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Antibody gene expression in CHO cells with recombinase mediated cassette exchange

Master Thesis

submitted by
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Abstract

In traditional transfections the transgene is randomly integrated into the chromosomes of the host cell line. Often, the expression level of traditionally transfected cells is variable and not stable. This is partly caused by an unpredictable influence of random integration sites, also called positioning effect. To overcome these major issues, a targeted gene integration tool is highly needed for comparing antibody producing cell lines under isogenic conditions. The recombinase-mediated cassette exchange (RMCE) concept with Flippase (Flp) enables the exchange of a target cassette which is flanked by two heterospecific Flippase recognition target (FRT) sites. The exchange is performed by co-transfection of Flp recombinase together with the donor cassette which is flanked by the same heterospecific sites. Within this study, three IgG1 antibodies (2G12, Ustekinumab, 4B3) and their associated germline variants (353/11, 554/12, 136/63) were expressed in the host cell line CHO RMCE I3 using the RMCE system. After co-transfection the clones were isolated by limiting dilution cloning and they were screened by enzyme-linked immunosorbent assay (ELISA). The subclones were compared in routine cultivations and in batch experiments. In addition intracellular product and GFP were measured by flow cytometry. Further emphasis was set on the isolation of the genomic DNA and the investigation of the donor plasmid integration by polymerase chain reaction (PCR). In general the antibody 2G12 and 353/11 had the highest product titers. The genetic investigation of 2G12 and 353/11 clones showed for the first time that the targeted RMCE exchange of the GTN fusion protein by the antibody donor cassette was successful. It was proven that targeted RMCE integration is possible with the new CHO K1 host CHO RMCE I3.

Zusammenfassung

Bei traditionellen Transfektionen wird das Transgen zufällig in die Chromosomen der Wirtszelllinie integriert. Das Expressionsniveau der transfizierten Zellen ist üblicherweise variabel und nicht stabil. Dies wird zum Teil durch den unvorhersehbaren Einfluss von zufälligen Intregrationsseiten verursacht, auch bekannt als Positionierungseffekt. Um diese bedeutenden Probleme zu beheben, wird eine Möglichkeit zur gezielten Genintegration dringend benötigt, um Antikörper-produzierende Zelllinien unter isogenen Bedingungen vergleichen zu können. Das Rekombinase mediierte Kassetten Austausch (RMCE) System mit Flippase (Flp) ermöglicht den gezielten Austausch einer Kassette, welche durch zwei heterospezifische Flippase Erkennungsziele (FRT) Seiten flankiert wird. Der Austausch wird durch die Co-Transfektion von Flp Rekombinase zusammen mit der Spender-Kassette durchgeführt. In der vorliegenden Arbeit wurden unter der Verwendung des RMCE Systems drei verschiedene IgG1 Antikörper (2G12, Ustekinumab, 4B3) und ihre zugeordneten Keimbahn-Varianten (353/11, 554/12, 136/63) in der Wirtszelllinie CHO RMCE I3 exprimiert. Nach den Co-Transfektionen wurden die Klone durch Grenzverdünnungsklonierung isoliert und durch Enzym-Immunoassay (ELISA) überprüft. Die Subklone wurden in der Routinekultur und Batch Experimenten verglichen. Zusätzlich wurde das intrazelluläre Produkt und GFP mittels Durchflusszytometrie gemessen. Ein weiterer Schwerpunkt wurde auf die Isolierung der genomischen DNA und die Untersuchung der Integrierung des Spender-Plasmids mittels Polymerase Kettenreaktion (PCR) gelegt. Im Allgemeinen wurden die höchsten Produktkonzentrationen bei den Antikörpern 2G12 und 353/11 festgestellt. Die genetische Untersuchung der 2G12 und 353/11 Klone zeigte zum ersten Mal, dass der gezielte RMCE Austausch des GTN Fusionsproteins durch ein Antikörper Spender-Kassette erfolgreich war. Es wurde bewiesen, dass die gezielte RMCE Integration in den neuen CHO K1 Wirt CHO RMCE I3 möglich ist.

Content

1 Abbreviations and Units

1.1 Abbreviations

bp	Base pairs
BSA	Bovine serum albumin
BX	Bromphenolblue - Xylencyanol
c/mL	Cells per milliliter
Cat. No.	Catalogue Number
CHO	Chinese hamster ovary
DAPI	4',6' - diamidino - 2 - phenylindol
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
EtBr	Ethidium bromide
EtOH	Ethanol
Fc	Fragment crystallizable
Flp	Flippase
FRT	Flippase recognition target
FS	Forward Scatter
GCV	Ganciclovir
gDNA	Genomic Deoxyribonucleic acid
GFP	Green fluorescent protein
GOI	Gene of interest
GTN	Green fluorescent protein/thymidine kinase/ neomycin phosphotransferase fusion protein
HBS	HEPES buffered Saline
HC	Heavy chain
IgG	Immunoglobulin G
L	Ladder
L-gln	L-glutamine
LC	Light chain

kbp	Kilo base pairs
mAb	Monoclonal antibody
OD	Optical density
Ori	Origin of replication
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
rEGF	Recombinant epidermal growth factor
RMCE	Recombinase mediated cassette exchange
RNA	Ribonucleic acid
RO-water	Reverse osmosis purified water
rpm	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SS	Side scatter
SV40	Simian Virus 40
Ta	Annealing Temperature
TAE	Tris-acetate- Ethylenediaminetetraacetic
TK	Herpes simplex Thymidine kinase
Tm	Melting temperature
TRIS	Tris(hydroxymethyl)-aminomethan
UV	Ultraviolet

1.2 Units

°C	Degree Celsius
d	Days
Da	Dalton [g/mol]
g	Gram
h	Hours
L	Liter
M	Molarity (mol/L)
mg	Milligram (10^{-3} g)
min	Minute
mL	Milliliter (10^{-3} L)
ng	Nanogram (10^{-9} g)

nm	Nanometer (10^{-9} m)
pg	Picogram (10^{-12} g)
u	Units
V	Volt
µg	Microgram (10^{-6} g)
µL	Microliter (10^{-6} L)

2 Introduction

In 1956 the isolation of the first Chinese Hamster Ovary (CHO) cells was done by Theodore Puck. The cell line was generated by recovery of spontaneously immortalized fibroblast cells which were isolated from cultured ovarian cells of a Chinese hamster [1].
For recombinant protein expression CHO cells have an established history of regulatory approvals. They are the preferred host expression system for complex therapeutics [2]. Within mammalian expression platforms, a small number of products are generated by various human cell lines, mouse myeloma cell lines and baby hamster kidney cells (BHK). The most common non-mammalian-based production cells are bacterial systems (e.g. *Escherichia coli*), systems based on yeast (e.g. *Saccheromyces cerevisiae*) and insect cells (e.g. *Spodoptera frugiperda*). Additionally, transgenic animal production with rabbits and goats are used for production of biologic products. Finally, the first US approval for a biologic product in plant cell culture was obtained in 2012 [3].

There are many advantages which suggest the use of CHO cells. First of all, the long regulatory history facilitates the approval of the therapeutic proteins on the market. In general a low specific productivity (qP) is known in mammalian production platforms, but this problem can be overcome by gene amplification, selection and screening procedures in CHO cells [4]. The biggest advantage is the human compatible post-translational modification (PTM). Correct PTMs, like glycosylation, are relevant for the therapeutic efficacy, protein longevity and to reduce safety concerns [2]. Finally, the adaptation to serum-free cultivation in suspension is possible. This leads to advantages for the further regulation and a reduction of costs which is especially relevant in large-scale cultures [4]. In highly optimized systems 3 to 10 g/L of high-value product can be generated by CHO cell lines [5].
CHO K1 is an ancestral cell line and many CHO cell lines derive from it [6]. CHO K1 contains 21 chromosomes and only 8 of them appear equivalent to an ordinary Chinese hamster chromosomes consisting of 22 chromosomes [5].

Monoclonal antibodies (mAbs) have an important status on the biopharmaceutical market and they still continue in their sophisticated role. Between 2010 and October 2014, 54 biologics were approved and the highest fraction with 17 were mAbs (27% of all approvals of biologic products). In the twenty-first century a focus was set on humanized or human forms over chimeric mAbs. Monoclonal antibodies as biopharmaceutical product are the most lucrative single product class. They reached $75.7 billion sales in 2013 including Fc fusion products. Concerning mammalian expression the Chinese hamster ovary cell line keeps the most commonly used expression system in the past 4 years [3].

In traditional transfections the transgene is randomly integrated into the chromosomes of the host cell line together with a selectable marker gene. Positive selection for stable transgenic cell lines is controlled by cell culture media supplemented with a cytotoxic antibiotic or using host cells lacking essential metabolic enzymes (e.g. dihydrofolate reductase) [7]. Often, the expression level of traditionally transfected cells is variable and not stable. This is partly caused by an unpredictable influence of random integration sites, also called positioning effect. Additionally, the presence of multiple integrated copies, chromosomal aberrations [8] and/or repeat-induced silencing [9] can cause complications. Furthermore, comparison of antibody expression levels and investigation of product influence on cellular behavior is complicated by unpredictable, multi-copy integration into different chromosomal loci. For these purposes a targeted gene integration tool is highly needed for comparing antibody producing cell lines under isogenic conditions.

The recombinase-mediated cassette exchange (RMCE) concept with Flippase (Flp) was first used in 1994 [10]. The target cassette is flanked by two heterospecific Flippase recognition target (FRT) sites and the exchange is performed by co-transfection of Flp recombinase together with the donor cassette which is flanked by the same heterospecific sites. The target cassette is integrated into the host cell genome, whereas the gene replacement cassette is part of the donor plasmid. It was shown that for the Cre/loxP system the chromosomal position of the integrated target cassette is critical for the efficiency of the RMCE system [11]. Common selection markers are hygromycin/thymidine kinase fusion gene variants for pre-selecting targetable chromosomal loci [12]. The isolation of the Flp gene from the *S. cerevisiae* 2µm circle was done by Broach and Hicks in 1980. Flippase is considered as the best-characterized eukaryotic member of the

tyrosine recombinase family. The biochemical and structural features has been studied extensively [13]. A FRT site consists of 48 bp with three individual Flippase recombinase binding elements (FBEs) with a size of 13 bp. Two of these form an inverted repeat around an 8 bp spacer. A direct repeat is formed by the third FBE and therefore it is separated from the other FBEs by a single bp [12]. Different heterospecific FRT-variants were designed and tested by introducing specific mutations within the FRT spacer sequence [10]. A loss of the integrated cassette might be possible due to spontaneous genetic rearrangements of the CHO cell line [14] [15].
The RMCE system was already proven in CHO cells in the RMCE host DUKX-B11 F3/F to express the antibody fragments 3D6scFv-Fc and the 2F5scFv-Fc. The antibody variants were stably integrated into the host cell line and thereby the success of targeted integration of a single antibody chain (scFv-Fc) was demonstrated. A predictable and reproducible expression in DUKX-B11 F3/F of scFv-Fc antibodies was performed [16]. However, for expressing full-length antibodies, such as IgG1, the targeted integration of two antibody chains (heavy and light) is required. Even more challenging might be the targeted integration for generating isogenic cell lines of IgA or IgM isotypes that additionally require expression of the J-chain.

There are five different isotypes of immunoglobulins: G, A, M, E, D naturally occurring in mammalians. Each isotype has its own structure and has different tasks in the immune system. Immunoglobulin G (IgG) is a four chain monomer and the major class of immunoglobulin in the human blood. The variability of antibodies in the human body is generated by the V(D)J recombination which is a site-specific recombination. It joins separate antibody gene segments together to form functional VH and VL regions. The V(D)J recombinase is responsible for the mediation. The RAG complex functions as endonuclease and thereby leads to double-strand breaks between the targeted gene segments. During this joining a variable number of nucleotides are often lost or added. This alteration of nucleotides is called junctional diversification. This effect can also lead to non-productivity and can therefore lead to clonal deletion [17]. The V(D)J recombination can be used to rebuild antibodies which are known for a certain binding specificity.

After immunization the affinity of the antibodies produced against the immunizing antigen is increasing. This is due to the accumulation of point mutations

in heavy and light chain variable region sequences, predominantly in the complementarity-determining regions (CDRs), but also, to a lesser extent, in the framework regions (FR). The process is called affinity maturation and the somatic hypermutation (SHM) is a major part of it. After the coding regions have been assembled, the mutations take place in the germinal centers. These centers are structures formed by activated B cells which are proliferating rapidly in the lymphoid follicles. A rate of about one mutation per variable region coding sequence per cell generation is achieved. This process is called somatic hypermutation, because it occurs in somatic cells rather than germ cells. Furthermore the rate of SHM is about a million times greater than the spontaneous mutation rate in other genes [17].

3 Objectives

A co-transfection of antibody donor plasmid and recombinase enzyme for the recombinase mediated cassette exchange system (RMCE) enables the comparison of antibody expression levels without the positioning effect in the genome [16]. Different genes of interest are integrated into the same genomic locus of the Chinese hamster ovary (CHO) cells using the flippase (FLP)/FLP recognition target (FRT) system. CHO K1 grows to high cell densities and leads therefore to high product concentrations. As host cell line a CHO K1 with integrated GTN fusion protein flanked by heterospecific FRT variants was chosen.

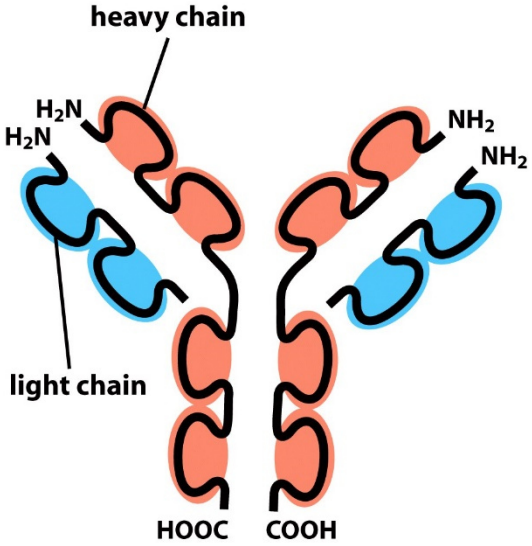


Figure 1: Structure of an IgG antibody [17]

In this project, three IgG1 antibodies (Figure 1) and their associated germline variants were expressed in the host cell line CHO RMCE I3 (in-house generation) using the RMCE system. As model antibodies 2G12, which is an IgG1 kappa anti HIV-1 antibody against gp120, and its germline variant were used [18]. In addition the therapeutic anti-IL12/23 IgG1 kappa human monoclonal antibody Ustekinumab and its germline variant were chosen. This therapeutic mAb is used as psoriasis treatment [19]. Furthermore the human monoclonal antibody

4B3 which is specific for HIV-1 gp41 and its germline variant were taken. The 4B3 antibody is an IgG1 lambda antibody [20].

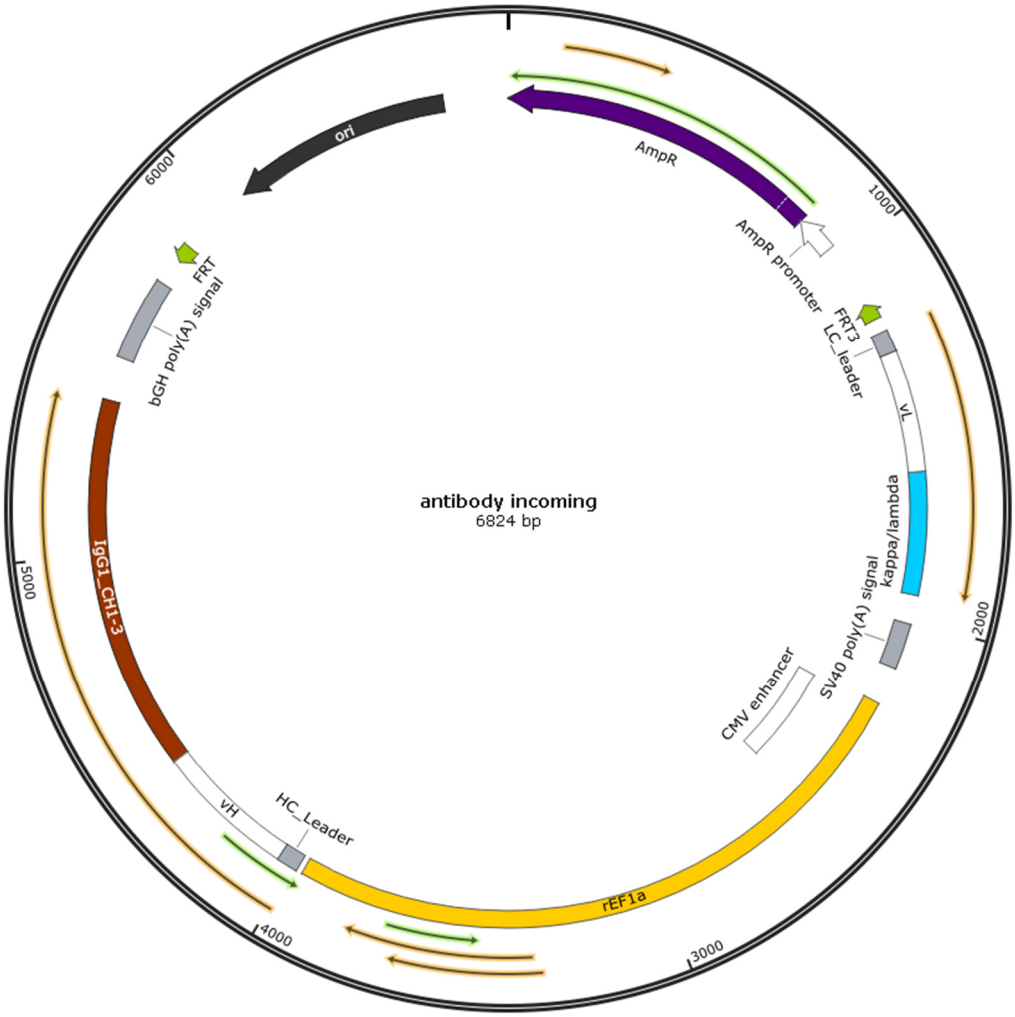


Figure 2: Donor plasmid for generation of antibody producing CHO cells. Light chain and heavy chain are located between FRT3 and FRT site. Arrows in green (antisense) and yellow (sense) on the outer rim mark the open reading frames.

The GTN fusion protein is already integrated into the CHO RMCE I3 cell line. It includes the enhanced green fluorescence protein (eGFP), the herpes simplex virus thymidine kinase and the neomycin phosphotransferase. The successfully co-transfected cells are selected via negative selection (Figure 2). The thymidine kinase functions as negative selection marker. Ganciclovir (GCV) is enzymatically phosphorylated by thymidine kinase which is expressed by the host cell line. The phosphorylation leads to an active triphosphate and is toxic for the

cell by mimicking dGTP and competing for mammalian DNA polymerases [21] [22]. An exchange of the GTN fusion protein by the antibody cassette mediated by the flippase enzyme removes the thymidine kinase. Successfully co-transfected cells are not able to phosphorylate ganciclovir and such preventing its toxicity. In order to ensure that the antibody expression is only possible if the donor plasmid is integrated in the FRT-sites, a "promoter trap" is constructed. An external CAGGS promoter is placed in front of the heterospecific FRT3 site. The donor plasmid does not include a promoter for the light chain of the IgG. In case of an exchange of the GTN fusion protein by the antibody cassette, the expression of the light chain is possible. If the integration of the donor plasmid takes place randomly, there is no promoter for the light chain available and the antibody cannot be transcribed.

Due to the fact that the positioning effect in the genome is removed by the RMCE system, a comparison of the different antibodies under isogenic conditions is possible. Three mature antibodies, which were generated by somatic mutations within the human body, were compared to their germline variants, which were constructed synthetically by combining the respective variable (V), diversity (D) and joining (J) segments.

Six different antibodies were integrated stably into the host cell line CHO RMCE I3 by RMCE. After co-transfection of antibody donor plasmid and FLP expression plasmid the clones were isolated by limiting dilution cloning and they were screened by enzyme-linked immunosorbent assay (ELISA).The specific growth rate and the specific productivity were determined from the routine cultivations of the generated cell lines and in batch experiments. Furthermore intracellular product and GFP were measured by flow cytometry. A purification of produced antibodies and an isolation of the genomic DNA of two cell lines were done. Further emphasis was set on the investigation of the donor plasmid integration by polymerase chain reaction (PCR).

4 Material and Methods

4.1 Material

4.1.1 Equipment

Centrifuge	Thermo Scientific Heraeus Megafuge 16 Centrifuge
Small centrifuge	Eppendorf Centrifuge 5415 R Eppendorf Centrifuge 5424
Microcentrifuge	VWR® Galaxy MiniStar
Pipettes	Gilson pipetman® neo P1000N 10-1000 µL Gilson pipetman® neo P200N 20-200 µL Gilson pipetman® neo P100N 10-100 µL Gilson pipetman® neo P20N 2-20 µL
Multichannel pipette	Thermo Scientific Finnpipette™ F2
Coulter Counter	Beckman Coulter™ Z2 Coulter™ Particle Count and Size Analyzer
Bottletop dispenser	BRAND Dispensette® III
Microplate Reader	Tecan Infinite® M1000 Pro
Flow Cytometer	Beckman Coulter™ Gallios™
Microscope	Leica DM IL LED
Hemocytometer	Labor Optik Neubauer
Incubator	Thermo Scientific Heracell™ 150i CO_2 Incubator
Laminar flow hood	Thermo Scientific MSC-Advantage™
Pipet boy	Pipethelp Accumax Matrix CellMate II®
Vortex	Vortex-Genie 2
Waterbath	GFL 1003
Shaker incubator	Kuhner Shaker Climo-Chaker ISF1-XC
PCR thermocycler	Bio Rad C1000™ Thermal Cycler

Thermoblock	Eppendorf Thermomixer comfort
Gel electrophoresis chamber	BioRad
Gel electrophoresis power supply	BioRad PowerPac™ Basic Power Supply
Agarose gel analyzer	BioRad Molecular Imager®
	Gel Doc™ XR+
Balance	Sartorius AW-4202
Analytical balance	Sartorius ME Micro Balance ME36S
Plate Washer	Tecan 96 Plate Washer™
Diafiltration	Millipore Labscale TFF System
Diafiltration Membrane	Merck Pellicon® XL
	Cat. No. PXB030A50
Shaker plate	Heidolph Rotamax 120
	VWR® symphony™ Incubating
	Microplate Shaker
Äkta	Amersham Biosciences ÄKTApurifier
Octét platform	fortéBIO Octét® QK System
SORT	Beckmann Coulter MOFLO
	ASTRIOS EQ
Nitrogen tank	CryoTherm Biosafe® MD
NanoDrop	PEQLAB NanoDrop™ 1000
Magnetic stirrer	VWR™ VS-C4
	VWR™ VS-C10
Chromatography column	GE Healthcare, HiTrap™ MabSelect
	SuRe™ Cat. No. 29-0491-04

4.1.2 Reagents

Coulter Cleaning Solution	Beckman Coulter™ Coulter Clenz®
	Cleaning Agent Cat. No. 8448222
Synth-a-freeze®	Gibco® by Life Technologies
	Cat. No. A12542-01
L-Alanyl-L-Glutamine	Merck Millipore Cat. No. K0302
	[200 mM]
Phenol red solution	Sigma® Life Science Cat. No. P0290
	0.5% in DPBS
G 418 – BC	Biochrom AG Cat. No. A 2912
Penicillin / Streptomycin (100x)	PAA The cell culture company
	Cat. No. P11-010

DAPI-stock solution	4',6'-Diamidino-2-Phenylindol in H_2O (L0009) [5 µg/mL]
TWEEN® 20	Roth® Polyoxyethylene-20-sorbitan monolaurate Cat. No. 9127.2
H_2SO_4	Roth® Sulphuric acide 25% Cat. No. 0967.1
BSA	Roth® Albumin, IgG free Cat. No. 3737.4
TMB	Invitrogen Stabilized Chromogen, TMB Cat. No. SB02
FBS	Biochrom FBS Superior Cat. No. S0615
Protein Ladder	Thermo Scientific PageRuler™ Pre-stained Protein Ladder, 10-180 kDa, Cat. No. 26616
DNA Ladder	Thermo Scientific GeneRuler™ DNA Ladder Mix, Cat. No. SM0331
Trypan blue	Sigma-Aldrich® Trypan Blue solution 0.4% Cat. No. T8154-100 ML
Ganciclovir	Sigma-Aldrich® Ganciclovir Cat. No. G2536-100MG
Formaldehyd 37%	Sigma-Aldrich® Cat. No. 252549
Glutaraldehyd	Sigma-Aldrich® Cat. No. G5882
Ethidium bromide	Sigma-Aldrich® Ethidium bromide E1510
PEI	Polysciences, Inc. Cat. No. 23966
dNTPs (10mM stocks)	KAPA Biosystems 10 mM KAPA dNTP Mix Cat. No. KN1009
PenStrep	PAA, Penicillin/Streptomycin (100x) Cat. No. P11-010
Transferrin	Merck Cell prime Cat. No. 9701-10 20 mg/mL
rEGF	Repligen Cat. No. 10-1021-1E 0.1 mg/mL
Albumin	Sigma Aldrich Cat. No. A7223 50 mg/mL
Nystatin	Sigma Aldrich Cat No. N1638 10000 U/mL
Triton X-100	Merck Cat. No. 1.08643.1000
EDTA	Merck Cat. No. 324503
Glycerin	Merck Cat. No. 104092

4.1.3 Disposables

Pipette tips	Micronic Cat. No. MP228C2 250 µL
	Greiner bio-one Ultratip Cat. No. 739290
	Greiner bio-one Ultratip Cat. No. 740290
	Biozym Scientific Tips 250 µL
	Cat. No. 720310
Filter	Merck Millipore Millex® Syringe-driven
	Filter Unit
Serological pipette 2 mL	Costar® Stripette® 4486
Serological pipette 5 mL	Costar® Stripette® 4487
Serological pipette 10 mL	Costar® Stripette® 4488
Serological pipette 25 mL	Costar® Stripette® 4489
Serological pipette 50 mL	Costar® Stripette® 4490
Shaker flasks 125 mL	Corning® 125 mL Erlenmeyer flask
Shaker flasks 250 mL	Corning® 250 mL Erlenmeyer flask
T25 flask	VWR® 734-2312 Tissue Culture flask
T80 flask	VWR® 734-2314 Tissue Culture flask
Plates 96 well	VWR® 734-2327 Tissue Culture Plates
Plates 384 well	Corning® Assay Plate 3701
Plates 24 well	VWR® 734-2325 Tissue Culture Plates
Epis	VWR® Micro-centrifuge tubes
	Cat. No. 211-0015
PCR tubes	BioRad PCR tube stips
	Cat. No. TBC0802
10 mL tubes	Nunc™ 10/11mL Polystyrene Centrifuge
	Tubes Cat. No. 347856
FACS tubes	FALCON® A Corning Brand
	5 mL Polystyrene Round-Bottom tube
50 mL tubes	Greiner bio-one Cellstar® tubes
	Cat. No. 227 261
30 mL tubes with white lid	Nunc™ Universal and Transport
	Containers with Caps Cat. No. 364238
Cryovials	Thermo Scientific Nunc™ CryoTube
	Vials 375418
50 mL tube reactor	TPP TubeSpin® Bioreactor
Gloves	VWR Latex Disposable gloves
Syringes	Braun Omnifix® Luer Lock Solo

ELISA plate	Thermo Scientice F96 Maxisorp Nunc-immuno plate
Dilution plate	Thermo Scientific 96F without lid sh microwell plate
Octet plate	Nunc™ F96 MicroWell™ Black Polystyrene Plate
Protein A tips	FortéBIO® Dip and Read™ Biosensors Protein A
Filtercup	Merck Stericup-GP, 0.22 µm Cat. No. SCGPU05RE
Centrifugal filter	Merck Amicon Ultra 0.5 mL for 10000 kDa
Desalting column	GE PD MidiTrap G-25
FACS tube	Falcon® 5mL Round Bottom Polystyrene Cat. No. 352054
SORT tube	Falcon® 5mL Round Bottom Polystyrene Test Tube Cat. No. 352235
Dialysis tube	Fisherbrand™ Regenerated Cellulose Dialysis Tubing Cat. No. 21-152-10

4.1.4 Kits

gDNA isolation	Qiagen QIAamp® DNA Mini Kit
GoTaq Polymerase Kit	Promega GoTaq® DNA Polymerase M300

4.1.5 Software

Plate reader	Magellan 6
Octét	fortébio data acquisition and analysis software
Coulter Counter	Z2 AccuComp
Data analysis of flow cytometer	Kaluza 1.2
Nanodrop	Nanodrop 1000

4.1.6 Chemicals

Sodium chloride	Roth® NaCl M = 58.44 g/mol Cat. No. P029.3

Sodium hydrogen carbonate	Merck $NaHCO_3$ M = 84.01 g/mol Cat. No. 6329.1000
Citric Acid	Sigma M = 192.12 g/mol Cat No. C0759
Triton X-100	Merck Triton® X-100 Cat. No. 108643
Di-Sodium hydrogen phosphate dehydrate	Roth® $Na_2HPO_4 \cdot 2\ H_2O$ M = 177.99 g/mol Cat. No. 49843
Potassium dihydrogen phosphate	Merck KH_2PO_4 M = 136.08 Cat. No. 104873
Potassium chloride	Roth® KCl M = 74.56 g/mol Cat. No. HN02.3
Sodium carbonate	Roth® Na_2CO_3 M = 105.99 g/mol Cat. No. A135.2
TRIS	Merck $H_2NC(CH_2OH)_3$ M = 121.13 g/mol Tris(hydroxymethyl)-aminomethan Cat. No. 1083822500
Ethanol	Merck Emplura® M=46.02 g/mol Cat. No. 8.18760.2500
Acetic Acid	Sigma 99% Cat. No. A6283
Sulphuric acid	Roth® M = 98.08 g/mol Cat. No. 0967.1
Magnesium chloride hexahydrate	Merck M = 203.3 g/mol Cat. No. 1.05833.1000
Sodium thiosulfate pentahydrate	Merck M = 248.21 g/mol Cat. No. 1.06516.0500
Agarose	Fermentas TopVision™ Agarose Cat. No. R0499
Sodium acetate $\cdot$ 3 H_2O	Merck M = 82.03 g/mol Cat. No. 1.06268.1000
Gycine	Merck M = 75.06 g/mol Cat. No. 104201
HEPES	Merck M = 238,3 g/mol Cat. No. 1603
Silver nitrate	Merck M = 169.87 g/mol Cat. No. 101510

4.1.7 Buffer

Coulter Incubation solution	0.1 M Citric Acid 2% w/w Triton X-100 Filtered 0.2 µm
Coulter dilution buffer	9 g/L NaCl in RO-H_2O

	Filtered 0.2 µm
10x PBS (5 Liter)	57.5 g $Na_2HPO_4 \cdot 2\ H_2O$
	10 g KH_2PO_4
	10 g KCl
	400 g NaCl
	Fill up to 5000 mL with RO-H_2O
ELISA: Coating buffer (500 mL)	4.2 g $NaHCO_3$
	2.1 g Na_2CO_3
	Fill up to 500 mL with RO-H_2O
	pH = 9.5 – 9.8
ELISA: Washing buffer (1 L)	100 g PBS 10x
	Fill up to 1000 g with RO-H_2O
	1 mL TWEEN 20
ELISA Dilution buffer (100 mL)	0.1 g BSA
	100 mL washing buffer
T PBS for Octét	PBS 1x
	0.1% TWEEN 20
FACS-buffer	100 mM TRIS
	0.1% TRITON
	2 mM $MgCl_2$
LDS-buffer	Thermo Scientific NuPAGE® LDS
	Sample Buffer (4x) Cat. No. NP0008
PCR buffer	Promega 5x Green GoTaq® Reaction
	Buffer Cat. No. M791A
Silver Stain: Fixation Solution	50% Ethanol / 10% Acetic Acid in H_2O
Silver Stain: Incubation Solution	150 mL Ethanol
	1.75 g $Na_2S_2O_3 \cdot 5\ H_2O$
	(Sodiumthiosulfate pentahydrate)
	56.4 g Na-acetate $\cdot$ 3 H_2O
	Filled up to 500 mL with H_2O
	(Add freshly 62.5 µl Glutaraldehyd /
	25 mL)
Silver Stain: Silver Solution	0.25 g $AgNO_3$ in 500 mL H_2O
	(Add freshly 5 µL Formaldehyd / 25 mL)
Silver Stain: Develop Solution	12.5 g Na_2CO_3 in 500 mL H_2O
	(Add freshly 5 µl Formaldehyd / 25 mL)
Silver Stain: Stop Solution	0.05 M EDTA in H_2O

ÄKTA: Buffer A	100 mM Glycine 100 mM NaCl pH 7.5
ÄKTA: Buffer B	100 mM Glycine pH 2.5
PBS Dulbecco sterile	Merck PBS solution without Ca^{2+} and Mg^{2+}
DSC: phosphate buffer	150 mM NaCl 0.02 M Na_2HPO_4 0.04 M NaH_2PO_4 pH = 6
TAE 50x	0.5M Tris Acetic acid 50mM EDTA
BX loading buffer	0.25%-w/v Bromphenolblue 0.25%-w/v Xylencyanol 30%-w/v Glycerin
HEPES Buffered Saline	150 mM NaCl 10 mM HEPES
HBS	4.5 g NaCl 1.19 g HEPES Filled up to 300 mL with H_2O

4.1.8 Gels

SDS-gel	Novex® 12% Tris-Glycine Gels
Agarose-gel	1% (w/v) agarose 1 x TAE (50 x) 200 ng/mL ethidium bromide HQ-water

4.1.9 Enzymes

Taq Polymerase	Promega GoTaq® DNA Polymerase M300

4.1.10 Antibodies

ELISA: Coating-antibody	Anti human IgG Gamma- chain 13382

	[1 mg/mL]
ELISA: IgG-Standard	3D6_IgG pur [200 ng/mL]
ELISA: HRP-conjugate	Invitrogen™ HRP-Goat Anti-Human IgG (Gamma)
FACS: Biotin-labeling	NOVEX GOXHU Fc BIO 1.5 mg Antibodies-online Anti-Kappa antibody (Biotin) Novus Biologicals pAb anti-Lambda light chain Antibody 1 mg
FACS: Alexa-conjugate	life technologies Streptavidin, Alexa Fluor® 647 1 mg

4.1.11 Plasmids

pRMCE_2G12_IgG_v2 #4 Midi	PM141216
pRMCE_HV3-21_HD5-12_HJ3/KV1-5_KJ1_IgG #4 Midi	PM141220
pRMCE_Ustekinumab_IgG #4 Midi	PM141216
pRMCE_HV5-51_HD5-5_HJ4/KV1D-16_KJ2_IgG #1 Midi	PM141220
pRMCE_4B3_IgG #1 Midi	PM141216
pRMCE136/63_v2 #1 Midi	AE150527
pPGKFLPobpA	PM120306

4.1.12 Medium

CD CHO Medium (1X)	Gibco® by life technologies™ Cat. No. 10743-029
ProCHO5	Lonza BioWhittaker® ProCHO5 Cat. No. BE12-766Q
MV3-2/6	In-house formulation (Master thesis of Mundsperger, P.)
MV3-2/6 (+ 30%)	In-house formulation (Master thesis of Mundsperger, P.)

