Platform development for human cytomegalovirus (HCMV) vaccine design to elicit cross-reactive neutralizing antibodies (nAbs)

by

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Abstract

Human cytomegalovirus (HCMV) can infect pregnant women, resulting in perinatal mortality or deafness in surviving infants. A protective vaccine is desirable. Most HCMV vaccine strategies aim to elicit neutralizing antibodies (nAbs) targeting surface glycoproteins, such as glycoprotein B (gB), which is required for infection. Previously assessed recombinant-gB has not been fruitful in eliciting nAbs. I constructed 14 gB-based antigens and evaluated their antigenicity with nAbs and non-nAbs specific to different gB epitopes. I identified an antigen, sgB-SS-HIS, to which nAbs bound strongly but non-nAbs did not. I also attempted to develop an HCMV pseudovirus assay platform to screen and categorize nAbs. Co-transfection of plasmids encoding HCMV glycoproteins and plasmids to generate lentiviral particles yielded pseudotyped HCMV, which infected representative epithelial and fibroblast cells. However, antibodies known to neutralize replication-competent HCMV did not neutralize the pseudotyped virus, suggesting that infection of cells by pseudotyped HCMV may not mirror the replication-competent virus.

Keywords: human cytomegalovirus; neutralizing antibodies; pseudovirus assay; recombinant antigens; vaccines

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List of Acronyms

Abs	Antibodies
AD	Antigenic domain
BSA	Bovine serum albumin
DMEM	Dulbecco’s minimal essential medium
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EGFRs	Epidermal growth factor receptors
Env	Envelope
FBS	Fetal bovine serum
gB	Glycoprotein B
gH	Glycoprotein H
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O
HBSS	Hank’s Balanced Salt Solution
HCMV	Human cytomegalovirus
HIG	Hyper-immunoglobulins
HIV	Human Immunodeficiency Virus
HSPGs	Heparan sulfate proteoglycans
HSV	Herpes simplex virus
Ig	Immunoglobulin
IVIG	Intravenous immunoglobulin
mAbs	Monoclonal antibodies
MW	Molecular weight
MPR	Membrane-proximal region
nAb(s)	Neutralizing antibody/antibodies
Ni-NTA	Nickel-nitrilotriacetic acid
NRP-2	Neuropilin-2

OD	Optical density
OR	Olfactory receptor
PBS	Phosphate buffer saline
PC	Pentamer complex
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
RLU	Relative luminescence units
RT	Room temperature
SNHL	Sensorineural hearing loss
TGFβR3	Transforming growth factor beta receptor III
TM	Transmembrane domain
VH	Variable heavy chain domain
VL	Variable light chain domain
VZV	Varicella-zoster virus

Chapter 1.

Introduction

1.1. Human cytomegalovirus (HCMV)

Human cytomegalovirus (HCMV) causes intrauterine infections globally, with an overall HCMV birth prevalence estimated at 0.7% as of 2007[1]. Infection can lead to perinatal mortality[2], long-term neurological damage[3], and hearing loss[4] or deafness[5] in 8-15% of surviving infants[6,7]. HCMV is also considered the leading non-genetic cause of sensorineural hearing loss (SNHL) in children[4], occurring in ~50% of symptomatic infants and 10% of asymptomatic children at birth[8]. An estimated 30-50% of women of reproductive age in the US and Europe are susceptible to HCMV infection[9].

Primary infection with HCMV during pregnancy in seronegative women, which can be defined as anti-HCMV IgG seroconversion during pregnancy[10], is usually due to direct care of young children[11]. Primary infection in early gestation carries the highest risk of symptomatic infection in fetuses and newborns[12], which differs from non-primary infection in women who were already seropositive for anti-HCMV IgG when first evaluated during pregnancy[13]. Seronegative HCMV women have a 5- to 25-fold increased risk of developing HCMV if exposed to children in day care[14], meaning that interaction with children leads to this increased risk. Increased exposure to individuals excreting HCMV is associated with non-primary infection, leading to intrauterine or vertical transmission of HCMV[8]. Exposure to a new strain of HCMV can also lead to reinfection of seropositive women, with subsequent intrauterine transmission resulting in congenital infection[15]. Vertical transmission is declared if the amniotic fluid contains HCMV or viral DNA, urine cultures are positive for HCMV, or when the fetus displays pathologic features of HCMV[16]. Congenital HCMV infection in neonates is detected in saliva or urine using polymerase chain reaction (PCR)-based assays[17].

Currently, there are no vaccines that can protect against HCMV infection. Vaccine-design approaches for highly variable viruses may inform strategies to design an effective HCMV vaccine. An effective vaccine given to women of childbearing age is considered the best way to prevent congenital infection[18].

1.1.1. Transmission and infection

HCMV is mainly transmitted through a mother-child cycle[19] (Figure 1-1), and effective interventions for the prevention of HCMV infection are lacking[4]. Mother-to-child transmission of HCMV predominantly occurs transplacentally, during birth, or after breast milk feeding[20]. The risk of vertical transmission to the fetus from primary maternal HCMV infection is ~40% (ref.[21]). At birth, virus particles in the cervix or vagina can infect the baby, and an infected child can transmit the virus via their saliva or urine to other children or their mother[20]. However, virus transmission via breast milk is the most common route. Infants who acquire HCMV in their first year of life may shed the virus in their saliva and urine for years, thus transmitting HCMV to their caregivers and other children[20]. Reinfection during pregnancy can also occur, such as when an infected child transmits the virus to a pregnant woman through saliva or urine, resulting in a congenital infection.

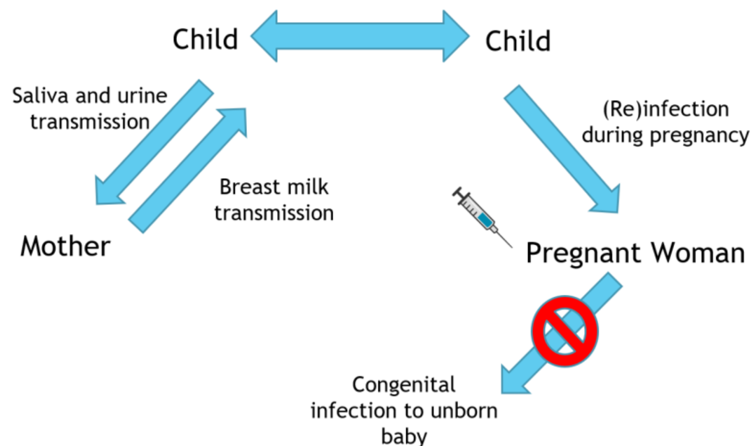

Figure 1-1: Mother-Child Cycle of Transmission
HCMV can be transferred from mother to child through saliva, urine, or breast milk transmission. The child can then pass it on to a pregnant woman, who could transmit it to the fetus, leading to a congenital infection. A vaccine given to women before or during pregnancy could stop this cycle.

1.2. HCMV glycoproteins

HCMV is an enveloped virus from the *Herpesviridae* family, with a DNA genome of approximately 236 kb[22]. The HCMV intra-host genomic variability at the nucleotide and amino acid level is comparable to many RNA viruses, including Human Immunodeficiency Virus (HIV)[23]. Glycoproteins on the virus surface form complexes important in virus attachment to target cells, fusion, and entry[24] (Figure 1-2).

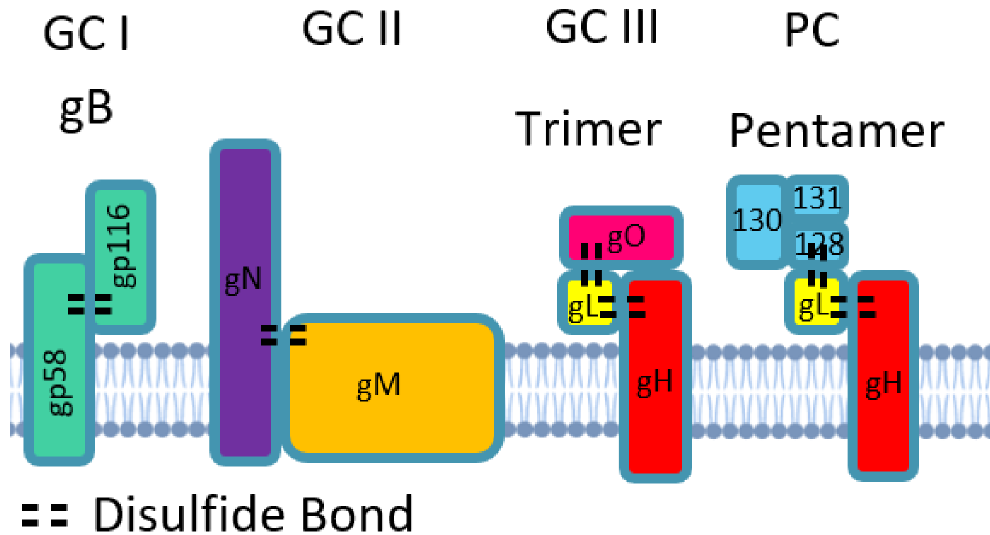

Figure 1-2: Glycoproteins found on HCMV
The image depicts three glycoprotein complexes (GC I-III) and a pentamer complex (PC) that occur on the surface of HCMV and are essential for virus attachment to target cells and entry. GC I is glycoprotein B (gB), shown in green, which comprises a surface subunit (gp116) and a transmembrane unit (gp58) covalently linked by disulfide bonds. GC II is a covalently linked dimeric complex of glycoprotein N (gN; purple) and M (gM; orange), and GC III is a trimer complex comprising glycoproteins H (gH; red), L (gL; yellow), and O (gO; magenta) covalently linked by disulfide bonds. The pentamer complex also consists of gH and gL, along with envelope proteins UL128, UL130, and UL131A (cyan). Schematic adapted from Gardner et al. (2016)[24]. The functions of the complexes are described in the text.

Glycoprotein B (gB), gH, and gL comprise the core fusion machinery required for attachment and entry[25]. HCMV gB is a fusion protein required for virus entry[26,27]. Two glycoprotein complexes can be formed with gB, either trimeric complex (GC III; gH, gL, and gO) or pentamer complex (PC; gH, gL, UL128, UL130, UL131A)[26]. The trimer complex promotes gB-mediated membrane fusion during entry into all cell types, but the pentamer complex provides a distinct function for entry into epithelial cells[28]. The complex used for virus entry into other cells depends on the cell type where the virus replication takes place[29]. Entry into fibroblast cells occurs through a pH-independent event by fusion through gB and the trimer complex (gL, gH, and gO) at the cell surface[24]. gB and the pentamer complex (gL, gH, and UL128/130/131A) enter epithelial cells through endocytosis after a pH-fusion event[24]. Therefore, to mediate attachment of HCMV to the cells, epithelial cells need to express receptors for the pentamer complex[30], whereas fibroblast cells need to express receptors for gH/gL/gO[31]. Research shows that

HCMV needs these glycoproteins (gB, gH, gL, gO, and UL128/130/131) to infect either epithelial or fibroblast cells efficiently[24].

1.2.1. Glycoprotein B (gB)

The HCMV gB ectodomain forms a trimer with an overall shape similar to herpes simplex virus (HSV-1) and Epstein-Barr virus (EBV) gB ectodomains[32,33] (Figure 1-3). There is also homology with gB with varicella-zoster virus (VZV)[34,35]. Five structural domains are colour assigned based on the structure of the HSV-1 gB homolog[36].

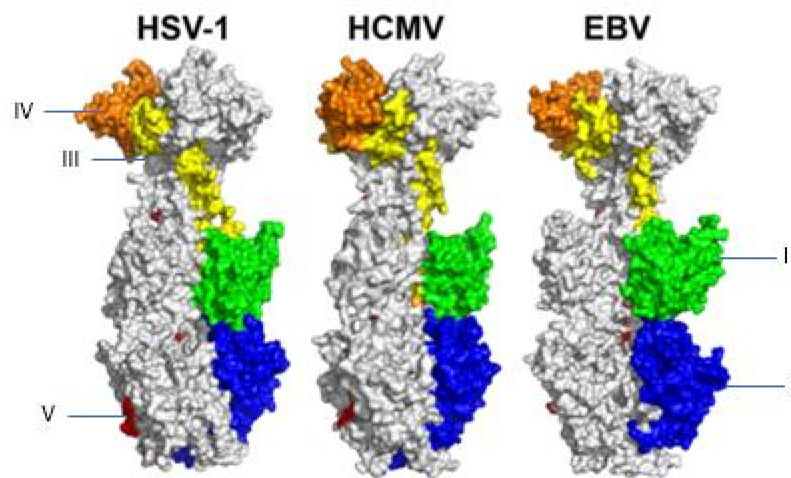

Figure 1-3: HSV-1, HCMV, and EBV gB ectodomains as trimers in surface highlighting domains I-V

gB ectodomains are structurally similar in HSV-1, HCMV, and EBV. Adapted from Burke et al. (2015)[33]. Structural domains: domain I (blue), domain II (green), domain III (yellow), domain IV (orange), and domain V (red).

HCMV gB is 907 amino acids long and contains a large ectodomain, a transmembrane domain (TMR), and a cytoplasmic domain (cyto) (Figure 1-4)[33,37]. HCMV gB has glycosylation sites that can protect neutralizing epitopes[33]. There are five distinct antigenic domains (AD 1-5; Figure 1-4 & Figure 1-5)[33], defined by gB-specific antibodies. AD-1 and AD-3 are mostly known sites of non-nAb vulnerability. Some antibodies that bind to AD-1 are present in all infected individuals and can be either neutralizing or non-neutralizing[37]. Anti-gB antibodies in human sera have a significant neutralizing activity that prevents HCMV infection in fibroblast cells[38]. A possible mechanism of virus neutralization would be to use nAbs to block the interaction between gB and gH/gL complexes[39]. Another mechanism is to have nAbs that recognize a pre-fusion conformation of gB that can block conformational changes in gB from a pre- to post-

fusion state[39]. Most nAbs from people target two protein domains: Domain I (AD-5) and Domain II (AD-4)[40] are also highly immunogenic[33]. More specifically, site 1 on AD-2, AD-4, and AD-5, are known to be vulnerable to nAbs[40], thus constituting obvious sub-targets within the overall gB antigen.

