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Production and characterization of IgM antibodies

Master Thesis

submitted by
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Abstract

The production of IgM antibodies which can be used for clinical applications is still in its beginnings and not very common. Compared to the available information about the production of IgG antibodies, the knowledge and experience about the production of IgM antibodies is still very limited.

Therefore, in this master thesis the production of three IgM antibodies (IgM617, IgM012 and IgM012_GL) is compared in a stable expression system (CHO DG44) as well as in a transient expression system (HEK-293-6E). The analysis of the transfected cell lines focuses on batch experiments where the cell growth, the productivity (ELISA) and the polymer distribution (SDS-PAGE) are determined. The highest growth and antibody titer was reached with the IgM617 antibody in stable expression.

In the transient expression form the transfection with different ratios of the heavy chain to light chain and joining chain plasmids of the antibodies is shown. A higher amount of light chain and joining chain plasmid seems to be advantageous for the product titer.

For further analysis the produced antibodies are purified via a two-step chromatography process which includes an affinity and a size exclusion column.

In addition the produced IgM617 antibody was characterized in respect to its binding to gangliosides on cell surfaces (flow cytometry and fluorescence microscopy). Based on the finding that gangliosides are overexpressed on tumour cell surfaces, several tumour cell lines and a negative non-tumorigenic cell line were used to evaluate if the IgM617 antibody can differentiate between cancerous and normal cells and could therefore be used as a cancer cell marker for the detection of circulating tumour cells (CTCs) in human blood.

In a storage experiment with the IgM617 antibody, three storage conditions were tested and the degradation of the antibody over time was shown.

// Zusammenfassung

Die Produktion von IgM Antikörpern, welche in der Klinik verwendet werden können, ist gerade in der Entwicklungsphase und wird noch nicht sehr häufig durchgeführt. Verglichen mit der Information, die für die Produktion der vielfach verwendeten IgG Antikörper vorhanden ist, ist das Wissen und die Erfahrung über die Produktion von IgM Antikörpern noch sehr gering.

Darum behandelt diese Masterarbeit die Produktion von drei verschiedenen IgM Antikörpern (IgM617, IgM012 und IgM012_GL). Die Produktion dieser Antikörper wurde sowohl in einem stabilen Expressionssystem (CHO DG44) als auch in einem transienten Expressionssystem (HEK-293-6E) durchgeführt und dann wurden diese beiden Bedingungen miteinander verglichen. Dafür wurden für beide Bedingungen Batch-Experimente durchgeführt, um anschließend das Zellwachstum, die Produktivität (ELISA) und die Polymer Verteilung (SDS-PAGE) zu bestimmen. Die höchste Wachstumsrate und der höchste Antikörpertiter wurde mit dem IgM617 Antikörper in dem stabilen Expressionssystem erzielt.

In der transienten Expressionsform wurden weitere Transfektionen mit verschiedenen Verhältnissen der Plasmide für schwere zu leichter Kette getestet. Eine größere Menge des Plasmids mit leichter Kette im Vergleich zur Menge des Plasmids mit schwerer Kette scheint dabei den Antikörpertiter zu erhöhen.

Mittels eines zweistufigen Chromatografie Prozesses, welcher eine Affinitätssäule und eine Größenausschlusssäule umfasst, wurde ein Teil der produzierten Antikörper für weiterführende Experimente aufgereinigt.

Zusätzlich wurde dann der aufgereinigte IgM617 Antikörper auf seine Bindung zu Ganglosiden auf Zelloberflächen verschiedener Zelllinien getestet (Durchflusszytometrie und Fluoreszenzmikroskopie). Basierend auf der Erkenntnis, dass bestimmte Ganglioside auf der Zelloberfläche von gewissen Tumorzellen überexprimiert werden, wurden verschiedene Tumorzelllinien und eine negative Kontrollzelllinie verwendet, um zu überprüfen ob der IgM617 Antikörper zwischen Tumorzellen und normalen Zellen unterscheiden kann und somit als Zellmarker für die Detektion von zirkulierenden Tumorzellen im menschlichen Blut verwendet werden kann.

In einem Lagerexperiment wurden verschiedene Lagerkonditionen für den IgM617 Antikörper getestet und die Degradation des Antikörpers über eine längere Zeitperiode gezeigt.

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1. Abbreviations and units

1.1. Abbreviations

Table 1: Abbreviations

BSA	Bovine serum albumin
c/mL	Cells per milliliter
Cat. No.	Catalogue number
CC	Cell concentration
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
CTC	Circulating tumor cell
DAPI	4’, 6’ – diamino - 2 - phenylindol
DHFR	Dihydrofolate reductase
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
EtOH	Ethanol
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FS	Forward scatter
HC	Heavy chain
HEK	Human Embryonic Kidney
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JC	Joining chain
L-gln	L-glutamine
LC	Light chain
MTX	Methothrexate
NGc	N-glycolyl
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PEI	Polyethyleneimine
RNA	Ribonucleic acid
$RO\text{-}H_2O$	Reverse osmosis purified water
rpm	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SS	Side scatter
Std	Standard

TN1	Tryptone N1
TRIS	Tris(hydroxymethyl)-aminomethan
VEGF	Vascular endothelial growth factor
VPA	Valproic acid

1.2. Units

Table 2: Units

°C	Degree Celsius
d	Days
kDa	Kilo-Dalton [kg/ mol]
g	Gram
h	hours
L	Liter
M	Molarity (mol/L)
mA	Milliampere (10^{-3} A)
mg	Milligram (10^{-3} g)
min	Minute
mL	Milliliter (10^{-3} L)
ng	Nanogram (10^{-9} g)
nm	Nanometer (10^{-9} m)
pg	Picogram (10^{-12} g)
s	Seconds
u	Units
V	Volt
W	Watt
µg	Microgram (10^{-6} g)
µL	Microliter (10^{-6} L)

2. Introduction

In this master thesis the recombinant production of three different IgM antibodies in stable and transient expression is compared. While there is a lot of knowledge available about the production of IgG antibodies, the knowledge and experience about the production of IgM antibodies is still very limited as is their use in clinical studies.

There are five classes of Immunoglobulins, called IgA, IgD, IgE, IgG and IgM. IgM is the first class of immunoglobulins that is produced by developing B cells in the bone marrow and is the major class of antibody that is secreted into the blood at early stages of antibody response when the human body is exposed to a foreign antigen (Alberts et al., 2002).

IgM occurs as monomeric, membrane-bound antibody on all naïve B cells and is secreted into the blood as pentamer in the human body (figure 1). The pentamers consist of five identical monomers (molecular mass of approximately 180 kDa), each of which is composed of two heavy chains (μ-chains) and two light chains (κ- or λ-chains) and a joining chain (J-chain). All together a pentameric IgM possesses 10 antigen binding sites. This high valency of IgM antibodies allows them to bind antigens within a wide range of avidities and causes agglutination or clumping, which facilitates the removal of pathogens or foreign antigens. IgM antibodies are 100 to 10,000 times more effective in mediating agglutination than IgG antibodies. Because of their high valency IgM antibodies bind antigens with repeating epitopes such as carbohydrates on pathogens and cell surfaces very efficiently (Racine and Winslow, 2009).
IgM molecules are very efficient in activating the complement cascade of the immune system. When IgM pentamers bind to the surface of a pathogen they are deformed so that they expose binding sites for the C1q which is a part of the first component of the classical pathway of complement activation (Janeway et al., 2001). In lower numbers IgM antibodies also occur as hexamers in the human blood without the J-chain and in other species also other polymer forms are common (Casali, 1998).

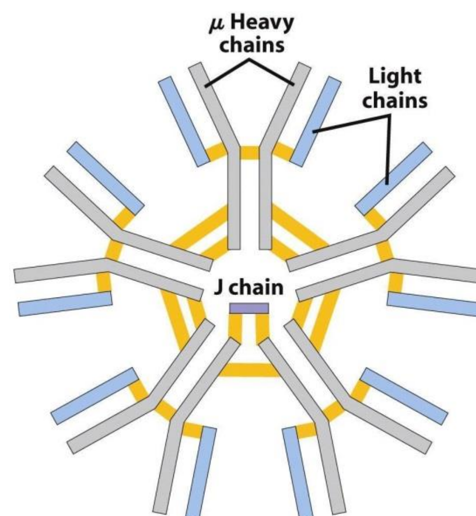


Figure 1: Structure of a pentameric IgM antibody (Lehninger et al., 2013)

The three IgM antibodies compared in this thesis are IgM617 (HB617), IgM012 (2G12) and IgM012_GL (2G12_GL). The IgM617 antibody binds to the gangliosides GM1, GM3 and GD3 which are frequently found on cell surfaces. The IgM617 antibody is produced by the HB617 cell line. This cell line is a transformed B cell line, responsible for activating a certain population of CD4+ cells which is responsible for tumour elimination (Jurisik et al., 2009). The latter two antibodies (IgM012 and IgM012_GL) are anti-HIV-1 antibodies which bind to a carbohydrate motif on the membrane protein gp120 of HI-1 viruses (Kunert et al., 1998). The IgM012 cell line was developed from the IgG2G12 cell line which was class-switched to IgM (Wolbank et al., 2003). The IgM012_GL antibody is a mutant of the IgM012 antibody in which 17 germline residues where introduced in order to make it more robust and improve the stability of the antibody (Chromikova et al., 2014, Chromikova et al., 2015). For all three IgM antibodies stably transformed CHO DG44 cell lines are available. Plasmids containing the genes of HC, LC and JC were transfected into CHO DG44 cells via DNA:poyethyleneiminie polyplexes (Chromikova et al., 2014).

Since it is important to ensure the right folding state and post-translational modifications to make bioactive antibodies, mammalian cell lines were used. The three antibodies are compared in a stable expression system in CHO DG44 cells and in a transient expression system in HEK-293-6E cells.

Stable expression means, that the gene of interest (GOI), in this case the genes for the heavy chain, the light chain and joining chain of the antibody, have been introduced and then permanently integrated into the host cell genome. They are under control of a constitutive promoter. For the generation of the stable cell lines used in these studies, the dihydrofolate reductase (DHFR) expression system was used. CHO DG44 cells lack both copies of the DHFR gene. They are DHFR deficient. The genes encoding the sequence for the HC, LC ad JC of the IgM antibodies were inserted together with the DHFR gene on the same plasmid. Through this approach it is possible to select transfected cells by the presence of the DHFR gene in the transfected CHO DG44 cells. Only successfully transfected cells can synthesize DHFR which is needed for purine and pyrimidine production and can survive in thymidine-lacking medium.

For gene amplification Methotrexate (MTX) is added to the culture medium of the transfected cells. MTX is a folate analogue and is used to inhibit the function of DHFR. Therefore, cell growth is inhibited when MTX is present. Only cells which overproduce DHFR and likely also cotransfected genes, here IgM coding genes, survive. Those cells contain hundreds to a few thousand copies of the introduced plasmid which leads to augmented production of the target protein (Wurm, 2004).

In case of transient expression, the DNA information for the production of the antibodies is transfected into the cell, in this case by means of polyethyleneimine (PEI) which makes holes in the cell membrane and makes it possible for the designed pCEP4 plasmids (figure 2) which harbour HC, LC and JC DNA information for the production of the antibody to enter into the cell. For a high transfection efficiency, the host cells must be in the exponential growth phase with high viability. As in stable transfection, these plasmids enter the cell nucleus but they do not get

integrated into the host genome. They remain episomal plasmids and use the Epstein-Barr virus (EBV) plasmid replication system. However, over the period of a few days the plasmids which have been transfected get diluted through cell divisions or degraded by nucleases. Consequently, the plasmids get lost over time and recombinant protein expression is time limited. Therefore, transient transfection is an easy method for quick generation of the desired product but not a permanent solution for production (Jäger et al., 2015).

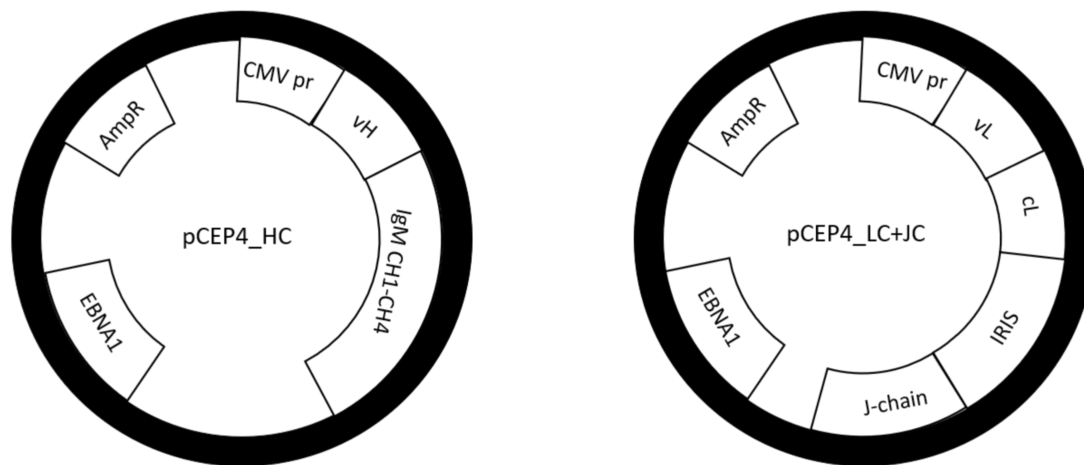


Figure 2: pCEP4 plasmids used for transient transfections

The HEK-293-6E cell line is a Human Embryonic Kidney cell line which was engineered to contain the Epstein-Barr virus nuclear antigen 1 (EBNA1). EBNA1 allows episomal amplification of plasmids which contain the viral EBV origin of replication, as it is the case for the used pCEP4 plasmids. Furthermore, the CMV promoter of the pCEP4 plasmids is highly active in HEK-293 cells because the constitutively expressed adenovirus E1a protein enhances the transcription of the CMV promoter. This ensures high expression rates of the recombinant proteins (Durocher et al., 2002).

The analysis focuses on batch experiments with the stable transformed CHO DG44 cell lines and transiently transfected HEK-293-6E cells. The cell growth is measured by determination of the cell concentration with a ViCell device, the productivity is measured by means of an indirect ELISA assay and the polymer distribution is elucidated by SDS-Page and Western blot analysis.

It is suspected that the polymer distribution plays a factor concerning the application of the antibodies as clinical products in humans. It can be regarded as a quality marker if the produced antibodies are mainly in the pentameric form and only few degradation products like dimers and monomers are contained, since those probably contribute to reduced biological activity. Also, the hexameric IgM form without the joining chain can be produced, however should not be included in clinical products as its precise function is still unknown and it is very reactive. Research has shown that the hexamer antibody activates the complement system of the immune system

about 1,000-fold stronger than the pentamer version. Therefore, it has been suggested that its production is under strict control in the human body, so that it is only produced when an extremely strong complement activation is needed (Petrusić et al., 2011).

The IgM617 antibody can potentially be used as a cancer detection system in human blood, as it targets the gangliosides GM1, GM3, N-glycolyl GM3 (NGcGM3) and GD3 (tested at Polymun Scientific GmbH) which are frequently found on cell surfaces of cancer cells.

Gangliosides are a family of sialic acid-containing glycosphingolipids. They function in several cell-surface events including modulation of growth factor receptors, cell-to-cell interactions and cell-to-matrix interactions (Daniotti et al., 2014). The transformation of normal cells into malignant cells often goes together with elevated expression of gangliosides such as GM2, GD2 and GD3 (Lee et al., 2001). Research showed that for example the gangliosides GD2 and GD3 are highly expressed in melanomas and small cell lung cancer cells (Furukawa et al., 2012). GD2 and GD3 are important gangliosides which play significant roles in tumour formation. They have functions in cell proliferation, migration, invasion, adhesion and angiogenesis of tumour cells (Liu et al., 2018). GD3, for example helps in the adhesion process and promotes malignancy of melanoma cells by recruiting integrins to glycolipid-enriched microdomains (Ohkawa et al., 2010). Moreover, researchers showed that GD3, which was isolated from ovarian cancer-associated ascites prevents innate activation of natural killer T cells (Webb et al., 2012). Furthermore, GD3 can also stimulate the expression of vascular endothelial growth factor (VEGF) and thereby regulate cell proliferation and differentiation (Malison & Testi, 2002). The ganglioside GD2 in turn has been shown to help in the process of attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins and may be involved in neuroblastoma metastasis (Cheresh et al., 1986).

Circulating tumour cells (CTCs) are cells which have detached from the primary tumour site and entered the blood stream. Most of the CTCs disappear within minutes but they also persist in small numbers in the blood stream (about 1 CTC in 1 mL of blood) (Telekoparan Akilliar & Yildiz, 2017). If it is possible to distinguish between cancerous CTCs and normal cells in human blood with IgM617, this antibody can be used to detect and target cancer cells. To establish and test a protocol for the detection of cancer cells with the IgM617 antibody, four tumour cell lines (COR-L88, MDA-MB-231, U87-MG and MEWO) as well as a non-cancerous cell line (HDF5) and a positive control cell line (CHO-K1) were used. The tumour cell lines originate form cancers in different tissues including lung, breast, brain and skin. The human dermal fibroblast primary cell line HDF5 was chosen as non-cancerous cell line to check if the IgM617 also binds to cells which show a normal number of gangliosides on their cell surface but no overexpression of gangliosides. An analysis of primary human fibroblast cells and skin samples with reversed-phase liquid chromatography coupled to mass spectrometry discovered gangliosides on the HDF5 cell surface however those gangliosides were barely quantifiable (Calvano et al. 2019). For this reason, HDF5 can be used as a sample which shows low ganglioside expression in this experiment. The positive control cell line CHO-K1 was

chosen as it is known to express the ganglioside GM3 on the cell surface (Ruggiero et al., 2013). GM3 is one of the gangliosides to which positive IgM617 binding was confirmed by Polymun Scientific GmbH. Therefore, CHO-K1 should show some signal when tested with the assay for fluorescence microscopy and flow cytometry for the detection of gangliosides on cell surfaces.

3. Objectives

This master thesis can be divided into two parts:

The first objective of this thesis is to find out if the expression system and the transfection with different ratios of HC:LC+JC has an impact on the cell growth and cell viability as well as on the titer and polymer distribution of the three produced IgM antibodies. All these mentioned factors are important criteria when it comes to large-scale production of IgM antibodies and their possible use in clinical studies for treatment of human diseases. On the one hand the production process of IgM antibodies still needs to be improved to increase the yield of antibodies to make them available for further large-scale analytical studies and clinical experiments. On the other hand, quality of the produced antibodies is an important factor as IgM can occur in different polymer forms and the correct glycosylation needs to be ensured. Therefore, the polymer distribution is assessed for each antibody, production system and transfection ratio.

The second objective is to design and establish a protocol to test if the IgM617 antibody binds to gangliosides on cell surfaces and if it is possible to distinguish between cancerous and non-cancerous cells with this antibody. Therefore, four tumour cell lines, one non-cancerous cell line and a positive control cell line were chosen to establish a flow cytometry protocol with which the binding of the IgM617 antibody to gangliosides on cell surfaces can be measured. If it is possible to clearly distinguish between cells with elevated gangliosides on their cell surfaces and even sort for those cells, this would be a very helpful tool in cancer detection and a valuable treatment target. In the first line CTCs in the human blood could be detected with this antibody and furthermore, if possible, also isolated. Then the CTCs can be characterized to monitor disease progression and provide targeted and personalized therapy as Telekoparan-Akillar and Yildiz already described this process for circulating breast cancer cells (Telekoparan-Akillar & Yildiz, 2017). A further future goal is to use CTCs to predict sites of future metastases through knowledge of the molecular architecture of isolated CTCs (Paterlini-Bréchot, 2011).

4. Materials and Methods

4.1. Material

4.1.1. Equipment

Equipment	Model
Centrifuge	Thermo Scientific Heraeus Megafuge 16 Centrifuge Thermo Scientific Heraeus Megafuge 40R Centrifuge
Small centrifuge	Eppendorf Centrifuge 5415 R Eppendorf Centrifuge 5428 Eppendorf Centrifuge 5424
Microcentrifuge	Grand-bio PCV-2400
Pipettes	Gilson pipetman® neo P1000N 100-1000 µL Gilson pipetman® neo P200N 20-200 µL Gilson pipetman® neo P100N 10-100 µL Gilson Pipetman® neo P20N 2-20 µL Pipette VWR™ P100-1000 µL Pipette VWR™ P20-200 µL
Multichannel pipette	Thermo Scientific Finnpipette™ F2
ViCell	ViCell™ XR Cell Viability Analyzer, Beckman Coulter™
Microplate Reader	Tecan Infinite® M1000 Pro
Microscope	Leica DM IL LED
Flourescence Microscope	Leica DM IL LED
Incubater	Thermo Scientific MSC Heracell™ 150i CO_2 Incubator
Laminar flow hood	Thermo Scientific MSC-Advantage™
Pipet boy	Pipethelp Accumax Matrix CellMate II® IBS Integra Biosciences Pipetboy acer
Vortex	Vortex-Genie 2
Shaker Incubator	Kuhner Shaker Climo-CHaker ISF1-XC
PCR thermocycler	Bio Rad C1000™ Thermal Cycler
Thermoblock	Eppendorf Thermomixer comfort
Gel electrophoresis chamber	BioRad
Gel electrophoresis power supply	BioRad PowerPac™ Basic Power Supply
Chemiluminiscence blot imaging	Fusion FX7, PEQLAB
Balance	Satorius AW-420
Plate Washer	Tecan 96 Plate Washer™
Shaker Plate	VWR® symphony™ Incubating Microplate Shaker
ÄKTA	ÄKTA™ start, GE Healthcare Biosciences AB, Cat. No. 1873855

NanoDrop	PEQLAB NanoDrop^{TM} 1000
Magnetic Stirrer	VWR^{TM} VS-C4 VWR^{TM} VS-C10
Capture Select Column	Thermo Scientific POROS^{TM} CaptureSelect^{TM} IgM Affinity Matrix Cat.No.195289005
Superose S6 Column	GE Healthcare Superose® 6 Prep Grade Cat.No. 17-0489-01
Flow cytometer	MoFlo Astorios, Beckman Coulter^{TM} CytoFLEX S Flow Analyzer 4L 13 Color, Beckman Coulter^{TM}

4.1.2. Reagents

L-Glutamine	Roth® Roti®-CELL Glutamine-solution Cat. No.9183.1
Phenol red solution	Sigma® Life Science, Cat.No. P0290 0.5% in DPBS
Methothrexate hydrate	Sigma-Aldrich®, Cat. No. M8407
Fetal Bovine Serum (FBS)	Biochrom, Cat. No. S0615
Trypsin	Gibco® by Life technologies Cat. No. 25300-054 0,05 % Trypsin-EDTA
G 418 - BC	Biochrom GmBH Cat. No. A 2912
DAPI-stock solution	4´,6´-Diamino-2-Phenylindol in H_20 (L0009) [5 µg/mL]
TWEEN® 20	Roth® Polyoxyethylene-20-sorbitan monolaurate, Cat.No. 9127.2
Valproic Acid Sodium Salt (VPA)	Sigma-Aldrich®, Cat. No. P4543, 500 mM
Tryptone N1 (20%)	7 g TN1 40 mL Freestyle F17 medium 0.8 mL L-Gln 0.4 mL Kolliphor P188 Cat.No. 9127.2
H_2SO_4	Roth® Sulphuric acide 25% Cat. No 0967.1
BSA	Sigma-Aldrich® Bovine serum albumin Cat.No. A7906-5006
TMB	Invitrogen Stabilized Chromogen TMB Cat. No. SB02
Protein Ladders	Thermo Scientific PageRuler^{TM} Pre-stained Protein Ladder, 10-180 kDa, Cat. No.26619 InvitrogenTM NativeMark^{TM} Protein Standard Cat. No. P/N 57030

Trypan blue	Sigma-Aldrich® Trypan Blue solution 0.4% Cat.No. T8154-100 ML
Formaldehyde 37%	Sigma-Aldrich® Cat. No. 252549
Glutaraldehyde	Sigma-Aldrich® Cat. No. G5882
PEI	Polysciences, Inc. Cat. No. 23966

4.1.3. Disposables

Pipette tips	VWR Pipet tips Cat.No. 53509-070 Greiner bio-one Ultratip Cat. No. 739290 Greiner bio-one Ultratip Cat. No. 740290 Biozym Scientific Tips 250 µL Cat. No. 720310
Serological pipette 2 mL	Costar® Stripette® 4486
Serological pipette 5 mL	Costar® Stripette® 4487
Serological pipette 10 mL	Costar® Stripette® 4488
Serological pipette 25 mL	Costar® Stripette® 4489
Serological pipette 50 mL	Costar® Stripette® 4490
Shaker flasks 125 mL	Corning® 125 mL Erlenmeyer flask
Shaker flasks 500 mL	Corning® 500 mL Erlenmeyer flask
Shaker flasks 1000 mL	Corning® 1000 mL Erlenmeyer flask
Epis	VWR® Micro-centrifuge tubes Cat. No. 211-0015
Plates 6 well	Greiner bio-one CELLSTAR® Cat.No. 657160
FACS tubes	FALCON® A Corning Brand 5 mL Polystyrene Round -Bottom tubes
50 mL tubes	Greiner bio-one Cellstar® tubes Cat. No. 227 261
50 mL Tube reactor	Corning® 50 mL Tube Reactor
T25 flask	VWR® 734-2312 Tissue Culture flask
T80 flask	VWR® 734-2314 Tissue Culture flask
ELISA plate	Thermo Scientific F96 Maxisorp Nunc-immuno plate
Dilution plate	Thermo Scientific 96F without lid sh microwell plate