Supplemented medium

Culture Medium	CD-CHO + 4 mM L-Gln + 15 mg/L phenol red + 0.5 mg/mL G418
Transfection Medium 1	Pro-CHO5 + 4 mM L-Gln + 15 mg/L phenol red

Transfection Medium 2	CD-CHO + 4 mM L-Gln + 15 mg/L phenol red
Selection Medium 1	CD-CHO + 4 mM L-Gln + 15 mg/L phenol red + 2 - 20 µM GCV
Selection Medium 2	CD-CHO + 4 mM L-Glutamine + 15 mg/L phenol red + 2 µM GCV + PenStrep (1:100)
Selection Medium 3	Pro-CHO5 + 4 mM L-Gln + 15 mg/L phenol red + 2 µM GCV
Production Medium 1	MV3-2/6 + 6 mM L-Gln + 15 mg/L phenol red
Production Medium 2	MV3-2/6 (+ 30%) + 6 mM L-Gln + 15 mg/L phenol red
Batch Medium 1	CD-CHO + 8 mM L-Gln + 15 mg/L phenol red
Batch Medium 2	MV3-2/6 + 8 mM L-Gln + 15 mg/L phenol red
Batch Medium 3	MV3-2/6 (+ 30%) + 8 mM L-Gln + 15 mg/L phenol red

4.2 Methods

4.2.1 Host cell line

As host cell line CHO K1: S1/0.3/I3 was chosen for all transfections. This suspension cell line was developed in-house (Mayrhofer, P.). It is abbreviated in the following as CHO RMCE I3.
The cells were cultured in a 125 mL shaker flask, pre-gased to 7% CO_2 at 37°C and 140 rpm at 25 mm orbital shaking amplitude or 220 rpm at 50 mm shaking amplitude. The shaker flask was filled with 24 mL cell suspension and the passaging was performed every 3 to 4 days. The seeding cell concentration was $2{\cdot}10^5$ cells/mL seeded in pre-warmed culture medium.

4.2.2 Cell culture methods

Shaker passaging

First of all the determination of cell concentration and viability was done. A defined aliquot for $2{\cdot}10^5$ cells/mL was transferred to a 10 mL tube. The remaining cell suspension in the shaker flask was discarded. Pre-warmed medium was

pipetted in the shaker flask and the determined aliquot was added. The flasks were cultivated in the shaker incubator at 37°C, 7% CO_2 and 140 rpm for 3 to 4 days.
The calculation for the sub-culturing steps was done with equation 1 and 2 [23].

$$V_{cells} = \frac{V_{intended} \cdot c_{intended}}{c_{sample}} \quad (1)$$

$$V_{medium} = V_{intended} - V_{cells} \quad (2)$$

V_{cells} …volume of cell suspension [mL]
$V_{intended}$ …intended volume after dilution [mL]
$c_{intended}$ …intended cell density [cells/mL]
c_{sample} …cell density of parental cell suspension [cells/mL]
V_{medium} …added medium for dilution [mL]

Passaging of T-flasks
The cell concentration of the cell suspension was determined. The pre-warmed medium was pipetted in a new T-flask. A defined aliquot for $2 \cdot 10^5$ cells/mL was transferred to the new T-flask. The incubation was performed static in the incubator at 37°C, 7% CO_2 for 3 to 4 days.

Passaging of plates
The passaging ratio was determined microscopically. The volume of pre-warmed medium was pipetted in the new plate. The cell suspension was carefully resuspended and the aliquot was transferred to the new plate which was already filled with medium.

4.2.3 Determination of selection pressure

The appropriate selection pressure for the transfections was determined with a comparison of CHO PG9 and CHO RMCE I3 at different ganciclovir (GCV) concentrations. The effect of GCV on the host cell line and the co-transfected cells should be elicited. Therefore the cell suspensions were cultured in selection medium 1 with 40, 20, 10 or 2 µM GCV. Two 96 well plates with 20000 c/Well/100 µL in lane H were diluted to lane A in 1:2 steps. Three wells in parallel were incubated with the same GCV concentration. The GCV concentrations 10 µM and 20 µM were checked after 15 days and the GCV concentrations 2 µM and 40 µM were analyzed after 11 days microscopically.

4.2.4 PEI-Transfection of CHO RMCE I3 cells

The cell suspension was passaged one day before transfection in the ratio 1:2 to ensure that the cell suspension is in the exponential growth phase. The determination of cell concentration and viability was done. The viability of the cell suspension should be greater than 95% to achieve a high transfection efficacy. An aliquot of $1 \cdot 10^6$ or $10 \cdot 10^6$ cells was centrifuged at 1000 rpm for 10 min and the pellet was resuspended in 4 mL transfection medium 1. The suspension was transferred to T25 or T80 flask. First of all 80 µL of PEI were added to 220 µL HBS and it was incubated for 10 min at RT. Then 6 µg of the pRMCE plasmid and 2 µg of the pPGKFLPopA plasmid were added to HBS to a total volume of 200 µL (Table 1). The DNA/HBS solution was incubated for 10 min at RT. The 300 µL PEI/HBS reaction was added to the 200 µL DNA/HBS solution and it was incubated for 10 min at RT. The DNA-PEI ratio is 1:10. The 500 µL transfection suspension was transferred to the 4 mL cell suspension in the T25 or T80 flask. After 4 h incubation time at 37°C a medium change was done from transfection medium 1 to transfection medium 2. Furthermore the volume of the cell suspension was enlarged to 10 mL. The T25 or T80 flask was incubated for 48 – 72 h at 37°C.

Table 1: Plasmids and designations for all co-transfected cell lines

Host cell line	Plasmid	Designation	Germline of
CHO-K1_S1_0.3_I3	pRMCE_2G12_IgG_v2 #4 Midi	RMCE2G12IgG	n.a.
	pRMCE_HV**3**-21_HD**5**-12_HJ**3**/KV**1**-5_KJ**1**_IgG #4 Midi	RMCE353/11/IgG	2G12
	pRMCE_Ustekinumab_IgG #4 Midi	RMCEUsteIgG	n.a.
	pRMCE_HV**5**-51_HD**5**-5_HJ**4**/KV**1**D-16_KJ**2**_IgG #1 Midi	RMCE554/12/IgG	Ustekinumab
	pRMCE_4B3_IgG #1 Midi	RMCE4B3IgG	n.a.
	pRMCE136/63_v2 #1 Midi	RMCE136/63/IgG	4B3

Transfection 1

The clones RMCE2G12IgG, RMCE353/11/IgG, RMCE554/12/IgG and RMCE4B3IgG were generated by transfection 1. A host cell aliquot of $1 \cdot 10^6$ cells was co-transfected in a T25 flask. The selection pressure was set after 48 h of incubation.

Transfection 2
The clone RMCEUsteIgG was generated by transfection 2. A host cell aliquot of $10 \cdot 10^6$ cells was co-transfected in a T80 flask and therefore the tenfold amounts of transfection medium 1, PEI, HBS and both plasmids were needed.

Transfection 3
The clone RMCE136/63/IgG was generated by transfection 3. A host cell aliquot of $1 \cdot 10^6$ cells was co-transfected in a T25 flask. The cell suspension was incubated 72 h after medium change.

4.2.5 Selection

Transfection 1
The limiting dilution subcloning was performed after 48 h in selection medium 1. The cell suspension was centrifuged and the pellet was resuspended in 20 mL selection medium 1, which contained 20 µM GCV for the selection of the successfully co-transfected cells. A 96 and a 384 well plate were used for subcloning of the co-transfected cells. The 96 well plate was seeded with 2000 cells/well/100 µL and the 384 well plate was plated out with 2000 cells/well/50 µL. The plates were incubated for 10 days statically at 37°C. After this time period the GCV concentration was stepwise reduced to 10 µM GCV. Selection medium 1 was used for the further passaging steps. The 384 well plate was passaged twice and all wells were transferred to 96 well plates. The GCV concentration was reduced from 10 to 2 µM and the plates were passaged three times. The viable clones of all plates were transferred to one 96 well plate. The 96 well plate was evaluated with qualitative ELISA and the best 12 clones, or all producing clones were transferred to an expansion plate. The expansion plate was screened with a quantitative ELISA. Due to a suspicion of yeast contamination the clones were pooled into a T25 flask and Nystatin was added for 3 passages.

Transfection 2
The cell suspension with the volume of 45 mL was centrifuged after an incubation time of 72 h. A slow media exchange from transfection medium 1 to selection medium 1 was done. The cell pellet was resuspended in 25 mL supernatant and 25 mL selection medium 1 containing 4 µM GCV to a total GCV concentration of 2 µM. Seven days later the T80 flask was split 1:2. The pool of co-transfected cells was sorted after 10 days (4.2.6 Cell sorting).

Transfection 3
The selection process was started after 72 h in selection medium 1 containing 20 µM GCV. Detailed description can be found in chapter 4.2.7 subcloning.

4.2.6 Cell sorting

As GFP positive control the cell line CHO RMCE I3 was used. The cell line CHO PG9 served as GFP negative control. $2 \cdot 10^6$ cells of both controls were centrifuged. The pellets were resuspended with 400 µL selection medium 2. A 24 well plate was prepared with 1 mL selection medium 2 per well. The T80 flask filled with 50 mL cell pool of transfection 2 was transferred to a 50 mL tube and it was centrifuged at 1000 rpm for 10 min. The sample pellet was resuspended with 2 mL selection medium 2. The prepared sample and both controls were filled in sort tubes. The suspensions were pressed directly through the sieve which is integrated in the blue lid of the sort tubes. The sort process was directed by GFP-negative cells. All GFP positive cells were discarded. The GFP negative cells were sorted into three wells of the 24 well plate. The seeding density for the co-transfected cells was 20000 cells/well/1 mL.

4.2.7 Subcloning

Subcloning of transfection 1
The cell pools in the 125 mL shaker flasks of RMCE2G12IgG, RMCE353/11/IgG, RMCE554/12/IgG and RMCE4B3IgG were subcloned in 384 well plates. Selection medium 1 containing 2 µM GCV was used supplemented with the growth factors: albumin (used: 1 mg/mL), transferrin (0.02 mg/mL) and rEGF (25 ng/mL). The fresh medium was mixed to same parts with filtered supernatant. The aliquot of the cell suspension was calculated for a seeding density of 1 cell/well/50 µL, centrifuged down and resuspended in conditioned medium. One half of each 384 well plate was fed after 13 days with 35 µL selection medium 3. All viable clones of one 384 well plate were picked and transferred to one 96 well plate per cell line. All four 96 well plates were screened with a qualitative ELISA. Six clones showing the highest titer were chosen of every cell line. The 6 clones were expanded in 24 well plates.
The subcloning of RMCE554/12/IgG and RMCE4B3IgG was repeated with the same medium conditions. As cell densities 4, 8 and 12 cells/well/50 µL were chosen in 384 well plates. Each cell concentration was plated out in 96 wells of the 384 well plate. Three clones of the first subcloning and three clones of the second subcloning were taken for the expansion into the 24 well plate.

Subcloning of transfection 2

14 days after the sorting process the sorted cells were fed with 1 mL selection medium 2. The 24 well plate was passaged twice with selection medium 1 (2 µM GCV) and the three wells were pooled in a T25 flask. A 125 mL shaker flask was prepared out of the T25 flask. The cells in the shaker flask were used for batch experiment 3. The T25 flask was kept over 10 passages in a ratio of 1:5 or 1:7 with selection medium 1 (2 µM GCV). A 384 well plate with the cell densities 1; 10 and 100 cells/well/50 µL was plated out with one half selection medium 1 and one half filtered supernatant (Figure 3). The subcloning of transfection 2 was performed without growth factors. After 7 days the 96 wells with 100 cells/well were fed with 35 µL selection medium 1. After 10, 14, 17 and 20 days all viable clones were transferred to one 96 well plate. The titers of the clones on the 96 well plate were determined with qualitative ELISA. The best 12 clones were transferred to an expansion plate. Each clone was passaged into 8 wells and each well had a volume of 300 µL. The 12 clones were tested with quantitative ELISA and the best 3 clones were expanded into T25 flasks.

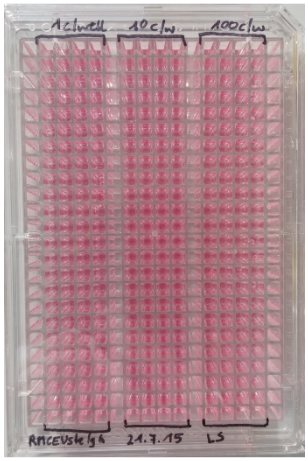


Figure 3: Subcloning of transfection 2 in 384 well plate, 96 wells with 1, 10, 100 c/well

Subcloning of transfection 3

The subcloning was done after 72 h in selection medium 1 containing 20 µM GCV. For the subcloning a 96 and a 384 well plate were taken. The 96 well plate was seeded with 2000 cells/well/100 µL and the 384 well plate was plated out with 2000 cells/well/50 µL. After 10 days the selection pressure was reduced from 20 µM to 10 µM GCV. The plates were passaged twice with selection medium 1 containing 10 µM GCV. All wells of the 384 well plate were transferred to 96 well plates. The concentration of GCV was reduced from 10 µM to

2 µM. The 96 well plates were passaged and all viable clones were collected in one 96 well plate over 3 passages. The quantification of the 96 well plate was done via qualitative ELISA. The highest producers were expanded into 8 wells in a 96 well plate. The expansion plate was screened with a quantitative ELISA. The best producing clones were passaged into T25 flasks.

4.2.8 Generation of product

The production vessels were 250 mL shaker flasks with 100 mL production medium 1 (for RMCE2G12IgG, RMCE353/11/IgG) or production medium 2 (for RMCE554/12/IgG, RMCE4B3IgG). The seeding density was 9-10·10^6 cells/mL. The shaker flask was incubated at 37°C, 7% CO_2, 140 rpm and 85% humidity. The production shakers were harvested after 3 to 4 days. The cell concentration and viability were determined and a sample of the supernatant was frozen. The calculated amount of cell suspension was centrifuged down, the supernatant was collected and the pellet was resuspended in fresh medium. The production shakers were kept for 3 passages. The supernatant was collected and stored at 4°C.

4.2.9 Batch experiments

Batch experiment 1

The first batch experiment was performed with the cell pools of the cell lines RMCE2G12IgG and RMCE353/11/IgG from transfection 1, before subcloning 1. The experiment was done in 125 mL shaker flasks with a volume of 20 mL/flask. The batch experiment was started with a seeding density of 1·10^6 cells/mL. Batch medium 1 and batch medium 2 were used for each cell line. The four shaker flasks were cultivated at 37°C, 7% CO_2, 140 rpm and 85% humidity. For the sampling 1 mL cell suspension were taken from the shaker flasks. The viability, cell concentration and product titer were measured.

Batch experiment 2

The second batch experiment was done with six cell lines from subcloning 1: RMCE2G12IgG/384/1B3, RMCE2G12IgG/384/1B11, RMCE2G12IgG/384/1E4, RMCE353/11/IgG/384/2A12, RMCE353/11/IgG/384/2C7 and RMCE353/11/IgG/384/2D7. The experiment was executed in 50 mL reactor tubes with a working volume of 10 mL with batch medium 3. The cultivation of the six reactor tubes was done at 37°C, 7% CO_2, 220 rpm and 90% humidity. The process was started with a seeding density of 2·10^6 cells/mL and each tube was sampled with 1 mL cell suspension. The

measurements of viability, cell concentration and product titer were performed for five days.

Batch experiment 3

For batch experiment 3 the cell line RMCEUsteIgG from transfection 2 was used. A comparison of the cultivation in 125 mL shaker flask and 50 mL reaction tube was taken. The batch process was started with the cell density of $1.5 \cdot 10^6$ cells/mL in both reaction vessels in batch medium 3. The working volume in the shaker flask was 20 mL and 10 mL in the reactor tube. The cells in the shaker flask were cultivated at 37°C, 7% CO_2, 140 rpm and 85% humidity. The reactor tubes were incubated at 37°C, 7% CO_2, 220 rpm and 90% humidity. The viability, cell concentration and product titer were measured daily.

4.2.10 Quantification of antibody titer

Qualitative IgG gamma-gamma ELISA

The ELISA plate was coated 1:2000 with the coating antibody. Each well was filled with 100 µL and the plate was incubated for 2 h at RT on the shaker plate or on 4°C overnight. A dilution plate was prepared with a minimum dilution of 1:4 in dilution buffer. The IgG-standard antibody was diluted to a concentration of 40 ng/mL. A dilution series was done from the H-well with 40 ng/mL in 1:2 steps. The pre-coated ELISA plate was washed with the plate washer, which was connected to the washing buffer. From the dilution plate 50 µL/well were transferred to the ELISA plate (Figure 4). The ELISA plate was incubated for one hour on the shaker plate. After incubation it was washed with the plate washer and knocked out. A volume of 50 µL of the conjugation antibody was applied to the ELISA plate in the dilution of 1:2000. The incubation on the shaker plate was done for one hour, the ELISA plate was washed and knocked out. The ELISA plate was stained with 100 µL TMB/well and the reaction was stopped with H_2SO_4. The antibody concentration was measured in the plate reader at the measurement wavelength of 450 nm and a reference wavelength of 620 nm. The concentrations were analyzed with MAGELLAN software.

	1	2	3	4	5	6	7	8	9	11	12
A		1:128	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
B		1:64	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
C		1:32	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
D		1:16	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
E		1:8	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
F		1:4	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
G		1:2	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
H			1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
	Blank	Std.	Samples								

Figure 4: Scheme for qualitative ELISA

Quantitative IgG gamma-gamma ELISA

The coating of the ELISA plate was performed like at qualitative ELISA. The samples were pre-diluted to the range of the IgG-standard. A dilution series was executed for the standard and all samples in 1:2 steps (Figure 5). The conjugation and staining reaction were done as for qualitative ELISA.

	1	2	3	4	5	6	7	8	9	11	12
A		1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128
B		1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
C		1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
D		1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
E		1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
F		1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
G		1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2
H											
	Blank	Std.	Std.	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample

Figure 5: Scheme for quantitative ELISA

Bio-layer interferometry

The protein A tips were activated in PBST for at least 10 min. The dilution of the samples was performed to a concentration of <100 µg/mL for IgG. Each sample was measured in two dilution steps. 200 µL sample were transferred to the octet plate air bubble-free. The lane 11 was filled with glycine buffer and lane 12 was filled with PBST for regeneration. The measurement was done for 300 s with 1000 rpm in the octét platform and it was analyzed with fortébio data acquisition and analysis software.

A280 & A260 quantification

The measurement was performed with NanoDrop and it was analyzed with the software "Nanodrop 1000". The measuring point was cleaned with 3 µL HQ-water. The blank was set by pipetting a drop of buffer to the measuring point and the A280 measurement was performed with the setting "1 Abs = 1 mg/mL". A standard curve is not required. It is from high importance that the sample is applied air bubble-free. With the settings "Other Protein E&MW" additional sample information, the extinction and the molecular weight, were added. 3 µL of the sample were measured. The A280 results were potentially influenced by the uncharacterized proteins or non-protein components. The A260 measurement was done with the setting "Nucleic Acid". [24]
The concentrations are calculated with the Beer-Lambert equation (Equation 3).

$$A = E \cdot b \cdot c \tag{3}$$

A …absorbance value
E …wavelength-dependent molar absorptivity coefficient [L/mol/cm]
b …path length [cm]
c …analyte concentration [mol/L]

4.2.11 Characterization of cell lines

Determination of viability

The cell suspension was carefully mixed and a sample was taken with a sterile pipette. The hemocytometer and the coverslip were cleaned with 70% EtOH. The coverslip was fixed on the hemocytometer with 70% EtOH on the side-bridges. The samples were prepared with a dilution factor of 1.2 with trypan blue. At a very high cell density the sample was diluted with PBS. The chamber was filled with the stained sample. The chamber has a volume of 0.1 µL. The

leaky cell membrane of dead cells enables the entry of trypan blue and therefore only the dead cells are stained blue. The viable and dead cells were counted with a clicker counter. The viability was calculated according to equations 4 - 7.

$$\frac{viable\ cells}{mL} = \frac{average\ of\ viable\ cells}{squares} \cdot 12000 \tag{4}$$

$$\frac{dead\ cells}{mL} = \frac{average\ of\ dead\ cells}{squares} \cdot 12000 \tag{5}$$

$$\frac{total\ cells}{mL} = \frac{viable\ cells}{mL} + \frac{dead\ cells}{mL} \tag{6}$$

$$viability = \frac{viable\ cells/mL}{total\ cells/mL} \cdot 100 \tag{7}$$

Determination of cell concentration

The cell suspension was mixed carefully and 2 mL sample aliquots were taken. The samples were centrifuged at 1000 rpm for 10 min. The supernatant was discarded or collected for determination of antibody titer and the pellet was resuspended with Coulter incubation solution. The samples were vortexed and incubated for at least 1 hour at room temperature. The blank was set with coulter dilution buffer. The samples were diluted to the range between 10,000 and 20,000 counts with the coulter dilution buffer in the bottletop dispenser. Each sample was measured twice and the capillary was cleaned with fresh coulter dilution buffer afterwards. After all samples the determination of the blank was repeated. The calculation was done according to equation 8.

$$\frac{cells}{mL} = \frac{\left(2 \cdot \sum \frac{x_i}{n}\right) \cdot (V_M + V_I) \cdot V_{T/C}}{V_M \cdot V_P} \tag{8}$$

x_i …i^{th} count result [particles/500 µL]
n …number of measurements
V_P …centrifuged sample volume [mL]
$V_{T/C}$ …used aliquot of coulter incubation solution [mL]
V_M …used aliquot of the incubated cell suspension [mL]
V_I …added aliquot of coulter dilution buffer [mL]

Calculation of relevant parameters

The specific growth rate (μ) was calculated according to equation 9.

$$\mu = \frac{\ln\left(\frac{X_2}{X_1}\right)}{t_2 - t_1} \tag{9}$$

X_1 …start cell concentration [cells/mL]
X_2 …end cell concentration [cells/mL]
t_1 …start time [d]
t_2 …end time [d]

The specific productivity (q_p) shows the generated product per cell per day [pg/cell/day]. The calculation is shown in equation 10.

$$q_p = \frac{(titer\ 2 - titer\ 1) \cdot \mu \cdot 10^6}{X_2 - X_1} \tag{10}$$

Flow cytometry

All centrifugation step were performed at 1000 rpm for 10 min.

Analyses of viable cells

A defined volume of the cell suspension for $1 \cdot 10^6$ cells was centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 1 mL PBS. The centrifugation step was repeated and the supernatant was discarded. The pellet was resuspended in 200 µL PBS + DAPI (1:100). The samples were analyzed in the flow cytometer with the protocol "GFP_DAPI_viable_CHO_PBS_all_param.PRO".

Analyses of fixed cells

A defined volume of the cell suspension for $2 \cdot 10^6$ cells was centrifuged from the samples and the positive and negative controls. The supernatant was discarded and 1 mL of 70% ice cold EtOH was added dropwise to the cell pellet under continuous vortexing. The samples were stored at 4°C for at least 20 min. The samples were split to enable a separate analysis of heavy and the light chain. It was centrifuged, the supernatant was discarded and the pellet was resuspended in 1 mL FACS-buffer. The centrifugation step was repeated and the pellet was resuspended in 100 µL FACS-buffer with 20% FCS for saturation to reduce unspecific binding. The samples were incubated for 30 min at 37°C. 100 µL of the biotin antibody 1:50 in FACS-buffer and 20% FACS was added. The next incubation step was done for 30 min at 37°C. The samples were centrifuged,

resuspended in 1 mL FACS-buffer and centrifuged again. The pellet was resuspended in 200 µL of the streptavidin-Alexa 647conjugate 1:100 in FACS-buffer and 20% FCS. The samples were incubated for 30 min at 37°C. They were centrifuged and the pellet was washed in 1 mL FACS-buffer. Finally the pellet was resuspended in 200 µL FACS-buffer + DAPI (1:100). All FACS analyses were measured on the flow cytometer. For the measurement the protocol "FL1_FL6_FL9_fixed_CHO_all param.PRO" was executed.

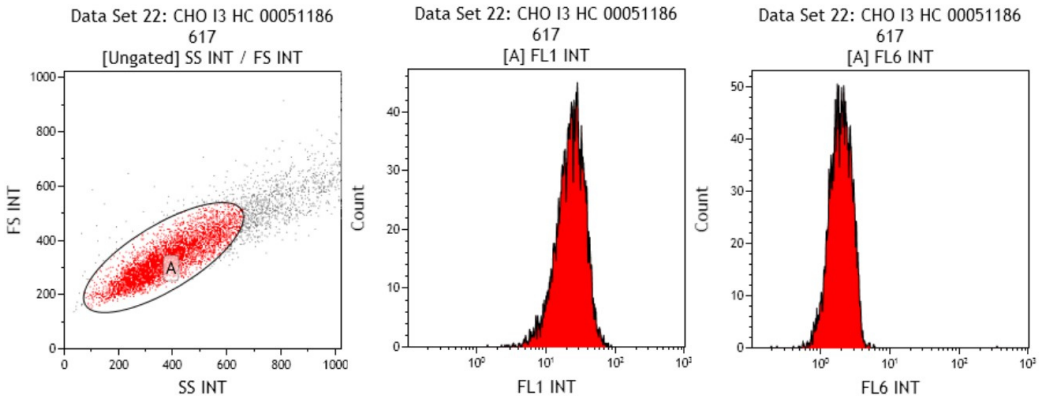


Figure 6: Setting a gate (A) in Forward Scatter (FS) and Side Scatter (SS) diagram of flow cytometer analyses of CHO RMCE I3 cells. In FL-1 and FL-6 laser channel only gated cells are evaluated.

Viable cells were gated according to their forward and side scatter properties and analyzed for GFP fluorescence by FL-1 laser channel or for Alexa Fluor 647 fluorescence, correlating to intracellular heavy or light chain accumulation, by the FL-6 laser channel using Kaluza 1.2 Analysis software (Figure 6). Only viable cells within gate A are considered for calculation.

Isolation of gDNA

An aliquot of $2 \cdot 10^6$ cells was centrifuged and washed in PBS. Liquid nitrogen was used to shock-freeze the washed pellet. The cell pellets were stored at -80°C. One pellet was resuspended in 200 µL PBS. The genomic DNA was isolated according to "QIAmp DNA Mini and Blood Mini Handbook".

