DEVELOPMENT OF ANTIBODIES AND CHARACTERISATION OF THE HUMORAL IMMUNE RESPONSES IN A SURROGATE ANIMAL MODEL FOR HEPATITIS C VIRUS (HCV)

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Abstract

Hepatitis C virus (HCV) infection has become a global public health concern with over 130 million people chronically infected and over 350,000 deaths every year from HCV-related liver diseases. GB virus-B (GBV-B) infection in tamarins is a surrogate model for acute HCV infection. Whilst HCV infection commonly leads to chronicity, GBV-B is naturally cleared. To better understand this natural clearance, this project aimed to study the associated humoral immune response to GBV-B. Additionally, GBV-B-specific antibodies were produced with the aim of characterising the pathology of the virus.

Previously, there was no available GBV-B neutralisation assay to identify antibodies in this animal model. Therefore, a GBV-B neutralisation assay, based on a method that is known to be successful for the closely-related HCV, was developed. This method involved producing pseudotyped retroviral particles (PV) expressing the GBV-B envelope that could infect a human hepatocarcinoma cell line. GBV-B PV production was confirmed by western blotting. Future studies can now test archived tamarin sera in this assay for the presence of neutralising antibodies. Neutralising antibodies found through this model could be epitope mapped, and incorporated into HCV vaccine design strategies.

To study the pathology of GBV-B infection, GBV-B-specific antibodies were also produced using two techniques in parallel- classical hybridoma technology and ribosome display. Antibodies targeting the nucleocapsid core protein of GBV-B have been previously detected in tamarins and served as the target for production of GBV-B antibodies using both aforementioned technologies. GBV-B core-specific antibodies were successfully isolated using both technologies and can now be used in downstream techniques, such as immunohistochemistry, to characterise the pathology of GBV-B infection thereby further validating the use of the animal model.

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Abbreviations

293T	Human Embryonic Kidney 293 T cells
ADCC	Antibody-dependent cell-mediated cytotoxicity
AID	Activation-induced cytidine deaminase
ALT	Alanine transaminase
APC	Antigen-presenting cell
ApoE	Apolipoprotein E
ARM	Antibody-ribosome-mRNA
BCR	B cell receptor
BHV	Bovine hepacivirus
BLAST	Basic Local Alignment Search Tool
BLyS	B Lymphocyte Stimulator
BSA	Bovine serum albumin
CD81	Cluster of Differentiation 81
cDNA	Complementary DNA
CDR	Complementarity-determining region
CHV	Canine hepacivirus
CLDN1	Claudin-1
CMV	Cytomegalovirus
CrFK	Crandell Rees feline kidney cells
CSR	Class-switch recombination
DAA	Direct-acting antiviral agents
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EHV	Equine hepacivirus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
FC	Fragment crystallisable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FR	Framework region
FRhK-4	Fetal Rhesus Kidney-4
G_4S	Glycine 4 serine
Gag	Polyprotein containing HIV-1 structural proteins containing matrix, capsid and nucleocapsid proteins
GAG	Glycosaminoglycan
GBV-B	GB virus B
GFP	Green fluorescent protein
GHV	Guereza hepacivirus
HA	Human influenza hemagglutinin
HAT	Hypoxanthine-aminopterin-thymidine
HCV	Hepatitis C virus
HCVcc	Cell-culture-derived infectious HCV
HCVpp	HCV pseudoparticles
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
His	Histidine tag (6xHis)
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSPC	Haematopoietic stem and progenitor cells
HT	Hypoxanthine-thymidine
HTLV-1	Human T-cell lymphotropic virus type 1
Huh	Human hepatoma
IFN	Interferon
IG	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside

IRES	Internal ribosome entry site
ITAM	Immunoreceptor tyrosine-based activation motif
JFH-1	Japanese Fulminant Hepatitis HCV genotype 2a strain
LB	Luria-Bertani
LDL	Low-density lipoprotein
LDLr	Low-density lipoprotein receptor
LTR	Long terminal repeats
mAb	Monoclonal antibody
MAC	Magnetic-Activated Cell Sorting
MAV	Mitochondrial antiviral-signalling protein
MHC	Major histocompatibility complex
miR-122	MicroRNA 122
MLV	Murine leukaemia virus
MOI	Multiplicity of infection
mRNA	MicroRNA
NANB	Non-A, non-B
NPC1L1	Niemann-Pick C1-Like 1
NS proteins	Non-structural proteins
OCLN	Occludin
PAMP	Pathogen-associated molecular patterns
PBS	Primer binding site
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PPT	Polypurine tract
PRR	Pattern recognition receptors
PV	Pseudotype viral particles
PVNA	PV neutralisation assay
R	Repeat region
RAV	Rous associated virus
RER	Rough endoplasmic reticulum

RFP	Red fluorescent protein
RHV	Rodent hepacivirus
RIPA	Radio immunoprecipitation assay
RLU	Relative light unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
scFv	Single chain variable region
SCID	Severe combined immunodeficiency
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRB1	Scavenger receptor class B member 1
SRFBP1	Serum Response Factor Binding Protein 1
STING	Stimulator of interferon genes
TCR	T cell receptor
TE	Tris-EDTA buffer
TLR	Toll-like receptor
tRNA	Transfer ribonucleic acid
U3	Unique region 3
U5	Unique region 5
uPA-SCID	Urokinase-type plasminogen activator-severe combined immunodeficiency
UTR	Untranslated region
V_H	Variable region from the immunoglobulin heavy chain
V_L	Variable region from the immunoglobulin light chain
vLDL	Very low density lipoprotein
VSV	Vesicular stomatitis virus
YT	Yeast extract-Tryptone

CHAPTER 1 : INTRODUCTION

1.1 HEPATITIS C VIRUS (HCV)

1.1.1 HEPATITIS VIRUSES

The most common aetiological agents of hepatitis in humans, an inflammation of the liver, is a group of hepatotropic viruses named hepatitis A, B, C, D, E, F and G (Shafran and Conly, 1996). Although these viruses have a common tropism for the liver and viral pathology, there are several differences between the viruses including the structure of each virus and the typical course, and subsequent outcome, of infection (Table 1-1). Whilst some of the hepatitis viruses, such as types A and E, typically cause acute, self-limiting infections, other hepatitis virus infections, such as types B and C, regularly progress to chronic infections. These chronic hepatitis B and C infections can lead to the development of liver cirrhosis and hepatocellular carcinoma. Globally, an estimated 399,000 people die every year as a result of these hepatitis C-related diseases (Lozano *et al*., 2012, World Health Organisation, 2017).

Hepatitis Virus	*Typical course of infection*	*Structure of virus*	*Family of virus*	*Reference*
A	Acute	Single-stranded RNA	Picornaviridae	(Koff, 1998)
B	Chronic	Partially double-stranded DNA	Hepadnaviridae	(Gitlin, 1997)
C	Chronic	Single-stranded RNA	Flaviviridae	(Simmonds, 2013)
D	Chronic	Single-stranded RNA	Unassigned	(Alvarado-Mora, 2013)
E	Acute	Single-stranded RNA	Hepeviridae	(Kamar, 2014)
F	Acute	Double-stranded DNA	Togavirus-like	(Deka *et al*., 1994; Fagan and Harrison, 1994)
G	Chronic, no associated disease	Single-stranded RNA	Flaviviridae	(Stapleton, 2003; Stapleton *et al*., 2011)

TABLE 1-1: SUMMARY OF DIFFERENT HEPATITIS VIRUSES IN HUMANS

1.1.2 DISCOVERY OF HEPATITIS C VIRUS

HCV is an enveloped RNA virus that can cause both acute and chronic hepatitis (Lindenbach and Rice, 2013). With an estimated 71 million people worldwide chronically infected, and at risk for developing liver cirrhosis or hepatocellular carcinoma, HCV has become a major public health concern. Prior to the discovery of the virus in 1989, blood and blood products unknowingly contaminated with HCV aided its spread (Alter, 1991; Donahue, 1992).

Although hepatitis A and hepatitis B were classified in the 1970s, there remained hepatitis of an unknown aetiologic agent termed non-A, non-B (NANB) hepatitis. Bradley and colleagues demonstrated that an infectious, transmissible agent was the cause of NANB hepatitis (Bradley *et al*., 1979) and that sera from affected patients resulted in acute and chronic hepatitis in chimpanzees (Alter *et al*., 1978; Hollinger *et al*., 1978; Tabor *et al*., 1978). In

1989, Choo and colleagues isolated an RNA virus from both the sera and tissues of a chimpanzee that had been inoculated with NANB serum and this was named Hepatitis C virus (HCV)(Choo *et al*., 1989).

1.1.3 CLASSIFICATION AND HETEROGENEITY

HCV belongs to the hepacivirus genus of the *Flaviviridae* family, a family of positive sense, single-stranded, enveloped RNA viruses. HCV is a highly genetically diverse virus. There are 7 identified genotypes, which can be further separated into 67 different subtypes (Smith *et al*., 2014). Approximately 10^{12} new virions can be produced daily, which in combination with highly error-prone viral RNA-dependent RNA polymerase (Powdrill *et al*., 2011), allows HCV to establish quasispecies in a single host.

HCV heterogeneity underpins its ability to successfully evade the immune system of its host permitting its persistence in the majority of cases (Forns, Purcell and Bukh, 1999). The highest level of diversity is seen within the envelope protein E2 (Figure 1-1, Weiner *et al*., 1992).

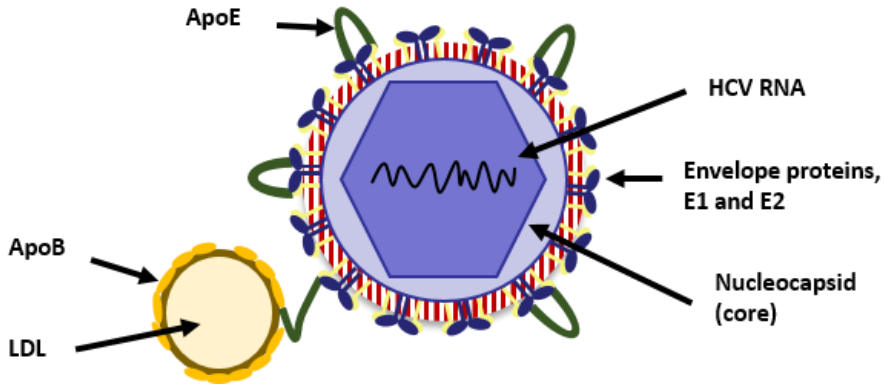


FIGURE 1-1: TYPICAL STRUCTURE OF AN HCV PARTICLE (ADAPTED FROM LINDENBACH, 2013).

HCV VIRIONS ASSOCIATE WITH SERUM LIPOPROTEINS, SUCH AS LDL (AS SEEN IN THIS DIAGRAM) OR HDL (HIGH DENSITY LIPOPROTEINS). THESE LIPOPROTEIN PARTICLES CONSIST OF TRIGLYCERIDES, CHOLESTEROL AND CHOLESTEROL ESTERS SURROUNDED BY A PHOSPHOLIPID MEMBRANE. THESE PARTICLES ALSO CONTAIN VARIOUS APOLIPOPROTEINS ON THEIR SURFACE, SUCH AS APOB. OTHER APOLIPOPROTEINS ARE THOUGHT TO ASSOCIATE WITH THE PARTICLE DIRECTLY, SUCH AS APOE. HCV USES THESE LIPOPROTEIN PARTICLES TO AID VIRUS ENTRY AND TO PROVIDE SOME PROTECTION AGAINST ANTIBODY NEUTRALIZATION (LINDENBACH, 2013). THE STRUCTURAL ORGANISATION OF THE HCV/LIPOPROTEIN PARTICLE IS CURRENTLY UNKNOWN; THE MODEL ABOVE IS A REPRESENTATION OF THE TWO PARTICLE MODEL WHICH SUGGESTS THAT HCV AND SERUM LIPOPROTEINS INTERACT TRANSIENTLY. IN CONTRAST, THE SINGLE PARTICLE MODEL SUGGESTS THAT THE TWO SHARE AN ENVELOPE.

E2 has 3 hypervariable regions that exhibit high numbers of amino acid substitutions (Kato *et al*., 1992; Troesch *et al*., 2006; Weiner *et al*., 1992). These regions are thought to be involved in viral persistence as they are typically targets of the immune system, in particular antibody targets (Torres-Puente *et al*., 2008; Troesch *et al*., 2006).

The heterogeneous nature of HCV also impacts the outcome of therapy as drug resistance can develop from viral mutations. Historically, it was thought that certain genotypes were more closely linked to a more severe outcome, such as hepatocellular carcinoma, although more recent studies dispute this (Hnatyszyn, 2005; Pawlotsky, 2003). A link between different genotypes and treatment outcome has also been investigated where genotype 1, the most prevalent genotype worldwide, and genotype 4 typically responded poorly to first generation

therapeutic regimens, such as peginterferon and ribavirin (Jackowiak *et al*., 2014; Messina *et al*., 2014).

1.1.4 ORIGINS

Although the origins of HCV are unknown, there are two possible scenarios. The first is a zoonotic origin. It was originally thought that HCV might have crossed the species barrier from chimpanzees. This could explain why the different genotypes of HCV are phylogenetically and geographically distinct. However, currently, there is no evidence of HCV, or HCV homologues, in wild chimpanzees (Makuwa *et al*., 2006).

More recently, several hepaciviruses that are closely related to HCV have been identified in a range of animals including horses, bats, rats and dogs, cows and monkeys. Figure 1-2 shows phylogenetic analysis of these closely related hepaciviruses from various species and Table 1-2 shows a summary of HCV related viruses and the pathogenesis in their host.

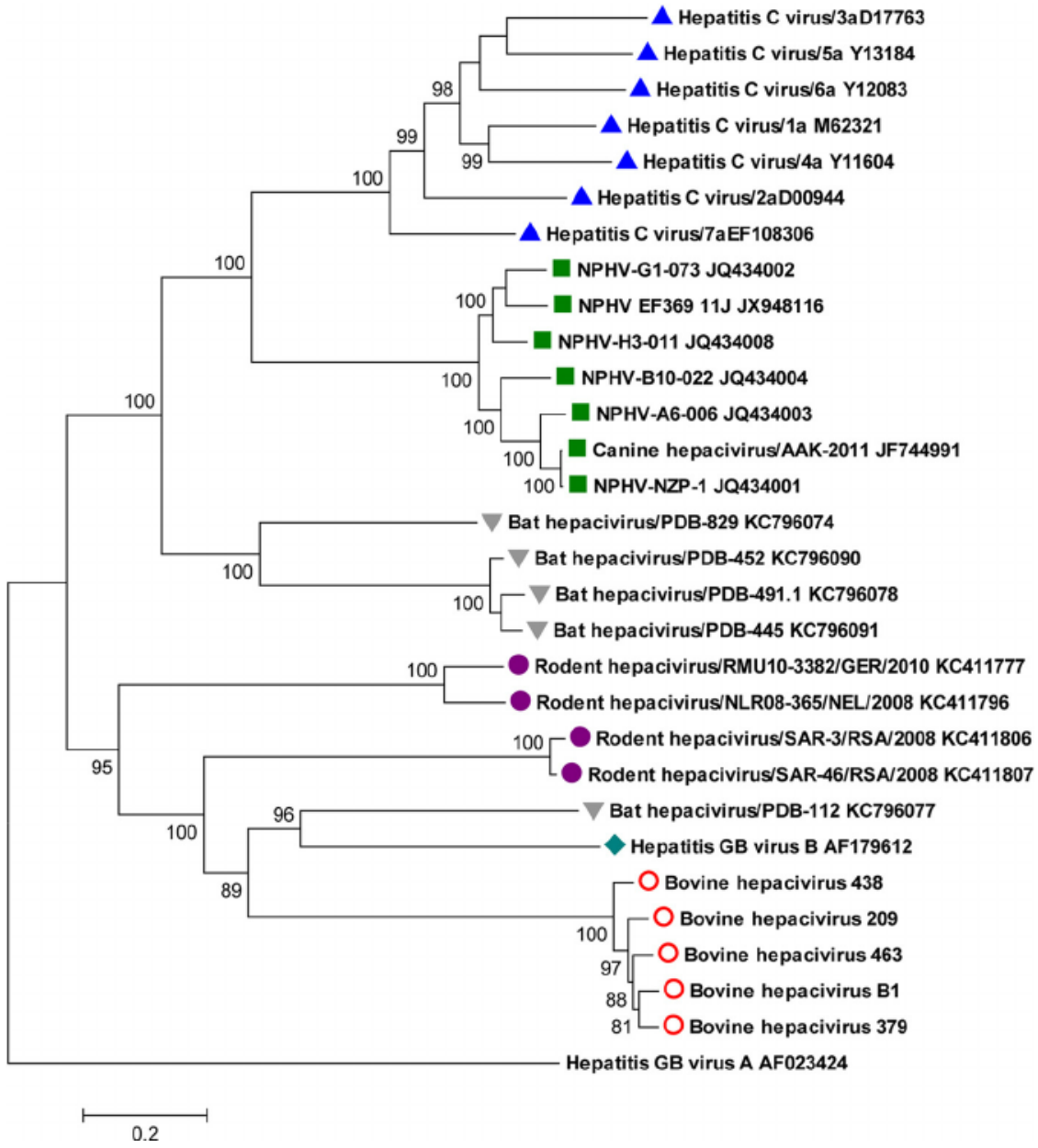


FIGURE 1-2: PHYLOGENETIC ANALYSIS OF HCV AND SEVERAL CLOSELY-RELATED HEPACIVIRUSES

THIS TREE WAS ROOTED TO GBV-A. FOR EACH SEQUENCE, THE GENBANK ACCESSION NUMBER IS INCLUDED. THIS FIGURE HAS BEEN TAKEN FROM BAECHLEIN *ET AL.*, 2015.

Host	*Name*	*Pathogenesis*	*Reference*
Human	Hepatitis C virus	Viral hepatitis	(Choo *et al*., 1989)
Canine	Canine hepacivirus, CHV	Respiratory disease	(Kapoor *et al*., 2011)
Bovine	Bovine hepacivirus, BHV	None	(Baechlein *et al*., 2015; Corman *et al*., 2015)
Equine	Equine hepacivirus, EHV	Viral hepatitis	(Burbelo *et al*., 2012; Lyons *et al*., 2012)
Rodent	Rodent hepacivirus, RHV	Evidence of liver inflammation	(Drexler *et al*., 2013; Kapoor *et al*., 2013)
Bat	Bat hepacivirus	None	(Quan *et al*., 2013)
New World monkey	GB virus B (GBV-B)	Viral hepatitis	(Simons *et al*., 1995b)
Old World monkey	Guereza hepacivirus (GHV)	None	(Lauck *et al*., 2013)

TABLE 1-2: SUMMARY OF HEPACIVIRUSES RELATED TO HCV AND THE PATHOGENESIS SEEN WITH THESE VIRUSES (ADAPTED FROM PFAENDER *ET AL*., 2014).

The broad range of animals that have been infected with these related viruses further support the potential for HCV jumping the species barrier. The hepaciviruses isolated from dogs and horses, commonly referred to as non-primate hepaciviruses, are highly genetically similar, with some equine hepacivirus (EHV) genomes being over 99% identical to canine hepacivirus (CHV) genomes. Cross-species transmission between these hosts has been previously suggested based on the episode of a dog which became seropositive to hepacivirus after being in close contact with an EHV-infected horse (Pfaender *et al*., 2014). For these viruses, cross-species transmission is likely due to the number of opportunities for routes of transmission, for example, the use of horse sera in canine vaccine manufacturing, the co-housing of these species or the consumption of horse meat by dogs (Scheel *et al*., 2015). Despite the possibility of cross-species transmission of EHV and CHV, a recent study

showed that EHV RNA was not detected in 172 people that are regularly exposed to horses (Pfaender *et al*., 2015). However, HCV transmission by either a single or multiple zoonosis events still remains a possibility and, with the continued discoveries of new hepaciviruses, these origins could soon be determined.

The second possibility is that HCV emerged as a human virus and co-evolved with its human host. If this is the case, HCV has always infected humans, similar to herpesviruses (Wertheim *et al*., 2014). Evidence supporting this is based on the fact that HCV can persist in humans for decades successfully. Further supporting this argument is HCV's requirement for human liver-specific microRNA-122 for replication (Jopling *et al*., 2005). As such, an increase in detection of HCV infections could be due to increased surveillance, increased opportunities for transmission and a greater life expectancy (Simmonds, 2013).

1.1.5 Structure

HCV virions consist of a single stranded RNA genome encapsulated by the nucleocapsid formed from the core protein (Figure 1-1). This nucleocapsid is then enveloped by a lipid bilayer containing the E1 and E2 glycoproteins (Gastaminza *et al*., 2010). The virions are associated with low-density and very-low-density lipoproteins (LDL and vLDL) and therefore vary in size and density (approximate density 1.1g/mL, Lindenbach, 2013, André *et al*., 2002).

HCV has a genome of around 9500 kilobases that encodes a single polyprotein that is then cleaved by both host peptidases and viral proteases into individual viral proteins (Forns *et al*., 1999). These viral proteins are either classed as structural proteins (this includes the core protein and both envelope proteins, E1 and E2) or non-structural proteins (p7, NS2, NS3,

NS4A, NS5A and NS5B). Individual roles for each of these proteins can be seen in Table 1-3. The open reading frame encoding these proteins has at either end an untranslated region (UTR) which is necessary for viral replication. The highly structured 5'UTR contains an internal ribosome entry site (IRES) allowing ribosomes to bind, beginning the translation of the viral polyprotein. The 3'UTR contains a polyuridine tract and is thought to interact with ribosomes to regulate the translation of the viral genome (Penin *et al*., 2004, Bai *et al*., 2013).

Viral protein	*Role*
Core	Forms the nucleocapsid which encapsulates the viral RNA. The N-terminal RNA binding domain allows the formation of the nucleocapsid through interaction with RNA. The membrane-binding domain allows mature core to locate to lipid droplets promoting viral assembly (Hope and McLauchlan, 2000; McLauchlan, 2000)
E1/E2	Envelope glycoproteins. The transmembrane domains for both function to retain the glycoproteins at the endoplasmic reticulum and help form the noncovalent E1E2 heterodimer (Cocquerel *et al*., 1998a). This heterodimer is held together by disulphide bonds in the ectodomains. They are critical for viral entry by binding to entry receptors (Pileri *et al*. 1998; Bartosch *et al*. 2003).
P7	P7 is a viroporin (Griffin *et al*., 2003). It protects nascent particles from low pH and is also involved in Envelopment and capsid assembly (Gentzsch *et al*., 2013; Wozniak *et al*., 2010).
NS2	A protease with a role in assembly by bringing together E1, E2, p7, NS3/4A (Jones *et al*., 2007; Popescu *et al*., 2011).
NS3	Serine protease that cleaves nonstructural proteins and some cellular proteins such as cellular antiviral signalling proteins involved in the innate immune response, RNA helicase and NTPase activities (Morikawa *et al*., 2011).
NS4A	NS3 cofactor by localizing NS3 and controlling its activities (Failla *et al*., 1994).
NS4B	Integral membrane protein and known as the 'master organizer of replication complex formation'. It's required to assemble the membranous web for RNA replication (Moradpour and Penin, 2013).
NS5A	Zinc containing phosphoprotein involved in regulating replication and particle production. It delivers HCV RNA to core protein (Macdonald and Harris, 2004).
NS5B	RNA-dependent RNA polymerase (Behrens *et al*., 1996).

TABLE 1-3: PROPERTIES AND FUNCTIONS OF HCV PROTEINS

1.1.6 LIFE CYCLE

Through the use of the various *in vitro* and *in vivo* models of HCV infection, the viral lifecycle has now been mostly characterised. Virions are blood borne and are transmitted most commonly intravenously, including through contaminated needles of drug users or blood products and transfusions. Transmission also occurs vertically (from an HCV positive mother) and, rarely, sexually (Dienstag, 1997; Yeung, 2001).

1.1.6.1 VIRUS ENTRY

The first stage in the viral life cycle, viral entry, has been of significant interest due to the inability to propagate almost all strains of the virus in cell culture. *In vitro* models of HCV, such as retroviral pseudotyped viruses bearing HCV envelope proteins or the only known isolate of HCV that can replicate and establish infection in cell culture, JFH-1 (Wakita, 2009), have been invaluable in understanding the viral life cycle.

Once the virus enters the body, it can infect a small range of cells that are most commonly hepatocytes. HCV entry into its host cell is an extremely complex, multi-component process focused on the interaction of HCV envelope proteins, associated lipoproteins and a range of host cell receptors, factors and regulators. This complexity surrounding HCV entry is most likely the contributing factor to the virus' limited tropism (Ding *et al*., 2014).

The initiation of HCV entry begins with a primary attachment of HCV particles to glycosaminoglycans (GAGs) present on the surface of hepatocytes as seen in Figure 1-3 (Popescu *et al*., 2014). HCV virions have been shown to bind to at least 2 types of heparin sulphate proteoglycans, syndecan-1 and syndecan-4 (Lefèvre *et al*., 2014; Shi *et al*., 2013), using both the viral glycoproteins (Barth *et al*., 2003) and the virion-associated apolipoprotein

apoE (Jiang *et al*., 2012). This interaction is thought to anchor the virion at the surface of the hepatocyte allowing further interactions between the virion and specific entry receptors.

As the HCV virion is associated with low-density lipoproteins (LDL) (Nielsen *et al*., 2006), virions can interact with lipoprotein receptors. Initially, the LDL receptor (LDLr) was thought to be involved in HCV entry (Agnello *et al*., 1999; Molina *et al*., 2007; Owen *et al*., 2009) however a recent study has suggested that interaction with this receptor would lead to a non-productive pathway and virion degradation (Albecka *et al*., 2012). Instead, virion glycoprotein E2 interaction with the lipoprotein receptor scavenger receptor 1 (SRB1), a receptor that typically binds a range of host lipoproteins (Shen *et al*., 2014), leads to a productive HCV entry pathway (Scarselli *et al*., 2002). Moreover, SRB1 appears to have a multifunctional role in HCV entry as it also is involved in virion attachment through virion-associated lipoproteins and a post-binding event (Dao Thi *et al*., 2012, 2011; Maillard *et al*., 2006; Zahid *et al*., 2013). From these studies, it is clear that the exact role of SRB1 in viral entry may not be completely understood. Current opinion is that SRB1 initially interacts with viral lipoproteins allowing a subsequent interaction between SRB1 and HCV E2. SRB1 could then modify the lipid composition of the virion or the plasma membrane of the hepatocyte to allow further interactions between the virion and other entry receptors (Popescu *et al*., 2014).

After the interaction between the hypervariable region 1 (HVR1) of HCV E2 and SRB1, the CD81 binding site of E2 is revealed (Bankwitz *et al*., 2010). This allows HCV to bind to the extracellular loop of CD81, a tetraspanin found on the plasma membrane of a variety of cells including hepatocytes and lymphocytes (Pileri *et al*., 1998). The binding of HCV E2 to CD81 induces signaling pathways involving epidermal growth factor receptor (EGFR) and the GTPase HRas that leads to the assembly of CD81-viral particle and tight-junction protein Claudin-1 (CLDN1) complexes (Diao *et al*., 2012; Douam *et al*., 2014; Harris *et al*., 2010;

Hopcraft and Evans, 2015; Lupberger *et al*., 2011; Zona *et al*., 2013). This interaction with CLDN1 has been shown to be essential for viral entry, along with another tight-junction protein Occludin (OCLN), although the exact role of OCLN is unknown (Evans *et al*., 2007; Ploss *et al*., 2009). The clustering of CD81-virions and CLDN1 is then thought to lead to internalisation of the virion via clathrin-dependent endocytosis (Blanchard *et al*., 2006; Farquhar *et al*., 2012). Numerous other additional factors have also been identified as to be involved in viral entry including the Niemann-Pick C1-like 1 receptor (NPC1L1), the transferrin-1 receptor and the serum response factor-binding protein 1 (SRFBP1) (Gerold *et al*., 2015; Martin and Uprichard, 2013; Sainz *et al*., 2012).

Following receptor-mediated binding to the cell surface, the virions are internalised into endosomes causing the viral envelope proteins to undergo the conformational changes required to promote fusion to the endosome membrane and the release of the viral genome (Blanchard *et al*., 2006; Tscherne *et al*., 2006). This process is briefly summarised in Figure 1-3, below.

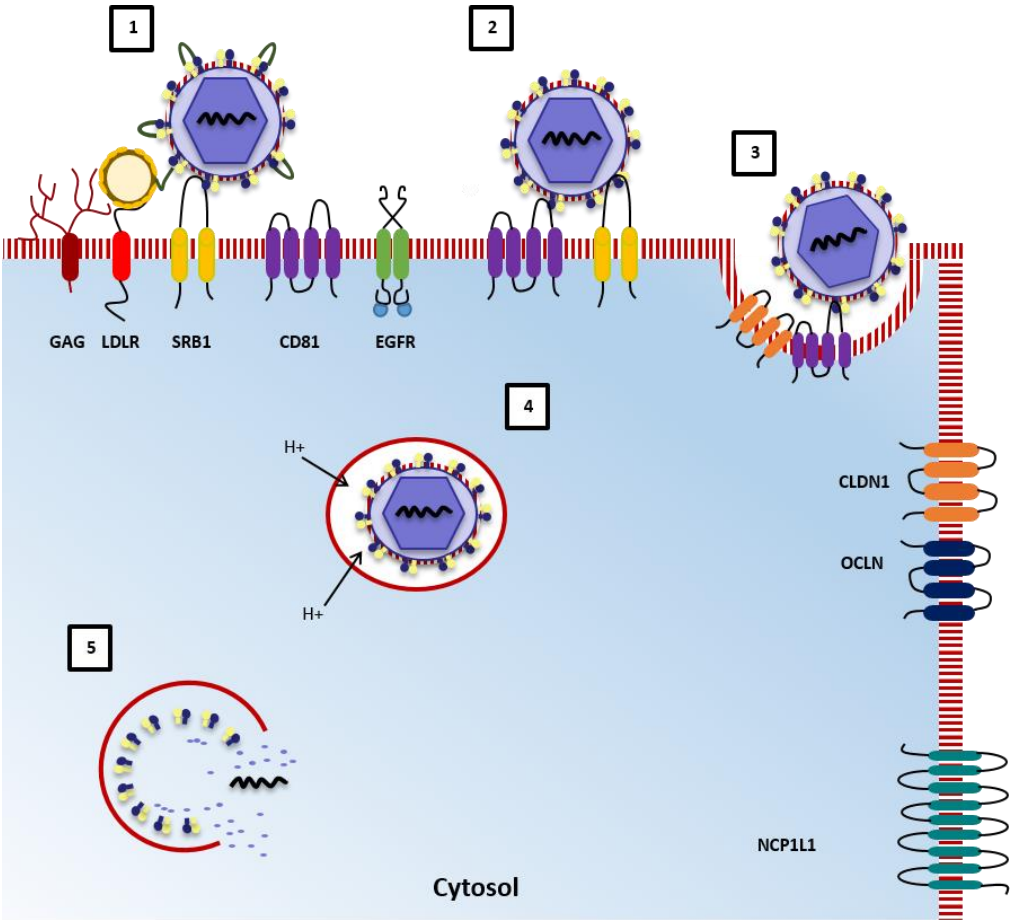


FIGURE 1-3: SUMMARY OF HCV ENTRY (ADAPTED FROM LINDENBACH AND RICE, 2013).

BRIEFLY, THE LIPOPROTEIN COMPONENT OF HCV LIPOVIRAL PARTICLES PROMOTES THE INITIAL INTERACTION BETWEEN THE HCV AND HCV ENTRY RECEPTORS GAG, LDLR AND SRB1 (1). THIS INTERACTION WITH SRB1 INDUCES A CONFORMATIONAL CHANGE IN HCV E2 ALLOWING THE SUBSEQUENT BINDING OF HCV E2 WITH CD81 (2). THIS IN TURN ACTIVATES A SIGNALLING CASCADE RESULTING IN THE REMODELLING OF ACTIN AND THE INTERNALISATION OF THE HCV PARTICLE (3). THE INTERACTION BETWEEN HCV-CD81 AND CLDN1 LEADS TO CLATHRIN-MEDIATED ENDOCYTOSIS. ONCE ENDOCYTOSED, H+ IONS FLOW INTO THE VESICLE LEADING TO HCV ENVELOPE FUSION WITH THE VESICLE MEMBRANE (4). THIS IN TURN LEADS TO THE RELEASE OF HCV RNA INTO THE CYTOSOL (5).

1.1.6.2 HCV REPLICATION

Once released in to the cytoplasm of the host cell, the positive-sense viral genome acts as messenger RNA allowing the translation of the viral genome. Translation of the viral genome

into a large polyprotein of around 3000 amino acids occurs at the rough ER and is initiated by the IRES within the 5'UTR of the viral genome (Dubuisson and Cosset, 2014). The HCV polyprotein is targeted to the ER membrane by internal ER signal peptides (Wu, 2001). Once translated, the polyprotein is processed. Host proteases, such as host-cell signal peptidases, work to cleave the structural proteins, core, E1 and E2, along with the non-structural p7. Cleaved E1 and E2 can then undergo glycosylation and conformational changes before mature E1E2 heterodimers are assembled (Penin *et al*., 2004). When separated from p7, the autoprotease NS2 is then able to cleave itself from NS3 (Grakoui *et al*., 1993). This cleavage allows NS3 to cleave the downstream NS4A/B junction leading to the association of NS3 and NS4A. This NS3/4A protease is then responsible for cleavage of the remaining HCV proteins (Penin *et al*., 2004). All HCV proteins are anchored at the ER via transmembrane regions, amphipathic α-helices or, in the case of NS3, by association with another membrane-bound protein (Bartenschlager *et al*., 2013).

After polyprotein processing, some of the non-structural HCV proteins NS3, NS4A, NS4B, NS5A and NS5B form a replication unit that, along with host factors, induces complex structural changes in the ER membrane forming what is known as the membranous web (Dubuisson and Cosset, 2014; Gosert *et al*., 2003; Lohmann *et al*., 1999). The membranous web, consisting of clusters of predominately double-membraned vesicles along with lipid droplets, is the site of HCV replication (Gosert *et al*., 2003). Host cell membrane rearrangements, such as the membranous web produced by HCV, is a common feature of the replication of positive-sense viruses (Miller and Krijnse-Locker, 2008). For example, both poliovirus and severe acute respiratory syndrome virus produce similar double-membraned vesicles.

HCV RNA synthesis, through the use of the RNA-dependent RNA polymerase NS5B and host factors such as miR-122, leads to the production of negative-sense HCV RNA from which

several positive-sense HCV RNA genomes can be produced. A summary of this process is seen in Figure 1-4 below. The RNA genomes formed are then either translated to more viral proteins, more RNA genomes or packaged in new virions (Bartenschlager *et al*., 2013).

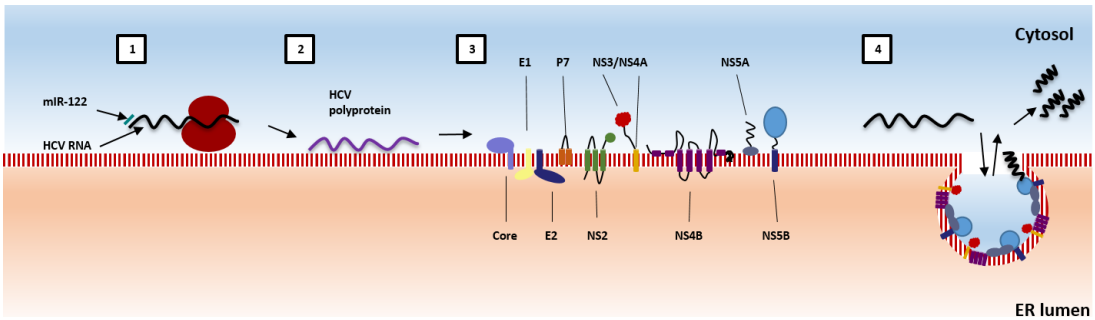


FIGURE 1-4: SUMMARY OF HCV RNA REPLICATION (ADAPTED FROM LINDENBACH AND RICE, 2013).

AFTER HCV RNA IS RELEASED FROM THE HCV PARTICLE IN THE CYTOSOL, THE RNA GENOME IS TRANSLATED ON RIBOSOMES LOCATED ON THE ROUGH ER (1) THAT RESULTS IN A LARGE POLYPROTEIN (2). THIS POLYPROTEIN IS THEN PROCESSED BY HOST AND VIRAL PROTEASES TO PRODUCE EACH INDIVIDUAL HCV PROTEIN, WHICH REMAIN LOCATED AT THE ER (3). ONCE PROCESSED, THE MEMBRANOUS WEB IS FORMED (4). NON-STRUCTURAL PROTEINS MIGRATE TO THIS AREA TO ALLOW THE REPLICATION OF THE HCV RNA GENOME. FOR EACH NEGATIVE-STRAND RNA GENOME PRODUCED, SEVERAL POSITIVE-SENSE RNA GENOMES ARE PRODUCED. SEE TABLE 1-3 FOR THE FUNCTION OF EACH HCV PROTEIN.

1.1.6.3 HCV ASSEMBLY

The assembly of HCV particles is a complicated process that is dependent on the interaction of both host and viral proteins (Figure 1-4, Lindenbach and Rice, 2013). The main structural components of HCV virions are the nucleocapsid core protein and the envelope proteins, E1 and E2. Core proteins are produced in the ER and transferred to lipid droplets, intracellular stores of neutral lipids in cells (Barba *et al*., 1997). These lipid droplets are thought to be the site of virion assembly as core is able to recruit the relevant HCV non-structural proteins and RNA genomes to nearby lipid droplet-associated membranes (Miyanari *et al*., 2007). Once core interacts with the RNA genome of HCV, core is able to oligomerise to produce the

nucleocapsid (Boulant *et al.*, 2006). NS5A can also bind RNA and deliver it to core further promoting virion assembly (Huang *et al*., 2005). Additionally, viral particles are often found nearby lipid droplets further implying that the lipid droplets are the site of HCV assembly (Miyanari *et al*., 2007).

The other major structural elements of HCV particles are the envelope proteins, E1 and E2. These proteins are also produced in the ER where they form heterodimers that are retained via an ER-retention signal, as described earlier. These heterodimers migrate to the site of viral assembly through the interaction with NS2 and P7 (Atoom *et al*., 2013; Stapleford and Lindenbach, 2011). As such, the envelope is acquired through the budding of the HCV particle through the ER membrane. In contrast to other viruses, HCV virions at this point are mature and infectious due to the budding from the ER rather than the cellular plasma membrane (Gastaminza *et al*., 2006).

This budding through the ER is linked to lipoprotein synthesis and assembly thereby allowing the association of lipoproteins with the new HCV particles (Catanese *et al*., 2013). This link between lipoprotein synthesis, lipoprotein assembly and HCV particle production was confirmed as inhibition of microsomal triglyceride transfer protein or ApoB prevented the generation of infectious HCV particles (Gastaminza *et al*., 2008; Huang *et al*., 2007).

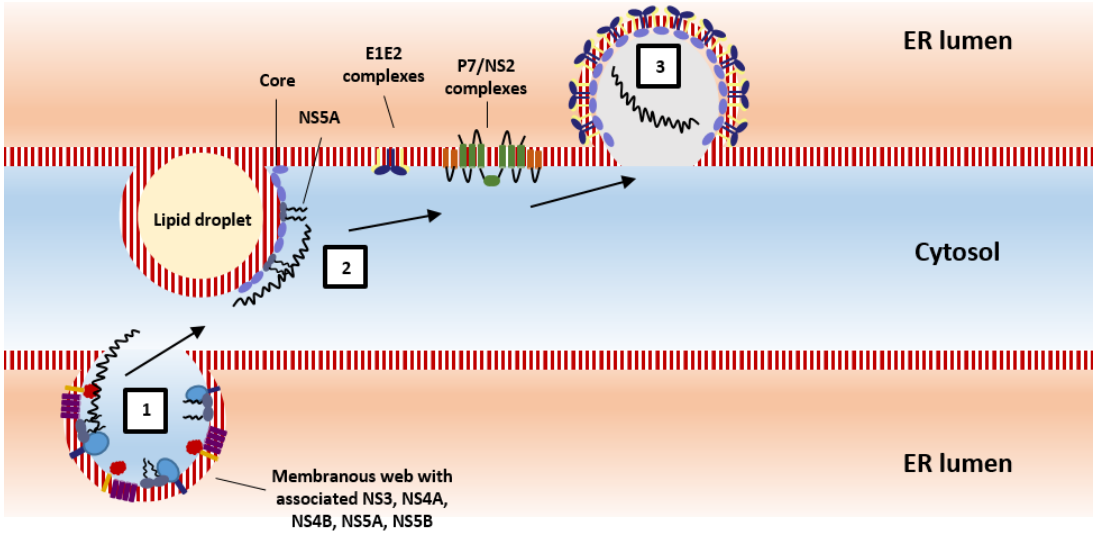


FIGURE 1-5: SUMMARY OF HCV ASSEMBLY (ADAPTED FROM LINDENBACH AND RICE, 2013).

AFTER REPLICATION OF HCV RNA GENOMES AT THE MEMBRANOUS WEB (1), THE RNA IS EITHER USED TO PRODUCE MORE RNA GENOMES, MORE HCV PROTEINS OR FOR PACKAGING IN NEW VIRIONS. CORE PROTEINS ARE TRANSFERRED TO LIPID DROPLETS ALLOWING THE RECRUITMENT OF NS5A AND HCV RNA (2). THE LIPID DROPLETS ARE THOUGHT TO BE THE SITE OF HCV ASSEMBLY AS CORE THEN OLIGOMERISES AROUND THE HCV RNA PRODUCING THE NUCLEOCAPSID. E1E2 COMPLEXES MIGRATE TO NEARBY LIPID DROPLETS, THROUGH INTERACTION WITH P7/NS2 COMPLEXES, ALLOWING THE BUDDING OF THE NUCLEOCAPSID FROM THE E1E2-STUDDED PHOSPHOLIPID ER MEMBRANE.

1.1.6.4 HCV PARTICLE EGRESS

As seen with other flaviviruses, HCV particle release is then thought to occur via the secretory pathway (Coller et al., 2012; Lindenbach and Rice, 2013b; Mackenzie and Westaway, 2001). This pathway is used to transport, among other proteins, lipoproteins including very LDL (VLDL, an LDL precursor) and HDL. As such, both very low density lipoprotein (vLDL) and HCV virion secretion can be blocked using the same reagents (Huang *et al.*, 2007; Nahmias *et al.*, 2008). Both vLDL and HCV particles are transported in VLDL transport vehicles (VTVs) through the Golgi apparatus and through the plasma membrane (Siddiqi, 2008). It has been previously suggested that the p7 viroporin protects the HCV particle from acidic conditions in these compartments (Wozniak *et al.*, 2010). Whilst the particles progress through the Golgi, the

HCV envelope proteins on these particles are post-translationally modified (Vieyres *et al*., 2010).

1.1.7 Regulation of HCV replication and assembly

Several microRNAs interact either directly or indirectly with HCV and have been shown to affect HCV replication. One particular microRNA, the liver-specific miR-122, is required for HCV replication (Jopling *et al*., 2005). This microRNA has been shown to have two separate functions by binding to two separate binding sites within the 5' untranslated region of the HCV viral genome. These two effects work to stabilise the viral RNA against degradation by host endonucleases and stimulating HCV RNA translation. By comparison, other microRNAs that are generally expressed in non-hepatic cells have the opposite effect of inhibiting viral replication (Conrad and Niepmann, 2014).

Another key regulator of HCV replication is lipid peroxidation, the process of oxidative degradation of lipids by reactive oxygen species (ROS). Viral infections typically induce the production of ROS (Peterhans, 1997); HCV is known to induce oxidative stress in the liver thereby promoting liver damage (Paracha *et al*., 2013). There is now evidence that HCV can sense lipid peroxides induced by infection and restrict viral replication with the aim to potentially facilitate virus persistence (Yamane *et al*., 2014).

1.1.8 Infection outcomes

HCV infection has two potential outcomes within its host. Of those infected, an estimated 30% will successfully clear the virus without any intervention or treatment (Miller and Dillon, 2015). Most of these cases are asymptomatic and therefore subclinical resulting in an estimated diagnosis rate of acute infections of around 35% in England. In the remaining

cases, HCV persists resulting in chronic infection that can last several decades. In addition, in some patients that have successfully cleared HCV (defined as having no detectable HCV RNA in sera and normal liver function tests), HCV RNA has been documented to persist at low levels in the liver and peripheral blood mononuclear cells. This type of infection is now known as occult HCV infection. Patients are still at risk of developing the same liver diseases seen in chronic HCV infection but at a slower rate (Carreño *et al*., 2012; Pham *et al*., 2004). Defining what immune response is needed to effectively clear the virus is therefore of particular interest to researchers in this field.

It is now thought that immunological events early in infection determine the outcome of infection. Distinct profiles defined by clinical and immunological parameters, including viral load, alanine transaminase (ALT) levels, HCV-specific $CD4^+$ and $CD8^+$ T cell responses, are seen for both acute and chronic infections thereby potentially predicting infection outcome (Lauer, 2013). However, these early immunological events affecting infection outcome are poorly understood and the subclinical nature of acute HCV infections has made their study difficult; understanding these events is, however, critical for vaccine development.

1.1.9 THERAPIES AND VACCINES

In the absence of a licensed vaccine, historical standard treatment was based on combination therapy with pegylated interferon-α and ribavirin (Shiffman, 2014). However, these drugs required a 24-48 week course of treatment, were only effective in 50% of patients and had severe side effects due to their non-specificity. Side effects included autoimmune diseases and haemolytic anaemia, and for this reason, many patients could not continue with treatment (Manns *et al*., 2006).

The next generation of HCV therapies, Telaprevir and Boceprevir, were first approved in 2011 for use with difficult to treat genotypes, such as genotype 1. These direct-acting antiviral agents (DAA) specifically targeted one of the viral proteins, the NS3-NS4A protease, and were effective in around 70% of patients. However, the treatments were compromised by side effects, resistance and drug-drug interactions (Au and Pockros, 2014).

More recently, new DAAs have been approved targeting not only the NS3-NS4B protease (Simeprevir) but also NS5A inhibitors (Daclatasvir, Ledipasvir) and NS5B inhibitors (Sofosbuvir). These new drugs have a high potency and can be combined to achieve over 90% effectiveness in around 12 weeks, even in patients who had previously not responded to treatment (Popescu *et al*., 2011; Younossi *et al*., 2014).

Despite these advancements in drug therapies, these are expensive and therefore not widely available to most people (Gornall *et al*., 2016). As the therapies can't protect against reinfection, resistance mutations could appear with repeated use of these drugs allowing HCV infections to persist. A vaccine would not have these problems and would therefore be a more tractable solution, especially in developing countries.

Vaccines based on recombinant proteins, peptides, DNA of HCV proteins or epitopes, viral vectors delivering HCV RNA, or dendritic cells expressing foreign proteins such as HCV epitopes have all been investigated and each has at least one vaccine candidate in either Phase I or II clinical trials (Table 1-4) (as reviewed in Ghasemi *et al*., 2015; Xue *et al*., 2014). These approaches all have technical issues such as weak immune responses, pre-existing immunity or the limited availability of dendritic cells from patients. Additionally, limited data is available for these vaccines and none have progressed to Phase 3 clinical trials yet.

Type of vaccine	*Components of vaccine*	*Clinical trial phase*	*Reference*
Recombinant protein	E1	Ceased in 2007	(Leroux-Roels *et al*., 2004)
	E1 or E2	I	(Frey *et al*., 2010)
	Core	I	(Drane *et al*., 2009)
	NS3-core fusion protein	II	NCT00606086*
Peptide	Core	II	(Yutani *et al*., 2015, 2009)
	NS3	I	NCT00445419*
	E1, E2, NS3, NS5A	I	(Yutani *et al*., 2007)
	Core, NS3, NS4	II	(Klade *et al*., 2008; Wedemeyer *et al*., 2009)
	T cell epitopes	II	(Firbas *et al*., 2010, 2006)
DNA	Core, E1, E2+ recombinant core protein	II	(Alvarez-Lajonchere *et al*., 2009; Amador-Cañizares *et al*., 2014)
	NS3, NS4A	II	NCT01335711*
Viral vector	Ad6/ChAd3 expressing NS3-NS5B	I	(Barnes *et al*., 2012)
	Adenoviral vectors expressing HCV NS3-NS5B	II	NCT01094873*
Dendritic cell	Core, NS3, NS4B, universal Th epitopes	I	(Gowans *et al*., 2010)

TABLE 1-4: LIST OF HCV VACCINES IN CLINICAL TRIALS (ADAPTED FROM GHASEMI *ET AL*., 2015; XUE *ET AL*., 2014).

*: CLINICALTRIALS.GOV IDENTIFIER.

HCV vaccine development is complicated due to the virus' high genetic variability, its association with lipoproteins and the glycosylation of the envelope proteins helping to shield the virus from neutralising antibodies (Maillard *et al*., 2006). The virus can also spread

directly by cell-to-cell transfer aiding its evasion of the immune response (Timpe *et al*., 2008). Despite this, at least partial protection against reinfection is documented in many cases in both humans and chimpanzees (Bassett *et al*., 2001; Mehta *et al*., 2002; Nascimbeni *et al*., 2003; Osburn *et al*., 2010). To develop a vaccine, the immune response behind viral clearance must be completely understood so that a vaccine can be designed that directly elicits this type of response. Currently, it is accepted that a vaccine could be used to prevent infections progressing to chronicity, the type of infection associated with liver cirrhosis and cancer, rather than inducing sterilising immunity.

1.1.10 HCV MODEL SYSTEMS

1.1.10.1 *IN VITRO* MODELS

One of the biggest hindrances to the study of HCV is its restricted host range and the inability to culture the virus *in vitro*. To address this issue, *in vitro* and *in vivo* model systems were developed. HCV replicons were the first established *in vitro* model of HCV. These viral RNA units of reduced length commonly contained the NS3-NS5B replication complex of HCV and a drug resistance gene replacing the rest of the genome (Lohmann *et al*., 1999). Unfortunately, it acquired several adaptive mutations in NS3 or NS5A which made the replicon ineffective *in vivo* and unable to produce infectious HCV particles (Blight *et al*., 2000; Bukh *et al*., 2002).

These HCV replicons were later replaced with a genotype 2a strain of HCV, named JFH-1, that can replicate efficiently in cell culture without acquiring adaptive mutations (Kato *et al*., 2003). Moreover, infectious virions could be produced that successfully infected chimpanzees (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). The infectivity titre remained low for this system until chimeric viruses were created containing the replication

complex of JFH-1 (NS3-NS5B) but the rest of the genome of a different HCV isolate allowing the study of different HCV genotypes. This cell cultured HCV (HCVcc) has been invaluable in studying the entire HCV lifecycle and testing of novel antivirals and candidate vaccines (Gottwein *et al*., 2009; Imhof and Simmonds, 2010; Pietschmann *et al*., 2006; Scheel *et al*., 2011). In combination with these developments, improved cell lines such as the human hepatocarcinoma 7-Lunet cells, which have low CD81 expression (an essential HCV entry receptor) but could be transduced to overexpress CD81 to support robust HCV replication, significantly aided the study of HCV's lifecycle (Koutsoudakis *et al*., 2007; Saeed *et al*., 2015).

The main other method to study HCV entry is using pseudotyped HCV (HCVpp). These consist of either a retroviral or lentiviral backbone encoding a reporter gene instead of its own genome and bearing functional HCV envelope proteins (Bartosch *et al*., 2003). This system has been crucial for studying viral entry and response of virus to neutralising antibodies. In particular, it has been used to discover entry receptors such as Claudin-1 (Evans *et al*., 2007). As the HCVpp is non-replicative, it can be used in lower containment level laboratories than the original wildtype virus. The obvious disadvantage with this system is that only viral entry can be studied as the envelope proteins of HCV are the only parts of the virus that are incorporated.

1.1.10.2 *IN VIVO* MODELS

Although *in vitro* models have provided methods for studying the life cycle of HCV and the testing of novel antiviral compounds and vaccines, an *in vivo* model is vital for studying host-virus interactions and novel vaccines and treatments. *In vivo* models also permit the study of the whole duration of infection in comparison to HCV infections in patients where important tissue samples and time points are difficult to access (Bukh, 2012).

Chimpanzees are the only animal model that can support HCV replication and produce a similar infection profile to that seen in humans. This model had several advantages: both acute and chronic infections are produced, liver tissue can be taken for biopsy, and the entire duration of infection can be observed (Lanford *et al*., 2001; Prince and Brotman, 1994; Walker, 1997). There are differences between HCV infection of humans and chimpanzees, such as a reduced rate of chronicity in chimpanzees, but they remain the best animal model of HCV infection (Lanford *et al*., 2001). The use of chimpanzees is now banned within the European Union due to ethical reasons so this model has not been viable for a number of years (Altevogt *et al*., 2011; Harrington, 2012).

Tree shrews (*Tupaia belangeri chinesis*) have also been used as an *in vivo* model for HCV infection. Of the animals infected, 20-35% tested positive for HCV by PCR (Xie *et al*., 1998). This model has not been widely accepted because studies showed that only certain isolates of HCV could cause infection in these animals (Xu *et al*., 2007; Zhao *et al*., 2002).

Other *in vivo* models are based on the use of mice. Both transgenic mice and mice with humanised livers have been created to study the HCV lifecycle. Although HCV replication at low levels in murine cells has been previously reported (Uprichard *et al*., 2006), murine cells are not naturally permissive for HCV entry. The reason for this was discovered in 2009 when it was identified that only murine hepatocytes expressing the human occludin and CD81 receptors could support HCV entry (Ploss *et al*., 2009). This was an important step forward for developing a small animal model of HCV infection in which to test new antiviral therapies or vaccines. The mouse model is still not ideal as HCV replication in mice is inefficient.

Transgenic mice were also produced stably expressing the four central HCV entry receptors: CD81, OCLN, SCARB1 and CLDN1, or combinations of these. These mice proved useful for studying viral entry, potential inhibitors of viral entry and vaccine candidates (Dorner *et al*., 2011). In addition to this, mice that express different parts of the HCV genome, for example

HCV core protein, have also been produced to study host-virus interactions (Lerat *et al*., 2011). These mice can develop liver co-morbidities similar to that seen in HCV infection in humans, but without inflammation in the liver or HCV replication (as reviewed in Mailly *et al*. 2013).

To further improve the mouse model, researchers developed urokinase-type plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice (Mercer *et al*., 2001). These immunodeficient mice have a transgene that caused liver damage. Liver of the mice can then be repopulated with human hepatocytes. These mice provide a useful model for studying the whole of the HCV lifecycle. Typical viral titres as seen in humans can be achieved, as well as chronic infections (Mercer *et al*., 2001). These mice have been an invaluable asset for studying the effect of neutralising antibodies on viral entry and assessing novel directly acting antivirals (Akazawa *et al*., 2013; Vanwolleghem *et al*., 2007). Despite the usefulness of this model, the mice are immunodeficient meaning that the immune response to HCV cannot be studied. Other murine model variations also exist, but all have significant limitations of either immunodeficiency, no viraemia or inefficient replication of HCV (Mailly *et al*., 2013).

Surrogate models of HCV infection have also been established to allow the study of the immune response. One such model is GBV-B infection in tamarins. HCV and GBV-B cluster together on their phylogenetic tree (see Figure 1-2) and therefore share many similarities including the same genome structure, organisation, tropism and functions (Beames *et al*., 2001). Although novel hepaciviruses have now been discovered that cluster closer to HCV, most of these viruses are not known to be hepatotropic or have other disadvantages. GBV-B infection in tamarins is therefore used as a surrogate animal model to study acute HCV infection which permits the study of the immune response, unlike most other HCV models.

1.2 GBV-B

1.2.1 IDENTIFICATION

Prior to the identification of HCV, Deinhardt and colleagues were aiming to discover a nonhuman primate that could be infected with the agent of NANB hepatitis for use as an animal model (Deinhardt *et al*., 1967). To achieve this, sera from a surgeon, who had the initials GB, with viral hepatitis was inoculated into tamarins, a type of New World monkey, which subsequently developed hepatitis. Passage of serum from the infected tamarins to naïve tamarins resulted in hepatitis in the recipients, thus initially the infectious agent in the serum was proposed as the cause of NANB hepatitis (Deinhardt *et al*., 1967). However, subsequently, Simons and colleagues (Simons *et al*., 1995a, 1995b) identified two flaviviruses in the sera and livers of the infected tamarins, termed GBV-A and GBV-B, the latter of which was later determined to be the aetiologic agent of hepatitis in these animals (Schaluder *et al*., 1995). By contrast, GBV-A has not been associated with any disease (Stapleton *et al*., 2011).

1.2.2 ORIGINS

The origin of GBV-B is unknown. The virus has never been isolated from wild tamarins, only from tamarins infected with passages of the original Deinhardt GB serum (Deinhardt *et al*., 1967; Simmonds, 2013). However, neither humans nor chimpanzees can support GBV-B infection suggesting that the GB serum cannot have been the source of GBV-B (Tabor *et al*., 1980). GBV-B has been shown to be able to infect a number of New World monkey species including several species of tamarins (Beames *et al*., 2001, 2000; Bukh *et al*., 1999; Deinhardt *et al*., 1967; Schaluder *et al*., 1995), owl monkeys (Bukh *et al*., 2001) and marmosets (Lanford *et al*., 2003). Infection profiles differ between each of these hosts leading to the hypothesis that GBV-B is most likely a tamarin virus as GBV-B replicates most efficiently in this host species (Bright *et al*., 2004; Bukh *et al*., 2001; Lanford *et al*., 2003). Further supporting this

hypothesis, the closely-related GBV-A has been determined to be an indigenous tamarin virus (Bukh and Apgar, 1997).

1.2.3 CLASSIFICATION AND HETEROGENEITY

Initially, GBV-B was considered a conserved virus that exhibited little genetic variation. The virus has only one genotype and nucleotide sequence identities of 99.6-99.9% have been documented by sequencing the whole genome of either the virus or infectious molecular clones (Bukh *et al*., 1999; Martin *et al*., 2003; Nam *et al*., 2004). In addition, the N-terminal end of E2 does not have the same hypervariability that is seen with HCV (Weiner *et al*., 1991). Mutations do occur within the GBV-B proteins and have been shown to accumulate more frequently within the non-structural proteins, in particular NS5A, and very rarely in the structural proteins (Kyuregyan *et al*., 2005; Martin *et al*., 2003; Nam *et al*., 2004; Rijnbrand *et al*., 2005). These are most likely adaptive mutations that could help the virus evade the immune system (Kyuregyan *et al*., 2005).

More recent studies have shown that GBV-B has a higher genetic variability than previously thought. Preliminary deep sequencing data has showed that mutations accumulate over the course of infection and are positively selected; these mutations also accumulate at a higher rate as the infection progresses (Bowen *et al*., manuscript in preparation). The molecular evolution of GBV-B and HCV is now thought to be similar (Fernandez *et al*., 2004; Takikawa *et al*., 2010). As GBV-B has only been identified from one source, it is entirely possible that different genotypes could also exist within other sources. However, all animal experiments have been undertaken with virus isolated from one source which significantly impacts on any estimation of genetic heterogeneity (Beames *et al*., 2001).

1.2.4 STRUCTURE AND LIFE CYCLE

Although the life cycle of GBV-B has not been directly studied, its genome encodes homologous proteins to HCV with corresponding functions. Despite the overall amino acid homology being only around 28%, some regions are more conserved than others, with the NS3 serine protease, the NS3 RNA helicase and NS5B having a much higher homology with their HCV counterparts (Muerhoff *et al*., 1995; Zhong *et al*., 2000, 1999). Functional homology also exists between the NS3 proteases of both viruses enabling the cleavage of both HCV and GBV-B polyproteins by either viral NS3 (Scarselli *et al*., 1997).

The natural host of GBV-B remains uncertain resulting in a lack of knowledge about both its origin and its life cycle as a virus. In addition, the lack of a robust cell culture system for GBV-B provides another barrier for determining its viral life cycle. However, the core protein of GBV-B is directed to lipid droplets, as is seen in HCV's life cycle, suggesting that GBV-B and HCV virions share a similar structure, function and life cycle (Hope *et al*., 2002).

1.2.5 GBV-B INFECTION AND PATHOLOGY

Typically, GBV-B causes an acute infection in its New World monkey host. Commonly, the infection profile consists of a high viraemia that appears within 2 weeks after inoculation, quickly peaking and then declining to an undetectable level at around weeks 16-18. These timings are dependent on the initial GBV-B inoculation dose used and the route of infection (Lanford *et al*., 2003). Alanine aminotransferase (ALT) levels, a measure of liver damage, have showed to peak immediately prior to clearance. Occasionally, viral recrudescence is seen in the last weeks of infection but is quickly cleared. Chronic infections are rarely seen in this animal model with most only occurring under immunosuppression or if infected

intrahepatically (Lanford *et al*., 2003; Martin *et al*., 2003). The rare natural chronic infections that have been documented do not appear to have a consistent infection profile (Iwasaki *et al*., 2011).

Therefore, whilst GBV-B infection in tamarins, or other New World monkeys, is not ideal to model chronic HCV infection, it remains a valuable animal model of acute HCV infection. This model could be used to investigate novel drugs, vaccines or other treatments that are developed over the coming years.

1.3 THE IMMUNE SYSTEM

The human immune system is a complex defense system that functions to recognise non-self molecules within the body to control infection and disease. It has two components: innate and adaptive immunity. Innate immunity is the initial non-specific response to infection with a foreign body, such as a pathogen. Adaptive immunity is the second branch of the immune system and is highly specialised. If the innate immune response cannot destroy the pathogen alone, the adaptive immune response is apparent several days post infection (Murphy and Weaver, 2016a). This lymphocyte-driven response is highly effective and also has the capacity for the development of immunological memory. If the pathogen is encountered for a second time, this memory response is much quicker and stronger, protecting the host from a second infection. Despite the separate branches, the innate and adaptive immune responses overlap throughout infection (Clark and Kupper, 2005).

1.3.1 INNATE IMMUNITY

Alongside the physical and chemical barriers, for example the ciliated respiratory epithelium and various molecules capable of degrading microbes (Gallo and Hooper, 2012), the innate immune system also involves several types of cells including antigen-presenting dendritic cells, phagocytic macrophages that can engulf and degrade microbes, cytotoxic natural killer cells (NK) and a specific subset of T cells called γδ (Banchereau and Steinman, 1998; Born *et al*., 2006; Iwasaki and Medzhitov, 2010; Topham and Hewitt, 2009). These components work together to limit the spread of infection and, if possible, to ultimately destroy the invading pathogen.

The first phase of the innate immune response to a pathogen includes enzymes such as lysozyme which digests bacterial cell walls, peptides, such as defensins, which are thought to disrupt the membrane integrity of pathogens, and complement proteins that directly lyse pathogens or recruit innate immune cells, such as phagocytes, to engulf and destroy the pathogen (Ganz, 2003; Nagl *et al*., 2002; Noris and Remuzzi, 2013; Sukhithasri *et al*., 2013). If this fails to control infection, the second phase of the innate immune response is activated.

This second phase response is reliant on the recognition of conserved features between pathogens, hence the non-specific element of this response. In general, pathogens contain conserved sequences called pathogen-associated molecular patterns (PAMPs) that are recognised by pattern-recognition receptors (PRRs, Takeda and Akira, 2005). Several families of PRRs exist to recognise the huge diversity of PAMPs, which can be derived from polysaccharides, glycolipids, lipoproteins, nucleotides and nucleic acids from a wide variety of pathogens (Mogensen, 2009). These families of PRRs are expressed not only intracellularly such as cytosolic sensors of viral RNA or DNA, but also extracellularly such as Toll-like receptors (TLRs) expressed on the plasma membranes of macrophages and dendritic cells. Indeed, most PAMPs can be recognised by both extracellular and intracellular PRRs (Iwasaki and Medzhitov, 2015).

Alongside the recognition of PAMPs, the innate immune system is also able to recognise functional effects of infection, such as the common effects of virulence factors (Chovatiya and Medzhitov, 2014). For example, influenza virus activates NLRP3 inflammasomes via its M2 ion channel resulting in the induction of an inflammatory response (Ichinohe *et al*., 2010). This detection of common functional effects reduces the number of specific receptors needed and also confirms that the invading microbe is a pathogen.

