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Characterization and cultivation of cell lines producing 3H6 antibodies

Master Thesis

submitted by
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Abstract

Different cultivation parameters such as the chosen media or cultivation vessel play a significant role in the success of mammalian cell based expression systems. In this thesis optimal process conditions were determined to cultivate recombinant cells expressing a chimeric (wt3H6), CDR-grafted (GA3H6) or superhumanized (su3H6) IgG1 variant of the anti-idiotypic antibody 3H6. Several cultivation strategies for recombinant Chinese hamster ovary (CHO) DUKX-B11 cells were tested to compare the performance in DMEM/Ham's F12 and CD CHO medium under static or dynamic conditions, supplemented by testing high cell-density seeding. Homogeneity of CHO DUKX-B11 cell pools expressing the aggressive grafted variant was improved by limiting dilution subcloning, leading to a four-fold higher specific productivity. The superhumanized variant was expressed by transient human embryonic kidney (HEK) cultures at 300 mL scale.

The combined use of optimal cultivation conditions for stable and transient cell cultures allowed the production of sufficient material for subsequent protein A affinity chromatography and additional analytical assays exemplified by two different binding assay setups by biolayer-interferometry using the Octet system. In total, 9603.2 µg of the su3H6, 4334.4 µg of the wt3H6 and 1102.2 µg of the GA3H6 antibody could be produced.

The binding assays enabled the characterisation of the kinetic properties of the antibodies, with wt3H6 showing the highest and GA3H6 a declined binding affinity, whereas su3H6 showed no binding to its epitope at all.

Zusammenfassung

Verschiedene Kultivierungsparameter wie das gewählte Medium oder Kultivierungssystem spielen eine signifikante Rolle für den Erfolg von auf Säugetierzellen basierten Expressionssystemen. In dieser Masterarbeit wurden die optimalen Prozessbedingungen für die Kultivierung rekombinanter Zellen bestimmt, die eine chimäre (wt3H6), eine „CDR-grafted“ (GA3H6) oder „superhumanized“ (su3H6) IgG1 Variante des anti-idiotypischen Antikörpers 3H6 exprimieren. Unterschiedliche Kultivierungsstrategien für rekombinante chinesische Hamsterovarien (CHO) DUKX-B11 Zellen wurden getestet um die Leistungsfähigkeit von DMEM/Ham’s F12 und CD CHO Medium unter statischen oder dynamischen Bedingungen zu vergleichen, unterstützt durch das Testen von hohen Zelleinsaaten. Die Homogenität von CHO DUKX-B11 Zellpools, die die „aggressive grafted“ Version exprimieren, wurde durch eine Subklonierung mit Hilfe limitierter Verdünnung verbessert, resultierend in einer vierfachen Steigerung der spezifischen Produktivität. Die „superhumanized“ Variante wurde transient durch menschliche embryonale Nierenzellkulturen (HEK Zellkulturen) im 300 mL Maßstab exprimiert.

Die kombinierte Verwendung optimaler Kultivierungsbedingungen für stabile und transiente Zellkulturen ermöglichte die Produktion von ausreichend Material für die nachfolgende Protein A Affinitätschromatographie und zusätzliche analytische Untersuchungen, veranschaulicht durch zwei unterschiedliche Bio-Layer-Interferometrie Bindungstest-Anordnungen, die mit dem Octet System durchgeführt wurden. Insgesamt konnten 9603,2 µg des su3H6, 4334,4 µg des wt3H6 und 1102,2 µg des GA3H6 Antikörpers produziert werden. Die Bindungstest-Anordnungen erlaubten die Charakterisierung der kinetischen Eigenschaften der Antikörper, wt3H6 zeigte die höchste, GA3H6 eine verminderte Bindungsaffinität, während su3H6 überhaupt keine Bindung zu dessen Epitop zeigte.

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1 Abbreviations

°C	Degree Celsius
µg	Microgram
µL	Microliter
AW	Anna Wachernig
C/mL	Cells per milliliter
Cat. No.	Catalogue Number
CHO	Chinese hamster ovary
Conc.	Concentration
D/H medium	DMEM / Ham's F-12 (1:1) medium
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Fc	Fragment crystallizable
g	Gram
h	Hours
HC	Heavy chain
HEK	Human embryonic kidney
IgG	Immunoglobulin G
L	Liter
LC	Light chain
L-gln	L-glutamine
M	Molar concentration (mol/L)
min	Minute
mL	Milliliter
MN	Martin Nagl
MTX	Methotrexate
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer

PBS	Phosphate-buffered saline
PEI	Polyethylenimine
PF	Protein-free supplement
pg	Picogram
rEGF	Recombinant epidermal growth factor
rpm	Rounds per minute
RT	Room temperature
scFv	Single-chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	Soy peptone
suppl.	supplements
TFF	Tangential flow filtration
TGE	Transient gene expression
TN1	Tryptone N1
Tris	Tris(hydroxymethyl)aminomethane
V	Volt
VPA	Valproic acid
wt	Wild type

2 Introduction

The 3H6 antibody panel

The anti-idiotypic 3H6 antibody mimics the epitope of the monoclonal antibody 2F5, which binds to the virus envelope glycoprotein gp41 of HIV-1. Anti-idiotypic antibodies like 3H6 stimulate the immune system of the host system and subsequently induce the formation of antibodies directed against their variable region. In case of the 3H6 antibody, antibodies against the paratope of the 3H6 antibody are developed, which is structurally similar to the 2F5 HIV-1 epitope. Therefore, the 3H6 antibody is of interest as HIV-1 vaccine. However, co-crystallization of the 3H6 antibody unveiled that the 3H6 binding site on 2F5 is only partially overlapping with the HIV-1 ELDKWA binding groove present on the 2F5 antibody, resulting in a weaker binding of the induced antibodies to the HIV-1 epitope[1].

The wild-type 3H6 antibody was developed in mice and consists of murine variable regions that were subsequently combined with a human constant region. The resulting chimeric wild-type 3H6 antibody can induce immunogenic reactions after application in human therapy, that can be reduced by humanization procedures. Several 3H6 antibody variants have been created, differing in their extent of humanization.

GA3H6 is the CDR-grafted version of the 3H6 antibody, which was created by transfer of the murine CDRs to a mature human framework. Because this procedure can result in a weaker or lost binding affinity, human-to-mouse backmutations that are required for the correct loop formation were integrated into the human framework, leading to the preservation of the binding capacity. The su3H6 antibody is another 3H6 variant, which was obtained through superhumanization. The CDR structures of human and mouse antibodies were compared with each other and human germline framework sequences with the same CDR canonical structure class and highest CDR similarity were chosen as acceptor framework for the transfer of the mouse 3H6 CDR regions.

After the humanization, the binding capacity of the 3H6 antibodies needs to be tested for revelation of any impairments caused by the humanization. The binding capacity of all 3H6 antibody variants was analysed via competitive ELISA. The GA3H6 variant showed a lower binding affinity to its target, whereas su3H6 completely lost its binding capacity[2].

Chinese hamster ovary (CHO) cells as expression systems

Mammalian cells are commonly used in biopharmaceutical production since they provide the machinery for proper folding, post-translational modifications and correct product assembly of therapeutic proteins. The most widely used mammalian cell line is the CHO cell line[3]. There are several reasons for the choice of CHO cells as the main work horse for therapeutic protein expression. CHO cells are recognised as safe hosts and market approval of therapeutic proteins can be easier obtained from regulatory agencies. Furthermore, powerful gene amplification systems like dihydrofolate reductase (DHFR) gene amplification are available that compensate the moderate specific productivity of CHO cells. Finally, CHO cells can perform the correct post-translational modifications that are required for the application in human therapy[4].

Several CHO clones exist and are readily available for the scientific community including the CHO-K1, CHO-S and dihydrofolate reductase (DHFR) deficient variants such as CHO-DUKX-B11 and CHO-DG44 [5]. DHFR is the enzyme responsible for the reduction of folate to tetrahydrofolate, which is required for the de novo synthesis of glycine, purine nucleotides and thymidylate. A DHFR deficiency strain can only survive if glycine, hypoxanthine and thymidine are provided within the cultivation medium. Methotrexate is an analogue of tetrahydrofolate and acts as an inhibitor of the DHFR enzyme[6]. DHFR deficient CHO cells are transfected with the gene of interest along with the DHFR gene. Glycine, hypoxanthine as well as thymidine are subsequently withdrawn from the medium, enabling only positively transfected cells to survive, since they received a copy of the DHFR gene. For further increase of the gene copy number, MTX can be added to the cultivation medium, allowing only cells with a high copy number of DHFR (and a high copy number of the gene of interest) to survive[7].

Human embryonic kidney (HEK) cells as expression systems

The HEK293 cell line was derived from human embryonic kidney cells and is one of the most commonly used human cell line for the expression of therapeutic proteins. The popularity of the cell line is related to the fact that HEK293 cells can perform human post-translational modifications and that they are highly efficient in protein production.

Several variants of the HEK293 cell line have been created. The HEK293.6E variant stably expresses the Epstein-Barr virus nuclear antigen 1 (EBNA-1) protein[8]. The EBNA-1 protein enables the episomal replication of plasmids containing the Epstein-Barr virus oriP. Using this system, each HEK293.6E cell receives a copy of the plasmid during cell division, which prolongs the duration of the transient expression, because the loss of the plasmid during the cell division is prevented. Furthermore, the EBNA-1 protein needs to be overexpressed by the host cell to achieve a positive influence on the duration of the expression[9]. The HEK293.6E cell line stably expresses a truncated form of the EBNA-1 protein and is used for transient gene expressions because of their ability to obtain high titers[10].

Selection of the appropriate cultivation medium

The choice of the appropriate cultivation medium is critical for the expression of recombinant proteins. Besides glucose and glutamine as the main energy and carbon source, mammalian cells require other amino acids, fatty acids, sugars, vitamins, inorganic ions and trace elements for their growth, all of which are provided by the cultivation medium. The medium also contains components for maintenance of the physiological pH and osmolarity of each cell. Cultivation media usually provide the energy source in form of glucose, however the concentration of which is depending on the used medium. Furthermore, phenol red is added to the medium to facilitate the assessment of the pH value of the medium or supplemental additives such as Kolliphor P188 to protect cells from hydrodynamic forces [11], [12].

Cultivation media can be divided into serum-containing and serum-free media. The use of serum has many disadvantages such as lack of reproducibility and a high risk of contaminations. Hence, serum-free media were developed containing completely defined components[13]. The Eagle's minimal essential medium (MEM) is the simplest medium, providing only 13 amino acids, 8 vitamins and basic organic salts. Dulbecco's modified Eagle's medium (DMEM) is a modified version with four times as much of the amino acids and vitamins as well as two to four times more glucose. Furthermore, DMEM includes phenol red and iron. Another cultivation medium is the Ham's F12 medium, which is an improvement of the Ham's F10 medium and traditionally used for the cultivation of primary rat hepatocytes and rat prostate epithelial cells[14].

The D/H medium is a 1:1 mixture of the Dulbecco's modified Eagle's and Ham's F12 medium, resulting in a medium with an increased concentration of nutrients compared to the original DMEM formula [15].

CD CHO is a chemically defined medium and was developed for the high-density suspension cultivation of mammalian cells, thus enabling the expression of recombinant proteins at higher concentrations[16]. Table 1 shows a comparison of the glucose, amino acid and vitamin concentrations between the glutamine-free versions of the D/H and CD CHO medium. The glucose concentration as well as the concentrations of all amino acids are higher in the CD CHO medium, except of L-alanine, L-cystine, glycine and L-proline, which are not present in the CD CHO medium. Furthermore, the concentrations of most vitamins are elevated in the CD CHO medium. A complete comparison of all ingredients between both media is provided in Table 60, which can be found in the appendix.

Table 1 Comparison of the amino acid and vitamin concentrations between the CD CHO and D/H medium

Component	**D/H medium [mg/L]** [15]	**CD CHO medium [mg/L]** [16]
Energy source		
D-glucose	3151	4500.00
Amino acids		
L-Alanine	4.50	0.00
L-Arginine	147.50	355.6
L-Asparagine	7.50	26.40
L-Aspartic acid	6.65	75.00
L-Cysteine	15.75	57.60
L-Cystine	24.00	0.00
L-Glutaminic acid	7.35	29.40
Glycine	18.75	0.00
L-Histidine	31.50	42.20
L-Isoleucine	54.50	190.00
L-Leucine	59.00	280.00
L-Lysine	91.25	204.0
L-methionine	17.24	115.00
L-Phenylalanine	35.50	70.00
L-Proline	17.25	0.00
L-Serine	26.25	250.00
L-Threonine	53.50	60.00
L-Tryptophane	9.00	20.00
L-Tyrosine	38.70	69.2
L-Valine	52.85	190.00
Vitamins		
Cholin chloride	9.00	14.00
Biotin	0.00365	0.097
Folic acid	2.65	5.00
D-Ca^{++}-pantothenate	2.24	1.19
I-Inositol	12.6	18.00
Nicotinamide	2.02	1.22
Pyridoxal	2.00	0.00
Pyridoxine	0.031	0.85
Riboflavin	0.05	0.22
Thiamine	2.17	1.00
Vitamin B_{12}	0.68	1.03

3 Objectives

The antibodies wt3H6, GA3H6 and su3H6 needed to be produced in large quantities. All three antibodies are variants of the 3H6 antibody.
The 3H6 antibody is directed against the human monoclonal antibody 2F5 and fits to the antigen binding site of 2F5, thus mimicking the gp41 epitope of HIV-1. Different variants of the 3H6 antibody are available, varying in their ability to induce immunogenicity. GA3H6 is the aggressive CDR-grafted version, whereas su3H6 is the superhumanized version of the 3H6 antibody. The GA3H6 variant and the chimeric wildtype version, wt3H6, of the antibody were already incorporated into the CHO DUKX-B11cell line, resulting in two different clones that are constantly producing antibodies during their cultivation. The su3H6 antibody was produced transiently using HEK293.6E as producing cell line.

During this master thesis, the medium and the optimal shaking conditions for the cultivation of wt3H6-CHO and GA3H6-CHO were assessed to enable an efficient production of both antibodies. Static cultivation systems like T25 and T80 roux flasks as well as dynamic systems like 125 mL shake flasks are available for the assessment. Also, higher start cell concentrations can improve the quantity of produced antibodies.

The GA3H6-clone showed a very low productivity and homogeneity. For the improvement of the antibody production and homogeneity of the clone, a subcloning procedure was performed. The obtained subclones were compared to each other and the subclone with the highest specific productivity was selected for further cultivation.

The kinetic properties of the wt3H6, GA3H6 and su3H6 antibodies were analysed via bio-layer interferometry using two different sensors, protein A and streptavidin. Therefore, wt3H6 and GA3H6 were purified and su3H6 was transiently expressed with the use of the HEK293.6E cell line. The association and dissociation curves of the antibodies were recorded and kinetic properties calculated to confirm the binding behaviour and identity of the antibodies by comparison with literature values.

4 Material and Methods

4.1 Material

4.1.1 Equipment

10 -100 µL pipette	Gilson pipetman neo® P100N
10 -1000 µL pipette	Gilson pipetman neo® P1000N
20-200 µL pipette	Gilson pipetman neo®P200N
2-20 µL pipette	Gilson pipetman neo® P20N
96 well plate washer	Tecan 96 PW
Balance	Sartorius AW-4202
Bio-layer interferometry system	Pall® fortéBio® Octét® QK
Centrifuges	Thermo Fisher Scientific Heraeus Megafuge 16 Thermo Fisher Scientific Heraeus Megafuge 40R Eppendorf® Centrifuge 5415 R
Chromatography column	GE Healthcare HiTrap MabSelect SuRe 1 mL Cat. No.: 29-0491-04
Coulter counter	Beckman Coulter® Counter Z2
Dialysis tubing	Fisherbrand Regenerated Cellulose Dialysis tubing Nominal MWCO 3500 d Cat. No.: 21-152-10
Flow cytometer	Beckman Coulter® Gallios™
Haemocytometer	Neubauer Labor Optik
Incubator	Thermo Fisher Scientific Heracell 150i CO_2 incubator
Magnetic stirrers	VWR® VS-C10 VWR® VS-C4
Microcentrifuge	VWR® Galaxy MiniStar
Microplate reader	Tecan Infinite® M1000
Microscope	Leica DMIL LED

Multichannel pipette 1	Thermo Fisher Scientific Finpipette F2 30-300 µL
Multichannel pipette 2	Integra Twelve Channel Evolve Manual Pipette. 20-200 µL Cat. No.: 3036
NanoDrop	NanoDrop™ 1000
Pipetboy 1	Matrix Cell Mate II
Pipetboy 2	Pipet Help Accumax
Protein A affinity chromatography system	GE Healthcare Äkta start
TFF membrane	Merck Pellicon™ XL Cassette Biomax 30 kDa Cat. No.: PXB030A50
TFF system	Millipore® Labscale TFF system
Vortex mixer	Scientific Industries Vortex-Genie® 2
Laminar flow hood	MSC Advantage Type MSC 1.2

4.1.2 Chemicals

Acetic acid	Sigma Aldrich® Cat. No.: A6283 M = 60.05 g/mol
Albumin, IgG free (BSA)	Carl Roth® Cat. No.: 3737.3 M ~ 66 000 g/mol
Citric acid monohydrate	Carl Roth® Cat. No.: 5110.1 M = 210.14 g/mol
Ethanol	Merck Emplura® Cat. No.: 8.18760.2500 M = 46.07 g/mol
Glycerol	Merck Cat. No.: 104201

	M = 75.06 g/mol
Glycine	Merck
	Cat. No.: 1.04201.5000
	M = 75.06 g/mol
NaCl	Carl Roth®
	Cat. No.: P029.3
	M = 58.44 g/mol
Potassium chloride	Carl Roth®
	Cat. No.: HN02.3
	M = 74.56 g/mol
Potassium dihydrogen phosphate	Merck
	Cat. No.: 1.04873.1000
	M = 136.08 g/mol
Silver nitrate	Merck
	Cat. No.: 101510
	M = 169.87 g/mol
Sodium acetate * 3 H_2O	Merck
	Cat. No.: 1.06268.1000
	M = 82.03 g/mol
Sodium carbonate	Carl Roth®
	Cat. No.: A135.2
	M = 105.99 g/mol
Sodium hydrogen carbonate	Merck
	Cat. No.: 1.06329.1000
	M = 84.01 g/mol
Sodium thiosulfate pentahydrate	Merck
	Cat. No.: 1.06516.0500
	M = 248.21 g/mol
Tris	Merck
	Cat. No.: 1083822500
	M = 121.14 g/mol
Valproic acid sodium salt	Sigma Aldrich®
	Cat. No.: P4543-10G
	M = 166.19 g/mol

4.1.3 Reagents

Sulphuric acid 25 %	Carl Roth® Cat. No.: 0967.1 M = 98.08 g/mol
1 x protein-free supplement (L0018)	Sodium selenite (L0012) Ascorbic acid (CA008) Ethanolamine (CE006)
10 % Kolliphor® P188	Sigma Aldrich® Cat. No.: K4894
200 mM alanyl-L-glutamine solution	Carl Roth® Cat. No.: 9183.1
50 x soy peptone	HV # L0021
Albumin, 50 mg/mL	Sigma Aldrich® Cat. No.: A7223
DAPI [5 µg/mL]	L0009
EDTA	Merck Cat. No.: 324503
Formaldehyde solution, 37 %	Sigma Aldrich® Cat. No.: 252549
G418	Biochrom GmbH G418-BC Cat. No.: A 2912
Glutaraldehyde solution, 25 %	Sigma Aldrich® Cat. No.: G5882
Kinetics buffer for Octet	Pall® FortéBio® Kinetics Buffer 10 x Cat. No.: 18-1092
MOPS buffer	Thermo Fisher Scientific NuPAGE® MOPS SDS Running Buffer (20x) Cat. No.: NP0001
Phenol red solution	Sigma Aldrich® Cat. No.: P0290

Protein ladder	Thermo Fisher Scientific PageRuler™ Prestained Protein Ladder Cat. No.: 26616
rEGF, 0.1 mg/mL	Repligen Cat. No.: 10-1021
TMB	Thermo Fisher Scientific TMB Stabilized Chromogen Cat. No.: SB02
Transferrin, 20 mg/mL	Merck CellPrime® Cat. No.: 9701-10
Triton® X 100	Carl Roth® Cat. No.: 3051.2 M = 624 g/mol
Trypan blue solution	Sigma Aldrich® Cat. No.: T8154-100ML
Tween® 20	Carl Roth® Cat. No.: 9127.2

4.1.4 Solutions

10 x PBS stock solution (5 L)	57.5 g Na_2HPO_4 * 2 H_2O 10 g KH_2PO_4 10 g KCL 400 g NaCl Filled up to 5 L with dH_2O
Äkta start buffer A	100 mM Glycine 100 mM NaCl pH 7.5
Äkta start buffer B	100 mM Glycine pH 2.5
Coating buffer for ELISA (1 L)	8.4 g $NaHCO_3$ 4.2 g Na_2CO_3 Filled up to 1 L with dH_2O pH 9.5-9.8

Coulter count incubation buffer	0.1 M Citric acid monohydrate
	2 % w/w Triton® X 100
Developing solution	12.5 g Na_2CO_3
	Filled up to 500 mL with H_2O
	(5 µL formaldehyde/25 mL)
Dilution buffer for ELISA (100 mL)	100 mL washing buffer
	1 g BSA
FACS buffer	0.1 M Tris HCl
	2 mM $MgCl_2$
	0.1 % Triton® X-100
	pH 7.5
	stored at 4 °C
FACS buffer + 20 % FCS	FACS buffer with added 20 % FCS
Fixation solution	50 % ethanol
	10 % acetic acid
	40 % dH_2O
Incubation solution	150 mL ethanol
	1.75 g $Na_2S_2O_3$ * 5 H_2O
	56.4 g Sodium acetate * 3 H_2O
	Filled up to 500 mL with H_2O
	(62.5 µL glutaraldehyde/25 mL)
PBS-t buffer	1 x PBS
	0.1 % Tween® 20
Silver solution	0.25 g $AgNO_3$
	Filled up to 500 mL with H_2O
	(5 µL formaldehyde/25 mL)
Stop solution	0.05 EDTA in H_2O
Washing buffer for ELISA (1L)	100 mL of 10 x PBS stock solution
	1 mL Tween
	Filled up to 1 L with dH_2O

4.1.5 Antibodies

2F5 IgG antibody	hm Ab 2F5 Lot: t58C703-A Conc.: 12.1 mg/mL
3D6 scFv-Fc antibody	LS 160621 Conc.: 5.36 mg/mL
Biotinylated 2F5 IgG antibody	2F5-B PM 20.05.14 Conc.: 0.45 mg/mL
ELISA capture antibody (anti-gamma)	Anti-human IgG (gamma-chain specific) capture antibody (1 mg/mL) Sigma Aldrich® Cat. No.: I3382-1MG
ELISA detection antibody (anti-gamma)	HRP-Goat anti-human IgG (Gamma) detection antibody (1 mg/mL) Invitrogen Cat. No.: 628420
ELISA detection antibody (anti-kappa)	Anti-human kappa light chain -Peroxidase detection antibody (1 mg/mL) Sigma Aldrich® Cat. No.: A7164-1ML
ELISA IgG standard	3D6 IgG (200 ng/mL) Affinity purified standard stock solution
FACS staining antibody (anti-gamma)	Anti-human IgG (gamma-chain specific) – FITC antibody Sigma Aldrich® Cat. No.: F0132-1ML
FACS staining antibody (anti-kappa)	Anti-human kappa light chain – FITC antibody Sigma Aldrich® Cat. No.: F3761-2ML

4.1.6 Disposables

10-200 µL pipette tips	Greiner Bio-One Pipette Tips. 10-200 µL Cat. No.: 739291
125 mL shake flask	Corning® 125 mL Polycarbonate Erlenmeyer Flask with Vent Cap Cat. No.:431143
15 mL centrifugal filters	Millipore® Amicon® Ultra-15 Centifugal Filter Concentrator with Ultracel® 10 Regenerated Cellulose Membrane. NMWL: 10.000 Cat. No.: UFC901024
200-1000 µL pipette tips	Greiner Bio-One Pipette Tips. 200-1000 µL Cat. No.: 740290
250 mL shake flask	Corning® 250 mL Polycarbonate Erlenmeyer Flask with Vent Cap Cat. No.:431144
384 well plate	Corning® 384-Well Clear Polystyrene Microplates Cat. No.: 3701
50 mL mini bioreactor tubes	Corning® 50 mL Mini Bioreactor Cat. No.: 431720
50 mL tubes	Greiner Bio-One CELLSTAR® Centrifuge Tubes Cat. No.: 227261
96 well plate	Thermo Fisher Scientific Nunc™ MicroWell™96- Well Microplate Cat. No.: 167008
Centrifuge tubes	Thermo Fisher Scientific Nunc™ 11 mL Polystyrene Centrifuge tubes Cat. No.: 347856
FACS tubes	Corning™ Falcon™ Round-Bottom Polystyrene Tubes

	Cat. No.: 352054
Octet microtiter plates	Thermo Fisher Scientific Nunc™ F96 MicroWell™ Black Polystyrene Plate Cat. No.: 137101
Pipette tips for twelve channel pipette	Integra Griptip tips. 300 µL Cat. No.: 4431
SDS-PAGE gel	Thermo Fisher Scientific NuPAGE™ 4-12% Bis-Tris Protein Gel Cat. No.: NP0321BOX
Serological pipette 10 mL	Corning® Costar® Stripette® Cat. No.: 4488
Serological pipette 2 mL	Corning® Costar® Stripette® Cat. No.: 4486
Serological pipette 25 mL	Corning® Costar® Stripette® Cat. No.: 4489
Serological pipette 5 mL	Corning® Costar® Stripette® Cat. No.: 4487
Serological pipette 50 mL	Corning® Costar® Stripette® Cat. No.: 4490
Sterile 5 mL syringe	Syringe 5 mL/Luer Lock Solo Braun Omnifix Cat. No.: 4617053V
T175 roux flask	Thermo Fisher Scientific Nunc™ Cell Culture Treated Flasks with Vent/Close Caps Cat. No.: 156502
T25 roux flask	Thermo Fisher Scientific Nunc™ Cell Culture Treated Flasks with Vent/Close Caps Cat. No.: 163371
T80 roux flask	Thermo Fisher Scientific Nunc™ Cell Culture Treated Flasks with Vent/Close Caps Cat. No.: 153732

Protein A sensors	Pall® FortéBio® Cat. No.: 18-5010
Streptavidin sensors	Pall® FortéBio® Cat. No.: 18-5019
0.5 mL centrifugal filters	Millipore® Amicon® Ultra-0.5 Centifugal Filter Concentrator with Ultracel® 10 Regenerated Cellulose Membrane. NMWL: 10.000 Cat. No.: UFC501096
1.5 mL microcentrifuge tubes	VWR Microcentrifuge tubes. 1.5 ml. SuperSpin™ Cat. No.: 211-0015
0.22 µm sterile syringe filter	Millipore® Millex-GP Syringe Filter Unit. 0.22 µm Cat. No.: SLGP033RS
Sterile 0.22 µm filter	Stericup 500 mL Millipore Express Plus 0.22 µm PES Cat. No.: SCGPU05RU

4.1.7 Media

CD CHO medium	Gibco™ CD CHO Medium Cat. No.: 10743 w/o L-Glutamine
D/H medium	DMEM/Ham's F-12 (1:1) w/o L-Glutamine Biochrom GmbH Cat. No.: F 4815
HEK293.6E medium	HyClone™ CDM4HEK293™ Media Cat. No.: SH30858.02
MV3-2/6 (+30%)	In-house formulation

4.2 Methods

4.2.1 Thawing of wt3H6-CHO and GA3H6-CHO

The strains wt3H6-CHO and GA3H6-CHO needed to be thawed before the cultivation, which was achieved by quickly warming the cryovials containing wt3H6-CHO or GA3H6-CHO to room temperature.
1 mL of the CHO cell suspension was then transferred from the cryovials into centrifuge tubes filled with 8 mL cold D/H medium including 4 mM L-glutamine, 1x soy peptone, 1x protein-free supplement and 0.1 % Kolliphor P188. For wt3H6-CHO 0.38 µM MTX was added, for GA3H6-CHO 0.096 µM MTX. The centrifuge tubes were centrifuged for 10 minutes at 1300 rpm, the media was discarded and the pellets were resuspended in centrifuge tubes containing 7 mL D/H Medium with the same supplements as mentioned above. The cell suspensions were then transferred into T25 roux flasks, already filled with 3 mL D/H medium including the same supplements. The T25 roux flasks with subsequently 10 mL total volume were incubated at 37°C and 5 % CO_2. On Mondays and Fridays, the wt3H6-CHO and GA3H6-CHO cultures were passaged to a start cell concentration of $2 * 10^5$ C/mL.

4.2.2 Sampling and passaging

Several metabolic products are formed during the cultivation of cell cultures, which can be toxic to the cells with increasing concentration. The release of by-products during the cultivation also lowers the pH medium, leading to the characteristic yellow colour of the cultivation medium, caused by the phenol red pH indicator. Simultaneously, necessary nutrients and growth factors of the medium are depleted. Passaging was performed twice a week to add fresh nutrients and to keep the cells in exponential phase[17].

First, 1 mL aliquot of cell suspension was transferred into a centrifuge tube. 100 µL of the sample was transferred to a micro centrifuge tube for the determination of the viability via haemocytometer. The residual 900 µL cell suspension was centrifuged for 10 minutes at 1000 rpm. After addition of 0.9 ml coulter count buffer, the suspension was incubated for 1 hour and the cell count determined with the Coulter Counter Z2. Saline solution was used as blank for the measurements.

Each measurement was done twice and an average value was calculated from both values. The resulting average cell count was used to calculate the cell concentration of the cells according to equation 1:

$$\frac{Cells}{mL} = \bar{x} * 10 * \frac{V_c}{V_{ib}} * \frac{V_b + V_s}{V_s} \quad \text{(Equation 1)}$$

$\bar{x}$…average value of the counted values (events/100 µL)
10…conversion factor
V_c…centrifuged cell suspension volume [mL]
V_{ib}...added coulter count incubation buffer [mL]
V_b…added saline solution volume [mL]
V_s…used sample aliquot for the measurement [mL]

The calculated cell concentration was used to determine the required passage ratio by utilizing equation 2:

$$Passage\ ratio = \frac{Calculated\ cell\ concentration\ [\frac{C}{mL}]}{Start\ cell\ concentration\ of\ the\ passage\ [\frac{C}{mL}]} \quad \text{(Equation 2)}$$

The desired media volume of the passage was then divided through the passage ratio to determine the required inoculation volume. After inoculation of the new passage, the missing media volume was added to the inoculation cell suspension, thereby leading to the dilution of the cells and the old media components.

4.2.3 Medium exchange

For adjusting the start cell concertation to 1 * 10^6 C/mL, the cells were centrifuged and the medium was exchanged. Therefore, the centrifugation volume to reach the necessary cell number was calculated using equation 3:

$$V_c = \frac{N_c}{C_m} \quad \text{(Equation 3)}$$

V_c…volume needed to be centrifuged
*N_c…required cell number for adjusting of the start cell concentration to 1 * 10^6 C/mL*
C_m…cell count of the culture

The required number of the cells depends on the intended cultivation volume. For example. for adjusting the start cell concentration of a culture with 30 mL volume to 1 * 10^6 C/mL, 30 * 10^6 cells were required.

The calculated centrifugation volume was centrifuged for 10 minutes at 1000 rpm and the cell pellet resuspended in the intended cultivation volume.

4.2.4 Characterisation of the passages/medium exchanges

Determination of the viability via haemocytometer

The viability was determined using the haemocytometer. Each haemocytometer consists of two counting chambers.100 µL cell suspension were transferred from the 1 mL sample aliquot into a micro centrifuge tube. 20 µL of trypan blue solution was subsequently added and after mixing by pipetting up and down, cell suspension was transferred into each of the two haemocytometer chambers until they were full. The number of living cells and the number of dead cells of two squares of each chamber were counted and average values determined. The number of viable, dead and the total cells per mL and the viability were calculated according to equations 4-7:

$$\frac{Viable\ cells}{mL} = counted\ viable\ cells * 1.2 * 10000 \quad \text{(Equation 4)}$$

$$\frac{Dead\ cells}{mL} = counted\ dead\ cells * 1.2 * 10000 \quad \text{(Equation 5)}$$

$$\frac{Total\ cells}{mL} = \frac{viable\ cells}{mL} + \frac{dead\ cells}{mL} \quad \text{(Equation 6)}$$

$$Viability\ [\%] = \frac{viable\ cells/mL}{total\ cells/mL} * 100 \quad \text{(Equation 7)}$$

1.2…dilution factor originated by diluting the sample with trypan blue
10000…factor for converting the volume of one square (0.1 µL) to 1 mL

Specific growth rate μ

The specific growth rate μ was calculated using equation 8:

$$\mu = \frac{\ln\frac{x_2}{x_1}}{t} \qquad \text{(Equation 8)}$$

μ…specific growth rate [1/d]
x_1…start cell concentration [C/mL]
x_2…end cell concentration [C/mL]
t…cultivation time [d]

Specific productivity Qp

The specific productivity Qp was calculated according to equation 9:

$$Q_p = \frac{(end\ titer - start\ titer) * \mu}{x_2 - x_1} * 1000 \qquad \text{(Equation 9)}$$

Q_p…specific productivity [pg/c/d]
Start titer…titer at the beginning of the passage [ng/mL]
End titer…titer at the end of the passage [ng/mL]
x_1…start cell concentration [C/mL]
x_2…end cell concentration [C/mL]
μ…specific growth rate [1/d]

Measurement of the antibody titer by quantitative gamma-gamma ELISA

The antibody titers of each passage were determined using a quantitative γ-γ ELISA. For the ELISA, the capture antibody I3382 was coated on a MaxiSorp™ 96 well microtiter plate. Therefore, the capture antibody was diluted 1:2000 in coating buffer and 100 µL were added to each well of the microtiter plate. The microtiter plate was subsequently incubated over night at 4°C or room-temperature for 2h under shaking at 220 rpm.
3D6 IgG was used as standard with a concentration of 200 ng/mL. Samples were diluted in micro centrifuge tubes with dilution buffer to a concentration of 200 ng/mL. As Blank, dilution buffer was used. Blank, standard and samples were diluted 8 times 1:2 by serial dilution in a separate 96 well microtiter plate, referred to as dilution plate.

The coated MaxiSorp™ 96 well microtiter plate was washed 3 times using the 96 well plate washer. 50 µL of each well were transferred from the dilution plate to the washed MaxiSorp™ 96 well microtiter plate, which was then incubated for 1 hour at 220 rpm for binding of the antibodies. After the binding of the antibodies, the MaxiSorp™ 96 well microtiter plate was again washed 3 times with the 96 well plate washer. The antibody HRP-Goat Anti Human IgG (Gamma) was used for the detection of bound sample antibodies. Therefore, the detection antibody was diluted 1:2000 with dilution buffer and 50 µL of the diluted antibody were added to each well of the MaxiSorp™ 96 well microtiter plate.

After 1-hour incubation at 220 rpm, the microtiter plate was again washed 3 times with the 96 well plate washer.
100 µL of the substrate TMB were added to the microtiter plate for staining. The microtiter plate was incubated until the most diluted standard showed blue colour. The reaction was stopped by adding 100 µL/well H_2SO_4 and the absorption of each well was measured at 450 nm using the Tecan Reader Infinite® M1000 Pro.

4.2.5 Stabilization of recombinant GA3H6-CHO

Because of the existence of a lower producing GA3H6 subculture, a subcloning procedure was started.

Day 0: Preparation of the 384 well plates

The subcloning procedure was started by inoculation of 384 well plates and subsequent 96 well plates. 4 different dilutions were prepared for the inoculation of two 384 well plates: 9 cells/well, 6 cells/well, 3 cells/well, 1 cell/well.
Two 384 well plates were inoculated: 1 plate with D/H and 1 plate with CD CHO as medium. The growth factors albumin, transferrin and recombinant EGF were added to both media. Table 2 and Table 3 give an overview about the added supplements and growth factor concentrations.

Table 2 Supplements added to the 384 well plates

Medium	**Prepared Volume**	**Supplements**
D/H	35 mL	4 mM L-gln, 1x SP, 1x PF, Kolliphor P188, 0.096 µM MTX
CD CHO	35 mL	4 mM L-gln, 15 mg/L phenol red, 0.096 µM MTX

Table 3 Growth factors added to the 384 well plates and their concentrations

Growth factors	**Stock [mg/mL]**	**Dilution factor**	**Volume added to 35 mL medium [µL]**	**Concentration in one well (50 µL medium)**
Albumin	50	50	700	1 mg/mL
Transferrin	20	1000	35	20 µg/mL
Recombinant EGF	0.1	4000	8.8	25 ng/mL

The GA3H6-CHO shake flask P4 ($2 * 10^5$ C/mL start cell concentration) with a concentration of $1 * 10^6$ C/mL was used for inoculation of the plates.
The cells were diluted 1:1000 by preparation of three 1:10 dilutions (500 µL cell suspension + 4.5 mL CD CHO + supplements).
Each well of the 384 well plate comprises a volume of 50 µL.

The exact number of cells needed to prepare the 9 cells/well dilution had to be calculated using equation 10:

$$\frac{9\ cells}{50\ \mu L} = 0.18 \frac{cells}{\mu L} = 180\ cells/mL \qquad \text{(Equation 10)}$$

For the 9 c/well dilution, 13 mL were required to make the serial dilutions. Therefore, following number of cells were needed:

$$180 \frac{cells}{mL} * 13\ mL = 2340\ cells\ needed$$

2.34 mL of the 1:1000 dilution of GA3H6-CHO shake flask P4 had to be added to the 9 c/well dilution. The other three dilutions were prepared by serial dilutions. Table 4 gives an overview about the preparation of the four dilutions.

Table 4 Preparation of the dilutions for the 384 well plates

Dilution	Total Volume [mL]	Preparation
9 cells/well	13	2.34 mL cell suspension + 10.66 mL medium
6 cells/well	10.5	7 mL of the 9 c/w dilution + 3.5 mL medium
3 cells/well	8	4 ml of 6 c/w dilution + 4 mL medium
1 cell/well	6	2 mL of 3 c/w dilution + 4 mL medium

The 384 well plate consisted of 16 rows, named A to P. Each row contained 24 wells, which were numbered 1-24.

The 384 well plates were divided into 4 areas, one area for each dilution. Each area contained 96 wells, which were filled up with 50 µL of each dilution. The wells of rows A-D were filled with the 9 cells/well, the rows E-H with the 6 cells/well, the rows I-L with the 3 cells/well and the rows M-P with the 1 cell/well dilution.

After the inoculation of the 384 well plates, the plates were incubated at 37°C and 5% CO_2.

Timetable of the stabilization after inoculation of the 384 well plates

Table 5 gives an overview about the timetable of the subcloning and the performed actions after inoculation of the 384 well plates.

