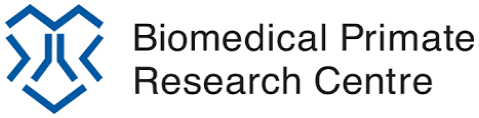
Bachelor´s thesis

Lina Velte



Hamburg University of Applied Sciences

Faculty of Life Sciences

Production of a therapeutically active form of the single chain variable fragment derived from the anti-malaria monoclonal antibody 4G2

in cooperation with the Biomedical Primate Research Centre (BPRC)

Bachelor of Science

in the study programme

Biotechnology

submitted by

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on 23. December 2022

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Affidavit

I, Lina Velte, hereby confirm that the present Bachelor´s thesis was independently written by me without any external or unauthorised aids.

All sources which have been used to finish this work are indicated as such.

Furthermore, I confirm that this thesis has not been submitted as part of another examination process neither in identical from.

22. December 2022, Rotterdam

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Lina Velte

Acknowledgement

Starting this acknowledgment, I first want to thank my professor from my university in Hamburg, Frau Dr. Prof. Gesine Cornelissen, for organizing and helping me with going abroad and establishing contact to the BPRC. Without her this internship and Bachelor´s thesis would not have been possible. Furthermore, she was one of the supervisors of this thesis and was more than helpful throughout this time in the nicest way possible.

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In addition to that he has the Dutch serenity one would expect.

Of course, not to forget, I was lucky to have more than qualified and nice colleagues, Erica, Roberto, and Nicole, who were there for me at all times and answered every of my questions with patience and understanding (even though some of these questions probably seemed ridiculous). In addition to that they were a big help proofreading this thesis.
All in all, I could have a glimpse into the everyday work of a non-profit research centre full of hard working, smart scientists, for which I am more than thankful and from that I will take a lot of new knowledge and experience home.

Lastly, I want to say "thank you" from the bottom of my heart to my family and close friends, who were always and will always be there for me and supported me no matter what and actually encouraged me and kept me sane throughout this whole time.
I am beyond grateful to call all of them my family.

Abstract

Multi-drug resistance has been observed for Plasmodium falciparum in many malaria-endemic regions. The intention is to develop a last-resort antibody-based therapeutic for patients in which drugs fail. For this, the rat-derived monoclonal antibody (mAB) 4G2 was selected as the primary candidate, as it broadly inhibits growth and invasion of all strains of Plasmodium falciparum.

As Fab fragments derived of mAB 4G2 have been shown to have a better inhibition profile compared to the intact antibody, a single chain variable fragments (scFv) of the mAB 4G2 was designed as a polypeptide comprising variable heavy (V_H) and light chain domain (V_L) of an immunoglobulin (IgG) linked through a polypeptide linker. Attempts to produce the scFv 4G2 in a therapeutically active form have failed so far. The protein is produced in high levels in *E. coli* but is deposited in insoluble form in inclusion bodies (IBs).

In this thesis, three approaches were implemented to obtain a therapeutically active form of the product. The first approach was to use the HEK293-F cells to express the scFv 4G2 protein fused to different secretion factors (CD5, Kappa, SEC, all in pSelect vectors) which should initiate a secretion of the protein into the medium. Although the transfection was successful, and the HEK cells expressed the scFv 4G2, it was and not successfully secreted into the medium, but retained intracellularly.

Secondly, it was tried to refold the *E. coli* produced, denatured protein via rapid dilution in various buffers, and lastly different combinations of chaperones were co-transfected to *E. coli* cells with the plasmid containing the scFv 4G2 gene, to increase the levels of properly folded scFv 4G2 protein.

Within the refolding process it could be shown, using a binding assay that was developed during the thesis, that isolated IBs refold properly via the method of rapid dilution in buffers containing high concentrations of arginine (0.66 M or higher).

Within testing various chaperones combinations (Skp in MCS1 and Trigger Factor in MCS2 = combination 1, Skp in MCS1 and GroEL = combination 2, GroEL/ES in MCS1 and Trigger Factor in MCS2 = combination 3, and grpE and clpB = combination 4, respectively) that were co-transfected to get a properly folded scFv 4G2 in the cytosol rather than intracellularly formed as IBs, chaperone combination grpE and clpB (combination 4) in pBB540 vector showed to most promising results, as co-transfection resulted in the strongest binding response on a spot blot. This indicates a higher yield of properly folded and active scFv 4G2.

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List of abbreviations

%	Percent
–	Minus
&	And
/	Per
~	About
+	Plus/positive
<	Smaller than
>	Bigger than
µg	Microgram
µL	Microliters
aa	Amino acids
AB	Antibody
AMA1	Apical membrane antigen 1
AU	Absorbance units
BCIP	5-Bromine-4-chlorine-3indoxylphosphate
bp	Base pairs
BSA	Bovine serum albumin
C	Celsius
c	Concentration
CD5	Cluster of differentiation 5
CO_2	Carbon dioxide
Demi. water	Demineralized water
DEPC	Diethylpyrocarbonate
D_f	Dilution factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DoE	Design of experiments
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FT	Flow through
Fv	Variable fragment
g	Gram
GFP	Green fluorescent protein
GIA	Growth inhibition assay
GSH	Glutathione (reduced)
GSHH	Glutathione (oxidized)
HEK cells	Human embryonic kidney cells
His-tag	Polyhistidine-tag
hr/ hrs	Hour/ hours
IB	Inclusion bodies
IgG	Immunoglobulin G
IMAC	Immobilized metal affinity chromatography
kDa	Kilo Dalton
L	Liter
LB	Luria Broth
LN_2	Liquid nitrogen
M	Mol
m	milli
mA	Milli Ampere
mAB	Monoclonal antibody
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
min	Minute(s)
mL	Milliliter
mM	Millimole
mS	Milli Siemens
NBT	Nitro blue tetrazolium chloride

ng	Nanogram
nm	Nano meter
OD	Optical density
PBS	Phosphate buffered saline
PEI	Polyethyleneimine
Pf	*Plasmodium falciparum*, P. *falciparum*
PI	Protein inhibitors
PNPP	p-Nitrophenyl phosphate
PVM	parasitophorous vacuole membrane
RBC	Red blood cells
RCF	Relative centrifugal force
rf	Refolded
RPM	Revolutions per minute
RT	Room temperature
sc	Single chain
SDS	Sodium dodecyl sulfate
TBE	Tris boric acid EDTA
TCEP	Tris(2-chlorethyl)phosphine
TE	Tris EDTA
TEV	Tobacco Etch Virus
TF	Trigger factor
UV	Ultraviolet
V	Volume/ Volt
V_H	Variable heavy chain
V_L	Variable light chain
x	Times
Zeocin	Phleomycin D1 – copper chelating glycopeptide antibiotic
α	Anti

Index

1. Introduction

Malaria is a tropical and one of the most important and dangerous infectious diseases worldwide. It occurs endemically in (sub-)tropical regions of almost all continents in about 100 countries. An article from 2015 of the Robert Koch Institute (RKI) states that about 40 % of the world's population lives in malaria endemic areas. An estimated 200 million people fall ill there every year. Furthermore, they state that worldwide about 600,000 people die of malaria every year, about three quarters of them are children under five. Malaria is predominantly acquired in countries as Africa, Asia and South America, with Africa being the most affected, accounting for about 90 % of cases. [Robert Koch Institue (RKI) 2015] Most of the severe morbidity and mortality occurs through infection with *Plasmodium falciparum*.

The Biomedical Primate Research Centre – BPRC, which is a non-profit research institute located in the Netherlands which, amongst many other important areas, investigate and develops possible vaccine candidates against malaria that prevent or reduce infection and minimize its morbidity and mortality.

Several potential molecular targets for vaccine development have been identified already, one of these being P. *falciparum* apical membrane antigen 1 (PfAMA1). [Remarque et al. 2008a]

It is a polymorphic, micronemal protein of apicomplexan parasites that appears to be essential during the invasion of red blood cells. Moreover, AMA1 is a merozoite membrane protein, whose function could be blocked by antibodies (AB) which initial studies in P. *falciparum* parasite cultures have shown. [Remarque et al. 2008b]

Meaning, naturally acquired ABs to PfAMA1 play an important role in protective immunity to infection. One of which is the rat heterohybridoma derived monoclonal antibody (mAB) 4G2, which recognizes PfAMA1 that inhibits red blood cell invasion by interfering with the reorientation and binding of the merozoite to the erythrocyte surface. [PhD Bart Faber, personal communication] Hereby, former studies have already proven that Fab fragments derived of the mAB 4G2 inhibit invasion markedly more efficient than intact ABs. [Collins et al. 2007] Which is why the focus in this thesis lies on the production of scFv mAB 4G2.

1.1 Objective

The aim of the project described in this thesis is to produce a therapeutically active form of the single chain variable fragment (scFv) derived from the anti-malaria monoclonal antibody 4G2. To this end, different approaches were implemented.

First approach was to express scFv 4G2 in HEK293-F cells which will properly fold and secret the protein into the medium. Therefore, various secretion factors will be used and tested.

Secondly, a DoE optimization strategy will be applied for refolding the scFv 4G2 produced in *E. coli*, as the *E. coli* product is found in inclusion bodies (IB) and not properly folded. This will be approached via a rapid dilution in 18 different buffers.

Lastly, a co-transformation of the scFv-construct with plasmids coding for a variety of chaperone proteins will be applied in order to increase the level of correctly folded 4G2 in *E. coli*.

To assess the latter two procedures, a binding assay will be developed, which will also be instrumental to test expression in the HEK cell approach. To assess secreted expression, a control plasmid will be constructed.

The above stated approaches will be described in four chapters: development of a binding assays using 4G2 Fab fragments, production of scFv 4G2 in HEK293-F cells, refolding using rapid dilution, and chaperones as helper-proteins for refolding scFv in *E. coli* cells.

2. Theoretical background

To give a better understanding of the in this thesis presented and described approaches and results, the next chapters will give a brief theoretical background, starting with PfAMA1.

2.1 *Plasmodium falciparum* apical membrane antigen 1 (PfAMA1)

Apical membrane antigen 1 is a micronemal, apicomplexan protein and derives from a single essential gene present in all *Plasmodium* parasite species.

Figure 1 shows a schematical overview of a P. *falciparum* invasion and egress.

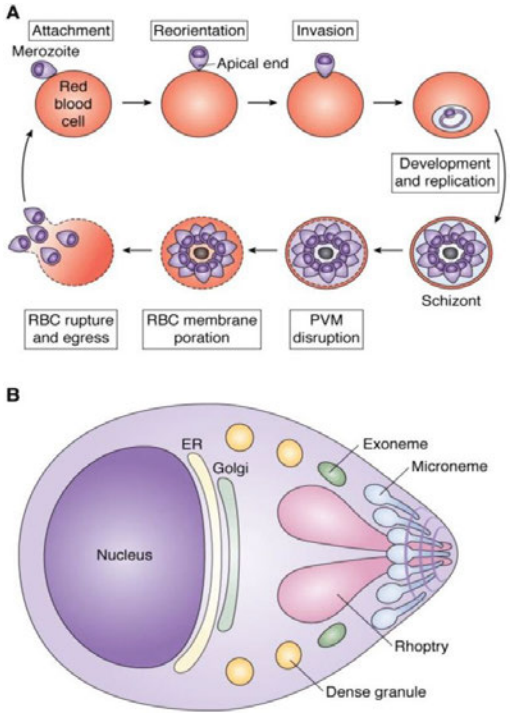


Figure 1: *Cycle of a P. falciparum invasion and egress.*

A: first, attachment of an infective merozoite to a red blood cell (RBC, top left). Then reorientation, so that its apical end is in contact with the host cell, and invades. It develops, replicates and divides, forming a schizont (bottom right). When ready to egress, it disrupts the parasitophorous vacuole membrane (PVM), then the RBC membrane and finally merozoites rupture out of the RBC (bottom left).

B: Overview of a merozoite. It´s showing its secretory organelles whose function is influenced by plasmepsins. First, exonemes (here:green) discharge to initiate egress. Then micronemes (here: blue) discharge to secrete RBC adhesion ligands onto the cell surface. Next, rhoptries (here: pink) discharge to prepare parasite and host cell for invasion and to condition the PVM. Lastly, dense granules (here: yellow) discharge to effect maturation of the parasitophorous vacuole.

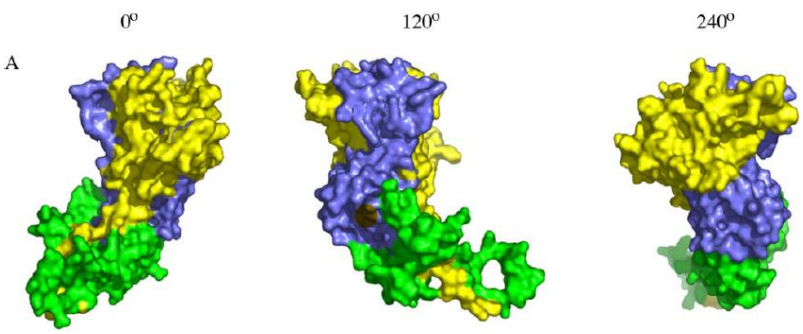
Source: https://www.researchgate.net/figure/Plasmodium-invasion-and-egress-A-An-infective-merozoite-top-left-attaches-to-an-RBC_fig8_341140296

During the invasion of host cells, AMA1 is an essential merozoite membrane protein.

Moreover, it is polymorphic which could be "a result of immune selection operating on an important target of naturally occurring immunity" as it is described in the article "Apical membrane antigen 1: a malaria vaccine candidate in review" from Remarque et al. 2008.

Furthermore, it is a structurally conserved type I integral membrane protein varying between 556 to 563 amino acids (aa) in most *Plasmodium* species. From that of ~ 50 aa are located in the cytosolic region. Meaning, the majority of AMA1 forms the ectodomain, within which 16 conserved cysteines contribute to disulfide bonding. Which led to the conclusion that the mature

ectodomain folds as an N-terminal pro-sequence and three domains (DI, DII and DIII) as shown in Figure 2, whereas DIII is known for direct binding to the RBC membrane proteins Kx on trypsin-treated erythrocytes. And DI and DII form PAN modules, often implicated in protein-protein and protein-carbohydrate interactions. [Remarque et al. 2008b]



Yellow: domain I.

Blue: domain II.

Green: domain III.

Figure 2: *A molecular surface representation of PfAMA1, showing its three domains from different angles (0 °, 120 °, 240 °).*

Source: [Collins et al. 2007]

AMA1 is processed proteolytically, as most micronemal proteins. Whereas, when merozoites are released and RBC are invaded, the pro sequence is cleaved away. In PfAMA1, the 83 kDa precursor protein $PfAMA1_{83}$ is converted to 66 kDa $PfAMA1_{66}$, an event preceding the re-localisation of AMA1 around the merozoites.

The in this thesis used antigen 'AMA1 FVO' was developed at the BPRC, whereas FVO is one of the naturally occurring alleles and has a MW of 66 kDa.

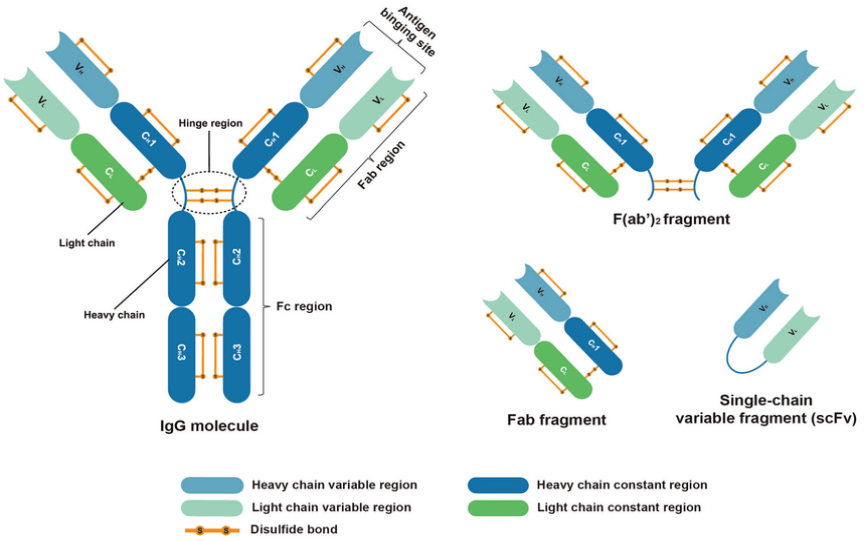
At or around the point of invasion, the bulk of the ectodomain is then shed quantitatively to a $PfAMA1_{48}$ component by the membrane-bound subtilisin-like 'sheddase' PfSUB2.

This proofs that AMA1 has a crucial role in the invasion biology of *Plasmodium* parasites. Sequestered initially in micronemes, AMA1 ectodomain then localises to the parasite surface, at which point its role in invasion can be interfered with by antibodies. [Remarque et al. 2008b]

Especially monoclonal antibodies (mAB) raised against PfAMA1 can inhibit erythrocyte invasion and protect against the disease. One of such mABs is 4G2. Since PfAMA1 is also expressed by the form of parasite injected by the mosquito vector (sporozoites), and anti-PfAMA1 mABs inhibit both hepatocyte and erythrocyte invasion, PfAMA1 is indeed an attractive target for vaccine development. [PhD Bart Faber, personal communication]

2.2 Single chain variable fragment derived from the mAB 4G2

In general, the structure of an immunoglobulin (IgG, antibody) is shown in Figure 3.



Left: whole IgG with all its heavy and light chain, constant and variable regions.

Right top: F(ab')$_2$ fragment. Right bottom: single Fab fragment and a single chain variable fragment linked together via a linkage.

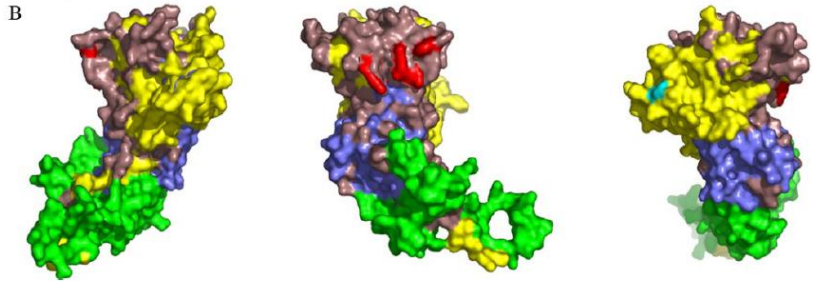
Figure 3: *Overview of a whole IgG and its fragments. Source: https://www.researchgate.net/figure/Structure-of-IgG-molecule-and-its-fragments-Fab-2-fragment-Fab-fragment-and_fig1_350057723*

On the left side of Figure 3 the whole structure of an IgG is shown. It consists of mainly three parts: Fab fragments with heavy and light chain variable and constant regions (x2) attached via disulfide bonds, the hinge region (consisting of disulfide bonds), and the Fc region only consisting of heavy chain constant regions. Whereas the Fab fragments show the antigen binding site.

On the right side of Figure 3, the three components in which the whole AB can be cleaved are pictured. First, the F(ab')$_2$ fragment, which can be produced via a pepsin-induced cleavage containing both Fab fragments of the AB still linked via the hinge region. Secondly, the Fab fragments which can be cleaved via the enzyme papain. This procedure was done in this thesis to use the Fab fragments as a binding (+) control. Thirdly, the single chain variable fragment (scFv) which are contiguous polypeptides, consisting of only the heavy and light chain variable regions of an intact AB, showing the antigen binding site, linked together through a polypeptide linker. Same as the in this produced and used scFv derived from the anti-malaria monoclonal AB 4G2. Lastly, the Fc region which will not play a further role in this thesis.

The monoclonal antibody 4G2 is an invasion-inhibitory AB that binds to a conserved epitope in the ectodomain of the essential P. *falciparum* microneme protein and vaccine candidate AMA1.

This epitope lies within a functionally critical region of the molecule. [Collins et al. 2007] Figure 4 shows the critical region coloured in red.



Yellow: domain I.
Blue: domain II.
Green: domain III.
Red: DII region for 4G2 binding.
Brown: DII region not required for 4G2 binding.

Figure 4: *A molecular surface representation of PfAMA1, showing its three domains from different angles (0 °, 120 °, 240 °).*
Source: [Collins et al. 2007]

It already has been proven that the Fab fragments of the whole IgG 4G2 inhibit invasion markedly more efficiently than the intact mAB, just as for scFvs. Meaning, the mechanism by which 4G2 interferes with the invasion, is neither due to cross-linking of exposed PfAMA1 molecules nor agglutination of free merozoites. Both of which can only be mediated by divalent intact ABs as Collins et al. states in their article about "Fine mapping of an epitope recognized by an invasion-inhibitory monoclonal antibody on the malaria vaccine candidate apical membrane antigen 1". This phenomenon has been observed in the case of some invasion-inhibitory anti-merozoite ABs before. But given the smaller mass of Fabs (and scFvs) compared to that of an intact IgG, initial studies have already discovered that mAB 4G2 interferes with invasion not simply as a result of steric effects but because it interferes directly with the function of a critical domain of PfAMA1. This critical domain lies exclusively within the loop of DII, which is conserved in all P. *falciparum* variants. [Collins et al. 2007]
Since it is known that Fab fragments and scFv give a higher inhibition level, it is hypothecianticipating that scFvs will have a similar efficacy. Therefore, this thesis dealt with the production of a therapeutically active form of the scFv derived from the anti-malaria mAB 4G2. The sequence of this scFv, starting with the heavy chain part followed by a linker and the light chain part of the epitope recognizing regions, is depicted below. For a better and easier purification of the produced scFv, a 6x-His-tag was attached to it, also useful for detection with anti-hexahistidine antibodies.

MGAQCEVQLVESGGGLVQPGRSLKLSCAASGFTYSNYVMAWVRQAPTKGLEWVASIST
GAYNTYYRDSVKGRFTISRDNAKNTLYLQMDSLRSEDTATYYCAKDIDNNYDYFDYW
GQGTSVTVSSGGGGSGGGGSGGGGSISIELTQSPSLLSASVGDRVTLNCKASQAFYIKLA
WYQQKLGEAPKLLIYDSNRLQTGIPSRFSGSGSGTDFTLTISSLQPEDVATYFCHQYHSGS
GTFGGGTKLELKRNVDHHHHHH

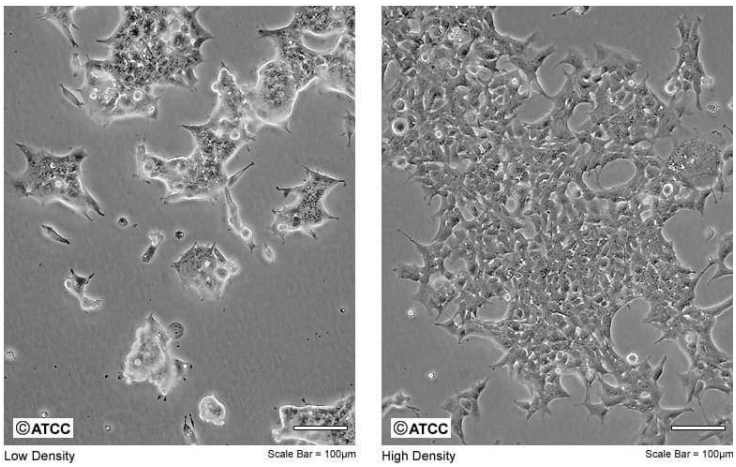
2.3 HEK293-F cells

HEK cells are human embryonic kidney cells, which have been immortalized due to a transformation with the sheard adenovirus 5 (Ad5) with E1A and E1B genes. The '293' refers to the number of experiments it took.

Since its establishment in the 1970s, this robust and fast-growing cell line and its derivatives have been used extensively in various fields of science e.g., cancer research or large-scale protein expression. As of today, it is one of the most widely used cell lines in research.

Initially the cell line was established from a female foetus of unknown parentage. Due to the addition of Ad5 E1A and E1B genes to the HEK293 genome, the cell line was immortalized, as aforementioned, by interfering with cell cycle control and preventing apoptosis, respectively.

Figure 5 shows a HEK293 cell culture under the microscope.



Left side: HEK293 cells at a low density.

Right side: HEK293 cells at a high density.

Figure 5:*Mircoscopic picture of a HEK293 cell line.*
Source: [Synthego]

The HEK293 cell line has in total twelve derivatives, one of which is the HEK293-F cell line used during the following experiments. Compared to HEK293 cell line which normally grows as an adherent monolayer, HEK293-F cells grow in high-density serum-free suspension cultures. [Synthego]

Since culturing the cells and the overall purification of the before transfected protein is cost-intensive and time consuming, in this approach various secretion factors are tested to help secrete the intracellularly expressed protein into the medium to be able to purify it easily. Therefore, from three different secreted proteins were genetically engineered in front of the scFv 4G2 gene: the one used by the CD5 molecule, the one used by the Kappa light chain of IgG, and the artificially designed SEC (secrecon) secretion factor, all in pSelect vectors.

Sequence maps of the secretion factors can be seen in the Appendix 8 – 10.

2.4 Inclusion bodies

Inclusion bodies (IB) are aggregated and dense proteins, often spherical granulates, and mostly formed in prokaryotes due to overexpression of extrinsic, recombinant, or eukaryotic proteins. Nevertheless, protein aggregation is generally a regulated phenomenon in most type of cells. Multiple factors contribute towards the formation of IBs, sometimes even as a pathological protection mechanism of the cell. Many more factors can contribute to an increase of formed IBs within the cells, for example a high copy number of the target gene or the reducing environment of bacterial cytosol as Singh et al. sated in their review form 2015 about “Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process”. [Singh et al. 2015]

In *E. coli* e.g., IBs occur normally intracellularly as a result of the aforementioned non-folded, misfolded or overexpressed non-native proteins. Especially for heterologous proteins, bacterial cells are often missing the right folding machinery. Such is the case for the in this thesis attempted production of the eukaryotic scFv 4G2 in *E. coli*.

Figure 6 shows briefly the formation of IBs in *E. coli* cells and already states a possibility to refold them via an in vitro rapid dilution which plays an important role in this thesis.

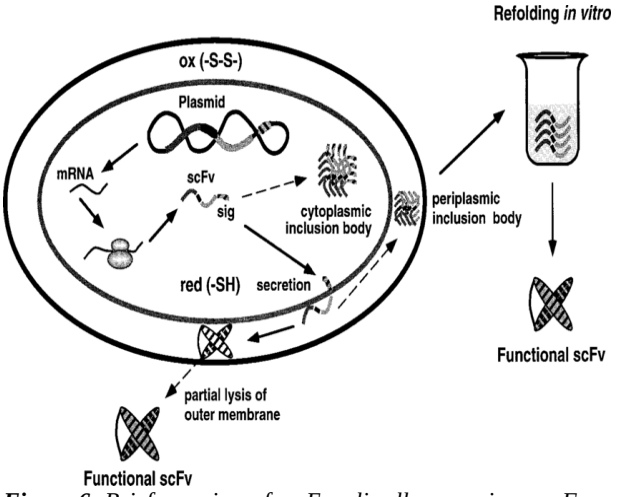


Figure 6: *Brief overview of an E. coli cell expressing a scFv and missfolding it to IBs.*

2.5 DoE

As in chapter 2.4 already indicated, a method of refolding proteins from IBs is via rapid dilution in different refolding buffers. Since in initial experiments (data here not shown) one buffer, here called “buffer 10”, showed the best results regarding refolded proteins from before isolated IBs, the aim was to optimize it regarding its composition (see 4.2.3). Therefore, DoE was used.

Design of Experiments (DoE) is a systematic, most cost-efficient, and fastest way to design effective experiments. It enables to get a more precise understanding of the relationship between multiple input variables or factors and key output variables or responses. It is a structured approach for collecting data and making discoveries.

DoE was firstly introduced by Ronald Fisher 1926. At that time, it was about the factorial principle, randomization, replication, and blocking. Generating and analysing these designs relied primarily on hand calculation. [JMP Statistical Discovery LLC]

For today, there are various computer-generated designs or systems to use for a more effective and efficient DoE, such as the here used system “MODDE” developed by Sartorius AG.

Generally, DOE proceeds as illustrated in Figure 7.

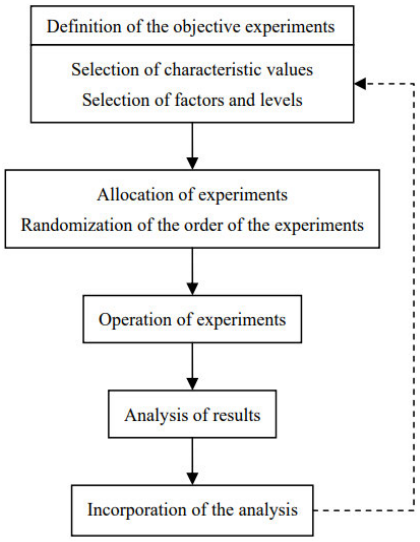


Figure 7:*Overview of a general DoE process.*
Source: [Gyung-Jin Park 2007]

There are three main approaches which can be implemented: one factor at a time (OFAT) or one-way factorial design, two-way factorial design, and three or more factors at a time.

