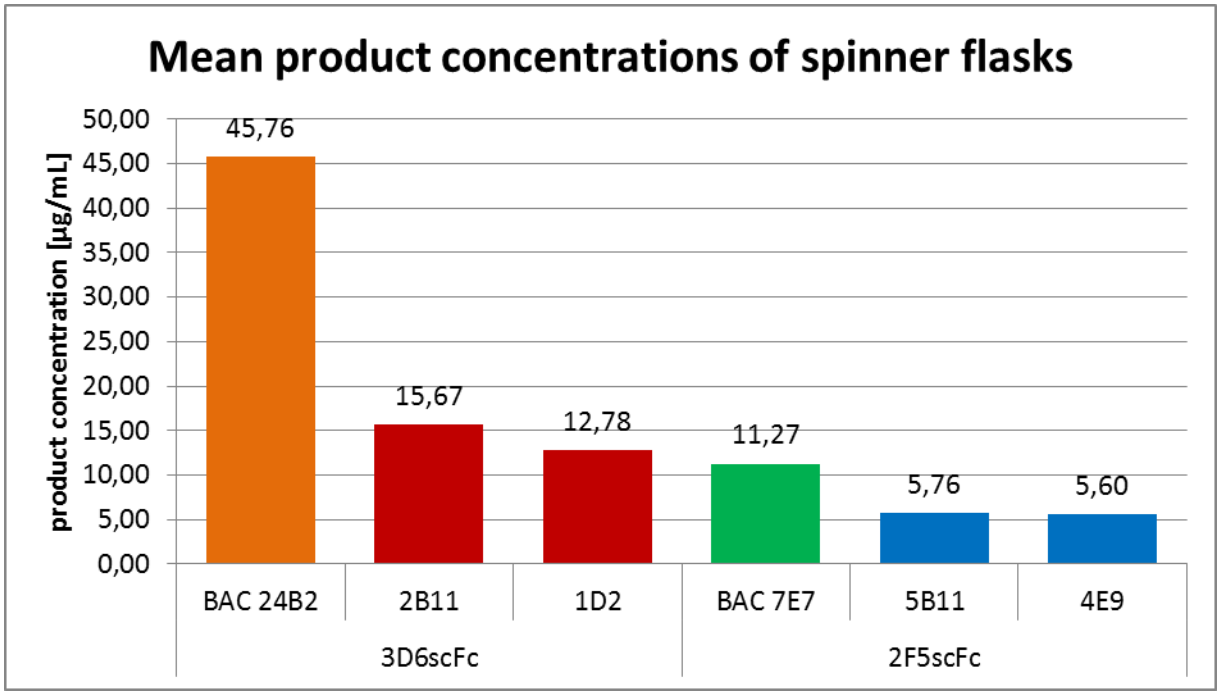

Fig. 3.21 Mean product conc. of clones in spinner flasks with 50 mL medium

3.2.2 Specific productivities

For specific productivities, the amount of accumulated product is related to a defined number of cells and time (Equ. 2.5). Therefore, if two cell lines have the same product concentrations in their supernatants, the slower growing cell line exhibits a higher qp. It does not need to build up as much biomass to reach the same product concentrations as the faster growing cell line.

Thus, the difference between BAC transfected and plasmid transfected cell lines is even more distinct than in chapter 3.2.1. With approximately 20 µg/(10^6 cells x day) 3D6scFc BAC had a four times higher qp than 3D6scFc clone 2B11 with 5 µg/(10^6 cells x day). For 2F5scFc, the BAC transfected clone had a nearly three times higher qp than the plasmid clone 5B11 (5 vs. 1.7 µg/(10^6 cells x day)).

Between the two antibodies, 2F5scFc BAC was approximately at the same level as plasmid transfected 3D6scFc clones. The low level for 3D6scFc 2B11 at passage number nine is probably due to a sampling or measurement error.

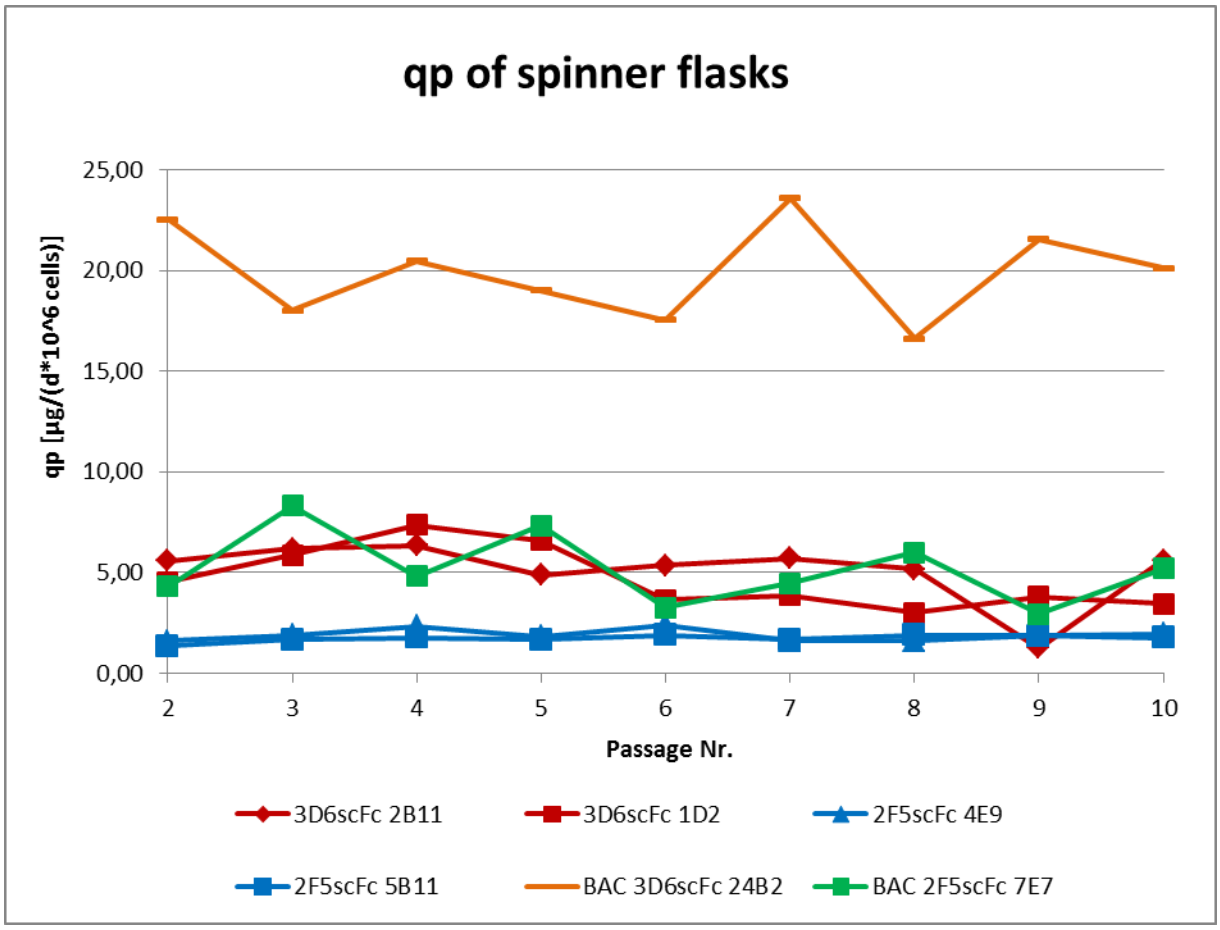

Fig. 3.22 Specific productivities of clones in spinner flasks with 50 mL medium

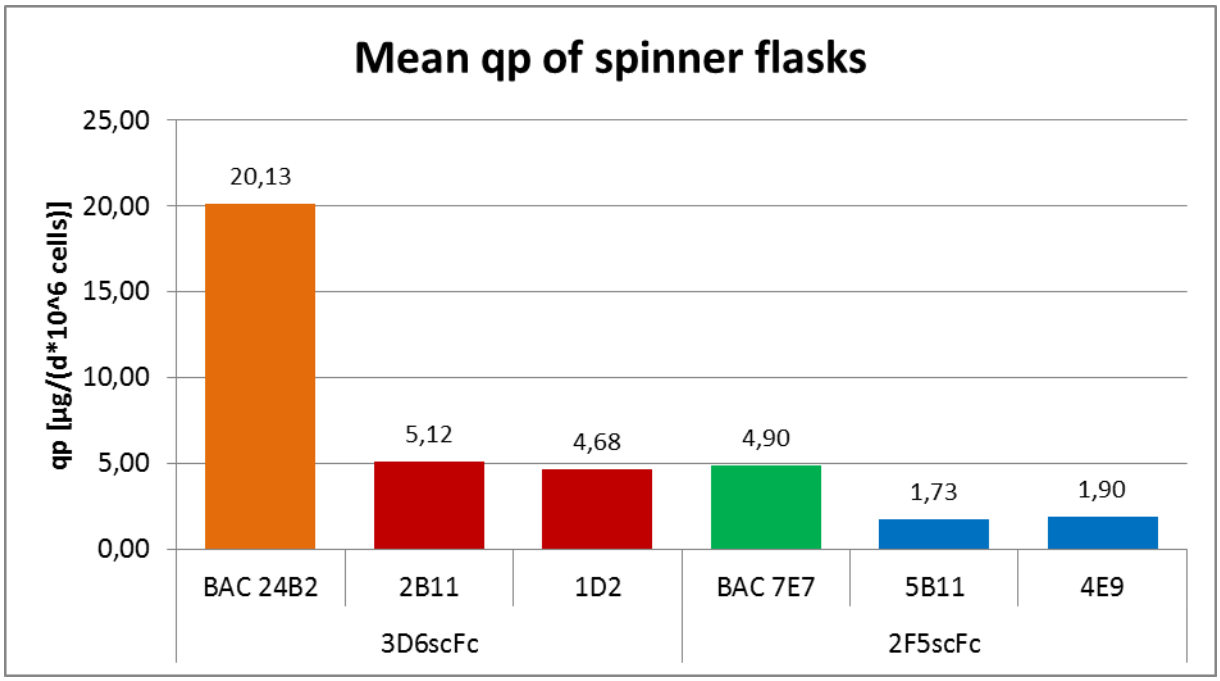

Fig. 3.23 Mean specific productivities of clones in spinner flasks with 50 mL medium

3.2.3 Subcloning

As mentioned before, the plasmid transfectants of both antibodies were cultivated in selection medium (without MTX) and amplification medium (with MTX). The amplified clones were not used for detailed comparison with the BACs, but cryopreserved for further experiments.

After increasing the MTX concentration from 0.048 µM to 0.096 µM, the clones were cultivated in T25 roux flasks for seven passages and in spinner flasks for five passages. The cell concentrations and viabilities directly before subcloning are presented in Tab. 3.4.

Tab. 3.4: Cell concentrations and viabilities before subcloning

Clone	Living cells [cells/mL]	Viability [%]
pCAGGS 3D6scFc/2B11 Amp.Medium 0.096 µM MTX	1,33E6	95
pCAGGS 2F5scFc/5B11 Amp.Medium 0.096 µM MTX	1,51E6	94

For both cell lines, the plates with seeding densities of 3 cells/well exhibited growth in over 50% of the wells. As these plates were most probable to have monoclonal colonies growing in their wells (in comparison with 9 cells/well and 27 cells/well seeding concentrations), they were selected for product screenings. In accordance with chapter 3.2.2, the best clones concerning product concentration and morphology were further cultivated.

The best subclone for 3D6scFc/2B11 was G7 and the best subclone for 2F5scFc/5B11 was D3. These clones were compared regarding specific growth rate (Fig. 3.24) and specific productivity (Fig. 3.25). By subcloning, qp of G7 could be raised to over 6.5 µg/(10^6 cells x day) and over 2 µg/(10^6 cells x day) for D3. Specific growth rates were in the range between BACs and plasmid transfectants without MTX amplification.

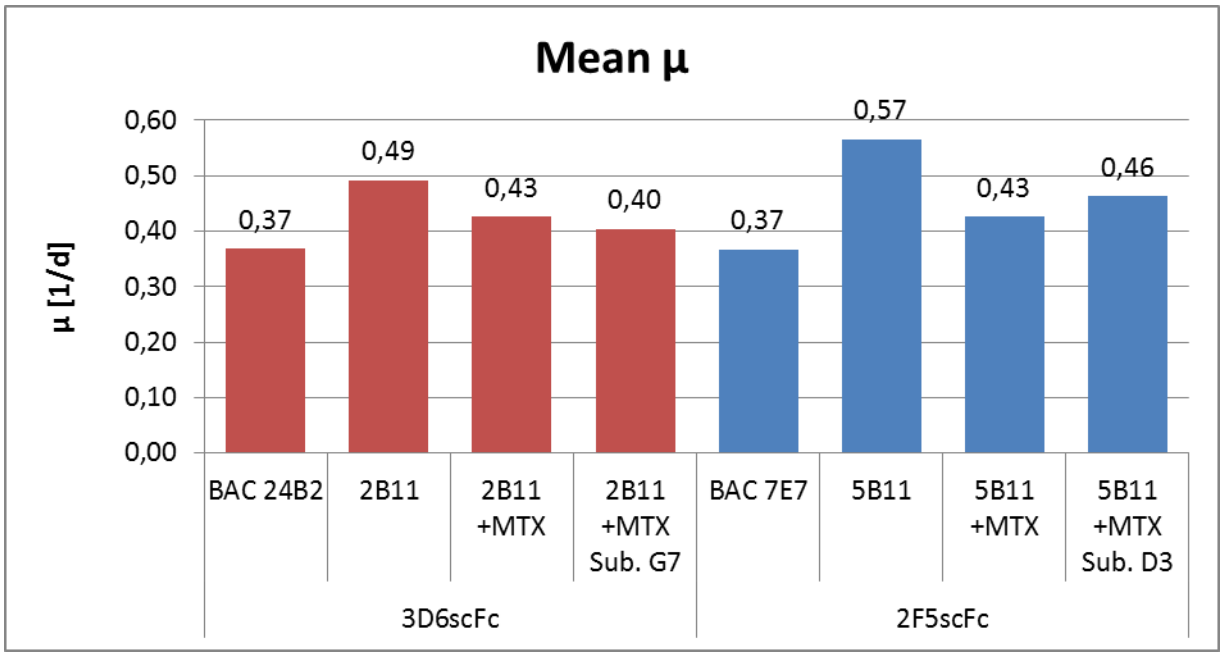

Fig. 3.24 Comparison of µ between BACs, selection medium, amplification medium and subcloned cell lines