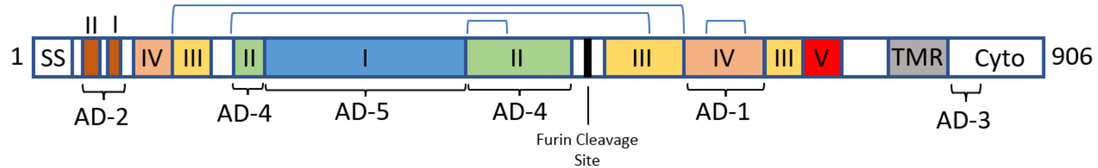

Figure 1-4: Linear antigenic map of HCMV gB with its antigenic domains (AD 1-5) sites

Full-length gB is synthesized as a ~906 aa precursor later cleaved by furin-like proteases to produce the soluble surface unit gp116 covalently attached to the cell surface anchored subunit gp58. The relative location of regions representing AD 1-5 is shown, including AD-2 sites I and II. Other regions denoted: SS, signal sequence; TMR, transmembrane region; Cyto, cytoplasmic domain. Schematic adapted from Schleiss et al. (2018)[41].

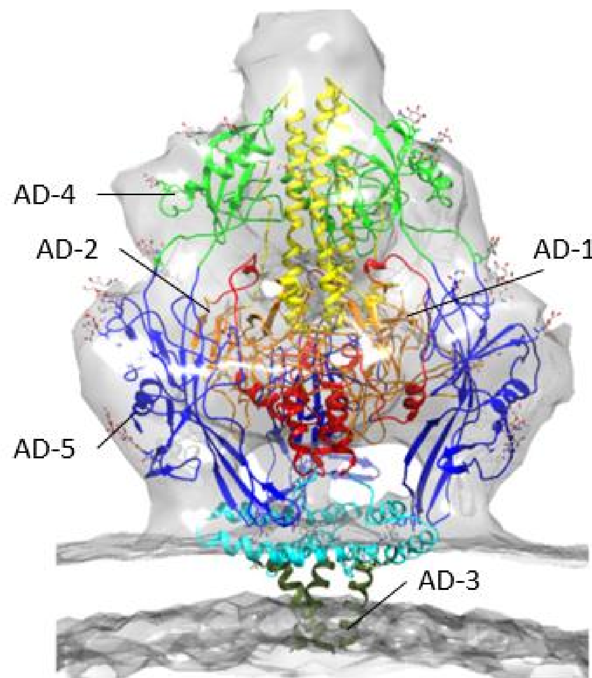

Figure 1-5: Purported pre-fusion structure of HCMV gB

The prefusion structure of HCMV gB can give insight into where the antibodies are binding to it Liu et al. (2021)[42]. Structural domains: Domain I (blue), domain II (green), domain III (yellow), domain IV (orange), and domain V (red).

AD-2 contains linear epitopes that are targets for nAbs from humans with natural HCMV infection[43]. Site I in AD-2 is a linear and conserved epitope on the N-terminal

domain of gB and is the target of some nAbs[44]. AD-2-specific antibodies can select escape variants of HCMV that are not affected by their neutralizing activity[45]. Antibodies against AD-4, a discontinuous epitope[46], were found in infected individuals and were able to neutralize HCMV infection[40]. These nAbs were potent[40], especially in comparison to AD-2, and were more diverse[47]. Through B cell repertoire analysis, Pötzch et al. hypothesize that antibodies against AD-4 may neutralize HCMV by blocking the interaction between gB and gH/gL[40]. AD-4 in gB is an attractive epitope for gB-based HCMV vaccines and a target for passive immunotherapy[45]. Neutralizing antibodies against AD-5 can prevent HCMV infection[40]. Furthermore, antibodies against AD-5 are less diverse than AD-4 but more diverse than AD-2 antibodies[45]. Therefore, site 1 on AD-2, as well as AD-4, and AD-5, are known to be vulnerable to nAbs[40].

1.2.2. Trimer complex (gH, gL, gO)

gL is linked to gH[48] and gO[49,50] via disulfide bonds, forming a trimeric complex. gO is highly glycosylated[51] and is attached to a site near the N terminus of gL[49]. gH/gL complexes carry out important functions in viral attachment and viral fusion with the host cell membrane[24]. The trimer complex is important for initial infection but seems less important for the subsequent spread of CMV to distal organs and tissues[52]. Another study suggests that the trimer complex alone is sufficient for infection of fibroblasts, but that infection of epithelial cells requires both the trimer and pentamer complex (discussed below)[53]. The trimer complex is believed to promote gB-mediated membrane fusion for entry into all cell types[28]. In a pH-independent fusion with the plasma membrane, gB fuses with the trimer complex, then HCMV enters fibroblast cells[24]. As mentioned in 1.2, for viral entry into epithelial cells, the trimer complex is also required[28]. Viral entry into epithelial cells involves micropinocytosis or endocytosis and low-pH-dependent fusion with endosomes[28]. gO is involved in receptor binding[49], such as PDGFR-α[54], as described in more detail in 1.3.1. The existence of several neutralizing epitopes on the trimer complex has also been noted[55].

1.2.3. Pentamer complex (PC; gH, gL, UL128, UL130, UL131A)

HCMV can enter epithelial, endothelial, dendritic cells, or monocytes via endocytosis after a low-pH-dependent fusion with gB and PC[24,56]. The gH and gL complex can interact with UL128/130/131A to construct the pentameric complex[57,58]. gL has a disulfide bond with UL128[49]. Some researchers found that coexpression of UL128, UL130, and UL131 with the gH/gL complex has increased the surface expression levels of gH/gL rather than gH/gL alone[31].

The pentamer complex is highly vulnerable to nAbs compared to gB, as evident from the relatively higher number of nAbs to PC compared to gB[57]. PC-specific serum antibodies can neutralize HCMV infection of epithelial cells[59]. Antibodies against the pentamer complex can interfere with binding HCMV to cell type-specific receptors and other signaling cascades[28]. Pentamer-specific antibodies that can neutralize HCMV infection in epithelial cells show no neutralizing activity on fibroblast cells[28]. Lilleri et al. found that antibodies binding to the pentameric complex could reduce the rate of vertical transmission of HCMV[12]. In mice immunization, Macagno et al. found that the pentamer complex could elicit extremely potent nAbs and cell entry in epithelial and endothelial cells[60]. The researchers also show that the antibodies bound to conformational epitopes required the expression of at least two proteins from the pentamer complex[60]. Some antibodies targeting the pentameric complex had an effective neutralizing infection of at least a thousand-fold higher than HCMV gB-specific antibodies[60]. Kabanova et al. immunized mice with the pentamer complex and were able to elicit HCMV nAb tiers that were consistently high levels[61]. The sera from mice immunized with the pentamer complex neutralized infection in epithelial and fibroblast cells[61].

1.2.4. Glycoproteins M and N (gM/gN)

Glycoproteins M and N form the gM/gN heterodimer, which is conserved in the herpesvirus family and is essential for viral replication[62]. gM is highly conserved with 99% mean identity across the different HCMV strains, whereas gN has 81% identity[63]. gM is important during virus-particle assembly[64], and gN is needed for virus replication[62]. gM and gN are linked by a disulfide bridge[24], forming a heterodimer that is the most abundant glycoprotein on the surface of HCMV[65]. The gM/gN complex binds to heparan sulfate proteoglycans (HSPGs) before entry of the cells, attaching HCMV to the cell[24].

There is extensive glycosylation of gN that can inhibit HCMV neutralization by antibodies[66], so the gM/gN complex is not considered a good target for nAbs.

1.3. Receptors that interact with the trimer or pentamer complex

HCMV can infect different types of cells that carry the appropriate receptors[24]. Initially, HCMV tethers to cells with either gB or gM/gN complexes and interacts with HSPGs[24]. A couple of significant receptors are present on cells, which directly interact with the HCMV trimer or pentamer complex. Some cell surface receptors are integrins, epidermal growth factor receptors (EGFR), or platelet-derived growth factor receptors (PDGFRα)[24]. Researchers suggest that entry of the HCMV pentamer complex, which involves gH, gL, and UL128/130/131A, into epithelial and endothelial cells requires receptors neuropilin-2 (NRP-2)[67] and olfactory receptor (OR)14I1[68]. Currently, researchers do not know how the trimer complex can bind to PDGFRα or how the pentamer complex can bind to NRP-2[69] or OR14I1[70], which can lead to activation of gB for HCMV entry into the cells.

Recent data suggest that transforming growth factor beta receptor III (TGFβR3)[67] interacts with the HCMV trimer complex to facilitate entry into fibroblast cells. Kschonsak et al. show that the trimer complex can bind to PDGFRα and TGFβR3 separately, suggesting that both receptors are mutually exclusive and can function as independent entry receptors[71]. Interestingly, PDGFRα and TGFβR3 bind to the trimer complex through a conserved surface on gO, gL, and the N-terminal region of gH[71]. Kschonsak et al. found that the pentamer complex has evolved to bind to different receptors, enabling HCMV to infect cells via different entry pathways[72]. There are other receptors on the cell where HCMV can still enter, although it is not widely understood how or through which mechanism. Researchers are studying PDGFRα and NRP-2 as these receptors seem to be the most important.

1.3.1. Platelet-derived growth factor receptor (PDGFRα)

The HCMV trimer (gH, gL, and gO) binds to PDGFRα on fibroblast cells[68]. Antibodies targeting PDGFRα do not inhibit HCMV entry into fibroblasts, epithelial, or endothelial cells, suggesting that HCMV might use PDGFRα in an alternative entry

pathway[73]. *In vitro* experiments have shown that PDGFRα directly interacts with gO[74]. It is most likely that the surface presentation of gO on the infected cells interacts with PDGFRα on neighboring cells to facilitate cell fusion and transfer the virus between the cells[75]. PDGFRα expressed in fibroblasts allows entry of the trimer complex as well as improves the efficiency of the pentamer complex[75]. Kschonsak et al. suggest that inhibiting the trimer complex (gO) interaction with PDGFRα may require blocking multiple interaction sites[71].

1.3.2. Neuropilin-2 (NRP-2)

Liu et al. found that the soluble trimer they designed could block HCMV entry into fibroblasts and epithelial cells, meaning that these were competing with HCMV to the cell surface receptors required for virus entry into the cell[76]. Their study found that HCMV requires both the trimer and pentamer complex to enter epithelial cells[76]. The HCMV pentamer complex (gH, gL, and UL128/130/131A) entry into epithelial and endothelial cells requires the neuropilin-2 (NRP-2) receptor[67]. Furthermore, Liu et al. found that the pentamer complex can promote egress from endosomes as it interacts later in the entry pathway of HCMV into the cells[76].

1.4. Current prevention of congenital HCMV infection

Most current strategies are focused on behavior measures during pregnancy, such as sanitization and hand hygiene, to avoid exposure to HCMV. Parents with young children at an increased risk of HCMV should be educated about transmission prevention through hygiene practices, avoiding sharing utensils, or kissing younger children on the mouth or cheek[21]. Additionally, most maternal HCMV infections are unrecognized clinically, and implementing a screening process to detect primary infections and prevent vertical transmission[20] would be beneficial.

Research on experimental antivirals and HIG for treatment[21] are undergoing. These measures would not likely successfully prevent HCMV infection as asymptomatic infants would not receive antiviral therapy. These asymptomatic infants are still at risk of developing late-onset sequelae[4], which is why a vaccine given to women of childbearing age is highly sought.

1.4.1. Hyper-immunoglobulins (HIG) and standard intravenous immunoglobulins

Two types of immunoglobulins are currently used to treat HCMV-infected patients but are not the standard therapy, and a case-to-case basis[45] is required. There are many studies on hyper-immunoglobulins and standard intravenous immunoglobulins, although there is currently more data for HIG therapies[45]. An HCMV-specific HIG is undergoing multiple clinical trials[4]. HIG has antiviral and immunomodulatory activities[77], which could help block HCMV glycoproteins and elevate anti-HCMV IgG titers[59]. HIG could be used as passive immunization to prevent congenital HCMV infection after primary infection from the mother[77]. Although, in one randomized trial reported in 2014, treatment with HIG did not significantly decrease primary HCMV infection during the pregnancy[78]. Other factors to consider are the availability of HIG, its unknown toxicity levels, and the detection of long-term deficits that might appear later on[77]. The other type is the standard intravenous immunoglobulin (IVIG) collected from donor pools with unknown HCMV serological status. A study by Schampera et al. showed that HIG- and IVIG-preparations have similar neutralization capacity when normalized for CMV IgG titer[79].

1.4.2. Antivirals: ganciclovir, valacyclovir, and cidofovir

Patients receive antivirals to treat HCMV infections, but some are prone to resistance and can also become toxic[76]. Ganciclovir, valacyclovir, and cidofovir are shown to slow down the replication of HCMV and can suppress clinical symptoms but cannot eliminate HCMV from the seropositive individual[22].

Ganciclovir was given to pregnant women to reduce the transmission of HCMV to the fetus[4]. In a randomized controlled trial, ganciclovir was given to neonates with symptomatic HCMV infection involving the central nervous system and prevented hearing deterioration at six months[80]. Unfortunately, ganciclovir gained resistance mutations after HCMV infection[81], which could lead to therapy failure as a standard treatment for HCMV infection.

