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Evaluation of recombinase mediated cassette exchange for recombinant protein production in CHO cells

Master Thesis

submitted by

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Abstract

Recombinant antibodies are the most important class of biopharmaceuticals. They are commonly produced in recombinant Chinese Hamster Ovary (CHO) cells. Traditional concepts for the creation of such cell lines rely on the random integration of the plasmid, containing the gene of interest, in the genome. To preselect a transcriptionally active genomic locus prior to transfection the recombinase mediated cassette exchange (RMCE) was developed. The recombinase Flipase recognises distinct DNA sequences and by the use of heterospecific recognition sites a cassette exchange is possible. Within this study, the two anti HIV-1 antibodies 2F5 and 3D6, both designed as single chain Fv-Fc (scFc) variant, were produced and compared by this technique. Therefore a RMCE competent CHO host cell line was cotransfected with either 2F5scFc or 3D6scFc and the enzyme Flipase. The twelve best producing clones of each scFc producing cell line were grown in T25 flask and the two best producers of each were selected for cultivation in spinner flasks. Those four cell lines were intensively characterised and special emphasis was laid on the detailed investigation of the RMCE. This characterisation was done by FISH and quantitative real time PCR. It turned out, that one cell line harboured an additional transgene copy, although the three other cell lines had the same gene copy number as the cell line prior to cassette exchange. Another difference was the higher specific productivity of the 3D6scFc producing cell lines, although the transcript level, specific growth rate and the metabolism remained almost the same in all scFc producing cell lines. Only the intercellular product formation was slightly higher for 3D6scFc producing cell lines than for 2F5scFc producing cell lines, but not in the dimension of the specific productivity. This study demonstrated the ability of RMCE for recombinant protein production and also the influence of the product itself on the specific productivity.

Zusammenfassung

Rekombinante Antikörper zählen zu den wichtigsten Biopharmazeutika. Diese werden häufig in rekombinanten Ovarienzellen des Chinesischen Goldhamsters produziert. Die traditionelle Entwicklung einer solchen Zelllinie basiert auf der zufälligen Integration eines Plasmid mit dem Gen für das rekombinante Protein in das Genom. Um jedoch das Gen gezielt an einem hochaktiven Ort im Genom zu integrieren, wurde der Rekombinase vermittelte Kassettenaustausch (RMCE) entwickelt. Dabei erkennt die Rekombinase Flipase bestimmte DNA Sequenzen und ermöglicht durch die Verwendung von heterospezifischen Erkennungsstellen einen Kassettenaustausch. In der vorliegenden Arbeit wurden die beiden Anti-HIV-1 Antikörper 2F5 und 3D6, beide als sc Fv-Fc (scFc) Varianten, mit dieser Technik produziert. Dafür erfolgte eine Kotransfektion der RMCE kompetenten Zelllinie mit 3D6scFc oder 2F5scFc und dem Enzyms Flipase. Die zwölf bestproduzierenden Klone je scFc Zelllinie wurden in T25 Flaschen kultiviert und jeweils die beiden höchstproduzierenden Klone in Spinnnerflaschen weiterkultiviert. Die so entstandenen vier Zelllinien wurden unter besonderer Berücksichtigung des Kassettenaustausches intensiv mittels FISH und quantitativer PCR charakterisiert. Dadurch zeigte sich, dass eine Zelllinie eine zusätzliche Transgenkopie aufwies, während alle anderen Ziellinien die gleiche Anzahl an Transgenkopien vor und nach dem Kassettenaustausch hatten. Außerdem wurde eine höhere spezifische Produktivität der Zelllinien für 3D6scFc ermittelt, obwohl die spezifische Wachstumsrate, der Metabolismus und die Transkriptmenge in all den Zelllinien annähernd ident waren. Nur die interzelluläre Produktbildung zeigte zwischen 3D6scFc und 2F5scFc produzierenden Zellen Unterschiede, nicht jedoch in dem Maße wie die spezifische Produktbildungsrate. Die durchgeführte Arbeit bestätigt die Möglichkeit von RMCE für die rekombinante Proteinexpression sowie den Einfluss des exprimierten Proteins auf die spezifische Produktivität.

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1. Abbreviations and Units

1.1. Abbreviations

ampR	Ampicillin resistance gene (β- Lactamase)
BLAST	Basic local alignment search tool
bp	base pairs
BSA	bovine serum albumine
BX	Bromphenolblue - Xylencyanol
Cat. No.	Catalogue Number
cDNA	complementary DNA
CHO	Chinese hamster ovary cells
CIP	Calf intenstine phosphatase
CMV	Cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	deoxy- Nucleotides (mixture of ATP, TTP, GTP and CTP)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked imuno sorbent assay
FAM	6-Carboxyfluorescein
Fc	fragment crystallisable
FCS	Fetal calf serum
FISH	Fluorescence in situ hybridisation
FITC	Flurorescein isothiocynate
FI.	Fluorescence
Flp	Flipase
FRT	Flipase recognition target
FS	Forward Scatter
GCN	Gene copy number
GFP	Green fluorescent protein
Glc	Glucose
GOI	Gene of interest
gp41	glycoprotein 41

GTN	GFP, thymidine Kinase, neomycin phosphotransferase
HBS	HEPES buffered saline
HT	Hypoxanthine and Thymidine
IgG	Immunoglobulin G
kbp	kilo base pairs (1000 base pairs)
L	Ladder (for agarose gel electrophoresis)
Lac	Lactate
M-MLV	Moloney Murine Leukemia Virus
neo^R	Neomycin phosphotransferase
OPD	ortho-Phenylenediamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-Phycoerythrin
PEI	Polyethylenimine
qPCR	quantitative real time polymerase chain reaction
RMCE	recombinase mediated cassette exchange
RNA	Ribonucleic acid
RO-water	Reverse osmosis purified water
rpm	rounds per minute
S/MAR	Scaffold/Matrix attachment regions
scFc	single chain Fragment variable-Fc
SS	Side scatter
TAMRA	Carboxytetramethylrhodamine
TK	Herpes simplex thymidine kinase
Tm	Melting temperature

1.2. Units

V	Volt
kV	Kilo Volt (10^3 V)
l	Liter
ml	Milliliter (10^{-3} L)
µl	Mikroliter (10^{-6} L)
g	Gramm
mg	Milligramm (10^{-3}g)
µg	Mikrogramm (10^{-6} g)

ng	Nanogramm (10^{-9} g)
pg	Pikogramm (10^{-12} g)
M	Molarity (mol/l)
mM	Millimolar (10^{-3} M)
µM	Mikromolar (10^{-6} M)
nM	Nanomolar (10^{-9} M)
g	relative centrifugal force
°C	degree Celsius
Ω	Ohm
µF	Microfarad (10^{-6} F)
u	Units
nm	Nanometer (10^{-9} m)
h	Hours
min	Minutes
d	Days
kDa	Kilodaltons (10^{3} Daltons)
rfu	relative fluorescence units
OD	optical density
% w/v	Percentage weight of volume
% w/w	Percentage weight of weight
% v/v	Percentage volume of volume

2. Introduction

The market for biopharmaceuticals is dominated by the production of monoclonal antibodies. (WALSH, 2010). Due to their complex structure and a certain glycosylation pattern, the production of these molecules relies on the use of mammalian cell culture. The most widely used cells therefore are Chinese Hamster Ovary (CHO) cells. (BUTLER and MENESES-ACOSTA, 2012).

CHO cells were isolated from a Chinese hamster (PUCK et al., 1958) and intensively used for recombinant protein production. There are many reasons, which make the CHO cells that popular for pharmaceutical protein production. First of all, the cells are regarded as save, because they are accepted since 20 years by the regulatory authorities for the production of biopharmaceuticals (KIM et al., 2012). Another big advantage is the easy culturing of these cells, because they can be adapted to serum free media and also to growth in suspension. This lowers on the one hand the costs for media as well as it improves the production process in terms of safety. The growth in suspension also enables an easier production process, because no mircrocarriers or other special equipment is needed in comparison to adherent mammalian cells. The use of mammalian cells further facilitates downstream process, because the cells are able to secret the product into the culture medium. For every production process a well characterised and stably transfected cell line is an important requirement.

Traditional concepts for the development of stable high producing CHO cell lines involve the transfection of cells, screening for high producers and gene amplification to further improve productivity (KIM et al., 2012). The commonly used gene amplification system relies on the dihydrofolate reductase deficient ($dhfr^-$) CHO cell lines described by (URLAUB and CHASIN, 1980). By integration of the dhfr gene into the CHO dhfr- genome the drug methotrexate can be used for gene amplification (KAUFMAN et al., 1985). The disadvantage of this technique is that the recombinant cell lines have to be screened intensively for high producing clones, because random integration of the gene of interest (GOI) will also result in insertion of GOI in transcriptionally in-active chromosomal loci. This is known as the position effect described by Wilson (WILSON et al., 1990).

Nowadays new strategies were developed to further improve the productivity of CHO cells. One approach is the transfection of large DNA vectors which provide their own transcriptionally active gene locus, like the Bacterial artificial chromosome system (BAC). The BAC system has shown to provide more specific productivity (qP) in comparison to conventional transfection strategies (MADER et al., 2013).

Another important system is the tag and exchange concept by the use of recombinase mediated cassette exchange (RMCE). In such a system, first of all a highly transcriptionally active genomic locus is selected by the transfection with a reporter gene, flanked by recognition sites for RMCE (QIAO et al., 2009). The basics for the use of such an advanced technique are the enzymes which catalyses this step (recombinases) and heterospecific RMCE sites, which enable the exchange of the reporter gene.

Different recombinases were tested for the purpose and the use for RMCE. Initially this system was developed by the use of the enzyme Flipase from yeast cells and the recognition target sites (FRT sites) for this enzyme (SCHLAKE and BODE, 1994). Also the phage derived enzyme Cre recombinase was used extensively for the purpose of RMCE, because of its higher activity. Later this enzyme turned out to trigger endonucleolytic activities inside the cell which result in cell toxicity (TURAN et al., 2011). Nowadays the Flipase based RMCE is most widely used.

2.1. Flipase based RMCE

The enzyme Flipase was originally isolated from the 2 µm plasmid of the yeast *Saccharomyces cerevisiae (S. cerevisae)* where it enables the amplification of this plasmid (FUTCHER, 1986).

The FRT sites are those sites, which are recognised by the enzyme and where the recombination takes place. Such a site consists of 48 bp, including three individual 13 bp Flipase recombinase binding elements, whereby two sites form an inverted repeat around an 8 bp spacer region. The third 13 bp Flipase recombinase binding element is separated only by a single base pair and facilitates the RMCE reaction (TURAN et al., 2011). Mutations in the inverted repeats and the spacer region allow the construction of heterospecific RMCE sites. The most suitable pair for these reactions consists of the wild type FRT site and the mutated FRT3 site (SCHLAKE and BODE, 1994). The use of this pair enables a full cassette exchange, because

only the FRT sites can recombine with each other as well as the FRT3 sites respectively. This means, that for a RMCE the vector and the preselected genome locus has to be flanked by these FRT pair, as it is shown in Figure 1 (taken from (TURAN et al., 2011)).

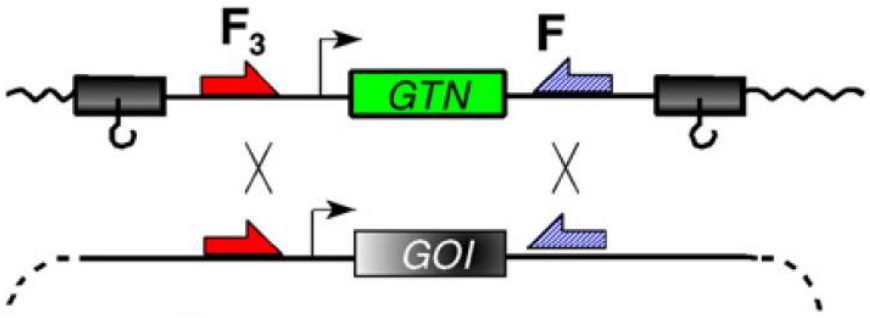


Figure 1: Scheme of RMCE by the use of heterospecific RMCE sites. The upper line represents the genome while in the lower section the plasmid is shown. The recombination event occurs between the corresponding FRT sites (abbreviated as F3 for FRT3 and F for the wild type FRT site).

The catalytic mechanism of the enzyme is based on the action of the amino acid tyrosine (Y). In Figure 2 the action of the enzyme Flipase is shown in detail for one FRT site.

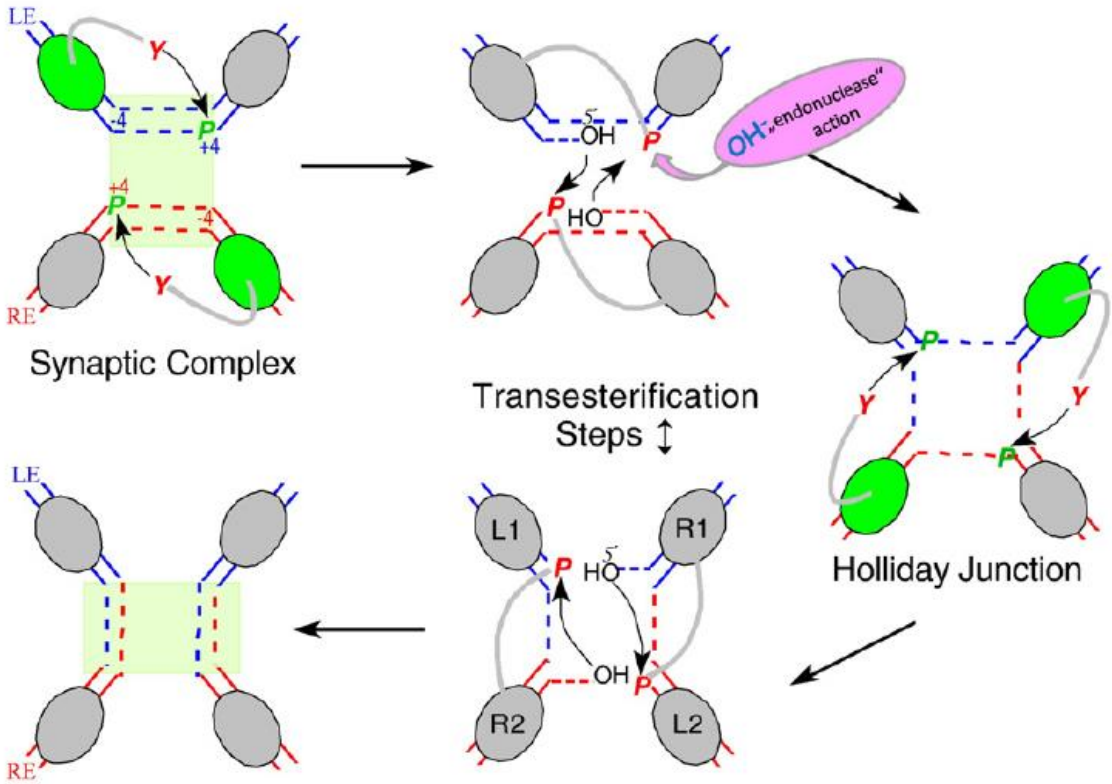


Figure 2: Detailed scheme of the Flipase enzymatic mechanism. Grey and green represents the protomers of the enzyme, where green always represents the active one. Red and blue lines represent the different DNA double strands, dashed lines the spacer region of the FRT site.

As first step, two Flipase protomers assemble at the inverted repeats of the FRT sites, one protomer at each DNA strand and together they form a synaptic complex. In such a complex, the corresponding FRT sites, e. g. the blue one in the genome and the red one in a plasmid, are located antiparallel to each other. The spacer of the FRT site is represented as dashed line. At this region the DNA is bent and exposes a phosphate residue, which is subsequently bound to the active site tyrosine of the enzyme and induces a single strand break. This step is catalysed by the two protomers in trans–position. The remaining free 5’ hydroxyl group acts as nucleophile for the trans-esterfication step to the corresponding FRT site to create a Holliday Junction intermediate. As last step now the remaining protomers of the enzyme also perform the same action which results in a complete rearrangement (TURAN et al., 2011).

3. Project Proposal

RMCE offers great possibilities for different purposes. It seems as powerful tool for the comparison of different recombinant cell lines while the influence of the position effect and the genomic locus is excluded.

Within this project, the two anti HIV-1 antibodies 2F5 and 3D6 were expressed in CHO cells by the use of RMCE. Both antibodies are specific for gp41, but only the antibody 2F5 possesses a broadly neutralising ability (KUNERT et al., 1998).

An IgG antibody is composed of two light and two heavy chains. Both chains contain variable and constant regions and are linked together via a disulphuric bond. The light chain consists of one variable and one constant domain, while the heavy chain consists of three constant domains and one variable domain. Both variable domains of the light and heavy chain are responsible for the binding to an antigen. For a better comparison, both antibodies are expressed as scFv-Fc antibodies (scFc). In such a case, the variable domains of the IgG antibody are linked via a polypeptide consisting of the amino acids glycine (G) and serine (S), designed as $(GGGGS)_3$. The variable domain of the heavy chain is still linked to two constant domains of the heavy chain. Therefore the only difference of both model proteins (3D6scFc and 2F5scFc) is the scFv region. In Figure 3 the difference in the structure between an IgG antibody and a scFc antibody is shown schematically.

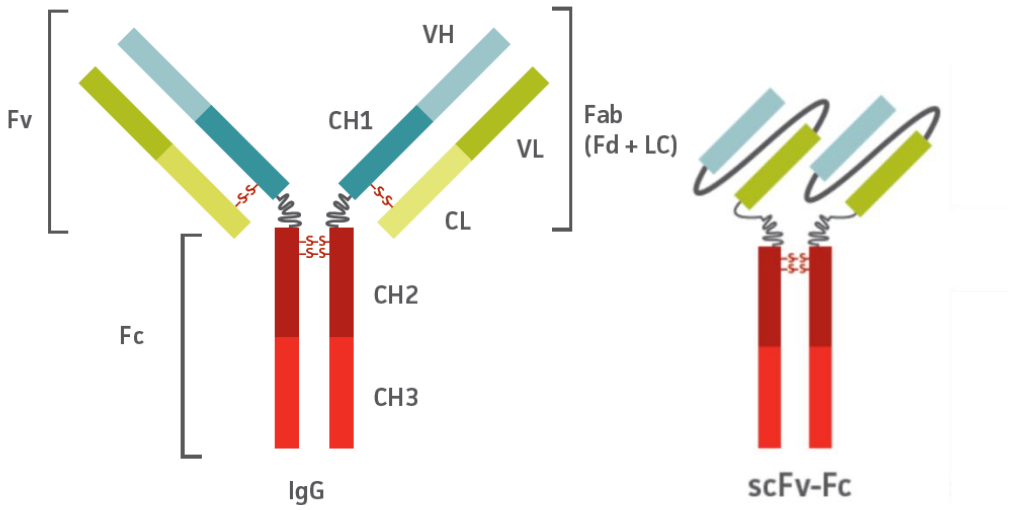


Figure 3: Differences between a human IgG and a ScFc antibody.

As previous studies indicate (MADER et al., 2013), both scFc antibodies show a difference in qP although the majority of their amino acids are the same. This phenomenon should now be investigated by the use of RMCE. Therefore an RMCE competent cell line will be used, which contains retargetable RMCE sites at a transcriptionally active chromosomal locus (MAYRHOFER, 2013). Within this locus a fusion gene containing the green fluorescence protein (GFP), the neomycin phosphotranspherase (as selection marker) and the herpes thymidine kinase as negative selection marker is integrated. For the creation of stable scFc producing cell lines by the use of RMCE the strategy of negative selection is used. Cell lines producing each antibody were developed and isolated using limited dilution as well as cell sorting methods. These cell lines were intensively characterised by flow cytometry for intercellular product formation and absence of GFP, transgene and transcript level, qP, growth rate and metabolism of glucose, ammonia and lactate.

Further emphasis was set on the detailed investigation of the RMCE by the determination of the gene copy number as well as chromosomal investigation of the recombination sites by the use of fluorescence in situ hybridisation (FISH). Therefore the newly scFc antibody producing cell lines were characterized and compared to the cell line prior to the cassette exchange (RMCE host cell line).

4. Materials and Methods

4.1. Molecular biology materials and methods

4.1.1. Molecular biology materials

Reagents

Agaraose: Peqlab, peq GOLD Universal Agarose, Cat No. 35-1020

Ethidium Bromid: Sigma Aldrich, Cat. No. E1510, 10 mg/ml

DNA Ladder: Fermentas, Gene ruler DNA Ladder Mix, Cat. No. SM0331/2/3,

BSA: New England BioLabs, Cat. No. B9001

dNTPs: Deoxynucleotide Solution Mix, New England BioLabs, Cat. No. N0447L

EDTA: 500 mM, New England Biolabs, Cat. No. B0255A

Liquid Nitrogen

Glycerin pro analysi, Merck, Cat. No. 1.04092.2500

Ampicillin, 100 mg/mlAgar-Agar: Merck, Cat No. 1.01614.1000

Primers: all synthesized by Sigma Aldrich, 100 µM Stock solution

Ultra-pure Water: Fluka Cat. No. 14211, autoclaved

Ethanol: Emplura Ethanol absolute, Merck, Cat. No. 8.18760.2500

Trizol: Tri Reagent solution, Ambion, Cat. No. 9736

Chloroform

Random Primers: Promega, Cat. No. C118A

Buffers

TAE buffer 50 x:

- 0.5 M Tris
- 1 M Glacial acetic acid
- 50 mM EDTA

Loading Dye: BX loading dye, 6x,

- 0.25 %-w/v Bromphenolblau
- 0.25 %-w/v Xylencyanol
- 30 %-w/v Glycerin

NEB 1 Buffer: New England BioLabs, Cat. No. R0142S

NEB 4 Buffer: New England BioLabs, Cat. No. R0142S

NEB 2 Buffer: New England BioLabs, Cat. No. B7002

Ligation Buffer: New England BioLabs, Cat. No. B0202

1 mM HEPES, sterile filtered

10 % Glycerin (v/v)

Thermo Pol Buffer (10 x): New England BioLabs, Cat. No. B9004S

5 x GC rich buffer for KAPA HiFi polymerase, Peqlab, supplied with the polymerase

70 % Ethanol (v/v)

Phosphate buffered saline (PBS):

2.0 mM KH_2PO_4,

10 mM $Na_2HPO_4 \cdot 2H_2O$,

2.7 mM KCl,

137 mM NaCl

Reverse Transcription buffer 5 x, Promega

Equipment

Casting Chamber: Biorad

Illumination: Gel Doc, Biorad

Photometer: Implen NanoPhotometer P-300

Centrifuge: Thermo Scientific, Heraeus Megafuge 40R

Eppendorf Centrifuge 5415 R (cooling)

Eppendorf Centrifuge 5424 (not cooling)

Electroporator: BioRad Gene Pulser Xcell

Thermo Cycler: BioRad C1000 Thermal Cycler

PCR tubes: Quiagen 0.2 mL, Cat. No. 981005

Quantitative real time PCR Cylcer: BioRad Mini Opticon

48 well plates: Low 48-well White, BioRad, Cat. No. MLL4851

Sealing tape: PCR Sealers Microseal ‚B' film, BioRad, Cat. No. MSB 1001

Enzymes

Restriction enzymes:

KpnI: New England BioLabs, Cat. No. R0142S

NheI-HF: New England BioLabs, Cat. No. R3131S

XhoI: New England BioLabs, Cat. No. R0146S

XbaI: New England BioLabs, Cat. No. R0145S

EcoRI-HF: New England BioLabs, Cat. No. R3101S

Alkaline phosphatase, Calf intestinal (CIP): New England BioLabs, Cat. No M0290S

DNA Polymerase I Large (Klenow) fragment: New England BioLabs, Cat. No. M0210S

T4 DNA Ligase: New England BioLabs, Cat. No. M0202S

Thermo stable polymerases:

Taq DNA polymerase: New England BioLabs, Cat. No. M0321S

Peqlab, Kapa Hifi Polymerase, Cat. No. KK2101

RNAse inhibitor: RNAse Out, Invitrogen Cat. No. 10777-019

Reverse Transcriptase: M-MLV, Promega, Cat. No. M1701

qPCR Supermix:

KAPA Probe fast Universal, Peqlab, Cat. No. 07-KK4701-02,

Kits:

Agarose gel extraction:

Promega Wizard Plus SV Gel and PCR clean up system, Cat. No. A9282

Plasmid Isolation:

Mini Prep Kit: Promega Wizard Plus SV Minipreps, Cat. No. A1460

Midi Prep Kit: Macherey Nagel, Nucleo Bond Xtra Midi EF, Cat. No. 740410.50

Genomic DNA Isolation:

Qiagen, QIAmp DNA Blood Mini Kit, Cat. No. 51104

DNA Digestion:

RNAse free DNAse set: Qiagen, Cat. No. 79254,

Bacteria:

Escherichia coli (*E.coli*)Top 10; F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-, Invitrogen, Cat. No. C4040-50

Media:

LB-Media:

10 g/l Bakto Trypton or Peptone out of Casein;

5 g/l Yeast-Extract;

170mM NaCl; pH 7,0

LB-Agar Plate with Ampicillin:

LB-Media

1.5 % Agar-Agar

100 µg/ml Ampicillin

SOC Media:

20 g/l Bacto Trypton;

5 g/l Yeast-Extract;

10 mM NaCl;

3 mM KCl;

10 mM $MgCl_2 \cdot 6H_2O$;

20 mM Glucose;

10 mM $MgSO_4 \cdot 7H_2O$

4.1.2. Agarose Gel electrophoresis

Gels with a concentration of 1% (w/v) agarose were used for gel electrophoresis. One gel typically consisted of 1.5 g agarose, 3 ml of 50 x TAE buffer and was filled up with RO-water to 150 ml. The solution was melted in a microwave by maximum power (about 2-3 minutes) until the agarose was completely dissolved and the solution appeared clear. After melting the solution was cooled down to approximately 65 °C and 3 µl of Ethidium Bromide were added, mixed well and poured into a prepared gel casting tray for cooling, until the gel looked milky and became solid. The slots for the samples were used according to the purpose of the gel, for analytical gels small slots were used while for preparative gels wide slots were used.

Samples for agarose gel electrophoresis were mixed with 6 x BX-loading dye and the electrophoresis was carried out in 1 x TAE buffer containing Ethidium Bromide for 45 minutes at 110 V.

For analytical gels 10 µl of the sample were used, while for preparative gels 60 µl were used.

Visualization of the separated DNA fragments was carried out in the Illumination chamber (Gel doc, Biorad) by illuminating the gel with UV light. The Ethidium Bromide is intercalating with the DNA and so the DNA is stained as bright bands under UV illumination, while the rest of the gel appears dark. For better contrast, an inverse color image, where dark appears light and light appears dark, was used.

4.1.3. DNA-Digestion

Digestion of DNA-fragments was performed for analytical and preparative reasons. The analytical gel was used to check the right plasmid pattern after DNA digestion and the preparative gel was used to isolate the desired DNA fragment after vector digestion.

An analytical digestion contained typically the following reagents as listed in Table 1.

Table 1: Composition of a restriction digestion for DNA plasmids

Component	**In 50µl:**
DNA template	500 ng
Restriction enzyme	10 Units
NEB 4 buffer (10x)	5 µl
Water	add to 50 µl

After incubation for 1 hour at 37°C the DNA digestion was mixed with loading dye and separated on an agarose gel.

A preparative DNA digest was carried out with the corresponding restriction enzymes, 3 µg plasmid DNA, BSA and the appropriate NEB buffer in 50 µl according to the protocols of the supplier (NEB). It was also possible to digest the plasmid simultaneously with two restriction enzymes, according to the protocols of the supplier. The choice of the appropriate buffer and enzyme concentration was therefore critical and was obtained from the New England biolabs homepage.

After incubation for 3 hours at 37°C, the DNA was separated on an agarose gel.

4.1.4. DNA Isolation from agarose gels

Purification of DNA fragments from agarose gels was carried out with the Promega Wizard Plus SV Gel and PCR clean up system, according to the manufacturer instructions. Before starting, the DNA was cut out from the gel, visualized under UV illumination. The cutting was carried out as fast as possible, as excessive illumination can damage the DNA by nicking and also the adherent agarose amount was kept as little as possible, as this improves the yield of the DNA isolation. After isolation of the DNA a spectrophotometric quantification was done.

4.1.5. Blunt End generating by Klenow DNA Polymerase

The large fragment of the DNA Polymerase I from *E. coli* (also called Klenow fragment) was used to generate blunt ends from digested vectors and inserts. This enzyme has a 3' to 5' polymerase activity and can fill in the 3' overhangs. This step was done immediately after the DNA purification from the agarose gel.

The isolated DNA fragments were mixed with 5 µl NEB Buffer 2 (10 x), 1.65 µl dNTP's and 2.5 Units of Klenow polymerase. The sample was incubated at 25 °C for 15 minutes. To inactivate the Enzyme 1 µl of 500 mM EDTA was added and the reaction was incubated at 75 °C for 20 minutes.

4.1.6. Dephosphorylation of the Vector

This step removes the phosphate groups of DNA ends and therefore prevents the vector from self-ligating.

The DNA was diluted with NEB-Buffer 2 to 50 ng/µl. Then 0.2 µl of the enzyme calf intestine phosphatase were added to the solution and incubated at 37 °C for 1 hour.

4.1.7. Ligation

The Ligation reaction was set up with three different molar ratios of insert to vector. The ratios were: insert to vector 1 to 1, 1 to 3 and 1 to 5. The needed amount of DNA insert respectively to the amount of vector backbone was calculated according to Equation 1.

Equation 1: Calculation of necessary amount of DNA insert (m_{insert}). The mass of the vector (m_{vector}) multiplied by the length of the vector (l_{vector}) multiplied with the excess factor (ef, determined by the ratio of vector to insert) divided by the length of the DNA insert (l_{insert})

$$m_{insert}\,[ng] = \frac{m_{vector}[ng] \cdot l_{vector}\,[bp] \cdot ef}{l_{insert}[bp]}$$

The reaction volume of each reaction was 20 µl in a PCR-tube, containing the DNA, 2 µl of 10 x ligation buffer, 1 µl of T4 ligase and water added up to a total volume of 20 µl. For the reaction itself, the enzyme was incubated over night at 16 °C and heat inactivated at 65 °C for 10 minutes.

4.1.8. Preparation of electro competent *E. coli* Top 10

The bacterial strain *E.coli* Top 10 was used to generate electro competent bacteria. First one aliquot (about 40 µl) from the bacterial stock was gently thawed on ice, suspended into 25 ml of LB-Media in a 250 ml Erlenmeyer flask and incubated over

night at 37 °C and 180 rpm. 2 ml of this culture were then transferred to 200 ml LB-Media in a 1000 ml Erlenmeyer flask. For one preparation four cultures were needed. The growth of this culture was monitored with a spectrophotometer at 600 nm until the culture reached an OD_{600} between 0.6 and 0.8. The four cultures were then pooled into two beakers for centrifugation. Every centrifugation step was carried out with a swing out rotor at 4769 g and 4 °C.

The bacteria were centrifuged for 30 minutes and the supernatant was discarded, the resulting pellet was suspended in 250 ml cold 1 mM HEPES buffer and centrifuged again for 15 minutes. Again the supernatant was discarded, and the bacteria were suspended in 150 ml of cold 1 mM HEPES and centrifuged for 10 minutes. After the supernatant was removed, the bacteria were suspended in 50 ml of cold 1 mM HEPES. Now the cells were centrifuged for 10 minutes and the supernatant was discarded again. The bacteria were suspended in 20 ml cold glycerin solution (10 % v/v) and pooled together. As last centrifugation step the bacteria were now centrifuged for 10 minutes, the supernatant was discarded and the pellet was suspended in 1.5 ml of cold glycerin solution (10% v/v). Finally the bacterial suspension was aliqouted into Eppendorf reaction tubes (40 µl each) and immediately snap-frozen in liquid nitrogen. One aliquot was used to test if the bacteria carry ampicillin or kanamycin resistances and another aliquot was used to test the transformation competency by electroporation with the pUC vector.

4.1.9. Transformation

For transformation the electroporation method was used. Therefore one frozen aliquot of the electro competent *E. coli* Top 10 was thawed gently on ice, 15 ng of Plasmid DNA were added and the electroporation was carried out using a self-made cuvette with 2 mm distance between the electrodes. The electroporation parameters were: 1.8 kV voltages, capacity of 25 µF, resistance of 200 Ω, which resulted in a typically time constant about 5 ms. After the electroporation the bacteria were quickly suspended into 250 µl of SOC media and incubated at 37 °C, 400 rpm for 45 minutes in an Eppendorf reaction tube.

After the incubation time 50 µl or 100 µl of the bacteria were plated on a LB-Agar plate with ampicillin.

4.1.10. Colony polymerase chain reaction (PCR)

To detect positive clones after gene cloning a colony PCR was used. This PCR was done with the Taq-polymerase and all components in Table 2 were mixed to a master mix. The total volume of one reaction was 30 µl, therefore in each tube 25.7 µl of water was pipetted first.

Table 2: Reaction mix for a colony PCR

Component	**Stock solution**	**Concentration in 25 µl**	**Used volume [µl]**
dNTPs	10 mM each	0.2 mM	0.6
Primer sense	10 µM	0.1 µM	0.3
Primer antisense	10 µM	0.1 µM	0.3
Taq DNA Polymerase	5 u/µl	0.025 u/µl	0.12
Thermopol buffer II	10x	1x	3

With an autoclaved toothpick one bacterial colony was picked from the agar plate, suspended in the water of the PCR reaction tube and stroked on a distinct place on another agar plate (Master Plate). Then 4.3 µl of the Master Mix were added to the reaction tube and a normal PCR with the reaction conditions listed in Table 3 was carried out. The annealing temperature for the used primer pair was calculated to be 53 °C. Steps 2 to 4 were repeated 35 times.

Table 3: Reaction conditions for a colony PCR

Temperature	**Duration**	**Description**	
95°C	30 seconds	Initial Denaturation	
95°C	20 Seconds	Denaturation	35 x
53°C	30 Seconds	Annealing of Primers	
68°C	1 Minute	Elongation	
72°C	5 Minutes	Final Elongation	
16°C	Forever	Storage	

4.1.11. Plasmid DNA preparation for sequencing

Bacteria containing the plasmid confirmed positive by colony PCR were chosen for an overnight culture and DNA isolation for a subsequent sequencing reaction. Therefore the selected bacterial clone was picked with a sterile toothpick from the

Master Plate and suspended in 10 ml of LB-Media with ampicillin, incubated overnight at 37 °C at 180 rpm shaking speed. The plasmid DNA was isolated with the Promega Wizard Plus SV Miniprep kit according to the manufacturers protocol. As final step, the DNA was eluted in 100 µl of endotoxin free water and the amount of DNA was quantified by spectrophotometric measurements as described in section 4.1.19.

4.1.12. Cryopreservation of transformed bacteria

One single colony of transformed bacteria was picked with a sterile toothpick and suspended into 5 ml of LB-Media, incubated overnight at 37 °C shaking with 180 rpm. To get 1 ml of a glycerol stock, 375 µl of 80 % glycerin were mixed with 625 µl of the overnight culture and stored at -80 °C.

4.1.13. Sequencing reaction

To proof the identity of the plasmid sequence the overnight sequencing service of the company microsynth was used. Therefore 80 ng/µl of plasmid DNA and 2 µM primer (3 µl of a 10 µM stock solution) were mixed and autoclaved distilled water was added up to a total volume of 15 µl.

