Co-expression of Factor VIII with anti-FVIII Camelid antibody ligands:

Effect on expression levels of bio-therapeutic FVIII

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification at the University of Kent or any other University or Institute of learning.

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Table 4. Abbreviations

Full name	Abbreviation
Baby hamster kidney cell line	BHK
B-domain deleted FVIII	BDD FVIII
Construct 1	C1
Construct 2	C2
Construct 3	C3
Chinese hamster ovary cell line	CHO
Confidence Interval	CI
Endoplasmic Reticulum retrieval motif	KDEL
ER-associated degradation	ERAD
Factor VIII	FVIII
Full length Factor VIII	FL-FVIII
Histidine tag	HIS
Immunoglobulin leader sequence	LEADER
International Unit	IU
Ligand 2, construct 1	L2C1
Ligand 2, construct 2	L2C2
Ligand 2, construct 3	L2C3
Ligand 6, construct 1	L6C1
Ligand 6, construct 2	L6C2
Ligand 6, construct 3	L6C3

Ligand 7, construct 1	L7C1
Ligand 7, construct 2	L7C2
Ligand 7, construct 3	L7C3
P-value	P
Recombinant Factor VIII	rFVIII
Unfolded protein response	UPR
Variable region of heavy chain of heavy chain only antibody (Camelid antibody)	VHH
von Willebrand Factor	vWF
Polyacrylamide gel electrophoresis	PAGE

Abstract

Production of recombinant FVIII, the protein that is missing or dysfunctional in haemophilia A patients, is highly inefficient compared to other recombinant clotting factors such as FIX. This is predominantly due to complex intracellular trafficking, short half-life and protein instability. This study aimed to increase the amount of functional FVIII produced in mammalian cells by co-expression with anti-FVIII Camelid antibody fragments (VHH). Three VHH ligands were supplied by BAC BV (as DNA constructs), two of which when expressed in yeast are known to bind recombinant FVIII (ligands 2 and 7) and are used commercially as FVIII purification tools. From these three constructs, nine new VHH plasmids constructs were designed and transiently expressed in a stable BHK-human FVIII-expressing cell line.

Of the nine VHH fragments that were co-expressed in the BHK FVIII cell line, four of these had a statistically significant impact on the 'clotting time' of the cell media as demonstrated by the activated partial thromboplastin time assay (aPTT). Two ligand 2 constructs (L2C1 and L2C2) prolonged the coagulation time by 4 seconds (P-value 0.0001, 95% confidence intervals 38.5-43.5), and 3.4 seconds (P-value 0.0072, 95% CI 36.5-40.3) respectively, indicating a decrease in functional FVIII activity versus media from the untransfected and null transfected BHK-FVIII cell line. Two ligand 7 constructs (L7C1 and L7C3) caused a decrease in coagulation time of 3.2 seconds (P=0.0057, 95% CI 30.5-33.3), and 4 seconds (P=0.0002, 95% CI 29.1-32.9) respectively, indicating an increase in functional FVIII activity versus media from the untransfected and null transfected BHK-FVIII cell line.

Ligand 7 and ligand 2 both bind to the FVIII light chain, albeit in different regions and with different affinities (data confidential to BAC BV). BAC studies showed that ligand 7 competes with vWF on the FVIII light chain, which is known to increase stability of FVIII *in vivo*, whereas ligand 2 does not compete for this binding site. The opposing effects of ligand 7 and ligand 2 on FVIII clotting times seen in this study could be due to their differences in FVIII binding properties, since it is known that binding location of FVIII ligands can have an impact on FVIII clotting activity.

1. Introduction

1.1 Haemostasis

Haemostasis is the tightly regulated response to vascular damage in the body which stops bleeding from smaller blood vessels. In a healthy human this mechanism is normally rapid and efficient and involves the blood vessel wall, platelets and blood coagulation factors (Tanaka, Key et al. 2009). It is a fine balance between procoagulant (formation of a fibrin clot) and anticoagulant (breakdown of fibrin) activity, and it is essential for survival.

There are three main mechanisms involved in haemostasis: vascular spasm whereby smooth muscle in damaged arteries contracts in order to limit blood loss, platelet plug formation at the site of injury, and blood clotting via the coagulation cascade (Tortora and Derrickson 2006).

The formation of a platelet plug begins when platelets are activated by von Willebrand Factor (vWF) and the exposed collagen fibres of damaged connective tissue. Binding of platelets to collagen occurs via platelet receptors alpha 2 beta 1 integrin and glycoprotein (GP)VI/Fc-receptor gamma-chain (FcR gamma) (Jackson, Ortar et al. 2013). The platelet release reaction whereby the platelets undergo dramatic changes in shape, interact with one another and release their contents via cytoskeletal reorganisation. This involves a multitude of proteins, many of which have only recently been characterised (Cerecedo 2013). The platelet cytoskeleton contains two actin filament-based components. Upon platelet activation one of these components undergoes a surge in polymerization. These polymerized actin filaments bind to myosin, and move from the periphery of the platelet towards the center of the platelet enabling it to contract (Fox, 1993). Activated platelets release ADP, thromboxane A_2 and serotonin, and in combination with thrombin produced at the platelet surface, result in the formation of platelet aggregates and the growth and stabilisation of the haemostatic plug (Schattner and Rabinovich 2013). A platelet plug may be sufficient to stop blood loss resulting from minor vascular damage, however more severe damage requires strengthening of the plug with fibrin threads which are generated during the blood clotting process (Tortora and Derrickson 2006).

Damage to vascular endothelium and the release of tissue factor (also known as Factor III) is the starting point of a complex series of reactions that is known as the coagulation cascade. The cascade involves the activation of blood coagulation factors via proteolysis, with the aim of generating sufficient levels of thrombin to convert plasma fibrinogen into fibrin. Fibrin, in cooperation with platelets forms a strengthened haemostatic plug at the site of vascular injury. There are several known bleeding disorders which result from defects in the haemostatic system including vascular and platelet abnormalities and defective or deficient coagulation factors (Hoffbrand, Moss et al. 2006).

The traditional model of the coagulation cascade is comprised of independent extrinsic and intrinsic pathways that later merge to a common pathway after the activation of factor X (FXa) - so called the 'MacFarlane Cascade' (MACFARLANE 1964), see figure 1.1. In the MacFarlane model, the extrinsic pathway begins with the release of tissue factor from the surface of damaged endothelial cells, into the blood and causes the activation of factor VII to factor VIIa. Factor VIIa forms a complex with tissue factor (TF-FVIIa) to activate factor X (factor Xa). Factor Xa and activated factor V (FVa) form prothrombinase. The intrinsic pathway, also known as the contact activation pathway, is slower than the extrinsic pathway and is more complex (more reaction steps are involved). It is call the contact activation pathway since it is activated when blood comes into contact with collagen fibres in connective tissue around a damaged blood vessel wall. Blood contact with collagen fibres causes activation of factor XII to factor XIIa via spontaneous cleavage of its single peptide chain due to surface contact (so called contact activation system) (Cool *et al*, 1985). Factor XIIa then activates factor XI to factor XIa, subsequently converting factor IX to factor IXa. Factor IXa works together with activated factor VIII (factor VIIIa) in a so-called 'tenase' complex to convert factor X to factor Xa, which can then go on to produce prothrombinase with activated FV.

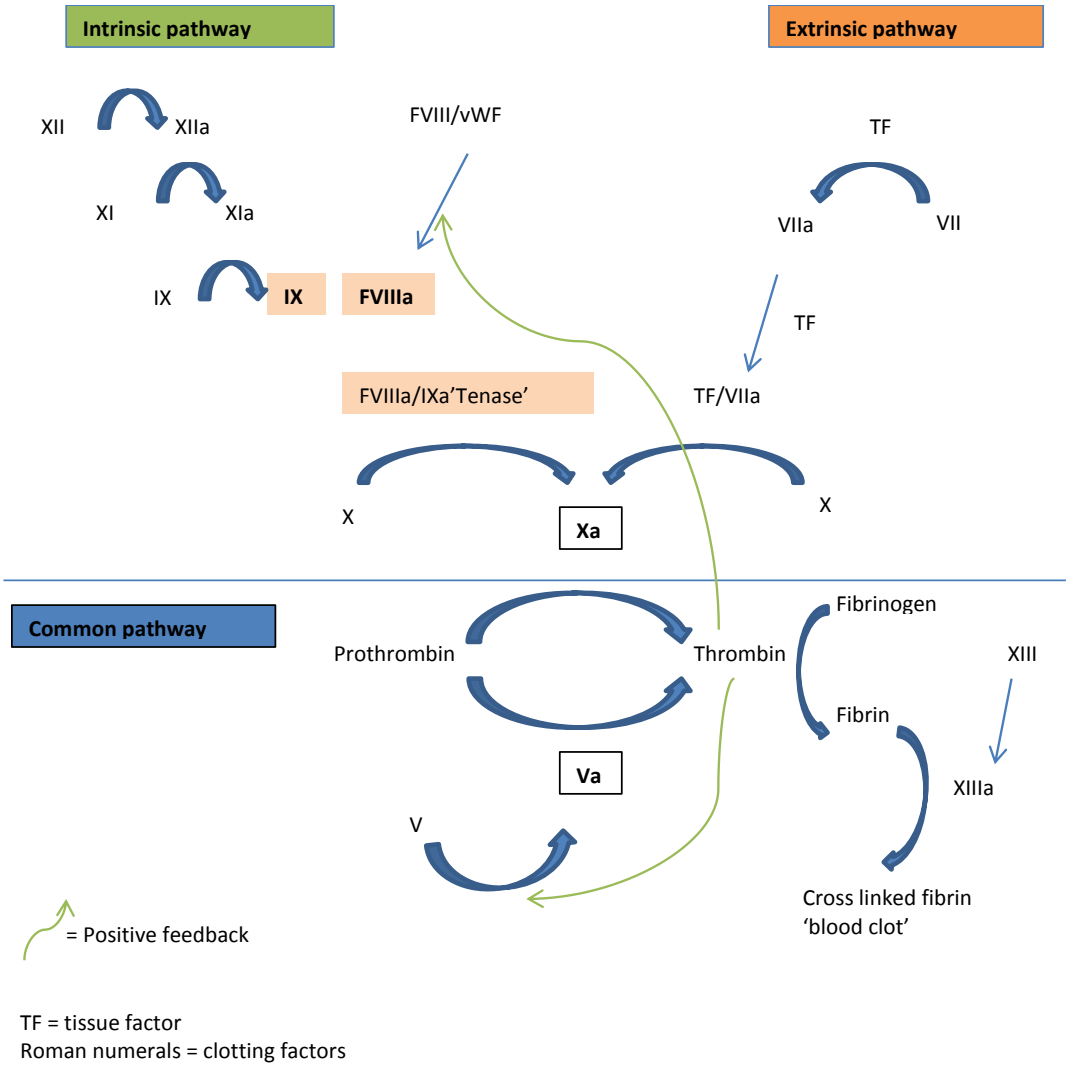


Figure 1-1 Schematic representation based on the traditional 'MacFarlane Cascade' coagulation model and the role of FVIII in forming a fibrin clot in humans.

However, a more contemporary view of a 'new cascade' has emerged and has recently been adopted internationally. The new cascade moves away from separate intrinsic and extrinsic pathways, and instead describes a common set of cell-surface based reactions with three overlapping phases of thrombin generation; initiation, amplification and propagation (Pérez-Gómez and Bover 2007).

The new cascade model describes a coagulation reaction which begins on tissue factor-exposing cells, and continues on the surfaces of activated platelets. Initiation encompasses the TF/FVIIa complex activation of coagulation factors IX to IXa and X to Xa, in order to generate trace amounts of thrombin. This initial thrombin burst can activate platelets and stop small bleeds by adhering to the site of injury and

forming a platelet plug as described earlier. Amplification of the initial burst of thrombin occurs via a positive feedback mechanism to stop larger bleeds, by activation of circulating FVIII and FV. Thrombin's proteolytic activity at ARG740, ARG1689, and ARG372, releases FVIII from von Willebrand factor to generate activated FVIIIa (Nogami, Shima et al. 2000), (Eaton *et al*, 1986). FVa and FVIIIa bind to the platelet surface and act as cofactors for the propagation phase whereby large-scale thrombin generation occurs. Thrombin also activates FXI which is bound to the platelet surface and plays a role in the propagation phase.

In the propagation phase, FVIIIa/FIXa 'tenase' complex and the FVa/FXa complex (prothrombinase) assemble to accelerate the generation of FXa and thrombin, respectively at the platelet surface. In addition, FXIa bound to the platelet surface activates FIX to allow the formation of additional 'tenase' complex. The resulting burst of thrombin allows the conversion of fibrinogen to soluble fibrin fibres, which polymerise to form fibrin protofibrils. Thrombin activated FXIIIa stabilises the protofibrils which stabilises the platelet plug to form a platelet/fibrin thrombus (De Caterina, Husted et al. 2013).

Due to the important amplification role of the 'tenase' complex, the cycle continues in a prothrombotic state until down-regulation occurs via anticoagulant pathways (Pallister and Watson 2010). Conversely, a deficiency or dysfunction in essential clotting factors can lead to a reduced ability to perform haemostasis, for example in the collection of disorders known as haemophilia.

1.1 Haemophilia types A, B and C

Haemophilia is a rare disorder which affects the blood coagulation pathway due to a deficiency in specific coagulation factors (Dahlbäck 2005). There are three types of Haemophilia, type A (classic Haemophilia) which is characterised by a deficiency in Factor VIII (Antonarakis, Kazazian et al. 1995), type B (Christmas disease) which is characterised by a deficiency in Factor IX (Bolton-Maggs and Pasi 2003) and type C which is caused by a deficiency in Factor XI (Bolton-Maggs 2013). The most common type is haemophilia A which affects 1 in 5000 males in the United States (Miao, Sirachainan et al. 2004), while type B is less common and affects 1 in 30,000 males (Bolton-Maggs and Pasi 2003). Haemophilia type C is reported to be found in

1 in 100,000 males in the United States, with a high incidence in Ashkenazi Jews (although it has been reported in all racial groups) (Bolton-Maggs 2013).

There is a long history of haemophilia with the first descriptions of the disease going back to the 10th century, and the first recorded case being treated by transfusion in London in 1840 (Farr 1981). Type C was first described in 1953 (Rosenthal, Dreskin et al. 1953), affects both genders and is the most mild of the inherited coagulation disorders (Gomez and Bolton-Maggs 2008).

Median life expectancy for those with severe haemophilia (A or B) has been estimated at 63 years, while for those with mild to moderate haemophilia it is 75 years, compared to the general population of males having a life expectancy of 78 years (Darby, Kan et al. 2007). This was calculated from data gathered over a period of 20 years from the NHS and haemophilia centres in the UK.

1.2 Acquired Haemophilia A (AHA)

While all three types of Haemophilia are inheritable via gene mutations (Peyvandi, Jayandharan et al. 2006), type A can also be acquired. Acquired hemophilia A (AHA) is associated with other diseases such as auto-immune disorders, cancers, reactions to medications or even as a severe complication of pregnancy. These AHA patients develop polyclonal auto-antibodies to FVIII via multiple mechanisms, resulting in an impaired intrinsic coagulation pathway (Franchini, Gandini et al. 2005). The mechanisms by which these inhibitors are formed have only recently come to light, and is still a topic that is not completely understand in the literature. Two pathways that have been suggested to play a role in AHA; 1) an increased frequency of a single nucleotide polymorphism of the CTLA-4 gene (which is found on the surface of activated and regulatory T-cells, and closely associated with auto-immune disorders) and 2) the elevation of B-cell activating factor belonging to the tumor necrosis factor family (BAFF) whose role has yet to be fully explained in AHA patients (for review see: Sakurai *et al*, 2014). The link between pregnancy and AHA is not clear, however since most of these cases occur postpartum, it has been suggested that the mother may develop autoantibodies due to exposure to fetal FVIII. It can also occur in mothers who are haemophilia carriers, who normally have low FVIII levels, but which rise during pregnancy. The sudden drop of FVIII levels after

childbirth can lead to severe postpartum haemorrhage (Hashimoto *et al* 2011). AHA is extremely rare (reported 0.2 - 2 per million population per year), and can occur in both sexes, whereas the inherited type A only affects males due to a recessive gene mutation on the X chromosome (Hay 1998).

1.3 Inherited Haemophilia A

The severity of this disease is determined by the blood plasma levels of FVIII, and this is expressed as a percentage relative to normal human plasma or in International Units relative to an International Standard, the details of which are updated periodically (Raut, Daniels et al. 2012). One International Unit (IU) is equivalent to that quantity of FVIII found in 1ml of normal human plasma (EMEA 2000).

The definitions of disease severity are as follows; severe (<1% of normal activity or < 0.01 IU/mL), moderate (1–5% of normal activity or 0.01-0.05 IU/mL) or mild (5–40% of normal activity or >0.05 to < 0.40 IU/mL) (Ljung 2009). Studies have shown that the half life of plasma FVIII is similar in all haemophilia patients, and that it is in fact the production rate of FVIII that determines the severity of the disease (Ghosh 2007).

Haemophilia A is currently treated by replacing the deficient coagulation factor by infusion of recombinant FVIII (Giangrande 2011). Although there is significant potential for a gene therapy treatment for haemophilia A, as of 2015 studies have not progressed beyond preclinical experiments in animals. Gene therapies for haemophilia B are however in phase 2 human clinical trials (review, Carr *et al* 2015) which offer some optimism for a gene therapy approach in haemophilia A.

Table 5 Gene therapy approaches in haemophilia A and B

Manufacturer	Indication	Vector	Construct	Phase
Baxter/Asklepios Bio	Hem B	AAV8	FIX Padua	Phase I (2014)
Pfizer/Spark	Hem B	Modified AAV8 (AAV8-hFIX19)	FIX Padua	Phase I (2015)
UniQure BV/Chiesi	Hem B	AMT-060 (AAV5-hFIX)	wt FIX	Phase I
St Jude	Hem B	scAAV 2/8-LP1-hFIXco	wt FIX	Phase II
Bayer/Dimension	Hem A		FVIII	Discovery
BioMarin/St Jude/UCL	Hem A	BMN270 (hFVIII)	BDD FVIII	Preclinical
Baxter/Chatham/ReGenX	Hem A	BAX 888 (BNP-FVIII)	BDD FVIII	Discovery
St Jude/Royal Free Hos	Hem A	AAV-HLP-codop-hFVIIIV3	BDD FVIII and glycos linker (HLP)	Preclinical
Emory Univ/Lentigen Ther	Hem A	Lentiviral HSC	FVIII-expressing stem cells	Preclinical
Biogen Idec/Fonda Tele/ Ospe San Raffaele	Hem A and B	Lentiviral		Discovery

Reproduced from Carr *et al*, 2015**.**

1.3.1 The symptoms

The most common symptom in inherited haemophilia A is bleeding into the joints (haemarthroses) (Rodriguez-Merchan 1996), and muscles, often causing the patient severe pain and rendering them incapacitated. Haemophiliacs can experience bleeding into a range of joints including the ankle, knee, elbows and shoulders, and this can occur recurrently into the same joint (target joint). Bleeding into the joints can affect their motion, muscle strength and appearance. Recurrent bleeding into a target joint can eventually lead to further complications such as osteoarthritis (Mulder and Llinás 2004). Haemophilic arthropathy (damaged joints) is the major cause of morbidity among haemophiliacs (Raffini and Manno 2007). Other types of bleeding such as intracranial haemorrhage can also be life-threatening (Ljung 2009).

Patients with acquired haemophilia (AHA) tend to have a different bleeding pattern to those who have inherited the disorder. They do not commonly bleed into their joints but they do suffer with large spontaneous life-threatening bleeds including intramuscular, epistaxes (nose bleeds), intracranial and sometimes major gastrointestinal bleeding (Hay 1998). As mentioned previously AHA can also occur in relation to pregnancy and in this instance tends to resolve itself spontaneously (Franchini, Gandini et al. 2005). It is much more frequently found in the elderly than the young and can be difficult to diagnose due the absence of patient/family history of bleeding episodes (Franchini, Targher et al. 2008).

1.3.2 Treatment of Haemophilia A

Since haemophilia A is caused by a deficiency in the coagulation protein Factor VIII (FVIII), the aim of the treatment for this disease is to replace this protein, so called 'replacement therapy'. Historically, haemophilia patients were treated with whole blood transfusions, the first reported case of this being in 1840 (Farr 1981). Replacement therapy evolved to the use of whole blood plasma up until the 1940s, and then more specifically concentrated from pooled human plasma in the 1950s. This was further improved in the 1960s when scientists were able to produce plasma enriched with FVIII and von Willebrand Factor (VWF) (Saenko, Ananyeva et al. 2003). However the concern for all of these types of replacement therapy was that they contributed to the transmission of viral diseases such as HIV and Hepatitis B and C (HBV and HCV) (Jankowski, Patel et al. 2007). Before the introduction of virus-inactivation procedures, most haemophiliac patients treated in the UK between 1969 and 1985 with blood products also contracted the hepatitis-C virus which put them at increased risk of death from liver cancer and liver disease (Darby, Ewart et al. 1997). The development of modern purification technology such as solvent/detergent (S/D) method, removed some serious pathogenic viruses from plasma derived clotting factors with the exception of non-enveloped viruses such as hepatitis A (HAV). These remained a risk factor to haemophiliacs and meant vaccinations were necessary alongside treatment (Chudy, Budek et al. 1999). Some countries have a zero risk policy on the use of blood derived products potentially containing infectious agents. Strict plasma-screening procedures together with an increase in the use of clotting factors globally, led to shortages in the availability of plasma-derived clotting factors (Evatt, Farrugia et al. 2002). Over the last 20 years, the risk of viral infection from plasma derived clotting factors has been virtually eliminated, however there are still theoretical concerns over the emergence of new strains of parvoviruses and prions since these cannot be 100% eliminated by current purification technologies (Norja, Lassila et al. 2012). The most significant improvement in the treatment of haemophilia came with the development of recombinant biotechnology and the cloning of the FVIII gene in 1984, which allowed the production of various recombinant FVIII (rFVIII) products in mammalian cells (Toole, Knopf et al. 1984). The sources of currently marketed FVIII products vary

from the plasma derived FVIII to recombinantly derived FVIII, and are manufactured by a variety of pharmaceutical companies globally. There are two types of recombinant products used today, produced using the full length FVIII (FL-FVIII) gene or B-domain deleted derivatives of FVIII (BDD FVIII), which is produced using a truncated version of the gene (the differences are discussed in more detail in section 1.6).

Historically, recombinant FVIII has been reportedly difficult and expensive to produce due to low protein expression, lengthy purification procedures and limited stability of the protein both *in vivo* and *in vitro* (Wang, Wang et al. 2003, Miao, Sirachainan et al. 2004). More recently there have been reports of much improved yields with Human-cl rhFVIII, a new-generation recombinant factor VIII protein which is the first to be produced from a human cell line (approved by the EMA in July 2014). The purification procedure of this product involves one cell removal step (centrifugation/filtration), two filtration and five chromatography column steps giving an overall yield of approximately 50% (Winge *et al* 2015). This yield of 50% compares favourably to the 15% yield reported 5 years previously for another rFVIII product produced CHO cells (Thim *et al* 2010).

Another limiting factor of haemophilia treatment is the immune response of patients to infused FVIII. Approximately 25% of haemophilia patients develop antibodies that are inhibitory to FVIII in response to treatment and this remains one of the most serious complications today (Ehrenforth, Kreuz et al. 1992). The European Medicines Agency (EMEA) reported in 2005 that occurrence of inhibitors to FVIII had been reported for all approved and marketed recombinant FVIII products (EMEA 2005). The appearance of anti-FVIII antibodies can also occur as a spontaneous event in people with normal levels of FVIII (acquired haemophilia) (Lubin, Healey et al. 1994). There is still an ongoing debate over the use of plasma-derived FVIII versus recombinant FVIII, and which type is safer in terms of the risk of FVIII inhibitor development. An ongoing international clinical study (SIPPET) hopes to provide an answer to this (Mannucci 2012).

It is widely recognised that prophylactic treatment in the form of regular infusions (as often as 2-3 times a week) of FVIII is superior to 'on-demand' treatment to treat a 'bleed event', especially for those with the severe form of the disease as it can

prevent arthropathy (chronic joint damage). Studies have shown prophylactic treatment can reduce the average number of bleeding episodes per year from 37 to 13 (Miners, Sabin et al. 2004). However prophylactic treatment is limited by the relatively high cost of FVIII (approximately $1700 per dose for a 150lb adult) and also the lack of universal agreement in the clinic of correct dosage and dose intervals (Ljung 2009). Haemophiliacs with the severe form of the disease have been treated prophylactically with the aim of keeping trough FVIII levels above 1 IU/dL^{-1} since this can prevent arthropathy. Studies have shown that prophylactic treatment reduces bleed events from approximately 23 bleeds per year to 14 per year, but at an additional cost of £547 per patient compared to on-demand treatment (Miners, Sabin et al. 1998). However, new recombinant FVIII products with an extended half-life are shortly due to be commercialised (around 15-18 hours half-life extension) (Spiller 2013). There are a number of different approaches to extending the half-life of FVIII including Fc fusion e.g. Eloctate (which has regulatory approval in US and Canada), addition of polyethylene glycol e.g. turoctocog alfa pegol [N8-GP], and a single-chain construct (CSL627) (for review of these technologies see: Tiede 2015). These new products will require less frequent dosing, potentially lowering the cost of treatment, improving patient adherence and creating more favourable conditions for prophylactic treatment (Oldenburg and Albert 2014).

The difficulty and high cost of producing rFVIII is also reflected in the limited use of the treatment in countries with economic constraints such as India, although many haemophiliacs in these countries often go undiagnosed and most likely die from the disease (Bolton-Maggs and Pasi 2003). Two-thirds of the world's haemophilia population still do not have access to treatment and it is predicted that countries such as China and India may go on to make biosimilar FVIII products in response to this demand (Mannucci 2012). Up until 2014, biosimilar clotting factors were not permissible for approval in the European Union. However this exclusion has recently been removed from the EMA biosimilar guidelines (Guideline on similar biological medicinal products, October 2014) so we could yet see a biosimilar FVIII approved in Europe.

Clearly there are still significant improvements to be made in the production of rFVIII if we are to increase the availability of haemophilia treatment globally, and enhance the quality of life of patients. In order to improve on the current treatments

available, it is first important to understand the nature of human FVIII production and function *in vivo*, as well as what has already been done in terms of bioengineering rFVIII.

1.4 Factor VIII and its function in the blood clotting cascade

Platelet adhesion to the subendothelium of a damaged blood vessel is mediated by von Willebrand Factor (vWF), a large glycoprotein (Kanaji, Fahs et al. 2012). This leads to activation and aggregation of platelets, localisation of inactive coagulation factors on the activated platelet surface, and their subsequent proteolysis and conversion to their active forms. This results in a burst of thrombin generation and conversion of fibrinogen to fibrin (Kaufman and Pipe 1999).

Generation of thrombin occurs in two stages. An initial short burst via the extrinsic pathway produces small amounts of thrombin. The second stage and amplification of the coagulation cascade via the intrinsic pathway produces larger amounts of thrombin (Hoffbrand, Moss et al. 2006). Initial generation of thrombin via the extrinsic pathway is activated by the release of tissue factor, which forms a complex with activated factor VII (FVIIa-TF complex). FVIIa-TF complex activates FIX to FIXa. FIXa cooperates with its cofactor, FVIIIa to activate FX to FXa. FXa acts with its co-factor FVa to convert prothrombin to thrombin. FXa and thrombin act via a positive feedback mechanism to activate more FVIII and FV in order to amplify the generation of thrombin (Kaufman and Pipe 1999).

The role of FVIII upon activation is to significantly increase the efficiency of FIXa in converting FX to FXa (Fang, Wang et al. 2007). Prior to activation by thrombin or FXa, FVIII circulates in the blood in a stable complex with vWF. vWF can exert both a regulatory and inhibitory effect on FVIII, as it prevents a number of activities including phospholipid binding which is essential for FXa mediated activation of FVIII and its binding to activated platelets. It also prevents inactivation of FVIII by activated protein C (Kaufman and Pipe 1999). VWF is also a co-factor in the thrombin-catalysed activation of FVIII (Hill-Eubanks and Lollar 1990).

Human FVIII is a large plasma glycoprotein that contains multiple glycosylation sites (25 N-linked glycans and 7 O-linked glycans), some of which are known to play a role in its intracellular trafficking and degradation (described in detail later).

1.5 The expression of factor VIII

FVIII expression at the molecular level

The FVIII gene is 186 kb in size, contains 26 exons and is located on the X chromosome (Toole, Knopf et al. 1984), see Figure 1.2. Haemophilia is caused by a broad range of mutations in the FVIII gene and over 900 mutations have been discovered (Castaman, Giacomelli et al. 2010).

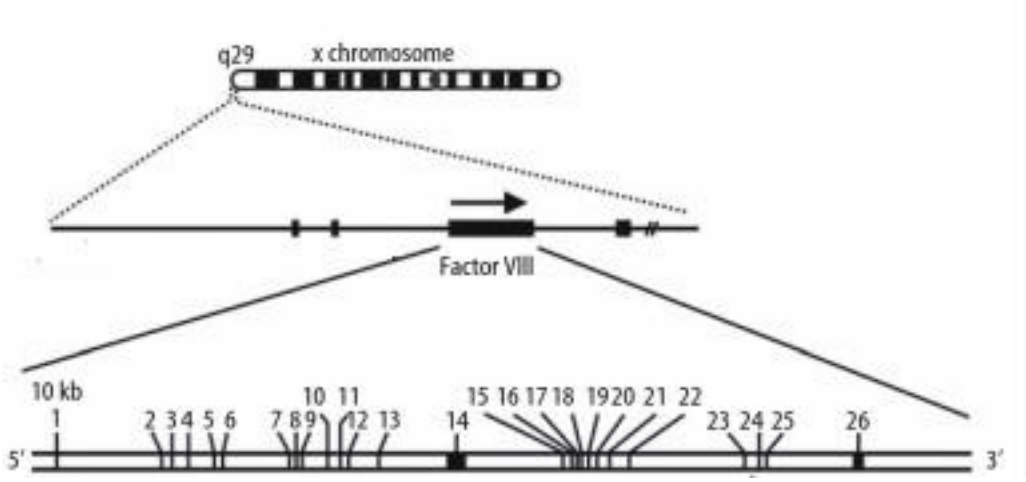


Figure 1-2 The structure of the Factor VIII gene containing 26 exons and located on the X chromosome. Reproduced from (Bagg 2007)**.**

FVIII mRNA is predominantly expressed in the sinusoidal cells of the liver, although it is also found at lower levels in the kidney, lung, brain and heart (Hollestelle, Thinnes et al. 2001). The liver has been shown to be the primary site of FVIII protein synthesis, and in fact liver transplantation in a haemophiliac has been shown to restore FVIII levels (Gordon, Mistry et al. 1998).

Around 50% of patients with severe haemophilia A have a mutation in their FVIII mRNA. It was suggested that this is due to insertion or inversion of a gene fragment in intron 22 of the FVIII gene (Naylor, Brinke et al. 1993). Other possible mutations include point mutations in the FVIII gene and a small proportion of severe haemophilia A patients (5%) have an inversion in intron 1 of the FVIII gene (Bolton-Maggs and Pasi 2003).

Approaches to increasing FVIII mRNA levels *in vitro* with a view to increasing the levels of FVIII expression have been tried including removing introns in the FVIII gene that contains transcriptional silencers, and replacing them with introns from other coagulation factor genes such as FIX that are known to have a positive effect on their secretion. For example splicing of a truncated FIX intron 1 into the site of FVIII introns 1 and 13 led to 13-fold higher levels of FVIII protein secretion versus the parent gene sequence (Plantier, Rodriguez et al. 2001).

The FVIII gene encodes for a single chain polypeptide of 2351 amino acids including a signal peptide of 19 amino acids (Wang, Wang et al. 2003). The signal peptide is cleaved from the protein upon translocation into the lumen of the Endoplasmic Reticulum (ER) (part of the secretory pathway) producing a single chain polypeptide containing 2332 amino acids (Kaufman and Pipe 1999), with a molecular weight of approximately 265 KDa, although this changes depending on the extent of glycosylation and/or sulfation of the protein, and has been detected up to 330 KDa in SDS-PAGE (Vehar, Keyt et al. 1984, Peake 1995). The single chain polypeptide consists of three domains, A, B and C of which there are three A, one B, and two C domains. These domains are connected by short acidic sequences a1, a2 and a3, see figure 1.3. This structure is abbreviated to NH2-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH (Vehar, Keyt et al. 1984, Wang, Wang et al. 2003). There is high homology among species for the majority of amino acids in FVIII except for the B domain (Fang, Wang et al. 2007).

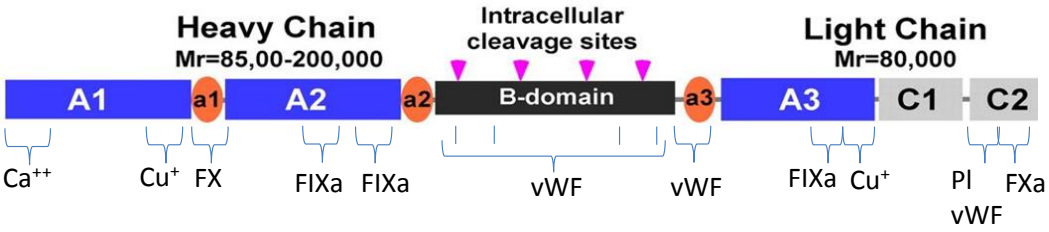


Figure 1-3 Schematic of FVIII domain structure, with binding sites indicated for calcium ions, copper ions, clotting factors IX and X, vWF and phospholipid surfaces (adapted from (Cao, Krishnaswamy et al. 2008) and (Lenting et al. 1998).

The A and C domains both have responsibility for coagulant function of FVIII, including binding sites for FIXa, FX, vWF and phospholipid surfaces (see figure 1.3). The role of the B domain is not as well studied as the A and C domain, although it is known to have roles throughout the life cycle of FVIII including the intracellular trafficking and secretion of the protein (Pipe 2009). Removal of the B domain in recombinant expression systems, so called B-domain deleted (BDD) rFVIII resulted in up to 20-fold higher expression of primary translation product (basic peptide not yet processed by ER/Golgi) versus the wild type, probably due to more efficient production of rFVIII mRNA (Saenko, Ananyeva et al. 2003). However, a 20-fold increase in primary translation product resulted in only a 30% increase in secreted FVIII, suggesting that there are other limiting factors in the production of FVIII protein, including the secretory pathway (Miao, Sirachainan et al. 2004).

Both full length FVIII and BDD-FVIII are currently marketed products, a summary of which can be found in table 2. It is important to note that FVIII produced by BDD-rFVIII cell lines is no more (or less) immunogenic than that produced by full length rFVIII cell lines (Saenko, Ananyeva et al. 2003), however there are reports that BDD-FVIII is less efficacious clinically than the FL-FVIII (Gruppo, Brown et al. 2003). A 2003 meta-analysis looking at clinical data for BDD-FVIII versus FL-FVIII in prophylactic treatment showed that in general higher doses of the BDD-FVIII were needed, the half-life of BDD-FVIII was shorter than FL-FVIII and the incidence of bleeding episodes/year was increased in those patients receiving BDD-FVIII (Gruppo, Brown et al. 2003).

Table 6. FVIII products marketed commercially for the treatment of Haemophilia A.

Product	Marketed by	Type of FVIII	Source	Production system
Advate	Baxter	Full length	Recombinant	CHO cells
Fanhdi®	Grifols	Full length / vWF complex	Plasma	Lyophilised concentrated human plasma
Kogenate®/Helixate®	Bayer	Full length	Recombinant	BHK cells
Recombinate®	Baxter/Pfizer	Full length	Recombinant	CHO cells
ReFacto®	Pfizer	B domain deleted	Recombinant	CHO cells
Replenate®	BioProducts Laboratory	Full length	Plasma	Lyophilised concentrated human plasma

FVIII at the protein level

Most of our knowledge regarding FVIII expression has come from recombinant mammalian cell lines due to the difficulties in finding a suitable native cell line to study (Kaufman and Pipe 1999). Only as recently as 2014 a liver study identified the exact cell type that manufactures FVIII as liver sinusoidal endothelial cells (LSECs), rather than liver hepatocytes in which FVIII could not be detected (Shahani T *et al*, 2014). To date, no successful attempts to immortalize cultured liver sinusoidal endothelial cells have been published. It has been demonstrated that rFVIII is expressed at levels up to 2 to 3 orders of magnitude lower compared to other similar sized recombinant coagulation proteins such as FIX (Doering, Healey et al. 2002), and up to 10,000-fold lesser production than is achieved for some monoclonal antibodies (Trent spencer *et al* 2011). In order to understand the reasons why FVIII is

such a difficult protein to express at high levels and to potentially improve the situation, it is important to explore and understand the pathways of secretory protein expression in mammalian cells.

1.5.1.1 The early secretory pathway

Since FVIII is a protein normally destined for secretion out of the cell, it is processed via the secretory pathway composed principally of the cell organelles the endoplasmic reticulum (ER) and the Golgi apparatus. Secretory proteins contain a leader sequence (also known as signal peptide), normally located on their N-terminus, which is recognised by a Signal Recognition Particle (SRP) (Walter, Ibrahimi et al. 1981). Upon binding of the SRP to the leader sequence, the protein is targeted to the SRP receptor on the ER membrane, initiating translocation through the Sec61 translocon (Nilsson, Lara et al. 2014). Sec61 is a pore that acts in partnership with another protein complex in the ER membrane called Sec62/Sec63, as well as a chaperone in the lumen of the ER called BiP, a member of the heat shock family of proteins (for review see (Park and Rapoport 2012)).

The ER is the first entry point for a protein into the secretory pathway. The ER is responsible for the correct folding and assembly of secreted proteins and also acts as a quality control check before the proteins go on to the Golgi apparatus for further processing. Almost a third of the open reading frames found in the human genome code for proteins that enter the ER. The ER is distinct from the cytosol of the cell and provides a compartment for proteins to be folded, assembled, modified or recycled (Braakman and Bulleid 2011). As the protein enters the ER lumen the signal recognition peptide is removed, and oligosaccharides are added on to specific asparagine residues of the polypeptide (consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline), an ER specific process known as *N*-glycosylation (Nilsson and Heijne 1993). These N-glycosylation sites provide recognition sites for chaperones that assist protein folding (Helenius and Aebi 2004).

Once in the ER lumen, the polypeptide chain is folded into a quaternary structure. Protein folding in the ER is assisted by a number of chaperones that belong to several well-known chaperone families including the heat shock proteins (Hsp)40, 70 and

90, as well as unique chaperones and enzymes like calnexin, calreticulin and thiol-disulphide reductases (Ellgaard and Helenius 2003).

FVIII contains 25 *N*-linked asparagine glycosylation sites, 19 of which are located in the B domain (Vehar, Keyt et al. 1984), as well as several sulphated tyrosine residues (Kaufman and Pipe 1999). Generally speaking, *N*-glycosylation patterns are unique to each protein and important for their biological function, their stability, solubility and their susceptibility to proteolytic attack (Roth, Zuber et al. 2010). Addition and initial processing of *N*-linked oligosaccharides takes place in the ER, with final processing completed in the Golgi apparatus (Lodish *et al*, 2000).

Within the ER there are many proteins and folding chaperones that FVIII has to interact with before the correctly folded protein can be released from the ER (Pipe, Morris et al. 1998). One such protein that interacts with FVII is GRP78, which upon overexpression in CHO cells was shown to reduce FVIII secretion (Dorner, Bole 1992). Interaction of FVIII with chaperones such as BiP in the ER has been shown to be a rate-limiting step in the production of FVIII in CHO cells (Dorner, Bole 1987). BiP (immunoglobulin heavy chain binding protein) is a major protein folding chaperone (Haas and Wable 1983), that can form a stable complex with FVIII via its B-domain. This interaction has been shown to limit the efficiency of secretion of FVIII as this stable complex with BiP is often retained in the cell, rendering the protein secretion – incompetent (Dorner, Bole et al. 1987). Stable complex formation with BiP can be dependent upon ATP hydrolysis (depending on the protein), which is regulated by ERdj1-5 proteins. Other regulatory proteins such as BAP allow the release of proteins from the BiP chaperone (Aridor 2007).

The interaction with BiP is increased in cases where FVIII is incorrectly folded due to incorrect glycosylation. Consequently the FVIII protein is targeted for degradation and exported from the ER to the proteasomal machinery, rather than continuing its journey on to the Golgi apparatus (Soukharev, Hammond et al. 2002). Deletion of a portion of the FVIII B-domain which contains the majority of the *N*-linked glycosylation sites resulted in a transient association with BiP and a higher amount of secreted FVIII (Toole, Pittman et al. 1986). Deletion of the putative FVIII BiP binding site by site-directed mutagenesis also resulted in enhanced secretion of FVIII (Saenko, Ananyeva et al. 2003). A BDD-FVIII CHO cell line containing a BiP

mutant which could not perform ATP hydrolysis resulted in inhibition of secretion of the BDD-FVIII hinting that the release of FVIII from BiP may be dependent on the conversion of ATP to ADP. This appeared to be specific to the FVIII protein as the secretion of another protein that interacted with BiP (M-CSF) was not affected by the loss of ATP hydrolysis (Morris, Dorner et al. 1997).

Two other protein chaperones calnexin (CNX) and calreticulin (CRT) which are endogenous lectin molecules found in the ER also interact with FVIII B domain. These two chaperones preferentially bind glycoproteins that contain N-linked oligosaccharides, and play a role in protein trafficking via degradative and/or secretory pathways (Pipe et al 1998). It has been suggested that CNX plays a positive role in promoting the release of FVIII from BiP and requires ATP. It is interesting to note that the secretion of another clotting factor, recombinant factor V, also involves interaction with CNX, CRT and BiP but this process is not dependant on ATP hydrolysis (Pipe, Morris et al. 1998). In mammalian cell lines that produce high amounts of recombinant proteins, these chaperones can become overloaded and this can lead to an accumulation of misfolded proteins and/or aggregated proteins (Jenkins, Murphy et al. 2008).

Aggregation of non-glycosylated proteins, in the ER can occur as a result of a disrupted *N*-glycosylation process. Subsequently these aggregated, disulphide bonded proteins cannot exit the ER, and are degraded over time (Kim, Bole et al. 1992). This has been demonstrated by multiple researchers on a variety of soluble and membrane bound proteins including Semliki Forest virus spike glycoproteins El and p62 and influenza hemagglutinin (Marquardt et al 1992). Aggregated proteins present in the ER can be transient and are sometimes simply folding intermediates (Pfeffer and Rothman 1987). It has been shown that FVIII can also form high molecular weight aggregates in the ER immediately after its synthesis, and that this is a rate limiting step for its secretion. These FVIII aggregates do not form disulphide bonds, and the process is reversible but requires ATP (Tagliavacca, Wang et al. 2000). Where the process cannot be reversed, misfolded FVIII is destined for degradation via proteasomal and lysosomal pathways (Plantier, Guillet et al. 2005).

Other chaperones exist that recognise misfolded or unassembled protein subunits which are then targeted for retrotranslocation out of the ER and back to the

cytoplasm of the cell. There are two pathways associated with this process called ER-associated degradation (ERAD), which occurs when proteins are terminally misfolded, and the unfolded protein response (UPR) which is associated with a stress response (Vasquez-Martinez, Diaz-Ruiz et al. 2012). Misfolded proteins can accumulate in the ER and aggregate causing the ER to dilate and activation of the UPR (Schroeder and Kaufman 2005). Proteins that are targeted for the ERAD pathway are ubiquitinated by Hrd1p. Another factor involved in this process is Yos9p, a lectin-like ERAD factor. Yos9p forms complexes with Hsp70, BiP and Hrd3p. Yos9p and Hrd1p together mediate the retention of proteins in the ER (Izawa, Nagai et al. 2012).

