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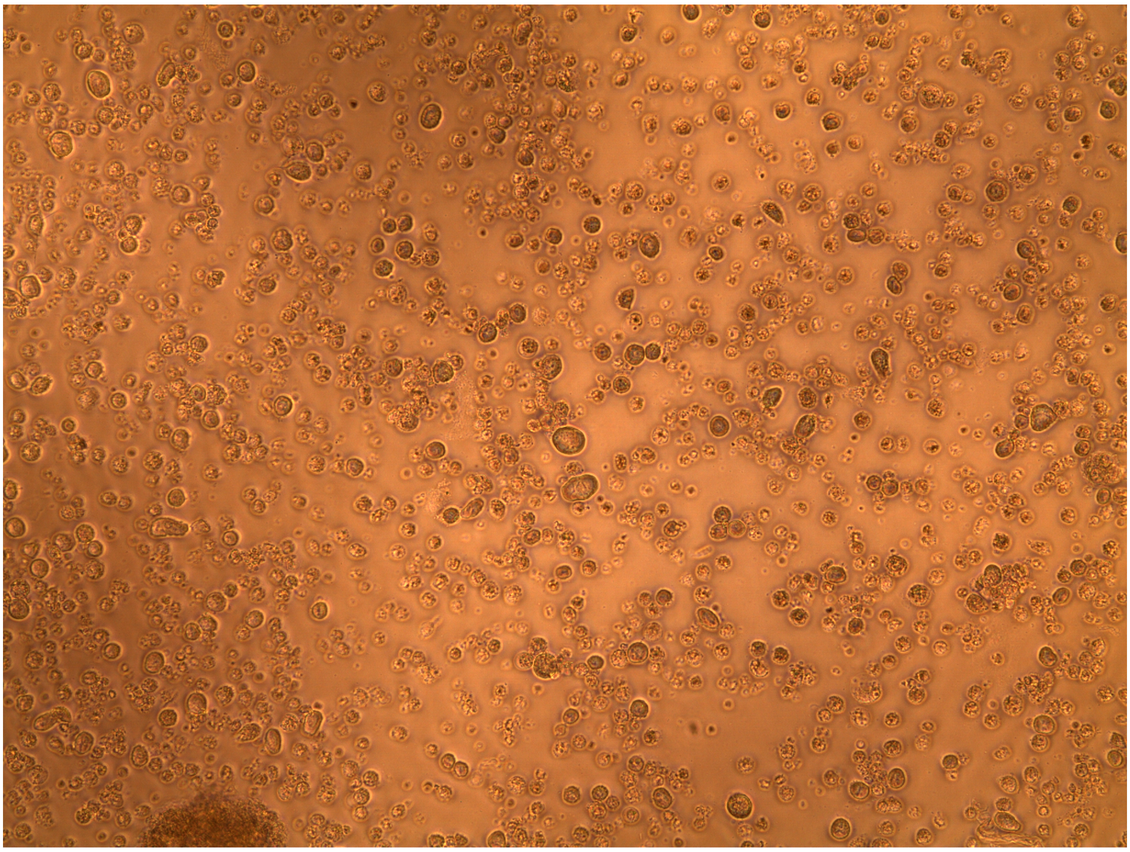

Development of Antibodies

Establishment and Characterisation of New Immunoreagents for Diagnosis of Neurodegenerative Disease

Master's thesis in Biotechnology

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CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2022
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Cover: Image of hybridoma cells 3 days after fusion, taken at 20x zoom with EVOS XL Base Miscroscopy System.

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Abstract

Neurodegenerative diseases are a collection of disorders which can develop any time in adulthood, but with the majority occurring in the elderly population. A common denominator for these diseases is the gradual degradation of neurons in the central nervous system, often taking place long before the manifestation of clinical symptoms. One of the proteins taking part in this process is called neurofilament light, which upon degradation can be found both in the cerebrospinal fluid as well as the blood.

In vivo diagnostic tools utilising antibodies have been developed for the detection of NF-L in the CSF, allowing for earlier diagnosis. However, for screening to be able to take place at local healthcare centers, a diagnostic test for NF-L in blood would be more beneficial. Since the concentration of the antigen is much lower in the blood compared to the CSF, antibodies with high specificity and sensitivity are necessary.

The aim of this project was to establish high affinity monoclonal antibodies against the protein neurofilament light, specifically when present in plasma, using classic hybridoma technology. Mice were immunised with full length bovine NF-L as well as two shorter custom made peptides, originating from the Coil-2 region of NF-L, having shown to be a valuable epitope sight for clinically effective antibodies. Over 20 hybridomas producing relevant antibodies were established, detected through ELISA screens. All succesfull hybridomas originated from the mice immunised with peptides. The majority of the established antibodies had the desired isotypes and a few had a strong response towards full length bovine NF-L, indicating clinical relevance. To conclude, a number of antibodies with the potential of being used in a sandwich ELISA diagnostic test for the detection of NF-L in blood have been established. These antibodies were proven to recognise the native form of bovine NF-L, epitope mapped to the clinically relevant Coil 2 region and were not reactive to other proteins present in clinical samples of CSF or serum.

This project was performed at Fujirebio AB located in Gothenburg, Sweden.

Keywords: Neurofilament light, biomarker, monoclonal antibodies, hybridoma technology, ELISA.

Acknowledgements

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Matilda Eriksson, Gothenburg, June 2 2022

Contents

1

Introduction

Neurodegenerative diseases are a vast collection of disorders having the common denominator of gradual degeneration of neurons in the central nervous system (CNS), resulting in disability and ultimately death [1] [2]. Some develop earlier in life such as multiple sclerosis (MS) and amythrophic lateral sclerosis (ALS), but many have age as the primary risk factor with the incidence increasing rapidly over the age of 60. Examples of such neurodegenerative diseases are Alzheimer's and Parkinson's disease, which are becoming more prevalent due to the growing elderly population [1]. As of today there is no cure, but it has been shown that the degradation of neurons can take place years before the manifestation of clinical symptoms and early detection and diagnosis enables medical intervention, slowing their progression [1] [3]. There is therefore a great need for clinical diagnostic tests, which could improve the treatment of patients tremendously both through diagnosis but also monitoring of the disease to optimise the treatment [4].

A biomarker of great interest in neurodegenerative diseases is neurofilament light (NF-L) [2]. It is the main constituent of neurofilaments, which are the major cytoskeletal components in the neurons, and gets it name from having the smallest molecular weight of the three neurofilament chains [1] [5]. When axonal damage occurs in the CNS, the concentration of NF-L greatly increases both in the cerebrospinal fluid (CSF) as well as the blood, explaining its value as a biomarker [6]. Many diagnostic tests have been developed for the detection of NF-L in CSF, but due to lumbar puncture being an invasive method it has not been extensively applied. Diagnostic tests for NF-L in blood would therefore allow for more extensive screening [2] [6]. The concentration of NF-L is however 40-fold lower in blood compared to CSF, making it a challenge to create diagnostic tests [6] [7].

One of the most widely used diagnostic tool in healthcare is ELISA, due to it being user friendly, inexpensive and highly sensitive. It is an immunoassay utilising antibodies who only bind to specific molecules, with the antibodies often being monoclonal. In order to create an ELISA that could detect NF-L in blood, antibodies with high specificity and affinity has to be developed. If succeeding in establishing such an ELISA, screening of neurodegenerative diseases at local healthcare centres could become a reality.

Fujirebio Diagnostics AB

Fujirebio Diagnostics AB (FDAB) is a company specialized in *in vitro* diagnostic products mainly targeted at cancer biomarkers. They started as a research project at the University of Gothenburg in the 1980s which later became the company CanAg. They established themselves as a highly respected biotechnology company with their antibodies often being regarded as the golden standard. In 2006 they where bought by the American company Fujirebio diagnostic Inc and joined the Fujirebio corporate group. Today there are multiple divisions over the world, such as the head office in Malvern, Pennsylvania and a division in the Netherlands. Although the main focus remains on cancer, FDAB has ongoing research projects targeting other fields of disease as well.

1.1 Aim

The aim of this project is to establish clinically relevant high affinity monoclonal antibodies against the protein neurofilament light. This is a research investment by Fujirebio Diagnostics AB, due to NF-L being of high interest as a biomarker for neurodegenerative diseases.

The ultimate goal is to establish a sandwich ELISA to be used as an *in vitro* diagnostic test for neurofilament light present in blood. This would result in more easily accessible diagnostic tools for detection and proper treatment of neurodegenerative diseases.

1.2 Limitations

Due to time limitations, the possibilities of tasks included in this project are restricted. The mice had been immunized for three months before the start of the project to attain proper immune response before fusion. Additionally, all handling of mice is carried out by authorised personnel at Experimental Biomedicine, core facility at the Universtity of Gothenburg. Remaining laboratory work will be performed at Fujirebio Diagnostics AB, using protocols, methods and material available there. Only antibodies responsive to NF-L will be evaluated.

1.3 Previous work

This is a continuation of a research project started in the spring of 2020 at Fujirebio Diagnostics AB. In this first project, mice were immunised with bovine NF-L, resulting in high affinity monoclonal antibodies towards bovine NF-L. These antibodies did however not recognise NF-L in humane clinical samples and could therefore not be utilised in diagnostic tests. This master thesis project is based on these previous results and continues with conclusions based on them.

1.4 Ethical Aspects

All animal experiments in this study were performed in accordance with the Animal Welfare Ordinance and the Animal Welfare Act of Sweden. It has approval number 5.8.18-19152/2019 issued by an ethical committee.

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Theory

The aim of this project was to establish monoclonal antibodies which can be used in an ELISA assay for detection of neurofilament light in blood. This chapter will therefore introduce NF-L and antibodies in more detail. The methods used both for production and characterisation of antibodies will also be described, starting with hybridoma technology, followed by ELISA and Western blot.

2.1 Neurofilament light

Neurofilaments are the main cytoskeletal components of neurons, found especially in axons where they are essential for the radial growth and the rapid transmission of signals. They consist of four subunits called neurofilament heavy, neurofilament medium, neurofilament light and alpha-internexin, when in the central nervous system. Neurofilament light (NF-L) is the main constituent in neurofilaments and is of great interest as a biomarker for neurodegenerative diseases due to its concentration in CSF and blood increasing rapidly following axonal injury [8] [1] [6].

The humane neurofilament light, presented in Figure 2.1, is 543 amino acids (aa) long with a molecular weight of 68 kDa. It can be divided into multiple regions, such as the Coil-1, Coil-2 and the Tail region [9]. A recent study by Budelier et al. examined NF-L present in CSF, characterising the different forms present at Alzheimer's disease. They found that there are multiple truncated species of NF-L present in CSF, with the fragment GMNEALEK (amino acids 324-331) being of extra interest. This fragment originating from the Coil-2 region, spanning between amino acid 253 to 396, has a strong correlation to the total NF-L concentration present in a sample, making it a promising target for biomarker assays [10] [11] [9]. This correlated with in-house knowledge, which found that clinically relevant antibodies have epitopes in the Coil-2 region. There is however also a cross reactive region between aa 360-380, which correlates with sequences in other neurofilaments. The anterior blue alpha helix seen in Figure 2.1 makes up the majority of the Coil-2 region in human NF-L. Neurofilament light is a protein which is highly conserved between species and bovine NF-L is therefor often used as an antigen instead of human, due to their strong resemblance [12] [13].

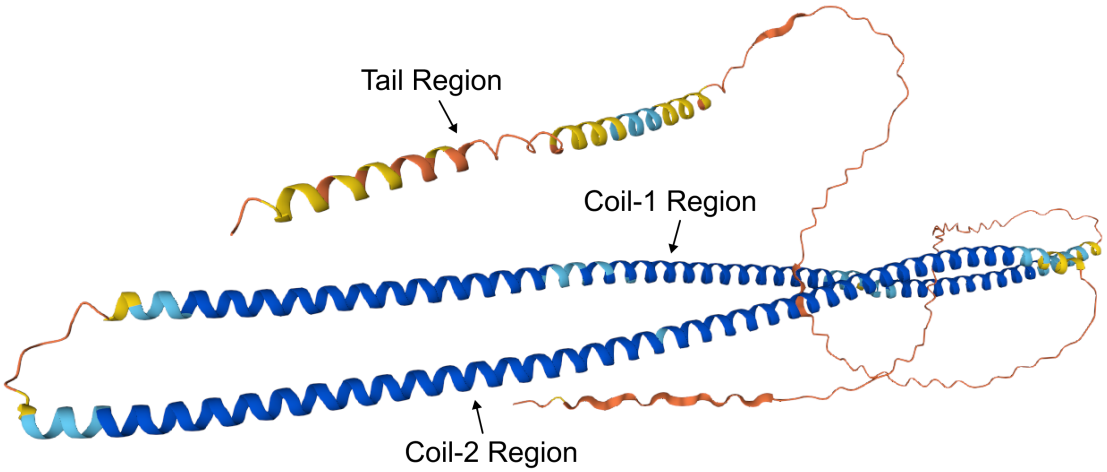


Figure 2.1: A 3D model of humane neurofilament light. The colours represent model confidence with blue being the highest and orange the lowest. The three main regions, namely Coil-1, Coil-2 and Tail, are indicated. Image retrieved from UniProt.com [9].

The concentration of NF-L present in both CSF and plasma is heavily dependent on age, as can be seen in Table 2.1. In disease, the level varies depending on the neurodegenerative disorder with dementia causing the highest levels. The determined concentration is also dependent on the method used to measure it and the chosen internal standard [14].

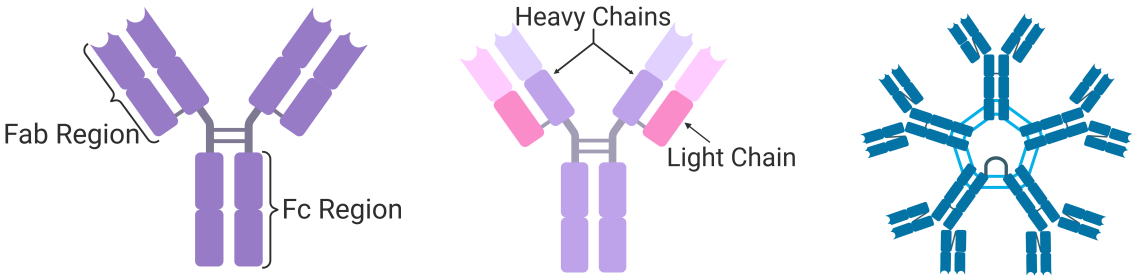
Age	**Normal Levels (ng/mL)**	
	CSF	**Plasma**
18-50	< 0.4	< 0.010
51-60	< 0.5	< 0.015
61-69	< 0.6	< 0.020
> 70	< 2.5	< 0.035

Table 2.1: Approximate levels of NF-L found in CSF and plasma in healthy individuals dependent on age. Data retrieved from Page et al. and Bridel et al. [7][14]

2.2 Antibodies

Antibodies, also called immunoglobulins, are proteins produced by B lymphocytes which help protect the body against pathogens and other foreign materials [15]. Their main function is to bind to molecules, called antigens, aiding other immune cells in identification and elimination of these foreign substances. Antibodies are Y-shaped proteins, consisting of two structural units. The tips of the Y are called Fragment antigen binding (Fab) regions due to their role of binding to the antigen, and they are identical. The third is called the Fragment crystalline (Fc) region and generates the response of other immune cells [16]. These regions are made up of polypeptide chains with varying molecular weight. The Fab regions consists of both

heavy and light chains whereas the Fc regions only consists of heavy chains. The three regions are bound together through disulfide bridges in a region called "the hinge". Schematic depictions of antibodies can be seen in Figure 2.2 a) and b).



