

**DEVELOPMENT OF MONOCLONAL ANTIBODIES
AGAINST INFECTIOUS SALMON ANAEMIA VIRUS**

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Dedicated to my wife Sarah,
and our children John & Anna

Declaration

I declare that this thesis has been compiled by myself, and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

Charles McGurk

Abstract

Infectious salmon anaemia has caused severe disease outbreaks in farmed Atlantic salmon, *Salmo salar* L. The development of monoclonal antibodies (MAbs) targeting infectious salmon anaemia virus (ISAV) would have multiple applications in diagnostics and vaccine development. Previously, one MAb has been produced against Norwegian ISAV, however this has shown assay restriction, limiting its use. The aim of this study was to develop MAbs to a Scottish isolate of ISAV, which would react under a variety of assay conditions.

The virus was cultured on a salmon head kidney (SHK-1) cell line of low passage number. SDS-PAGE analysis of the ISAV isolate demonstrated major protein components of molecular weight 20 kDa, 27 kDa, 33 kDa, 45 kDa, 70-72 kDa and 224 kDa. Comparison with previously described protein distributions of Norwegian and Canadian strains demonstrated a degree of similarity between these geographically separated isolates.

Following the immunisation of two mice with samples of ISAV, fusion of mouse spleen cells with myeloma cells resulted in the successful production of antibody-producing hybridoma cells. To assess the potential range of anti-ISAV antibodies available for the development of MAbs, serum from the mouse used for hybridoma production was tested against the Scottish ISAV isolate, using an enzyme-linked immunosorbent assay (ELISA) and Western blot immunoassay. Binding was observed to viral antigens of 45 kDa and 70 kDa. Simultaneous analysis of antibody binding to uninfected SHK-1 cells displayed cellular antigens of 25 kDa, 68 kDa and 75 kDa.

Five hybridoma lines producing antibodies targeting Scottish ISAV were successfully identified by ELISA. Western blot analysis demonstrated the *in vitro* production of antibodies targeting antigens of approximately 27 kDa & 71 kDa.

Cloning of the 'parent' hybridoma cell lines, however, resulted in poor growth of the cells, with loss in production of anti-ISAV antibodies, as detected by ELISA. In future projects, in order to increase the murine immunological response, it may prove necessary to administer increased concentrations of ISAV, or modify the ISAV antigen during the immunisation protocol. Re-cloning of the 'parent' hybridoma cell lines may potentially lead to the successful production and characterisation of MAbs targeting ISAV.

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1. Introduction

1.1. Infectious Salmon Anaemia

1.1.1. Overview

Infectious salmon anaemia (ISA) is an economically important disease of farmed Atlantic salmon, *Salmo salar* L. The condition has been diagnosed on fish farms in Norway (Thorud & Djubvik 1988), Canada (Groman 1998; Mullins, Groman & Wadowska 1998; Kibenge, Whyte, Hammell, Rainnie, Kibenge & Martin 2000b), Scotland (Rodger, Turnbull, Muir, Millar & Richards 1998), the Faroe Islands (Anonymous 2000b), Chile (Kibenge, Gárate, Johnson, Arriagada, Kibenge & Wadowska 2001), and recently in the United States of America (Bouchard, Brockway, Giray, Keleher & Merrill 2001). The disease has led to severe losses on infected premises from mortalities, ill thrift and disease control measures. The condition has been characterised by severe anaemia, alongside distinctive pathological findings (Nylund, Hovland, Watanabe & Endresen 1995). The pathogen involved in the syndrome has been named infectious salmon anaemia virus (ISAV), and has been reported to resemble members of the *Orthomyxoviridae* family (Koren & Nylund 1997; Krossøy, Hordvik, Nilsen, Nylund & Endresen 1999). As with many infectious diseases, effective control measures would be greatly facilitated by the availability of reliable rapid diagnostic techniques.

1.1.2. History of distribution

In the autumn of 1984, an apparently new disease syndrome led to substantial losses among farmed Atlantic salmon in Nordland County on the Southwest coast of Norway (Thorud & Djubvik 1988). Mortalities reaching 80% were experienced

owing to this condition, which was initially named Bremnes syndrome due to the geographical location. At that time, a mixture of salt water and fresh water was used to aid acclimatisation during the smolting process when young salmon were moved from fresh to salt water. Control measures were based on the assumption of a viral aetiology, and drastic protocols were implemented. The condition was viewed as a major threat to salmon aquaculture and was the first disease to be classified as List 1 under the European Commission's fish health regime, Council Directives 91/67/EEC & 93/53/EEC (Anonymous 2000a). This categorisation required the total destruction of the entire stock of fish on confirmed infected premises. The disease was considered exotic outwith Norway, and the movement of fish and products was restricted to limit potential translocation of the pathogen. Despite implementation of control measures, the disease became more widespread throughout Norway in the 1980s until more than 80 farms were positively diagnosed by the end of the decade.

Although originally the disease was only diagnosed in Norway, few aquatic diseases remain geographically isolated, as has been noted in the past with nodavirus infection, amoebic gill disease and rickettsiosis (Roberts 1998). A previously unrecognised disease affected Atlantic salmon farms situated in the Bay of Fundy, New Brunswick, Eastern Canada, in the summer of 1996. High mortalities occurred, but the histopathological appearance of this syndrome did not display the characteristic findings associated with ISA (Simko, Brown, Mackinnon, Byrne, Ostland & Ferguson 2000). Due to the post mortem findings, the condition was initially named haemorrhagic kidney syndrome (HKS) (Byrne, Macphee, Ostland, Johnson & Ferguson 1998). It was demonstrated that this seemingly new condition was indeed caused by ISAV, although no epidemiological correlation between

Norwegian and Canadian isolates could be shown (Lovely, Dannevig, Falk, Hutchin, MacKinnon, Melville, Rimstad & Griffiths 1999). In 1998, severe losses were experienced in Atlantic salmon farms in Western Scotland, and ISA was diagnosed (Rodger *et al.* 1998), having profound economic repercussions on the aquaculture industry (O'Hagan 1999).

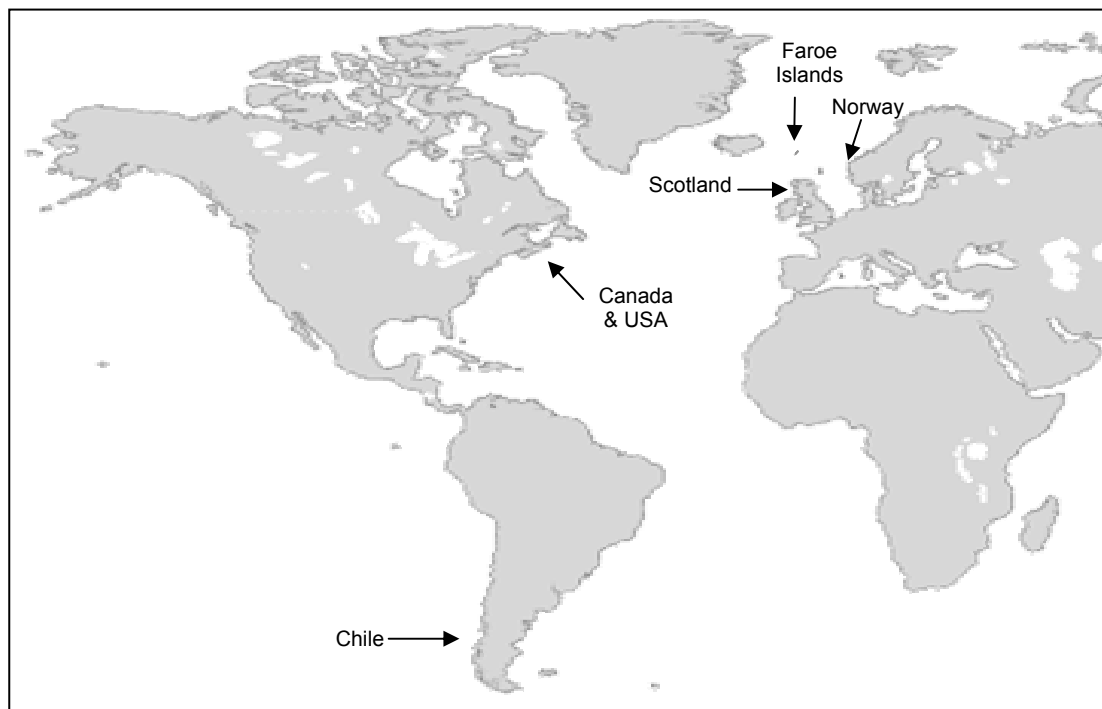


Figure 1.1: Geographical distribution of outbreaks of disease attributed to ISA (areas denoted by arrows.)

Diagnosis of the disease on a sea farm in the Fuglafjordur region of the Faroe Islands (Anonymous 2000b) led to a ban on the export of slaughtered salmonids to the European Union (EU) unless head, gills and viscera were removed during the slaughter process. ISA was suspected on a farm of Coho salmon, *Oncorhynchus kisutch* (Walbaum) in Chile (Kibenge *et al.* 2001), and this was confirmed by molecular techniques. Further North American outbreaks were diagnosed in Nova Scotia (Ritchie, Cook, Melville, Simard, Cusack & Griffiths 2001), and for the first

time in the United States of America on a salmon farm in Maine, close to the Canadian border (Bouchard *et al.* 2001). Reported outbreaks have shown a wide geographical presence of ISAV (Figure 1.1).

1.1.3. Disease signs

Clinically, ISA has manifested itself in a number of ways ranging from classical peracute mortalities to inapparent subclinical infection, and has most commonly been seen in Atlantic salmon after one year in marine conditions. In prolonged cases, stock may be noticeably lethargic, congregate in the upper parts of the cages, gasp for air at the surface, hang motionless near the cage walls before sinking to the bottom, and show marked inappetence. Recently, less characteristic presenting signs have been noted, including non-specific mortalities and ill thrift (Poppe 1998). The disease has also been identified in first feeding Atlantic salmon fry, although no firm evidence of vertical transmission has been proven (Nylund, Krossøy, Devold, Aspehaug, Steine & Hovland 1999; Melville & Griffiths 1999). High levels of mortality have been seen over the chronic course of spread through a unit, with anaemia and ascites being evident in dead fish (Thorud & Djubvik 1988). Severe branchial pallor, exophthalmos, congestion of the spleen, foregut and liver, with concurrent petechiation of the viscera and fat have been noted (Thorud & Djubvik 1988; Rodger *et al.* 1998; Falk, Namork & Dannevig 1998; Opitz, Bouchard, Anderson, Blake, Nicholson & Keleher 2000).

In European outbreaks, under histological examination, distinctive changes were seen in the liver and spleen (Thorud & Djubvik 1988; Rodger *et al.* 1998). Focally extensive areas of acute hepatocellular necrosis, often accompanied by

haemorrhaging, have been seen in addition to splenic congestion with haemosiderin deposition. Vacuolation of the hepatic perisinusoidal macrophages and endothelial degeneration have been observed by transmission electron microscopy (Speilberg, Evensen & Dannevig 1995). Changes have also been noted in various other organs including myocardial tissue, the gills, and the intestines. Renal involvement was also reported as an additional pathological finding in a limited number of cases (Rodger *et al.* 1998). In Canada, HKS presented a different pathological picture, the predominant changes being in the posterior kidney, including marked interstitial haemorrhage and acute tubular necrosis and casting (Byrne *et al.* 1998; Simko *et al.* 2000). In the original cases of HKS, the typical hepatic changes associated with European outbreaks of ISA were not reported. A togavirus-like virus was isolated from outbreaks, but it was later concluded that it was not involved in the pathogenesis of the syndrome (Kibenge *et al.* 2000b). In later Canadian outbreaks in New Brunswick, a combination of renal and hepatic lesions was recorded. An experimental study involving infection of Atlantic salmon with a Canadian ISAV isolate resulted in pathognomonic hepatic lesions in more than 90%, and pathognomonic renal lesions in over 78% of challenged fish which died due to infection (Simko *et al.* 2000).

In ISA outbreaks, biochemical and haematological analyses demonstrated profound microcytic anaemia - packed cell volume (PCV) below 10% - with erythrocytic abnormalities (Falk, Press, Landsverk & Dannevig 1995), leucopaenia, and increased levels of plasma potassium & liver enzymes (Dannevig, Falk & Krogsrud 1993; Falk *et al.* 1998). Hepatic damage led to increased plasma levels of glutathione, while in the liver levels fell, possibly compromising hepatic function in the transformation and excretion of xenobiotics from the body, with deleterious health implications

(Hjeltnes, Samuelson & Svardal 1992). Plasma cortisol and lactate levels have also been found to rise (Olsen, Falk & Reite 1992). Apparent suppression of the immune response has been found to be independent of the development of clinical anaemia (Dannevig *et al.* 1993). Vascular endothelium has been identified as the target tissue for the virus, resultant shutdown leading to multiple systemic effects due to impaired endothelial endocrine functions (Nylund *et al.* 1995). During a disease outbreak, ISAV has been found to be present in most organs of infected fish (Rimstad, Falk, Mikalsen & Teig 1999).

1.1.4. Infectious salmon anaemia virus

Although a viral aetiology was suspected soon after the first reports of the disease in Norway, it was not until more than a decade later that the pathogen was identified. Treating infected plasma samples with a lipid solvent showed that the suspected aetiological agent contained essential lipids, most likely in the form of a viral envelope (Christie, Hjeltnes, Uglenes & Winton 1993). Using electron micrography, apparently enveloped virus particles of approximate diameter 100 nm, were observed budding from endothelial cells in heart blood vessels (Hovland, Nylund, Watanabe & Endresen 1994). Within the slightly pleomorphic unit membrane, a number of granules of diameter 10 – 12 nm were seen, apparently arranged in 2 concentric spheres (Nylund *et al.* 1995). The virus was seen to consist of a single-stranded RNA genome of negative polarity, with a total molecular weight of 14.5 kb (Inglis, Bruce & Cunningham 2000). Although budding was seen only from blood vessels and sinuses, virus was also found intracellularly in endothelial cells and leucocytes. The virus was not isolated until 1995 when it was successfully cultured on a newly founded cell line, designated salmon head kidney 1 (SHK-1) (Dannevig, Falk &

Namork 1995). It was noted that optimal growth occurred at 15°C, with none seen above 25°C. The virus was assumed to be capable of replication in ectothermic species only, with no agglutination of mammalian red blood cells nor growth on mammalian cell lines possible (Falk, Namork, Rimstad, Mjaaland & Dannevig 1997). The virus was also seen to be sensitive to lowered pH conditions (Falk *et al.* 1997). It was found that the morphology and morphogenesis of this spherical virion resembled that of the orthomyxoviruses (Koren & Nylund 1997).