Table 5 Timetable of the subcloning with conducted actions

Day	**Action**
Day 0	cells added to 384 well plates in 4 dilutions (96 wells/dilution): 9,6,3,1 c/w 50 µL medium/well
Day 13	384 well D/H plate under microscope 1 c/w: 2/96 = 2%, 3 c/w: 12/96 = 13%, 6 c/w: 19/96 = 20%, 9 c/w: 24/96 = 25% no growth on CD CHO plate, only D/H plate used for further actions
Day 14	30 µL medium without growth factors added to D/H plate
Day 17	yellow wells of 3 and 1 c/w clones (45/192 clones = 23%) transferred to a 96 well plate (80 µL cell suspension + 220 µL D/H medium)
Day 21	additional 10 clones transferred to 96 well plate and already dense clones of the 96 well plate passaged 1:3 on a second 96 well plate (100 µL cell suspension + 200 µL D/H medium)
Day 25	qualitative γ-κ ELISA to determine best producing clones Clones produced GA3H6 ranging from 0.01 to 3.4 µg/mL 10 best producing clones selected and transferred to 96 well plate (expansion plate): **clones with wells on two 96 well plates:** 100 µL cell suspension from second 96 well plate + 50 µL from first 96 well plate + 150 µL D/H medium (1:2). 3 wells/row/clone Clones: I10, I13, J5, L4, J20, L18, L15 **clones with wells on one 96 well plate:** 100 µL cell suspension from 96 well plate + 200 µL D/H medium (1:3). 2 wells/row/clone Clones: I4, L23, M5 Clones passaged 1:2 until enough volume for transfer to T25 roux flasks (150 µL cell suspension in new wells + 150 µL medium to all wells)

Day 31	Clones with 6 wells on the expansion plate were transferred to T25 roux flasks (1800 µL cell suspension + 1800 µL D/H medium) Clones: I10, I13, J5, L4, J20, L18, L15 (7 Roux Flasks)
Day 35	**7 T25 roux flasks**: passaged 1:2 by adding 3.6 mL D/H medium **Clones I4, L23:** 150 µL out of 4 wells of the expansion plate in 4 new wells and 150 µL D/H medium added to all 8 wells **Clone M5**: all 8 wells transferred to T25 roux flasks (2400 µL cell suspension + 2400 µL D/H medium)
Day 38	**7 T25 roux flasks:** 1:2 passage (5 mL cell suspension + 5 mL D/H medium) **M5 T25 roux flask:** 1:2 passage (4.8 mL D/H medium added) **Clones I4, L23:** 8 wells in T25 roux flasks (2400 µL cell suspension + 2400 µL D/H medium)
Day 41	**Clones L4, L18, L15, J5, J20, I13, I10, M5:** Passaged 1:3 (3.3 mL cell suspension + 6.6 mL D/H medium + supplements) **Clones I4, L23:** Passaged 1:2 (3.8 mL D/H medium + supplements added)
Day 45	**Clones L4, L15, J5:** Passaged 1:3 (3.3 mL cell suspension + 6.6 mL D/H medium + supplements) **Clones L18, I13, J20, I10, M5, I4, L23:** Passaged 1:2 (5 mL cell suspension + 5 mL D/H medium + supplements)
Day 49	Comparison of the T25 roux flask cultivations and selection of four best producing subclones for further cultivations
Day 74	Comparison of the four remaining T25 roux flask cultivations and selection of two subclones for further cultivations

Day 17 and Day 21: Transfer of subclones to 96 well plates

The D/H 384 well plate was analysed under the light microscope. Wells with two thirds of their area overgrown by the subclone and showing a yellow colour due to the acidification of the medium were transferred to a 96 well plate. 45 clones were transferred on day 17 and additional 10 clones were added on day 21. The layout of the 96 well plate is shown in Table 6. The clones, which were transferred on day 21 are highlighted in red.

Table 6 First 96 well plate of the GA3H6-CHO subcloning

	H	**G**	**F**	**E**	**D**	**C**	**B**	**A**
1								
2								
3	I3	I4	I7	I10	I13	I14	I15	I16
4	I20	J20	J24	J4	J5	J14	J15	J16
5	J18	I21	I22	K2	K4	K6	K10	K12
6	K15	K18	K20	L4	L5	L9	L10	L13
7	L15	L16	L18	L19	L20	L21	L22	M13
8	N7	N9	N22	O15	P17			
9	M5	M8	O7	O8	O9	I2	J17	J21
10	K7	K11	K21	L23				

Some of the clones transferred on day 17 were already very dense on day 21 and were therefore passaged 1:3 on a second 96 well plate. The transferred clones and the layout of the second 96 well plate is shown in Table 7.

Table 7 Second 96 well plate of the GA3H6-CHO subcloning

	H	**G**	**F**	**E**	**D**	**C**	**B**	**A**
1								
2								
3				I10	I13	I14		
4		J20		J4	J5	J14	J15	
5	J18	I21	I22	K2	K4	K6	K10	K12
6				L4				
7	L15	L16	L18	L19			L22	
8		N9		O15				
9								
10								
11								
12								

Day 25: Qualitative ELISA for determination of best producing subclones

For the determination of the best subclones, a qualitative γ-κ ELISA was performed. The different steps for the assay were nearly identical to the quantitative ELISA. Instead of the HRP-goat anti human IgG (gamma) antibody, which is directed against the gamma fraction of the antibody, the anti-human kappa light chain peroxidase antibody, directed against the kappa fraction, was utilized for detection. Dilution buffer was used as blank for the qualitative ELISA. 200 ng/mL of 3D6 IgG functioned as standard, which was serial diluted 1:2 to a final concentration of 1.56 µg/mL.

The supernatant of the different subclones, which were already transferred to the 96 well plate on day 17, was diluted 1:50. However, the supernatant of clones transferred on day 21, was diluted 1:20. The layout of the qualitative ELISA is shown in Table 8.

Table 8 Layout of the qualitative ELISA

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
2	200 ng/mL STD	100 ng/mL STD	50 ng/mL STD	25 ng/mL STD	12.5 ng/mL STD	6.25 ng/mL STD	3.13 ng/mL STD	1.56 ng/mL STD
3	I3	I4	I7	I10	I13	I14	I15	I16
4	I20	J20	J24	J4	J5	J14	J15	J16
5	J18	I21	I22	K2	K4	K6	K10	K12
6	K15	K18	K20	L4	L5	L9	L10	L13
7	L15	L16	L18	L19	L20	L21	L22	M13
8	N7	N9	N22	O15	P17			
9	M5	M8	O7	O8	O9	I2	J17	J21
10	K7	K11	K21	L23				

4.2.6 Flow cytometry (FACS)

The wt3H6-CHO and GA3H6-CHO cultivations, as well as the transient gene expressions of su3H6 were analysed via flow cytometry. The intracellular 3H6 antibody fractions were stained by addition of either an antibody directed against the light chain of the 3H6 antibody or an antibody against the heavy chain. The staining antibodies were marked with FITC, allowing the detection of the staining antibodies in each cell through excitation of FITC and measuring the emission via flow cytometer.

First, the cells of the different cultures needed to be fixed. Therefore, 1 mL of each cell suspension was centrifuged in a centrifuge tube for 10 minutes at 1000 rpm and subsequently fixed by addition of 70 % ethanol, which was performed drop-wise under vortexing. The fixed cells were incubated at 4 °C for at least 1 hour.

The staining of the cells was started by removal of the ethanol through centrifuging the centrifuge tube for 10 minutes at 1000 rpm. The resulting pellet was resuspended in 1 mL FACS buffer, which was added drop-wise under vortexing. Afterwards, the FACS buffer was removed by centrifuging the suspension for 10 minutes at 1000 rpm and 100 µL FACS buffer + 20 % FCS was added drop-wise under vortexing. After 30 minutes of incubation at 37 °C, 100 µL of 1:50 diluted FITC antibody, directed against either the heavy or light chain, were added drop-wise under vortexing and the solution was incubated for 60 minutes at 37 °C, allowing the antibodies to bind to the intracellular 3H6 fractions. Afterwards, 1 mL of FACS buffer was added to the solution for washing. The buffer was removed by centrifuging the centrifuge tube for 10 minutes at 1000 rpm. Finally, 200 µL of FACS buffer containing 1:100 diluted DAPI was added for resuspension of the pellet and the solution was transferred from the centrifuge tube to the FACS tube. The FACS measurement was performed using the Beckman Coulter® Gallios™ flow cytometer. Each measurement was stopped after recording 10000 events or after expiration of 120 seconds. As negative control either CHO K1 or HEK293.6E was used.

4.2.7 Antibody Production

Transient gene expression of su3H6

The su3H6 antibody was expressed in four different transient gene expression experiments using HEK293.6E as host cell line. Each transient gene expression was comprised of a set of 50 mL bioreactor tubes. Each tube was inoculated with 30 * 10^6 cells. One day before transfection, cells were passaged 1:2 to keep them in exponential growth phase. At the time of transfection, HEK293.6E cultures were centrifuged for 10 minutes at 1000 rpm and the cell pellet resuspended in 8 mL CD CHO containing 8 mM L-glutamine and 15 mg/L phenol red.

15 µg of the light chain and 15 µg of the heavy chain su3H6 plasmid were added to 1500 µL of CD CHO medium without supplements in a 50 mL greiner tube and incubated for 3 minutes. Additionally, 60 µg of PEImax were added to 1500 µl of CD CHO without supplements in a second 50 mL greiner tube and incubated for 3 minutes. The contents of both tubes were subsequently mixed together, incubated for 3 minutes as well and added to the bioreactor tube, already containing 8 mL of cell suspension. After incubating the bioreactor for 4 hours at 220 rpm, 19 mL of CDM4HEK medium containing 8 mM L-glutamine and 15 mg/L phenol red was added. The tubes were incubated at 37 °C. 5 % CO_2 and 220 rpm. Additionally, 5 mM VPA and 0.5 % TN1 were added on day 2.

Samples were taken on every day of the transient gene expression for determination of the cell concentration, viability and antibody titer. Furthermore, 1 mL sample was obtained on day 2 for analysis via FACS. The expression was stopped after the viability value dropped to 60 % or lower. The bioreactor tube was harvested by centrifugation for 10 minutes at 1300 rpm and the supernatant was collected in a T80 roux flask.

The transient gene expression 1 also included a separate tube as negative control, containing all the ingredients described above, but without addition of plasmid DNA. The number of tubes of each transient gene expression and the used plasmid preparations are shown in Table 9 and Table 10.

Table 9 Number of tubes of each transient gene expression

	TGE 1	**TGE 2**	**TGE 3**	**TGE 4**
Tubes	1	10	10	10

Table 10 Plasmid preparations used for the transient gene expressions of su3H6

Plasmid	**TGE 1**	**TGE 2**	**TGE 3**	**TGE 4**
pCEP4_SU3H6_HC #3	MN 8.9.16	MN 8.9.16	AW 6.10.16	AW 4.1.17
pCEP4_SH3H6_LC_v2	MN 8.9.16	MN 8.9.16	AW 20.9.2016	AW 4.1.17

4.2.8 Antibody Purification

4.2.8.1 Concentration and buffer exchange via TFF

For concentration and buffer exchange into buffer A of the antibodies present in the culture supernatant, tangential flow filtration (TFF) was utilized using a 30 kDa membrane cut-off. In tangential flow filtration, the feed stream is pumped across the face of the membrane, leading to the back transport of the fouling fractions to the feed stream. The applied transmembrane pressure causes fractions bigger than the membrane molecular cut off to pass the membrane, whereas the cross flow is responsible for the back transport of retained fractions such as antibodies to the feed[18]. For the concentration of the 3H6 antibodies, the Millipore Labscale TFF system with the Pellicon XL Biomax 30 kDa membrane was utilized. Because of the low pore size of the membrane, the process can also be referred to as ultrafiltration.

First, the TFF system was cleaned before the concentration. Therefore, the permeate tubings were disconnected from each other and the pump was activated to remove the 0.05 M NaOH, which was stored in the tank. Then the system was flushed twice with filtrated 500 mL dH_2O.

The culture supernatant was filtrated before the concentration process for the removal of particles from the solution. Therefore, the Stericup 500 mL Millipore Express Plus 0.22 µm filter was utilized. The supernatant was filled into the 0.22 µm filter and the full filter was weighted. After the filtration, the filtrated supernatant was filled into the tank of the TFF system and the empty 0.22 µm filter was weighted to determine the applied supernatant volume.

The concentration process was started by activating the pump of the TFF system and adjusting the transmembrane pressure to 1 bar. Permeate was collected in a 500 mL flask. When only approximately 10 mL supernatant solution were left in the tank, 5 x 25 mL of buffer A were added to suspend the antibodies in buffer A. The solution was filtrated until only little antibody solution was remaining. The retentate was subsequently collected into a 50 mL greiner tube by pipetting the antibody solution out of the tank. Remaining antibody solution in the tubings of the system was extracted by connecting a syringe to the pump outlet, flushing 2 x 6 mL of buffer A and finally air through the system into the collection tube.

Finally, the system was cleaned by filtrating 2x 500 mL of filtrated dH_2O. The tank was subsequently filled with 500 mL 0.05 M NaOH and 50 mL were flushed through the system. 100 µL sample was obtained from the retentate and 200 µL from the permeate solutions. The retentate tube and the permeate flask were weighted to determine their volume.

4.2.8.2 Purification via protein A affinity chromatography

The purification of the antibody was performed via protein A chromatography using the Äkta start system with the HiTrap MabSelect SuRe column. The column is packed with a resin containing protein A molecules. The antibodies bind with a high affinity and specificity to the protein A molecules through their Fc region. Because impurities can attach to the bound antibodies and co-elute with them, a washing step is necessary before the elution. The elution of the antibodies is performed by applying low pH to the protein A column[19].

First, the concentrated and buffer exchanged antibody solution (retentate) was filtrated through an 0.45 µm filter to remove particles before the application to the protein A column. The Äkta start system was stored in 20 % ethanol. For removal of the ethanol, the system was cleaned by applying 0.5 M NaOH with a flow rate of 5 mL/min to the sample inlet tubing. After washing the system with NaOH, the protein A column was installed and the entire system was flushed with dH_2O through the pump A and B inlets with a flow rate of 1 mL/min. Also, the sample inlet tubing was washed with dH_2O to remove residual NaOH, which could denaturate the antibodies of the sample. Subsequently, the buffers A and B were connected to the pump A and B inlets and the system was first flushed with buffer A, then B and finally with buffer A again for restoring the pH value of the system to 7.5.

The system was prepared for absorption of the concentrated antibody solution and the recording of the UV and conductivity sensor data was initiated. The concentrated antibody solution was applied to the sample inlet tubing and simultaneously the collection of the flow through fraction into a 50 mL greiner tube was started.
During this step, the 3H6 antibodies were loaded onto the protein A column through their heavy chain, whereas other present protein fractions were not retained. The indistinct protein fractions of the retentate led to an increase of the UV signal.

After adsorption of the antibodies to the protein A column, the column was washed with buffer A and the resulting wash fraction was collected in a different 50 mL greiner tube. The UV signal was decreasing as result of absent protein fractions. The wash fraction was collected for approximately three column volumes, afterwards the elution of the antibodies was initiated by pumping buffer B through the column. Simultaneously the elution fraction was collected in a new 50 mL greiner tube. The elution of the antibody resulted in a rapidly increasing and subsequently falling UV signal, forming a distinct peak. After the peak. the column was flushed with buffer A and the collection of the clean fraction was started.
The elution fraction was neutralized by dropwise addition of 0.1 M Tris with a pH of 9.5. The pH of 7 of the purified antibody solution was controlled via pH paper.

The column was cleaned by pumping 0.5 M NaOH through the sample inlet tubing. The high ion content of the NaOH solution led to an increase of the measured conductivity. Residual antibody fractions bound to the column were denaturated by the NaOH, eventually causing a temporary and slight increase of the UV signal. The recording of the sensor data and collection of the clean fraction was stopped and the entire system was flushed with dH_2O by connecting dH_2O to the pump A and B inlets. The sample inlet tubing was also cleaned with dH_2O for removal of residual NaOH.

Finally, the system was flushed with 20 % ethanol and the column was removed. 200 µL sample was collected of all fractions, except of the elution fraction, of which only 100 µL sample was obtained. Figure 1 shows the recorded UV and conductivity data of the su3H6 transient gene expression 1 & 2 purification, illustrating the described behaviour of each step.

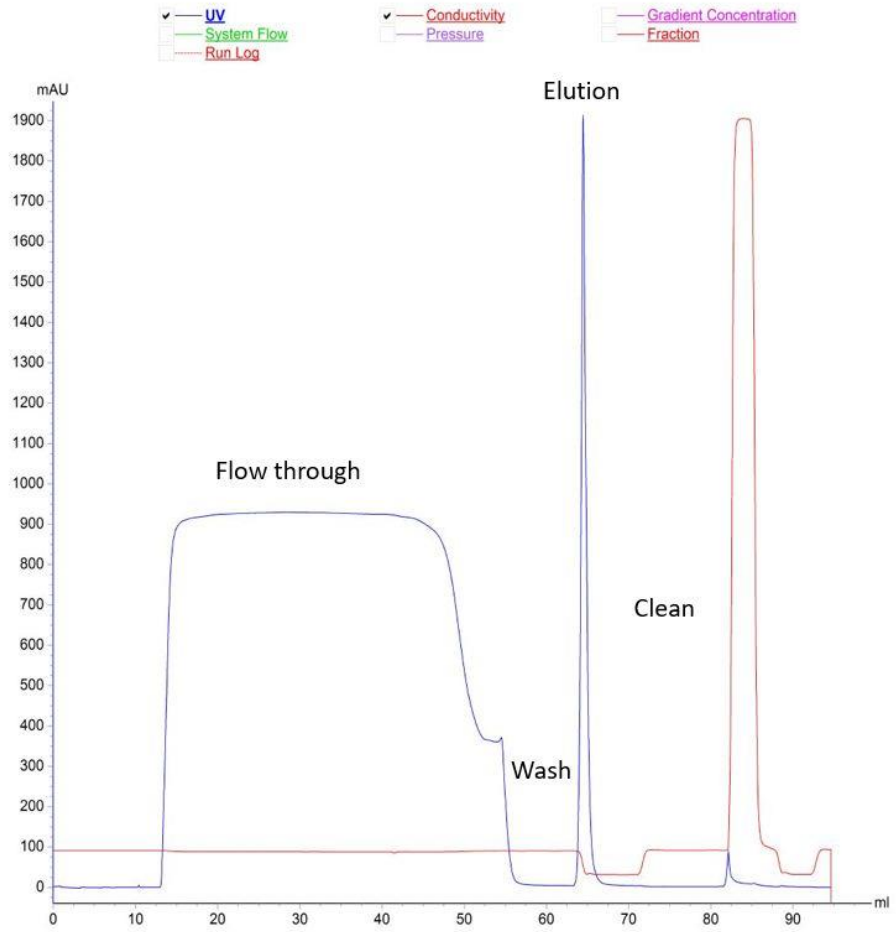


Figure 1 Chromatogram of the TGE 1 & 2 purification

4.2.8.3 Dialysis of the purified antibody solution

The purified antibody solution was eluted with buffer B after the purification, which contains 100 mM glycine. For bringing the sample into PBS, the buffer was changed via dialysis. The antibody solution was filled into a dialysis tubing, which consists of a semipermeable membrane. Molecules with a molecular weight below the molecular weight cut-off of the membrane can pass through the pores of the membrane and enter the surrounding dialysis buffer. This movement is driven by the concentration difference between the solutes in the dialysis buffer and the protein solution and therefore the movement stops after reaching an equilibrium concentration. After reaching the equilibrium concentration, the dialysis buffer (PBS) needs to be changed to continue the dialysis[20].

The buffer of the 3H6 antibody solution was changed to 1 x PBS via dialysis. Therefore, the solution was transferred into a dialysis tubing of appropriate length (1 cm/mL antibody solution + 2 cm space for the clips sealing the tubing), which was subsequently incubated in a beaker for 2 hours in 500 mL 1 x PBS under slow stirring. After 2 hours, the dialysis tubing was transferred to fresh 500 mL 1 x PBS to continue the dialysis and incubated over night at 4 °C. The dialysed product was filtrated through a sterile 0.22 µm filter into a pre-weighted centrifuge tube, using a sterile 5 mL syringe. After filtration, the full centrifuge tube was weighted for determination of the product volume and samples were collected for measurement of the product concentration via ELISA and NanoDrop. Finally, the product was stored at 4 °C.

4.2.8.4 Determination of the antibody concentration via NanoDrop

The concentration of the purified and dialysed product was measured using the A280 method via the NanoDrop ND-1000 spectrophotometer. For initialization and blank measurement, 3 µL of 1x PBS was used. Afterwards, the device was loaded with 3 µL of purified antibody sample and the absorbance of the 3H6 antibody at 280 nm was measured twice. The NanoDrop software subsequently calculated the concentration according to equation 11:

$$A = E * b * c \qquad \text{(Equation 11)}$$

A…absorbance in absorbance units
E…extinction coefficient (wt3H6, su3H6:204505, GA3H6: 198545)
b…path length in cm (1 cm)
c…analyte concentration in mol/L

Finally, an average value of the two measurements of the antibody solution was calculated.

4.2.8.5 Analysis of the collected fractions during purification via SDS-PAGE

The collected fractions of the su3H6 transient expression 1 & 2 purification were analysed via SDS-PAGE. A NuPAGE 4-12 % Bis-Tris gel was used for this purpose. 5 µL of the PageRuler prestained protein ladder was applied to the first slot of the gel. The target amount of 3H6 sample to be loaded on the goal was 1 µg, which was calculated based on the ELISA concentrations of each fraction. If the concentration of a fraction was too low to reach 1 µg, 20 µL were loaded onto the gel, if the concentration was higher, the sample was diluted to 1 µg with dH_2O. 20 µL of each sample was loaded onto the gel, which was then run at 120 V for 90 minutes. Afterwards, the gel was silver stained for visualization of the different protein bands.

Silver Staining

The SDS-PAGE gel was first incubated in fixation solution for 1 hour and afterwards in incubation solution for 20 minutes. After washing the gel three times with dH_2O, the gel was incubated in silver nitrate solution for 15 minutes. The gel was washed in dH_2O and suspended in developing solution until bands became visible. The developing reaction was stopped using the stop solution, the gel stored between a plastic wrap with 10 % glycerol on top of the gel and subsequently scanned.

4.2.9 Kinetics

The kinetic properties of 3H6 antibodies were analysed via bio-layer interferometry using the ForteBio® Octet® QK system. Bio-layer interferometry is a label-free method, which uses light, that is directed towards two interfaces, the surface layer on the sensor tip and an internal reference layer. The binding of an antibody to the surface of the sensor tip leads to a shift in the interference pattern of the reflected light, as the surface layer moves away from the reference layer. The shift depends on the number of ligands binding to the surface layer[21].

After the analysis via bio-layer interferometry the k_{obs}, k_{on}, k_{dis}, and K_d values could be calculated. K_{obs} is the observed rate constant, because as the antibody is binding to its target, it is simultaneously also dissociating. K_{on} describes only the association of the antibody to its epitope, whereas k_{dis} the dissociation. The dissociation constant K_d, also called "binding affinity", is calculated by dividing the k_{dis} through the k_{on} value and gives information about the strength of the binding capacity. The unit of the K_d value is stated in molar concentrations (M) [22].

Furthermore, the free binding energy ΔG of each antibody can be calculated with a given dissociation constant using equation 12:

$$\Delta G = -RT \ln K_d \qquad \text{(Equation 12)}$$

ΔG…free binding energy [J/mol]
R…gas constant [J/·mol·K]
T…temperature [K]
K_d…dissociation constant [M]

Binding of an antibody to its epitope only occurs with a negative free binding energy value[23].

4.2.9.1 Protein A assisted binding assay

The kinetics of the wt3H6, GA3H6 and su3H6 antibodies were determined using the protein A assisted binding assay. Because only the su3H6 antibody was purified at the time of the assay, supernatants containing wt3H6 and GA3H6 antibodies, respectively, were concentrated using Amicon® Ultra 15 mL and 0.5 mL columns.

Concentration of wt3H6 supernatant

For the wt3H6 antibody, an antibody concentration of 15 µg/mL was assumed in the supernatant. The supernatant was therefore concentrated by factor 5 to reach a final concentration of 75 µg/mL. 200 µL of antibody solution were required for each well of the microtiter plate. The wt3H6 antibody was analysed with three different concentrations of 2F5 IgG, therefore 600 µL of wt3H6 antibody solution were required in total. Additional 200 µL of wt3H6 were prepared for quantification by bio-layer interferometry.
4 mL of wt3H6 supernatant were concentrated using a Amicon® Ultra 0.5 mL column. The column was centrifuged for 5 minutes at 14 000 g and refilled with supernatant as many times as the 4 mL of supernatant were loaded completely and only 92 µL of concentrated antibody solution were left. The 92 µL were then filled up to 800 µL with kinetics buffer.

Concentration of GA3H6 supernatant

For the GA3H6 antibody, an antibody concentration of 2 µg/mL was assumed in the supernatant. The supernatant was therefore concentrated by factor 37.5 to reach a final concentration of 75 µg/mL. 200 µL of antibody solution were required for each well of the microtiter plate. The GA3H6 antibody was analysed with three different concentrations of 2F5 IgG, therefore 600 µL of GA3H6 antibody solution were required in total. Additional 200 µL of GA3H6 were prepared for quantification by bio-layer interferometry.
30 mL of GA3H6 supernatant were concentrated using a Amicon® Ultra 15 mL column. The column was centrifuged for 20 minutes at 4 000 g and refilled with supernatant as many times as the 30 mL of supernatant were loaded completely and only 500 µL of concentrated antibody solution were left. The remaining 500 µL were then transferred to a Amicon® Ultra 0.5 mL column and centrifuged for 5 minutes at 14000 g as many times as only 340 µL were left. The solution was then filled up to 800 µL using kinetics buffer.

Preparation of su3H6 TGE 3

TGE 3 of su3H6 with a concentration of 0.98 mg/mL was diluted to 75 µg/mL. Because the kinetics of su3H6 was analysed only with one concentration, only 400 µL antibody solution were required, 200 µL for the kinetic assay and 200 µL for quantification using bio-layer interferometry. 31 µL of su3H6 TGE 3 were added to 369 µL kinetics buffer which corresponds to a 1:13.1 dilution.

Preparation of 3D6 scFv-Fc antibody

For blocking, 100 µg/mL 3D6 scFv-Fc antibody was used, which was already available at this concentration.

Preparation of 2F5 IgG

The 2 F5 IgG was available in a concentration of 12.1 mg/mL. The prepared 2F5 concentrations are shown in Table 11.

Table 11 Prepared 2F5 IgG concentrations for the protein A assisted assay

2F5 IgG concentration [µg/mL]	Prepared volume [µL]	Preparation
30	650	2.5 µL of 2F5 stock were added to 997.5 µL kinetics buffer
15	450	350 µL of the 30 µg/mL dilution were added to 350 µl kinetics buffer
7.5	500	250 µL of the 15 µg/mL dilution were added to 250 µL kinetics buffer

A detailed overview of all prepared antibodies and their concentrations is provided in Table 12.

Table 12 Overview over all antibodies used for the protein A assisted assay

Antibody	Concentration [µg/mL]	Prepared volume [µL]
wt3H6	75	800
GA3H6	75	800
su3H6	75	400
3D6 scFv-Fc	100	1500
2F5 IgG	30	650
2F5 IgG	15	450
2F5 IgG	7.5	500

Quantification of the used antibodies via bio-layer interferometry

To be able to make an exact calculation of the kinetic characteristics of the 3H6 antibodies, all prepared antibodies were analysed using bio-layer interferometry to measure the actual concentrations using protein A sensor tips. The wt3H6 and su3H6 samples were diluted 1:4 and 1:8, the GA3H6 sample was diluted 1:5 and 1:10, whereas the 2F5 IgG 30 µg/mL sample was diluted twice 1:2. All dilutions were prepared with PBS-t buffer. 200 µL of all dilutions were added to the octet microtiter plate. Additionally, 200 µL of PBS-t and glycine (pH 2.5) were pipetted in separate wells of the microtiter plate. Each sample was subsequently measured for 300 seconds with the same protein A tip. The protein A tip was regenerated after each sample by moving the tip three times to the glycine buffer for 5 seconds for removal of bound antibodies and three times to the PBS-t buffer for 5 seconds for neutralization. The concentration of the samples was calculated by making a linear fit with the 3D6 IgG standard curve ranging between 0.39 and 25 µg/mL.

Plate layout of the protein A assisted assay

Table 13 shows the plate layout of the protein A assisted assay. Each row corresponds to a separate protein A sensor tip.

Table 13 Plate layout of the protein A assisted assay

	1	2	3	4	5
A	kinetics buffer	wt3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (200 nM)	kinetics buffer
B	kinetics buffer	wt3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (100 nM)	kinetics buffer
C	kinetics buffer	wt3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (50 nM)	kinetics buffer
D	kinetics buffer	GA3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (200 nM)	kinetics buffer
E	kinetics buffer	GA3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (100 nM)	kinetics buffer
F	kinetics buffer	GA3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (50 nM)	kinetics buffer
G	kinetics buffer	su3H6 (75 µg/mL)	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (200 nM)	kinetics buffer
H	kinetics buffer	kinetics buffer	3D6 scFv-Fc (100 µg/mL)	2F5 IgG (200 nM)	kinetics buffer

Steps of the protein A assisted assay

The assay comprised of different steps. First, the protein A sensors A-H were incubated for 60 seconds in column 1 for recording of the baseline. Afterwards the 3H6 antibodies were loaded onto the protein A sensors through their heavy chain by moving the sensor tips to column 2 for 1200 seconds. The sensors were subsequently moved to column 1 for 60 seconds to record another baseline. To prohibit the binding of 2F5 IgG to the protein A sensors, remaining free protein A entities were blocked by moving the sensor tips to column 3 for 1200 seconds and allowing 3D6 scFv-Fc to bind to residual protein A molecules. After the blocking step, another baseline recording step was performed by incubating the tips in column 1 for 120 seconds. The protein A sensors were subsequently moved to column 4 for 600 seconds, allowing the 2F5 IgG antibody to bind to the 3H6 antibodies. As last step, the protein A sensors were incubated for 1200 seconds in column 5, containing fresh kinetics buffer, leading to the dissociation of 2F5 IgG. Table 14 gives an overview over the described assay steps.

Table 14 Overview over the protein A assisted assay steps

Step	Column	Time [s]	Purpose
step 1	1	60	Baseline (kinetics buffer)
step 2	2	1200	Antigen loading
step 3	1	60	Baseline 2 (kinetics buffer)
step 4	3	1200	Blocking (3D6scFv-Fc)
step 5	1	120	Baseline 3 (kinetics buffer)
step 6	4	600	Association (2F5 IgG)
step 7	5	1200	Dissociation (kinetics buffer)

4.2.9.2 Streptavidin assisted binding assay

The kinetic values of the antibodies were also determined by performing a streptavidin binding assay. The antibodies wt3H6 and GA3H6 were prepared in three different concentrations, whereas su3H6 only in one concentration. For the streptavidin assay, also biotinylated 2F5 IgG antibody was required, which was already available. The used concentrations and the way they were prepared are shown in Table 15.

Table 15 Overview over all antibodies used for the streptavidin assisted assay

Antibody	**Molar concentration [nM]**	**Prepared volume [µL]**	**Preparation**
wt3H6 puri 1 60 µg/mL	400	250	19.9 µL of wt3H6 puri 1 were added to 480.1 µL kinetics buffer
wt3H6 puri 1 30 µg/mL	200	250	250 µL of the 60 µg/mL dilution were added to 250 µL kinetics buffer
wt3H6 puri 1 15 µg/mL	100	500	250 µL of the 30 µg/mL dilution were added to 250 µL kinetics buffer
GA3H6 puri 1 60 µg/mL	400	250	89.6 µL of GA3H6 puri 1 were added to 410.4 µL kinetics buffer
GA3H6 puri 1 30 µg/mL	200	250	250 µL of the 60 µg/mL dilution were added to 250 µL kinetics buffer
GA3H6 puri 1 15 µg/mL	100	500	250 µL of the 30 µg/mL dilution were added to 250 µL kinetics buffer
su3H6 TGE 4 60 µg/mL	400	700	50.6 µL of su3H6 TGE 4 were added to 649.4 µL kinetics buffer
biotinylated 2F5 IgG 20 µg/mL		1600	already available

Quantification of the used antibodies via bio-layer interferometry

The antibody solutions used for the streptavidin assay were quantified using bio-layer interferometry to enable the exact calculation of the kinetic values.
The 15 µg/mL wt3H6 and GA3H6 samples were measured undiluted and as 1:2 dilutions, whereas the su3H6 60 µg/mL sample was first diluted twice 1:2 to reach a concentration of 15 µg/ml, the 15 µg/mL concentration was then measured as well as two subsequent 1:2 dilutions. The concentration of the biotinylated 2F5 IgG antibody was not measured.
Only the 15 µg/mL concentrations could be measured directly, because the 3D6 IgG standard used for the quantification was only ranging to 25 µg/mL, the concentrations of the higher concentrated samples were back calculated based on the 15 µg/mL results.

All dilutions were prepared with PBS-t buffer. 200 µL of all dilutions were added to the octet microtiter plate. Additionally, 200 µL of PBS-t and glycine (pH 2.5) were pipetted in separate wells of the microtiter plate. Each sample was subsequently measured for 300 seconds with the same protein A tip. The protein A tip was regenerated after each sample by moving the tip three times to the glycine buffer for 5 seconds for removal of bound antibodies and three times to the PBS-t buffer for 5 seconds for neutralization. The concentration of the samples was calculated by making a linear fit with the 3D6 IgG standard curve ranging between 0.39 and 25 µg/mL.

Plate layout of the streptavidin assisted assay

Table 16 shows the plate layout of the streptavidin assisted assay. Each row corresponds to a separate streptavidin sensor tip.

Table 16 Plate layout of the streptavidin assisted assay

	5	6	7	8
A	kinetics buffer	2F5 IgG (20 µg/mL)	wt3H6 (60 µg/mL, 400 nM)	kinetics buffer
B	kinetics buffer	2F5 IgG (20 µg/mL)	wt3H6 (30 µg/mL, 200 nM)	kinetics buffer
C	kinetics buffer	2F5 IgG (20 µg/mL)	wt3H6 (15 µg/mL, 100 nM)	kinetics buffer
D	kinetics buffer	2F5 IgG (20 µg/mL)	GA3H6 (60 µg/mL, 400 nM)	kinetics buffer
E	kinetics buffer	2F5 IgG (20 µg/mL)	GA3H6 (30 µg/mL, 200 nM)	kinetics buffer
F	kinetics buffer	2F5 IgG (20 µg/mL)	GA3H6 (15 µg/mL, 100 nM)	kinetics buffer
G	kinetics buffer	2F5 IgG (20 µg/mL)	su3H6 (60 µg/mL, 400 nM)	kinetics buffer
H	kinetics buffer	kinetics buffer	su3H6 (60 µg/mL, 400 nM)	kinetics buffer

Steps of the streptavidin assisted assay

The streptavidin sensor tips were first incubated in column 5 for 60 seconds for recording of the baseline. Afterwards, the sensor tips were moved to column 6 for 1200 seconds for loading of biotinylated 2F5 IgG onto the streptavidin sensor tips. The sensors were moved to column 5 for 60 seconds to record another baseline and were subsequently incubated in column 7, containing the different 3H6 antibodies, allowing them to bind to the 2F5 IgG antibody. As last step, the sensors were moved to column 8, containing fresh kinetics buffer, for dissociation of the 3H6 antibodies from 2F5 IgG. Table 17 gives an overview over the described assay steps.

Table 17 Overview over the streptavidin assisted assay steps

Step	Column	Time [s]	Purpose
step 1	5	60	Baseline (kinetics buffer)
step 2	6	1200	2F5 IgG loading
step 3	5	120	Baseline 2 (kinetics buffer)
step 4	7	600	Association (wt3H6, GA3H6, su3H6 IgG)
step 5	8	1200	Dissociation (kinetics buffer)

5 Results

5.1 Cultivation of HEK293.6E for the transient expression of su3H6

For the transient expression of su3H6, HEK293.6E needed to be cultivated.
Because cultivations of HEK293.6E with 20 passages or more could already have lost a significant amount of the EBNA-1 protein and therefore result in lower productivities when used for transient expressions, a total number of three shake flasks were cultivated in parallel or sequentially to guarantee high productivities.

All three shake flasks contained 30 mL of CDM4HEK293 with 4 mM L-glutamine, 15 mg/L phenol red and 25 µg/mL G418 as supplements. All shake flasks were passaged to a start cell concentration of 2 * 10^5 C/mL and were cultivated at 37 °C and 5 % CO_2

The measured viabilities, cell concentrations and specific growth rates are shown in Figure 2 and Figure 3. It can be noticed, that the measured cell concentrations of all shake flasks in Figure 2 show mostly up and down movements, which can be explained by the differing cultivation times of the passages, alternating between three and four days. The cell concentrations, viabilities and growth rates of shake flask 1 (220 rpm) were very low during the first eleven passages, which can be explained by the necessity of the HEK293.6E culture to adapt to the high shaking speed of 220 rpm. After the adaption, the shake flask 1 at 220 rpm showed slightly higher cell concentrations and growth rates compared to the other two shake flasks at 140 rpm. The cell concentrations, viabilities and growth rates of the shake flasks 2 and 3 remained constant over the cultivation time.

Average values of the viabilities, cell concentrations and growth rates were calculated and are highlighted in Table 18. The first eleven passages of shake flask 1 (220 rpm) were not included into the calculation of the average values.