(a) IgG Antibody 1 **(b)** IgG Antibody 2 **(c)** IgM Pentamer

Figure 2.2: Structure of IgG antibodies. a) A simplified image of an IgG antibody, depicting the Y-shape and the different regions. b) An image of an IgG1 antibody with the light chains (pink) and heavy chains (lilac) highlighted. c) IgM monomers organised into a pentamer with exposed Fab regions. All images created with Biorender.

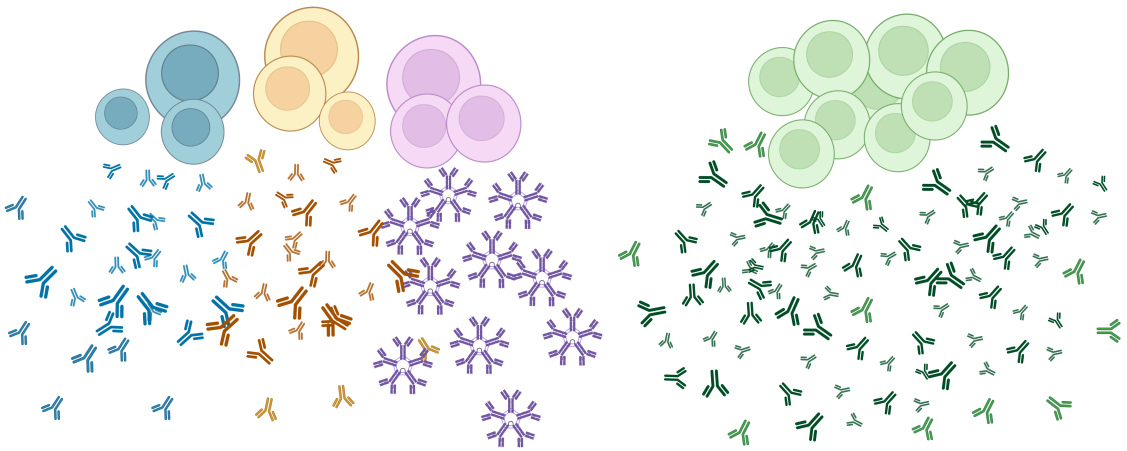
An antibody can only bind to a specific antigen, and only to a certain region called epitope, typically spanning between 5 to 8 amino acids in proteins [17][18]. Many different immunoglobulins can therefore bind to the same antigen but at different sites, something which can be utilised in diagnostic tests, see subsection 2.4. The strength by which an antibody binds to an antigen is called affinity whereas the strength of many affinities are called avidity. An antibody can therefore have a low affinity but high avidity if appearing in polymeric form, for example like IgM which will be discussed later on. Another important characteristic of an antibody is its specificity, meaning its capability to bind to the right antigen. The specificity relies heavily on the epitope of the antibody, since resemblance of the epitope to an amino acid sequence in another protein can lead to cross-reactivity. Specificity is not to be confused with sensitivity, meaning the lowest concentration of antigen the antibody can detect.

The production of antibodies is, as previously mentioned, performed by B lymphocytes [19]. When an immunogen enters the body it will eventually through different mechanisms end up in a secondary lymphoid organ, such as the spleen. There it will be degraded by a phagocytic cell, such as a dendrite or a macrophage. These are also called antigen presenting cells (APCs) since they process and then present certain parts of the antigen for T-cells, activating them. The T-cells will then secrete cytokines, activating the B-cells to produce antibodies binding to the antigens [19]. There will therefore be an abundance of antibodies binding to the antigen, at different sites and with different success at triggering an immune response [20]. If exposed a second time to the same antigen, a secondary immune response will oc-

cur. This response is both quicker and more extensive, due to the number of specific lymphocytes increasing in the secondary lymphoid organs by each exposure [19].

2.2.1 Antibodies derived from animals

The antibodies used for diagnostics are most often produced using the hybridoma technology, see section 2.3. They are raised by immunising animals with an antigen and harvesting their B-cells. The derived antibodies are called polyclonal antibodies (pAbs) and can often detect the antigen at varying conformations and orientations. This is due to them originating from many different B-cells, all having their own epitope and specificity [21]. By cloning one of these B-cells, monoclonal antibodies (mAbs) are created. These are only able to bind to one epitope since they are derived from clones all stemming from the same B cell, making them identical. These are the antibodies often used for diagnostic tests due to them being alike, leading to only small batch variations. mAbs are also favorable in diagnostics due to them having high sensitivity, specificity and affinity for their single epitopes [21]. An image depicting the difference between polyclonal and monoclonal antibodies can be seen in Figure 2.3.



(a) Polyclonal Antibodies **(b)** Monoclonal Antibodies

Figure 2.3: a) Polyclonal antibodies of different isotype and origin B cell b) Monoclonal antibodies all derived from the same B cell or clones from it, making them identical. Both images created with Biorender

2.2.2 Isotypes

Antibodies are categorised both according to their structure as well as components. The five main classes of immunoglobulins (Ig) in mammalians are IgA, IgD, IgE, IgG and IgM, with the primary difference being the structure of their Fc region [16]. They also appear in different stages of polymerisation, with IgG and IgE often being monomers, IgA differing between monomeric or dimeric form depending on environment and IgM often being a pentamer. These five classes can be further

categorised into subclasses, depending on the disulfide bridges in the hinge regions as well as which form of heavy chains they consist of [16].

IgM

When the immune system is subjected to an immunogen, IgM will be the first antibody isotype produced by the B-cells. These are vital for many functions of the immune system and plays a crucial role in the first few days of an infection. An immune response can therefore be said to be naive if the main type of antibody produced is IgM [15].

When being freely dispersed and not membrane bound, IgM is most often found in pentameric form. This means that five monomers assemble themselves in a circle formation, with the Fab regions facing outwards, see Figure 2.2. IgM typically has a low affinity towards antigens but has high avidity in pentameric form, due to the ten available binding sites. IgMs are therefore often deemed "sticky" since their low affinity but high avidity lead to unspecific binding [15].

IgG

IgG is the smallest and most common out of all antibody classes, often comprising 70-80% of the total antibodies. It is part of the secondary immune response, indicating a more matured immunity [19]. IgG consist of four different chains, two light and two heavy, appearing as a monomer in mammalians. In mice, it can be categorised into four subclasses termed IgG1, IgG2, IgG3 and IgG4 depending on form of heavy chain present in the antibody [16]. As previosly mentioned, immunglobulins are also categorised according to their hinge region and its difulfide bridges. Due to this the subclasses can be divided further, for example into IgG2a and IgG2b [22].

2.3 Production of Antibodies

Antibodies have been an important biomedical research tool for many years, both within diagnostics as well as in therapeutic treatments [21]. It started in 1975 when Köhler and Milstein developed the hybridoma technology, allowing large scale production of mAbs [23]. Since then, many new techniques have emerged, mostly relying on recombinant production. Additional to not requiring animal studies, these recombinant techniques can be of more advantage when trying to raise antibodies against an antigen which is not immunogenic or lack 3D conformation. The antigen will also not be degraded in any way, avoiding the antibody to not recognise the antigen in its' native form [24]. Despite this, hybridoma technology is still the most favoured for producing monoclonal antibodies, especially if they are to be used for analytical purposes. It is a primitive and robust process, often being more successful in the establishment of new antibodies compared to other techniques. Due to the antibodies being produced by naturally matured B-cells, they also attain high affinity and the natural combination of light and heavy chains [21].

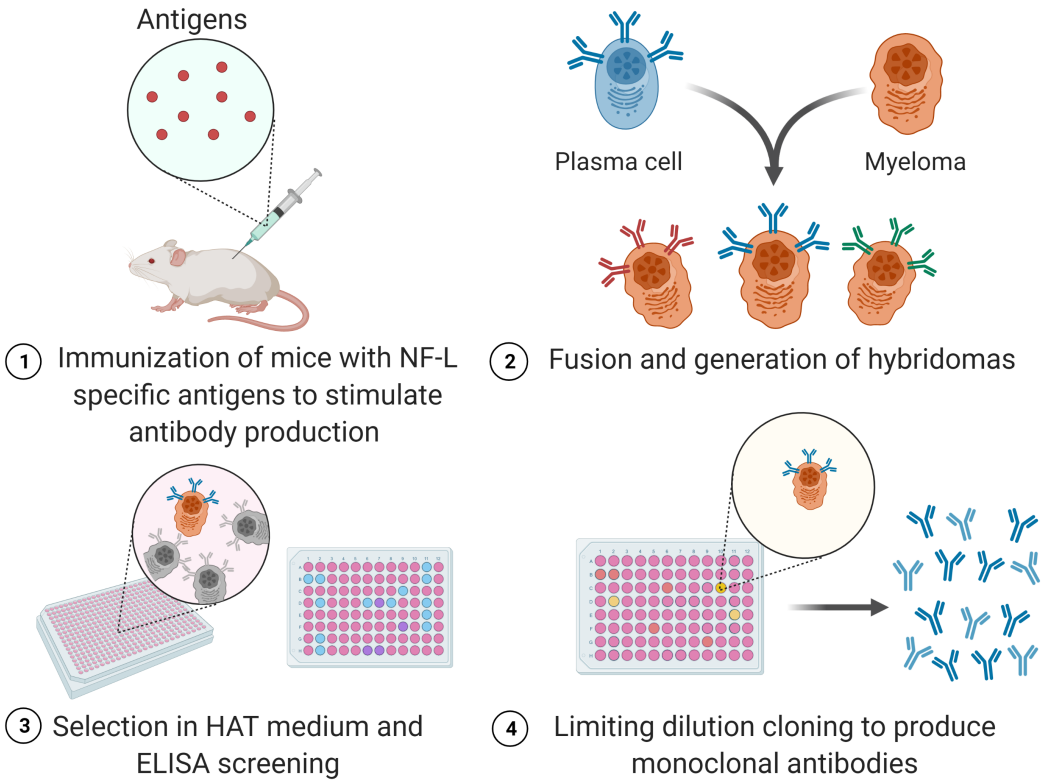


Figure 2.4: Schematic representation of hybridoma technology, specific for this project.

2.3.1 Hybridoma Technology

The hybridoma technology follows a set of predetermined steps where the animals and cells used may differ. A schematic representation of the hybridoma technology can be seen in Figure 2.4.

Immunisation

The first step in hybridoma technology is immunisation [25]. Many different animals can be used, such as rat or chicken, but the most common is mouse due to the mAbs then being more specific and easier to produce [21]. Mouse was therefore also used in this project and the hybridoma technology will from here on be presented as it is performed when using mice.

The animal is injected with the antigen multiple times over several weeks, maturing the immune response. An adjuvant is included in the injection, which is needed to increase the immune response towards the antigen [21] [26]. For proper immune response towards shorter antigens such as peptides, they are conjugated with additional tags, see section 2.3.2.

Fusion

The second step is fusion, which is performed when a proper immune response has been established. The matured B-cells, called plasma cells in this stage, are harvested from the mouse by extracting the spleen and straining it. These are then fused together with metastatic plasma cells from a type of blood cancer, called myeloma cells [21]. The two cell types are fused with the help of polyethylene glycol (PEG), which enables the plasma membranes to merge, creating a cell with two nuclei [25]. The myeloma cells are altered so that they can not express the gene for the enzyme HGPRT while B-cells do not divide in cultivation. By cultivating them in hypoxanthine-aminopterin-thymidine (HAT) medium, a selective pressure is created where only fused cells can survive since they have the functional HGPRT gene from the B-cell but lso can divide indefinitely as the myeloma cells. The successfully merged cells are called hybridomas [25].

Cloning

Lastly, there is cloning. The attained hybridomas are screened to find those producing the right antibodies, more details can be found in Section 2.4. Once the successful hybridomas have been distinguished, they are cloned using the limiting dilution technique. This means that they are sparsely cultured and examined to confirm the presence of only one hybridoma in each well, producing mAbs.

2.3.2 Antigens for immunisation

Antigens are molecules that invoke an immune response in the body through the binding of antibodies [16]. The choice of immunogens is important when performing hybridoma technology, since they determine the immune response as well as the epitopes of the raised antibodies. If an antigen has high immunogenicity, a smaller amount is needed in order to evoke an immune response and the affinity of the antibody/epitope interaction will be greater [27]. In order for an antigen to be immunogenic it has to possess a set of characteristics. It has to be foreign, have a relatively high molecular weight, be degradable and have a certain grade of chemical complexity. This means not being native to the host, not be too small and be degradable by APCs. It also needs to have a conformation, the more complex the better [19]. The conformation of the antigen is also of importance for determining type of epitope the antibodies will bind to. The two main types of epitopes are linear or conformational. The linear eptiopes bind to the primary sequence of an antigen, meaning its' amino acid sequence. The antibody can still be able to recognise the antigen in its native structure, but there is a risk of it only detecting it in denatured state. Immunising with denatured proteins or short peptide fragments will therefore only result in antibodies with linear epitopes. Antibodies with conformational epitopes can on the other hand only recognise proteins when they are properly folded and would not recognise a primary structure [28]. Antibodies binding to linear epitopes are often used as detector proteins or in Western blots. Capture antibodies need to be able to bind to a proteins native structure, meaning that it can either have a linear or conformational epitope [29].

Antigens can either be whole proteins or shorter peptides sequences, depending on desired epitope regions. When using peptides, it has to be conjugated with a carrier molecule to elicit a sufficient immune response. One of the most common and effective carrier molecule is keyhole limpet hemocyanin (KLH), which is a large protein containing copper that stimulates a strong immune response [30]. If using the same peptide for both immunisation and screening, it is however important to use two different tags to avoid isolation of antibodies with reactivity towards the carrier [29].

2.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay used routinely all over the world, both in research and diagnostics. It is considered the gold standard among immunoassays, having high sensitivity while inexpensive [31]. ELISA is widely used within diagnostics, successfully detecting many different molecules related to diseases [32]. It is therefore commonly used in the healthcare system, playing an important role in patient diagnosis and treatment planning. Due to the relatively simple method, there is no need for additional training in order for usage, making it an easily accessible diagnostic tool [31].