4.1.4. Kits

Miniprep Kit	VWR PeqLab Plasmid Miniprep Kit I (C-Line)
Midiprep Kit	Machery Nagel NucleoBond Xtra Midi EF
Gel Extraction Kit	VWR PeqLab PeqGOLD DNA Gel Extraction Kit
BCA Assay Kit	QuantiPro™ BCA Assay Kit, Merck

Cat. No. QPBCA-1KT

4.1.5. Software

Plate reader	Magellan 6
Data analysis of flow cytometer	Kaluza 1.2

4.1.6. Chemicals

L-Arginine Monohydrochloride	Roth® C6H14N4O2HCl M = 120.67 g/mol Cat. No. 1689.3
Sodium Chloride	Roth® NaCl M = 58.44 g/mol Cat. No. P029.3
Sodium hydrogen carbonate	Merck $NaHCO_3$, M = 84.01 g/mol Cat. No. 1.06329.1000
Triton X-100	Merck Triton® X-100 Cat. No. 108643
Di-Sodium hydrogen phosphate dehydrate	Roth® Na2HP04· 2H2O M = 177,99 g/mol Cat. No. 49843
Potassium dihydrogen phosphate	Merck KH_2PO_4 M = 136.08 g/mol Cat. No. 104873
Potassium chloride	Roth® KCl M = 74.56 g/mol Cat. No. HN02.3
Sodium carbonate	Roth® Na_2CO_3 M = 105.pp g/mol Cat.No. A135.2
TRIS	Merck $H_2NC(CH_2OH)_3$ M = 121.13 g/mol Cat. No. 1083822500
Ethanol	Merck Emplura® C_2H_6O M = 46.02 g/mol Cat. No. 8.18760.2500
Methanol	Merck M = 32.04 g/mol Cat.No. 1.06007.2500
Isopropanol	Merck M = 50.01 g/mol Cat.No. 1.01040.2500
Acetic Acid	Sigma 99% Cat. No. A6283
Sulphuric acid	Roth® M = 98.08 g/mol Cat. No. 0967.1
Magnesium Chloride hexahydrate	Merck $MgCl_2{\cdot}6H_2O$ M = 203.3 g/mol Cat. No. 1.05833.1000
Milk powder	Roth® Powdered milk Cat.No. T145.2
Sodium thiosulfate pentahydrate	Merck M = 248.21 g/mol Cat. No. 1.06516.0500
Sodium acetate · 3 H_2O	Merck M = 82.03 g/mol Cat. No. 1.06268.1000
Silver nitrate	Merck M = 169.87 g/mol Cat. No. 101510
Kolliphor P 188 (10%)	Sigma-Aldrich® Cat.No. K4894

4.1.7. Buffer

Buffer	Composition
10 x PBS (5 Liter)	57.5 g $Na_2HPO_4 \cdot 2H_2O$
	10 g KH_2PO_4
	10 g KCl
	400 g NaCl
	Fill up to 5000 mL with RO-H_2O
ELISA Coating buffer (500 mL)	4.2 g $NaHCO_3$
	2.1 g Na_2CO_3
	Fill up to 500 mL with RO-H_2O
	pH = 9.5 – 9.8
ELISA Washing buffer (1 L)	100 mL PBS 10 x
	Fill up to 1000 mL with RO-H_2O
	1 mL TWEEN20
ELISA Dilution buffer (100 mL)	1 g BSA
	100 mL Washing buffer
FACS buffer	100 mM TRIS
	0,1 % TRITON
	2 mM $MgCl_2$
Silver Stain Fixation solution	50 % Ethanol / 10 % Acetic Acid in H_2O
Silver Stain Incubation solution	150 mL Ethanol
	1.75 g $Na_2S_2O_3 \cdot 5\ H_2O$
	56.4 g Na-acetate $\cdot$ 3 H_2O
	Filled up to 500 mL with H_2O
	(Add freshly 62.5 µL Glutaraldehyd / 25 mL)
Silver Stain Silver solution	0.25 g $AgNO_3$ in 500 mL H_2O
	(Add freshly 5 µL Formaldehyd / 25 mL)
Silver Stain Develop solution	12.5 g Na_2CO_3 in 500 mL H_20
	(Add freshly 5 µL Formaldehyd / 25 mL)
Silver Stain Stop solution	0.05 M EDTA in H_2O
Capture Select buffer A	1 x PBS (10 x PBS diluted with HQ-H_2O)
Capture Select buffer B	1 M arginine
	2 M $MgCl_2$
	dissolved in HQ-H_2O pH = 3.5
ÄKTA regeneration buffer	0.2 M Na_2CO_3
	dissolved in HQ-H_2O pH = 11
SEC buffer	0,37363 g/L $Na_2HPO_4 \cdot 2H_2O$
	13.2282 g/L $NaH_2PO_4 \cdot H_2O$
	11,688 g/L NaCl
	dissolved in HQ-H_2O pH = 5.5
PBS Dulbecco sterile	Biochrchrom GmbH Cat. No. L1820
TAE 50 x	0.5 M Tris
	Acetic acid
	50 mM EDTA
SDS-Loading Buffer (5x)	250 mM Tris.HCl, pH = 6.8
	10% SDS
	50% glycerine

	0,5% bromphenolblue
Western blot blotting buffer	20 mL NuPAGE® Transfer buffer (20 x)
	100 mL methanole
	ad 500 mL RO-H_2O
RIPA buffer	25 mM Tris
	150 mM NaCl
	0.1 % SDS
	0.5 % sodium deoxycholate
	1 % Triton X-100
	1 tablet Complete Mini, EDTA-free protease inhibitor, Roche

4.1.8. Gels

SDS-Gel	Novex® 2-12% Bis Tris Gel

4.1.9. Antibodies

ELISA coating antibody	Sigma-Aldrich® anti-hu IgM µ-chain specific antibody, Cat. No. I1636
ELISA HRP conjugate 1	Sigma-Aldrich® anti-hu kappa chain HRP antibody Cat. No. A7164
ELISA HRP conjugate 2 (= Western blot HRP conjugate 2)	Thermo scientific anti-hu kappa chain HRP antibody Cat. No. A18853
Western blot HRP conjugate 1	Sigma-Aldrich® Anti-hu µ-chain HRP antibody Cat.No. A0420
FACS FITC conjugate 1	Sigma-Aldrich® Anti-hu kappa-specific FITC antibody Cat. No. F3761-2ML
FACS FITC conjugate 2	Sigma-Aldrich® anti-hu µ-FITC antibody Cat. No. F5384-1ML

4.1.10. Media

ActiPro	GE Healthcare HyClone™ Cat.No. SH31037.01
MV3-2/6 (+30%)	In-house formulation (Master thesis of Mundsperger P.)
ProCHO5	Lonza Bio Wittaker® ProCHO5 Cat. No. BE12766Q
CDM4HEK293E	HyClone™ CDM4HEK293TM Cat. No. SH30858.02
Freestyle F17 Medium	Gibco® Freestyle™ F17 Medium Cat. No. A13835-01

Supplemented media:

Culture Medium 1 IgM617	ActiPro + 4 mM L-Gln + 15 mg/L phenol red + 0.096 µM MTX
Culture Medium 2 IgM617	MV3-2/6 (+30%) + 4 mM L-Gln + 15 mg/L

	phenol red + 0.096 µM MTX
Culture Medium IgM012_2I5 + IgM012_GL	ProCHO5 + 4 mM L-Gln + 15 mg/L phenol red +0.096 µM MTX
HEK culture medium	CDM4HEK293E + 4 mM L-Gln + 15 mg/L phenol red + 1:2500 G418
Transfection medium 1	Freestyle F17 Medium + 8 mM L-Gln + 15 mg/L phenol red
Transfection medium 2	CDM4HEK293E + 8 mM L-Gln + 15 mg/L phenol red
Culture medium U-87 MG + HDF5	DMEM/HAMs F12 1:1 + 4 mM L-Gln + 10% FBS
Culture medium COR-L88 + MDA-MB-231 + MEWO	RPMI 1640 + 4 mM L-Gln + 10% FBS
Culture medium CHO-K1	CD CHO + 4 mM L-Gln + 15 mg/L phenol red

4.2. Methods

4.2.1. Cell culture methods

Passaging of stable cell lines:

The stable CHO-DG44 cell lines of the antibodies IgM617, IgM012 and IgM012_GL were routinely cultivated for the period of 6 months. The cultures were kept in 50 mL tube reactors and passaged every 3-4 days. Before passaging 1 mL of cell suspension was taken from the tubes and the cell concentration was determined on the ViCell device. According to the cell concentration an aliquot of the cells was seeded into the tube reactor and filled up to 15 mL with prewarmed (37°C) medium resulting in a starting cell concentration of $0.3*10^6$ cells/mL in the tube reactor. The cell line of the IgM617 antibody was cultivated in ActiPro medium and the cell lines of IgM012 and IgM012_GL were cultivated in ProCHO5 medium. The tubes were then incubated in a shaker incubator under following conditions: 37°C, 7% CO_2, 85% humidity, 220 rpm;

Host cell line for transient transfections:

For all transient transfections HEK-293-6E cells were used as host cells. Over the period of 6 months the HEK-293-6E culture was kept in 125 mL shaker flasks and passaged every 3-4 days. Prior to passaging 1 mL of cell suspension was taken from the culture for the determination of the cell concentration on the ViCell device. According to the cell concentration cells were seeded into the shaker flask and filled up to 20 mL with prewarmed (37°C) HEK culture medium so that the starting cell concentration was $0.2*10^6$ cells/mL. The shaker flask was incubated in a shaker incubator under following conditions: 37°C, 7% CO_2, 85% humidity, 140 rpm;

4.2.2. PEI transfection of HEK293-6E cells

The HEK-293-6E cell suspension obtained from the routine culture was passaged in the ratio 1:2 with HEK culture medium one day before the transfection to ensure that the cells are in the exponential growth phase at the time of transfection. Prior to the transfection process the viability and cell concentration of the cell suspension was evaluated with the ViCell device. The viability of the cells should be above 95% to result in high transfection efficiency. According to the cell concentration an aliquot of $30*10^6$ cells for each transfection was taken from the cell suspension and centrifuged at 1000 rpm for 10 min. The obtained pellet was then resuspended in 8 mL prewarmed (37°C) transfection medium 1, resulting in a starting cell concentration of $3.75*10^6$ cells/mL for the transfection process. Then the DNA/PEI mixture was prepared in 50 mL tubes. For each transfection two pCEP4 plasmids were used, one carrying the heavy chain of the respective antibody and the other one carrying the light chain and joining chain. An overall amount of 75 µg DNA was used for each DNA solution consisting either of equal amounts of HC and LC+JC or other ratios (see table 3).

Table 3: Amounts of HC and LC+JC plasmid used for different transfection ratios

Ratio HC:LC+JC	HC plasmid [µg]	LC+JC plasmid [µg]
1:1	37.5	37.5
1:2	25	50
2:1	50	25
1:5	15	60
5:1	60	15

To produce the DNA solutions, the respective amounts of HC and LC plasmid shown in table 3 were taken according to the concentrations of the plasmid solutions and then filled up to 250 µL with dH_20. After that, 3500 µL transfection medium (F17 without supplements) were added to the DNA mixtures in 50 mL tubes. The PEI solution consisting of 800 µg PEI (= 800 µL) and 19200 µL transfection medium (F17 without supplements) was prepared in a 50 mL tube. PEI and DNA solutions were incubated 3 minutes each. Then 1.5 mL DNA mixture and 1.5 mL PEI solution were mixed and incubated for 3 minutes. Afterwards in the transfection process 3 mL DNA/PEI mixture (20 µg PEI and 10 µg DNA per ml) were added to each tube reactor containing 8 mL HEK-239-6E cell suspension dropwise and under constant shaking. This resulted in the transfection of 10^6 HEK cells with 0.3 µg DNA, the ratio of PEI:DNA was 2:1 and the starting cell concentration in the tubes was $3.75*10^6$ cells/mL. The tubes were incubated for four hours on the shaker incubator (37°C, 7% CO_2, 85% humidity, 220 rpm). After this incubation time the tube reactors were filled up to a final volume of 30 mL with prewarmed (37°C) Trafe 2 medium resulting in a cell concentration of $1*10^6$ cells/mL in the tube reactors.

To ensure a high product production the transfected cells were supplemented 48 h post-transfection with 0.3 mL of 500 mM VPA and 0.75 mL of 20% TN1 so that each reaction tube contained 5 mM VPA and 0.5 % TN1. Sampling was performed daily for determination of the cell concentration and the viability on the ViCell. On day 2 post-transfection an additional sample of 1 mL was taken for flow cytometry analysis. The tubes were cultivated until the viability dropped below 60%. Then the tubes were centrifuged at 1000 rpm for 10 min and the supernatant, containing the produced antibodies was collected.

4.2.3. Batch experiments

For the batch experiments with the stable cell lines, $30*10^6$ cells were seeded in 50 mL tube reactors and filled up to a volume of 30 mL with fresh medium to reach a cell concentration of 10^6 cells/mL. The tube reactors were incubated in a shaker incubator (37°C, 7% CO_2, 85% humidity, 220 rpm). Daily sampling for determination of cell concentration and viability and antibody titer was performed. The cultures were harvested when the viability dropped below 60%. Then the tubes were centrifuged at 1000 rpm for 10 min and the supernatant containing the produced antibodies was collected for further analysis.

Calculation of growth rate: $\mu = \frac{\ln(CCend) - \ln(CCstart)}{days}$

Calculation of specific productivity: $qP = \frac{(titer\ end - titer\ start) * \mu}{(CC\ end - CC\ start)}$

4.2.4. Measurement of cell concentration and viability

For the determination of the cell concentration and viability a sampling volume of 1 mL was taken from the cell suspension. After mixing 700 µL were applied in the containers for the ViCell device and measured with the programme CHO DG44 for the stable cell lines and HEK-293-6E for the transient cell lines.

4.2.5. Quantification of antibody titer

For the determination of the antibody titer a cell suspension sample of 1 mL was taken daily from the batch experiment tubes and centrifuged for 10 min with 1000 rpm and stored at 4°C until the day of ELISA analysis. The ELISA plate was coated with 100µl/well of 1:3000 diluted coating antibody in coating buffer. Wells were incubated either at 4°C overnight or at room temperature for 2 h on the plate shaker. The dilution plate was prepared with a minimal dilution of 1:4 of the culture supernatants. The supernatant samples were diluted according to the estimated titer on the respective day. The diluted cell supernatants were applied in the row H of the dilution plate. In the well H1 of the dilution plate a blank in form of dilution buffer was applied and, in the wells, H2 and H3 204 µL of an in-house produced IgM standard with the concentration of 200 ng/mL IgM were applied. The remaining wells of the plate were filled with 150 µL dilution buffer. Then the dilution plate was used to dilute the contents of all wells in row H 1:2 according to the dilution scheme (table 4).

Table 4: Dilution scheme for ELISA samples

	1	2	3	4	5	6	7	8	9	10	11	12
A		1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128
B		1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
C		1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
D		1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
E		1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
F		1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
G		1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2
H												
	Blank	Std.	Std.	Samples								

In the next step the coated ELISA plate was washed with the plate washer which has been connected to a bottle of washing buffer and knocked out to dry the wells. Then 50 µL of all wells of the dilution plate were transferred onto the ELISA plate starting with row A, which is the row with the highest dilution factor. In this way only one set of pipette tips for the multichannel pipette was used for the transfer process since the

samples were pipetted in ascending order according to their IgM concentration. Afterwards the plate was incubated for 1 h at the plate shaker at room temperature. After the incubation step, the plate was washed again with the plate washer and knocked out. The conjugation antibody was applied in a dilution of 1:2000 in dilution buffer. 50 µL diluted conjugation antibody were applied per well and the plate was incubated again for 1 h on the plate shaker at room temperature. Then the plate was washed again with the plate washer and knocked out. After that, the plate was stained with 100 µL TMB substrate starting with row A. Subsequently the chromogenic reaction was stopped by adding 100 µL sulphuric acid to the wells, again starting with row A. After the staining procedure the plate was measured in the plate reader at a measurement wavelength of 450 nm and a reference wavelength of 620 nm. The concentrations were calculated with MAGELLAN software.

4.2.6. SDS-PAGE

For the SDS-PAGE the culture supernatant samples, and the cell samples which were prior to that treated with RIPA buffer as described in 4.2.9. were diluted so that the applied volume per lane of the gel contained approximately the amount of 200 ng IgM. For samples of the transfections and batch experiments 24 µL of sample were mixed with 6 µL of 5 x SDS loading buffer and for samples of the storage experiment 8 µL of sample and 2 µL of 5 x SDS loading buffer were mixed. 800 mL of Tris-Acetate SDS running buffer were prepared. The gel was put in the gel apparatus and the chamber of the apparatus was filled with running buffer. Then after removal of the comb the prepared mixtures of sample and SDS loading butter were loaded onto the gel. In the first lane 15 µL of the pre-stained protein marker were loaded if the gel was subsequently used for a Western blot and 5 µL of unstained protein marker were loaded if the gel was afterwards used for silver staining. The gel chamber was then attached to the power supply and the gel was run at 200 V for 1 h. Then the gel was removed from the chamber and shortly rinsed with RO-H_2O.

4.2.7. Western blot

For the Western blot 500 mL of transfer buffer, a membrane and 2 filter papers in the right size were prepared. Sponges and filter papers were shortly equilibrated in transfer buffer prior to use. The gel was removed from the gel cassette and one piece of filter paper was carefully placed on the gel. Then the membrane was shortly activated in methanol and placed on the other side of the gel and the second piece of filter paper was placed above the membrane. This process was performed without touching the gel with the hands and trying to avoid entrained air bubbles between the gel, membrane and filter papers. Two equilibrated sponges were put in the blotting apparatus followed by the pile of filter paper, gel, membrane and filter paper. Above this pile another two equilibrated sponges were placed and the apparatus was closed with the lid and quickly put in the running cassette. The blotting cassette was then filled with transfer buffer and the outer part of the running cassette was filled with RO-H_2O. After this the blotting apparatus was attached to the power supply and

the blotting procedure was carried out with the conditions 25V, 125 mA, 17 W for 1 h. After the blotting procedure the membrane was taken out of the cassette and blocked in 3 % milk powder in 0.1 % PBST solution for 1 h at room temperature on a shaker platform at 100 rpm or at 4 °C over night. Then the membrane was incubated with the detection antibody (μ-chain HRP-conjugate or κ-chain HRP-conjugate) for 1h at 100 rpm on a shaker platform. The membrane was washed three times for 10 min in PBST solution at 100 rpm. Then the membrane was dried on cellulose paper without laying the membrane onto it. The membrane was put in a plastic foil and 0.8 mL ECL reagent were pipetted onto it followed by a 5 min incubation step in the dark. After that the bands on the membrane could be visualized with the Fusion chemiluminescence system.

4.2.8. Silver Staining

For silver staining the gel was taken out of the gel cassette and incubated in fixation solution for 1 h. All incubation steps were carried out under shaking on a shaker platform at 100 rpm. Then the fixation solution was replaced by incubation solution and the gel was shaken for 20 min. After that the gel was washed three times for 5 min in RO-H_2O. Then the gel was incubated for 15 min in silver solution and washed shortly with RO-H_2O. The gel was incubated with develop solution until the protein bands were visible and washed again shortly with RO-H_2O. As a final step the gel was incubated in stop solution for at least 15 min.

4.2.9. Determination of total protein content

4.2.9.1. Cell sampling

A cell suspension of $2*10^6$ cells of the respective sample was centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and the cell pellet was washed with 1 mL of PBS buffer (4°C). After another washing step the cells were shock-frozen in liquid nitrogen and could be kept in a -80°C freezer until analysis.

4.2.9.2. Cell lysis

The thawed cell pellet was resuspended in 0.4 mL RIPA buffer and incubated for 15 min at 4 °C. After the incubation the suspension was centrifuged at 8000 x g for 10 min at 4°C. The supernatant which contained the cell lysate was then transferred into a separate reaction tube.

4.2.9.3. Determination of protein content

For the determination of the total protein content a BCA Assay Kit was used. The QuantiPro working reagents of the kit were mixed in the ratio 25 parts Buffer A + 25 parts Buffer B + 1 part 4% Copper (II) sulphate pentahydrate solution. For the standard series of BSA, dilutions with the concentrations 30, 20, 10, 5 and 0.5 μg/mL were prepared. The samples were diluted 1:200 before 150 μL of diluted sample and 150 μL of QuantiPro working reagents mixture were mixed. The samples were then incubated for 1 h at 60°C in a thermoblock. After cooling of samples, the absorption

at 562 nm wavelength was measured on the plate reader and the concentrations were calculated manually with the help of the calibration curve obtained by the BSA dilutions and a blank value.

4.2.10. IgM Purification (ÄKTA)

Before use the ÄKTA system was rinsed with ddH_2O. After use the system was rinsed with ddH_2O again and stored in 20% EtOH.

1st step: Capture Select IgM affinity matrix column:

Capture Select buffer A and buffer B were filtered with a 0.2 µm filter and attached to the system. The capture select column for affinity chromatography was installed in the system and equilibrated with capture select buffer A. The harvested supernatant of the respective antibody was filtered with a 0.2 µm filter and then injected into the system via a sample loop. Elution of the IgM antibodies was started by setting the pump to 100% capture select buffer B. It was carried out with a flow rate of 0.5 mL/min and could be observed through A280 measurement. IgM was eluted and the eluate was neutralized immediately with 1M Tris-HCl (pH 8.5) and stored at 4°C until the second chromatography step. The capture select column was flushed with regeneration buffer, then rinsed with ddH_2O and stored in 20%EtOH.

2nd step: Superose S6 column:

The SEC buffer was filtered with a 0.2 µm filter and attached to the system. The superpose S6 column was installed in the system and equilibrated with SEC buffer. The eluate from the previous chromatography step was injected into the system with a syringe and loaded onto the column. Elution was started with a flow rate of 0.5 mL/min and monitored via A280 measurement. The eluted antibodies were collected in fractions of 2 mL. Through measurement of A280 on Nanodrop, the fractions which contained antibodies were pooled. The S6 column was rinsed with ddH_20 and consecutively stored in 20% EtOH.

4.2.11. Spectrophotometric determination of antibody concentration

The determination of the antibody standards used for the SDS gels and ELISA was determined spectrophotometrically. The purified antibody solution was filled into a cuvette and its absorption was measured at a wavelength of 280 nm and 320 nm in the spectrophotometer located in Muthgasse II (working group Obinger). The A280 value was corrected by subtracting the A320 value. To calculate the concentration of the sample the Lambert-Beer law was used. This law describes the correlation between absorption and concentration of a solution which contains a protein.

Lambert-Beer law: $A = \varepsilon * c * L$

Calculation of the concentration: $c = \frac{A}{\varepsilon * L}$

A…absorption of the sample (unitless), ε…molar extinction coefficient [$M^{-1}cm^{-1}$], c…concentration of the protein [M], L…light pathlength [cm]

The light pathlength is 1 cm which is the length of the cuvette. The molar extinction coefficient of the antibody was calculated theoretically by entering the primary amino acid sequence into the ProtParam™ tool (web.expasy.org/protparam). HC, LC and JC were entered separately into the tool and then the values of those building blocks were multiplied in order to obtain the value for the IgM pentamer since purification results in mainly the pentameric form of the antibody. The obtained ε value for the IgM617 pentamer is 1228915 $M^{-1}cm^{-1}$. The molecular weight of the IgM617 pentamer was as well calculated with the ProtParam™ tool and was 892,741.59 g/mol. Glycosylation of the antibody was not considered in the value for the molecular weight.

4.2.12. Storage experiment

For the storage test aliquots of 1 mL of purified IgM617 antibody were stored at 4°C, -20°C and -80°C for 1 month and for 3 months. The antibody was produced in stable expression, a concentration of 450µg/mL was measured after purification and was stored in 0.1 M Na_2PO_4 and 0.2 M NaCl (pH 5.5). After the respective time the antibody titer was determined by ELISA measurement and the polymer distribution was assessed by SDS-PAGE and Western blotting.

4.2.13. Cultivation of cell lines for ganglioside determination

4.2.13.1. Cultivation of tumour cell lines

The U-87 MG (glioblastoma), COR-L88 (lung cancer), MDA-MB-231 (breast cancer) and MEWO (skin cancer) cell line were obtained from Polymun Scientific GmbH in T25 flasks in passage one. The cells are adherently growing and were routinely cultivated in T25 flasks. For bigger experiments the cells were split into a T25 flask for routine culture and a T80 flask for the experiment. The cells were passaged every 3 to 4 days. Therefore, the cells were detached from the flask bottom with Trypsin/EDTA (0.05/0.02%) solution and an aliquot of the cell suspension was transferred into a new T25/T80 flask according to the confluency seen under the microscope before the process of detaching the cells. Then the bottom of the T25/T80 flask was filled with prewarmed (37°C) fresh medium (about 10 mL for T25 flask and 30 mL for T80 flask). The Cells were incubated static in an incubator at 37°C and 7% CO_2.Table 5 shows the media and passage ratios for each cell line.