PCR

The DNA-concentrations of the samples were determined with an A260 measurement. The dilutions of the samples were prepared to a concentration of 800 ng/30 µL in HQ water. 15 µL of the diluted sample were aliquoted into PCR tubes. A master mix for all reactions was prepared.

Master mix:	6 µL	5x Green GoTaq buffer
(per reaction)	0.6 µL	dNTPs
	0.3 µL	Sense primer (Table 2)
	0.3 µL	Antisense primer (Table 2)
	0.15 µL	GoTaq DNA Polymerase
	Ad HQ water to a volume of 30 µL	

The PCR reactions are controlled with three plasmids. The positive control for the primers for parental integration, CAGGS_s and GFP_as, was the plasmid "L-series F3GTNF" which is integrated in the host cell line. The plasmid "L-series F32G12F #1" was used as targeted control for the primers CAGGS_s and SV40pA_as. For the PCR specific for random integration, with pMG433_FSHB_2465_as and SV40pA_as, the "pRMCE2G12_IgG" plasmid which was taken for transfection was used as positive control. Every PCR reaction was performed with all three controls, which means that the other two controls served as negative controls. The controls were used in a concentration of 0.8 pg in PCR for parental and targeted integration. For PCR for random integration the concentration of the three controls was increased to 5 ng.
The master mix was added to the template DNA samples (400 ng/15 µL) in the ratio 1:2. Each sample had a total volume of 30 µL, was vortexed and the reaction tube was spinned down before the PCR thermal cycles. The thermal cycles started by heating to 95°C for 10 min as initial denaturation step. The following 3 temperature steps were performed for 30 to 40 cycles. First the DNA strands were denatured at 95°C for 30 seconds. In the second step, the annealing temperature of 50-55°C for 30 seconds was set. Once the primers annealed to the denatured strands, the elongation temperature of 73°C for 60 seconds per kbp was reached. Finally after these cycles a longer elongation step at 73°C for 5 minutes was done. The optimal annealing temperature was determined by a temperature gradient. The samples were applied to a 1% agarose gel with TAE-buffer. The gel was loaded with 6 µL DNA Ladder and 15 µL of gDNA PCR-samples (200 ng). In addition 15 µL of the parental, targeted and random control PCR samples and 200 ng of gDNA without PCR were applied to the gel. The samples were separated with 110 V for 20 min.

Table 2: PCR Primer sequences

Primer	Sequence	Tm [°C]
CAGGS_s	GGA CTT CCT TTG TCC CAA ATC	63.5
SV40pA_as	TGG TTT GTC CAA ACT CAT CAA	62.9
GFP_as	GGT CAG GGT GGT CAC GAG	64.6
pMG433_FSHB_2465_as	GGA AAT GTT GAA TAC TCA TAC TC	55.8

The calculation for the corresponding amount of plasmid serving as control is shown in equations 11-13. It is assumed that one genome has 5.4 pg of DNA [25].

$$200\,ng\ gDNA = 200000\,pg = \frac{200000\,pg}{5.4\,pg/genome} = 3.7 \cdot 10^4\ genomes \quad (11)$$

$$9890\,bp = 6.11 \cdot 10^6 \frac{g}{mol} = \frac{6.11 \cdot 10^6 \frac{g}{mol}}{6.022 \cdot 10^{23}\ mol^{-1}} = 1.01 \cdot 10^{-17}\ g/Plasmid \quad (12)$$

$$amount\ plasmid = 1.01 \cdot 10^{-17} g/pl. \cdot 3.7 \cdot 10^4\ genomes = 0.37\,pg \quad (13)$$

Agarose gel electrophoresis

The agarose was weight and filled up with TAE and HQ water. The suspension was melted in the microwave and cooled to 50°C in the water bath. The ethidium bromide was supplemented and the gels were poured out. When they were cooled down, they were stored in TAE buffer with EtBr on 4°C.
Before the application to the gel, the gDNA samples without PCR were supplemented with BX-buffer. The GoTaq PCR samples do not need a supplementation for visualization on the gel. The separation was done at 110 V in TAE buffer and the imaging of the DNA bands took place under UV illumination at 254 nm.

4.2.12 IgG purification via Protein A Affinity Chromatography

Diafiltration

The filtered culture supernatant from the production was concentrated with the diafiltration TFF device. As membrane the pellicon XL membrane was used. The filtration system was stored in 0.05 M NaOH and it was washed twice with 500 mL HQ-H_2O before use. The retentate was collected and the system was rinsed with buffer A.

Affinity Chromatography

The ÄKTA system was stored in 20% EtOH and before usage it was rinsed with water. First of all the system was filled with buffer A and buffer B. An installation of the superloop and the column were done and the column was equilibrated. The sample was loaded on the column and it was monitored via an A280 measurement. After loading of the sample the column was washed with buffer A. By setting the pump gradient to 100% buffer B the elution was started. The A280 measurement enables the monitoring of the elution of the IgGs. Neutralization of the eluate was performed with 0.1 M Tris (pH 9.5) and it was cooled down to 4°C. The ÄKTA system was cleaned and it was filled with 20% EtOH. A sample of each fraction was collected.

Dialysis

The dialysis tube was equilibrated in H_2O for about 20 min and it was washed very carefully. The tube was closed with a locking clamp on one end and the retentate was filled into it. A locking clamp was placed on the other side of the tube and it was incubated in 500 mL PBS shaking for 2 h on room temperature.

SDS-PAGE

The samples had a volume of 15 µL, which contained approximately 1 µg antibody protein or less. They were mixed with 5 µL LDS-buffer and were preheated for 2 minutes at 85°C. The tris-glycine gel wells were filled with 20 µL and the separation was done at 270 V for 90 min.

Silver staining

The SDS-PAGE gel was placed in the Fixation Solution for 1 h or overnight. After the remove of the Fixation Solution the gel was incubated in Incubation Solution for 20 min. It was washed three times for 5 min in H_2O. The washed gel was incubated in Silver Solution for 15 min and then it was flushed briefly with H_2O. The development was done with Develop Solution until Bands were visible. Then the gel was washed briefly with H_2O and the reaction was stopped with Stop Solution. An incubation time of 15 min to 1 h is possible for the stopping step. The stained gel was visualized by a scanner.

4.2.13 Cryopreservation

Freezing

The cryovials were labeled and pre-cooled at 4°C. A defined volume of the cell suspension was pipetted in a centrifuge tube and it was centrifuged 1000 rpm for 10 min. After removal of the supernatant the pellet was resuspended with synth-a-freeze cryopreservation medium. Then the pre-cooled cryovials were filled with 1 mL per vial. At a cooling rate of 1°C per minute the samples were frozen to a temperature of -80°C overnight to prevent damage. On the following day the cryovials were transferred from -80°C to a liquid nitrogen tank. The samples were registered in the database and a thawing control was performed.

Thawing

Centrifugation tubes with a volume of 10 mL were filled with 8 mL medium and pre-cooled at 4°C. For each cryovial, a universal tube was filled with approximately 30 mL EtOH and the vials were transferred from the liquid nitrogen to the EtOH. The universal tubes were emptied on paper towels in the laminar flow hood. The volume of the thawn cryovials was added to the 8 mL pre-cooled medium to remove DMSO present in the cryopreservation medium. The tubes were centrifuged at 1000 rpm for 10 min. In the meantime a T25 flask filled with 5 mL medium was prepared. The supernatant was removed and the pellet was resuspended with 5 mL medium and transferred to the T25 flask (total volume 10 mL).

5 Results

5.1 Determination of selection pressure

CHO PG9 and CHO RMCE I3, originate from CHO K1 were used for positive and negative control in this experiment. In place of co-transfected cells, CHO PG9 was used, because it has no recombinant thymidine kinase integrated. It corresponds to the behavior of co-transfected cell lines, where the TK-containing fusion protein is exchanged by the antibody of interest. After co-transfection followed by RMCE the heterologous expression of thymidine kinase is replaced by the expression of antibody. As CHO RMCE I3 includes the GTN fusion protein it shows heterologous expression of thymidine kinase and converts Ganciclovir (GCV) into its toxic phosphorylated form (negative selection).

Table 3: Comparison of GCV concentrations with CHO PG9 and CHO RMCE I3 cells in 96 well plate. Plated out in selection medium 1 and checked after 11 days (2 µM and 40 µM GCV) and 15 days (10µM and 20 µM GCV); +…high viability; -…low viability

		Ganciclovir [µM]						Ganciclovir [µM]			
PG9	c/mL	40	20	10	2	I3	c/mL	40	20	10	2
	20000	+	+	+	+		20000	-	-	-	+
	10000	-	+	+	+		10000	-	-	-	-
	5000	-	+	+	+		5000	-	-	-	-
	2500	-	-	+	+		2500	-	-	-	-
	1250	-	-	-	+		1250	-	-	-	-
	625	-	-	-	-		625	-	-	-	-

The results of the determination of selection pressure are shown in Table 3. The cell line CHO PG9 was able to grow at 40 µM GCV concentration at 20000 c/mL. The PG9 cells were not viable at a lower cell concentration at 40 µM GCV. At 20 µM GCV the PG9 cells were viable in the densities 20000, 10000 and 5000 c/mL. At 10 µM GCV the critical cell concentration of CHO PG9 was below 2500 c/mL. The most viable colonies were recorded at 2 µM GCV where a seeding density up to 1250 c/mL showed viable cells.
The host cell line CHO RMCE I3 showed viable cells at a cell concentration of 20000 c/mL in 2 µM GCV. At a cell density of 10000 c/mL and below, no CHO

I3 cells were viable at 2 µM GCV. At the GCV concentrations of 40-10 µM GCV no CHO RMCE I3 cells were viable indicating a higher sensitivity of the RMCE host cell line in contrast to the antibody producing cell line.

5.2 Cell sorting

The sorting process is determined by GFP negativity (Figure 7). A successful co-transfection leads to the exchange of the GFP-containing fusion protein with the antibody of interest by RMCE. After transfection, GFP expressing cells did not undergo an exchange with the antibody donor plasmid and thus have still integrated the GTN fusion protein. The cell sorting experiment resulted in three wells with 20,000 cells in a 24 well plate. Only GFP-negative RMCEUsteIgG cells were placed in the 24 well plate.

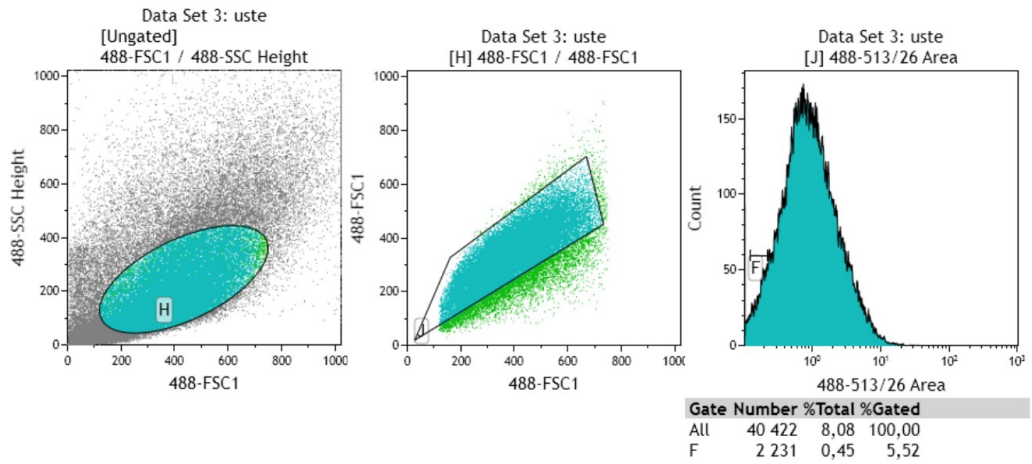


Figure 7: Example for gating at sort process

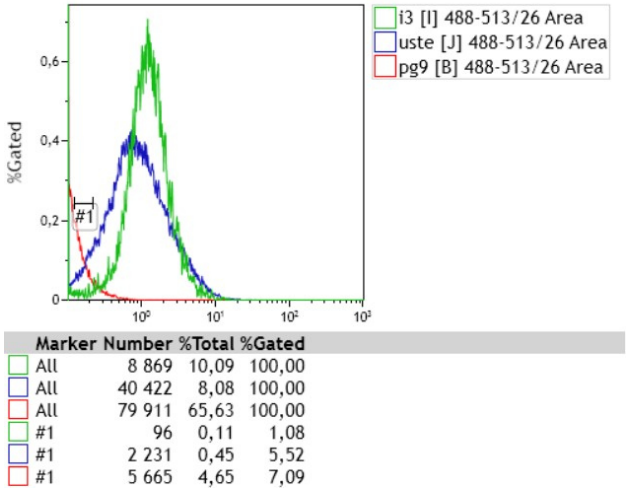


Figure 8: Cell Sorting of GFP-negative RMCEUsteIgG cells. GFP positive control: CHO RMCE I3; GFP-negative control: CHO PG9. Gate #1 contains the sorted cell population

According to Figure 7 the gates for the analysis of RMCEUsteIgG were set. The first gate in the FS versus SS diagram was used to gate the living cells. In the FS-height versus FS-area graph the gate was set on single cells, cell doublets were excluded. The GFP negative cells were gated in the 488-513/26 area versus counts diagram. In Figure 8 the overlay shows that the controls overlap to approximately 35%. This rather big overlap complicates the sorting process. The sample was analyzed in the fluorescence microscope after one day of incubation. In all three wells green fluorescence cells were visible, which were bigger than the surrounding cells. The sorted cells were used for batch experiment 3 and subcloning 2 was performed.

5.3 Subcloning

1st subcloning

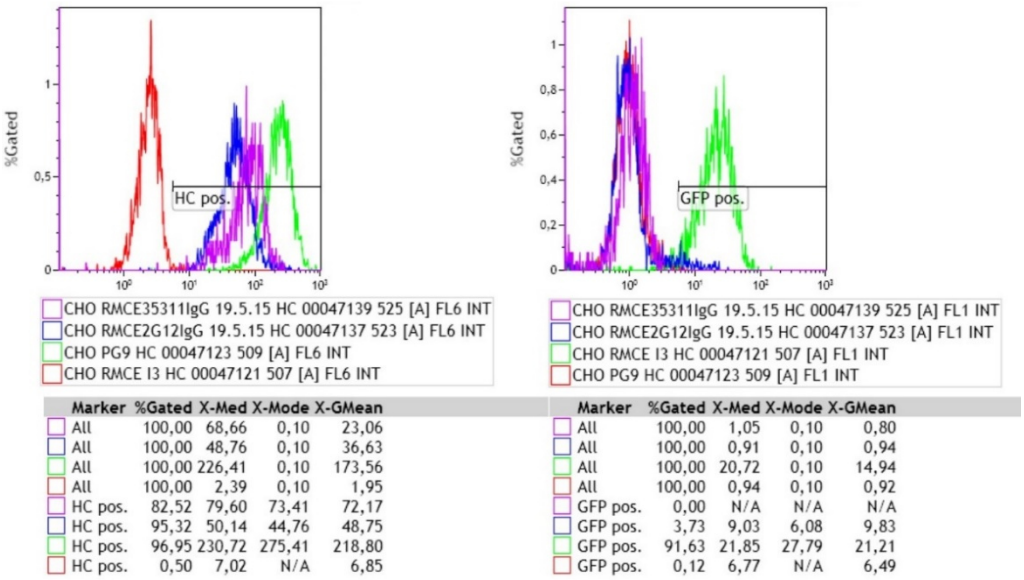
Cell lines: RMCE2G12IgG, RMCE353/11/IgG, RMCE554/12/IgG, RMCE4B3IgG (cell pools in shaker flask from transfection 1)

Medium: Selection Medium 1: CD-CHO + 4 mM L-Glutamine + 15 mg/L phenol red + 2 µM Ganciclovir + Growth factors: 0.02 mg/mL Transferrin, 25 ng/mL rEGF, 1 mg/mL Albumin
Filtered supernatant: 1:1 with selection medium

Cell density: 1 c/well/50 µL in 384 well plates

Feeding: half of 384 well plate after 13 days with selection medium 3 without growth factors

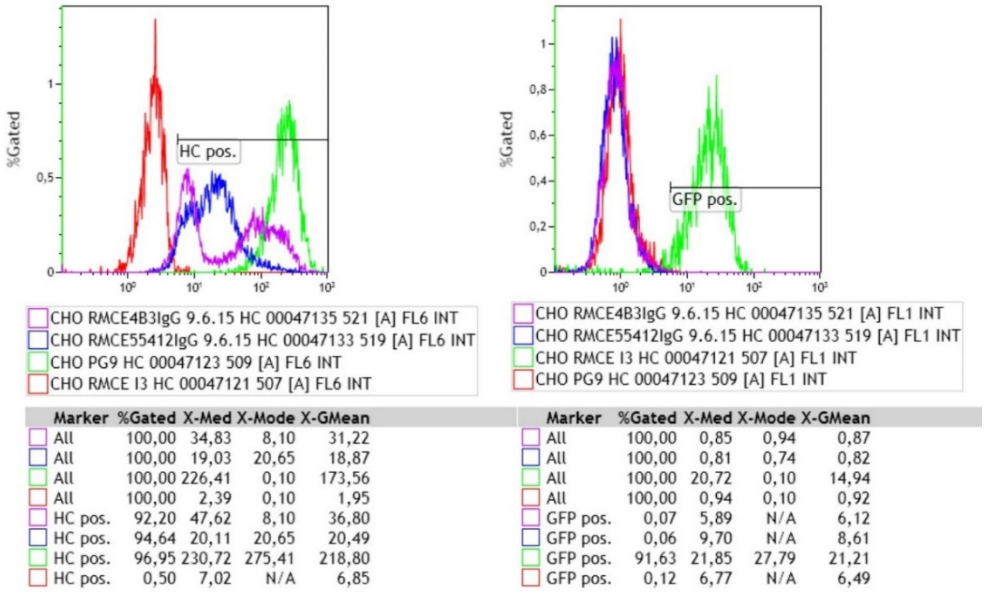
From transfection 1 resulting cell pools showed a heterogeneous population. In Figure 9 and 10 the heavy chain of the cell pools before subcloning 1 were analyzed.



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	68,66	0,10	23,06
All	100,00	48,76	0,10	36,63
All	100,00	226,41	0,10	173,56
All	100,00	2,39	0,10	1,95
HC pos.	82,52	79,60	73,41	72,17
HC pos.	95,32	50,14	44,76	48,75
HC pos.	96,95	230,72	275,41	218,80
HC pos.	0,50	7,02	N/A	6,85

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	1,05	0,10	0,80
All	100,00	0,91	0,10	0,94
All	100,00	20,72	0,10	14,94
All	100,00	0,94	0,10	0,92
GFP pos.	0,00	N/A	N/A	N/A
GFP pos.	3,73	9,03	6,08	9,83
GFP pos.	91,63	21,85	27,79	21,21
GFP pos.	0,12	6,77	N/A	6,49

Figure 9: FL6 and FL1 laser channel of RMCE2G12IgG and RMCE353/11/IgG cell pools before subcloning 1 - staining of intracellular heavy chain (left) and GFP (right) of EtOH fixed cells. Negative control HC: CHO RMCE I3, positive control HC: CHO PG9, negative control GFP: CHO PG9, positive control GFP: CHO RMCE I3



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	34,83	8,10	31,22
All	100,00	19,03	20,65	18,87
All	100,00	226,41	0,10	173,56
All	100,00	2,39	0,10	1,95
HC pos.	92,20	47,62	8,10	36,80
HC pos.	94,64	20,11	20,65	20,49
HC pos.	96,95	230,72	275,41	218,80
HC pos.	0,50	7,02	N/A	6,85

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	0,85	0,94	0,87
All	100,00	0,81	0,74	0,82
All	100,00	20,72	0,10	14,94
All	100,00	0,94	0,10	0,92
GFP pos.	0,07	5,89	N/A	6,12
GFP pos.	0,06	9,70	N/A	8,61
GFP pos.	91,63	21,85	27,79	21,21
GFP pos.	0,12	6,77	N/A	6,49

Figure 10: FL6 and FL1 laser channel of RMCE4B3IgG and RMCE554/12/IgG cell pools before subcloning - staining of intracellular heavy chain (left) and GFP (right) of EtOH fixed cells. Negative control HC: CHO RMCE I3, positive control HC: CHO PG9, negative control GFP: CHO PG9, positive control GFP: CHO RMCE I3

RMCE2G12IgG has 95.32% antibody producer cells in the population. Its germline RMCE353/11/IgG has a fraction of 82.52% of the population which are heavy chain producers. The peaks of both cell lines are broad which indicates heterogeneity, compared to the homogenous PG9 producer. Even broader peaks and clear subpopulations are seen in the FACS analysis of the cell pools RMCE554/12/IgG and RMCE4B3IgG. Although 92.2% of RMCE4B3IgG and 94.64% of RMCE554/12/IgG of their cell populations are antibody producers, the peaks shown in Figure 10, are clearly separated. There is no GFP positive subpopulation in the cell pools of RMCE353/11/IgG, RMCE554/12/IgG and RMCE4B3IgG. A subpopulation of 3.7% of GFP positive cells is seen in the cell pool of RMCE2G12IgG.

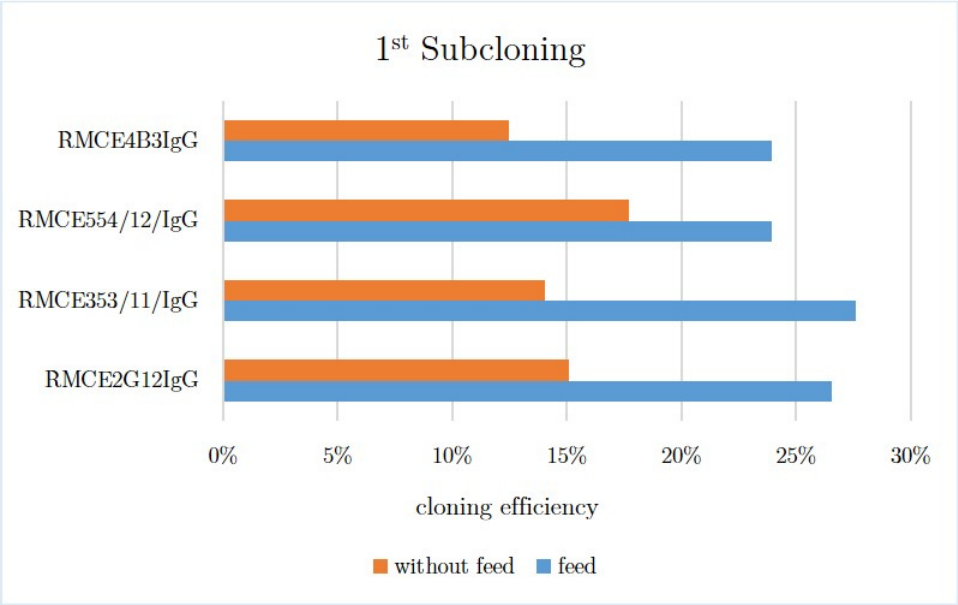


Figure 11: Transferred wells from 384 to 96 well plate of 1st subcloning

The number of grown clones of RMCE2G12IgG into a 96 well plate with feed was 51 and without feed 29 clones (Figure 11). The transfer of fed clones of RMCE353/11/IgG was done for 53 clones, without feed 27 clones were picked. The number of clones of RMCE554/12/IgG which were transferred was 46 of fed wells and 34 of wells without feed. In total 80 wells of RMCE2G12IgG, RMCE353/11/IgG and RMCE554/12/IgG were transferred from 384 to 96 well plate. 70 clones of RMCE4B3IgG were chosen, 46 fed wells and 24 wells without feed.

Six clones per cell line were chosen from RMCE2G12IgG and RMCE353/11/IgG. From RMCE554/12/IgG and RMCE4B3IgG three clones were expanded into

24 well plate. The other three clones were from the second transfection with the cell density of 4 c/well.

The resulting clones of the 1st subcloning (Figure 12):

RMCE2G12IgG/384/1B3,
RMCE2G12IgG/384/1E4,
RMCE2G12IgG/384/1B11,

RMCE353/11/IgG/384/2A12,
RMCE353/11/IgG/384/2C7,
RMCE353/11/IgG/384/2D7,

RMCE554/12/IgG/384/3A11,
RMCE554/12/IgG/384/5H1,
RMCE554/12/IgG/384/5F7,

RMCE4B3IgG/384/4A9,
RMCE4B3IgG/384/4F7,
RMCE4B3IgG/384/6B1

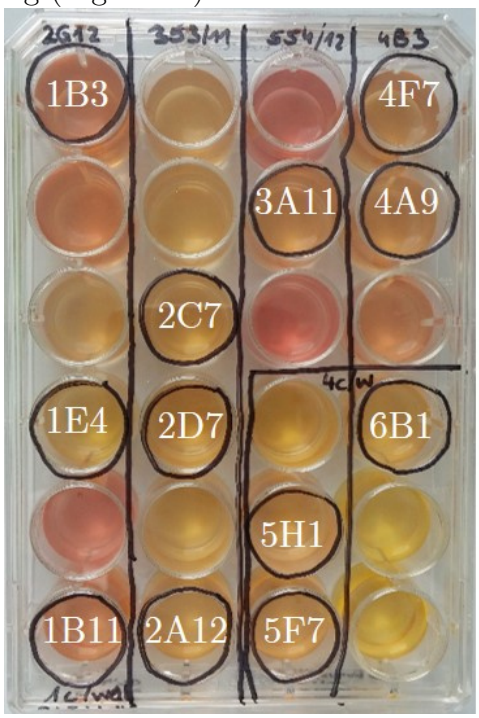
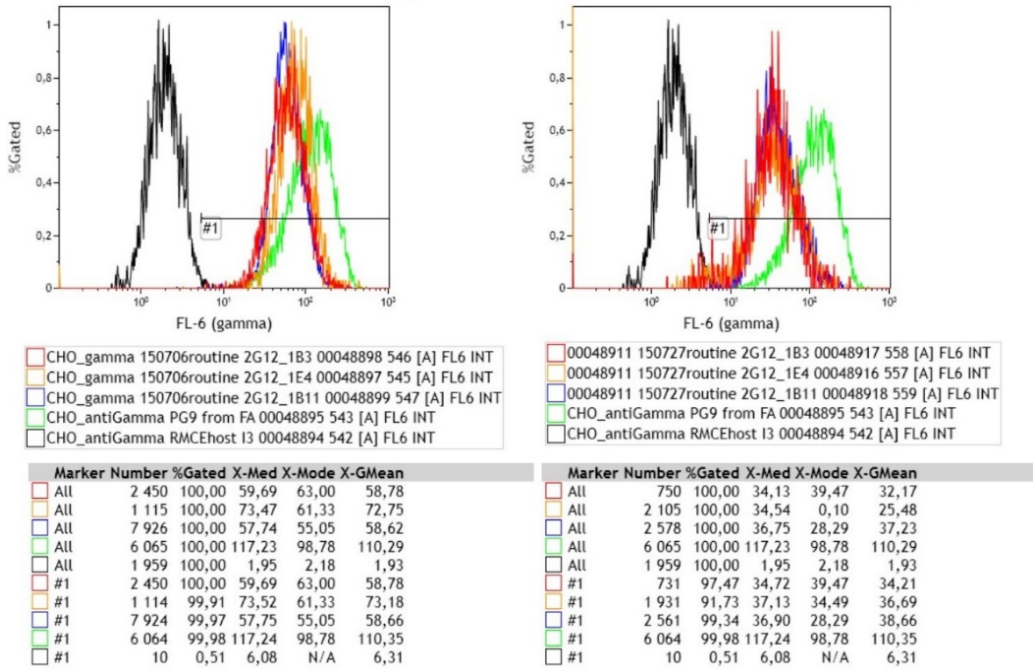


Figure 12: Expansion of 1st subclones

The clones were cryopreserved according to 4.2.13. They were used for batch experiment 2 and the product generation of RMCE554/12/IgG and RMCE4B3IgG.

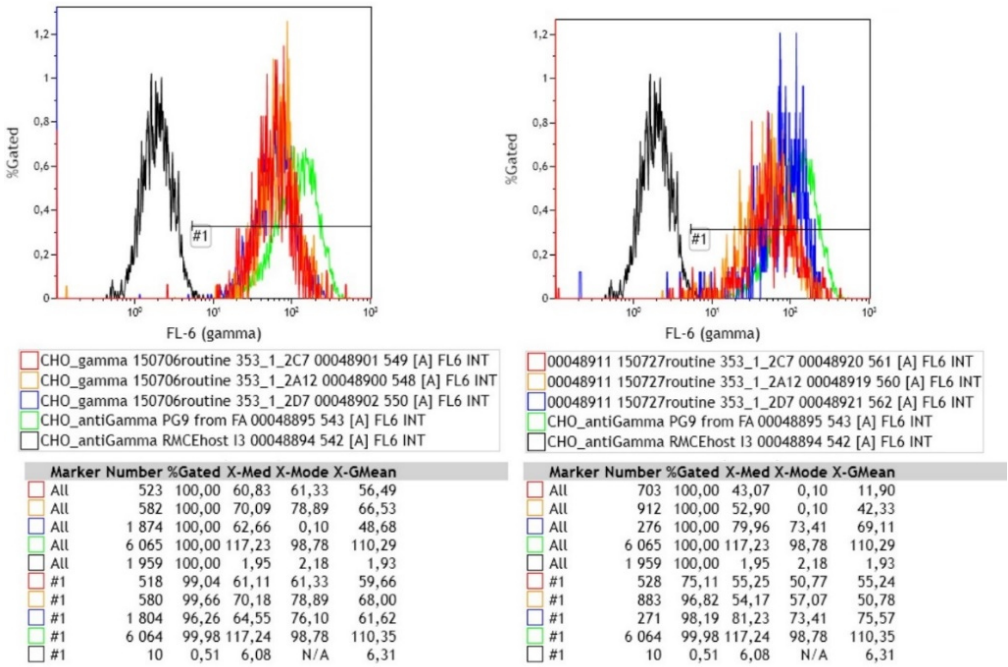


Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	2 450	100,00	59,69	63,00	58,78
All	1 115	100,00	73,47	61,33	72,75
All	7 926	100,00	57,74	55,05	58,62
All	6 065	100,00	117,23	98,78	110,29
All	1 959	100,00	1,95	2,18	1,93
#1	2 450	100,00	59,69	63,00	58,78
#1	1 114	99,91	73,52	61,33	73,18
#1	7 924	99,97	57,75	55,05	58,66
#1	6 064	99,98	117,24	98,78	110,35
#1	10	0,51	6,08	N/A	6,31

Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	750	100,00	34,13	39,47	32,17
All	2 105	100,00	34,54	0,10	25,48
All	2 578	100,00	36,75	28,29	37,23
All	6 065	100,00	117,23	98,78	110,29
All	1 959	100,00	1,95	2,18	1,93
#1	731	97,47	34,72	39,47	34,21
#1	1 931	91,73	37,13	34,49	36,69
#1	2 561	99,34	36,90	28,29	38,66
#1	6 064	99,98	117,24	98,78	110,35
#1	10	0,51	6,08	N/A	6,31

Figure 13: Staining of intracellular heavy chain analyzed by FACS. RMCE2G12IgG/384/1B3, 1E4 and 1B11 after subcloning 1 (left) and after cultivation for one month (right). Negative control HC: CHO RMCE I3, positive control HC: CHO PG9

After subcloning 1 the cell populations of RMCE2G12IgG/384/1B3, 1E4 and 1B11 were homogenous with an amount of over 99.9% of antibody producing cells (Figure 13). The fraction of antibody producing cells is reduced after one month of cultivation in shaker flasks in all three subclones. RMCE2G12IgG/384/1E4 shows the highest subpopulation with a reduction of 8.18% of antibody producing cells. Furthermore, a small lower producing subpopulation appeared after one month.