Once a pathogen has been detected through a PRR, a signaling cascade is triggered that involves the activation of a range of adapter molecules, kinases and transcription factors (Takeda and Akira, 2005). These pathways lead to the activation of gene expression and the production of cytokines, chemokines, cell adhesion molecules and immunoreceptors which in turn leads to a rapid and potent inflammatory response (Akira *et al*., 2006). Important proinflammatory cytokines include tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6. Proinflammatory molecules have a range of roles. These include the modification of vascular endothelial permeability, cell death, recruitment of leukocytes at the site of infection, the appearance of selectin proteins allowing leukocyte adherence to inflamed tissue and the induction of acute phase proteins leading to the well-defined symptoms of inflammation (redness, swelling, heat, pain and loss of tissue function) (Takeuchi and Akira, 2010). Non-specific phagocytes (including dendritic cells) that have now migrated to the site of infection can then go on to engulf pathogens, degrade it into peptides and present the remaining peptides on protein complexes called major histocompatibility complexes (MHC) class II (Roche and Furuta, 2015). These activated antigen-presenting cells (APC) then migrate to lymphoid tissue to present these peptides, or antigens, to lymphocytes. This antigen presentation leads to the initiation of the adaptive immune response (Medzhitov, 2007).

1.3.2 ADAPTIVE IMMUNITY

Once activated, APC engulf the pathogen, degrade it into peptides and present these peptides on their surface. Antigen presentation occurs via one of two types of highly polymorphic MHC molecules: class I molecules activate the cytotoxic CD8 subset of T cells whereas class II molecules activate the helper CD4 subset of T cells (Doyle and Strominger, 1987; Norment *et al*., 1988). As such, the MHC molecules present different types of peptides. To combat intracellular infections, MHC class I molecules are produced in the ER where they are able to bind to intracellular microbial peptides. These peptides are produced from microbial proteins that have been degraded via the proteasome. In contrast, MHC class II molecules obtain their peptides from within intracellular endocytic vesicles containing digested pathogens in APC (Neefjes *et al*., 2011; Vyas *et al*., 2008). Once peptides are bound to MHC molecules, they are transported to the cell surface where T cells are able to recognise the complexes.

In the case of an extracellular pathogen, this initial antigen presentation on APC activates the cell which in turn results in the upregulation of MHC class II molecules on the APC allowing the efficient presentation of antigens (Roche and Furuta, 2015). This activation also leads to the expression of co-stimulatory molecules, such as CD80 and CD86, on the cell's surface (Zhang *et al*., 2004). These activated APC migrate to the lymphoid tissue, due to chemokines, whereby they encounter naïve antigen-specific CD4 T helper cells (CD4 T cells, Sprent, 1995).

APCs are responsible for delivering up to three types of signals required for the efficient activation, expansion and differentiation of CD4 T cells (Goral, 2011). The first activation signal comes from CD4 co-receptor after the binding of the MHC: peptide complex to the TCR and CD4. A second, co-stimulatory signal is delivered to ensure the survival and proliferation of T cells that have received the first signal. The last signal determines the pathway of differentiation of the now activated CD4 T cell. Typically, the final signal is a cytokine. Once activated, the CD4 T cells proliferate and differentiate into one of several types of effector

CD4 T cells. Each subset is specialised for the control and destruction of different types of pathogens. The type of effector CD4 T cells produced during infection depends on the type of antigen presented on the initial APC (Goral, 2011; MacLeod *et al*., 2010; Smith-Garvin *et al*., 2009; Zhu and Paul, 2008).

Another important subset of T cells, CD8 T cells, are also involved in the adaptive immune response and require activation (Wiesel *et al*., 2009). These cells are specialised to destroy host cells infected with intracellular pathogens through apoptosis, otherwise known as programmed cell death (Elmore, 2007). As such, these cells recognise peptides bound to MHC class I molecules on infected cells (Norment *et al*., 1988). CD8 T cells also require co-stimulatory activation which they can receive in two possible ways: direct activation by an activated dendritic cell or through CD4 T cell help (Ruedl *et al*., 1999; Zhang *et al*., 2009). This CD4 T cell help occurs if a CD4 and CD8 T cell recognise antigens presented on the same APC. The CD4 T cell can deliver additional co-stimulatory signals to promote CD8 T cell proliferation and differentiation (Zhang *et al*., 2009).

Once both CD4 and CD8 cells have become activated effector T cells, these cells can migrate from the lymphoid tissues to perform specific effector functions (Berstein and Abraham, 2008). Effector CD8 T cells are able to destroy infected cells that display peptides from an intracellular pathogen on an MHC class I molecule. These effector functions are due to the release of two types of effector molecules into an infected cell: perforin to form pores in the cellular membrane and granzymes to activate apoptosis (Trapani and Smyth, 2002). Other effector molecules that are produced by this cell type include interferon (IFN)-γ which can block viral replication and activate macrophages along with Fas ligand which can trigger apoptosis in a cell bearing Fas on its surface (Refaeli *et al*., 2002; Schroder *et al*., 2003).

In contrast, CD4 T cells will recognise peptides bound to MHC class II molecules (Doyle and Strominger, 1987). Effector CD4 T cells can activate macrophages to allow the destruction of

infected cells and activate B cells to produce antibodies to label pathogens (Murphy and Weaver, 2016a). Macrophages act to clean up cellular debris from destroyed cells but also can destroy pathogens by a process known as phagocytosis. This involves the ingestion of a pathogen, fusion of this pathogen with a lysosome containing various enzymes and the subsequent degradation of that pathogen (Murphy and Weaver, 2016a). CD4 T cells can also activate B cells to proliferate, produce antibodies and effector molecules. These molecules include IFN-γ to activate macrophages, CD40 ligand to activate B cells and macrophages and the B cell activation factors IL-4 and IL-5 (Luckheeram *et al*., 2012).

Another highly-specific lymphocyte type involved in the adaptive immune response is the B cell, also known as antibody-producing cells. These cells are activated via a signal supplied from either an effector CD4 T cell that has the same antigen-specificity as the B cell (T cell-dependent activation) or certain types of antigen that can directly provide the second activation signal (T cell-independent activation) (Murphy and Weaver, 2016; Parker, 1993; Vos *et al*., 2000). Once activated, the B cell begins to proliferate and differentiate into plasma cells that are able to secrete antibodies (Nutt *et al*., 2015).

Antibodies have several important functions within the adaptive immune response, including neutralisation of pathogen, opsonisation of a pathogen, complement activation or antibody-dependent cell-mediated cytotoxicity (Forthal, 2014, Huber and Trkola, 2007). Neutralisation occurs when antibodies bind to a pathogen, coating it and preventing it from infecting host cells. Binding of antibodies to pathogens also promotes the uptake of these pathogens by phagocytes, also known as opsonisation. Antibodies can also activate the complement proteins through the activation of the complement protein C1 (Melis *et al*., 2015). This activation of C1 can either lead to further opsonisation or the destruction of pathogens, such as bacterial cells, through the insertion of the membrane-attack complex which inserts pores into cellular membranes. The last effector mechanism of antibodies is antibody-dependent

cell-mediated cytotoxicity. This mechanism of killing infected cells relies on the coating of these cells with antibodies. The Fc domain of the antibodies can then be recognised by NK cells and the NK cells act to initiate apoptosis of the infected cells (Huber and Trkola, 2007). More recent studies suggest further functions of antibodies including acting as direct effector molecules (example: directly bactericidal or fungicidal antibodies) and modifying microbial signal transduction or microbial physiology (Casadevall and Pirofski, 2012).

In the case of viral infection (relevant to this thesis), antibodies specific for viral envelope proteins can bind to these proteins thereby neutralising the virus, activate the complement system to lyse viral particles alongside infected cells, label the pathogen for destruction by phagocytes and interact with effector cells via the fragment crystallisable (Fc) receptors leading to antibody-dependent cellular cytotoxicity (ADCC) (Burton, 2002; Huber and Trkola, 2007; Murphy and Weaver, 2016a). This is summarised in Figure 1-6.

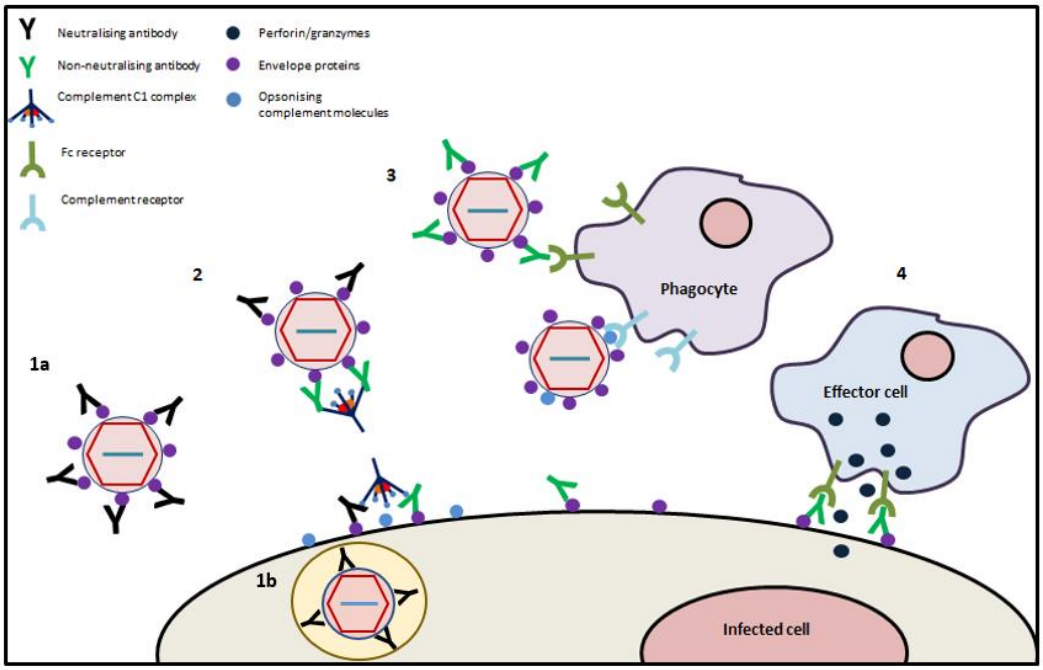


FIGURE 1-6: SUMMARY OF ANTIBODY FUNCTIONS IN RESPONSE TO A VIRAL INFECTION (ADAPTED FROM HUBER AND TRKOLA, 2007).

ANTIBODIES FUNCTION TO CLEAR VIRAL INFECTIONS THROUGH FOUR MAIN PATHWAYS. 1: NEUTRALISING ANTIBODIES TARGET VIRAL ENVELOPE PROTEINS TO STOP VIRUSES FROM ENTERING HOST CELLS (1A) OR BY BLOCKING VIRUS UNCOATING OR BUDDING (1B). 2: ANTIBODIES CAN CAUSE THE LYSIS OF EITHER FREE VIRUS OR INFECTED CELLS VIA THE C1 COMPLEMENT COMPLEX AND THE CLASSICAL COMPLEMENT PATHWAY. 3: COMPLEMENT PROTEINS AND ANTIBODIES OPSONISE VIRUSES ENHANCING THE PHAGOCYTOSIS OF VIRAL PARTICLES USING COMPLEMENT OR FC RECEPTORS. 4: EFFECTOR CELLS, INCLUDING NATURAL KILLER CELLS, MACROPHAGES AND DENDRITIC CELLS, RECOGNISE INFECTED CELLS LABELLED WITH ANTIBODIES LEADING TO THE RELEASE OF CYTOTOXIC GRANULES OR EXPRESSION OF CELL-DEATH INDUCING MOLECULES (TEILLAUD *ET AL.*, 2012).

1.3.3 ANTIGEN SPECIFICITY OF ADAPTIVE IMMUNE CELLS

Pathogen recognition capabilities of the adaptive immune system is based on the presentation of antigens by MHC molecules on APC and cell surface receptors on B cells and T cells, named T cell receptors (TCRs) and B cell receptors (BCRs) respectively (Choudhuri *et al.*, 2005; Edelman, 1991). Whilst TCRs and MHC molecules remain on the cell surface, BCRs can also be secreted as an effector molecule of the immune response (Kehry *et al.*, 1980). These soluble receptors are known as antibodies. Despite their different functions, B and T cells are

required to recognise a huge array of foreign antigens via receptors on their cell surface; both sets of cells achieve this by rearranging their genes that encode the receptors for antigen recognition during development (Murphy and Weaver, 2016b).

Both BCRs and TCRs are complexes of multiple proteins: the heavy and light chain of the BCR or α and β chains of the TCR (Pleiman *et al*., 1994; Wucherpfennig *et al*., 2010). Invariant accessory proteins are also associated with these complexes that primarily play a role in signalling during antigen recognition. In the case of the BCR, two proteins, Igα and Igβ, are associated with the heavy chain of the BCR (Treanor, 2012). These proteins are required for the BCR to be expressed at the cell surface and for signalling post antigen recognition as these proteins contain cytoplasmic domains that contain immunoreceptor tyrosine-based activation motifs (ITAMs). As the heavy and light chains do not contain cytoplasmic domains, these polypeptide chains alone can only recognise and bind antigen, they cannot provide any signal to activate transcription factors and gene expression downstream which in turn supports the control of the adaptive immune response. In comparison, the TCR is also associated with several accessory protein chains named the CD3 complex. Similarly, to Igα and Igβ, the CD3 complex contains ITAMs for downstream signalling after antigen recognition (Al-Lazikani *et al*., 2000; Choudhuri *et al*., 2005; Wucherpfennig *et al*., 2010).

Both receptors also recognise a unique antigen, although B cells and T cells differ in the type and form of antigen presented; T cells recognise antigens that have been processed into peptides and presented on MHC molecules on infected cells whilst B cells recognise both conformational or continuous epitopes within intact antigens alone in the form of protein, polysaccharide or lipid. The final major similarity between the receptors is their need to recognise a huge range of antigen specificities (Batista and Harwood, 2009; Carbone and Gleeson, 1997; Choudhuri *et al*., 2005).

1.3.4 BCR/TCR REARRANGEMENT IS ESSENTIAL FOR ANTIGEN RECEPTOR DIVERSITY

To provide the required level of antigen receptor diversity needed to respond to all potential pathogen encounters, each BCR and TCR chain consists of a highly variable region and several constant regions (Edelman, 1991; Ely *et al*., 2005; Yoshikai *et al*., 1985). Whilst the constant regions are involved in effector functions, the highly variable regions recognise and bind the specific antigen. The basic structure of a BCR/antibody is shown below in Figure 1-7. Each immunoglobulin is constructed from two heavy chains and two light chains, further increasing the antigen receptor diversity. These chains are encoded on three separate loci: the heavy chain loci, the κ light chain loci and the λ light chain loci (Matsuda *et al*., 1998). The only structural difference between the BCR and a secreted antibody is that the C-terminus of the constant region of the heavy chain can contain either a hydrophobic sequence (BCR) or a hydrophilic sequence (antibody). This is achieved through alternate splicing of the mRNA molecule encoding the heavy chain of the immunoglobulin (Kehry *et al*., 1980).

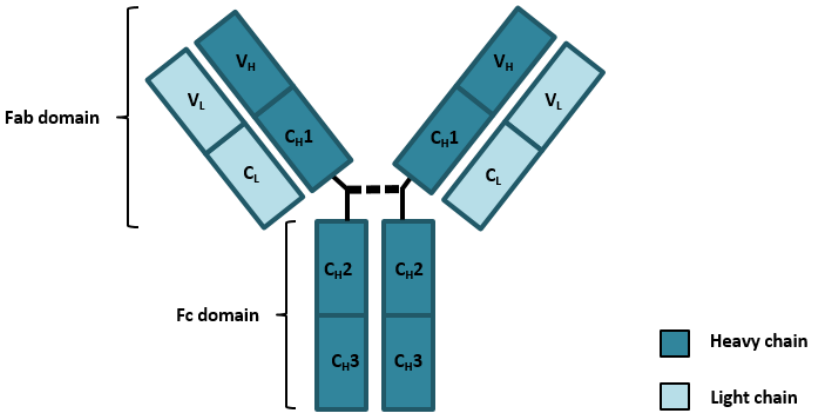


FIGURE 1-7: THE BASIC STRUCTURE OF AN ANTIBODY (ADAPTED FROM MURPHY AND WEAVER, 2016A).

EACH IMMUNOGLOBULIN CONSISTS OF 4 POLYPEPTIDE CHAINS- 2 IDENTICAL LIGHT CHAINS AND 2 IDENTICAL HEAVY CHAINS, BOUND TOGETHER BY DISULPHURIC BONDS. THE FRAGMENT ANTIGEN BINDING DOMAIN (FAB) CONTAINS THE V REGION AND THE FIRST CONSTANT DOMAIN AND IT IS INVOLVED IN ANTIGEN BINDING. THE FRAGMENT CRYSTALLISABLE (FC) DOMAIN CONTAINS THE SECOND AND THIRD CONSTANT DOMAINS AND IT IS INVOLVED IN MEDIATING EFFECTOR FUNCTIONS.

As shown in Figure 1-7, the variable and constant regions of immunoglobulin chains form two basic units: the Fragment antigen binding (Fab) domain and the Fragment crystallisable (Fc) domain. The Fab region contains the highly variable (V) region and one constant (C) region of both the heavy and light chain (Edelman, 1991). Consequently, there are two Fab regions in each complete immunoglobulin molecule. This V region confers the high variability of the immunoglobulin receptors. To achieve this level of variability, a V region is encoded by gene segments, rather than whole genes, which are combined via somatic recombination (Fugmann *et al*., 2000; Herbst *et al*., 1986; Lewis, 1994; Schatz and Ji, 2011; Tonegawa, 1983). This type of diversity is called combinatorial diversity (Boehm, 2011).

To produce a complete immunoglobulin light chain, two segments are combined: a V segment of around 95-101 amino acids and a joining (J) segment of up to 13 amino acids (Fugmann *et al*., 2000; Lewis, 1994; Murphy and Weaver, 2016c). These segments encode the variable region of the light chain. The recombination of V region segments is required as the J gene segments are located close to the C region thereby bringing the V, J and C sequences together. Through RNA splicing to remove introns, the V region is joined to the C region to allow translation and downstream expression. In comparison, to produce a complete immunoglobulin heavy chain gene, three segments are combined: V, J and a diversity (D) segment. First, recombination occurs to join the D and J segment together; later, a V segment is combined. Again, RNA splicing removes introns in the VDJ segment to combine the V region sequence to the C region sequence (Fugmann *et al*., 2000; Murphy and Weaver, 2016c; Schatz and Ji, 2011). An overview of these processes is shown in Figure 1-8 below.

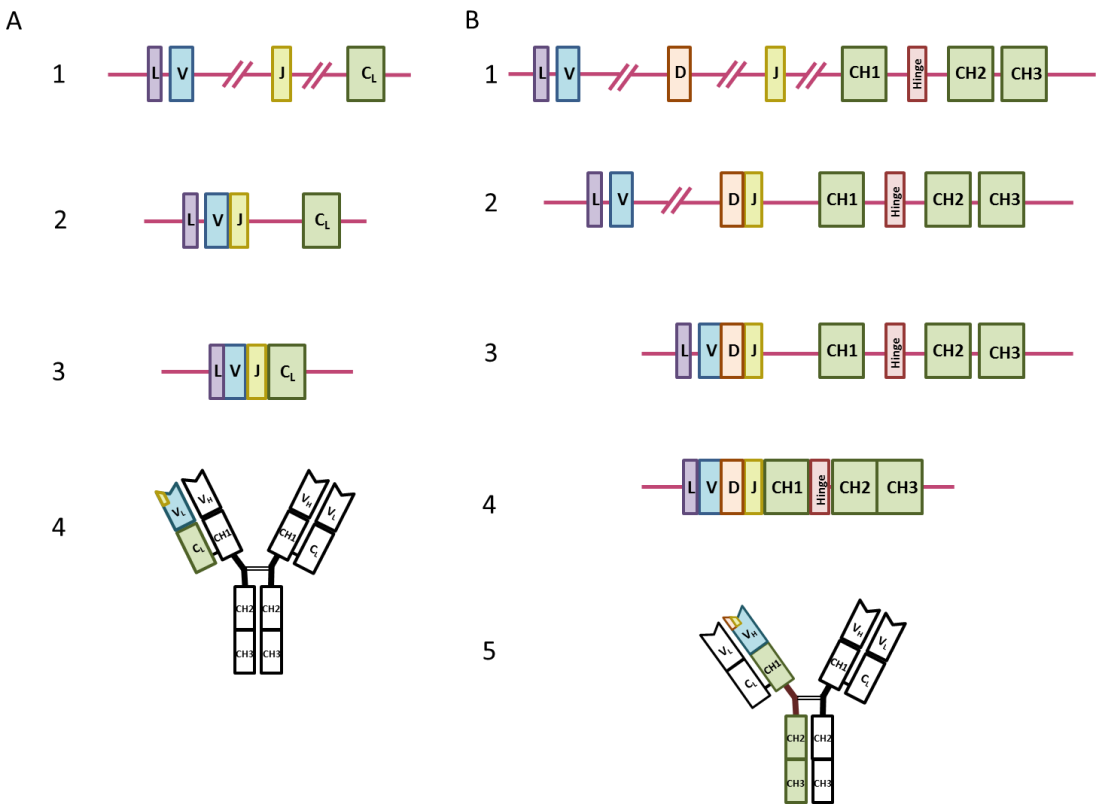


FIGURE 1-8: REARRANGEMENT OF IMMUNOGLOBULIN GENE LOCI TO PRODUCE IMMUNOGLOBULINS.

VARIABLE (V), JOINING (J) AND DIVERSITY (D) GENE SEGMENTS ON THE LIGHT CHAIN (A) AND HEAVY CHAIN (B) IMMUNOGLOBULIN LOCI ARE REARRANGED AND JOINED TO A C REGION TO PRODUCE IMMUNOGLOBULINS (adapted from Murphy and Weaver, 2016c). A LEADER SEQUENCE (L) IS NECESSARY FOR THE SECRETION OF IMMUNOGLOBULIN CHAINS. A HINGE (H) REGION IS ALSO INCLUDED IN THE CONSTANT REGION OF HEAVY CHAINS TO ALLOW FLEXIBILITY FOR BINDING TO ANTIGENS.

A: THE PROCESS OF GENE SEGMENT REARRANGEMENT ON THE LIGHT CHAIN IMMUNOGLOBULIN LOCI, TERMED IGκ AND IGλ. (1) SEVERAL COPIES OF V AND J GENE SEGMENTS ARE PRESENT IN THE GENOMIC DNA WITH ONLY ONE CONSTANT REGION ENCODED ON EACH LIGHT CHAIN LOCI. (2) THROUGH RECOMBINATION, ONE V AND ONE J GENE SEGMENT ARE JOINED. (3) THE VJ SEGMENTS ARE JOINED TO THE C REGION AFTER TRANSCRIPTION OF THE PRIMARY RNA TRANSCRIPT AND SUBSEQUENT SPLICING OF RNA TO REMOVE INTRONS. (4) THIS MRNA IS THEN TRANSLATED INTO THE LIGHT CHAIN OF AN IMMUNOGLOBULIN MOLECULE.

B: THE PROCESS OF GENE SEGMENT REARRANGEMENT ON THE HEAVY CHAIN IMMUNOGLOBULIN LOCUS, TERMED IGH. (1) SEVERAL COPIES OF V, D AND J GENE SEGMENTS ARE PRESENT IN THE GENOMIC DNA ALONG WITH SEVERAL CONSTANT REGION EXONS. A MATURE HEAVY CONSTANT REGION TRANSCRIPT IS GENERATED BY THE JOINING OF THREE EXONS: CH1, CH2 AND CH3 (2) THROUGH RECOMBINATION, A D AND J SEGMENT ARE JOINED. (3) THROUGH FURTHER RECOMBINATION, A V SEGMENT IS JOINED TO THE DJ SEGMENTS. (4) THE VDJ SEGMENTS ARE JOINED TO THE C REGION AFTER TRANSCRIPTION OF THE PRIMARY MRNA AND SUBSEQUENT SPLICING TO REMOVE INTRONS. (5) AFTER TRANSLATION OF THE MRNA MOLECULE, A HEAVY CHAIN OF AN IMMUNOGLOBULIN IS PRODUCED.

The combinatorial diversity that arises by immunoglobulin gene segments increases antigen recognition specificities as there are many copies of each segment of each immunoglobulin loci (Boehm, 2011). In humans, for example, the heavy chain locus consists of 65 V, 27 D and 6 J whilst the κ light chain locus consists of 40 V, and 5J and the λ light chain locus consists of 30V and 4J (Li *et al*., 2004; Matsuda *et al*., 1998; Murphy and Weaver, 2016c). In humans, the V segments are grouped in families- there are 7 families for the heavy chain and κ chain and 11 families for the λ chain. In each family, the V segments share at least 80% sequence identity with each other (Matsuda *et al*., 1998).

To ensure that gene segment recombination occurs in the correct order and location, rearrangement is directed by the presence of a recombination signal sequence (RSS) that flanks gene segments (Schatz and Ji, 2011). This sequence consists of three elements: a conserved non-coding heptamer sequence, a non-conserved spacer of 12 or 23 nucleotides and finally another conserved sequence of 9 nucleotides. The presence of a spacer brings the heptamer and nonamer to the same side of the DNA helix allowing the recognition of the sequences by a complex of enzymes called the V(D)J recombinase. The presence of two different length spacers also has another important role. Recombination relies on the 12/23 rule that stipulates that a gene segment with a 12 base pair spacer can only be joined to a segment with a 23bp spacer. Therefore, V, D and J segments contain the relevant length of spacer to ensure that the order of recombination remains VDJ or VJ (Hiom and Gellert, 1998).

The RSS also play a role in the rearrangement of the gene segments (Schatz and Ji, 2011). Segments are typically rearranged by the looping out of DNA and the subsequent deletion of the extra gene segments. To delete the extra gene segments, the VDJ recombinase complex causes a break in the DNA. However, the location, or junction, of where this DNA is re-joined is imprecise. Additionally, the enzymes involved in DNA repair remove nucleotides and also

randomly add nucleotides further increasing the diversity seen in the V region. This type of diversity is known as junctional diversity (Jeske *et al*., 1984).

All of these stages of recombination occur early during B cell development. Once the loci are successfully rearranged to encode complete immunoglobulin genes, another form of combinatorial diversity is seen. The pairing of different heavy chains with light chains provides a further enhancement of diversity. Using these three methods alone, up to 10^{11} different receptors could be produced, although this is somewhat unlikely due to instability of immunoglobulins with particular heavy and light chain combinations and the fact that V gene segments are not used at the same frequency (Jayaram *et al.*, 2012; Murphy and Weaver, 2016c).

1.3.5 Affinity maturation of B cells

A further increase of the diversity of the antibody repertoire occurs in mature B cells in the peripheral lymphoid tissue (Riss, 2013; Weiser *et al*., 2011). These processes are called somatic hypermutation and class switch recombination (Maul and Gearhart, 2010; Stavnezer *et al*., 2008). These mature B cells already express functional BCRs and have encountered a foreign antigen along with signals from activated T cells. Whilst in the peripheral lymphoid tissue, random point mutations occur in the V regions as this affects antigen binding (Maul and Gearhart, 2010). The enzyme activation induced deaminase (AID) is responsible for this process as it converts cytosines to uracils. A uracil in DNA is mutagenic thereby initiating error-prone DNA repair (Maul and Gearhart, 2010). As B cells proliferate and their BCRs are mutated, they are still subjected to selection and those B cells with improved antigen binding may be positively selected. This process of increasing antigen binding affinity is also known as affinity maturation (Siskind and Benacerraf, 1969). The process of somatic hypermutation is

in fact the predominant method of diversity for a number of other animals including birds, rabbits, cows, pigs, sheep and horses (Sitnikova and Su, 1998).

1.3.6 CLASS SWITCH RECOMBINATION

Whilst somatic hypermutation occurs in the V regions of mature B cells, class switch DNA recombination (CSR) occurs in the C regions (Stavnezer *et al*., 2008). Both mechanisms are crucial in the development of an effective antibody response. As the C region determines the class and functionality of the antibody produced, the switching of these C regions can change the effector functions of the antibody (Stavnezer *et al*., 2008).

The process of CSR is similar to the recombination seen between gene segments in the V region, although all recombination events are productive (Murphy and Weaver, 2016c; Xu *et al*., 2012). In this case, the different exons, relating to the different possible isotypes of antibodies, are located in the immunoglobulin heavy chain locus sequentially: Cμ, Cδ, Cγ, Cε and Cα. The switching of Cμ (IgM) with Cγ, Cε or Cα produces the corresponding IgG, IgE or IgA whilst generation of IgD is through alternate splicing of the IgM transcript. Using the AID enzyme, double-stranded breaks in the DNA are made in Switch (S) regions, G-rich regions located upstream of Cμ, Cγ, Cε and Cα. Once broken, the strands are repaired resulting in the VDJ cassette now preceding a different C region (Xu *et al*., 2012).

1.3.7 ANTIBODY CLASSES

Different classes of antibodies have different functions (Schroeder *et al*., 2010; Ward and Ghetie, 1995). Pre-B cells begin their development by expressing IgM on their surface due to the proximity of the Cμ exon to the J gene segments of the VDJ cassette (Nishimoto *et al*.,

1991). Directly downstream of the Cμ exon lies the Cδ exon. B cells produce one long primary transcript that includes both Cμ and Cδ exons, which is then spliced to produce one of two mRNA molecules- one encoding an IgM molecule, the other an IgD molecule. This means that mature B cells are able to express both IgM and IgD receptors, however, IgD receptors only appear at the later stages of B cell development (Geisberger *et al*., 2006; Yuan *et al*., 1996).

Despite this co-expression, IgM is always the first antibody produced during infection whilst a limited amount of IgD is secreted and the function of these remains elusive (Chen and Cerutti, 2010). The secreted IgM is typically of a lower affinity as the B cells have not yet undergone affinity maturation (Eisen, 2014). The other classes, IgG, IgA and IgE, are produced after antigen recognition and the subsequent CSR. Each of the subtypes has specific functions and locations (Table 1-5). For example, IgG has a larger cytoplasmic domain and increased B cell signaling and therefore a B cell carrying an IgG responds to antigens faster (Xu *et al*., 2014); IgA and IgE do not efficiently activate complement whilst particular IgG subclasses, such as IgG1 and IgG3, activate complement very efficiently which is important in infection (Bogers *et al*., 1991; Sörman *et al*., 2014; Vidarsson *et al*., 2001).

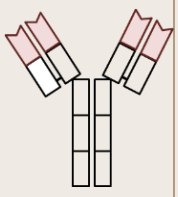
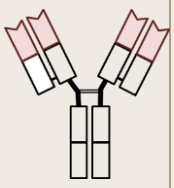
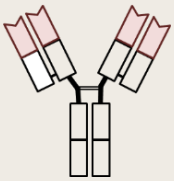
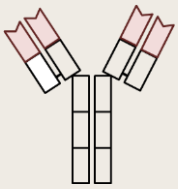
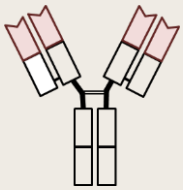
	IgM	*IgD*	*IgG*	*IgE*	*IgA*
C exon used	μ	δ	γ	ε	α
Number of subtypes	1	1	4	1	2
Structure	 Can form pentamer, no hinge region, extra C domain.			 No hinge region, extra C domain.	 Can form dimer.
Size (kDa)	970 (pentamer)	180	150	190	160 (monomer)
Location	Mainly in serum.	Very low levels found in serum.	Blood and extracellular fluid.	Surface of basophils and mast cells in lungs, skin and mucous membranes.	Blood and extracellular fluid as monomers, mucosa epithelium if dimeric, secreted in saliva, tears and milk.
Main role	Activate complement, agglutination as able to bind to multivalent antigens due to ability to polymerise.	Unclear, possibly different type of activation.	Neutralisation of pathogens, opsonisation, activation of complement. Can cross placenta.	Role in allergy, hypersensitivity and response to parasites. Cause vasodilation and vessel permeability.	Neutralisation on epithelial surfaces.

TABLE 1-5: THE MAIN PROPERTIES OF THE DIFFERENT IMMUNOGLOBULIN CLASSES (ADAPTED FROM MURPHY AND WEAVER, 2016C, CHEN AND CERUTTI, 2010).

1.3.8 IMMUNOLOGICAL MEMORY

One of the most important features of the adaptive immune response is the memory response. This response is coordinated by a subset of B and T cells, called memory cells. Once B and T cell activation during infection occurs, both cell types begin to proliferate and differentiate. B cells differentiate into plasma cells that can secrete antibodies or long-lived memory B cells. On the other hand, T cells, when activated, typically become short-lived effector T cells (Murphy and Weaver, 2016b). A small subset of these cells is able to express cytokine receptors, such as IL-7 and IL-15 receptors, that allows their survival for decades (Tan *et al*., 2002). Therefore, if a pathogen has been previously encountered, the memory response enables a faster and more efficient clearing of the same pathogen from the host as these cells already exist.

In the case of antibody production, a typical primary antibody response consists of mostly IgM and, after class-switching, a delayed IgG response (Murphy and Weaver, 2016d). In contrast, the secondary response generally consists of a lower level production of IgM antibodies and much higher levels of IgG antibodies, alongside lower levels of IgA and IgE. Memory B cells formed after a primary infection are responsible for this secondary antibody response (Kurosaki *et al*., 2015). Memory B cells express higher levels of MHC class II molecules and co-stimulatory molecules which allows more efficient antigen presentation and subsequent activation of effector T cells (Murphy and Weaver, 2016d). The effector T cells can then aid in promoting antibody production which leads to an earlier antibody response in secondary infection in comparison to primary infection. Furthermore, the level of plasma cells produced is higher and launches much earlier in secondary infection contributing to the antibody response seen. Along with a higher level of antibody production in secondary infections, the affinity of antigen-specific antibodies also increases as memory B cells are able

to undergo additional somatic hypermutation and affinity maturation. This means that the affinity of antibodies produced increases with each subsequent infection.

More recently, immunological memory has also been associated with IgM-positive, CD4 T cell-dependent memory B cells that are generated following immunization, although their physiological role has not yet been determined (Yates *et al*., 2013). Interestingly, these cells do not secrete antibodies *ex vivo*, have a differentiated profile of cell surface markers, are generally located in the spleen rather than the blood or lymphoid organs. Data from a recent study suggests that these cells are required for humoral immunity as when depleted *in vivo*, the IgG memory responses to specific antigen challenge was impaired (Yates *et al*., 2013).

Memory T cell responses are equally important for immunological memory. Memory T cells can be activated by lower amounts of antigen, proliferate to higher levels and can exert their effector functions much earlier than naïve T cells (Rogers *et al*., 2000; Slifka and Whitton, 2001; Veiga-Fernandes *et al*., 2000). This is important for promoting a fast and efficient secondary immune response.

The induction of the immunological memory response is the basic concept behind vaccine design (Castellino *et al*., 2009). By eliciting an initial priming of the immune response with a vaccine (the primary response), if the vaccinee encounters the same pathogen again, a higher affinity, more effective and faster response to this pathogen will be raised.

1.4 IMMUNE RESPONSE TO HCV AND GBV-B INFECTION

1.4.1 IMMUNE RESPONSE TO HCV

The initial immune response to HCV infection is provided by the innate immune system. Detection of HCV infection leads to the activation of two distinct types of receptors thereby initiating the production of anti-viral and proinflammatory cytokines: Toll-like receptor-3 (TLR-3) and RIG-1-like receptors (RIG-1 and MDA-5) (Li *et al*., 2012). Polymorphisms in MDA-5 have shown to influence the clearance of HCV infection, further confirming the importance of this innate response in viral infection outcome (Hoffmann *et al*., 2015). Indeed, a human hepatocarcinoma cell line that has a single mutation in the RIG-1 gene supports much higher levels of HCV RNA replication (Blight *et al*., 2002).

To overcome this innate immune response, HCV proteins are able to block signalling cascades that result in IFN production. For example, HCV NS3-4A serine protease is able to cleave host proteins, such as mitochondrial anti-viral-signalling protein (MAVS), leading to the inhibition of the signalling cascade resulting in the release of anti-viral IFN (Li *et al*., 2005). NS4B has also recently been implicated in blocking IFN production by interacting with the stimulator of interferon genes (STING) protein (Ding *et al*., 2013; Nitta *et al*., 2013).

HCV escape from the innate response leads to the second branch of the immune response, the highly-specific adaptive immune response (Larrubia *et al*., 2014). This leads to the induction of both cellular and humoral immune responses. Cellular immunity involves the generation of HCV-specific CD8+ cytotoxic T cells and CD4+ helper T cells, whilst the humoral immune response leads to the production of both non-neutralising and neutralising antibodies. This highly-specific response is usually detectable at 6-8 weeks after initial viral infection (Netski *et al*., 2005; Thimme *et al*., 2002). The reason for this delay, however, is unknown (Rehermann, 2013; Shin *et al*., 2011; Su *et al*., 2002; Thimme *et al*., 2012, 2002). Defining the adaptive immune response that leads to viral clearance is essential for vaccine development, however current understanding is lacking in this area.

In cellular immunity, naïve CD4+ helper and CD8+ cytotoxic T cells are activated by antigen-presenting cells and play a hugely important role in orchestrating the adaptive immune response through the production of cytokines and destruction of infected cells, respectively (Larrubia *et al*., 2014). A multi-specific and long-lasting T cell response is vital for controlling HCV infection in both patients and animal models (Dustin and Rice, 2007; Lechner *et al*., 2000; Thimme *et al*., 2002).

Studies investigating T cell dysfunction in patients with chronic viral infections support the theory that continuous antigenic stimulation and exposure to proinflammatory cytokines leads to the upregulation of inhibitory receptors on cytotoxic CD8+ T cells (Wherry and Kurachi, 2015). It is of note however that these CD8+ T cells are not inert but maintain a suboptimal level of effector functions allowing viral load to be controlled, but not cleared. Blocking of these inhibitory receptors on CD8+ T cells leads to an increase in functional cytotoxic T cells and improved viral control (Fuertes Marraco *et al*., 2015). In a clinical setting, blocking the signalling pathways of inhibitory receptors is known to be successful in restoring T cell function (Gardiner *et al*., 2013; Nguyen and Ohashi, 2014). This correlation between viral clearance and reversal of T cell exhaustion has also been seen in a patient that spontaneously cleared HCV infection (Raghuraman *et al*., 2012).

Along with T cell exhaustion, T cell effector functions can also be impacted by the significant expansion of T regulatory cells seen in HCV infection. One study in chimpanzees found that after an initial exposure to HCV, subsequent exposure to subinfectious doses of HCV lead to expansion of T regulatory cell populations and suppressed T cell effector responses (Park *et al*., 2013). This study suggests that repeated exposure to HCV could increase the propensity for chronic HCV infection. Additional evidence confirming HCV's ability to affect T cell effector functions came from a recent study that showed HCV envelope protein E2 and HCV RNA can inhibit T cell activation by inhibiting T cell receptor signalling (Bhattarai *et al*., 2015).

A humoral immune response, leading to the production of antibodies specific to the invading pathogen, is also seen in HCV infection (Cashman *et al*., 2014). HCV-specific antibodies are likely to play a role in controlling infection, in particular with regards to controlling initial viral load and protection against reinfection. Early studies focusing on the identification of neutralising antibodies (antibodies that block virus entry on a permissive cell) were largely performed in chimpanzees (Choo *et al*., 1994; Farci *et al*., 1996). These studies determined that HCV-specific neutralising antibodies are produced in infection and have some protective capacity to limit future infections. A more recent study in chimpanzees showed that transfusion of a human monoclonal antibody against HCV E2 protected a naïve chimpanzee from infection and reduced HCV RNA replication in an animal acutely infected with the virus (Morin *et al*., 2012). Human studies have also confirmed that a human monoclonal anti-E2 antibody could delay viral rebound in liver transplant HCV patients (Chung *et al*., 2013).

Further confirming the role of antibodies in control of HCV infection, hypogammaglobulinaemic patients with dramatically reduced immunoglobulin levels have a severe and rapid disease progression (Bjoro *et al*., 1994). Additionally, patients treated with rituximab to deplete B cells experience an increase of HCV viral load which reduces as therapy is completed (Ennishi *et al*., 2008).

Although antibodies are known to be produced in both acute and chronic HCV infections, it is important to define their role in both outcomes of infection. In 1997, a study found that antibodies specific to the hypervariable region 1 (HVR1) in HCV glycoprotein E2 generated in the first six months of infection were detected in 43% of patients who spontaneously cleared infection in comparison to only 13% of patients who failed to clear HCV (Zibert *et al*., 1997). Antibody profiles to other viral proteins did not differ between the two groups indicating that neutralising antibodies early in infection are involved in viral clearance. Similar antibody profiles were seen in other patients with acute HCV infections (Allander *et al*., 1997).

Recent studies indicate that neutralising antibodies may play more of an important role in viral clearance than originally thought if induced early in infection. In a reported case of spontaneously resolved chronic HCV infection, a neutralizing antibody response appeared before the decline in viral titre and reversal of T cell exhaustion, eventually leading to viral clearance (Raghuraman *et al*., 2012). Additionally, in two separate studies of acutely infected HCV patients, a neutralising antibody response early in infection was thought to correlate with viral clearance (Lavillette *et al*., 2005; Pestka *et al*., 2007). An early, strong and broad neutralising antibody response will most likely be needed for viral control and clearance, although successful viral clearance is almost certainly dependent on a balance of cellular and humoral immunity (Giang *et al*., 2012; Law *et al*., 2008; Osburn *et al*., 2014; Osburn *et al*., 2010; Raghuraman *et al*., 2012).

1.4.2 IMMUNE RESPONSE TO GBV-B

Data surrounding the precise role of both the humoral and cellular immunity in GBV-B clearance is still relatively limited. The humoral immune response in GBV-B infection is poorly understood due to the limited availability of immunological assays covering this area. There are few data on the identification and role of neutralising antibodies during GBV-B infection and clearance, yet information regarding this would enhance the value of the GBV-B/tamarin model of HCV infection. Limited *in vivo* work has been carried out in tamarins. Convalescent sera from tamarins mixed with GBV-B at a low dose and then inoculated into naïve tamarins was not found to neutralise GBV-B (Bukh *et al*., 2008). However, no definitive answer to whether neutralising antibodies exist in GBV-B infection can be attained without the development of an *in vitro* neutralisation assay.

The presence of antibodies targeting other GBV-B proteins has been identified in previous studies in both marmosets and tamarins, in particular against GBV-B core, NS3 and NS5B (Beames *et al*., 2000; Bukh *et al*., 2008; Woollard *et al*., 2008). Antibodies targeting core are not commonly identified but have been previously detected during both acute and chronic infections (Bukh *et al*., 2008; Nam *et al*., 2004). In contrast, reports of antibodies targeting the non-structural proteins of GBV-B vary: NS3 antibodies have been found in several animals whilst NS5B antibodies are rare (Beames *et al*., 2000; De Tomassi *et al*., 2002; Lanford *et al*., 2003; Martin *et al*., 2003; Sbardellati *et al*., 2001; Woollard *et al*., 2008). Although these antibodies are unlikely to be neutralising as they are not specific for the envelope protein (Bukh *et al*., 2008), their role has yet to be clearly defined in GBV-B infection. Similar to HCV, antibodies targeting non-structural proteins typically appear first and at high levels, peak prior to viral clearance and then decrease to undetectable levels after viral clearance implicating a role in elimination (Lanford *et al*., 2003).

Cellular immunity also exists in GBV-B infection and may be necessary, but not sufficient, for viral clearance. Virus-specific T cell responses to non-structural GBV-B proteins have been documented in marmosets, in particular towards NS3 and NS4A, which appear around the period when the virus is cleared suggesting a role in control and clearance (Woollard *et al*., 2008). Cellular immunity may also be involved in protection against reinfection via memory T cells as a rapid spike in virus-specific T cell responses targeting the non-structural proteins appears more quickly in secondary infection than in primary (Bukh *et al*., 2008). However, viral clearance is most likely a multifactorial process, as with HCV, involving both cellular and humoral immunity. Supporting this hypothesis, the percentage of all T cells increases whilst B cell numbers decrease during high viraemia in tamarins yet reverses immediately prior to viral clearance and NS3 antibody peaks (Hood *et al*., 2014).

If the immune mechanisms surrounding viral clearance in the GBV-B/tamarin model can be delineated, this may be translatable to acute HCV infection which could help to elucidate how HCV infections so readily progress to chronicity.

1.5 MONOCLONAL ANTIBODY PRODUCTION AND RECOMBINANT DNA TECHNOLOGIES

1.5.1 MURINE HYBRIDOMA SYSTEM

Antibodies are regularly used as diagnostic tools and therapeutically. Consequently, production of high specificity and high affinity antibodies is of great importance. In 1975, monoclonal antibodies (mAb) were produced for the first time using the hybridoma system (Köhler & Milstein, 1975). The procedure consists of immunizing mice with a target antigen followed by one or more boosts (Goodall, 1999). After several weeks, B cells are harvested from the spleen and immortalized by fusion, using either chemical or virus induced methods, with a myeloma cell line that lacks the hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) gene. These cells are grown in a selective medium containing hypoxanthine-aminopterin-thymidine (HAT) that allows only fused cells to survive. Fused cells are able to grow as they are immortal due to the myeloma cell line and have the ability to synthesize purine nucleotides due to the HGPRT gene from the primary B cells. Fused cells are then isolated by single cell titration, cultured and screened for production of antibodies against the target antigen (Little *et al*., 2000). This method is summarized in Figure 1-9 below.

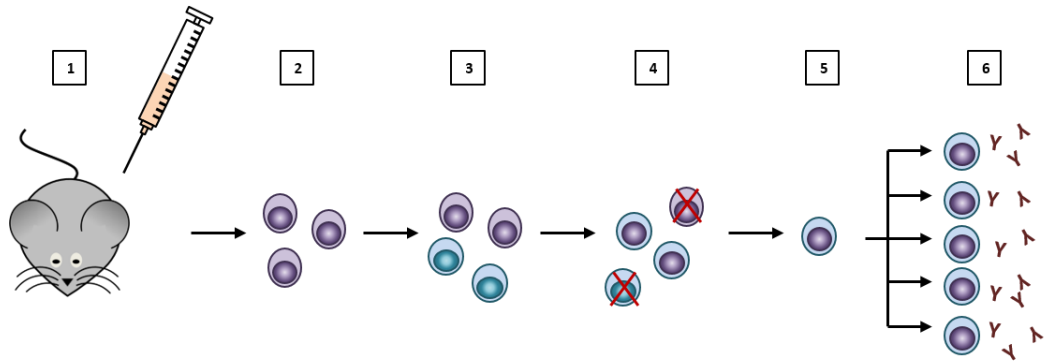


FIGURE 1-9: PRODUCTION OF MONOCLONAL ANTIBODIES USING THE HYBRIDOMA SYSTEM (ADAPTED FROM ALKAN, 2004).

THE PRODUCTION OF MONOCLONAL ANTIBODIES STARTS WITH THE IMMUNIZATION OF A MOUSE WITH THE ANTIGEN OF INTEREST (1). AFTER THIS IMMUNIZATION PERIOD, SPLEEN CELLS, INCLUDING B CELLS (SHOWN IN PURPLE), ARE COLLECTED (2) AND MIXED WITH MYELOMA CELLS (SHOWN IN BLUE) THAT HAVE A MUTATION IN THE HGPRT GENE (3). CELLS ARE FUSED USING POLYETHYLENE GLYCOL (YANG AND SHEN, 2006). AS THE MYELOMA CELLS LACK THE HGPRT GENE AND PRIMARY SPLEEN CELLS CAN ONLY SURVIVE IN CULTURE FOR A WEEK OR SO, AFTER 10-14 DAYS ONLY SPLEEN CELL-MYELOMA CELL FUSIONS SURVIVE AND REPLICATE (4). FUSED CELLS ARE THEN TITRATED TO ENSURE SINGLE CELL COLONIES (5). SINGLE CELL COLONIES CAN BE EXPANDED IN CELL CULTURE AND THE SUPERNATANT COLLECTED AND TESTED FOR MONOCLONAL ANTIBODIES PRODUCTION (6).

From the first approved anti-CD3 mAb in 1986 (Thistlethwaite *et al*., 1984), there are now over 40 mAbs approved for therapeutic use targeting a range of diseases including various cancers and autoimmune diseases (Ecker *et al*., 2015). Nevertheless, the hybridoma technology is not without its pitfalls. The reliance on immunizing mice does not allow the production of mAb targeting toxic or conserved self-antigens. Other limitations include the time constraints and the uncertain target/affinity of the antibodies produced. However, the most obvious issue with this technique is the ethical issues around use of animals in research, as it is now widely accepted that the number of animals used in research should be reduced due to the principles of human experimental technique (Balls and Straughan, 1996).

1.5.2 RECOMBINANT DNA TECHNOLOGIES

After the development of polymerase chain reaction (PCR) techniques and the identification of the sequence of the immunoglobulin genes that encode antibodies, DNA recombinant technologies were developed such as phage, yeast and ribosome display. All display technologies are based on the physical connection of the genotype (gene coding for an antibody) and the phenotype (the characteristics of the expressed antibody) displayed on the surface of a recombinant microorganism or particle and screened against the antigen of interest (Bradbury *et al*., 2011).

Another important observation was that the specificity of an antibody lies in the antigen-binding site, which consists of the variable regions of the heavy chain and light chain of an immunoglobulin (Figure 1-10). The variable regions of the heavy and light chain can be cloned by PCR and linked by a short peptide, which is typically a number of repeats of 4 Glycine and 1 Serine. The linked variable regions are then known as single chain variable fragments (scFv) (Hammers and Stanley, 2014). Each display technology then has a unique method for identifying and isolating antibodies that specifically target the antigen of choice.

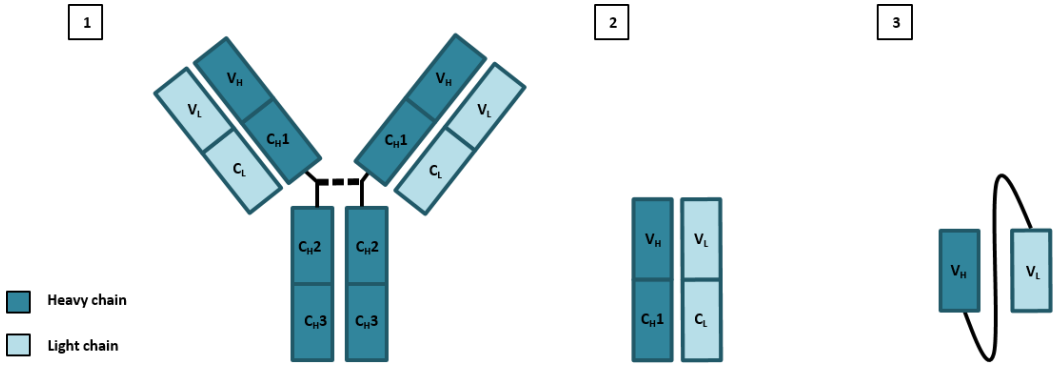


FIGURE 1-10: STRUCTURE OF AN ANTIBODY IN COMPARISON TO THE STRUCTURE OF SCFV (ADAPTED FROM DE GENST *ET AL.*, 2014).

THE BASIC STRUCTURE OF A WHOLE IMMUNOGLOBULIN MOLECULE (1), THE ANTIGEN BINDING FAB FRAGMENT (2) AND A SCFV (3).

The most well-known display technologies are described below. Whilst each method can be, and is indeed commonly, used to isolate all types of proteins, the techniques will only be described in the context of scFv isolation.

1.5.2.1 PHAGE DISPLAY

In phage display, the scFv library is cloned into phagemids which express the scFv fused to a surface protein of the phage such as the pIII capsid protein of M13 phages (Barbas *et al*., 1991). These phagemids, containing the scFv library, are introduced by transformation into permissive *E.coli*. Infection of these bacteria with helper phages is required because the phagemids do not contain all of the necessary bacteriophage genes for propagation. This leads to the production of phages expressing the pIII minor coat protein fused to each of the scFv from the library. The scFv, displayed on phages, are then screened against the antigen of choice, which is usually immobilized on a plate or on beads. If a phage binds, the scFv must be targeting the antigen of interest and can therefore be isolated. Following purification washes,

phages that do not possess a scFv that targets the antigen are removed. Bound phages can then be eluted, used to infect *E.coli* for bulk production, their phagemids isolated and sequenced (Smith and Scott, 1993). To enrich the population of positive phages, several rounds of panning against the immobilized target antigen are performed. Each time, phages that bind the antigen of interest are selected by washing the non-binding phages away resulting in a population of highly antigen-specific scFv expressed on phage.

Whilst the development of phage display was a leap forward in the production of high affinity, high specificity antibodies, the technology still has limitations. There is some expression bias due to a different codon usage between the prokaryotic host and the eukaryotic scFv (Rodi *et al*., 2002). This could lead to the loss of high affinity antigen-specific scFv. Additionally, the different fitness of bacteria producing different scFv could lead to the loss of high affinity antigen-specific scFv that were toxic to the prokaryotic host. The advantages of phage display over other display technologies include the ease of use and the rapid isolation of scFv (Pande *et al*., 2010).

Examples of approved therapeutic antibody therapies that have been isolated through the use of phage display include adalimumab (AbbVie Inc., North Chicago, IL) and belimumab (GlaxoSmithKline, London, UK). The antibody adalimumab binds to TNFα blocking the activation of its receptors. This treatment is approved for several diseases including rheumatoid arthritis and Crohn disease. Belimumab binds to the human B-lymphocyte stimulator (BLyS); the function of BLyS is to promote B cell survival and differentiation. This antibody has been approved for use as a treatment for systemic lupus erythematosus (Nixon *et al*., 2014).

1.5.2.2 YEAST AND BACTERIAL DISPLAY TECHNOLOGIES

Other display technologies such as yeast or bacterial display can also be used for the screening of a scFv library. In both cases, the scFv are fused to a protein attached to the cell wall which is followed by selection using both magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) (Bessette *et al*., 2004; Boder and Wittrup, 1997). The screening in this case works by incubating yeast/bacteria expressing scFv with a target antigen that is either fluorescently tagged or bound to magnetic beads. After washing, only the yeast/bacteria expressing scFv that bind to the target antigen with the highest affinity remain fluorescent/magnetic and can be isolated by FACS/MACS (Boder *et al*., 2012). This method of selection is highly discriminative and therefore produces the highest affinity antibodies (Boder *et al*., 2000). However, the bacterial display method still has the same disadvantages as phage display in that the scFv has to be translocated across the inner membrane and out onto the surface of the bacteria. Whilst yeast display does not have the secretion limitation or expression bias, the library size is limited and requires fluorescence-activated cell sorting (FACS) for sorting the scFv-expressing cells. The other major advantage of yeast display is that this eukaryotic system allows protein folding and processing in a similar way to that in mammalian cells (Arbabi-Ghahroudi *et al*., 2005).

1.5.2.3 RIBOSOME DISPLAY

Ribosome display, which takes place entirely *in vitro*, is a further development of the display technologies (Figure 1-11). Relying on the same principle of coupling genotype and phenotype, this technique has several advantages to other display technologies including the production of scFv against toxic proteins and the reduction of bias from using a host organism. This technique is also much faster and a larger library can be screened as the only limitation is the number of ribosomes available in the reaction (Zahnd *et al*., 2005).

For this technique, a scFv library is constructed. During the construction of this library, scFv are fused to a spacer that lacks a stop codon to ensure that after scFv transcription and translation, the ribosome holds on to both the translated scFv and the mRNA encoding it. These Antibody-Ribosome-mRNA (ARM) complexes can then be screened against immobilized antigen. Non-specific scFv are washed away through several wash steps allowing any bound complexes expressing target antigen-specific scFv to be eluted. The mRNA from these eluted complexes is isolated and reverse transcribed. It has been suggested that for each round of ribosome display, any specific scFv are enriched between 100-1000-fold therefore multiple rounds of screening are generally performed (Chen *et al*., 2010; J. Hanes *et al*., 1998; Hanes and Plückthun, 1997; Schaffitzel *et al*., 1999a; Takahashi *et al*., 2002; Xin *et al*., 2013; Zhao *et al*., 2009a).

One of the benefits of using display technologies, including ribosome display, is the ability to select for antibodies with a higher affinity, higher stability or with a broader spectrum by changing the selection conditions. Previously, antibodies have been isolated from ribosome display with enhanced stability or affinity through the addition of DTT into the selection step or by using off-rate selections over several days (Jermutus *et al*., 2001). Additionally, random mutations can be introduced into scFv libraries, the scFvs screened and high affinity antigen-specific scFv isolated thereby mimicking affinity maturation of antibodies (Groves *et al*., 2006).

The final stage of ribosome display involves isolating the antigen-specific scFv cDNA and subsequent cloning into expression vectors. The encoded antibodies can then be expressed in protein expression systems, such as prokaryotic hosts. Antibodies are later analysed to determine their specificity and affinity against the initial target antigen (He and Khan, 2005).

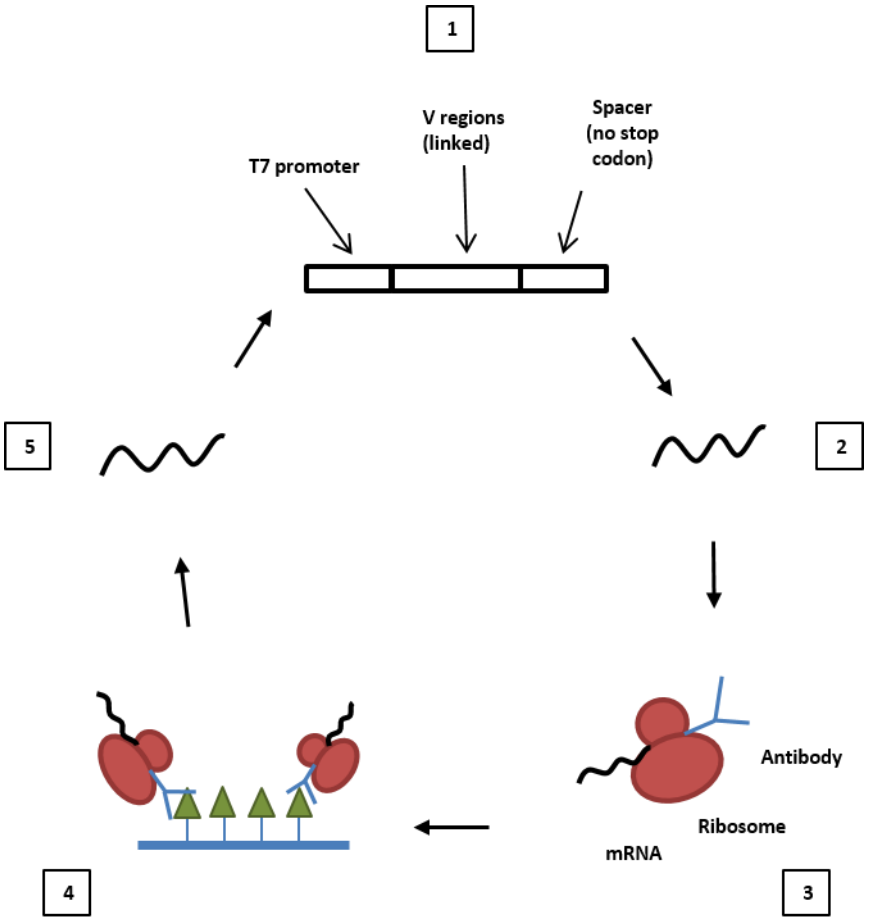


FIGURE 1-11: SCHEMATIC REPRESENTATION OF RIBOSOME DISPLAY TECHNOLOGY (ADAPTED FROM HE AND KHAN, 2005).

IN RIBOSOME DISPLAY, A SCFV LIBRARY IS CONSTRUCTED FROM IMMUNOGLOBULIN GENES (1). THIS LIBRARY CONTAINS THE V REGIONS FROM THE HEAVY AND LIGHT CHAIN THAT ARE JOINED BY A LINKER. THE T7 PROMOTER AND KOZAK SEQUENCE IS ADDED TO 5' ENDS OF SCFV TO AID WITH DOWNSTREAM EXPRESSION OF SCFV. A SPACER IS ADDED TO 3' END OF SCFV TO ENSURE THE COMPLETE PROTRUSION OF THE SCFV FROM THE RIBOSOME. THIS LIBRARY IS THEN TRANSCRIBED (2) AND TRANSLATED IN VITRO (3). ARM COMPLEXES ARE SCREENED AGAINST A TARGET ANTIGEN (4). ANY UNBOUND, AND NON-SPECIFIC COMPLEXES ARE LOST DURING WASH STEPS. THE MRNA ENCODING THE SCFV THAT ARE ABLE TO BIND TO TARGET ANTIGEN IS ISOLATED AND REVERSE TRANSCRIBED (5). THIS SCFV CDNA IS USED TO EXPRESS THE SCFV FOR CHARACTERISATION STUDIES.

1.5.2.4 ALTERNATE DISPLAY TECHNOLOGIES

Other display-based technologies have been developed but are not commonly used, especially for antibody selection. These include selecting antibody fragments displayed on mammalian cells, retroviruses, microbeads or biotin labels in emulsion compartments (Hoogenboom, 2005). Other methods such as a protein fragment complementation assay and the yeast two-hybrid system have also been used to determine interactions between proteins and therefore have the potential to be used for antibody selection. These techniques are based on linking the two proteins to be studied with domains of a protein that can interact and result in an output that can be measured, for example, resulting in gene activation and yeast survival (Fields and Song, 1989). However, the three main platforms for antibody production and evolution remain phage, yeast and ribosome display. Each of these technologies has different advantages and disadvantages and are chosen based on the antibody requirements (Table 1.6).

	Advantages	*Disadvantages*	*Antibodies produced*
Phage display	• Robust method with many protocols available • Easy to use	• Harder to introduce diversity Larger libraries hard to produce (generally 10^7-10^{10} members) Expression bias • Limited by transformation efficiency	• Anti-gp120 (Ditzel *et al.*, 1995) • Anti-TNFα (Salfeld *et al.*, 2001) • Anti-PA from *B. anthracis* (Harvey *et al.*, 2004)
Yeast display	• Fast Direct screening for high affinity antibodies • Eukaryotic protein folding and processing	• Cell sorting equipment needed • Limited by transformation efficiency • Larger libraries hard to produce (generally 10^7-10^{10} members) • Affinity maturation difficult	• Anti-Huntington protein (Swers *et al.*, 2004) • Anti-FITC (Boder *et al.*, 2000)
Ribosome display	• Fastest Largest library size (generally 10^{12}-10^{14} members) • Easy to introduce diversity	• Technically sensitive due to RNA handling and stability • Heavily reliant on PCR, which introduces mutations	• Anti-GCN4 (Zahnd *et al.*, 2004) • Anti-IL13 (Douthwaite *et al.*, 2006)

TABLE 1-6: ADVANTAGES AND DISADVANTAGES OF THE MAIN THREE DISPLAY TECHNOLOGIES (HE AND KHAN, 2005, BRADBURY *ET AL.*, 2011).

1.6 PSEUDOTYPED RETROVIRAL VECTORS

1.6.1 RETROVIRUS GENOME STRUCTURE

The family *Retroviridae* has 7 genera: alpharetroviruses, betaretroviruses, gammaretroviruses, deltaretroviruses, epsilonretroviruses, lentiviruses and spumaretroviruses (Linial *et al*., 2005). Retroviruses have single stranded, positive sense RNA

genomes of around 7-11 kilobases in size; two copies of the viral genome are encapsulated in each virion within the protein core and wrapped in a lipid envelope from which the envelope proteins protrude (see Figure 1-12). Typically, the viral genome encodes 4 basic genes: *gag* (encoding the structural proteins such as the matrix, capsid and nucleoproteins), *pro* (encoding viral protease), *pol* (encoding reverse transcriptase and integrase), and *Env* (encoding the envelope proteins) (Coffin *et al*., 1997, Pedersen, Pyrz and Duch, 2011).

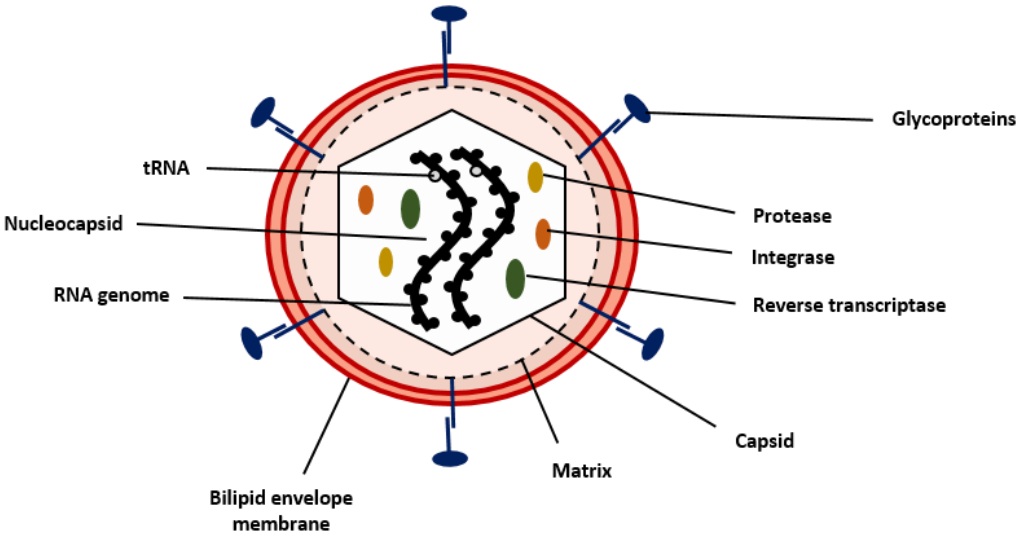


FIGURE 1-12: TYPICAL STRUCTURE AND COMPONENTS OF A RETROVIRAL PARTICLE (ADAPTED FROM PEDERSEN *ET AL*., 2011).