Table 5: Media and passage ratios for tumour cell lines

Cell line	Medium	Passage ratio
U-87 MG	DMEM/HAMs F12 1:1 + 4 mM L-Gln + 10 % FBS	1:2, 1:3 or 1:4
COR-L88	RPMI 1640 + 4 mM L-Gln + 10 % FBS	1:2 or 1:3
MDA-MB-231	RPMI 1640 + 4 mM L-Gln + 10 % FBS	1:2, 1:3 or 1:4
MEWO	RPMI 1640 + 4 mM L-Gln + 10 % FBS	1:4 or 1:5

4.2.13.2. Cultivation of CHO-K1 cell line

The CHO-K1 cells were cultivated in suspension culture in 50 mL tube reactors. The medium for the CHO-K1 cells was CD CHO with 4 mM L-Gln and 15 mg/L phenol red. The cells were passaged every 3-4 days with a seeding cell concentration of $2*10^5$ cells/mL. Before passaging 1 mL of cell suspension was taken from the tubes and the cell concentration was determined on the ViCell device. According to the cell concentration an aliquot of the cells was seeded into the new tube reactor and filled up to a volume of 15 mL with prewarmed (37°C) fresh medium. The tubes were incubated in a shaker incubator with following conditions: 37°C, 7% CO_2, 85% humidity, 220 rpm;

4.2.13.3. Cultivation of HDF5 cell line

The HDF5 cell line was obtained as a deep-frozen ampulla from AG Grillari. The cells were thawn and then routinely cultivated in T25 and later in T80 flasks. The cell line is adherently growing. For passaging, the cells were detached from the bottom of the flask with Trypsin/EDTA (0.05/0.02%) solution and an aliquot of the cell suspension was passaged into a new T25/T80 flask in the ratio 1:2. Then the bottom of the flask was filled up with fresh, prewarmed (37°C) (DMEM/HAMsF12 1:1 (+ 4 mM L-Gln + 10% FBS) medium. The cells were static incubated in an incubator at 37°C and 7% CO_2.

4.2.14. Cell surface staining for fluorescence microscopy

For the fluorescence microscopy experiment the tumour cell lines U-87 MG and MDA-MB-231 were chosen since they adapted very quickly to the growth conditions and showed good growth characteristics in routine culture. As positive control CHO-K1 was used. Cells of the three different cell lines were seeded into 6-well plates with their respective medium (see 4.2.13) and grown in an incubator at 37°C and 7%CO_2 until they were about 80% confluent. To be able to grow the CHO-K1 cell line adherently 10 % FCS was added to its medium. The staining procedure was also performed in these 6 well plates. First, the cell culture medium was sucked off with a pipette at the side of the well. Then the following washing step was performed two times: 4 mL of PBS were added to each well, the plate was shortly and carefully shaken by hand before the liquid was sucked off again with a pipette at the side of the well. Then 4 mL of PBS/20% FBS were added to each well to block unspecific binding sites and the plate was incubated at 4°C in the dark for 30 min. Afterwards

the supernatant was discarded and 1 mL of purified IgM617 with concentration of 50 µg/ml diluted in PBS/20 % FBS were added to each well. The plate was again incubated for 30 min at 4°C in the dark. Afterwards the supernatant was discarded, and the above-mentioned washing step was repeated. In the next step 1 mL of anti-human µ-chain specific FITC antibody, diluted 1:200 in PBS/20% FBS was added to the wells and the plate was incubated for 30 min at 4°C in the dark. The supernatant was sucked off afterwards and the washing step was performed again. Then one drop (20 µL) of 50 % Glycerine in PBS was pipetted onto the middle of every well and a cover glass was placed above it. For the time until the plates were placed under the microscope they were stored in the dark.

4.2.15. Flow cytometry

4.2.13.1 Intracellular IgM detection by flow cytometry

The transiently transfected cell samples for flow cytometry analysis were taken on day two post-transfection. An aliquot of the sample cell suspension containing 10^6 cells was centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and the cell pellet was shaken until it spread. Then 1 mL of 70 % ice cold ethanol was added to the pellet. All pipetting steps for flow cytometry analysis were carried out dropwise and under constant mixing on a vortex to prevent clump formation. The sample was then stored at 4°C for at least 20 min. The ethanol fixed sample was centrifuged at 1000 rpm for 10 min and the pellet was washed by pipetting 1 mL of FACS buffer dropwise and under constant mixing onto the pellet and was then centrifuged again for 10 min with 1000 rpm before the supernatant was discarded. Then 100 µL FACS buffer/20 % FBS were pipetted to the sample to saturate unspecific binding sites and the sample was incubated 30 min at 37 °C. After that 100 µL of anti-human µ FITC antibody and anti-human κ FITC antibody, both diluted 1:50 in FACS buffer/ 20 % FBS, were added to the sample. The sample was then incubated again for 30 min at 37 °C. After this step the sample was centrifuged again 10 min at 1000 rpm and washed as mentioned above with FACS buffer. Then the pellet was re-suspended in 200 µL FACS buffer with DAPI, diluted 1:100 in buffer. As negative control and for adjusting the measurement parameters of the flow cytometer an equally treated sample of the host cell line HEK-239-6E was used.

4.2.13.2 Detection of gangliosides on cell surfaces by flow cytometry

Since one of the objectives of this thesis was to establish a flow cytometry protocol to measure the binding of IgM617 to gangliosides on the cell surfaces of cancer cells, the improvement of this protocol was a continuous process and only the final protocol can be found here in the methods part. The initial protocol which was used for the first try of this flow cytometry experiment can be found in 9.1 in the appendix.

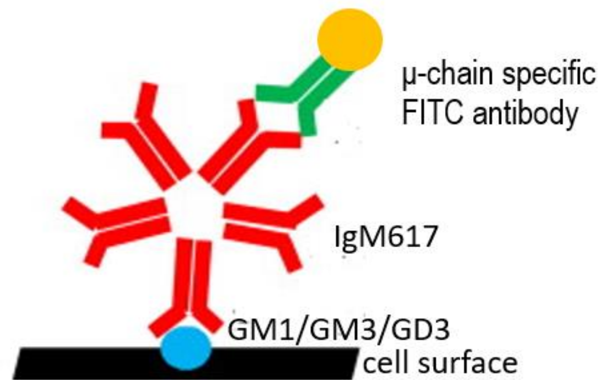


Figure 3: Principle of antibody assay for the detection of gangliosides on cell surfaces of cancer cells with flow cytometry

For the experiments with the tumour cell lines it was essential to keep the cell membrane intact to be able to detect gangliosides on the cell surface. So, a great focus was put on keeping the cells alive and membranes intact during the whole staining procedure for flow cytometry.

First, the adherently growing tumour cells and HDF5 cells were trypsinized with Trypsin/EDTA (0.05/0.02%) solution to loosen them from the cell culture flask. Then the cells were resuspended in their respective cell culture medium and FBS to stop trypsin activity. After this, the cell concentration and viability of this cell suspension was assessed on the ViCell device. In the next step an aliquot of 10^6 cells of each cell suspension was taken and centrifuged for 5 min at 1000 rpm and 4°C. Then the cells were washed with 1 mL PBS-buffer, centrifuged for 5 min at 1000 rpm and 4°C. All pipetting steps for the flow cytometry analysis were carried out dropwise and under constant mixing on a vortex to prevent clump formation. After washing 100 µL PBS-buffer/20% FBS were pipetted onto the cell pellet and the sample was incubated 30 min at 4°C in the dark on a rotor to constantly mix the sample. After this step 100 µL PBS-buffer/20% FBS with the purified IgM617 antibody in the concentration of 100 µg/mL were added to the sample and the sample was again incubated for 30 min at 4°C in the dark on a rotor. Then the sample was centrifuged, 5 min, 1000 rpm, 4°C, and the supernatant was taken off and discarded. After that the cell pellet was washed in 100 µL PBS-buffer/20% FBS as mentioned above. For the next incubation for 30 min at 4°C in the dark, 100 µL PBS-buffer/20%FBS with antibody anti-human µ FITC in the dilution 1:80 was added. This step was then followed by another washing step and in the last step of the staining procedure the pellet was re-suspended in 200 µL PBS buffer. The samples were then measured as soon as possible on the flow cytometer to keep the cells alive and avoid bleaching of the FITC antibody. Furthermore, the samples were kept on ice and in the dark as long as possible during the whole procedure and until measurement.

As all cell lines used for this experiment had different measurement parameters (different forward scatter (FS) and side scatter (SS) behaviour) as well as different autofluorescence on the flow cytometer, for each tumour cell line a separate

negative control was prepared to set a threshold for fluorescence signal which is due to binding of IgM617 to gangliosides the cell surface. The negative control of each cell line was treated identically to the sample of the respective tumour cell line with the difference that for the second incubation step 100 µL of PBS-buffer/20 % FBS were added without addition of the IgM617 antibody. With the help of this negative control of every cell line it was possible to identify which part of the fluorescence was due to binding of the IgM617 to gangliosides and which amount of the fluorescence was due to autofluorescence, unspecific binding of the FITC-conjugated antibody or FITC conjugate which was eventually not entirely removed after the last washing step.

Table 6: Overview of used cell lines for testing if IgM617 can distinguish between cancerous and non-cancerous cells

Cell line:	Description:	Use:
COR-L88	Lung cancer	Sample
MDA-MB-231	Breast cancer	Sample
U87-MG	Glioblastoma	Sample
MEWO	Skin cancer	Sample
HDF5	Human dermal fibroblasts	Sample
CHO-K1	Chinese hamster ovary	Positive control

5. Results

5.1. Testing different HC:LC+JC ratios in transient transfection

This chapter shows the transient transfection of HEK 293-6E cells with different ratios of HC:LC+JC of the antibodies IgM617 and IgM012. The purpose of this experiment was to see if different ratios yield in different antibody production and perhaps also a different polymer distribution of the produced antibodies.

In the following figures the flow cytometry analysis of cell samples of the transient transfections with different HC:LC+JC ratios is shown. The samples were taken on day 2 post-transfection. Each transfection ratio for the antibodies IgM012 and IgM617 was performed in duplets. The first number in the ratio stands for the amount of HC plasmid and the second number in the ratio stands for the amount of LC+JC plasmid used in the transfection process. Everything which is under the "#1" gate in the figures 4-7 is positive for the respective HC or LC. The more the peaks of the cell samples are shifted to the right in comparison to the control peak of the untransfected host cell line in red, the more fluorescence they show and the more of the respective antibody chain they contain.

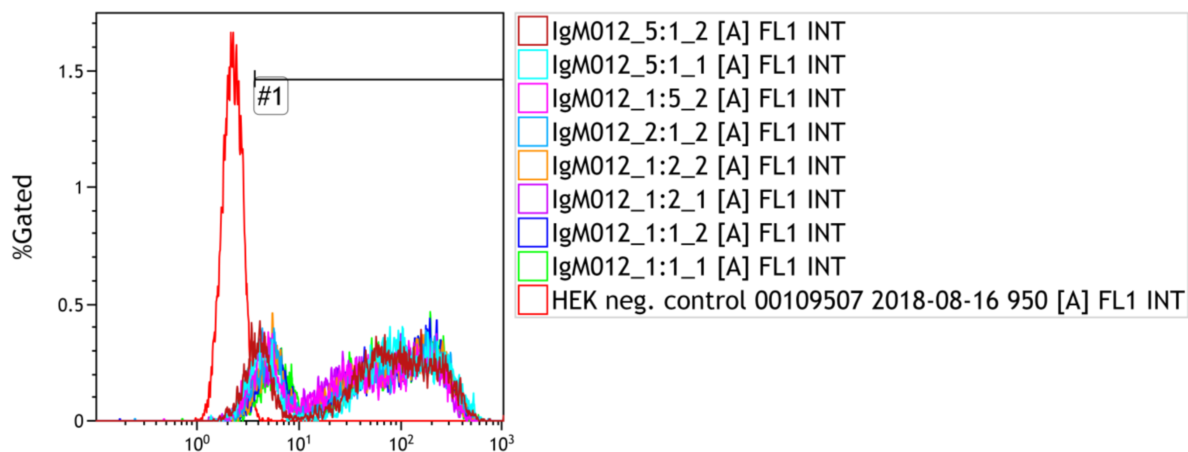


Figure 4: Intracellular HC of the IgM012 cell samples analysed by flow cytometry (µ - FITC antibody)

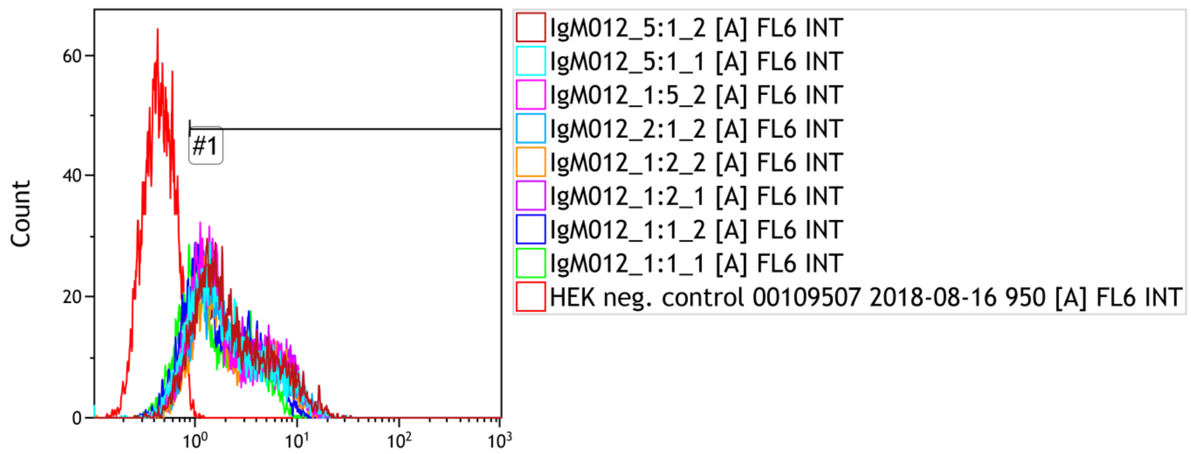


Figure 5: Intracellular LC of the IgM012 cell samples analysed by flow cytometry (κ - Biotin + Alexa-Fluor-647-Streptavidin antibodies)

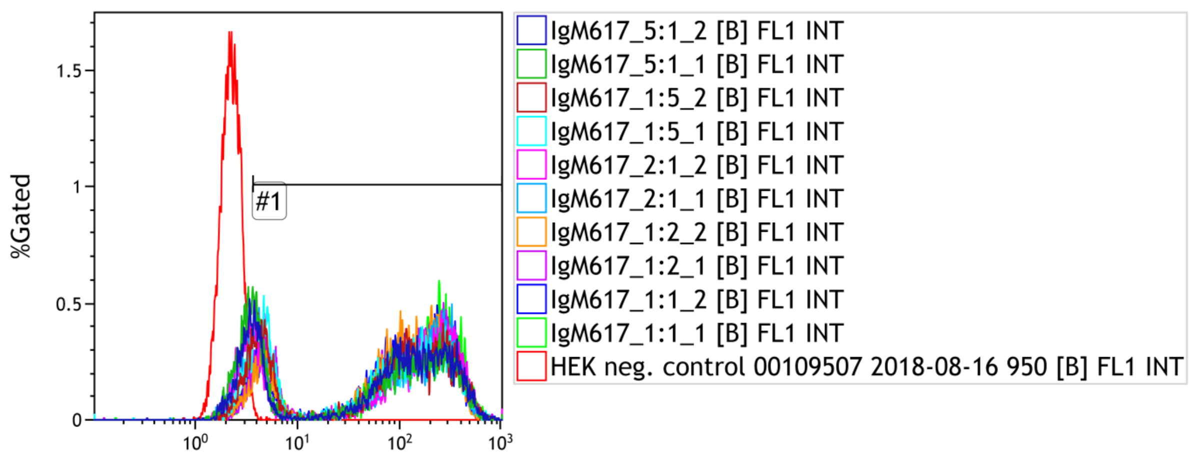


Figure 6: Intracellular HC of the IgM617 cell samples analysed by flow cytometry (µ - FITC antibody)

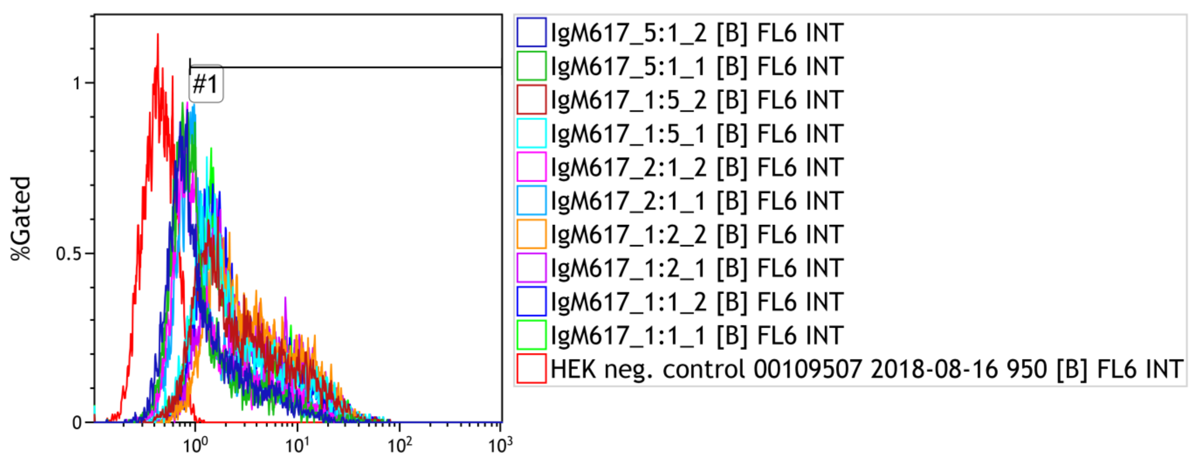


Figure 7: Intracellular LC of the IgM617 cell samples analysed by flow cytometry (κ - Biotin + Alexa-Fluor-647-Streptavidin antibodies)

The analysis of intracellular HC and LC proves that the plasmids have been successfully transfected into the HEK293-6E cells as the peaks for the transfected cells are right of the host cell control sample which is shown in red in each diagram. In comparison to the transfections in experiment 5.2 more cells remained untransfected which can be seen under the host cell control peak. Furthermore, the intracellular light chain peak of both, IgM012 and IgM617, shows an unexpected shape especially for the IgM617 LC which suggests the conclusion that there possibly went something wrong with the transfection of the LC+JC IgM617 plasmid, or that this plasmid has been degraded.

Growth curves and antibody titer curves for the transfection of both antibodies with all transfection ratios were generated by daily sampling and determination of cell concentration and antibody titer. For better visibility the mean value of the transfection duplets is shown in figure 8.

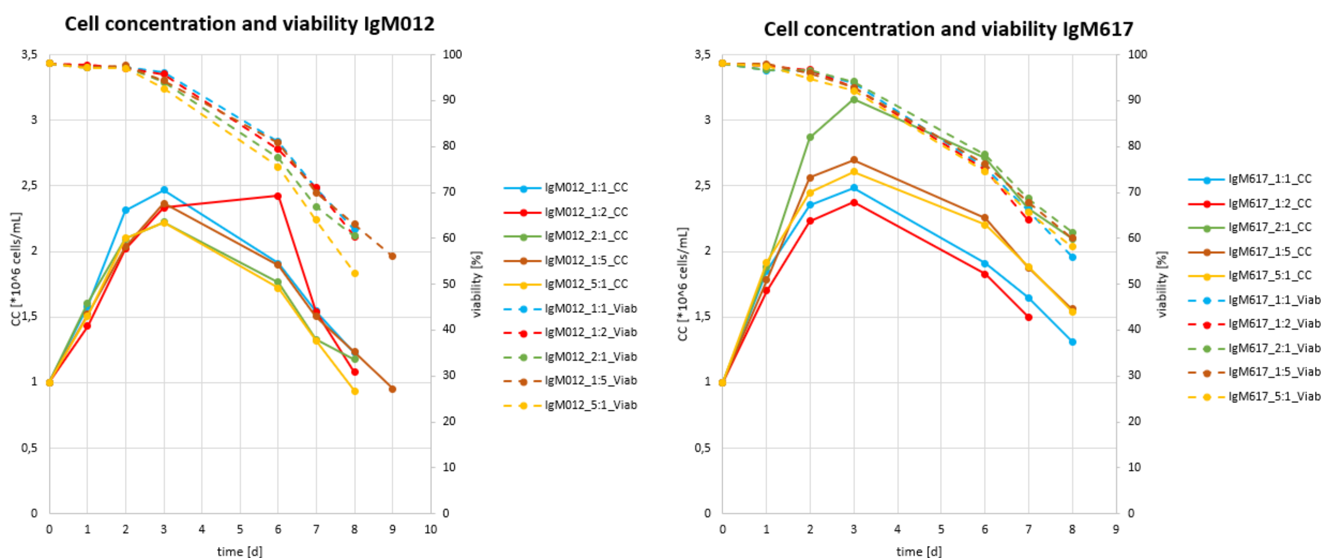


Figure 8: Cell concentration and viability of transfections with different IgM012 and IgM617 HC:LC+JC ratios

The transient transfections with different ratios of HC to LC+JC yielded in peak cell concentrations of 2.2 - 2.5*10^6 cells per mL for the IgM012 antibody and cell concentrations of 2.4 - 3.2*10^6 cells per mL for the IgM617 antibody. Concerning the cell concentration and viability no big differences between the different transfection ratios could be observed. In comparison to the transient transfection performed in 5.2. the maximal cell concentrations in the transfections with different HC: LC+JC ratios were here observed on day 3 and in the transient transfection in 5.2 they were observed on day 5.

The culture supernatants of every sampling day were measured with ELISA to determine the antibody titers (figure 9) and see which transfection ratios resulted in an improved product titer and which were lower than the common 1:1 ratio of HC:LC+JC.

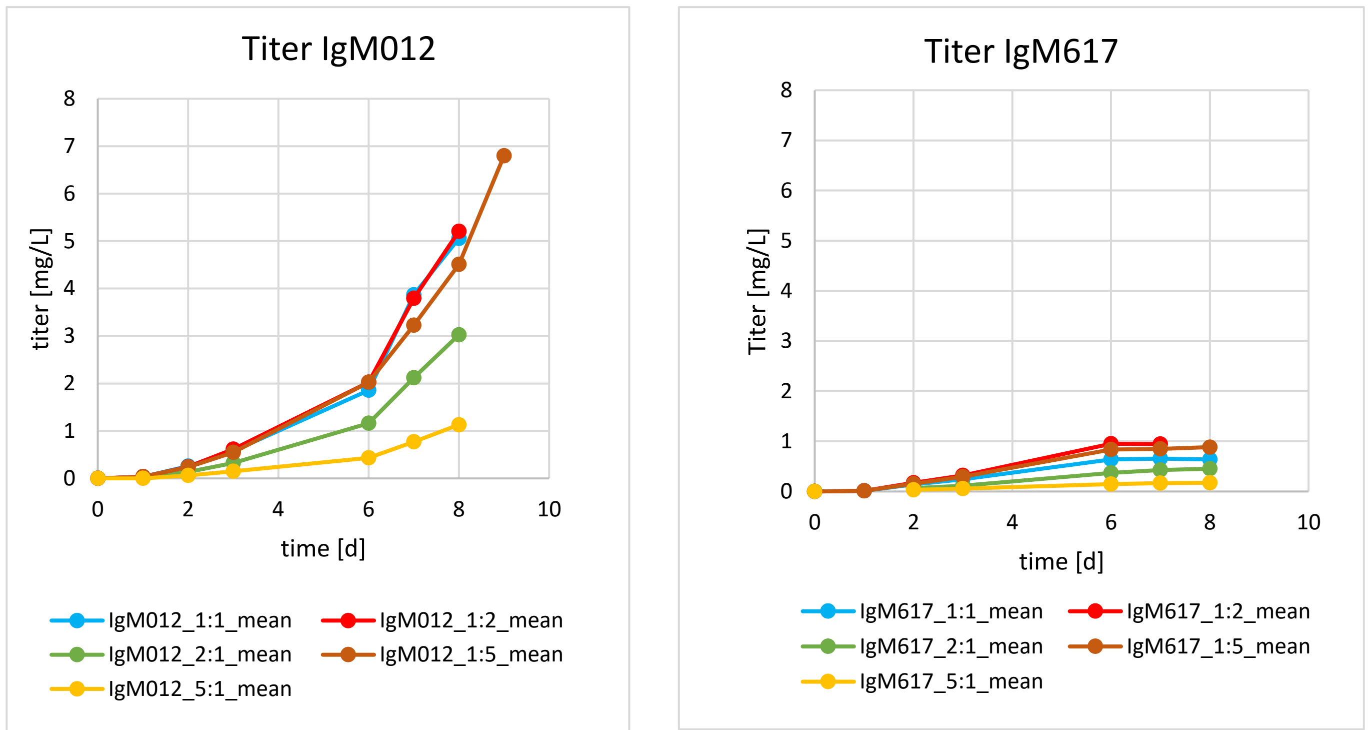


Figure 9: Antibody titers of transfection with different IgM012 and IgM617 HC:LC+JC ratios

The graphs with the antibody titers show that the transfection ratios 1:1, 1:2 and 1:5 yield in the highest antibody titers for both antibodies while the transfection ratios 2:1 and 5:1 have lower antibody titers. The product titers for the transfections with the IgM617 antibody plasmids were much lower than expected from previous experiments with transient transfection with IgM617. This leads to the conclusion that either there happened a mistake in the transfection procedure or the used IgM617 plasmids for the transfection were degraded or otherwise damaged. Another explanation would be that the HEK293-6E cells have been cultured too long at the time-point of transfection.

To check if the transfection with different ratios of HC:LC+JC affects the polymer distribution, cell supernatants of the produced IgM012 antibody were analysed by SDS PAGE gel electrophoresis and Western blot. This analysis could not be carried out with the IgM617 antibody since the antibody product titer in the cell supernatants was too low to be applied on a SDS gel.