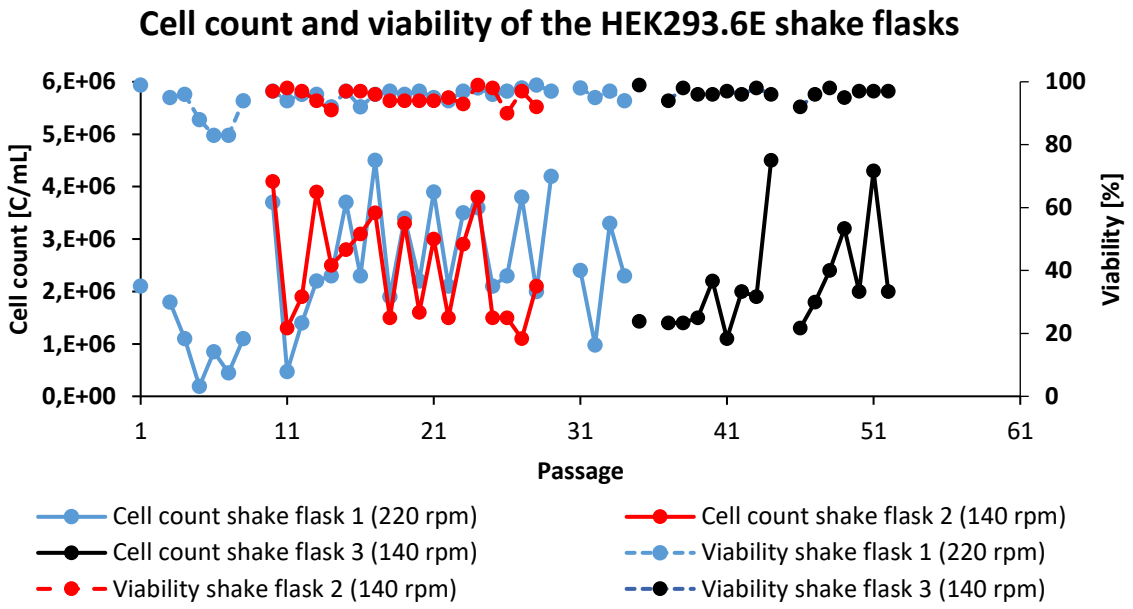


Figure 2 Measured viabilities and cell concentrations of the HEK293.6E shake flasks

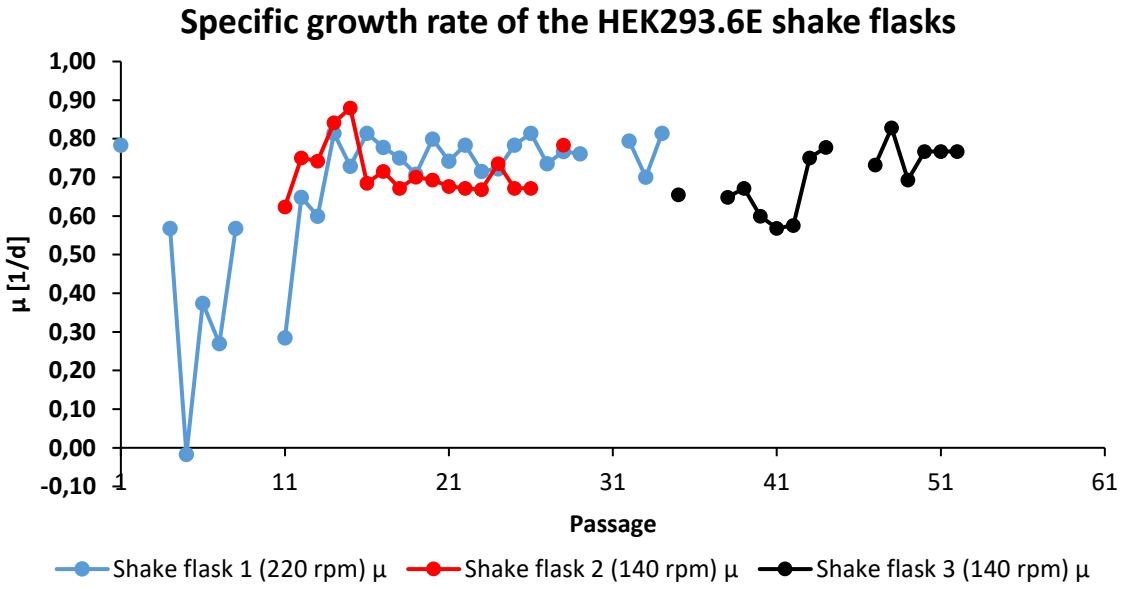


Figure 3 Specific growth rates of the HEH293.6E shake flasks

Table 18 Average values of the viabilities. cell concentrations and growth rates of the HEK293.6E shake flasks

Shake flask	**Cell concentration [C/mL]**	**Viability [%]**	**Growth rate μ [1/d]**
Shake flask 1 (220 rpm)	2.74E+06	96	0.75
Shake flask 2 (140 rpm)	2.44E+06	95	0.72
Shake flask 3 (140 rpm)	2.15E+06	96	0.70

Shake flask 1 (220 rpm) shows the highest cell concentration and growth rate value, indicating that the high shaking speed has no negative effects on the cultivation of HEK293.6E. However, the growth rates of all three shake flasks are similar. The average cell concentration of shake flask 3 (140 rpm) is the lowest of all three shake flasks, which can be explained by the short cultivation time of the culture, the HEK2936.E culture may have needed more time to adapt to the cultivation conditions.

5.2 Cultivation of wt3H6-CHO

5.2.1 Definition of the cultivation medium

The CHO DUKX-B11 strain CHO-3H6/7C6/6B9 SF #131204 has the gene for the human IgG1 antibody wt3H6 stably integrated and constantly produces the antibody during cultivation. For an efficient production of the wt3H6 antibody, several cultivation media were tested. Table 19 gives an overview about the different tested cultivation media. The analysis of wt3H6-CHO under the light microscope showed the formation of clusters composed of wt3H6-CHO cells, making the determination of the viability via haemocytometer more difficult.

Table 19 Tested media for the optimal cultivation of wt3H6-CHO

Medium	**Supplements**	**Culture system**	**Culture volume [mL]**	**Shaking [rpm]**	**Start cell concentration [C/mL]**
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.38 µM MTX	T25 roux flask	10	static	$2 * 10^5$
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.38 µM MTX	T80 roux flask	30	static	$2 * 10^5$
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.38 µM MTX	T80 roux flask	30	static	$1 * 10^6$
CD CHO	4 mM L-gln 15 mg/L phenol red 0.38 µM MTX	T80 roux flask	30	static	$2 * 10^5$

The cell concentrations, viabilities, antibody titers. growth rates and specific productivities of all tested cultivation media are shown in Figure 4, Figure 5 and Figure 6.

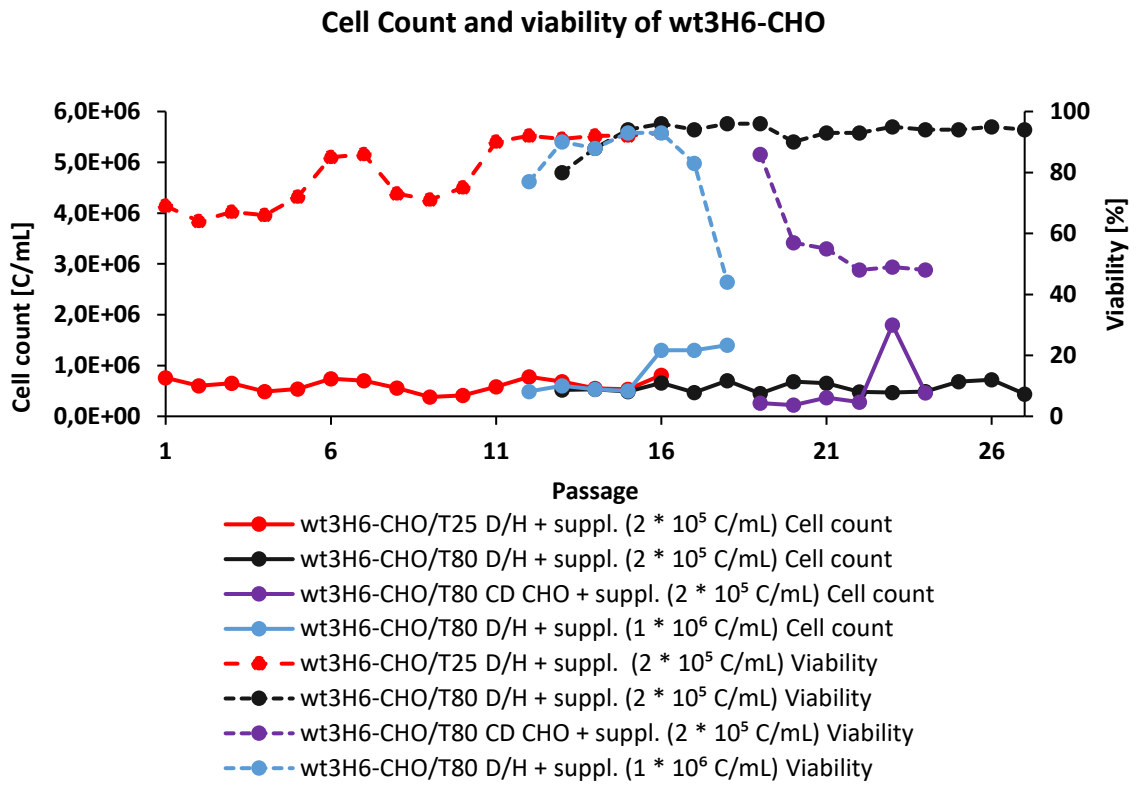


Figure 4 Cell count and viability of the wt3H6-CHO cultivations using different media

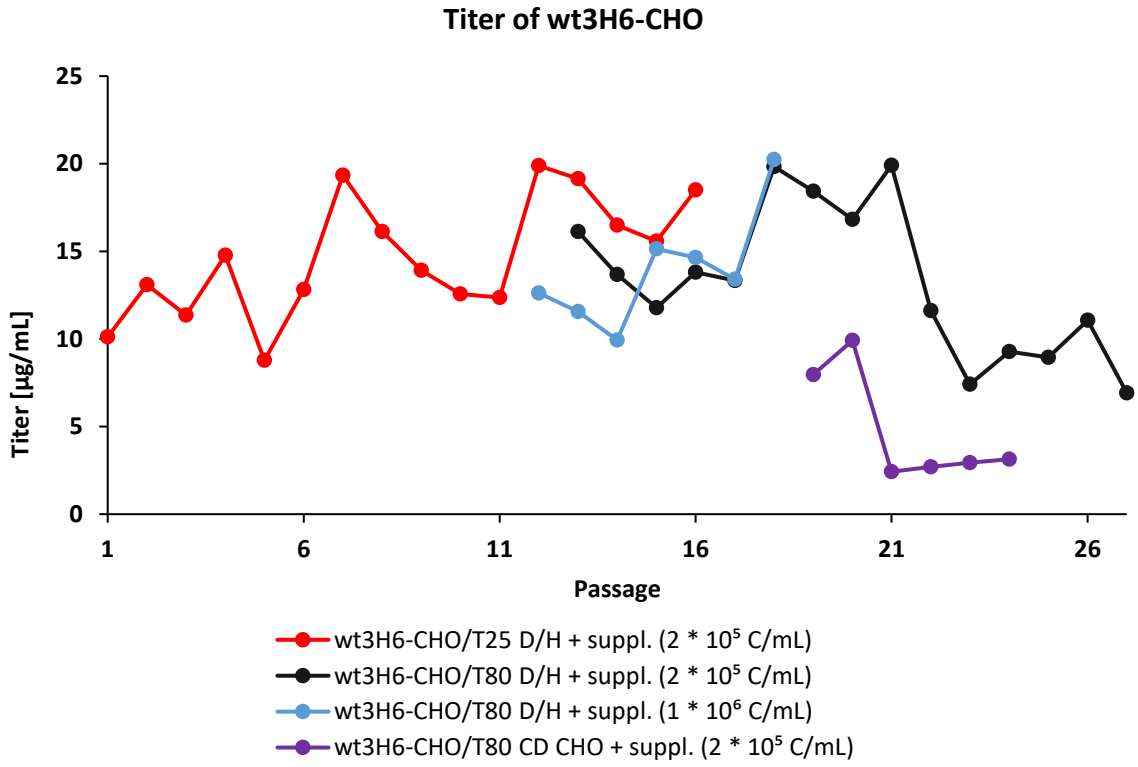


Figure 5 Antibody titer of the wt3H6-CHO cultivations using different media

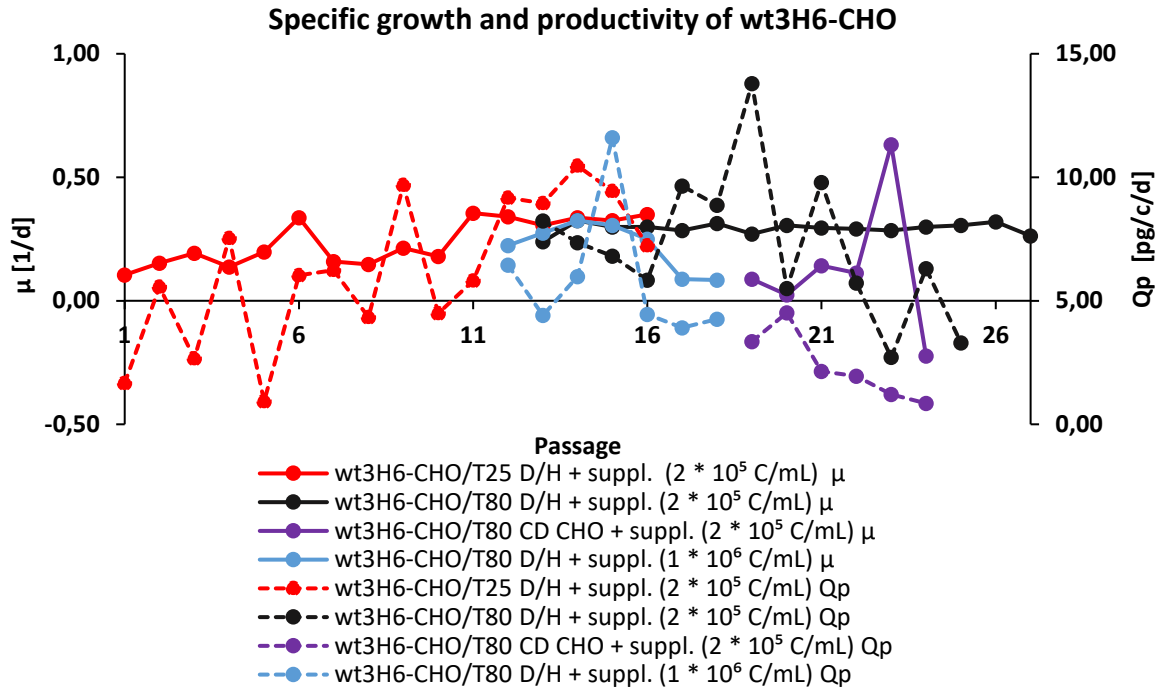


Figure 6 Growth rate and specific productivity of the wt3H6-CHO cultivations using different media

The figures outline the inability of wt3H6-CHO to grow in CD CHO medium, as the viability of the CD CHO culture was decreasing to 57 % after the first passage (Figure 4). Hence, D/H medium was defined as the medium of choice and used for further examinations. The D/H T80 roux flask culture with a start cell concentration of 1 * 10^6 C/mL exhibited rapidly falling viability values, which could be related to the non-existent mixing of the nutrient components or insufficient oxygen supply. Therefore, it was decided to test the cultivation of wt3H6-CHO in shake flasks at 140 rpm.

wt3H6-CHO cultivation in T25 roux flasks containing D/H medium (2 * 10^5 C/mL start cell concentration)

The cultivation was first tested in D/H medium. Therefore, a T25 roux flask culture was started with a start cell concentration of 2 * 10^5 C/mL. The viability of the T25 roux flask culture started at 69 % and slowly increased to 90 % after the 12^{th} passage. After the 12^{th} passage, the viability stayed above 90 % for the rest of the cultivation time (Figure 4). The antibody titer of the T25 roux flask culture was at 10 µg/mL after the first passage and constituted of 18.5 µg/mL, when the T25 roux flask culture was stopped (Figure 5).
An upward trend of the antibody titer, specific productivity and growth rate is observable, which correlates with an improvement of viability.

The growth rate was at 0.10 [1/d] after the first passage and improved to 0.35 [1/d] at the 17th passage. The T25 roux flask culture showed a productivity of 1.67 [pg/c/d] after the first passage and 7.24 pg/c/d after the 17th passage (Figure 6).
Table 20 gives an overview about the average values of the viability, cell concentration, antibody titer, growth rate and the specific productivity of the T25 roux flask culture.

Table 20 Average values of the wt3H6-CHO T25 roux flask culture

Medium	Start cell concentration [C/mL]	Viability [%]	Cell count [C/mL]	Titer [µg/mL]	Growth rate [1/d]	Specific productivity [pg/c/d]
D/H	2 * 10^5	80	6.10E+05	14.68	0.24	6.26

wt3H6-CHO cultivation in T80 roux flask containing D/H medium (2 *10^5 C/mL start cell concentration)

It could be observed, that wt3H6-CHO could be cultivated in 10 mL D/H medium using T25 roux flasks. As next step, the cultivation of wt3H6-CHO was moved to T80 roux flasks to be able to use a higher volume of D/H medium. The first passage of the T80 roux flask culture was started with 22 mL D/H medium with supplements, the volume was then increased to 30 mL.

The viability was at 80 % after the first passage and then increased to 94 % after the third passage. The viability stayed over 90 % after the third passage for the rest of the cultivation time (Figure 4). Although the viabilities, cell concentrations and growth rates remained constant after the third passage, the antibody titers and therefore the specific productivity values were falling during the last six passages (Figure 4, Figure 5 and Figure 6).

Table 21 gives an overview about the average values of the viability, cell concentration. antibody titer, growth rate and the specific productivity of the T80 roux flask culture.

Table 21 Average values of the wt3H6-CHO T80 roux flask culture

Medium	Start cell concentration [C/mL]	Viability [%]	Cell count [C/mL]	Titer [µg/mL]	Growth rate [1/d]	Specific productivity [pg/c/d]
D/H	$2 * 10^5$	93	5.62E+05	13.26	0.29	6.89

Although the average cell count of the T80 culture is lower than that of the T25 culture, the average antibody titer and specific productivity is higher, which can be explained by the lower viability of the T25 culture.

wt3H6-CHO cultivation in T80 roux flask containing D/H medium (1 *10^6 C/mL start cell concentration)

To be able to produce more wt3H6 antibody, a T80 culture was started with D/H as medium with the goal to increase the start cell concentration to $1 * 10^6$ C/mL as soon as possible. The culture was started with 15 mL medium and the volume was increased to 30 mL after the third passage.

The viability of the wt3H6-CHO T80 ($1 * 10^6$ C/mL) culture started at 77 % and increased to 93 % after the 5th passage. After the 5th passage the start cell concentration was increased to $1*10^6$ C/mL. The viability dropped to 83 % after the 6th and to 44 % after the 7th passage (Figure 4). The falling viability of the culture can be explained by extinguishing D/H media components or poor O_2 availability due to the lack of shaking.

wt3H6-CHO cultivation in T80 roux flask containing CD CHO medium (2 * 10^5 C/mL start cell concentration)

Because of the higher nutrient concentration of the CD CHO medium (Table 60), the cultivation of wt3H6-CHO using a T80 roux flask containing 30 mL of CD CHO medium with 4 mM L-glutamine, 15 mg/mL phenol red and 0.38 µM MTX was evaluated.

The viability started at 86 %, then it dropped to 57 % after the second and to 48 % after the 6th passage (Figure 4). The measured cell concentration of $1.8 *10^6$ C/mL in Figure 4 is an outlier and can be related to a false value. The cell concentrations, antibody titers, growth rates and specific productivities correlate with the falling viability values.

The examination of different cultivation conditions revealed, that the wt3H6-CHO clone could not be cultivated in CD CHO medium and therefore, it was decided to cultivate wt3H6-CHO in D/H medium at $2*10^5$ C/mL seeding cell density.

5.2.2 Adaptation of wt3H6-CHO to shaking conditions

As next step, the cultivation of wt3H6-CHO under shaking conditions was evaluated, since the cultivation at 140 rpm shaking speed could improve O_2 and nutrient availability and hence allow the cultivation of wt3H6-CHO at $1 * 10^6$ C/mL start cell concentration using D/H medium. Table 22 gives an overview about the tested shaking conditions. Two shake flasks were started, differing in the start cell concentration.

Table 22 Tested shaking conditions for the optimal cultivation of wt3H6-CHO

Medium	**Supplements**	**Culture system**	**Culture volume [mL]**	**Shaking [rpm]**	**Start cell concentration [C/mL]**
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.38 µM MTX	125 mL shake flask	30	140	$2*10^5$
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.38 µM MTX	125 mL shake flask	30	140	$1*10^6$

The cell concentrations, viabilities, antibody titers, growth rates and specific productivities of both shake flask cultures are shown in Figure 7. Figure 8 and Figure 9.

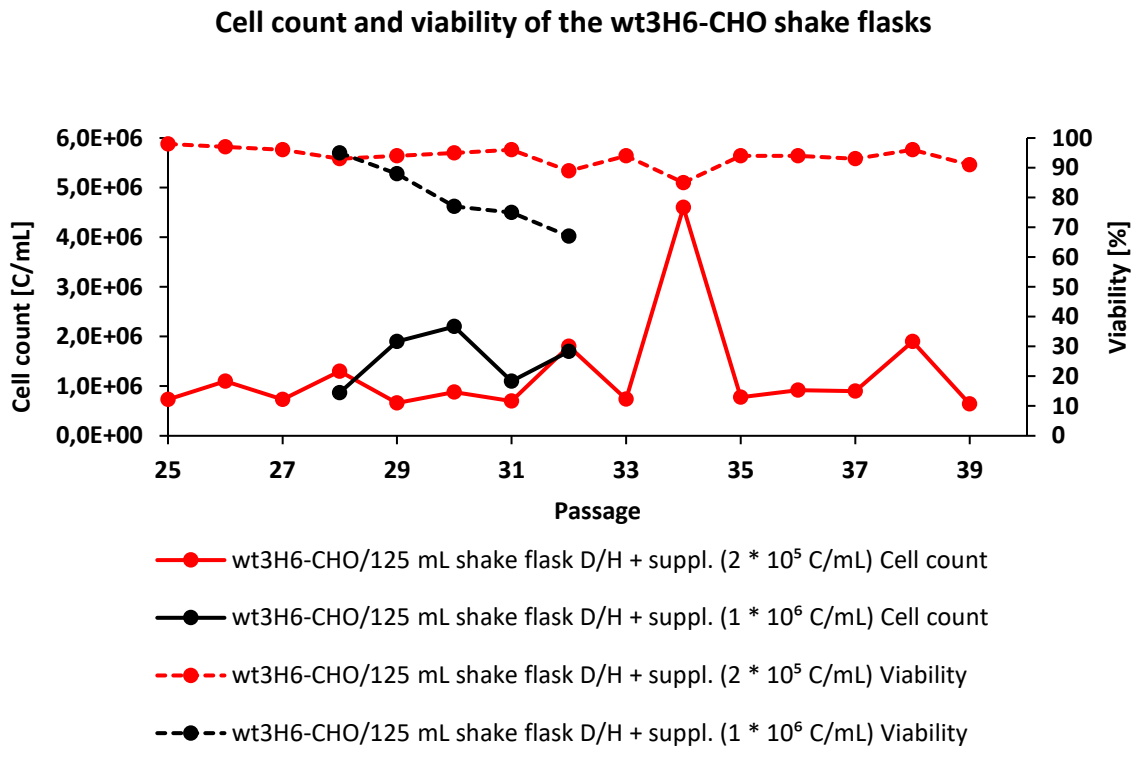


Figure 7 Cell count and viability of the wt3H6-CHO shake flasks

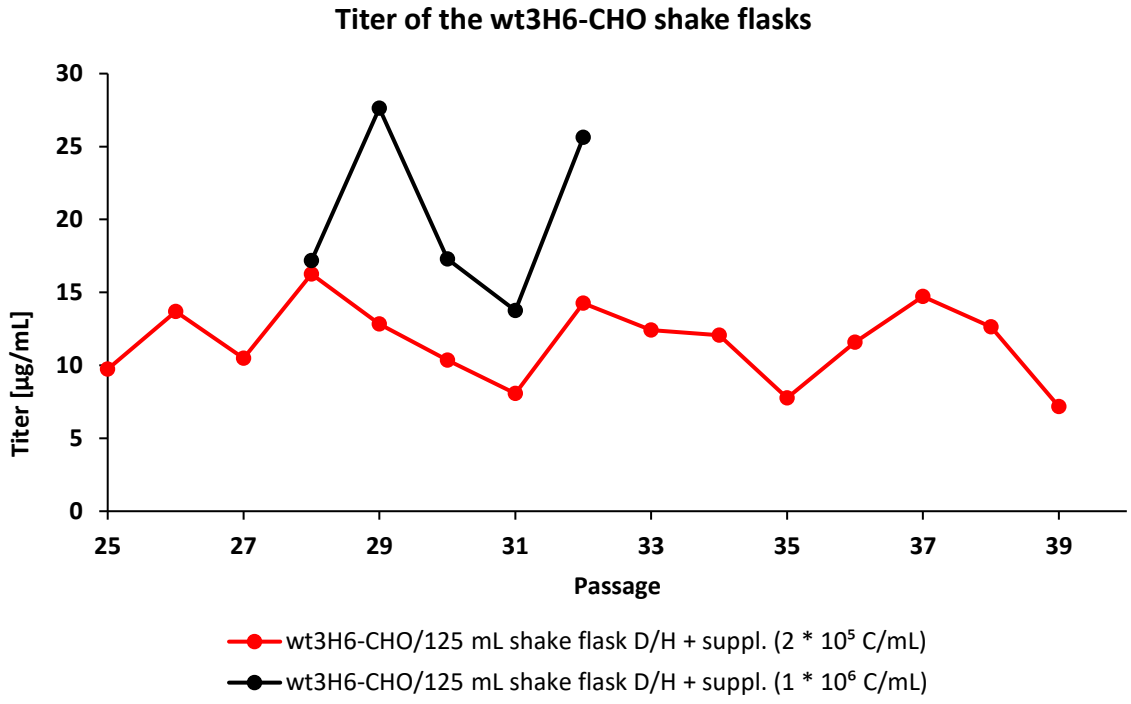


Figure 8 Antibody titer of the wt3H6-CHO shake flasks

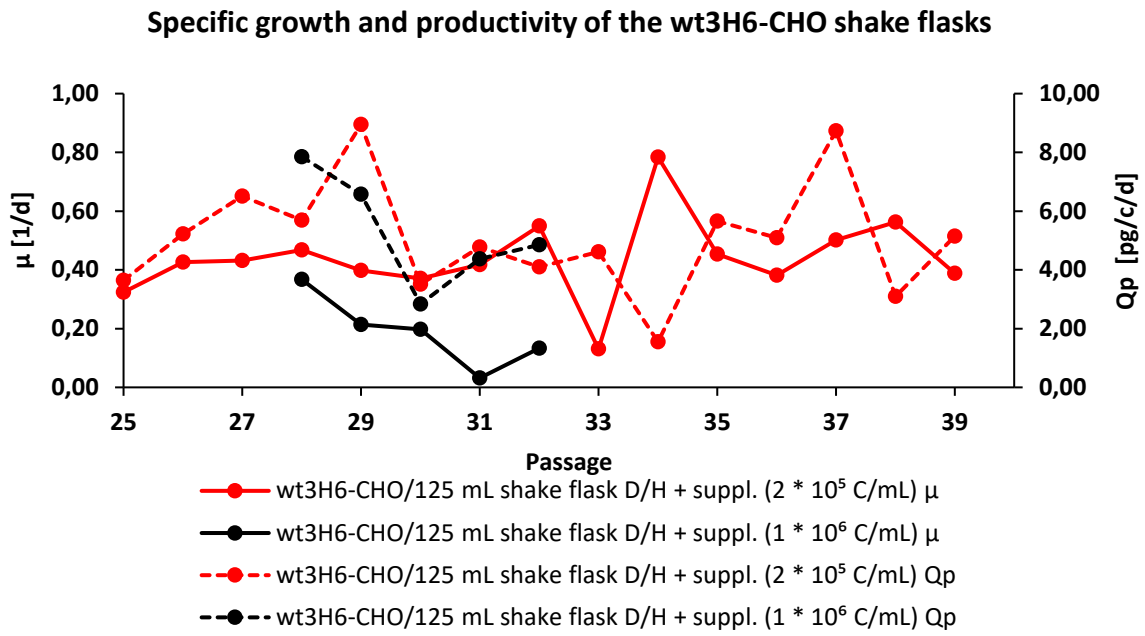


Figure 9 Specific growth and productivity of the wt3H6-CHO shake flasks

The cultivation of wt3H6-CHO in a 125 mL shake flask at a start cell concentration of 2 * 10^5 C/mL resulted in higher cell concentrations and growth rates (Figure 7 and Figure 9). However, the viability values of the shake flask with a start cell concentration of 1 * 10^6 C/mL were decreasing from passage to passage (Figure 7), even though not so fast as in the T80 roux flask cultivation. To further investigate this behaviour, it was decided to record a batch growth curve of the 1 * 10^6 C/mL shake flask.

wt3H6-CHO cultivation in 125 mL shake flask containing D/H medium (2 * 10^5 C/mL start cell concentration)

A 125 mL shake flask culture was started with 30 mL D/H medium and a start cell concentration of 2 * 10^5 C/mL to test if the shaking of wt3H6-CHO has negative effects on the cultivation. The shake flask was cultivated at 140 rpm.

The viability of the new shake flask culture started 98 % and stayed over 90% over the whole cultivation time (Figure 7). Table 23 gives an overview about the average values of the viability, cell concentration, antibody titer, growth rate and specific productivity of the wt3H6-CHO shake flask culture with 2 * 10^5 C/mL start cell concentration. The cell count of the 34th passage can be considered as an outlier and was not included in the calculation, as well as the specific growth rate and productivity value. The values also remained constant over the whole cultivation time, there is no trend recognizable (Figure 7, Figure 8 and Figure 9).

*Table 23 Average values of the wt3H6-CHO D/H shake flask with 2 * 10^5 C/mL start cell concentration*

Medium	Start cell concentration [C/mL]	Viability [%]	Cell count [C/mL]	Titer [µg/mL]	Growth rate [1/d]	Specific productivity [pg/c/d]
D/H	2 * 10^5	94	9.84E+05	11.60	0.41	5.34

In comparison to the T25 roux flask culture, the average cell count and growth rate is much higher, however the antibody titer and specific productivity is lower in the shake flask culture.

wt3H6-CHO cultivation in 125 mL shake flask containing D/H medium (1 * 10^6 C/mL start cell concentration)

The T80 roux flask cultivation with D/H as medium and 1 * 10^6 C/mL start cell concentration showed, that the viability and the growth rate were rapidly decreasing after raising the start cell concentration to 1 * 10^6 C/mL (Figure 7 and Figure 9). This observation can be explained by extinguishing nutrient components of the D/H medium or inefficient O_2 availability.

To evaluate if shaking of the culture can improve the availability of the nutrients and O_2 and thus enable the cultivation at a higher cell concentration, a shake flask with 1 *10^6 C/mL start cell concentration was started, containing 30 mL of D/H medium. The shake flask was cultivated at 140 rpm. The first passage started with 2 * 10^5 C/mL start cell concentration, then the start cell concentration was raised to 1 *10^6 C/mL. The viability was decreasing very fast after a cultivation time of 4 days, but remained constant or was falling only slowly after a cultivation time of only 3 days (Figure 7). This observation could be an indication of extinguishing nutrients after more than 3 days of cultivation and the medium may need to be exchanged before reaching 4 days of cultivation.

Batch growth curve of the wt3H6-CHO shake flask with 1*10^6 C/mL start cell concentration

To determine the time when the stationary phase is reached and thus the medium of the culture needs to be exchanged, a batch growth curve of the wt3H6-CHO shake flask was recorded. Therefore, a new shake flask was started with 1*10^6 C/mL start cell concentration and 30 mL D/H as cultivation medium. Samples were taken on every day of the cultivation for measurement of the development of the cell concentration, antibody titer, growth rate and specific productivity. The results are shown in Figure 10, Figure 11 and Figure 12.

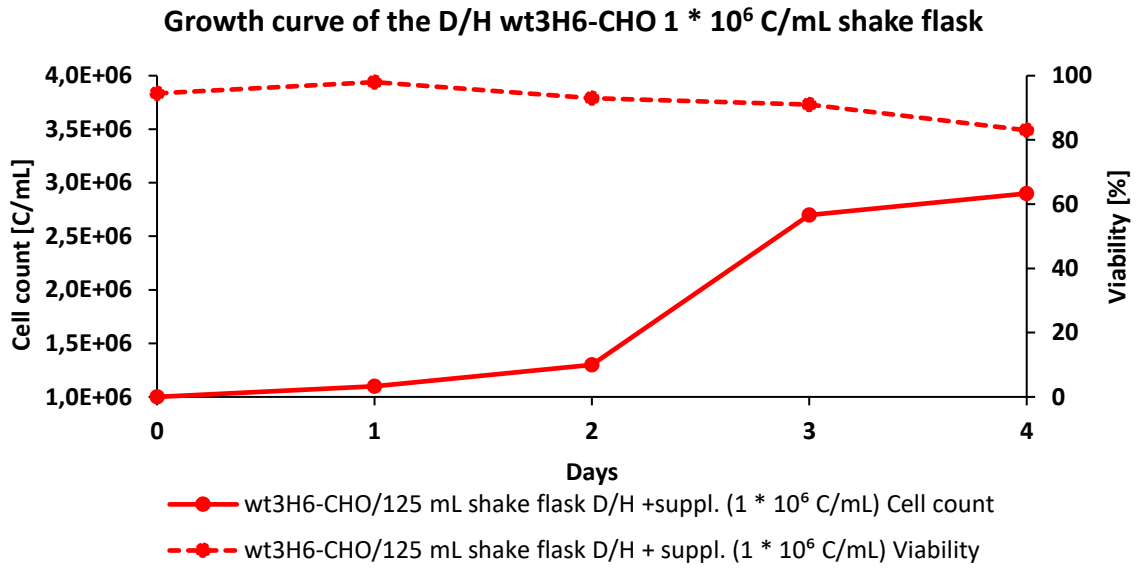


Figure 10 Development of the cell concentration and viability during a 4-day passage of wt3H6-CHO

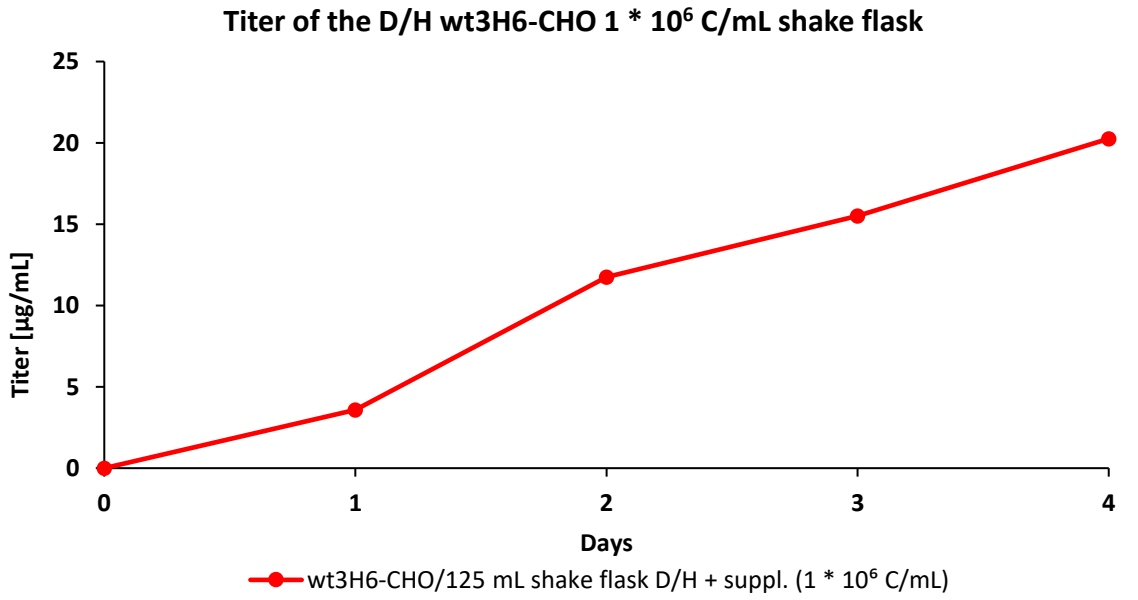


Figure 11 Development of the antibody titer during a 4-day passage of wt3H6-CHO

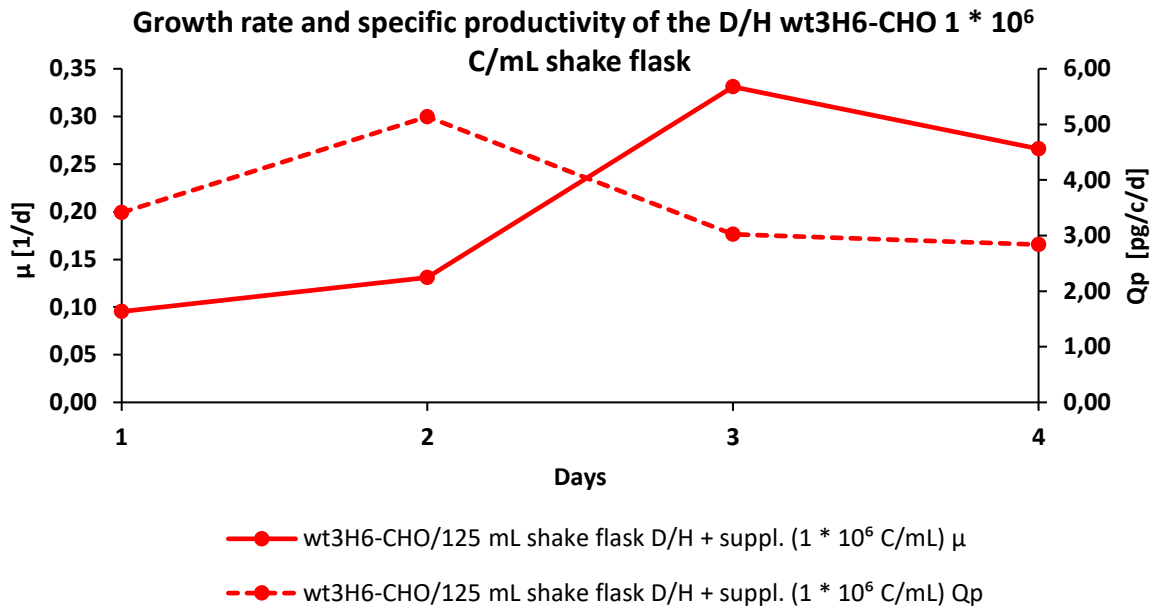


Figure 12 Development of the growth rate and specific productivity during a 4-day passage of wt3H6-CHO

The viability of the culture started at 94.5 % and was still at 91 % after 3 days of cultivation. After 4 days of cultivation the viability decreased to 83 % and the cell concentration began to become static, indicating the reach of the stationary phase (Figure 10). The antibody titer was at 20.3 µg/mL at the end of the cultivation and was still rising (Figure 11).

Although the antibody titer was still rising after 3 days of cultivation, it is not recommended to cultivate these cultures with start cell concentrations of $1*10^6$ C/mL more than 3 days, because of the rapidly decreasing viability.

Because medium exchange every 3 days would require working on weekends, it is recommended to produce the wt3H6 antibody in batches instead and to start a new batch after a batch is cultivated for more than 3 days.

5.3 Cultivation of GA3H6-CHO

5.3.1 Definition of the cultivation medium

The CHO DUKX-B11 strain CHO-3H6/GA/D10 has the gene for the human IgG1 antibody GA3H6 incorporated and constantly produces the antibody during cultivation. The cultivation of GA3H6-CHO was tested using different media. Table 24 gives an overview about the different tested cultivation media.

