CHALMERS
UNIVERSITY OF TECHNOLOGY

Establishment and Characterisation of new Immunoreagents for diagnosis of Ovarian cancer

Master's thesis in Biotechnology

FANNY CARLSSON

DEPARTMENT OF BIOLOGY AND BIOLOGICAL ENGINEERING

CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2022
www.chalmers.se

Master's thesis 2022

Establishment and Characterisation of new Immunoreagents for diagnosis of Ovarian cancer

FANNY CARLSSON

CHALMERS
UNIVERSITY OF TECHNOLOGY

Department of Biology and Biological Engineering
Chalmers University of Technology
Gothenburg, Sweden 2022

Establishment and Characterisation of new Immunoreagents
for diagnosis of Ovarian cancer
FANNY CARLSSON

Supervisors: Catharina Wising, Fujirebio Diagnostics AB and Maria Lidqvist, Fujirebio Diagnostics AB
Examiner: Verena Siewers, Department of Biology and Biological Engineering

Master's Thesis 2022
Department of Biology and Biological Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Typeset in LaTeX
Printed by Chalmers Reproservice
Gothenburg, Sweden 2022

Establishment and Characterisation of new Immunoreagents
for diagnosis of Ovarian cancer
FANNY CARLSSON
Department of Biology and Biological Engineering
Chalmers University of Technology

Abstract

Ovarian cancer is the most lethal of all gynaecological cancers and it is often diagnosed at an advanced stage due to diffuse and only mild symptoms at early stages. Early detection is crucial to increase survival but high-grade serous ovarian cancer (HGSOC) often presents non-specific symptoms, such as loss of appetite, bloating of abdomen and tiredness. This, in combination with no screening for ovarian cancer, results in over 80% of patients being diagnosed late. At this stage chemotherapy is crucial for survival and targeted therapies are often more efficient. Studies suggest that PARP inhibitors and EZH2 inhibitors are synergistic in vivo in tumours with high levels of Coactivator-associated arginine methyltransferase 1 (CARM1). Overexpression of CARM1 could therefore be a promising biomarker for precision treatment with the two substances PARP and EZH2 in combination.

This project is a collaboration between Fujirebio Diagnostics AB and an academic institute which have requested an antibody towards the biomarker CARM1. The overall goal is to establish high affinity monoclonal antibodies to the CARM1 biomarker to be used in immunohistochemistry and ultimately serum detection.

The results in this thesis indicates that creating an antibody with high specificity towards CARM1 is possible and further immunisations resulted in high serum titers in all mice. Two fusions were successfully performed and resulted in 17 hybridomas producing antibodies against CARM1. However, further work is needed to obtain antibodies with the desired properties, such as IgG isotype and high affinity against CARM1. The C-terminus GST-tagged antigen developed to screen the produced antibodies is functional and can be used for further screenings whilst cloning of the GST-tagged N-terminus antigen will need investigation and optimisation.

Keywords: Ovarian, cancer, monoclonal, antibodies, biomarkers, CARM1, PARPi, EZH2i

Acknowledgements

I would like to express my gratitude to my supervisors Catharina Wising and Maria Lidqvist for guidance and expertise throughout this thesis. Your support and feedback has been invaluable for the completion of this thesis. I also would like to thank Åsa Agapiev for helping me with the protein purification process and to everyone at Fujirebio Diagnostics AB for not hesitating to help me whenever I have needed it. Thank you also to my examinor Verena Siewers for taking the time to review this report.

Finally, thank you to my family and to Sam for always believing in me.

Fanny Carlsson, Gothenburg, January 2022

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis listed in alphabetical order:

Ab	Antibody
Ag	Antigen
BSA	Bovine serum albumin
BT	Biotin
CARM1	Coactivator-associated arginine methyltransferase 1
ELISA	Enzyme-linked immunosorbent assay
EOC	Epithelial ovarian cancer
EZH2i	Enhancer of zeste 2 inhibitor
GST	Glutathione S-transferase
HAT	Hypoxanthine-Aminopterin-Thymidine
HGSOC	High-Grade Serous Ovarian Cancer
HRP	Horseradish peroxidase
HT	Hypoxanthine-Thymidine
Ig	Immunoglobin
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KLH	Keyhole limpet hemocyanin
LB	Lysogeny broth
mAb	Monoclonal antibody
pAb	Polyclonal antibody
PARPi	Poly(ADP-ribose) polymerase inhibitors
PBS	Phosphate-Buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PRMT4	Protein arginine N-methyltransferase 4
TMB	3,3',5,5'-Tetramethylbenzidine

Contents

1
Introduction

Ovarian cancer is the most fatal female gynaecologic malignancy and it is usually diagnosed at a late stage due to only mild or diffuse symptoms at an early stage. Due to late stage diagnosis, the survival is less than 50% [1]. Therefore, it is of great importance to find novel strategies to improve the survival rate, this can be done both by diagnosing the cancer at an earlier stage but also by improving and individualising the treatment.

There are different types of ovarian cancers with High-Grade Serous Ovarian Cancer (HGSOC) being the deadliest, accounting for 70-80% of deaths from ovarian cancer [2]. Research has shown that the biomarker coactivator-associated arginine methyltransferase 1 (CARM1) can be an indicator to how patients with HGSOC will respond to certain treatments [3]. CARM1 is a protein arginine methyltransferase (PRMT) responsible for modifications that have an impact on signal transduction, gene transcription, DNA repair and mRNA splicing [3]. CARM1 is overexpressed in many types of human cancers, such as breast, colon and prostate [3]. Studies have shown that poly(ADP-ribose) polymeras inhibitors (PARPi) and enhancer of zeste 2 inhibitor (EZH2i) are synergistic in vivo in CARM1-high, but not CARM1-low, tumours [4] making CARM1 a promising target biomarker for precision medicine in patients with HGSOC.

1.1 Aim

This project is a collaboration between Fujirebio Diagnostics AB and an academic institute which have identified CARM1 for potential use in precision medicine and have requested an antibody. The overall goal of the current project is to establish high affinity monoclonal antibodies to the CARM1 biomarker to be used in immunohistochemistry and ultimately serum detection.

The ultimate goal is to use CARM1 antibodies with high specificity and affinity to stratify patients for precision treatment by EZH2i alone or in combination with PARPi. The goal for this project is to establish CARM1 antibodies and screening methods with the following objectives:

- Establish new monoclonal antibodies towards CARM1 using classic hybridoma technology.
- Establish methods for hybridoma screening and antibody characterisation, in-

cluding production of glutathione S-transferase (GST) fusion protein as an antigen.
- Select antibodies with the desired properties such as high specificity and affinity.

1.2 Limitations

Because of time limitations, the scope of the project has to be limited. Certain items, such as gene blocks for cloning and peptides for immunisation and screening, had been prepared and ordered before the start of the master thesis project. Immunisation of animals had also started ahead of time in order for the animals to gain immunity in time before fusion. The laboratory work will as far as possible consist of protocols, materials and methods available at Fujirebio Diagnostics AB. During this project monoclonal antibodies for only the biomarker CARM1 will be established and evaluated.

2
Theory

In the following chapter, clinical background and theory behind used methods are presented.

2.1 Background

In the following subsections backgrounds of ovarian cancer and treatments, the biomarker CARM1 and antibodies as a diagnostic tool are presented.

2.1.1 Ovarian cancer and treatment

Ovarian cancer is the most fatal female gynaecologic malignancy and is usually diagnosed at a late stage with a five year survival of less than 50% [1]. Ovarian cancer is however not one disease but has several subtypes with about 90 % originating from epithelial cells and therefore deemed as epithelial ovarian cancer (EOC). There are four different histological subtypes of EOC, serous, mucinous, clear-cell and endometrioid, where high grade serous (HGSOC) is the most dominant type diagnosed clinically accounting for over 70% of all ovarian cancer deaths [2].

Because of diverse and non-specific symptoms related to HGSOC, about 80 % of patients will become symptomatic and diagnosed at an advanced stage of the disease [2], thus differing from other subtypes of EOC where patients normally become symptomatic at a far earlier stage. This means the most common way to treat HGSOC after diagnosis is initially surgically removing the tumour followed by adjuvant chemotherapy [2]. Many new types of chemotherapy have been developed since the 1970s with the latest developments being in more targeted therapies [2].

Chemotherapy targeting abnormal pathways during cancer growth without targeting normal cells would be an optimal strategy. Two promising drugs doing just that are PARPi and EZH2i. PARPi works by inhibiting the DNA repairing enzyme poly(ADP-ribose) polymerase and thereby reduce the tumours ability to repair damaged DNA, leading to cell death [6]. EZH2i instead works by inhibiting EZH2, which acts as a histone methyltansferase of tumour suppressor genes, thus reducing cell proliferation and cell survival of the cancer cell [8]. Overexpression of EZH2 occurs in many humane cancers, including HGSOC.

The two inhibitors are synergistic in vivo in suppressing tumours overexpressing

CARM1, where EZH2i sensitises the cells to PARPi, see figure 2.1. Inhibition of EZH2 will in response to a DNA break upregulate the genes responsible for choosing non-homologous end-joining (NHEJ) over homologous recombination when repairing it [4]. When upregulating the error prone NHEJ repair with EZH2i there is a significant increase in chromosomal abnormalities [4]. This makes the tumour cells sensitive to PARP inhibition in CARM1-high, but not CARM1-low, tumours. Indicating that the EZH2 inhibition is CARM1 dependent [4].

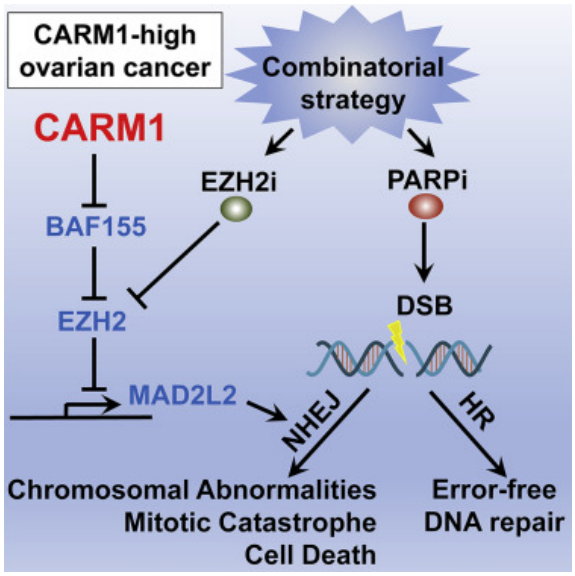

Figure 2.1: A graphical abstract of how EZH2i and PARPi work synergistic in CARM1-high ovarian cancer [4].

2.1.2 Biomarkers & CARM1

Biomarkers are molecules that can be measured and used clinically for detection and diagnosis of a disease or for prediction of therapeutic response. There are currently only two FDA approved biomarkers used for detection of ovarian cancer, CA125 and HE4, with many more being studied [9].

Studies have shown that PARP inhibitors, such as Olaparib, and EZH2 inhibitors, such as GSK126, are synergistic in vivo in CARM1-high, but not CARM1 low, tumours [4]. This makes CARM1 a promising target biomarker for precision treatment strategies in patients with HGSOC. Overexpression of CARM1 occurs in about 20% of patients with HGSOC [4] and by being able to measure the CARM1 levels produced by a tumour it could be possible to predict the therapeutic response to PARPi and EZH2i in combination.

CARM1, also known as PRMT4, is a type I protein arginine methyltransferase. Findings indicate that PRMTs in general play an important role in cancer since

arginine methylation is a key process hijacked by the cancer cell to ensure survival [10]. Arginine methylation is associated with many processes in the cell, but is especially critical in the RNA-associated processes of splicing, translation and stability regulation [5]. Evidence suggests that specifically CARM1 is an oncogene for various types of cancer [3], meaning it is a gene that when mutated can contribute to the development of cancer.

There are nine mammalian PRMTs all with unique signature sequences but with individual parts, see figure 2.2(a). It is therefore of great importance when designing a diagnostic test to make it specific to only the PRMT of interest, CARM1.

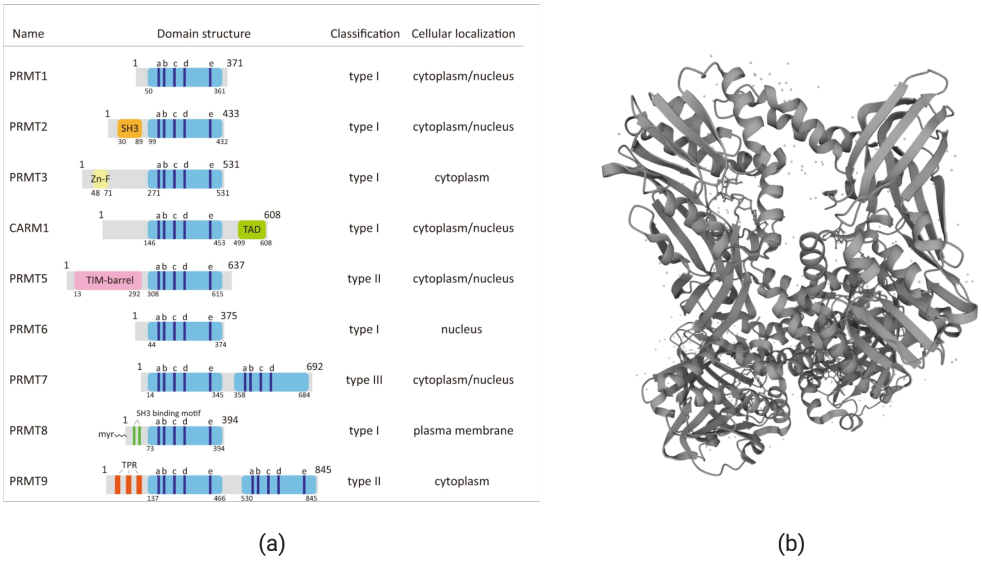

Figure 2.2: (a) Comparison of proteins PRMT1-9, including CARM1. Dark blue areas indicate high sequence similarity [13]. (b) Structure of CARM1 protein [14]

2.1.3 Antibodies as a diagnostic tool

Immunoglobulins (Ig), also known as antibodies (Ab), are the most important part of the body's adaptive immune response with the purpose of identifying foreign objects, known as antigens (Ag). Antibodies have become an invaluable tool in diagnostics, therapy and research because of their ability to have high specificity and affinity against a specific target.

As visualised in figure 2.3a, antibodies are proteins with a Y-shaped structure. They are made up of two identical light chains and two identical heavy chains linked to each other through disulfide bonds [11]. The chains have one constant and one variable domain [12]. The constant region decides the isotype and can be divided into five classes in mammals, IgA, IgD, IgE, IgG and IgM each with independent characteristics and functions [11]. Isotypes and subtypes of them vary in between

species but in mice, IgG has the subtypes IgG1, IgG2a, IgG2b, IgG3 [11].

IgG is the most abundant isotype, accounting for around 80 % of antibodies in the blood. IgM accounts for only about 5-10 % but it is the first type to be secreted when first exposed to any antigen [15]. IgM has the ability to form pentamer structures, visualised in figure 2.3b, while IgG antibodies are monomeric, meaning they consist of one single antibody [11]. IgM is a part of the early response and normally have relatively low affinity binding sites compared to IgG making them less suitable for diagnostics [16].