The OFAT is one of the simplest DoE theories. It is a method for allocation of experiments when the number of factors is one and the number of values of the factors is more than one. For each, experiments are repeatedly performed. [Gyung-Jin Park 2007]

The two-way factorial design is about changing two factors at the same time, which provides a better information about the location of the optimum. Since the latter two approaches are not used during this thesis, it will be focused on the DoE with three variables. Regarding the rapid dilution and the buffers in which the dilution is implemented and its composition, three variables were set: pH, arginine concentration in M and sucrose concentration in M (see Appendix 1). Via the DoE program "MODDE" a D-optimal design was established. Meaning, from three primary experimental objectives the "optimization" design was chosen. As the "screening" was already done in previous experiments to identify influential factors and to determine a range in which these should be investigated. Now, the interest lies in defining which combination of the important factors will result in optimal operating condition. Since optimisation is more complex than screening, it requires more experiments per factor. [Prof. Dr. Gesine Cornelissen, personal communication]

Figure 8 shows the D-optimal design of the experiments. Hereby, establishing a cube model with a symmetrical distribution of experimental points.

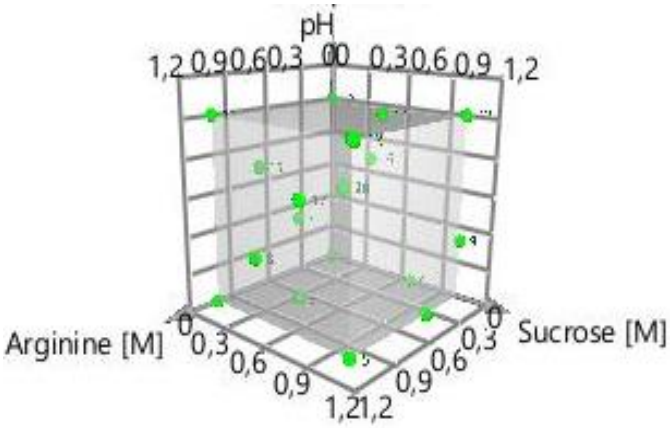


Figure 8: *D-optimal design region of buffer compositions in correlation with number of refolding experiments.*

Y-axis: pH, varying from 6.0 over 6.8, 7.7, and 8.5.

X-Axis: Arginine [M] varying from 0 over 0.33, 0.66 and 1.0 M.

Z-Axis: Sucrose [M] varying from 0 over 0.33, 0.66 and 1.0 M.

Source: MODDE program.

Figure 8 will be described in detail in chapter **Error! Reference source not found.**.
For detecting the binding response of the properly folded protein, a spot blot is foreseen.

2.6 Chaperones

Another and the last approach described in this thesis is the use of co-transfected chaperones. Molecular chaperones are proteins that bind to non-native conformations of the proteins and assist with the non-covalent folding reaching the native steric structure. [Sonoda et al. 2011] They are present in all organisms and are essential for cell survival.
Figure 9 shows an overview of chaperones and their function within the cells.

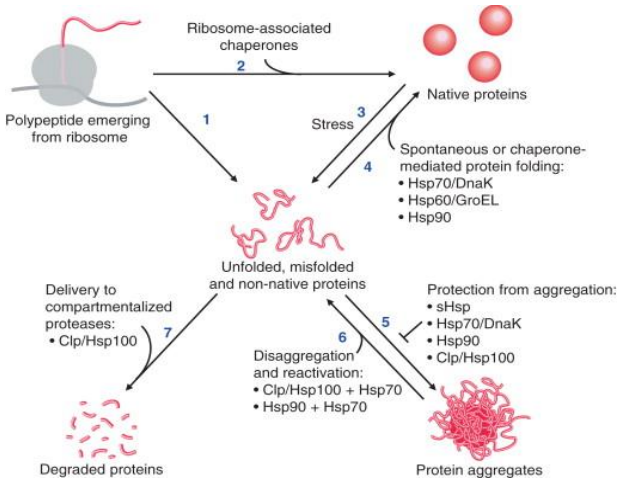


***Figure 9**: Overview of chaperones and their function within the cells.*
Source: [Brenner and Miller 2014]

The figure demonstrates that native proteins will be correctly folded, since their amino acid sequences contain the right information required to adopt their proper conformation.
In case of the in chapter 2.4 mentioned reasons (e.g., overexpression of heterologous proteins), protein aggregates often form, resulting in IBs. This happens due to missing folding machineries or the limitation of their pre-existing chaperone capacity. [Marco et al. 2007]
Other reasons for the emergence of unfolded proteins could be due to cellular stress (e.g., heat shock), oxidative stress or pathological conditions as Marco et al. explain in their article "Chaperone-based procedure to increase yields of soluble recombinant proteins produced in *E. coli*" from 2007.
Therefore, a combination of various molecular chaperones are used to decrease mis- or non-folded proteins which lead to IBs or degraded proteins, while increasing the yield of proper folded proteins and minimizing harmful aggregates. [Brenner and Miller 2014]
All these advantages are also used in this thesis by co-transfecting four different combinations of chaperones: Skp in MCS1 and Trigger Factor in MCS2 which will be stated as combination 1, Skp in MCS1 and GroEL which will be stated as combination 2, GroEL/ES in MCS1 and Trigger Factor in MCS2 which will be stated as combination 3, all in in the pACYCDuet-1 derivative plasmid, and lastly GrpE and ClpB in the plasmid pBB540 which will be stated as combination 4. Each co-transfected with scFv 4G2 to *E. coli* DE3 cell cultures. Whereas e.g., Skp is a major periplasmic chaperone helping to increase solubility and yields of scFv when co-transfected, and GroEL/GroES and trigger factor (TF) are on the other hand cytoplasmatic chaperones. [Sonoda et al. 2011]

All plasmids were designed to allow for IPTG-inducible expression of chaperone genes.

IPTG is hereby a chemical reagent, that acts as allolactose. When it enters the cell, it removes a repressor from the lac operon. As a result, T7 RNA polymerase binds to the T7 promotor and initiates gene transcription. Which will eventually lead to the synthesis of the protein of interest. [PhD Tyasning Kroemer]

Sequence maps of each chaperone combination can be seen in the Appendix 2 – 5.

3. Material

The following chapter lists any material, that has been used during the different attempts to produce a correctly folded scFv derived from mAB 4G2, starting with the list of chemicals.

Table 1: *List of all used chemicals.*

Chemical	Name	Manufacturer
$(C_2H_5N)_n$, linear form	PEI, Polyethyleneimine 0.5 mg/mL	-
AB buffer (dilution buffer)	PBS buffer with 0.3 % BSA, 0.05 % TWEEN®20	Self-mixed
Agarose	UltraPure™ Agarose LOT: DO22309 CAT: 15510-022 CAS: 9012.36.6	invitrogen™ by Thermo Fisher Scientific
Anti-6x His-tag mAB	Clone: 4A12E4 Concentration: 0.5 mg/mL donor: mouse LOT: UD283823 REF: 37-2900	invitrogen™ by Thermo Fisher Scientific
Apical membrane antigen 1	AMA1 FVO, 3 mg/mL without a His-tag	-
Apical membrane antigen 1	Alexa 488, fluorescent	-
Blocking buffer	PBS buffer with 0.05 % TWEEN®20, 3 % BSA	Self-mixed
$C_{11}H_{12}Cl_2N_2O_5$	Chloramphenicol LOT: SLBR8868V CAS: 56-75-7	Sigma-Aldrich Co. LLC Safety
C_2H_4INO	Iodoacetamide CAS: 144-48-9	Merck KGaA, Darmstadt

C_2H_6O	Ethanol 70 % LOT: I1153783 123 CAS-No: 64-17-5	Merck KGaA, Darmstadt, EMD Millipore Corporation
$C_{34}H_{28}N_6O_{14}S_4$	Trypan blue, 4%	Sigma-Aldrich Co. LLC Safety
C_3H_8O	2-Propanol (Isopropanol) LOT: K51198634 912 CAS-No: 67-63-0	Merck KGaA, Darmstadt, EMD Millipore Corporation
$C_{40}H_{30}Cl_2N_{10}O_6$	Nitro blue tetrazolium chloride (NBT), 50 mg/mL LOT: 0000365950 REF: S380C	Promega Corporation
$C_{47}H_{48}N_3NaO_7S_2$	SimplyBlue™ SafeStain, Coomassie blue stain LOT: 2264057 CAT: LC6065	novex® by life technologies™
$C_{55}H_{86}N_{20}O_{21}S2$	Zeocin™ Antibiotic, 100 mg/mL LOT: 1257932 CAS-No: 46-0509	invitrogen™ by Thermo Fisher Scientific
$C_{58}H_{114}O_{26}$	TWEEN®20 LOT: 029K01853 CAS: 9005-64-5	Sigma -Aldrich Co. LLC Safety
$C_6H_6NO_6P$	SIGMAFAST™ p-Nitrophenylphosphate tablets LOT: SLCB2822	Sigma-Aldrich Co. LLC Safety
$C_8H_8BrClNO_4P$	5-Bromine-4-chlorine-3indoxylphosphate (BCIP), 50 mg/mL LOT: 0000356063 REF: S381C	Promega Corporation
$C_9H_{18}O_5S$	IPTG, Isopropyl-β-D-1-thiogalactopyranoside 100 mM/L	-
Expression Medium	FreeStyle™ 293 LOT: 2470378 REF: 12338-018	gibco
Gel Filtration Standard	#1511901	Bio-Rad Laboratories, Inc.
LB	LURIA BROTH (Miller´s LB Broth Base) LOT: 206161 REF: 12795027	invitrogen™ by Thermo Fisher Scientific
LDS sample buffer, loading buffer	NuPAGE® LDS sample buffer (4x), pH 8.4 LOT: 2201423	invitrogen™ by Thermo Fisher Scientific

MES SDS Running buffer	NuPAGE® MES SDS Running buffer (20x) LOT: 2364979	novex® by life technologies™
NuPAGE™ 4-12% Bis-Tris Gel	Electrophoresis gel LOT: 22051310 REF: NP0322BOX	invitrogen™ by Thermo Fisher Scientific
Papain	Papain from papaya latex 16 units/mg, 24 mg/mL CAS: 9001-73-4	Sigma -Aldrich Co. LLC Safety
PBS buffer	pH 7.4 LOT: 2156430 REF: 10010-015	gibco
Pierce® goat anti-mouse IgG (H+L)	Alkaline Phosphatase Conjugated LOT: OC182745	Thermo Scientific
Pierce® goat anti-rat IgG (H+L)	Alkaline Phosphatase LOT: OH1747021	Thermo Scientific
Protease inhibitor cocktail tablets	C0mplete tablets, mini EDTA-free, EASYpack LOT: 53002700 REF: 04693159001	Roche Diagnostics GmbH, Mannheim
SeeBlue® Plus2 Standard (1x)	Pre-stained LOT: 2126102 CAT: LC5925	invitrogen™ by Thermo Fisher Scientific
TE buffer	Tris EDTA buffer, pH 8	self-mixed
Transfer buffer	Self-mixed (100 mL 10x transfer buffer, 200 mL methanol, 700 mL water)	-

The next table lists all cell lines, that were used for the experiments.

Table 2: *List of all used cell lines.*

Cell line	Name	Manufacturer
E. coli	*Escherichia coli* DH5α	-
E. coli	*Escherichia coli* DE3	-
HEK293-F cells	Human embryonic kidney cells, FreeStyle™ 293-F cells, 1×10^7 cells in one mL	invitrogen™ by Thermo Fisher Scientific

For all experiments using both E. *coli* DE3 and DH5α cells, pre-cultivating was followed by the same procedure, only the working volumes could vary from 3 mL up to 16 mL pre-cultures.

Bacteria were stored as glycerol stocks in a – 80 °C freezer.

For pre-culturing, the cells were prepared in centrifuge tubes with LB medium (25 g/L) and the proper antibiotic to keep the selective pressure high. They were then incubated over night at 37 °C and 180 RPM.

The next day, cell cultures were handled individually depending on the protocol/ experiments which followed.

The HEK293-F cells were cultivated differently, as described in chapter 4.6.
They were frozen on December 12th, 2019. Since then, the cells were correctly stored in liquid nitrogen, approximately 1×10^7 cells/mL in 90 % FreeStyle™ 293 Expression Medium and 10 % DMSO.

The next table is an overview of all used plasmids.

Table 3: *List of all used plasmids.*

Name	Inserted Sequence	Resistance gene	Purpose
p.Jexpress412	scFv4G2	Zeocin*	Expression in *E. coli* cells
pACYCDuet-1	Skp + Trigger factor (TF)	Chloramphenicol**	Chaperones to induce correct folding of scFv 4G2
pACYCDuet-1	Skp + GroEL	Chloramphenicol	Chaperones to induce correct folding of scFv 4G2
pACYCDuet-1	GroEL/ES + TF	Chloramphenicol	Chaperones to induce correct folding of scFv 4G2
pBB540	grpE + clpB	Chloramphenicol	Chaperones to induce correct folding of scFv 4G2
pSelect	scFv4G2	Zeocin	Expression in HEK293-F cells
pSelect	CD5 leader-scFv4G2	Zeocin	Promote secretion into the medium
pSelect	Kappa light chain leader-scFv4G2	Zeocin	Promote secretion into the medium
pSelect	Secrecon (SEC) leader-scFv4G2	Zeocin	Promote secretion into the medium
pSelect	GFP	Zeocin	+ control to validate the transfection method

**Zeocin concentration 1:4000*
***Chloramphenicol concentration 1:1000*

The plasmids containing the 4G2 gene with different secretion signals, were already available. These were transfected to *E. coli* DH5α bacterial cells and stored in a − 80 °C freezer.

The knowledge and help for setting up a DoE, to do the refolding of scFv 4G2 (in p.Jexpress412 vector) produced in *E. coli* DE3 cells was provided from Dr. Prof. Gesine Cornelissen from the HAW – Life Sciences, Bergedorf.

The chaperones were acquired via the "addgene" repository as bacterial stabs. They were grown, plasmids were isolated and subsequently transfected to bacteria at BPRC as described in chapter 4.3.

The next table shows which equipment was used during the lab work.

Table 4: *List of all used equipment.*

Equipment	Type	Manufacturer
Autoclave	-	Omega™ Media
Bunsen burner	Fuego SCS	WLD-TEC GmbH, Göttingen
Cell counter	TC10™ Automated Cell Counter SN: 506BR1845	Bio-Rad Laboratories, Inc.
Centrifugal Devices	Nanosep with 3K Omega 100/pk LOT: FJ3198 REF: OD003C34	PALL Corporation
Centrifuge	Avanti JXN-26	Beckman Coulter
Centrifuges vessels	Different sizes	Greiner bio-one GmbH, Kremsmünster
Column, HPLC	Superose™ 6 Increase 10/300 GL, volume 24 mL LOT: 10308792	Cytiva, Marlborough
Column, IMAC	HiTran™ IMAC FF, 5 mL LOT: 10284516	-
Counting slides	Dual Chamber for cell counting CAT: 145-0011	Bio-Rad Laboratories, Inc.
Cryovials	1 mL cryovials	-
Dounce homogenizer	-	-
Electro blotter	Ancos semi-dry electroblotter model A with electrophoresis Power Supply EPS 500/400	Pharmacia Fine Chemicals
Electrophoresis system	XCell SureLock™	Bio-Rad Laboratories, Inc.
Erlenmeyer flasks	Duran, different sizes	SCHOTT

Fluorescence microscope	DFC365 FX, Type DMI6000 B	Leica Microsystems CMS GmbH
Glas pipettes	Different sizes	VWR & Greiner bio-one GmbH
Heating block	UBD	Grant
HPLC	Aglient 1100 Series	Aglient Technologies
Humidified CO_2 Incubator	MCO-19AIC (UV) SN: 10050227	SANYO Electric Co., Ltd.
IMAC	Biologic DuoFlow	Bio-Rad Laboratories, Inc.
Incubator	innova® 44 – Incubator series	New Brunswick Scientific Co., Inc.
Microplate washer	405 LS microplate washer REF: 405LSRS SN: 2110209	BioTek Instruments, Inc.
Microplates, 96 well	Modified high binding LOT: E21033NG REF: 655092	Greiner Bio-One GmbH, Frickenhausen
Modern blot & gel imaging system	ChemiDoc™ MP Imaging System, Universal Hood III SN: 731BR01080	Bio-Rad Laboratories, Inc.
Nitrocellulose Blotting Membrane	Amersham™ Protan™ Premium 0.45 µm, 300 mm x 4 m LOT: G9998437 CAT: 10600003	GE Healthcare Life Sciences
Pipette tips	Different sizes	RAININ™
Pipettes	Pipet·light, different sizes	RAININ™
Pipetting aid	Pipetboy 2	Integra Biosciences, Biebertal
Platform shaker	innova™ 2000	New Brunswick Scientific Co., Inc.
Roller	Roller 6 basic	IKA®
Scale	CP3202P	Sartorius AG, Göttingen
Scale	572	Kern
Shaker	See-saw rocker, SSL4	Stuart®
Six well plate	Cellstar® 6 well suspension culture plate; sterile, with lid LOT: E1303032 CAT: 657 185	Greiner bio-one GmbH, Kremsmünster
Spectrophotometer/ Fluorometer	DS-11 FX	DeNovix
Sterile Erlenmeyer flasks	With vented cap, different sizes REF: 431143	CORNING
Sterile workbench	Clean Air by BAKER	The BAKER Company, Benelux B.V.

Ultracentrifuge	Optima XE-90 ultracentrifuge	Beckmann Coulter
Ultrasonic Cleaner	Branson 1210	Marshall Scientific
Vacuum pump	Speed VacConcentrater	SAVANT
Vortex	Vortex-Genie® 2	Scientific Industries
Water bath	Water bath 26 L, 260 V SN: BR1819002	VWR, Cambridge

The last table shows all used kits during the experiments.

Table 5: *List of all used kits.*

Kit	Type	Manufacturer
NucleoSpin® Plasmid EasyPure	Plasmid DNA purification Kit LOT: 2209-002 REF:740727-250	Macherey-Nagel GmbH & Co. KG, Düren
QIAfilter Plasmid Maxi Kit	MaxiPrep (25) LOT: 169044201 REF: 12263	QIAGEN GmbH, Hilden

All material and chemicals were provided from the BPRC.

4. Methods

The next chapters give an overview and describe all four approaches as well as all used methods and assays to purify, test and analyse the properly folded and therefore active form of the derived single chain variable fragment (scFv) from the anti-malaria monoclonal antibody (mAB) 4G2, starting with the IMAC.

4.1 IMAC

Immobilized metal affinity chromatography (IMAC) is a common technique used to purify recombinant proteins fused to a short peptide affinity tag. It can be done under denaturing or nondenaturing conditions, relies on the interaction between multiple electron donors on the affinity tag with a transition metal ion (e.g. Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) chelated to a solid-phase support. The typically used affinity tag is Polyhistidine, with a ranging length of 6 – 12 residues fused to the N- or C-terminus of the target, where 6-His is most common and the electron donor is the histidine imidazole ring, which is also the case of in this thesis purified scFv. [2013; Falke and Corbin 2013]

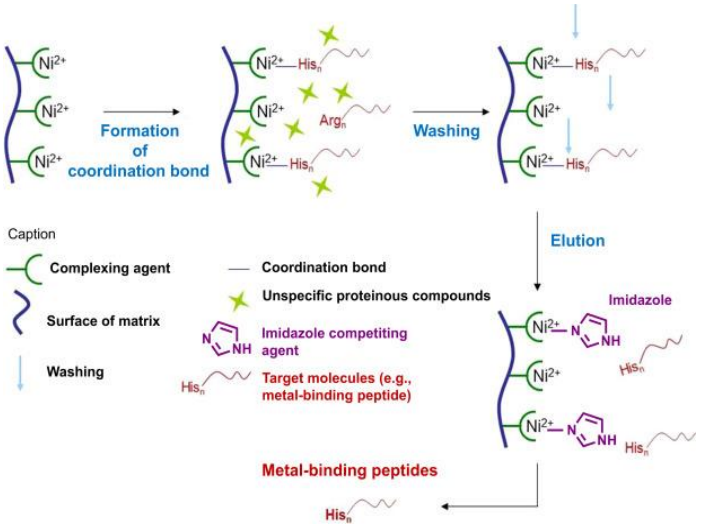


***Figure 10**:Purification process of a recombinant protein fused with a His-tag via a nickel binding IMAC.*
Source: [Falke and Corbin 2013]

First, nickel is bound to the column. To that the protein fused with a 6-His-tag, which is a metal-binding peptide, bind. All the remaining proteins in the sample get washed of the column. The protein of interest gets eluted with a high concentration of imidazole (100 mM), which is a competing agent to the His-tag.

The column used for these experiments is the HiTran™ IMAC FF, 5 mL which binds nickel. Every purification process using IMAC starts with rinsing the column, which is stored in 20 % ethanol, with demi. water until the conductivity is at 0 mS/cm. Flow rate is normally put on 3 mL/min.

Next, the nickel is brought onto the column, which will later bind the 6-His-tag of the target protein 4G2. The flowrate will be put down to 1 mL/min for one minute for this step. Column will turn green.

After, the column is washed again for a few minutes with demi. water at normal flow rate of

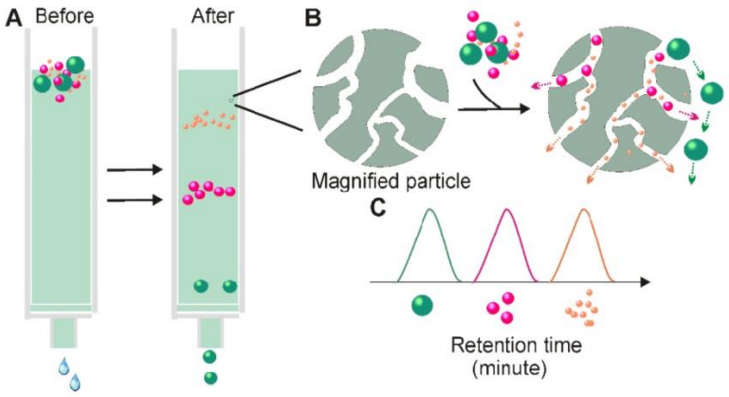
3 mL/min. Column will turn blue, due to pH changes.
Next, the column gets equilibrated with buffer A, containing 20 mM Tris, pH 8.0 and 500 mM NaCl or 8.0 M urea, which should be the same buffer as the buffer the protein is in. Conductivity will increase.
Normal flow rate until conductivity is stable again.
Next step is turning on the UV light for detection and putting on the sample containing the to be purified protein. From the flow-through (FT) a sample is taken (protein-FT).
After, the column is washed with IMAC buffer B containing 20 mM Tris, pH 8.0, 10 mM imidazole and 500 mM NaCl or 8.0 M urea. This step washes off the proteins that haven´t bound to the column. Therefrom, another sample will be taken (protein W10).
The elution of the desired protein is achieved with IMAC buffer C containing 20 mM Tris, pH 8.0, 100 mM imidazole and 500 mM NaCl or 8.0 M urea at normal flow rate until blue protein detection line is back to base. The higher concentration of imidazole elutes the 4G2 because it is a competing agent to the 6-His-tag and will take its binding sites. Therefrom, a sample must be taken (protein E-100), containing the desired protein.
The column is then washed with an EDTA buffer until it appears white again. This step washes off the bound nickel. Another sample is taken (protein EDTA).
As a final step, the column is rinsed with demi. water until conductivity goes back to 0 mS/cm. Here from, a last sample is taken (H_2O). For storage, the column is lastly rinsed again with 20 % ethanol for at least 10 minutes.

All the taken samples are afterwards put on a gel (see chapter 4.3) and the rest was stored in the freezer.

4.2 SEC

Size exclusion chromatography (SEC) or high-performance liquid chromatography (HPLC) is common method of determining the average molecular weight of polymers. It separates on the basis of molecular hydrodynamic volumes or sizes, rather than by enthalpic interactions with the stationary phase or surfaces, which is the case with, for example the prior described IMAC.

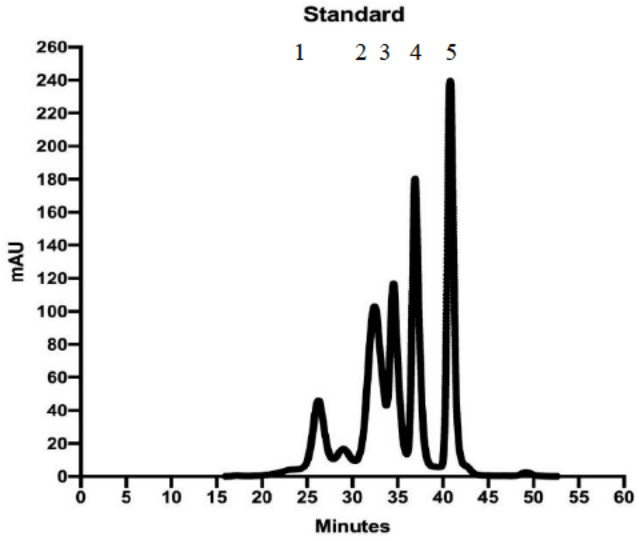
In SEC, the polymer is dissolved in an appropriate dissolvent, which is generally also the mobile phase, and injected onto a column packed with porous particles of fairly defined pore size.
The here used column is the Superose™ 6 Increase 10/300 GL, 24 mL total volume.
As the polymer elutes through the column, molecules that are too large to penetrate the pores of the packing elute in the void volume or interstitial of the column. As the molecular size of the polymer decreases regarding the average pore size of the packing, molecules penetrate into the pores and as a result elute at a later time. Very small molecules, which can freely diffuse into and out of the pores and can sample the total pore volume, elute at the total elution volume of the column. Meaning, that high molecular weight material elutes first from a SEC column, followed by low molecular weight components as shown in Figure 11. [Mori 1999]



Column is packed with porous material, in which molecules can enter. Depending on their size a separation takes place, because big particles are not entering the pores of the column and elute earlier compared to the small ones, which flow through the pores.

***Figure 11**:Principal of a size exclusion chromatography (SEC).*
Source: https://www.researchgate.net/figure/Principle-for-Size-exclusion-chromatography-based-exosome-isolation-When-passing-a_fig5_339544937

Since SEC is not an absolute molecular weight technique rather than a relative, columns must be calibrated with polymer standards of known molecular weight.
The here used standard for calibrating the column was the 'Gel Filtration Standard #1511901' from Bio-Rad Laboratories and samples were normally run with PBS buffer, pH 7.0.
Figure 12 shows the chromatogram of the standard to which the results are compared to.



First peak: Thyroglobulin.
Second peak: Bovine γ-globulin.
Third peak: chicken ovalbumin.
Fourth peak: Equine myoglobin.
Fifth peak: Vitamin B12.

***Figure 12**: Chromatogram of SEC from Gel Filtration Standard.*
Source: private picture, HPLC BPRC.

The standard contained a lyophilized mix of thyroglobulin (MW of 670 kDa with a retention time of ~ 26 minutes), bovine γ-globulin (MW of 158 kDa with a retention time of ~ 32 minutes), chicken ovalbumin (MW of 44 kDa with a retention time of ~ 34.5 minutes), equine myoglobin (MW of 17 kDa with a retention time of ~ 37 minutes), and vitamin B12 (MW of 1.35 kDa with a retention time of ~ 41 minutes), which can be seen from first to fifth peak in Figure 12, in that order.

When compared to other chromatographic methods, the instrumentation requirements for SEC are simpler since mobile phase gradients are not used, and it is easy to handle. However, the separation of proteins with similar molecular weight is by far not as precise as other techniques, because the elution time of similar sized proteins does not differ much. In addition, much more material is required to work with SEC compared to other techniques. [Mori 1999]

4.2.1 SEC – Testing Fab fragments

Table 6 shows an overview of the injected samples, their concentrations and running time.

***Table 6**: Overview of injected samples in SEC to test Fab fragments.*

Protein sample	MW [kDA]	Sample concentration [mg/mL]	Total mass [µg]	Injection volume [µL]	Time [hr]
calibration mix	/	/	/	20	1
AMA1	60	3	30	10	1
4G2	150	10	75	7.5	1
Fab	25	4	15	8	1
Mix 1: AMA1 + mAB4G2*	210	/	/	18	1
Mix 2: AMA1 + Fab fragments*	85	/	/	18	1

**same amount of molecules, meaning 1:1 ratio.*
The column "Time" states here the running time of each sample.

As Table 6 shows, not only the Fab sample was injected, but also a mixture of it with AMA1, to test if after an hour of incubation, before injecting the sample, they bind together and form the desired complex. Therefore, AMA1 had to be injected onto the column, as well as a bound complex of mAB 4G2 and AMA1 as a positive control (same incubation conditions as with AMA1 and Fab fragments).

To get a more precise result to compare and do a proper analysis with, all the injected samples had to have the same number of molecules. Therefore, pre-calculations were made as follows.

> AMA1 served as reference value with 10 µL injection volume and a MW of 60 kDa. Using a 3 mg/mL solution, makes 30 µg as a total mass, which will be injected. Since mAB 4G2, as a + control, has a MW of ~ 150 kDa (sample was taken from the before concentrated solution of IgGs), the following calculation was made to get to the needed amount of injection volume to end up with the same number of molecules:

$$\frac{150\ kDa}{60\ kDa} \times 30\ \mu g\ = 75\ \mu g$$

Meaning, that of a diluted 10 mg/mL solution, 7.5 µL had to be injected to get the same number of molecules as AMA1.