Pregnant women can receive another antiviral called valacyclovir[4]. This antiviral would have some advantages as it is low cost and can be orally administered[82]. Shaher-Nissan et al. did a randomized, double-blind, placebo-controlled study[83]. They gave 8 g per day of valacyclovir or a placebo to pregnant women, reducing fetal HCMV infection

after primary maternal infection by 71%[83]. A case report where patients were given valacyclovir as an early treatment for maternal primary HCMV infection showed that in maternal blood, the virus was no longer detected[84]. The neonate was not infected and remained asymptomatic, suggesting that the 8 g per day of valacyclovir effectively reduces the maternal viral load, so there would be less risk of vertical HCMV transmission to the fetus[84]. A phase II clinical study found that valacyclovir given during pregnancy effectively improves the outcome of symptomatically infected fetuses[85]. Although these results look promising, a randomized clinical trial involving a larger cohort of patients might not be accessible to organize[82].

Furthermore, Bravo et al. looked at an antiviral for oral administration of hexadecyloxypropyl-cidofovir (HDP-CDV) therapy in pregnant guinea pigs. It is an excellent antiviral candidate for the treatment of HCMV infection[86]. However, in humans, HDP-CDV has not been tested.

1.4.3. Current vaccine strategies

Due to the mother-child transmission cycle, an effective vaccine given to women of childbearing age is widely considered the best way to prevent HCMV infection[18,87,88]. Studies show that pre-existing HCMV immunity is protective against congenital HCMV infection as there were significantly reduced vertical transmission rates in women with nonprimary infections[38,89]. An effective vaccine would need to induce HCMV-neutralizing antibodies (nAbs) that either provide sterilizing immunity by inhibiting HCMV acquisition or reduce systemic viral replication, viral seeding of the placenta, and subsequent fetal infection[4,90] and block the maternal-fetal transmission of HCMV[91]. However, no candidate vaccine has shown the desired efficacy in human clinical trials; at least 60% efficacy is required to curb congenital infection[90]. Vaccine candidates that induce nAbs against glycoproteins found on the surface of HCMV are desireable[18,37,38,87,90,92,93].

Developing a successful HCMV vaccine may benefit from a more systematic approach to immunogen design and evaluation. HCMV strains are genetically diverse, and a vaccine would need to elicit broadly cross-reactive nAbs[94]. Defining the HCMV immunogens which elicit the most robust protective immune responses and block infection of both fibroblast and epithelial cell types is critical for eliminating all HCMV infections[95]. Several vaccines are in clinical trials. These vaccines can induce both

humoral and cellular immune responses that closely mimic those induced by natural infection by expressing a full or nearly full complement of viral antigens[96].

Most HCMV vaccine strategies focus on the virus envelope gB antigen to induce nAbs, antibodies that could blunt virus infection and protect against disease and HCMV acquisition[27]. HCMV gB is considered an attractive target for vaccine design because of its central role in HCMV infection of all cell types[27]. Vaccine strategies focusing on nAbs are preferred because nAbs protect against HCMV more effectively than non-nAbs in murine HCMV models[97]. The nAbs reduced the viral burden more strongly than non-nAbs. Two notable recombinant gB vaccines are studied intensely[98]. Most HCMV vaccine strategies have focused on the HCMV envelope gB from lab strains AD169 (GlaxoSmithKline laboratories) and gB/MF59 (Sanofi) or other viral envelope glycoproteins to induce anti-viral nAb[37,87,95,99,100]**.** In the case of the gB-AD169 vaccine, N- and C-terminal segments of gB, including the intracellular domain, are replaced with equivalent segments from herpes simplex virus 1 (HSV-1) glycoprotein D and fused together[87]. The gB/MF59 vaccine contained HCMV gB adjuvanted with microfluidic adjuvant 59 (MF59)[92]. In the case of the gB/MF59 vaccine, the remaining parts, including the nominally intracellular domain of the glycoprotein, are then stitched together genetically to produce a single-chain construct[87]. The gB/MF59 vaccine had a 50% efficacy against primary HCMV infection in seronegative women[18].

An internal protease site and the membrane-spanning domain are removed in both recombinant versions of gB used for clinical trials to facilitate the manufacture of gB[37,87]. From ELISA data and neutralizing antibody titers, gB-specific responses were higher in vaccinated seropositive women. Furthermore, in another study with healthy HCMV seronegative adolescent women, a 43% efficacy was found in preventing HCMV primary HCMV infection, but this was not statistically significant compared to the placebo[93].

Pre-existing maternal immunity can reduce maternal-fetal transmission[101]. Two vaccine candidates developed by ModernaTX Inc., mRNA-1647 and mRNA-1443, are undergoing clinical trials (NCT04232280 and NCT03382405. Both candidates are based on the mRNA technology used for the SARS-CoV-2 vaccine (mRNA-1273). The mRNA-1647 vaccine candidate combines six mRNAs that encode the pentamer complex and

gB of HCMV[102]. NAbs to the pentamer complex and gB would be expected to prevent HCMV infection of various cell types.

Cui et al. produced a novel trimeric recombinant HCMV gB protein in Chinese ovary cells (CHO), which were able to elicit serum titers of nAbs and anti-HCMV gB-IgG antibodies at a higher amount in comparison with monomeric HCMV gB[101]. This data shows that vaccines containing only gB might not be enough and that the vaccine may need to include the trimeric or pentameric complex in conjunction with gB[101]. Furthermore, Kabanova et al. found that in human antibody responses to HCMV infection, the anti-gB antibodies were mostly non-neutralizing[61]. However, antibodies against the pentamer complex were nAbs and neutralized HCMV infection with high potency[61]. *In vitro* studies found that antibodies against the pentamer complex were thousands of folds more potent than gB-specific antibodies for neutralizing HCMV infection in epithelial cells[103]. A combination of epitopes on gB as well as the pentamer complex would probably be good candidates for developing therapeutic antibodies and preventing infection of various cell types[45]. Therefore, a combination of antigens may be required for HCMV vaccine candidates to maximize the protection against HCMV infection[38]. Experiments with gB-specific or pentamer complex-specific antibodies could help aid vaccine advances to see which glycoproteins on the surface would be needed.

1.5. Statement of the problem and thesis overview

Past HCMV vaccines have failed because they cannot induce high-titer persistent nAb responses that can effectively prevent the infection of epithelial cells[104]. These recombinant HCMV vaccines have not been successful at boosting immune responses in seropositive individuals and have failed to prevent primary HCMV infection among seronegative women exposed to young children who were actively shedding HCMV[95]. Current neutralization assays rely on live viruses. A sensitive platform amenable to high throughput might help to categorize HCMV nAbs better and, by extension, vaccine candidates.

I aimed to construct and evaluate the antigenicity of gB-based antigens designed to preserve conserved nAb-sensitive epitopes to elicit cross-reactive antibodies. In Chapter 2, I discuss the construction and evaluation of gB-based antigens made in the lab to induce nAbs for future immunizations in mice. Furthermore, I detail how I designed the

gB-based antigens and the justification for the mutations in each construct. Then I evaluate and characterize 14 recombinant gB-based antigens in terms of antigenicity and if they are multimeric and oligomeric. Finally, I discuss and analyze the different antigens. I aimed to develop an in vitro virus assay platform to screen and categorize cross-reactive anti-HCMV/gB nAbs. In Chapter 3, I discuss the development of a pseudovirus platform assay that can measure infection and, in the future, be used to evaluate nAbs of HCMV. I detail the pseudovirus's construction and different conditions for the pseudovirus platform to screen and categorize antibodies. In Chapter 4, I discuss the future directions for the gB-based antigens generated in Chapter 2 and the nAb evaluation platform developed in Chapter 3.

Chapter 2.

Construct and evaluate the antigenicity of gB-based antigens designed to preserve conserved nAb-sensitive epitopes for the elicitation of cross-reactive antibodies

2.1. Abstract

HCMV is a ubiquitous pathogen that can cause severe disease in infants. An effective maternal vaccine for HCMV is considered a significant health priority and should reduce the incidence and severity of congenital HCMV infection. Vaccine strategies that target the virus envelope gB protein would need to induce cross-reactive nAbs to have adequate protection against HCMV. Past vaccines have not been able to induce high-titer persistent nAb responses that can effectively prevent infection. In my work, I have generated and assessed a panel of 14 recombinant gB-antigens with five different antibodies (2F12, HCMV37, 1G2, SM5-1, and ITC88). From my results, I have selected three different recombinant gB-antigens, the best from each group. I have assessed their binding to the nAbs as a first step in identifying prospective gB-based vaccine candidates. The first recombinant is sgB-SS-His, a soluble gB antigen ending at the furin cleavage site and a His-tag with two serine mutations to disrupt cysteines that form a disulfide bridge. The second recombinant gB-based antigen is sgB-SS-Hpbf, which contains the same serine mutations as the previous antigen, except it has Hpbf; a particle formed from *Helicobacter pylori* (Hp) and the first five residues from a bullfrog (bf). The third gB-based antigen is gB.ecto-R6-P-HIS-T4 and is made from the gB ectodomain and contains various mutations: R6 stands for six arginine amino acids, which are in the furin cleavage site, as well as an A501P point mutation. Three nAbs: SM5-1, 1G2, and ITC88 were able to bind to these three recombinant gB-based antigens along with the commercial recombinant gB.

2.2. Introduction

Most vaccine strategies target the virus envelope gB and aim to induce cross-reactive nAbs. The functional importance of gB makes it an attractive vaccine target.

Approximately 70% of the HCMV neutralizing capacity of HCMV immune sera can recognize gB[105]. Vaccine strategies should focus on eliciting these nAbs as opposed to non-nAbs. Unfortunately, prior subunit vaccines, such as gB/MF59, mostly elicited non-neutralizing antibody responses and only achieved 50% of primary protection in HCMV seronegative women[106].

This project aims to inform the development of HCMV vaccine strategies focused on gB, a protein molecule on the surface of HCMV that is a known target for nAbs in infected individuals. gB is one of the main HCMV antigens considered for vaccine design because of its central role in virus entry of all cell types. gB can mediate virus and host cell membrane fusion. Researchers have shown structural data comparing other herpesviruses with HCMV, where the gB ectodomain is presented as a trimer on the virus surface[32,34], as mentioned previously (Figure 1-3). Vaccine strategies pursued by researchers for Human Immunodeficiency Virus (HIV) helped inform the gB protein designs used in this project.

Five antigenic domains (AD1-5) on gB (Figure 1-4) are defined, and a linear epitope ("site I") in AD-2 and epitopes in AD-4 and AD-5 have known nAb targets. Some researchers believe that engineering a gB vaccine that does not have AD-1 and AD-3 generates a higher neutralizing titer than the vaccine candidates used with full-length gB[63]. This way, the vaccine would primarily elicit nAbs rather than non-nAbs. Maternal nAbs are essential in preventing HCMV infection and the progression of the disease. Furthermore, pentamer-specific antibodies correlate with protection against transmission from the mothers to the babies[107]. Passively administered monoclonal nAbs protect against infection, which supports the pursuit of an effective HCMV vaccine that elicits such antibodies. Vaccine candidates containing recombinant gB elicit predominantly non-nAbs, particularly against AD-3. Antibody responses directed against a crucial gB-neutralizing epitope, AD-2 site I, are not elicited by a leading recombinant gB-based vaccine candidate. Most nAbs from people target two protein domains: Domain I (AD-5) and Domain II (AD-4)[40], which are also highly immunogenic[33]. Thus, site 1 on AD-2, AD-4, and AD-5 are vulnerable to nAbs[40] constituting obvious sub-targets within the overall gB antigen.

The first objective is to select recombinant antigen candidates that would best evoke a nAb response to HCMV upon immunization. To this end, a series of gB-based

antigens were genetically designed and expressed. I have constructed and produced a series of gB-based proteins and assessed which ones were best recognized by anti-gB nAbs relative to non-nAbs. By doing this, I can select which gB-based antigens might best be able to evoke nAbs upon immunization. Promising gB-based antigens were purified once, then assessed and characterized. Furthermore, evaluating nAbs and non-nAbs can characterize the antigenicity of these neo-constructed gB candidates and screen for potential immunogens.

2.3. Methods

2.3.1. Schematic representation of recombinant gB-based antigens

These recombinant gB-based antigens were designed from the VR1814 clinical isolate sequence. There were three main groups of the recombinant gB-based antigens: sgB-His, sgB-Hpbf, and gB.ecto (Figure 2-1). These contained conserved nAb-sensitive epitopes and no or few non-neutralizing ones, such as in the gB.ecto series. After designing, cloning, and expressing these antigens, an ELISA was used to screen to evaluate antigenicity. Lastly, the 3 top antigens were purified once for further development as potential vaccine candidates.