4.1.14. Plasmid DNA preparation for transfection

For transfection of mammalian cells large amounts of DNA were needed and plasmid isolation with a Midi Prep kit was performed. Therefore one pipet tip was put into the glycerol stock of the transformed bacteria or if no glycerol stock was available a single colony was picked from an agar plate containing the transformed bacteria and suspended into approximately 150 ml of LB media containing ampicillin for selection. The bacteria were incubated overnight at 37 °C shaking at 180 rpm. On the next day, 100 ml of the bacteria culture were centrifuged at 4769 g for 10 minutes. For the plasmid DNA preparation the Nucleo bond Xtra Midi EF- Kit from the company Macherey Nagel was used and the preparation was done according to the protocol of the manufacturer. As last step of the manufacturer's protocol, an ethanol precipitation was performed, and the DNA-Pellet was solubilized in 500 µl of Endotoxin free water. After the preparation, the amount of plasmid DNA was quantified by spectrophotometric measurements according to section 4.1.19 and checked prior to a transfection by an analytical plasmid digestion.

4.1.15. Analytical polymerase chain reaction (PCR)

A standard PCR was performed in a total volume of 25 µl according to the protocol for the KAPA HiFi Polymerase. One reaction consisted of the following components listed in Table 4.

Table 4: Reaction mix for an analytical PCR with genomic or cDNA as template

Component	**Stock solution**	**Concentration in 25 µl**	**Used volume [µl]**
dNTPs	10 mM each	0.3 mM	0.75
Primer sense	10 µM	0.3 µM	0.75
Primer antisense	10 µM	0.3 µM	0.75
KAPA HiFi Polymerase	1 u/µl	0.02 u/µl	0.5
GC rich-Buffer	5 x	1 x	5
Ultrapure Water (UPW)		add up to 25 µl	

The amount of template relied on the DNA-Source. For plasmid DNA 5 ng was enough, while for the detection of genes in genomic DNA 200 ng were used. For PCR with cDNA the 1:10 diluted reverse transcription product from 500 ng RNA solution was used.

For more than one reaction, every component in Table 4 was mixed to a Master Mix and added to the template in the PCR reaction tubes. All components of the Master Mix were pipetted and stored on ice until the reaction was started in the thermocycler. The reaction was performed according to the conditions listed in Table 5. Steps 2 to 4 were repeated for 35 cycles. The annealing temperature had to be determined for the corresponding primer pair and it was usually in the range of the melting temperature. This is higher as for other polymerases, such as the Taq-Polymerase, because the KAPA Hifi polymerase needs a higher salt concentration and also the GC-rich buffer has an even higher ionic strength to improve the reaction. Therefore the calculated annealing temperature was too low. The distinct conditions for every used primer pair are listed in section 8.1.

Table 5: Reaction conditions for an analytical PCR with genomic or cDNA as template

Temperature	Duration	Description	
95°C	3 Minutes	Initial Denaturation	
98°C	20 Seconds	Denaturation	35 x
Tm °C	15 Seconds	Annealing of Primers	
72°C	30 Seconds per kbp	Elongation	
72°C	5 Minutes	Final Elongation	
16°C	Forever	Storage	

4.1.16. Ribonucleic acid (RNA) – Isolation

The isolation of RNA from the cells was performed with $2 \cdot 10^6$ cells. Cells were taken from the culture, centrifuged for 10 minutes at 188 g at room temperature. Afterwards, the supernatant was discarded, and the cells were suspended in 1 ml PBS, transferred to a 1.5 ml reaction tube and centrifuged for 10 minutes with 200 g.

Again the supernatant was discarded and the cells were suspended in 1 ml TRIzol reagent. 200 µl of Chloroform was added, shaken thoroughly and incubated on ice for 15 minutes. Then a centrifugation step with 16,100 g for 15 minutes at 4 °C was performed and the clear upper aqueous phase was transferred into a fresh 1.5 ml reaction tube. 500 µl of Isopropanol were added and incubated at -20 °C for 15 minutes. After the incubation a centrifugation step with 16,100 g for 15 minutes at 4 °C was performed. The supernatant was discarded and the white pellet on the bottom of the reaction tube was washed with 1 ml 70% Ethanol. Then a centrifugation step with 16,100 g for 5 minutes at room temperature was performed. The supernatant was again discarded and the pellet was dried at room temperature.

4.1.17. DNA-Digestion

To digest the DNA in the RNA-isolation, a digestion with RNAse free DNAse was performed. Therefore the dried pellet of the RNA-isolation was solubilized in 70 µl RNAse free DNAse buffer, 4 µl RNAse Inhibitor (RNAase out) and 10 µl RNAse free DNAse was added to the preparation. For the digestion the preparation was incubated 30 minutes at room temperature and the enzyme was then heat inactivated by incubation at 75 °C for 10 minutes.

4.1.18. RNA Precipitation

The RNA after the DNA digestions was precipitated by adding 250 µl Isopropanol and incubated at -20° C for 20 minutes. Afterwards a centrifugation step was performed at 16,100 g, 4 °C for 15 minutes. The supernatant was discarded and the colorless RNA pellet was washed with 1 ml 70 % Ethanol. Then a centrifugation step with 16,100 g for 5 minutes at room temperature was done, the supernatant was removed and the pellet was air dried at room temperature.

The dried pellet was suspended in 23.5 µl ultra-pure water and 1.5 µl RNAse inhibitor (RNAse out).

4.1.19. Quantification of Nucleic acids

The amount of nucleic acids in solution was quantified by spectrophotometric measurements with the Implen nanophotometer P-300. Therefore 3 µl of the sample were pipetted in the center of the cuvette, the cap for the expected concentration was put on the drop and the absorption at 260 nm was measured. The amount of the nucleic acids was calculated according to the Equation 2.

Equation 2: Quantification of nucleic acids based on A_{260}. ε is a proportional constant, which has a value of 50 ng/µl for double stranded DNA and 40 ng/µl for RNA

$$Concentration\left[\frac{ng}{\mu l}\right] = A_{260} \cdot \varepsilon$$

Additionally the absorption at 280 nm was measured and the ratio between the absorption at 260 nm and 280 nm gave an idea of the purity of the DNA. The calculation of this value is shown in Equation 3.

Equation 3: Ratio between Absorption at 260 and 280 nm. It determines the purity of the nucleic acid preparation.

$$Ratio = \frac{A_{260}}{A_{280}}$$

Usually values between 1.8 and 2.2 were in the acceptable range, because lower values indicate protein impurities and higher values DNA or respectively RNA impurities.

4.1.20. Reverse Transcription

Reverse transcription was used to obtain cDNA from the isolated mRNA. 500 ng of RNA, 1 µl of dNTPs (10mM each dNTP), 250 ng of random primers (0.5 µg random primers per µg RNA) and water to a total volume of 14 µl were pipetted to a reaction tube. Then the mixture was incubated at 70 °C for 5 minutes and immediately cooled

down on ice for the annealing of the random primers. Afterwards 4 µl reverse transcription buffer (5 x), 1 µl M-MLV reverse transcriptase and 1 µl RNAse inhibitor (RNAse out) were added. For the reverse transcription the reaction mix was incubated at 37 °C for 1 hour, following 30 minutes at 42 °C and finally for heat inactivation of the enzyme 5 minutes at 95 °C.

4.1.21. Quantitative real-time PCR

Quantitative real time PCR (qPCR) was carried out with the MiniOpticon Thermocycler. For the real time detection of the amplified DNA the TaqMan method with hydrolysation probes was used. Therefore a DNA-probe, conjugated with the fluorescent dye FAM at the 5' end and TAMRA at the 3' end was used. These two dyes quench each other, as they are in close proximity. The DNA-sequence of the probe was designed in such a way, that it matches with the target DNA between the forward and backward primer. As now the amplification of the DNA is carried out by the polymerase, the 5' exonuclease activity degrades the annealed probe and the two fluorescent dyes are released and therefore no quenching occurs anymore. The fluorescence is measured as a sign for amplification. The results of the qPCR are fluorescence curves of the different genes, which rely on the number of genes or transcript present.

A qPCR was performed in a 48 well plate, where one reaction was carried out in 20 µl. This consisted of the following reagents listed in Table 6.

Table 6: Composition of one reaction in a quantitative real-time PCR

Component	**Stock solution**	**Concentration in 20 µl**	**Used volume [µl]**
Primer sense	10 µM	0.3 µM	0.6
Primer antisense	10 µM	0.3 µM	0.6
Probe	10 µM	0.2 µM	0.4
KAPA Probe fast Universal	2x	1 x	10
Ultrapure Water (UPW)		add up to 15 µl	

For gene copy number determination, a Master Mix containing all these reagents was prepared and pipetted carefully into the wells. For the target DNA 5 µl were used. Using genomic DNA, 5 ng of total DNA was used. Therefore the DNA had to be diluted to a concentration of 1 ng/µl. Afterwards the genomic DNA was denatured at 95 °C for 10 minutes. This step improves the accuracy by the degradation of the DNA according to (WILHELM et al., 2000).

For transcript level analysis, cDNA was diluted 1 by 10 and 5 µl thereof was used for the reaction as template. Every sample was measured in triplicates per run and two technical runs were performed. This results in 6 values for each sample. The qPCR conditions, which were used for all samples are listed in Table 7. A two-step protocol was used. Step 2 and 3 was repeated for 45 cycles and after step 3 the fluorescence of the hydrolyzed probes was measured.

Table 7: Reaction conditions for qPCR

Step	**Temperature**	**Duration**	
1	95°C	5 Minutes	
2	95°C	15 Seconds	} 45 x
3	55 °C	1 Minute	

The calculation of the relative gene copy number or transcript level in comparison to a reference gene was done according to the $2^{-\Delta\Delta Cq}$ method described in (LIVAK and SCHMITTGEN, 2001). Therefore, the –ΔCp value was calculated according to Equation 4. Subsequently the $2^{-\Delta Cp}$ value was calculated according to Equation 5. This value results in the relative gene copy number respectively the relative transcript level in comparison to the reference gene assuming a PCR efficiency of 100 %. To compare two clones of the same cell line, the $2^{-\Delta\Delta Cp}$ value according to Equation 6 was calculated.

Equation 4: Calculation of the –ΔCp value to the reference gene β-actin.

$$-\Delta Cq = Cq_{\beta-actin} - Cq_{GOI}$$

Equation 5: Ratio of the gene copy number/ transcript level relative to β-actin

$$\frac{GOI}{\beta - actin} = 2^{-\Delta Cp}$$

Equation 6: Ratio of the gene copy number/ transcript level relative of the same GOI between two samples

$$\frac{GOI_{sample1}}{GOI_{sample2}} = \frac{2^{-\Delta Cp_{GOI1}}}{2^{-\Delta Cp_{GOI2}}} = 2^{-\Delta\Delta Cp}$$

4.2. Biochemical materials and methods

4.2.1. Biochemical materials

Reagents

Tween 20: Roth, Cat. No. 9127.2

Hydrogen Peroxide (H_2O_2): 35 %, Merck, Cat. No. 1.08600.1000

ortho-Phenylenediamine (OPD): 100 mg/mL

25 % Sulfuric acid: Roth, Cat. No. 0967.1

Bovine serum albumin (BSA)

Buffers

Coating buffer:

8.4 g $NaHCO_3$

4.2 g Na_2CO_3

filled up to one liter with RO-water, pH = 9.5-9.8

Phosphate buffered saline (PBS):

2.0 mM KH_2PO_4

10 mM $Na_2HPO_4 \cdot 2H_2O$

2.7 mM KCl

137 mM NaCl

Washing buffer: PBS with 0.1 % Tween 20

Dilution buffer: PBS with 0.1 % Tween 20 and 1 % BSA

Coloring buffer:

7.3 g Citric acid $\cdot$ H_2O,

11.86 g $Na_2HPO_4 \cdot 2\ H_2O$

filled up to one liter with RO-water, pH = 4.8-5.0

Detection buffer (for one plate, prepare immediately before use):

10 ml Coloring buffer

100 µl OPD

10 µl H_2O_2

Neutralisation buffer: PBS with 0.1 % Tween 20

Regeneration buffer: 10 mM Glycine, pH 2.0

Sample buffer: PBS with 0.1 % Tween 20

Equipments

Plate washer: Tecan, Microplate Washer, 96 Plate Washer™ (96 PW)

Multichannel photometer: Tecan, Infinite® M1000 PRO

Bio-Layer interferometer: fortébio, Octet QK

Consumables

ELISA plates: Nunc™, Immuno 96 MicroWell™ Solid Plates, MaxiSorp™ Cat. No. 442404

Dilution plates: Nunc™, 96 MicroWell™ plate Cat. No. 269620

96 well plates for Bio-Layer interferometry: Nunc™ F96 MicroWell™ Plate Cat. No. 137101

Sensors for Bio-Layer interferometer: Protein A dip and read sensors, fortébio

Antibodies and antigens

Capture Antibody:

Anti-Human IgG (γ-chain specific) antibody produced in goat, affinity isolated antibody, lyophilized powder solubilized in 1 ml of 0.135 M NaCl solution, Sigma Aldrich, Cat. No. I3382

Detection Antibody:

HRP-Goat Anti-Human IgG (Gamma), Invitrogen, Cat. No. 628420

Standard:

3D6scFc protein A affinity purified, diluted in dilution buffer to 40 ng/ml

Capturing antigen for specificity testing:

Linear epitope for antibody 2F5 containing following amino acids: GGGELDKWASL, Polymun Scientific

Software

Magellan 6

fortébio data acquisition and analysis software

4.2.2. Determination of product concentration by ELISA

An Enzyme linked immunosorbent assay (ELISA) was used to detect the amount of product in the cell culture supernatant. The assay was set up as double antibody sandwich ELISA.

First of all the ELISA plates were coated with capture antibody, then incubated with the culture supernatant, and detected with an enzyme coupled antibody. The coupled enzyme was the horseradish peroxidase, which catalyzes the reaction from the colorless OPD in the presence of hydrogen peroxide to an orange color product. The absorption of this product at 492 nm is therefore proportional to the amount of bound enzyme conjugated antibody and therefore to the concentration of the product in the supernatant. ELISA tests were used for different purposes. A qualitative ELISA for the determination of the producing clones after transfection and a quantitative ELISA for the precise quantification of the product concentration in the supernatant.

Qualitative ELISA

As capture antibody an anti-human IgG gamma chain antibody developed in goat from the company Sigma-Aldrich (Cat. No. I3382) was used. This antibody was diluted 1:2000 in coating buffer and 100 µl per well were transferred into the 96 well ELISA plate. For a stable equilibrium of adsorption and desorption plates were incubated for 2 hours at room temperature or overnight at 4 °C. After incubation the plate was washed three times with washing buffer, soak time 5 s, 300 µl per well. Every washing step was carried out with these parameters automatically using a plate washer. The culture supernatant was diluted 1:5 in dilution buffer in an extra 96 well plate (dilution plate). As standard, affinity purified 3D6scFc was used, diluted to 40 ng/ml in dilution buffer and subsequently diluted in a 1:2 dilution series in the dilution plate. Additionally one row of the plate was reserved for the reaction blank by the use of dilution buffer. From this dilution plate 50 µl of each well were transferred in the corresponding well of the ELISA plate. This plate was incubated for 1 hour at room temperature at 300 rpm on a shaker. After washing the plate again, 50 µl per well of 1:3000 diluted detection antibody were added and incubated for 1 hour at room temperature at 300 rpm. After the incubation the plates were washed again three times and 100 µl of detection buffer were applied to each well and incubated until the most diluted standard showed a slight color. Now the reaction was stopped with 100 µl of 25% H_2SO_4 and the absorption values were measured with a Tecan Infinite 1000 reader at 492 nm with a reference wavelength of 620 nm.

Quantitative ELISA

The quantitative ELISA was performed in the same way like the qualitative ELISA with following exceptions.

All samples were diluted into the concentration range of the standard with dilution buffer (40 ng/ml until 0.3125 ng/ml). 100 µl of dilution buffer was transferred into each well of the dilution plate except for the column H. In this column, 200 µl of blank (row 1), standard in duplicates with a starting concentration of 40 ng/ml (row 2 and 3) and the diluted samples were pipetted (row 4 to 12). For the 1:2 dilution series 100 µl of the column H were aspirated with the 12 channel pipette, mixed by pipetting up and down for 5 times and then transferred to the column G. This was repeated until the column A of the dilution plate was reached. Now the different incubation steps were performed liked in the qualitative ELISA.

Finally the absorption at 492 nm (reference wavelength 620 nm) was measured with the multichannel photometer and the standard curve and the average sample concentration was calculated by the Magellan 6 software package (Tecan).

4.2.3. Determination of product concentration by Bio-Layer interferometry

The Octet QK from the company fortébio was used for quantification of the protein concentration. To get comparable results to the ELISA biosensors coated with protein A were used. The detection method was based on the kinetics of the binding of the Fc part of the scFc protein to the protein A, as this is a concentration depended process. The concentrations of bound antibodies were detected by a wavelength shift of the incoming light to the biosensor, which is proportional to the thickness of the boundary surface between the sensor and the solution. More bound antibodies lead to an increase of the thickness of the surface and also the shift in the wavelength increases.

For measurement a volume of 200 µl per well was needed. To regenerate the protein A sensors, after each measurement they were moved into row 11 (filled with regeneration buffer) and row 12 (neutralization buffer) 3 times for 5 seconds each. Samples were diluted 1:2 and 1:4 with sample buffer. The measurement itself was done at 30 °C, shaking with 1000 rpm and 300 seconds for each sample.

The quantitation is based on the binding properties of the unknown samples with a defined standard curve. This includes known concentration of purified 3D6scFc ranging from 15.3 µg/ml to 0.476 µg/ml in 1:2 serial dilution steps. The binding kinetics was used for the determination of the antibody concentration, because with higher concentration more molecules bind in a distinct period of time. For the

calculation the initial slope of the binding curve was taken. The calculation of the concentration was based on a linear point to point standard curve, which only had to be run once and data were saved for further experiments. As final result the average of the two dilutions per sample was calculated.

4.2.4. Specificity testing for epitope ELDKWA

The specificity testing for the epitope ELDKWA was done according to an ELISA test. Therefore a 96 well ELISA plate was coated with 100 µl of 1 µg/ ml diluted antigen epitope GGGELDKWASL in coating buffer overnight at 4°C. Afterwards the plate was washed 3 times with 300 µl of washing buffer using the TECAN plate washer.

Samples and controls were diluted in dilution buffer to a concentration of 1 µg/ml. As positive control the purified monoclonal anti-HIV-1 antibody 2F5 supplied by Polymun Scientific was used. Starting from this concentration a 1:2 dilution series for each sample and control was prepared in a fresh 96 well plate (dilution plate).

From the dilution plate 50 µl per sample were transferred to the ELISA plate, starting with the lowest concentration. The samples were incubated for 1 hour at room temperature shaking at 300 rpm. Afterwards the plate was washed 3 times with 300 µl washing buffer per well.

Then 50 µl per well of a 1:3000 diluted anti-human IgG γ-chain specific antibody conjugated to horse radish peroxidase was pipetted and incubated again for one hour at room temperature shaking with 300 rpm.

After washing the plate again, detection was done by applying 100 µl detection buffer per well and incubation until the most diluted control showed a visible color. The reaction was then stopped with 100 µl per well 25 % sulfuric acid. For the measurement of the absorption the TECAN infinite 3000 plate reader was used. The measurement was done at a wavelength of 492 nm with a reference wavelength of 620 nm.

4.3. Cell culture materials and methods

4.3.1. Cell culture materials

Reagents

Hypoxanthine, Sigma-Aldrich, Cat. No. H9636-84

Thymidine, Sigma-Aldrich, Cat. No. T1898-14

Phenol red: Sigma-Aldrich, Cat. No. P0290, 0.5% in DPBS, Sterile filtered and Endotoxin tested

G-418 Sulphate Solution (50 mg/ml): PAA, Cat. No. P11-012

Ganciclovir: Sigma-Aldrich, Cat. No. G2536-100 mg, 4 mM Stock solution in 0.1 M HCl

L-Glutamine: Biochrome AG, L-Alanyl-L-Glutamine, 200 mM, Cat. No. K 0302

ProCHO5: Lonza BioWhittaker, w/Pluronic F68, w/o L-Glutamine or Nucleosides (HT), Cat. No. BE12-766Q

Trypan blue: Sigma-Aldrich, Trypan blue solution 0.4 %, Cat. No. T8154

Polyethylenamine (PEI): Polysciences Inc., Polyethylenimine 1 mg/mL, Cat.No. 23966-2.

Ethanol: Emplura Ethanol absolute, Merck 8.18760.2500

Media

Cryo Preservation: Cryo Max II PAA, CryoMaxx II Cryopreservation Medium, Cat. No. J05-012

HT-Supplement: HT Stock solution (100 x), L0026, 10 mM Hypoxanthine, 1.6 mM Thymidine

Media for CHO/RMCE/2-4/E/Sp125, CHO/RMCE/2-4/E/F12, CHO/RMCE/2-4/E/B1:

- Pro CHO5
- 4 mM L-Alanyl-L-Glutamine
- HT supplement 1x
- 0.5 µg/ml G418
- 15 mg/L Phenol red

Media for transfection:

- Pro CHO5
- 4 mM L-Alanyl-L-Glutamine
- HT supplement 1x
- 15 mg/L Phenol red

Selection medium after transfection:

- Pro CHO5
- 4 mM L-Alanyl-L-Glutamine
- HT supplement 1x
- 1 µM or 2 µM Ganciclovir
- 15 mg/L Phenol red

Consumables:

T25 Flasks: Nunc Nunclon Δ surface T25 flasks, Cat. No. 163371

T80 Flasks: Nunc Nunclon Δ surface T80 flasks, Cat. No. 153732

96 well plates: Nunc Nunclon Δ surface 96 well plates, Cat. No. 167008

24 well plates: Greiner Bio One Cellstar 24 well plates, Cat. No. 6651870

Cryotubes: Nunc CryoTubes™, 1.8 mL, Cat. No. 377267

Centrifugation tubes 10ml: 110 x 16 x v x cent tube with screw cap, thermo scientific Nunc, Cat. No. 347856

Centrifugation tubes 50ml: Cell star tubes, greiner bio-one, Cat. No. 227261

Centrifugation tubes 50ml: Cell star tubes, greiner bio-one, Cat. No. 210261

Reaction Tubes: VWR micro-centrifuge tubes for high G-forces, Cat. No. 20170-038

Universalcont 25 ml with screw cap: thermo scientific Nunc, Cat. No. 364238

Cell strainer: BD Falcon, Cat. No. 352340

Buffers

Phosphate buffered saline (PBS):

2.0 mM KH_2PO_4

10 mM $Na_2HPO_4 \cdot 2H_2O$

2.7 mM KCl

137 mM NaCl

Coulter Counter Incubation buffer: 0,1M Citric acid, 2 % w/w Triton X-100

Coulter Diluent: Beckmann Coulter isotone II diluent, Cat No. 8448011

HEPES buffered saline (HBS):

10 mM HEPES,

150 mM NaCl

Equipment

Spinner Flasks 125 ml: Techne complete culture vessel 125 mL spinner flask, Cat. No. F7987

Counting chamber: Optik Labor, Neubauer-chamber, 0.100 mm depth Pro-fondeur 0.0025 mm²

Cover Glass for haemocytometer: Marienfeld, 22*22*0.4 mm, Cat. No. 03 510 00

Centrifuge: Thermo Scientific, Heraeus Megafuge 16

Particle Counter: Beckmann Coulter, Z2 Coulter counter

Microscope: Leica DMIM LED microscope, Leica Microsystems GmbH, Type 11090137001

Mr. Frosty, Naglene

Bioprofiler 100 plus, Nova Biomedical, Rödermark, Germany

4.3.2. Viability testing

To assess the viability of the cells in culture, a viable cell counting using a hemocytometer was performed. Therefore 500 µl of the cell suspension were mixed with 100 µl of trypan blue solution. Trypan blue can only pass the cell membrane of dead cells and therefore they appear blue in the light microscope. As next step, the Neubauer chamber was cleaned with 70 % Ethanol, dried and assembled in such a way, that at the edges of the coverslip Newton's rings appear. For the counting, one drop of the mixture was put at the edge of the coverslip outside of the chamber. The capillary force sucks the necessary volume (0.1 µl) into the chamber. One counting chamber shows 9 large squares, which are divided into 16 small squares each. Only cells and dead cells, which could be determined by the round shape, were counted to get reliable results. In total 8 large squares in two different counting chambers were counted. To get an idea of the cell concentration, the dilution and the volume of the counting chamber has to be taken into account and result in Equation 7. This calculation was also performed with the average cell count.

Equation 7: Viable Cell concentration (VCC) or total cell concentration (TCC) was determined by the average of counted cells (MCC) multiplied with the dilution factor 1.2 of the trypan blue staining and multiplied with 10^4, to take the volume of the counting chamber into account.

$$VCC\left[\frac{cells}{ml}\right] \; or \; TCC\left[\frac{cells}{ml}\right] = MCC \cdot 1.2 \cdot 10^4$$

After the determination of the total and viable cell concentration, the viability was calculated according to Equation 8.

Equation 8: Percentage of viable cell (VCC) from the total cell concentration (TCC). Both values were counted in the Neubauer chamber.

$$Viability\ V\ [\%] = \frac{VCC}{TCC} \cdot 100$$

4.3.3. Cell counting

As the cell counting by the hemocytometer gives imprecise results for cell aggregates, the coulter counter principle was used to obtain more valid data. Therefore the cells were lysed and only the cell nuclei were counted. For a typical

measurement, 2 ml of a cell culture suspension in a T25 flask or 4 ml of cell suspension in a spinner flask were taken, centrifuged for 10 minutes at 188 g. The supernatant was either discarded or collected for further analysis. The cell pellet was suspended in 1 ml of coulter counter buffer, mixed thoroughly and incubated for at least 2 hours at room temperature. To obtain a result in the optimal range between 10,000 and 20,000 counts, either 500 µl (of a 2 ml sample) or 200 µl (of a 4 ml sample) were diluted with 9 ml of coulter counter isotone II diluent. Then the analysis was performed and the size distribution of the counted particles was assessed with the corresponding software. All particles in the desired range (usually lager than 3 µm) were regarded for the calculation of the total cell concentration by using Equation 9. In total 2 analysis per sample were done and the cell count was calculated as the average thereof.

Equation 9: Calculation of the total cell concentration (TCC) with the coulter counter. Mean particle count (MPC) multiplied with the total volume used (V_t, 9ml + Volume of the aliquot V_a) multiplied by 10 (depends on the used volume for analysis which is sucked in the capillary), divided through the concentration factor (CF, 2 for 2 ml cell culture suspension, 4 for 4 ml) and V_a (Volume of the aliquot used for dilution of the analysis).

$$TCC\left[\frac{cells}{ml}\right] = \frac{MPC \cdot V_t \cdot 10}{CF \cdot V_a}$$

4.3.4. Culturing of cells

T flasks

The different cell lines were usually cultured into a T flasks during the cell line development. The size of the flask depended on the volume of the needed cell suspension. A T25 flask was used up to 15 ml of cell suspension, while for larger volume up to 50 ml a T80 flask was used.

For cell splitting in T flasks, first of all the splitting ratio was calculated based on the total cell concentration and viability of the culture. This ratio was based on the fact, that a cell concentration of 2 to 3 $\cdot 10^5$ cells per ml should be present after the splitting. As next step, the right amount fresh media was pipetted into a fresh T flask and as second step the corresponding amount of cell culture suspension was taken and transferred to the fresh media. The cells were then incubated for 3 or 4 days at 37 °C with 7.0 % CO_2 and humid atmosphere in an incubator. The cap of the T flask was not tightly closed to allow a gas exchange.

Spinner Flask

Cells cultured in Spinner flasks profit from the better gas supply due to the agitation of the culture. For the suspension culture this also enhances the accessibility to nutrition and therefore most of the time it results in higher growth rates and specific productivities. This cultivation was usually used for cell lines to obtain always enough cells for different analysis.

Cultivation in the spinner flask was carried out in a volume of about 50 ml and agitation at 50 rpm at 37 °C. The seeding density after each splitting was 2 to $3 \cdot 10^5$ cells per ml. At the beginning of the spinner cultivation (after the cell seeding) the spinner had to be pre-gassed with 60 ml of sterile CO_2 to ensure optimal buffering of the medium. Splitting of the spinner cultivation was done in the following way. First of all the splitting ratio was determined by total cell concentration and viability. After the splitting a cell concentration about $2.5 \cdot 10^5$ cells/ml had to be present. As next step the appropriate amount of cell suspension was taken out of the spinner and transferred into a sterile tube. Now the remaining culture was discarded and the necessary amount of fresh medium was transferred into the spinner flask. The last step was the transfer from the appropriate amount from the sterile tube back into the spinner flask. If heavy clumping of the cells occurs, they were passed through a cell strainer to disaggregate the cells and promote growing in suspension at this stage.

Cells cultivated in a spinner flask were split twice a week, so either after three or four days of cultivation.

4.3.5. Transfection

The transfection of the recombinant cell line was done with PEI. PEI is a polymer with an average size of 25 kDa and contains lots of positively charged amino groups. These groups are able to bind to DNA, form Nano complexes and introduce it in the mammalian cells (CLAMME et al., 2003).

The transfection of a RMCE cell lines needs two plasmids, one plasmid containing the GOI and a different one containing the enzyme Flipase. For $1 \cdot 10^6$ cells 8 µg of total DNA and 80 µg of PEI were used. The total DNA contained 6 µg with pF32F5scFcF or pF33D6scFcF and 2 µg of pFlpo plasmid DNA. This ratio of plasmid containing GOI and plasmid containing the Flipase was based on the experiments described in (QIAO et al., 2009). The concentration of the cells was adjusted to $5 \cdot 10^5$

cells per ml if they were used for sorting. For the limited dilution approach in 96 well plates a concentration of $1 \cdot 10^6$ cells per ml was used for transfection. For more cells, the relation of plasmid DNA to PEI to cells should be kept constant, as well as the cell concentration. The next important factor which had to be taken into account is that the volume of the PEI/DNA solution to the total volume of the cell suspension should not exceed 1: 10, so for 2 ml of cell suspension the PEI/DNA solution should not exceed 200 µl. The different transfection conditions which were used are summarized in Table 8.

Table 8: Summary of the different transfection conditions

Plasmid DNA [µg]	**Flipase DNA [µg]**	**PEI [µg]**	**Total volume of PEI/DNA solution [µl]**	**Total cell number**	**Media Volume**
6	2	80	200	$1 \cdot 10^6$ cells	2 ml
12	4	160	200	$2 \cdot 10^6$ cells	2 ml
18	6	240	300	$3 \cdot 10^6$ cells	3 ml

For the transfection itself, the necessary amount of cells had to be taken, centrifuged at 188 g for 10 minutes and suspended in the appropriate amount of media according to Table 8. The DNA solution as well as the PEI solution was kept separately and incubated for 10 minutes in HBS buffer, so an equal volume of both had to be present. As next step the PEI reagent was transferred to the DNA and also incubated for 10 minutes to form the Nano-complexes. This solution was then put to the cells and they were incubated for 4 hours at 37 °C under humid atmosphere containing 7.0 % CO_2. After this incubation time the cell suspension was filled up to 10 ml total volume with fresh media to protect the cells from the toxic effects of the PEI (FISCHER et al., 1999). The used protocol was based on the research described in (REISINGER et al., 2009).

4.3.6. Strategy of cell line establishment

The strategy to develop and monitor the recombinant cell line is outlined in the following section. 24 hours after the transfection, the cells were centrifuged for 10 minutes at 188 g and suspended in selection media (1 or 2 µM Ganciclovir) to obtain a cell concentration of $1 \cdot 10^5$ cells per ml. As next step the cells were seeded into a 96 well plate with a density of 10^4 cells per well (therefore 100 µl per well were

transferred). The cells were grown for 2 to 3 weeks (fed every week) and checked microscopically for growth. As the first clones started to grow in the whole well, a qualitative ELISA was done and the 12 best clones were selected. These clones were split into a new 96 well plate in a ratio of 1:2. This was continued for 3 subsequent passages until these clones grow in a row of 8 wells. After the desired clone was present in a row of 8 wells, it was split into a T25 flask, where growth and productivity was monitored for a couple of passages in a volume of 10 ml. The two best clones in terms of growth, product concentration and qP were then selected to grow in the spinner flask. Additionally flow cytometry analysis to check the cell population for intercellular product formation was used to determine the most suitable cell clones. In the spinner flask growth and qP as well as some genetic parameters were further monitored. The cells were cultured in a volume of approximately 50 ml in the spinner vessel.

4.3.7. Bioprofiler

For the determination of the different glucose consumption rates as well as lactate and ammonia production rates the Bioprofiler 100 plus, Nova Biomedical, Rödermark, was used. Therefore 1 ml cell culture supernatant was collected before each splitting and analyzed for the concentration of these three metabolic key molecules.

4.3.8. Thawing up of cells

To preserve established cell lines, cells were stored in liquid nitrogen. For the recovery of these cells, one ampoule was removed from liquid nitrogen and put into a tube containing 70 % ethanol for disinfection. After the evaporation of the residual liquid nitrogen, the tube was warmed to room temperature, so that no ice appeared and the complete cell suspension in the ampoule was transferred into a centrifugation tube containing 8 ml of fresh media and centrifuged for 10 minutes at 188 g. The supernatant was then discarded and the cell pellet was suspended in 5 ml of fresh media and grown in a T25 flask.

4.3.9. Cryopreservation of cells

The cryopreservation of the cells was done to ensure the long term preservation of the developed cell line. Usually about 4 to 6 ampoules containing $5 \cdot 10^6$ cells per ampoule are prepared. For one ampoule the necessary amount of cells were taken, centrifuged at 188 g for 10 minutes and the supernatant was discarded. The cell

pellet was suspended in 1 ml of the cryopreservation medium, pipetted into an ampoule and transferred to the Mr. Frosty (Naglene). This device was then put on -80 °C and kept there for at least 24 hours. This step is important, as the isopropanol in this device ensures a cooling rate of 1 °C per minute. Faster freezing results in uncontrollable ice forming, which leads to cell destruction and can therefore reduce the recovery after freezing. After the 24 hours at -80 °C the cells were put into liquid nitrogen.

4.3.10. Cell culture parameters

In the following section equations for the calculation of important cell culture parameters used for monitoring of cell lines are shown. The calculation of the specific growth rate μ was done according to Equation 10.

Equation 10: The calculation of the specific growth rate is based on the logarithmic difference of the Cell concentration CC [cells/ml] due to exponential growth divided by the difference of the culturing time t [d].

$$\mu\,[d^{-1}] = \frac{\ln(CC_1) - \ln(CC_0)}{t_1 - t_0}$$

The specific productivity qP was calculated according to Equation 11.

Equation 11: Calculation of specific productivity. Difference in product concentration P [µg/ml] multiplied with the specific growth rate μ [d^{-1}] and a proportional factor divided by the difference of the cell concentration CC.

$$qP\left[\frac{pg}{d \cdot cell}\right] = \frac{(P_1 - P_0) \cdot \mu \cdot 10^6}{CC_1 - CC_0}$$

The calculation of the Glucose consumption rate qGlc is shown in Equation 12.

Equation 12: Calculation of the qGlc. Difference of Glucose concentration cGlc [g/l] multiplied by the specific growth rate μ [d^{-1}] and multiplied with the proportional factor 10^9 divided by the difference of the cell concentration CC [cells/ml]

$$qGlc\left[\frac{pg}{d \cdot cell}\right] = \frac{(cGlc_0 - cGlc_1) \cdot \mu \cdot 10^9}{CC_1 - CC_0}$$

In Equation 13 the determination of the lactate production rate qLac is shown.