Same for the Fabs with a MW of ~ 28 kDa:

$$\frac{28\ kDa}{60\ kDa} \times 30\ \mu g\ = 15\ \mu g$$

However, the Fabs were cleaved from a whole IgG, leaving 2x Fab fragments and 1x Fc tail in the solution, if every mAB was cut.

So, in a $V_{end}\ =\ 0.2$ mL are 2 mg/mL IgGs in total. Rounding up the MW of Fab fragments to 30 kDa compared to the whole IgG with ~ 150 kDa leads to $\frac{2\times30\ kDa}{150\ kDa} = 0.4$, which makes a total of 4 mg/mL Fab fragments in the solution. Meaning, at least 4 µL needed to be injected to get the same number of molecules. However, this amount was multiplied by two (= 8 µL) to even out probable loss of Fab fragments during the purification or uncut mABs.

For Mix 1, 20 µL of AMA1 were incubated with 15 µL of mAB 4G2 for 1 hr at RT. Injection volume was then 18 µL.

For Mix 2, again 20 µL of AMA1 were incubated with 16 µL of Fab fragments under same incubation conditions.

The run time for each sample was ~ 1 hr.

4.2.2 SEC – refolded scFv 4G2

Table 7 shows an overview of the injected samples, their concentrations and running time.

Same pre-calculations took place here as in the before stated chapter 4.2.1. Only now instead of Fab fragments, the refolded scFv 4G2 was tested, with a MW of ~ 28 kDa.

Table 7: *Overview of injected samples in SEC to test refolded sc4G2.*

Protein sample	MW [kDA]	Sample concentration [mg/mL]	Total mass [µg]	Injection volume [µL]	Time [hr]
calibration mix	/	/	/	20	1
AMA1	60	3	30	10	1
Refolded sc4G2	28	~ 0.25**	15	60	1
Refolded sc4G2 in PBS buffer	28	~ 0.20**	15	60	1
Mix 1: AMA1 + refolded scFv 4G2*	88	/	/	35	1
Mix 2: AMA1 + refolded scFv 4G2 in PBS buffer*	88	/	/	35	1

**same amount of molecules, meaning 1:1 ratio. **approximation of protein concentration to know how much to inject onto the column. Measured via DeNovix.*
The column "Time" states here the running time of each sample.

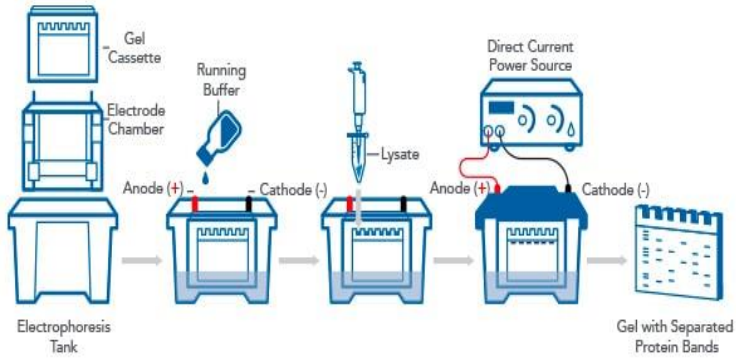
Samples were measured before injection to have an approximation about their protein concentration.

For Mix 1 and 2, 10 µL AMA1 were incubated with 30 µL refolded scFv 4G2 under same conditions.

4.3 Protein gel electrophoresis and Coomassie staining

Protein gel electrophoresis in general is an often-used technique to separate proteins for purification, characterization, and expression analysis. In this approach, charged protein molecules are transported through a polyacrylamide gel by an electrical field. Their mobility through the electric field is dependent on their size, charge, and shape.

Polyacrylamide gel matrices serve as a sieve, allowing smaller proteins to travel more rapidly than larger proteins. It is possible to separate proteins ranging in size from 5 kDa to 2 kDa via this technique.



First a ready gel cassette is placed into its chamber and electrophoresis tank filled with running buffer. This gets then connected to an electric field, which pulls apart the proteins and separate them by their size. After, gel gets filled with samples (20 µL/ well) and connected to the power source. Gels run normally at 200 V for ~ 45 minutes. After protein bands are separated, gels get stained in Coomassie blue.

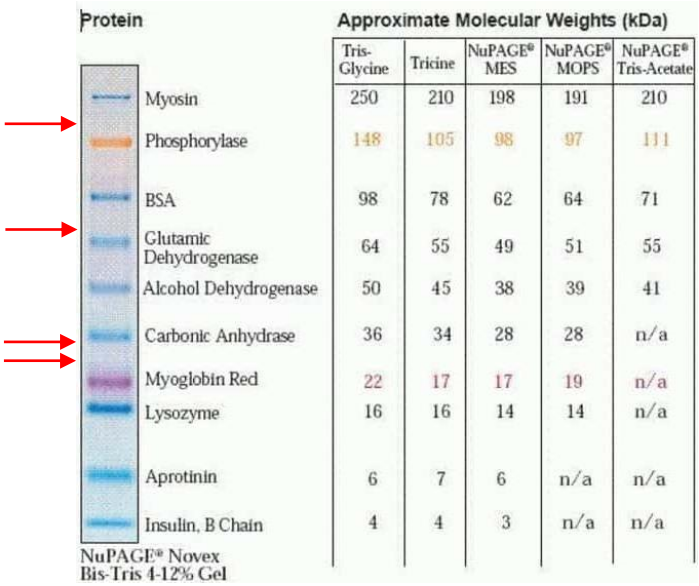
***Figure 13**:Overview of a gel electrophoresis system.*
Source: https://www.novusbio.com/support/support-by-application/western-blot-sds-page

Throughout this thesis, SDS page gels (sodium dodecylsulfate polyacrylamide gel electrophoresis) were used to separate proteins based on their molecular mass. Hereby, SDS imparts a net negative charge on proteins by masking their intrinsic charge. By incubating the protein-SDS mixture for 10 minutes at 70 °C, the proteins denature which breaks their hydrogen bonds. Meaning, their globular form becomes more linear. In addition to that, DTT (dithiothreitol) as a reduction component can be added which breaks the protein´s disulfide bonds.

As a result, the rate at which SDS-bound proteins migrate through the gel is primarily dependent on their size, enabling estimation of molecule weight by comparing to protein standards.

The here used standard for all SDS pages is SeeBlue® Plus2 Standard (1x), 5 µL). [Merck KGaA 2022]

Figure 14 shows the division of the bands. Herby, only column three ‘NuPAGE® MES’ is important to look at, regarding analysation.



Protein	Approximate Molecular Weights (kDa)				
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS	NuPAGE® Tris-Acetate
Myosin	250	210	198	191	210
Phosphorylase	148	105	98	97	111
BSA	98	78	62	64	71
Glutamic Dehydrogenase	64	55	49	51	55
Alcohol Dehydrogenase	50	45	38	39	41
Carbonic Anhydrase	36	34	28	28	n/a
Myoglobin Red	22	17	17	19	n/a
Lysozyme	16	16	14	14	n/a
Aprotinin	6	7	6	n/a	n/a
Insulin, B Chain	4	4	3	n/a	n/a

NuPAGE® Novex Bis-Tris 4-12% Gel

Figure 14: *Molecular weight estimation of proteins by gel electrophoresis using SeeBlue® Plus2 Pre-Stained marker.*
Source: https://www.thermofisher.com/order/catalog/product/LC5925

Red arrows are indiating the expected MW of the different parts of the mAB 4G2 (listing from the top).

First arrow: 150 kDa – whole mAB 4G2

Second arrow: 50 kDA – Fc tail

Third arrow: 28 kDa – scFv 4G2

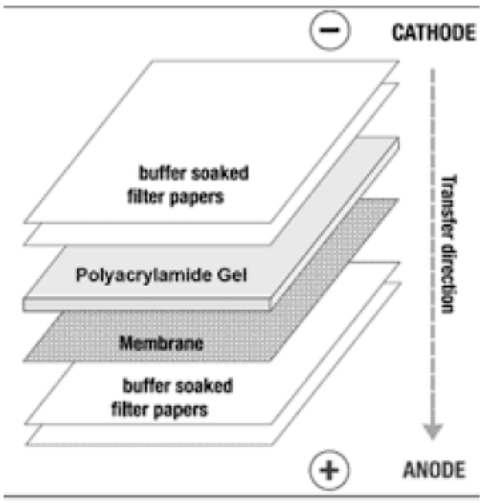
Fourth arrow: 25 kDa – Fab fragments

All runs are performed in electrophoresis systems from invitrogen with NuPAGE™ 4-12% Bis-Tris gels (20 µL/ well) in MES SDS Running buffer, for 45 minutes at 200 V. Samples were prepared with 18 µL sample (equal amounts of protein, 20-60 µg) mixed with 6 µL loading buffer. If loading buffer contained DTT, a mixture was made of 20 µL 1 M DTT and 180 µL loading buffer. After, gels get stained in SimplyBlue™ SafeStain, Coomassie blue stain until fully developed bands are visible.

4.4 Western and spot blotting

To get a more precise result, whether the desired protein was expressed or not, the Western blot is a common technique to use. Here for, the protein bands from a (unstained) SDS page will be blotted onto a membrane, then incubated with antibodies or antigens that will bind to the desired protein and can furthermore be detected.

Therefore, after the electrophoresis, two pieces of cardboard are drenched in transfer buffer, as is a piece of cellulose membrane, which got activated in demi. water beforehand. After that, the gel will be put in between the cardboards and the cellulose membrane as Figure 15 shows.



In between the two cardboards, which were drenched in a special transfer buffer, a nitrocellulose membrane gets placed and on top of that the unstained polyacrylamide gel. This is then placed into an electro blotter which is the power source, that will transfer the protein bands from the gel onto the membrane via an electric field from cathode to anode.

***Figure 15**: Different layers of a western blot.*
Source:https://www.uni-due.de/imperia/md/content/water-science/ws0910/1652_ws0910_molekularbiologie5__immunoblot__proteinbestimmung.pdf

This is then put onto the electro blotter for 45 minutes if one gel, and for 1 hr 15 minutes if two gels at 180 mA.

After the run, the blot must be blocked in blocking buffer for 30 minutes at RT, swirling gently on a rocking platform to block all free binding sites on the membrane, which cannot be recognized by ABs. This is followed by a briefly wash in washing buffer. Then, in AB buffer diluted primary AB (mostly 1:500, 14 mL) is added to react with the blot for 1 hr at RT under gentle agitation. 5x five minutes washing steps with washing buffer follow. After that, the diluted secondary AB (usually 1:1000, 14 mL) is added to the blot and incubated for another hr at RT under gentle agitation. If there is a third AB, the 5x five minutes washing steps and the 1 hr of incubation must be repeated. If not, only 4x five minutes washing steps are following at RT. Lastly, the blot is developed in development buffer, containing the chemicals BCIP and NBT which colour the bound complex, at RT until the desired signal has been achieved. The development of the blot is stopped with demi. water.

For a spot blot the same procedure is implemented, except no gel must be run and therefore no electro blotter is used. Instead ~ 5 µL of (protein) sample is directly spotted onto the cellulose membrane, air dried and after putted into the blocking buffer for 30 minutes. The following steps are identical to a Western blot.

4.4.1 Spot blotting plan for refolded scFv 4G2

Here for, the cellulose membranes were divided up into "rows" and "columns". The first column, controls in a concentration of 1 mg/mL were spotted on plus every step (primary, secondary and third AB) of the technique of the spot blot to exclude the possibility of one binding-step or the development not working or not binding properly. The rest of the membrane was used to spot on the 18 different buffers containing the refolded protein (columns 2 – 5, rows 1 – 6). For each sample 5 µL were spotted onto the membrane and washing steps were reduced to 2x five minutes for each step.

The first blot was incubated with a fluorescent AMA1 (AMA1 – Alexa488) as a primary AB in a concentration of 1:30 (500 µL AMA1 into 15 mL AB buffer). This blot only needed one, primary, AB, because the fluorescent AMA1 could be detected right after the first incubation step if bound.

***Table 8**: Spotting plan to test binding capacity of properly folded 4G2 to a fluorescent AMA1.*

Cellulose Membrane	**Columns**				
Rows	**1**	**2**	**3**	**4**	**5**
1	4G2 (+ control)	1	7	13	19
2	FAB (+ control)	2	8	14	20
3	AMA1 fluorescent	3	9	15	
4	AMA1 fluorescent (1:10)	4	10	16	
5	AMA1 fluorescent (1:100)	5	11	17	
6	/	6	12	18	

Column 1: controls (5 µL each). Columns 2-5: different refolding buffers containing the refolded 4G2 (5 µL each).

The second blot was incubated with an AMA1 without a His-Tag (AMA1 FVO) as a primary AB (c = 1 µL/mL). Onto that the secondary AB called 62F12 (donor: mouse) was bound (c = 1 µg/mL). The third AB had to be an α-mouse alkaline phosphorated (AP labelled, 1:3000), which binds to the secondary AB. Because of the AP label the binding can be detected by incubating the blot with the development buffer which colours the bound complex.

***Table 9**: Spotting plan to test binding capacity of properly folded 4G2 to an AMA1 witout a His-Tag.*

Cellulose Membrane	**Columns**				
Rows	**1**	**2**	**3**	**4**	**5**
1	4G2 (+ control)	1	7	13	19
2	FAB (+ control)	2	8	14	20
3	AMA1 FVO	3	9	15	
4	62F12	4	10	16	
5	α-mouse	5	11	17	
6	/	6	12	18	

Column 1: controls (5 µL each). Columns 2-5: different refolding buffers containing the refolded 4G2 (5 µL each).

As third, 18 small cellulose strips were cut out and spotted with AMA1. Each individually was then incubated at RT on a roller bank with 50 µL refolded sample diluted in 950 µL AB buffer in 2 mL tubes. Onto that an anti-6-His-tag AB (donor: mouse) was bound and onto that an α-mouse AP, which was detected afterwards.

All three ways of spot blotting and testing the sample were implemented to ensure, that every possible binding scope is working, minimizing mistakes. [PhD Bart Faber, personal communication]

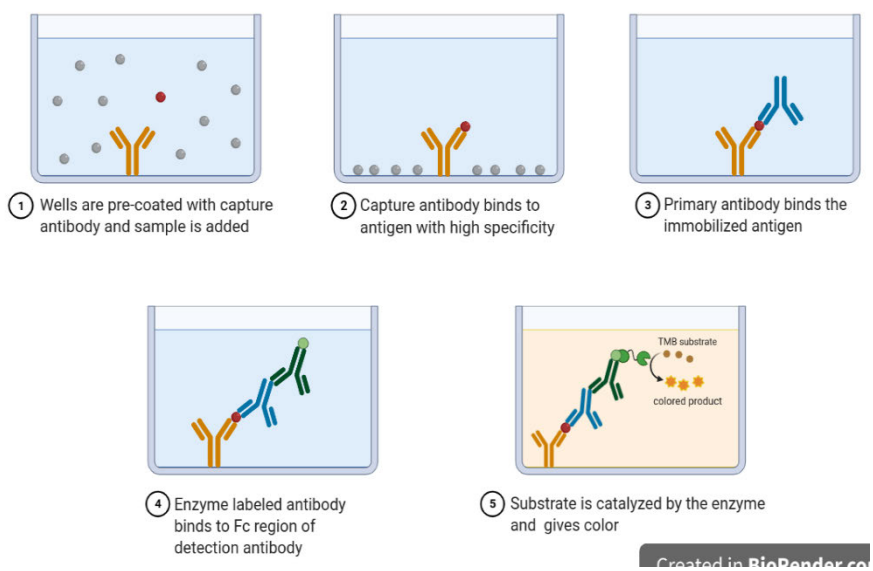
After that, another spot blot was done with only the samples from buffers that gave the strongest response (see results chapter 6.2). Only difference in the samples was, that the buffers have been exchanged right after the refolding to PBS, pH 7.0.

4.5 ELISA

Another way of testing if the desired protein is properly folded and therefore in its active form is establishing an ELISA.

ELISAs (enzyme-linked immunosorbent assays) are immunological assays commonly used to measure antibodies, antigens, proteins, and glycoproteins in biological samples. They generally

carried out in 96 well plates, allowing multiple samples to be measured in a single experiment. These plates need to be special absorbent plates to ensure the antigen or AB sticks to the surface. Samples are mostly added in a duplicate or triplet to allow statistical analysis. [Claire Horlock, Imperial College London, UK]



First, the microplates get coated with the antigen and incubated overnight at 4 °C. To that the sample (AB) will bind. To that an enzyme labelled AB binds, which will later be detected by a substrate that gets catalysed by the enzyme.

***Figure 16**: Steps of an enzyme-linked immunosorbent (ELISA).*
Source: https://microbeonline.com/elisa-test-for-antigenantibody-detection/

The ELISAs that were implemented during this thesis, were carried out in 96 well microplates as follows.

First, coated with 1 µg/mL AMA1 FVO in coating buffer (PBS). Therefore, 100 µL are pipetted in each well, followed by an incubated at 4 °C overnight, covered with a plastic lid.

The next day the coting is removed from microplate by inverting it with a vigorous wrist action. After, the wells get blocked with 150 µL/ well of blocking buffer. This is then incubated for 1 hr at 37 °C. After, the blocking buffer is removed the same way as the coting.

Then, the samples are pipetted in and get diluted out per each well (100 µL). This is then again incubated for 1 hr at 37 °C and after washed by the microplate washer (3 – 6 washing steps). This ensures to get rid of all excess sample. After, 100 µL of the first conjugate (1:5000) is added, diluted in AB buffer, followed by another hour of incubation at 37 °C. The washing steps are repeated and 100 µL of the second conjugate (1:3000) is added afterwards. This as well is followed by an incubation and washing steps. If there is a third conjugate, the coting, incubation and washing steps must be repeated once more.

Afterwards, 100 µL of 1 mg/mL para nitrophenyl phosphate hexahydrate (PNPP) is added, followed by an incubation of 20 min at RT. The results are read out by a microplate reader at 405 nm.

4.5.1 ELISA of scFv 4G2 expressed in HEK293-F cells

Table 10 shows the ELISA format of the scFv 4G2 expressed in HEK293-F cells.

***Table 10**: Plate format for the ELISA of 4G2 from HEK 293-F cells.*

	Intracellular Pre-diluted (1:20)					**Extracellular (supernatant) undiluted**					**+ control Pre-diluted (1:425)**	**BLANK**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	sample 1 (1:1)	sample 2 (1:1)	sample 3 (1:1)	Sample 4 (1:1)	sample 5 (1:1)	sample 1 (1:1)	sample 2 (1:1)	sample 3 (1:1)	sample 4 (1:1)	sample 5 (1:1)	sample 0 (1:1)	N 0 SAMPLE
B	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	
C	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	
D	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	
E	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	
F	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	
G	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	
H	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	

1. *HEK 293-F cells (blank – not transfected)*
2. *HEK 293-F cells with transfected 4G2 p.Selecet vector (without secretion factor)*
3. *HEK 293-F cells with transfected 4G2 p.SelecetCD5*
4. *HEK 293-F cells with transfected 4G2 p.SelecetKappa*
5. *HEK 293-F cells with transfected 4G2 p.SelecetSEC*
6. *Medium-supernatant of HEK 293-F cells (blank)*
7. *Medium-supernatant of HEK 293-F cells, 4G2 p.Selecet vector (without secretion factor)*
8. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelecetCD5*
9. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelecetKappa*
10. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelecetSEC*
11. *Positive control (4G2 AB from a rat)*
12. *BLANK – no sample*

Each well contains 100 µL of (diluted) sample.

The first conjugate was a 6x-His-tag monoclonal AB, 1:5000, diluted in AB buffer.

The second conjugate was a 1:3000 goat anti-mouse IgG (H+L) for lane 1 – 10 and 12 and for lane 11 a goat anti-rat IgG (H+L) in the following concentration: 0.47 µL of a 4.3 mg/mL solution into 200 µL for the first well and was then diluted each well down to 1:128, both from Pierce®.

4.5.2 ELISA of refolded scFv 4G2

Table 11 shows the ELISA format for testing the binding response of the refolded scFv 4G2 from the rapid dilution in buffers 2, 3, 5, 8, 11, 13, and 17.

***Table 11**: Plate format for the ELISA of refolded samples in buffer 2, 3, 5, 8, 11, 13 and 17.*

	Refolded (pre- diluted) samples in buffer 2, 3, 5, 8, 11, 13 and 17							**+ control**	**BLANK (No samples)**			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	sample 1 (1:1)	sample 2 (1:1)	sample 3 (1:1)	Sample 4 (1:1)	sample 5 (1:1)	sample 1 (1:1)	sample 2 (1:1)	sample 3 (1:1)				
B	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2				
C	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4				
D	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8				
E	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16				
F	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32				
G	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64				
H	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128				

1. *Refolded sample in buffer 2 (50 µL sample diluted in 150 µL AB buffer)*
2. *Refolded sample in buffer 3 (50 µL sample diluted in 150 µL AB buffer)*
3. *Refolded sample in buffer 5 (50 µL sample diluted in 150 µL AB buffer)*
4. *Refolded sample in buffer 8 (50 µL sample diluted in 150 µL AB buffer)*
5. *Refolded sample in buffer 11 (50 µL sample diluted in 150 µL AB buffer)*
6. *Refolded sample in buffer 13 (50 µL sample diluted in 150 µL AB buffer)*
7. *Refolded sample in buffer 17 (50 µL sample diluted in 150 µL AB buffer)*
8. *mAB 4G2 (0.5 mg/mL)*

The plate was first incubated with AMA1 FVO (1 µg/mL) and to the refolded scFv 4G2 was bound. Since all refolded proteins, as well as the + control, were fused with a 6-His-tag, the second conjugate, was an anti-6-His-tag AB (donor: mouse) and the third conjugate was an α-mouse AB, AP-labelled which could then be detected.
Duplicates were avoided in this assay.

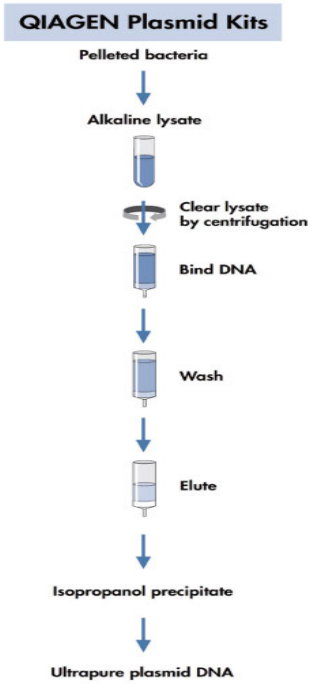
4.6 Production of 4G2 in HEK293-F cells

Since HEK293 cells are mammalian cells, they have the folding machinery to properly fold eukaryotic proteins, such as ABs like 4G2. This is not a guarantee for the scFv 4G2 to properly fold. Mammalian cell culture is more expensive and laborious than microbial cultivation and also yields may be less, which is problematic if lengthy purification procedures are needed. That is why within the first attempt using HEK293-F cells are transfected with different secretion factors to attempt secretion of the scFv protein out of the cell into the medium.

The next subchapters sum up the plasmid purification of scFv 4G2 with the secretion factors, thawing and establishing, subculturing, cryopreservation and freezing and the transfection on the HEK293-F cells, starting with a plasmid purification.

4.6.1 Purification of plasmids from *E. coli* DH5α cells

All following steps refer to the isolation and purification of the scFv 4G2 pSelect, pSelectCD5, pSelectKappa, pSelectSEC, and the pSelectGFP (without scFv 4G2) plasmids in *E. coli* DH5α cells using the QIAfilter Plasmid Maxi Kit, according to the instructions of the manufacturer (pages 23 et sqq., quantities = 500 mL). [QIAGEN® 2021]
Figure 16 shows a brief overview of the process.
Before the purification of the plasmids, the *E. coli* DH5α were pre-cultivated as described previously (V = 3 mL each).
The next day 150 µL were transferred into 1 L Erlenmeyer flasks with 150 mL LB medium and 37,5 µL Zeocin per culture to enhance their growth. This was then again incubated overnight, under same conditions. To prevent contamination of the cultures, work was done above a Bunsen burner.



First, the bacteria culture gets centrifuged. The pellet will then be lysed and centrifuged again in various steps. After, supernatant is brought onto the column, spun down, washed, spun down again, and eluted in isopropanol.

Figure 17: *Process of plasmid purification with QIAGEN Plasmid Kits. Source:[QIAGEN® 2021]*

4.6.2 Thawing and establishing HEK293-F cells

Before transfecting the HEK293-F cell culture, cells had to be carefully thawed and cultivated for at least 2 weeks. Therefore, the 'FreeStyle 293-F cells User Guide' was followed from pages 9 et sqq. [invitrogen by Thermo Fisher Scientific 2015]

To avoid contamination work was done exclusively in a sterile flow cabinet.

4.6.3 Life and death cell counts

After the first day of cultivation, the viable and total cell counts were determined via Bio-Rad TC10 cell counter as follows.

50 µL of pre-filtered Trypan blue and 50 µL of cell culture (1:1) were mixed.

The Trypan blue colours live cells light blue and dead cell (clumps) dark blue, which then can be electronically detected by the cell counter.

Therefore, 10 µL were pipetted onto each side of a counting slide (A & B) and put into the cell counter for determination.

The upper described method was used every time live cells were detected (meaning on day 1, 4, 7, 11, 14 as shown in Table 12).

After thawing and cultivation over night the viability of the cells was detected between 76 % and 78 % the first day, which is within the normal range (everything > 70 % is acceptable). Therefore, the culture kept on growing until the first sub-cultivation on day four.

Table 12 shows the viability of the HEK cells during the two-week cultivation. Since the counting slides have two chambers, measurements were done in duplicate (A & B). For further subculturing cells the average of the live cells in the culture was an important indicator.

For calculations and further dilutions that were made, see following chapter 4.6.4.

***Table 12**: Overview cell viability during two-week cultivation.*

	Days	**1**	**4**	**7***	**11**		**14**		**15*****
					(1)**	(2)	(2.1)	(2.2)	
A	**Total count: [10^5 cells/mL]**	4.20	12.1	5.05	10.5	8.64	9.00	13.8	3.39
	Live count: [10^5 cells/mL]	3.18	1.11	4.95	9.86	8.06	8.59	12.2	3.18
	Live cells: [%]	76	92	98	94	94	96	88	94
B	**Total count: [10^5 cells/mL]**	-	0.13	7.33	0.12	8.34	0.11	0.14	3.29
	Live count: [10^5 cells/mL]	1.21	0.13	7.23	0.12	7.73	9.65	0.13	3.13
	Live cells: [%]	78	94	99	97	93	94	91	95
	Average of live cells [10^5 cells/mL]	4.39	0.25	0.12	0.22	0.16	0.18	0.25	0.62
	Live cells in the culture [10^6 cells]	13.20	76.0	36.5	64.1	63.3	54.7	74.1	1.86
	D_f	-	10	10	-	6.4	3.65	20	-

A & B are indicating each side of the counter slides.
**On day 7 the culture was spilt into two (first culture (1) was the working culture, with which the transfection was made, second culture (2) was the back-up culture).*
***Culture (1) was used for freezing in five new stocks as described in following chapter. Culture (2) was split again: (2.1) working culture & (2.2) back-up culture.*
****Day 15: day of transfection.*

4.6.4 Subculture HEK293-F cells

When the cells reach a density between $1 - 3 \times 10^6$ live cells/mL they were subcultivated as stated in the 'FreeStyle 293-F cells User Guide', pages 11 et seqq. [invitrogen by Thermo Fisher Scientific 2015]

This happened every three to four days as Table 13 shows, meaning every Monday & Friday.

On day seven the culture has been split into two. Subculture (1) was later used to freeze in new stocks and subculture (2) was then again split into two: (2.1) as working culture in terms of the transfection and (2.2) as a back-up culture.

Since on day 4 the culture had already established over 90 % of live cells and kept that percentage high during the two-week cultivation, it was refreshed as follows on days 4, 7, 11 & 14.

Table 13: *Dilution compounds of refreshed cultures.*

Day	**4**	**7**	**11**	**14**	
			(2)*	(2.1)	(2.2)
Amount of old culture added [mL]	3	3	4.5	5.6	1.5
Amount of new medium added [mL]	27	27	25.5	15.4	28.5
Concentration of refreshed cell culture [10^5 cells/mL]	2.5	1.25	2.5	2.5	1.25

**Culture (1) was used to freeze in new stocks, whereas (2) was the only working culture and split again into two: (2.1) working culture & (2.2) back-up culture.*

As on day seven the culture has been split into two, one of the cultures (1) was used to freeze new stocks. The second culture (2) was again split into two (2.1) and (2.2). After the minimum of two-weeks cultivation the culture with a higher percentage of viable cells, which in this case was (2.1), was prepared for the transfection as follows.

> 5.6 mL culture was mixed with 15.4 mL fresh medium to get a final $c = 0.5 \times 10^6$ cells/mL, so the cells will have a density of approximately 1.0×10^6 cells/mL the next day.
>
> 3 mL were then pipetted onto each well of a sterile 6 well suspension culture plate with a lid. Afterwards, it was cultivated over night as usual. [invitrogen by Thermo Fisher Scientific 2015]

Culture (2.2) was handled as a back-up culture and refreshed as before mentioned.