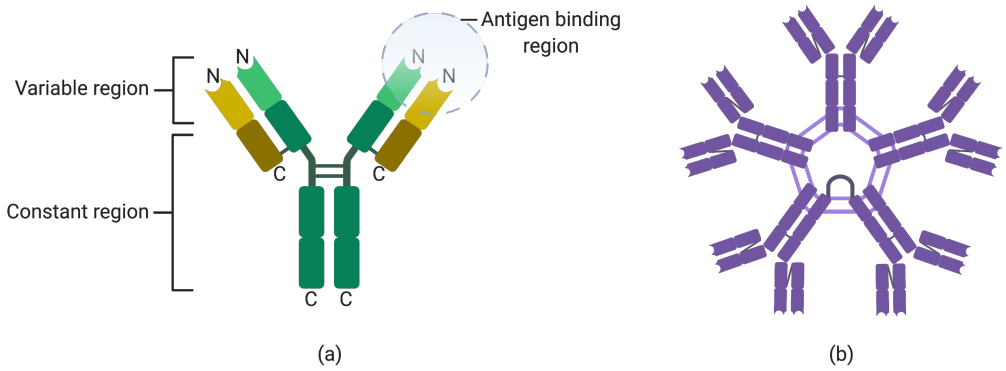

Figure 2.3: (a) An IgG antibody where green indicates the heavy chain, yellow the light chain, N the N-terminal end and C the C-terminal end. (b) IgM antibodies in a pentamer structure. Figures created with BioRender.

There are different kinds of antibodies where monoclonal antibodies (mAbs) bind to one epitope on the antigen, as opposed to polyclonal antibodies (pAbs) which can recognise multiple epitopes [17]. MAbs are produced from a single clone of identical cells grown in the laboratory whereas pAbs can be obtained directly from the serum of an immunised animal [15].

2.2 Method theory

An overview of the different methods used will be displayed in the following section, including classic cloning technique, Enzyme-linked immunosorbent assay (ELISA), SDS PAGE, Western blot and hybridoma technology.

2.2.1 GST-tagged proteins

GST is a protein used as an affinity tag, which enables purification and detection of any protein attached to it. The molecular weight of GST is 26 kDa and it is frequently integrated in the expression vector used to produce a recombinant protein

and is in this case attached to the N-terminal part of the protein, see figure A.1 [18].

The vector used for expression of the recombinant protein also contains an ampicillin resistant gene as a selection marker and the lacI system for induction with Isopropyl β D 1 thiogalactopyranoside (IPTG), see figure A.1. The vector as well as the genes blocks are digested with digestion enzymes creating sticky ends which will be ligated together in the presence of ligase.

The assembled plasmid is transformed into competent *E. coli* cells for plasmid amplification and PCR is used to select colonies of bacteria containing the plasmid with the insert. The insert may also be sequenced to assure correct assembly. The plasmid is also purified and transformed into competent cells optimised for protein expression. IPTG is used to induce expression of the recombinant protein.

The cells are then lysed and the lysate is tested to detect the protein. The protein with the GST-tag can be purified against glutathione immobilised on agarose beads in a column which captures the GST-tagged protein via enzyme-substrate binding. The rest of the lysate is washed off and the GST-tagged protein is eluted and collected.

2.2.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a commonly used assay used to detect protein. It works by coating a 96 well plate with an antigen or antibody, or using a plate already coated, then adding the antibody or antigen of interest. Then a primary antibody is added, followed by a secondary antibody conjugated with a detection label, such as horseradish peroxidase (HRP), which recognises the primary antibody and produces a signal when adding a substrate, such as 3,3',5,5'-Tetramethylbenzidine (TMB).

2.2.3 SDS PAGE and Western blot

SDS-PAGE and Western blot are methods to separate and identify specific proteins. SDS-PAGE refers to the method of separating proteins on a gel based on size using an electric current, normally accompanied by a standard of known protein sizes. Western blot then refers to the transferring of the protein from the gel to a membrane. The membrane is blocked with for example milk protein before antibodies are added to bind the protein of interest, washing in between each step, starting with a primary antibody which binds to the protein and then a secondary, labelled antibody which binds to the primary antibody. A detecting reagent is then added to the membrane and the protein can be detected using an imaging system.

2.2.4 Hybridoma technology

The hybridoma method to produce antibodies was first invented in 1975 [19] and has been widely used to produce stable antibody producing cells ever since. The process, visualised in figure 2.6, starts by inducing a specific immune response in mice through injecting them with an antigen together with an adjuvant according

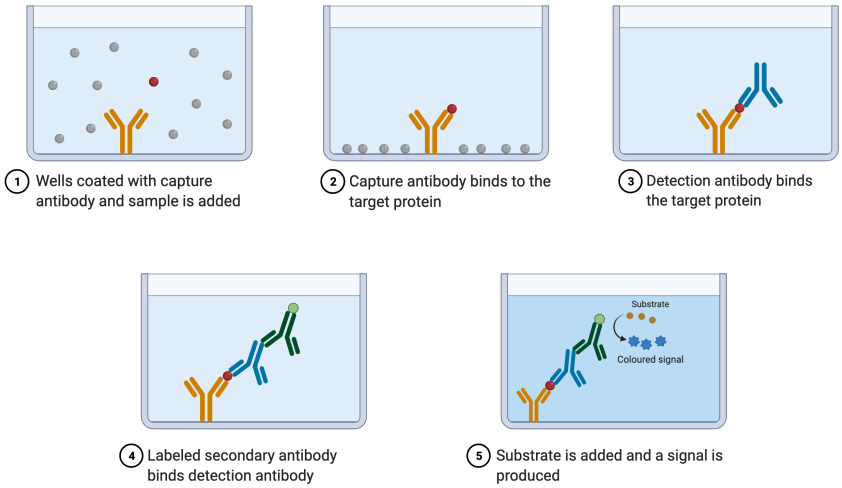

Figure 2.4: A visualisation of the ELISA experimental procedure. Figure created with BioRender.

to an immunisation schedule. The spleens from the mice are then harvested and fused with myeloma cells which are immortal cancer cells. The new fused cells called hybridoma will thereby have characteristics of both antibody producing B-cells and myeloma cells, meaning the cells will be immortal, antibody producing cells.

Not all cells will be successfully fused so by culturing the cells in the selection medium HAT (hypoxanthine-aminopterin-thymidine) the positive hybridomas will survive while the non-fused myeloma cells will die from not being able synthesise nucleotides due to the presence of aminopterin which blocks the de novo pathway. The non-fused B-cells will die due to a short life span.

Screens using ELISA will then be performed to select the hybridoma which produces antibodies of desired specificity. The positive hydridoma will also be isotyped to screen for desired antibody isotype. The hybridomas with the desired isotype are then cloned in order to produce identical antibody producing cells. These cells will then produce identical antibodies indefinitely which can then be purified and used for many different purposes.

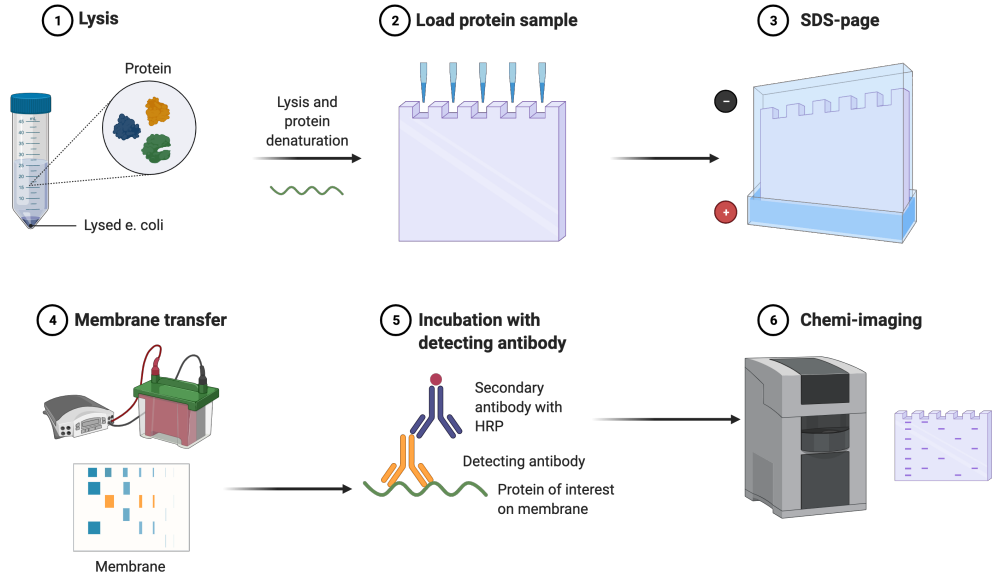

Figure 2.5: A visualisation of the SDS-PAGE and western blot procedure. Figure created with BioRender.

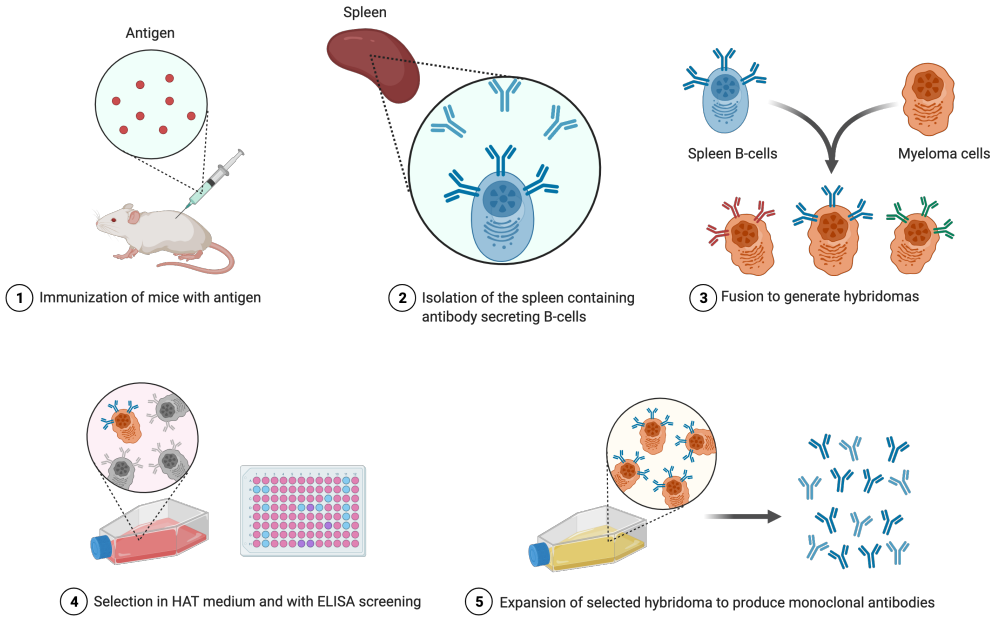

Figure 2.6: A visualisation of hybridoma method of producing mAbs. Figure created with BioRender.

3
Methods

In this chapter the procedures performed during the project will be presented. Details, such as exact amounts and times, were documented and stored internally at the company and will not be included in this thesis due to confidentiality.

The project can be divided into two parts that run in parallel. The first part of the project was to produce GST-tagged fusion proteins to be used in screens as antigen to the produced antibodies and the second part was to establish mAbs specific to CARM1 through hydridoma technology.

3.1 Production of CARM1 antigen for screening

In this section methods for production of CARM1 antigen will be presented, including cloning, production and purification of the GST-tagged protein.

3.1.1 Cloning of GST-tagged protein

The GST-tagged CARM1 fusion proteins used as antigen to screen the hybridomas after fusion were synthesised in *E. coli* after using classic cloning techniques. The two gene blocks used were synthesised by IDT. The C-terminus gene is 450 base pairs and 150 amino acids long and from the C-terminus end of the CARM1 protein while the N-terminus gene is 453 base pairs and 151 amino acids long and from the N-terminus end of the CARM1 protein. The full CARM1-protein is 1824 base pairs and 608 amino acids long.

The two gene fragments coding for the N- and C-terminus of the CARM1 protein were cloned into the vector pGEX-6P-3, see figure A.1, containing the gene coding for the GST protein and ampicillin resistance as a selection marker. The gene blocks and vector were initially digested with restriction enzymes EcoRI/BamHI (Thermo Scientific) according to the FastDigest protocol (Thermo Scientific) [20]. The digested fragments were run on an agarose gel for 40 minutes at 90 V and purified with QIAquick gel extraction kit (QIAGEN) according to the manufacturer protocol [21].

The purified fragments were then ligated together in the presence of T4 ligase (Thermo Scientific) according to the manufacturer protocol for sticky ends [22] at

an insert:vector ratio of 3:1. When the ligation had to be performed multiple times, ratios of 5:1 and 10:1 were also evaluated.

The assembled plasmids were then transformed into *E. coli* JM109 competent cells (Agilent) according to the manufacturer protocol for plasmid amplification [23]. After growing on a LB-plate containing ampicillin overnight, colonies were screened for correct insert using Polymerase chain reaction (PCR) with Taq DNA Polymerase (VWR) according to the manufacturer protocol [24] and then run on agarose gel for 40 minutes at 90 V. Three of the colonies with a correct insert on the PCR were purified using Plasmid Mini Kit (QIAGEN) according to the manufacturer protocol [25]. The purified plasmids were also sent for Sanger sequencing to Eurofins genomics to ensure correct assembly.

3.1.2 Production of GST-tagged protein

In order to produce larger quantities of the GST-tagged protein with high level protein expression and easy induction, *E. coli* BL21 Competent Cells (Agilent) were transformed with the plasmids. The transformation and induction with IPTG were performed according to the manufacturers protocol [26]. The cells were then harvested and lysated with lysozyme. The lysate was then processed with MgCl2 and DNase before screened both with western blot and ELISA.

Cell debris and lysate from cells induced and not induced with IPTG was screened with SDS PAGE and Western blot. The gels used for SDS PAGE were 4-15 % 15 well Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad) and Trans-Blot Turbo Transfer System (BioRad) was used to blot the gels. Both the SDS PAGE and blot was performed according to the manufacturer protocols [27] [28]. The primary antibodies used were goat anti-GST pAb (Cytiva), PRMT4/CARM1 (3H2) Mouse mAb (Cell Signaling) and PRMT4/CARM1 (C31G9) Rabbit mAb (Cell Signaling), they were all used at a dilution of 1:1000. The secondary antibodies used were rabbit anti-goat (Agilent), rabbit anti-mouse (Agilent) and goat anti-rabbit (Agilent). The detection reagent used was ECL™ Prime Western Blotting Detection Reagent (Cytiva) and detection was done with the ChemiDoc Imaging system (BioRad)

To evaluate the presence of GST-tagged fusion protein in the lysate an ELISA with glutathione coated microplate was performed. A visualisation and setup of the experiment can be seen in figures 3.2 and A.2. The glutathione-coated microplate was first washed before adding lysate from the production of C- and N-terminus CARM1 protein with a GST-tag for 1 h. If present and correctly folded, the GST tag of the fusion protein binds to the coated surface. The plate was again washed before primary antibodies, PRMT4/CARM1 (3H2) Mouse mAb (Cell Signaling) and PRMT4/CARM1 (C31G9) Rabbit mAb (Cell Signaling), were added and incubated for one hour on a shaker. The plate was washed and secondary antibodies were added, in this case rabbit anti-mouse and goat anti-rabbit conjugated with HRP, and incubated for one hour on a shaker and then washed. TMB substrate was finally added to all wells and the plate was incubated for 5-30 minutes until the

absorbance was measured at 620 nm using a BioTek ELx808 Absorbance Reader.