Some retroviruses have a more complex genome structure. Whilst these complex retroviruses, such as lentiviruses including human immunodeficiency virus (HIV), contain all of the basic retroviral proteins and associated retroviral genes, they also have several accessory and regulatory proteins (Rajarapu, 2014). These proteins often play an important role in pathogenesis. This is clearly demonstrated by null mutations in these accessory proteins of simian immunodeficiency virus that lead to the attenuation of the virus in vivo (Desrosiers *et al*., 1998).

HIV, an example of a complex retrovirus, has two regulatory proteins, Tat and Rev, and four accessory proteins, Nef, Vpu, Vif and Vpr (Rajarapu, 2014). The Tat protein, a transcriptional activator, acts to stimulate the efficient elongation of HIV gene transcripts (Gaynor, 1995; Jones and Peterlin, 2003). Indeed, without this protein, there is a build-up of prematurely terminated HIV transcripts (Adams *et al*., 1994). Rev, on the other hand, transports unspliced viral mRNAs from the nucleus to the cytoplasm (Felber *et al*., 1989; Malim *et al*., 1989; Rosen *et al*., 1988). In eukaryotic cells, mRNA must be processed before nuclear export (Libri *et al*., 2002; Maniatis and Reed, 2002); Rev therefore allows the export of viral RNA that is not processed and contains introns. The accessory proteins are also known to have a range of functions including immune evasion, suppression of restriction factors that limit viral entry and ensuring efficient release of virus from infected cells (Malim and Emerman, 2008).

In addition to these viral genes, there are repeat regions (R) and unique regions called U3 and U5 at either end of the viral genome (Pedersen *et al*., 2011). During reverse transcription, these regions are duplicated to form long terminal repeats (LTR) consisting of U5, R and U3 at either end of the viral genome. A primer binding site (PBS) lies after U5 to prime synthesis of the first DNA strand. The polypurine tract (PPT), which lies before U3, primes the synthesis of the second DNA strand (Coffin *et al*., 1997). The psi signal sequence (ψ) is also encoded in the 5'- untranslated region (UTR) and is involved in retroviral particle packaging (Kuzembayeva *et al*., 2014). This genome organisation is seen in Figure 1-13.

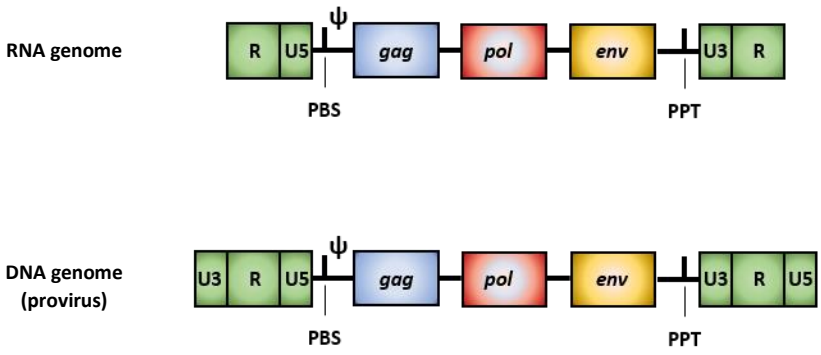


FIGURE 1-13: TYPICAL GENOME ORGANISATION OF A SIMPLE RETROVIRUS (ADAPTED FROM COFFIN, 1997).

THIS FIGURE SHOWS TWO FORMS OF THE VIRAL GENOME: THE INITIAL RNA GENOME AND THE DNA-BASED FORM THAT IS CREATED DURING REVERSE TRANSCRIPTION TO ALLOW THE SUBSEQUENT INSERTION OF THE VIRAL GENOME INTO THE HOST CELL GENOME.

1.6.2 RETROVIRAL LIFECYCLE

The retrovirus lifecycle begins with virus attachment to the host cell surface and to specific receptors, which leads to conformational changes of the envelope protein and host cell surface receptors and the subsequent fusion of the host cell membrane with the viral membrane (Nisole and Saïb, 2004). Once fused, the capsid core of the virus is released into the cytoplasm. The retrovirus can then begin reverse transcription, using its error-prone viral reverse transcriptase (RT), to create a double stranded DNA (dsDNA) copy of the viral genome (Hizi and Herschhorn, 2008).

The process of reverse transcription is primed by a host transfer RNA (tRNA) that binds to the PBS within the 5'UTR promoting reverse transcription of the RNA genome (Eckwahl *et al.*, 2016). This host tRNA is packaged within the viral particle and can be different for different

viruses: e.g. wild-type HIV use $tRNA^{Lys}$, murine leukaemia viruses (MLV) use $tRNA^{Pro}$ and avian leucosis viruses use $tRNA^{Trp}$ (Harada *et al*., 1975; Peters *et al*., 1977; Rhim *et al*., 1991).

Initially, R and U5 at the 5'end of the viral genome are transcribed due to the annealing of the host tRNA at the PBS (Coffin *et al*., 1997; Gilboa *et al*., 1979). After the RNase H activity of the RT has digested the corresponding RNA to this segment (Schultz and Champoux, 2008), the newly synthesised minus-strand DNA is transferred to the 3' end where transcription continues (Gilboa *et al*., 1979). This transfer occurs due to homology between the two repeat regions and results in the creation of the first long terminal repeat (LTR) region (Dang and Hu, 2001). RNase H removes the corresponding RNA segment except the PPT which can then act as a primer for synthesising the second complementary strand of DNA (plus-strand). This plus-strand DNA synthesis continues until reaching the initial tRNA primer at the 5' end leading to its removal but also the creation of the second LTR. The plus strand DNA is then transferred to the PBS in the minus-strand DNA whereby both strands are completed to produce double stranded DNA (Nisole and Saïb, 2004). An overview of this process is shown in Figure 1-14.

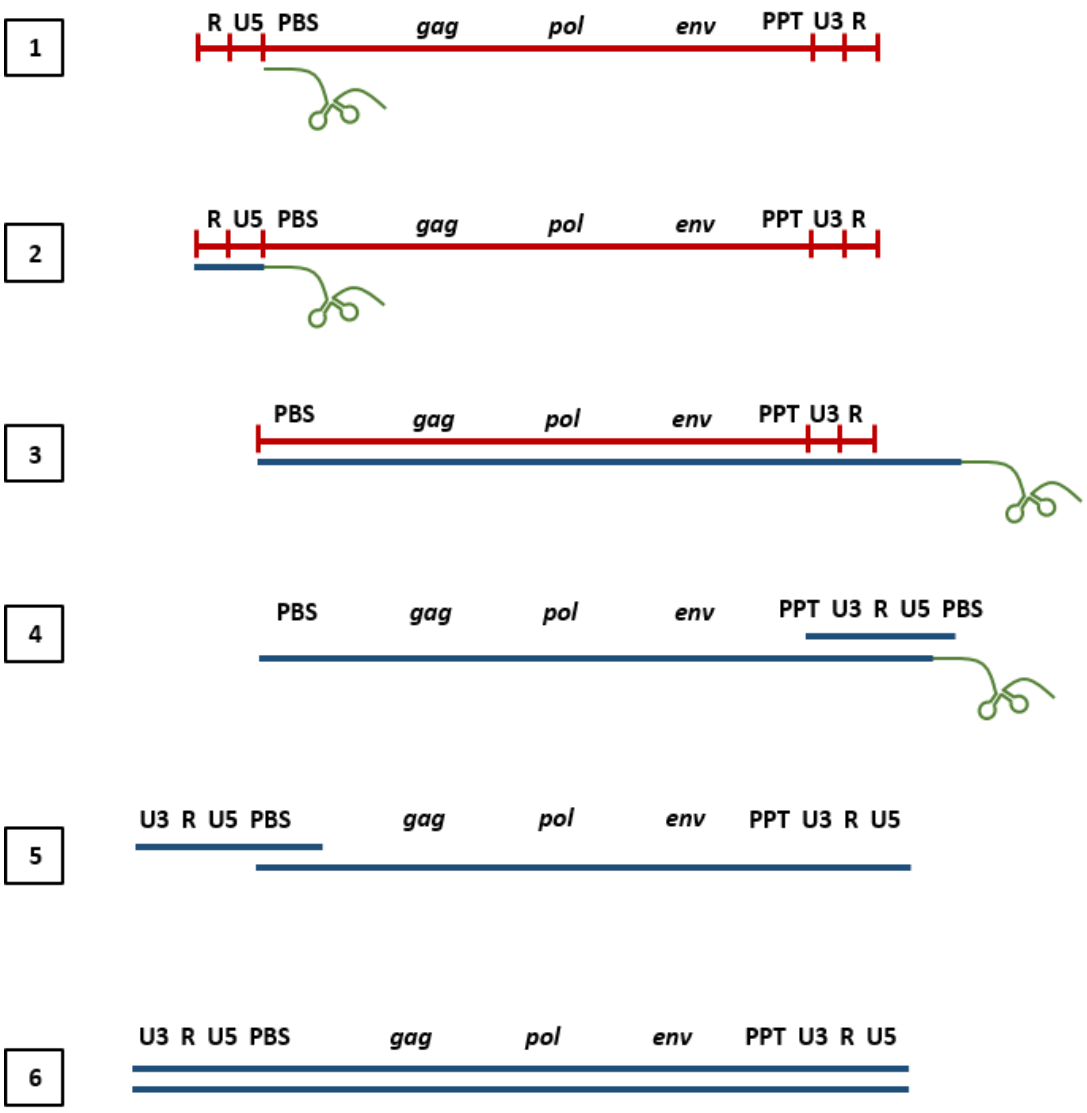


FIGURE 1-14: THE BASIC SEVEN STAGES OF REVERSE TRANSCRIPTION OF RETROVIRAL GENOMES (ADAPTED FROM PEDERSEN, PYRZ AND DUCH, 2011).

REVERSE TRANSCRIPTION IS INITIATED BY THE BINDING OF THE HOST TRNA (GREEN) TO THE PBS OF THE VRNA GENOME (RED) (1). WITH THE TRNA SERVING A PRIMER, THE R AND U5 REGION ARE TRANSCRIBED TO DNA (BLUE) (2). RNASEH DEGRADES THE VRNA ONCE THE VIRAL DNA IS SYNTHESIZED. THE DNA-TRNA COMPLEX IS THEN TRANSFERRED TO THE 3' END OF THE VRNA AND TRANSCRIPTION CONTINUE (3). RNASEH REMOVES THE VRNA EXCEPT FOR THE HIGHLY-RESISTANT PPT SITE WHICH THEN SERVES AS A PRIMER FOR SECOND DNA STRAND SYNTHESIS (4). THIS SYNTHESIS CONTINUES UNTIL THE TRNA SITE WHEREBY IT IS TRANSFERRED TO THE PBS SITE ON THE VIRAL DNA MOLECULE (5) ALLOWING THE TRANSCRIPTION OF BOTH STRANDS TO BE COMPLETED (6).

Alongside the process of reverse transcription, the viral genome is thought to shed its capsid coat (Nisole and Saïb, 2004). Historically, it was thought that once inside a host cell, the virus is uncoated immediately to free the viral genome however, for at least HIV, this is now disputed (Campbell and Hope, 2015; Nisole and Saïb, 2004). There are three possible theories for how HIV particles are uncoated: the particle immediately uncoats as suggested by early biochemical studies, partial uncoating of the viral particle as suggested by imaging studies or uncoating at the nuclear pore complex. Supporting the latter theories, the capsid protein has been found associated with the pre integration complex within the nucleus (a complex of viral DNA, host factors and viral proteins such as integrase) (Campbell and Hope, 2015). Additionally, it is known that the capsid core of the virus, containing the viral genome, is trafficked to the nucleus of the host cell via the microtubule network further suggesting that some copies of the capsid protein remain associated with the viral genome in the host cell cytoplasm (Naghavi and Goff, 2007).

Ultimately, the dsDNA viral genome arrives at the nucleus and must be inserted into the host cell genome (Matreyek and Engelman, 2013). Mitosis is generally required for import of the viral DNA into the nucleus as the nuclear membrane is temporarily degraded during this process; lentiviruses, such as HIV, however, are able to use the cellular nuclear import pathways to enter the nucleus (Bukrinsky *et al*., 1992; Matreyek and Engelman, 2013). Through the use of the viral integrase, the viral DNA is integrated permanently into the host cell genome forming a stable provirus (Andrake and Skalka, 1996). Integrase recognises a very short integration sequence within the LTRs of the retrovirus allowing the processing of the viral ends and subsequent joining within the target cell genome (Hindmarsh and Leis, 1999).

The provirus is transcribed by the host cellular RNA polymerase II, which is promoted by the strong viral promoter within the 5' LTR (Berry *et al*., 1988; Gorman *et al*., 1982; Klaver and Berkhout, 1994). The transcribed viral mRNA molecules, which are either full length genomes

or spliced mRNA, are transported to the cytoplasm and translated (Pedersen *et al*., 2011). Full length viral mRNA molecules are used to produce the Gag precursor polyprotein (containing the structural proteins) and the GagPol precursor polyprotein (containing structural proteins and enzymes) whereas the spliced mRNA is translated at the RER into the envelope proteins. The translated envelope proteins are then transported to the plasma membrane of the host cell via the Golgi apparatus and secretory pathway. Gag recruits full length viral RNA through the recognition of the ψ packaging element, oligomerises and locates to the host cell plasma membrane. The retroviral particles assemble at the plasma membrane and bud off leaving the particle coated in the plasma membrane and envelope proteins (Goff, 2006).

1.6.3 RETROVIRAL VECTORS

Retroviral vectors have a wide range of applications from cancer immunology and gene therapy to studying the biology of highly pathogenic viruses and serology assays (Cavazzana-Calvo *et al*., 2000, King *et al*., 2016). Their use stemmed from understanding that retroviruses could integrate their genome into a host cell genome but also the discovery that oncogenic retroviruses had often acquired sequences from host proto-oncogenes and could therefore support insertions in their genome (Sinkovics, 1984). Several laboratories produced retroviral vectors encoding foreign genes, however, these insertions in the retroviral genome often impacted viral replication and meant that these retroviral vectors could only be produced in the presence of replication-competent helper viruses (Shimotohno and Temin, 1981; Tabin *et al*., 1982; Wei *et al*., 1981). Due to the use of helper viruses, these vectors could not be used in gene therapy because of safety concerns, primarily based on the possibility of generation of replication-competent retroviruses (Miller and Rosman, 1989; Otto *et al*., 1994).

Improvements in the safety of these retroviral vectors were achieved by reducing the number of viral coding regions and elements to the minimum required for high-efficiency transfer of a foreign gene (Miller and Rosman, 1989). These vectors are based on the fact that viral protein synthesis is not required for the early steps of the viral lifecycle. An example of this generation of vectors was the vector used for gene therapy of patients with X-linked Severe Combined Immunodeficiency (SCID). In this study, the genes for *Gag*, *Pol* and *Env* are replaced with the gene of interest, the gamma subunit of the interleukin-2 receptor. By encoding this gamma subunit gene in a murine leukemia virus (MLV)-based vector, hematopoietic stem progenitor cells (HSPC) could be transduced *ex vivo* with this vector and reinfused into the patient. These progenitor cells, with the correct version of the gamma subunit gene, allow the HSPC cells to differentiate into functional immune cells such as T cells (Cavazzana-Calvo et al., 2000). This treatment was initially thought to be successful although three of the children developed T cell leukemia as the vector had inserted near the LMO2 oncogene, activating its expression (Cavazzana-Calvo and Fischer, 2007; Hacein-Bey-Abina *et al*., 2008). Lentiviral vectors are now more commonly used in clinical trial applications as these do not integrate near transcription start sites reducing this safety issue (Yi *et al*., 2011).

To further increase the safety of using these retroviral vectors, the components of the viral genome could be split between several plasmids to reduce the possibility of recombination resulting in replication-competent virus and therefore a complete viral genome (Naldini *et al*., 1996; Soneoka *et al*., 1995). This is particularly important in gene therapy. More recent generations of viral vectors have further improved the safety of using these vectors (Sakuma *et al*., 2012). These improvements include the deletion of accessory genes in HIV-based vectors and the generation of self-inactivating vectors. Self-inactivating vectors contain a U3 deletion at the 3’ end resulting in the U3 promoter only supporting one round of replication.

1.6.4 PSEUDOTYPED VIRAL PARTICLES

A further use of retroviral vectors has been to create pseudotyped viral particles (PV). The first pseudotyped viral particles, based on Rous sarcoma virus (RSV), were produced by Rubin and colleagues (Hanafusa *et al*., 1963; Rubin, 1965). They determined that infectious RSV particles could only be produced if permissive cells were co-infected with a helper virus, Rous associated virus (RAV). Infectious viral particles were produced from both viruses but RSV particles could express the envelope of RAV instead of its native envelope. Other retroviruses were also seen to exhibit the same ability, for example, HIV can incorporate Human T lymphotropic virus, type I, (HTLV-1) envelope proteins and vice versa (Landau *et al*., 1991). This incorporation of other viral envelopes increased the tropism of the virus and changed the course of disease.

PV are based on, typically, either a retrovirus (e.g. Moloney mouse leukaemia virus, MLV) or lentivirus (e.g. HIV) that expresses a reporter gene instead of its own genome and the envelope proteins of another virus (Zavada *et al*., 1972). To produce these PV, a producer cell line is transfected with three plasmids: one containing the *gag-pol* genes, one or two containing the *Env* gene of the virus desired and the last containing either the reporter gene, or transgene to be delivered, flanked by LTRs which is known as the retroviral vector (Figure 1-15). Expression of this transgene can be measured in the case of a therapeutic gene or reporter gene. The packaging signal is also included on the vector containing the gene of interest to ensure it's incorporated into the PV (Coffin *et al*., 1997). Whilst lentiviruses and gammaretroviruses are the most commonly used in pseudotyping studies, other families of viruses, such as VSV from the *Rhabdoviridae* family, have also been used (Mebatsion, 1996).

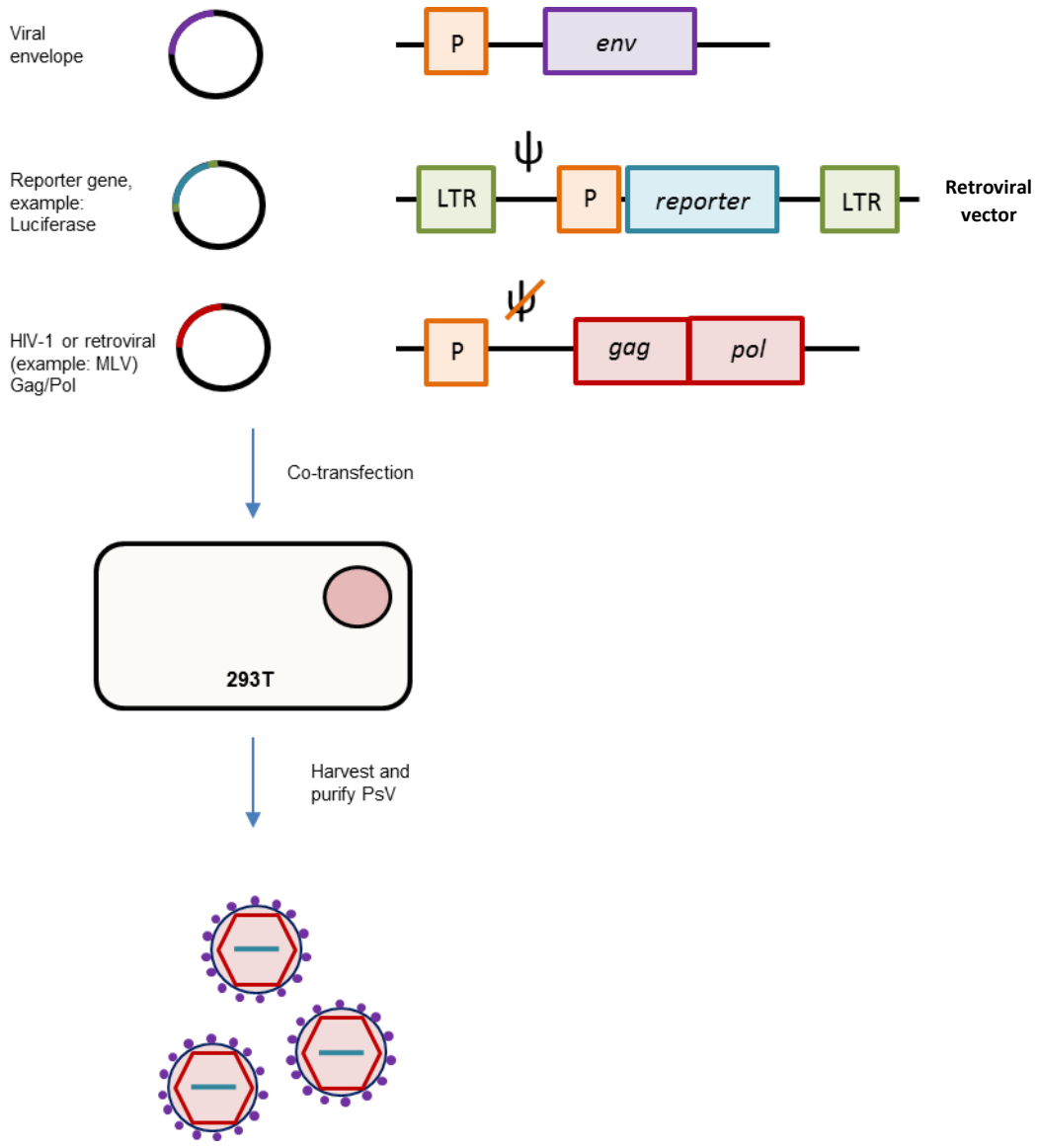


FIGURE 1-15: PV PRODUCTION (ADAPTED FROM TEMPERTON, WRIGHT AND SCOTT, 2015).

PV PRODUCTION IS TYPICALLY BASED ON THE CO-TRANSFECTION OF A PRODUCER CELL LINE, SUCH AS HUMAN EMBRYONIC KIDNEY 293T CELLS, WITH 3 PLASMIDS: A PLASMID CONTAINING THE ENVELOPE GENE, A PLASMID CONTAINING THE *GAG-POL* GENES AND A PLASMID CONTAINING THE REPORTER GENE FLANKED BY LTRS FOR DOWNSTREAM INTEGRATION IN THE HOST CELL AND THE PACKAGING SIGNAL (Ψ) TO ENSURE PACKAGING OF THE GENE IN THE PV. PROMOTERS ARE INCLUDED IN ALL PLASMIDS AND ARE USUALLY CONSTITUTIVE. AFTER 48-72 HOURS, SUPERNATANT FROM TRANSFECTED CELLS IS COLLECTED, PURIFIED AND USED IN DOWNSTREAM APPLICATIONS.

1.6.5 APPLICATIONS OF PSEUDOTYPED VIRAL PARTICLES

Pseudotyped viral particles have been used successfully in a range of fields including cancer immunology, gene therapy, virology, serosurveillance, antiviral drug testing and vaccine immunogenicity testing. Examples of several fields can be seen below.

1.6.5.1 GENE THERAPY

One of the disadvantages of using standard retroviral vectors in gene delivery is their limited host range. This issue of limited tropism was solved by using different viral envelopes such as the G protein of vesicular stomatitis virus (VSV-G), which is able to facilitate entry into a wide range of cell types from different species. This has been utilised successfully in gene delivery (Cronin *et al*., 2005; Lee *et al*., 2001). The VSV-G envelope is also highly stable allowing concentration of retroviral particles by ultracentrifugation (Burns *et al*., 1993).

1.6.5.2 VIROLOGICAL STUDIES

PV are regularly used in the study of highly pathogenic viruses or viruses that are difficult to propagate in cell culture. The use of PV eliminates the need for high-containment laboratories for highly pathogenic viruses, such as Ebola or rabies (Bentley *et al*., 2015). This use of different viral envelope proteins allows the study of the biology of virus interaction with the host of enveloped pathogenic viruses such as the influenza virus or enveloped viruses that do not replicate efficiently in cell culture such as HCV. For example, CD81 was discovered as one of the host receptors used for HCV entry through the use of PV bearing an HCV envelope (Cormier *et al*., 2004). The tropism of viruses and the effect of innate antiviral responses, such as restriction factors, can also be confirmed using PV (Bae and Jung, 2014; Desmaris *et al*., 2001).

Alongside the study of different viruses, PV also allow the study of the virus that the PV is based on, e.g. HIV or MLV. For example, HIV-based PV pseudotyped with different envelopes have been used to study HIV biology and the effects of antiviral drugs (Harrison *et al*., 2011; Spector *et al*., 1990; Yu *et al*., 2009).

1.6.5.3 ANTIVIRAL DRUG SCREENING AND VACCINATION STUDIES

Several studies screening antiviral drugs against a variety of viruses using PV have been employed in the last 10 years. These reports include a recent study testing 1,012 Food and Drug Administration - approved drugs against Ebola PV (Madrid *et al*., 2013) and another study that involved screening 100,000 chemical compounds against influenza pseudotypes (Basu *et al*., 2014). These studies led to the discovery that chloroquine could protect mice from Ebola infection *in vivo* and the identification of two new influenza virus entry inhibitors. The use of PVs are well-suited for high-throughput screening due to their relative simplicity to produce, safety and scalability (Bentley, Mather and Temperton, 2015).

In addition to being used as immunogens in vaccines for both influenza and HIV (Breckpot *et al*., 2010; Szécsi *et al*., 2006), PV are also used to monitor the immune response to vaccines through the development of PV neutralisation assay (PVNA). Previous studies include those based on the use of rabies PV for studying antibody neutralisation in vaccinated individuals/animals and various studies based on using PV to monitor vaccine response by using vaccinated patient sera (Alberini *et al*., 2009; Ewer *et al*., 2016; Wright *et al*., 2008). PVNAs are of particular interest to this study and are summarised in Figure 1-16.

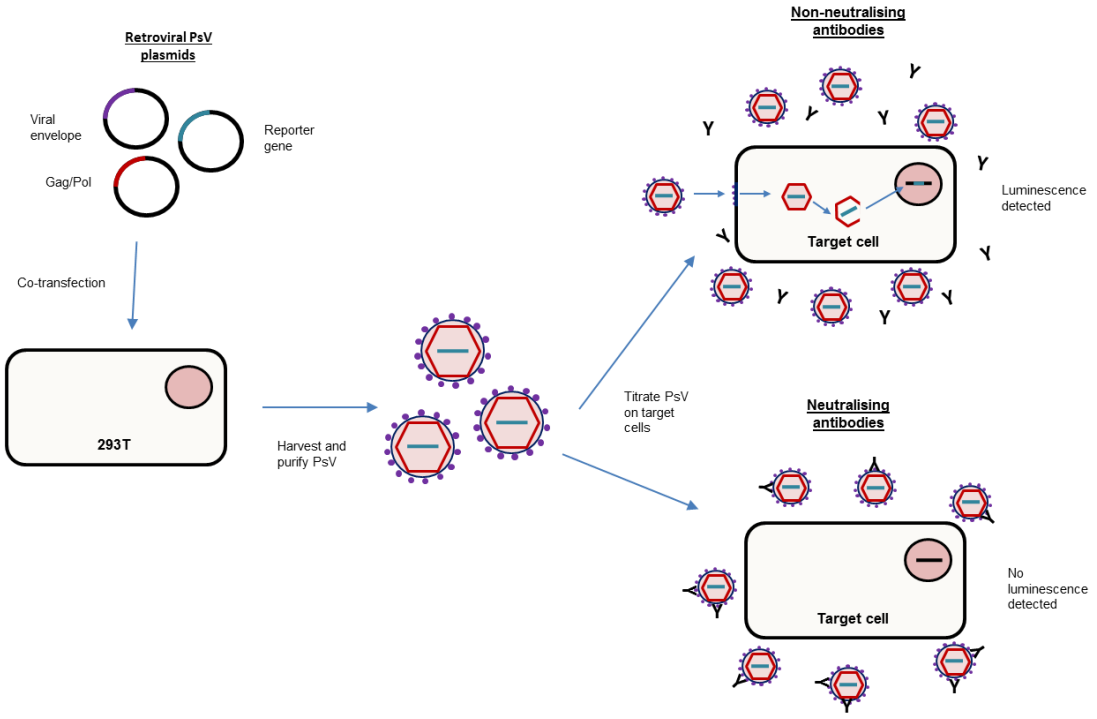


FIGURE 1-16: SUMMARY OF A PV-BASED NEUTRALISATION ASSAY (ADAPTED FROM BENTLEY, MATHER AND TEMPERTON, 2015).

A PRODUCER CELL LINE IS CO-TRANSFECTED WITH 3 PLASMIDS TO PRODUCE PV BEARING THE SPECIFIC VIRAL ENVELOPE OF THE VIRUS TO BE STUDIED. PV ARE COLLECTED AND PURIFIED FROM THE SUPERNATANT OF TRANSFECTED PRODUCER CELLS. PURIFIED PV ARE PRE-INCUBATED WITH THE ANTIBODIES TO BE TESTED AND THIS MIXTURE IS THEN TITRATED ON TARGET CELLS. AFTER 72 HOURS, TARGET CELLS ARE WASHED, LYSED AND THE REPORTER GENE EXPRESSION IS MEASURED. IF THE ANTIBODIES TO BE TESTED ARE NEUTRALISING, NO REPORTER GENE EXPRESSION IS SEEN. IF THE ANTIBODIES ARE NOT NEUTRALISING, PV WILL INFECT THE TARGET CELL, INCORPORATE THE REPORTER GENE INTO THE TARGET CELL GENOME AND REPORTER GENE EXPRESSION IS SEEN.

1.6.6 CHALLENGES ASSOCIATED WITH PV PRODUCTION

Despite the success of pseudotyping a variety of viruses, some have proven difficult to pseudotype (King *et al.*, 2016). The pseudotyping of flaviviruses has proven to be particularly challenging with HCV being one of the few successes. This is likely to be due to the difference in the lifecycle of flaviruses in comparison to retroviruses (see section 1.1.6 and 1.6.2 for

further details on each respective family of viruses). Even so, the generation of HCV PV from clinical samples remains challenging with one study reporting a success rate of just 78 out of 900 primary isolates (Urbanowicz *et al*., 2015).

Viral envelope incorporation appears to be an important factor for high titre PV production. Several factors can affect the efficient incorporation of viral envelope protein into PV. For example, viral envelope incorporation onto the PV requires the envelope to be expressed on the producer cell plasma membrane for the retroviral PV system (King *et al*., 2016; Murakami and Murakami, 2012). Some viral envelope proteins, such as those that belong to viruses in the *Flaviviridae* family, contain endoplasmic reticulum(ER) retention signals in the transmembrane domain (Cocquerel *et al*., 1998a; Op De Beeck *et al*., 2004). These signals lead to the retention of the viral envelope proteins at the ER, therefore PV will not incorporate these proteins into the particle. This can occasionally be overcome if the envelope protein is overexpressed causing 'leaky expression', as seen with HCV (Bartosch *et al*., 2003). Additionally, the truncation of some envelope proteins is required for efficient incorporation into the PV, as seen with PV bearing Measles virus envelope proteins (Frecha *et al*., 2008). Indeed, some envelope proteins are not efficiently incorporated into lentiviral or retroviral-based PV (Takeuchi *et al*., 1992). For example, MLV PV can efficiently incorporate by C-type, but not D-type, retroviral envelope proteins. A similar system based on using vesicular stomatitis virus (VSV) can be used although this system is more complex (King *et al*., 2016, Whitt, 2010). Due to the high efficiency of VSV-G incorporation, PV can often be produced with a VSV-G envelope instead of the desired foreign envelope. Additionally, this system requires more components, for example, a larger number of plasmids.

1.7 AIMS

The first part of this project aimed to investigate the pathology of GBV-B through the production of antibodies that could be used in immunohistochemistry to analyse the presence of GBV-B in various tamarin organs. This immunohistochemistry can now be used to track the virus throughout infection and aid in determining the tropism of GBV-B. These pathology studies will help to further validate GBV-B infection in tamarins as a useful animal model of acute HCV infection.

The second part of this project aimed to understand the immunological events that lead to GBV-B clearance via the development of a neutralisation assay based on GBV-B PV, as GBV-B does not replicate in cell culture. The identification of neutralising antibodies will help to determine the role of the humoral immune response in viral clearance and may potentially inform future HCV vaccine design.

CHAPTER 2 : METHODS AND MATERIALS

2.1 MATERIALS

BUFFERS/SOLUTIONS	RECIPE
MOLECULAR BIOLOGY	
AVE BUFFER (QIAGEN)	RNase-free water with 0.04% NaN3
1X PBS	1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.4
1X TAE	40 mM Tris (pH 7.8), 20 mM sodium acetate, 1 mM EDTA
BLUE/ORANGE 6X LOADING BUFFER (PROMEGA)	0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0)
SOLUTION A FOR PREPARATION OF CHEMICALLY COMPETENT BACTERIA	10 mM $MnCl_2$, 50 mM $CaCl_2$, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH6.3
LURIA-BERTANI (LB) BROTH	1% (w/v) bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 1% NaCl, pH 7.0
2X YT MEDIUM (SIGMA-ALDRICH-ALDRICH)	16 g/L Tryptone, 10 g/L Yeast Extract, 5 g/L NaCl
LB AGAR	1% (w/v) bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, 1.5% (w/v) bacto-agar, pH 7.0
EB BUFFER (QIAGEN)	10 mM Tris-Cl, pH 8.5
WASH BUFFER FOR PROTEIN PURIFICATION	0.02M Tris pH 8.0, 0.5M NaCl, 10mM imidazole
ELUTION BUFFER FOR PROTEIN PURIFICATION	0.02M Tris pH 8.0, 0.5M NaCl, 500mM imidazole
LYSIS BUFFER FOR PROTEIN PURIFICATION UNDER NATIVE CONDITIONS	1x PBS with 0.25% N-Lauroylsarcosine sodium salt
CELL CULTURE	

RIPA BUFFER	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Igepal ca-630, 0.5% (w/v) sodium deoxycholic acid, 10% (w/v) SDS, 1% (v/v) Triton X-100
4X LAEMMLI BUFFER	200 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol
2X LAEMMLI BUFFER	4x Laemmli buffer diluted 1:1
10X TRIS-GLYCINE SDS RUNNING BUFFER (LIFE TECHNOLOGIES)	25 mM Tris Base, 192 mM Glycine, 0.1% SDS pH 8.3
1X TRANSFER BUFFER	10% (v/v) Tris-Glycine SDS running buffer, 20% (v/v) methanol

TABLE 2-1: BUFFERS AND SOLUTIONS COMPOSITION

2.2 MOLECULAR BIOLOGY METHODS

All primers and plasmids used throughout the project are included in Tables 7-1—7-5.

2.2.1 RNA EXTRACTION

Archived splenocytes from an acutely GBV-B infected tamarin that had cleared its infection were washed twice in PBS and resuspended in 350µl RTL lysis buffer (Qiagen) containing guanidine isothiocycanate supplemented with 1% (v/v) ß-mercaptoethanol. After vortexing cells for 1 minute, samples were frozen at -80°C. Cell lysate was then thawed, an isovolume of 70% (v/v) ethanol added and the RNA extracted using an RNeasy mini kit (Qiagen) following manufacturer's instructions. The suggested optional step to remove cellular DNA using DNase I (Qiagen) was always performed. RNA was eluted in 50µl RNase-free water and the concentration and purity of the preparation was determined using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific).

2.2.2 VIRAL RNA EXTRACTION

A purpose-bred red-bellied tamarin (*Saguinus labiatus*) had been previously infected with 1 × 10^7 genome equivalents of GBV-B in serum. Sera samples were collected weekly. Of these sera samples, 140µl of a sample from week 20 (post viral clearance) was lysed by adding 560µl AVL buffer (Qiagen) containing guanidine thiocyanate and 5.6µl of carrier RNA diluted at 1µg/µl in AVE buffer (Qiagen). Viral RNA was then extracted from the sample using a QIAamp Viral RNA Mini kit (Qiagen) following manufacturer's instructions. Viral RNA was eluted in 60µl AVE buffer and the concentration determined as above.

2.2.3 cDNA SYNTHESIS

cDNA was synthesised from 200ng of extracted RNA or vRNA using AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene) following manufacturer's instructions using either random primers or, for single chain variable fragment (scFv) library production, dT29VN primer. DTT, RNase block and High Fidelity Reverse Transcriptase were added after the denaturation step to avoid heat inactivation. A PCR Express thermal cycler HBPX110 (Thermo Hybaid) was used to generate the cycle parameters for cDNA synthesis.

Components	RNA	vRNA
AccuScript RT buffer	2µl	2µl
Oligo(dT) primer (0.5µg/µl)	1µl	
Random primers (0.1µg/µl)		3µl
dNTP (100mM)	0.8µl	0.8µl
DTT (100mM)	2µl	2µl
RNase Block (40U/µl)	0.5µl	0.5µl
AccuScript High Fidelity RT µl	1µl	1µl
RNase-free water up to a final volume of 20µl		

TABLE 2-2: THE COMPONENTS OF THE CDNA SYNTHESIS REACTION

Cycle parameters		**Function**
65°C	5 minutes	Denaturation
Room temperature	5 minutes	Annealing of primers
25°C	10 minutes	Extension of primers*
42°C	60 minutes	cDNA Synthesis
70°C	15 minutes	Terminate synthesis

TABLE 2-3: THE CYCLE PARAMETERS USED FOR CDNA SYNTHESIS

* THIS STEP IS ONLY CARRIED OUT WHEN USING RANDOM PRIMERS.

2.2.4 POLYMERASE CHAIN REACTION (PCR)

PCRs were carried out using either KOD Hot Start (Novagen), FastStart Taq (Roche), AmpliTaq Gold (Roche) or Phusion High-Fidelity (New England Biolabs) DNA polymerases in a 50µl reaction. The required components of each reaction are stated below:

Component	KOD Hot Start (1U/µl)	FastStart Taq (5U/ µl)	AmpliTaq Gold (5U/µl)	Phusion High-Fidelity (1U/µl)
PCR buffer	5µl (10x)	5µl (10x)	5µl (10x)	10µl (5x)

dNTP	5µl (2mM each)	8µl (1.25mM each)	8µl (1.25mM each)	1µl (10mM each)
Forward primer	1.5µl of 10µM primer	2.5µl of 5µM primer	2.5µl of 5µM primer	2.5µl of 10µM primer
Reverse primer	1.5µl of 10µM primer	2.5µl of 5µM primer	2.5µl of 5µM primer	2.5µl of 10µM primer
Mg source	3µl of 25mM $MgSO_4$	4µl of 25mM $MgCl_2$	4µl of 25mM $MgCl_2$	Included in PCR buffer at a final concentration of 1.5mM
DNA polymerase	1µl	0.4µl	0.25µl	0.5µl
Distilled water	Up to a final volume of 50µl			

TABLE 2-4: THE REACTION MIXTURES FOR EACH PCR REACTION

Cycle conditions:

Once set up, a T3 thermocycler (Biometra), with a heated lid, was used to produce the cycle conditions below.

KOD DNA polymerase			
Step	**Temp (°C)**	**Time (s)**	**Use**
1. Initialisation	95	120	Cloning of GBV-B E1E2 envelope proteins
2. Denaturation	95	20	
3. Annealing	Table 7-1	10	
4. Extension	70	40	
Number of cycles (steps 2-4): 30			
FastStart Taq DNA polymerase			
1. Initialisation	95	240	

2. Denaturation	95	30	Cloning of GBV-B core, NS3 and NS5B proteins
3. Annealing	Table 7-1	30	
4. Extension	72	60	
5. Final extension	72	420	
Number of cycles (steps 2-4): 30			

AmpliTaq Gold DNA polymerase

Stage of scFv production	**Step**	**Temp (°C)**	**Time (s)**	**Aim**
1	1. Initialisation	94	600	Cloning of variable regions, Kozak sequence (red line), part of linker (green line) and part of spacer (purple line).
	2. Denaturation	94	30	
	3. Annealing	54/56	30	
	4. Extension	72	60	
	5. Final extension	72	600	
	Number of cycles (steps 2-4): 30			
2	1. Initialisation	94	600	Elongating linker and adding part of T7 promoter (yellow line).
	2. Denaturation	94	30	
	3. Annealing	56	30	
	4. Extension	72	60	
	5. Final extension	72	600	
	Number of cycles (steps 2-4): 16			
3	1. Initialisation	94	600	Joining variable regions together via a complete linker.
	2. Denaturation	94	30	
	3. Annealing	52	45	
	4. Extension	72	90	
	5. Final extension	72	600	
	Number of cycles (steps 2-4): 16			
5	1. Initialisation	94	600	Adding spacer (C_H) to scFv.
	2. Denaturation	94	30	
	3. Annealing	52	45	
	4. Extension	72	180	
	5. Final extension	72	600	

	Number of cycles (steps 2-4): 16			
6	1. Initialisation	94	600	Completing scFv by elongating T7 promoter (yellow line).
	2. Denaturation	94	30	
	3. Annealing	52	45	
	4. Extension	72	180	
	5. Final extension	72	600	
	Number of cycles (steps 2-4): 20			
Phusion High-Fidelity DNA Polymerase				
Step	**Temp (°C)**	**Time (s)**		**Use**
1. Initialisation	98	180		Amplification of CDR3 sequences within immunoglobulin variable regions for deep sequencing.
2. Denaturation	98	10		
3. Annealing	Table 7-1	15		
4. Extension	72	30		
Number of cycles (steps 2-4): 25				

TABLE 2-5: THE CYCLE PARAMETERS USED FOR PCR

For recovery of scFv after the screening of a scFv library against GBV-B proteins using ribosome display, a combined reverse transcription reaction and PCR was performed. Recovered, eluted mRNA was cleaned up using the RNeasy Mini kit (Qiagen) to remove EDTA and used as the input mRNA for the One-Step RT-PCR kit (Qiagen). This kit includes the enzymes Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq® DNA Polymerase which allows reverse transcription and PCR to take place in the same tube. Reactions were performed in 50µl volumes and were kept on ice. A T3 thermocycler (Biometra), with a heated lid, was used to produce the cycle conditions below. The components for this reaction are included below alongside the conditions used:

Component	Volume/reaction
PCR buffer (5x)	10µl
dNTP (10mM of each dNTP)	2µl
Forward primer (10µM)	3µl
Reverse primer (10µM)	3µl
Enzyme mix	2µl
Template mRNA	Varied
Distilled water	Up to a final volume of 50µl

TABLE 2-6: THE COMPONENTS OF THE ONE-STEP RT-PCR REACTION

Step	Temperature (°C)	Time (seconds)	Use
1. Reverse transcription	50	1800	Reverse transcription and amplification of recovered scFv from ribosome display.
2. Initial PCR activation	95	900	
3. Denaturing	95	30	
4. Annealing	Table 7-1	30	
5. Extension	72	120	
6. Final extension	72	600	
Number of cycles (steps 3-5): 30, unless stated			

TABLE 2-7: THE CONDITIONS USED FOR THE ONE-STEP RT-PCR REACTION

2.2.5 AGAROSE GEL ELECTROPHORESIS

Agarose gels were produced by dissolving agarose powder (Invitrogen) in 50-100ml 1× TAE. Gels were supplemented with 5µl SafeView (NBS biologicals) per 100ml agarose suspension for nucleic acid visualisation using a UltraBright LED Transilluminator (Applied Biological

Materials). Loading buffer to 1× concentration (6× stock, Promega) was added to each sample prior to gel electrophoresis. Approximately 500ng of a 100bp or a 1Kb DNA ladder (Promega) was also run with samples to allow for size determination of DNA fragments.

In the case of CDR3 PCR products, the Safe-Green (Applied Biological Materials) nucleic acid stain was used at a dilution of 1:5 (dye: sample). Agarose gels were prepared in the manner above minus the addition of dye. The remainder of the protocol remains as described above.

2.2.6 PURIFICATION OF DNA FROM AGAROSE GEL

DNA was purified from agarose gel using the QiaQuick Gel Extraction kit (Qiagen) following manufacturer's instructions. DNA was eluted in 30µl elution buffer and the concentration determined using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific).

2.2.7 PURIFICATION OF DNA FROM PCR SAMPLES

DNA was purified directly from PCR samples using the High Pure PCR Cleanup Micro Kit (Roche), following manufacturer's instructions, to maximise the recovery of DNA. DNA was eluted in 10µl Elution buffer (kit supplied). Again, the concentration was determined using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific).

2.2.8 PREPARATION OF CHEMICALLY COMPETENT BACTERIAL CELLS

To prepare chemically competent bacteria, 5µl of Competent Subcloning Efficiency DH5α *Escherichia Coli* bacterial cells (Invitrogen) were inoculated in 5ml of LB broth and incubated in a shaker incubator at 250rpm overnight at 37°C. This culture was then diluted into 500ml LB broth containing 15mM $MgCl_2$ and further incubated at 37°C in the shaker incubator until the OD_{600} reached 0.5. Bacterial cells were pelleted at 4000rpm for 15 minutes at 4°C using a

Sorvall Super T21 centrifuge. Pellets were then resuspended in 125ml of ice-cold solution A, spun down as before and resuspended in 12.5ml of solution A with 15% (v/v) glycerol. Bacterial cells were aliquoted and stored at -80°C.

2.2.9 TRANSFORMATION OF COMPETENT BACTERIA

Aliquots of chemically competent bacteria were transformed following the manufacturer's instructions. For home-made competent bacterial cells, 50µl of bacterial cell suspension was incubated with 100-200ng of plasmid DNA or 5µl of a ligation reaction on ice for 30 minutes. Bacteria were heat shocked at 42°C for 45 seconds. After the addition of 450µl of LB broth, samples were incubated for 1 hour at 37°C shaking at 250rpm and then 120µl spread on LB agar plates supplemented with 100µg/ml of carbenicillin (Sigma-Aldrich-Aldrich). Commercial bacterial cells were purchased in single use aliquots and transformed using the manufacturer's provided protocols. Commercial bacterial cells were purchased from the following companies: BL21 (New England Biolabs), SHuffle® T7 Express (New England Biolabs), Rosetta-Gami B DE3 (Novagen) and NiCo21 (New England Biolabs).

2.2.10 PLASMID DNA MINI PREPARATIONS

Individual bacterial colonies, picked from LB agar plates, were grown overnight in 5ml LB broth containing an appropriate concentration of antibiotic at 37°C in a shaker incubator at 200-250rpm. Bacteria were then pelleted and plasmid extraction was performed using Qiaprep Spin miniprep kit (Qiagen) following the manufacturer's instructions. Purified plasmid DNA was eluted in 30µl EB buffer and the concentration determined using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific). If the absorbance ratio at wavelength 260nm/280nm was 1.8-2.0, purified plasmid samples were considered good quality. Please see Appendix for plasmid maps of protein production plasmids (Figure 7-1-7-6).

2.2.11 ENZYMATIC REACTIONS

Restriction digests were carried out with the required enzymes (New England Biolabs), in the appropriate buffer, and distilled water at a final volume of 20µl for single digestions or 50µl for double digestions. Digestion reactions were then incubated at 37°C overnight in a PCR Express thermal cycler HBPX110 (Thermo Hybaid) with a heated lid.

DNA ligations were performed using 1µl of T4 DNA ligase (3U/µl, Promega) in the supplied buffer, using 50ng backbone and the insert at a ratio of 1 backbone: 3 insert in a final volume of 10µl overnight at 16°C in a PCR Express thermal cycler HBPX110 (Thermo Hybaid). After this incubation period, ligations were inactivated at 65°C for 10 minutes and used to transform a suitable bacterial host.

2.2.12 SANGER SEQUENCING

Samples to be sequenced were set up in 8 x 0.2ml tube strips (Thermo Scientific) with the following components: 2.5µl of BigDye terminators (Applied Biosystems), 2µl of 5× sequencing buffer (Applied Biosystems), 1µl of 5mM sequencing primer (forward or reverse), 400-500ng of template DNA and PCR-grade H_20 up to 10µl. A T3 thermocycler (Biometra) with a heated lid was then used to produce 25 cycles of the following conditions: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Reactions were then either stored at -20°C or cleaned up.

To clean up PCR products for sequencing, 10µl of PCR grade H_20, 5µl of 125mM EDTA and 80µl of 100% ethanol were added to samples and mixed by inversion. After a 15-minute incubation at room temperature, samples were centrifuged at 3800rpm for 30 minutes using the ST-H750 rotor in the Sorvall Super T21 centrifuge. The supernatant was then discarded and spun inverted for 1 minute at 800rpm. The DNA pellet was then washed with 200µl of

70% ethanol. Samples were centrifuged for a further 15 minutes at 3800rpm before the inversion spin step was repeated.

DNA pellets were then resuspended in highly deionized (Hi-Di) Formamide before loading on to a 3130 Genetic Analyser (Applied Biosystems).

2.1.13 Analysis of DNA sequences

All DNA sequence files obtained from the 3130 Genetic Analyser were processed using DNADynamo (Blue Tractor Software). Forward and reverse reads for each sequence were imported into this software and aligned using the default settings. As the sequences are linked with chromatogram data, a visual check of each sequence can confirm the called sequence is correct.

2.1.14 Analysis of immunoglobulin sequences

Once immunoglobulin sequences were gathered by Sanger sequencing, IgBLAST (accessed online at: http://www.ncbi.nlm.nih.gov/igblast/) was used to screen each nucleotide sequence against the human germline immunoglobulin gene database using the 'blastn' program. All search parameters and formatting options were kept at the default setting. As tamarin immunoglobulins appear to be highly similar to human immunoglobulin sequences (see table 4-4), this allows the relevant V/D/J segments and CDR3 sequences to be identified.

2.2.15 DEEP SEQUENCING OF SCFV LIBRARIES

The highly-variable CDR3 sequences of PCR-generated scFv libraries before and after screening against an antigen in ribosome display, were deep sequenced. This led to the sequencing of 4 libraries. As scFv consist of both the variable region from the heavy chain and the variable region from the light chain of antibodies, CDR3 sequences were obtained for both regions. Additionally, all reactions were duplicated to increase the reliability and accuracy of the obtained sequences. This resulted in 16 reactions.

The first step, prior to deep sequencing, was the amplification of CDR3 regions by PCR using the high fidelity polymerase, Phusion (see **2.2.4**). Degenerate primers were designed to capture as many CDR3 regions as possible (see table 7-1). Three primers were designed to cover the region preceding the CDR3 sequence and one primer to cover the sequence succeeding the CDR3 sequence.

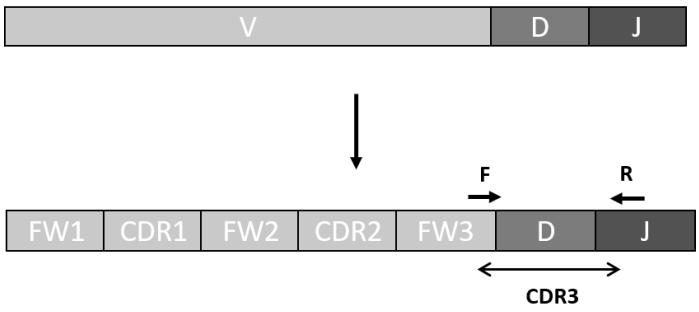


FIGURE 2-1: DESIGN OF CDR3 PRIMERS

IN THE HEAVY CHAIN OF AN IMMUNOGLOBULIN, THE CDR3 REGION ORIGINATES FROM THE SEQUENCES FROM THE END OF THE V GENE SEGMENT, THE D GENE SEGMENT AND THE BEGINNING OF THE J GENE SEGMENT, AND BY NUCLEOTIDES ADDED OR LOST WHEN THESE GENE SEGMENTS ARE JOINED DURING LYMPHOCYTE DEVELOPMENT. AS SUCH PRIMERS WERE DESIGNED TO COVER THIS AREA (F: FORWARD, R: REVERSE). THE EXON FOR THE CDR3 LOOP OF THE LIGHT CHAIN IS FORMED BY THE JOINING OF SEQUENCES FROM V AND J GENE SEGMENTS (NOT SHOWN). FW: FRAMEWORK REGION, CDR: COMPLEMENTARITY DETERMINING REGION

These primers were designed by comparing previously sequenced variable regions containing CDR3 sequences (see table 7-6). PCR products were then run in a 3% agarose gel (see **2.2.5** for details) and purified (see **2.2.6** for details). Purified PCR products were then submitted to the Deep Sequencing group at NIBSC for sequencing on the MiSeq platform.

2.2.16 Antibody Mining Tool

Although forward and reverse reads of scFv sequences were produced for each sample from deep sequencing, only the quality-filtered forward reads from CDR3 samples for each library were obtained from the Deep Sequencing group at NIBSC. Due to the short length of CDR3 sequences (approximately 250 basepairs), only forward sequences were required for analysis and these were analysed using the Antibody Mining Tool (downloadable, D'Angelo *et al*., 2013).

The files containing the filtered forward reads for both the heavy chain and light chain CDR3 sequences for each scFv library were analysed using the 'cdr3_pipeline' or 'lcdr3_pipeline' python script using the default settings, as these are recommended and have been determined experimentally by D'Angelo and colleagues using the MiSeq platform (D'Angelo *et al*., 2013). The output of this script is an Excel file containing all CDR3 sequences within each library along with the number of appearances of this sequence in each library. All 16 samples (4 libraries, heavy and light chain and duplicates) were run in these pipelines. To analyse this data, the number of unique CDR3 sequences were categorised by frequency and presented in a stacked column chart. A logarithmic scale was used due to the high number of sequences analysed.

To further analyse this data, the enrichment of the top ten CDR3 sequences for each sample were investigated. The percentage of each CDR3 sequence in each library was calculated by

dividing the average frequency of a particular CDR3 sequence (as they were duplicated) by the total number of CDR3 sequences. The percentages for each of the top ten CDR3 sequences for each library (before and after panning) were then plotted on a stacked line graph.

2.2.17 Ribosome Display

The ribosome display protocol was from Zhou and colleagues (Zhou *et al*., 2009). Plates were coated overnight with 10µg of recombinant target antigen or BSA diluted in RNase-free Tris pH8.0 (Ambion). After 16 hours, the plate was washed with PBS containing 5mM $MgCl_2$ (PBS-M) and blocked with 5% powdered milk (w/v, Marvel) in PBS-M for 30 minutes. After this time, the plate was washed with PBS-M and blocked for a further 2 hours with PBS-M containing 1% BSA (Miltenyi Biotec). Three washes with PBS-M were then performed and the plate was incubated for 10 minutes on ice.

During the two hour blocking stage, the scFv library was transcribed and translated using the TnT T7 Quick-Coupled Transcription Translation system (Promega). This reaction was set up in a 0.5-ml RNase-free, protein lo-bind tube (Eppendorf) as follows: 40µl TnT T7 Quick Master Mix, 1µl DNA enhancer, 1µl 1mM methionine, 4µl of the scFv library at a concentration of at least 100ng/µl, and 4µl of RNase free H_20. This mixture was incubated at 30°C for 90 min; 6µl of RNase-free DNase I (Roche) (10,000 U/ml) was then added and the mixture was incubated for a further 20 min at 30°C. Immediately after this stage, 50µl of ice-cold PBS-M containing 1% BSA was added to stop the reaction and the mixture was added to the BSA-coated well for 1 hour at 4°C. Later, this mixture was transferred to the target antigen-coated well and incubated at 4°C for a further hour.

Finally, the mixture was discarded and the well washed three times with ice-cold PBS-M containing 0.05% Tween-20 and two times with ice-cold PBS-M. Bound scFv were dissociated

with PMBS-M containing 20mM EDTA (Invitrogen) for 10 minutes on ice. ScFv mRNA purification was performed using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions and was then used immediately in the OneStep RT-PCR kit (Qiagen).

For further rounds of ribosome display panning, scFv DNA was then elongated by repeating the PCR for the 6th stage of scFv library construction, however 30 cycles were needed to obtain a sufficient amount of scFv DNA. This DNA was then purified and used as the input library for ribosome display.

2.3. PROTEIN PRODUCTION METHODS

2.3.1 INDUCTION OF TARGET PROTEINS IN BACTERIAL CELLS

Bacterial cells were transformed with protein expression plasmids containing genes of proteins to be expressed, such as His-tagged GBV-B proteins. Colonies of bacteria containing these plasmids were picked after 24 hours and grown overnight in 2× yeast extract-tryptone (YT) broth supplemented with 100µg/ml Carbenicillin. Cultures were then used to inoculate either 50ml (for scFv expression) or 300ml (GBV-B protein expression) of 2x YT broth supplemented with 100µg/ml Carbenicillin and 0.5% glucose. These cultures were grown to an OD_{600} of 0.6 and protein expression was induced using 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 hours at 20°C (for scFv) or 4 hours at 25°C for GBV-B proteins. Cells were harvested by centrifugation at 4000rpm for 15 minutes using the ST-H750 rotor and the Sorvall Super T21 centrifuge.

2.3.2 SDS-PAGE AND COLLOIDAL BLUE STAINING

Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-12% Tris-Glycine polyacrylamide gels in the XCell SureLock Mini-Cell system and 1x Novex Tris-Glycine SDS Running Buffer (Life Technologies). Samples were heated for 5 minutes at 95°C (or incubated for 15 minutes at 37°C for viral envelope proteins), loaded along with SeeBlue Plus2 Pre-Stained Protein Standard into gels and run at 130 volts for 90 minutes (Life Technologies). Proteins were then visualized by staining gels for 3 hours with the Colloidal Blue Staining Kit (Life Technologies) and de-stained for a minimum of 7 hours in distilled water, following manufacturer's instructions.

2.3.3 DISRUPTION OF BACTERIAL MEMBRANES

Bacterial pellets were frozen at -80°C, thawed, and resuspended in PBS containing 0.25% (w/v) N-Lauroylsarcosine sodium salt (Sarkosyl, Sigma-Aldrich-Aldrich) at 1/30 of its original volume. Cells were sonicated at 30% amplitude (volumes of 10ml) or 60% amplitude (volumes over 10ml) for 6 times at 30 seconds, with 45 second rest intervals, on ice. Triton X-100 (Sigma-Aldrich-Aldrich) was added to a final concentration of 0.5% v/v before incubating lysate for 60 minutes on a rolling spin. Cell debris was removed by centrifugation at 5000rpm for 30 minutes at 4°C in an Sorvall Legend RT centrifuge using the Swinging Bucket Rotor 7500 6445.

2.3.4 PURIFICATION OF HIS-TAGGED PROTEINS

To purify His-tagged proteins produced in bacterial cells under native conditions using a Nickel affinity column, supernatant from 2.3.3 was applied to a chromatography column (Sigma-Aldrich-Aldrich) containing 1ml Ni-NTA sepharose (Sigma-Aldrich-Aldrich) and incubated for 1 hour at room temperature on a rolling spin. After washing the gel with 30ml wash buffer (Table 2-1), His-tagged proteins were eluted with elution buffer (Table 2-1). Column elutions and washes were run on an SDS-PAGE and stained as above to confirm successful purification.

To purify and concentrate His-tagged proteins produced in mammalian cells, the Ni-NTA Spin kit (Qiagen) was used following the manufacturer's instructions.

2.3.5 QUANTIFICATION OF PROTEIN

To calculate the concentration of proteins produced in a prokaryotic system, eluates containing His-tagged protein were electrophoresed by SDS-PAGE along with serial dilutions of bovine serum albumin of known concentration. Protein concentrations were calculated using GeneTools software (Syngene) by comparison to the BSA standard curve.

Purified monoclonal antibodies were quantified using the Mouse IgG Total Ready-SET-Go! (eBioscience), which uses NUNC 96-well plates (eBioscience), following manufacturer's instructions. A mouse IgG standard (provided in the kit) was serially diluted and also included in the assay. Plates were read at both 570nm and 450nm using a FLUOstar Omega microplate reader (BMG Labtech). The 570nm values were then subtracted from the 450nm values to account for background fluorescence. A standard curve was created from the values obtained from the mouse IgG standard of known concentration and used to calculate the concentration of monoclonal antibody in each sample.

2.3.6 ISOTYPING OF MONOCLONAL ANTIBODIES

The isotype of monoclonal antibodies was determined using the Mouse Monoclonal Antibody Isotyping Test Kit (Bio-Rad) following the manufacturer's instructions. This kit is based on a lateral flow membrane that has specific antibodies targeting each mouse isotype or light chain on specific strips of the membrane. Briefly, concentrated monoclonal antibody samples to be tested were diluted in a solution containing colloidal gold particles. These particles, which bind to the monoclonal antibodies in the samples, were then exposed to the membrane allowing the migration of the antibody-colloidal gold particle complexes to the specific band of the membrane that contains the corresponding anti-mouse isotype/light chain antibodies. A band is seen due to the colloidal element that indicates the isotype of the

monoclonal antibody and which light chain is used.

2.3.7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs were performed in 96-well plate formats using Nunc MaxiSorp plates (eBioscience). Plates were coated overnight with 1µg/ml of antigen diluted in PBS. The next day, plates were washed with PBS containing 0.05% Tween-20 (Sigma-Aldrich-Aldrich) and blocked for 2 hours with PBS containing 5% (w/v) skimmed milk powder (Marvel). Plates were then washed again using the same wash buffer containing Tween-20. Primary antibodies were then added to the plate for 1 hour at room temperature; scFv antibodies and monoclonal antibodies were generally used undiluted, unless stated otherwise. After a further five rounds of washing, the secondary antibody, diluted in PBS containing 2% (w/v) skimmed milk powder (Marvel), were added for 45 minutes at room temperature at the following dilutions:

- 1:5000 of anti-human IgG-HRP, Fc-specific, Sigma-Aldrich-Aldrich.
- 1:5000 of anti-mouse IgG-HRP, Sigma-Aldrich-Aldrich.
- 1:5000 of anti-His-HRP, Cambridge Biosciences. This antibody was also used at 1:20000 in Chapter **4.2.7**.

Excess secondary antibody was removed through a further five rounds of washing before enhanced K-Blue TMB substrate (Neogen Corporation) was added and the plates were incubated for 10 minutes at room temperature. Peroxidase reactions were stopped using sulphuric acid and the intensity of each well at 450nm was read using a FLUOstar Omega microplate reader (BMG Labtech).

2.4 CELL CULTURE METHODS

2.4.1 CELL LINES USED

CELL LINES USED	CELL TYPE	CULTURE MEDIA	REFERENCE
293T CELLS	Human embryonic kidney cells expressing the SV40 large T antigen	Dulbecco's Modified Eagle's medium containing GlutaMAX supplement (GIBCO) with added 10%(v/v) Foetal Calf Serum (FCS) and 1%(v/v) Penicillin/Streptomycin (Sigma-Aldrich-Aldrich). To stop N-glycosylation, 0.5µg/ml tunicamycin was added into the medium	DuBridge *et al*., 1987
NS-0 CELLS	Murine myeloma cells that do not secrete immunoglobulins	RPMI 1640 medium (GIBCO) containing L-Glutamine with added 10% (v/v) Foetal Calf Serum (FCS) and 1% (v/v) Penicillin/ Streptomycin (Sigma-Aldrich-Aldrich). Prior to fusion with splenocytes, medium was supplemented with Hypoxanthine-Aminopterin-Thymidine (HAT) (Sigma-Aldrich).	Galfre and Milstein, 1981, Clark and Milstein, 1981
HYBRIDOMA CELLS	Splenocytes fused to NS-0 cells	RPMI 1640 medium (GIBCO) containing L-Glutamine with added	Galfrè and Milstein., 1981

		10% (v/v) Foetal Calf Serum (FCS) and 1%(v/v) Penicillin/ Streptomycin (Sigma-Aldrich-Aldrich).	
STAR CELLS	293T cells that are stably transfected with HIV-1 Gag and Pol proteins	See 293T cells	Ikeda *et al*., 2003
HUH7	Human hepatoma cells	See 293T cells	Nakabayashi *et al*., 1982
TAMARIN FIBROBLASTS	Primary fibroblast cells isolated from tamarin	RPMI 1640 medium (GIBCO) with added 10% (v/v) Foetal Calf Serum (FCS) and 1%(v/v) Penicillin/Streptomycin (Sigma-Aldrich-Aldrich).	n/a
FRHK-4	Rhesus monkey kidney cells	Dulbecco's Modified Eagle's medium (GIBCO) with added 10% (v/v) Foetal Calf Serum (FCS) and 1%(v/v) Penicillin/Streptomycin (Sigma-Aldrich-Aldrich).	Wallace *et al*., 1973.
VERO	African Green monkey kidney cells		Yasumura and Kawakita, 1963.
CRFK	Cat kidney cells		Crandell, Fabricant and Nelson-Rees, 1973.
HUH7.5	Human hepatoma cells		Blight, McKeating and Rice, 2002.