Equation 13: Calculation of the qLac. Difference of lactate concentration cLac [g/l] multiplied by the specific growth rate μ [d^{-1}] and multiplied with the proportional factor 10^9 divided by the difference of the cell concentration CC [cells/ml]

$$qLac\left[\frac{pg}{d \cdot cell}\right] = \frac{(cLac_1 - cLac_0) \cdot \mu \cdot 10^9}{CC_1 - CC_0}$$

The determination of the ammonia production rate qNH_4 was done according to Equation 14

Equation 14: Calculation of the qNH_4. Difference of Ammonia concentration c [g/l] multiplied by the specific growth rate μ [d^{-1}] and multiplied with the proportional factor 10^9 divided by the difference of the cell concentration CC [cells/ml]

$$qNH_4 \left[\frac{pg}{d \cdot cell}\right] = \frac{(c_1 - c_0) \cdot \mu \cdot 10^9}{CC_1 - CC_0}$$

4.4. Fluorescence in situ hybridization materials and methods

The described approach is based on (LATTENMAYER et al., 2006).

4.4.1. Fluorescence in situ hybridization materials

Reagents:

Dextran sulphate: Roth, Cat. No. 5956.3

Herring Sperm DNA, Roche, Cat. No. 223646

Ribonucleic acid, transfer from baker's yeast (*S. cerevisiae*), Sigma Aldrich, Cat. No. R5636, 10 mg/ml

Ethanol: Emplura Ethanol absolute, Merck, Cat. No. 8.18760.2500

Demecolcine, Sigma, Cat. No. D-6165

Pepsin, Roche, Cat. No. 108057

Tween 20: Roth, Cat. No. 9127.2

Blocking Reagent, Roche, Cat. No. 11096176001

Formaldehyde 37 %, histological grade stabilized with Methanol, Sigma Aldrich, Cat. No. 533998

Formamide, Merck, Cat. No. K.4012.9584 940

Propidium Iodide Stock Solution 25µg/ml

Mounting Medium: Invitrogen Slow Antifade Kit, Cat. No. S-2828

Kits:

DIG High Prime, Roche, Cat. No. 11585606,

Antibodies:

Mouse Anti DIG Antibody, Roche, Ca. No. 1333062

Anti-Mouse FITC conjugated antibody developed in Goat, Sigma, Cat. No. F-2012

Anti-Goat FITC conjugated antibody developed in rabbit, Sigma, Cat. No. F-7367

Buffers:

1M Sodiumphosphate pH 7.0

TE-Buffer:

10 mM Tris

1 mM EDTA

pH 7.5

MAB Buffer:

150 mM NaCl

100 mM Malic acid

pH 7.5

Washing buffer for dot blot: MAB buffer + 0.3% Tween 20

Blocking buffer for dot blot: MAB buffer + 1% (w/v) blocking reagent

20 x SSC:

3 M NaCl

0.3 M Sodium Citrate·$2H_2O$

Probe Stock solution:

50% (v/v) Formamid

10% (w/v) Dextransulphate

50 mM Sodium Phosphate pH 7.0

10 % (v/v) 20 x SSC

Herring sperm DNA solution 10 µg/µl:

10 mg Herring Sperm DNA dissolved in 1 ml of HQ-Water

3 M Sodium acetate pH 5.2

Phosphate buffered saline (PBS):

2.0 mM KH_2PO_4

10 mM $Na_2HPO_4 \cdot 2H_2O$

2.7 mM KCl

137 mM NaCl

Demecolcine Solution (Demecolcine, adjusted with PBS to a final concentration of 100 µg/ml)

75 mM KCl solution

Fixation Solution: 3 parts methanol with 1 part glacial acetic acid, both p. a. grade

6 M HCl

70 % Ethanol

90 % Ethanol

0.02 % Pepsin in 10 mM HCl (0.01 g Pepsine dissolved in 50 ml of 10 mM HCl)

2 x SSC:

0.3 M NaCl

30 mM Sodium Citrate·$2H_2O$

PBS containing 50 mM $MgCl_2$

PBS containing 50 mM $MgCl_2$ with 1% of Formaldehyde

TNT – Buffer:

150 mM NaCl

100 mM TrisCl

0.05 % Tween 20

pH 7.5

TNB – Buffer:

150 mM NaCl

100 mM TrisCl

0.5 % (w/v) Blocking Reagent

pH 7.5

2 x SSC / 50 % Formamide Buffer

Antibody Solution 1: Mouse Anti DIG Antibody (diluted 1:250 in TNB Buffer)

Antibody Solution 2: Anti Mouse FITC conjugated antibody developed in Goat diluted 1:64 in TNB Buffer

Antibody Solution 2: Anti Goat FITC conjugated antibody developed in rabbit diluted 1:64 in TNB Buffer

Equipment

Centrifuge: Thermo Scientific, Heraeus Megafuge 16

Slides: Carl Roth, Cat No. H686

Cover slip:

20 x 20 mm: Menzel Gläser, Thermo scientific

24*60 mm VWR, Cat. No. ECN 631-1575

Coplin jars

Shaker: Heidolph Rotamax 120

Water bath

Rubber cement: Fixogum, Marabu

Oven: Thermo scientific, Heraeus T6

Typhoon fluorescence scanner

Confocal microscope: Leica SP5 II laser scanning confocal microscope

4.4.2. Preparation of the Probes by DIG-Klenow-labeling

For the labelling of the FISH probes, 3 µg of plasmid DNA were used and diluted with UPW to a final volume of 16 µl. This probe was then denatured for 10 minutes at 95°C. After the denaturation step, the sample was immediately chilled on ice, carefully mixed with 4 µl of DIG-High-Prime mix and incubated at 37 °C overnight.
The reaction was stopped by incubation for 10 minutes at 65 °C. As next step the probes were precipitated by the addition of 50 µg of herring sperm DNA and 50 µg of Yeast t-RNA per µg of plasmid DNA in a total volume of 54 µl water. Then 6 µl of 3 M sodium acetate pH 5.2 followed by 150 µl of absolute alcohol was added and everything was incubated on ice for 30 minutes. After this incubation step, it was centrifuged for 30 minutes at 4 °C at 13,000 g. The supernatant was discarded and the pellet was air dried until no ethanol was left. To reconstitute the labelled DNA, the pellet was dissolved in 300 µl of TE-Buffer and stored at 4°C in the dark.

4.4.3. Dot Blot

Dot Blot assay was used to test the labelling of the DNA probes as wells as the detection setup with fluoresceinisothyocyanate (FITC) conjugated antibodies.

For a dot blot, a 1:10 dilution series (10 / 100 / 1000 / 10000) of the labeled DNA was prepared and 1 µl of each dilution step was spotted on a nylon membrane. The DNA was then cross-linked with the membrane at 120 °C for 30 minutes in the oven. As next step, the membrane was briefly washed with washing buffer and then incubated in blocking buffer for 30 minutes. Every further incubation step was carried out at room temperature and under light protection. The membrane was washed two times with washing buffer and incubated with antibody solution 1 for 30 minutes, washed two times with washing buffer, incubated with antibody solution 2 for 30 minutes, washed two times with washing buffer, incubated with antibody solution 3 for 30 minutes and again washed two times with washing buffer. The membrane was then analyzed by fluorescence emission after excitation with the 488 nm laser with the typhoon fluorescence scanner.

4.4.4. Preparation of metaphase spreads

For the preparation of metaphase spreads, a dense cell culture was split 1:2 or 1:3 to a resulting volume of 50 ml and left overnight to reach a population with maximum growth rate. To stop the dividing cells in the metaphase of the cell cycle, 0.2 µg

Demecolcine per ml cell culture suspension were added and the cells were incubated for 4 to 6 h.

After the incubation the cells were harvested into a 50 ml centrifugation tube, centrifuged at 188 g for 10 minutes and the supernatant was discarded. The cell pellet was suspended by drop wise addition of 5 ml 75 mM KCl solution and incubated at 37 °C for 20 minutes. Now the cell suspension was carefully transferred into a 10 ml centrifugation tube, centrifuged with 188 g for 5 minutes and the supernatant was discarded. From now on, the cell suspension had to be handled with great care, to avoid bursting of the swollen cells during the preparation. The cells were as next step fixated by the drop wise addition of 5 ml fixation solution and incubation at 4 °C for 20 minutes. After the incubation time the cells were centrifuged (180 g for 5 minutes), suspended in 5 ml fixation solution by drop wise addition, centrifuged (180 g for 5 minutes) again and suspended again by the drop wise addition of 5 ml fixation solution. As last step, the cell suspension was centrifuged (180 g for 5 minutes) and the pellet was suspended in 1 or 2 ml of fixation solution, to reach a density of $1\text{-}2 \cdot 10^6$ cells per ml. This suspension was stored at -20 °C.

For the metaphase spreads, the slides were washed for 30 minutes in 6 M HCl, washed three times with water and incubated in absolute ethanol for 30 minutes and air dried. A critical parameter for the quality of the metaphase spread is the evaporation of the fixation medium and therefore the cold cell suspension was dropped on the cleaned and angular positioned slides from 20 cm height and air dried. The quality of the metaphase spread was assured by a view on them via the light microscope. Additionally the place of the spot was marked by graving into the slide with a diamond knife.

4.4.5. Pretreatment of the Metaphase-Spreads

RNAse treatment

RNAse treatment was performed to reduce the background. Therefore RNAse A was diluted to 100 µg/ml in 2 x SSC buffer and 100 µl were spotted on every slide, covered with a coverslip and incubated in humid atmosphere for 1 hour at 37°C. After the incubation the slides were washed three times for 5 minutes in 2 x SSC buffer. Every washing step was carried out in a coplin jar at room temperature and under shaking. As next step the slides were dehydrated in an ethanol series by the incubation in 70 % ethanol for 3 minutes, 90 % ethanol for 3 minutes and absolute

ethanol for 3 minutes. Now the slides were air dried and a pepsin treatment was performed to digest cellular proteins.

Pepsin treatment

100 µl of prewarmed (37 °C) 0.02 % Pepsin solution in 10 mM HCl were spotted on each slide, covered with a cover slip and incubated in humid atmosphere for 10 minutes at 37 °C. After this treatment the slides were washed two times for 5 minutes in PBS, 5 minutes in PBS with 50 mM $MgCl_2$ and 10 minutes in PBS with 50 mM $MgCl_2$ and 1% formaldehyde and briefly two times with PBS. Now the slides were again dehydrated in ethanol series as described before and air dried.

4.4.6. Hybridisation

For the hybridisation the labelled DNA probes were diluted 1:5 in probe stock solution, well mixed and 25 µl of the probe were spotted on the metaphase spread. The area was then covered with a cover slip and sealed with rubber cement. For denaturation the slides were heated in the oven for 5 minutes at 90 °C and incubated overnight in humid atmosphere at 37 °C.

4.4.7. Detection

For the detection of the probes first of all the rubber cement sealing and the cover slips were removed from the slides, and the slides were washed four times with 2 x SSC buffer containing 50 % formamide under shaking at 45 °C in the coplin jar in the water bath. All further washing steps were also carried out in coplin jars under shaking at room temperature. Now the slides were washed five times for 2 minutes with 2 x SSC buffer and 5 minutes in TNT buffer. To block unspecific binding of the antibodies 100 µl of blocking buffer were spot on the slide, covered with a coverslip and incubated in humid atmosphere at 37 °C under shaking. After 30 minutes the coverslips are removed, 100 µl of antibody solution 1 were spot on the slides and incubated under shaking at 37 °C for 30 minutes in humid atmosphere. All further steps were from now carried out under light protection. The slides were washed three times for 5 minutes in TNT buffer, 100 µl of antibody solution 2 were spot on the slide, covered with a cover slip, incubated for 30 minutes under shaking at 37 °C in humid atmosphere, washed three times with TNT buffer, 100 µl of antibody solution 3 were spot on the slide and also covered with a cover slip and incubated at 37 °C for 30 minutes under shaking in humid atmosphere. As last steps the slides were

washed three times for 5 minutes in TNT washing buffer, dehydrated in an ethanol series as described in the RNAse treatment and air dried.

For detection chromosomes were counterstained by the addition of 100 µl Propidium Iodide solution on the slide, covered with a cover slip and incubated for 10 minutes at 37 °C under shaking and in humid atmosphere.

Finally slides were air dried, covered with 10 µl mounting medium sealed with a cover slip and examined with the Leica SP5 II laser scanning confocal microscope. For the excitation of the FITC the 488 nm laser was used, while for the excitation of the Propidium Iodide the 561 nm laser was used.

4.5. Flow cytometry based materials and methods

4.5.1. Materials for flow cytometry

Reagents

DAPI: 4',6-Diamidino-2-phenylindoledihydrohloride, Sigma Aldrich, Cat. No. D9542, Prepared as 5 µg/ml stock solution (100 x)
Fetal calf serum (FCS): Fetal bovine serum Gold, PAA, Cat. No. A15-152
Antibiotics: Penicillin/Streptomycin (100 x), PAA, Cat. No. P11-010
Ethanol: Emplura Ethanol absolute, Merck, Cat. No. 8.18760.2500

Buffers:

Phosphate buffered saline (PBS):

- 2.0 mM KH_2PO_4
- 10 mM $Na_2HPO_4 \cdot 2H_2O$
- 2.7 mM KCl
- 137 mM NaCl

Flow cytometry buffer:

- 100 mM Tris,
- 0.1 % Triton X-100,
- 2 mM $MgCl_2$,
- pH 7.40

70 % Ethanol
Sheath Liquid: Coulter Isotone II, Beckmann Coulter, Cat No. 8448011
Coulter Clenz, Beckmann Coulter

Consumables

Centrifugation tubes 10ml: 110 x 16 x v x cent tube with screw cap, thermo scientific
FACS tubes: BD Falcon, Polystyrene Round Bottom Centrifuge Test Tube with Dual-Position Snap Cap, 12mm Diameter x 75mm Length, 5mL Capacity Cat. No. 352054
FACS Tubes with mesh: BD Falcon Polystyrene Round Bottom Centrifuge Test Tube with Cell Strainer Cap, 12mm Diameter x 75mm Length, 5mL Capacity, Cat. No. 352235

Equipment

Centrifuge: Thermo Scientific, Heraeus Megafuge 16
Flow cytometer: Gallios™, Beckmann Coulter
Cell sorter: MoFlo® Astrios™ Beckmann Coulter

Antibodies

Anti-Human IgG (γ-chain specific) − R-Phycoerythrin antibody produced in goat affinity isolated antibody, buffered in aqueous solution, Sigma Aldrich, Cat. No. P9170

Software

Kaluza V1.2, Beckmann Coulter

4.5.2. Intracellular product formation using immunofluorescence staining

For this analysis $2 \cdot 10^6$ cells were taken, centrifuged at 188 g for 10 minutes and the supernatant was discarded. As next step the cells were fixed by the addition of 70 % ethanol. Therefore the cell pellet was shaken up and 1 ml of 70 % ethanol was added slowly under gently shaking to avoid clumping. After fixation the cells were stored at 4 °C.

The next step in immunofluorescence staining was the remove of the 70 % ethanol by centrifugation. All centrifugation steps were carried out at 188 g for 10 minutes. The cell pellet itself was suspended in 1 ml of flow cytometry buffer, centrifuged again and the supernatant was discarded. Now the cell pellet was suspended in 1 ml of flow cytometry buffer containing 10 % FCS, centrifuged again and the supernatant was discarded. As blocking step to inhibit unspecific binding of the staining antibody, the pellet was suspended in 100 µl of flow cytometry buffer containing 10 % FCS and incubated at 37 °C for 30 minutes. After the incubation time 100 µl of a 1:50 diluted anti human IgG γ-chain conjugated to R-Phycoerythrin (PE) in flow cytometry buffer

containing 10 % FCS was added under gently shaking and incubated for 1 hour at 37°C. As next step 1 ml of flow cytometry buffer was added and the samples were centrifuged, the supernatant was discarded and the cell pellet was finally suspended in PBS supplemented with 50 µg/ml DAPI and transferred into Flow cytometer tubes for analysis.

For the flow cytometry analysis the Gallios Flow cytometer from Beckman Coulter was used. PE was excited with the 488 nm laser and detected in fluorescence channel 2 (575 nm, bandwidth 30 nm), while the DAPI was excited with the 405 nm laser and detected at fluorescence channel 9 (450 nm, bandwidth 40 nm), both on a logarithmic scale. The power of the laser and the gain of the photomultipliers were adjusted in such a way, that the negative control was located between 10^0 and 10^1. The signal in forward and side scatter was also adjusted in a way, so that the sample was located in the first quadrant on a linear scaled plot of these two parameters. For the evaluation of the data the software Kaluza V1.2 (Beckmann Coulter) was used.

The living cells were gated based on the signal in the forward and in the side scatter channel with an elliptic gate. As next step, an overlay plot of the different measured samples was created out of the different fluorescence intensities. Additionally a second gate was set in such a way, that only 1% of the negative control was contained within. This was done in the overlay plot. The higher the percentage of the samples within this second gate, the more living cells remain producing. Applying this gate to the fluorescent intensity, the geometric mean fluorescence was determined. The calculation of this value is shown in Equation 15.

Equation 15: The geometric mean Gmean for a sample size of n is defined as the n^{th} root of the product of n samples.

$$Gmean = \sqrt[n]{\prod_{i=1}^{n} n_i}$$

4.5.3. Determination of GFP positive cells

For the determination of GFP positive cells, $2 \cdot 10^6$ cells were taken, centrifuged for 10 minutes at 188 g, the supernatant was discarded and the cells were suspended in 1 ml of PBS, centrifuged again for 10 minutes at 188 g and the supernatant was again discarded. Finally the cells were suspended in 500 µl of PBS containing 50 ng/ml DAPI.

As the GFP itself can be excited at 488 nm, this laser was used for the determination of the forward and side scatter as for the GFP fluorescence excitation. The detection for GFP was done by fluorescence channel 1 at 525 nm with a bandwidth of 40 nm, while the DAPI was excited with the laser of 405 nm and detected at 450 nm with a bandwidth of 40 nm.

The data analysis was done with the Kaluza software according to the determination of the intercellular product content.

4.5.4. Sorting of GFP negative cells

Within this study, bulk cell sorting was tested. After the RMCE no GFP should be present in the resulting recombinant cells. Therefore $2 \cdot 10^6$ cells were transfected, as described in the transfection section. They were kept in T25 flasks for 1 or 2 weeks and media exchanges were performed according to the growth of the cells. The cells were split once before they were sorted. Directly before sorting, antibiotics were added to the media (Penicillin and Streptomycin, 1 x). As preparation for sorting, cells were first centrifuged for 10 minutes at 180 g and suspended in 1 or 2 ml of antibiotic containing medium, so that the total cell concentration did not exceed $2 \cdot 10^7$ cells per ml. For the sorting procedure itself, the cells were pressed through a cell strainer and sorted into a 50 ml tubes, containing 5 ml of cell culture medium with antibiotics.

To determine the desired population, a combination of three gates was used. The first gate was set on the living cells, determined by forward and side scatter signal according to the flow cytometry analysis without sorting. A second gate was used to eliminate the doublets, which was set on fluorescence signal intensity plotted against the signal area. The third gate was set on the fluorescence signal, where it contained all GFP negative cells. The GFP fluorescence was determined with the same parameters as in flow cytometry analysis.

After the sorting, the cells were kept for one hour under shaking in the 50 ml tube and transferred afterwards into a T25 flask.

5. Results

5.1. Verification of the vector

The vectors used for the transfection of the mammalian cells contain only the GOI between the two FRT sites without a promoter. This is known as the promoter trap strategy (TURAN et al., 2011) because the product should only be produced, if the RMCE occurs. The scheme of this strategy is shown in Figure 4, where the strong Cytomegalovirus promoter (pCMV) is located outside of the left and right FRT site. Only the part between these two sites will be exchanged, so the pCMV can enable the transcription of the GOI. If the GOI would be integrated anywhere else in the genome, no product should be transcribed because of the lack of a promoter.

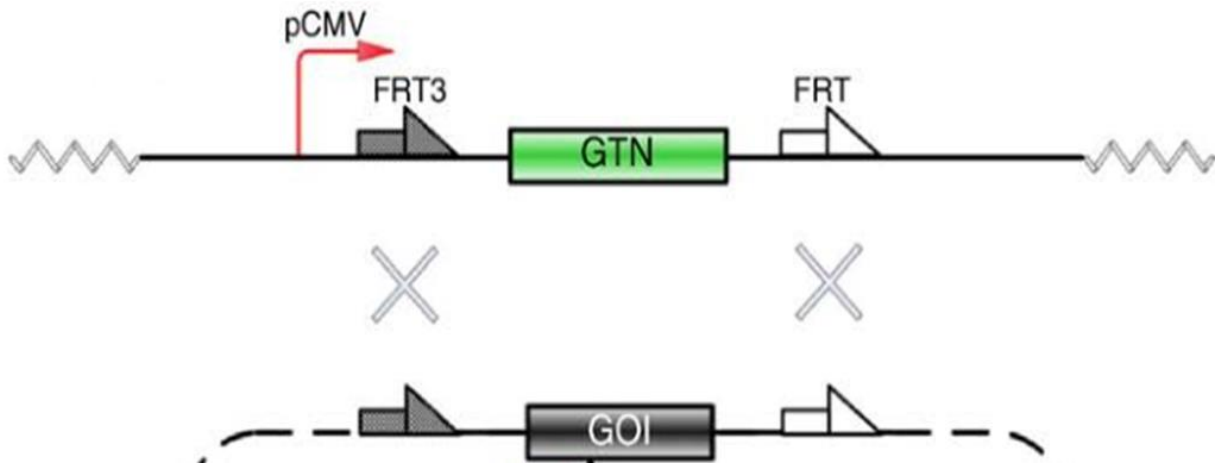


Figure 4: Scheme of the Promoter trap strategy: The strong constitutive promoter of the Cytomegalovirus (pCMV) is set outside of the two Flipase recognition target sites (FRT3 and FRT). Sufficient expression of the GOI should only occur after a sufficient cassette exchange, because the GOI is then placed under the control of the strong promoter.

This strategy was done by the use of a special integration plasmid for the two heterospecific FRT sites (FRT3 and FRT) with the pCMV placed upstream to the FRT3 site, which flanks the 5' border of the recombination cassette. Additionally the integration plasmid also harbors two Scaffold/matrix attachment regions (S/MAR) outside of the recombination cassette, which should create an open chromatin region and prevent gene silencing through chromatin condensation (MIELKE et al., 1990). Another positive effect of these elements is an easier access of the enzyme Flipase to these recombination sites, which results in an improvement of the RMCE reaction (QIAO et al., 2009).

The vector for transfection contains a FRT3 and a FRT site and in between them the GOI. The GOI are the two scFc antibodies 2F5 and 3D6 which are both directed against gp41 of HIV-1. The detailed plasmid map of these two vectors is demonstrated in Figure 5.

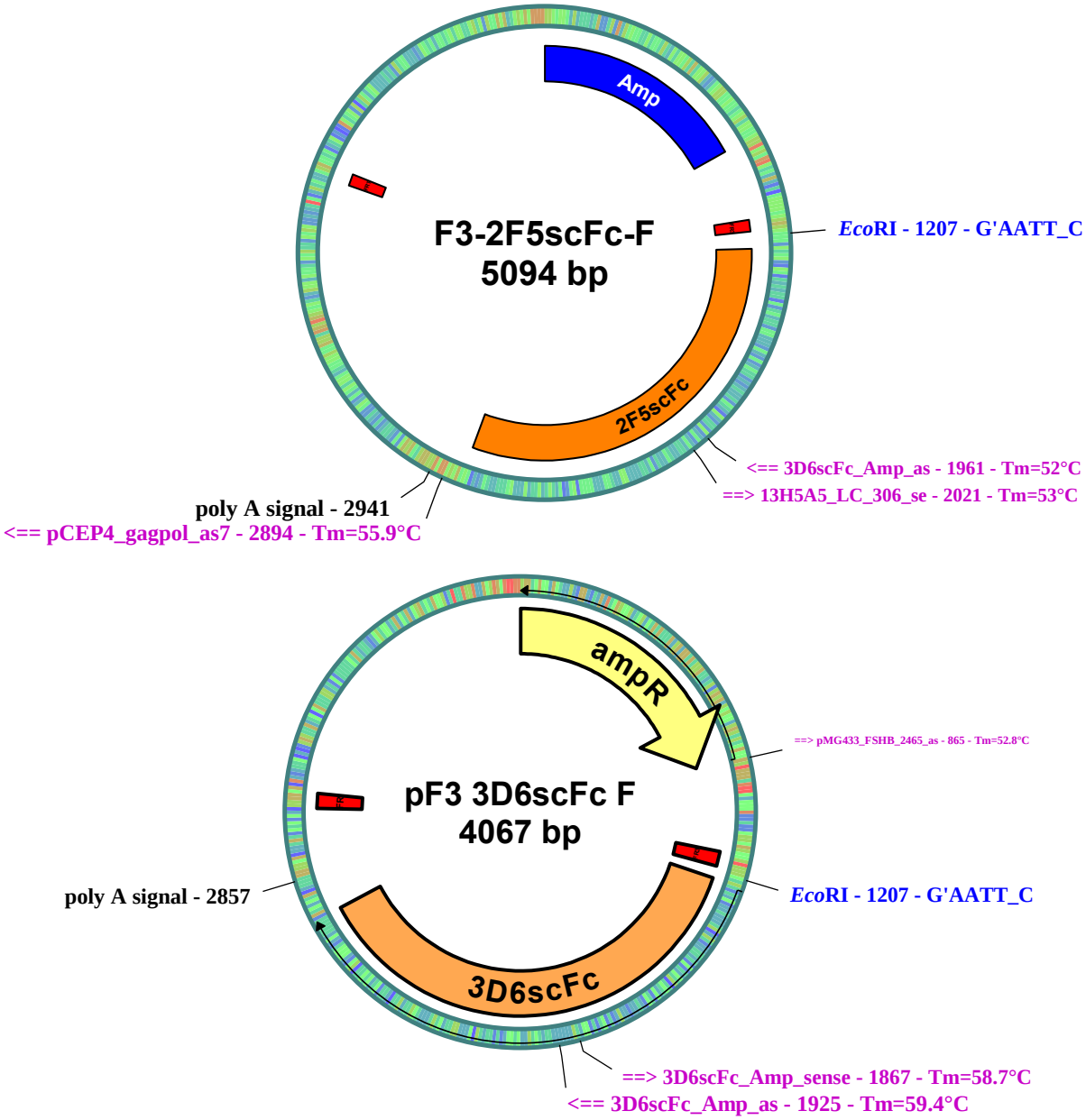


Figure 5: Detailed plasmid maps of the two plasmids used for the RMCE to produce either 2F5scFc or 3D6scFc. All used binding sites for primers and cutting sites for restriction enzymes are marked.

Before the RMCE was performed, these two vectors were checked with a control digestion and also by sequencing. The linearized form of the vector pF32F5scFcF had a size of 5094 bp while the vector pF33D6scFcF had a size of 4067 bp. The result of this control digest can be seen in Figure 6. For the control digestion the enzyme EcoRI-HF was used. The restriction site is shown in the plasmid map.

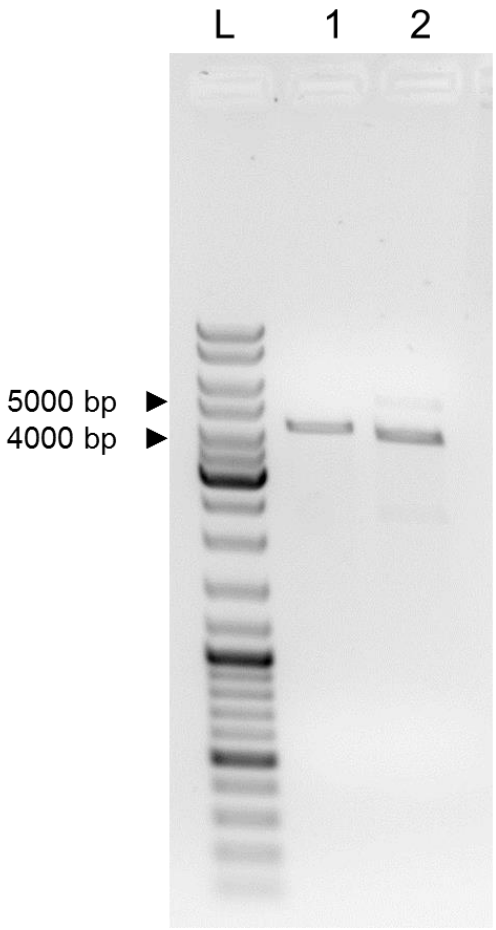


Figure 6: Control digestion of both vectors with the enzyme EcoRI. Both vectors show a length between 4 and 5 kbp, which match the theoretical size.

For the sequencing reaction the primers listed Table 9 were used.

Table 9: List of all primers used for the sequencing reaction of the two plasmids. The binding position is shown in the detailed plasmid map

Name	**Sequence**	**Tm**
pMG433_FSHB_2465_as	GGAAATGTTGAATACTCATACTC	52.8 °C
3D6scFc_Amp_sense	CCCAAGCTGCTGATCTACAA	59 °C
3D6scFc_Amp_as	GATGGTCAGGGTGAACTCG	59 °C
13H5A5_LC_306_se	CGCCACCTACTACTGCCAG	64 °C
pCEP4_gagpol_as7	GCAATAGCATCACAAATTTCACA	64 °C

The obtained sequences were aligned to the sequences of the plasmid map by using the web application clustal w, provided by the European Bioinformatics Institute.

The sequence of the vector pF32F5scFcF was confirmed by the sequencing reaction, while the vector pF33D6scFcF showed some deletions. The lost bases are

highlighted in Figure 7. The FRT3 site is indicated in blue, while the destroyed start codon as well as the missing part of the leader sequence is indicated in red.

```
Theoretic       TGAAGTTCCTATACTATTTGAAGAATAGGAACTTCGGAATAGGAACTTCAAGATCCCC-T 1199
Sequencing      TGAAGTTCCTATACTATTTGAAGAATAGGAACTTCGGAATAGGAACTTCAAGATCCCCCT 462
                ************************************************************ *

Theoretic       GGCGAAGGAATTCGATATCGGTACCGTCGAGGCCGCCACCATGGACTGGACCTGGCGCAT 1259
Sequencing      GGCGAAGGAATTCGATATCGGTACCGTCGAGTCC-------CGGATCGGAATTG----AT 511
                ****************************** **        ***  ***  **    **

Theoretic       CCTGTTTCTGGTCGCCGCAGCCACCGGTGTCCACTCCGAGGTGCAGCTGGTCGAGTCTGG 1319
Sequencing      CC------------------------------------------------------------ 513
                **

Theoretic        CGGAGGACTGGTGCAGCCTGGCAGATCCCTGAGACTGTCTTGCGCCGCCTCCGGCTTCAC 1379
Sequencing       ------ACCGGTGCAGCCTGGCAGATCCCTGAGACTGTCTTGCGCCGCCTCCGGCTTCAC 567
                       ** ************************************************
```

Figure 7: Alignment between the theoretic DNA sequence of the vector and the sequence obtained by a sequencing reaction. Matches are marked with a star (*) while differences are marked by a dot (.). In blue the FRT 3 region is marked and the red marked parts shows the start codon (ATG) and the missing part of the 3D6scFc gene.

The deletion of these few base pairs was not able to detect by the control digestion because the exact size of the vector could not be determined on a 1 % agarose gel.

Furthermore the base pairs were missing at the beginning (N-terminus) of the gene, which results in the destruction of the start codon ATG. A start codon serves as starting point of the translation, which is needed for efficient protein synthesis. Therefore a new vector had to be constructed.

5.2. Vector construction

To construct the vector pF33D6scFcF again, a vector with pre-existing FRT sites, proper restriction sites and already containing poly A signal was used. This vector is named, pF3GTNF and contains a triple fusion gene consisting of GFP, thymidine kinase and neomycin phosphotransferase between the heterologous FRT sites. This is also the same vector, which was used to create the RMCE host cell lines.

The detailed plasmid map can be seen in Figure 8 where all necessary primer and restriction sites are marked. The gene for the antibody 3D6scFc, which should replace the GTN fusion gene was checked by sequencing of the vector pCIneo3D6scFc. For the sequencing reaction, the primer pair listed in Table 10 was used. As the alignment of the DNA sequences was perfect, this insert was chosen to be cut out.

Table 10: List of primers used for sequencing of the insert 3D6scFc in pCIneo

Name	Sequence	Tm
pCIneo_976sense	GGCACCTATTGGTCTTACTGA	58.1
3D6scFc_Amp_sense	CCCAAGCTGCTGATCTACAA	58.7 °C

The vector F3GTNF was digested with the enzymes NheI and KpnI. The second restriction site of the enzyme KpnI within the GTN fragment was needed, because otherwise two fragments with similar size were created, which could not be separated by agarose gel electrophoresis using 1 % agarose gels. The insert was cut out of the pCIneo3D6scFc vector by a digestion with XhoI and XbaI. The detailed plasmid map of the vector pCINeo containing the gene 3D6scFc is shown in Figure 9.

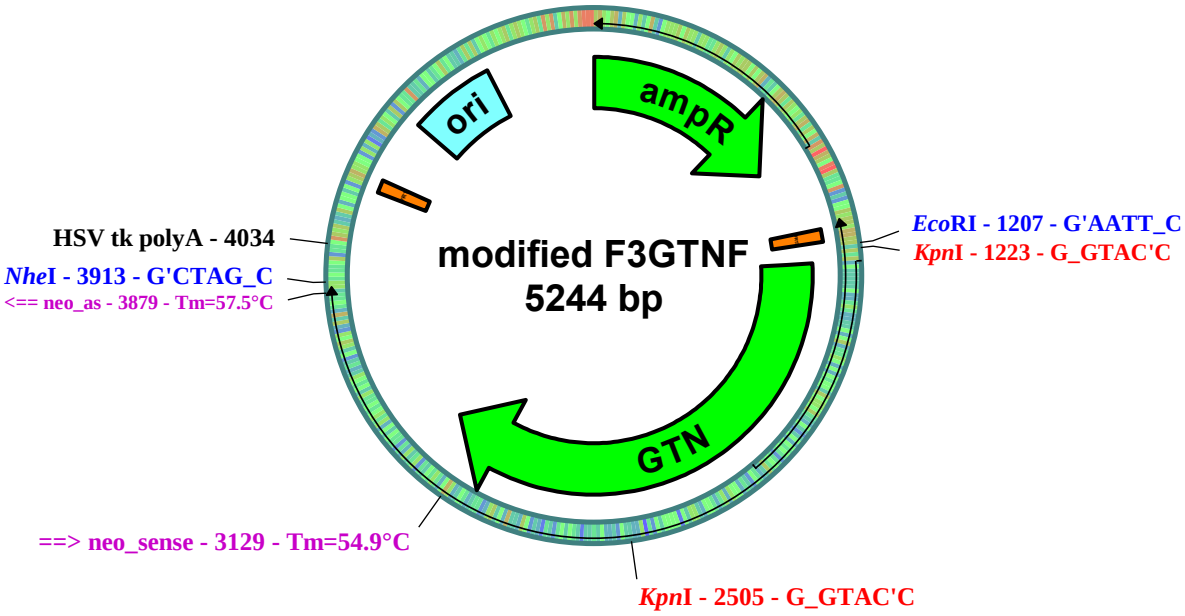


Figure 8: Detailed plasmid map of the vector pF3GTNF which was used as vector backbone for the construction of the new plasmid pF33D6scFcF. The important restriticton sites and primer binding sites are shown within this map.