3.1.3 Purification of GST-tagged protein

To purify GST-tagged protein from the *E. coli* lysate, GSTrap FF Columns (Cytiva) were used. The purification was performed according to manufacturers protocol [29] with a manual chromatography system, 1 ml fractions were collected. An SDS PAGE and a Western blot was then performed with the purified protein as well as unpurified lysate as a control.

Both the SDS PAGE and blot was performed according to the manufacturer protocol. The primary antibodies used were goat anti-GST mAb (Cytiva) and PRMT4/CARM1 (3H2) Mouse mAb (Cell Signaling), they were used at a dilution of 1:1000. The secondary antibodies used were rabbit anti-goat (Agilent) and rabbit anti-mouse (Agilent).

3.2 Production and characterisation of CARM1 antibodies

In this section procedures for production and characterisation of CARM1 antibodies will be presented, including immunisation, determining the immune response of immunised mice, fusion, screening for positive hybridoma cells and isotyping.

3.2.1 Immunisation of mice

Female Balb/c mice (BK Universal) were used and kept at the Experimental Bio medicine facility, Sahlgrenska Academy at the University of Gothenburg. All experiments were approved by the Ethical Committee for Animal experimentation in Gothenburg, Sweden, approval 5.8.18-19152/2019, and performed by trained personnel.

Four groups of five mice each were intraperitoneally immunised with four different keyhole limpet hemocyanin (KLH) conjugated peptides: GPG32, LLT44, KCS36 and CQG43 in buffer together with Sigma Adjuvant System (Sigma Aldrich) to provoke an immune response. The first three are sequences from the N-terminal part of the CARM1 protein and the last peptide is a sequence from the C-terminal part of the CARM1 protein, see table 3.1. The peptides are chosen based being part of unique and exposed regions of the protein. The mice were immunised day 1, 22, 51 and 104 and the mice used for fusion were additionally immunised one week in advance. Serum samples were taken before the first immunisation (0-serum) and a few days after each immunisation 1-4.

Table 3.1: An overview of the antigens used for immunisation and their position in the CARM1 protein. The three letters indicate the three first amino acids in the sequence and the number indicates the number of amino acids the peptide is made up of.

Ag for immunisation	Position in CARM1 protein
GPG32	N-terminus
LLT44	N-terminus
KSC36	N-terminus
CQG43	C-terminus

3.2.2 Determination of immune response of immunised mice

In order to test the immune response of the immunised mice, ELISA was used to measure the presence of CARM1 antibodies in the serum. Serum is taken from each animal after each immunisation and tested in an ELISA.

The setup of the experiment, visualised in figure 3.1, is a microplate coated with streptavidin which was first washed with EIA wash solution before the biotinylated peptides at a concentration of 1 µg/ml were added to bind to the streptavidin and coat the surface during one hour incubation on a shaker in room temperature. Both the corresponding peptide and a different peptide were used as control to test cross reactivity. The plate was again washed before serum and controls were added according to figure A.4 and incubated for one hour on a shaker. The serum was diluted in 1% BSA/PBS and the dilution was optimised to 1:400. The plate was washed and a secondary antibody was added, in this case rabbit anti-mouse conjugated with HRP, and incubated for one hour on a shaker and then washed. TMB substrate was added to all wells and the plate was incubated for 5-30 minutes until the absorbance was measured at 620 nm using a BioTek ELx808 Absorbance Reader.

3.2.3 Fusion of spleen and myeloma cells

All cells were handled in a sterile environment in a LAF bench using sterile technique. The cells were cultured in an incubator at a constant humidity, temperature of 37 degrees and 8% CO2.

In preparation of the fusion, cells from the cell line 5637 were thawed and suspended in T-bottles in cultivation medium. The cells grew until confluent and were then trypsinised and expanded. The spent medium from the confluent 5637 cultures was harvested to be used as supplement in the upcoming fusion.

A mouse myeloma cell line was also thawed and suspended in T-bottles in cultivation medium. The cells were counted and expanded. When the desired number of cells with high viability had been reached, they were used in the fusion. Media and solutions were also prepared ahead of time.

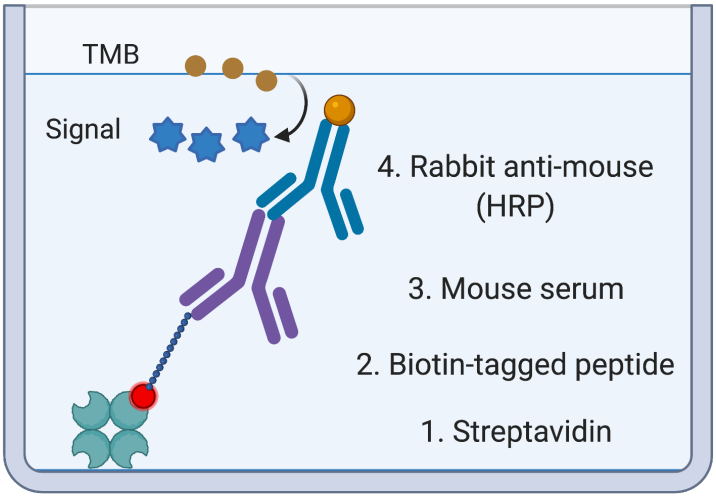

Figure 3.1: A visualisation of the ELISA screen performed to test the immune response of the immunised mice and to test the hybridoma medium after fusion. The microplate is coated with streptavidin, which binds to the biotinylated peptides. When adding mouse serum, the specific CARM1 antibodies binds to the peptide if present and a secondary antibody, rabbit anti-mouse conjugated with HRP, binds to the CARM1 antibody and produce a signal when adding TMB substrate. Figure created with BioRender.

When fusing the myeloma cells with the spleen cells, the mice were first sacrificed and the spleens harvested. Two mice from the group immunised with GPG32 and two mice from the group immunised with CQG43 were sacrificed, meaning two separate fusions were performed at the same time. These mice were chosen because one group of mice was immunised with a peptide from the N-terminus and one group with a peptide from the C-terminus. The mice had also shown a high and specific immune response. The other groups of mice continued to be immunised for later fusions. The spleens were then mashed over a petri dish and the cells were counted. In parallel, the myeloma cells were prepared for fusion by gathering the cells and counting them. The myeloma and spleen cells were then combined in a tube and polyethylene glycol (PEG) solution was slowly added at 37 degrees during agitation. The cells were then suspended in HAT-medium and distributed in 20 96-well microplates.

Four days after the performed fusion, the medium was replaced. Without disturbing the cells, the medium was discarded and replaced with new HAT-medium.

Ten days after the fusion the medium was replaced again but this time the medium was collected instead of being discarded and replaced with HT-medium. The collected hybridoma medium was used for screen 1. The hydridomas positive in screen 1, cut off A620 0.09, and with a 50 % confluency were moved to 6-well plate in HT-medium and named CARM1-n (n = 1-28). When the cells had grown to cover a maximum of 2/3 of the surface the cells were frozen and hybridoma medium was taken for screen 2. The hybridoma positive in screen 2, cut off A620 0.1, were then isotyped.

3.2.4 Screening for positive hybridoma cells

The screening for positive hybridoma cells, meaning hybridoma cells secreting CARM1 antibodies, can be performed in an ELISA with spent medium from the hybridomas, here called screen 1 and 2. Table 3.2 shows the antigen used for immunisation, titer tests, screen 1 and screen 2 for the two fusions.

Table 3.2: An overview of the antigen used for screen 1 and 2 for the screens of hybridomas after fusion.

Ag for immunisation	Ag in titer test	Screen 1 ag	Screen 2 ag
GPG32	Biotin-GPG32	Biotin-GPG32	Biotin-GPG32
CQG43	Biotin-CQG43	Biotin-CQG43	Biotin-CQG43 and C-terminus GST-fusion protein

Both screen 1 and screen 2 were performed with an ELISA with the same experimental setup for the fusion with the mice immunised with the GPG32 and CQG43 peptides. An overview of the ELISA is presented in figure 3.1. A microplate coated with streptavidin was first washed with EIA wash solution before the biotinylated peptides with a concentration of 1 µg/ml were added to bind to the streptavidin and coat the surface for one hour incubation on a shaker in room temperature. The

plate was again washed before hybridoma medium and controls were added according to figures A.5 and A.6 and incubated for one hour on a shaker. The plate was washed and a secondary antibody was added, in this case rabbit anti-mouse conjugated with HRP, and incubated for one hour on a shaker and then washed. TMB substrate was added to all wells and the plate was incubated for 5-30 minutes until the absorbance was measured at 620 nm using a BioTek ELx808 Absorbance Reader.

Additionally, screen 2 was performed with the produced GST-tagged C-terminus antigen. The screen was performed as an ELISA with a glutathione coated microplate, visualised in figure 3.2. The plate was first washed before adding lysate from the production of C-terminus CARM1 protein with a GST-tag. The coating binds to the fusion protein via the GST-tag to coat the surface for one hour incubation on a shaker in room temperature. The plate was again washed before hybridoma medium and controls were added according to figure A.7 and incubated for one hour on a shaker. The plate was washed and a secondary antibody was added, in this case rabbit anti-mouse conjugated with HRP, and incubated for one hour on a shaker and then washed. TMB substrate was added to all wells and the plate was incubated for 5-30 minutes until the absorbance was measured at 620 nm using a BioTek ELx808 Absorbance Reader.

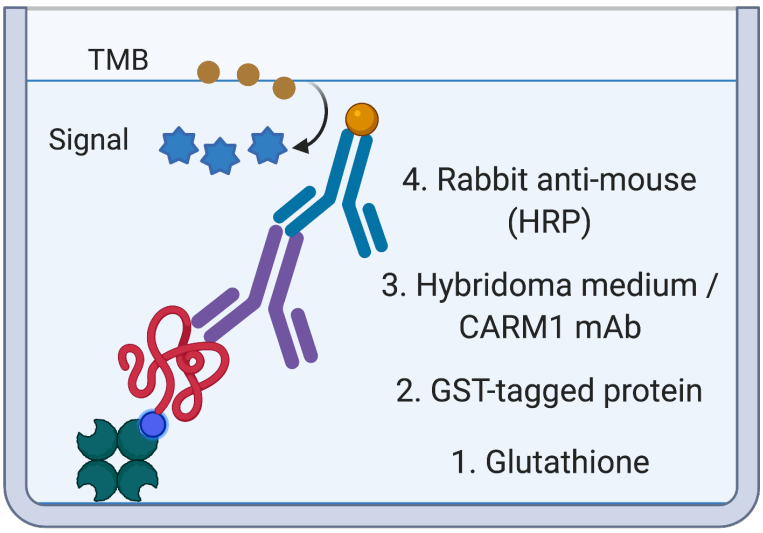

Figure 3.2: A visualisation of the ELISA screen performed to test the hybridoma medium after fusion and to evaluate the presence of GST-tagged fusion protein after production. The microplate is coated with glutathione, which binds to the GST-tagged protein. When adding hybridoma medium, the specific CARM1 antibodies binds to the C-terminus CARM1 protein if present and a secondary antibody, rabbit anti-mouse conjugated with HRP, binds to the CARM1 antibody and produce a signal when adding TMB substrate. Figure created with BioRender.

3.2.5 Isotyping of antibodies

ELISA was used to isotype the antibodies in the hybridomas positive in screen 2. A visualisation of the experimental setup can be seen in figure 3.3 and started with a microplate coated with goat anti-mouse IgG+M. The microplate was first washed before hybridoma medium from the hybridomas positive in screen 2 was added together with five controls containing known antibodies of isotypes IgG1, IgG2a, IgG2b, IgG3 and IgM in quintuplicates, as seen in figure A.8, and incubated for one hour incubation on a shaker in room temperature. The plate was again washed before isotype specific goat anti-mouse conjugated with HRP, one per the five identical test setups, was added and the plate was incubated for one hour on a shaker and then washed. TMB substrate was added to all wells and the plate was incubated for 5-30 minutes until the absorbance was measured at 620 nm using a BioTek ELx808 Absorbance Reader.

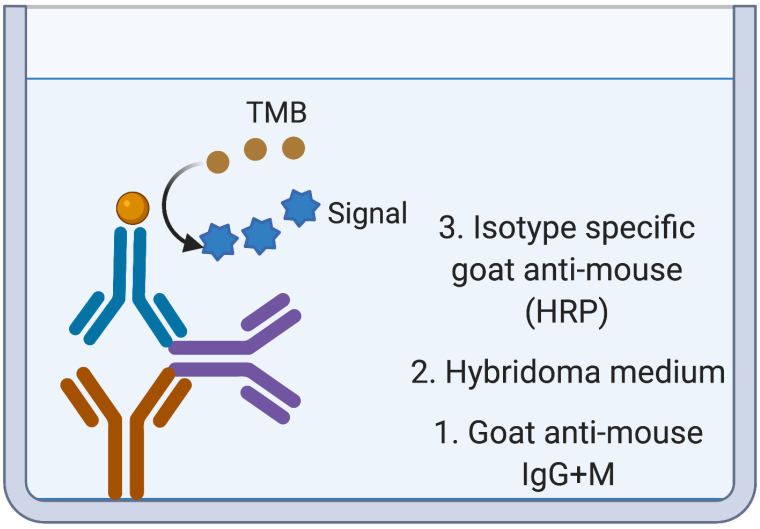

Figure 3.3: A visualisation of the ELISA screen performed to test the isotype of the antibodies after fusion and screen 1 and 2. The microplate is coated with goat anti-mouse IgG+M. When adding hybridoma medium, the mouse antibodies bind to the goat anti-mouse antibodies if present. HRP conjugated isotype specific secondary antibodies, goat anti-mouse, binds to the mouse antibody of the specific isotype and produce a signal when adding TMB substrate. Figure created with BioRender.

4

Results

In this chapter results from all experiments will be displayed, including production and characterisation of CARM1 antibodies and production of CARM1 antigen for screening.

4.1 Production of CARM1 antigen for screening

In this section results from production of CARM1 antigen will be presented, including cloning, production and purification of GST-tagged protein.

4.1.1 Cloning of GST-tagged protein

The C-terminus coding fragment was inserted into a vector containing the GST protein, *E. coli* was transformed with the vector and then screened. Figure 4.1 shows the image taken of the gel electrophoresis run with the PCR products of 10 *E. coli* colonies transformed with the cloned C-terminus of CARM1 and a negative control of the vector without any insert. Colonies 11, 12, 13, 14, 15, 17, 18 and 19 appeared to have the correct insert of 560 base pairs. Colonies 11 and 12 were chosen for sequencing and further growth.

The N-terminus coding fragment was inserted into a vector containing the GST protein, *E. coli* was transformed with the vector and then screened. Figure 4.2 shows the image taken of the gel electrophoresis run with the PCR products of 10 *E. coli* colonies transformed with the cloned N-terminus and a negative control of the vector without any insert. Colonies 5, 8 and 10 appeared to have the correct insert of 560 base pairs. Colonies 5 and 8 were chosen for sequencing and further growth.

Sequencing of the initial cloning of the N-terminus coding fragment indicated that it had not been successful. Figure 4.3 shows the image taken of the gel electrophoresis run with the PCR products of 3 *E. coli* colonies transformed a second time with the cloned plasmid containing the N-terminus coding fragment and a negative control of the vector without any insert. All colonies appeared to have the correct insert of 560 base pairs. Colonies 1, 2 and 3 were all chosen for sequencing.