TABLE 2-8: LIST OF CELL LINES USED

ALL CELLS WERE GROWN AT 37°C WITH 5% CO_2.

2.4.2 HYBRIDOMA PRODUCTION

To produce monoclonal antibodies, mice were immunised with recombinant GBV-B core protein. This was carried out by the Biological Services Division at NIBSC, where mice were housed and maintained in accordance with UK Home Office guidelines. Briefly, two mice were immunised with 200µl of a mixture containing 0.2mg/ml recombinant GBV-B core protein and Sigma-Aldrich Adjuvant System oil at a ratio of 1:1. Mice were immunised at day 1, 28 and 56 and terminated at day 59. Spleens were collected at termination and maintained on ice for same-day use.

Harvested spleens were placed in sterile Petri dishes with 5ml of culture medium (serum-free RPMI with glutamine and 1% penicillin/streptomycin, GIBCO). Splenocytes were collected by scraping spleens with forceps and scissors; cells were then collected in a Falcon tube and centrifuged for 5 min at 1,000rpm using a Hettich Rotanta 460R centrifuge and the 5624 swinging bucket rotor. Cells were then resuspended in 10ml media and the wash stage was repeated a further two times. NS-0 cells were washed in an identical manner in parallel to splenocytes.

To fuse splenocytes and NS-0 cells together, both cells suspensions were mixed together at a 1:4 (splenocyte: NS-0) ratio. The mixed cell suspension is then centrifuged at 1000rpm for 10 minutes, the supernatant discarded and the cell pellet partially air-dried. This pellet was loosened by gentle tapping and 1ml polyethylene glycol (50% w/v in distilled water, Sigma-Aldrich) was added slowly over one minute, whilst gently stirring the cells. The mixture was left for 1 min and serum-free media was added slowly over a period of 10 min to a final volume of 10ml. Cells were then centrifuged again for 15 minutes at 1000rpm and the supernatant discarded.

The fused cell pellet was resuspended in 200ml RPMI supplemented with 20 % FCS, 20 pg/ml of human IL-6 and 2% HAT Media Supplement Hybri-Max (50x, all Sigma-Aldrich) and dispensed into 12 96-well tissue culture plates at 150µl/well. Fused cells were incubated for around 3 weeks at 37°C in 5% CO_2. After this period, HAT supplement was substituted for HT media supplement Hybri-Max (50x, Sigma-Aldrich) for a further month.

2.4.3 HYBRIDOMA SINGLE CELL TITRATION

Following the 7-8 week hybridoma growth period after the initial fusion of mouse splenocytes and NS-0 cells, colonies of hybridomas contained within the 96-well plates were screened for the presence of GBV-B core-specific antibodies by ELISA. Any wells that showed evidence of core-specific binding were then cloned by limiting dilution and screened by ELISA again.

Limiting dilutions of hybridomas were performed by removing hybridoma cells from an individual well of the tissue culture 96-well plate and diluting them to a concentration of $2x10^4$ cells/ml. To stain cells, cells were mixed at a 1:1 ratio with trypan blue stain (0.4%, Life Technologies). Stained cells were then counted using the Countess II Fl Automated cell counter.

Of these diluted cells, 200µl was placed in well A1 of Figure 2-1 for each plate. The remaining wells were filled with 100µl of medium. Two dilutions series were performed to obtain single cells dilutions; the first from wells A1 to H1 and the second from columns 1-12 using a multichannel pipette (see Figure 2-1). Cells were then incubated for two weeks at 37°C in 5% CO_2. Cells in wells containing single colonies were isolated and cell-growth was scaled up.

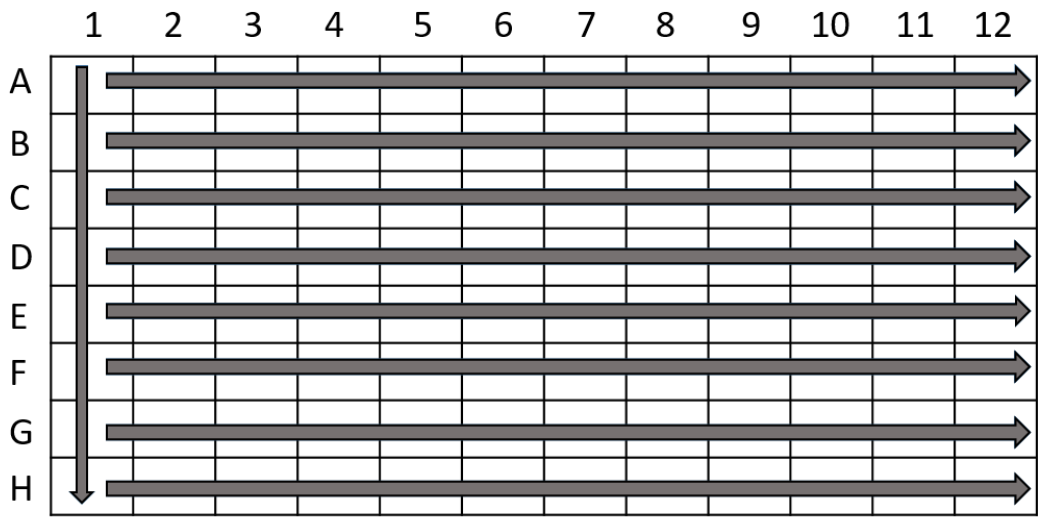


FIGURE 2-2: 96-WELL PLATE SETUP FOR SERIAL DILUTION OF HYBRIDOMA CELLS

2.4.4 PURIFICATION OF HYBRIDOMA CELLS

Hybridoma cell supernatant was collected and cells were removed by centrifugation at 3000rpm for 20 min at 4°C in an Sorvall Legend RT centrifuge using the Swinging Bucket Rotor 7500 6445. The pellet was disregarded and the supernatant mixed at a 1:1 ratio with 50% ammonium sulphate, to a final volume of 50ml, to allow precipitation of monoclonal antibodies. Supernatant was rotated overnight at 4°C. Precipitated antibodies were collected by centrifugation 3000rpm for 30 min at 4°C. The pellet was then resuspended in 1ml of PBS.

2.4.5 CELL TRANSFECTION

To express proteins in a mammalian cell line, 293T cells or STAR cells were seeded in a 6 well plate at a density of either 1×10^6 or 2.5×10^6, respectively, one day before transfection to achieve 80-90% confluence. To transfect cells, plasmid DNA, diluted in TE buffer (Sigma-

Aldrich) to a final volume of 5µl, was mixed with an appropriate transfection reagent (Fugene 6, Fugene HD or XtremeGeneHP) diluted in Optimem (Life Technologies). Transfections were then carried out according to manufacturer's instructions.

2.4.6 PSEUDOTYPED VIRUS PRODUCTION

Pseudotyped viruses were produced through the co-transfection of 293T or STAR-HV cells with a mix of two or three plasmids encoding the necessary elements: Gag and Pol genes, a retroviral vector containing a reporter gene flanked by LTRs and the viral envelope to be studied (see Table 2-7).

Initially, 293T cells were transiently co-transfected using Fugene-6 (Promega) with three plasmids: pCMV8.91 containing HIV-1 *gag* and *pol*, HIV-based vector pCSGW encoding the reporter gene, Green Fluorescent Protein (GFP) and a plasmid containing the desired envelope protein at a ratio of 1:1:1.5 respectively (Besnier *et al*., 2002). Plasmids containing envelope proteins included pcDNA3_E1E2, pCAGG_E1E2, and pMDG containing vesicular stomatitis virus glycoprotein G (VSV-G) (Naldini *et al*., 1996).

Similarly, STAR cells that were stably transfected with pCMV8.91 were transduced with self-inactivating pHV (Ikeda *et al*., 2003) encoding GFP and then transfected with a plasmid containing the envelope protein. To produce pseudotyped viral particles with no envelope protein as a negative control, the native plasmid without the cloned envelope protein/s was used. A mix of the 3 plasmids at the correct ratio and TE buffer to a final volume of 15µl was added to 18µl of Fugene-6 diluted in 200µl Optimem. This mix was incubated at room temperature for 15 minutes and added to cells that had been seeded in a 6 well plate that were 80% confluent. The medium of the transfected cells was replaced after 24 hours. After both 48 and 72 hours, pseudotyped viruses (PV) were harvested, filtered through a 0.45µm

Whatman membrane (GE healthcare) to remove cell debris from the culture medium and stored at -80°C.

An altered PV production protocol was later adopted (Tarr *et al*., 2007; Urbanowicz *et al*., 2015). For this protocol, 293T cells were seeded in a 10cm dish at a density of $1.2x10^6$ one day before transfection to achieve 40-50% confluence. This should help to reduce the level of cellular debris due to overgrowth of cells and the subsequent leaking of envelope proteins.

Plasmids used to transiently transfect to create PV are as follows:

- Lentiviral three-plasmid system: 2µg of pCMV8.91 containing HIV-1 *gag* and *pol*, 2µg of HIV-based vector pCSFLW that was previously created in the lab by substituting the *GFP* gene from pCSGW for the *Firefly luciferase* gene and varying concentrations of the plasmid containing the desired envelope protein, pCAGG_E1E2 or pcDNA3.1D_HCVE1E2. The plasmid containing GBV-B envelope proteins was used at a concentration of either 2µg, 400ng, 80ng or 16ng. The plasmid containing HCV envelope proteins was used at concentration of 2µg.
- Lentiviral two-plasmid system: 2µg of a packaging vector, pNL4-3.Luc.R^-E^-, containing the HIV-1 genome with the *Firefly luciferase* gene inserted into the *nef* gene, and a plasmid containing the viral envelope of interest, 400ng of pCAGG_E1E2 or 2µg of pcDNA3.1D_HCVE1E2.
- Retroviral three-plasmid system: 1µg pCMVi containing Moloney murine leukaemia virus *Gag* and *Pol* genes, 1µg of pCFCR_luc containing the Firefly luciferase gene and a plasmid containing the viral envelope of interest, 80ng of pCAGG_E1E2 or 2µg of pcDNA3.1D_HCVE1E2.

As a negative control, PV bearing no envelope were created in all three systems through the omission of the plasmid containing envelope proteins.

Cells were transfected using TurboFect transfection reagent following manufacturer's instructions (Thermofisher). Supernatant, containing PV, were collected at 48 and 72 hours or 72 hours alone, filtered through a 0.45µm Whatman membrane (GE healthcare) and stored at -80°C, as before.

2.4.7 CELL TRANSDUCTION

STAR cells were transduced with pHV (Ikeda *et al*., 2003), a lentiviral vector containing the GFP gene flanked by wild type HIV LTRs, at a Multiplicity of Infection (MOI, number of particle available to infect each cell) of 5 in the presence of 8µg/ml polybrene onto cells that had been seeded the previous day at a density of $2.5x10^6$. Culture medium was replaced with fresh DMEM supplemented with 10% FCS after 24 hours. After 72 hours, a sample of these cells was isolated, fixed in 2% paraformaldehyde (Sigma-Aldrich-Aldrich) in PBS and analysed for GFP expression by flow cytometry (FACSCalibur, BD Biosciences) using the Flowjo software (FlowJo, LLC).

2.4.8 CELL LYSATE COLLECTION

Transfected 293T/STAR cells from a 6 well plate were lysed in 100µL of radio immunoprecipitation assay (RIPA) buffer with 1x proteinase inhibitor cocktail, Complete mini (Roche), for 30 minutes on ice. Samples were then spun down at 13000rpm in a Biofuge Pico bench-top centrifuge (Heraeus) with the 7500 3328 microlitre rotor at 4°C for 8 minutes and the supernatant saved and frozen at -80°C.

2.4.9 PV CONCENTRATION

On a small scale, 900µl of 293T transfected cell supernatant containing PV was concentrated by centrifugation at 13,000rpm for 4 hours in a Biofuge Pico bench-top centrifuge (Heraeus) with the 7500 3328 microlitre rotor at 4°C. PV were then resuspended in 25µl of 2x Laemmli buffer containing 10% β-mercaptoethanol. To concentrate supernatant by 2000x, PV were instead resuspended in 900µl of 293T cell culture media (Table 2-8) and the centrifuge step repeated.

For large scale purification, collected supernatant from transfected 293T cells was pooled and layered on top of 5ml 50nm sodium phosphate containing 20% sucrose (w/v; Sigma-Aldrich-Aldrich) in polyallomer tubes (Beckmann). These tubes were then loaded into a SW28 rotor in a Beckman Optima LE-80K Ultracentrifuge and centrifuged at 23,000rpm for 2 hours at 4°C. After this, supernatant was discarded and PV were resuspended in 500µl 293T cell supernatant (Table 2-8). PV were then used immediately or frozen at -80°C.

2.4.10 WESTERN BLOTTING

PV pellets or all other proteins were resuspended in 2x or 4x Laemmli buffer, respectively, containing 10% β-mercaptoethanol. Once resuspended, protein or PV samples were heat treated at either 95°C for 5 minutes or 37°C for 15 minutes. Samples could then be separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) using 4-12% Tris-Glycine Mini Gels with 1x Tris-Glycine SDS running buffer on the XCell SureLock Mini platform (Life Technologies). Gels were run at 130V for 2 hours.

A wet blotting system based on a Mini Trans-Blot electrophoretic transfer cell was used to transfer proteins from the Tris-Glycine gel onto a nitrocellulose membrane (Amersham Biosciences) at a 100V for 1 hour. This membrane was subsequently blocked in 5% (w/v) non-fat dried milk (Marvel) in PBS and washed in PBS. The following primary antibodies were

diluted in PBS containing 2% skimmed milk powder (w/v; Marvel) and added to the membranes for 1 hour at room temperature:

Antibody target	HA	GBV-B E2	VSV-G	HIV-1 p55/p24	HCV E2	Moloney MLV p30	6xHis tag
Working dilution	1:1000	1:2000	1:1000	1:1000	1:1000	1:1000	1:2000, or 1:5000-1:20:000 if used as a secondary antibody
Details	HA.11 clone 16B12 monoclonal.	Polyclonal sera	P5D4 monoclonal	ARP319	AP33 monoclonal	Polyclonal sera targeting MLV capsid	HRP Conjugated
Host	Mouse	Rabbit	Mouse	Mouse	Mouse	Mouse	Mouse
Source	Biolegend	A kind gift from Annette Martin; Marnata *et al*., 2015	Sigma-Aldrich-Aldrich	Medical Research Council AIDS Directed Programme	A generous gift from Ania Owsianka; Clayton *et al*., 2002	A generous gift from Yasu Takeutchi	Bethyl Laboratories

TABLE 2-9: DETAILS OF PRIMARY ANTIBODIES USED IN THIS THESIS

After washing the membrane three times in 0.05% (v/v) Tween-20 in PBS, an anti-mouse A4416 or anti-rabbit W4011 IgG horse radish peroxidase (HRP) conjugated secondary antibody (Sigma-Aldrich-Aldrich and Promega, respectively) diluted 1:3000 in 2% (w/v) milk in PBS was added to membranes and incubated for 45 minutes at room temperature. The membrane was washed a further three times with 0.05% (v/v) Tween-20 in PBS, once in PBS alone. In the case of detecting tamarin antibodies, the anti-IgG 2B11 (Kerafast) antibody was used by diluting it 1:1000 in 2% (w/v) milk in PBS, which in turn could be detected by the anti-mouse IgG-HRP antibody.

Chemiluminescence was detected by incubation with ECL Prime or the highly sensitive ECL Select western blotting detecting reagent following the manufacturer's instructions (Amersham Biosciences).

2.4.11 Immunostaining for flow cytometry analysis

Prior to flow cytometry analysis, transfected 293T cells were fixed with 2-4% paraformaldehyde for 20 minutes at room temperature and then permeabilised with 0.2% saponin for a further 10 minutes at room temperature. From this point, all buffers contained 0.2% saponin to maintain membrane permeabilisation. Samples were then blocked on ice for 30 minutes with PBS supplemented with 10% FCS and washed with wash buffer (PBS containing 2% FCS). An anti-HA primary antibody, diluted 1:100 in wash buffer, was added to samples and kept on ice for an hour. After two washes, samples were incubated with a fluorescein isothyocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (BD Pharmingen) diluted 1:100 in wash buffer for an hour in ice. After a further two washes, samples were analysed on a FACSCanto using FACSDiva software (BD Biosciences).

2.4.12 In vitro Infection Assays

Target cell lines were seeded at 1×10^4 per well in culture medium in a 96-well cell culture plate (Corning) and incubated for 24 hours at 37°C. Culture medium was then removed and replaced with 50µl PV diluted either three-fold or five-fold in red phenol free culture medium in the presence of polybrene (final concentration 4µg/ml, hexadimethrine bromide, Sigma). Cells were either incubated for a further six hours or centrifuged for 30 minutes at 2500rpm using a Hettich Rotanta 460R centrifuge and the 5624 plate rotor before a further 100µl of red phenol free medium was added; cells were then incubated for 48 hours. After the 48-

hour incubation, culture medium was removed and replaced with 100µl of fresh culture medium and 100µl of BrightGlo reagent, which serves to both lyse cells and provide the luciferase substrate (Promega). Cells were pipetted, incubated for 5 minutes in the dark and 150µl was transferred to a white 96-well plate. The luminescence was then read on a FLUOstar Omega microplate reader (BMG Labtech).

CHAPTER 3 : PRODUCTION OF GBV-B PROTEINS AND CONSTRUCTION OF A TAMARIN SINGLE CHAIN ANTIBODY LIBRARY

3.1 INTRODUCTION

The study of viral pathogenesis is crucial for the development of vaccine candidates to effectively control viral infections. As the pathology of acute HCV infection is not well understood, this project aims to investigate the pathology of the closely-related GBV-B in tamarins. To contribute to the definition of the pathology caused by GBV-B, diagnostic reagents are required, such as virus-specific antibodies. Currently, the availability of diagnostic reagents for GBV-B is limited; no commercial antibodies exist and therefore laboratories must produce their own.

Ribosome display is an *in vitro* display-based technology used to isolate and evolve proteins against a specific ligand. Display technologies, such as ribosome, phage and yeast display, have been used successfully to produce antibodies for a variety of targets and applications (Bradbury *et al*., 2011). These methods have several advantages over the traditional method of producing antibodies through the immunisation of animals, such as the ability to control selection conditions, the affinity maturation of isolated antibodies and the availability of the sequence of the selected antibodies. For example, a human antibody targeting HIV gp120 was successfully mutated in the complementarity-determining regions (CDRs) by phage display leading to a 420-fold increase in affinity (Yang *et al*., 1995). However, phage display is limited by bacterial cell transformation efficiency whereas ribosome display is only limited by the number of ribosomes in the reaction. Additionally, antibodies isolated in phage display must be expressed in bacteria introducing an element of codon usage bias whilst ribosome display is cell-free; isolated antibodies from ribosome display can be expressed in a variety of

expression systems to overcome codon usage bias. For these reasons, ribosome display was chosen to produce recombinant tamarin antibodies that can target GBV-B proteins.

To produce recombinant tamarin antibodies, GBV-B proteins and a library of scFvs were first produced. Previous studies have shown that tamarins produce antibodies against various GBV-B proteins; these proteins include the nucleocapsid of the virus, Core, the serine protease used by the virus in polyprotein processing, NS3, and the RNA-dependent RNA polymerase used for viral replication, NS5B (Ishii *et al*., 2007; Iwasaki *et al*., 2011; Pilot-Matias *et al*., 1996; Sbardellati *et al*., 2001). These three GBV-B proteins, Core, NS3 and NS5B, were therefore chosen as the screening targets for ribosome display. Additionally, all three proteins had been previously produced in a prokaryotic system in other laboratories thereby simplifying protein production.

In parallel to GBV-B protein production, a scFv library was constructed. Antibodies consist of four polypeptide chains − two heavy chains and two light chains- that are held together by disulphide bonds. Each of these polypeptide chains comprise of variable regions that are involved in antigen binding and constant regions that are involved in determining the class and functionality of the antibody. Historically, producing whole recombinant antibodies has proved to be difficult due to improper protein folding and solubility issues in prokaryotic systems (Boss *et al*., 1984; Cabilly *et al*., 1984). However, the various constituents of antibodies can be produced in bacteria such as the fragment antibody-binding (Fab) region, the two variable regions of an antibody linked together (scFv) and the fragment crystallisable (Fc) region (Jendeberg *et al*., 1997; Shibui and Nagahari, 1992; Skerra *et al*., 1991). As Skerra and Plückthorn had previously determined that the two variable regions, one from the heavy chain and one from the light chain, otherwise known as scFvs, were the minimal unit required for antigen recognition, display methods are based on producing these types of antibodies (Skerra and Plückthun, 1988). Therefore, a library made from millions of combinations of the

variable regions of tamarin antibodies was produced to be screened against GBV-B proteins to isolate any tamarin antibodies that recognise either GBV-B core, NS3 or NS5B. Any antibodies isolated would be used in immunohistochemistry to help define the pathology of GBV-B with the aim to further validate the use of GBV-B infection in tamarins as an animal model of acute HCV infection.

3.2 GBV-B PROTEIN PRODUCTION

3.2.1 CLONING OF CORE, NS3 AND NS5B

To select antibodies against GBV-B proteins, GBV-B core, NS3 serine protease and NS5B were chosen as target antigens (see Figure 3-1A for genome organisation of GBV-B). The non-structural proteins NS3 serine protease and truncated NS5B (NS5BΔ23) were amplified by PCR as described in Chapter **2.2.4** (Figure 3-1B). Restriction sites were included in primer design for both proteins to allow subsequent cloning into a suitable expression vector. The RNA-dependent RNA polymerase NS5B was cloned as a truncated version of the protein with the last highly hydrophobic 23 amino acids removed to aid with protein purification, as described previously (Ranjith-Kumar *et al*., 2003). PCR products of the correct size were visualised on a 1% agarose gel and later cloned into the pGem T easy vector for safekeeping (see Chapter **2.2.5** for further details). The correct identity of the fragments in this vector was confirmed by Sanger sequencing (as described in Chapter **2.2.12**). The structural core protein of GBV-B had been cloned previously into the expression vector pET22b+; Sanger sequencing of this vector confirmed the genes' correct sequence.

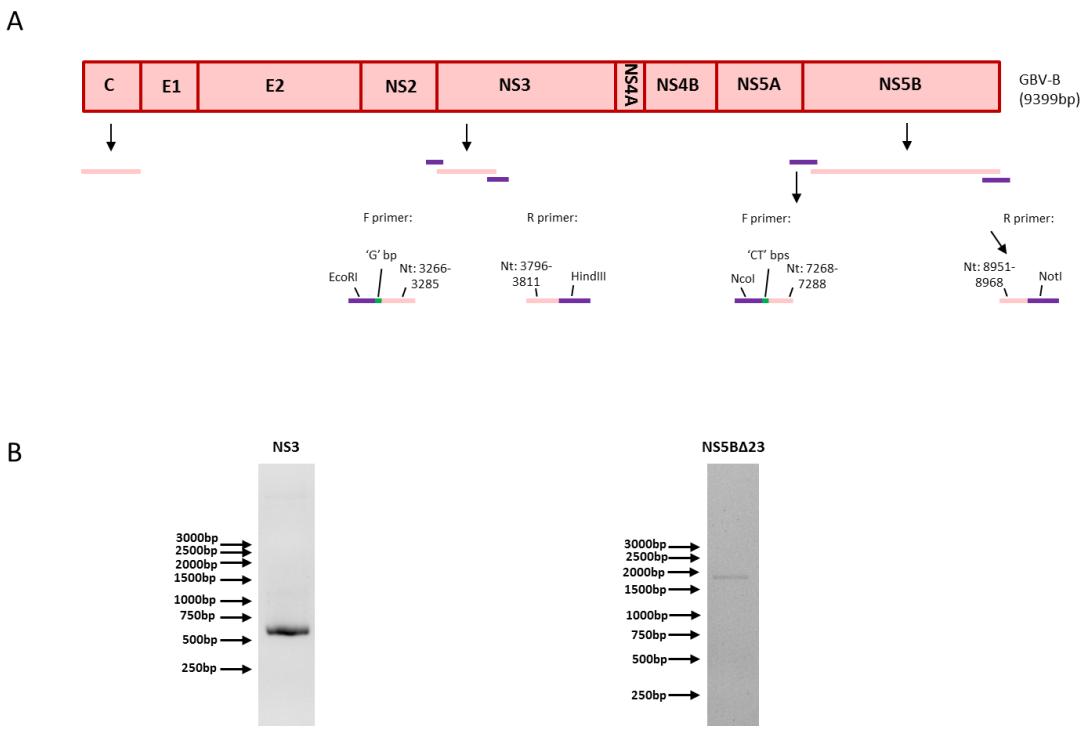


FIGURE 3-1: GBV-B PROTEIN CLONING STRATEGY

A: DESIGN OF PRIMERS FOR CLONING OF GBV-B PROTEINS. IN THIS DIAGRAM, THE THREE GBV-B PROTEINS TO BE GENERATED ARE SHOWN ALONGSIDE THE GENOME STRUCTURE OF GBV-B. CORE HAD BEEN PREVIOUSLY CLONED AND THEREFORE PRIMER DESIGN IS NOT INCLUDED. FOR NS3, THE RESTRICTION SITES ECORI AND HINDIII ARE ADDED TO THE FORWARD OR REVERSE PRIMER, RESPECTIVELY. THE FORWARD PRIMER ALSO CONTAINS A RANDOM ADDITIONAL 'G' NUCLEOTIDE BEFORE THE START OF THE GENE TO ENSURE THAT NS3 IS KEPT IN FRAME. LIKEWISE, NS5BΔ23 PRIMERS ALSO CONTAIN THE NCOI AND NOTI RESTRICTION SITES NECESSARY FOR DOWNSTREAM SUBCLONING INTO AN EXPRESSION VECTOR. THE NS5BΔ23 FORWARD PRIMER CONTAINS 2 RANDOM ADDITIONAL NUCLEOTIDES, A C AND T, TO ENSURE NS5BΔ23 IS KEPT IN FRAME.

B: CLONING OF GBV-B PROTEIN GENES BY PCR.
1% AGAROSE GELS VISUALISING PCR PRODUCTS OF GBV-B NS3 SERINE PROTEASE (545BP) AND GBV-B NS5BΔ23 (1700BP).

3.2.2 EXPRESSION OF CORE, NS3 AND NS5BΔ23

For the production of GBV-B proteins in a prokaryotic host, GBV-B core, NS3 and NS5BΔ23 were subcloned into suitable expression vectors. GBV-B NS3 serine protease gene was cloned into the vector pET32b+ using restriction enzymes *Eco*RI and *Hin*dIII whilst GBV-B core and

NS5BΔ23 genes were cloned into the modified expression vector p4D5 using restriction enzymes NcoI and NotI (Markiv *et al*., 2011, see Chapter **2.2.11** for further details). This vector, p4D5, had been previously created by digesting pET32b+ using *Nde*I and *Not*I to remove all but one C-terminus His tag to simplify protein purification as additional tags would not have to be cleaved downstream in the purification procedure. Sanger sequencing confirmed that each GBV-B protein had been successfully moved into the correct expression vector.

Expression vectors containing GBV-B core, NS3 and NS5BΔ23 were used to transform BL21 *E.coli* bacterial cells (as described in Chapter **2.2.9**). One colony of BL21 containing either core, NS3 or NS5BΔ23 was grown overnight and used to inoculate 1 L of 2*YT broth. Once each bacterial culture had reached an OD of 0.6, GBV-B protein expression was induced using 1mM IPTG (as described in Chapter **2.3.1**).

Non-induced and induced bacterial cultures (500 µl samples) were lysed using 2× Laemmli buffer containing 10% β-mercaptoethanol and boiled; bacterial proteins were separated by SDS-PAGE using 4-12% Tris-Glycine polyacrylamide gels and subsequently stained using a Colloidal Blue staining kit (Figure 3-2, methods described in Chapter 2.3.2). All three proteins were seen to be induced in the BL21 *E.coli* strain and electrophoresed at their expected molecular weight of 20kDa for core, 38kDa for NS3 serine protease and 65kDa for NS5BΔ23.

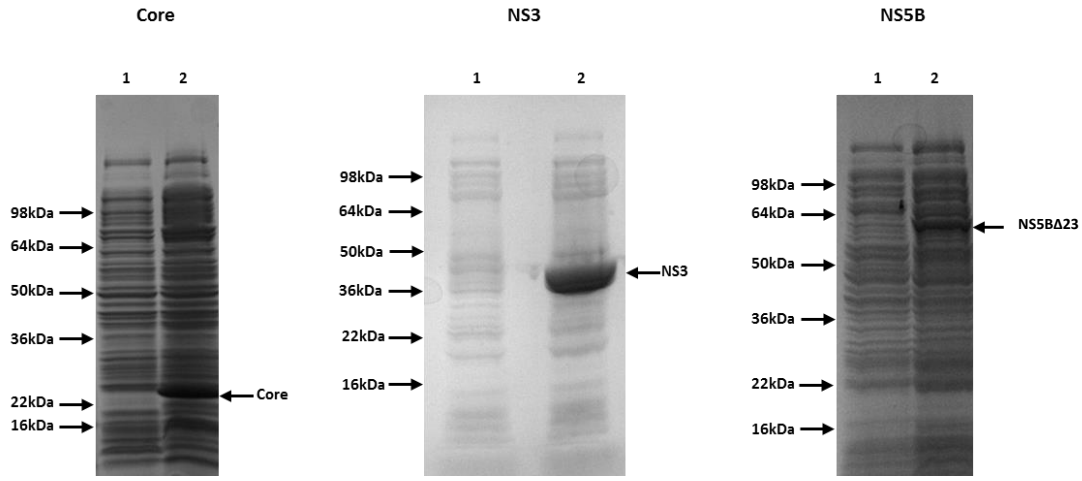


FIGURE 3-2: INDUCTION OF GBV-B PROTEIN EXPRESSION IN BL21 BACTERIAL STRAIN.

SAMPLES OF NON-INDUCED (LANE 1) AND INDUCED (LANE 2) BL21 TRANSFORMED WITH EITHER P4D5-CORE (ESTIMATED PROTEIN SIZE: 20KDA), PET32B-NS3 (ESTIMATED PROTEIN SIZE: 39KDA) OR P4D5-NS5BΔ23 (ESTIMATED PROTEIN SIZE: 65KDA) WERE LYSED AND DENATURED BEFORE RUNNING ON AN SDS-PAGE TO SEPARATE PROTEINS BY SIZE.

3.2.3 SMALL-SCALE PURIFICATION OF GBV-B CORE, NS3 SERINE PROTEASE AND NS5BΔ23 UNDER NATIVE CONDITIONS

C-terminal His-tagged GBV-B core, NS3 serine protease and NS5BΔ23 produced in a prokaryotic system (see Chapter **2.1.2**) were purified under native conditions using a nickel affinity column. After induction of GBV-B protein expression in BL21 cells, proteins were isolated through lysis in a buffer containing mild detergents Sarkosyl and Triton-X100 and sonication to disrupt bacterial membranes (Tao *et al*., 2010). His-tagged GBV-B proteins were subsequently purified under native conditions on a nickel affinity column. For each protein, each stage of the purification process was analysed by SDS-PAGE and Colloidal Blue staining (Figure 3-3A, D and F). Western blots of each protein using an anti-His antibody (Figure 3-3B, E and G) or tamarin sera (Figure 3-3C) confirmed each protein's identity by size (as described in Chapter **2.4.10**).

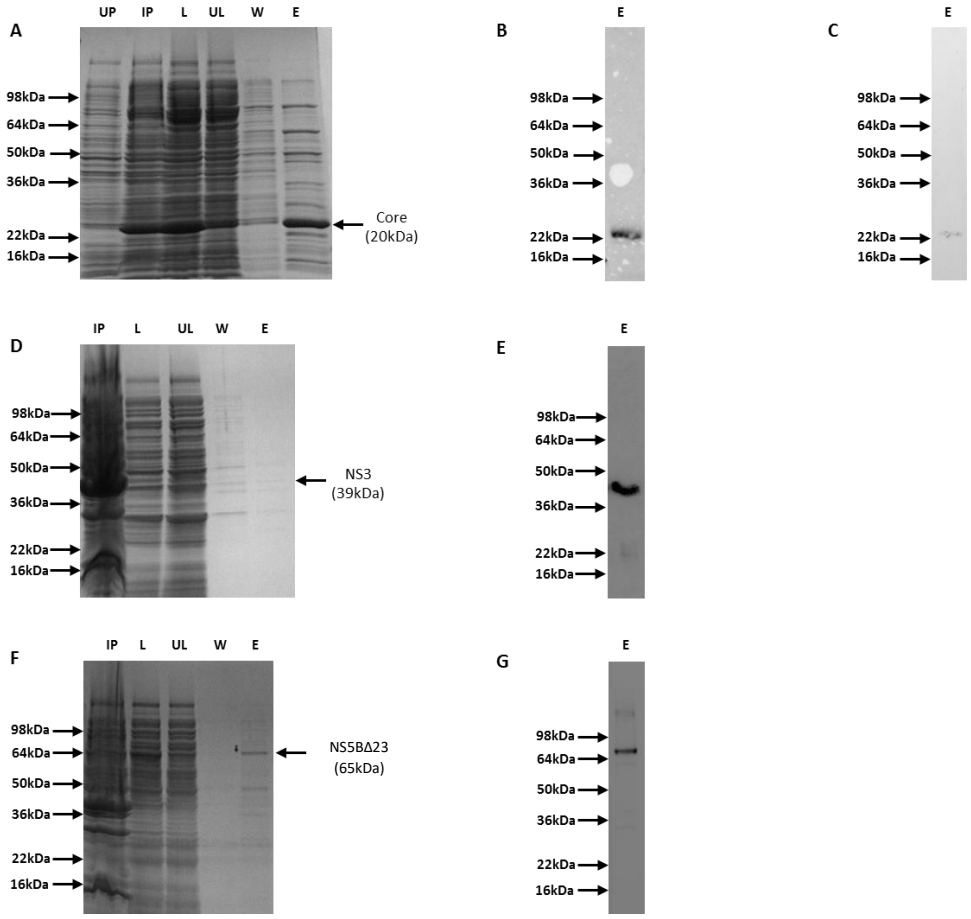


FIGURE 3-3: PRODUCTION OF SOLUBLE GBV-B PROTEINS

SDS-PAGE SHOWING INDUCTION AND PURIFICATION OF GBV-B CORE IN THE P4D5 EXPRESSION VECTOR(A), NS3 SERINE PROTEASE IN THE EXPRESSION VECTOR PET32B (D) AND NS5BΔ23 IN P4D5 (F). CORE, NS3 AND NS5BΔ23 ELUTION SAMPLES WERE THEN RUN SEPARATELY BY SDS-PAGE, TRANSFERRED TO A NITROCELLULOSE MEMBRANE AND THE MEMBRANE BLOTTED WITH ANTI-HIS ANTIBODIES (B, E, G RESPECTIVELY). THE MEMBRANE CONTAINING THE CORE ELUTION SAMPLE WAS LATER STRIPPED WITH 0.1M GLYCINE-HCL AND REPROBED WITH TAMARIN SERA (C) TO DETERMINE THE BIOLOGICAL RELEVANCE OF THE RECOMBINANT GBV-B CORE PROTEIN GENERATED. UP: NON-INDUCED PELLET, IP: INDUCED PELLET, L: LYSATE, UL: UNBOUND LYSATE, W: WASH, E: ELUATE.

3.2.4 LARGE SCALE PRODUCTION OF GBV-B PROTEINS

Once the protocols for native purification of GBV-B proteins in a prokaryotic host (see Chapter **3.1.3**) were established, a larger scale protein production was carried out at the Protein Facility at Queen Mary University of London with the following modifications to the

protocols to optimise the upscaling. Core was produced in a different bacterial strain, NiCo21, that limits contaminating bacterial proteins in the eluate from a nickel affinity column. NS3 serine protease was produced in a BL21 derivative, pLysS, and purified on a nickel affinity column with additional glycerol, CHAPS, DTT and EDTA. The bacterial strain BL21 containing the plasmid pLysS was chosen as this plasmid encodes T7 lysozyme. T7 lysozyme inhibits basal expression of proteins under the control of the T7 promoter, such as NS3 in the expression vector pET32b+. Leaky basal expression is known to slow the growth of bacteria if the target protein is toxic (Rosano and Ceccarelli, 2014). Glycerol, CHAPS and EDTA were used during the nickel affinity purification procedure to aid with protein stability, solubility and maximise protein elution whilst DTT was added to limit protein aggregation (Bornhorst and Falke, 2000; Gräslund *et al*., 2008). Identical protocols were used for production and purification of NS5BΔ23 as described in Chapter **3.2.3**. Protein samples from each stage of GBV-B protein purification were then separated by SDS-PAGE and visualised by Colloidal Blue staining (Figure 3-4).

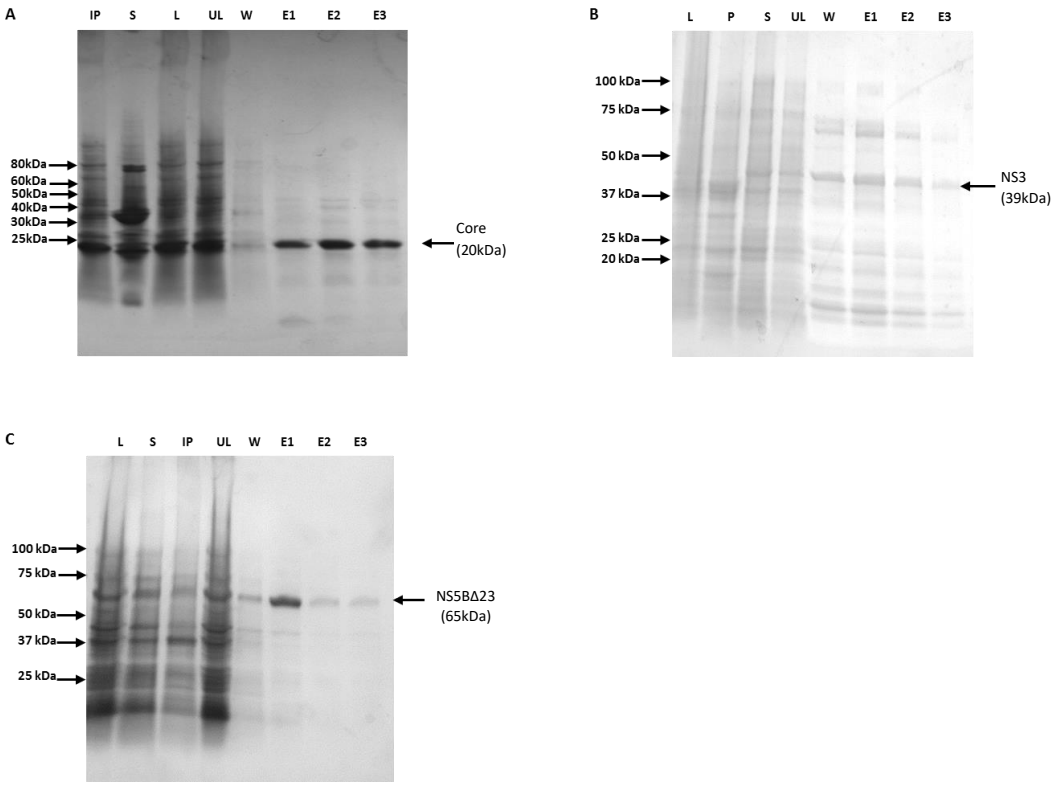


FIGURE 3-4: LARGE SCALE PRODUCTION OF GBV-B PROTEINS

SDS-PAGE SHOWING INDUCTION AND PURIFICATION PROCESS OF GBV-B CORE (A), NS3 SERINE PROTEASE (B) AND NS5BΔ23 (C) FOLLOWING NICKEL AFFINITY PURIFICATION. UP: NON-INDUCED PELLET, IP: INDUCED PELLET, S: SUPERNATANT, L: LYSATE, UL: UNBOUND LYSATE, W: WASH, E: ELUATE.

3.3 SINGLE CHAIN VARIABLE REGION LIBRARY CONSTRUCTION

3.3.1 CLONING OF VARIABLE REGIONS FROM THE HEAVY AND LIGHT CHAIN OF TAMARIN IMMUNOGLOBULIN GENES

To isolate recombinant tamarin antibodies directed against GBV-B proteins, a library of single chain variable fragments (scFv) was created by recombinant DNA technology, as previously described in (see Chapter **1.5.2**). This consists of a series of six consecutive PCRs whereby a fragment is produced that contains variable regions of tamarin immunoglobulins from both

the heavy and lights chains along with several essential elements. The basic structure is seen in Figure 3-5. A scFv consists of a variable region from the heavy chain and light chain, linked by a 20-mer $(G_4S)_4$, with the T7 promoter and Kozak sequence at the 5' end for downstream expression of the scFv and a spacer at the 3' end to allow for complete protrusion of the scFv from the ribosome. A library of up to 10^{14} recombinant tamarin scFv can be produced using this technology and be used to screen against GBV-B proteins allowing the isolation of GBV-B protein-specific antibodies.

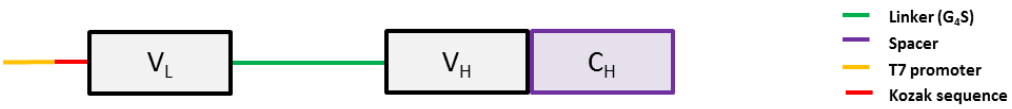


FIGURE 3-5: THE BASIC STRUCTURE OF A COMPLETE SINGLE CHAIN VARIABLE FRAGMENT

THE SPACER IN THIS DIAGRAM IS NOTATED AS C_H AS THE SPACER USED IN SCFV LIBRARY CONSTRUCTION WAS A CONSTANT REGION FROM THE HEAVY CHAIN OF A HUMAN IGG.

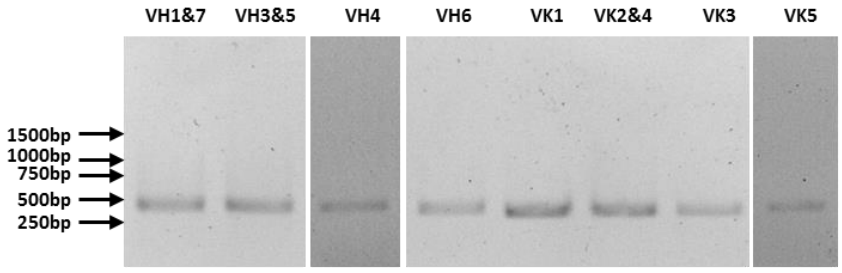
The first step of scFv library construction is the cloning of variable regions from tamarin immunoglobulin genes. Total RNA was extracted from splenocytes of a tamarin experimentally infected with GBV-B which subsequently had cleared its infection naturally, and was reverse transcribed using random primers (as described in Chapter **2.2.2** and **2.2.3**). The resulting cDNA was used as a template for the first round of PCR to clone the variable regions from both the heavy chain (V_H) and light chain (V_L) from tamarin immunoglobulin genes using degenerate primers. Degenerate primers were designed to allow complete coverage of the highly diverse families of variable regions (Matsuda *et al.*, 1998; Schäble and Zachau, 1993). However, as the tamarin genome has yet to be sequenced, primers were designed based on the closely-related marmoset equivalent (von Büdingen *et al.*, 2002). Primers were therefore designed on the characterised immunoglobulin heavy chain variable

regions (containing V gene segment from families VH1, 3, 4, 5, 6 and 7) and kappa light chain variable regions (containing V gene segments from families Vκ1-5).

These PCR products were visualised on agarose gels (Figure 3-6). Generation of PCR products for each known family was successful and were shown to be of the expected size of 400-500bp. During this initial cloning step, the primers used contain a Kozak sequence, part of the linker and part of the spacer as represented in the scheme of the design of scFv shown in (see Chapter **2.2.4** and Table 2-2) and Figure 3-5. More specifically, the Kozak sequence was added to the 5' end of the V_L region, 1 G_4S repeat to both the 3' end of the V_L region and 5' end of the V_H region and part of the spacer was added to the 3' end of the V_H region (Figure 3-6B).

Variable sequences were then cloned into the pGem T-easy vector and used to transform DH5α bacterial cells. For each V_H and V_K family of variable regions, 12 individual colonies, containing 12 unique plasmids, were cultured overnight and minipreparations of the 96 plasmids were performed using the QIAprep Spin Miniprep kit (Qiagen). Sanger sequencing was performed for each of these plasmids to confirm the identity and diversity of the isolated tamarin variable regions as no repetition of variable region sequences was identified.

A



B

FIGURE 3-6: PCR PRODUCTS OF THE TAMARIN V_H AND V_L REGIONS

A: 2% AGAROSE GEL OF TAMARIN VARIABLE REGIONS BELONGING TO DIFFERENT V_H OR V_K FAMILIES. SOME PRIMERS COVERED 2 FAMILIES OF V_H REGIONS, FOR EXAMPLE, V_H1&7, DUE TO THE SIMILARITY OF SEQUENCES AT THE BEGINNING AND END OF THE VARIABLE REGIONS.

B: DIAGRAM SHOWING ADDITIONAL ELEMENTS ADDED THROUGH PCR TO THE VARIABLE REGIONS DURING THE FIRST STAGE OF SCFV LIBRARY PRODUCTION.

3.3.2 PRODUCTION OF A TAMARIN SINGLE CHAIN IMMUNOGLOBULIN LIBRARY

After the initial cloning of the tamarin variable regions (Chapter **3.3.1**), five consecutive PCRs were performed incorporating various elements required for ribosome display. The aims of these PCRs were as follows:

1. Stage 2: addition of part of the T7 promoter at the 5' end of the V_L region, extension of the linker by addition of G_4S repeat at both the 3' of the V_L region and the 5' end of the V_H region.
2. Stage 3: overlapping PCR to join the G_4S linker of both variable regions together.
3. Stage 4: cloning of the human constant region (CH1) for use as a spacer.

4. Stage 5: addition of the spacer to the scFv constructs.
5. Stage 6: elongation of the T7 promoter and amplification of the complete scFv constructs.

In advance of stage 2 scFv production, all cloned tamarin variable regions (Chapter **3.3.1**) were electrophoresed through 2% agarose gels, the correct bands excised and purified using the Qiagen QIAquick Gel Extraction kit. These purified PCR products were then pooled into either V_H or V_L pools and used as the templates for the stage 2 PCRs for either the V_H or V_L regions, respectively. An equimolar mix of stage 2 gel purified PCR products were added as templates for the stage 3 PCR of scFv production. To confirm that the variable regions had been successfully joined together, stage 3 PCR product was cloned into the vector pGem-T easy. Twenty-four of these plasmids were used to transform DH5α *E.coli*, individual colonies were cultured overnight and a minipreparation of plasmid DNA from each colony was prepared using the Qiagen QIAprep kit. Sanger sequencing was performed on these samples. This revealed that the stage 3 scFv were in the correct conformation in 83% of samples.

In parallel, the spacer, the human constant region (CH1), was amplified. This spacer was chosen as it had been previously cloned and would allow easy identification of scFv as several commercially available antibodies exist to this region. Once cloned, this spacer was gel-purified, as described previously, and used alongside the gel-purified stage 3 PCR product as the template for stage 5 of scFv construction. To obtain an equimolar mix of both PCR products for use as the template for stage 6 of library construction, this formula was used:

$$\text{Volume of spacer to add} = \frac{10\mu l \text{ scFv} \times (\text{concentration of scFv in ng}/\mu l) \times \left(\frac{\text{Size of scFv in bp}}{\text{Size of spacer in bp}}\right)}{\text{Spacer concentration in ng}/\mu l}$$

Using gel-purified stage 5 PCR product as a template, the final stage of scFv production, stage 6 PCR, was performed, gel-purified and quantified. Further rounds of stage 6 scFv production were performed, gel-purified and pooled to ensure a final concentration of > 100ng/µl, as required for ribosome display.

The correct identity of full-length scFv was confirmed before starting ribosome display. As such, stage 6 PCR products were cloned in the vector pGem T easy. These vectors were used to transform DH5α *E.coli* and individual colonies were cultured overnight. Plasmid minipreparations from these colonies were once again prepared and the generation of full length scFv confirmed by Sanger sequencing.

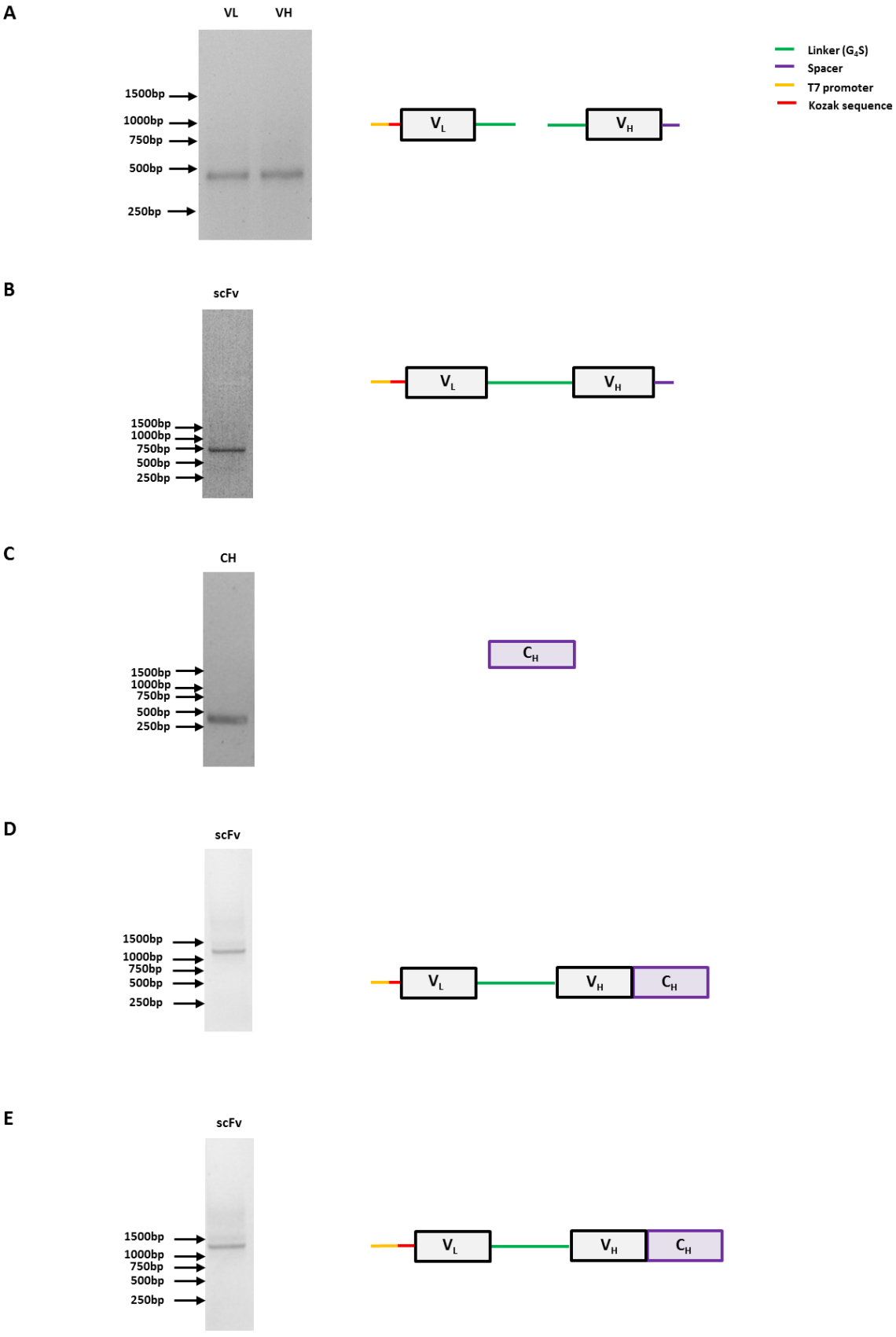


FIGURE 3-7: PCR PRODUCTS FOR EACH STAGE IN THE GENERATION OF THE SCFV LIBRARY.

All PCR products from each stage of scFv library construction were visualised on 2% agarose gels and shown to be of the correct size. The scheme for library construction is shown alongside each stage of PCR.

A: 2% agarose gel showing stage 2 PCR product. Pooled V_H and V_L regions are amplified separately to elongate the linker and add part of the T7 promoter to the 5' end of the V_L region. Each V_H and V_L are expected to be 400-500bp.

B: 2% agarose gel of stage 3 PCR product. VH and VL regions have now been linked together; PCR product size expected to be ~800bp.

C: 2% agarose gel of stage 4 PCR product. Amplification of human CH1 region. Expected size: ~300bp.

D: 2% agarose gel of stage 5 PCR product. Addition of CH1 to scFv construct. Expected size: ~1100bp.

E: 2% agarose gel of stage 6 PCR product. Elongation of T7 promoter and final amplification of library. Expected size: ~1150bp.

3.4: DISCUSSION

Studies of GBV-B pathology have been hampered by the lack of specific reagents against GBV-B such as monoclonal antibodies. Greater characterisation of the pathology of this virus would further validate the use of this animal model as a model of acute HCV infection. In this Chapter, GBV-B target proteins were generated and they will be used in the next chapter to screen a scFv library of recombinant tamarin antibodies.

Despite previous successful attempts at producing soluble GBV-B protein in the literature (Ishii *et al*., 2007; Ranjith-Kumar *et al*., 2003; Scarselli *et al*., 1997; Zhong *et al*., 1999), it was found that GBV-B proteins, produced in similar prokaryotic systems, were difficult to purify under native conditions. For large scale protein production, GBV-B core was produced in NiCo21 cells to limit the contaminating proteins from the bacteria eluted using a nickel affinity column. These cells have four proteins that commonly bind to nickel (GlmS, SlyD, ArnA and Can) deleted or tagged to aid removal of contaminating proteins. GBV-B NS3 serine protease was only produced to low levels with relatively high levels of contaminating bacterial host proteins in nickel column eluates in both small scale and large scale protein production. As NS3 was problematic to purify, additional glycerol and CHAPS to aid with protein stability and solubility, DTT to limit protein aggregation and EDTA to maximise protein elution was used during the purification procedure (Bornhorst and Falke, 2000; Gräslund *et al*., 2008). GBV-B NS5BΔ23 was successfully produced in both small scale and large scale protein production. Mass spectrometry data (Appendix, Table 7-1) determined that NS5BΔ23 had been successfully produced as a truncated version of the protein.

Although GBV-B proteins were produced in a prokaryotic system, and will therefore lack glycosylation, previous studies have confirmed that the proteins are still produced in an active, enzymatic form (Ranjith-Kumar *et al*., 2003; Scarselli *et al*., 1997). Western blots have

also confirmed that the recombinant GBV-B core is recognised by convalescent tamarin sera thereby confirming its biological relevance (Figure 3-3C, as described in Chapter **2.4.10**).

In parallel to the production of soluble GBV-B proteins, the scFv library was successfully constructed (see Chapter **2.2.4**). Degenerate primers allowed the coverage of as many potential families of immunoglobulins as possible. However, these primers were partly designed based on a study that used marmosets (von Büdingen *et al*., 2002). This study failed to amplify variable regions from the VH2 family. This could be due to a failed PCR or that the marmosets do not contain VH2 family immunoglobulins. Unfortunately, the tamarin genome has not been sequenced meaning that the scFv library construction primers were based on the primers used in von Budingen *et al*. and are therefore not thought to cover VH2 family immunoglobulins. The other possible limitation of this library is due to the separate amplification of V_H and V_L regions from tamarin splenocytes; the combinations of VH and VL regions in this scFv library will not represent what would be seen in nature. However, this is not an issue for this particular study as any recombinant antibodies isolated from ribosome display will be used directly in immunohistochemistry for diagnostic purposes.

CHAPTER 4 : ISOLATION AND CHARACTERISATION OF RECOMBINANT GBV-B-SPECIFIC ANTIBODIES

4. 1 INTRODUCTION

Monoclonal antibodies (mAbs) are a key research tool for the understanding of vaccine function through identification of epitopes that are critical for a vaccine's efficacy (such as neutralising epitopes), viral or bacterial pathogenesis by facilitating the detection and tracking of the pathogen in tissues and throughout infection, and as therapeutics (Alamares *et al*., 2005; Burton, 2002; Chow and Casadevall, 2012; Lin *et al*., 2006; Tabll *et al*., 2015; Tang *et al*., 2015). Monoclonal antibodies can be generated in different ways. Traditionally, mAbs are produced by hybridoma technology which is based on the immunisation of animals with a target antigen, followed by immortalisation of the antibody-producing cells (Köhler and Milstein, 1975). Cloning of the antibody genes led to the construction of combinatorial libraries of scFv, generated by PCR-directed fusion of the variable regions of heavy (V_H) and light (V_L) chains of antibodies to recreate the antigen-recognition domain (Hoogenboom, 2005). Isolation of recombinant antibodies from scFv libraries offers several advantages in comparison with the traditional hybridoma technology, such as a wider choice of target, the possibility to easily "humanise" the antibodies for human therapy, and the improvement of the characteristics of the antibody by affinity maturation (Bradbury *et al*., 2011; Hoogenboom, 2005).

Monoclonal antibodies targeting HCV proteins have been used successfully as serological markers in a huge range of studies, including those investigating the effect of current therapy regimens in chronic HCV infection (Kurt *et al*., 2015), the effect of novel anti-viral therapies (Zhang *et al*., 2013), HCV lipoviral formation (Boyer *et al*., 2014), HCV protein interaction (Eyre *et al*., 2014; Jones *et al*., 2011) and the identification of novel virus-host interactions

(Germain *et al*., 2014). These studies have been crucial for the characterisation of the lifecycle and pathology of HCV, along with aiding the understanding of the progression of infection and the investigation of the effectiveness of novel therapies. In the case of GBV-B, the limited availability of reagents has resulted in a lack of similar studies, despite the need for them to validate GBV-B infection in tamarins as an ideal animal model of acute HCV infection.

Antibodies against GBV-B proteins would be of high value to further the study of the tissue tropism and pathology of the virus. Two different approaches were used to generate GBV-B-specific antibodies: ribosome display technology and hybridoma technology. Initially, ribosome display was used to screen a library of scFv antibodies created from a GBV-B infected tamarin, constructed in Chapter **3.3.2**, against recombinant GBV-B core or NS5BΔ23 protein~~s~~, produced in Chapter **3.2.4**. Technical difficulties and delays in the production of specific anti GBV-B antibodies by this method prompted the use of the hybridoma technology by immunisation of mice with the GBV-B core protein, as an alternative system.

In both cases, GBV-B core-specific antibodies were produced and will have future use in immunohistochemistry for studying the localisation of GBV-B in infected tamarin tissues, which will allow the comparison of the pathology (distribution and kinetics of the viral infection) in the GBV-B animal model with literature data from HCV infection in humans. However, this is only one of the possible applications of these antibodies; future studies can now be designed to investigate the lifecycle and pathology of GBV-B, for example the role of lipids in the GBV-B life cycle, which are known to be bind to HCV core protein, or the tracking of virus in virus entry receptor studies (Benedicto *et al*., 2009; Boyer *et al*., 2014). Understanding the lifecycle of GBV-B is of particular interest as most of what is known of the GBV-B lifecycle is inferred from the knowledge of the HCV lifecycle.

4.2 RESULTS

4.2.1 ISOLATION OF SCFV ANTIBODIES AGAINST RECOMBINANT GBV-B NS5BΔ23 FROM THE SCREENING OF A GBV-B INFECTED TAMARIN-DERIVED ANTIBODY LIBRARY

The scFv antibody library, created in Chapter **3.3.2** from GBV-B infected tamarin, was probed against recombinant GBV-B NS5BΔ23 protein produced in Chapter **3.2.4**. Presence of recombinant NS5BΔ23 in the protein preparation had been confirmed by both western blot and mass spectrometry (Figure 3-3, Table 7-1, see Chapter **2.4.10** for further details on western blotting). NS5BΔ23 was chosen as target because antibodies reactive to this protein in tamarins have been reported in the literature (Martin *et al*., 2003; Sbardellati *et al*., 2001). The screening was conducted on a 96-well plate coated with 10µg of NS5BΔ23 protein. The scFv library was transcribed and translated *in vitro* using TNT T7 Quick Coupled Transcription/Translation System and pre-adsorbed in a well coated with 10ug of BSA for an hour. This step was performed to reduce the amount of non-specific antibodies within the translated library mix. The translated scFv library mix was then transferred to the well coated with GBV-B NS5BΔ23. After several washes to eliminate non-binding, non-specific scFv antibodies, antibody-ribosome-mRNA complexes were dissociated with EDTA, and the mRNA purified using RNeasy Mini kit. This protocol is described in detail in Chapter **2.2.17**.

The RNA recovered was then amplified by PCR using the OneStep RT-PCR Kit, as described in Chapter **2.2.4**. Specific bands were seen at the correct size (1100bp) confirming scFv antibodies had been recovered from the ribosome display screening (Figure 4-1). To determine if the number of amplification cycles during this PCR stage was sufficient for the efficient amplification of scFv, PCR reactions were performed in triplicate, with an increase in the number of amplification cycles. Specific bands were seen at the correct size for all PCR

samples, however, the highest number of amplification cycles (40 cycles) led to a reduced yield of amplified scFv (Figure 4-1).

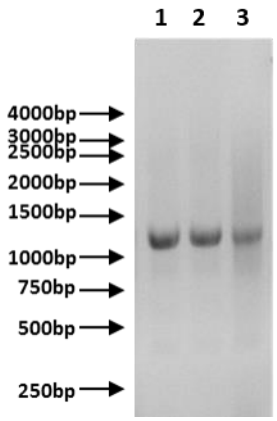


FIGURE 4-1: AMPLIFICATION OF MRNA ENCODING SCFV RECOVERED AFTER SCREENING OF A SCFV LIBRARY AGAINST GBV-B NS5BΔ23 USING RIBOSOME DISPLAY

RECOVERED MRNA FROM ONE ROUND OF SCREENING AGAINST GBV-B NS5BΔ23 WAS AMPLIFIED BY PCR IN TRIPLICATE WITH A VARYING NUMBER OF AMPLIFICATION CYCLES. PRODUCTS WERE VISUALISED ON A 1% AGAROSE GEL; THE PRODUCTS CORRESPONDING TO THE FUSION OF VH AND VL WERE DETECTED AT THE EXPECTED SIZE OF 1100BP. LANE 1: 30 CYCLES, LANE 2: 35 CYCLE, LANE 3: 40 CYCLES.

To confirm that the amplified mRNA coded for scFv sequences, the PCR product from 30 cycles (Figure 4-1, lane 1) was subcloned into a pGEM-T Easy vector for sequencing (see Chapter **2.2.12**). Plasmids derived from 96 single colonies were initially assessed by enzymatic restriction using the restriction enzymes *Nco*I and *Not*I, which lie at the 5' and 3' end of each scFv antibody (Chapter **3.3.2**). Of these 96 plasmid preparations, only 20 contained an insert of the correct size of 1100bp, corresponding to the V_H region fused to the V_L region.