The data from an ELISA can either be quantitative, semiquantitative or qualitative depending on the purpose. The quantitative gives exact measurements through the use of standard curves, the qualitative can only say if the analyte is present in the sample and the semiquantitative compares the relative amount between samples.

ELISA is performed in polystyrene plates, typically 96-wells, and there are four major types of ELISAs, called direct ELISA (dELISA), indirect ELISA (iELISA), sandwich ELISA and competitive ELISA [31]. The indirect ELISA and sandwich ELISA will be presented in more detail, and a schematic representation can be seen in Figure 2.5.

Indirect ELISA

Indirect ELISAs are used for the detection of primary antibodies that can bind the antigen of interest. The first step is therefore to coat the wells with the antigen. A primary antibody is then added, followed by a wash step removing all unbound antibodies. A secondary antibody is added which binds to the primary. This secondary antibody is conjugated with an enzyme, for example Horseradish Peroxidase (HRP). After another wash step, a chromophore acting as a substrate to the enzyme is added, in the case of HRP being 3,3'5,5'-Tetramethylbenzidine (TMB). When the substrate is degraded by the enzyme, a blue colour is generated which can be measured using an absorbance reader. The absorbance is proportional to concentration of primary antibodies bound in the well. Although being more time consuming then direct ELISA, iELISA is both more sensitive and less expensive. A problem that can occur in indirect ELISA is cross reactivity taking place between the secondary

antibodies [31]. There is also a concern when used for screening of hybridomas that the majority of selected antibodies will have linear epitopes, due to conformational change of the antigen when immobilised. This is however not true for all antigens, stressing the importance of knowing the characteristics of chosen protein [29]

Sandwich ELISA

In the sandwich ELISA, the wells are coated with capture antibodies. The antigen is added, for example through the addition of a clinical sample if used in diagnostic tests. The plate is washed to remove any unbound antigen and then a detector antibody is added. After another wash, a secondary antibody with a conjugated enzyme is added. A chromophore substrate is added lastly, resulting in a colour change if antigen was present in the sample [31].

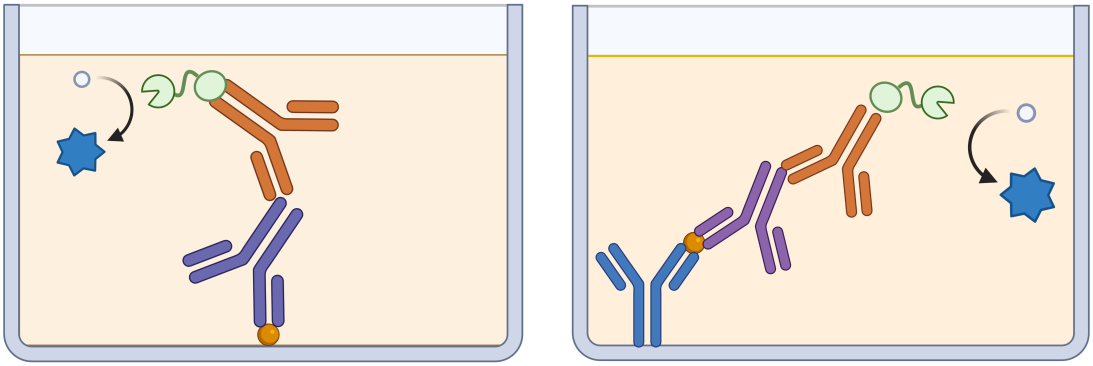
The sandwich ELISA is the most sensitive out of all ELISA designs, making it possible to detect minute concentrations of analytes in a solution. The major disadvantage with this ELISA is that it requires a matched pair of antibodies, having separate epitope sites on the antigen allowing simultaneous binding [31]. The capture antibody must also recognise the antigen when in native conformation [29].

Buffers

An important part of ELISA is the addition of buffers to prevent false positive results. The buffer is generally used to dilute antigens and antibodies to evade an additional blocking step. The buffer is often composed of phosphate-buffered saline (PBS) and an additional blocking agent such as bovine serum albumin (BSA). The BSA is a protein solution which block any sites in the well that might be unbound, preventing unspecific binding [31].

Biotinylation

The sensitivity of an ELISA can be improved by increasing the amount of immobilised antibodies or antigens on the plate. An effective way of increasing the binding to the well is through the use of streptavidin-biotin technology. When using this technology in iELISA, the plate is coated with streptavidin and the antibody/antigen is conjugated with biotin, allowing high affinity binding and immobilisation [32]. Biotin, also called vitamin H, is a molecule which can interact with the protein streptavidine and the binding is in fact one of the strongest and most specific among biological molecules. This has been utilised by researchers for many years, by tagging molecules through the interaction of biotin with an amine on the targets. When biotinylating antigens, a linker is often used to prevent steric hindrance [33]. A concern with biotinylation is that a too high biotin to antigen ratio can lead to altered protein conformation and the masking of epitopes. If on the other hand too low, there will be no efficient screening [34].



(a) Indirect ELISA **(b)** Sandwich ELISA

Figure 2.5: Schematic representation of two different ELISA setups. **a)** Indirect ELISA: The well is coated with immobilised antigen (orange sphere), which the primary antibody (purple) binds to. The primary antibody may for example be obtained from a clinical specimen, such as blood. A secondary enzyme conjugated antibody (orange) then binds to the primary antibody (purple). At the addition of a substrate (white sphere), the enzyme conjugated to the secondary antibody (green) catalyses a reaction leading to a colour shift (blue star). **b)** Sandwich ELISA: The well is coated with immobilised capture antibodies (blue), which bind to the antigen (orange sphere) dispersed in a sample. A detector antibody (purple) then binds to the antigen simultaneously at another epitope site. An enzyme conjugated antibody (orange), binds to the detector antibody. At the addition of a substrate (white sphere), the enzyme conjugated to the secondary antibody (green) catalyses a reaction leading to a colour shift (blue star). Both images created with Biorender.

2.5 Western Blot

Western blot is an analytical technique combining electrophoretic protein separation with an immunoassay. It is used routinely within basic research, often for identification and characterisation of proteins in complex mixtures [35]. It is robust and dependable, but time consuming due to the many steps. The many steps also affects the reproducibility and reliability of the technique, with sample preparation and amount and source of primary antibody being of high importance. New techniques such as microchip electrophoresis, cell-resolution western blot and automated microfluid Western blotting among others have been developed in an attempt of improving the original Western blot [36].

The Western blot is divided in two main steps, the first being a classic gel electrophoresis. Here, the proteins are first reduced by the addition of an anionic detergent, for example sodium dodecyl sulphate (SDS), which denatures the proteins into their primary structure. The detergent also binds to them, resulting in a net charge of the proteins proportional to their molecular mass. The protein sample is then loaded onto a polyacrylamide gel and a voltage is applied. The protein solution will then migrate through the gel towards the positive cathode, separating the

proteins depending on their size. A protein standard is often loaded in one of the wells, allowing to determine the molecular weights of the analysed proteins [37].

The second part of Western blot is the immunoassay. The proteins are first transferred from the gel to a membrane, which is a labor intensive step if not automated. The membrane is then incubated in blocking agent, often consisting of 5%BSA or non-fat dried milk, to prevent unspecific binding of antibodies to the membrane. The primary antibodies are then added, all binding to linear epitopes as the protein is denatured before characterisation. After a wash step, the secondary enzyme conjugated antibodies are added, that upon addition of substrate allows imaging of the membrane.

3

Method

The method section describes all the performed experiments, from immunisation to characterisation of the attained antibodies. The order in which they are presented follows the chronological order in which they were performed during the project. All experiments containing animals had an ethical permit and were handled at the site of Experimental Biomedicine, an animal facility at the University of Gothenburg. The animals were only handled by trained personnel and no details on how the animals were kept or managed which are outside the relevance for this project will be described.

3.1 Antigens

A vital part when trying to raise antibodies are the antigens, both in immunisation and screening. Since the previous project did not raise clinically useful antibodies, see subsection 1.3, other antigens were considered. As previously mentioned, antibodies with epitopes lying within the Coil-2 region have been shown to be clinically applicable, see subsection 2.1, and the strategy was thus to establish antibodies targeting this region. Three peptide sequences lying within this region were therefore chosen as antigens, called KNT45, EAC41 and LQE45 where the name stems from the first three amino acids and the length of the peptide. The lengths were chosen in hopes of a conformation resembling that found in the native protein. A schematic picture of the three peptides and their arrangement in the full length protein can be seen in Figure 3.1.

The antigens chosen for immunisation were KNT45, EAC41 and full length bovine NF-L. The peptide LQE45 was not chosen to avoid cross reactivity, but was still used for epitope characterisation. All peptides were ordered from *Innovagen* as custom peptides. To be able to use them both in immunisation and screening, they were ordered with two different tags. For proper immunisation response, the peptides were conjugated with keyhole limpet hemocyanin (KLH) and for use in ELISA they were conjugated with biotin. The full length bovine NF-L was ordered from *OriGene* without any conjugates, see Section 3.4.1 for later modifications.

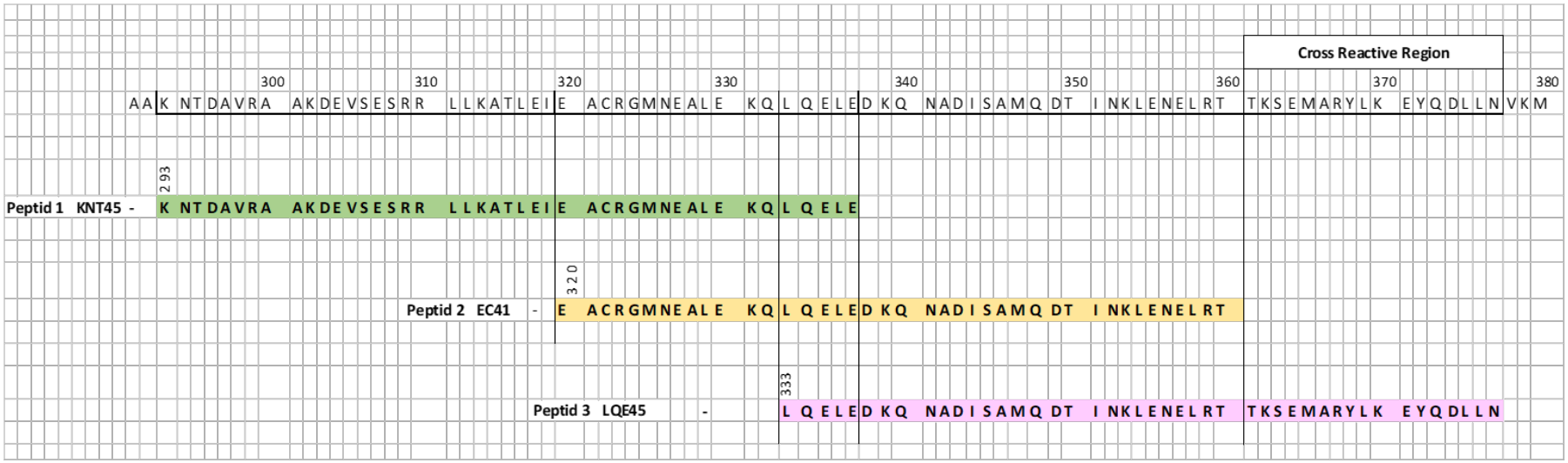


Figure 3.1: A part of the amino acid sequence in the Coil-2 region found in the protein bovine NF-L is listed at the top of the figure. The numbers show the location in the full length protein. The three peptides KNT45, EAC41 and LQE45 are highlighted in colour, where the letters represent the three first amino acids and the number the length of the peptide. These peptides are derived from bovine NF-L and their placement in the figure corresponds to their sequence placement in the original protein. As can be seen there is an overlap, with LQE45 overlapping both other peptides with more than 10 amino acids while KNT45 and LQE45 only overlap each other with 5 amino acids. The peptide LQE45 also span over a region which is not suitable as an epitope site, as it may lead to cross reactivity.

3.2 Immunisation and titer test

The project started with the immunisation of mice to attain an immune response and raise antibodies towards NF-L. In this section the exact method is described, as well as the screening of blood samples to evaluate the immune response.

3.2.1 Immunisation

The immunisation of mice commenced two months before the official start of this master thesis project to ensure mature immune responses were achieved, hopefully shifting the main antibody isotype from IgM to IgG. Three groups of mice, containing five mice each, were immunised. Group one was immunised with the full length bovine NF-L protein, while group 2 and 3 were immunised with the KLH conjugated peptides EAC41 and KNT45 respectively. The mice were immunised with approximately 4 week intervals, through intraperitoneal injection of a solution containing antigen and the *Sigma Adjuvant System*® with catalog number S6322. Blood samples were drawn in connection to each immunisation and the first sample was collected before the immunisation process started to serve as reference. The proceeding blood samples were collected 5-10 days after each new booster, and a final sample at euthanisation. A detailed immunisation schedule can be seen in Table 3.1.

3.2.2 Titer Test

To evaluate the production of desired antibodies in the mice, an indirect ELISA was performed using blood collected after boost 2, see Figure 2.5 a) for a schematic representation. Biotinylated forms of the peptides KNT45, EAC41 and LQE45 were used as antigens, see Section 3.1. First, the biotinylated ELISA plates were prewashed using the *Tecan HydroFlex™* Microplate Washer, which was also used for all proceeding wash steps. The antigens were added with a concentration of 1 µg/ml and incubated shaking at room temperature and then washed. The blood samples, diluted 1:400, and controls were added with 1%BSA/ PBS buffer as negative control. The positive control was a blood sample collected from a mouse immunised with bovine NF-L in the previously performed project known to have an immune response towards NF-L, also diluted 1:400. The incubation and wash step was repeated before HRP-conjugated Rabbit-anti-Mouse antibody, diluted 1:15 000 in 1%BSA/ PBS buffer, was added. After another incubation and wash, HRP substrate (TMB) was added and the plates incubated ten minutes before the absorbance was measured at 620 nm using the *ELx808™* Incubating Absorbance Plate Reader from Lonza.

Immunisation	Day	Antigen	Adjuvant
Group 1 : NF-L			
First Immunisation	0	25µg bovine NF-L in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Boost 1	22	25µg bovine NF-L in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Boost 2	63	25µg bovine NF-L in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Perfusion boost	105	25µg bovine NF-L in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Group 2 : EAC41			
First Immunisation	0	50µg peptide EAC41 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Boost 1	22	50µg peptide EAC41 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Boost 2	63	50µg peptide EAC41 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Perfusion boost	123	50µg peptide EAC41 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Group 3 : KNT45			
First Immunisation	0	50µg peptide KNT45 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Boost 1	22	50µg peptide KNT45 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Boost 2	63	50µg peptide KNT45 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322
Perfusion boost	123	50µg peptide KNT45 in 100 µL PBS	100 µL *Sigma Adjuvant System* S6322

Table 3.1: Immunisation schedule for three groups of mice, receiving a specific antigen each. The table lists the different immunisations, at what day they were given relative the first injection and the composition of the injection. The first group received the full length protein bovine NF-L, the second group the peptide EAC41 and the third group the peptide KNT45.