By comparison of the conserved motifs with corresponding areas of other segmented negative stranded RNA viruses, it was suggested that ISAV was most closely related to the members of the *Orthomyxoviridae* family (Krossøy *et al.* 1999). In common with this group of viruses, the growth of ISAV was inhibited by actinomycin D, but not by 5-bromo-2-deoxyuridine treatment (Sommer & Mennen 1997). The virus exhibited other consistent properties, including haemagglutinating activity and receptor destroying enzyme activity (Falk *et al.* 1997; Falk *et al.* 1998). Orthomyxoviruses have been identified in mammals and birds, with orthomyxovirus-like viruses seen in ticks, European eels, *Anguilla anguilla* (Linnaeus), and the bluegill, *Lepomis macrochirus* Rafinesque (Falk *et al.* 1997). The viral particles seen with ISAV have been found to be larger than the orthomyxovirus viruses, making it distinct from influenza A, B & C viruses. It was proposed that ISAV represented a new fifth genus of the *Orthomyxoviridae* (Krossøy *et al.* 1999). The members of this viral family exhibit rapid mutation rates of some genes (Inglis *et al.* 2000), antigenic variations in influenza viruses being primarily recognised in surface glycopeptides (Falk *et al.* 1998).

It was noted that genetic differences existed between different isolates of ISAV (Blake, Bouchard, Keleher, Opitz & Nicholson 1999). Even amongst the European isolates, homology was not seen, differences in nucleotide sequences and amino acids being noted between Norwegian and Scottish strains (Cunningham & Snow 2000). Similarities of 98 – 100% were seen between the European isolates, while comparison of nucleotide sequences between these and Canadian isolates from New Brunswick showed 84 – 88% constancy for the 2 segments analysed (Krossøy, Nilsen, Falk, Endresen & Nylund 2001). Phylogenetic analysis suggested that divergence of Norwegian and Canadian isolates may have occurred around the year 1900 (Krossøy *et al.* 2001), coinciding with the beginning of the trade in salmonids between North America and Europe. One theory that has been proposed is that the pathogen may have been translocated across the Atlantic Ocean at the turn of the 20th Century. Whether possible spread was eastward with imported rainbow trout, *Oncorhynchus mykiss* (Walbaum) or westward with sea trout, *Salmo trutta* L. could not be discerned (Krossøy *et al.* 2001).

A more recently discovered isolate from Nova Scotia appeared to bear more similarities to European strains than to the neighbouring New Brunswick isolate (Ritchie *et al.* 2001). Canadian isolates have been found to fall into 2 discrete groups, categorised on whether they possess the ability to replicate in Chinook salmon embryo 214 (CHSE-214) cell lines producing discernible cytopathic effects (CPE) (Kibenge *et al.* 2001). The strain isolated from Nova Scotia was not associated with the typical clinical or pathological findings of ISA. It could not be concluded whether this strain was introduced to America more recently than the New Brunswick strain, or if the latter was atypical of a generalised transatlantic virus (Ritchie *et al.* 2001).

Although the Nova Scotia strain bore structural similarities to the European strains, it differed functionally by not causing the pathognomonic pathology of ISA. Isolates from Chile were analysed at a molecular level by reverse-transcriptase polymerase chain reaction (RT-PCR), and nucleotide sequence analysis showed similarities to New Brunswick virus (Kibenge *et al.* 2001).

1.1.5. Disease transmission

It is now thought that the main route of transmission of ISA is via sea water from infected salmonid sources to clean sites (Jarp & Karlsen 1997). The emergence of the disease suggested the existence of natural reservoir hosts for the virus in the wild. The most likely fish to be involved would be coastal salmonids, and indeed although only salmon appear to be clinically affected, other salmonids including sea trout and rainbow trout have been shown to harbour the virus (Devold, Krossøy, Aspehaug & Nylund 2000). It was demonstrated that the virus could be experimentally transmitted from sea trout to Atlantic salmon (Nylund & Jakobsen 1995). Sea trout are abundant along the Norwegian coast, and are common in areas that have suffered from ISA (Devold *et al.* 2000). Sea trout have been shown to develop no clinical signs following injection of infected ascitic fluid, but the virus was seen to propagate and PCV dropped (Nylund & Jakobsen 1995). Brown trout, *Salmo trutta* L. have been shown to harbour ISAV for up to 7 months post-exposure, suggesting latent or persistent infection (Nylund, Kvenseth, Krossøy & Hodneland 1997). Experimentally infected rainbow trout did not develop clinical disease, but again PCV dropped and hepatic haemorrhage was noted histologically in some specimens (Nylund *et al.* 1997). It was found that Arctic char, *Salvelinus alpinus* L. could harbour the virus post exposure, but cleared the pathogen more rapidly than the 2 trout species (Snow,

Raynard & Bruno 2001). It has been suggested that an evolutionary balance may exist between these salmonids and the virus, which has not been developed by farmed stocks of Atlantic salmon (Nylund 1998).

The high levels of mortality witnessed in farmed Atlantic salmon stocks, coupled with the lack of disease in infected trout suggest that the virus may be a novel pathogen to the salmon, or the fish may have become more susceptible to ISAV due to genetic changes stemming from domestication (Nylund *et al.* 1997). Influenza viruses have been shown to be highly subject to mutation (Falk *et al.* 1998), potentially leading to a harmless agent becoming pathogenic. With many aquatic infectious diseases, a balance exists between the host, the pathogen, and the environment. It seems feasible that farming practices, resulting in high stocking densities and increased stress, may have contributed to the emergence of the disease (Håstein 1998).

Although the presence, and indeed replication of ISAV had been demonstrated in other salmonids, clinical disease was recognised only in Atlantic salmon, until an outbreak was diagnosed in coho salmon in Chile (Kibenge *et al.* 2001). In this instance neighbouring Atlantic salmon did not exhibit signs of disease. Analysis of Atlantic salmon infected with the strain of ISAV present in Nova Scotia, Canada showed a lack of typical ISA pathology or mortalities. These were the first reported examples of Atlantic salmon being exposed to the pathogen without exhibiting clinical disease, and could support the hypothesis of the disease originating from mutation of non-pathogenic strains of virus. Attempts have been made to reduce susceptibility of farmed salmon by use of breeding programmes (Anonymous 1999).

Other vectors of the agent have been suggested, including sea lice, *Lepeophtheirus salmonis* (Krøyer) & *Caligus elongatus* (Nordmann) (Nylund & Jakobsen 1995). Sea lice have been thought to be important vectors of ISAV during epidemic and endemic phases (Nylund, Hovland, Hodneland, Nilsen & Løvik 1994). Strategic control policies have been recommended to reduce risks from the increased populations of copepod parasites around salmon farms (Anonymous 2000a). Bivalves have previously been shown to harbour viruses, including aquatic birnaviruses (Hill & Way 1995), therefore concerns have been expressed regarding the duoculture of great scallops, *Pecten maximus* (L.) with Atlantic salmon. However, there has been no evidence to show that scallops could filter and concentrate ISAV to act as carriers, despite exposure to infective material (Bjoershol, Nordmo, Falk & Mortensen 1999). There is uncertainty as to whether the virus is capable of replication in herring, *Clupea harengus* L., freshwater eels, *Anguilla spp.*, and saithe, *Pollachius virens* (L.) (Anonymous 2000a).

It has been revealed through epidemiological studies that the risk of transfer was intimately related to farming practices, and that an important risk factor was from waste originating from the slaughtering and processing of salmonids. The virus has been shown to be associated with blood material, epidermal mucus and urine (Hjeltnes, Flood, Totland, Christie & Kryvi 1994). Disease has been experimentally transmitted following 100 nm filtration of homogenised tissue from infected fish (Sommer & Mennen 1996). No evidence of transmission following ingestion of the virus has been shown, and the gills are thought to be the most likely entry port (Totland, Hjeltnes & Flood 1996).

1.1.6. Control measures

The Norwegian Department of Agriculture proposed regulations requiring that slaughter effluents be disinfected to give a 3 times logarithmic reduction in the number of pathogens present, in an attempt to control both furunculosis and ISA (Flogstad, Schei, Torgersen, Roettereng, DePauw & Joyce 1991). Viscera and trimmings from slaughter waste have been found to be highly contagious even from infected fish exhibiting no clinical signs. Although muscle tissue was much less infective, the entire fish should be considered as potentially contagious (Torgersen 1998). Disinfection has been achieved by exposing material to temperatures in excess of 50°C for more than 2 minutes, formic acid (pH \leq 4) for over 8 hours, sodium hydroxide (pH 11.5) for 48 hours, sodium hypochlorite (100 mg l⁻¹) for 15 minutes, or ultra violet light doses at 4mJ cm⁻¹ or higher (Torgersen 1998).

In the United Kingdom, strict regulatory procedures were put in place to reduce the risks of entry of the disease, and to control spread in the event of an outbreak (Anonymous 2000a). Measures instigated included a ban on the use of sea water in production phases in fresh water, the use of separate equipment (or thorough disinfection) between sites including wellboats and diving equipment. Production units could not be situated within 5 km or one tidal excursion of a harvest station, escapes from farms should be minimised, and other biological vectors considered including fish, sea lice and birds. Sound management practices were gauged to be of great importance, including single year class stocking, fallowing periods, high health status of new smolts, good husbandry techniques, and good communication & co-operation with other local producers. Marine trout farms were included within these

control strategies, although the accelerated viral clearance in Arctic char compared with trout may have implications on future regulations of culture of the former in Scotland.

In the case of an infectious disease such as ISA, where no chemotherapeutic agents are applicable during a disease outbreak, prophylaxis is a critical component of disease control. A protective immune response has been demonstrated in Atlantic salmon that had recovered from infection 75 days previously (Falk & Dannevig 1995), suggesting that vaccination against the disease may be feasible. An attempt to produce a vaccine, consisting of inactivated ISAV injected intraperitoneally, achieved some success (Jones, Mackinnon & Salenius 1999). An efficacious vaccine could play a crucial role in control of this economically and clinically damaging disease, although limitations due to diversity of strains and possible mutations would have to be resolved.

1.1.7. Diagnostic techniques

Effective control measures and an increased epidemiological understanding of the syndrome rely heavily on accurate swift diagnosis of disease outbreaks. Diagnostic techniques used include analysis of epidemiological and clinical findings (Jarp & Karlsen 1997), gross pathology and histopathology (Simko *et al.* 2000), virus culture (Opitz *et al.* 2000), indirect fluorescent antibody test (IFAT), and RT-PCR (Devold *et al.* 2000). Ideally, all diagnostic tests would be utilised for each case, but this may prove prohibitively expensive. Confirmation has been found to be particularly difficult in mild or early cases in an outbreak of disease (Falk *et al.* 1998). In the UK, confirmation of disease has only been made following fulfilment of the following

criteria: distinctive clinical signs, significant macroscopic lesions, evidence of anaemia, pathognomonic histopathological changes, and identification of ISAV by isolation, IFAT and / or RT-PCR (Anonymous 2000a). RT-PCR has been found to be more sensitive in detecting infection in both trout and salmon than cell culture (Opitz *et al.* 2000; Devold *et al.* 2000). Parallel isolation on SHK-1 & CHSE-214 cell lines has proved more sensitive than using one alone, while immunoassay by IFAT has been found to be least sensitive (Opitz *et al.* 2000). Recently, a new salmonid cell line (designated TO) has been developed, apparently allowing higher yields of ISAV to be cultured than on conventional cell lines (Wergeland & Jakobsen 2001).

Immunoassays including slide agglutination, co-agglutination / latex agglutination, immunodiffusion, direct and indirect fluorescent antibody tests (FAT & IFAT), immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), dot blot / dip stick and Western blot assays can allow the rapid and accurate confirmation of the presence of pathogens (Adams, Thompson, Morris, Farias & Chen 1995). Attempts have been made to develop specific antibody probes against ISAV, including the production of monoclonal antibodies (MAbs) (Christie, Hjeltne, Totland, Flood & Spasowska 1994; Falk *et al.* 1998). However, the application of such probes developed for the diagnosis of ISA have so far proved significantly less sensitive than the other available techniques (Devold *et al.* 2000; Opitz *et al.* 2000).

1.2. The use of antibody probes in the study of aquatic disease

1.2.1. Antibodies as a component of the humoral immune response

In vertebrates, antibodies are composed of immunoglobulins, a family of structurally related proteins that are produced by lymphoid cells, particularly plasma cells. In

higher mammals, 5 classes of immunoglobulin (Ig) have been recognised, designated IgG, IgA, IgM, IgD & IgE (Turner 1981). Antibodies circulate systemically, being involved in the humoral immune response. Additionally, some Ig molecules may become bound to the surface membranes of lymphocytes and macrophages, acting as antigen receptors with a role in cell-mediated immunity. In fish, it has traditionally been believed that the only class of Ig present is IgM, although structural differences of unknown significance have been noted (Jurd 1985). In teleost fish, IgM is tetrameric, as opposed to the pentameric structure in mammals.

Once a B-lymphocyte has been stimulated by foreign material to differentiate into an antibody-producing plasma cell, that line of cells may produce only one single type of antibody molecule, binding to a particular site (epitope) on the antigen. In the vertebrate, a vast array of antibody-producing cells is active, each clone contributing one particular antibody molecule to the overall heterogeneous response. Thus, it could be considered that the humoral immune response is composed of a number of monoclonal cell pools, which together constitute a polyclonal response.

1.2.2. Antibody probes

Polyclonal antibody probes have long been used as diagnostic tools for fish and shellfish diseases (Adams *et al.* 1995). They are prepared by serial immunisation of laboratory animals (often rabbits) with a particular antigen and harvesting the anti-serum. The serum would contain many antibodies, some of which would be specific to the antigen injected, but some would cross-react with other substances. The purity of the antigen used for the immunisation protocol would be paramount in determining the specificity of the resulting antibody probes. In addition to the limitations of

specificity, the use of polyclonal antibodies (PAb) would require the continued use of laboratory animals. It was realised that the ideal solution would be to culture *in vitro* a clonal line of antibody-producing cells, all having specificity for the same epitope and thus producing MAbs.

Extreme difficulties were experienced in attempting to culture antibody-producing cells *in vitro* for any meaningful length of time. Köhler and Milstein (1975) developed a technique for the reliable long-term production of MAbs, the importance of this discovery being rewarded in 1986 with the presentation of a Nobel Prize (Eryl Liddell & Cryer 1991). The ingenious solution involved the fusion of plasma cells with tumour cells (myeloma cells) resulting in the production of immortal antibody-producing hybridoma cells. A technique was thus developed allowing indefinite production of MAbs *in vitro*. The procedure for the production of MAbs (Figure 1.2) involves initial immunisation of a laboratory animal (normally a mouse or rat), subsequently screening its serum for significant antibodies, production of a hybridoma cell line, cloning, harvesting and storage of the products.