Table 24 Tested media for the optimal cultivation of GA3H6-CHO

Medium	**Supplements**	**Culture system**	**Culture volume [mL]**	**Shaking [rpm]**	**Start cell concentration [C/mL]**
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.096 µM MTX	T25 roux flask	10	static	$2 * 10^5$
MV3-2/6 (+30%)	4 mM L-gln 15 mg/mL phenol red 0.096 µM MTX	50 mL tube	15	220	$2 * 10^5$
CD CHO	4 mM L-gln 15 mg/mL phenol red 0.096 µM MTX	125 mL shake flask	30	140	$2 * 10^5$
D/H	4 mM L-gln 1x PF 1x SP 0.1 % Kolliphor 0.096 µM MTX	T80 roux flask	45	static	$1 * 10^6$

The determined cell counts, viabilities, antibody titers, growth rates and specific activities of all tested cultivation media are highlighted in Figure 13, Figure 14 and Figure 15.

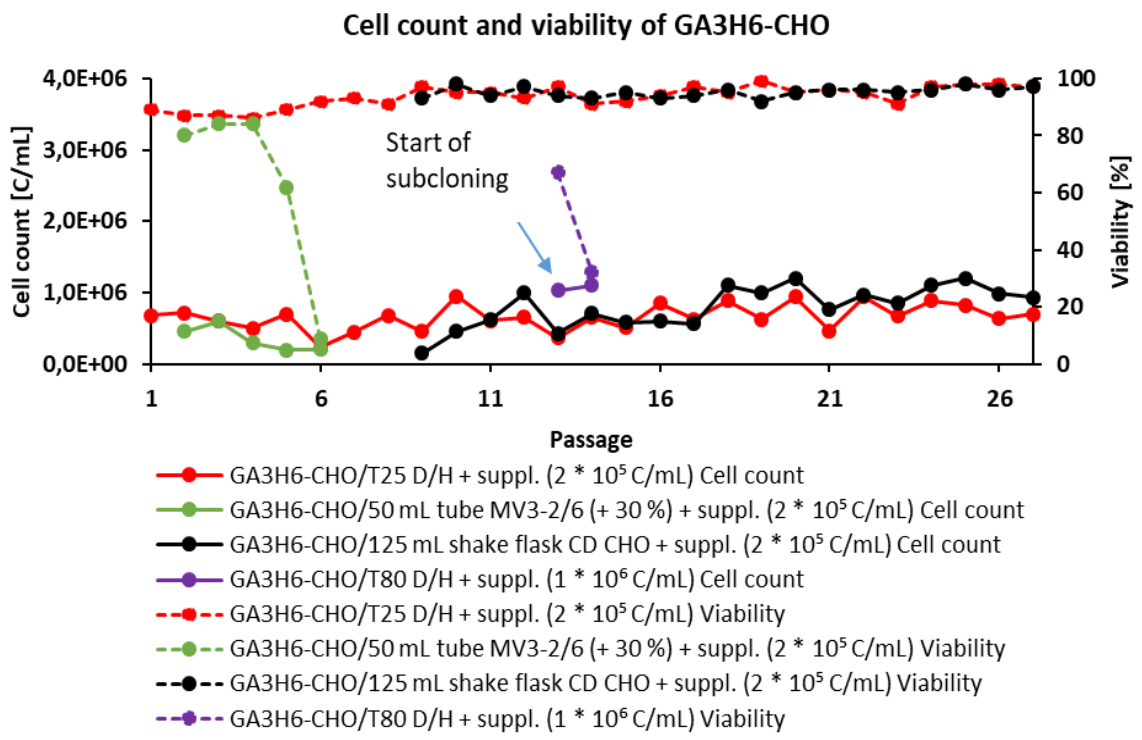


Figure 13 Cell count and viability of the GA3H6-CHO cultivations using different media

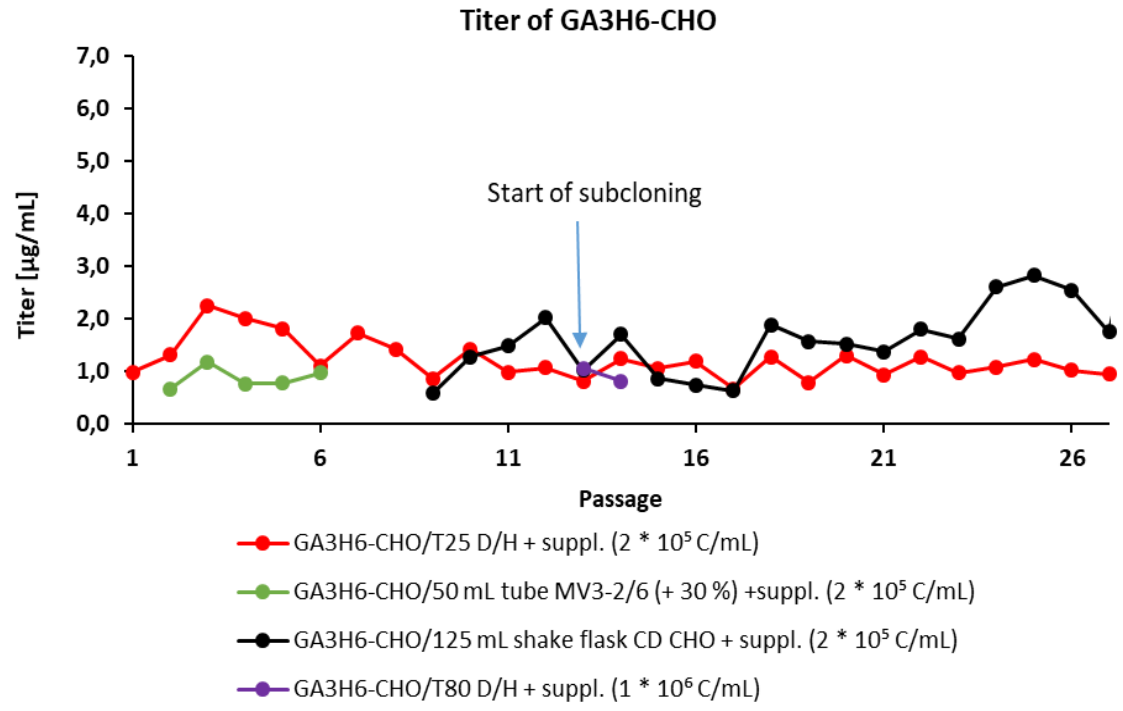


Figure 14 Antibody titer of the GA3H6-CHO cultivations using different media

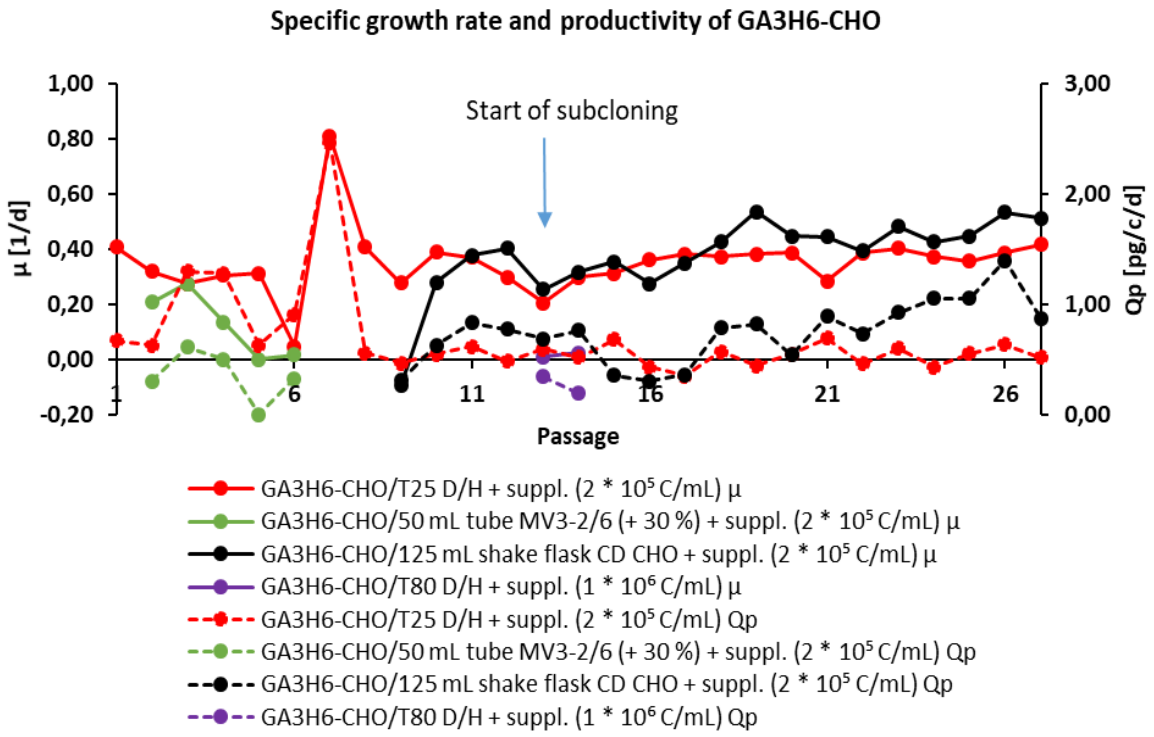


Figure 15 Growth rate and specific productivity of the GA3H6-CHO cultivations using different media

The figures illustrate the inability of GA3H6-CHO to grow in the in-house medium MV3-2/6 (+30 %) at a seeding concentration of 2 * 10^5 C/mL as well as in D/H medium at a start cell concentration of 1 * 10^6 C/mL, both expressed by the decreasing viability values (Figure 13). The cultivation of GA3H6-CHO in a 125 mL shake flask containing CD CHO medium was successful, hence it was decided to choose CD CHO as preferred medium, since the nutrient concentrations of the CD CHO medium are higher than those of the D/H medium (Table 60), and to test the cultivation of GA3H6-CHO in CD CHO medium at the higher seeding cell concentration of 1 * 10^6 C/mL.

GA3H6-CHO cultivation in T25 roux flask containing D/H medium (2 * 10^5 C/mL start cell concentration)

The cultivation was first tested in D/H medium. Therefore, a T25 roux flask culture was started with a start cell concentration of 2 * 10^5 C/mL. The viability of the T25 roux flask culture started at 89 % and improved to 92 % after the 6th passage (Figure 13). After the 6th passage the viability of the culture always stayed above 90 %.

There is no trend recognisable when looking at the measured cell concentrations. However, there is a downward tendency of the antibody titer values recognizable, which are falling until the 16th passage (Figure 14). The values of the specific growth rate and the specific productivity were quite erratic within the first ten passages, but then stabilized and remained constant over the rest of the cultivation time (Figure 15).

Table 25 gives an overview about the average values of the viability, cell concentration, antibody titer, growth rate and specific productivity of the culture. The values of the seventh passage can be considered as outliers and were not included in the calculation of the average value of the productivity.

Table 25 Average values of the GA3H6-CHO T25 roux flask culture

Medium	**Start cell concentration [C/mL]**	**Viability [%]**	**Cell count [C/mL]**	**Titer [µg/mL]**	**Growth rate [1/d]**	**Specific productivity [pg/c/d]**
D/H	2 * 10^5	94	6.70E+05	1.19	0.33	0.62

GA3H6-CHO cultivation in a 50 mL mini bioreactor tube containing MV3-2/6 (+ 30%) medium (2 * 10^5 C/mL start cell concentration)

To evaluate, if the cell density and the culture volume can be improved by using MV3-2/6 (+30%) medium, a tube culture containing 15 mL of MV3-2/6 (+ 30 %) was started and incubated at 220 rpm.

The viability of the culture started at 80 % and decreased to 62 % and 8.9 % after the 4th and 5th passage, respectively (Figure 13). The cell concentration and the specific growth rate values were decreasing along with the viability values, but the antibody titers remained constant and therefore the specific productivity (Figure 13, Figure 14 and Figure 15). Because of the low viability, the cultivation needed to be stopped, indicating the inability of GA3H6-CHO to adapt to the MV3-2/6 (+ 30 %) medium.

GA3H6-CHO cultivation in 125 mL shake flask containing CD CHO medium (2 * 10^5 C/mL start cell concentration)

Because of the higher nutrient concentration of the CD CHO medium, a shake flask culture was started with a start cell concentration of 2 * 10^5 C/mL containing 30 mL of CD CHO medium. The culture was cultivated at 140 rpm, 5 % CO_2 and 37 °C.

The viability of the shake flask culture started at 93 % and stayed above 90 % over the whole cultivation time (Figure 13). After the first passage, the cell suspension was left in the old medium, because of the growth rate of -0.10 [1/d] (Figure 15). The growth rate then increased to 0.28 [1/d] after the second and to 0.38 [1/d] after the third passage, which is an indication that time for adaptation to the CD CHO medium was needed. The adaptation time can also be observed by looking at the cell concentrations (Figure 13) and antibody titers (Figure 14), which needed three passages for stabilization. There is also a slight upward trend of the cell concentration, antibody titer, specific growth rate and productivity values observable.
The average values of the CD CHO shake flask culture with a start cell concentration of 2 * 10^5 C/mL are shown in Table 26.

*Table 26 Average vlaues of the GA3H6-CHO CD CHO shake flask culture with a start cell concentration of 2 * 10^5 C/mL*

Medium	Start cell concentration [C/mL]	Viability [%]	Cell count [C/mL]	Titer [µg/mL]	Growth rate [1/d]	Specific productivity [pg/c/d]
CD CHO	2 * 10^5	96	9.46E+05	2.0	0.41	0.84

GA3H6-CHO cultivation in T80 roux flask containing D/H medium (1 * 10^6 C/mL start cell concentration)

It could be determined, that GA3H6-CHO could be cultivated in 10 mL D/H medium using T25 roux flasks. As next step, a T80 roux flask culture with D/H medium and a start cell concentration of 1*10^6 C/mL was started to evaluate if GA3H6-CHO can be cultivated in D/H medium with a higher start cell concentration.

The viability of the T80 production culture decreased to 67 % after the first medium exchange and to 32 % after the second exchange (Figure 13). The antibody titer was at 0.82 µg/mL at the end of the cultivation (Figure 14). The culture was stopped after the second medium exchange due to the low viability. The falling viability can be explained by extinguished nutrients of the D/H medium and insufficient mixing due to the static cultivation.

D/H medium is therefore not suitable for the cultivation of GA3H6-CHO with a start cell concentration of 1 *10^6 C/mL. Because of the higher nutrient concentration of the CD CHO medium, CD CHO was chosen for further cultivations.

5.3.2 High density seeding of GA3H6-CHO in CD CHO medium

The examination of various cultivation media revealed, that GA3H6-CHO can be cultivated in CD CHO medium at 140 rpm shaking speed using a 125 mL shake flask. As next step, the start cell concentration was raised to 1 * 10^6 C/mL to be able to produce a higher amount of GA3H6 antibody. The results were compared to the shake flask with 2 * 10^5 C/mL start cell concentration. Table 27 gives an overview about the cultivated GA3H6-CHO shake flasks.

Table 27 GA3H6-CHO shake flasks

Medium	**Supplements**	**Culture system**	**Culture volume [mL]**	**Shaking [rpm]**	**Start cell concentration [C/mL]**
CD CHO	4 mM L-gln 15 mg/mL phenol red 0.096 µM MTX	125 mL Shake flask	30	140	2 * 10^5
CD CHO	4 mM L-gln 15 mg/mL phenol red 0.096 µM MTX	125 mL Shake flask	25.5 - 49.5	140	1 * 10^6

The cell concentrations, viabilities, antibody titers, growth rates and specific productivities of both shake flask cultures are shown in Figure 16. Figure 17 and Figure 18.

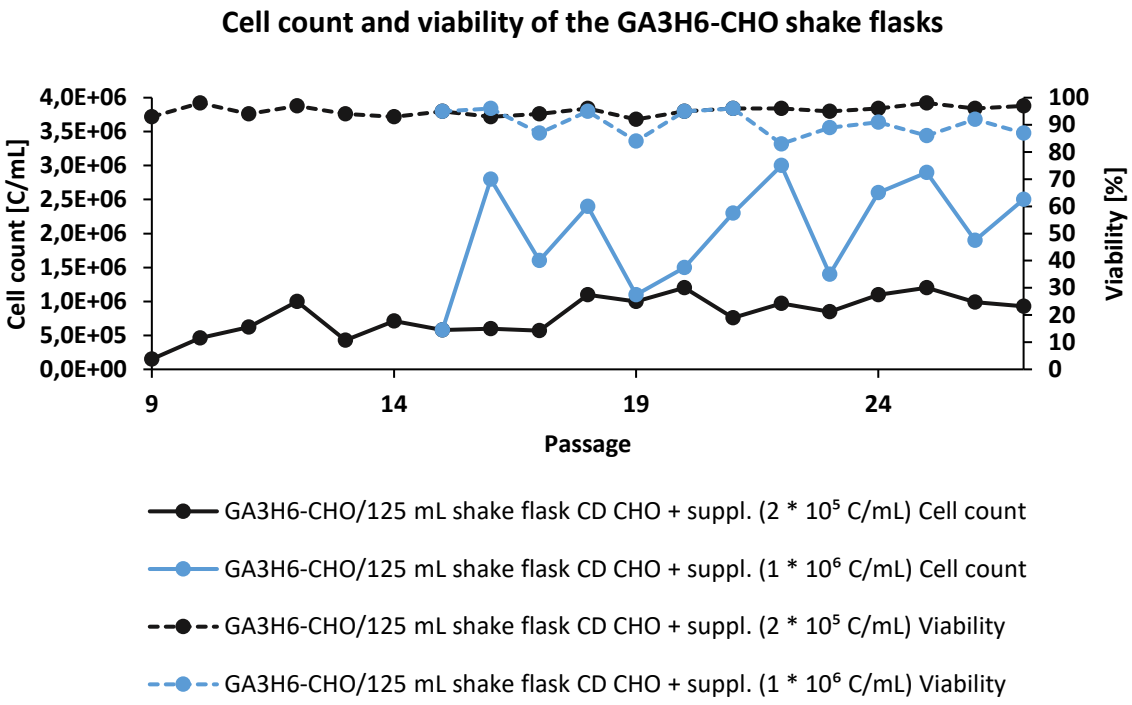


Figure 16 Cell count and viability of the GA3H6-CHO shake flasks

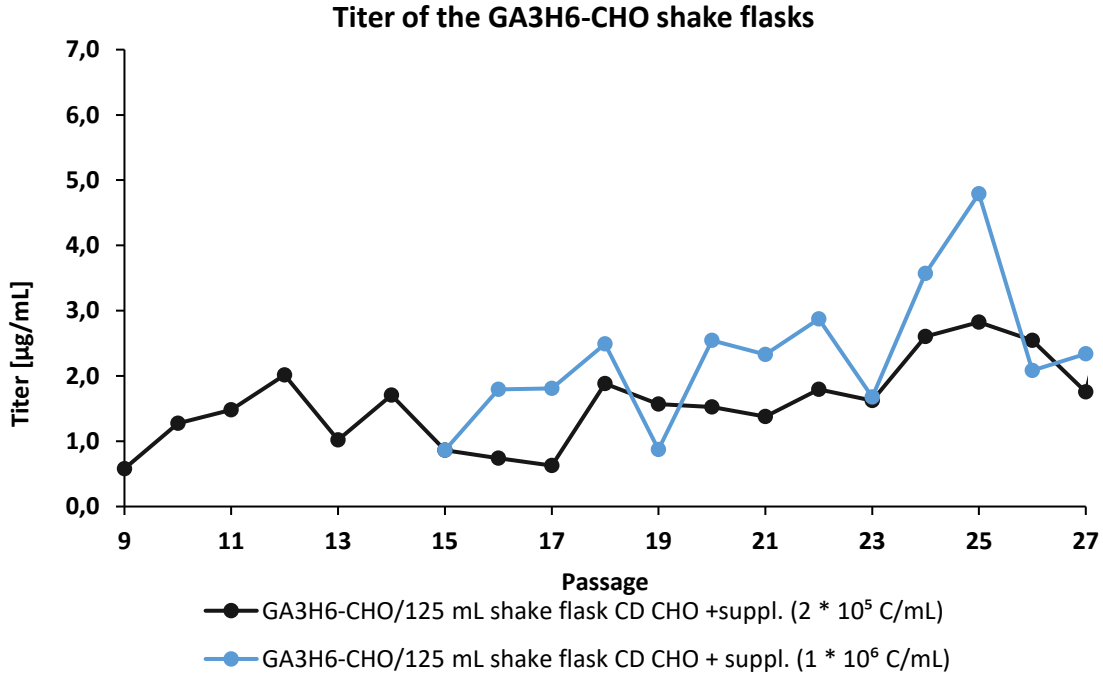


Figure 17 Antibody titer of the GA3H6-CHO shake flasks

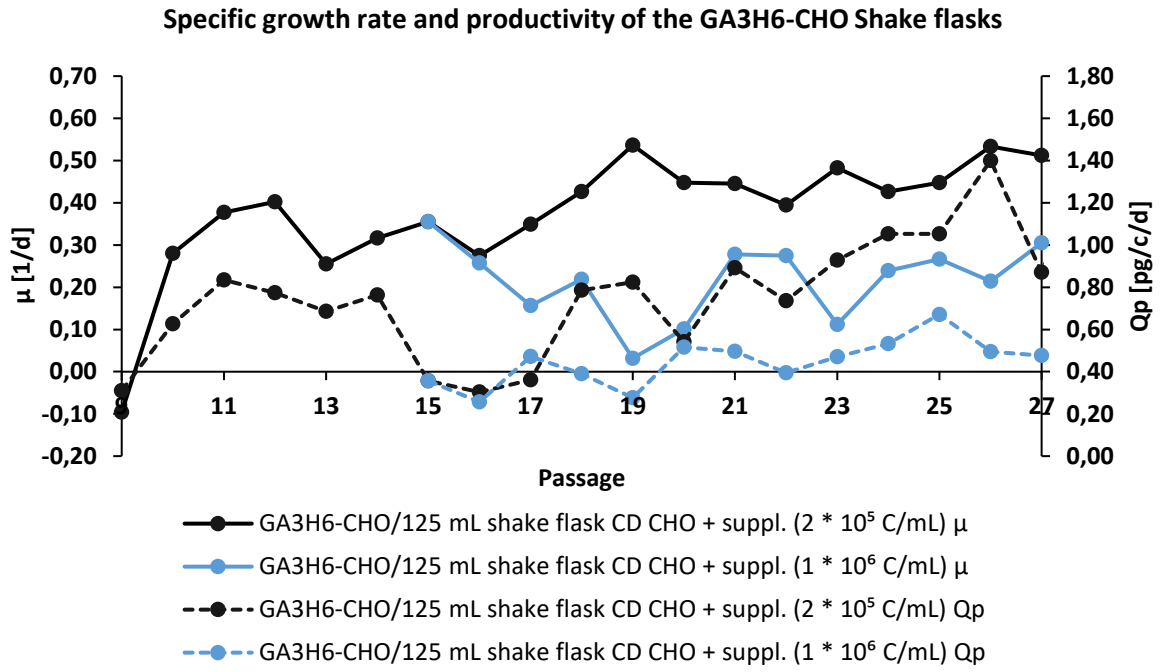


Figure 18 Specific growth and productivity of the GA3H6-CHO shake flasks

GA3H6-CHO cultivation in 125 mL shake flask containing CD CHO medium (1 * 10^6 C/mL start cell concentration)

To increase the antibody production and to evaluate if GA3H6-CHO can also be cultivated in CD CHO medium with a higher cell concentration, a 125 mL shake flask was started with CD CHO as medium and 1*10^6 C/mL as start cell concentration. The volume of the culture was ranging between 25.5 and 49.5 mL CD CHO.

The average values of the CD CHO shake flask culture with a start cell concentration of 1 * 10^6 C/mL are shown in Table 28. Because the passages 4-7 were cultivated with a volume higher than 35 mL, which is significantly higher than the working volume of 30 mL for a 125 mL shake flask, these passages were not considered for the calculation of the average values. Cultivations with a higher cultivation volume than the working volume can lead to a lack of oxygen and subsequently lower cell concentrations, as actually possible.

*Table 28 Characteristics of the GA3H6-CHO CD CHO shake flask culture with a start cell concentration of 1 * 10^6 C/mL*

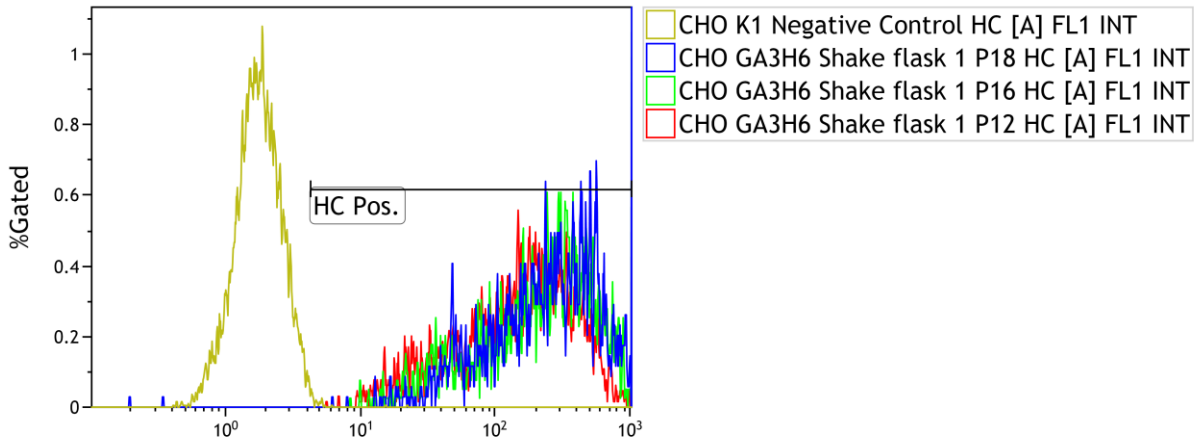
Medium	Start cell concentration [C/mL]	Viability [%]	Cell count [C/mL]	Titer [µg/mL]	Growth rate [1/d]	Specific productivity [pg/c/d]
CD CHO	1 * 10^6	90	2.14E+06	2.42	0.24	0.46

The viability of the culture was falling slightly under 90 %, when cultivating the shake flask for 4 days, but was returning to values over 90 % after only 3 days of cultivation (Figure 16). This behaviour could be the indication of extinguishing nutrient components of the CD CHO medium or insufficient O_2 supply.

There is also an upward tendency of the antibody titer and specific productivity values recognizable (Figure 17 and Figure 18). In comparison with the 2 * 10^5 C/mL shake flask, the 1 * 10^6 C/mL shake flask showed higher cell concentration and antibody titer values, but the specific growth rate and productivity values were lower than those of the 2 * 10^5 C/mL shake flask.

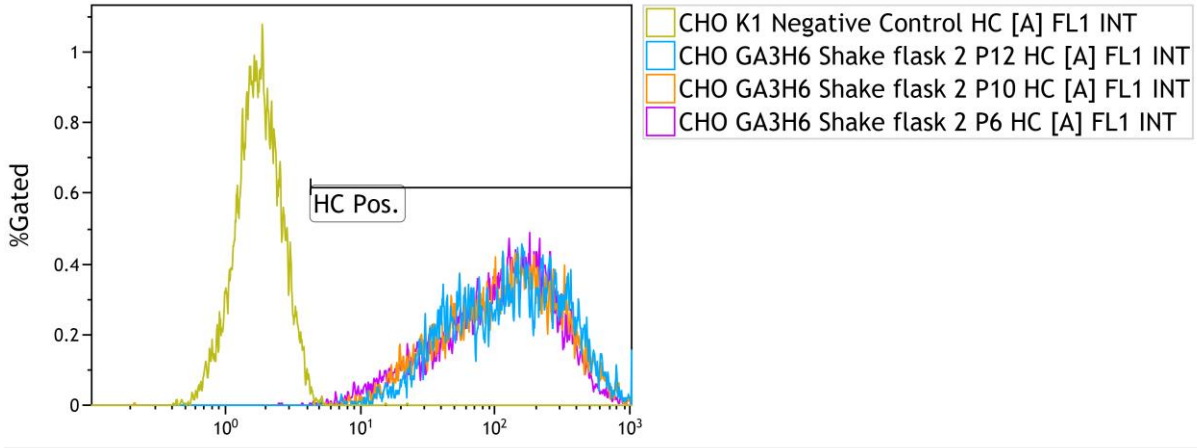
5.3.3 Analysis of the homogeneity of the GA3H6 shake flasks

The intracellular product homogeneity of both GA3H6 shake flasks was analysed using flow cytometry. Figure 19 shows the results of the 125 mL shake flask with 2 * 10^5 C/mL start cell concentration, Figure 20 the results of the 125 mL shake flask with 1 * 10^6 C/mL start cell concentration. As negative control, the strain CHO K1 with a cell number of 1 * 10^6 cells was used, the strain does not produce any antibodies. For both shake flasks, the median and geometric mean values of the different passages were increasing over time, indicating an improvement of the intracellular product content. Furthermore, an inhomogeneous population can be assumed, visualized by the peak shape.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	6,424	100.00	1.76	1.89	1.70
All	1,146	100.00	254.38	1,014.83	199.41
All	1,314	100.00	246.13	1,014.83	187.80
All	2,150	100.00	161.79	148.07	127.17
HC Pos.	31	0.48	4.70	4.36	5.10
HC Pos.	1,144	99.83	254.76	1,014.83	205.15
HC Pos.	1,314	100.00	246.13	1,014.83	187.80
HC Pos.	2,150	100.00	161.79	148.07	127.17

*Figure 19 FACS analysis of the GA3H6-CHO 125 mL shake flask (2 * 10^5 C/mL start cell concentration)*



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	6,424	100.00	1.76	1.89	1.70
All	3,211	100.00	133.78	114.07	115.16
All	5,791	100.00	122.61	146.74	104.04
All	6,137	100.00	119.96	210.28	95.82
HC Pos.	31	0.48	4.70	4.36	5.10
HC Pos.	3,211	100.00	133.78	114.07	115.16
HC Pos.	5,790	99.98	122.64	146.74	104.32
HC Pos.	6,133	99.93	120.09	210.28	96.12

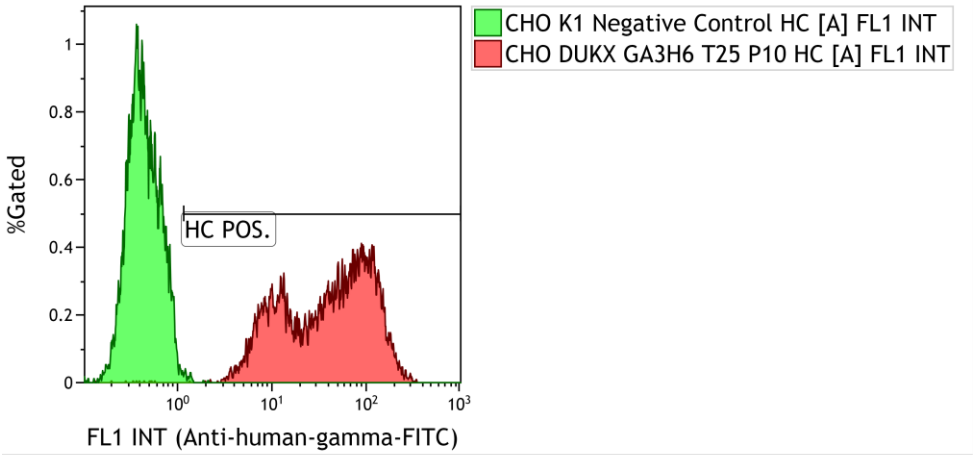
*Figure 20 FACS analysis of the GA3H6-CHO 125 mL shake flask (1 * 10^6 C/mL start cell concentration)*

5.4 Stabilization of recombinant GA3H6-CHO

5.4.1 FACS of the GA3H6-CHO T25 roux flask culture

It could be observed, that the antibody titer of the GA3H6-CHO T25 roux flask culture was falling within the first 10 passages (Figure 14). The specific productivity values were decreasing as well, however, the growth rate was slightly increasing (Figure 15). This effect could be the indication of the existence of a subculture with a better growth rate, but lower antibody production. The high producing subculture could then be overgrown by the better growing low producing subculture, leading to decreasing antibody titers over time.

To evaluate the homogeneity of the routine culture, a FACS analysis of GA3H6-CHO T25 roux flask P10 was performed. As negative control, the CHO strain K1 with a cell number of $1*10^6$ cells was used. The results of the FACS analysis are shown in Figure 21.



Marker	%Gated	X-Med	X-Mode	X-GMean
All	100.00	0.43	0.37	0.41
All	100.00	44.73	95.29	32.97
HC POS.	0.57	1.29	1.19	1.31
HC POS.	99.85	44.88	95.29	33.59

Figure 21 FACS analysis of GA3H6-CHO T25 P10

Two distinct peaks are visible, when looking at GA3H6-CHO T25 roux flask P10, which is coloured in red. The first and left peak of the GA3H6-CHO T25 roux flask P10 has a weaker intensity than the second peak, indicating a subculture with lower intracellular product content. This could translate into lower productivities but higher growth rates of this low-producing subpopulation. To eliminate the low producing subculture, a subcloning procedure was performed for stabilization of the GA3H6-CHO clone. The green peak on the left-hand side is originating from the strain CHO-K1 used as the negative control.

5.4.2 Subcloning efficiency

The subcloning procedure was started by inoculation of two 384 well plates using two different cultivation media: D/H and CD CHO. Each 384 well plate was divided into four areas containing 96 wells for four different dilutions: 9,6,3 and 1 cells/well.
The D/H medium was supplemented with 4 mM L-gln, 1x SP, 1x PF, Kolliphor P188 and 0.096 µM MTX, whereas the CD CHO medium with 4 mM L-gln, 15 mg/L phenol red and 0.096 µM MTX. Furthermore, growth factors were added to each 384 well plate: albumin (1 mg/mL), transferrin (20 µg/mL) and recombinant EGF (25 ng/mL).

On day 13, the two 384 well plates were analysed under the light microscope. The D/H 384 well plate showed no grown clones at all and was therefore discarded, whereas the CD CHO plate revealed several clones with a different extent of growth. The number of wells of each dilution demonstrating a yellow cultivation medium was noted for calculation of the subcloning efficiency: 1 c/w: 2/96 = 2%, 3 c/w: 12/96=13%, 6 c/w: 19/96 = 20%, 9 c/w: 24/96= 25%.

On Day 17, the CD CHO plate was analysed again and clones of wells showing a yellow medium and two third of the well overgrown, were transferred to a 96 well plate. However, only clones of the 1 and 3 c/well dilutions were used for the transfer to obtain clones with a preferably high homogeneity. In total, 45 clones were transferred corresponding to a subcloning efficiency of 23 %. 10 additional clones were transferred on Day 21, resulting in a final subcloning efficiency of 29 %.

5.4.3 Qualitative ELISA for selection of clones for transfer to expansion plate

Table 29 shows the results of the qualitative ELISA of the 96 well plate subclones, which was performed on day 25 of the subcloning procedure. The dilutions were already included in the calculation of the concentrations of the different clones. Clones transferred to the 96 well plate on day 17 were diluted 1:50, whereas clones transferred on day 21 were diluted 1:20 and are shown in red colour. Clones, which were selected for transfer to the expansion plate and subsequent cultivation in T25 roux flasks are marked with a blue background.

Table 29 Qualitative ELISA of the subclones with the dilutions already included into the calculation of the GA3H6 concentration

	H	**G**	**F**	**E**	**D**	**C**	**B**	**A**
3	I3 916.65 ng/mL	I4 2661.65 ng/mL	I7 273.51 ng/mL	I10 3420.55 ng/mL	I13 889.45 ng/mL	I14 <Min	I15 <Min	I16 <Min
4	I20 940.45 ng/mL	J20 2252.90 ng/mL	J24 1642.30 ng/mL	J4 95.05 ng/mL	J5 2791.25 ng/mL	J14 1707.45 ng/mL	J15 1529.45 ng/mL	J16 1972.55 ng/mL
5	J18 1607.90 ng/mL	I21 2150.75 ng/mL	I22 1754.85 ng/mL	K2 324.12 ng/mL	K4 1566.55 ng/mL	K6 <Min	K10 <Min	K12 1461.20 ng/mL
6	K15 1683.3 ng/mL	K18 585.25 ng/mL	K20 1162.20 ng/mL	L4 <Min	L5 2413.70 ng/mL	L9 <Min	L10 2049.20 ng/mL	L13 352.81 ng/mL
7	L15 2937.35 ng/mL	L16 2089.70 ng/mL	L18 2640.75 ng/mL	L19 975.95 ng/mL	L20 1948.60 ng/mL	L21 1152.10 ng/mL	L22 1298.00 ng/mL	M13 <Min
8	N7 976.70 ng/mL	N9 <Min	N22 1292.8 ng/mL	O15 879.00 ng/mL	P17 <Min			
9	M5 477.28 ng/mL	M8 195.85 ng/mL	O7 <Min	O8 <Min	O9 <Min	I2 <Min	J17	J21 130.69 ng/mL
10	K7 207.00 ng/mL	K11 207.00 ng/mL	K21 198.05 ng/mL	L23 392.86 ng/mL				

Although the I13 and L4 clones have shown very low antibody titer values, they were selected, because of a pipetting mistake.

The antibody titers of clones M5 and L23 of the day 21 transfer also have shown lower antibody titer values compared to the day 17 clones. They were chosen, because the cultivation time of the day 21 clones was lower, however the clones could still have a higher productivity.

5.4.4 Comparison of the T25 roux flask subclone cultures

The growth rates and specific productivities of the different T25 roux flask subclone cultivations are shown in Table 30. The values were compared to each other and 4 clones were selected for further cultivation. Clones with the highest specific productivities were chosen, but only clones with a growth rate over 0.35 were considered.

*Table 30 Growth rates and specific productivities (measured over the first two passages) of the T25 roux flask subclone cultures. Clones selected for further cultivation marked with *.*

Clone	Growth rate [1/d]	Specific productivity [pg/c/d]	Best clones
L4	0.38	0.71	
L18	0.27	1.10	
L15	0.35	0.41	
J5	0.42	0.53	
I13	0.40	0.22	
J20*	0.37	1.13	4
I10*	0.41	3.36	1
M5*	0.39	1.96	2
I4*	0.36	1.77	3
L23	0.40	0.99	

The cultivation of all T25 roux flasks was stopped except of the four best subclones which were further cultivated: I10, M5, I4, J20. The best clones were ranked according to the highest specific productivity values.

After the 11th passage, the viabilities, growth rates, antibody titers and specific productivities of all four subclones were compared to each other and two clones were selected for further cultivation (Table 31).