These 20 plasmids were then sequenced by the Sanger method. Consensus sequences obtained from two reactions using a forward and reverse primer were processed using

DNADynamo software. These consensus sequences were then analysed by comparison to a database of human antibody (Ig BLAST, Table 4-1). Whilst 11 samples (55% of sequenced samples, 11% of all prepared samples) were shown to represent antibody sequences, only one scFv (5% of sequenced samples, 1% of all prepared samples) contained a full-length sequence corresponding to a complete full-length linker fusing the V_L to the V_H region. Linker length plays a role in the association of the two variable regions in scFv antibodies and as a result, the minimum linker length used is generally 15 amino acids (Huston *et al*., 1988; Schaefer *et al*., 2010). For this reason, the specific binding properties of only two scFv were investigated- one scFv with a full linker, NS5BΔ23.11, and one with a truncated linker, NS5BΔ23.12.

scFv		Top V match	Top D match	Top J match	Number of G_4S repeats	CDR3
NS5BΔ23.1	VH	IGHV1-8*02, IGHV1-46*03, IGHV1-46*01	IGHD1-7*01	IGHJ4*03, IGHJ4*02	1	ARSHSWNYRPFDY
	VK	IGKV1-27*01	-	IGKJ1*01		QKYNSVPWT
NS5BΔ23.4	VH	IGHV1-8*01, IGHV1-8*02	IGHD1-26*01	IGHJ4*02	2	ARVVGAHYYFEY
	VK	IGKV1-27*01	-	IGKJ1*01		QQYYSTWT
NS5BΔ23.5	VH	IGHV1-46*01	IGHD3-3*02, IGHD2-15*01, IGHD3-10*02	IGHJ2*01	1	ARGYVSYYWYFDV
	VK	IGKV1-39*02	-	IGKJ1*01		QYGYRTPYT
NS5BΔ23.6	VH	IGHV1-8*02	IGHD5-12*01	IGHJ2*01	1	ARSGYVRFFDV
	VK	IGKV3D-15*01	-	IGKJ2*02, IGKJ2*01		QQYNDRPTLT
NS5BΔ23.7	VH	IGHV4-38-2*02	IGHD6-13*01	IGHJ4*03, IGHJ4*02	1	ARWGIRSSWYTIQY
	VK	IGKV3D-15*01	-	IGKJ4*01		QQYYSTPLT
NS5BΔ23.8	VH	IGHV3-48*03, IGHV3-48*04	IGHD4-23*01, IGHD4-17*01	IGHJ4*03, IGHJ4*02	2	ARIAYGTYPPFEY
	VK	IGKV6D-41*01	-	IGKJ1*01		QQTNYWPLA
NS5BΔ23.9	VH	IGHV1-46*01	IGHD2-15*01, IGHD5-12*01, IGHD4-23*01	IGHJ4*03	2	ARDRFSWHYEGYFEF
	VK	IGKV3D-15*01	-	IGKJ1*01		QQYNNRRT
NS5BΔ23.10	VH	IGHV3-23D*02, IGHV3-23*04	IGHD4-11*01, IGHD1-7*01, IGHD4-4*01	IGHJ2*01	2	ARGGNYLRVWYFDV
	VK	IGKV1-27*01	-	IGKJ2*02, IGKJ2*01, IGKJ3*01		QHGYSSLT
NS5BΔ23.11	VH	IGHV5-10-1*01	IGHD3-3*02, IGHD3-16*02, IGHD3-3*01	IGHJ4*03, IGHJ4*02	4	AKVGGLEYFEY
	VK	IGKV1-NL1*01	-	IGKJ1*01		QHGYSTPYA
NS5BΔ23.12	VH	IGHV1-8*02, IGHV1-46*01	IGHD7-27*01, IGHD1-1*01	IGHJ3*02	2	ARVFSNWADAFDI
	VK	IGKV6-21*02	-	IGKJ2*02, IGKJ2*01		QQSNNWPLT
NS5BΔ23.20	VH	IGHV4-38-2*01	IGHD2-8*01	IGHJ3*02	2	LRWHCTSSHCSPSDALDI
	VK	IGKV1-27*01	-	IGKJ2*02, IGKJ2*01		QQYYSTPLT

TABLE 4-1: CHARACTERISATION OF SCFV ANTIBODIES RECOVERED AFTER THE SCREENING OF THE SCFV LIBRARY AGAINST GBV-B NS5BΔ23

PLASMIDS CONTAINING POTENTIAL SCFV WERE ANALYSED BY SANGER SEQUENCING. CONSENSUS SEQUENCES WERE CREATED FROM REACTIONS WITH A FORWARD AND REVERSE PRIMER AND THEN ANALYSED USING THE BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST).

4.2.2 One round of screening of the GBV-B infected tamarin-derived antibody library against GBV-B NS5BΔ23 did not produce anti-GBV-B NS5BΔ23-specific scFv antibodies

The two scFv (NS5BΔ23.11 and NS5BΔ23.12) that were isolated from the screening of a GBV-B infected tamarin-derived antibody library against recombinant GBV-B NS5BΔ23 were expressed in a prokaryotic system to allow the investigation of their binding properties.

Antibody sequences were subcloned into the prokaryotic expression vector, p4D5_Fc plasmid, using restriction enzymes *Nco*I and *Not*I, as previously described in Markiv *et al*., 2011 (Figure 4-2). The vector p4D5 had been modified from pET32b+ through the removal of all N-terminal tag sequences, retaining only the C-terminal 6x His-tag in frame with the restriction site *Xho*I, and by the addition of human C_H2/C_H3 regions inserted into the vector using *NotI* and *XhoI* (Markiv *et al*., 2011). ScFv antibodies were designed to have a *Nco*I site at their 5' end and a *Not*I site at the 3' end, which allows the subcloning of scFv antibodies into this plasmid, creating a scFv-C_H2/C_H3/His-tag construct. Human C_H2 and C_H3 regions from IgG, otherwise known as the Fc domain are also in frame after NotI site allowing the fusion to the scFv antibody; this endows the scFv antibody with several advantages including an increase in the solubility and stability, and the use of the Fc domain as a tag for identification and purification of the scFv antibody in downstream applications such as immunohistochemistry (Czajkowsky *et al*., 2012; Liu, 2003; Moutel *et al*., 2009). Sanger sequencing confirmed the correct identity of scFv sequences within the p4D5_Fc vector.

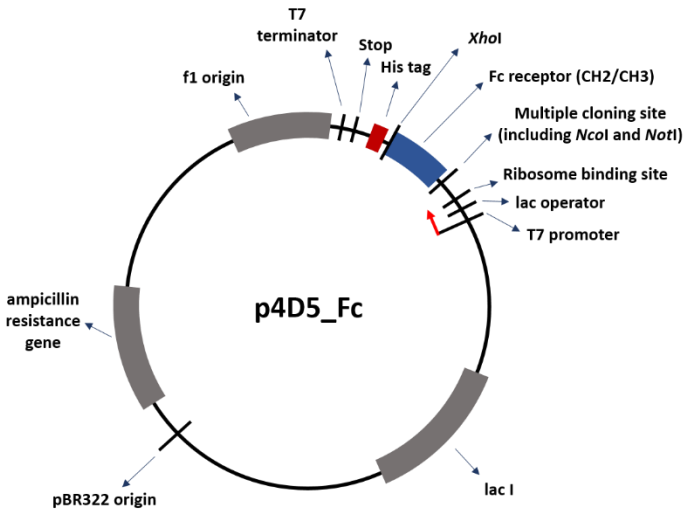


FIGURE 4-2: SCHEMATIC REPRESENTATION OF THE VECTOR USED FOR THE EXPRESSION OF THE SCFV IN A PROKARYOTIC HOST (ADAPTED FROM MARKIV *ET AL.*, 2011).

NS5BΔ23.11 and NS5BΔ23.12 scFv antibody expression was induced in BL21 bacterial cells using IPTG (see Chapter **2.3.1**). BL21 bacterial cells were chosen as they are routinely used for recombinant protein expression under the T7 promoter. Samples pre- and post-induction were analysed by SDS-PAGE and Colloidal Blue staining (Figure 4-3). Both NS5BΔ23.11 and NS5BΔ23.12 scFv antibodies were expressed at a relatively high level in these bacterial cells.

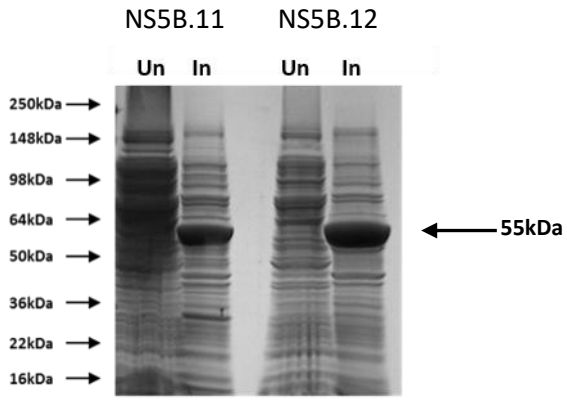


FIGURE 4-3: EXPRESSION OF THE SCFV RECOVERED AFTER PANNING AGAINST NS5BΔ23 IN A PROKARYOTIC SYSTEM

NS5BΔ23.11 AND NS5BΔ23.12 SCFV ANTIBODIES WERE PRODUCED IN BL21 CELLS AND THE BACTERIAL CELL LYSATES WERE RUN THROUGH A 4-12% SDS-PAGE AND STAINED WITH COLLOIDAL BLUE DYE. UN: NON-INDUCED, IN: INDUCED. EXPECTED SIZE: 55KDA.

The binding specificity of the isolated NS5BΔ23.11 and NS5BΔ23.12 scFv antibodies was then explored using an enzyme-linked immunosorbent assay (ELISA, as described in Chapter **2.3.7**). The assay was performed on a 96-well plate coated with either 1µg/ml of recombinant GBV-B NS5BΔ23 protein or bacterial cell lysate from untransformed BL21. Unpurified supernatant from induced BL21 containing scFv NS5BΔ23.11 and NS5BΔ23.12 were applied to the plate and detected with an human IgG HRP-conjugated antibody, which recognises the Fc domain. Neither of the two scFv antibodies isolated bind specifically to recombinant GBV-B NS5BΔ23 protein as the absorbance values for each scFv antibody were similar for both the NS5BΔ23-coated and BL21 cell lysate-coated plate (Figure 4-4).

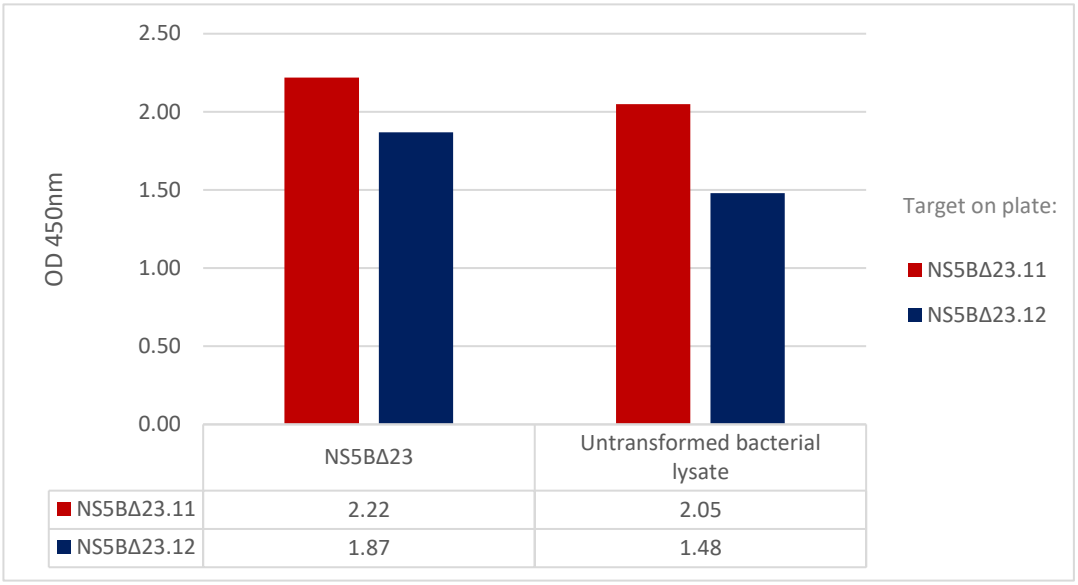


FIGURE 4-4: SCFV ANTIBODIES ISOLATED FROM SCREENING THE SCFV LIBRARY AGAINST NS5BΔ23 DURING RIBOSOME DISPLAY DO NOT SPECIFICALLY BIND NS5BΔ23.

BACTERIAL CELL LYSATE CONTAINING TWO UNIQUE SCFV ANTIBODIES WERE USED IN AN ELISA TO ASSESS THEIR BINDING SPECIFICITIES. PLATES WERE COATED WITH GBV-B NS5BΔ23 OR UNTRANSFORMED BL21 CELL LYSATE. THE MEAN ABSORBANCE OF TWO REPLICATES, NORMALISED TO BLANK WELLS, WERE PLOTTED. BELOW THE GRAPH ARE THE OD VALUES.

4.2.3 ISOLATION OF SCFV ANTIBODIES AGAINST GBV-B CORE FROM THE SCREENING OF A GBV-B INFECTED TAMARIN-DERIVED ANTIBODY LIBRARY

As the isolated anti-GBV-B NS5BΔ23 scFv antibodies did not specifically bind to the target protein, the original scFv library was also used to screen against a different recombinant GBV-B viral protein, the nucleocapsid protein, Core, produced in Chapter **3.2.4**. GBV-B core was chosen as the target protein as it is the nucleocapsid of the virus and therefore likely to be a prominent target for antibodies. Indeed, GBV-B core-specific antibodies have previously been more readily identified than GBV-B NS5BΔ23-specific antibodies *in vivo* (Bukh *et al*., 2008; Ishii *et al*., 2007; Iwasaki *et al*., 2011; Pilot-Matias *et al*., 1996; Sbardellati *et al*., 2001;

Woollard *et al*., 2008). Additionally, tamarin antibodies targeting GBV-B core were previously identified by western blot in Figure 3-3 in Chapter **3.2.3**.

Similar to the panning against GBV-B NS5BΔ23 (Chapter **4.2.1**), the library screening was performed on a 96-well plate coated with 10µg of recombinant GBV-B core. After *in vitro* transcription and translation of the scFv library using the TNT T7 Quick Coupled Transcription/Translation System, the scFv library was adsorbed on a BSA-coated well before being transferred to the well coated with GBV-B core. After several washes ARM complexes were then dissociated with EDTA and the mRNA purified using RNeasy Mini kit.

The RNA recovered was then amplified by PCR using the OneStep RT-PCR Kit. The presence of recovered scFv antibody sequences was confirmed by gel electrophoresis (Figure 4-5).

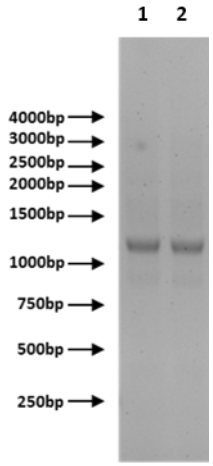


FIGURE 4-5: AMPLIFICATION OF THE MRNA RECOVERED FROM THE SCREENING OF SCFV LIBRARY AGAINST GBV-B CORE

MRNA PURIFIED FROM THE FIRST ROUND OF PANNING AGAINST GBV-B CORE WAS AMPLIFIED BY 30 CYCLES OF RT-PCR. MRNA SAMPLES WERE RUN IN DUPLICATE (LANE 1 AND 2) AND VISUALISED ON A 1% AGAROSE GEL. EXPECTED SIZE OF PRODUCT: ~1100BP.

The identity of recovered scFv sequences was then investigated using Sanger sequencing after subcloning into a pGEM-T easy plasmid. From 72 cultures, plasmid preparations were analysed by enzymatic restriction using *Nco*I and *Not*I enzymes (see Chapter **2.2.11**): 29 cultures contained pGEM T-easy plasmids encoding an insert of the correct size, 1100bp.

The identity of these 29 scFv antibodies was investigated using Sanger sequencing and then analysed using Ig BLAST. Only six scFv antibodies (21% of sequenced samples, 8% of all prepared samples) were confirmed to represent antibody sequences that were in frame and had unique sequences (Table 4-2A). A further five scFv antibodies (17%) were also confirmed to represent antibody sequences; however, these contained early stop codons and shorter linkers (Table 4-1B). One of these scFv antibodies (Core.5 in Table 4-2A) was chosen as a negative control for downstream expression and characterisation of scFv antibody. These seven scFv antibodies (Core.1- Core.7 in Table 4-2A) were then expressed to investigate their binding property towards GBV-B core specifically.

A

ScFv		Top V gene match	Top D gene match	Top J gene match	Number of G_4S repeats	CDR3
Core.1	VH	IGHV4-38-2*01, IGHV4-38-2*02	IGHD1-26*01	IGHJ2*01	4	ARSGSGSFYWYFDV
	VK	IGKV3D-7*01	-	IGKJ1*01		QHGSHTPPWT
Core.2	VH	IGHV1-8*01, IGHV1-8*02	IGHD1-26*01	IGHJ4*03, IGHJ4*02	4	ARPIVGTLAFEY
	VK	IGKV1-27*01	-	IGKJ2*01		QQYYSTPYT
Core.3	VH	IGHV1-8*02, IGHV1-46*01	IGHD4-23*01, IGHD4-17*01	IGHJ4*03, IGHJ4*02	4	ANTYGNLFEY
	VK	IGKV1-27*01	-	IGKJ1*01		QQCYSTPPT
Core.4	VH	IGHV3-48*03	IGHD5-12*01	IGHJ6*02	4	ARDGGGYYGLDL
	VK	IGKV3D-15*01	-	IGKJ3*01		QQYYSTPFI
Core.5	VH	IGHV4-38-2*01	IGHD1-7*01, IGHD1-20*01, IGHD1-1*01	IGHJ4*03	2	ARSNTWKRDAFGF
	VK	IGKV1-27*01	-	IGKJ2*02, IGKJ2*01		QQYYSTPLT
Core.6	VH	IGHV1-8*01, IGHV1-8*02	IGHD3-10*02, IGHD3-10*01	IGHJ2*01	4	ARELFGTVVDWYFDV
	VK	IGKV1-27*01	-	IGKJ1*01		QHGYSTPYT
Core.7	VH	IGHV1-8*01, IGHV1-8*02	IGHD3-16*02, IGHD3-16*01	IGHJ4*03, IGHJ4*02	4	ARYDTVGVEWRYDY
	VK	IGKV1-27*01	-	IGKJ2*04, IGKJ2*03, IGKJ2*02		QQYYSTPFI

B

ScFv		Top V gene match	Top D gene match	Top J gene match	Number of G_4S repeats	CDR3
Core.8	VH	IGHV1-46*01	IGHD5-24*01	IGHJ4*03, IGHJ5*02, IGHJ4*02	1	AEADGLT
	VK	IGKV1-27*01	-	IGKJ4*02		QHGHTIPWT
Core.9	VH	IGHV1-8*02	IGHD1-26*01	IGHJ4*03, IGHJ4*02	2	ARGQVGAIDY
	VK	IGKV6D-41*01	-	IGKJ1*01		QQSNNWASD
Core.10	VH	IGHV4-38-2*01	IGHD3-10*01	IGHJ4*03, IGHJ4*02	2	ARYLHRHYYGTNSYFEY
	VK	IGKV1-27*01	-	IGKJ4*02		QHGRTIPWT
Core11	VH	IGHV1-46*03, IGHV1-46*01	IGHD2-8*02	IGHJ3*02	2	ARFFLILAAFDI
	VK	IGKV1-NL1*01	-	IGKJ2*02, IGKJ2*01		QHGYSSPLT
Core.12	VH	IGHV4-38-2*01	IGHD1-26*01, IGHD1-7*01, IGHD5-12*01	IGHJ3*02, IGHJ3*01	2	ARSENSWKSVDAFDL
	VK	IGKV3D-15*01	-	IGKJ1*01		HHYYSTPWT

TABLE 4-2: CHARACTERISATION OF RECOVERED SCFV AFTER ONE ROUND OF RIBOSOME DISPLAY PANNING AGAINST GBV-B CORE PROTEIN

ISOLATED SCFV ANTIBODIES WERE SEQUENCED BY SANGER SEQUENCING AND ANALYSED USING THE IMMUNOGLOBULIN BASIC LOCAL ALIGNMENT SEARCH TOOL (IG BLAST) TO FIND THE CLOSEST GENE SEGMENT MATCHES FOR EACH SCFV. IN-FRAME SCFV ARE INCLUDED IN TABLE 4-1A WHILST OUT-OF-FRAME SCFV WERE INCLUDED IN TABLE 4-1B.

4.2.4 LACK OF SPECIFIC ANTI-GBV-B CORE SCFV ANTIBODIES AFTER ONE ROUND OF SCREENING OF THE GBV-B INFECTED TAMARIN-DERIVED ANTIBODY LIBRARY

The six in-frame scFv antibodies, and the negative control scFv (Core.5) that was out of frame due to an early stop codon in the V_L region, isolated from one round of panning of the GBV-B-infected tamarin-derived antibody library against recombinant GBV-B core, were produced in a prokaryotic system and tested for GBV-B core binding specificity. To permit this, scFv were cloned into the p4D5_Fc vector using *Nco*I and *Not*I restriction enzymes.

Production of eukaryotic proteins in a bacterial host can potentially impact their solubility and functionality as proteins can incorrectly fold in a prokaryotic system (Arbabi-Ghahroudi *et al*., 2005; Vendel *et al*., 2012). For this reason, different prokaryotic hosts were investigated for their scFv antibody production. One of the scFv antibodies, p4D5_Core.3_Fc was arbitrarily chosen to setup the conditions for the optimal production of the scFv. Three bacterial strains were compared: BL21, SHuffle T7 Express and Rosetta-Gami. Whilst BL21 is routinely used for recombinant protein expression under the T7 promoter, SHuffle T7 express and Rosetta-Gami strains both have additional advantages for scFv antibody expression. Shuffle T7 Express cells have enhanced disulphide bond formation via the expression of the isomerase DsbC. The Rosetta-Gami cells also have improved disulphide bond formation through the mutation of two reductase genes, but also a better expression of eukaryotic proteins due to the expression of rare tRNAs in bacteria. Antibody expression in prokaryotic cells was induced using IPTG and samples of bacterial cultures taken before and after induction were analysed by SDS-PAGE and subsequent Colloidal Blue staining. Although the

expression levels were very low with all of the bacterial strains, the BL21 strain appears to be the best suited to scFv antibody expression of the three strains investigated (Figure 4-6A).

The remaining six scFv antibodies were expressed in BL21 bacterial cells alongside a positive control, the scFv 4D5-8RFP (Markiv *et al*., 2011), kindly donated by Dr. A. Kang. This scFv contains a red fluorescent protein (RFP) gene between the V_H and V_L sequence thereby providing a rapid, visual confirmation of protein expression in bacteria. After IPTG induction, antibody expression for each scFv protein was analysed by SDS-PAGE. Unfortunately, Core.1-7 scFv antibody expression could not be confirmed as no clear induced band was visible for any of the scFv antibodies. This is most likely due to low protein expression levels rather than the scFv not being induced; indeed, scFv 4D5-8RFP could not be seen by SDS-PAGE, but the antibody was induced as indicated by the pink colour of the pelleted, induced bacteria culture, which could be seen with naked eye (Figure 4-6B-C).

To determine whether the expression of the scFv antibodies was lower than the detection limit of the Colloidal blue staining, bacterial cell lysates were analysed in a western blot with an anti-His tag antibody. Core.2, 3, 4, 6, 7 scFv antibodies and scFv 4D5-8RFP had been successfully induced in BL21 cells, whilst the Core.5 scFv antibody had not been expressed as expected due to the early stop codon in the scFv antibody sequence. The Core.1 scFv antibody was also unexpectedly not expressed (Figure 4-6D).

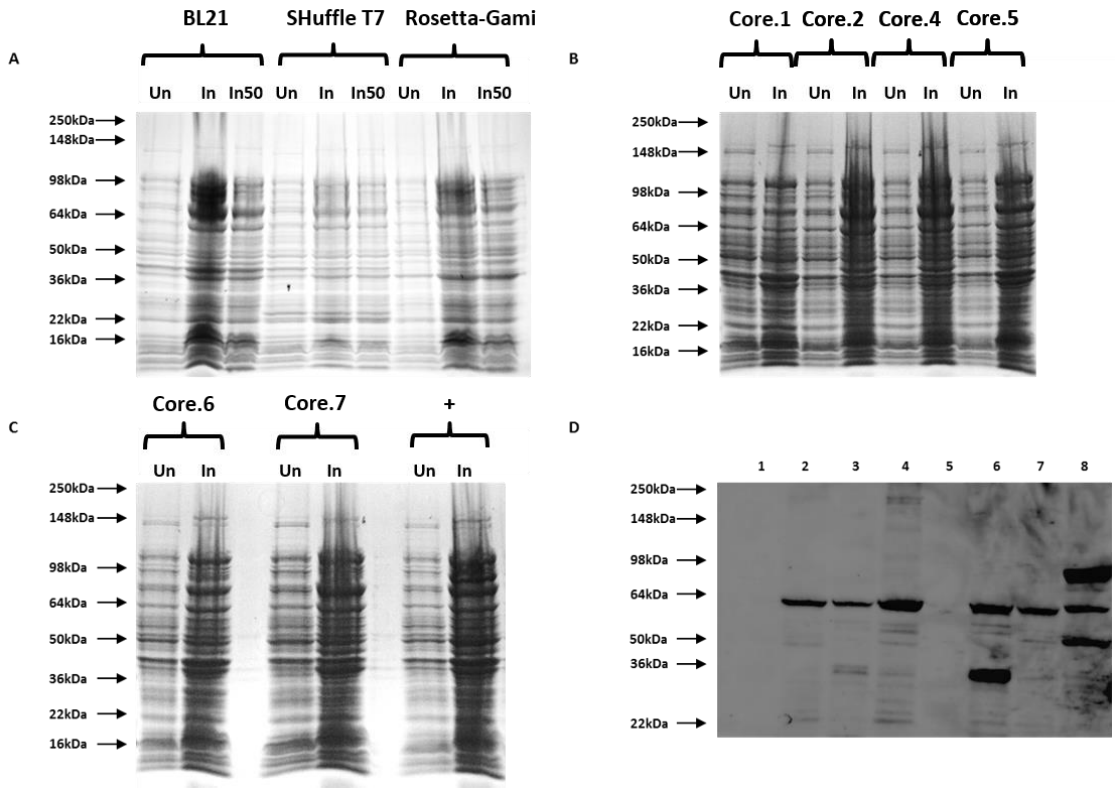


FIGURE 4-6: EXPRESSION OF THE ISOLATED ANTI-CORE SCFV ANTIBODIES IN BACTERIAL CELLS

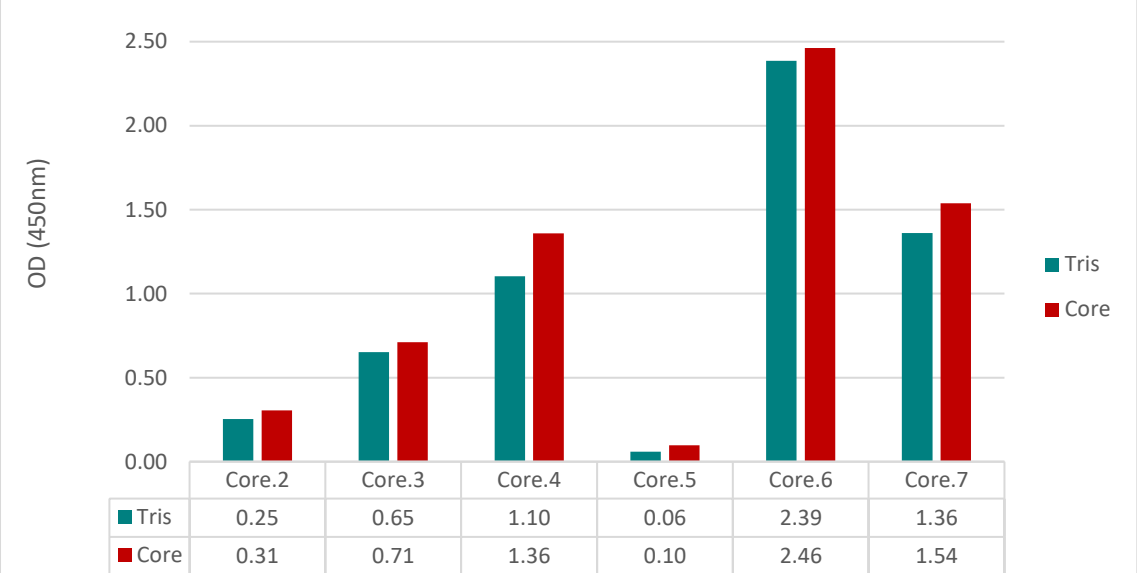
CELL LYSATES FROM BACTERIAL CULTURES BEFORE AND AFTER IPTG INDUCTION WERE PROCESSED IN A 4-12% SDS-PAGE AND PROTEINS VISUALISED BY COLLOIDAL BLUE STAINING. (A) CORE.3 SCFV ANTIBODIES WERE EXPRESSED IN DIFFERENT BACTERIAL CELLS TO DETERMINE THE MOST SUITABLE STRAIN. (B) CORE.1, 2, 4 AND 5 SCFV ANTIBODIES AND (C) CORE.6 AND 7 SCFV ANTIBODIES ALONGSIDE THE POSITIVE CONTROL PROTEIN WERE PRODUCED IN BL21 BACTERIAL CELLS. (D) THE LYSATE FROM ALL INDUCED BACTERIAL CELL CULTURES WERE RUN THROUGH AN SDS-PAGE, TRANSFERRED TO A NITROCELLULOSE MEMBRANE AND IMMUNOBLOTTED WITH AN ANTI-HIS ANTIBODY. LANE 1-7: CORE.1-CORE.7, LANE 8: POSITIVE EXPRESSION CONTROL.

THE ESTIMATED SIZE OF SCFV ANTIBODY-FC FUSION PROTEINS BASED ON THEIR NUCLEOTIDE SEQUENCES WAS 55KDA AND 76 KDA FOR THE POSITIVE CONTROL, USING THE SOFTWARE DNADYNAMO (BLUETRACTORSOFTWARE, UK).

UN: INDUCED; IN: IPTG INDUCED; IN50: INDUCED SAMPLES DILUTED BY 50% TO ENSURE A CLEAR SCFV ANTIBODY STAINING.

The binding specificity of the bacterial cell lysates containing scFv antibodies was then explored by ELISA: 96-well plates were coated overnight with 1µg/ml of recombinant GBV-B core or coating buffer alone. ScFv antibodies, Core.2, Core.3, Core.4, Core.6 and Core.7, along with the negative control supernatant that contains no expressed recombinant protein

(Core.5), were added to the plate and detected with an anti-His HRP-conjugated antibody. No specific binding to GBV-B core was observed (Figure 4-7).



	Core.2	Core.3	Core.4	Core.5	Core.6	Core.7
Tris	0.25	0.65	1.10	0.06	2.39	1.36
Core	0.31	0.71	1.36	0.10	2.46	1.54

FIGURE 4-7: LACK OF SPECIFIC BINDING TO GBV-B CORE OF SCFV ANTIBODIES ISOLATED FROM RIBOSOME DISPLAY

SUPERNATANT CONTAINING FIVE UNIQUE SCFV ANTIBODIES WERE RUN IN AN ELISA ALONGSIDE THE NEGATIVE CONTROL UNEXPRESSED CORE.5 SCFV ANTIBODY. PLATES WERE COATED WITH EITHER GBV-B CORE (RED) OR WITH JUST THE COATING BUFFER TRIS (BLUE). RESULTS REPRESENT THE MEAN ABSORBANCE VALUE OF TWO REPLICATES, NORMALISED TO BLANK WELLS. THE OD VALUES ARE PROVIDED BELOW THE CHART.

4.2.5 EXPRESSION OF SCFV ANTIBODIES IN A MAMMALIAN CELL LINE DID NOT RESCUE SPECIFIC BINDING OF THE SCFV TO GBV-B CORE PROTEIN

Whilst the binding of scFv antibodies, Core.2-Core.7, were non-specific if expressed in a prokaryotic host, this may be due to improper protein folding in the prokaryotic cell and a difference in codon usage (Arbabi-Ghahroudi *et al.*, 2005). It has been previously documented that bacterial scFv antibody expression can lead to the improper folding of scFv antibodies which impacts their functionality and specificity (Vendel *et al.*, 2012). ScFv were therefore expressed in a mammalian cell line to determine if their lack of specific binding

properties was due to improper protein folding.

All seven scFv antibodies were subcloned into the eukaryotic expression plasmid pSF_CMV_NH2_InsulinSP1 (pSF) using the restriction enzymes *Nco*I and *Xho*I (Figure 4-8). These enzymes were used as this allowed the transfer of both the scFv antibody gene and the Fc receptor. This plasmid adds a secretory signal peptide to the N-terminus of the protein of choice allowing scFv secretion into the cell culture supernatant thereby simplifying scFv purification. The vector had been previously modified through the addition of annealed oligonucleotides to encode a 6x His tag and stop codon at the C-terminus of the protein of choice for downstream identification and purification. Sanger sequencing confirmed that most of the scFv had been successfully subcloned into the pSF plasmid, however, the negative control Core.5 scFv subcloning was not successful and therefore excluded from the study.

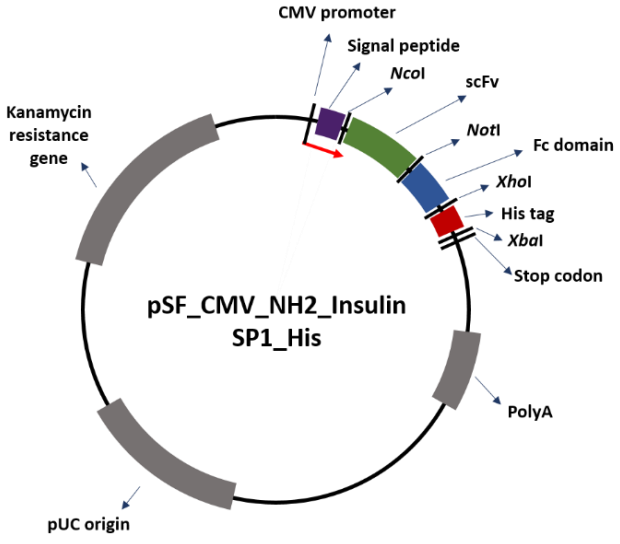


FIGURE 4-8: SCHEMATIC REPRESENTATION OF THE VECTOR USED FOR THE EXPRESSION OF THE SCFV IN A EUKARYOTIC HOST

To express scFv in mammalian cells, pSF_scFv_Fc plasmids were transfected into HEK 293T cells using the transfection reagent XtremeGeneHP (as described in Chapter **2.4.5**). As a negative control, 293T cells were treated with the XtremeGeneHP alone. Cell culture supernatant was collected, purified and concentrated using a Ni-NTA Spin Kit (as described in Chapter **2.3.4**). To confirm that scFv antibodies were indeed present in the purified, concentrated supernatant, supernatant was analysed by western blot with an anti-His antibody. This western blot confirmed that scFv antibodies had been expressed, albeit at a very low level (Figure 4-9).

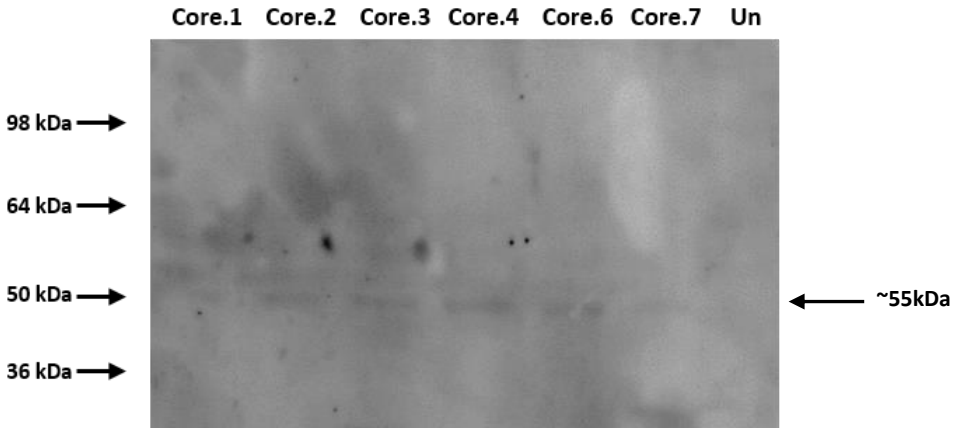


FIGURE 4-9: SCFV-FC PROTEINS, ISOLATED FROM RIBOSOME DISPLAY, CAN BE EXPRESSED AND SECRETED FROM HEK 293T CELLS

SCFV-FC-TRANSFECTED HEK 293T CELL CULTURE SUPERNATANT WAS RUN ON A 4-12% SDS-PAGE, THE PROTEINS TRANSFERRED TO A NITROCELLULOSE MEMBRANE AND DETECTED WITH AN ANTI-HIS TAG ANTIBODY. UN: UNTRANSFECTED 293T CELL SUPERNATANT. EXPECTED SIZE OF SCFV: 55KDA.

Cell culture supernatant was then tested by ELISA to confirm the binding properties of the scFv. A 96-well plate was coated overnight with 1µg/ml of either recombinant GBV-B core protein or recombinant His-tagged HIV-1 p24 protein as a negative control (Centre for AIDS reagents, NIBSC). Cell culture supernatant containing the six scFv or the negative control cell culture supernatant was added and detected with both an anti-His tag or an anti-human IgG HRP-conjugated antibody. In both cases, no binding of scFv to either GBV-B core or the His-tagged p24 was seen (OD values <0.1).

4.2.7 A SMALLER PROPORTION OF SCFV ANTIBODIES ARE ISOLATED AFTER THREE ROUNDS OF PANNING AGAINST GBV-B CORE

As non-specific scFv antibodies have been isolated from one round of screening of the GBV-B infected tamarin-derived antibody library against GBV-B antigens, ribosome display was repeated with three rounds of screening, as it is well documented that antigen-specific scFv antibody populations within scFv libraries can be enriched by a factor of 100-1000 after each round of ribosome display (J Hanes *et al*., 1998; Hanes and Plückthun, 1997).

To further screen the scFv antibody library, the recovered cDNA amplified in Chapter **4.2.3** was elongated to reconstitute the 3' end of the spacer of the scFv antibodies, which was lost in the ARM complex attached to the ribosome. After the elongation of the scFv by PCR, the scFv library is then purified and used as the input library for another round of screening against recombinant GBV-B core protein using ribosome display.

For each round of screening against recombinant GBV-B core protein, the intensity of the band representing amplified, recovered scFv sequences decreased and non-specific bands were also present suggesting either a poor amplification of recovered mRNA or a decrease in the input mRNA through the loss of non-specific scFv sequences (Figure 4-10A, B and C). To increase the efficiency of the mRNA amplification of the recovered scFv library, input mRNA was diluted 50 times which gave a stronger signal (Figure 4-10D, lane 3-4). To further optimise the recovery PCRs, a range of annealing temperatures were also evaluated (Figure 4-10E), showing that an annealing temperature of 62°C produced the most intense band, although without increase in specificity (Figure 4-10E, lane 4).

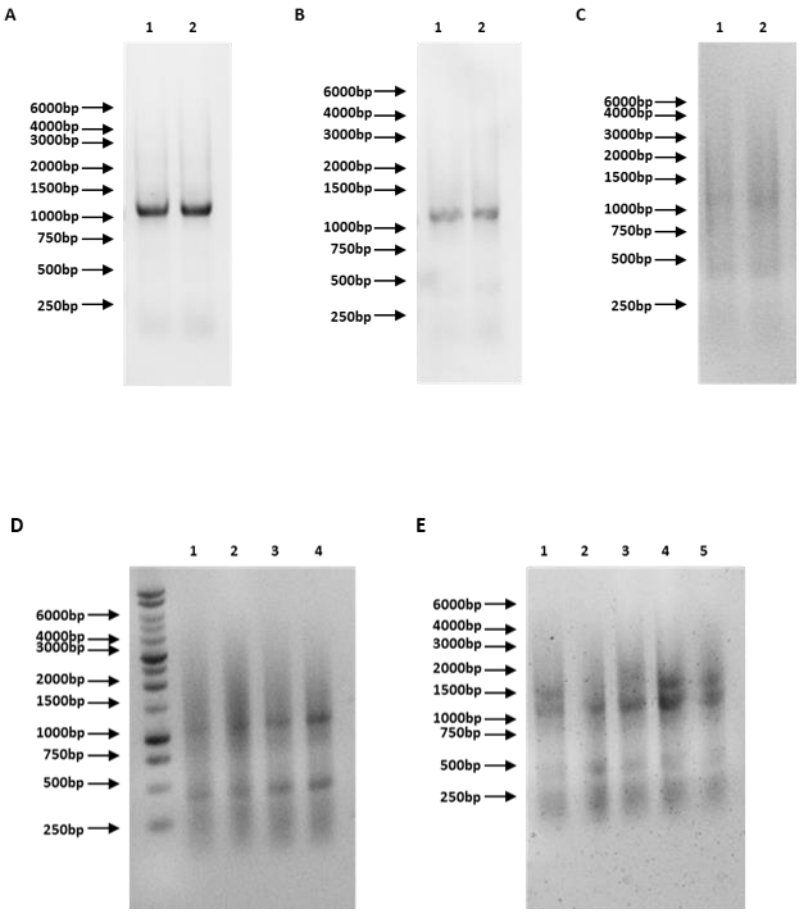


FIGURE 4-10: DIFFERENT EFFICIENCIES OF AMPLIFICATION OF RECOVERED MRNA AFTER 3 ROUNDS OF SCREENING OF GBV-B ANTIBODY LIBRARY AGAINST GBV-B CORE.

MRNA WAS RECOVERED FROM THE SCREENING OF THE SCFV ANTIBODY LIBRARY AGAINST GBV-B CORE AND AMPLIFIED USING A ONESTEP RT-PCR KIT. THESE TRUNCATED PCR PRODUCTS, REPRESENTING SCFV ANTIBODY GENES, WERE VISUALISED ON A 1% AGAROSE GEL. PCR PRODUCTS, CONSISTING OF RECOVERED SCFV AFTER ONE ROUND (A), TWO ROUNDS (B) AND THREE ROUNDS (C), OF PANNING. ALL PCR REACTIONS WERE RUN IN DUPLICATE (LANES 1 AND 2 FOR EACH GEL, RESPECTIVELY). D) DUPLICATES OF RT-PCRS WERE PERFORMED WITH EITHER NEAT, RECOVERED MRNA (LANES 1 AND 2) OR A 1:50 DILUTION OF MRNA (LANES 3 AND 4). E) USING A 1:50 DILUTION OF INPUT MRNA, THE RT-PCR WAS CONDUCTED WITH A RANGE OF DIFFERENT ANNEALING TEMPERATURES: 54°C, 58°C, 60°C, 62°C AND 64°C (LANES 1-5 RESPECTIVELY).

The PCR product from the third round of screening against GBV-B core was subcloned into the pGEM T-easy vector for downstream sequencing. Plasmids from 96 overnight cultures were isolated and analysed by enzymatic digestion using the restriction enzymes *Nco*I and *Not*I; only four (4%) were possible scFv antibodies, represented by an insert size of

approximately 1100bp. This recovery rate is much lower than that seen after the first round of screening (30%), and could suggest enrichment of antigen-specific scFv antibodies and therefore less scFv diversity.

These four plasmids were sequenced using Sanger technology and analysed using Ig BLAST (Table 4-3). All four sequences were in-frame antibody sequences, although scFv.27 and scFv.59 could potentially be the same scFv sequence as both scFv have very similar sequences. Additionally, scFv.65 appeared to have a truncated V_L region which lacked a CDR3 sequence. This type of sequence, a variable region lacking a CDR3 paired with an in-frame constant region, has been reported previously by a laboratory in China (GenBank: AAS88328.1). For this reason, the binding properties of all four scFv sequences was explored.

scFv		Top V match	Top D match	Top J match	Number of G4S repeats	CDR3
scFv.13	VH	IGHV3-48*03, IGHV3-48*01, IGHV3-48*04	IGHD3-9*01	IGHJ4*03, IGHJ4*02	2	GRHLNTIPAGRFDY
	VK	IGKV1-NL1*01, IGKV1-27*01, IGKV1-16*01	-	IGKJ4*01		QQYYSTPLT
scFv.27	VH	IGHV4-38-2*01, IGHV4-38-2*02	IGHD1-26*01	IGHJ2*01	4	ARSGSGSFYWYFDV
	VK	IGKV3D-7*01	-	IGKJ1*01		QHGSHTPPWT
scFv.59	VH	IGHV4-38-2*01, IGHV4-38-2*02	IGHD1-26*01	IGHJ2*01	4	ARSGSGSFYWYFDV
	VK	IGKV3D-7*01	-	IGKJ1*01		QHGSHTPPWT
scFv.65	VH	IGHV1-46*02, IGHV1-46*01	-	IGHJ4*03, IGHJ4*02	1	ASPDFDY
	VK	IGKV1-27*01, IGKV1-39*02, IGVK1-9*01	-	-		-

TABLE 4-3: CHARACTERISATION OF SCFV SEQUENCES ISOLATED FROM THREE ROUNDS OF SCREENING OF AN ANTIBODY LIBRARY AGAINST GBV-B CORE PROTEIN

FOUR SCFV RECOVERED FROM THREE ROUNDS OF PANNING AGAINST GBV-B CORE PROTEIN USING RIBOSOME DISPLAY WERE SEQUENCED BY SANGER SEQUENCING. DNA SEQUENCES WERE ANALYSED BY COMPARISON TO HUMAN IMMUNOGLOBULIN GENES USING IGBLAST.

4.2.8 THE SCFV ANTIBODIES RECOVERED FROM THREE ROUNDS OF PANNING USING RIBOSOME DISPLAY ARE GBV-B CORE-SPECIFIC

The four scFv antibodies recovered from three rounds of panning against GBV-B core were then expressed in a prokaryotic system to investigate their binding specificities. The antibody sequences were first cloned into the expression plasmid, p4D5_Fc, which contains the human Fc receptor, using the restriction enzymes *Nco*I and *Not*I (Figure 4-2).

Expression of scFv.13, scFv.27, scFv59 and scFv.65 was induced using IPTG and protein production confirmed by SDS-PAGE (Figure 4-11A) and western blot analysis using an anti-His antibody (Figure 4-11B).

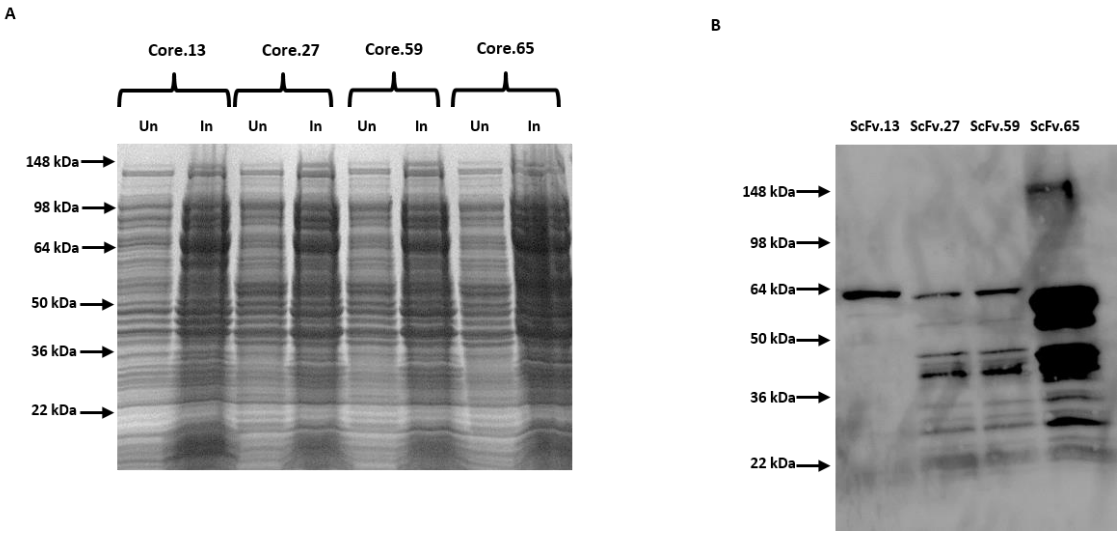
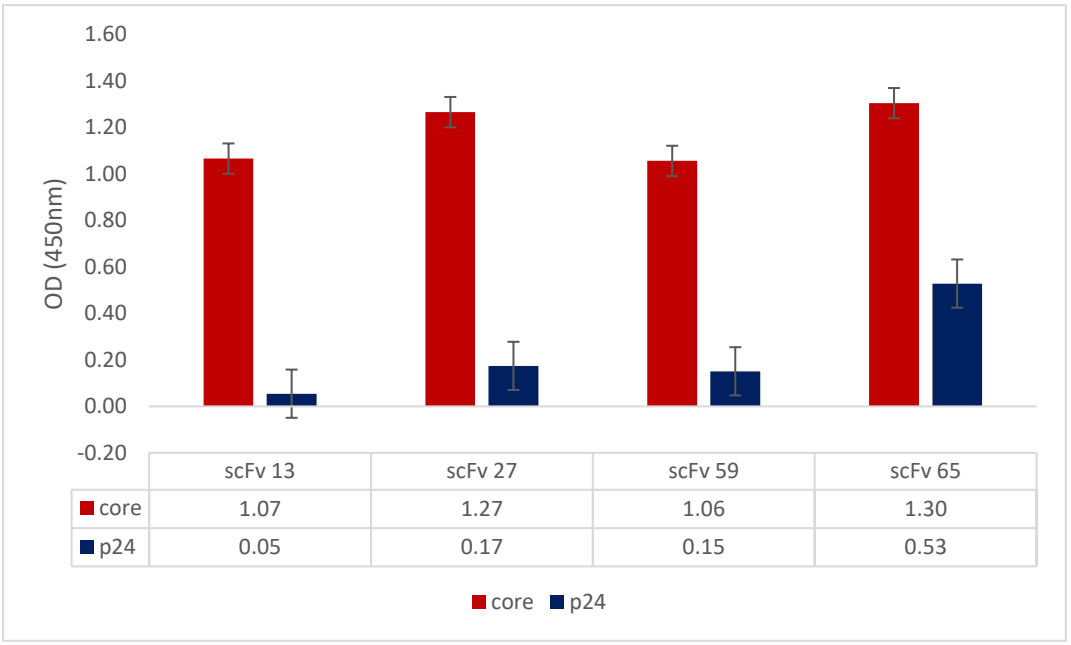


FIGURE 4-11: EXPRESSION AND ISOLATION OF SCFV_FC RECOVERED FROM THREE ROUNDS OF RIBOSOME DISPLAY USING GBV-B CORE PROTEIN IN BACTERIAL CELLS

(A) BL21 BACTERIAL CELL LYSATE BEFORE AND AFTER IPTG INDUCTION WERE RUN BY SDS-PAGE AND PROTEINS WERE STAINED WITH COLLOIDAL BLUE DYE. UN: NON-INDUCED, IN: IPTG INDUCED. (B) INDUCED BACTERIAL CULTURES BY LYSED AND RUN ON A SEPARATE SDS-PAGE. PROTEINS WERE TRANSFERRED TO A NITROCELLULOSE MEMBRANE AND DETECTED WITH AN ANTI-HIS ANTIBODY. ESTIMATED SIZE OF SCFV_FC: 55KDA, USING DNADYNAMO SOFTWARE (BLUETRACTORSOFTWARE, UK).

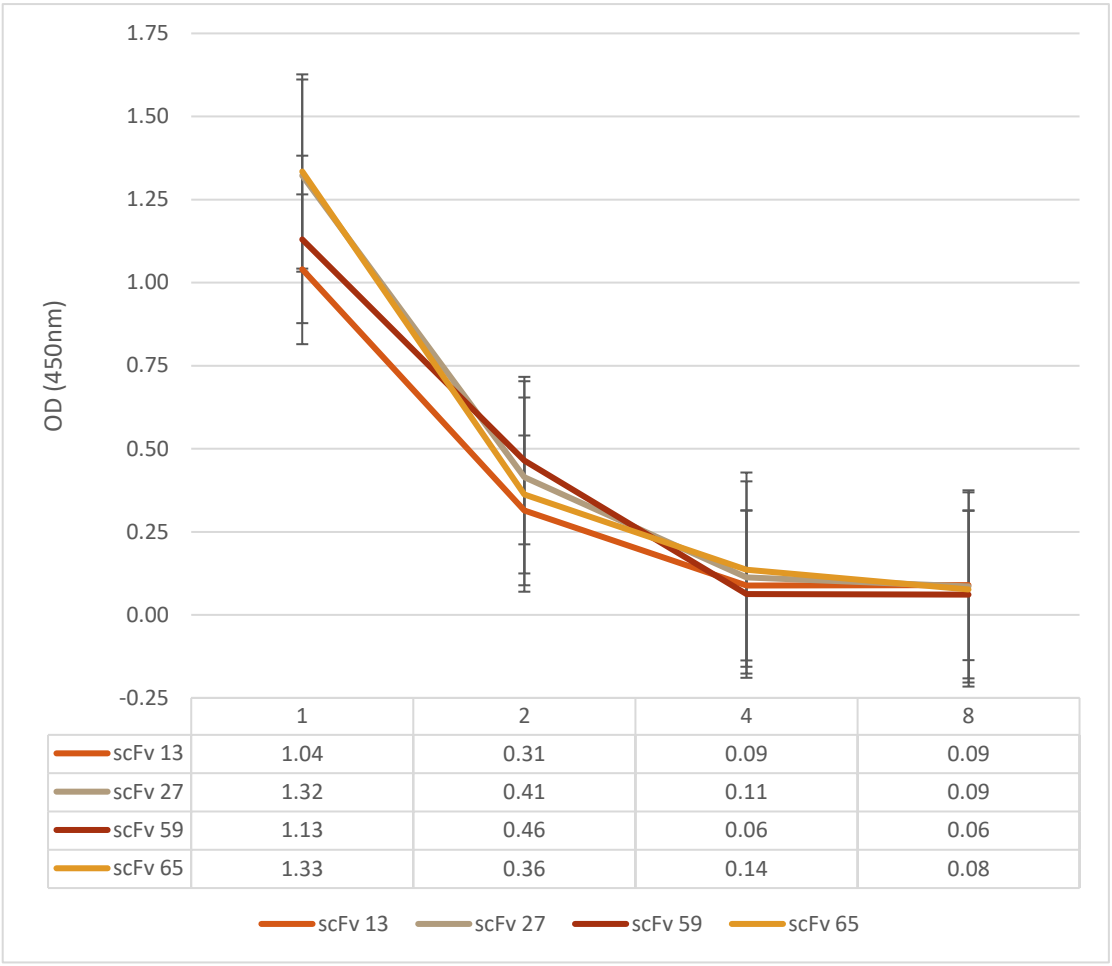
Binding specificity of each scFv antibody was investigated by ELISA using GBV-B core protein as target or His-tagged HIV-1 p24 protein, as negative control. Bacterial lysate samples, containing isolated scFv.13, scFv.27, scFv.59 and scFv.65, were added to the plate and subsequently detected with an anti-His HRP-conjugated antibody. All scFv antibodies were able to bind to specifically to GBV-B core protein (Figure 4-12A). ScFv.65, which has a truncated V_L region, showed binding specificity to GBV-B core but with higher background than the other scFvs. Specificity of the antibodies was also confirmed by serial dilutions of the scFv (Figure 4-12A). Additionally, all of these scFv produced a dose-response curve (Figure 4-12B)

A



	scFv 13	scFv 27	scFv 59	scFv 65
core	1.07	1.27	1.06	1.30
p24	0.05	0.17	0.15	0.53

B



	1	2	4	8
scFv 13	1.04	0.31	0.09	0.09
scFv 27	1.32	0.41	0.11	0.09
scFv 59	1.13	0.46	0.06	0.06
scFv 65	1.33	0.36	0.14	0.08

FIGURE 4-12: GBV-B CORE-SPECIFIC SCFV ANTIBODIES ISOLATED AFTER THREE ROUNDS OF PANNING AGAINST GBV-B CORE LOSE THEIR BINDING SPECIFICITY IF DILUTED FOUR-FOLD

A: CELL LYSATE SUPERNATANT CONTAINING SCFV ANTIBODIES WERE RUN IN AN ELISA. PLATES WERE COATED WITH EITHER GBV-B CORE (RED) OR WITH HIS-TAGGED HIV-1 P24 (BLUE). RESULTS REPRESENT THE MEAN ABSORBANCE VALUE OF TWO REPLICATES, NORMALISED TO THE VALUES FROM WELLS ANTI-HIS ANTIBODY ALONE. THE OD VALUES ARE PROVIDED BELOW THE CHART. THIS ASSAY WAS REPEATED THREE TIMES IN TOTAL, THE RANGE OF ABSORBANCE VALUES IS REPRESENTED ON THE GRAPH BY ERROR BARS.

B: THE BINDING SPECIFICITY OF BACTERIAL CELL LYSATES, CONTAINING SCFV.13 (ORANGE), SCFV.27 (BEIGE), SCFV.59 (RED) AND SCFV.65 (YELLOW), WERE EVALUATED BY ELISA. PLATES WERE COATED WITH EITHER GBV-B CORE PROTEIN OR HIS-TAGGED HIV-P24 AND THE MEAN ABSORBANCE VALUE OF TRIPLICATES, NORMALISED VALUES FROM WELLS CONTAINING EACH PROTEIN WITH THE ANTI-HIS ANTIBODY ALONE ARE SHOWN. THE OD VALUES ARE PROVIDED BELOW THE CHART. THIS ASSAY WAS REPEATED THREE TIMES IN TOTAL, THE RANGE OF ABSORBANCE VALUES IS REPRESENTED ON THE GRAPH BY ERROR BARS.

4.2.10 Deep sequencing of complementarity determining region 3 (CDR3) of scFv antibodies libraries before and after panning against GBV-B core protein shows a weak selection of scFv against GBV-B core

Within each variable region of an antibody, there are three highly conserved framework regions (FR) and three highly variable complementarity determining regions (CDR) (Sela-Culang *et al*., 2013). The CDR regions are thought to be involved in antigen recognition whilst the FR regions are considered to primarily act as a scaffold for CDRs. In particular, it is thought that the diversity of antigen specificity of antibodies is largely due to the third CDR (CDR3) of the heavy chain variable region (Xu and Davis, 2000). Consequently, to investigate the diversity of the scFv libraries before and after panning against GBV-B core protein, the CDR3 regions of scFv libraries were deep sequenced with the aim to visualise the selection of core-specific scFv.

4.2.10.1 Design of primers for the amplification of tamarin CDR3 regions

To perform the deep sequencing of the scFv libraries, the CDR3 within the tamarin variable regions were amplified (see Chapter **2.2.4**). As the sequence of tamarin CDR3 are unknown, the PCR products of the variable regions of tamarin antibodies used to construct the initial scFv library in Chapter **3.3.1** were sequenced by Sanger sequencing to allow the design of primers. Four different variable regions for each family from both the heavy chain and light chain were sequenced (32 sequences in total). Each sequence was analysed by comparing the sequence to the database of human antibodies using the Ig BLAST tool, and the FR and CDR identified. To design primers to amplify the CDR3 regions, all sequences were aligned and a consensus sequence of the sequences before the CDR3 regions was obtained. The sequences used to design the primers are provided in Table 4-4 below. Using this information, four degenerate primers were designed for both the CDR3 of the heavy chain and the light chain due to the diversity within the different variable region families.

A

SEQUENCE	NUCLEOTIDE BASES	AMINO ACIDS
VH1-7.1	ACA GCC GTC TAT TAC TGT TCT AGA	TAVYY**CSR**
VH1-7.2	ACG GCC GTG TAT TAC TGT GCG AGA	TAVYY**CAR**
VH1-7.3	ACG GCC GTG TAT TAC TGT GCA AGA	TAVYY**CAR**
VH1-7.4	ACA GCC GTC TAT TAC TGT AGT AAT	TAVYY**CSN**
VH3-5.1	ACG GCC GTG TAT TAC TGT GCA AGA	TAVYY**CAR**
VH3-5.2	ACG GCC GTC TAT TAC TGT GCA GGA	TAVYY**CAG**
VH3-5.3	ACG GCC GTA TAT TAC TGT GCA GGA	TAVYY**CAG**
VH3-5.4	ACG GCC ATG TAT TAC TGT GCG AGA	TAMYY**CAR**
VH4.1	ACG GCC ATC TAT TAC TGT GCG AGA	TAIYY**CAR**
VH4.2	ACG GCC GTG TAT TAC TGT GCG AGA	TAVYY**CAR**
VH4.3	ACG GCC GTG TAT TAC TGT GCG ACA	TAVYY**CAT**
VH4.4	ACG GCC GTC TAT TAC TGT GCA TAT	TAVYY**CAY**
VH6.1	ACG GCC GTA TAT TAC TGT GCG AGA	TAVYY**CAR**
VH6.2	ACG GCC GTG TAT TAC TGT GCT GGG	TAVYY**CAG**
VH6.3	ACG GCC GTA TAT TAC TGT GCG AGA	TAVYY**CAR**
VH6.4	ACG GCC AGA TAT TAC TGT GCG AGG	TARYY**CAR**

B

SEQUENCE	NUCLEOTIDE BASES	AMINO ACIDS
VK1.1	GTT TCG ATT TAT TAC TGT CAC CAC	VSIYY**C**HH
VK1.2	TTT GCC ACT TAT TAC TGT CAA CAT	FATYY**C**QH
VK1.3	TTT GCC ACT TAT TTC TGT CAA CAT	FATYF**C**QH
VK1.4	GTT GCA ATT TAT TAC TGT CAA CAG	VAIYY**C**QQ
VK2-4.1	TTT GCC ACT TAT TAC TGT CAA CAT	FATYY**C**QH
VK2-4.2	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK2-4.3	GTT GCG ATT TAC CAC TGT CAA CAG	VAIYH**C**QQ
VK2-4.4	GTT GCA ATT TAT TAC TGT CAA CAG	VAIYY**C**QQ
VK3.1	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK3.2	TTT GCC GCT TAT TAC TGT CAA CAT	FAAYY**C**QH
VK3.3	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK3.4	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK5.1	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK5.2	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK5.3	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYY**C**QQ
VK5.4	TCT GCA GTT TAT TAC TGT CAC CAG	SAVYY**C**HQ

TABLE 4-4: AMINO ACID SEQUENCES IMMEDIATELY BEFORE TAMARIN CDR3 SEQUENCES ARE SIMILAR TO THEIR HUMAN COUNTERPARTS

TAMARIN VARIABLE REGIONS, CLONED IN CHAPTER 3.3.1, WERE SEQUENCED BY SANGER SEQUENCING AND ANALYSED USING DNA DYNAMO SOFTWARE (BLUE TRACTOR SOFTWARE) AND IGBLAST. IGBLAST SUCCESSFULLY IDENTIFIED THE CDR3 SEQUENCE FOR EACH VARIABLE REGION. THE NUCLEOTIDE AND AMINO ACID SEQUENCES IMMEDIATELY BEFORE THE IDENTIFIED CDR3 REGIONS ARE SHOWN IN THE TABLE ABOVE FOR EACH FAMILY OF VARIABLE REGIONS (VH=HEAVY CHAIN, VK=LIGHT CHAIN), WITH THE CONSERVED AMINO ACID KNOWN TO BE IN HUMAN SEQUENCES ARE HIGHLIGHTED IN BOLD. FOUR VARIABLE REGIONS FROM EACH FAMILY WERE INVESTIGATED TO ACCOUNT FOR SEQUENCE VARIATION.

From the IgBLAST analysis of tamarin variable region, all of the sequences appeared to follow similar patterns to human variable region sequences (see table 7-6). For example, it was seen that immediately before the heavy chain CDR3 region, a consensus amino acid sequence is seen, C-X-X (e.g. C-A-R was seen in 56% of sequences analysed).

4.2.10.2 AMPLIFICATION OF TAMARIN CDR3 REGIONS

To investigate the enrichment of scFv antibodies within scFv libraries before and after panning against GBV-B core antigen, CDR3 regions from each library were deep sequenced to investigate their diversity, as described in Chapter **2.2.15**. CDR3 regions from both the heavy chain and light chain of scFvs from each library, before and after each of the 3 rounds of panning, were separately amplified by PCR using a high-fidelity polymerase enzyme: library 1 represents the input scFv library, pre-panning, library 2 is the recovered antibodies sequences after the first round of panning and so on. Each PCR was performed in duplicate as biological replicates. Specific bands were seen at the correct size of approximately 200bp; the CDR3 regions amplified from the light chain variable regions (VK) appear to be slightly smaller at around 170bp (Figure 4-13).