1.2.3. Monoclonal antibody probes as investigative tools

Monoclonal antibodies have been widely used as diagnostic and investigative probes in the study of numerous aquatic diseases of diverse aetiology. MAb probes have been developed against an ever-increasing array of bacterial pathogens, facilitating improved diagnostic capabilities coupled with advances in the understanding of the structure and behaviour of disease causing agents. A MAb probe was prepared and used in IHC & ELISA tests against *Mycobacterium marinum* (Puttinaowarat, Thompson & Adams 2000), although its use was limited by an inability to recognise

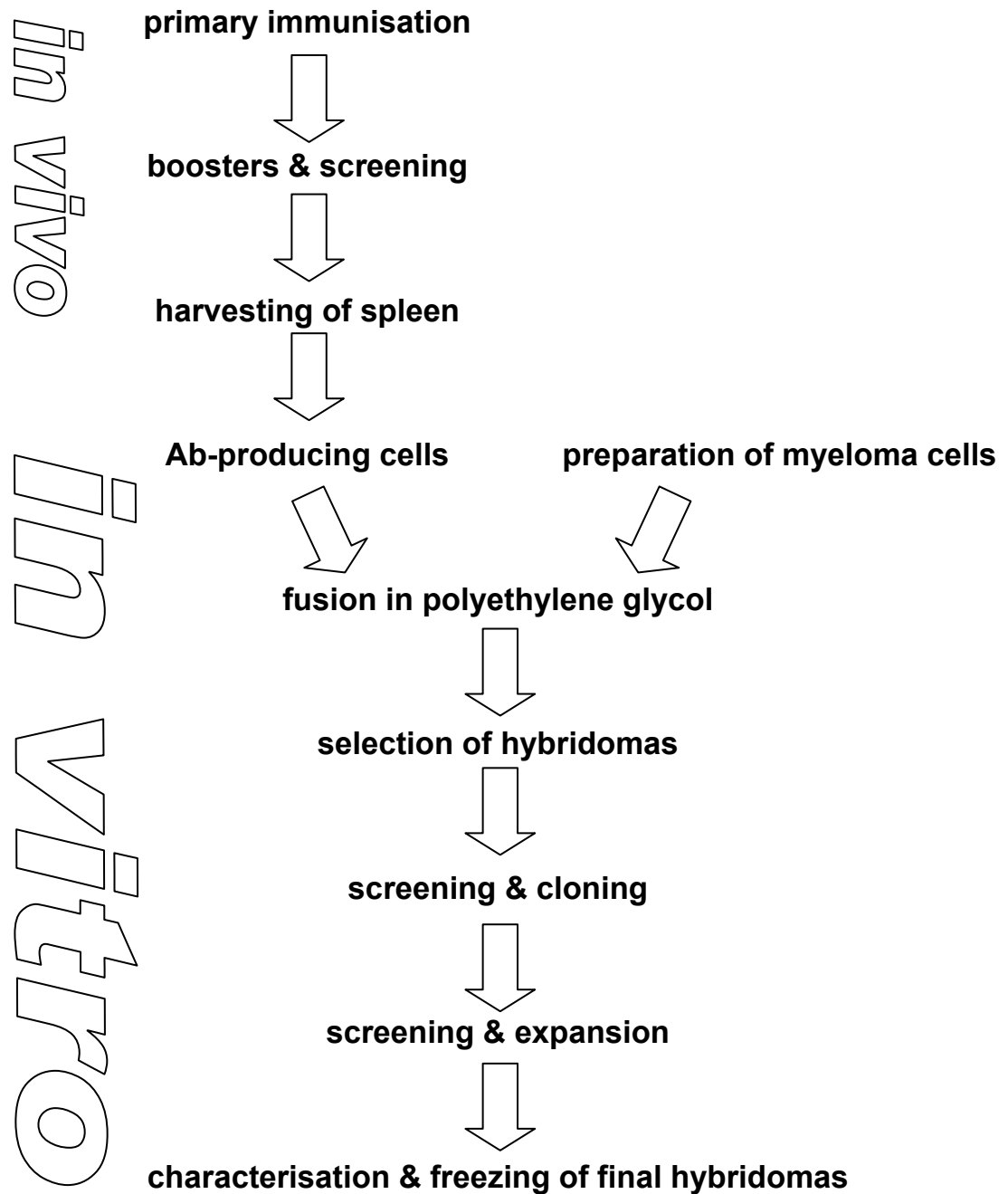


Figure 1.2: The major steps in the production of monoclonal antibodies

all isolates of the species. Some other MAbs against *Mycobacterium* species have been shown to exhibit a phenomenon known as assay restriction by only reacting with certain immunoassays, for example Western blot but not ELISA (Chen, Adams, Thompson & Richards 1997). MAbs have been incorporated into rapid ELISA tests for use on farms to screen for enteric redmouth and furunculosis (Austin, Bishop, Gray, Watt & Dawes 1986), greatly speeding up diagnosis. MAbs developed for studies of *Photobacterium damsela* subspecies *piscicida* identified different antigens in European and Japanese strains (Bakopoulos, Adams & Richards 1997a), demonstrating a heterogeneity of the pathogen. Positive reactions to extracellular products using MAbs, suggested that the antigens targeted were either sloughed from the cell membranes or secreted into the culture medium (Bakopoulos, Adams & Richards 1997b). MAbs targeting a major exotoxin of *Aeromonas salmonicida* subspecies *achromogenes* were developed to aid investigations into the phenotypic differentiation of atypical *Aeromonas salmonicida* isolates (Wagner, Gudmundsdottir & Droessler 1999). MAbs were developed against extracellular proteins of *Listeria monocytogenes* and had possible application in determining the infectivity of samples (Wiens, Chien, Winton & Kaattari 1999). In the investigation of bacterial kidney disease (BKD), MAbs were used in the serological comparison of isolates of *Renibacterium salmoninarum* (Arakawa, Sanders & Fryer 1987; Hsu, Bowser & Schachte 1991). As work has progressed, libraries of MAbs have been accumulated, potentially allowing speciation to be carried out diagnostically, as has reportedly been achieved with some *Vibrio* species (Hanna, Altmann, Chen, Smith, Cosic, Moon & Hammond 1991).

The technology has also been increasingly applied to studies of aquatic parasitic diseases. In the investigation of proliferative kidney disease (PKD), MAbs have been produced against *Tetracapsula bryosalmonae* (Adams *et al.* 1995), different MAbs targeting separate life cycle stages of this parasite (Marin de Mateo, Adams, Richards, Castagnaro & Hedrick 1993). Such uses of MAbs in elucidating life cycle details could have profound benefits in the understanding of the transmission of diseases, and further applications including potential vaccine development. In salmonids, comparisons between *T. bryosalmonae* and the myxosporean parasites of *Sphaerospora* species, were also made involving the use of MAbs (Marin de Mateo, McGeorge, Morris & Kent 1996). A MAb was developed against the haemoflagellate parasite *Cryptobia (Trypanoplasma) salmositica* that appeared to lead to agglutination of the parasite (Woo 1999), potentially affecting pathogenicity.

The development of MAbs for use in the identification of fish viruses has allowed an increase in the speed and ease of use of diagnostic tests (Nicholson 1993; Nicholson 1995), coupled with potentially improved sensitivity and specificity to pathogens (Sanz & Coll 1992). Various aquatic viruses have been targeted, including red sea bream iridovirus (Nakajima, Maeno, Fukudome, Fukuda, Tanaka, Matsuoka & Sorimachi 1995), birnaviruses (Novoa, Blake, Nicholson & Figueras 1995), and yellow head virus in *Penaeus monodon* Fabricius (Sithigorngul, Chauyuchwong, Sithigorngul, Longyant, Chaivisuthangkura & Menasveta 2000). Channel catfish virus (CCV) has been studied with MAbs, and differences between strains have been discerned (Arkush, McNeill & Hedrick 1992). MAbs have also been developed against infectious pancreatic necrosis (IPN) virus (Vazquez Branas, Morales & Estepa 1994), viral haemorrhagic septicaemia (VHS) virus (Fichtner, Bergmann,

Enzmann, Weiland & Granzow 1998) and infectious haematopoietic necrosis (IHN) virus (Wang, Lee, Shieh, Wi, Huang & Chien 1996) for use in isolate characterisation.

1.2.4. The use of monoclonal antibodies in vaccine development

Traditionally, in mammalian medicine, vaccines have been based on inactivated or live attenuated pathogens. But shortcomings in success and worries over reverse-mutation of attenuated strains have led to increased interest in subunit recombinant vaccines. Both polyclonal and monoclonal antibodies have been seen to be extremely useful in the development of recombinant vaccines (Lorenzen 1999). Antibody probes have been used to screen pathogen DNA libraries for potential vaccine antigens (Adams *et al.* 1995). Some success has been achieved in the production of subunit vaccines against viral infections, including CCV (Awad, Nusbaum & Brady 1981), IPN (Harvarstein, Kalland, Christie & Endressen 1990; Frost & Ness 1997; Vaughan, Liljestroen, Todd, Lillehaug & Ramstad 1998), and VHS (Thiery, Lecocq-Xhonneux, Dheur, Renard & de Kinkelin 1990; Lorenzen, Einer-Jensen, Martinussen, LaPatra & Lorenzen 2000). Work has also continued against many other diseases including PKD (Adams *et al.* 1995), IHN (Enzmann, Fichtner, Schuetze & Walliser 1998; Cain, LaPatra, Shewmaker, Jones, Byrne & Ristow 1999), and BKD (Adams *et al.* 1995).

1.2.5. Novel uses of monoclonal antibodies

The use of MAbs has also been explored in other areas, including the characterisation of morphological features of organisms (Van de Braak, Taverne, Botterblom, Van der Knaap & Rombout 2000), and in investigating pathogen ultrastructural characteristics

(Morris, Adams & Richards 2000). In human public health, MAbs have been developed against domoic acid (the cause of amnesic shellfish poisoning) to be used in the screening of shellfish (Kawatsu, Hamano & Noguchi 1999), and against *Helicobacter pylori* for potentially screening water samples (Hegarty, Dowd & Baker 1999). Future possibilities include the use of MAbs in passive immunisation against infectious disease (Morimoto, Schnell, Pulmanusahakul, McGettigan, Foley, Faber, Hooper & Dietzschold 2001), in malignancy therapy (Glennie & Johnson 2000; Nelson, Reynolds, Waldron, Ward, Giannopoulos & Murray 2000) and the development of antibodies that catalyse chemical reactions, known as abzymes (Rodkey, Gololobov, Rumbley, Rumbley, Schourov, Makarevich, Gabibov & Voss 2000). Recently, advances in recombinant technology have allowed the production of specific immunoglobulin molecules, utilising a procedure known as phage display (Hoogenboom & Chames 2000; Miescher, Zahn-Zabal, De Jesus, Moudry, Fisch, Vogel, Kobr, Imboden, Kragten, Bichler, Mermod, Stadler, Amstutz & Wurm 2000). MAbs have been shown to have the potential for a wide variety of novel applications.

1.3. Objectives of this project

The objectives of this study were to develop and characterise MAbs against the Scottish isolate of ISAV. If successful, these probes could have multiple applications in diagnostics and future studies of the virus, including working towards recombinant vaccine formation.

2. Materials and Methods

2.1. Culture and purification of infectious salmon anaemia virus

Two 25 cm³ tissue culture flasks were lined with salmon head kidney cells (SHK-1, passage 57, 3-4-01), and 0.5 ml aliquots of ISAV isolates from Scottish outbreaks (obtained from FRS Marine Laboratory Aberdeen, Scotland) were added to each flask on the following day. These were placed in an incubator at 15°C. Sixteen days later, once a recognisable CPE was seen, both flasks were harvested, the contents centrifuged at 1,000 x g (Denley, BR 40 centrifuge) for 10 minutes to crudely clarify the virus by separating cellular debris. The supernatant containing virus was removed and stored at -70°C in 3 ml aliquots until further use.

Four days later, a 5 ml sample was thawed, clarified at high speed and finally pelleted as follows. An ultracentrifuge (Beckman, L-80), its rotor and buckets (SW 41Ti) were cooled to 4°C. Ultracentrifuge tubes were sterilised in 70% ethanol for 10 minutes, allowed to air dry and inverted on a disinfectant wipe soaked with 70% ethanol (Spectrum ®, Johnson & Johnson™) to allow final drying. The virus (5 ml) was added to one of these tubes, and the volume made up to 12 ml using TNE buffer (Appendix I). This sample and a balancing tube were then ultracentrifuged at 12,000 x g for 35 minutes at 4°C to allow clarification of the virus by removing cellular material. The supernatant, containing virus was then decanted and pipetted into a new sterile tube and the volume made up to 12 ml with TNE buffer. The virus was pelleted by ultracentrifugation at 100,000 x g for 95 minutes at 4°C. The supernatant was then discarded and the pellet allowed to dry slightly by inverting the tube on a 70% ethanol-soaked disinfectant wipe for 1-2 minutes. TNE buffer was added to the

tube to resuspend the pellet, and the sample ultracentrifuged again at 100,000 × g for 95 minutes at 4°C.

The supernatant was decanted and discarded, and the pellet allowed to dry slightly before 200 µl of TNE buffer was added to the tube. The tube was covered with film (Nescofilm™) and kept overnight at 4°C, being manually agitated periodically by gently flicking. The pooled sample tube was placed in a sonication bath for 2 minutes to aid in the separation of aggregates of virus material. The sample was placed in a sterile cryovial, then placed in 50 µl aliquots in Eppendorf tubes and stored at -70°C.

2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Initially, to investigate viral protein components, a Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) assay was conducted on serial dilutions of Scottish ISAV ranging from undiluted to 1/8 in TNE buffer. The method of Laemmli (1970) was used with modifications. Two pre-cast 4-20% gradient gels (see Section 2.2.2.) were used, one was stained with Coomassie blue and one with silver stain. Subsequently, three 12% gels were made (as in Section 2.2.1.), virus diluted 50:50 in TNE buffer and negative controls of SHK-1 cells (dilutions from 1 to 1/100) were added. Two of these gels were used for staining, the other was used for a Western blot immunoassay, using the method of Wiens, Turaga and Kaatari (1990), with modifications as described in Section 2.4.3.

2.2.1. 12% Gel formation protocol

Samples of the Scottish isolate of ISAV were analysed by electrophoresis. A gel holder apparatus (Hoefer Dual Gel Caster, SE245) was assembled. The separating gel (Appendix II) was prepared: firstly separating gel buffer was added to distilled H₂O and acrylamide solution. This mixture was placed in a Buchner flask and attached to a pump to facilitate degassing for 15 minutes. Then, ammonium persulphate and N,N,N,N-Tetramethylethylenediamine (TEMED) were added and the solution was immediately poured between the mounted plates of the gel casting apparatus. One hundred µl of butanol was layered over the separating gel to prevent evaporation from the free surface. The gel was allowed to polymerise for 60 minutes at room temperature. The butanol was washed away with distilled H₂O, then the stacking gel (Appendix II) was poured over the solidified separating gel. Placing plastic combs in the stacking gel made sample wells. This was allowed to set for 60 minutes at room temperature, after which the combs were removed, allowing sample addition.

2.2.2. Electrophoresis procedure

The gels used for electrophoresis were either made as in Section 2.2.1., or were pre-cast 4%-20% gradient Mini-Polyacrylamide gels (Sigma™, P-5466; 6.8 cm x 8.1 cm).