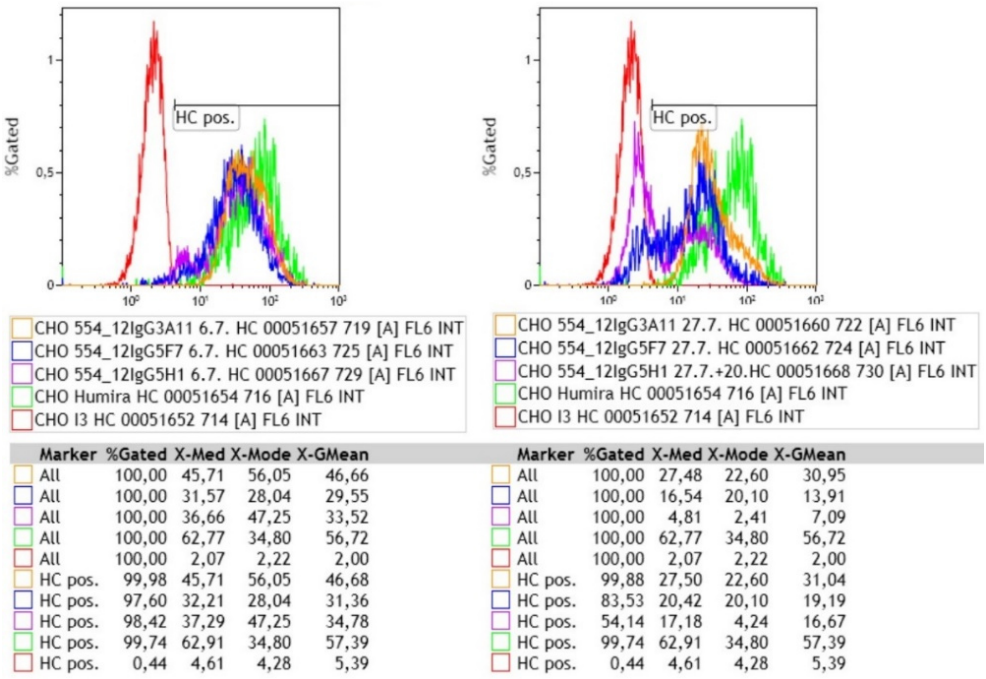


Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	523	100,00	60,83	61,33	56,49
All	582	100,00	70,09	78,89	66,53
All	1 874	100,00	62,66	0,10	48,68
All	6 065	100,00	117,23	98,78	110,29
All	1 959	100,00	1,95	2,18	1,93
#1	518	99,04	61,11	61,33	59,66
#1	580	99,66	70,18	78,89	68,00
#1	1 804	96,26	64,55	76,10	61,62
#1	6 064	99,98	117,24	98,78	110,35
#1	10	0,51	6,08	N/A	6,31

Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	703	100,00	43,07	0,10	11,90
All	912	100,00	52,90	0,10	42,33
All	276	100,00	79,96	73,41	69,11
All	6 065	100,00	117,23	98,78	110,29
All	1 959	100,00	1,95	2,18	1,93
#1	528	75,11	55,25	50,77	55,24
#1	883	96,82	54,17	57,07	50,78
#1	271	98,19	81,23	73,41	75,57
#1	6 064	99,98	117,24	98,78	110,35
#1	10	0,51	6,08	N/A	6,31

Figure 14: Staining of intracellular heavy chain analyzed by FACS. RMCE353/11/IgG/384/2C7, 2A12 and 2D7 after subcloning 1 (left) and after cultivation for one month (right). Negative control HC: CHO RMCE I3, positive control HC: CHO PG9

The subclones RMCE353/11/IgG/384/2C7 and 2A12 are homogeneous with an amount of over 99% of antibody producing cells (Figure 14). The population of RMCE353/11/IgG/384/2D7 has 96.26% antibody producing cells. After one month of cultivation the antibody producing population of RMCE353/11/IgG/384/2C7 is reduced to 75.11%. The population of RMCE353/11/IgG/384/2A12 and 2D7 stay over 96.8% of antibody producing cells.

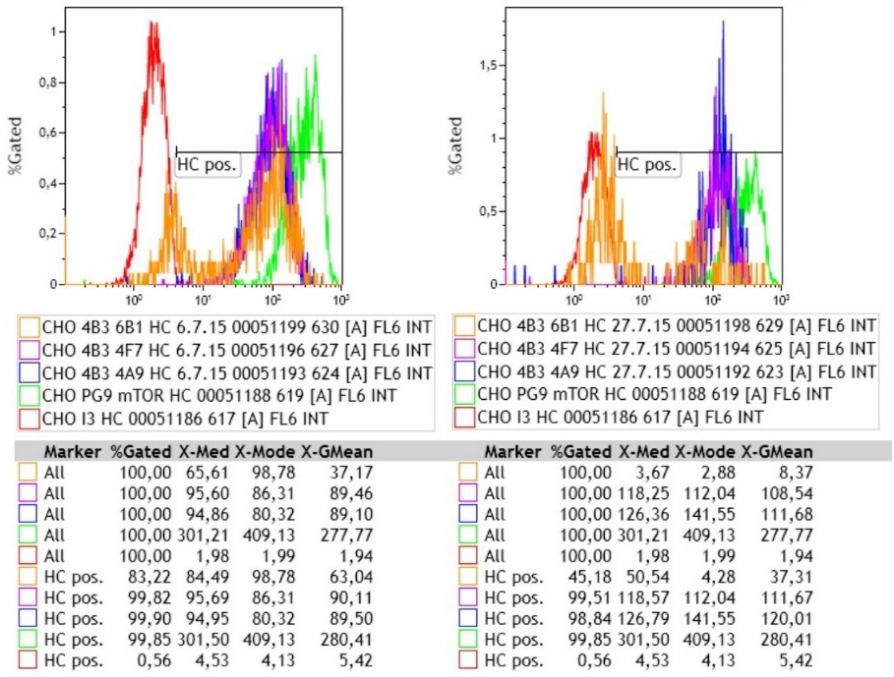


Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	45,71	56,05	46,66
All	100,00	31,57	28,04	29,55
All	100,00	36,66	47,25	33,52
All	100,00	62,77	34,80	56,72
All	100,00	2,07	2,22	2,00
HC pos.	99,98	45,71	56,05	46,68
HC pos.	97,60	32,21	28,04	31,36
HC pos.	98,42	37,29	47,25	34,78
HC pos.	99,74	62,91	34,80	57,39
HC pos.	0,44	4,61	4,28	5,39

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	27,48	22,60	30,95
All	100,00	16,54	20,10	13,91
All	100,00	4,81	2,41	7,09
All	100,00	62,77	34,80	56,72
All	100,00	2,07	2,22	2,00
HC pos.	99,88	27,50	22,60	31,04
HC pos.	83,53	20,42	20,10	19,19
HC pos.	54,14	17,18	4,24	16,67
HC pos.	99,74	62,91	34,80	57,39
HC pos.	0,44	4,61	4,28	5,39

Figure 15: Staining of intracellular heavy chain analyzed by FACS. RMCE554/12/IgG/384/3A11, 5F7 and 5H1 after subcloning 1 (left) and after cultivation for one month (right). Negative control HC: CHO RMCE I3, positive control HC: CHO Humira

After subcloning 1 the cell populations of RMCE554/12/IgG/384/3A11 and 5H1 were homogenous with an amount of over 98.4% of antibody producing cells (Figure 15). The clone RMCE554/12/IgG/384/5F7 showed 97.6% of antibody producing cells. The fraction of antibody producing cells is reduced after one month of cultivation in shaker flasks in two subclones. The subclones RMCE554/12/IgG/384/3A11 remained stable with over 99.88% of heavy chain positive cells.



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	65,61	98,78	37,17
All	100,00	95,60	86,31	89,46
All	100,00	94,86	80,32	89,10
All	100,00	301,21	409,13	277,77
All	100,00	1,98	1,99	1,94
HC pos.	83,22	84,49	98,78	63,04
HC pos.	99,82	95,69	86,31	90,11
HC pos.	99,90	94,95	80,32	89,50
HC pos.	99,85	301,50	409,13	280,41
HC pos.	0,56	4,53	4,13	5,42

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	3,67	2,88	8,37
All	100,00	118,25	112,04	108,54
All	100,00	126,36	141,55	111,68
All	100,00	301,21	409,13	277,77
All	100,00	1,98	1,99	1,94
HC pos.	45,18	50,54	4,28	37,31
HC pos.	99,51	118,57	112,04	111,67
HC pos.	98,84	126,79	141,55	120,01
HC pos.	99,85	301,50	409,13	280,41
HC pos.	0,56	4,53	4,13	5,42

Figure 16: Staining of intracellular heavy chain analyzed by FACS. RMCE4B3IgG/384/6B1, 4F7 and 4A9 after subcloning 1 (left) and after cultivation for one month (right). Negative control HC: CHO RMCE I3, positive control HC: CHO PG9

After subcloning 1 the cell populations of RMCE4B3IgG/384/4F7 and 4A9 were homogenous with an amount of over 99.8% of antibody producing cells (Figure 16). The clone RMCE4B3IgG/384/6B1 showed 83.22% of antibody producing cells and a clear subpopulation consisting of low/non-producers. The fraction of antibody producing cells is reduced after one month of cultivation in shaker flasks in RMCE4B3IgG/384/6B1 and the number of non/low-producers exceeds the number of high-producers. The subclones RMCE554/12/IgG/384/4F7 and 4A9 remained stable with over 99.5% of heavy chain positive cells.

2nd subcloning

Cell line: RMCEUsteIgG
Medium: Selection Medium 1: CD-CHO + 4 mM L-Glutamine
+ 15 mg/L phenol red + 2 µM Ganciclovir
Filtered supernatant: 1:1 with selection medium 1
Cell density: 1; 10; 100 c/well/50 µL in 384 well plate
Feeding: after 7 days

At the subcloning of the 2nd transfection 58 clones of RMCEUsteIgG were generated. 20.7% of the total resulting clones were grown at a cell density of 10

c/well. At a cell density of 100 c/well 79.3% of the transferred clones were chosen. All resulting clones after the quantitative ELISA were seeded with 100 c/well. They were cryopreserved according to 4.2.13.

The resulting clones of the subcloning of the 2nd transfection:
RMCEUsteIgG/H5
RMCEUsteIgG/H4
RMCEUsteIgG/H12

3rd subcloning

Cell line:	RMCE136/63/IgG
Medium:	Selection Medium 1: CD-CHO + 4 mM L-Glutamine
	+ 15 mg/L phenol red + 20 µM Ganciclovir
	Filtered supernatant: 1:1 with selection medium 1
Cell densities:	2000 cells/well/100 µL in 96 well plate
	2000 c/well/50 µL in 384 well plate
Feeding:	after 10 days

In total 39 clones from RMCE136/63/IgG in 384 and 96 well plate were transferred to one 96 well plate. To enable the comparison of 384 and 96 well plates, the number of transferred clones to a 96 well plate was calculated per well (equation 14 and 15).

$$\frac{33\ grown\ clones}{384\ wells} = 0.0859 \frac{clones}{well} = 9\ \%\ \text{cloning efficacy} \qquad (14)$$

$$\frac{6\ grown\ clones}{96\ wells} = 0.0625 \frac{clones}{well} = 6\%\ \text{cloning efficacy} \qquad (15)$$

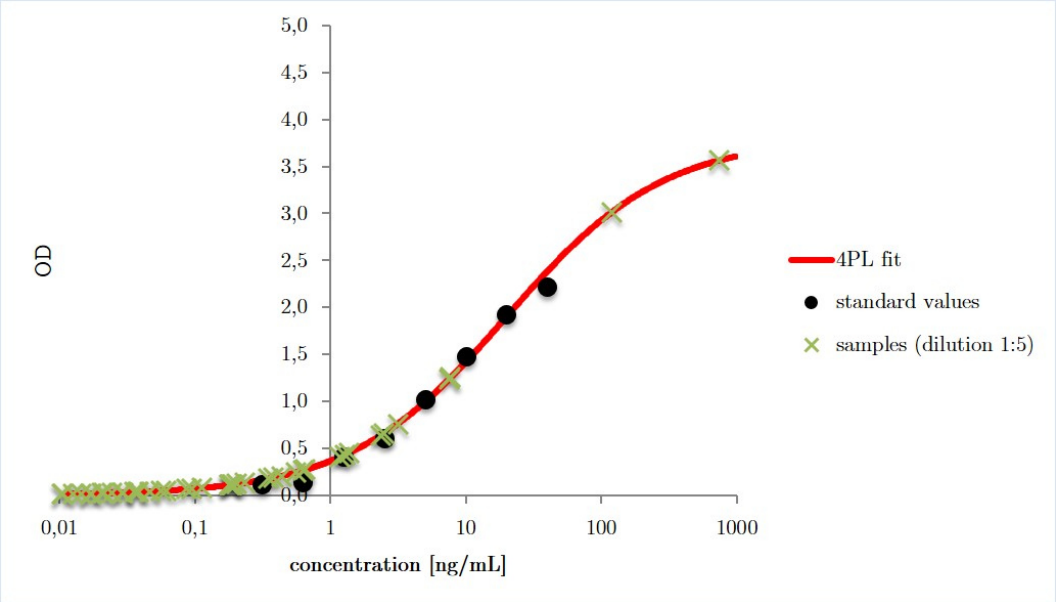


Figure 17: Qualitative ELISA values for the transfection of RMCE136/63/IgG. The highest standard value is 40 ng/mL. The samples were diluted 1:5. The qualitative ELISA was performed after 45 days with 2 µM GCV in the selection medium. The standard curve is extrapolated before the lowest standard value and after the highest standard value.

Only the OD of one dilution point is known, the antibody concentration has to be calculated with this information. The correlation between the OD and the antibody concentration is non-linear, and therefore a 4 Parameter Logistic (4PL) nonlinear regression model fitted to the standard OD-values has to be found for the calculation of the concentration (Figure 17).
11 best producing clones, of 39 clones, were expanded in a 96 well plate. After 10 days a quantitative ELISA was done. Only two subclones of the 11 in the expansion plate produced 35 ng/mL and 2.4 µg/mL. The two subclones were transferred into T25 flasks and they were cryopreserved according to 4.2.13.

The resulting clones of 3rd subcloning:
RMCE136/63/G3 from 96 well plate
RMCE136/63/E10 from 384 well plate

5.4 Cell line establishment

Subcloning 1 resulted in a total of 12 subclones for 4 different cell lines (3 subclones for each variant). All clones were cultivated in 125 mL shaker flasks with 24 mL culture volume with selection medium 1 containing 2 µM GCV. The

shaker flasks were passaged every 3 to 4 days for 9 passages. The seeding density of the routine shakers were $2 \cdot 10^5$ c/mL.

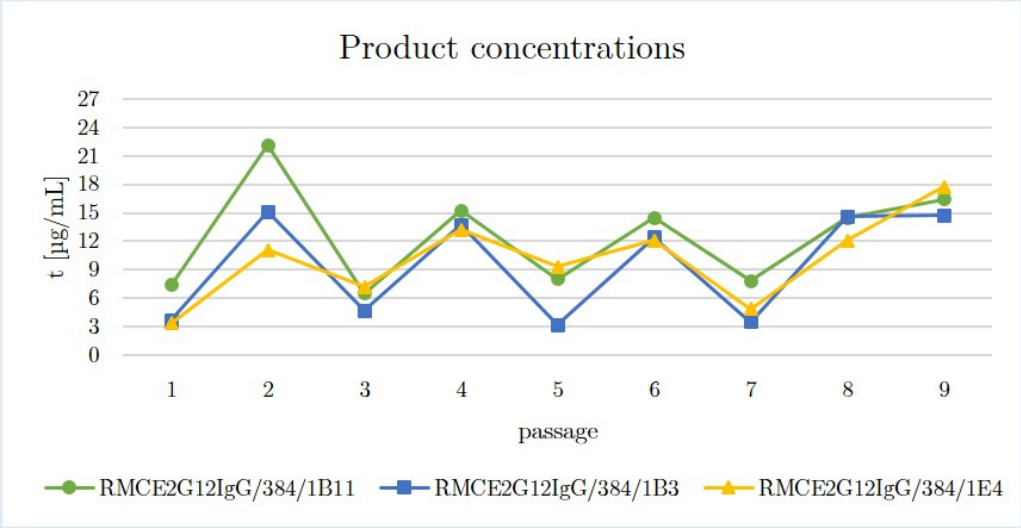


Figure 18: Product concentrations of RMCE2G12IgG/384/1B11, 1B3 and 1E4 over 9 passages

The average product concentration of RMCE2G12IgG/384/1B11 is 12.5 µg/mL and of RMCE2G12IgG/384/1B3 is 9.5 µg/mL (Figure 18). The cell line RMCE2G12IgG/384/1E4 shows an average titer of 10.1 µg/mL.

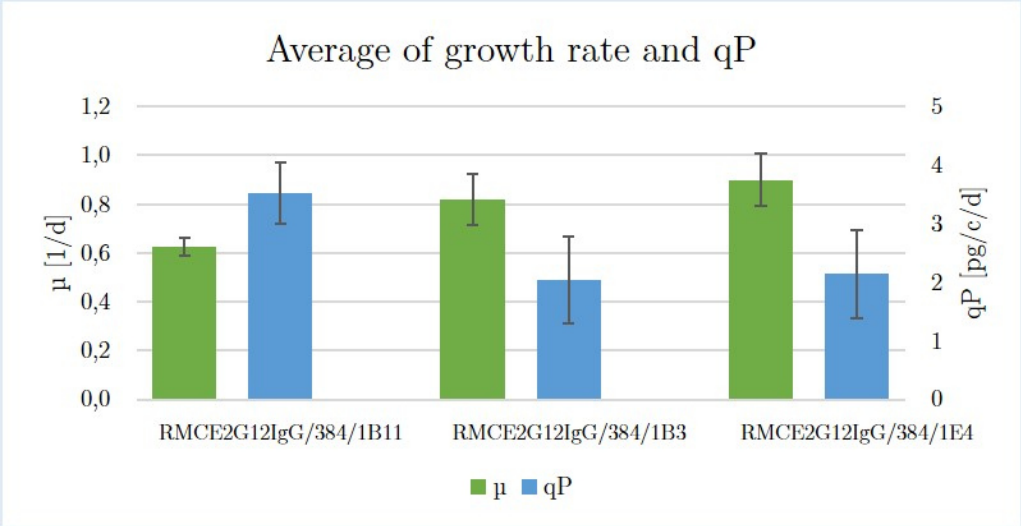


Figure 19: Average of growth rate and specific productivity of RMCE2G12IgG/384/1B11, 1B3 and 1E4 over 8 passages. Error bars represent ± standard deviation.

The cell line RMCE2G12IgG/384/1B11 shows a maximum growth rate of 0.67 d^{-1} and an average of 0.63 d^{-1} (Figure 19). The maximum growth rate of

RMCE2G12IgG/384/1B3 is 1.02 d^{-1} and the average growth rate results in 0.82 d^{-1}. The average growth rate of RMCE2G12IgG/384/1E4 is 0.90 d^{-1} and the highest reached growth rate is 1.10 d^{-1}. The average of growth rate is compared to the average of specific productivity in all three subclones. The cell line RMCE2G12IgG/384/1B11 has the lowest average of growth rate and the highest average of specific productivity. RMCE2G12IgG/384/1B3 and 1E4 have a higher average of growth rate, but a lower average of specific productivity.

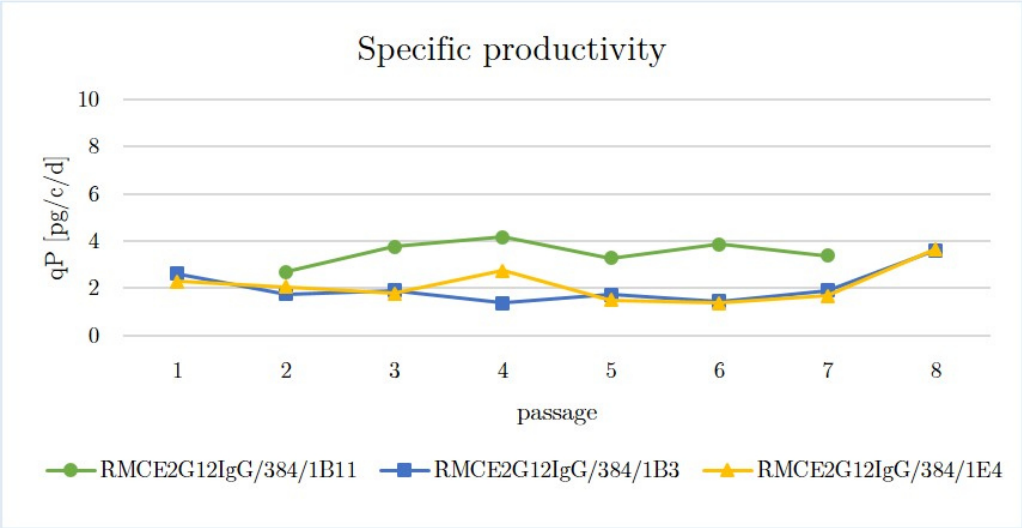


Figure 20: Specific productivity of RMCE2G12IgG/384/1B11, 1B3 and 1E4 over 8 passages

The specific productivity of RMCE2G12IgG/384/1B11 is on average 3.53 pg/c/d (Figure 20). The average qP of RMCE2G12IgG/384/1B3 is 2.04 pg/c/d. The cell line RMCE2G12IgG/384/1E4 has a specific productivity of 2.14 pg/c/d on average.

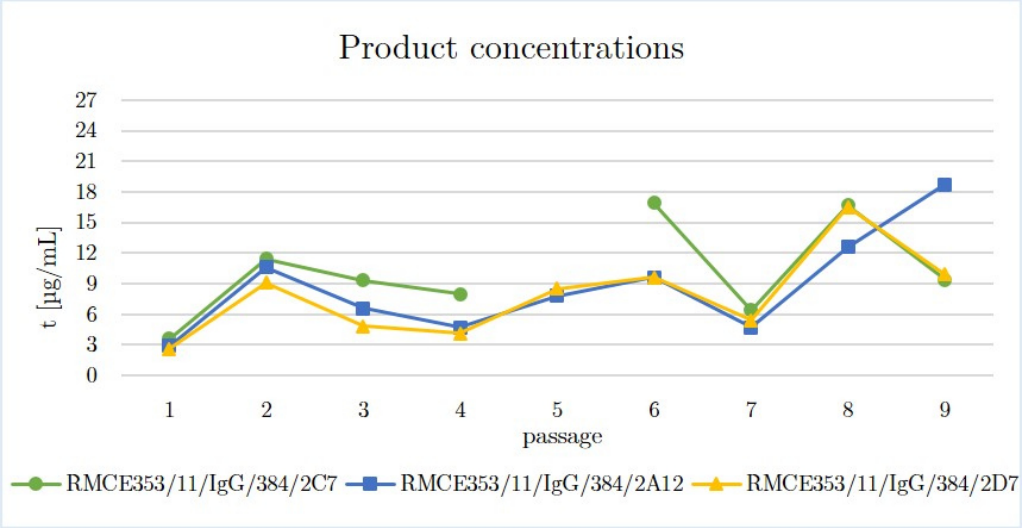


Figure 21: Product concentrations of RMCE353/11/IgG/384/2C7, 2A12 and 2D7 over 9 passages

The average product concentration of RMCE353/11/IgG/384/2C7 is 10.2 µg/mL and of RMCE353/11/IgG/384/2A12 is 8.7 µg/mL (Figure 21). The cell line RMCE353/11/IgG/384/2D7 shows an average titer of 7.8 µg/mL.

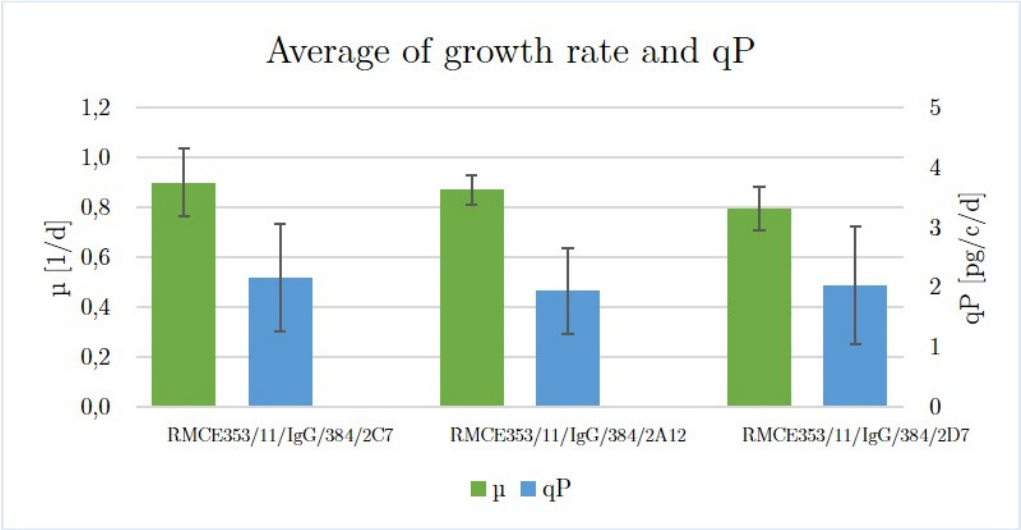


Figure 22: Average of growth rate and specific productivity of RMCE353/11/IgG/384/2C7, 2A12 and 2D7 over 8 passages. Error bars represent ± standard deviation.

The cell line RMCE353/11/IgG/384/2C7 shows a maximum growth rate of 1.14 d^{-1} and an average of 0.87 d^{-1} (Figure 22). The maximum growth rate of RMCE353/11/IgG/384/2A12 is 0.84 d^{-1} and the average growth rate results in 0.78 d^{-1}. The average growth rate of RMCE353/11/IgG/384/2D7 is 0.81 d^{-1} and

the highest reached growth rate is 0.94 d^{-1}. A comparison of the average of growth rate to the average of specific productivity is shown in all three subclones. The cell line RMCE353/11/IgG/384/2A12 has the lowest average of growth rate and the lowest average of specific productivity.

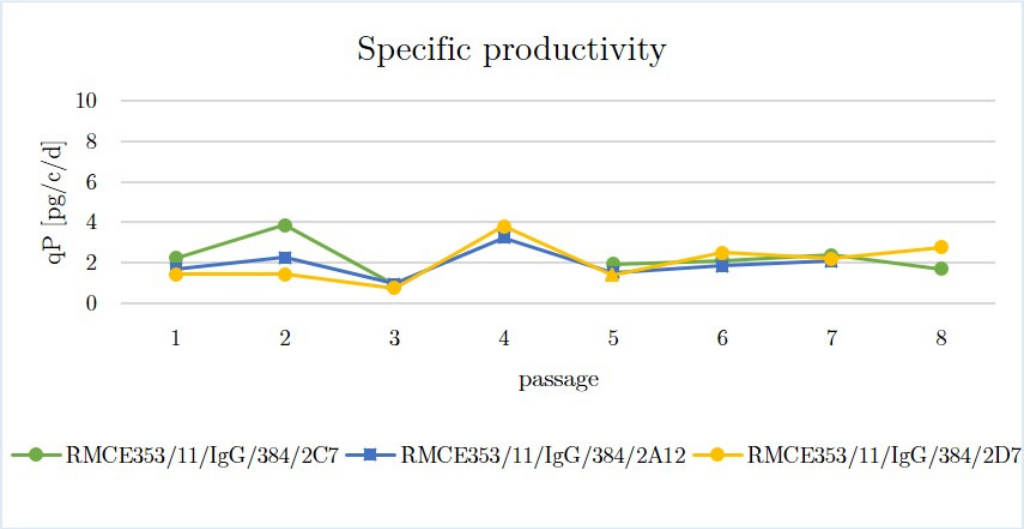


Figure 23: Specific productivity of RMCE353/11/IgG/384/2C7, 2A12 and 2D7 over 8 passages

The specific productivity of RMCE353/11/IgG/384/2C7 is in average 2.16 pg/c/d (Figure 23). The average qP of RMCE353/11/IgG/384/2A12 amounts to 1.94 pg/c/d. The cell line RMCE353/11/IgG/384/2D7 has a specific productivity of 2.03 pg/c/d in average.

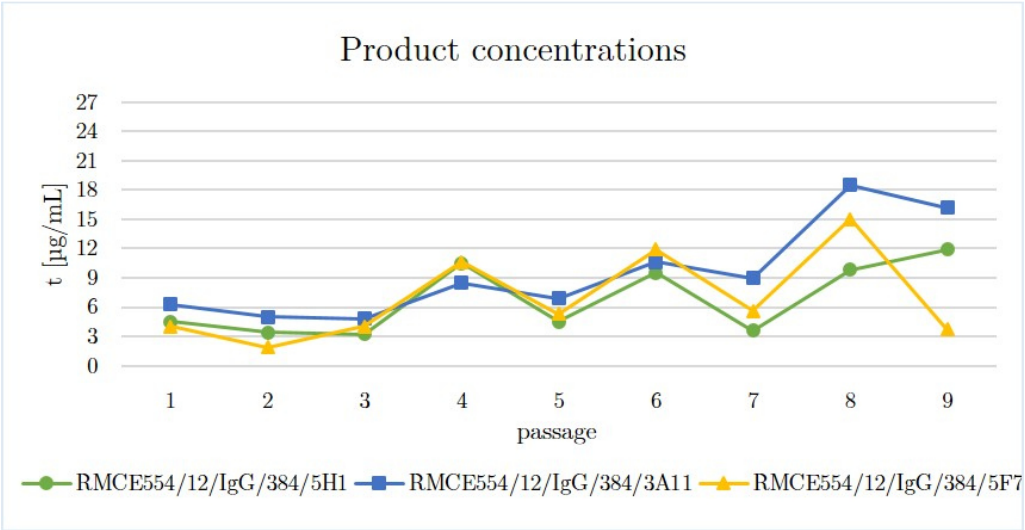


Figure 24: Product concentrations of RMCE554/12/IgG/384/5H1, 3A11 and 5F7 over 9 passages

The average product concentration of RMCE554/12/IgG/384/5H1 is 6.8 µg/mL and of RMCE554/12/IgG/384/3A11 is 9.5 µg/mL (Figure 24). The cell line RMCE554/12/IgG/384/5F7 shows an average titer of 6.9 µg/mL.

