Universität für Bodenkultur Wien
University of Natural Resources
and Applied Life Sciences, Vienna

MASTER'S THESIS

Establishment of a high-throughput screening protocol for identification of high-yield CHO cell lines

In partial fulfilment of the requirements for the degree of
Diplom-Ingenieurin (Dipl.-Ing.^{in} or DI^{in})

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2 Abstract

Chinese Hamster ovary cells (CHO) are the main host for the production of complex proteins such as monoclonal antibodies. Despite having been used for decades, the establishment and selection of a stable high producing cell line is still a major bottleneck in pharmaceutical and biotechnological development.

This is due to the fact that transgenesis and selection with expression constructs, especially when based on random integration, leads to a highly diverse population of cells which can change their expression and growth behavior during the lengthy development process.

While recent advantages like bacterial artificial chromosome (BAC) based vectors dramatically improve cell line stability, early identification of highly productive clones remains a challenge with potentially huge impacts on development timelines and required resources.

The aim of this thesis was to establish a cost-effective method that allows predicting the productivity of clonal cell lines early in the development process, thus significantly reducing the number of clones that have to be characterized in more detail.

Based on the link between BAC copy numbers integrated into the host and expression levels, a two-step protocol was developed which uses qPCR combined with ELISA to drastically narrow down the number of clonal cell lines which are then further characterized in parallel fed-batches.

To test this approach we generated cell lines expressing two different commercial antibodies performing all steps from vector generation to final fed-batch in shaker flasks.

It was found that BAC copy number evaluation by qPCR is an efficient and robust tool to generate reasonably precise predictions of specific productivity, but that a second step is required to determine final volumetric yield. Nonetheless, it is a fast method that reduces the time and work-load to obtain high producing cell lines, thus dramatically increasing the throughput and allowing to easily screen a much larger number of clones in less time.

3 Zusammenfassung

Chinese hamster ovary (CHO)-Zellen sind die am häufigsten verwendeten Wirtszellen für die Produktion von komplexen Proteinen wie monoklonaler Antikörper. Trotz jahrzehntelanger Nutzung ist die Etablierung und Selektion von stabilen, hochproduzierenden Zelllinien immer noch ein Flaschenhals in der pharmazeutischen und biotechnologischen Entwicklung.

Dies ist unter anderem der Tatsache geschuldet, dass die Integration des zu exprimierenden Konstrukts, insbesondere wenn sie auf zufälliger Integration in das Wirtsgenom basiert, zu einer sehr unterschiedlichen Population stabiler Zellen führt, was während der langen Entwicklungsprozesse zu Veränderungen in deren Expressions- und Wachstumseigenschaften führen kann.

Während neueste Entwicklungen, wie Vektoren auf der Basis von künstlichen Bakterienchromosomen (BAC), erheblich zur Verbesserung der Stabilität von Zelllinien beigetragen haben, bleibt dennoch die Schwierigkeit der Identifikation von hoch produktiven Klonen, was einen erheblichen Einfluss auf Zeit- und Ressourcenaufwand haben kann.

Das Ziel dieser Arbeit war die Etablierung einer kosteneffizienten Methode zur Prognose der Zellproduktivität im frühen Entwicklungsprozess, wodurch die Zahl der später näher zu untersuchenden Klone wesentlich reduziert werden kann.

Basierend auf dem Zusammenhang der BAC-Kopienzahl im Wirtszellgenom und den Expressionslevels, wurde ein zwei-stufen Protokoll entwickelt, das es mithilfe von qPCR und ELISA erlaubt die Anzahl der klonalen Zelllinien, die dann in parallelen Fed-Batches weiter charakterisiert werden, signifikant zu verringern.

Dafür wurden Zelllinien generiert, die zwei kommerzielle Antikörper exprimieren und alle Prozessschritte von der Generierung des Vektors bis zur endgültigen Fed-Batch Produktion in Schüttelkolben damit durchgeführt.

Die Ergebnisse zeigten, dass die mittels qPCR ausgewerteten BAC-Kopienzahlen ein zuverlässiges Mittel zur Vorhersage der spezifischen Produktivität sind, jedoch einen zweiten Schritt zur Bestimmung der endgültigen volumetrischen Ausbeute erfordern. Nichtsdestotrotz ist es eine schnelle Methode, die die Zeit und den Arbeitsaufwand für die Gewinnung von Zelllinien mit hohem Produktionserfolg verringert, wodurch der Durchsatz wesentlich erhöht und das Screening einer viel größeren Anzahl von Klonen in kürzerer Zeit ermöglicht wird.

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5 Abbreviations

°C	Degree Celcius
µg	Mikrogram (10^{-6} g)
µL	Microliter (10^{-6} L)
µM	Micromolar (10^{-6} mol/L)
ABS	Antigen binding site
Amp	Ampicillin
BAC	Bacterial artificial chromosome
BHK	Baby hamster kidney
BSA	Bovine serum albumin
c/mL	Cells/milliliter
caggs	Cytomegalovirus (CMV) enhancer fused to the chicken beta-actin promoter
Cat No.	Catalogue Number
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CN	Copy number
CO2	Carbondioxide
d	day(s)
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dwp	deep well plate
E. coli	Escherichia coli
e.g.	Example given
Ef1a	Elongation factor-1 alpha promoter
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
FC	Fragment crystallizable
Flp	Flippase recombinase
FRT	FLP recognition target
FSC	Forward scatter
g	Gram or gravitational force
G418	Geneticin
gDNA	Genomic DNA
GFP	Green fluorescent protein
GOI	Gene of interest
GS	Glutamine synthetase
h	Hour(s)
HC	Heavy chain
HEK	Human embryonic kidney cells
HRP	Horse radish peroxidase
Hygro	Hygromycin
Ig	Immunoglobulin
IL-6	Interleukin 6
kb	Kilo base pairs

kDa	Kilodaltons
L	Liter
LC	Light chain
M	Molarity (mol/L)
mAb	Monoclonal antibodies
mg	Milligram (10^{-3} g)
min	Minutes
mL	Milliliter (10^{-3} L)
mM	Millimolar (10^{-3} mol/L)
mRNA	Messenger RNA
MSX	Methionine sulphoximine
MTX	Methotrexate
Neo	Neomycin
ng	Nanogram (10^{-9} g)
nm	Nanometer (10^{-9} m)
NS0	Mouse myeloma cells
NTC	No-template control
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
(q)PCR	(quantitative) Polymerase chain reaction
q_P	Specific productivity
RMCE	Recombinase-mediated cassette exchange
RNA	Ribonucleic acid
rpm	Rounds per minute
SDS	Sodium dodecyl sulfate
sec	Seconds
SSC	Side scatter
STD	Standard Deviation
TALEN	Transcription activator-like effector nuclease
TMB	3,3',5,5'- tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane
V	Volt
VCD	Viable cell density
wt	Wild type

6 Introduction

6.1 Recombinant protein production in CHO cells

In 1957, the first Chinese Hamster Ovary (CHO) cell line was generated by recovery of spontaneous immortalized cells, which were isolated by Theodore Puck in 1956 [1]. CHO cells are still the preferred expression system for complex recombinant therapeutics [2, 3]. The CHO cell system provides a variety of advantages when it comes to expression of proteins. The first one is their ability to grow in suspension and reach high cell densities. This and the fact that CHO cells are able to grow in serum-free media, allow safe large-scale production of proteins [5, 6]. Another advantage is the correct folding of recombinant proteins and that CHO cells are able to do post-translational modifications, including at least the 'human-like' glycosylation of proteins [5, 7]. Additionally, CHO cells are resistant to many human viruses [8]. One of their biggest advantages is their adaptability, which however is also linked to phenotypic and genomic instability, which is one of the main drawbacks for usage of CHO cells [9]. Over the last years, the percentage of biopharmaceuticals produced in mammalian systems has been increasing. In the period from 2015 to 2018, 79 % of all approved biopharmaceutics were produced in mammalian expression systems [3, 4].

Besides CHO cells, there are several other mammalian expression systems for recombinant protein production, such as Baby hamster Kidney (BHK) cells, mouse myeloma cells (NS0) or various human cell lines (e.g. HEK cells), as well as bacterial (e.g. *Escherichia coli*), yeast (e.g. *Saccharomyces cerevisiae*), insect cell (e.g. *Spodoptera frugiperda)* and plant (e.g. *Arabidopsis thaliana*) expression systems, to name only the most common ones.

6.2 Immunoglobulins

Antibodies or immunoglobulins (Ig) are glycoproteins that defend vertebrates against pathogens and infections. They have a key role in the humoral immune response by recruiting the complement system and different types of white blood cells [10]. Monoclonal antibodies (mAbs) represent the biggest group of recombinant proteins for biopharmaceutical applications. They are commonly used in human therapy for treatment of various diseases and cancer types [11]. In the period from 2010 to 2014, they comprised about over a quarter (27%) of all first-time approvals. This number increased to 53% in the period from 2014 to 2018 [3]. Furthermore, mAbs are not only used for therapeutic applications, but also for *in-vivo* imaging of different diseases [7].

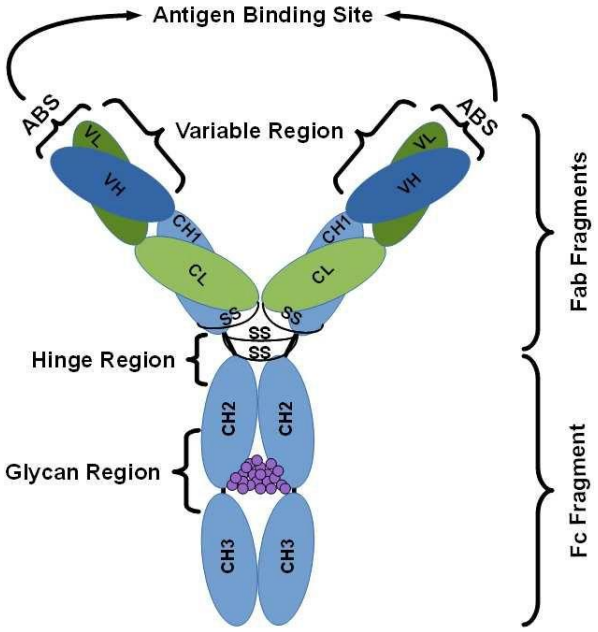


Figure 1: Schematic figure of an antibody molecule [12]. Heavy chains (CH1, CH2, CH3, VH) are shaded in blue, light chains (CL, VL) in green. Heavy and light chains are bound covalently with disulfide bonds. Darker shades indicate variable domains (VL, VH), which form two identical antigen binding sites [10].

A typical antibody has a Y-shaped structure and consists of four polypeptide chains, two identical heavy chains (50 kDa) and two identical light chains (25 kDa), which both have variable regions that together form the pocket for antigen binding [10]. In most Ig types both heavy chains also contain a flexible hinge region, which increases efficiency of antigen binding and cross-linking [10]. The Ig molecule consists of a Fc (fragment crystallizable) region, which facilitates the binding of the complement as well as immune cells, and a Fab (fragment antigen-binding) region. All antibodies are glycosylated on their CH2 domain. These *N*-linked oligosaccharides vary between the different antibody types and have influence on the biological properties [10, 12, 13].

6.3 Types of Immunoglobulins

In mammals, there are five different isotypes of immunoglobulins naturally occurring, some of which have various subclasses. The five major isotypes are: IgM, IgD, IgG, IgA and IgE, which can be distinguished by their constant regions (μ, δ, γ, α, and ε, respectively). All of them have distinct effector functions [10, 15].

In humans, IgG is the most abundant immunoglobulin in plasma and has several subtypes. These subtypes are listed according to their abundancy (IgG1, 2, 3 and 4) and vary in their γ-chain sequences and in their disulfide bridging patterns [16]. IgG is also the most frequently used Ig for human cancer therapy. Antibodies used in therapy are typically fully human, humanized or chimeric [11]. IgG is always found in its monomeric form and together with IgM has the primary role when it comes to activating the complement system, where IgG1 and IgG3 play the most important role [11].

IgM is always the first class of antibody that is produced in a humoral immune response. It forms pentamers, connected with another polypeptide chain, called joining (J) chain. These pentameric IgMs constitute ten antigen-binding sites emphasizing the crucial role in activation of the

complement system. Besides IgM, IgD is part of the primary antibody repertoire of the body. It can mainly be found on B cell surfaces and makes up only a small amount (0.2%) of total serum immunoglobulin [10, 15].

IgA is the principal class of immunoglobulin in secretions, such as milk, tears, intestinal and respiratory secretions. It has two subclasses (IgA1 and IgA2) and it is present as monomer in serum or can form dimers. In serum, there is predominantly monomeric IgA, but also dimeric IgA present. The dimeric IgA contains besides the two IgA molecules also of an extra J chain. When the dimeric IgA is secreted, it binds to the polymeric Ig receptor and is transported to the extracellular matrix where it forms a disulfide linkage with the secretory component. Once it is bound, the latter is cleaved from the polymeric Ig receptor to be released as secretory IgA. Finally, IgE is the least abundant antibody in blood. It binds to receptors on mast cells and basophils, which triggers the release of chemical mediators, like different cytokines or amines (e.g. histamine) [10, 15].

6.4 Anti-IL-6 antibodies

Interleukin 6 (IL-6) is a cytokine and has been shown to have multiple biological activities with influence on the innate and the adaptive immune system. It is produced in various cell types, including B cells, T cells, monocytes, endothelial cells, fibroblasts, keratinocytes and some tumor cells [17]. Due to its pleiotropic character, the deregulation of IL-6 levels is connected with numerous diseases, such as rheumatoid arthritis, systemic juvenile idiopathic arthritis, and Crohn's disease, as well as different malignant diseases [18]. Therefore, the demand of anti-IL-6 therapies is high and many monoclonal anti-IL-6 antibodies are on the market [18, 19, 20].

6.5 Transfection methods

The choice of the transfection method plays a major role for recombinant protein production. For screening of a panel of variants, transient gene expression is the method of choice. When a gene is transiently expressed, the foreign DNA is delivered into the nucleus, but not incorporated into the host cell genome. This can result in a high amount of product in short time. However, due to environmental factors and cell division the cells eventually will lose the genetic material [4, 21].

For large-scale production stable transfected cell lines are required. When a gene is transfected stably, it is integrated into the host cell genome, ensuring product expression over a long period of time. When using traditional methods, the gene of interest (GOI) is randomly integrated into the host genome, leading to unpredictable expression results, depending on the site of integration [22]. Consequently, methods using selection and gene amplification have been developed. One of the most commonly used methods includes the amplification of the dihydrofolate reductase (DHFR) gene together with the GOI in DHFR deficient CHO cells [23, 24, 4]. The DHFR lacking cells need to be cultivated in a medium supplemented with glycine, hypoxanthine and thymidine [4]. Additional amplification is achieved by using methotrexate (MTX), which is a DHFR inhibitor, leading to further amplification of the DHFR gene and thus amplification of the GOI [23]. Similar to this, gene amplification can also be obtained using glutamine synthetase (GS) deficient cells in glutamine lacking media with methionine sulphoximine (MSX) as GS inhibitor for further gene amplification [4].

However, gene amplification and subsequent screening is very labor- and time-intensive [25] and can lead to genetic instability and often silencing of the genes [26].

To overcome this problem, methods for targeted integration of the GOI have been established. The frequently used recombinase-mediated cassette exchange (RMCE) system gives the opportunity to insert the expression cassette with the GOI into a defined, highly transcribed region in the genome. This offers the significant advantage that major complications of random integration, like epigenetic silencing as well as strong, potentially negative effects on the genetic landscape at the site of integration can be largely mitigated [27, 22].

For this method, the yeast derived Flippase recombinase (Flp) is used. Flp is a site-specific recombinase, which recognizes FLP recognition target sites (FRT sites) for recombination and therefore exchange or integration of the GOI cassette into the host genome. The target cassette in the host genome is hereby flanked by two heterospecific FRT sites. The Flp is co-transfected with the GOI cassette, containing the same heterospecific FRT sites, inducing exchange of both cassettes [28]. Moreover, the Cre/loxP system can be applied for targeted integration of a transgene [29]. These methods support the integration of only one copy of the GOI into the genome, but a recent study showed multiple transgene integration in a predefined site of the CHO cell genome by Cre recombinase-incorporating integrase-defective retroviral vectors [30]. Further, site-specific integration of transgenes was accomplished by transcription activator-like effector nucleases (TALENs) [31] and CRISPR/Cas9 [32, 33], whereby TALENs are expensive and give a high frequency of insertion/deletion mutations [31] and CRISPR/Cas9 can have off-target effects [32, 33].

An ideal approach would combine the advantages of both random small vector and targeted integration, namely the expression from a large number of copies of the cassette and the protection from potentially negative effects of the integration site.

6.6 Bacterial artificial chromosomes (BACs)

Bacterial artificial chromosomes (BACs) are large cloning vectors that ensure a high cloning capacity of up to 300 kb and can harbor mammalian open chromatin loci [34]. This is an advantage, because the integration site of the transgene in the host cell genome has a huge impact on the transcription rate [22]. The integration of the GOI into a densely packed region of DNA, described as heterochromatin, leads to lower transcription efficiency. Random integration of small vectors constitutes the risk of so called ‘chromatin positional effects’ [22]. In the past, BACs had been widely used for generation of transgenic mice [35]. Due to their beneficial properties, they are a useful tool for the production of recombinant proteins. Previous studies showed an increase in specific productivity and also in transgene transcription in comparison to methods based on random integration of plasmids. Also they showed a good correlation between gene copy number and transcription level [34, 36, 37].

The aim of this thesis was to establish a fast and convenient process for identification of high-producing cell lines. Therefore, CHO cells were engineered to produce two anti-IL-6 antibodies, one IgG1 and one IgG4 using the BAC expression system.

Therefore, a full-length BAC with the Rosa26 locus was used, which has previously been proven to be the most efficient expression construct [34, 37]. Additionally, to the common approach with the full-

length BAC (~200 kb), all work steps were performed in parallel with a smaller, improved BAC of approximately 70 kb, generated in our lab [Zboray, unpublished data].

