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Characterization of Monoclonal Mouse IgG Antibodies to Cod IgM

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Abstract

The unique immune system of the Atlantic cod (Gadus morhua) has attracted interest since the groundbreaking genomic sequencing study by Bastian Starr et al. in 2011, which revealed the absence of the MHC class II locus and the CD4 genes in Atlantic cod, unlike most jawed vertebrates. Given the increasing interest in Atlantic cod in the aquaculture industry, understanding their immune system is important. This knowledge is essential to effectively protecting cod populations from pathogens, as farmed fish species are vulnerable to various infectious agents present in their environment. High-quality reagents, such as antibodies, are necessary to support research and vaccine development. Recently, Qiao and Johansen's group developed mouse monoclonal antibodies (mAbs) and a chicken polyclonal antibody (pAb) targeting cod IgM, which holds great promise for the research community and aquaculture industry. However, further characterization of these antibodies and the development of a protocol for their use in immunological studies in cod are required.

To characterize these antibodies, several immunological methods were used, such as Enzyme-linked immunosorbent assay (ELISA), Western blot (WB), and Immunohistochemistry (IHC). The ELISA results revealed varying signal intensities among the mAbs compared to a mouse pAb cocktail. However, clone 19.3 consistently generated strong signals at different concentrations. In contrast, the other three mAbs showed weaker signals. Competitive ELISA experiments revealed no significant interference between the different mAbs, indicating they are likely to bind to different epitopes. In WB, only clone 19.3 showed effective binding and was revealed to bind the heavy chain of cod IgM. However, in IHC, the mAbs that were ineffective in WB, such as clones 1.1, 9.2, and 13E4, successfully stained tissue sections.

In conclusion, the optimal antibody conditions for generating a high signal-to-noise ratio in ELISA, WB, and IHC have been described. Characterizing these antibodies will enhance our understanding of their usefulness in different immunological assays and can serve as a guideline for future research and vaccine development for cod.

Abbreviations

Ab	Antibody
APC	Antigen presenting cells
BCR	B cell receptor
BSA	Bovine serum albumin
dH_2O	Distilled water
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystallizable
HGPRT	Hypoxanthine-guanidine phosphoribosyl transferase
HRP	Horseradish peroxidase
Ig	Immunoglobulins
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MAC	Membrane Attack Complex
MHC	Major histocompatibility complex
pAb	Polyclonal antibodies
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptors
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TCR	T cell receptor
WB	Western blot

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1.Introduction

1.1 The Immune System

In all animals, the immune system is a network of cells, tissues, and organs that functions as a defense mechanism against potential pathogens such as bacteria, viruses, and fungi. It is divided into two components: the innate immune system and the adaptive immune system [1].

The innate immune system

The innate immune system is often referred to as the first line of defense against pathogens, as it is the initial response to infections and can respond quickly, within hours or days. The rapid response of the innate immune system is due to immune cells, such as phagocytes and dendric cell's ability to recognize conserved molecular patterns called pathogen-associated molecular patterns (PAMPs) through their germline-encoded pattern recognition receptors (PRRs) without the need for prior exposure [2]. PAMPs are found on a wide range of pathogens and an example is lipopolysaccharide (LPS), which is present in the outer membrane of all gram-negative bacteria. Because the innate immune response is not aimed at a specific pathogen, it is considered non-specific [3]. But, the innate immune system is important for instructing specific responses generated by the adaptive immune system against pathogens [4].

The adaptive immune system

The adaptive immune system responds more slowly but with greater specificity than the innate immune system [3]. The adaptive immune response is mediated by lymphocytes, a type of white blood cell that expresses surface receptors that are not encoded in the germline. Instead, these receptors are assembled from DNA building blocks in the genome by somatic rearrangement [5]. This process is referred to as somatic diversification, and it results in each developing lymphocyte having a unique receptor that is specific to a given molecular structure found on a specific pathogen, generally referred to as an antigen. As a result, the lymphocytes in our bodies are capable of recognizing a wide range of antigens, including new mutant forms and those already present in our bodies [1]. Autoimmune diseases, such as Type I diabetes, are caused by immune cells attacking healthy cells and tissue as if they were infected by a pathogen. During development, lymphocytes recognizing our self-antigens are removed or altered during development to prevent autoimmune responses **(Fig.1)**. This

process, known as central tolerance and ensures that lymphocytes only recognize and respond to foreign antigens and not self-antigens [5].

There are two types of lymphocytes, called T lymphocytes and B lymphocytes, responsible for cell-mediated and humoral immunity, respectively [3]. Lymphocytes are abundant in both the blood and lymph organs. Lymph organs include the primary lymphatic organs bone marrow and thymus, where lymphocytes develop and the secondary lymphatic organs spleen, and lymph nodes where lymphocytes meet foreign antigens [5]. Lymphocytes are considered naïve or antigen-inexperienced until they encounter a foreign antigen. Once a naïve lymphocyte recognizes an antigen through its receptors in the lymph nodes, an immune response is activated. During this activation, lymphocytes will proliferate and differentiate. After repeated exposure to the same antigen, lymphocytes will rapidly recognize and respond to it. This is referred to as "immunological memory" and serves as the foundation for protective immunity [3]. Vaccines work by stimulating the adaptive immune system to develop immunological memory without causing the experience of the actual disease, thereby giving immunity against future exposure to the antigen [6].

B lymphocytes

B lymphocytes, or B cells, are derived from hematopoietic precursor cells in the bone marrow. After their production, B cells undergo several stages of maturation and selection before they become fully functional **(Fig.1).** The maturation process of B cells involves both positive and negative selection mechanisms to ensure the development of functional B cells that can respond to antigens while avoiding self-reactivity. This process is known as central tolerance, as mentioned above. During negative selection, immature B cells that recognize self-antigens with high affinity are eliminated or undergo receptor editing, a process in which their B cell receptors (BCRs) are modified to reduce self-reactivity [5]. After passing through the negative selection process, naïve B cells migrate via the blood from the bone marrow to secondary lymphoid organs, such as the spleen and lymph nodes. In these secondary lymphoid organs, the B cells encounter antigens and proliferate and differentiate [1].

Each B cell has a unique antigen receptor on its surface known as BCR, which is composed of two heavy chains and two light chains that form a Y-shaped molecule. When the naïve B cell first recognizes an antigen, B cells undergo an activation process. This activation generates a signal that induces the proliferation and differentiation of B cells into initial plasma cells that are short-lived. The short-lived plasma cells are specialized to produce and secrete large

amounts of antibodies that are specific to the antigen that initially stimulated their production [1]. These antibodies are identical to the BCR on the surface of the B cell that recognized the antigen and circulates in the bloodstream and other body fluids, neutralizing the antigen that activated their production [3]. This process is referred to as humoral immunity, which is associated with the immune response in body fluids. Some of the antigen-stimulated naïve cells differentiate into memory cells, which are more easily and rapidly induced to become effector cells by later exposure to the same antigen and have a longer lifespan [1, 6]. Therefore, upon exposure to the same pathogen with the same antigen, memory cells divide and differentiate into plasma cells, resulting in a rapid response and the production of antibodies [7].

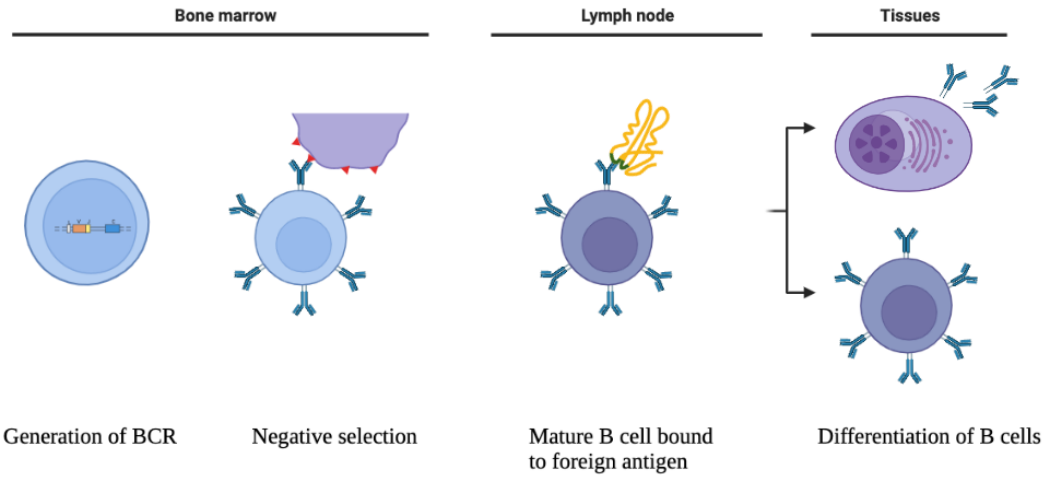

Figure 1: B cell maturation.
B cell maturation is a complex process that occurs in the bone marrow and lymphoid tissues. It involves several stages, starting from the development of B cells in the bone marrow to their activation and differentiation upon encountering antigens in the lymph node. Created with BioRender.com.

Activation of B cells can occur through two different pathways, depending on the nature of the antigen and the involvement of T cells [3]. T-independent activation occurs when B cells directly recognize an antigen and initiate an immune response without T cell help. This is typically observed when the antigen has a repeated pattern, allowing BCRs to become clustered on the surface of the pathogen. Then the B cell can divide and differentiate into plasma cells. This type of response is rapid and relatively simple and mostly results in the production of low-affinity antibodies [3]. In contrast, T-dependent activations require the assistance of a type of T cell called a T helper cell. T helper cells recognize the same antigen

as the B cells, but they recognize a different epitope of the antigen. An epitope, also called an antigenic determinant, is a specific antigen region to which an antibody binds to [5].

In this pathway, an antigen recognized by the BCR must be presented to a T helper cell. The antigen is endocytosed and processed by B cells into small peptides that bind to a protein called major histocompatibility complex II (MHC II) and are presented to a T helper cell through their T cell receptor (TCR) (**Fig. 2**) [8]. This process, called linked recognition, leads to the activation of the T helper cell. Once activated, the T helper cells then provide necessary signals to the B cells, such as cytokines, which promote B cell proliferation and differentiation into plasma cells and memory cells. The resulting antibodies produced have a higher affinity and are longer-lived than those produced through T-independent activation [3, 9].

T-lymphocytes

T lymphocytes, also known as T cells, are also derived from hematopoietic stem cells like B cells, but they mature in the thymus, which is one of the primary lymphoid organs. The primary function of T cells is to support the immune response by assisting phagocytes in the destruction and elimination of infected cells. They also contribute to the production of long-lived plasma cells by assisting B cells [10].

T cells have a restricted specificity for antigens; they only recognize peptides derived from foreign proteins that are bound to MHC molecules. Therefore, T cells only recognize and respond to antigens associated with the cell surface of professional antigen-presenting cells (APCs), not soluble antigens [3]. There are two classes of MHC molecules: MHC class I and MHC class II. MHC class I molecules are found on the surface of all nucleated cells in the body and present antigens from intracellular pathogens. T cells that can recognize antigens presented by MHC I are called cytotoxic T cells. On the surface of APCs, such as dendritic cells, macrophages, and B cells, MHC class II molecules present antigens from extracellular pathogens. T helper cells are T cells that can recognize antigens presented by MHC class II molecules. The presence of the cell-surface proteins CD8 and CD4 assists to distinguish cytotoxic T cells from T helper cells. CD8 and CD4 are known as T cell co-receptors as they work together with the TCR to recognize complexes of peptide antigens and MHC I and MHC II molecules, respectively [1].

The main function of CD8 cytotoxic T cells is to eliminate infected cells caused by a virus, a bacterium, or some other intracellular pathogen. The primary function of CD4 T helper cells is to assist the adaptive immune response by, among other functions, activating B cells by providing essential signals for the activations such as, which leads to the production of antibodies [5, 11]. **Fig. 2** shows the interaction between a B cell and a CD4 T helper cell. CD40 ligand (CD40L), is a protein that is primarily expressed on activated T cells. It binds to CD40 on B cells, triggering a cascade of signaling that provides the necessary signal for further activation, differentiating, and antibody production [5].

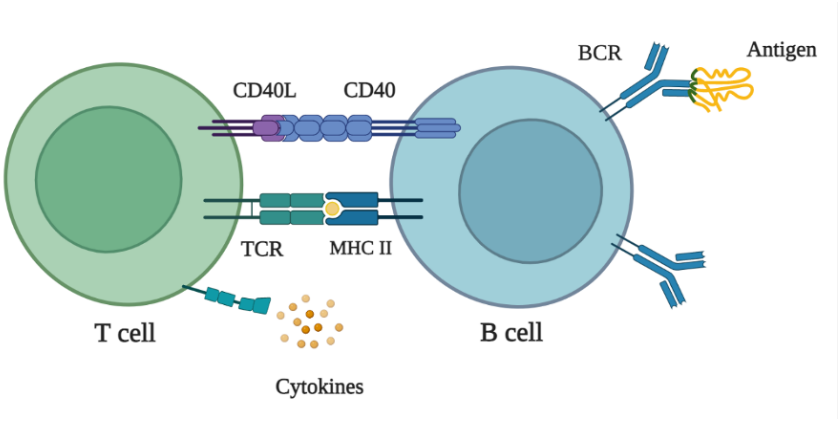

Figure 2: B cell and T cell interaction in linked recognition.
B cell endocytose antigens bind to the BCR and present the processed antigen to T helper cells. When a T helper cell recognizes the peptide presented by the B cell, the CD40 ligand (CD40L) on the T helper cell binds to the B cell's CD40, causing B cell activation. Adapted from "CD40 Ligand Deficiency" by Biorender (2020).