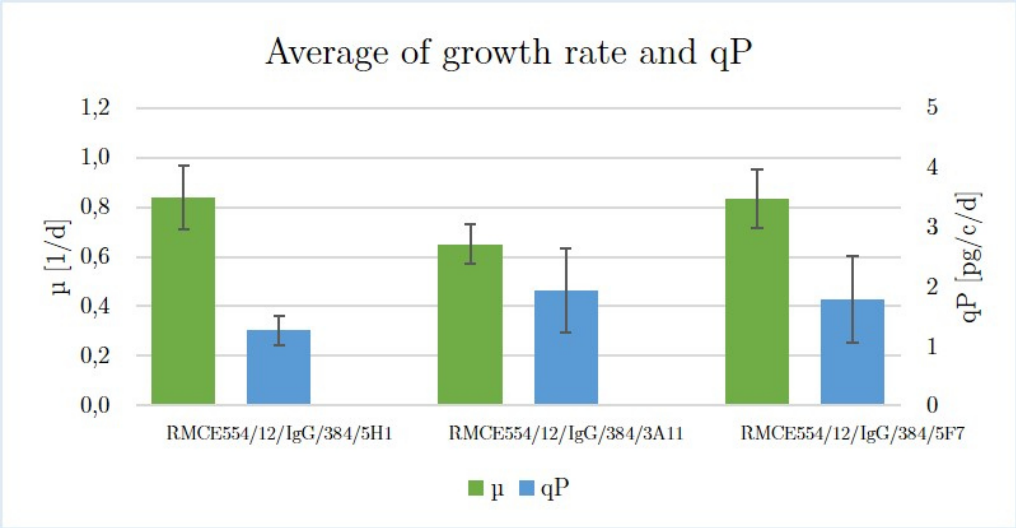


Figure 25: Average of growth rate and specific productivity of RMCE554/12/IgG/384/5H1, 3A11 and 5F7 over 8 passages. Error bars represent ± standard deviation.

The cell line RMCE554/12/IgG/384/5H1 shows a maximum growth rate of 0.92 d^{-1} and an average of 0.84 d^{-1} (Figure 25). The maximum growth rate of RMCE554/12/IgG/384/3A11 is 0.78 d^{-1} and the average growth rate results in 0.65 d^{-1}. The average growth rate of RMCE554/12/IgG/384/5F7 is 0.83 d^{-1} and the highest reached growth rate is 1.02 d^{-1}. The average of growth rate is compared to the average of specific productivity in all three subclones. The cell line RMCE554/12/IgG/384/3A11 has the lowest average of growth rate and the highest average of specific productivity. RMCE554/12/IgG/384/5F7 and 5H1 have a higher average of growth rate, but a lower average of specific productivity.

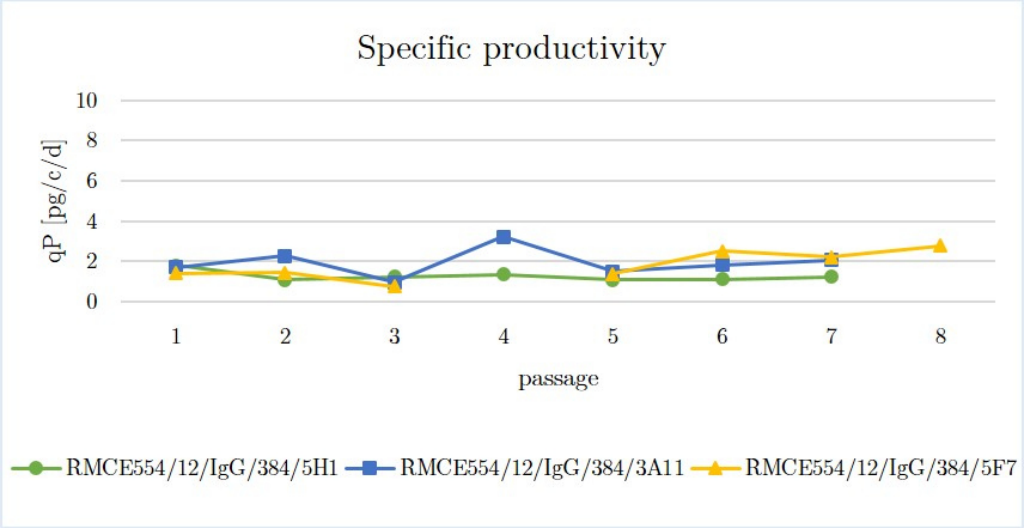


Figure 26: Specific productivity of RMCE554/12/IgG/384/5H1, 3A11 and 5F7 over 8 passages

The specific productivity of RMCE554/12/IgG/384/5H1 is in average 1.27 pg/c/d (Figure 26). The average qP of RMCE554/12/IgG/384/3A11 amounts to 1.94 pg/c/d. The cell line RMCE554/12/IgG/384/5F7 has a specific productivity of 1.78 pg/c/d in average.

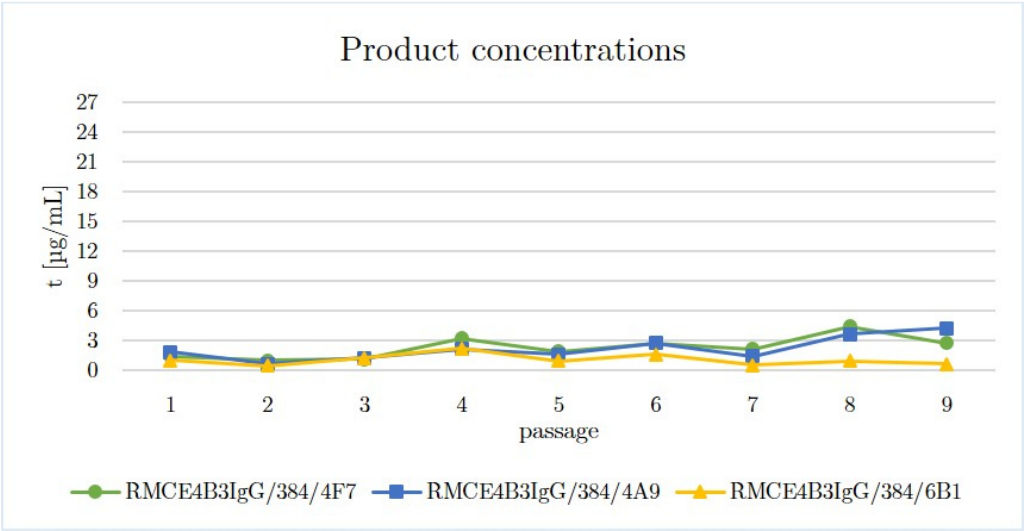


Figure 27: Product concentrations of RMCE4B3IgG/384/4F7, 4A9 and 6B1 over 9 passages

The average product concentration of RMCE4B3IgG/384/4F7 is 2.3 µg/mL and of RMCE4B3IgG/384/4A9 is 2.1 µg/mL (Figure 27). The cell line RMCE4B3IgG/384/6B1 shows an average titer of 1.0 µg/mL.

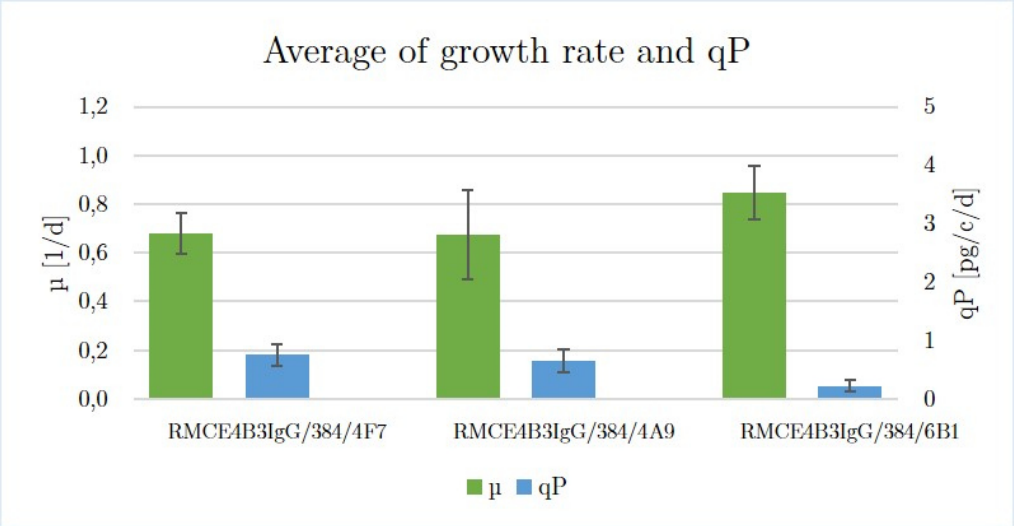


Figure 28: Average growth rate and specific productivity of RMCE4B3IgG/384/4F7, 4A9 and 6B1 over 8 passages. Error bars represent ± standard deviation.

The cell line RMCE4B3IgG/384/4F7 shows a maximum growth rate of 0.79 d^{-1} and an average of 0.68 d^{-1} (Figure 28). The maximum growth rate of RMCE4B3IgG/384/4A9 is 1.03 d^{-1} and the average growth rate results in 0.67 d^{-1}. The average growth rate of RMCE4B3IgG/384/6B1 is 0.85 d^{-1} and the highest reached growth rate is 0.97 d^{-1}. A comparison of the average of growth rate to the average of specific productivity is drawn in all three subclones. The cell line RMCE4B3IgG/384/4A9 has the lowest average of growth rate and a high average of specific productivity. RMCE4B3IgG/384/4F7 has very similar values to 4A9. The cell line RMCE4B3IgG/384/6B1 shows the highest average of growth rate, but the lowest average of specific productivity.

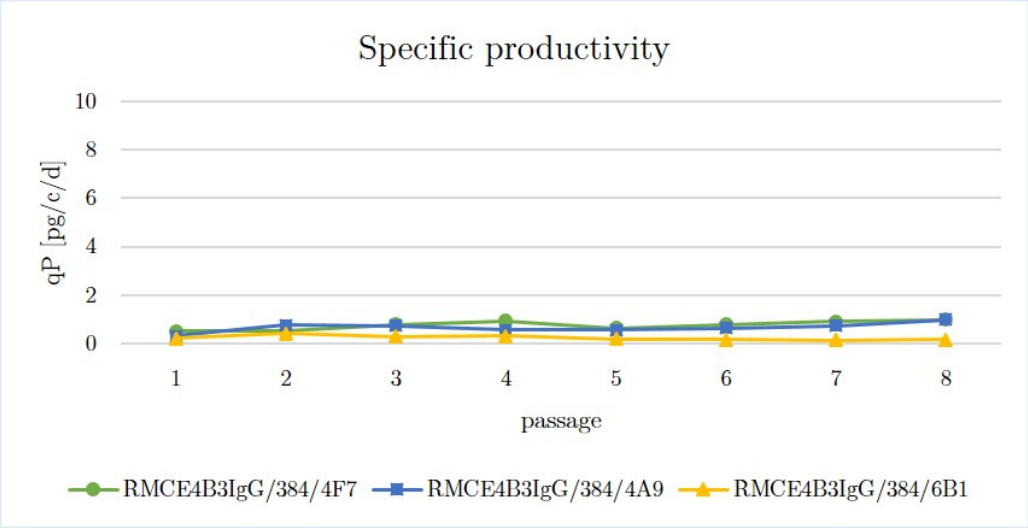


Figure 29: Specific productivity of RMCE4B3IgG/384/4F7, 4A9 and 6B1 over 8 passages

The specific productivity of RMCE4B3IgG/384/4F7 is in average 0.75 pg/c/d (Figure 29). The average qP of RMCE4B3IgG/384/4A9 amounts to 0.66 pg/c/d. The cell line RMCE4B3IgG/384/6B1 has a specific productivity of 0.23 pg/c/d in average.

5.5 Generation of product

The clones RMCE2G12IgG and RMCE353/11/IgG from transfection 1 were used for the product generation according to 4.2.8. The supernatant was harvested six times. Figure 30 shows all harvests of both cell lines. Harvest 2, 4 and 6 were done after three days. Harvest 1, 3 and 5 were performed after four days. The product titer of both cell lines is very similar after three days, after four days a bigger difference is shown. The titer is 7.5 µg/mL higher in RMCE2G12IgG than in RMCE353/11/IgG in harvest 1. In harvest 3 RMCE353/11/IgG produced 4.2 µg/mL more than RMCE2G12IgG.

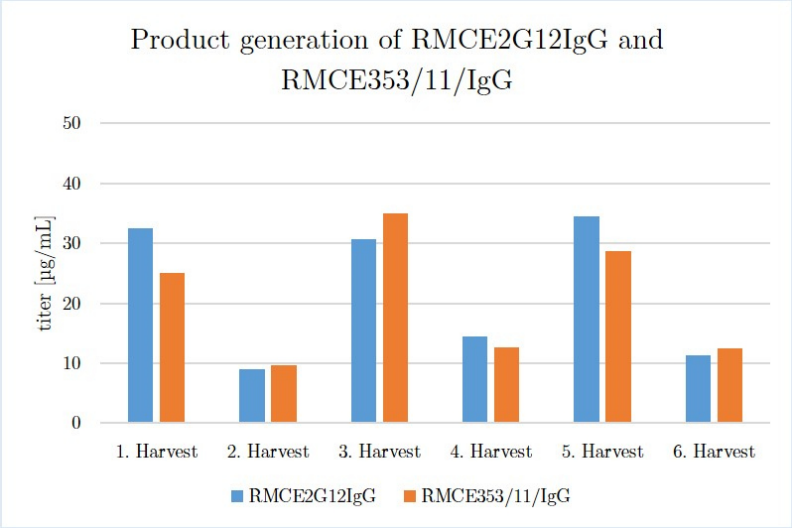


Figure 30: Product generation of RMCE2G12IgG and RMCE353/11/IgG

A staining of intracellular heavy and light chain of both cell lines on time point of the second harvest is shown in Figure 31. In the set gate, 87% of RMCE2G12IgG and 98% of RMCE353/11/IgG show higher intracellular heavy chain content compared to the RMCE host I3. The amount of light chain positive cells in gate "#1" is lower with 14% in RMCE2G12IgG and 31% in RMCE353/11/IgG. Overall, the geometric mean, median and mode values for RMCE2G12IgG is minimally lower for heavy chain or minimally higher for light chain product compared to RMCE353/11/IgG.

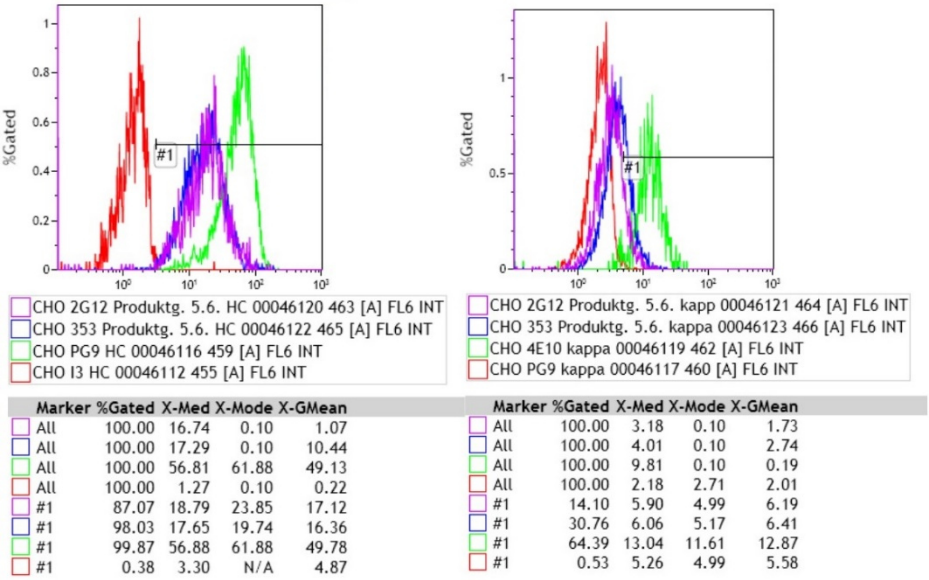


Figure 31: Staining of intracellular heavy and light chain of RMCE2G12IgG and RMCE353/11/IgG at 2nd harvest of product generation. HC positive control: CHO PG9,

HC negative control: CHO RMCE I3, LC kappa positive control: CHO 4E10, LC kappa negative control: CHO PG9

The three subclones of RMCE554/12/IgG of transfection 1 were passaged for product generation according to 4.2.8. Each subclone was used in one 250 mL production shaker, but in the end all supernatants were pooled. RMCE554/12/IgG/384/3A11 has the highest titer in all three harvests (Figure 32).

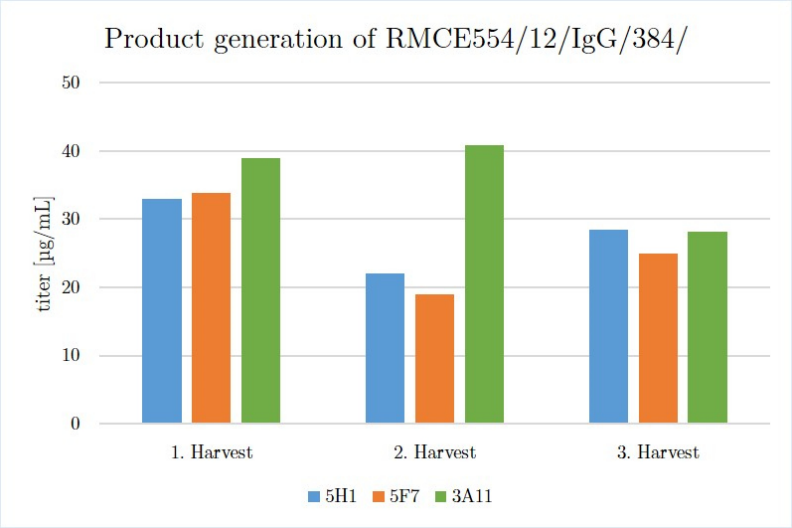


Figure 32: Product generation of RMCE554/12/IgG/384/5H1, RMCE554/12/IgG/384/5F7 and RMCE554/12/IgG/384/3A11

The product generation according to 4.2.8 with three subclones of RMCE4B3IgG from transfection 1 is shown in Figure 33. The highest titer is 12.4 µg/mL which is reached at the first harvest of RMCE4B3IgG/384/4A9.

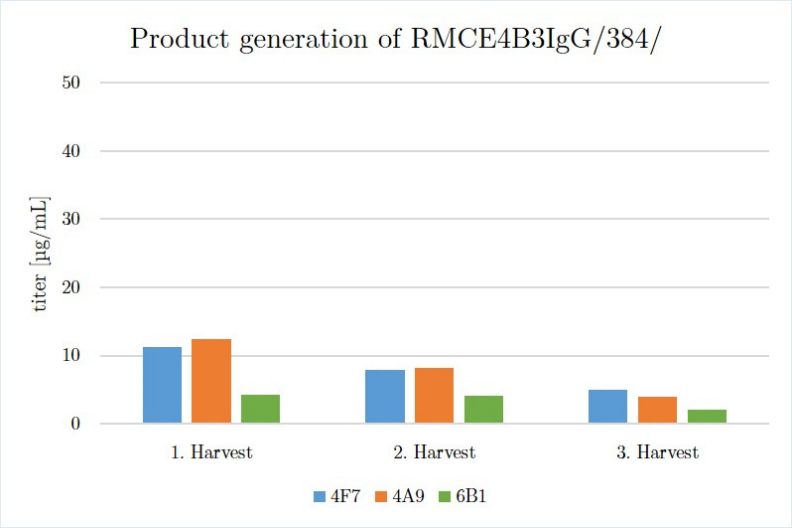


Figure 33: Product generation of RMCE4B3IgG/384/4F7, RMCE4B3IgG/384/4A9 and RMCE4B3IgG/384/6B1

5.6 Batch experiments

Batch experiment 1

Cell line:	RMCE2G12IgG and RMCE353/11/IgG (cell pools from transfection 1)
Seeding density:	$1 \cdot 10^6$ cells/mL
Medium:	batch medium 1 (CD-CHO) and batch medium 2 (MV3-2/6)
Vessel:	20 mL/125 mL shaker flasks

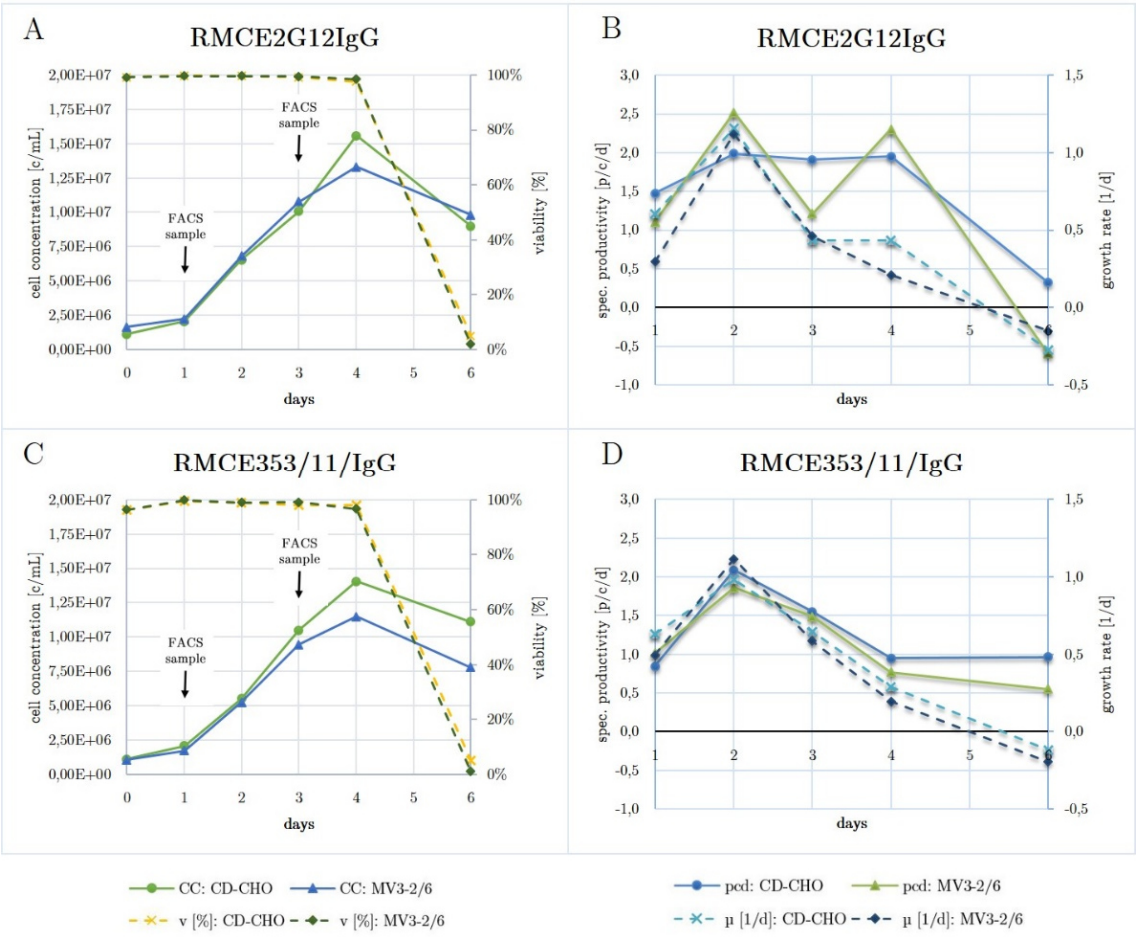


Figure 34: Results batch experiment 1: cell concentration, viability, specific productivity and growth rate

The maximum of the total cell concentration is reached on day 4 with $1.56 \cdot 10^7$ c/mL in batch medium 1 and $1.33 \cdot 10^7$ c/mL in batch medium 2 with RMCE2G12IgG (Figure 34, A). RMCE2G12IgG shows in both batch media very similar cell densities and therefore the lower cell density of $1.33 \cdot 10^7$ c/mL in batch medium 2 seems like an outlier. With the cell line RMCE353/11/IgG the maximum cell concentration is reached on day 4 as well. The total cell concentrations amount to $1.41 \cdot 10^7$ c/mL in batch medium 1 and $1.15 \cdot 10^7$ c/mL in batch medium 2 (Figure 34, C). The viability on day 4 of RMCE2G12IgG is 97.6% in batch medium 1 and 98.44 in batch medium 2. The amount of viable cells of RMCE353/11/IgG is 98.11% in batch medium 1 on day 4 and 96.68% in batch medium 2. However, since samples were not taken at day 5 we cannot conclude that maximal cell density was already reached at day 4. The specific productivity of RMCE2G12IgG until day 4 is between 1.48 and 1.99 pg/c/d in batch medium 1 and between 1.10 and 2.52 pg/c/d in batch medium 2 (Figure

34, B). The cell line RMCE353/11/IgG reaches a specific productivity between 0.85 and 2.09 pg/c/d in batch medium 1 and between 0.77 and 1.86 pg/c/d in batch medium 2 until day 4 (Figure 34, D). The growth rate in batch medium 1 has its maximum at 1.16 d^{-1} with RMCE2G12IgG and 0.98 d^{-1} with RMCE353/11/IgG. Batch medium 2 show a maximum growth rate of 1.12 d^{-1} with both cell lines.

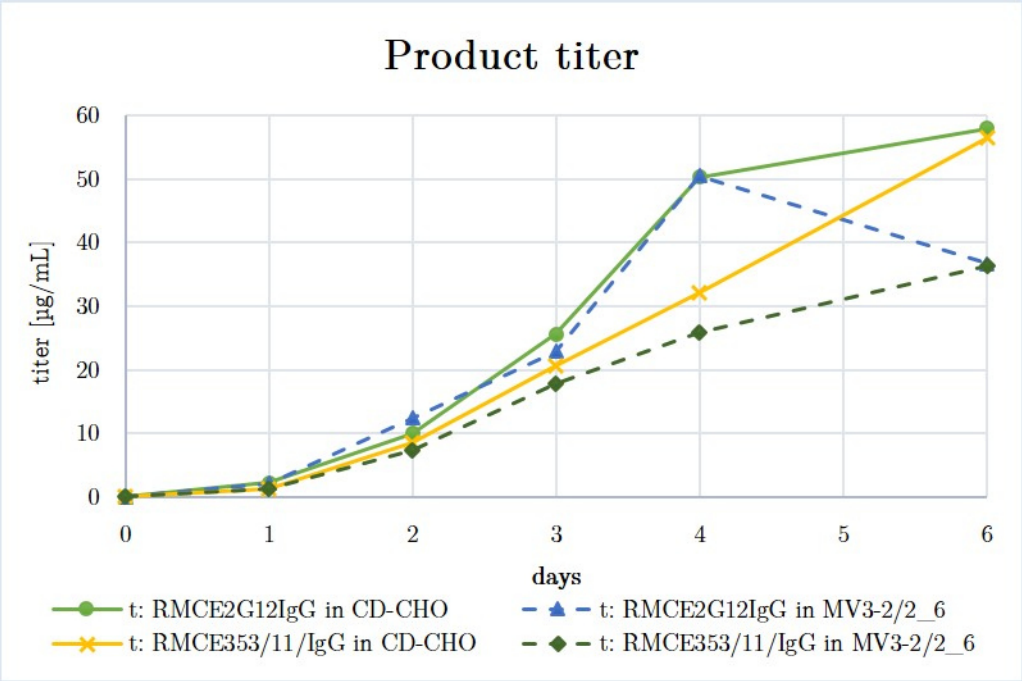
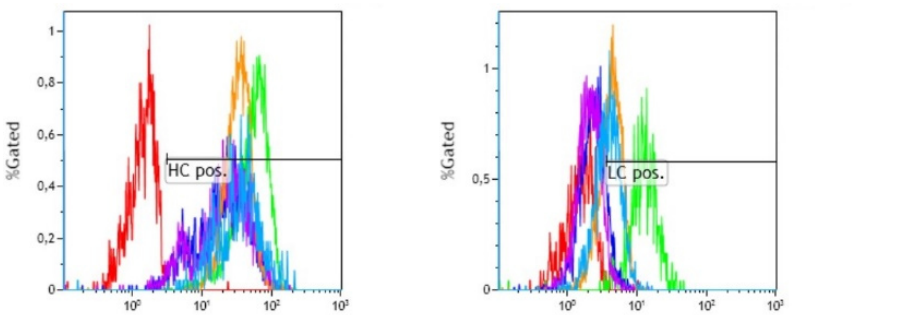


Figure 35: Product titer batch experiment 1

The maximum product titer of batch medium 1 is reached on day 6 with 57.97 µg/mL in RMCE2G12IgG and 56.39 µg/mL in RMCE353/11/IgG (Figure 35). The end titer of batch medium 2 is 36.69 µg/mL in RMCE2G12IgG and 36.39 µg/mL in RMCE353/11/IgG on day 6.