As well as molecular chaperones such as BiP, calnexin and calreticulin, there are also 'chemical chaperones' in the cell such as osmolytes that can inhibit aggregation and restore trafficking of misfolded proteins (Leandro and Gomes 2008). The chemical chaperone betaine (amino acid derivative) has been shown to restore intracellular trafficking of a trafficking-defective FVIII mutant both *in vivo* in murine models of haemophilia A and B and *in vitro* in cell based models (Roth, Schüttrumpf et al. 2012). In this study, betaine increased FVIII solubility, facilitated movement of FVIII from ER to the Golgi and increased FVIII plasma levels in gene-corrected FVIII knockout mice. This demonstrates the importance and also the therapeutic potential for chaperones to improve FVIII secretion.

A critical step for a correctly folded protein to exit the ER and be transported to the Golgi, is packaging into vesicles called coat protomer type II (COPII) in response to an ER export signal. The ER has specific exit points (tER sites) which are characterised by the presence of these vesicles (Barlowe 2003). The COPII coat is comprised of five proteins Sar1, Sec23/24 and Sec13/31. These are recruited to the ER and induce the ER membrane to curve and capture newly synthesised proteins that are to be exported (Gillon, Latham et al. 2012). Misfolded proteins mostly fail to enter the COPII vesicles although some proteins may make it out of the ER and into the Golgi before being retrieved into the ERAD pathway (Pagant, Kung et al. 2007). COPII vesicles bud away from the ER, lose their coat and fuse with pre-Golgi intermediates where they release their protein cargo (Dancourt and Barlowe 2010). Fusion of the vesicles is mediated by SNARE proteins (v-SNAREs) and target-SNAREs (t-SNAREs) (Brandizzi, Barlowe 2013). These intermediates then move

along microtubules towards the Golgi apparatus where the proteins are delivered (Lippincott-Schwartz, Roberts et al. 2000).

1.5.1.2 FVIII and the Golgi apparatus

The Golgi apparatus is a dynamic organelle comprised of multiple cisternal membranes and an array of different enzymes, chaperones, lectins and transporters. The main roles of the Golgi consists of sorting proteins, providing a gateway for secretory proteins to move from the ER to the extracellular environment (Palade 1975), O-glycosylation and final processing of N-linked glycans (Lodish *et al*, 2000). Besides mature glycosylation, sulfation and phosphorylation by Golgi enzymes, proteins also undergo post-translational modifications such as oligomerization and lipidation to aid protein transport to the cell membrane (Reynders, Foulquier et al. 2011). Proteins such as FVIII can undergo proteolytic action in the Golgi, to convert them from inactive proproteins to biologically active mature proteins (Lodish, Berk et al. 2000). Proteins destined for secretion from the cell move from the Golgi towards the cell membrane packed inside secretory granules, with both microtubules and actin filaments playing a role in moving these granules (Vasquez-Martinez, Diaz-Ruiz et al. 2012). With regard to FVIII, N-glycans are modified in the Golgi apparatus; O-glycosylation and sulfation of tyrosine residues occurs in the trans-Golgi. FVIII has six locations of tyrosine sulfation at positions 346, 718, 719, 723, 1664, and 1680 respectively, and these surround the sites where FVIII is cleaved by thrombin (Orlova *et al* 2013).

With regard to the FVIII protein, provided it has folded correctly in the ER, the protein then moves to the Golgi apparatus via a facilitated transport mechanism involving a molecular chaperone, ERGIC-53 (also described as mannose-binding lectin LMAN1), which is a type 1 transmembrane protein found in the ER-Golgi intermediate compartment. ERGIC-53 also plays a role in retrograde transport, movement of proteins back to the ER as a recycling mechanism. It has been shown that a mutation in the gene encoding ERGIC-53 can cause a combined deficiency of both FVIII and FV (Nichols, Seligsohn et al. 1998). Binding FVIII to ERGIC-53 occurs via the FVIII B domain, is dependent on the calcium concentration in the ER.

ERGIC-53 forms a complex with another protein called MCFD2 (LMAN1-MCFD2 complex) which recruits FVIII to sites of transport vesicle budding within the ER lumen (Zhang et al, 2005). Miao *et al* developed a recombinant BDD-FVIII cell line which contained a short B-domain spacer with the oligosaccharides essential for ERGIC-53 mediated transport. This resulted in a cell line with 15-25 fold increased FVIII protein expression levels versus a full length wild type FVIII (Miao, Sirachainan et al. 2004). Kolind *et al* also investigated BDD-FVIII expression by varying the B-domain length and found that expression levels of recombinant FVIII decreased with increasing B-domain length (Kolind, Norby et al. 2010).

Transport of proteins through the Golgi relies on vesicle carriers called COPI. The COPI coat is comprised of seven subunits which assemble on the Golgi membrane and form COP-I coated vesicles (Kondylis, Pizette et al. 2009). Besides the COPI coat proteins, there are many other proteins involved in regulating protein biosynthesis at the Golgi including Rabs, tethers, SM proteins and SNAREs (for review see (Cottam and Ungar 2012)). Sorting of proteins into COPI coated vesicles for movement around the Golgi occurs with all proteins regardless of whether they are Golgi resident proteins that move to different cisternae, proteins destined for secretion or proteins destined to return to the ER. Most proteins move in an anterograde (forward) fashion, but some move retrograde between the Golgi cisternae, and also back to the ER, thus ensuring a strict quality control system for all proteins (Lippincott-Schwartz, Roberts et al. 2000).

An additional quality control mechanism exists for resident ER proteins that make it to the Golgi. ER-resident proteins contain a KDEL-like motif at their C-terminus which normally prevents them being secreted. There are KDEL receptors in the ER-Golgi intermediate compartment and also in the Golgi itself, and binding of proteins to these receptors triggers retrieval of a protein back to the ER via COPI coated vesicles (Raykhel, Alanen et al. 2007). This is a highly selective and efficient process demonstrated by the fact that the receptor is capable of retaining a tenfold molar excess of KDEL-tagged protein. This is the case even when the protein is a KDEL-tagged secretory protein normally destined to leave the ER (Pelham 1996). The KDEL motif has been used in genetic engineering to enhance the accumulation of proteins intracellularly. Schouten *et al* added a C-terminal KDEL sequence to an ScFv construct containing a secretory signal sequence, which caused an increase in

expression levels in tobacco plant cells compared to a construct without a KDEL sequence, probably due to an increase in ScFv protein stability (Schouten, Roosien et al. 1996).

1.4.2.3 FVIII secretion from the cell as an inactive heterodimer

FVIII is secreted from the cell as an inactive heterodimer following cleavage in the Golgi between the B and A3 domains, as well as several cleavages within the B domain (after residues 1313 and 1648) (Marder et al. 2013). The heterodimer consists of a 90-200KDa heavy chain (A1-A2-B) and an 80kDa light chain (A3-C1-C2) associated by a metal ion (Yonemura, Sugawara et al. 1993). The metal ion linking the two chains has been identified as copper, and is released upon dissociation of the dimer. It has been shown that addition of copper is able to reconstitute FVIII activity from dissociated FVIII chains (Wang, Wang et al. 2003). In some recombinant cell lines it has been noted that the intracellular cleavage of the FVIII polypeptide chain in the B domain to form a heterodimer (two polypeptide chains) either does not occur, or is incomplete. To overcome this issue, several groups have investigated the co-expression of heavy and light chains using separate constructs. Yonemura *et al* co-expressed heavy and light chains using two separate plasmids and co-transfected these into CHO cells. Each chain was produced linked to an amino acid sequence that resembled the FVIII precursor sequence (signal peptide), and the heavy chain contained Kozak consensus sequence ((G/A)NNATGG, plays a major role in the initiation of the translation process) flanking the translation initiation codon. These conditions resulted in a mammalian cell line expressing functional FVIII at levels of 15 IU/day/10^6 cells, which is around 10-fold higher than other full length FVIII expression systems (Yonemura, Sugawara et al. 1993).

In vivo, FVIII heterodimer circulates in the blood complexed with a chaperone protein, von Willebrand factor (vWF), which acts as a stabiliser by preventing its proteolytic degradation and promoting the association of heavy and light chains (Wang, Wang et al. 2003). vWF is a large glycoprotein containing 2813 amino acids arranged into four homologous domains (A-D), of which the 'D' domain is responsible for non-covalent interactions with FVIII. It is produced as a pro-

polypeptide in megakaryocytes and vascular endothelial cells, and is converted to a mature form by proteolysis (Wagner 1990). vWF is only activated during high blood flow conditions and shear stress (Siediecki, Lestini et al. 1996). FVIII contains several binding sites for vWF which can be found in the C2 domain and the A3 domain of the FVIII protein and the D'D3 region of vWF (Saenko and Scandella 1997).While it is well understood that FVIII and vWF are synthesised in different cell types and assemble in plasma after secretion, recent evidence suggests that additional cell types exist *in vivo* where these two proteins are co-expressed (Van Den Biggelaar, Bierings et al. 2007). vWF is stored in elongated secretory organelles specific to endothelial cells called Weibel-Palade bodies (WPBs) (Weibel and Palade, 1964). WPBs contain a variety of other proteins that contribute to inflammation, angiogenesis, and tissue repair such as tissue-type plasminogen activator (tPA), P-selectin, interleukin-8 (IL-8), eotaxin-3, angiopoietin-2, osteoprotegerin, endothelin-1, endothelin-converting enzyme, and calcitonin gene-related peptide (Valentijn et al, 2011). vWF is initially produced as multimers in the Golgi of endothelial cells. These multimers go on to fold into elongated tubular structures, the presence of which drives the formation of WPBs. Biogensis of WPBs is also dependent on Clathrin and the heterotetrameric adaptor protein (AP) complex AP1, which help to form vesicles that transport proteins between post-Golgi compartments (Metcalf et al 2007). It has been demonstrated that FVIII is also trafficked to the WPBs and that the FVIII:vWF complex can be stored here until required (van den Biggelaar, Meijer et al. 2009).

In recombinant systems expressing FVIII, the absence of vWF in the cell culture media results in the secretion of dissociated heavy and light chains that are rapidly degraded. Research groups have overcome this issue by adding vWF to the culture medium (containing serum) or alternatively in serum-free systems, co-expressing vWF and FVIII together in CHO cells in order to increase the stability of the FVIII protein (Kaufman and Pipe 1999), (Kaufman, Wasley et al. 1989). Co-expression of vWF and FVIII in CHO cells also increased the total amount of FVIII secreted from the cell compared to the expression of FVIII alone. The secretion rate of FVIII was however much slower in the presence of vWF and this was thought to be due to an increase in BiP expression in the VWF/FVIII cell line which slowed the intracellular trafficking of the FVIII protein. Interestingly it was demonstrated that vWF had a

transient association with BiP and therefore its secretion rate was unaffected by the increase in BiP expression (Kaufman, Wasley et al. 1989).

1.5 Activation and inactivation of FVIII

Activation of the inactive protein FVIII to FVIIIa is induced by thrombin or FXa and results in an unstable heterotrimer that is susceptible to proteolytic degradation by activated protein C (APC), FIXa or FXa (Saenko, Ananyeva et al. 2003). The activation step cleaves the B domain from the heavy chain and releases vWF from the light chain, leaving FVIII free to exert its coagulant properties by interacting with FIXa (Kaufman and Pipe 1999). The heterotrimer consists of the A1 domain, A2 domain and the A3-C1-C2 domain (Vehar, Keyt et al. 1984). The light chain (A3-C1-C2) and the A1 domain are linked by a stable divalent metal (copper) ion bond whereas the A2 subunit is weakly associated to the A1 domain via electrostatic interaction (Fang, Wang et al. 2007). Dissociation of the A2 domain occurs readily in wild type FVIIIa at physiological pH and results in loss of coagulant activity (Wang, Wang et al. 2003).

Several attempts have been made by research groups at engineering parts of the A2 domain in order to increase the stability and extend the half-life of the FVIII protein. Radtke *et al* produced a B-domain deleted cell line that contained an additional disulfide bond between the A2 domain and the A3 domain in order to prevent A2 dissociation and prolong the half-life of activated FVIII. Compared to wild type FVIII, only 10% of the FVIII with an extra disulphide bond was required to produce the same clotting activity in whole blood bioassays (Radtke, Griffin et al. 2007). This approach of prolonging the half-life of FVIII seems promising when you consider the needs of haemophilia patients in terms of the frequency of FVIII infusions and the preference for prophylactic treatment with a longer half-life, meaning fewer doses would be required to elicit the same level of clotting activity. Pipe *et al* developed a cell line producing FVIII that was resistant to A2 domain dissociation, by deleting residues 794-1689 so that the A2 domain was covalently attached to the light chain, as well as introducing missense mutations at thrombin and activated protein C inactivation cleavage sites providing resistance to proteolysis. Both of these steps

resulted in increased protein stability demonstrated by FVIII activity remaining after 4 hours in the mutated version, versus thrombin inactivation of FVIII within 10 minutes in the wild type FVIII. (Pipe and Kaufman 1997).

Aside from the dissociation of the A2 domain, there are other mechanisms of inactivating FVIII *in vivo*. Activated FIX, activated protein C (APC), activated FX and plasmin are all proteases capable of cleaving FVIII. The effect of APC can be enhanced by protein S and there is also evidence to suggest a role for FV in this process.

A number of receptors are also involved in the clearance of FVIII including low density lipoprotein (LDL) receptor, the LDL receptor related protein-1 (LRP1), the asialoglycoprotein receptor (ASGPR) via the glycosylated regions of the B-domain, and the macrophage mannose receptor (CD206) (review, (Lenting, Christophe et al. 2010)). In terms of the receptors involved in FVIII clearance, this appears to be a relatively recent area of research and the mechanisms of action need to be more fully understood before these receptors can be exploited with regard to extending the half-life of FVIII.

A high level summary of the steps that the FVIII protein goes through as it is secreted from the cell, activated by thrombin and then inactivated can be seen in figure 1.4.

1. Partial cleavage of the FVIII polypeptide in the B domain by an intracellular protease in the Golgi results in secretion of an inactive heterodimer consisting of an 80 kDa light chain composed of the A3-C1-C2 domains and a heterogeneous 90–210 kDa heavy chain of the A1-A2-B domains.

2. The unactivated FVIII heterodimer circulates in blood plasma with its carrier protein, von Willebrand factor (VWF), to form a tight non-covalent complex.

3. Thrombin activates FVIII by proteolysis at three residues: Arg372, Arg740 and Arg1689. Arg372 and Arg1689 are known to be essential sites for FVIII activation (Fig. 1) [34]. Cleavage at Arg372 bisects the A1 and A2 domains and at Arg1689 the acidic a3 region from the light chain to create the FVIIIa heterodimer (A1-a1/ A2-a2/ A3-C1-C2)

3. FVIII activity is rapidly lost either through spontaneous dissociation of the A2 subunit from the rest of the molecule or through proteolytic degradation by a number of enzymes, such as thrombin, FIXa, FXa and activated protein C (aPC). The main physiological inactivator of FVIIIa, aPC , cleaves FVIIIa in the A1 (Arg336) and A2 domains (Arg562).

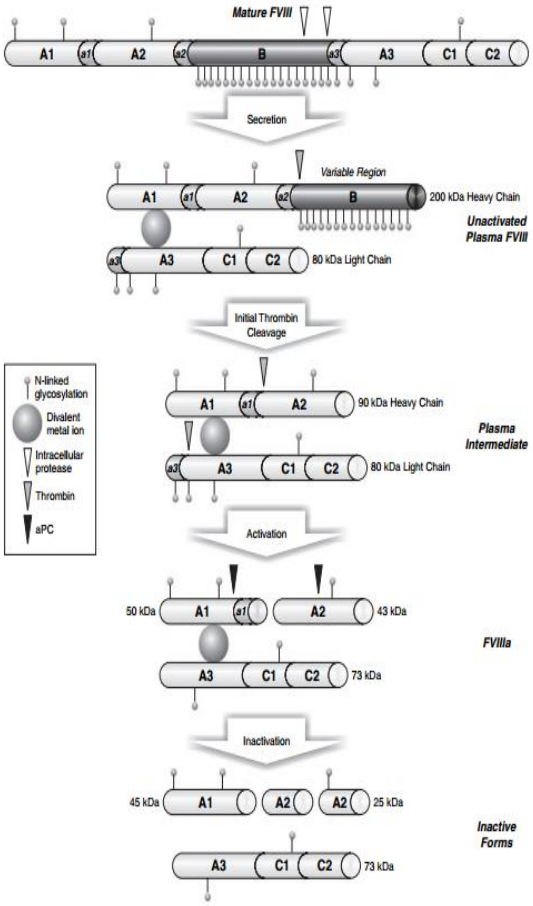


Figure 1-4. A summary of the steps that FVIII goes through including secretion, activation and inactivation (image reproduced from Pipe et al, 2009).

1.6 The formation of anti-factor VIII antibodies

A serious complication of haemophilia treatment is the development of antibodies which are inhibitory to FVIII as a result of FVIII infusion (Hoffbrand, Moss et al. 2006). The formation of these polyclonal alloantibodies occurs in around 25% of haemophilia A patients who receive repeated infusions of FVIII (Saenko, Ananyeva et al. 2003). Polyclonal autoantibodies to FVIII can also occur in people with normal FVIII levels (acquired haemophilia, AHA) (Lubin, Healey et al. 1994). Both allo- and autoantibodies bind to the A2 (454–509), A3 (1804–1819), or C2 domains (2181–2243) of the FVIII protein with varying modes of action. The anti-C2 antibodies interfere with the binding of FVIII to phospholipids and VWF, A2 and A3 inhibitors block the binding of FVIII to factor X (FX) and FIXa, respectively,

obstructing the formation of the Xase complex (Sakurai, 2014). Alloantibodies occur in response to infusion of FVIII into the patient whereas autoantibodies form spontaneously against the patient's own plasma FVIII. Interestingly it has been shown in several studies that alloantibodies against FVIII can demonstrate proteolytic activity although the clinical relevance of this has not yet been elucidated. In one such study, plasma purified from 13 of 24 FVIII inhibitor-positive patients contained IgG alloantibodies that demonstrated significant proteolytic activity, when incubated with biotinylated factor VIII, whereas FVIII that had been incubated with normal human IgG had no detectable proteolytic activity (Lacroix-Desmazes et al, 2002).

It is apparent that there is a link between the type of FVIII gene mutation a patient has and the likelihood of developing inhibitors. For those patients with mutations that prevent the production of FVIII e.g. intron 22 inversion, the likelihood of developing inhibitors is around 35% versus 5% for those patients who are able to produce small amounts of FVIII (Bolton-Maggs and Pasi 2003).

The incidence of inhibitor formation in congenital haemophilia appears to be linked to the type of treatment given, either prophylactic or on-demand. There is evidence to suggest that patients who are given FVIII on demand are more likely to develop these inhibitors than those receiving prophylactic treatment prior to a bleeding event (Ljung 2009). There are also differences in the type of inhibitors formed in response to different commercial FVIII products. There is an ongoing debate over the immunogenicity of plasma derived versus recombinant FVIII products and which is safer in terms of inhibitor development. The European Medicines Agency published a report in 2007 on FVIII products and inhibitor development and stressed the need for clinical data comparisons between the two product types (EMEA 2007). Infusion of vWF along with FVIII in plasma derived FVIII products may be preferable to infusing FVIII alone since vWF appears to regulate immune recognition by concealing antibody binding epitopes (Terraube, O'Donnell et al. 2010).

For recombinant FVIII products, even a small change to the manufacturing process can affect their immunogenicity profile. This has been seen with manufacturing changes to KOGENATE® and its successor, Kogenate® FS/Bayer (Bayer) that were made in order to improve pathogen safety. These included removal of albumin,

addition of an S/D viral inactivation step (to inactivate enveloped viruses) and a shortening of the protein residence time in the fermenter which lowers the probability of immunogenic FVIII degradation products forming. Improvements in the successor product have led to potentially improved immunogenicity profile in previously untreated or minimally treated patients with severe haemophilia A (Lusher and Scharrer 2009). An assessment by the EMA concluded that the product did not appear to be different from other FVIII products in terms of efficacy and safety in the treatment of PUPs (previously untreated patients) and MTPs (minimally treated patients), had sufficient viral safety due to the various viral removal/inactivation steps, and the sourcing of bovine products from countries without any reported cases of BSE (EMEA 2004).

Other groups have looked at inducing immune tolerance to specific inhibitory epitopes. Rawle *et al* demonstrated this concept in a mouse model of haemophilia A by exposing the mice to purified FVIIII C2 domain via their mucosa. When dosed with whole FVIII, lower levels of antibodies to FVIII C2 domain were seen in the pre-exposed mice compared to those mice who had not been previously exposed (Rawle, Pratt et al. 2006).

1.7 Porcine FVIII versus human FVIII

One approach to overcome the problem of anti-FVIII antibodies takes advantage of the fact that these inhibitors of human FVIII have limited cross-reactivity (often negligible) with porcine FVIII (Kernoff 1991). Early attempts at treating haemophiliacs with porcine FVIII were abandoned due to allergic reactions and the presence of platelet aggregating factor which caused thrombocytopenia. However, a highly purified version of plasma-derived porcine FVIII (Hyate, Speywood) was used successfully to achieve hemostatic FVIII levels in patients in whom human FVIII was ineffective (Mayne, M et al. 1981), but possible residual viral contamination and immunogenicity prevented its routine use. More recently, OBI-1, a B-domain deleted recombinant porcine FVIII (manufactured by Ipsen and Inspiration Biopharmaceuticals Inc), showed promise in both animal and human haemophilia subjects. In a murine model of haemophilia A, OBI-1 demonstrated less

immunogenicity than plasma derived FVIII, and in cynomolgus monkeys, OBI-1 did not generate detectable inhibitors. In a human trial (phase II, open-label), patients with hemophilia A and inhibitors against porcine FVIII showed OBI-1 was well tolerated, without drug-related serious adverse events and showed promise for further studies (Toschi 2010). A phase 2/3 study also demonstrated that OBI-1 is safe and effective in treating bleeding episodes in patients with acquired haemophilia A (Kruse-Jarres, R. et al 2015). A Biologics License Application (BLA) was submitted in 2013 to the U.S. Food and Drug Administration (FDA) for the approval of OBI-1, as a treatment for patients with acquired hemophilia A (Baxter 2013), and received FDA approval in 2014.

Although porcine and human FVIII have the same domain structure (A1-A2-B-A3-C1-C2), human FVIII has weaker coagulant activity than porcine FVIII due to faster dissociation of the A2 subunit (Lollar, Parker et al. 1992). Porcine FVIII has limited cross-reactivity with human FVIII inhibitory antibodies, therefore, purified porcine FVIII has been used in the treatment of haemophilia patients who have developed inhibitors to human FVIII since 1980 (Kernoff 1991). Hyate:C, a plasma-derived porcine FVIII, was used in humans for approximately 20 years until it was discontinued due to suspected viral contamination of the porcine blood supply (Healey, Parker et al. 2009). Treatment with porcine FVIII results in cessation of bleeding in haemophiliacs with inhibitors to human FVIII, and can even be used when the patient has porcine FVIII inhibitors, as demonstrated in a case study (Gribble and Garvey 2000).

Recombinant versions of porcine FVIII have also been investigated since it was noted that porcine FVIII exhibits high-level expression due to enhanced transit through the secretory pathway (Dooriss, Denning et al. 2009).

Lubin *et al* developed a B-domain deleted human/porcine FVIII hybrid by eliminating the human FVIII A2 inhibitor epitope and replacing this with the homologous porcine sequence. The coagulant properties of the protein in a functional assay were unchanged but anti-A2 inhibitor binding was reduced (Lubin, Healey et al. 1994). The approach of deleting inhibitor epitopes on FVIII could be promising in terms of personalised medicine and tailoring treatment to the individual patient depending on the inhibitor type present.

In 2002, Doering *et al* looked at a recombinant B-domain deleted (BDD) porcine FVIII producing cell line in comparison with a recombinant BDD human FVIII cell line and saw expression levels 14-fold greater in the porcine system (Doering, Healey et al. 2002). The same group later went on to investigate the regions of porcine FVIII gene that are responsible for these increased expression levels. They developed a human/porcine BDD FVIII hybrid containing the porcine A1 and a3-A3 domains and were able to achieve protein expression levels comparable to that of the native BDD porcine FVIII system. Human FVIII contains two potential glycosylation sites that are not present in porcine FVIII, at Asn-42 within A1 domain and at Asn-1685 within a3-A3 domain. Also, porcine FVIII contains a potential glycosylation site at Asn-213 in the A1 domain that is not conserved in human FVIII. It was hypothesised that due to the differences in the glycosylation patterns between the porcine and human molecules in the A1 and a3-A3 domains, and the fact that these sites normally affect the passage of FVIII through the endoplasmic reticulum/Golgi apparatus, the hybrid porcine/human protein had more efficient folding and passage through the ER-Golgi secretory pathway (Doering, Healey et al. 2004). This is known to be a rate-limiting step for the production of human FVIII due to interactions with the BiP molecular chaperone as described earlier.

1.8 Purification of FVIII

Due to previous safety concerns about the pathogenicity of blood derivatives, the regulatory approval process for recombinant FVIII products requires that all human and animal protein additives have to be removed and of course this includes vWF. In a manufacturing setting, a complicated series of purification steps have to be taken to separate FVIII from a large volume of cell media before it can be infused into patients. In order to remove all medium components, host cell residues, and potential pathogens such as viruses, many techniques are employed. These vary depending on the manufacturer in question but can include crude physical restriction on the basis of protein size, immunoaffinity (using an anti-FVIII antibody), ion-exchange chromatography (based on charged side chains), pasteurization (heat treatment to remove viruses), solvent-detergent (to remove viruses) (Pipe 2008), and nano-

filtration with more recent products (remove prions and viruses) (Lee, Berntorp et al. 2010).

Purification processes for FVIII vary according to the source of the protein and the manufacturer (for high level summary see table 7). The FVIII products listed in the table below can be classified into three groups ranging from the intermediate purity products obtained through precipitation/adsorption, concentrates purified through ion exchange chromatography and concentrates purified through the use of monoclonal antibodies. The recombinant FVIII products are all obtained using immunoaffinity chromatography (see review, Franchini, 2013).

Table 7 Examples of commercialised FVIII products and their manufacturing processes (reproduced from Franchini 2013).

Product	Manufacturer	Production characteristics	
		Purification	Viral inactivation
Plasma-derived FVIII concentrates			
Alphanate	Grifols	Heparin ligand chromatography	S/D, dry heat
Beriate	CSL Behring	Ion exchange chromatography	Pasteurisation
Emoclot D.I.	Kedrion	Ion exchange chromatography	S/D, dry heat
Fanhdi	Grifols	Heparin ligand chromatography	S/D, dry heat
Haemate P	CSL Behring	Multiple precipitation	Pasteurisation
Haemoctin	Biotest	Ion exchange chromatography	S/D, dry heat
Hemofil M	Baxter	Immunoaffinity chromatography	S/D
Immunate Stim Plus	Baxter	Ion exchange chromatography	Detergent, vapour
Recombinant FVIII concentrates			
Advate	Baxter	Immunoaffinity chromatography	S/D
Helixate NexGen	CSL Behring	Immunoaffinity chromatography	S/D, ultrafiltration
Kogenate Bayer	Bayer Healthcare	Immunoaffinity chromatography	S/D, ultrafiltration
Recombinate	Baxter	Immunoaffinity chromatography	-

Refacto	Wyeth Pharmaceuticals	Immunoaffinity chromatography	S/D
Refacto AF	Wyeth Pharmaceuticals	Immunoaffinity chromatography	S/D, nanofiltration

Legend: S/D = solvent/detergent.

Purification of FVIII using immunoaffinity chromatography is an expensive procedure involving many steps, and is complicated by the use of antibody ligands which can elute along with FVIII and contaminate the product (Burnouf and Radosevich 2001). The antibody ligands also present other problems as they need to be virus-inactivated to minimise risk of contamination and are expensive to produce. Nord *et al* looked at an alternative to using antibodies by using phage display to produce FVIII specific-affibodies which are much smaller than antibodies and have an improved affinity to FVIII (Nord, Nord et al. 2001). Knör *et al* also looked at alternatives to using antibodies by developing a small synthetic peptide ligand with high affinity to FVIII. This ligand offered several advantages over the use of antibodies as it was stable, non-toxic, cheap to produce and enabled the elution of FVIII under mild conditions (Knör, Khrenov et al. 2008).

1.8.1 Antibody fragments as tools in the purification of proteins

Another approach to the purification of large proteins such as FVIII is the use of small antibody fragments instead of whole antibodies. These include 'Fab' which is the antigen binding fragment composed of one constant and one variable domain from each heavy and light chain of the antibody or 'Fv', containing just the variable domain from the heavy and light chain (V_H and V_L) (Janeway, Travers et al. 2005). These fragments have been successfully expressed in bacteria, yeast and fungi and offer advantages over the use of whole antibodies such as improved stability. However these fragments are difficult to produce on an industrial scale as their heavy and light chains have a tendency to dissociate upon dilution. To overcome this issue, three strategies were tried including linking the two chains with glutaraldehyde, disulphide bonds or a peptide linker (Glockshuber et al 1990). However, the option of synthetically linking the chains to form a single chain fragment, so-called ScFv, lowered their affinity for their antigen compared to their parent antibody

(Muyldermans 2001). Also, the synthetic link used to form the ScFv caused the fragments to aggregate in the ER of the cell, resulting in ineffective secretion (Thomassen, Meijer et al. 2002). Ward *et al* also tried producing single chain VH antibody fragments in a mouse (Ward and Gussow 1989) however these are less soluble and have a lower antigen binding affinity than ScFv (Muyldermans 2001).

1.8.2 Camelid antibodies

An exciting new development in this field came with the discovery that Camelids (camels, dromedaries, llamas, alpacas, guanacos and vicunas) have a unique ability to produce not only conventional antibodies but also antibodies that are devoid of light chains and CH1 domains (constant heavy chain domain 1) (Omidfar, Rasaee et al. 2007), see figure 1.5. Despite the absence of the light chain and CH1 domain, these heavy chain only antibodies are able to bind to antigens as efficiently as conventional antibodies (Maass, Sepulveda et al. 2007). The antigen binding domain of these heavy chain only antibodies is denoted VHH (variable domain of the heavy chain of a heavy-chain only antibody), and at approximately 12-15 kDa is the smallest fragment known to derive from a functional antibody (Muyldermans 2001).

The VHH and the VH region of a conventional antibody contain domains that are comparable in their basic structure, containing four conserved framework regions, and three hypervariable regions, the complementarity determining regions (CDR) (Muyldermans 2009). Although these VHH have adapted to compensate for the lack of a light chain, the VHH demonstrate a high degree of homology in their amino acid sequences with VH in conventional antibodies, particularly those amino acids that determine the typical immunoglobulin folding (Muyldermans, Cambillau et al. 2001). The VHH also show a close homology to human VH domains which could be particularly useful for *in vivo* applications of antibody fragments when considering immunogenicity issues (Omidfar, Rasaee et al. 2007).

Although close homology exists between VH and VHH, there are four specific amino acids that are normally present in VH that are constitutively replaced in VHH (residues Val37Phe (or Tyr), Gly44Glu (or Gln), Leu45Arg (or Cys) and Trp47Gly (or Ser, Leu, Phe)) and this makes it easier to distinguish between the two (Muyldermans, Cambillau et al. 2001). These amino acid substitutions are in regions

normally that normally bind to VL, renders this part of VHH more hydrophilic and less likely to bind to VL (Muyldermans 2001). The hydrophilic nature of VHH contributes to the increased solubility of these fragments compared to VH.

In addition, the VHH CDR3 region is longer (17 amino acids), than in human VH (9 amino acids) (Vu et al 1997). It is thought that the enlarged hypervariable regions of VHH results in a larger antigen binding site and compensates for the absence of the VL domain which in an Fv fragment would provide additional antigen contact sites. The enlarged hypervariable regions are responsible for many structural differences between VH and VHH, primarily the presence of loop structures in the CDRs of their antigen binding sites. The structural diversity and small size of VHH allows additional freedom with regard to the epitopes they recognise and can bind to (Muyldermans 2001).

VHH fragments offer many advantages over other antibody fragments such as Fab, Fv or scFv due to their increased stability, solubility and antigen binding properties. This has led to interest in these VHH for research and commercial purposes (Maass, Sepulveda et al. 2007). Originally heavy chain antibodies were sourced from immunised Camelid, from which the VHH domain could be separated via proteolysis (Muyldermans 2001). More recently, research groups have looked into the production of Camelid derived ligands on a large scale using recombinant systems to express the VHH domains. Thomassen *et al* were able to produce VHH fragments on a large scale using a yeast expression system (*Saccharomyces cerevisiae*) and a Llama VHH gene (Thomassen, Meijer et al. 2002).

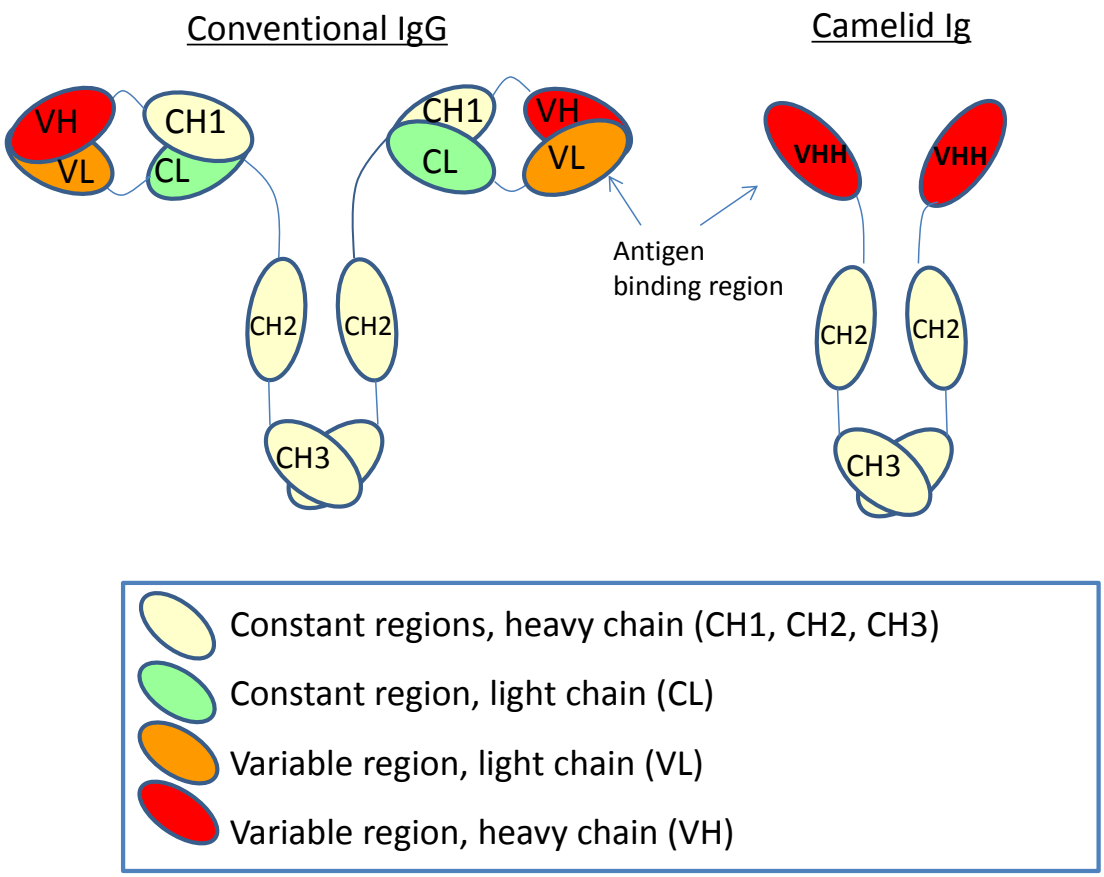


Figure 1-5 The structure of a conventional antibody compared with a Camelid heavy chain only antibody.

The Camelid immunoglobulin is devoid of the light chain and the CH1 region of the heavy chain that are both present on the conventional immunoglobulin G. The antigen binding region of the heavy chain of the Camelid antibody is referred to as VHH (variable region of the heavy chain of a heavy chain only antibody).

1.8.2.1 Anti-FVIII specific VHH

BAC BV in the Netherlands use a yeast expression system, free of animal derived components to produce recombinant VHH fragments (CaptureSelect® ligands). These ligands have been developed specifically as tools in the purification of recombinant proteins. More specifically, BAC has developed several ligands that

bind specifically to FVIII. Currently, these ligands are commercially available as tools for the purification of recombinant FVIII (FVIIISelect, GE Healthcare).

Previous experiments (unpublished) were conducted at the University of Kent by Dr Kerry Tappenden and Dr Wynne Jones, who used these camelid ligands in a 2-stage purification of FVIII from cell culture media at pH7 and showed that they are robust and highly selective. In the first stage of the method, FVIII (Advate) was purified from AIM-V cell culture media using Mustang® Q (Pall Corporation) anion exchange chromatography. FVIII was then eluted from the Mustang® Q coin and the sample loaded directly on to a FVIII Select™ affinity chromatography column (GE Healthcare). FVIII Select™ contains anti-FVIII camelid antibody linked to a porous membrane. Given the current complex multi-step procedure of purifying FVIII in an industrial manufacturing setting, these camelid ligands provide some hope in simplifying the process. Also, harsh elution conditions can often prove harmful to the final protein product so the elution at neutral pH also offers advantages (McCue, Selvitelli et al. 2009). BAC has also demonstrated (confidential/unpublished) that these anti-FVIII VHH have high affinity binding to FVIII, which has contributed to their commercial value as simple purification tools.

Based on the unpublished data generated by BAC and the University of Kent, it is possible that these ligands not only bind FVIII, but also increase the stability of the protein. This project will explore a targeted approach of co-expression of these anti-FVIII VHH ligands with FVIII to investigate the possibility that these ligands do increase the stability of the FVIII protein upon binding, and subsequently impact the levels of functional FVIII protein secreted into the cell culture media of mammalian cells.

Targeting of VHH ligands to specific proteins (membrane, secretory, intracellular, cell surface and others) has been reported in many studies in the literature (Wesolowski, Alzogaray et al. 2009). An example includes an anti-vWF VHH in development for detection of vWF in serum samples for the diagnosis of von Willebrand's disease (Hulstein, Groot et al. 2005). Also, a VHH specific for hepatitis B particles has been targeted intracellularly (also known as an intrabody) by the addition of KDEL which led to the intracellular accumulation of hepatitis B particles in mice (Serruys, Houtte et al. 2009). The strategy of co-expressing VHH with a

target protein in the same cellular compartment has also been tried with Bax, a member of the Bcl-2 family. Bax-specific VHH were developed and used to inhibit Bax induced permeabilisation of mitochondria and resulted in cell lines that were resistant to oxidative stress (Gueorguieva, Li et al. 2006).

In the present study, three Camelid-derived VHH ligands, named ligand 2, ligand 6 and ligand 7 respectively have been supplied by BAC BV, Netherlands. Two of these ligands are reported by BAC to have anti-FVIII activity (ligands 2 and 7). The third ligand, (ligand 6) binds to FII and will therefore be used as a negative control. Both ligand 2 and ligand 7 bind to the light chain of FVIII (exact binding locations confidential to BAC), with ligand 7 demonstrating the best binding affinity (low nM) to FVIII. Previous competition ELISA experiments (conducted by BAC) indicated that anti-FVIII ligand 7 competes with vWF for the same epitope on the FVIII protein (personal communication from BAC). It is possible ligand 7 may have similar properties to vWF such as stabilising the FVIII protein and/or protecting it from proteolysis since it binds the same epitope on the FVIII protein. Since vWF stabilises FVIII when the two proteins are co-expressed *in vivo*, it is hypothesised that co-expression of FVIII with ligand 7 could provide a route to producing increased levels of stable recombinant FVIII. Conversely, ligand 2 has a binding epitope on FVIII that does not compete with that of vWF so the present will also compare the effect of co-expression with FVIII between ligand 2 and ligand 7. In the present study, ligand 2, ligand 6 and ligand 7 will be co-expressed with FVIII, and the resultant effect on FVIII expression will be assessed using a gold standard coagulation assay.

It is important to note here that an increase in total FVIII protein expression does not necessarily translate to an increase in the amount of functional FVIII i.e. that which is correctly folded and fully functional in terms of procoagulant activity. Therefore any differences in the amount of functional FVIII must be tested using a functional coagulation assay, which is currently widely used in a clinical setting to diagnose bleeding abnormalities and monitor anticoagulant therapies (Bates and Weitz 2005). Conversely, an increase in the amount of functional FVIII does not necessarily equate to an increase in total protein so both must be assessed.

In summary, the literature surrounding FVIII and haemophilia research is immense. It is clear that many different cell lines, expression systems and engineered versions of FVIII have been investigated, all with the aim of improving on current treatments for haemophilia. Since those products which are currently in clinical use are manufactured under confidentiality (at least the full chemistry, manufacturing and controls data is not normally published by regulatory agencies), it is difficult to say which of these areas of research have made the most impact. What is clear however is that there is still significant room for improvement to be made with FVIII expression, given the fact that it is expressed at much lower levels than other recombinant clotting factors, and the subsequent effect this is having on availability and cost of treatment for haemophiliacs worldwide. Any future FVIII products need to have a fine balance between safety, clinical efficacy and cost effectiveness if they are to have any impact on the current situation.

This project aims to add to and complement this vast area of research with an alternative means of increasing the amount of functional FVIII that is expressed in a BDD-FVIII BHK cell line by using Camelid antibody fragments as molecular chaperones, an approach that has not previously been investigated in the production of FVIII.

Aims of the project

The overall aim of this project is to co-express three different Camelid antibody ligands (VHH ligands 2, 6 and 7) with FVIII. Two of these ligands are anti-FVIII and the third ligand is anti-FII (negative control). Upon co-expression, FVIII production in mammalian cells will be tested using both quantitative and functional assays in order to demonstrate any changes in FVIII protein expression and/or coagulant activity.

Experimental aims:

1. Using a variety of molecular techniques nine plasmid constructs will be created and cloned into a pcDNA3.1 vector that is suitable for mammalian transfection and expression. Each plasmid construct has either an immunoglobulin leader sequence, a HIS tag, a KDEL sequence (endoplasmic reticulum retrieval motif) or a combination of these (see figure 2.1). Plasmid constructs to be created include:
    a. Ligand 2, constructs 1, 2 and 3
    b. Ligand 6, constructs 1, 2 and 3
    c. Ligand 7, constructs 1, 2 and 3
2. Transiently transfect Camelid antibody constructs into mammalian cells and confirm expression using ELISA or western blotting
3. Co-express Camelid antibody ligand and FVIII in mammalian cells
4. Test the pro-coagulant activity of FVIII protein secreted into cell media using a gold standard coagulation assay before and after co-expression with VHH.

It is hypothesised that VHH ligands produced from construct 1 containing a leader sequence will be secreted from the cell; VHH ligands produced from construct 2 (containing no leader sequence and a HIS tag) will possibly not be secreted from the cell and stay in the cytosol due to the absence of a leader sequence. For construct 3 which contains both a leader sequence and KDEL, the hypothesis is more complicated. The presence of the leader sequence will direct the protein via the ER/Golgi pathway to be secreted from the cell. However, since KDEL is an endoplasmic reticulum retrieval motif, it is possible that a high

proportion of this protein will bind to the KDEL receptor in the Golgi apparatus and be rendered secretion incompetent, despite the presence of the leader sequence. Interaction of the VHH ligand at the KDEL receptor may have important consequences when considering those constructs that have anti-FVIII activity. If the VHH ligand is bound to FVIII when it binds to the KDEL receptor, this may also prevent FVIII being secreted, and result in both proteins being recycled back to the ER. The impact of this could be a reduction in secreted FVIII protein since excess FVIII accumulates in the ER, potentially resulting in overloading the system, the formation of aggregates and the degradation of the protein.

Chapter 2: Methods

2.1 Overview

In order to meet the aims of this project, multiple laboratory techniques and experimental approaches were employed. The first aim of this project was to produce plasmids containing the insert for Camelid antibody (VHH) ligands supplied by BAC BV (The Netherlands) that were suitable for transfection into mammalian cells. Once the plasmids were produced and the gene sequence confirmed, they were transfected into a selection of mammalian cell lines, and the expression of VHH protein confirmed.