7 Material and Methods

7.1 Cloning

The sequences coding for heavy and light chain for both, the IgG1 and the IgG4 antibody were retrieved from the IMGT database (www.Imgr.org). The codon sequence was optimized with Thermo Scientific Web Tool (www.thermofisher.com) for expression in CHO cells and ordered as linear DNA fragments (gBlocks) from IDT.

The gBlocks were cloned into vectors for recombination into the BAC using In-fusion HD EcoDry Cloning Kit (Takara Bio, Cat No.: 638909) according to manufacturer's protocol. The plasmids were digested with FastDigest SgsI (AscI) restriction enzymes (Thermo Fisher Scientific, Cat No.: FD1894) and contained promoters (Ef1a or caggs) for the transcription of the gene of interest as well as selection markers (NeoR or HygroR) together with bacterial and eukaryotic promoters for these genes.

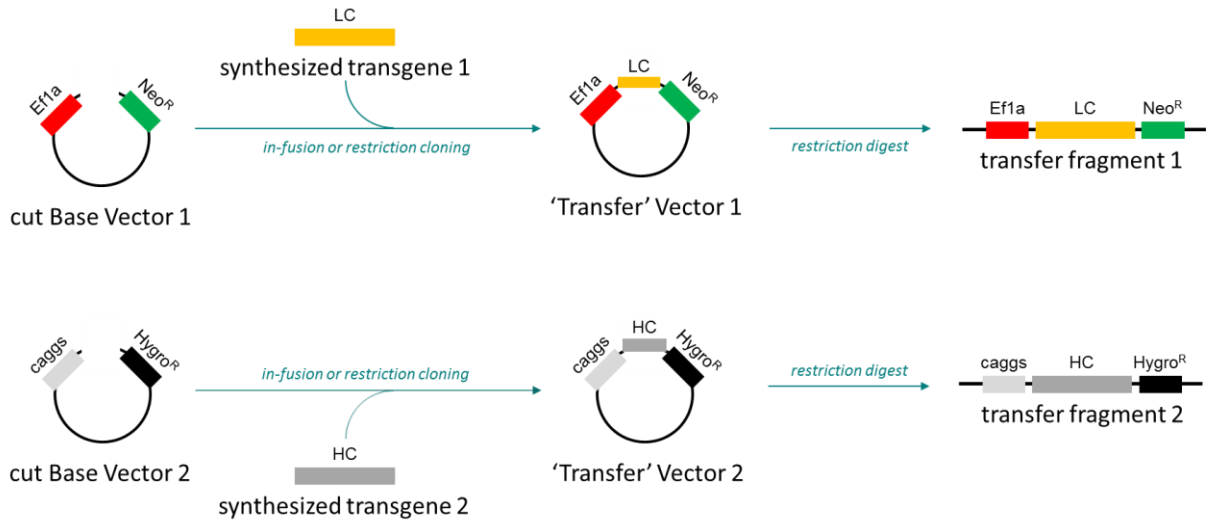


Figure 2: Workflow from transgene to transfer fragment. Synthesized gBlocks (LC and HC) were cloned into the transfer plasmids with in-fusion. After successful in-fusion cloning the plasmid was digested for recombination into the BAC and the desired fragment was purified via gel elution.

For recombination the synthesized gBlock and the plasmid contain overlapping homology arms of 15 bp length. 50 ng of linearized vector and 50 ng of gBlock were used for the In-fusion reaction. The reaction mix was incubated 15 min at 37 °C and 15 min at 50 °C.

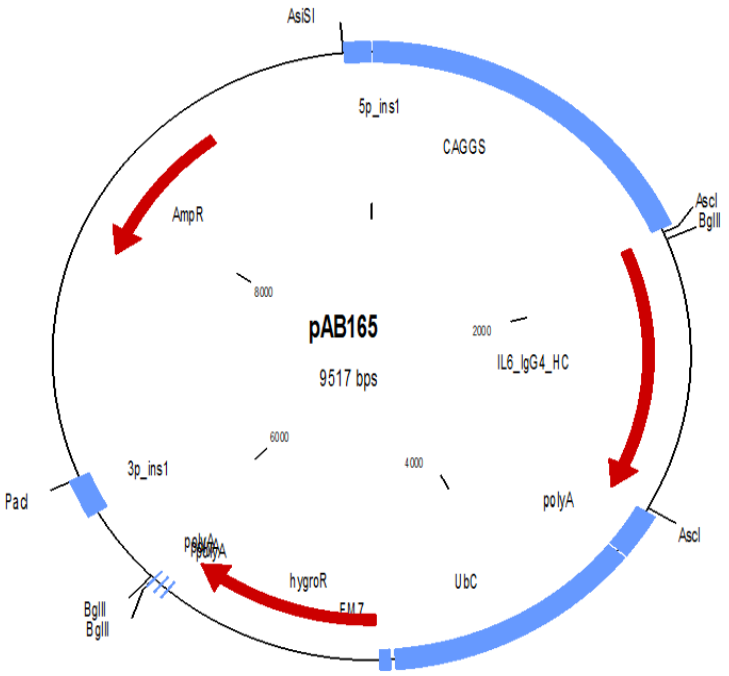


Figure 3: Example of a transfer vector. The plasmid contains a caggs promoter upstream the IgG4 HC sequence. Moreover, the plasmid contains Hygro and Amp resistance genes as well as bacterial sequences for amplification in bacteria and screening for positive clones. Furthermore, it contains two homology arms (5p_ins1 and 3p_ins1) for homologous recombination into insertion site 1 of the BAC. The cleavage sites of the restriction enzymes used during cloning procedure are indicated on the map.

7.2 Transformation

The plasmids were amplified in *E.coli* for further use. Therefore the DNA was transfected into *E. coli* Stellar competent cells (Clontech, Cat No.: 636766) via heat shock at 42 °C for exactly 45 sec. The cells were selected under respective antibiotic conditions on agar plates. After 24 h, 6 colonies of each plate were picked and 5 mL LB-medium were inoculated to perform a Miniprep after another 24 h. For the DNA purification Miniprep High Pure Plasmid Isolation Kit (ROCHE, Cat No.: 11754785001) was used. The identity of the plasmids was confirmed by a restriction digest with BglII (Thermo Fisher Scientific, Cat No.: FD0083) and Sanger sequencing. (Sequence and primers are listed in the appendix)

Further, a Midiprep was performed with NucleoBond Xtra Midi Plus (Macherey-Nagel, Cat No.: 740412.50). The obtained plasmids were digested with FastDigest PacI and AsiSI (Thermo Fisher Scientific, Cat No.: FD2204; FD2094) cutting at the 3’ and 5’ homologous arms. The fragments were separated via gel electrophoresis and the desired cassettes were eluted from the gel with Gel Elute Extraction Kit (Sigma, Cat No.: NA1111).

7.3 BAC recombination

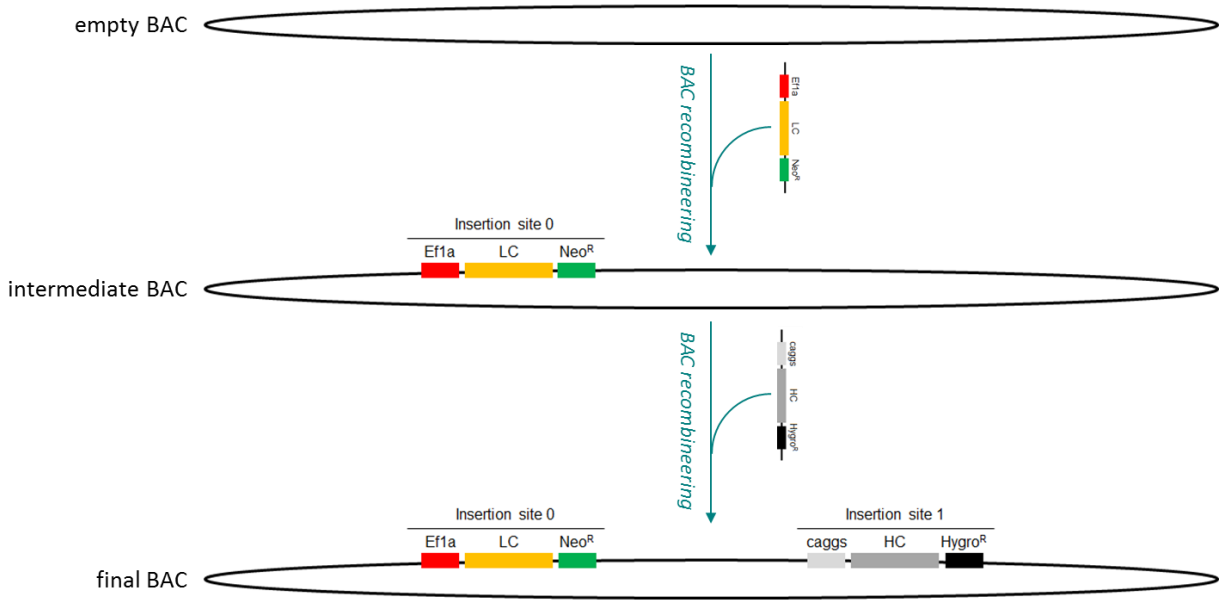


Figure 4: Integration of LC and HC expression cassettes into the BAC

In a first step, the cDNA for the antibody to be expressed was introduced into a regular plasmid containing all the required elements for selection and expression in eukaryotic cells as well as short DNA sequences homologous to the BAC. One plasmid was generated for the HC and one for the LC (see point 7.1, Fig. 2, Fig. 3).

In a second step, these expression cassettes were isolated from the plasmids and introduced into bacteria containing the BAC using Red/ET recombination. Red/Et recombination or also referred to as λ Red recombination was developed to inactivate chromosomal genes as described by Datsenko and Wanner [38]. It can also be used for insertion and deletion of genes or DNA fragments as well as for point mutations.

The Red/ET recombination plasmid (pRed/ET) contains besides an arabinose promoter and a resistance gene, two genes important for recombination, Redα and Redβ. Redα encodes for an exonuclease for digesting DNA. Redβ encodes for a DNA annealing protein [39]. By co-expressing pRed/ET, recombination between the homology arms and the corresponding site in the BAC was induced, resulting in the integration of the expression cassette (Fig. 4, first step).

In detail, the expression cassettes for the LC were introduced into DH10B *E. coli* harboring the 200 kb or 70 kb version of the BAC. Typically 100 µL of *E. coli* suspension were electroporated with 100 ng expression vector using Gene Pulser Xcell (Bio-Rad, Cat No.: 165-2660). Both BAC containing cell lines also contain the pRed/ET helper plasmid for homologous recombination. After recovery, *E. coli* were plated on agar plates containing selection pressure for the expression cassette, for the BAC backbone as well as for the pRed/ET plasmid and incubated at 30°C.

After isolation of bacterial clones in which this process had been successful, the integration was repeated in a similar manner for the HC (Fig. 4, 2nd step).

7.4 BAC prep

For isolation of the BACs, 1 L of bacterial overnight culture was harvested 15 min at 4400 rpm and the NucleoBond Xtra BAC Kit (Macherey-Nagel, Cat No.: 740436.25) was used. The final DNA pellet was resuspended in 100 µL 10 mM Tris buffer. The final identity of the BAC was confirmed by PCR and EcoRI restriction digest.

7.5 Quantification of Nucleic Acids

Nucleic acid quantification is based on the absorbance at 260 nm. All quantification measurements were done on Tecan Microplate Reader Infinite M1000 pro with NanoQuant Plate (Tecan), individually blanked with 10 mM Tris buffer or RNase free water.

7.6 Polymerase Chain Reaction (PCR)

Quality controls of the correct insertion of the gene of interest into the base vectors and further into the BAC were done by PCR. The PCR reaction contained 1 µL of DNA or 1 µL of bacterial culture, 1 µL forward and reverse primers (10 µM), 10 µL 2x Dream Taq Green PCR mix (Thermo Fisher Scientific, Cat No.: K1082) and water to a total volume of 20 µL. All PCRs were performed under following conditions.

Table 1: PCR conditions

	Temperature	Time	Cycles
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	
Annealing	57 °C	30 sec	35
Extension	72 °C	60 sec	
Final Extension	72 °C	7 min	1

7.7 Linearization

For transfection of the CHO cells, linearized BAC DNA was used. For this purpose the BAC was digested with PI-SceI homing endonuclease (New England Biolabs, Cat No.: R0696S) over night at 37 °C. For confirmation of the proper digestion, the digested BACs were loaded on a 0.7% agarose gel together with undigested BACs.

7.8 Cell Culture Methods

7.8.1 Host cell line

CHO-K1 wild type (wt) cells were maintained in suspension in chemically defined CD CHO Medium (Gibco, Cat No.: 10743029) supplemented with 4 mM Ultraglutamine 1 (Lonza, Cat No.: BE17-605E/U1), Anti-Clumping Agent (Gibco, Cat No.: 01-0057DG) and phenol red (Sigma, Cat No.: P0290) which is in the following referred to as stock medium. The cells were cultivated in a volume of 30 mL medium at 37 °C at 5% CO_2 at 125 rpm shaking in a 125 mL shaker flask with vented cap (Corning, Cat No.: 431143) and were passaged every 3-4 days at a seeding concentration of $3*10^5$ c/mL.

7.8.2 Cell counting and viability

Cell concentration and viability were either determined with BioRad counting chamber (Cat No.: 1450011) on Bio-Rad T10 automated cell counter based on trypan blue staining or with flow cytometry using Cytoflex (Beckman Coulter). The Cytoflex measurement was performed in 96 V bottom plates either directly with livings cells or with fixed cells. Therefore the cells were 1:2 diluted in 4% paraformaldehyde (PFA, Thermo Fisher Scientific, Cat No.: 12777847). The PFA prevents the cells from sticking to the bottom of the plate while keeping the parameters for viability. The number of events in a defined volume was determined and used for calculation of the viable cell count in the culture based on forward and side scatter.

7.8.3 Transfection into CHO-K1 cells

To transfect the BAC into CHO-K1 cells and subsequent integration into the host cell genome electroporation was used. It was performed on the Amaxa Nucleofector II device and the Amaxa Cell Line Nucleofector Kit V (Lonza, Cat No.: VCA-1003).

On the day of transfection, cell concentration and viability were determined. An aliquot of 10^5 cells per transfection was centrifuged for 5 min at 300 g and resuspended in 100 µL Cell Line Nucleofector Solution per reaction and aliquoted into Eppendorf reaction tubes. Before every electroporation 10 µL (5 µg) of linearized BAC was added to the cells. The mix was transferred into a 100 µL aluminum cuvette and electroporated using program U-23. After electroporation 1 mL of fresh pre-warmed stock medium was added to the cells immediately and the cells were transferred into a 6 well plate with another 2 mL of pre-warmed stock media for recovery. The cells were incubated at 37 °C, 5% CO_2, static in a humidified incubator.

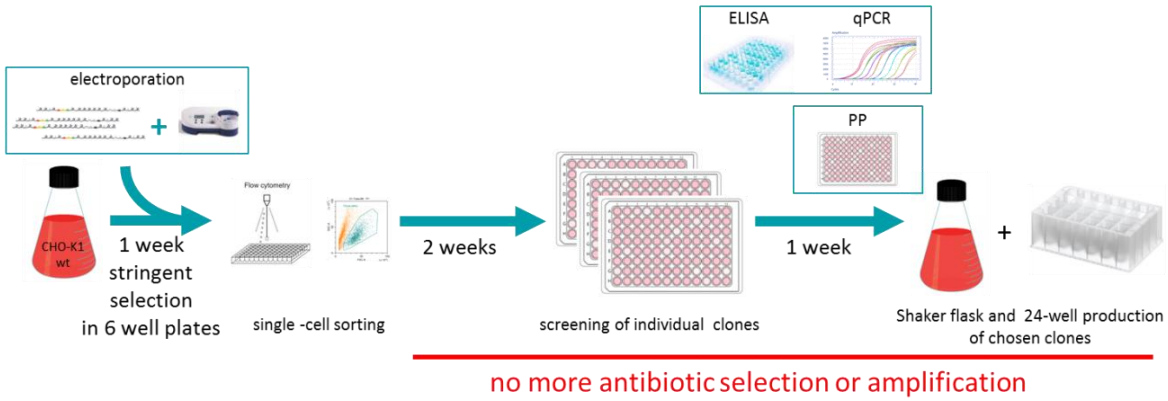


Figure 5: Workflow from transfection to production

7.8.4 Selection pressure

After 24 h of recovery, selection pressure was applied by adding 60 µL of 50 mg/mL geneticin disulfate (G418)-solution (Carl Roth, Cat No.: CP11.3) to obtain a final concentration of 1000 µg/mL.

7.8.5 Conditioned medium

For conditioned medium generation, CHO K1 wt cells were seeded at a density of 10^5 c/mL in fresh stock medium. After 24 h the cells were centrifuged at 300 g for 5 min and the medium was sterile filtered (0.22 µm). For single cell sorting the conditioned media was plated in 96 well U-bottom plates at a volume of 100 µL/well.

7.9 Single cell sorting

Single cell clones were obtained by fluorescence-activated cell sorting (FACS). Seven days after transfection the cell pools in the 6 well plates together with a CHO K1 wt control were resuspended and 2.5 mL of cell suspension were centrifuged at 300 g for 5 min. In the 6 well plates, the remaining 500 µL were topped up with 2.5 mL fresh stock medium with 1000 µg/mL G418. About 2 mL medium were removed from the centrifuge tube, the pellet was resuspended in the remaining 500 µL and pipetted through the cell strainer cap of a FACS tube (Falcon).

The sorting procedure was performed at St. Anna Children's Cancer Research Institute under supervision of Ing. Dieter Printz. The setting of the gate for sorting was based on the forward and side scatter of the CHO K1 wt control. There were no cells sorted in the outer wells of the plate because of the higher evaporation rate.

Two weeks after single-cell sorting the recovered colonies were transferred into a new 96 well U bottom plate and were topped up with 100 µL fresh CD CHO stock medium to give the cells another week for growth. Afterwards, a prescreen ELISA was performed to qualitatively test whether the recovered clones are expressing antibody.

7.10 ELISA (qualitative and quantitative)

All ELISA plates were pre-coated. Therefore the plates were incubated 1 h shaking at room temperature with 1:2000 Goat anti-human IgG, blocked with 5 % BSA in PBS and stored dry at 4 °C.