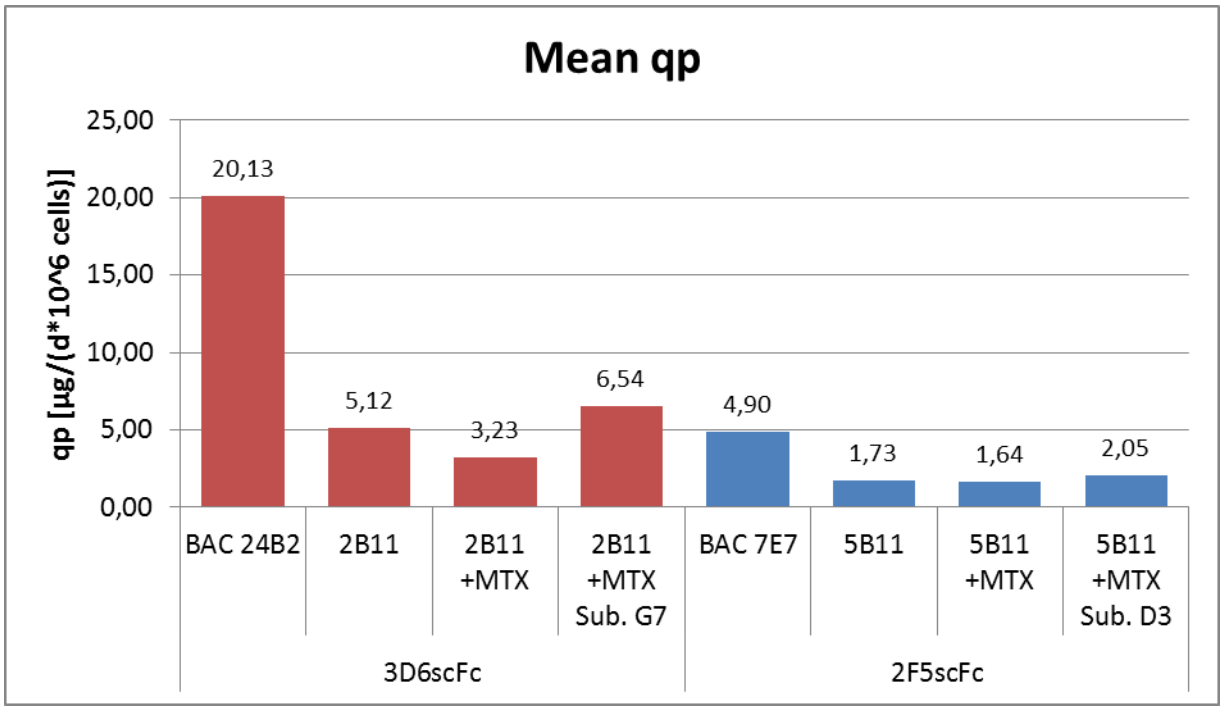

Fig. 3.25 Comparison of qp between BACs, selection medium, amplification medium and subcloned cell lines

3.3 Intracellular product formation (flow cytometry analysis)

3.3.1 Samples

Samples used for flow cytometry analysis were taken at three different time points (passages) to minimize sampling errors and have a better indication for the reproducibility of the results. For all cell lines, sampling was performed every second passage, except for BAC 2F5scFc (Tab. 3.5). This cell line was already in culture and samples were provided, but one of the three samples of the BAC 2F5scFc cell line could not be measured due to loss of cell pellet during sample preparation. For the overlay plots, one representative sample of each cell line was used.

Tab. 3.5 Samples used for determination of intracellular product formation

Sample	Date	Passage N°	Label
3D6scFc Selection Medium / 2B11	20.01.2012	4	A
	27.01.2012	6	B
	03.02.2012	8	C
2F5scFc Selection Medium / 5B11	20.01.2012	4	A
	27.01.2012	6	B
	03.02.2012	8	C
BAC 3D6scFc / 24B2	03.02.2012	3	A
	10.02.2012	5	B
	17.02.2012	7	C
BAC 2F5scFc / 7E7	24.01.2012	16	A
	27.01.2012	17	B

3.3.2 **Overlay plots**

The overlay plots demonstrate the distribution of intracellular product over a population of measured cells. 10000 events were analysed for each sample and the population was gated to be below 400 forward scatter (FS) and 400 side scatter (SS). An example gate for 2F5scFc/5B11 is presented in Fig. 3.26. From these gated populations, the intracellular product concentration was determined by fluorescence detection.

The negative control (NC, host cell line) has a low fluorescence intensity due to unspecific fluorescence of the cells and background noise. A shift of the peak to the right side (higher intensity) represents fluorescence due to intracellular labelled product.

Fig. 3.27 represents the overlay plots of BAC and plasmid transfectants producing the same scFc antibody. The negative control is presented in red colour. BAC transfectants are marked orange and plasmid transfectants yellow. The horizontal axis represents fluorescence intensity at logarithmic scale. On the vertical axis, the number of counted events out of the gated population is depicted. For all samples, a distinct peak shift to higher intensity was observed. Therefore, the product was detected in all cell lines.

For 3D6scFc, the peaks of BAC and plasmid transfectants had a similar distribution, but the BAC peak had a higher fluorescence intensity. Thus, both cell lines appeared to have a similar homogeneity with the BAC transfectant exhibiting a higher internal product concentration.

Regarding 2F5scFc, the BAC cell line also had a higher peak shift towards increasing intensity than the plasmid cell line. Hence, it had a higher internal product concentration. The peak height from the BAC transfectant was lower than from the plasmid cell line. This was contributed to the distribution of cells in the sample. From 10000 measured events, less cells were beyond 400 FS and 400 SS. Therefore, the gated population consisted of fewer total counts than the other samples and the peak was not as high. Probably this is due to sampling or ethanol conservation issues. However, as the peak had a normal distribution comparably to 2F5scFc plasmid, also the 2F5scFc BAC cell line exhibited characteristics of a homogenous cell line. Summarizing, BAC transfectants had a higher internal product concentration compared to plasmid transfectants and all cell lines had a homogenous distribution.

In Fig. 3.28, the overlay plots for the same transfection method and different product are shown. For the BAC transfectants, internal product concentration seemed to be similar, although 2F5scFc BAC had a wider distribution to higher intensity. Concerning plasmid transfectants, the peak of 3D6scFc had a distinct shift towards higher intensity compared to 2F5scFc. Therefore, more intracellular product was found for 3D6scFc.

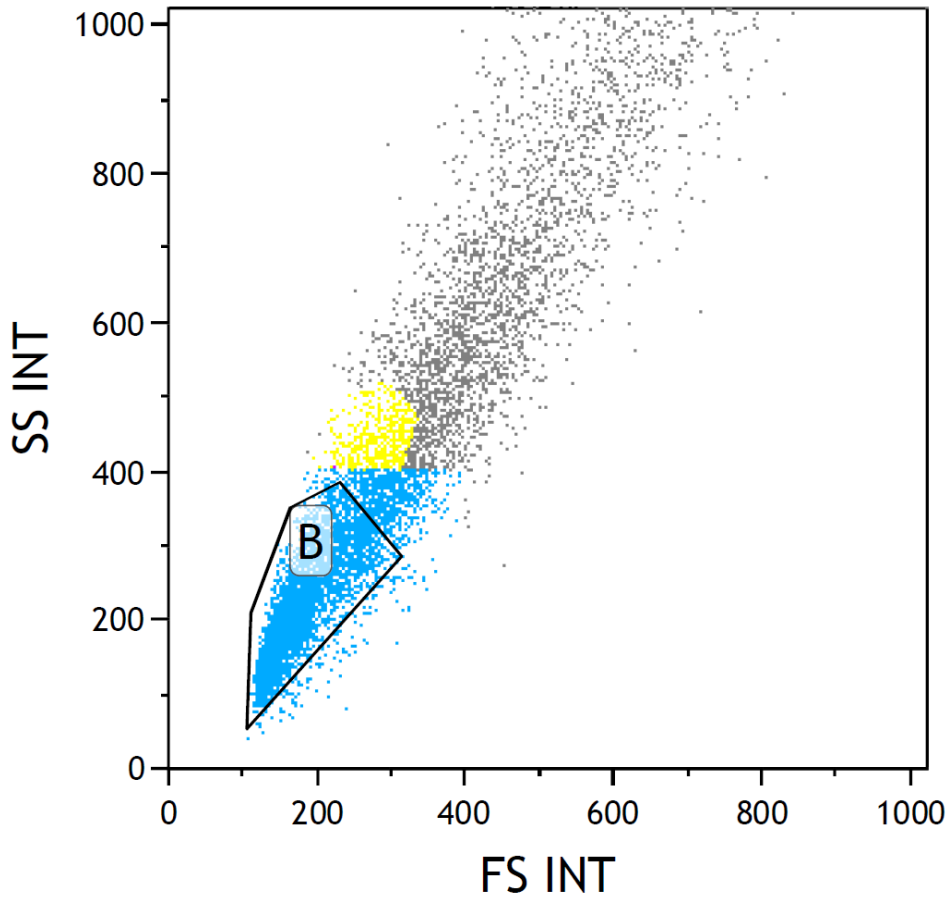

Fig. 3.26 Scatterplot and gated population of 2F5scFc/5B11 sample
Gate B (inside the black borders) was used for the analysis, the events marked in blue (square gate up to 400 FS and 400 SS) and yellow (large gate up to 500 SS, partly overlapped) were also tested but considered less representative for the population.

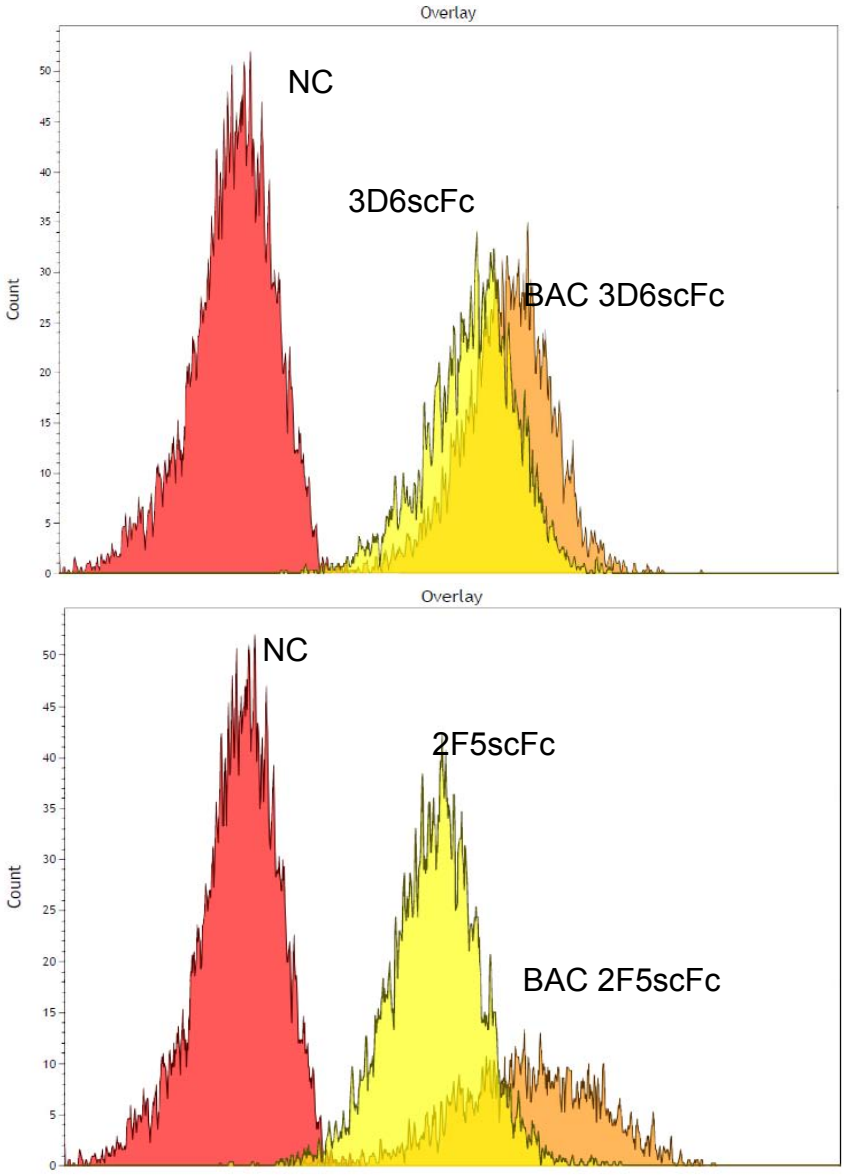

Fig. 3.27 Overlay plots of BAC vs. plasmid

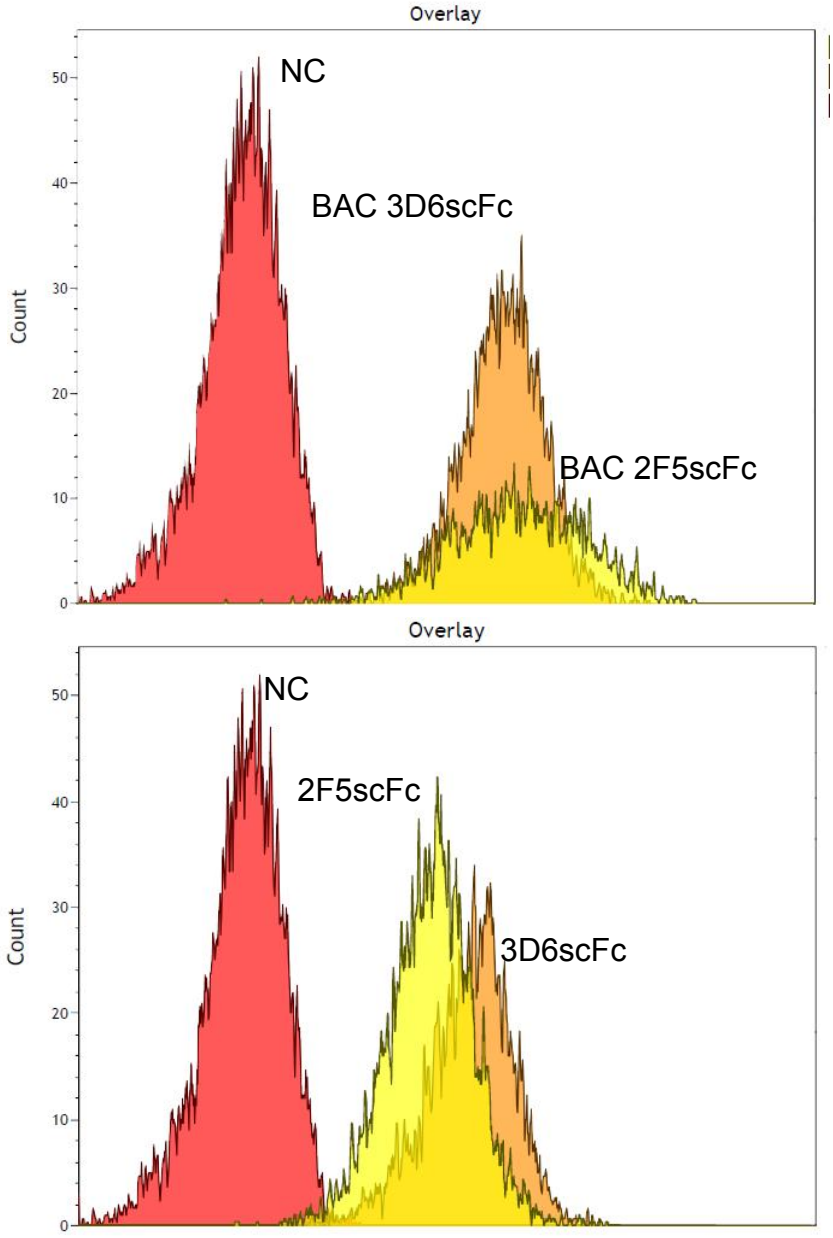

Fig. 3.28 Overlay plots of 3D6scFc vs. 2F5scFc

3.3.3 Quantification

For quantitative comparison of the cell lines, the median fluorescence intensity (MFI) for each sample was used (Fig. 3.29). This MFI was corrected for autofluorescence by subtracting the MFI of the negative control. An example for the estimation of the median fluorescence from the histogram is presented in Fig. 3.30. Compared to the overlay plots, the same tendencies were also visible over all samples. BAC transfectants had the highest intracellular product concentration for all passages. With an average of 22 FIU, the 2F5scFc BAC had the highest intracellular product content, followed by 3D6scFc BAC (18 FIU). However, an additional sample for 2F5scFc BAC would be beneficial for assessment. 3D6scFc plasmid (15 FIU) had a higher internal product concentration than 2F5scFc plasmid (7 FIU).