Antibodies

Function

Together with T cells and B cells, antibodies are essential components of the adaptive immune system, working together to produce effective immune responses against extracellular pathogens. Antibodies, also known as immunoglobulins (Ig), are proteins produced by B cells. There are two types of antibodies: membrane-bound antibodies on the B cells as BCR and secreted antibodies [3]. Plasma cells are terminally differentiated B cells dedicated to secreted antibody production. Antibodies are highly specific, and this specificity makes antibodies valuable reagents in various applications (see later).

Antibodies play a crucial role in the immune system by serving multiple functions. The primary functions of antibodies include the neutralization of pathogens, opsonization, and complement activation [12]. Neutralization occurs when antibodies recognize and bind specifically to antigens, forming antigen-antibody complexes. This binding process can prevent the proliferation, replication, or receptor interaction of pathogens with their target cells [13]. In addition to neutralization, antibodies facilitate the phagocytic destruction of extracellular pathogens and toxins through a process called opsonization. Opsonization is particularly effective against pathogens that have a surface containing a limited variety of molecules but a high density of antigenic molecules. When a pathogen's surface has a high concentration or repetition of antigenic molecules, antibodies can bind and coat the entire surface, serving as opsonins [5]. As opsonins, antibodies act as molecular markers or tags that enhance the process of phagocytosis [14]. They act as bridges between the pathogen and phagocytic cells such as neutrophils and macrophages. The Fc region of the antibody, accessible to phagocyte receptors, facilitates the binding of phagocytes to the antibody. This recognition and binding process initiates phagocytosis, resulting in the engulfment and subsequent destruction of the pathogen by the phagocytic cell [5].
Furthermore, when antibodies bind to antigens on the pathogen's surface, it triggers a complement cascade. A series of proteins, is activated, leading to the formation of the Membrane Attack Complex (MAC). MAC is a cytolytic effector of innate and adaptive immunity that causes cell death by forming pores in the plasma membrane of pathogens or targeted cells [15].

Structure

Antibodies are Y-shaped and consist of four polypeptide chains, two heavy chains, and two light chains **(Fig. 3)** [16]. The two heavy chains and the two light chains of an antibody are identical. Disulfide bridges connect the heavy chain with the light chain. In addition, disulfide bridges also connect the two heavy chains [17]. The antibody molecule consists of two functional components: the fragment antigen-binding portion (Fab) and the crystallizable fragment (Fc). The Fab region is located at the tips of the “Y” of the antibody molecule and is responsible for the epitope recognition and binding to the antigen, allowing antibodies to specifically target and neutralize pathogens for example. The Fab region consists of variable domains, known as the variable heavy (V_H) and variable light (V_L) chains, which contribute to the diversity and specificity of antibody molecules. On the other hand, the Fc portion is the

tail region of the antibody and is responsible for mediating the activity of antibodies. The Fc region can interact with various components of the immune system because it contains binding sites for Fc receptors present on the surface of, for instance, phagocytic cells.

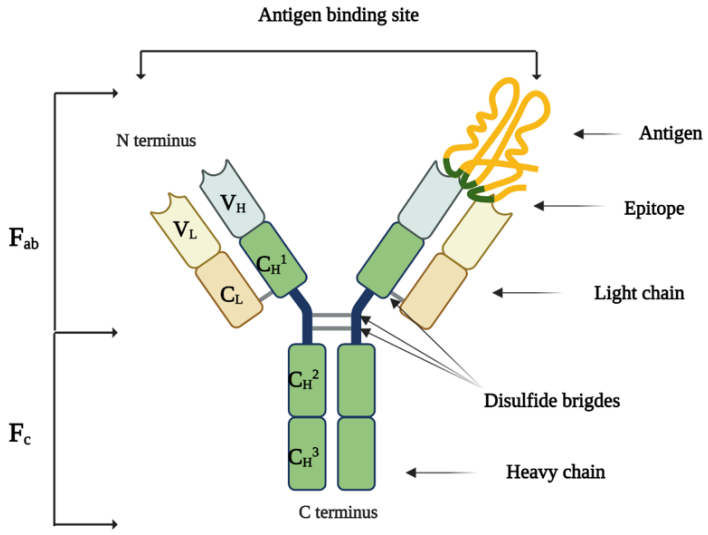

Figure 3: The antibody molecule structure of an IgG
Antibodies are composed of four polypeptide chains, consisting of two heavy chains (green) and two light chains (yellow). Together, these chains form a Y-shaped structure. Each chain is comprised of variable (V) and constant (C) regions. Created with BioRender.com.

Differences in the heavy chain C regions define five classes or isotypes of antibodies IgG, IgM, IgA, IgD, and IgE in humans [5]. Each class has distinct functions and distribution in the body. IgG is the most abundant antibody, while IgM is the first antibody produced during an initial immune response. IgA is found in mucosal areas. IgD is primarily found on the surface of B cells and IgE is involved in allergic responses and defense against helminths [3].

The immune system of the Atlantic cod (*Gadus morhua*)

The adaptive immune system common to all jawed vertebrates arose about 500 million years ago during a period that has been called “the big bang of adaptive immunity” [18]. Thus, all species from sharks to humans share the genes needed to make MHC molecules, B cells, and T cells, and have similar mechanisms for somatic diversification of BCR and TCR genes. Most immunological research has been performed on human subjects or model organisms such as mice. It has been generally assumed that the basic workings of the adaptive immune system are conserved across all jawed vertebrates.

However, in 2011, Bastian Starr and colleagues conducted groundbreaking research on the genomic sequencing of Atlantic cod (*Gadus morhua*), revealing a surprising and unique aspect of its immune system. They discovered that the entire MHC class II locus and CD4 genes were lost in Atlantic cod. Before this discovery, no other jawed vertebrate lacking these genes had been identified [19]. This loss raises questions about the specific immune mechanisms employed by Atlantic cod in recognizing and combating pathogens, particularly those requiring MHC class II-mediated antigen presentation, and how Atlantic cod in their natural environment are not vulnerable to diseases [20]. Even though the fact that the predominant type of Ig in bony fish is IgM, many species can produce effective humoral antibody responses against a variety of antigens. Despite efforts to induce a specific antibody response in cod, such as vaccination or injection with killed pathogenic bacteria, these attempts have been unsuccessful [21]. This finding potentially explains the poor antibody response to pathogen exposure observed in previous studies on Atlantic cod [20]. While there are relatively high levels of natural or non-specific antibodies in serum [21], the absence of the MHC class II genes could hinder the antigen presentation required for effective and specific antibody production through the T-dependent pathway, which depends on MHC class II and CD4 (**Fig. 2**).

Currently, our knowledge about how the cod immune system functions is incomplete. For centuries, Atlantic cod have been an economically important fish species to the countries around the North Atlantic [22] and as the interest in Atlantic cod grows within the aquaculture industry, it is important to have a better knowledge of their unique immune system. This knowledge is important because Atlantic cod, like other farmed fish species, can be exposed to various pathogens in their environment. In addition, the knowledge of the immune system can provide a better understanding of human innate and adaptive immunity.

1.2 Practical uses of antibodies

Monoclonal antibodies and their production

Antibodies can bind with high affinity to a wide range of molecules, making them valuable tools in scientific research and clinical medicine. In natural immune responses, the antibodies produced are polyclonal, meaning they are produced by B cells of different clonal origins, and hence recognize multiple epitopes on a target molecule [5]. Polyclonal antibodies can be useful for various applications; however, they also have limitations. One challenge with polyclonal antibodies is the potential for cross-reactivity, due to the fact they may bind to similar epitopes on unintended targets [3]. To overcome these limitations, César Milstein and George Köhler developed monoclonal antibodies (mAbs) using the hybridoma technique in 1975 [23]. They were awarded the Nobel Prize in 1984 in recognition of their development of the hybridoma technique [24]

The production of mAbs typically follows a series of steps (**Fig. 4**). Initially, an animal, often a mouse, is immunized with the target antigen mixed with an adjuvant, which stimulates an immune response. BCR on B cells in the mouse recognizes and binds to the antigen, activating the production of specific antibodies. These B cells are later from the spleen and fused with immortal myeloma cells, cancerous B cells capable of continuous growth and proliferation. The fusion of these two cell types generates hybrid cells known as hybridomas.

The hybridomas are then cultured in a selective medium called HAT (Hypoxanthine, Aminopterin, and Thymidine). In the HAT medium, cells rely on an alternative survival pathway that requires the presence of the enzyme HGPRT (hypoxanthine-guanidine phosphoribosyl transferase). Under these culture conditions, unfused myeloma cells or hybrids of myeloma cells with myeloma cells cannot survive due to the absence of HGPRT. Similarly, unfused B cells or hybrids of B cells are also unable to survive as they lack the ability for continuous growth. Only the hybridomas produced by the fusion of B cells and myeloma cells contain the HGPRT gene, allowing them to proliferate in the HAT medium [25]. Each hybridoma produces a single Ig, which was derived from a B cell of the immunized mouse. These antibodies secreted by numerous hybridoma clones are then screened for their ability to bind to the target antigen, and the clone with the desired specificity is selected and expanded. The products of these individual clones are mAbs, and each is specific for a single epitope on the antigen used to immunize the mouse [3].

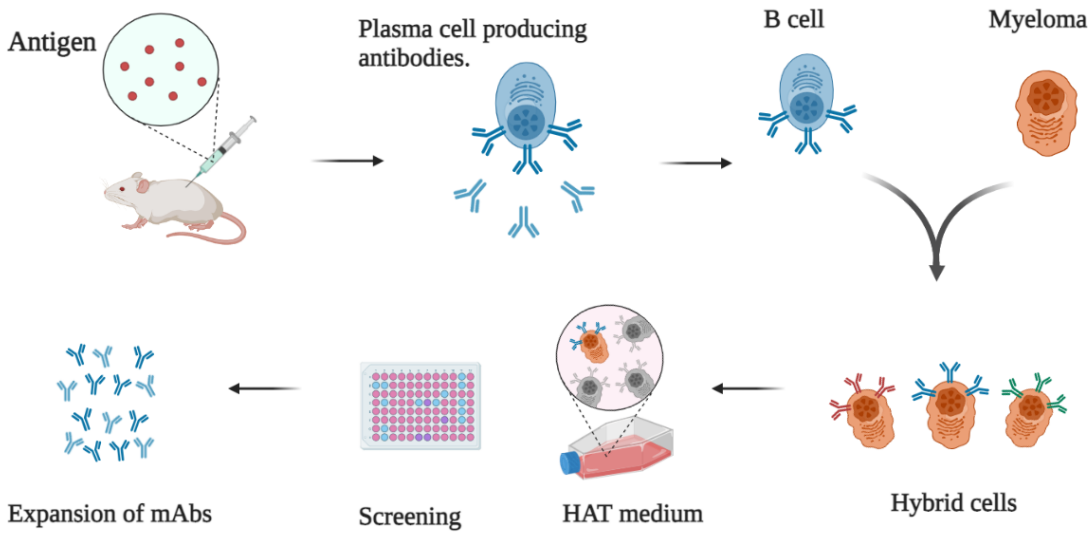

Figure 4: The production of mAbs.
Known antigens mixed with an adjuvant are used to stimulate the immune system of mice through immunization. This causes the B cell to differentiate into plasma cells that produce antigen-specific antibodies. The B cells are then extracted from the spleen of the mouse and fused with the myeloma cell to produce hybrid cells. The hybrid cells are then placed in a hybridoma-specific HAT medium. Hybridomas are then screened for their ability to bind to the antigen. The selected hybridoma clones are then expanded in culture to produce large amounts of mAbs. Adapted from "Monoclonal Antibody Production" by Biorender (2020).

Applications of monoclonal antibodies

MAbs have proven to have high specificity towards specific epitopes, making them a useful tool for various applications in research and medical diagnosis, and therapy.

In biological research, mAbs are an important tool for analyzing the cell surface and secreted molecules, as they stimulate or inhibit cellular functions by binding to cell surface molecules. This knowledge is essential for determining the functions of these molecules and the antigen receptors [3].

For immunodiagnostic purposes, mAbs serve as an important tool in a laboratory setting for biomarker detection, providing information about biological conditions. Techniques such as ELISA, Western blot, and immunohistochemistry are used [26]. Moreover, mAbs are used beyond the laboratory, such as in home-testing kits for pregnancy tests, ovulation testing or COVID-19 testing [27]. These kits can be used to perform tests on body fluids, including blood and urine samples. For instance, mAbs can detect pregnancy as early as a week or two

after conception by binding with human chorionic gonadotrophin (HCG), a hormone produced by the placenta and present in the urine of pregnant women [28].

The advancement of medical research has led to the identification of cells and molecules associated with the pathogenesis of different diseases. Given the specificity of mAbs, they are used to target and interact precisely with these cells and molecules [3]. For immunotherapy, mAbs can be used to interact with specific target molecules to induce various responses, such as apoptosis, inhibition of cell growth, or blocking of other molecules [29].

One notable example of immunotherapy is ipilimumab, a human anti-CTLA4 mAbs that was the first immunotherapy approved by the U.S. Food and Drug Administration (FDA) in 2011 [30]. Ipilimumab works by blocking the cytotoxic T lymphocyte antigen-4 (CTLA4), which is a molecule that inhibits the immune response. CTLA-4 competes with CD28, a co-stimulatory receptor, for binding to B7 (a.k.a. CD80/CD86) on antigen-presenting cells. B7 binding to CD28 activates the T cell, whereas B7 binding to CTLA-4 inhibits the T cell. Thus, ipilimumab will favor B7 interaction with CD28. By doing so, ipilimumab improves the antitumor response and strengthens the persistence of T cell co-stimulation in patients with malignant melanoma [5, 31].