*Table 31 Average values from passage 4 to 11 of selected subclones of the T25 roux flask cultivations in comparison with the initial GA3H6 T25 culture. Clones selected for further cultivation marked with *.*

Subclone	µ [1/d]	Titer [µg/mL]	Viability [%]	Specific Productivity [pg/c/d]
I10*	0.34	5.23	99	2.63
M5	0.30	1.40	96	0.72
I4*	0.23	3.37	94	1.56
J20	0.29	1.85	96	0.94
Initial GA3H6 clone	0.35	1.21	94	0.62

The subclones I10 and I4 were selected, because of the higher specific productivities. The cultivation of the subclones M5 and J20 was then stopped. The viabilities, cell concentrations, antibody titers, growth rates and specific productivities of the four T25 subclone cultivations are shown in Figure 22. Figure 23 and Figure 24.

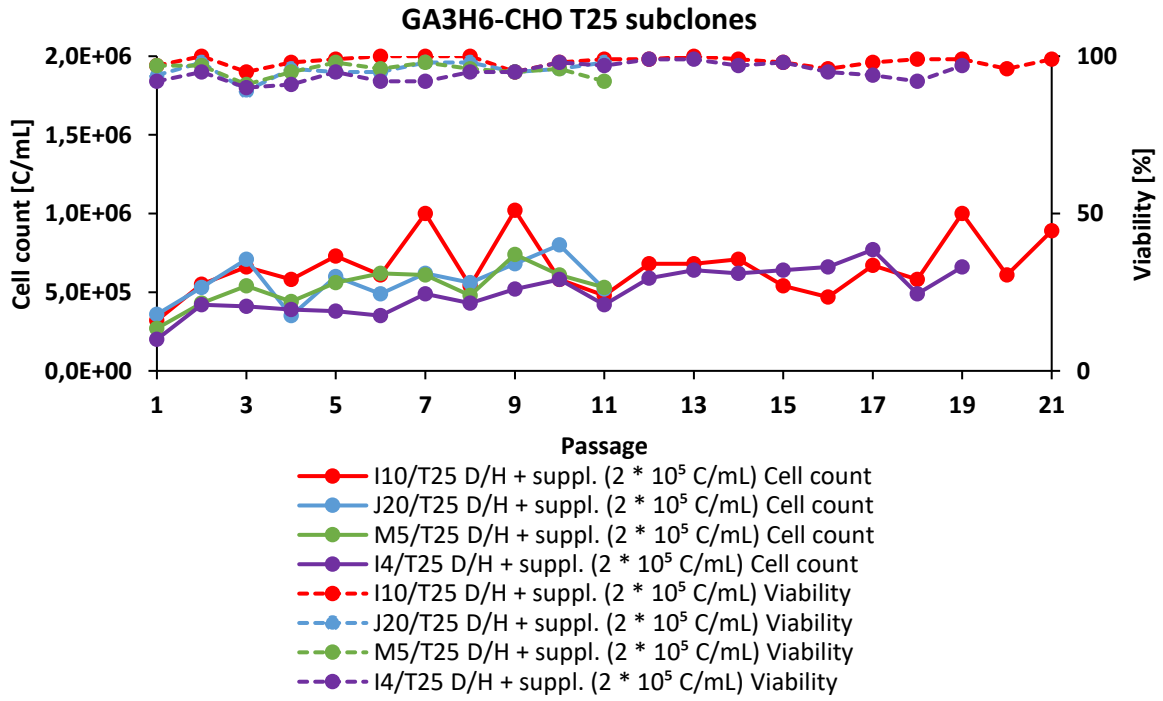


Figure 22 Cell count and viability of the T25 roux flasks of the four subclones

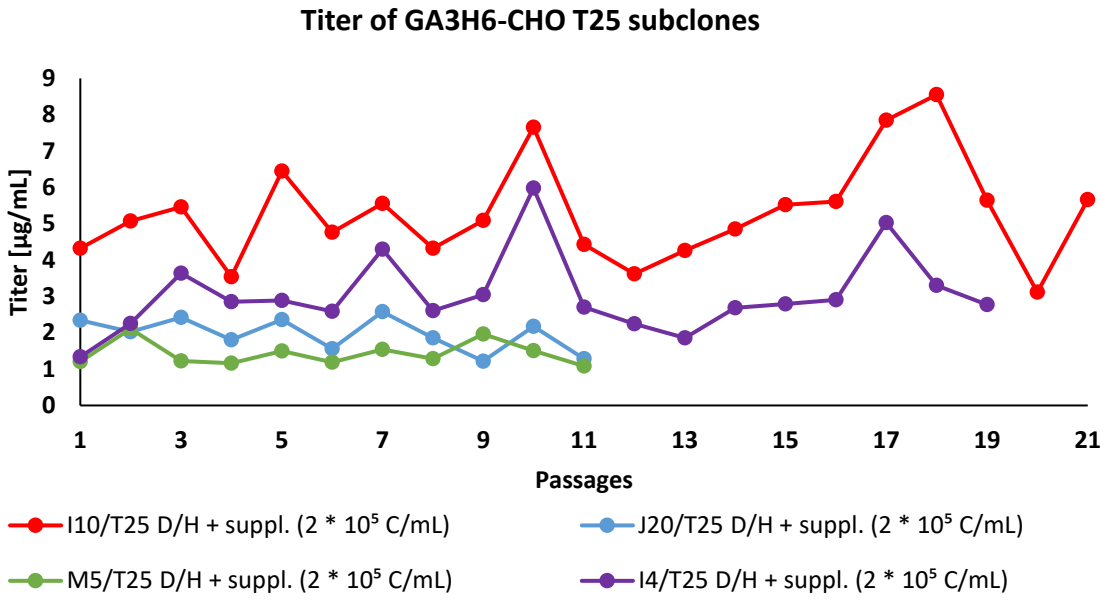


Figure 23 Antibody titer of the T25 roux flasks of the four subclones

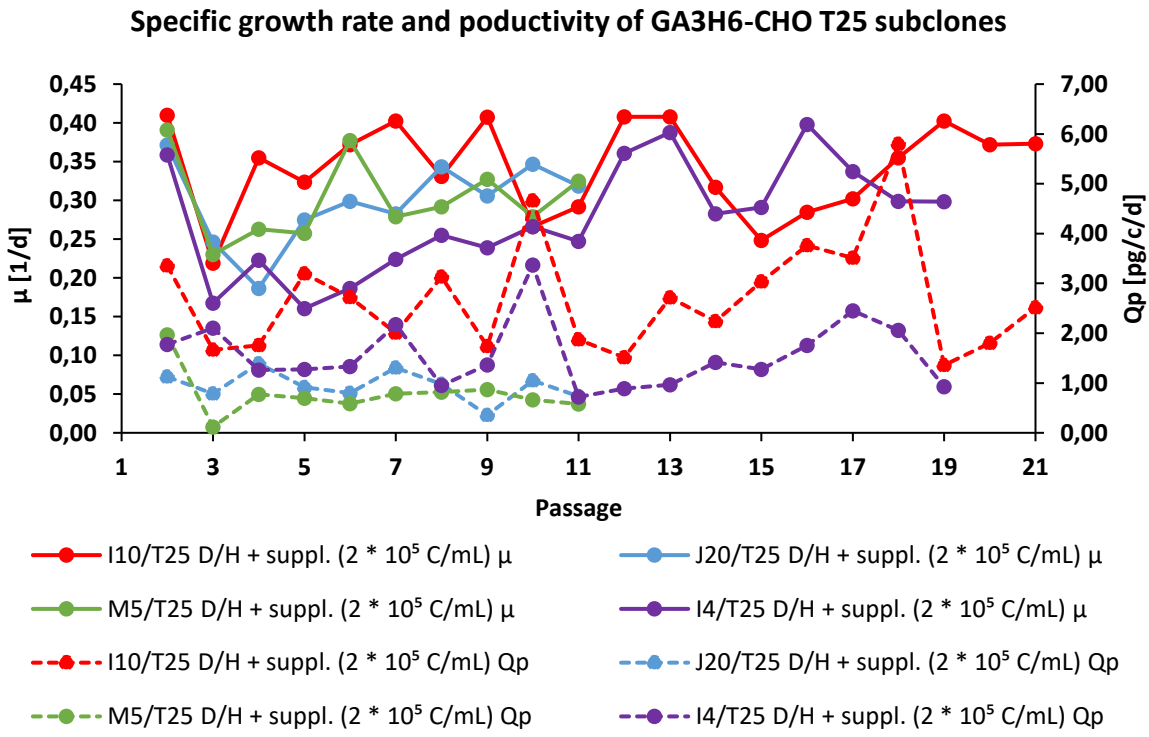


Figure 24 Growth rate and specific productivity of the T25 roux flasks of the subclones

The subclone I10 showed the highest cell concentration, antibody titer, specific growth rate and productivity values. Clone I4 demonstrated the second highest values, although the specific growth rate values of I4 were lower than those of the M5 and J20 clones, which can be explained by the lower antibody production of the M5 and J20 clones, leading to a growth benefit.

5.4.5 Adaptation of the selected GA3H6-CHO subclones to shaking conditions

To obtain higher cell concentrations and to test the cultivation of the subclones at 140 rpm, shake flask cultivations using 125 mL shake flasks with a start cell concentration of 2 * 10^5 C/mL of the I10 and I4 subclones were started. As medium 30 mL CD CHO was used containing 4 mM L-glutamine, 15 mg/L phenol red and 0.096 µM MTX.

Additionally, a shake flask cultivation with a start cell concentration of 1 * 10^6 C/mL of the I10 subclone using a 250 mL shake flask was started to evaluate possible negative effects of the higher productivity on the cultivation and to analyse the stability of the clone at higher cell concentrations. The shake flask was started with a volume of 100 mL CD CHO to have enough cells for the increase of the start cell concentration. The start cell concentration was increased to 1 * 10^6 C/mL by medium exchange after the second passage and the culture volume decreased to 60 mL CD CHO for the rest of the cultivation time. The CD CHO medium contained the same supplements as described above and the shake flask was also cultivated at 140 rpm.

The viabilities, cell counts, antibody titers, growth rates and specific productivities of the shake flasks are shown in Figure 25, Figure 26 and Figure 27. Calculated average values are highlighted in Table 32.

Table 32 Average values of the I10 and I4 shake flasks

Subclone	**Start cell concentration [C/mL]**	**µ [1/d]**	**Titer [µg/mL]**	**Viability [%]**	**Specific productivity [pg/c/d]**
I10 shake flask 1	2 * 10^5	0.61	9.91	98	3.56
I10 shake flask 2	1 * 10^6	0.31	15.80	97	2.49
I4 shake flask	2 * 10^5	0.31	2.32	97	1.25

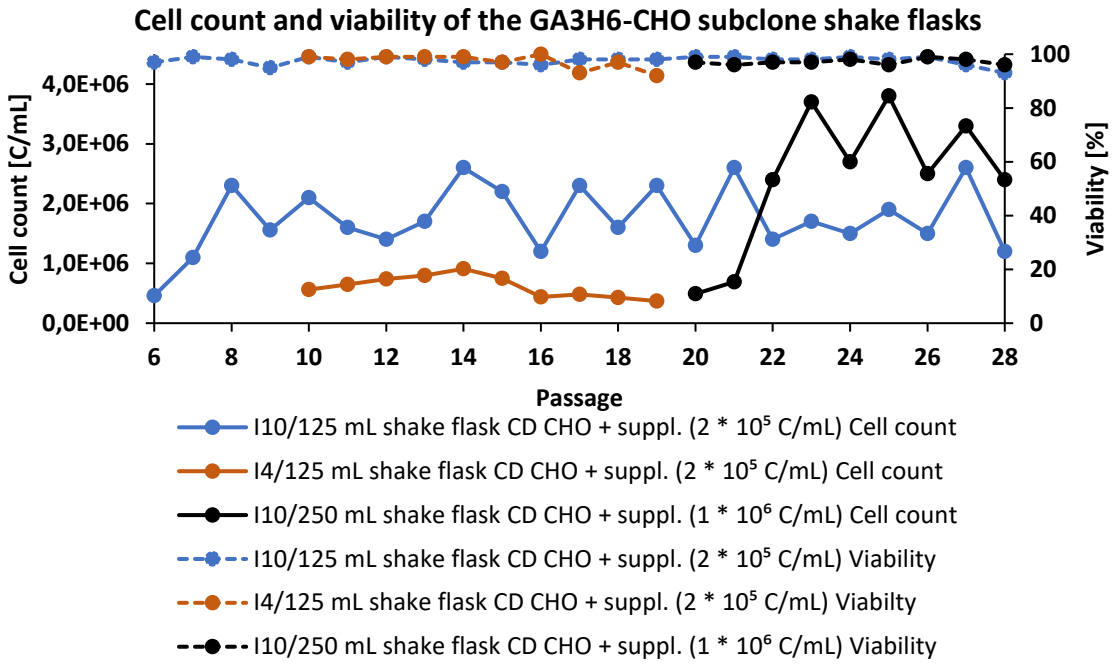


Figure 25 Cell count and viability of the GA3H6-CHO subclone shake flasks

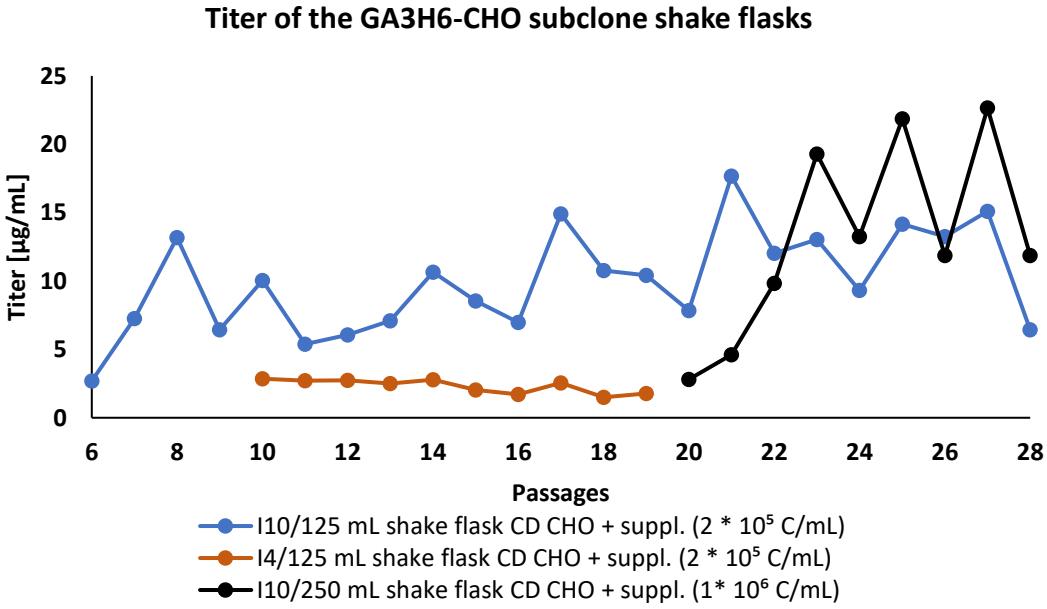


Figure 26 Antibody titer of the GA3H6-CHO subclone shake flasks

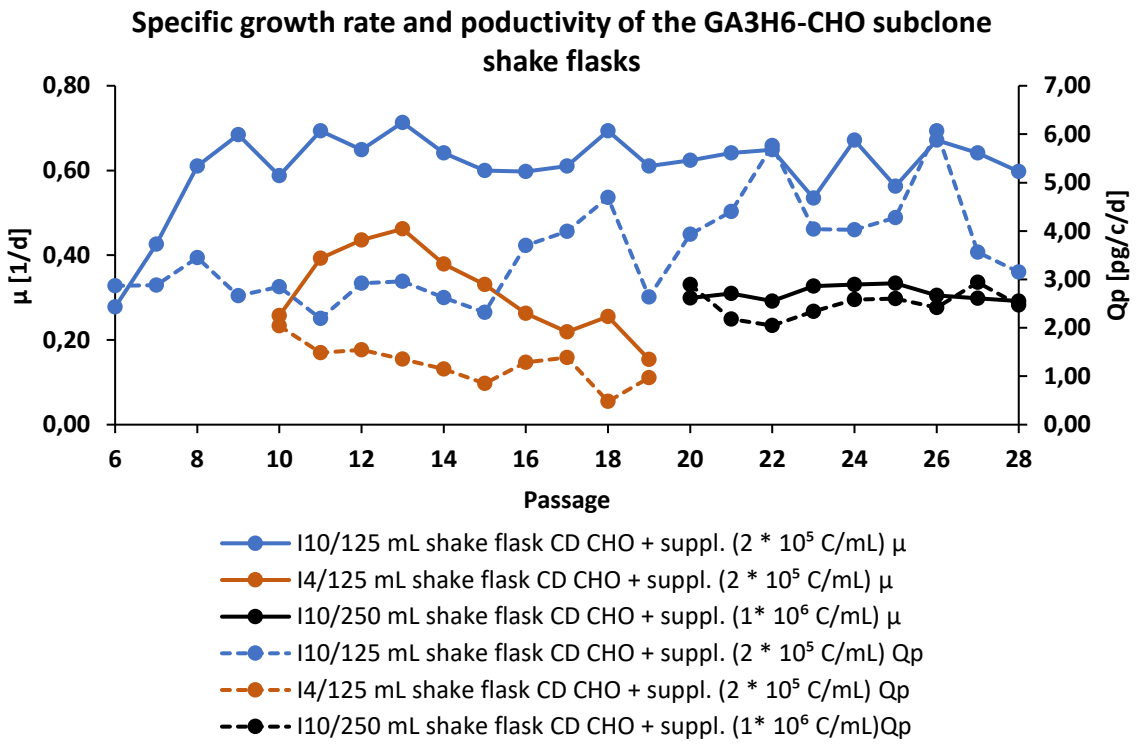
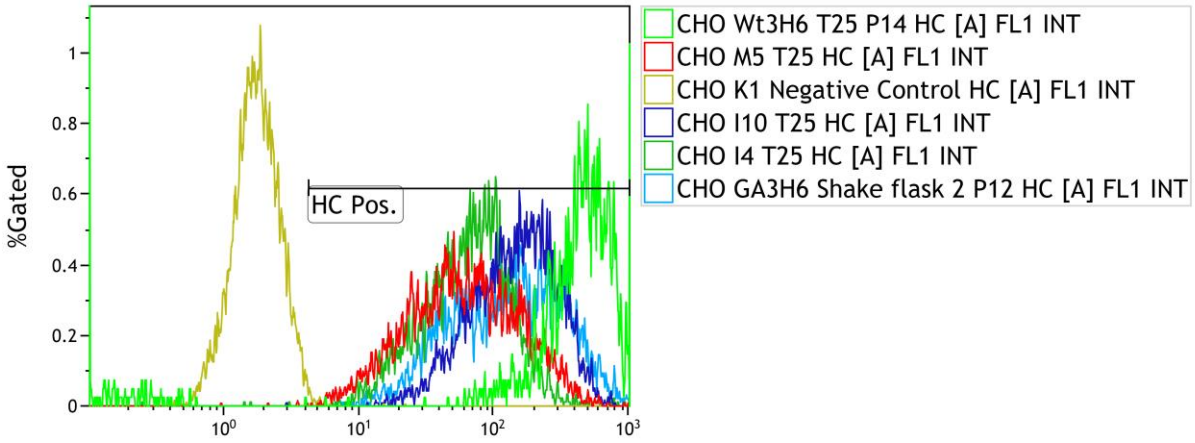


Figure 27 Growth rate and specific productivity of the GA3H6 subclone shake flasks

The subclone I10 showed higher cell concentration, antibody titer, specific growth rate and productivity values than the I4 subclone. The antibody titer values of both I10 shake flasks correlate with the cell concentrations, indicating a dependence on the cell concentration. It could be observed, that when raising the start cell concentration of I10 to 1 * 10^6 C/mL, the specific growth rate and productivity values were decreasing.

5.4.6 Analysis of the homogeneity of the T25 roux flask subclone cultures

A FACS analysis of the third passage of the T25 roux flasks of the subclones I10, I4 and M5 was performed to compare the homogeneity of the chosen subclones with each other and with the initial GA3H6 subclone. As negative control, the CHO strain K1 with a total number of 1 * 10^6 cells was used. The total cell number of all samples were adjusted to 0.5 * 10^6 cells to allow comparison. The FACS data of the clone J20 was damaged and is therefore excluded from the comparison. The results of the FACS analysis are shown in Figure 28.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	1,363	100.00	408.34	0.10	1.95
All	4,383	100.00	59.48	80.32	54.90
All	6,424	100.00	1.76	1.89	1.70
All	3,933	100.00	149.94	156.28	133.94
All	2,974	100.00	67.55	104.26	59.43
All	3,211	100.00	133.78	114.07	115.16
HC Pos.	1,120	82.17	465.23	494.19	411.28
HC Pos.	4,378	99.89	59.63	80.32	55.17
HC Pos.	31	0.48	4.70	4.36	5.10
HC Pos.	3,932	99.97	149.97	156.28	134.13
HC Pos.	2,970	99.87	67.63	104.26	59.98
HC Pos.	3,211	100.00	133.78	114.07	115.16

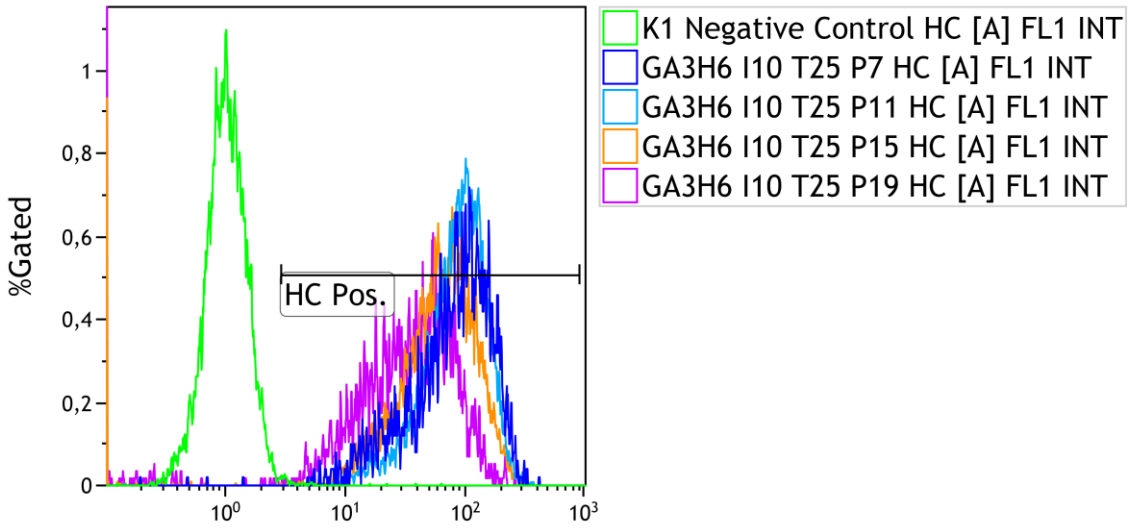
Figure 28 FACS analysis of the T25 roux flask subclone cultivations

The peak of the M5 subclone is very broad, which is an indication of a very poor homogeneity. The subclones I10 and I4 show a narrow peak and therefore exhibit a better homogeneity. Subclone I10 has a median of 149.94 and demonstrates the highest value of all subclones, it is therefore the subclone with the highest intensity. The initial GA3H6 clone with the shake flask P12 and the wt3H6 clone with T25 P14 were also analysed for comparison. The initial GA3H6 clone also shows a very broad peak. indicating a poor homogeneity.

5.4.7 Analysis of the homogeneity of the GA3H6 subclones

I10 T25 roux flask

The homogeneity of the I10 T25 roux flask culture was analysed using flow cytometry. As negative control, the CHO strain K1 with a cell number of $1*10^6$ cells was used. The results are shown in Figure 29. It is noticeable, that the intensity was slightly increasing from passage 7 to P11, which can be deviated by looking at the median values of P7, 86.98, and P11, 90.68, but significantly decreasing after the 11^{th} passage, which can be derived by the median values of passage 15, 63.72, and passage 19, 34.02.



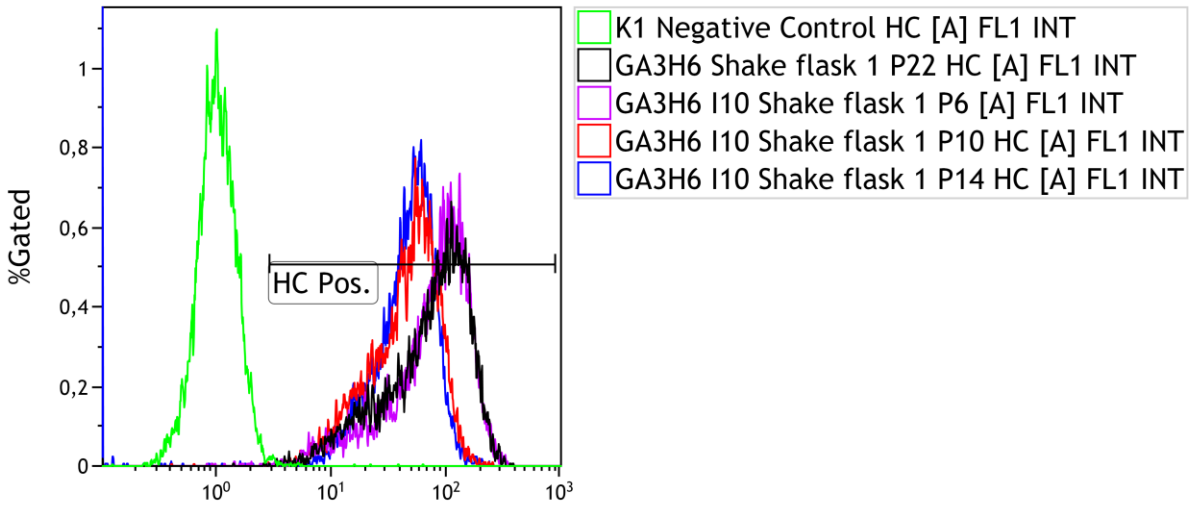
Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	8.385	100,00	1,02	1,01	0,98
All	1.672	100,00	86,98	95,29	69,42
All	8.382	100,00	90,68	97,90	80,23
All	6.862	100,00	63,72	0,10	38,07
All	1.919	100,00	34,02	0,10	3,78
HC Pos.	44	0,52	3,36	3,01	4,16
HC Pos.	1.668	99,76	87,09	95,29	71,03
HC Pos.	8.381	99,99	90,69	97,90	80,42
HC Pos.	6.782	98,83	64,43	75,42	57,19
HC Pos.	1.755	91,45	37,95	53,59	32,39

Figure 29 FACS analysis of different I10 T25 roux flask passages

I10 shake flask 1 (2 * 10^5C/mL start cell concentration)

The homogeneity of the I10 125 mL shake flask 1 (2 * 10^5 start cell concentration) was analysed using flow cytometry. As negative control, the CHO strain K1 with a cell number of 1*10^6 cells was used. The results are shown in Figure 30. The intensities of passages 6, 10 and 14 were compared to each other and to the initial GA3H6 shake flask 1 P22.

The intensity of I10 shake flask 1 P6 was higher than the initial GA3H6 shake flask 1 P22, which can be deviated by looking at the median values of P6, 88.96, and P22, 83.89. When looking at I10 shake flask 1 passages 6, 10 and 14. the intensities were decreasing over time, expressed by median values of 88.96, 49.79 and 49.65.

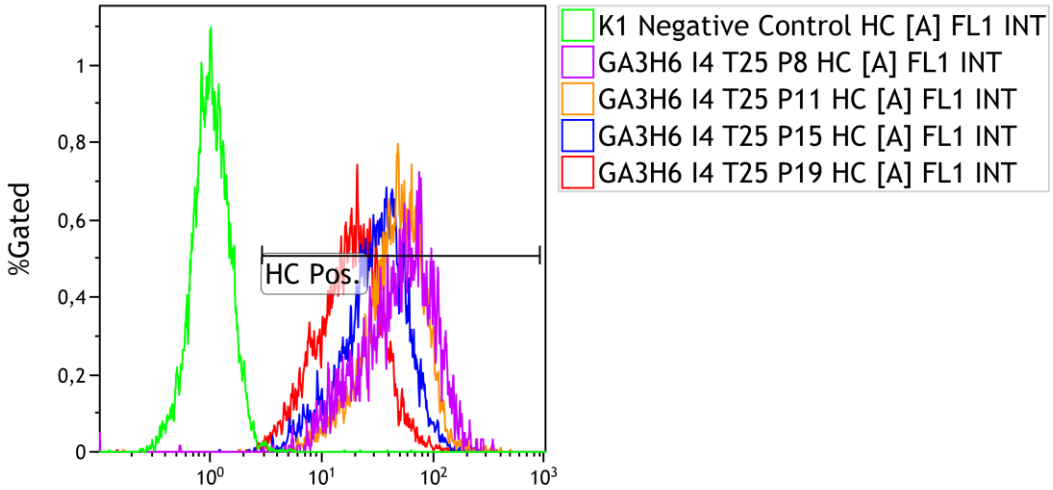


Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	8.385	100,00	1,02	1,01	0,98
All	5.421	100,00	83,89	111,03	62,91
All	5.943	100,00	88,96	132,92	66,32
All	6.854	100,00	49,79	54,07	40,90
All	5.616	100,00	49,65	0,10	26,80
HC Pos.	44	0,52	3,36	3,01	4,16
HC Pos.	5.416	99,91	83,96	111,03	63,62
HC Pos.	5.925	99,70	89,19	132,92	67,43
HC Pos.	6.847	99,90	49,84	54,07	41,34
HC Pos.	5.526	98,40	50,19	61,33	43,92

*Figure 30 FACS analysis of different I10 shake flask 1 passages (2 * 10^5 C/mL start cell concentration)*

I4 T25 roux flask

The homogeneity of the I4 T25 roux flask culture was analysed using flow cytometry. As negative control, the CHO strain K1 with a cell number of 1 * 10^6 cells was used. The results are shown in Figure 31. Passages 8, 11,15 and 19 of the I4 T25 roux flask culture were compared to each other. It can be noticed, that the intensity was decreasing from passage 8 to 19, which can be identified by looking at the median values of 53.01, 47.80, 32.51 and 18.50.



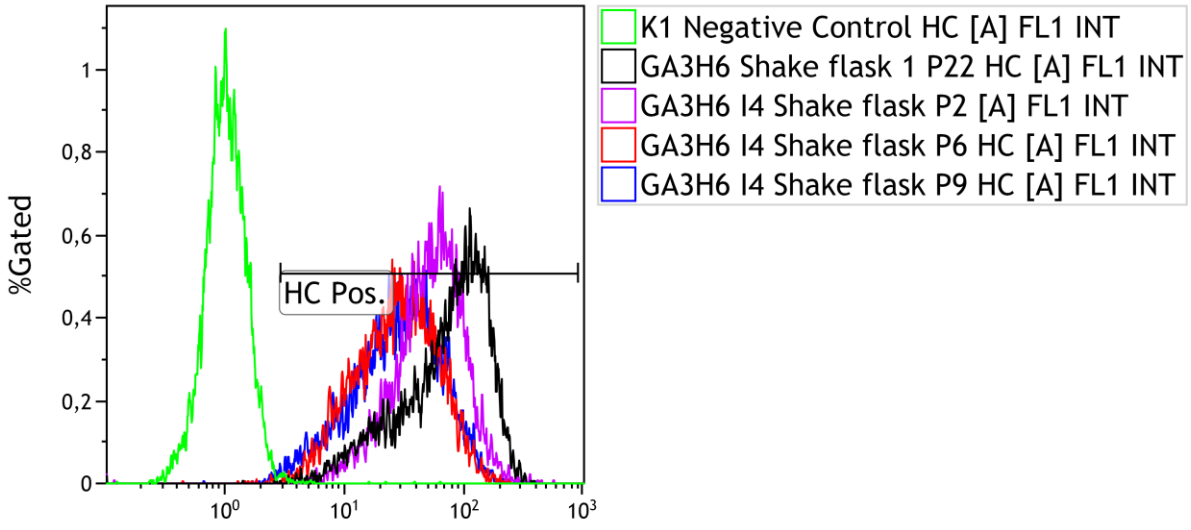
Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	8.385	100,00	1,02	1,01	0,98
All	2.028	100,00	53,01	55,55	44,06
All	6.281	100,00	47,80	48,54	42,92
All	6.538	100,00	32,51	37,73	28,83
All	7.365	100,00	18,50	20,65	16,82
HC Pos.	44	0,52	3,36	3,01	4,16
HC Pos.	2.026	99,90	53,06	55,55	45,10
HC Pos.	6.279	99,97	47,81	48,54	43,05
HC Pos.	6.533	99,92	32,54	37,73	28,91
HC Pos.	7.342	99,69	18,53	20,65	16,97

Figure 31 FACS analysis of different I4 T25 roux flask passages

I4 shake flask culture (2 * 10^5 C/mL start cell concentration)

The homogeneity of the I4 125 mL shake flask culture was analysed using flow cytometry. As negative control, the CHO strain K1 with a cell number of $1*10^6$ cells was used. The results are shown in Figure 32. The intensities of passages 2, 6 and 9 were compared to each other and to the initial GA3H6 shake flask 1 P22.

The intensities of I4 shake flask 1 P6 are significantly lower than the initial GA3H6 clone, which can be deviated by looking at the median values of 83.89, 54.05, 28.15 and 29.96. The intensities of passages 2 and 6 are decreasing, expressed by median values of 54.05 and 28.15 while the median value of passage 9, 29.96, has only slightly increased.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	8.385	100,00	1,02	1,01	0,98
All	5.421	100,00	83,89	111,03	62,91
All	4.510	100,00	54,05	62,44	47,84
All	6.416	100,00	28,15	38,76	25,30
All	6.235	100,00	29,96	46,40	25,41
HC Pos.	44	0,52	3,36	3,01	4,16
HC Pos.	5.416	99,91	83,96	111,03	63,62
HC Pos.	4.508	99,96	54,07	62,44	48,52
HC Pos.	6.402	99,78	28,20	38,76	25,51
HC Pos.	6.189	99,26	30,17	46,40	25,99

Figure 32 FACS analysis of different I4 shake flask 1 passages

5.5 Antibody Production

5.5.1 Transient gene expression of su3H6

The su3H6 antibody was transiently produced using HEK293.6E as host cell line. The antibody su3H6 was produced in 4 distinct transient gene expressions.

5.5.1.1 Transient expression 1 (TGE 1)

The first transient gene expression (TGE 1) was performed using two 50 mL mini bioreactor tubes, containing 30 mL medium. One tube was used for the transient expression of su3H6, the second as negative control. The aim of these transfection was to evaluate the integrity of the plasmids, by analysing the produced su3H6 product via ELISA and to check if the negative control is not producing any su3H6 antibody as intended.

The transient gene expression lasted for 7 days and was stopped after the viability value dropped to 60 % or lower. The cell concentrations, viabilities, antibody titers, growth rates and specific productivities were determined and the average values of all three sampled tubes are shown in Figure 33, Figure 34 and Figure 35.

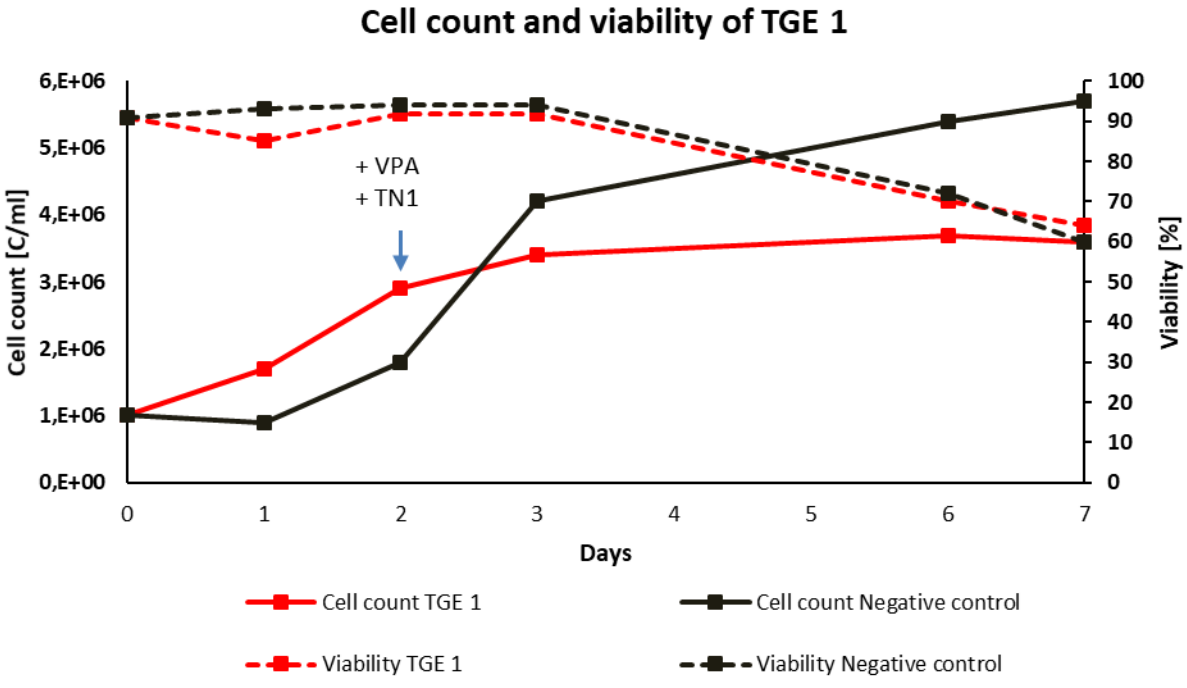


Figure 33 Cell count and viability of TGE 1

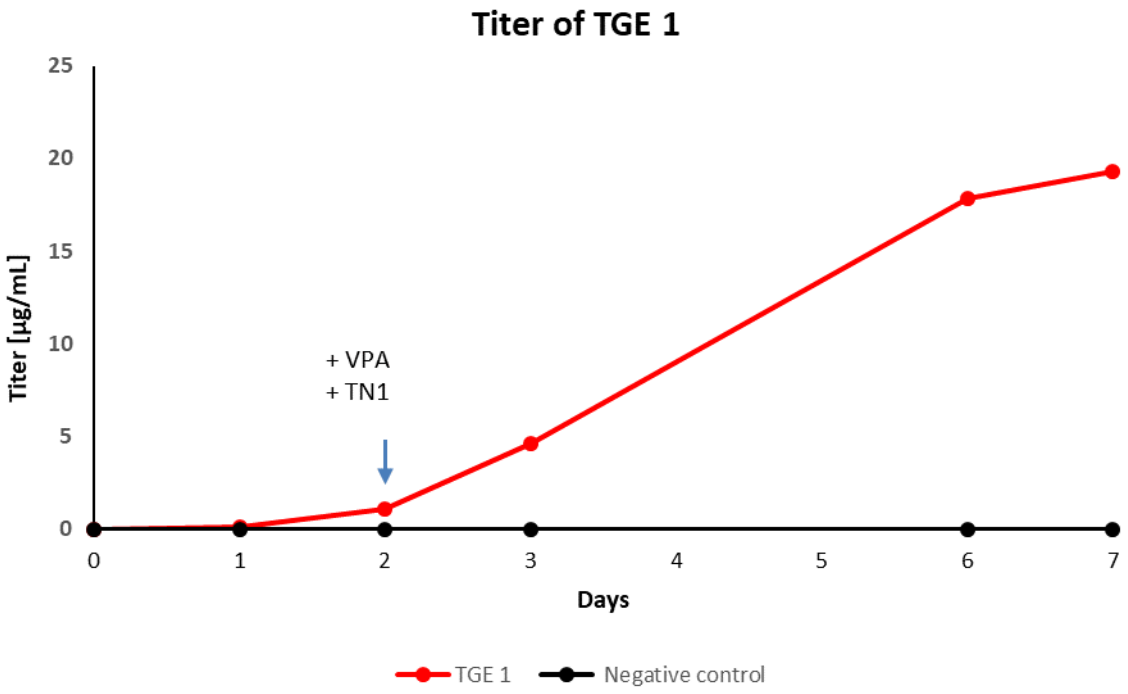


Figure 34 Antibody titer of TGE 1

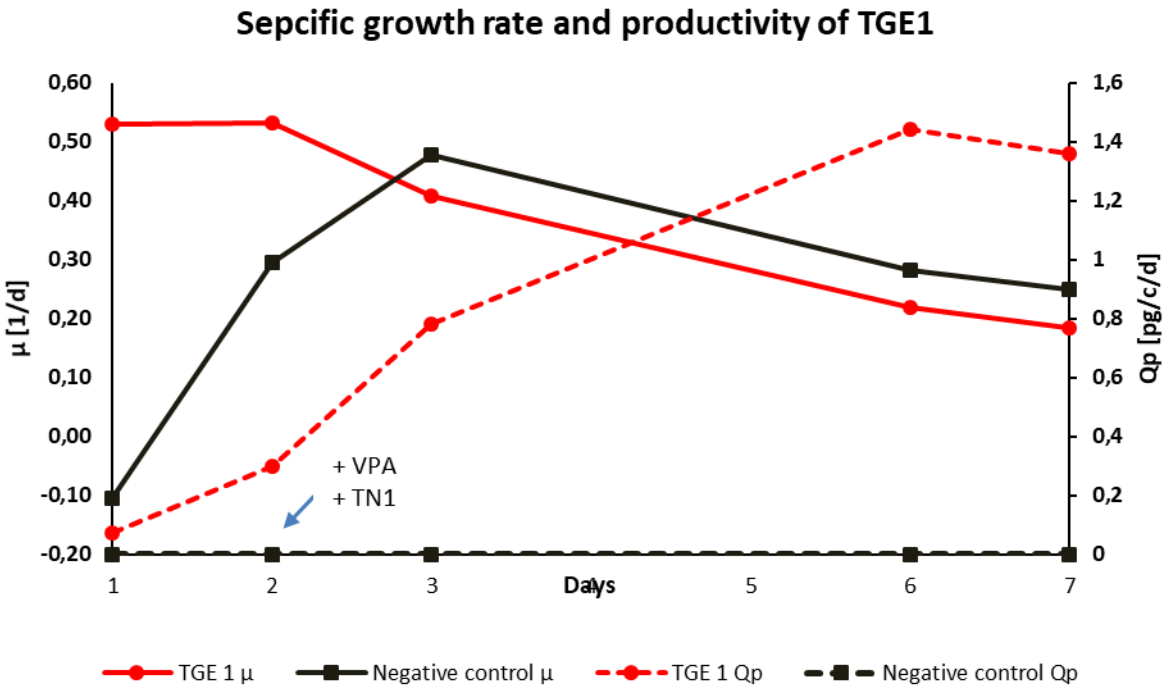


Figure 35 Specific growth rate and productivity of TGE 1

The first tube was producing su3H6 antibody, which was confirmed by detection of the antibody via ELISA. The antibody titer was at 19.3 µg/mL on the last day of the transient gene expression (Figure 34). The negative control did not produce any antibody as expected. It also showed a higher cell concentration and growth rate than the producing tube, although the growth rate was very low after the first day of the cultivation (Figure 35). The result of the producing tube indicates, that the heavy and light chain su3H6 plasmids were intact and led to the formation of the su3H6 antibody as intended.