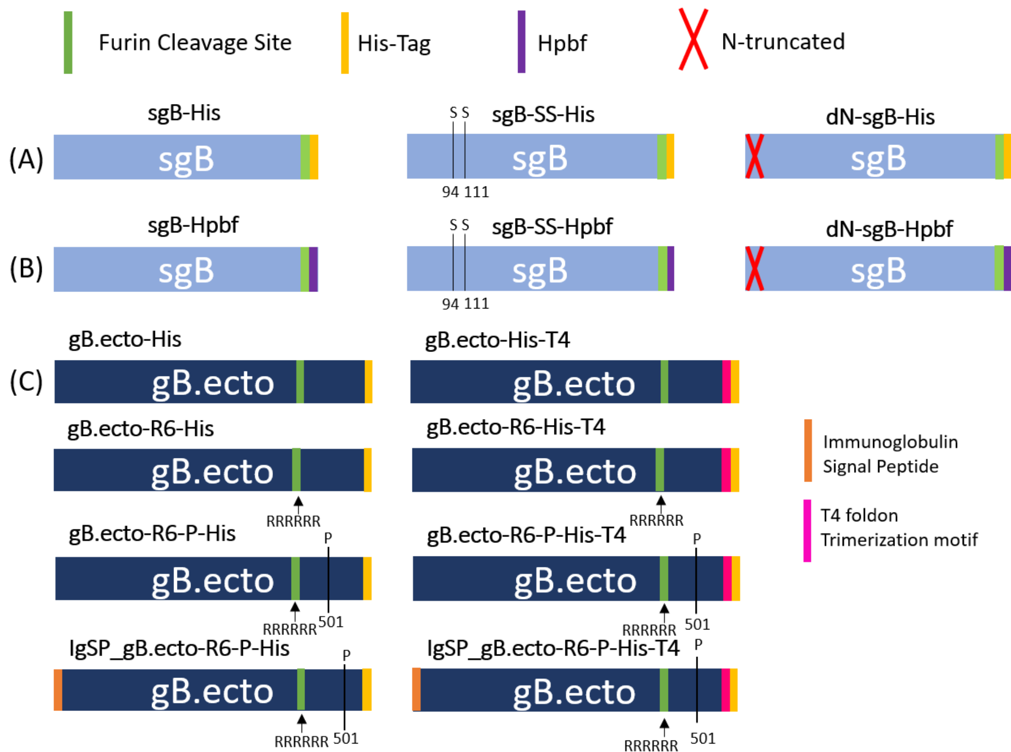

Figure 2-1: Panel of HCMV gB-derived recombinant antigens
All antigens were derived from the gB of HCMV strain VR1814. *(A)* His-tagged soluble gB (sgB) constructs. sgB is equivalent to the gp116 subunit of gB. *(B)* sgB-ferritin fusion constructs. The ferritin segment is *Helicobacter pylori* (Hp); the first five residues are from bullfrog (bf) ferritin. The sgB and sgB-Hpbf antigens include a construct with unpaired Cys94 and Cys111 replaced by serine (S) and a truncated N terminus (dN). *(C)* gB ectodomain constructs. RRRRRR, Hexa-arginine replacement of furin cleavage motif to increase gB processing. P, A501P substitution to potentially stabilize trimeric gB.

The first group contains soluble gB-based antigens with a His-tag, called the “sgB-His” constructs, where the gB-based antigen was truncated at the furin cleavage site (Figure 2-1A). The His-tag can provide a method for detecting and purifying the tagged soluble gB. The second group also contains the soluble gB-based antigens but with Helicobacter pylori (Hp) ferritin-gB instead of a His-tag, known as the “sgB-Hpbf” group (Figure 2-1B). For the sgB-ferritin fusion constructs, the ferritin segment is mainly from Helicobacter pylori (Hp), where the first five residues are from bullfrog (bf) ferritin.

Because of the disulfide bonds, C94-C550 and C111-C506, found in the original full-length version of gB, some unpaired cysteine (C) residues found in AD-1 potentially be an issue in forming the structure of the protein. One sgB-His construct and one sgB-Hpbf construct also have C94S and C111S mutations. These were replaced with serine

(S) to ensure no structural issues upon forming the gB-based antigen. Furthermore, on one sgB-His construct and one sgB-Hpbf construct, the N-terminus was truncated.

The third group, which contains the gB ectodomain only, is now termed “gB.ecto” (Figure 2-1C). This group contains various mutations: “R6” stands for six arginine amino acids in the furin cleavage site, an A501P point mutation “P” before AD-1, “T4” a T4 fold on trimerization motif, as well as “IgSP,” an immunoglobulin signal peptide. The six arginine residues (R6 or RRRRRR) have enabled better cleavage of recombinant HIV envelope glycoprotein[108] into the two subunits (gp120, gp41). These six arginine residues would help cleave HCMV gB into its two subunits (gp90, gp58). The proline substitution (A501P) also stems from HIV experiments, stabilizing the metastable conformation of envelope glycoproteins[109]. Furthermore, some antigens contained a T4 fold-on trimerization motif (T4), which can append a C-terminal fold-on trimerization domain[110] to potentially approximate the envelope glycoprotein's natural configuration. Lastly, some antigens contain a secretory immunoglobulin signal peptide (IgSP) that can be cleaved effectively from the protein of interest (gB).

These gB antigens were expressed transiently in 293T cells, as described in 2.3.2. After that, gB antigens from transfectants were screened for antigenicity by ELISA using mAbs, also as described in 2.3.4.

2.3.2. Construction and expression of gB antigens in 293 cells

A PCR (sequences are in Appendix A) with *Pfu* DNA polymerase (ThermoFisher) was used to generate the constructs. These modified antigen PCR products were analyzed on a 1% agarose gel (Biorad) and extracted with the QIAEXII Gel extraction kit (Qiagen) to ensure the correct size. These were digested with *KpnI*-HF (Invitrogen) and *XhoI* (Invitrogen) restriction enzymes and then ligated together using T4 DNA Ligase (Invitrogen; 1 U/μl) and 5X concentrated ligase buffer (Invitrogen). They were cloned into pcDNA-Zeo+ (Invitrogen/Fisher) and then transformed into bacterial cells (NEB 10). Then the plasmid DNA was extracted using minipreps (Qiagen), and a NanoVue spectrophotometer (GE) determined the concentration before being sent for sequencing (Eurofins). After that, the pure construct with the correct sequence was cloned into NEB 10 cells, and colonies were picked for midipreps (Qiagen) to get more DNA plasmid and then stored at -20 °C for future use. Then 200 μl of OptiMEM (Gibco), FuGENE

(Promega), and 6 µg of pure DNA plasmid were mixed and incubated at room temperature for 15 min. Then the mixture was transfected into half of a 6-well plate with 1×10^5 293T cells seeded the day before with Gibco Dulbecco's Modified Eagle Medium (DMEM) with an initial concentration of 1% Penicillin/Streptomycin (P/S) and 10% Fetal Bovine Serum (FBS). The supernatant was collected after incubating for three days at 37 °C, supplemented with 0.05% NaN_3 then stored at 4 °C for future use.

2.3.3. Recombinant antibody (Ab) expression

Sequences were from GenBank, and the antibodies were made internally or bought commercially. The antibody sequences were digested with enzymes and ligated into either IgG1 or IgG2 vector backbones. 1G2, ITC88, and SM5-1 were cloned into IgG1- and IgG2- expression vectors, whereas 8I21, 3G16, and 10F7 were cloned only into an IgG1 vector backbone. Regions encoding light chain domains were subcloned into pFUSEss-CLIg-hk or pFUSEss-CLIg-hL (Invivogen). Regions encoding heavy chain domains were subcloned into pFUSEss-CHIg-hG1 or pFUSEss-CHIg-hG2 (Invivogen) for expression of soluble antibody.

Cloning antibody heavy and light chain genes into expression vectors is performed as described above. A restriction enzyme digest was done and ligated to heavy chain vectors pFUSEss-CHig-hG1 or pFUSEss-CHig-hG2 or light chain vectors pFUSEss-hL or pFUSEss-hK. These were digested with either *EcoRI*-HF (Invitrogen) or *NheI*-HF (Invitrogen) for heavy chains or *EcoRI*-HF (Invitrogen) or *BsiWi*-HF (Invitrogen) restriction enzymes and then ligated with their respective parts together using T4 DNA Ligase (Invitrogen; 1 U/µl) and 5x concentrated ligase buffer (Invitrogen). They transformed into bacterial cells (NEB 10), and then the plasmid DNA was extracted using minipreps (Qiagen) and a NanoVue spectrophotometer (GE) to determine the concentration before being sent for sequencing (Eurofins). After that, another transformation of the pure construct was cloned, and colonies were picked for midipreps (Qiagen) to get more DNA plasmid and then stored at -20 °C for future use.

The 293F cells were maintained in 293 Freestyle media (Life Technologies) and pelleted by centrifugation (1000 rpm for 5 min at 15 °C), re-suspended in 293 Freestyle media, counted, and then seeded into one or more 125-ml shaker flasks (Corning) at a density of 5×10^5 cells/ml in 50-ml volumes. Following overnight incubation (at 37 °C,

10% v/v CO_2) on an orbital shaker at 120-150 rpm, cells were transfected with 25 µg of DNA (12.5 µg plasmid of each light chain and heavy chain plasmid) formulated in 2.5 ml OptiMEM containing 50 µl of Expifectamine (Invitrogen). Six days post-transfection, the supernatant was then harvested, with 0.05% Sodium azide (NaN_3) added, then centrifuged (3000xg for 30 min at 10 °C), then filtered (0.22-µm filters) and concentrated using 50 MWCO concentrators (Amicon). The raw supernatant was stored at 4 °C until ready to be purified.

The supernatant was concentrated and purified on an affinity column (NAb protein A+ spin columns, Thermo Scientific) per the manufacturer's recommendations. Sample purity was verified on a 7.5% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad) with a Coomassie Blue stain to ensure purity and that a single band could be seen at the correct molecular weight ~150 kDa for IgG antibodies. A BCA protein assay kit (Pierce) accurately measured the antibodies' concentration, and I followed the manufacturer's protocol. The standard used was Bovine Gamma Globulin (BGG). Antibodies were stored at 4 °C until needed.

The anti-gB monoclonal antibodies I used in the ELISA experiments were 2F12, HCMV37, ITC88, SM5-1, and 1G2 IgG. These antibodies target different sites of gB. 2F12 is a commercial monoclonal antibody from Abcam against an unspecified region of gB[111] but was non-neutralizing. HCMV37 is a commercially (Abcam) neutralizing gB-specific monoclonal antibody bound to gB in an unspecified region[104]. ITC88 is an anti-AD2 antibody demonstrated to prevent gB fusion post-binding[111]. SM5-1 targets and neutralizes two contiguous segments of Dom-II (AD-4) and was expected to bind to VR1814 gB[112]. 1G2 is a neutralizing human monoclonal antibody isolated from a seropositive subject on AD-5[113]. 1G2 IgG and ITC88 IgG bound to VR1814 gB[113]. 2F12 and HCMV37 are commercially bought from Abcam and serve as either non-neutralizing or poorly neutralizing antibodies.

For the constructs with the His-tag, the ELISA method was more straightforward and could bind to a primary antibody with a His-tag. Another antibody with a different serotype was generated for the constructs without a His-tag. SM5-1 IgG2 was the capture antibody, and the recombinant gB-based antigens could bind to it. The differences between the two types of ELISAs were followed as outlined in 2.3.4.

2.3.4. Enzyme-Linked Immunoassay (ELISA)

ELISA for His-tagged constructs

The schematic representation in Figure 2-2 shows the ELISA method. ELISA screened supernatants, and the best-bound antigens to the three nAbs were evaluated by the half-maximal effective concentration (EC_{50}) values. The EC_{50} value is the concentration that gives a half-maximal response[114], meaning fewer antibodies would be needed to produce 50% of the maximum effect. As stated previously, 2F12 is a non-neutralizing antibody, HCMV37 is a poorly neutralizing antibody, and SM5-1, ITC88, and 1G2 are nAbs known to bind to a different nAb epitope on gB. The ELISA experiment assessed supernatants with the antigens from 293T cells for binding to antibodies 1G2, ITC88, SM5-1, 2F12, and HCMV37. I performed an ELISA with the antibodies to measure the binding affinity and EC_{50} values. The antibody was coated on the plate the day before in PBS by titrating it 1:2 across the plate starting at 5 µg/ml and leaving the last column blank (only filled with PBS) and then stored at 4 °C overnight. The next day, washes with PBS-T (0.05% Tween) were performed between steps after incubating for an hour, rotating at room temperature. The 3% BSA was the blocking solution, and the antigen from the raw supernatant from 293T cells was added to the plate. After washing, 1 µg/ml of Penta-HIS-Biotin (Qiagen) supplemented with 1% bovine serum albumin (BSA) and 0.02% Tween (Bioshop) (PBS-BT) was added. After incubating and washing, alkaline phosphatase-conjugated (AP)-Streptavidin (Jackson ImmunoResearch) was diluted 1:1000 in PBS-BT and added. Finally, after incubating and washing, nitrophenyl phosphate substrate (Sigma; AP substrate) was added and incubated for 30 min in the dark, then measured at 405 nm on the SpectraMax.

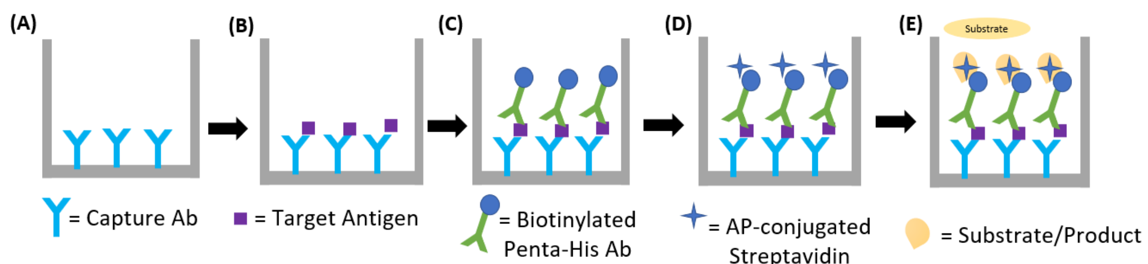

Figure 2-2: ELISA method for His-tagged gB antigen constructs
The plate was coated with the antibody (2F12, HCMV37, 1G2 IgG, ITC88 IgG, or SM5-1 IgG) at 4 °C overnight. The next day, washes were done at each step after incubating for 1 hr at RT. The supernatant of the gB-based antigens was added, then biotinylated penta-His Ab, and then AP-conjugated Streptavidin was added as the secondary antibody. Finally, the AP substrate was

added after incubating for 30 min in the dark and measured on a spectrometer at an optical density of 405 nm.