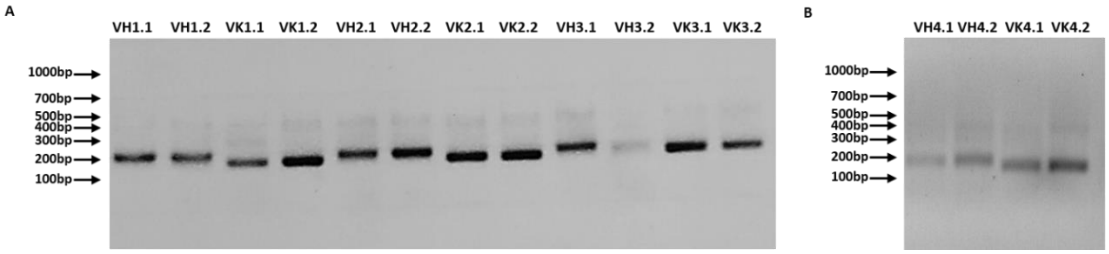


FIGURE 4-13: AMPLIFICATION OF CDR3 REGIONS FROM SCFV LIBRARIES BEFORE AND AFTER PANNING AGAINST GBV-B CORE PROTEIN

THE CDR3 REGIONS OF SCFV WITHIN THE GBV-B INFECTED TAMARIN-DERIVED ANTIBODY LIBRARIES, BEFORE AND AFTER EACH ROUND OF PANNING AGAINST GBV-B CORE (LIBRARY 1-4), WERE AMPLIFIED BY PCR AND VISUALISED IN A 3% AGAROSE GEL. (A) THE CDR3 REGIONS AMPLIFIED FROM THE NAÏVE SCFV LIBRARY (LIBRARY 1), THE SCFV LIBRARY RECOVERED AFTER ONE ROUND OF PANNING (LIBRARY 2) AND THE SCFV LIBRARY RECOVERED AFTER TWO ROUNDS OF PANNING. (B) THE CDR3 REGIONS AMPLIFIED FROM THE SCFV LIBRARY RECOVERED AFTER THREE ROUNDS OF PANNING. VH: CDR3 AMPLIFIED FROM VARIABLE REGION FROM HEAVY CHAIN OF ANTIBODY. VK: CDR3 REGION AMPLIFIED FROM VARIABLE REGION FROM LIGHT CHAIN OF ANTIBODY. ALL PCRS WERE PERFORMED IN DUPLICATE (FOR EXAMPLE, VH1.1 AND VH1.2).

4.2.10.3 DEEP SEQUENCING OF CDR3 REGIONS OF SCFV ANTIBODIES WITHIN SCFV ANTIBODY LIBRARIES BEFORE AND AFTER GBV-B ANTIGEN PANNING

The purified PCR products were processed using the NexTera XT kit (Illumina) and sequenced on a Miseq platform. The raw data files were analysed using the Antibody Mining Toolbox, developed by D'Angelo and colleagues (D'Angelo *et al.*, 2013) as described in Chapter **2.2.16**. The software recognises conserved amino acid sequences immediately before and after human CDR3 regions. The same settings were used to analyse the data retrieved for the tamarin antibodies sequences as they share similar conserved amino acids.

For each library, the number of unique CDR3 sequences for the heavy chain and light chain were analysed separately. If ribosome display was successful, the complexity of the library at each stage should decrease as the library should be enriched in GBV-B core-specific antibodies. The number of unique sequences in each library were separated into categories ranging from 1-5 repeats to 64,001-128,000 repeats). A very limited level of selection against

GBV-B core took place, as visually seen by lack of difference between each round of selection (Figure 4-14); for the heavy chain CDR3 sequences, there is a minor enrichment of CDR3 sequences (as seen by the appearance of the 16,001-32,000 repeats category). However, the complexity of the scFv libraries is not decreasing. A similar minor enrichment of CDR3 is also seen with the light chain CDR3 analysis, with no significant decrease in library complexity.

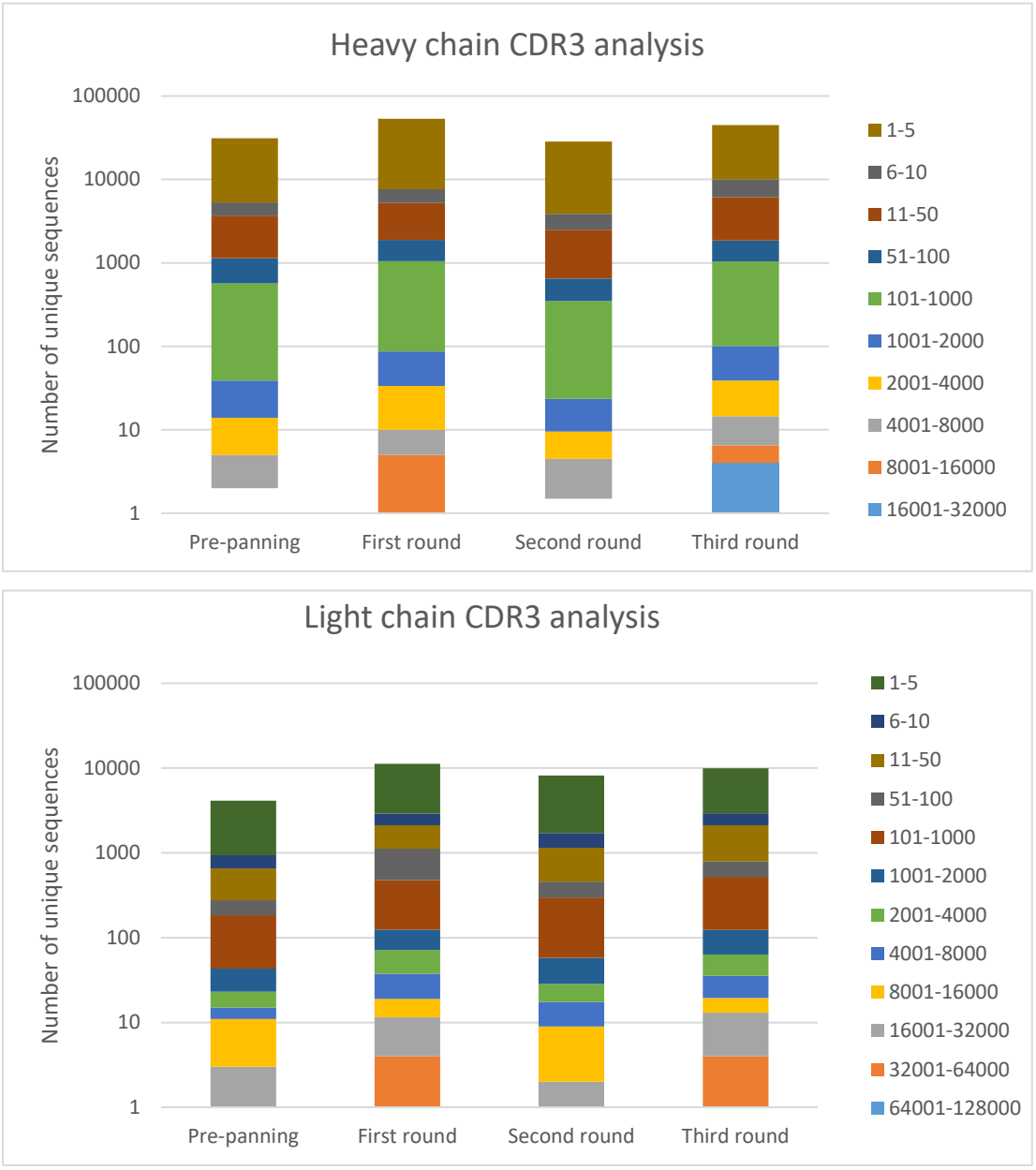


FIGURE 4-14: THE DIVERSITY OF SCFV LIBRARIES, AS REPRESENTED BY NUMBER OF UNIQUE CDR3 SEQUENCES, MINIMALLY DECREASES AFTER EACH ROUND OF SCREENING AGAINST GBV-B CORE PROTEIN

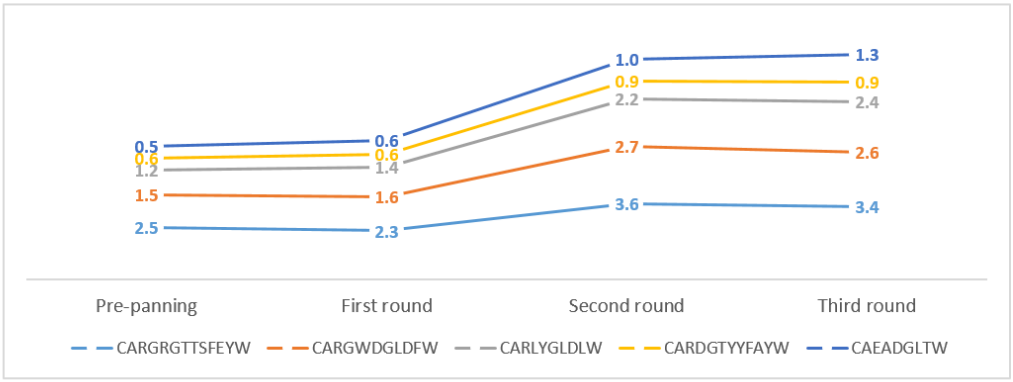
CDR3 REGIONS WERE DEEP SEQUENCED USING THE MISEQ PLATFORM AND SUBSEQUENTLY ANALYSED USING THE 'CDR3-PIPELINE' IN THE ANTIBODY MINING TOOLBOX. THE AMINO ACID SEQUENCES OF CDR3 REGIONS WERE CATEGORISED BY THE NUMBER OF OCCURRENCES FOR EACH SCFV LIBRARY INTO 10 CATEGORIES FOR THE HEAVY CHAIN AND 11 CATEGORIES FOR THE LIGHT CHAIN. EACH BARREL REPRESENTS THE MEAN NUMBER OF UNIQUE SEQUENCES OF TWO DUPLICATES.

To further analyse the minor enrichment of CDR3 regions within scFv libraries, the most highly repeated CDR3 sequences for all scFv libraries, for both the heavy and light chain, were investigated. These sequences were tracked through all four scFv libraries to see whether they were positive selected after each round of panning against GBV-B core protein using ribosome display. The percentage of each CDR3 sequence in relation to the entire CDR3 sequenced population was calculated and visualised in a stacked line graph (Figure 4-15 and Figure 4-16).

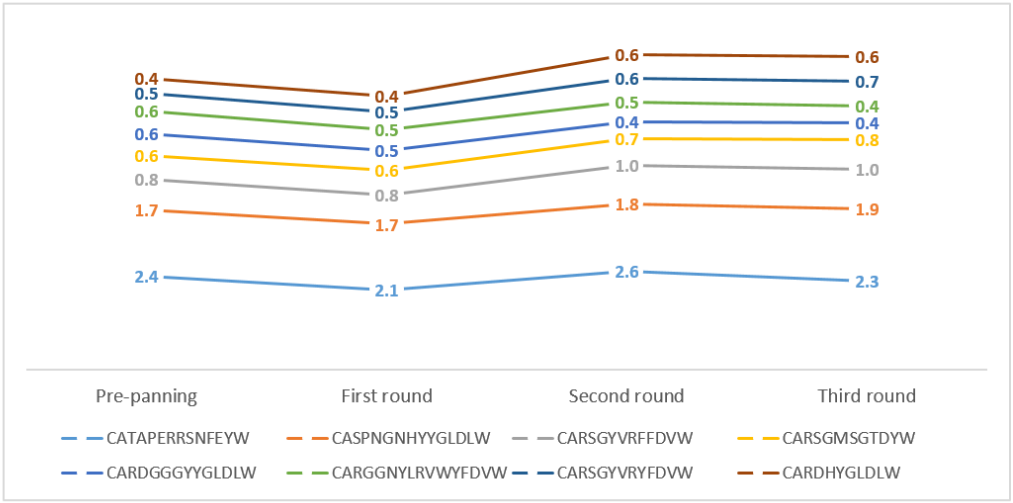
For heavy chain CDR3 sequences, there was a minor enrichment seen within the population investigated. For example, the number of CDR3 regions with the amino acid sequence CAEADGLTW in relation to the whole sequenced CDR3 population increased by around 0.8% over the course of 3 rounds of panning against GBV-B core. The highest enrichment of a specific heavy chain CDR3 (CARLYGLDLW) only resulted in an increase of around 1.2% in relation to the entire sequenced CDR3 population (Figure 4-15A). As only a minor enrichment is seen, the disappearance of non-specific CDR3 sequences was difficult to track, with only one CDR3 sequence (of the sequences investigated) decreased in the number of occurences (Figure 4-15C). Most CDR3 sequence occurences appeared to remain stable throughout ribosome display suggesting no enrichment of these CDR3 regions and possibly no enrichment of GBV-B core-specific scFv throughout ribosome display (Figure 4-15B).

In comparison to the heavy chain CDR3 sequences, the enrichment of CDR3 of the light chain populations appeared to be even lower as most CDR3 sequences showed no enrichment (Figure 4-16B). A minor enrichment was seen for two similar, specific CDR3 sequences, CQHGYSTPYTF and CQHGYSTPWTF (Figure 4-16A) whilst three CDR3 sequences appeared to decrease in the number of occurrences (Figure 4-16C).

A



B



C

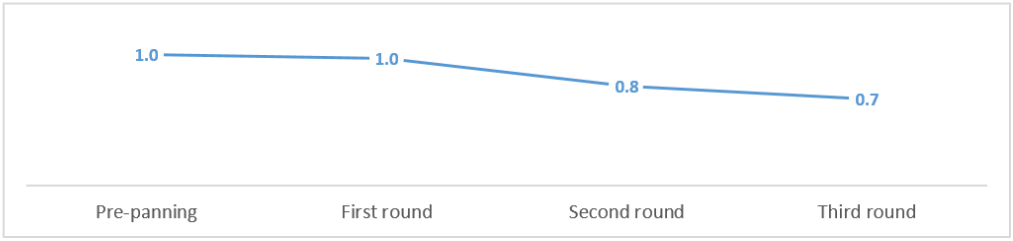
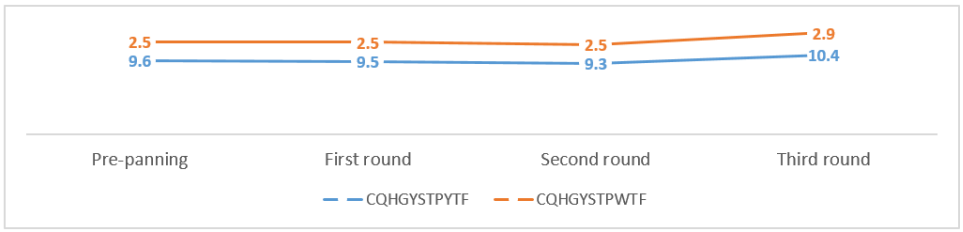


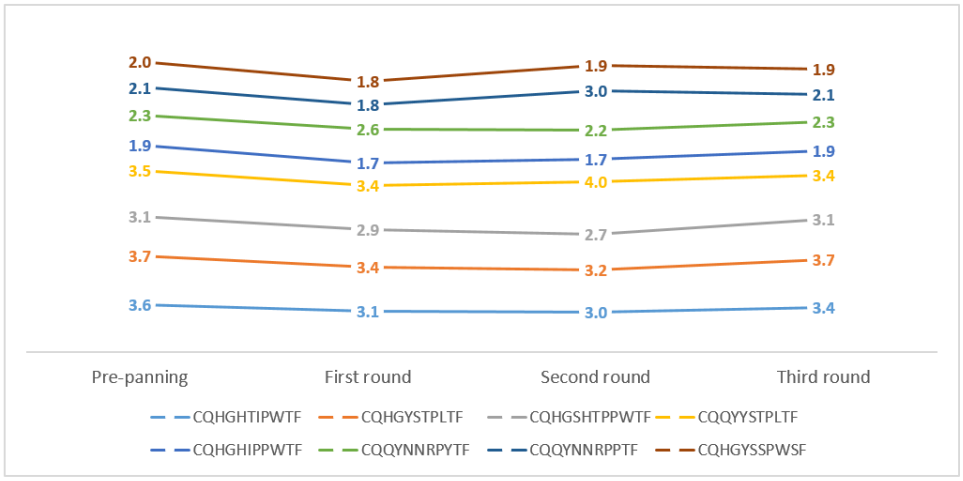
FIGURE 4-15: VARIATION IN THE PROPORTION OF THE MOST ABUNDANT HEAVY CHAIN VARIABLE REGION CDR3 SEQUENCES AFTER EACH ROUND OF SCREENING AGAINST GBV-B CORE PROTEIN USING RIBOSOME DISPLAY

HEAVY CHAIN VARIABLE REGION CDR3 SEQUENCES WERE SEQUENCED ON THE MISEQ SEQUENCING PLATFORM AND ANALYSED USING THE ANTIBODY MINING TOOL. THE ENRICHMENT OF THE TEN SCFV WITH THE HIGHEST NUMBER OF OCCURRENCES FOR EACH SCFV LIBRARY (BEFORE AND AFTER PANNING) WAS INVESTIGATED BY CALCULATING THE PERCENTAGE OF EACH SCFV IN RELATION TO THE ENTIRE SCFV LIBRARY. THESE FIGURES WERE VISUALISED ON A STACKED LINE GRAPH TO SHOW THE EVOLUTION OF CDR3 ENRICHMENT THROUGHOUT RIBOSOME DISPLAY. THREE TYPES OF ENRICHMENT WERE SEEN: A POSITIVE ENRICHMENT OF CORE-SPECIFIC SCFV (A), NO ENRICHMENT (B) AND A DECREASE IN ENRICHMENT (C).

A



B



C

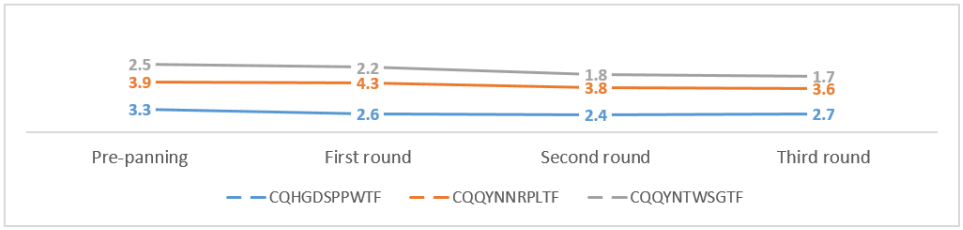


FIGURE 4-16: VARIATION IN THE PROPORTION OF THE MOST ABUNDANT LIGHT CHAIN VARIABLE REGION CDR3 SEQUENCES AFTER EACH ROUND OF SCREENING AGAINST GBV-B CORE PROTEIN USING RIBOSOME DISPLAY

LIGHT CHAIN VARIABLE REGION CDR3 SEQUENCES WERE SEQUENCED ON THE MISEQ SEQUENCING PLATFORM AND ANALYSED USING THE ANTIBODY MINING TOOL. THE TOTAL PERCENTAGE OF THE TEN SCFV WITH THE HIGHEST NUMBER OF OCCURRENCES FOR EACH SCFV LIBRARY (BEFORE AND AFTER PANNING) IN COMPARISON TO ALL CDR3 SEQUENCES FOR EACH LIBRARY WERE CALCULATED. THESE FIGURES WERE VISUALISED ON A STACKED LINE GRAPH TO SHOW THE EVOLUTION OF CDR3 ENRICHMENT THROUGH RIBOSOME DISPLAY. THREE TYPES OF ENRICHMENT WERE SEEN: A POSITIVE ENRICHMENT OF CORE-SPECIFIC SCFV (A), NO ENRICHMENT (B) AND A NEGATIVE ENRICHMENT (C).

4.2.11 MONOCLONAL ANTIBODIES CAN BE ISOLATED FROM MICE IMMUNISED WITH GBV-B CORE PROTEIN

GBV-B core-specific murine monoclonal antibodies were also produced using the traditional hybridoma technology (see Chapter **2.4.2**). Despite the lack of novelty in using this system, it has been proven to be a reliable and consistent method to obtain monoclonal antibodies. An overview of the immunisation schedule and generation of single clone anti GBV-B core antibodies producing hybridomas is provided in Figure 4-17.

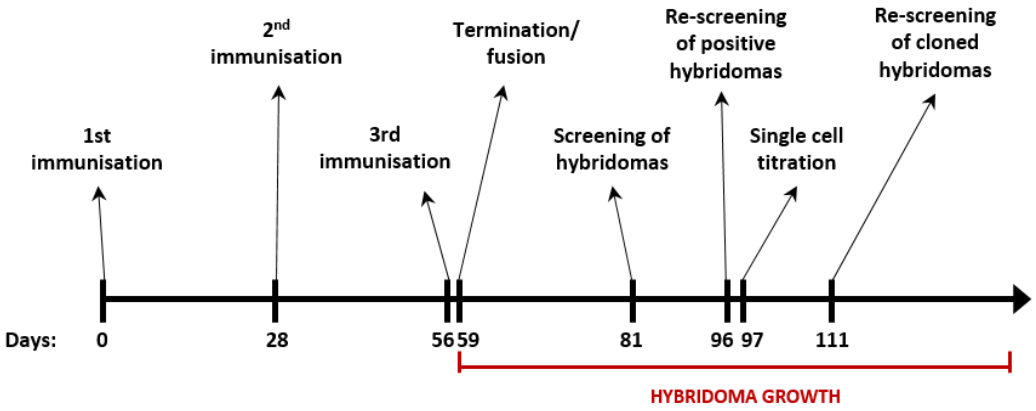


FIGURE 4-17: TIMELINE FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES TARGETING GBV-B CORE

TWO MICE WERE IMMUNISED AT DAY 1 WITH GBV-B CORE PROTEIN AND SIGMA ADJUVANT. BOOSTS TOOK PLACE AT DAY 28 AND 56. MICE WERE TERMINATED AT DAY 59 AND SPLEENS WERE HARVESTED. SPLENOCYTES WERE COLLECTED AND FUSED WITH MYELOMA CELLS. AFTER A GROWTH PERIOD OF AROUND THREE WEEKS, CULTURE SUPERNATANT WAS SCREENED TO TEST FOR HYBRIDOMAS SECRETING MONOCLONAL ANTIBODIES TARGETING GBV-B CORE. POSITIVE CULTURES WERE THEN CLONED BY SINGLE CELL TITRATION BEFORE RE-SCREENING CULTURE SUPERNATANT. AT THE END OF THIS PROCESS, BULK SUPERNATANT WAS COLLECTED AND STORED WHILST ANY GBV-B CORE-SPECIFIC CLONED HYBRIDOMA CELLS WERE FROZEN FOR STORAGE.

Mice were first immunised with 20µg of His-tagged recombinant GBV-B core protein, generated in a prokaryotic system in Chapter **3.2.4**, mixed with Sigma adjuvant (as described

in Chapter 2.4.2). This immunisation was repeated a further two times to increase the antibody response. After the third immunisation at approximately two months, spleen cells were collected and fused to murine myeloma cells, NS-0, using polyethylene glycol (PEG). Cells were then seeded in 96-well plates in selection culture medium, containing hypoxanthine-aminopterin-thymidine (HAT) that allows only fused cells to grow.

After 3 weeks, the supernatant from each culture was tested for the presence of GBV-B core-specific monoclonal antibodies by ELISA. Plates were coated with 1µg/ml of GBV-B core protein or His-tagged HIV-1 p24 protein, as negative control. Out of 576 cultures tested, 43 cultures (1A1-6G11 in Figure 4-18) contained hybridoma cells that were secreting GBV-B core-specific antibodies.

FIGURE 4-18: SCREENING OF THE HYBRIDOMAS PRODUCING GBV-B CORE-SPECIFIC MONOCLONAL ANTIBODIES

HYBRIDOMA CELL CULTURE SUPERNATANT, CONTAINING SECRETED ANTIBODIES, WAS TESTED BY ELISA. PLATES WERE COATED WITH EITHER GBV-B CORE (RED) OR WITH HIV-P 24 (BLUE). OF THE 576 CULTURES TESTED, ONLY THE ONES PRODUCING AN OD ABOVE BACKGROUND WERE PLOTTED IN THE GRAPH. RESULTS REPRESENT THE ABSORBANCE VALUE, NORMALISED TO BLANK WELLS.

The 12 colonies that produced monoclonal antibodies with the highest absorbance values from the initial screening were cloned by serial dilution to ensure that the hybridomas culture only contain one type of cell producing anti-GBV-B core antibodies. Only seven hybridoma cultures continued to proliferate and secrete GBV-B core-specific monoclonal antibodies once tested by ELISA (Figure 4-19).

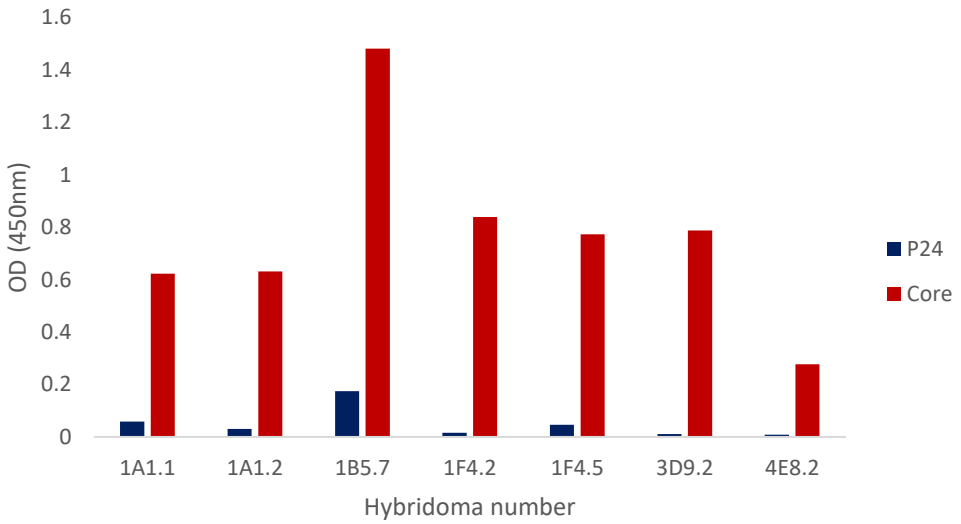


FIGURE 4-19: SPECIFIC BINDING TO GBV-B CORE OF THE 7 MONOCLONAL ANTIBODIES PRODUCED BY SINGLE CLONED HYBRIDOMAS

SUPERNATANT WAS COLLECTED FROM SINGLE HYBRIDOMA CELL CLONES AND TESTED BY ELISA TO DETERMINE THE SPECIFICITY OF THE MONOCLONAL ANTIBODIES WITHIN THE CULTURE SUPERNATANT. THIS GRAPH ONLY SHOWS THE SINGLE CELL CULTURE SUPERNATANTS THAT TESTED POSITIVE FOR MONOCLONALS THAT TARGET GBV-B CORE (RED). HIV-1 P24 (BLUE) WAS USED AS NEGATIVE CONTROL. RESULTS REPRESENT THE MEAN ABSORBANCE VALUES OF DUPLICATED WELLS, NORMALISED TO BLANK WELLS.

4.2.12 CHARACTERISATION OF GBV-B CORE-SPECIFIC MONOCLONAL ANTIBODIES

GBV-B core-specific monoclonal antibodies were collected from cell culture supernatant of expanded hybridoma cell lines, and purified by ammonium sulphate purification. Purification by ammonium sulphate is commonly used to purify monoclonal antibodies from cell culture

supernatant by altering the solubility of the antibodies (Duong-Ly and Gabelli, 2014). This method also allows the concentration of samples as antibodies can be resuspended in a much smaller final volume.

Once purified and concentrated, the concentration of monoclonal antibodies in each sample was quantified using the Mouse IgG total ELISA Ready-SET-Go! Kit. Antibody concentrations are summarised in Table 4-5. One monoclonal antibody, 4E8 was negative in this ELISA.

The monoclonal antibodies were also isotyped using the Mouse Monoclonal Antibody Isotyping Test Kit (Biorad, see Chapter **2.3.6**). Most of the antibodies were IgG1, with only one IgG2a. All of the light chains were Kappa chain (Table 4-5). Isotyping revealed that the monoclonal antibody, 4E8, consisted of IgM antibodies, explaining the negative result of the quantification ELISA as this ELISA only detects IgG antibodies. Due to time restrictions and the fact that IgM antibodies are generally low affinity antibodies produced first during infection, the study of this antibody was not continued (Racine and Winslow, 2009).

Hybridoma	*Concentration (µg/ml)*	*Isotype*	*Light chain*
1A1.1	432	G1	Kappa
1A1.2	1233	G1	Kappa
1B5.7	128	G2a	Kappa
1F4.2	184	G1	Kappa
1F4.5	79	G1	Kappa
3D9.2	503	G1	Kappa
4E8.2	0	M	Kappa

TABLE 4-5: CHARACTERISATION OF PURIFIED GBV-B CORE-SPECIFIC MONOCLONAL ANTIBODY SAMPLES

To further confirm the core-specificity of the monoclonal antibodies produced, western blots were performed. For each blot, 50ng of either GBV-B core and HIV-1 P24 protein was run on

agarose gel and transferred onto a nitrocellulose membrane. These membranes were blotted with each monoclonal antibody sample (Figure 4-20). All but one sample were shown to recognise GBV-B core, whilst no antibodies were shown to recognise the P24 protein. However, as GBV-B core has been denatured during the process of western blotting, this indicates that the monoclonal antibody 1A1.1 may recognise only conformational epitopes within GBV-B core.

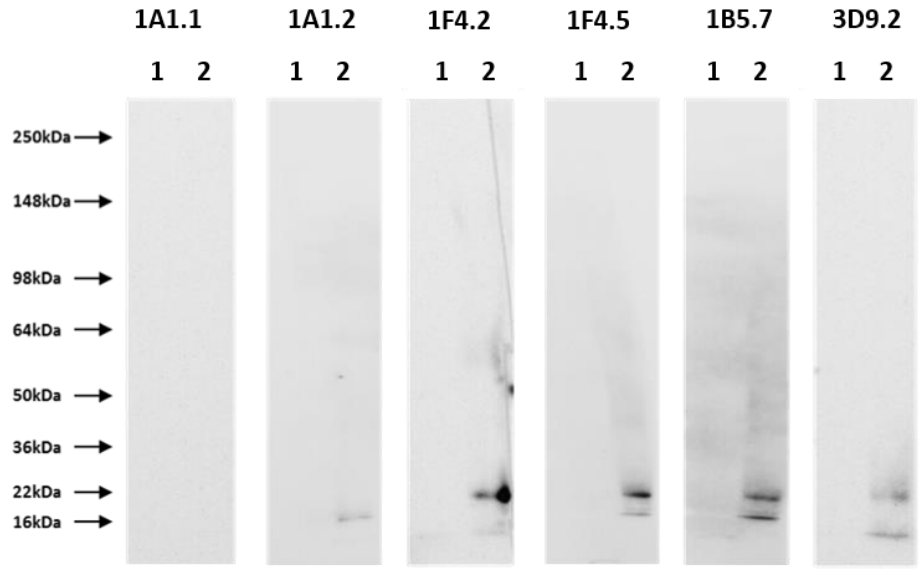


FIGURE 4-20: GBV-B CORE-SPECIFIC MONOCLONAL ANTIBODIES CAN RECOGNISE DENATURED LINEAR EPITOPES WITHIN RECOMBINANT GBV-B CORE

HIV-1 P24 (LANE 1) AND GBV-B CORE (LANE 2) WERE RUN ON AN SDS-PAGE AND TRANSFERRED TO A NITROCELLULOSE MEMBRANE. THIS MEMBRANE WAS THEN PROBED WITH 10µG/ML OF MONOCLONAL ANTIBODIES: 1A1.1, 1A1.2, 1B5.7, 1F4.2, 1F4.5 AND 3D9.2. MONOCLONAL ANTIBODIES WERE THEN DETECTED USING AN ANTI-MOUSE IGG-HRP-CONJUGATED ANTIBODY.

4.4: DISCUSSION

Antibodies have been produced as reagents for the study of GBV-B infection of tamarins as an animal model of acute HCV infection. Two different approaches have been used, the classical hybridoma technology and the screening of a tamarin antibody library by ribosome display. Although hybridoma technology is simple and reliable, *in vitro* display technologies, including ribosome display, offer multiple benefits in comparison to this technology. These benefits include a greater level of control over the nature of isolated antibodies, through the careful control of selection and screening conditions, and the high throughput nature of the technologies, which allows for the isolation of huge numbers of antibodies in a short space of time (Bradbury *et al*., 2011). For these reasons, ribosome display was first used to isolate GBV-B specific antibodies.

The tamarin scFv library produced in the previous Chapter (Chapter **3.3.2**) was screened against two GBV-B proteins, NS5BΔ23 and core (as described in Chapter **2.2.17**). GBV-B NS5BΔ23 was first chosen as the target antigen for ribosome display, as its production had been confirmed by both western blot and mass spectrometry (Figure 3-4, Table 7-1). GBV-B core was later chosen as tamarin sera was shown to contain antibodies that recognised this protein.

Initially, only one round of selection was performed, as it has been previously shown that this could be sufficient for the selection of specific antibodies (Tang *et al*., 2012). However, for both proteins, although scFv were isolated, they were either non-specific or non-functional (Figures 4-4 and 4-7). Changing the host for the expression of the scFv from prokaryotic to mammalian cells did not improve the functionality of the antibodies isolated (Figure 4-9). Of note, full-length scFv antibodies were isolated from the GBV-B-infected tamarin-derived antibody library more readily after screening against GBV-B core protein (Table 4-2 and 4-3) in comparison to GBV-B NS5BΔ23 protein (Table 4-1), which could support the hypothesis

that antibodies targeting GBV-B core are produced at a higher level in tamarins *in vivo* in comparison to antibodies targeting NS5BΔ23. Indeed, NS5B antibodies are less regularly documented in the literature, in comparison to core antibodies (Bukh *et al*., 2008; Iwasaki *et al*., 2011; Martin *et al*., 2003; Woollard *et al*., 2008). Of note, the scFv antibodies isolated after screening against GBV-B core (Figure 4-7) were much more difficult to express in comparison to those isolated from screening against GBV-B NS5BΔ23 (Figure 4-3).

Following other examples in the literature (Hanes *et al*., 1998; Yuan *et al*., 2009; Zhao *et al*., 2009b), the tamarin scFv library was screened three consecutive times against GBV-B core protein. This led to the successful isolation of four GBV-B core-specific scFv (Figure 4-12A). Two of these antibodies appear to be of the same sequences and showed similar binding property. Additionally, one of the other two antibodies had a truncated V_L region and a very short linker, which could impact its ability to bind to GBV-B core. A previous study suggested that a linker this length may inhibit VH and VL pairing, thereby inducing dimer formation (Kortt *et al*., 1997). These dimers may account for the high OD values in ELISAs with this scFv (Figure 4-12A). Further characterisation of these scFv should include large-scale production and purification, and the use of BIACORE to determine affinity of these antibodies. If the affinity of these scFv is shown to be weak, affinity maturation could be employed in further ribosome display experiments. A competition ELISA could also be performed to characterise epitope binding of these scFv to GBV-B core.

The isolation of only four scFv antibodies after the three rounds screening of scFv library against GBV-B core could suggest an inefficient protocol either during the panning or in the recovery of scFv mRNA. To investigate the effectiveness of the selection procedure, the diversity of the antibody library was investigated after each round of panning against GBV-B core by deep sequencing of the scFvs CDR3 sequences (as described in Chapter **2.2.15** and Chapter **2.2.16**). CDR3 sequences analysis showed a very minimal enrichment of any scFv,

further suggesting an issue with the ribosome display. Optimisation of the screening or recovery protocol of scFv using ribosome display is therefore required and may include further washes, the use of different blocking reagents, the use of different selection techniques (such as protein-coated magnetic beads), an increased spacer length of scFv, and the use of proof-reading polymerases during the elongation steps of scFv (Schaffitzel *et al*., 1999a).

Due to the technical difficulties with ribosome display, monoclonal antibodies were produced using hybridoma technology to obtain essential reagents for the study of GBV-B infection in tamarins (see Chapter **2.4.2**). Mice were immunised three times, 4 weeks apart using recombinant His-tagged GBV-B core, and after fusion with an immortalised myeloid cell line, specific antibodies-producing cells were isolated. Although some monoclonal antibodies seem to recognise the His-tag at the C-terminus of GBV-B core (Fig 4.18: 1E5, 5D4, 6A8, 6C9, 6F10, 6G11), the majority of the binding antibodies were specific to GBV-B core. Seven single cloned hybridomas were amplified and the monoclonal antibodies purified and concentrated for downstream applications. Both monoclonal antibodies and scFv antibodies targeting GBV-B core will now be used in immunohistochemistry to investigate the pathology of GBV-B. For example, these antibodies will be used to identify GBV-B in a range of different archived tamarin tissues with the aim to define the tropism of the virus. This is of particular interest as these data exist for other HCV animal models, such as nonprimate hepacivirus infection of horses, and could help to further validate GBV-B infection of tamarins as an effective animal model of acute HCV infection.

CHAPTER 5 : IDENTIFICATION OF ANTIBODIES INVOLVED IN THE NATURAL CLEARANCE OF GBV-B THROUGH THE DEVELOPMENT OF A VIRUS NEUTRALISATION ASSAY

5.1 INTRODUCTION

HCV, which is an important public health concern, has a high propensity for chronicity leading to sequelae including hepatocellular carcinoma (Lozano *et al*., 2012). Determining the mechanisms surrounding viral clearance would therefore have a significant impact on disease burden. However, due to the subclinical nature of acute HCV infections, the mechanisms involved in viral clearance are currently incompletely understood, including the role of the humoral immune response. To study the immunological events surrounding viral clearance, GBV-B infection of tamarins has been used as an animal model of acute HCV infection in this study.

GBV-B clearance is also poorly understood. A combined cellular and humoral response is likely important in the clearance of virus but there is a lack of appropriate immunological assays for the latter. T cell responses targeting GBV-B viral proteins have been described previously in the literature and are thought to play a role in viral clearance (Woollard *et al*., 2008). Additionally, memory T cell responses are thought to be induced in secondary infection (Woollard *et al*., 2008).

Little is known about the role of the humoral immune response in GBV-B clearance. Antibodies have been detected against a range of GBV-B proteins, including NS3-specific antibodies that have been found to increase and peak at viral clearance but decline shortly after (Beames *et al*., 2000). In the rare cases of extended GBV-B viraemia, it has been suggested that this 'persistence' could be linked to a delayed antibody response (Iwasaki *et al*., 2011; Martin *et al*., 2003). HCV neutralising antibodies have been readily detected in both

acute and chronic HCV infections (Cashman *et al*., 2014). In recent years, neutralising antibodies have been linked to spontaneous HCV clearance and are therefore of particular interest (Giang *et al*., 2012; Law *et al*., 2008; William Osburn *et al*., 2010; Osburn *et al*., 2014). However, due to the lack of an appropriate immunological assay, it has not been possible to investigate whether neutralising antibodies can be detected in New World monkeys experimentally infected with GBV-B.

To investigate the neutralising antibody response in GBV-B infection as a model of what might happen in an acute HCV infection, a GBV-B neutralisation assay is required. Retroviral-based PV consist of a retroviral core with the envelope proteins of the virus substituted for a foreign virus' envelope. This permits the study of viruses that are difficult to propagate in cell culture, such as HCV and GBV-B, or highly pathogenic viruses that require high level biocontainment facilities (Bentley, Mather and Temperton, 2015, King *et al*., 2016). Additionally, the retroviral particle genome encodes a reporter gene, flanked by LTRs, instead of its original viral genome making the particle replication-deficient. For these reasons, PV have been employed in both research and clinical applications, including the study of virus-host interactions, determining antibody responses, serosurveillance, for use as immunogens and for the testing of novel antiviral therapies (Temperton *et al*., 2015). As HCV is difficult to propagate in cell culture, HCV PV have been used extensively to characterise the envelope glycoproteins, the receptors required for virus entry, host range, tropism and the neutralising capabilities of patient sera (Bartosch *et al*., 2003; Bartosch and Cosset, 2009; Ploss *et al*., 2009; Tarr *et al*., 2011).

Based on the method used for HCV (Bartosch *et al*., 2003), this project aimed to produce retroviral-based PV expressing GBV-B envelope proteins that can transduce a susceptible cell line. Once this neutralisation assay is established, archived GBV-infected tamarin sera will be

tested in this system. Neutralising antibodies found through this model could be epitope mapped, and potentially incorporated into HCV vaccine design strategies.

5.2 RESULTS

5.2.1 GBV-B ENVELOPE PROTEINS, E1 AND E2 CAN BE CLONED AND EXPRESSED IN A MAMMALIAN CELL LINE

To produce PV expressing the GBV-B envelope, the envelope proteins, E1 and E2, were first cloned. A cDNA fragment containing the first 3000bp of the GBV-B genome had been previously generated in the lab from vRNA isolated from a GBV-B-infected tamarin. Using this fragment as a template, a PCR was performed to amplify the final 60 nucleotides of GBV-B core, for use as a signal peptide for the E1E2 genes, along with the E1E2 genes (Figure 5-1A, see Chapter **2.2.4**).

The primers – HINDIIIE1E2_F and E1E2CLAI_R (Table 2-2)- used for E1E2 amplification include the restriction sites *Hind*III and *Cla*I for sub-cloning into an expression vector, a Kozak sequence in the forward primer to increase E1E2 protein synthesis and a nt→nt mutation in the reverse primer to introduce an amino acid substitution (A→R) at the C-terminal amino acid of E2 (Figure 5-1A,B). The Alanine to Arginine mutation was required to disrupt the cleavage site and allow the fusion of an HA tag downstream of the E2 sequence. The C-terminal HA tag sequence was included for detection of E1E2 as no GBV-B envelope antibodies are commercially available; it was created by subcloning the E1E2 fragment into a pcDNA3 vector containing an HA tag sequence downstream of the *Cla*I restriction sites, available in the lab (Figure 5-1C). The E1E2 construct was cloned into pcDNA3 using the restriction enzymes, *Hind*III and *Cla*I and its identity was confirmed by Sanger sequencing.

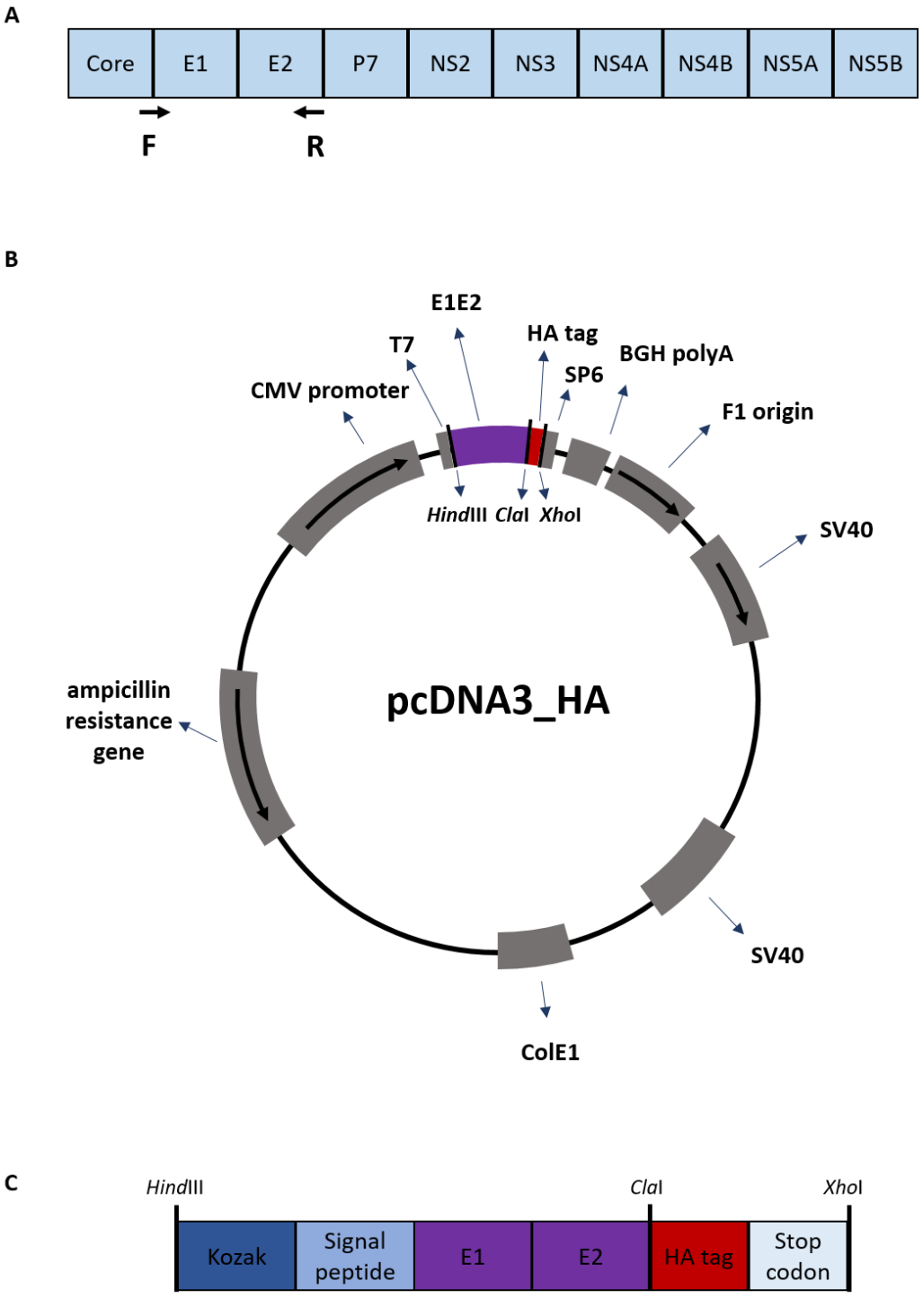


FIGURE 5-1: THE CLONING STRATEGY OF GBV-B ENVELOPE PROTEINS, E1 AND E2

(A) GBV-B GENOME STRUCTURE WITH ALIGNED PRIMERS – HINDIIIE1E2_F AND E1E2CLAI_R - FOR THE CLONING OF E1 AND E2.

(B) MAP OF THE PLASMID PCDNA3 CODING GBV-B E1E2 SEQUENCES.

(C)) THE FINAL PRODUCT SCHEME OF E1E2, AFTER PCDNA3_HA SUBCLONING, INCLUDING THE NECESSARY ELEMENTS FOR DOWNSTREAM APPLICATIONS.

The CMV promoter-driven expression vector pcDNA3 containing GBV-B E1E2 was used to transfect Human Embryonic Kidney 293T (293T) cells using the transfection reagent, Fugene HD (as described in Chapter **2.4.5**). As a positive control for the western blot, pcDNA3 containing an irrelevant HA-tagged protein (human PERV-A receptor 2, huPAR-2, Mattiuzzo *et al*., 2009) was transfected in parallel. After 72 hours, transfected cells were lysed using radioimmunoprecipitation assay (RIPA) buffer and separated by size through an SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with an HA antibody (Figure 5-2A). As showed in Figure 5-2A, GBV-B E1E2 is expressed in 293T cells in a similar manner to that seen in a recent study (Marnata *et al*., 2015).

GBV-B E1 and E2 proteins are heavily glycosylated proteins (Ghibaudo *et al*., 2004) and this prevents the correct size determination and causes smearing when visualised in a western blot. Therefore, transfected 293T cells were treated with tunicamycin, a N-linked glycosylation inhibitor (Powell, 2001), and the correct size of the proteins was verified (Figure 5-2B).

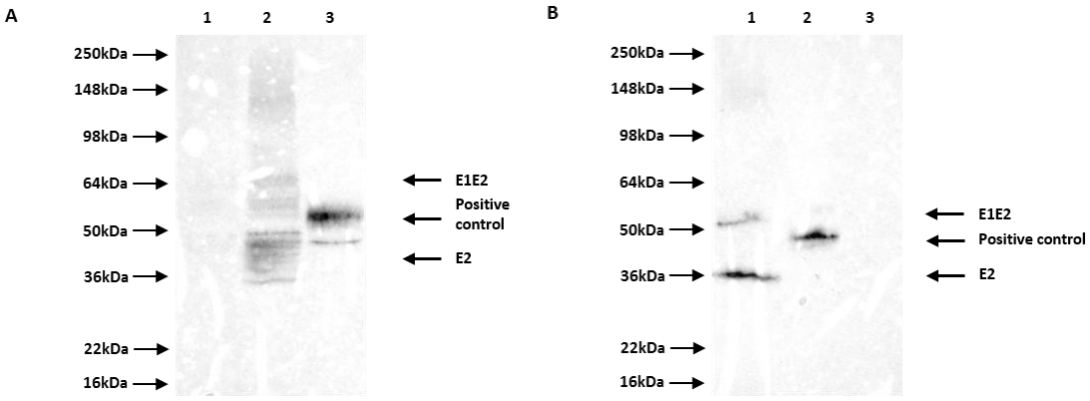


FIGURE 5-2: EXPRESSION OF GBV-B E1E2 IN TRANSFECTED 293T CELL LYSATE

WESTERN BLOT OF LYSATE OF 293T CELLS TRANSFECTED WITH EITHER PCDNA3_GBV-B E1E2_HA OR AN IRRELEVANT HA TAGGED PROTEIN, USING AN HA ANTIBODY. (A) LANE 1: UNTRANSFECTED CELLS, LANE 2: GBV-B E1E2 TRANSFECTED CELLS, LANE 3: CELLS TRANSFECTED WITH POSITIVE CONTROL PLASMID. (B) LYSATE OF 293T CELLS TREATED WITH TUNICAMYCIN 24HR POST TRANSFECTION TO INHIBIT N-LINKED GLYCOSYLATION. LANE 1: GBV-B E1E2 TRANSFECTED CELLS, LANE 2: CELLS TRANSFECTED WITH POSITIVE CONTROL PLASMID, LANE 3: UNTRANSFECTED CELLS. GBV-B E2 MOLECULAR WEIGHT IS APPROXIMATELY 36KDA, WHILST UNCLEAVED E1E2 IS APPROXIMATELY 55KDA.

5.2.2 CO-TRANSFECTION OF 293T CELLS LEADS TO THE PRODUCTION OF LENTIVIRAL PARTICLES LACKING A GBV-B ENVELOPE

Once GBV-B E1E2 protein expression was confirmed, lentiviral-based PV were produced through the co-transfection of 293T cells with three plasmids: the packaging plasmid pCMV8.91 containing the structural and enzymatic HIV-1 genes *gag* and *pol*, an expression plasmid containing the envelope proteins of GBV-B and a vector, pCSGW, containing the reporter gene Green Fluorescent Protein (GFP) between the two HIV LTRs, which will be incorporated in the particles as genomic RNA (ratio: 1:1:1.5, see Chapter **2.4.6**). In parallel, PV were also produced containing no envelope, through the omission of the Env-expressing plasmid as a negative control, or with the VSV envelope, by substituting the GBV-B envelope for the protein G of VSV (VSV-G) as a positive control. VSV-G was chosen as a positive control

Env because it produces high titre PV, is very stable and has a wide tropism (Hastie *et al*., 2013).

To check for PV production, 48 and 72 hours post transfection, supernatant from transfected cells containing PV was collected and concentrated by centrifugation at 13000rpm in a Biofuge Pico bench-top centrifuge at 4°C for 4 hours, as described in Chapter **2.4.9**; proteins were separated in an SDS-PAGE and samples analysed by immunoblotting (Figure 5-3, see Chapter **2.4.10**). Lentiviral particle formation was verified for all the three samples by anti-HIV p24 staining (Figure 5-3A); p24 or capsid, is the main component of HIV core, and it is present in its three forms: as part of the unprocessed HIV-1 Gag polyprotein (p55), as the partially processed p41 and the fully processed form, p24. As p24 is present in all supernatant samples and can self-assemble into viral particles (Ehrlich *et al*., 1992), it can be assumed that all samples contain lentiviral particles. To verify whether the VSV or GBV-B envelope proteins had been successfully incorporated into PV, the same samples were probed with either an anti-VSV-G antibody (Figure 5-3B) or an anti-HA antibody (Figure 5-3C). Whilst a band of the correct size for VSV-G was visualised in only the lane containing VSV-G PV (lane 2 Fig 5-3B), HA-tagged GBV-B E1E2 was not detected by anti-HA staining (lane 3, Fig 5-3C), suggesting that GBV-B Env has not been incorporated in the lentiviral particles.

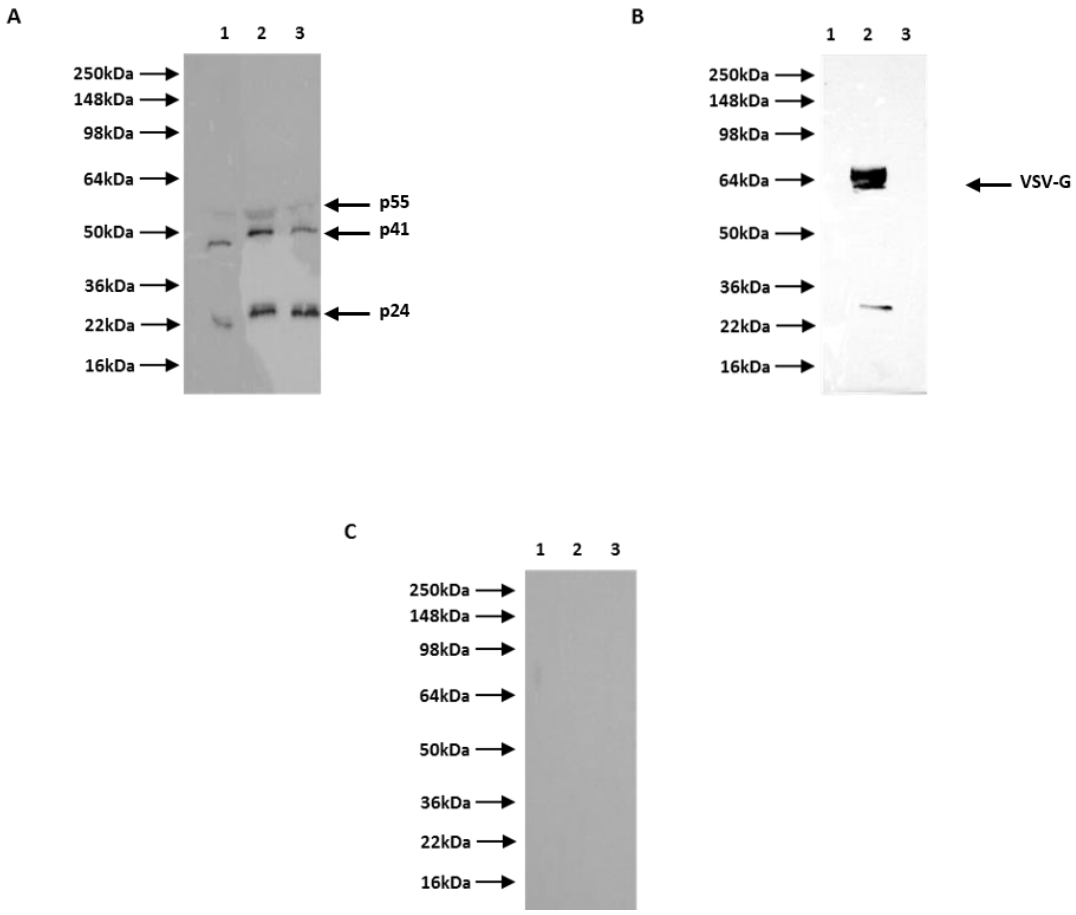


FIGURE 5-3: PRODUCTION OF RETROVIRAL-BASED LENTIVIRAL PARTICLES EXPRESSING VSV-G OR GBV-B ENVELOPE

WESTERN BLOT OF THE CONCENTRATED SUPERNATANT OF TRANSFECTED 293T CELLS, PROBED WITH AN ANTI-HIV P24 ANTIBODY (A), AN ANTI-VSV-G ANTIBODY (B), WHERE A BAND AT THE CORRECT SIZE OF ~57KDA IS SEEN ONLY IN THE LANE CONTAINING THE VSV-G PV, OR AN ANTI-HA ANTIBODY (C). NO SIGNAL IS SEEN INDICATING NO GBV-B ENVELOPE IS PRESENT.

LANE 1: NO ENVELOPE; LANE 2: VSV-G; LANE 3: GBV-B E1E2.

5.2.3 GBV-B E1E2 EXPRESSION IS SUBOPTIMAL WHEN CO-TRANSFECTED WITH OTHER PLASMIDS

HCV envelope proteins contain ER retention signals that act through charged amino acids in the transmembrane domain of the proteins to ensure their retention (Cocquerel *et al.*, 1998a). As GBV-B envelope proteins have a similar organisation to HCV envelope proteins

and charged residues within the transmembrane domains, GBV-B envelope proteins are also presumed to contain similar signals. This is an issue for lentiviral PV production as lentiviruses bud from the cellular membrane and will therefore not obtain the GBV-B envelope during pseudotype virus production. However, 'leaky' expression of the HCV envelope proteins on the cellular membrane has been seen if the expression level is sufficiently high (Bartosch *et al*., 2003; Drummer *et al*., 2003).

The concentration of pcDNA3_E1E2_HA needed for a sufficient E1E2 expression level was investigated. This was determined by co-transfection of 293T cells with pcDNA3_E1E2_HA in combination with an empty plasmid, pcDNA3.1. E1E2 expression was then determined by visibility in western blot. Cell lysate from 293T transfected with either pcDNA3_E1E2_HA alone or in combination with pcDNA3.1 at different ratios were analysed in a western blot using an anti-HA antibody (Figure 5-4A). This blot indicated that GBV-B E1E2 expression was suboptimal if co-transfected with any other plasmid.

To confirm this effect, intracellular staining for the C-terminal HA tag of GBV-B E2 was performed on the same transfected cells and analysed by flow cytometry (Fig 5.4B, see Chapter **2.4.11**). HA-tagged E2 was detected when co-transfected with pcDNA3.1 but at a lower level than a single plasmid transfection: 16% positive cells in comparison to 12%. Additionally, a decrease in concentration of pcDNA3_E1E2_HA below 1.25µg resulted in only 2% HA-positive cells.

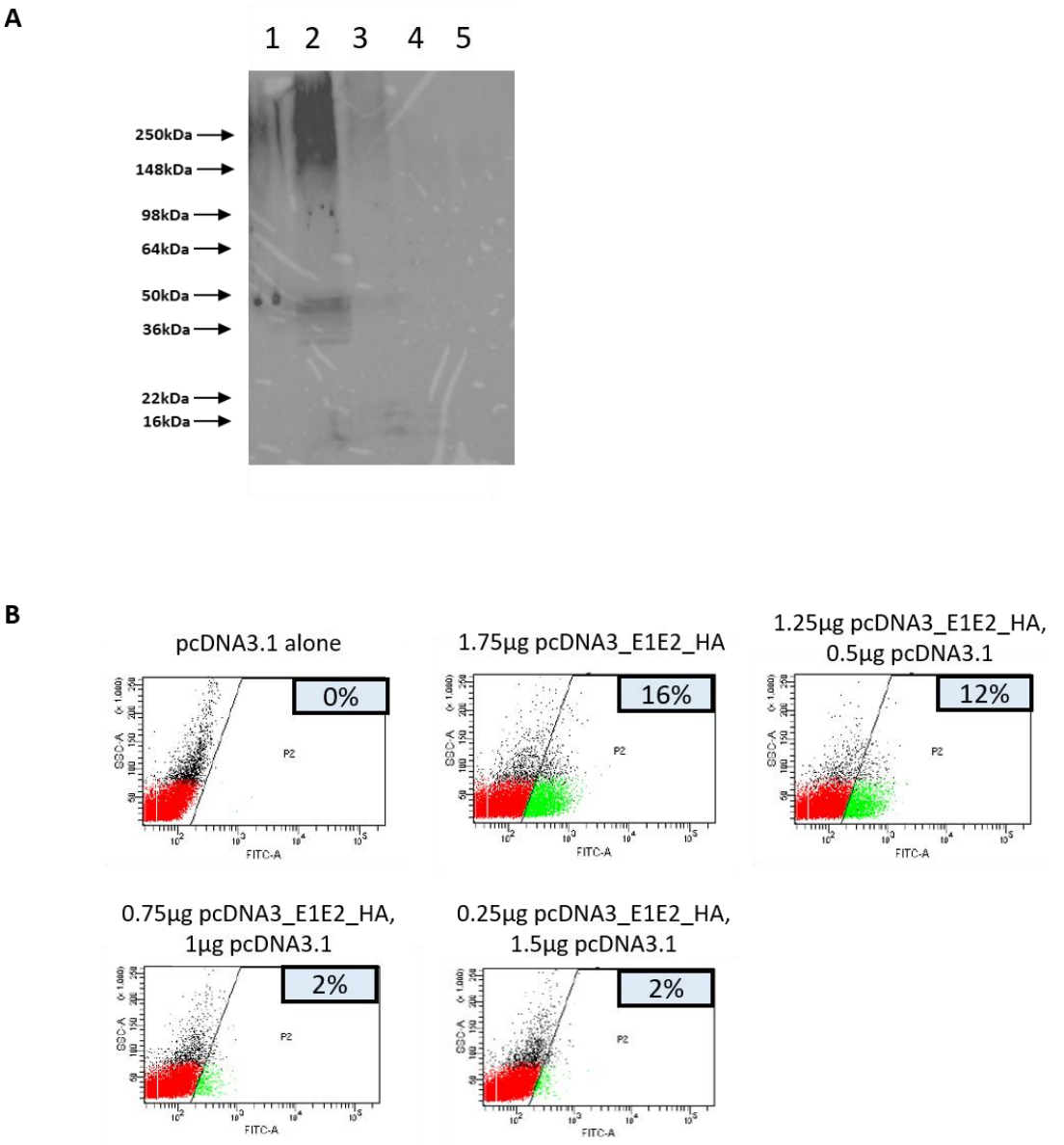


FIGURE 5-4: CO-TRANSFECTION OF A PCDNA3-E1E2 WITH AN EMPTY PLASMID RESULTS IN A SEVERELY REDUCED GBV-B E1E2 EXPRESSION LEVEL

A: WESTERN BLOT SHOWING THE REDUCTION IN PROTEIN EXPRESSION OF GBV-B E1E2 IN TRANSFECTED 293T CELL LYSATE DURING CO-TRANSFECTION WITH ANOTHER PLASMID. CELLS WERE TRANSFECTED WITH LANE 1: 1.75MG OF PCDNA3.1 ALONE, LANE 2: PCDNA3-E1E2 ALONE, LANE 3: 1.25MG PCDNA3-E1E2 AND 0.5MG PCDNA3.1, LANE 4: 0.75MG PCDNA3-E1E2AND 1MG PCDNA3.1, LANE 5: 0.25MG PCDNA3-E1E2 AND 1.5MG PCDNA3.1.

B: THE ABOVE EXPERIMENT WAS REPEATED AND CELLS WERE INTRACELLULARLY STAINED WITH AN HA ANTIBODY, DETECTED WITH AN ANTI-MOUSE IGG ANTIBODY CONJUGATED WITH FITC AND ANALYSED BY FLOW CYTOMETRY.

5.2.4 THE TRANSFECTION OF A STABLE PACKAGING CELL LINE, BASED ON STAR CELLS, DID NOT LEAD TO THE INCORPORATION OF GBV-B E1E2 PROTEINS ON PV

As GBV-B E1E2 expression is suboptimal if co-transfected alongside another plasmid, a stable packaging cell line was generated that continuously produces high titres of PV lacking an envelope (ΔEnv PV). The expectation was that GBV-B envelope could later be transfected into these cells alone potentially enabling a much higher expression level.

HIV-1 packaging cells, STAR cells (Ikeda *et al*., 2003), have been kindly donated by Dr Y Takeuchi, UCL. These cells were produced by co-transfecting 293T cells with codon-optimised HIV-1 Gag-Pol and Rev within gammaretroviral vectors, which resulted in the stable insertion of HIV-1 *gag* and *pol* sequences into chromosomal DNA and its subsequent high-level, stable production. To stably express the reporter gene, STAR cells were transduced with lentiviral particles, produced by transient transfection of 293T cells, containing as its genome an HIV-1 vector carrying the *gfp* gene under the transcription control of wild type HIV-1 LTRs (see Chapter **2.4.7**). The lentiviral particles were generated using the Env of RDpro to allow for a high efficiency of transduction. Upon infection, the viral genome of the transducing particles integrates into genome of the STAR cells, allowing for a continuous transcription of the vector. The rate of transduction was measured as percentage of GFP-positive cells using flow cytometry analysis; after one round of transduction with the RDpro-HV virus, there was GFP reporter gene expression from 67% of STAR cells (Figure 5-5B). A second round of transduction was performed and resulted in 98% of cells expressing the reporter gene (Figure 5-5C).

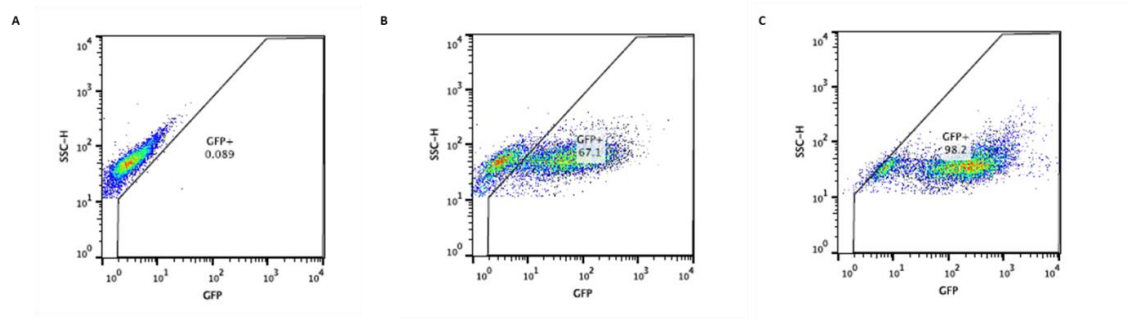


FIGURE 5-5: PRODUCTION OF HIV-1 PACKAGING CELLS STAR CELLS STABLY EXPRESSING HIV-GFP PARTICLES

FLOW CYTOMETRY ANALYSIS OF RDPRO-HV TRANSDUCED STAR CELLS MEASURING GFP EXPRESSION. A) UNTRANSDUCED STAR CELLS, (B) STAR CELLS AFTER A SINGLE AND (C) SECOND ROUND OF TRANSDUCTION WITH THE RDPRO-HV VIRUS.