5.5.1.2 Transient expression 2 (TGE 2)

The transient gene expression 2 was comprised of ten 50 mL bioreactor tubes containing 30 mL of medium. For the measurement of the changes of the viability, cell concentration and antibody titer, 1 mL sample was obtained from tube 1, 5 and 10 on every day of the transfection.

The transient gene expression lasted for 7 days and was stopped after the viability value dropped to 60 % or lower. The cell concentrations, viabilities, antibody titers, growth rates and specific productivities were determined and the average values of all three sampled tubes are shown in Figure 36, Figure 37 and Figure 38.

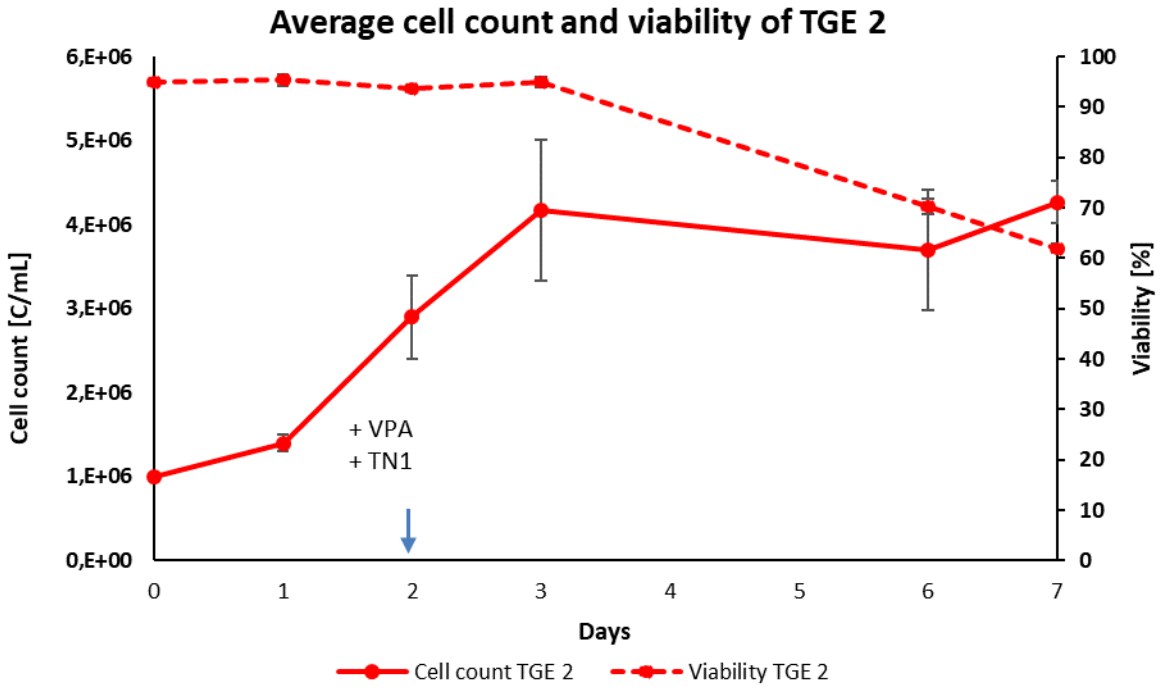


Figure 36 Average cell count and viability of TGE 2

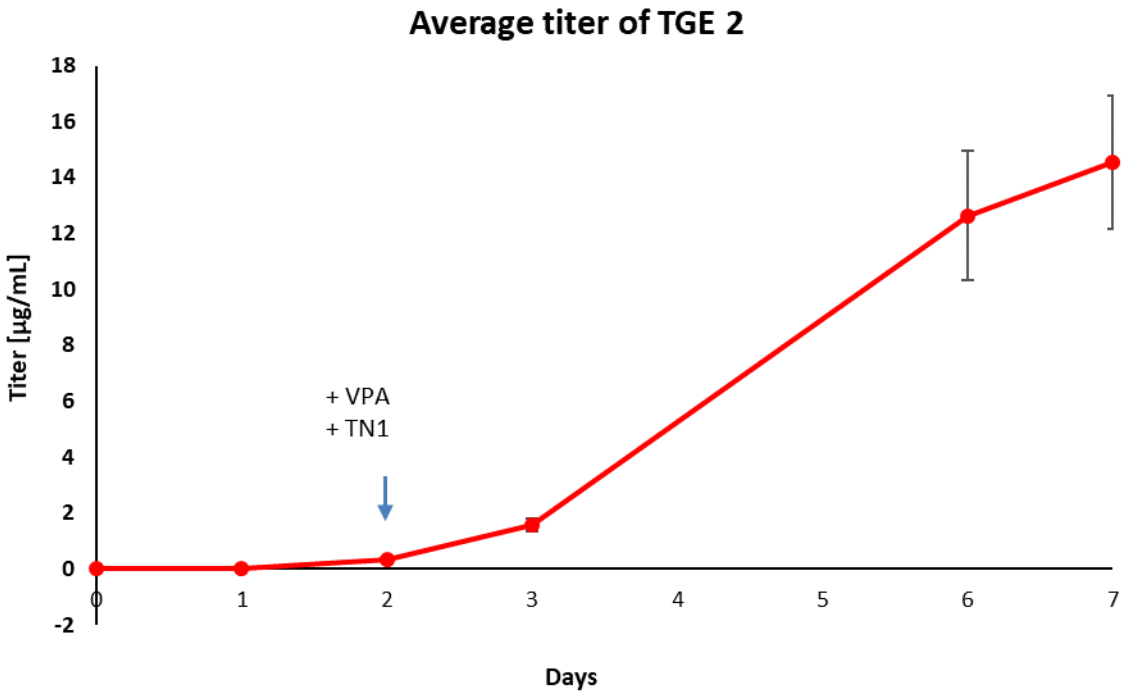


Figure 37 Average antibody titer of TGE 2

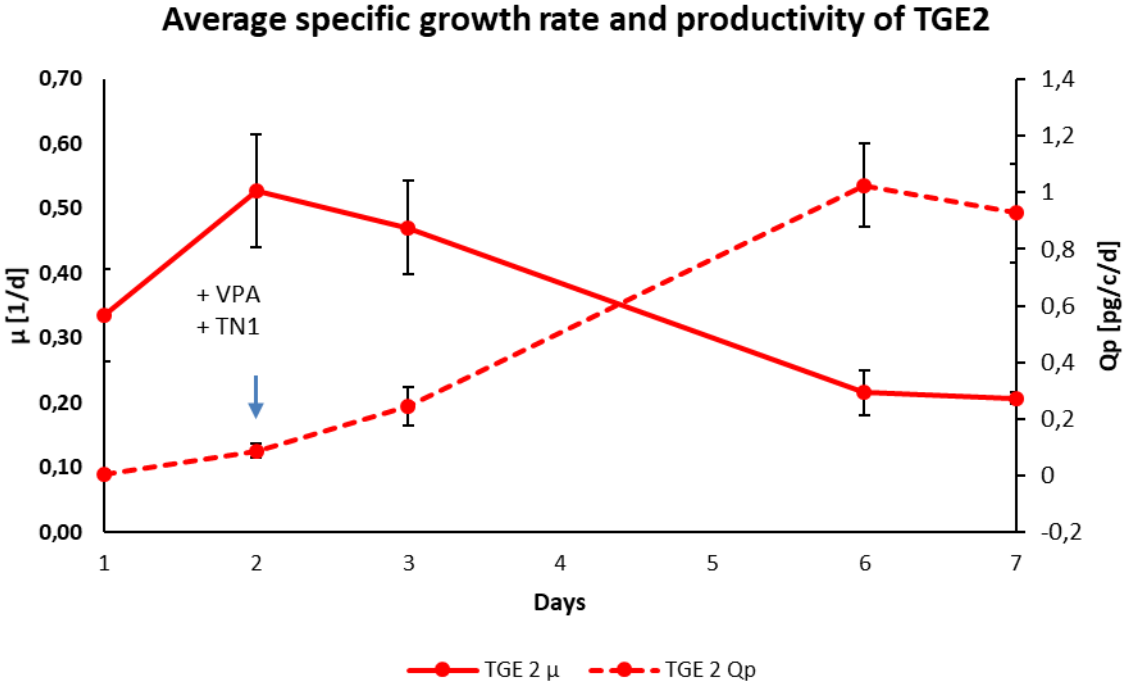


Figure 38 Average growth rate and specific productivity of TGE 2

The specific growth rate and productivity was remaining static after the sixth day in Figure 38, although the antibody titer was still rising. The average antibody titer was at 14.5 µg/mL on the last day of the transient gene expression (Figure 37).

5.5.1.3 Transient expression 3 (TGE 3)

The transient gene expression 3 was comprised of ten 50 mL bioreactor tubes containing 30 mL of medium. For the measurement of the changes of the viability, cell concentration and antibody titer, 1 mL sample was obtained from tube 1. 5 and 10 on every day of the transfection.

The transient gene expression lasted for 7 days and was stopped after the viability value dropped to 60 % or lower. The cell concentrations, viabilities, antibody titers, growth rates and specific productivities were determined and the average values of all three sampled tubes are shown in Figure 39, Figure 40 and Figure 41.

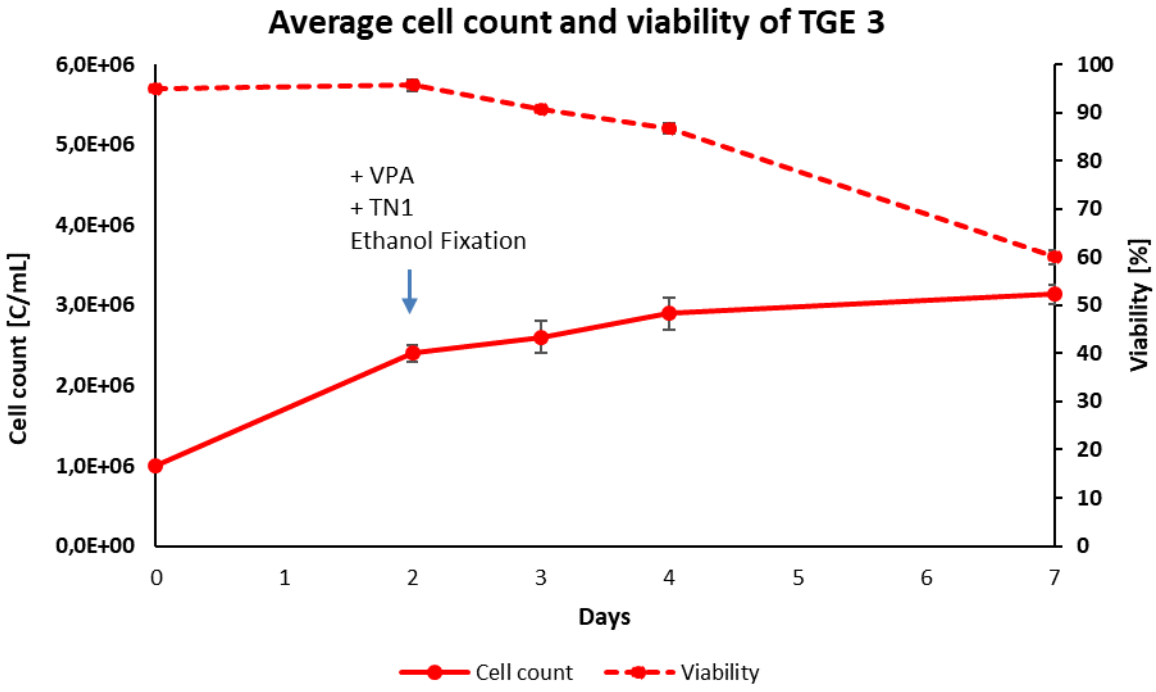


Figure 39 Average cell count and viability of TGE 3

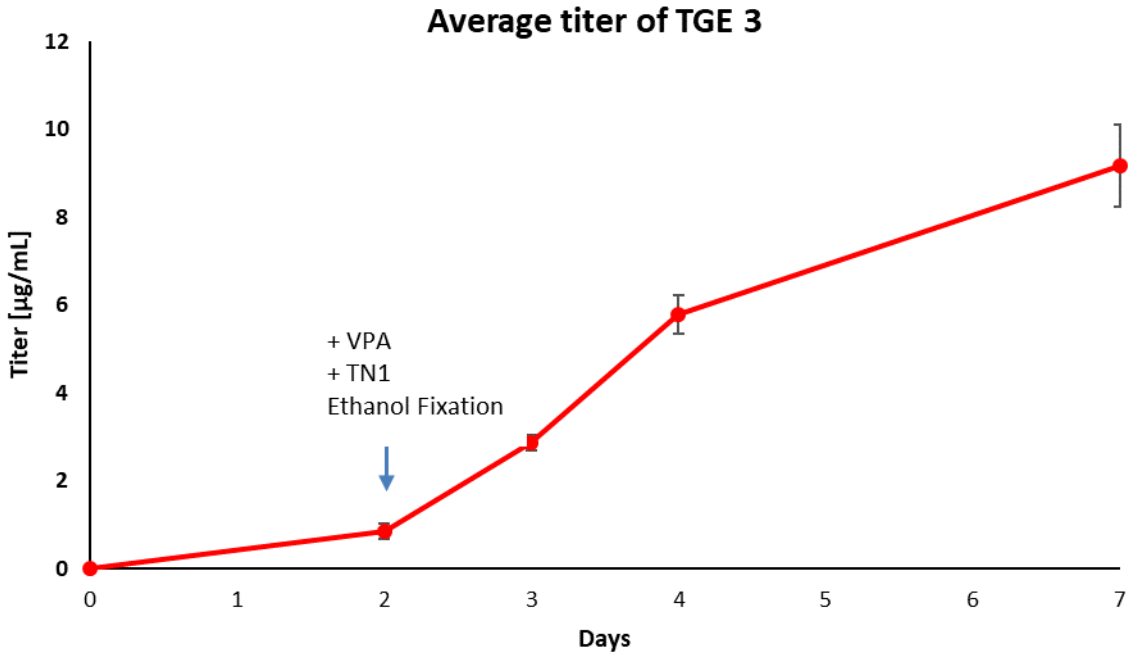


Figure 40 Average antibody titer of TGE 3

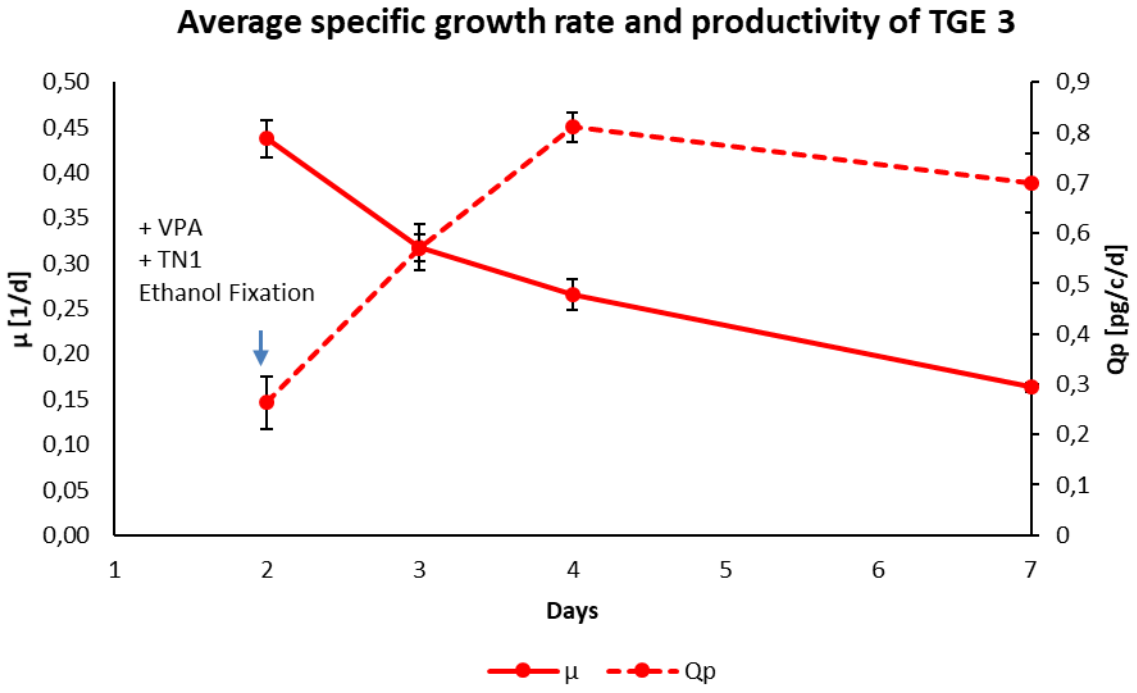


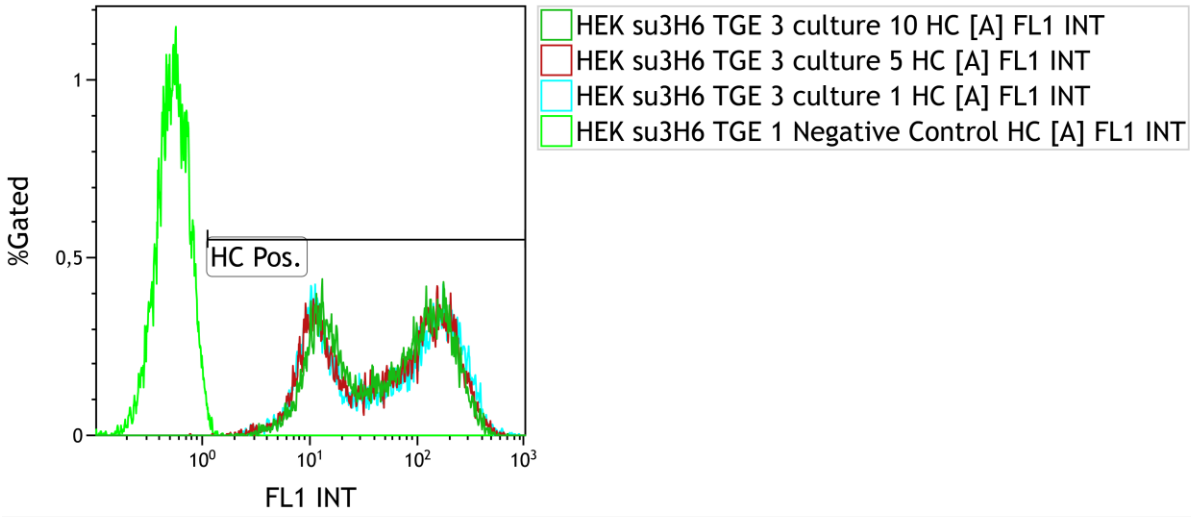
Figure 41 Average growth rate and specific productivity of TGE 3

No samples were obtained on day 1 of the transient gene expression due to a free day, which explains that the specific growth rate and productivity starts on day 2. The average antibody titer was at 9.2 µg/mL on the last day of the transient gene expression (Figure 40) .

Analysis of the homogeneity of TGE 3 HEK293.6E

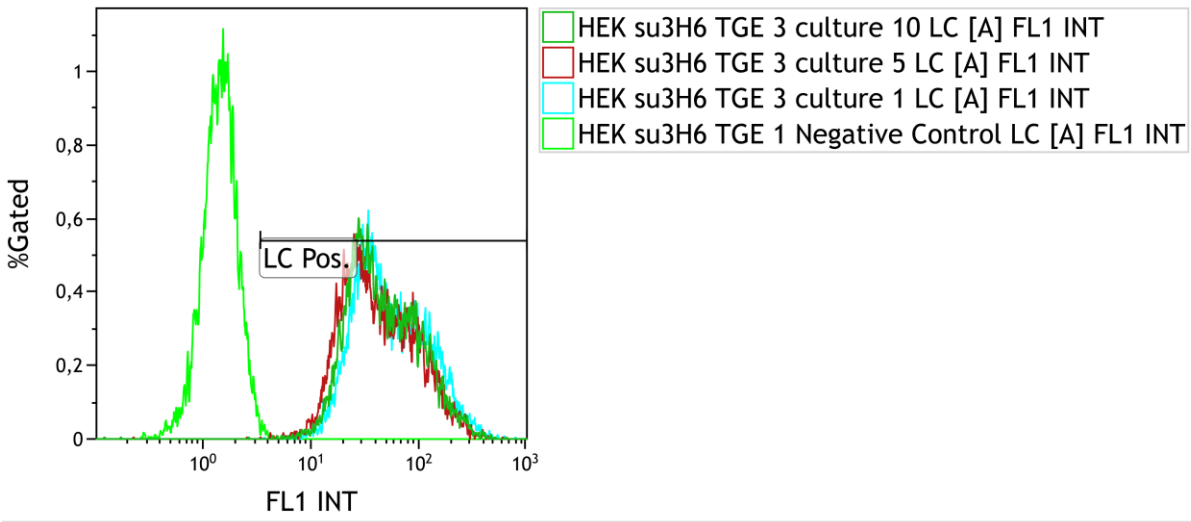
A FACS analysis of the transient expression 3 was performed to examine the homogeneity of the transfection. As negative control, the negative control of TGE 1 was used. For all samples 0.8 * 10^6 cells were stained to allow the comparison of all results. Different cell numbers could lead to higher intensities, caused by the higher cell number and not by the higher productivity of a clone. The FACS analysis was done twice, once with an antibody directed against the heavy chain and once with an antibody directed against the light chain of the su3H6 antibody.

The results of the FACS analysis, with an antibody directed against the heavy chain are shown in Figure 42. Two different peaks are visible, indicating two subpopulations producing the heavy chain of the antibody in a different extent.
Figure 43 illustrates the results of the FACS analysis with an antibody directed against the light chain. There are also two peaks with different intensities visible, suggesting two subpopulations, one producing the light chain with a lower productivity.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	8.100	100,00	60,28	12,94	49,13
All	8.161	100,00	59,37	153,49	47,94
All	8.621	100,00	64,99	11,10	50,58
All	5.939	100,00	0,54	0,50	0,53
HC Pos.	8.100	100,00	60,28	12,94	49,13
HC Pos.	8.160	99,99	59,40	153,49	47,96
HC Pos.	8.621	100,00	64,99	11,10	50,58
HC Pos.	35	0,59	1,20	1,15	1,26

Figure 42 FACS analysis of TGE 3, directed against the heavy chain



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	7.817	100,00	42,68	27,79	47,13
All	7.947	100,00	38,42	25,17	42,48
All	8.678	100,00	48,99	34,18	54,47
All	5.883	100,00	1,45	1,52	1,42
LC Pos.	7.814	99,96	42,70	27,79	47,22
LC Pos.	7.944	99,96	38,45	25,17	42,54
LC Pos.	8.678	100,00	48,99	34,18	54,47
LC Pos.	31	0,53	3,70	3,48	3,74

Figure 43 FACS analysis of TGE 3, directed against the light chain

5.5.1.4 Transient expression 4 (TGE 4)

The su3H6 transient gene expression 4 was comprised of ten 50 mL bioreactor tubes containing 30 mL of medium. For the measurement of the changes of the viability, cell concentration and antibody titer, 1 mL sample was obtained from tube 1 and 10 on every day of the transfection.

The transient gene expression lasted for 7 days and was stopped after the viability value dropped to 60 % or lower. The average values of the determined cell concentrations, viabilities, antibody titers, growth rates and specific productivities are shown in Figure 44, Figure 45 and Figure 46.

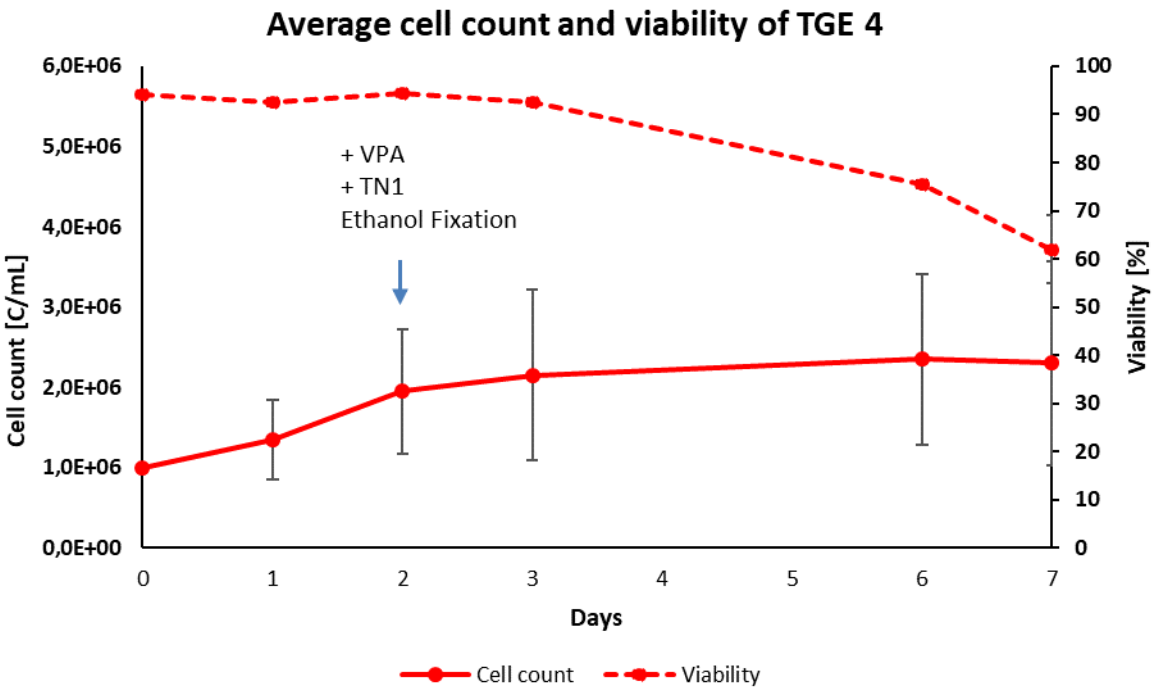


Figure 44 Average cell count and viability of TGE 4

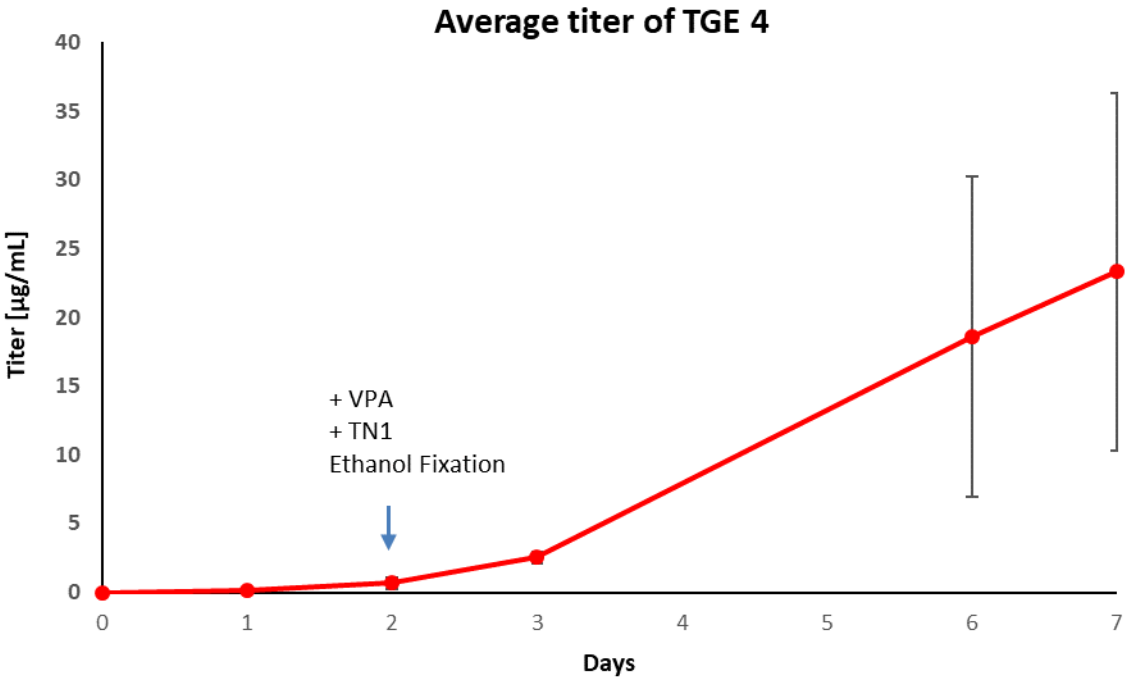


Figure 45 Average antibody titer of TGE 4

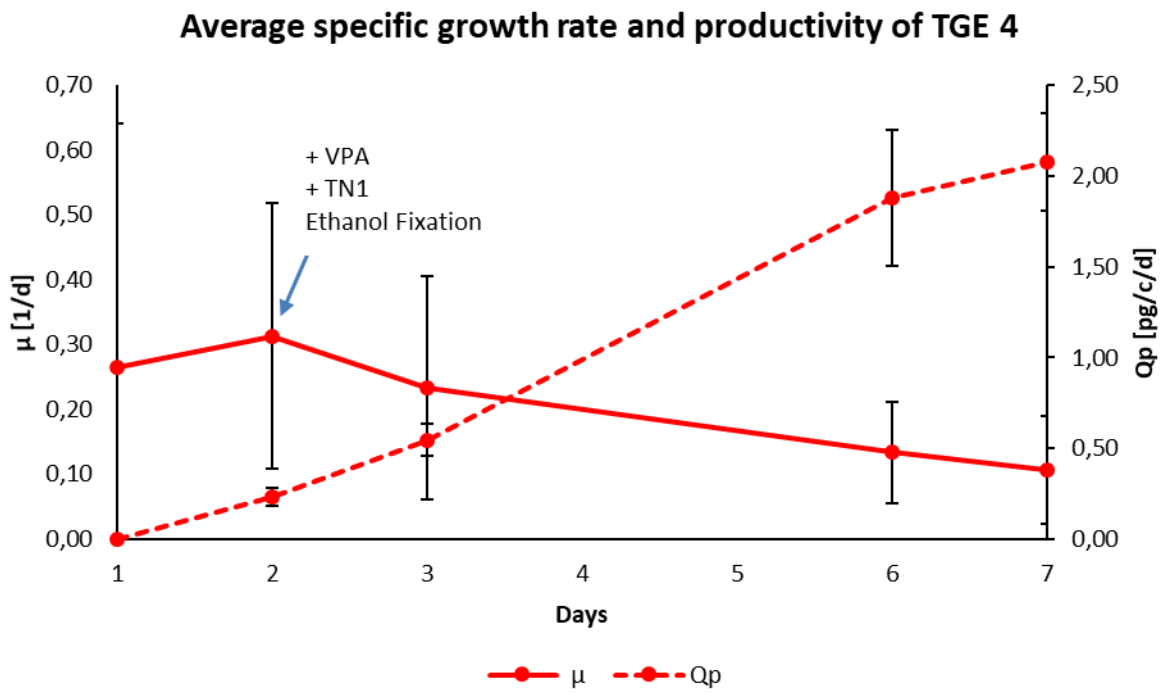


Figure 46 Average growth rate and specific productivity of TGE 4

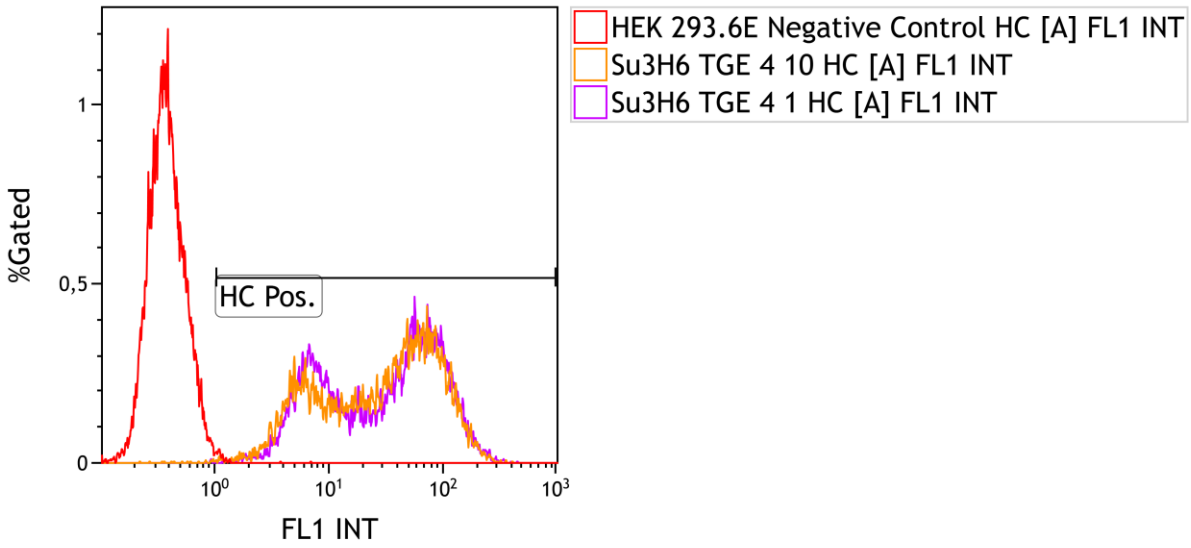
The standard deviations of all samples are very high, especially the standard deviations of the antibody titers of day 6 and 7 (Figure 45) as well as the standard deviations of the growth rate of day 2 and 3 (Figure 46). This result can be explained by insufficient mixing of the HEK293.6E culture, used for the inoculation of the tubes, leading to different cell concentrations in the tubes. Another explanation could be the addition of too much complexed DNA solution in some of the tubes. The average antibody titer was at 23.3 µg/mL at the last day of the transfection (Figure 45), supporting the assumption that the cell concentration or the amount of added complexed DNA was too high.

The average specific productivity was still rising on the seventh day of the transfection, although the growth rate was declining since the third day (Figure 46).