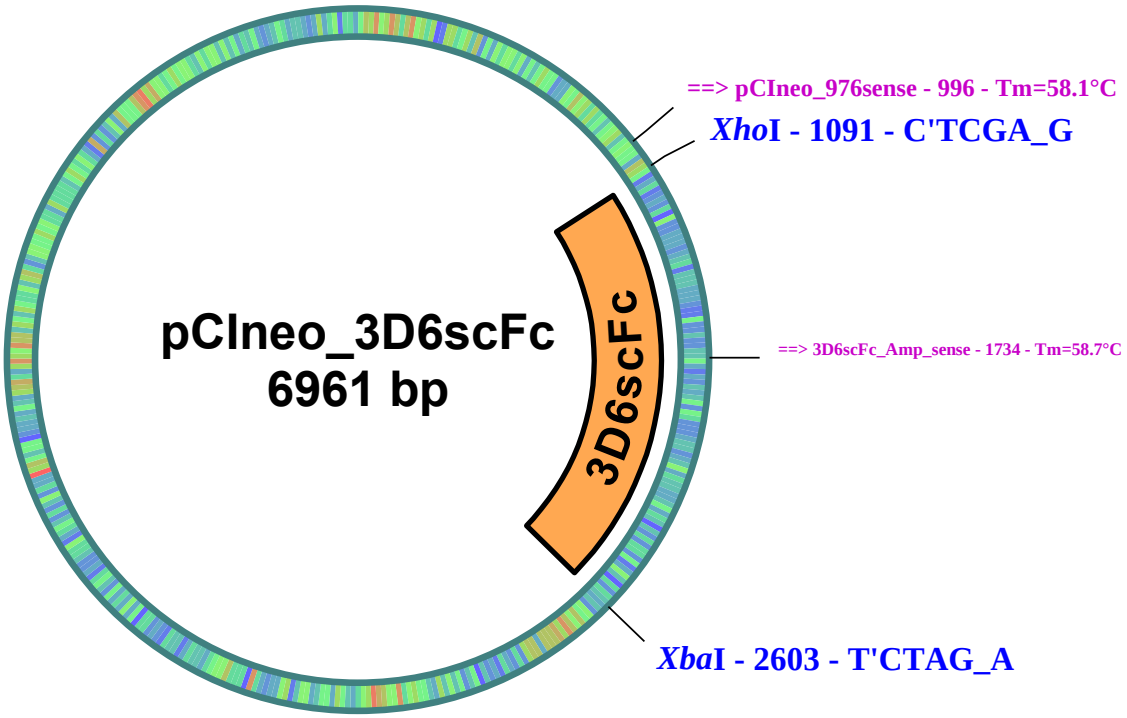


Figure 9: Detailed plasmid map of the vector pCINeo_3D6scFc. The insert 3D6scFc with the primer binding sites used for sequencing and the restriction sites are shown within this figure.

In Figure 10 the digested insert and the vector backbone can be seen in an agarose gel electrophoresis. For vector backbone and insert two lanes were used to obtain enough digested DNA for the following reactions.

The plasmid pF3GTNF was cut in three fragments, with a size of 2554 bp, 1408 bp and 1282 bp. The vector backbone which was used for the further cloning had a size 2554 bp. The pCINeo_3D6scFc was cut in two fragments with a size of 5449 bp and 1505 bp. The part containing the 3D6scFc gene had a size of 1505 bp. The desired fragments were cut out of the gel and isolated.

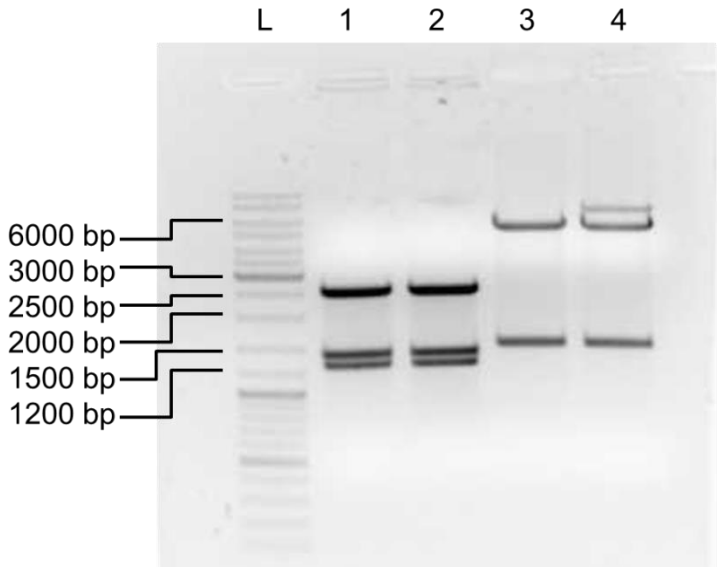


Figure 10: Picture of the preparative 1 % agarose gel electrophoresis for the construction of the vector. 1 and 2 shows the digested plasmid pF3GTNF with the 3 fragments of 2554 bp, 1408 bp and 1282 bp. The fragment of 2554 bp length represents the desired fragment for the further vector construction. 3 and 4 show the digested vector pCINeo containing the insert 3D6scFc. The fragments have a length of 5449 bp and 1505 bp. Any longer fragment shows undigested vector. The fragment with 1505 bp represents the gene for the 3D6scFc antibody and used for further vector construction. Both digestions were loaded to double the amount of digested plasmids.

The restriction enzymes of the vector backbone and the insert did not produce compatible ends and so a blunt end ligation had to be done. For a blunt end ligation the Klenow fragment of the DNA polymerase I was used, as it fills up the 5' overhang and digests the 3' overhang of the cut DNA. To prevent the blunt ends of the vector from self-ligation a dephosphorylation step was performed using the enzyme calf intestine phosphatase (CIP).

The ligation reaction was performed with three different molar ratios between vector backbone and insert. The transformation of the bacteria was done with all three ratios, but for further cloning the focus was on the ratio 1:1, because with higher ratios such as 1:3 or 1:5 the probability to ligate more inserts in the vector backbone increases. This is not wanted for this plasmid, as it is the purpose of the plasmid to carry only one copy of the 3D6scFc gene.

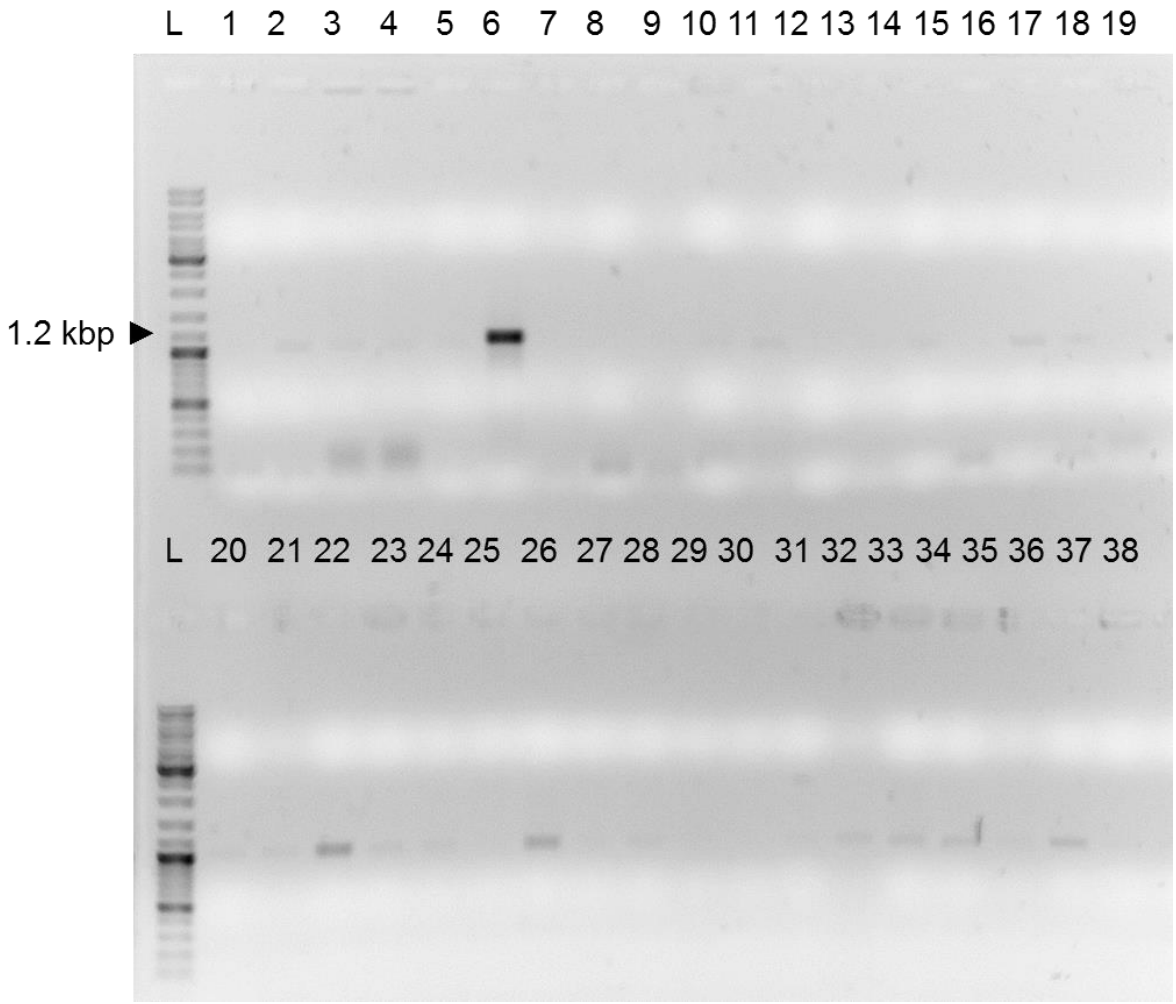


Figure 11: Agarose gel electrophoresis of the colony PCR. The desired band with a lenght of 1061 bp can be seen very well for clone 6, 19 (partly out of the focus), 22, and 26. Some of the other lanes also show very weak positive bands, which were not taken into account for further analysis.

The transformed bacteria were checked by the use of a colony PCR using the primer pair pMG433_FSHB_2465_as and 3D6scFc_Amp_as. The amplicon had a calculated length of 1061 bp. In Figure 11 the picture of the agarose gel of the colony PCR is shown. It can be clearly seen, that four clones showed a strong signal between 1000 bp and 1200 bp, which fits to the theoretical amplicon size. These clones were further selected to be grown in LB-Media supplemented with antibiotics overnight. The DNA was isolated and checked by sequencing. One clone containing the proper sequence could be identified and the DNA was used for amplification. After the plasmid DNA preparation with the midi prep kit a control digestion was done to check the quality and estimate the concentration of the DNA. The picture of these control digestion can be seen in Figure 12.

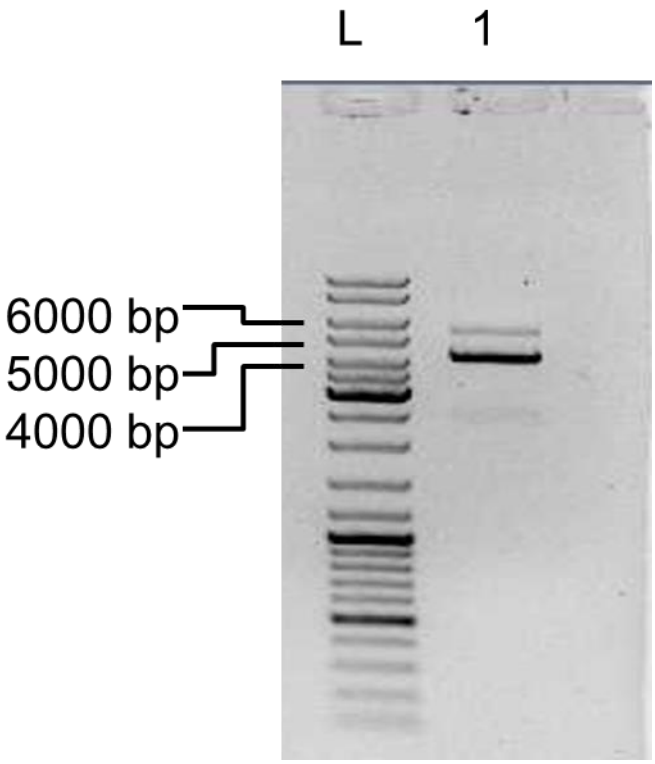


Figure 12: Control digestion of the plasmid pF33D6scFcF with the enzyme EcoRI-HF. A clear band at 4067 bp can be seen. The band above this band represents undigested plasmid.

5.3. Characterisation and cultivation of the host cell line

The used cell line for the experiments was a recombinant CHO DUKX B11 cell line, the first dihydrofolate reductase negative CHO cell line developed by (URLAUB and CHASIN, 1980). Therefore the cultivation medium (Pro CHO5, 4 mM L-Glutamine, 15 mg/L phenol red) had to be supplemented with hypoxanthine and thymidine.

To select the proper cell line for the transfection and the testing of the RMCE for protein production three different cell lines were cultivated and characterised. These cell lines already contained the heterospecific FRT sites in the genome, as they were designed according to the redefined strategy described in (QIAO et al., 2009). One major difference to the development is the use of a limited dilution subcloning approach. The details and challenges of the development are described in (MAYRHOFER, 2013). The selected clones were named CHO/RMCE/2-4/E. From this cell line three clones were compared prior to transfections, which were named Sp125, F12 and B1. The cell line Sp125 was adapted to the spinner flask, and the two cell lines F12 and B1 were the subclones out of the Sp125 cell line.

These cell lines contained a fusion gene consisting of GFP, neomycin phosphotransferase and a herpes virus thymidine kinase gene. All three genes were inserted to enable different purposes. The GFP was inserted as reporter gene, as it can be easily detected in the fluorescence microscope as well as with the flow cytometer. Additionally this gene should be replaced after a successful RMCE with the gene of the antibody 2F5scFc or 3D6scFc, so these cells should not show GFP fluorescence anymore. This is especially useful for sorting, because all cells without GFP fluorescence signal can be sorted and should contain the GOI. Furthermore the positive replacement of the GOI can be seen in the fluorescence microscope and also enables quantitative analysis by flow cytometry.

The neomycin phosphotransferase was used as a selection marker in the development of the cell line and it is also used to maintain selection pressure during the cultivation. This is needed because CHO cells are known for genomic instability (WURM and HACKER, 2011). Another challenge is the loss of productivity which is reported for CHO cells in the absence of selection pressure (CHUSAINOW et al., 2009). For this reason, the media contains additionally 0.5 mg/ml G418 to keep up the selection pressure.

The third gene is the thymidine kinase of the herpes simplex virus. This gene offers the possibility of negative selection, because this enzyme is 30 to 120 fold faster in taking up the drug gancoclovir than the cellular one (FYFE et al., 1978). The drug ganciclovir is an analogue to guanine as the chemical structure in Figure 13 shows. It is phosphorylated by the herpes simplex thymidine kinase, taken up by the cell and further phosphorylated by intercellular enzymes. The triphosphate form is incorporated instead of guanosine in the DNA and leads to chain determination in the synthesis of the DNA. The result is a growth arrest of cell carrying the herpes virus thymidine kinase in the presence of ganciclovir.

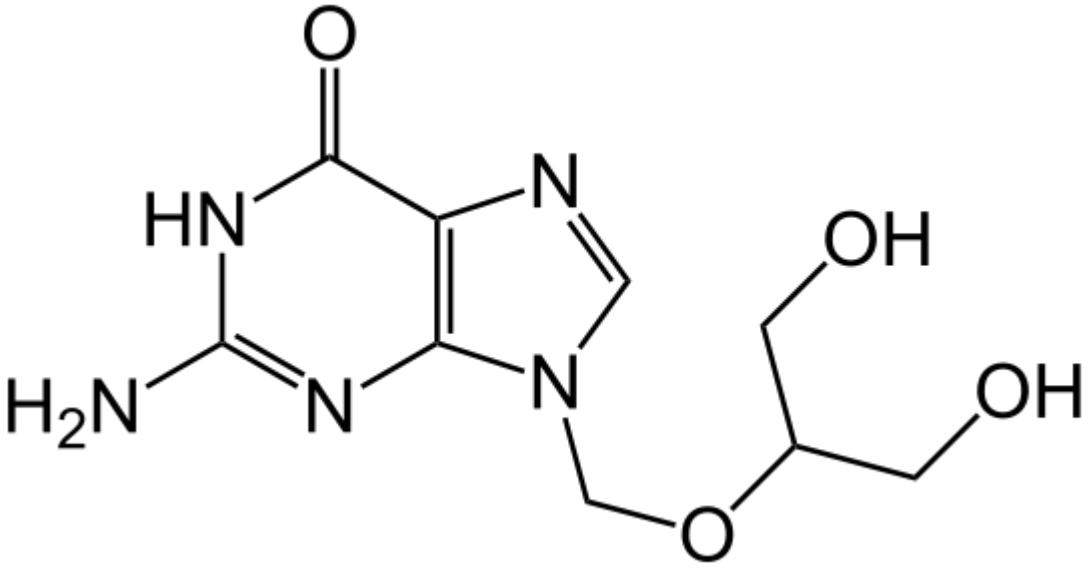


Figure 13: Structure of Ganciclovir. The phosphorylation of the hydroxyl group leads to uptake of the drug via the herpes simplex thymidine kinase inside of the cell.

This three cell lines were cultivated in spinner flasks and the specific growth rate and the viability was monitored. All three cell line showed comparable viabilities around 90 % while the mean specific growth rate differed a lot. The mean specific growth rate of the three cell lines is shown in Figure 14. The cell line Sp125 showed a significant higher mean specific growth rate than the two subclones. Although the subclone F12 showed also a higher mean specific growth rate than the clone B1. This clear ranking was also observable during the whole cultivation in the spinner flask, although all three cell lines adapted more and more to the cultivation in the spinner flask and therefore the specific growth rate generally increased within the culturing time.

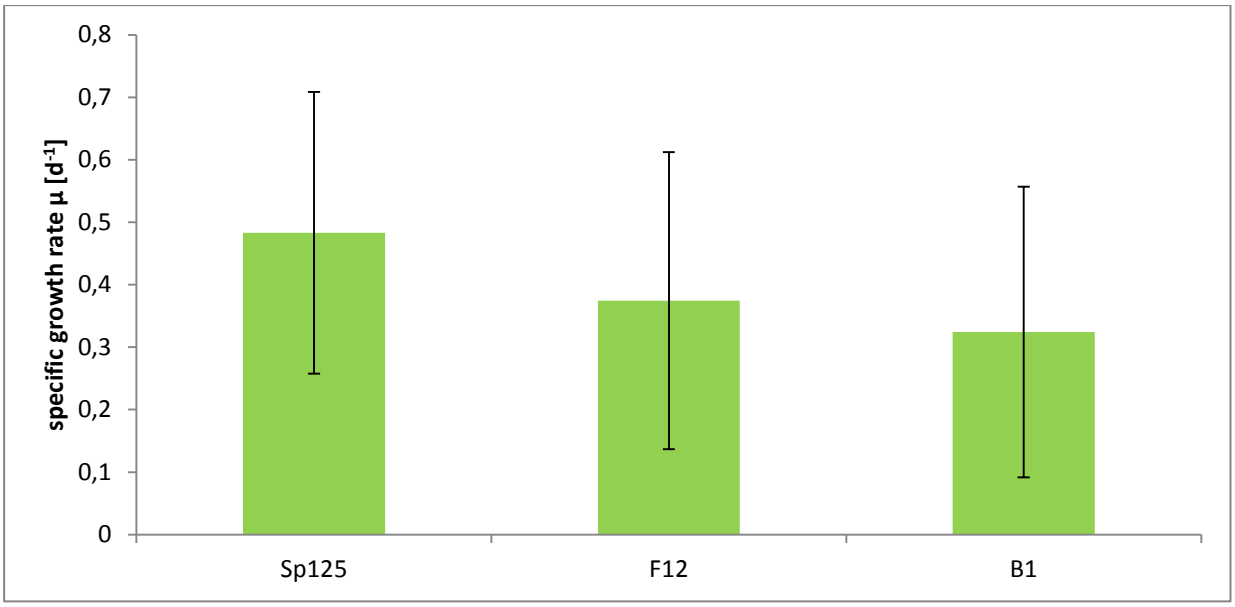


Figure 14: Mean specific growth rate of the three cell lines during the 53 days of cultivation in the spinner flask. The error bars represent the standard deviation.

This insert was also verified with molecular biology techniques. Therefore genomic DNA was isolated with the Qiagen blood mini kit, and a PCR with specific primers for the neomycin phosphotransferase was done. The sequence of the primer pair is shown in Table 11. The amplified gene segment of the neomycin phosphotransferase had a size of 791 bp. In Figure 15 the PCR products were separated by an agarose gel electrophoresis, and all three cell lines contained the desired product.

Table 11: Primer pair used for the detection of the neomycin phosphotransferase in the genomic DNA

Name	**Sequence**	**Tm**
neo_sense	ATTGAACAAGATGGATTGCAC	61.4 °C
neo_as	TCAGAAGAACTCGTCAAGAAGG	63 °C

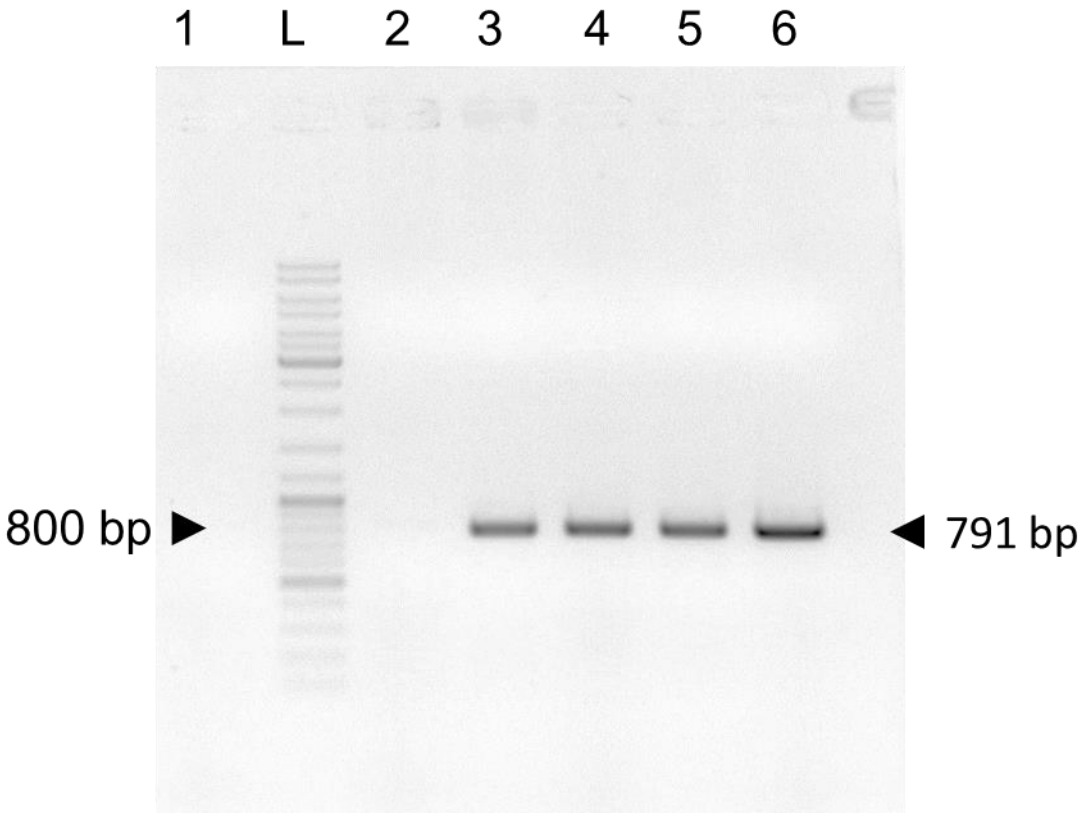


Figure 15: Agarose gel electrophoresis of the PCR products with neomycin phosphotransferase specific primers. 1 contains the non template control and 2 the cell line DUKX B11, which show no product. 3 is clone Sp125, 4 clone F12 and 6 clone B1. In 6 the plasmid pF3GTNF, which was used to transfect the cell and therefore as positive control is shown.

Another interesting point to consider was the presence of Flipase in the RMCE host cell lines. Previous experiments as described in (MAYRHOFER, 2013) also showed a resistance to the selection pressure without cotransfection of the plasmid encoding for the enzyme Flipase. Basically there are two theories to describe this, first it was a random integration behind an endogenous promoter or the Flipase was still present as protein after the previous rounds of RMCE. To test the random integration hypothesis, RNA was isolated from all three cell lines, reverse transcribed into cDNA and a PCR reaction with specific primers was done. In Table 12 the sequence of the used primer pair is listed. The detailed plasmid map containing the position of the primers is shown in Figure 16. The used enzyme was an optimized variant of the enzyme Flipase as described in (RAYMOND and SORIANO, 2007). The optimized Flipase further shows enhanced activity at 37 °C due to some point mutations in the amino acid sequence.

Table 12: Primer pair used for the detection of the Flipase cDNA

Name	Sequence	Tm
Flp_sense	GGCCACCTTCATGAGCTACA	65.2 °C
Flp_as	GAATGTGGCCAGGAACAGGA	67.4 °C

In Figure 17 the agarose gel electrophoresis of the PCR with the Flipase specific primers is shown. The amplicon should have a size of 358 bp. Clearly no Flipase could be detected in any cDNA of the clones. As positive control the plasmid was used in the reaction. As a control of the cDNA preparation also a neomycin phosphotransferase specific PCR was done and in every preparation the neomycin phosphotransferase gene was detectable (data not shown).

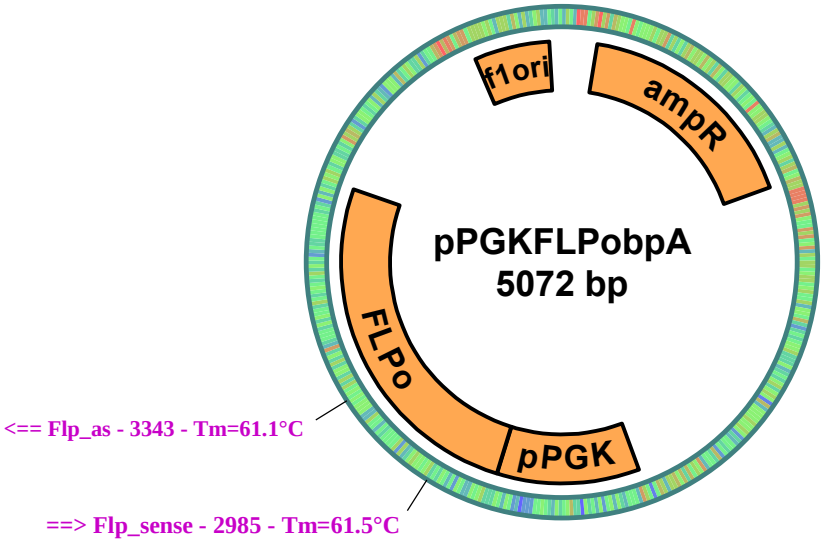


Figure 16: Plasmid map for the Flipase containing plasmid obtained by Addgene (plasmid 13793). AmpR encodes for the β-Lactamase and f1 is a phage origin to amplify the plasmid by the use of bacteriophages. The origin for amplification in *E.coli* is located within the ampR site. The promoter pPGK is a eukaryotic promoter for the phosphoglycerolkinase. The sequence Flpo encodes for the optimised variant of the enzyme Flipase. The two primers used for the amplification are shown in violet and the amplicon has a size of 358 bp.

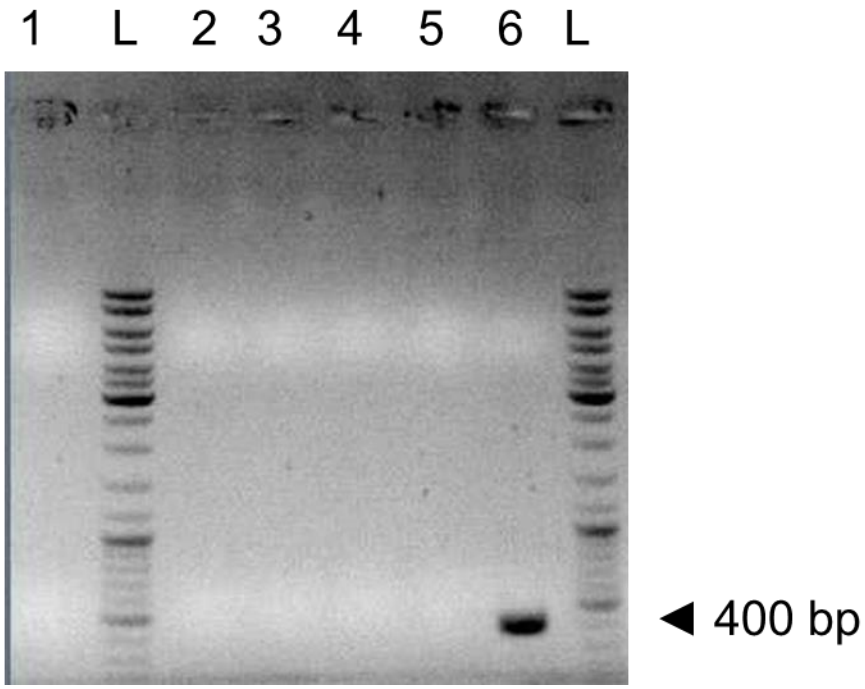


Figure 17: Agarose gel electrophoresis of the PCR reaction with the isolated cDNA and Flipase specific primer. In 1 the non-template control was put, lane 2 contained the cell line DUKX B11 as negative control, 3 contained Sp125, 4 F12 and 5 B1. In lane 6 the pFlpo plasmid is put on the gel and gives a signal at 358 bp.

To get further insight into the distribution of the GFP in the entire population, flow cytometry analysis was done. The basis of the analysis was first of all to determine living cells, based on the signal for forward and side scatter. The chosen region represents the living cells, as they have a distinct size, which is represented in the forward scatter signal as well as a distinct granularity, which is represented in the side scatter signal. Both of them should be within the first quadrant of the plot. In Figure 18 the plot is shown and the region, which represents the living cells, is marked in red. The clustering of the region is called a gate.

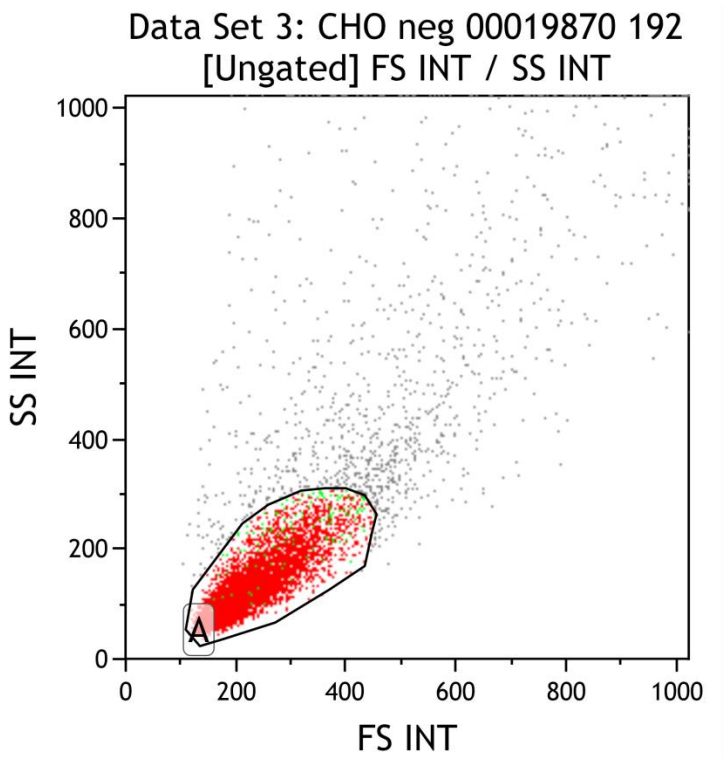
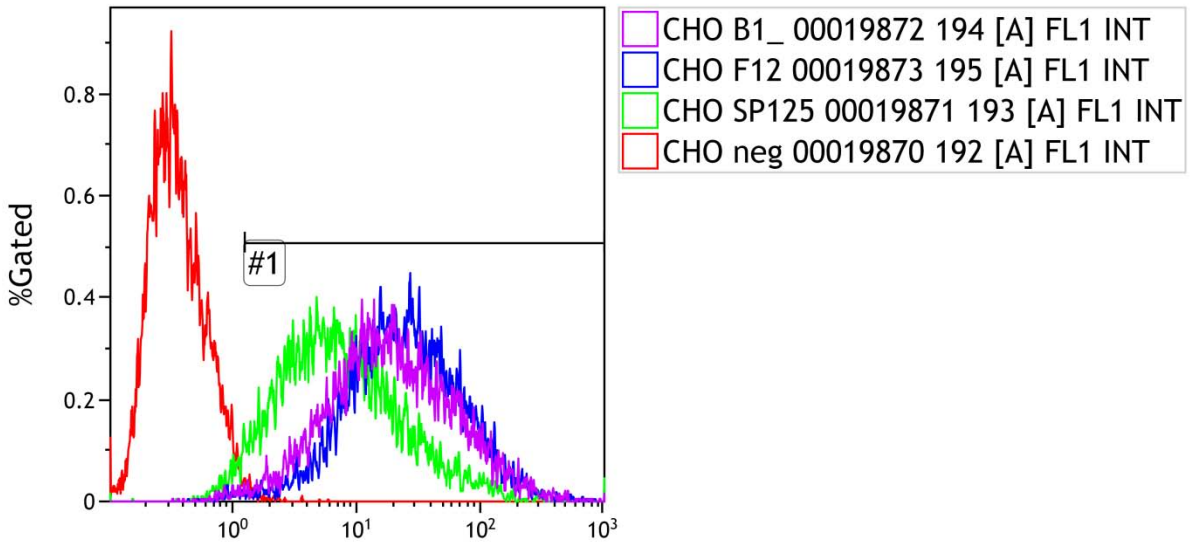


Figure 18: Plot of forward and side scatter. The gate A, represented by everything within the black border and all red dots represents living cells for further analysis. Green dots represent the cell within the overlay marker used for further analysis.

For detailed analysis the fluorescence intensity according to the GFP signal of the living cells of the different samples was plotted together in one figure and an overlay marker was set. This marker was set in such a way, that it only contained about 1 % of the negative control. The percentage of the positive populations in this second gate represented on the one hand how many cells contained GFP and on the other hand how uniformly distributed the population is. The uniformity of the distribution is represented with the three values x-mode, x-median and the geometric mean. The x-median value represents the value which is at 50 % of all values; x-mode represents the value which occurs most in the dataset and the geometric mean reflects the average for a logarithmic dataset. If all three values are similar, the population is uniformly distributed. The results of the analysis can be seen in Figure 19. According to these results the subclone F12 was used for the further transfection experiments because it showed a homogenous population with the highest mean fluorescence intensity.