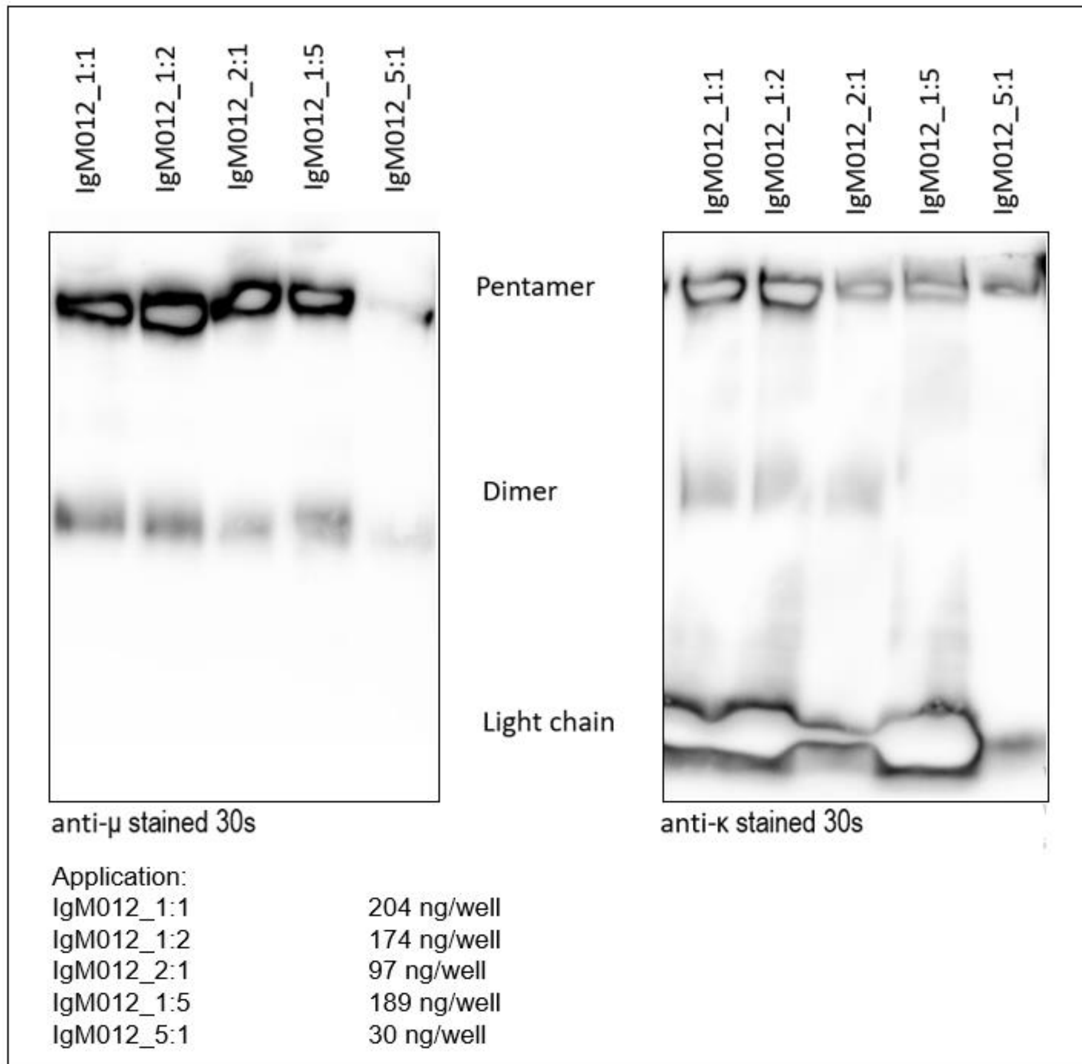


Figure 10: Polymer distribution of the IgM012 antibody in the culture supernatant (extracellularly) analysed by SDS-Page and Western blot

For all IgM012 samples the pentameric form is the most abundant polymer form of the antibody. Dimers can also be observed for all samples and also free light chain can be seen in the κ-stained Western blot, especially in the ratio 1:1 and ratios 1:2 and 1:5 which contain a higher amount of light chain plasmid compared to heavy chain plasmid but those supernatant samples were also applied in higher concentration since they produced more antibodies.

In addition to SDS gels and Western blots of the HEK cell supernatants containing the produced antibodies also gels and Western blots of cell samples of the transfected HEK cells were made, to see what happened inside the cells. Samples of $2*10^6$ cells of each transfection ratio and each antibody were taken on day three after the transfection and lysed with RIPA buffer as described in 4.2.9.2. The supernatant obtained after removal of the cell debris of the lysed cells was applied undiluted onto the SDS gels.

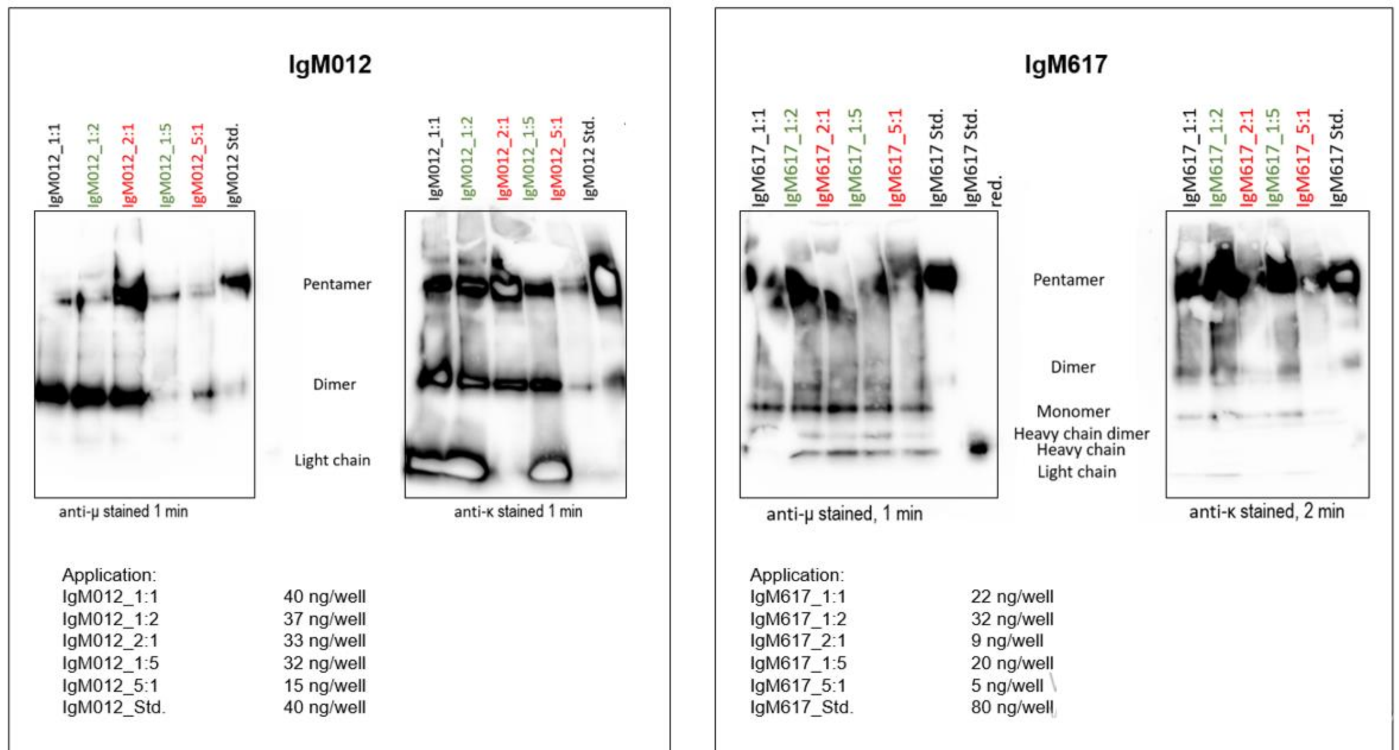


Figure 11: Polymer distribution of the IgM012 and IgM617 antibody of cell samples (intracellularly): the samples marked in green were transfected with an excess of LC plasmid compared to the amount of HC plasmid and the samples marked in red are limited in their amount of LC, compared to the amount of HC.

Figure 11 shows, that the samples which were transfected with an excess of light chain still show a band for free LCs in the cell, whereas the samples which were limited in their amount of LC do not show this band (visible in the anti-κ stained blot). This observation concerns the transfection with the IgM012 antibody as well as the transfection with the IgM617 antibody.

In advantage to the Western blots, the total protein concentration and the intracellular IgM concentration of the cell samples taken on day three post-transfection were measured and the total protein concentration per cell was calculated (table 7 + 8) to check if some of the transfection ratios lead to intracellular accumulation of IgM or IgM protein parts inside the cell.

Table 7: Total intracellular protein concentration and IgM concentration of the IgM012 cell samples of the transfection with different ratios of HC:LC+JC

IgM012 sample	Total protein concentration [µg/mL]	Total protein concentration per cell [pg]	IgM concentration [µg/mL]
IgM012_1:1	1577.6	315.5	1.7
IgM012_1:2	1287.3	257.5	1.3
IgM012_2:1	1615.7	323.1	1.3
IgM012_1:5	1615.7	321.1	1.3
IgM012_5:1	1417.1	283.4	0.5

Table 8: Total intracellular protein concentration and IgM concentration of the IgM617 cell samples of the transfection with different ratios of HC:LC+JC

IgM617 sample	Total protein concentration [µg/mL]	Total protein concentration per cell [pg]	IgM concentration [µg/mL]
IgM617_1:1	1615.6	265.2	0.7
IgM617_1:2	2066.8	358.5	1.1
IgM617_2:1	2435.8	434.9	0.3
IgM617_1:5	2313.8	409.7	0.7
IgM617_5:1	2799.2	510.2	0.2

By determination of the total protein concentration and the IgM concentration inside the transfected cells it can be shown if there is an intracellular accumulation of IgM in the cells which is not the case for the two transfections with different ratios of HC: LC+JC of IgM012 and IgM617. Even the extreme transfection ratios of 1:5 and 5:1 show no intracellular IgM accumulation compared to the total protein concentration in the cell.

5.2. Comparison of stable and transient expression system

The batch experiments with the stable and the transient expression system were performed in duplets for each antibody (IgM617, IgM012 and IgM012_GL). In the stable expression system ActiPro medium (+ 4mM L-Gln + 15 mg/L phenol red + 0.096 µM MTX) was used for the IgM617 cell line and ProCHO5 medium (+ 4 mM L-Gln + 15 mg/L phenol red + 0.096 µM MTX) was used for the IgM012 and IgM012_GL cell lines. For the transient transfection system CDM4HEK293E medium (+ 4 mM L-Gln + 15 mg/L phenol red + 1:2500 G418) was used for all transfections. In both expression systems each batch was started with a cell density of 10^6 cells/mL and was performed in duplets for every antibody. The values given for CC, antibody titers, growth rate and specific productivity were calculated with the mean of the duplets.

In the following graphs IgM617 batches are displayed in blue, IgM012 batches in red and IgM012GL batches in green. The y-axis for the cell concentration and product titer has the same scaling for better comparability of the two expression systems.

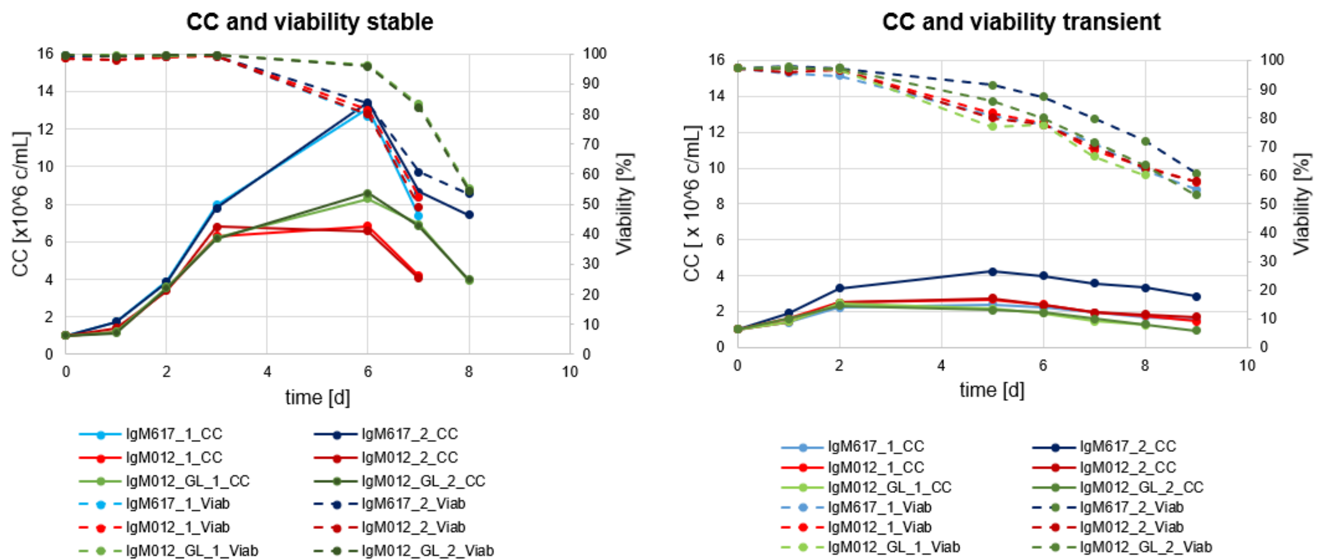


Figure 12: Cell concentration and viability of the stable and transient expression system

The batches with the stable cell lines reached higher cell densities with the peak cell concentration on day 6 (13.3*10^6 cells/mL for IgM617, 6.7*10^6 cells/mL for IgM012 and 8.4 *10^6 cells/mL for IgM012_GL) while transient expression resulted in lower cell concentrations with the peak on day 5 (IgM617 and IgM012) and day 2 (IgM012_GL) (3.3*10^6 cells/mL for IgM617, 2.7*10^6 cells/mL for IgM012 and 2.4*10^6 cells/mL for IgM012_GL) . The viability stayed above 80 % for the first three days in both expression systems and then started to drop.

Due to a pipetting mistake in the transient transfection of the HEK293-6E cells with the IgM617 antibody plasmids the values for this duplet show more variance than the values of the other duplets. IgM617_1 was transfected with approximately 4 mL DNA/PEI mixture and IgM617_2 was transfected with approximately 2 mL DNA/PEI mixture instead of the usually used 3 mL amount.

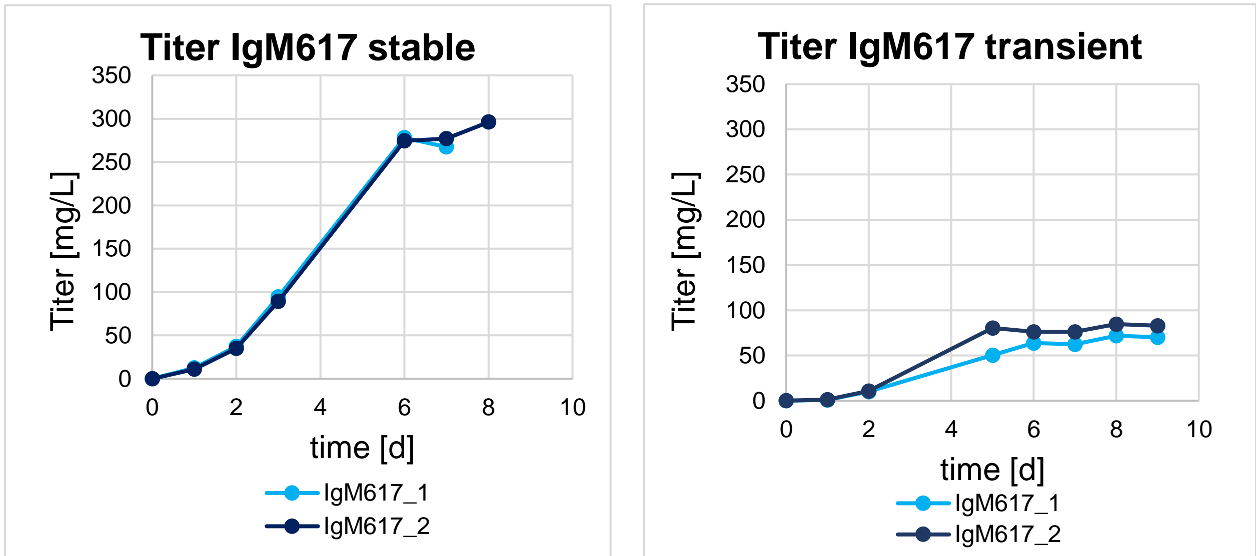


Figure 13: Titer of IgM617 in stable and transient expression system

In stable expression the end product titer of IgM617 was 272 mg/L and in the transient expression batch it was 77mg/L. The antibody production was 3.5 times less in transient production compared to the stable production for IgM617.

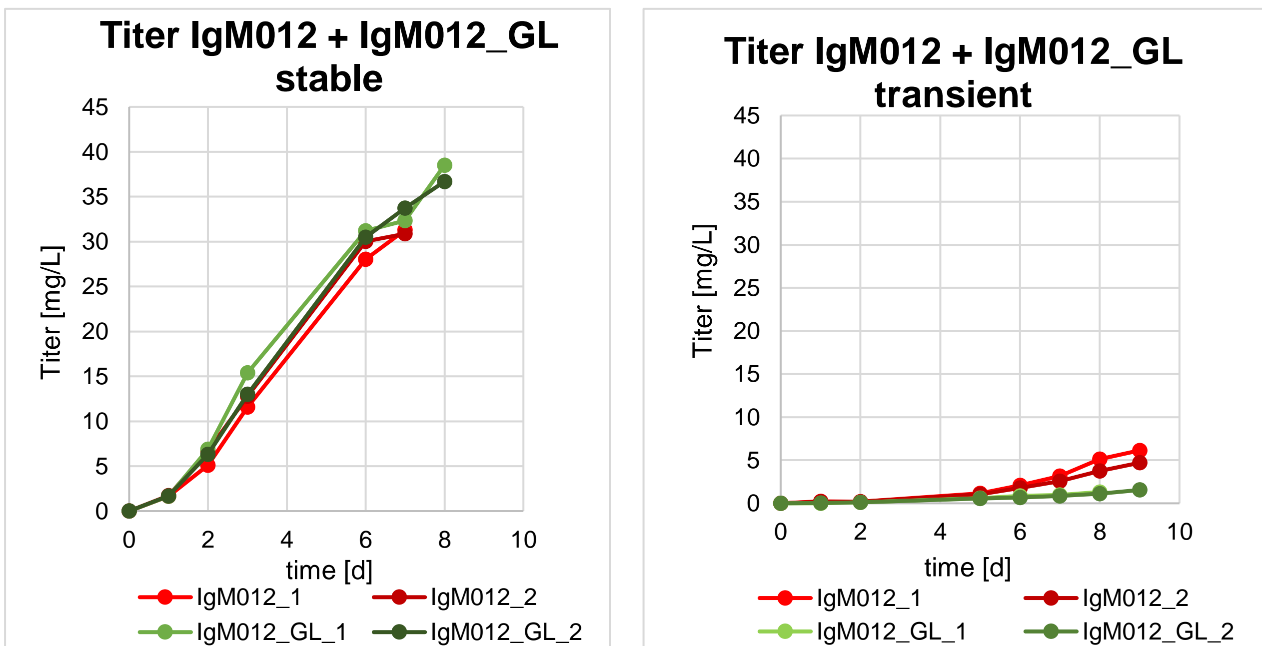


Figure 14: Titer IgM012 and IgM012_GL instable and transient expression system

IgM012 and IgM012_GL are lower antibody producing cell lines. Therefore, they are here shown in separate graphs. In stable expression an end product titer of 31 mg/L was reached for the IgM012 antibody and a titer of 38 mg/L was reached for the IgM012_GL antibody. In the transient batches the titers for these two antibodies were as low as 5 mg/L for IgM012 and 1 mg/L for IgM012_GL.

Table 9: Growth rate and specific productivity of antibodies in stable and transient expression system

Clone	µ [1/d]	qP [pg/c/d]
Stable expression:		
IgM617	0.86	19.40
IgM012	0.63	3.23
IgM012_GL	0.71	2.95
Transient expression:		
IgM617	0.36	12.83
IgM012	0.29	0.40
IgM012_GL	0.22	0.22

In table 9 the growth rate (µ) and specific productivity (qP) of the two expression systems and the three antibodies are compared. In each expression system the growth rate and specific productivity for the IgM617 antibody is highest. It can also be seen that the growth rate and the specific productivity are higher in the stable expression system for all three antibodies. IgM012 and IgM012_GL show only small differences in µ and qP.

To show possible differences in the polymer distribution between the three different antibodies and between stable and transient expression system culture supernatants of the last day of the batch experiments were loaded onto two SDS-PAGE gels. The supernatants of stable expression were diluted so that 300 ng IgM were applied to the gels. As can be seen in figure 13 and 14 the antibody titers in transient expression were lower, so supernatants of the batches of the transient expression system were applied undiluted onto the gels. Afterwards one gel was used for a Western blot and the other one was silver-stained.

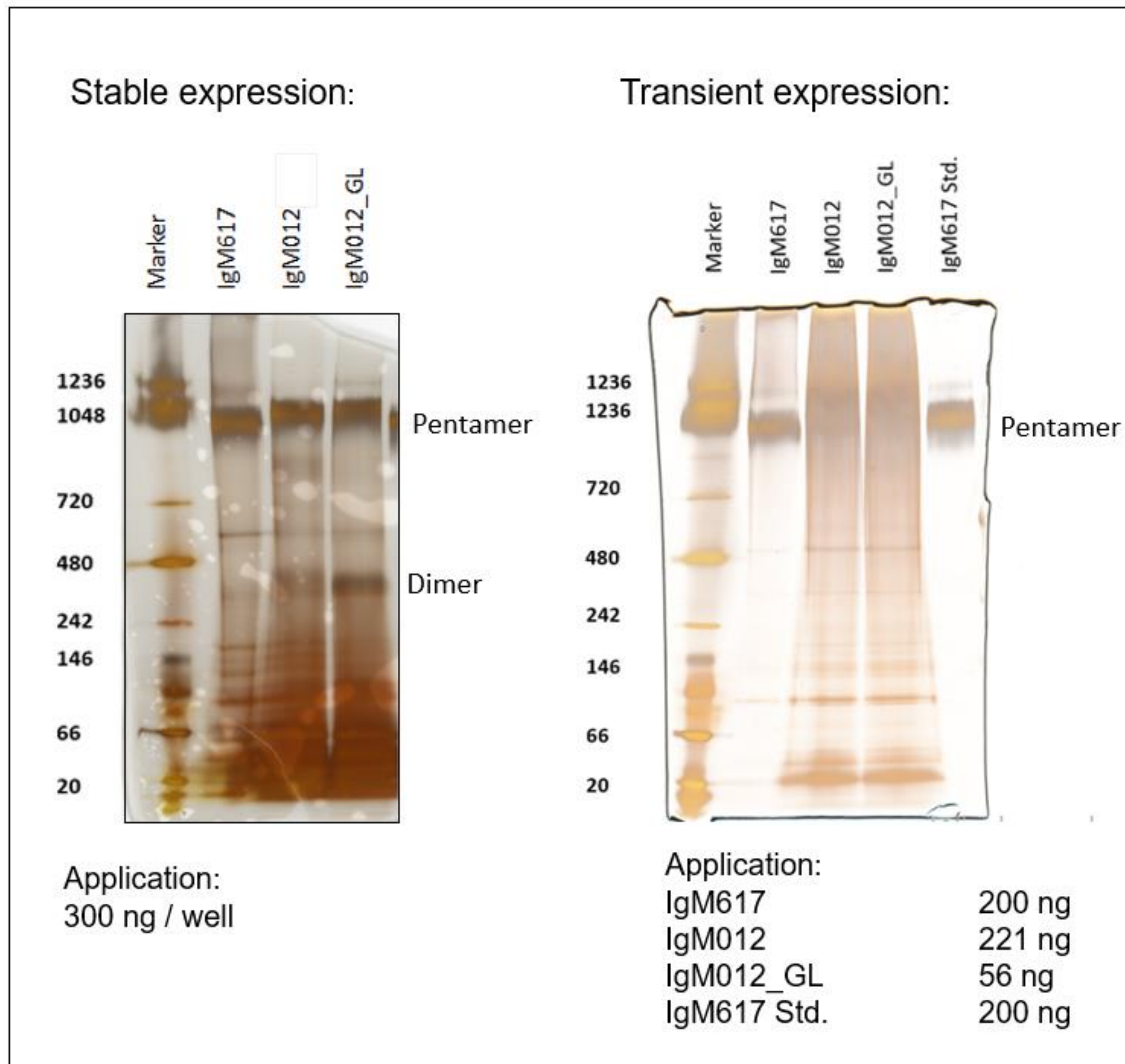


Figure 15: Silver stained SDS gels of stable and transient expression of IgM617, IgM012 and IgM012_GL; IgM617 Std. produced from stably transformed CHODG44 cell line

The pentameric form (950 kDa) is the dominant form in all three antibodies produced in both expression systems. In stable expression also the dimeric form (340 kDa) is clearly visible for all antibodies, however IgM012 and IgM012_GL produce more dimers than IgM617. Probably also a weak band for the hexamer form (1100 kDa) of

all three antibodies is visible in the silver stained gel of the stable expression system, but this band does not appear in the Western blot so it cannot be stated definitely that this band is the hexamer form of the antibodies. In the gel of the transient expression the dimer and hexamer forms are only very weakly visible.