Analysis of the homogeneity of TGE 4 HEK293.6E

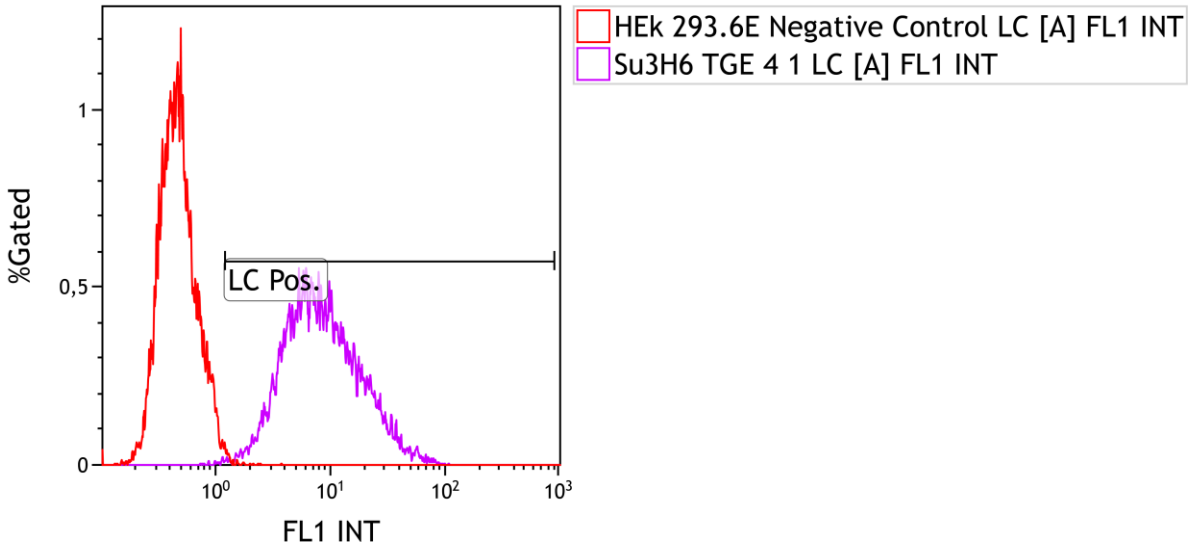
A FACS analysis of the transient expression 4 was performed to examine the homogeneity of the transfection. As negative control, passage 9 of the HEK293.6E routine culture with a concentration of $1.5*10^6$ C/mL was used. The number of cells of all samples was adjusted to $0.9*10^6$ cells to allow comparison of the samples of the tubes 1 and 10. The FACS analysis was done twice, once with an antibody directed against the heavy chain and once with an antibody directed against the light chain of the su3H6 antibody. Because the cell concentration of tube 10 was not high enough for both procedures, only the FACS analysis for the heavy chain was performed.

The results of the FACS analysis, with an antibody directed against the heavy chain, are shown in Figure 47. Two different peaks are visible, indicating two subpopulations producing the heavy chain of the antibody in a different extent. Figure 48 illustrates the results of the FACS analysis with an antibody directed against the light chain. There is only one peak visible, indicating a population that homogenous produces the light chain.



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	9.506	100,00	0,37	0,35	0,38
All	8.521	100,00	34,68	72,10	26,62
All	9.037	100,00	37,16	57,07	28,10
HC Pos.	50	0,53	1,12	1,11	1,21
HC Pos.	8.500	99,75	34,86	72,10	26,87
HC Pos.	9.031	99,93	37,21	57,07	28,16

Figure 47 FACS analysis of TGE 4, directed against the heavy chain



Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	9.417	100,00	0,46	0,50	0,47
All	8.967	100,00	8,01	8,25	8,46
LC Pos.	51	0,54	1,31	1,24	1,42
LC Pos.	8.939	99,69	8,04	8,25	8,52

Figure 48 FACS analysis of TGE 7, directed against the light chain

5.5.2 Production of wt3H6

During the cultivation of wt3H6, supernatant of several passages was collected by centrifuging the cell suspensions for 10 minutes at 1000 rpm. Table 33 gives an overview about the collected passages, which were used for concentration and purification.

Table 33 wt3H6-CHO passages used for concentration and purification

wt3H6-CHO culture	**Medium**	**Passages**
T80 roux flask ($2 * 10^5$ start cell concentration)	D/H	P1-P14
T80 roux flask ($1 * 10^6$ start cell concentration)	D/H	P2-P7
T80 roux flask CD CHO ($2 * 10^5$ start cell concentration)	CD CHO	P1-P6
125 mL shake flask ($2 * 10^5$ start cell concentration)	D/H	P1-P5
125 mL shake flask ($1 * 10^6$ start cell concentration)	D/H	P1-P2

5.5.3 Production of GA3H6

During the cultivation of GA3H6, supernatant of several passages was collected by centrifuging the cell suspensions for 10 minutes at 1000 rpm. Table 34 gives an overview about the collected passages, which were used for concentration and purification.

Table 34 GA3H6-CHO passages used for concentration and purification

GA3H6-CHO culture	**Medium**	**Passages**
T80 roux flask ($2 * 10^5$ start cell concentration)	D/H	P1
125 mL shake flask ($2 * 10^5$ start cell concentration)	CD CHO	P5-P19
125 mL shake flask ($1 * 10^6$ start cell concentration)	CD CHO	P2, P4, P5-P13
I10 shake flask ($2 * 10^5$ start cell concentration)	CD CHO	P4

5.6 Antibody Purification

5.6.1 Purification of su3H6 transient gene expression 1 & 2 (TGE 1 & 2)

During the purification process, different fractions were collected, which were analysed via ELISA. The results are shown in Table 35.

Table 35 Collected fractions during the TGE 1 & 2 purification

Fraction	Volume [mL]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
Supernatant	283.55	16.17	4585.00	100.00
Permeate	336.39	0.04	14.77	0.32
Retentate	39.80	93.37	3716.21	81.05
Flow through	44.32	0.74	32.75	0.71
Wash	8.99	0.06	0.50	0.01
Elution	6.37	340.17	2166.88	47.26
Clean	20.60	0.12	2.54	0.06
Product	5.82	189.84	1104.89	24.10

The measured concentrations of the product are shown in Table 36.

Table 36 Measured product concentrations of TGE 1 & 2

TGE 1 & 2 Product	Volume [ml]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
ELISA	5.82	189.84	1104.89	24.10
NanoDrop	5.82	640.00	3724.80	81.24

5.6.2 Analysis of the collected fractions of su3H6 TGE 1 & 2 via SDS-PAGE

The purified su3H6 product of the TGE 1 & 2 purification and all collected fractions during the purification process were analysed via non-reducing SDS-PAGE for the evaluation of the specificity of the purification process. The result of the SDS-PAGE is shown in Figure 49.

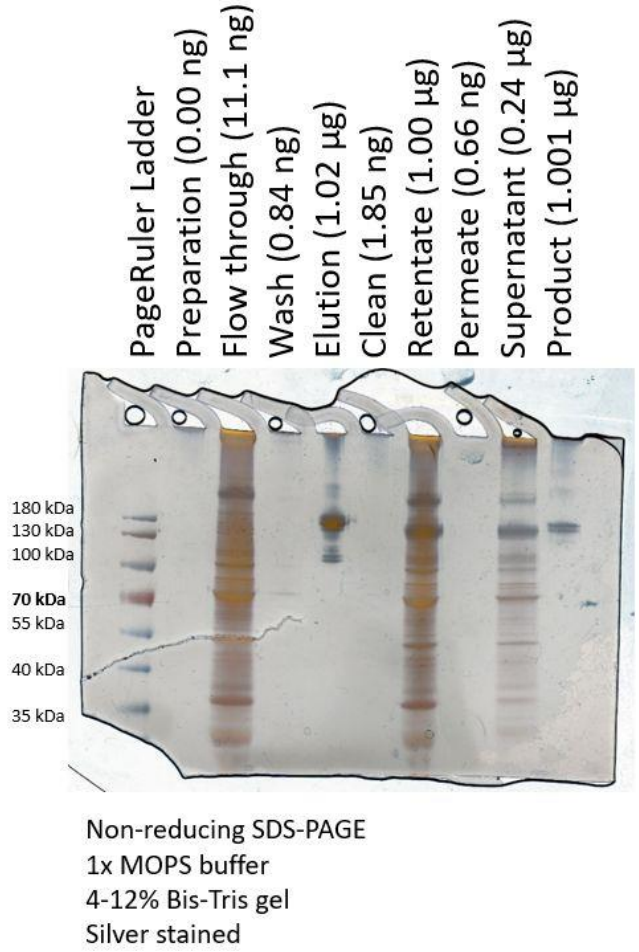


Figure 49 SDS-PAGE of the su3H6 TGE 1 & 2 purification. The amount of loaded su3H6 antibody is stated in the brackets of each fraction (based on the measured ELISA concentration).

5 µL of the PageRuler prestained protein ladder was applied to the first slot, whereas 20 µL of each sample was loaded onto the gel.

The experiment revealed, that indistinct protein fractions are visible in the retentate, flow-through and supernatant fraction. As expected, no protein fractions were present in the preparation, wash and permeate fractions. The su3H6 antibody has a size of approximately 150 kDa. The bands of the elution and product fraction in Figure 49, comprise mostly of the su3H6 antibody, demonstrating a very good specificity of the protein A column.

Furthermore, the intensity of the product was lower than the elution fraction. The amount of loaded su3H6 antibody was based on the ELISA concentrations, which is connected to certain inaccuracies that can explain the discrepancies.

5.6.3 Purification of su3H6 transient gene expression 3 (TGE 3)

During the purification process, different fractions were collected, which were analysed via ELISA. The results are shown in Table 37.

Table 37 Collected fractions during the TGE 3 purification

Fraction	Volume [mL]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
Supernatant	268.95	14.50	3899.24	100.00
Permeate	380.82	0.03	10.64	0.27
Retentate	25.05	108.51	2718.18	69.71
Flow through	24.36	0.28	6.90	0.18
Wash	11.32	0.09	1.07	0.03
Elution	3.20	810.60	2593.92	66.52
Clean	21.59	0.29	6.27	0.16
Product	2.78	978.93	2721.43	69.79

The measured concentrations of the product are shown in Table 38.

Table 38 Measured product concentrations of TGE 3

TGE 3 Product	Volume [ml]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
ELISA	2.78	978.93	2721.43	69.79
NanoDrop	2.78	980.00	2724.4	69.87

5.6.4 Purification of su3H6 transient gene expression 4 (TGE 4)

During the purification process, different fractions were collected, which were analysed via ELISA. The results are shown in Table 39.

Table 39 Collected fractions during the TGE 4 purification

Fraction	Volume [mL]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
Supernatant	268.22	18.18	4876.51	100.00
Permeate	385.27	0.03	12.93	0.27
Retentate	19.53	259.99	5077.55	104.12
Flow through	19.15	0.81	15.47	0.32
Wash	6.72	0.69	4.62	0.09
Elution	4.57	1037.01	4739.14	97.18
Clean	6.12	2.38	14.59	0.30
Product	3.80	1223.13	4647.89	95.31

The measured concentrations of the product are shown in Table 40.

Table 40 Measured product concentrations of TGE 4

TGE 4 Product	Volume [ml]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
ELISA	3.80	1223.13	4647.89	95.31
NanoDrop	3.80	830.00	3154.00	64.68

5.6.5 Purification of wt3H6

During the purification process, different fractions were collected, which were analysed via ELISA. The results are shown in Table 41.

Table 41 Collected fractions during the wt3H6 purification

Fraction	Volume [mL]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
Supernatant	621.73	8.20	5098.43	100.00
Permeate	745.43	0.16	116.95	2.29
Retentate	26.93	175.58	4728.48	92.74
Flow through	26.54	0.09	2.33	0.05
Wash	7.78	0.13	0.99	0.02
Elution	3.53	1005.12	3548.07	69.59
Clean	18.10	0.31	5.54	0.11
Product	2.88	943.20	2716.42	53.28

The measured concentrations of the product are shown in Table 42.

Table 42 Measured product concentrations of wt3H6 puri 1

wt3H6 puri 1 Product	Volume [ml]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
ELISA	2.88	943.20	2716.42	53.28
NanoDrop	2.88	1505.00	4334.40	85.01

5.6.6 Purification of GA3H6

During the purification process, different fractions were collected, which were analysed via ELISA. The results are shown in Table 43.

Table 43 Collected fractions during the GA3H6 purification

Fraction	Volume [mL]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
Supernatant	733.84	2.20	1617.68	100.00
Permeate	857.31	0.02	21.38	1.32
Retentate	25.65	49.12	1260.03	77.89
Flow through	24.11	0.07	1.58	0.10
Wash	6.08	0.03	0.17	0.01
Elution	3.72	260.58	969.36	59.92
Clean	13.12	0.86	11.30	0.70
Product	3.29	260.52	857.11	52.98

The measured concentrations of the product are shown in Table 44.

Table 44 Measured product concentrations of GA3H6 puri 1

GA3H6 puri 1 Product	Volume [ml]	Concentration [µg/mL]	Amount [µg]	Percentage of the supernatant [%]
ELISA	3.29	260.52	857.11	52.98
NanoDrop	3.29	335.00	1102.15	68.13

5.7 Kinetics

5.7.1 Protein A assisted binding assay

To be able to make an exact calculation of the kinetic characteristics of the 3H6 antibodies, all prepared antibodies were analysed using bio-layer interferometry to measure the actual concentrations. The determined concentrations are highlighted in Table 45.

Table 45 Actual protein concentrations for the protein A assisted assay

Antibody	**Measured concentration [µg/mL]**	**Molar concentration [nM]**
wt3H6	61.5	410
GA3H6	Concentration too low	-
su3H6 TGE 3	60.1	401
2F5 IgG 30 µg/mL	41.6	275
2F5 IgG 15 µg/mL	20.2	133
2F5 IgG 7.5 µg/mL	9.2	61

Figure 50 shows the recorded raw data curve of the protein A assisted binding assay. The raw data was aligned to the baseline to allow the comparison between the different association curves. For correction of misalignments between the association and dissociation step, interstep correction was applied to the data. Then the raw data was processed using Savitzky-Golay filtering, which removes high-frequency noise.

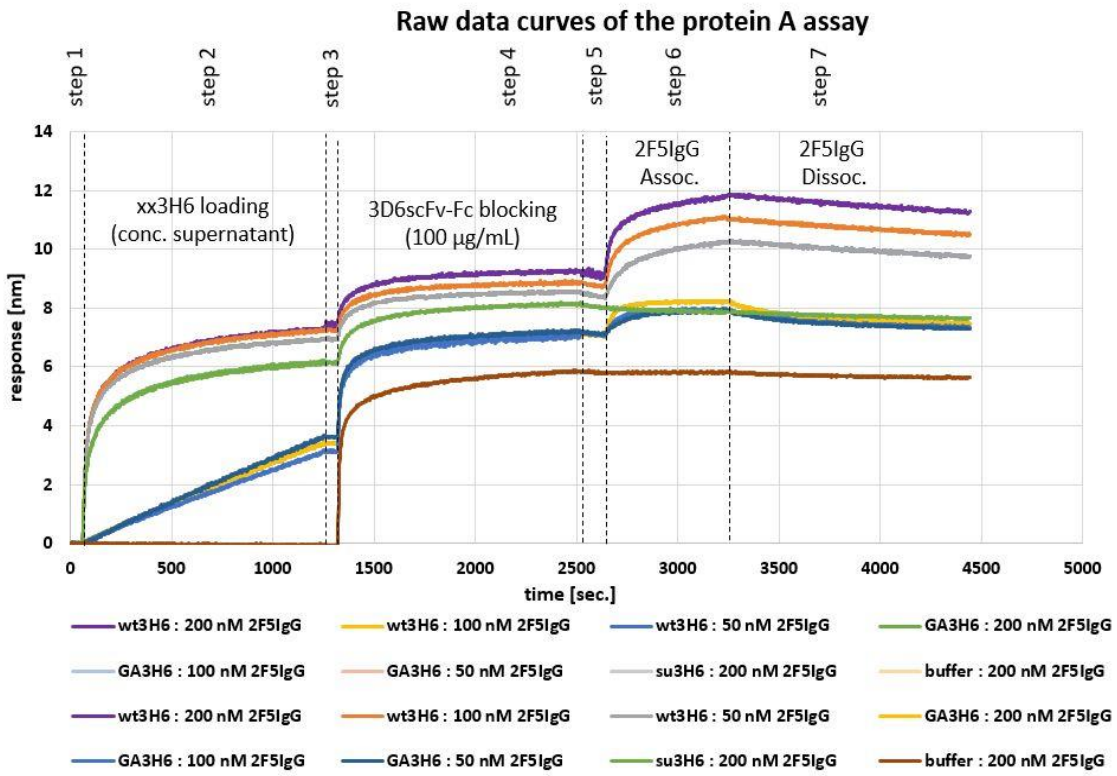


Figure 50 Raw data curve of the protein A assisted assay

The GA3H6 antibody was loaded according to a linear function in contrast to wt3H6 and su3H6, which can be explained by the low concentration of GA3H6. As expected, the buffer control showed no binding to the protein A sensor at all, therefore the sensor was loaded entirely with 3D6 scFv-Fc and no 2F5 IgG could bind.

After the processing of the raw data, the association and dissociation curves were fitted using a 1:1 binding model for the calculation of k_{obs}, k_{on}, k_{dis}, and K_d. The fitted curves are shown in Figure 51.

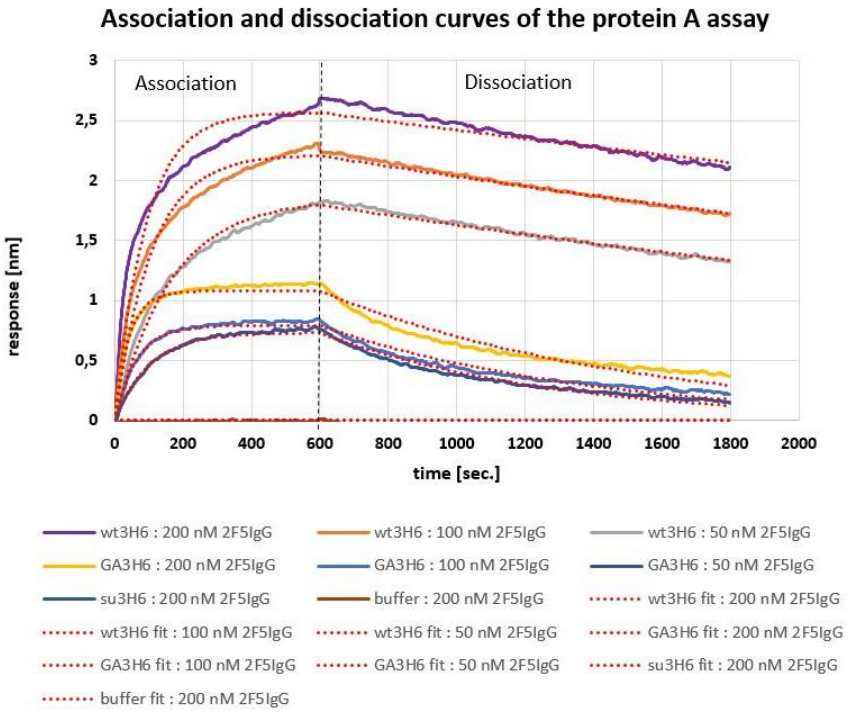


Figure 51 Association and dissociation curves including fitted 1:1 binding model of the protein A assisted assay

The responses of the association and dissociation curves of all three GA3H6 concentrations were lower than those of wt3H6 and GA3H6 shows also a higher dissociation rate, confirming the lower binding affinity of the GA3H6 antibody variant. As expected, su3H6 was binding to the protein A sensor tip through its heavy chain, but there was no association of the 2F5 IgG antibody to the epitope of su3H6 at all, because su3H6 lost its binding ability during the humanization process.

For the calculation of the kinetic values, the measured concentrations of 2F5 IgG were used to obtain more accurate results. The calculated kinetic values are highlighted in Table 46.

Table 46 Calculated kinetic values of the protein A assisted assay

Antibody	2F5 IgG Conc. [nM]	K_d [M]	k_{on}[1/Ms]	k_{dis}[1/s]	k_{obs}[1/s]	Full X^2	Full R^2
wt3H6	275	3.71E-09	3.96E+04	1.47E-04	1.10E-02	2.47	0.90
wt3H6	133	2.97E-09	6.88E+04	2.04E-04	9.35E-03	0.79	0.96
wt3H6	61	2.56E-09	9.64E+04	2.47E-04	6.13E-03	0.29	0.99
GA3H6	275	1.31E-08	8.28E+04	1.08E-03	2.39E-02	0.59	0.97
GA3H6	133	1.16E-08	1.09E+05	1.27E-03	1.58E-02	0.24	0.98
GA3H6	61	1.07E-08	1.38E+05	1.47E-03	9.88E-03	0.12	0.99
su3H6	275	4.41E-10	2.02E+04	8.89E-06	5.55E-03	2.14	0.00
kinetics buffer	275	4.00E-07	4.65E+04	1.86E-02	3.14E-02	2.42	0.00

The antibody su3H6 does not bind to 2F5 anymore and therefore cannot fit to the model, which can be identified by looking at the full R^2 value of 0.00. Average values of the different kinetic values of the wt3H6 and GA3H6 antibodies were calculated. The average values are shown in Table 47.

Table 47 Average kinetic values and calculated ΔG values of the protein A assisted assay

Antibody	K_d [M]	k_{on}[1/Ms]	k_{dis}[1/s]	k_{obs}[1/s]	ΔG [kJ/mol]
wt3H6	3.1E-09	6.8E+04	2.0E-04	8.8E-03	-48.6
GA3H6	1.2E-08	1.1E+05	1.3E-03	1.7E-02	-45.2

The wt3H6 antibody shows the lowest dissociation constant value (K_d) and therefore the strongest binding affinity, indicating that the wild-type conformation with the completely murine variable region is most favourable for binding of the antibody to its epitope. This assumption is also supported by the ΔG value of -48.6 kJ/mol, which is lower than that of GA3H6. In fact, the GA3H6 antibody shows a 3.9 times higher dissociation constant than wt3H6, whereas the ΔG value is 3.4 kJ/mol higher, expressing a more unfavourable free binding energy.

The association rate of GA3H6, expressed by the k_{on} value, is 1.6 times higher than that of wt3H6, indicating beneficial conformational changes of the GA3H6 variant that led to improvements of the association. However, the dissociation rate k_{dis} of GA3H6 is 6.5 times higher than that of wt3H6, leading to a significantly faster dissociation of the antibody from its epitope.

5.7.2 Streptavidin assisted binding assay

The 3H6 antibodies were quantified using bio-layer interferometry to enable the exact calculation of the kinetic values. The measured protein concentrations are shown in Table 48.

Table 48 Actual antibody concentrations of the streptavidin assisted assay

Antibody	**Measured concentration [µg/mL]**	**Molar concentration [nM]**
wt3H6 puri 1 60 µg/mL	59.2	395
wt3H6 puri 1 30 µg/mL	29.6	197
wt3H6 puri 1 15 µg/mL	14.8	99
GA3H6 puri 1 60 µg/mL	50.6	337
GA3H6 puri 1 30 µg/mL	25.3	169
GA3H6 puri 1 15 µg/mL	12.7	84
su3H6 TGE 4 60 µg/mL	47.5	317

Figure 52 shows the recorded raw data curve of the streptavidin assisted binding assay. The raw data was aligned to the baseline to allow the comparison between the different association curves. For correction of misalignments between the association and dissociation step, interstep correction was applied to the data. Then the raw data was processed using Savitzky-Golay filtering, which removes high-frequency noise.

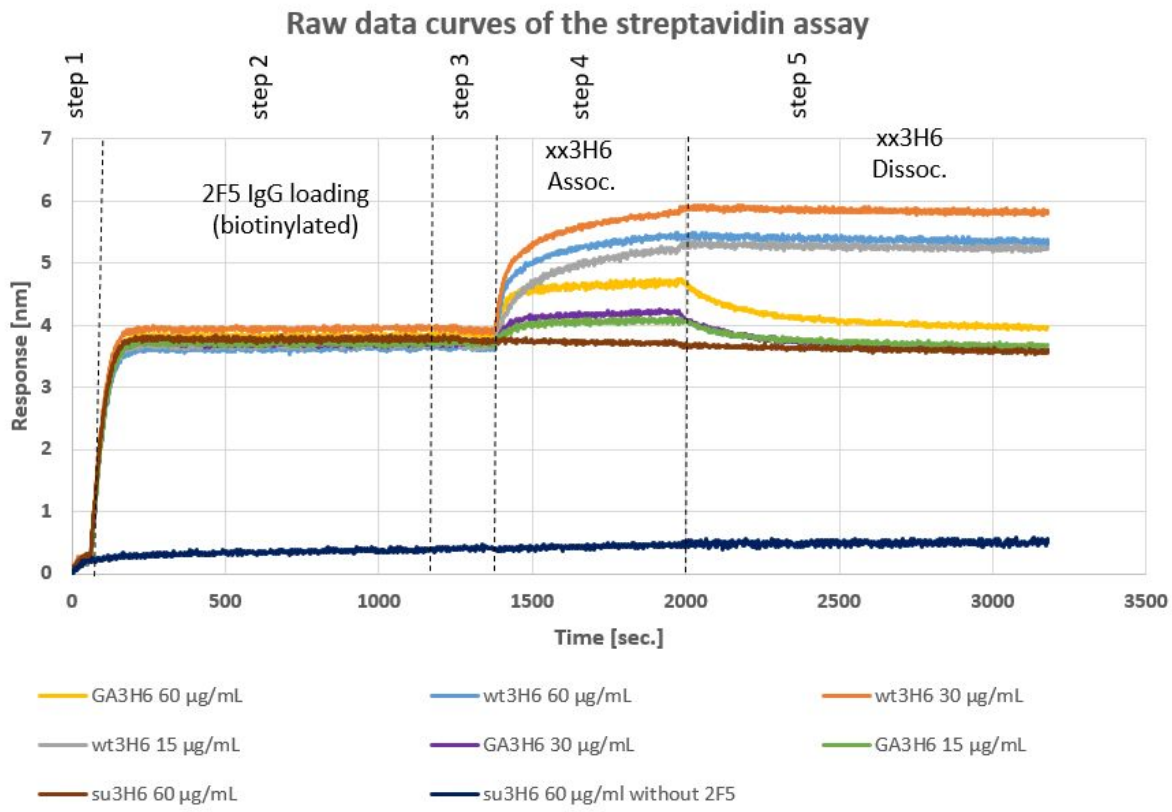


Figure 52 Raw data curves of the streptavidin assisted assay

The wt3H6 30 µg/mL association and dissociation curve is higher than that of 60 µg/mL. 30 µg/mL of wt3H6 antibody equals a molar concentration of 200 nM. The wt3H6 antibody has a literature K_d value of 0.4 nM [2]. Therefore, 30 µg/mL of wt3H6 antibody correspond to a concentration, 500 times higher than the K_d value. Hence, the limit of the binding capacity could already have been reached and no more antibodies could bind after addition of a higher concentration of wt3H6. The higher curve of the wt3H6 30 µg/mL in Figure 52 can be explained by minimal discrepancies between the different channels of the octet system. As expected, the buffer shows no binding at all.

After the processing of the raw data, the association and dissociation curves were fitted to a 1:1 binding model for the calculation of k_{obs}, k_{on}, k_{dis}, and K_d. The fitted curves are shown in Figure 53.

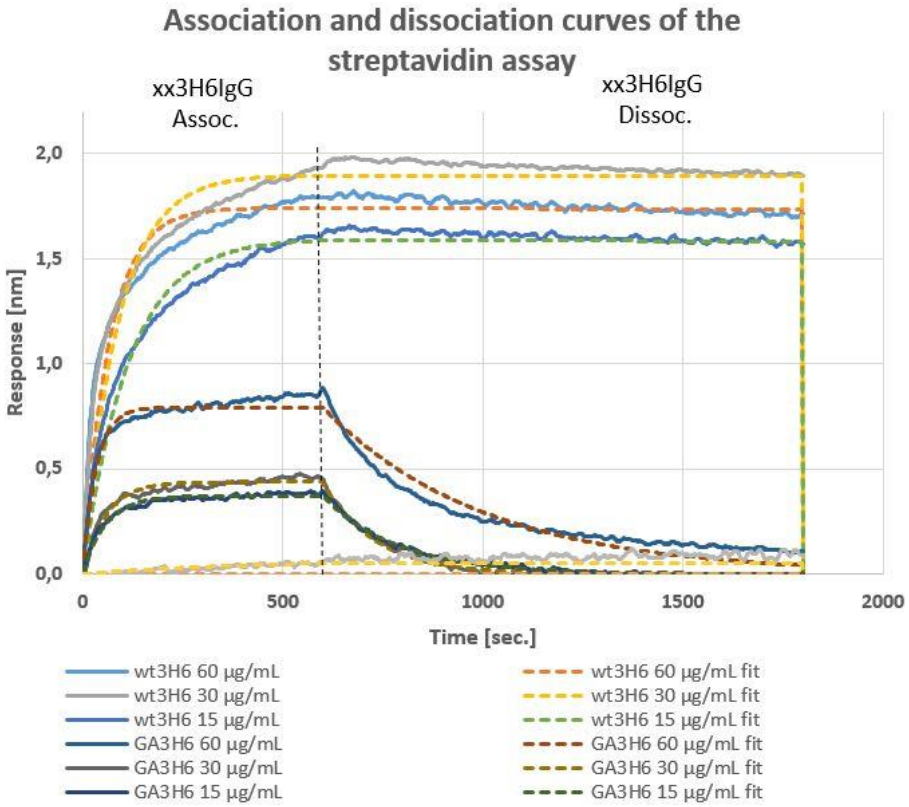


Figure 53 Association and dissociation curves including fitted 1:1 binding model of the streptavidin assisted assay

The responses of the association and dissociation curves of all three GA3H6 concentrations are lower than those of wt3H6 and GA3H6 shows also a higher dissociation, confirming the lower binding affinity of the GA3H6 antibody variant. As expected, there is no binding of the su3H6 antibody at all, because su3H6 lost its binding ability during the humanization process. The calculated kinetic values are highlighted in Table 49.

Table 49 Calculated kinetic values of the streptavidin assisted assay

Antibody	Conc. [nM]	K_d [M]	k_{on}[1/Ms]	k_{dis}[1/s]	k_{obs}[1/s]	Full X^2	Full R^2
wt3H6 60 µg/mL	395	7.85E-11	3.83E+04	3.01E-06	1.51E-02	1.03	0.89
wt3H6 30 µg/mL	197	2.25E-11	5.71E+04	1.29E-06	1.13E-02	1.23	0.91
wt3H6 15 µg/mL	99	1.92E-11	8.82E+04	1.69E-06	8.74E-03	0.50	0.96
GA3H6 60 µg/mL	337	3.07E-08	8.09E+04	2.49E-03	2.97E-02	0.49	0.97
GA3H6 30 µg/mL	169	1.32E-07	5.83E+04	7.70E-03	1.76E-02	0.63	0.94
GA3H6 15 µg/mL	84	3.70E-08	1.45E+05	5.38E-03	1.76E-02	0.06	0.99
su3H6 60 µg/mL	317	3.29E-10	3.16E+04	1.04E-05	1.00E-02	1.01	0.00
su3H6 60 µg/mL (kinetics buffer instead of 2F5)	317	4.06E-15	6.46E+03	2.63E-11	2.05E-03	0.07	0.25

The antibody su3H6 does not bind to 2F5 anymore and therefore cannot fit to the model, which can be identified by looking at the full R^2 value of 0.00. Average values of the different kinetic values of the wt3H6 and GA3H6 antibodies were calculated. The average values are shown in Table 50.

Table 50 Average kinetic values and calculated ΔG values of the streptavidin assisted assay

Antibody	K_d [M]	k_{on}[1/Ms]	k_{dis}[1/s]	k_{obs}[1/s]	ΔG [kJ/mol]
wt3H6	4.0E-11	6.1E+04	2.0E-06	1.2E-02	-59.8
GA3H6	6.7E-08	9.5E+04	5.2E-03	2.2E-02	-41.5

The wt3H6 antibody demonstrates the lowest K_d and ΔG values and therefore expresses the highest binding affinity. The K_d value of GA3H6 is 1675 times higher than the K_d value of wt3H6, illustrating a significantly lower binding capacity. The ΔG value of GA3H6 is 18.3 kJ/mol higher than that of wt3H6, making the binding of GA3H6 to its epitope energetically more unfavourable. It also can be observed, that the k_{on} value of GA3H6 is 1.6 times higher than that of wt3H6 and therefore GA3H6 shows a higher association rate. However, the k_{dis} value of GA3H6, expressing the dissociation rate, is 2600 times higher than that of wt3H6.

6 Discussion

6.1 Cultivation of wt3H6-CHO

The cultivation of wt3H6-CHO was tested using different media, cultivation systems and shaking speeds. Table 51 gives an overview over all performed wt3H6-CHO cultivations.

Table 51 Overview over all performed wt3H6-CHO cultivations

Medium	**Culture system**	**Culture volume [mL]**	**Shaking [rpm]**	**Start cell concentration [C/mL]**	**Comment**
D/H	T25 roux flask	10	static	$2 * 10^5$	Cultivation possible
D/H	T80 roux flask	30	static	$2 * 10^5$	Cultivation possible
D/H	T80 roux flask	30	static	$1 * 10^6$	Cultivation not possible. viability dropped very fast
CD CHO	T80 roux flask	30	static	$2 * 10^5$	Cultivation not possible. viability dropped very fast
D/H	125 mL shake flask	30	140	$2 * 10^5$	Cultivation possible
D/H	125 mL shake flask	30	140	$1 * 10^6$	Cultivation possible. if cultivation time stays under 3 days

The wt3H6-CHO strain could be cultivated in D/H medium using T25 and T80 roux flasks at a start cell concentration of $2 *10^5$ C/mL, however, the cultivation in T80 roux flasks at a start cell concentration of $1 * 10^6$ C/mL was not possible. This behaviour can be explained by extinguished nutrient components of the D/H medium. The wt3H6-CHO strain was unable to grow in CD CHO medium with a much higher nutrient concentration. Hence, D/H medium was defined as the appropriate choice of medium for the cultivation of wt3H6-CHO.

Shaking of the culture could lead to an improvement of the O_2 availability and nutrient accessibility and thus enable the cultivation using D/H medium at a start cell concentration of 1 * 10^6 C/mL. Higher start cell concentrations are adjusted through medium exchange, thus replacing the old medium completely, which is beneficial for high density cultivations because of the higher nutrient demand.

However, the cultivation of the strain in a 125 mL shake flask led to increased cell concentrations and growth rates, but could not enable the continuous cultivation of wt3H6-CHO at a start cell concentration of 1 * 10^6 C/mL. The recording of the growth curve of the wt3H6-CHO 1 * 10^6 C/mL culture revealed dropping viability values after the third day of the cultivation, which can be related to extinguishing nutrient components of the D/H medium. To confirm this theory, additional tests are necessary such as analysis of the glucose, lactose, glutamine, glutamate, ammonium, sodium and potassium level of the D/H medium.

6.2 Cultivation of GA3H6-CHO

The cultivation of GA3H6-CHO was tested using different media, cultivation systems and shaking speeds. Table 52 gives an overview over all performed GA3H6-CHO cultivations.

Table 52 Overview over all performed GA3H6-CHO cultivations

Medium	**Culture system**	**Culture volume [mL]**	**Shaking [rpm]**	**Start cell concentration [C/mL]**	**Comment**
D/H	T25 roux flask	10	static	$2 * 10^5$	Cultivation possible
D/H	T80 roux flask	45	static	$1 * 10^6$	Cultivation not possible. viability dropped very fast
MV3-2/6 (+30%)	50 mLtube	15	220	$2 * 10^5$	Cultivation not possible. viability dropped very fast
CD CHO	125 mL shake flask	30	140	$2 * 10^5$	Cultivation possible
CD CHO	125 mL shake flask	25.5 - 49.5	140	$1 * 10^6$	Cultivation possible

The GAH6-CHO strain could be cultivated in D/H medium using T25 roux flasks at a start cell concentration of $2 *10^5$ C/mL however, the cultivation in T80 roux flasks at a start cell concentration of $1 * 10^6$ C/mLwas not possible. This result can be related to extinguished components of the D/H medium. Furthermore, the cultivation of GA3H6-CHO in MV3-2/6 medium was not successful.

In contrast to wt3H6-CHO, the cultivation of GA3H6-CHO in 125 mL shake flasks using CD CHO medium was possible. The wt3H6-CHO clone could be more susceptible to changes of the medium composition because of the higher specific productivity. GA3H6-CHO could also be cultivated at a start cell concentration of $1 * 10^6$ C/mL using CD CHO medium, however the viability was dropping under 90 % after a cultivation duration of 4 days and returning to values over 90 % after only 3 days of cultivation.

This behaviour can be explained by extinguishing medium components of the CD CHO medium or insufficient O_2 availability. To confirm this theory, additional tests are necessary such as analysis of the glucose, lactose, glutamine, glutamate, ammonium, sodium and potassium level of the CD CHO medium as well as measurement of the pO_2.

6.3 Stabilization of recombinant GA3H6-CHO

A comparison of the average values of the cell concentration, viability, antibody titer, growth rate and specific productivity of the I4 and I10 T25 roux flasks with the initial GA3H6 T25 roux flask is shown in Table 53.
Despite similar growth rates, the specific productivity of the I10 subclone is 4.4 times, that of I4 2.4 times higher than that of the initial GA3H6-CHO clone.

Table 53 Comparison of the average values of the subclone T25 Roux Flasks with the initial T25 roux flask culture

Clone	**Cell concentration [C/mL]**	**Viability [%]**	**Antibody titer [µg/mL]**	**Growth rate [1/d]**	**Specific productivity [pg/c/d]**
GA3H6 T25 roux flask	6.61E+05	94	1.21	0.35	0.62
GA3H6 I10 T25 roux flask	6.87E+05	98	5.36	0.35	2.74
GA3H6 I4 T25 roux flask	5.39E+05	95	3.16	0.28	1.51

Different consecutive passages of the I10 T25 roux flask cultivation were analysed via FACS, revealing a dropping intracellular content of the heavy chain over time, however the specific productivity of the T25 roux flask cultivation improved over time. This behaviour suggests that the I10 subclone needed an adaption phase for improvement of the antibody secretion.

The subclones I4 and I10 were subsequently cultivated in 125 mL shake flasks containing 30 mL CD CHO. Table 54 gives an overview about the average values of the cell concentration, viability, antibody titer, growth rate and specific productivity of the I4 and I10 shake flasks.

The specific productivity of the I10 shake flask with a start cell concentration of 2 * 10^5 C/mL is 4.2 times, that of the I4 shake flask 1.5 times higher than the initial GA3H6-CHO clone. The growth rate of the I4 shake flask is 24 % lower than the initial GA3H6-CHO clone and 49% lower than the I10 shake flask.

*Table 54 Comparison of the average values of the subclone shake flasks with a start cell concentration of 2 * 10^5 C/mL with the initial GA3H6-CHO shake flask*

Culture	**Cell concentration [C/mL]**	**Viability [%]**	**Antibody titer [µg/mL]**	**Growth rate [1/d]**	**Specific productivity [pg/c/d]**
GA3H6-CHO shake flask	9.46E+05	96	1.97	0.41	0.84
GA3H6-CHO I10 shake flask	1.74E+06	98	9.97	0.61	3.56
GA3H6-CHO I4 shake flask	6.13E+05	97	2.32	0.31	1.25

6.4 Transient gene expression of su3H6

Table 55 gives an overview about the number of tubes, end-titer and specific productivity of all performed su3H6 transient gene expressions.

Table 55 Number of tubes, end-titer and specific productivity of all su3H6 transient gene expressions

	Tubes	**End-titer [µg/mL]**	**Specific productivity [pg/c/d]**
TGE 1	1	19.3	1.36
TGE 2	10	14.5	0.93
TGE 3	10	9.2	0.70
TGE 4	10	23.3	2.08

The average antibody titer at the end of each transient gene expression is differing between the various transient gene expressions, although all tubes were started under the same conditions. There are several explanations for this behaviour. The antibody titer depends on the age of the used HEK293.6E culture as the probability of lost EBNA-1 protein is rising with increasing cultivation time of HEK293.6E.