ELISA for non-His-tagged constructs and to observe oligomerization

Schematic representation, as seen in Figure 2-3, was used for non-His-tagged constructs and oligomerization, where the antigen can bind to many antibodies. The antibody (SM5-1 IgG2) was coated on the plate the day before in PBS, titrated 1:2 across the plate starting at 5 µg/ml, and then stored at 4°C overnight. The next day, plates were blocked with 3% BSA in PBS (1 hour at room temperature), and a supernatant-containing antigen was added. After incubating (1 hour at room temperature) and washing with PBS-T (0.05% Tween), 1 µg/ml of Penta-HIS-Biotin (Qiagen), supplemented with 1% bovine serum albumin (BSA) and 0.02% Tween (Bioshop) (PBS-BT), was added. After incubating (1 hour at room temperature) and washing, mouse anti-Human IgG1-HRP (horseradish peroxidase) was added. After another incubation (1 hour at room temperature) and washing, 3,3′,5,5′-tetramethylbenzidine (TMB) was added and incubated in the dark for 30 min. Sulfuric acid (2 M) was added to stop the color reaction. Finally, the plate was measured on the SpectraMax at an optical density of 450 nm.

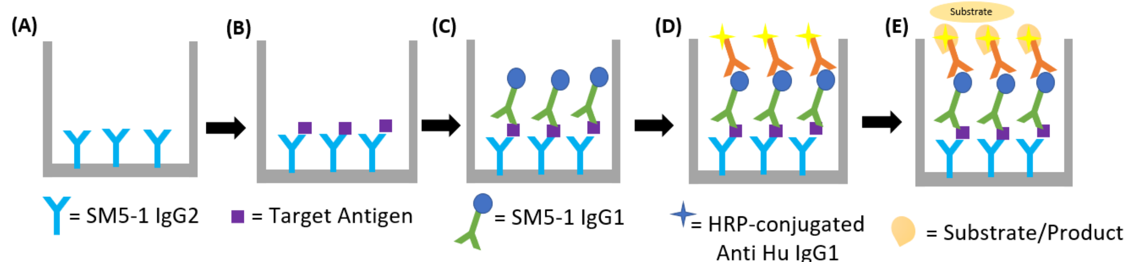

Figure 2-3: ELISA method for Hpbf gB-antigen-based constructs and for determining if the antigens are oligomeric
The plate was coated with SM5-1 IgG 2 at 4 °C overnight. The next day, washes between each step were performed after incubating for 1 hr at RT. Afterward, the gB-based antigens supernatant was used, SM5-IgG1 was used as the secondary antibody, and HRP-conjugated anti-Hu IgG1 was added. After incubation with the substrate, color reactions were stopped with H_2SO_4, and absorbance was measured on a spectrometer at 450 nm.

2.3.5. Purification strategies for recombinant gB-based antigens

Nickel Nitriloacetic acid (Ni-NTA) affinity chromatography

DNA encoding all constructs were cloned into the expression plasmid or pcDNA-Zeo+ (Invitrogen/Fisher) and verified by sequencing (Eurofins), as explained previously. 293F cells were transfected with the resulting plasmids. The His-tagged derived

constructs (either sgB-SS-His or gBectoR6PHisT4) were purified by using nickel-nitrilotriacetic acid agarose resin (Ni-NTA) columns (50% suspension in 30% EtOH; Qiagen). The wash buffer contained 10 mM imidazole, and the elution buffer contained 200 mM imidazole. The eluted sample was then buffer-exchanged into phosphate buffer saline (PBS) and stored at 4 °C for future use.

Diethylaminoethyl (DEAE) purification of antigen by High-Performance Liquid Chromatography (HPLC)

For sgB-SS-Hpbf, a transfection was done in 293F cells and then harvested after six days of incubating at 37 °C, 10% v/v CO_2. Immediately after harvesting, the cells and supernatant were put directly through a DEAE column (Sigma-Aldrich) in the AKTA machine following the manufacturer's protocol. Every 5 ml, fractions were collected, and then specific fractions were analyzed on a 7.5% SDS-PAGE (Bio-Rad).

2.3.6. Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl-sulfate Polyacrylamide gel electrophoresis (SDS-PAGE)

The supernatant, which contained the gB-based antigens, was concentrated and purified on an affinity column (NAb protein A+ spin columns, Thermo Scientific) per the manufacturer's recommendations. Sample purity was verified on a 7.5% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad) with a Coomassie Blue stain to ensure purity and that a single band could be seen at the correct molecular weight ~150 kDa for IgG antibodies. Fixing solution was made with 50% MeOH (50 ml), 10% acetic acid (10 ml), 40% water (40 ml). The Coomassie solution was 0.1% Coomassie Blue R-250 dissolved in the fixing solution. The destaining solution was 20% MeOH (20 ml) and 10% acetic acid (10 ml) in water (70 ml). BioRad Laemmli Sample Buffer (2x concentrated) was added before heating the sample for 5 min at 95 °C. Gels ran at a constant 150 V for 45 min.

Native Polyacrylamide gel electrophoresis (Native PAGE)

A 4-14% native polyacrylamide gradient gel (Bio-Rad) was used to analyze if the gB antigen was monomeric and estimate the size and folding. Set up and run were done according to NativePAGE Bis-Tris gels and XCell Surelock mini-cell system (Novex/Life Technologies). Coomassie stain was performed as stated previously above.

2.4. Results

2.4.1. ELISA was used to determine the antigenicity of gB-based antigens with anti-gB monoclonal antibodies

I used five antibodies for my experiments to assess the recombinant gB-based antigens from raw supernatant. I screened these gB-based antigens that I have generated with non-nAbs and nAbs. Afterward, data was graphed on Prism Graphpad, the Bmax values changed to 4, and the EC_{50} values were calculated. The cut-off for "No Binding" was determined by observing a maximum value of <0.4. My results reveal that the gB-derived protein construct sgB-SS-His is the best at preserving nAb epitopes and presenting them to the antibodies.

	Non-/Poorly Neutralizing Ab		(Broadly) Neutralizing Antibody		
	2F12	HCMV37	1G2	ITC88	SM5-1
gB (Towne)	0.14	No Binding	0.24	1.68	0.12
sgB-His	No Binding	No Binding	No Binding	No Binding	No Binding
sgB-SS-His	No Binding	No Binding	0.04	0.42	0.09
dN-sgB-His	No Binding	No Binding	12.53	No Binding	1.49
sgB-Hpbf	ND	ND	8.71	11.08	43.57
sgB-SS-Hpbf	ND	ND	0.29	0.27	37.60
dN-sgB-Hpbf	ND	ND	7.01	No Binding	40.95
gB.ecto-His	No Binding	3.87	0.78	8.10	0.80
gB.ecto-R6-His	No Binding	No Binding	1.10	No Binding	3.23
gB.ecto-R6-P-His	No Binding	No Binding	0.99	6.04	7.78
IgSP_gB.ecto-R6-P-His	No Binding	No Binding	1.07	9.88	5.87
gB.ecto-His-T4	No Binding	5.71	0.80	6.28	6.62
gB.ecto-R6-His-T4	No Binding	No Binding	No Binding	No Binding	No Binding
gB.ecto-R6-P-His-T4	No Binding	3.47	0.65	7.75	5.79
IgSP_gB.ecto-R6-P-His-T4	No Binding	7.06	0.98	1.66	2.99

EC_{50} (µg/ml)
< 0.01 – 0.1
< 0.1 – 1
< 1 – 10
> 10

Figure 2-4: Binding of non-neutralizing and neutralizing gB-specific mAbs to gB antigens by ELISA

Tabulated ELISA EC_{50} (µg/ml) values. sgB-SS-His was bound best by all three nAbs (i.e., 1G2 (AD-5), ITC88 (AD-2 site I), and SM5-1 (AD-4)) and not by the poorly neutralizing monoclonal antibodies: 2F12 and HCMV37. sgB-SS-Hpbf and gB.ecto-R6-P-His-T4 were bound second- and third-best. ND = no data.

gB (Towne strain; SinoBiological) was used as the control antigen and is the full-length version of gB. Interestingly, 2F12 is a non-neutralizing antibody found to bind to gB (Towne strain) but not to the VR1814. There is currently no research stating whether 2F12 can bind to the VR1814 strain. Moreover, with HCMV37, a neutralizing antibody, there is no available information on whether it should bind to the VR1814 strain. In these ELISA experiments, HCMV37 could bind to some constructs from the gB.ecto series but none of the sgB series. Furthermore, some epitopes did not seem appropriately presented on the antigen but could present correctly in the mutated version. For example, none of the nAbs can bind to sgB-His, but after two serine mutations, such as in sgB-SS-His, the antibodies could bind to the antigen.

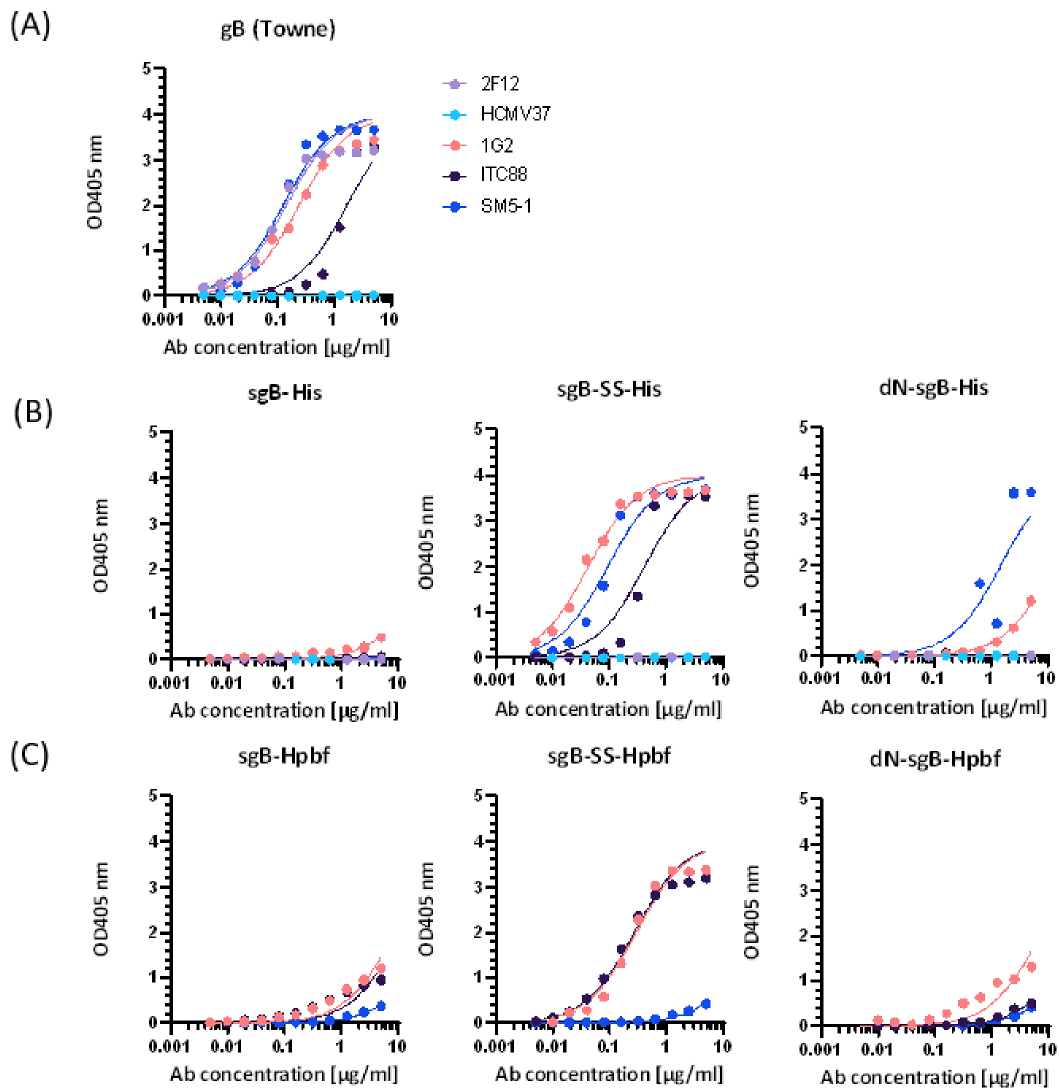

Figure 2-5: Binding of non-neutralizing and neutralizing gB-specific mAbs to soluble gB antigens by ELISA

Antigens were expressed from transiently transfected 293T cells and harvested supernatants screened by capture ELISA with serially titrated gB-specific antibodies. (*A*) Soluble recombinant full-length gB (Towne strain; SinoBiological) served as a control. (*B*) Soluble gB with His tag group. (*C*) Soluble gB with Hpbf.