For all assays HRP-Goat anti-human IgG F(ab')2 secondary antibody (Invitrogen, Cat No.: 31482) diluted 1:30000 was used for detection. Developing was performed with 100 µL TMB and stopped with 100 µL 3% H_2SO_4.

For the prescreen ELISA after single-cell sorting, 50 µL of supernatant were directly transferred into a pre-coated ELISA plate and 1:2 diluted with 1x PBS. The plate was incubated shaking for 1 h at room temperature and after washing incubated with secondary antibody at the same conditions. Developing was done as described previously.

After the final production, all supernatants were quantified by ELISA. The supernatants were diluted 1:20 000 and 1:100 000 in dilution buffer (PBS (Applichem, Cat No.: A0965,9010), 1% BSA (Sigma-Aldrich, Cat No.: A7906-500G), 0.05% Tween (Sigma, Cat No.: P9416-100ML) and the ELISA was performed as described above. As standard previously purified and quantified antibody (produced in-house) was used. Calculation of antibody titers was based on standard curves.

7.11 qPCR

The gene copy numbers of the BAC integrated into the host cell genome, as well as the relative mRNA levels were determined using real-time PCR.

The copy number of the BAC was determined using sequence-specific PrimeTime qPCR probe Assays (IDT) probes. The expression level determination with mRNA was done using SYBR Green qPCR.

7.12 Cell Lysis

For gDNA isolation, the cells were lysed with DirectPCR Lysis Reagent (Viagen Biotech, Cat No.: 302-C) with Proteinase K (Merck, Cat No.: 1073930010). Cell lysis was performed at 55 °C for 2 h and 85 °C for 5 min for Proteinase inactivation. The crude lysate was directly used as template for qPCR.

7.13 Copy number

Analysis of BAC copy number was performed using multiplex PrimeTime qPCR probe Assays from IDT. In purchased probes, FAM and HEX served as reporter dyes.

The reaction contained 5 µL Master mix (IDT, Cat No.: 1055771), 0.5 µL of each qPCR probe (HEX and FAM), 3 µL template and 1 µL water. All qPCRs were performed on CFX Connect Real-Time PCR Detection System (Bio-Rad) and under following conditions (Table 2).

Table 2: qPCR conditions

	Temperature	Time	Cycles
Polymerase activation	95 °C	3 min	1
Denaturation	95 °C	5 sec	40
Annealing/Extension	60 °C	30 sec	

Calculation of BAC integration numbers was done according to double delta Ct method. Number of BAC integrations were determined using a probe binding both to the backbone of the BAC and the endogenous Rosa26 sequence. As a reference, wt CHO cells of the same strain were use, for which the number of binding sites of the Rosa26 specific probe had previously been determined as two. A second probe binding to a housekeeping gene served as a normalizer for the amount of total template genome.

7.14 Expression Levels

7.14.1 RNA isolation

RNA isolation was done of frozen cell pellets centrifuged from 1 mL culture sampled during production and was performed with RNeasy Mini Kit (Qiagen, Cat No.: 74104) according to manufacturer's protocol. Additionally, an on-column digestion of gDNA was performed during mRNA isolation using RNase-Free DNase Set (Qiagen, Cat No.: 79256). RNA was quantified with NanoQuant Plate on a Tecan Microplate Reader Infinite M1000 pro individually blanked with RNase free water.

7.14.2 cDNA synthesis

For mRNA analysis with qPCR, cDNA was synthesized from mRNA templates using All-in-One cDNA Synthesis Supermix (bimake.com, Cat No.: B24408). RNA samples were diluted to 500 ng/µL. 1 µL of RNA, 2 µL of 5x qRT SuperMix and 7 µL of RNase-free water were used for one reaction. This was mixed on ice, incubated at 25 °C for 10 min, 42 °C for 30 min and 85 °C for 5 min.

7.14.3 RNA analysis

Samples for mRNA level determination were taken on day 0, 4, 6, 8 and 10 of production and measured in 2 technical replicates using probes specific for the HC and the LC sequence of the 2 different antibodies. mRNA expression levels were determined using goTaq qPCR Master Mix (Promega, Cat No.: A600A). For each reaction 10 µL of Master Mix, 0.4 µL of each primer, 8.2 µL water and 1 µL of cDNA template (see above) were used. The qPCRs were performed on CFX Connect Real-Time PCR Detection System (Bio-Rad) using same conditions as in 7.13 (Table 2).

β-Actin was used as internal reference. To determine relative expression levels, the double delta Ct method was used. Relative expression levels are shown as $2^{-\Delta Ct}$ values. The mean $2^{-\Delta Ct}$ values were

calculated based on the difference of Ct values of HC respectively LC sequences to the β-Actin reference.

7.15 Expansion of clones

For the established protocol 3 expansion steps were necessary. From 96 well U bottom plate to 96 deep well plate (500 µL/well) 2-3 weeks after sorting; from 96 deep well plate into 24 deep well plate (3 mL/well) 1-2 weeks later and from 24 deep well plate into 125 mL shaker flask (30 mL volume).

When the cells were expanded into the 24 deep well plate, incubation was switched from static to shaking (225 rpm).

For each expansion step pre-warmed stock medium was provided in the new vessels. The cells were resuspended in the entire volume and directly transferred into the new vessel.

7.16 Power Production (PP)

Power production is an assay that has been previously established in the lab that aims at determining specific productivity of clones under conditions similar to fed-batch in a miniaturized format.

For this purpose, 10000 cells are seeded per 96-well in media spiked with feeds [40] and cultivated for 3 days. At the end of this period, cell number and antibody titer are determined and the specific productivity is calculated.

For the PP 42.3 mL of CD CHO stock medium were supplemented with 7.5 mL Efficient feed B (Thermo Fisher Scientific, Cat No.: A1024001) and 165 µL Function MAX titer enhancer (Thermo Fisher Scientific, Cat No.: A1501002).

Each clone was seeded at 10^4 cells per well in PP medium in a 96 well flat bottom plate.

After 3 days, cell numbers were determined by Cytoflex and antibody titers were evaluated by ELISA.

7.17 Fed-batch Production

Antibody production was performed in 24 deep well plates in 4 mL volume as well as in 125 mL shaker flasks in 30 mL volume.

A few days before starting the production the cells were expanded into 125 mL shaker flasks. On the day the production was started (day 0) the cells were counted on Cytoflex to determine the cell density and to ensure that the viability was above 95%. If both parameters were in the desired range, the cells were passaged to a cell density of $3 \cdot 10^5$ c/mL and split into two 24 deep well plates and a 125 mL shaker flasks.

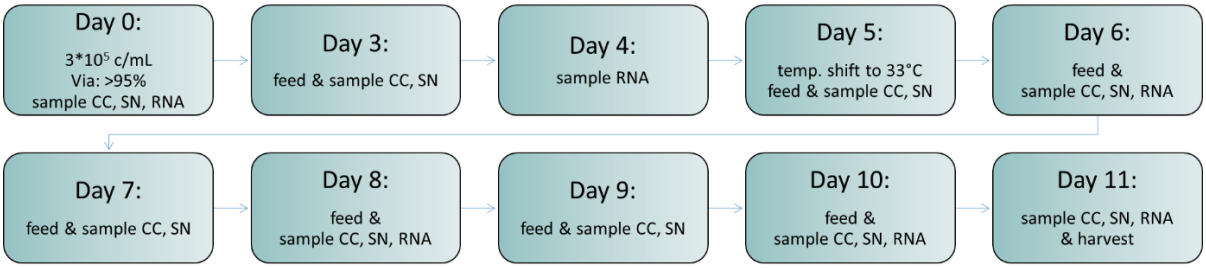


Figure 6: Production workflow. CC: samples for determination of cell concentration; SN: supernatant samples for PAGE and ELISA; RNA: sampling cell pellets for transcript level evaluation.

For feeding 4% (v/v) HyClone Cell Boost 7a (GE, Cat No.: SH31026.07) and 0.4% (v/v) HyClone Cell Boost 7b (GE, Cat No.: SH31027.01) were supplemented to the cells.

The 24 well plates were put on a scale every other day and filled up with the required amount of WFI (Gibco, Cat No.: A1287301) to compensate the evaporated liquid.

For sampling, 100-150 µL were taken out of plates/flasks. 50 µL cell suspension were fixed with 50 µL PFA for cell counting. The rest was spun down for 5 min at 300 g and the supernatant was transferred to a new plate for SDS-PAGE and quantitative ELISA analysis. The samples were stored at 4 °C.

Every other day a sample for RNA isolation was taken from the 125 mL shaker flasks. For this, 1 mL culture was centrifuged at 300 g for 5 min, the supernatant was removed and the pellet was stored at -80 °C.

On day 11 the culture supernatant was harvested. For this, the culture was centrifuged at 4400 rpm and sterile filtered (0.22 µm).

Production related parameters like specific growth rate (µ) and specific production rate (q_P), were calculated from acquired data. Respective formulas as are given below.

$$\mu = \frac{\ln(x_1) - \ln(x_0)}{t}$$

The specific growth rate μ [d^{-1}] was calculated with the seeding cell concentration x_0 and final cell concentration x_1 [c/mL] achieved during time t [d].

$$q_P = \frac{(T_1 - T_0) * \mu * 10^6}{x_1 - x_0}$$

The specific production rate q_P [pg/c/d] was calculated with the initial T_0 and final T_1 antibody titer [µg/mL], respectively.

7.18 SDS-PAGE

To assess quality and quantity of the produced antibody, the crude supernatants were analysed with (non-reducing) sodium dodecyl sulfate polyacrylamide gel electrophoresis. Therefore, 30 µL of the supernatant were mixed with 10 µL of 4x Laemmli Sample buffer (Biorad, Cat. No.: 1610747) and brought to a temperature of 65 °C for 5 min.

10 µL of a 1:10 dilution of boiled samples were loaded on a 4-12% precast polyacrylamide gel (Invitrogen, Cat No.: WG1402BOX) and run 50 min at 200 V in 1x Nu Page MOPS SDS running buffer (Novex, Cat No.: NP0001). PageRuler pre-stained protein ladder (Thermo Fisher Scientific, Cat No.: 26616) was used as marker and protein A purified Rituximab (produced in-house) in different concentrations as standard.

After running, the gel was extracted from the chamber and incubated 15 min in InstantBlue Coomassie protein stain (Expedeon, Cat No.: ISB1L). After staining, the gel was destained in MQ water over night.

Gels were imaged with a Li-Cor Odyssey CLx scanner. For quantification, the area under the curve for each band was determined and compared to loaded antibody standard using ImageJ software.

8 Results and Discussion

8.1 Cloning

Four base vectors were generated, two containing the two different light chains and two containing the two different heavy chains for expression of 2 anti-IL-6 antibodies, one IgG1 and one IgG4.

First, the synthesized DNA fragments (gBlocks) were introduced into plasmids containing homology arms for later recombination in the BAC, promoters, selection markers and required restriction sites.

Table 3: Generated plasmids

	Insertion site	**Fragment**	**Promoter**	**Selection marker**
pAB164	Ins0	IgG4 light chain	PGK; GB3	Amp; Neo
pAB165	Ins1	IgG4 heavy chain	Ubc; EM7	Amp; Hygro
pAB166	Ins0	IgG1 light chain	PGK; GB3	Amp; Neo
pAB167	Ins1	IgG1 heavy chain	Ubc; EM7	Amp; Hygro

To confirm their identity, the plasmids were digested with BglII. The expected sizes of the fragments as shown in Table 3 are well visible on the gel (Figure 7).

*Table 4: Expected fragment sizes of digest with BglII, * bands cannot be properly visualized under the conditions used*

Plasmid	**Fragment size [bp]**
pAB164	5020
	1621
	672
pAB165	5386
	4114
	17*
pAB166	5020
	1618
	672
pAB167	5386
	3928
	192*
	17*

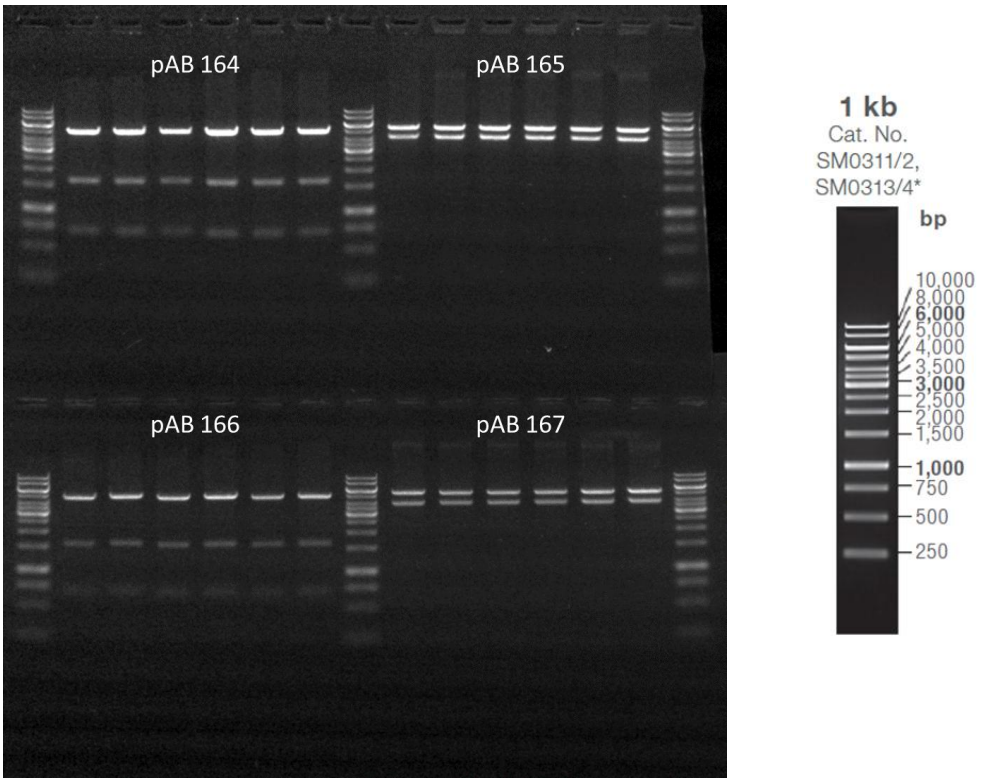


Figure 7: Confirmation of plasmid identity by BglII digest and used ladder (Thermo Scientific)

Figure 7 shows the digestion pattern of the plasmids digested with BglII. All four plasmids show bands at the expected sizes.

In order to obtain the required expression cassette with homology arms for integration in the BAC, the plasmids were digested with PacI and AsiSI and the desired bands were eluted from the gel (Figure 8).

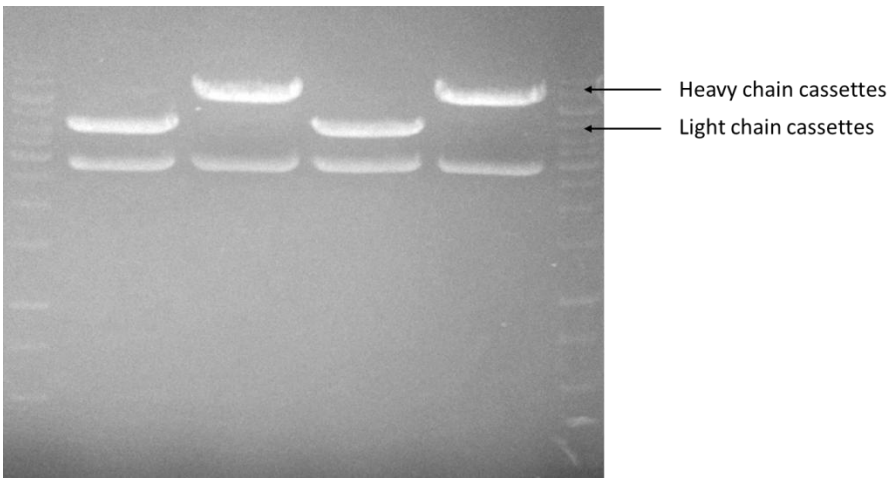


Figure 8: Agarose gel after PacI/AsiSI digest to obtain expression selection cassettes

The expression cassettes were electroporated into *DH10B E. coli* strain harboring the Rosa26 BAC and the pRed/ET plasmid which carries the recombinase genes for homologous recombination.

To verify successful recombination, a colony PCR was done on resulting bacterial colonies. The primer pair was chosen to screen the overlapping end of the BAC backbone and the inserted fragment. (Sequences are listed in the appendix.)

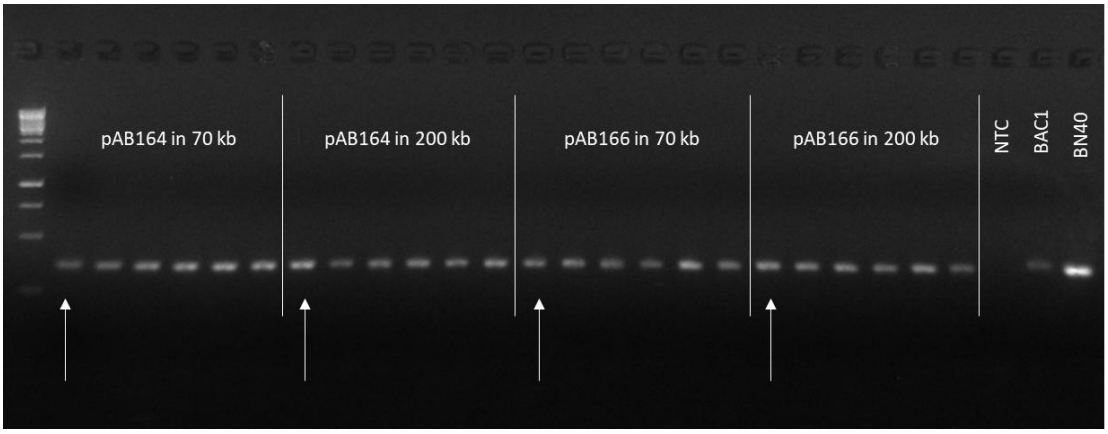


Figure 9: Verification of insertion of LC fragment of the two different antibodies into 70 kb and 200 kb BAC by PCR. NTC and BAC1 served as negative control, BN40 as positive control.