Concerning the distribution of values, 3D6scFc BAC seems to have an increase in intracellular product over the passages. This trend is also visible for product concentrations at the passages (3, 5 and 7) of sampling (Fig. 3.20). However, overall the product concentrations of 3D6scFc BAC were not significantly increasing and the three samples appear representative for the cell line. Least variations were found for the 3D6scFc plasmid cell line, followed by the 2F5scFc plasmid cell line (Fig. 3.30). 2F5scFc BAC had the highest variation in fluorescence intensity, but without further samples it is not possible to assess the reasons. Probably, there were issues with sampling or ethanol fixation, as for both measured samples, gated populations consisted of fewer suitable events (chapter 3.3.2) and an additional third sample, which was provided, could not be measured at all (chapter 3.3.1).

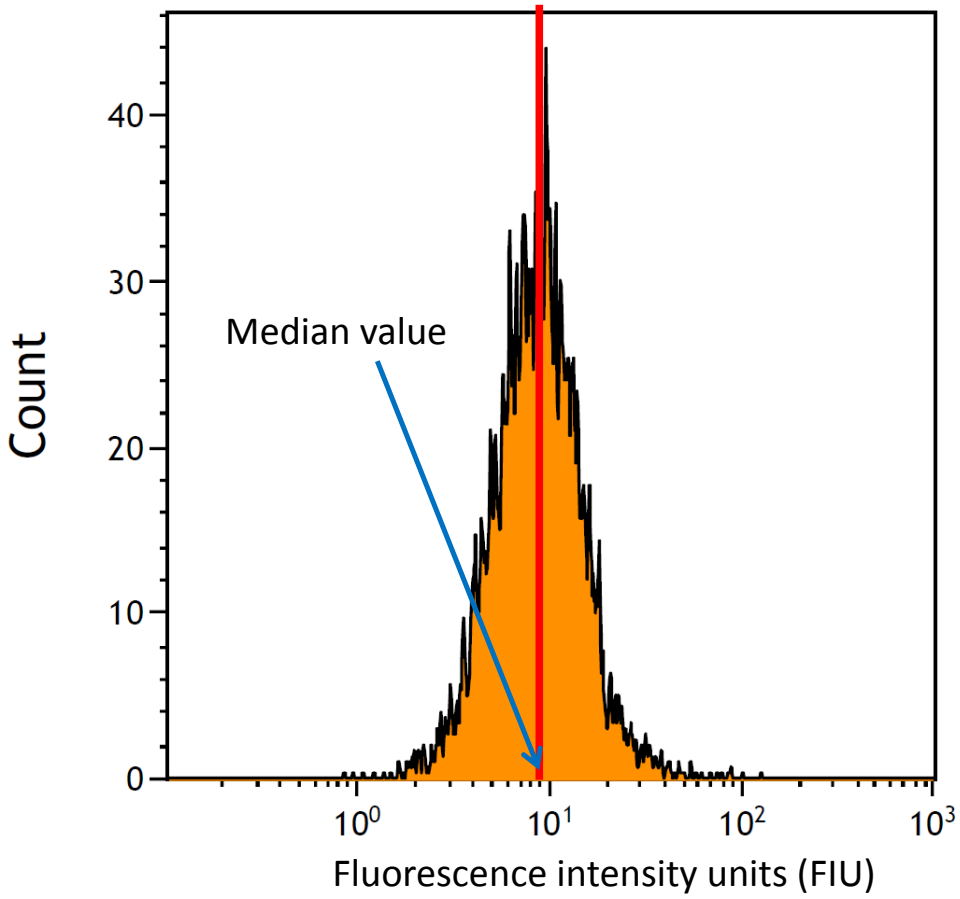

Fig. 3.29 Chosen median value for quantification

Example for 2F5scFc 5B11 sample A

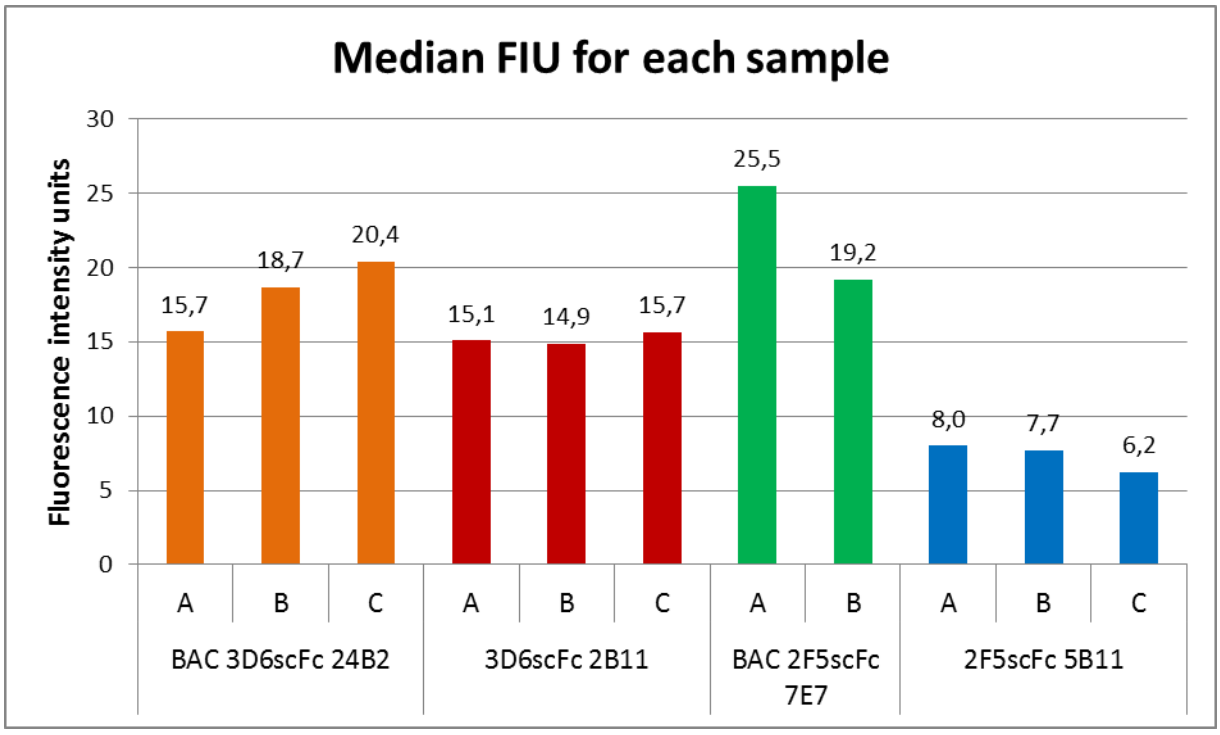

Fig. 3.30 Median flourescence intensities of measured samples

3.4 Preliminary considerations for qPCR

3.4.1 Principle

The qPCR analyses were conducted for determination of gene copy numbers and transcript levels. Therefore, samples from the gDNA isolation (chapter2.8) and the reverse transcription (chapter2.10) were measured in various qPCR runs. After first experiments, a relative quantification approach was applied, as it led to the best reproducibility and most reliable results. Thus, not only the amount of gene of interest (scFc) but also the quantity of ß-actin was measured. The ß-actin gene, one of the housekeeping genes, was used as an internal reference (Thellin et al., 1999) and for each sample the levels of transgene were referred to this reference.

3.4.2 Design of primers and probes for qPCR

As 3D6scFc and 2F5scFc had quite similar structures, the approach was to find a set of primers and a corresponding probe that would work for both antibody genes. Both sequences were codon-optimized for CHO and consequently there were differences between the two sequences on the genetic level. Although the constant regions have the same amino acid sequence, there can be different “optimal” codons, according to the applied algorithms and subsequently variations in the gene sequence.

For this reason, the sequence of 3D6scFc was BLASTed against 2F5scFc to find the best alignments between them (Tatusova and Madden, 1999). The longest perfect alignment was 194 nucleotides long and is presented in Fig. 3.31. In chapter 6.1 the complete sequences of 3D6scFc and 2F5scFc can be found.

This aligned sequence was used for the design of primers and probe. Therefore, the software application Primer3Plus was applied (Untergasser et al., 2007). The chosen primers were also controlled with OligoCalc (Kibbe, 2007), to assure that no secondary structures would inhibit correct PCR reaction.

The primers and probe for ß-actin were already available (chapter 2.11).

```
5’ – AAGGACACCC TGATGATCTC CCGGACCCCC GAAGTGACCT GCGTGGTGGT
   GGACGTGTCC CACGAGGACC CTGAAGTGAA GTTCAATTGG TACGTGGACG
   GCGTGGAAGT GCACAACGCC AAGACCAAGC CCAGAGAGGA ACAGTACAAC
    TCCACCTACC GGGTGGTGTC CGTGCTGACC GTGCTGCACC AGGA – 3’
```

Fig. 3.31 Longest alignment between 3D6scFc and 2F5scFc
Primers are highlighted in grey, the probe in green

3.4.3 Selection of sampling volume

There was a certain reason for using 3 ng of gDNA template in the beginning. First experiments did not use a relative quantification approach and the results were not related to ß-actin but to a certain number of cell genomes. It was assumed that the CHO-DUKX-B11 genome had a similar genome size compared to the CHO-K1 cell line, which was already published to be 2.45Gbp (Xu et al., 2011). Therefore, the DNA content of one cell was calculated to be 2.7 pg according to Equ. 2.8. In 3 ng of genomic DNA, there would be around 1110 cell genomes.

Nevertheless, for the relative quantification approach, which was applied, minor changes in gDNA template amount would not influence the outcome, as the relation of tested gene to the reference gene would not be changed. Primers and probes have to be present in the right amount, not to inhibit the qPCR cycles.

For cDNA, quantification of the template DNA is not as simple, as the mRNA has to be reverse transcribed and eventually DNA and RNA are present in the reaction tube. Therefore, the DNA concentration cannot be accurately determined by photometric measurement without having absorbance from the RNA. For this case, the relative quantification approach benefits from its independence from exactly known template concentrations.

3.4.4 Selection of samples

For analysis of gene copy numbers and transcript amounts, the samples were taken at the same passages as for analysis of intracellular product formation (Tab. 3.5). For 2F5scFc BAC, an additional third sample was provided (Tab. 3.6).

Tab. 3.6 Samples used for analysis of gene copy numbers and transcipt amounts

Sample	Date	Passage N°	Label
3D6scFc Selection Medium / 2B11	20.01.2012	4	A
	27.01.2012	6	B
	03.02.2012	8	C
2F5scFc Selection Medium / 5B11	20.01.2012	4	A
	27.01.2012	6	B
	03.02.2012	8	C

BAC 3D6scFc / 24B2	03.02.2012	3	A
	10.02.2012	5	B
	17.02.2012	7	C
BAC 2F5scFc / 7E7	20.01.2012	15	A
	24.01.2012	16	B
	27.01.2012	17	C

3.4.5 Replicates and controls

From every clone, three samples were taken on three different time points as depicted in chapter 3.4.4. Every clone was measured on two independent plates for each plate in triplicate. Therefore, in total six technical replicates were measured for one clone at one certain time point. Additionally to the samples, every plate was measured with three no template controls (NTC, dH_2O as a template) and three negative controls (NC, host cell line as a template). For cDNA runs, there were also no reverse transcriptase controls (NRT, according to chapter 2.10) used to check for potential DNA contaminations in the RNA solutions and proper function of the reverse transcription.

3.4.6 Quantification of data

For the evaluation of the results, a relative quantification method was applied. Thereby, data derived from measurement of the scFc (which equals product) amplicon was correlated with data from an internal reference gene. This reference gene was ß-actin (Thellin et al., 1999), a housekeeping gene which is also present in CHO cells. For each sample, the difference between the Cq values for product and reference was calculated. This difference is referred to as the ΔCq value.

A higher Cq value resembles a lower concentration of the gene of interest in the sample (there are more qPCR cycles needed to reach enough fluorescent signal to overcome the threshold). Therefore, according to Equ. 2.9 and Equ. 2.10, a negative ΔCq value corresponds to a higher initial concentration of scFc genes compared to ß-actin genes in the sample.

Assuming 100% PCR-efficiency (the amount of amplicon genes is doubled at every PCR cycle), the ratio of scFc genes to ß-actin genes can be calculated according

to Equ. 2.11. A ratio of 2 therefore stands for twice as much product copies than ß-actin copies in the sample. Taking the individual ΔCq values, the ratios could be calculated for all the samples.

As the intention of the qPCR measurements was a comparison between different samples, the approach was extended to the ΔΔCq method. It is among the most popular ways of determining concentration differences between different samples with a reference gene (Bustin et al., 2009). Maintaining the assumption of 100% PCR-efficiency, the comparison can be done with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). The ratio between two different samples can be calculated according to Equ. 2.12. As ß-actin is a housekeeping gene, the gene copy numbers and amounts of transcript should be similar between the different CHO cell lines. Therefore, a ratio of 2 equals twice the amount of product copies in sample 1 compared to sample 2.