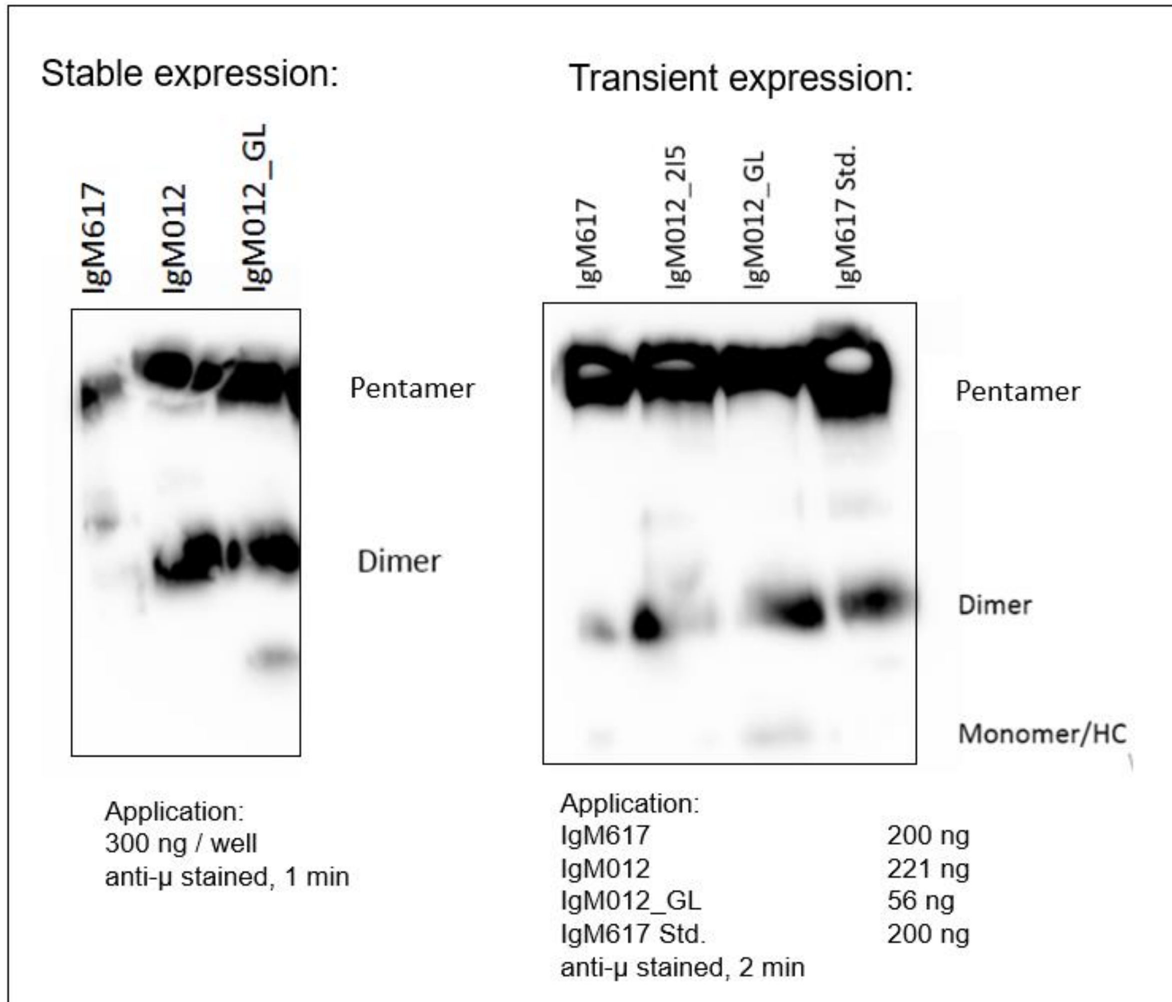
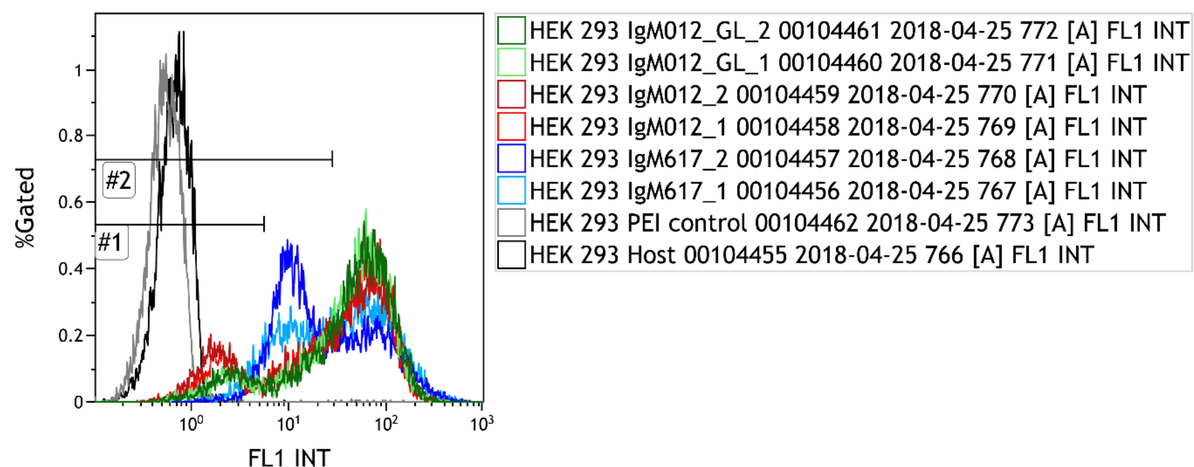


Figure 16: Western blot of stable and transient expression of IgM617, IgM012 and IgM012_GL; IgM617 Std. produced from stably transformed CHODG44 cell line.

The Western blot of the SDS gels again clearly shows that the pentamer form of the antibodies is the predominant form for all three antibodies. To a lower amount the dimeric form is present in all samples. In the blot more dimeric form is observed for the IgM012 and IgM012_GL antibody than for the IgM617 antibody in both expression forms.

The IgM617 standard applied on the gels was in-house produced with the stable IgM617 cell line and purified by the two-step chromatography process explained in 4.2.10. After the purification process the concentration was determined spectrophotometrically as explained in 4.2.11.

To check if the plasmids for transient protein expression have been successfully transfected into the cells and produce heavy and light + joining chains, cell samples of the transfected cells were taken on day 2 post-transfection and the heavy chain was stained with a μ-FITC antibody and the light chain was stained with a κ-biotin antibody which was then detected with streptavidin which was coupled to Alexa Fluor-647. The black and grey peaks are controls of the host cell line HEK 293-6E. The black control are untransfected HEK cells and the grey control are HEK cells treated with PEI but no plasmid has been introduced. The more the peaks of the cell samples are shifted to the right in comparison to the control peaks in the figure 17 and 18, the more fluorescence they show and the more of the respective antibody chain they contain.



Marker	%Gated	X-Med	X-AMean	X-Mode
#1	12.47	2.49	2.65	3.01
#1	12.20	2.30	2.54	2.07
#1	21.71	1.83	2.08	1.52
#1	17.70	2.00	2.25	1.71
#1	5.57	4.51	4.23	5.46
#1	8.54	4.09	3.95	5.46
#1	99.76	0.54	0.57	0.53
#1	99.99	0.68	0.70	0.74
#2	30.78	9.28	11.13	25.17
#2	31.72	9.94	11.50	27.79
#2	44.79	6.20	9.21	24.07
#2	39.21	7.58	10.02	26.09
#2	57.56	10.76	12.09	10.15
#2	45.37	10.62	12.27	10.43
#2	99.78	0.54	0.58	0.53
#2	100.00	0.68	0.70	0.74

Figure 17: Intracellular HC content; cells were fixed in ethanol and stained with anti-μ -chain FITC conjugate

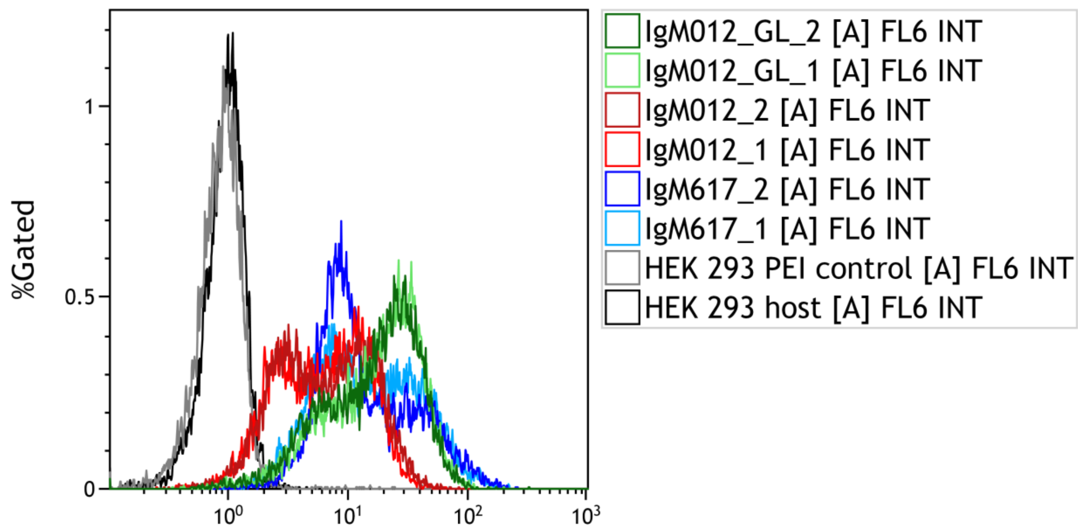


Figure 18: Intracellular LC content; cells were fixed in ethanol and stained with anti- κ -biotin and streptavidin coupled to Alexa-Fluor 647

For the heavy chain plasmid there are two populations of cells as there are two peaks especially for the cell samples of the transfection with the IgM012 HC and IgM012_GL HC. The first peak of IgM012 samples contains about 20% of the gated cells and the first peak of the IgM012_GL samples contains about 12% of the gated cells. This could indicate that cells in the first peak have taken up less plasmid and produce less heavy chain and cells of the second peak have taken up more plasmid and therefore produce more heavy chain. Another explanation could also be intracellular accumulation of HCs in the cell population of the second peak The IgM617 samples also show two peaks, each of which contains about half of the gated cells but their difference in fluorescence intensity is not as big as seen for IgM012 and IgM012_GL samples.

Furthermore, the peaks for the IgM617 duplet show a difference in HC and LC analysis as they were transfected with different amounts of DNA/PEI because of the pipetting mistake mentioned above. Interestingly, figure 13 shows that the IgM617_2 sample which was treated with a lower amount of plasmid DNA and PEI shows more HC and LC producing cells than the IgM617_1 sample which was treated with more plasmid DNA and PEI.

In figure 18 which shows the LC analyses, only for the IgM012 samples two separated peaks, indicating two cell populations, can be seen. The other samples show very broad peaks which indicate heterogeneity of the transfected cells. Only a small part of the sample peaks are located under the control peaks, which indicates a high transfection efficacy.

5.3. Purification of IgM617 for storage and ganglioside binding experiments

For the experiments shown in 5.4, 5.5 and 5.6 IgM617 antibodies produced in a batch of the IgM617 stable cell line, which has been harvested before the viability of the batch dropped below 60 % was purified by the double-step chromatography process described in 4.2.10. In the following figures the purification of the antibody used for the experiment is shown.

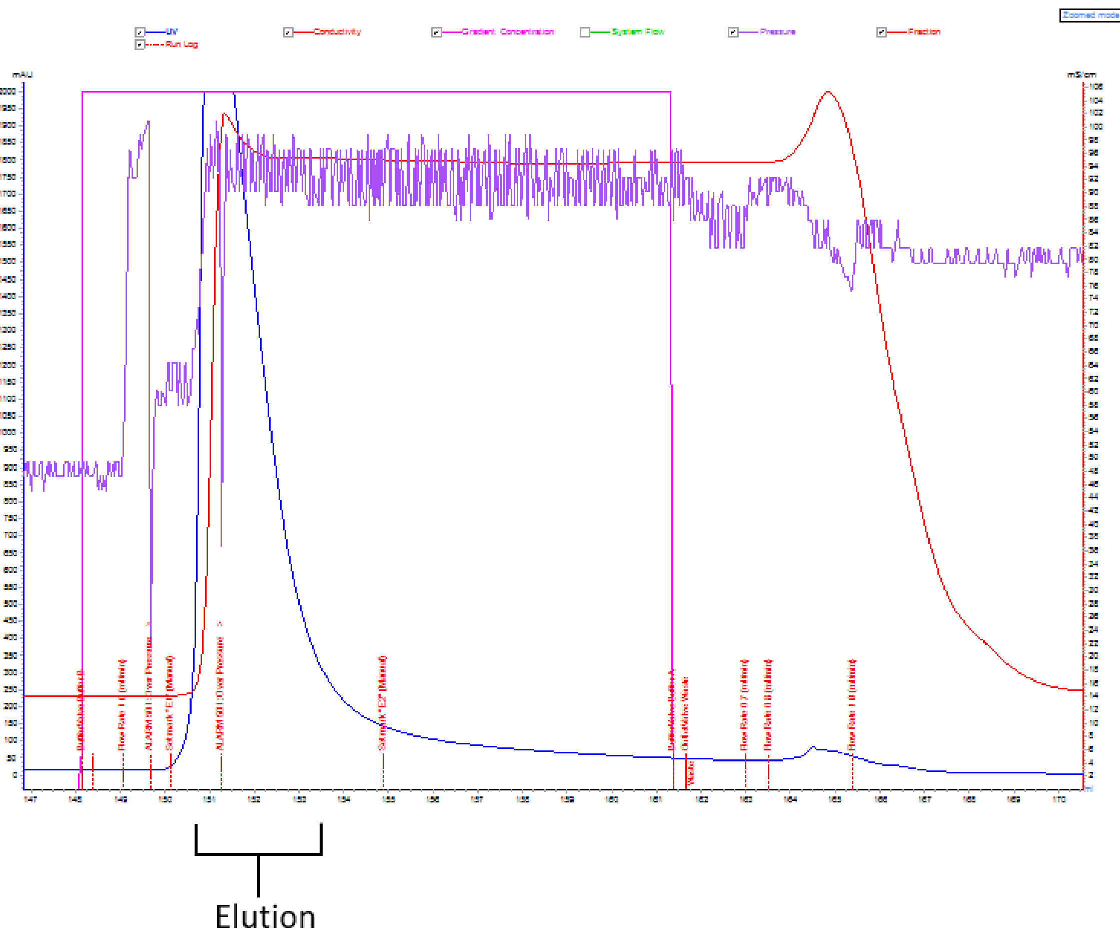


Figure 19: A280 profile (blue graph) of elution of capture select column

The blue peak in figure 19 shows the elution of the IgM617 antibody from the Capture select column. Flow through and wash out, the elution fraction and the solution from the column recovery were collected in separate tubes.

Table 10: Overview of the purification process with Capture Select column

No	Peak name	Retention [ml]	Retention at start [ml]	Retention at end [ml]	Area [ml*mAU]	Height [mAU]	Peak end point height at start [mAU]	Peak end point height at end [mAU]
1	FT1	6.269	4.36	53.36	96045.9100	2001.026	5.477	1999.558
2	FT2	53.818	53.51	98.74	87613.7000	1999.548	1999.548	242.956
3	WO	98.876	98.88	119.76	1639.0610	234.246	234.246	28.674
4	E1	150.880	150.14	154.90	3816.6590	1997.364	25.605	140.074
5	E2	154.920	154.92	161.67	509.0742	139.182	139.182	45.703

FT1…flow through 1, FT2…flow through 2, WO….wash out, E1…elution 1, E2…elution 2;

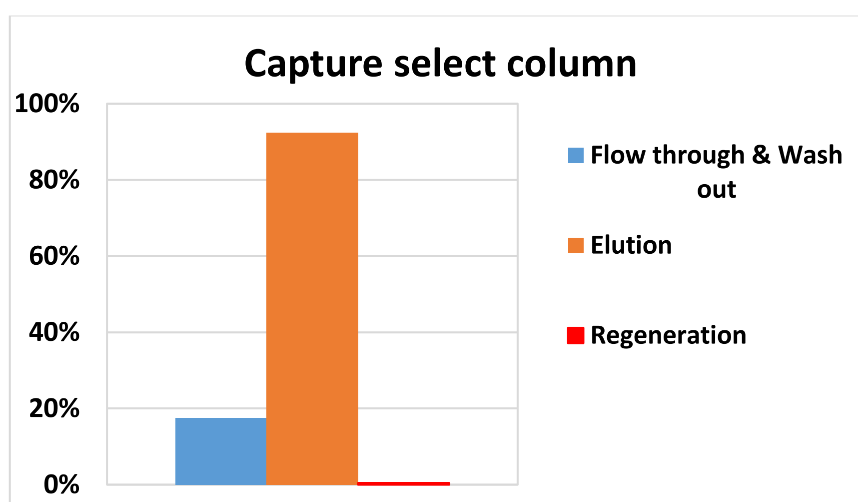


Figure 20: IgM617 recovery after the first chromatography step

After the first chromatography step with the capture select column a recovery of 90% of the applied antibody could be reached in the elution fraction. This was measured by determining the antibody titer in the elution, regeneration and flow through and wash out fractions of the 1st chromatography step with the capture select column. The elution fraction was then further applied to the Suparose 6 column.

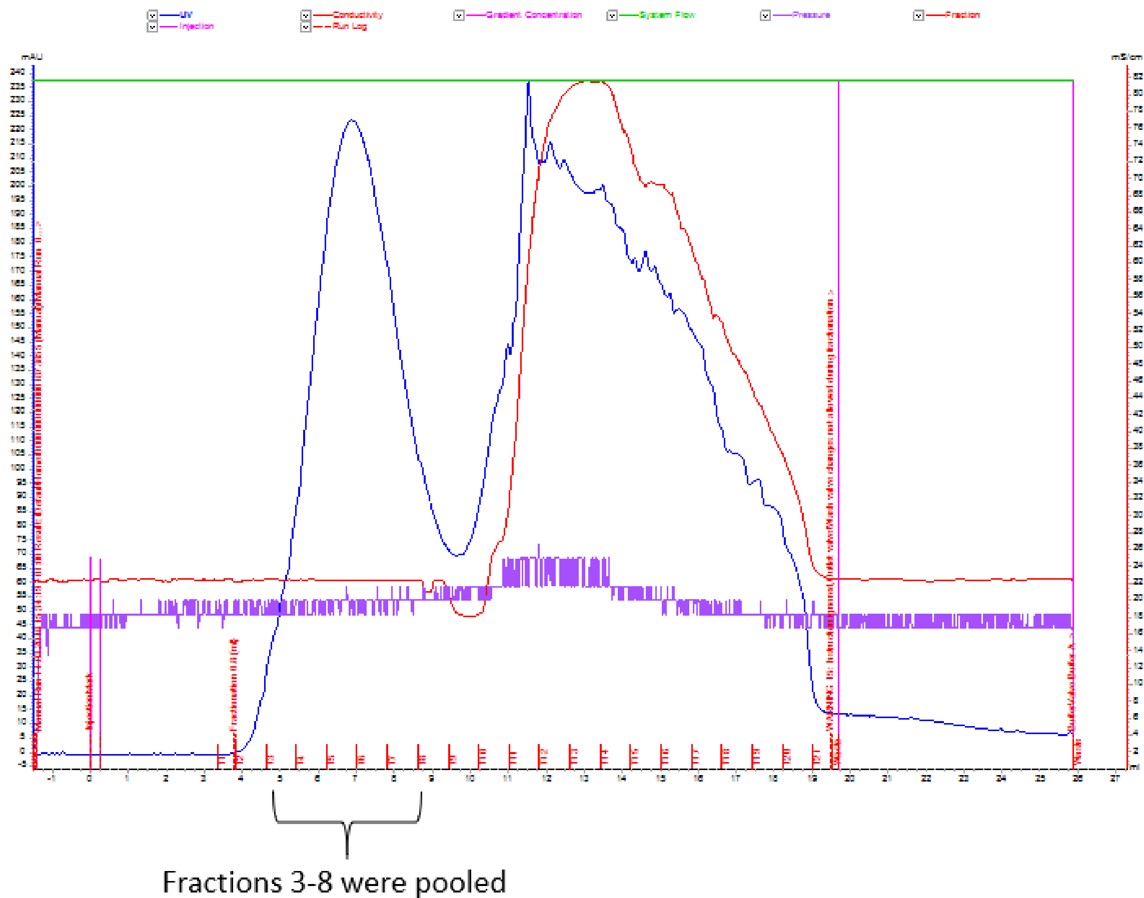


Figure 21: A280 profile (blue graph) of elution of Suparose 6 column

Figure 21 shows the elution of the 2nd chromatography step with the Suparose 6 column. This column was used to purify the antibody from free DNA which is also contained in the harvested culture supernatant. The first peak in the profile shows the eluted IgM617 antibody and the second peak shows DNA and other impurities. The fractions 3 to 8 were pooled and the antibody concentration was determined photometrically as described in 4.2.11. 76% of the applied IgM617 antibody could be recovered in the pooled fractions after chromatography step two which was again determined by titer measurement with ELISA. The concentration of the purified IgM617 antibody which was then used for the storage, flow cytometry and fluorescence microscopy experiments was 450 µg/mL.

5.4. Storage experiment

For the storage experiment purified IgM617 samples (c = 450 µg/mL) were used and stored in lots of 0.5 mL in Eppendorf reaction tubes at the temperatures 4°C in the fridge and -20°C and -80°C in the freezer.

Table 11: IgM concentration determined by ELISA after storage at different temperatures for one and three months

Sample	Titer 1 month [mg/L]	Titer 1 month [% of start titer]	Titer 3 months [mg/L]	Titer 3 months [% of start titer]
IgM617 4°C	410.6	91.2	375.2	83.4
IgM617 -20°C	436.3	97.0	352.9	78.4
IgM617 -80°C	433.5	96.3	410.2	91.2

After the storage time of one and three months the concentration of the samples was determined with the ELISA assay. Overall it can be stated that the antibody titer decreases over time. The biggest loss of antibodies (21.6 % after 3 months) was recorded for the storage of the antibody at -20°C, followed by the storage at 4°C (16.6 % after 3 months). According to this small storage experiment storage of the antibody at -80°C is best (loss of 8.8 % after 3 months) but further research is needed to confirm this data since this experiment was only carried out with single samples and the ELISA titers show a big variation for these measurements.

In figure 22 the polymer distribution of the IgM617 antibody is shown before storage, after 1 month of storage and 3 months of storage at different conditions. The antibody samples were applied on SDS gels and then blotted.

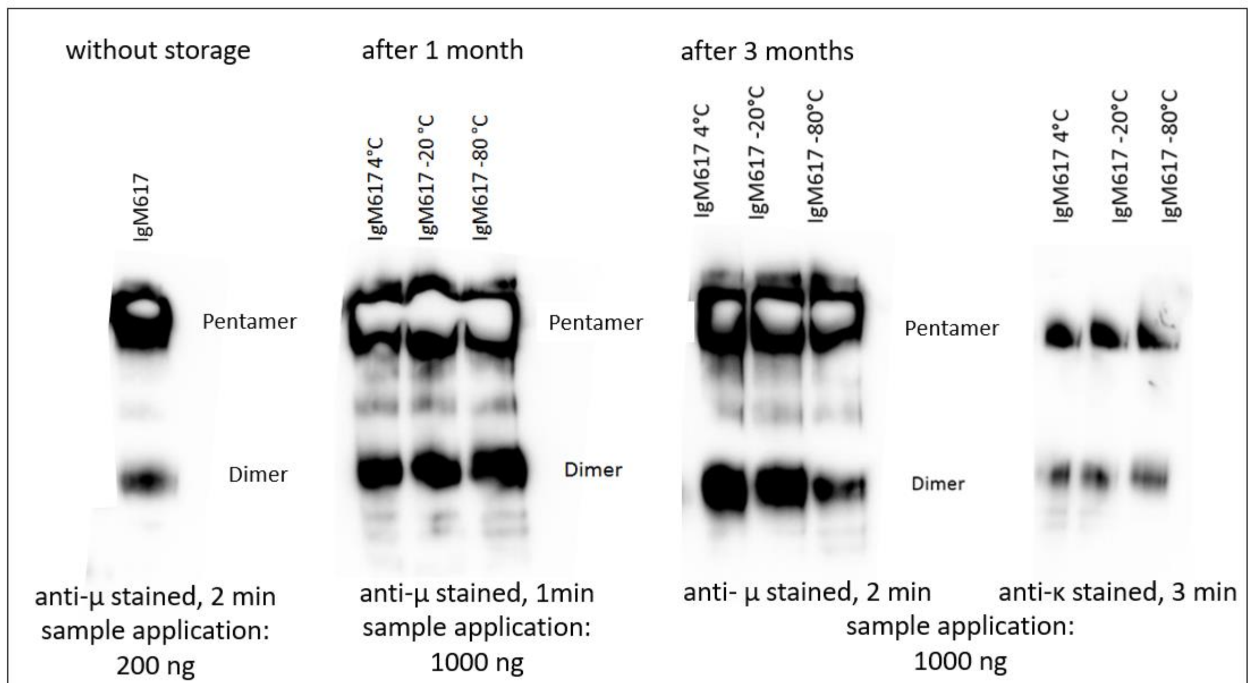


Figure 22: Western blot of stored IgM617 samples at different temperatures for one and three months

There is no difference in the polymer distribution between storage for one and three months and also the different conditions have no influence on the polymer distribution. The IgM617 sample which was not stored shows less bands for degradation products in comparison to the stored samples but was also applied in a lower amount onto the gel.