Co-expression of VHH and FVIII in mammalian cells was then carried out and both VHH and FVIII protein expression was investigated at length. Lastly, since the ultimate aim of this project, was to investigate the effect of co-expressing VHH and FVIII on the functional activity of FVIII, the two proteins were co-expressed in BHK cells. A gold standard clotting assay and statistical analysis were then used to investigate any changes in FVIII functional activity when FVIII was co-expressed with VHH.

Overall, a series of molecular, microbiological, cell biology, analytical and assay development techniques were utilised in order to achieve these aims in a stepwise fashion and will be described in detail in this chapter.

2.2 Plasmid production

In the present study, three Camelid antibody VHH ligands were tested, ligand 2, ligand 6 and ligand 7 respectively. For each ligand, three unique constructs were designed, with a combination of features in their gene sequence such as an immunoglobulin leader sequence, a histidine tag and KDEL (Endoplasmic Reticulum retrieval motif) (see figure 2.1 and table 8). Each of these constructs was cloned into a pcDNA3.1 vector (Invitrogen) ready for transfection into mammalian cell lines.

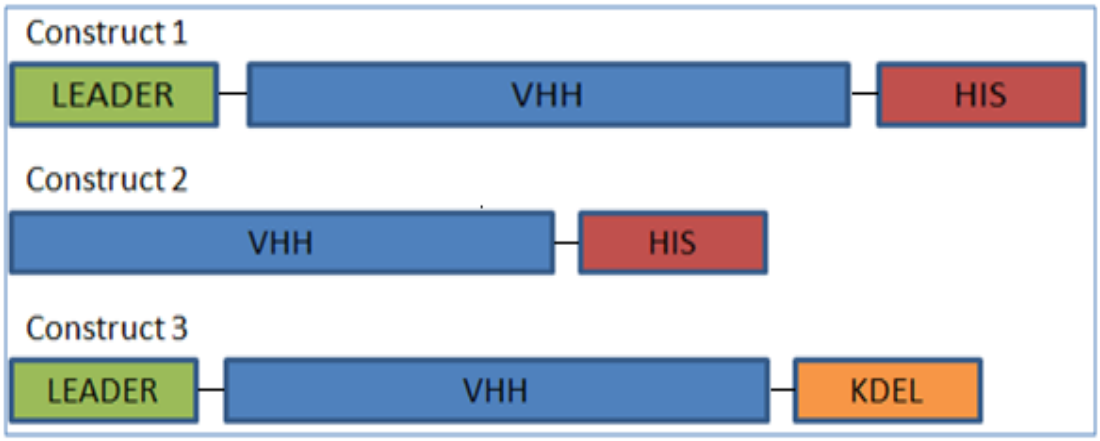


Figure 2.1. VHH construct design.

Construct 1 (C1) contains an immunoglobulin leader sequence from the commercialised monoclonal antibody herceptin (LEADER) and a histidine tag (HIS). Construct 2 (C2) contains a histidine tag (HIS). Construct 3 (C3) contains an immunoglobulin leader sequence from the commercialised monoclonal antibody herceptin (LEADER) and an endoplasmic reticulum retrieval motif (KDEL).

Table 8 Design elements of VHH constructs

Construct number	**1**	**2**	**3**
Leader sequence	Yes	No	Yes
HIS tag	Yes	Yes	No
KDEL	No	No	Yes
Secreted extracellularly?	Yes	No	Maybe

For simplification, each ligand construct was given an abbreviated name, for example, ligand 2, construct 1 was abbreviated to L2C1, as shown in table 9.

Table 9. Naming of plasmids.

	Ligand 2	**Ligand 6**	**Ligand 7**
Construct 1	L2C1	L6C1	L7C1
Construct 2	L2C2	L6C2	L7C2
Construct 3	L2C3	L6C3	L7C3

2.2.1 Primer design

The Camelid antibody gene sequences and partial template DNA were provided by BAC to the University of Kent for use in this project under a confidentiality agreement, and consequently this thesis will not disclose these gene sequences.

The template DNA that BAC supplied was in fact a digested fragment that had to be 'rebuilt' in order to produce the gene encoding a fully functional VHH protein. BAC had digested the VHH template DNA from a plasmid vector using two restriction enzyme sites (*PstI x BstEII).* This meant that the first 15 nucleotides were missing from the full VHH gene sequence and therefore primers were designed in such a way to 'rebuild' the full gene sequence using PCR. Forward primers, incorporated an 'ATATA' clamp, a restriction enzyme cutting site (*BamH1*), a translation initiation codon, a leader sequence (for constructs 1 and 3 only), the first 21 nucleotides of the template DNA sequence and the 15 'missing' nucleotides that had been digested from the template provided by BAC.

Reverse primers incorporated an 'ATATA' clamp, a restriction enzyme cleavage site (*EcoR1*), a translation termination codon, the last 36 nucleotides of the template DNA and either a HIS tag (constructs 1 and 2 only) or KDEL (construct 3 only). The reverse primer was ordered as the reverse complement of the desired PCR product.

Forward and reverse primers were designed manually and supplied by Eurofins.

Each Camelid VHH gene of interest was supplied by BAC BV, Netherlands and was cloned into the vector pcDNA3.1 (Invitrogen V790-20/V795-20). The following considerations were taken into account when designing primers for cloning of each gene.

1. Kozak consensus sequence (G/A)NNATGG which is needed to increase the likelihood of successful translation initiation
2. Translation termination codon TCTAGA
3. +/- immunoglobulin leader sequence from the monoclonal antibody 'Herceptin'
4. +/- endoplasmic reticulum retrieval motif.
5. Restriction enzymes sites were carefully selected so that they did not disrupt the gene of interest or any vital sections of the plasmid vector for gene expression.

2.2.1.1 Ligand 2 primers

Ligand 2, construct 1 (L2C1):

Forward primer format: ATATA/*BamH1*/START/LEADER SEQ/VHH

Sequence: ATATA GGA TCC ATG TCT GTC CCC ACC CAA GTC CTC GGA CTC CTG CTG CTG TGG CTT ACA GAT GCC AGA TGC CAA GTT CAA CTT CAA GAG AGT GGG GGA GGC TTG GTG

Reverse primer format: VHH/H6 tag/STOP/*EcoR1*/ATATA

Sequence: ATATA GAA TTC TTA GTG ATG GTG ATG GTG ATG AGA AGA AAC AGT AAC CTG GGT CCC CTG GCC CCA GTA

Ligand 2, construct 2 (L2C2):

Forward primer format: ATATA/*BamH1*/START/VHH

Sequence: ATATA GGA TCC ATG CAA GTT CAA CTT CAA GAG AGT GGG GGA GGC TTG GTG

Reverse primer format: VHH/H6 tag/STOP/*EcoR1*/ATATA

Sequence: ATATA GAA TTC TTA GTG ATG GTG ATG GTG ATG AGA AGA AAC AGT AAC CTG GGT CCC CTG GCC CCA GTA

Ligand 2, construct 3 (L2C3):

Forward primer format: ATATA/*BamH1*/START/LEADER SEQ/VHH
Sequence: ATATA GGA TCC ATG TCT GTC CCC ACC CAA GTC CTC GGA CTC CTG CTG CTG TGG CTT ACA GAT GCC AGA TGC CAA GTT CAA CTT CAA GAG AGT GGG GGA GGC TTG GTG

Reverse primer format: VHH/KDEL/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA AAG TTC ATC TTT AGA AGA AAC AGT AAC CTG GGT CCC CTG GCC CCA GTA

2.2.1.2 Ligand 6 primers

Ligand 6, construct 1 (L6C1):
Forward primer format: ATATA/*BamH1*/START/LEADER SEQ/VHH
Sequence: ATATA GGA TCC ATG TCT GTC CCC ACC CAA GTC CTC GGA CTC CTG CTG CTG TGG CTT ACA GAT GCC AGA TGC CAA GTT CAA CTT CAA GAG AGT GGG GGA GGA TTG GTG

Reverse primer format: VHH/H6 tag/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA GTG ATG GTG ATG GTG ATG AGA AGA AAC AGT AAC CTG AGT CCC CTG GCC CCA GTA

Ligand 6, construct 2 (L6C2):
Forward primer format: ATATA/*BamH1*/START/VHH
Sequence: ATATA GGA TCC ATG CAA GTT CAA CTT CAA GAG AGT GGG GGA GGA TTG GTG

Reverse primer format: VHH/H6 tag/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA GTG ATG GTG ATG GTG ATG AGA AGA AAC AGT AAC CTG AGT CCC CTG GCC CCA GTA

Ligand 6, construct 3 (L6C3):
Forward primer format: ATATA/*BamH1*/START/LEADER SEQ/VHH

Sequence: ATATA GGA TCC ATG TCT GTC CCC ACC CAA GTC CTC GGA CTC CTG CTG CTG TGG CTT ACA GAT GCC AGA TGC CAA GTT CAA CTT CAA GAG AGT GGG GGA GGA TTG GTG

Reverse primer format: VHH/KDEL/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA AAG TTC ATC TTT AGA AGA AAC AGT AAC CTG AGT CCC CTG GCC CCA GTA

2.2.1.3 Ligand 7 primers

Ligand 7, construct 1 (L7C1):
Forward primer format: ATATA/*BamH1*/START/LEADER SEQ/VHH
Sequence: ATATA GGA TCC ATG TCT GTC CCC ACC CAA GTC CTC GGA CTC CTG CTG CTG TGG CTT ACA GAT GCC AGA TGC CAA GTT CAA CTT CAA GAG TCT GGG GGA GGC TTG GTG

Reverse primer format: VHH/H6 tag/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA GTG ATG GTG ATG GTG ATG AGA AGA AAC AGT AAC CTG GGT CCC CTG GCC CCA GGT

Ligand 7, construct 2 (L7C2):
Forward primer format: ATATA/*BamH1*/START/VHH
Sequence: ATATA GGA TCC ATG CAA GTT CAA CTT CAA GAG TCT GGG GGA GGC TTG GTG

Reverse primer format: VHH/H6 tag/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA GTG ATG GTG ATG GTG ATG AGA AGA AAC AGT AAC CTG GGT CCC CTG GCC CCA GGT

Ligand 7, construct 3 (L7C3):
Forward primer format: ATATA/*BamH1*/START/LEADER SEQ/VHH

Sequence: ATATA GGA TCC ATG TCT GTC CCC ACC CAA GTC CTC GGA CTC CTG CTG CTG TGG CTT ACA GAT GCC AGA TGC CAA GTT CAA CTT CAA GAG TCT GGG GGA GGC TTG GTG

Reverse primer format: VHH/KDEL/STOP/*EcoR1*/ATATA
Sequence: ATATA GAA TTC TTA AAG TTC ATC TTT AGA AGA AAC AGT AAC CTG GGT CCC CTG GCC CCA GGT

2.2.2 PCR

In order to produce each VHH construct ready for cloning into a pcDNA3.1 vector, PCR experiments were carried out using the primers previously designed.

2.2.2.1 PCR for ligand 2, constructs 1, 2 and 3.

Each PCR experiment was set up as a 50 µL reaction. Initially Taq DNA polymerase was used according to manufacturer's instruction (Roche). For specific reaction conditions see table 10.

Table 10. Typical PCR Reaction set up for ligand 2 using Taq DNA polymerase.

Reagent	**Stock concentration.**	**Final concentration.**	**Vol. (µL) in 50 µL reaction**
Filtered MilliQ H_20			30.05
PCR reaction buffer	10 x	1 x	5
dNTP mix	10 mM	0.2 mM	1
Forward primer	2 µM	0.2 µM	5
Reverse primer	2 µM	0.2 µM	5
DNA (ligand 2)	2.9 ng/µL	10 ng	3.45
Taq DNA polymerase	5 U/µL	2.5 U/reaction	0.5

A negative control was run alongside all PCR reactions with no DNA template present, and the volume of H_2O increased to ensure a total reaction volume of 50 µL.

Cycle conditions for Taq Polymerase:

1. 94 °C for 2 minutes (denaturation)
2. 25 cycles
    a. 94 °C for 30 seconds (denaturation)
    b. 60 °C for 60 seconds (annealing)
    c. 72 °C for 60 seconds (extension)
3. Hold at 4 °C

Each PCR product was purified using a PCR purification kit according to the manufacturer's instructions (QIAGEN) and then run on a 1.5 % agarose gel. The gel showed smeared bands at 250 bp, 500 bp and 1000 bp indicating the presence of primer-dimers and other unspecific by-products (see figure 3.2, panel A) and therefore an alternative enzyme was sought in order to improve the quality of the PCR product.

A proofreading enzyme, Platininum *pfx* DNA polymerase (Invitrogen) was tested as an alternative to *Taq* Polymerase. The reaction conditions used are shown in table 11.

Also an additional reaction was run with an 'enhancer' which was suggested in the Invitrogen protocol to lower the melting temp of the primer which is good for long primers. In this instance, 5 µL of 10 x enhancer was used to give a final concentration of 1 x enhancer. In this instance the MilliQ H_2O was reduced by 5 uL.

Cycle conditions for Platinum® Pfx:

1. 94 °C for 5 minutes (denaturation)
2. 30 cycles
    a. 94 °C for 15 seconds (denaturation)
    b. 55 °C for 30 seconds (annealing)
    c. 68 °C for 30 seconds (extension)
3. Hold at 4 °C

Table 11. Typical PCR reaction set up for ligand 2 using Platinum® Pfx DNA polymerase

Reagent	**Stock concentration.**	**Final concentration.**	**Vol. (µL) in 50 µL reaction**
Filtered MilliQ H_20			23.65
Pfx amplication buffer	10 x	1 x	5
dNTP mix	10 mM	0.3 mM	1.5
$MgSO_4$	50 mM	1 mM	1
Forward primer	2 µM	0.3 µM	7.5
Reverse primer	2 µM	0.3 µM	7.5
DNA (ligand 2)	2.9 ng/µL	10 ng	3.45
Platinum® Pfx	2.5 U/µL	1 U/reaction	0.4

PCR products were purified using a PCR purification kit according to manufacturer's instructions (QIAGEN) before running products on a 1.5 % agarose gel. Bands at the expected sizes were excised using a scalpel and a UV light box and the DNA purified using a gel purify kit (QIAGEN). The same conditions were used to successfully amplify each of L2C1, L2C2 and L2C3.

2.2.2.2 PCR for ligand 6, constructs 1, 2 and 3.

Platinum® Pfx was used to amplify each of the constructs 1, 2 and 3 for ligand 6 in the same way as ligand 2 constructs (see table 12 for reaction set up). The PCR cycle conditions were identical to those used for amplification of ligand 2 constructs.

Table 12. Typical PCR reaction set up for ligand 6 using Platinum® Pfx.

Reagent	Stock concentration.	Final concentration.	Vol. (µL) in 50 µL reaction
Filtered MilliQ H20			24.5
Pfx amplication buffer	10 x	1 x	5
dNTP mix	10 mM	0.3 mM	1.5
$MgSO_4$	50 mM	1 mM	1
Forward primer	2 uM	0.3 uM	7.5
Reverse primer	2 uM	0.3 uM	7.5
DNA (ligand 6)	6.25 ng/µL	10 ng	1.6
Platinum® Pfx	2.5 U/µL	1 U/reaction	0.4

The PCR products were purified as described previously above.

2.2.2.3 PCR for ligand 7, constructs 1, 2 and 3.

Based on results for ligand 2 and 6, initial PCR reactions for ligand 7 were carried out using Platinum® Pfx, however this produced no PCR product. Several optimisation steps were carried out including varying the $MgCl_2$ concentration, running the reaction with and without enhancer, changing primer concentrations, adjusting the annealing temperature, none of which proved successful. A new polymerase enzyme was tested, Phire Hot Start DNA Polymerase (Finnzymes, NEB) which is only activated once the PCR reaction begins to heat up. See table 13 for typical reaction set up.

Table 13. Typical PCR reaction set up for ligand 7 using Phire Hot Start DNA polymerase.

Reagent	Stock concentration.	Final concentration.	Vol. (µL) in a 50 µL reaction
Milli Q H20			10.1
PCR reaction buffer	5 x	1 x	10
dNTP mix	10 mM	0.2 mM	1
Forward primer	2 µM	0.5 µM	12.5
Reverse primer	2 µM	0.5 µM	12.5
DNA (ligand 7)	6.9 ng/µL	20 ng	2.9
Phire Hot Start		1 unit	1

A negative control was run alongside each PCR experiment where no DNA was added and the Milli Q H_2O was increased to 13 µL.

Cycle conditions for Phire Hot Start DNA polymerase:

1. 98 °C for 1 minute (denaturation)
2. 30 cycles
    a. 98 °C for 5 seconds (denaturation)
    b. 70 °C for 5 seconds (annealing)
    c. 72 °C for 10 seconds (extension)
3. 72 °C for 1 minute
4. Hold at 4 °C

The PCR products were purified as described previously above.

2.2.3 Restriction digest of purified PCR products

The PCR products were double digested using restriction enzymes *BamH1* and *EcoR1* according to manufacturers' instructions (Promega) which included 3

incubation steps; 37 °C for 1 hour, 65 °C for 15 minutes and hold at 4 °C (see table 14) .

Table 14. Typical reaction set up for restriction digest of PCR products.

Reagent	Amount
MilliQ H_2O	15.23 µL
Buffer E	2 µL
Acetylated BSA	0.2 µL
DNA (concentration 638 µg/mL)	1.57 µL (1 µg)
BamH1	0.5 µL
EcoR1	0.5 µL
Total volume	**20 µL**

These products were then separated using agarose gel electrophoresis and the DNA purified using a Gel Extraction Kit according to manufacturer's instructions (QIAGEN). The plasmid vector pcDNA3.1 was also cut with the same enzymes (*BamH1* and *EcoR1*) according to manufacturer's instructions, run on an agaroser gel and then purified using a Gel Extraction Kit ready to be ligated with the digested and purified PCR products.

2.2.4 Ligation of pcDNA3.1 and PCR products (VHH)

For six out of the nine gene inserts (L2C2, L2C3, L6C1, L6C2, L6C3 or L7C1), an overnight ligation was carried out using ligase enzyme (Promega), the digested plasmid pcDNA3.1 and one of the respective gene inserts.

An equation was used to calculate how much plasmid and gene insert should be used in each ligation reaction:

$$\frac{\text{ng of vector} \times \text{kb of insert}}{\text{Kb size of vector}} \times \text{molar ratio vector: insert} = \text{ng of insert}$$

The following is a typical reaction set up, in this instance for L6C1:

$$\frac{\text{100 ng vector}}{\text{5.4 Kb vector}} \times \frac{\text{0.45 Kb gene}}{} \times 3{:}1 = 24.9 \text{ ng of insert}$$

If the concentration of the plasmid vector pcDNA3.1 =14 µg/ml, and the concentration of the gene insert L6C1 = 7.6 µg/ml, the reaction required:

100 ng vector DNA = 7.14 µL
24.99 ng L6C1 DNA = 3.3 µL
H_2O = 7.56 µL
Total reaction volume = 18 µl

Once the reaction mixture was assembled, the reaction was heated to 45°C for 5 minutes, and then cooled immediately on ice. Once cooled, 2 µL of buffer and 0.5 µL ligase enzyme was added. The reaction was incubated at 4°C overnight.

In order to inactivate the ligase enzyme, the reaction mixture was heated to 65 °C for 10 minutes, and then the sample could be frozen at -20 °C until needed, or used immediately.

2.2.5 TA cloning

For three of the nine VHH constructs (L2C1, L7C2 and L7C3), the overnight ligation procedure described above resulted in unsuccessful transformations in *E. coli*, therefore a TA cloning® kit (Invitrogen) was used according to the manufacturer's instructions. TA cloning® is a one-step cloning process which does not require the restriction digest of PCR products prior to ligation into a suitable vector. When carrying out a PCR reaction using Taq polymerase, PCR products would be produced that have a single adenosine at the 3'-end (overhangs). These can then be cloned into a linearized plasmid vector that possesses a 3'-T overhangs on both ends, resulting in complementarity between the PCR product 3'-A overhangs and vector 3'-T overhangs (Zhou and Gomez-Sanchez 2000). However in this case the PCR products were generated using Platinum® Pfx, resulting in blunt ends that are not suitable for

TA cloning. Instead, a single adenosine is added to each of the PCR products as 'overhangs' and then a ligation reaction carried out with a plasmid vector that has 'T' overhangs.

Each of the PCR products were treated with Taq polymerase in order to add a 3'-A overhang which would complement the 3'-T overhang of plasmid pCR2.1 used in TA cloning as per the Invitrogen protocol. Table 15 describes a typical reaction set up for a TA cloning ligation.

Table 15. Typical TA cloning ligation reaction set up for L2C1.

Reagent	Amount (µl)
Milli Q H_20	5.24
10 x Ligation buffer	1
pCR2.1 vector (25 ng/µl)	2
PCR product	0.76
Ligase	1

For TA cloning ligation the insert:vector molar ratio was recommended 1:1 for the best efficiency. The reaction was incubated at 14 °C overnight as per the Invitrogen protocol. Ligated plasmids were transformed into DH5α competent *E. coli* cells (using method 2.2.6). Plasmids were then purified from these cells using a miniprep kit (method 2.2.7). The insert was then digested out of the TA vector using *BamH1* and *EcoR1* and ligated into pcDNA3.1 using the ligation procedure as detailed in section 2.2.4.

2.2.6 pcDNA3.1 transformation into XL-1 blue competent cells

Initially commercially available XL-1 blue competent cells (Stratagene) were used for transformation experiments. However, it proved difficult to purify high enough DNA levels from either a miniprep or a maxiprep kit (QIAGEN) for transfection into mammalian cells so an alternative strain was sought. A strain of DH5α competent

cells was obtained from Mark Smales lab (University of Kent) and these were used to generate a large batch of competent cells for all transformation experiments. In order to prepare a large batch of competent cells, *E. coli* was grown on antibiotic free LB agar plates at 37 °C overnight. A single colony was selected from the plate using sterile technique and grown as a starter culture in 2 ml LB media without antibiotics in a 15 ml Falcon tube at 37 °C overnight, with shaking at 200 rpm. Sterile cryotubes were chilled on ice in preparation for the cells. 40 ml of fresh LB media (no antibiotics) was inoculated with 0.5 ml of the starter culture and incubated at 37 °C with shaking at 200 rpm. The OD_{600} was measured in a 1 ml cuvette (Fisher Scientific) using LB media as a blank. The OD was expected to be between 0.3-0.5 when ready and this took approximately 2-3 hours. Once the OD reached the desired value, the cells were centrifuged in a sterile 50 ml Falcon tube at 6000 g in a table-top centriguge for 5 minutes at room temperature. The supernatant was discarded before re-suspending the pellet in 20 ml of sterile 50 mM $CaCl_2$ and storing on ice for at least 30 minutes. The cells were centrifuged again at 6000 g for 5 minutes and the supernatant discarded. The cells were re-suspended in 4 ml of ice cold 50 mM $CaCl_2$ and stored on ice for at least 30 minutes. After this the cells were deemed to be competent and ready for transformation experiments. The cells were diluted in LB media containing 15 % glycerol for storage at -70 °C. All nine plasmids were transformed successfully and propagated in these DH5α cells.

Prior to transformation, the DH5α cells were thawed on ice and gently mixed before aliquoting 100 µl of cells into pre-chilled 15 ml Eppendorf tubes. 25 ng of DNA was added (or no DNA for negative control) to the cells and the tubes swirled gently. The tubes were incubated on ice for 30 minutes before heat-pulsing the tubes in a 42 °C water bath for 45 seconds. The tubes were then incubated on ice for 2 minutes before adding 0.9 ml of preheated SOC medium and incubating at 37 °C for 1 hour with shaking at 200 rpm. The transformation mixture was spread on to LB agar plates using sterile glass beads and grown overnight at 37 °C.

The host vector pcDNA3.1 carries the gene for Ampicillin resistance so transformed cells were selected in the presence of Ampicillin at 50 µg/ml.

2.2.7 Miniprep and restriction digest

Single clones were picked from successfully transformed *E.coli* cells growing on LB agar plates using sterile swabs. Each colony was grown up in a 15 ml Eppendorf tube containing 5 ml LB media, for 12 hours at 37 ºC with shaking at 200 rpm. Plasmids were then purified using a QIAprep Spin Miniprep Kit according to manufacturer's instructions (QIAGEN). The DNA was digested using *BamH1* and *EcoR1* (see table 16 for reaction set up) and run on a 1.5 % agarose gel to check for the presence of the Camelid antibody insert. Samples that showed the appropriate band size for the Camelid insert when run on a gel (for example. 450 bp for construct 1), were sent off for sequencing at either CoGenics or GATC Biotech.

Table 16. Restriction enzyme digest reaction set up (L2C3 construct).

Reagent	Volume (µl)
H_20	6.8
Buffer E (see appendix 5.4)	2
BSA (10 µg/µl)	0.2
DNA	10 (1 µg)
BamH1 (10 u/µl)	0.5
EcoR1(12 u/µl)	0.5

2.2.8 Sequencing

L2C2, L2C3, L6C1-C3 and L7C1 plasmids were sequenced by CoGenics using universal primer pairs T7P and BGHR. L2C1, L7C3 and L7C2 plasmids were sequenced by GATC Biotech using a T7 primer only as their sequencing process includes up to 1000 nucleotides.

All sequencing data was analysed using a website SDSC biology workbench http://workbench.sdsc.edu/. Primer pair data was aligned using BL2SEQ which compares nucleotides to each other using BLAST. The forward primer and the desired construct sequence were aligned using BL2SEQ. All sequence data was blasted against the entire expected gene sequence to check for 100 % homology.

2.2.9 Maxiprep

After confirmation of the correct plasmid sequence, *E. coli* containing the correct VHH plasmid was grown up in 100 ml LB media ready for purification using an Endofree Maxiprep kit according to manufacturer's instructions (QIAGEN).The Endofree Maxiprep kit removes endotoxin, which is a component of bacterial cell walls, from plasmid DNA preparations. If not removed, these endotoxins can have a negative impact on transfection efficiency and cell viability in mammalian cells upon transfection of plasmid DNA (Butash, Natarajan et al. 2000).

Purified DNA stocks were stored at -20 °C and 15 % glycerol stocks of the bacteria containing the plasmid with the correct VHH gene sequence were prepared and stored at -80 °C for future use.

2.2.10 Plasmid linearization

All VHH plasmids were linearised using restriction enzyme *Sca1* according to manufacturer's instructions (Fermentas) prior to transfection into mammalian cells (see table 17). It has been demonstrated that transfecting mammalian cells with a linearised plasmid as opposed to an intact plasmid can improve levels of protein expression (Stuchbury and Münch 2010).

Sca1 cleaves DNA in the Ampicillin resistance site at nucleotide 4984 of the pcDNA3.1 vector which is not needed for mammalian transfection experiments, and none of the inserted VHH genes contained *Sca1* restriction sites.

Table 17. Typical reaction set up for linearising 8µg plasmid DNA:

Reagent	Volume (200 µl reaction)
DNA	16 µL (8 µg of L2C1 plasmid at 485 µg/ml)
Sca1 enzyme (10 U/µL)	0.8 µL (8 units)
Buffer H (see appendix 5.4)	20 µL
Milli Q H_2O	163.2 µl

Restriction digests using *Sca1* enzyme were incubated at 37 °C for 1 hour.

The DNA was then purified using a Fermentas PCR purification kit (up to 25 µg DNA can be loaded on to each column). To cut 50 µg of plasmid DNA the volumes were increased as follows: 10 µl enzyme, 100 µl buffer H and make up to 1 ml with H_2O. This reaction was incubated for 1 hour at 37 °C (as advised by Fermentas technical support).

2.2.11 Full length FVIII plasmid

PcDNA3.1 plasmids containing the full length human FVIII gene were available in-house from previous work that had been done in the Peter Nicholls lab (University of Kent). For this project, these plasmids were transformed into DH5α cells using the same method as described in section 2.2.6. The host vector pcDNA3.1 carries the gene for Ampicillin resistance so transformed cells were selected in the presence of Ampicillin at 100 µg/ml. Selected colonies were grown up in 100 ml LB media ready for purification using an Endofree Maxiprep kit according to manufacturer's instructions (QIAGEN) and as described in section 2.2.9. Purified DNA stocks were stored at -20 °C and 15 % glycerol stocks of the bacteria containing the plasmid were prepared and stored at -80 °C for future use.

2.3. Protein expression studies in mammalian cells

Mammalian cells were used as a biological factory to express VHH and FVIII proteins, initially in separate cell lines. They were also used to co-express FVIII and VHH proteins in the same cell line, to assess the effect of co-expression on FVIII protein expression levels.

2 approaches were adopted in order to test the effect of co-expressing VHH with FVIII on FVIII expression levels:

1. Create a cell line stably expressing VHH and transiently transfect in the FVIII gene
2. Use (or create) a cell line stably expressing FVIII and transiently transfect in the VHH gene

A summary of experiments carried out can be seen in table 18.

Table 18. Summary of mammalian cell lines and their application in the current study

Cell line → / **Protein ↓**	**CHO-K1 cell line (ECACC)**	**BHK BDD-FVIII cell line (Pete Lollar)**	**HEK cell line (ECACC)**
Full length FVIII	Transient transactions to produce FL-FVIII (with the aim of producing a new stable cell line if the transient transfection worked)	N/A	Transient transfections to produce FL-FVIII (with the aim of producing a new stable cell line if the transient transfection worked)
B domain deleted FVIII	N/A	Stable cell line available at the start of the project used for co-expression studies	N/A
VHH	Transient transfection and stable cell line development	Transient transfection to co-express BDD-FVIII and VHH	Transient transfection (with the aim of producing a new stable cell line if the transient transfection worked)

Three cell lines were available in-house (University of Kent) for use in this project. One of which was a Baby Hamster Kidney cell line (BHK) which stably expressed B-domain deleted FVIII (BDD-FVIII) and was kindly provided by John S. (Pete) Lollar, III, M.D., Atlanta, USA. The other cell lines available included a Chinese Hamster Ovary cell line (CHO-K1) originally obtained from ECACC, and a Human Epithelial Kidney (HEK) cell line that was donated by Mike Geeves of the University of Kent (originally from ECACC).

The BHK cell line was originally developed by the Pete Lollar laboratory in the USA to stably express B-Domain Deleted FVIII to investigate the effect of removing the B domain on the function and expression of the FVIII protein. In this project, this cell line was transiently transfected with VHH constructs, and the effect on FVIII protein expression analysed.

The CHO-K1 cell line was used to test if the VHH constructs would be expressed as proteins and a number of transfection techniques were utilised in this process. The potential of these CHO-K1 cells to develop a stable cell line expressing VHH was also investigated, with the aim of using this cell line to transiently transfect FVIII and assess the effect of co-expression on FVIII production. The CHO-K1 cells were also used to transiently transfect a full length FVIII plasmid with the aim of producing a new stable cell line if the transient expression proved successful. A stable full length FVIII cell line could then be used to co-express VHH and assess the effect on FVIII expression levels.

The HEK cell line was transiently transfected with a plasmid encoding the full length FVIII gene, with the aim of developing a stable full length FVIII cell line that could be transiently transfected with the VHH constructs, and then any effect on FVIII protein expression analysed. HEK cells were also transiently transfected with VHH plasmids since BAC had previously had some limited success with the expression of their VHH proteins in this cell line.

A number of analytical techniques were utilised to investigate expression levels of FVIII and VHH in BHK, CHO-K1 and HEK cell lines respectively, at both the mRNA and protein level.

2.3.1 Cell culture

2.3.1.1. Reagents and consumables

Tables 19 and 20 give a summary of all cell culture reagents and consumables used in the mammalian cell biology experiments.

Table 19. Reagents for cell culture

Item	Supplier	Code	Amount
CHO-K1 cells	ECACC	85051005	1 ml
DMEM/F-12	Gibco	21331020	500 mls
Hepes	Sigma- Aldrich	H3375	100 g
L-Glutamic acid	Sigma-Aldrich	G8415	100 g
Dialysed Foetal Bovine Serum	Invitrogen	26400-044	500 mls
Penicillin/Streptomycin	Invitrogen	15140-122	100 mls
AIM-V media	Invitrogen	12055-083	1 L
L-Asparagine	Sigma-Aldrich	A4159	25 g
Adenosine	Sigma-Aldrich	A4036	5 g
Guanosine	Sigma-Aldrich	G6264	1 g
Cytidine	Sigma-Aldrich	C4654	1 g
Uridine	Sigma-Aldrich	U3003	5 g
Thymidine	Sigma-Aldrich	T1895	1 g
Phosphate Buffered Saline	OXOID	BR0014G	100 tablets
0.25 % Trypsin –EDTA	Gibco	25200-056	100 ml
Genetecin	Invitrogen	10131-019	20 ml
TurboFect™ transfection reagent	Thermo scientific	R0531	1 ml
Lipofectamine® LTX	Invitrogen	15338-100	1 ml
Polyplus jetPEI®®	Source Bioscience	101-01	0.1 ml

Table 20. Materials and equipment for cell culture.

Item	Supplier
6 well cell culture plates	Sigma-Aldrich
T-25 cm^2, T-75 cm^2 and T-175 cm^2 vented tissue culture flasks	Sarstedt
Sterile serological pipettes 1 ml, 2 ml, 5 ml, 10 ml, 25 ml, 50 ml	Sarstedt
Sterile pipette tips 1 ml, 200 µl, 20 µl	Fisherbrand
Eppendorf pipettes	Sigma-Aldrich
BioAir Aura 2000 M.A.C. Class II Safety Cabinet	Bioair Instruments, Italy
Cell culture incubator	Panasonic
Waterbath	Thermoscientific
Gene Pulser Xcell total system	Bio-Rad Laboratories
Gene Pulser cuvettes 0.2 cm gap (50)	Bio-Rad Laboratories
1.5 ml Eppendorf microcentrifuge tubes	Sigma-Aldrich
0.22 uM Millipore express 500 ml funnel/receiver bottle	Fisher

2.3.1.2 Cell culture growth media

All cell culture media ingredients were combined using sterile pipettes in a Class II safety cabinet, then sterile filtered using a vacuum pump and a 0.22 µM Millipore express 500 ml funnel/receiver bottle. For composition details see appendix.

2.3.1.3 Resuscitating cells

Mammalian cells were resuscitated from a liquid nitrogen-filled cryostat and thawed quickly using a water bath at 37 °C. The thawed cells were diluted in 10 ml media warmed to 37 ºC and centrifuged at 150 g. The supernatant was removed in order to remove any residual DMSO. The cell pellet was then resuspended in 10 ml media

warmed to 37 °C. All cells were then placed into a T-25 flask in an incubator at 37 °C with 5 % CO_2. Cells were left to grow for 2-3 days until confluent and then sub-cultured into a T-75 flask for future experiments.

2.3.1.4 Sub-culture of mammalian cells

Cells were sub-cultured approximately every 48 hours, once cells reached approximately 90 % confluency. The correct seeding cell density and timings for sub-culture of cells was defined for each cell line to ensure consistency across experiments. For each cell line, cells were seeded at a variety of densities to find the optimal growth conditions for future experiments. For example in table 21, a cell density experiment was conducted with BHK-FVIII cells. Cells seeded at 1.5 x 10^6 in a T75 cell culture flask reached approximately 90 % confluency within 48 hours, at which point the media was changed from DMEM/F12 to serum free AIM-V. The concentration selected meant that each co-expression study could be conducted within the space of 3 days which is a practical amount of time for managing laboratory work that requires multiple repetitions. It also meant that cells were left too long in the same media as this may lead to nutrient depletion and a decrease iin viability of the cells. Cell media was sampled later for use in the coagulation experiments that measured FVIII activity. Although subjective, this approach of changing the cell media when the cells were at 90 % confluency ensured a consistent approach and approximately the same number of cells in the flask every time the AIM-V media was added.

To sub-culture cells, all cell media was removed from the flask before washing cells with sterile PBS warmed to 37 °C. Sterile PBS was removed before addition of 2 ml of trypsin (room temperature). Cells were incubated for 2-3 minutes at 37 °C. 10 mls cell culture media was added to the cells in order to neutralise the trypsin. Using a sterile pipette, cells were resuspended in order to break up any cells that had clumped together. Between 0.5 ml to 1 ml of counted cells were transferred into a new T-75 cell culture flask containing 20 mls of cell media and placed in an incubator at 37 °C with 5 % CO_2.

Table 21. Cell density experiment (BHK-FVIII cells)

Day 1 cell density:	Confluency (%) at Day 2 (+ 24 hrs)	Confluency (%) at Day 3 (+ 48 hrs)	Confluency (%) at Day 4 (+ 72 hrs)	Confluency (%) at Day 5 (+ 96 hrs)
$1x10^5$	<10 %	20 %	30 %	60 %
$5x10^5$	10-20 %	50 %	90 % (switch media to AIM-V)	100%
$1x10^6$	20-30 %	60 %	90 % (switch media to AIM-V)	100%
$1.5x10^6$	60-70 %	90 % (switch to AIM-V)	100%	Not viable (cells clumped)
$2x10^6$	70-80 %	Not viable (cells clumped)	Not viable (cells clumped)	Not viable (cells clumped)

2.3.2 Transient transfections

Transient transfection was used as a method of introducing either VHH or FVIII genes into a mammalian cell line.

VHH: plasmids encoding the VHH constructs were initially carried out in CHO-K1 cells to see if the VHH proteins would be expressed. Later on in the project, VHH constructs were transiently transfected into the BHK cell line that was stably expressing B domain deleted FVIII, and the effect on FVIII expression analysed. VH proteins were also transiently transfected into HEK cells.

FVIII: The full length FVIII gene was transiently transfected into either CHO-K1 or HEK cells to see if the full length protein could be produced and function in a gold standard coagulation assay (described in 2.4) If the transient transfection worked then the aim was to produce a stable cell line which could then be transfected with VHH and the effect on FVIII expression levels analysed.

Two different methods were tested and optimised for transfection experiments; either chemical transfection or electroporation.

For all transfection experiments, a mock transfection was carried out (cells plus transfection reagents, no DNA), as well as a control transfection (plasmid containing no gene of interest), to make sure that any effects on protein expression were due to the gene of interest and not the transfection reagents.

2.3.2.1 Transfection using electroporation (CHO-K1 example)

This procedure required a Gene Pulser Xcell main unit and CE Module. Firstly, electro-competent cells were prepared as follows for the CHO-K1 cells:
Cells were seeded into a T-75 flask ready for transfection after 2 days (70 % confluent). After 2 days the growth media was removed from the flask and cells were washed using 5 ml of phosphate buffered saline (PBS) pre-warmed to 37 ºC. The PBS was removed and 2 ml of trypsin was added before incubating the cells 2–3 minutes at 37 °C. The flask was then tapped gently to detach the cells from the surface. The trypsin was neutralised by adding 10 ml of growth media.

Cells were then counted manually using a haemocytometer, and resuspended in electroporation buffer (PBS). The cells were transferred to a 50 ml sterile centrifuge tube and centrifuged at 400 x g for 5–7 minutes at room temperature. The supernatant was discarded and then the cell pellet resuspended in PBS at a density of 5×10^6 cells/ml. The cells were gently pipetted to obtain a single-cell suspension.

The pulse conditions were predefined according to the cell line, by the manufacturer. For CHO-K1, (square wave protocol), Voltage 160, and Pulse length 15 ms were used.

CHO-K1 cells and VHH plasmid DNA (4 µg plasmid DNA per cuvette) were pre-mixed before adding to the cuvette and electroporating as per the Bio-Rad protocol. Each electroporation cuvette (0.2 cm gap) contained 5×10^6 cells in 1 ml electroporation buffer (PBS).

Once the cells had been electroporated, the cells were transferred to a 6 well plate using 0.5 ml of media and a plugged pasture pipette. Each cuvette full of cells was added to 1 well of 6 well plate containing 2 ml full growth media. The plates were rocked gently to assure even distribution of the cells over the surface of the plate, and

then incubated at 37 °C and 5 % CO_2. VHH gene expression was analysed 48 hrs following electroporation in at least one of the following ways:

(i) cells were viewed on a Leica MZFL III microscope under GFP2 filter to detect fluorescence level (for GFP transfected cells)

(ii) the cell media was collected for protein expression analysis in a dot blot or western blot

(iii) the cells were lysed for further analysis in a dot blot or western blot

2.3.2.2 Transfection using chemical methods (CHO-K1 example)

Four different reagents were tested to assess the optimal method for transfecting mammalian cells; TurboFect™ (Fermentas), Lipofectamine® LTX (Invitrogen), FuGENE® and PEI.

Initial experiments were carried out using a plasmid encoding for green fluorescent protein to determine the optimal reagent for transfecting CHO-K1 cells. The two best performing reagents were TurboFect™ and Lipofectamine® LTX. TurboFect™ is a cationic polymer which forms a stable complex with DNA, protecting the DNA from degradation and facilitating gene delivery into eukaryotic cells by endocytosis. Lipofectamine® LTX is a cationic lipid-based plasmid transfection reagent that according to the manufacturer provides the highest level of transfection efficiency in primary cells of any lipid- or polymer-based reagent. In lipid based transfection, a positively charged liposome (phospholipid bilayer) forms a complex with negatively charged DNA, which then binds to the cell membrane. The mechanism by which the DNA enters the cell has been investigated extensively and has shown that the cationic/DNA complex enters the cell by endocytosis. This causes the endosomal membrane to destabilise and this in turn induces movement of anionic lipids from the cytoplasmic-facing monolayer into the complex forming a charge neutral ion pair with the cationic lipids, displacing the DNA from the complex and releasing it into the cytoplasm of the cell (Xu and Szoka 1996).

(i) TurboFect™

Cells were seeded into a 6 well plate at a concentration of 2.4 x 10^5 cells/well with 4 mls growth media at 37 °C and 5 % CO_2 to ensure 90-95 % confluency within 24 hours. After 24 hours cells were transfected with the test DNA.

Transfections were carried out in Optimem media which is a serum free media from Invitrogen. This was pre-warmed in a 37 °C water bath.

Plasmid DNA-TurboFect™ complex solutions were prepared in sterile 2 ml Eppendorf tubes as follows:
Per well of a 6 well plate: 4 µg DNA + 400 µL Optimem + 6 µL TurboFect™.
Stock DNA concentration is 100 µg/ml, final concentration 10 µg/ml.
The DNA/TurboFect™ complex was incubated for 20 minutes at room temperature.
Meanwhile the cells previously plated in 6 well plates were washed with 2 mL PBS (pre-warmed to 37 °C) and then 4 mL of fresh growth media (without antibiotics) was added to each well, before adding the DNA/TurboFect™ complex dropwise and swirling the plates gently. The plates were incubated at 37 °C in 5 % CO_2 for 48 hours prior before detecting protein expression.

(ii) Lipofectamine® LTX

Cells were seeded into a 6-well plate at a concentration of 2 x 10^5 cells/well in 2 ml complete cell media ready for plasmid transfection into the cells 24 hours later.
Per well of a 6 well plate: 4 µg (40 µl) DNA was mixed with 210 µL fresh cell media in a sterile 2 ml Eppendorf tube. 10 µl Lipofectamine® was mixed with 240 µl fresh cell media in a separate sterile 2 ml Eppendorf tube. Both of these mixtures were incubated for 5 minutes at room temperature before combining the two together and mixing gently. This was incubated for a further 20 minutes at room temperature, and then added to cells drop wise followed by gently rocking the plate. The plate was incubated at 37 °C in 5 % CO_2 and after 48 hours, protein expression studies were carried out.

(iii) FuGENE®

Cells were seeded into a 6-well plate at a concentration of 2 x 10^5 cells/well in 2 ml complete cell media ready for plasmid transfection into the cells 24 hours later. Per well of a 6 well plate: FuGENE® was mixed directly with plasmid DNA in various ratios according to manufacturer's instructions (see table 16). The mixture was vortexed immediately before incubating at room temperature for 15 minutes. Again the mixture was vortexed briefly before adding the complex directly to the cells. The cells were then placed into a 37 ºC incubator, and then checked for GFP expression 48 hours later by viewing on a MZRFL III microscope under GFP2 filter to detect fluorescence level.