Additionally, the PCR efficiencies were also calculated with the software LinRegPCR (Ruijter et al., 2009). Thereby, the software makes a linear regression in the exponential phase of the PCR amplification curve to determine the efficiencies of all sample runs. These can be used to quantify with an approach from Pfaffl, that expands the $2^{-\Delta\Delta Cq}$ method for an efficiency correction (Pfaffl, 2001).

Both methods produced similar results and therefore the $2^{-\Delta\Delta Cq}$ method was chosen for the presented results, as it facilitates a statistical analysis of the derived Cq values.

3.5 Gene copy numbers

Mean –ΔCq values and $2^{-\Delta Cq}$ values of all measured samples are presented in Tab. 3.7. As mentioned before, the $2^{-\Delta Cq}$ values represent the amount of genes of interest relative to the amount of ß-actin genes. BAC 3D6scFc was the only cell line with values higher than one (more scFc genes present). All other cell lines had values below one, indicating that there were more ß-actin genes present in the cells. The meaning of these numbers regarding absolute copy numbers is discussed in chapter 4.3.1.

For relative comparison of the cell lines, -ΔΔCq values are related to 3D6scFc 2B11 / sample C and also presented in Tab. 3.7. The $2^{-\Delta\Delta Cq}$ values (Fig. 3.32) represent the amount of GOI copy numbers relative to the selected sample. BAC 3D6scFc samples exhibited the highest relative gene copy numbers, in average approximately threefold higher than the other cell lines.

Tab. 3.7 Quantification of gene copy analysis

Clone	Sample	-ΔCq mean	scFc relative to ß-actin (2^-ΔCq)	-ΔΔCq referred to 3D6scFc 2B11 / C
BAC 3D6scFc 24B2	A	1,16	2,23	1,53
	B	0,70	1,62	1,07
	C	1,63	3,09	2,00
3D6scFc 2B11	A	-0,02	0,99	0,35
	B	-0,56	0,68	-0,19
	C	-0,37	0,77	0,00
BAC 2F5scFc 7E7	A	-0,36	0,78	0,01
	B	-0,45	0,73	-0,08
	C	-0,65	0,64	-0,28

2F5scFc 5B11	A	-0,37	0,77	0,00
	B	-0,59	0,67	-0,22
	C	-0,91	0,53	-0,54

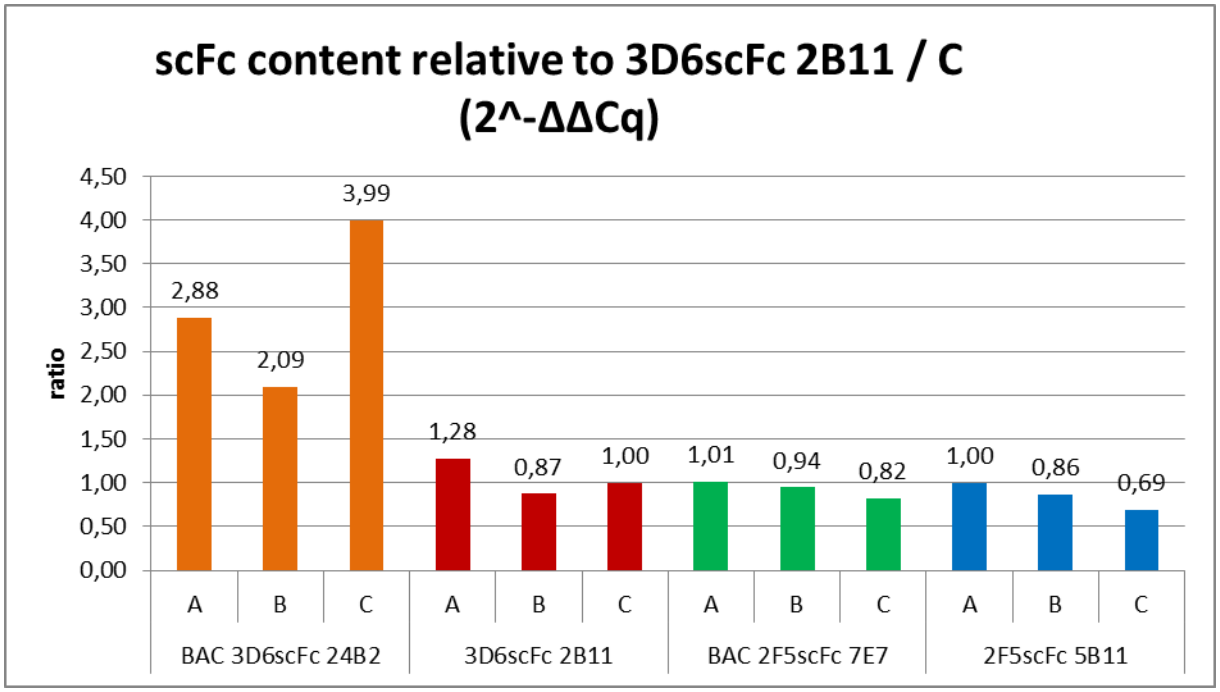

Fig. 3.32 Relative comparison of GCN related to 3D6scFc 2B11 / C

3.6 Amount of transcript

Higher diversities between the cell lines were observed for transcript levels. –ΔCq, $2^{-\Delta Cq}$ and -ΔΔCq values (Tab. 3.8) indicate the advantages of BAC mediated integration in highly transcribed genome areas. For BAC 2F5scFc, scFc transcripts were approximately fivefold higher present than ß-actin transcripts ($2^{-\Delta Cq}$ values) and for BAC 3D6scFc, the difference was between 13x to 26x (with a higher variation between the samples). For plasmid transfectants, the amount of scFc transcripts was below the amount obtained for ß-actin.

For comparison between the samples, the amount of scFc transcript copies is related to 3D6scFc 2B11 / sample C (Fig. 3.33), analogous to derived GCN (chapter 3.5). With a ratio of 15x to 31x, BAC 3D6scFC illustrated its high transcript amount,

followed by BAC 2F5scFc with 5x to 7x. Lowest transcript amounts were found for plasmid transfected 2F5scFc.

Variation between the samples is further investigated in chapter 3.7.

Tab. 3.8 Quantification of transcript amount analysis

Clone	Sample	-ΔCq mean	scFc relative to ß-actin (2^-ΔCq)	-ΔΔCq referred to 3D6scFc 2B11 / C
BAC 3D6scFc 24B2	A	4,68	25,61	4,94
	B	3,66	12,65	3,92
	C	3,82	14,11	4,08
3D6scFc 2B11	A	-0,28	0,82	-0,02
	B	-0,57	0,67	-0,31
	C	-0,26	0,83	0,00
BAC 2F5scFc 7E7	A	2,63	6,21	2,90
	B	2,39	5,24	2,65
	C	2,16	4,46	2,42
2F5scFc 5B11	A	-0,69	0,62	-0,43
	B	-0,18	0,89	0,09
	C	-1,29	0,41	-1,02

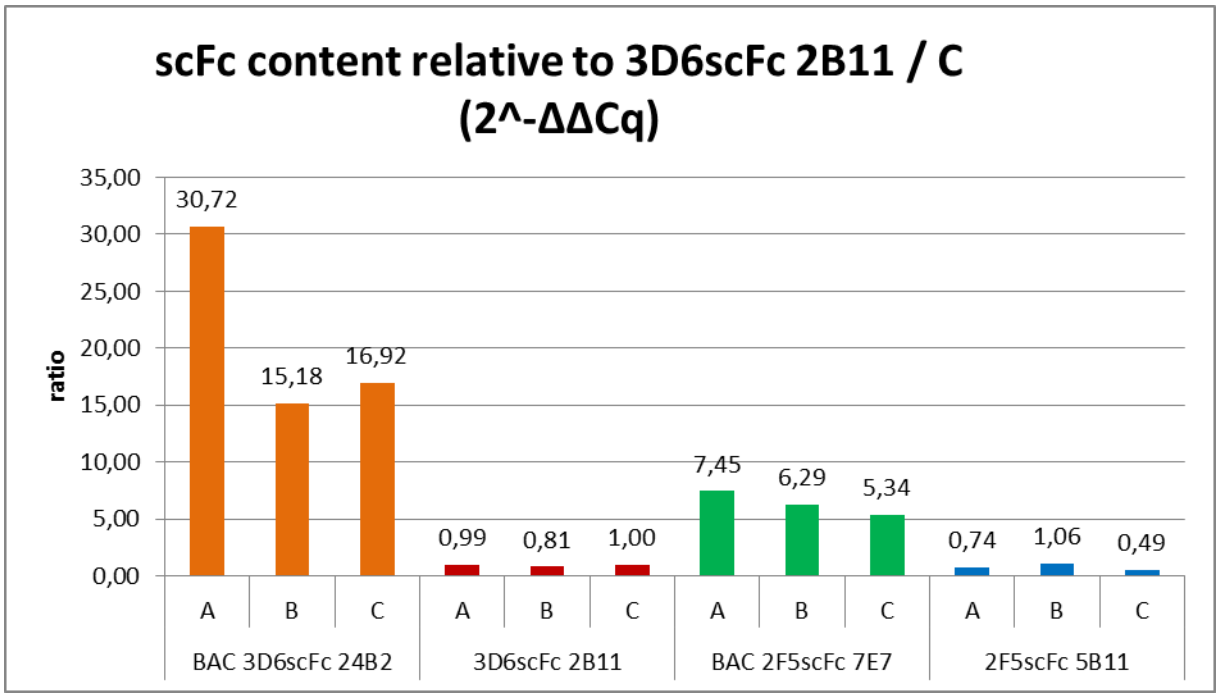

Fig. 3.33 Relative comparison of transcript amounts related to 3D6scFc 2B11 / C

3.7 Statistical Analysis (qPCR)

3.7.1 Input Data

At first, mean values were calculated for the ß-actin Cqs of each sample. For each measurement of scFc, the -ΔCq was obtained according to Equ. 2.10 with the mean ß-actin Cq value. An example for BAC 2F5scFc cell line of the cDNA runs is depicted in Tab. 3.9 and Tab. 3.10. The –ΔCq values, as presented in Tab. 3.10, were calculated for all cell lines and used as input data for STATGRAPHICS. A complete list and graphical visualisation of variations can be found in chapter 6.3.

Tab. 3.9 Cq values of BAC 2F5scFc measurements with cDNA

Sample	Cq values			
	scFc P1	scFc P2	ß-actin P1	ß-actin P2
BAC 2F5scFc Sample A	19.17	19.18	21.81	21.76
	19.04	19.08	21.66	21.66
	19.05	19.13	21.73	21.85
BAC 2F5scFc Sample B	20.45	20.51	22.90	22.81
	20.40	20.35	22.80	22.83
	20.38	20.42	22.76	22.75
BAC 2F5scFc Sample C	21.48	21.46	23.72	23.66
	21.40	21.35	23.44	23.53
	21.32	21.37	23.40	23.57

P1 = first qPCR plate, P2 = second qPCR plate

Tab. 3.10 BAC 2F5scFc values used for statistical analysis

Sample	ß-actin mean Cq	-ΔCq P1	-ΔCq P2
BAC 2F5scFc Sample A	21.74	2.57	2.56
		2.71	2.66
		2.70	2.61
BAC 2F5scFc Sample B	22.81	2.36	2.30
		2.41	2.46
		2.43	2.39
BAC 2F5scFc Sample C	23.55	2.07	2.09
		2.15	2.20
		2.23	2.18

P1 = first qPCR plate, P2 = second qPCR plate

3.7.2 gDNA Results

An overview of the distribution of –ΔCq values for the different cell lines is presented in the scatterplot (Fig. 3.34). Thereby, the three biological samples for each cell line are grouped together. For all cell lines, the distribution is within a –ΔCq range of 0.6 to 1.1, with BAC 2F5scFc having the lowest and BAC 3D6scFc the highest variation between the values. A –ΔCq increase of one results in a doubling of the relative scFc amount ($2^{-\Delta Cq}$).

The three separated value groups for BAC 3D6scFc result from the different biological samples and therefore, the individual results are spread within a range of 2.1 to 4.0 (chapter 3.5). This variation is discussed in chapter 4.3.2. For the other cell lines, groups resulting from the different samples cannot be clearly distinguished.

In the Box-and-Whisker plot, mean, median, quartiles and extremes are graphically presented (Fig. 3.35). In a normal distribution, mean and median have the same value. For all measured cell lines, derived –ΔCq values had a small deviation between mean and median over measured samples (Fig. 3.36). No outliers were found.