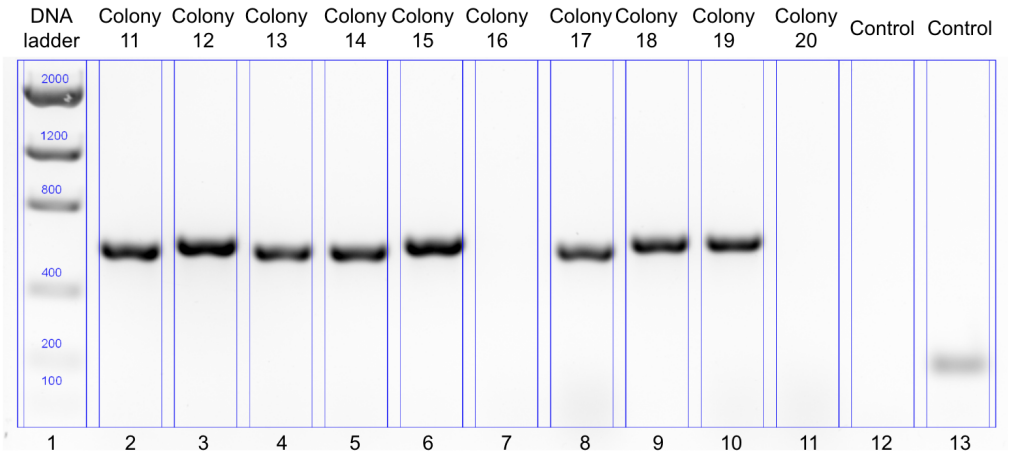

Figure 4.1: The results of an agarose gel run with the PCR products of 10 *E. coli* colonies transformed with the cloned the plasmid containing the C-terminus coding fragment and a negative control of the vector without any insert. The size of the PCR fragment with the insert is 560 base pairs and 150 base pairs without the insert.

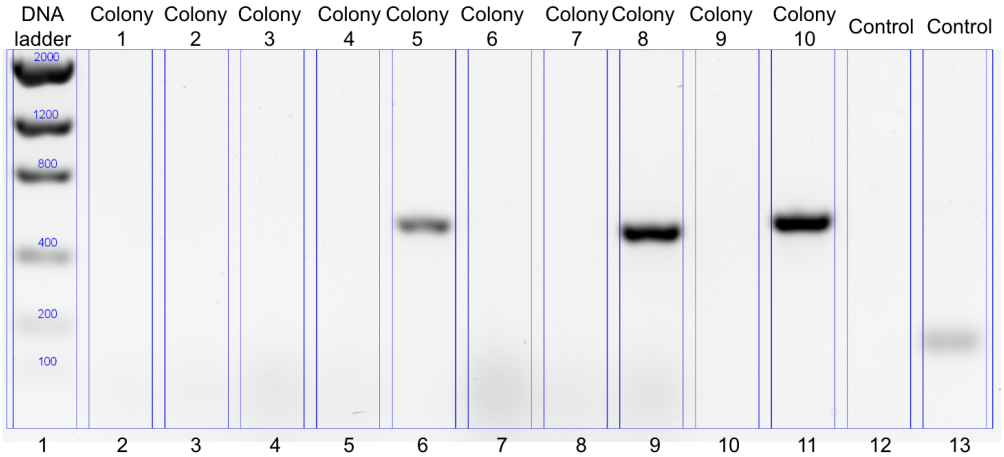

Figure 4.2: The results of an agarose gel run with the PCR products of 10 *E. coli* colonies transformed with the cloned plasmid containing the N-terminus coding fragment and a negative control of the vector without any insert. The size of the PCR fragment with the insert is 560 base pairs and 150 base pairs without the insert.

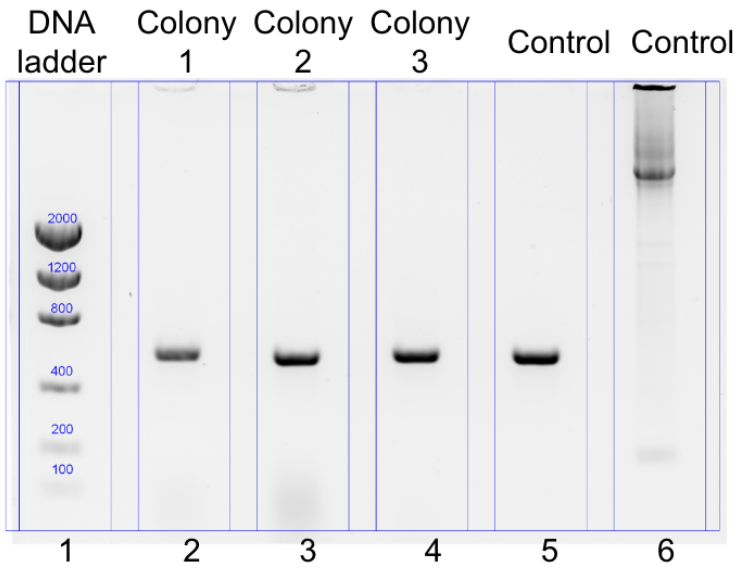

Figure 4.3: The results of an agarose gel run with the PCR products of 3 *E. coli* colonies transformed with the cloned plasmid containing the N-terminus coding fragment and a negative control of the vector without any insert. The size of the PCR fragment with the insert is 560 base pairs and 150 base pairs without the insert.

4.1.2 Production of GST-tagged protein

The colonies that looked to have the correct insert on the DNA gel electrophoresis were cultivated in a small scale, induced with IPTG, lysated and run on an SDS PAGE, see figure 4.4.

The protein gel was then blotted in three different sections. Columns 1-5 are blotted with primary antibody goat anti-GST and secondary antibody rabbit anti-goat, columns 6-10 are blotted with primary antibody CARM1 mouse and secondary antibody rabbit anti-mouse, columns 11-15 are blotted with primary antibody CARM1 rabbit and secondary antibody goat anti-rabbit. Figure 4.5 shows the results from the Western blot. The weight of the GST-tag is 26 kDa and the weight of the GST-tagged N- and C-termini 40 kDa. Column 2 and 3, N-termini colony 5 and 8 blotted with goat anti-GST, show a band at 26 kDa, indicating that the GST-tag without the N-terminus protein is present in the samples. In column 5 and 10, the C-terminus coding protein blotted with goat anti-GST and CARM1 mouse antibody respectively, a band above 37 kDa is visible, indicating that the full GST-tagged C-terminus protein is present in the sample.

Since the C-terminus protein was detected in figure 4.5 and sequencing results confirmed correct assembly, the protein was produced at a larger scale. The N-terminus protein was also produced at a larger scale while also being re-cloned since it was not detected in the initially detected clones. Figure 4.6 shows the results of a SDS

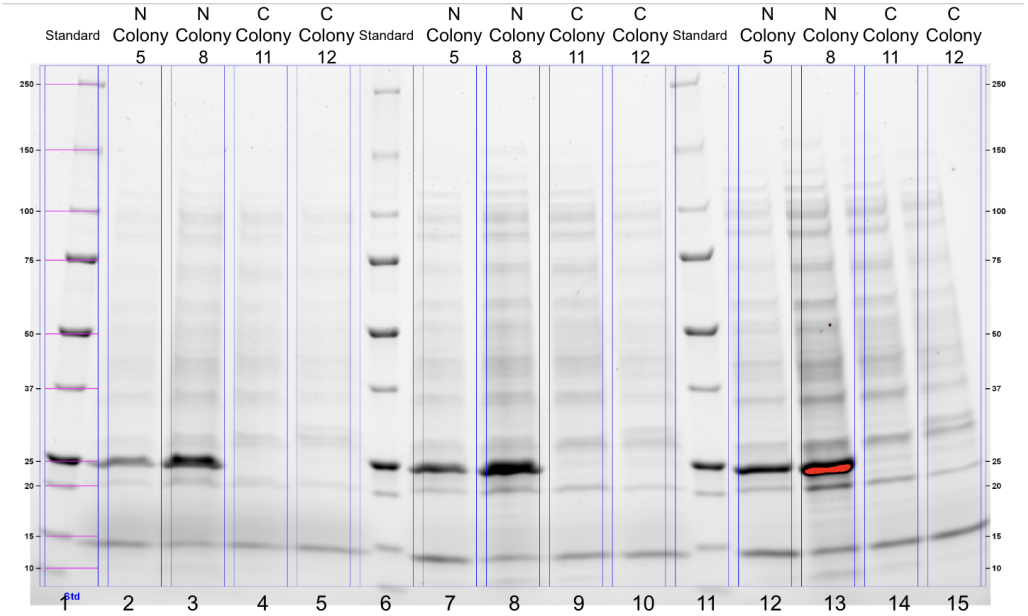

Figure 4.4: The results of a stain free SDS PAGE run with one Precision Plus Protein WesternC Standards (BioRad), two N-terminus (5 and 8) and two C-terminus (11 and 12) colonies. The samples were reduced and run in triplicate.

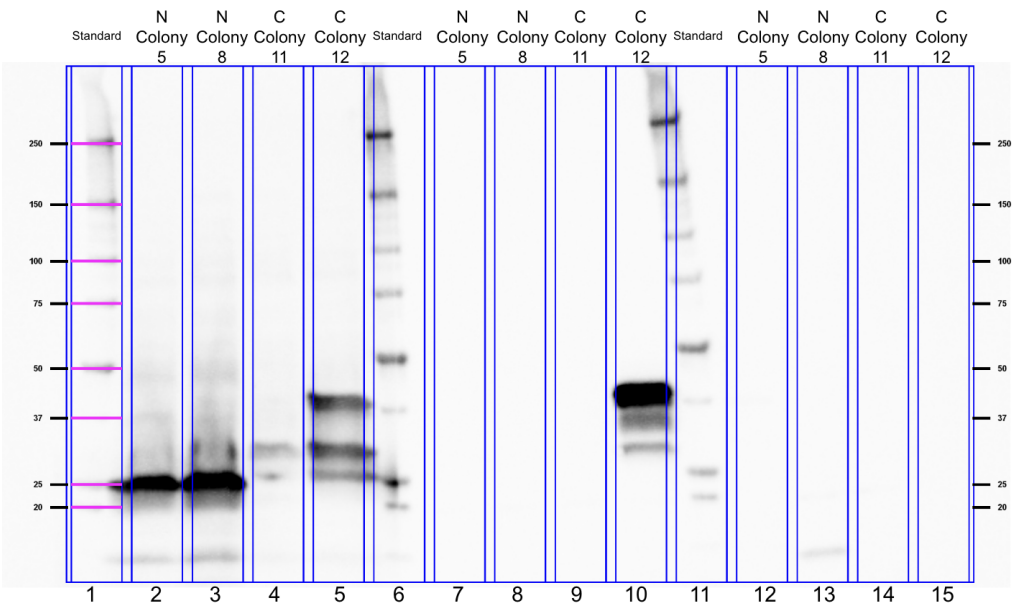

Figure 4.5: The results of a Western blot with two N-terminus (5 and 8) and two C-terminus (11 and 12) colonies. Columns 1-5 are blotted with primary antibody goat anti-GST and secondary antibody rabbit anti-goat, columns 6-10 are blotted with primary antibody CARM1 mouse and secondary antibody rabbit anti-mouse, columns 11-15 are blotted with primary antibody CARM1 rabbit and secondary antibody goat anti-rabbit. The weight of the GST-tag is 26 kDa and the weight of the GST-tagged N- and C-termini 40 kDa.

PAGE run after cultivation at a larger scale, induction and the cells were lysed. Both the lysate and the remaining cell debris was run to test whether some of the protein remains in the cell debris when lysed.

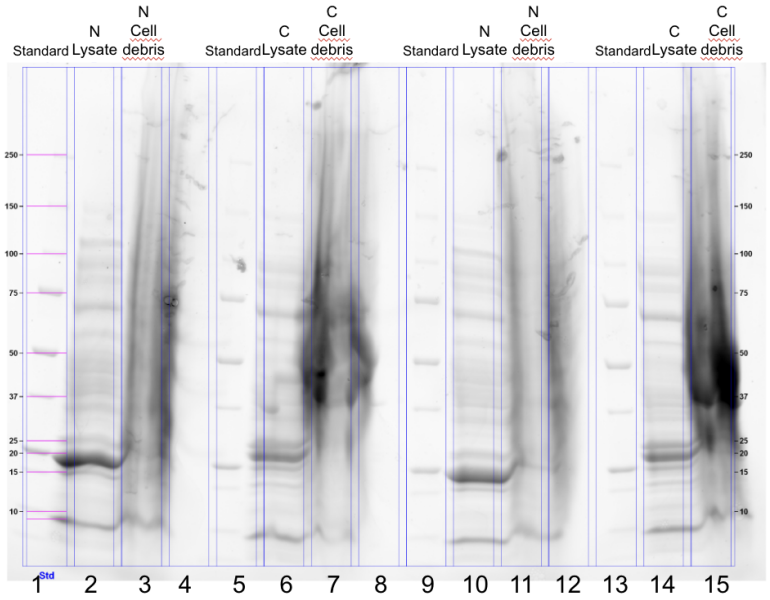

Figure 4.6: The results of a stain free SDS PAGE run with one Precision Plus Protein WesternC Standards (BioRad), lysate and cell debris containing the N-terminus protein and lysate and cell debris containing the C-terminus protein and cell debris. The samples were reduced and run in duplicate.

The protein gel was then blotted in three different sections. Columns 1-8 were blotted with primary antibody goat anti-GST and secondary antibody rabbit anti-goat, columns 9-12 were blotted with primary antibody CARM1 rabbit and secondary antibody goat anti-rabbit, columns 13-15 were blotted with primary antibody CARM1 mouse and secondary antibody rabbit anti-mouse. Figure 4.7 shows the results of the Western blot. Columns 1-8 and 13-15 show background noise which could be due to the blotting taking place in vessels used previously with other antibodies. The lysate containing the C-terminus protein seem to contain a protein at about 40 kDa, indicating that the GST-tagged C-terminus protein is present. The lysate containing the N-terminus protein seem to contain mainly a protein with a size around 20 kDa which could be the GST-tag but not any clear bands at 40 kDa, again indicating that the GST-tagged N-terminus protein is not produced.