Table 22. Optimisation of transfection using FuGENE®

Well number	**1**	**2**	**3**	**4**	**5**	**6**
Ratio of FuGENE® to DNA	4:1	3.5:1	3:1	2.5:1	2:1	1.5:1
Serum free media (µL)	155	155	155	157	158	160
GFP plasmid DNA total 3 µg (µL)	33	33	33	33	33	33
Volume of FuGENE® reagent (µL)	13	12	9.9	8.3	6.6	5.0

(iv) PEI

Cells were seeded into a 6-well plate at a concentration of 2 x 10^5 cells per well in 2 ml complete cell media ready for plasmid transfection into the cells 24 hours later. Per well of a 6 well plate: 2 Eppendorf tubes were made up, 1 containing 30 µl DNA (3 µg) and 70 µl NaCl (supplied with PEI kit), and the other tube containing 6 µl of PEI (PolyPlus) and 96 µl NaCl. The two solutions were then mixed, vortexed and centrifuged briefly, before incubating for 15-30 minutes at room temperature. The mixture was then added drop wise to cells before gently swirling the plate, then incubating at 37 ºC. GFP expression was checked 48 hours later by viewing cells on a MZRFL III microscope under GFP2 filter to detect fluorescence level.

Transient transfections were also carried out in HEK cells with a plasmid encoding full length FVIII, with the aim of creating a stable full length FVIII cell line that could later be transiently transfected with VHH.

Lipofectamine® LTX (Invitrogen) was used in these experiments as described below:

Cells were seeded into a 6-well plate, at a concentration of 2 x 10^5 cells/well in 2 ml complete cell media ready for transfection 24 hours later.

Per well of a 6 well plate: 4 µg (40 µl) DNA was mixed with 210 µL fresh cell media in a sterile 2 ml Eppendorf tube. 10 µl Lipofectamine® was mixed with 240 µl fresh cell media in a separate sterile 2 ml Eppendorf tube. Both of these mixtures were incubated for 5 minutes at room temperature before combining the two together and mixing gently. This was incubated for a further 20 minutes at room temperature, and then added to cells drop wise followed by gently rocking the plate. The plate was incubated at 37 °C in 5 % CO_2 and after 48 hours, the cell media was removed and tested in a coagulation assay (as described in section 2.4).

2.3.3 Stable cell line development

Since expression of VHH protein was seen in CHO-K1 cells (albeit at very low levels), this cell line seemed a good choice to go on and try to develop stable cell lines expressing each of the nine VHH plasmids. Although the literature predominantly reports VHH expression in microorganisms such as yeast and bacteria, there are limited examples of their expression in mammalian cell lines. However there are many examples of other antibodies produced in CHO cells so this seemed a logical choice.

The aim was to produce a stable VHH cell line that could then be used for further experiments to:

1. Transiently transfect a FVIII plasmid and then test the effect of co-expression of FVIII with VHH

2. Develop a cell line stably expressing both VHH and FVIII at optimal levels for maximising FVIII production which would have therapeutic and therefore commercial value

CHO-K1 cells were transfected using Lipofectamine® as described in methods section 2.3.2. All plasmids were linearised prior to transfection experiments. 24 hours after transfecting the cells, the cell media was changed to media containing 400 µg/ml genetecin (G418) to select for those cells which had taken up the VHH plasmid which contained a neomycin resistance gene (see appendix 3 for plasmid diagram). Control cells (untransfected) were also treated with genetecin. A range of genetecin concentrations were tested (200 µg / ml to 800 µg / ml) in both transfected and untransfected cells to see if the concentration used (400 µg / ml) was cytotoxic. After 24 hours the media was changed to fresh media containing genetecin and after another 2 days the cells were reseeded into a new T-75 flask. This procedure of changing the media every 2 days and then reseeding the cells every 2 days was repeated until all of the control (un-transfected) cells were completely eliminated.

Once all the control cells were eliminated, a single cell cloning/limited dilution protocol was followed. Sub-cultured transfected cells were then counted and the cell density adjusted to 1 x 10^4 cells / ml by adding the appropriate volume of cell media. The cells were then diluted into three separate concentrations that contained an average of 0.5, 3 and 10 cells per 100 µL respectively. 96 well plates were then seeded with 100 µl cells per well. The plates were left for 1-2 hours before examining each well for those that had only a single cell per well (hence the term single cell cloning). Wells containing only a single cell were observed for several days to see if they started to proliferate. Those wells where colonies formed were sub-cultured and bulked into 24 well plates initially, and then when the cells were growing sufficiently, they were transferred to 6 well plates, then subsequently T25 flasks in order to grow enough cells to test for VHH protein expression.

2.3.4. Co-expression of VHH and FVIII in BHK cells using Lipofectamine® LTX

Conditions for transient transfection of VHH plasmids into BHK cells were firstly optimised using Lipofectamine® LTX and a plasmid coding for expression of green fluorescent protein (GFP). A 6 well plate was seeded with 2 x 10^5 cells per well. After 24 hours growth, cells were transfected with a pcDNA3.1 plasmid containing the gene for green fluorescent protein (GFP) using Lipofectamine® LTX in 2.5ml of AIM-V (serum free) media. Varying concentrations of Lipofectamine® and PLUS reagent were investigated to determine the optimal concentration for the highest transfection efficiency, as shown in table 23. Transfection efficiency was determined by viewing the cells under a MZRFL III microscope using a GFP2 filter to detect fluorescence levels. The percentage of cells expressing GFP was assessed by eye and the cells showing the highest percentage of fluorescing cells were deemed to have been transfected with the optimal Lipofectamine® and PLUS reagent concentrations. These were subsequently the concentrations used in all further transient transfection experiments in BHK cells.

Table 23. Optimisation of transfection conditions using Lipofectamine® LTX and GFP plasmid

Well of 6 well plate	DNA (µg)	Lipofectamine® LTX (µl)	PLUS reagent (µl)
1	4	7.5	0.5
2	4	10	2.5
3	4	12.5	5
4	4	15	7.5
5	4	20	8
6	4	25	10

For transfection of BHK cells using VHH plasmids, the media was changed to AIM-V (serum free) prior to the transfection to remove serum proteins that may affect the outcome of the coagulation assay to be run later. For every experiment there was a 'control' well which received no transfection reagents or DNA and was treated as the negative control for VHH expression and the baseline FVIII expression for coagulation assays.

Each well of a 6 well plate was treated as an independent transfection i.e. one transfection mixture was prepared per well. Each well contained 2 mls AIM-V media prior to transfection. The transfection mix contained 500 µl AIM-V media, 4 µg DNA, 0.5 µl PLUS reagent and this was incubated at room temperature for 5 minutes before adding 7.5 µl LTX reagent. This was incubated for 20 minutes at room temperature before adding the whole mixture drop wise to cells. The plates were gently rocked before incubating at 37 °C and 5 % CO_2 for 48 hours before analysis of protein expression and coagulation assays.

2.3.5 mRNA analysis of VHH gene expression by Reverse Transcriptase-PCR (RT-PCR)

BHK cells were analysed for the presence or absence of beta-actin mRNA (control housekeeping gene) and VHH mRNA after transfection with pcDNA3.1 plasmids containing the VHH gene. Cells were transfected as described in method section 2.3.4. Cells from one well of a 6 well plate were harvested using trypsin either 24 or 48 hours after transfection. Cells were centrifuged briefly to form a pellet before removing the supernantant and homogenising the cells using a QIAshredder (QIAGEN) and extracting the RNA using an RNeasy mini kit (QIAGEN).

RNA was quantified using a spectrophotometer and the quality of the RNA checked using the ratio of the absorbance at 260 nm and 280 nm ($A_{260/280}$). For pure RNA the $A_{260/280}$ is around 2. The RNA sample was then treated for the removal of genomic DNA using a TURBO DNase kit (Invitrogen). DNase was then removed from the RNA sample using an RNeasy mini kit (QIAGEN). RNA was then reverse transcribed into cDNA using a First Strand cDNA synthesis kit (Novagen). 1 µL of cDNA for each original RNA sample was taken through to PCR experiments in order to amplify either the VHH or beta-actin gene.

PCR experiments were carried out as described in method section 2.2.2. PCR primers for detection of VHH genes were designed as described in method section 2.2.1. Primers for the detection of the hamster beta-actin housekeeping gene were reproduced from Ley *et al* (Ley, Balmanno et al. 2003).

Beta-actin forward primer: 5' -CCGGATGCAGAAGGAGATCA-3'

Beta-actin Reverse primer: 5' -GCCACCGATCCACACAGAGTA-3'

PCR products were run on a 1 % agarose / TAE gel to assess the size of the DNA band and that it corresponded with the expected sizes of the VHH gene constructs and the beta-actin gene fragment (approximately 100 bp).

2.3.6 Protein expression analysis

In order to determine that the VHH ligands were being expressed by the cells, dot blotting and western blotting experiments were carried out. FVIII protein expression was also investigated in a similar manner.

2.3.6.1 Treatment of cells prior to western blotting/dot blotting

48 hours after all transient transfections, both cell supernatant and lysed cells were assessed for VHH expression in western blotting. Cell supernatant was collected in 50 ml Falcon tubes. Cells were harvested as described in methods section 2.3.4 and resuspended in serum free media (AIM-V). Cells were centrifuged for 5 minutes at 400 g. The cell pellet was resuspended in 0.5 ml lysis buffer (see composition in table 24) and used straight away or the sample was frozen at -20 °C until western blot.

Alternatively cells were lysed whilst still adhered to the plate by adding 200 µl cold lysis buffer (containing protease inhibitors) to each well. The plate was left on ice for 1-2 minutes with occasional rocking before using a cell scraper to loosen all of the cells. These were then collected and samples frozen at -20 °C prior to western blotting or used straight away.

Table 24. Lysis buffer composition

Reagent	Amount (to make 50 ml)
200 mM Hepes pH7.2	0.24 g
100 mM NaCl	0.294 g
1 % Triton X-100	500 µL
EDTA-free Protease inhibitor tablet (add at time of use)	1 tablet per 10 ml buffer

The lysis buffer composition was taken from Smales *et al*. Biochem Biophys Res Com 306 (2003) 1050-1055.

Prior to western blotting, samples were syringed through a 19 gauge needle to break up nucleic acids then centrifuged at 3000 x g for 10 minutes. The cell pellet was discarded and the supernatant used in the western blot.

2.3.6.2 Western blotting protocol

SDS-Polyacrylamide Gel electrophoresis (PAGE)

Electrophoresis experiments were conducted under either denaturing or non-denaturing (native) conditions.

For denaturing electrophoresis, pre-cast NuPAGE® Bis-Tris Mini Gels (Life Technologies) were used according to manufacturer's instructions. To prepare 500 ml of 20X NuPAGE® MES SDS Running Buffer, reagents were dissolved in ultrapure water as shown in table 25. The buffer was stored at 4 °C to ensure its stability for 6 months. For use in electrophoresis experiments, the buffer was diluted to 1 X with water.

Table 25. NuPAGE® MES SDS running buffer composition

Reagent	Concentration	Amount (500 ml 20 x).
MES	50 mM	97.6 g
Tris base	50 mM	60.6 g
SDS	0.1 %	10 g
EDTA	1 mM	3 g
Ultrapure water	n/a	Up to 500 ml

20 µL samples were made containing 5 µl LDS loading buffer (NuPAGE) and 15 µl of lysed cell or cell supernatant (cell media). These samples were heated at 70 °C for 10 minutes before loading on to a NuPAGE Bis-Tris gel (1 mm containing 10 wells). Lane 1 of each gel always contained a protein size marker such as BIORAD kaleidoscope marker and 10 µl of this was loaded.

XCell SureLock™ Mini-Cells were used for the electrophoresis which required 200 ml running buffer for the Upper (inner) Buffer Chamber and 600 ml running buffer for the Lower (outer) Buffer Chamber. Conditions for electrophoresis were 200 V for 35 minutes.

For non-denaturing (native) electrophoresis, pre-cast NuPAGE® Tris-Acetate Mini Gels (Life Technologies) were used according to manufacturer's instructions. Native electrophoresis also required a different running buffer (Tris-Glycine Native running buffer) which was made up to a 10 x solution and diluted in water to 1 x for use in experiments (see table 26).

Table 26. NuPAGE® Tris-Glycine Native Running Buffer composition (pH 8.3)

Reagent	Concentration	Amount (1 L 10 x).
Tris base	25 mM	98 g
Glycine	192 mM	144 g
Ultrapure water	n/a	Up to 1 L

20 µL samples were made containing 5 µl Tris-Glycine Native Sample Buffer (NuPAGE) and 15 µl of lysed cell or cell supernatant (cell media). These samples were NOT heated before loading on to a NuPAGE Tris-Acetate Mini Gel (1mm containing 10 wells). Lane 1 of each gel always contained a protein size marker such as BIORAD kaleidoscope marker and 10 µl of this was loaded.

XCell SureLock™ Mini-Cells were used for the electrophoresis which required 200 ml running buffer for the Upper (inner) Buffer Chamber and 600 ml running buffer for the Lower (outer) Buffer Chamber. Conditions for electrophoresis were 150 V for 2 hours.

Transfer of proteins to PVDF membrane

After the electrophoresis was complete, proteins were transferred to PVDF membrane using a BIORAD Trans-blot SD Semi-Dry Electrophoretic Transfer Cell. Towbins transfer buffer was used at pH 8.3 (see composition in table 27).

Table 27. Towbin transfer buffer composition

Reagent	Concentration	Amount (for 1 L)
Tris	25 mM	3.03 g
Glycine	192 mM	14.4 g
Methanol	20 %*	200 ml
Distilled water	n/a	Up to 1 L

*For native proteins, methanol concentration in Towbin buffer was reduced to 10%.

Following electrophoresis, gels were equilibrated for 30 minutes in transfer buffer. PVDF membrane (Invitrogen LC2002) and blotting paper (Bio-Rad 170-3969) was cut to the same dimensions of the gel and soaked in transfer buffer. The sandwich was assembled according to manufacturer's (BIORAD) instructions for semi-dry transfer. Proteins were transferred for 20 minutes at 20 V.

All gels were stained (post protein transfer) with Coomassie Blue, and the PVDF membranes stained with Ponceau S to detect if proteins had transferred from the gel.

Immunoprobing of Western Blotted proteins

Once proteins had been transferred from the gel to the PVDF membrane, the PVDF membrane was placed in approximately 30 ml of blocking buffer (see appendix 6) for 1 hour at room temperature or overnight at 4 ˚C in order to minimize the amount of non-specific binding.

The blocking buffer was removed from the PVDF membrane before the addition of a primary antibody diluted in 10 ml fresh blocking buffer. This was incubated at room temperature for 1 hour with gentle shaking.

After 1 hour the primary antibody was removed and the PVDF membrane washed 3 times, 10 minutes each time with approximately 30 ml wash buffer at room temperature with gentle shaking.

If a secondary antibody was not necessary, i.e. if a HRP conjugated primary antibody was used then the antigen detection method could be used immediately. In this instance, 5-10 ml Pierce Supersignal West Pico Chemiluminescent substrate (Thermo scientific 34080) was used to detect antigen according to manufacturer's instructions.

If a secondary antibody was used, the membrane was incubated with 10 ml of antibody diluted in blocking buffer. This was incubated for 1 hour at room temperature with gentle shaking.

After 1 hour the secondary antibody was removed and the PVDF membrane washed 3 times, 10 minutes each time with approximately 30 ml wash buffer at room temperature with gentle shaking, and followed by a brief wash with 10 ml PBS. After this the antigen was detected by covering the membrane with 5-10 ml Supersignal West Pico Chemiluminescent substrate for 5 minutes at room temperature (for HRP conjugated antibodies).

Excess substrate solution was poured away and the membrane covered with cling film or a piece of plastic pocket and placed in a film cassette. Any excess solution was removed and air bubbles expelled by wiping over the cling film or plastic with a piece of tissue. In a dark room the membrane was exposed to ECL Hyperfilm and the image developed using a hyper-processor. Films were exposed for various times until a good image was obtained.

All antibodies (and their sources) used for anti-VHH and anti-FVIII western blotting are shown in table 28.

All antibodies were used at a dilution of 1:2000 and were diluted in 2 % BSA in 0.05 % Tween-20 in PBS.

Detection of AP conjugated antibodies was done using AP colour development reagents from BIORAD, BCIP and NBT (170-6539 BCIP (5-bromo-4-chloro-3-indolyl phosphate and 170-6532 NBT (Nitroblue Tetrazolium)) and used according to manufacturer's instructions.

Table 28. Antibodies used in western blotting, dot blotting and ELISA experiments

Antibody	Supplier	Code
Rabbit polyclonal anti-VHH antibody	BAC	Not available commercially
Anti-rabbit IgG (Fc) AP conjugate	Promega	5373B
Goat anti-VHH (biotinylated)	BAC	Not available commercially
Sheep polyclonal anti-human FVIII	Abcam	ab20946-1
Rabbit polyclonal anti-sheep IgG AP conjugate (secondary)	Abcam	ab6748-1
Anti-human FVIII	Affinity Biologicals	SAFC-AP
Anti-human FVIII HRP	Affinity Biologicals	SAFC-HRP
Goat anti-rabbit HRP (GARPO)	Dako	P044801-2
Swine anti-rabbit peroxidise (SWARPO)	Dako	P0217
Streptavidin HRP	Dako	P0397
6x-His Epitope Tag Antibody	Thermofisher scientific	4E3D10H2/E3

2.3.7 Immunoprecipation of VHH proteins

Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. The concept is shown in the diagram below (figure 2.2).

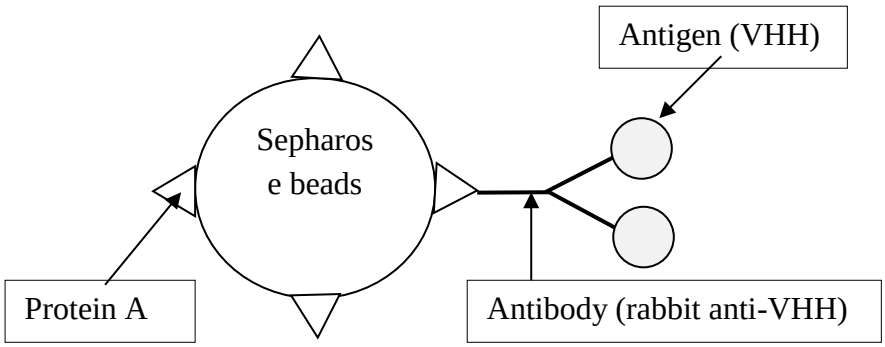


Figure 2.2. Immunoprecipitation using sepharose beads coated with protein A

Immunoprecipitation was used to increase the concentration of VHH protein in the cell supernatant of cells transfected with VHH plasmid. This was carried out prior to western blotting, in order to increase the likelihood of detecting these proteins at low expression levels. One sample from each transfection of ligand 2, 6 and 7 constructs was tested, (a total of 9 samples) plus one sample from untransfected cells as a negative control. Two positive control samples were tested; VHH ligand 2 and VHH ligand 7, both diluted in 1 x PBS.

For each sample to be tested the following mixture was made:

- 150 µl supernatant
- 450 µl buffer (0.1 % BSA, 0.05 % Tween-20 in 1 x PBS)
- 4 µl of rabbit anti-VHH antibody (BAC in–house reagent, not available commercially)

Each sample was incubated overnight at 4 °C on a rotating wheel, which allowed any VHH protein present in the test samples to bind to the rabbit anti-VHH antibody.

After the overnight incubation, Protein A Sepharose beads were used to capture all of the anti-VHH in the sample. Prior to use the sepharose beads were washed in 20 % ethanol, 0.05 % Tween-20 in distilled H_2O.

2.5 µg sepharose beads were diluted in 50 µl of 0.05 % Tween-20/1 x PBS. This was added to each sample before placing on a rotating wheel at 4 °C for 1 hour.

After incubating for 1 hour, each sample was centrifuged at 500 x g for 1 minute. The supernatant was discarded and the pellet resuspended in 800 µl 0.05 % Tween-20 in 1 x PBS in order to wash the beads. The centrifugation and washing step was repeated three times with the third wash step using 500 µl 1 x PBS. After washing the beads, each sample was centrifuged and the supernatant removed. The pellet was air-dried in a bench top heater in order to remove excess PBS. When the pellet was almost dry the pellet was resuspended in 45 µl SDS-PAGE sample buffer (50 nM, diluted in H_2O). 10 µl of each sample was run on a Tris-glycine SDS-PAGE gel, 140 V for 1 hour.

3 SDS-PAGE gels were run in total, 1 to be used for anti-VHH western blot, 1 to be used for anti-FVIII western blot, and 1 for SYPRO® staining (section 2.5.5).

Transfer of proteins to PVDF membrane was carried out at 35 V for 90 minutes. The PVDF membrane was blocked overnight in 4 % protifar (milk protein powder) in 1 x PBS at 4 °C before immunoprobing the membrane for either VHH or FVIII.

2.3.8 Dot blotting

Dot blotting was used as a crude experiment to check for protein expression in mammalian cells. 100 µl samples (either harvested and lysed cells, or spent cell media) were applied to methanol-activated PVDF membrane via a 96 well manifold. A vacuum pump was applied in order to allow the samples to enter the membrane. After this the PVDF membrane was left to air dry before Immunoprobing as described in 2.3.6.2 using anti-FVIII, anti-VHH or anti-His antibodies to check for VHH or FVIII expression in cell media after transient transfections and during the development of stable cell lines.

2.3.9 SYPRO® Ruby Protein PAGE Gel Stain

SYPRO® Ruby Protein PAGE Gel stain (Molecular Probes, Invitrogen) is a sensitive fluorescent stain that can detect proteins separated by SDS-PAGE. It has the same low nanogram sensitivity as silver staining but detects more proteins over a broader size range, making it ideal for detection of proteins which are expressed at low levels (Lopez, Berggren et al. 2000).

The basic protocol for SYPRO® staining involved 3 steps; fixing, staining and washing.
Firstly the fixing and washing solutions were prepared as follows:
Fix Solution: 50 % methanol, 7 % acetic acid. 200 mL fix solution was prepared per mini gel.
Wash Solution: 10 % methanol, 7 % acetic acid. 100 mL wash solution was prepared per mini gel.

After electrophoresis, the gel was placed into a clean container with 100 mL of fix solution and agitated on an orbital shaker for 30 minutes. This was repeated once more with fresh fix solution. After removing the fixing solution, 60 mL of SYPRO® Ruby gel stain was added. This was agitated on an orbital shaker overnight at room temperature and the gel covered with foil to exclude light. The next step was to wash the stain away. The gel was transferred to a clean container and washed in100 ml of wash solution for 30 minutes with gentle agitation at room temperature.
The transfer of the gel to a clean container and the washing step helped to minimize background staining irregularities and stain speckles on the gel. Before imaging the gel, it was rinsed in ultrapure water a minimum of two times for 5 minutes to prevent possible corrosive damage to the imager. The gel was covered to exclude light in each of these steps. SYPRO® Ruby protein gel stain has two excitation maxima, one at ~280 nm and one at ~450 nm, and has an emission maximum near 610 nm. Proteins stained with the dye were visualized using a UV transilluminator and the image saved for analysis of protein expression.

2.3.10 Nickel column purification of histidine-tagged proteins

Since some of the VHH constructs encoded a histidine tag in their gene sequence, it was deduced that these proteins could be purified using a nickel column in order to increase the protein concentration of samples and enhance the likelihood of detecting these proteins in dot blotting and/or western blotting.

The GE Healthcare HisTrap HP system was used according to the manufacturers instructions (protocol 71-5027-68 AF, 1ml column). The HisTrap HP system is an immobilized metal affinity chromatography (IMAC) system, which in this case relies on the bonds formed between histidine amino acids and immobilised nickel ions. This technique is designed for one-step purification of histidine-tagged proteins using columns that are pre-packed with Ni Sepharose, which has high binding capacity, and does not biological activity of the target antigen. Peptides containing sequences of consecutive histidine residues are efficiently retained on the Ni Sepharose column, and can be eluted by either adding imidazole to the column buffer or adjusting the pH of the column buffer (Porath, 1992).

In the present study, several buffers were prepared (see appendix) including a binding buffer and elution buffer at a variety of imidazole concentrations. The binding buffer was used to equilibrate the column and the elution buffer was tested at a variety of concentrations to ensure that the VHH protein was eluted.

Prior to loading the sample on to the column, samples were passed through a 0.45 µM filter to remove large host cell proteins that may have contaminated the procedure.

When eluting proteins a range of imidazole concentrations was tested in order to find the optimal concentration for eluting VHH. 10 mls of elution buffer was run through the column at each concentration, and 1ml samples were collected.

All eluted samples collected from the HisTrap column were analysed on a dot blot.

2.3.11. Enzyme Linked Immuno-Sorbent Assay (ELISA)

Several ELISA formats were investigated for the detection of VHH or FVIII protein in the cell culture media of transfected or untransfected cells. The aims for each experiment were different depending on the protein being detected. Ultimately these were the main goals

1. The FVIII ELISAs were developed in order to detect any increase or decrease in protein levels (quantitative) between cells transfected with VHH constructs and untransfected cells.
2. The VHH ELISAs were developed in order to detect the presence or absence of protein (qualitative), to give an indication whether the proteins were being expressed or not in transfected cell lines.
3. Detection of 'VHH:FVIII complex' in BHK FVIII cell line transfected with VHH (qualitative) to see if the VHH was bound to FVIII

Typical assay format (per 96 well plate) as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BHK	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3		
B	BHK	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3		
C	BHK	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3		
D	BHK	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3		
E	BHK	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3		
F	BHK	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3		
G	Std	Std	Std	Std	Std	Std	Std	Std	Std			
H	Std	Std	Std	Std	Std	Std	Std	Std	Std			

Std = Standard; BHK = Baby Hamster Kidney cell media (untransfected cells); L2C1, L2C2, L2C3, L6C1, L6C2, L6C3, L7C1, L7C2, L7C3 = Test samples (cell media or lysate from BHK cells transfected with VHH plasmid).

The development of these assays was restricted by the limited selection and specificity of both anti-VHH and anti-FVIII antibodies currently available. At the time of the experiments, no antibodies (primary, conjugated) were available for the direct detection of either VHH or FVIII and therefore a direct ELISA was not

possible. Therefore all ELISAs investigated were designed for an indirect detection of protein.

All antibodies used in the detection of either VHH or FVIII are listed in table 28 and all buffers used in ELISA are listed in appendix 8.

2.3.11.1 ELISA protocol

Most of the ELISA tested followed the protocol as described below. Some of the ELISA formats deviated from the standard protocol to accommodate different assay formats and this will be described later.

The first step in the ELISA protocol is to coat a 96 well microtiter plate. 100 µl of antigen (antibody or cell supernatant) was added to each well and incubated for 1 hour at room temperature with gentle shaking. The antigen was then washed from the plate 5 times using wash buffer. The plate was blocked by adding 200 µl of 2% blocking buffer and incubating for 1 hour at room temperature with gentle shaking. Samples and controls (or antibody depending on format of assay) were then added to the plate in the desired wells. 100 µl of sample or control in 1 % sample buffer was added to each well and incubated for 1 hour at room temperature with gentle shaking. The plate was then washed 5 times with wash buffer. Antibody was then added to the plate, 100 µl per well diluted in sample buffer and incubated for 1 hour at room temperature with gentle shaking. The plate was then washed 5 times with wash buffer. If necessary, a secondary antibody (conjugated to AP or HRP) was then added to the plate, 100 µl per well in sample buffer and incubated for 1 hour at room temperature with gentle shaking. The plate was then washed 5 times with wash buffer and then twice with demineralised water. In order to detect either the HRP or AP, 100 µl of enzyme substrate was added to each well. To stop the reaction from proceeding, 50 µl of 1 M H_2SO_4 was added to each well, causing a colour change from blue to yellow. The OD was measured at 450 nm using a plate reader and a spreadsheet used to process the raw data.

2.3.11.2 Commercially available ELISA

At the time of the project there was a commercially available anti-VHH ELISA, the CaptureSelect™ VIII Select Leakage ELISA. This was originally designed for the detection of less than 1 ng/mL Factor VIII ligand (VHH ligand 7) that may be present in FVIII product purified with GE-Healthcare's VIII Select. This ELISA kit was used to test cell media and lysed cell samples from cells transfected with VHH plasmid (these experiments were conducted in the BAC laboratory in the Netherlands).

The CaptureSelect™ leakage assay enabled detection of the VHH in solutions with and without the presence of the target protein i.e. VHH alone or VHH bound to FVIII (see figure 2.3). This assay involved the following steps:

- A microtiter plate was coated with either anti-VHH antibody (goat)
- Samples containing the affinity ligand (VHH or VHH bound to FVIII) were incubated in the coated plate wells.
- Bound affinity ligand (VHH or VHH bound to FVIII) was detected using a biotinylated affinity ligand (either anti-VHH or anti-FVIII).
- Streptavidin horseradish peroxidase conjugate was added to bind to the biotinylated antibody in the sandwich complex.
- Substrate reactive with horseradish peroxidise (tetramethylbenzidine-hydrogen peroxide) was added.
- The amount of hydrolyzed substrate was determined (directly proportional to the concentration of VHH or VHH bound to FVIII present).

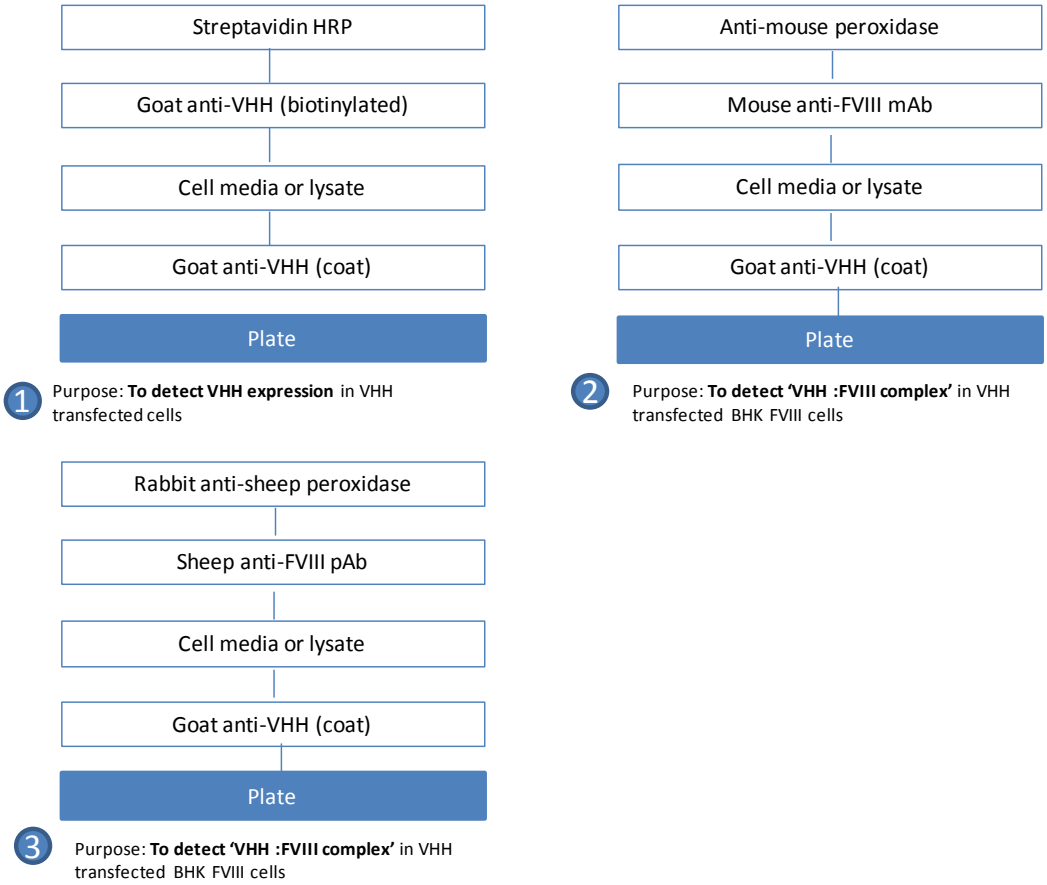


Figure 2.3. ELISA formats using CaptureSelect VIII Leakage ELISA coating antibody

Assay 1: Original manufacturer specified assay format to detect VHH in the cell media or cell lysate of cells transfected with VHH plasmid;

Assay 2: Modified manufacturer specified assay format to detect VHH:FVIII complex in cell media or cell lysate of BHK cells transfected with VHH plasmids using a mouse anti-FVIII antibody as a secondary antibody;

Assay 3: Modified manufacturer specified assay format to detect VHH:FVIII complex in cell media or cell lysate of BHK cells transfected with VHH plasmids using a sheep anti-FVIII antibody as a secondary antibody.

In addition, it is worth noting that there are commercially available ELISA kits for detection of FVIII; however these were prohibitively expensive and therefore were not able to be used for the purpose of this project. For example it costs approximately £1500 per FVIII ELISA kit from Affinity Biologicals, which is an FDA approved kit used to assess FVIII concentrates, as well as distinguishing patients with FVIII inhibitors.

2.3.11.3 Bespoke ELISA

The limited selection and prohibitive cost of the commercial ELISA kits for VHH and FVIII meant that a bespoke solution was sought. Several ELISA formats were designed and optimised in-house. Multiple formats were tested for both VHH and FVIII proteins and these are summarised in figure 2.4

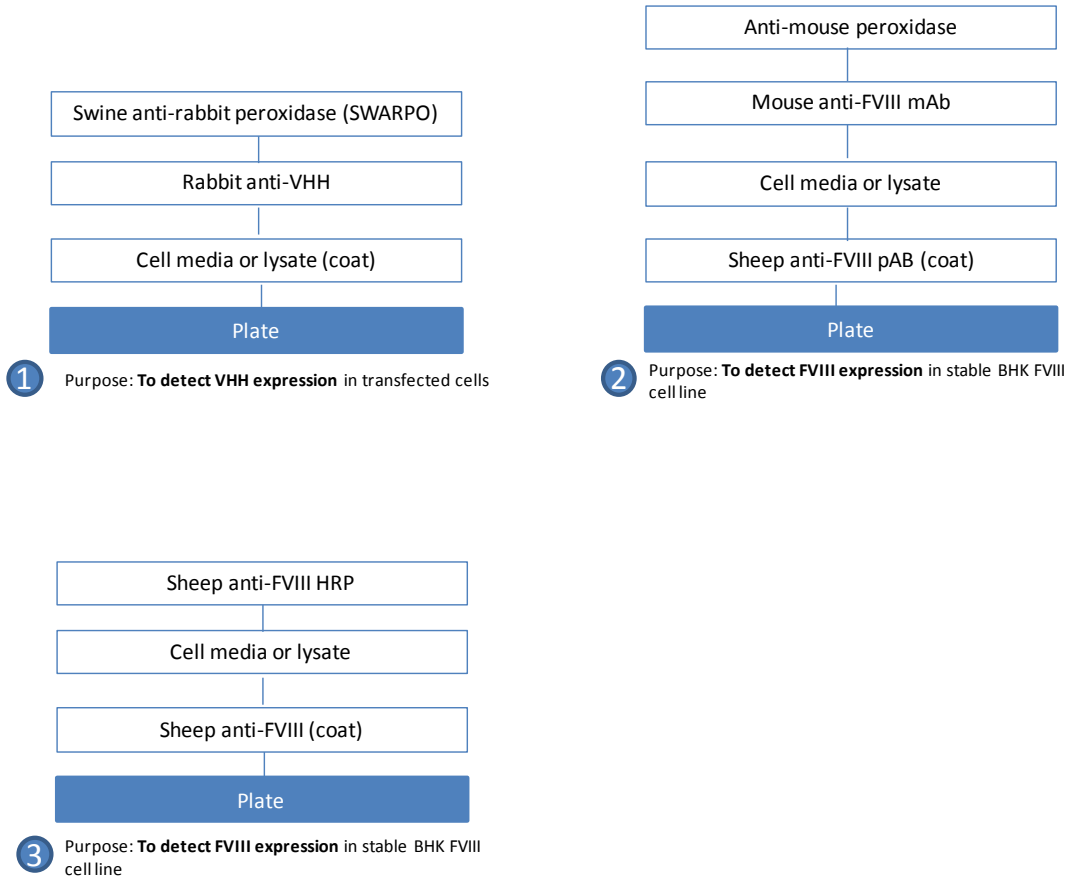


Figure 2.4. ELISA formats tested

Assay 1: For the detection of VHH in cell media or cell lysate of cells transfected with VHH plasmid;

Assay 2 and 3: For the detection of FVIII in BHK cell media or cell lysate

2.4. Detection of FVIII activity using Ceveron automated coagulation assay (One stage clotting assay)

There are many methods available for testing the functional activity of clotting factors and more specifically the activity of FVIII including clot-based assays, chromogenic, and immunoassay methods. The most common method used for measuring FVIII activity is the 1-stage activity (clot-based) assay based on the activated partial thromboplastin time (aPTT), which is a highly sensitive and precise way of measuring FVIII activity, even at low levels (Chandler, Ferrell et al. 2003). Platelet poor plasma [PPP] is incubated at 37°C then phospholipid and a contact activator are added followed by calcium (also at 37°C). Addition of calcium initiates clotting, and the aPTT is the time taken from the addition of calcium to the formation of a fibrin clot. In the clinic, the aPTT time can be normal with very mild FVIII deficiency, and in general FVIII levels have to be less than 20-40% of a normal level before the aPTT is prolonged. A mild FVIII deficiency can be detected around 0.35-0.4 IU/ml (Lee *et al*, 2010).

Ceveron® alpha, a machine manufactured by Technoclone, is a sophisticated and fully automated blood coagulation analyzer (figure 2.3). The Ceveron® alpha consists of an analyzer, a personal computer and an optional printer. Ceveron® alpha operates on the photometric measurement principle. This measurement method finds the coagulation time by an optical detection of the change of turbidity caused by the formation of fibrin fibres during an *in-vitro* 'clot' formation.

Alternative methods exist for testing FVIII activity. These include chromogenic methods which measure the change of absorbance after adding a chromogenic substrate after plasma and reagent incubation. Immunological methods measure the change of absorbance during the reaction of antigen and antibody complex formation.

For this study, the machine was regularly calibrated as recommended by the manufacturer (Technoclone), using the coagulation reference plasma, to ensure reproducible and accurate data. All reagents and consumables required for the coagulation assay are listed in table 29.

Table 29. Reagents and consumables for Ceveron coagulation assay (all supplied by Technoclone)

Reagent, equipment and consumables
Ceveron Alpha machine
Dapttin aPTT reagent with sulfatides and silica as surface activators
25 mM $CaCl_2$
Imidazole buffer
FVIII deficient plasma
Control plasma (normal/abnormal)
Coagulation reference
Ceveron Alpha cuvettes
Ceveron Alpha sample cups (3 ml)

Samples, controls and calibrators were loaded into the sample rotor of the analyzer. Samples, controls, calibrators and reagents could be identified by two independent bar code readers, one on the outside of the machine, and one built into the sample rotor, and allowing for quality control of 'use-by' dates and batch numbers. After loading all samples and reagents manually, the machine was fully automated according to a pre-defined assay protocol.

In order to avoid damaging the pipettor arms, the position of the cover (open or closed) was automatically checked during operation. These positions were indicated on the screen and on the LED display that indicates when an error has occurred, when it is busy (the cover is locked) and when it is ready to go (the cover is

unlocked). The measuring rotor is where the assay took place. There were two dilutors fitted with 500 µL syringes that performed all dilutions and dispensing of samples and reagents. Samples, controls and reagents were assembled into cuvettes by the pipettor arms, into the measuring rotor, and the wavelength measured. The temperature of the reagent pipettor was pre-set to 37 °C, which also had a liquid volume sensor. The computer attached automatically converted collected data including the number of seconds taken to form a 'plasma clot'. Each sample or control was tested twice, and an average clotting time calculated (the system/software was pre-designed by the manufacturer to do this and it could not be changed by the user). Acceptable values for control samples were provided in ranges by the manufacturer with each new batch. The barcodes on the control vials were scanned each time they were used so that the software could conduct quality control calculations each time they were used. If a control value fell outside the expected range then the whole experiment would be abandoned (this only happened once during the entire study and a new control batch was opened).

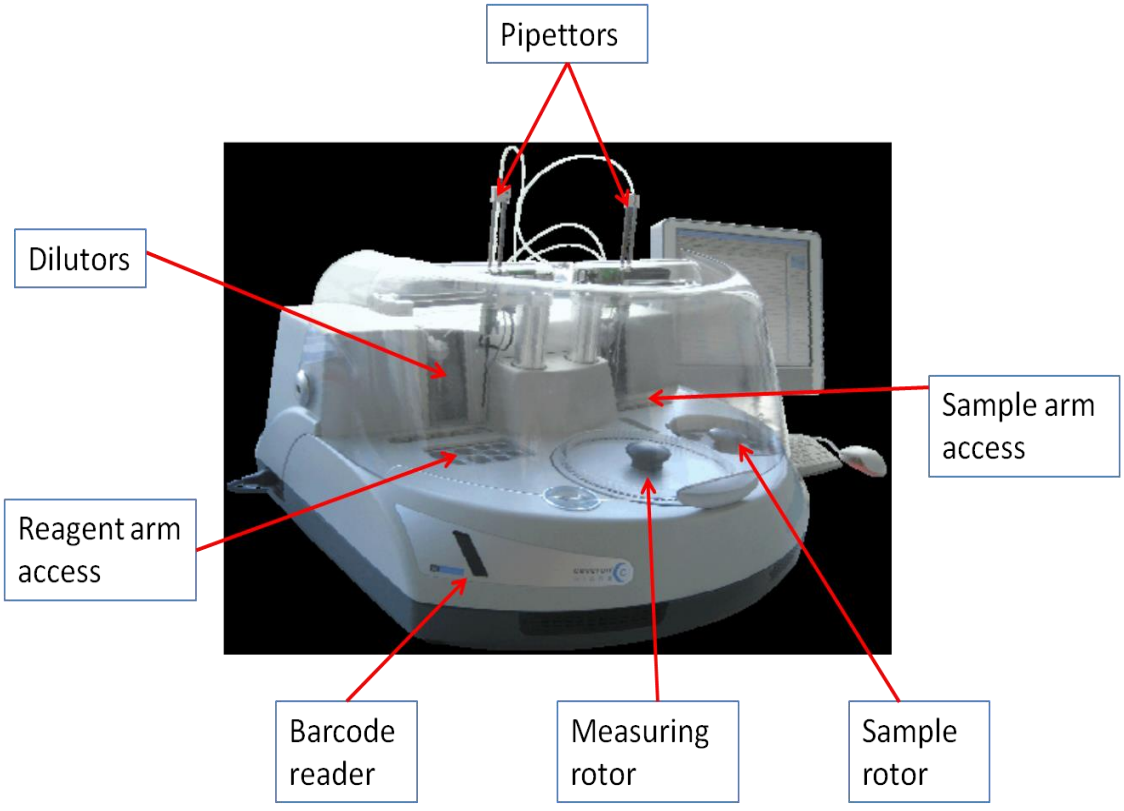


Figure 2.5. Ceveron® alpha, an automated blood coagulation analyzer

Marketed FVIII products were tested in the coagulometer to compare their clotting times with the FVIII produced in the stable BHK cell line, and also in an attempt to correlate FVIII protein concentration or international units with clotting times. Media (cell supernatant) was taken from each of the nine VHH ligand plasmid transfections in the BHK cell line stably expressing FVIII, 48 hours after transfection, and used immediately in the Ceveron assay, which was carried out according to the manufacturer's instructions. Media from transfected cell lines was compared with media from the untransfected cells (stable FVIIL cell line) to assess if there was a difference in coagulation time.