4.6.5 Cryopreservation and freezing HEK293-F cells

As already mentioned, on day eleven subculture (1) was used to freeze in new stocks. Therefore, the 'FreeStyle 293-F cells User Guide' was followed according to pages 12 et seqq. [invitrogen by Thermo Fisher Scientific 2015]

4.6.6 Transfection of HEK293-F cells

Before transfection, cells were counted again as previously described, to verify their quantity. Even though cell density was a little lower than the needed density of 1×10^6 cells/mL, the transfection was still proceeded as stated in the 'JoVe' protocol 'Recombinant protein expression for structural biology in HEK 293F suspension cells: a novel and accessible approach'. The thereby stated quantities were divided by 10, since the here used working volumes were only 3 mL each. [Raymond et al. 2011]

Table 14 shows the amount of the before purified plasmid DNA (in µL) needed for each transfection.

Table 14: *Calculated amount of DNA solution needed for the transfection.*

Plasmids	DNA [µL]
4G2 pSelectSEC	3.9
4G2 pSelectKappa	6.9
4G2 pSelectCD5	3.5
4G2 pSelect	4.3
pSelectGFP	5.4

The following equation was used to calculate the upper shown values.

$$3\ \mu g \Big/ Yield\ [\frac{\mu g}{\mu L}]$$

The here mentioned $Yield\ [\frac{\mu g}{\mu L}]$ can be found in Table Table 17.

Afterwards, this was added into each well to the cell culture and incubated under same conditions as before, for a further 48 hrs. Results can be seen under 6.1.

4.6.7 Harvesting the transfected HEK293-F cells

After 48 hrs cultivation post transfection, cells were harvested as follows.

Due to the secretion sequences on the plasmids, the location of the desired protein is expected extracellularly, meaning in the medium. So, started harvesting extracellular proteins by centrifuging cells at 3000 RCF for five minutes. Supernatant was kept for further analysis.
To be able to compare and do the complete analysis, intracellular proteins were also harvested to see whether the protein of interest was even expressed or not. Therefore, the cell pellet was resuspended in 120 µL pre-chilled lysis buffer (100 mM potassium acetate, 50 mM Tris pH 7.5, 5 % glycerol, 0.3 % Titron X-100) containing one tablet of protein inhibitors by carefully pipetting up and down to avoid foaming. This was then sonicated for three cycles (15 seconds on, 15 seconds off) to fully break open cells. Afterwards, it was centrifuged again at 12,700 RCF for 25 minutes at 4 °C. Supernatant, containing the proteins, was retained.

Both cultivation medium as well as the cells were analysed trough SDS gels (chapter 4.3) and Western blotting (chapter 4.4).
Before starting the gel electrophoresis, each sample was diluted 1:1 with PBS buffer and mixed with 6 µL of loading buffer.
This was done twice to stain one gel in Coomassie blue and to do a Western blot with the other as described in chapter 4.4. Here by, the primary AB was an anti-6-His-tag AB (1:500, V = 14 mL) from a mouse. Therefore, the second AB was α-mouse AP conjugated (1:1000, V = 14 mL) for detection.

4.1 Development of a binding assay, using Fab fragments of mAB 4G2

For the next approach, validating the methods and assays to detect the scFv 4G2, Fab fragments were generated and purified according to chapter 4.1.2 and 4.1.3.
They were tested in the binding assays, as follows.

4.1.1 Purification of 4G2 mAB from a rat hybridoma culture

Starting the Fab fragment production by purifying whole IgGs from a rat hybridoma culture. These were grown in DMEM medium which contains a pH indicator which turned the cultivation medium pink.

First, the solution was spun down by centrifuging it for 5 minutes at 5000 RCF. The supernatant was then carefully collected and diluted 1:1 with binding buffer, which was also later used during the chromatography. This caused a pH shift from 7.4 to 5.5, which turned solution yellow.

For the chromatography the column HiTrap™ protein G HP was used, which binds whole ABs and Fc tails.

Starting by cleaning the column with demi. water. Then equilibrating the column with binding buffer. Next, bringing the IgG 4G2 solution onto the column. Of the flow-through a sample was taken (S1). Afterwards, shortly washed again binding buffer. Here from, a sample was taken as well (S1.1). Then, the bound antibody was eluted with elution buffer. Here, 5 mL Tris buffer (pH 9) were filled in a 50 mL Tube (10 %) before collecting the eluate to get a more neutral pH for storage, since the 4G2 is present in an acid solution (S2). After, the column was washed with demi. water (sample S3) and for storage of the column with PBS + azide (sample S4).

For concentration and rebuffering of the eluted antibody, sample 2 was put into a concentrator tube (15 mL, 30 kDa cut-off) and centrifuged for over 2 hr at 4 °C and 5000 RCF. The same was done again with the new buffer (here called “Fab buffer”) containing 0.075 M Na-phosphate, 0.075 M NaCl, 0.01 M Cysteine, in which the digestion later found place. Form the flow-through of the centrifugation steps a sample was taken as well (sample 5). After concentrating, the protein concentration in the solution was detected with the DeNovix and was c = 56.727 mg/mL.

All samples were put on gel to check for results and correctness.

For the digestion, the cutting proteolytic enzyme protease papain which is specific for cleaving the ABs into Fc tails and Fab fragments as shown in Figure 18, was added to the solution.

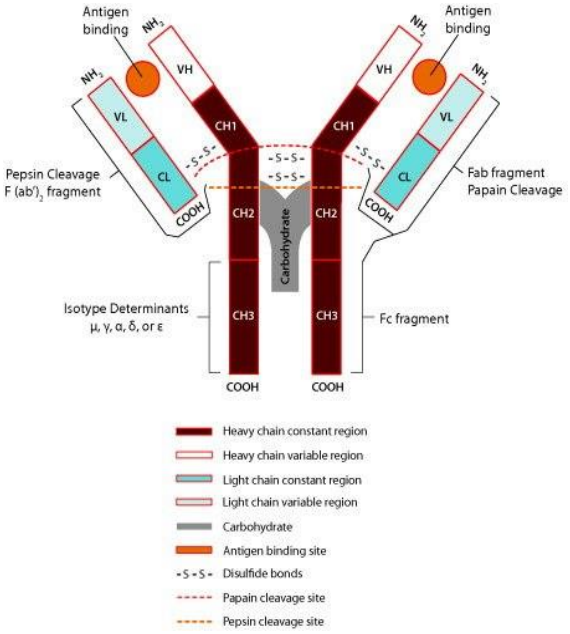


Figure 18: *Antibody and its different (cleavage) regions.*
Source: https://www.sigmaaldrich.com/NL/en/technical-documents/technical-article/protein-biology/western-blotting/antibody-basics

For the next approach, only the method using pepsin is important since Fab fragments are desired. Papain has a different cleavage site compared to pepsin, which is between CH1 and CH2 producing Fab fragments. Compared to the pepsin cleavage site which is within the CH2 producing F(ab')$_2$ fragments.

4.1.2 Digestion of mAB 4G2 using papain to obtain Fab fragments

For the digestion, the concentrated mAB 4G2 from sample 2 was firstly diluted to a concentration of 10 mg/mL, for a total volume of 200 µL in the Fab buffer. Papain as cutting enzyme was added in an enzyme:protein ratio of 1%. Meaning, that 0,1 mg/mL papain was added, which got immediately activated through the Cysteine concentration in the buffer. This was then digested for 1 hr at 37 °C, which lead to an almost complete cleavage into Fab and Fc fragments.
The digestion was ended with 0.015 M iodoacetamide and afterwards put on gel. [Rousseaux et al. 1983]

4.1.3 Fab fragment purification

Next step, before testing the Fab fragments, was to purify them from the rest of the solution. Meaning, purifying them from uncut ABs, Fc tails and the added papain and iodoacetamide, to ensure the binding assays are done with only cleaved Fabs and not whole Abs. Which are already known for their binding capacity.

First attempt to purify the Fab fragments was done with a self-poured DEAE column.

Since this did not achieve the wanted separation and therefore purification it will not be further discussed in this thesis. Same applies for the presentation of results.

Second attempt to purify the fragments, using a protein G agarose column, which binds bigger fragments such as whole Abs and Fc tails. Whereas smaller molecules, such as the Fab fragments don´t bind and therefore elute immediately.
To run the sample, it was first diluted 1:10 with column specific binding buffer, with which the column was also washed before putting on the sample.
As soon as the sample was put onto the column, the flow-through was collected, containing the Fab fragments. After, the flow-through was concentrated again and put on gel. Results can be seen in chapter 6.2.
The Fab fragments were also tested in the binding assays via spot blotting as described in chapter 4.4. Therefore, undiluted Fab sample was spotted on (5 µL), plus a 1:10 dilution and a 1:100 dilution of it. As a control, AMA1 FVO was taken along. Incubated it with AMA1 FVO as the first AB (c = 1 µg/mL), then with 62F12 as the second AB (c = 1 µg/mL) and lastly with α-mouse AB AP-labelled (1:3000). Washing steps in between reduced to 2 – 3x instead of 5x five minutes.
A SEC was done as well, which is described in chapter 4.2.1.

With ending these experiments and proofing that the binding assays work and are functioning, the next approaches were implemented. Starting with the Refolding experiments.

4.2 Refolding of in *E. coli* expressed scFv 4G2 using rapid dilution

For this next approach DE3 cells were used, which were already transfected with the plasmid containing the scFv 4G2 gene. These were than cultivated as previously described underneath Table 2.
Since DE3 cells are *E. coli* cells and therefore procaryotic bacteria, are missing proper refolding mechanisms to correctly folded eukaryotic proteins, the scFv 4G2 is found in intracellular IBs.
Within these next chapters, the process of isolating these IBs, purify and refold them, is described.

4.2.1 Expression of 4G2 in *E. coli* DE3 cells

Beginning with a pre-culture of *E. coli* DE3 as previously described ($V = 16$ mL).

The next day, 2 L LB medium was divided into four 2 L Erlenmeyer flasks with $V = 0.5$ L each containing 125 µL Zeocin (1:4000). To this 4 mL pre-culture was added each, followed by three hours of incubation with same conditions as pre-culture. After that, 2.5 mL IPTG ($c = 0.1$ M) were added to each flask to reach a concentration of 0.5 mM in total

After adding IPTG, another three hours of incubation followed, same conditions.

After incubation, the cultures were divided over centrifuges pods to spin down cells for 20 minutes at 4 °C and 4000 RFC. The supernatant was decanted.

Pellet was collected and resuspended with 10 mL of PBS buffer to wash, same centrifuge conditions. Again, the supernatant was decanted, and the pellet was frozen in – 20 °C freezer and stored until isolation process of the inclusion bodies (IB). [Heijmans, personal communication]

4.2.2 Isolation of 4G2 inclusion bodies

The isolation process began with defrosting the cell pellet, which was then kept on ice.

First 16 mL lysis buffer were added and homogenised with a dounce homogeniser. After, 4 mL of lysis buffer, which contained 20 mg of lysozyme and one, already dissolved, tablet of protein inhibitors, were added and mixed. This was then put on the roller for 10 minutes at RT, which induced the breaking off the cells (solution becomes more viscous).

Next, 200 µL of 1 M $MgCl_2$, 20 µL RNase, 20 µL DNase and 400 µL of 10 % NP40, which is a detergent, were added and again mixed for 10 minutes on the roller at RT. The end concentration of DNase and RNase should always be 10 mM. After 10 minutes of mixing, the solution should not appear viscous anymore. If so, more lysis buffer should be added, or the mixing time should be increased.

Next, 20 mL of detergent buffer and 200 µL 1 M DTT were added and again mixed with the dounce homogeniser. This was then centrifuged for 20 minutes at 9000 RPM and 4 °C. From the supernatant a sample was taken (S1), the rest was decanted.

The pellet was then fully resuspended in 20 mL GII (containing 10 mM Tris, pH 8.0 and 0.5 % Triton x100) and 20 µL 1 mM DTT containing protein inhibitors (PI). After, it was centrifuged

again for 10 minutes at 9000 RPM and 4 °C. From the supernatant a sample was taken (S2), the rest was decanted.

After that, the pellet was again fully resuspended in 20 mL GII containing 1.5 M KCL, PI and 20 µL of 1 mM DTT. This was followed by another centrifuge step (10 minutes, 9000 RPM and 4 °C) and from this supernatant a sample was taken as well (S3).

Next, the pellet was fully resuspended in another 20 mL of GII with PI and 20 µL DTT, followed by another centrifuge step and sample taking (S4).

Lastly, 16 mL of 8.0 M buffered Urea (RT) was added to the pellet and fully resuspended. This was then centrifuged in the ultracentrifuge for 1 hr at 100,000 RCF at 20 °C, which opened up the IBs. After that, the supernatant, containing desired proteins, was kept, and mixed with Urea (1:1) so it could be further processed and purified with IMAC. Therefrom another sample was taken (Inb). [Heijmans, personal communication]

The samples containing the eluted IBs was purified via IMAC as stated in chapter 4.1 using buffers with 8.0 M urea. Every taken samples, as well the samples from the isolation process were afterwards put on a gel (see results, chapter 6.3). Since samples contained a high amount of urea, they were diluted 1:1 with demi. water before putting them on gel. Also, DTT was added to the sample buffer to properly unfold the IBs.

4.2.3 Buffer preparation for rapid dilution

To implement the refolding, a buffer was chosen (buffer 10), which has shown success in proper folding of the scFv 4G2 expressed in *E. coli* DE3 in former experiments (data not shown).

To optimize that result, three variables have been chosen: concentration of arginine in the buffer, the concentration of sucrose in the buffer and pH, since they have a major impact.

With the help from Dr. Prof. Gesine Cornelissen and via the system called “MODDE” (total overview is attached in the Appendix 1), a concept of experiments has been designed (DoE) in which the three variables vary as follows:

***Table 15**: Variation of the three different variables for refolding experiments*

Variable	Unit	Settings
Arginine	M	0, 0.33, 0.66, 1
Sucrose	M	0, 0.33, 0.66, 1
pH	-	6, 6.8, 7.7, 8.5

To adjust pH 6 and 6.8, a 50 mM MES buffer, and for pH 7.7 and 8.5 a 50 mM Tris buffer was used. Other than that, buffer 10 contained 9.6 mM NaCl, 0.4 mM KCL, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM GSH and 0.1 mM GSSH. These ingredients did not vary. [PhD Bart Faber, personal communication]

In the end, the system came up with 18 different refolding experiments (+ 2 replicates), which means 18 different buffers had to be prepared. Therefore, see **Appendix 1**.

4.2.4 Rapid dilution

Since sample 9 "protein E100" contained the wanted, yet unfolded protein scFv 4G2, it was used and prepared for the rapid dilution to achieve a proper folding of the protein.
First, the solution was rebuffered from an 8.0 M urea buffer to 4.0 M urea using 20 mM Tris buffer. After, the protein concentration in the sample was determined via the spectrophotometer DeNovix (~ 0.631 mg/mL). Since this concentration was too low for a desired final concentration for the experiment of 1 mg/mL, the protein solution had to be concentrated in a concentrator tube (50 mL, 10 kDa cut-off) and centrifuged for at least 3 hrs at 4 °C and 4000 RCF. After that, the concentration was ~ 1.153 mg/mL. To that, 1 mM DTT (final concentration) as a detergent was added, to help unfolding the IBs before the proper folding of the protein.

To perform the rapid dilution, 0.5 mL of each buffer were pipetted into cryovials. To that, 25 µL of sample solution with the added DTT was rapidly pipetted into each buffer, shortly inverted, and then incubated at RT for 45 minutes. The protein concentration after the rapid dilution should be approximately 0.05 mg/mL.

After incubation, the mixtures were pipetted into concentrator tubes (0.5 mL, 3 kDa cut-off) and centrifuged at 4 °C and 14,000 RCF. The centrifugation was stopped after 2 hrs. In the concentrated fluids, the proper folded 4G2 was expected. [PhD Bart Faber, personal communication]
To test if the scFv 4G2 refolded properly a spot blotting plan was established as described in chapter 4.4.1.
A SEC and an ELISA were implemented as well with the samples that gave the strongest response, described each in chapter 4.2.2 and 4.5.2. Hereby the ELISA was done first, to distinguish if one of the sample responses are higher compared to others. From that result was passed to implementing the SEC.
Furthermore, the rapid dilution and refolding experiment was done once more, the same way previously described with samples 2, 3, 5, 8, 11, 13 and 17 that gave the strongest response before. Only, the sample buffers were directly changed after the refolding into PBS, to test if proteins would stay refolded if buffer exchanges took place. Therefore, a third mix was injected onto the HPLC column to test if that would bind as well.
A spot blot with only these samples was done in the same way as described in chapter 4.4.1, as a second control.
Results can be seen in chapter 6.3.

For the third and last approach, *E. coli* cells were used again since the grow faster and are easier to handle than HEK293 cells.

4.3 Chaperones as helper-proteins for refolding scFv in *E. coli* cells

For this experiment, the cells were co-transfected with plasmids coding for different chaperones, which are proteins, that support the refolding machinery in cells. Especially bacteria cells are most likely to miss certain folding apparats to properly fold recombinant proteins or eukaryotic AB such as scFv 4G2.
By using four different chaperone combinations, a higher yield of correctly folded scFv 4G2 should be expressed in the cytosol rather than put into intracellularly IBs.

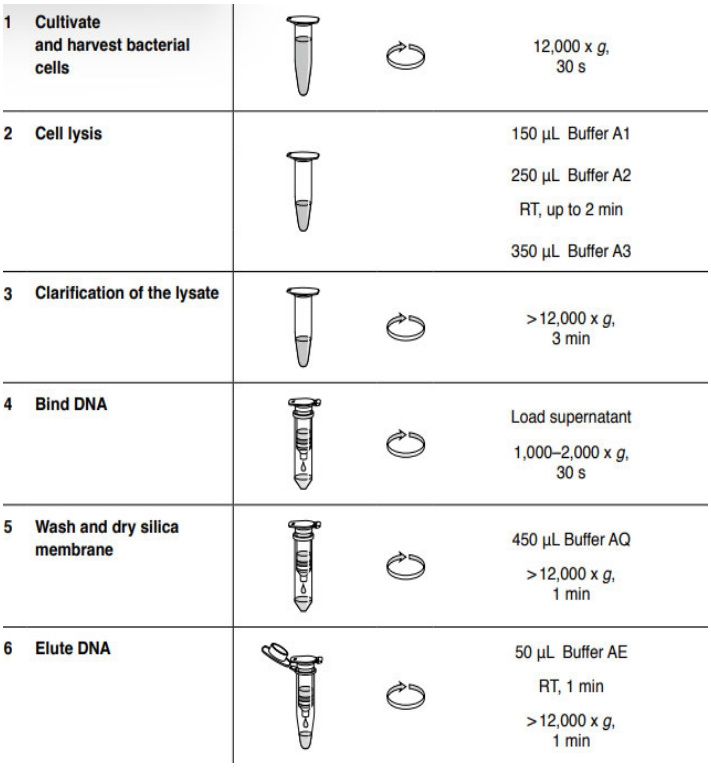
4.3.1 Plasmid purification chaperones

Before starting the expression test, the plasmids of the chaperones were purified using the 'NucleoSpin® Plasmid EasyPure' kit following the instructions according to the 'Plasmid DNA purification User manual' pages 13 et seqq. from Mancherey-Nagel GmbH & Co. KG. [Mancherey-Nagel GmbH & Co. KG 2014]

Sequence maps from all plasmids are attached in the appendix (**Appendix 2** – **Appendix 5**).

The cells were then each plated out in freshly made Agar plates with 1:1000 chloramphenicol (25 mg/mL in EtOH). Therefrom two colonies each were picked and cultivated in 3 mL pre-cultures. 4G2 was taken along as well, grown with Zeocin (1:4000), as a negative control to compare the outcome with the co-transfected chaperones to the outcome without.

After, the plasmid purification (MiniPrep) was done as Figure 19 shows.



First, 1.5 mL of cell culture gets harvested and centrifuged as picture shows. After, cell pellet gets lysed by three different buffers and then poured onto the column that comes with the kit. There the plasmid binds. After that a centrifuge step follows again, as well as a washing step, and lastly plasmids get eluted with elution buffer.

***Figure 19**: Overview of a plasmid purification (MiniPrep).*
Source: [Mancherey-Nagel GmbH & Co. KG 2014]

Yields are shown in the result section in **Table 19**.

4.3.2 Co-transfection of DE3 cells via heat-shock method

Next, *E. coli* DE3 cells, which came in the One Shot® kit from Thermo Fischer Scientific, were double transfected with 10 ng of each plasmid DNA in a total volume of 5 µL, coding for chaperones and scFv 4G2. Therefore, the heat-shock method from the 'One Shot® User manual' (page 6) was implemented. [Thermo Fischer Scientific 2010]
In addition to that, two single transformations were done with only the scFv 4G2 and only chaperone combination 4.

4.3.3 Expression test

For the expression test, five colonies (Clone 1 – 5) were picked from each transformation, which was implemented as follows.

> First, colonies were pre-culture in a small scale (V = 3 mL + both antibiotics) overnight. Then, 500 µL were pipetted out into 10 mL fresh LB medium with both antibiotics to keep selective pressure high, followed by an incubation for 3 hrs at 37 °C and 180 RPM. After, 1 mL was taken out, spun down for 20 minutes at full speed and the pellet frozen. The rest of the culture was divided into 2 cultures. One, 5 mL in which 10 µL 100 mM IPTG was added. And the other 4 mL culture was taken along as a negative control without added IPTG. Both was cultivated for another 3 hrs at 37 °C and 180 RPM. After that, again 1 mL of each culture was harvested, centrifuged, and spun down for 20 minutes at full speed.

Those pellets were then analysed and put on gel as follows.

> First to every pellet, 100 µL of MilliQ filtered water was added and vortexed until fully resuspended. To that, 50 µL loading buffer were added, vortexed for 1 minute and incubated for 3 minutes at 70 °C. This was done twice which breaks open the cells. Then, the samples were spun down at maximum speed for at least 10 minutes and afterwards 5 µL each put on gel.

4.3.4 Large-scale expression

Since the expression test did not show any clear results about which chaperone compared to the others expressed the most scFv 4G2, the large-scale expression was repeated with all four chaperones.

Therefore, pre-cultures were set up with a starting volume of 16 mL from the co-transfected bacteria cells. From there, each culture was divided in four 2 L Erlenmeyer flasks, each filled with 500 mL LB medium and 4 mL of culture, plus both antibiotics.

The expression was implemented the same way as previously described, only 2.5 mL sterile IPTG was added to each flask, and instead of putting the culture on a gel, cells were harvested after the incubation time with a centrifugation step for 20 minutes at 4000 RCF.

From there, the protein of interest was isolated the same way as described in chapter 4.2.2. Only now, the sample S1, after the first centrifugation step, was the sample of interest since this is the cytosol sample in which the properly folded scFv 4G2 should occur.

This was then also the sample which was further used for purification via IMAC as described in chapter 4.1. All used buffers did not contain urea, but 500 mM NaCl, since the cytosol sample does not contain any urea to begin with. After that the eluted cytosol samples were concentrated in a concentrator tubes (with a cut-off of 5 kDa) for ~ 45 minutes.

***Table 16**: Overview of expressed protein concentrations from folded 4G2 with chaperones in mg/mL after isolation, purification, and concentration.*

Cytosol sample from each co-transfected chaperone combination + scFv 4G2	Protein concentration [mg/mL]
Combination 1 + scFv 4G2	1.068
Combination 2 + scFv 4G2	0.921
Combination 3 + scFv 4G2	2.411
Combination 4 + scFv 4G2	2.376
scFv 4G2	2.428

 # = number of each chaperone.

All samples of each co-transfected chaperone combinations + scFv 4G2 culture from the isolation and purification steps were put on gel.

With the concentrated samples, a spot blot was done. Therefore 5 µL each were spotted on after diluting all samples to 1 mg/mL. Also, a 1:2 and a 1:4 dilution was each spotted on the blot. Incubated with AMA1 FVO as primary AB (1 µg/mL), then incubated with 62F12 as secondary AB (1 µg/mL) and for developing incubated with α-mouse AB AP conjugated.
To compare, the scFv 4G2 culture without co-transfected chaperones was taken along as a negative control.
Results can be seen in chapter 6.4.

5. Implementation plan

To give a summarized overview, the implementation plan to get to the desired results of a properly folded and active scFv 4G2, was established as follows.

The three different attempts to produce a therapeutically active form of the scFv derived from the anti-malaria mAB 4G2, were implemented parallelly to be able to compare their outcome in the end.

Therefore, on the one hand a mammalian cell line was established (HEK293-F) and transfected with the scFv 4G2 plasmid, since they have the folding machinery to properly fold eukaryotic proteins, such as mABs like 4G2. However, this is not a guarantee for the scFv 4G2 properly folds. In addition, mammalian cell cultures are more expensive and laborious than microbial cultivation and also yields may be less, which is problematic if lengthy purification procedures are needed. That is why using HEK293-F cells are transfected with different secretion factors to attempt secretion of the scFv protein out of the cell into the medium for a short and easy purification.

On the other hand, by mentioning all the laborious effort and time mammalian cell lines require, bacterial cultures (*E. coli*) were established and transfected with the scFv 4G2 plasmid. Their doubling time is way shorter plus the laborious efforts are markedly less compared to the HEK293-F cells. However, the scFv 4G2 is a eukaryotic mAB for which prokaryotic cell lines are mostly missing the right folding machinery. That is why, eukaryotic proteins are often transferred to intercellular IBs, which makes the isolation and purification of the proteins more difficult. Plus, they are missing their active form.

Therefore, two further approaches using the *E. coli* cell cultures were implemented parallelly. First, attempting to refold the isolated and purified IBs of scFv 4G2 through a rapid dilution in different refolding buffers and secondly, co-transfecting four chaperone combinations which assist the cells in properly folding the eukaryotic protein rather than putting it into IBs.

Since all these attempts need a proper analysis, a binding assay was developed with Fab fragments derived from mAB of a rat hybridoma culture, cleaved via the cutting enzyme papain, to test all used methods and assays for their validity beforehand. Fab fragments were used because of their similar characteristics compared to scFvs.

6. Results

This chapter presents the results of all experiments described in previous chapters.

For a better overview, the results will also be divided into sub-chapters from each approach, starting with the results from the production of scFv 4G2 in HEK293-F cells.

6.1 Production of scFv 4G2 in HEK293-F cells

HEK cells were cultivated according to chapter 4.6.

After two weeks of cultivation, cells were transfected with 5 different expression plasmids. Four of them expressing scFv 4G2, one of them without a secretion signal and the others with different secretion signals. And the last one expressing only GFP as a positive control, as shown in Table 17.

Table 17: *Yield of double stranded DNA of each plasmid.*

Plasmids	**Yield** [ng/µL]	**Total yield** [µg]
scFv 4G2 pSelectSEC	770	385
scFv 4G2 pSelectKappa	433	216.5
scFv 4G2 pSelectCD5	866	433
scFv 4G2 pSelect	695	347.5
pSelectGFP (+ control)	560	280

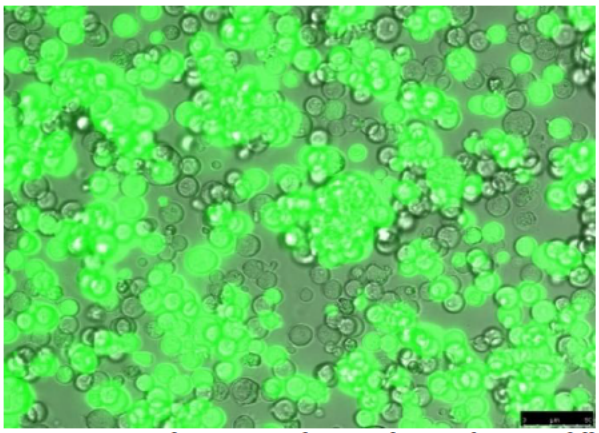
The last column states the yield of 150 mL culture.

For measurements, the DeNovix UV spectrophotometer was used at $\lambda = 260$ nm to determine the double stranded DNA in each case. Therefore 1 µL from each sample was pipetted onto its sensor, after a blank measurement with demi. water.

All pre-settings were already programmed.

Measurement was automatic.

After 48 hours of cultivation, the GFP control culture was checked under the fluorescence microscope to validate if the transfection method worked as seen in Figure 20.



Green-fluorescent cells show successfully transfected cells, whereas grey cells in the background are showing not successfully transfected cells.

Figure 20: *Overlap picture of GFP culture with successfully transfected and not successfully transfected cells.*
Source: private shot

Observing GFP-expressing cells indicates that the transfection procedure was successful, implying that all the other plasmids were also successfully transfected, and warrant further analysis. Therefore, the other transfected cells were harvested and both cells and supernatants were analysed using SDS-page and Western blotting. See Figure 21 and 22.