Marker	Number	%Gated	X-Med	X-AMean	X-Mode	X-GMean
All	6,304	100.00	18.31	37.18	20.47	16.37
All	7,283	100.00	24.24	41.42	27.05	21.28
All	6,557	100.00	6.69	16.98	4.81	6.36
All	8,773	100.00	0.34	0.41	0.23	0.32
#1	6,229	98.81	18.62	37.62	20.47	17.26
#1	7,218	99.11	24.49	41.79	27.05	22.21
#1	6,289	95.91	7.10	17.66	4.81	7.14
#1	93	1.06	1.47	1.75	1.38	1.62

Figure 19: Flow cytometry analysis of the three cell lines Sp125, F12 and B1. The overlay marker is set to contain about 1% of the negative control. The statistical values and the shape of the peak determine clone F12 as best suitable for further RMCE experiments.

5.4. Transfection

As described in 5.3 the cell line CHO/RMCE/2-4/E/F12 was chosen for the transfections with the scFc antibodies 2F5 or 3D6. A parameter to investigate was the appropriate concentration of the selection drug ganciclovir. In literature (MORTENSEN and KINGSTON, 2001) a concentration of 2 µM is recommended. This concentration was used as a starting point and attempts with 1 µM and 2 µM of ganciclovir were tested. A summary of the results of the different transfection experiments with varying ganciclovir concentration can be found in Table 13.

Table 13: Summary of the different transfection experiments in dependency of the ganciclovir concentration.

Antibody	2F5scFc	3D6scFc	2F5scFc	3D6scFc
Ganciclovir concentration	1 µM	1 µM	2 µM	2 µM
Seeded wells	480	288	192	192
Growing wells	258	196	24	120
Percentage	53.75 %	68.10 %	12.50 %	62.50 %

For every transfection condition, also a negative control was prepared with $1 \cdot 10^6$ cells treated with 80 µg of PEI without any DNA. In none of these experiments any product could be detected by qualitative ELISA. Further the cells in the negative control seemed to be dead by microscopic assessment. 10^4 cells per well were seeded and in all grown wells the production of antibodies could be confirmed by a qualitative ELISA. The results clearly indicate, that 1 µM of ganciclovir is enough to put up sufficient selection pressure. The comparison of the two products even showed, that for the antibody 2F5scFc 2 µM ganciclovir drastically decreases the number of living cells. In the case of the antibody 3D6scFc no significant difference between both concentrations could be detected. Another interesting comparison between the two products can be observed by a closer look into the results of the qualitative ELISA. A typical result for the antibody 2F5scFc is shown in Figure 20. The absorption values represent the antibody concentration in the well. It is clearly shown, that all wells were positive tested for the product, as the values were higher than the reaction blank, but only few wells showed values higher values than 1 absorption unit. In contrast in Figure 21 the results for the antibody 3D6scFc are shown. There it can be clearly seen that all wells contained higher product concentration than for the antibody 2F5scFc and also more wells with a higher product concentration than 1 absorption unit could be detected. This trend was observable regardless of the ganciclovir concentration.

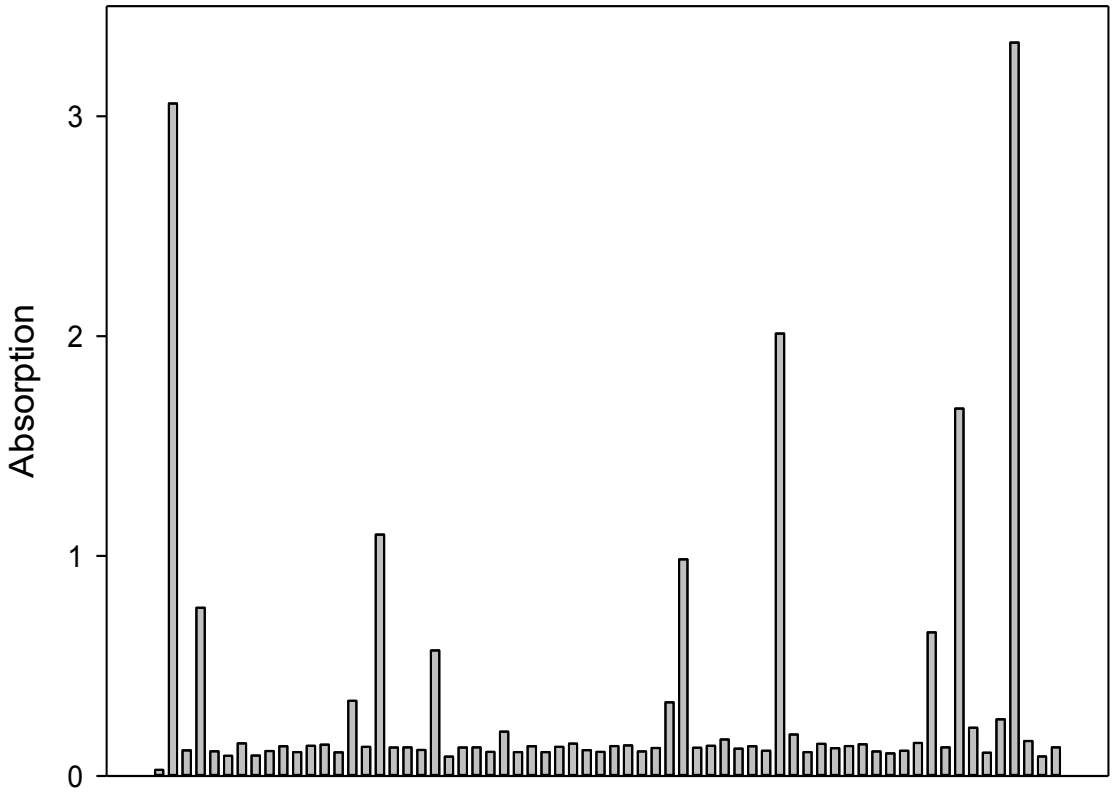


Figure 20: Qualitative ELISA values for the transfection with 2F5scFc. The first bar represents the reaction blank and the second bar the standard with a concentration of 100 ng/ml. The absorption values were measured at 492nm. The results were taken in the presence of 1 µM ganciclovir 3 weeks after transfection.

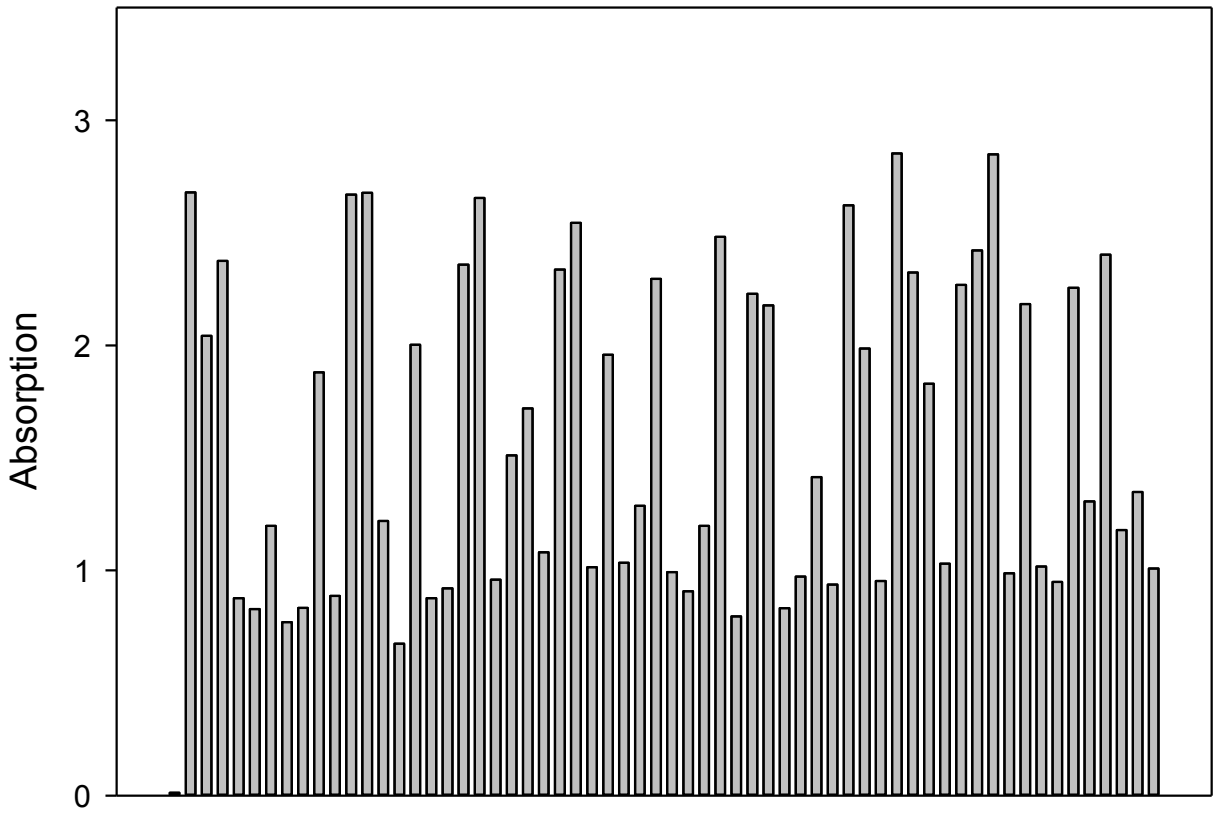


Figure 21: Qualitative ELISA values for the transfection with 3D6scFc. The first bar represents the reaction blank and the second bar the standard with a concentration of 100 ng/ml. The absorption values were measured at 492nm. The results were taken in the presence of 2 µM ganciclovir 3 weeks after transfection

5.5. Cell line establishment

According to the qualitative ELISA the clones with highest product concentrations were expanded into 96 well plates and subsequently in T25 flasks. In the T25 flask the clones were further expanded, until 10 ml cell culture volume was reached and then monitored in terms of cell concentration, product concentrations, qP and specific growth rate. These values were calculated by cell counting and product concentrations at every splitting. The different clones were monitored over several passages. For further analysis and comparison only the 12 best clones of each scFc antibody producing cell lines as well as the sorted cells were considered. For the product 2F5scFc the twelve best clones out of 23 and for the product 3D6scFc the twelve best clones out of 21 were taken. Additionally also the sorted cells were monitored by the described parameters. In the case of the 2F5scFc cell line also some sorted clones, which were immediately split in a 96 well plates, were treated as single clones and taken into account for the best twelve. In case of the antibody 2F5scFc all 12 clones were cultivated in the presence of 1 µM ganciclovir. In contrast, for the antibody 3D6scFc the clones 1C9, 1F11, 1E6 and 1F7 were cultivated in the presence of 2 µM ganciclovir, while all other clones were cultivated in the presence of 1 µM ganciclovir.

The purpose of monitoring the cell clones was to find the two best producers for each product in terms of growth and qP and select them for growth in the spinner flask. Also the comparison between the clones was interesting, as by the RMCE the GOI should integrate at the same genomic locus and therefore no position effect should occur. According to this concept, all the different clones should have comparable qP. In the following figures the bars represent one distinct value at the splitting while the dots represents the calculated mean values.

In Figure 22 and Figure 23 the specific productivities and the product concentration of the twelve best clones and the sorted cells producing 2F5scFc are shown. A special case is the clone sE7, because it was selected from a 96 well plate, where sorted cells were plated out soon after recovering from sorting. It was the only clone of this 96 well plate which produced antibodies in a comparable concentration. The abbreviation susp and ad also represents sorted cell, but these two were kept in a T25 flask, so they both represents the bulk number of sorted cells. Susp and ad result from the same transfection experiment, but they were split before sorting into

the suspension aggregates (named susp) and the population which stick at the bottom and some of them even developed an adherent phenotype (named ad). In this comparison it can be seen, that the sorted cells which were kept in bulk produce only very little amount of antibody and also showed very low qP. In general it can be seen, that only four of the clones showed a mean qP about 1 pg/c·d and a mean product concentration above 2 µg/ml. Those clones were the best producing ones for the antibody 2F5scFc

In Figure 24 the mean specific growth rates are shown. All clones had specific growth rates between 0.28 and 0.41 per day. In general no correlation between the specific growth rate and the qP was found. To get further insight into the distribution of the cell population flow cytometry analysis with immunofluorescence staining for intercellular product formation was done. On the basis of all of this analysis the clones 1C3 and 3E5 were chosen for further cultivation in the spinner flask and more detailed analysis.

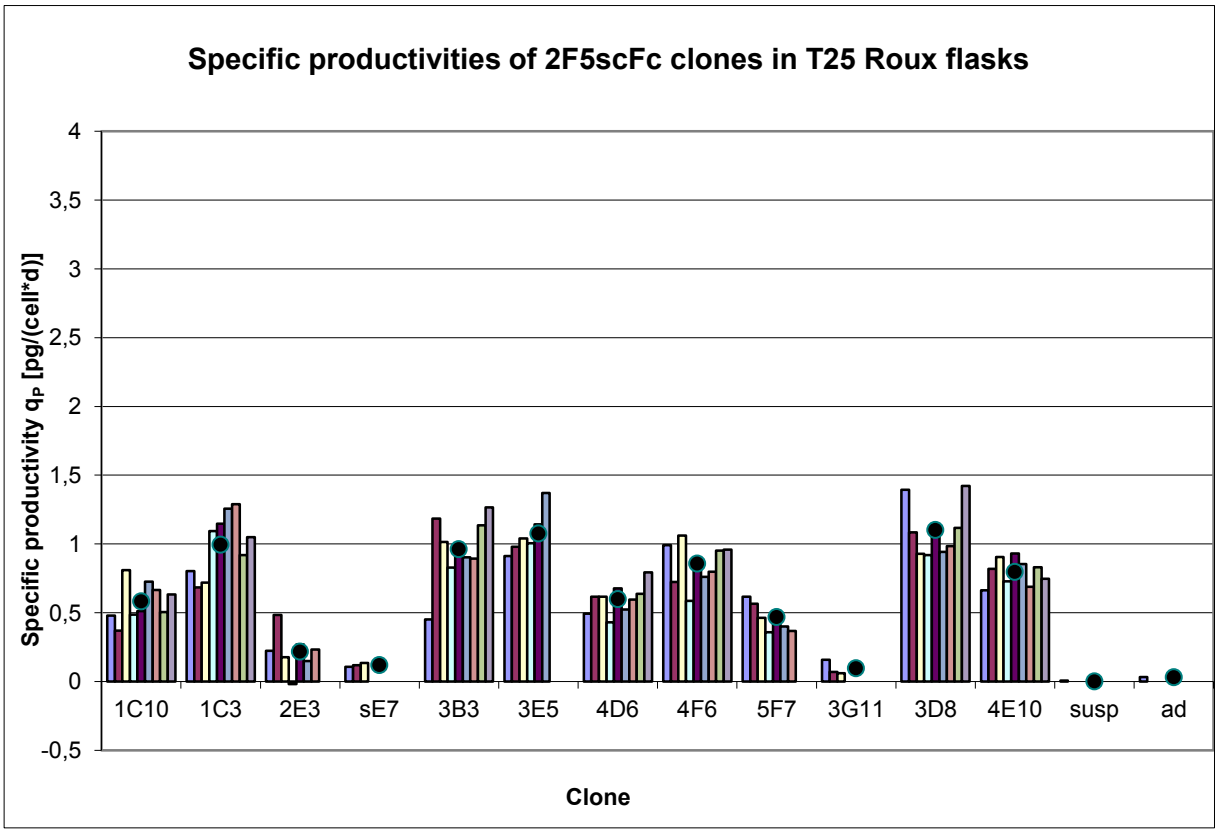


Figure 22: Specific productivity of the twelve best clones and sorted cells producing 2F5scFc. Bars represent the qP determined before every splitting and the dot marks the mean value.

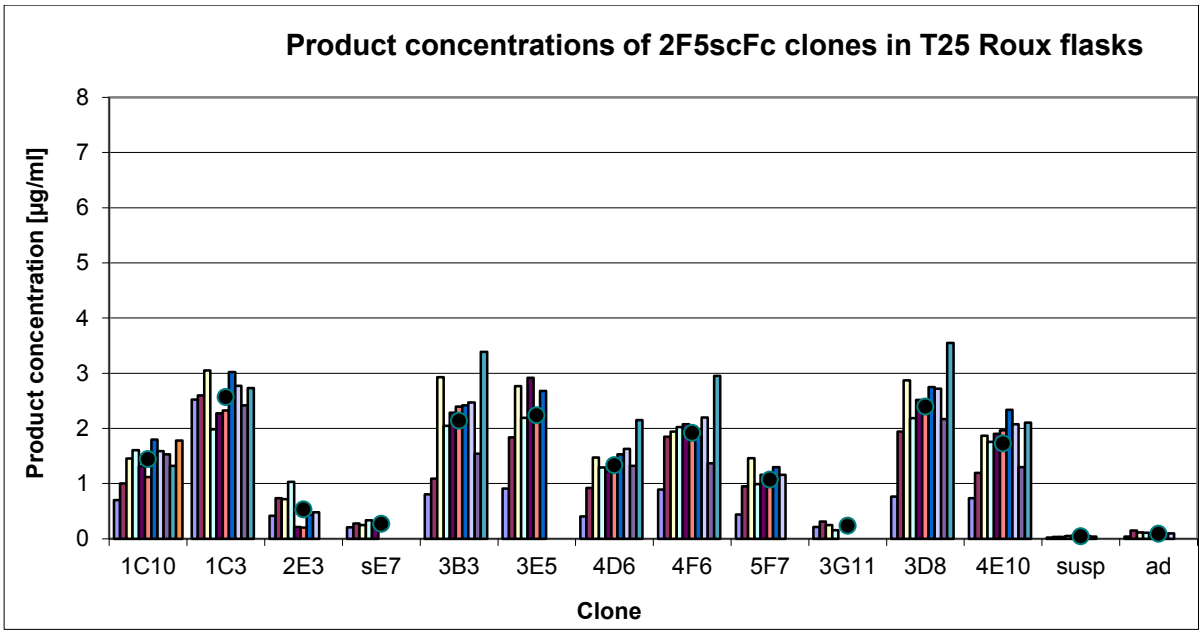


Figure 23: Product concentration of the twelve best clones and sorted cells producing 2F5scFc. Bars represent the actual concentration determined before every splitting and the dot marks the mean value.

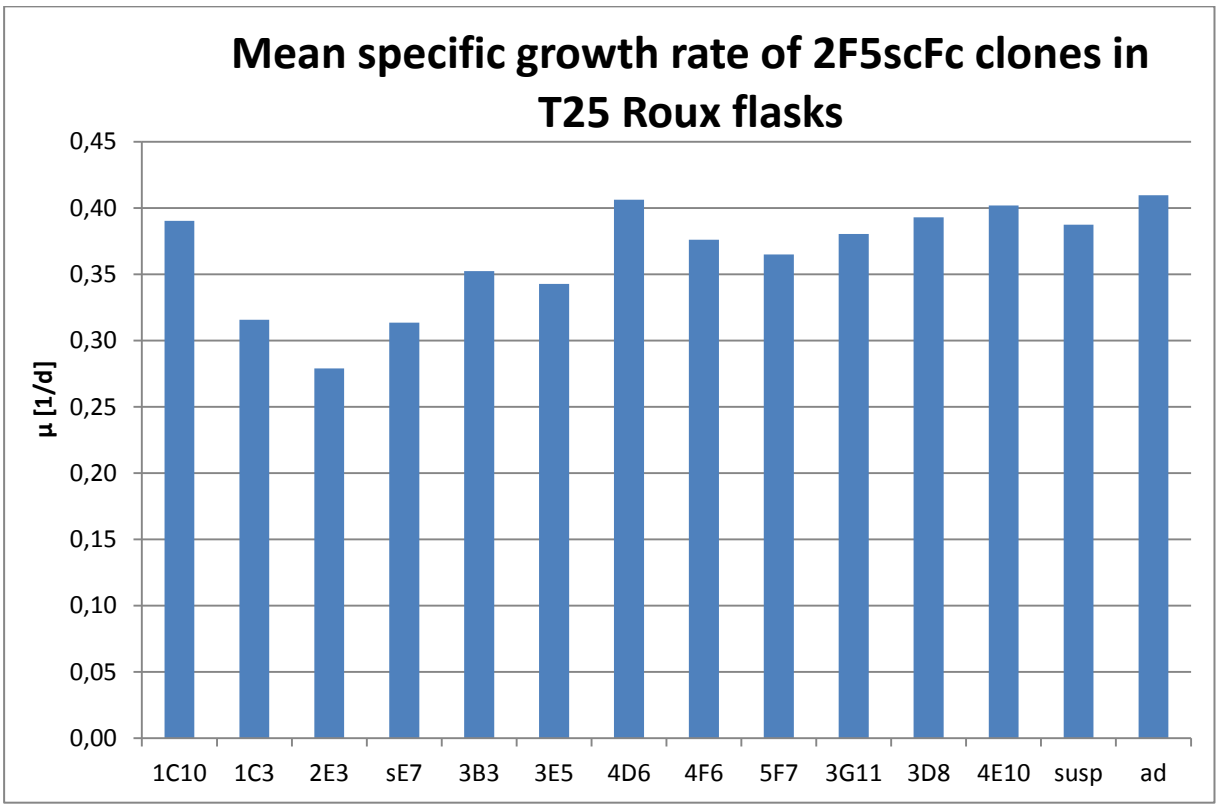


Figure 24: Mean specific growth rates of all clones producing 2F5scFc in T25 flasks.

In Figure 25 and Figure 26 the specific productivities and the product concentration of the twelve best clones producing 3D6scFc are shown. The named clones susp2 and ad2 were prepared in the same way, as those producing 2F5scFc with the same name. Also for this product the clones and the sorted cells showed a huge difference

in the qP and also in the product concentration. Another parallel to the data produced of the 2F5scFc producing clones is, that four out of the twelve clones show a superior qP. For the product 3D6scFc this range was about 2 pg/c·d. In general 3D6scFc clones produced more product than those producing 2F5scFc.

Figure 27 shows the mean specific growth rate for the clones in the T25 flasks. These values also showed no correlation to the qP. The values were also in the same range as they are for the clones producing 2F5scFc. Also for the 3D6scFc producing clone flow cytometry analysis for intercellular product formation by immunofluorescence staining were done to get further insight into the cell population. On the basis of all this data the clones 1F11 and 3B9 were chosen for further cultivation in the spinner flask.

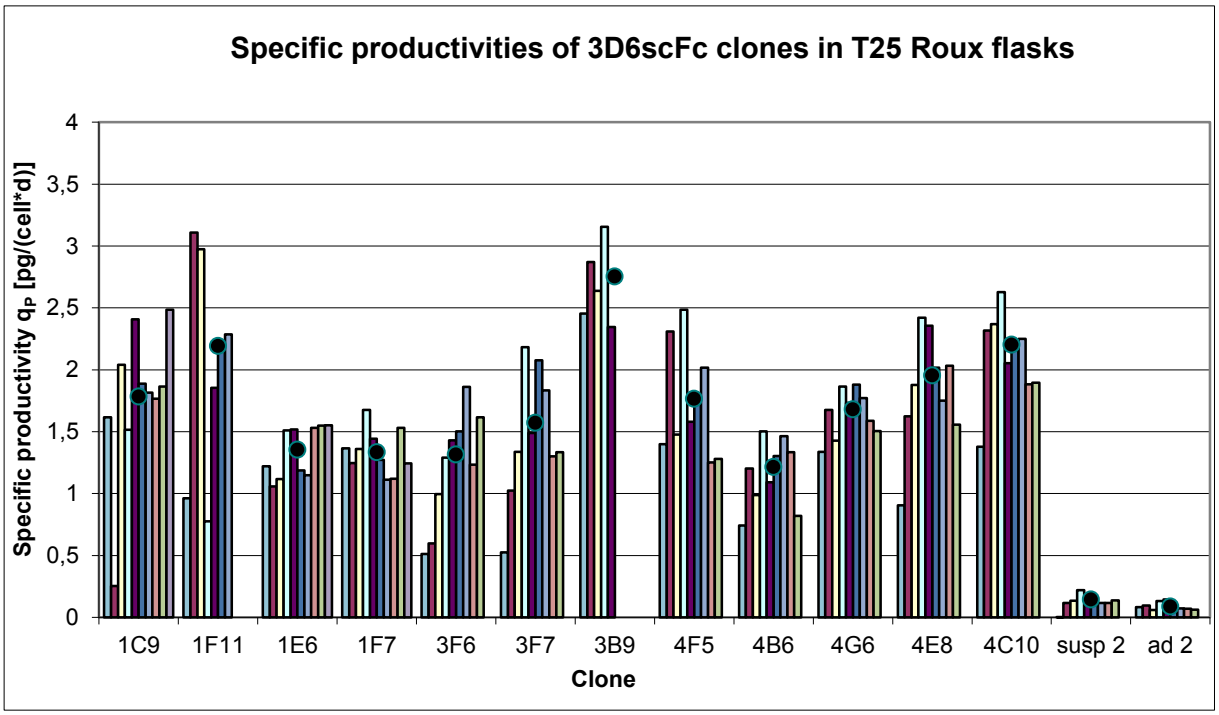


Figure 25: Specific productivity of the twelve best clones and sorted cells producing 3D6scFc. Bars represent the qP determined before every splitting and the dot marks the mean value.

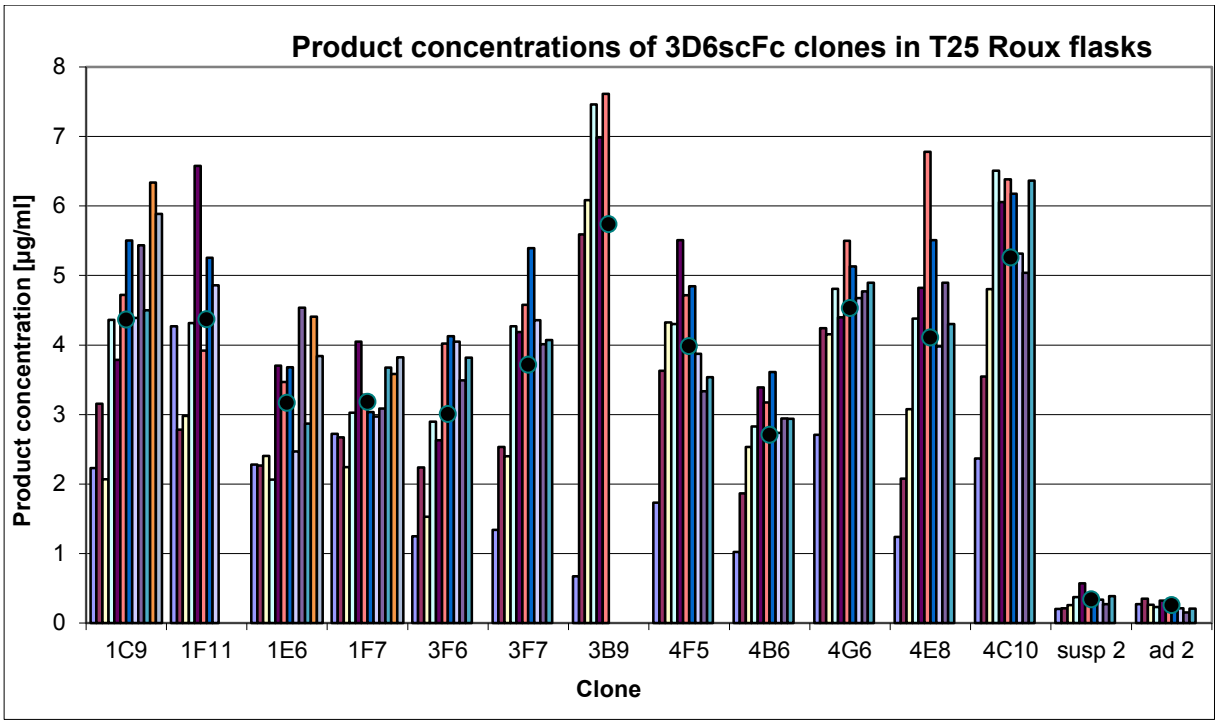


Figure 26: Product concentration of the twelve best clones and sorted cells producing 3D6scFc. Bars represent the actual concentration determined before every splitting and the dot marks the mean value.

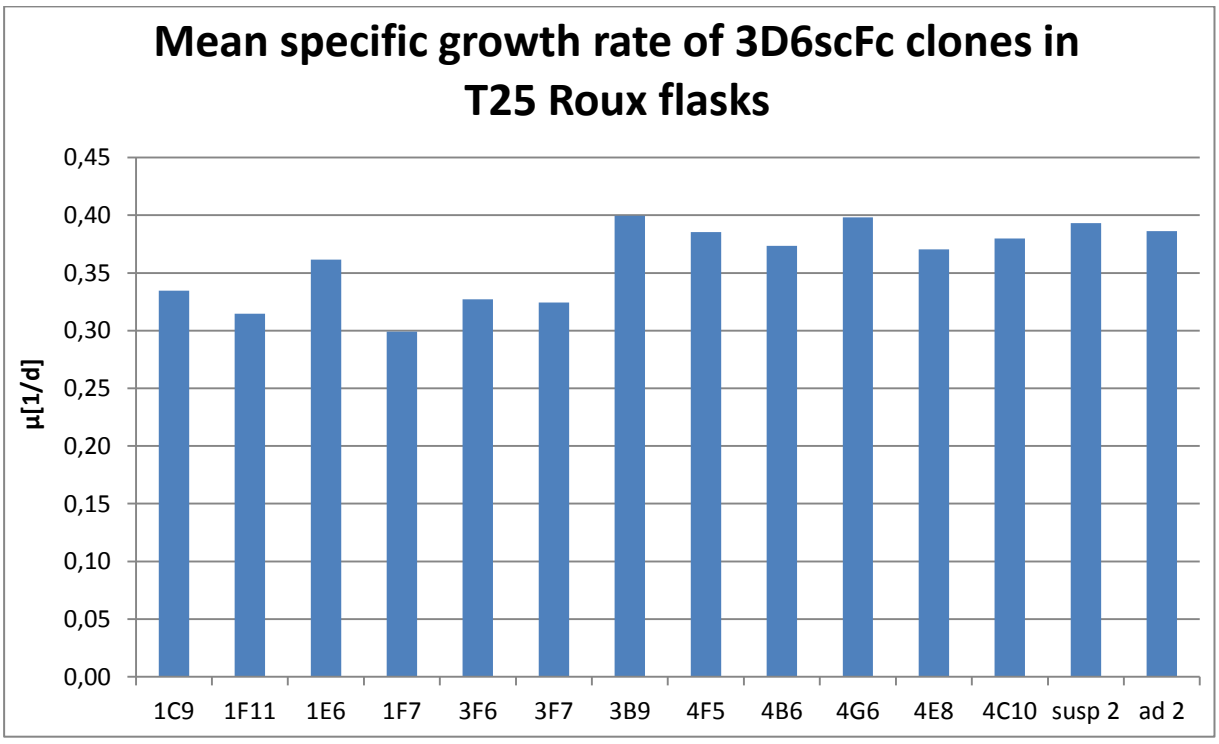


Figure 27: Mean specific growth rates of all clones producing 3D6scFc in T25 flasks.

A direct comparison of the mean qP is shown in Figure 28. The box plots were drawn from the mean specific productivities of the twelve best clones. It can be clearly seen, that the productivity of the clones, even if they integrate the gene on the same genomic locus, as intended with the RMCE, showed significant differences between

the two scFc antibodies. Both boxes show outliners on the upper and on the lower side. The upper outliners are the clones with the outstanding qP in comparison to the other clones and they were chosen to further cultivation in the spinner flask. The length of the whiskers in the plots represents an unequal distribution. For the antibody 2F5scFc an almost equal distribution could be seen, while for the antibody 3D6scFc some clones with a higher qP than the majority occurred.

Comparison of mean specific productivity of 12 best clones each

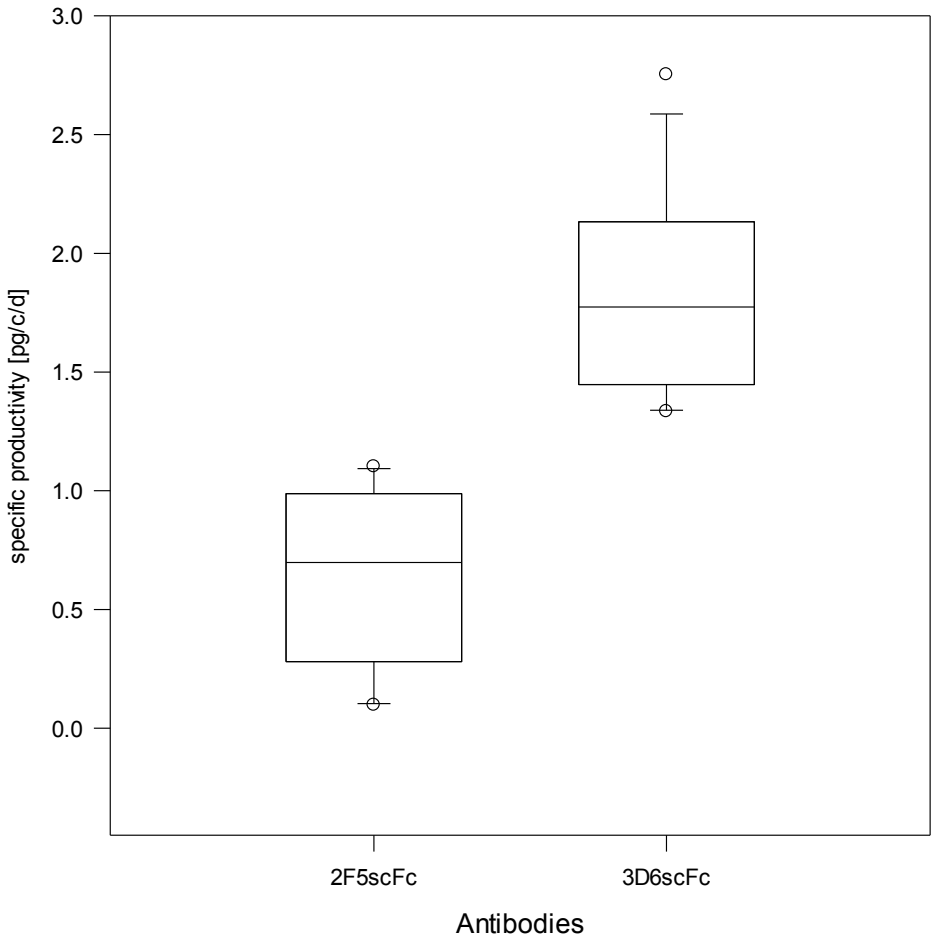
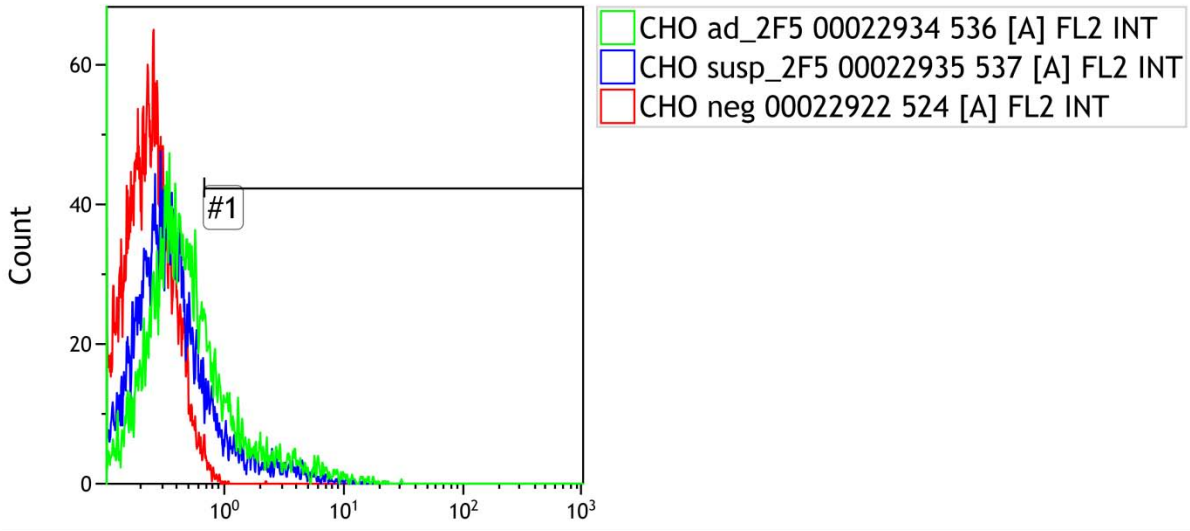


Figure 28: Box plot drawn out the mean specific productivity in pg/c·d. The box represents all values starting with the 25 % confidence interval until the 75 % confidence interval, the line inside represents the median value. The round dots outside represent the outliners. The upper outliners showed superior specific productivities and were therefore chosen for further cultivation in the spinner flask.