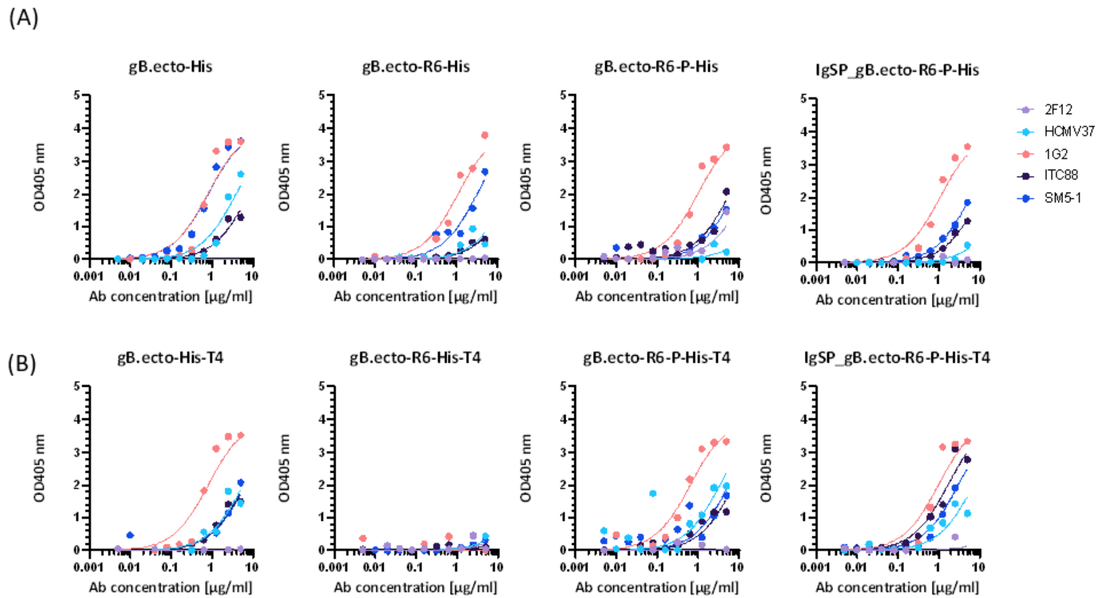

Figure 2-6: Binding of non-neutralizing and neutralizing gB-specific mAbs to gB ectodomain antigens by ELISA

Antigens were expressed from transiently transfected 293T cells and harvested supernatants screened by capture ELISA with serially titrated gB-specific antibodies. Soluble recombinant full-length gB (Towne strain; SinoBiological) served as a control. (*A*) gB ectodomain group. (*B*) gB ectodomain with T4 (T4 fold on trimerization motif).

In Figure 2-5 and Figure 2-6, some recombinant gB-based antigens from each group preserve nAb epitopes. The best gB antigen from each group that preserves all nAb epitopes (AD-2, AD-4, and AD-5) was chosen to be purified and further characterized to evaluate vaccine strategies and the nAbs response to HCMV. I was able to observe which constructs had the best binding of different antibodies, more specifically, the three nAbs that can bind to each domain: 1G2 IgG1 (binds to AD-5), ITC88 IgG1 (binds to AD-2), and SM5-1 IgG1 (binds to AD-4). The lowest EC_{50} value shows that the binding is the best between the respective antibody and the generated gB-based antigen. One from each group with the lowest EC_{50} values of the three antibodies was purified at a small scale and assessed more closely. In this case, the best from each group was: sgB-SS-His, gB.ecto-R6-P-His-T4, and sgB-SS-Hpbf.

2.4.2. Coomassie-stained SDS-PAGE was used to determine MW after Ni-NTA purification or DEAE

To determine the theoretical molecular weight (MW) of the recombinant gB-based antigens, ProtParam[115] was used to estimate the weight based on the protein

sequence. The VR1814 sequence of gB (GenBank accession number: ACZ79977.1) is 905 amino acids[116]. Additionally, one average molecular weight glycan is approximately 2.05 kDa[117]. The number of glycosylation sites was determined from N-GlycoSite[118]. The schematic concepts are in Figure 2-1. The gB.ecto-R6-P-His-T4 construct only contains the ectodomain, and the sgB constructs are only up to the furin cleavage site. According to the N-GlycoSite[118], the gB.ecto-R6-P-His-T4 construct has 18 glycosylation sites, and sgB has 15 glycosylation sites. Furthermore, depending on the construct, the His tag, comprised of 8-His residues, had to be considered. Moreover, Hpbf is a 24mer, so the molecular weight is multiplied by 24. For gB.ecto-R6-P-His-T4, the weight for the extra arginine residues and the T4 motif was added. The following theoretical molecular weights were calculated: gB.ecto-R6-P-His-T4: 110-134 kDa, sgB-SS-His: 75-94 kDa, sgB-SS-Hpbf: 83-143 kDa (x 24mer) = 1992-3432 kDa.

I had to transfect and purify these gB-based antigens in 293F cells, but a small-scale version to first determine which protein purification process to follow. For sgB-SS-His and gB.ecto-R6-P-His-T4, a Ni-NTA column was used for protein purification as these recombinant gB-based antigens have the His-tag. Afterward, fractions were collected and then resolved on a 7.5% SDS-PAGE and Coomassie stain to see the proper band size, prediction of the molecular weights, and if the protein of interest was able to elute a pure protein. On the 7.5% SDS-PAGE, multiple bands are seen for both antigens, meaning that the purification process needs to be refined. Potentially, another purification step can be included, such as using a size exclusion chromatography column (SEC) and filter before loading it on the Ni-NTA column to get rid of the extra bovine serum albumin (BSA) that is approximately 66 kDa[119]. There are also faint bands around 250 kDa; we are unsure what that is. The band around 75 - 150 kDa could potentially be the proteins of interest.

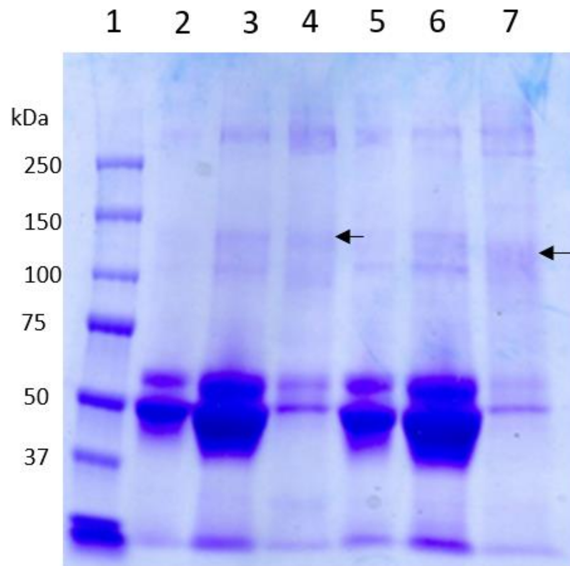

Figure 2-7: SDS-PAGE of gB.ecto-R6-P-His-T4 and sgB-SS-His following Ni-NTA purification

7.5% SDS-PAGE of gB.ecto-R6-P-His-T4 and sgB-SS-His following NiNTA purification. Arrows indicate bands where the protein of interest is at the predicted molecular weight. gB.ecto-R6-P-His-T4 is predicted to have a molecular weight of 110-134 kDa, and sgB-SS-His is predicted to have a molecular weight of 75-94 kDa. Lane 1 is the precision protein ladder, and lanes 2-4 are gB.ecto-R6-P-His-T4: 1:100 dilution of flow-through, wash, elution. Then lane 5-7 is sgB-SS-His.

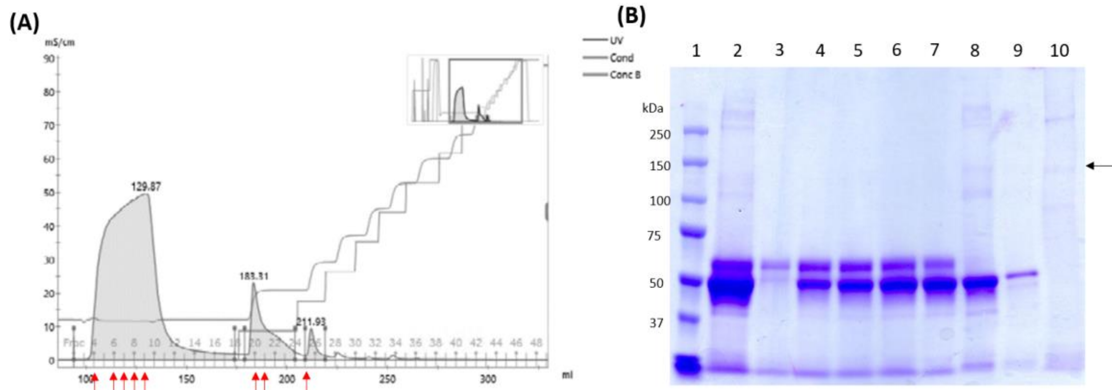

Figure 2-8: SDS-PAGE sgB-SS-Hpbf fractions following DEAE AKTA results

Red arrows indicate fractions chosen to run on the 7.5% SDS-PAGE. Black arrows indicate bands where the protein of interest is at the predicted molecular weight. sgB-SS-Hpbf is predicted to have a molecular weight of 83 - 145 kDa for its monomeric form. Lane 1 is the precision plus protein ladder, and lane 2 is the raw supernatant, then lanes 3-10, where fractions 4, 6, 7, 8, 9, 20, 21, and 25 were loaded onto the SDS-PAGE.

2.4.3. Coomassie-stained Native PAGE was used to determine if recombinant gB-based antigens are expressed as multimeric proteins

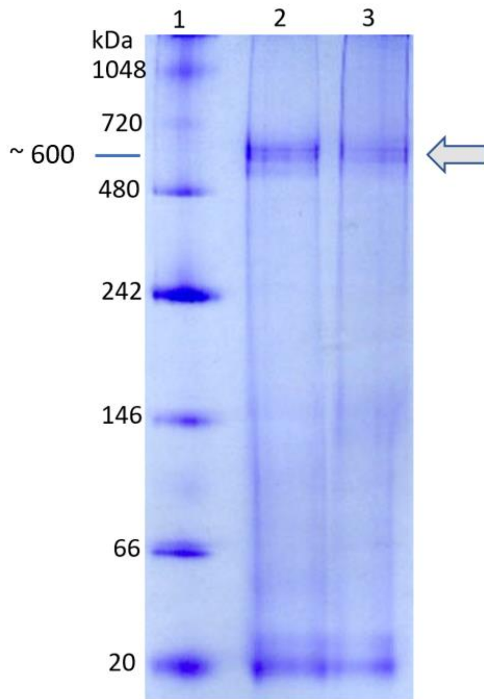

Figure 2-9: Native PAGE of gB.ecto-R6-P-His-T4 and sgB-SS-His
4-14% Native PAGE of gB.ecto-R6-P-His-T4 and sgB-SS-His. The black arrow indicates the protein of interest. The most prominent bands are around 600 kDa. gB.ecto-R6-P-His-T4 is predicted to have a molecular weight of 110-134 kDa, and sgB-SS-His is predicted to have a molecular weight of 75-94 kDa.

To further characterize and evaluate the recombinant gB-based antigens, gB.ecto-R6-P-His-T4 and sgB-SS-His ran on a 4-14% Native PAGE. The results show that the recombinant gB-based antigens are expressed in 293F cells as multimeric as the bands are around 600 kDa, which is approximately six times more than the predicted monomeric molecular weight.

2.4.4. ELISA was used to determine if gB-based antigens were oligomeric

Along with the Native PAGE (Figure 2-9), the results from the ELISA indicate that sgB-SS-His is unwantedly multimeric. The ELISA protocol used was outlined in 2.3.4.

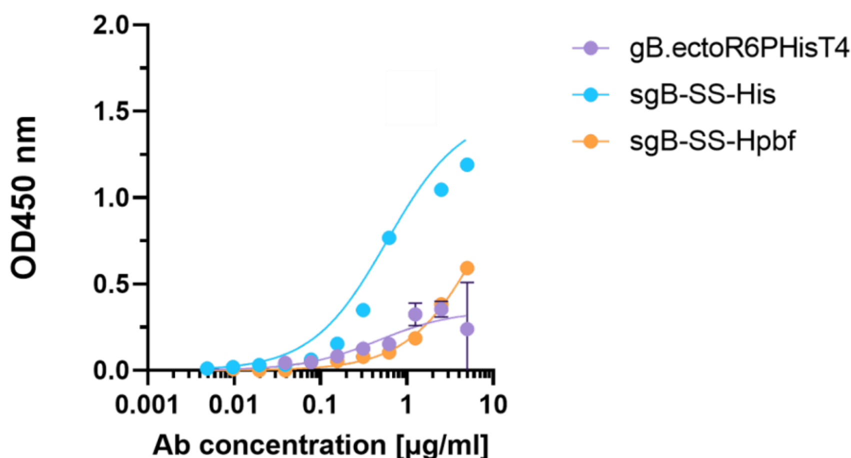

Figure 2-10: ELISA reveals that gB-derived constructs generated in this study are highly oligomerized
Capture ELISA of gB.ecto-R6-P-His-T4, sgB-SS-His, and sgB-SS-Hpbf using a human IgG2 version of anti-gB antibody SM5-1 to capture antigen on the ELISA plate. Bound antigen was detected with a human IgG1 version of the same antibody. sgB-SS-His shows the most significant binding, which supports the notion that it is highly multimerized.

2.5. Discussion

Some vaccine strategies focus on gB, a target for nAbs in infected individuals. Evaluating potential recombinant gB-based antigens that present the conserved epitopes for nAbs could be the first step toward vaccine candidates before immunization experiments. From the panel of recombinant gB-based antigens, sgB-SS-His was able to preserve and present nAb epitopes better than the full-length gB (SinoBiological) used as a control.

The construction of recombinant gB-based antigens (Figure 2-1) was established from protein designs informed by HIV vaccine strategies. Mutations such as the six arginine residues enable better cleavage of the envelope glycoprotein into the two subunits[108]. The proline substitution helps stabilize the metastable conformation of envelope glycoproteins[109]. Lastly, the C-terminal fold on the trimerization domain to

approximate the natural configuration of the envelope glycoproteins[110] can help increase the antigens' expression. The HCMV recombinant gB-based antigens designed for this project were derived from the VR1814 strain, which is a clinical isolate, where its complete sequence is known and is extensively studied by researchers. Engineering a recombinant gB vaccine that does not have AD-1 and AD-3 generates higher neutralizing titers than vaccine candidates with full-length gB to elicit nAbs rather than non-nAbs[63].