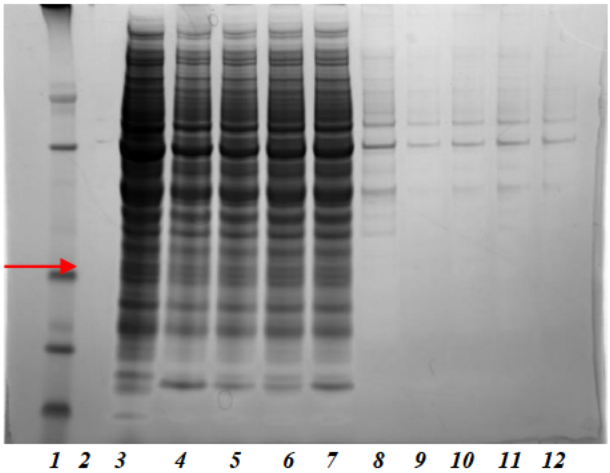


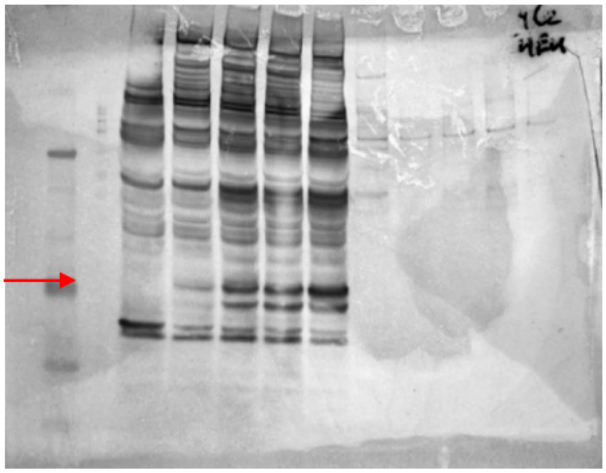
Figure 21: *Electrophoresis gel of proteins from transfected HEK 293-F cells after harvesting.*
Source: private shot

1. *NuPAGE® MES marker*
2. */*
3. *HEK 293-F cells (blank – not transfected)*
4. *HEK 293-F cells with transfected 4G2 p.Select vector (without secretion factor)*
5. *HEK 293-F cells with transfected 4G2 p.SelectCD5*
6. *HEK 293-F cells with transfected 4G2 p.SelectKappa*
7. *HEK 293-F cells with transfected 4G2 p.SeleceSECRECON*
8. *Medium-supernatant of HEK 293-F cells (blank)*
9. *Medium-supernatant of HEK 293-F cells, 4G2 p.Select vector (without secretion factor)*
10. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelectCD5*
11. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelectKappa*
12. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelectSECRECON*

The red arrow is indicating the height at what the MW of scFv 4G2 is expected (28 kDa).
From the above figure, no bands are visible at the expected size of 28 kDa, nor anywhere else in the 4G2 producing cells compared to the non-transfected control (compare lane 3 with lane 4-7, and lane 8 with lanes 9-12.

As the sensitivity of Coomassie staining is not very high, a western blot was performed using the same samples (Fig. 22), using antibodies against the hexa-histidine tag at the C-term as a positive identification for the protein-of-interest (ScFv 4G2).

Fig. 22 shows no signal in the medium samples of any cells, suggesting that the ScFv 4G2 is not secreted into the medium. However, it shows, apart from a high level of a-specific binding of the hexa-histidine antibodies in solubilized cells that there is a band present in the 4G2 transfected cells that is absent from the control. Moreover, the cells transfected with a secretion factor containing the ScFv 4G2 gene showed a slightly more intense band at the expected apparent molecular weight compared to the control ScFv 4G2 plasmid (compare lane 4 with lanes 5-7).



1 2 3 4 5 6 7 8 9 10 11 12

***Figure 22**: Band pattern of the Western blot of proteins from transfected HEK 293-F cells.*
Source: private shot

1. *NuPAGE® MES marker*
2. */*
3. *HEK 293-F cells (blank – not transfected)*
4. *HEK 293-F cells with transfected 4G2 p.Select vector (without secretion factor)*
5. *HEK 293-F cells with transfected 4G2 p.SelectCD5*
6. *HEK 293-F cells with transfected 4G2 p.SelectKappa*
7. *HEK 293-F cells with transfected 4G2 p.SelectSECRECON*
8. *Medium-supernatant of HEK 293-F cells (blank)*
9. *Medium-supernatant of HEK 293-F cells, 4G2 p.Select vector (without secretion factor)*
10. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelectCD5*
11. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelectKappa*
12. *Medium-supernatant of HEK 293-F cells, 4G2 p.SelectSECRECON*

To check all possible ways, two more Western blots were done. Where in one half of the samples DTT was added to compare the differences to the ones without.

Only samples from solubilized cells were considered, since samples of the medium did not show any response.

Figure 23 shows a Western blot incubated with anti-6-His-tag AB (1:500) as primary AB and α-mouse AB AP labelled as secondary AB (1:1000).

Figure 24 shows a Western blot incubated with AMA1 FVO (1:200) as primary AB, BG98 (1:2000, donor: rabbit) as secondary AB, and α-rabbit AB AP labelled (1:5000) as third AB to detect the binding.

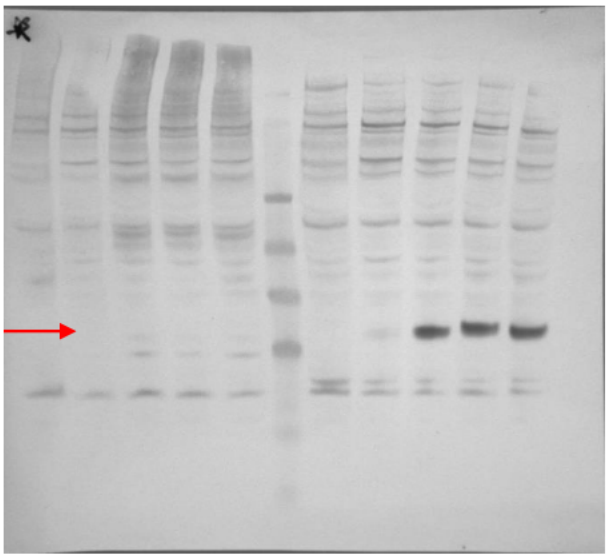


***Figure 23**: Western blot of cellular fractions from transfected HEK 293-F cells, either non-reduced (lanes 1-5) or reduced (lanes 7-11), visualized with an anti-hexahistidin AB.*
Source: private shot, ChemieDoc™ BPRC.

1. *HEK 293-F cellular fraction (blank – not transfected), no DTT*
2. *HEK 293-F cellular fraction with transfected 4G2 p.Select vector (without secretion factor), no DTT*
3. *HEK 293-F cellular fraction with transfected 4G2 p.SelectCD, no DTT*
4. *HEK 293-F cellular fraction with transfected 4G2 p.SelectKappa, no DTT*
5. *HEK 293-F cellular fraction with transfected 4G2 p.SelectSECRECON, no DTT*
6. *NuPAGE® MES marker*
7. *HEK 293-F cellular fraction (blank – not transfected) + DTT*
8. *HEK 293-F cellular fraction with transfected 4G2 p.Select vector (without secretion factor)*
9. *HEK 293-F cellular fraction with transfected 4G2 p.SelectCD5 +DTT*
10. *HEK 293-F cellular fraction with transfected 4G2 p.SelectKappa +DTT*
11. *HEK 293-F cellular fraction with transfected 4G2 p.SelectSECRECON + DTT*
12. */*

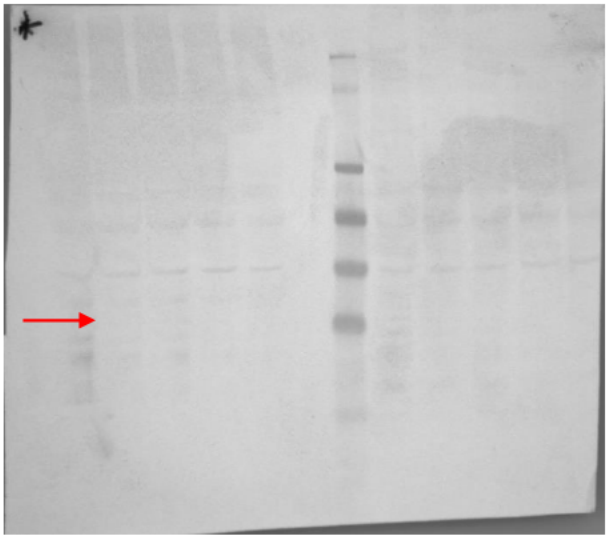


***Figure 24**: Western-assay blot of cellular fractions from transfected HEK 293-F cells, either non-reduced (lanes 1-5) or reduced (lanes 7-11), using an PfAMAM1 based binding assay to visualize the scFv 4G2 protein. PfAMA1 FVO was added to the blot, followed by the rabbit polyclonal BG98 that was detected using its AP-conjugate.*
Source: private shot, ChemieDoc™ BPRC.

Blot was incubated with AMA1 FVO (1:200, 0.15 mg/mL) as primary binding molecule, BG98 (1:2000, donor: rabbit) as secondary AB recognizing PfAMA1, and α-rabbit AB AP labelled (1:5000) as detector AB.

1. *HEK 293-F cellular fraction (blank – not transfected), no DTT*
2. *HEK 293-F cellular fraction with transfected 4G2 p.Select vector (without secretion factor), no DTT*
3. *HEK 293-F cellular fraction with transfected 4G2 p.SelectCD, no DTT*
4. *HEK 293-F cellular fraction with transfected 4G2 p.SelectKappa, no DTT*
5. *HEK 293-F cellular fraction with transfected 4G2 p.SelectSECRECON, no DTT*
6. */*
7. *NuPAGE® MES marker*
8. *HEK 293-F cellular fraction (blank – not transfected) + DTT*
9. *HEK 293-F cellular fraction with transfected 4G2 p.Select vector (without secretion factor)*
10. *HEK 293-F cellular fraction with transfected 4G2 p.SelectCD5 +DTT*
11. *HEK 293-F cellular fraction with transfected 4G2 p.SelectKappa +DTT*
12. *HEK 293-F cellular fraction with transfected 4G2 p.SelectSECRECON + DTT*

The blot from Figure 23 shows clearly on the right side, where DTT is added to the samples with the secretion factors, thick bands with the expected size for scFv 4G2. Compared to the left side of

the blot without added DTT, only very slight bands are visible in the samples transfected with the secretion factors.
However, the blot incubated with AMA1 (Figure 24), to test the binding capacity and therefore active and properly folded form of scFv 4G2 did not show any signal, suggesting that the scFv 4G2 did not bind to the PfAMA1, and was likely not properly folded.

At the same time an ELISA was done with the cellular fraction samples, to double check the binding capacity of the expressed scFv 4G2 in the HEK cells.
Since this did not give any binding signal either, the results of the ELISA will not be presented here.

6.2 Development of a binding assay, using Fab fragments of mAB 4G2

As proof for the correct folding of the scFv 4G2, binding to the target molecule, PfAMA1, is considered to be a prerequisite. As correctly folded scFv 4G2 was not available (as this is the goal of the project), Fab fragments derived of the mAB were considered to be the best mimic for the scFv 4G2, as these are considerably smaller than the intact mAB 4G2 itself and size could be of influence on the behaviour of the molecule under assay conditions.
Fab fragments may be obtained from the mAB 4G2, which can be purified from a rat hybridoma culture, as described below.

Purification of the rat mAB 4G2 using Protein G Sepharose was performed as described in chapter 4.1.1).

From each step (as described in chapter 4.1.1) a sample was taken, put on gel, and stained in Coomassie blue (Figure 25).

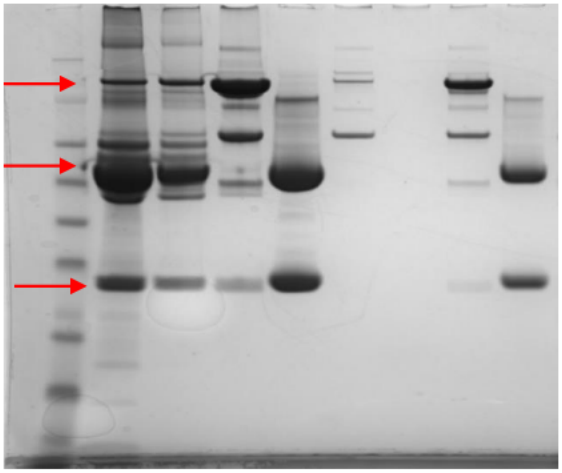


Figure 25: Band pattern of the purified IgG 4G2 from a rat. Source: private shot, ChemieDoc®™ BPRC.

1. *NuPAGE® MES marker*
2. *S1 – FT of column*
3. *S1.1 – washing step*
4. *S2 – eluted fraction*
5. *S2 – eluted fraction, reduced*
6. *S3 – washing step*
7. *S4 – PBS + azide*
8. *S5 – FT concentrating step*
9. *S5 – FT concentrating step, reduced*

The red arrows are indicating the expected MW of the parts from the whole mAB 4G2 (describing from the top).
First arrow: 150 kDa – whole mAB
Second arrow: 50 kDa – partially degraded 4G2/ Fc -tails
Third arrow: 25 kDa – Fab fragments

Lanes 2 and 3 show the FT of the hybridoma medium sample i.e., with proteins that did not bind onto the column. A dominant band is present in the FT, which has a molecular size of approximately 50 kDa, which may resemble partially degraded 4G2 protein. It is unlikely that this fraction resembles free Fc-tails, as these are expected to bind to the column. Also, a band is present at 25 kDa, that may represent free light chains, which were already washed out within the binding and washing step. Since the IgGs have a MW of ~ 150 kDa, their protein band should occur between the Myosin band (188 kDa) and Phosphorylase band (98 kDa) compared to the marker. Since there is a small, yet clear band visible in lanes 2 and 3, not all IgGs did bind. The mAB 4G2 was eluted using elution buffer, illustrated by the presence of a protein of 150 kDa in fraction S2 (lanes 4 + 5). After reduction with DTT, the 150 kDa protein is split up in Fc-tail and Fab, with size of 50 and 25 kDa, respectively (lane 5).

The purified mAB 4G2 was digested with papain, according to chapter 4.1.24.1.2. As shown in figure 26, lanes 3 and 5, the digestion resulted in a clear band at 25 kDa, with or without the addition of DTT.

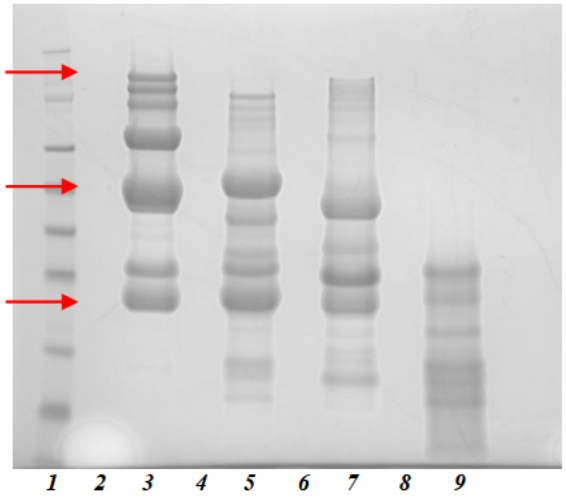


Figure 26: Band pattern of digested IgG solution stopped with different chemicals.
Source: private shot, ChemieDoc®™ BPRC.

1. *NuPAGE® MES marker*
2. */*
3. *Fabs – digestion stopped with Iodoacetamide*
4. */*
5. *Fabs – digestion stopped with Iodoacetamide + DTT*
6. */*
7. *Fabs – digestion stopped with 1mM ascorbic acid and 50µM Cuso4*
8. */*
9. *Fabs – digestion stopped with 1mM ascorbic acid and 50µM Cuso4, reduced*

The red arrows are indicating the expected MW of the parts from the whole mAB 4G2 (describing from the top).
First arrow: 150 kDa – whole mAB
Second arrow: 50 kDa – Fc tail
Third arrow: 25 kDa – Fab fragments

Figure 26 shows not only the digestion that was stopped by the addition of Iodoacetamide, but also a digestion stopped by the addition of 1 mM ascorbic acid and 50 µM $CuSO_4$ (lane 7 and 9) to compare. Since the reduced protein (lane 9) showed that more cuts were present in the protein, the digestion with Iodoacetamide was further used to obtain the Fab fragments.
Focusing on lanes 3 and 5, the samples from the digestion stopped with Iodoacetamide without and with DTT, it clearly shows the cleaved parts of the IgGs. The bands with higher MW, that are visible, can be considered as uncut IgGs. In the sample with the added DTT, the expected shift to mainly Fc tails and Fabs, occurs, since DTT is a reduction reagent which breaks protein´s disulfide bonds.

After a successful digestion, the Fabs were purified via a Protein G Agarose column as described in chapter 4.1.1. Figure 28 shows the SDS page of the Fab fraction after purification.

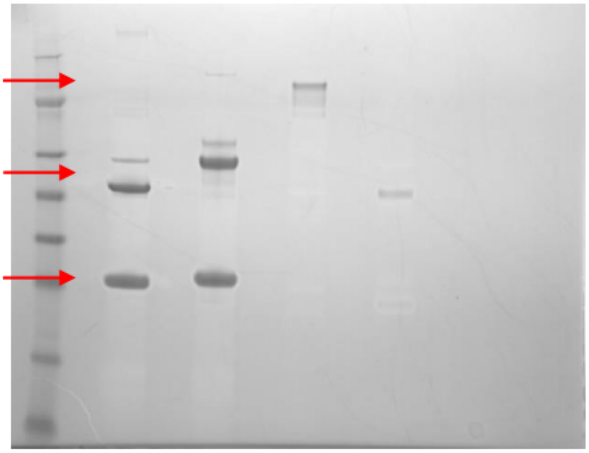


Figure 27: Band pattern of purified Fabs.
Source: private shot, ChemieDoc®™ BPRC.

1. *NuPAGE® MES marker*
2. */*
3. *Eluted Fab fragments*
4. */*
5. *Eluted Fab fragments + DTT*
6. */*
7. *FT (containing uncut IgGs)*
8. */*
9. *FT (containing uncut IgGs) + DTT*

The red arrows are indicating the expected MW of the parts from the whole mAB 4G2 (describing from the top).
First arrow: 150 kDa – whole mAB
Second arrow: 50 kDa – partially degraded 4G2/ Fab multimeres
Third arrow: 25 kDa – Fab fragments

Lanes 3 and 5 show the eluted Fab fragments, which were not retained on the column, at their expected MW of ~ 25 kDa. The upper bands showing at a higher MW resemble either partially degraded 4G2 or formed Fab multimeres, which couldn´t and were not further purified.
This purification step was intended primarily to remove intact antibody. So that, for further tests and analysation of the Fab fragments, results will not be falsified by the strong binding signal of the whole remaining 4G2. Moreover, Fc tails are not considered as interference factors, as they will not be able to bind to the target antigen, PfAMA1.

After the purification, the binding capacity of the Fabs were tested via a spot blot (Fig. 28) and Size Exclusion Chromatography (Fig 29) as described in chapter 4.2.2 and 4.4.

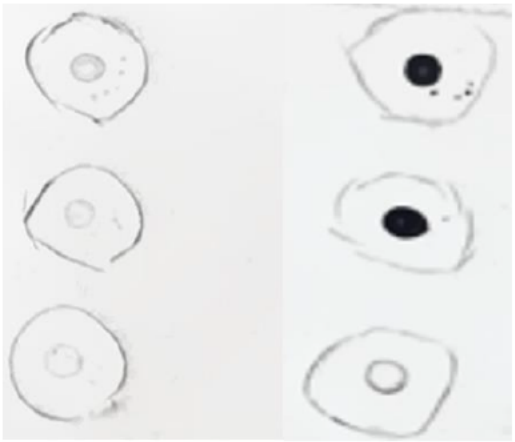


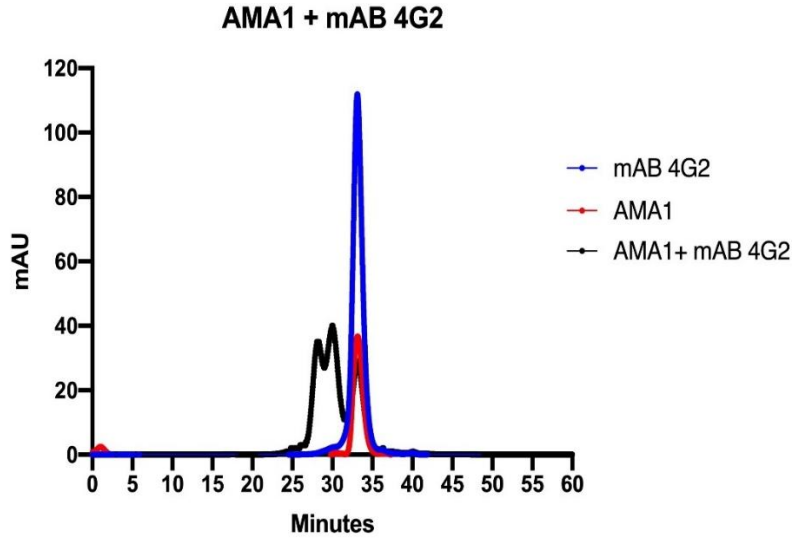
Figure 28: Spot blot of purified Fab fragments (left) and AMA1 as a control (right). Source: privat shot.

First row: 1 mg/mL.

Second row: 1:10 dilution.

Third row: 1:100 dilution

On the left side of Figure 28, the response of the Fab sample is shown. Here 5 µL of undiluted Fab-fragment sample was spotted on (top left) and from that two dilutions were made (1:10 and 1:100). Same applies for AMA1 FVO as a control, shown on the right side of the blot, with a starting concentration of 1 mg/mL (top right). First, the blot was incubated with 1 µg/mL AMA1 FVO (without a His-tag) as binding antigen. After, it was incubated with 62F12 as a secondary AB (donor: mouse, 1 µg/mL) and lastly with an anti-mouse AB AP labelled for detection. Since the blot gave a signal and binding and detection can be considered successful, a SEC followed for further analysis (Figure 29).



Upper chromatogram: Graphs of 4G2 (blue lain), AMA1 FVO (red lain), and incubated mix of AMA1 and 4G2 (black lain), as control.

Bottom chromatogram: Graphs of AMA1 FVO (red lain), Fab fragments (pink lain), and incubated mix of AMA1 and Fab fragments (black line).

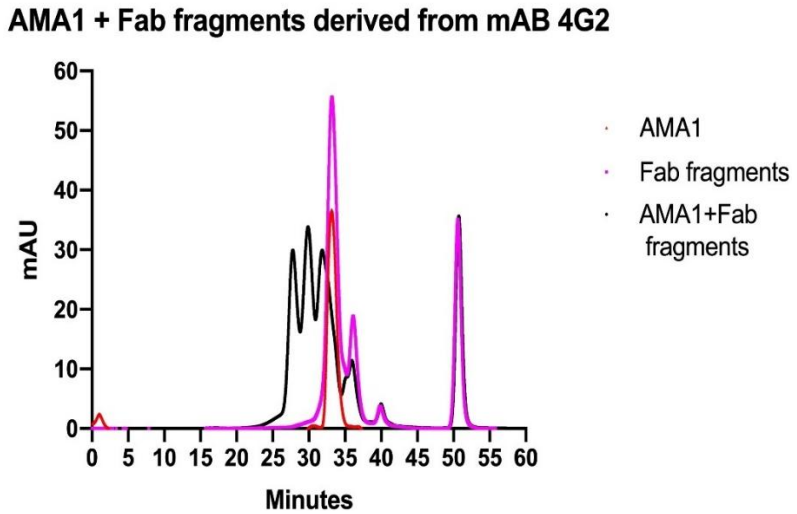


***Figure 29**: Chromatograms of binding experiments with Fabs and AMA1 FVO. Source: private image, HPLC BPRC.*

The upper superimposed chromatograms show the analysis of mAB 4G2 (in blue), PfAMA1 (in red) and the mixture of the two, incubated at RT for 1 hour (in black), serving as a positive control. It is already known that the active form of 4G2 binds to AMA1 and forms a complex. This complex is bigger than the individual components and should therefore elute at a smaller retention time compared to the solution with only the AB (blue graph) or the AMA1 (red graph).
From the upper chromatogram of Figure 29 it is evident that the antibody forms a binding complex with PfAMA1, as peaks appear at higher sizes, respectively they elute before both the mAB 4G2 and AMA1. However, there are some peculiar aspects to the chromatograms that will be discussed in the next chapter.
Similarly, the Fab fragments incubated with AMA1 show that larger complexes are formed. Again, also in this case there are peculiarities that will be discussed in the next chapter.
Regardless, it is clear that the Fab fragments may be used for assay-development, as illustrated in Fig 29, where there is a signal, although weak, of the Fab fragments binding to AMA1.

After methods and assays were found to be valid, the refolding experiments and expression in *E. coli* cells co-transfected with chaperones, were implemented as described in chapter 4.2 and 4.3.

6.3 Refolding of in *E. coli* expressed scFv 4G2 using rapid dilution

After growing the *E. coli* culture as described in chapter 4.2.1 and before starting the rapid dilution, the produced scFv 4G2 present in the IBs needed to be isolated and purified as described in chapter 4.2.2.

As the scFv 4G2 is present in the IBs, the first step of the purification of scFv was to solubilize the IBs. This was done using 8.0 M urea, a chaotropic agents that basically unfolds proteins, in this way making them water soluble.
After solubilization, the scFv 4G2 was purified using affinity chromatography in the presence of 8.0 M urea, using the C-terminal hexahistidine tag of the scFv 4G2 to bind to the IMAC column, loaded with Nickel as described in chapter 4.1.

Figure 30 shows the chromatogram of the purifying process of the scFv 4G2 via IMAC.

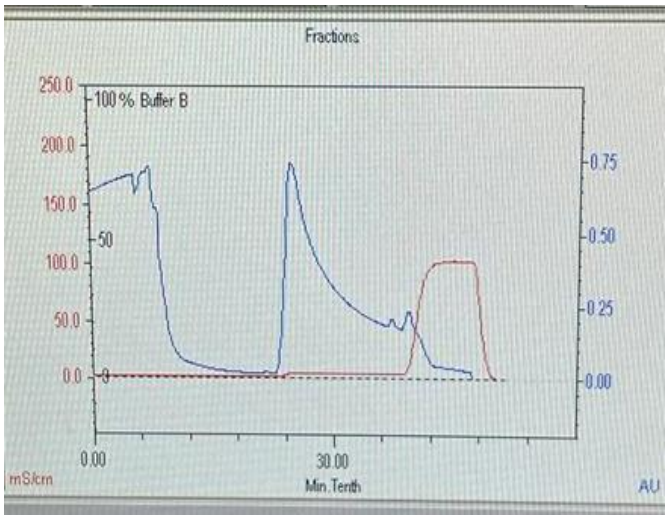


***Figure 30**: Purification of the isolated 4G2 IBs via IMAC.*
Source: private shot, IMAC system BPRC.

Blue line (right y-axis): protein detection via UV detector in AU.

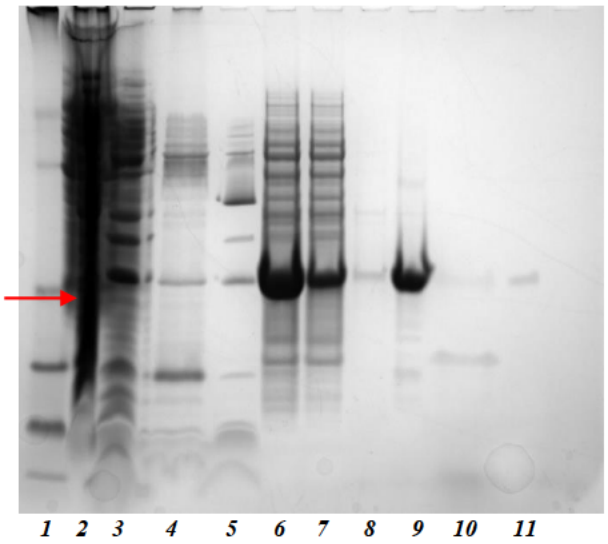
Red line (left y-axis): conductivity in mS/cm.

Peak in the middle (at minute 30): elution of IB.

The red line shows the conductivity in mS/cm and the blue graph shows the absorbance at 280 nm, basically detection proteins.

The peak in the middle of the chromatograph, appearing at a retention time of 20 minutes with 0.75 AU at 280 nm is the elution step of the urea solubilized scFv 4G2. Since the peak shows strong tailing, the washing step was indicated before the blue detection line was back to baseline. Which can be seen at the increase of the conductivity line and the following peaks. Moreover, sufficient amounts of the scFv 4G2 was collected already, and a further dilution of that sample was not preferable. Samples of the purification steps were analysed by SDS-PAGE.

Figure 31 shows a picture of the Coomassie-stained gel.



1. *NuPAGE® MES marker*
2. *S1*
3. *S2*
4. *S3*
5. *S4*
6. *IB*
7. *Protein FT*
8. *Protein W 10 mM Imidazole*
9. *Protein E 100 mM Imidazole*
10. *Protein EDTA*
11. *H_2O.*

Red arrow is indicating the expected height of the MW from scFv 4G2 IBs (28 kDa).
All buffers contained 8.0 M urea.