Besides the many applications of mAbs for immunotherapy, there are some limitations due to the immune response that mAbs can induce in humans [3]. As mentioned, the majority of mAbs are produced by immunizing mice. Patients treated with mouse antibodies will make antibodies against the mouse Ig, called human antimouse antibody (HAMA). As a result, patients will have an immune response such as immune complex hypersensitivities, rapid clearance of the antibody, serum sickness, and reduced clinical usefulness [29]. To reduce the generation of HAMAs, genetic engineering techniques have been used to “humanize” mAbs, thereby also expanding their usefulness [3].

In the field of scientific research, the identification of the loss of MHC II and CD4 genes has sparked interest in comparative and evolutionary immunology, as well as in vaccine development and vaccine strategies in cod aquaculture [22]. To support the research of the Atlantic cod immune system and the development of vaccines, high-quality reagents such as mAbs are needed. Reagents can be used to provide valuable insights and knowledge into the

immune response of cod. However, it is important to note that there is currently a shortage of such tools in cod research, highlighting the need for further development in this field.

Immunological assays

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is a technique based on an enzyme-labeled antibody capable of detecting and quantifying an immobilized biomarker of interest. It was first described by Engvall and Perlmann [32]. ELISA relies on antibodies to detect a target antigen through highly specific antibody-antigen interactions. Traditionally, ELISA is performed in 96-well or 384-well polystyrene plates. There are four different types of ELISA: direct, indirect, sandwich, and competitive. In an indirect sandwich ELISA (**Fig. 6**), the target biomarker, or analyte, is captured between two different antibodies: an immobilized capture antibody and a primary detection antibody, creating an "antibody sandwich" [33].

This method begins with the immobilization of the capture antibody onto a solid phase, typically a well plate. This immobilized capture antibody specifically binds to the target analyte present in the analyzed sample. Then, the primary antibody binds to the analyte. To detect the primary antibody, a secondary antibody is added. Typically, the secondary antibody is conjugated to an enzyme, such as horseradish peroxidase (HRP), and detected with e.g., 3,3',5,5'-tetramethylbenzidine (TMB). The HRP conjugated to the secondary antibody catalyzes the oxidation of the TMB substrate, resulting in the development of a blue-colored solution [34].

To stop the oxidation and inactivate the enzymes within the solution, an acid such as sulfuric acid or hydrochloric acid is added. The addition of acid leads to a pH change, causing the protonation of the substrate. This results in the color change from blue to yellow. The intensity of the color is directly proportional to the concentration of the analyte present in the sample. To quantify the concentration of the analyte, the absorbance of the solution at a specific wavelength is measured [34].

To ensure the quantitative nature of the ELISA assay, a set of standards with known concentrations is required. These standards serve as a reference point for the construction of a calibration curve, allowing the precise quantification of the analyte within the samples being analyzed.

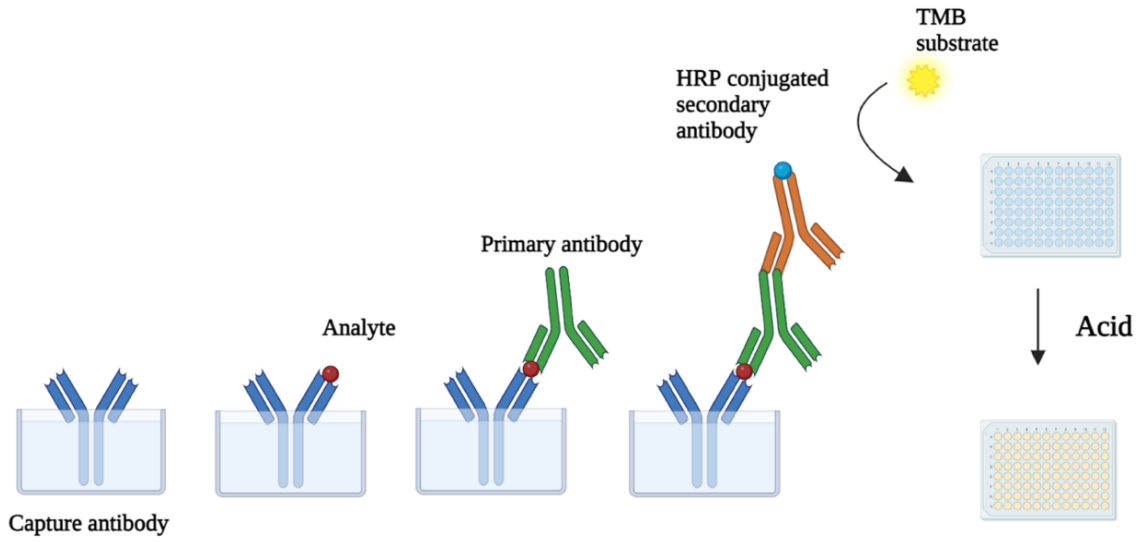

Figure 5: The principle of indirect sandwich ELISA.
The target protein (red) is captured by a capture antibody (blue)and primary antibody (green). The secondary antibody is HRP-conjugated (orange) and is used for detection. HRP oxidizes the TMB substrate causing the solution to become blue, and after the addition of acid the blue color changes to yellow. Created with BioRender.com.

Competitive ELISA

The competitive ELISA can be used to determine whether two antibodies compete for the binding to the same epitope. It follows the same principles as the indirect sandwich ELISA already described [34]. In this assay, biotin is commonly used to label primary antibodies. Biotin has a high affinity for streptavidin, which can be conjugated to HRP, enabling a color reaction [35].

When both the unlabeled antibody and the biotinylated antibody recognize and bind to the same antigen binding site, a competition to bind to the epitope occurs **(Fig. 6, b)**. When competition occurs, the biotinylated antibody is unable to bind effectively due to the presence of the unlabeled antibody, resulting in fewer biotinylated antibodies being bound and a weakened signal [36]. However, if the two antibodies recognize and bind to different binding sites on the antigen, both antibodies can bind simultaneously (**Fig. 6, a**). As a result, the signal is not affected by the unlabeled antibody.

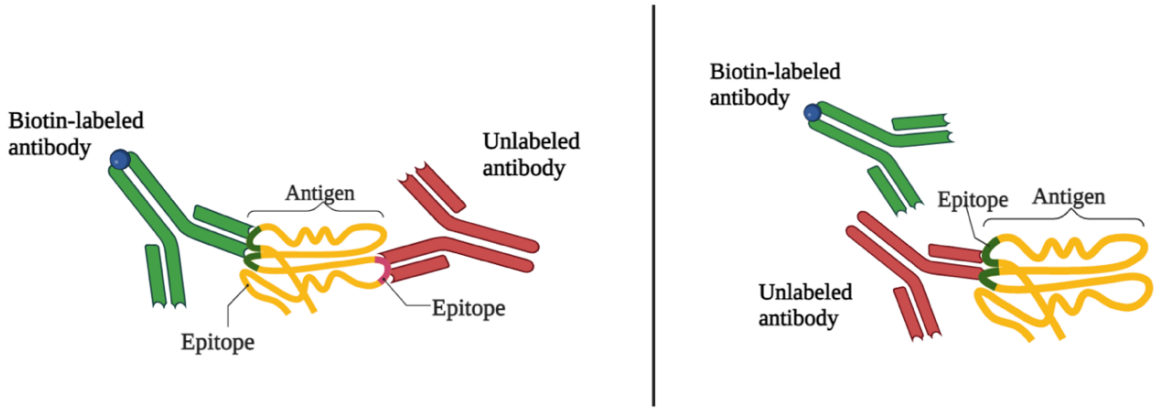

Figure 6: The principle of competitive ELISA using a biotinylated antibody
The biotinylated and unlabeled antibodies are both added to the well. To the right, the two antibodies do compete, and the unlabeled binds to the binding site of the analyte, inhibiting the biotinylated antibody from binding. To the left, the biotinylated antibody is bound because the two antibodies don't share the same binding site. Created with BioRender.com.

Western blot

Western blot (WB) is a common technique used in molecular biology and biochemistry that was introduced in 1979 by Neal Burnette [37]. The purpose of WB is to detect and analyze specific proteins within a sample, as well as to determine their size. It is particularly valuable for studying protein expression levels and the localizations of target proteins. The technique consists of two main steps: gel electrophoresis and immunoblotting.

The first step of WB involves the separation of target proteins within a sample through electrophoresis, typically using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [38]. This separation is based on the molecular weight and charge of the proteins. SDS-PAGE involves the use of Sodium Dodecyl Sulfate (SDS), which is a denaturing detergent. SDS binds to proteins, unfolding them and imparting a negative charge. When the SDS-treated proteins are loaded into the polyacrylamide gel, an electric field is applied, causing the negatively charged proteins to migrate through the gel matrix.

During electrophoresis, the proteins separate based on their size, as the smaller proteins move more quickly through the gel, while larger proteins migrate more slowly [39]. This separation results in distinct bands representing different proteins within the sample.
To estimate the molecular weight of the target protein, molecular weight markers are used. These markers consist of proteins with known sizes and are run alongside the sample proteins.

By comparing the migration distance of the target protein to that of the molecular weight markers, the approximate molecular weight of the target protein can be determined.

After gel electrophoresis, the proteins are transferred from the gel onto a supporting matrix, e.g., nitrocellulose (NC) membrane or polyvinylidene fluoride (PVDF) membrane [38]. To detect the target protein on the membrane, the membrane is probed with a primary antibody that specifically recognizes and binds to the target protein of interest. To visualize the presence of the primary antibody, a secondary antibody is added. As in ELISA, the secondary antibody is also conjugated to an enzyme, such as HRP. To generate a detectable signal, a chemiluminescent substrate is added to the membrane. The HRP conjugated to the secondary antibody catalyzes the oxidation of the substrate, resulting in the production of the light-emitting pathway [38]. This signal can be captured and visualized using a light-sensitive camera.

Immunohistochemistry

Immunohistochemistry (IHC) is a widely used technique in biological and medical research for identifying and visualizing specific protein localization within cells, both at the cellular and subcellular levels, in tissue samples. IHC involves the use of antibodies that specifically recognize and bind to the target protein of interest within the tissue.

The steps of IHC with fixed-frozen tissues involve cryoprotection, sample preparation, embedding, sectioning, blocking, antibody incubation, and mounting **(Fig. 7)**. During the sample preparation, the tissue is preserved with formaldehyde to preserve its morphology. The sample is then embedded in an embedding medium, such as OCT (Optimal Cutting Temperature), which facilitates cryosectioning. The embedding medium is applied around the sample to create a block, which is then rapidly frozen with liquid nitrogen. After the sample has been frozen, it is sectioned using a cryostat and positioned on a microscope slide. The sections are then incubated with primary antibodies specific to the protein of interest. To visualize the bound primary antibody, a secondary antibody is applied. The secondary is conjugated to a detectable marker, such as an enzyme (e.g., HRP) or/and a fluorescent dye. When the samples are excited by a light at a specific wavelength range, a fluorescent molecule emits light at longer wavelengths, which a microscope can detect.

In addition to the secondary antibody, multiple stains can be applied in IHC to aid with orientation and enhance the visualization of specific proteins within the tissue. These additional stains can provide valuable information about cellular and tissue architecture, as well as identify different cell types or subcellular structures. For example, DAPI is a fluorescent dye that binds to DNA, allowing the cell nucleus to be visualized. Phalloidin is a fluorescent dye that binds to actin filaments, allowing the cytoskeleton of a cell to be visualized. Before examining the stained tissue sections under a microscope, tissue samples are mounted with a protective mounting medium [40].

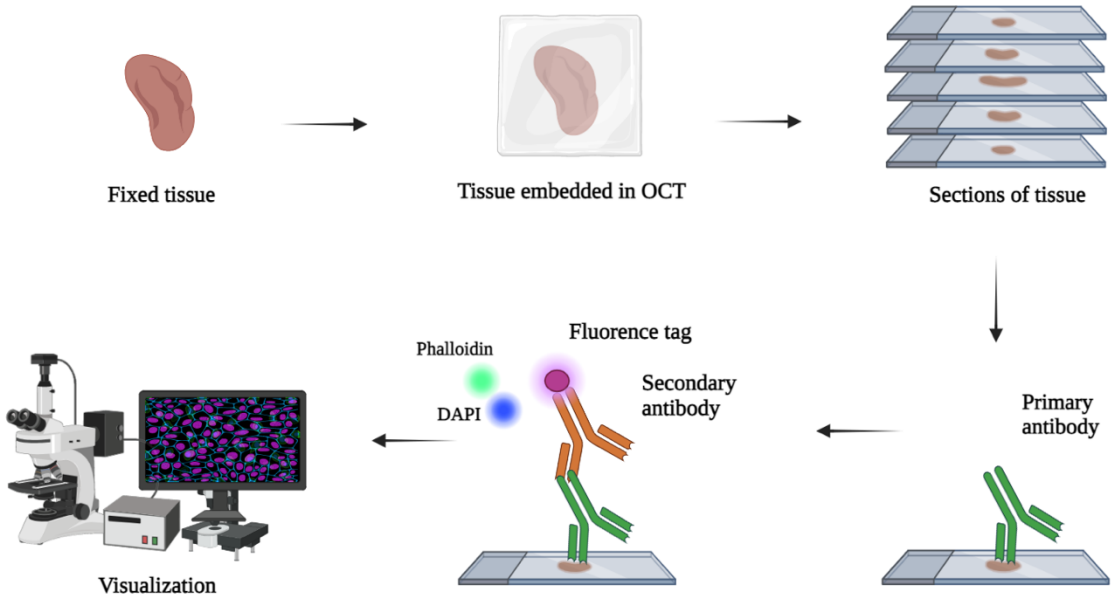

Figure 7: Principle of IHC.
After the tissue has been fixed, it is embedded in an OCT medium and sectioned before being stained with primary and secondary antibodies. Adding multiple stains together with the secondary antibody allows the detection of multiple targets within the same tissue. Created with BioRender.com.