From the data in

	Non-/Poorly Neutralizing Ab		(Broadly) Neutralizing Antibody		
	2F12	HCMV37	1G2	ITC88	SM5-1
gB (Towne)	0.14	No Binding	0.24	1.68	0.12
sgB-His	No Binding	No Binding	No Binding	No Binding	No Binding
sgB-SS-His	No Binding	No Binding	0.04	0.42	0.09
dN-sgB-His	No Binding	No Binding	12.53	No Binding	1.49
sgB-Hpbf	ND	ND	8.71	11.08	43.57
sgB-SS-Hpbf	ND	ND	0.29	0.27	37.60
dN-sgB-Hpbf	ND	ND	7.01	No Binding	40.95
gB.ecto-His	No Binding	3.87	0.78	8.10	0.80
gB.ecto-R6-His	No Binding	No Binding	1.10	No Binding	3.23
gB.ecto-R6-P-His	No Binding	No Binding	0.99	6.04	7.78
IgSP_gB.ecto-R6-P-His	No Binding	No Binding	1.07	9.88	5.87
gB.ecto-His-T4	No Binding	5.71	0.80	6.28	6.62
gB.ecto-R6-His-T4	No Binding	No Binding	No Binding	No Binding	No Binding
gB.ecto-R6-P-His-T4	No Binding	3.47	0.65	7.75	5.79
IgSP_gB.ecto-R6-P-His-T4	No Binding	7.06	0.98	1.66	2.99

EC_{50} (µg/ml)
< 0.01 – 0.1
< 0.1 – 1
< 1 – 10
> 10

Figure 2-4 and Figure 2-5, the antigenicity for 14 recombinant gB-based antigens was evaluated by supernatants screened through an ELISA and calculated EC_{50} values. The lower the EC_{50} value, the stronger the binding between the antigen and antibody. Five different antibodies against gB were used: 2F12 (a non-nAb), HCMV37 (a poorly nAb), ITC88 (nAb against AD-2), SM5-1 (nAb against AD-4), and 1G2 (nAb against AD-5). For the full-length gB (SinoBiological), all antibodies except for HCMV37 could bind with an EC_{50} value < 2 µg/ml, meaning the binding interaction is very strong. For the sgB series, 2F12 and HCMV37 could not bind to the soluble gB His group, so the soluble gB

with Hpbf antigens were not evaluated. The two antigens with the serine mutations, sgB-SS-His, and sgB-SS-Hpbf, had lower EC_{50} values than those without the serine mutations (sgB-His and sgB-Hpbf). Furthermore, the antigens with the Hpbf mutation increase epitopes found on the surface to encourage more antibody binding. For example, sgB-Hpbf could bind the antibodies, whereas the sgB-His antigen was not. Some of the Hpbf antigens had higher EC_{50} values than their soluble gB counterparts. For example, SM5-1 was poorly bound to the soluble gB Hpbf antigens with an EC_{50} value > 30 μg/ml, whereas the soluble gB His antigens had EC_{50} values < 2 μg/ml. The Hpbf addition did not aid in decreasing the EC_{50} values. For the gB.ecto series, the T4 motif was able to help stabilize the protein as hypothesized. The IgSP helped cleave the signal peptide and improved the antigen's expression. The IgSP for the soluble gB antigens could help increase expression levels and decrease EC_{50} values. Antigens with the six arginine residues could not properly fold until there was a proline mutation. Potentially having a construct with the proline mutation without the six arginine residues would assist with the binding of the antibodies to the antigen.

Some recombinant gB-based antigens could not correctly present the different antigenic domains. For example, dN-sgB-His contained a truncated version of the N-terminus region close to site I on AD-2. ITC88 is an AD-2-specific antibody that effectively neutralizes HCMV infection in various cell types[45]. ITC88 can bind to site I on AD-2 near the N-terminus region. Since the N-terminus region was truncated, the epitope was presumably not correctly presented on the surface for ITC88 to bind.

Another example is the difference between the sgB-His and the sgB-SS-His antigens. The full-length gB protein was shortened and made soluble by ending at the furin cleavage site. The disulfide bonds were disrupted from before the furin cleavage site to after the cleavage site (Figure 1-4). Once it was truncated, cysteines on AD-2 were supposed to form a disulfide bond but could not. The free cysteines presented on sgB-His and sgB-Hpbf would potentially ruin the proper folding of the antigen. The antigenic domains could adequately present themselves on the surface through these different mutations so the antibodies could bind.

A recombinant gB-based antigen that can bind many nAbs has a strong binding interaction with known nAbs and can preserve the conserved nAb-sensitive epitopes to elicit cross-reactive antibodies upon immunization be beneficial. To determine if the

recombinant gB-based antigens were expressed as monomers or multimers, Native PAGE (Figure 2-9) and ELISA (Figure 2-10) experiments were performed. In Figure 2-9, the most prominent bands were seen at ~600 kDa, even though gB.ecto-R6-P-His-T4 was theoretically predicted to have a molecular weight of 110-134 kDa and sgB-SS-His was theoretically predicted to have a molecular weight of 75-94 kDa. Since the most prominent band was ~5-7 fold more than the theoretical monomer molecular weight, these recombinant gB-based antigens are considered multimers. Furthermore, in Figure 2-10, the antigens are oligomeric and have many epitopes on the surface for nAbs to bind. Out of the three antigens evaluated, sgB-SS-His presented many epitopes.

The proteins needed to be purified further to evaluate the antigenicity of the recombinant gB-based antigens. Despite efforts, it was not easy to obtain a pure antigen. For sgB-SS-His and gBectoR6PHisT4, a Ni-NTA column was used and analyzed on a Coomassie-stained SDS-PAGE (Figure 2-7). For sgB-SS-Hpbf, since it did not contain a His tag, a DEAE column on the AKTA was used, and fractions were analyzed on a Coomassie-stained SDS-PAGE (Figure 2-8). Each protein purification did not elicit a pure protein. A different process of purifying each protein must be determined before using it for other experiments that need pure protein. For protein purification, other columns can be used; for example, a *Galanthus nivalis* lectin (GNL) column can purify the glycoproteins first and then an SEC afterward.

After evaluating the antigenicity of the panel of 14 different recombinant gB-based antigens, one gB-based antigen: sgB-SS-His, appears to be the most promising. sgB-SS-His is a multimeric and oligomeric antigen that appears to preserve the conserved nAb epitopes (AD-2, AD-4, and AD-5) and elicit low EC_{50} values. By designing the gB-based antigen only to present the antigenic domains that nAbs bind to, a more significant nAb titer response can be induced through immunization. Past vaccines have not been able to induce high-titer persistent nAb responses that can effectively prevent infection. To help combat this issue, I have generated promising immunogen candidates, especially sgB-SS-His, that can potentially evoke a great nAb response upon immunization.

Chapter 3.

Develop an *in vitro* virus assay platform to screen and categorize cross-reactive anti-HCMV/gB nAbs

3.1. Abstract

An effective vaccine given to women of childbearing age that elicits nAbs is considered the best way to prevent congenital HCMV infection. A platform to reliably categorize nAbs and screen sera from vaccinees would be beneficial in supporting such endeavors. HCMV neutralization assays rely heavily on live viruses, which may explain the limited assessment of antibody cross-neutralizing activity. A platform that is amenable and sensitive to high throughput might help to categorize HCMV nAbs and vaccine candidates.

Here, we report on developing a single-round HCMV pseudovirus infectivity assay. As stated previously, gB, gH, gL, and gO are required for infection of fibroblasts, while the gB, gH, gL, and UL128, UL130, and UL131A mediate entry into endothelial, epithelial, and myeloid cells[12]. Because of this, the infectivity of the pseudovirus was evaluated with two different cell lines: MRC-5 (human male lung fibroblast) and ARPE-19 (human retinal pigment epithelium). For infection of fibroblast cells (MRC-5), pseudovirus was produced by co-transfecting 293T cells with plasmids encoding gB, gH, gL, and gO in conjunction with a lentiviral packaging plasmid (pCMV-dR8.2 dvpr; Addgene) and a plasmid with a luciferase reporter gene. This pseudovirus is called "PSV-Trimer." Pseudovirus for infection of epithelial cells (ARPE-19) was made by combining the gB-expressing plasmid with plasmids encoding for proteins of the pentamer complex (gH, gL, UL128, UL130, UL131A) along with the packaging and luciferase reporter plasmids. This pseudovirus is called "PSV- Pentamer." Results show infectivity ($5x10^5$ – $1x10^6$ RLU) of each pseudovirus 'type' for the intended target cell. The pseudovirus types were also able to infect the alternate target cell, albeit at reduced levels. Pseudovirus infectivity was abolished upon omission of gB, gH, and gL with both target cells, and pseudovirus infectivity was not abolished upon omission of either gO or UL128/130/131A. In summary, none of the known nAbs exhibited neutralizing activity in the pseudovirus-based neutralization assays.

3.2. Introduction

My goal was to develop a platform to successfully produce a pseudovirus version of HCMV in the lab in a manner that circumvents the need for virus cultures so that researchers can reliably screen and categorize cross-reactive anti-HCMV/gB nAbs. Developing a reproducible assay that is widely used is essential to enable more rapid identification of potential nAb candidates for the following stages of vaccine development. As previously discussed, nAbs against glycoproteins in HCMV can provide adequate protection. Stopping the virus from spreading and infecting other cells could be done by using antiviral antibodies that can inhibit virus entry into the target cells effectively[120]. Although discovering antiviral antibodies that inhibit virus entry is the most useful, there are other ways antibodies can inhibit the virus, for example, through budding inhibition, where the virus can be inhibited from being released from the infected cells[120]. However, no candidate vaccine for HCMV has achieved the level of efficacy deemed minimal to prevent congenital disease.

Pseudotyped lentiviral vectors have already been used in other laboratories to study viruses such as retroviruses, HIV-1, Ebola viruses, SARS coronavirus, or vesicular stomatitis virus (VSV). Evaluating the efficiency of other pseudotypes is always compared to VSV, which is the pseudotype standard[121]. These generally consist of vector particles expressing glycoproteins derived from other enveloped viruses[121]. Transfecting these DNA plasmids encoding glycoproteins into cells elicit a pseudotyped virus particle, also known as a pseudovirus. Furthermore, an advantage of pseudotyping is using the envelope proteins from different viruses and studying different variants for the desired cell population[122]. The advantage of the pseudotyped lentiviral platform is that it can be studied in a level 2 biosafety laboratory and would result in a non-pathogenic and replication-defective pseudotyped virus[123]. Researchers can comfortably use the pseudovirus platform rather than wild-type viruses and still be able to study many different aspects of the glycoproteins, such as conformational structures, or can also be used to evaluate promising nAbs for the virus[124]. Other researchers may look at viral particle stability, host-cell specificity, toxicity, and titer[121]. Other advantages of the pseudovirus are that there is high sensitivity and specificity, sera from immunizations can be screened, and only small volumes of sera are needed in neutralization assays[123].

The glycoproteins from the VR1814 strain were used in designing the pseudovirus platform. VR1814 is a clinical isolate of HCMV from a cervical swab of a pregnant woman and was previously studied and cloned in *E. coli*[125]. The VR1814 strain is widely used to study HCMV and is also completely sequenced. Researchers found that wild-type VR1814 uses two different pathways into cells. One depends on PDGFRα with the trimer complex, and the other involves the epidermal growth factor receptor B (ErbB) family members from the pentamer complex[126]. gL is linked to different glycoproteins on HCMV via disulfide bonds[48], and as seen in Figure 1-2, gL has disulfide bonds that link gH and gO or UL128 to it. These disulfide bonds are essential to creating the trimer complex or pentamer complex. Without the disulfide bonds, the complexes would not be able to form correctly.

Additionally, I wanted to evaluate a different HCMV strain for the pseudovirus platform and chose the Merlin strain. Merlin is the World Health Organization (WHO) International Standard for HCMV[127]. Merlin was previously isolated from neonatal urine samples and was the first complete HCMV genome sequence determined[128]. I only used Merlin-gB but kept the other glycoproteins derived from VR1814.

Figure 3-1 shows the pseudovirus platform for HCMV that was developed, based on the pseudotyped lentiviral vector, to rapidly test and categorize various nAbs.

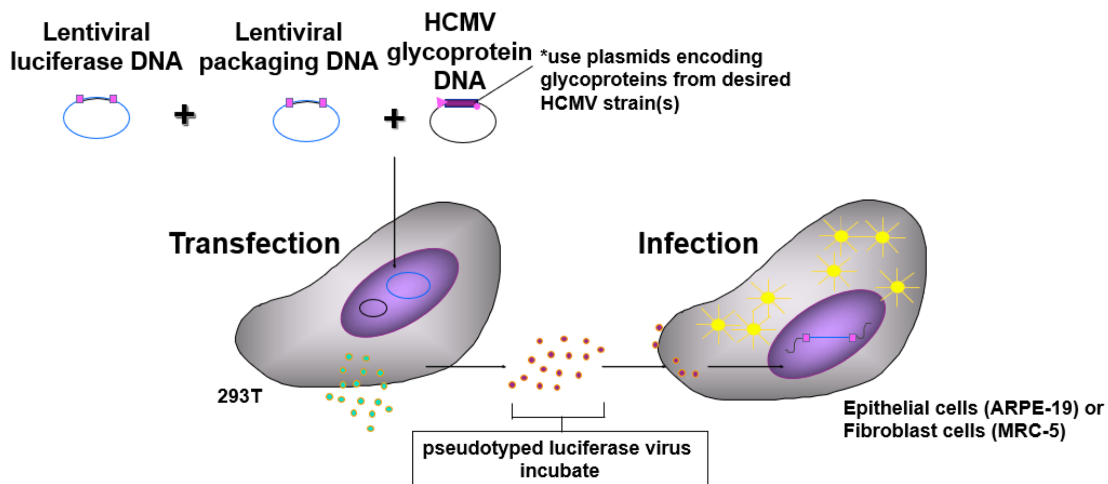

Figure 3-1: Schematic of pseudovirus-based luciferase reporter assay
Plasmids (lentiviral luciferase DNA, lentiviral packaging DNA, and HCMV glycoprotein DNA) are incubated together and transfected into 293T cells, where pseudotyped luciferase virus is

collected (form supernatant) and incubated to allow time for infection on epithelial (ARPE-19) or fibroblast (MRC-5) cells.