***Figure 31**: Band pattern from IBs of 4G2 expressed in E. coli DE3 cells.*
Source: private shot, ChemieDoc™ BPRC.

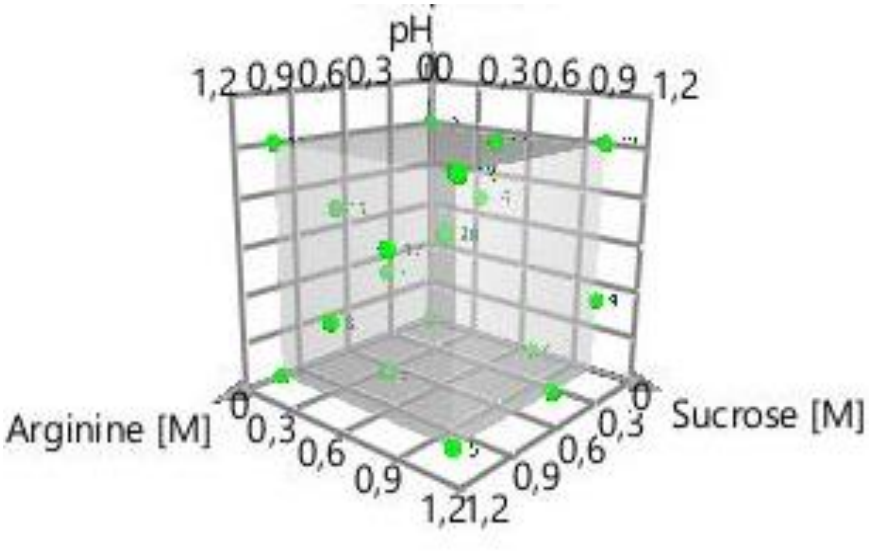
Hereby lane 9 ('Protein E 100 mM Imidazole') is important, since this contains the protein of interest, the scFv 4G2. A clear band occurs at the MW of ~ 28 kDa, which indicates a successful purification of the inclusion bodies. However, the bands of well 6 and 7 also show a notable amount of IBs that flow through the column without binding.

Nevertheless, the amount of purified IBs is enough to continue with further experiments. Moreover, it is also clear to see, that the purification was successful, because not many other protein bands are visible. Those who are, are at a remarkably low concentration. Which means, that the IBs are pure enough for the following experiments.

After, the 18 different buffers were prepared as shown in Appendix 1 and as described in chapter 4.2.3.

PH was adjusted with either a stock solution of HCl or NaOH.

Figure 32 shows again the overview of the D-optimal design of the experiments, which was programmed via 'MODDE'. Buffer 18, respectively experiment 18, as centre point was done in a triplicate.



Y-axis: pH, varying from 6.0 over 6.8, 7.7, and 8.5.

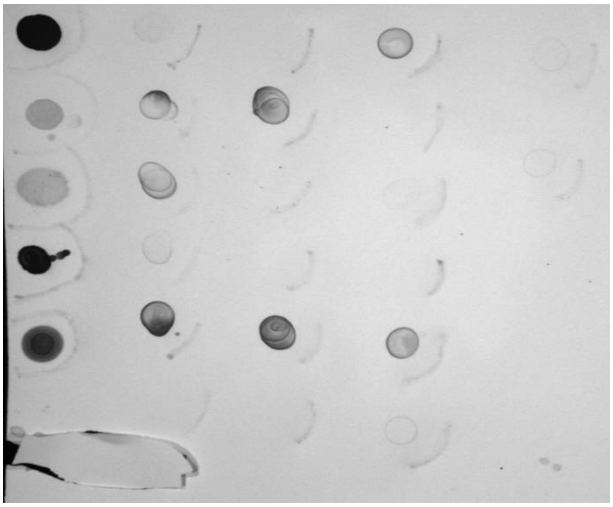
X-Axis: Arginine [M] varying from 0 over 0.33, 0.66 and 1.0 M.

Z-Axis: Sucrose [M] varying from 0 over 0.33, 0.66 and 1.0 M.

Figure 32: *D-optimal design region of buffer compositions in correlation with number of refolding experiments.*
Source: MODDE program.

The y-axis shows the pH, which varied between 6.0, 6.8, 7.7, and 8.5. The x-axis shows the arginine concentration in molar and the z-axis shows the sucrose concentration in the buffers also in molar. Both ingredients vary from 0 to 0.33, 0.66 and 1.0 M in the buffers.

Figures 33 and 34 show the result of the rapid dilution of the refolding experiments.
All blots were implemented and developed as previously described in chapter 4.4.1.



First column: every incubation step as controls.

Rest of the blot: buffers 1 – 18 containing refolded scFv 4G2 (beginning from the top of column 2, counting down) + sample 19 and 20 as a triplicate to buffer composition 18 (top on the very right).

Figure 33: *Spot blot of refolded 4G2 in buffers 1 – 18 incubated with AMA1 FVO.*
Source: private shot, ChemieDoc™ BPRC.

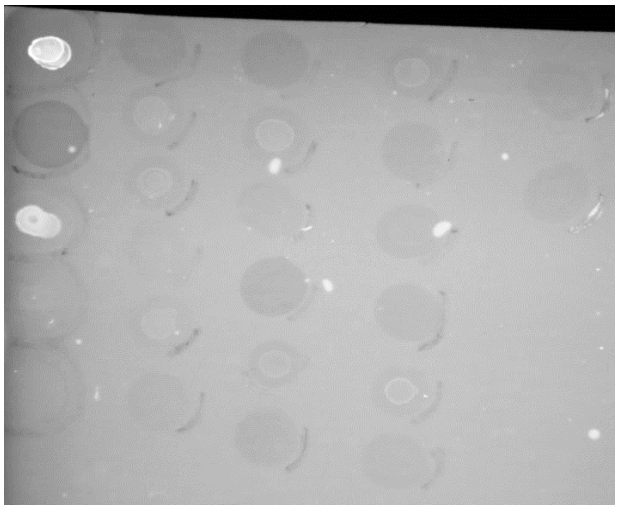


Figure 34: *Spot blot of refolded 4G2 in buffers 1 – 18 incubated with fluorescent AMA1 Alexa488.*
Source: private shot, ChemieDoc™ BPRC.

First column: every incubation step as controls.

Rest of the blot: buffers 1 – 18 containing refolded scFv 4G2 (beginning from the top of column 2, counting down) + sample 19 and 20 as a triplicate to buffer composition 18 (top on the very right).

.

Comparing the signal from blot shown in Figure 33 (incubated with AMA1 FVO) with the signal from blot shown in Figure 34 (incubated with AMA1 Alexa488) it is clear to see, that all the control steps worked, which ensures the correctness of the blots. Moreover, every signal that has been achieved, meaning signals from buffers 2, 3, 5, 8, 11, 13, and 17, are seen in both blots simultaneously. That ensures that those buffers contain a correctly folded active form of scFv 4G2 which binds to the antigen. Therefore, these samples were further used to do more analysis.

Figure 35 shows the blot of the samples previously mentioned, that gave the strongest signal, but rebuffered to PBS (pH 7.0). The buffer exchange was directly done after the rapid dilution, to show that supposedly once the protein is properly folded, it should stay in its active, correctly folded form despite the buffer it is in.

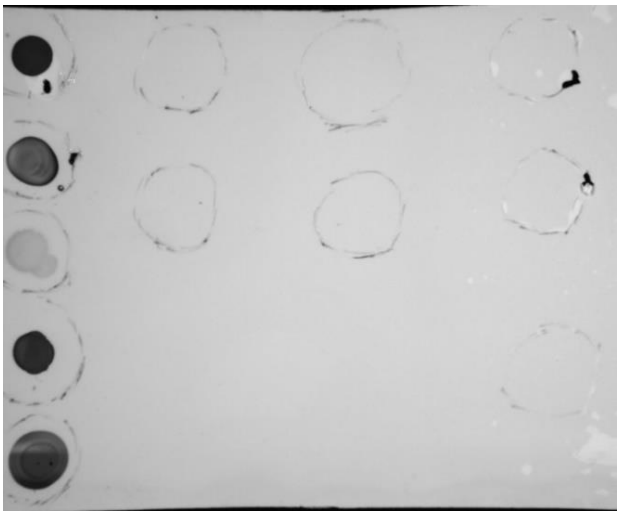


Figure 35: *Spot blot of refolded 4G2 from buffers 2, 3, 5, 8, 11, 13, and 17 after buffer exchange to PBS.*
Source: private shot, ChemieDoc™ BPRC

First column: every incubation step as controls.

Column two: buffers 2, and 3.
Column three: buffers 5 and 8.
Column four: buffers 11, 13, and 17
All rebuffered to PBS directly after the refolding in the aforementioned buffers.

Looking at the blot, the control steps show strong response as before, which proofs the correctness of the blot. However, no signal has been achieved after developing the blot in neither of the samples.

Concluding that, the ELISA and SEC were implemented with only the correctly folded samples from blot shown in Figure 33.

The purpose of doing the ELISA was only to examine, if one of the buffers shows a stronger signal compared to others and therefore contains a higher concentration of refolded scFv 4G2.
Results were automatically measured in an ELISA plate reader at 405 nm after developing as described in chapter 4.5.2. Triplicates have been omitted.

Table 18 shows the results.

Table 18: ELISA results from buffers 2, 3, 5, 8, 11, 13, and 17 containing refolded scFv 4G2, measured at 405 nm.

Samples	Buffer 2	Buffer 3	Buffer 5	Buffer 8	Buffer 11	Buffer 13	Buffer 17	4G2 (+ control)	Negative control
Wells	1	2	3	4	5	6	7	8	9
A	0.745	0.864	0.685	0.872	0.931	1.249	0.902	3.381	0.026
B	0.578	0.704	0.475	0.809	0.635	0.588	0.946	3.283	0.026
C	0.362	0.402	0.363	0.614	0.476	0.577	0.561	3.281	0.025
D	0.241	0.301	0.272	0.436	0.429	0.363	0.379	3.241	0.026
E	0.220	0.273	0.258	0.342	0.298	0.294	0.401	3.333	0.029
F	0.214	0.241	0.234	0.305	0.282	0.327	0.340	3.034	0.026
G	0.226	0.237	0.224	0.250	0.234	0.253	0.288	3.362	0.025
H	0.247	0.257	0.217	0.249	0.238	0.264	0.278	3.353	0.025

Column 1: buffer 2. Column 2: buffer 3. Column 3: buffer 5. Column 4: buffer 8. Column 5: buffer 11. Column 6: buffer 13. Column 7: buffer 17. Column 8: 4G2 as positive control. Column 9: / as negative control, only incubated with AMA1 FVO.

Figure 36 shows the measured OD of each sample in relation to the dilutions that were made, starting with an undiluted sample.

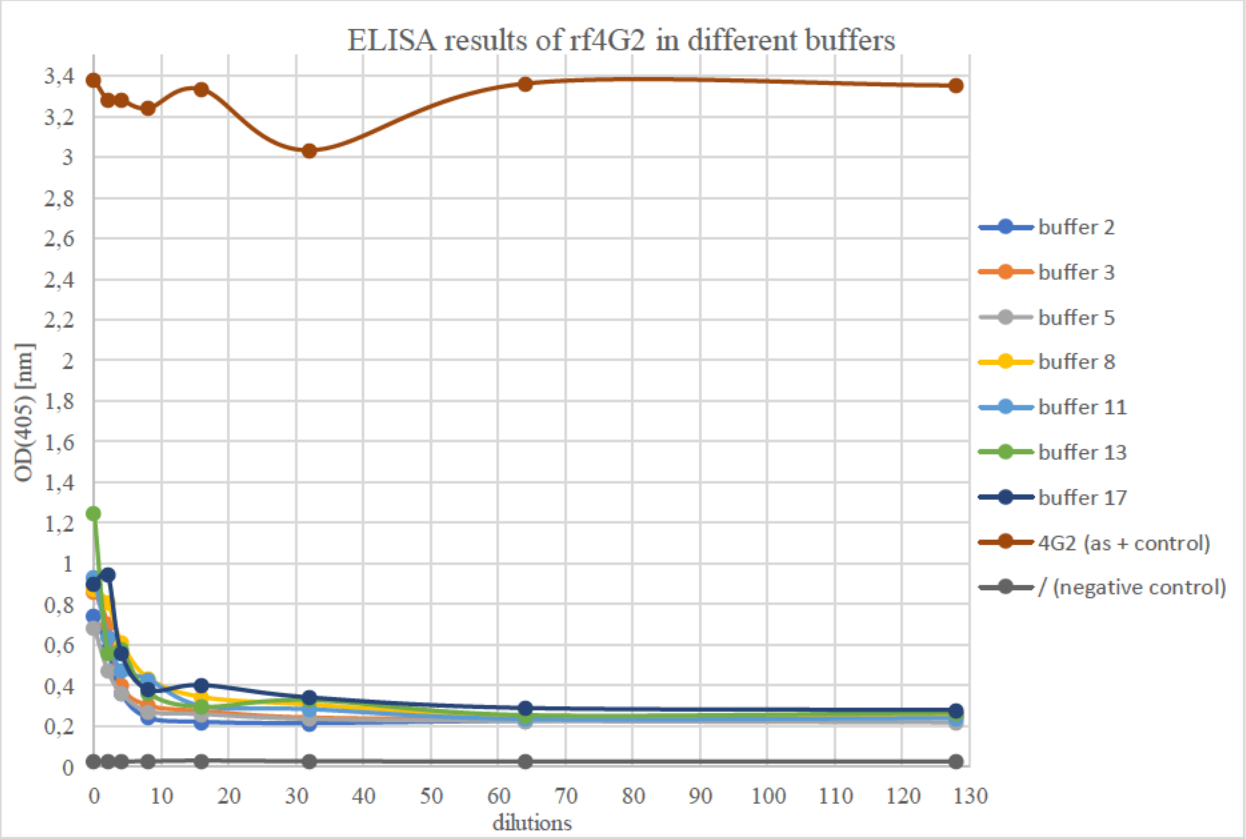
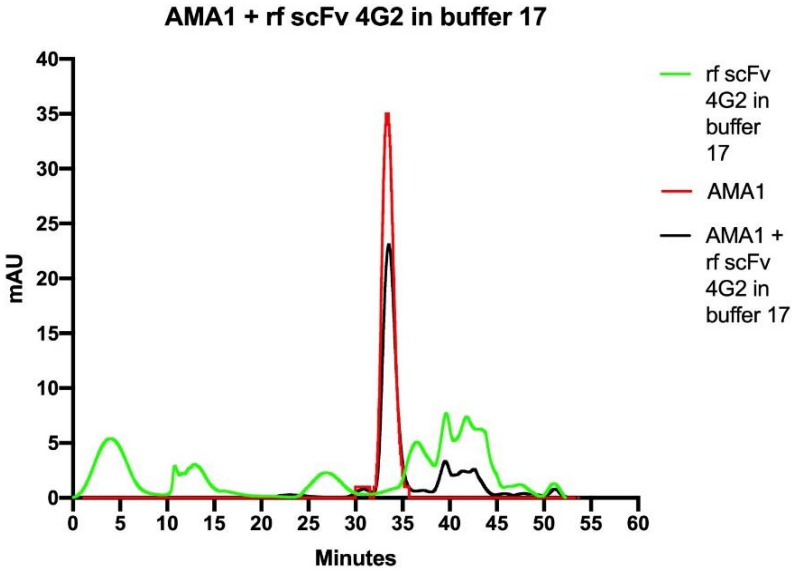
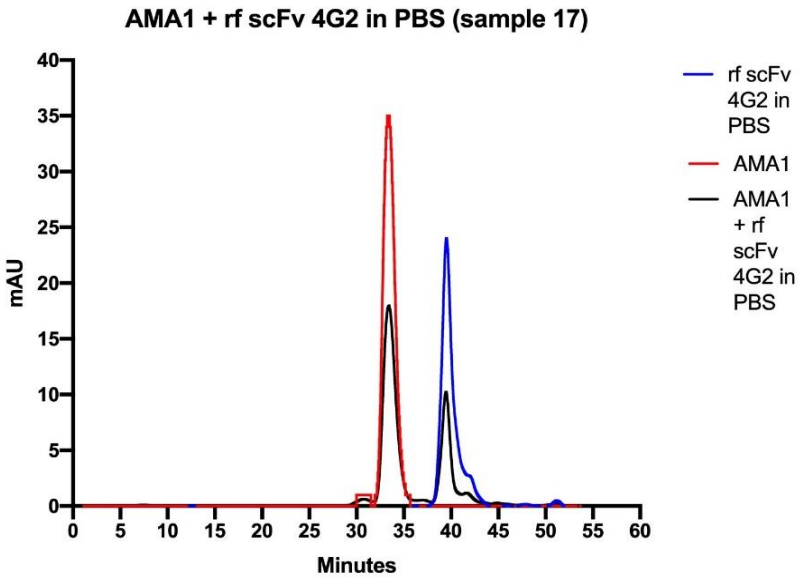


***Figure 36**: ELISA results of refolded scFv 4G2 in buffers 2, 3, 5, 8, 11, 13, and 17.*
Y-axis: OD measured at 405 nm. X-axis: dilutions starting from undiluted to a D_f= 128.

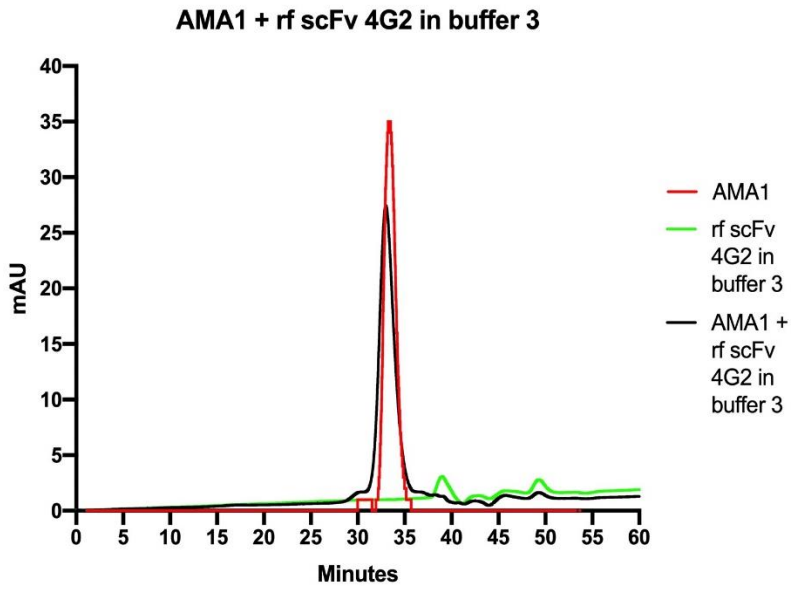
By looking at the results, it is clear to see that the positive control was too highly concentrated (starting with c = 0.5 mg/mL). Meaning, its graph was saturated throughout the dilutions which makes it incomparable. Whereas the negative control, which was only incubated with AMA1, shows no signal as expected. By looking at the response from the other graphs, no clear difference in the developed binding signal is visible. Meaning, that the concentration of the properly folded scFv 4G2 is about the same in all tested buffers, emitting the same signal. Therefore, buffer 17, which shows comparably a slightly stronger signal throughout its dilution, giving its higher measured OD, and buffer 3 were chosen to do further analysis using SEC (Figure 37) as described in chapter 4.2.2.



Upper chromatogram: Graphs of rf4G2 in buffer 17 (green lain), AMA1 FVO (red lain), and incubated mix of AMA1 and rf4G2 (black lain).



Middle Chromatogram: Graphs of rf4G2 from buffer 17 rebuffered to PBS (blue lain), AMA1 FVO (red lain), and incubated mix of AMA1 and rf4G2 (black lain).



Bottom chromatogram: Graphs of AMA1 FVO (red lain), rf4G2 in buffer 3 (green lain), and incubated mix of AMA1 and rf4G2 in buffer 3 (black line).

Figure 37: *Chromatograms of binding experiments with refolded scFv 4G2 via rapid dilution in buffers 17 and 3 + incubation mix with AMA1 FVO.*
Source: private image, HPLC BPRC.

As in chapter **Error! Reference source not found.** samples were run the same way. First, AMA1 FVO represented in each superimposed chromatograms which elutes at a retention time of ~ 34 minutes. Then refolded scFv 4G2 in buffer 17 (green graph, top chromatogram), in buffer 3 (green graph, bottom chromatogram) and refolded scFv 4G2 from buffer 17 rebuffered to PBS. Black graphs show the mix of AMA1 with each buffer, incubation time 1 hr at RT.

Since during the run of buffer 17, an interference signal occurred (upper chromatogram 'AMA1 + rf4G2 in buffer 17', green graph), the experiment was repeated with sample buffer 3 due to sample shortage. However, by looking at the black graphs of each chromatogram, which shows the incubated mixture of AMA1 and refolded scFv 4G2, no binding has occurred in neither of the samples. Each component is coming off individually, which the superimposed chromatograms clearly show. However, the absorbance measured for peaks appearing from the mixture has decreased by ~ 5 to 10 mAU in each chromatogram compared to the peaks from the individual components. This could be due to the amount of sample injected onto the column, which was less for the mixture than it was for each sample individually, due to sample shortage. Nevertheless, keeping in mind the molecule similarity.

Since rebuffering the refolded scFv 4G2 to PBS indicated no binding as well as the upper chromatogram shows no binding behaviour, for sample in buffer 3 the SE column was run with its

refolding buffer to keep the conditions as equal as possible to avoid possible unfolding, yet still no binding behaviour.
In regard to the standard, AMA1 elutes at an expected retention time for its size. However, the samples of the refolded scFv 4G2 is eluting much later. All about a retention time between 39 and 40 minutes, which indicates a size much smaller than they are supposed to be.
Comparing all three chromatograms from Figure 37, it can be concluded that no specific binding occurred with the 1 hr incubation at RT method in neither of these cases.

Therefore, the next approach was implemented, using chaperones to help *E. coli* cells to properly fold the scFv 4G2 rather than transferring the protein into IBs.

6.4 Chaperones as helper-proteins for refolding scFv 4G2 in *E. coli* cells

Before co-transfection and starting the expression test, the plasmids coding for scFv 4G2 and chaperones were purified as described in chapter 4.3.1.
The DeNovix UV spectrophotometer was used again at λ = 260 nm to determine the double stranded DNA each. Therefore 1 µL from each sample was pipetted onto its sensor, after a blank measurement with demi. water.

Table 19 shows the determined plasmid concentration for each purification.

***Table 19**: Yields of MiniPreps from all used chaperones and 4G2 as negative control.*

Plasmid combinations	**Yield** [ng/µL]
Combination 1 – Clone 1	15.757
Combination 1 – Clone 2	20.409
Combination 2 – Clone 1	19.285
Combination 2 – Clone 2	17.395
Combination 3 – Clone 1	23.764
Combination 3 – Clone 2	20.831
Combination 4 – Clone 1	8.676
Combination 4 – Clone 2	10.688
scFv 4G2 – Clone 1	79.187
scFv 4G2 – Clone 2	54.363

After a successful co-transfection and growing pre-cultures, the expression test was implemented as described in chapter 4.3.3.

The following Figures 38 – 41 show SDS page gels after the expression test of scFv 4G2, co-transfected with each chaperone combination. Each gel has the same format, which is:

Lane 1: marker
Lane 2: /
Lane 3: Clone 1 (-)
Lane 4: Clone 1 (+)
Lane 5: Clone 2 (-)
Lane 6: Clone 2 (+)
Lane 7: Clone 3 (-)
Lane 8: Clone 3 (+)
Lane 9: Clone 4 (-)
Lane 10: Clone 4 (+)
Lane 11: Clone 5 (-)
Lane 12: Clone 5 (+)

In this case (-) indicates the culture ‘without added IPTG’ and (+) the culture ‘with added IPTG’.

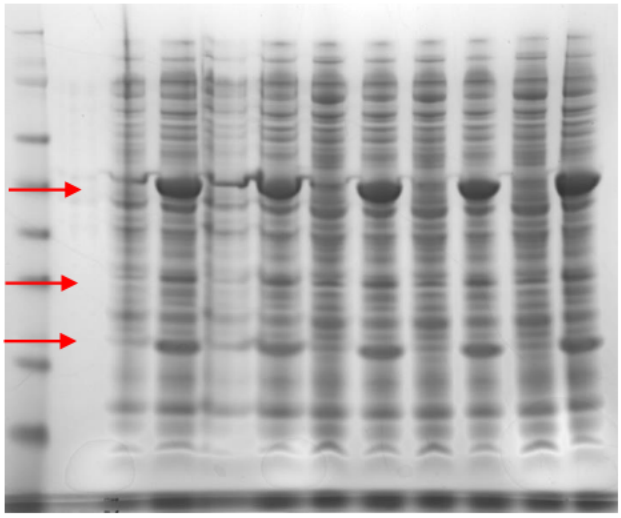


Figure 38: *Band pattern of the expression test of chaperon combination 1 co-transfected with scFv 4G2.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 48 kDa – Trigger factor
Second arrow: 28 kDa – scFv 4G2
Third arrow: 17 kDa – Skp
Source: private shot, ChemieDoc™ BPRC.

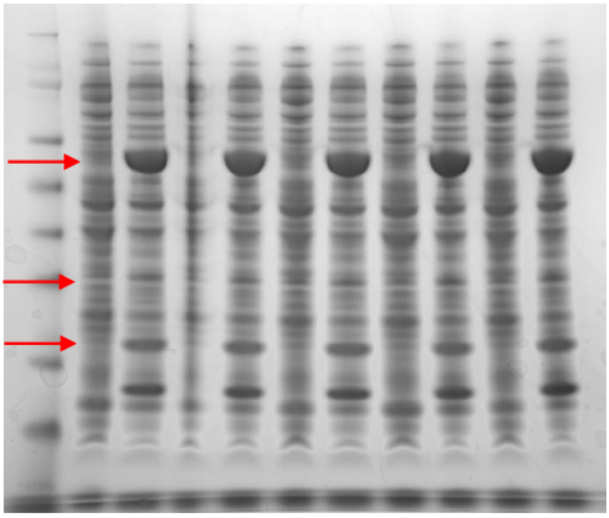


Figure 39: *Band pattern of the expression test of chaperon combination 2 co-transfected with scFv 4G2.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 58 kDa – GroEL
Second arrow: 28 kDa – scFv 4G2
Third arrow: 17 kDa – Skp
Source: private shot, ChemieDoc™ BPRC.

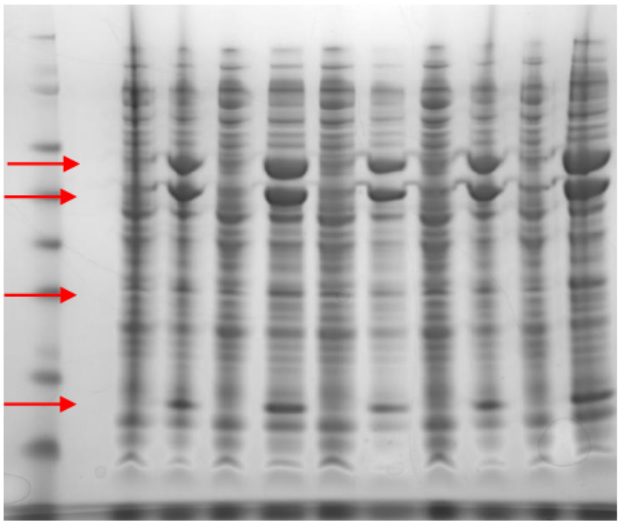


Figure 40: *Band pattern of the expression test of chaperon combination 3 co-transfected with scFv 4G2.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 58 kDa – GroEL
Second arrow: 48 kDa – Trigger factor
Third arrow: 28 kDa – scFv 4G2
Fourth arrow: 10 kDa - GroES
Source: private shot, ChemieDoc™ BPRC.