5.6. Cell sorting

In section 5.5, it was shown, that all of the sorted cells, showed in general less qP and less product concentration than the other clones. It was also shown, that the specific growth rate always was among the highest for all clones grown in a T25 flask, although in general no correlation between specific growth rate and qP was found. In contrast, the strategy of cell sorting and recovering after RMCE was successfully described in literature (QIAO et al., 2009).

A reason therefore is on the one hand, that the clones were grown in a T25 flask after sorting and so a mixed population was created. Only some cell produce antibodies in larger quantity, because in the case of the antibody 2F5scFc one of the clones, which were separated in a 96 well plate after sorting, showed a qP comparable to the twelve best. An example therefore is the clone sE7, but it was the only clone of this plate which produces comparable amounts of antibodies. A solution to this problem would be direct sorting into 96 well plates or a consequently separation of the cells after sorting by a limited dilution approach. Also by the use of flow cytometry analysis for intercellular product formation only a small population showed the formation of intercellular product. As example, in Figure 29, the analysis for the sorted 2F5scFc clones is shown. One possible reason is the loss of the herpes virus thymidine kinase but no replacement with the transfected antibody containing expression cassette occurs. Therefore further investigation has to be done, why these cells remain alive in the presence of ganciclovir.

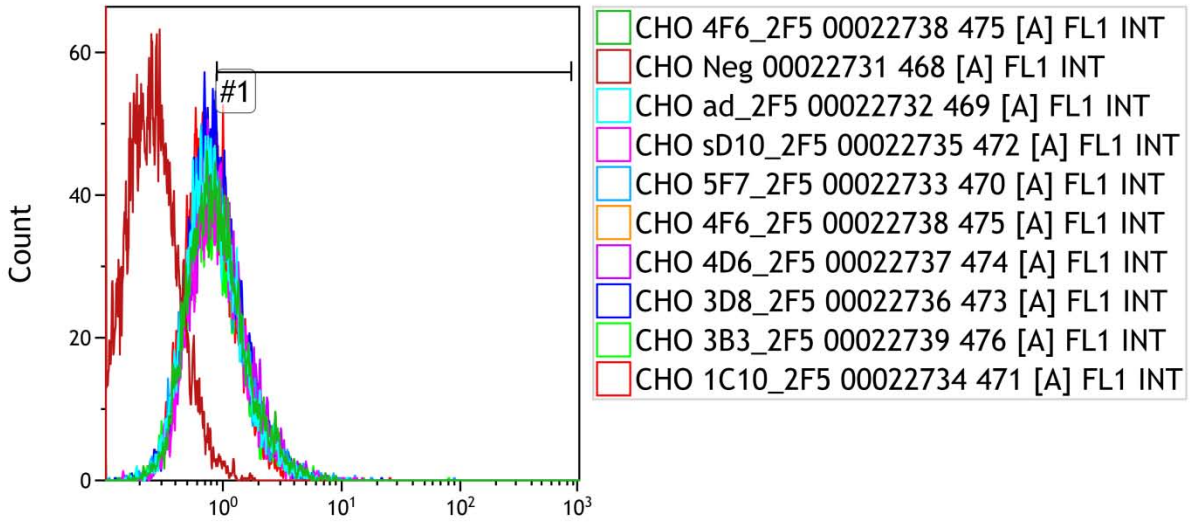


Marker	Number	%Total	%Gated	X-Med	X-AMean	X-Mode	X-GMean
All	6,468	64.68	100.00	0.43	0.87	0.10	0.37
All	6,256	62.56	100.00	0.33	0.59	0.10	0.27
All	7,177	71.77	100.00	0.22	0.25	0.10	0.16
#1	1,751	17.51	27.07	1.16	2.24	0.70	1.39
#1	1,045	10.45	16.70	1.19	1.96	0.69	1.35
#1	69	0.69	0.96	0.75	0.79	0.70	0.77

Figure 29: Flow cytometry analysis for intercellular product formation with bulk sorted 2F5scFc producing cells. Only a small population inside of the overlay marker can be determined, which show intercellular product formation.

Another problem is the discrimination between GFP positive and negative cells. For every cell sorting, the cell line CHO/RMCE/2-4/E/F12 was used as positive control, as this cell line expresses the GFP protein and this cell line was also used as host cell line for the RMCE. As negative control, the cell line CHO DUKX B11 was used, because it should represent the non GFP containing host cell line. After screening the antibody producing cell line for GFP fluorescence signal, this cell lines also showed a higher auto fluorescent signal than the cell line CHO DUKX B11. In Figure 30 the GFP signal of the CHO DUKX B11 cell line and of the clones producing 2F5scFc antibodies are shown. All of these clones show a higher fluorescence signal than the cell line CHO DUKX B11 although none of them contains any GFP, which is demonstrated in section 5.8. The same data were measured for the 3D6scFc producing clones. The absence of the GFP signal was checked by a fluorescence microscope and for some clones also by a PCR with specific primers for the neomycin phosphotransferse (Figure 36). A solution therefore would be a better discrimination by the use of the appropriate negative control and better adjustment of the instrumental setup for sorting. This negative control should be a cell line

described in (MAYRHOFER, 2013), which contains no GFP but the retargetable FRT sites and also show a higher auto fluorescence.



Marker	Number	%Total	%Gated	X-Med	X-AMean	X-Mode	X-GMean
All	5,807	58.07	100.00	0.84	1.04	0.88	0.82
All	7,684	76.84	100.00	0.25	0.29	0.10	0.20
All	6,043	60.43	100.00	0.79	0.97	0.84	0.77
All	5,411	54.11	100.00	0.84	1.02	0.71	0.81
All	5,944	59.44	100.00	0.80	1.03	0.59	0.78
All	5,807	58.07	100.00	0.84	1.04	0.88	0.82
All	5,978	59.78	100.00	0.87	1.08	0.73	0.85
All	6,540	65.40	100.00	0.83	1.02	0.69	0.81
All	5,360	53.60	100.00	0.81	1.01	0.88	0.78
All	6,145	61.45	100.00	0.76	0.90	0.71	0.73
#1	2,631	26.31	45.31	1.28	1.56	1.10	1.38
#1	85	0.85	1.11	1.04	1.12	0.91	1.09
#1	2,460	24.60	40.71	1.26	1.49	0.89	1.34
#1	2,432	24.32	44.95	1.25	1.52	0.94	1.34
#1	2,502	25.02	42.09	1.27	1.61	0.91	1.37
#1	2,631	26.31	45.31	1.28	1.56	1.10	1.38
#1	2,879	28.79	48.16	1.29	1.57	0.97	1.39
#1	2,878	28.78	44.01	1.26	1.52	0.93	1.35
#1	2,259	22.59	42.15	1.26	1.57	0.93	1.35
#1	2,243	22.43	36.50	1.20	1.39	0.99	1.27

Figure 30: Flow cytometry analysis for GFP signal of 2F5scFc producing clones and cell line CHO DUKX B11 as negative control. All producing clones show a sharp peak at a higher fluorescent intensity than the negative control.

5.7. Characterisation of established cell lines

The clones 1C3 and 3E5, which produce 2F5scFc antibodies and the clones 1F11 and 3B9, which produce 3D6scFc antibodies were selected as new cell lines for further characterisation. They were grown in a spinner flask in approximately 50 ml, split twice a week with a seeding density between 2 -3·10^5 c/ml. All clones were cultivated in ProCHO5 containing 4 mM L-Glutamine, HT supplement and 15 mg/l phenol red. Also the selection pressure with ganciclovir was kept. Only for the cell line 3D6scFc 1F11 2 µM ganciclovir was used, while the other three cell lines were grown in the presence of 1 µM ganciclovir. At every splitting the viability, the total cell concentration and the product concentration was measured. Out of these values the specific growth rate and the qP was calculated.

In Figure 31 the specific growth rate of all four clones are shown over the entire cultivation time in the spinner flask. The zigzag course of the values is due to the alternate cultivation time of 3 respectively 4 days, because the cultures are split twice a week. The differences between the values result because after three days, the cultures are in exponential growth phase, while after four days the cultures might already enter the stationary phase, which results in a lower specific growth rate. It was also observed, that the growth rate increased with time. This might be due to the better nutrition supply in the spinner flask than in the T25 flask. The increase in specific growth rate was rather slow, because the adaptation of the cells to the conditions in the spinner flask seems to be a rather slow process. After approximately 60 days in culture, the maximum growth rate was reached and the specific growth rate decreased. Further research or longer culture times would be needed to investigate this behaviour.

As the curves indicate, there is no difference in the growth rate between these four clones. Therefore, in Figure 32 the mean specific growth rate with the standard deviation is shown. No significant difference between the four clones could be detected, because the slightly higher values of the 3D6scFc producing clones are within the error bars. This leads to the conclusion that neither the product 3D6scFc nor 2F5scFc affected the growth of the recombinant cell lines.

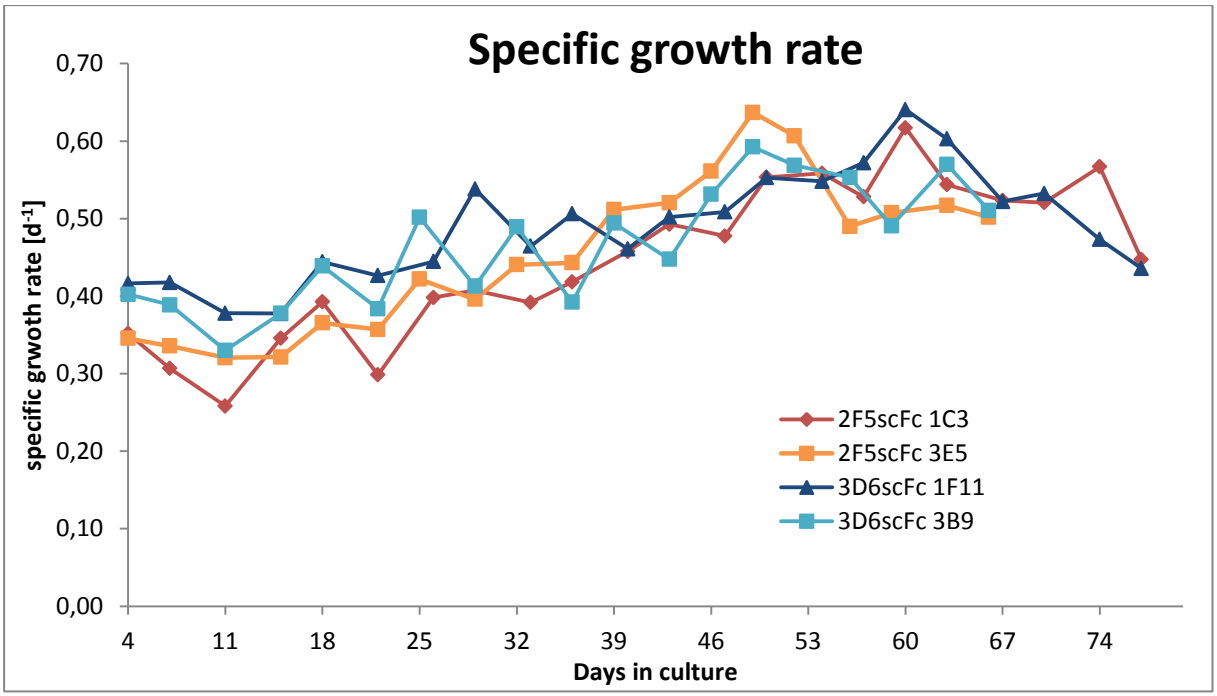


Figure 31: Specific growth rate of the four selected clones during the cultivation in the spinner flask. As interval on the x-Axis 7 days (one week) was chosen. In this time two splitting's were done, which are represented by the marks.

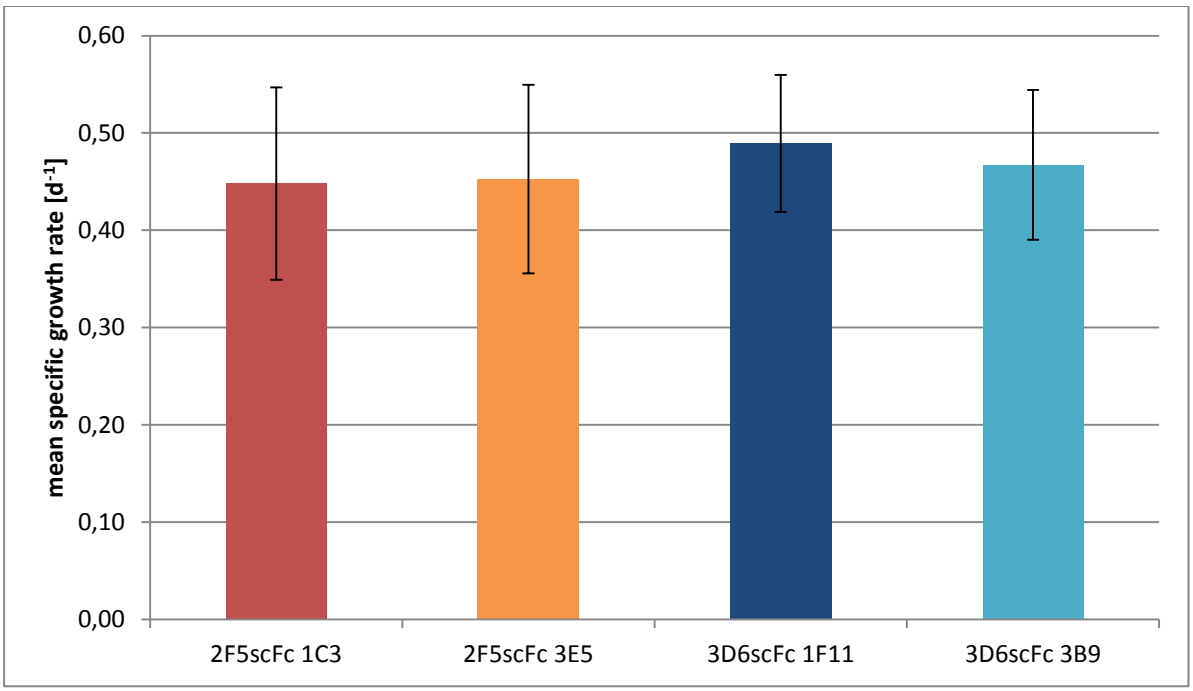


Figure 32: Mean specific growth rate of the clones during the spinner cultivation. The error bars represent the standard deviation of the mean value. No significant difference between the clones can be seen.

In Figure 33 the product concentration at every splitting is shown. The analysis was done at the same sampling points as the cell counts. Using the values for the product concentration and the specific growth rate the qP was calculated. The zigzag of the curves is also due to the alternate cultivation time between three or four days.

In the graph for the product concentration it can be seen, that the 3D6scFc clones showed most of the time a higher product concentration (between 3 µg/ml and 6 µg/ml) than the 2F5scFc clones over the entire cultivation time. In the case of the 2F5scFc producing clones the product concentration increased within cultivation time. This might be due to the adaption of the cell line to the spinner flask, because the cells showed also an increase in the specific growth rate. As the productivity of a cell line is dependent on the growth rate, this resulted in higher product concentration.

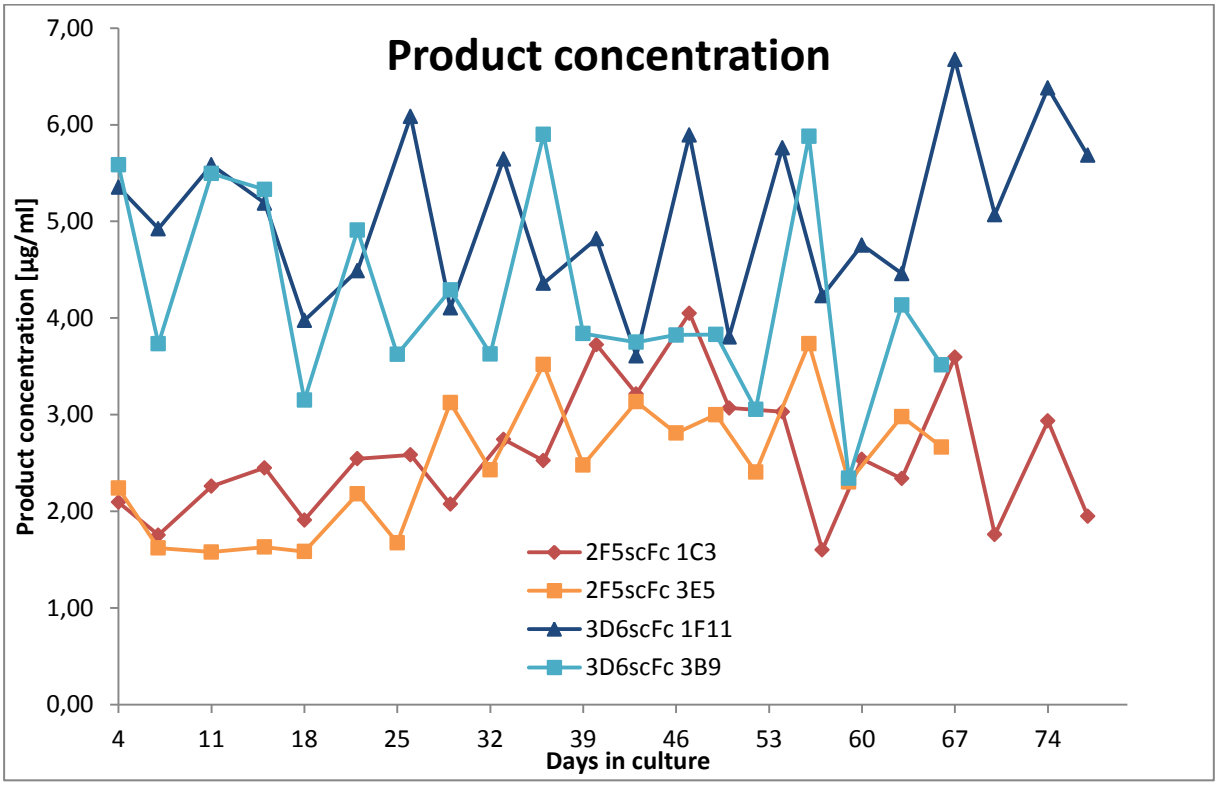


Figure 33: Product concentration over the entire cultivation in the spinner flask.

Another interesting part is the graph of cell line 3D6scFc 3B9 from day 49 on. From this value the cell line showed really huge variability in the product concentration. A reason therefore was the culturing of the cells in the spinner flask, because high product concentration always occurred together with a high cell density. This caused higher splitting ratios. Because of the alternate 3 to 4 days between a splitting, the following situation occurred: After 4 days very high cell densities resulted in a higher splitting ratio and also in a lower product concentration after the splitting. The next splitting was done after 3 days, where a rather low cell density was measured and of course a lower product concentration. This resulted in a very low splitting ratio. If now the next splitting was done after 4 days again a higher cell and product concentration

was measured. This phenomenon always occurred, if the cell concentration showed unusual high values and a higher splitting ratio than usual had to be used.

In Figure 34 the qP of the four cell lines over the entire cultivation time in the spinner flask is shown. This parameter takes the specific growth rate as well as the product concentration into account. The qP for the 3D6scFc producing clones remained rather constant over the cultivation time. The clone 3D6scFc 1F11 showed an increase in the qP at the end of the cultivation time, while the clone 3B9 showed a decrease. An explanation for these low values of the clone 3B9 is shown in Figure 33, because those were exactly these values, where the high variance between product concentrations occurred.

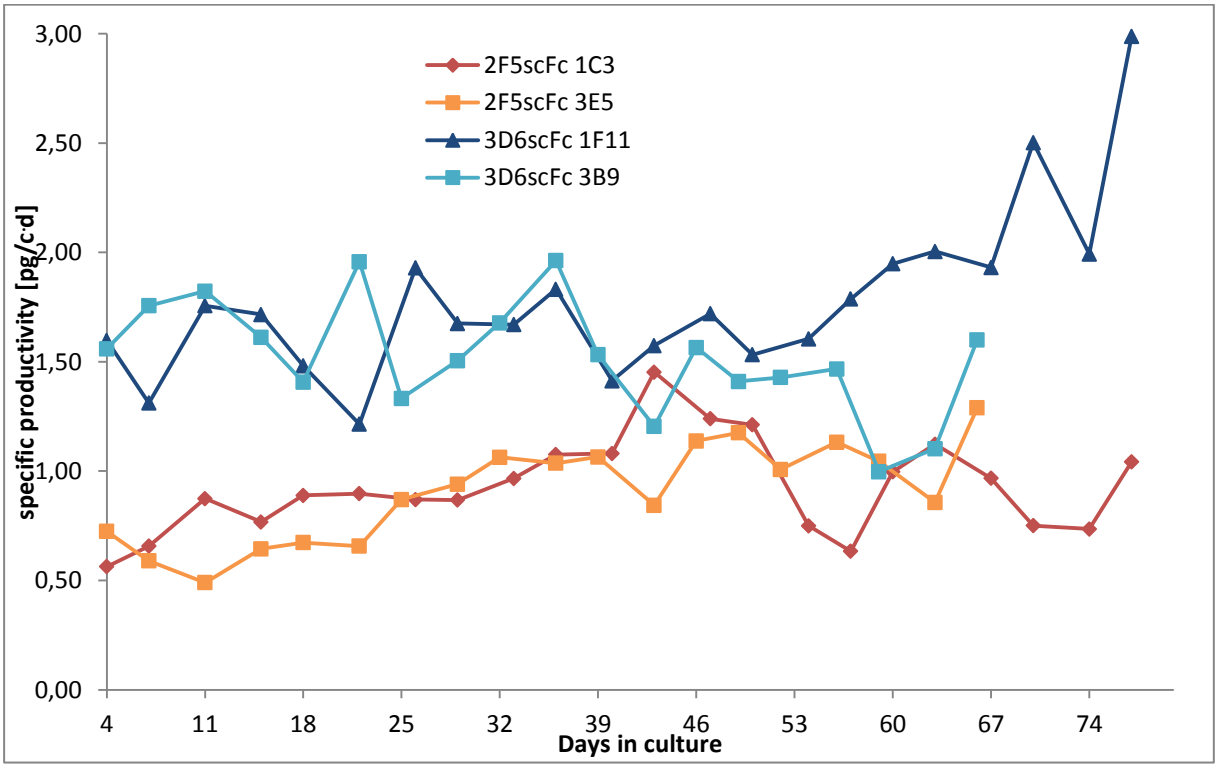


Figure 34: Specific productivity of the four cell lines over the entire cultivation time in the spinner flask

The clones producing 2F5scFc showed an increase in qP for the first 30 days in culture, which could be due to the adaption of growth in spinner flask. After this adaptation phase the qP remained rather the same. The clone 1C3 showed a strong increase around day 43 which was followed by a strong decrease until day 57. This might be due to a gradual increase in the specific growth rate. Another reason could be that at this sampling point another adaption occurred and the cells grew to a higher final cell density.

A comparison of the mean specific productivities is shown in Figure 35. The clones producing 2F5scFc showed a mean qP about 0.9 pg/cd, while the 3D6scFc producing clones showed a mean qP of 1.5 and 1.7 pg/cd, respectively.

The comparison of those 4 clones also showed interesting facts, because the 3D6scFc producing clones showed a more than 1.5 times higher qP than the 2F5scFc producing clones. This is a general trend for these two antibodies, which was also reported previously for the conventional as well as for the BAC based transfections (MADER et al., 2013).

Another interesting fact was that the mean qP is rather low for both antibodies, so a positive effect of the S/MAR regions could not be seen. Although it has to be mentioned, that the purpose of RMCE was to introduce only one gene copy, while with conventional plasmid transfer and the selection of the highest producers the clones with more gene copies have an advantage.

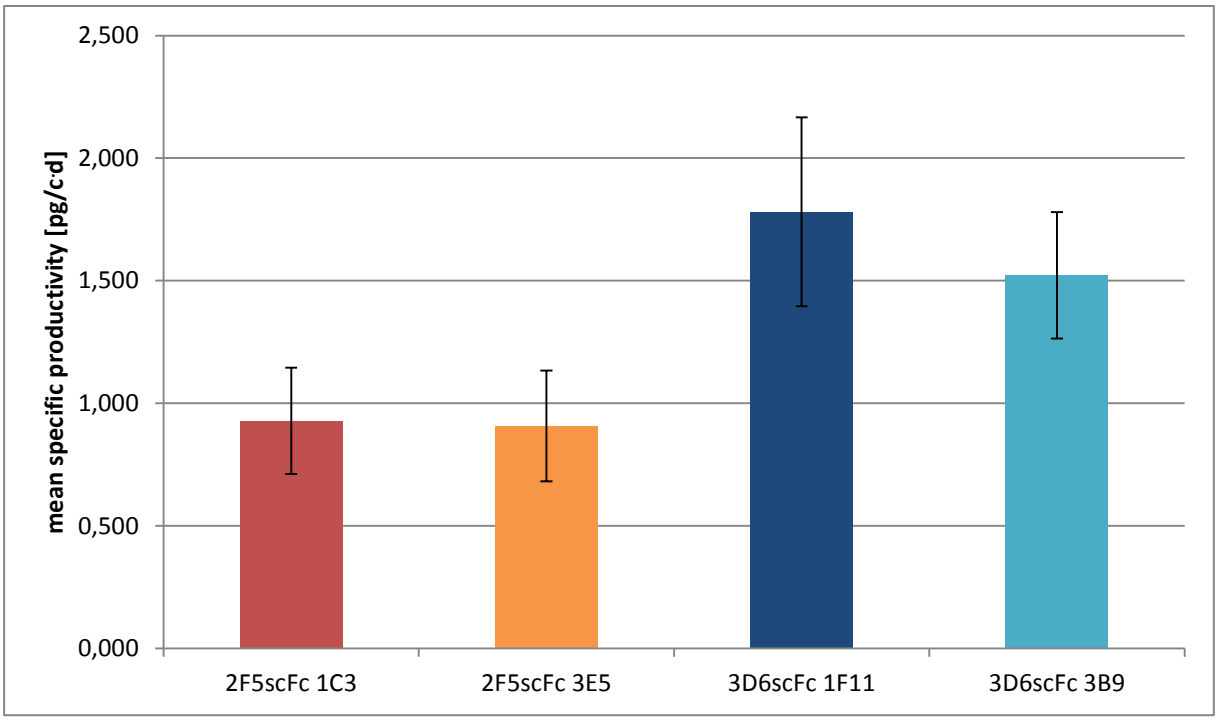


Figure 35: Mean specific productivity shown with standard deviation.

5.8. Detailed investigation of RMCE

In literature the proof of the RMCE was done with PCR and the investigation of the integrated gene copies was done by Southern blot. None of these methods can show if the RMCE really occur on the specific site and takes place at the same chromosomal location.

The first step for a detailed investigation was a PCR with specific primers for the neomycin phosphotransferase gene. This should additionally to the flow cytometry and fluorescence microscope proof, if any cells are present, which still contain the genes prior to the cassette exchange. In Figure 36 the agarose gel with the PCR products is shown. It can be clearly seen, that only the control cell line CHO/RMCE/2-4/E/F12 showed the specific amplicon with a size of 791 bp while for all of the other cell lines no PCR product was obtained. This indicates a clean and successful cassette exchange with no genes left. It also indicates that the observation of higher auto fluorescence described earlier is not due to leftover GFP gene in the genome or that only the thymidine kinase is lost and the residual genes are leftover in the genome.

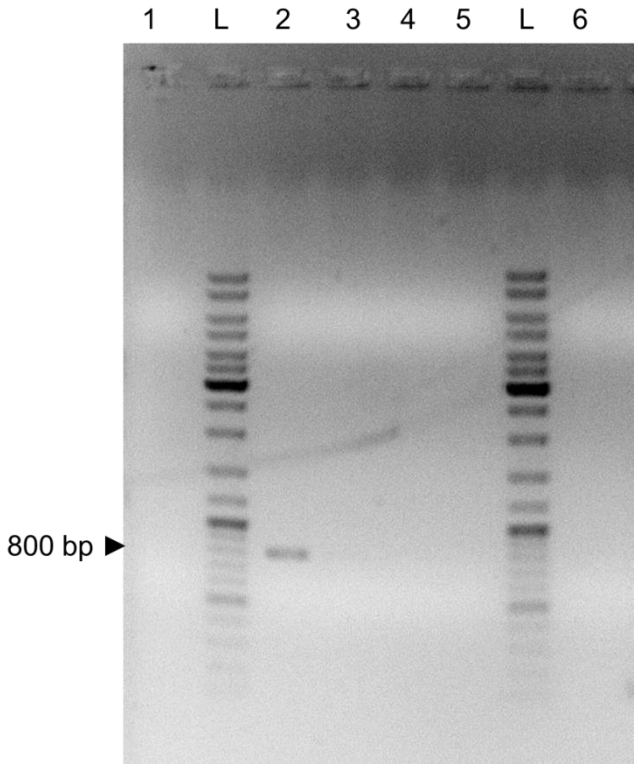


Figure 36: Agarose gel electrophoresis of the PCR products with neomycin phosphotransferase specific primers. Lane 1 contains the non template cotrol, 2 contains the cell line CHO/RMCE/2-4/E/F12 as positive control, 3 contains cell line 2F5scFc 1C3, 4 2F5scFc 3E5, 5 3D6scfc 1F11 and 6 3D6scFc 3B9.

The further investigation was done on the chromosomal level by FISH. The idea was to detect the gene on the chromosome before the cassette exchange and after it. The used probes were labelled with digoxygenin (DIG) and as detection an immune staining with three different antibodies, whereas two thereof were labelled with FITC was used. For the labelling reaction the plasmids used for transfection of the cell lines were used. In Table 14 an overview of the used probes with the corresponding plasmids is given.

Table 14: Overview of the probes, detected genes and plasmids used for the labelling reactions

Probe	**Plasmid**	**Detected genes**
GTN	pF3GTNF	GFP, Neomycin phosphotransferase, thymidine kinase
2F5scFc	pF32F5scFcF	2F5scFc
3D6scFc	pF33D6scFcF	3D6scFc

The Klenow labelling reaction was performed with the DIG High Prime labelling Kit (Roche) containing random primers. According to the manufacturer 200 to 1000 bp in length will be created by a labelling efficiency of nearly 100 % overnight. After the labelling reaction a co precipitation with herring sperm DNA and yeast t-RNA was done.

To check if the labelling reaction and also the detection reaction worked, a dot blot was performed. The principal setup of the detection reaction for the FISH can be seen in Figure 37.

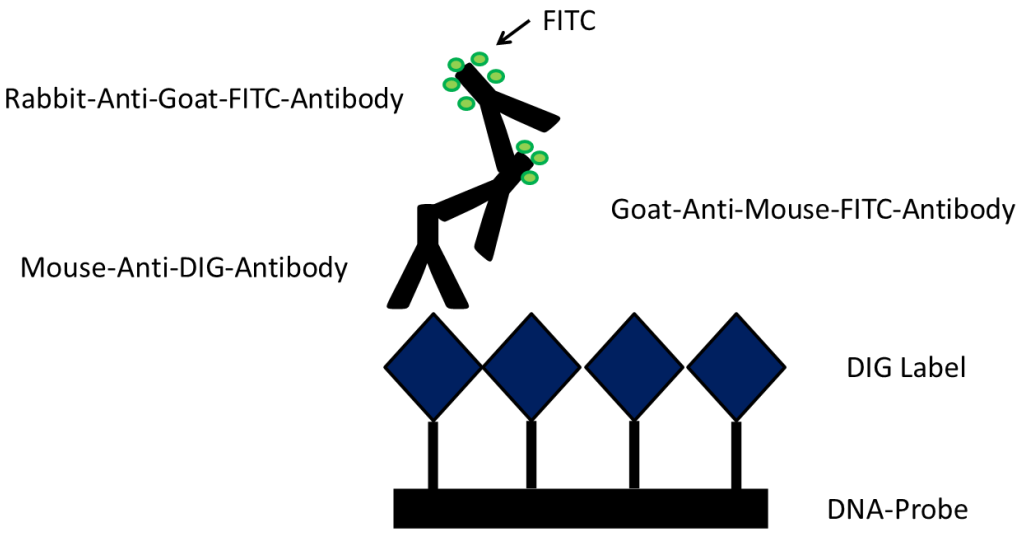


Figure 37: Schematic setup of the detection reaction for chromosomal FISH

The DNA probes for the dot blot were spotted in a 1:10 dilution series and detected by fluorescence. The starting concentration of the labelled DNA was 100 ng/µl for the dilution series. A picture of the dot blot is shown in Figure 38. For all three different probes the 100 ng/µl and 10 ng/µl dots were visible. This indicates the proper function of the detection system and also a sufficient labelling reaction. The blot showed high background, which was due to insufficient washing at the different incubation steps.

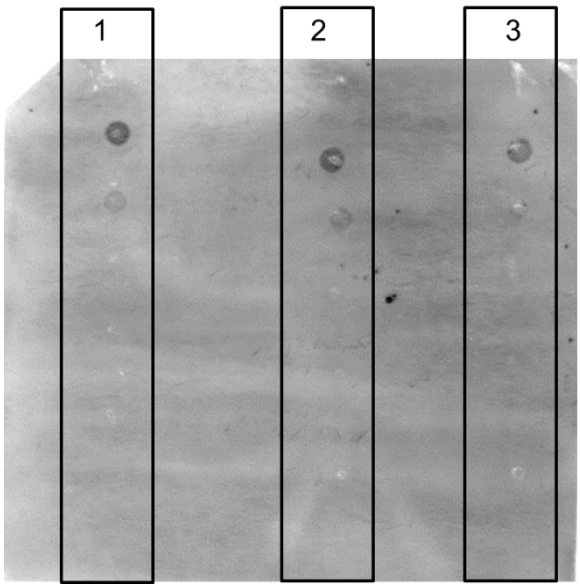


Figure 38: Dot blot with DIG labelled probes and fluorescence detection cascade. 100 ng/µl starting concentration of labelled DNA for the 1:10 dilution series. 1 contains probe GTN, 2 2F5scFc and 3 3D6scFc. 100 ng/µl and 10 ng/µl are visible.

The same detection system was also used for the FISH detection of the integrated cassettes in the genome. The chromosomes were counterstained with Propidium Iodide. For any analysed clone at least four pictures were taken and the cell line CHO DUKX B11 was used as negative control.