The results from the ELISA screening for the GST proteins are shown in figure 4.8. The screen was performed on a glutathione coated microplate with three different dilutions of the lysate, 1:2, 1:10 and 1:100. The screen indicated that the C-terminus GST-tagged protein is present and functional in a screen and was subsequently used to screen hybridoma medium for antibodies after cloning, with results presented in

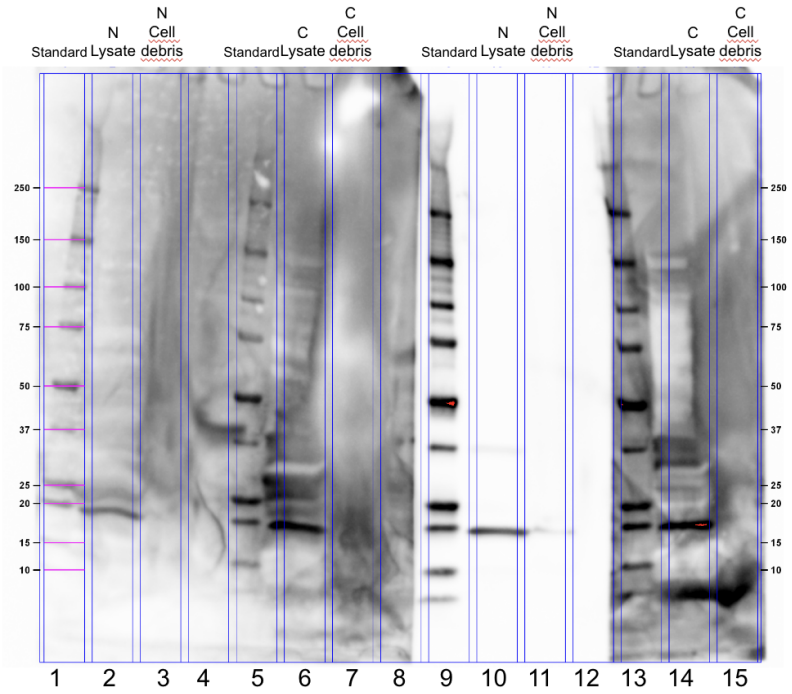

Figure 4.7: The results of a Western blot with lysate containing the N-terminus and C-terminus and cell debris respectively. Columns 1-8 were blotted with primary antibody goat anti-GST and secondary antibody rabbit anti-goat, columns 9-12 were blotted with primary antibody CARM1 rabbit and secondary antibody goat anti-rabbit, columns 13-15 were blotted with primary antibody CARM1 mouse and secondary antibody rabbit anti-mouse. The weight of the GST-tag is 26 kDa and the weight of the GST-tagged N- and C-termini proteins is 40 kDa.

figure 4.8, as well as purified. The screen of the N-terminus fragment again indicated that the full protein was not present and the cloning had to be performed again. From the sequencing results it appear that the insert was present but with an error in the PreScission site, causing a codon shift.

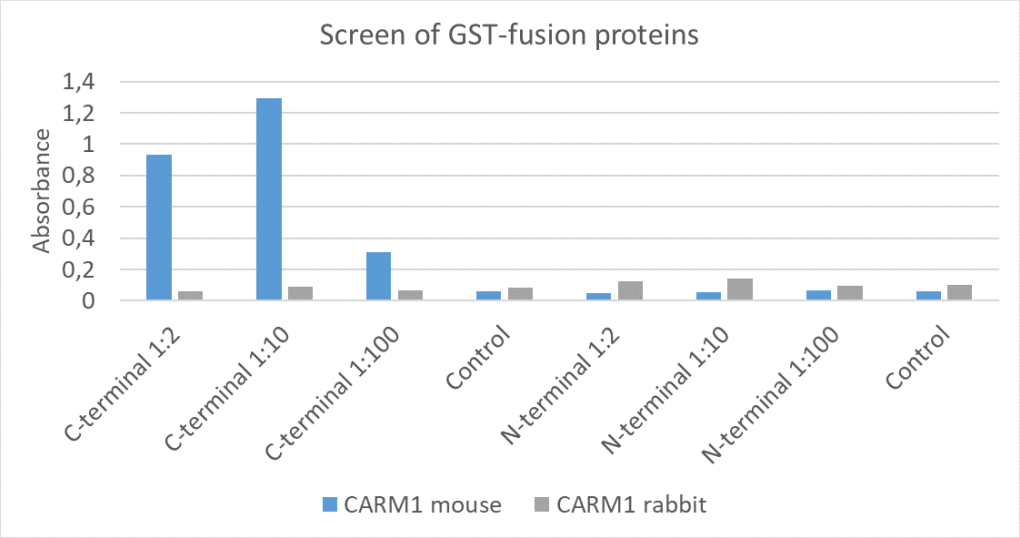

Figure 4.8: The results of an ELISA performed on the lysate produced containing C-terminus and N-terminus GST-tagged fusion protein. The screen was performed on a glutathione microplate with three different dilutions of the lysate, 1:2, 1:10 and 1:100. The primary antibodies used were CARM1 mouse mAb binding to the C-terminus fragment and CARM1 rabbit mAb binding to the N-terminaus fragment. The lysates were tested for cross reactivity with both antibodies.

4.1.3 Purification of GST-tagged protein

Table 4.1 shows the concentration of the fractions collected during the purification of the GST-tagged C-terminus fragment. Fractions 2-9 were chosen for further testing based on the high concentrations indicated by a peak in the chromatogram shown in Appendix 1 A.3.

Figure 4.9 shows the results of an SDS PAGE run with eight fractions of the purified C-terminus protein together with standards and not purified lysate, all samples were reduced with DTT. The bands indicate a successful purification, as most of the proteins present in the non-purified lysate are not present in the purified. The GST-tag seems to be present at 26 kDa and the C-terminus protein seems to be present at 40 kDa but at a lower concentration. The double bands around 26 kDa could be a result of the GST protein not being fully reduced when run on the gel. The GST-tag only could be a results of it being cleaved off from some of the fragments.

Table 4.1: The total protein concentrations of the fractions collected during the purification of the GST-tagged C-terminus fragment.

Fraction	Concentration (mg/ml)
1	0.074
2	0.107
3	0.538
4	0.515
5	0.471
6	2.505
7	1.358
8	0.538
9	0.253
10	0.152
11	0.133

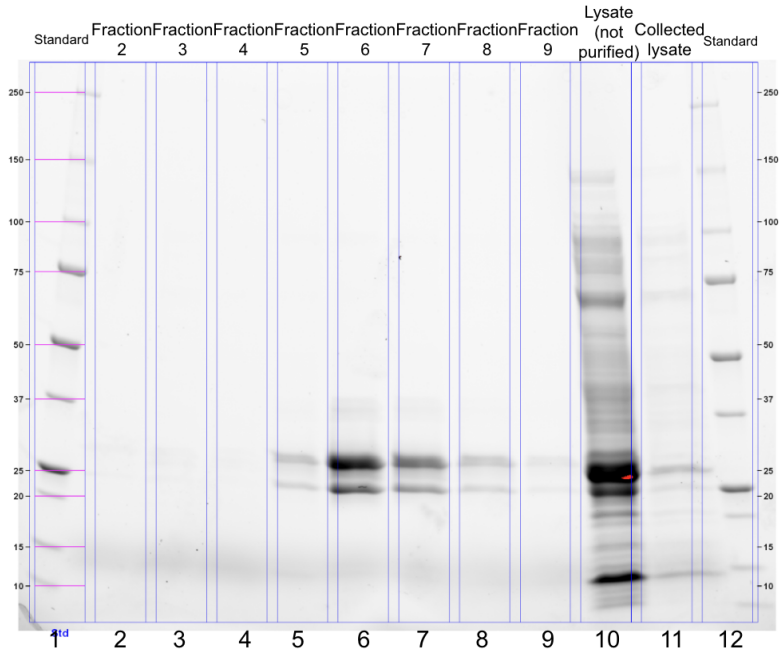

Figure 4.9: The results of a stain free SDS PAGE run with one Precision Plus Protein WesternC Standards (BioRad), eight fractions of purified GST-tagged C-terminus fragment and controls of unpurified lysate run in duplicate. One of the gels is presented in the figure. The weight of the GST-tag is 26 kDa and the weight of the GST-tagged C-terminus fragment 40 kDa.

The protein gels were then blotted with different antibodies. The first gel, shown in figure 4.10(a), was blotted with primary antibody goat anti-GST and secondary antibody rabbit anti-goat. The second gel, shown in figure 4.10(b), was blotted with primary antibody CARM1 mouse and secondary antibody rabbit anti-mouse. Again, the GST-tagged C-terminus protein seem to be present at weight 40 kDa. When blotted with anti-GST mAb a band is also visible at around 26 kDa indicating that the GST-tag without the C-terminus also is present in the sample.

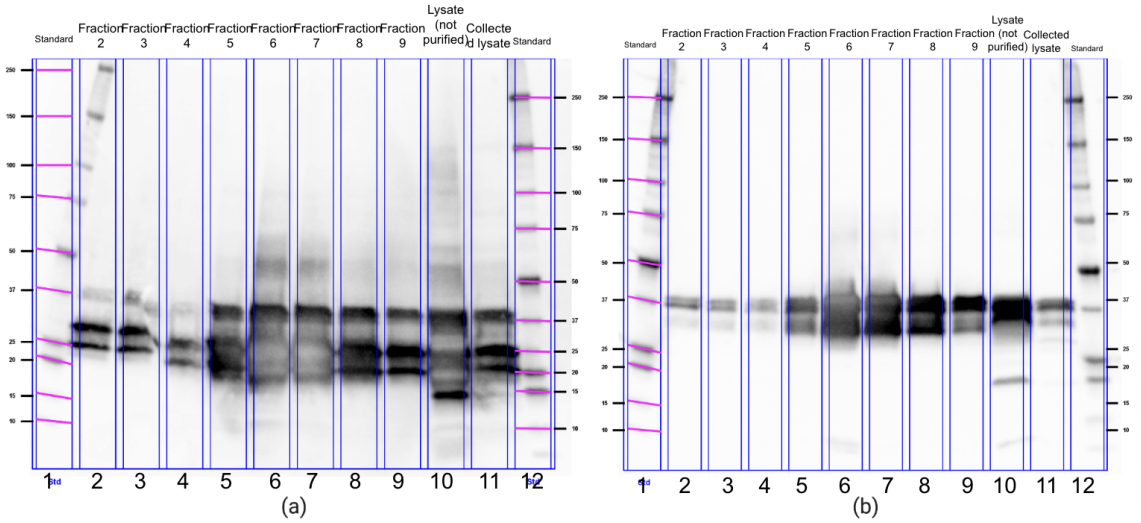

Figure 4.10: The results of western blots with Precision Plus Protein WesternC Standards (BioRad), eight fractions of purified GST-tagged C-terminus fragment and controls of unpurified lysate. Blotted with (a) anti-GST as a primary antibody and rabbit anti-goat as a secondary antibody (b) CARM1 mouse mAb as a primary antibody and rabbit anti-mouse as a secondary antibody. The weight of the GST-tag is 26 kDa and the weight of the GST-tagged C-terminus fragment 40 kDa.

4.2 Production and characterisation of CARM1 antibodies

In this section results from production and characterisation of CARM1 antibodies will be presented, including determining the immune response of immunised mice, screening for positive hybridoma cells and isotyping.

4.2.1 Determination of immune response of immunised mice

ELISAs were performed to test the blood of the immunised mice by using the peptides the mice were immunised with to evaluate if antibodies binding to the peptides

were present in the blood. The results from the ELISA performed to test the immune response of the immunised mice are presented in figures 4.11, 4.12, 4.13 and 4.14. The serum was collected before immunisation as well as after immunisation number 1, 2, 3 and 4 for all five mice from all four groups. The results from the ELISA indicate an increased immune response in the mice after immunisation with a slight increase after each booster immunisation. It also indicated very low cross reactivity when tested against a different peptide than the one used for immunisation as a control. The mice immunised with GPG32 were tested against KCS36 as a control, the mice immunised with KCS36 were tested against GPG32 as a control, the mice immunised with LLT44 were tested against CQG43 as a control and the mice immunised with CQG43 were tested against LLT44 as a control.

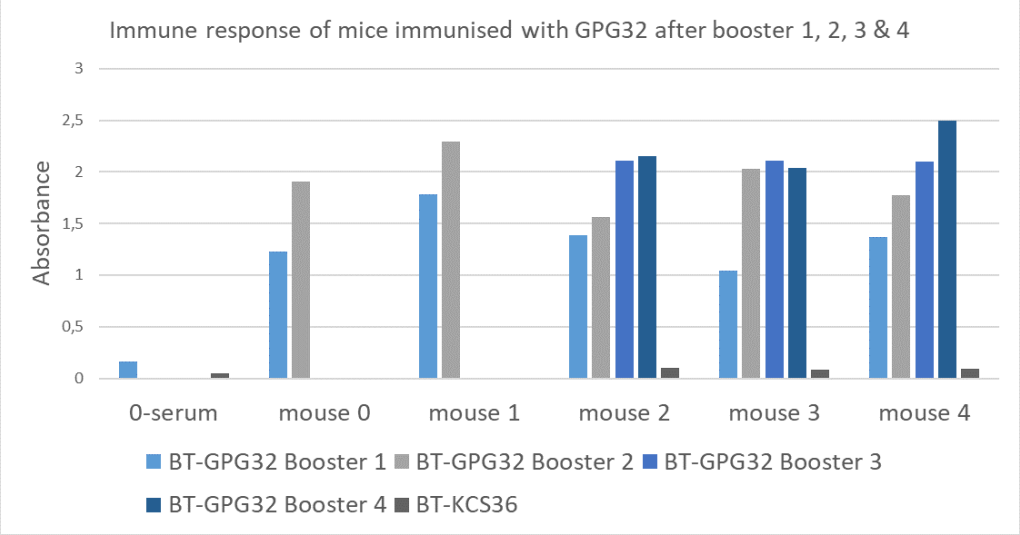

Figure 4.11: The results of an ELISA performed on serum collected before immunisation (0-serum) and after immunisation 1, 2, 3 and 4 with GPG32 from five mice numbered 0-4. The serum was diluted to 1:400 and tested against BT-GPG32 and the cross reactivity was tested against BT-KCS36 as a control. Mouse 0 and 1 were used for fusion after booster 2.

4.2.2 Selection of antibody producing hybridoma cells

The following section presents results from screens 1 and 2 of the hybridoma cells created through fusion of mice immunised with peptides GPG32 and CQG43.

4.2.2.1 Selection of hybridomas after immunisation with peptide GPG32

Screen 1 performed 10 days after the fusion was performed according to the setup described in section A.5, 10 96-well microtiter plates of hybridoma medium were tested. Mouse serum from the immunised mice was used as a positive control and HAT-medium was used as a negative control. With a cut off value at 0.09 11 wells

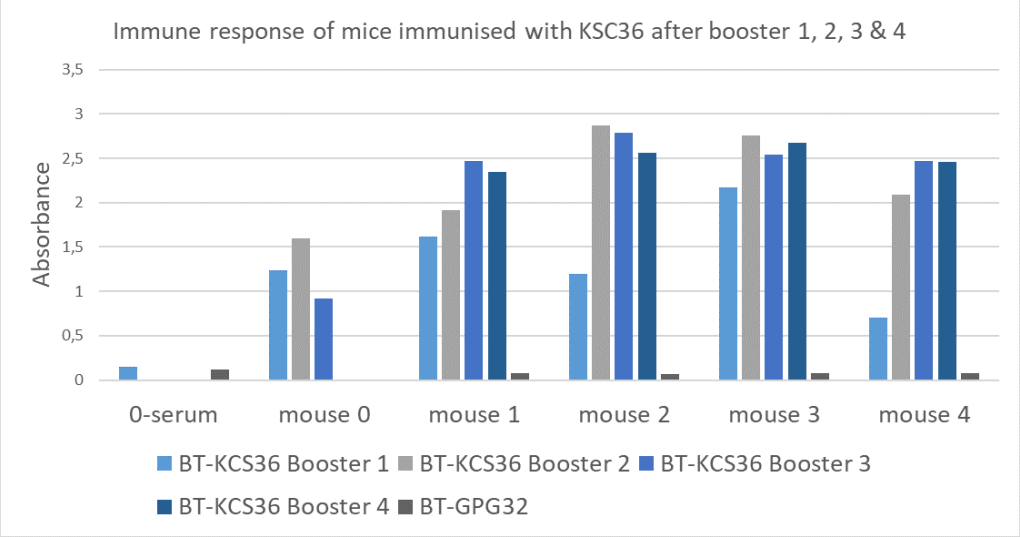

Figure 4.12: The results of an ELISA performed on serum collected before immunisation (0-serum) and after immunisation 1, 2, 3 and 4 with KCS36 from five mice numbered 0-4. The serum was diluted to 1:400 and tested against BT-KCS36 and the cross reactivity was tested against BT-GPG32 as a control. Mouse 0 died after booster 3.

were determined to be positive. The average value of the positive controls was 1.42 and of the negative controls 0.04. The positive hybridomas were named CARM1-n (n = 1-11).