3.3 Fusion

In preparation of fusion, myeloma cells from the cell line P3x63Ag8.653 (ATCC: CRL-1580) were cultivated. Approximately two weeks before the first planned fusion, one vial of myeloma cells where thawed and vortexed using the *Jouan C41* from Thermo Scientific. The supernatant was discarded and the cell pellet resuspended in standard medium. To keep the cells healthy, they were passaged every few days and the *Cellometer K2* from Nexcelom was used to measure the cell density and viability at each passage.

Three fusions were performed, one for each group of mice. Fusion 1 was the group of mice immunised with full length bovine NF-L and Fusion 2 and 3 the groups of mice immunised with EAC41 and KNT45 respectively. For each fusion, two mice were chosen based on their immune response in the titer test after boost 2. At the morning of the fusion, the mice were euthanised and their spleens extracted and strained. The attained B-lymphoctyes were fused with the myeloma cells using PEG, essentially as described by de StGroth and Scheidegger in 1980 [38]. Vital for this process are temperatures and addition times, due to the newly formed myeloma cells being very sensitive. After fusion, the cells were washed in Iscove's medium and then suspended in HAT-medium. This cell suspension was added to 20 previously prepared 96-well plates, leaving the first row empty to facilitate upcoming screens. The plates were then incubated at 37°C in 8 % CO_2.

3.4 Screen and Epitope Mapping

After the fusion, the attained hybridomas were screened in order to find those, if any, producing the correct antibodies. Two iELISA screens were performed, the first to find successfully fused hybridomas in the 20 96-well plates that should be further cultivated, and the second to confirm that the cultivated hybridoma produced the correct antibodies. The second screen was also designed to give a rough estimation of the epitope sites of the antibodies. Streptavidine coated ELISA plates were used for all screens and the antigens therefore had to be biotinylated. The full length bovine NF-L therefore had to be tagged, as described in the first section.

3.4.1 Biotinylation of bovine NF-L

The purified bovine Neurofilament-Light attained from OriGene with catalogue number BA1012 had to be biotinylated before utilised in iELISA screens. The protein was first reconstituted in 200 µL Milli-Q water with a final volume of 250 µL and a concentration of 1 mg/mL. To facilitate the biotinylation, the 6M urea original buffer which the protein was suspended in was exchanged for a 2M urea buffer using the *PD Spintrap*™G-25 from Cytiva according to the included manual. The protein concentration was determined using the Thermo Scientific NanoDrop™2000C. The biotin, EZ-Link™NHS-PEG4-Biotin No-Weigh™format, was added to the protein solution with a 1:15 biotin to antigen ratio and incubated for 30 minutes. The buffer was then changed back to 6M urea, once again using the Spintrap™. The

final protein concentration was determined and the solution spiked with BSA for stabilisation.

3.4.2 Selection of Hybridomas

When the 20 hybridoma plates had been incubated for 7-10 days after fusion, a first screen was performed. For the hybridomas from mice immunised with bovine NF-L, a mixture of EAC41 and KNT45 was used. Hybridomas from Fusion 2 and 3 were screened against the same peptide as the mice they were stemming from were immunised with. Each hybridoma plate had a corresponding streptavidin coated ELISA plate, marked with the same number from 1 to 20, which the medium was transferred to. The plates were prewashed and the antigens were added with a total concentration of 1 µg/mL and incubated for one hour at room temperature in a humidified chamber. After incubation, the plates were washed before the hybridoma medium and controls were added. For negative control, HAT medium was used. For positive control, a blood sample taken at euthanisation of one of the two mice was used, diluted 1:400. After another incubation and wash step, HRP-conjugated Rabbit-anti-Mouse antibodies were added, diluted 1:15 000. The plates were incubated and washed again before the addition of HRP substrate (TMB). The absorbance was then measured at 620 nm using the *ELx808™* Incubating Absorbance Plate Reader from Lonza. The hybridomas with an absorbance distinctly higher than the background were given a serial number and further cultivated in 6-well plates in HT-medium.

Further, the overall success of the fusion was estimated by looking at attained hybridomas. This was performed after the first screen, approximately 10 days after fusion. Five plates were examined and all wells with growth were counted. The average percentual growth per plate was then calculated.

3.4.3 Second Screen and Epitope Mapping

When a hybridoma cultivated in a 6-well plate reached a confluency of 2/3, the hybridoma was frozen for preservation. The well was then left until the supernatant turned yellow, indicating a lowered pH due to cell death. The supernatant was collected and 1%MIT was added as a preservative. The tubes with hybridoma supernatant was stored in the fridge until all had been collected.

In screen 2 several antigens were used, namely full length bovine NF-L, KNT45 and EAC41 as well as an unrelated peptide. Fusion 2 and 3, immunised with peptides, were also screened against the LQE45 peptide. The plates were prewashed before the antigens were added with a concentration of 1 µg/mL and then incubated on a shake for one hour. The plates were washed before the hybridoma supernatants and controls were added, with the positive control being the blood samples taken at euthanisation and the negative control HT-medium. The positive control was diluted 1:600. After another incubation and wash, HRP-conjugated Rabbit-anti-Mouse antibodies were added, diluted 1:15 000. The plates were incubated and

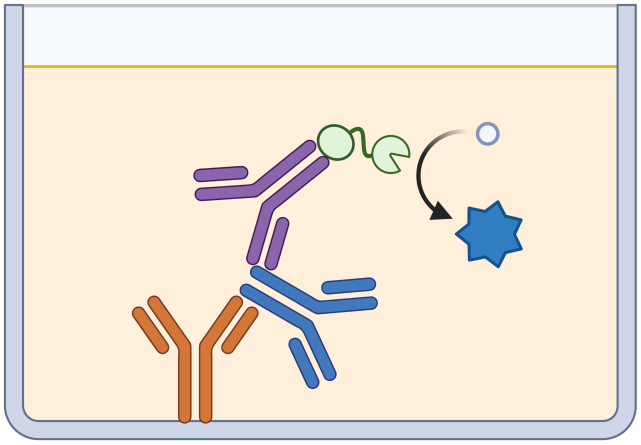


Figure 3.2: The ELISA setup of the isotype determination. The immobilised antibodies (orange) are the capture Goat-anti-Mouse antibodies, the primary antibodies (blue) are controls and sample antibodies while the secondary antibodies (purple) are the isotype specific HRP-conjugated antibodies.

washed again before the addition of HRP substrate (TMB). The absorbance was then measured at 620 nm.

3.5 Characterisation

The relevant antibodies which were detected in the screen of the hybridoma media were further characterised to evalutate their possible application in diagnostic tests in the future. The characterisation was performed using the methods described below.

3.5.1 Isotype Determination

The isotypes of antibodies present in the hybridoma supernatants were determined using an ELISA following the design described in Figure 3.2. The Goat-anti-Mouse coated plates were prewashed before the supernatants and controls were added. The positive controls were antibodies of known isotypes, added with a concentration of 1 µg/ml, while the negative control was 1%BSA/ PBS buffer. The hybridoma media was diluted 1:5 in 1%BSA/ PBS. The plates were incubated shaking for one hour and then washed before the addition of HRP-conjugated isotype specific antibodies. Incubation and wash was repeated before the addition of TMB. The absorbance was read at 620 nm.

3.5.2 Western Blot

Western blot was used both to see if the antibodies could bind non-conjugated antigen, recognise linear epitopes as well as how low concentrations they could detect. The same equipment was used for both experiments, being the Mini-PROTEAN®

for gel electrophoresis, the Trans-Blot Turbo transfer system for transfer to membrane and the ChemiDoc for imaging, all provided by BioRad. The same wash and blocking agents were also used, being 10% PBS and 0.2% Tween20 mixed with RO-water for wash and the same solution with the addition of dry milk powder for blocking.

Western blot with bovine NF-L

In this Western blot, all hybridoma supernatants were analysed. The Mini-Protean TGX strain-free gels, containing 15 wells, were loaded with the Precision Plus unstained protein standard as a ladder in the first and last well and 0.2 µg reduced bovine NF-L with the volume 10 µL in every other well between them. The gel electrophoresis was run and the proteins then transferred to PVDF membranes, all according to the BioRad manual. An image was taken of one of the gels before transfer. The membranes were cut in strips where each had one protein sample, which were then incubated in blocking agent for an hour on shake. The same procedure was repeated but with each strip being incubated in a hybridoma supernatant or blocking medium as negative control. The strips were washed 3x5min before being incubated in HRP-conjugated Rabbit-anti-Mouse antibodies on shake for on hour. The wash step was repeated before the strips were assembled and ECL™ detection reagent from Cytiva was added. All membranes were then photographed.

Detection Limit Characterisation

The hybridoma antibodies with promising results in both the ELISA screen and previously performed Western blot were chosen for a second Western blot to determine their detection limit. Eight Mini-Protean TGX strain-free gels, with 10 wells each, were loaded with the Precision Plus unstained protein standard as a ladder in the first well and then reduced bovine NF-L with a concentration from 100 to 0.01 ng in the following five wells, reducing it ten fold each time. The last two wells were loaded with humane samples of CSF and serum as a reference. Each well was loaded with a volume of 10 µL. The gel electrophoresis was run and the protein then transferred to PVDF membranes. All gels were incubated on shake in blocking agent for one hour which was then discarded before the addition of hybridoma supernatant to seven of them while the eight remained in blocking agent as a negative control. They were incubated for another hour and then washed 3x5 minutes before the addition of HRP-conjugated Rabbit-anti-Mouse antibodies. Incubation and wash step was repeated before the addition of ECL™ detection reagent and imaging.

4

Results

In this chapter, results from performed experiments are presented in chronological order. The establishment of antibodies are presented first and characterisations of them last.

4.1 Titer test

To evaluate the immune response of the immunised mice, their blood was screened in an ELISA. The results from the titer test after boost 2 are presented in the figures below. The absorbance of the blood samples taken from the mice in Group 1, immunised with bovine NF-L, can be seen in Figure 4.1. The reference was the blood sample taken before the start of immunisation, the negative control was buffer and the positive control blood from a mouse known to have an immune response towards NF-L. As can be seen in the graph, the mice seemed to have an even immune response between all three peptides and the absorbance was in the same magnitude as the positive control.

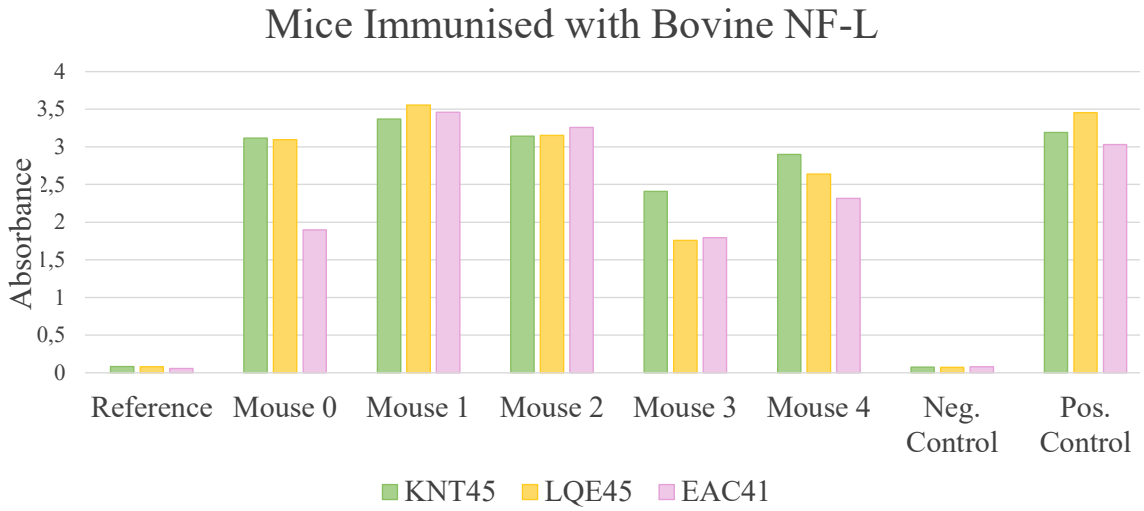


Figure 4.1: The absorbance detected in an indirect ELISA with the samples being blood collected from mice who had received booster two of bovine NF-L. A high absorbance indicates that a high concentration of antibodies or antibodies with high affinity are present in the sample. The reference was blood from one of the mice before the start of the immunisation. The negative control was buffer and the positive control blood from a mice known to have antibodies against bovine NF-L.

In Figure 4.2 the titer results of the mice immunised with the peptide EAC41 are presented. As can be seen, the mice in this group seemed to have a stronger immune response towards the peptide EAC41 relative to the other two peptides KNT45 and LQE45. Mouse 1 was found deceased and could not be sampled. Relative to the positive control, the mice had a more uneven response to the different peptides and a stronger response towards EAC41.

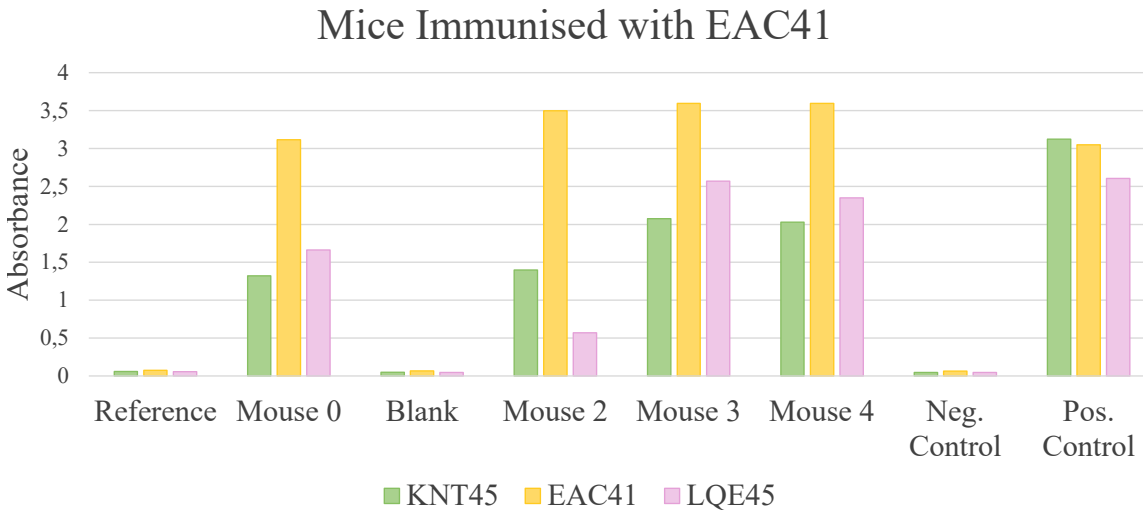


Figure 4.2: The absorbance of blood samples collected from mice who had received booster two of the peptide EAC41. A high absorbance indicates that a high concentration of antibodies or antibodies with high affinity are present in the sample. The reference was blood from one of the mice before the start of the immunisation. The negative control was buffer and the positive control blood from a mice known to have antibodies against bovine NF-L.