STAR-HV cells, stably expressing the pHV plasmid containing *gfp*, were subsequently transfected using Fugene 6 with 1µg of either a plasmid containing the VSV or GBV-B envelope with the aim of producing PV expressing the aforementioned viral envelopes on their surface. Supernatant from transfected cells was collected at 48 and 72 hours, filtered and concentrated by centrifugation. Viral particles in the concentrated supernatant were run on an SDS-PAGE to separate proteins by size and immunoblotted with one of three antibodies: an anti-p24 (Figure 5-6A), an anti-VSV-G (Figure 5-6B) and an anti-HA (Figure 5-6C).

Lentiviral particles were successfully produced as evidenced by the presence of p24, the processed HIV capsid protein in all three viral samples at similar levels (Figure 5-6A). Additionally, PV could be produced expressing VSV-G (Figure 5-6B). Although data in Figure 5-6C appear to show that GBV-B E2 was incorporated into PV, this result could not be repeated. A possible explanation for the signal in the GBV-B Env lane could be free E2 protein in the supernatant released from dying cells, as the transfected STAR cell lysate from PV production had been confirmed to contain expressed E1E2 (Figure 5-6D).

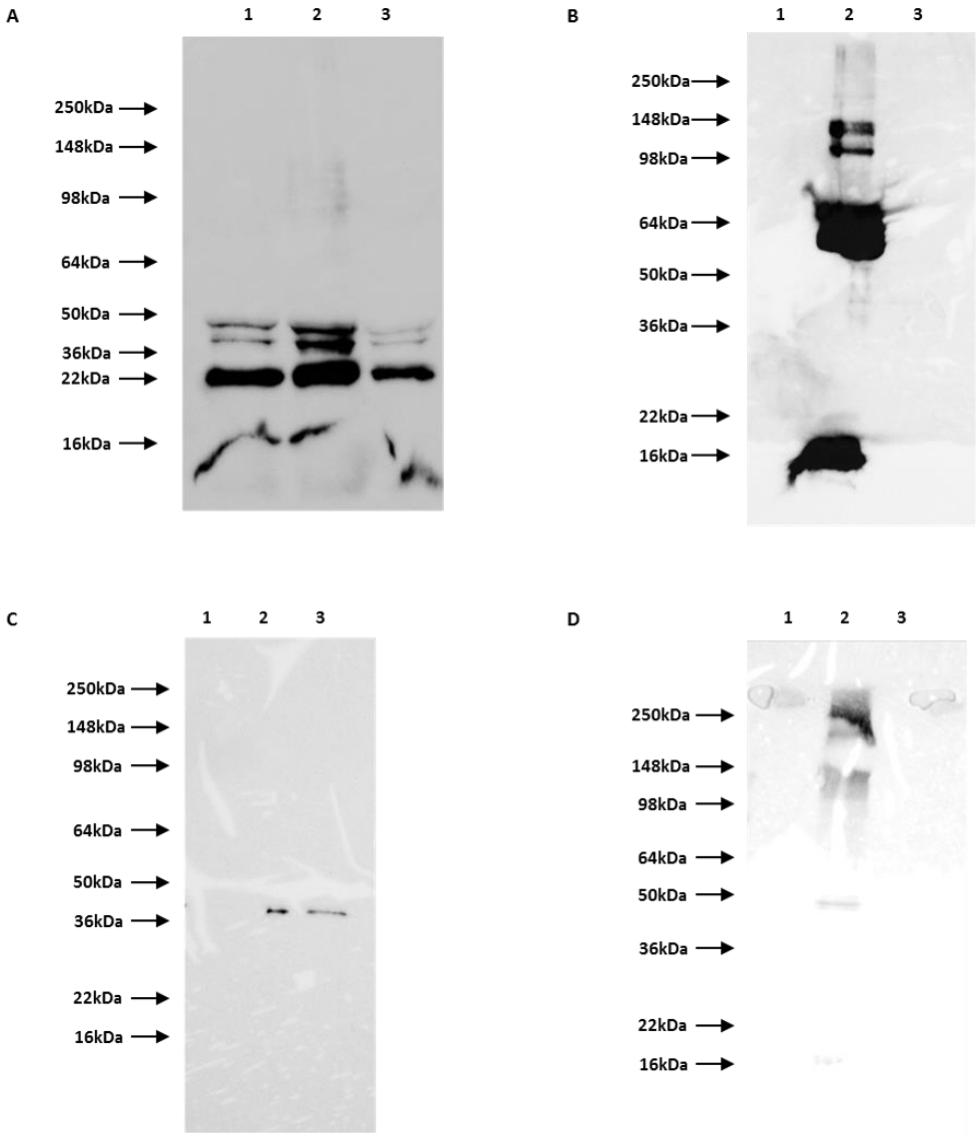


FIGURE 5-6: ANALYSIS OF THE ENV INCORPORATION ONTO PV PRODUCED FROM STABLE CELL PACKAGING CELL LINE, STAR-HV

PV IN THE SUPERNATANT OF TRANSFECTED STAR-HV CELLS WERE IMMUNOBLOT WITH AN ANTI-P24 ANTIBODY (A) AN ANTI-VSV-G ANTIBODY (B) OR AN ANTI-HA TAG ANTIBODY (C) LANE 1: NO ENVELOPE, LANE 2: VSV-G, LANE 3: GBV-B ENVELOPE.

(D) WESTERN BLOT OF TRANSFECTED STAR CELL LYSATE USING AN ANTI-HA TAG ANTIBODY.

LANE 1: UNTRANSFECTED CELLS; LANE 2: GBV-B E1E2 TRANSFECTED STAR CELLS; LANE 3: VSV-G TRANSFECTED STAR CELLS.

5.2.5 THE TRANSFECTION OF HCV AND GBV-B ENVELOPE PROTEINS INTO A PACKAGING CELL LINE DID NOT RESULT IN THE PRODUCTION OF INFECTIOUS PV

To understand why GBV-B E1E2 could not be detected on the PV by western blots, an infectivity assay was set up using human hepatoma cell line, Huh7. These cells are known to be permissive for HCV PV entry (Bartosch *et al*., 2003). HCV PV were produced through the transfection of a plasmid containing HCV E1E2 (kindly supplied by Dr B. Bartosch) into STAR-HV cells, for use as a positive control. This positive control is more relevant as the biology, including the life cycle, of the two viruses is much more similar than retroviruses. In parallel to the production of PV in STAR-HV cells, PV were also produced by transient transfection in 293T cells as described in Chapter **5.2.2**.

Filtered supernatant from both transfected STAR-HV and 293T cells were then used to infect Huh7 cells, in the presence of polybrene. After 48 hours, GFP expression in cells was analysed by flow cytometry. None of the samples was positive for GFP expression, and therefore infection. This was somewhat surprising as HCV PV production has been well documented in the literature which indicates that there is a more general issue with PV production (Bartosch *et al*., 2003; Bartosch and Cosset, 2009; Pestka *et al*., 2007; Tarr *et al*., 2011; Vieyres *et al*., 2010).

5.2.6 OPTIMISATION OF PRODUCTION OF GBV-B PV

5.2.6.1 INCREASE OF ENVELOPE PROTEIN EXPRESSION IN PRODUCER CELLS

To increase the expression level of GBV-B envelope proteins, the *E1E2* genes were sub-cloned into a eukaryotic expression vector pCAGGS that contains a different promoter (see Figure 7-6), which has been previously successfully used for the expression of viral Env protein (Niwa

et al., 1991, Kuhl *et al*., 2011). GBV-B Env was amplified using the NSIIE1E2_F primer (Table 2-2) which contains a *Nhe*I site instead of *Hind*III site, and the SP6 universal primer (Table 2-2). The fragment was then cloned into pCAGGS using *NheI* and *Xho*I (a restriction site downstream of *Cla*I in the vector pcDNA3). Sanger sequencing confirmed the correct identity of the E1E2 fragment in this plasmid. Transfection of 293T cells was then performed with pCAGGS-GBV-B E1E2 using the transfection reagent XtremeGeneHP. Cell lysate was immunoblotted with an anti-HA tag antibody, which showed a good level of expression (Figure 5-7).

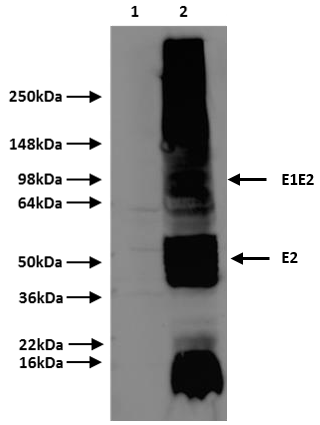


FIGURE 5-7: WESTERN BLOT SHOWING THE INCREASED EXPRESSION LEVEL OF GBV-B ENVELOPE PROTEINS IN TRANSFECTED CELL LYSATE

LANE 1: UNTRANSFECTED CELLS, AND LANE 2: GBV-B E1E2 TRANSFECTED CELLS. GLYCOSYLATED GBV-B E2 SIZE APPROXIMATELY 44KDA WHILST GLYCOSYLATED E1E2 SIZE IS 75KDA.

PV production was repeated with the three-plasmid transfection using the following plasmids: pCMV8.91 (HIV *gag* and *pol*), pCSFLW (*firefly luciferase* reporter gene) and the relevant viral envelope proteins (HCV and GBV-B). Plasmids were transfected into 293T cells in a 1:1:1.5 ratio using the XtremeGeneHP transfection reagent. PV were then concentrated

by centrifugation and run in a western blot. Although GBV-B E1E2 expression had increased, PV production remained unsuccessful (data not shown).

5.2.6.2 USE OF ALTERNATIVE REPORTER GENE AND DETECTION METHOD

To further optimise the PV production the reporter gene GFP was substituted for the more sensitive firefly luciferase gene (*luc*) using the restriction enzymes EcoRI and NotI. Bioluminescent reporter products, including luciferase, are known to be more sensitive and have a broader dynamic range than reporter genes based on fluorescence, such as GFP (Paley and Prescher, 2014). An alternative protocol was used to produce GBV-B PV, based on published work on HCV PV, as described in Chapter **2.4.6** (Tarr *et al*., 2007; Urbanowicz *et al*., 2015). Lentiviral PV were created from a first generation packaging plasmid which contains the HIV genome with the firefly luciferase gene inserted instead of the *nef* gene. This plasmid contains wild type LTRs, with intact HIV U3 promoters, and therefore replicates as normal wild-type virus would. In comparison, self-inactivating vectors, due to the deletion of a region in the U3 promoter at the 3'LTR, can only undergo one round of replication. In addition to wild-type LTRs which yield higher titres of PV, frameshifts ensure that the virus produced does not express the HIV envelope.

HEK293T cells were transfected using PEI with the first-generation packaging plasmid, pNL4.3luc+Env-, which contains HIV-1 *gag-pol* and the *luc* gene in the place of the HIV envelope. Different amount of plasmid pCAGGS expressing GBV-B envelope proteins were added to the transfection mix. As controls, HCV PV were produced as above (as positive control) alongside PV lacking the envelope through the omission of the envelope-containing plasmid (ΔEnv PV, as negative control). HCV envelope proteins were provided through a plasmid containing the synthetic construct isolate UKN1A20.8 envelope glycoprotein gene

(kindly donated by Alex Tarr, University of Nottingham, GenBank number: ABV82434.1) as these HCV envelope proteins have been well documented to efficiently incorporate into lentiviral-based particles (Tarr *et al*., 2013, 2011).

Supernatant from transfected cells was collected, filtered using a 0.45µm filter and serial dilutions were used to infect the human hepatocellular carcinoma cell line Huh7, in the presence of polybrene (Figure 5-8, see Chapter **2.4.12**). Infected cells were detected by measuring the level of luciferase production using the Bright-Glo Luciferase Assay System. Lentiviral GBV-B PV, made with either 400ng or 80ng of the plasmid containing GBV-B envelope proteins generated a signal higher than the background (PV delta Env) but only the PV produced with 400ng of pCAGGS-E1E2 showed a dilution response titration (Figure 5-8).

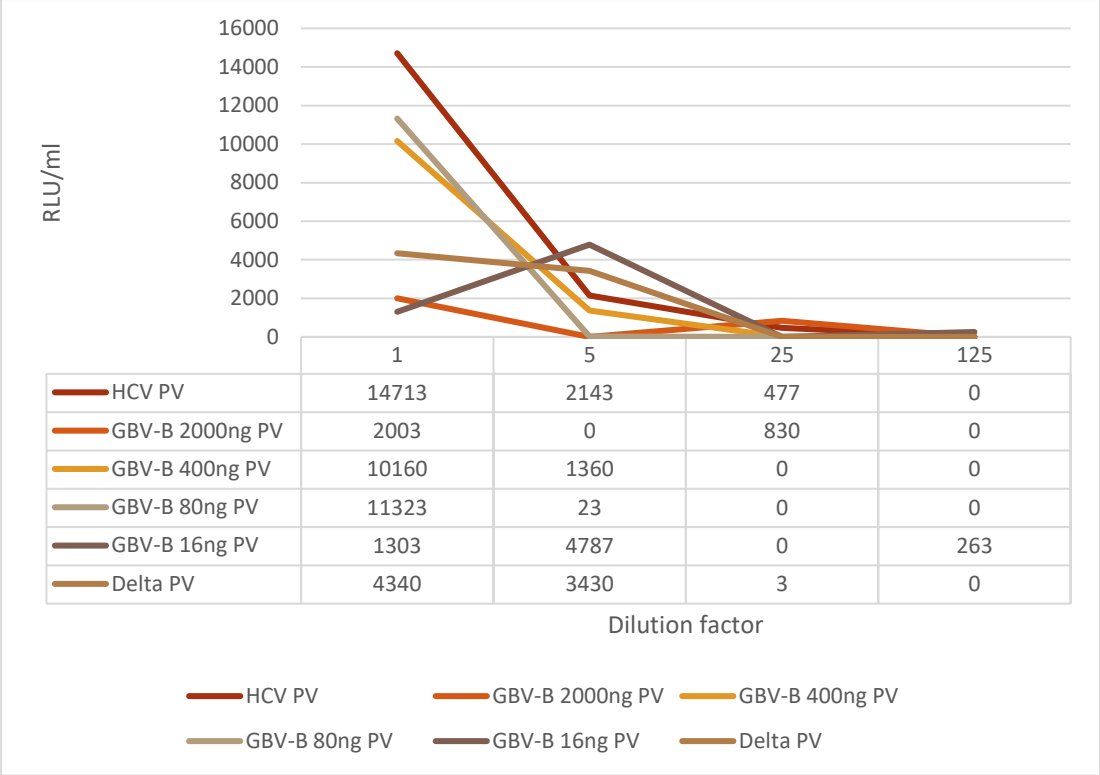


FIGURE 5-8: INFECTION OF HUH7 CELLS WITH LENTIVIRAL-BASED PV

LENTIVIRAL-BASED PV BEARING EITHER HCV, GBV-B OR NO ENVELOPE (ΔENV) WERE GENERATED THROUGH CO-TRANSFECTION OF 293T CELLS. PV WERE PRODUCED USING 2µG OF HCV E1E2- EXPRESSING PLASMID OR DIFFERENT AMOUNTS OF PCAGGS-GBVB E1E2 (16NG, 80NG, 400NG OR 2 µG). SERIAL DILUTION OF THE SUPERNATANT COLLECTED AT 72 HOURS WERE USED TO INFECT HUH7 CELLS. INFECTION WAS MEASURED THROUGH THE DETECTION OF BIOLUMINESCENCE AND REPORTED AS RELATIVE LUMINESCENCE UNIT PER ML (RLU/ML). RESULTS ARE MEAN VALUES OF TRIPLICATES READINGS AND THE LUMINESCENCE VALUES ARE SHOWN BELOW.

PV based on a lentiviral core are highly suitable for use in a GBV-B neutralisation assay as they are able to infect non-dividing cells, such as hepatocytes, and are typically produced at high titres. However, the luminescence readout (RLU/ml) obtained with GBV-B PV were lower than for the HCV PV. Some primary isolates of HCV envelope proteins from patients are known to only incorporate onto retroviral-based PV (Alex Tarr, personal communication), therefore pseudotyping of GBV-B Env on to retroviral particles was tested in the attempt to increase the titre of GBV-B PV. The retroviral-based PV were created using the three plasmid co-transfection system and PEI. The producer cell line, 293T, were seeded in a 10cm-dish and

transfected with 1µg of a plasmid containing Moloney MLV (MoMLV) *gag-pol* (pCMV), 1 µg of retroviral vector containing firefly luciferase flanked by MLV LTRs (pCFCR_luc) and different amounts of pCAGG-GBVB E1E2: 80ng, 400ng or 2µg. As a negative control, ΔEnv PV were produced through the omission of the Env plasmid. Supernatant from transfected cells was collected after 72 hours, filtered and serial dilutions were used to infect Huh7 cells in the presence of polybrene. After 3 days, the production of firefly luciferase was measured as before. When compared to lentiviral GBV-B PV production, infection of Huh7 cells using the retroviral GBV-B PV was less efficient (Fig 5-9). Only PV made with 80ng of the pCAGGS-GBV-BE1E2 plasmid gave a good signal above ΔEnv PV background luminescence.

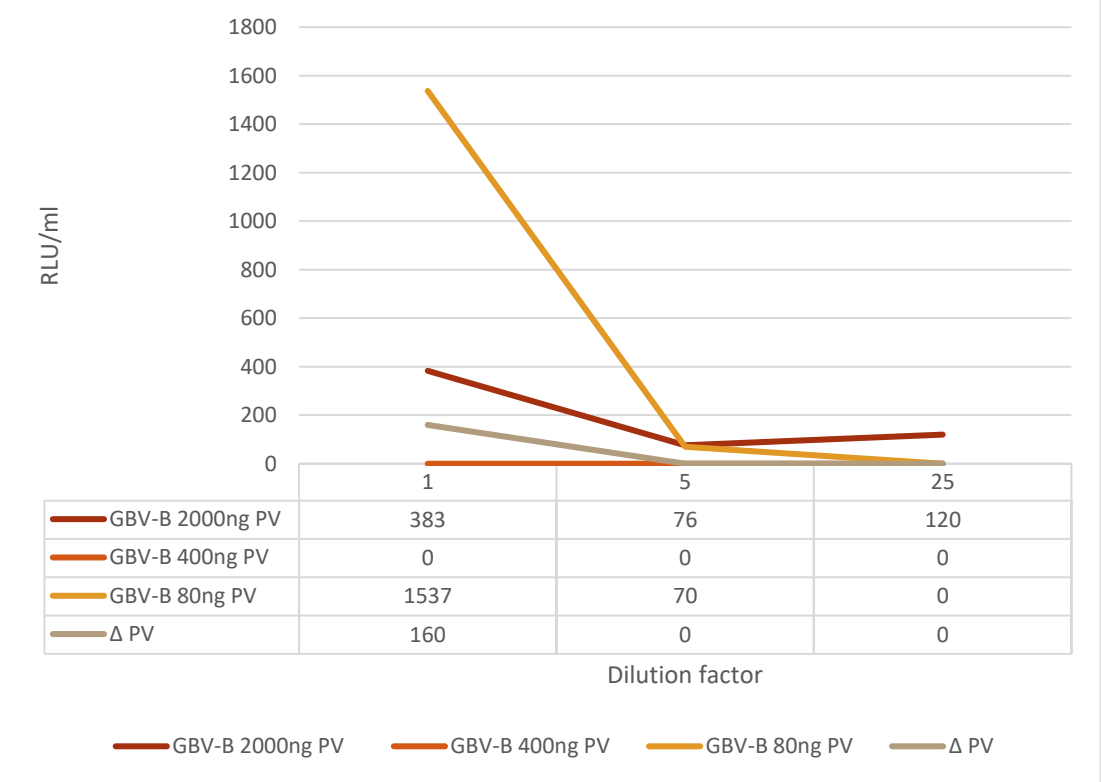


FIGURE 5-9: INFECTION OF HUH7 CELLS WITH RETROVIRAL-BASED PV

RETROVIRAL-BASED PV BEARING EITHER GBV-B OR NO ENVELOPE (ΔENV) WERE GENERATED THROUGH CO-TRANSFECTION OF 293T CELLS AND TITRATED BY ADDING SERIAL DILUTION TO HUH7 CELLS. GBV-B PV WAS PRODUCED USING DIFFERENT AMOUNT OF PCAGGS-GBVB E1E2: 80NG, 400NG OR 2µG. THE RESULTS REPRESENT THE AVERAGE OF THREE REPLICATES AND THE LUMINESCENCE VALUES ARE SHOWN BELOW.

5.2.6.3 DETECTION OF THE ENVELOPE PROTEIN ON PV SURFACE BY IMMUNOSTAINING

Western blots were performed to confirm the presence of the correct viral envelope proteins on the surface of the HCV and GBV-B PV. Lentiviral-based HCV, GBV-B (made with 400ng of pCAGGS-GBV-B_E1E2) and ΔEnv PV were concentrated 63-fold by overnight centrifugation using a slow spin (5,500rpm for 4 hours in a Biofuge Pico bench-top centrifuge with the 7500 3328 microlitre rotor at 4°C, see Chapter **2.4.9**). PV proteins were then separated on an SDS-PAGE, transferred to a nitrocellulose membrane and then blotted with an anti- HIV-1 p24 antibody. All the samples contained lentiviral cores present in the supernatant (Figure 5-10A).

This membrane was stripped and blotted with AP33, an anti-HCV E2 antibody. Although there was some non-specific binding, a band was visible only in the sample containing the HCV PV. HCV E2 is 64KDa (as estimated by DNA Dynamo software), however it is heavily glycosylated and the band seen in the blot is likely to be the E1E2 dimer (Figure 5-10B). This result strongly suggested the incorporation of HCV E2 on the surface of the PV.

The blot was then stained with an anti-HA to detect GBV-B E2, however there was no signal (not shown). Therefore, supernatant containing PV was further concentrated to a final factor of 2000 times through further centrifugation of previously concentrated PV. The concentrated PV samples were then processed as above and immunoblotted with an anti-HA antibody (Figure 5-10C) or an anti-GBV-B E2 polyclonal rabbit sera (Marnata *et al*., 2015) (Figure 5-10D). In both cases, a band representing glycosylated E2 was detected only in the sample containing GBV-B PV, suggesting that GBV-B Env is incorporated onto the lentiviral particles.

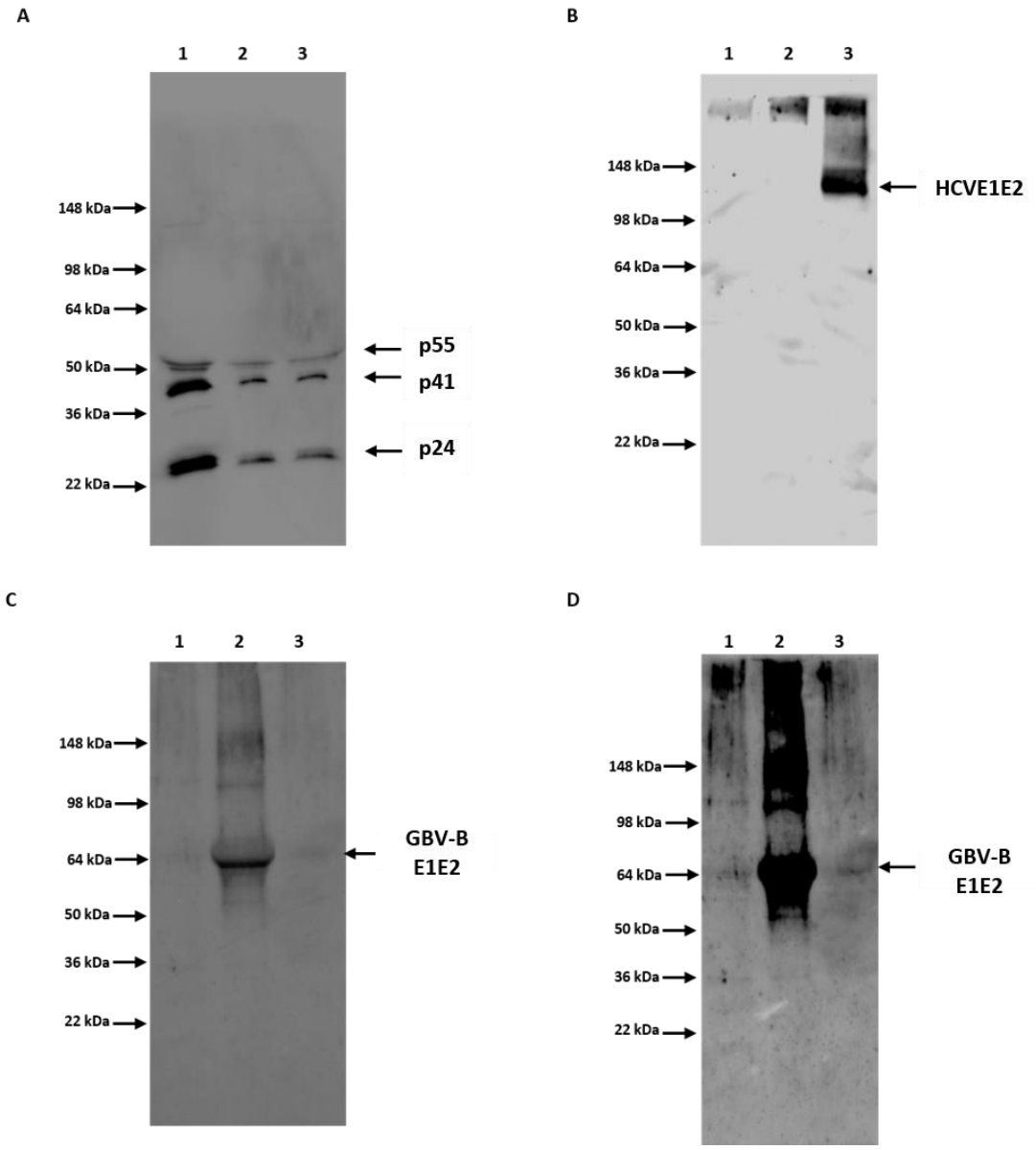


FIGURE 5-10: INCORPORATION OF HCV AND GBV-B ENV PROTEINS ON THE LENTIVIRAL PV

PV PRODUCED WITHOUT ENV (LANE 1), WITH GBV-B E1E2 (LANE 2) OR HCV E1E2 (LANE 3) WERE CONCENTRATED 63-FOLD AND ANALYSED BY WESTERN BLOT USING AN ANTI-P24 ANTIBODY (A) OR AP33 AGAINST HCV E2 ANTIBODY (B).
THE SAME SAMPLES WERE FURTHER CONCENTRATED UP TO 2000 TIMES AND ANALYSED BY WESTERN BLOT WITH AN ANTI-HA ANTIBODY (C) OR A RABBIT ANTI-GBV-B E2 POLYCLONAL SERA (D).

The infectivity of retroviral-based GBV-B PV was much lower than the infectivity seen with lentiviral-based GBV-B PV. To investigate whether this could be due to a less efficient GBV-B

envelope incorporation into the retroviral particle the same analysis by western blot as above was performed on the retroviral-based HCV, GBV-B (made with 80ng of pCAGGS-GBV-B E1E2) and ΔEnv PV. Similar to the results obtained with the lentiviral PV, 63-fold concentrated PV were detected using a polyclonal sera targeting MLV p30 in all the samples (Figure 5-11A). The membrane was then stripped and re-probed with the HCV E2-specific AP33 antibody for the detection of HCV E2 on PV particles. An HCV-specific band was visible indicating incorporation of the HCV Env on the retroviral-based PV (Figure 5-11B). GBV-B PV concentrated 2000-fold were tested using an anti-GBV-B E2 polyclonal rabbit sera, but no specific band was detected in sample containing GBV-B PV (Figure 5-11C). This result suggests that a lower number of infectious, envelope-bearing particles were obtained with the retroviral-based system in comparison with the lentiviral system, which could be due to an inefficient incorporation of the Env onto the particles surface.

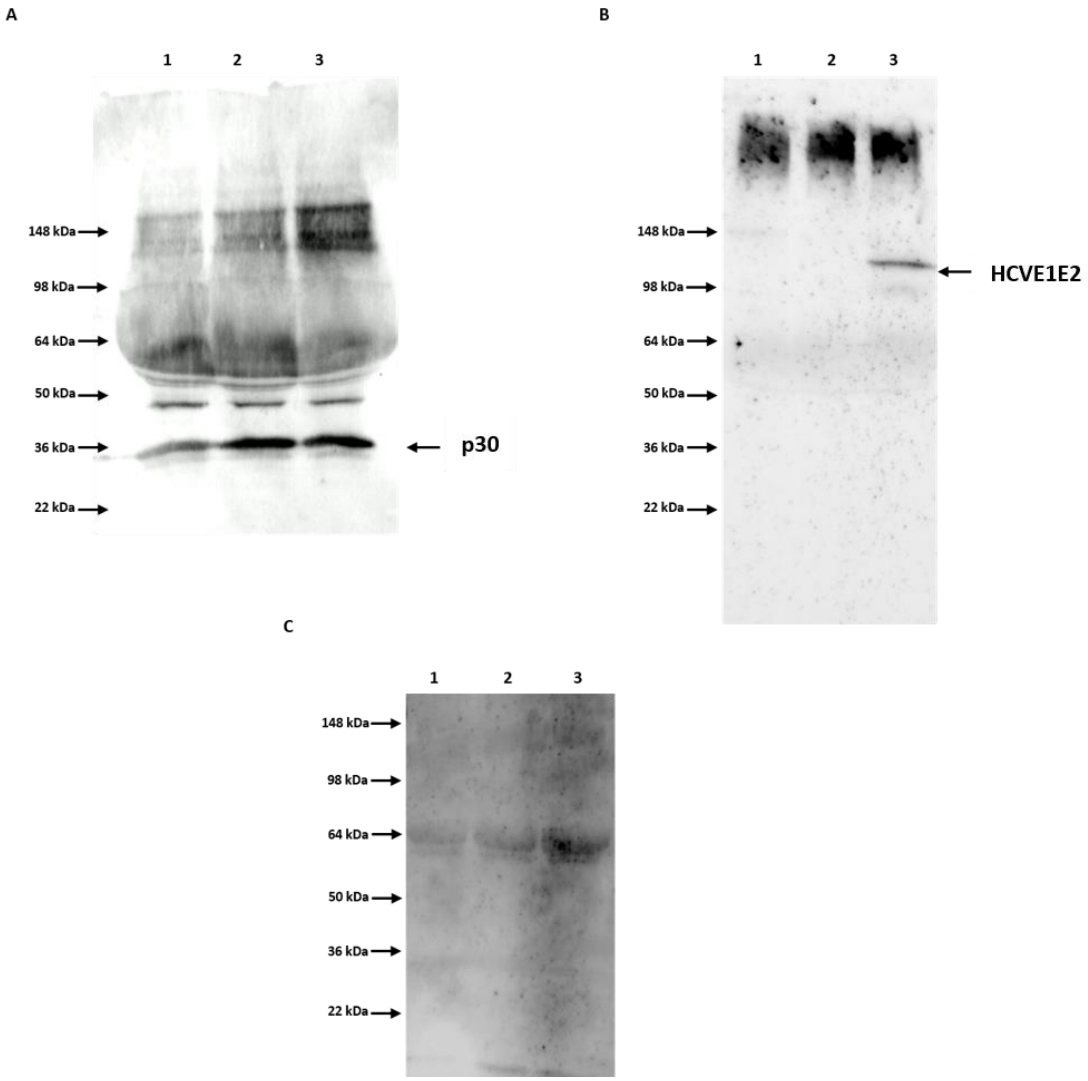


FIGURE 5-11: INCORPORATION OF HCV AND GBV-B ENV PROTEINS ONTO RETROVIRAL-BASED PV

PV PRODUCED WITHOUT ENV (1) WITH GBV-B E1E2 (2) OR HCV E1E2 (3) WERE CONCENTRATED 63-FOLD AND ANALYSED BY WESTERN BLOT USING A RABBIT ANTI-MLV P30 SERA (A) OR AP33 ANTIBODY AGAINST HCV E2 (B). THE SAME SAMPLES CONCENTRATED 2000-FOLD WERE PROBED WITH A RABBIT ANTI-GBV-B E2 POLYCLONAL SERA (C).

5.2.7 OPTIMISATION OF THE PV INFECTION ASSAY

HCV and GBV-B lentiviral-based and retroviral-based PV were successfully produced through the co-transfection of 293T cells with a two-plasmid or three-plasmid system, respectively, and PEI. The infectivity of these particles were determined through the use of an infection assay based on Huh7 cells, however, the infectivity was relatively low and therefore requires optimisation. The incorporation of GBV-B Env could only be confirmed for lentiviral GBV-B PV so these PV were used preferentially.

5.2.7.1 THE STABILITY OF PV CANNOT BE IMPROVED THROUGH THE MODIFICATION OF THE PV PRODUCTION PROTOCOL

It has been previously observed that PV stability is affected by storage temperature (Ghani *et al*., 2009; Strang *et al*., 2005; Wright *et al*., 2009). For example, Wright and colleagues saw a sharper decrease in the titre of lentiviral PV bearing the rabies virus envelope if PV were stored at room temperature in comparison to -20°C (Wright *et al*., 2009). The stability of retroviral PV bearing retroviral envelope proteins, such as VSV-G, has also been described to be poor at 37°C in other similar studies (Ghani *et al*., 2009; Strang *et al*., 2005). Indeed, the infectivity of HCVcc, the JFH-1 isolate of HCV able to replicate in cell culture, is completely lost by 48 hours if stored at 37°C in culture medium (Song *et al*., 2010). Using the protocol from Chapter **5.2.5**, PV are collected at 72 hours post transfection. However, PV can be collected 48 hours post transfection. It is therefore possible that some HCV or GBV-B PV are being degraded before collection. To investigate this hypothesis, HCV and ΔEnv PV were collected from transfected 293T cells at both 48 and 72 hours post transfection. HCV PV were used as these produce higher levels on infectivity than GBV-B PV. PV were then filtered, diluted five-fold and these dilutions were then used to infect Huh7 cells in the presence of

polybrene. After 72 hours, the production of luciferase was measured using Bright Glo reagent (Figure 5-12).

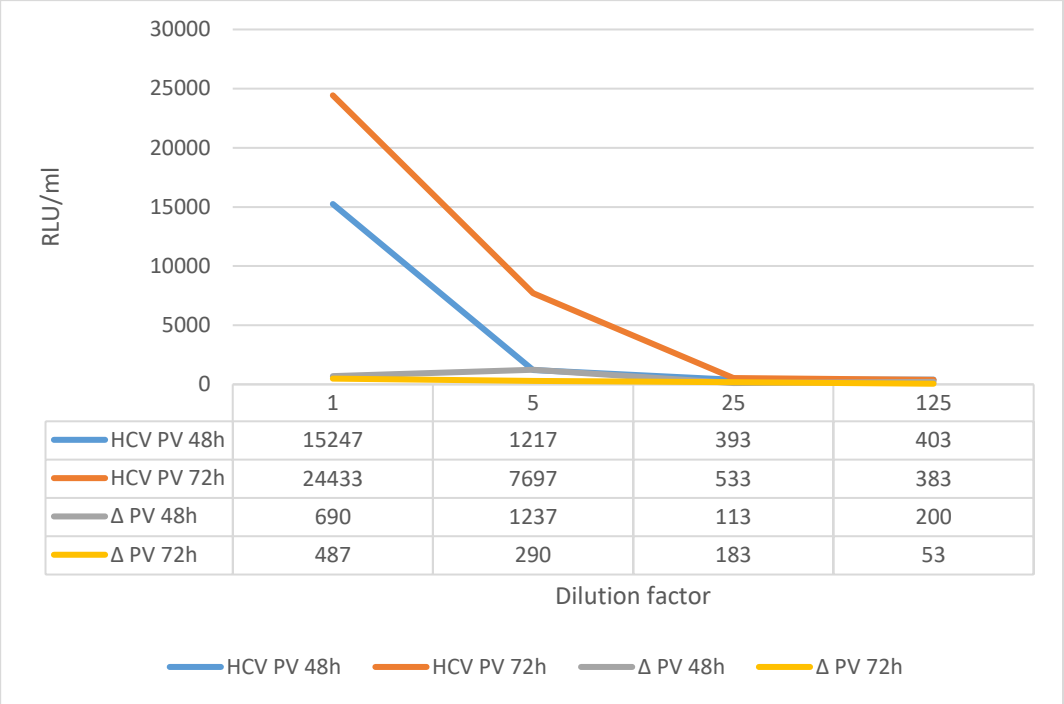


FIGURE 5-12: THE INFECTIVITY OF HCV AND ΔENV PV PRODUCED AT 48 AND 72 HOURS AFTER TRANSFECTION OF 293T CELLS

LENTIVIRAL-BASED PV BEARING EITHER HCV OR NO ENVELOPE (ΔENV) WERE GENERATED THROUGH CO-TRANSFECTION OF 293T CELLS AND COLLECTED AT BOTH 48 HOURS AND 72 HOURS POST TRANSFECTION. THIS SUPERNATANT WAS FILTERED, TITRATED BY SERIAL DILUTION AND ADDED TO HUH7 CELLS. THE RESULTS REPRESENT THE AVERAGE OF THREE REPLICATES AND THE LUMINESCENCE VALUES ARE SHOWN BELOW.

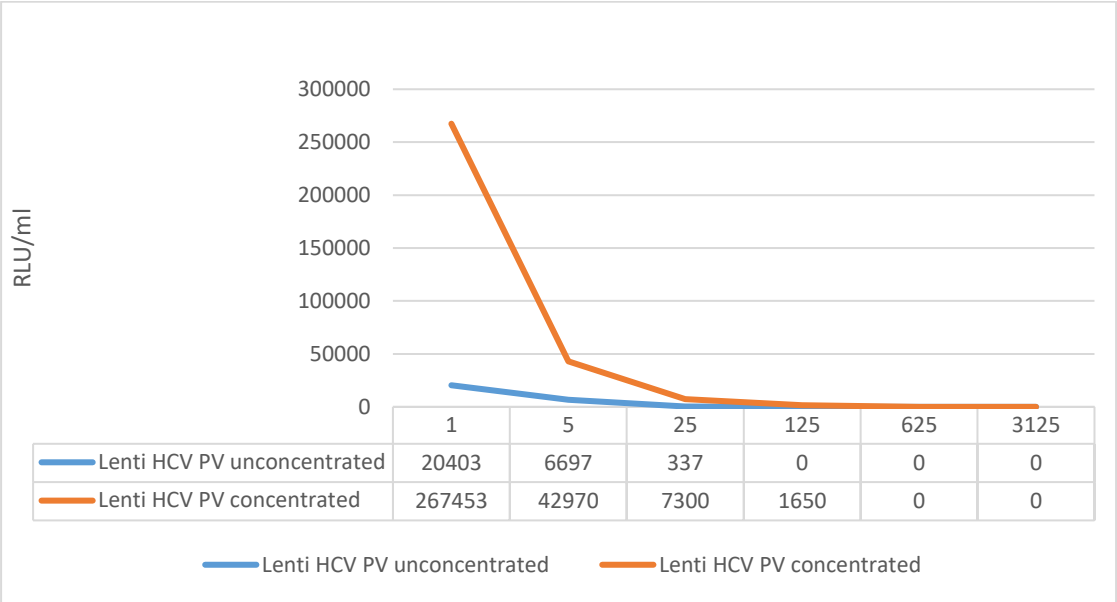
5.2.7.2 LENTIVIRAL-BASED AND RETROVIRAL-BASED GBV-B PV PARTICLES CAN BE CONCENTRATED BY ULTRACENTRIFUGATION

To enhance the infectivity of PV (represented by the production of the luciferase reporter gene), supernatant from transfected 293T cells was concentrated by a factor of 50 by ultracentrifugation through a sucrose cushion (see Chapter **2.4.9**). This concentrated supernatant was then serially diluted and used to infect Huh7 cells, using the established

protocol. This was shown to successfully increase the level of HCV PV infection of Huh7 cells by approximately 10-fold (Figure 5-13A).

After the successful optimisation of HCV PV infectivity of Huh7 cells, these modifications were applied to the protocol for lentiviral and retroviral-based GBV-B PV infection of Huh7 cells. This showed that GBV-B PV could be concentrated, however the final infectivity of GBV-B PV remained lower than HCV PV (Figure 5-13B). Additionally, the production of luciferase from Huh7 cells was higher after infection with lentiviral GBV-B PV than retroviral GBV-B PV.

A



B

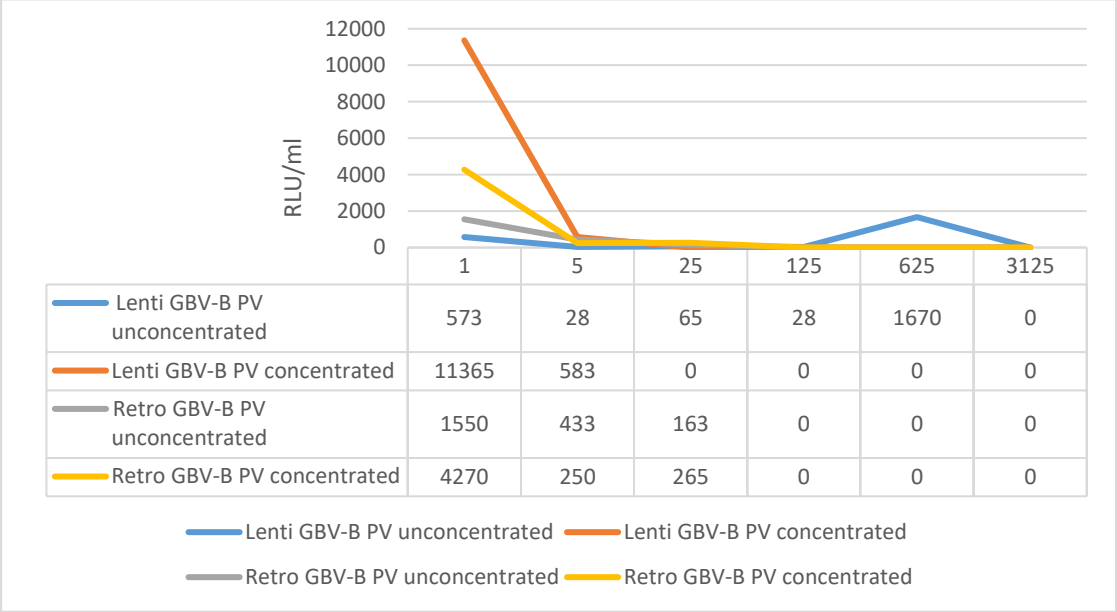


FIGURE 5-13: INFECTIOUS HCV AND GBV-B CAN BE CONCENTRATED BY ULTRACENTRIFUGATION

LENTIVIRAL-BASED PV BEARING EITHER THE HCV (A) OR GBV-B ENVELOPE (B) OR RETROVIRAL-BASED PV BEARING THE GBV-B ENVELOPE (B) WERE GENERATED THROUGH CO-TRANSFECTION OF 293T CELLS. THE SUPERNATANT WAS COLLECTED, FILTERED AND CONCENTRATED BY ULTRACENTRIFUGATION THROUGH A SUCROSE CUSHION. CONCENTRATED PV WERE THEN TITRATED BY SERIAL DILUTION AND ADDED TO HUH7 CELLS. THE RESULTS REPRESENT THE AVERAGE OF THREE REPLICATES AND THE LUMINESCENCE VALUES ARE SHOWN BELOW.

5.2.7.3 INVESTIGATION OF DIFFERENT TARGET CELLS TO ENHANCE GBV-B INFECTIVITY

Huh7 cells were chosen as the target cells for the infectivity assay to gauge the infectivity of GBV-B PV as they have been extensively used to determine the infectivity of HCV PV. To enhance the infectivity seen, different target cells were investigated for their permissiveness for GBV-B PV infection. Primary tamarin fibroblasts, African green monkey cells (Vero), feline kidney cells (CrFK), fetal rhesus monkey kidney cells (FRhK-4) and human hepatocarcinoma (Huh7.5) cells were all tested. These cell lines were tested for the following reasons:

- tamarin fibroblasts as HCV has been previously shown to infect a human fibroblast cell line (Zibert *et al*., 1995);
- FRhK-4 and Vero cells as they are of Old World monkey origin and therefore more closely related to the natural host range of GBV-B (New World monkeys) than human cells lines;
- CrFK as they are known to be permissive for a number of viruses including retroviruses, coronaviruses and herpesviruses (Baldinotti *et al*., 1994; Ikeda *et al*., 1997; Klumperman *et al*., 1994);
- Huh7.5 cells as these have shown a greater susceptibility to HCV infection than Huh7 cells (Bartenschlager and Pietschmann, 2005);

Serial dilutions of both concentrated and unconcentrated GBV-B PV were used to infect cells in the presence of polybrene. After 72 hours, luciferase production was measured (Table 7-2); a summary of cell line permissibility for GBV-B PV is shown in Table 5-1. Whilst lentiviral GBV-B PV are preferable for the development of the GBV-B PV neutralisation assay due to the higher titre of virus produced and higher infectivity, retroviral GBV-B PV was used in the infection assays with primate cells. This is due to the presence of restriction factors in both

Old World monkeys and New world monkeys that either block or limit lentiviral infection (Song *et al*., 2005).

Target cells of Old World monkey origin were shown to not support GBV-B PV transduction whilst the feline CrFK and human Huh7.5 cells were shown to support efficient GBV-B PV infection. Despite the high level of permissiveness seen with CrFK cells, GBV-B PV infection of these cells was not stable and PV entry into this cell line was unlikely to be due to receptor-mediated endocytosis. For this reason, Huh7.5 cells were chosen as the most appropriate target cell for the GBV-B PV neutralisation assay.

	LENTIVIRAL-BASED GBV-B PV	**RETROVIRAL-BASED GBV-B PV**
TAMARIN FIBROBLAST	Not tested	Not permissive
FRHK-4	Not tested	Not permissive
VERO	Not tested	Not permissive
CRFK	Highly permissive	Low level permissibility
HUH7.5	Highly permissive	Not tested

TABLE 5-1: CRFK AND HUH7.5 CELLS SUPPORT EFFICIENT GBV-B PV INFECTION

ALL CELL LINES INVESTIGATED WERE INFECTED WITH SERIAL DILUTIONS OF EITHER LENTIVIRAL-BASED OR RETROVIRAL-BASED PV PRODUCED THROUGH THE CO-TRANSFECTIONS OF 293T CELLS WITH THE TWO OR THREE PLASMID SYSTEM AND PEI. HUH7.5 WERE INFECTED WITH ONLY LENTIVIRAL PV DUE TO TIME RESTRICTIONS AS IT WAS PRESUMED THAT LENTIVIRAL PV HAVE HIGHER LEVELS OF INFECTIVITY. LUMINESCENCE VALUES FOR EACH CELL LINE TESTED ARE SHOWN IN THE APPENDIX (TABLE 7-2), WHILST A SUMMARY OF PERMISSIBILITY (DETERMINED BY THE RESULTS SEEN IN TABLE 7-2) FOR EACH EXPERIMENT IS SHOWN ABOVE.

5.2.7.4 REMOVAL OF THE GBV-B E2 C-TERMINAL HA TAG ENHANCES GBV-B PV INFECTIVITY IN A MODIFIED INFECTION ASSAY PROTOCOL

In the case of HCV PV, a C-terminal HA tag was shown to reduce the infectivity by 65% (Op De Beeck *et al*., 2004)(Beeck *et al*., 2004). The HA tag was therefore deleted from the C-terminus

of GBV-B E2 by PCR. Additionally, the use of centrifugal inoculation, or spinoculation, has been shown to enhance viral infection in previous studies (O'Doherty *et al*., 2000; Yan *et al*., 2015). Due to time restrictions, these optimisations were performed at the same time.

To remove the HA tag from the C-terminus of GBV-B E2, GBV-B *E1E2* genes were re-amplified by PCR with modified primers, using pCAGGS_GBV-BE1E2_HA as the template. For this reaction, the forward primer NSIIE1E2_F was designed that contained an *Nsi*I restriction enzmye site and the reverse primer NHEIE1E2NOTGA_R containing the C-terminal amino acids of GBV-B E2, a stop codon and a *Nhe*I restriction enzmye site. This PCR product produced a specific band at the correct size by gel electrophoresis, which was then subcloned into the pCAGGS vector using the restriction enzymes *Nsi*I and *Nhe*I. Sanger sequencing confirmed this cloning was successful.

Lentiviral GBV-B PV were produced by co-transfection of 293T cells with the first generation packaging plasmid, pNL4.3Luc+Env-, and the plasmid containing GBV-B envelope proteins E1E2 without an HA tag (pCAGG_E1E2noHA). Supernatant from these cells, containing PV, was collected and concentrated by ultracentrfugation. A small amount of supernatant from transfected cells was not concentrated to act as a control. Lentiviral GBV-B PV were used to infect Huh7.5 cells in the presence of polybrene. After GBV-B PV were added to cells, the cells were spun at 2500rpm using a Hettich Rotanta 460R centrifuge and the 5624 plate rotor for 30 minutes, as described in Chapter **2.4.12**. Spinoculation is well known to increase the infectivity of viruses by increasing the chance that the virus interacts with the host cell entry receptor on the surface of the target cell (O'Doherty *et al*., 2000). After three days, luminescence was measured using a luminometer. This infection assay shows that through the removal of the HA tag and the use of spinoculation, the permissiveness of Huh7.5 cells for GBV-B PV increased by around 70% (Figure 5-14). Consequently, these modifications will be adopted for the GBV-B neutralisation assay.

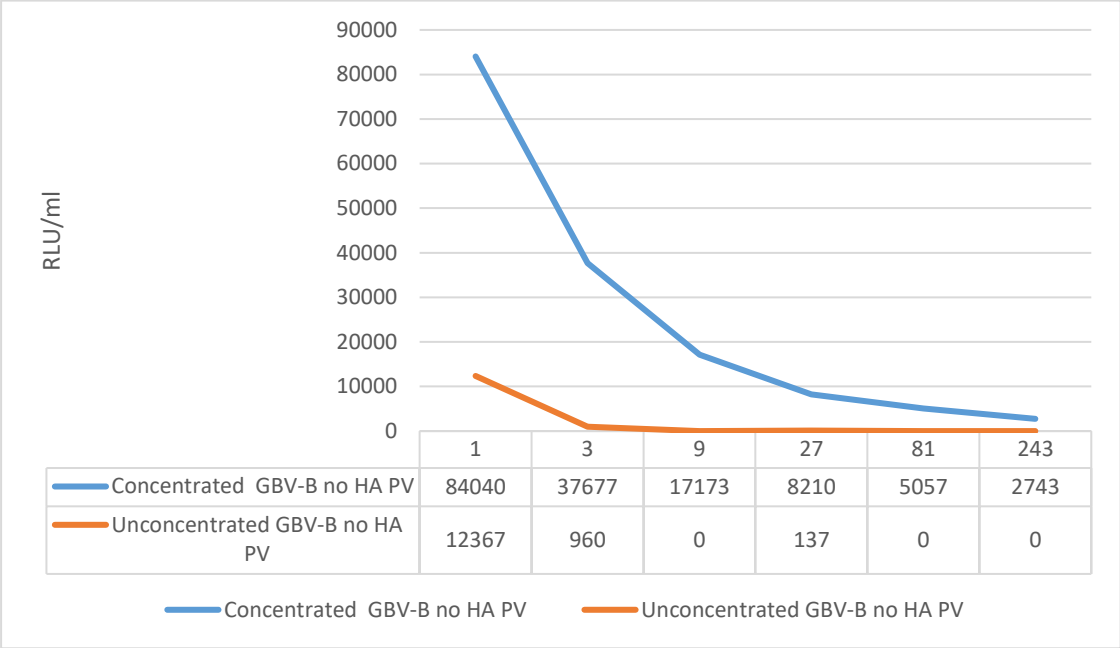


FIGURE 5-14: THE PERMISSIVENESS OF HUH7.5 CELLS FOR GBV-B PV IS ENHANCED AFTER THE REMOVAL OF THE C-TERMINAL GBV-B E2 HA TAG AND THE USE OF A SPINOCULATION-BASED PROTOCOL

LENTIVIRAL-BASED PV BEARING EITHER THE GBV-B ENVELOPE LACKING THE C-TERMINAL E2 HA TAG WERE GENERATED THROUGH CO-TRANSFECTION OF 293T CELLS. THE SUPERNATANT WAS COLLECTED, FILTERED AND CONCENTRATED BY ULTRACENTRIFUGATION. AS A CONTROL, A PROPORTION OF THE SUPERNATANT WAS NOT CONCENTRATED. PV-CONTAINING SUPERNATANT WAS THEN TITRATED BY SERIAL DILUTION, ADDED TO HUH7.5 CELLS AND THE CELL CULTURE PLATE CENTRIFUGED FOR 30 MINS AT A LOW SPEED (2500RPM). THE RESULTS REPRESENT THE AVERAGE OF THREE REPLICATES AND THE LUMINESCENCE VALUES ARE SHOWN BELOW.

5.3 DISCUSSION

To better understand the immunological events surrounding GBV-B clearance as a model for acute HCV clearance, a GBV-B neutralisation assay was designed based on the use of GBV-B pseudotyped viral particles (PV). This would allow the testing of GBV-B-infected tamarin sera in this assay to detect any neutralising antibodies present. These neutralising antibodies are likely to play an important role in GBV-B clearance, as a strong association has been described between HCV neutralising antibodies and clearance of the virus (Cashman *et al.*, 2014).

PV are a highly useful tool for the study of viruses that are difficult to propagate in cell culture, such as GBV-B. These viruses consist of a viral core encapsulating a reporter gene flanked by LTRs. Due to the ability of retroviruses to incorporate foreign viral envelopes onto their viral cores, the envelope of a target virus can be expressed on these PV. The development of HCV PV has enabled the study of several different fields including HCV biology and the characterisation of the humoral immune response using patient sera. As such, GBV-B PV production, and the subsequent development of a GBV-B neutralisation assay, would be of high value.

To produce GBV-B PV, the GBV-B envelope was first cloned and expressed in mammalian cells (Figure 5-1 and 5-2, respectively). Although GBV-B Env protein cloning and expression was relatively straight-forward, their incorporation onto PV particles proved to be difficult. PV bearing the VSV envelope protein were successfully produced and the VSV-G envelope protein was detected by immunoblotting; in comparison, the GBV-B PV particle could be detected but the GBV-B envelope on PV could not. This was most likely due to a lack of incorporation of the GBV-B envelope on PV, which was not unexpected as there is a significant difference between the life cycle of flaviviruses and retroviruses. Retroviruses bud through the plasma membrane, whilst flaviviruses bud through the ER (Morita and Sundquist,

2004; Welsch *et al*., 2007). As such, there is no opportunity for PV to gain GBV-B envelope proteins from the plasma membrane as they are predicted to contain ER retention signals (Cocquerel *et al*., 1999, 1998a).

Despite these differences between retroviruses and flaviviruses, retroviral PV can incorporate HCV envelope proteins if these proteins are expressed at extremely high levels due to 'leaky expression' on the plasma membrane (Bartosch *et al*., 2003). Unfortunately, it was shown that GBV-B Env expression was suboptimal if transfected alongside other plasmids, such as those used for PV production (Figure 5-4). To address this issue, the STAR-HV cell line, based on STAR cells (Ikeda *et al*., 2003), was produced which generated PV with no envelope protein (Figure 5-5). STAR-HV cells could then be transfected with the plasmid containing the GBV-B envelope alone, thereby bypassing the issue of suboptimal expression. Unfortunately, PV bearing the GBV-B envelope were not produced using these cells (Figure 5-6). This could be due to the fact that these cells are already expressing other stably transfected proteins.

Finally, an alternative PV production protocol was adopted, based on incorporating a different reporter gene into PV particles (Tarr *et al*., 2013, 2011; Urbanowicz *et al*., 2015). This led to the successful detection of both lentiviral-based and retroviral-based GBV-B PV, as both of these PV could successfully infect Huh7 cells, a cell type known to be permissive for HCV PV infection (Figure 5-8 and Figure 5-9).

GBV-B Env incorporation onto PV particles was confirmed by immunoblotting PV with antibodies that could detect the C-terminal HA tag of GBV-B E2 and a polyclonal antibody recognising GBV-B E2 (Figure 5-10 and Figure 5-11). These Env proteins were not incorporated efficiently on PV and were subsequently only detected by immunoblotting after being concentrated 2000-fold. In contrast, HCV Env could be detected by western blot after a 63-fold concentration suggesting that the HCV envelope is more easily incorporated onto PV, perhaps due to a greater degree of 'leaky expression' on the plasma membrane.

Furthermore, the GBV-B envelope could not be detected by western blot on retroviral particles, most likely due to a combination of poor incorporation of envelope proteins on to the particles and a lower titre of virus produced in comparison to lentiviral particles. As infection assays were performed prior to western blots, infectious retroviral particles bearing GBV-B envelopes can be produced but not detected by western blot.

Despite this success, GBV-B PV infection was shown to be inefficient in comparison to HCV PV infection (Figure 5-8). Attempts to optimise the infection were therefore made using several approaches including the incorporation of two PV collection time points in the production protocol, the concentration of PV before use in infection assays, investigation of different target cells, removal of the HA tag from GBV-B E2 and the use of spinoculation. The incorporation of two PV collection time points was a relatively minor modification to ensure that PV were stored at the correct temperature to limit degradation, although it was seen that most HCV PV were produced between 48 and 72 hours post transfection (Figure 5-12). The effect of concentration, however, had a substantial effect, with lentiviral GBV-B PV being concentrated by a factor of approximately 20 and retroviral GBV-B PV by a factor of 3 (Figure 5-13).

Another modification to the infection protocol was a change in the target cell used (Table 5-1). Huh7.5 cells were adopted as these appeared to give the highest level of luminescence read out and therefore infection. This is in line with the literature that states that these cells support a higher level of HCV infection, possibly due to the mutation in a protein that is involved in innate antiviral defences (Bartenschlager and Pietschmann, 2005). Whilst the feline kidney cells used were permissive for GBV-B PV, these cells were not used as it is unlikely that PV entry was via the typical, as yet unidentified, GBV-B entry receptors.

The final, and most effective, change to the infection protocol to increase the level of GBV-B PV infection in Huh7.5 cells, was the deletion of the HA tag at the C-terminus of GBV-B E2 and

the use of a spinoculation step during infection (Figure 5-14). Unfortunately, due to time restraints, these modifications were adopted together so it is not possible to identify which of these adjustments led to a 70% increase in infectivity of GBV-B PV or whether the combination was critical. It is likely that both had some impact as both are documented in the literature to have a significant effect (Bartosch *et al*., 2003; O'Doherty *et al*., 2000; Yan *et al*., 2015).

This infection assay can now be used as a basis of a GBV-B neutralisation assay to test archived tamarin sera to detect any possible neutralising antibodies, although biological repeats of the infection assay are first required. Additionally, these PV could be used in a range of other studies including identifying the GBV-B entry receptors and confirming the tropism of the virus.

CHAPTER 6 : DISCUSSION

HCV is a major public health concern with around 500,000 deaths per year from HCV-related liver disease (Lozano *et al*., 2012). Despite this large disease burden, there is currently no available vaccine and the highly effective HCV treatments available have been significantly restricted by the NHS due to their high cost (Gornall *et al*., 2016). Even if this highly effective treatment were to reduce in cost, treatment does not protect against reinfection or provide a remedy for the HCV-associated liver disease (Freeman and Cox, 2016). Additionally, the largely asymptomatic nature of HCV infection means that many infected people do not realise they are infected and therefore would not know to seek treatment. In fact, Public Health England have estimated that only 5% of people with chronic viral hepatitis are aware of their positive status (Public Health England, 2016). To reduce the disease burden of this virus, the development of a prophylactic vaccine is necessary.

The development of an HCV vaccine has been extremely challenging, partly due to the virus' genetic variability (Naderi *et al*., 2014). Moreover, clearance of the virus is not thought to provide protection against reinfection, but rather protect against chronic disease (Osburn *et al*., 2010). Due to the subclinical nature of acute HCV infection, an animal model is necessary to investigate the immunological events that occur surrounding HCV clearance (Gerdts *et al*., 2007). Understanding these events could then inform the design of a novel vaccine for HCV.

Aside from humans, HCV is known to only infect chimpanzees, which, due to ethical constraints, limited availability and high study costs, are no longer used in the study of HCV (Vercauteren *et al*., 2015). Despite this, there are a number of experimental animal models available that involve either investigating specific parts of the viral life cycle/pathogenesis or the study of closely-related hepaciviruses (Billerbeck *et al*., 2013).

In this project, GBV-B infection of tamarins has been used as an animal model of acute HCV infection, as spontaneous clearance of GBV-B occurs in most cases (Stapleton *et al*., 2011). However, there is a limited amount of data available on GBV-B. This project therefore had two aims:

1. To investigate the pathology of GBV-B to further validate GBV-B infection in tamarins as a useful animal model of acute HCV infection.
2. To understand the immunological events that lead to viral clearance to potentially inform HCV vaccine design.

Alongside hepatocytes, HCV is also known to infect other extrahepatic cells, such as lymphocytes and brain cells, resulting in extrahepatic manifestations (Ikeda *et al*., 1997; Weissenborn *et al*., 2009). It is therefore of interest to determine if GBV-B can also infect these cells, as extrahepatic manifestations have only been potentially demonstrated in transgenic mice models (Koike *et al*., 1997). In the case of GBV-B, a previous study showed that GBV-B RNA could be isolated from several different extrahepatic tissues, including lymphocytes, splenocytes and lymph nodes (Ishii *et al*., 2007). Immunohistochemistry with GBV-B specific antibodies could then be used to determine if viral proteins are also present in these cells along with other relevant cells, such as brain cells.

Before immunohistochemistry could be performed, antibodies targeting specific GBV-B proteins had to be produced. The production of these antibodies was required as no GBV-B antibodies were commercially available. Initially, ribosome display was employed, as described in Chapter **2.2.17**, as this *in vitro* display technology allows the rapid selection of highly-specific antibodies from a hugely diverse library of antibodies (Zahnd *et al*., 2007). This diversity is only limited by the number of ribosomes and unique antibody mRNA molecules within the system. Moreover, due to the amplification of the antibody library by PCR after

each round of screening, random mutations can be introduced into antibodies allowing the direct evolution of GBV-B-specific antibodies throughout the rounds of selection.

In Chapter 3, the set-up required for ribosome display was described. GBV-B proteins, for use as target antigens for the screening of the antibody library, and the antibody library itself was produced. The GBV-B proteins core, NS3 and NS5BΔ23 were chosen as initial screening targets as these proteins had been successfully produced within other laboratories and therefore protein production protocols already existed (Pilot-Matias *et al*., 1996; Ranjith-Kumar *et al*., 2003; Scarselli *et al*., 1997; Zhong *et al*., 2000). Additionally, antibodies targeting these proteins *in vivo* were described in the literature (Ishii *et al*., 2007; Iwasaki *et al*., 2011; Pilot-Matias *et al*., 1996; Sbardellati *et al*., 2001). GBV-B E2 was not included as a target antigen as recombinant, soluble GBV-B E2 has never been produced and therefore GBV-B E2-specific antibodies have never been identified.

Production of these GBV-B proteins in a prokaryotic system and purification using nickel affinity columns was relatively straightforward (see Chapter **2.3.4**). Prokaryotic expression was chosen over eukaryotic expression as large amounts of protein are easy to produce in this system and, as stated above, the protocols already existed and did not require time-consuming protocol optimisation. Western blots with an anti-His tag antibody confirmed each protein was present in each preparation (Figure 3-3). Tamarin sera, from two different tamarins, was also applied to each of the three blot membranes, although only GBV-B core was recognised (Figure 3-3C). This could be due to a range of reasons including, but not limited to, GBV-B core antibodies being produced at a higher volume than NS3/NS5BΔ23 antibodies (core is the nucleocapsid of the virus), the tamarins tested not producing antibodies targeting NS3 or NS5BΔ23 or that antibodies targeting core and antibodies targeting non-structural proteins are produced at different times during infection.