A multiple range test was performed to examine if the means were significantly different from each other on a 95% least significant difference (LSD) interval (chapter 6.4.1). BAC 3D6scFc was significantly different from all other cell lines and difference

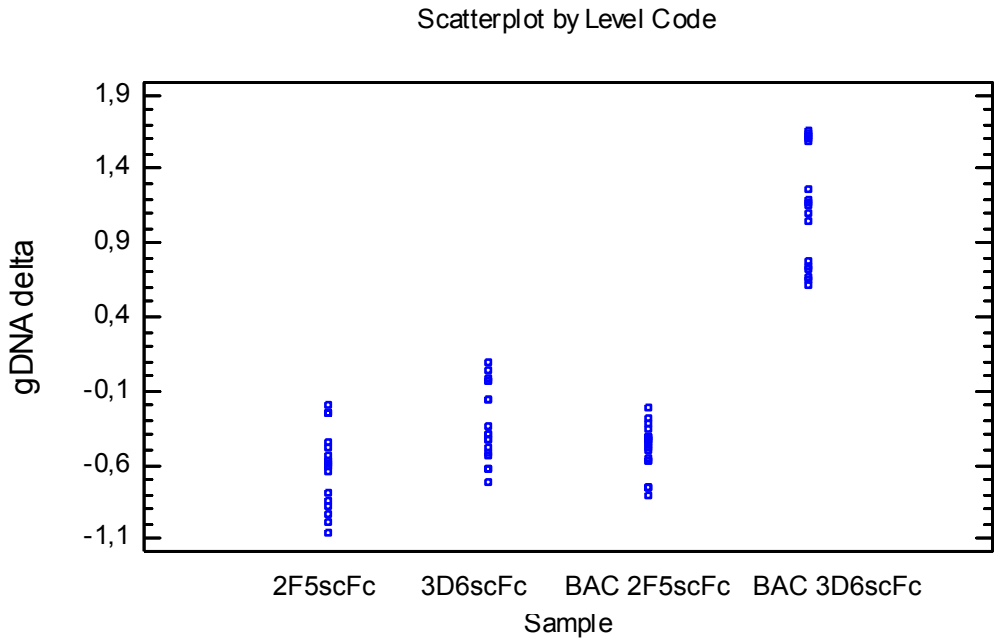

Fig. 3.34 Scatterplot of gDNA –ΔCq values

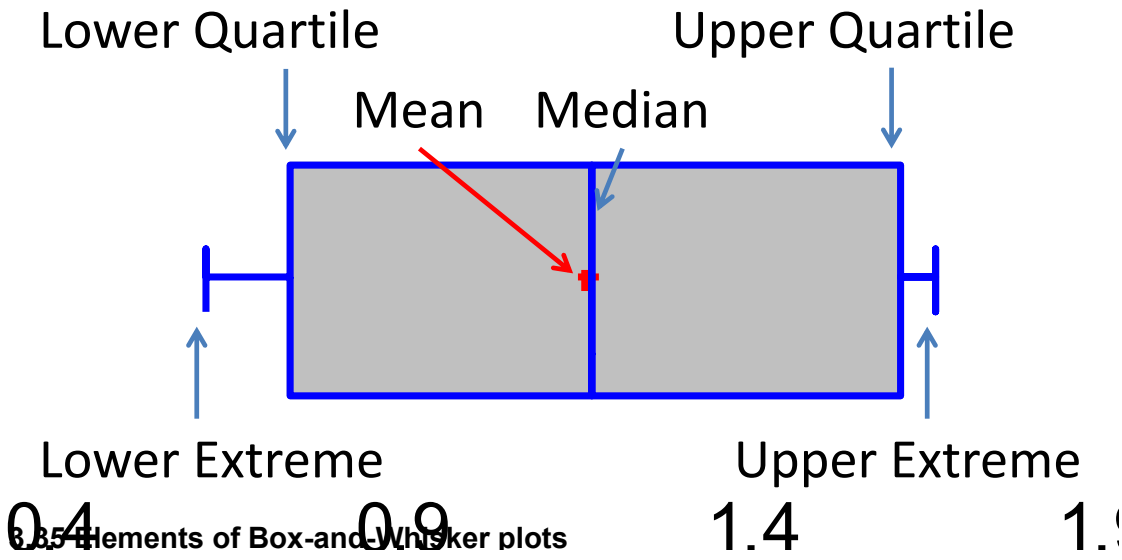

Fig. 3.35 Elements of Box-and-Whisker plots

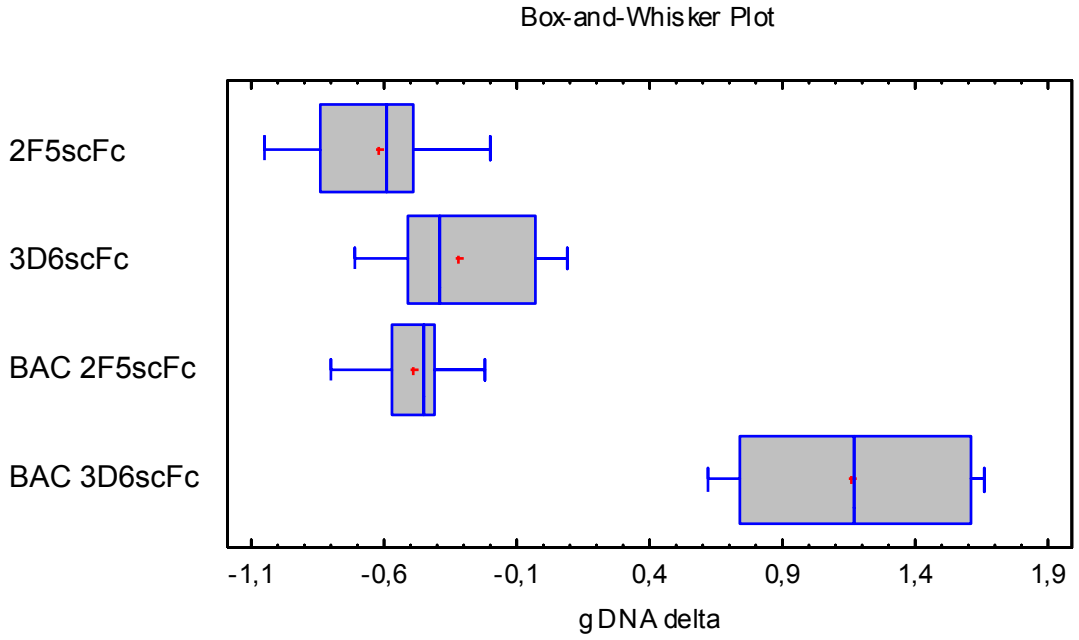

Fig. 3.36 Box-and-Whisker plot of gDNA –ΔCq values

3.7.3 cDNA Results

For cDNA, the –ΔCq values are spread over a range of approximately 0.5 (for 3D6scFc) to 1.5 (for BAC 3D6scF) (Fig. 3.37). The individual biological samples cannot be clearly distinguished, except for plasmid transfected 2F5scFc (chapter 4.3.2).

Only minor differences between medians and means were observed and BAC 2F5scFc values were considered as an optimal distribution for qPCR results (Fig. 3.37). Correlation of median and mean, centred between the quartiles, indicates an

optimal distribution, which had small variations between biological or technical replicates. For no cell line, outliers were found.

The multiple range tests (chapter 6.4.1) resulted in significant differences between

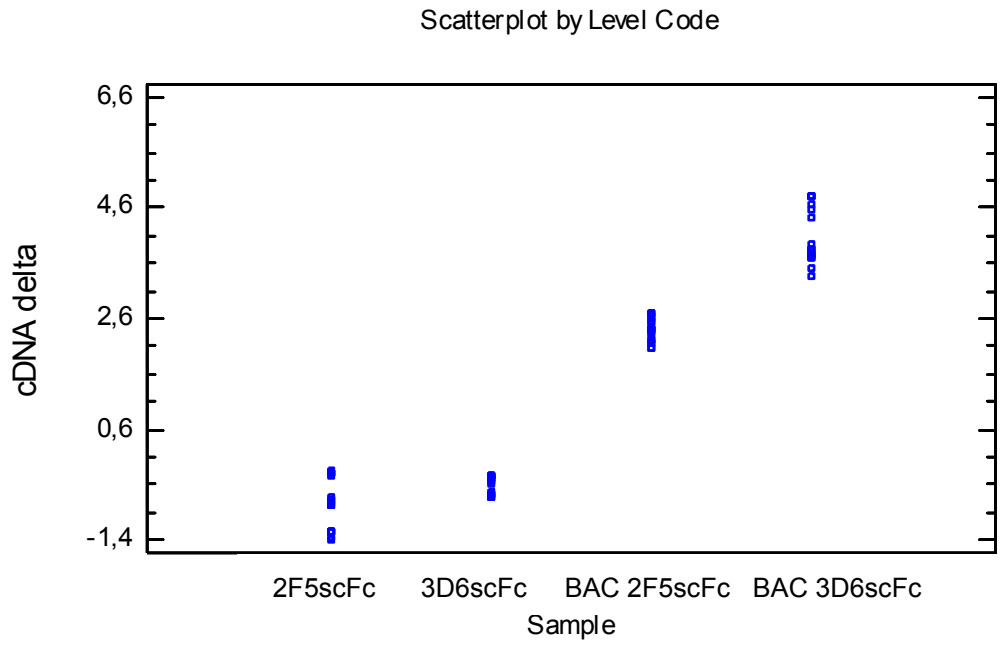

Fig. 3.37 Scatterplot of cDNA –ΔCq values

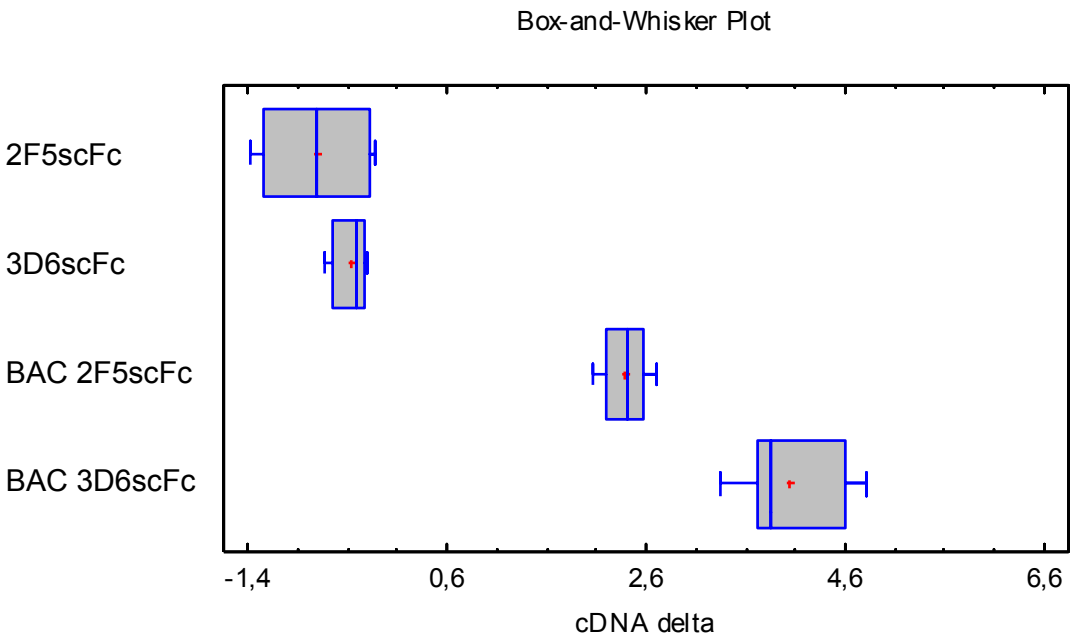

Fig. 3.38 Box-and-Whisker plot of cDNA –ΔCq values

3.8 Correlation of analyses

For relating the results with each other, means and standard deviations were calculated for each analysis and cell line (Tab. 3.11). Hence, a relative comparison between the transfection methods (Fig. 3.39) and different products (Fig. 3.40) was established by calculating the quotients of the denoted cell lines. For example, the value 3 indicates a three-fold increase in BAC over plasmid transfectants (Fig. 3.39) or 3D6scFc over 2F5scFc producers (Fig. 3.40), respectively.

Additionally, the transcript amount, qp and GCN were related to each other (Fig. 3.41). Standard deviations (Fig. 3.39 - Fig. 3.41) were propagated by the law of error propagation (Doerffel, 1990).

The correlations are discussed in chapter 4.2.

Tab. 3.11: Means and standard deviations (SD) of conducted analyses

		BAC 3D6scFc 24B2	**3D6scFc 2B11**	**BAC 2F5scFc 7E7**	**2F5scFc 5B11**
µ [1/d]	Mean	0.37	0.49	0.37	0.57
	SD	0.05	0.06	0.08	0.06
qp [µg/(d*10^6 cells)]	Mean	20.13	5.12	4.90	1.73
	SD	2.30	1.52	1.82	0.17
Intracell. Prod. [FIU]	Mean	18.26	15.21	22.35	7.31
	SD	2.39	0.41	4.45	0.95
GCN ($2^{-\Delta Cq}$)	Mean	2.31	0.81	0.72	0.66
	SD	0.74	0.16	0.07	0.12
Transcript ($2^{-\Delta Cq}$)	Mean	17.46	0.78	5.30	0.64
	SD	7.10	0.09	0.88	0.24

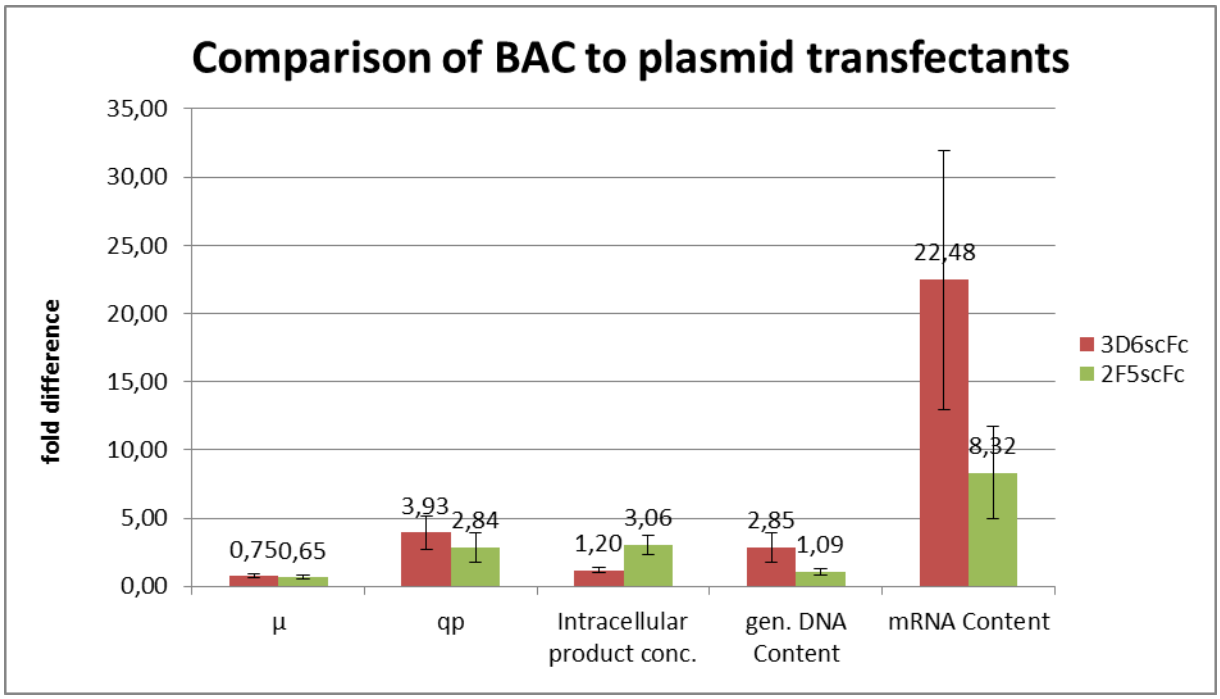

Fig. 3.39 Relative comparison of BAC to plasmid cell lines
Mean values of cell line characteristics are related from BAC to plasmid transfectants.