Figure 9 shows the colony PCR analysis to confirm the proper integration of the light chain fragment into the base vector by determining the status of the 5' homology arm. For all screened colonies, a band at the expected size of 340 bp is visible. Empty BAC (BAC1) and a no template control (NTC) were used as negative controls, BN40, a BAC containing the same type of expression cassettes was used as positive control. BAC1 showed a positive signal with different migration behaviour on the gel. Follow up showed that the band was unspecific probably due to unspecific primer binding.

The final confirmation of the purified BACs was done by PCR. Primer sets for checking the status of 5' and 3' homology arms in both insertion sites and also for the unmodified insertion site were used. (Sequences can be found in appendix.) The lengths of the expected fragments are listed in Table 5.

Table 5: Expected fragment size of PCR products for final BAC

Primer pair		**Binding site**	**Size correct integration**	**Size empty BAC**
AB11, AB72	(Ins0 5' homology arm)	Ins0, Ef1a	340 bp	-
AB13, AB14	(Ins0 3' homology arm)	Ins0, Neo	680 bp	-
AB11, AB14	(Ins0 unmodified)	Ins0	>4000 bp;	501 bp
AB267, AB12	(Ins1 5' homology arm)	Ins1, Caggs	256 bp	-
AB71, AB268	(Ins1 3' homology arm)	Ins1, Hygro	987 bp	-
AB267, AB268	(Ins1 unmodified)	Ins1	>6000 bp	537 bp

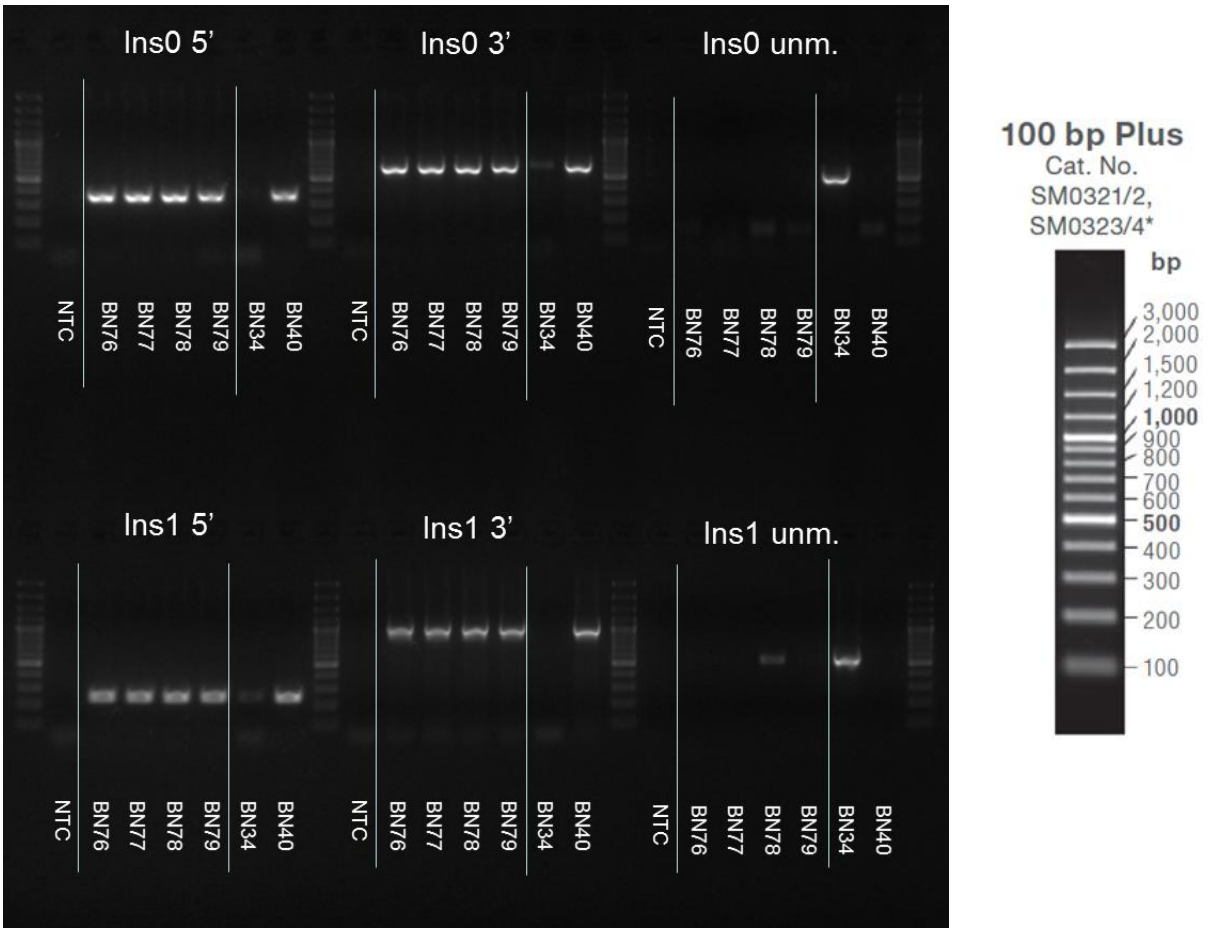


Figure 10: Confirmation of identity of purified BACs by PCR and used ladder (Thermo Scientific)

Figure 10 shows the final PCR analysis on the BACs used for transfecting CHO cells. All showed the expected size at 3' and 5' homology arms.

The primers for the unmodified site are flanking the insertion site which would result in a theoretical PCR product of 4000/6000 bp which cannot be amplified under the conditions used.

Additionally to the PCR, the purified BAC was digested with EcoRI.

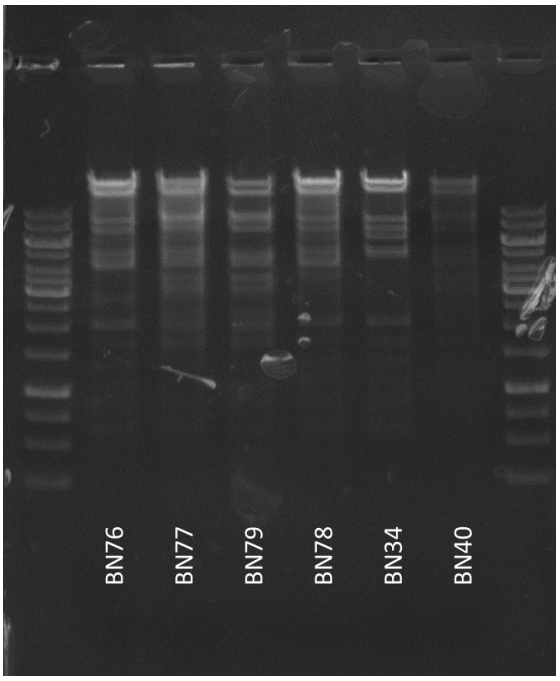


Figure 11: EcoRI digest of purified BACs

Figure 11 shows the EcoRI digested BACs. For the 200 kb BACs (BN77 and BN79), there are clearly some additional bands visible. BN34 served as control for 70 kb and BN40 for the 200 kb BAC. For BN40 an insufficient amount of DNA was loaded to be properly seen on the gel.

Table 6: Final BAC constructs

	Insertion site 0	**Insertion site 1**	**BAC Size**	**Promoters**	**Selection marker**
BN76	IgG4 light chain	IgG4 heavy chain	70 kb	EF1a; Caggs	Cam; Neo; Hygro
BN77	IgG4 light chain	IgG4 heavy chain	200 kb	EF1a; Caggs	Cam; Neo; Hygro
BN78	IgG1 light chain	IgG1 heavy chain	70 kb	EF1a; Caggs	Cam; Neo; Hygro
BN79	IgG1 light chain	IgG1 heavy chain	200 kb	EF1a; Caggs	Cam; Neo; Hygro

8.2 Linearization

To improve genomic integration efficiency, the BACs were linearized prior to transfection. Due to the large size of the BAC and the large number of potential cleaving sites for standard restriction enzymes, a specifically engineered homing endonuclease (PI-SceI) was used for linearization. This endonuclease has a DNA recognition sequence of about 40 bp, which ensures a single cut in the Rosa26 BAC. In figure 12 the single band of the linear BAC can be clearly observed.

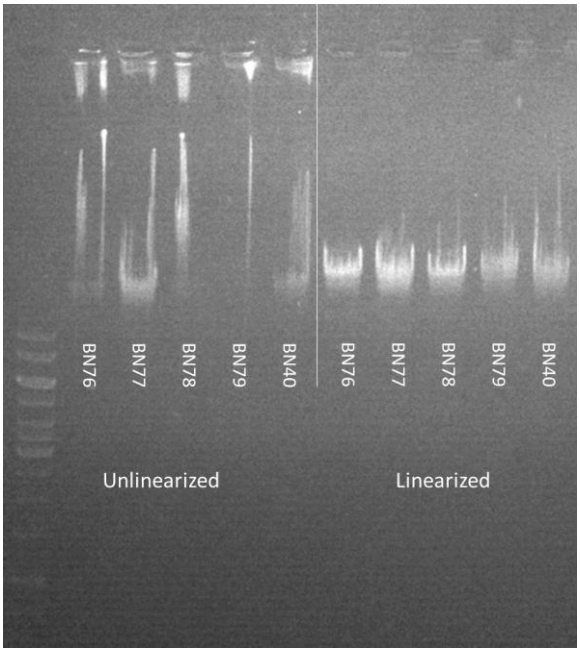


Figure 12: Verification of linearization

A large amount of the undigested BAC remained in the gel pocket, whereas the linearized BAC fully migrated into the gel.

8.3 Transfection and selection

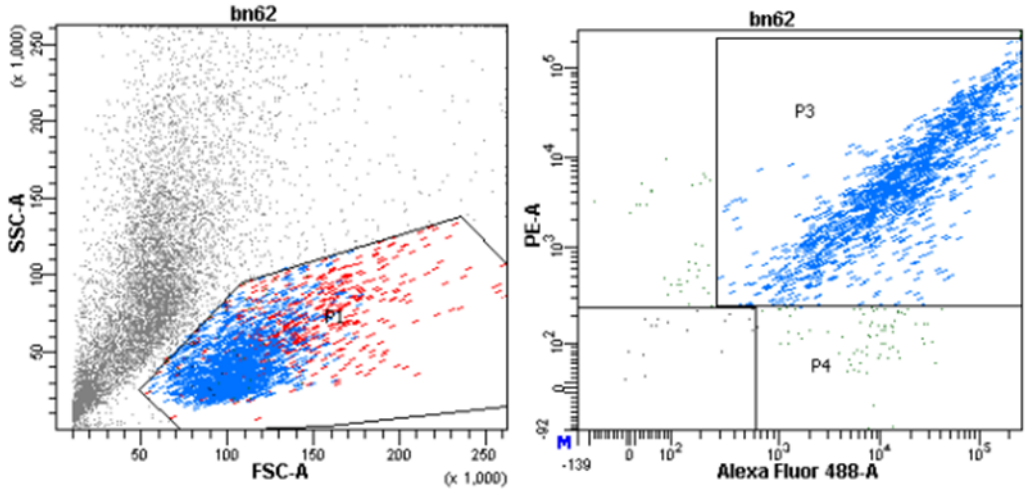
The linear BAC was stably transfected into the host cell genome by electroporation. After a recovery time of 24 h, a selection pressure of 1000 µg/mL G418 was applied. The strong selection should provide a stable pool of transfected cells as soon as possible and should therefore ensure earliest possible single cell sort.

As seen in the following figure (Figure 13) of the FACS data of a previously performed experiment, most of the cells are expressing the transgene after 7 days of selection pressure.

8.4 Single cell sorting

Monoclonal recombinant cell lines were established using fluorescence-activated cell sorting (FACS).

All sorting processes were performed at St. Anna Children's Cancer Research Institute. In the established protocol, the selected cell pools were single-cell sorted after 7 days. As previous data showed, most living cells express the transgenes after 7 days of selection (Figure 13).



Tube: bn62

Population	#Events	%Parent	%Total
All Events	10,589	####	100.0
P1	2,171	20.5	20.5
P2	1,747	80.5	16.5
P3	1,610	92.2	15.2
P4	23	1.3	0.2

Figure 13: Positive control. CHO K1 cells expressing GFP and dsRed. Fluorescent proteins were introduced into host cell genome using one BAC (Ins0: GFP, Ins1: dsRed). Sorted 8 days post transfection, 7 days of selection. Most of the living cells express the transgenes (P3) after 7 days of selection.

Sorting gates for viable cells were set on a dot plot based on forward and side scatter properties of a CHO K1 wt control (Figure 14). P2 was set in a SSC-A/FSC-W plot to discriminate single cells from doublets.

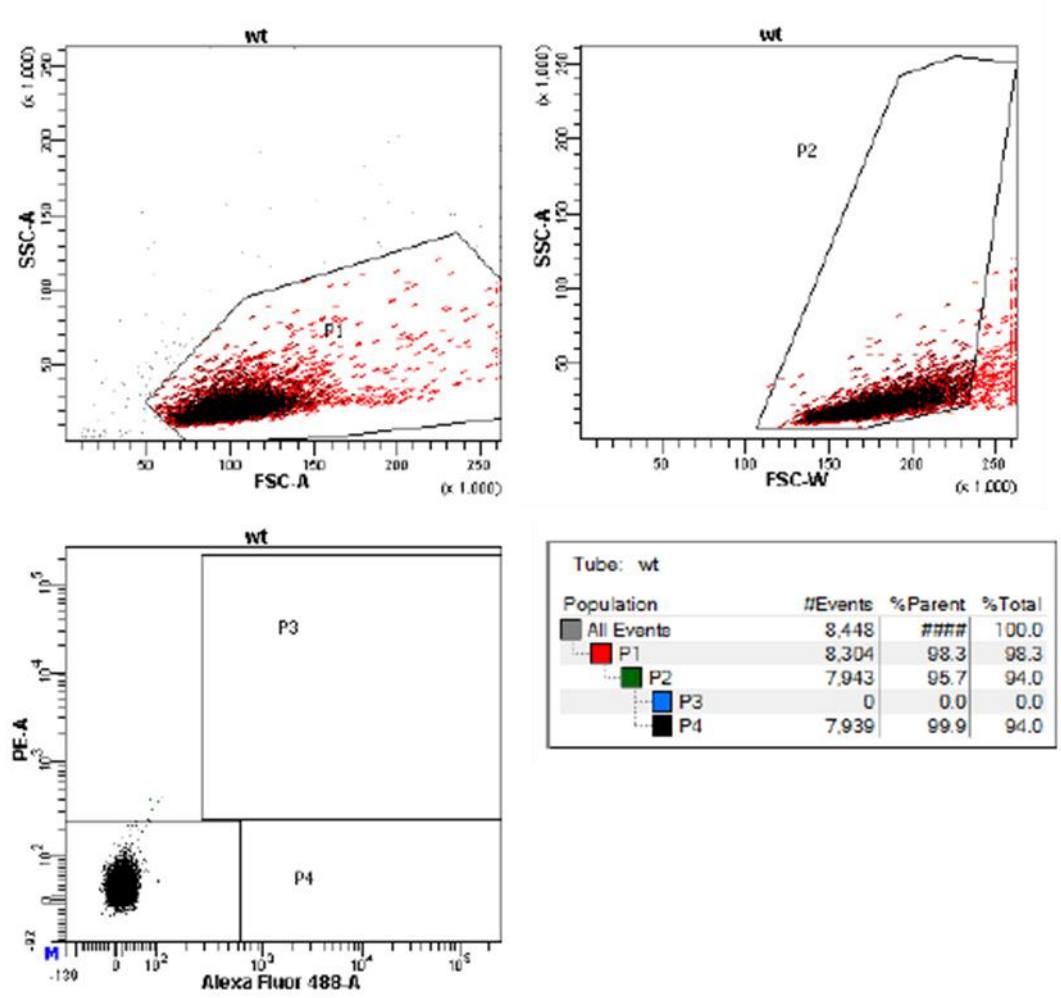
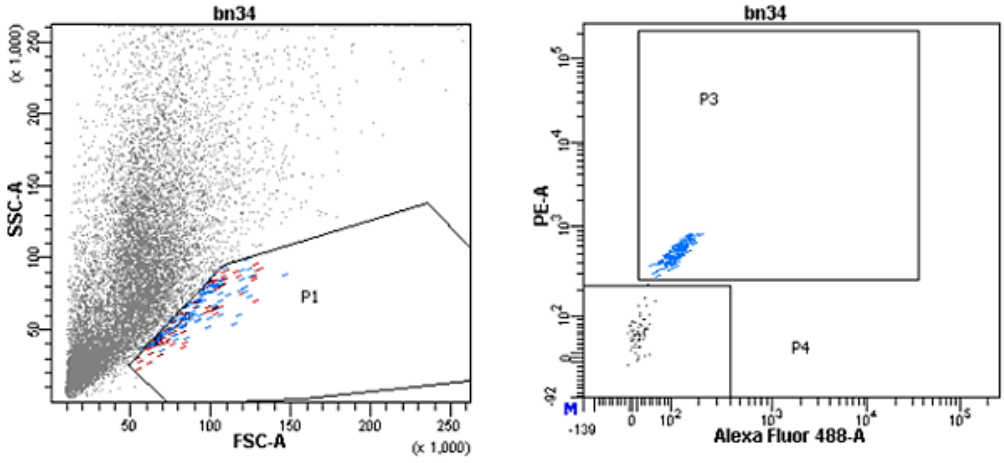


Figure 14: Gating CHO K1 wt control

Cells were sorted based on gate P4 to exclude all cells with high autofluorescence, since cells with high autofluorescence are considered dead. As can be observed in Figure 15, most cells of the negative control were dead after 7 days of selection. The remaining 1.1% that were not gated out based on FSC and SSC can be further separated into two distinct populations, one of which showed high autofluorescence (P3).



Tube: bn34

Population	#Events	%Parent	%Total
All Events	16,335	####	100.0
P1	196	1.2	1.2
P2	174	88.8	1.1
P3	109	62.6	0.7
P4	65	37.4	0.4

Figure 15: Negative control. CHO K1 cells transfected with a BAC with no inserts and no selection markers. Sorted 8 days post transfection, 7 days of selection. Most cells are dead after 7 days of selection.