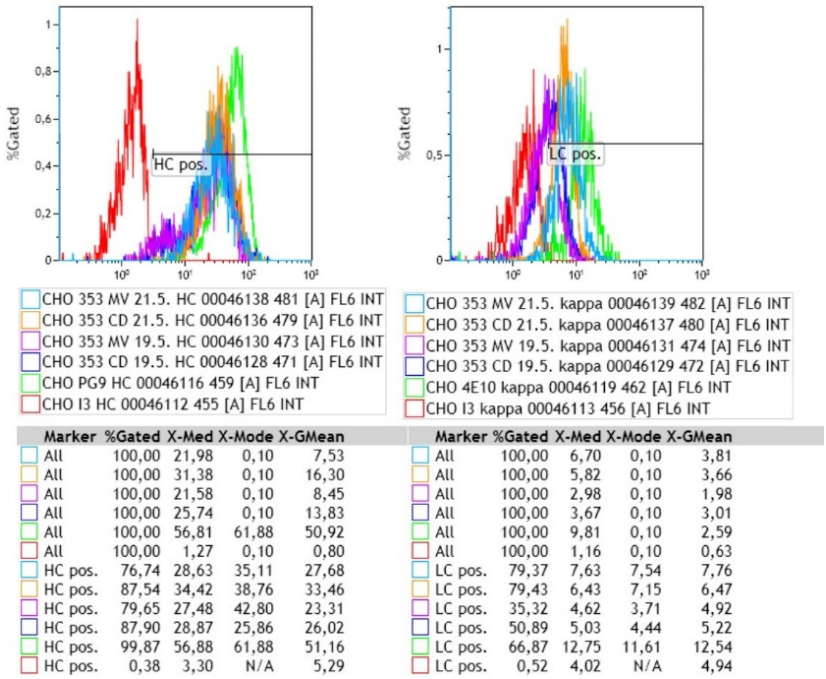


Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	25,09	0,10	6,43
All	100,00	33,72	0,10	27,76
All	100,00	20,31	0,10	11,03
All	100,00	19,04	0,10	10,97
All	100,00	56,81	61,88	50,92
All	100,00	1,27	0,10	0,80
HC pos.	71,64	35,75	45,58	33,09
HC pos.	96,89	34,32	39,82	33,22
HC pos.	86,80	23,40	20,10	20,98
HC pos.	87,23	22,61	23,22	20,26
HC pos.	99,87	56,88	61,88	51,16
HC pos.	0,38	3,30	N/A	5,29

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	3,65	0,10	2,57
All	100,00	4,27	0,10	3,55
All	100,00	2,04	0,10	1,69
All	100,00	2,33	0,10	1,99
All	100,00	9,81	0,10	2,59
All	100,00	1,16	0,10	0,63
LC pos.	50,74	4,90	3,98	5,07
LC pos.	66,02	4,97	4,48	5,09
LC pos.	5,96	4,15	3,84	4,47
LC pos.	12,71	4,17	3,64	4,40
LC pos.	66,87	12,75	11,61	12,54
LC pos.	0,52	4,02	N/A	4,94

Figure 36: Batch experiment 1 - Staining of intracellular heavy and light chain of RMCE2G12IgG. Negative control HC: CHO RMCE I3, positive control HC: CHO PG9, negative control kappa LC: CHO RMCE I3, positive control kappa LC: CHO 4E10

The cell line CHO PG9 serves as positive control and CHO RMCE I3 as negative control for intracellular product content. The FACS samples were taken on the first and the third day of batch experiment 1. The cell line RMCE2G12IgG in batch medium 1 is intracellular heavy chain positive to 87.23% on day 1 and 96.89% on day 3. In batch medium 2 the population consists of 86.80% producers on day 1 and 71.64% on day 3 (Figure 36). It can be seen that the peaks of intracellular heavy chain are very broad, meaning that it is a heterogeneous population. A second, smaller peak indicates a subpopulation. The FL1 laser channel of this samples shows that the subpopulations are GFP negative (not shown). For batch experiment 1 the cell pool from transfection 1 were taken.



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	21,98	0,10	7,53
All	100,00	31,38	0,10	16,30
All	100,00	21,58	0,10	8,45
All	100,00	25,74	0,10	13,83
All	100,00	56,81	61,88	50,92
All	100,00	1,27	0,10	0,80
HC pos.	76,74	28,63	35,11	27,68
HC pos.	87,54	34,42	38,76	33,46
HC pos.	79,65	27,48	42,80	23,31
HC pos.	87,90	28,87	25,86	26,02
HC pos.	99,87	56,88	61,88	51,16
HC pos.	0,38	3,30	N/A	5,29

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	6,70	0,10	3,81
All	100,00	5,82	0,10	3,66
All	100,00	2,98	0,10	1,98
All	100,00	3,67	0,10	3,01
All	100,00	9,81	0,10	2,59
All	100,00	1,16	0,10	0,63
LC pos.	79,37	7,63	7,54	7,76
LC pos.	79,43	6,43	7,15	6,47
LC pos.	35,32	4,62	3,71	4,92
LC pos.	50,89	5,03	4,44	5,22
LC pos.	66,87	12,75	11,61	12,54
LC pos.	0,52	4,02	N/A	4,94

Figure 37: Batch experiment 1 - Staining of intracellular heavy and light chain of RMCE353/11/IgG. Negative control HC: CHO RMCE I3, positive control HC: CHO PG9, negative control kappa LC: CHO RMCE I3, positive control kappa LC: CHO 4E10

The controls for the FACS analyses of RMCE353/11/IgG and the sampling period are identical to the analyses of RMCE2G12IgG (Figure 36). The population of RMCE353/11/IgG shows 87.9% of heavy chain producers on day 1 and 87.54% on day 3 in batch medium 1. In batch medium 2 79.65% of the population consist of heavy chain positive cells on day 1 and 76.74% on day 3 (Figure 37). It is shown that the peaks of intracellular heavy chain are very broad and form a second peak which indicates a subpopulation. The cell pool is heterogeneous and there is a need of subcloning (subcloning 1). The FL1 laser channel of this samples shows that the subpopulations are GFP negative (not shown).

Batch experiment 2

Cell line:	RMCE2G12IgG/384/1B3, RMCE2G12IgG/384/1B11, RMCE2G12IgG/384/1E4, RMCE353/11/IgG/384/2A12, RMCE353/11/IgG/384/2C7 and RMCE353/11/IgG/384/2D7
Seeding density:	$2 \cdot 10^6$ cells/mL
Medium:	batch medium 3

Vessel: 10 mL/50 mL reactor tubes

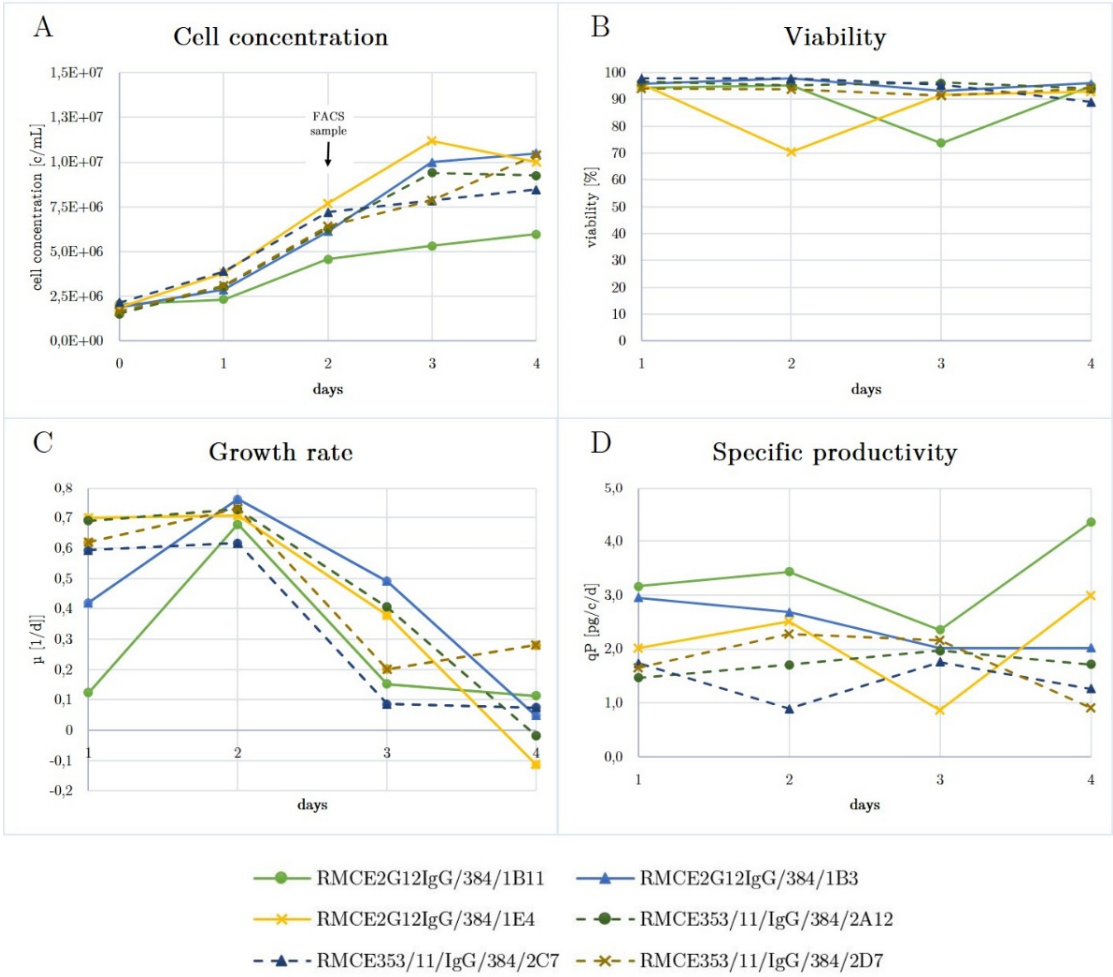


Figure 38: Batch experiment 2 - cell concentrations, viability, growth rate and specific productivity

The three RMCE2G12IgG subclones show cell concentrations of $5.96 \cdot 10^6$ c/mL in 1B11, $1.05 \cdot 10^7$ c/mL in 1B3 and $1.00 \cdot 10^7$ c/mL in 1E4 at day 4. The cell concentrations of the three RMCE353/11/IgG subclones on day 4 amount to $9.25 \cdot 10^6$ c/mL in 2A12, $8.46 \cdot 10^6$ c/mL in 2C7 and $1.04 \cdot 10^7$ c/mL in 2D7 (Figure 38, A). The viability of all six cell lines did not drop below 90% (Figure 38, B). The growth rate of RMCE2G12IgG is the highest on day 2 with 0.68 d^{-1} in 1B11, 0.76 d^{-1} in 1B3 and 0.71 d^{-1} in 1E4 (Figure 38, C). On day 2 the growth rate of the RMCE353/11/IgG subclones was 0.73 d^{-1} in 2A12, 0.62 d^{-1} in 2C7 and 0.73 d^{-1} in 2D7. The value of the specific productivity of RMCE2G12IgG on day 4 is 4.35 pg/c/day in the subclone 1B11, 2.02 pg/c/d in 1B3 and 2.98 pg/c/day in 1E4. RMCE353/11/IgG shows a specific productivity of 1.71 pg/c/day in 2A12, 1.26 pg/c/day in 2C7 and 0.90 pg/c/d in 2D7 on day 4 (Figure 38, D).

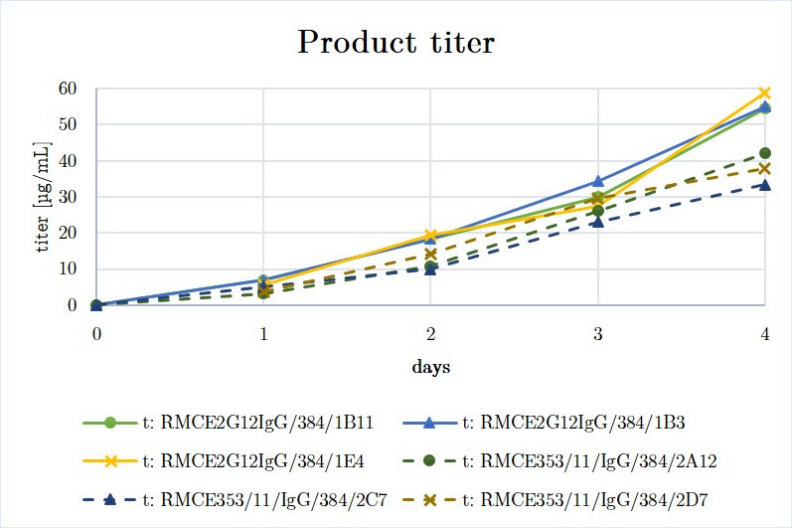
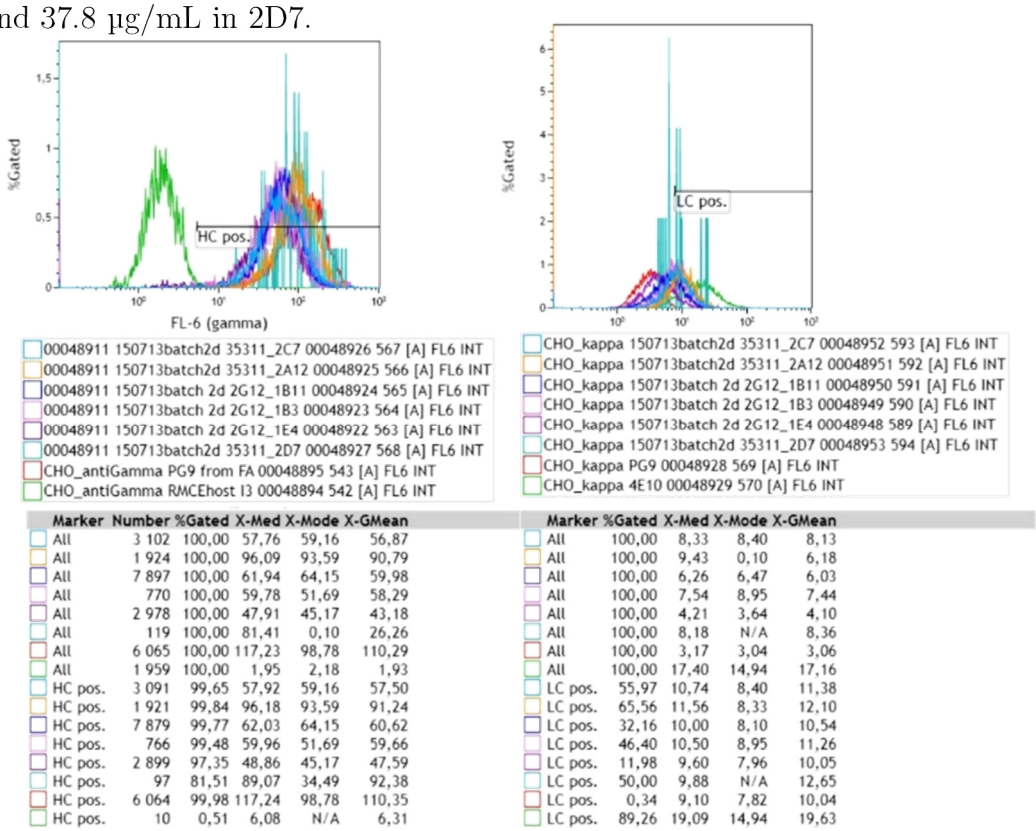


Figure 39: Product titers of batch experiment 2

The product titer of RMCE2G12IgG is 54.4 µg/mL in 1B11, 55.0 µg/mL in 1B3 and 58.9 µg/mL in 1E4 on day 4 (Figure 39). The maximum titers of the three subclones of RMCE353/11/IgG are 42.0 µg/mL in 2A12, 33.4 µg/mL in 2C7 and 37.8 µg/mL in 2D7.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	3 102	100,00	57,76	59,16	56,87
All	1 924	100,00	96,09	93,59	90,79
All	7 897	100,00	61,94	64,15	59,98
All	770	100,00	59,78	51,69	58,29
All	2 978	100,00	47,91	45,17	43,18
All	119	100,00	81,41	0,10	26,26
All	6 065	100,00	117,23	98,78	110,29
All	1 959	100,00	1,95	2,18	1,93
HC pos.	3 091	99,65	57,92	59,16	57,50
HC pos.	1 921	99,84	96,18	93,59	91,24
HC pos.	7 879	99,77	62,03	64,15	60,62
HC pos.	766	99,48	59,96	51,69	59,66
HC pos.	2 899	97,35	48,86	45,17	47,59
HC pos.	97	81,51	89,07	34,49	92,38
HC pos.	6 064	99,98	117,24	98,78	110,35
HC pos.	10	0,51	6,08	N/A	6,31

Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	8,33	8,40	8,13
All	100,00	9,43	0,10	6,18
All	100,00	6,26	6,47	6,03
All	100,00	7,54	8,95	7,44
All	100,00	4,21	3,64	4,10
All	100,00	8,18	N/A	8,36
All	100,00	3,17	3,04	3,06
All	100,00	17,40	14,94	17,16
LC pos.	55,97	10,74	8,40	11,38
LC pos.	65,56	11,56	8,33	12,10
LC pos.	32,16	10,00	8,10	10,54
LC pos.	46,40	10,50	8,95	11,26
LC pos.	11,98	9,60	7,96	10,05
LC pos.	50,00	9,88	N/A	12,65
LC pos.	0,34	9,10	7,82	10,04
LC pos.	89,26	19,09	14,94	19,63

Figure 40: FACS results of batch experiment 2 - heavy and light chain. HC negative control: CHO RMCE I3, HC positive control: CHO PG9, LC kappa negative control: CHO PG9, LC kappa positive control: CHO 4E10

As controls served the cell lines CHO RMCE I3 and CHO PG9 in the heavy chain analysis. In the light chain analysis the controls CHO PG9 and CHO 4E10 were chosen. The FACS samples were fixed on day 2 of batch experiment 2. The number of counted events was lower than 10000. The cell lines RMCE2G12IgG/384/1B11, RMCE2G12IgG/384/1B3, RMCE353/11/IgG/384/2C7 and RMCE353/11/IgG/384/2A12 show a population of over 99% of heavy chain producers. The population of RMCE2G12IgG/384/1E4 consists of 97.35% of heavy chain producers and the population of RMCE353/11/IgG/384/2D7 of 81.51% producers (Figure 40). The light chain analysis shows that RMCE353/11/IgG/384/2C7, 2A12 and 2D7 have 50% or more light chain positive cells. RMCE2G12IgG/384/1B3, 1B11 and 1E4 are below 47% of light chain positive cells.

Batch experiment 3

Cell line:	RMCEUsteIgG (transfection 2)
Seeding density:	$1.5 \cdot 10^6$ cells/mL
Medium:	batch medium 3
Vessel:	20 mL/125 mL shaker flask (140 rpm)
	10 mL/50 mL reaction tube (220 rpm)

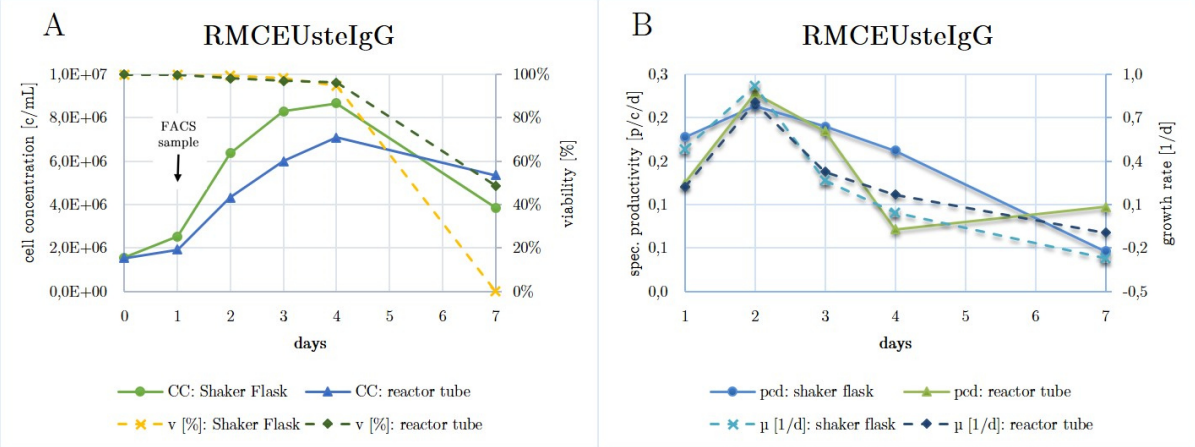


Figure 41: Batch experiment 3 - cell concentration and viability (A), specific productivity and growth rate (B)

The viability in shaker flask is 94.7% and in the reactor tube 96.3% on day 4 (Figure 41, A). The difference increases on day 7 where the viability in the shaker flask drops to 0% and the viable cell population in the reactor tube is at 48.7%. The total cell concentration was higher in the shaker flask up to day 4.

On day 7 the cell concentration in the reactor tube is $5.35 \cdot 10^6$ c/mL and in the shaker flask it is decreased to $3.85 \cdot 10^6$ c/mL. The specific productivity decreases in both vessels during the process. The highest specific productivity was measured on day 2. In the shaker flask it amounts to 0.21 pg/c/d and in the reactor tube the specific productivity is 0.23 pg/c/d (Figure 41, B). The specific growth rate is 0.48 d^{-1} on day 1 and 0.92 d^{-1} on day 2 in the shaker flask. It decreases on day 3 to 0.26 d^{-1} and on day 4 to 0.04 d^{-1}. In the reactor tube the specific growth rate on day 1 is 0.22 d^{-1} and on day 2 0.81 d^{-1}. On day 3 and 4 a decrease to 0.33 d^{-1} and 0.17 d^{-1} was recorded in the reactor tube.

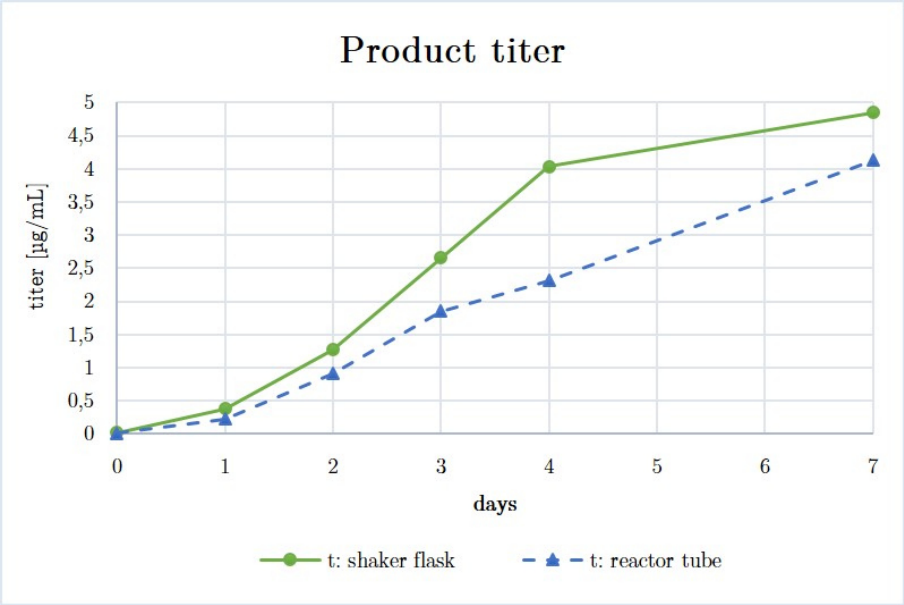
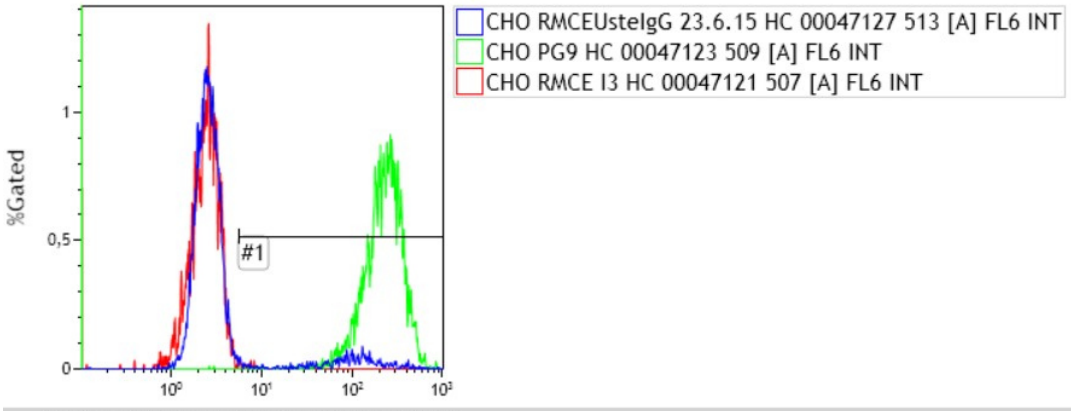


Figure 42: Batch experiment 3 - product titer

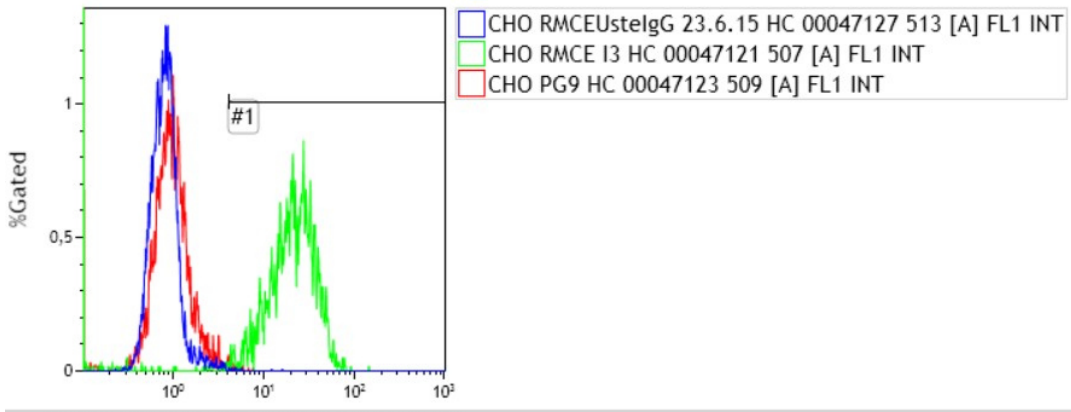
The product titer of RMCEUsteIgG in the shaker flask rises faster than in the reactor tube (Figure 42). The difference is the highest at day 4 with 4.03 µg/mL in the shaker flask and 2.32 µg/mL in the reactor tube. The end titer of batch experiment 3 amounts to 4.85 µg/mL in the shaker flask and 4.13 µg/mL in the reactor tube.



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	2,61	2,36	3,43
All	100,00	226,41	0,10	173,56
All	100,00	2,39	0,10	1,95
#1	9,13	98,12	99,67	79,73
#1	96,95	230,72	275,41	218,80
#1	0,50	7,02	N/A	6,85

Figure 43: FACS results of batch experiment 3: intracellular heavy chain. Negative control HC: CHO RMCE I3, positive control HC: CHO PG9

The cell line CHO PG9, which is an antibody producer, functions as positive control. The cell line CHO RMCE I3 serves as negative control. The sample RMCEUsteIgG was analyzed from day 1 of batch experiment 3. The sample was taken from the shaker flask. The gate is set at 0.5% of the negative control. 9.13% of the RMCEUsteIgG population are heavy chain positive (Figure 43).



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100,00	0,80	0,83	0,80
All	100,00	20,72	0,10	14,94
All	100,00	0,94	0,10	0,92
#1	0,21	5,08	N/A	5,87
#1	93,03	21,60	27,79	20,74
#1	0,49	4,85	4,40	5,12

Figure 44: FACS results of batch experiment 3: GFP expression. Negative control GFP: CHO PG9, positive control GFP: CHO RMCE I3

The FACS analyses of the intracellular GFP shows that 0.21% of the RMCEUsteIgG population is GFP positive (Figure 44). This is less than the negative control with 0.49% of CHO PG9 cells. The GFP positive CHO RMCE I3 cells have 93.03% of the population in gate#1.

5.7 Polymerase Chain Reaction

5.7.1 Isolation of genomic DNA

The isolation of the genomic DNA of RMCE2G12IgG/384/1E4, RMCE2G12IgG/384/1B3 and RMCE2G12IgG/384/1B11 was done. Additionally the genomic DNA was isolated from RMCE353/11/IgG/384/2A12, RMCE353/11/IgG/384/2C7, RMCE353/11/IgG/384/2D7 and CHO RMCE I3. The amount of isolated gDNA of $2 \cdot 10^6$ cells was determined with A260 measurement. The resulting concentrations are shown in Table 4.

Table 4: Isolation of genomic DNA of CHO RMCE I3, RMCE2G12IgG/384/subclones and RMCE353/11/IgG/384/subclones

Cell line	gDNA [ng/mL]	A260/280
CHO RMCE I3	73	2.06
RMCE2G12IgG/384/1E4	88	2.03
RMCE2G12IgG/384/1B3	72	2.03
RMCE2G12IgG/384/1B11	92	2.02
RMCE353/11/IgG/384/2A12	95	2.09
RMCE353/11/IgG/384/2C7	86	2.04
RMCE353/11/IgG/384/2D7	122	2.08

5.7.2 Taq-PCR

PCR for parental integration

As primers for the parental fusion protein CAGGS_s and GFP_as were used. CAGGS_s binds the CAGGS promoter before the FRT3 site and GFP_as binds to eGFP in the GTN fusion protein. The resulting fragment is 697 bp long. The primers were used to screen if the GTN fusion protein is still present in the cell population.