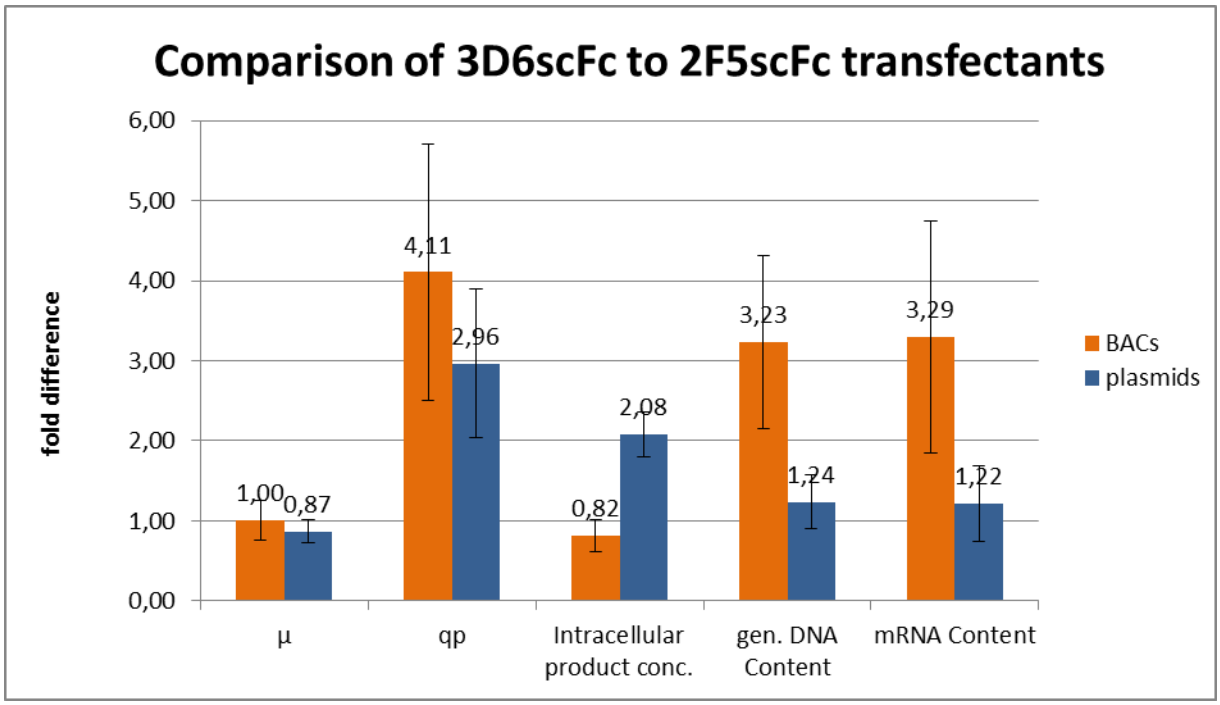

Fig. 3.40 Relative comparison of 3D6scFc to 2F5scFc cell lines
Mean values of cell line characteristics are related from 3D6scFc to 2F5scFc transfectants.

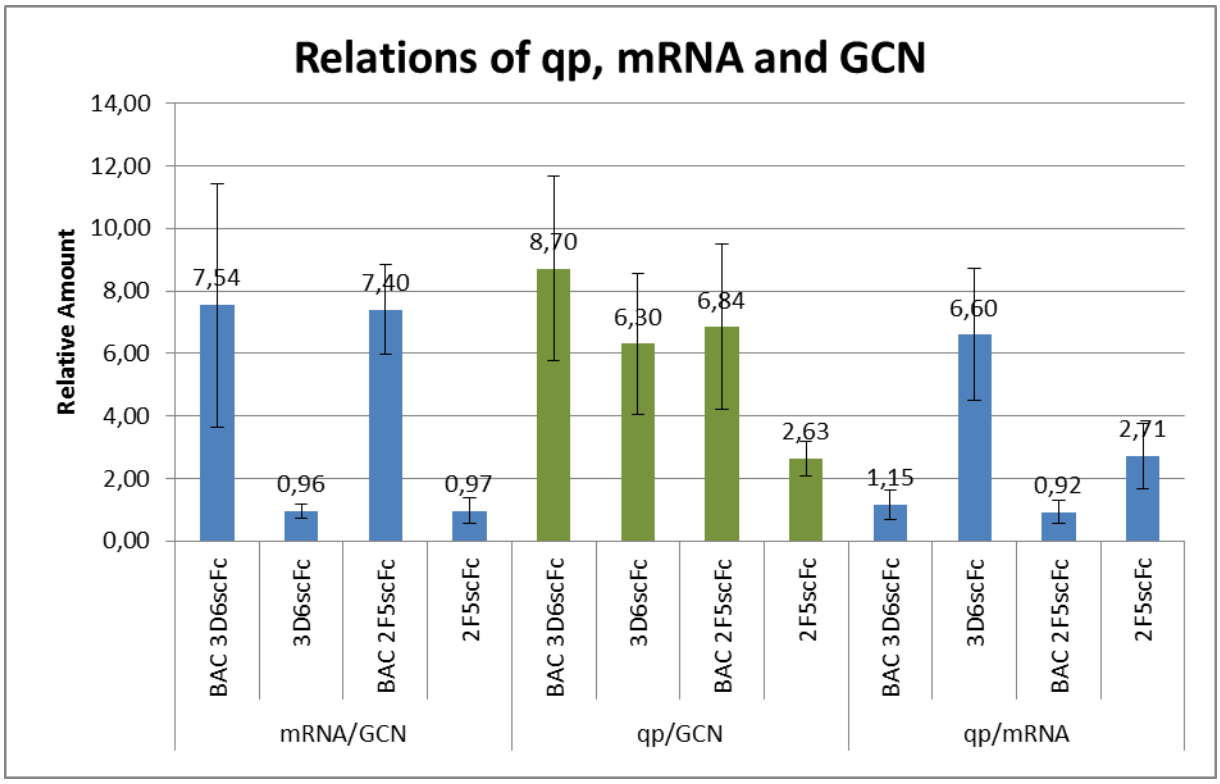

Fig. 3.41 Relations of cell line characteristics
Mean values of cell line characteristics are related to each other.

4 Discussion

4.1 Cell culture

Within this project, it was possible to obtain stable cell lines producing 3D6scFc and 2F5scFc antibodies. Co-transfection with pCAGGS and DHFR plasmids was successful and the best producing clones were selected from initially 480 wells in a three-step process. After screening of grown wells, the twelve best clones for each antibody were monitored in T25 flasks and the two best clones were further cultivated to select one best clone for additional experiments.

The best plasmid transfectants in selection medium (without MTX amplification) and best BAC transfectants were cultivated in spinner flasks over ten passages. Specific growth rates and end cell concentrations before splitting were increasing for the plasmid derived cell lines over the monitoring phase of ten passages (Fig. 3.18). One possible reason for this increase is adaption to growth in spinner flasks. BAC transfectants were already cultivated in these flasks, but plasmid transfectants were cultivated in static cultures (microtiter plates, RO-flasks) before. Adaption to increased nutrient and oxygen supply in agitated culture could be the explanation for the increase in μ. Moreover, also inhomogeneous sampling would influence the calculated cell concentrations. Due to high cell densities, derived samples might contain cell aggregates or accumulated cells. Although effort was put on homogenization of the cell suspension and taking a representative sample (not directly from the bottom of the flasks, as cells sink down due to gravity), there could still be influence due to high cell concentrations. Thereby, derived concentrations could be biased to even higher values.

For a project with a longer timeframe, it would be beneficial to further monitor these cell lines until they reach their maximum specific growth rates and assess the stability over time.

Specific productivity was stable for all cell lines (Fig. 3.22), except for BAC 2F5scFc, a slight decrease could be noticed. If this tendency continues, subcloning might be considered.

Gene amplification with an initial concentration of 0.048 μM MTX and subsequent increase to 0.096 μM MTX was successful. Because of this treatment, the cell lines became heterogeneous and subcloning was essential for deriving a high producing

clone. Within this study, no further evaluations of amplified clones could be performed.

4.2 Potential of BAC transfection

For obtaining an overview of cell line characteristics, relative comparison between the cell lines was considered (Fig. 3.39, Fig. 3.40). Both BAC transfectants exhibited a similar specific growth rate, which was below growth rates of plasmid transfectants. Every production cell line has to portion provided energy in a part used for cell maintenance and cell growth and another part used for product generation. Therefore, high producers are often not growing as fast as low producers. Plasmid 2F5scFc was growing with highest μ but had the lowest productivity.

BAC transfectants exhibited a three- to four-fold increase in specific productivity compared to plasmid transfectants. This was observed for two different antibodies, which were independently transfected. In HEK 293 cells, even an eleven-fold increase was reported for the less complex Fc part as a model product (Blaas et al., 2009). These values indicate the high potential of BAC transfection compared to conventional plasmid transfection with the same expression cassettes.

Gene copy numbers and transcript amounts were determined to assess the reasons for the qp increase in BAC transfectants. For 2F5scFc, GCN was approximately at the same level, but transcript amounts were eight-fold higher in BAC transfectants. For 3D6scFc, a three-fold GCN increase resulted in a 22-fold transcript increase. Comparing the two BAC transfectants with each other, an increase in GCN led to a proportional increase in transcript amount (three-fold GCN to three-fold mRNA), although different antibodies were used. Therefore, cDNA results were directly related to gDNA results to further assess the correlation (Fig. 3.41). For both BAC transfectants, the transcript quantity per one relative gene copy was seven to eight times higher than for plasmid transfectants. Thus, higher qps can possibly be related to increased transcript amounts, induced by chromosomal position effects. Thereby, qp related to relative GCN is enhanced in BAC clones. However, due to elevated transcript numbers, the qp per relative mRNA unit is lower in BAC transfectants. Therefore, an increase in mRNA does not automatically result in a direct proportional enhancement of qp in BACs compared to plasmid clones.

Probably, other factors such as translation, protein folding, modifications or secretion are the limiting factors on productivity for clones with a rather high amount of

transcript. Those could possibly have more influence on the production of rather complex products (more post translational modifications, secretion problems). This would be one explanation why, with BAC transfection, Fc productivity in HEK 293 cells could be further improved than scFc (more complex) productivity in CHO cells.

Furthermore, despite of the applied transfection method, 3D6scFc clones had a three- to four-fold higher overall qp than 2F5scFc clones, although the only differences between the two products are the variable regions and variations in codon optimization.

Flow cytometry was applied to get information about intracellular product content. The BAC 3D6scFc transfectant exhibited just a minor increase compared to the plasmid clone. Although productivity is significantly enhanced, BAC 3D6scFc might not have issues in secreting the product, as no accumulation occurs. There may even be a possibility of further improving productivity by increasing the GCN and correlated transcript amounts.

Contrarily, BAC 2F5scFc exhibited a far higher intracellular product content compared to plasmid 2F5scFc. Although having similar productivities, BAC 2F5scFc also had a higher intracellular product content than plasmid 3D6scFc, which could be an indication for accumulation of unprocessed product or problems with product secretion. Perhaps, productivity of BAC 2F5scFc can also be improved by enhancement of GCN and transcript amount, but limitations due to bottlenecks at other stages of the product development pathway might become even more pronounced. When the GCN exceeds its theoretical optimum, a further increase would stop to be beneficial and could even be harmful for the cells (for instance, stress due to high amount of transcript or unprocessed product). Without other indications, it is not possible to assess if this optimum is already reached for BAC 2F5scFc. Therefore, further studies should additionally concentrate on the pathway from translation to secretion of final product and analyze markers indicating stress (for example KDEL structures for stress with secretion from the endoplasmic reticulum (Munro and Pelham, 1987)). If available, analyzing different BAC 2F5scFc clones with varying GCN would help to better correlate GCN to qp and intracellular product content.

Additional studies with BAC transfectants should also focus on more complex products, such as for example complete IgGs, IgAs or IgMs. Because of the high cloning capacity, BACs have the potential to incorporate various chains or large constructs.

4.3 qPCR Outlook

4.3.1 Gene copy numbers

Assuming perfectly homogenous cell lines (all cells have the same copy numbers of GOI and ß-actin), no DNA breakage during extraction and storage, no measurement inaccuracies and variations, the gene copy numbers should have integer values. Therefore, a $2^{-\Delta Cq}$ value of 2 could indicate for example 2/1 or 4/2 copies of GOI/ß-actin. A $2^{-\Delta Cq}$ of 0.5 would indicate e.g. 1/2 or 2/4 copies, a value of 0.66 could refer to 2/3 or 4/6 copies of GOI/ß-actin.

Currently, CHO K1 is the only fully sequenced CHO genome (Xu et al., 2011). A BLAST (Altschul et al., 1990) of ß-actin against this genome results in 1 alignment, indicating that there is 1 copy present. However, genetic heterogeneity and continuing diversification are reported between the CHO cell lines (Wurm and Hacker, 2011). As most $2^{-\Delta Cq}$ values were below 1 (Tab. 3.7), a ß-actin copy number of 1 is less probable for the used host cell line CHO DUKX-B11 (this would mean that the GOI was integrated less than once) and therefore can be assumed to be higher. If the ß-actin gene was present 2 – 3 times in the used cell lines, BAC 3D6scFc would have approximately 4 – 6 GOI copies and all other used cell lines 1 – 2 GOI copies.

However, for more accurate determination of gene copy numbers, more information is needed. If the genome of CHO DUKX-B11 was sequenced, the accurate amount of ß-actin copies could be determined. Another approach would be the use of several internal standards (not only ß-actin, but additional housekeeping genes). Fluorescence in situ hybridization (FISH) could be performed to assess the copy numbers and additionally gain information about the loci of integration.

4.3.2 Variations between the samples

Every analysing method has a bias and variances between different measurements can occur. Nevertheless, for qPCR experiments, variation between biological replicates (different samples of same cell line) were observed to be generally higher than variations between technical replicates (multiple measurements of the same sample) (chapter 6.3). For some cell lines, these differences could be clearly distinguished in the Scatterplots (chapter 3.7), for others, variations in biological samples were overlapping with variations of technical replicates.

Higher differences between the biological samples may be due to gDNA / RNA extraction, preparation (boiling of gDNA, reverse transcription of RNA) and storage. DNA breakage in the scFc or ß-actin amplicons results in different –ΔCq values. Another possible cause of variation might be the qPCR machine and plate layout. The same sample was always analysed in the same wells and therefore inhomogeneous heating or detection in different wells would always influence the measurements in the same way. This cause of error could be investigated by analysing one sample in all wells and examining variations or excluded by randomizations in plate layout.

As there was no amplification pressure applied, it is improbable that the actual gene copy number changed within the passages of sampling. No correlation of gDNA variations with changes in μ (chapter 3.2.3), qp (chapter 3.2.2) or intracellular product concentration (chapter 3.3) were observed. Therefore, the differences between gDNA samples are unlikely to be caused by variations of the cell lines.

Theoretically, changes between different sampling dates are rather possible for transcript amounts (according to cell density, product concentration or slight cultivation changes the cells might adapt their transcriptome). Nevertheless, observed variations were comparable to gDNA results and could not be related to cell line characteristics.

In future studies, GCN and transcript amounts of a cell line could be monitored over a longer time of cultivation. With more samples, it would be further possible to comprehend tendencies and fluctuations of biological replicates in different passages. Cell lines could be tested for potential changes in transcription rate with different cultivation methods. Storage of gDNA could be further improved by restriction digestion to obtain smaller DNA fragments, which are split apart in defined regions.