In order to minimise variability in the assay, cells were counted prior to seeding and the exact same seeding density was used for every experiment. In addition, the cells were incubated for exactly the same time after every transfection, and after 48 hours the media was extracted and immediately tested in the coagulation assay as it was known that FVIII can be unstable in cell media. For every experiment, a null transfection (no DNA) was carried out and the media tested alongside those transfected with VHH. Coagulation assays were repeated at least 16 times (individual transfection experiments) for each of the transfected cell lines as well as the untransfected cells. This was following advice sought from a qualified statistician at the School of Maths, University of Kent on experimental design and statistical analysis methods to ensure that the data produced was of high quality.

Controls were included in every experiment using a 'normal' and 'abnormal' reference plasma provided by Technoclone, which represented healthy human plasma and that of a human with an abnormal blood clotting time. Because the Technoclone system was programmed to compare all samples against the controls (% activity versus the 'normal' reference sample), those experiments where the control data did not fall into specified brackets (as specified by Technoclone) meant that coagulation results for samples was not reliable, and were excluded from the data analysis.

Samples and reagents were loaded into the appropriate parts of the machine according to Ceveron instructions. Using the programme 'FVIII Dapttin TC 10ml_[V001]' each sample was tested in duplicate and the time taken to form a 'clot' measured in seconds. Each reagent had a lot number and expiry date that was entered into the Ceveron before each experiment to confirm that they were viable. For each new batch of reagents a calibration curve was generated to ensure that the expected clotting times of the control reagents were correct.

The difference in clotting times between media from untransfected cells and transfected cell lines was statistically analysed using an unpaired t-test and a graphpad calculator website http://www.graphpad.com/quickcalcs/ttest1.cfm.
An unpaired t-test compares the means of two data sets from independent sources (two batches of cells in this case) and indicates if they are statistically significantly different. It assumes that the data follows a Gaussian (normal) distribution. As well as a t-test the standard deviation, 95 % confidence intervals and the p-values were calculated in order to determine if the data were statistically significant. In addition, Analysis of Variance (ANOVA) (Cochran and G.M.Cox 1957) was carried out on the entire dataset of clotting times. The null hypothesis was that all media samples tested had the same amount of functional FVIII (clotting times used as an indirect measure). Statistical evaluation was conducted under the guidance of a qualified statistician (Jian Zhang, University of Kent).

Chapter 3: Production of plasmids containing VHH genes suitable for mammalian expression

3.1 Introduction

In order to investigate the effect of co-expression of anti-FVIII VHH and FVIII on FVIII production, the VHH genes needed to be placed into a vector suitable for mammalian expression. Three anti-FVIII VHH ligands were used to make nine constructs with a variety of design elements including a leader sequence, a histidine tag and/or KDEL, an endoplasmic reticulum retrieval motif. All nine constructs were designed, cloned into a mammalian expression vector pcDNA3.1 and sequenced ready for transfection into mammalian cells.

3.2 Aims

The first aim of this project was to produce nine plasmids, each encoding the gene for one Camelid antibody (VHH) ligand 2, 6 or 7 respectively. The genes encoding each of these ligands were supplied by BAC BV in the Netherlands and these had to be manipulated to ensure expression and include various design elements.

3.3 Plasmids and primer design

For each of the 3 VHH ligands, 3 different constructs were designed (see methods 2.2). Each construct was cloned into a pcDNA3.1 vector (Invitrogen) ready for transfection into mammalian cells.

3.4 PCR to generate VHH ligand constructs

In order to generate the VHH constructs previously designed, PCR experiments needed to be carried out. Primers were designed in house as described in 2.2. The expected sizes of PCR products were as follows:

Construct 1 = 496 bp

Construct 2 = 446 bp

Construct 3 = 490 bp

3.4.1 PCR for ligand 2 constructs 1, 2 and 3.

Initially Taq DNA polymerase (Roche) was used to amplify L2C1. The PCR product was purified using a QIAquick PCR purification kit (QIAGEN) and then run on a 1.5% agarose gel. The gel showed smeared bands at approximately 250bp, 500bp and 1000bp indicating the presence of primer-dimers and other nonspecific by-products (see Figure 3.1, Panel A). Therefore, a proofreading DNA polymerase, Platinum® Pfx DNA polymerase (Invitrogen) was used as it is claimed to have a much higher fidelity rate (lower error rate) than *Taq* polymerase. This is important when the DNA sequence needs to be 100% correct after amplification, as it needed to be in this project. Also, an additional reaction was run using an 'enhancer' suggested in the Invitrogen protocol to lower the melting temperature of the primers. Lowering the melting temperature below 65 ºC generally reduces the potential for secondary annealing of primers to the DNA template. Also, since the primers being used in these experiments were relatively long, and the longer the primer the more inefficient the annealing step in PCR, lowering the melting temperature may have improved the annealing efficiency.

The use of Platinum® Pfx gave improved results over the Taq DNA polymerase with distinct bands in the agarose gel (see Figure 3.1, Panel B) for L2C1 and was used to generate L2C2 and L2C3. The ‘enhancer’ did not appear to make a significant difference to the performance of the PCR and was not used in any future PCR experiments.

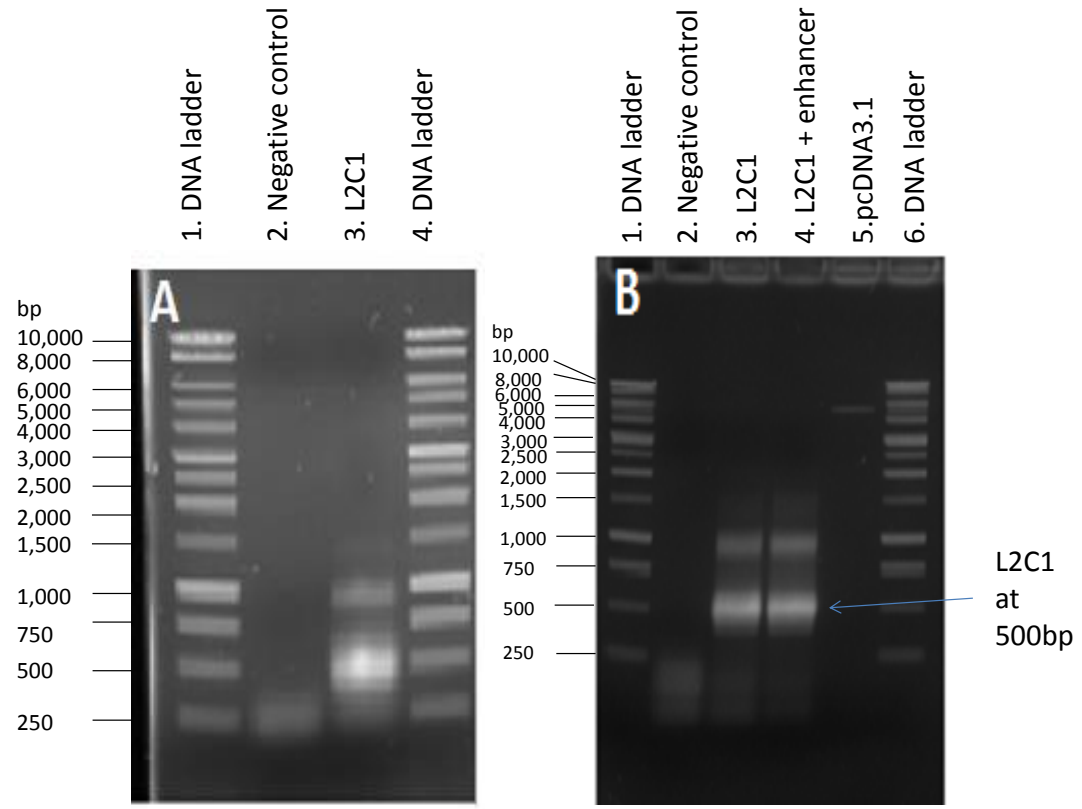


Figure 3.1. PCR products for ligand 2, construct 1.

1.5% agarose (Melford) gels.

A. *Taq* polymerase results, lane 1 and 4 DNA ladder, lane 2 negative control, lane 3 L2C1. Cycle conditions: 94 °C for 2 minutes followed by 25 cycles of 94 °C for 30 seconds, 60 °C for 60 seconds, 72 °C for 60 seconds, hold at 4 °C.

B. Platinum® Pfx results, lane 1 and 6 DNA ladder, lane 2 negative control, lane 3 L2C1, lane 4 L2C1 + 'enhancer', lane 5 plasmid vector pcDNA3.1. Thermocycler conditions: 94 °C for 5 minutes followed by 30 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds, 68 °C for 30 seconds, hold at 4 °C. Lane 3 and 4 show L2C1 construct at approximately 500 bp.

After purification, the PCR products were run on another agarose gel to check their purity (see figure 3.2 for example).

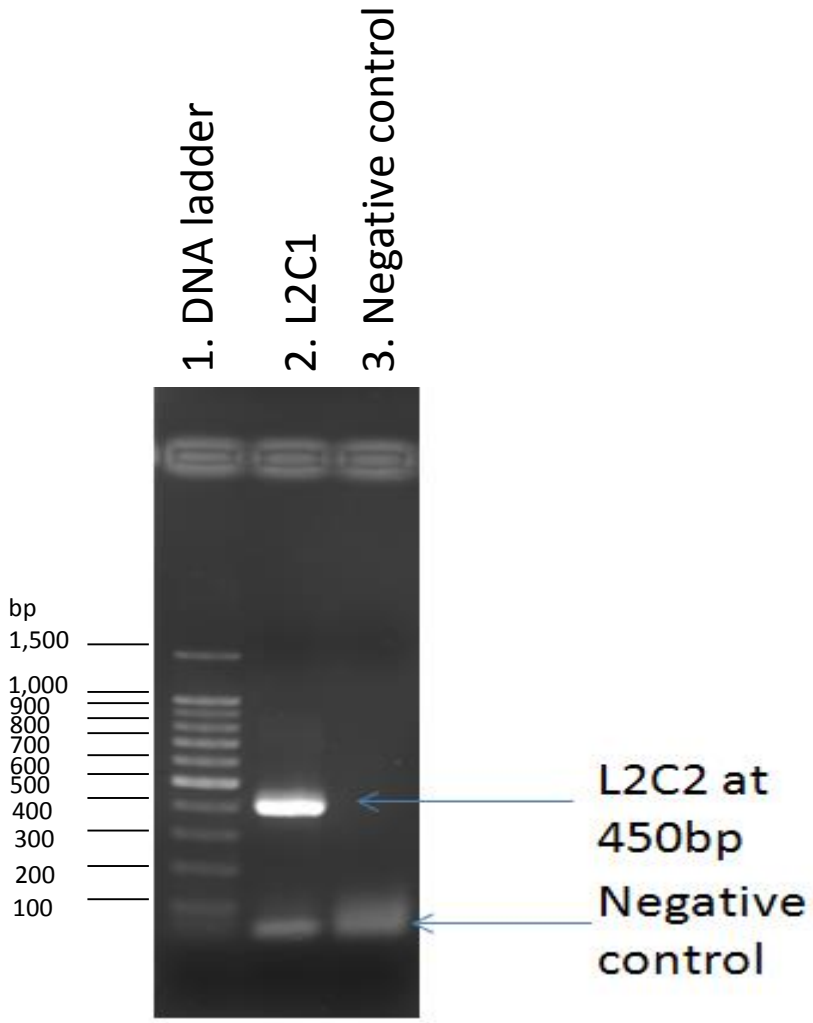


Figure 3.2 Purified PCR product Ligand 2, construct 2

Purified PCR products (L2C2) run on 1.5 % agarose gel. Lane 1 DNA ladder, lane 2 L2C2, lane 3 negative control (PCR). Lane 2 contains L2C2 construct at approximately 450 bp.

3.4.2 PCR for ligand 6, constructs 1, 2 and 3.

Platinum® Pfx was used to amplify each of the constructs for ligand 6 in the same way as ligand 2 constructs. The PCR products were purified using QIAquick PCR Purification kit (QIAGEN) before running on a 1.5 % agarose gel (see Figure 3.3).

Bands of the appropriate size (approximately 500 bp for L6C1 and L6C3, and approximately 450 bp for L6C2) were excised using a scalpel and UV light box and the DNA purified using QIAquick Gel Extraction Kit according to the manufacturer's instructions (QIAGEN). After purification, the PCR products were run on another agarose gel to check their purity (data not shown).

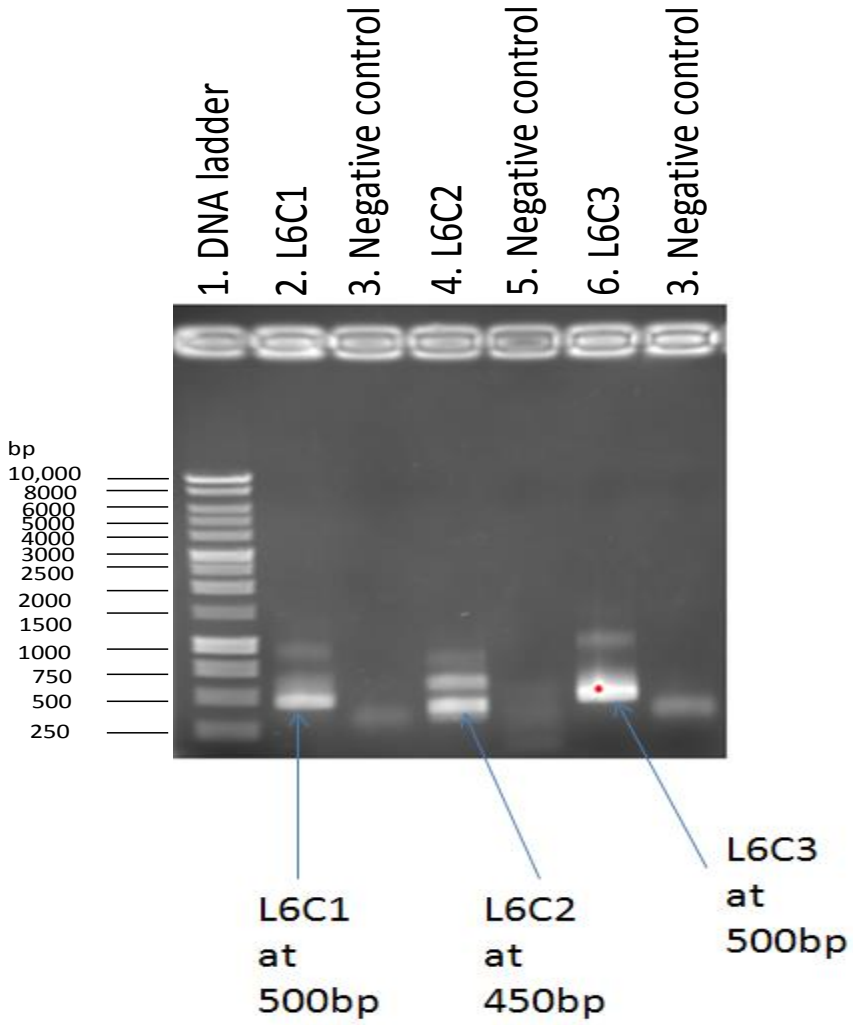


Figure 3.3. PCR products for ligand 6 constructs.

Lane 1 DNA ladder, lane 2 L6C1, lane 3 negative control, lane 4 L6C2, lane 5 negative control, lane 6 L6C3, lane 7 negative control. Cycle conditions: 94 °C for 5 minutes followed by 30 cycles of 94 °C for 15 seconds, 55°C for 30 seconds, 68 °C for 30 seconds, hold at 4 °C. As expected L6C1 in lane 2 is approximately 500 bp, L6C2 in lane 4 is approximately 450 bp and L6C3 in lane 6 is approximately 500 bp.

3.4.3. PCR for ligand 7, constructs 1, 2 and 3.

Based on previous results for ligand 2 and 6, initial PCR reactions for ligand 7 were carried out using Platinum® Pfx, however this produced no PCR product. Several optimisation steps were carried out including varying the $MgCl_2$ concentration, running the reaction with/without enhancer, changing primer concentrations, adjusting the annealing temperature, none of which proved successful.

An alternative enzyme was used, Phire Hot Start DNA Polymerase (Finnzymes, NEB) which is only activated once the PCR reaction begins to heat up and this proved successful. All 3 ligand 7 constructs were produced using this enzyme. The PCR products were purified using QIAquick PCR Purification Kit according to the manufacturer's instructions (QIAGEN) before running on a 1.5 % agarose gel (see Figure 3.4). Bands were excised using a scalpel and UV lightbox and the DNA purified using QIAquick Gel Extraction Kit (QIAGEN). After purification, the PCR products were run on another agarose gel to check their purity (data not shown).

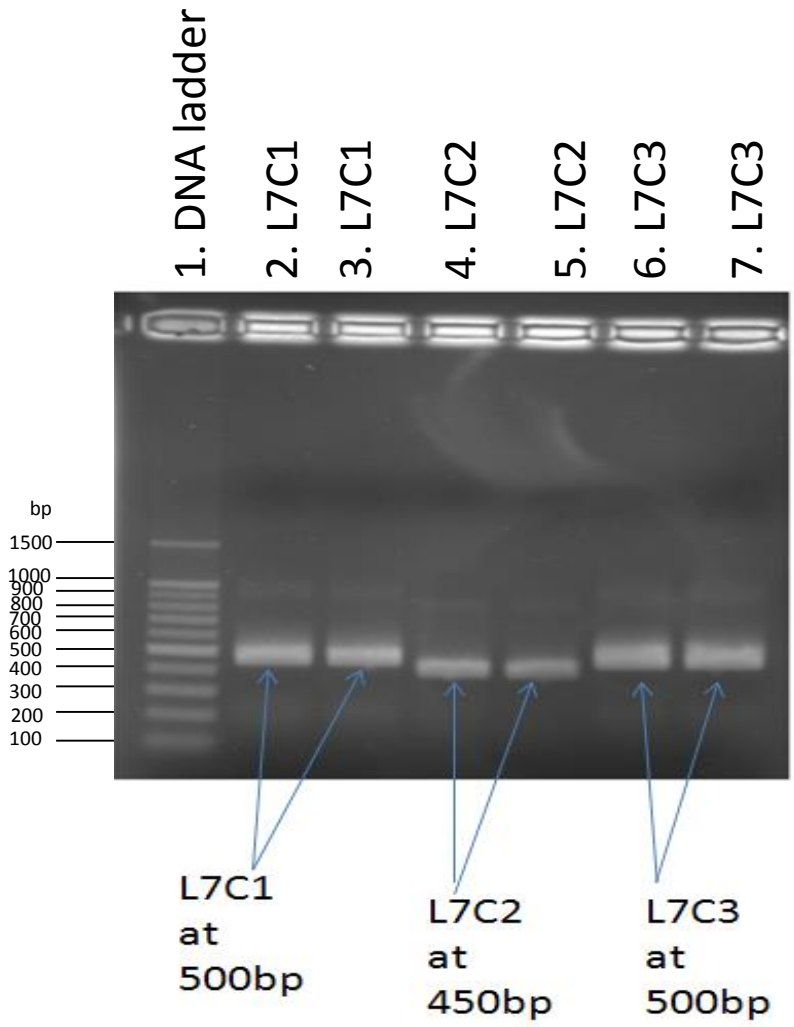


Figure 3.4 PCR products for ligand 7 constructs.

Lane 1 DNA ladder, lane 2 and 3 L7C1, lane 4 and 5 L7C2, lane 6 and 7 L7C3. Cycle conditions: 98 °C for 1 minute, 30 cycles of 98 °C for 5 seconds, 70 °C for 5 seconds, 72 °C for 10 seconds, hold at 72 °C for 1 minute, hold at 4 °C. As expected L7C1 in lane 2 and 3 is approximately 500 bp, L7C2 in lane 4 and 5 is approximately 450 bp and L7C3 in lane 6 and 7 is approximately 500 bp.

3.5 Plasmid ligation

3.5.1 Restriction digest

All PCR products were digested using the restriction enzymes *BamH1* and *EcoR1* ready for ligation into pcDNA3.1. These products were then run on a gel and the DNA re-purified using a Gel Extraction Kit (QIAGEN). The vector pcDNA3.1 was also digested with the same restriction enzymes and purified ready to be ligated with the digested PCR products (see Figure 3.5).

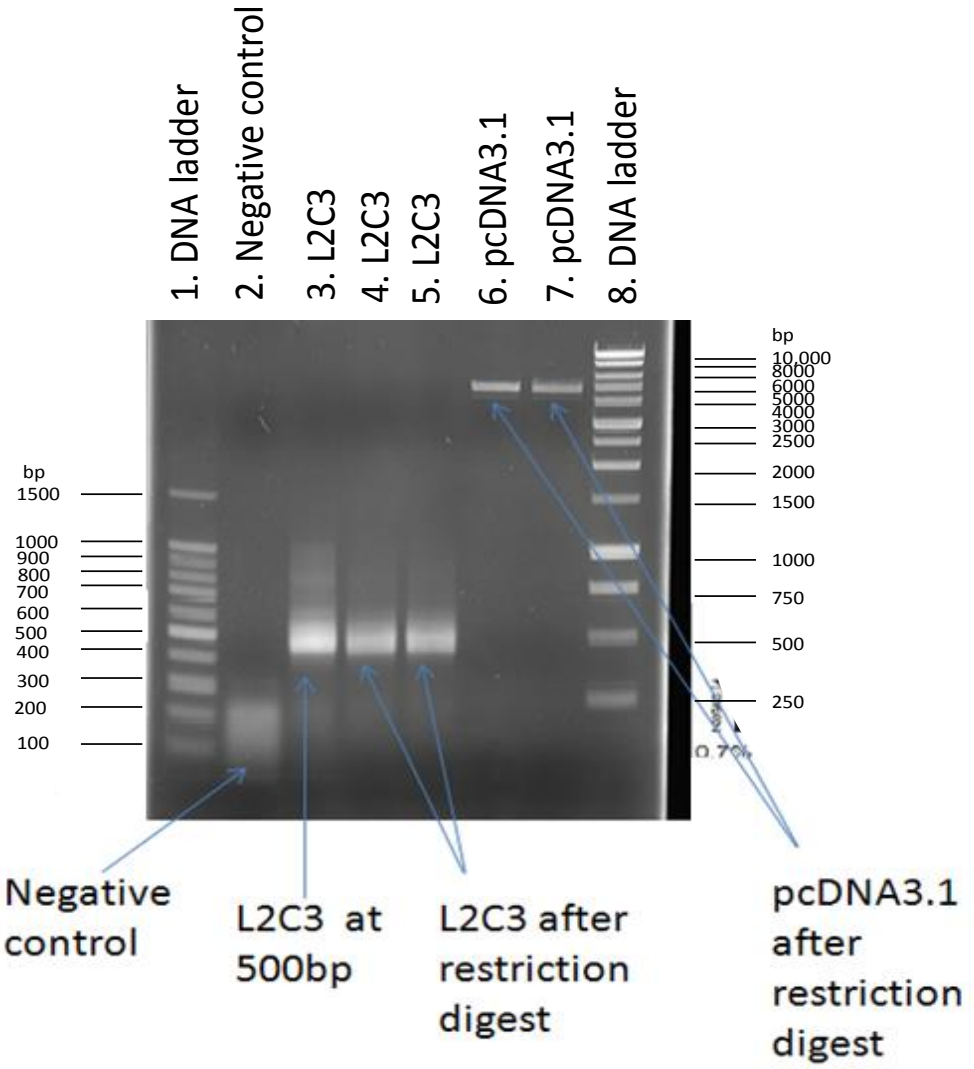


Figure 3.5. Purified and restriction enzyme digested PCR products (L2C3).

1.5% agarose gel. Lane 1 DNA ladder 100 bp, lane 2 negative control, lane 3 purified PCR product L2C3, lane 4 and 5 L2C3 digested with *BamH1* and *EcoR1* restriction enzymes, lane 6 and 7 pcDNA3.1 digested with *BamH1* and *EcoR1* restriction enzymes, lane 8 DNA ladder 1 Kb. L2C3 bands in lanes 3, 4 and 5 are

approximately 500 bp, and pcDNA3.1 bands in lanes 6 and 7 are approximately 5 Kb.

3.5.2 DNA ligation (pcDNA3.1 + VHH)

A standard overnight ligation was carried out using ligase enzyme and pcDNA3.1 for inserts L2C2, L2C3, L6C1-3 and L7C1.

For the other inserts L2C1, L7C2 and L7C3, standard ligation procedures resulted in unsuccessful transformations in *E. coli*, therefore a TA cloning kit (Invitrogen) was used (see 2.2.5). TA cloning resulted in successful ligations for all three of these constructs, confirmed by transformation into *E. coli*.

Single *E. coli* colonies were picked and placed into an overnight starter culture (LB media). The plasmids were purified from the bacteria using QIAGEN miniprep kits and the DNA subsequently digested using *BamH1* and *EcoR1* restriction enzymes. An example of 2 positive colonies containing a ligated plasmid with the L2C1 gene insert can be seen in figure 3.6, lanes 3 and 5.

After propagation of the TA plasmid in DH5α *E. coli* cells, the insert was then digested out of the TA vector using *BamH1* and *EcoR1* and successfully ligated into pcDNA3.1 using standard procedures.

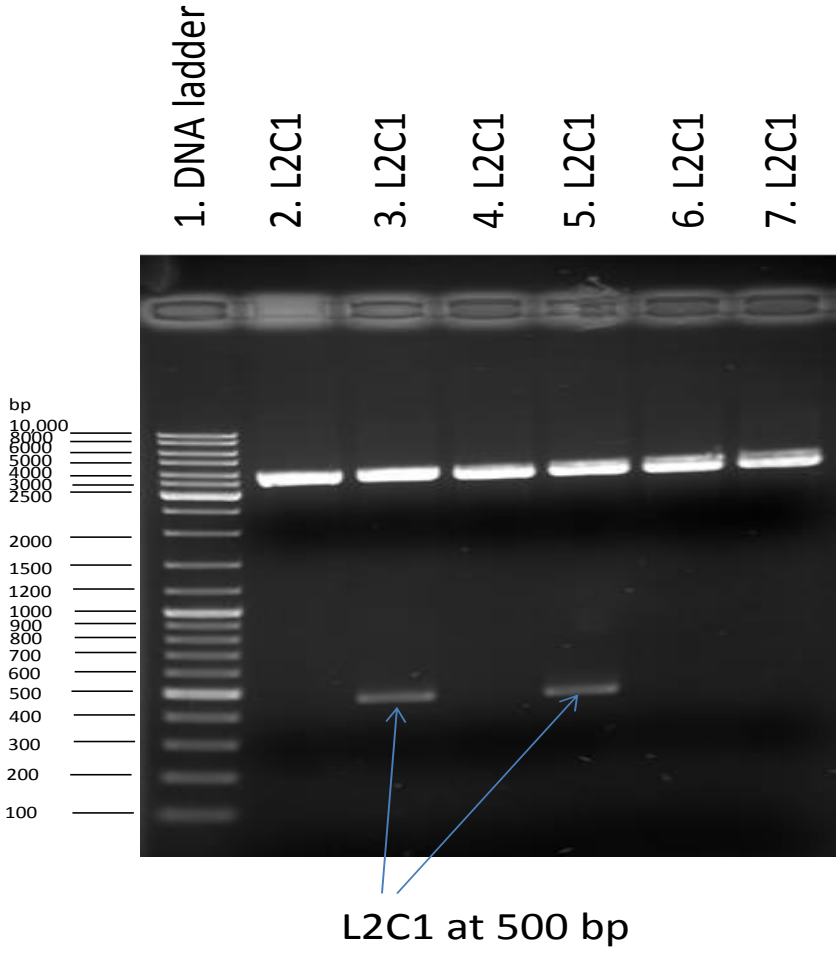


Figure 3.6. Plasmids digested with restriction enzymes (L2C1).

1.5 % agarose gel. Lane 1 DNA ladder, lanes 2-7 L2C1 miniprep DNA digested with *EcoR1* and *BamH1* restriction enzyme. Lanes 3 and 5 show the presence of L2C1 gene insert at approximately 500 bp.

3.6 Transformation of VHH plasmids into bacterial cells

All plasmids were transformed successfully into competent DH5α bacterial cells and propagated in these cells under Ampicillin selective pressure.

3.6.1. Sequencing

DNA samples that showed the appropriate band size for the VHH gene insert when run on an agarose gel were sent off for sequencing at either CoGenics or GATC Biotech. Eight out of nine plasmids showed a 100% homology with the desired sequence at the first sequencing attempt. L7C2 however had to be sequenced 3 times (3 individual clones were analysed) before a plasmid containing the correct sequence was found.

The first L7C2 construct to be sequenced had a cytosine base missing in the 3' end of the gene sequence which encoded the histidine tag. The second L7C2 construct to be sequenced contained a base change from a cytosine to a thymine residue, also at the 3' end of the gene sequence in the region encoding the histidine tag. The third attempt at sequencing resulted in a L7C2 construct that was 100% homologous with the expected gene sequence and this was the plasmid selected for cloning.

3.7 Discussion

All nine VHH constructs were designed, built and inserted into the mammalian expression vector pcDNA3.1 ready for transfection into mammalian cells. Each plasmid encoded the gene for one Camelid antibody (VHH) ligand 2, 6 or 7 respectively, with 3 different constructs designed per ligand giving a total of nine unique plasmids. Since the genes encoding each of these ligands were supplied by BAC BV in the Netherlands as an incomplete DNA template, several molecular techniques had to be employed in order to produce a plasmid containing the full length gene sequence as well as the various design features of each construct, such as the region encoding a histidine tag. Several important design features were included such as the Kozak sequence which is critical for initiation of the translation process (Kozak 1987). For successful initiation, the small ribosomal subunit must bind to the translational start site (Kozaks DNA consensus sequence in eukaryotes) and recruit the large ribosomal subunit to form a functional ribosome (Grzegorski et al, 2014). A translation termination codon was included so that translation would stop

at the end of the mRNA sequence, and no more amino acids added to the polypeptide chain. An immunoglobulin leader sequence was added to VHH constructs 1 and 3 in order to encourage secretion of the VHH into cell media. The leader sequence used was an exact copy of that used in the production of the antibody Herceptin (supplied by Peter Nicholls, University of Kent). An endoplasmic reticulum retrieval motif was added to construct 3 in order to influence the intracellular trafficking of the protein (binding to KDEL normally results in proteins being retained in the ER and subsequently degraded). Restriction enzymes sites were added to ensure that the VHH gene could be digested and ligated into the plasmid of interest.

In order to make the constructs and produce enough DNA for ligating the genes into a plasmid, the genes were amplified by PCR. Initially *Taq* Polymerase was used; however the error rate appeared to be high with the formation of primer-dimers and smeared band sizes when running PCR products on an agarose gel. The constructs for ligand 2 and ligand 6 were amplified using, however a different enzyme had to be used for the ligand 7 constructs (Phire hotstart) despite several optimisation steps being taken and the manufacturer's advice being followed. It was expected that all of the PCR experiments would be successful using the same enzyme as the gene sequences are all of a similar length (450-500 bp) and fairly homologous. The only difference between each of the three ligand gene sequences is in the antigen binding region where one or two base pairs are different (data not shown, confidential to BAC BV). It is still unexplained why ligand 7 constructs could not be amplified successfully using Platinum® Pfx.

When it came to ligating the VHH gene constructs into pcDNA3.1, six of the nine VHH constructs were successfully ligated using a standard ligase enzyme reaction. However the remaining 3 VHH gene constructs (L2C1, L7C2 and L7C3) were not successfully ligated using a standard ligase enzyme reaction, and an alternative method had to be used, TA cloning. Again, there does not seem to be a logical explanation for this as the 3 constructs that required TA cloning are not from the same ligand family, for example, all ligand 7, nor are they all of the same construct design, for example all construct 1.

Sequencing of the plasmids was relatively straight forward apart from L7C2, where 3 individual colonies were analysed before the correct sequence was found. The first

two attempts at sequencing L7C2 showed a base change or a base deletion in the 3'end of the gene which encoded the histidine tag. Interestingly this is where the reverse primer would bind at the 3' end of the gene. A possible explanation for this is that there was some variation within the primer solution provided by the manufacturer. This could be due to an error in the manufacturing process and was therefore beyond the control of the experimenter.

In summary, all nine VHH plasmids were confirmed to have 100 % homology with the expected gene sequence and were therefore deemed ready for transfection into mammalian cell lines (chapter 4).

Chapter 4: Expression studies of VHH and FVIII

4.1 Introduction

In the previous chapter it was described how nine unique plasmids were made encoding VHH proteins, ready to be transfected into mammalian cells. This chapter describes the VHH expression studies that were conducted in mammalian cells, including co-expression in a FVIII expressing cell line. The expression of the VHH ligands was investigated at both the RNA and at the protein level, the results of which will be discussed in this chapter.

Prior to investigating RNA and protein expression of the VHH ligands, both physical and chemical transfection techniques were investigated. A GFP expressing plasmid was used in order to ensure that transfection conditions were optimal to allow introduction of the plasmids into the cells and to ensure that the cells were not exposed to toxic levels of reagents. The transfection methods tested included electroporation, where an electrical field was applied to the mammalian cells in order to increase the permeability of the plasma cell membrane and allow the introduction of the plasmid into the cell. Several chemical-based transfection methods were also tested including liposomal and non-liposomal cationic reagents that form a positively charged complex with the plasmid which then binds to the negatively charged cell surface residues. The DNA is then displaced from the cationic complex and enters the cell by endocytosis (see 2.3.5). The chemical method, in particular the cationic lipid-based reagent Lipofectamine®, proved to be the optimal transfection method and this was then employed for all further VHH transfection experiments.

Confirmation that the VHH were being expressed in mammalian cells was demonstrated by a series of experiments investigating both RNA and protein expression by PCR and western blotting respectively. Attempts at manufacturing stable cell lines expressing VHH were unsuccessful, so the majority of the VHH expression studies were carried out using transient transfections.

Upon confirmation that the VHH were successfully expressed in mammalian cells, the VHH plasmids were then transfected into a stable BHK-FVIII expressing cell line. In this cell line both VHH and FVIII was expressed at levels too low to be

detected in a standard western blotting experiment. Therefore immunoprecipitation was used in order to concentrate the sample (cell media and cell lysate tested) prior to carrying out western blotting again, as well as SYPRO® protein staining (see methods 2.3.6 and 2.3.7) to detect both the VHH and FVIII proteins.

Given the negligible or non-detectable levels of protein expression seen in the BHK-FVIII/VHH co-expression studies, an alternative assay method was sought in order to detect both the VHH and FVIII proteins. Several different ELISA based formats (both commercially available and in-house versions) were tested but unfortunately these experiments either did not work or did not provide any statistically significant results.

Studies investigating the function of FVIII and any effect co-expression with anti-FVIII VHH ligands may have had on this were also carried out (see chapter 5).

4.2 Experimental Aims

One of the experimental aims in this project was to successfully transfect Camelid antibody (VHH) plasmid constructs into mammalian cells. This initial step was taken in CHO-K1 cells to confirm VHH expression prior to co-expressing the VHH with FVIII. This was investigated using several techniques including RT-PCR and western blotting. Once this was confirmed, the two proteins (VHH and FVIII) could be co-expressed in BHK cells.

4.3 Optimisation of transfection conditions in CHO-K1 using a GFP plasmid

Transfections conditions were optimised in CHO-K1 cells by using a plasmid encoding for the reporter gene, green fluorescent protein before proceeding to any work using VHH plasmids. CHO-K1 cells were either electroporated with a GFP plasmid or transfected using chemical methods as described in 2.3.2. These experiments were carried out in order to ensure that the conditions imposed on the cell were not so toxic as to cause cell death before gene expression could be investigated.

4.3.1. Electroporation using a GFP plasmid in CHO-K1 cells

Efforts to optimise GFP expression included varying the amounts DNA plasmid per electroporation and testing various buffers (see appendix for composition). A variety of buffer compositions were found in the literature so it was important to find the optimal conditions for CHO-K1 cells. The best electroporation conditions of those tested for expression of GFP was found to be 10 µg plasmid DNA in PBS (see figure 4.1). To improve upon this further, a number of chemical transfection methods were tried to see if the amount of GFP-expressing cells could be further increased over and above what was seen in the electroporation experiments.

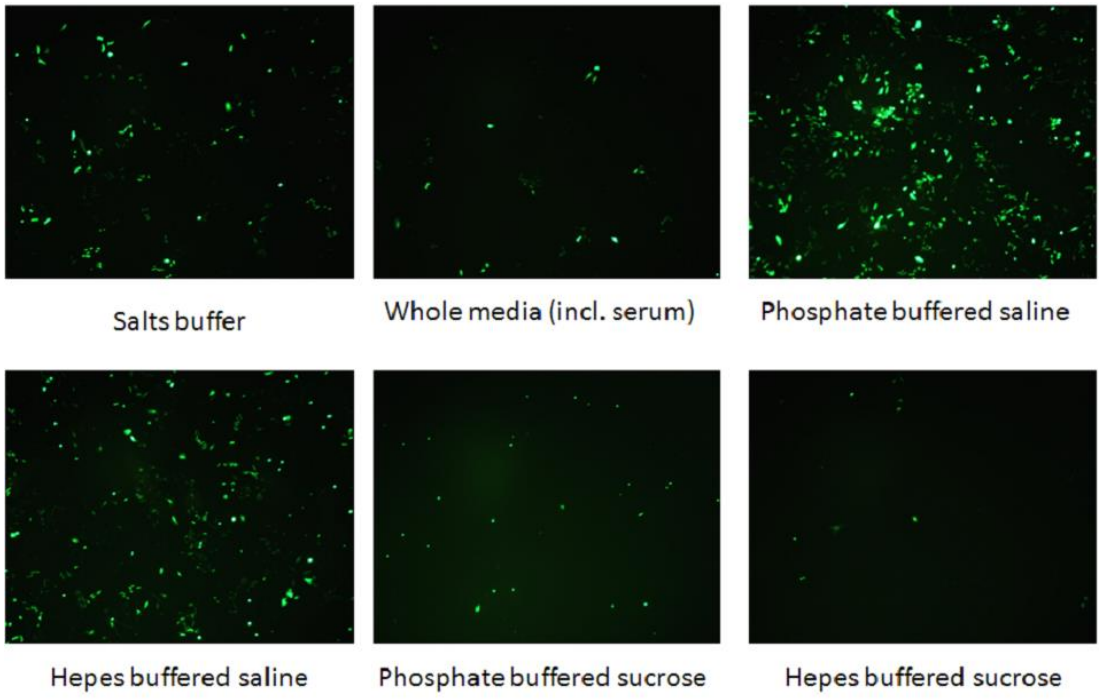


Figure 4.1. CHO-K1 cells transfected with GFP plasmid. A comparison of electroporation buffers and their effect on GFP expression in CHO-K1 cells.

A comparison of 6 different electroporation buffers on GFP expression in CHO-K1 cells showed that PBS was the best buffer in terms of the number of fluorescing cells. 10 µg plasmid DNA was used for each experiment. Magnification x 400.

4.3.2. Chemical transfection of GFP plasmid in CHO-K1

4 different commercially available chemical transfection reagents were tested as described in methods section 2.3.2.2, including cationic polymers (FuGENE® , TurboFect™ and PEI) and a cationic lipid-based product (Lipofectamine®).

FuGENE® was tested at various ratios to DNA as recommended by the manufacturer with the 4:1 ratio giving the highest number of fluorescing cells. However, none of the concentrations tested gave a high level of fluorescing cells indicating low transfection efficiency. This was also the case with PEI which was also tested according to the manufacturers' instructions (data not shown).

Both TurboFect™ treated cells and Lipofectamine® treated cells showed a higher percentage of fluorescing cells when compared with the FuGENE® and PEI treated cells (figure 4.2). Lipofectamine® appeared to be the best reagent out of all four chemical methods based on the highest proportion of fluorescing cells.

Having tested both the electroporation and chemical methods in CHO-K1 cells, it was clear that the chemical method (Lipofectamine®) gave the greatest GFP expression and this was therefore the method of choice for all future transfection experiments.

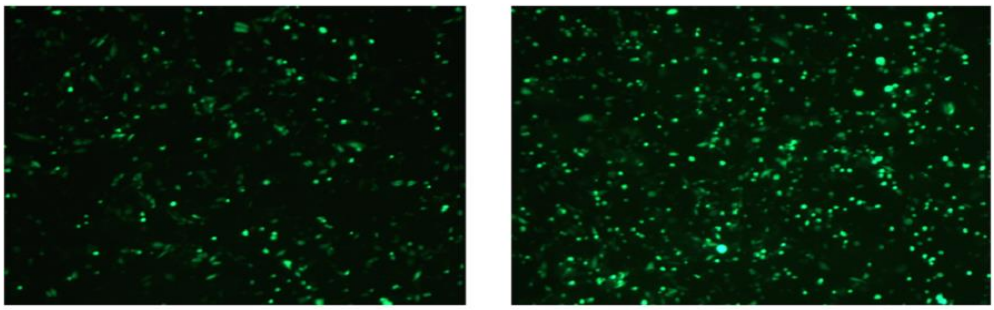


Figure 4.2. Comparison of TurboFect™ and Lipofectamine® transfection reagents of GFP expression in CHO-K1 cells.

A comparison of TurboFect™ treated cells and Lipofectamine® treated cells shows a higher number of fluorescing cells indicating higher transfection efficiency in the

Lipofectamine® treated cells. 4 µg plasmid DNA was used for each experiment Magnification x 400.

4.3.3. Chemical transfection of GFP plasmid in BHK cells

The results of GFP plasmid transfection experiments in CHO-K1 cells indicated that Lipofectamine® was the best performing reagent of the four tested, however the manufacturer had made a change to the product with the introduction of Lipofectamine® LTX (and PLUS reagent). Therefore an additional optimisation experiment was carried out in order to determine the optimal ratio of Lipofectamine® LTX: PLUS reagent. This was carried out as described in methods section 2.3.2.2. The optimal concentrations were found to be 7.5 µl of Lipofectamine® LTX, 0.5 µl of PLUS reagent and 4 µg plasmid DNA (see figure 4.3 and table 30)

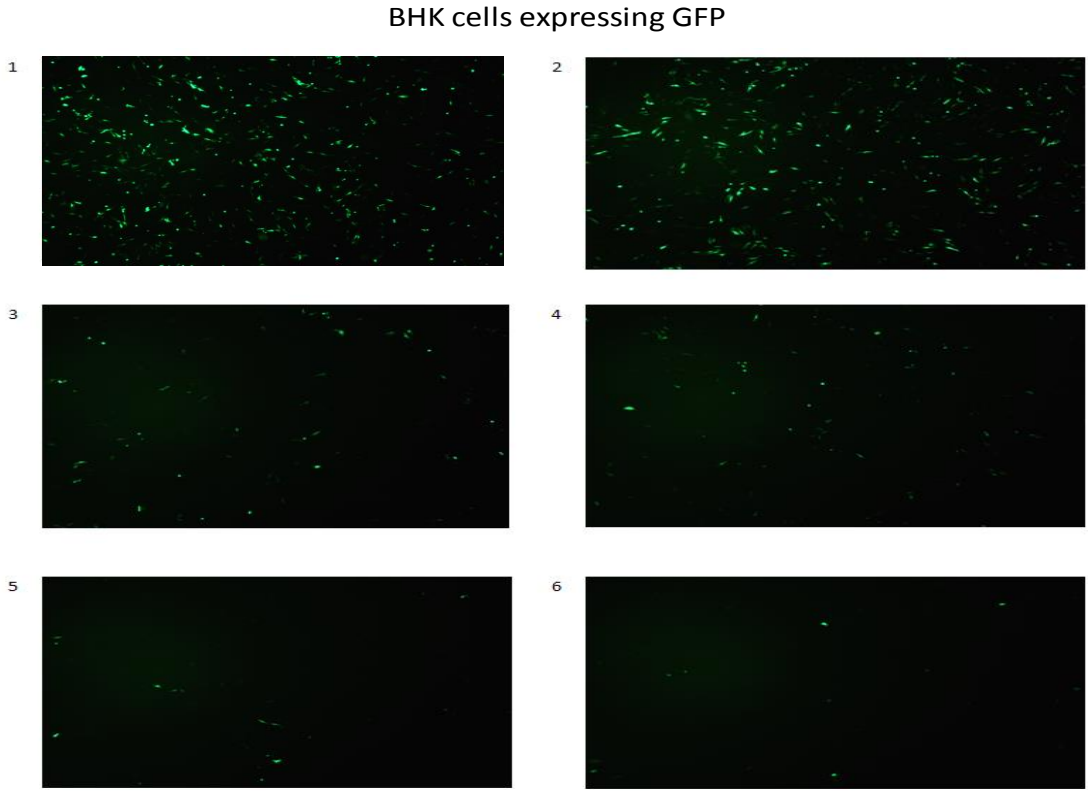


Figure 4.3. Optimisation of Lipofectamine® LTX transfection in BHK cells

Panels 1 to 6 represent wells 1 to 6 of a 6 well plate (each well was seeded at 2 x 10^5 cells/well). Panels 1 and 2 show the greatest GFP signal when compared to all other panels. Panel 1 was transfected with 7.5 µl of Lipofectamine® LTX and 0.5 µl of PLUS reagent. Wells 2 to 6 contain increasing amounts of both Lipofectamine® LTX and PLUS reagent as shown in table 30. Magnification x 400.