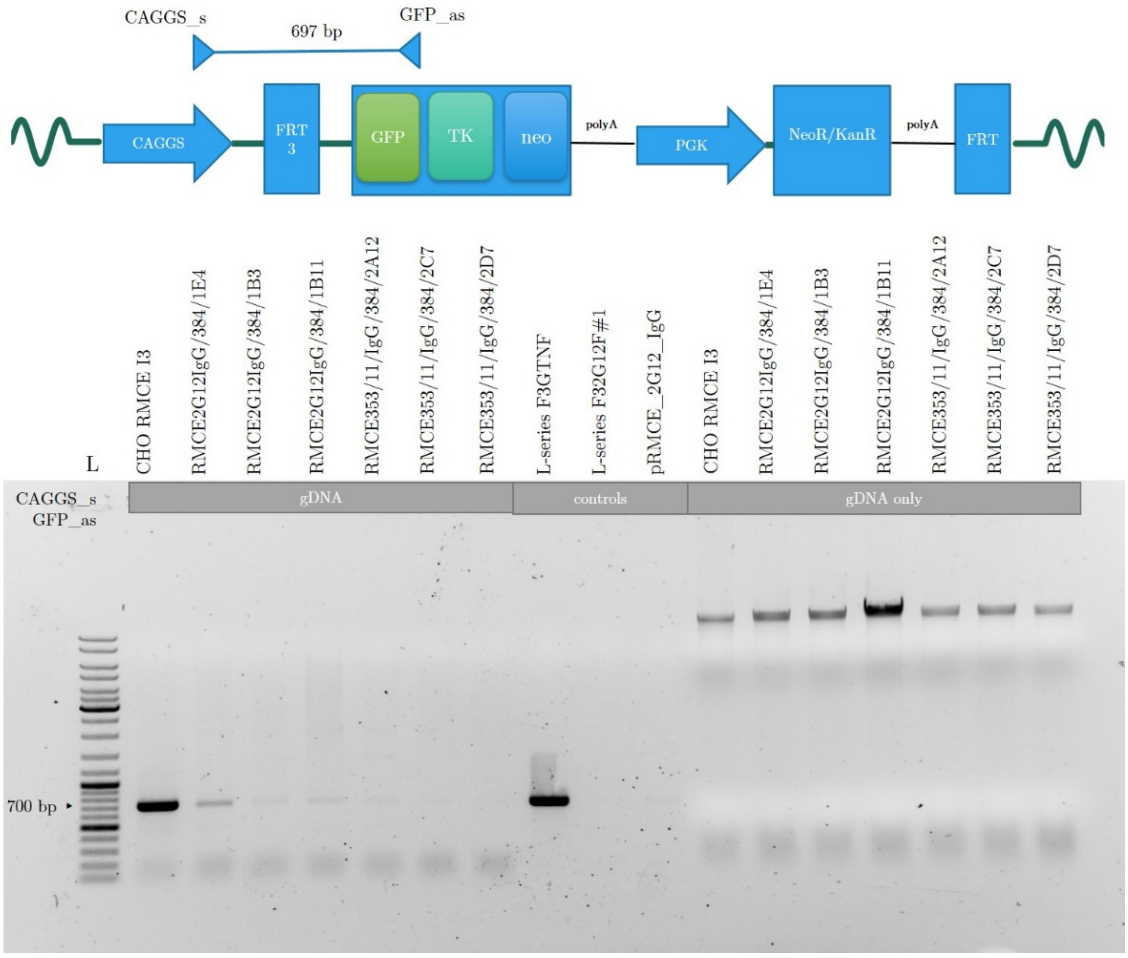
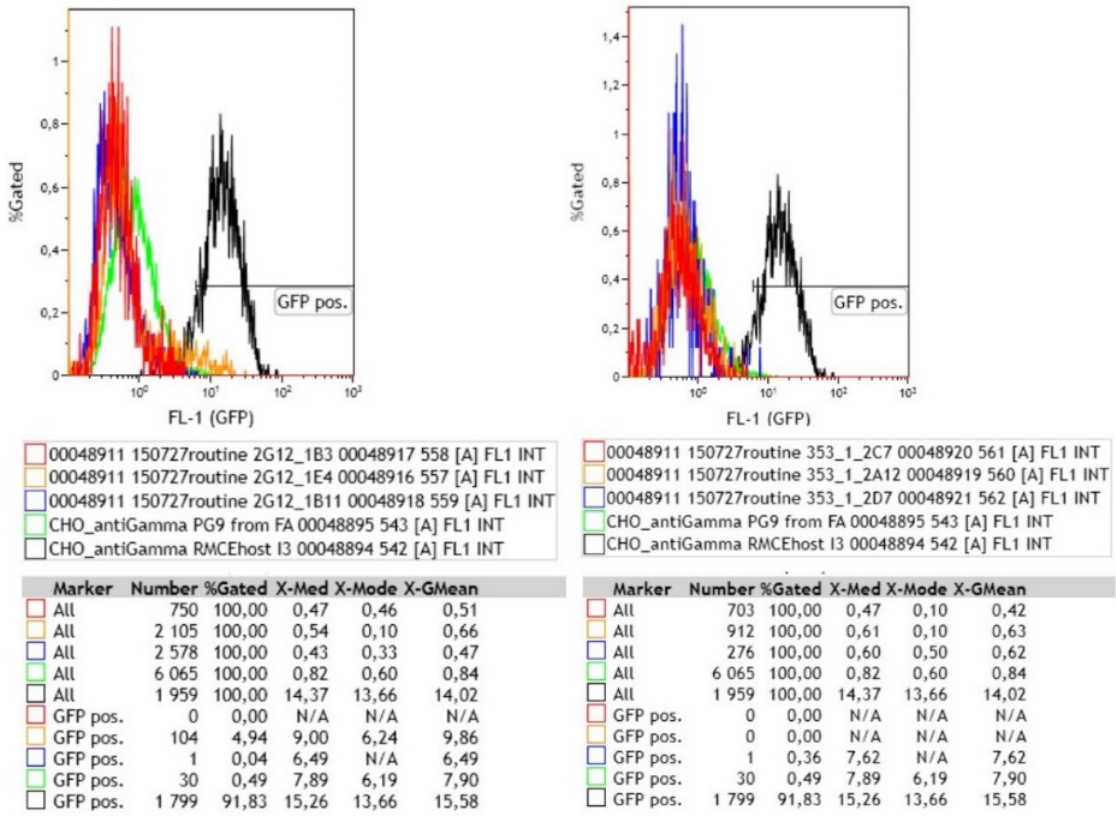


Figure 45: PCR with primers for the GTN fusion protein of RMCE2G12IgG/384 clones and RMCE353/11/IgG/384 clones on 1% agarose gel at the PCR conditions: 95/10', 40 cycles (95/30, 55/30, 73/45), 73/5'

In Figure 45 a PCR with the genomic DNA of all six clones, the host cell line and three controls was performed. There are bands visible at the parental positive control "L-series F3GTNF" and the host cell line CHO RMCE I3. There is a very light band at the PCR samples of RMCE2G12IgG/384/1E4, RMCE2G12IgG/384/1B3 and RMCE2G12IgG/384/1B11. The last seven bands are from samples without PCR. They show that there has been functional template DNA in sufficient amounts in the PCR samples. In addition the integrity of the gDNA is shown.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	750	100,00	0,47	0,46	0,51
All	2 105	100,00	0,54	0,10	0,66
All	2 578	100,00	0,43	0,33	0,47
All	6 065	100,00	0,82	0,60	0,84
All	1 959	100,00	14,37	13,66	14,02
GFP pos.	0	0,00	N/A	N/A	N/A
GFP pos.	104	4,94	9,00	6,24	9,86
GFP pos.	1	0,04	6,49	N/A	6,49
GFP pos.	30	0,49	7,89	6,19	7,90
GFP pos.	1 799	91,83	15,26	13,66	15,58

Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	703	100,00	0,47	0,10	0,42
All	912	100,00	0,61	0,10	0,63
All	276	100,00	0,60	0,50	0,62
All	6 065	100,00	0,82	0,60	0,84
All	1 959	100,00	14,37	13,66	14,02
GFP pos.	0	0,00	N/A	N/A	N/A
GFP pos.	0	0,00	N/A	N/A	N/A
GFP pos.	1	0,36	7,62	N/A	7,62
GFP pos.	30	0,49	7,89	6,19	7,90
GFP pos.	1 799	91,83	15,26	13,66	15,58

Figure 46: FL1 laser channel of FACS analysis of RMCE2G12IgG/384/1B3, 1E4 and 1B11 and of RMCE353/11/IgG/384/2C7, 2A12 and 2D7 fixed in EtOH. Negative control GFP: CHO PG9, positive control GFP: CHO RMCE I3

The population of RMCE2G12IgG/384/1E4 has an amount of 4.94% GFP positive cells also visible as a smaller second peak (Figure 46). The remaining RMCE2G12IgG/384 subclones show an amount of below 0.05% of GFP-positive cells in their populations. All three subclones of RMCE353/11/IgG/384 are below 0.4% of GFP-positive cells.

PCR for targeted integration

As primers for the antibody RMCE integration CAGGS_s and SV40pA_as were used. CAGGS_s binds the CAGGS promoter in 5' of the FRT3 site and SV40pA_as binds to the polyA sequence of SV40 in the antibody cassette. The resulting fragment is 1284 bp long. The primers were used to screen if the GTN fusion protein is exchanged with the antibody donor plasmid.

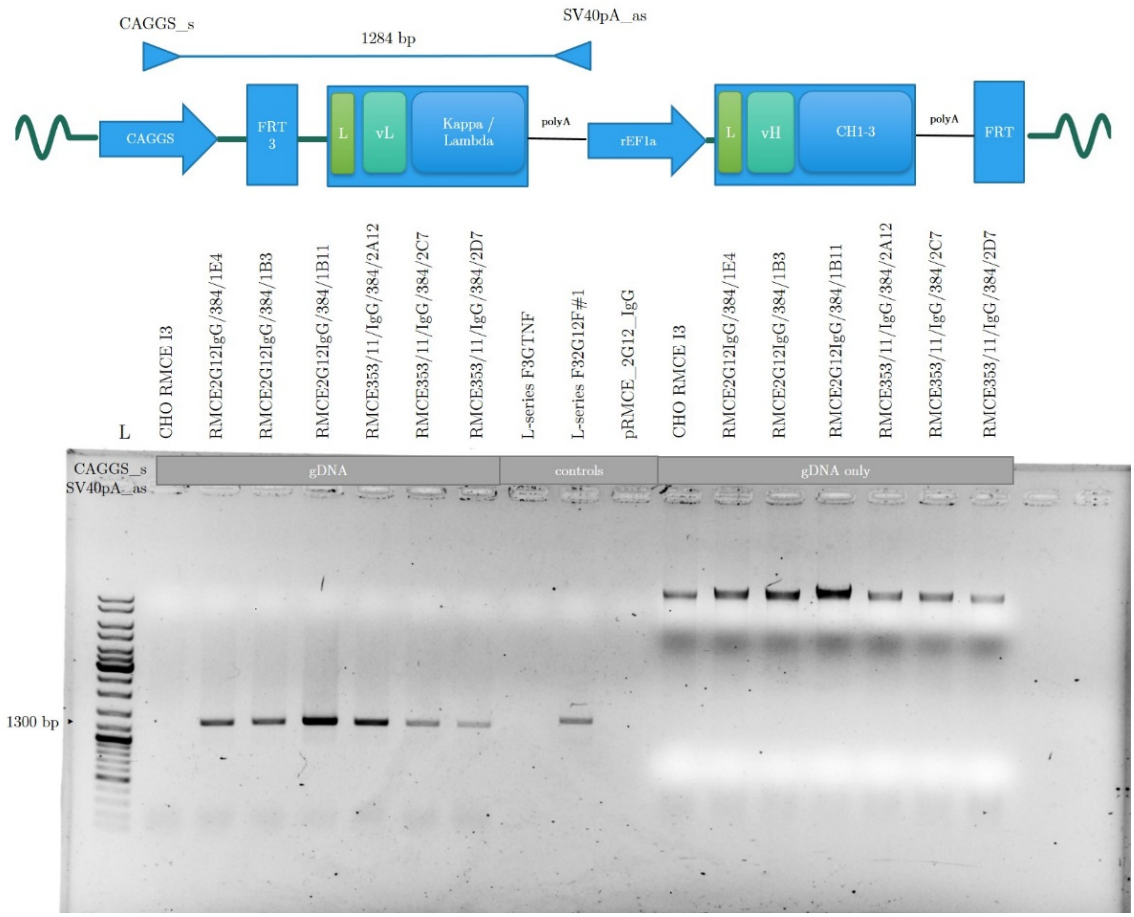


Figure 47: PCR for targeted antibody integration of RMCE2G12IgG/384 clones and RMCE353/11/IgG/384 clones on 1% agarose gel at the PCR conditions: 95/10', 40 cycles (95/30, 55/30, 73/80), 73/5'

In Figure 47 a PCR with the genomic DNA of all six clones, the host cell line and three plasmid controls was done. All PCR samples show a band at ~1300 bp, except the gDNA of CHO RMCE I3. The positive control "L-series F32G12F#1" for targeted integration shows a specific band with the correct size. All seven gDNA-only samples demonstrate integrity of the used genomic DNA.

PCR for random integration

As primers for the random donor plasmid integration pMG433_FSHB_2465_as and SV40pA_as were used. pMG433_FSHB_2465_as binds the complementary DNA sequence of the donor plasmid 299 bp before the first FRT3 site and SV40pA_as binds to the polyA sequence of SV40 present in the antibody cassette. The resulting fragment is 1197 bp long. The primers were used to screen

any possible random integration of the antibody donor plasmid into the RMCE host genome.

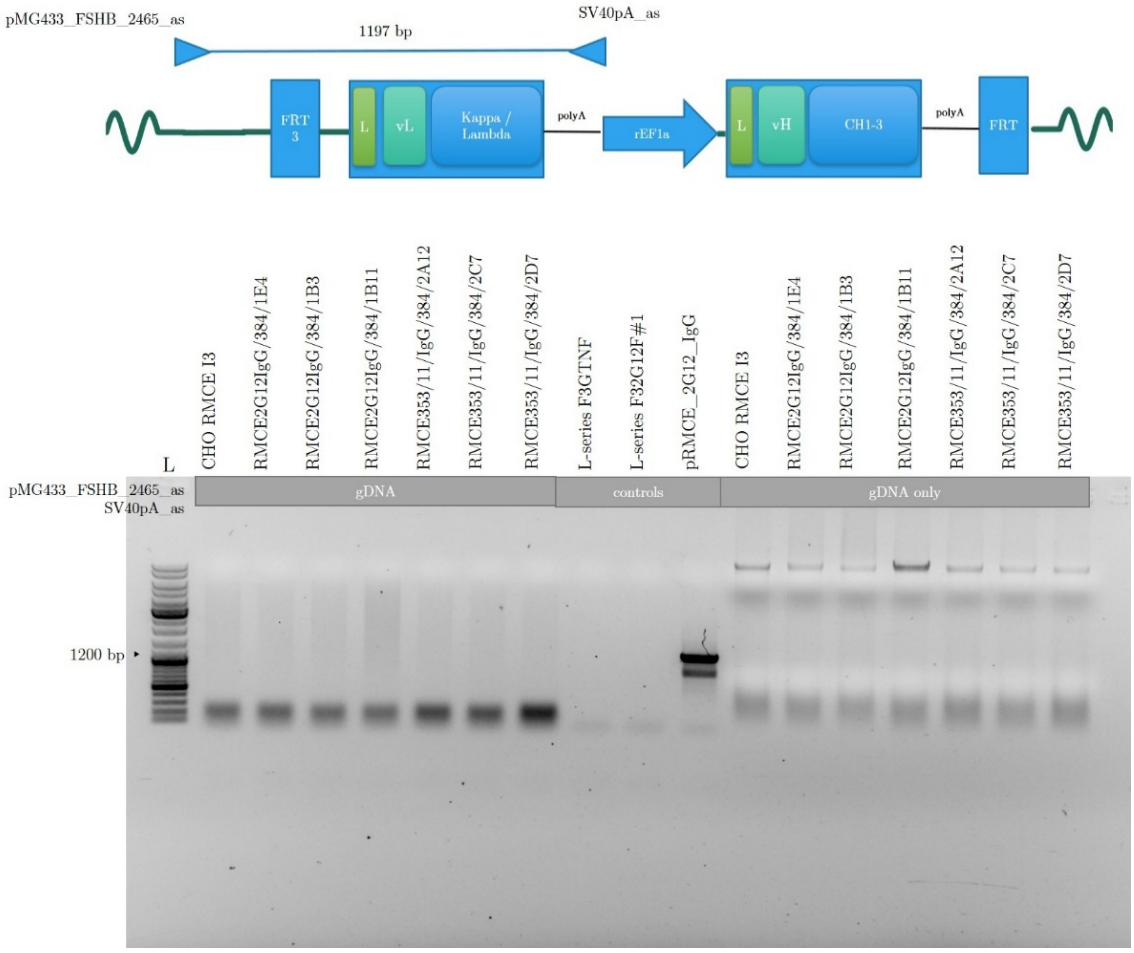


Figure 48: PCR for random integration of RMCE2G12IgG/384 clones and RMCE353/11/IgG/384 clones on 1% agarose gel at the PCR conditions: 95/10', 30 cycles (95/30, 50/30, 73/72), 73/5'

The PCR with the primers specific for random integration pMG433_FSHB_2465_as and SV40pA_as results in two bands at the positive control pRMCE_2G12_IgG. Genomic DNA (gDNA) only samples demonstrate presence of undegraded gDNA used for PCR reactions in correct concentrations (Figure 48). The positive control gave a band with the correct size together with a band of unspecific size.

5.8 IgG purification

Seven sections of the purification steps were collected and the antibody concentration was detected with biolayer interferometry (Octét). In cases of lower product concentrations, which were below the detection limit of biolayer interferometry (about 0.4 µg/mL), samples were analyzed with quantitative ELISA. In addition the volume of the collected fractions was determined to enable the calculation of the purification process.

Table 5: IgG purification of RMCE2G12IgG

RMCE2G12IgG	Volume [g=mL]	Octét [µg/mL]	Concentration with ELISA [µg/mL]	Total amount [mg]	Percent of total amount [%]
Culture supernatant	490.37	18.20	-	8.9	100.0
Concentrate	38.24	282.95	-	10.8	121.2
Permeate	667.14	<0.39	0.4	0.3	3.3
Flow through	37.71	0.42	-	0.0	0.2
Wash	8.31	<0.39	0.3	0.0	0.0
Eluate	3.13	1129.15	-	3.5	39.6
Dialyzed product	2.84	1174.45	-	3.3	**37.4**

The purification of RMCE2G12IgG supernatant is successful for 37.4% of the total amount of antibody (Table 5). 3.3% of the product remains in the permeate fraction, 0.2% ends up in the flow through and the wash fraction shows a product concentration of 0.3 µg/mL. The eluate includes 39.6% of the total product amount. After dialysis the purified product is 62.6% lower than in the culture supernatant.

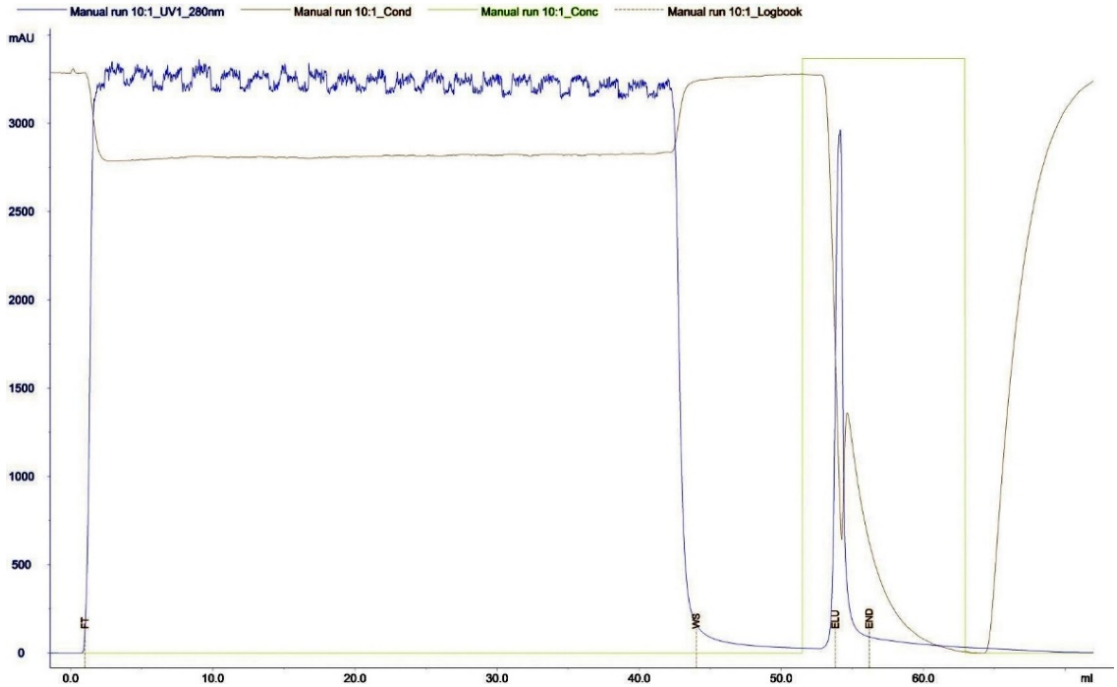


Figure 49: Chromatogram of RMCE2G12IgG purification

The affinity chromatography of RMCE2G12IgG is depicted in Figure 49. In this chromatogram milli absorption units are on the y-axis versus milliliter on the x-axis. The UV 280 nm measurement is pictured in blue. At start of the flow through, the UV 280 nm signal is rising because non-antibody protein compounds (e.g. host cell proteins) that cannot bind to the protein A column pass the detector. During the washing step, the signal decreases back to the baseline. The elution is started with 100% buffer B. Due to a lower salt concentration in buffer B, the conductivity decreases during elution. The conductivity is visualized in brown. The UV 280 nm signal has a clear peak at the elution of pure antibody product, which dropped to the baseline before buffer B gradient was set to 0. The conductivity signal again rises with the use of buffer A.

Table 6: IgG purification of RMCE353/11/IgG

RMCE353/11/IgG	Volume [g=mL]	Octét [µg/mL]	Concentration with ELISA [µg/mL]	Total amount [mg]	Percent of total amount [%]
Culture supernatant	467.29	21.45	-	10.0	100.0
Concentrate	34.03	301.5	-	10.3	102.4
Permeate	652.44	1.51	-	1.0	9.8

Flow through	43.55	<0.39	0.3	0.0	0.1
Wash	9.9	<0.39	0.1	0.0	0.0
Eluate	2.76	2281	-	6.3	62.8
Dialyzed product	2.7	1874.1	-	5.1	**50.5**

The purification of RMCE353/11/IgG is successful for 50.5% of the total amount of antibody (Table 6). The permeate fraction includes 9.8% of the product, 0.1% remains in the flow through fraction and the wash fraction collects 0.1 µg/mL. The eluate fraction results in 62.8% of antibody concentration. The product concentration is 49.5% reduced after the purification process.

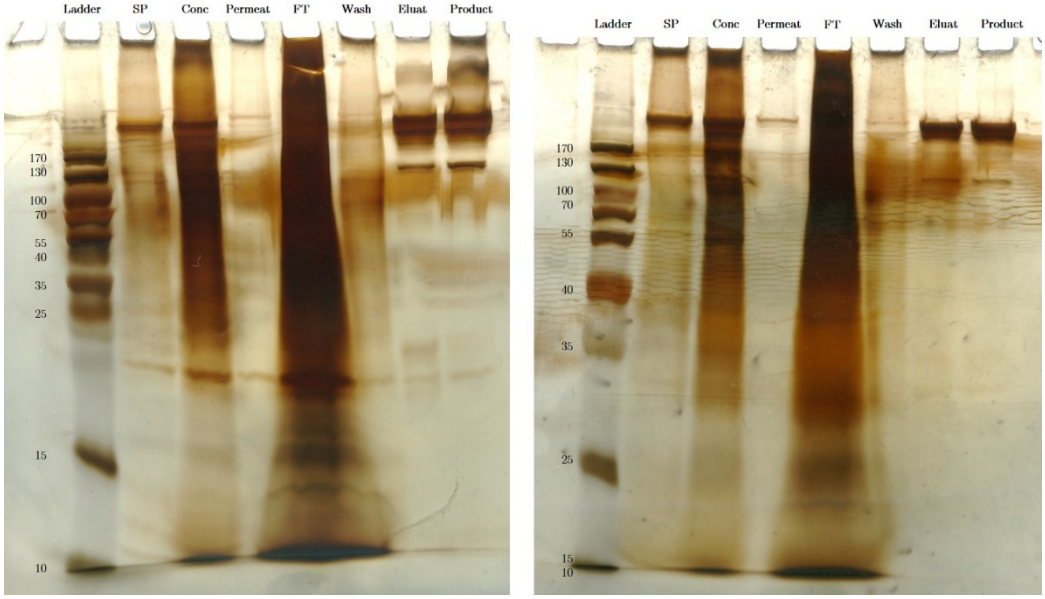


Figure 50: SDS-PAGE and silver staining of purification steps of RMCE2G12IgG (left) and RMCE353/11/IgG (right); SP…Supernatant, Conc…Concentrate, FT…Flow through

The collected fractions of the affinity chromatography were electrophoretically separated by denaturing non-reducing SDS-PAGE and subsequently analyzed by silver staining. The obtained fractions after chromatography of RMCE2G12IgG and RMCE353/11/IgG samples are shown in Figure 50. In both purification experiments the fractions concentrate and flow through show high amounts of impurities. A whole IgG antibody has a molecular weight of ~150 kDa. One heavy chain of an IgG has a molecular weight of 50 kDa and a light chain 25 kDa. There are three product bands visible in both purification experiments.

6 Discussion

The flowchart in Figure 51 provides an overview of the entire contents.

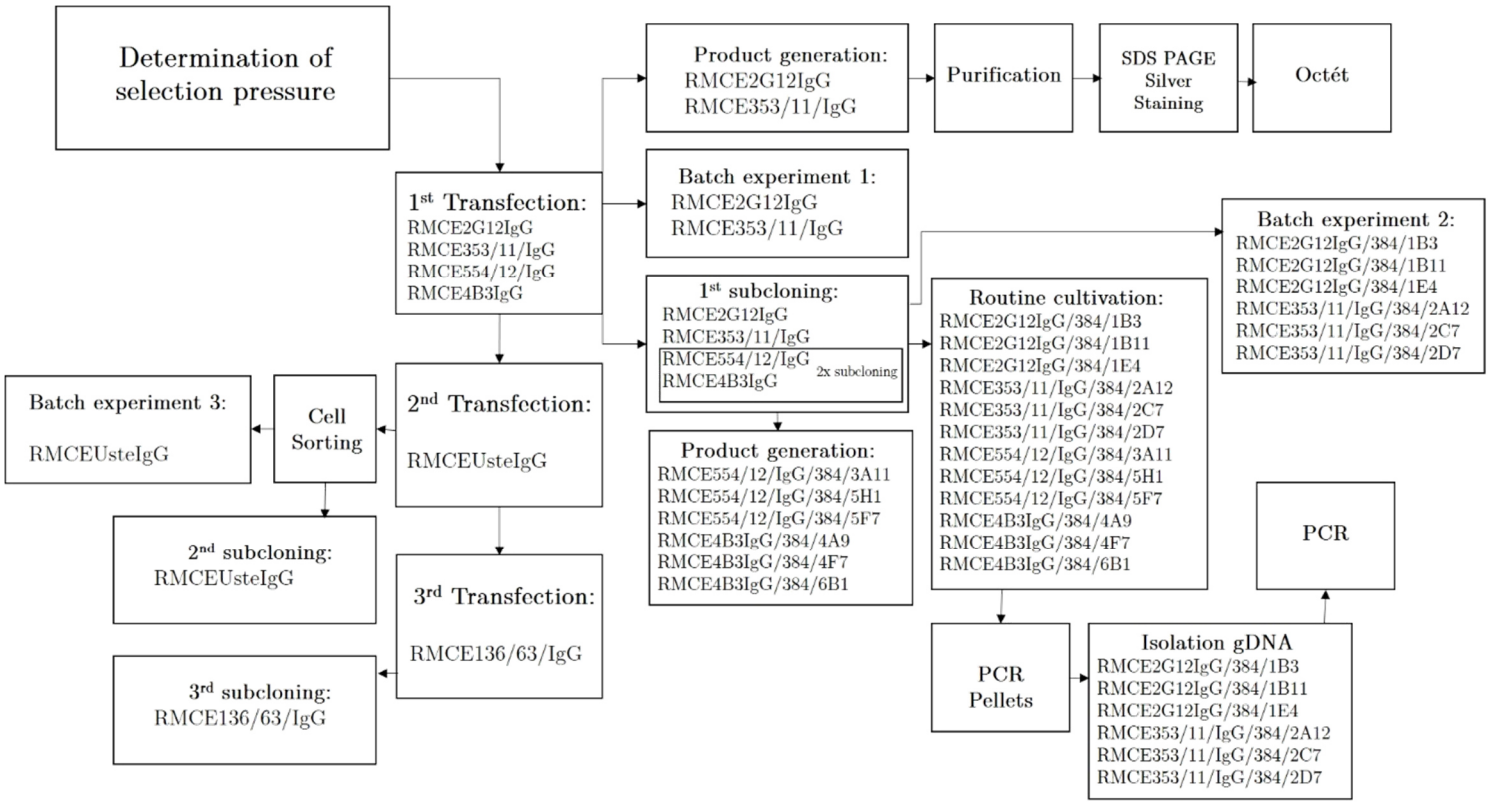


Figure 51: Flow diagram over the entire thesis

6.1 Determination of selection pressure

The cell line CHO PG9 has no exogenous thymidine kinase and therefore no toxic effect of GCV was expected. A toxic effect of GCV on the host cell line CHO RMCE I3 should be observed, because it has stably integrated the recombinant thymidine kinase (HSV-TK).

In contrast to that, CHO PG9 does not express the recombinant HSV-TK gene and no toxic effect of GCV is expected. The experiment showed that the effect of GCV on the cells is depending on the cell concentration (Table 3). At a seeding density of 2500 c/mL and a concentration of 20 µM GCV the CHO PG9 cells were affected by the GCV. The higher the seeding concentration of the

CHO PG9 cells, the lower is the toxic effect of GCV on the cells. The unexpected effect of GCV on CHO PG9 cells could be referred to endogenous thymidine kinase of CHO K1. Another possibility would be that GCV itself has toxic properties at higher concentrations. At a high seeding density of 20000 c/mL the CHO RMCE I3 cells are viable at a concentration of 2 µM GCV. A loss of toxicity could be caused by the exhaustion of the available GCV in the medium. A high consumption of GCV could be followed due to high cell density. This shows that the growth is possible at very high cell densities despite presence of a heterologous thymidine kinase.
It was noticed that neighboring, bystander cells which do not express HSV-TK are also affected by the toxic phosphorylated GCV product [26]. In mixed cell populations, like a co-transfected cell pool, it is thought that GCV is phosphorylated with HSV-TK and the phosphorylated GCV can be taken up by neighboring untransfected cells. Therefore, successfully co-transfected cells could be influenced by phosphorylated GCV from CHO RMCE I3 cells. This is called the bystander effect [26].
For the following experiments the selection pressure was set at 20 µM GCV and was stepwise reduced during the selection process. A transfection efficacy of 10% was considered and that was the reason for a seeding density of 2000 c/well in 96 well plate and 2000 c/well in 384 well plate. The total seeding density was ten times higher than the ideal seeding density (marked light grey in Table 3) in the selection pressure experiment.

6.2 Characterization of cell performance and antibody product

The cell pools of RMCE2G12IgG and RMCE353/11/IgG resulting from transfection 1 are heterogeneous (Figure 9, 36 and 37). Before subcloning 1 the FL1 laser channel, which indicates intracellular GFP, is positive in RMCE2G12IgG with 3.7% and negative in RMCE353/11/IgG. This shows that there are no cells which include the GTN fusion protein in the RMCE353/11/IgG pool.
A comparison of the cell culture media batch medium 1 (CD-CHO) and batch medium 2 (MV3-2/2_6) is drawn in batch experiment 1 (Figure 34). The media is compared in 125 mL shaker flasks with a seeding density of $1 \cdot 10^6$ cells/mL in a total volume of 20 mL. The cell concentrations are slightly higher in batch medium 1 for RMCE353/11/IgG after day 3. This difference could not be observed for RMCE2G12IgG. Additionally, the specific productivity and the growth rate are very similar in both media. The high viability of

RMCE2G12IgG and RMCE353/11/IgG was comparable in both media and could be maintained for more than the first four days in batch culture. There is a difference of the product titers in RMCE353/11/IgG. In RMCE353/11/IgG the titer in batch medium 1 is 20 µg/mL higher than in batch medium 2 at day 6 which might be due to the minimally higher cell densities for batch medium 1 (CD-CHO). The titers in RMCE2G12IgG are very similar in both media until day 4. The value of RMCE2G12IgG in batch medium 2 on day 4 is probably an outlier. The FACS results indicate that the amount of intracellular heavy chain in batch medium 2 is decreasing during batch experiment 1 (Figure 36 and 37). The producer populations of both cell lines seem stable in batch medium 1. Both cell lines show a rather broad peak with a lower producing subpopulation. This indicates that the cell populations are heterogeneous and therefore subcloning 1 was performed.