The gel apparatus (mini-Protean II, Biorad Labs Ltd.) was filled carefully with reservoir buffer (Appendix II), avoiding the entrapment of any air bubbles. Samples of ISAV and SHK-1 cells were added to sample buffer (Appendix II) at a ratio of 4 parts sample to 1 part buffer. These samples were heated to 98°C for 4 minutes in a water bath before being centrifuged at 13,000 x g for 5 minutes. Approximately 20 µl

of sample was then added to each well, known molecular weight standards were also applied: broad range protein markers (New England BioLabs Inc., P7702S) for gels subsequently stained; recombinant protein molecular weight markers (Amersham Life Science™, Rainbow RPN 800) for gels used for Western blot assays. An electrical potential difference of 100 volts was then applied across the gel until the dye front (visible due to the presence of bromophenol blue) approached the bottom of the separating gel.

The gel was then either stained or subsequently used for Western blot analysis.

2.2.3. Coomassie blue staining of the gel

Two gels were placed in glass dishes and covered with Coomassie blue stain (Appendix II). Overnight incubation at room temperature under continuous agitation followed. The next morning, successive changes of de-stain solution (Appendix II) were used until reasonable contrast could be achieved. The gels were stored in petri dishes containing distilled H₂O.

2.2.4. Silver staining of the gel

Two gels were stained using the reagents of a Silver Staining Kit (Sigma™ AG-25, Appendix II). The solutions were added to the gels in a glass dish. Initially, Fixing Solution was added for 3 washes of 20 minutes. Three washes of 10 minutes each in deionised H₂O were followed by a 30 minute soak in the Silver Equilibration Solution. The gels were then rinsed for 15 seconds with deionised H₂O. Development Solution was added for two incubations of 6 minutes and was immediately followed by a 5 minute bathe in Stop Solution. Three 10 minute soaks in deionised H₂O

followed. Reducer Solution was then added for 15 seconds (until background colouration diminished), followed by a 1 minute rinse in running tap H₂O. Three 10 minute soaks in deionised H₂O completed the procedure. The gels were stored in petri dishes containing distilled H₂O.

2.2.5. Regression plot analysis of standard molecular weight markers

2.2.5.1. Coomassie blue stained 4-20% gradient gel

The relative electrophoretic migration (R_f) of each of the bands of the standard molecular weight (MW) markers was calculated by dividing the measured migration of each band by the full length of the run of the gel.

Using the Minitab™ computer statistical package, a fitted line regression plot was formulated, allowing the MW in kilo Daltons (kDa) of sample bands to be interpreted from measured R_f :

$$\text{MW (kDa)} = 555.963 - 2558.22 R_f + 4199.98 R_f^2 - 2366.4 R_f^3$$

$$r^2 = 0.994 \quad F = 512.691 \quad P < 0.001$$

2.2.5.2. Silver stained 4-20% gradient gel

Regression plot from Minitab™:

$$\text{MW (kDa)} = 570.099 - 2690.33 R_f + 4531.3 R_f^2 - 2624.4 R_f^3$$

$$r^2 = 0.996 \quad F = 779.87 \quad P < 0.001$$

2.2.5.3. Silver stained 12% gel

Regression plot from Minitab™:

$$\text{MW (kDa)} = 4.98998 - 85.5865 \log R_f - 69.2946 \log R_f^2 - 52.985 \log R_f^3$$

$$r^2 = 0.999 \quad F = 1640.4 \quad P < 0.001$$

2.3. Mouse immunisation

Two 5-6 week old Balb/c female mice were immunised for production of antibodies against ISAV. Before any antigen was administered, 0.5 ml of blood was taken from their tail veins and stored for future use as pre-immune serum samples. Each mouse was given an intraperitoneal injection of 100 µl of ISAV of approximate protein content 200 µg ml⁻¹, diluted 50:50 with Titermax adjuvant (Vaxcel™ Inc., USA). Four weeks later, to boost the mice, the immunisation procedure was repeated. Ten days later, 0.5 ml of blood was collected from each mouse to assess, by ELISA, which mouse gave the greater immune response (as described in Section 2.4.2.). The mouse with the higher titre of anti-ISAV antibodies was given the final boost of 100 µl of virus (without adjuvant) intravenously. The mouse spleen was ready for use in hybridoma production 4 days later.

2.4. Screening mouse test bleeds

To assess the antibody titres before commencing fusion, test bleeds from the two mice immunised with ISAV were screened by an indirect ELISA test, using the method of Adams (1992) with modifications. An overview of the method is shown in Figure 2, with a full description in Section 2.4.2.

2.4.1. Assessment of the protein content of the ISAV samples

In order to assess the concentration of ISAV present in the aliquots for subsequent use in immunoassays, protein assays were carried out.

The optical density of the sample was measured at 280 nm (OD₂₈₀) in a spectrophotometer (Jenway™) to allow an estimation of the protein content to be made using the equation: protein content (mg ml⁻¹) = OD₂₈₀ / 1.4 (Bollag, Rozycki & Edelstein 1996).

A protein assay kit (Biorad Protein Assay; 500-0001) was also utilised to give a measure of soluble protein present in the virus sample. Protein standards comprising bovine serum albumin (BSA) were made for dilutions (in distilled H₂O) from 0.05 – 1 mg ml⁻¹. Quantities of 0.1 ml of sample buffer, each BSA standard, and the sample were placed in clean dry Bijoux. Five ml of dye reagent (diluted ¼ in distilled H₂O & filtered) was added to each tube, and mixed by gentle inversion. After 10 minutes, the OD₅₉₅ was measured firstly for the sample buffer to allow calibration, then for each protein standard and for the virus sample. Plotting OD₅₉₅ versus concentration of the known protein levels made a standard curve, and the sample concentration was read from it.

2.4.2. ELISA procedure for screening mouse test bleeds

Initially, an attempt was made to coat a 96 well ELISA plate (Immulon, Dynex™) following the method of Dixon & de Groot (1996). ISAV was dissolved in coating buffer (Appendix I) to a concentration of 1 µg ml⁻¹, the plate coated with ISAV at 200 µl well⁻¹, and incubated overnight at room temperature.

Following lack of success with the initial method, the coating procedure was modified to follow the technique of Adams *et al.* (1995). An ELISA plate was

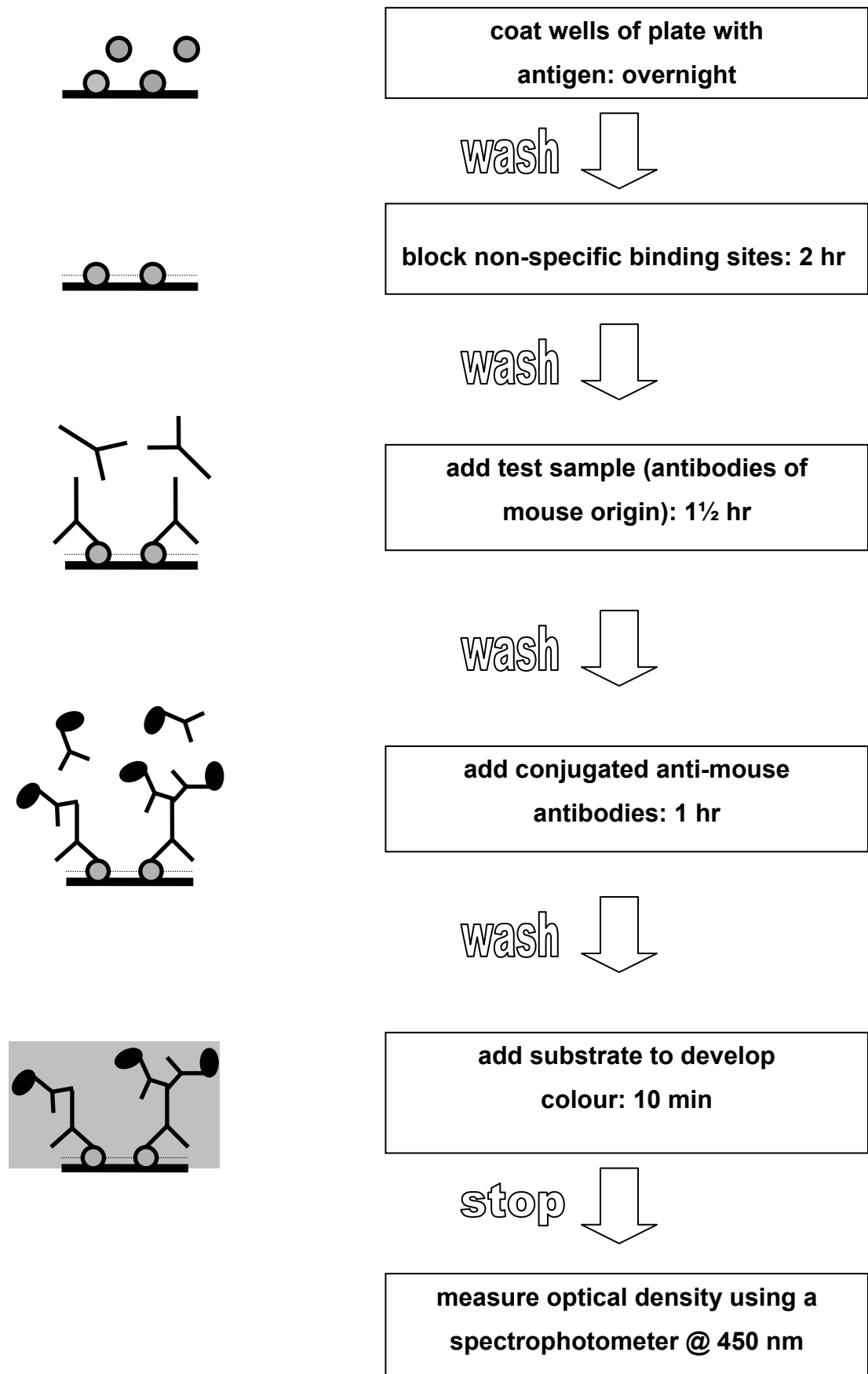


Figure 2: Overview of method of an indirect antibody capture ELISA

coated with 50 $\mu\text{l well}^{-1}$ of 0.01% (w/v) poly-L-lysine in coating buffer (Appendix I). An incubation period of 60 minutes at room temperature was followed by triple washing with low salt wash buffer (LSWB, Appendix I). ISAV was diluted in TNE buffer (Appendix I) to give a concentration of 1 $\mu\text{g ml}^{-1}$ and added to the plate at 100 $\mu\text{l well}^{-1}$, overnight incubation at 4°C followed, allowing the plate to be coated with the antigen. Four negative control wells: 2 containing neat TNE buffer, and 2 with SHK-1 cells dissolved in TNE buffer were included. The following morning, 50 $\mu\text{l well}^{-1}$ of 0.05% (v/v) glutaraldehyde in phosphate buffered saline (PBS, Appendix I) was added for 20 minutes at room temperature to aid in antigen fixation.

Following triple washing with LSBW, the plate was post-coated with 250 $\mu\text{l well}^{-1}$ of 3% casein (w/v) in distilled H₂O for 2 hours at room temperature to block non-specific binding sites. Triple LSBW washing followed this. Serial dilutions of pre-immune and post-immunised mouse sera were made from 1/200 to 1/6400 dilution, in antibody buffer (Appendix I). MAb produced against Norwegian ISAV, (MAb 3H6F8 obtained from Dr. K. Falk, National Veterinary Institute, Oslo, Norway (Falk *et al.* 1998)), was diluted 1/100 in antibody buffer; antibody buffer was used as a negative control. Each sample and control was added in duplicate to the plate at 100 $\mu\text{l well}^{-1}$ and incubated at room temperature for 90 minutes to allow binding between fixed antigens and soluble antibodies. To remove unbound antibodies, this was followed by 5 washes in high salt wash buffer (HSWB, Appendix I) and a 5 minute soak on the last wash. Goat anti-mouse immunoglobulin-G labelled with horseradish peroxidase (HRP) (Scottish Antibody Production Unit, Lanark, Scotland) was diluted 1/1000 in conjugate buffer (Appendix I), and added at 100 $\mu\text{l well}^{-1}$, followed by a 1 hour incubation at room temperature. Following HSWB washing as described

previously, 100 µl of substrate (Appendix I) was added to each well, and incubated at room temperature for 10 minutes, until colour developed. Stop solution (2M H₂SO₄ in distilled H₂O) was added at 50 µl well⁻¹ to the plate, and the results were read at 450 nm using an ELISA reader (Dynex™ MRX II). Values were taken as positive if the OD₄₅₀ exceeded triple that of the average negative control value.

2.4.3. Western blot immunoassay using mouse serum from time of fusion

Western blot analysis of the polyclonal anti-serum from the mouse was performed to determine the potential MAbs that might be produced following fusion. Samples of ISAV were processed for SDS-PAGE as described in Section 2.2., but the gel was not stained. Instead, the gel was placed in transblot buffer (Appendix III) for 20 minutes. Nitrocellulose paper, filter papers & filter pads were also equilibrated in the buffer at the same time. The gel and nitrocellulose paper were then mounted onto the transblotting device (Biorad™) in the following order: outer envelope, negative electrode, filter pad, 3 sheets of filter paper, gel, nitrocellulose paper, 3 sheets of filter paper, filter pad, positive electrode, outer envelope. Any air bubbles were carefully removed by rolling each layer with a Pasteur pipette. The cassette was placed into the transblotting chamber and this was filled with transblot buffer. The buffer was kept well mixed by placing the device over a magnetic stirrer which rotated a magnet sited in the bottom of the chamber.

The transfer was achieved by applying a potential difference across the apparatus of 60 volts for 60 minutes. The device was then taken apart and non-specific binding sites were blocked by soaking the nitrocellulose paper in 1% (w/v) BSA in TBS (Appendix III) for 60 minutes at room temperature. The nitrocellulose paper was

washed 3 times for 5 minutes each with HSWB. The paper was then cut into strips, each corresponding to one lane of the initial gel, and these were placed in a mini incubation tray. The strip containing the standard molecular weight marker lane was stored in a universal tube containing distilled H₂O. One ml of 1/100 mouse serum (diluted in antibody buffer), was added to each lane, and incubated overnight at room temperature under constant agitation. Three washes as above followed. Conjugate in the form of 1 ml of biotin anti-mouse immunoglobulin-G (1/100 in antibody buffer) was added to each well for 90 minutes at room temperature, followed by washing as above. Next, 1 ml of streptavidin peroxidase (1/100 in antibody buffer) was added to each well, and left for 90 minutes at room temperature. Washing as above was followed by a 1 minute soak in TBS. Adding chromogen and substrate (2 ml of stock substrate solution (Appendix III) plus 10 ml of PBS & 10 µl of H₂O₂) until the bands materialised developed the blot. Soaking the strips in distilled H₂O for 10 minutes stopped the reaction. The strips were then realigned and glued to a piece of white card alongside the standard molecular weight marker strip to allow interpretation of the results.

2.4.3.1. Regression plot analysis of standard molecular weight markers

Regression plot from Minitab™:

$$\log \text{MW (kDa)} = 1.03256 - 2.65073 \log R_f - 4.54631 \log R_f^2 - 3.29977 \log R_f^3$$

$$r^2 = 0.979$$

$$F = 76.76$$

$$P < 0.001$$

2.5. Hybridoma production

Hybridomas were produced following the method of Adams, Richards and Marin de Mateo (1992) with modifications as follows.