The immune response of mice immunised with the peptide KNT45 can be seen in Figure 4.3. These mice seemed to have a high response towards KNT45 which then decrease towards EAC41 and even more so towards LQE45. Relative to the positive control, the response towards KNT45 was higher and the response to EAC41 and LQE45 lower.

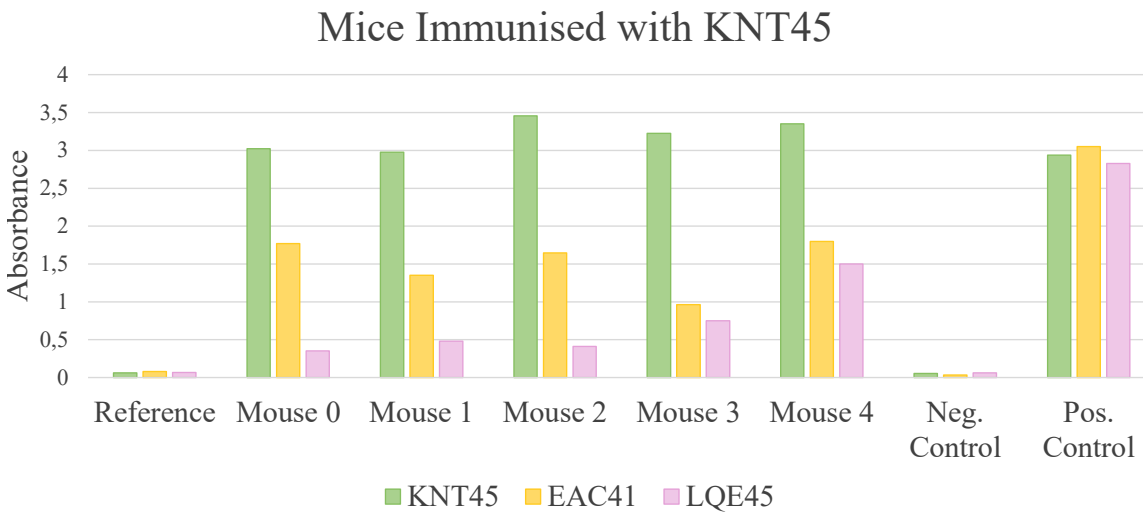


Figure 4.3: The absorbance of blood samples collected from mice who had received booster two of the peptide KNT45. A high absorbance indicates that a high concentration of antibodies or antibodies with high affinity are present in the sample. The reference was blood from one of the mice before the start of the immunisation. The negative control was buffer and the positive control blood from a mice known to have antibodies against bovine NF-L.

4.2 Selection of hybridomas

To find successfully fused hybridomas producing the correct antibodies, a screen was performed aiding in the selection for further cultivation. In Table 4.1, the selected hybridomas from each fusion can be found. In the screen of the bovine NF-L hybridomas, established in Fusion 1, the max absorbance was 0.18 and the cut off value for when they were deemed positive was set at 0.1. This resulted in 8 hybridomas being chosen for further cultivation, given the denominations NFL-53 to NFL-60. The hybridomas created from Fusion 2, with mice immunised with EAC41, had a max absorbance of 1.64, almost being as high as the positive control. The cut of value for this fusion was set at 0.5, and resulted in 18 hyridomas being chosen for further cultivation. They were denominated NFL-61 to NFL-78. Lastly, 17 hybridoma cells produced from Fusion 3 were chosen for further cultivation, denominated NFL-79 to NFL-95. The highest achieved absorbance was 1.32 and the cut of was set at 0.4. The cut off values were chosen arbitrarily so that number of selected hybridomas were around 20, since this was estimated as an appropriate amount of work.

Fusion 1		Fusion 2		Fusion 3	
Sample	**Abs.**	**Sample**	**Abs.**	**Sample**	**Abs.**
Positive Control	1,51	Positive Control	1.83	Positive Control	1.87
Negative Control	0.04	Negative Control	0.04	Negative Control	0.04
NFL-53	0.14	NFL-61	1.03	NFL-79	0.53
NFL-54	0.16	NFL-62	1.64	NFL-80	0.50
NFL-55	0.15	NFL-63	0.62	NFL-81	0.48
NFL-56	0.18	NFL-64	0.82	NFL-82	0.54
NFL-57	0.15	NFL-65	0.55	NFL-83	0.94
NFL-58	0.11	NFL-66	1.24	NFL-84	0.47
NFL-59	0.11	NFL-67	1.34	NFL-85	1.25
NFL-60	0.12	NFL-68	1.63	NFL-86	0.647
-	-	NFL-69	0.69	NFL-87	0.712
-	-	NFL-70	0.54	NFL-88	0.841
-	-	NFL-71	0.68	NFL-89	0.674
-	-	NFL-72	1.56	NFL-90	1.08
-	-	NFL-73	1.45	NFL-91	1.32
-	-	NFL-74	1.24	NFL-92	1.05
-	-	NFL-75	1.34	NFL-93	0.44
-	-	NFL-76	1.25	NFL-94	1.24
-	-	NFL-77	1.34	NFL-95	0.80
-	-	NFL-78	1.25	-	-

Table 4.1: The hybridomas listed in the table where chosen for further studies due to their absorbance values making them of interest. A high absorbance either indicates a high concentration of antibodies or antibodies with high affinity. Fusion 1 are the bovine NF-L hybridomas while Fusion 2 and 3 are the EAC41 and KNT45 hybridomas respectively. The positive control was blood collected from the immunised mice at euthanisation and the negative control was HAT-medium.

4.2.1 Success of fusion

The average percentual growth per plate from each fusion was calculated to estimate the overall success. Fusion 1, from bovine NF-L, had an average growth of 17%. Fusion 2, from mice immunised with EAC41, had an average growth of 26% and Fusion 3, from mice immunised with KNT45, had an average growth of 10%.

4.3 Biotinylation

The bovine NF-L was biotinylated to enable usage in iELISA. The concentration determination after the two desalting steps can be seen in Table 4.2. After the last desalting, BSA was added resulting in a final volume of 152 µL and concentration 0.40 mg/mL bovine NF-L. The total protein recovery was therefore 40 %.

No.	Start Vol. (µL)	Start Conc. (mg/mL)	End Vol. (µL)	Exp. Conc. (mg/mL)	Conc. (mg/mL)	Recovery
1	125	1.00	140	0.674	0.89	75%
2	140	0.67	145	0.439	0.65	67%

Table 4.2: Details of the solution used in biotinylation of the protein bovine NF-L before and after the two desalting steps, with the steps indicated by No. The concentration describe the amount of bovine NF-L present in the solution.

4.4 Second Screen and epitope mapping

The hybridoma cells selected for further cultivation did not all make it to the second screen due to poor growth or no growth at all. This resulted in the number of hybridomas from Fusion 2 to be reduced from 18 to 13 and the number of hybridomas from Fusion 3 to be reduced from 17 to 11.

The hybridomas who were successfully cultivated were screened again, to ensure the continued production of relevant antibodies. The results from the second screen of the selected hybridomas are presented in Figure 4.4 to 4.6. In Figure 4.4, the results of the NF-L hybridomas from Fusion 1 are presented. Comparing to the positive controls, being blood samples collected at euthanisation from the mice, the antibody response towards all peptides were low, with an absorbance below 0.5. There seem to be no difference in response between any of the peptides, including the unrelated.

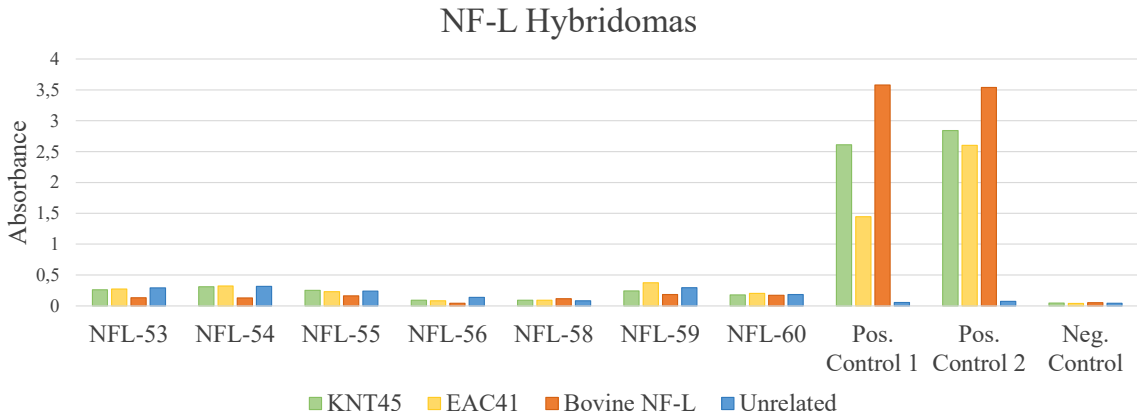
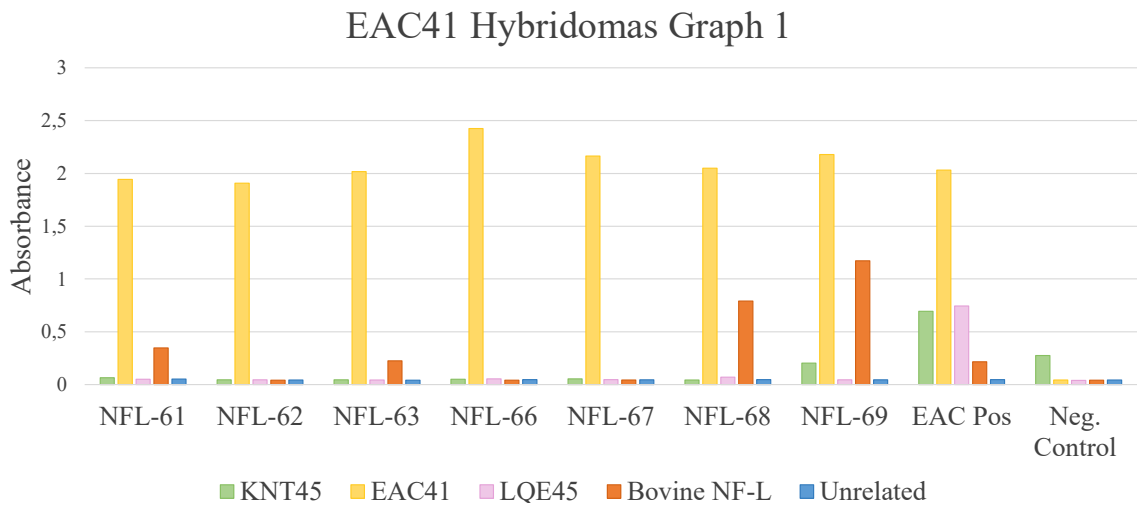


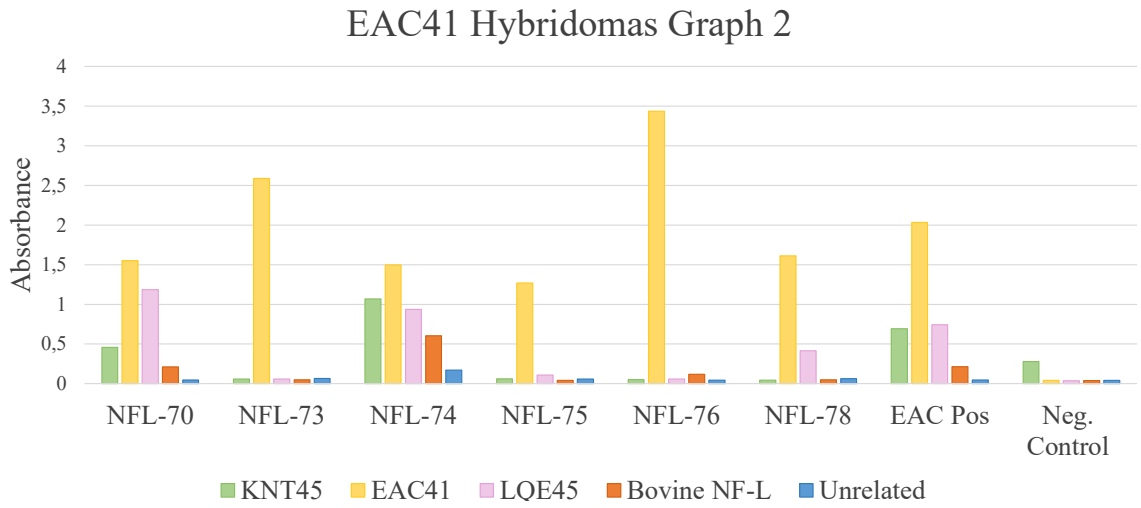
Figure 4.4: The further cultivated hybridoma cells from Fusion 1 and the absorbance of their collected supernatants. A high absorbance either indicates that a high concentration of antibodies or antibodies with high affinity are present in the sample. The positive controls were the blood samples collected from the mice at euthanisation and the negative control was HT-medium.

In Figure 4.5, two graphs with the results from the second screen of the EAC41 hybridomas are presented. Overall, all hybridomas seemed to produce antibodies towards the peptide EAC41, with absorbance within the same range as the positive

control. A few hybridomas seemed to produce antibodies who were more responsive towards bovine NF-L relative the others, being NFL-68, NFL-69 and NFL-74.