Moreover, a study of GCN and transcript variations after gene amplification and subcloning would be beneficial. Regarding this study, an additional comparison to amplified plasmid transfectants would further evaluate the potential of BAC based transfection.

5 Conclusion

Within this study, successful adaption of BAC based transfection for recombinant production of scFc antibodies in CHO cells was demonstrated. Compared to conventional plasmid transfection with an identical expression cassette, specific productivities could be enhanced up to a three- to four-fold difference.

Investigation of the reasons with qPCR and flow cytometry affirmed the hypothesis, that up-regulated transcription due to chromosomal position effects is responsible for this increase. A seven- to eight-fold enhancement in mRNA levels, related to gene copy numbers, was found for BAC transfectants. Thereby, intracellular product concentrations were higher and specific growth rates lower for BAC clones. Except for BAC 3D6scFc, no major differences in gene copy numbers were observed between the cell lines.

Furthermore, for plasmid transfectants, the genes of interest were amplified and subcloned to obtain high producing clones for subsequent studies.

BACs exhibited their potential for time- and labor-efficient transfection and cell line establishment, as gene amplification is not necessary for deriving a high producer. Moreover, due to their size of more than 200 kbp, production of more complex products would benefit from the possibility to insert various large constructs into the backbones.

6 Appendix

6.1 Genetic Sequence of 3D6scFc and 2F5scFc

Primers used for qPCR are marked in grey colour and the probe in black.

6.1.1 **3D6scFv-Fc: 1497 bp**

ATGGACTGGACCTGGCGCATCCTGTTTCTGGTCGCCGCAGCCAC-
CGGTGTCCACTCCGAGGTGCAGCTGGTCGAGTCTGGCGGAGGACTGGTGCAGCCTGGCA-
GATCCCTGAGACTGTCTTGCGCCGCCTCCGGCTTCACCTTCAACGACTACGCCAT-
GCACTGGGTCCGCCAGGCTCCAGGCAAGGGCCTGGAATGGGTGTCCGGCATCTCCTGG-
GACTCCTCCAGCATCGGCTACGCCGACTCCGTGAAGGGCCGGTTCACCATCTCCCGG-
GACAACGCCAAGAACTCCCTGTACCTGCAGATGAACTCCCTGCGGGCCGAGGACATGGCCCTG-
TACTACTGCGTGAAGGGCAGGGACTACTACGACTCCGGCGGCTACTTCACCGTGGCCTT-
CGACATCTGGGGCCAGGGCACCATGGTGACCGTGTCTAGCGGCGGAGGCGGAAGTGGAGGCG-
GAGGAAGCGGAGGCGGCGGATCTGACATCCAGATGACCCAGTCCCCCTCCAC-
CCTGTCTGCCTCCGTGGGCGACAGAGTGACCATCAC-
CTGTCGGGCCTCCCAGTCCATCTCCCGGTGGCTGGCCTGGTATCAGCAGAAAC-
CTGGCAAGGTGCCCAAGCTGCTGATCTACAAGGCCTCCTCCCTGGAATCCGGCGTGCCTT-
CCCGGTTCTCCGGCTCTGGCTCCGGCACCGAGTTCACCCTGACCATCTCCAGCCTGCAGCCT-
GACGACTTCGCCACCTACTACTGCCAGCAGTACAACTCCTACTCCTTCGGCCCTGGCACCA-
AGGTGGACATCAAAGAGCCCAAGAGCTCTGACAAGACCCACAC-
CTGTCCCCCCTGCCCTGCCCCTGAACTGCTGGGCGGACCTTCCGTGTTCCTGTT-
CCCCCCAAAGCCCAAGGACACCCTGATGATCTCCCGGACCCCCGAAGTGAC-
CTGCGTGGTGGTGGACGTGTCCCACGAGGACCCTGAAGTGAAGTTCAATTGGTACGTG-
GACGGCGTGGAAGTGCACAACGCCAAGACCAAGCCCAGAGAGGAACAGTACAACTCCAC-
CTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAAGAG-
TACAAGTGCAAGGTGTCCAACAAGGCCCTGCCTGCTCCCATCGAAAAGACCA-
TCTCCAAGGCCAAGGGCCAGCCCCGCGAGCCTCAGGTGTACACCCTGCCTCCATCCCGG-
GACGAGCTGACCAAGAACCAGGTGTCCCTGACCTGCCTGGTCAAAGGCTTCTACCCCTCCGA-
TATCGCCGTGGAATGGGAGTCCAACGGCCAGCCCGAGAACAACTACAAGACCAC-
CCCCCCTGTGCTGGACTCCGACGGCTCATTCTTCCTGTACTCCAAGCTGACCGTG-
GACAAGTCCCGGTGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTGAT-
GCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCCCTGAGCCCCGGCAAGTGATAG

6.1.2 **2F5scFv-Fc: 1521 bp**

ATGGATTGGACTTGGAGAATCCTGTTCCTGGTGGCCGCTGCCACCGGCGTGCACTCTAGAAT-
CACCCTGAAAGAGTCCGGCCCTCCCCTCGTGAAACCCACCCAGACCCTGACACTGAC-
CTGCAGCTTCTCCGGCTTCTCCCTGTCCGATTTCGGCGTGGGCGTGGGCTGGATCA-
GACAGCCTCCTGGCAAGGCCCTGGAATGGCTGGCCATCATCTACTCCGACGACGACAAGCGG-
TACTCCCCCTCCCTGAATACCCGGCTGACCATCACCAAGGACACCTCCAAGAACCA-
GGTGGTGCTCGTGATGACCCGGGTGTCCCCTGTGGACACCGCCACCTACTTTTGCGCCCATCG-
GAGAGGCCCCACCACCCTGTTTGGAGTGCCTATCGCTCGGGGCCCTGTGAACGCCATGGAT-
GTGTGGGGCCAGGGAATTACCGTGACCATCTCCTCTGGCGGCGGAGGATCTGGGGGAGGCGG-
TAGCGGAGGTGGTGGATCTGCTCTGCAGCTGACCCAGTCCCCTT-
CCAGCCTGTCTGCCTCTGTGGGCGACCGGATCACCATCACCTGTAGAGCCTCTCAGGGCGT-
GACCTCTGCCCTGGCCTGGTACAGACAGAAGCCTGGCTCTCCACCCCAGCTGCT-
GATCTACGACGCCTCCTCCCTGGAATCTGGCGTGCCCTCCAGATT-
CTCTGGCTCCGGCTCTGGCACCGAGTTCACCCTGACAATCTCCACCCTGCGGCCCGAG-
GACTTCGCTACCTACTACTGCCAGCAGCTGCACTTCTACCCCCACACCTTTGGCGGAGGCAC-
CAGAGTGGATGTGCGCGAGCCTAAGAGCTCTGACAAGACCCACAC-
CTGTCCCCCCTGTCCTGCTCCTGAACTGCTGGGCGGACCTTCCGTGTTCCTGTT-
CCCCCCAAAGCCTAAGGACACCCTGATGATCTCCCGGACCCCCGAAGTGAC-
CTGCGTGGTGGTGGACGTGTCCCACGAGGACCCTGAAGTGAAGTTCAATTGGTACGTG-
GACGGCGTGGAAGTGCACAACGCCAAGACCAAGCCCAGAGAGGAACAGTACAACTCCAC-
CTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGATTGGCTGAACGGCAAAGAG-
TACAAGTGCAAGGTGTCCAACAAGGCTCTGCCTGCCCCCATCGAAAAGACCA-
TCTCTAAGGCCAAGGGCCAGCCCCGCGAGCCCCAGGTGTACACACTGCCTCCATCTCGG-
GACGAGCTGACAAAGAATCAGGTGTCCCTGACCTGTCTCGTGAAGGGCTTCTACCCTTCTGA-
TATCGCCGTGGAATGGGAGTCCAACGGCCAGCCTGAGAACAACTACAAGACCAC-
CCCCCCTGTGCTGGACTCCGACGGCTCATTCTTCCTGTACTCCAAGCTGACAGTG-
GACAAGTCCAGATGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTGAT-
GCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCCCTGAGCCCCGGCAAGTGATGA

6.2 Detailed plasmid maps

Plasmids with attp sites, primers and *Eco*RV restriction site (Fig. 6.1, Fig. 6.2).

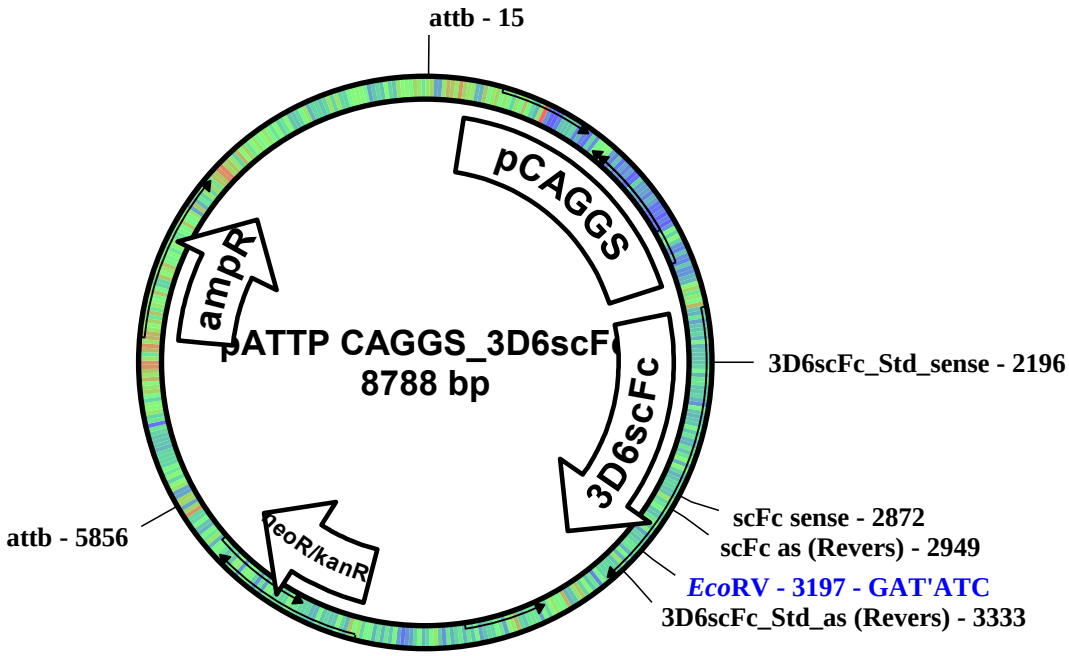

Fig. 6.1 pCAGGS 3D6scFc detailed plasmid map

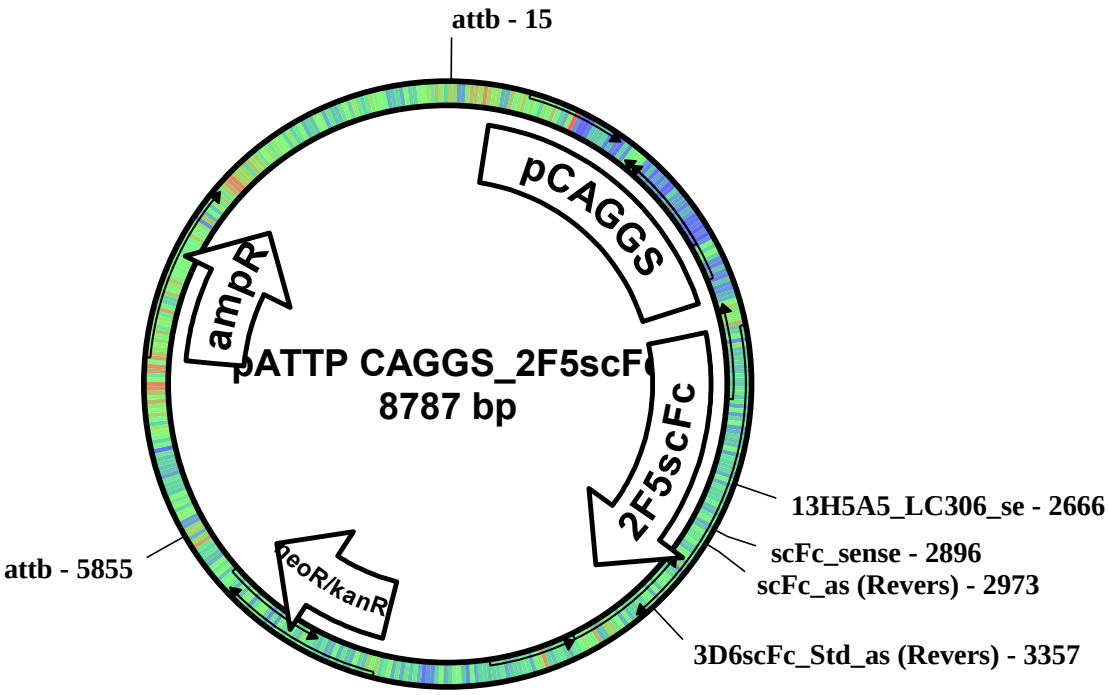

Fig. 6.2 pCAGGS 2F5scFc detailed plasmid map

6.3 qPCR –ΔCq distributions

Tab. 6.1 –ΔCq values derived from gDNA and cDNA analsis

Sample	gDNA –ΔCq		cDNA –ΔCq	
BAC3D6scFc/24B2/A	1,11	1,05	4,43	4,59
	1,2	1,26	4,64	4,8
	1,15	1,18	4,8	4,8
BAC3D6scFc/24B2/B	0,66	0,74	3,35	3,53
	0,62	0,72	3,72	3,76
	0,67	0,78	3,83	3,77
BAC3D6scFc/24B2/C	1,59	1,66	3,72	3,72
	1,62	1,61	3,94	3,86
	1,65	1,63	3,85	3,82
BAC2F5scFc/7E7/A	-0,44	-0,35	2,57	2,56
	-0,41	-0,32	2,71	2,66
	-0,41	-0,22	2,7	2,61
BAC2F5scFc/7E7/B	-0,58	-0,5	2,36	2,3
	-0,49	-0,42	2,41	2,46
	-0,29	-0,43	2,43	2,39
BAC2F5scFc/7E7/C	-0,75	-0,56	2,07	2,09
	-0,76	-0,57	2,15	2,2
	-0,8	-0,47	2,23	2,18
3D6scFc/2B11/A	-0,16	-0,03	-0,38	-0,33
	-0,03	-0,01	-0,24	-0,27
	0,04	0,09	-0,21	-0,24
3D6scFc/2B11/B	-0,71	-0,53	-0,62	-0,52
	-0,62	-0,39	-0,6	-0,57
	-0,62	-0,51	-0,56	-0,57
3D6scFc/2B11/C	-0,49	-0,33	-0,27	-0,22
	-0,43	-0,15	-0,27	-0,33
	-0,4	-0,42	-0,24	-0,25
2F5scFc/5B11/A	-0,44	-0,2	-0,76	-0,62
	-0,6	-0,24	-0,75	-0,73