2.5.1. Thawing of myeloma cells

Seven days before fusion, a vial of cells from a mouse myeloma cell line (SP2) was removed from storage in liquid nitrogen. It was placed in a water bath at 37°C to allow rapid thawing within 5 minutes. Nine ml of Dulbecco's Modified Eagles Medium (DMEM, Sigma™ D-5671), supplemented with 1% L-glutamine, 0.5% penicillin-streptomycin, and 1% sodium pyruvate was added to a 10 ml sterile conical tube. Using a sterile 1 ml Pasteur pipette, 1 ml of 20% foetal calf serum (FCS, previously heat inactivated for 30 minutes at 56°C) was placed into the tube beneath the DMEM. Using a sterile 1 ml pipette, the cells were layered onto the medium, and the tube centrifuged at 150 x g for 7 minutes. The pellet was then gently resuspended in 5 ml supplemented DMEM & FCS, transferred into a 25 cm³ tissue culture flask and kept at 37°C in a CO₂ incubator. Cells were checked on the following day with an inverted light microscope to confirm survival. The cells were checked regularly, and counted with a haemocytometer before fusion commenced.

2.5.2. Preparation of mouse splenocytes

Four days after the final intravenous boost with virus, the immunised mouse was killed by exposure to CO₂ and immediately exsanguinated by cardiac puncture using a 23 gauge needle attached to a 2 ml syringe. This sample was placed in an Eppendorf tube and then centrifuged at 300 x g for 15 minutes. This serum sample was used for a Western blot assay to assess the antibody content at the time of fusion

(Section 2.4.3.). The ventral surface of the mouse was swabbed with 70% alcohol and the spleen was carefully removed aseptically using sterile instruments. Care was taken to trim the edges of the spleen avoiding penetration of the intestinal lumen. The spleen was immediately placed in a universal tube containing 20 ml of serum-free DMEM. Under a sterile hood, it was placed in a petri dish containing DMEM and any remaining fat was aseptically trimmed off, followed by 3 washes in serum-free DMEM (warmed to 37°C). In order to release the antibody-producing cells from the organ, the spleen was flushed by cutting both ends with scissors and passing 4 washes of 5 ml of warmed DMEM through the interior. The cell suspension was then placed in a universal tube and allowed to settle for 1 minute. The supernatant was collected and made up to a volume of 50 ml with warmed DMEM.

2.5.3. Preparation of the mouse red blood cell feeder layer

A mouse red blood cell feeder layer was used in the culture medium for hybridoma cells, allowing growing clones to be seen easily.

A non-immunised mouse (of the same strain as the mouse used for the fusion) was killed by exposure to CO₂, and immediately exsanguinated by cardiac puncture using a 23 gauge needle attached to a 1 ml syringe containing 10 iu of sterile heparin as an anticoagulant. One ml of blood was added directly to 300 ml of DMEM with supplements, & 2% hypoxanthine aminopterin thymidine (HAT, Sigma™ H-0262) solution. This flask was stored in an incubator at 37°C until needed for the fusion procedure.

2.5.4. Fusion of immunised mouse spleen cells with myeloma cells

Both splenocytes and myeloma cells were removed from their tissue culture flasks and centrifuged for 7 minutes at 150 x g. The cells were resuspended in 50 ml warmed DMEM (serum-free) and centrifuged again at 150 x g for 7 minutes. The cells were resuspended, this time in 10 ml DMEM, and the number of cells present was assessed using a haemocytometer. The cells were mixed at a ratio of 1:10 myeloma cells : splenocytes. The mixed cell suspension was centrifuged at 100 x g for 7 minutes, and the supernatant was aspirated to leave a dry pellet. One ml of 50% w/v polyethylene glycol (PEG, Sigma™ P-7181) was slowly added to the pellet and allowed to stand for 90 seconds. Then, 1 ml of PEG was added over a period of 30 seconds, then 3 ml PEG over 30 seconds and finally 16 ml PEG over 1 minute. This was then allowed to stand undisturbed for 5 minutes before being centrifuged at 100 x g for 7 minutes. The supernatant was aspirated and the pellet very gently resuspended in 10 ml supplemented DMEM, 20% FCS (Sigma™ F-2442) & 2% HAT. This was then placed in a CO₂ incubator for 2 hours at 37°C. Following this, the suspension of fused cells was centrifuged for 7 minutes at 100 x g. The supernatant was aspirated and the volume was made up to 200 ml using the mouse red blood cell feeder layer (as prepared in Section 2.5.3.). The cell suspension was pipetted at 0.5 ml well⁻¹ into 24 well tissue culture plates (Costar® cell culture cluster dish). Negative control wells of myeloma cells (which die due to the HAT medium), and splenocytes were also set up. The plates were placed in a CO₂ incubator at 37°C. The cells were checked under a microscope after 7 days, and screening commenced at 10 days following fusion using an ELISA.

2.5.5. Screening of 'parent' hybridoma cells by ELISA

Ten days after the fusion procedure, the tissue culture plates were examined microscopically, and 55 wells were seen to contain defined colonies of hybridoma cells. Supernatant from each of these was removed and was screened for the presence of anti-ISAV antibodies by an indirect ELISA procedure.

The assay followed the same general protocol as that described in Section 2.4.2., with the following differences. On this occasion, 3 ELISA plates were used. Wells were coated with either virus or SHK-1 cells. Previously tested mouse anti-serum (Section 2.4.2.) was used as a positive control, antibody buffer as a negative control. Hybridoma supernatants from the chosen 55 wells were added at a rate of 100 μ l well⁻¹ in duplicate to wells pre-coated with ISAV or SHK-1 cells.

2.5.6. Screening of 'parent' hybridoma cells by Western blot immunoassay

A Western blot procedure was conducted to discern the range of antibody production of the 'parent' hybridoma cells, using the procedure described in Section 2.4.3., with the following modifications. In addition to mouse anti-serum, pre-immune mouse serum (1/100 in antibody buffer) was added to one lane, and neat supernatant from the 'parent' hybridoma cells that were screened positive by ELISA was added to each of 5 lanes.

2.5.6.1. Regression plot analysis of standard molecular weight markers

Regression plot from Minitab™:

$$\log \text{MW (kDa)} = 2.03942 - 1.23664 \log R_f + 0.532737 \log R_f^2$$

$$r^2 = 0.998$$

$$F = 969.47$$

$$P < 0.001$$

2.5.7. Cloning of hybridoma colonies

Supernatant from the 5 wells that proved positive by ELISA screening were cloned onto 96 well tissue culture plates (Greiner, Cellstar®). Serial dilution of the supernatant was implemented in attempting to achieve clonal colonies producing single MAbs.

One hundred ml of the mouse red blood cell feeder layer was made up (as described in Section 2.5.3.), and using a multi-channel pipette, 100 µl was added to each well of five 96 well plates. The 5 wells which were screened positive by ELISA were selected, and 100 µl of supernatant from each was added to the top left well (A1) of a 96 well plate, and mixed with the mouse red blood cell feeder layer by pipetting up & down. Removing 100 µl from well A1, adding it to the well below (B1) & mixing made a doubling dilution. This was continued down column 1 of the plate. Using a multi-channel pipette, 100 µl was removed from each well in column 1, added to the adjacent well in column 2 and mixed. This was continued across the plate to achieve serial dilution of the supernatant across each row. The 5 plates were then stored in a CO₂ incubator. Screening of the contents of the wells was carried out 10 days later.

The original 'parent' hybridoma cells were cloned a second time, following loss in the production of antibodies by the cells cloned on the first occasion. The method was followed as above, except that on this occasion 50 µl of L-glutamine was added to each well as additional nutrition. Screening was again carried out 10 days later.

2.5.8. Screening of cloned hybridomas by ELISA

Each well of the 5 plates was examined microscopically to assess growth of hybridoma colonies. Supernatant from each well with noticeable hybridoma growth was removed and an ELISA assay was carried out on a single plate as described in Section 2.4.2. Norwegian MAb (MAb 3H6F8 courtesy of Dr. K. Falk) at 1/25 dilution was also included. Duplicates on SHK-1 lined wells were not carried out due to insufficient supernatant being available.

This procedure was repeated following the second cloning.

3. Results

3.1. SDS-PAGE analysis of ISAV

The Scottish ISAV isolate was analysed by SDS-PAGE. Four gels were completed, two 4-20% gradient gels, and two 12% gels, one of each being stained with Coomassie blue, and one with silver stain. The MW of the bands was estimated using the equations in Section 2.2.5. On the Coomassie blue stained 4-20% gradient gel (Figure 3.1), one band was observed for ISAV, equating to a MW of 71 kDa. The silver stained gradient gel (Figure 3.2) revealed 14 viral bands, the most prominent being of MW 20 kDa, 27 kDa, 70 kDa, and 224 kDa. The Coomassie blue stained 12% gel revealed no bands in the virus lanes, while the silver stained 12% gel (Figure 3.3) showed 10 viral bands, the most prominent being at 33 kDa, 45 kDa, and 72 kDa.

Nine bands were seen in the SHK-1 cell lanes of the silver stained 12% gel, the most prominent corresponding to 75 kDa and a group of bands at 15-19 kDa (Figure 3.3).

3.2. Determination of protein content of ISAV samples

The protein content of ISAV samples was determined by measuring the OD₂₈₀ to calculate total protein, and using a protein assay kit to estimate soluble protein concentration.

Measuring the OD₂₈₀ of samples of virus gave readings between 0.280 and 0.323, approximating to a total protein level in the range of 200 µg ml⁻¹ - 230 µg ml⁻¹. Soluble protein content was estimated to be 33 µg ml⁻¹.

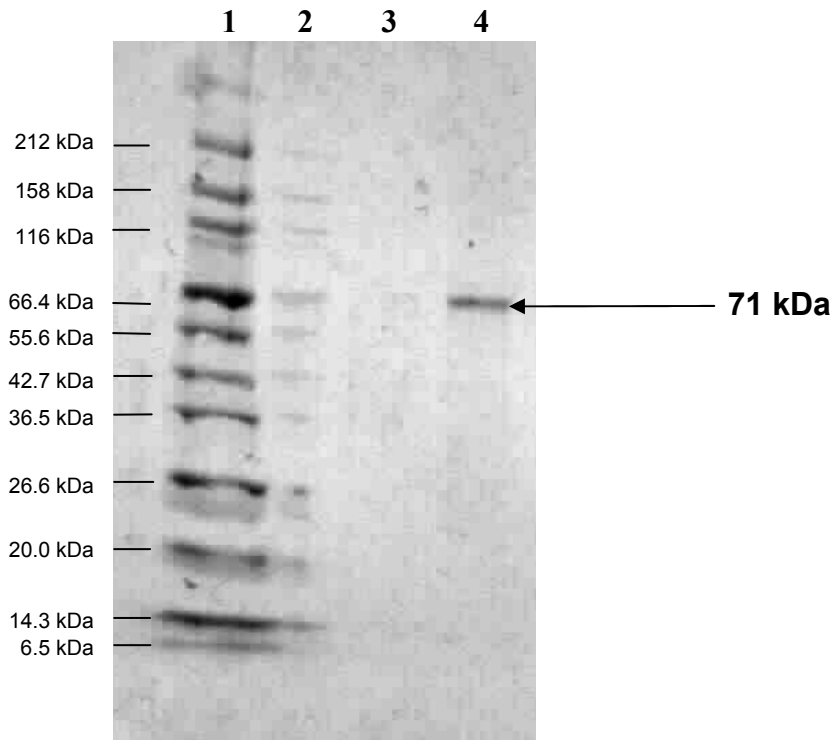


Figure 3.1: Coomassie blue stained gradient SDS-PAGE gel of Scottish ISAV
Lanes 1 & 2 = standard Molecular Weight (MW) markers; lanes 3 & 4 = ISAV at concentrations of $25 \mu\text{g ml}^{-1}$ & $50 \mu\text{g ml}^{-1}$ respectively. MW of bands shown in kilo Daltons.

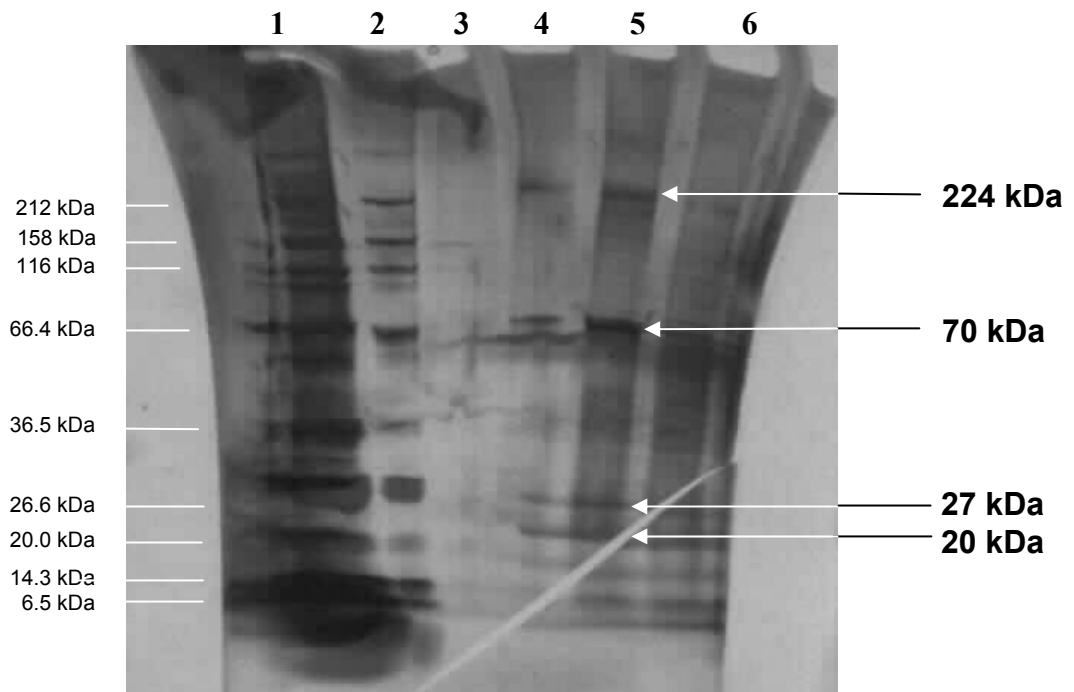


Figure 3.2: Silver stained gradient SDS-PAGE gel of Scottish ISAV
Lanes 1 & 2 = standard Molecular Weight (MW) markers; lanes 3 - 6 = ISAV at concentrations of $25 \mu\text{g ml}^{-1}$, $50 \mu\text{g ml}^{-1}$, $100 \mu\text{g ml}^{-1}$, & $200 \mu\text{g ml}^{-1}$ respectively. MW of bands shown in kilo Daltons.