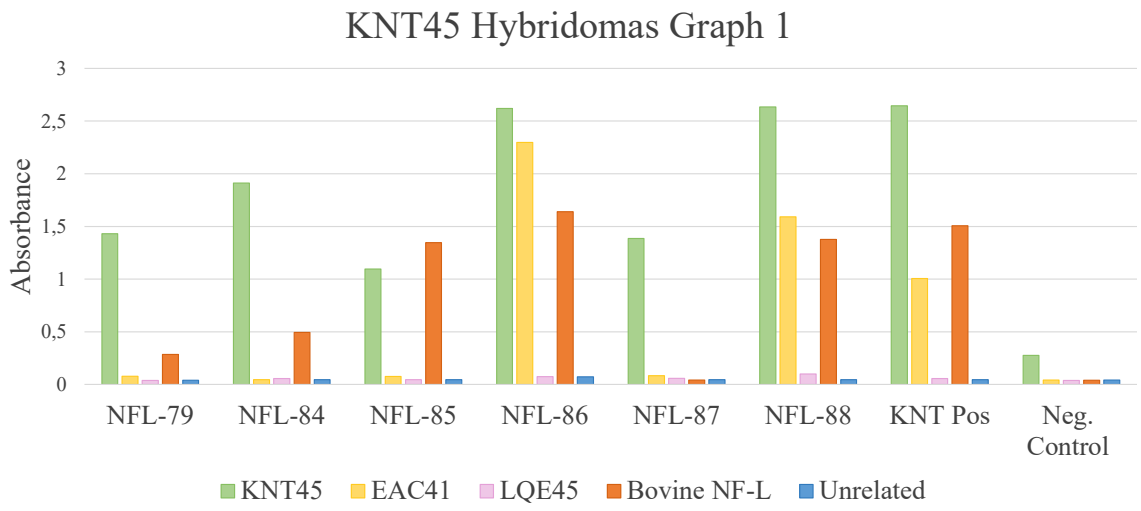
(a)



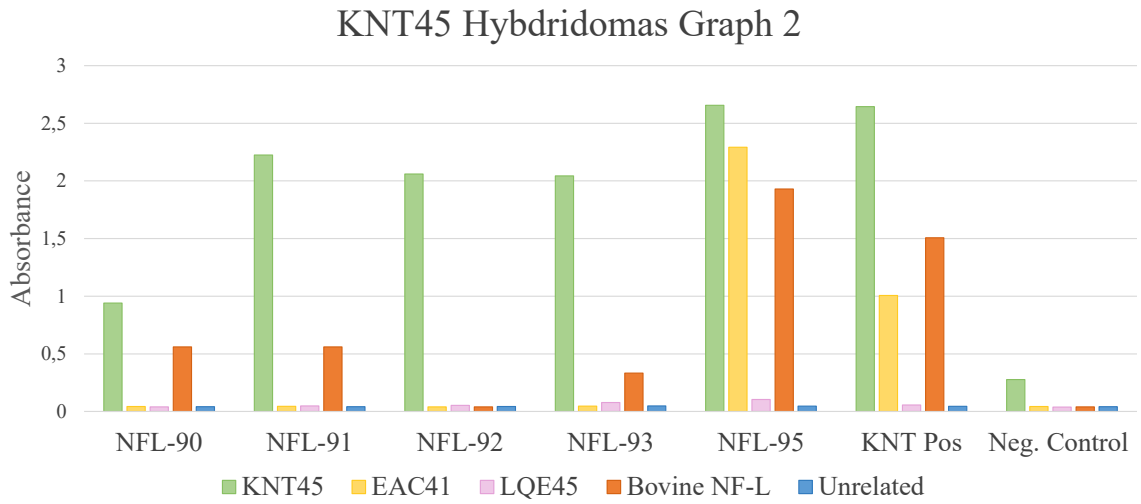
(b)

Figure 4.5: The further cultivated hybridoma cells from Fusion 2 and the absorbance of their collected supernatants. A high absorbance either indicates that a high concentration of antibodies or antibodies with high affinity are present in the sample. The positive control was a blood sample collected from the mice at euthanisation and the negative control was HT-medium. The results are presented in two graphs, a) and b), for easier visualisation

The results from the second screen of the KNT45 hybridomas are presented in Figure 4.6. The antibodies produced by the hybridomas seemed to be most responsive against the KNT45 peptide, with the absorbance being in the range of the positive control. A number of hybridomas also had antibodies responsive against bovine NF-L with an absorbance above 1, being NFL-85, NFL-86, NFL-88 and NFL-95. The antibodies of NFL-86, NFL-88 and NFL-95 also had a response towards the peptide EAC41, with an absorbance above 1.5.



(a)



(b)

Figure 4.6: The further cultivated hybridoma cells from Fusion 3 and the absorbance of their collected supernatants. A high absorbance either indicates that a high concentration of antibodies or antibodies with high affinity are present in the sample. The positive control was a blood sample collected from the mice at euthanisation and the negative control was HT-medium. The results are presented in two graphs, a) and b), for easier visualisation

4.5 Isotype Determination

The antibodies produced by relevant hybridomas, selected from the second screen, were characterised in an isotype determination. The results from the isotype ELISA are presented in Table 4.3. As can be seen, the majority of the antibodies produced have an IgG isotype. Some of the tested supernatants were positive for multiple isotypes, indicating multiple clones present in the cultivation. Examples of this can be seen in NFL-61, NFL-86 and NFL-88.

Hybridoma	Isotype				
	IgG1	IgG2a	IgG2b	IgG3	IgM
NFL-61	X			X	
NFL-62	X				
NFL-63	X				
NFL-66		X			
NFL-67	X				
NFL-68	X				
NFL-69	X				X
NFL-70	X				
NFL-73		X			X
NFL-74	X				
NFL-75				X	X
NFL-76	X	X			
NFL-78			X		
NFL-79	X				
NFL-84	X				
NFL-85	X				
NFL-86	X	X			
NFL-87	X				
NFL-88	X	X			
NFL-90					
NFL-91	X				
NFL-92	X				
NFL-93		X			X
NFL-95		X			

Table 4.3: Results from the isotype characterisation of the hybridomas who survived further cultivation. An X means that there was sufficient absorbance values to indicate the presence of an antibody with the corresponding isotype in the sample, in this case being the supernatant from the cultivated hybridomas. Multiple X on the the same row indicates the presence of multiple antibodies with different isotypes.

4.6 Western Blot

Two different Western blots were performed, the first to see if the antibodies could recognise NF-L and the second to test the concentration sensitivity. All image processing was performed in the Image Lab tool from BioRad, version 5.2.

Western Blot with bovine NF-L

To control proper loading of NF-L in all wells, the gels were visualised using the stain free technology prior to transfer to the PVDF membrane for Western blotting. Figure 4.7 is one of the gels after electrophoresis. The faint bands in every other well is the loaded sample of 0.2µg reduced bovine NF-L.

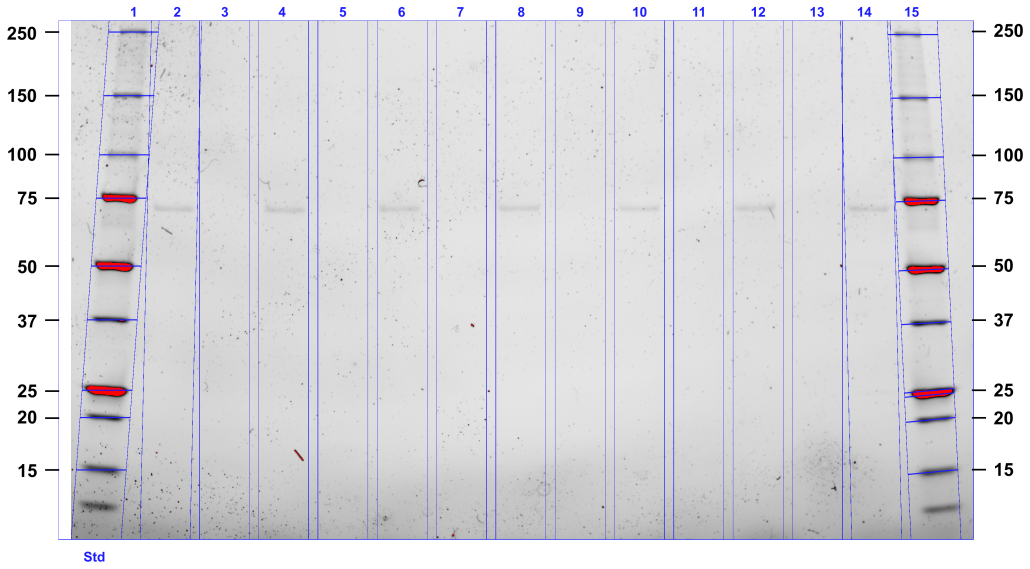


Figure 4.7: Picture of one of the Mini-Protean TGX stain-free gels used in the first Western blot after gel electrophoresis. The first and the last well were loaded with Precision Plus unstained protein standard. Every other well between them were loaded with 0.2 µg reduced bovine NF-L. The placement and width of the band indicates the length and the state of the protein.

To investigate the epitope type and sensitivity to conjugates, a Western blot with all hybridomas from the second screen was performed under reduced conditions. Figure 4.8 shows an example of a membrane which was cut in pieces and incubated in the spent medium of seven different hybridomas. It can be seen that there are bands for NFL-70, NFL-74 and NFL-79. The remaining blot images can be found in Appendix A. All hybridomas producing antibodies that could detect NF-L in the Western blot are listed in Table 4.4. No bands were detected for the negative control.

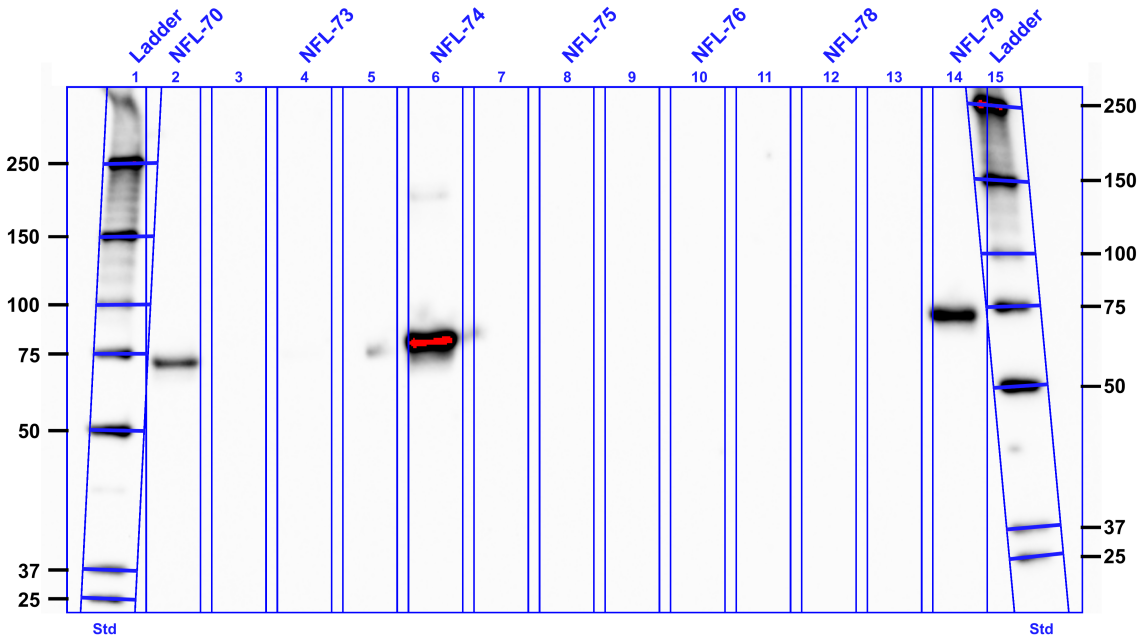


Figure 4.8: Picture of PVDF membrane 2 after Western blot. The membrane was cut and incubated in supernatant from NFL-70, NFL-73, NFL-74, NFL-75, NFL-76, NFL-78 and NFL-79, with placement stated in the image. A band indicates the presence of antibodies which bind to denatured bovine NF-L.

Hybridomas producing bands	
NFL-61	NFL-79
NFL-62	NFL-84
NFL-63	NFL-85
NFL-66	NFL-86
NFL-67	NFL-88
NFL-68	NFL-90
NFL-69	NFL-91
NFL-70	NFL-95
NFL-74	

Table 4.4: Hybridomas listed in the table produced antibodies which gave detectable bands in Western blot against 0.2 µg denatured bovine NF-L.

Detection Limit Characterisation

To control proper loading of NF-L in all wells, the gels were visualised using the stain free technology prior to transfer to the PVDF membrane for Western blotting. The result from one of the gel electrophoresis can be seen in Figure 4.9. The NF-L concentration is too low to be detected but it can be seen that both the humane CSF and serum samples were loaded correctly. The proteins in the serum created a gel when denatured, which can be seen in the sample distribution in the SDS-page.

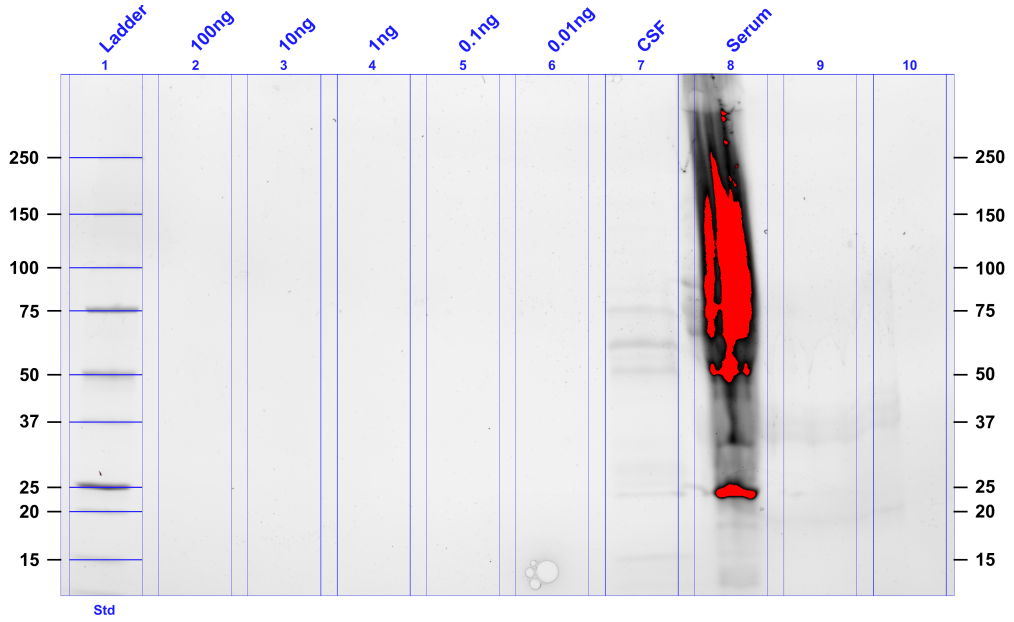


Figure 4.9: Picture of one of the Mini-Protean TGX stain-free gels used in the detection limit test after gel electrophoresis. The first well is loaded with Precision Plus unstained protein standard. The following five wells were loaded with denatured bovine NF-L, ranging from 100 to 0.01 ng as indicated in the image. Well 7 and 8 were loaded with denatured clinical samples of cerebrospinal fluid (CSF) and serum respectively.

The detection limit of the antibodies were tested through a second Western blot. The results can be found in Table 4.5. The hybridomas chosen for the test were those with promising results from both the second ELISA screen as well as the first Western blot. The X indicates at which concentrations the antibodies from the hybridomas could identify NF-L. As can be seen, almost all hybridomas had antibodies who could detect the denatured antigen at 10ng and NFL-86 could also detect it at 1ng. Additionally, the negative control gave no bands and none of the antibodies bound to neither CSF or serum.