In Figure 39 a metaphase spread of a CHO/RMCE/2-4/E/F12 cell is shown. One single spot, where the probe hybridised can be clearly seen. So it seems that before the cassette exchange was performed, only one integration locus was there.

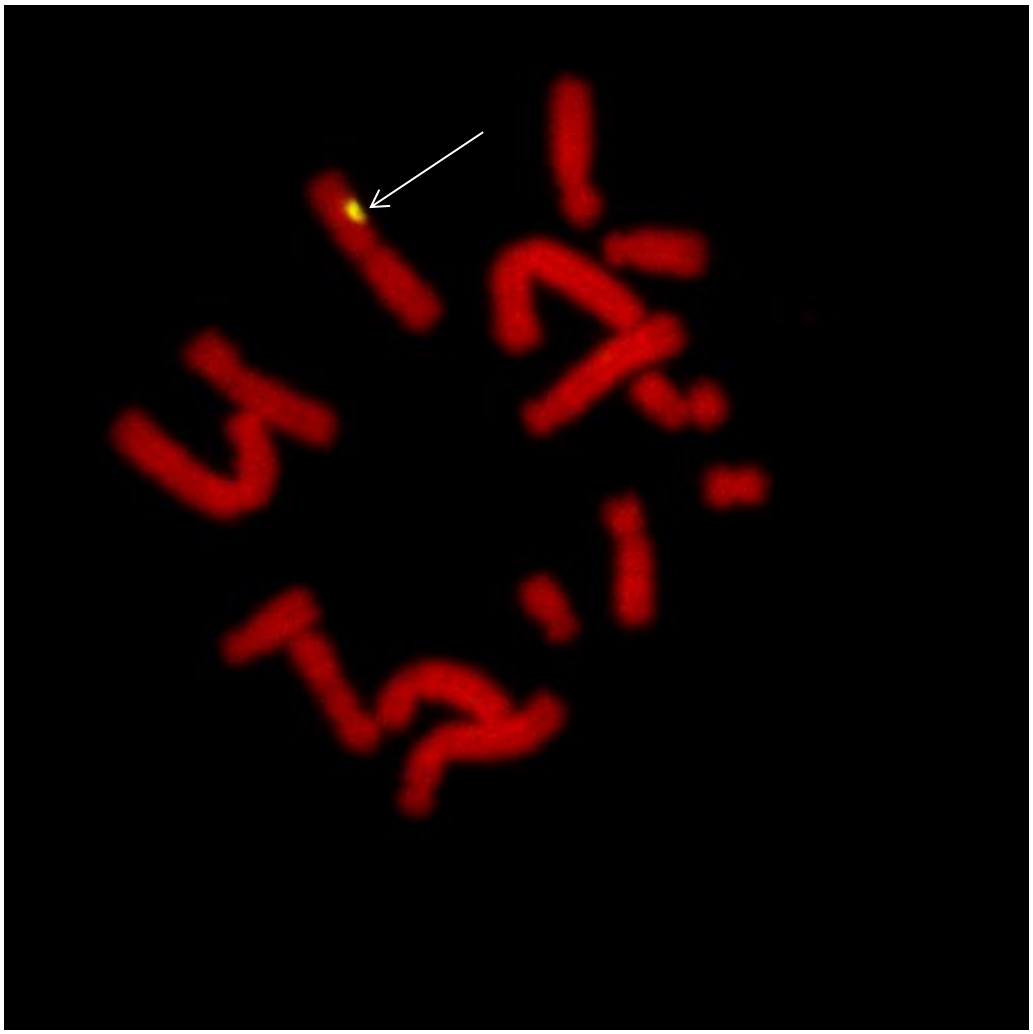


Figure 39: Metaphase spread of a CHO/RMCE/2-4/E/F12 and GTN used as probe. One single spot with the GOI can be seen.

In Figure 40 a metaphase spread of a 2F5scFc 1C3 clone is shown. This shows the hybridisation locus after the cassette exchange. It also shows one locus as for the cell line prior to the cassette exchange although it seems that the locus in the chromosome had changed. The picture also contains not bursted cell, which are an artefact of the chromosome preparation.

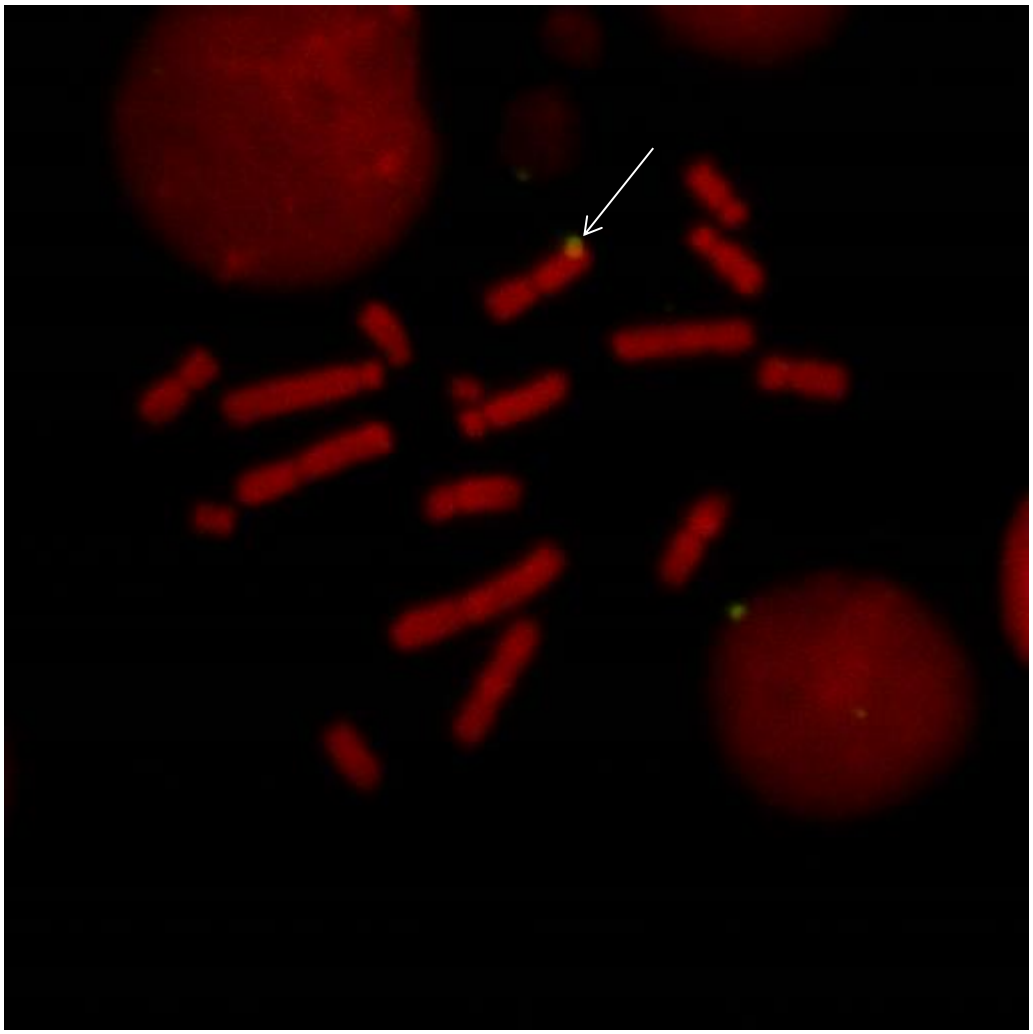


Figure 40: Metaphase spread of a 2F5scFc 1C3 cell hybridised with a 2F5scFc probe. One single spot is seen. The round cells in the upper and lower half are not bursted cells.

In figure Figure 41 a metaphase spread of a 3D6scFc 1F11 clone is shown. This clone showed two integration sites, as on two chromosomes the probe was hybridised. Further it also seems that the integration sites were not on the same site as in the cell line prior to the cassette exchange.

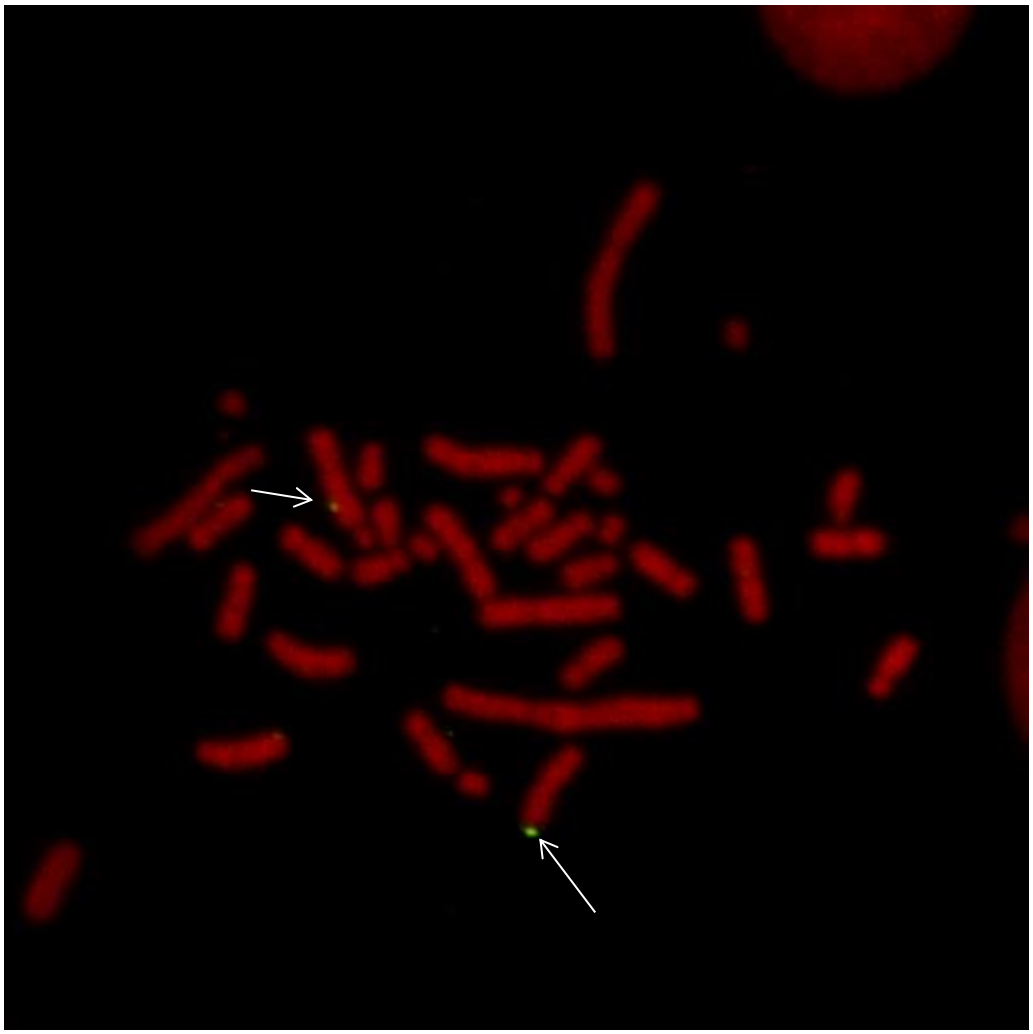


Figure 41: Metaphase spread of a 3D6scFc 1F11 cell hybridised with a 3D6scFc probe. Two marked genes can be seen.

In general the results of the FISH give a first insight into the RMCE. For further results more metaphase spreads per cell line has to be investigated and also more cell lines should be used. The analysis of the cell line 3D6scFc 1F11 indicates more than one integration site. As additional method a qPCR with specific primers for the scFc parts of the antibodies was performed. Additionally, the neomycin phosphotransferase in the F12 RMCE host cell line was compared to this data. A problem of the FISH data was the laborious and time consuming preparation of the samples as well as the microscopic analysis. Further none of the pictures showed a comparable number of chromosomes and also the condensation of the different chromosomes was not comparable.

For the qPCR the four cell lines after the cassette exchanges were investigated in detail. Therefore three consecutive biological samples were taken at three different passages, every time after 3 days in culture after the last splitting. The exact time

points of the taken samples are listed in Table 15. For the cell line F12 only one sample was taken. Additionally the cell line CHO DUKX B11 was taken as negative control In general every sample was measured in triplicates and in two technical runs.

Table 15: Overview of the biological samples taken for the analysis of the gene copy number.

Sample name	**Cell line**	**Date**
1C3_1	2F5scFc 1C3	24.05.2013
1C3_2	2F5scFc 1C3	31.05.2013
1C3_3	2F5scFc 1C3	07.06.2013
3E5_1	2F5scFc 3E5	24.05.2013
3E5_2	2F5scFc 3E5	31.05.2013
3E5_3	2F5scFc 3E5	07.06.2013
1F11_1	3D6scFc 1F11	24.05.2013
1F11_2	3D6scFc 1F11	31.05.2013
1F11_3	3D6scFc 1F11	07.06.2013
3B9_1	3D6scFc 3B9	24.05.2013
3B9_2	3D6scFc 3B9	31.05.2013
3B9_3	3D6scFc 3B9	07.06.2013

The calculation of the qPCR result was done relative to a reference gene. For this analysis the reference gene β-Actin was used. Calculations were done according to the $2^{-\Delta\Delta Cq}$ method of (LIVAK and SCHMITTGEN, 2001). The calculation was done without efficiency correction because all samples were treated in the same way and also the same set of primers was used for the analysis of the antibody producing clones. According to (SOMMEREGGER et al., 2013) this does not affect the results of the comparison between the clones.

The calculation of Cq values was done with the Software package CFX manager 3.0 facilitating the baseline subtraction and the regression of the curves for threshold setting.

The further calculation was done according to the equations described in section 4.1.21. The correct size of the amplified DNA was checked with an agarose gel electrophoresis and confirmed for all qPCR samples.

In Figure 42 the relative gene copy number to the reference gene β-actin is shown. Although the reference gene should only be present once in the genome of the cell line, it is known, that the used set of primers bind more than once in the genome. So it does not mean that less than one copy of the GOI is present, although the low values indicate that only one copy is present. A BLAST analysis of the primers and amplicons showed at least 9 possible copies in the genome. For more accurate determination an absolute quantification with defined gene copy numbers or relative quantification with the use of additional reference genes or optimized primer/probes has to be done.

Therefore the results were related to the gene copy number of the GTN gene, which was present prior to the cassette exchange. If this shows comparable gene copy numbers, a complete cassette exchange can be assumed.

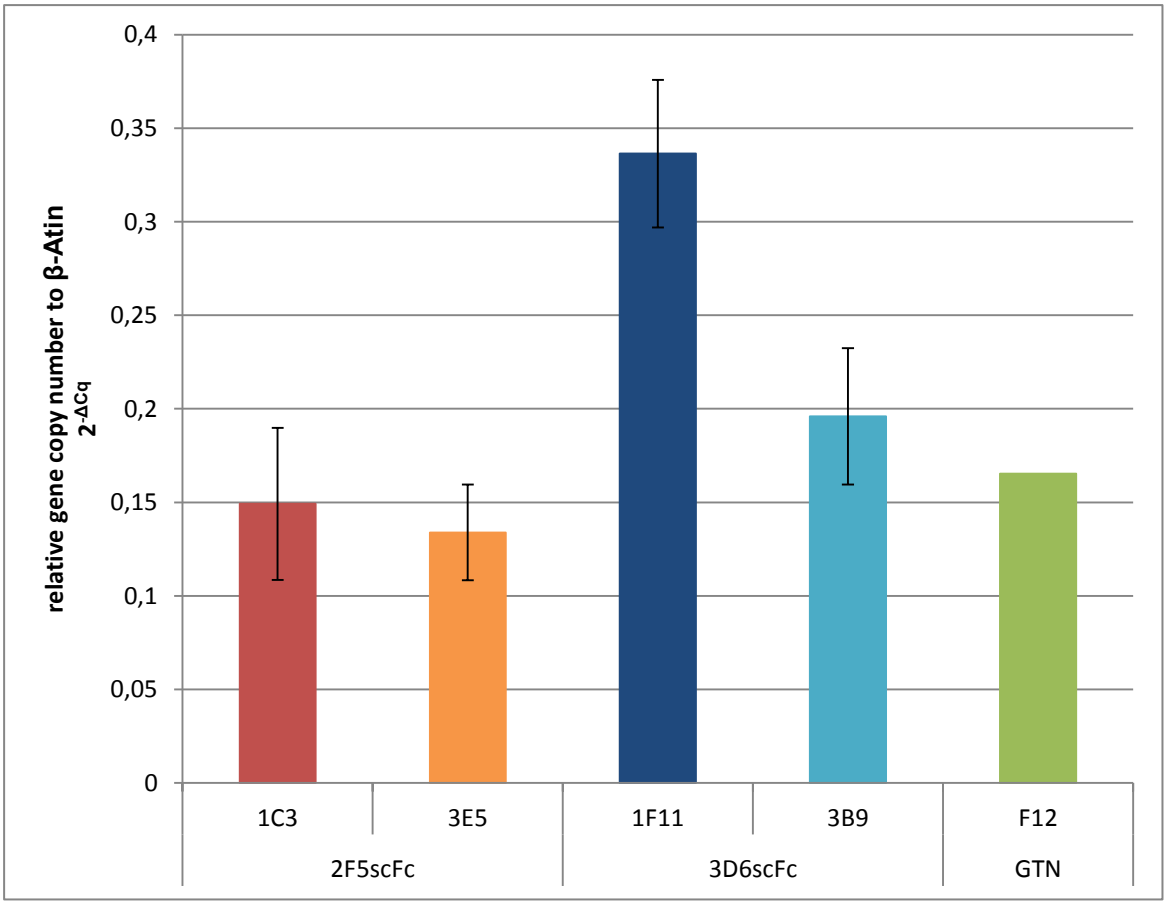


Figure 42: Relative gene copy to the reference gene β-actin. It can be clearly seen, that clone 1F11 shows double amount in contrast to all other clones.

In Figure 43 the relative gene copy number of the scFc producing cell lines to the GTN gene harbouring cell line is shown. The three clones 1C3, 3E5 and 3B9 showed a relative gene copy number of one. This indicates that the same gene copy number

after the cassette exchange is present. According to the FISH pictures, also only one integration locus was shown. In contrast, clone 1F11 showed the double amount of gene copy numbers than the GTN gene. This is also confirmed by the FISH data, which showed two integration sites.

Additionally to confirm this data by statistical methods, a one way analysis of variance was performed using the program SIGMA Plot 12.0. The result also indicated no significant differences between the clones 1C3, 3E5 and 3B9, but showed a significant difference for the clone 1F11.

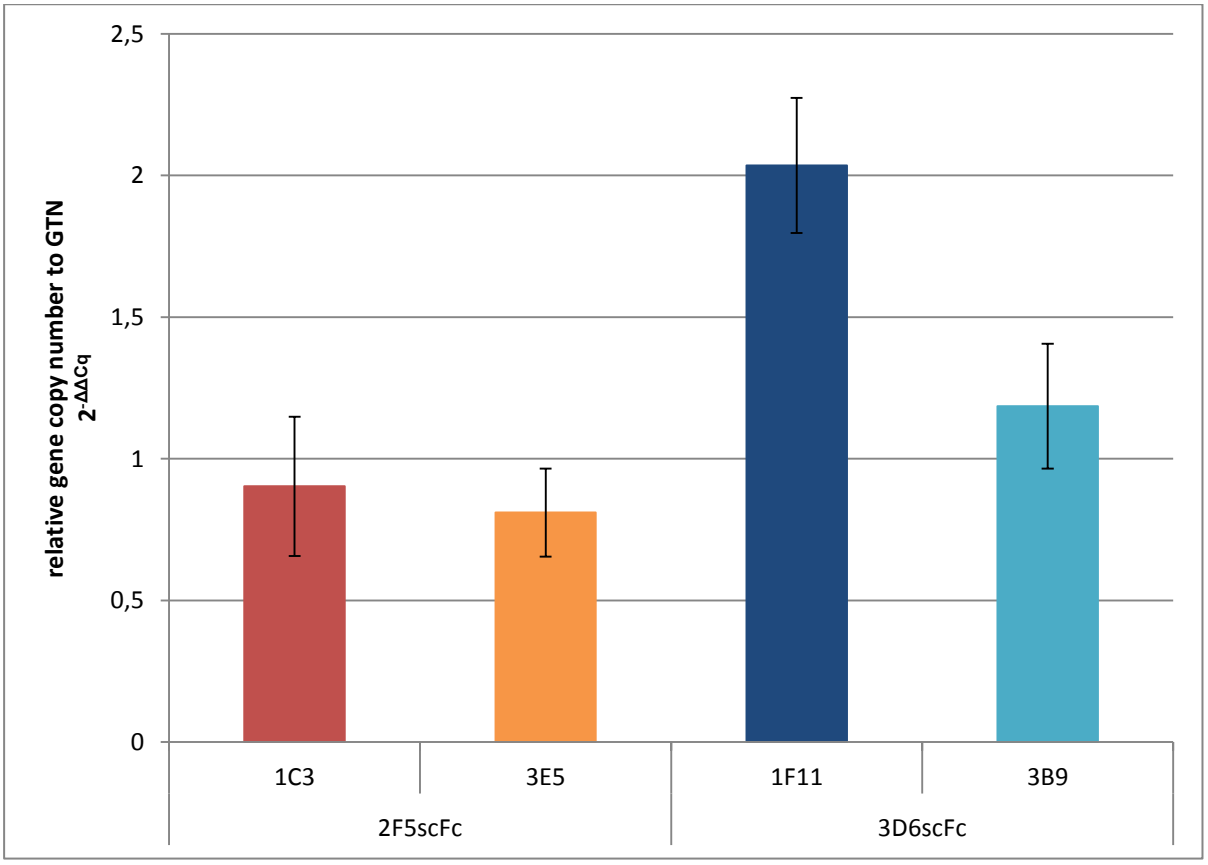


Figure 43: Relative gene copy number compared to the GTN gene. Only clone 3D6scFc 1F11 shows the double amount.

5.9. Analysis of the transcript level

The investigation of the RMCE showed an additional integration site for the clone 3D6scFc 1F11. The next logical step for analysis was the investigation of the transcript level. This should on the one hand confirm an equal transcript level independent from the produced antibody as it was reported prior (MADER et al., 2013). On the second hand, the promoter trap strategy was used, so the second transgene copy, which was determined for the clone 3D6scFc 1F11 should not be transcribed in relevant amounts.

For the determination of the transcript level for each cell line three biological samples were collected, in the same way as described for the determination of the gene copy number. After the RNA isolation DNA digestion and reverse transcription was performed. The determination of the transcript level itself was therefore performed with the cDNA obtained by the reverse transcription with random primers. The amount of cDNA represents the total RNA amount.

In Figure 44 the relative transcript level to the reference gene β-actin is shown. The values of all four different clones showed the same transcript level. This was also confirmed by a one way analysis of variance. The values below one in comparison to β-actin also indicate the presence of only one transcriptionally active gene copy. This is especially important for the clone 3D6scFc 1F11, because this clone contained two integration sites. It seemed, that the second integration site lies outside of the FRT sites, which had no promoter, is transcriptionally inactive or contributes only in such low amounts, that it was not detectable with qPCR. Therefore the promoter trap strategy is confirmed to work well. If even more guaranty for the replacement reaction is needed, also the possibility for a poly-A side trap was developed. (TURAN et al., 2011). For the analysis it was not possible to determine the relation of the transcript level to the GTN gene, as a different set of primers was used and therefore the results are not comparable due to different efficiencies in the qPCR. (SOMMEREGGER et al., 2013).

This analysis also confirmed that the different specific productivities of the 2F5scFc and 3D6scFc clones did not depend on the transcription of the genes, because the transcription level was the same for both products. This further indicates that the transcription is not the bottleneck for recombinant protein expression. It further indicates that the integrated DNA sequences do not have a different initiation of the

transcription or one of the genes has a lower mRNA half-life, as both had to contribute to the total RNA amount. If the way of a recombinant product through the protein expression system is followed, the next logical step is to investigate the internal product formation by flow cytometry.

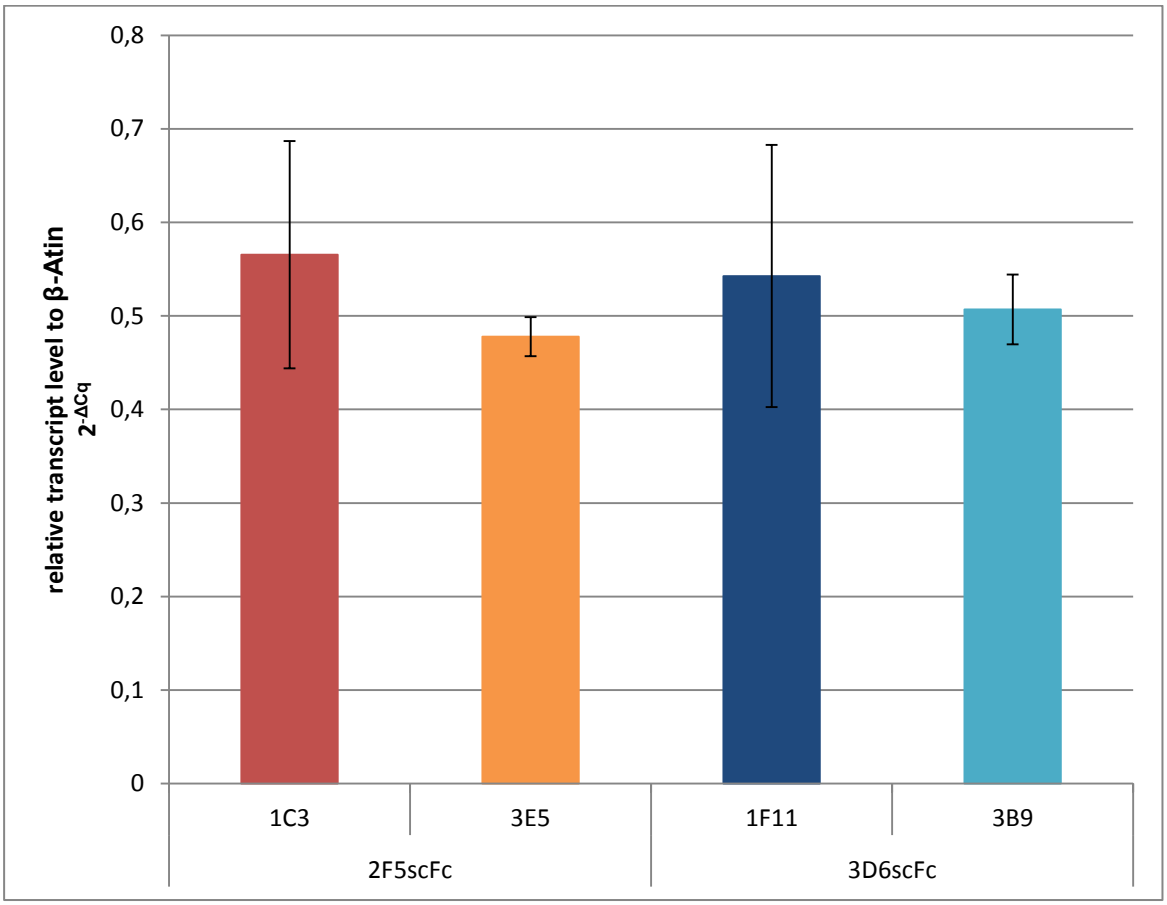


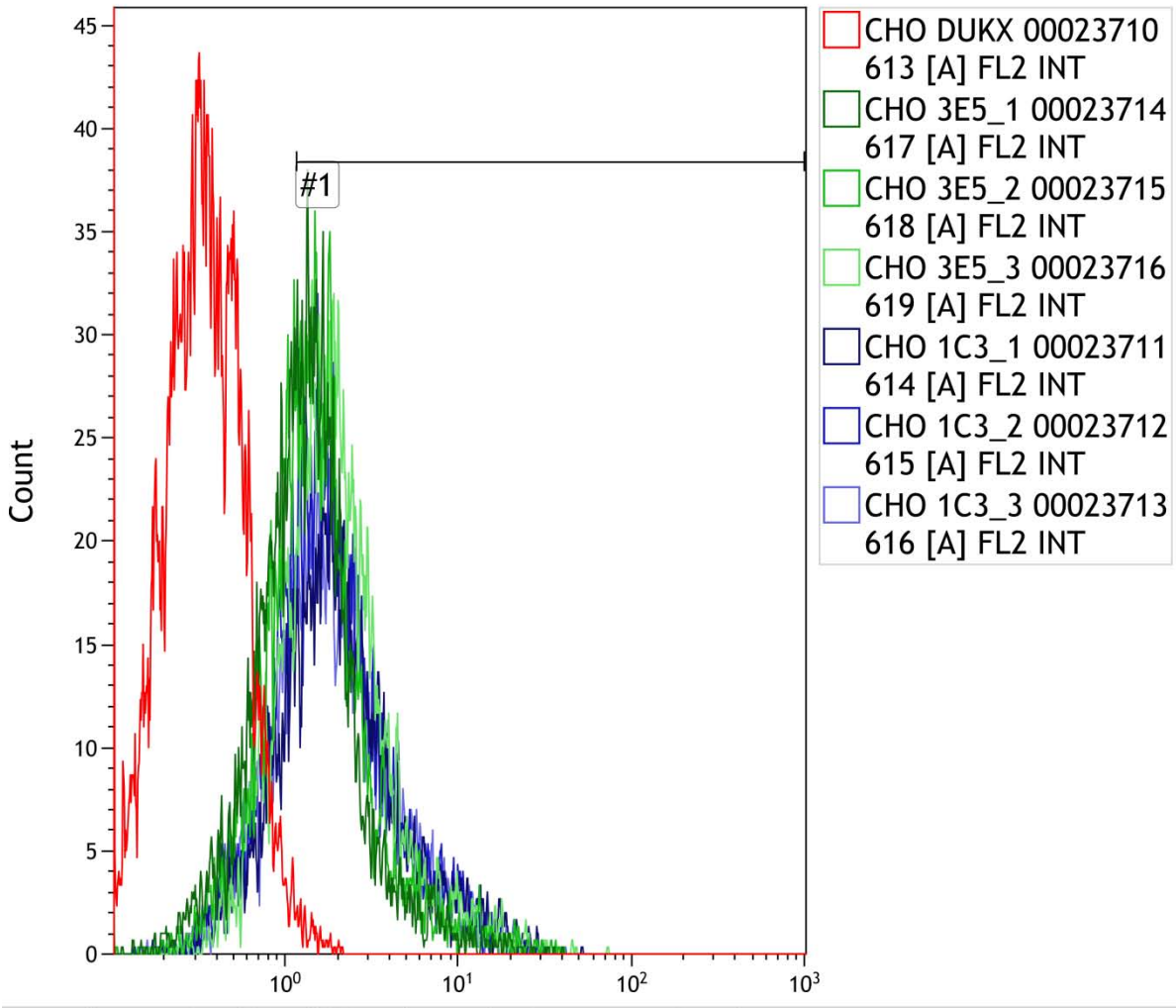
Figure 44: Relative transcript level in comparison to β-Actin. All four clones show the same amount.

5.10. Analysis of internal product formation by immune-fluorescence flow cytometry

The internal product formation was investigated by flow cytometry. Therefore from each cell line three different biological samples were analysed. The samples were taken at the same time points as described in section 5.8 for the determination of the gene copy numbers. The use of ethanol fixed cells allowed immunofluorescence detection, because the fixation enables the PE conjugated antibodies to pass through the membrane. For the detection an anti-human IgG gamma chain conjugated to PE developed in goat was used.

In Figure 45 an overlay plot of the 2F5scFc producing clones is shown. It shows the fluorescence intensity of every sample of the clones. Both clones showed a symmetrical peak, which is indicated by the similar values for x-mode, x- median and the geometric mean. The symmetrical peak shape represented a homogenous population with no subpopulation of low or high producers.

An interesting detail can be seen by the dataset of the clone 2F5scFc 3E5. The geometric mean values increased from sample 1 to sample 3 as well as the percentage of cells in gate #1. This is consistent with the increase of the qP, which also took place at this time. This increase could be related due to the better nutrition supply in the agitated spinner flask and showed the adaptation to it.

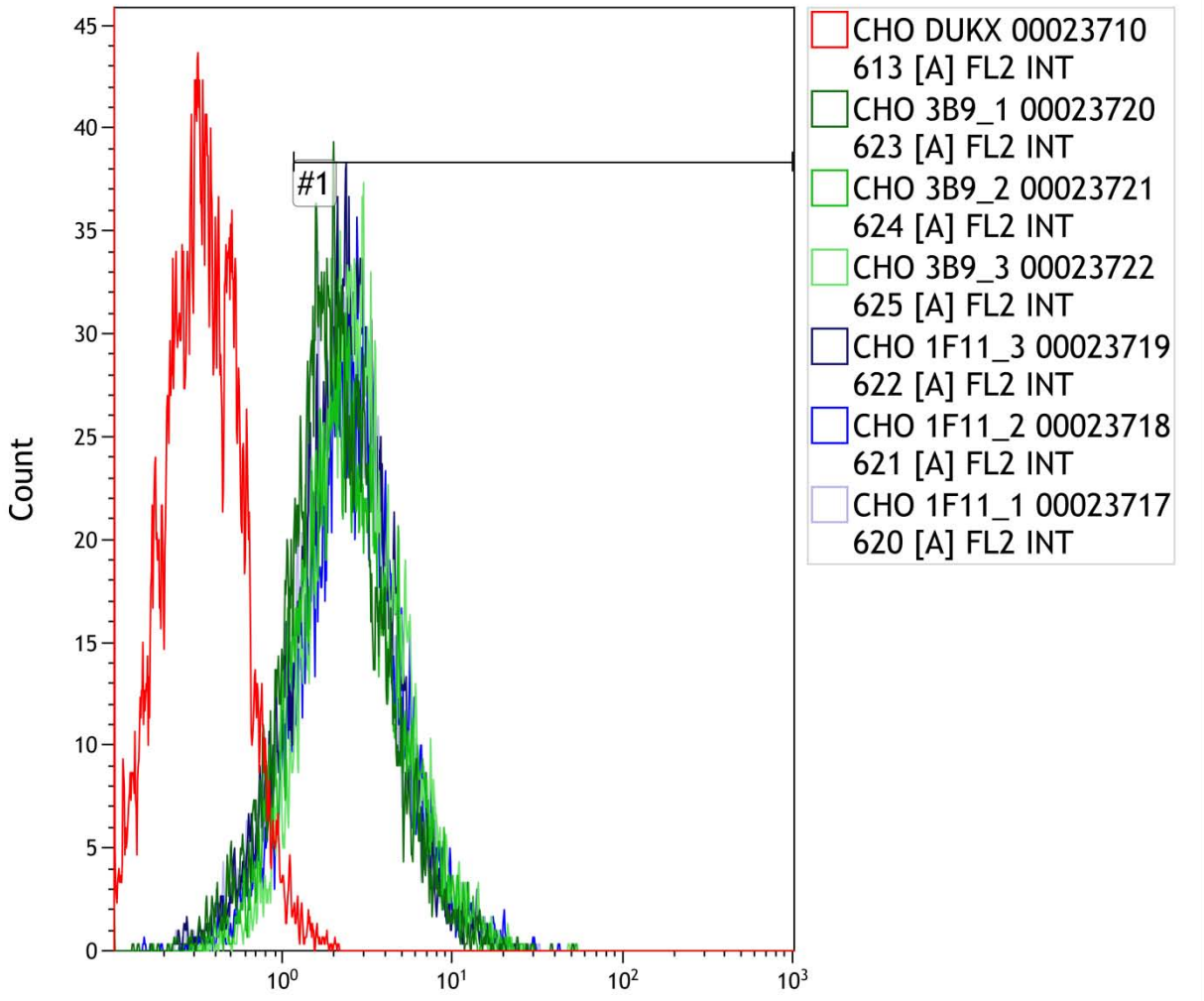


Marker	Number	%Total	%Gated	X-Med	X-AMean	X-Mode	X-GMean
All	5,489	54.89	100.00	0.33	0.38	0.10	0.27
All	4,500	45.00	100.00	1.31	1.80	1.35	1.19
All	4,452	44.52	100.00	1.44	2.12	1.49	1.36
All	4,553	45.53	100.00	1.80	2.66	1.43	1.72
All	3,587	35.87	100.00	1.85	2.99	1.69	1.78
All	4,144	41.44	100.00	1.69	2.55	1.55	1.62
All	3,783	37.83	100.00	1.75	2.63	1.80	1.64
#1	56	0.56	1.02	1.41	1.45	1.21	1.42
#1	2,609	26.09	57.98	1.76	2.54	1.35	1.96
#1	2,866	28.66	64.38	1.84	2.84	1.49	2.08
#1	3,499	34.99	76.85	2.13	3.21	1.43	2.34
#1	2,722	27.22	75.89	2.31	3.69	1.69	2.57
#1	3,007	30.07	72.56	2.13	3.21	1.55	2.37
#1	2,735	27.35	72.30	2.25	3.33	1.80	2.46

Figure 45: Fluorescence intensity overlay of the three samples for every 2F5scFc producing clone after gating the living cells.