5.5. Binding of IgM617 to gangliosides on cell surfaces via fluorescence microscopy

Three cell lines were grown adherently in 6-well plates and tested with the staining protocol for fluorescence microscopy (see 3.2.12). Fluorescence microscopy was used in the course of this project to test if the set-up of the staining assay for detection of gangliosides (which is basically the same for fluorescence microscopy and flow cytometry) is working and if a fluorescence signal due to IgM617 binding to gangliosides on cell surfaces can be seen. The tumour cell lines U87-MG and MDA-MB-231 were chosen for this experiment because they proved to be fast growing and grew to the highest cell densities out of the four cultivated tumour cell lines in routine culture. CHO-K1 cells were used as a positive control since they are known to contain the ganglioside GM3 on their cell surface (Ruggiero et al., 2013) for which positive IgM617 binding has already been proved by Polymun Scientific GmbH. The IgM617 antibody used in this experiment, was in-house produced and purified (5.3.).

Figure 23 to 25 show the results of the staining protocol with the IgM617 antibody to detect gangliosides on cell surfaces. The figures show the fluorescence and light microscope pictures. For every cell line one well was treated with the IgM617 antibody and one well only with buffer/FCS to have a negative control for each cell line which shows that the fluorescence seen is due to the IgM617 antibody and is higher than the autofluorescence of the cells which were not treated with IgM617.

CHO-K1 (positive control cell line):

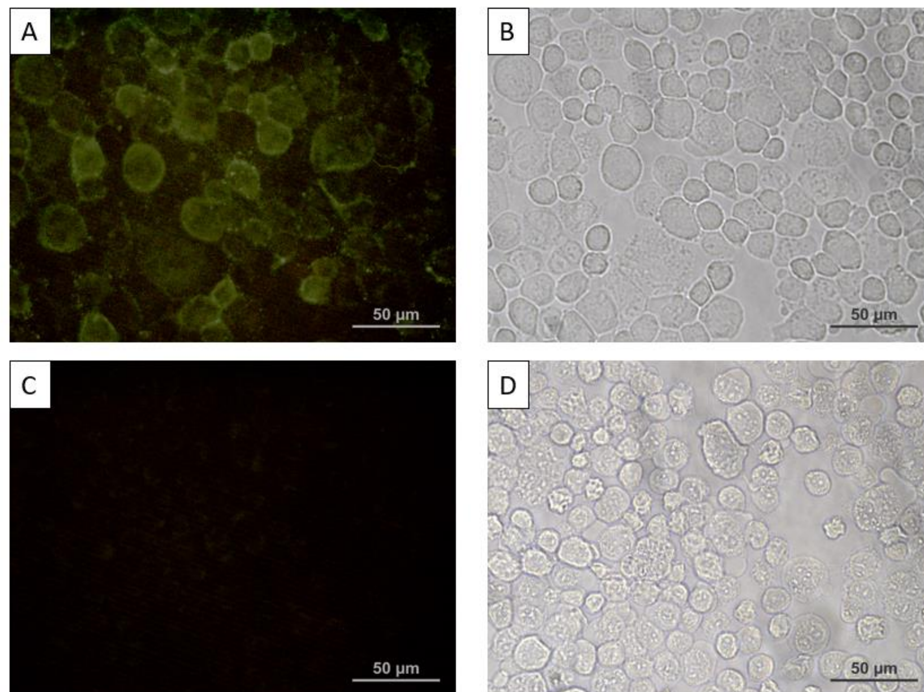


Figure 23: Fluorescence and light microscope pictures (40 x objective) of stained CHO-K1 cells: A+B with IgM617, C+D negative control (without IgM617);

U87-MG (glioblastoma):

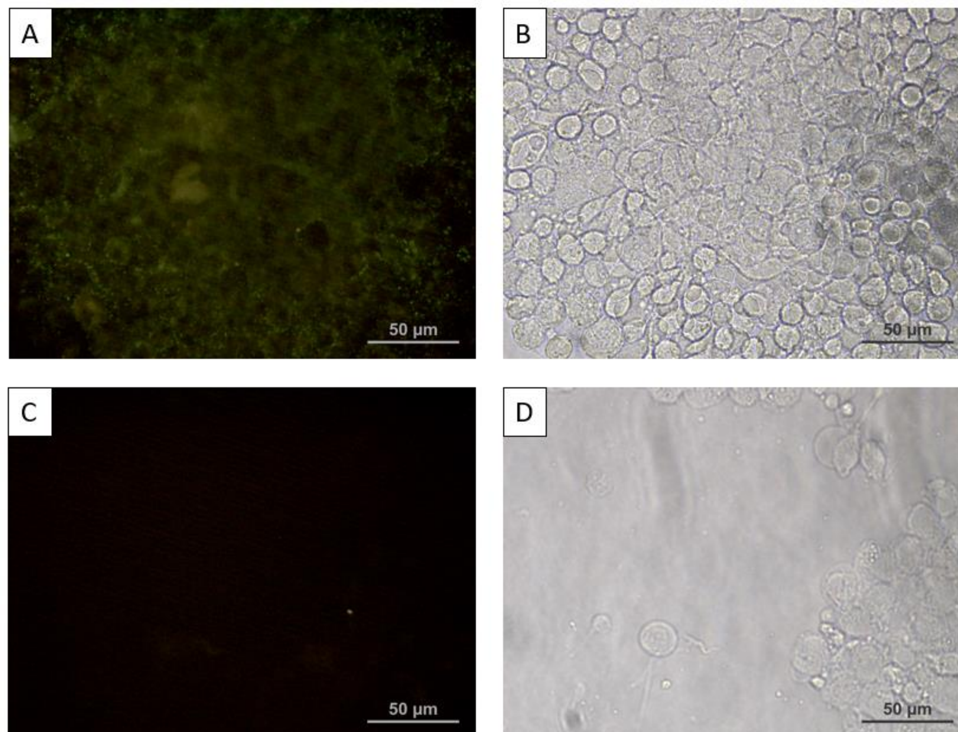


Figure 24: Fluorescence and light microscope pictures (40 x objective) of stained U87-MG cells: A+B with IgM617, C+D negative control (without IgM617);

MDA-MB-231 (breast cancer):

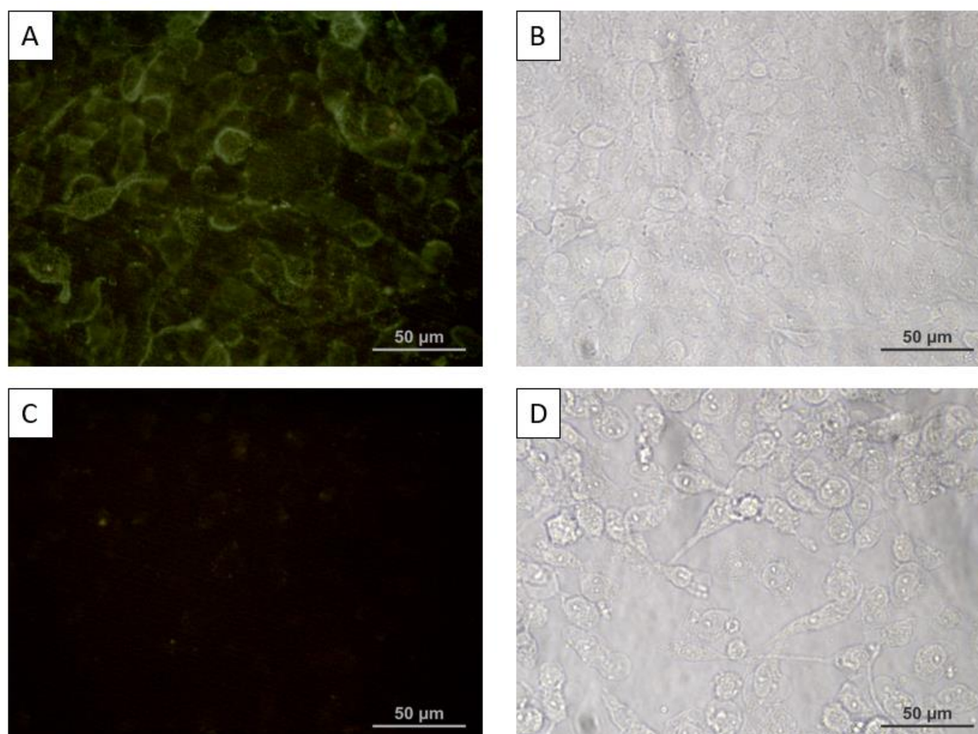


Figure 25: Fluorescence and light microscope pictures (40 x objective) of stained MDA-MB-231 cells: A+B with IgM617, C+D negative control (without IgM617);

All three tested cell lines were adherently grown in 6-well plates and could therefore also be stained in the plates. The CHO-K1 cells served as positive control cell line, since they are known to contain the ganglioside GM3 on their cell surface (Ruggiero et al., 2013). Of each cell line also a negative control was generated. For this purpose, one well of each cell line was incubated with buffer/FCS instead of the IgM617 antibody, to ensure that the fluorescence seen is due to the IgM617 antibody which binds to gangliosides.

The two tested tumour cell lines U87-MG and MDA-MB-231 both show fluorescence, whereas the negative control wells of these cell lines, which have not been incubated with IgM617 antibody, show almost no fluorescence (the weak signal seen may be due to autofluorescence of the cells or the antibody FITC conjugate which has not been entirely washed away by the last washing step). The positive control cell line CHO-K1 also shows a good fluorescence signal.

Besides the in-house produced and purified IgM617 antibody (purification described in 5.3) which was used for staining in figure 23-25, also a deep-frozen lot of IgM617 which was produced by Polymun Scientific GmbH and purified with a Superose 6 size exclusion chromatography column by the working group of Rainer Hahn (purification protocol attached in 9.2. in appendix) was analysed with ELISA and SDS Page and tested with the fluorescence microscopy protocol with the MDA-MB-231 cell line. This was done to test which of the antibody lots shows the highest fluorescence signal and is therefore the best antibody to use for the flow cytometry experiments.

Figure 26 shows a Western blot and a silver stained gel of the electrophoretic analysis of the in-house produced IgM617 antibody which was used for the fluorescence microscope and flow cytometry experiments for the detection of gangliosides on cell surfaces. In addition, the IgM617 batch obtained by Polymun Scientific GmbH, which was purified by the working group of Rainer Hahn, was analysed. This batch was purified in 2 runs. In each run 2 fractions were eluted from the column. Fraction 1 is supposed to contain the pentamer version of the antibody and fraction 2 is supposed to contain the dimeric IgM617.

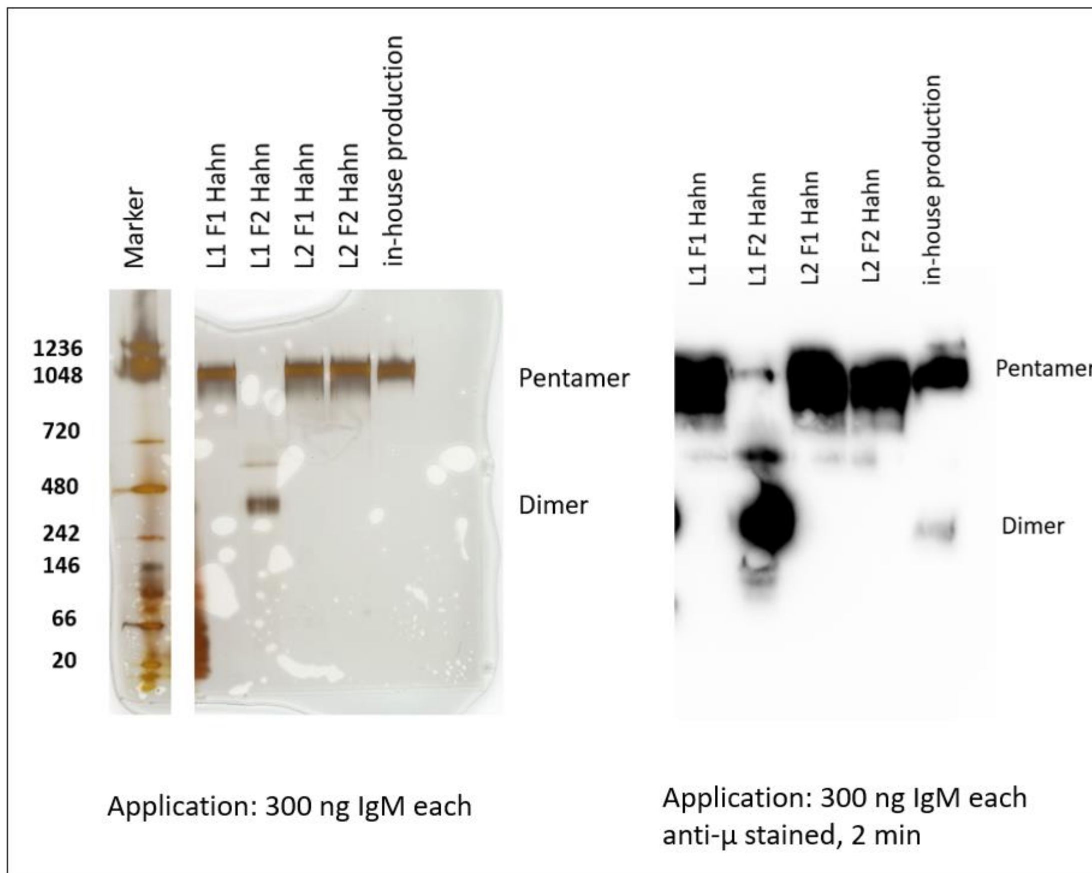


Figure 26: Silver-stained SDS gel and Western blot of the in-house produced IgM617 antibody and the IgM617 lots of Polymun Scientific GmbH, purified by the working group of Rainer Hahn (4 samples obtained) ; The four samples obtained from the working group of Rainer Hahn derived from two independent purification runs (run 1 and run 2) in which two separate peaks were collected as “IgM617” each (fraction 1 and fraction 2; see Appendix 9.2). Abbreviations: L1F1…run 1 fraction 1, L1F2…run 2 fraction 2, L2F1…run 2 fraction 2, L2F2…run 2 fraction 2;

The analysis showed that the in-house produced IgM617 antibody contains mainly the pentamer version of the IgM617 antibody and a smaller amount of dimer. Furthermore, the in-house produced lot shows fewer impurities than the IgM617 lot from Polymun. Therefore, this antibody lot was chosen for the flow cytometry experiments.

As suspected both fractions 1 (run 1 and run 2) of the purification of IgM617 obtained from Polymun which were carried out by the working group Hahn showed the pentamer version of the antibody but also several impurities. Run 1 fraction 2 contains the dimeric version of the antibody and also impurities but run 2 fraction 2 again shows the pentameric version although it is supposed to contain the dimer. There is no explanation why run 2 fraction 2 contains the pentamer although it is supposed to contain the dimer of the IgM617 antibody.

The different IgM617 lots were tested on the MDA-MB-231 cell line (figure 27). This tumour cell line was chosen because it proved to grow to a high cell density and showed a high viability in routine culture. This experiment was done to check which lot shows the strongest fluorescence signal and is best suited for detection of gangliosides to establish the flow cytometry protocol.

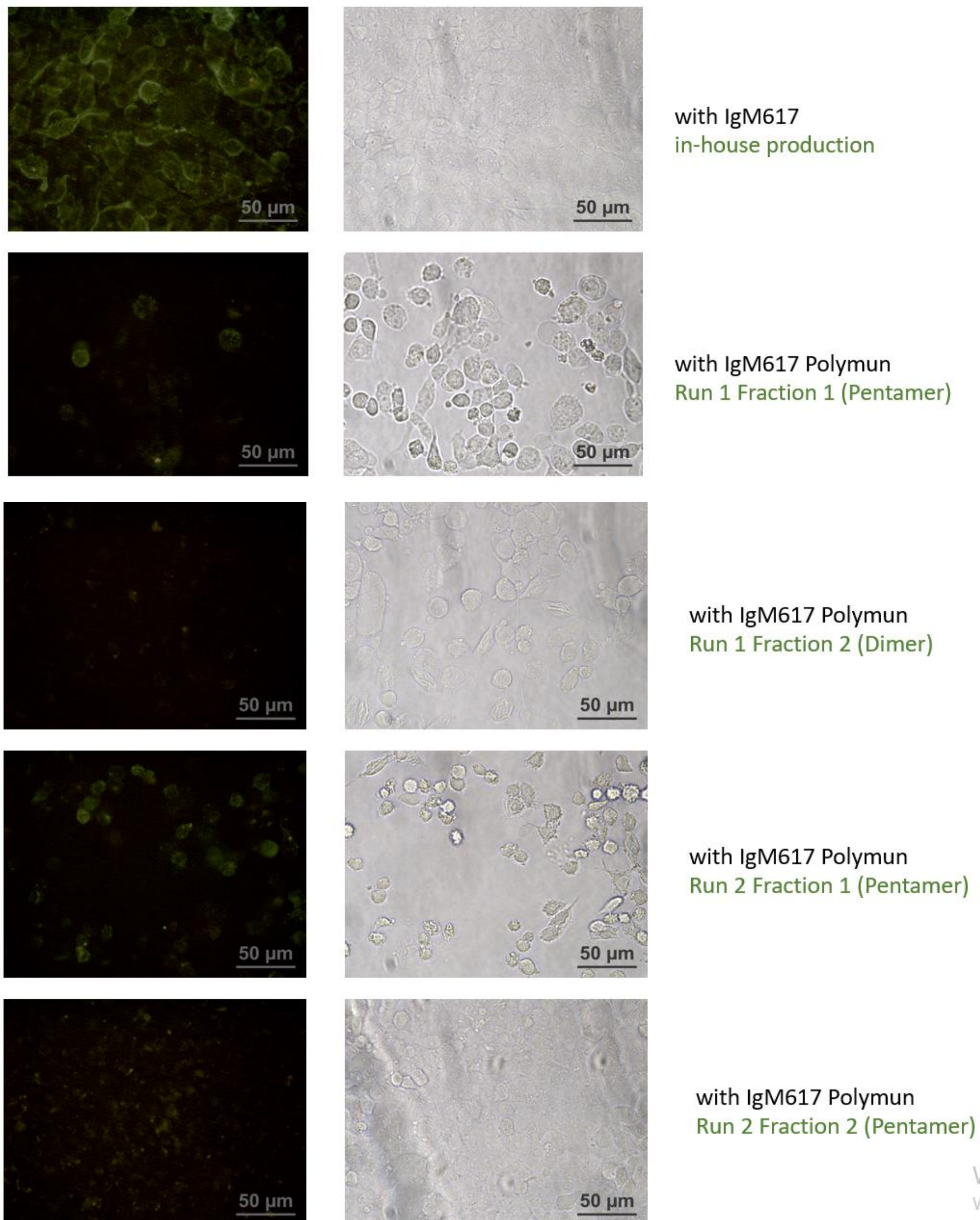


Figure 27: Fluorescence and light microscope pictures of different IgM617 lots on MDA-MB-231 cell line (40 x objective)

The strongest fluorescence signal was obtained with the in-house produced and purified IgM617 antibody. Therefore, this antibody lot was used for the experiments performed in 5.6 to establish the flow cytometry protocol. Unfortunately, the other IgM617 lots obtained by purification of the batch produced by Polymun were applied on a separate 6-well plate and measured after the first plate. Adjusting the microscope and trying to add a scale bar to the pictures on the first plate took very long, so maybe the weaker signal seen and the bad condition of the cells may be due to bleaching of the FITC conjugate due to the long waiting time before the measurement. Nevertheless, for all antibody lots fluorescence could be seen. The weakest signal resulted from the run 1 fraction 2 lot which only contained the IgM617 dimer.

5.6. Establishment of flow cytometry protocol for assessing binding of IgM617 to gangliosides on cell surfaces

To see if the overexpression of gangliosides on cancer cells can be observed by binding of the IgM617 antibody a flow cytometry protocol was established and later on improved.

As can be seen in the fluorescence microscope pictures of the stained tumour cell lines in 5.5, they all have different sizes and shapes. So, for every cell line used in the experiment a separate negative control was produced by applying the same staining procedure to cells of the respective cell line but instead of the incubation step with IgM617 they were incubated with buffer/FCS. For this purpose, two sample vials of each cell line were set up. One treated with the IgM617 antibody, here referred to as the sample and one which was not treated with the IgM617 antibody, referred to as the negative control of the cell line. This procedure was necessary because the cell lines showed different FS and SS behaviour as well as different autofluorescence intensities due to their different appearance and could not directly be compared. The negative controls of the cell lines were used to set the measurement parameters on the flow cytometer and to determine a boundary in the flow cytometry diagrams for the fluorescence signal which is due to binding of IgM617 to gangliosides on the cell surface and has to be discriminated from autofluorescence or unspecific binding of the antibody carrying the FITC conjugate of the cells of every cell line.

For measurement of the fluorescence signal two different flow cytometer were used. First the Astorios Flow cytometer was used and for the last experiment the CytoFLEX flow cytometer was used, since the previously used Astorios Flow cytometer was out of order for more than a month. The type of Flow cytometer is indicated under the figures. The cells were gated to exclude duplets, dead cells and cell debris.

5.6.1. First flow cytometry round with the initial flow cytometry protocol

As a first try the initial flow cytometry protocol which can be found in the appendix in 9.1 was applied to stain the cell surfaces of the tumour cell lines COR-L88, MDA-MB-231 and U87-MG as well as to the non-cancerous HDF5 cell line. As a positive control CHO-K1 cells were used.

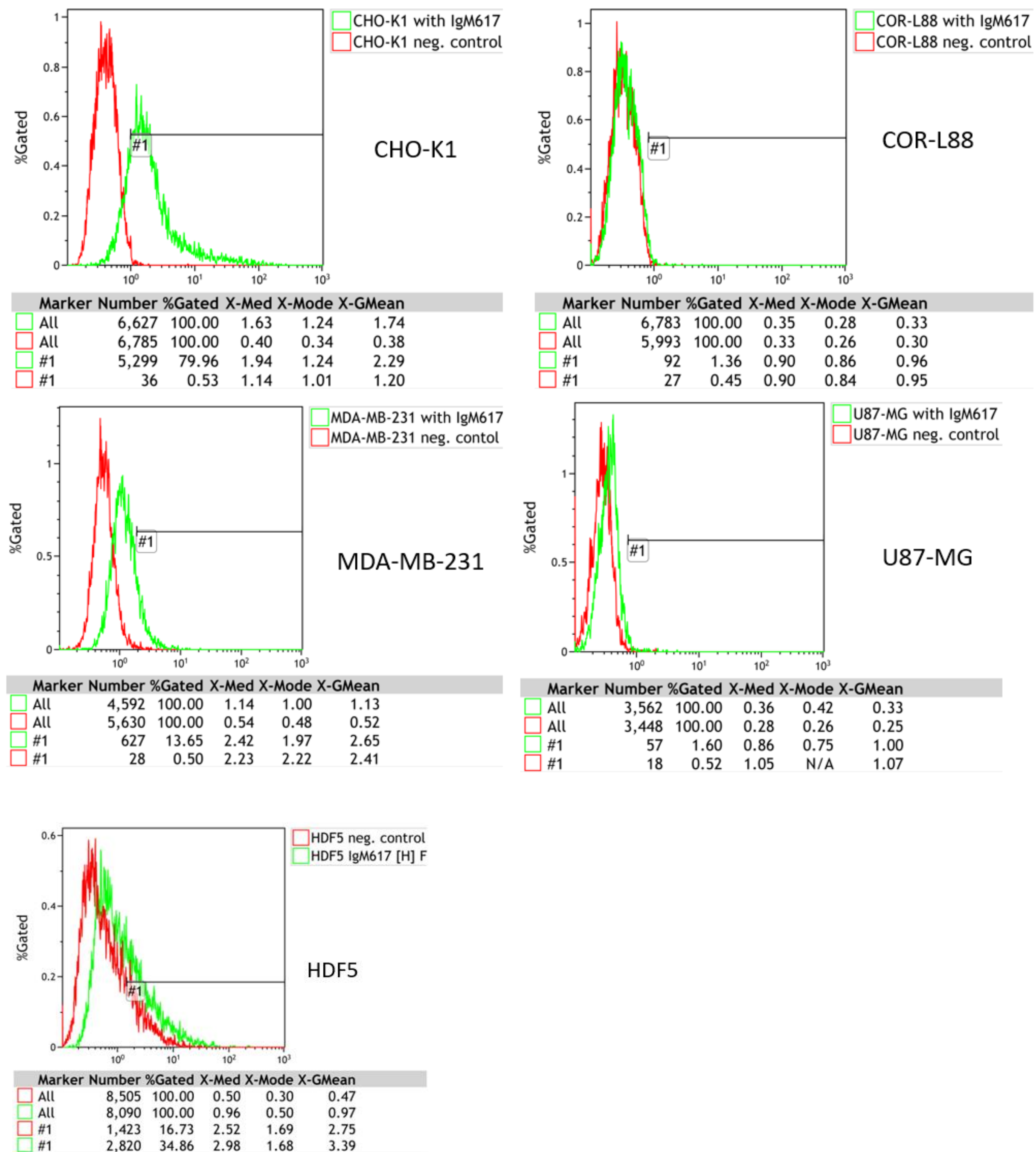


Figure 28: Flow cytometry data of the first try of the staining protocol, measured on Astorios;

The red peak of each diagram signifies the negative control of the cell line which has not been incubated with IgM617 and the green peak the sample with IgM617. The overlay marker “#1” shows the number of cells which are positive for gangliosides on their cell surfaces. For comparability reasons the marker was set for every cell line in the same way so that approximately 0.5 % of the gated cells of the negative control are in the gate for positive ganglioside binding. CHO-K1 was used as a positive control in this experiment and the sample peak of this cell line shows the greatest shift of the green peak to the right which means the greatest fluorescence signal in comparison to the negative control. The samples of the following three tumour cell lines also show fluorescence signals. MDA-MB-231 shows a little shift to the right whereas the green peaks of the cell lines COR-L88 and U87-MG almost overlap with the red peak which signifies the negative control of the cell lines which was not treated with IgM617. This outcome was not suspected as according to the hypothesis that cancer cells overexpress gangliosides on the cell surface the fluorescence shift of the positive peak of the tumour cell samples should be greater than that for CHO-K1 cells. The sample of the non-cancerous cell line HDF5 also showed a small shift in the fluorescence signal to the right compared to its negative control which has not been treated with IgM617. This small fluorescence signal is likely due to gangliosides which are present on the HDF5 cell surface but not overexpressed, therefore the shift is only very small.