Figure 16 shows the FACS data of one of the four sorted cell lines.

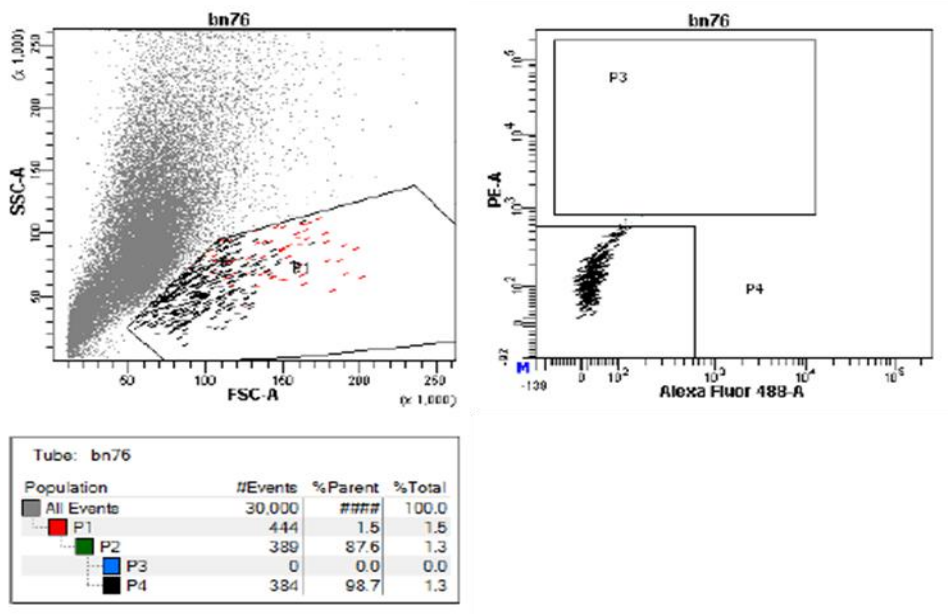


Figure 16: Example of one of the four antibodies for production. CHO K1 cells transfected with a BAC of 70 kb size, expressing IgG4.Sorted 8 days post transfection, 7 days of selection.

Table 7: Summary single-cell sorting data. P1:Cells gated as living. P4: Sorting gate, after excluding doublets and cells with high autofluorescence

		P1 (%Total)	P4 (%Total)
IgG4	BN76 (70 kb)	1.5	1.3
	BN77 (200 kb)	1	0.7
IgG1	BN78 (70 kb)	0.7	0.5
	BN79 (200 kb)	0.7	0.5

Recovery rates at the time point of single cell sorting were between 0.5 and 1.3 %. These rates seem to be very low. During the time we performed this experiment, we observed that other projects with different constructs using the same cell lines and conditions, also showed to have lower recovery rates than usual. This probably indicates a problem with the used cell lines and/or that a new aliquot of CHO-K1 wt cells should have been thawed.

8.5 Pre-screen

After single cell sorting, the cells recovered for 14 days. During that time the cells were regularly checked under the microscope and filled up with fresh stock medium if required. After 2 weeks, all colonies of a designated size were transferred into a new 96 well plate. Secretion of antibody was checked by performing ELISA and BAC copy numbers were determined using qPCR of crude cell lysates.

8.5.1 Productivities

Table 8: Recovery rates and pre-screen ELISA

	Sorted Cells	Recovered Colonies	Recovery Rate	Secreting (Pre-screen)	Secreting Rate
IgG4 70 kb	240	43	**17,9%**	38	88,4%
IgG4 200 kb	120	3	**2,5%**	2	66,7%
IgG1 70 kb	180	23	**12,8%**	12	52,2%
IgG1 200 kb	120	5	**4,2%**	3	60,0%

As shown in Table 8, the recovery rate for cells transfected with the 70 kb BAC was 12.8% IgG1 clones and 17.9% for IgG4 clones. For cells with the 200 kb BAC the recovery rate of 2.5% and 4.2% was much lower. After pre-screen ELISA, most of the colonies transfected with the IgG4 construct were secreting antibody, but only half of the colonies for IgG1 had detectable antibody in the supernatant.

At the time of single cell sort, both of the different constructs for IgG1 showed the same recovery rate, whereas the 70 kb construct for IgG4 showed to be approximately twice as high as the one for the 200 kb construct (Table 8). Two weeks after the sort, it was observed that many more of the 70 kb clones for both of the antibodies made it into stable growing colonies. This might indicate, that the bigger construct of 200 kb constitutes much more stress for the cells than the 70 kb BAC.

Due to the low number of secreting clones with the 200 kb BAC and the fact, that none of them showed gene copy numbers higher than 1 (see point 8.5.2), we decided not to include the 200 kb clones in those chosen for final production.

8.5.2 Gene Copy Number

The number of integrations of the BAC (copy number, CN) into the host cell genome was determined with a real-time PCR and calculated from three independent experiments.

Independent experiments in the research group had shown that copy numbers determined from crude lysates correlated well with those measured in gDNA from the same colonies at a later stage using a commercial kit.

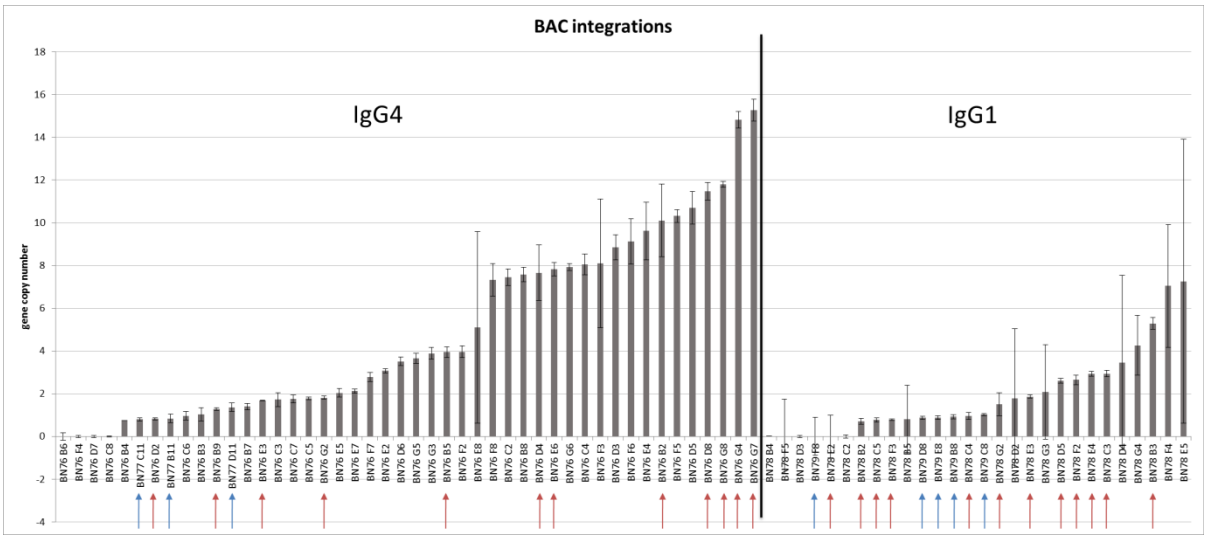


Figure 17: Gene copy number results for integration of the IgG4 and IgG1 BAC into the host cell genome. Red arrows show clones that were further chosen for production. Blue arrows are copy number results for the 200 kb BACs. Error bars indicate standard deviation. Individual results can be found in the appendix.

Gene copy numbers of clones expressing IgG4 appear to be reproducible with low standard deviations. Clones transfected with the IgG1 gene showed overall much lower gene copy numbers and very high standard deviations between the individual replicates. All samples were prepared together and under the same conditions. This variance might be caused by the construct, as the cloning showed differences in the uptake of the IgG1 construct, which was not observed for the IgG4 construct. Additionally, very low cell concentrations can influence the results and can lead to very low or even negative copy numbers due to the calculation method. Lower growth rates resulted in much higher Ct values in the qPCR raw data, which in turn leads to lower confidence in the copy number determination. Since calculating the number of integrations of the BAC is based on relating the Ct for the transgene obtained in one qPCR to the Ct value of a gene with a known number of copies in the CHO genome ("housekeeping gene") obtained in a second qPCR, small difference in PCR efficiencies can have a high impact in the final calculation. These issues are highly amplified when the Ct values are high as the error is repeated in every cycle. Indeed, the Ct values of the IgG1 clones were 3 to 4 cycles higher compared to the IgG4 clones (see appendix).

8.6 Power Production

To assess the productivity of the clones, we performed the previously established approach of Power Production in which a defined number of cells is seeded in a 96 well plate, cultivated in media with feeds and then assessed for the amount of secreted antibody and cell proliferation after several days.

While this has been shown to give a good indication for productivity in actual fed-batch production (see discussion), the method is very time- and labor-intensive and suffers from dramatic interoperator variabilities. Thus, it was aimed to compare the results obtained by this method with the much simpler and more robust qPCR approach.

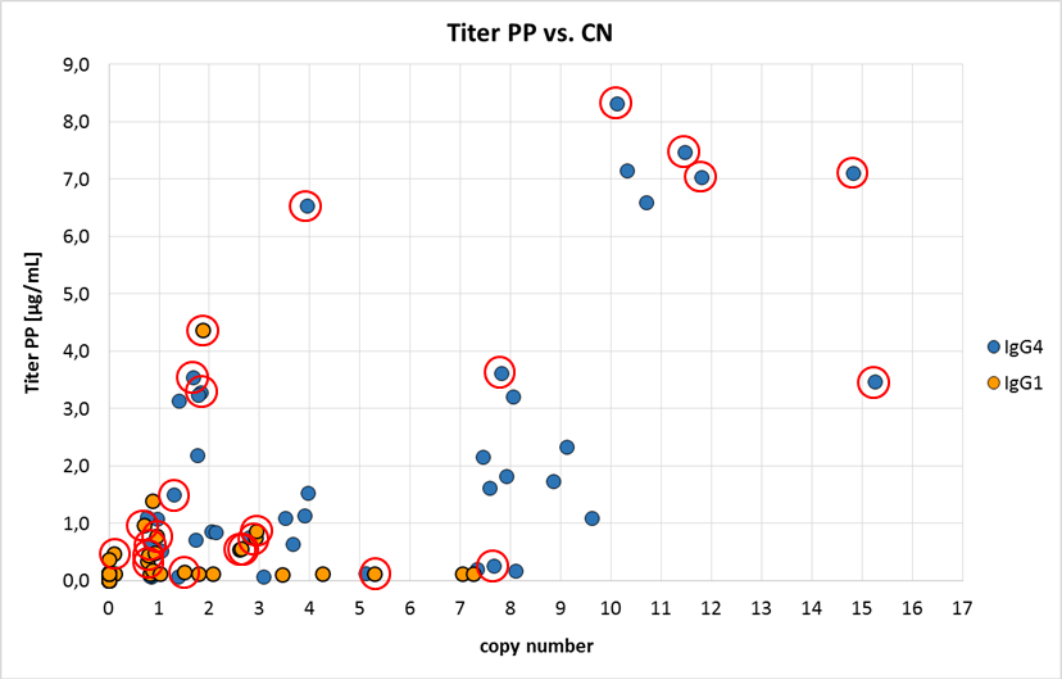


Figure 18: Power Production (PP) results: Titer PP [µg/mL] vs. CN of IgG4 (blue) and IgG1 (orange). Red circles indicate clones, which were subsequently chosen for fed-batch production.

Figure 18 shows the titer of the PP assay vs. the gene copy number. For IgG4, the clones can roughly be divided into three populations. Clones with low copy numbers (CN<5) showed low titers, which was to be expected, except for one clone with a copy number of 4. Clones with high copy numbers (CN>10) showed high titers, except for one outlier with over 15 copies. The population in between (CN=7-10) also show low titers in the Power Production. Ideally we would expect a positive correlation from copy number and titer, which is not supported by this data. The copy number seems to have no influence on the titer except for very high copy numbers of over 10.

The results for the IgG1 clones give similar results as for the IgG4 clones, although only clones with low copy numbers are present. Most of the clones show very low or even no antibody titer in the power production. In the range of very low titers and low copy numbers it is difficult to predict how they are going to behave during the actual production. Only one clone with about 2 copies showed a significant titer value. Due to the lack of high copy numbers, it is not possible to make a reliable statement for the IgG1 clones.

For the IgG1 construct only clones in the range of the lowest copy numbers for IgG4 were present. Most of the clones exhibited no or extremely low antibody titers in the supernatant in comparison to the IgG4 clones. For very low titers it is difficult to come to any useful conclusions. Only one clone with about 2 copies showed a significant titer value. To assess a (potential) correlation between copy numbers and titers, both variables would need to be present in the sample in a significantly wider range. Alternatively, a much larger sample size might solve the problem that even small uncertainties inherent in the methods used can easily mask any potential correlation.

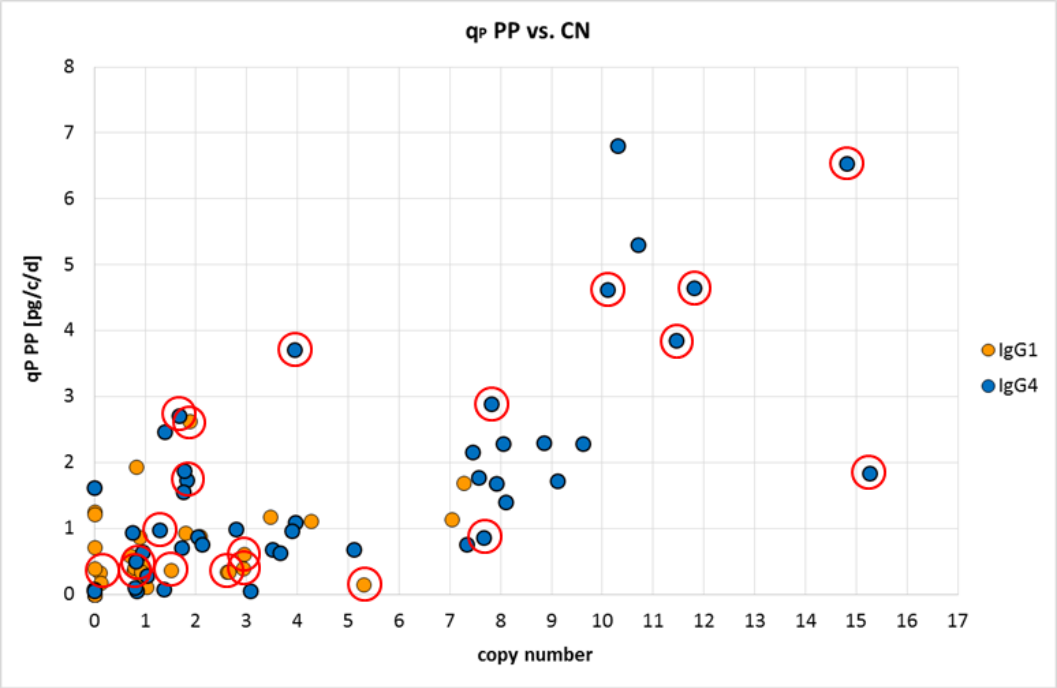


Figure 19: PP results: q_P [pg/c/d] vs. CN of IgG4 (blue) and IgG1 (orange). Red circles indicate clones, which were subsequently chosen for fed-batch production.

Figure 19 shows again the gene copy number on the x-axis, but now plotted against the specific productivity on the y-axis. As can be observed, the cells showed about the same distribution as in the CN/titer plot. Most cells with a high titer and a high copy number also showed a high specific productivity. The outlier with a copy number of about 15 showed a clear decrease in the q_P plot, which indicates that the titer was a results of a high cell number and not high productivity as would have been expected.

The population in the middle (CN=7-10) and showed to have higher q_P values compared to the population with low copy numbers. This suggests that the low titer in this population was a result of a low cell density. Furthermore, the clone with about 4 copies is less of an outlier here, which again indicates that the high titer was a result of a high cell density.

For the IgG4 clones the q_P/CN plot seems to fit slightly better into a linear correlation than the titer/CN plot.

For the IgG1 construct, it could be observed that most of the clones showed to be located a bit higher than in the titer/CN plot, which suggests that the lack of antibody is a result of poor growth of the clones. The clone with about two copies, which was the only one that showed a significant titer developed towards the population of the weakest clones, which suggests that the titer was only a result of the high cell number. Is has to be noted however that for IgG1, the clones with the highest q_P/CN were in the range of the weakest clones for IgG4, making it difficult to draw conclusions from this experiment. It would be interesting to compare 'better' clones harboring this construct at a later point. Besides that, it is also important that the cells have the ability to reach high cell concentrations and thus produce more product.

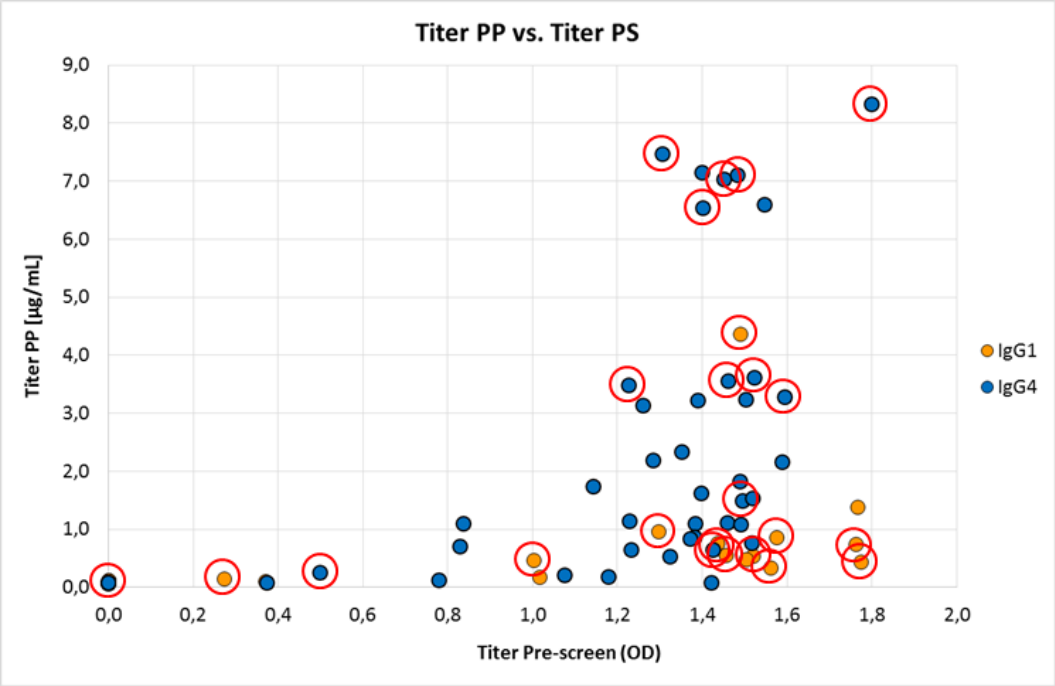


Figure 20: PP results: Titer PP vs. Titer Pre-screen of IgG4 (blue) and IgG1 (orange). Red circles indicate clones, which were subsequently chosen for fed-batch production. The OD values from the pre-screen were not normalized to the cell number.