2. Aim of the thesis

Recently, the group of Qiao and Johansen have developed several mouse IgG mAbs (Appendix) and a chicken IgY polyclonal antibody against cod IgM. These reagents will be a major asset to the research community and aquaculture industry. The preliminary result with these reagents is promising. However, to fully use these antibodies for immunological studies in cod, further characterization is needed. Once fully characterized, these antibodies could be used to study the immune response of Atlantic cod and bring the development of a vaccine for the Atlantic cod one step closer.

Thus, the aim of this project is:

1. Determine optimal conditions for the use of the antibodies in immunological assays such as Western blot, ELISA, and Immunohistochemistry
2. To determine where on the cod IgM molecule the established reagents bind. Whether each reagent binds to the light chain or the heavy chain and if they bind to the same epitope on IgM.

3. Methods

3.1 Sandwich ELISA protocol

To determine the usefulness of the four mAbs (Appendix) in ELISA and which concentration is needed to give an optimal signal-to-noise ratio compared to an "in-house" mouse polyclonal antibody mix, designated pAb in this thesis. A chicken IgY anti-cod IgM (commissioned production by NABAS; https://www.nabas.no/; concentration 20 mg/mL) was used as a capture antibody. The primary detection antibodies mAbs 1.1, 9.2, 13E4, and 19.3 were purified from hybridoma supernatants by protein G affinity columns prior to the work in this thesis. The secondary antibody was HRP-conjugated goat anti-mouse (Thermo Fisher Scientific, G-21040).

Coating: The 96-Well Microtiter Microplates (Thermo Fisher Scientific, 442404) were coated with 150 µL/well chicken IgY anti-cod IgM diluted 1:4000 in carbonate buffer (pH 9.6) or phosphate-buffered saline (PBS) and incubated overnight at room temperature. *Blocking:* The coating solution was removed, and the plate was blocked with 200 µL/well PBS containing 5% skim milk for a minimum of one hour at room temperature. The plate was blocked to reduce background interference and prevent unspecific binding. From here, all incubation was done in ELISA buffer (PBS + 0.05% Tween-20 (PBS-T) + 0.5% skim milk), and the plates were washed three times in PBS-T using an automated plate washer (Thermo Fisher Scientific) to remove unbound antibodies between each step. *Sample addition*: The blocking solution was removed, and the plate was incubated with 100 µL/well diluted cod serum in triplicates and incubated overnight at room temperature on a plate shaker set to 30 rpm. A 3-fold dilutions series was used, starting with 1:9000 as the least diluted samples. *Primary detection antibody:* Primary antibodies (mAb) were diluted in ELISA buffer to concentrations of 10, 3, and 1 µg/mL and incubated for one hour at room temperature on a plate shaker at 30 rpm. *Secondary antibody:* Secondary antibody, HRP-conjugated goat anti-mouse was diluted 1:2000 in ELISA buffer and incubated for one hour at room temperature. *Substrate reaction:* After the final wash, 50 µL/well TMB substrate (Thermo Fisher Scientific, 34022) was added to the plates. The plates were placed on a plate shaker at 50 rpm and inspected regularly for color development. When a clear blue color appeared in the strongest wells (after about 3 minutes), the reaction was terminated by the addition of 50 µL/well 1M HCl or 50 µL/well 0.8 M H_2SO_4. The plates were read in a plate reader (Thermo Fisher Scientific) with Ascent

software at 450 nm wavelength. OD_{450} values from negative control wells (without sample) were subtracted from all OD values before analysis in Microsoft Excel or GraphPad Prizm.

3.2 Biotinylation of mAbs protocol

Biotinylation was performed according to the manufacturer's protocol (Abcam, ab201795). A maximum of 100 µg mAb was used and PBS was used as diluent when necessary.

Briefly, 9 µL of modifying reagent was added to 81 µL of mAb. The mixture was added to one vial containing Biotin Conjugation Mix and was incubated for 15 min at room temperature before the reaction was stopped with 10 µL of quencher.

3.3 Competitive ELISA protocol

The coating, blocking, serum, substrate, and stop solution incubation protocols for competitive ELISA were the same as described under section 4.1 Sandwich ELISA.

Primary detection antibody: The serum samples were removed, and the plate was washed. The unlabeled antibodies were diluted in ELISA buffer to concentrations of 20, 6, 2, and 0 µg/mL. The biotinylated antibodies were diluted in ELISA buffer to 2 µg/mL. The biotinylated antibodies were added to the different unlabeled antibodies at a 1:1 ratio and mixed well so the final concentrations of unlabeled antibodies were 10, 3, or 1 µg/mL and the final concentration of biotinylated antibody was 1 µg/mL. 80 µL/well of this mixture of unlabeled and biotinylated mAbs were added to the plate and incubated for one hour at room temperature on a plate shaker at 30 rpm. *Secondary antibody:* After the plates were washed, 100 µL/well of Streptavidin-HRP (Thermo Fisher Scientific) diluted 1:2000 in ELISA buffer were added and incubated for one hour at room temperature.

3.4 Antigen-specific ELISA

To determine the usefulness of clone 19.3 in an antigen-specific ELISA, an indirect sandwich ELISA was performed. The plate was coated with 4-Hydroxy-3-nitrophenylacetic (NP) hapten conjugated to BSA (Bovine serum albumin). Serum samples added were made from a pool of sera from Atlantic cod immunized with NP-ficoll (gift from A.L. Porras). The

blocking, secondary, substrate, and stop solution incubation protocols are the same as described under 3.1 Sandwich ELISA.

Coating: The 96-Well Microtiter Microplates were coated with 150 µL/well with antigen NP-BSA (1 mg/ml, Biosearch Technologies, N-5050XL-10) and diluted in 1:100 with carbonate buffer and incubated overnight at room temperature. *Sample addition:* The block solution was removed and 150 µl/well of the NP pool serum (In house) and naïve serum (In house) were added to the plate in triplicates. A 3-fold dilutions series was used, starting with 1:10 as the least diluted samples. The plate was incubated overnight at room temperature. *Primary detection antibody:* The plate was washed. The primary detection antibody (19.3) was diluted in ELISA buffer to 10, 3, and 1 µg/mL. 100 µL/well of diluted 19.3 was added to the plate and was incubated for one hour at room temperature.

3.5 Western blot protocol

Sample preparation: The sample for WB analysis was prepared by adding 64 µL PBS, 1 µL normal cod serum, 10 µL 1 M DTT (Dithiothreitol), and 25 µL loading buffer (Thermo Fisher Scientific, B0007) and mixed well. The sample was heated to 75 °C for 10 minutes. *Gel electrophoresis:* 10 µL of the protein standard (Merck, RPN800E) was loaded into the first well of 4-12% Bis-Tris Plus mini gel (Thermo Fisher Scientific, NW04125BOX). 10 µL of the prepared samples were added to every second well of the gel. To separate the proteins, the gel was run at 200 volts until the dye front reached the bottom of the gel. *Blotting:* Following electrophoresis, the PVDF membrane (Merck, IPVH00005) was briefly activated with 100% ethanol. It was then washed three times with milliQ water and equilibrated in a transfer buffer (Thermo Fisher Scientific, 20x NuPAGE Transfer buffer, NP00061). The gel and filter papers (Merck Millipore) were soaked in transfer buffer for 10 minutes to equilibrate. The PVDF membrane, the gel, and the filter papers were placed to form a "sandwich" **(Fig 8)**. A rolling tube was used to gently remove the air bubbles between each layer and ensure proper contact. Finally, the sandwich was placed in the semi-dry blotter (Trans-Blot Turbo^{TM}). The proteins were transferred from the gel to the membrane by applying a constant voltage of 20 V and a current of 0.5 A for 20 minutes.

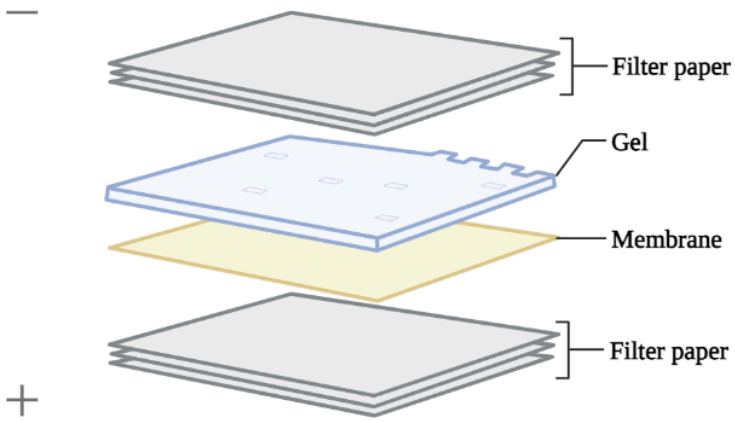

Figure 8: WB sandwich
The gel and membrane are layered to form a "sandwich" between three sheets of filter paper, which protect the gel and membrane and ensure that their surfaces remain in close contact. Placing the PVDF membrane between the gel and the positive electrode allows the negatively charged protein to migrate from the gel onto the PVDF membrane. Adapted from "Membrane Transfer Schematics" Biorender (2022).

Immunodetection: For immunodetection, the blocking, incubations, and washings were all done on a rocking platform. The PVDF membrane was blocked in PBS containing 5% skim milk for one hour at room temperature. After blocking, the membrane was divided into six sections and placed in six separate 15 mL Falcon tubes. This was done because each section was incubated with different primary antibodies. For each Falcon tube, 5 mL of the specific primary antibodies were diluted in PBS-T with 0.5% skim milk. The primary antibodies included the four mAbs and two chicken pAbs. The membrane sections were incubated for one hour at room temperature. Following incubation, the membrane section was washed three times for five minutes each with PBS-T. Then the secondary antibodies were added. The four mAbs were detected using HRP goat anti-mouse (1:10 000, Thermo Fisher Scientific, G-21040), and the two pAbs were detected using HRP goat anti-chicken (1:5000, Thermo Fisher Scientific, A16054). Both secondary antibodies were diluted in PBS-T and 0.5% skim milk. The membrane sections were incubated with secondary antibodies for one hour. After the final wash, the membrane sections were placed together in a black box, and the ECL substrate (Merck, GERPN2109) was added for chemiluminescent detection. Imaging was performed using a light-sensitive camera (BioRad Touch Chemidoc).

3.6 IHC protocol

For this study, the spleen of a euthanized adult cod was collected and previously fixed in a solution of methanol-free formaldehyde 4% in HEPES 60 mM (pH 7.2) for a week. Since the fixation is done to preserve the tissue and minimize degradation of tissue, the fixation is typically performed at 4 °C. The tissue was washed three times for 15 minutes each with HEPES 60mM and washed with dH_2O for 5-10 minutes at room temperature. The sample was then cryoprotected with a solution of sucrose 30% until the specimens sunk to the bottom of the recipient. Then the sample was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, 4583) in a cryomold and placed in a beaker containing isopentane (2-methyl butane) that was cooled with liquid nitrogen until the OCT is completely white and frozen. The samples were stored at -20 °C.

Sectioning: The frozen sample was processed using a cryostat (Leica biosystems, CM1950) at -18 °C to produce serial 30-µm thick cryosections that were collected on a microscope slide (SuperFrost Plus, Thermo Fisher Scientific). Since the blocking step was not performed on the same day, the slides were stored at -20 °C to preserve the samples. *Blocking:* The samples were first washed twice for 10 minutes each using 1x PHEM buffer to remove the OCT medium. Then the samples were blocked by adding 180 µL of blockaid solution (Thermo Fisher Scientific, B10710) per section and incubated for one hour at room temperature. *Primary detection antibody:* The blockage was removed by tilting the microscope slide allowing the liquid to drain. The primary antibodies were diluted in 1x PHEM buffer to the following concentrations: 10, 3, and 1 µg/mL. 180 µL of primary antibodies to each section were added and incubated for one hour at room temperature. *Secondary antibody:* The secondary antibody, Goat anti-mouse, Alexa Fluor 647 (Jackson Immunoresearch, AB_2338902) was diluted in a ratio of 1:250 in 1x PHEM buffer. 180 µL of the secondary antibodies were added to the sections. Additionally, the color stains such as DAPI (Thermo Fisher Scientific, D1306) and fluorescent Phalloidin-FITC (Sigma Aldrich, P5282) were added to the mix containing the secondary antibody. DAPI was diluted at a ratio of 1:200 in 1x PHEM, and Phalloidin was diluted at a ratio of 1:100. The tissue section was incubated for one hour at room temperature. After the incubation of secondary antibodies, the sections were washed three times with 1x PHEM buffer for 15 minutes each. Lastly, the glass slides were mounted with prolong-glass mounting medium (Thermo Fisher Scientific, P36984) and cured for 24h at room temperature, and stored at 4 °C to preserve the sample before imaging.

Imaging and analysis were performed at the NorMIC imaging platform using a Dragonfly 500 spinning-disk confocal microscope (Oxford instruments) and the image analysis software Fiji.

4. Results

4.1 ELISA analysis

Comparison of four mAbs and mouse pAbs in indirect sandwich ELISA

To determine the usefulness of the different mAbs in ELISA and which concentration is needed to give an optimal signal-to-noise ratio, a comparative analysis was performed using all four mAbs as primary detection antibodies in four different indirect sandwich ELISAs. The mAbs were tested at concentrations of 10, 3, and 1 µg/mL, and their signal generated was compared to a polyclonal mouse antibody cocktail **(Fig. 9)**.