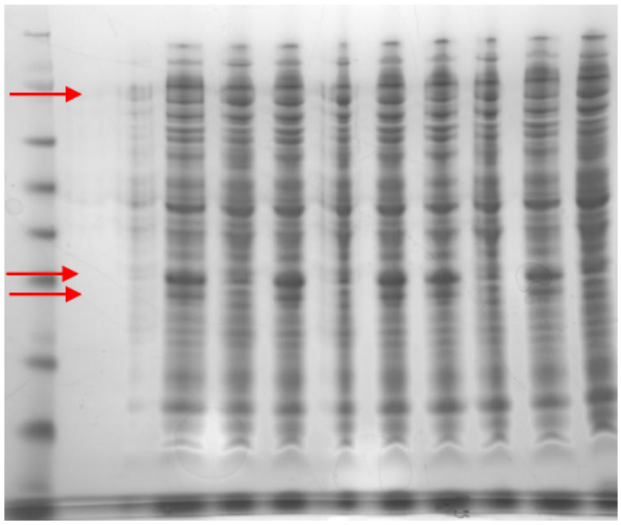
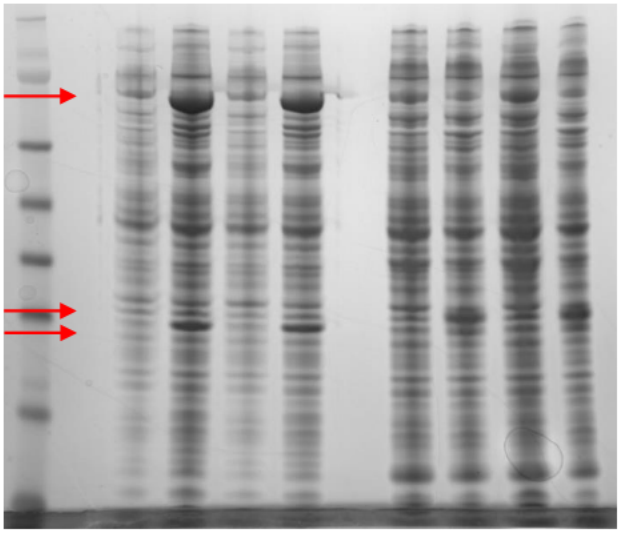


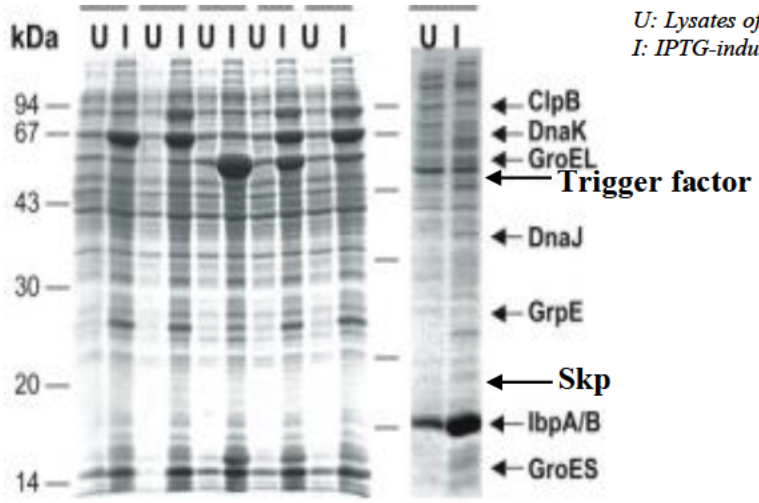
Figure 41: *Band pattern of the expression test of chaperon combination 4 co-transfected with scFv 4G2.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 93 kDa – ClpB
Second arrow: 28 kDa – scFv 4G2
Third arrow: 22 kDa – GrpE
Source: private shot, ChemieDoc™ BPRC.



Well 1: marker
Well 2: /
Well 3: clone 1 (-)
Well 4: clone 1 (+)
Well 5: clone 2 (-)
Well 6: clone 2 (+)
Well 7: /
Well 8: clone 1 (-)
Well 9: clone 1 (+)
Well 10: clone 2 (-)
Well 11: clone 2 (+)

The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 93 kDa – ClpB
Second arrow: 28 kDa – scFv 4G2
Third arrow: 22 kDa – GrpE

Figure 42: *Band pattern of the expression test of chaperon combination 4 without co-transfected scFv 4G2 on the left and scFv 4G2 only as a control on the right side of the gel.*
Source: private shot, ChemieDoc™ BPRC.



U: Lysates of non-induced cells
I: IPTG-induced cells

Figure 43: *Overview of standard MW of the different chaperones (for comparism).*
Source: [Marco et al. 2007]

Figures 38 – 41 show almost the same band pattern between the different chaperone cultures. However, a clear difference is visible between the cultures with and without the added IPTG as expected. Seeing that difference, the method does express the proteins and can be indicated as valid. However, no clear bands, or only slightly, are visible at the height of 28 kDa, indicating clearly that scFv 4G2 was expressed, rather than seeing all kinds of other bands at different MWs from as well cellularly expressed proteins.

The visible clear, dark bands in the lanes with the added IPTG are representing the different chaperones, which are indicated by the red arrows pointing at their corresponding MW.
The rest of the bands are other cell proteins that were expressed, since the method does not include a purification step.
Comparing that to the in Figure 42 presented controls (only transfected chaperone combination 4 without scFv 4G2 on the left side and only transfected scFv 4G2 without chaperones on the right side), it is no very clear which culture and therefore chaperone combination was most successful in assisting the folding process of scFv 4G2. That is why, from each culture the large-scale expression was set up followed by a purification as described in chapter 4.1, and a binding assay via spot blotting.

Since the properly folded scFv 4G2 is expected in the cytosol, rather than represented in intracellularly IBs, the cytosol sample S1 was further purified (chromatograms here not shown) and put on gel, as well as a spot blot was implemented.

The following Figures 44 – 47 show SDS page gels from the isolated and purified cytosol samples of scFv 4G2, co-transfected with chaperones. Each gel has the same format, which is:

Lane 1: marker	Lane 7: S2 (from isolation protocol)
Lane 2: IBs	Lane 8: S3 (from isolation protocol)
Lane 3: IBs + DTT	Lane 9: S4 (from isolation protocol)
Lane 4: Purified cytosol sample S1	Lane 10: IMAC – Protein FT from S1
Lane 5: Purified cytosol sample S1 +DTT	Lane 11: IMAC – 1. wash (protein W10)
Lane 6: /	Lane 12: /

Whereas band patterns from lane from 2 – 5 which confront the amount of IBs to the purified scFv 4G2 in the cytosol are the most interesting and important to look at.

The red arrows here, are indicting again the MW of the chaperones and the scFv 4G2.

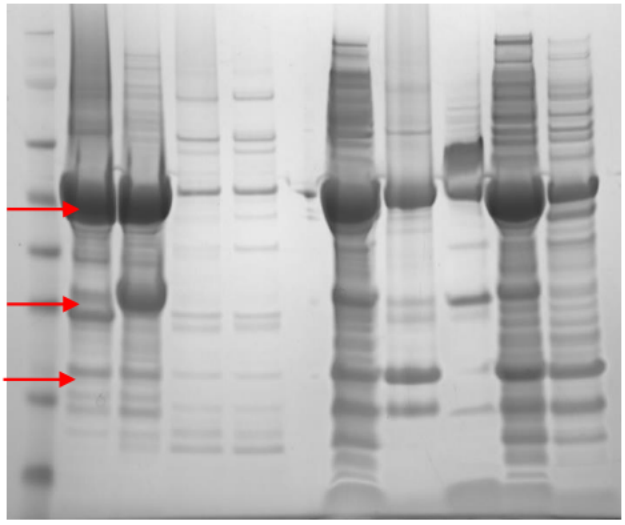


Figure 44: *Band pattern of samples from the isolation protocol and the purified cytosol sample S1 from chaperone combination 1.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 48 kDa – Trigger factor
Second arrow: 28 kDa – scFv 4G2
Third arrow: 17 kDa – Skp
Source: private shot, ChemieDoc™ BPRC.

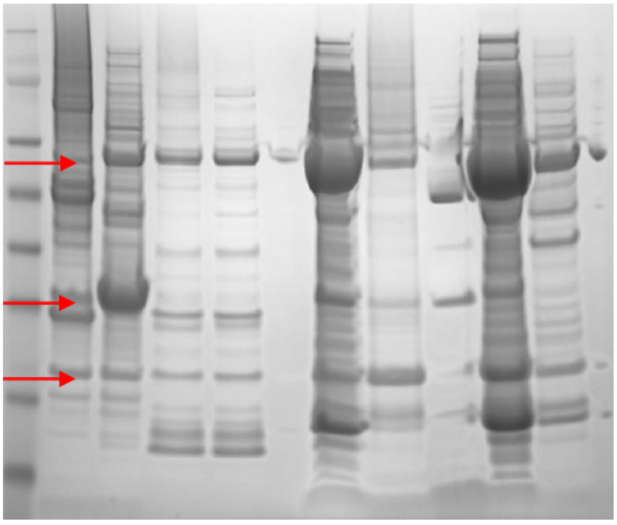


Figure 45: *Band pattern of samples from the isolation protocol and the purified cytosol sample S1 from chaperone combination 2.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 58 kDa – GroEL
Second arrow: 28 kDa – scFv 4G2
Third arrow: 17 kDa – Skp
Source: private shot, ChemieDoc™ BPRC.

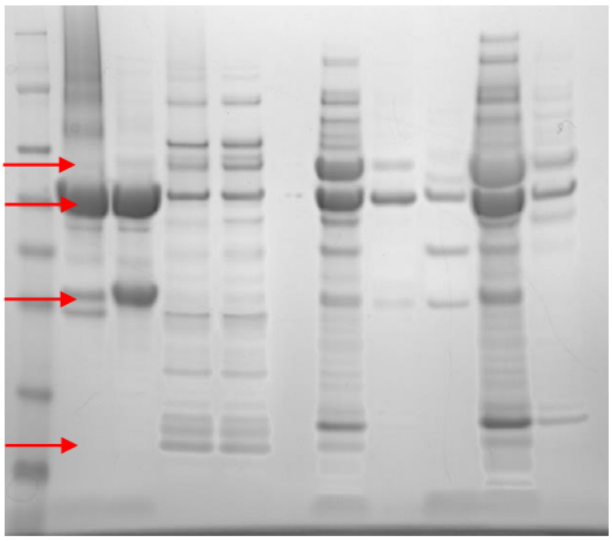


Figure 46: *Band pattern of samples from the isolation protocol and the purified cytosol sample S1 from chaperone combination 3.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 58 kDa – GroEL
Second arrow: 48 kDa – Trigger factor
Third arrow: 28 kDa – scFv 4G2
Fourth arrow: 10 kDa - GroES
Source: private shot, ChemieDoc™ BPRC.

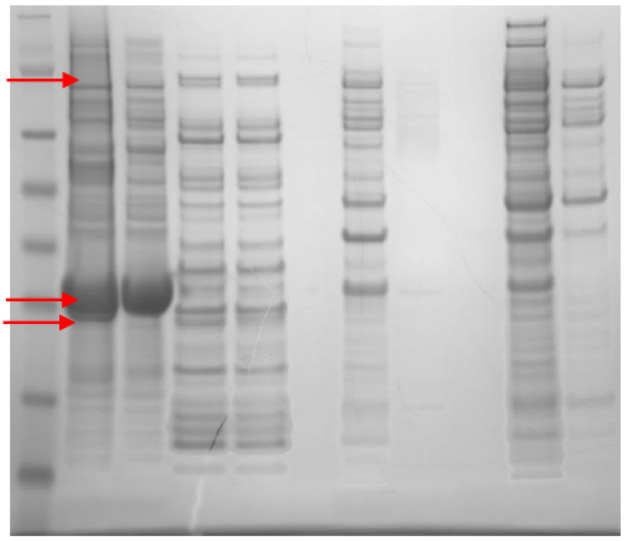
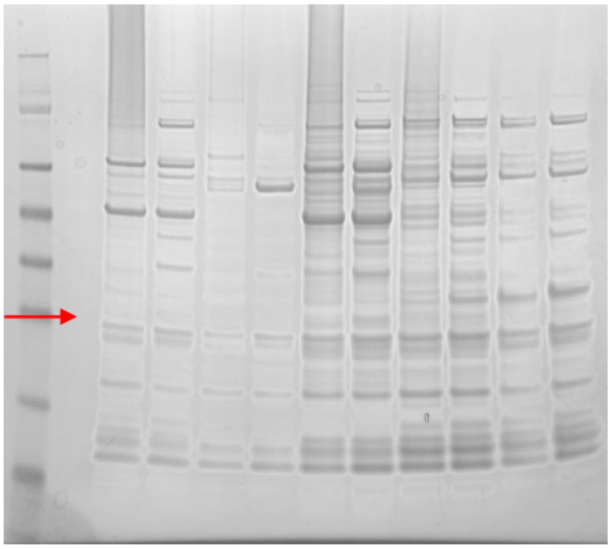


Figure 47: *Band pattern of samples from the isolation protocol and the purified cytosol sample S1 from chaperone combination 4.*
The red arrows are indicating the expected MW of the scFv 4G2 and the chaperones (describing from the top).
First arrow: 93 kDa – ClpB
Second arrow: 28 kDa – scFv 4G2
Third arrow: 22 kDa – GrpE
Source: private shot, ChemieDoc™ BPRC.

By looking at wells 2 and 3 from all cultures it is clearly visible that still a big part of the protein is put into IBs. Nevertheless, by looking at wells 4 and 5 of every culture, a band occurs at the hight of ~ 28 kDa from the protein of interest.
By looking at the FT and first wash from the IMAC, there are also bands appearing at that MW. This indicates a loss of the protein. Which means, a little percentage of the protein does not bind and gets washed of the column.

Figure 48 shows an overview of all S1 samples from the chaperone cultures starting with chaperone combination 1 in lane 3 and ending with control culture scFv 4G2 only. Every sample was put twice on gel, first non-reduced and secondly reduced in alternation.
As well as in previous gels, a lot of other bands are visible despite the purification. Nevertheless, in all of them a band appears at a MW of ~ 28 kDa from the scFv 4G2. Whereas in chaperone combinations 3 and 4 (lanes 7 and 8 and lanes 9 and 10) the strongest signal is visible compared to combinations 1 and 2 (lanes 3 and 4 and lanes 5 and 6).



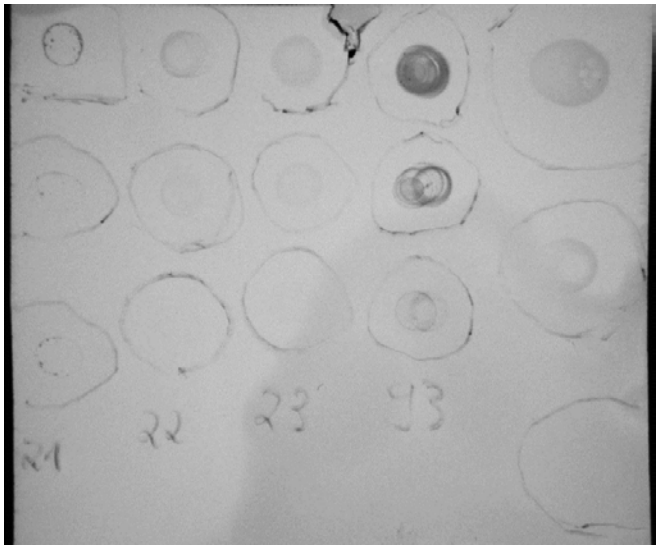
Starting in lane 3 with chaperone combination 1 co-transfected with scFv 4G2.
Lane 4: sample from lane 3, reduced.
Same applies for other chaperone combinations following (combination 2, 3, and 4 all co-transfected with scFv4G2)
Lane 11: scFv without co-transfected chaperones.
Lane 12: sample from lane 11, reduced.

Red arrow is indicating the height of the MW from scFv 4G2.

***Figure 48:** Overview of the band pattern from cytosol sample S1 of all chaperone cultures co-transfected with scFv 4G2 + the scFv 4G2 culture without the chaperones. Source: private shot, ChemieDoc™ BPRC.*

To test if the produced and purified scFv 4G2 is properly folded and therefore in its active form, a spot blot was done (Figure 49) with the S1 sample of every co-transfected chaperone culture. To

see if the chaperones improve the amount of properly folded the scFv 4G2, the scFv 4G2 culture without co-transfected chaperones was spotted as a comparison.



1 ***2*** ***3*** ***4*** ***5***

Figure 49: *Spot blot of properly folded 4G2, co-transfected with chaperones and expressed in E. coli cells.*
Source: private shot, ChemieDoc™ BPRC.

Column 1: scFv 4G2 with chaperone combination 1.
Column 2: scFv 4G2 with chaperone combination 2.
Column 3: scFv 4G2 with chaperone combination 3.
Column 4: scFv 4G2 with chaperone combination 4.
Column 5: scFv 4G2 without a co-transfected chaperone

First row 5 µL of 1 mg/mL solution was spotted on

Second row: 5 µL of a 1:2 dilution

Third row: 5 µL of a 1:4 dilution.

Column 1 represents the cytosol sample with chaperone combination 1 and scFv 4G2. Column 2 represents the cytosol sample with chaperone combination 2. Column 3 represents the cytosol sample with chaperone combination 3. Column 4 represents the cytosol sample with chaperone combination 4 and column 5 shows the cytosol sample of scFv 4G2 without any chaperones co-transfected.

All spotted on samples gave a results, at least at the higher concentration. But, compared to the control the culture of scFv 4G2 without co-transfected chaperones, only chaperone combination 4 (GrpE and ClpB) gave the strongest response.

7. Discussion

For a better overview to discuss the in chapter 6 stated results, this chapter will also be sectored into the three different approaches, plus the establishing binding assays via Fab fragments, staring with production of scFv 4G2 in HEK293-F cells.

7.1 Production of scFv 4G2 in HEK293-F cells

The transfection of HEK293-F cells with expression plasmids containing the scFv 4G2 protein preceded by three different secretion signals did apparently not result into the secretion of the protein, while was observed in the cellular fractions (Figure 22 and 23). Many other proteins have been shown to be secreted by these secretion factors. However, it is not easy to predict which protein will secrete with what signal, if at all. Obviously, the scFv 4G2 fails to be secreted by the secretion signals that were selected. Even by testing the medium sample again (reduced, data not shown), after seeing that the anti-histidine AB only gave a signal when the scFv 4G2 protein was reduced by DTT, via SDS page gel, it did not show any band or signal. Since Western blots are more precise, this would be another opportunity to repeat, since in SDS page gel from Figure 21 also did not show any clear band at the MW of the protein (28 kDa) at first. Moreover, other secretion signals could be tried, although there is a fair chance that these will not work either due to possible secretion-resistances of the protein of interest.

The absence of the anti-histidine signal in non-reducing conditions is of concern. It shows that the His-tag is not available for the AB and may interact with the rest of the scFv 4G2 protein, potentially interfering in binding PfAMA1. This could be verified by looking at the Western blot (Figure 24), that shows the absence of binding with its antigen AMA1, as no signal is observed. As it has been shown that scFv 4G2 is present on the gel and blot, the most likely explanation is that it the protein is not properly folded. Whether this is due to the denaturing conditions in the gel (SDS, or DTT) or the conditions during the sample preparation (e.g., the sample was heated to 70˚C for 10 min.) is not clear, and should be addressed by performing a spot blot, directly on the cell fractions, in the absence of denaturing agents. Alternatively, an SEC could be done in the presence and absence of PfAMA1, which may lead to a shift in peaks in the chromatogram.

In addition to that, expression levels of the scFv 4G2 were low, as Coomassie stained SDS page gels of cell fractions (Figure 21) did not show additional bands after transfection. Protein bands are only visible on the Western blot using anti-hexahistidine ABs (Figure 23) after addition of DTT. Interestingly, higher signals were obtained for the cells transfected with 4G2 scFv with secretion factors. If confirmed, it would be interesting to find out how they help produce the protein of interest in a higher rate than the culture without the secretion factors when comparing those.

Lastly, expression levels could be improved by increasing transfection efficiency. Since the cells gave a green glowing response under the fluorescence microscope, the transfection can be considered as prolific. The hereby used PEI is a transfection reagent, which involves the formation of nanoparticles (polyplexes) which are obtained by its mixing with plasmid DNA. It condenses DNA into positively charged particles, which then bind to the anionic cell surface residues and via endocytosis are brought into the cells. Due to the prior incubation step, polyplexes can reach their optimal size, which has a direct impact on transfection efficiency. [Raymond et al. 2011]

However, it is seen that not all the cells were successfully transfected which is quite normal since never a full 100 % transfection rate is reachable (an estimation of the exact transfection efficiency was not done for this experiment due to its early state). Still, to improve this, another transfection medium and reagent could be used for example PEI MAX in order to increase the transfection rate of the cells. It is known that PEI MAX exhibits a higher solubility and a relatively lower cytotoxicity. Thus, it should be a better gene delivery vehicle compared to other transfection reagents. [Li et al. 2013]

Another way to improve the transfection rate is to increase the incubation time post-transfection for example from the here given 48 hrs up to 96 hrs. The higher the post-transfection incubation, the more time the cells have to properly express and fold the protein of interest. [Longo et al. 2013]

7.1 Development of a binding assay, using Fab fragments of mAB 4G2

To set up a binding assay to assess the amount of properly folded scFv 4G2, Fab fragments of the mAB 4G2 were used. First, the mAB 4G2 was isolated from culture supernatants of the heterohybridoma cell line 4G2.

A small percentage of the 4G2 ended up in the FT of the column (Figure 25). This indicates that either a small percentage didn´t bind at all to the column due to e.g., a modification at the Fc tail or that the capacity of the column was not large enough, and all binding sites of the column were already occupied with ABs. Meaning, that the amount put onto the column was too high. Since it is only an unremarkable amount of FT, it is not further discussed.
Digestion of the purified mAB resulted into the expected Fab fragments (~ 25 kDa) and their Fc tails (~ 50 kDa) (Figure 26). This shows that the method to digest IgGs for 1 hr with the enzyme papain in a cysteine buffer at pH 7.0, stopping the digestion with Iodoacetamide, works nearly perfectly.
Nevertheless, removal of the uncut mAB the Fab fragment fraction (Figure 27) was successful. All uncut ABs, which would have falsified the results on the spot blot when still present, could be removed, as shown in the band in sample 4 on the gel with a MW of ~ 150 kDa as expected (Figure 27). Since the spot blot from Figure 28 also showed a binding response, the assay development could commence, using the Fabs fragments as control.

The SEC analyses in Figure 30 also showed some promising results. Comparing the graphs of either the mAB 4G2 as positive control, the Fab fragments, or the AMA1 to those from the incubated mixture, it is clearly visible that the ABs as well as the Fab fragments bound to AMA1 and formed a bigger complex, which therefore had a smaller retention time.
AMA1 elutes at a retention time suitable for its size (60.6 kDa) by comparing the retention time to the standard. However, the peak for the individual mAB 4G2 sample occurred at the same time as AMA1, which should not be the case since AMA1 FVO has a MW of 60.6 kDa and 4G2 a MW of 150 kDa, which is more than double the size. However, the detected absorbance (in mAU) is much higher for the 4G2 sample with about 115 mAU compared to AMA1 with only about 35 mAU even though the same number of molecules were injected.

When looking at the the chromatogram of the control mixture of AMA1 + mAB 4G2 shows however three peaks (peak one: at minute 27.85, peak two: at minute 30.00 and peak three: at minute 31.98) instead of one representing the bound complex. The peak occurring at minutes 30 could indicate, comparing to the retention times of the standard, the bound complex between mAB 4G2 and AMA1 with a MW of 210 kDa. The peak occurring at minute approximately 27 could be formed multimers in between the mABs (~ 300 kDa) or even two mAB binding to AMA1 at the

same time forming an even bigger complex, since it has been found for that AMA1 and Fab fragments of the mAB R3/1C2, in which one of the two Fabs bound to more or less a-specific, yet tightly, to the AMA1 molecule. [Vulliez-Le Normand et al. 2015] Moreover, there is a supposedly still unbound protein in the mixture, represented by peak three.

By looking at the chromatogram of 'AMA1 + Fab fragments', similar patterns occur. The Fab fragments show multiple peaks in their sample (pink graph, peak one: at ~ minute 34, peak two: at ~ minute 36, peak three: at ~ minute 40, and peak four: at ~ minute 51), confirming the observation in the SDS-gel (Figure 27) that two main bands are present, apparently the Fab fragments, the other of unknown constitution, but likely not containing the Fc-tail, as this would have been removed in the Protein G agarose purification step. The two additional bands could therefore be complexes of AMA1 with the two constituents of the Fab fraction. The observation that both the original peaks present in the Fab fraction diminish in intensity after addition of the PfAMA1, support this hypothesis.

7.2 Refolding of in *E. coli* expressed scFv 4G2 using rapid dilution

Figures 30 and 31 show a successful isolation and purification of the scFv 4G2 formed inclusion bodies produced in *E. coli*.

After designing the DoE via MODDE, which established the D-optimal design shown in Figure 32, the rapid dilution was implemented as described in chapter 4.2.4. The composition of the buffer 10, which was used as an outlet, will not be further discussed in this thesis since it has been shown from previous experiments (data not shown) that e.g., guanine and GSH and GSSH also have an essential influence on the refolding.

The spot blots shown in Figures 33 and 34 show the binding of AMA1 to the refolded scFv 4G2 after the development of all conditions that were tested. From these blots, that showed comparable results in duple, it was deduced that buffer containing high concentrations of arginine, gave the highest signals, indicative of the presence of properly refolded scFv 4G2.
The sucrose concentration in the buffers had little to no impact on the result, since e. g. buffers 2 and 11 hat a sucrose concentration of 0.0 M.

Also, the pH range of the buffers seemed to have no effect on the refolding since the ones that gave a binding response varied between all before set pH values.

Concluding that, the variable that seems to matter the most is a high concentration of arginine, which is already known for its important role in the refolding process of other scFv´s.
It is also used e.g., to prevent protein-protein or protein-surface interactions during refolding and purification. In addition, as Tsumoto et al. state in their article "Role of arginine in protein refolding, solubilization, and purification" it is used to "extract active, folded proteins from insoluble pellets obtained after lysing *E. coli* cells. Moreover, it is known that arginine increases the yield of proteins secreted to the periplasm and stabilizes proteins during storage. All these arginine effects are due to suppression of protein aggregation." Yet, they say little is known about the mechanism. "Various effects of solvent additives on proteins have been attributed to their preferential interaction with the protein, effects on surface tension, or effects on amino acid solubility. However, the suppression of protein aggregation by adding arginine cannot be readily explained by either surface tension effects or preferential interactions which." [Tsumoto et al. 2004]

Given those results, the refolding was repeated with the buffers that showed the strongest signal exactly as before with an additional buffer exchange directly after the concentrating step of the rapid dilution. The buffer exchange was done with PBS (pH 7.0), in which proteins should supposedly stay refolded. Since the spot blot shown in Figure 35 shows no binding signal in any of these samples, it can be stated that either the repeated refolding did not work which is highly unlikely since it was carried out the same way as initially, or due to the exchange of buffers the properly refolded scFv 4G2 from the rapid dilution unfolded again and therefore lost its native, active form.
Therefore, the SEC experiment that followed was only done with the correctly folded samples from the previous trial.
Before that, an ELISA was implemented to detect a difference between the binding capacity of the different buffers, which should give an indication of what buffer worked the best and has therefore the highest concentration of correctly folded scFv 4G2.
When looking at the ELISA results in Figure 36, it is clearly visible that all buffers emitted about the same response. Moreover, the graphs showed all an exponential decrease directly after the first

dilution (D_f = 2), what indicates that the signals were not saturated, and the protein concentration for the rapid dilution can be increased for further experiments, since also several unfolded proteins in the solution can be expected. However, by looking at buffer 17, it shows a slightly higher signal throughout its dilutions. This could indicate that 1.0 M arginine and 0.66 M sucrose at a pH of 7.7 is the best composition for the refolding so far.

It is also the reason, why the refolded scFv 4G2 from buffer 17 was used for implementing the SEC. In addition to that, sample buffer 17, which was rebuffered to PBS was also injected to the HPLC to sum up the experiment.

Comparing each superimposed chromatogram from Figure 37 with each other and moreover to the ones from Figure 30 it is obvious that no binding has occurred. Even though the incubation conditions were equal in comparison to the Fab fragment experiments, no peak shift occurs regarding a smaller retention time, indicating a bound complex with a higher MW.

AMA1 still elutes at all chromatograms at its usual retention time of approximately 34 minutes.

By looking at all the graphs representing the refolded scFv 4G2, a peak (respectively the first peak) occurs at ~ minute 39, which indicates a much lower MW of the protein as expected.

However, by comparing the graph of the refolded scFv 4G2 in PBS to the upper chromatogram 'AMA1 + rf4G2 in buffer 17', it shows a better, more precise peak detected at a comparably high absorbance of ~ 25 mAU.

Since the upper two to chromatograms in Figure 37 gave inexplicably no binding signal and due to the interference signal within sample 'rf scFv 4G2 in buffer 17', refolded scFv 4G2 from buffer 3 was injected onto the column as well using its buffer composition to run the column in order not to dilute the protein in PBS buffer, risking the change of its conformation.

However, by looking at the last chromatogram of Figure 37 where its results are shown, it is also clearly visible that no binding has occurred. Moreover, the sample of buffer 3 was detected at a very low absorbance with only ~ 3 mAU. This, compared to the other graphs is the lowest detected absorbance.

Since the SE chromatograms showed inexplicably no response of bound complexes from the incubated mixtures, it was passed to experiments chaperones as helper-proteins for properly folding the scFv 4G2 expressed in *E. coli* DE3 cells.

7.3 Chaperones as helper-proteins for refolding scFv 4G2 in *E. coli* cells

After a successful co-transfection of the chaperones and scFv 4G2 to the *E. coli* DE3 cells, an expression test was started. Its results are shown in Figures 38 – 41.

By looking at the gels, they show all similar band patterns. The dark, thick bands that are visible in samples with the added IPTG, indicated by the red arrows, are representing the overexpression chaperones. This gets clear by comparing the gels to Figure 43 in which the different MWs of the chaperones are marked. Therefore, in each chaperone cultures thick bands occur at the suitable height, stating that the induction and expression of the chaperones was successful.
The rest of the bands can be explained as are other cell proteins that were expressed, since the method does not include a purification step.
However, a clear band at a MW of ~ 28 kDa was hardly visible in neither of these cultures which makes it difficult to state, whether the scFv 4G2 was properly expressed. The reason for this could be, that many other proteins got overly expressed comparing to the scFv 4G2 overlying the results, since every band pattern shows smears rather than singled out, clear bands. In addition to that, instead of at least adding 20 µL of 0.1 M IPTG, only 10 µL were added. Which is only half the amount and could be the reason of poorly expressed scFv 4G2.