The plasmids containing GBV-B protein sequences were sent for large scale production and purification by the Protein Production facility at Queen Mary University. This was largely successful, although NS3 remained difficult to produce in large enough quantities despite using the identical, previously successful, protocols to the small-scale production and purification of NS3 (Figure 3-4). To solve this issue, different bacterial strains, different bacterial promoters or perhaps a different protocol (such as that described in Ishii *et al*., 2007) could have been adopted, however, due to time restrictions, the NS3 protein was not used as an antibody screening target so this optimisation wasn't required. Before use as a target antigen, NS5BΔ23 (as this was to be used as a screening antigen first) was sent for mass spectrometry analysis. This showed that NS5BΔ23 was indeed present in the protein preparation (Appendix, Table 7-1).

After the successful production of GBV-B proteins in a prokaryotic system, the antibody library was generated for use in ribosome display. This antibody library was based on the heavy and light chain variable regions of the tamarin immunoglobulin genes which could be modified to produce scFvs (Figure 3-5). To permit the cloning of tamarin immunoglobulin genes, tamarin RNA was isolated from splenocytes from a tamarin that had recently cleared its acute GBV-B infection. In this way, successfully rearranged immunoglobulin genes can potentially be isolated and the V_H and V_L regions cloned. However, the cloning of the V_H and V_L regions was somewhat challenging as the tamarin genome has not currently been sequenced. As such, the only viable option was to design primers using marmoset immunoglobulin sequences as tamarins and marmosets are closely-related (Lanford *et al*., 2003).

Degenerate primers were designed to cover known heavy and light chain variable region sequences within the immunoglobulin genes (von Büdingen *et al*., 2002). For example, only kappa light chain variable sequences have been identified in marmoset immunoglobulin

genes and were therefore cloned. This may indicate that kappa chains are more abundantly used in the B cell receptor (BCRs) complex than lambda and are therefore more readily isolated. Indeed, it is well known that light chain usage varies between mammals, with human BCRs expressing 70% kappa light chains, cattle expressing 95% lambda light chains and rhesus macaques expressing 50% of each (Tizard, 2013). However, the lack of lambda chain isolation from marmosets may simply be due to the fact that kappa sequences were more readily cloned than lambda sequences. Due to the huge number of antibody sequences that were already available with only kappa light chain usage, this was deemed sufficient for this study's purpose.

All tamarin variable regions were cloned by PCR successfully (see Chapter **2.2.4**) and the identity and diversity of these variable regions was confirmed using Sanger sequencing. To confirm the identity of tamarin variable regions, sequences were compared against their marmoset equivalent using the Basic Local Alignment Search Tool (BLAST, available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi) as this is the first time that tamarin variable regions have ever been cloned. This analysis confirmed that the sequences were closely related to marmoset immunoglobulin sequences thereby corroborating the approach taken in this project. Diversity was also confirmed by isolating around 100 variable region sequences from different V_H and V_K families and comparing intrafamilial sequence variation using DNA Dynamo. All identified variable regions were unique.

The remaining necessary elements (the T7 promoter sequence, the linker and the spacer) required for scFv library construction were added through a further five rounds of PCR (see Chapter **2.2.4**). Five PCRs were required for the addition of three elements due to the length of each of the additional element sequences. The T7 promoter was chosen as scFv expression is required downstream in the process and the human constant region (CH1) was chosen as

the spacer for the identification of scFv after downstream expression. Sanger sequencing was performed at both the 3rd and 6th PCR stage to ensure the correct identify of scFv.

Once the scFv library had been constructed and the GBV-B target antigens had been produced, ribosome display was performed. GBV-B NS5BΔ23 was used as the first target antigen as this is the viral polymerase. If NS5BΔ23 protein was therefore detected in extrahepatic tamarin tissues, this would suggest active replication of the virus in these tissues. Although scFv sequences were successfully isolated from screening of the scFv library against NS5BΔ23, only 5% of sequences corresponded to full length scFv. Full length scFv were readily identified within the scFv library by Sanger sequencing before the screening against NS5BΔ23 so this suggested that either the tamarin that was used to create the scFv library had never made NS5BΔ23 antibodies or that the ribosome display protocol required further optimisation.

Despite this, two scFv sequences were identified (one with a full-length linker and one with a truncated linker) and these scFv were expressed in prokaryotic cells (Figure 4-3, as described in Chapter **2.3.1**). To aid with downstream scFv stability, solubility and identification, scFv were cloned into the p4D5_Fc vector that contains human CH2 and CH3 regions (Czajkowsky *et al*., 2012; Liu, 2003; Moutel *et al*., 2009). Although both scFv could be produced at high levels, neither scFv was NS5BΔ23-specific by ELISA (Figure 4-4). This may be due to the improper folding of NS5BΔ23 in prokaryotic cells (mass spectrometry only proved that the protein was present), improper folding of scFv in prokaryotic cells or that there was an issue with the selection stage of ribosome display.

Although it is possible that there was an issue with the selection stage of ribosome display due to the isolation of only one full length scFv sequence, ribosome display was repeated with a different screening antigen- GBV-B core. Core was chosen as the screening antigen as core-specific antibodies have been more readily identified *in vivo* than NS5B-specific

antibodies (Bukh *et al*., 2008; Ishii *et al*., 2007; Iwasaki *et al*., 2011; Pilot-Matias *et al*., 1996; Sbardellati *et al*., 2001; Woollard *et al*., 2008). Furthermore, core antibodies had been identified by western blot in Chapter 3 (Figure 3-3C).

After this screening, six full-length unique scFv sequences were isolated. Additionally, an out-of-frame scFv was also isolated and used as a protein expression control. The most appropriate host for scFv expression was selected through the small-scale expression of one scFv in various prokaryotic hosts. Induction of scFvs proved to be difficult due to low expression levels (Figure 4-6). Protocol optimisation was attempted, such as using different growth temperatures, IPTG concentrations, and culture mediums, but scFv induction remained low. This could be due to the expressed scFv being toxic to the bacterial cells, although this was not the case with NS5BΔ23 scFv (Wang *et al*., 2013). Despite low induction levels, the expressed scFv could be identified by western blot using an anti-His antibody (Figure 4-6).

Once expressed, these scFv were tested for their GBV-B core-binding capabilities by ELISA as described in Chapter **2.3.7** (Figure 4-7). Unfortunately, all scFv were non-specific for core, as they also bound to the plastic 96-well plate. This could have been due to improper folding of scFv in the bacterial cells so scFv were later expressed in a mammalian cell line (Figure 4-9). Unfortunately, the mammalian expressed scFv then lost all binding capabilities, as determined by ELISA, perhaps due to correct protein folding. It may be the case that these scFv, once folded properly, would only bind to their specific target, such as one of the ribosome display components.

As it has been well documented that antigen-specific scFv are enriched in the scFv library with each round of screening against the target antigen (Hanes and Plückthun, 1997; Schaffitzel *et al*., 1999), ribosome display was repeated. Three rounds of screening were performed to increase the chance of isolating core-specific scFv. Of note, the recovery of the

scFv library after each round of screening was increasingly less efficient with each round of screening (Figure 4-10). This could be due to a difficulty in amplifying scFv sequences (as recovery is PCR-based) or it could have been that non-specific scFv were being lost with each round of screening and therefore the scFv library was reducing in size. Due to these difficulties, only four antibodies were isolated after three rounds of screening the scFv library against core, of which only two were full-length (Table 4-3). These full-length antibodies were also likely to be the same antibody as Sanger sequencing and IgBLAST analysis of the nucleotide sequence revealed that both scFv had the same VDJ gene segments and CDR3 sequences.

As seen with the previous scFv antibodies, prokaryotic expression of these scFv was challenging as protein induction levels were low. Expressed scFv were eventually isolated from BL21 cells and the binding properties of all four scFv were investigated by ELISA. In comparison to the scFv isolated after one round screening, scFv isolated after three rounds of screening all bound specifically to core (Figure 4-12A). These scFv may well prove to be relatively low affinity as they do not maintain their binding capabilities when diluted (Figure 4-12B), although affinity maturation is a possibility using ribosome display.

To investigate the issue with the scFv selection stage of the ribosome display protocol, the CDR3 sequences of the scFv libraries, before and after antigen panning, were deep sequenced, as described in Chapter **2.2.15**. Only the CDR3 regions were sequenced as it is thought that the majority of the diversity of antigen-specificity of antibodies is due to this sequence (Xu and Davis, 2000). Degenerate primers covering all possible scFv CDR3 sequences were designed from previously sequenced tamarin variable regions from Chapter **3.1.1** (Table 4-4). These primers were based on recognising conserved sequences before and after the CDR3 sequences, which were remarkably similar to their human counterparts (see Table 7-6). CDR3 sequences from both the heavy chain and light chain of tamarin variable

regions were amplified by PCR and deep sequenced. PCRs were performed in duplicate for increased reliability of results.

To analyse CDR3 sequences within the scFv libraries, the Antibody Mining Toolbox was used (see Chapter 2.2.16, D'Angelo *et al*., 2013). CDR3 sequences were readily identified from all libraries. This sequencing showed that there was indeed an issue with the selection stage of ribosome display as there is no significant selection of specific scFv sequences and the libraries were not decreasing with complexity (Figure 4-14-4-16). This data suggests that the ribosome display protocol needs to optimised. For example, the use of different selection techniques (such as protein-coated magnetic beads), an increased spacer length for scFv, and the use of proof-reading polymerases during the elongation steps of scFv could all impact the selection stage of ribosome display and may impact the number of antigen-specific scFv isolated (Schaffitzel *et al*., 1999).

Due to the difficulties encountered with ribosome display, monoclonal antibodies (mAbs) were also produced using the hybridoma method using a standard protocol previously established in the laboratory. This protocol was effective and several core-specific mAbs were produced with similar binding specificities as seen with the scFv isolated after three rounds of panning using ribosome display (Figure 4-12, 4-18). Although these mAbs were easier and quicker to produce, the ribosome display method offers an animal-free method for obtaining antibodies. Therefore, if successfully optimised, ribosome display would be a more appropriate method to adopt in the future for the production of antibodies. Due to time constraints, neither core-specific scFv or mAbs were evaluated in immunohistochemistry studies.

The other aspect of this project was the investigation of immunological events surrounding GBV-B clearance. Although it is likely that a combined cellular and humoral immune response is responsible for successful viral clearance, it has been suggested in previous GBV-B studies

that a delayed humoral immune response, particularly the neutralising antibody response, can cause extended, or chronic, viraemia (Iwasaki *et al*., 2011; Martin *et al*., 2003). As such, the humoral immune response to GBV-B was investigated. To facilitate this, a neutralisation assay was needed to determine if neutralising antibodies are present during GBV-B clearance.

However, HCV, and therefore most likely GBV-B, does not replicate well in cell culture (Steinmann and Pietschmann, 2013). A neutralisation assay based on pseudotyped viral particles (PV) was therefore developed, as this bypasses the need for GBV-B replication in cell culture (Temperton *et al*., 2015). Additionally, this method has been used successfully to investigate the neutralising antibody response to HCV (Bartosch *et al*., 2003; Lavillette *et al*., 2005; Meunier *et al*., 2005; Tarr *et al*., 2011). For this, GBV-B E1 and E2 envelope proteins are expressed on a retroviral core that encapsulates a reporter gene. During infection, this reporter gene is incorporated into the host cell's genome and expressed thereby providing a simple infectivity measurement (Temperton, Wright and Scott, 2015).

To produce PV expressing GBV-B envelope proteins, the envelope proteins were first cloned by PCR (Figure 5-1, see Chapter **2.2.4**). An HA tag was added at the C-terminus for identification purposes as no commercial GBV-B antibodies to E1 or E2 are currently available. These proteins were then expressed in a mammalian cell line, 293T, isolated and detected by western blot using an anti-HA antibody (Figure 5-2A). For further confirmation (as no E1E2 antibody is available), the envelope proteins were expressed again in the 293T cell line that had been treated with an N-glycosylation inhibitor. The proteins were then isolated and detected by western blot, which showed that both proteins were approximately the correct size (Figure 5-2B).

PV production was then attempted through the transfection of 293T cells (as described in **2.4.6**). As a positive control, PV bearing VSV-G envelope protein were also produced as this can be easily generated at very high titres. Western blots were performed to detect the

relevant components of the PV: the envelope protein and the retroviral core. This showed that although PV bearing VSV-G were successfully produced, PV did not bear GBV-B E1E2 on their surface (Figure 5-3). This was originally thought to be due to a suboptimal expression level of the envelope proteins, most likely due to the difference between the life cycle of retroviruses and flaviviruses (such as GBV-B). In the case of retroviruses, the retrovirus buds through the plasma membrane and therefore the envelope proteins need to be in this location. However, flaviviruses bud internally from the ER. As such, GBV-B E1 and E2 proteins contain ER retention signals and would therefore not be at the plasma membrane (Cocquerel *et al*., 1998). It is thought that HCV PV are able to be produced if the expression level is high enough to allow leaky protein expression from the ER permitting a proportion of the envelope proteins to relocate to the plasma membrane (Bartosch *et al*., 2003; Drummer *et al*., 2003).

To determine if GBV-B E1E2 was being suboptimally expressed during PV production, 293T cells were transfected with the GBV-B E1E2 plasmid in combination with varying amounts of an empty plasmid (representing PV production plasmids). This showed that E1E2 expression levels were significantly reduced when E1E2 was transfected with another plasmid, even if this plasmid was empty, although it is unclear why this would be the case (Figure 5-4).

To circumvent this issue, a cell line that stably expressed lentiviral core proteins was obtained. These STAR cells, were transduced with lentiviral particles containing the *gfp* reporter gene (Figure 5-5, see Chapter **2.4.7**). Two rounds of transduction were required to ensure approximately 100% (98.2%, Figure 5-5) of cells were GFP-positive. These cells were shown to be successfully producing PV, as evidenced by western blots of the STAR cell supernatant (Figure 5-6). These STAR cells could then be transfected with the GBV-B E1E2 plasmid alone to enhance the level of protein expression. Disappointingly, this still resulted in PV not bearing E1E2 proteins. This may have been due to an issue with suboptimal expression

or that GBV-B envelope protein are more tightly retained in the ER. It is likely that E1E2 was still expressed at a suboptimal level as 293T E1E2-transfected cell lysate contained a low level of E1E2 (Figure 5-6D).

Finally, through the adoption of a different PV protocol and the substitution of the *gfp* reporter gene for the *luciferase* reporter gene (see Chapter **2.4.6**), PV expressing either HCV and GBV-B were successfully produced as determined by the infection of Huh7 cells (Figure 5-8 and 5-9), a cell type known to support HCV PV infection (Bartosch *et al*., 2003). HCV PV appeared to have a higher infectivity, which could be due to a higher level of HCV envelope incorporation on to PV than GBV-B envelope proteins, a higher titre of HCV PV being produced or Huh7 cells having a low susceptibility to GBV-B infection at the same efficiency as HCV infection.

Both lentiviral (HIV) and retroviral (MLV)- based PV were produced in this study as lentiviral PV are unable to infect cells of New World monkey origin due to pre-existing viral entry restriction factors (Song *et al*., 2005). Additionally, some viral envelope proteins incorporate efficiently onto retroviral-based PV, some onto lentiviral-based PV and some onto both particles (Personal communication, Dr Alexander Tarr, University of Nottingham). Retroviral PV expressing GBV-B envelope proteins were shown to have a much lower infectivity than their lentiviral counterparts (Figure 5-9). This was somewhat expected as lentiviruses are able to infect non-dividing cells, whilst gammaretroviruses cannot (Matreyek and Engelman, 2013).

In parallel to performing infection assays, western blots of concentrated HCV and GBV-B PV were performed to confirm that the envelope proteins were indeed incorporated onto the PV particle (Figure 5-10). To detect the relevant envelope proteins, PV had to be significantly concentrated (see Chapter **2.4.9**). This is most likely due to an inefficient incorporation of the envelope protein onto the PV. Of note, HCV envelope proteins could be detected on PV after

63-fold concentration. In comparison, GBV-B PV had to be concentrated up to 2000-fold before any signal on the western blot could be detected. Additionally, whilst GBV-B envelope proteins could be detected on lentiviral-based PV, GBV-B envelope proteins could not be detected on retroviral-based PV (Figure 5-11). This is likely to be due to a lower titre of virus being produced in the case of retroviral PV, possibly in combination with a less efficient envelope incorporation onto retroviral PV. This would also explain the reduced infectivity seen with retroviral GBV-B PV.

The successful HCV and GBV-B PV production protocol was then optimised again; successful modifications included the adoption of ultracentrifugation, the use of a modified target cell line, the removal of the E2 C-terminus HA tag and the use of spinoculation.

Ultracentrifugation was successful for both lentiviral-based and retroviral-based PV. It increased the infectivity readout by a factor of greater than ten in the case of HCV PV. GBV-B PV infectivity still remained much lower than HCV PV infectivity; the lentiviral GBV-B PV infectivity readout was increased by around a factor of 20 in comparison to an increase of only three-fold for retroviral-based GBV-B PV (Figure 5-13). Of note, lentiviral GBV-B PV production was relatively unstable as the infectivity readout ranged between around 10,000 RLU/ml to only 500 RLU/ml (Figure 5-8 and Figure 5-13). This suggests either inefficient PV production, inefficient incorporation of the envelope proteins on to the PV or that the cells are not particularly permissive for GBV-B infection.

To determine the role of permissibility of target cells for the development of the GBV-B PV neutralisation assay, several different target cells were investigated. Cells of both New World and Old World monkey origin were tested as they may have been more permissive for GBV-B infection than human cell lines. No cells of monkey origin that were tested in this study proved highly permissive for GBV-B, although this may be due to the poorly infectious retroviral GBV-B PV. Interestingly, the cell lines Huh7.5 and CrFK seemed to be highly

permissive for lentiviral GBV-B PV. Huh7.5 cells were chosen as the target cell line as they are hepatocytes and are more likely to express the relevant entry receptors in comparison to the feline cell line.

The final optimisation stages, the removal of the HA tag and the use of spinoculation during the infectivity assay, were adopted at the same time due to time restrictions but together increased the permissiveness of Huh7.5 cells for GBV-B PV by around 70% (Figure 5-14). The removal of the HA tag, in the case of HCV PV, was shown to increase their infectvity by around 65% (Op De Beeck *et al*., 2004). It has been suggested that the HA tag affects the function of the transmembrane domains of E1E2 during the fusion process, which would explain why infectivity was affected. Supporting this theory, when mutations were introduced into the the transmembrane domain of the VSV-G protein, viral fusion was severly affected (Cleverley and Lenard, 1998). It therefore may the case that by altering the C-terminus of GBV-B E2, viral fusion is severely reduced. Additionally, the use of centrifugal inoculation, or spinoculation, has been shown to enhance viral infection in previous studies by increasing the chance encounters between the virus and the host (O'Doherty *et al*., 2000; Yan *et al*., 2015).

Interestingly, in the middle of this study in September 2015, Marnata and colleagues published a study which involved producing GBV-B PV. As they used codon-optimised GBV-B envelope proteins (which enhances the translation of proteins in mammalian cells), these envelope proteins appeared to be incoporated more efficiently onto PV (Marnata *et al*., 2015). It would therefore be of interest to include this protocol modification in future GBV-B PV production. Despite this, the GBV-B infection assay developed during this project could form the basis of any future GBV-B neutralisation assay study. Unfortunately, due to time restrictions, tamarin sera was not assessed in this assay.

In summary, this project has led to both the production of GBV-B specific antibodies for use in the investigation of GBV-B pathology and the development of a GBV-B neutralisation assay. In the near future, these GBV-B core-specific antibodies could be used in immunochemistry studies to determine the tropism of the virus in comparison to HCV. As the conditions for ribosome display are now set up, other GBV-B proteins could also be used as ribosome display screening targets. These could be used in various studies, including the tracking of the viral lifecyle (which is still largely unknown for GBV-B) and vaccine development. Additionally, the production of tamarin antibody libraries at different stages of infection and either the monitoring of the diversity of the immunoglublin repertoire through deep sequencing or isolation of scFv antibodies through ribsome display could help to determine the differences between the antibody response to HCV and the antibody response to GBV-B.

To aid in the investigation of the antibody response to GBV-B, a GBV-B neutralisation assay was also developed. This now simply requires the testing of tamarin sera to determine if neutralising antibodies to GBV-B exist. Human sera from HCV-infected individual could also be tested to determine if any cross-reactivity exists, which could potentially help inform vaccine design. The infection assay that forms the basis of the neutralisation could also be used to determine the entry receptors for GBV-B, for example through the use of antibodies that block known HCV receptors.

CHAPTER 7 : APPENDIX

ADDITIONAL MATERIALS AND METHODS

NAME OF PRIMER	SEQUENCE (5' TO 3')	ANNEALING TEMP (°C)	NOTES	USE
GBV-B PROTEIN PRODUCTION				
CORE_F	GCG AGG GGA TCT GGG AG	55	Forward primer for cloning GBV-B core (previously done in lab and therefore not cloned in this project)	
CORE_R	GTC TGG GTC AGT GAC CCG C	55	Reverse primer for cloning GBV-B core (previously done in lab and therefore not cloned in this project)	
NS3RBS_F	TCC ATC TAG AAA GAA GGA GAT ATA CCA TGG CAC CTT TTA CGC TGC AGT G	55	Ribosome binding site and XbaI restriction site	Forward primer for cloning GBV-B NS3 serine protease
NS3NOTI_R	TTG CGC GGC CGC TGT GTA CTG GGG ATG G	55	NotI restriction site	Reverse primer for cloning GBV-B NS3 serine protease
NS5BNCO1_F	TCC ACC ATG GCT AGC ATG AGC TAC ACC TGG ACC	60	NcoI restriction site	Forward primer for cloning GBV-B NS5B
NS5B-23_R	GCA AGC GGC CGC CTT CTG CAA TCT TCT CTG	60	NotI restriction site	Reverse primer for cloning GBV-B NS5B without its C-terminal 23 amino acids
HINDIIIE1E2_F	TGG TAA GCT TGC CAC CAT GGG TTG GTT CGG TGT CCA CCT TTT TG	67	HindIII restriction site	Forward primer for cloning GBV-B E1E2 into pcDNA3
E1E2CLAI_R	AAC CAT CGA TCC GAG CCA TGG GCA CAA ACC	65	ClaI restriction site	Reverse primer for cloning GBV-B E1E2 into pcDNA3. Mutates the last amino acid of E2 from Alanine to Arginine
NSIIE1E2_F	TGG TAT GCA TGC CAC CAT GGG TTG	55	NsiI restriction site	Forward primer for cloning GBV-B E1E2 without an HA tag

	GTT CGG TGT CCA CCT TTT TG			
NHEIE1E2NOTGA_R	AAC CGC TAG CTC ACG CAG CCA TGG GCA CAA ACC	55	NheI restriction site	Reverse primer for cloning GBV-B E1E2 without the HA tag.
SP6 UNIVERSAL	ATT TAG GTG ACA CTA TAG	55		Reverse primer used for cloning GBV-B E1E2 into pCAGGS.
SCFV LIBRARY CONSTRUCTION PRIMERS				
$DT_{29}VN$	TTT TTT TTT TTT TTT TTT TTT TTT TTV N	n/a		cDNA synthesis
VH1/7_F	GGT GGT GGT GGT TCT CAG GTG CAG CTG GTG CAG TCT GG	54	Stage 1 scFv production	Forward primer for cloning tamarin variable region of heavy chain (families 1 and 7). It includes 1x G_4S.
VH3/5_F	GGT GGT GGT GGT TCT GAG GTG CAG CTG GTG SAG TCT GG	54	Stage 1 scFv production	Forward primer for cloning tamarin variable region of heavy chain (families 3 and 5). It includes 1x G_4S.
VH4_F	GGT GGT GGT GGT TCT CAG GTG CAG CTG CAG GAG TCG GA	56	Stage 1 scFv production	Forward primer for cloning tamarin variable region of heavy chain (family 4). It includes 1x G_4S.
VH6_F	GGT GGT GGT GGT TCT CAG GTG CAG CTG CAG GAG TCA GG	54	Stage 1 scFv production	Forward primer for cloning tamarin variable region of heavy chain (family 6). It includes 1x G_4S.
VH_R	GGA ACG CGG CCG CGA CAT CTG GGT TCT TGG TGG AGG CTG A	54/56	Stage 1, 2 and 3 scFv production, NotI restriction site	Reverse primer for cloning tamarin variable region of heavy chain. Includes part of spacer.
VK1_R	CGA ATT CCA CCA TGG CCC AGA TGA CCC AGT CTC C	54	Stage 1 scFv production, EcoRI and NcoI restriction sites	Forward primer for cloning tamarin variable region of kappa light chain (family 1). It includes Kozak sequence.
VK2/4_R	CGA ATT CCA CCA TGG CCG TGA	54	Stage 1 scFv production,	Forward primer for cloning tamarin variable region of

	TGA CYC AGT CTC C		EcoRI and NcoI restriction sites	kappa light chain (families 2 and 4). It includes Kozak sequence.
VK3_R	CGA ATT CCA CCA TGG CCG TGW TGA CRC AGT CTC C	54	Stage 1 scFv production, EcoRI and NcoI restriction sites	Forward primer for cloning tamarin variable region of kappa light chain (family 3). It includes Kozak sequence.
VK5_R	CGA ATT CCA CCA TGG CCA CAC TCA CGC AGT CTC C	56	Stage 1 scFv production, EcoRI and NcoI restriction sites	Forward primer for cloning tamarin variable region of kappa light chain (family 5). It includes Kozak sequence.
VK_R	GAA CCA CCA CCA CCG AAG ACA GAC GGC GCA GCC ACA GC	54/56	Stage 1 scFv production	Reverse primer for cloning tamarin variable region of kappa light chain. It includes 1x G_4S.
RHVHFLINK	GTA ATA CGA CTC ACT ATA GGG CGA ATT CCA CCA TGG	52	Stage 2, 3 and 5 scFv production, EcoRI and NcoI restriction sites	Forward primer adding part of the T7 promoter to the variable region of the light chain.
BAMH1LINK	GGA GCC GCC GCC GCC GGA TCC ACC ACC ACC AGA ACC ACC ACC ACC	52	Stage 2 scFv production, BamHI restriction site	Reverse primer for elongating linker of the variable region of the light chain.
RHVLFLINK	GGC GGC GGC GGC TCC GGT GGT GGT GGT TCT	52	Stage 2 scFv production	Forward primer elongating linker of heavy chain variable region by adding a repeat of G_4S.
HUCH1_F	CGT CGC GGC CGC GTT CCC CCT GGY RCC CT	52	Stage 4 scFv production, Not1 restriction site	Forward primer for cloning constant region of heavy chain
HUCH2_XHOI	GTT CCT CGA GGG TRT CCT TGG GTT TTG GGG GGA A	52/56	Stage 4, 5 and 6 scFv production, XhoI restriction site	Reverse primer for cloning constant region of heavy chain

RDT7	CTA TAG AAG GGT AAT ACG ACT CAC TAT AG	56	Stage 6 scFv production	Forward primer completing T7 promoter of scFv library
RECMRNA_F	GTA ATA CGA CTC ACT ATA GGG CGA ATT C	56		Forward primer used in the recovery of scFv mRNA after ribosome display
HURECMRNA_R	AGG TGC TGG GCA CGG GGG CAT G	56		Reverse primer used in the recovery of scFv mRNA after ribosome display
SEQUENCING PRIMERS				
T7 PROMOTER	TAA TAC GAC TCA CTA TAG GG	50		Forward sequencing primer for
SP6 UNIVERSAL	ATT TAG GTG ACA CTA TAG	50		Reverse sequencing primer for pcDNA3 vector.
PET TERMINATOR	GCT AGT TAT TGC TCA GCG G	50		Reverse sequencing primer for p4D5 vector.
PSF_F	ATC GTT GCG TTA CAC ACA C	50		Forward sequencing primer for pSF.
PSF_R	GCT GCC TTG TAA GTC ATT GGT C	50		Reverse sequencing primer for pSF.
PCAGGS_F	TTC TCC ATC TCC AGC CTC GGG	50		Forward primer for sequencing pCAGGS.
PCAGGS_R	CCC ATA TGT CCT TCC GAG TGA	50		Reverse primer for sequencing pCAGGS.
M13_F	GTT TTC CCA GTC ACG AC	50		Forward sequencing primer for pGEM T-Easy vector.
M13_R	AAC AGC TAT GAC CAT G	50		Reverse sequencing primer for pGEM T-Easy vector.
DEEP SEQUENCING PRIMERS				
VH1_F	*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG* ACG GCC GTV TAT TAC TGT GCD DVD	55	Illumina adapter in italic.	Forward deep sequencing primer for amplifying CDR3 regions of tamarin heavy chain variable regions (1/3).
VH2_F	*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG* ACG	55	Illumina adapter in italic.	Forward deep sequencing primer for amplifying CDR3 regions of tamarin heavy chain variable regions (2/3).

	GCC AKV TAT TAC TGT GCG AGR			
VH3_F	*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG* ACA GCC GTC TAT TAC TGT WST ARW	55	Illumina adapter in italic.	Forward deep sequencing primer for amplifying CDR3 regions of tamarin heavy chain variable regions (3/3).
VH_R	*GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G*GA CAT CTG GGT TCT TGG TGG AGG CTG A	55	Illumina adapter in italic.	Reverse deep sequencing primer for amplifying CDR3 regions of tamarin heavy chain variable regions.
VK1_F	*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG* GTT KCR ATT TAY YAC TGT CAM CAS	55	Illumina adapter in italic.	Forward deep sequencing primers for amplifying CDR3 regions of tamarin light chain variable regions (1/3).
VK2_F	*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG* TTT GCC RCT TAT TWC TGT CAA CAT	55	Illumina adapter in italic.	Forward deep sequencing primer for amplifying CDR3 regions of tamarin light chain variable regions (2/3).
VK3_F	*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG* TYT GCA GTT TAT TAC TGT CAS CAG	55	Illumina adapter in italic.	Forward deep sequencing primer for amplifying CDR3 regions of tamarin light chain variable regions (3/3).
VK_R	*GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G*GA AGA CAG ACG GCG CAG CCA CAG C	55	Illumina adapter in italic.	Reverse deep sequencing primer for amplifying CDR3 regions of tamarin heavy chain variable regions.

TABLE 7-1: LIST OF PRIMER SEQUENCES.

RESTRICTION SITES ARE UNDERLINED.

GBV-B PROTEIN PRODUCTION PLASMIDS

NAME	USE	GENE	REFERENCE
4D5_CORE_HIS	GBV-B core production	GBV-B core,	See 3.2.1
PET32B_NS3_HIS	GBV-B NS3 production	GBV-B NS3 protease	See 3.2.1
P4D5_NS5BΔ23_HIS	GBV-B NS5BΔ23 production	GBV-B NS5B, truncated (last 23 amino acids deleted)	See 3.2.1

TABLE 7-2: LIST OF PLASMIDS USED TO PRODUCE RECOMBINANT GBV-B PROTEINS

ANTIBIOTIC CONCENTRATION WAS 100 μG/ML FOR AMPICILLIN IN LB BROTH AND AGAR PLATE.

SCFV PRODUCTION PLASMIDS

NAME	USE	GENE	REFERENCE
PGEM T-EASY_CH1	Spacer in scFv library	Human CH1 region	n/a
P4D5_8RFP_FC	Positive control for scFv expression, backbone for scFv prokaryotic expression	RFP flanked by a VH and VL on either side	Markiv *et al*., 2011
P4D5_NS5BΔ23.SCFV_11_FC	NS5BΔ23-specific scFv production in prokaryotic cells	scFv isolated after panning against NS5BΔ23, with human Fc receptor	See 4.2.2
P4D5_NS5BΔ23.SCFV_12_FC	NS5BΔ23-specific scFv production in prokaryotic cells	scFv isolated after panning against NS5BΔ23, with human Fc receptor	See 4.2.2
P4D5_CORE.SCFV_1_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.4
P4D5_CORE.SCFV_2_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.4
P4D5_CORE.SCFV_3_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.4
P4D5_CORE.SCFV_4_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.4

P4D5_CORE.SCFV_5_FC	Core-specific scFv, negative control for scFv production	scFv isolated after panning against core, with human Fc receptor. Truncated due to early stop codon	See 4.2.4
P4D5_CORE.SCFV_6_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.4
P4D5_CORE.SCFV_7_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.4
PSF_CMV_NH2_INSULINSP1	Backbone for eukaryotic cell protein expression	Empty plasmid, with secretory signal peptide of insulin	Oxford Genetics
PSF_CORE.SCFV_1_FC	Core-specific scFv production in eukaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.5
PSF_CORE.SCFV_2_FC	Core-specific scFv production in eukaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.5
PSF_CORE.SCFV_3_FC	Core-specific scFv production in eukaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.5
PSF_CORE.SCFV_4_FC	Core-specific scFv production in eukaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.5
PSF_CORE.SCFV_6_FC	Core-specific scFv production in eukaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.5
PSF_CORE.SCFV_7_FC	Core-specific scFv production in eukaryotic cells	scFv isolated after panning against core, with human Fc receptor	See 4.2.5
P4D5_CORE.SCFV_13_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after 3 rounds of panning against core, with human Fc receptor	See 4.2.8
P4D5_CORE.SCFV_27_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after 3 rounds of panning against	See 4.2.8

		core, with human Fc receptor	
P4D5_CORE.SCFV_59_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after 3 rounds of panning against core, with human Fc receptor	See 4.2.8
P4D5_CORE.SCFV_65_FC	Core-specific scFv production in prokaryotic cells	scFv isolated after 3 rounds of panning against core, with human Fc receptor	See 4.2.8

TABLE 7-3: LIST OF PLASMIDS USED TO EXPRESS SCFV PROTEINS ISOLATED FROM RIBOSOME DISPLAY

ANTIBIOTIC CONCENTRATION WAS 100 μG/ML FOR AMPICILLIN AND 50 μG/ML FOR KANAMYCIN IN LB BROTH AND AGAR PLATE.

TRANSFECTION PLASMIDS

NAME	USE	GENE	REFERENCE
C-HA PCDNAHUPAR-2	Transfection positive control	C-terminal HA tagged huPAR-2	Mattiuzzo *et al*., 2007
PCDNA3.1	Empty plasmid used as transfection negative control		Thermofisher

TABLE 7-4: LIST OF PLASMIDS USED TO TRANSFECT MAMMALIAN CELL LINES

ANTIBIOTIC CONCENTRATION WAS 100 μG/ML FOR AMPICILLIN IN LB BROTH AND AGAR PLATE.

PSEUDOTYPE VIRUS PRODUCTION PLASMIDS

NAME	USE	GENE	REFERENCE
PCMV8.91	First attempt at GBV-B PV production	HIV-1 GagPol	Zufferey *et al*., 1997
PCSGW		HIV-1 derived vector expressing GFP	Bainbridge *et al*., 2001, Demaison *et al*., 2002
PCDNA3.1_E1E2_HA		GBV-B E1E2 with C-terminus HA tag	See 5.2.1
PMDG		VSV-G	Naldini *et al*., 1996
PHV	Transduction of STAR cells	HIV-1 derived vector expressing GFP	Ikeda *et al*., 2003, Sanber *et al*., 2015
PHCMV-7A	Production of HCV PV in STAR-HV cells	HCV E1E2	Bartosch *et al*., 2003

PCSFLW	Third attempt at GBV-B PV production	HIV-1 derived vector expressing the firefly luciferase gene. Made previously in the lab by substituting GFP.	Bainbridge *et al*., 2001, Demaison *et al*., 2002
PCMV INTRON	Fourth, successful attempt at GBV-B PV production	MoMLV GagPol	Collins *et al*., 1995
PCFCR_LUC		This plasmid was created from pCFCR-huPAR2 by substituting the huPAR-2 gene with firefly luciferase	Ylinen *et al*., 2005, Mattiuzzo, 2009
PNL4.3LUC.R$^-$ENV$^-$		Replication-defective HIV vector containing luciferase	Connor *et al*., 1995, NIRRRP catalog # 3417
PCAGGS_E1E2_HA		GBV-B E1E2 with C-terminus HA tag	See 5.2.6.1
PCDNA3.1D_HCVE1E2		Synthetic HCV E1E2	Johansson *et al*., 2007
PCAGGS_E1E2_NOHA	Production of GBV-B PV with no HA tag	GBV-B E1E2	See 5.2.7.4

TABLE 7-5: LIST OF PLASMIDS USED TO CREATED PV EXPRESSING EITHER VSV, HCV OR GBV-B ENVELOPE PROTEINS.

ANTIBIOTIC CONCENTRATION WAS 100 µG/ML FOR AMPICILLIN IN LB BROTH AND AGAR PLATE.

Sequence	Nucleotide sequence immediately before tamarin CDR3 region	Amino acids	Alignment with human Ig sequences
V_H SEQUENCES			
VH1-7.1	ACA GCC GTC TAT TAC TGT TCT AGA	TAVYYCSR	92% alignment to IGHV3-49*01
VH1-7.2	ACG GCC GTG TAT TAC TGT GCG AGA	TAVYYCAR	100% alignment to IGHV1-69*04
VH1-7.3	ACG GCC GTG TAT TAC TGT GCA AGA	TAVYYCAR	96% alignment to IGHV1-69*04
VH1-7.4	ACA GCC GTC TAT TAC TGT AGT AAT	TAVYYCSN	91% alignment to IGHV3-49*01
VH3-5.1	ACG GCC GTG TAT TAC TGT GCA AGA	TAVYYCAR	96% alignment to IGHV1-69*04
VH3-5.2	ACG GCC GTC TAT TAC TGT GCA GGA	TAVYYCAG	95% alignment to IGHV1-24*01
VH3-5.3	ACG GCC GTA TAT TAC TGT GCA GGA	TAVYYCAG	100% alignment to IGHV3-23*01
VH3-5.4	ACG GCC ATG TAT TAC TGT GCG AGA	TAMYYCAR	96% alignment to IGHV1-69*04
VH4.1	ACG GCC ATC TAT TAC TGT GCG AGA	TAIYYCAR	92% alignment to IGHV1-69*04
VH4.2	ACG GCC GTG TAT TAC TGT GCG AGA	TAVYYCAR	100% alignment to IGHV1-69*04
VH4.3	ACG GCC GTG TAT TAC TGT GCG ACA	TAVYYCAT	100% alignment to IGHV1-69*11
VH4.4	ACG GCC GTC TAT TAC TGT GCA TAT	TAVYYCAY	95% alignment to IGHV1-69-2*01
VH6.1	ACG GCC GTA TAT TAC TGT GCG AGA	TAVYYCAR	96% alignment to IGHV1-69*12
VH6.2	ACG GCC GTG TAT TAC TGT GCT GGG	TAVYYCAG	100% alignment to IGHV3-72*01
VH6.3	ACG GCC GTA TAT TAC TGT GCG AGA	TAVYYCAR	96% alignment to IGHV1-69*04
VH6.4	ACG GCC AGA TAT TAC TGT GCG AGG	TARYYCAR	91% alignment to IGHV3-23*01
V_L SEQUENCES			
VK1.1	GTT TCG ATT TAT TAC TGT CAC CAC	VSIYYCHH	81% alignment to IGKV1-13*01
VK1.2	TTT GCC ATT ATT ACT GTC AAC AT	FATYYCQH	100% alignment to IGKV1-8*01
VK1.3	TTT GCC ACT TAT TTC TGT CAA CAT	FATYFCQH	92% alignment to IGKV1-13*01
VK1.4	GTT GCA ATT TAT TAC TGT CAA CAG	VAIYYCQQ	96% alignment to IGKV1-13*01
VK2-4.1	TTT GCC ACT TAT TAC TGT CAA CAT	FATYYCQH	96% alignment to IGKV1-8*01
VK2-4.2	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-11*01
VK2-4.3	GTT GCG ATT TAC CAC TGT CAA CAG	VAIYHCQQ	87% alignment to IGKV1-39*01
VK2-4.4	GTT GCA ATT TAT TAC TGT CAA CAG	VAIYYCQQ	96% alignment to IGKV1-13*01
VK3.1	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-15*01
VK3.2	TTT GCC GCT TAT TAC TGT CAA CAT	FAAYYCQH	91% alignment to IGKV1-13*02
VK3.3	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-15*01
VK3.4	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-11*01
VK5.1	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-11*01
VK5.2	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-11*02
VK5.3	TTT GCA GTT TAT TAC TGT CAG CAG	FAVYYCQQ	100% alignment to IGKV3-11*01
VK5.4	TCT GCA GTT TAT TAC TGT CAC CAG	SAVYYCHQ	96% alignment to IGKV3-11*01

TABLE 7-6: ANALYSIS OF TAMARIN V REGION SEQUENCES

TAMARIN SCFV SEQUENCES, CONTAINING VH AND VL SEQUENCES, WERE OBTAINED BY SANGER SEQUENCING. CDR3 REGIONS WERE IDENTIFIED USING IGBLAST SOFTWARE (DOWNLOADABLE) AND THE SEQUENCES IMMEDIATELY PRIOR TO THE CDR3 REGIONS WERE ALIGNED TO THEIR HUMAN COUNTERPARTS SHOWING SIGNIFICANT HOMOLOGY.

PLASMID MAPS OF PROTEIN PRODUCTION PLASMIDS

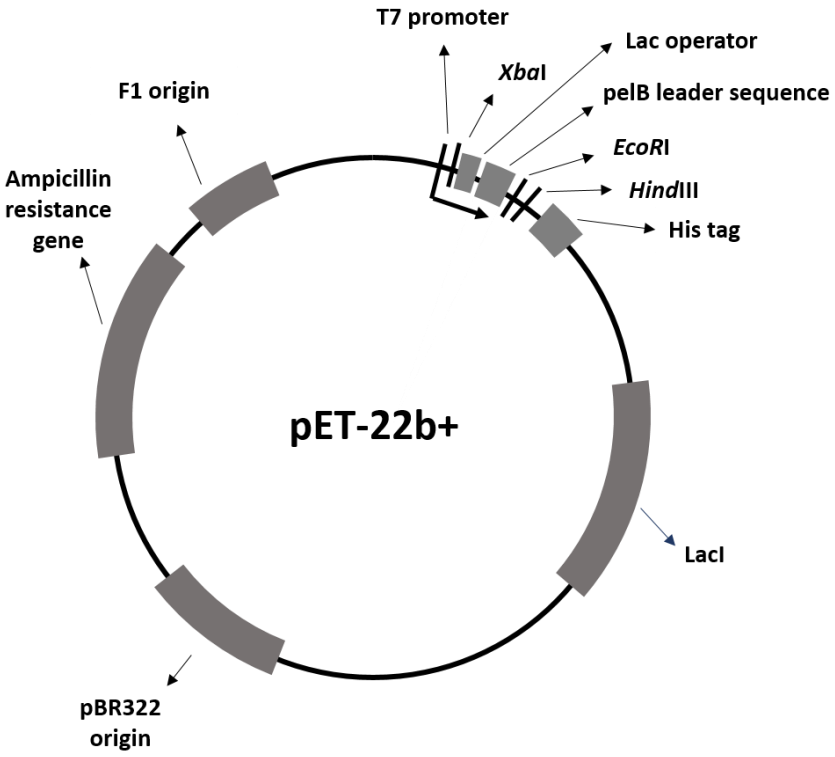


FIGURE 7-1: PLASMID MAP OF PET22B+

THIS PLASMID CONTAINS A PELB LEADER SEQUENCE TO DIRECT THE RECOMBINANT PROTEIN TO THE BACTERIAL PERIPLASM, THEREBY ALLOWING PROTEIN SECRETION. THIS PLASMID IS DESIGNED FOR EXPRESSION OF RECOMBINANT PROTEINS IN BACTERIAL CELL LINES.

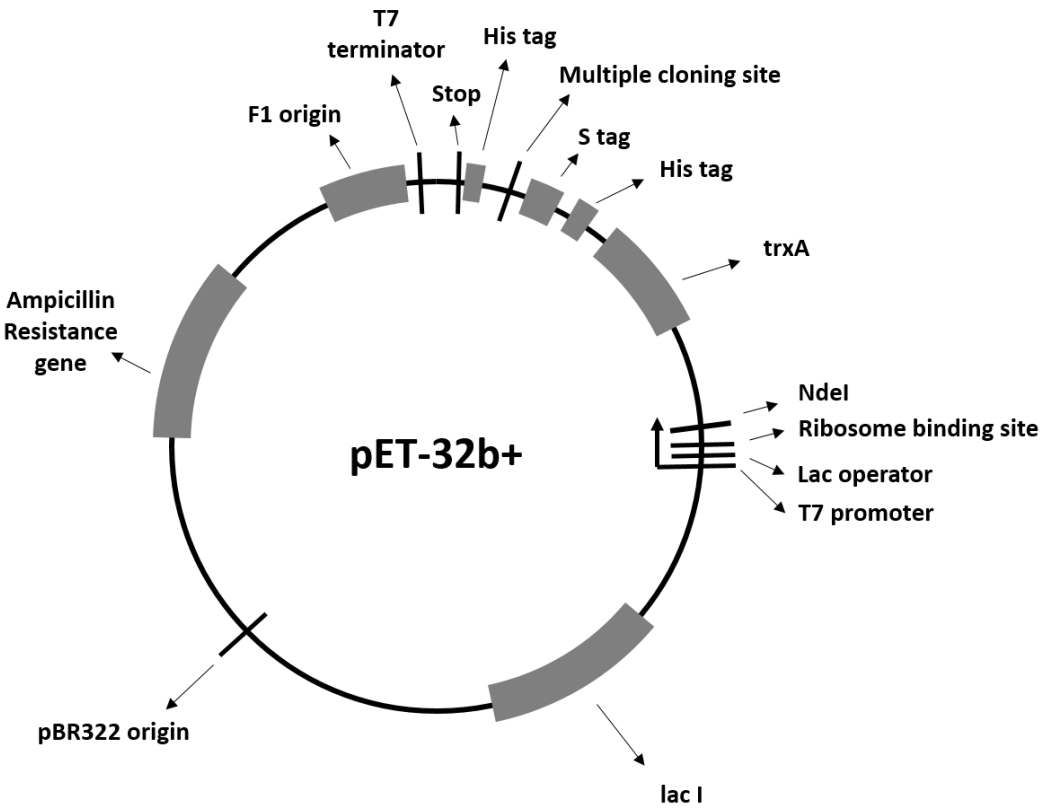


FIGURE 7-2: PLASMID MAP OF PET32B+

THIS PLASMID CONTAINS SEVERAL TAGS FOR PROTEIN PRODUCTION, INCLUDING AN S TAG, A TRXA TAG AND TWO HIS TAGS. THIS PLASMID IS DESIGNED FOR EXPRESSION OF RECOMBINANT PROTEINS IN BACTERIAL CELL LINES.

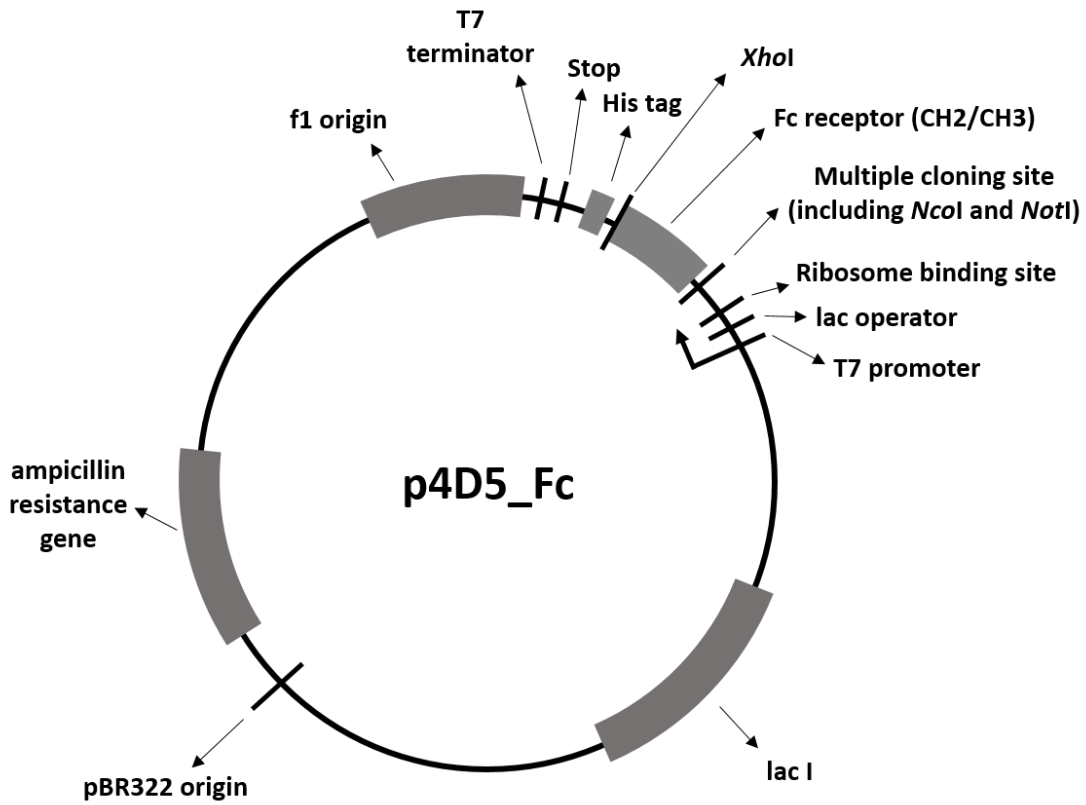


FIGURE 7-3: PLASMID MAP OF P4D5

THIS PLASMID IS A DERIVATIVE OF PET32B THAT CONTAINS ONLY ONE TAG- A C-TERMINAL HIS TAG. THIS PLASMID WAS ALTERED BY MARKIV AND COLLEAGUES TO SIMPLIFY PROTEIN PURIFICATION, AS SOME TAGS ARE KNOWN TO BE HIGHLY HYDROPHOBIC (MARKIV *ET AL.*, 2011). LATER, THE FC DOMAIN OF IGG WAS CLONED INTO THIS VECTOR FOR EXPRESSION OF SCFV ANTIBODIES. IT IS DESIGNED FOR EXPRESSION OF RECOMBINANT PROTEINS IN BACTERIAL CELL LINES.

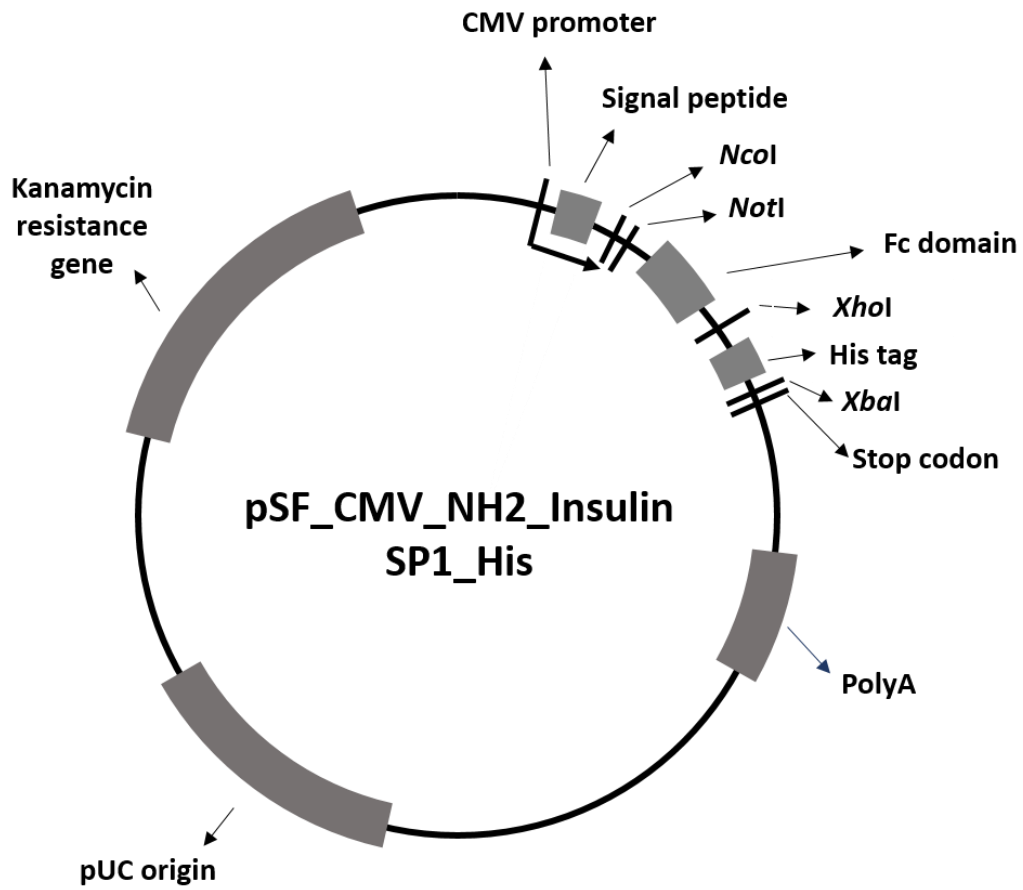


FIGURE 7-4: PLASMID MAP OF PSF

THIS PLASMID IS DESIGNED FOR EXPRESSION OF RECOMBINANT PROTEINS IN MAMMALIAN CELL LINES. IT CONTAINS A SECRETORY SIGNAL PEPTIDE TO ALLOW PROTEINS TO BE EXPORTED FROM THE CYTOSOL. DURING THE COURSE OF THIS PROJECT, THE FC DOMAIN WAS ALSO CLONED INTO THIS VECTOR TO ALLOW DOWNSTREAM EXPRESSION OF FUNCTIONAL SCFV.

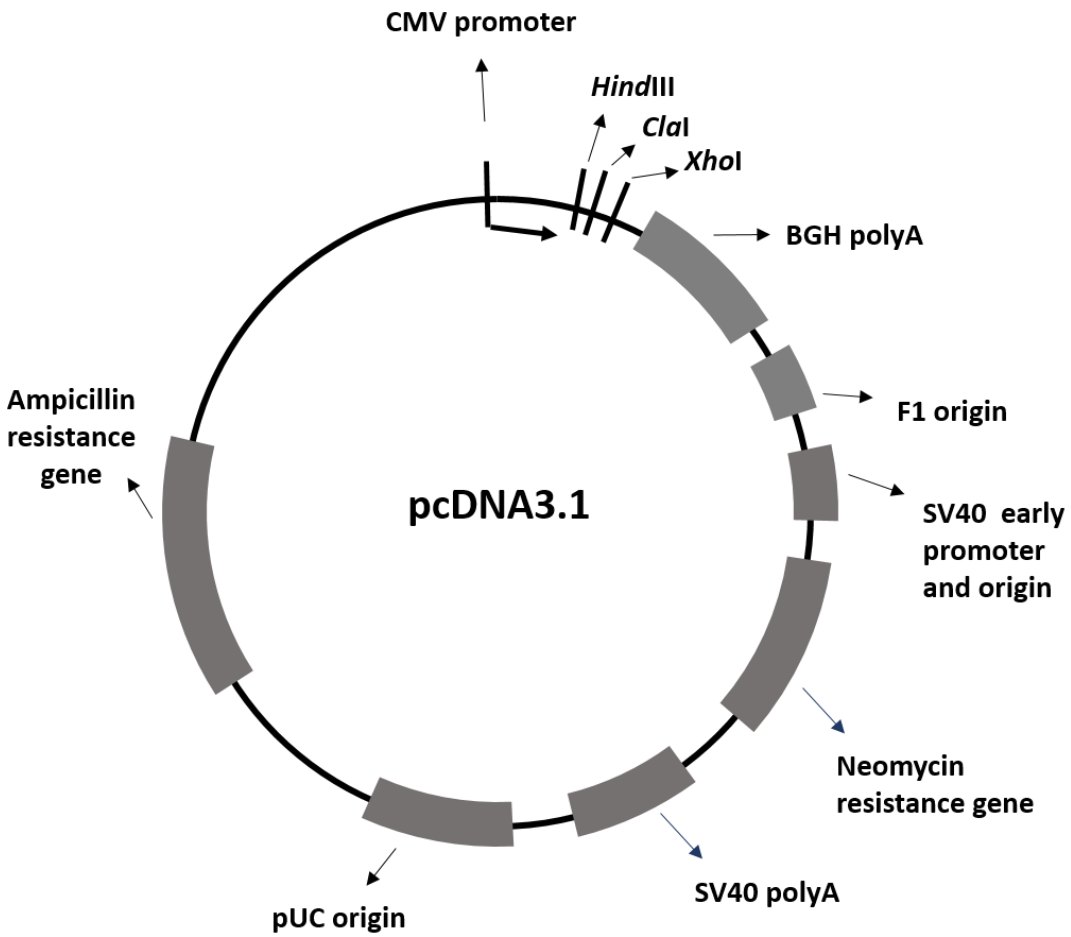


FIGURE 7-5: PLASMID MAP OF PCDNA3.1

THIS PLASMID IS DESIGNED FOR EXPRESSION OF RECOMBINANT PROTEINS IN MAMMALIAN CELL LINES. THIS PLASMID IS A DERIVATIVE OF PCDNA3 (WHICH IS NO LONGER COMMERCIALLY AVAILABLE); IT HAS AN ALTERED MULTIPLE CLONING SITE AND LACKS AN SP6 PROMOTER BINDING SITE.

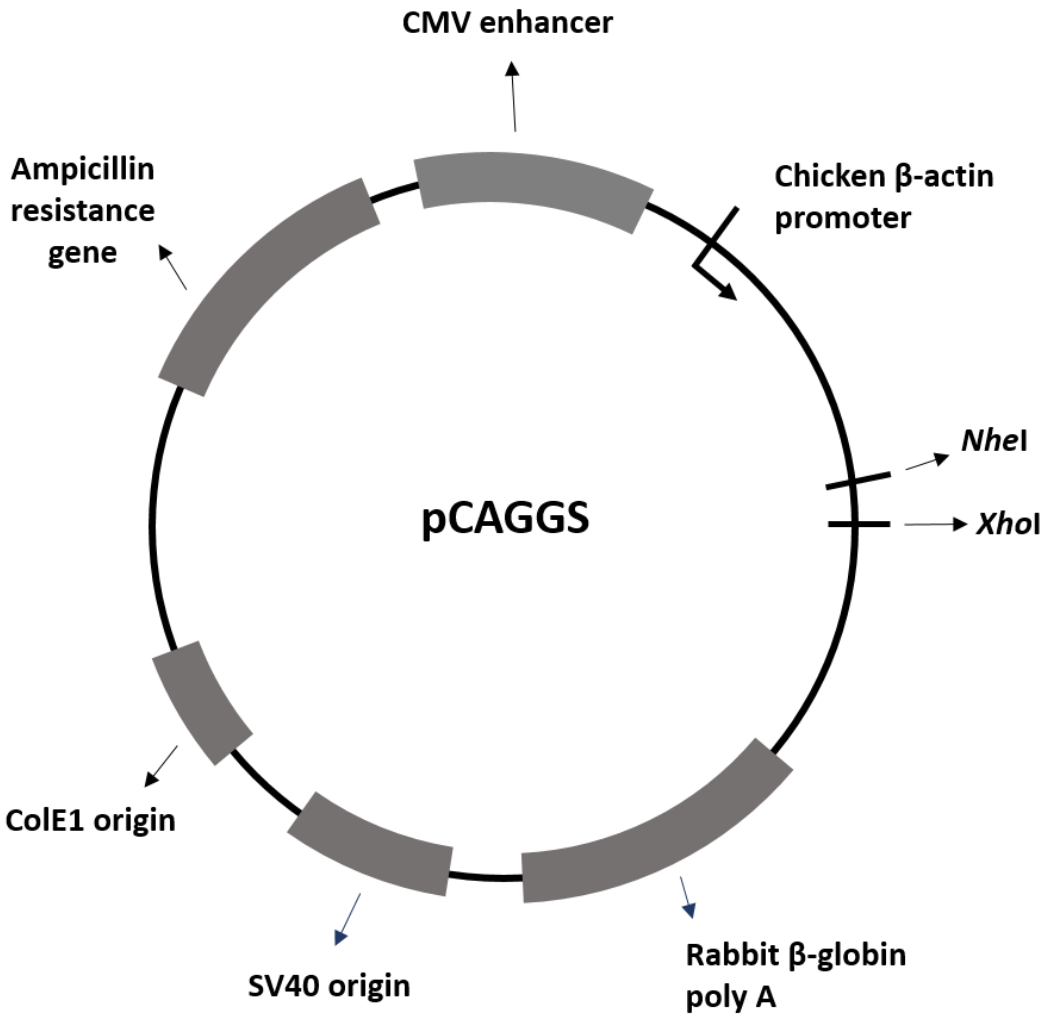


FIGURE 7-6: PLASMID MAP OF PCAGGS

THIS PLASMID IS DESIGNED FOR EXPRESSION OF RECOMBINANT PROTEINS IN MAMMALIAN CELL LINES. IT HAS A CMV ENHANCER, THE STRONG CHICKEN ß-ACTIN PROMOTER FOLLOWED BY A CHICKEN ß-ACTIN INTRON SEQUENCE WHICH DRIVES EXPRESSION OF RECOMBINANT PROTEIN GENES INSERTED INTO THE MULTIPLE CLONING SITE (WHICH CONTAINS *NHE*I AND *XHO*I).

ADDITIONAL RESULTS:

Protein	Accession number	Area	Score	Coverage	No. of peptides
GBV-B polyprotein	Q69422	2.563E10	782.90	9.50	21
Chaperone protein DnaK (*E.coli* O139:H28)	A7ZHA4	6.086E8	174.49	40.60	17
Cysteine desulfurase IscS (*E.coli* O139:H28)	A7ZPX4	1.431E9	166.18	41.58	12
Polyribonucleotide nucleotidyltransferase (*E.coli* O139:H28)	A7ZS61	7.834E8	164.84	27.57	15
Elongation factor Tu 1 (*E.coli* O139:H28)	A7ZSL4	1.374E9	105.34	40.10	12

TABLE 7-7: MASS SPECTROMETRY DATA FOR THE RECOMBINANT NS5BΔ23 PREPARATION

THE NS5BΔ23 PREPARATION ISOLATED FROM *E.COLI* WERE FIRST DIGESTED BY TRYPSIN AND LATER ANALYSED USING MASS SPECTROMETRY BY JUN WHEELER AT NIBSC. THE SEQUENCES OBTAINED FROM THIS MACHINE WERE THEN RUN AGAINST THE PUBMED GENBANK DATABASE. THE TOP FIVE RESULTS FROM THIS ANALYSIS ARE SHOWN IN THE TABLE ABOVE, WHICH SHOWS THE MOST ABUNDANT SEQUENCES IN THIS PROTEIN PREPARATION WERE PART OF THE GBV-B POLYPROTEIN.

	LENTIVIRAL-BASED GBV-B PV		RETROVIRAL-BASED GBV-B PV	
	UNCONCENTRATED *(RLU/ML)*	CONCENTRATED *(RLU/ML)*	UNCONCENTRATED *(RLU/ML)*	CONCENTRATED *(RLU/ML)*
TAMARIN FIBROBLAST	Not tested		0	0
FRHK-4	Not tested		23	2023
VERO	Not tested		127	2892
CRFK	1853	1198	8070	35833
HUH7.5	4800	26400	Not tested	

TABLE 7-8: PERMISSIBILITY OF DIFFERENT CELL LINES WITH GBV-B PV

ALL CELL LINES INVESTIGATED WERE INFECTED WITH SERIAL DILUTIONS OF EITHER LENTIVIRAL-BASED OR RETROVIRAL-BASED PV PRODUCED THROUGH THE CO-TRANSFECTIONS OF 293T CELLS WITH THE TWO OR THREE PLASMID SYSTEM AND PEI. UNCONCENTRATED AND CONCENTRATED SAMPLES OF BOTH LENTIVIRAL-BASED AND RETROVIRAL-BASED GBV-B PV WERE USED FOR EACH INFECTION AS I HAVE SHOWN THAT UNCONCENTRATED GBV-B PV HAVE LOW INFECTIVITY. THE LUMINESCENCE FROM THE PRODUCTION OF THE REPORTER GENE, LUCIFERASE, INCORPORATED INTO TARGET CELL GENOMES THROUGH SUCCESSFUL INFECTION WAS DETECTED FOR EACH CELL LINE. HUH7.5 WERE INFECTED WITH ONLY LENTIVIRAL PV DUE TO TIME RESTRICTIONS AS IT WAS PRESUMED THAT LENTIVIRAL PV HAVE HIGHER LEVELS OF INFECTIVITY. LUMINESCENCE VALUES FOR EACH CELL LINE TESTED ARE SHOWN ABOVE.

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