Table 30: Lipofectamine® LTX: PLUS regent ratios used for optimisation of transfection conditions

Well number of a 6 well plate	DNA (µg)	Lipofectamine® LTX (µl)	PLUS reagent (µl)
1	4	7.5	0.5
2	4	10	2.5
3	4	12.5	5
4	4	15	7.5
5	4	20	8
6	4	25	10

4.4 mRNA analysis by RT-PCR to confirm successful transfection of VHH in BHK cells

In order to assess the presence or absence of VHH mRNA in BHK cells, mRNA analysis was carried out using the beta-actin housekeeping gene as a positive control.

All cells were transfected using Lipofectamine® LTX (Invitrogen) as described in methods section 2.3.2.2. RNA was extracted from the cells as described in methods 2.3.5, and untransfected cells were used as a negative control for VHH expression. RNA was converted to cDNA and amplified using PCR. The PCR products were then run on an agarose gel to check for VHH or Beta-actin expression.

In each of the samples from cells transfected with the VHH plasmids, as well as the untransfected BHK cell line, the beta-actin housekeeping gene was detected as seen in figure 4.4 where bands can be seen at approximately 100 bp as expected.

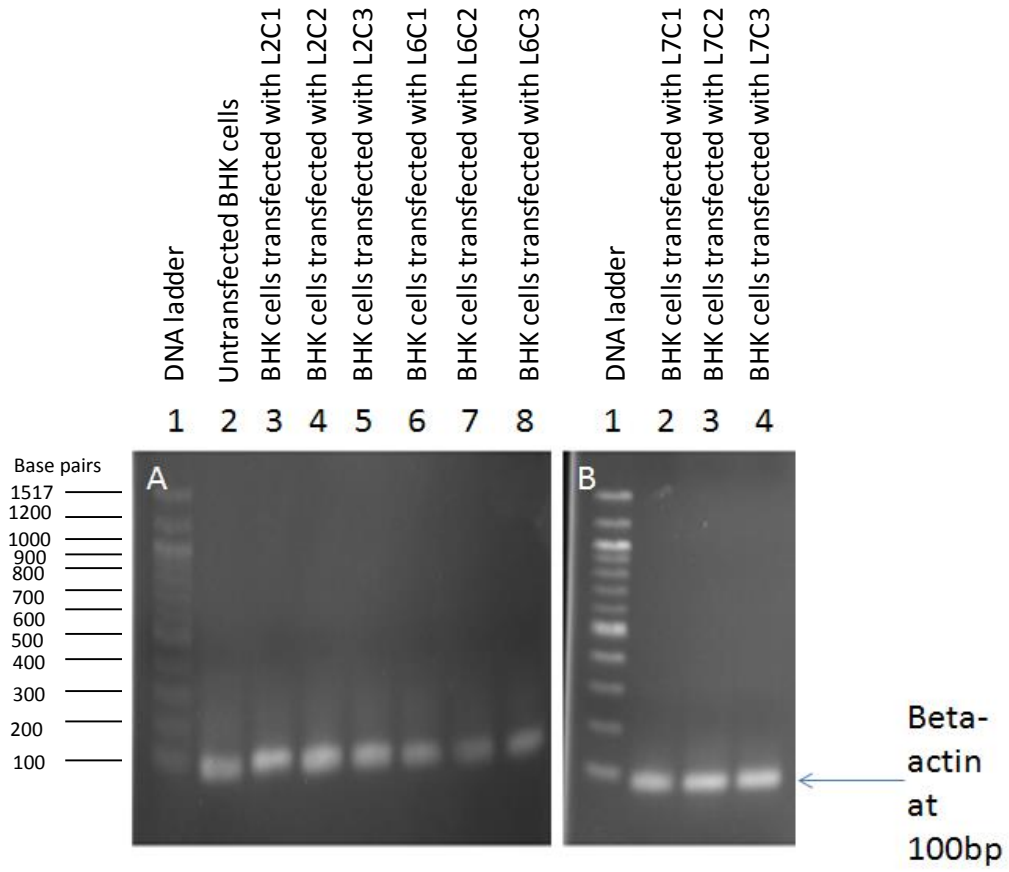


Figure 4.4. PCR products using primers for beta-actin gene after 48 hours cell growth.

RNA extracted from BHK-FVIII cells. **A:** Lane 1 DNA ladder, lane 2 untransfected BHK cells, lanes 3 to 8 cells transfected with L2C1, L2C2, L2C3, L6C1, L6C2 and L6C3 respectively. **B:** Lane 1 DNA ladder, lane 2 to 4 cells transfected with L7C1, L7C2 and L7C3.

Cells that were transfected with VHH plasmids were assessed for presence of VHH mRNA 48 hours after transfection. The 48 hour time point was chosen because this is how long BHK cells were grown before testing the cell media in the coagulation

assay. This is relevant because it was planned to co-express VHH and FVIII in the BHK cell line so it was imperative to check that VHH was still being expressed at this time point. For 6 of the VHH constructs (L2C2, L6C1, L6C2, L6C3, L7C2, L7C3) PCR products indicated the presence of VHH mRNA at 48 hours post transfection (see figure 4.5). However, for the remaining 3 VHH constructs (L2C1, L2C3 and L7C1), mRNA was not detected at 48 hours. For these 3 constructs RNA was extracted at an earlier time point of 24 hours after transfection instead of 48 hours and the PCR products were then successfully amplified (figure 4.6). It was thought that perhaps at 48 hours, the mRNA for these 3 constructs may have degraded and therefore could not be detected (see discussion 4.9).

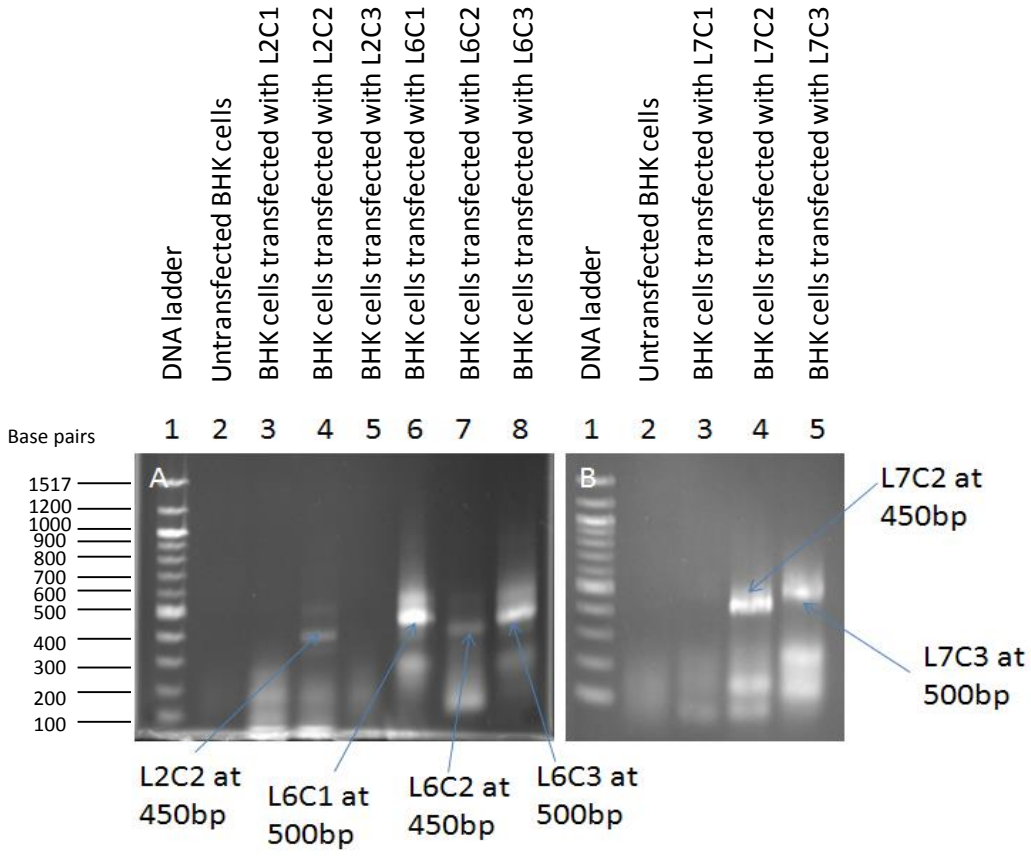


Figure 4.5. VHH PCR products, mRNA analysis 48 hours after transfection

A: Lane 1 DNA ladder, lane 2 untransfected BHK cells (negative control), lane 3 cells transfected with L2C1 (not detected), lane 4 cells transfected with L2C2 (band at approximately 450 bp), lane 5 cells transfected with L2C3 (not detected), lane 6 cells transfected with L6C1 (band at approximately 500 bp), lane 7 cells transfected with L6C2 (band at approximately 450 bp), lane 8 cells transfected with L6C3 (band

at approximately 500 bp). **B**: Lane 1 DNA ladder, lane 2 untransfected BHK cells (negative control), lane 3 cells transfected with L7C1 (not detected), lane 4 cells transfected with L7C2 (band at approximately 450 bp), lane 5 cells transfected with L7C3 (band at approximately 500 bp).

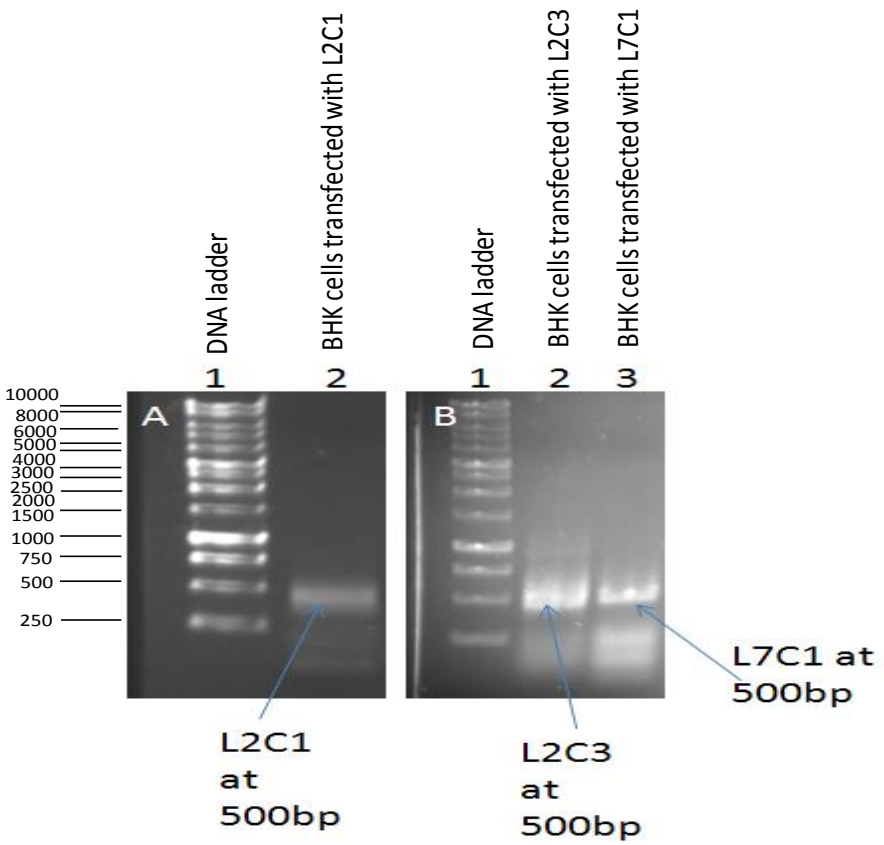


Figure 4.6 VHH PCR products, mRNA analysis 24 hours after transfection

A: Lane 1 DNA ladder, lane 2 cells transfected with L2C1 (band detected at approximately 500 bp). **B:** Lane 1 DNA ladder, lane 2 cells transfected with L2C3 (band detected at approximately 500 bp), lane 3 cells transfected with L7C1 (band detected at approximately 500 bp).

All nine VHH constructs were successfully transfected into the BHK cell line and subsequently expression of mRNA was detected by RT-PCR experiments either 24 or 48 hours after transfection. In all of the transfected cell samples, as well as the untransfected cells, the housekeeping gene Beta-actin was also detected.

4.5 Expression and detection of VHH ligands in CHO-K1 cell line

4.5.1 Western blotting to detect VHH (using anti-VHH and anti-His antibodies)

VHH plasmids were transfected into CHO-K1 cells using Lipofectamine® as described in 2.3.2.2. In order to detect if the VHH were being expressed at the protein level, several dot blots and western blotting experiments were carried out as described in sections 2.3.6. All nine VHH constructs were expressed in CHO-K1 cells, with variable results. An example of a western blot showing possible L2C1 protein expression can be seen in figure 4.7. There are 4 bands seen at approximately 12 KDa, which is the size expected for the VHH protein (12-15 KDa). 2 VHH standard solutions were also run on the same agarose gel (lane 5 and 6, both supplied by BAC) and gave bands at the expected sizes. There are also some other bands on the top half of the blot which are unidentified and could potentially be aggregated VHH protein.

This experiment also compared the effect of linearising the plasmid (see 2.2.10) prior to transfection. When comparing media samples from cells transfected with a linearised plasmid to those that were transfected with a whole plasmid, the highest VHH expression can be seen with the linearised plasmid (lane 4 of figure 4.9).

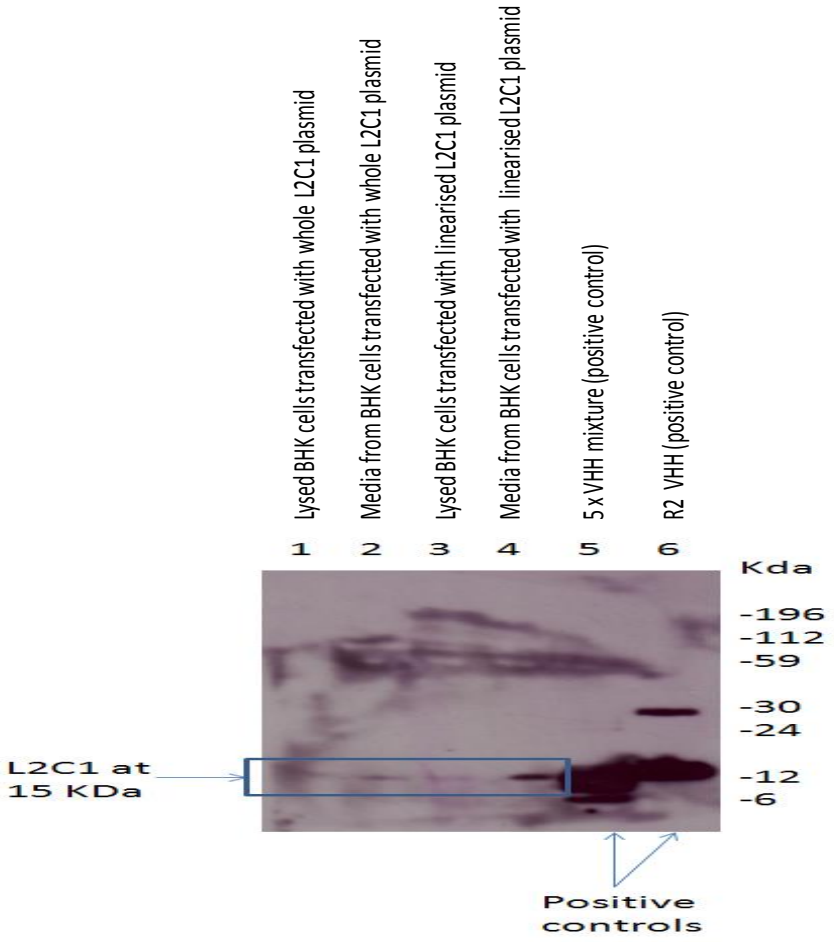


Figure 4.7. Anti –VHH western blot to detect VHH expression in CHO-K1 cells

Western blotted PVDF membrane using rabbit anti-VHH antibody as the primary antibody and goat anti-rabbit antibody as the secondary antibody. Lane 1; Lysed cells transfected with whole L2C1 plasmid, 2; Media from cells transfected with whole L2C1 plasmid, 3; Lysed cells transfected with linearised L2C1 plasmid, 4; Media from cells transfected with linearised L2C1 plasmid, 5; 5 x VHH mixture (positive control), 6; R2 VHH (positive control). Lanes 1 to 4 show bands at approximately 12 KDa which is close to the approximate size expected for the L2C1 VHH (12-15 KDa). The media sample from cells transfected with linearised plasmid (lane 4) shows the highest expression of L2C1. A negative control was not included in this blot.

In addition to western blots using anti-VHH antibodies as a detection method, anti-His antibodies were also used to detect those six VHH ligands that had a Histidine tag encoded in their gene sequence (constructs 1 and 2). An example of an anti-His western blot can be seen in figure 4.8. Only one of the 4 L2C1 samples tested (media

from cells transfected with linearised L2C1 plasmid, lane 4) gave a faint band indicating very low protein expression. It may be that the histidine tag had been cleaved by the cell during protein production and therefore could not be detected by western blot. Cleavage of histidine tag was not investigated further in this study. This experiment was repeated on several occasions and at no point was the anti-His signal improved, for any of the histidine tagged VHH proteins.

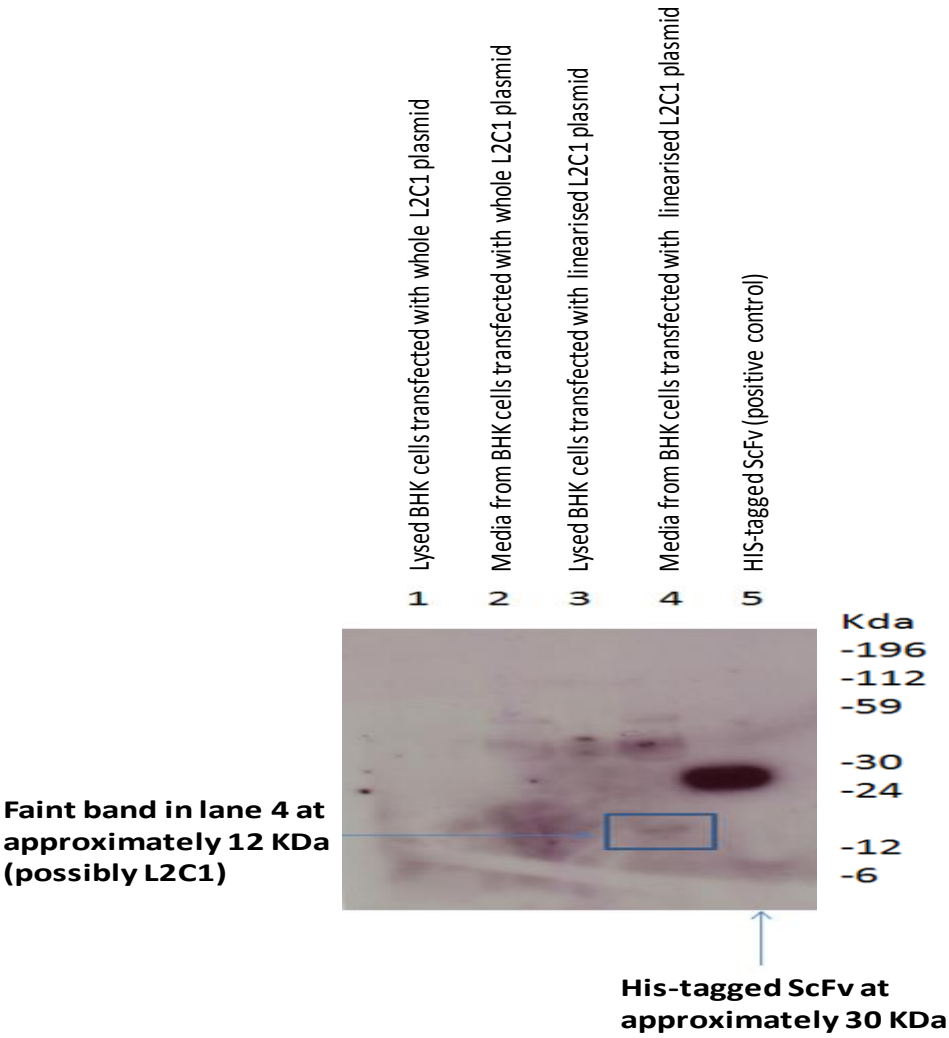


Figure 4.8. Anti-His western blot to detect L2C1 expression in CHO-K1 cells

Western blotted PVDF membrane using Anti-His antibody (conjugated to HRP). Lane 1; Lysed cells transfected with whole L2C1 plasmid, 2; Media from cells transfected with whole L2C1 plasmid, 3; Lysed cells transfected with linearised L2C1 plasmid, 4; Media from cells transfected with linearised L2C1 plasmid, 5; His-tagged ScFv. Lanes 4 shows a very faint band at approximately 12 KDa which is

the size expected for L2C1 protein (12-15 KDa). No negative control was included in this experiment.

4.5.2 Nickel column purification of His-tagged proteins

Since the histidine-tagged constructs could barely be detected in an anti-His western blot, cell culture media from cells transfected with plasmids encoding for histidine-tagged VHH was purified on a nickel column (see methods 2.5.6). Samples were collected from the column over a range of imidazole eluting buffer concentrations. Each of these samples were analysed in a dot blot using an anti-His antibody as described in methods 2.3.8.

For cell culture media from cells transfected with L2C2 plasmid (construct encodes for histidine tag at 3' end), a signal was seen in samples eluted at 150mM imidazole. These samples were tested in two western blots under both denaturing and non-denaturing conditions using either anti-VHH or anti-His antibodies. The anti-VHH western blot showed no bands at the expected VHH protein size in both the denaturing and non-denaturing conditions. The non-denaturing anti-His western blot did show one band at approximately 12KDa which is likely to be a histidine tagged VHH (see figure 4.9).

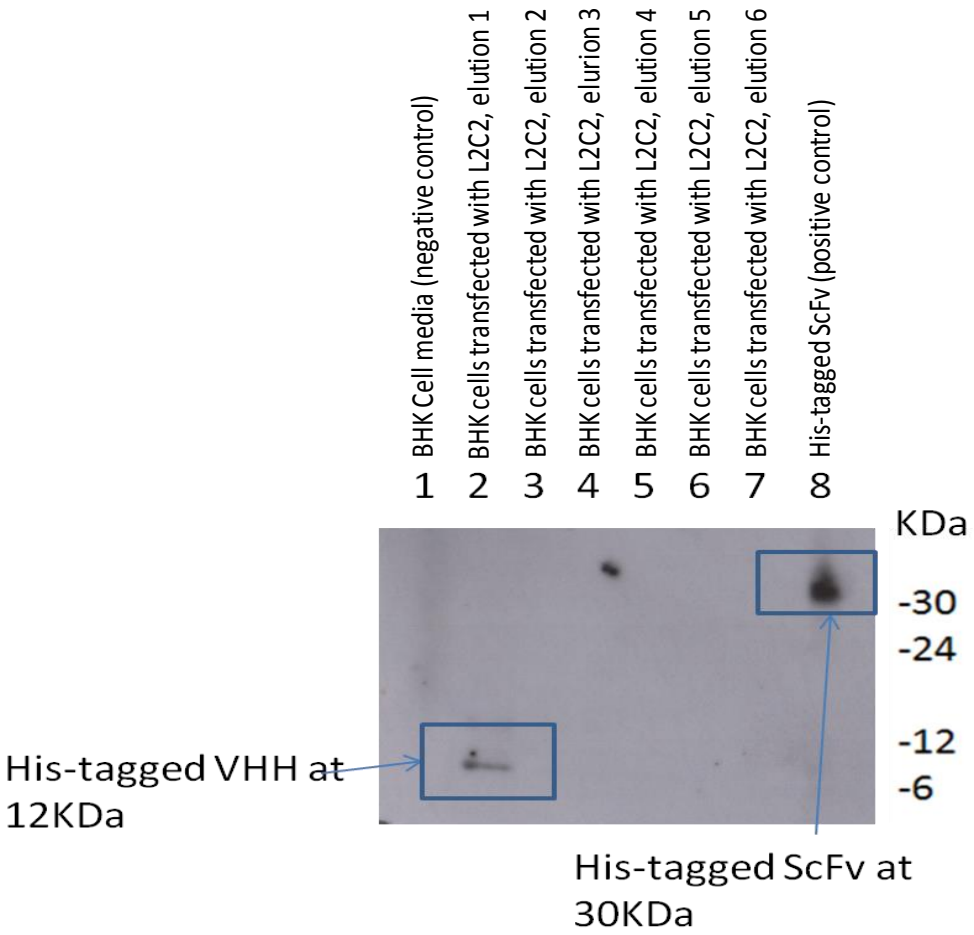


Figure 4.9 Anti-His western blot (nickel column eluted samples)

Lane 1: BHK cell media (untransfected cells); lane 2: L2C2 transfected cell media, elution sample 1; lane 3: L2C2 transfected cell media, elution sample 2; lane 4: L2C2 transfected cell media, elution sample 3; lane 5: L2C2 transfected cell media, elution sample 4; lane 6: L2C2 transfected cell media, elution sample 5; lane 7: L2C2 transfected cell media, elution sample 6; lane 8: positive control (his-tagged ScFv, 30 KDa).

4.6 Development of stable CHO-K1 cell lines expressing VHH

Multiple attempts were made at developing stable CHO-K1 cell lines expressing each of the nine VHH proteins as described in 2.3.3. Both electroporation and chemical methods of transfection were tested (as described in 2.3.2) in order to try and produce a stable cell line. During the course of developing a stable cell line, different conditions were tested, similar to the GFP expression studies including both

electroporation and chemical transfection methods. Various buffers were tested, a range of VHH plasmid DNA concentrations, as well as trying both linearised and whole versions of the VHH plasmids. It is regretful and disappointing that despite all of the resources and attempts employed, none of the approaches were successful in producing a stable VHH cell line, however, the steps taken and the limited results will be outlined here.

Once the cells had been transfected with VHH plasmid, the cell media and lysed cells were tested at regular intervals in a dot blot using anti-VHH antibodies to detect expression of VHH protein. Although VHH protein expression could be detected 24 hours after transfecting the cells (by dot blot analysis using an anti-VHH antibody), subsequent VHH protein expression was lost (see 3 examples in figure 4.10). The loss of VHH protein expression was evident just 10 days after transfection, as the VHH could not be detected in cell culture media or lysed cells, in neither a dot blot nor a western blot (both denaturing and non-denaturing conditions were tested).

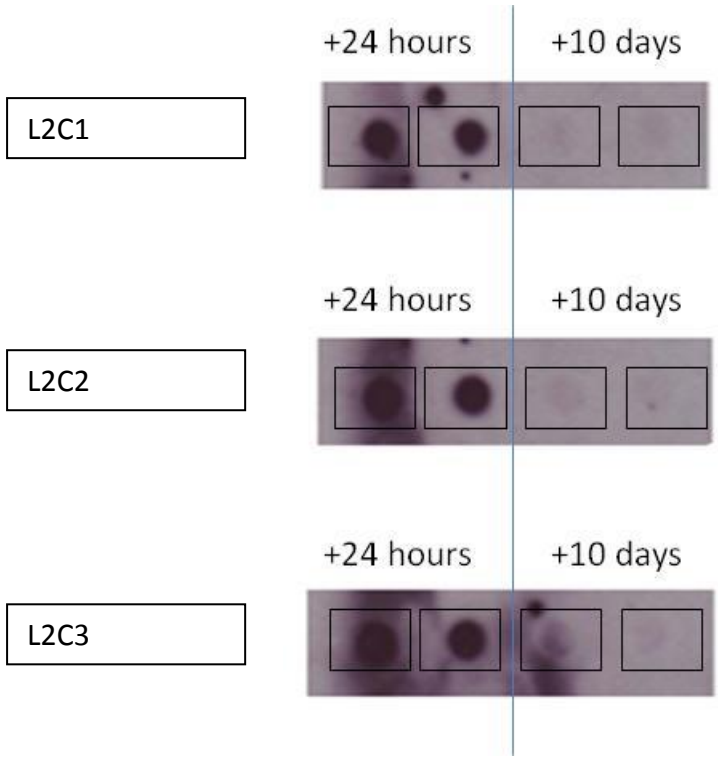


Figure 4.10. Dot blot using anti-VHH antibodies to detect VHH expression in CHO-K1 cell media (in the presence of G418).

VHH expression can clearly be seen 24 hours after addition of genetecin to the media (dots 1 and 2), but cannot be seen after 10 days (dots 3 and 4). 100 µl of cell media in each well.

No VHH protein expression was seen after 10 days for any of the VHH plasmids tested, and this could have been due to a very low percentage of cells incorporating the VHH gene into their genome. Consequently the single cell cloning method was used to try and grow any cells which may have incorporated the VHH gene.

Transfected cells were grown in the presence of genetecin and consequently any cells that had not taken up the VHH gene would eventually die. As expected, the majority of the cells died in the presence of 400 µg / ml genetecin after a couple of weeks. Those few cells that survived the presence of genetecin were taken through to single cell cloning. None of the clones that were picked and bulked up showed any sign of VHH expression in dot blots or western blots, despite the fact they were growing in the presence of genetecin.

Due to project time constraints, it was decided that a stable CHO-K1 cell line expressing VHH, although interesting to study, was not an essential element to fulfilling the aims of this project, and therefore transient transfections became the method of choice for all future investigations into VHH expression.

4.7 Co-expression of VHH and FVIII in BHK cells

The ultimate aim of this project was to co-express VHH and FVIII together in mammalian cells in order to assess the effect of VHH on FVIII expression levels. Two approaches were investigated; one being the development of a stable CHO-K1 cell line expressing VHH which could then be transfected with a plasmid encoding the FVIII protein; the second approach was to take a stable BHK cell line that expresses FVIII (available in-house) and transiently transfect these cells with plasmids encoding the VHH ligands.

The first approach was unsuccessful as a stable CHO-K1 cell line expressing VHH could not be developed as previously described.

The second approach was more promising as VHH mRNA expression was detected between 24 and 48 hours after transfection into the BHK – FVIII cell line as described previously in section 4.4. Lysed cells and cell media were tested for VHH protein expression after a 48 hour incubation period using western blotting as described in 2.3.6.2. The VHH proteins could not be detected in a western blot (under reducing and non-reducing conditions) in any of the transfected cell lines, and this was thought to be due to low expression levels. VHH detection in a western blot was also limited by the availability and specificity of anti-VHH antibodies (see communication 2 from Professor Serge Muyldermans).

FVIII protein expression was also investigated in the BHK cell line. FVIII protein could not be detected in a western blot despite several optimisation attempts. Although the BHK cell line had been developed to express B domain deleted FVIII by Pete Lollar et al, the levels were so low that they could not be detected in a western blot. In the many publications where FVIII is detected, it is widely acknowledged that a functional coagulation assay is the preferable method of FVIII

detection as it is more sensitive than traditional protein detection methods such as western blot. This is described in detail in chapter 5.

4.7.1 Immunoprecipitation of VHH

An alternative approach was sought in order to detect the VHH and FVIII proteins using immunoprecipitation (2.3.7), followed by western blotting and SYPRO® protein staining (2.3.9). Immunoprecipitation was carried out using an anti-VHH antibody in order to purify VHH proteins from the cell media of transfected cells (plus untransfected cell samples for a negative control). These samples were then analysed in both an anti-VHH western blot (Figure 4.11) and an anti-FVIII western blot (Figure 4.12). The anti-VHH western blot showed some faint bands between 6 KDa and 15 KDA for each of the media samples collected from the cell lines transfected with ligand 2, ligand 6 and ligand 7 VHH constructs. This could indicate VHH expression, albeit at a very low level, as the expected size of these proteins is between 12-15KDa. No bands at the VHH protein size were seen in the untransfected cell line. A darker band can be seen in the samples from the cells transfected with L7C3 compared to samples from the cell lines transfected with the other 8 VHH constructs, which could simply be carrying over from the other lanes or be an indication of a greater amount of protein expression versus the other VHH. But, since this was not a quantitative assay conclusive comparisons could not be made on protein expression levels between the different VHH.

It was not expected that construct 2 for each of the ligands would be found in the cell culture media as they did not have a leader sequence encoded in their plasmid and therefore should not have been secreted into the cell culture media via the ER/Golgi pathway. However it may be that a small amount of cells had lysed, thereby releasing the contents of their cytosol into the surrounding media, potentially explaining the presence of construct 2 in the cell culture samples.

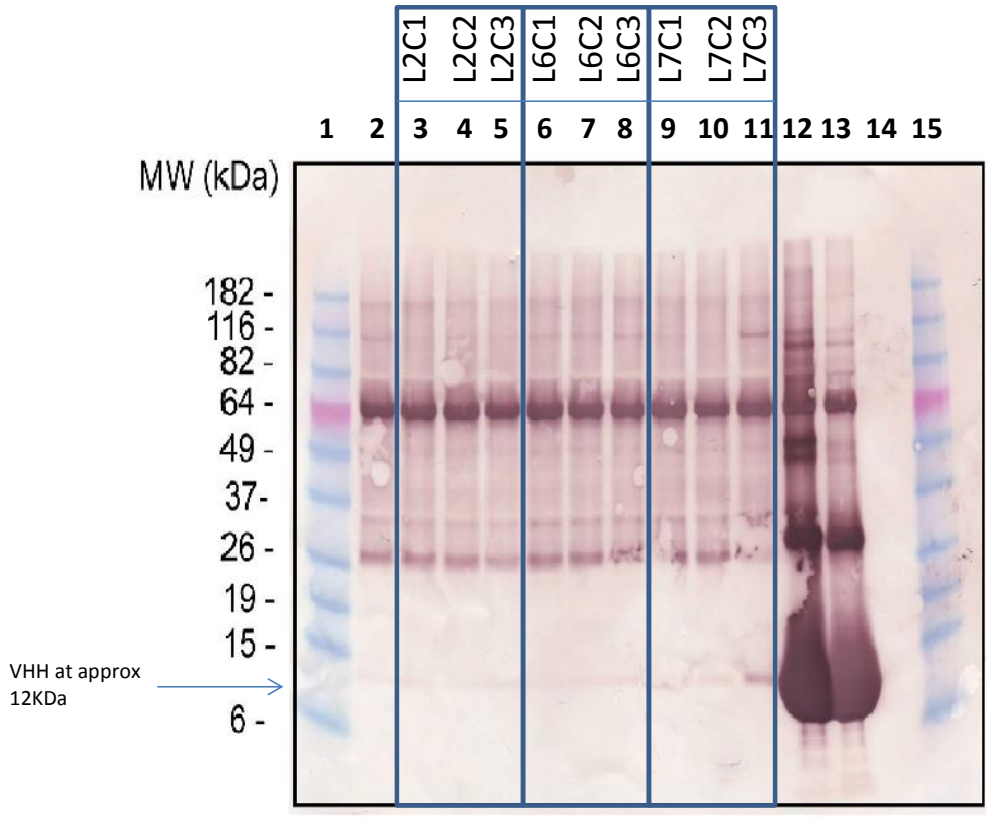


Figure 4.11 Anti-VHH western blot after immunoprecipitation of BHK-FVIII cell media samples.

Lane 1 and 15 protein size ladder, lane 2, untransfected cells (negative control). lane 3, L2C1 transfected cells, lane 4, L2C2 transfected cells, lane 5, L2C3 transfected cells, lane 6, L6C1 transfected cells, lane 7, L6C2 transfected cells, lane 8, L6C3 transfected cells, lane 9, L7C1 transfected cells, lane 10, L7C2 transfected cells, lane 11 L7C3 transfected cells, lane 12 and lane 13 positive controls (VHH), lane 14 is empty. Faint bands can be seen between 6 - 15 KDa for some of the VHH transfected cell lines. The band for L7C3 is darker than for the other VHHs, which could indicate a higher amount of VHH protein expression in this sample, or carryover from another lane.

The anti-FVIII western blot (Figure 4.12) was carried out as it was known that some of the VHH ligands have anti-FVIII activity (ligand 2 and ligand 7) and therefore they may have been co-purified along with the VHH ligands. Several bands could

clearly be seen at 80 KDa, the size of the FVIII light chain for cell samples transfected with ligand 2 and ligand 7 constructs. No bands for the FVIII light chain could be seen for any of the cell samples transfected with ligand 6, which was expected since ligand 6 does not bind FVIII.

For those cells transfected with L2C2, L7C1, and L7C3, darker bands could be seen compared to the L2C1, L2C3 and L7C2 transfected samples. Given that the immunoprecipitation was carried out on cell media, it was surprising to see that the sample from L2C2 transfected cells gave a strong signal. This is because the plasmid encoding construct 2 does not contain a leader sequence, and should not be secreted from the cell. Therefore the presence of L2C2/FVIII in the cell media suggests some degree of cell lysis. It is also interesting to see that the cell media from L7C3 transfected cells gave a strong signal since the plasmid encoding L7C3 contains KDEL, and could have resulted in the L7C3/FVIII complex being retained inside the cell. There are two possible reasons why L7C3 may be secreted into the media even in the presence of KDEL; the KDEL receptor may have been saturated with VHH causing the excess to be secreted, although this seems unlikely given the low expression levels previously demonstrated. Alternatively, the binding of VHH to the FVIII protein may be interfering with the way the VHH interacts with the KDEL receptor, resulting in the protein being secreted due to the presence of the leader sequence.

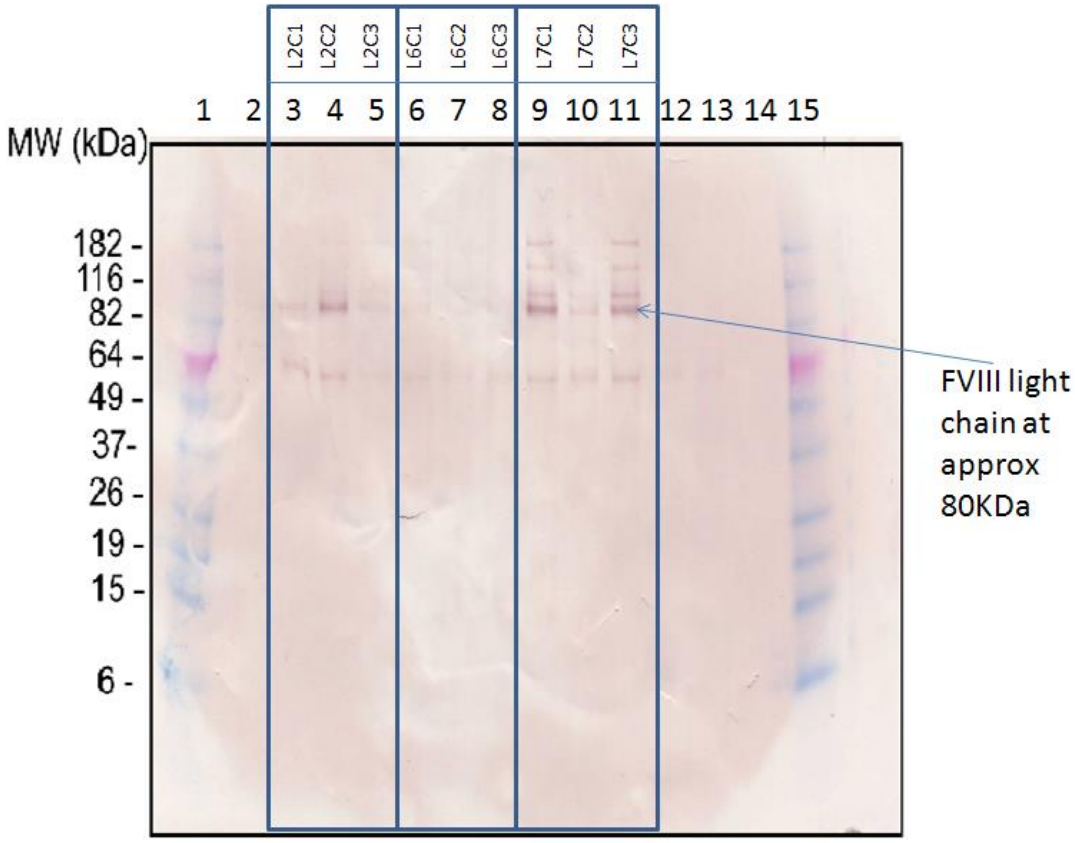


Figure 4.12. Anti-FVIII western blot after immunoprecipitation of cell media samples.

Lane 1 and 15 protein size ladder, lane 2, untransfected cells (negative control). lane 3, L2C1 transfected cells, lane 4, L2C2 transfected cells, lane 5, L2C3 transfected cells, lane 6, L6C1 transfected cells, lane 7, L6C2 transfected cells, lane 8, L6C3 transfected cells, lane 9, L7C1 transfected cells, lane 10, L7C2 transfected cells, lane 11 L7C3 transfected cells, lane 12, 13 and 14 empty wells. Several bands can clearly be seen at approximately 80 KDa (the size of the FVIII light chain) for cell samples transfected with L2C2, L7C1, and L7C3. Faint bands can be seen at 80 KDa for L2C1 and L7C2 transfected samples. No bands for the FVIII light chain can be seen for any of the cell samples transfected with ligand 6 (as expected).

A protein staining technique, SYPRO® Ruby stain was used to detect proteins in all of the samples resulting from the immunoprecipitation experiment (2.5.5). Since SYPRO® is a fluorescent stain that is sensitive to light it was important to analyse the stained gel as quickly as possible once the incubation time was reached as any exposure to light would reduce the signal. Figure 4.13 shows the best image that was

captured using a fluorescent imager. This was the third image to be captured after the SYPRO® incubation time, since some of the parameters of the imager had to be changed in order to capture the best image. The corner of the gel had to be covered up as the signal from the positive controls was so strong that it was impacting the quality of the image. Due to exposing the gel three times to capture the best image, some of the fluorescent signal was lost, however it was still possible to see some bands between 6 - 15 KDa in the media samples from transfected cell lines, which is the approximate size of the VHH proteins (between 12-15 KDa). It is difficult to pinpoint which bands on the gel could be the FVIII light chain due to multiple bands in the size region expected (approximately 80 KDa). Also it looks as though there may have been a loading error with carryover from lane 3 into lane 2 as there are some unexpected bands in the negative control.