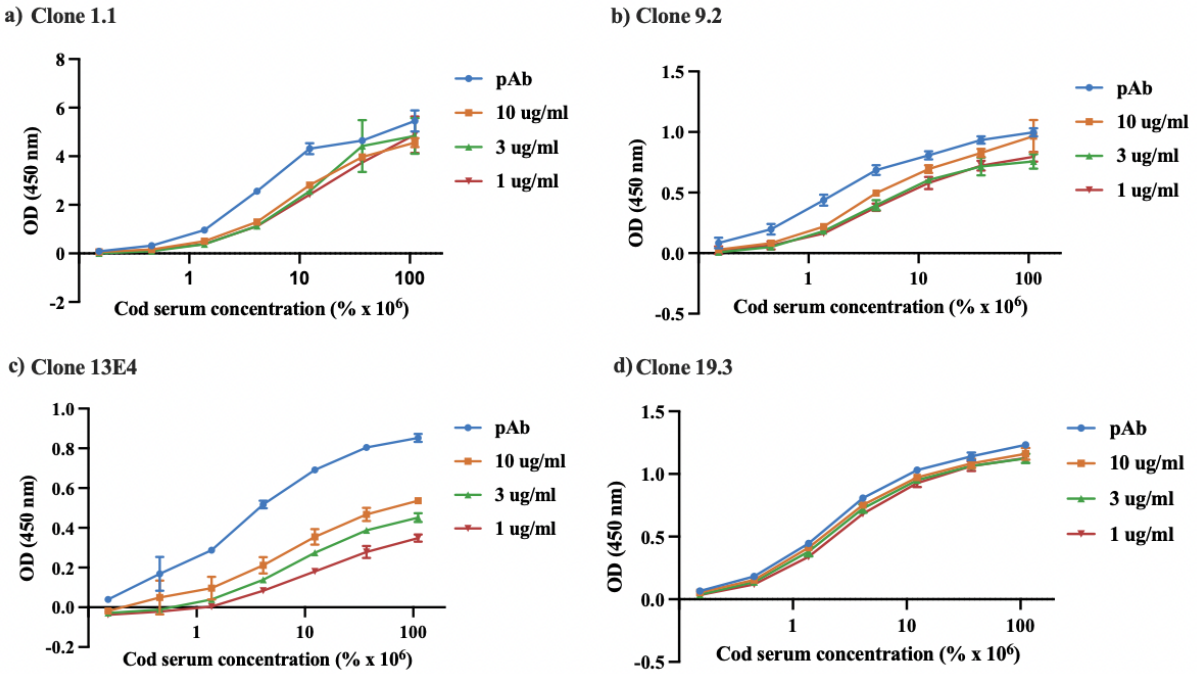

Figure 9:Indirect ELISA analysis of the four mAbs against mouse pAbs.
The figure shows aligned graphs representing the outcome of an indirect ELISA used to study the usefulness of the four mAbs in producing a signal compared to the mouse pAbs. The x-axis represents the concentration of the cod serum in %x10⁶, while the y-axis represents the optical density (OD) value. In the figure, graph a) represents the results for clone 1.1, b) corresponds to 9.2, c) indicates clone 13E4, and d) corresponds to clone 19.3. Blue represents mouse pAbs; orange represents 10 ug/mL of mAb; green represents 3 ug/mL of mAb; and red represents 10 ug/mL of mAb. The error bars show the standard deviation for each data set.

Optimizing dilutions of clone 19.3

Due to its strong signal observed in comparison to the mouse pAbs, 19.3 was selected for further development and improvement. As a result, several dilutions were carried out. **Fig.10, a** illustrates the repeated experiment with 10, 3, and 1 µg/mL of 19.3. **Fig. 10, b** illustrates the result when diluting 19.3 to concentrations of 0.33 and 0.11 µg/mL.

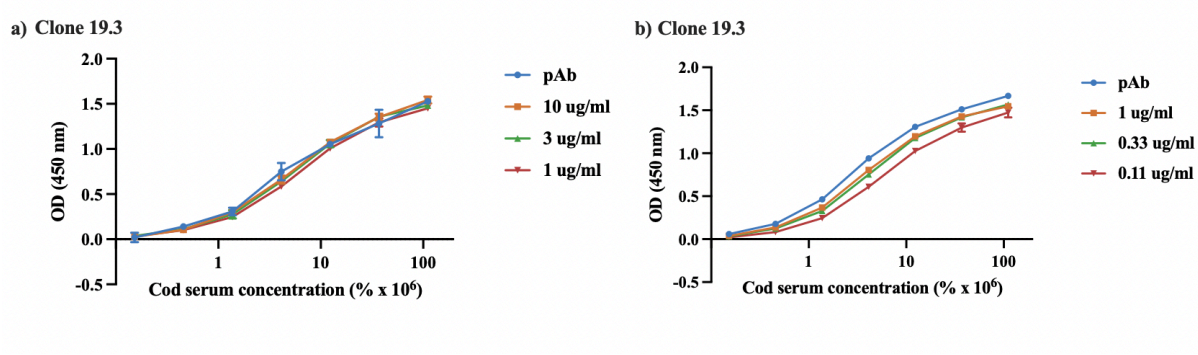

Figure 10: Indirect ELISA Analysis of Diluted 19.3.
The figure shows aligned graphs representing the results of an indirect ELISA with various dilutions of 19.3 compared to a mouse pAb. a) 19.3 at concentrations of 10, 3, and 1 µg/mL with signal intensities comparable to the SN. Blue represents mouse pAbs; orange represents 10 µg/mL of mAb; green represents 3 µg/mL of mAb; and red represents 10 µg/mL of mAb. The error bars show the standard deviation for each data set.

Titration of IgY as coat

A titration experiment was performed to determine the optimal dilution factor for optimal signal detection of chicken IgY anti-cod IgM for coating purposes in ELISA with the 19.3 mAb. The titration findings are shown in **Fig. 11**.

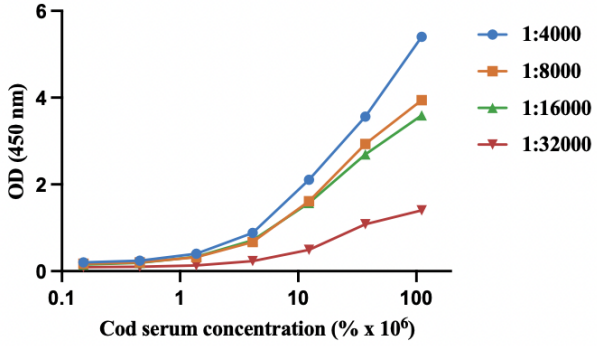

Figure 11. Titration of Chicken IgY Anti-Cod IgM for ELISA Coating.
The figure presents the results of a titration experiment performed to determine the optimal concentration of chicken IgY anti-cod IgM for coating purposes in ELISA. The x-axis represents the concentration of the cod serum in %x10⁶, while the y-axis represents the optical density (OD) value. Blue indicates 1:4000, orange indicates 1:8000, green indicates 1:16000, and red indicates 1:32000. The error bars represent the standard deviation associated with each data point.

Purified IgM analysis

To determine the sensitivity of the ELISA (in ng/mL), clone 19.3 were tested against purified IgM of known concentration (4.10 mg/mL) with the mouse pAbs as a reference. The concentration tested for 19.3 were 10 and 1 µg/mL.

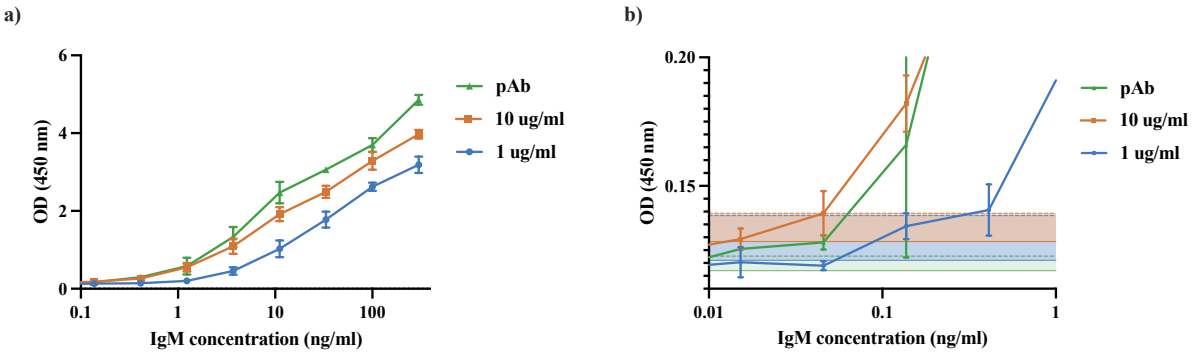

Figure 12: Purified IgM Analysis.
The x-axis in these graphs represents the IgM concentration in ng/mL, while the y-axis represents the optical density (OD) value. Graph a) shows the results of an indirect sandwich ELISA in which purified IgM was detected at two different concentrations of 19.3: 10 µg/mL (indicated in orange) and 1 µg/mL (indicated in blue). In addition, the mouse pAbs (indicated in green) were used, b) the graph is zoomed in to provide a more detailed view of the data. The average background signal (solid line), along with two standard deviations (dashed line), for 1 µg/mL, 10 µg/mL, and pAbs were added in their respective colors. The error bars represent the standard deviation associated with each data point.

Competitive ELISA

A competitive ELISA was performed to determine if the mAbs bind to the same epitope. The biotinylated mAbs 1.1, 13E4, and 19.3 were tested against their respective unlabeled antibodies. **Table 1** provides additional information on the specific antibody pairings tested in the competitive ELISA.

Table 1: A schematic table demonstrating the pairing of biotinylated antibodies.
The three biotinylated mAbs with their corresponding unlabeled antibodies for the competitive ELISA.

	Biotinylated 1.1	**Biotinylated 13E4**	**Biotinylated 19.3**
Unlabeled 1.1	X		
Unlabeled 9.2	X	X	X
Unlabeled 13E4	X	X	
Unlabeled 19.3	X	X	X
	Shown in Figure 13	Shown in Figure 14	Shown in Figure 15

Biotinylated 1.1

To determine if any of the four unlabeled mAbs compete with the biotinylated 1.1 for binding to the same epitope, the four mAbs were tested against the biotinylated 1.1.

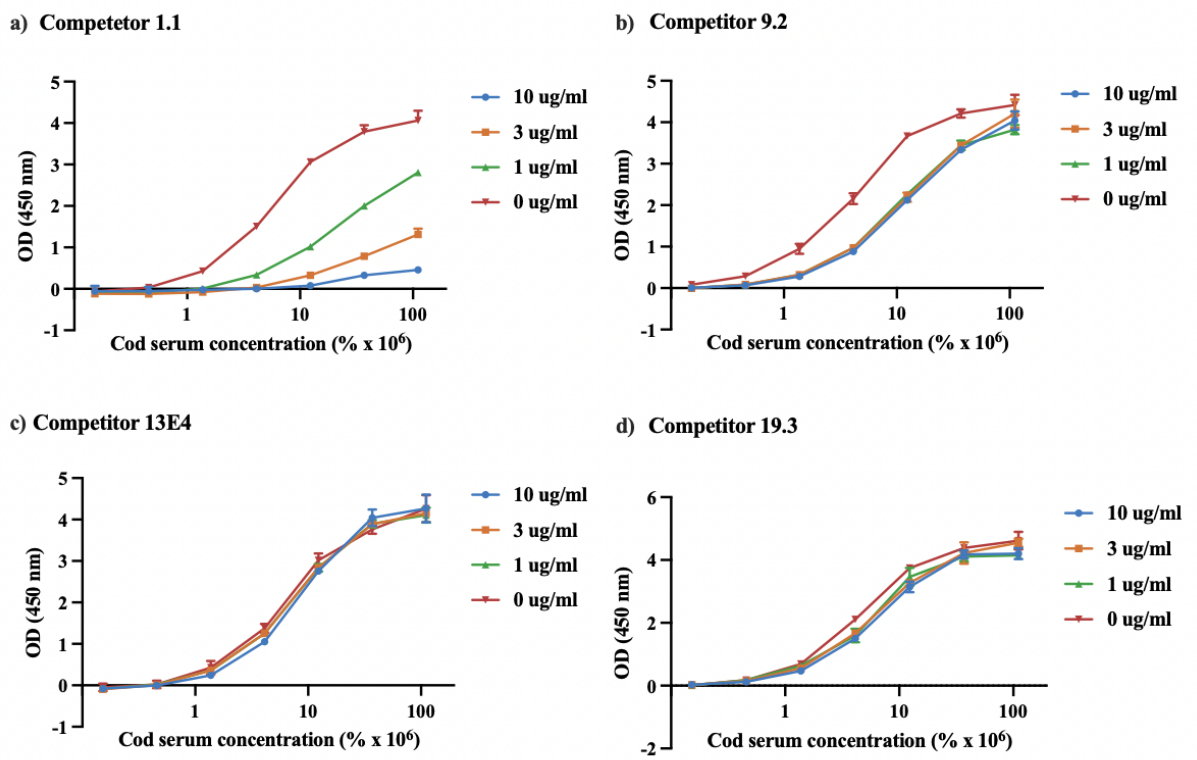

Figure 13: Analysis of biotinylated-1.1 in Competitive ELISA.
The figure shows aligned graphs representing the result of a competitive ELISA using biotinylated 1.1. a) Biotinylated 1.1 competes with itself for binding to IgM. b) Indicates the result of biotinylated 1.1 with unlabeled 9.2, c) Presents the result of unlabeled 13E4 and biotinylated 1.1, d) Result of biotinylated 1.1 with 19.3. Blue represents 10 µg/mL of unlabeled mAb; orange represents 3 µg/mL of unlabeled mAb; green represents 1 µg/mL of unlabeled mAb; and red represents absence (0 µg/mL) of unlabeled mAb. The error bars represent the standard deviation associated with each data point.

Biotinylated 13E4

The competitive ELISA with biotinylated 13E4 was tested against unlabeled 19.3 and 9.2, and the results are presented in **Fig. 14**.