Eight of the hybridoma positive in screen 1 had grown to be screened in a second screen. Mouse serum from the immunised mice was used as a positive control and HT-medium was used as a negative control. The results from the ELISA can be seen in figure 4.15. The antibodies do not show a specific response to the peptide GPG32, 7 of 8 hybridomas show significant binding to both peptides. The antibodies showing a response to GPG32, CARM1-1, CARM1-3, CARM1-4, CARM1-5, CARM1-8, CARM1-10 and CARM1-11 also show a response to CQG43, indicating that the response is not very specific. The negative control and background signal was around 0.07.

4.2.2.2 Selection of hybridomas after immunisation with peptide CQG43

Screen 1 performed 10 days after the fusion was performed according to the setup described in section 4.2.2.1, 10 96-well microtiter plates of hybridoma medium were tested. Mouse serum from the immunised mice was used as a positive control and HAT-medium was used as a negative control. With a cut off value at 0.09 17 wells were determined to be positive. The average value of the positive controls was 1.69 and of the negative controls 0.04. The positive hybridomas were named CARM1-n (n = 11-28).

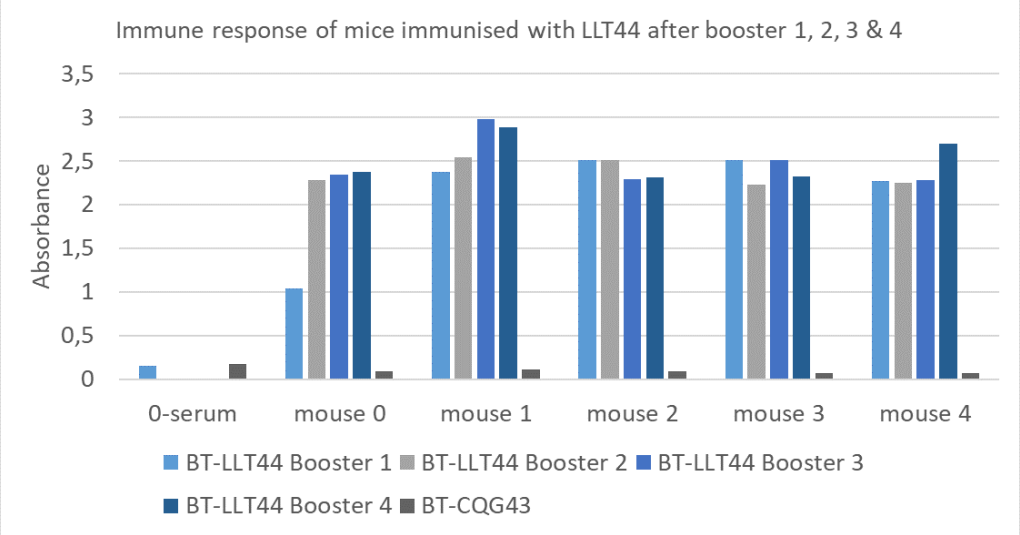

Figure 4.13: The results of an ELISA performed on serum collected before immunisation (0-serum) and after immunisation 1, 2, 3 and 4 with LLT44 from five mice numbered 0-4. The serum was diluted to 1:400 and tested against BT-LLT44 and the cross reactivity was tested against BT-CQG43 as a control.

14 of the hybridoma positive in screen 1 had grown to be screened in a second screen. Mouse serum from the immunised mice was used as a positive control and HT-medium was used as a negative control. The results from the ELISA can be seen in figure 4.16 The antibodies produced from the hybridoma appear to be less specific than the antibodies in the mouse serum. The antibodies do not show a specific response to CQG43, 10 of 14 hybridomas show significant binding to both peptides. The antibodies showing a response to CQG43, CARM1-12, CARM1-14, CARM1-20, CARM1-21, CARM1-23, CARM1-24, CARM1-25, CARM1-26 and CARM1-27 also show a response to GPG32, indicating that the response is not very specific. The negative control and background signal was around 0.06.

Screen 2 for the antibodies specific to the C-terminus part of CARM1 (CQG43) were also tested against the C-terminus GST-fusion protein produced in section 4.1. Since the N-terminus protein was not successfully produced there is no test for cross reactivity. Mouse serum from the immunised mice was used as a positive control and HT-medium was used as a negative control. The results from the ELISA can be seen in figure 4.17. The same hybridomas show reactivity in both screens shown in figures 4.16 and 4.17, indicating that the screen with the produced C-terminus GST fusion protein is functional and produces a true signal.

4.2.3 Isotyping of antibodies

The hybridomas tested in screen 2 that had grown in the wells were also isotyped and the results from that isotyping are presented in table 4.2. As shown in the

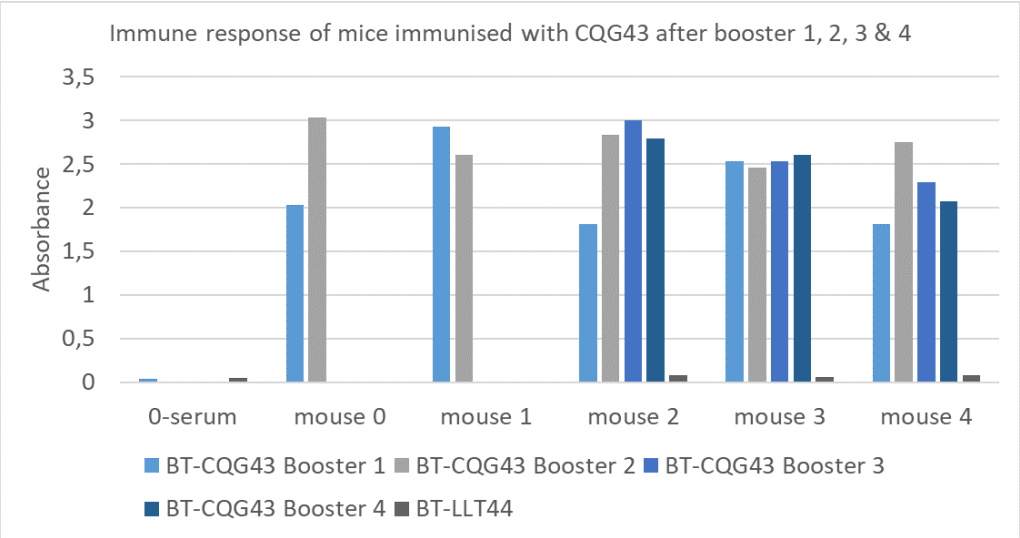

Figure 4.14: The results of an ELISA performed on serum collected before immunisation (0-serum) and after immunisation 1, 2, 3 and 4 with CQG43 from five mice numbered 0-4. The serum was diluted to 1:400 and tested against BT-CQG43 and the cross reactivity was tested against BT-LLT44 as a control. Mice 0 and 1 were used for fusion after booster 2.

table the results indicate all hybridomas being of isotype IgM. CARM1-5 produced a signal for isotypes IgM and IgG2b.

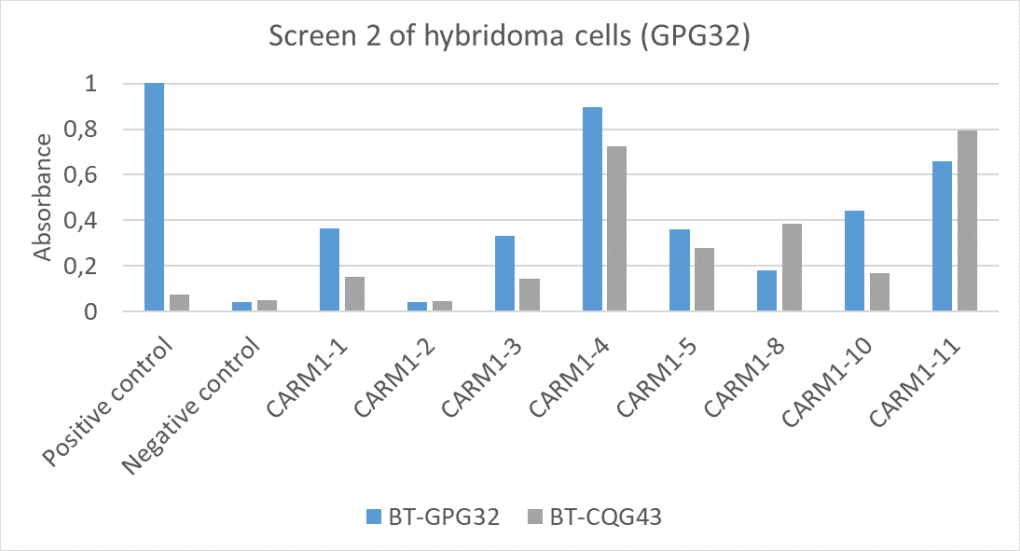

Figure 4.15: The results of an ELISA performed on hybridoma medium collected for screen 2 on a streptavidin coated microplate. The medium was tested against BT-GPG32 and the cross reactivity is tested against BT-CQG43. Mouse serum was used as positive control and HT-medium as negative control.

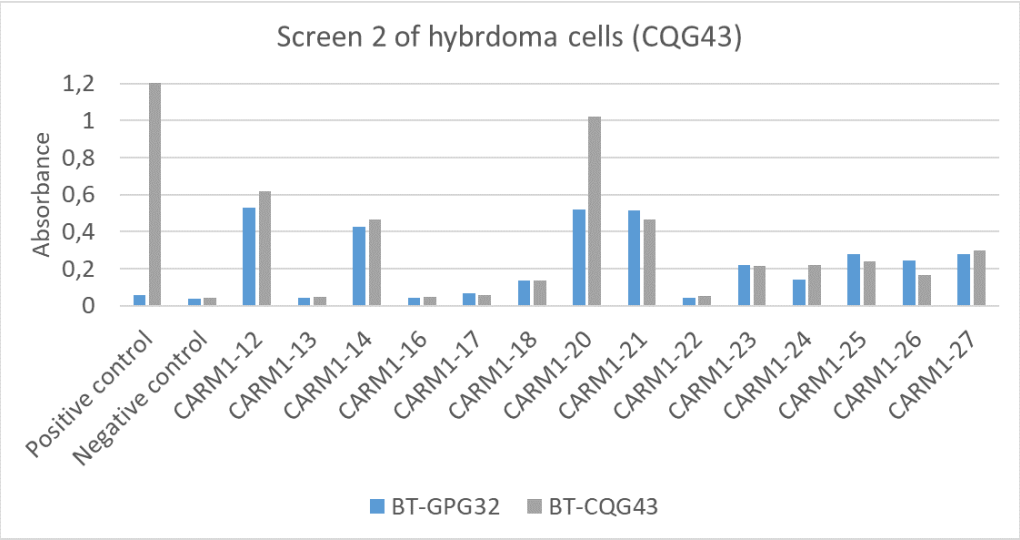

Figure 4.16: The results of an ELISA performed on hybridoma medium collected for screen 2 on a streptavidin coated microplate. The medium was tested against BT-CQG43 and the cross reactivity is tested against BT-GPG32 as a control. Mouse serum was used as positive control and HT-medium as negative control.

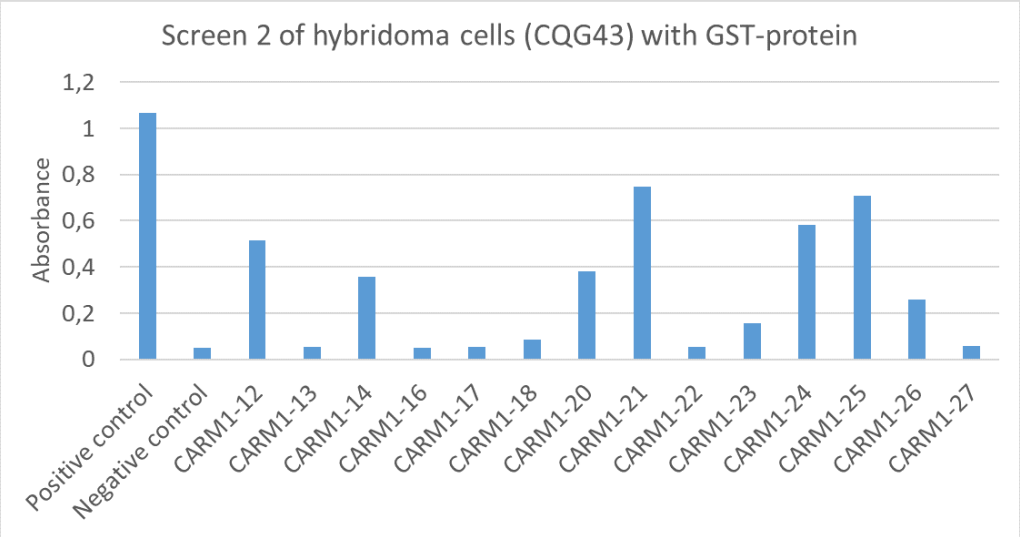

Figure 4.17: The results of an ELISA performed on hybridoma medium collected for screen 2 on a glutathione coated microplate. The medium was tested against C-terminus GST-tagged fusion protein, diluted 1:25. No cross reactivity was tested. CARM1 mouse mAb was used as positive control and casein buffer as negative control.

Table 4.2: The results of an ELISA performed to test the isotype of the antibodies produced by the hybridoma.

	IgG1	IgG2a	IgG2b	IgG3	IgM
CARM1-1					x
CARM1-3					x
CARM1-4					x
CARM1-5			x		x
CARM1-8					x
CARM1-10					x
CARM1-11					x
CARM1-12					x
CARM1-14					x
CARM1-18					x
CARM1-20					x
CARM1-21					x
CARM1-23					x
CARM1-24					x
CARM1-25					x
CARM1-26					x
CARM1-27					x

5
Discussion

In this report, the aim was to establish antibodies targeting CARM1 using classical hybridoma technique. The first step is to raise an immune response in mice by immunising them with CARM1, in this study specific exposed CARM1 peptides to raise specific and usable antibodies. To measure the immune response after immunisations, blood was drawn from the tail of the immunised mice and antibody titre was measured in an ELISA.

The results of the ELISAs performed on the mouse serum collected after booster 1 and 2 showed an increased and specific immune response to CARM1, which lead to two fusions being performed after booster 2. All groups of mice show a difference in immune response in between individual mice which is expected due to differences in individuals, but the immune response was more or less steady after booster 2. The largest increase in immune response seems to happen after booster 2. However, by immunising the mice repeatedly, the immune response gets more mature and specific, thereby increasing the chance of getting good monoclonals.

Two fusions were performed within this study using animals immunised with one N-terminus (GPG32) and one C-terminus (CQG43) peptide. Both fusions resulted in a total of 17 CARM1 antibody producing hybridoma cells.

The production of CARM1 antibodies through the fusions were successful with the antibodies showing a specific response to the biotinylated peptides when tested in ELISAs for screen 1. Screen 2 however showed a less specific response for the medium from both the GPG32 and the CQG43 hybridoma cells as presented in figures 4.15 and 4.16. The fact that the hybridomas show less reactivity than the mouse serum may have several explanations, but the most probable one is that the immune response was not mature enough, and too few B-cells producing CARM1 were present in the mouse spleen at the time of the fusion. Additional immunisations would thus result in clonal expansion of the CARM1 specific B-cells and increase the possibility of getting IgG isotype.