Hybridoma	**Concentration**				
	100 ng	10 ng	1 ng	0.1 ng	0.01 ng
NFL-69	X	X			
NFL-74	X	X			
NFL-79	X				
NFL-85	X	X			
NFL-86	X	X	X		
NFL-88	X	X			
NFL-95	X	X			

Table 4.5: Results from the second Western blot, analysing the detection limit of antibodies present in hybridoma supernatants. The X indicates at which amount the antibodies from the hybridomas could identify denatured bovine NF-L.

Table 4.5 was compiled from analysing the images of the membranes. The membrane incubated in spent medium from NFL-86 is visualised in Figure 4.10 as an example of how they looked. It can be seen that the bands gets gradually weaker as the concentration decreases. It can also be seen that no bands are detected in well 7 and 8, indicating that the antibodies do not bind humane CSF or serum.

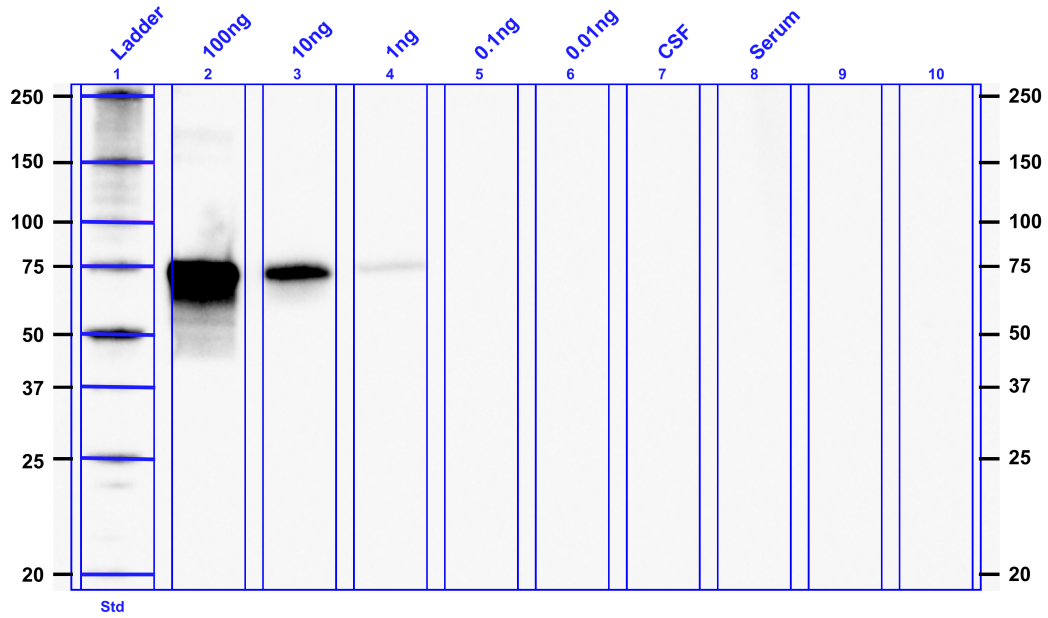


Figure 4.10: Picture of the PVDF membrane incubated in supernatant from NFL-86 in the detection limit test Western Blot. The first well is loaded with Precision Plus unstained protein standard. The following five wells were loaded with denatured bovine NF-L, ranging from 100 to 0.01 ng as indicated in the image. Well 7 and 8 were loaded with denatured clinical samples of cerebrospinal fluid (CSF) and serum respectively. A band indicates the presence of antibodies which bind to the denatured protein sample of that well.

5
Discussion

In this chapter, all results are discussed in the order that they were attained. The possible clinical applications of the established antibodies are also reviewed. Lastly, the study is discussed in terms of methods used and ethical considerations.

5.1 Immune response in mice

The results of the titer test is presented in Section 4.1. Even though the first group of mice were immunised with the whole protein, they produced antibodies with epitopes within the desired region. They had an overall equal response to all three peptides, indicating that they have similar immunogenicity. The mice immunised with EAC41 had a higher response towards EAC41 and lower but overall equal towards KNT45 and LQE45. As can be seen in Figure 3.1, the EAC41 peptide is located so that it overlaps both other, explaining the equal response towards KNT45 and LQE45. The last group of mice were immunised with the peptide KNT45. It can be seen in Figure 3.1 that it is located upstream of the other two peptides, overlapping almost 20 amino acids of EAC41 and only five of LQE45, which is reflected in the immune response wich is highest towards KNT45, then EAC41 and lastly LQE45.

The positive control in the titer test was blood from a mouse from the previous project, immunised with bovine NF-L. Accordingly, the group immunised with bovine NF-L had the most alike response compared to the positive control. The mice immunised with peptides did on the other hand have a stronger response towards their peptide, with an absorbance above the positive control. The directed immunisation was therefore deemed successful, with the mice producing either a higher concentration of antibodies with epitopes within the peptides or antibodies with higher affinity towards the peptides. The overall immune response was also considerably higher after boost 2 compared to the reference, as seen in Figures 4.1 to 4.3, and the mice were therefore deemed to have an immune response which sustained fusion.

5.2 Fusion and Selection of Hybridomas

Three fusions were performed, Fusion 1 with mice immunised with full length bovine NF-L and Fusion 2 and 3 with mice immunised with EAC41 and KNT45 respectively. Just over a week after fusion, hybridomas were screened for the production of

antibodies with epitopes in the clinically interesting region using an inderect ELISA. The results are seen in Table 4.1, which presents the absorbance of the chosen hybridomas, the fusion they were from and their given serial number. The hybridomas were chosen based on if their antibodies were responsive to the clinically interesting region covered by the peptides.

The cut off value for the hybridomas from Fusion 1, attained from mice immunised with bovine NF-L, was set to 0.1. The negative control had a value of 0.04, so 0.1 was deemed sufficiently high to indicate an antibody response of some sort. However, the highest achieved absorbance was 0.18, being 8-fold lower than the positive control. This could either be due to slow growth of the hybridomas which produce the correct antibodies resulting in low concentrations, or unspecific binding by IgMs. Only 8 hybridomas passed the bar, which suggests very few fused B-cells produced responsive antibodies. The low number of positive hybridomas can be explained by the antigens chosen for screening. Since the mice were immunised with full length bovine NF-L, their immune system would produce antibodies with epitopes that can be located anywhere on the protein. Antibodies with epitopes in the desired part of the Coil-2 region would therefore be many less compared to the total. As a result, there will be countless B-cells producing antibodies with other epitopes and the probability of fusion succeeding with a "correct" B-cell is lower compared to in the directed immunisation. When comparing the average growth, presented in Subsection 4.2.1, it can be seen that Fusion 1 actually attained more hybridomas compared to Fusion 3, which were from the mice immunised with KNT45. Regardless, Fusion 3 resulted in 17 hybridomas with an overall higher absorbance. This shows the effectiveness of directed immunisation. The reason why full length bovine NF-L immunisation was still performed was due to the possibility of attaining antibodies with epitopes within the clinically relevant region that also recognised the protein in native conformation. As seen in the titer test, Figure 4.1, the mice had an immune response towards the peptides and therefore also produced antibodies of interest. It seems that the correct B-cells were unfortunately not successfully fused.

The max absorbance of hybridomas from Fusion 2 were significantly higher than that of Fusion 1. The cut of value for the EAC41 hybridomas was set to 0.5 and resulted in 18 hybridomas. The max absorbance was 1.62, being almost as high as the positive control. This indicates a high concentration of antibodies in the wells or high affinity of the antibodies present. Fusion 2 attained the highest number of hybridomas producing antibodies within the desired epitope region, and as can be seen in Subsection 4.2.1 it had the highest percentual growth per plate of all fusions at 26%. The last fusion, with the KNT45 hybridomas, resulted in 17 hybridomas with the cut off value set to 0.4. The obtained max absorbance from this fusion was 1.32, being relatively high compared to the positive control. The percentual growth per plate from this fusion was however only 10%, being much lower compared to the 26% of Fusion 2. Despite the 10 %, Fusion 3 resulted in many more hybridomas of interest compared to Fusion 1. Overall the growth was very low, as it usually lands on over 50% utilising the followed protocol. The reason is thought to be that the myeloma cells were cultivated at a too high cell density and therefore had a lower

viability when performing the fusion, resulting in fewer hybridomas.

5.3 Biotinylation

The bovine NF-L was biotinylated to enable usage in indirect ELISA, and the results can be seen in Section 4.3. 60% of the protein was lost during the biotinylation, which is not a desirable yield. It was probably due to the small sample volume, and up scaling of the experiment would improve the results. Most of the protein was lost during desalting, at the change of buffer. These steps were however deemed necessary as the original buffer included 6M urea, being a compound containing two amine groups. As biotin react with amines, proper biotinylation of the protein could not be ensured at such high concentrations. The buffer used during biotinylation did however have 2M urea, to prevent precipitation of the protein. The presence of urea explain the high ratio of biotin used, which was 15:1.

Despite the low yield, the biotinylation was deemed successful as enough biotinylated antigen for an ELISA was obtained.

5.4 Second screen and epitope mapping

A second iELISA, here called screen 2, was performed to find the hybridomas that had continued to grow and produce the correct antibodies. The results are presented in Figure 4.4 to 4.6. The NF-L hybridomas had an average absorbance below 0.5 and an equal response towards all antigens, even the unrelated. It was therefore concluded that none of the hybridomas produced the proper antibodies but rather unspecific IgMs. There could have existed hybridomas producing the correct antibodies which were unstable or overgrown, or the positive results in screen 1 were also due to unspecific binding of IgMs. None of the hybridomas from Fusion 1 were further examined but discarded. It could however be seen that the positive controls, being blood samples from the immunised mice, had a strong response towards both peptides. This indicates that the immunisation was successful, since the mice produced antibodies with epitopes within the desired region. Since the antibodies also had a strong response towards the bovine NF-L, the biotinylation was deemed successful with the biotin not changing the native conformation of the protein.

The EAC41 hybridomas had much better results, with absorbance around 2. The produced antibodies responded primarily to the EAC41 peptide but some also bound to KNT45 and LQE45, although with lower affinity. Some hybridomas had a response against full length bovine NF-L, with NFL-68, NFL-69 and NFL-74 having the highest relative affinity. This indicates that they recognise the native conformation protein. Compared to the positive control, the hybridomas had a generally lower response towards the other peptides. The positive control is however the response of polyclonal antibodies, which can have more spread epitopes compared to the supernatants who at least contain fewer if not monoclonal antibodies. It is therefore reasonable that the hybridomas bind more specifically to EAC41 compared to

the other antigens, especially since they were selected and screened for only EAC41 in the first screen.

The results of the second screen of the KNT45 hybridomas can be seen in Figure 4.6. Their highest response was towards KNT45, which was expected. They had no response towards LQE45, which is reasonable since they only overlap with 5 amino acids. The probability of an epitope being situated right at that sequence is low. The hybridomas who bound to EAC41 also had the highest affinity towards bovine NF-L, indicating that an epitope site which is relevant for the detection off full length protein in native conformation is located in the overlapping sequence between KNT45 and EAC41. The KNT45 hybridomas had overall a more strong response to bovine NF-L compared to EAC41. It can either be that the antibodies produced by these got more relevant epitopes by chance or that the epitope sight which is significant for whole protein detection spans both the overlapping sequence but also KNT45 by itself.

The addition of the full length bovine NF-L in screen 2 was to further characterise the antibodies produced by the hybridomas. Those who bind the full length bovine NF-L in ELISA do most probably recognise the antigen in native form, being of high importance in a diagnostic tests. The KNT45 hybridomas were generally better on binding to NF-L and a possible relevant epitope sight for the recognition of NF-L in native conformation is located around the overlapping sequence between KNT45 and EAC41. This site of interest was able to be characterised through screening against multiple peptides, allowing epitope mapping.

The hybridomas could not be compared to each other based on the absorbance values from the second screen. The sample material was supernatants with unknown antibody concentration which depended on when they were sampled and the selection of this was arbitrary. The absorbance therefore reflect both concentration and affinity, preventing the comparison of hybridomas. The antigen response within one hybridoma could however be compared, due to the concentration of the antigens being equal. The hybridomas having a higher response towards bovine NF-L can therefore be found. The response towards bovine NF-L can, and should, be further characterised after cloning and purification.

5.5 Isotype determination

The isotype determination showed that all hybridomas, now only from Fusion 2 and 3, produced IgG antibodies. The results can be seen in Table 4.3. This shows that the immune response in the mice was mature and that they had been immunised and getting boosters over a sufficient amount of time. Some hybridomas produced multiple IgGs of different subtypes, for example NFL-86 and NFL-88. Hybridomas NFL-69, NFL-73, NFL-75 and NFL-93 produced both IgG and IgM antibodies. Additional hybridomas must be present in all wells were multiple epitopes were detected. The hybridomas with only one isotype can not be said to be monoclonal either, as there could be two hybridomas present producing the same isotype. The

probability off this is however low. Once again, the hybridomas must be cloned to allow for further characterisation and to identify which of the produced antibodies that have the desired properties, if not both.

5.6 Western blot

In the first Western blot, all hybridomas were tested towards 0.2 µg / mL of reduced bovine NF-L. The image of a protein gel after electrophoresis can be seen in Figure 4.7. All loaded wells contained visible bands, located just below the 75 kDa line in the ladders. This shows that the gel was properly run and that the protein was intact, since its molecular weight is 68 kDa. Any lines that were not visible in the Western blot would therefore be due to the antibodies failing to bind. The hybridomas with antibodies capable of binding the denatured full length NF-L are listed in Table 4.4, which shows that 17 of the total 24 created a band. An example image of a membrane can be seen in Figure 4.8. Even though NFL-74 creates a stronger band than the other two, nothing can be said about affinity of the antibodies since the concentrations of the supernatants were unknown, making it a qualitative assay. It can however be concluded that the ones who bind have linear epitopes and recognises full length bovine NF-L without any conjugates, thus making them interesting candidates to move forward with in the project.

Detection limit

A selection of hybridomas were selected for a detection limit test. Seven were chosen, based on them having a high response towards bovine NF-L in the iELISA and producing bands in the Western blot. The antibodies who did not bind the protein in Western did most likely have conformational epitopes and could not be further characterised with this method.

The results from the detection limit test can be seen in Table 4.5. Most hybridomas could detect NFL down to the concentration 10 ng and NFL-86 could even detect it at 1ng. The membrane incubated in spent medium from NFL-86 can be seen in Figure 4.10. The intensity of the band correspond to amount of antibodies bound and as can be seen, the intensity decreases with the concentration of antigen. In this Western blot, patient samples of CSF and serum were loaded as controls, and no hybridoma medium bound to it. They had no reactivity towards it even though it contained a multitude of proteins, as can be seen in Figure 4.10, which indicates high specificity of the antibodies towards NF-L. The next step would be to test their response towards human NF-L in a patient sample.

Again, the results from this Western are only an indication. The concentration of antibodies in the supernatant is still unknown and does not allow proper comparison of performance between the hybridomas.