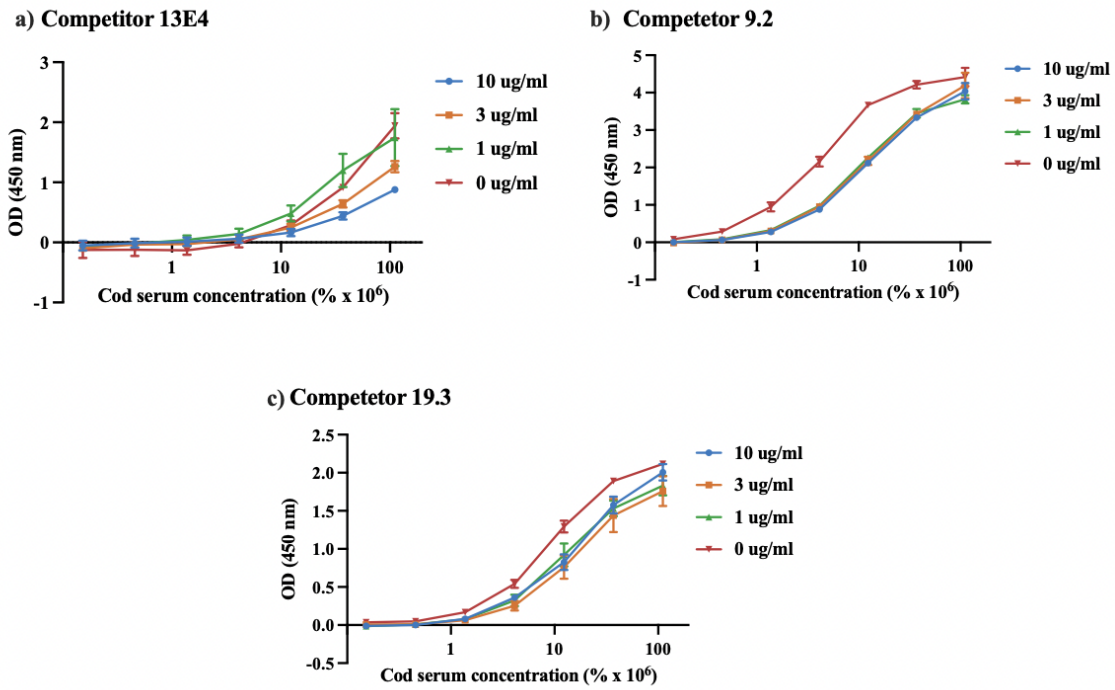

Figure 14:Analysis of Biotinylated -13E4 in Competitive ELISA.
The figure shows aligned graphs representing the result of a competitive ELISA using biotinylated 13E4. a) shows the result of biotinylated 13E4 against unlabeled 13E4, b) indicates biotinylated 13E4 against unlabeled 9.2, and c) corresponds to biotinylated 13E4 against unlabeled 19.3 Blue represents 10 µg/mL of unlabeled mAb; orange represents 3 µg/mL of unlabeled mAb; green represents 1 µg/mL of unlabeled mAb; and red represents absence (0 µg/mL) of unlabeled mAb. The error bars represent the standard deviation associated with each data point.

Biotinylated 19.3

Biotinylated 19.3 was tested against unlabeled 19.3 and 9.2 (**Fig. 15)**.

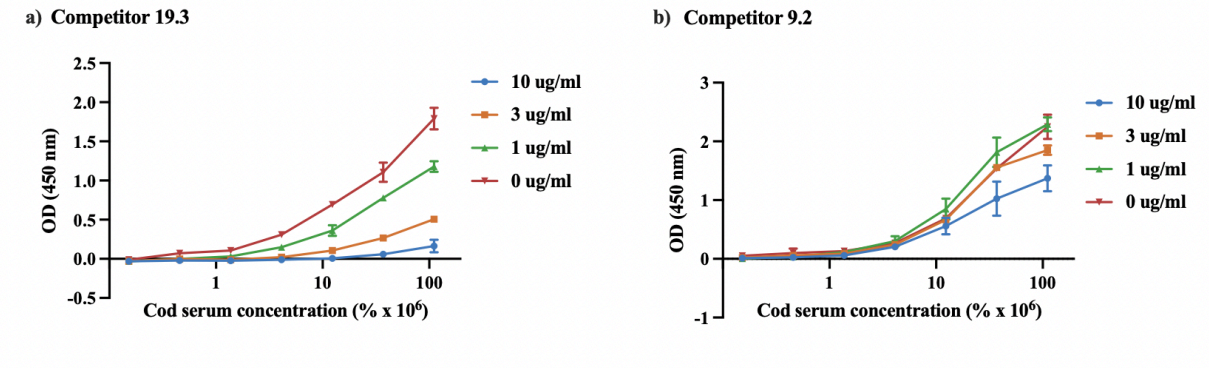

Figure 15:Analysis of Biotinylated 19.3 in Competitive ELISA.
The figure shows two aligned graphs representing the result of a competitive ELISA using biotinylated 19.3. a) shows the result of biotinylated 19.3 against unlabeled 19.3, b) indicates biotinylated 19.3 against unlabeled 9.2. Blue represents 10 µg/mL of unlabeled mAb; orange represents 3 µg/mL of unlabeled mAb; green represents 1 µg/mL of unlabeled mAb; and red represents absence (0 µg/mL) of unlabeled mAb. The error bars represent the standard deviation associated with each data point.

Antigen-specific ELISA

An antigen-specific ELISA was performed using NP-BSA as a coat. In addition, the NP pool and the cod serum (naïve) were used as a sample.

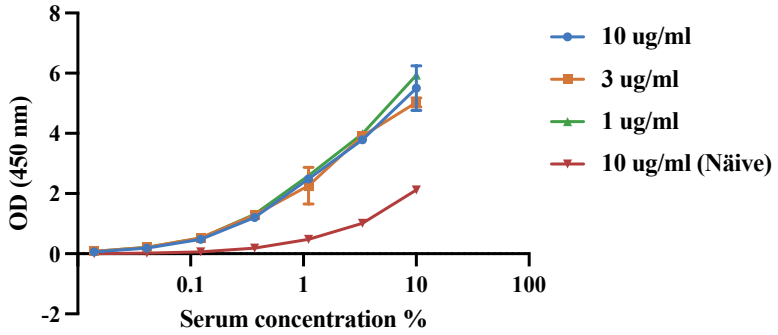

Figure 16: Analysis of Antigen-Specific ELISA.
The figure shows the result of an antigen-specific indirect sandwich ELISA. As primary detection antibodies, concentrations of 19.3 at 10 µg/mL (in blue), 3 µg/mL (in orange), and 1 µg/mL (in green) were used. In addition, 10 µg/mL of 19.3(in red) was added to the naïve serum.

4.2 Western blot analysis

To determine if the mAb could recognize denatured cod IgM and identify if they bind the heavy or light chain, we performed WB on normal serum from Atlantic cod. The serum was reduced and denatured with DTT and SDS, respectively, and separated on 4-12% polyacrylamide gel in six identical lanes before transfer to the PVDF membrane. After blocking, the membrane was cut longitudinally and each lane was probed with a different mAb, the chicken IgY anti-Cod IgM and non-immune IgY. Only 19.3 and the chicken IgY anti-cod IgM gave positive signals in the WB. Based on the size of the revealed bands, we determined that mAb 19.3 bound the IgM heavy chain while the chicken IgY anti-cod IgM bound both heavy and light chains.

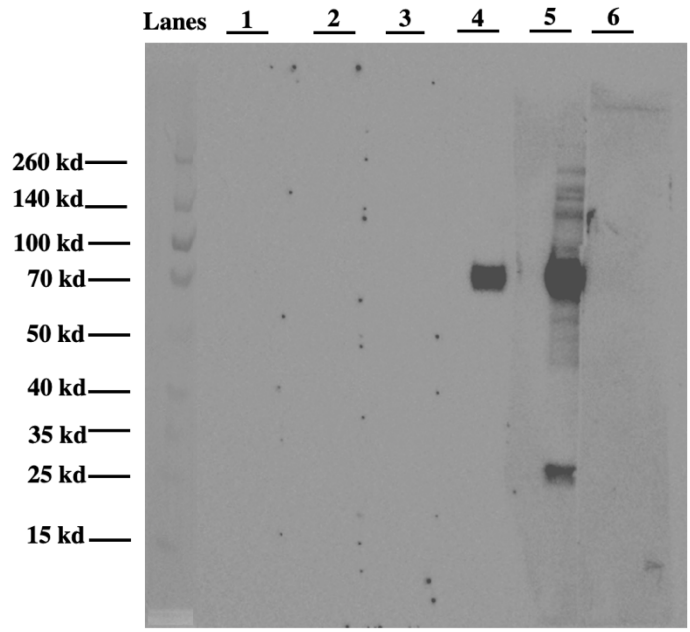

Figure 17: WB Analysis of The Binding of mAbs to IgM.
After immunodetection and imaging, three bands are observed. Lane 1) Clone 1.1, Lane 2) Clone 9.2, Lane 3) 13E4, Lane 4) Clone 19.3, Lane 5) chicken IgY anti-cod IgM and Lane 6) chicken non-immune IgY.

4.3 Immunohistochemistry

In order to determine the suitability of the different antibodies for IHC, three concentrations of the mAbs were tested on fixed-frozen spleen tissue to identify the optimal concentration giving the best signal-to-noise ratio: 1, 3, and 10 µg/mL. The results for all three concentrations of mAbs, together with a graph illustrating the relationship between the concentrations and the staining pattern, are presented in the figures. **Fig. 18** represents the results for clone 1.1, **Fig. 19** demonstrates the findings for clone 9.2, **Fig. 20** showcases the results for clone 13E4, and **Fig. 21** presents the results for clone 19.3.

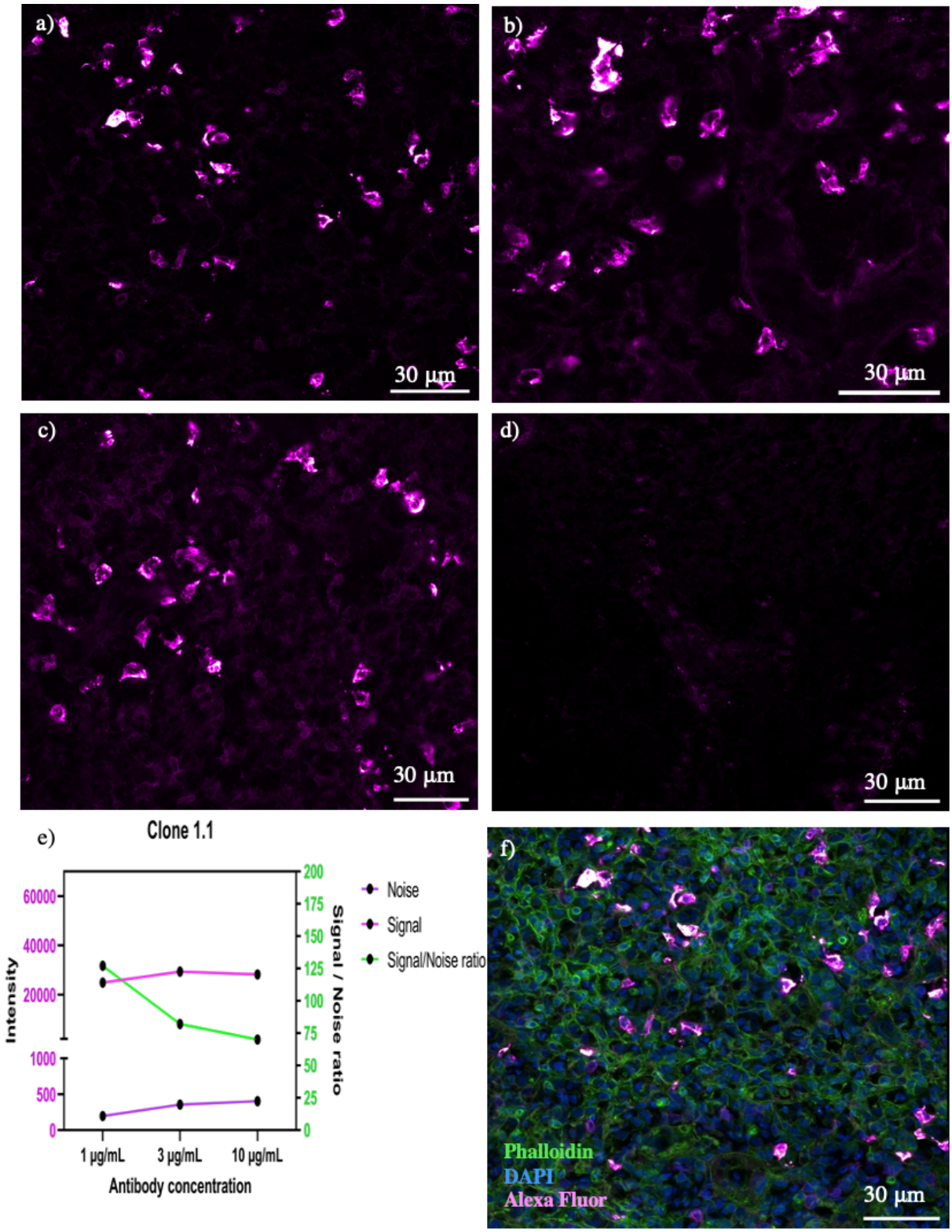

Figure 18: Cod spleen stained with clone 1.1.

a) Staining IgM-producing cells in the cod spleen with 1 µg/mL of 1.1, b) demonstrate the staining of IgM-producing cells using 3 µg/mL, c) shows the staining of IgM-producing cells using 10 µg/mL, d) presents the background noise, e) indicates a graph giving information about the signal, noise and the signal/noise ratio, f) presents the pattern of all the images stitched together. Blue represents the nuclei, magenta hot represents the IgM-producing cells, and the green represents actin structures.