In Figure 46 the overlay plot for the 3D6scFc producing clones is shown. These cell lines also showed homogenous peaks, indicated with the statistical values. As well as the cell line 2F5scFc 3E5 the clone 3D6scFc 3B9 also showed an increase in the geometric mean and the percentage of gated cells. The reason therefore is also the

adaptation phase to the spinner vessel, because this cell line was at the same time in culture in the spinner flask as the 2F5scFc 3E5 clone



Marker	Number	%Total	%Gated	X-Med	X-AMean	X-Mode	X-GMean
All	5,489	54.89	100.00	0.33	0.38	0.10	0.27
All	5,157	51.57	100.00	1.99	2.56	1.99	1.85
All	4,605	46.05	100.00	2.33	3.04	2.16	2.20
All	4,849	48.49	100.00	2.59	3.26	2.96	2.50
All	5,301	53.01	100.00	2.34	2.83	2.34	2.07
All	4,648	46.48	100.00	2.51	3.13	2.73	2.29
All	4,994	49.94	100.00	2.27	2.87	2.03	2.08
#1	56	0.56	1.02	1.41	1.45	1.21	1.42
#1	4,222	42.22	81.87	2.28	2.94	1.99	2.39
#1	3,978	39.78	86.38	2.59	3.38	2.16	2.68
#1	4,469	44.69	92.16	2.74	3.46	2.96	2.80
#1	4,491	44.91	84.72	2.61	3.19	2.34	2.64
#1	4,085	40.85	87.89	2.75	3.44	2.73	2.78
#1	4,245	42.45	85.00	2.56	3.23	2.03	2.62

Figure 46: Fluorescence intensity overlay of the three samples for every 3D6scFc producing clone after gating the living cells.

In contrast to the 2F5scFc clones these cell lines showed a slightly higher geometric mean as well as a higher percentage of gated cells in gate #1. A comparison of the geometric mean values for all four clones is shown in Figure 47. The 3D6scFc

producing cell lines showed a slightly, but significant, higher geometric mean value for the intercellular product formation than the 2F5scFc producing cell lines. This indicates that the antibody 3D6scFc is better formed inside the cell than the antibody 2F5scFc. Although the differences between the four cell lines was very low compared to the relative fluorescence of 1.42 of the gated negative control. So the difference in the intercellular product formation is rather low in comparison to the high difference of the qP, which indicates other bottlenecks in recombinant protein production like protein maturation or ER stress. These are also described bottlenecks in the literature (LATTENMAYER et al., 2007), (REISINGER et al., 2008).

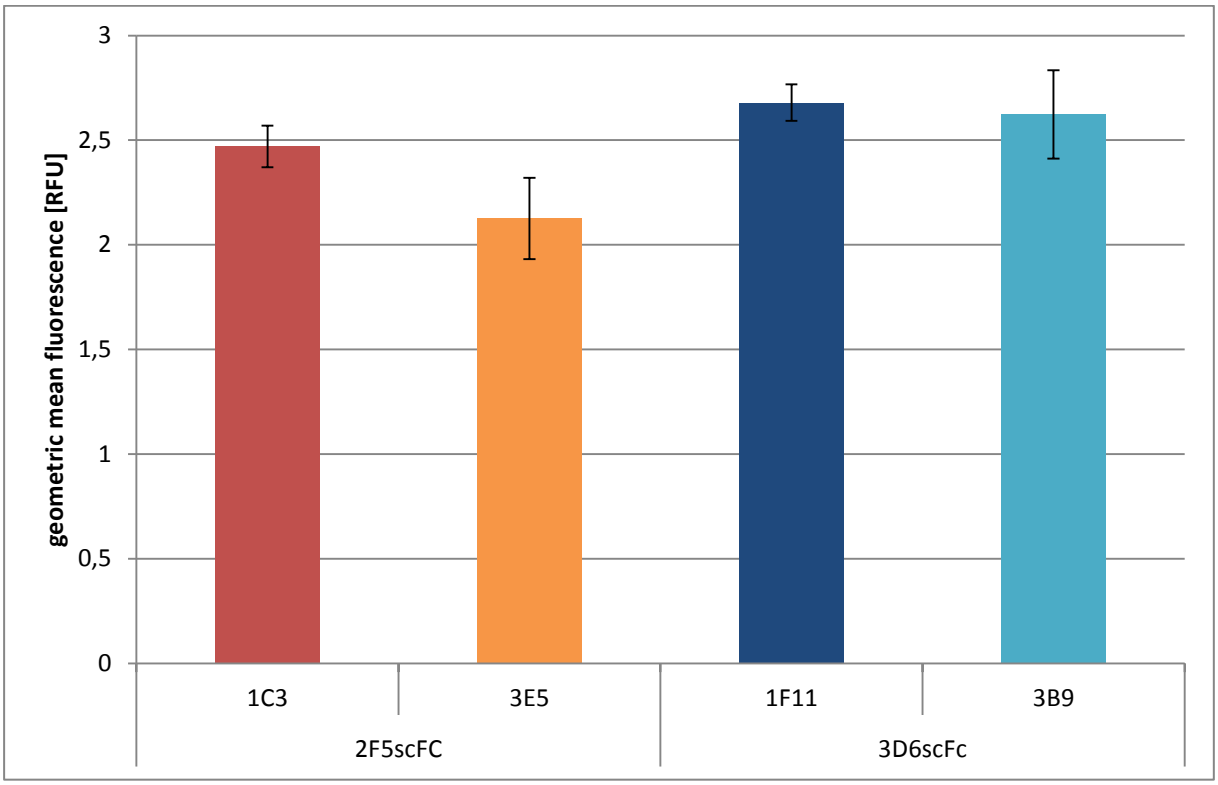


Figure 47: Geometric mean fluorescence intensity of all three samples without correction of the reaction blank. Clone 3E5 shows lower mean fluorescence intensity in comparison to the other clones.

5.11. Metabolic analysis of recombinant cell lines

To get further insight in the biology of the cell lines, the production of lactate and ammonia as well as the consumption of glucose was investigated. The analysis was done with the Bioprofiler 100 plus, Nova Biomedical, which measures all three parameters simultaneously. The determination of residual glutamine in the media was not possible, because the media for the cell lines was supplemented with stable glutamine (L-alanyl-glutamine).

The samples of the four cell lines were taken at the beginning of the cultivation in the spinner flasks, after the adaptation and at the end of the cultivation. For every sampling point three consecutive samples were taken to calculate at least two production rates per metabolic molecule respectively glucose consumption rates. In Table 16 the date and the passage number of the taken samples for the cell lines 2F5scFc 1C3 and 3D6scFc 1F11 are listed. In Table 17 the same information for the cell lines 2F5scFc 3E5 and 3D6scFc 3B9 are listed.

Table 16: Overview over the taken samples for the cell lines 2F5scFc 1C3 and 3D6scFc 1F11

Sample	Date	Passage Nr.	Days in culture
1	29.04.2013	2	4
2	02.05.2013	3	7
3	06.05.2013	4	11
4	31.05.2013	11	36
5	04.06.2013	12	40
6	07.06.2013	13	43
7	24.06.2013	18	60
8	27.06.2013	19	63
9	01.07.2013	20	67

Table 17: Overview over the taken samples for the cell lines 2F5scFc 3E5 and 3D6scFc 3B9

Sample	Date	Passage Nr.	Days in culture
1	10.05.2013	2	4
2	13.05.2013	3	7
3	17.05.2013	4	11
4	04.06.2013	9	29

Sample	Date	Passage Nr.	Days in culture
5	07.06.2013	10	32
6	11.06.2013	11	36
7	24.06.2013	15	49
8	27.06.2013	16	52
9	01.07.2013	17	56

In Figure 48 the average glucose consumption rate for all four cell lines in pg per cell per day is shown. No significant difference between the four cell lines could be detected. For the cell line 2F5scFc 1C3 one value for the glucose concentration had to be eliminated from the dataset, because it would result in negative glucose consumption rate. This was due to a measurement error of the Bioanalyser for the glucose concentration.

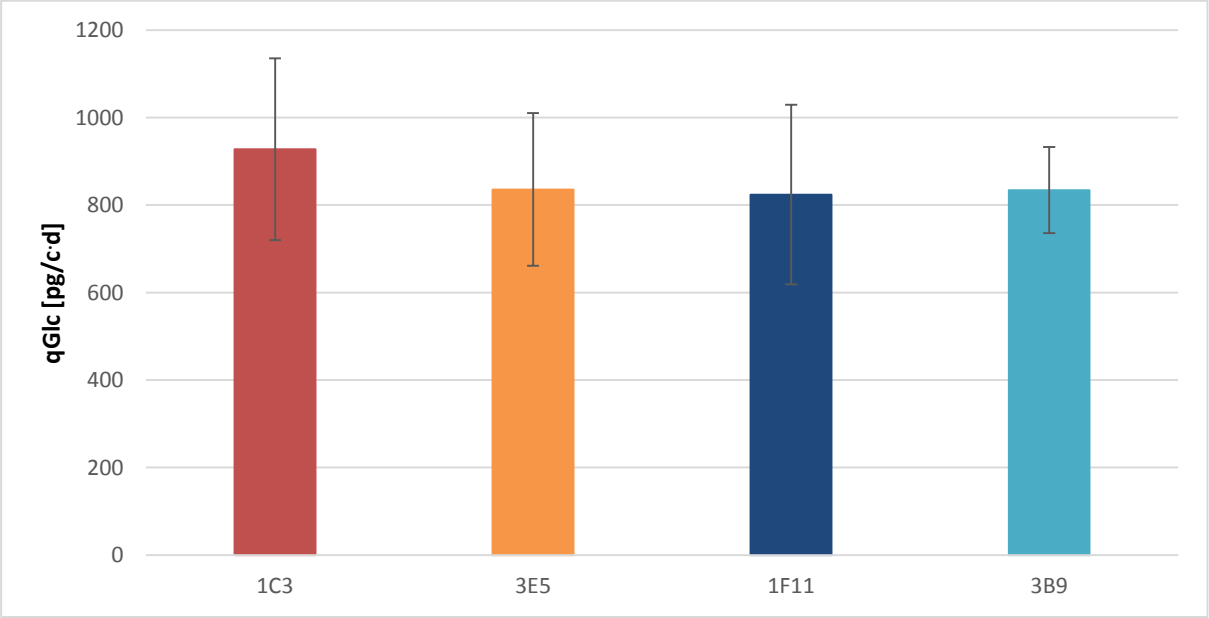


Figure 48: Average glucose consumption rate for all four cell lines during the cultivation in the spinner flask.

Figure 49 shows the average lactate production rate for all four cell lines. For the four clones also no significant difference could be detected.

In Figure 50 the average ammonia production rate for all four cell lines are shown. It also seems to be the same for all four cell lines although the clone 3D6scFc 3B9 showed a quite constant ammonia production rate in contrast to the other cell line.

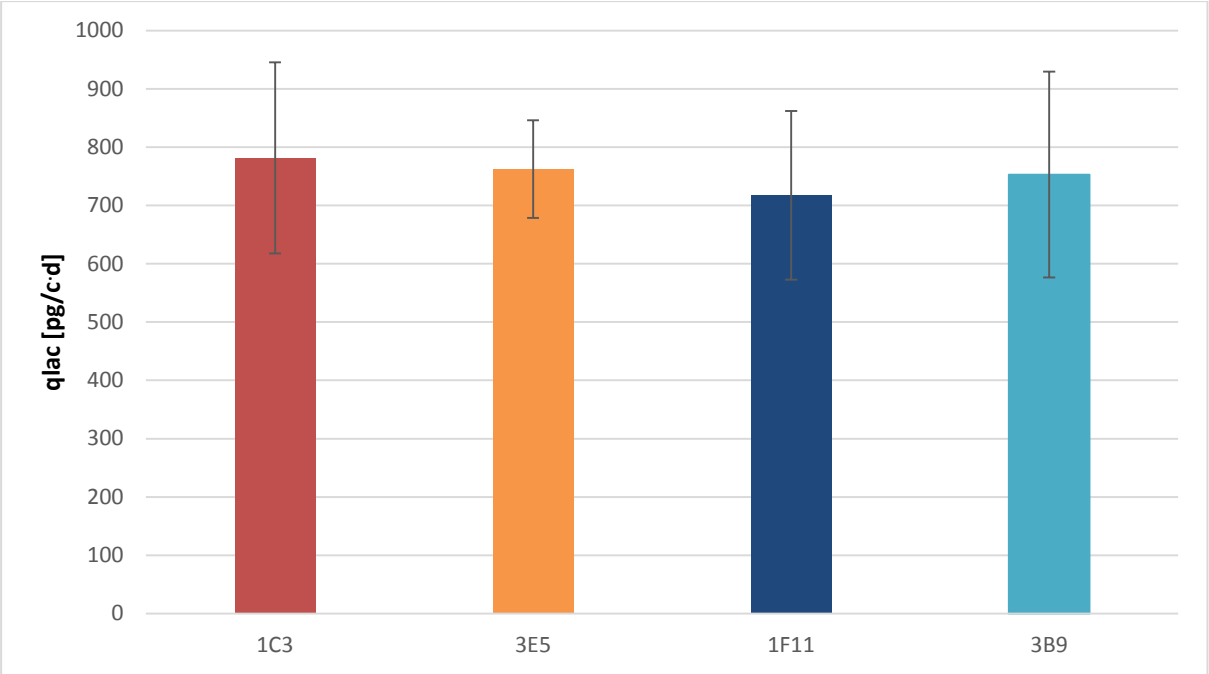


Figure 49: Average lactate production rate for all four cell lines during the cultivation in the spinner flask

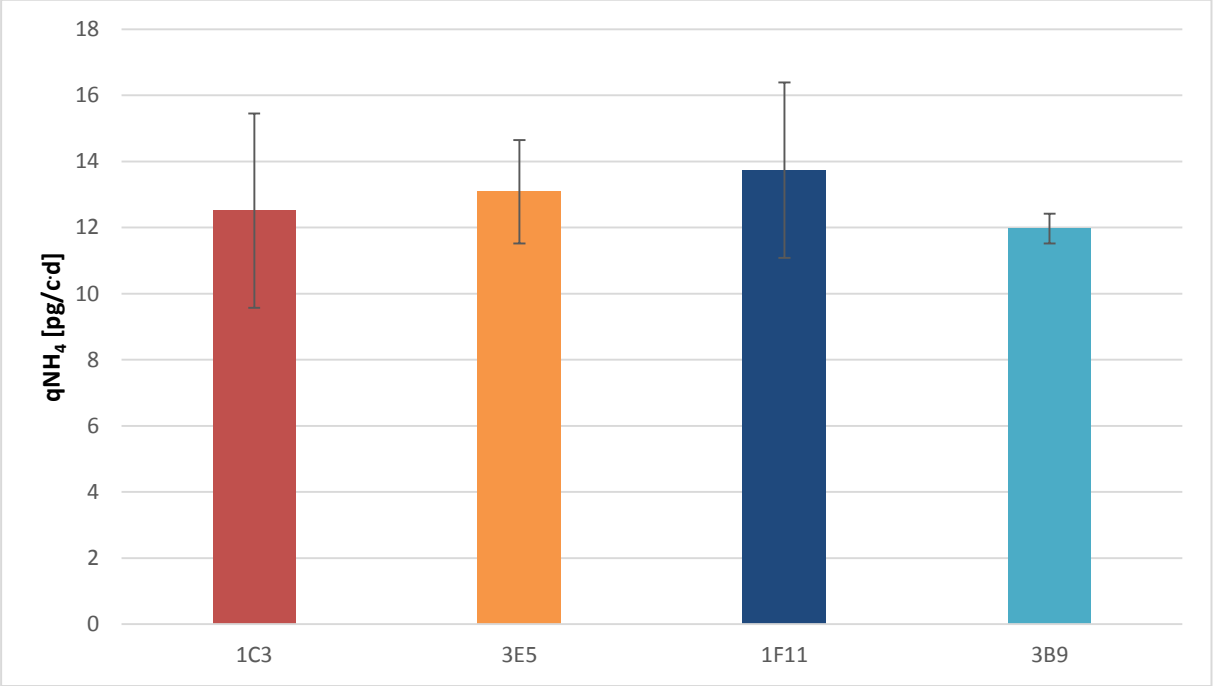


Figure 50: Average ammonia production rate for all four cell lines during the cultivation in the spinner flask.

In general no significant difference between the four cell lines could be detected. To get further insight into the metabolism of the antibody production additional samples or batch cultivation has be done. Also the analysis of glutamine especially with the information about the ammonia metabolism would enable a better insight. For very detailed investigation also labelling experiments together with advanced analytical methods like mass spectrometry could be performed.

5.12. Specificity testing of the produced antibodies

For every cell line four samples were taken for the specificity tests and all of them showed comparable curves. Therefore only the sample, which was taken at the end of the cultivation in the spinner flask, was used. In Figure 51 the plot of the absorption versus the logarithmic molar concentration of the used antibodies is shown. In this figure the absorption represents the concentration of bound antibodies. The values should show a sigmoid shape of the curve because of the logarithmic x-Axis. The absorption value for the highest concentration was lower compared to the 1:2 diluted values. This could be due to the maximum binding capacity per well and the large excess of the antibodies. The human monoclonal IgG anti-HIV-1antibody 2F5 was used as a control because it has a known affinity for the epitope ELDKWA (MUSTER et al., 1993). This means, that this specificity is only valid for the antibody 2F5 and not for the antibody 3D6, which binds to a different epitope. This has also be seen for the scFc variants, because only the curves of the 2F5scFc producing cell lines showed binding to the epitope and not the 3D6scFc producing cell lines This leads to the conclusion, that after the whole cultivation time no change in the specificity of the antibodies occurred.

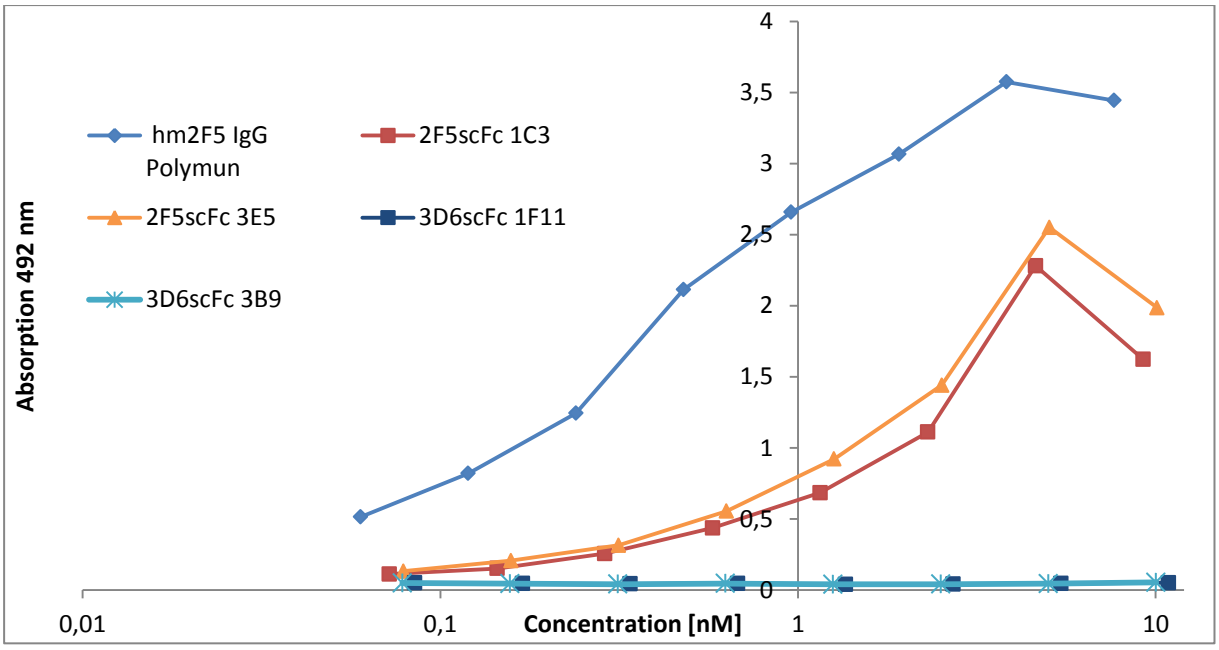


Figure 51: Specificity assay with the produced antibodies of the four recombinant cell lines. The human antibody hm 2F5 IgG was used as standard.

The binding affinity to the specific epitope is determined by the shape of the obtained curve. This means, the stronger the increase, the higher is the affinity to the epitope. The human IgG 2F5, which was used as control, showed a higher affinity than the

2F5scFc antibodies. This might be due to the structure of the antibody, because in a single chain antibody the variable light and the variable heavy chain were linked together with the $(GGGGS)_3$ linker. This linkage could affect the binding properties of the whole antibody, as the structure around the CDR regions becomes less flexible. Especially for the antibody 2F5scFc this might be a problem, because an unusual long CDR 3 region plays an important role for the binding (KUNERT et al., 1998). So a minor change in the structure of the antibody might affect the whole binding properties.

The antibody 2F5 is one of the few neutralising anti HIV antibodies. Further tests like neutralisation assays have to be performed to test, if the scFc variant of this antibody is still able to neutralise HIV.

6. Discussion

6.1. Investigation of RMCE

The development of antibody producing cell lines by the use of RMCE was demonstrated within this study. To get a sufficient cassette exchange the negative selection with the drug ganciclovir for the absence of the herpes simplex thymidine kinase was done. The detailed investigation of the RMCE by the use of FISH and determination of the gene copy number displayed some problems of the technique. In the case of the cell line 3D6scFc 1F11 two transgene integration sites by the use of FISH as well as the double amount of gene copy number in comparison to all other cell lines were shown. This did not result in any higher qP or higher transcript amount. Another problem is due to the laborious and time consuming procedure of the FISH analysis. Only four metaphase spreads of the distinct clones could be investigated in detail by this technique. More investigated metaphase spreads would be beneficial to obtain more precise results. In the obtained pictures, the integration locus did not seem to be the same prior to the cassette exchange because it sometimes seemed to be located at the telomere region and sometimes more in the middle. Additionally the different condensation status of the chromosomes complicated a clear interpretation. One solution therefore is the ordering of the chromosomes according to their size, although more metaphase spreads would also be beneficial for clearer results. Further it has to be determined, if this is due to chromosomal aberration during time. Also a Giemsa banding for a detailed chromosomal investigation could be done, to overcome the limitations. Another method for the identification of the chromosomes is according to the AT and GC content by flow cytometry and subsequent sorting and sequencing of the desired chromosomes (BRINKROLF et al., 2013). Also alternatives to this strategy were developed, like sorting chromosomes on a slide and then perform a FISH, or amplification of the GOI out of sorted chromosomes. Although, none of these techniques has a comparable resolution like advanced FISH methods (DOLEŽEL et al., 2012).

Another problem is the precise determination of gene copy number. In the qPCR analysis, only a comparison between the clones can be done, relative to β-actin. This is not an absolute quantification, although the low values in comparison to β-actin

indicate a low gene copy number. No distinct answer could be given, if really only one gene copy number is present in the genome and also the use of FISH can only determine if more integration loci are present. This method does not distinguish between the different gene copy numbers in one locus (LATTENMAYER et al., 2006).

In general, the use of the promoter trap strategy showed to be beneficial for the comparison of two different products by the use of RMCE, because the second integration site for this clone did not result in any higher transcript amount or outstanding qP. It is still possible, that the second transgene copy is active and a weak endogenous promoter drives the transcription of the gene in such low levels, that the used analytical methods were not able to detect it.

The use of S/MAR regions to facilitate RMCE events and also to augment transcription does not show similar effects as gene amplification or the highly open chromatin regions in the BAC system. The specific productivities of the developed cell lines are still below the plasmid or the BAC cell lines (MADER et al., 2013). This also might confirm the presence of only a low gene copy number.

The use of RMCE for comparative studies between different products, like the two scFc antibodies within this study is still given and RMCE is a valuable tool for cell biological investigations.

6.2. Cell line development

The development of the RMCE cell lines for recombinant protein production was done by the cotransfection of the enzyme Flipase and the GOI. The transfection method was based on the cationic polymer PEI and showed comparable results for both antibodies. The subsequent negative selection with the drug ganciclovir however displayed big differences between both products. It turned out, that 1 µM of ganciclovir is enough to obtain sufficient selection pressure. In the case of the antibody 2F5scFc 2 µM lead to a growth arrest of the most cells, although the 3D6scFc producing cell lines tolerate 2 µM. The attempts of sorting for the absence of the reporter gene GFP only showed inferior results in comparison to the clones grown in 96 well plates. This was due to technical limitations of the cell sorter, because the possibility for single cell sorting was not given. Another problem was the

enhanced auto fluorescence of the recombinant cell line and the resulting problems with discrimination of GFP positive and negative cells.

From the conventional transfections seeded into 96 well plates, the 12 best clones per produced antibody were selected and further cultivated into T25 Roux flasks. Out of these twelve clones two clones were selected for further cultivation in a spinner flask. The selection of these two clones was based on antibody production, growth, intercellular product formation and qP. These clones (two for each antibody) were cultivated for at least 66 days and split twice a week. At every splitting the cell density, the product concentration and the viability was determined and the specific growth rate and qP was calculated out the obtained values. Additionally samples for metabolic analysis were taken. In total, the four cell lines showed similar growth, glucose consumption, ammonia formation and lactate formation rates. A difference between the four cell lines was the qP, which was 1.5 to 2 fold higher for the cell lines producing 3D6scFc than for the 2F5scFc producing cell lines. Another interesting fact was the steady increase of specific growth rate during the spinner cultivation due to the adaptation. The agitation in the spinner flask offers further better nutrition supply. This behaviour was also observed for the qP for the first splittings, although only one cell line (3D6scFc 1F11) showed a steady increase over the entire cultivation time.

6.3. Comparison in the production of the two antibodies

The newly developed cell lines were used for different comparison. As described before, the cell lines producing the antibody 3D6scFc always showed a higher qP in comparison to the cell lines producing 2F5scFc. Because both cell lines were constructed by the use of RMCE, they harbour in principal the gene for antibody production on the same locus. Further analyses were done by the investigation of the transcript level for both antibodies by reverse transcription and qPCR analysis relative to β-Actin. The results therefore showed no difference in the transcript level of both antibodies. The next step was the analysis of the intercellular product concentration by flow cytometry. In this analysis all four cell lines exhibited a homogenous antibody producing population. The 3D6scFc producing cell lines showed slightly higher geometric mean fluorescence in this analysis, which indicates a slightly higher intercellular product formation. This difference was not in the magnitude as for the qP. The 1.5 to 2 fold higher qP can not only be due to a slightly improved intercellular product formation. This data indicates that the main differences

in the recombinant antibody production between these two molecules result from protein maturation, folding and ER stress response.

6.4. Specificity testing of both antibodies

At the end of the cultivation in the spinner flask both antibodies were used for a specificity assay for the epitope ELDKWA. This epitope is only binding to the antibody 2F5. This was also confirmed by the samples taken from the four cell lines. Only the produced antibody 2F5scFc showed any binding to this epitope, although the affinity was lower compared to the 2F5 IgG antibody. This can be due to small differences in the molecular structure, because of the peptide linker between the two variable domains. Further tests have to be done, if the 2F5scFc antibody still possesses the ability to neutralize HIV. This test indicated that during the cultivation no interchange of the cell lines occurred.

7. Conclusion

The use of RMCE for recombinant protein production could be demonstrated within this study. This concept provides an excellent tool to compare the production of different antibodies. In this study, the two antibodies 2F5scFc and 3D6scFc were compared and the antibody 3D6scFc showed a higher qP, although no difference in the specific growth rate, metabolism or transcript level could be detected. Also a slightly higher intercellular product formation was observed for the antibody 3D6scFc.

The detailed investigation of the RMCE by the use of FISH and qPCR showed the possibility of an additional transgene copy in the genome in one case. The use of the promoter trap strategy to prevent the active transcription of the additional site also worked, because this cell line did not show any higher transcription level. The precise integration locus could not be determined within this study due to time limitations.

8. Appendix

8.1. Different conditions for each PCR reaction

Primer pair	Poymerase	Annealing temperature
pMG433_FSHB_2465_as &9 3D6scFc_Amp_as	Taq Polymerase	53 °C
neo_sense & neo_as	KAPA HIFI	60 °C
Flp_sense & Flp_as	KAPA HIFI	64 °C

8.2. Primers used for qPCR

Primer	Sequence	Amplicon size
scFc_sense	ACGAGGACCCTGAAGTGAAG	100 bp
scFc_Probe	AAGTGCACAACGCCAAGACCAAGC	
scFc_as	CGGTAGGTGGAGTTGTACTGTTC	
β-actin_sense	TGAGCGCAAGTACTCTGTG	78 bp
β-actin_probe	CCATCCTGGCCTCACTGTCCACCT	
β-actin_as	TTGCTGATCCACATCTCCTG	
Neo_sense	ATGGCCGCTTTTCTGGATTC	74 bp
neo_probe	GACTGTGGCCGGCTGGGTG	
Neo_as	AGCCAACGCTATGTCCTGATAG	

8.3. Molecular weight of antibodies used for specificity testing

Antibody	Weight [kDa]
hm2F5IgG	147.6
2F5scFc	105.6
3D6scFc	104.7

8.4. References

BRINKROLF, K. et al. (2013): Chinese hamster genome sequenced from sorted chromosomes. Nat Biotechnol 31: 694-5.

BUTLER, M.; MENESES-ACOSTA, A. (2012): Recent advances in technology supporting biopharmaceutical production from mammalian cells. Applied Microbiology and Biotechnology 96: 885-894.

CHUSAINOW, J. et al. (2009): A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng 102: 1182-96.

CLAMME, J. P.; AZOULAY, J.; MÉLY, Y. (2003): Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy. Biophys J 84: 1960-8.

DOLEŽEL, J. et al. (2012): Chromosomes in the flow to simplify genome analysis. Funct Integr Genomics 12: 397-416.

FISCHER, D. et al. (1999): A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 16: 1273-9.

FUTCHER, A. B. (1986): Copy number amplification of the 2 micron circle plasmid of Saccharomyces cerevisiae. J Theor Biol 119: 197-204.

FYFE, J. A. et al. (1978): Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J Biol Chem 253: 8721-7.

KAUFMAN, R. J. et al. (1985): Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Mol Cell Biol 5: 1750-9.

KIM, J. Y.; KIM, Y. G.; LEE, G. M. (2012): CHO cells in biotechnology for production of recombinant proteins: current state and further potential. Applied Microbiology and Biotechnology 93: 917-30.

KUNERT, R.; RÜKER, F.; KATINGER, H. (1998): Molecular characterization of five neutralizing anti-HIV type 1 antibodies: identification of nonconventional D segments in the human monoclonal antibodies 2G12 and 2F5. AIDS Res Hum Retroviruses 14: 1115-28.

LATTENMAYER, C. et al. (2006): Identification of transgene integration loci of different highly expressing recombinant CHO cell lines by FISH. Cytotechnology 51: 171-82.

LATTENMAYER, C. et al. (2007): Characterisation of recombinant CHO cell lines by investigation of protein productivities and genetic parameters. J Biotechnol 128: 716-25.

LIVAK, K. J.; SCHMITTGEN, T. D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-8.

MADER, A. et al. (2013): Exploration of BAC versus plasmid expression vectors in recombinant CHO cells. Appl Microbiol Biotechnol 97: 4049-54.

MAYRHOFER, P. (2013): Integration of retargetable FLP/FRT sites into DUKX-B11 CHO cells for Recombinase Mediated Cassette Exchange (RMCE). Institute for applied microbiology. Vienna, University of Natural Resources and Life Sciences, Vienna.

MIELKE, C. et al. (1990): Hierarchical binding of DNA fragments derived from scaffold-attached regions: correlation of properties in vitro and function in vivo. Biochemistry 29: 7475-85.

MORTENSEN, R. M.; KINGSTON, R. E. (2001): Selection of Transfected Mammalian Cells. Current Protocols in Molecular Biology. John Wiley & Sons, Inc.

MUSTER, T. et al. (1993): A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J Virol 67: 6642-7.

PUCK, T. T.; CIECIURA, S. J.; ROBINSON, A. (1958): Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J Exp Med 108: 945-56.

QIAO, J. et al. (2009): Novel tag-and-exchange (RMCE) strategies generate master cell clones with predictable and stable transgene expression properties. J Mol Biol 390: 579-94.
RAYMOND, C. S.; SORIANO, P. (2007): High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. PLoS One 2: e162.
REISINGER, H. et al. (2009): Serum-free transfection of CHO cells with chemically defined transfection systems and investigation of their potential for transient and stable transfection. Cytotechnology 60: 115-23.
REISINGER, H. et al. (2008): The absence of effect of gene copy number and mRNA level on the amount of mAb secretion from mammalian cells. Appl Microbiol Biotechnol 81: 701-10.
SCHLAKE, T.; BODE, J. (1994): Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. Biochemistry 33: 12746-51.
SOMMEREGGER, W. et al. (2013): Transgene copy number comparison in recombinant mammalian cell lines: critical reflection of quantitative real-time PCR evaluation. Cytotechnology.
TURAN, S. et al. (2011): Recombinase-mediated cassette exchange (RMCE): traditional concepts and current challenges. J Mol Biol 407: 193-221.
URLAUB, G.; CHASIN, L. A. (1980): Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proceedings of the National Academy of Sciences of the United States of America 77: 4216-4220.
WALSH, G. (2010): Biopharmaceutical benchmarks 2010. Nature Biotechnology 28: 917-924.
WILHELM, J.; HAHN, M.; PINGOUD, A. (2000): Influence of DNA target melting behavior on real-time PCR quantification. Clin Chem 46: 1738-43.
WILSON, C.; BELLEN, H. J.; GEHRING, W. J. (1990): Position effects on eukaryotic gene expression. Annu Rev Cell Biol 6: 679-714.
WURM, F. M.; HACKER, D. (2011): First CHO genome. Nature Biotechnology 29: 718-720.

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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.

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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 Kratzer, September 2013)