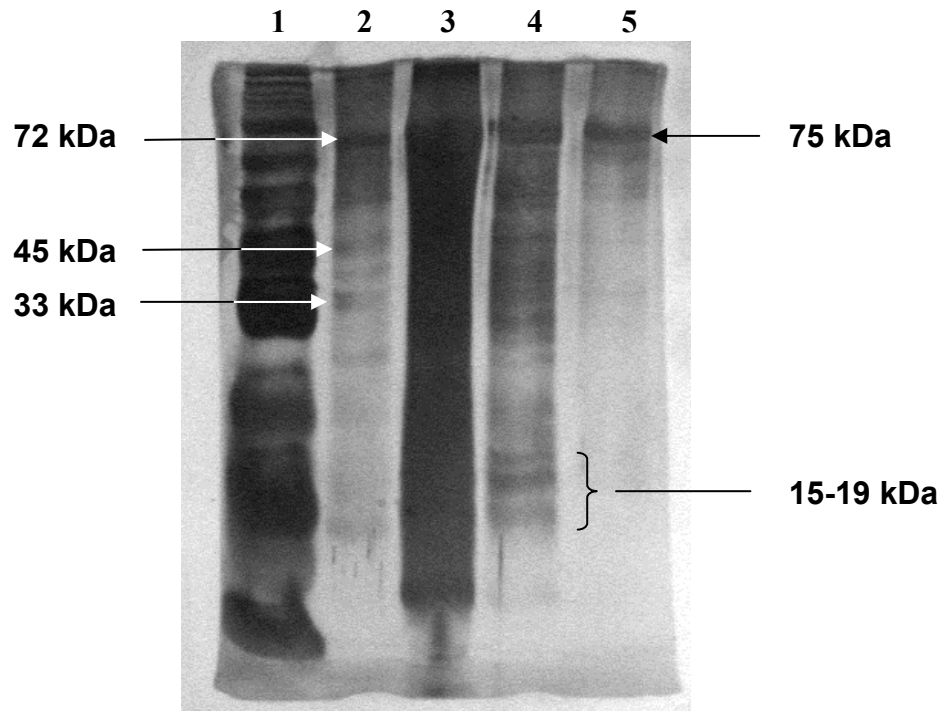


Figure 3.3: Silver stained 12% SDS-PAGE gel of Scottish ISAV & SHK-1 cells
Lane 1 = standard Molecular Weight (MW) markers; lane 2 = ISAV of concentration $200 \mu\text{g ml}^{-1}$; lanes 3 - 5 = SHK-1 cells: undiluted, 1/10, 1/100 dilutions respectively. MW of bands shown in kilo Daltons.

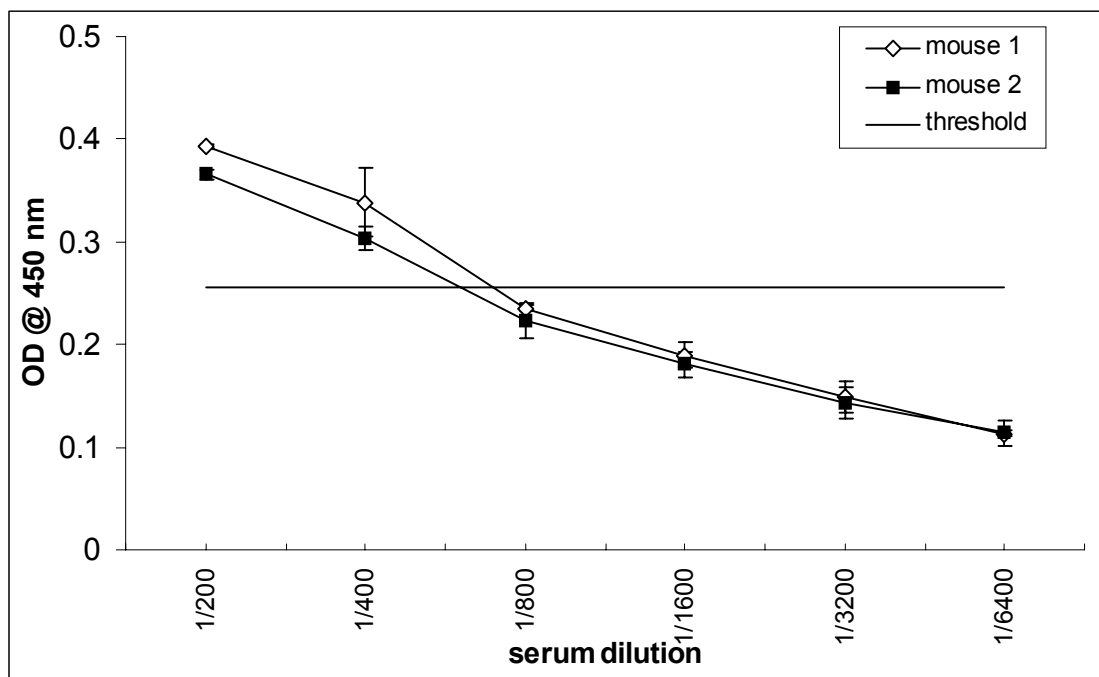


Figure 3.4: Graph of Optical Densities (OD_{450}) from ELISA of mice test bleeds following immunisation with ISAV

Mouse ODs shown \pm standard deviation, threshold level represents triple the negative control OD.

3.3. Screening of mouse test bleeds by ELISA

The initial method attempted for coating the ELISA plates was unsuccessful, the OD₄₅₀ for the immunised mouse sera (1/100) giving readings (0.101 & 0.072) insignificantly greater than the negative control value (0.073). The method was then modified, in an attempt to increase sensitivity.

With the modifications, the titre of the tested mouse sera was estimated to be 1/400 for both mouse 1 & mouse 2 by ELISA (OD₄₅₀ of 0.338 & 0.304 respectively) (Figure 3.4). The average negative control OD₄₅₀ value was 0.085. Pre-immune sera from the 2 mice, & the SHK-1 cells both gave negative results.

MAb 3H6F8 (obtained from Dr. K. Falk) gave a negative result (0.115) against Scottish ISAV in this assay.

3.4. Western blot assay using mouse serum

Western blot analysis of mouse anti-serum collected at the time of the fusion was conducted (Figure 3.5). Three bands were observed for ISAV, corresponding to MWs of 45 kDa, 60 kDa (faint) & 70 kDa.

Four bands were apparent in the SHK-1 cell lanes, corresponding to MWs of 25 kDa, 35 kDa (faint), 68 kDa & 75 kDa.

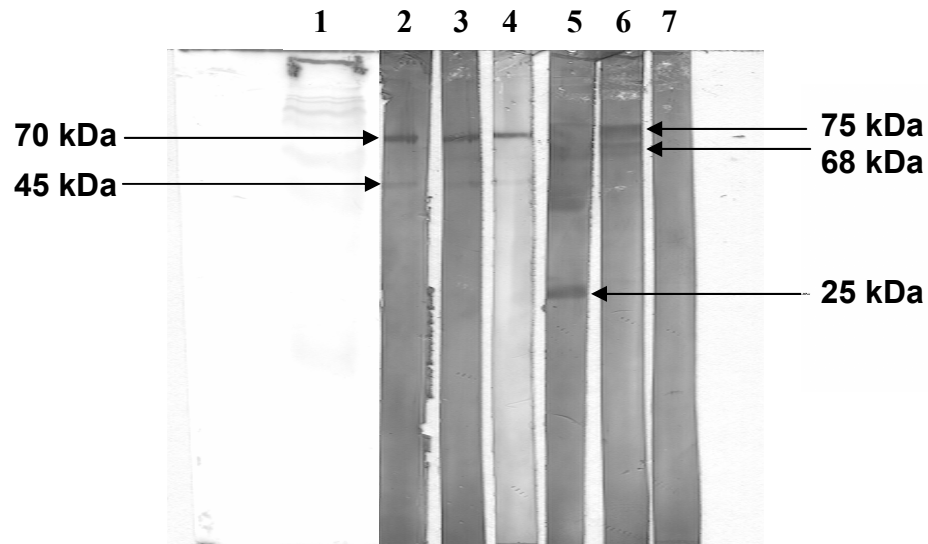


Figure 3.5: Western blot immunoassay of Scottish ISAV isolate & SHK-1 cells using mouse anti-serum

Lane 1 = standard MW markers; lanes 2 - 4 = ISAV of concentration 200 µg ml⁻¹, lanes 5 - 7 = SHK-1 cells: undiluted, 1/10, 1/100 dilutions respectively. Subsequent addition of mouse anti-serum to lanes 2 - 7. MW of bands shown in kilo Daltons.

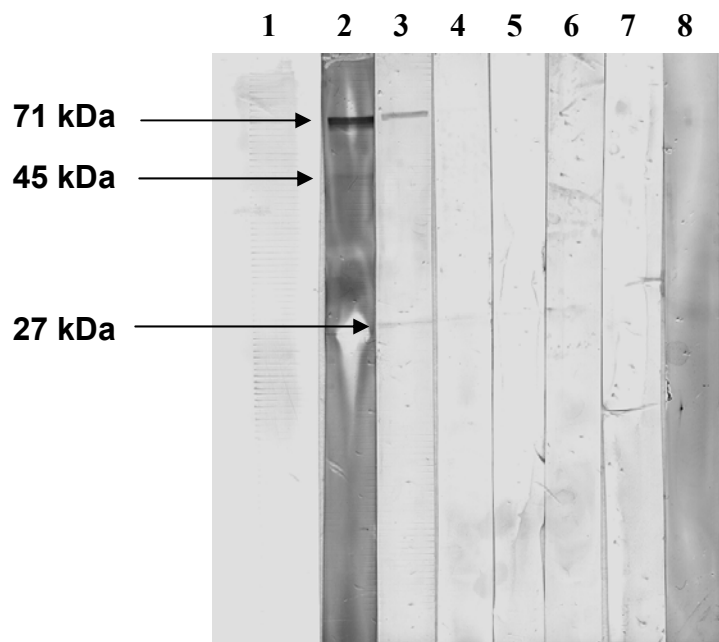


Figure 3.6: Western blot immunoassay of Scottish ISAV, using mouse anti-serum & hybridoma supernatants

Lane 1 = standard MW markers; lanes 2 - 8 coated with ISAV, with subsequent addition to: lane 2 of immunised mouse serum; lanes 3 – 7 of hybridoma supernatants (denoted by well origin): lane 3: 17 R3, lane 4: 1 A3, lane 5: 8 B5, lane 6: 9 D3, lane 7: 11 A2; lane 8 of pre-immunised mouse serum (negative control).

3.5. Fusion of mouse spleen cells with myeloma cells

3.5.1. Screening of 'parent' hybridoma cells by ELISA

Microscopically, growth was evident in 55 wells ten days after fusion. Supernatant from these wells was assayed against ISAV, uninfected SHK-1 cells and TNE buffer by ELISA. The positive control wells consisting of virus and anti-ISAV mouse anti-serum gave an average reading (0.895), in excess of triple the negative control of 0.104. On the virus-coated wells, 5 of the 55 supernatants gave OD₄₅₀ readings of more than double the negative control level, none gave triple the value. On the SHK-1 cell coated wells, 1 supernatant gave a reading of more than triple, and 4 more than double the negative control value. On the TNE buffer coated wells to which anti-ISAV mouse anti-serum was added, a reading (0.386) more than double the negative control was obtained.

3.5.2. Screening of 'parent' hybridoma cells by Western blot immunoassay

Western blot analysis using the supernatant from the 5 'parent' hybridoma cell colonies was conducted, revealing 2 bands with hybridoma supernatant 17 R3 at 27 kDa and 71 kDa (Figure 3.6). A faint band was seen with supernatant 1 A3 at 27 kDa. The positive control lane (mouse anti-serum) showed bands at 45 kDa and 71 kDa. The negative control lane (ISAV and pre-immune mouse serum) showed no bands.

3.5.3. Screening of cloned hybridomas by ELISA

Microscopic examination of the five 96 well plates revealed growth in 19 wells of plate 1 A3, and in 7 wells of plate 17 R3. No growth was evident on the remaining 3 plates. By ELISA, the positive control wells gave an average reading (0.397) in excess of triple the negative control value of 0.060, however, none of the 26 tested

hybridoma supernatants gave a positive result. Norwegian MAb 3H6F8 also gave a negative result (0.086). The wells initially coated with TNE buffer with subsequent addition of mouse anti-serum gave a positive result (0.217).

The 'parent' cell lines were therefore re-cloned and more apparent cell growth was observed than with the first cloning. Hybridoma clones were seen in 4 wells of plate 8 B5, and 2 wells from plate 17 R3. However, supernatant from these 6 wells tested negative against ISAV by ELISA. No growth was seen on the other plates.

4. Discussion

Infectious salmon anaemia has proven to be a disease of immense political, economical and clinical significance (Håstein 1998). The threat of the disease has led to the implementation of severe regulatory measures, not only affecting the international movement of salmonids and products, but also having serious consequences for owners of infected premises. The first outbreaks of ISA in Scotland resulted in the destruction of fish on confirmed and suspected sites, followed by mandatory 6 month fallowing periods (Anonymous 1998). Legal restrictions have led to severe losses to businesses, involving the compulsory slaughter of stock without compensation (Hill 1994; Hill 1996; Håstein, Hill & Winton 1999). The association of ISAV with members of the *Orthomyxoviridae*, which cause fatal human diseases, has caused additional concern.

Orthomyxoviruses have been held responsible for a massive level of human mortality worldwide (Ginsberg 1988). The influenza viruses (A, B & C) belonging to this family, have been found to be highly prone to mutation, exhibiting both antigenic drift and antigenic shift. Whereas the former results in limited changes, the latter – occurring approximately once a decade – has led to devastating pandemic disease outbreaks due to lack of cross-strain immune protection. Haemagglutinins have been found to be the major antigenic surface glycoproteins, with neuraminidase also being important in influenza A & B. Each of these is thought to be involved in antigenic changes, mutations having been witnessed in both the haemagglutinin and neuraminidase genes. Various diagnostic techniques have been utilised in human medicine, including virus isolation, neutralisation, complement fixation,

haemagglutination, haemagglutination inhibition, ELISA, solid state radioimmunoassays and immunofluorescence.

The association of ISAV with the *Orthomyxoviridae* has caused concern over potential zoonotic risks from the consumption of infected fish. Although evidence exists that the propagation of ISAV requires an initial low pH (4.5) step (Eliassen, Froystad, Dannevig, Jankowska, Brech, Falk, Romoren & Gjoen 2000), the virus has been found to be inactivated below pH 3.0 (Torgersen 1998), and therefore ISAV would be unlikely to survive human gastric secretions (pH 2.0). Also, the virus has not been seen to replicate above 25°C, and to be inactivated at 37°C making mammalian infection unlikely (Falk *et al.* 1997). Additional morphological differences between the orthomyxoviruses and ISAV include the limited haemagglutination potential of the latter (Falk *et al.* 1997), varying nucleoprotein composition (Snow & Cunningham 2001), different specificities of the receptor destroying enzyme, different polypeptide profiles (Falk *et al.* 1997), and evidently, the host species range. Coupled with the failure of propagation of ISAV on mammalian cell lines, these factors make the risk of infection to mammals apparently minimal. However, the potent mutation rate of these viruses (Inglis *et al.* 2000) must be considered, meaning that possible future alterations leading to cross-species infection could not be disregarded. ISAV may have previously mutated from a harmless to pathogenic strain (Falk *et al.* 1998), therefore there exists the risk of future changes, such as those that led to the transmission of influenza viruses between poultry and humans (Ginsberg 1988).