That is why the large-scale expression was done with all four chaperone combinations.

Hereby, the cytosol sample of the cells from the isolation protocol was of high interest.
Since, if the protein gets properly folded via the chaperones, the cells should not form intracellularly IBs, or at least, should produce more scFv 4G2 in the cytosol.
SDS pages 44 – 47 show the purified cytosol sample of each culture (lanes 4 and 5), plus the rest of the samples from the isolation protocol (lanes 6 – 10) and the IB samples (lanes 1 and 2).
In all the gels, a band is visible in the cytosol sample at the height expected for the properly folded scFv 4G2 which indicates, it was expressed and correctly folded. Nevertheless, clear bands occur in the IB samples as well, which indicates that even with co-transfected chaperones, forming IBs cannot be prevented.
All the other bands visible, despite purification, could be either formed multimers of the protein or aggregates containing their His-tag, smaller molecules that were still remaining in the column from previous purifications, or as well the chaperones.

To test if the scFv 4G2 that was expressed in the cytosol is firmly properly folded, a spot blot was done shown in Figure 49. Hereby, a culture with scFv 4G2 without co-transfected chaperones as a control was taken along to compare if chaperones do improve the expression of correctly folded protein.

All spots on the blot from Figure 49 gave a binding response, indicating that the expressed scFv 4G2 is properly folded. Hereby chaperone combination 4 (GrpE and ClpB) shows the strongest response comparing to the other spot on the blot. This states that this particular chaperone combination does improve the folding of the protein of interest and therefore increases its yield.

For future experiments it would be interesting to see, if adding the IPTG and incubating at a lower temperature would increase the properly folded scFv 4G2. Since the lower temperature slows down the expression process and therefore its folding, what should decrease the level of folding mistakes. [PhD Tyasning Kroemer]

With other chaperones, which compared to the control did not increase the properly folded scFv 4G2 since their binding response is partly even lower, no further experiments should be implemented. Reasons for their weak response could be that sometimes unwanted side effects occur when co-transfecting chaperones. Examples here for are reduced yields, reduced solubility, reduced specific activity, and many more. [Martínez-Alonso et al. 2010]

8. Conclusion and outlook

To conclude this thesis, despite all promising outcomes and an important step in the right direction, the production of scFv derived from the anti-malaria monoclonal AB 4G2 needs to be further investigated and improved to get a higher amount of properly folded protein. All results have so far not been sufficient to go into the next round which would be, establishing and implementing growth inhibition assays (GIAs) with parasite cultures to aim the goal of producing a new vaccine variant against one of the most dangerous viruses in the world 'malaria'.

However, to sum up all achievements and to give an outlook on what can and should be further investigated, it was still possible to produce correctly folded 4G2 in its active form in *E. coli* cells. Therefore, the method of refolding via rapid dilution in buffers containing a high concentration of arginine was expediently. For future experiments, the results should first be incorporated into the MODDE system for analysation and a new DoE could be established in which even higher concentrations than 1 M of arginine could be tested. Or, AMA1 could be added to the refolding buffers as another component to test, if the protein properly refolds when its antigen is present. Another variable that could be included instead of the pH for example, is the incubation temperature after the rapid dilution.
This could then be implemented in a larger scale with higher amounts of refolded scFv 4G2, to test the binding capacity via the incubation with AMA1 again. Followed by another SEC or even GIAs if binding via the incubation method worked.
Furthermore, it was proven that chaperones do help in the refolding process to produce more properly folded scFv 4G2 in the cytosol rather than intracellularly IBs. Herby chaperone combination (GrpE and ClpB) was working expediently. For future experiments, the expression of scFv 4G2 in *E. coli* cells with this particular chaperone combination could be implemented again, adding the IPTG and cultivating at a lower temperature e.g., 18 °C. The lower temperature will slow down the expression and therefore improve the refolding. In addition, a combination with other chaperones could be co-transfected and tested for better results. Moreover, there are more chaperones (e.g., chaperones DnaK, DnaJ, GroESL inserted in the pBB542 plasmid, spectinomycin resistance), which could be co-transfected and tested.

The results of the HEK293-F cells were not as promising as the rest. To improve the outcome a larger scale experiment could be implemented, producing a higher amount of scFv 4G2 for better detection. Moreover, a different transfection buffer in combination with adding PEI MAX instead of PEI could increase the number of transfected cells. Which also leads to a higher amount of scFv 4G2 production if more cells are correctly transfected. In addition to that, anther Western blot with reduced medium samples should be repeated. Despite that, there are more secretion factors to be investigated. However, this method from all the above stated is the most time consuming and expensive one, since working with mammalian cells is quite laborious and requires compared to bacteria cells.

Bearing in mind that antibody treatments are generally very expensive and therefore almost impossible to afford in third world countries, where it is needed the most. Additionally, regarding the half-live of a scFv AB in the body is way shorter compared to full-sized ABs. That means that a treatment, respectively vaccination would need to be refreshed regularly. Which simply cannot be implemented in the countries that would need it the most.

9. References

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Appendix

Investigation FirstTryREFOLDINGPROCESS

Date: 08.09.2022. User: Lina Velte

Introduction and background

Factors

Name	Abbreviation	Units	Type	Settings
Arginine	Arg	M	Multilevel	0; 0,33; 0,66; 1
Sucrose	Suc	M	Multilevel	0; 0,33; 0,66; 1
pH	pH		Multilevel	6; 6,8; 7,7; 8,5

Responses

Name	Abbreviation	Units	Condition	Objective	Min	Target	Max
Optical density	Opt		Desired	Target	0,2	1,5	2

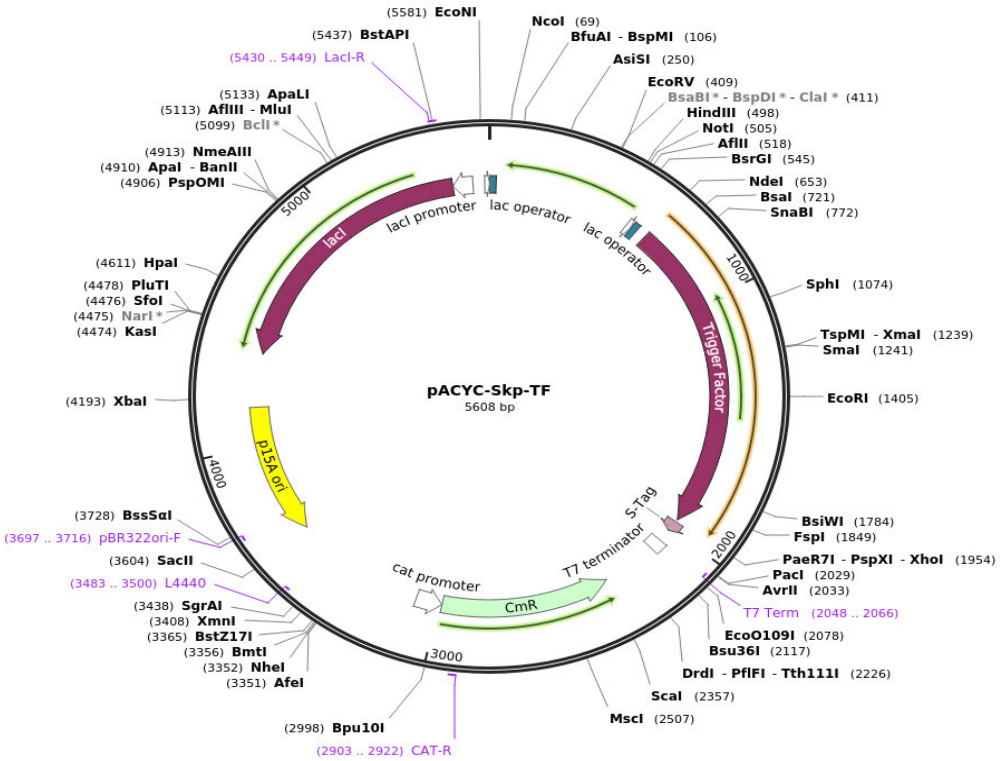
Objective, model and design

Objective	Optimization (RSM)	
Process model	Quadratic	
Mixture model	--	
Design	**D-Optimal**	
Runs in design	17*	
Center points	3	
Replicated runs	0	
Replicates	0	
N = actual runs	20*	
Maximum runs	12000	
Constraints	No	
Candidate set		
Extreme vertices	64	
Total runs	64	
D-Optimal		
Potential terms		
Number of inclusions	0	
Constraints	No	
Selected design number	2	
Design statistics	G-efficiency	61,8583
	log(Det. of X'X)	8,99515
	Norm. log(Det. of X'X)	-0,401515
	Condition number	4,61854

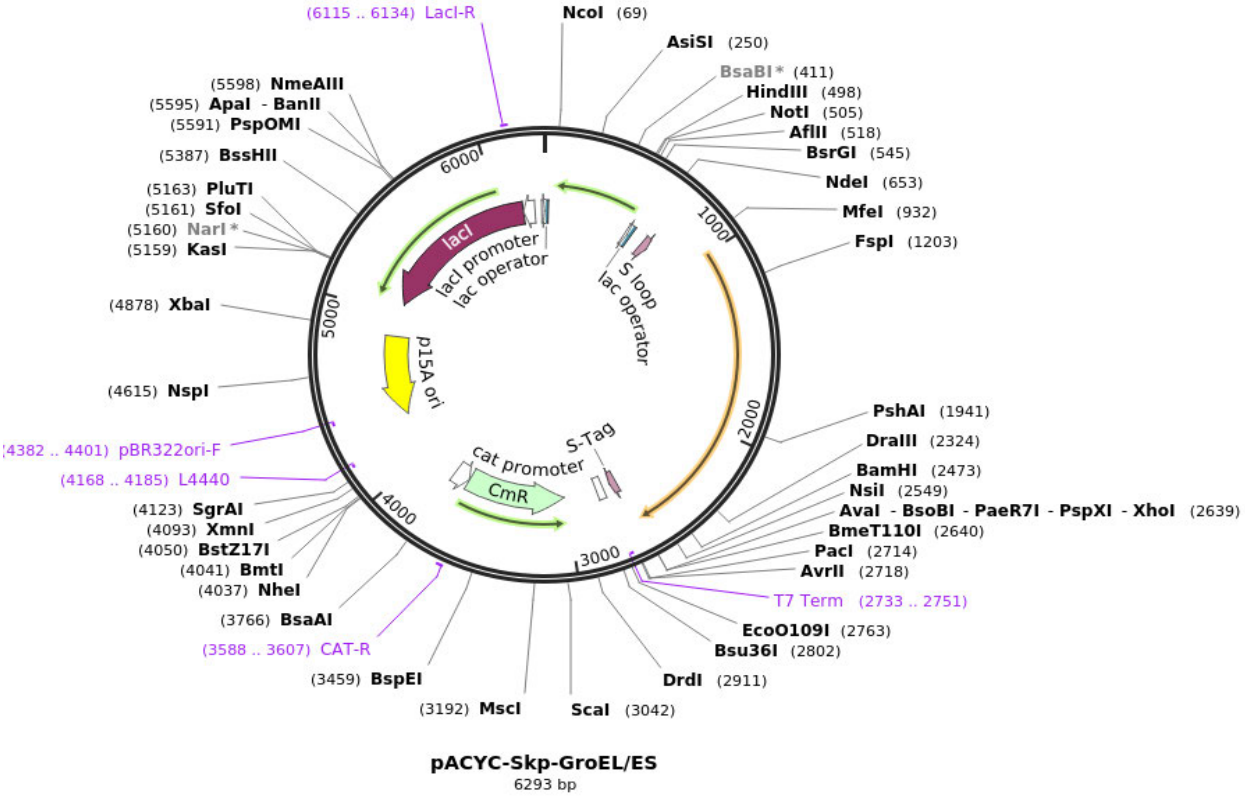
Worksheet

Exp No	Exp Name	Run Order	Incl/Excl	Arginine	Sucrose	pH	Optical density
1	N1	10	Incl	0	0	6	
2	N2	4	Incl	1	0	6	
3	N3	16	Incl	0,66	0,33	6	
4	N4	20	Incl	0,33	1	6	
5	N5	11	Incl	1	1	6	
6	N6	17	Incl	0	0,66	6	
7	N7	13	Incl	0,33	0	6,8	
8	N8	3	Incl	1	0,33	6,8	
9	N9	7	Incl	0	1	6,8	
10	N10	12	Incl	0	0	8,5	
11	N11	19	Incl	1	0	8,5	
12	N12	6	Incl	0	1	8,5	
13	N13	2	Incl	1	1	8,5	
14	N14	8	Incl	0,33	0,66	8,5	
15	N15	5	Incl	0,66	0	7,7	
16	N16	9	Incl	0	0,33	7,7	
17	N17	1	Incl	1	0,66	7,7	
18	N18	14	Incl	0,66	0,66	7,7	
19	N19	15	Incl	0,66	0,66	7,7	
20	N20	18	Incl	0,66	0,66	7,7	

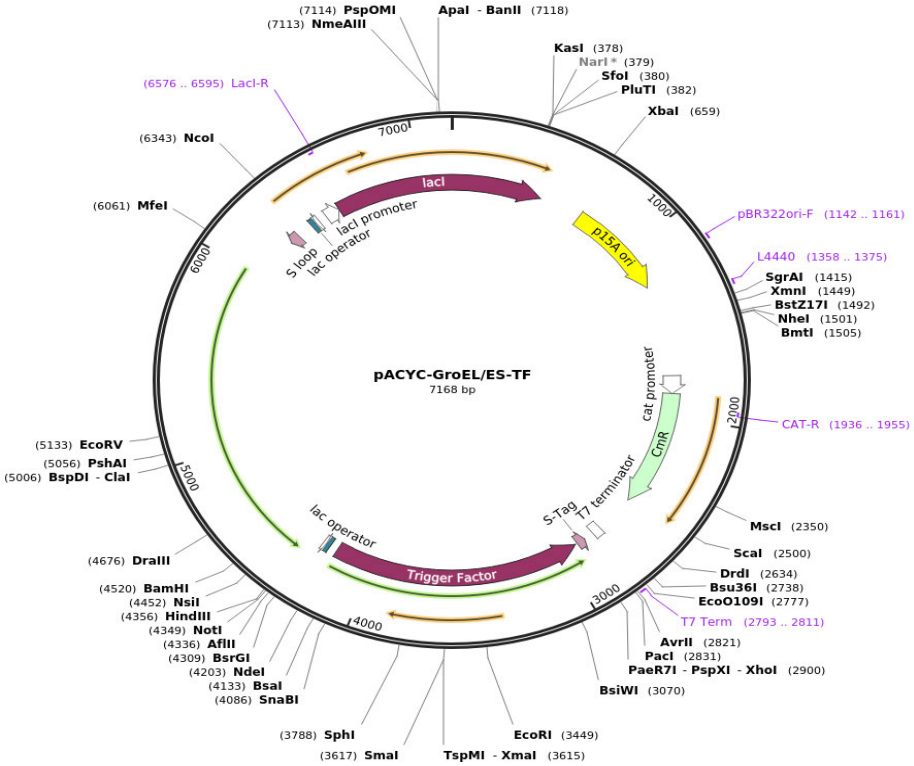
Appendix 1: *Overview of buffer composition from DoE ' Refolding of in E. coli expressed 4G2 using rapid dilution'. Source: self-programmed, MODDE*



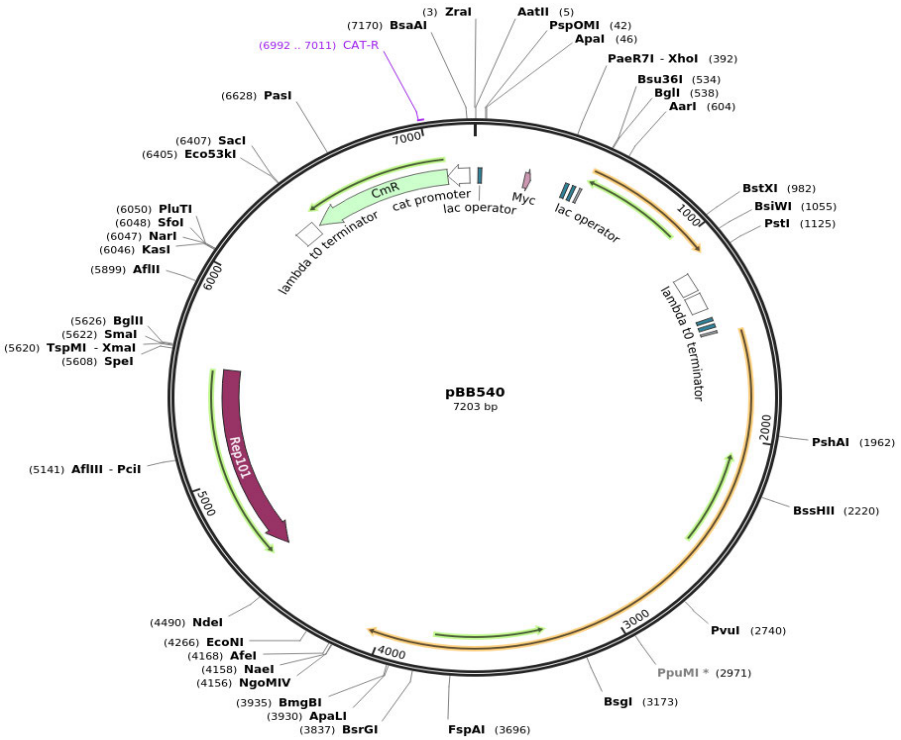
Appendix 2:*Sequence map of chaperone combination 1 (pACYC-Skp-TF).*
Source: https://www.addgene.org/



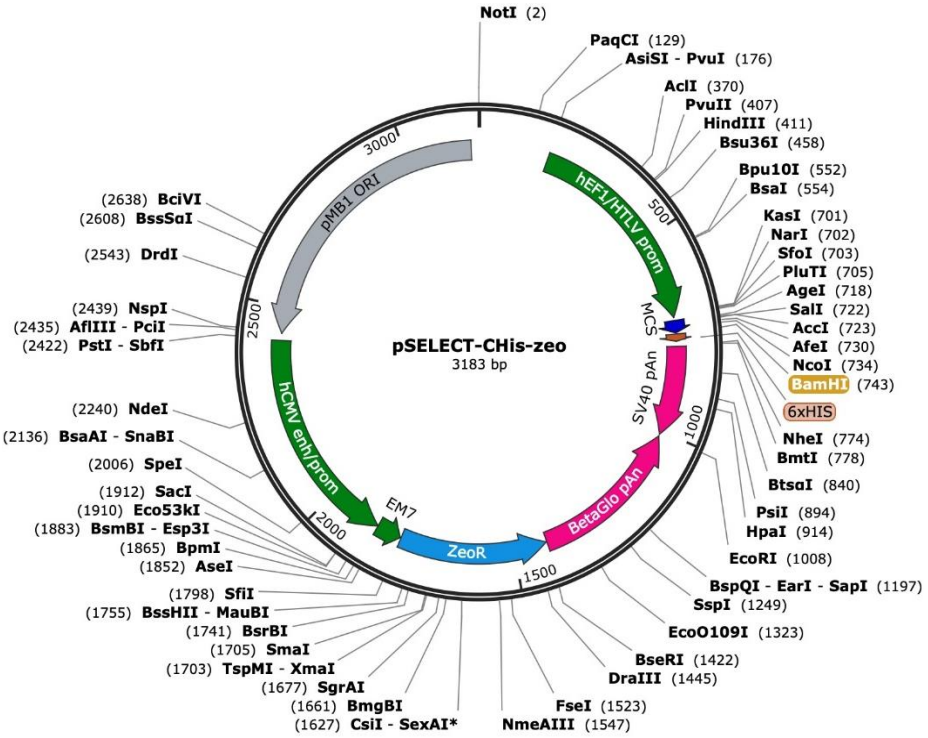
Appendix 3: *Sequence map of chaperone combination 3 (pACYC-Skp-GroEl).*
Source: https://www.addgene.org/



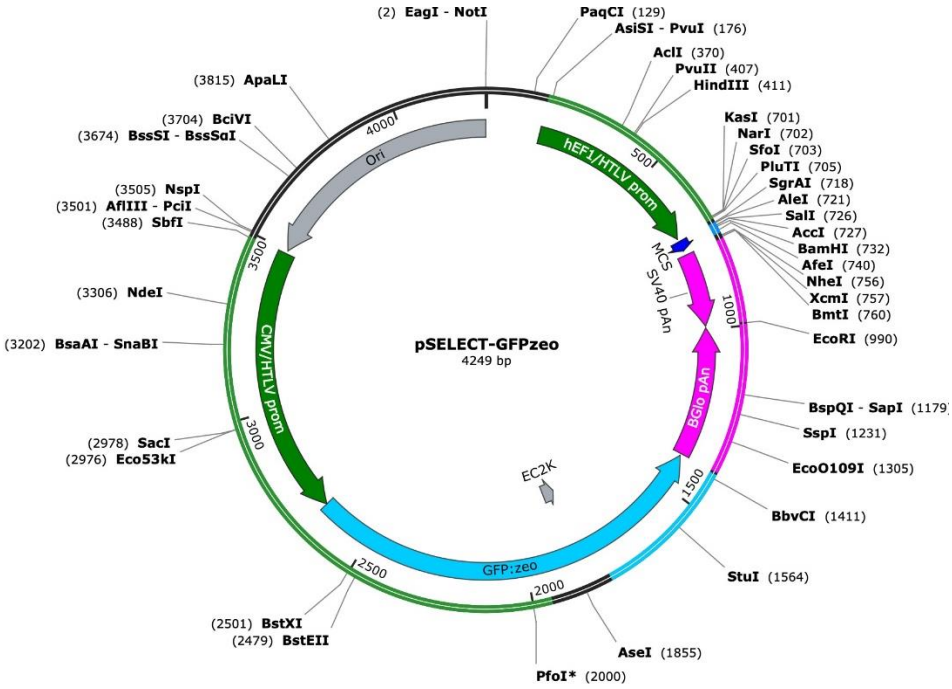
Appendix 4: *Sequence map of chaperone combination 2 (pACYC-GroEL/ES-TF).*
Source: https://www.addgene.org/



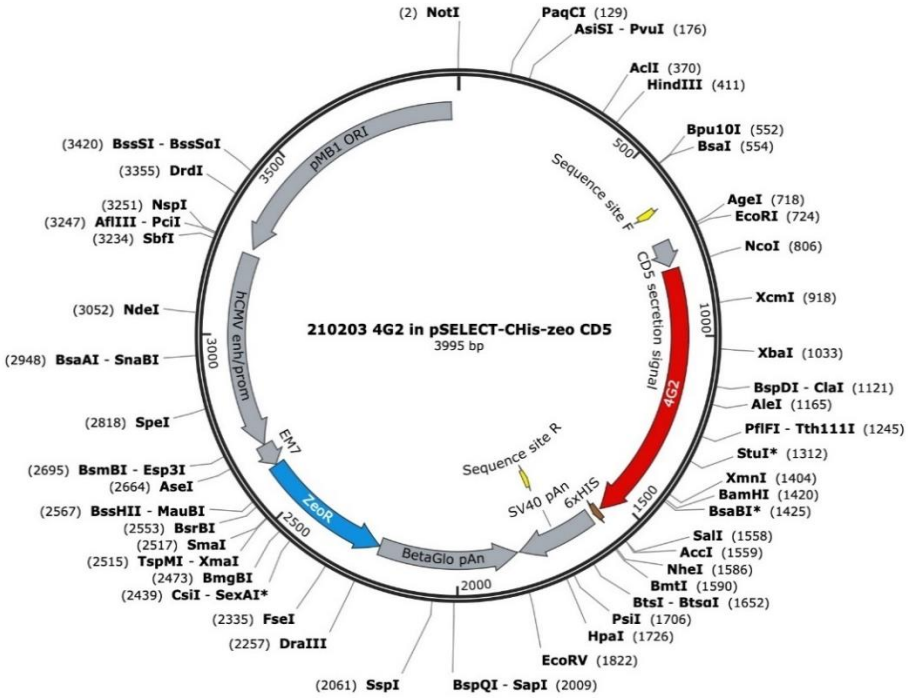
Appendix 5: *Sequence map of chaperone combination 4 (pBB540).*
Source: https://www.addgene.org/



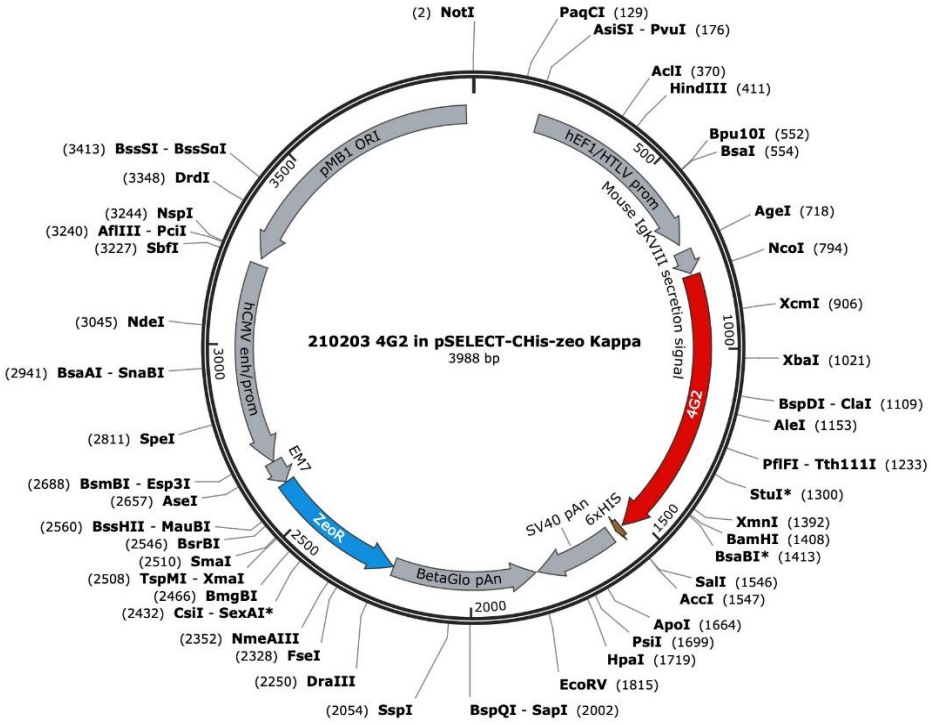
Appendix 6: *Sequence map of pSelect standard plasmid.*
Source: SnapGene, BPRC.



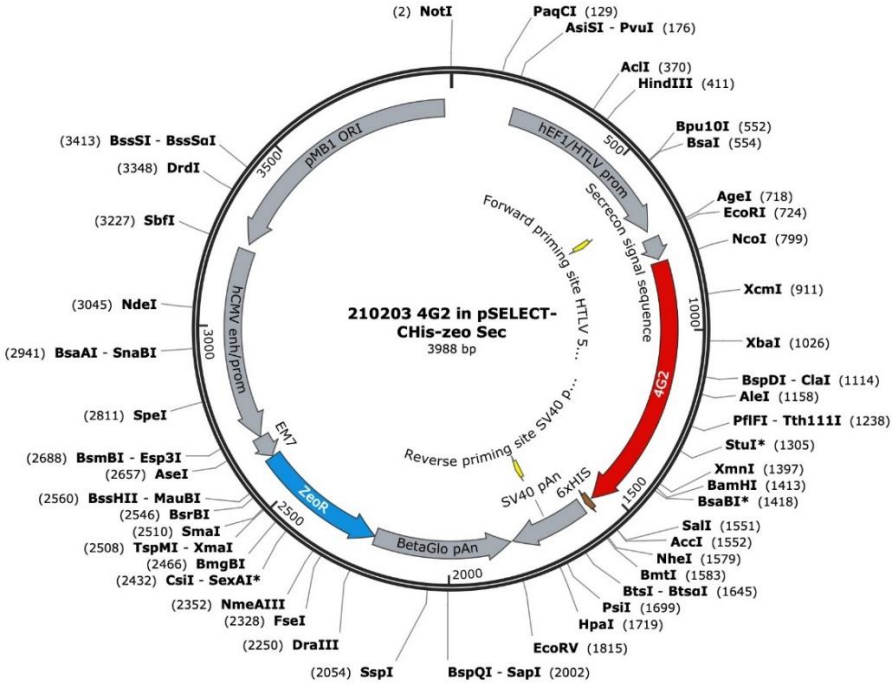
Appendix 7: *Sequence map of GFP in pSelect standard plasmid without His-tag.*
Source: SnapGene, BPRC.



Appendix 8: *Sequence map of scFv 4G2 in pSelect standard plasmid with secretion factor CD5. Source: SnapGene, BPRC.*



Appendix 9: *Sequence map of scFv 4G2 in pSelect standard plasmid with secretion factor Kappa. Source: SnapGene, BPRC.*



Appendix 10: *Sequence map of scFv 4G2 in pSelect standard plasmid with secretion factor SEC. Source: SnapGene, BPRC.*