Figure 20 shows the titer of the Power Production vs. the titer of the pre-screen. It is important to emphasize that the pre-screen can only serve as a yes/no assay for secretion of antibody as it is not normalized for the amount of cells per well or the time these cells spend producing. Thus, one would also expect colonies to correlate between assays in so far as they are determined to be (non-) producers in both approaches, but high titer in the pre-screen does not guarantee high productivity in the Power Production. Yet, elimination of clones that showed low yield in the pre-screen could be a suitable method to lower the number of clones to be screened in the Power Production, providing cheaper and more sustainable means for clone identification. This approach becomes significantly more important with a larger number of colonies emerging after the single cell sort.

8.7 Selection of production clones

For establishments of high producing cell lines, 12 clones of each antibody were picked for production. Clones that didn't show reasonable cell growth during Power Production as well as clones, that didn't show antibody in the supernatant in the pre-screen were excluded. Additionally, the influence on the gene copy number on growth and protein expression behavior was of interest. Therefore, clones with high as well as with lower copy numbers were selected.

8.8 Fed-batch production

All clones were expanded to 125 mL shaker flasks 2 days before starting the production. All clones showed sufficient growth, except for clone 18 (IgG1), which dropped in viability during the course of the expanson procedure and therefore was excluded.

In the established production protocol the cells are kept at 37 °C for the first 5 days and are then shifted to 33°C. The first feed is given on day 3, the second on day 5 and after that cells were fed every day and harvested on day 11.

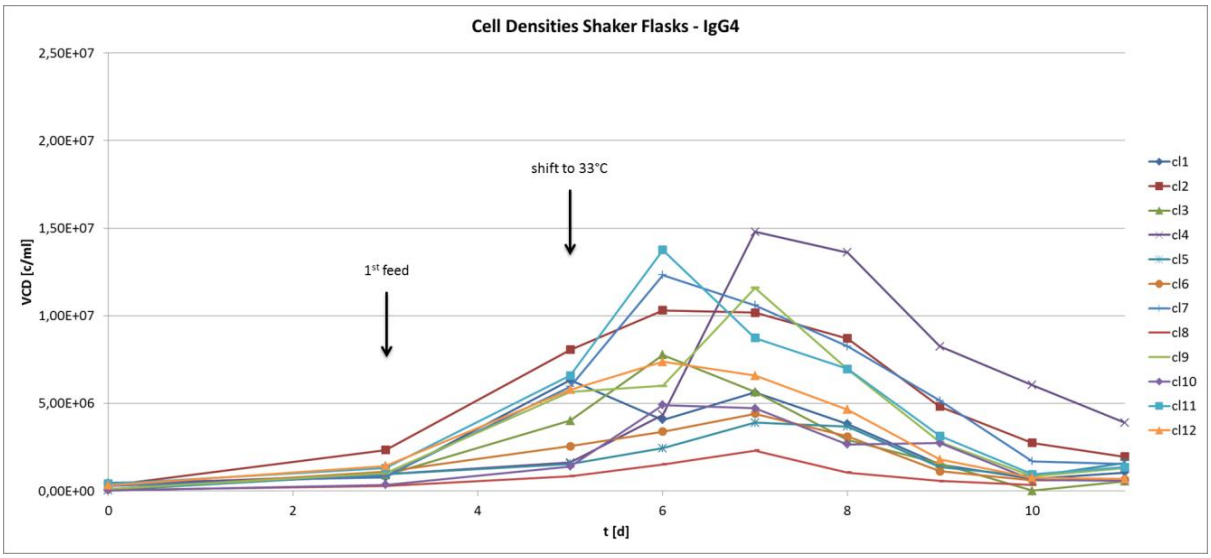


Figure 21: Production results: Living cell count [c/mL] IgG4

Figure 21 shows the viable cell concentration of the IgG4 clones during the production in the shaker flasks. After the first feed on day 3, a clear increase in cell density can be observed. After the temperature shift to 33 °C, all clones still increased in their cell numbers until the maximum cell densities was reached between day 6 and day 7, which were in the range of $2.3 \cdot 10^6$ and $1.5 \cdot 10^7$ c/mL.

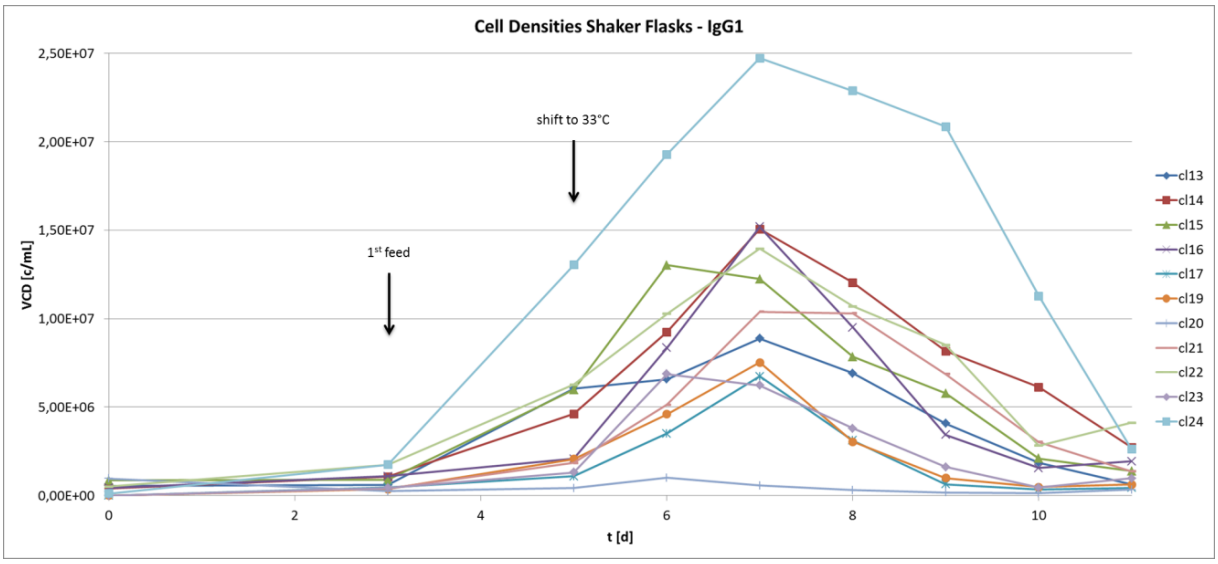


Figure 22: Production results: Living cell count [c/mL] IgG1

Figure 22 shows the viable cell densities of the IgG1 clones. They continued to grow after the temperature shift until all clones reach their peak density on day 7, except for clone 20, which seemed to be in a growth arrest. The peak cell densities were between $1 \cdot 10^6$ (clone 20) and $2.5 \cdot 10^7$ c/mL (clone 24).

It can be observed that most of the clones grew until day 7 of production and started to transit into decline phase after that time point. The highest viable cell densities were between $1 \cdot 10^6$ and $2.5 \cdot 10^7$ c/mL.

The graphs indicate that the IgG1 clones reached slightly higher cell densities than the IgG4 clones. Additionally the viable cell count of the IgG1 clones seems to drop slightly later than the cell count of the IgG4 clones.

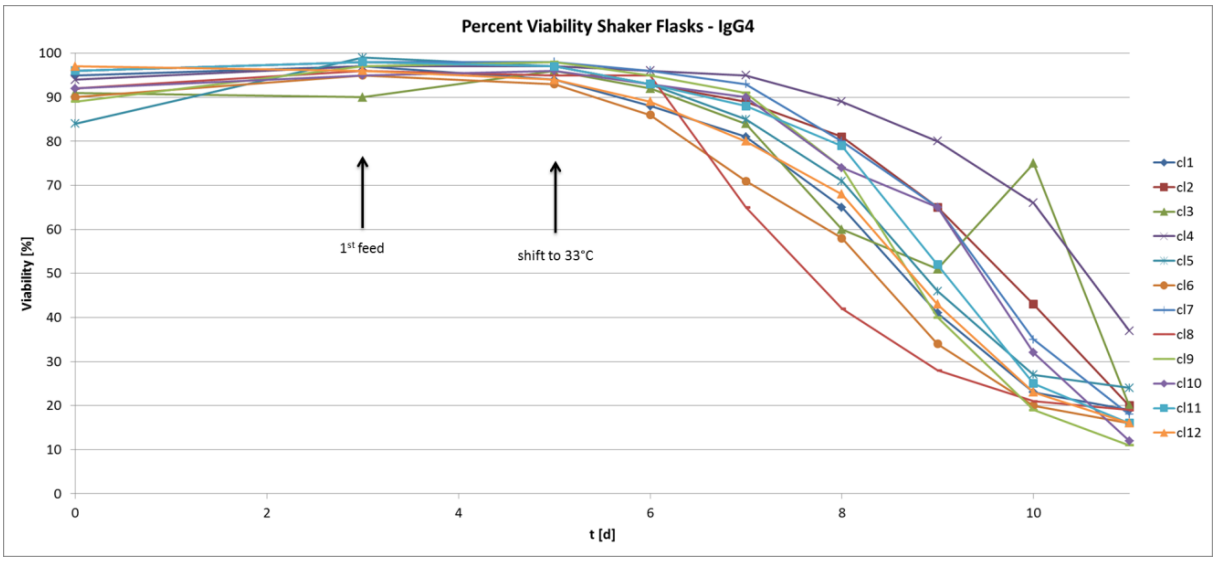


Figure 23: Production results: Viability IgG4

The viability on the first days of production was between 90 and 99%. After temperature shift on day 5, the viabilities declined rapidly every day. On the day of harvest the viabilities had dropped to a value of 11 to 37%.

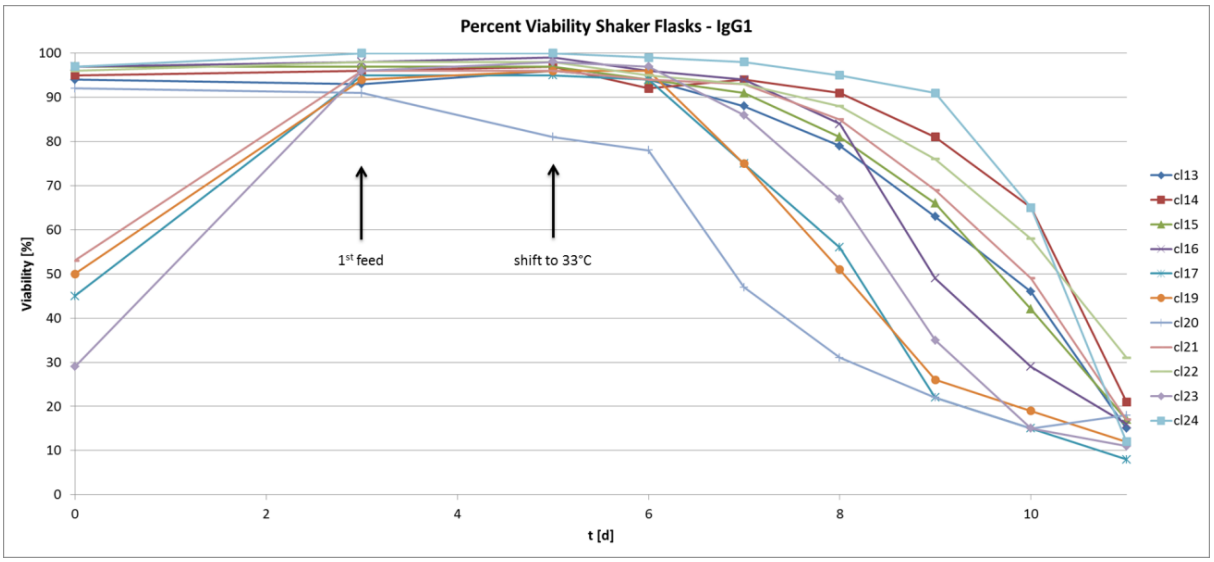


Figure 24: Production results: Viability IgG1

On the first days of production the viability of all clones was in the range of 93 to 100%, except for clone 20, where the viability dropped quickly after day 3, and which showed poor growth during the whole production. The other clones kept stable viabilities until day 6 and declined after that. On the last day, the viabilities dropped to values between 8 and 31%. The viabilities under 60% on day 0 are assumed to be a measurement error, since the values were normal for these clones before seeding. Also on day 3 they are again in the normal range between 90 and 100%.

The figures show that the IgG1 clones remained at higher viabilities for approximately one day longer.

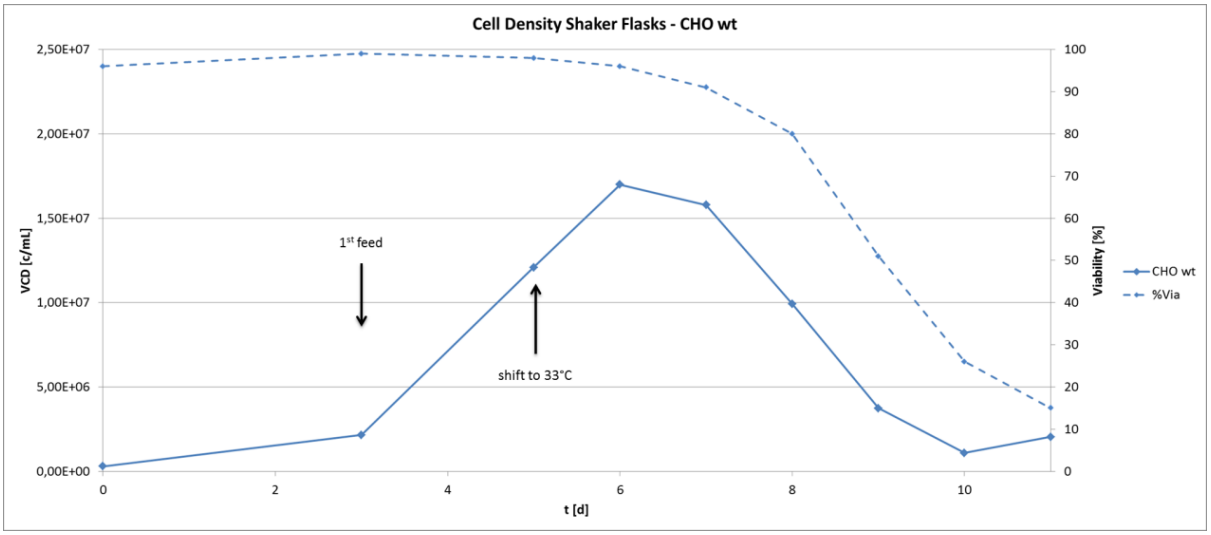


Figure 25: Viable cell count and viability of CHO wt control

The CHO wt control reached the highest cell density on day 6 with a concentration of $1.7 \cdot 10^7$ c/mL. The viability on the first days ranged between 96 and 99% until day 6 and declined until it reached a value of 15% on the day of harvest.

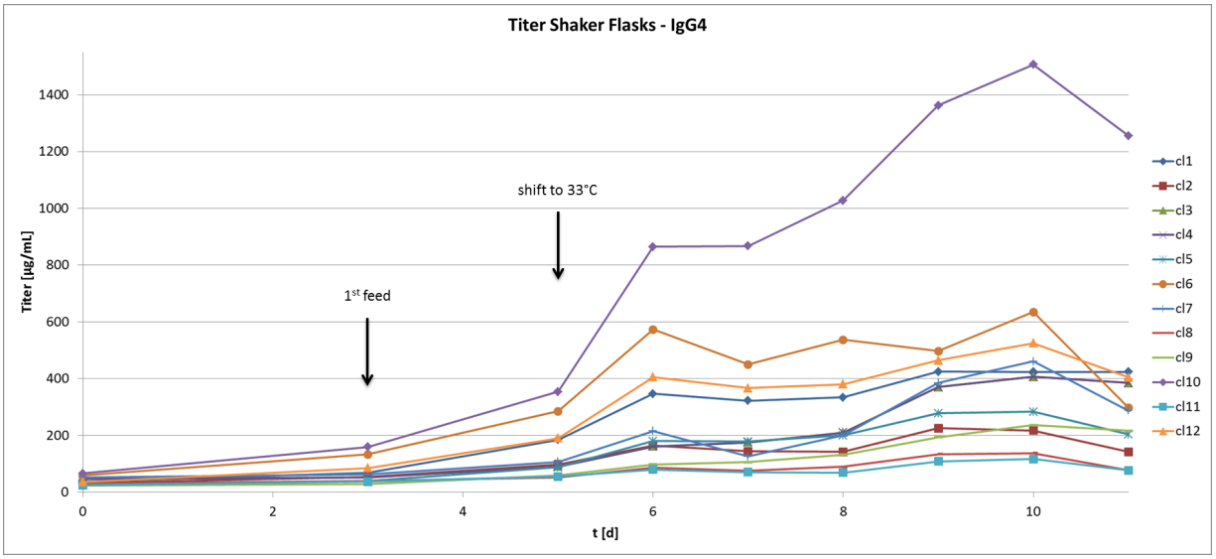


Figure 26: Production results: Titer curves IgG4

The end titer of the highest producing clone (clone 10) is about 1.2 mg/mL. The final titer values of the other IgG4 clones at the day of harvest ranged between 77 and 423 µg/mL (clone 14 and 16).