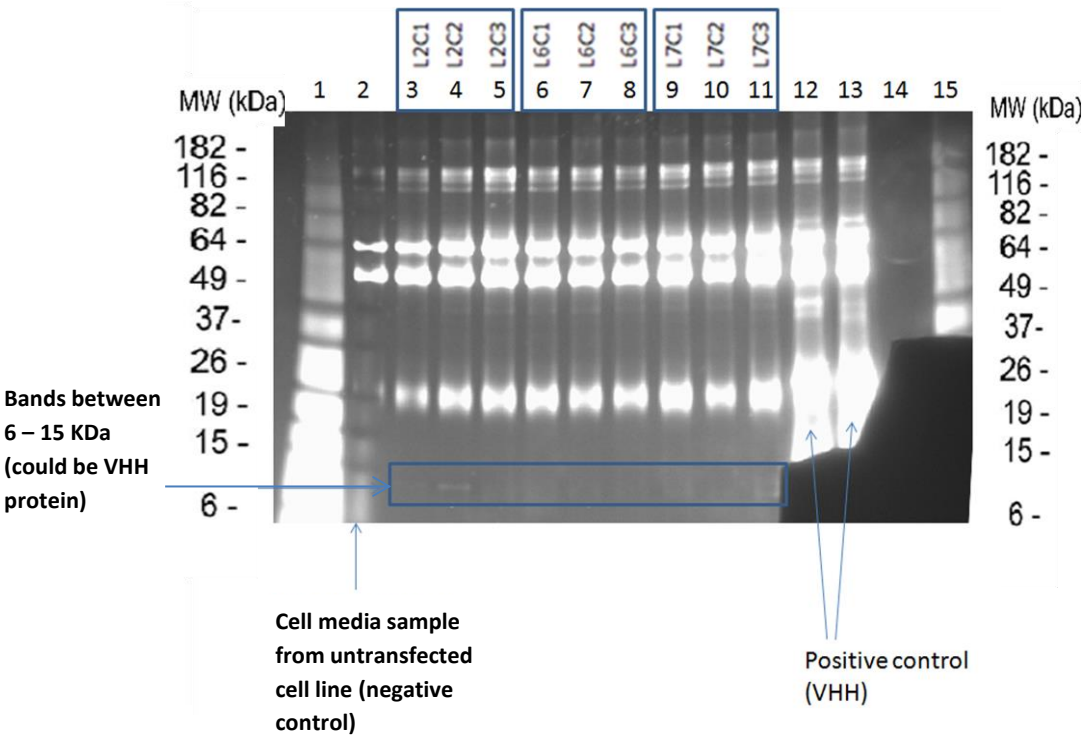


Figure 4.13. SYPRO® stained SDS-PAGE agarose gel of immunoprecipitated proteins.

Lane 1 and 15 protein size ladder, lane 2, untransfected cells (negative control). lane 3, L2C1 transfected cells, lane 4, L2C2 transfected cells, lane 5, L2C3 transfected cells, lane 6, L6C1 transfected cells, lane 7, L6C2 transfected cells, lane 8, L6C3

transfected cells, lane 9, L7C1 transfected cells, lane 10, L7C2 transfected cells, lane 11 L7C3 transfected cells, lane 12 and lane 13 positive controls (VHH), lane 14 is empty. Very faint bands can be seen at approximately 15 KDa for samples transfected with L2C2 and L7C3.

4.8 Enzyme Linked Immuno-Sorbent Assay (ELISA)

4.8.1 CaptureSelect™ VIII Leakage ELISA

At the time of the project there was a commercially available anti-VHH ELISA, the CaptureSelect™ VIII Leakage ELISA (ThermoFisher). This was originally designed for the detection of less than 1 ng/mL Factor VIII ligand (BAC's VHH ligand 7) that may be present in FVIII product purified with GE-Healthcare's VIII Select product. This is a sandwich assay where anti-ligand 7 is bound to a microtiter plate, to which samples containing ligand 7 bind, followed by a biotinylated affinity ligand, which is then detected by a streptavidin horseradish peroxidase substrate reaction.

This ELISA kit was used to test cell media and lysed cell samples from cells transfected with VHH plasmid (these experiments were conducted in the BAC laboratory in the Netherlands).

The CaptureSelect™ VIII leakage assay enabled detection of the VHH in solutions with and without the presence of the target protein i.e. VHH alone or VHH bound to FVIII (see figure 4.14).

Figure 4.14. ELISA format, Capture Select Leakage ELISA

Assay 1: Original manufacturer specified assay format to detect VHH in the cell media or cell lysate of cells transfected with VHH plasmid;

Assay 2: Modified manufacturer specified assay format to detect VHH:FVIII complex in cell media or cell lysate of BHK cells transfected with VHH plasmids using a mouse anti-FVIII antibody as a secondary antibody;

Assay 3: Modified manufacturer specified assay format to detect VHH:FVIII complex in cell media or cell lysate of BHK cells transfected with VHH plasmids using a sheep anti-FVIII antibody as a secondary antibody.

Standard curves were generated as advised by the manufacturer. An example standard curve can be seen in Figure 4.15.

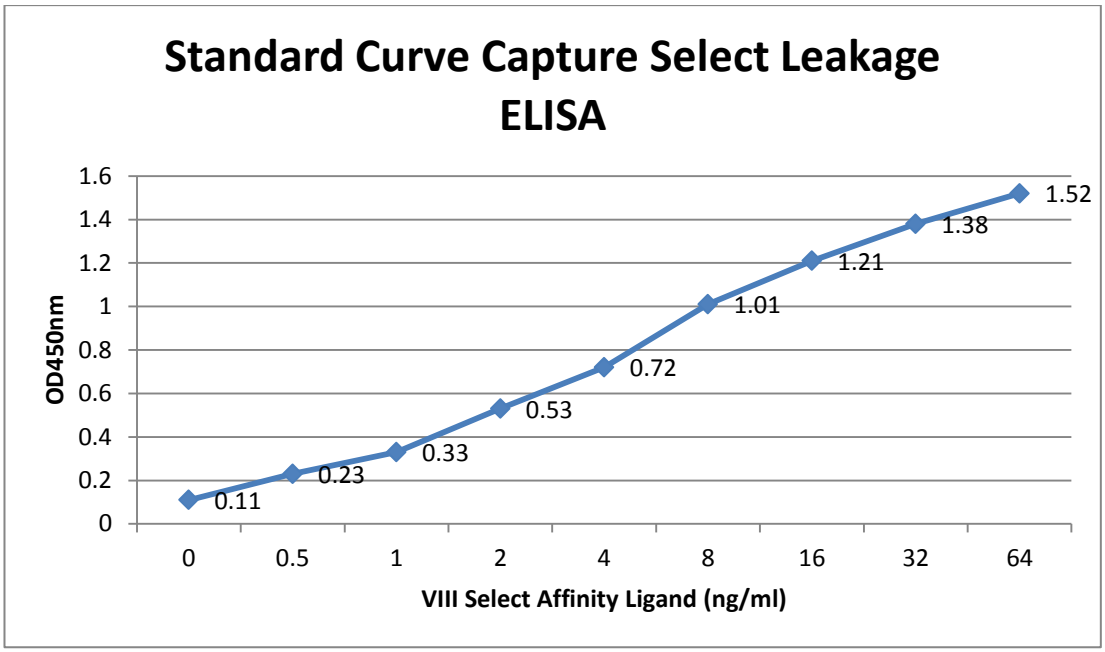


Figure 4.15. Example Standard Curve, CaptureSelect VIII Leakage ELISA

In this experiment, it was expected that the cell media samples from BHK cells transfected with ligand 7 would be detected in the ELISA.

Under the conditions specified by the manufacturer, none of the samples (tested both undiluted cell media and cell lysate) taken from BHK cells transfected with ligand 7 showed an absorbance reading above 0.06 nm. Therefore none of the data could show conclusive evidence that ligand 7 VHH was being expressed and alternative assay formats were investigated. Given that the ELISA kit was designed by the manufacturer to detect 1 ng/mL VHH ligand 7, it would indicate that if VHH ligand 7 was present in these samples, the concentration is less than 1ng/mL.

Assays 2 and 3 were developed to use the same coating antibody from the CaptureSelect FVIII leakage ELISA; however the secondary antibodies were anti-FVIII not anti-VHH. The aim of these experiments was to detect any VHH ligand 7 that may have formed a complex with FVIII in the cell media of BHK cells transfected with ligand 7. The anti-FVIII secondary antibodies tested were either from a mouse (monoclonal), or sheep (polyclonal). It was known from personal communication with the manufacturer (see Communication) that the polyclonal anti-FVIII antibody recognises variable epitopes between lots of product. The immunogen used to prepare these antibodies is full-length Factor VIII, and therefore epitopes throughout the protein including the B-domain will likely be recognised to

varying degrees by different lots. This is why it was decided to test the mouse monoclonal antibody first and then the sheep polyclonal antibody.

In this experiment, it was not possible to generate a standard curve and use it measure protein concentration since a solution containing VHH: FVIII complex was not available. However, it may have been possible to detect a difference between the control sample (cell media from untransfected BHK cells that contained FVIII) and the samples from BHK cells transfected with ligand 7.

Assay format 2 using mouse monoclonal anti-FVIII as the secondary antibody:

It was hypothesised that the cell media samples taken from BHK cells transfected with ligand 7 may generate a positive result in the ELISA (a higher absorbance reading than the untransfected sample). However none of the samples gave absorbance readings above 0.06 nm and therefore were not able to discern any difference between the untransfected and transfected samples.

Assay format 3 using sheep polyclonal anti-FVIII as the secondary antibody:

Although this assay was able to produce substantial absorbance readings (OD above 0.8 for test samples), there was variability in the data even with a small sample size (n=6), and no significant difference in the absorbance readings between the untransfected and transfected cell media samples (see figure 4.16). Therefore it was concluded that this assay format would not be suitable for confirming the presence or absence of a ligand 7: FVIII complex in cell media samples.

These three assays were not able to confirm ligand 7 expression in any of the samples tested.

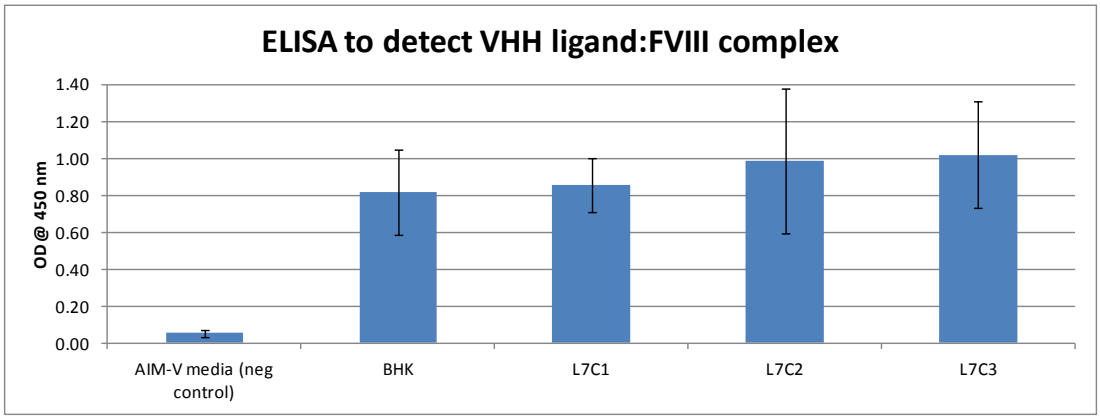


Figure 4.16. Assay format 3 to detect ligand 7:FVIII complex (n=6 per sample type)

4.8.2 ELISA

Due to the limited selection and prohibitive cost of the commercial ELISA kits, several ELISA formats were also designed and optimised in-house. Multiple formats were tested for both VHH and FVIII proteins and these are summarised in figure 4.17.

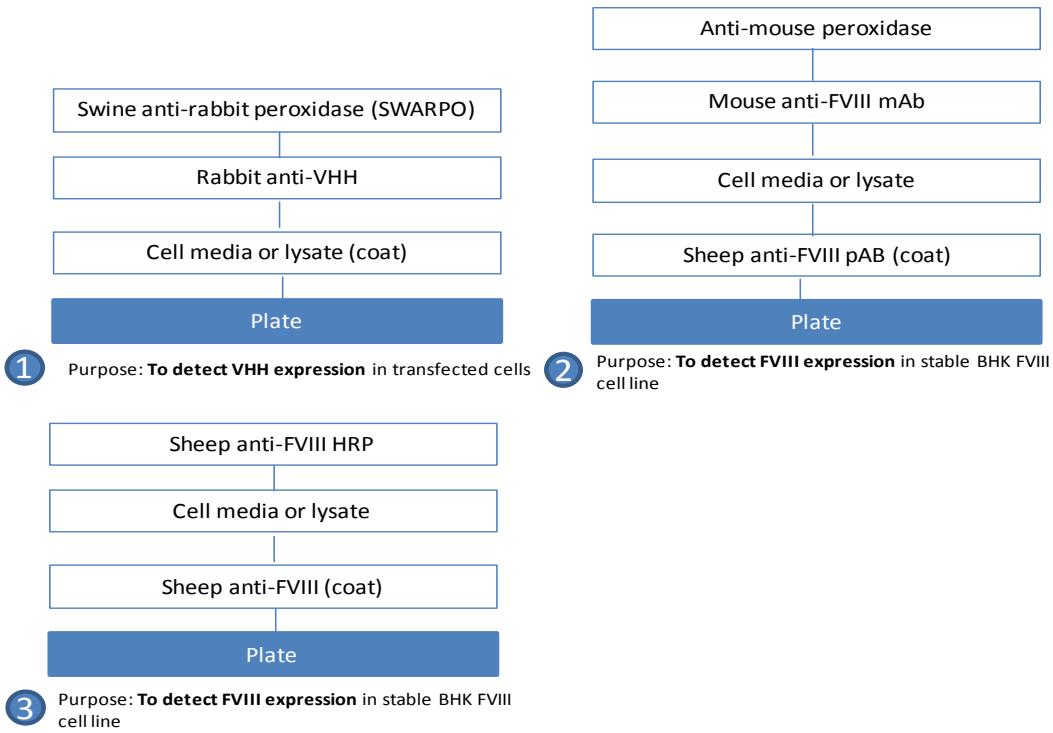


Figure 4.17. ELISA formats to test for VHH or FVIII protein expression

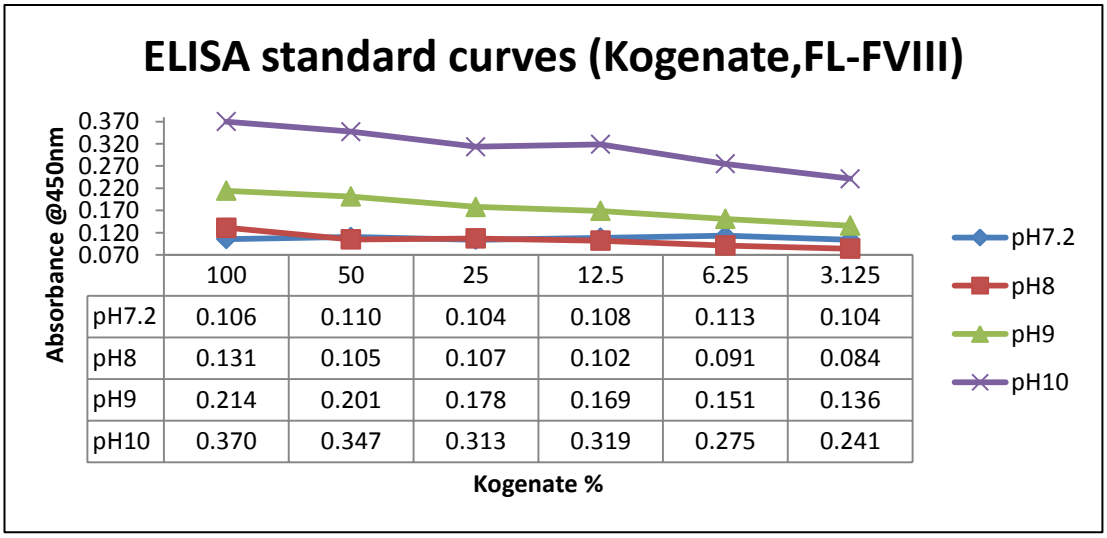
Assay 1: Detection of VHH in cell media or cell lysate of cells transfected with VHH plasmid; Assay 2 and 3: For the detection of FVIII in BHK cell media or cell lysate

Assay 1: Detection of VHH in cell media or cell lysate of cells transfected with VHH plasmid:

The microtiter plate was coated directly with cell culture media from either untransfected BHK cells, or cells transfected with VHH plasmid. The untransfected cell culture media samples were treated as the baseline (no VHH expression = 1) and data from transfected cells normalised to this. Out of the 54 transfected cell samples tested only 2 of those showed a positive fold change compared to the untransfected cells and these were one L7C1 sample and one L7C3 sample. However these data were not reproducible for any other samples tested and therefore this assay format was not developed any further.

Assay 2: For the detection of FVIII in BHK cell media or cell lysate:

The first step was to generate a standard curve. This was done using dilutions of Kogenate® which is a human full length FVIII product currently registered for treatment of haemophilia. The plates were coated at a range of pH including 7.2, 8, 9 and 10. The best 'standard curve' (that which gave the greatest difference between the lowest and highest data points) was generated at pH 10 (see Figure 4.18). However when cell culture supernatants from transfected and untransfected BHK cells were tested, no signal was seen, probably due to levels of FVIII expression below the limit of detection in this assay.



	100	50	25	12.5	6.25	3.125
pH7.2	0.106	0.110	0.104	0.108	0.113	0.104
pH8	0.131	0.105	0.107	0.102	0.091	0.084
pH9	0.214	0.201	0.178	0.169	0.151	0.136
pH10	0.370	0.347	0.313	0.319	0.275	0.241

Figure 4.18. The effect of coating at different pH on the standard curve for Kogenate in FVIII ELISA.

On the advice of the manufacturer of the anti-FVIII antibody (Affinity Biologicals Inc. – see communication 1), it was advised to develop the assay using the same type of FVIII contained within test samples i.e. B-domain deleted FVIII. Therefore a standard curve was generated using Advate® (see figure 4.19) which is a human B-domain deleted FVIII also approved for the treatment of haemophilia. A standard curve was generated using this reagent; however, when testing samples from transfected and untransfected BHK cells, no difference in absorbance values between the negative control (AIM-V media) and the samples were seen. This indicated that the assay was not sensitive enough for the low levels of BDD-FVIII expressed in the BHK cells.

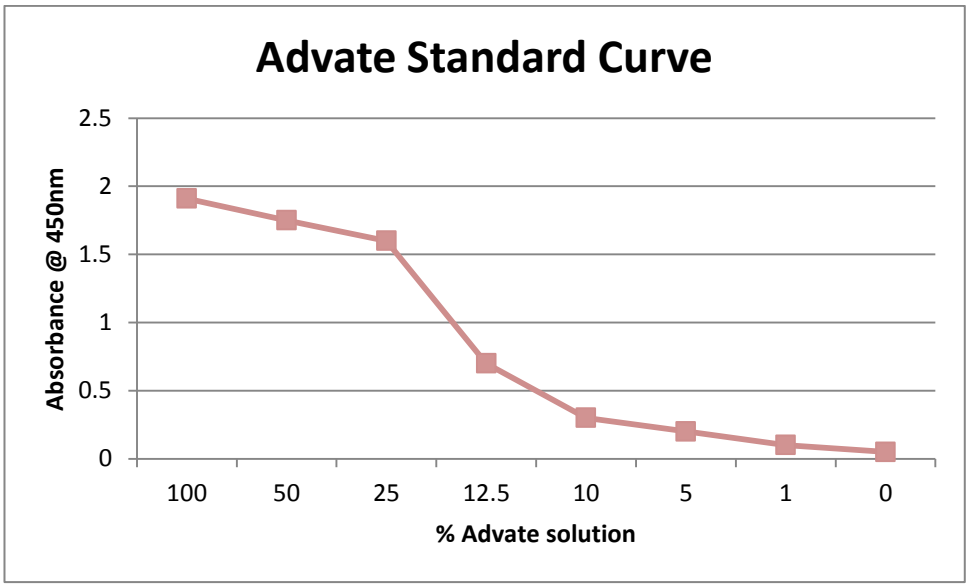


Figure 4.19. Example of Advate® standard curve, FVIII ELISA.

Advate® (BDD FVIII) was used to generate a standard curve in the FVIII ELISA.

Assay 3: For the detection of FVIII in BHK cell media or cell lysate

Standard curves were generated for the assay using Advate®. Cell culture media samples were tested for each of the transfected (with VHH ligand) and untransfected BHK cell lines. The absorbance at 450 nm was calculated for each sample and the mean absorbance plotted on a bar chart (see figure 4.20). The error bars show that

this assay had high variability across the plate as was observed in previous FVIII ELISA experiments. Samples from L2C1 transfected cells gave an absorbance value lower than the untransfected cell samples, which could indicate a reduction in FVIII concentration, however this was not statistically significant. Samples from L7C1 transfected cells gave an absorbance value higher than the untransfected cell samples, which could indicate an increase in FVIII concentration; however this was not statistically significant. Only n=6 of each sample (6 x untransfected, 6 x L2C1, etc.) were tested in this experiment, so if it had been repeated with a larger number of samples, the results may have had more statistical power.

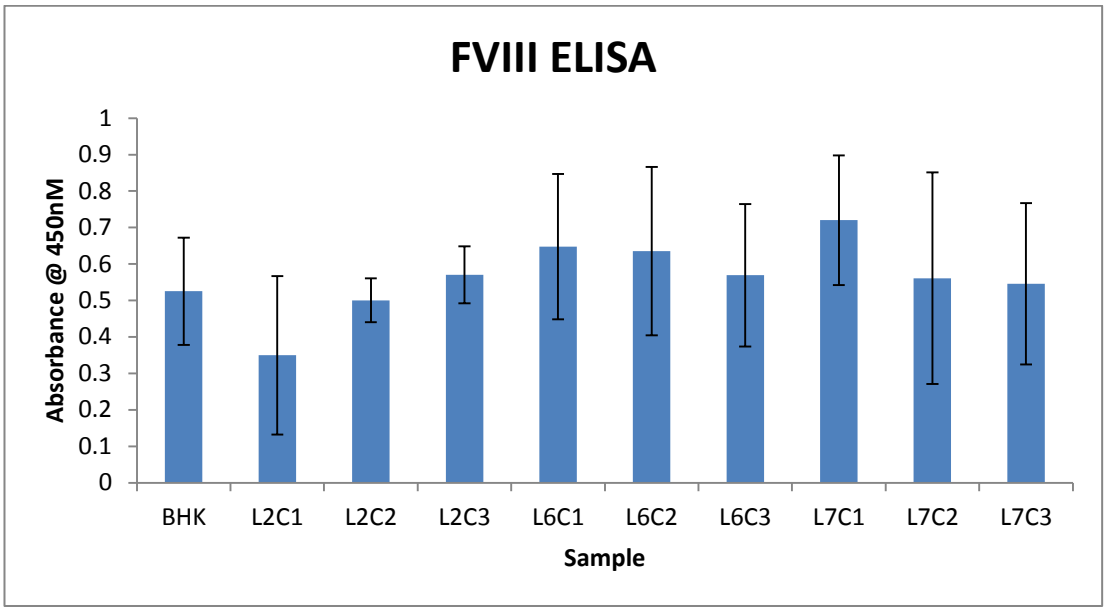


Figure 4.20. FVIII ELISA

BHK = untransfected cells (positive control). Mean absorbance plotted for n=6 samples. L2C1 samples have a lower mean absorbance when compared to the untransfected BHK cell line (not significant). L7C1 samples had an increased mean absorbance versus the untransfected BHK cell line (not significant). Error bars indicate one standard deviation.

4.9 Discussion

This chapter explains in detail the expression studies carried out for both VHH expression and VHH and FVIII co-expression in mammalian cells. Nine VHH plasmids were investigated in total, six of which encoded an anti-FVIII VHH ligand and various design elements.

Transfection conditions were tested using a plasmid encoding the reporter gene, Green Fluorescent Protein (GFP). This was important to ensure that the transfection method of choice was not excessively toxic to the cells, and also that the transfection efficiency was of a reasonable level to perform protein expression studies. In CHO-K1 cells both electroporation and chemical methods were tested and it was clear after testing both methods that the chemical method using Lipofectamine® gave the greatest GFP expression levels. This was therefore the method chosen for all future transfection experiments in CHO-K1 cells. Lipofectamine® was also the most successful transfection reagent in BHK cells for GFP expression. Of course there are many more potential methods of transfection available commercially that were not tested as it was concluded that Lipofectamine® was sufficient to fulfil the experimental aims. These preparation experiments could have been significantly improved by using flow cytometry to measure the number of fluorescing cells, instead of judging by eye through a microscope which is highly subjective and variable.

Reverse Transcriptase-PCR (RT-PCR) remains the most commonly used tool in the confirmation of gene expression by comparing mRNA levels in different samples (Bustin 2002). This method was used to determine if the VHH genes were being transcribed in BHK cells upon transfection with Lipofectamine®. In all nine transfected cell lines and in an untransfected (negative control) cell sample, beta-actin mRNA was detected. Although mRNA from 6 of the VHH constructs was positively identified in cell samples that were assessed 48 hours after transfection, mRNA from the remaining 3 VHH transfected cells was not found at this timepoint. These 3 VHH constructs were however detected in cell samples assessed 24 hours after transfection. There could be a number of reasons why these constructs were found after 24 hours but not 48 hours. These include operator error in one of the

steps from transfection to RNA extraction and the production of a PCR product. It is also possible that a loss in cell viability in response to transfection and expression of VHH may have resulted in mRNA degradation at the later time point. Low transfection efficiency could also have been an issue in some of these samples as mammalian cells are variable by their biological nature and hence the mRNA expression levels may have been too low to detect on these occasions. However since the expression of the beta actin housekeeping gene was also assessed in the 48 hour samples (untransfected and transfected cells) and appears to be expressed at a similar level in all samples, it is thought that cell viability was not the main issue. Several steps could have been taken to investigate VHH mRNA levels further. Firstly this experiment was conducted to an n=1, so it should have been repeated to confirm the results. Secondly, it would have been prudent to test for presence of mRNA at various time points after transfection, not just at 24 or 48 hours. This may have helped to inform protein expression studies, since a lack of mRNA at a certain time point ultimately equals no VHH protein expression and may have helped to explain some of the results from the protein studies discussed later. Lastly, it would have been useful to conduct quantitative real time RT-PCR (qRT-PCR) studies to compare mRNA expression levels at different time points and between the different VHH transfected cell samples.

A protein expression experiment was carried out to compare the effect of transfecting cells with a linearised plasmid encoding VHH rather than a whole plasmid. The reason this was tried was because of a publication that showed tranfecting with a linearised plasmid rather than an intact plasmid may incur a positive effect on protein expression levels, even in transient transfections (Stuchbury and Münch 2010). In a western blot of lysed cell samples, a stronger band appeared for those cells transfected with a linearised plasmid rather than an intact plasmid which may indicate a positive effect on protein expression levels as has been reported previously in the literature. The reason for this is that when transfecting with whole plasmids, it has to be linearised before the cell can process the DNA. Since this is a random process, some of the plasmids may not be linearised and this may have an adverse effect on protein production. Also when transfecting with a whole plasmid, there is no control over where in the sequence the plasmid is linearised by the cell, and could therefore result in the gene of interest being interrupted and a lack of protein

produced. Transfecting with a linearised plasmid increases the likelihood of the DNA being transcribed, as well as allowing control over where in the plasmid the sequence is digested. Although this is commonly only carried during the creation of a stable cell lines, where the gene of interest has to integrate with the genome and therefore it is desirable to control where the plasmid is digested, it was thought that it could have a positive on transient transfections in the present study due to the published paper mentioned earlier. Also the protein expression studies were not proving successful at that time so it seemed a sensible strategy to try and increase expression levels.

It is known from personal communication with Serge Muyldermans who discovered VHH (see communication 2), that VHH don't perform very well in Western blot experiments, as anti-VHH antibodies recognise conformational epitopes that are disrupted upon SDS treatment. Therefore both denaturing and non-denaturing conditions were tested to try to optimise the binding conditions. Western blotting experiments showed very low or negligible expression of VHH proteins in CHO-K1 cells. Although some of the constructs were designed in a manner that would render them secretion incompetent and therefore retained inside the cell (construct 2 does not have a leader sequence, construct 3 encodes KDEL), these VHH were detected in cell media. This could have been due to apoptosis and cell lysis, with the cell contents being released into the cell culture media. A study to detect apoptosis in these cells could have confirmed this, perhaps using flow cytometry (Wlodkowic et al, 2009). The presence of construct 3 in the cell media are somewhat more difficult to interpret since the plasmid encodes for both a leader sequence and KDEL. The presence of a leader sequence normally results in the extracellular secretion of a protein, whereas the presence of KDEL should cause a protein to bind to the KDEL receptor in the ER-Golgi secretory pathway and be retained intracellularly and subsequently degraded. Construct 3 was found in the cell media and this could be due to the VHH protein folding in a manner that KDEL receptor binding is somehow inhibited. The binding of the VHH to FVIII could also be blocking KDEL interaction. An alternative hypothesis is that the KDEL receptor was saturated, causing excess protein to bypass KDEL and be secreted, but this is unlikely given the low expression levels demonstrated.

In the immunoprecipitation experiments, the cell media from cells transfected with L7C3 gave the strongest band. This could either be spillover from one of the other

lanes or it could indicate a higher level of protein expression than for the other VHH investigated. When harvesting cell media from the transfected cells, it was not obvious to the eye that a large amount of cell lysis had occurred and therefore it is suggested that in this situation KDEL is in fact being bypassed in some way and the leader sequence is causing construct 3 to be actively secreted. It is more than likely that the cause is spill over from another lane since the ELISA experiments conducted do not confirm any ligand 7 expression as discussed later.

Some of the VHH ligand constructs (1 and 2) were designed to encode a His-tag at their 3' end. It was anticipated that the VHH proteins expressing a His-tag could then be detected in western blotting experiments using an anti-His antibody. Despite repeated attempts at detecting His-tagged VHH ligands in cell culture media of transfected cells, only one sample (L2C1) gave a very faint band on a western blot. For the other VHH ligands that should have contained a His-tag it may be that the tag was cleaved by the cell during protein production and therefore could not be detected by western blot. It may also have been the low expression levels of the protein meant that the His-tag was below the levels of detection, and beyond the sensitivity level of a western blot. Another reason could be that the conformation of the VHH was such that the His tag was 'hidden' in a protein fold, and therefore could not be detected.

A further step was carried out in an attempt to purify His-tagged proteins from the cell culture media using a column containing immobilised nickel ions, which bind to the histidine tag. All untagged proteins should pass straight through the column allowing the bound His-tagged proteins to be eluted separately. Eluted samples were analysed for His-tag expression and a positive signal was seen on an anti-His dot blot for cell culture media from cells transfected with L2C2 plasmid. However, further analysis of these samples on an anti-VHH western blot revealed that the positive signal on the dot blot was not a VHH ligand, but possibly just other cellular proteins that contained histidine-like residues. Following this result, no further work was carried out on the basis of the HIS-tag. Potential further work could be carried out to investigate what is happening to the HIS-tag in the VHH proteins.

One of the strategies for co-expressing VHH and FVIII was to attempt to make a stable cell line expressing VHH which could then be transfected with a plasmid encoding full length FVIII. Since VHHs are easily produced in bacteria and yeasts,

mammalian cell lines have not been routinely been evaluated for this purpose (De Mayer *et al* 2014). However there are examples noted in the literature showing that it is possible to produce VHH-fusion proteins in mammalian cell lines. A 6xVHH-Fc fusion protein has been expressed transiently in a HEK293 cell line (Zhang, Liu et al. 2009). The Zhang *et al* study used expression vectors bearing the Epstein–Barr virus origin of replication (oriP), and a pTT plasmid expression vector with protein yield ranging from 20 to 136 mg per liter of culture. An example of a stable CHO cell line expressing another fusion protein (single domain VHH and a green fluorescent protein as a reporter gene) was demonstrated by Bazl *et al* (Bazl, Rasaee et al. 2007). Similarly another stable cell line was developed expressing VHH-GFP fusion protein in Human neuroblastoma cells using a pEGFP-N1 vector and a Fugene 6 transfection reagent (Gueorguieva *et al*, 2006). Therefore the current study results were somewhat surprising in that attempts to express VHH either transiently or stably in CHO-K1 or BHK-FVIII cell lines produced very little or no VHH protein. This could be due to a number of reasons. In the stable cell line attempts, it may be that the relatively few cells that were stably expressing VHH were simply not selected and cultivated effectively using the methods employed in this project. Another reason may be that the plasmid and the promoter contained within was not the most ideal for stable insertion of the VHH gene into the genome of the cell, but this seems unlikely given the frequent use of pcDNA3.1 in mammalian expression systems. Perhaps a specialised expression system which is optimised for the generation of constitutive expression cell lines such as FlpIn™ (Invitrogen) would be better for this aim. The majority of the literature surrounding VHH expression explores the use of microorganisms such as *E.coli* (Rahbarizadeh, Rasaee et al. 2005) or *Pichia pastoris* (Rahbarizadeh, Rasaee et al. 2006), and even in plants (Teh and Kavanagh 2010) rather than mammalian cells and therefore it is difficult to make a recommendation. The use of microorganisms was not suitable for this study since FVIII is a large complicated protein that requires extensive post-translational modification which is not supported in microorganisms such as *E.coli*. One more possibility is that the VHH investigated were toxic to the mammalian host and therefore integration into the cell genome resulted in cell death. However, several reports in the literature state that VHH are non-toxic to mammalian cells, therefore this an unlikely concept (Li, Bourgeois et al. 2012). The VHH gene sequences that were supplied by BAC were

optimised for expression in yeast. It may be that adapting the codons for expression in hamster cell lines would have resulted in more successful expression.

Studies investigating the expression of FVIII protein upon co-expression with VHH ligands in BHK cells included western blotting and ELISA. It was known that this cell line does produce functional FVIII protein, as the cell media was tested in a gold standard functional coagulation assay at the start of this study. However, the exact amount of FVIII being expressed was not able to be confirmed in this study (see chapter 5). The western blotting experiments initially proved unsuccessful due to very low levels of FVIII protein expressed by the BHK cell line. However following immunoprecipitation of VHH protein from cells co-expressing VHH and FVIII, and anti-FVIII western blot showed bands at the expected size of the FVIII light chain for the cell samples transfected with VHH ligand 2 and VHH ligand 7. This was reassuring as it is known that these two ligands bind FVIII (BAC BV, Netherlands). However the results of this experiment are limited by the fact that no positive FVIII control was included in the western blot. The purpose of the ELISA was to determine if co-expressing VHH and FVIII together had any effect on FVIII protein expression levels. Unfortunately it was not possible to determine this using an ELISA due to the low protein levels, sensitivity limits of the assay and restricted availability of monoclonal anti-FVIII antibodies specifically for the detection of B-domain deleted FVIII (see communication 1).

In conclusion, expression studies in mammalian cells have produced highly variable results. VHH ligands were expressed at the RNA level as confirmed by RT-PCR; however the evidence for VHH protein expression is weak with many results showing little or no VHH expression. Future work could address this by codon optimisation, and alternative transfection systems. The immunoprecipitation and the western blot of the precipitated samples indicated that the ligand 2 and ligand 7 VHH bind FVIII; however it was not possible to show if these ligands cause an increase in the amount of total FVIII protein using proteomic methods due to consistently low levels of protein.

It was deemed that the best way forward from this point was to investigate the functional activity of FVIII produced in BHK cells since the proteomic studies did not yield any conclusive results. Functional FVIII activity could be tested using a

coagulation assay (clotting assay, see chapter 5), with or without transfecting those cells with the plasmids encoding VHH. This work will be discussed in the next chapter.

Chapter 5: Co-expressing FVIII and VHH: the effect on the functional activity of FVIII in a coagulation assay

5.1 Introduction

The aim of this study was to assess the effect of anti-FVIII VHH ligands on FVIII expression when the two proteins are co-expressed in a mammalian cell line. Plasmids encoding different VHH ligands were transfected into BHK-BDD FVIII expressing cell line, and the effect on FVIII expression investigated using a coagulation assay. Previously, VHH expression was confirmed at the messenger RNA level, however there was little or inconclusive evidence of expression at the protein level (see chapter 4). In addition, the level of total FVIII protein was also investigated by both western blot and ELISA, without success. However, any change in total FVIII protein would not have directly translated into a change in the amount of functional (active) FVIII protein, which is necessary for coagulation. Therefore it was deemed an essential part of this study to investigate a functional coagulation assay as a way of determining if the transfection of plasmids encoding VHH into the BHK-FVIII cell line would have any impact on the functional activity of FVIII.

In a clinical setting, the amount of active FVIII available for blood clotting can be assessed using an array of different coagulation assays, one of which is the aPTT assay (activated partial thromboplastin time – see section 2.4). The aPTT measures the efficacy of both the intrinsic and extrinsic pathways of the coagulation cascade, and is one of the assays used in diagnosing haemophilia. The 'partial' part of the aPTT is described as such due to the absence of tissue factor that would be needed for the *in vivo* cascade reaction. Clinically a prolonged aPTT time may indicate a coagulation factor deficiency. In this project, the aPTT assay was employed in order to determine the amount of active FVIII protein expressed in a BHK cell line.

As part of fulfilling the study aims, statistical analysis on any data produced was carried out to see if any change in active FVIII levels as demonstrated by a change in the coagulation time in the aPTT assay was statistically significant.

5.2 Coagulation assay

The Ceveron® alpha analyser, a machine manufactured by Technoclone, is a fully automated coagulation measuring instrument used for clotting, chromogenic and turbidimeric assays. For clotting assays with an endpoint of fibrin clot formation, it measures the time taken to form a 'blood clot' *in vitro* via a change in turbidity caused by the formation of fibrin fibres. In a clinical setting FVIII deficiency in humans can be tested using a clotting assay called aPTT (activated partial thromboplastin time). An aPTT assay was used in this project to detect changes in active FVIII protein expression; where the faster the 'clot' was formed (in seconds) the more active FVIII is present in the sample (serum-free cell media in this project).

According to the manufacturer's instructions, a calibration curve was carried out for each new set of reagents to be used in the aPTT assay. An example of a valid calibration curve can be seen in figure 5.1. In addition to this, Technoclone supplied a sample of normal and abnormal plasma samples (containing levels of active FVIII which were considered normal or deficient for a human) which were used as internal controls for each experiment, as well as a calibration reagent. If these plasma samples gave clotting times outside of the specified times for the normal or abnormal samples (different for every batch, specified by manufacturer on batch documents), all data from that experiment was to be disregarded. Fortunately, of the many experiments carried out on the Ceveron machine, this only happened once due to a technical failure, and data from that experiment was not included in the results of this study.

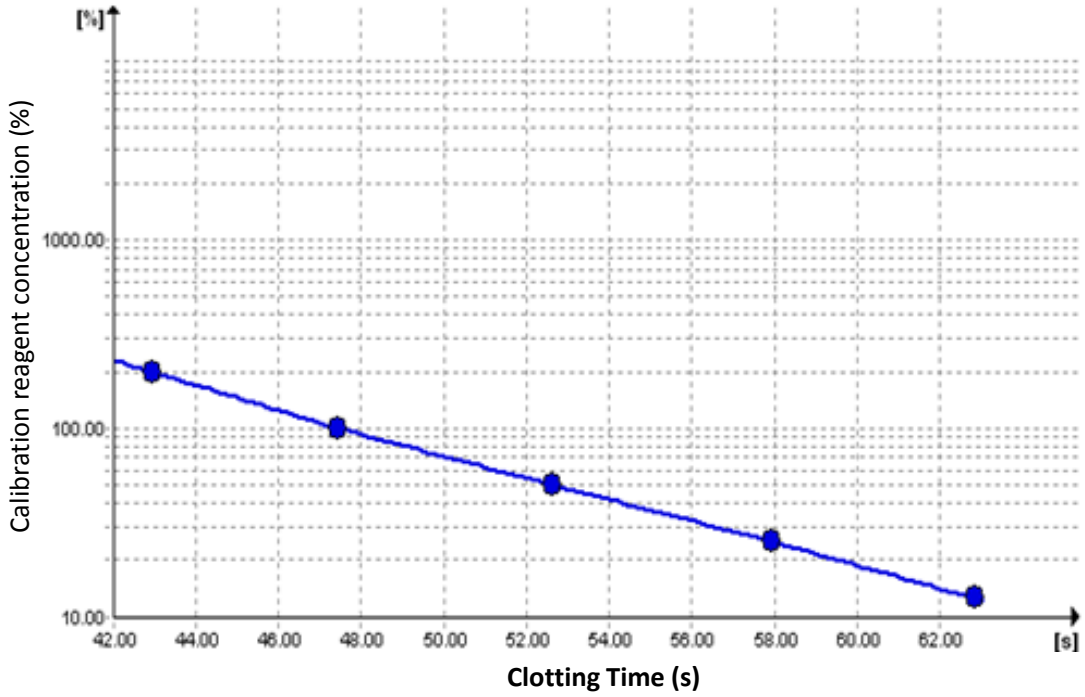


Figure 5.1. An example calibration curve for the aPTT assay.

A calibration curve was carried out using predefined dilution steps (automated) for each new batch of reagents for the aPTT assay and validated according to Technoclone instructions.

AIM-V media was taken from B-domain deleted FVIII expressing cells and tested in the aPTT assay. The cells were either untransfected or transfected with plasmids encoding nine different VHH ligands. To allow enough time for FVIII to accumulate into the cell media, all samples were taken 48 hours after addition of AIM-V media (media changed from full media to AIM-V at time of transfection). Cells were seeded at the same density at the start of each experiment and monitored under the microscope to detect any major differences at transfection and at the time of sampling the cell media. Under a light microscope, the cell density did not appear to be any different between those cells that were untransfected versus transfected at the time of sampling the cell media. The cells were not counted at the time of media sampling; however in retrospect this should have been done in order to see if there were any differences in cell growth between the transfected and untransfected cells.

AIM-V media taken from untransfected BHK cells was tested in the aPTT assay and treated as the standard (baseline) to which all transfected cell samples were compared to. Data analysis of the standard samples gave a mean clotting time of 35 seconds (n=48 samples) as can be seen in table 33.

AIM-V media samples taken from BHK cells transfected with either ligand 2, 6 or 7 constructs, and tested in the aPTT assay at least 20 times per construct. In order for the experiments to be statistically significant, the minimum number of times each ligand should have been tested was 16, in order to produce enough data that comparisons could be accurately drawn between transfected and untransfected cell lines. This number was calculated in conjunction with the advice of Jian Zhang, Professor of Statistics in the School of Mathematics, Statistics and Actuarial Sciences at the University of Kent. This advice was based on a power analysis and long established principles of experimental design (Cochran and G.M.Cox 1957). A power analysis is where a research question is phrased as two different statistical hypotheses and gives you an estimation of the sample size you should be testing in order to detect statistical significance. The null hypothesis (H_0) would be the negative result and the alternative hypothesis (H_a) would be the positive result (or the finding that is of interest to the researcher) (Steidl and Thomas 2001). In this study the null hypothesis was that there would be no difference between untransfected cell lines and transfected cell lines in terms of the amount of FVIII secreted. The alternative hypothesis was that there would be a difference between the untransfected and transfected cell lines i.e. the amount of FVIII secreted would increase or decrease when co-transfected with Camelid antibody ligands. Initial data sets that were generated were analysed for the standard deviation, and were assumed to have a normal distribution. The significance level of the test (the *P*-value) was assigned '0.05' (5%) which is the probability of rejecting the null hypothesis even if it is true. Degrees of freedom were also calculated which is the number of values in a distribution that are free to vary (Healey 1990). Based on these calculations the minimum sample size to be tested for statistical significance was 16.

All data generated in the aPTT assay was vigorously statistically analysed. The recommended statistical test was the student (unpaired) t-test, which was used to directly compare clotting times of media taken from transfected with that of untransfected cells. An unpaired student t-test is used to compare the mean of two

independent data sets (you would only use paired if you were looking at the same sample before and after treatment, or at two time points for example). The student t-test was selected as no assumptions are placed on the data (non-parametric), so it does not introduce any bias or restriction of data points into any parameters.

5.2.1 Marketed FVIII products

Fahndi®, Replenate®, and Advate® FVIII samples were kindly donated by the Kent Haemophilia centre in Canterbury, Kent. These were samples that had expired and therefore were no longer able to be used in humans. These samples were tested in the coagulation assay alongside cell media samples from the BHK-FVIII cell line. The aim of this experiment was to try to correlate clotting time (seconds) with either protein concentration (ng) or international units (IU), so that the FVIII concentration of BHK-FVIII cell media samples could be calculated using their clotting times.