The HEK293.6E cultures also need to be mixed sufficiently before taking the sample for determination of the cell concentration and prior to the inoculation of each tube. Also, the precipitated plasmid solution must be mixed carefully before the inoculation and the exact same volumes must be added to each tube.

6.5 Antibody Purification

Table 56 gives an overview over the amount of purified 3H6 antibodies. The amount of purified 3H6 antibody was determined via ELISA as well as NanoDrop and compared with the amount of antibody dissolved in the supernatant, which was measured via ELISA.

Table 56 Overview over the obtained antibody amount of all performed antibody purifications

Purification	**Supernatant (ELISA) [µg]**	**Product (ELISA) [µg]**	**Product (NanoDrop) [µg]**
su3H6 TGE 1 & 2	4585.00 (100 %)	1104.89 (24.10 %)	3724.80 (81.24 %)
su3H6 TGE 3	3899.24 (100 %)	2721.43 (69.79 %)	2724.4 (69.87 %)
su3H6 TGE 4	4876.51 (100 %)	4647.89 (95.31 %)	3154.00 (64.68 %)
wt3H6 puri 1	5098.43 (100 %)	2716.42 (53.28 %)	4334.40 (85.01 %)
GA3H6 puri 1	1617.68 (100 %)	857.11 (52.98 %)	1102.15 (68.13)

In total, 9603.2 µg of the su3H6, 4334.4 µg of the wt3H6 and 1102.2 µg of the GA3H6 antibody could be purified based on the NanoDrop measurements. In contrast, 8474.2 µg of the su3H6, 2716.4 µg of the wt3H6 and 857.1 µg of the GA3H6 antibody could be produced depending on the ELISA concentrations.

The products of all 3H6 purifications exhibit a lower amount of antibody in comparison with the original amount dissolved in the supernatant. The purification process consists of several steps, offering various possibilities for product loss.

The supernatant was applied to a 0.22 µm filter before the ultrafiltration via TFF and the concentrated antibody solution was filtrated through a 0.45 µm filter afterwards. After dialysis of the eluted antibody solution, the product is finally filtrated through a 0.22 µm filter. These filtrations steps are possible causes for product loss if antibody fractions form aggregates bigger than the filter pore size, which subsequently lead to the retention of the antibody fractions in the filter. The SDS-PAGE revealed additional bands in the elution fraction, which could be caused by aggregated 3H6 antibody fractions (Figure 49).

There are different causes of antibody aggregation. The ultrafiltration process puts a lot of stress on the antibodies due to pumping, air bubbles and microcavitation, which can lead to protein aggregation. Another possibility of antibody aggregation is the subjection of the antibody solution to low pH (2.5) during protein A chromatography for the detachment of the antibodies from the protein A column. The low pH can damage the structure of the antibodies, leading to aggregation[24].
Finally, some remaining antibody fraction could still be bound to the column after the elution.

There is also a difference between the determined ELISA and NanoDrop antibody amount observable. Using ELISA, the 3H6 antibody is measured via a detection antibody directed against the heavy chain of the 3H6 antibody, which is a very specific method. The determination using NanoDrop is performed by measuring the extinction at 280 nm, which is caused by all proteins in the sample solution and not only by the 3H6 antibody. Although the purification of the 3H6 antibody via protein A column is very specific, as the SDS-PAGE showed (Figure 49), other protein fractions could still be present in the final product, leading to a higher value.

The fractions of the TGE 1 & 2 purification were analysed via SDS-PAGE (Figure 49). Indistinct protein fractions were visible in the supernatant, retentate and flow through fractions. These indistinct proteins could descend from host cell proteins and the addition of TN1, which was added on the second day of the transient gene expression.

6.6 Kinetics

The protein A assisted and the streptavidin assisted affinity assays give information about the kinetics of the wt3H6, GA3H6 and su3H6 antibodies. In contrast to the ELISA method, bio-layer interferometry allows the calculation of the k_{obs}, k_{on}, k_{dis}, and K_d values, which is the main advantage for usage of bio-layer interferometry.

Table 57 and Table 58 give an overview about the calculated kinetic values of the protein A and streptavidin assisted affinity assays. The measured values are compared to the values found in literature (Table 59).

Table 57 Average kinetic values and calculated ΔG values of the protein A assisted assay

Antibody	**K_d [M]**	**k_{on}[1/Ms]**	**k_{dis}[1/s]**	**k_{obs}[1/s]**	**ΔG [kJ/mol]**
wt3H6	3.1E-09	6.8E+04	2.0E-04	8.8E-03	-48.6
GA3H6	1.2E-08	1.1E+05	1.3E-03	1.7E-02	-45.2

Table 58 Average kinetic values and calculated ΔG values of the streptavidin assisted assay

Antibody	**K_d [M]**	**k_{on}[1/Ms]**	**k_{dis}[1/s]**	**k_{obs}[1/s]**	**ΔG [kJ/mol]**
wt3H6	4.0E-11	6.1E+04	2.0E-06	1.2E-02	-59.8
GA3H6	6.7E-08	9.5E+04	5.2E-03	2.2E-02	-41.5

Table 59 Literature values of Kd, k_{obs} and ΔG for wt3H6 and GA3H6

Antibody	**K_d [M]**	**k_{obs}[1/s]**	**ΔG [kJ/mol]**
wt3H6	4.0E-10[2]	6E-02 [2]	Ca. -50[25]
GA3H6	1.4E-09[2]	4E-02 [2]	

The two assays show varying results. The values of the protein A assisted assay are the closest to the literature values with an K_d value of wt3H6 7.8 times higher than the literature value, whereas the K_d value of the streptavidin assisted assay is 10 times lower. The wt3H6 ΔG value of the protein A assisted assay is also near the literature value of -50 kJ/mol, whereas the ΔG value of the streptavidin assisted assay is too low. The GA3H6 ΔG values of both assays are higher than the literature value of wt3H6, indicating that the binding to its epitope is energetically more unfavourable.

The wt3H6 k_{obs} literature value of the protein A assisted assay is 6.8 times, that of the streptavidin assisted assay 5.0 times higher than the measured results, whereas the GA3H6 literature value of the protein A assisted assay is 2.4 times, that of the streptavidin assisted assay 1.8 times higher.

The discrepancies could have been caused by several reasons. The used GA3H6 concentration of the protein A assisted assay was very low, which resulted in a linear loading curve. The amount of GA3H6 antibody could have been too low for an exact determination of the kinetic values.

Furthermore, the streptavidin assisted assay resulted in a wt3H6 30 µg/ml curve, slightly higher than the 60 µg/mL curve. The limit of the binding capacity at a wt3H6 concertation of 30 µg/ml could already have been reached and a higher amount of wt3H6 was not able to change the equilibrium concentration anymore. It is therefore recommended to repeat the assay with lower concentrations of wt3H6 for more accurate results.

7 Conclusion

The appropriate cultivation media for wt3H6-CHO and GA3H6-CHO could be determined. For wt3H6-CHO, D/H medium could be defined as the medium of choice, although the cultivation time at a start cell concentration of 1 * 10^6 C/mL should not exceed more than 3 days. GA3H6-CHO could be cultivated in CD CHO medium either with a start cell concentration of 2 * 10^5 C/mL or 1 * 10^6 C/mL.

The FACS analysis of the GA3H6-CHO clone revealed a very poor homogeneity. Furthermore, the GA3H6-CHO T25 roux flask culture showed a very low specific productivity, unsuitable to produce high amounts of GA3H6 antibody. Both properties could be improved by the subcloning procedure. The resulting GA3H6-CHO clone I10 demonstrated a 4.4 times higher specific productivity in the T25 roux flask cultivation and a 4.2 times higher specific productivity in the 125 mL shake flask cultivation. In comparison to the original GA3H6-CHO clone, the FACS analysis of the I10 subclone exhibited a narrower peak and a higher overall intensity.

The su3H6 antibody was produced in four distinct transient gene expressions using HEK293.6E as host system with specific productivities ranging from 0.70 to 2.08 pg/c/d. The discrepancies between the different transient gene expressions strengthen the importance of careful mixing of either the DNA mixture and the HEK293.6E culture prior to the inoculation.

The wt3H6, GA3H6 and su3H6 antibodies were purified via protein A chromatography. The analysis of the obtained fractions revealed discrepancies between the 3H6 antibody amount in the supernatant and the final product. This product loss could be explained by formation of aggregates, but further tests need to be conducted to confirm this theory.

Kinetic properties of all three 3H6 antibodies were analysed via bio-layer interferometry using two different sensors, protein A and streptavidin. The protein A assisted binding assay demonstrated the closest values to the literature values. In both assays, wt3H6 showed the highest binding and therefore lowest K_d values. GA3H6 demonstrated a much lower binding capacity to its epitope, whereas su3H6 showed no association at all.

8 List of figures

9 List of tables

10 Bibliography

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11 Appendix

11.1 Comparison of the ingredients of the D/H and CD CHO medium

Table 60 Complete comparison of the ingredients of the D/H and CD CHO medium

Component	**D/H medium [mg/L]** [15]	**CD CHO medium [mg/L]** [16]
Energy source		
D-glucose	3151	4500.00
Inorganic Salts		
Calcium salt (e.g. CaCl2)		11.10
Manganese salt (e.g. MnCl2)		0.0001
Selenium salt (e.g. Na2SeO3)		0.0000067
Vanadium salt (e.g. NH4VO3)		0.0006
Zinc salt (e.g. ZnSO4)		0.0874
NaCl	6999.5	4410.00
KCl	311.8	276.30
Na_2HPO_4	71	125.00
NaH_2PO_4	62.5	
$MgSO_4$	100	24.10
$MgCl_2$	61	76.20
$CaCl_2$	116.61	
$Fe(NO_3)_3$	0.05	0.810
$FeSO_4$	0.417	
$CuSO_4$	0.00125	
$ZnSO_4$	0.432	
$NaHCO_3$	2438	2400.00
Amino acids		
L-Alanine	4.50	0.00
L-Arginine	147.50	355.6
L-Asparagine	7.50	26.40
L-Aspartic acid	6.65	75.00
L-Cysteine	15.75	57.60
L-Cystine	24.00	0.00
L-Glutaminic acid	7.35	29.40
Glycine	18.75	0.00
L-Histidine	31.50	42.20
L-Isoleucine	54.50	190.00

L-Leucine	59.00	280.00
L-Lysine	91.25	204.0
L-methionine	17.24	115.00
L-Phenylalanine	35.50	70.00
L-Proline	17.25	0.00
L-Serine	26.25	250.00
L-Threonine	53.50	60.00
L-Tryptophane	9.00	20.00
L-Tyrosine	38.70	69.2
L-Valine	52.85	190.00
Vitamins		
Cholin chloride	9.00	14.00
Biotin	0.00365	0.097
Folic acid	2.65	5.00
D-Ca^{++}-pantothenate	2.24	1.19
I-Inositol	12.6	18.00
Nicotinamide	2.02	1.22
Pyridoxal	2.00	0.00
Pyridoxine	0.031	0.85
Riboflavin	0.05	0.22
Thiamine	2.17	1.00
Vitamin B_{12}	0.68	1.03
Other Components		
Ethanolamine		3.2
HEPES		2980.00
Insulin		10.00
PLURONIC F68		300.00
Transferrin		5.00
Dextran Sulfate. MW 5000		100
Sodium-pyruvate	55	110.00
Phenol red	12.5	1.00
Lipoic acid	0.11	2.00
Linoleic acid	0.042	0.06
Putrescine	0.081	0.087
Hypoxanthine	2.05	
Thymidine	0.37	

11.2 Raw data

11.2.1 Cultivation of HEK293.6E

Table 61 Raw data of HEK293.6E shake flask 1 (220 rpm)

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]
0		91		1.60E+06	
1	3	99	8.0	2.10E+06	0.78
2	3		10.5		
3	1	95		1.80E+06	
4	3	96	9.00	1.10E+06	0.57
5	3	88	5.50	1.90E+05	-0.02
6	4	83	1.00	8.50E+05	0.37
7	3	83	4.25	4.50E+05	0.27
8	3	94	2.25	1.10E+06	0.57
9	4		5.5		
10	1	97		3.70E+06	
11	3	94	18.5	4.70E+05	0.28
12	3	96	2.35	1.40E+06	0.65
13	4	96	7.00	2.20E+06	0.60
14	3	92	11.00	2.30E+06	0.81
15	4	97	11.50	3.70E+06	0.73
16	3	92	18.50	2.30E+06	0.81
17	4	96	11.50	4.50E+06	0.78
18	3	97	22.5	1.90E+06	0.75
19	4	96	9.5	3.40E+06	0.71
20	3	97	17.0	2.20E+06	0.80
21	4	95	11.0	3.90E+06	0.74
22	3	94	19.50	2.10E+06	0.78
23	4	97	10.50	3.50E+06	0.72
24	4	98	17.50	3.60E+06	0.72
25	3	96	18.00	2.10E+06	0.78
26	3	97	10.50	2.30E+06	0.81
27	4	98	11.50	3.80E+06	0.74
28	3	99	19.00	2.00E+06	0.77
29	4	97	10.00	4.20E+06	0.76
30	4		21.00		
31	1	98		2.40E+06	
32	2	95	12.00	9.80E+05	0.79
33	4	97	4.90	3.30E+06	0.70
34	3	94	16.50	2.30E+06	0.81

Table 62 Raw data of HEK293.6E shake flask 2 (140 rpm)

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]
8	3	99		1.80E+06	
9	4		9.0		
10	1	97		4.10E+06	
11	3	98	20.50	1.30E+06	0.62
12	3	97	6.50	1.90E+06	0.75
13	4	94	9.50	3.90E+06	0.74
14	3	91	19.50	2.50E+06	0.84
15	3	97	12.50	2.80E+06	0.88
16	4	97	14.00	3.10E+06	0.69
17	4	96	15.50	3.50E+06	0.72
18	3	94	17.50	1.50E+06	0.67
19	4	94	7.50	3.30E+06	0.70
20	3	94	16.50	1.60E+06	0.69
21	4	94	8.00	3.00E+06	0.68
22	3	95	15.00	1.50E+06	0.67
23	4	93	7.50	2.90E+06	0.67
24	4	99	14.50	3.80E+06	0.74
25	3	98	19.00	1.50E+06	0.67
26	3	90	7.50	1.50E+06	0.67
27	1	97	2.00	1.10E+06	
28	3	92	5.50	2.10E+06	0.78

Table 63 Raw data of HEK293.6E shake flask 3 (140 rpm)

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	μ [1/d]
35	3	99		1.43E+06	0.66
36	4		7.2		
37	1	94		1.40E+06	
38	3	98	7.0	1.40E+06	0.65
39	3	96	7.0	1.50E+06	0.67
40	4	96	7.5	2.20E+06	0.60
41	3	97	11.0	1.10E+06	0.57
42	4	96	5.5	2.00E+06	0.58
43	3	98	10.0	1.90E+06	0.75
44	4	96	9.5	4.50E+06	0.78
45	3		22.5		
46	1	92		1.30E+06	
47	3	96	6.5	1.80E+06	0.73
48	3	98	9.0	2.40E+06	0.83
49	4	95	12.0	3.20E+06	0.69
50	3	97	16.0	2.00E+06	0.77
51	4	97	10.0	4.30E+06	0.77
52	3	97	21.5	2.00E+06	0.77

11.2.2 Cultivation of wt3H6-CHO

*Table 64 Raw data of the wt3H6-CHO T25 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	5	58		5.70E+05		6.81	
1	4	69	1.14	7.60E+05	0.10	10.11	1.67
2	3	64	2.00	6.00E+05	0.15	13.10	5.57
3	4	67	2.00	6.50E+05	0.19	11.37	2.66
4	3	66	2.00	4.90E+05	0.14	14.77	7.54
5	4	72	2.00	5.40E+05	0.20	8.78	0.93
6	3	85	2.00	7.40E+05	0.34	12.83	6.04
7	4	86	2.00	7.00E+05	0.16	19.34	6.24
8	7	73	3.50	5.60E+05	0.15	16.14	4.33
9	3	71	2.80	3.80E+05	0.21	13.91	9.68
10	4	75	1.90	4.10E+05	0.18	12.57	4.49
11	3	90	2.05	5.80E+05	0.35	12.36	5.81
12	4	92	2.90	7.80E+05	0.34	19.89	9.17
13	4	91	3.90	6.80E+05	0.31	19.14	8.95
14	3	92	3.40	5.50E+05	0.34	16.50	10.47
15	3	92	2.75	5.30E+05	0.32	15.58	9.44
16	4	93	2.65	8.10E+05	0.35	18.51	7.24

*Table 65 Raw data of the wt3H6-CHO T80 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
13	4	80	3.90	5.20E+05	0.24	16.14	8.24
14	3	88	2.60	5.30E+05	0.32	13.68	7.36
15	3	94	2.65	4.90E+05	0.30	11.77	6.81
16	4	96	2.45	6.60E+05	0.30	13.81	5.84
17	3	94	3.30	4.70E+05	0.28	13.34	9.65
18	4	96	2.35	7.00E+05	0.31	19.84	8.87
19	3	96	3.50	4.50E+05	0.27	18.43	13.80
20	4	90	2.25	6.80E+05	0.31	16.82	5.50
21	4	93	3.40	6.50E+05	0.29	19.91	9.80
22	3	93	3.25	4.80E+05	0.29	11.62	5.72
23	3	95	2.40	4.70E+05	0.28	7.41	2.71
24	3	94	2.35	4.90E+05	0.30	9.27	6.30
25	4	94	2.45	6.80E+05	0.31	8.95	3.29
26	4	95	3.40	7.20E+05	0.32	11.07	5.19
27	3	94	3.60	4.40E+05	0.26	6.92	4.21

*Table 66 Raw data of the wt3H6-CHO T80 roux flask culture using D/H medium (1 * 10^6 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
12	4	77	2.90	4.90E+05	0.22	12.61	6.45
13	4	90	2.45	6.00E+05	0.27	11.57	4.41
14	3	88	3.00	5.30E+05	0.32	9.93	5.98
15	3	93	2.65	5.00E+05	0.31	15.14	11.60
16	4	93		1.30E+06	0.25	14.65	4.45
17	3	83		1.30E+06	0.09	13.41	3.91
18	4	44		1.40E+06	0.08	20.24	4.26

*Table 67 Raw data of the wt3H6-CHO T80 roux flask culture using CD CHO medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
19	3	86	3.50	2.60E+05	0.09	7.97	3.35
20	4	57	1.30	2.20E+05	0.02	9.91	4.51
21	4	55		3.70E+05	0.14	2.42	2.14
22	3	48	1.85	2.80E+05	0.11	2.70	1.95
23	3	49		1.80E+06	0.63	2.93	1.21
24	3	48	2.00	4.60E+05	-0.22	3.13	0.85

*Table 68 Raw data of the wt3H6-CHO 125 mL shake flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
25	4	98	2.45	7.30E+05	0.32	9.75	3.64
26	4	97	3.65	1.10E+06	0.43	13.70	5.22
27	3	96	5.50	7.30E+05	0.43	10.49	6.51
28	4	93	3.65	1.30E+06	0.47	16.26	5.69
29	3	94	6.50	6.60E+05	0.40	12.84	8.95
30	4	95	3.30	8.80E+05	0.37	10.35	3.52
31	3	96	4.40	7.00E+05	0.42	8.07	4.77
32	4	89	3.50	1.80E+06	0.55	14.26	4.10
33	3	94	3.60	7.40E+05	0.13	12.43	4.61
34	4	85	3.70	4.60E+06	0.78	12.06	1.55
35	3	94	23.00	7.80E+05	0.45	7.76	5.66
36	4	94	3.90	9.20E+05	0.38	11.59	5.09
37	3	93	4.60	9.00E+05	0.50	14.72	8.74
38	4	96	4.50	1.90E+06	0.56	12.63	3.10
39	3	91	9.50	6.40E+05	0.39	7.17	5.15

*Table 69 Raw data of the wt3H6-CHO 125 mL shake flask culture using D/H medium (1 * 10^6 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
28	4	95	3.65	8.70E+05	0.37	17.19	7.85
29	3	88		1.90E+06	0.21	27.63	6.57
30	4	77		2.20E+06	0.20	17.28	2.84
31	3	75		1.10E+06	0.03	13.76	4.37
32	4	67		1.70E+06	0.13	25.62	4.86

*Table 70 Raw data of the wt3H6-CHO 125 mL shake flask culture used for record of the growth curve (1 * 10^6 C/mL start cell concentration. D/H medium)*

Days [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-iter [µg/mL]	Qp [pg/c/d]
0	94.5	1.00E+06		0.00	
1	98	1.10E+06	0.10	3.59	3.42
2	93	1.30E+06	0.13	11.75	5.14
3	91	2.70E+06	0.33	15.51	3.02
4	83	2.90E+06	0.27	20.26	2.84

11.2.3 Cultivation of GA3H6-CHO

*Table 71 Raw data of the GA3H6-CHO T25 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	4	60		6.80E+05		0.66	
1	3	89	3.40	6.80E+05	0.41	0.98	0.67
2	4	87	3.40	7.20E+05	0.32	1.31	0.63
3	4	87	3.60	6.00E+05	0.27	2.25	1.30
4	3	86	3.00	5.00E+05	0.31	2.01	1.28
5	4	89	2.50	7.00E+05	0.31	1.81	0.63
6	3	92	3.50	2.30E+05	0.05	1.11	0.91
7	1	93	1.15	4.50E+05	0.81	1.72	2.47
8	3	91	2.25	6.80E+05	0.41	1.42	0.56
9	3	97	3.40	4.60E+05	0.28	0.86	0.47
10	4	95	2.30	9.50E+05	0.39	1.43	0.55
11	3	95	4.75	6.10E+05	0.37	0.98	0.62
12	4	93	3.05	6.60E+05	0.30	1.06	0.48
13	3	97	3.30	3.70E+05	0.21	0.81	0.59
14	4	91	1.85	6.60E+05	0.30	1.24	0.52
15	3	92	3.30	5.10E+05	0.31	1.05	0.68
16	4	94	2.55	8.50E+05	0.36	1.19	0.43
17	3	97	4.25	6.30E+05	0.38	0.67	0.35
18	4	95	3.15	8.90E+05	0.37	1.27	0.57
19	3	99	4.45	6.30E+05	0.38	0.79	0.45
20	4	95	3.15	9.40E+05	0.39	1.29	0.55
21	3	96	4.70	4.70E+05	0.28	0.93	0.69
22	4	95	2.35	9.40E+05	0.39	1.27	0.46
23	3	91	4.70	6.70E+05	0.40	0.97	0.60
24	4	97	3.35	8.90E+05	0.37	1.08	0.43
25	4	98	4.45	8.30E+05	0.36	1.22	0.55
26	3	98	4.15	6.40E+05	0.39	1.02	0.63
27	3	97	3.20	7.00E+05	0.42	0.94	0.52

*Table 72 Raw data of the GA3H6-CHO tube using MV3-2/6 (+ 30%) medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
2	4	80	3.4	4.60E+05	0.21	0.67	0.30
3	4	84	2.3	6.00E+05	0.27	1.18	0.61
4	3	84	3.00	3.00E+05	0.14	0.77	0.50
5	4	62	1.50	2.00E+05	0.00	0.77	
6	3	8.9	1.00	2.10E+05	0.02	0.98	0.33

*Table 732 Raw data of the GA3H6-CHO 125 mL shake flask using CD CHO medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
9	3	93	3.40	1.50E+05	-0.10	0.58	0.31
10	4	98	1.00	4.60E+05	0.28	1.27	0.63
11	3	94	2.30	6.20E+05	0.38	1.48	0.83
12	4	97	3.10	1.00E+06	0.40	2.02	0.77
13	3	94	5.00	4.30E+05	0.26	1.02	0.69
14	4	93	2.15	7.10E+05	0.32	1.70	0.76
15	3	95	3.55	5.80E+05	0.35	0.86	0.36
16	4	93	2.90	6.00E+05	0.27	0.74	0.30
17	3	94	3.00	5.70E+05	0.35	0.63	0.36
18	4	96	2.85	1.10E+06	0.43	1.88	0.79
19	3	92	5.50	1.00E+06	0.54	1.57	0.82
20	4	95	5.00	1.20E+06	0.45	1.52	0.54
21	3	96	6.00	7.60E+05	0.45	1.38	0.89
22	4	96	3.80	9.70E+05	0.39	1.80	0.74
23	3	95	4.85	8.50E+05	0.48	1.62	0.93
24	4	96	4.25	1.10E+06	0.43	2.61	1.05
25	4	98	5.50	1.20E+06	0.45	2.82	1.05
26	3	96	6.00	9.90E+05	0.53	2.55	1.40
27	3	97	4.95	9.30E+05	0.51	1.76	0.87
28	4	96	4.65	2.10E+06	0.59	5.02	1.44
29	3	99	10.50	1.10E+06	0.57	2.69	1.40
30	4	98	5.50	2.40E+06	0.62	5.91	1.53

*Table 74 Raw data of the GA3H6-CHO T80 roux flask culture using D/H medium (1 * 10^6 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
13	3	67	1.03E+06	0.01	1.06	0.35
14	4	32	1.10E+06	0.02	0.82	0.19

*Table 75 Raw data of the GA3H6-CHO 125 mL shake flask using CD CHO medium (1 * 10^6 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
15	3	95	3.55	5.80E+05	0.35	0.86	0.36
16	4	96		2.80E+06	0.26	1.80	0.26
17	3	87		1.60E+06	0.16	1.81	0.47
18	4	95		2.40E+06	0.22	2.49	0.39
19	3	84		1.10E+06	0.03	0.87	0.28
20	4	95		1.50E+06	0.10	2.54	0.52
21	3	96		2.30E+06	0.28	2.33	0.50
22	4	83		3.00E+06	0.27	2.88	0.39
23	3	89		1.40E+06	0.11	1.68	0.47
24	4	91		2.60E+06	0.24	3.57	0.53
25	4	86		2.90E+06	0.27	4.79	0.67
26	3	92		1.90E+06	0.21	2.08	0.49
27	3	87		2.50E+06	0.31	2.34	0.48

11.2.4 Stabilization of recombinant GA3H6-CHO

*Table 76 Raw data of the GA3H6-CHO J20 T25 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
1	3	94	2.00	3.60E+05		2.34	
2	4	98	3.00	5.30E+05	0.37	2.02	1.13
3	4	89	2.00	7.10E+05	0.25	2.43	0.78
4	3	96	3.55	3.50E+05	0.19	1.80	1.39
5	4	95	1.75	6.00E+05	0.27	2.36	0.91
6	3	95	3.00	4.90E+05	0.30	1.56	0.80
7	4	98	2.45	6.20E+05	0.28	2.58	1.31
8	3	98	3.10	5.60E+05	0.34	1.86	0.98
9	4	95	2.80	6.80E+05	0.31	1.22	0.35
10	4	96	3.40	8.00E+05	0.35	2.18	1.05
11	3	98	4.00	5.20E+05	0.32	1.28	0.74

*Table 77 Raw data of the GA3H6-CHO I10 T25 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
1	3	97	2.00	3.20E+05		4.33	3.36
2	4	100	3.00	5.50E+05	0.41	5.07	1.66
3	4	95	2.00	6.60E+05	0.22	5.46	1.76
4	3	98	3.30	5.80E+05	0.35	3.54	3.19
5	4	99	2.90	7.30E+05	0.32	6.45	2.72
6	3	100	3.65	6.10E+05	0.37	4.77	2.01
7	4	100	3.05	1.00E+06	0.40	5.56	3.13
8	3	100	5.00	5.40E+05	0.33	4.33	1.73
9	4	95	2.70	1.02E+06	0.41	5.09	4.66
10	4	98	5.10	5.80E+05	0.27	7.65	1.87
11	3	99	2.90	4.80E+05	0.29	4.43	1.51
12	3	99	2.40	6.80E+05	0.41	3.62	2.71
13	3	100	3.40	6.80E+05	0.41	4.26	2.24
14	4	99	3.40	7.10E+05	0.32	4.86	3.03
15	4	98	3.55	5.40E+05	0.25	5.52	3.76
16	3	96	2.70	4.70E+05	0.28	5.61	3.51
17	4	98	2.35	6.70E+05	0.30	7.85	5.80
18	3	99	3.35	5.80E+05	0.35	8.55	1.36
19	4	99	2.90	1.00E+06	0.40	5.65	1.80
20	3	96	5.00	6.10E+05	0.37	3.11	2.51
21	4	99	3.05	8.90E+05	0.37	5.66	3.36

*Table 78 Raw data of the GA3H6-CHO M5 T25 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
1	3	97	2.00	2.70E+05		1.21	
2	4	97	3.00	4.30E+05	0.39	2.11	1.96
3	4	91	2.00	5.40E+05	0.23	1.22	0.12
4	3	95	2.70	4.40E+05	0.26	1.16	0.77
5	4	98	2.20	5.60E+05	0.26	1.50	0.69
6	3	96	2.80	6.20E+05	0.38	1.19	0.59
7	4	98	3.10	6.10E+05	0.28	1.54	0.79
8	3	96	3.05	4.80E+05	0.29	1.29	0.81
9	4	95	2.40	7.40E+05	0.33	1.97	0.87
10	4	96	3.70	6.10E+05	0.28	1.50	0.66
11	3	92	3.05	5.30E+05	0.32	1.08	0.58

*Table 79 Raw data of the GA3H6-CHO I4 T25 roux flask culture using D/H medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
1	3	92	2.00	2.00E+05		1.34	
2	4	95	2.00	4.20E+05	0.36	2.25	1.77
3	4	90	2.00	4.10E+05	0.17	3.64	2.10
4	3	91	2.05	3.90E+05	0.22	2.85	1.26
5	4	95	1.95	3.80E+05	0.16	2.89	1.27
6	3	92	1.90	3.50E+05	0.19	2.59	1.33
7	4	92	1.75	4.90E+05	0.22	4.29	2.17
8	3	95	2.45	4.30E+05	0.26	2.61	0.95
9	4	95	2.15	5.20E+05	0.24	3.04	1.37
10	4	98	2.60	5.80E+05	0.27	5.98	3.37
11	3	97	2.90	4.20E+05	0.25	2.70	0.72
12	3	99	2.10	5.90E+05	0.36	2.25	0.89
13	3	99	2.95	6.40E+05	0.39	1.86	0.97
14	4	97	3.20	6.20E+05	0.28	2.68	1.42
15	4	98	3.10	6.40E+05	0.29	2.80	1.27
16	3	95	3.20	6.60E+05	0.40	2.90	1.76
17	4	94	3.30	7.70E+05	0.34	5.03	2.45
18	3	92	3.85	4.90E+05	0.30	3.30	2.06
19	4	97	2.45	6.60E+05	0.30	2.77	0.92

*Table 80 Raw data of the GA3H6-CHO I10 125 mL shake flask using CD CHO medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
6	3	97	3.65	4.60E+05	0.28	2.69	2.87
7	4	99	2.30	1.10E+06	0.43	7.26	2.88
8	4	98	5.50	2.30E+06	0.61	13.18	3.45
9	3	95	11.50	1.56E+06	0.68	6.44	2.66
10	4	99	7.80	2.10E+06	0.59	10.03	2.85
11	3	97	10.50	1.60E+06	0.69	5.38	2.19
12	3	99	8.00	1.40E+06	0.65	6.07	2.92
13	3	98	7.00	1.70E+06	0.71	7.09	2.96
14	4	97	8.50	2.60E+06	0.64	10.66	2.62
15	4	97	13.00	2.20E+06	0.60	8.55	2.32
16	3	96	11.00	1.20E+06	0.60	6.98	3.70
17	4	98	6.00	2.30E+06	0.61	14.90	3.99
18	3	98	11.50	1.60E+06	0.69	10.77	4.69
19	4	98	8.00	2.30E+06	0.61	10.41	2.63
20	3	99	11.50	1.30E+06	0.62	7.84	3.94
21	4	99	6.50	2.60E+06	0.64	17.68	4.40
22	3	98	13.00	1.40E+06	0.65	12.02	5.76
23	4	98	7.00	1.70E+06	0.54	13.05	4.04
24	3	99	8.50	1.50E+06	0.67	9.32	4.02
25	4	98	7.50	1.90E+06	0.56	14.17	4.28
26	3	99	9.50	1.50E+06	0.67	13.24	6.07
27	4	96	7.50	2.60E+06	0.64	15.09	3.56
28	3	93	13.00	1.20E+06	0.60	6.44	3.15

*Table 81 Raw data of the GA3H6-CHO I4 125 mL shake flask using CD CHO medium (2 * 10^5 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
10	4	99	2.60	5.60E+05	0.26	2.85	2.04
11	3	98	2.80	6.50E+05	0.39	2.72	1.49
12	3	99	3.25	7.40E+05	0.44	2.75	1.54
13	3	99	3.70	8.00E+05	0.46	2.50	1.36
14	4	99	4.00	9.10E+05	0.38	2.78	1.15
15	4	97	4.55	7.50E+05	0.33	2.03	0.85
16	3	100	3.75	4.40E+05	0.26	1.72	1.29
17	4	93	2.20	4.80E+05	0.22	2.55	1.39
18	3	97	2.40	4.30E+05	0.26	1.50	0.48
19	4	92	2.15	3.70E+05	0.15	1.77	0.97
10	4	99	2.60	5.60E+05	0.26	2.85	2.04
11	3	98	2.80	6.50E+05	0.39	2.72	1.49
12	3	99	3.25	7.40E+05	0.44	2.75	1.54
13	3	99	3.70	8.00E+05	0.46	2.50	1.36
14	4	99	4.00	9.10E+05	0.38	2.78	1.15
15	4	97	4.55	7.50E+05	0.33	2.03	0.85
16	3	100	3.75	4.40E+05	0.26	1.72	1.29
17	4	93	2.20	4.80E+05	0.22	2.55	1.39
18	3	97	2.40	4.30E+05	0.26	1.50	0.48
19	4	92	2.15	3.70E+05	0.15	1.77	0.97

*Table 82 Raw data of the GA3H6-CHO I10 125 mL shake flask using CD CHO medium (1 * 10^6 C/mL start cell concentration)*

Passage	Days [d]	Viability [%]	Passage 1: ...	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
20	3	97	11.50	4.90E+05	0.30	2.81	2.89
21	4	96	2.45	6.90E+05	0.31	4.60	2.18
22	3	97		2.40E+06	0.29	9.82	2.05
23	4	97		3.70E+06	0.33	19.30	2.34
24	3	98		2.70E+06	0.33	13.25	2.58
25	4	96		3.80E+06	0.33	21.86	2.61
26	3	99		2.50E+06	0.31	11.87	2.42
27	4	98		3.30E+06	0.30	22.65	2.94
28	3	96		2.40E+06	0.29	11.87	2.47

11.2.5 Transient gene expression of su3H6

TGE 1

Table 83 Raw data of the su3H6 transient gene expression 1 tube 1

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	91	1.00E+06		0.0	
1	85	1.70E+06	0.53	0.1	0.07
2	92	2.90E+06	0.53	1.1	0.30
3	92	3.40E+06	0.41	4.6	0.78
6	70	3.70E+06	0.22	17.8	1.44
7	64	3.60E+06	0.18	19.3	1.36

Table 84 Raw data of the su3H6 transient gene expression 1 negative control

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	91	1.00E+06		0.0	
1	93	9.00E+05	-0.11	0.0	0.00
2	94	1.80E+06	0.29	0.0	0.00
3	94	4.20E+06	0.48	0.0	0.00
6	72	5.40E+06	0.28	0.0	0.00
7	60	5.70E+06	0.25	0.0	0.00

TGE 2

Table 85 Raw data of the su3H6 transient gene expression 2 tube 1

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	95	1.00E+06		0.0	
1	96	1.50E+06	0.41	0.0	0.00
2	94	2.90E+06	0.53	0.4	0.12
3	96	4.70E+06	0.52	1.6	0.22
6	69	3.90E+06	0.23	10.9	0.86
7	62	4.00E+06	0.20	15.7	1.04

Table 86 Raw data of the su3H6 transient gene expression 2 tube 5

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	95	1.00E+06		0.0	
1	96	1.40E+06	0.34	0.0	0.00
2	94	3.40E+06	0.61	0.3	0.08
3	94	3.20E+06	0.39	1.8	0.32
6	72	4.30E+06	0.24	15.3	1.13
7	61	4.30E+06	0.21	16.2	1.02

Table 87 Raw data of the su3H6 transient gene expression 2 tube 10

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	95	1.00E+06		0.0	
1	94	1.30E+06	0.26	0.0	0.02
2	93	2.40E+06	0.44	0.2	0.07
3	95	4.60E+06	0.51	1.4	0.19
6	70	2.90E+06	0.18	11.7	1.09
7	63	4.50E+06	0.21	11.8	0.72

TGE 3

Table 88 Raw data of the su3H6 transient gene expression 3 tube 1

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	95	1.00E+06		0.0	
2	98	2.50E+06	0.46	0.8	0.23
3	92	2.80E+06	0.34	3.0	0.58
4	88	2.90E+06	0.27	5.8	0.82
7	60	3.20E+06	0.17	9.1	0.69

Table 89 Raw data of the su3H6 transient gene expression 3 tube 5

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	95	1.00E+06		0.0	
2	94	2.30E+06	0.42	0.7	0.23
3	93	2.40E+06	0.29	2.8	0.59
4	86	2.70E+06	0.25	5.3	0.78
7	64	3.00E+06	0.16	8.3	0.65

Table 90 Raw data of the su3H6 transient gene expression 3 tube 10

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	95	1.00E+06		0.0	
2	95	2.40E+06	0.44	1.0	0.32
3	87	2.60E+06	0.32	2.7	0.54
4	86	3.10E+06	0.28	6.2	0.84
7	56	3.20E+06	0.17	10.1	0.76

TGE 4

Table 91 Raw data of the su3H6 transient gene expression 4 tube 1

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	94	1.00E+06		0.0	
1	93	1.70E+06	0.53	0.2	0.12
2	94	2.50E+06	0.46	1.0	0.30
3	92	2.90E+06	0.35	3.6	0.67
6	76	3.10E+06	0.19	26.9	2.41
7	67	3.20E+06	0.17	32.5	2.46

Table 92 Raw data of the su3H6 transient gene expression 4 tube 10

Day [d]	Viability [%]	End-CC [C/ml]	µ [1/d]	End-titer [µg/mL]	Qp [pg/c/d]
0	94	1.00E+06		0.0	
1	92	1.00E+06	0.00	0.1	
2	95	1.40E+06	0.17	0.4	0.16
3	93	1.40E+06	0.11	1.5	0.43
6	75	1.60E+06	0.08	10.3	1.35
7	57	1.40E+06	0.05	14.1	1.69

STATUTORY DECLARATION

I hereby declare that I am the sole author of this work; no assistance other than that permitted has been used and all quotes and concepts taken from unpublished sources, published literature or the internet in wording or in basic content have been identified by footnotes or with precise source citations.

Vienna, September 2017 Martin Nagl