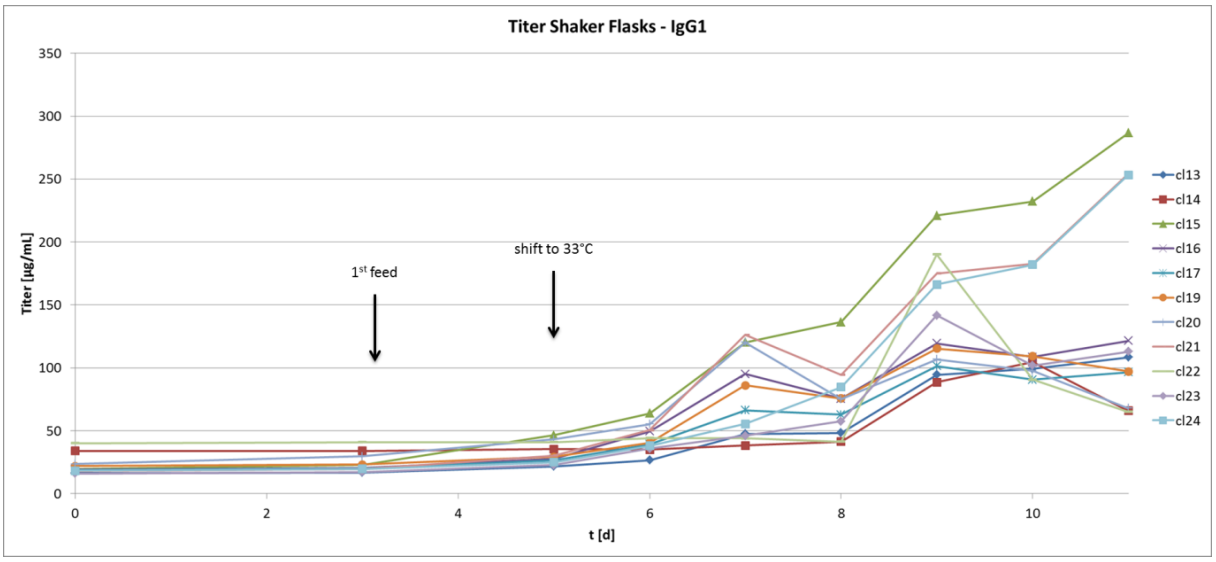


Figure 27: Production results: Titer curves IgG1

For IgG1, the final product titer of the three highest producing clones (clone 15, 21 and 24) were 286, 254 and 253 µg/mL respectively. The titer values of the other clones varied between 66 and 121 µg/mL, which is much lower than for IgG4.

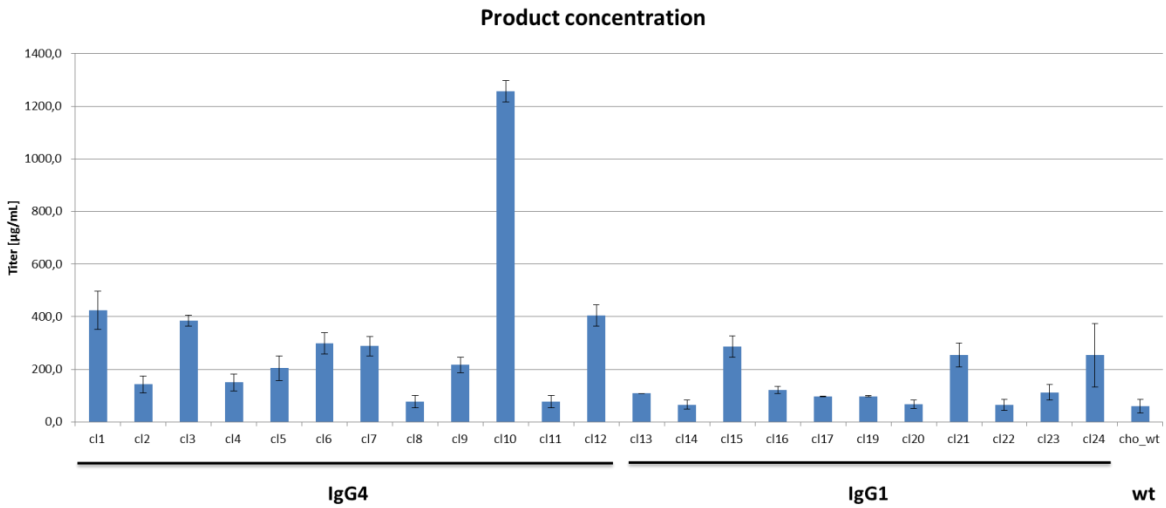


Figure 28: Production results: Final antibody titer. Titers were determined by Coomassie staining measured in three technical replicates. Error bars indicate standard deviation. Results are shown without subtraction of background; therefore the CHO-K1 wt control also shows a small signal.

Figure 28 shows end titer values of all clones. In general, the IgG4 clones showed higher titers than the IgG1 clones. Despite comparable cell densities in fed-batch production, final titers for IgG4 clones were significantly higher (median: 252 µg/mL) than for IgG1 clones (median: 109 µg/mL), many of which were barely above the blank. This is in agreement with the image that emerged after Power Production and pre-screen.

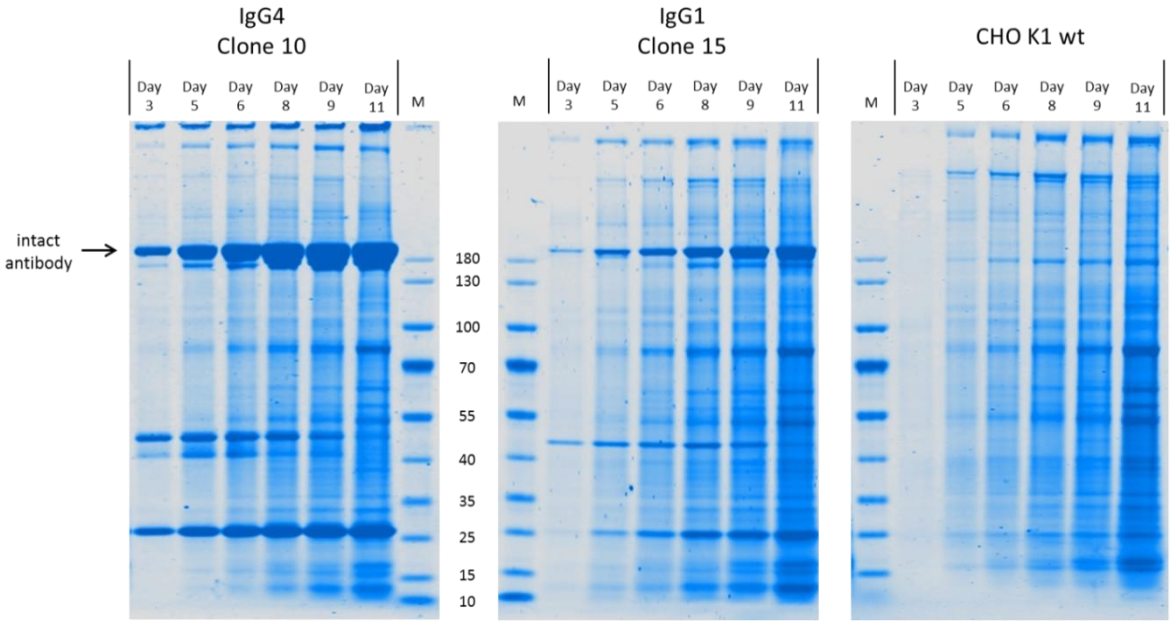


Figure 29: SDS-PAGE results of Clone 10 (IgG4), Clone 15 (IgG1) and CHO K1 wt control. Marker: PageRuler pre-stained protein ladder, Thermo Fisher Scientific.

Figure 29 shows the non-reducing SDS-PAGE results of the supernatants of the two highest producing clones for each antibody and also for the CHO wt. Especially clone 10 from IgG4 shows 2 significant bands in addition to the one for the intact antibody, one around 25 and around 50 kDa.

The smaller band in particular is difficult to identify as some semblance of it can also be seen in the CHO wt. It might however indicate the presence of unincorporated light chain. It has been shown previously that CHO cells may secrete free light chain [38]. While the size of the larger band would be in agreement with heavy chain, its behavior during the course of the production is somewhat unusual as its intensity decreases over time. One possible explanation for this would be the degradation of this protein by enzymes released during lysis.

A significant amount of host cell protein is seen, probably released from dying cells, which increases over time of the production, which also correlates with a significant drop of viability beginning on day 7.

The antibody band is here shown to be above 180 kDa. We observed that the highest molecular weight band for this marker did not fit the stated molecular weight compared to other markers (data not shown).

8.9 24 deep well plate production

The data from the 24 deep well plate (dwp) productions turned out to be inconclusive. As seen in Figure 30, the cells in the 24 dwp showed poor growth in comparison to the cells in shaker flasks. The viabilities (data not shown) dropped rapidly to 60% and below after production start.

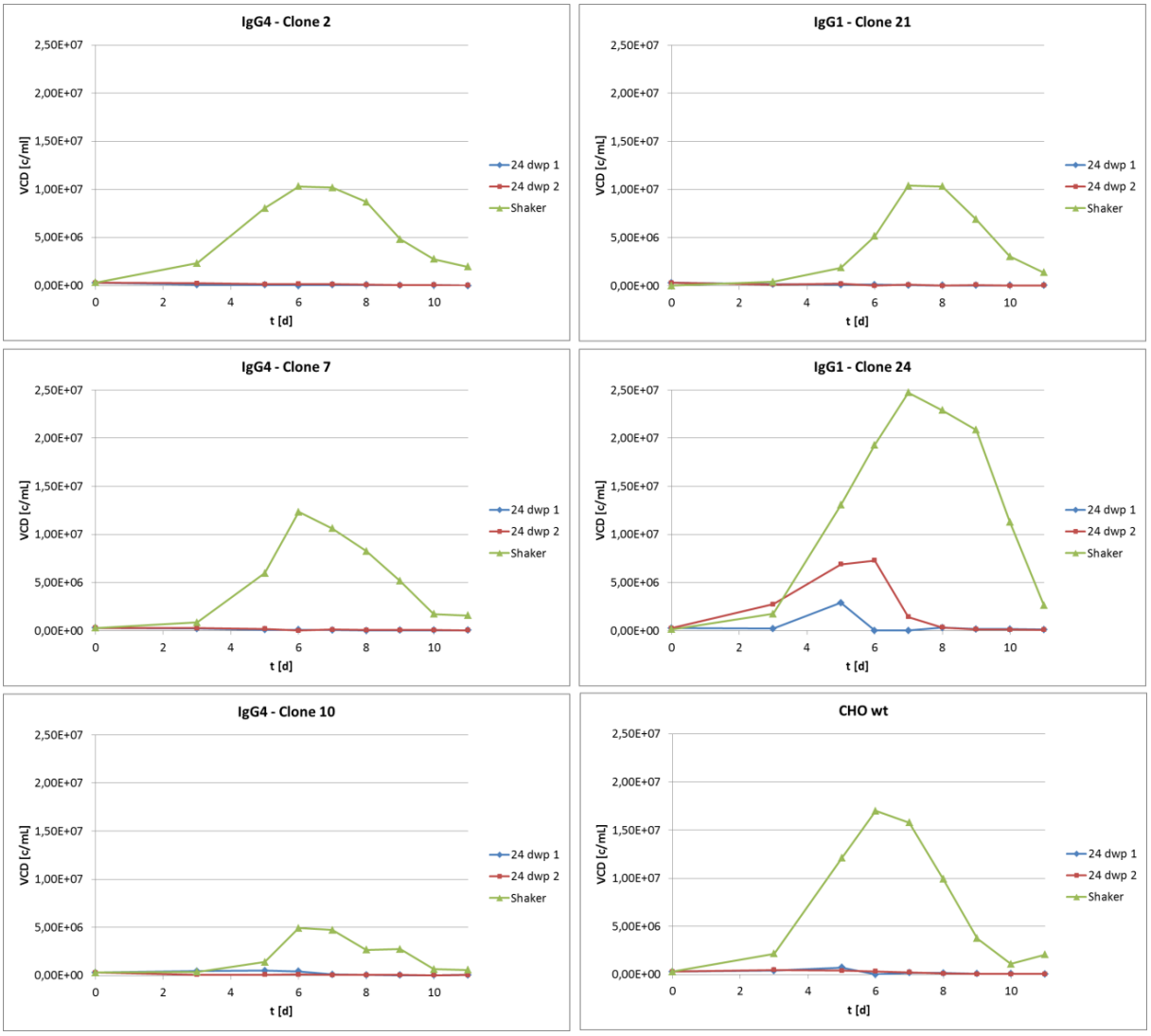


Figure 30: Production results: Comparison of growth behavior between 24 dwp and shaker flask. Exemplary data of growth behavior of 6 clones. The other 18 clones also showed no growth under the conditions used for 24 dwp production.

Figure 30 shows the comparison of the growth behaviors of cells in shaker flasks (green line) and cells in 24 dwps (red and blue lines). It can be clearly seen that the cells did not grow properly in the deep well plates. Only clone 24 reached a maximum living cell concentration of $7 \cdot 10^6$ c/mL but dropped immediately after that.

The data shows that it is not possible to grow cells under the applied conditions. Possible reasons could be that due to the low shaking rate, the cells sedimented on the bottom of the plate, respectively the lack of required oxygen due to insufficient mixing. The conditions for a 24 dwp production need to be optimized in further experiments considering these facts.

8.10 mRNA Levels

qPCR analysis of mRNA levels was performed for three of the IgG4 clones and four of the IgG1 clones as well as the CHO-K1 wt control. Samples were drawn on 5 different days and analyzed in two technical replicates with β-Actin as internal standard.

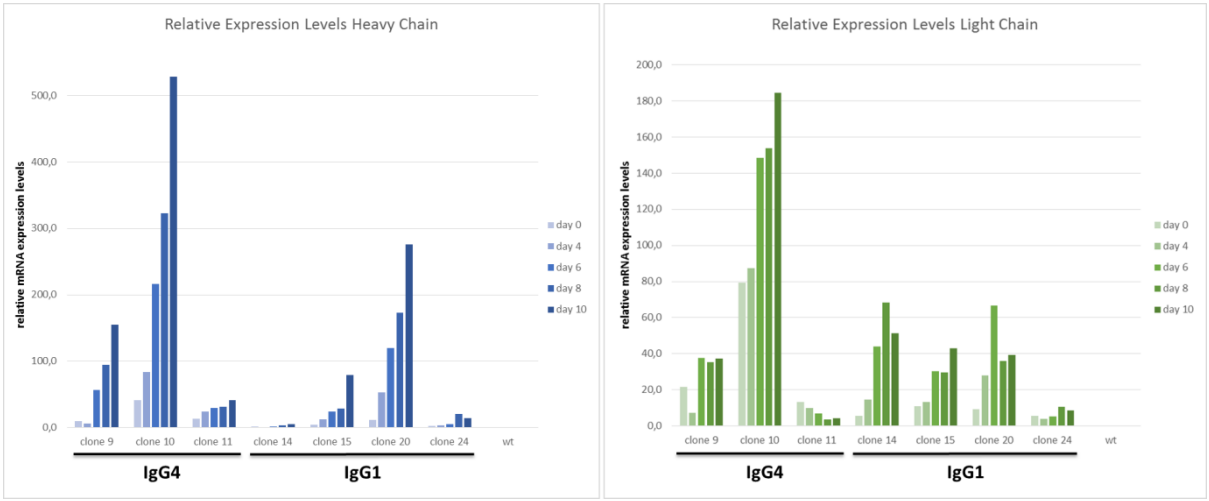


Figure 31: Production results: Relative Expression Levels of HC and LC normalized to β-Actin for IgG4 (left) and IgG1 (right). Relative expression levels are shown as $2^{-\Delta Ct}$

For the majority of clones, the relative expression levels compared to β-Actin of both heavy and light chain increased significantly over the course of the fed-batch process.

IgG4 clone 10, which was the highest producing clone during production, showed the highest values of relative mRNA expression of all tested clones and showed an increase in both heavy and light chain expression during production. The relative expression data of clone 9 and clone 11 correlates with the titer data, since clone 9 showed more than twice as much antibody in the supernatant at the end of production than clone 11. The low relative expression levels of clone 11 indicate a major problem with transcription of the construct in this clone, due to the fact that clone 10 and clone 11 had the same copy number in the qPCR.

For clone 15, the highest producing IgG1 clone, the increase in both heavy and light chain can be observed during production. The final titer value of clone 15 showed to be in the range of the titer of clone 9 and so did the relative expression levels. Clone 14, which was the IgG1 clone with the lowest titer value, showed good light chain expression but almost no heavy chain expression. When heavy and light chain levels are unbalanced the assembly and thus secretion of antibody is limited, which is finally reflected in low titer values. Clone 20 showed good relative expression levels, which is also supported by a relatively high q_P value, but due to the poor growth of the cells, almost no antibody could be detected in the supernatant. For clone 24 the relative mRNA expression levels were very low, which results in a low q_P. The decent titer value, which was the second highest of the IgG1 clones, is the results of the high cell density.

8.11 Summary

In this project, we used two different methods to identify the highest producing clones of antibody-producing CHO clones as rapidly and conveniently as possible. There are various approaches to identify high producing clones. Besides selection and amplification methods, there are also FACS and flow cytometry-based methods and fully automated approaches. The drawbacks are that these methods are often very time consuming and/or need very expensive equipment [41, 42].

The established protocol included a Power Production assay, which involves seeding all clones with the same cell number in a 96 well plate format, incubating it for 3 days and testing for antibody and cell concentration afterwards.

In the new approach, the aim was to show whether this technically challenging, long and labor-intensive process could be replaced by analyzing only the gene integration numbers of our gene of interest. Therefore, the protocol of gDNA analysis was adapted. The new approach using crude cell lysate instead of isolated gDNA resulted in robust results.

Table 9 shows an overview of the old and new approach and the expected time intensities.