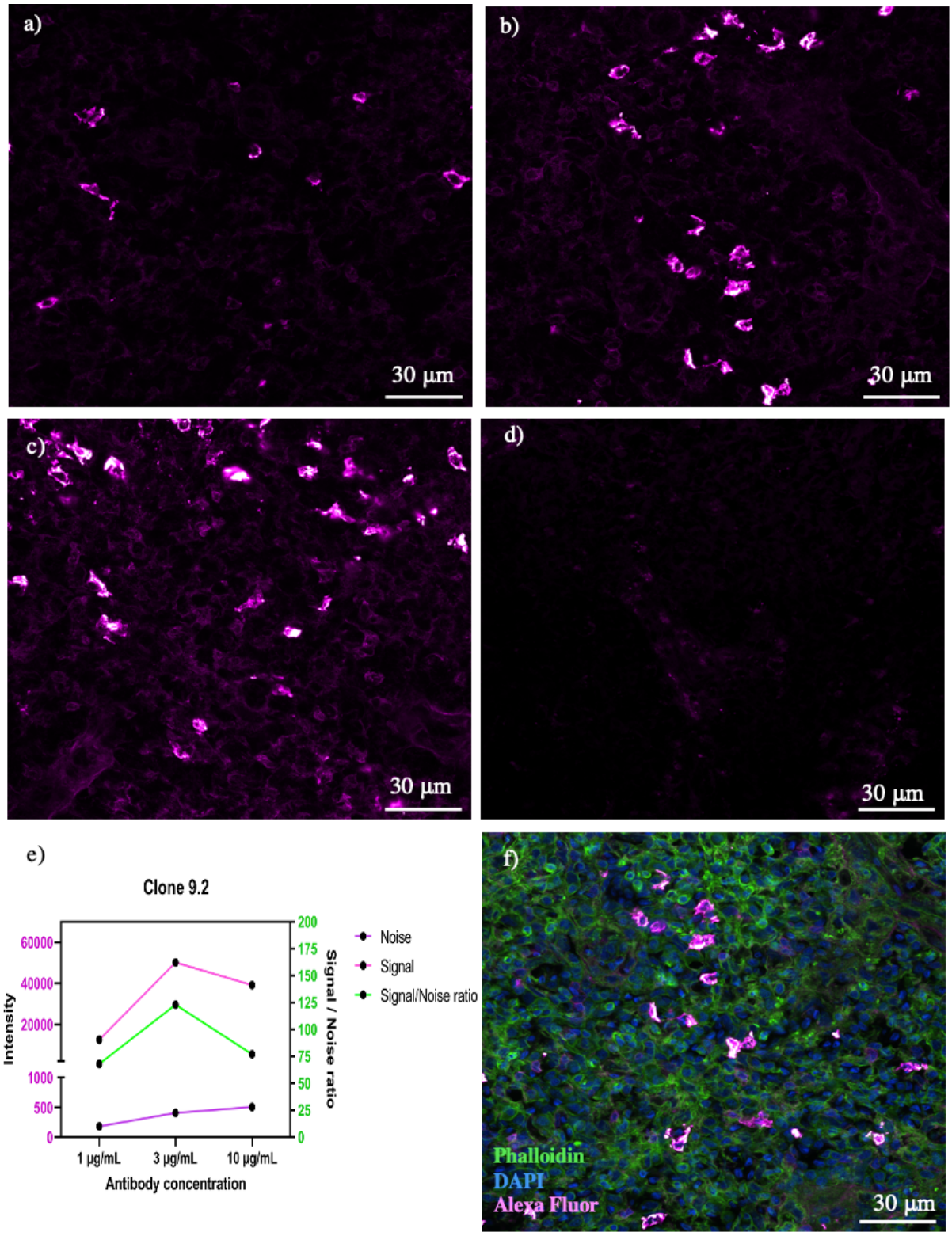

Figure 19: Cod spleen stained with clone 9.2.

a) Staining IgM-producing cells in cod spleen with 1 µg/mL of 9.2, b) demonstrates the staining of IgM-producing cells using 3 µg/mL, c) shows the staining of IgM-producing cells using 10 µg/mL, d) shows the background noise, e) present a graph giving information about the signal, noise and the signal/noise ratio, f) represent the pattern of all the images stitched together. Blue represents the nuclei, magenta hot represents the IgM-producing cells, and the green represents actin structures.

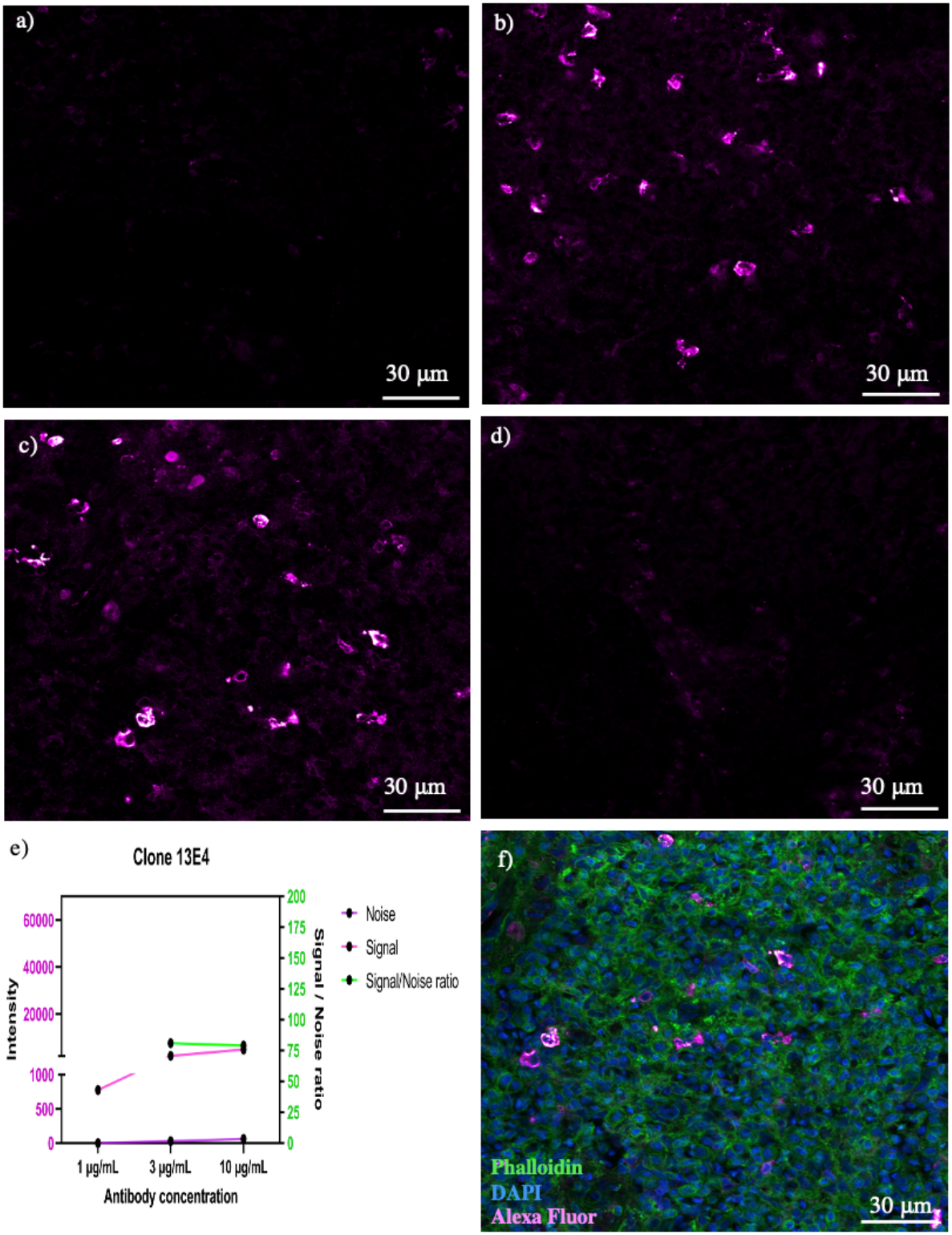

Figure 20 Cod spleen stained with clone 13E4.
a) Staining IgM-producing cells in the cod spleen with 1 µg/mL of 13E4, b) shows the staining of IgM-producing cells using 3 µg/mL, c) demonstrates the staining of IgM-producing cells using 10 µg/mL, d) shows the background noise, e) present a graph giving information about the signal, noise and the signal/noise ratio, f) represent the pattern of all the images stitched together. Blue represents the nuclei, magenta hot represents the IgM-producing cells, and the green represents actin structures.

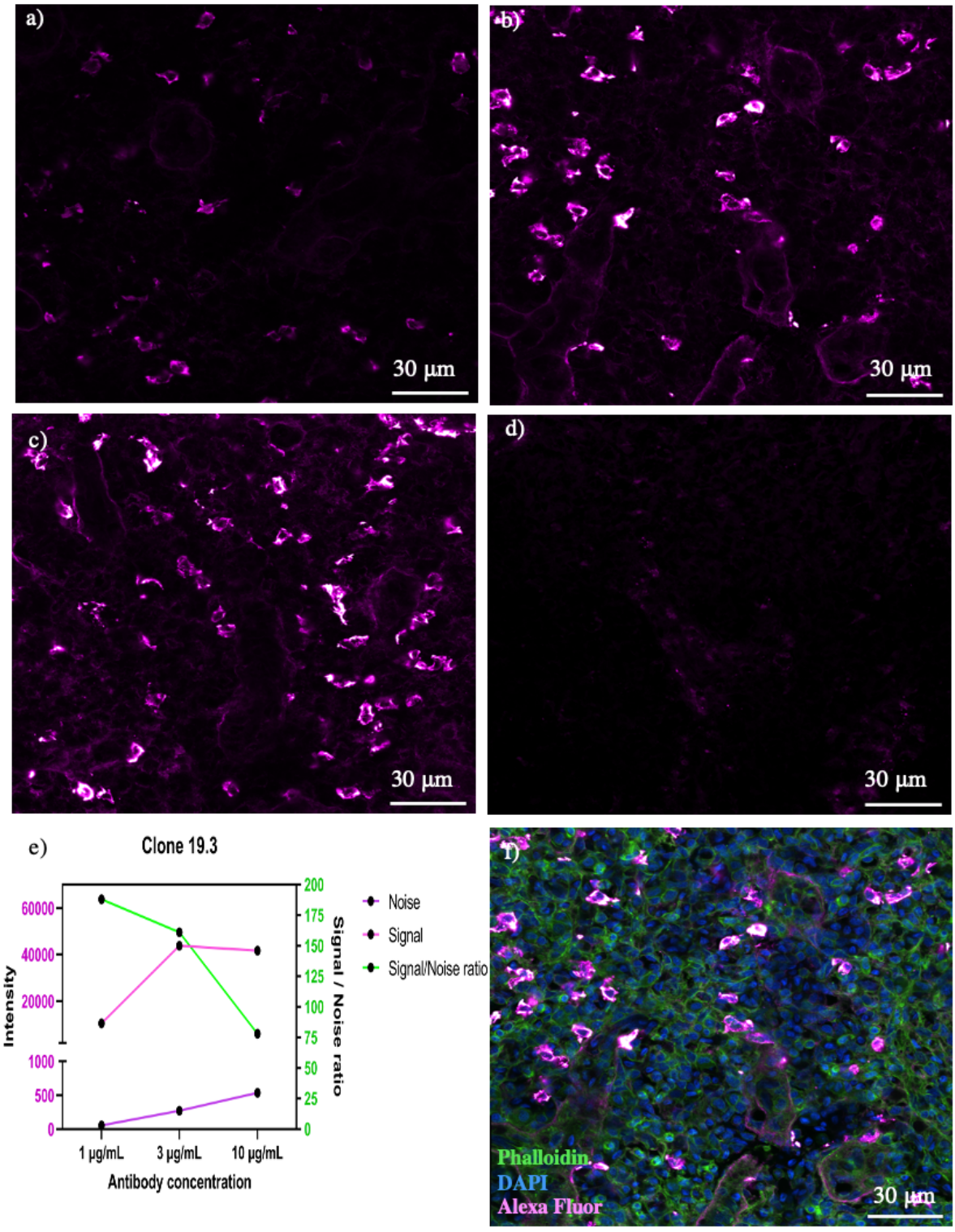

Figure 21: Cod spleen stained with clone 19.3.

a) Staining IgM-producing cells in the cod spleen with 1 µg/mL of 19.3, b) indicates the staining of IgM-producing cells using 3 µg/mL, c) demonstrates the staining of IgM-producing cells using 10 µg/mL, d) shows the background noise, e) present a graph giving information about the signal, noise and the signal/noise ratio, f) represent the pattern of all the images stitched together. Blue represents the nuclei, magenta hot represents the IgM-producing cells, and the green represents actin structures.

5. Discussion

5.1 ELISA

Comparison of Four mAbs and Mouse pAbs in Indirect Sandwich ELISA

This study analyzed the usefulness of four different mAbs in ELISA, and the optimal concentration needed for producing a strong signal-to-noise ratio was determined. Using all four mAbs at three different concentrations (10, 3, and 1 µg/mL) as primary antibodies and the signal from the mouse pAb cocktail as a reference, an indirect sandwich ELISA was performed.