With this initial protocol it would not be possible to sort for cells with overexpressed gangliosides as they can even not be easily distinguished from cells which have not been treated with the IgM617 antibody and therefore do not show fluorescence due to ganglioside binding. COR-L88 and U87-MG even showed a lower shift in the fluorescence signal than the non-cancerous cell line HDF5.

Since this first try of the flow cytometry protocol was not very satisfying, various experiments were done and improvements of the protocol were made to get a better separation of the negative control peaks and positive sample peaks of the cell lines.

5.6.2. Testing the effect of trypsinization

First it was tested if the process of Trypsinization which is used to detach the cells from the bottom of the culture flasks has an effect on the fluorescence signal seen as well as on the FS and SS behaviour. Therefore, CHO-K1 cells were used since they can be grown in suspension and do not need the trypsinisation step to detach them form the culture flask. First, CHO-K1 cells were grown in suspension in CD CHO medium (+ 4 mM L-Gln + 15 mg/L phenol red). Then one sample of CHO-K1 cells was treated with Trypsin/EDTA like it is necessary for the tumour cell lines and stated in the protocol in 4.2.13.2 in the methods part. The other sample was not treated with trypsin. After this, both samples were centrifuged, the supernatant discarded, and the cells taken up in about 5 mL of fresh CHO-K1 medium. This step ensures trypsin inactivation of the sample which was treated with trypsin. Both samples were then subjected to the staining protocol with the IgM617 antibody for flow cytometry analysis.

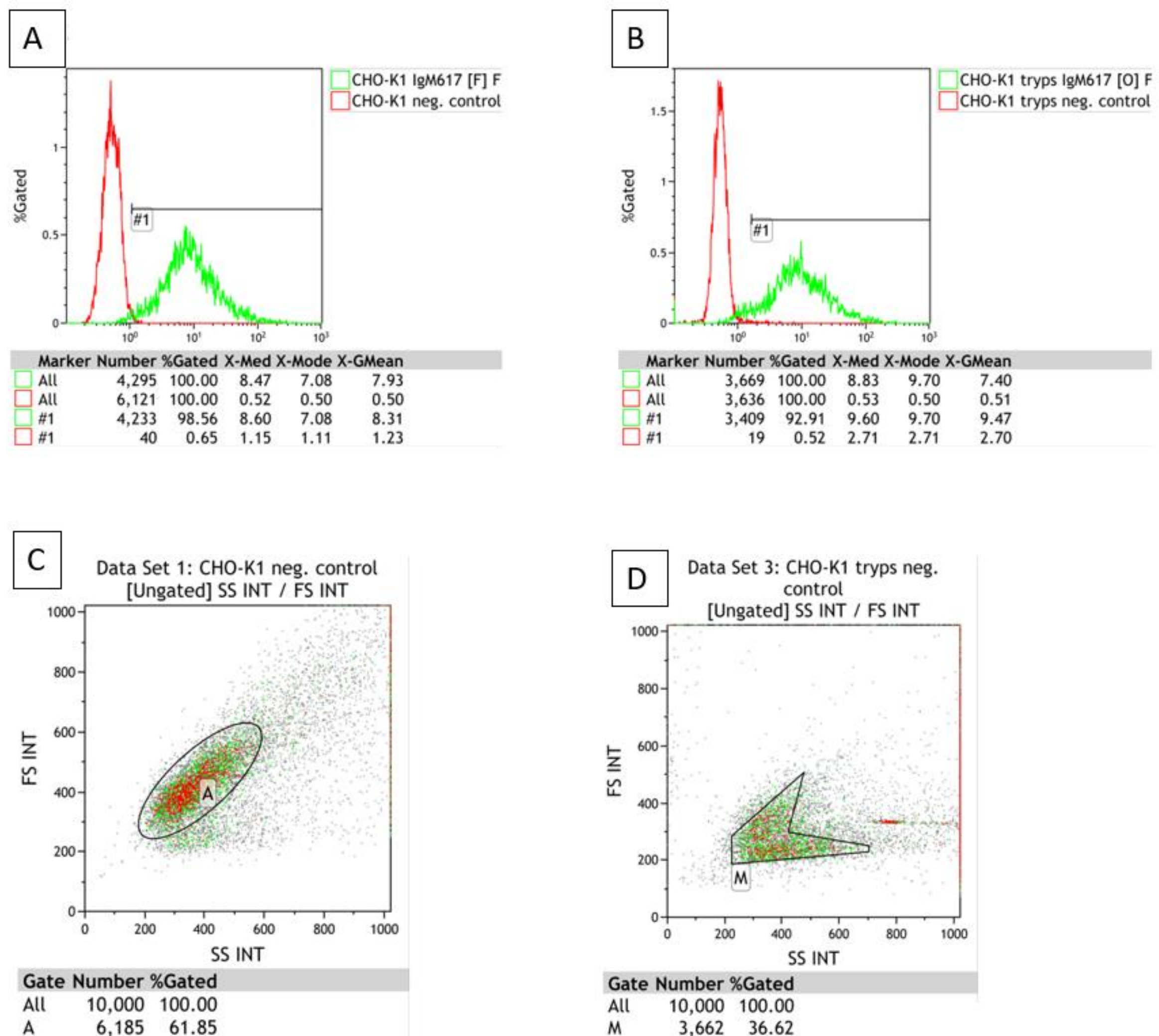


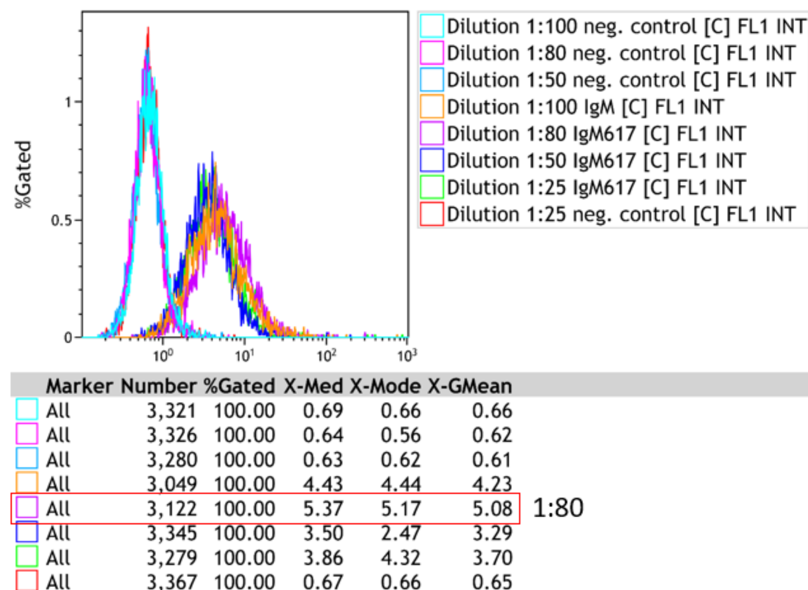
Figure 29: Fluorescence signal and scatterplots of CHO-K1 cell line treated with and without trypsin, measured on Astorios; A+C without trypsin, B+D with trypsin;

It can be seen from figure 29 that trypsinization does not change the pattern of the fluorescence peaks. This proved that trypsinization does not have an influence on the separation of positive and negative peak and can further on be used to detach the tumour cells from the culture flasks. However figure 29 also shows that trypsinization has an effect on the FS and SS behaviour of the cells but since only the fluorescence of gangliosides on the cell surface is the parameter with which we aim to sort for cancer cells and not the morphology of the cells this impact of trypsin is not relevant for the flow cytometry protocol.

5.6.3. Protocol optimization with MDA-MB-231 cell line

The MDA-MB-231 cell line was chosen for protocol optimization since its sample peak already showed positive shift to the right in the fluorescence signal compared to its negative control peak in the first try with the initial flow cytometry protocol (figure 28). The goal of the protocol optimization was to separate the positive and the negative peaks of all tested cell lines in such a way that positive and negative cells can be seen clearly separated. For this purpose, in two separate experiments various concentrations of IgM617 (primary antibody) and anit-µ FITC antibody (secondary antibody) were tested to find out which concentration resulted in the best peak separation.

First the concentration of anti-µ FITC antibody (secondary antibody) was varied. Dilutions of 1:25 to 1:100 were used while the concentration of IgM617 stayed the same as in the initial protocol (50 µg/mL).

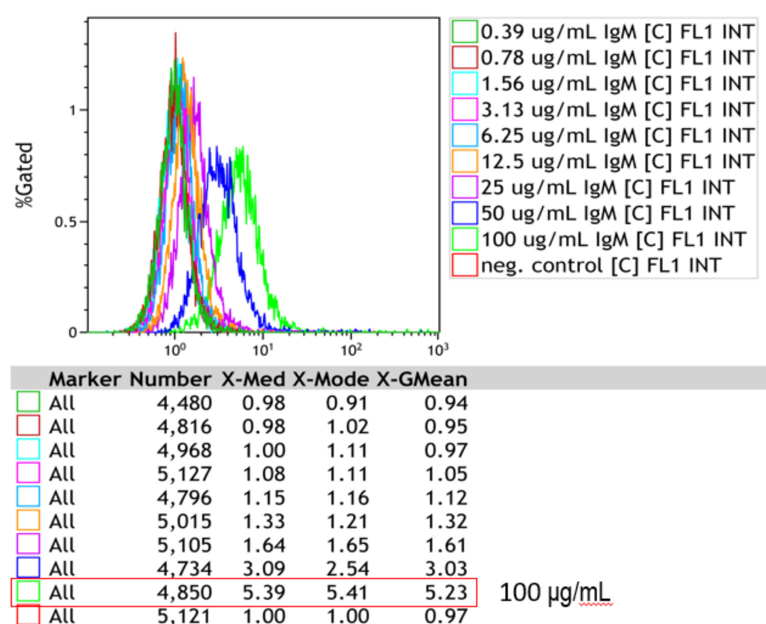


Marker	Number	%Gated	X-Med	X-Mode	X-GMean
All	3,321	100.00	0.69	0.66	0.66
All	3,326	100.00	0.64	0.56	0.62
All	3,280	100.00	0.63	0.62	0.61
All	3,049	100.00	4.43	4.44	4.23
All	3,122	100.00	5.37	5.17	5.08
All	3,345	100.00	3.50	2.47	3.29
All	3,279	100.00	3.86	4.32	3.70
All	3,367	100.00	0.67	0.66	0.65

Figure 30: Effect of different concentrations of the anti-µ FITC antibody on peak separation, measured on Astorios;

For the secondary antibody dilutions from 1:100 to 1:25 were tried (end concentration not known because there was no concentration indication on the antibody flask). For every concentration also a negative control without addition of the IgM617 antibody was prepared. It can be seen in figure 30 that the peaks of all negative controls are almost identical whereas the peaks of the samples with different dilutions of the anti-µ FITC antibody show fluorescence in slightly different intensities. The anti-µ FITC antibody concentration 1:80 resulted in the highest fluorescence signal of the sample and therefore also the best peak separation and was then used in the final FACS protocol.

In the next step the concentration of IgM617 antibody was varied. Concentrations of 0.39 µg/ml to 100 µg/mL were used while the concentration of the anti-µ FITC antibody stayed the same (dilution 1:80).



Marker	Number	X-Med	X-Mode	X-GMean
All	4,480	0.98	0.91	0.94
All	4,816	0.98	1.02	0.95
All	4,968	1.00	1.11	0.97
All	5,127	1.08	1.11	1.05
All	4,796	1.15	1.16	1.12
All	5,015	1.33	1.21	1.32
All	5,105	1.64	1.65	1.61
All	4,734	3.09	2.54	3.03
All	4,850	5.39	5.41	5.23
All	5,121	1.00	1.00	0.97

Figure 31: Effect of different concentrations of the IgM617 antibody on peak separation, measured on Astorios;

Figure 31: Effect of different concentrations of the IgM617 antibody on peak separation, measured on Astorios

Also for testing the different concentrations of IgM617 a negative control of the MDA-MB-231 cell line was used without the addition of the IgM617 antibody. Figure 31 shows that the use of the 100 µg/mL concentration resulted in the highest fluorescence signal of the sample cells and therefore best peak separation and was then used in the improved, final flow cytometry protocol.

Additional to the testing of different concentrations of the used antibodies, the time of the numerous centrifugation steps was shortened form 10 to 5 minutes to shorten the staining procedure to maintain a good condition of the cells and especially cell surfaces until the time point of measuring the fluorescence in the flow cytometer. In addition the whole flow cytometry protocol for detection of gangliosides on the cell surface was performed on 4°C. A further change was the switch to a rotor in a cooling room for the incubation steps with the antibodies to ensure that the antibody solutions are well mixed with the cell suspensions and reach as many cells as possible.

5.6.4. Applying the optimized protocol

After these improvements the optimized flow cytometry protocol was tested on all tumour cell lines and HDF5 cell line. Furthermore, cells of the tumour cell line MDA-

MB-231 and negative, non-cancerous control cell line HDF5 were mixed in the ratio 1:1 to see if these two cell populations can be distinguished because of different fluorescence signals given by different ganglioside expression in the two different cell types.

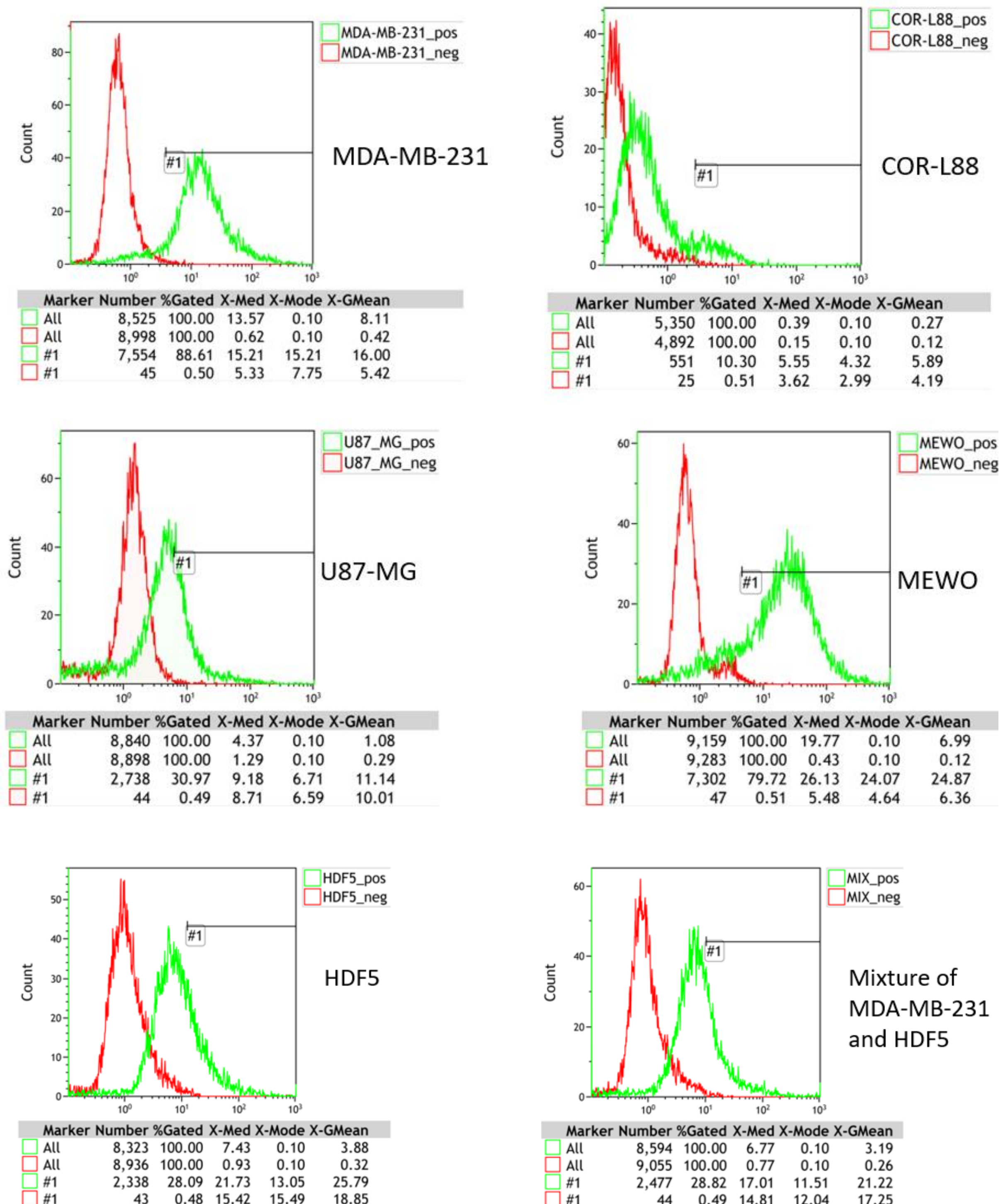


Figure 32: Flow cytometry data of the optimized staining protocol performed on all available tumour cell lines as well as on HDF5 cell line and a mixture of MDA-MB-231 cells and HDF5 cells (ratio 1:1), measured on CytoFLEX;

Figure 32 shows that the peak separation between sample peak and negative control peak for all available cell lines could be improved with the protocol optimization. For the MDA-MB-231 cell line for example 88.6% of the stained cells are now positive for gangliosides on their cell surfaces in comparison to only 13.7% which were positive in the first flow cytometry round. However, it has to be noted that this last round of flow cytometry analysis was carried out on the CytoFLEX flow cytometer in the core facility of Muthgasse III since the previously used Astorios flow cytometer was out of order. This change to a more powerful flow cytometer might also be partly responsible for the better peak separation.

The two cell lines COR-L88 and U87-MG which were already used for the first try of the flow cytometry protocol (figure 28) also showed a better peak separation with the improved protocol on the CytoFLEX flow cytometer. The in this experiment newly introduced tumour cell line MEWO showed 79.7% cells which are positive for gangliosides on their cell surfaces. For the negative control cell line HDF5 the peak separation did not improve (34.8 % of stained cells positive for gangliosides in first FACS round and 28.9 % cells positive with the improved protocol).

Table 12: Improvement of peak separation with adjusted flow cytometry protocol. The percentages given are cells of the respective sample which show fluorescence signal above that of their respective negative control.

Cell line	Initial protocol	Improved protocol
CHO-K1	80.0 %	
MDA-MB-231	13.7 %	88.6 %
COR-L88	1.4 %	10.3 %
U87-MG	1.6 %	31.0 %
MEWO		79.7 %
HDF5	34.9 %	28.0 %
Mix: MDA-MB-231 + HDF5		28.8 %

Furthermore, it was tested in this experiment if a discrimination of cancer cells (MDA-MB-321 cells) and normal (non-cancerous) cells (HDF5 cells) is possible with the improved protocol by mixing cells of these two cell lines in the ratio 1:1.

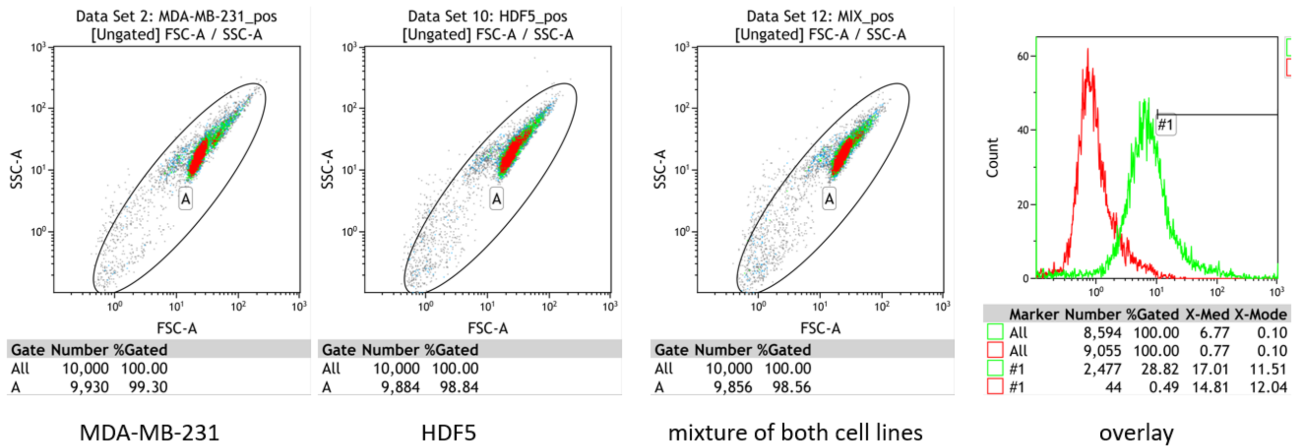


Figure 33: left: scatterplots of MDA-MB-231 cells and HDF5 cells and mixture of both; right: overlay of fluorescence signal of mixture, negative control in red and sample incubated with IgM617 in green, measured on CytoFLEX;

Figure 33 shows that it is not possible to discriminate between the two cell lines MDA-MB-231 and HDF5 according to their fluorescence signal. In the overlay of the fluorescence signals only one peak for the cells treated with IgM617 and one peak for the negative control cells not treated with IgM617 can be seen but none of these two peaks is separated in a way that we can see the two different cell populations (cancerous MDA-MB-231 cells and non-cancerous HDF5 cells). Therefore, sorting for cancer cells in this mixture according to the fluorescence signal given is not possible. Furthermore, also their FS and SS behaviour it too similar as to be able to gate for one cell line only as can be seen in the scatterplots in figure 33.

Figure 34 shows an overlay of the fluorescence signal of all tumour cell lines and HDF5 cell line.

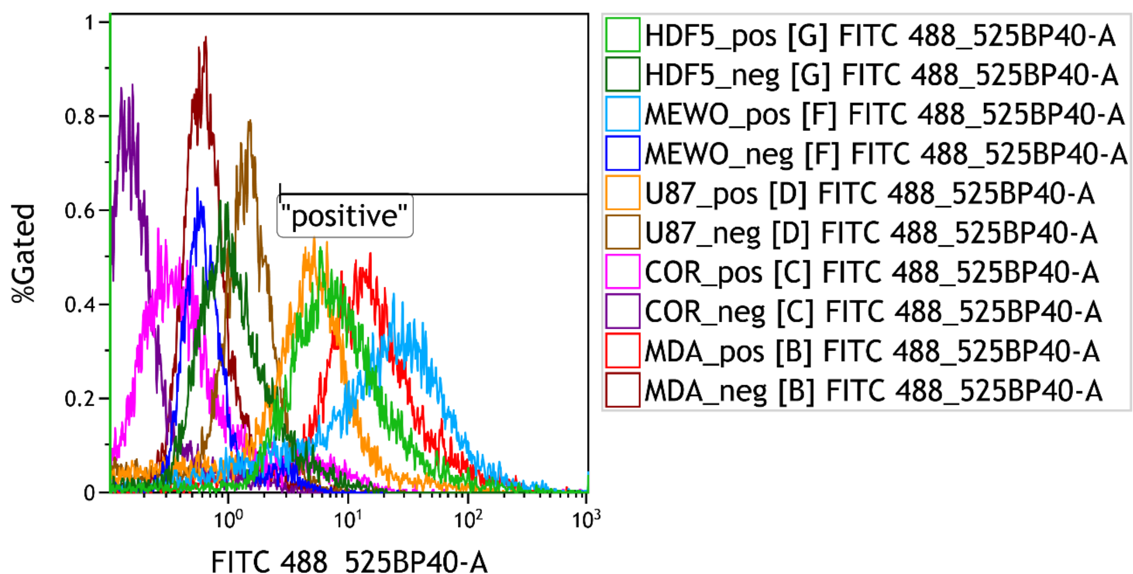


Figure 34: Overlay of fluorescence signal of all tumour cell samples and the negative control cell line samples, measured on CytoFLEX;

The overlay in figure 34 shows that every sample which has been treated with the IgM617 antibody (pos) shows a higher fluorescence signal than its corresponding sample which is the negative control and was not treated with the IgM617 antibody (neg). But as can be seen in the diagram, the peaks of the samples overlap each other and no absolute cutoff-value between ganglioside-positive and ganglioside-negative cells is possible. In figure 34 the overlay marker “positive” is shown to prove that it is not possible to set a definite cutoff-value for the fluorescence signal which could show us on which cell surfaces gangliosides are overexpressed and which cells are normal.