In preparation of and during the actual fusion process it is important to optimise the environment for the cells used in order for viability to be as high as possible when fusion is performed. Unfortunately, the cell count during both fusions were low based on experience which is why only 10 microplates were filled with hybridoma per fusion instead of the normal 20 per fusion. Myeloma cells are sensitive to high confluency and it is optimal to use them in growth phase. Too high confluency

might lead to low viability which could be a reason for a less successful fusion.

The isotyping confirmed that all antibodies were of type IgM which is the less mature antibody. The characteristics of the IgM antibody does not suit well for a diagnostic test due to e.g. low affinity and their pentamere structure which is why IgG antibodies are more desirable. A decision, based on previous experience with IgM antibodies as unstable, was made to not continue working with the antibodies but instead continue immunising the mice as an attempt to provoke a more mature immune response containing IgG antibodies. The one hybridoma which showed a signal for both IgM and IgG2b isotypes, CARM1-5, was most likely a hybrid with different hybridoma producing antibodies of two different isotypes in the same well. This hybridoma was the most promising candidate but because of low specificity in screen 2 and low signal in the isotyping the mAbs are not likely to have the desired properties of high specificity and affinity.

The other part of the project was to produce GST-tagged C- and N-terminus parts of the CARM1 protein to use for screens. The cloning performed to assemble the pGEX-6P-3 vector with the C-terminus and N-terminus fragments respectively seemed initially successful as presented in figures 4.1 and 4.2, where 8 out of 10 and 3 out of 10 colonies for the C- and N-terminus fragments respectively indicated a correct length on the gel electrophoresis with the PCR products. Two colonies of each protein were selected for further cultivation and production as well as sent to sequencing for assuring correct sequences.

The lysate from inducing the bacteria containing the GST-tagged C- and N-terminus fragments with IPTG was then screened for the presence of the protein. Initially a western blot was performed using three different antibodies for blotting shown in figure 4.5. The anti-GST mAb should in theory bind to both proteins as they both have the GST tag, the CARM1 mouse mAb should in theory bind to an epitope on the C-terminus end of the CARM1 protein and the CARM1 rabbit mAb should in theory bind to an epitope on the N-terminus end of the CARM1 protein.

The bands for the colonies cloned with the N-terminus fragments indicated that it was not present in full when blotted with either the anti-GST or CARM1 rabbit mAb but when blotted with anti-GST the 26 kDa GST-tag seems to be present. This, together with results from further western blot, figures 4.7, where the N-terminus fragment is not present, and the ELISA screen performed on a glutathione plate, figure 4.8, indicates that the N-terminus fragment is not present in the lysate or the cell debris and not likely produced by the *E. coli*. The sequencing results shows an error in the PreScission site situated in between the GST tag and the multiple cloning site (MCS), indicating that the restriction digestion of the cloning was not successful. This resulted in a codon shift causing the fragment to not contain the right amino acids.

The cloning of the N-terminus fragment was therefore performed again with three colonies growing after digestion, ligation and transformation. These colonies again

showed an error in the PreScission site when sequenced indicating an error with restriction digestion and/or ligation. Multiple other cloning attempts resulted in no colonies. This could be a result of multiple different factors, such as an error being made when performing the experiment, inactive enzymes or an error in the sequence of the gene block. Further investigation and trouble shooting would be needed to examine the errors when cloning the N-terminus fragment. Another possible explanation could be that the gene is toxic to the cell but this does not explain the complete absence of colonies after cloning.

The cloning of the C-terminus fragment on the other hand showed that the protein was present on the western blot in figure 4.5 for colony 12 when blotted with CARM1 mouse mAb and anti-GST mAb as a visible band at 40 kDa. When blotted with the anti-GST mAb there is also a band visible at 26 kDa indicating the presence of the GST-tag. This could be a result of some of the protein being detached from the GST-tag during cultivation. Because of the background noise in figure 4.7 the presence of the C-terminus protein is not as clear but a band seems to be present at 40 kDa in the lysate but not in the cell debris, indicating that the protein is not remaining in the cell debris when lysated.

The C-terminus protein was used in an ELISA to screen for the presence of it as well as examine the dilution needed for a screen of the hydridoma medium. The screen showed a specific response to the C-terminus protein which meant it could be used to screen the hybridoma medium. The results indicated that a dilution in between 1:10 and 1:100 would be optimal which resulted in the use of dilution 1:25 in screen 2. The results from screen 2 with the GST and glutathione plate, figure 4.17, show a response in the same hybridoma as the screen with the biotinylated peptides and streptavidin plate, figure 4.16, suggesting that the screen is functional and can be used to screen upcoming fusions. No cross reactivity was however tested since no lysate containing the N-terminus protein was available so no conclusions can be drawn about specificity.

The expression and purification of the C-terminus protein could also be optimised in order to achieve a higher yield. Whilst different variables, such as culture temperatures, times and IPTG concentrations could be explored further, this is outside of the scope for this thesis.

5.1 Future perspectives

The continued immunisations indicate a steady or increased immune response among the mice which is promising for another fusion. A more mature immune response should result in hybridomas producing IgG antibodies as opposed to IgM, since IgM antibodies are produced as a first response to an antigen while IgG are more abundant over time.

Since the final goal is to use the antibodies for immunohistochemistry, in addition to the testing performed in this thesis, further testing should also be performed to

ensure the antibodies of the desired isotype can bind to the entire CARM1 protein. Next step would be to test the affinity of the antibodies to the antigen with a biosensor. Further testing could first include immunocytochemistry on human cells which expresses the CARM1 protein natively, such as HeLa cells, before performing tests on CARM1-high tissue with immunohistochemistry.

Since it is not ideal to screen for antibodies using the same antigen as the mice were immunised with, the GST-tagged protein is a good alternative for initial screens before moving on to the whole CARM1 protein. Having the fusion proteins in a plasmid also means more protein can be produced on demand. The GST-tagged protein could also be used to immunise the mice if the antibodies produced from the mice immunised with peptides does not prove to be successful.

The GST-tagged C-terminus protein is prepared and ready to be used for screens of the new antibodies but the expression could be optimised to produce more protein. The GST-tagged N-terminus fragment was not successfully cloned. Further investigation would need to be done into why the cloning was not successful and try different strategies to optimise the process.

5.2 Conclusion

In conclusion, it is possible to produce specific antibodies towards CARM1 but further maturation of the immune response is necessary to achieve antibodies of isotype IgG instead of IgM. The next step in the project is therefore to perform additional immunisations and fusions. Since all groups of mice show an increased or stable immune response, they are all similarly suitable for fusion. The C-terminus GST-tagged protein can be used to screen for positive hybridomas whilst the cloning of the N-terminus GST-tagged protein will have to be investigated and optimised. Further screening techniques to test the affinity and compatibility with immunocytochemistry and immunohistochemistry will need to be established.

Bibliography

[1] Majidi, A., Na, R., Dixon-Suen, S., Jordan, S. J., and Webb, P. M. (2020). Common medications and survival in women with ovarian cancer: A systematic review and meta-analysis. Gynecologic Oncology, 157(3), 678–685. http://doi.org/10.1016/j.ygyno.2020.03.028

[2] Lisio, M.-A., Fu, L., Goyeneche, A., Gao, Z.-H., Telleria, C. (2019). High-Grade Serous Ovarian Cancer: Basic Sciences, Clinical and Therapeutic Standpoints. International Journal of Molecular Sciences, 20(4), 952. https://doi.org/10.3390/ijms20040952

[3] Karakashev, S., Zhu, H., Wu, S., Yokoyama, Y., Bitler, B. G., Park, P. H., ... Zhang, R. (2018). CARM1-expressing ovarian cancer depends on the histone methyltransferase EZH2 activity. Nature Communications, 9(1). https://doi.org/10.1038/s41467-018-03031-3

[4] Karakashev, S., Fukumoto, T., Zhao, B., Lin, J., Wu, S., Fatkhutdinov, N., ... Zhang, R. (2020). EZH2 Inhibition Sensitizes CARM1-High, Homologous Recombination Proficient Ovarian Cancers to PARP Inhibition. Cancer Cell, 37(2), 157-167.e6. https://doi.org/10.1016/j.ccell.2019.12.015

[5] Wu, Q., Schapira, M., Arrowsmith, C.H., Barsyte-Lovejoy, D. (2021). Protein arginine methylation: from enigmatic functions to therapeutic targeting. Nat Rev Drug Discov, 20, 509-530. https://doi.org/10.1038/s41573-021-00159-8

[6] Lord, C. J., Ashworth, A. (2017). PARP inhibitors: Synthetic lethality in the clinic. Science, 355(6330), 1152–1158. urlhttps://doi.org/10.1126/science.aam7344

[7] Kaelin, W. G. (2005). The concept of synthetic lethality in the context of anticancer therapy. Nature Reviews Cancer, 5(9), 689–698. https://doi.org/10.1038/nrc1691

[8] Duan, R., Du, W., Guo, W. (2020). EZH2: A novel target for cancer treatment. Journal of Hematology and Oncology, 13(1), 1–12. https://doi.org/10.1186/s13045-020-00937-8

[9] Bonifácio, V. D. B. (2020). Ovarian Cancer Biomarkers: Moving Forward in Early Detection. In J. Serpa (Ed.), Tumor Microenvironment : The Main Driver of Metabolic Adaptation (pp. 355–363). Cham: Springer International Publishing. urlhttps://doi.org/10.1007/978-3-030-34025-4$_1$8

[10] Jarrold, J., Davies, C. C. (2019). PRMTs and Arginine Methylation: Cancer's Best-Kept Secret? Trends in Molecular Medicine, 25(11), 993–1009. urlhttps://doi.org/10.1016/j.molmed.2019.05.007

[11] Chiu, M. L., Goulet, D. R., Teplyakov, A., and Gilliland, G. L. (2019). Antibody Structure and Function: The Basis for Engineering Therapeutics. Antibodies, 8(4), 55. https://doi.org/10.3390/antib8040055

[12] Ma, H., O'Kennedy, R. (2015). The Structure of Natural and Recombinant Antibodies. Peptide Antibodies. Methods in Molecular Biology, vol 1348. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-2999-3_2

[13] Hwang, J. W., Cho, Y., Bae, G. U., Kim, S. N., Kim, Y. K. (2021). Protein arginine methyltransferases: promising targets for cancer therapy. Experimental and Molecular Medicine, 53(5), 788–808. urlhttps://doi.org/10.1038/s12276-021-00613-y

[14] UniProt. UniProtKB - Q86X55 (CARM1_HUMAN). urlhttps://www.uniprot.org/uniprot/Q86X55

[15] Tortora, G. J., Derrickson, B. (2019). Introduction to the Human Body. 11th ed., Wiley, .

[16] Heyman, B., Shulman, M. J. (2016). Structure, Function, and Production of Immunoglobulin M (IgM). Encyclopedia of Immunobiology, 2, 1–14. https://doi.org/10.1016/B978-0-12-374279-7.05001-3

[17] Siddiqui, M. Z. (2010). Monoclonal antibodies as diagnostics; An appraisal. Indian Journal of Pharmaceutical Sciences, 72(1), 12–17. https://doi.org/10.4103/0250-474X.62229

[18] Thermo Fisher Scientific. GST-tagged Proteins – Production and Purification. https://www.thermofisher.com/se/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/gst-tagged-proteins-production-purification.html

[19] Köhler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256(5517), 495–497. urlhttps://doi.org/10.1038/256495a0

[20] Thermo Fisher Scientific. Fast Digestion of DNA. https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012413_Fast_Digestion_DNA_UG.pdf&title=VXNlciBHdWlkZTogRmFzdCBEaWdlc3Rpb24gb2YgRE5B

[21] QIAGEN. Extraction of DNA fragments from polyacrylamide gels using the QIAquick® Gel Extraction Kit. https://www.qiagen.com/us/resources/resourcedetail?id=a72e2c07-7816-436f-b920-98a0ede5159a&lang=en

[22] Thermo Fisher Scientific. DNA Insert Ligation (sticky-end and blunt-end) into Vector DNA. https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011906_DNAsert_Ligation_Vector_DNA_UG.pdf

[23] Agilent. JM109 Competent Cells. https://www.agilent.com/en/product/mutagenesis-cloning/competent-cells-competent-cell-supplies/competent-cells-for-routine-cloning/jm109-competent-cells-233092#literature

[24] VWR. VWR Taq DNA Polymerase. https://es.vwr.com/assetsvc/asset/es_ES/id/12367445/contents

[25] QIAGEN. QIAGEN Plasmid Kits. https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiagen-plasmid-kits/

[26] Agilent. BL21(DE3) Competent Cells, BL21(DE3)pLysS Competent Cells, and BL21 Competent Cells. https://www.agilent.com/cs/library/usermanuals/public/200133.pdf

[27] BioRad. Mini-PROTEAN® TGX™ Precast Gel Quick Start Guide. https://www.bio-rad.com/sites/default/files/webroot/web/pdf/lsr/literature/10026447.pdf

[28] BioRad. Trans-Blot Turbo Transfer System. https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10020688.pdf

[29] Cytiva. GSTrap FF, 1 ml and 5 ml. https://cdn.cytivalifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=11484

[30] Addgene. Plasmid: pGEX-6P-3. Vector Database. https://www.addgene.org/vector-database/2887/

A

Appendix 1

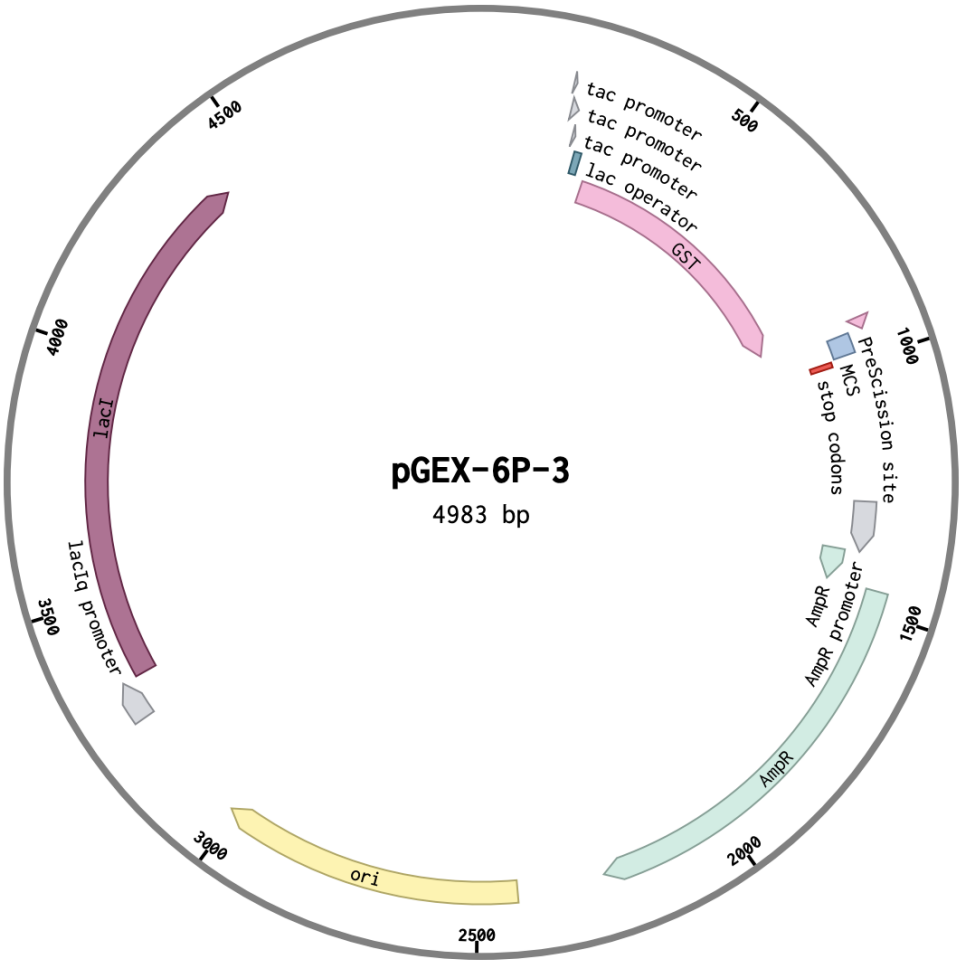

Figure A.1: The pGEX-6P-3 vector used for cloning with annotations of the coding parts. Data from [30]. Figure created with Benchling.