As with the majority of diseases of viral aetiology, avoidance measures have been shown to be of critical importance. A wide range of measures has been devised in the control of spread of the syndrome (Anonymous 2000a). Prompt eradication and heightened biosecurity have been found to be essential in limiting the spread of ISA (Vagsholm, Djupvik, Willumsen, Tveit & Tangen 1994). An array of reliable and preferably rapid diagnostic techniques must be intrinsic in all control strategies.

Presently, unequivocal diagnosis of ISA relies upon a range of diagnostic measures and criteria. To satisfy the EU Community Reference Laboratory for Fish Diseases and L' Office International des Epizooties Reference Laboratory for ISA, a number of clinical, pathological and diagnostic techniques must be employed (Anonymous 2000a). The use of a wide range of tests could incur not only a large financial burden, but could also lead to possible delays in the confirmation of outbreaks. In the case of a highly infective pathogen, any postponement in instigating control measures could result in extensive disease spread. Also, epidemiological studies of the disease, including incidence and prevalence assessments, rely on accurate diagnostic techniques. The development of immunological probes has been found to be of great benefit in improving the understanding of disease pathogenesis (Nelson *et al.* 2000). Therefore, an antibody probe-based diagnostic test of high sensitivity, specificity and ease of use would be particularly desirable for ISAV.

Polyclonal antibodies (PAb) have been widely used as antibody probes for many years. In the short term, it has been relatively straightforward and inexpensive to produce great volumes of PAb using larger animals including horses, goats and rabbits (Nelson *et al.* 2000). PAb have been found to be more sensitive than MAb

when used in certain assays, including ELISA tests (Jansson, Hongslo, Hoeglund & Ljungberg 1996), PAbs have the ability to detect a multiplicity of epitopes, therefore recognising three-dimensional antigens from various orientations. Polyclonal anti-serum also has the capacity to form large insoluble immune complexes with antigen, allowing reactions to be easily measured visually or photometrically. However, the heterogeneity of immunoglobulin molecules that on one level allows increased sensitivity, could also lower specificity of a probe by increasing the chance of cross-reactivity with other substances. Problems can also arise when the supply of polyclonal anti-serum from a particular donor animal is exhausted, potentially leading to batch-to-batch variations in antibody reactivity and titre, reducing experimental reproducibility. Animal welfare also has to be taken into account when considering the continued production of PAbs involving the use of laboratory animals. These factors have resulted in the more widespread use of MAbs.

The development of MAbs has allowed a continuous supply of consistent monospecific antibodies. MAbs have been used widely for diagnostic purposes (Adams *et al.* 1995). They have been applied to a wide range of immunological techniques, although a particular MAb may be limited by assay restriction, only being of use in a limited spectrum of assays, depending on whether experimental conditions allow the specific target epitope to be recognisable (Catty 1988). MAbs have allowed the elucidation of previously unknown information, with many research applications. MAbs have been used in studies of disease pathogenesis, including visualising the location of antigen binding sites using transmission electron microscopy with immunogold labelling (Bartholomew, Yamamoto, Rohovec & Fryer 1990), and the immunohistochemical staining of histological sections (Adams & Marin de Mateo

1994). MAbs have been used widely in the development of subunit vaccines (Lorenzen 1999), and in an ever increasing number of novel applications including the morphological characterisation of pathogens (Van de Braak *et al.* 2000) and chemotherapeutics (Nelson *et al.* 2000).

In this study, the aim was to produce and characterise MAbs directed against a Scottish isolate of ISAV. Such antibodies could have a considerable number of applications, depending on various criteria including specificity and sensitivity. Ideally, anti-ISAV MAbs could be employed in reliable diagnostic tests, reacting with the various strains of the virus, but having limited cross-reactivity with other agents. On this occasion however, it did not prove possible to take the production process through to its natural conclusion within the available time.

Infectious salmon anaemia virus has been found to be notoriously demanding to culture to high levels *in vitro*, having taken over a decade from the first descriptions of the disease to the successful isolation of virus on the specially developed SHK-1 cell line (Dannevig *et al.* 1995). In this study, the virus was propagated on SHK-1 cells, with growth being evident only on younger cell lines of lower passage number. It was found that although the virus did replicate, causing a distinctive CPE, the growth was slow. Upon measuring the OD₂₈₀ of the produced ISAV, a protein range of 200 µg ml⁻¹ - 230 µg ml⁻¹ was calculated. Using the protein assay kit, a reading of only 33 µg ml⁻¹ was obtained. This apparent discrepancy may be explained by the fact that the Biorad™ protein assay kit quantified only soluble proteins; the difference between the assays could be accounted for by insoluble protein content. Attempts were made to propagate the virus on CHSE-214 cell lines, in the hope that

faster growth would occur. The Scottish isolate used in this study has not been found to cause a distinctive CPE on CHSE-214 lines, unlike certain other strains (Kibenge, Lyaku, Rainnie & Hammell 2000a). Therefore, it was difficult to gauge potential viral replication in the CHSE-214 cells. Supernatant from the CHSE-214 cells was removed and added to SHK-1 cells to look for development of CPE, but none was evident. Haemadsorption techniques have been used to detect the presence of ISAV in SHK-1 cells in cases where a CPE had not developed (Smail, Grant, Ross, Bricknell & Hastings 2000). Possibly such a procedure could be attempted with CHSE-214 propagated virus in the future. Recently, a newly developed cell line – designated TO – has been produced, apparently allowing higher yields of virus to be obtained which may be of great future value (Wergeland & Jakobsen 2001).

With orthomyxoviruses, it has been found that following disruption of the virion with sodium dodecyl sulphate (SDS), the haemagglutinin and neuraminidase subunits could be separated by electrophoresis. At least 7 separate structural and 2 non-structural proteins have been found (Ginsberg 1988). SDS-PAGE analysis of the Scottish ISAV isolate was conducted to reveal proteins for potential antibody targeting. The virus and uninfected SHK-1 cellular material were treated with SDS to dissociate multichain proteins, and mercaptoethanol to break down disulphide linkages, disrupting the secondary structure on subsequent heating to 100°C. The electrophoresis procedure involved separation of particles according to their molecular weight and charge by the application of an electrical field across a polyacrylamide gel. The shape of a 4-20% gradient gel (Figure 3.2) became deformed during the silver staining procedure, appearing flared towards the wells. The gel also became increasingly friable, and tore during the protocol. Staining of the gels

revealed 6 main bands at approximate MW of 20 kDa, 27 kDa, 33 kDa, 45 kDa, 70-72 kDa and 224 kDa (Figures 3.1-3.3). Silver staining of the gels proved more highly sensitive than Coomassie blue staining, the former having been used in previous studies of ISAV (Falk *et al.* 1997; Wergeland & Jakobsen 2001). Falk *et al.* (1997) described four polypeptides (MW 24 kDa, 43 kDa, 53 kDa and 71 kDa) of putative viral origin on analysis of Norwegian ISAV isolates, and a minor polypeptide (15 kDa) of suspected cellular origin. Wergeland & Jakobsen (2001) described distinct protein bands related to a Norwegian ISAV isolate at MW of 26 kDa, 40-48 kDa, 53 kDa and 71 kDa, as well as some low MW bands (between 14 kDa and 21 kDa). A similar distribution of four main polypeptide bands was also described using Canadian ISAV isolates, with some differences being seen on comparison of Canadian and Norwegian isolates (Kibenge *et al.* 2000a).

The results of the present study demonstrated a similarity in protein composition between the Scottish strain analysed and previously studied Norwegian and Canadian isolates. The band of MW 33 kDa seen here (Figure 3.3) had not been formerly described, possibly representing a structural difference between the current isolate and previously studied strains. The highest MW band recognised (224 kDa, Figure 3.2) was evident only on the 4-20% gradient gel, due to a greater spread of high MW products. This band had not been identified in the past, perhaps due to structural differences between strains, or owing to its failure in being identified on much used standard 12% polyacrylamide gels. Low MW bands have been identified in the past, using non-ISAV viral isolates or non-infected cells, suggesting a non-viral origin (Kibenge *et al.* 2000a; Wergeland & Jakobsen 2001). In the current study this finding was confirmed, the uninfected SHK-1 cell lanes showing a group of bands in the

range of 15-19 kDa. A clear band was also seen at 75 kDa with the SHK-1 material, but this seemed to be unrelated to the viral band at 72 kDa (Figure 3.3).

Immunoblotting was carried out to assess the potential range of MAb production. Western blot analysis of the protein band pattern of the Scottish isolate of ISAV, using mouse anti-serum (collected at the time of fusion), determined the MW of antigens targeted by the murine antibodies. This, in turn, provided an indicator of which MAbs could potentially be developed. The use of biotin and streptavidin during consecutive stages increased the sensitivity of the assay by amplifying the colour change. Two main bands were observed with ISAV at 45 kDa and 70 kDa (Figures 3.5 & 3.6). These bands were consistent with SDS-PAGE findings (Figures 3.1 - 3.3). Products in the range 40-43 kDa had previously been reported with Norwegian and Canadian ISAV, thought to be associated with the viral envelope, and the likely target of MAb 3H6F8 produced by Falk *et al.* (Falk *et al.* 1997; Falk *et al.* 1998; Griffiths, Cook, Mallory & Ritchie 2001). Sequencing of this protein showed significant differences between Norwegian and North American strains (Griffiths *et al.* 2001), and it could be expected therefore that the Scottish isolate may also show variation at this level.

On analysis of Canadian ISAV, Griffiths *et al.* (2001) described an antigen of MW 72 kDa being associated with autolytic behaviour, and the possibility of some lower MW peptides being derived from this band. Similarities were drawn between this antigen and the autolytic behaviour of the 57 kDa major antigen of *Renibacterium salmoninarum*. Further investigation of these antigens could prove significant in the understanding of the mechanisms of host cell determinants leading to internalisation

of pathogens in salmonid hosts (Griffiths & Lynch 1991; Griffiths *et al.* 2001). The SHK-1 cell antigens showed antibody binding to proteins of MW 25 kDa, 68 kDa and 75 kDa (Figure 3.5). The 75 kDa band seemed to be distinct from the viral product at 70 kDa, being previously identified by silver staining (Figure 3.2). Other bands of putative cellular origin have been described in the range of 55-66 kDa, and at low MW in earlier studies (Kibenge *et al.* 2000a; Wergeland & Jakobsen 2001). The immunoblotting technique demonstrated that there was the potential for the development of MAbs from the immunised mouse, specifically targeting ISAV antigens of MW 45 kDa and 70 kDa.

In common with many previous studies involving the production of MAbs, enzyme-linked immunosorbent assays were used as the primary screening method for the specific antibody content of both immunised mouse serum and hybridoma supernatants (Arakawa *et al.* 1987; Hanna *et al.* 1991; Le Gall, Mourton, Boulo, Paolucci, Pau & Mialhe 1992; Arkush *et al.* 1992; Bakopoulos *et al.* 1997b; Falk *et al.* 1998; Erdenlig, Ainsworth & Austin 1999; Al-Harbi, Truax & Thune 2000; Jamett, Aguayo, Miquel, Muller, Arriagada, Becker, Valanzuela & Burzio 2001). The assays used in this project involved the coating of 96 well plates with ISAV, and subsequent measurement of the level of immunological binding between the virus and the constituents of the test material. ELISAs have been found to be particularly well suited to rapidly screening large numbers of samples, producing quantitative results, and offering high sensitivity (Crowther 1995; Adams *et al.* 1995).

An ELISA test was used in this study initially for the screening of test bleeds from the two immunised mice after their second injections of ISAV. The first protocol

attempted, following the method of Dixon & de Groot (1996) was unsuccessful. This method had been shown to be suitable for the coating of plates with preparations containing soluble antigens. In this case, in light of the protein analysis data suggesting a significant proportion of insoluble protein, a modification was made involving the pre-coating of the wells with poly-L-lysine and subsequent addition of glutaraldehyde (Adams *et al.* 1995). These compounds act as bridging molecules to aid in antigen fixation to the solid state. This protocol proved successful, giving positive results for the sera of both mice.

Prior to their final intravenous immunisation of ISAV, the titre of the immunised mouse sera was found to be 1/400 for both mice. Ideally, at this stage a much greater titre would be preferable, in the order of 1/10,000 (Eryl Liddell & Cryer 1991). Due to animal welfare regulations limiting the number of doses administered to individual mice, it was not possible within the time span available to repeat the immunisation procedure in pursuit of a better response. In addition, the screening ELISA did not appear to be highly sensitive, so the possibility existed that the assay was underestimating the anti-ISAV antibody levels in the mice. It was therefore concluded that the best course of action was to continue with hybridoma production.

Although evidence of a protective anti-ISAV immune response has been noted in salmon (Falk & Dannevig 1995), some viruses have been found often to be poorly immunogenic, giving them a competitive advantage (Dulbecco 1988). Chemical modifications, including the cross-linking of monomers into supramolecular complexes and the conjugation of molecules to carrier proteins or haptens have been shown to increase the immune response to certain agents (Eryl Liddell & Cryer

1991). In mammals, the B-lymphocytes of the immune system have been shown to recognise free viral antigens, whereas the T cells usually recognise either denatured antigens or fragments of the native antigen in combination with cellular proteins of the major histocompatibility complex. Thus, B cells and T cells often recognise different viral epitopes, antibodies frequently targeting unaltered proteins whose conformation depends upon a folded three-dimensional polypeptide chain (Dulbecco 1988). Therefore, antibodies have the ability to be more specific than T cells, leading to less cross-reactivity. For example, in the immune response to influenza haemagglutinin in mice, antibodies have regularly distinguished between different strains within a common subtype, while T cells showed extensive cross-reactivity, even bridging across subtypes (Dulbecco 1988). The development of monoclonal antibodies targeting ISAV would allow the production of highly specific immunological tools.

The fusion of ISAV-immunised mouse spleen cells with mouse myeloma cells resulted in the successful production of 'parent' hybridoma cells, visible microscopically ten days later. Recognisable growth was seen in 55 wells (out of a total of 400) of the tissue culture plates. An ELISA procedure, following the protocol devised during screening of the mouse serum, was employed to assess the presence of anti-ISAV antibodies in supernatants from the 55 wells with hybridoma growth. Five of the wells were considered positive, giving an OD₄₅₀ in excess of double the negative control level. Normally, a value exceeding triple the negative control would be considered positive. However, with the screening of hybridomas there is a need to keep the cells nutritionally viable using sufficient volumes of culture medium, resulting in dilution of the antibody concentration (Eryl Liddell & Cryer 1991).

Considering this, coupled with the limited sensitivity of this assay, the threshold was reduced to twice the negative control value for the purpose of screening hybridoma supernatants.

Five different supernatants gave positive results in the ELISA wells pre-coated with uninfected SHK-1 cells. This suggested that mouse antibodies had been produced against cellular debris included in the virus preparation used for immunisation. An increased OD₄₅₀ was also seen in the ELISA wells pre-coated with the virus buffer only, and subsequently incubated with positive control mouse anti-serum. In the use of ELISA tests, the matrix containing the analyte (TNE buffer in this case) has been found to produce non-specific reactions (Dixon 1985). In this assay, although an increase in absorbance was measured with the buffer-coated wells plus anti-serum, it was less than half the value achieved with the positive control wells. Therefore, the readings achieved, in combination with subsequent immunoblotting, demonstrated the production of anti-ISAV antibodies by the newly created 'parent' hybridoma cells.