5.7 Clinical applicability

In order for antibodies to be clinically applicable in a sandwich ELISA for detection of NF-L in blood, they have to fulfil a set of criteria. They need to have a high specificity and affinity in order to get a sensitive test, and also recognise NF-L in native conformation. This is best achieved by the antibodies having epitopes within a specific part of the Coil-2 region, as indicated by previous studies. They should also be of the isotype IgG, due to their general high affinity and specificity. Antibodies of IgG isotype also facilitates easier production and down stream processing. They have to form a pair with another antibody, having two separate epitope sites that allow simultaneous binding in order to design a sandwich ELISA. Lastly, a high sensitivity is acquired which allows for detection of concentrations that are relevant for neurodegenerative diseases. It is difficult to set a value on these relevant concentrations as data listed in literature use different methods and standards, preventing comparison.

All hybridomas from Fusion 2 and 3 produced IgG antibodies with epitopes within the desired region of Coil-2, and none had a response to the unrelated peptide in ELISA. Some of them were responsive to the full length bovine NF-L, which indicates that they can detect the protein in its native conformation. They therefore also have the potential of detecting NF-L in human clinical samples which is desirable for use in diagnostic test of neurodegenerative diseases. The antibodies must however be evaluated using clinical samples containing human NF-L to verify their clinical relevance. In general, the KNT45 hybridomas had a higher response towards bovine NF-L. The hybridomas with a relative high NF-L response when compared to the overall absorbance of the hybridoma were NFL-69, NFL-74, NFL-85, NFL-86, NFL-88 and NFL-95. They all produced bands in Western blot, meaning they have linear epitopes and can recognise the protein without conjugates. When their detection limit was tested, all could detect 10 ng and NFL-86 could also detect 1 ng, corresponding to 100 ng/mL. As the samples were supernatants, the detection limit is most likely lower and need to be measured again with purified mAbs of known concentration. Additionally, the detection limit values attained from the Western Blot can not be compared to relevant concentration values found in literature, since varying methods and internal standards are used. In order to evaluate if the antibodies are sufficiently sensitive, clinical samples containing NF-L has to be concentration determined at Fujirebio Diagnostics AB to allow for proper comparison. When it comes to pairs, NFL-85 and NFL-68 may be of interest as they seem to have separate epitopes as well as affinity to the native conformation, as seen in the second ELISA screen, Figure 4.5 and 4.6. Further tests would however have to be performed.

In conclusion, many anibodies with potential of being clinically applicable have been established. NFL-86 is of extra interest, but contains more than one type of antibody. To attain quantitative results which allows for comparison, the hybridomas must be cloned and purified, and the antibody concentration determined. The indications of the Coil-2 region as a site of interest was further supported, and the

choice of peptides from this region was profitable.

5.8 Methods

In this section, the utilised methods will be discussed in the chronological order that they were used.

5.8.1 Hybridoma Technology

The main ethical aspect of this method is the use of animals. As discussed in the theory, there are plenty of other methodologies that rely on recombinant production instead. Hybridoma technology is however superior in producing antibodies with optimised epitopes. The variety achieved when utilising a natural immune response can never be mimicked when using recombinant techniques, such as recombinant antibody libraries. Additionally, hybridoma technology is the standard practice at Fujirebio Diagnostics AB, with over 20 years of experience within the field. Their protocols are therefore optimised within this methodology. Recombinant technology does however continue to develop and may be the superior alternative in the future.

5.8.2 ELISA

As mentioned in the theory section, there can occur a few problems when using indirect ELISA for screen of hybridomas. One of them is the cross reactivity between secondary antibodies. Negative controls where therefore always used as references. Another is the proneness of selection of linear epitopes over conformational. This may however not be a problem since an antibody with a linear epitope may recognise an antigen in both denatured and conformational form. Further, sandwich ELISA is optimal to use as an easy screening method that can be performed at any local healthcare centres. This is of course why the antibodies are developed for this particular diagnostic test.

5.8.3 Western blot

As discussed in the theory section, many methods have been developed after Western blot, being more dependable in the characterisation of proteins. Western blot was however not used for protein characterisation but antibody characterisation in this study. It was ideal in this project, as no additional tag or caption antibody is required for the immobilisation of the protein, avoiding cross contamination. Further, it can be established that it is the full length protein that the antibodies bind to and nothing else.

5.9 Ethical Aspects

As any study containing animal experiments, the ethical aspects have to be discussed. The first consideration is if the end goal is of such importance that it is

justified? Neurodegenerative diseases are disorders which lessens the life quality tremendously and often lead to death. Due to the degradation of neurons occurring many years before the first clinical symptoms, early diagnosis could slow the progression as well as allow further characterisation of the disease, enabling the development of cures. As of today, there exist no easily accessible and user friendly diagnostic tests that allow for screening of these diseases. The establishment of one would therefore help countless people around the world. This would therefore justify the use of animals to achieve. The other consideration is if there are alternative methods without the need of animal experiments? The answer is yes, antibodies can be established using recombinant techniques. However, as discussed in Section 5.8.1 hybridoma technology is still considered superior.

Since animal experiments are necessary for this methodology, the handling of mice have of course been optimised to lessen the suffering of the animals. As few mice as possible are used in the experiments and they are kept in groups and in environments that allow them to have an as natural behaviour as possible. Additionally, the handling of mice in the experiments is strictly controlled and performed by trained personnel to avoid pain as far as it goes. The adjuvant used is for example developed so that is does not cause as much inflammation and pain as more traditional adjuvants. The reasons listed above are part of the material submitted to the ethical border, which considered it sufficient and issued a permit.

6
Conclusions

The aim of this project was to establish high affinity monoclonal antibodies towards neurofilament light. A number of antibodies were developed with great potential of becoming high affinity monoclonal antibodies towards NFL. This may have been achieved if the project had encompassed more time.

The hybridomas which produced desirable antibodies originated from the mice immunised with peptides. The mice immunised with bovine NF-L did not results in any hybridomas producing antibodies with epitopes within the desired region. This confirms the importance and success of the directed immunisation.

Mice immunised with KNT45 recognised bovine NF-L to a greater extent than those immunised with EAC41. Additionally, an epitope site of interest was located in the overlapping sequence between KNT45 and EAC41, in accordance with previous literature. Overall, immunisation and screening using peptides from the Coil-2 region was effective for the establishment of antibodies which could be clinically useful for the detection of NF-L.

A few hybridomas were of higher interest due to their produced antibodies response to full length bovine NF-L. All of these antibodies had linear epitopes, as they could detect the reduced protein in Western blot. They could all detect the concentration 10 ng in the sensitivity test and NFL-86 could also detect 1 ng in 10 µL per well, corresponding to 100 ng/mL. As the samples used were supernatants with unknown concentration, the detection limit is presumably lower. A pair for use in sandwich ELISA could potentially be NFL-85 and NFL-68.

To conclude, a number of antibodies with the potential of being used in a sandwich ELISA diagnostic test for the detection of NF-L in blood have been established. These antibodies were proven to recognise the native form of bovine NF-L, epitope mapped to the clinically relevant Coil-2 region and were not reactive to other proteins present in clinical samples of CSF or serum. However, additional purification and characterisation steps are required in order to get quantitative results.

6.1 Future Work

The next step would be to clone and purify the hybridomas to attain monoclonal antibodies. This will allow for further characterisation, enabling comparison and

definite results. It would also be of interest to characterise the epitopes in further detail and especially the overlapping sequence between KNT45 and EAC41. Methods such as bio-layer interferometry (BLI) or surface plasmon resonance (SPR) could be used to characterise the kinetics, specificity and affinity of the antibodies, as well as identify pairs that could be used together in a sandwich ELISA. Ultimately, the antibody response towards NF-L in clinical samples such as serum and CSF has to be investigated.

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A

Appendix 1

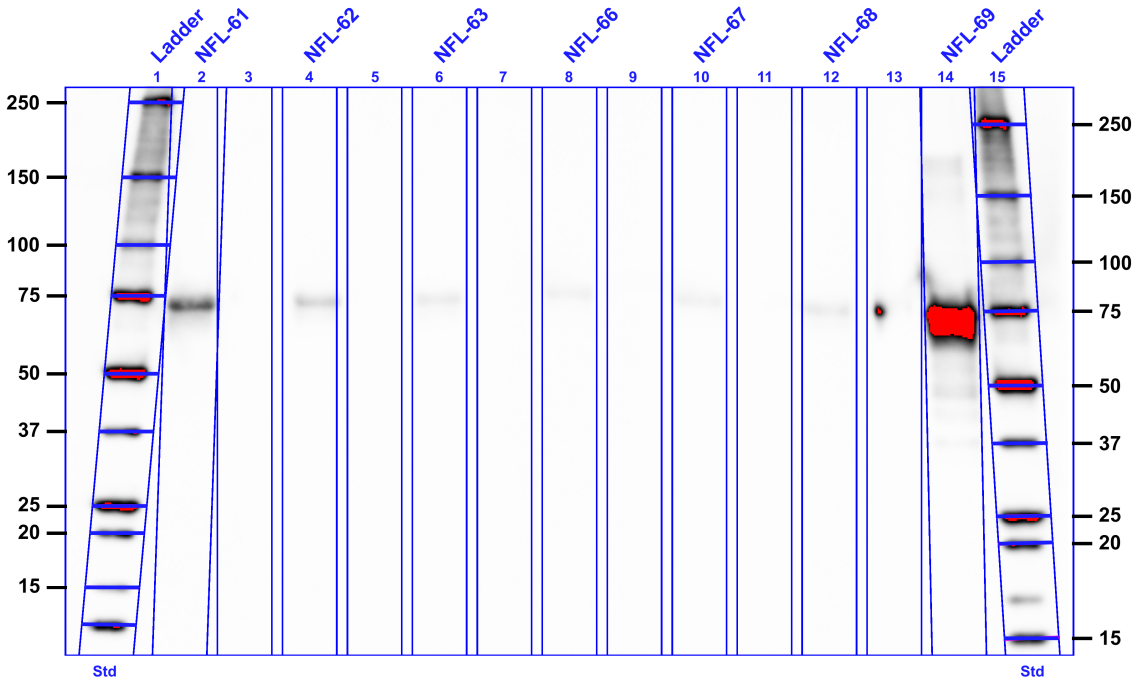

Figure A.1: Picture of PVDF membrane 1 after Western blot. The membrane was cut and incubated in supernatant from NFL-61, NFL-62, NFL-63, NFL-66, NFL-67, NFL-68 and NFL-69, with placement stated in the image. A band indicates the presence of antibodies which bind to denatured bovine NF-L.

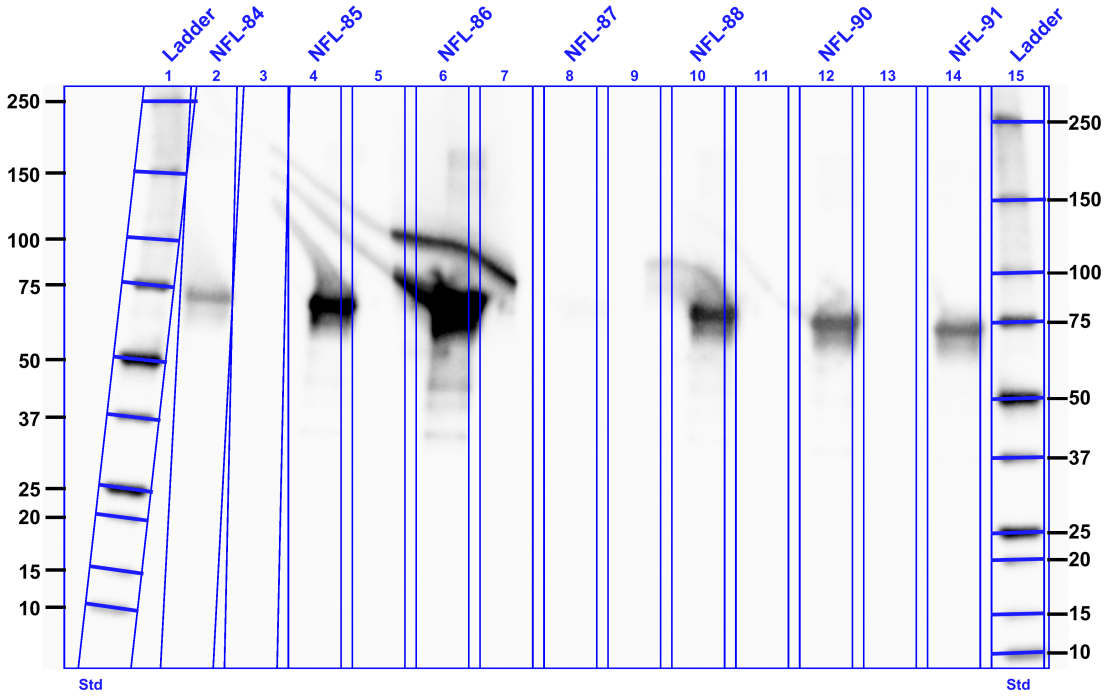

Figure A.2: Picture of PVDF membrane 3 after Western blot. The membrane was cut and incubated in supernatant from NFL-84, NFL-85, NFL-86, NFL-87, NFL-88, NFL-90 and NFL-91, with placement stated in the image. A band indicates the presence of antibodies which bind to denatured bovine NF-L.

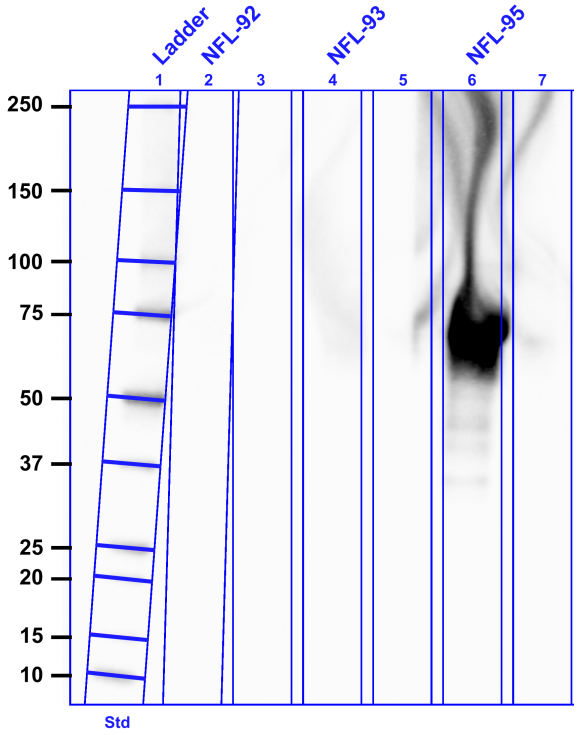

Figure A.3: Picture of PVDF membrane 4 after Western blot. The membrane was cut and incubated in supernatant from NFL-92, NFL-93, NFL-95 and negative control. A band indicates the presence of antibodies which bind to denatured bovine NF-L. No bands were detected in the negative control where hybridoma medium was omitted (data not shown.)

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