	-0,49	-0,25	-0,66	-0,64
2F5scFc/5B11/B	-0,64	-0,59	-0,17	-0,17
	-0,6	-0,54	-0,24	-0,17
	-0,57	-0,58	-0,13	-0,18
2F5scFc/5B11/C	-0,98	-0,79	-1,38	-1,23
	-0,93	-0,87	-1,24	-1,26
	-1,05	-0,84	-1,35	-1,26

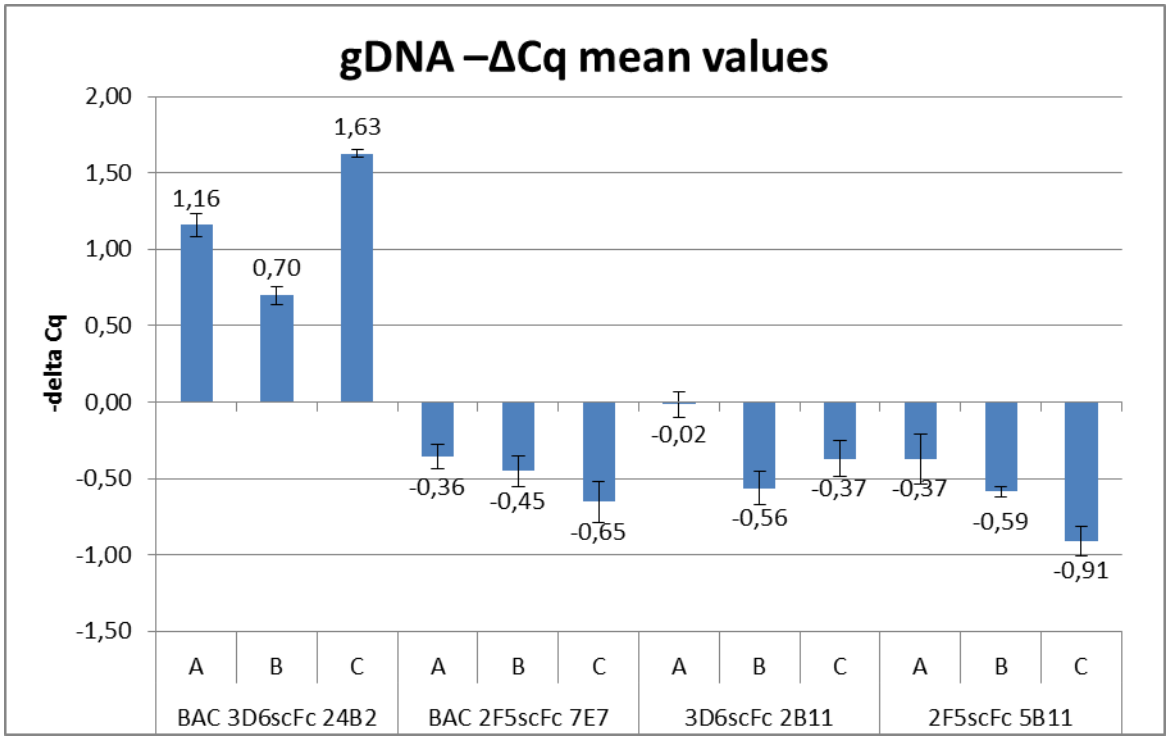

Fig. 6.3 Means and standard deviations for gDNA analysis

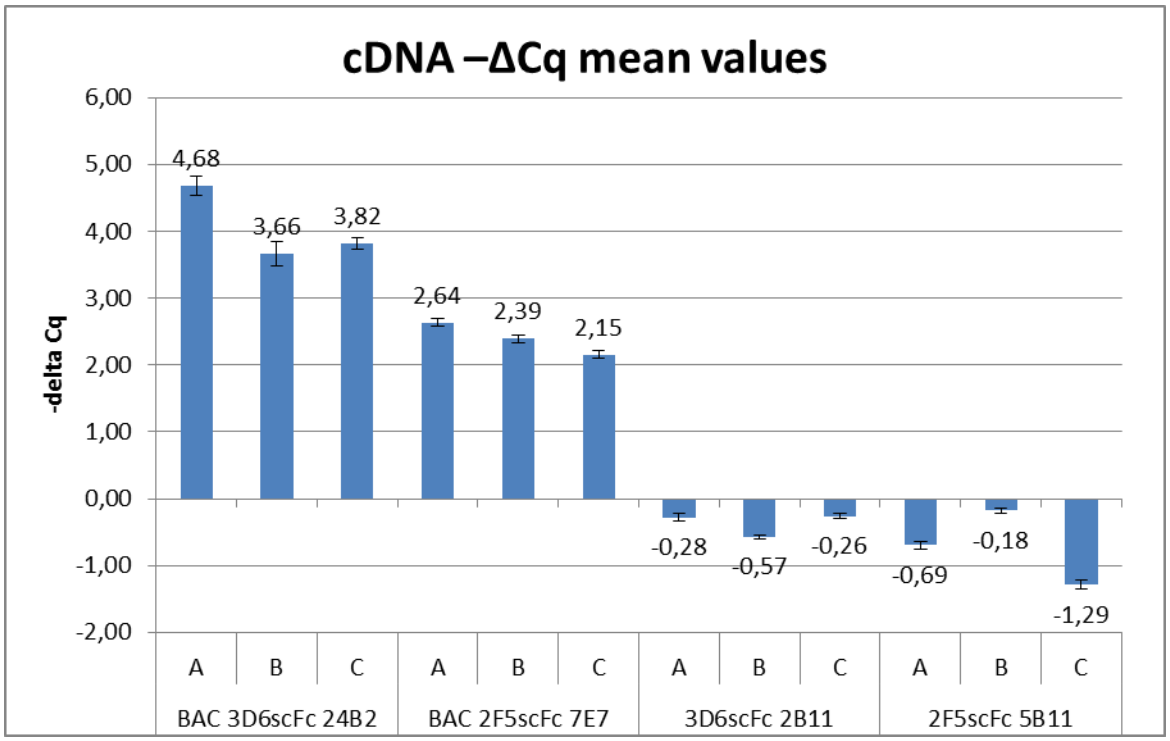

Fig. 6.4 Means and standard deviations for cDNA analysis

6.4 Statistical analysis

Tab. 6.2 Sample Codes

Code	Cell line
2	2F5scFc / 5B11
3	3D6scFc / 2B11
22	BAC 2F5scFc / 7E7
33	BAC 3D6scFc / 24B2

6.4.1 **gDNA**

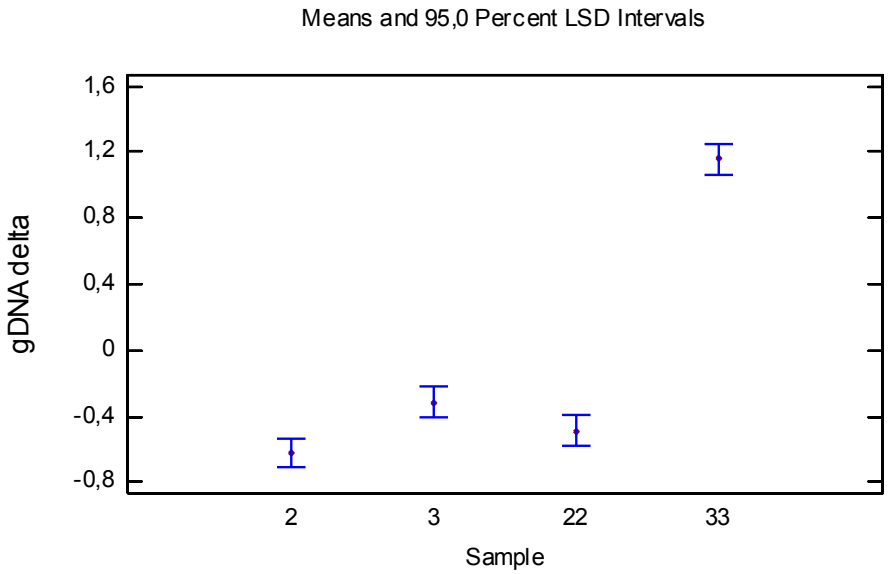

Fig. 6.5 gDNA –ΔCq means with 95% least significant difference intervals

Tab. 6.3 Multiple Range Tests for gDNA –ΔCq on a 95% LSD interval

Sample	Count	Mean	Homogeneous Groups
2	18	-0,622222	X
22	18	-0,487222	XX
3	18	-0,316667	X
33	18	1,16111	X
Contrast	**Sig.**	**Difference**	**+/- Limits**
2 - 3	*	-0,305556	0,184561
2 - 22		-0,135	0,184561
2 - 33	*	-1,78333	0,184561
3 - 22		0,170556	0,184561
3 - 33	*	-1,47778	0,184561
22 - 33	*	-1,64833	0,184561

* denotes a statistically significant difference.

6.4.1 cDNA

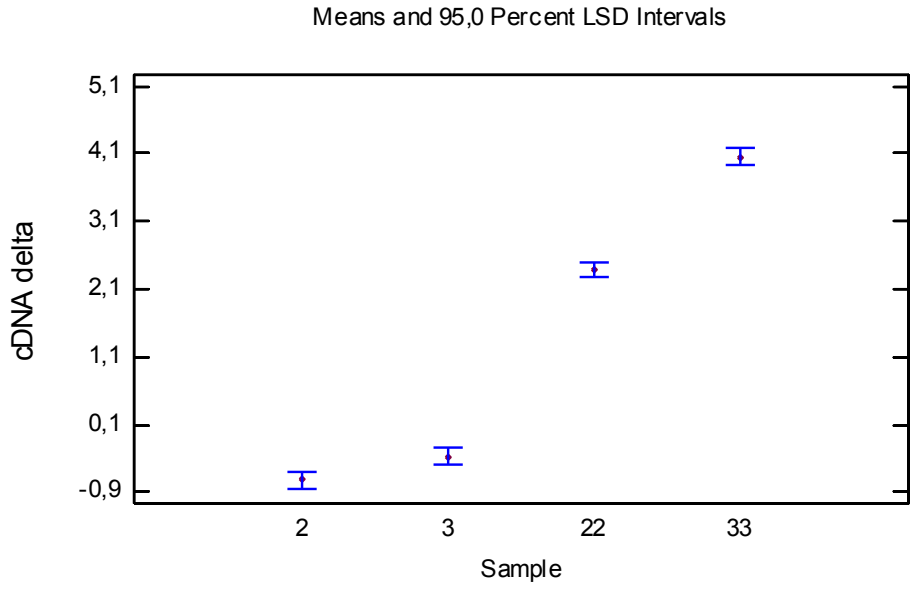

Fig. 6.6 cDNA –ΔCq means with 95% least significant difference intervals

Tab. 6.4 Multiple Range Tests for cDNA –ΔCq on a 95% LSD interval

Sample	Count	Mean	Homogeneous Groups
2	18	-0,718889	X
3	18	-0,371667	X
22	18	2,39333	X
33	18	4,05167	X
Contrast	**Sig.**	**Difference**	**+/- Limits**
2 - 3	*	-0,347222	0,23942
2 - 22	*	-3,11222	0,23942
2 - 33	*	-4,77056	0,23942
3 - 22	*	-2,765	0,23942
3 - 33	*	-4,42333	0,23942
22 - 33	*	-1,65833	0,23942

* denotes a statistically significant difference.

7 Statutory Declaration

(Eidesstattliche Erklärung)

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

(Bernhard Prewein, July 2012)

8 Curriculum Vitae

Bernhard Prewein

* 5. Februar 1987 in Wien
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Ausbildung

10/2009-heute	**Masterstudium** der Biotechnologie an der Universität für Bodenkultur, Wien
10/2011-05/2012	**Masterarbeit** am Institut für angewandte Mikrobiologie bei Prof. Dr. Kunert und Dr. Mader zum Thema: *BAC Based vs. Conventional Plasmid Transfection - A Comparison Study in CHO Cells*
12/2009-06/2010	**Auslandssemester** an der Wageningen University and Research centre, Niederlande, im Rahmen des ERASMUS Programms
10/2006-10/2009	**Bachelorstudium** der Lebensmittel- und Biotechnologie an der Universität für Bodenkultur, Wien Abschluss als Bakk. techn., Schnitt 1.6
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07/2005-09/2006	**Präsenzdienst** bei der Gardemusik, Maria Theresien-Kaserne, Wien
09/1997-06/2005	**Konrad Lorenz Gymnasium**, Gänserndorf Matura mit ausgezeichnetem Erfolg

Praktische Tätigkeiten

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07/2009-08/2009	**Marchfelder Storchenbräu,** Untersiebenbrunn, bei DI Bauer: Produktion von Bier und Softdrinks, Wasseruntersuchungen und Charakterisierung von Hefen
08/2008-09/2008	**AGES,** Mödling, bei Dr. Eichinger: Serologische Arbeiten, Antikörpernachweise und ELISA
07/2003, 07/2004	**Leithäusl,** Schönkirchen-Reyersdorf, bei Ing. Weindl: Vermessungen, Abrechnungen und EDV

Kenntnisse

Sprachen	Deutsch – Muttersprache Englisch – fließend in Wort und Schrift (Univ. of Cambridge, FCE) Französisch, Latein - Maturaniveau
EDV	MS Office, OpenOffice, LaTex Bioinformatic Tools (u.a. BLAST, Primer3, OligoCalc, LinRegPCR) CorelDRAW, Photoshop, GIMP Linux, Netzwerktechnik, Hardware
Führerschein	Klasse B

Zusatzqualifikationen und Stipendien

Auszug Wahlfächer	Molekularbiologische Arbeitsmethoden (in Eng.), Tierische Zellkultur (in Eng.), Bioprozesstechnik (in Eng.), Cell Biology (in Eng.), Zellbiologische Übungen, Kältetechnik und Kühllagerung, Energie- und verfahrenstechnisches Seminar, Energietechnisches Praktikum, Umwelttechnisches Praktikum, Instrumentelle Analytische Chemie, Chemisches Rechnen
Stipendien	Top-Stipendium des Landes Niederösterreich, 2010 Stipendium des Siegfried Ludwig-Fonds, 2010 Leistungsstipendium der Univ. für Bodenkultur, 2009

Interessen

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9.2 Figures

Fig. 1.1 Antibody structures: From IgG to scFv-F:

Modified excerpt from the publication *Production systems for recombinant antibodies* (Schirrmann et al., 2008)