Table 13: Percentages of ganglioside-positive gated cells and geometric mean of fluorescence signal of the peaks of the samples in figure 34

Cell line	% positive [%]	X-Gmean	Incubation with IgM617 [+/-]
HDF5	88.14	9.85	+
HDF5	10.27	4.70	-
MEWO	84.95	23.56	+
MEWO	2.85	3.76	-
U87-MG	69.12	6.68	+
U87-MG	7.14	3.67	-
COR-L88	10.32	6.13	+
COR-L88	0.51	4.33	-
MDA-MB-231	91.17	16.27	+
MDA-MB-231	1.21	3.94	-

As can be seen in table 13, the fluorescence intensity of the sample of the COR-L88 cell line (6.13) is much lower than the negative samples of the other cell lines and even lower than the non-cancerous cell line HDF5 (9.85). HDF5 was initially intended to be the non-cancerous control cell line which does not show an excessive number of gangliosides on its cell surface. The tumour cell lines were suspected to show overexpression of gangliosides on their cell surfaces and would therefore show a much higher fluorescence signal than HDF5. But as can be seen in figure 34 and table 13 only the tumour cell lines MDA-MB-231 (16.27) and MEWO (23.56) show an increased fluorescence signal compared to the HDF5 sample (9.85). The tumour cell lines U87-MG (6.68) and COR-L88 (6.13) show a lower fluorescence signal than the HDF5 sample (9.85). The experiment shows that already the autofluorescence of the different tumour cell lines used varies greatly as can be seen in the negative control peaks of the cell lines (marked with “_neg” in figure 34). The fluorescence signal seen is dependent on the cell type and therefore it is not possible to set a boundary to discriminate between overexpression of gangliosides and a normal number of gangliosides on the cell surface, especially not without a negative control of the cell line which shows the autofluorescence of the respective cell type.

Therefore, it is not possible to sort with this flow cytometry protocol for circulating tumour cells in the human blood, especially, if it is not clear which tumour site the CTCs originate from as in the human blood lots of different cell types with different autofluorescence can be contained. Figure 34 makes it clear that not even a boundary fluorescence value can be determined which would separate the cells which were treated with the IgM617 antibody and those who were not treated with the antibody and show only autofluorescence or fluorescence due to the FITC conjugate which might not have been entirely removed by the last washing step or unspecifically bound.

6. Discussion

6.1. Testing different HC:LC+JC ratios in transient transfection

The goal of this experiment was to find out if the ratio of HC:LC+JC used in the transfection process has an influence on the antibody production especially on the polymer distribution.

The different ratios seem to have no influence on cell growth and viability. It seems that excess of heavy chain (ratios 2:1 and 5:1) corresponds to a lower product titer.

It must be noted at this point, that with the ELISA setup used to quantify the IgM antibodies in the culture supernatant only antibodies with light chain and heavy chain are detected. If due to the different transfection ratios of HC:LC+JC IgM antibodies with only heavy chains or only light chains are produced and secreted into the culture supernatant they are not included in the antibody titer measured by ELISA.

The Western blot of the culture supernatant as well as the Western blot of the cell samples did not show a different polymer distribution between the ratio 1:1 and the other ratios. But what can be seen in the Western blot of the cell samples of both antibodies is, that in the ratios 2:1 and 5:1 which contain more HC than LC+JC plasmid, there seems to be a limitation in light chain, as no free light chains can be detected in the cell, which would be a reason for the low antibody titers observed of those ratios. The possible explanation is, that there is not enough light chain available to build complete assembled IgM antibodies which can then be detected with the ELISA essay.

It is not clear why the transfections with the IgM617 antibody in this experiment yielded in lower antibody titers compared to the titers observed in the transient transfection in experiment 5.2. In general, the transient transfections showed a high variability. Maximal cell concentrations and titers could not be reproduced from transfection to transfection. This fact may be due to variations in the transfection process or aging of the HEK293-6E host cell line (De Los Milagros Bassani Molinas et al., 2014). Another explanation would be that the plasmids which were already used for previous transfections got degraded or destroyed by the freeze-thaw-freeze process.

The flow cytometry analysis of intracellular HC and LC suggests that there might have happened something to the light chain plasmid as the pattern of the IgM617 LC peaks is different to those previously observed from transient transfections which were performed in 5.2.

In general, the flow cytometry analysis was not as satisfactory as it was for the transient transfections performed in 5.2. as the transfection efficacy was lower.

6.2. Comparison of stable and transient expression system

As can be seen from figure 13 and 14, IgM617 is a higher antibody producer than IgM012 and IgM012_GL, in both stable and transient expression system. As suspected, the production in stable expression is higher for all three antibodies compared to the production in the transient expression form.

Although VPA and TN1 were added to the transient transfected cells the antibody yield in transient expression remained lower than in stable expression. VPA and TN1 are thought to increase protein production in transient expression up to yields which can be expected in stable expression (Blackiwal et al., 2008, Pham et al., 2005).

It was observed that the amount of DNA/PEI mixture used in the transfection process has an influence on the growth rate and antibody production rate of the transfected cells.

Table 14: IgM617 transfections with different DNA/PEI mixture amounts

sample	µ [1/d]	qP [pg/c/d]
IgM617_1 (4 mL DNA/PEI mixture)	0.27	13.92
IgM617_2 (2 mL DNA/PEI mixture)	0.46	11.73

Table 14 shows the difference in growth rate and specific productivity of transfections with the IgM617 antibody where different amounts of DNA/PEI mixture were added in the transfection process. A lower amount of DNA/PEI mixture leads to an increased growth rate because the cells have a lower burden of plasmids but at the same time also shows a lower productivity than the cells transfected with a higher amount of DNA/PEI mixture. Therefore, the difficulty in performing transient transfections for protein production is, to find the right ratio of PEI to DNA as well as the right amount of DNA/PEI mixture to transfect the cells to yield the maximal possible amount of protein.

The silver gels and Western blots show that all three cell lines produce mainly the pentameric IgM form. The Western blots of stable and transient expression show that IgM012 and IGM012_GL produce higher amounts of dimers than the IgM617 antibody. Overall, it could be shown that neither the host cell line nor the expression system influence the polymer distribution of the three IgM antibodies.

The flow cytometry analysis of the intracellular HC and LC showed that there are two peaks which correspond to two cell populations which have taken up and produce different amounts of HC and LC. However, only one broad peak would have been suspected with cells which all contain different amounts of HC and LC instead of two separate peaks. Only a very small part of the sample peaks is underneath the control peaks which corresponds to a high transfection efficacy.

6.3. Binding of IgM617 to gangliosides on cell surfaces via fluorescence microscopy

Fluorescence microscopy was used in this project to check if the setup of the staining protocol which is basically the same as for the flow cytometry protocol to detect gangliosides on cell surfaces is working and if fluorescence due to the binding of IgM617 to gangliosides can be observed. In advantage, several IgM617 lots were tested before the start of the flow cytometry experiments. Besides the in-house produced and purified antibody through stable expression with the IgM617 cell line, also a deep-frozen lot of IgM617 which was produced by Polymun Scientific GmbH company and purified by the working group of Rainer Hahn was tested with the fluorescence microscopy protocol. The in-house produced IgM617 antibody thereby showed the best fluorescence signal. In the analysis of a silver-stained SDS gel and Western blot of the different IgM617 lots the in-house produced antibody showed the least impurities. Because of the high fluorescence signal observed and the purity of the in-house produced IgM617 antibody this one and was used for the flow cytometry experiments to detect gangliosides on the cell surface.

The differences of the fluorescence microscopy protocol to the flow cytometry protocol for the detection of gangliosides on cell surfaces are listed in table 15.

Table 15: Differences of the staining protocol for fluorescence microscopy and flow cytometry

Fluorescence microscopy	Flow cytometry
Cells were grown and stained in 6-well plates	Cells were stained in suspension in tubes
No trypsinization	Trypsinization
Shorter staining procedure	Longer staining procedure
More antibodies used in total (IgM617 and µ-FITC)	Less antibodies used in total (IgM617 and µ-FITC)

Although fluorescence could be observed for all three tested cell lines, the signal for U87-MG was weaker than that for the positive control cell line CHO-K1 and MDA-MB-231 cell line.

For the fluorescence microscope two plates were stained. The second plate showed weaker fluorescence compared to the first plate. In this context it is questionable if the long incubation time (adjusting the microscope and taking pictures with the first plate took time) lead to internalization of cell surface receptors and decrease of the fluorescence signal or if the weaker signal was really due to the different IgM617 lot and purification method.

6.4. Establishment of flow cytometry protocol for assessing binding of IgM617 to gangliosides on cell surfaces

In the first round of flow cytometry analysis, the results were not unambiguous and the positive and negative control peaks were not separated well. Therefore, the staining procedure was tested with fluorescence microscopy (see 5.4). After it could be proven with the fluorescence microscope that the antibody assay for binding of IgM617 to gangliosides works, different experiments were made to improve the initial flow cytometry protocol and improve peak separation.

As it was suggested in various protocols for flow cytometry that trypsinization might alter the cell surface the effect of trypsin was tested on CHO-K1 cells. The objective of this experiment was to see if trypsin changes the pattern of gangliosides on the cell surface. The analysis with the in suspension growing CHO-K1 cell line showed that trypsin has no influence on the fluorescence signal seen but only on the FS/SS behaviour. It was important to see in this experiment that trypsin does not modulate the cell surface of the cells in a way that it destroys gangliosides or leads to their internalization.

Further experiments with the MDA-MB-231 cell line with different concentrations of IgM617 and anti-µ FITC antibody resulted in the following improvement of the flow cytometry protocol:

- The separation of the peaks is best for the 1:80 dilution of the µ-FITC antibody
- The separation of the peaks is best for the 100 µg/ mL concentration of the IgM617 antibody

These two points contributed to the improvement of the flow cytometry protocol. Further changes in the protocol were that the incubation steps were carried out at 4°C and with end-over- end mixing. Centrifugation times were shortened form 10 min to 5 min and also carried out at 4°C to shorten the whole staining procedure. The low temperature throughout the procedure and shorter time should help to keep the cell membranes intact. In addition, from the timepoint of addition of the µ-FITC conjugate the stained cells were kept in the dark to avoid bleaching and reduction of the fluorescence signal.

From the experiments with different IgM617 concentrations could be seen that with increase of the IgM617 concentration also the peak separation increased. 100 µg/mL was the highest concentration tested and lead to the best separation. But saturation has not been reached yet with this antibody concentration. As it is not economic to further increase the IgM617 concentration, it would be interesting to decrease the amount of cells used for flow cytometry analysis to for example $0.5*10^6$ cells instead of $1*10^6$ cells in further experiments.

Table 16: Improvement of peak separation with the optimized flow cytometry protocol

Cell line	Δ fluorescence signal	Ganglioside-positive cells
MDA-MB-231	11.84	88.6 %
COR-L88	0.31	10.3 %
U87-MG	2.24	31.0 %
MEWO	13.70	79.7 %
HDF5	6.47	28.0 %
Mix: MDA-MB-231 + HDF5	-	28.8 %

The second column in table 16 shows the difference in fluorescence signal between the positive and negative peak of each cell line (seen in figure 34) and therefore the peak separation. This delta value of fluorescence signal was calculated by using the geometric mean of each peak. The best peak separation could be reached with the MEWO (13.70) and MDA-MB-231 (11.84) cell line. COR-L88 (0.31) shows almost no separation between positive sample and negative control peak. The percentages given in the third column are cells of the respective sample which show fluorescence above that of their respective negative control. As can be seen in the flow cytometry diagrams from the improved protocol (figure 32) the separation of the peaks could be improved through the changes made in the staining protocol and maybe also partly due to the more powerful flow cytometer which was used for this analysis.

However, the problem remains that the non-cancerous cell line HDF5 which does not overexpress gangliosides on its cell surface shows more fluorescence signal than the tumour cell lines U87-MG and COR-L88 (figure 34 and table 13). As already mentioned, gangliosides are also expressed on normal cells and overexpressed on various tumour cells. Maybe this overexpression is not sufficient enough to be able to discriminate between normal and cancerous cells. Another explanation is, that the tumour cell lines which were chosen for the project do not show significant overexpression of gangliosides (especially U87-MG and COR-L88) or that long term cultivation of tumour cells changes the cell surface. Furthermore, the different tumour cell lines chosen showed differently strong fluorescence signal for their positive sample and negative controls. With these cell lines it was not possible to determine a cutoff-value of fluorescence signal which would have been a tool to discriminate between cancerous and non-cancerous cells and could have been tested in further experiments also in human blood.

7. Conclusion

It could be shown in this thesis that that IgM012, IgM012_GL and IgM617 can be produced in stable and transient expression. The IgM617 cell line is a higher antibody producing cell line than IgM012 and IgM012_GL in both expression modes. Furthermore, the hypothesis was proven that the IgM cell lines produce a higher antibody yield in stable expression when directly compared to transient expression with the same starting cell densities and culture conditions. If the cell concentrations of the transient batches could be increased to those of the batches with the stably transformed cells and can be performed in a reproducible way, it might be possible to reach as high antibody titers as in the stable production system. The transient transfections with different ratios of HC:LC+JC showed that transfection of less LC+JC plasmid compared to the amount of HC plasmid results in a reduced product titer but no difference in the polymer distribution between the different ratios could be observed.

The stable expression system of CHODG44 produced reproducible cell concentrations and antibody titers, while the titers in the transient expression system in HEK293-6E cells could not be reproduced from transfection to transfection. Nevertheless, IgM antibodies remain hard-to-express proteins because of their pentamer assembly, complex glycosylation and product titers and as high product titers as seen in IgG antibody production cannot be reached yet.

The analysis of the polymer distribution showed that in both expression systems mainly the pentameric form of the IgM antibodies is produced. IgM012 and IgM012_GL produce more dimeric antibody form than IgM617.

The storage experiment with a purified antibody lot of IgM617 suggested that storage at -80°C is the preferred storage form to avoid antibody losses and degradation products of the antibody.

It was proven with the staining protocol for fluorescence microscopy that the in-house produced IgM617 antibody which was produced by the stable CHO-DG44 cell line and purified via a two-step chromatography process binds to gangliosides on cell surfaces. A flow cytometry protocol was established to compare the amount of gangliosides on cell surfaces of tumour cell lines and one non-cancerous cell line. This protocol did not prove to be effective in discriminating between tumour and non-tumour cells by means of their fluorescence signal. Whether this is due to the cell lines used for the experiments, the different autofluorescence of different cell types or the IgM617 antibody cannot be stated definitely and remains a subject to further investigation.

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9. Appendix

9.1. Initial staining protocol for detection of gangliosides on cell surfaces with flow cytometry

Cell preparation:

1. Trypsinization
2. Suspension in Medium with FCS
3. Determination of cell concentration on ViCell

Staining:

1. Centrifuge 1*10^6 cells per sample (10 min, 1000 rpm)
2. Wash in 1 ml PBS-buffer. Add buffer slowly and drop-wise
3. Centrifuge (10 min, 1000 rpm)
4. Wash in 100 µl PBS-buffer/20% FCS
5. Incubate 30 min
6. Re-suspend in 100 µL PBS-buffer/20% FCS with antibody IgM617 50 µg/mL
7. Incubate 30 min
8. Centrifuge (10 min, 1000 rpm)
9. Wash in 100 µL PBS-buffer + 20% FCS
10. Centrifuge (10 min, 1000 rpm)
11. Re-suspend in 100 µl PBS-buffer/20% FCS with antibody anti-hu µ-FITC, 1:50 dilution
12. Incubate 30 min
13. Centrifuge (10 min, 1000 rpm)
14. Wash in 1 ml PBS-buffer
15. Centrifuge (10 min, 1000 rpm)
16. Re-suspend in 200 µl PBS-buffer + DAPI (1:100)

9.2. Purification protocol of working group Hahn for the purification of IgM617 obtained form Polymun

The cell culture supernatant containing IgM was first concentrated by ultrafiltration (UF) and further purified with size exclusion chromatography (SEC).

First 1 L of supernatant was concentrated using a Kvick crossflow ultrafiltration cassette with a 100 kDa cut – off. The volume was reduced to 35 mL, which is equivalent to a 28 fold concentration. The initial flow rate of 6.5 mL/min decreased to 5 mL/min during the process, resulting in a total process time of 3 hours.

Prior to purification an initial run with a smaller column (Superose 6 10 300 GL; CV: 23.562 mL) was conducted to test process parameters, mainly the flow rate. A flow rate of 30 cm/h resulted in sufficient peak separation. 200 µL of concentrated cell culture supernatant were loaded on the column and isocratically eluted with 1x PBS. Cell culture supernatant was filtrated through a 0.22 µm filter prior to application onto the column.

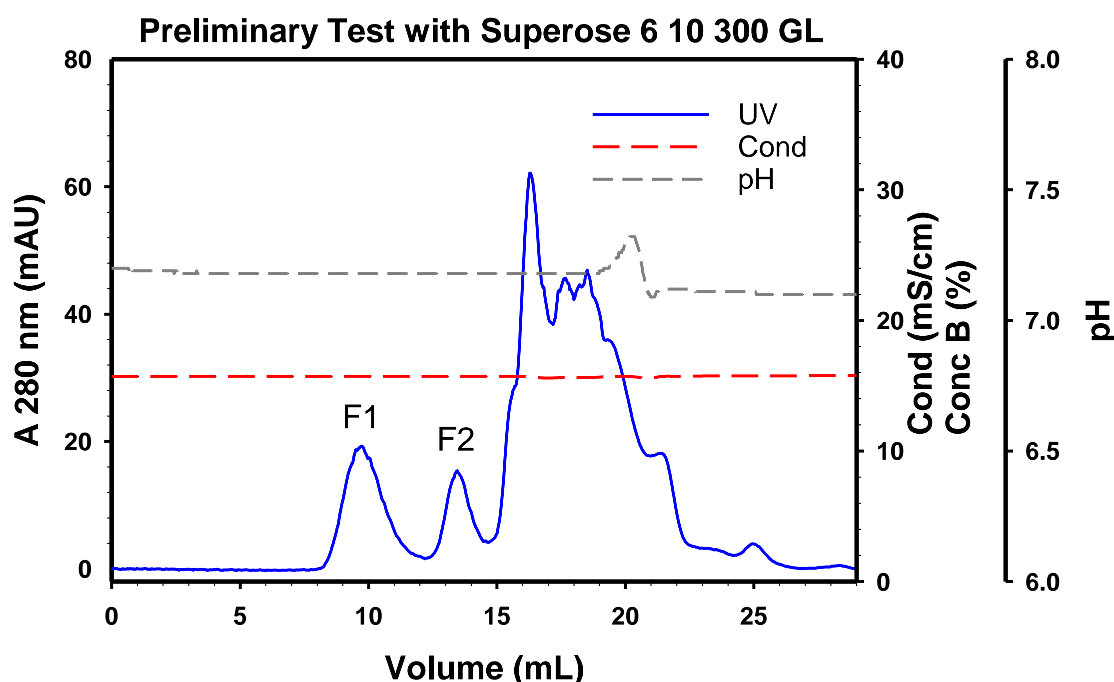
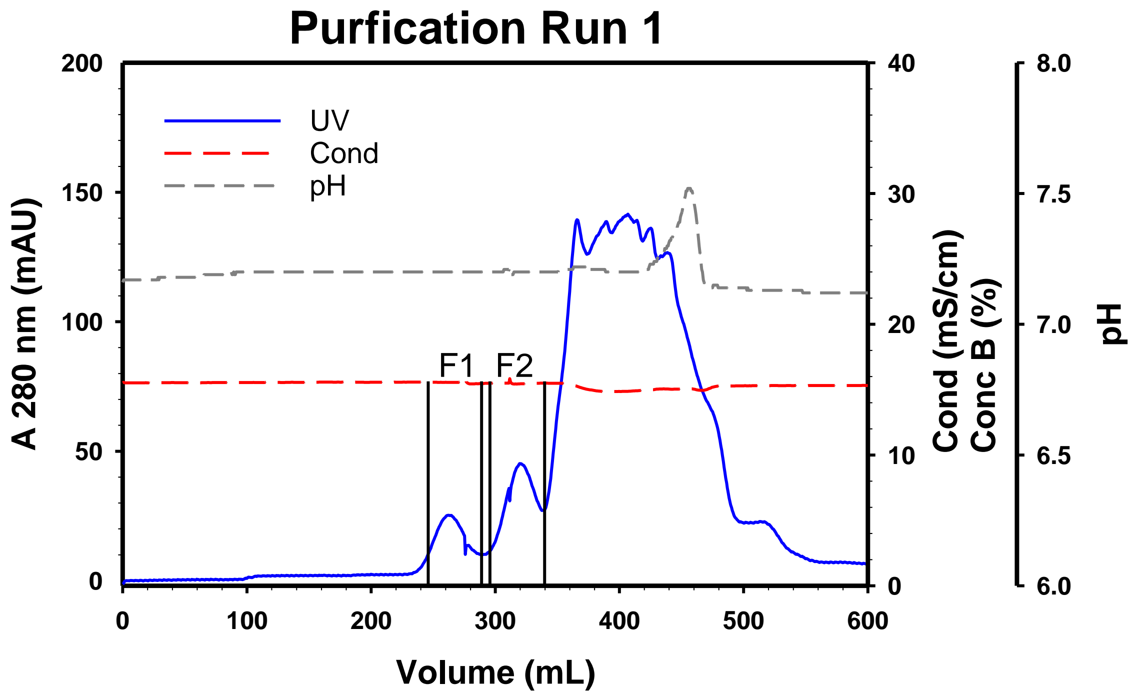


Figure 1: Preliminary test with small SEC column

Comparison of the peak elution time with data from Superose 6 data sheet, provided by the supplier (GE Healthcare), suggests that IgM elutes in the first fraction (F1). For safety, it was decided that the second fraction (F2) will be collected as well.

Next, concentrated cell culture supernatant was purified in two subsequent runs on Superose 6 26x920mm (CV: 488 mL). 15 mL of concentrated cell culture supernatant were loaded on the column in each run. Flow rate was set to 30 cm/h. Isocratic elution was carried out using 1x PBS. Cell culture supernatant was filtrated through a 0.22 µm filter prior to application onto the column.



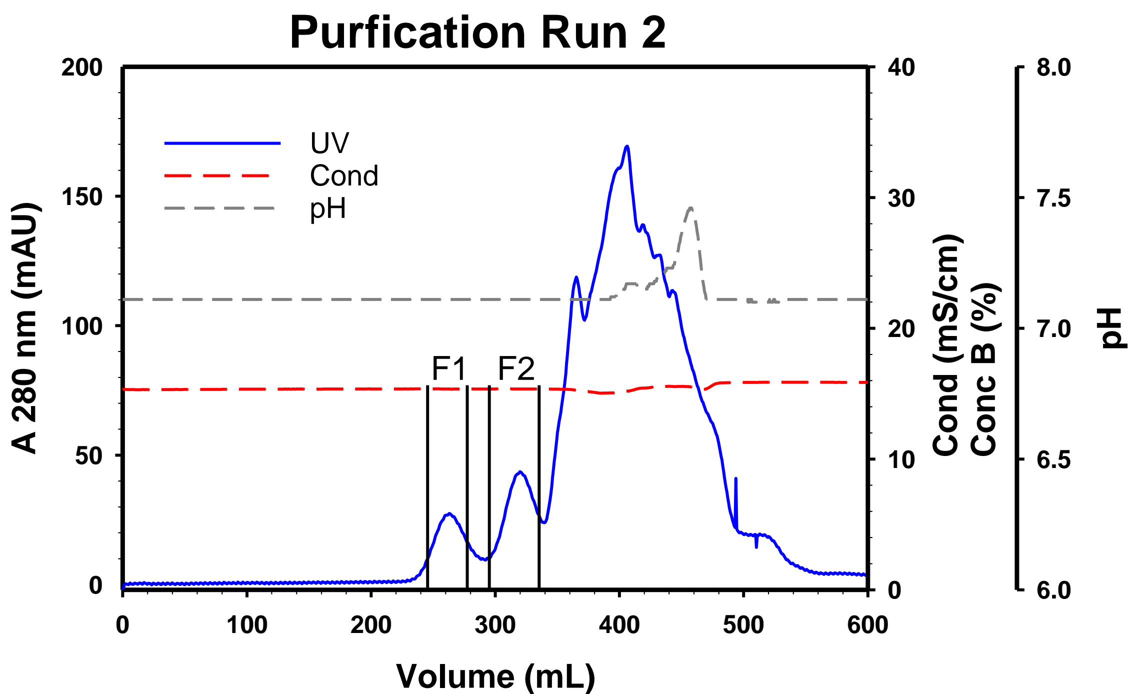


Figure 2: IgM purification on Superose 6 (prep grade)

Integration of the peaks was carried out with Peakfit v4, using an exponential modified Gaussian (EMG) function. Results from peak integration and an estimation of the respective protein concentrations are shown in Table 1.

Table 1: Peak Areas and respective protein concentrations in collected eluates

Run and Fraction	Peak Area [mAU*mL]	Protein [mg]	Concentration [mg/ml]
Run 1 F1	639	2.4	0.05
Run 1 F2	1131	4.2	0.09
Run 2 F1	877	3.3	0.08
Run 2 F2	1241	4.6	0.10

The UV cell of the chromatographic system was calibrated by multiple injections of a defined volume of polyclonal IgG of known concentration into the system and integrating the resulting peak. As a consequence, peak area and protein amount can be related. Protein concentration was obtained by dividing the protein amount by the elution volume of the respective peak. Extinction coefficients of IgG and IgM were assumed to be equal. Unknown variations led to higher peak areas in the second run. Note that these values should be considered as good approximation rather than exact concentrations.

Statutory declaration

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

Vienna, November 2019 Viktoria Trommet

Eidesstattliche Erklärung

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