The results of clone 1.1 (**Fig. 9, a**) revealed that all three dilutions of 1.1 generated a weaker signal compared to the mouse pAb. Even though the graph for the pAb in blue declines at the end, the pAb still generated a stronger signal than 1.1.
For clone 9.2 (**Fig. 9, b**), the signals were observed to be weaker than the mouse pAb.
At all three concentrations, the signals from clone 13E4 (**Fig. 9, c**) were consistently weaker than those from the mouse pAb. In contrast, clone 19.3 (**Fig. 9, d**) showed high signal intensities at all three concentrations and was the only mAb able to generate a signal comparable to the mouse pAb, even at lower concentrations.

Further indirect sandwich ELISA was performed to identify the minimum concentration of 19.3 required to produce a signal comparable to the mouse pAb. Repetition of the concentrations of 10, 3, and 1 µg/mL revealed that all three concentrations generated a high signal intensity (**Fig. 10, a**), supporting the selection of 19.3 for further titration. When the concentration was decreased below 1 µg/mL, a minor decrease in signal intensity was observed (**Fig. 10, b**). Consequently, it was determined that 1 µg/mL was the minimum concentration of 19.3 to use in ELISA experiments.

Since clone 19.3 produced the strongest signal intensity among the four mAbs, it was chosen for an antigen-specific ELISA. In this ELISA, NP-BSA was used as an antigen, and serum from an NP pool was used. As a negative control, serum from a naïve pool was used. Three different concentrations of 19.3 were tested (10, 3, and 1 µg/mL). The result indicates that 19.3 were effective in the antigen-specific ELISA, and a concentration of 1 µg/mL of 19.3 produced the same signal intensity as the higher concentrations of 19.3 (**Fig.16**).

Titration of the Chicken Anti-Cod IgM as coat

In the ELISA experiments, the coated chicken IgY anti-cod IgM was diluted at various ratios (1:4000, 1:8000, 1:16000, and 1:32000). The goal was to determine the optimal dilution for producing the strongest signal with clone 19.3. Through titration of chicken IgY anti-cod IgM, it was found that a 1:4000 dilution with 1 µg of 19.3 yielded the most robust signal and the other dilution factors yield relatively weaker signals (**Fig. 11).** Consequently, the 1:4000 dilution was selected for the rest of the ELISA experiments.

It was possible to observe the signal intensity of the 1:8000 dilution overlapped with the 1:16000 dilution, while the 1:32000 dilution showed the weakest signal. This outcome was expected because reducing the concentration of the coat leads to a decrease in the number of immobilized antibodies, resulting in a weaker signal.

Notably, the 1:4000 dilution corresponds to a concentration of 5 µg/mL, given that the initial concentration of the chicken IgY anti-cod IgM was 20 mg/mL. It's important to note that the recommended maximum antibody concentration, as indicated by NUNC maxisorp, is 10 µg/mL. Therefore, concentrations greater than 1:4000 were not tested in any subsequent sandwich ELISA experiments.

Purified IgM

To determine the sensitivity of the ELISA in ng/mL, an indirect sandwich ELISA was performed using two different concentrations (1 and 10 µg/mL) of clone 19.3 to detect dilutions of a purified cod IgM with a known stock concentration of 4.10 mg/mL. The mouse pAb was used as a reference **(Fig. 12, a)**.

The lowest sensitivity of the ELISA assay was determined by analyzing the signal-to-noise ratio at various concentrations, where the signal decreases and becomes undetectable from the background noise and where there are more than two standard deviations.

Based on the result of the indirect sandwich ELISA with purified IgM, it was observed that the lowest sensitivity for the mouse pAbs, 10 µg/mL of 19.3 and 1 µg/mL of 19.3, was greater than 50 pg/mL, 50 pg/mL, and 137 pg/mL, respectively **(Fig. 12, b)**. For comparison, the sensitivity of the ELISA was compared to two commercial kits, the Acram Human IgM

ELISA Kit (ab 137982), which according to the manufacturer has a sensitivity greater than 270 pg/ml, and the Mouse/Rat IgM ELISA kit (ab215085) with a sensitivity of 385 pg/ml. These results suggest that the sensitivity of the cod IgM ELISA assay is comparable to or greater than that of the commercial kit. Additionally, the cod IgM ELISA using 19.3 at 10 µg/mL provided a significantly higher sensitivity in detecting IgM.

Competitive ELISA

To determine if the mAbs bind to the same epitope on IgM, a competitive ELISA was performed. In the competitive ELISA, three of the mAbs (1.1, 13E4, and 19.3) were biotinylated and tested against the unlabeled versions of mAbs 1.1, 9.2, 13E4, and 19.3.

Biotinylated 1.1

Signal interference was observed when biotinylated 1.1 was tested against unlabeled 1.1 for binding to the cod IgM molecule (**Fig. 13, a**). This interference suggests that biotinylated 1.1 competes with itself for binding to cod IgM, decreasing the detected signal for each concentration as expected. The presence of unlabeled 1.1 interferes with the binding of biotinylated 1.1 to IgM because unlabeled 1.1 binds to the available binding sites.
For biotinylated 1.1 where it was tested with 9.2, a lower signal was observed at concentrations of 1, 3, and 10 µg/mL, and in the absence of 9.2 (0 µg/mL), a high signal was observed. This indicates that the decrease in signal is independent of the concentration and there is no indication of competition between the antibodies (**Fig.13, b**).
Based on the result from biotinylated 1.1 tested with 13E4, 13E4 did not affect the biotinylated 1.1 (**Fig. 13, c**). The signal of biotinylated 1.1 remained unaffected by the presence of unlabeled 19.3 as well **(Fig. 13, d**).

Biotinylated 13E4

Biotinylated 13E4 was tested against unlabeled 13E4, 9.2, and 19.3. The observed signal interference (**Fig. 14, a**) suggested that biotinylated 13E4 competed with itself. When analyzing the results of biotinylated 13E4 against 9.2 and 19.3 (**Fig. 14, a, and b**), both showed the highest signal in the absence of unlabeled 9.2 and 19.3 (0 µg/mL). However, the presence of unlabeled 9.2 and 19.3 had minimal interference on the signal generated.

Biotinylated 19.3

In the competitive ELISA, biotinylated 19.3 was tested against unlabeled 19.3 and 9.2. As expected, the unlabeled 19.3 caused signal interference by competing with the biotinylated 19.3 (**Fig. 15, a**). However, the conclusion is inconclusive when analyzing the signal generated by biotinylated 19.3 against 9.2 (**Fig. 15, b**). The graph indicates that the presence of 9.2, most notably at a concentration of 3 and 10 µg/mL, may interfere with the signal generated by biotinylated 19.3. Despite this, the signals generated by the lower concentration of 9.2 are minimally affected.

In summary, the results of the competitive ELISA results suggest that the mAb binds to different epitopes of IgM. However, the results obtained from the competition between biotinylated 19.3 and 9.2 antibodies revealed inconclusive due to interference observed at 3 and 10 µg/mL of 9.2 **(Fig. 15, b)**.

5.2 Western Blot

Binding of the mAbs to IgM using WB

With the aim to determine the binding sites of mAbs on the cod IgM, WB was performed. The result reveals that only 19.3 mAb demonstrated an efficient binding, as seen by the presence of a visible band on the WB **(Fig. 17**). In contrast, the other three remaining mAbs and the non-immune IgY did not produce visible bands.

Epitopes, which are antibody-binding sites on antigen molecules, can be linear, a short sequence of amino acids, or conformational, which refers to the three-dimensional (3D) structure, which is composed of amino acids from different parts of the polypeptide chain that are brought together when the chain folds [5]. Denaturation of the protein, such as during gel electrophoresis, can alter the epitope's conformation, altering its ability to interact with an antibody. Only linear epitopes can be recognized in the case of denatured proteins [41]. The specific binding of 19.3 suggests that it recognizes a linear epitope on the heavy chain of IgM, whereas the lack of binding of the remaining three mAbs indicates that they most likely recognize conformational epitopes.

In an attempt to partially preserve some conformational epitopes, a WB was performed without DTT during electrophoresis. Thus, the disulfide bonds between the heavy chain and light chain, as well as between the two heavy chains, stayed intact. The result showed a high molecular band was observed which corresponds to a tetrameric IgM (data not shown).

A WB with DTT was performed to further investigate the protein's structure. The membrane was then denatured with 6M Guanidine HCl and renatured with a constant lower concentration of Guanidine. However, this did not result in additional bands (data not shown). Therefore, we cannot conclude regarding the epitopes recognized by the other mAbs than clone 19.3.

The non-immune IgY, which is not specifically produced against cod IgM, was found to be ineffective in detecting cod IgM in the WB. This is because non-immune IgY does not have a specific binding affinity towards cod IgM. On the other hand, the pAbs chicken anti-cod IgM was revealed to be efficient in WB, where it was seen with two visible bands: one on the heavy chain and one on the light chain **(Fig. 17).** PAbs are derived from diverse populations of B cells [5]. The diverse nature of pAbs allows them to bind to both discontinuous and linear epitopes. This means that even if some epitopes on the IgM molecule are partially or completely denatured during the electrophoresis process, pAbs still have a higher chance of binding to different epitopes that may be accessible in the denatured state.

5.3 Immunohistochemistry

The suitability of four different mAbs for IHC was also evaluated by determining the concentration that provides the best compromise between a high signal-to-noise ratio and clear labeling patterns. Three different concentrations were tested (1, 3, and 10 µg/mL) for each mAb. These are typical antibody concentrations usually recommended by antibody manufacturers for IHC applications. The result of IHC shows IgM-producing cells, revealing the presence of IgM, plasma cells, and B cells.

Based on the previous finding in ELISA, clone 19.3 was initially expected to produce a brighter and stronger signal at the lowest concentration (1 µg/mL). In ELISA experiments, 19.3 have consistently demonstrated a stronger signal compared to other mAbs, relative to the mouse pAbs. Whereas clone 19.3 presented a satisfactory suitability for IHC at 3 µg/mL, it is

however clone 1.1 that generated the best signal-to-noise ratio and labeling pattern at the lowest concentration used (1 µg/mL) **(Fig. 18).** A strong signal indicates the successful binding of the antibody to the target in the tissue section, allowing for its visualization and detection. On the other hand, noise refers to any non-specific binding of the antibody, which can interfere with the interpretation and analysis of the results.

Interestingly, although clones 1.1, 9.2, and 13E4 were ineffective in WB, all four of them demonstrated a specific signal in IHC. This suggests that the antibody-antigen interaction may be influenced by different sample preparations between WB and IHC. One possible explanation of all mAbs' success in IHC could be attributed to the preservation of the epitope site within the tissue. In the IHC procedure, the tissue undergoes fixation which maintains the conformational structure of the antigen at the price of epitopes being slightly less accessible because of the formaldehyde cross-linking of proteins. In contrast, the proteins are denatured during WB, which means their conformation structure is lost, resulting in a linearization of the protein's amino-acids sequence. Therefore, these three mAbs clones recognize conformational epitopes rather than linear amino-acid sequences.

6. Conclusion

This thesis aims to characterize and determine optimal conditions for the use of the four mouse mAbs developed by the group of Qiao and Johansen in immunological assays such as ELISA, Western blot, and Immunohistochemistry.

The results of ELISA revealed variations in signal intensity among the different mAbs. Clone 19.3 consistently generated strong signals at different concentrations (0.11–10 µg/mL), whereas clones 1.1, 9.2, and 13E4 produced weaker signals compared to the mouse pAb. The competitive ELISA results indicated no significant interference between the different mAbs, suggesting that they likely bind to different epitopes. In WB, only clone 19.3 demonstrated effective binding and was found to bind the heavy chain of the cod IgM. However, in IHC, the mAbs that did not yield results in WB, such as 1.1, 9.2, and 13E4, successfully stained the tissue sections. This suggests the importance of preserving the epitope site within the tissue for effective antibody-antigen interaction, and the interaction between antibodies and IgM may be affected by different sample preparations between WB and IHC. **Table 2** presents the optimal concentrations for generating strong signal-to-noise ratios in each of the three assays. The developed protocols for ELISA and IHC provide guidelines for optimal antibody concentrations, facilitating future research and applications relating to the cod immune system.

Table 2: Summary of Monoclonal Antibody Concentrations for Immunological Assays.
The optimal amount to use in different immunological assays for each of the mAbs.

mAbs	Assay		
	ELISA	**IHC**	**WB**
1.1	10 µg/mL	1 µg/mL	-
9.2	10 µg/mL	3 µg/mL	-
13E4	10 µg/mL	10 µg/mL	-
19.3	1 µg/mL	1-3 µg/mL	3 µg/mL

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8. Appendix

Table 3: Heavy and light chain sequences of the CDR3 of the four monoclonal antibodies.
The heavy and light chain (IgG) of 11 hybridomas scored as positive for producing IgG reactive with Atlantic cod IgM were sequenced. Three sets of sister clones were identified, thus there were only eight unique clones. Clones 1(1) and 19(3) both had a sister clone. The subclass and CDR3 sequence of the four clones characterized in this thesis is shown.

Clone	Subclass	H-CDR3	L-CRDR3
1 (1)	IgG2a	IGHV3-2*02_CARSVLRYWSFDVW_IGHJ1*01	IGKV3-1*01_CQQSRKFPWTF_IGKJ1*01
9 (2)	IgG1	IGHV5-9-3*01_CARQALSYGNYAFMDYW_IGHJ4*01	IGKV1-117*01_CFQNSHVPPTF_IGKJ1*01
13(E4)	IgG1	IGHV5-9-3*01_CARQALSYGNYAFMDYW_IGHJ4*01	IGKV6-23*01_CQQYSRYPWTF_IGKJ1*01
19 (3) *	IgG2b	IGHV1-66*01_CARHFRRDAVDYW_IGHJ4*	IGKV10-96*01_*RQGDEVY##TF_IGKJ1*02

*The sequencing quality was too poor to unambiguously resolve the CDR3 of the light chain.