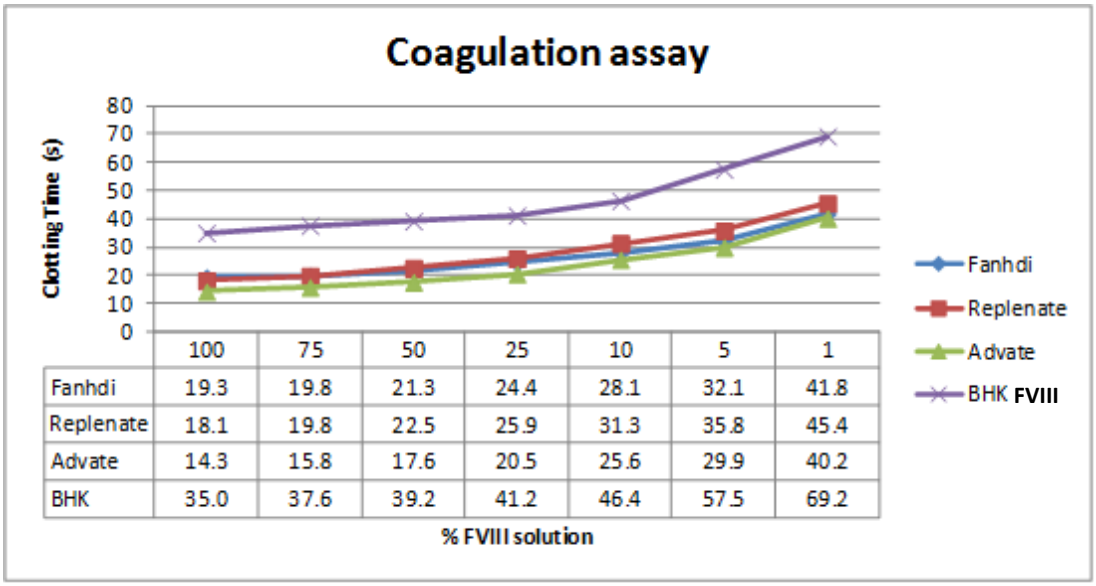
FVIII samples were tested at a range of concentrations (%), in triplicate (3 lots of materials tested) in the coagulation assay. Table 31 shows the differences in international units, protein concentration and the mean clotting times between the products tested.

Table 31. Characteristics of marketed FVIII products (all full length FVIII)

	Fandhi®	**Replenate®**	**Advate®**
IU FVIII in 100 µL of 100% solution	10	5	2.5
ng protein in 100 µL of 100% solution	100	5	355
Mean coagulation time (n=3) (seconds)	19.3 (18.7 – 20.3)	18.1 (17.6 -19.0)	14.3 (13.7 – 15.4)

Each product varied in terms of their FVIII protein concentration (ranging from 5 ng/100 µL to 355 ng / 100 µL) and international units, and their mean clotting times ranged from 14.3 seconds to 19.3 seconds (see figure 5.2). Considering that the clotting time is indicative of functional FVIII content, it was expected that the product with the highest concentration of international units (Fandhi) would perform

the fastest in the coagulation assay; however this product gave the slowest clotting time of the three products. It may be some level of degradation had occurred with these products since they were all past their expiry date. When comparing the profile of the marketed FVIII products to that of the BHK cell media (containing BDD-FVIII), the BHK cell media gave the slowest clotting time, at least 15 seconds slower versus any of the marketed FVIII product solutions.



	100	75	50	25	10	5	1
Fanhdi	19.3	19.8	21.3	24.4	28.1	32.1	41.8
Replenate	18.1	19.8	22.5	25.9	31.3	35.8	45.4
Advate	14.3	15.8	17.6	20.5	25.6	29.9	40.2
BHK	35.0	37.6	39.2	41.2	46.4	57.5	69.2

Figure 5.2. Performance of marketed FVIII products and BHK cell media in the coagulation assay.

For every log reduction in FVIII solution concentration (e.g. 100% to 10%), the clotting time slowed by approximately 10-15 seconds. Given that the BHK media clotting time was 15 to 20 seconds slower than any of the marketed FVIII products; it could be concluded that the FVIII concentration of the BHK media was at least a log factor less than any of the marketed FVIII products. However, due to the large variance of the FVIII protein concentration of the marketed FVIII products, it was not possible to estimate the FVIII protein concentration of the cell media.

5.2.2 FVIII co-expression with VHH ligands

BHK-FVIII cells (untransfected) gave a mean clotting time of 35.0 seconds (n=48) with a standard deviation of 4.6 seconds. BHK-FVIII cells were also null transfected

i.e. transfected without any DNA plasmid present, to see if the transfection reagents had any effect on clotting time (35.3 seconds, n=30). The two datasets were analysed and compared using the student t-test, and the clotting times were not statistically significantly different from one another (p value = 0.74), confirming that the transfection reagents used in this study did not impact FVIII activity.

BHK-FVIII cells were transfected with plasmids encoding one of the nine VHH ligands. Cell media samples were taken 48 hours after transfection and tested in the coagulation assay. Media samples from those cells transfected with plasmids encoding L2C1, L2C2, L7C1, or L7C3 VHH ligands showed a statistically significant change in FVIII clotting time when compared to untransfected BHK-FVIII cell media samples (see figure 5.3).

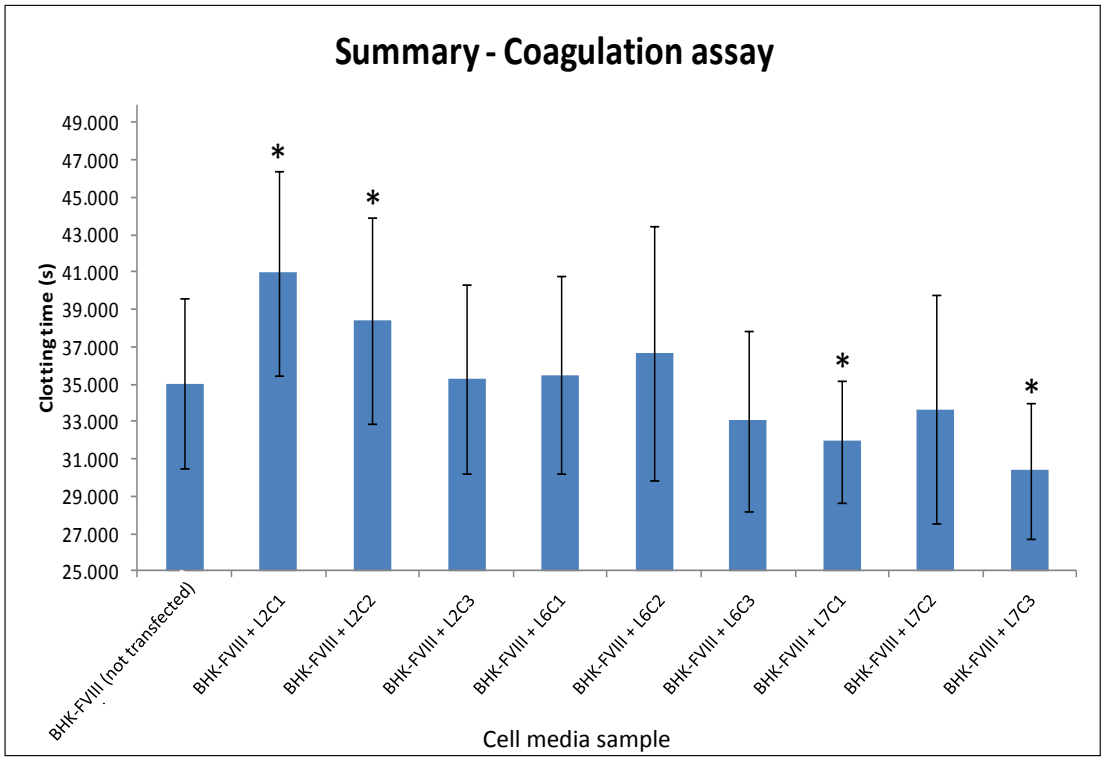


Figure 5.3. A summary of the FVIII clotting times for each VHH transfected cell line (48 hours after transfection).

A bar chart showing the results of co-expressing each of nine VHH ligands with FVIII in a BHK cell line and the resulting cell culture media tested in a coagulation assay. L2C1, L2C2, L7C1 and L7C3 all had a significant effect (individual t-test) on

clotting time compared to cell culture media from untransfected cells (BHK) (indicated by *). Error bars indicate one standard deviation. Minimum n=20 per test construct. Untransfected sample n=48.

Analysis of Variance (ANOVA) (Cochran and G.M.Cox 1957) was carried out on the entire dataset of clotting times. The null hypothesis was that all media samples tested had the same amount of functional FVIII (clotting times used as an indirect measure). The 'F ratio' was equal to 7.22, and the critical value for F at the 0.05 significance level was equal to 1.87. Because the calculated value (F ratio) is greater than or equal to the critical value, the null hypothesis can be rejected, which means that at least 2 groups of samples are different from each other.

Student t-test analysis was then carried out to see which of the clotting times for each of the VHH ligands tested were significantly different from one another (see table 32). When comparing the VHH transfected cells against each other, both L2C1 and L2C2 were statistically significantly different from L2C3 but not from each other. L2C1 was also statistically significantly different to all of the other VHH tested. In addition to the above, L2C2 was statistically significantly different to all other VHH with the exception of L6C1 and L6C2. L7C1 is statistically significantly different to all other VHH with the exception of L6C3, L7C2 and L7C3. L7C3 is statistically significantly different to all VHH with the exception of L7C1 and L7C2.

Table 32. Comparison of VHH ligands to show if they statistically significantly different to one another

	BHK (untransfected)	L2C1	L2C2	L2C3	L6C1	L6C2	L6C3	L7C1	L7C2	L7C3
BHK (untransfected)										
L2C1	Yes									
L2C2	Yes	No								
L2C3	No	Yes	Yes							
L6C1	No	Yes	No	No						
L6C2	No	Yes	No	No	No					
L6C3	No	Yes	Yes	No	No	No				
L7C1	Yes	Yes	Yes	Yes	Yes	Yes	No			
L7C2	No	Yes	Yes	No	No	No	No	No		
L7C3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	

Yes = statistically significantly different from one another; No = not statistically significantly different from one another

5.2.2.1 FVIII co-expressed with VHH ligand 2

BHK-FVIII cells were transfected with a plasmid encoding an anti-FVIII VHH ligand 2. Three different constructs of ligand 2 were tested; constructs 1, 2 and 3 (see 2.2). The cell media from each of these three transfected cell lines was tested along with media taken from mock transfected (minus VHH DNA) BHK-FVIII cells in an aPTT coagulation assay.

The transfection of plasmid encoding ligand L2C1, into the BHK-FVIII cell line, had a negative effect on the clotting time of cell media samples in the aPTT coagulation assay when compared with cell media samples from the untransfected BHK-FVIII cell line. This prolonged clotting time indicated a reduction in the amount of active FVIII being expressed when compared to the untransfected cell line. Cell media from the transfected cells gave a clotting time of 41 seconds compared to a clotting time of 35 seconds for the untransfected cells, a difference of 6 seconds. Statistical analysis of the data using an unpaired student t-test indicated that this difference in clotting time was statistically significant, with a P-value of 0.0001 (summarised in table 33).

The transfection of plasmid encoding ligand L2C2 into the BHK-FVIII cell line, had a similar effect as the L2C1 transfection, in that this construct also had a negative effect on the clotting time of BHK-FVIII cell media samples, when compared to untransfected cell media samples. Again, the prolongation of the clotting time of 3.4 seconds indicated a reduction in the in the amount of functional FVIII. Although this difference in clotting time was not as large of an effect as that of the L2C1 transfection, this difference was also statistically significant, giving a P-value of 0.0072 (see table 33).

Cell media samples from that of BHK-FVIII cells transfected with L2C3 resulted in a clotting time very similar to that of cell media from untransfected cells, with a difference of 0.3 seconds. Statistical analysis of the data showed that this change in clotting time was not statistically significant.

Table 33. Coagulation assay results from BHK-FVIII cells +/- ligand 2 constructs

	Media from BDD-FVIII cell line (control - no transfection)	Media from BDD-FVIII cell line transfected with L2C1	Media from BDD-FVIII cell line transfected with L2C2	Media from BDD-FVIII cell line transfected with L2C3
Mean clotting time (seconds)	35.0	41.0	38.4	35.3
Standard deviation	4.6	5.5	5.5	5.1
Sample Size (n)	48	19	32	23
Standard Error of Mean	0.7	1.3	1.0	1.1
Significant vs control?	n/a	Yes	Yes	No
P-value	n/a	0.0001	0.0072	0.9921
95% Confidence intervals	33.7-36.3	38.5-43.5	36.5-40.3	33.2-37.4

5.2.2.2 FVIII co-expressed with VHH ligand 6

FVIII was co-expressed with an anti-FII VHH called ligand 6 in BHK cells. Three different constructs of ligand 6 were tested; constructs 1, 2 and 3 (see 2.2). The cell media from each of these three transfected cell lines was tested alongside media from untransfected cells in an aPTT coagulation assay. Since ligand 6 does not bind to FVIII it was expected that these ligands would have no effect of FVIII expression. L6C1 and L6C2 caused a prolongation of the clotting time (plus 1.2 seconds and 2.9

seconds respectively) whereas L6C3 caused a shortening in the clotting time (minus 1.2 seconds) when comparing transfected and untransfected cells. None of these clotting times was statistically significantly different to cell media from untransfected cells (summary in table 34).

Table 34. Coagulation assay results from BHK-FVIII cells +/- ligand 6 constructs

	Media from BDD-FVIII cell line (control - no transfection)	Media from BDD-FVIII cell line transfected with L6C1	Media from BDD-FVIII cell line transfected with L6C2	Media from BDD-FVIII cell line transfected with L6C3
Mean clotting time (seconds)	35.0	36.2	37.9	33.8
Standard deviation	4.6	6.1	8.5	5.7
Sample size (n)	48	20	20	20
Standard Error of Mean	0.7	1.4	1.9	1.3
Significant vs control?	n/a	No	No	No
P-value	n/a	0.8466	0.3319	0.0911
95% Confidence intervals	33.7-36.3	33.5-38.9	34.2-41.6	31.3-36.3

5.2.2.3 FVIII co-expressed with VHH ligand 7

FVIII was co-expressed with an anti-FVIII VHH called ligand 7 in BHK cells. Three different constructs of ligand 7 were tested; constructs 1, 2 and 3 (see 2.2). The cell media from each of these three transfected cell lines was tested alongside media from untransfected cells in an aPTT coagulation assay.

Transfection of BHK-FVIII cells with L7C1, had a positive effect on the clotting time media samples when compared with untransfected cells. Cell media from the transfected cells gave a clotting time of 31.9 seconds compared to a clotting time of 35 seconds for the untransfected cells, a difference of 3.1 seconds. Statistical analysis of the data using an unpaired student t-test indicated that this difference in clotting time is statistically significant, with a P-value of 0.0057 (summarised in table 35), and indicates an increase in functional FVIII.

Transfection of BHK-FVIII cells with L7C2, had a very small effect on the clotting time of media samples when compared with untransfected cells giving a difference in the clotting time of minus 0.2 seconds. The data was analysed using an unpaired student t-test which indicated that this difference in clotting time is not statistically significant (see table 35).

Transfection of BHK-FVIII cells with L7C3 had a positive effect on the clotting time of media samples when compared with untransfected cells. Cell media from the transfected cells gave a clotting time of 31 seconds compared to a clotting time of 35 seconds for the untransfected cells, a difference of 4 seconds faster in the transfected cells. Statistical analysis of the data using an unpaired student t-test indicated that this difference in clotting time is statistically significant, with a P-value of 0.0002 (summarised in table 35).

The shortened clotting time using cell media from transfected cells may indicate an increase in the amount of functional FVIII being expressed when compared to the untransfected cell line.

Table 35. Coagulation assay results from BHK-FVIII cells +/- ligand 7 constructs

	Media from BDD-FVIII cell line (control - no transfection)	**Media from BDD-FVIII cell line transfected with L7C1**	**Media from BDD-FVIII cell line transfected with L7C2**	**Media from BDD-FVIII cell line transfected withL7C3**
Mean clotting time (seconds)	35.0	31.9	34.8	31.0

Standard deviation	4.6	3.2	7.8	4.3
Sample size (n)	48	20	19	19
Standard Error of Mean	0.7	0.7	1.8	1.0
Significant vs control?	n/a	Yes	No	Yes
P-value	n/a	0.0057	0.2665	0.0002
95% Confidence intervals	33.7-36.3	30.5-33.3	31.3-38.3	29.1-32.9

5.3 Discussion

Plasmids encoding nine unique VHH constructs were transfected into BHK-FVIII cells. The media taken from those cells was tested in an aPTT coagulation assay and the clotting times compared to cell media taken from untransfected BHK cells (see figure 5.2). The clotting assay used in this study is currently considered the gold standard method for detection of FVIII and the diagnosis of haemophilia A and screening for thrombophilia (Gouws, Botha et al. 2014). Of the nine BHK cell lines that were transfected with VHH constructs, four of those VHH resulted in a statistically significant effect on the clotting times of FVIII; L2C1, L2C2, L7C1 and L7C3 respectively. All four of these VHH constructs are anti-FVIII Camelid antibody ligands (ligand 2 and ligand 7) as confirmed by BAC BV (Netherlands).

Co-expression of FVIII with the ligand 2 constructs L2C1 and L2C2 gave a prolonged clotting time, potentially indicating a reduction in the amount of

functional FVIII expressed into the cell media when compared to that of untransfected cells. However, this was not able to be supported by proteomic studies due to the inability to detect total FVIII protein (chapter 4). Hypothetically speaking, if there was a decrease in functional FVIII levels, this could be due to a number of potential reasons, for example, a reduction in total FVIII protein, increased FVIII degradation, or a masking of binding sites required for FVIII clotting activity.

Co-expression of FVIII with the ligand 7 constructs L7C1 and L7C3, gave a shortened clotting time, potentially indicating an increase in the amount of functional FVIII in the cell media when compared to that of untransfected cells. Again, this was not supported by proteomic studies in chapter 4. If an increase in functional FVIII had been confirmed, it could have been due to a number of potential reasons, for example, an increase in total FVIII protein, or a decrease in FVIII degradation (prolonged half-life).

The difference between the negative effect of transfecting with the ligand 2 constructs and the positive effect of ligand 7 on clotting times may be explained by their anti-FVIII activity. Firstly it is known that ligand 7 and ligand 2 bind to different regions of the FVIII light chain consisting of A3, C1 and C2 domains as well as having different binding affinities. Ligand 7 competes with vWF for the same binding site whereas ligand 2 binds to a region other than the vWF binding site (data not shown, confidential to BAC). Since FVIII is known to circulate in blood plasma bound to vWF which has been shown to stabilize FVIII, it could be that ligand 7 is behaving in a similar protective fashion thereby preventing degradation of FVIII and preserving the amount of FVIII available for clotting activity. Two regions on the FVIII light chain have been implicated in binding VWF: the N-terminal acidic a3 domain and the C-terminal C2 domain (Shiltagh, Kirkpatrick et al. 2014). It would be interesting to conduct epitope mapping to find out exactly where the ligand 7 VHH binds to FVIII in comparison with vWF. There are several methods available for epitope mapping ranging from the gold standard of x-ray co-crystallography to site-directed mutagenesis, and the chosen method depends on whether you are analysing a linear or conformational epitope. Since it is known that both ligand 7 and ligand 2 bind to the light chain, deletions could be introduced in the FVIII light chain to examine the binding profile of these ligands. Similar experiments have been noted in the literature looking at the impact of anti-FVIII antibodies on the clotting time of

FVIII, albeit with the use of monoclonal antibodies rather than VHH. One particular study demonstrated that an anti-FVIII monoclonal antibody bound to FVIII heavy chain enhanced FVIII coagulant activity, demonstrated by a shortened coagulation time in the aPTT assay, by approximately 1.5-fold. The monoclonal antibody in that study bound to the A2 domain of the heavy chain and was shown to enhance thrombin-induced activation of the FVIII protein, as well as improve the interaction of thrombin with the A2 domain (Takeyama, Nogami et al. 2010). Another study demonstrated that an anti-FVIII monoclonal antibody that bound to FVIII light chain blocked vWF binding in a dose dependent manner as well as acting as an inhibitor in FVIII coagulation assays (both aPTT and chromogenic), thought to be due to the prevention of thrombin activation of FVIII light chain (Precup, Kline et al. 1991)

When comparing the three L7 VHH constructs, the effect of L7C1 and L7C3 on the clotting time was statistically significant, whereas the effect of L7C2 was not. L7C3 had a more significant effect on the clotting time than L7C1. L7C1 reduced the clotting time by 3.1 seconds and L7C3 reduced the clotting time by 4 seconds. Both L7C1 and L7C3 contained a leader sequence which meant they should have been secreted into the cell media along with FVIII. The only difference between these two constructs is that L7C3 has a KDEL sequence encoded within the construct. It is possible that the interaction of L7C3 at the KDEL receptor, if bound to FVIII could slow down the processing and secretion of FVIII through the ER/Golgi secretory pathway. It is known that the folding/misfolding of the FVIII protein is a rate limiting step in FVIII protein expression, so it is possible that this could result in a net increase of protein. You could test this by comparing the rate of FVIII production in the presence and absence of this construct. Although exact amounts of total protein could not be calculated in this study, the clotting assay is the gold standard for FVIII detection.

Co-expression of L7C2 with FVIII had no significant effect on the FVIII clotting time when compared to the untransfected BHK cells. The difference between L7C2 and the other two L7 constructs (L7C1 and L7C3) is that the plasmid does not encode a leader sequence and thereby should be not be secreted via the ER/golgi pathway in the same way as the L7C1 and L7C3. If L7C2 was retained intracellularly, it would not be co-secreted with FVIII and would not be able to provide the same protective effect to the FVIII protein in the cell media. However it

was seen in the immunoprecipitation/anti-FVIII western blot experiment (figure 4.13) that FVIII was co-purified with L7C2 from cell media indicating that at least a small amount of L7C2 was present in the cell culture media, but perhaps not enough to elicit a protective effect on FVIII. The presence of L7C2 in the cell media may be due to an accumulation inside the cell, resulting in cell lysis and causing a release of the contents of the cytosol into the surrounding cell culture media.

It is hypothesised that the negative effect that the ligand 2 constructs had on the clotting time of functional FVIII in the cell media may be due to the binding region on the FVIII protein. This may be a region that affects its activity in the coagulation assay, similar to that demonstrated in the Precup *et al* study (Precup *et al*, 1991).

When comparing the effect of the different ligand 2 constructs, whereby construct 1 and 2 had a statistically significant negative effect on the clotting time of FVIII in the cell culture media, and construct 3 (L2C3) which had no significant effect on the clotting time of FVIII in the cell culture media it is important to consider the design of each of these constructs VHH. Despite only 2 of these constructs containing a leader sequence (construct 1 and 3), it appears from the immunoprecipitation experiments that all 3 constructs were present in the cell culture media of transfected cells (as shown in immunoprecipitation/western blotting experiments, see 4.7.1). Also that FVIII was co-purified with each of these ligands in the same experiment suggests that they were bound together in the cell culture media. The similar feature of construct 1 and 2 (that construct 3 does not contain) is a HIS tag. It could be hypothesised that the HIS tag of the bound ligand 2 VHH is somehow affecting the functional activity of the FVIII protein, perhaps by masking an important coagulation assay co-factor epitope.

The differences in the binding sites of these two anti-FVIII VHH (ligand 2 and ligand 7) may impact the way the cell processes the FVIII protein (if they were bound intracellular), including the way FVIII interacts with other chaperones. This in turn could lead to many differences including the way the protein is folded (affecting its activity), how much protein is sent for degradation and how much is secreted from the cell into the media. This of course is hypothetical and has not been tested in the present study.

Co-expression of FVIII with the ligand 6 constructs had no significant effect on the amount of FVIII expressed in BHK cells. This result was expected as ligand 6 has no anti-FVIII activity and was used as negative control in this study.

In conclusion, although statistically significant differences in cell media clotting times have been demonstrated in BHK-FVIII cells transfected with plasmids encoding anti-FVIII VHH, it is not clear as to the reasons why. Especially as the proteomic studies did not give convincing evidence of VHH or FVIII protein expression in the BHK cells. If it had been possible to categorically confirm VHH and FVIII protein expression, the evidence generated in the coagulation studies would have shown much more promise than they currently do.

Chapter 6: General discussion

The field of research on the FVIII protein is vast and continues to remain a hot topic of research due to the enduring problem of low levels of expression and poor stability. Indeed prior to this study, many attempts have been made to improve the expression of FVIII include tweaking the intracellular trafficking of the protein, removing sections of the B domain which are not required for coagulant activity, improving the stability of the protein, improving the manufacturing/purification conditions and also extending the half-life of the protein with more modern fusion proteins. Attempts have also been made at optimising the gene sequence encoding the protein, and making porcine/human hybrids that confer higher expression levels. The present study aimed to build on what has previously been done in this area by testing a novel approach with regard to influencing FVIII expression levels by co-expression with camelid derived heavy chain antibodies. Had this study been successful, the principles could have been applied to the expression of other recombinant proteins.

The present study aimed to improve the production and yield of biologically active FVIII from mammalian cell lines, via co-expression with camelid derived anti-FVIII antibody ligands (VHH). VHH ligands are 12 – 15 KDa heavy chain only antibody fragments that are highly specific for their antigen, very stable and can be easily separated from their antigen at neutral pH. Antibody fragments, such as the VHH ligands used in this study are relatively new to research when compared to the body of work that has been done with conventional antibodies, and they are currently being tested in human clinical trials as treatments in their own right. Because of their desirable properties, the potential of VHH ligands to be used as molecular tools is vast and there are many approaches currently being investigated to harness this potential (De Meyer, Muyldermans et al. 2014). Co-expression of VHH with a protein of interest has previously been successful in bacterial cells (de Marco 2014). There is also an example of VHH being directed to intracellular compartments of mammalian cells using KDEL-specific VHH that bind to ER resident proteins in human umbilical vein endothelial cells (HUVECS) (Klooster, Eman et al. 2009). However, co-expression of antibodies, antibody fragments or VHH with the FVIII

protein in mammalian cells has not previously been explored as a mechanism for increasing the levels of functional FVIII produced in mammalian cells.

3 VHH gene sequences were provided for use in this study by BAC BV (The Netherlands). From these, nine unique plasmids encoding VHH were designed and produced using a variety of molecular biology techniques. These VHH plasmids were transiently expressed in mammalian cells including a BHK-FVIII expressing cell line. However there were significant hurdles along the way, not least that both VHH and FVIII proteins were expressed at very low levels, as seen in proteomic studies (chapter 4). This was to be not a surprise for the FVIII protein, despite the use of a BHK cell line that was previously optimised for FVIII expression by Lollar *et al*, since FVIII is renowned for expressing at low levels and that is the foundation for the purpose of this study. However, it was expected that the VHH would be simple to express, given their small size, supposed high solubility and non-toxic properties that has been reported several times in the literature. It is likely that this particular study did not employ optimal methods for VHH protein expression. Aternative expression systems such as FlpIn could be explored, and also gene sequences with optimised codons could be tested in future studies.

Significant problems were encountered in this study with low or no VHH protein expression, meaning that is was not possible to confirm VHH expression using proteomic methods. However, since the function of FVIII can be tested in gold standard coagulation assays, these were carried out to investigate clotting times of the cell media samples from cells transfected with plasmids encoding VHH. An automated coagulometer was used to test the time taken to form a ‘plasma clot’ using the cell culture media containing FVIII, either in the presence or absence of VHH. The coagulation assay employed in this study is the gold standard method which is currently used clinically to diagnose coagulation disorders. Four out of the nine VHH ligand constructs tested had a statistically significant effect on FVIII clotting times. These four VHH were all anti-FVIII ligands (ligand 2 and ligand 7 provided by BAC BV). Two ligand 2 constructs had a negative effect (slowing down) on FVIII clotting times and two of the ligand 7 constructs had a positive effect (speeding up) on FVIII clotting times. The other five VHH ligands tested had no significant effect on FVIII clotting times. It was already known prior to this study that ligand 2 and 7 had a high binding affinity for the FVIII light chain due to these ligands being available

commercially for the purification of FVIII. However these ligands had not previously been co-expressed with FVIII. Ligand 2 and ligand 7 VHHs bind to different areas of the FVIII light chain, with ligand 7 binding to one of the vWF binding sites and ligand 2 binding to a site dissimilar to the vWF binding site. It is proposed that the effect these two ligands have on FVIII expression is opposing due to their different binding sites on the FVIII protein. vWF is well known to have a protective effect on FVIII (Takeyama, Nogami et al. 2009) so it is possible that ligand 7 demonstrates similar protective properties due to its binding location.

It is possible that the four VHH ligands that had a statistically significant effect on FVIII clotting times, if bound to the protein intracellularly, could be molecular chaperones to the FVIII protein. Immunoprecipitation studies demonstrated that all the VHH tested were secreted into the cell media along with FVIII and therefore it is reasonable to assume that the two proteins could have been bound when secreted into cell media. It is well known that one of the reasons that FVIII is expressed at low levels is due to the way it is trafficked through the cell, and is limited by its interactions with molecular chaperones such as BiP. It would be worth further investigation to decipher why the ligand are having the effect on clotting times.

In conclusion, this study has shown:

1. The selected methods for mammalian expression of these VHH ligands are suboptimal in this instance and require significant optimisation.
2. In order to informed make conclusions about FVIII clotting times in co-expression studies it is useful to have some proteomic data to back up the findings.
3. FVIII protein is notoriously difficult to detect in conventional proteomic assays due to low expression levels and the lack of availability of specific and cost effective detection methods.

Future work

It is clear that there is the potential for many more studies to be done to confirm the findings of this study as well as enhance it.

It was very difficult to detect FVIII protein in standard proteomic assays such as western blotting and ELISA due to low expression levels, as well as the large multimeric nature of the protein. The only way to reliably confirm its presence was to test the cell media in a functional assay, which has limitations in itself since the functional activity of FVIII is not directly related to protein concentration but correct folding/post-translational modification of the protein. The study was also limited by the lack of availability of specific tools for detection of VHH and FVIII. Those that were available commercially such as the VisuLize™ FVIII Antigen Kit (Affinity Biologicals) for the quantitative determination of Factor VIII antigen in human plasma samples and Factor VIII concentrates were prohibitively expensive for an academic research project.

The BHK-FVIII cell line used in this study was not generating enough protein to detect in traditional proteomic studies such as western blot or ELISA. Therefore additional profiling studies should be carried out on this cell line to confirm if it is still expressing FVIII protein. RT-PCR could be used to test for presence of FVIII mRNA. You could also concentrate the cell media to increase the concentration of any FVIII present – this could be done very simply using centrifugation and a sample concentrator such as Vivaspin (GE Healthcare). The concentrated samples could then be used in traditional proteomic studies, perhaps using a validated commercial ELISA. It would also be possible to try and purify the FVIII protein from cell media using an affinity chromatography column designed for the purification of recombinant b domain deleted factor VIII, for example VIIISelect (GE Healthcare).

The VHH protein did not express in sufficient concentrations in any of the cell lines tested so that they could be consistently detected in proteomic assays like western blotting and ELISA. It would be interesting to take the VHH gene sequences and see if the protein would express in yeast cells, in the same way that BAC produced these ligands originally. There are many reports in the literature of VHH expression in yeast for industry usage, much less so in mammalian cells.

The leader sequence selected for this study was optimised for production of a monoclonal antibody (Herceptin) in CHO cells. It would be useful to consider the use of a VHH optimised leader sequence for future expression studies since the leader sequence can have a major impact on protein synthesis and secretion (review, see Stern *et al*, 2007).

It would be useful to try purifying the VHH protein from cell media by methods other than those tried already (nickel column purification of his-tagged VHH, and also immunoprecipitation) in order to increase the concentration of expressed protein. Crude methods such as centrifugal filter concentration was an option explored in this project however, due to the small size of the VHH protein (12-15 KDa), and the manufacturer recommendation of choosing a filter one-half to one-third smaller than the protein to be concentrated (the molecular weight cut-off being 5 KDa), this was not considered as a reliable method. Zhong et al was able to purify a 45 KDa VHH protein produced in *E.Coli* via gel filtration on a Sephadex G-200 column (Amersham Biosciences, United Kingdom) so that is an option that could be explored (Zhong, Zhang et al. 2006).

It could be perceived that another limitation to this study was the fact that the cells were not counted post-transfection in the experiments where FVIII was co-expressed with VHH. Instead of counting, all the cells were harvested and lysed to test for intracellular VHH and FVIII expression. However, if this study was to be repeated then of course the cells could be counted using a cell counting machine or a simple haemocytometer to confirm that the results seen in the clotting assay were not attributed to differences in cell numbers, but in fact the presence or absence of VHH.

There is also room for optimisation of the expression of VHH ligands in order to achieve higher protein levels. It would be interesting to investigate a variety of VHH expression levels in a stable cell line to see if this has any effect on FVIII expression i.e. a high VHH expressing cell line versus a low VHH expressing cell line co-expressed with FVIII.

In the present study, all clotting experiments were carried out using B-domain deleted FVIII. It would be desirable to repeat the experiments with full length FVIII to see if ligand 7 and ligand 2 co-expression has the same effect on FVIII clotting times, since many of the FVIII products on the market are derived from the full

length protein. It would also be good to study the full-length FVIII protein since it is viewed as the gold standard treatment for haemophilia versus B-domain deleted FVIII, and therefore would be preferable to work on from a clinical perspective.

It would also be interesting to consider the interaction of VHH with chaperones in the secretory pathway when co-expressed with FVIII, and indeed whether the interaction of FVIII with classical chaperones such as BiP is affected.

Competition binding studies could be conducted, for example ligand 7 VHH vs. vWF binding site on FVIII. In order to do this you would need either a labelled form of vWF or VHH to this. Potential labels could be fluorescent or radiolabeled to allow dose-dependent detection.

Epitope mapping could be carried out to find out exactly where the ligand 7 and ligand 2 VHH bind to FVIII. A much larger question is how the difference in binding location of ligand 2 and ligand 7 could affect FVIII coagulant activity.

The coagulation experiments could be repeated without co-expressing FVIII and VHH proteins together. VHH could be produced in another system such as yeast, purified and then added to the BHK cell media and measure any subsequent effect on clotting times to see if the effect is the same i.e. no transfection necessary.

Microscopy studies could be conducted to investigate VHH trafficking in the BHK cell line, to see where in the secretory pathway VHH binds to FVIII if at all. Fluorescent tagging could be used for this as demonstrated in the literature (Bazl, Rasaee et al. 2007).

1. FVIII proteolytic activity – could be used to investigate FVIII activity +/- VHH (does L2 block FVIII activity?). Peptide cleavage = fluorescence change. Is ligand 7 protecting FVIII from proteolysis inside the cell?

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Appendices

1. Amino acid codes

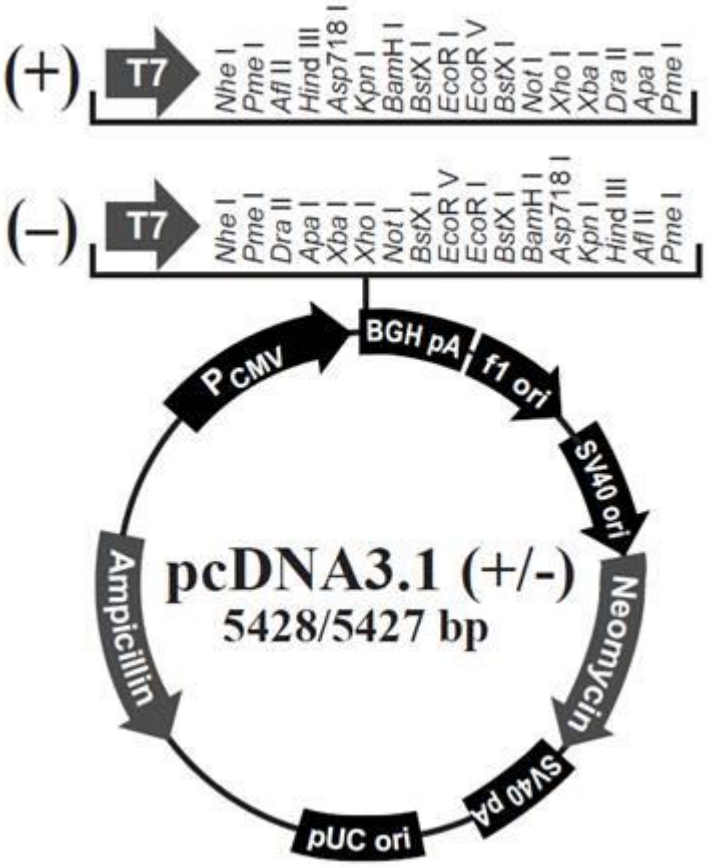
Table 36. Amino acid codes

A	**Ala**	**Alanine**
C	**Cys**	**Cysteine**
D	**Asp**	**Aspartic Acid**
E	**Glu**	**Glutamic Acid**
F	**Phe**	**Phenylalanine**
G	**Gly**	**Glycine**
H	**His**	**Histidine**
I	**Ile**	**Isoleucine**
K	**Lys**	**Lysine**
L	**Leu**	**Leucine**
M	**Met**	**Methionine**
N	**Asn**	**Asparagine**
P	**Pro**	**Proline**
Q	**Gln**	**Glutamine**
R	**Arg**	**Arginine**
S	**Ser**	**Serine**
T	**Thr**	**Threonine**
V	**Val**	**Valine**
W	**Trp**	**Tryptophan**
Y	**Tyr**	**Tyrosine**

2. VHH ligand gene sequences

The gene sequences for the VHH ligands utilised in this project are of a commercially sensitive nature (owned by BAC BV, The Netherlands) and therefore have been omitted from this thesis.

3. pcDNA3.1 sequence



Comments for pcDNA3.1 (+)

5428 nucleotides

CMV promoter: bases 232-819

T7 promoter/priming site: bases 863-882

Multiple cloning site: bases 895-1010

pcDNA3.1/BGH reverse priming site: bases 1022-1039

BGH polyadenylation sequence: bases 1028-1252

f1 origin: bases 1298-1726

SV40 early promoter and origin: bases 1731-2074

Neomycin resistance gene (ORF): bases 2136-2930

SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

4. Electroporation buffers

Salts buffer (20 mM Hepes, 135 mM KCl, 2 mM $MgCl^2$, 0.5 % FiColl 400, pH 7.6 with NaOH, filter sterilised)

DMEM/F12 with serum

Phosphate Buffered Saline, autoclaved

Hepes buffered saline (10 mM Hepes, 140 mM NaCl at pH 7.3, autoclaved).

Phosphate buffered sucrose (272 mM sucrose, 10 mM sodium phosphate, pH 7.3, filter sterilised).

Hepes buffered sucrose (272 mM sucrose, 10 mM Hepes, pH 7.3, filter sterilised).

5. Media and buffer formulations

1. **LB media composition:**

5 g NaCl
5 g tryptone
2.5 g yeast extract
Make up to 500 ml with Milli Q H_2O
pH 7 with 5 M NaOH
Autoclave
After autoclaving cool to 55 °C before adding antibiotics

2. **LB agar plates composition**

5 g NaCl

5 g tryptone

2.5 g yeast extract

10 g agar

Make up to 500 ml with Milli Q H_2O

pH 7 with 5 M NaOH

Autoclave

After autoclaving cool to 55 °C before adding antibiotics

Pour out into sterile petri dishes (20 ml media per plate)

Leave to set for 30 minutes and store at 4 °C

3. SOB medium

10 g tryptone

2.5 g yeast extract

0.25 g NaCl

Make up to 500 ml with milliQ H^2O

Autoclave

Add 5 ml of filter-sterilized 1 M $MgCl^2$

Add 5 ml of filter-sterilized 1 M $MgSO^4$

4. Restriction enzyme buffer

Buffer E: 6 mM Tris-HCl, 6 mM $MgCl^{2,}$ 100 mM NaCl, pH 7.5.

Buffer H: 90 mM Tris-HCl, 10 mM $MgCl^{2,}$ 50 mM NaCl, pH 7.5.

Cell culture regents:

1. **BHK cell media:**

500 ml DMEM/F-12

15 mM Hepes (50 ml of 150 mM stock)

2 mM L-Glutamine (5 ml of 200 mM stock)

10 % heat inactivated Fetal Bovine Serum (50 ml of stock)

5 ml 100 x Penn/Strep into 500 ml media

For FVIII expression studies in BHK cells, media is switched to AIM-V (no serum).

2. **CHO-K1 cell media:**

415 ml DMEM/F12

5 ml L-Glutamic acid/Asparagine stock

10 ml Nucleoside stock

15ml L-Glutamine stock

50ml dialysed Foetal Calf Serum (10%)

5ml Non-essential amino acids (Gibco x100)

Vacuum cap filter media into 1 L sterile bottle.

3. **HEK cell media:**

500 ml DMEM

50 ml Foetal Calf Serum (10%)

5 ml L-Glutamine stock

5 ml Penicillin/streptomycin stock

4. **Phosphate Buffered Saline:**

1 PBS tablet dissolved in 100ml distilled water. PBS was sterilised by autoclaving.

5. **Hepes buffer:**

150mM made using autoclaved water and then sterile filtered using a vacuum pump and a 0.22 uM Millipore express 500 ml funnel/receiver bottle.

6. **Nucleoside stock:**

175 mg Adenosine

175 mg Guanosine

175 mg Cytidine

175 mg Uridine

60 mg Thymidine

In 500 ml ddH_2O

Store -20 °C

7. **L-Glutamine stock:**

4.383 g in 150 ml ddH20

Store -20°C

8. **L-Glutamic acid/L-Asparagine stock:**

1.5g L-Glutamic acid

1.5g L-Asparagine

In 250ml ddH2O

Store -20oC

9. **Heat-inactivation of foetal bovine serum/foetal calf serum:**

Defrost dialysed Foetal Bovine Serum (dFBS) or Foetal Calf Serum (dFCS), heat to 56°C for 30 minutes. Aliquot in sterile cabinet into 50 ml sterile containers and store at -20°C until use.

6. Western blotting buffers

Table 37. Western blotting buffers

Reagent	Blocking buffer	Wash buffer
PBS	1x	1x
Marvel milk powder	5%	none
Tween	0.2%	0.2%

7. Purification of his-tagged proteins on a nickel column

Binding buffer (equilibration): 20mM sodium phosphate, 0.5M NaCl, 20mM imidazole, pH 7.4

Elution buffer: 20mM sodium phosphate, 0.5M NaCl, imidazole varying from 50mM to 500mM on 50mM increments, pH 7.4.

8. ELISA buffers

Blocking buffer: 2% BSA (or protifar or marvel) in PBS pH 7.4

Sample buffer: 1% blocking agent in PBS pH 7.4 with 0.05% Tween 20

Wash buffer: PBS with 0.05% Tween 20

Communications

Communication 1. Regarding the use of anti-FVIII antibodies

Hi Caroline,
Sorry for the delay in responding. The antibodies you are using are polyclonal therefore there may be variable recognition of epitopes between lots of product. The immunogen used to prepare these antibodies is full-length Factor VIII therefore epitopes throughout the protein including the B-domain will likely be recognized to varying degrees by different lots. If you are using as your sample a recombinant FVIII-deleted protein, such as a B-domain deleted FVIII, it is recommended that the calibration curve be prepared using the same type of protein in order to minimize the observed variability from lot-to-lot. We do not perform epitope mapping on these polyclonal antibodies therefore cannot guarantee that each lot recognizes the exact same epitopes on the Factor VIII.

I hope that this is helpful.

Best regards,

Ada Blythe
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Fax: 905-304-9897

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08.03.12

Communication 2. Regarding the use of anti-VHH antibodies

Dear Caroline,

The best thing to do is to clone a tag at the C end of the VHH and to detect the VHH via this tag. You can use His6 tag or heamaglutinin tag or c-myc tag or whatever. You can also biotinylate the VHH and then detect with HRP or AP conjugated streptavidin.

Rabbit antibodies against VHH do not work that well. The outcome for ELISA detection is rather unpredictable.
In addition, please remember that VHH don't perform very well in Western blot, normally as they recognize conformational epitopes that are disrupted upon SDS treatment.

Kind regards,
Serge Muyldermans

svmuylde@vub.ac.be
23.03.12