Table 9: Time overview of the old Power Production approach and the new crude lysate qPCR approach

	Power Production		**qPCR screening**	
Setup	Transfer cells	0.5 h	Transfer cells	0.5 h
	Count cells	2 h		
	Seed at same density	2 h		
Run	Incubate	3 days	Lyse cells	1 h
			qPCR	2 h
Obtain results	Count cells	2 h		
	ELISA	4 h		
Analyse	Combine data	1 h	Analyse qPCR data	1 h
		83.5 h		4.5 h

This crude high throughput approach was later confirmed to give consistent results in also biological replicates (personal communication), but nonetheless does not offer the same degree of confidence as more sophisticated approaches. Many of these advanced approaches are however not feasible to perform economically in a high throughput manner.

Based on the results, the clones for fed-batch antibody production were selected. Here, clones throughout the full range of copy numbers were chosen. After the production, the data of the Power Production and the gene copy number analysis were compared with the production results.

Table 10: Summary table of all production clones, sorted from highest to lowest q_P (fed-batch). VCD_{max}: maximum viable cell density during fed-batch. GCN: gene copy number. Titer: Antibody in the supernatant on day 11. q_P fed-batch: average q_P from day 0 to day 7, because after day 7 the viability and cell concentration drops so drastically, that q_P values decrease to values below zero.

	Clone	VCD_{max} [c/ml]	GCN	q_P PP [pg/c/d]	q_P fed-batch [pg/c/d]	Titer [µg/mL]	HC	LC
IgG4	cl 10	4.9E+06	15	6.5	111.8	1256.0	528.8	184.4
	cl 6	4.4E+06	11	3.9	33.6	297.9		
	cl 12	7.4E+06	12	4.6	19.5	405.2		
	cl 1	6.3E+06	10	4.6	9.8	423.8		
	cl 8	2.3E+06	8	2.9	9.8	77.3		
	cl 3	7.8E+06	1	1.0	8.8	384.7		
	cl 7	1.2E+07	2	2.7	8.2	288.2		
	cl 5	3.9E+06	8	0.9	8.1	204.2		
	cl 9	1.2E+07	2	1.7	4.9	216.7	155.2	37.3
	cl 11	1.4E+07	15	1.8	2.7	77.4		
	cl 2	1.0E+07	4	3.7	2.3	142.6		
	cl 4	1.5E+07	1	0.5	1.0	150.1	41.0	4.3
IgG1	cl 20	1.0E+06	2	2.6	11.0	68.0	275.6	39.2
	cl 21	1.0E+07	3	0.4	6.1	254.1		
	cl 17	6.7E+06	1	0.4	5.3	96.5		
	cl 15	1.3E+07	3	0.6	3.8	286.7		
	cl 23	6.9E+06	1	0.4	3.7	112.8		
	cl 19	7.5E+06	1	0.3	3.3	97.1	79.3	43.0
	cl 16	1.5E+07	1	0.4	3.2	121.5	14.3	8.7
	cl 13	8.9E+06	1	0.6	1.4	108.5		
	cl 24	2.5E+07	2	0.4	0.9	253.4		
	cl 14	1.5E+07	5	0.1	0.3	65.9		
	cl 22	1.4E+07	3	0.3	0.0	65.0	5.3	51.2

Table 10 shows the summary of the data gathered during fed-batch production. The specific productivity of IgG4 and IgG1 clones is shown in comparison to the GCN, maximum VCD and titer, and the corresponding q_P result of the Power Production.

For IgG4, it was shown that all clones with high q_P also showed high gene copy numbers. However, there were also high copy number clones that did not show high q_P which results in low product yield. Since transcription, measured by qPCR is only the most upstream step in protein production, low protein expression despite high expression levels are to be expected in some clones due to potential bottlenecks in more downstream steps like folding or secretion.

Clones with low copy numbers reached slightly higher viable cell densities, which may be caused by the stress of protein production. Still, it is an important factor for the overall antibody yield in the supernatant and needs to be considered. This can be seen for clone 20, which shows the highest q_P for the IgG1 clones, but almost no antibody due to its poor growth.

The IgG1 clones showed no correlation between gene copy number and q_P, which is presumably caused by problems with the construct/antibody sequence, which was mentioned previously. Also IgG1 clones had in general much lower antibody titers, despite almost similar cell densities.

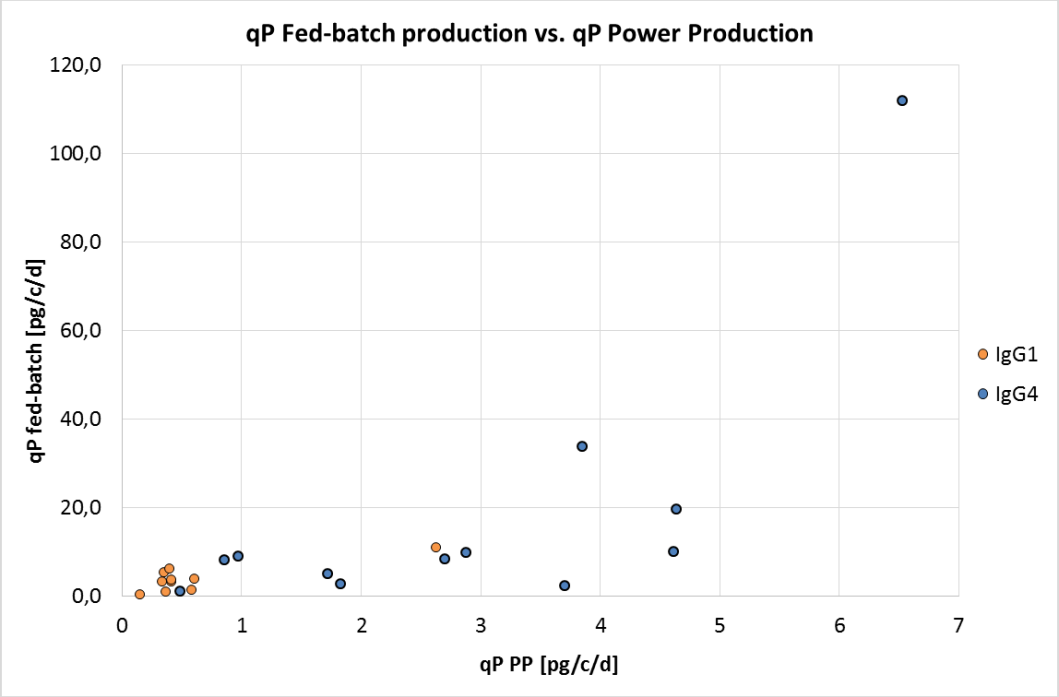


Figure 32: q_P fed-batch production vs. q_P Power Production

Figure 32 shows the specific productivity of IgG1 and IgG4 clones in the power production compared to the productivity of those clones in the fed-batch. The q_P of the IgG1 clones in the PP was generally low and the data for this antibody was inconclusive. As mentioned earlier, this underlines the conclusion, that there might be a problem with the construct. IgG4 clones that were highly productive in the PP (q_P > 3 pg/c/d) showed a tendency to be productive in the fed-batch. While there is some level of correlation, the limited number of clones makes it difficult to determine whether there is a clear linear relationship.

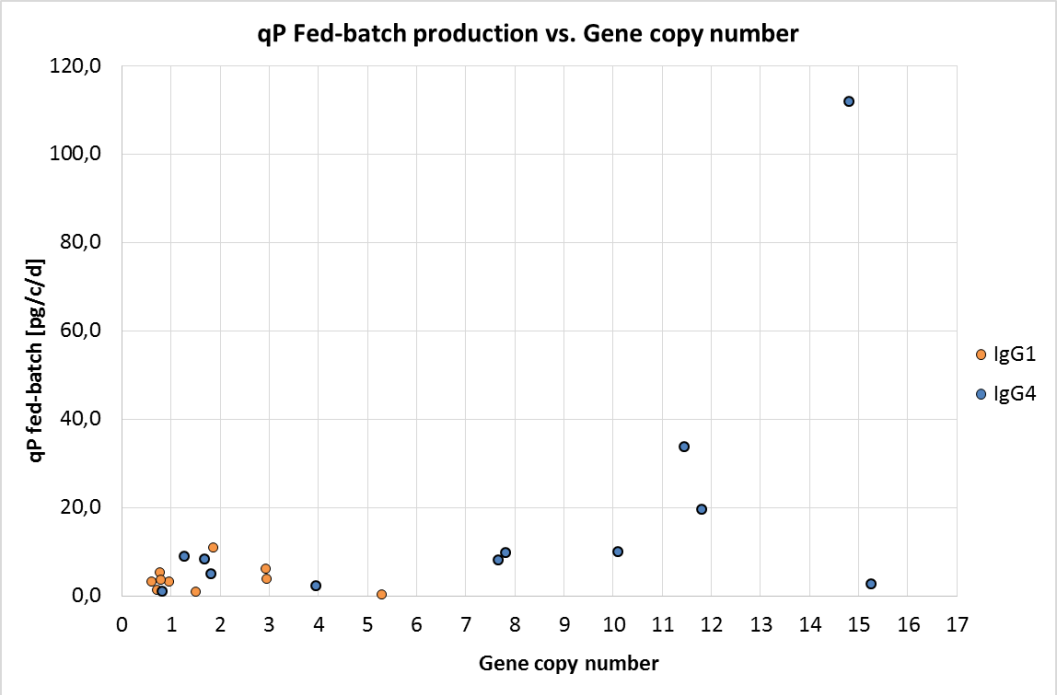


Figure 33: q_P fed-batch production vs. gene copy number.

Figure 33 compares the number of BAC copies in the cells with the specific productivity in the fed-batch. Clones with low copy numbers of IgG4 turned out to have a low specific productivity whereas all highly productive clones were having also high GCN, except for one clone with 15 copies. This clone (clone 11) also showed to have almost no mRNA, which suggests a problem during transcription. This indicates that the copy number is a valuable parameter to sort out clones with low productivity without losing the highest performing ones, thus reducing the necessity of extensive screening.

9 Conclusion

This thesis describes the successful expression of two different antibodies using bacterial artificial chromosomes (BACs) and selection of high-producing clones.

The implemented protocol includes the Power Production assay, which is based on determining the specific production rate over 3 days. Previously, this assay showed to be a reasonable tool for the selection of clones. Nonetheless, it is very labor and time-consuming, particularly when the number of clones is very high. Therefore, a new approach was used that increased the efficiency significantly.

The new approach is based on screening the gene copy number of the gene of interest using real-time PCR of crude cell lysate. It has previously been shown that expression levels correlate well with the number of integrations of the BAC in the host cell genome [37]. This is due to the fact that BACs are large cloning vectors with an open chromatin locus. This prevents the silencing of the integrated gene section and is believed to isolate against effects of the host cell genome at the site of integration, which is often the problem when using smaller vectors, such as plasmids. In addition, BACs are stable over time and do not need gene amplification [37, and unpublished data], whereas plasmid-derived clones have high variability over time, resulting in a need to assess expression levels over different generations [38].

Here, it was demonstrated that this technique generates robust results and permits the identification of highly productive clones. Although the results did not show consistent correlation between gene copy number and specific productivity, the method is a suitable tool to reduce the number of clones that need to be screened subsequently. Furthermore, it facilitates to increase the initial number of clones after transfection, thus increasing the likelihood of identifying high producers with the desired properties in subsequent steps.

It should also be considered that too high copy numbers may also not be favorable, since protein production means a lot of stress to the cells, and high producers often have a lower growth rate than low- or non-producers [42]. Also, translation, maturation and secretion play a major role in context of cell productivity and are most likely protein specific [36].

In the experiments described here, CHO cells transfected with the full-length (200 kb) BAC did not recover properly after sorting. These results differ from other 200 kb BAC derived clones [unpublished data], which can indicate a problem with the host cell line resulting in higher sensitivity to the stress induced by the bigger construct or alternatively a problem with the BAC preparation or transfection in this particular case.

Additionally, it should be considered that both model antibodies have never been tested in the lab before and the new approach should be optimized and adapted. Furthermore, unusual problems with lower recovery rates were observed. This was also seen in other projects with different constructs during the time of performing these experiments. This might indicate problems with the thawn vial of the host cell line used during this time. As other factors like DNA quality, transfection and culture conditions can influence the quality and quantity of the antibody production process, these results should be considered as preliminary.

Nonetheless, using copy numbers to perform a preliminary ranking of production clones appears to be an efficient tool, greatly reducing the amount of effort required for this critical step.

Finally, it should be kept in mind that recombinant protein production is a complex process, which can be influenced by various factors and that no single method can give us a complete picture yet.

10 References

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13 Appendix

13.1 Primer list

Table 11: Primer sequences for PCR on BAC

AB11	CCAACACAGATGAGCCTAAGCC
AB12	AACTAATGACCCCGTAATTGATTAC
AB13	CATCGCCTTCTATCGCCTTCTTG
AB14	AACCTGAGCCAGACTTTCCACTGCAATATC
AB71	CTGGACCGATGGCTGTGTAGAA
AB72	CTCTAGGCACCGGTTCAATTGCCG
AB267	CTTGGTAGAAGCAGAGGACACG
AB268	CTTCCAGAGCCGACATCCAGATG

Table 12: Primer for mRNA PCR

IgG1 IgG4 LC rev	GGCGTACACCTTGTGCTTCT
IgG1 IgG4 LC for	CCAAGAGTCTGTGACCGAGC
IgG1 IgG4 HC rev	ATCACGGAGCAGGAGAACAC
IgG1 IgG4 HC for	TCCGATATCGCCGTGGAATG

Table 13: Primer for β-Actin

AB73 fw	ACTB CGTACCACTGGCATTGTGAT
AB74 rv	ACTB GGCAACATAGCACAGCTTCT

13.2 qPCR raw data

Table 14: Gene copy number analysis. qPCR raw data of production clones. Negative copy numbers resulting from high Ct values were set to 0.

Clone	Ct qPCR1	Ct qPCR2	Ct qPCR3	Ct mean	GCN qPCR1	GCN qPCR2	GCN qPCR3	GCN mean
cl 1	21,8	21,8	21,6	21,7	11,2	7,7	11,4	**10**
cl 2	23,9	23,9	23,6	23,8	4,1	3,6	4,1	**4**
cl 3	24,1	23,8	24,3	24,0	1,2	1,3	1,4	**1**
cl 4	25,7	25,4	25,5	25,5	0,9	0,8	0,9	**1**
cl 5	31,6	29,7	31,0	30,8	6,0	7,9	9,1	**8**
cl 6	22,9	22,5	23,0	22,8	12,0	11,1	11,3	**11**
cl 7	23,2	22,6	23,1	23,0	1,7	1,7	1,7	**2**
cl 8	22,7	22,4	22,9	22,7	8,3	7,6	7,6	**8**
cl 9	23,3	22,8	23,0	23,0	1,9	1,7	1,9	**2**
cl 10	22,8	22,7	22,8	22,8	15,2	14,9	14,3	**15**
cl 11	23,5	23,4	23,4	23,5	15,4	14,6	15,8	**15**
cl 12	23,2	22,6	23,2	23,0	12,0	11,6	11,8	**12**
cl 13	26,1	25,6	25,9	25,9	0,8	0,5	0,8	**1**
cl 14	27,7	28,2	27,5	27,8	5,4	5,5	4,9	**5**
cl 15	24,4	24,3	24,4	24,4	2,9	2,8	3,1	**3**
cl 16	26,0	25,2	26,2	25,8	1,2	0,8	0,9	**1**
cl 17	25,7	25,4	25,9	25,7	0,9	0,7	0,8	**1**
cl 19	29,9	26,9	26,8	27,9	0,0	0,7	0,9	**1**
cl 20	24,0	23,8	24,1	23,9	2,0	1,9	1,8	**2**
cl 21	23,6	23,3	23,5	23,5	3,0	2,8	3,0	**3**
cl 22	25,0	25,0	25,3	25,1	2,6	2,4	2,9	**3**
cl 23	25,3	24,8	25,2	25,1	0,8	0,7	0,8	**1**
cl 24	30,6	31,4	30,7	30,9	1,6	0,8	2,1	**2**

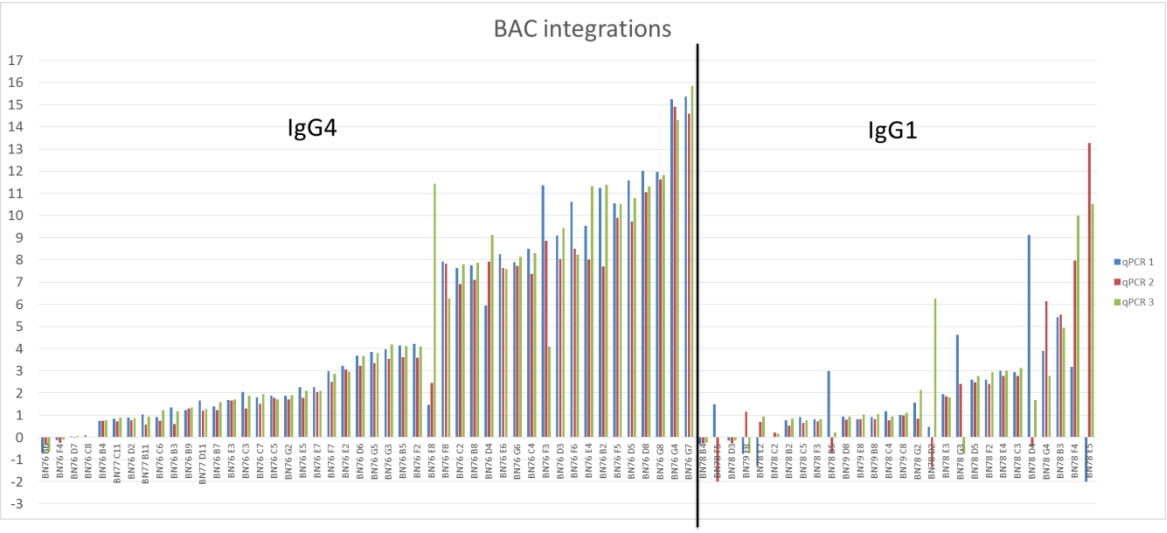

Figure 34: Gene copy number, qPCR results for all clones. Negative copy numbers derive from high cycle values and were further set to 0.

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, March 2020 Laura Stangl

EIDESSTATTLICHE ERKLÄRUNG

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

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