Each well containing the 'parent' hybridoma cells was seen to contain multiple separate colonies of cells, each derived from individual plasma cell lines. Therefore, the supernatant of each would be polyclonal, containing a group of monoclonal cell lines. Cloning, consisting of the serial dilution of the 'parent' hybridoma cells was conducted with the aim of producing wells containing single colonies, producing solitary MAbs. Each of the 5 positively-screened 'parent' cell supernatants was cloned by dilution on a separate 96 well tissue culture plate, but after 10 days incubation, growth was seen only in 26 wells spread over 2 of the 5 plates. These

were screened by ELISA, but all proved negative on reaction with ISAV. Poor growth of certain hybridoma cells has been previously reported as a common complication of MAb production (Eryl Liddell & Cryer 1991; Nelson *et al.* 2000). Infection of plates with bacteria, fungi, yeasts, and mycoplasma has also been previously reported (Eryl Liddell & Cryer 1991), but no evidence of such contamination was seen.

Cloning a second time from the original 'parent' hybridoma cells was performed, additional quantities of the essential nutrient L-glutamine being added due to its short half-life (7 days at 37°C) (Eryl Liddell & Cryer 1991). After 10 days, more prolific hybridoma growth was evident microscopically than with the previous cloning, but screening by ELISA still proved negative. Although growth of hybridoma cells was seen at this point, the number of cells potentially producing antibodies into the supernatant was not high, and the antibody concentration may therefore have been insufficient, coupled with limited sensitivity of the ELISA, to allow detection of anti-ISAV antibodies.

Western blot analysis using the supernatant from the 'parent' hybridoma cells confirmed the production of anti-ISAV antibodies *in vitro*. Bands were observed at 71 kDa and 27 kDa from well 17 R3, and at 27 kDa from well 1 A3 (Figure 3.6). The higher MW band correlates with a viral antigen previously recognised in this study by Western blot using the mouse anti-serum, and during previous studies (Falk *et al.* 1997; Griffiths *et al.* 2001; Wergeland & Jakobsen 2001). Earlier, on the Western blot measuring mouse anti-serum against SHK-1 cells, a band was seen at approximately 25 kDa (Figure 3.5). Therefore, the 27 kDa antigen may well be due to

the binding of antibody to SHK-1 cellular material, which would explain the faintness seen, because of the low concentration of cellular material being present in the pelleted viral preparation. It was possible that additional antibodies may have been produced by the hybridomas, but not recognised due to their target viral epitopes having been denatured during the SDS-PAGE protocol. Although on the first attempt at cloning the hybridomas, there were no positive results by ELISA, the plates derived from the 'parent' hybridoma cells of wells 17 R3 and 1 A3 showed the best growth upon microscopic examination. This finding would be consistent with these wells containing hybridomas of greater vigour than the others. These two wells would be the best candidates for future attempts at cloning, well 17 R3 having the best potential due to the presence of anti-71 kDa antibodies, demonstrated by Western blot. Future efforts of cloning these cells would require meticulous attention to the health of the hybridoma lines, with optimal nutrition being maintained.

Falk *et al.* (1998) succeeded in making six hybridoma lines which produced antibodies against Norwegian ISAV, but only one of these was successfully stabilised. This was designated MAb 3H6F8, and was used in ELISA assays during the current study. Upon isotyping, this MAb was determined to be of the class IgG₁ and thought to bind to the 43 kDa surface haemagglutinin glycoprotein on the virion surface. This MAb has been found to demonstrate assay restriction, not reacting on Western blot or standard formalin-fixed paraffin embedded tissue sections. These findings were presumably due to attenuation of the target antigen occurring during the assay procedures, making the epitope unrecognisable to the MAb. In the current study, MAb 3H6F8 did not give positive results when reacted with Scottish ISAV in ELISA tests, even at a relatively low dilution (1/25). The haemagglutinin molecule of

orthomyxoviruses has been shown to be prone to mutation (Ginsberg 1988; Falk *et al.* 1997), and this result could therefore reflect differences at this level between this Scottish isolate and the Norwegian isolate, although MAb 3H6F8 has been successfully used with Scottish ISAV in the past (Anonymous 2000a). Alternatively, this result could reflect limited sensitivity of the ELISA, or attenuation of the target epitope during the assay procedure.

Production of anti-ISAV MAbs that function in a variety of immunological assays would have a wide range of applications, particularly for disease diagnosis. MAb 3H6F8 has been used with some success as a diagnostic tool utilising indirect fluorescent antibody techniques, but its sensitivity has been found to be significantly lower than other diagnostic methods (Opitz *et al.* 2000). Although the possible sensitivity of immunological assays is limited, and would be unlikely to be comparable with molecular techniques (Puttinaowarat *et al.* 2000), successful diagnostic MAbs have been produced against aquatic viruses. MAbs against VHS virus have been applied with some success in the development of an antigen capture ELISA. This has been found to be sensitive enough to detect non-purified virus particles (Mourton, Romestand, de Kinkelin, Jeffroy, Le Gouvello & Pau 1992), allowing rapid quantitative screening of large numbers of samples. The availability of antibody preparations of suitable titre and avidity (overall stability of the antibody-antigen complex) is of prime importance in the development of a reliable ELISA test (Dixon 1985). Such an immunological tool could have great implications in simplifying the diagnosis of ISAV.

MAbs have also been used in the development of subunit vaccines against viruses including IHN, IPN, VHS, and CCV as previously mentioned in Section 1.2.4. Much research has been carried out regarding the production of DNA vaccines for aquatic organisms. Some protective immunity has been demonstrated with IHN in salmonids (Corbeil, LaPatra, Anderson, Jones, Vincent, Hsu & Kurath 1999; Corbeil, Kurath & Lapatra 2000), although there is much consumer scepticism, no DNA vaccines having been licensed in aquaculture thus far. It has been found with influenza viruses that immune stimulating complexes (ISCOMs) consisting of purified glycoproteins, were a more efficient antigen formulation than whole virus particles or glycoprotein micelles (Bogoyavlenskiy, Berezin, Tolmacheva, Khudyakova & Ogneva 1999). Much work is ongoing in the field of subunit vaccines, and the development of future anti-ISAV MAbs would be highly prized.

In conclusion, although this attempt at production of anti-ISAV MAbs has not been successfully completed, hybridomas were produced, apparently producing antibodies targeting ISAV antigens. The titre of anti-ISAV antibodies from the immunised mouse serum appeared to be relatively low. This may have been a result of insufficient antigen being present in the initial samples used for injection of the mice or may reflect a lack of sensitivity of the ELISA used in screening. Growth of ISAV was found to be slow and low yielding using SHK-1 cells, and unsuccessful with CHSE-214 cell lines. Future attempts could possibly involve use of the newly developed TO cell line, with its reportedly higher levels of yield (Wergeland & Jakobsen 2001). Other methods, including chemical modifications involving the conjugation of molecules to haptens could be used in attempting to increase the immune response to the agent.

Further cloning of the existing 'parent' hybridoma cells could result in the production and isolation of monoclonal cell pools. Of the wells that were screened positive by ELISA, the two with the greatest recognised growth (17 R3 & 1 A3) would be the best candidates for future cloning. Vigilance would need to be exercised in optimising cell growth, with particular attention to nutrition. Reliance upon one screening method could result in the lack of identification of certain antibodies due to assay restriction (Nelson *et al.* 2000), therefore multiple methods, as used here involving ELISA, Western blot and possibly indirect fluorescent antibody tests could be advantageous. Isotype analysis would allow definition of the murine Ig class, and could also be applied to demonstrate the presence of a single isotype. Such knowledge would also be applied in the choice of purification method for culture supernatant.

Upon purification of a particular MAb it would be essential to assess potential cross-reactivity with other material, to lower the possibility of false positive results in future assays. Although a hybridoma is the fused product of a single B cell, and produces antibody of exquisite sensitivity, it has been found that a single antibody could have the potential to cross-react with other antigens, thus exhibiting dual specificity (Nelson *et al.* 2000). Epitope mapping could alleviate such limitations by determining key amino acid residues involved in antibody recognition and binding.

This study has demonstrated that antibodies targeting a Scottish isolate of ISAV could be produced *in vitro*. It would be interesting to use Western blotting techniques to screen isolates from other locations (such as Norway and Canada) with mouse anti-

serum or hybridoma cell supernatants from this study, to compare and contrast findings with the Scottish ISAV profile. As the antibody-producing cells are hybrids of 2 cell types, instability of the cells can be a complication in culture conditions (Eryl Liddell & Cryer 1991).

Assuming that the lines of 'parent' hybridoma cells already developed could be maintained, the possibility would exist to successfully clone MAbs specifically targeting viral epitopes. Future attempts at immunising mice with ISAV could involve the production of more concentrated viral samples and / or chemical modification to enhance antigenicity. Realisation of the goal of producing monoclonal antibodies against infectious salmon anaemia virus would mean the production of immunological probes with numerous significant applications.

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APPENDIX I

Virus Buffer

TNE Buffer (pH 7.2)

0.01M Tris (hydroxymethyl) methylamine; 0.1M NaCl;

1mM Ethylenediaminetetra-acetic acid (EDTA)

ELISA Buffers

Coating Buffer (carbonate-bicarbonate solution, pH 9.6)

Sodium carbonate	1.5 g l ⁻¹
Sodium bicarbonate	2.93 g l ⁻¹
Sodium azide	0.2 g l ⁻¹

(1 buffer tablet (Sigma C-3041) in 100 ml distilled H₂O)

Low Salt Wash Buffer (LSWB) (pH 7.3)

0.02 M Tris; 0.38 M NaCl; 0.05% Tween 20

Trisma base	2.42 g l ⁻¹
NaCl	22.2 g l ⁻¹
Tween 20	0.5 ml l ⁻¹
Merthiolate	0.1 g l ⁻¹
HCl (concentrated)	used to adjust pH

High Salt Wash Buffer (HSWB) (pH 7.7)

0.02 M Tris; 0.5 M NaCl; 0.1% Tween 20

Trisma base	2.42 g l ⁻¹
NaCl	29.2 g l ⁻¹
Tween 20	1 ml l ⁻¹
Merthiolate	0.1 g l ⁻¹
HCl (concentrated)	used to adjust pH

Phosphate Buffered Saline (PBS) (pH 7.2)

0.02M Phosphate; 0.15M NaCl

NaH ₂ PO ₄ ·2H ₂ O	0.876 g l ⁻¹
Na ₂ HPO ₄ ·2H ₂ O	2.56 g l ⁻¹
NaCl	8.77 g l ⁻¹
1M NaOH	used to adjust pH

Antibody Buffer

1% (w/v) Bovine Serum Albumin (BSA) in PBS

Conjugate Buffer

1% (w/v) BSA in LSWB

Substrate Buffer (pH 5.4)

0.1M Citric acid; 0.1 M Sodium acetate

Citric acid	21 g l ⁻¹
Sodium acetate	8.2 g l ⁻¹
1M NaOH	used to adjust pH

Stock Chromogen Solution

42mM of 3'3'5'5'-Tetramethylbenidine dihydrochloride (TMB) in 1:2 acetic acid:distilled H₂O.

Stored @ 4°C in darkness.

Substrate Solution

Stock Chromogen Solution	150 µl
Substrate Buffer	15 ml
30% H ₂ O ₂	5 µl

Stop Reagent

2M H₂SO₄

APPENDIX II

Electrophoresis Buffers & Gels

Separating Gel Buffer (pH 8.7)

1.5 M Tris; 0.4% (w/v) Sodium Dodecyl Sulphate (SDS)

Trisma base	182 g l ⁻¹
SDS	4 g l ⁻¹
HCl (concentrated)	used to adjust pH

Stacking Gel Buffer (pH 6.8)

0.5 M Tris; 0.4% (w/v) SDS

Trisma base	60.5 g l ⁻¹
SDS	4 g l ⁻¹
HCl (concentrated)	used to adjust pH

Sample Buffer (x5)

1M Tris HCl (pH 6.8)	0.6 ml
50% (v/v) Glycerol	5 ml
10% (w/v) (SDS)	2 ml
2-β Mercaptoethanol	0.5 ml
1% (w/v) Bromophenol blue	1 ml
Distilled H ₂ O	0.9 ml

Reservoir Buffer (x5) (pH 8.3)

Tris	15 g l ⁻¹
Glycine	43.2 g l ⁻¹
SDS	5 g l ⁻¹
HCl (concentrated)	used to adjust pH

Ammonium Persulphate Solution: 10% (w/v)

Ammonium persulphate solution	100 g l ⁻¹
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Acrylamide Stock Solution

30% (w/v) Acrylamide; 0.8% (w/v) Bis-acrylamide

Stored in darkness @ 4°C.

Separating Gel (12% Acrylamide)

Separating Buffer	5 ml
Distilled H ₂ O	7 ml
Acrylamide	8 ml
N,N,N,N-Tetramethylethylenediamine (TEMED)	0.015 ml
10% Ammonium persulphate	0.07 ml

Stacking Gel

Stacking Gel Buffer	2.5 ml
Distilled H ₂ O	6.1 ml
Acrylamide	1.34 ml
TEMED	10 µl
10% Ammonium persulphate	50 µl

Coomassie blue solutions

Coomassie blue stain

0.25% (w/v) Coomassie brilliant blue R-250

50% (v/v) methanol

10% (v/v) acetic acid

Coomassie blue De-stain

40% (v/v) methanol; 10% (v/v) acetic acid

Silver stain solutions (SIGMA AG-25)

Fixing Solution

10% Acetic acid; 30% Ethanol

100% Glacial acetic acid	90 ml
100% Ethanol	270 ml
Deionised H ₂ O	540 ml

Silver Equilibration Solution

Silver concentrate	1.5 ml
Deionised H ₂ O	300 ml

Development Solution

Developer 1 concentrate	30 ml
Developer 2 concentrate	0.17 ml
Deionised H ₂ O	300 ml

Stop Solution

1% (v/v) Acetic acid

Reducer Solution

Reducer A concentrate	2 ml
Reducer B concentrate	4 ml
Reducer C concentrate	0.7 ml
Deionised H ₂ O	293.3 ml

APPENDIX III

Western Blot Buffers

Tris Buffered Saline (TBS) (pH 7.5)

0.02 M Tris; 0.5 M NaCl

Trisma base	2.42 g l ⁻¹
NaCl	29.24 g l ⁻¹
HCl	to adjust pH

TBS with Tween (TTBS) (pH 7.5)

Tween 20	0.5 ml added l ⁻¹ of TBS
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Transblot Buffer (pH 8.3)

Glycine	14.4 g l ⁻¹
Tris base	3.03 g l ⁻¹
Methanol	200 ml l ⁻¹
Distilled H ₂ O	800 ml l ⁻¹

Stock Substrate Solution (x 10)

4-chloro-naphthol	0.15 g
Methanol	50 ml

Stored in darkness @ -20°C.