Immediately after transfection all plated clones were viable. The selection process was started very slowly. That was the reason for the stepwise selection via passaging of the plates. The selection process was prolonged via the slow effect of GCV. Throughout the subcloning and expansion process a loss of clones could not have been excluded. Especially the relation between cell density and toxic effect of GCV on the cells could be the reason for the instability of the subcloned cell line. The feed of 35 µL selection medium 3 shows in all four cell lines a higher number of viable clones before transfer from 384 into 96 well plates. The feed strategy with 35 µL selection medium in the 384 well plate was kept in the following subcloning experiments. Intracellular staining of the heavy chain shows that subcloning 1 resulted in homogeneous populations. After one month of routine culture, subpopulations were formed in at least one subclone of every cell line (Figure 13, 14, 15 and 16; Table 7). It is reported that if a non- or low-producer subpopulation appear, the percentage of them will rise in comparison to high-producers [27] [28]. A reason for this could be that non- or low-producing cells do not need to spend energy on antibody production.

Table 7: Overview of titer, growth rate, specific productivity and percentage of heavy chain (HC) and GFP positive cells of established cell lines of routine culture over 8 passages

	Average		Average growth		Average		HC positive [%]		GFP positive [%]	
cell line	titer [µg/mL]	Standard deviation	rate [1/d]	Standard deviation	qP [pg/c/d]	Standard deviation	Passage 1	Passage 7	Passage 1	Passage 7
RMCE2G12IgG/384/1B11	12.49	+/- 5.47	0.63	+/- 0.04	3.53	+/- 0.52	99.97	99.34	0.00	0.04
RMCE2G12IgG/384/1B3	9.51	+/- 5.51	0.82	+/- 0.11	2.04	+/- 0.73	100.00	97.47	0.12	0.00
RMCE2G12IgG/384/1E4	10.11	+/- 3.64	0.90	+/- 0.11	2.14	+/- 0.76	99.91	91.73	1.79	4.85
RMCE353/11/IgG/384/2C7	12.02	+/- 7.38	0.87	+/- 0.14	2.16	+/- 0.90	99.04	75.11	0.19	0.00
RMCE353/11/IgG/384/2A12	8.69	+/- 3.33	0.78	+/- 0.04	1.94	+/- 0.71	99.66	96.82	0.00	0.00
RMCE353/11/IgG/384/2D7	7.83	+/- 4.41	0.81	+/- 0.09	2.03	+/- 0.98	96.26	96.82	0.21	0.36
RMCE554/12/IgG/384/5H1	6.77	+/- 3.20	0.84	+/- 0.13	1.27	+/- 0.25	98.42	54.14	0.16	0.82
RMCE554/12/IgG/384/3A11	9.53	+/- 4.43	0.65	+/- 0.08	1.94	+/- 0.71	99.98	99.88	0.06	0.32
RMCE554/12/IgG/384/5F7	6.89	+/- 4.62	0.83	+/- 0.12	1.78	+/- 0.72	97.60	83.53	0.10	1.43
RMCE4B3IgG/384/4F7	2.28	+/- 1.16	0.68	+/- 0.08	0.75	+/- 0.19	99.82	99.51	3.62	3.57
RMCE4B3IgG/384/4A9	2.13	+/- 0.93	0.67	+/- 0.18	0.66	+/- 0.20	99.90	98.84	2.77	4.63
RMCE4B3IgG/384/6B1	1.04	+/- 0.57	0.85	+/- 0.11	0.23	+/- 0.09	83.22	45.18	3.25	1.32

The summary in Table 7 shows the average numbers of titer, growth rate, specific productivity and heavy chain (HC) GFP positive cells of the generated subclones of RMCE2G12IgG/384, RMCE353/11/IgG/384, RMCE554/12/IgG/384 and RMCE4B3IgG/384.

The subclone RMCE2G12IgG/384/1B11 has the highest average product concentration with 12.5 µg/mL of all 12 subclones in the shaker flask concomitant with the lowest specific growth rate resulting in the highest specific productivity (Figure 18). RMCE2G12IgG/384/1B3 shows the lowest product titer of the three 2G12 subclones. The average growth rate is the highest in RMCE2G12IgG/384/1E4, but it shows an increasing GFP-positive subpopulation (Table 7). RMCE2G12IgG/384/1B11 has the slowest growth with 0.63 d^{-1} in average over 8 passages (Figure 19). This leads to a specific productivity of 3.53 pg/c/d in RMCE2G12IgG/384/1B11 (Figure 20). It is the highest qP in RMCE2G12IgG. A limitation of growth in RMCE2G12IgG/384/1B11 could be due to the energy spent on antibody production.

The highest product concentration of the RMCE353/11/IgG clones is RMCE353/11/IgG/384/2C7, the second highest producer in total (Figure 21). It is about 0.5 µg/mL lower than the highest average product concentration of RMCE2G12IgG/384/1B11. The lowest antibody production is shown by RMCE353/11/IgG/384/2D7. The range of the average growth rate over 8 passages is very similar in all three clones (Figure 22). The highest specific productivity over 8 passages shows RMCE353/11/IgG/384/2C7, but the staining of

intracellular heavy chain indicates that this cell line is not stable. The subpopulation in RMCE353/11/IgG/384/2C7 is GFP negative (Table 7). The highest average qP of RMCE2G12IgG/384 is about 1.4 pg/c/d higher than in RMCE353/11/IgG/384.

The subclone RMCE554/12/IgG/384/3A11 shows the highest average product titer of 9.5 µg/mL over 9 passages (Figure 24). The remaining subclones of RMCE554/12/IgG/384 have very similar product concentrations of 6.8 and 6.9 µg/mL. RMCE554/12/IgG/384/3A11, which has the highest titer, has the lowest average growth rate over 8 passages. Considering both parameters with the calculation of the specific productivity, the slower growing RMCE554/12/IgG/384/3A11 is the most productive one of RMCE554/12/IgG/384. This result is also applicable in the product generation of RMCE554/12/IgG/384 (Figure 32). The growth limitation, like in RMCE2G12IgG/384/1B11, could be due to a high production. In RMCE554/12/IgG/384/5H1 nearly one half of the population loses the ability of antibody production. This could be due to silencing of the antibody cassette. There is no indication for the existence of a GFP-positive subpopulation (Table 7).

All three RMCE4B3IgG/384 subclones showed a far less product concentration compared to the other three cell lines. This indicates that the 4B3 antibody generation seems harder to synthesize, assemble and secrete for the CHO RMCE I3 cells than the other antibodies. The highest average product concentration over 9 passages is 2.3 µg/mL of RMCE4B3IgG/384/4F7. The lowest is shown by RMCE4B3IgG/384/6B1 with 1.0 µg/mL, which is 12.5 times lower than the highest producing subclone of RMCE2G12IgG/384. The growth rates are comparable to the remaining cell lines. Taking both parameters into consideration RMCE4B3IgG/384/4F7 shows the highest specific productivity with 0.75 pg/c/d in average. The lowest specific productivity, not only of RMCE4B3IgG/384 clones, shows RMCE4B3IgG/384/6B1 with 0.23 pg/c/d. All three RMCE4B3IgG/384 clones have a GFP-positive subpopulation and RMCE4B3IgG/384/6B1 does not remain a stable antibody producer cell line over 8 passages.

In batch experiment 2 three subclones of RMCE2G12IgG and three subclones of RMCE353/11/IgG were compared (Figure 38). The cultivation was done in batch medium 3 in 50 mL reactor tubes with a volume of 10 mL. The seeding density was $2 \cdot 10^6$ cells/mL for each cell line. The cell line RMCE2G12IgG/384/1B3 shows the highest cell concentration and

RMCE2G12IgG/384/1B11 reaches the lowest cell concentration. The measurement of the viability leads to an outliers at day 2 of RMCE2G12IgG/384/1E4 and day 3 of RMCE2G12IgG/384/1B3. The growth rate was very similar for all six cell lines, only RMCE2G12IgG/384/1B11 seems very low in comparison to the others. All three subclones of RMCE2G12IgG show a higher specific productivity than the RMCE353/11/IgG subclones. The product titer is higher in the RMCE2G12IgG cell lines on day 4 than in the RMCE353/11/IgG cell line. The staining of intracellular heavy chain shows that all subclones have a homogeneous population (Figure 40). The number of 10000 events is not reached at this FACS measurement, but a trend can still be identified. The cell line RMCE353/11/IgG/384/2D7 has the lowest amount of producer cells with 81.51%.

For the product generation the 250 mL shaker flasks served as easy to maintain and effective production vessels. The desired product amount was reached with a working volume of 100 mL in MV3-2/3_6 or MV3-2/6 (+30%) over 6-9 harvests (Figure 30). The harvest after 4 days is more than two times higher than after three days which is illustrated in the production of RMCE2G12IgG and RMCE353/11/IgG. The subclone RMCE554/12/IgG/384/3A11 clarifies as the highest producer of the RMCE554/12/IgG/384 cell lines, which confirms the results of the cell line establishment (5.4). The product generation of RMCE4B3IgG/384 subclones is consistent with the results of the cell line establishment.

The IgG purification of RMCE2G12IgG was successful for 37.4% of the total amount of product (Table 5). The loss of product may be attributed to variation in product quantification and/or precipitation after concentration followed by removal by a 0.22 µm filter during loading of the superloop. Additionally losses because of void volumes should be minimized. The IgG purification of RMCE353/11/IgG was successful for 50.5% of the product (Table 6). In both purification experiments a large product loss before the elution step is observed, but the product could not be detected in the fractions permeate, flow through or wash. The SDS-PAGE with silver staining shows that the impurities could be removed by the IgG purification (Figure 50). The bands show a higher molecular weight than expected. This could be due to modifications like for example glycosylation or phosphorylation. In summary, the IgG purification is possible by affinity chromatography, but the loss of product should be examined more closely.

As selection criteria, the GFP-negative properties of successfully co-transfected cells were chosen for the sorting process. The sorting process itself was accomplished under unsterile conditions and therefore the antibiotic mixture PenStrep needs to be added. The results are GFP-negative cells in a 24 well plate. The FACS results show that RMCEUsteIgG has 37% heavy chain positive cells in its population (not shown).
As vessels 125 mL shaker flasks and 50 mL reactor tubes are compared in batch experiment 3 (Figure 41). The cell line RMCEUsteIgG was used for the comparison in batch medium 3 with the same seeding density in both vessels. It seems as if the batch process in the reactor tube is performing slower than in the shaker flask. Between day 4 and 7 of the process the viability and the cell concentration in the shaker flask drop fast. Whereas these parameters are more slowly decreasing in the reactor tube. The titer with a maximum of 4.85 µg/mL and 4.13 µg/mL is very low. The maximum values of 0.18 pg/c/d and 0.12 pg/c/d for the specific productivity are very low. The cell line performs very similar in both reactor vessels.
The FACS results indicate the reason for the low process parameters. Only 9.13% of the population of RMCEUsteIgG do have intracellular heavy chain (Figure 43). The remaining 90.87% of the cell population are non-producers. The outcome for the intracellular GFP expression shows that the entire cell population is GFP-negative (Figure 44). Therefore the cell population do not have integrated the GTN-cassette or the GFP is downregulated. The cell sorting of transfection 2 was directed to sort GFP-negative cells. The FACS results indicate the necessity for a second subcloning step by limiting dilution. The limiting dilution cloning was performed without growth factors (as subcloning 1). At a seeding density of 1 c/well no clones were viable.
In subcloning 3 the cloning efficacy is higher in 384 well plate than in 96 well plate (equation 14 and 15). The antibody producers at the qualitative ELISA lost their producer properties within 10 days to the quantitative ELISA. Of 11 clones chosen, only two clones RMCE136/63/G3 and RMCE136/63/E10 were able to be expanded. RMCE136/63/G3 originates from the 96 well plate and RMCE136/63/E10 was plated out in 384 well plate. The comparison of the both plates is not significant, because of the poor producer stability.

6.3 Genetic characterization of producer cell lines

The PCR specific for parental integration confirms that the parental GTN cassette is exchanged in the RMCE-subclones, but present in the CHO RMCE I3 host cell line (Figure 45). The positive control "L-series F3GTNF" gave a specific band at the expected size of 700 bp. The light bands of the subclones indicate that there might be a part of the population where the GTN cassette is still integrated. The FACS results of the GFP laser channel confirm the results of the PCR for parental integration. RMCE2G12IgG/384/1E4, which shows the most defined band of the PCR samples, has an amount of about 5% of GFP-positive cells in the population (Figure 46). This implies that 5% of the population did not exchange the parental GTN cassette by the antibody cassette. The "gDNA only" bands of RMCE2G12IgG and RMCE353/11/IgG clones ensure that there has been functional template gDNA used as PCR samples. After amplification of the PCR samples, the bands of the genomic DNA are not visible. The genomic DNA is probably degraded during the PCR.

The PCR for targeted integration shows that the replacement of the host cell cassette by the antibody cassette took place at least in one part of the population in the RMCE subclones (Figure 47). The CHO RMCE I3 host cell line has no antibody cassette. The targeted control "L-series F32G12F#1" is positive at the expected size of 1300 bp. This is the first proof that targeted RMCE integration is possible with the new CHO K1 host CHO RMCE I3.

It is assumed that random integration of the antibody cassette would not take place exactly at the FRT-sites (Figure 48). Therefore the primers for random integration bind before the FRT3 site on the donor plasmid. It is expected that the fragment spanning range of pMG433_FSHB_2465_as binding site to the first FRT3 site was also integrated during random integration. To best of our knowledge no random integration was detected using PCR primers specific for the antibody donor plasmid. The positive control "pRMCE_2G12_IgG" has a specific band at 1197 bp and additionally an unspecific band. The unspecific band could be removed via PCR optimization, but since there are no bands at the samples, it can be shown that the primers have no corresponding template present in the genomic DNA of antibody producers.

7 Conclusio

The RMCE system for generation of recombinant antibody production was used successfully in CHO K1 cells. Different IgGs were integrated into the same genomic locus and were compared in the host cell line CHO RMCE I3 within this study. Three different antibodies and their associated germlines were integrated and expressed. They were 2G12 and the associated germline 353/11, Ustekinumab and the respective germline 554/12 and finally 4B3 and the associated germline 136/63. It could be shown that the selection process using ganciclovir is dependent on the cell concentration. The established selection procedure was done with stepwise reduction of GCV. The duration of the selection process was rather long and would need further improvement.
A comparison of the different IgGs without the positioning effect showed a huge difference in the specific productivities. In general the antibody 2G12 and 353/11 had the highest product titers. The therapeutic antibody Ustekinumab and its germline are ranked closely after 353/11. Far behind is the antibody 4B3 which seems far harder to synthesize and secret than the others. It was possible to generate homogeneous and stable clones of four cell lines.
Furthermore, the medium CD-CHO was compared to the in-house generation MV3-2/6. There has been no significant difference between both media. In addition a comparison of production vessels was taken. Shaker flasks with a cultivation volume of 20 mL were compared to reactor tubes with a cultivation volume of 10 mL. The experiment was done with different cultivation conditions. To sum up, the reactor tube is very well suitable for routine cultivation and batch experiments.
Genetic investigation of 2G12 and 2G12 germline clones showed that the targeted RMCE exchange of the GTN fusion protein by the antibody donor plasmid was successful. It was proven that targeted RMCE integration is possible with the new CHO K1 host CHO RMCE I3. There is no indication for additional random integration of the antibody donor plasmid, assuming that 299 bp before the FRT3 site of the antibody donor plasmid are also co-integrated.

8 References

8.1 List of Figures

8.2 List of Tables

8.3 Bibliography

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9 Appendix

Table 8: Transferred wells from 384 to 96 well plate at 1st subcloning

Cell lines	1st transfer		2nd transfer		3rd transfer		4th transfer		Total clones
	no feed	feed	no feed	feed	no feed	feed	no feed	feed	
RMCE2G12IgG	10	10	2	18	9	15	8	8	80
RMCE353/11/IgG	11	25	15	24	1	4	-	-	80
RMCE554/12/IgG	7	16	3	11	9	13	15	6	80
RMCE4B3IgG	5	13	3	11	8	14	8	8	70

Table 9: Transferred wells from 384 to 96 well plate at 2nd subcloning

Cell line	1st transfer		2nd transfer		3rd transfer		4th transfer		Total clones
	10 c/well	100 c/well	10 c/well	100 c/well	10 c/well	100 c/well	10 c/well	100 c/well	
RMCEUsteIgG	1	36	7	6	3	4	1	0	58

Table 10: Transferred wells from 384 to 96 well plate at 3rd transfection

Cell line	96 plate from 384 well plate					96 well plate	Total clones	total 384	total 96	from 384 [%]	from 96 [%]
	331	332	333	334	335	31					
RMCE136/63/IgG	4	2	12	7	8	6	39	33	6	84.6%	15.4%

Table 11: Data of qualitative ELISA of RMCE136/63/IgG at subcloning 3

	Blank	Standard [40 ng/mL]	RMCE136/63/IgG samples with dilution of 1:5							
	1	2	3	4	5	6	7	8	9	10
A	0.030	0.149	0.046	0.050	0.047	0.058	0.074	0.056	0.298	0.033
B	0.032	0.173	0.237	0.043	0.062	0.045	0.041	0.067	0.102	0.038
C	0.043	0.443	0.038	0.037	0.062	0.055	0.142	0.219	0.145	0.068
D	0.033	0.642	0.464	0.041	0.049	0.041	0.097	0.083	0.152	0.053
E	0.028	1.051	0.079	0.036	0.034	0.035	0.142	0.672	3.046	3.596
F	0.028	1.513	0.040	0.037	0.033	0.053	0.277	0.146	0.483	0.690
G	0.038	1.963	1.285	0.036	0.036	0.111	0.035	0.047	0.438	1.268
H	0.040	2.251	0.068	0.038	0.164	0.145	0.036	0.306	0.208	0.794

Table 12: Data of product concentrations over 9 passages of subcloning 1

cell line	Product concentrations of passage [µg/mL]									Average [µg/mL]
	1	2	3	4	5	6	7	8	9	

RMCE2G12IgG/384/1B11	7.4	22.1	6.5	15.2	8.0	14.5	7.8	14.5	16.4	**12.5**
RMCE2G12IgG/384/1B3	3.7	15.1	4.7	13.7	3.2	12.4	3.5	14.6	14.7	**9.5**
RMCE2G12IgG/384/1E4	3.4	11.1	7.2	13.2	9.3	12.1	4.9	12.1	17.7	**10.1**
RMCE353/11/IgG/384/2C7	3.6	11.4	9.3	8.0	26.5	16.9	6.4	16.7	9.4	**12.0**
RMCE353/11/IgG/384/2A12	2.9	10.6	6.6	4.7	7.8	9.6	4.7	12.6	18.7	**8.7**
RMCE353/11/IgG/384/2D7	2.6	9.1	4.8	4.1	8.5	9.6	5.4	16.5	9.9	**7.8**
RMCE554/12/IgG/384/5H1	4.5	3.4	3.2	10.5	4.5	9.5	3.6	9.8	11.9	**6.8**
RMCE554/12/IgG/384/3A11	6.3	5.0	4.8	8.5	6.9	10.6	9.0	18.5	16.2	**9.5**
RMCE554/12/IgG/384/5F7	4.0	1.9	4.0	10.6	5.3	11.9	5.6	15.0	3.7	**6.9**
RMCE4B3IgG/384/4F7	1.4	1.0	1.1	3.2	1.9	2.7	2.1	4.4	2.7	**2.3**
RMCE4B3IgG/384/4A9	1.8	0.6	1.2	2.1	1.6	2.7	1.4	3.6	4.2	**2.1**
RMCE4B3IgG/384/6B1	1.0	0.5	1.2	2.2	0.9	1.6	0.5	0.9	0.6	**1.0**

Table 13: Data of growth rate over 8 passages of subcloning 1

cell line	Growth rate [1/d] of passage								Average [1/d]
	1	2	3	4	5	6	7	8	
RMCE2G12IgG/384/1B11	0.58	0.58	0.64	0.59	0.67	0.62	0.66	0.67	**0.63**
RMCE2G12IgG/384/1B3	0.77	0.75	0.86	0.69	0.86	0.72	0.88	1.02	**0.82**
RMCE2G12IgG/384/1E4	0.71	0.92	0.87	0.92	0.89	0.93	0.86	1.10	**0.90**
RMCE353/11/IgG/384/2C7	0.73	0.75	0.90	0.83	0.89	0.86	0.85	1.14	**0.87**
RMCE353/11/IgG/384/2A12	0.80	0.84	0.70	0.76	0.80	0.77	0.80	0.90	**0.80**
RMCE353/11/IgG/384/2D7	0.81	0.90	0.74	0.73	0.82	0.69	0.87	0.94	**0.81**
RMCE554/12/IgG/384/5H1	0.57	0.77	0.92	0.91	0.92	0.89	0.90	0.89	**0.85**
RMCE554/12/IgG/384/3A11	0.55	0.60	0.58	0.69	0.65	0.78	0.71	0.88	**0.68**
RMCE554/12/IgG/384/5F7	0.63	0.78	0.81	0.81	0.87	0.86	0.85	1.02	**0.83**
RMCE4B3IgG/384/4F7	0.55	0.62	0.66	0.64	0.66	0.79	0.72	0.79	**0.68**
RMCE4B3IgG/384/4A9	0.49	0.50	0.49	0.76	0.71	0.68	0.72	1.03	**0.67**
RMCE4B3IgG/384/6B1	0.60	0.85	0.89	0.85	0.91	0.89	0.83	0.97	**0.85**

Table 14: Data of qP over 8 passages of subcloning 1

cell line	qP [pg/c/d] of passage								Average [pg/c/d]
	1	2	3	4	5	6	7	8	
RMCE2G12IgG/384/1B11	6.43	2.69	3.77	4.17	3.28	3.87	3.38	8.01	**4.45**
RMCE2G12IgG/384/1B3	2.61	1.76	1.90	1.37	1.72	1.46	1.92	3.60	**2.04**
RMCE2G12IgG/384/1E4	2.29	2.06	1.78	2.75	1.50	1.39	1.67	3.67	**2.14**
RMCE353/11/IgG/384/2C7	2.23	3.87	0.88	9.84	1.94	2.10	2.37	1.71	**3.12**
RMCE353/11/IgG/384/2A12	1.70	2.27	0.96	3.22	1.51	1.84	2.07	5.89	**2.43**
RMCE353/11/IgG/384/2D7	1.41	1.44	0.76	3.79	1.38	2.51	2.21	2.77	**2.03**
RMCE554/12/IgG/384/5H1	1.78	1.09	1.23	1.34	1.09	1.11	1.22	7.63	**2.06**
RMCE554/12/IgG/384/3A11	1.70	2.27	0.96	3.22	1.51	1.84	2.07	5.89	**2.43**
RMCE554/12/IgG/384/5F7	1.41	1.44	0.76	3.79	1.38	2.51	2.21	2.77	**2.03**
RMCE4B3IgG/384/4F7	0.51	0.52	0.77	0.92	0.62	0.77	0.91	0.99	**0.75**
RMCE4B3IgG/384/4A9	0.31	0.75	0.73	0.56	0.56	0.63	0.72	0.99	**0.66**

RMCE4B3IgG/384/6B1	0.20	0.41	0.27	0.30	0.19	0.15	0.14	0.16	**0.23**

Table 15: Data of product generation of RMCE2G12IgG and RMCE353/11/IgG

Cell line	Harvest	Titer [µg/mL]	Cell line	Harvest	Titer [µg/mL]
RMCE2G12IgG	1	32.5	RMCE353/11/IgG	1	25.0
	2	9.0		2	9.6
	3	30.8		3	35.0
	4	14.4		4	12.7
	5	34.5		5	28.8
	6	11.4		6	12.4

Table 16: Data of product generation of RMCE554/12/IgG and RMCE4B3IgG

Cell line	day	CC [c/ml]	µ [1/d]	Titer [µg/ml]	q_p [pg/c/d]	viability [%]
RMCE554/12/IgG/384/5H1	0	1.66E+06	#NV	#NV	#NV	91.5
	7	5.60E+06	0.48	33	2.91	95.9
	4	5.97E+06	0.45	22	1.62	97
	3	6.73E+06	0.64	28.4	2.71	95
RMCE554/12/IgG/384/5F7	0	1.55E+06	#NV	#NV	#NV	97
	7	5.70E+06	0.48	33.9	2.95	95.7
	4	5.98E+06	0.45	19	1.31	96
	3	6.33E+06	0.62	24.9	2.49	97
RMCE554/12/IgG/384/3A11	0	1.11E+06	#NV	#NV	#NV	94
	7	3.20E+06	0.40	38.9	5.14	93
	4	4.29E+06	0.36	40.9	3.75	95
	3	3.65E+06	0.43	28.2	2.51	97
RMCE4B3IgG/384/4F7	0	1.25E+06	#NV	#NV	#NV	91.6
	7	5.40E+06	0.47	11.2	1.01	95.3
	4	6.90E+06	0.48	7.95	0.50	95
	3	5.37E+06	0.56	4.92	0.44	95
RMCE4B3IgG/384/4A9	0	1.62E+06	#NV	#NV	#NV	97
	7	5.30E+06	0.47	12.4	1.14	93.9
	4	6.76E+06	0.48	8.17	0.55	94
	3	6.51E+06	0.62	3.94	0.27	98
RMCE4B3IgG/384/6B1	#NV	1.57E+06	#NV	#NV	#NV	94
	3	2.98E+06	0.90	#NV	#NV	97
	4	1.00E+07	0.48	4.19	#NV	95.7
	4	7.93E+06	0.52	4.04	0.15	97
	3	6.99E+06	0.65	2.09	0.18	97

Table 17: Data Batch experiment 1

Cell line	day	CC [c/ml]	µ [1/d]	Titer [µg/ml]	q_p [pg/c/d]	viability [%]

RMCE2G12IgG CD-CHO	0	1.13E+06	#NV	0.00	#NV	99.2
	1	2.06E+06	0.60	2.28	1.48	99.8
	2	6.55E+06	1.16	9.99	1.99	99.6
	3	1.01E+07	0.43	25.63	1.91	99.2
	4	1.56E+07	0.44	50.28	1.95	97.6
	6	8.98E+06	-0.28	57.97	0.32	5.0
RMCE2G12IgG MV3-2/6	0	1.65E+06	#NV	0.00	#NV	99.20
	1	2.22E+06	0.30	2.12	1.10	99.66
	2	6.80E+06	1.12	12.42	2.52	99.62
	3	1.08E+07	0.46	22.88	1.21	99.42
	4	1.33E+07	0.21	50.50	2.30	98.44
	6	9.82E+06	-0.15	36.69	-0.60	2.00
RMCE353/11/IgG CD-CHO	0	1.10E+06	#NV	0.00	#NV	96.30
	1	2.06E+06	0.63	1.29	0.85	99.41
	2	5.52E+06	0.98	8.63	2.09	98.79
	3	1.05E+07	0.64	20.64	1.55	97.99
	4	1.41E+07	0.29	32.19	0.95	98.11
	6	1.11E+07	-0.12	56.39	0.97	5.00
RMCE353/11/IgG MV3-2/6	0	1.05E+06	#NV	0.00	#NV	96.30
	1	1.72E+06	0.49	1.38	1.02	99.83
	2	5.26E+06	1.12	7.28	1.86	98.96
	3	9.46E+06	0.59	17.91	1.48	99.18
	4	1.15E+07	0.20	25.92	0.77	96.68
	6	7.78E+06	-0.20	36.39	0.55	1.00

Table 18: Data Batch experiment 2

cell line	day	CC [c/ml]	µ [1/d]	Titer [µg/ml]	q_p [pg/c/d]	viability [%]
RMCE2G12IgG/384/1B11	0	2.05E+06	#NV	0	#NV	#NV
	1	2.32E+06	0.12	6.9	3.16	94.5
	2	4.57E+06	0.68	18.3	3.43	95.1
	3	5.32E+06	0.15	29.9	2.35	73.7
	4	5.96E+06	0.11	54.4	4.35	95
RMCE2G12IgG/384/1B3	0	1.88E+06	#NV	0.00	#NV	#NV
	1	2.86E+06	0.42	6.90	2.95	95.7
	2	6.12E+06	0.76	18.40	2.68	97.9
	3	1.00E+07	0.49	34.30	2.01	93.3
	4	1.05E+07	0.05	55.00	2.02	96
RMCE2G12IgG/384/1E4	0	1.88E+06	#NV	#NV	#NV	#NV
	1	3.78E+06	0.70	5.5	2.02	95.9
	2	7.67E+06	0.71	19.3	2.51	70.5

	3	1.12E+07	0.38	27.3	0.86	91.6
	4	1.00E+07	-0.11	58.9	2.98	93
RMCE353/11/IgG/384/2A12	0	1.52E+06	#NV	0.00	#NV	#NV
	1	3.03E+06	0.69	3.20	1.46	96.6
	2	6.27E+06	0.73	10.80	1.71	95.3
	3	9.41E+06	0.41	26.00	1.97	96.3
	4	9.25E+06	-0.02	42.00	1.71	94
RMCE353/11/IgG/384/2C7	0	2.15E+06	#NV	0	#NV	#NV
	1	3.89E+06	0.59	5.1	1.74	97.9
	2	7.20E+06	0.62	9.9	0.89	97.9
	3	7.85E+06	0.09	23.1	1.76	95.6
	4	8.46E+06	0.07	33.4	1.26	89
RMCE353/11/IgG/384/2D7	0	1.67E+06	#NV	#NV	#NV	#NV
	1	3.10E+06	0.62	3.8	1.64	94.2
	2	6.43E+06	0.73	14.2	2.28	93.9
	3	7.86E+06	0.20	29.6	2.16	91.4
	4	1.04E+07	0.28	37.8	0.90	94

Table 19: Data batch experiment 3

cell line	day	CC [c/ml]	µ [1/d]	Titer [µg/ml]	q_p [pg/c/d]	viability [%]
RMCEUsteIgG	0	1.56E+06	#NV	0.02	#NV	100.0
Shaker flask	1	2.54E+06	0.48	0.38	0.18	99.7
	2	6.38E+06	0.92	1.27	0.21	99.3
	3	8.31E+06	0.26	2.65	0.19	98.4
	4	8.68E+06	0.04	4.03	0.16	94.7
	7	3.85E+06	-0.27	4.85	0.05	0.0
RMCEUsteIgG	0	1.54E+06	#NV	0.01	#NV	100.0
Reactor tube	1	1.93E+06	0.22	0.23	0.12	99.7
	2	4.32E+06	0.81	0.91	0.23	98.3
	3	6.00E+06	0.33	1.85	0.18	97.0
	4	7.10E+06	0.17	2.32	0.07	96.3
	7	5.35E+06	-0.09	4.13	0.10	48.7

STATUTORY DECLARATION

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.

Vienna, November 2015 Linda Schwaigerlehner

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