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	C-terminal lysat 1:2	C-terminal lysat 1:2	C-terminal lysat 1:2	C-terminal lysat 1:2								
B	C-terminal lysat 1:10	C-terminal lysat 1:10	C-terminal lysat 1:10	C-terminal lysat 1:10								
C	C-terminal lysat 1:100	C-terminal lysat 1:100	C-terminal lysat 1:100	C-terminal lysat 1:100								
D	Caseinbuffert	Caseinbuffert	Caseinbuffert	Caseinbuffert								
E	N-terminal lysat 1:2	N-terminal lysat 1:2	N-terminal lysat 1:2	N-terminal lysat 1:2								
F	N-terminal lysat 1:10	N-terminal lysat 1:10	N-terminal lysat 1:10	N-terminal lysat 1:10								
G	N-terminal lysat 1:100	N-terminal lysat 1:100	N-terminal lysat 1:100	N-terminal lysat 1:100								
H	Caseinbuffert	Caseinbuffert	Caseinbuffert	Caseinbuffert								

Figure A.2: The ELISA setup for testing the presence of GST-tagged protein in lysate on a glutathione plate. The primary antibodies used are CARM1 mouse mAb (blue), CARM1 rabbit mAb (green), mouse serum from mouse immunised with N-terminal peptide (orange) and mouse serum from mouse immunised with C-terminal peptide (yellow). Casein buffer was used as negative control and the samples were tested for cross reactivity.

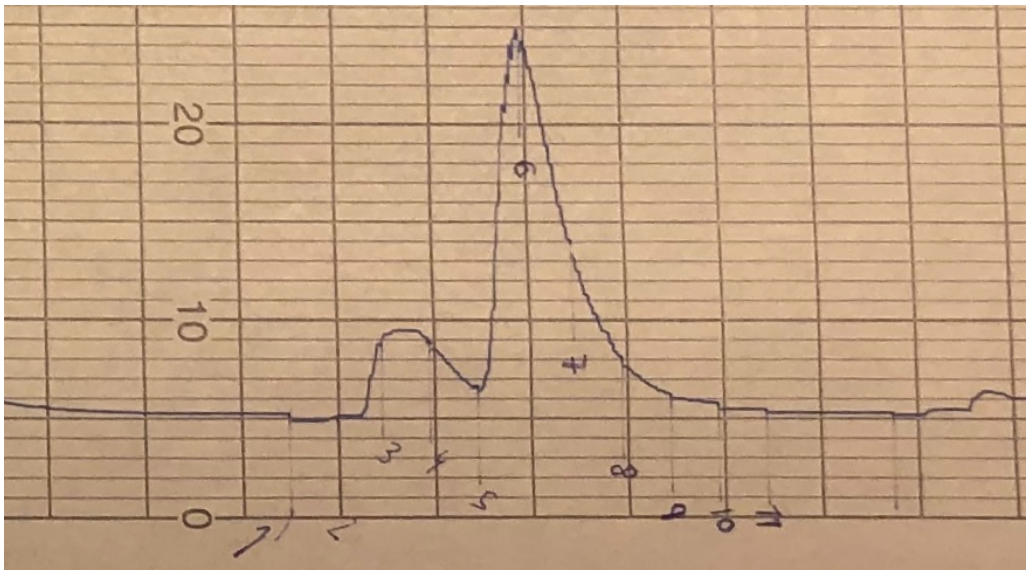

Figure A.3: The chromatogram from the purification of the C-terminal GST fusion protein through a GST column. Numbers 1-11 indicates the times of the collected fractions.

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	GPG32 0-serum pool 1:400	GPG32 mouse2 booster2 1:400	GPG32 mouse4 booster4 1:400	GPG32 0-serum pool 1:400	KCS36 0-serum pool 1:400	KCS36 mouse2 booster2 1:400	KCS36 mouse1 booster4 1:400	KCS36 0-serum pool 1:400	LLT44 0-serum pool 1:400	LLT44 mouse2 booster2 1:400	LLT44 mouse0 booster4 1:400	LLT44 0-serum pool 1:400
B	GPG32 mouse0 booster1 1:400	GPG32 mouse3 booster2 1:400	1% BSA/PBS	GPG32 mouse2 booster4 1:400	KCS36 mouse0 booster1 1:400	KCS36 mouse3 booster2 1:400	KCS36 mouse2 booster4 1:400	KCS36 mouse1 booster4 1:400	LLT44 mouse0 booster1 1:400	LLT44 mouse3 booster2 1:400	LLT44 mouse1 booster4 1:400	LLT44 mouse0 booster4 1:400
C	GPG32 mouse1 booster1 1:400	GPG32 mouse4 booster2 1:400	1% BSA/PBS	GPG32 mouse3 booster4 1:400	KCS36 mouse1 booster1 1:400	KCS36 mouse4 booster2 1:400	KCS36 mouse3 booster4 1:400	KCS36 mouse2 booster4 1:400	LLT44 mouse1 booster1 1:400	LLT44 mouse4 booster2 1:400	LLT44 mouse2 booster4 1:400	LLT44 mouse1 booster4 1:400
D	GPG32 mouse2 booster1 1:400	GPG32 mouse2 booster3 1:400	1% BSA/PBS	GPG32 mouse4 booster4 1:400	KCS36 mouse2 booster1 1:400	KCS36 mouse0 booster3 1:400	KCS36 mouse4 booster4 1:400	KCS36 mouse3 booster4 1:400	LLT44 mouse2 booster1 1:400	LLT44 mouse0 booster3 1:400	LLT44 mouse3 booster4 1:400	LLT44 mouse2 booster4 1:400
E	GPG32 mouse3 booster1 1:400	GPG32 mouse3 booster3 1:400	1% BSA/PBS	1% BSA/PBS	KCS36 mouse3 booster1 1:400	KCS36 mouse1 booster3 1:400	1% BSA/PBS	KCS36 mouse4 booster4 1:400	LLT44 mouse3 booster1 1:400	LLT44 mouse1 booster3 1:400	LLT44 mouse4 booster4 1:400	LLT44 mouse3 booster4 1:400
F	GPG32 mouse4 booster1 1:400	GPG32 mouse4 booster3 1:400	1% BSA/PBS	1% BSA/PBS	KCS36 mouse4 booster1 1:400	KCS36 mouse2 booster3 1:400	1% BSA/PBS	1% BSA/PBS	LLT44 mouse4 booster1 1:400	LLT44 mouse2 booster3 1:400	1% BSA/PBS	LLT44 mouse4 booster4 1:400
G	GPG32 mouse0 booster2 1:400	GPG32 mouse2 booster4 1:400	1% BSA/PBS	1% BSA/PBS	KCS36 mouse0 booster2 1:400	KCS36 mouse3 booster3 1:400	1% BSA/PBS	1% BSA/PBS	LLT44 mouse0 booster2 1:400	LLT44 mouse3 booster3 1:400	1% BSA/PBS	1% BSA/PBS
H	GPG32 mouse1 booster2 1:400	GPG32 mouse3 booster4 1:400	1% BSA/PBS	1% BSA/PBS	KCS36 mouse1 booster2 1:400	KCS36 mouse4 booster3 1:400	1% BSA/PBS	1% BSA/PBS	LLT44 mouse1 booster2 1:400	LLT44 mouse4 booster3 1:400	1% BSA/PBS	1% BSA/PBS

Strip	1	2	3	4
A	CQG43 0-serum pool 1:400	CQG43 mouse2 booster2 1:400	CQG43 mouse4 booster4 1:400	CQG43 0-serum pool 1:400
B	CQG43 mouse0 booster1 1:400	CQG43 mouse3 booster2 1:400	1% BSA/PBS	CQG43 mouse2 booster4 1:400
C	CQG43 mouse1 booster1 1:400	CQG43 mouse4 booster2 1:400	1% BSA/PBS	CQG43 mouse3 booster4 1:400
D	CQG43 mouse2 booster1 1:400	CQG43 mouse2 booster3 1:400	1% BSA/PBS	CQG43 mouse4 booster4 1:400
E	CQG43 mouse3 booster1 1:400	CQG43 mouse3 booster3 1:400	1% BSA/PBS	1% BSA/PBS
F	CQG43 mouse4 booster1 1:400	CQG43 mouse4 booster3 1:400	1% BSA/PBS	1% BSA/PBS
G	CQG43 mouse0 booster2 1:400	CQG43 mouse2 booster4 1:400	1% BSA/PBS	1% BSA/PBS
H	CQG43 mouse1 booster2 1:400	CQG43 mouse3 booster4 1:400	1% BSA/PBS	1% BSA/PBS

Figure A.4: One example of an ELISA setup for testing the immune response of mouse serum after each immunisation on a streptavidin plate. The plate is coated with biotin-GPG32 (orange), biotin-KCS36 (green), biotin-LLT44 (blue) and biotin-CQG43 (yellow). Mouse serum before immunisation was used as a negative control and all samples were tested for cross reactivity.

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	M (0)											
B	M (1)											
C	HAT											
D	HAT											
E	HAT											
F	HAT											
G	HAT											
H	HAT											

Figure A.5: One example of an ELISA setup for testing hybridoma medium for screen 1 after fusion on a streptavidin plate. The plate was coated with biotin-GPG32 and biotin-CQG43 respectively when testing the different hybridoma. Mouse serum was used as positive control and HAT-medium as negative control.

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	Mouse GPG32 (0)	CARM1-10	Mouse CQG43 (0)	CARM1-20	Mouse CQG43 (0)	CARM1-20	Mouse GPG32 (0)	CARM1-10				
B	HT	CARM1-11	HT	CARM1-21	HT	CARM1-21	HT	CARM1-11				
C	CARM1-1	HT	CARM1-12	CARM1-22	CARM1-12	CARM1-22	CARM1-1	HT				
D	CARM1-2	HT	CARM1-13	CARM1-23	CARM1-13	CARM1-23	CARM1-2	HT				
E	CARM1-3	HT	CARM1-14	CARM1-24	CARM1-14	CARM1-24	CARM1-3	HT				
F	CARM1-4	HT	CARM1-16	CARM1-25	CARM1-16	CARM1-25	CARM1-4	HT				
G	CARM1-5	HT	CARM1-17	CARM1-26	CARM1-17	CARM1-26	CARM1-5	HT				
H	CARM1-8	HT	CARM1-18	CARM1-27	CARM1-18	CARM1-27	CARM1-8	HT				

Figure A.6: The ELISA setup for testing the hybridoma medium of hybridomas positive in screen 1 for screen 2 after fusion on a streptavidin plate. The plate was coated with biotin-GPG32 (blue) and biotin-CQG43 (green) when testing the different hybridoma. Mouse serum was used as positive control and HT-medium as negative control. The samples are tested for cross reactivity.

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	CARM1 mAb mouse (1:2000)	CARM1-20	CARM1 mAb mouse (1:2000)	CARM1-20								
B	Caseinbuffer	CARM1-21	Caseinbuffer	CARM1-21								
C	CARM1-12	CARM1-22	CARM1-12	CARM1-22								
D	CARM1-13	CARM1-23	CARM1-13	CARM1-23								
E	CARM1-14	CARM1-24	CARM1-14	CARM1-24								
F	CARM1-16	CARM1-25	CARM1-16	CARM1-25								
G	CARM1-17	CARM1-26	CARM1-17	CARM1-26								
H	CARM1-18	CARM1-27	CARM1-18	CARM1-27								

Figure A.7: The ELISA setup for testing the hybridoma medium of hybridomas positive in screen 1 for screen 2 after fusion on a glutathione plate. The plate was coated with unpurified lysate containing C-terminal GST-fusion protein, diluted 1:25. CARM1 mouse mAb was used as positive control and casein buffer as negative control.

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	IgG1	CARM1-5	CARM1-21	IgG1	CARM1-5	CARM1-21	IgG1	CARM1-5	CARM1-21	IgG1	CARM1-5	CARM1-21
B	IgG2a	CARM1-8	CARM1-23	IgG2a	CARM1-8	CARM1-23	IgG2a	CARM1-8	CARM1-23	IgG2a	CARM1-8	CARM1-23
C	IgG2b	CARM1-10	CARM1-24	IgG2b	CARM1-10	CARM1-24	IgG2b	CARM1-10	CARM1-24	IgG2b	CARM1-10	CARM1-24
D	IgG3	CARM1-11	CARM1-25	IgG3	CARM1-11	CARM1-25	IgG3	CARM1-11	CARM1-25	IgG3	CARM1-11	CARM1-25
E	IgM	CARM1-12	CARM1-26	IgM	CARM1-12	CARM1-26	IgM	CARM1-12	CARM1-26	IgM	CARM1-12	CARM1-26
F	CARM1-1	CARM1-14	CARM1-27	CARM1-1	CARM1-14	CARM1-27	CARM1-1	CARM1-14	CARM1-27	CARM1-1	CARM1-14	CARM1-27
G	CARM1-3	CARM1-18	1%BSAPBS	CARM1-3	CARM1-18	1%BSAPBS	CARM1-3	CARM1-18	1%BSAPBS	CARM1-3	CARM1-18	1%BSAPBS
H	CARM1-4	CARM1-20	1%BSAPBS	CARM1-4	CARM1-20	1%BSAPBS	CARM1-4	CARM1-20	1%BSAPBS	CARM1-4	CARM1-20	1%BSAPBS

Strip	1	2	3	4	5	6	7	8	9	10	11	12
A	IgG1	CARM1-5	CARM1-21									
B	IgG2a	CARM1-8	CARM1-23									
C	IgG2b	CARM1-10	CARM1-24									
D	IgG3	CARM1-11	CARM1-25									
E	IgM	CARM1-12	CARM1-26									
F	CARM1-1	CARM1-14	CARM1-27									
G	CARM1-3	CARM1-18	1%BSAPBS									
H	CARM1-4	CARM1-20	1%BSAPBS									

Figure A.8: The ELISA setup for testing the isotype of the antibodies produced by hybridoma. The secondary antibodies are IgG1-HRP (blue), IgG2a-HRP (yellow), IgG2b-HRP (green), IgG3-HRP (orange) and IgM-HRP (grey).

DEPARTMENT OF BIOLOGY AND BIOLOGICAL ENGINEERING
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden
www.chalmers.se

CHALMERS
UNIVERSITY OF TECHNOLOGY