3.3. Methods

3.3.1. Construction and expression of antibodies and antigens

A PCR (sequences can be found in Appendix A) mixture with *Pfu* DNA polymerase (ThermoFisher) was used to generate the DNA. These modified antigen PCR products were analyzed on a 1% agarose gel (Biorad) and extracted with the QIAEXII Gel extraction kit (Qiagen) to ensure the correct size. These were digested with *KpnI*-HF (Invitrogen) and *XhoI* (Invitrogen) restriction enzymes and then ligated together using T4 DNA Ligase (Invitrogen; 1 U/µl) and 5X concentrated ligase buffer (Invitrogen). They were cloned into pcDNA-Zeo+ (Invitrogen/Fisher) and then transformed into bacterial cells (NEB 10). The plasmid DNA was extracted using minipreps (Qiagen), and a NanoVue spectrophotometer (GE) was used to determine the concentration before being sent for sequencing (Eurofins). After that, another transformation of the pure construct with the correct sequence was done, and colonies were picked for midipreps (Qiagen) to get more DNA plasmid and then stored at -20 °C for future use. Then 200 µl of OptiMEM (Gibco), FuGENE (Promega), and 6 µg of pure DNA plasmid were mixed and incubated at room temperature for 15 min. Then the mixture was transfected into half of a 6-well plate with $1x10^5$ 293T cells seeded the day before in Gibco Dulbecco's Modified Eagle Medium (DMEM) with 1% Penicillin/Streptomycin (P/S) and 10% Fetal Bovine Serum (FBS). The supernatant was collected after incubating for three days at 37 °C, supplemented with 0.05% NaN_3 then stored at 4 °C for future use.

Sequences (found in Appendix A) were gathered from GenBank, and the antibodies were made internally or bought commercially. The antibody sequences were digested with enzymes and ligated into either IgG1 or IgG2 vector backbones. 1G2, ITC88, and SM5-1 were ligated into IgG1 and IgG2 vector backbones, while 8I21, 3G16, and 10F7 were ligated into IgG1 vector backbones only. Regions encoding light chain domains were subcloned into pFUSEss-CLIg-hk or pFUSEss-CLIg-hL (Invivogen). Regions encoding heavy chain domains were subcloned into pFUSEss-CHIg-hG1 or pFUSEss-CHIg-hG2 (Invivogen) for expression of soluble antibody.

Cloning of antibody heavy and light chain genes into expression vectors were performed as described above. A restriction enzyme digest was done and ligated to heavy chain vectors pFUSEss-CHig-hG1 or pFUSEss-CHig-hG2 or light chain vectors pFUSEss-hL or pFUSEss-hK. These were digested with either *EcoRI*-HF (Invitrogen) or *NheI*-HF (Invitrogen) for heavy chains or *EcoRI*-HF (Invitrogen) or *BsiWi*-HF (Invitrogen) restriction enzymes and then ligated with their respective parts together using T4 DNA Ligase (Invitrogen; 1 U/µl) and 5x concentrated ligase buffer (Invitrogen). They were transformed into bacterial cells (NEB 10), and then the plasmid DNA was extracted using minipreps (Qiagen). A NanoVue spectrophotometer (GE) was used to determine the concentration before being sent for sequencing (Eurofins). After that, another transformation of the pure construct with the correct sequence was done, and colonies were picked for midipreps (Qiagen) to get more DNA plasmid and then stored at -20 °C for future use.

3.3.2. Pseudovirus types that were generated

The two pseudovirus types were called the "PSV-Trimer" or "PSV-Pentamer" (Figure 3-2). The PSV-Trimer contained gB, gL, gH, and gO, which infected fibroblast cells, and the PSV-Pentamer contained gB, gL, gH, and UL128/130/131A, which infected epithelial cells. Combinations of gB and the trimer complex (gH, gL, gO) or gB and pentamer complex (gH, gL, UL128/130/131A) were transfected with a packaging plasmid and luciferase into 293T cells and evaluated with a luciferase assay. These were transfected into their respective cell lines either in MRC-5 (fibroblast cells) or ARPE-19 cells (epithelial cells). The supernatant was harvested from the 293T cells and infected into the correct cell lines to determine how infectious each pseudovirus was (Figure 3-3). Different plasmids (gB, gH, gL, and either gO or UL128/130/131A) with the lentiviral packaging plasmid (pCMV-dR8.2 dvpr; Addgene) and the lentiviral luciferase transfer plasmid (either BrightGlo or NanoGlo), along with Transporter 5 transfection reagent (Polysciences, Inc) and 1X OptiMEM (Gibco) mixed then transfected into 293T cells and grown for three days at 37 °C & 10% v/v CO_2 for three days. The pseudovirus contained in the culture supernatant was collected, and the pseudovirus was frozen by adding 20% of FBS and then stored at -80 °C for later use.

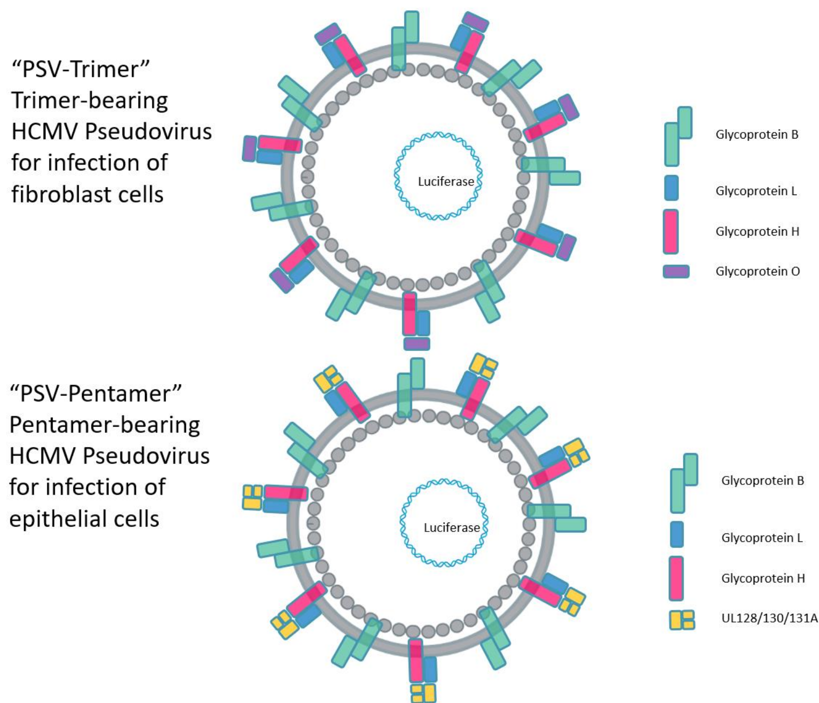

Figure 3-2: Schematic of pseudovirus types
(*Top*) Fibroblast cell-specific HCMV pseudovirus consists of gB and the trimeric complex (gH, gL, gO) for infecting fibroblast cells (e.g., MRC-5). (*Bottom*) Epithelial cell-specific HCMV pseudovirus consists of gB and the pentamer complex (gH, gL, UL128/130/131A) for infecting epithelial cells (e.g., ARPE-19).

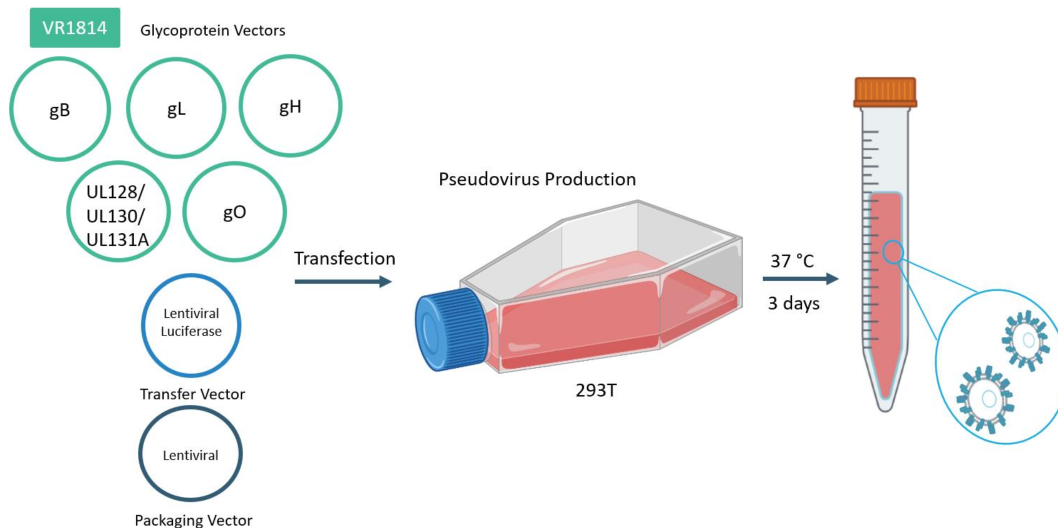

Figure 3-3: Schematic of lentiviral-based production of HCMV pseudovirus
Different plasmids gB, gH, gL, and gO (for infecting MRC-5 cells) or UL128/130/131A (for infecting ARPE-19 cells) with the lentiviral packaging vector and the lentiviral luciferase transfer vector are transfected into 293T cells and incubated at 37 °C for three days.

3.3.3. Flow cytometry

293T cells were seeded $1x10^5$ and incubated overnight in 6-well plates with DMEM. The next day, various HCMV antigens at 2 µg were transfected with OptiMEM (Gibco) and FuGENE (Promega). These mixtures were left at room temperature for 15 min and then added to the cells drop-wise. They were incubated at 37 °C & 10% v/v CO_2 for three days and then harvested. These HCMV antigen-expressing 293T cells were then trypsinized with TryPLE Express (Gibco) and pelleted by centrifugation at 1500 rpm at 4 °C for 5 min. The cells were then re-suspended in Hank's Balanced Salt Solution (HBSS; Lonza) supplemented with 10% (v/v) FBS (Thermo Fisher) (staining buffer) and EDTA. 5mM EDTA followed by staining with 1 µM of Zombie aqua viability dye (BioLegend). The cells were centrifuged and resuspended in HBSS with FBS twice to wash the cells, then resuspended in HBSS without FBS. Samples were then incubated with Zombie dye (live/dead stain) in the dark for 15 min at room temperature, then centrifuged at 1500 rpm for 5 min at 4 °C for a wash step in HBSS with EDTA and FBS. After that, different antibodies (3G16, 8I21, or SM5-1) were added at 1 µg/ml and incubated on ice for 30 min in the dark. Afterward, the PE-conjugated anti-human IgG antibody (Jackson ImmunoResearch) was added and incubated on ice for 30 min in the dark. They were then collected and put through a filter and collected in tubes. Samples were washed and re-suspended in a staining buffer between steps. Following staining, the cells were washed and subjected to data analysis on BD Fortessa X20, and Flojo (version 10) was used to analyze the data.

3.3.4. Titration of Pseudovirus

Fresh pseudovirus or frozen pseudovirus, which was thawed, was used to infect either MRC-5 or ARPE-19 cells by titrating the pseudovirus 2-fold down the 96 half-well plates. At three days post-infection, the media was discarded, and the cells were lysed with cell lysis buffer (12.2 g Tris, 5.85 g NaCl, pH 7.4, 10 ml IGEPAL (NP-40; Bioshop), water to 1 L) and pipetted up and down vigorously to ensure the breaking of cells, then half of the cell lysis buffer in the well was transferred to a black plate. After that, the luciferase reagent (BrightGlo or NanoGlo Luciferase; Promega) was added to the cells, then the infectivity was measured on the luminometer, and the relative luminescence units (RLU) were recorded.

3.3.5. Pseudovirus-based neutralization assay

An entry neutralization assay determined HCMV-specific neutralizing titers in the samples. Cell control (media only) and virus control (HCMV pseudovirus) are in the first two rows. Then the antibody made at 25 µg/ml final with polybrene (Sigma; 10 µg/ml final) and DMEM mix was titrated 1:2 down the plate, and then the pseudovirus was added (a mix of antibody and pseudovirus). These were incubated at 37 °C & 10% v/v CO_2 for 1 hr. Afterward, the mixture was transferred to the plates with either ARPE-19 or MRC-5, seeded the day before at $1x10^5$. Then the mixture was left at 37 °C & 10% v/v CO_2 for another three days. Afterward, the media was removed from the plate, and the cells were lysed with cell lysis buffer and measured as described above. Percent neutralization is determined by calculating the difference in RLU between sample wells (cells, serum sample, virus) and the mean RLU of cell control wells (cells only) and dividing this result by the difference in mean RLU between virus control (cell and virus) and mean RLU of cell control wells, subtracting from 1 and multiplying by 100.

3.4. Results

3.4.1. Flow cytometry was used to show the expression of glycoproteins

Antibodies (8I21, 3G16, and SM5-1) were used to bind to glycoproteins (gB, gL/gH, gO or gB, gL, gH, UL128/130/131) that were transfected. 8I21 binds an epitope on the HCMV pentamer involving gH/gL/UL128/130[91]. 3G16 binds mainly to gH, as described by other researchers[129]. SM5-1 binds to gB as described previously. Flow cytometry verified that antigen expression of different glycoproteins was expressed (Figure 3-4).

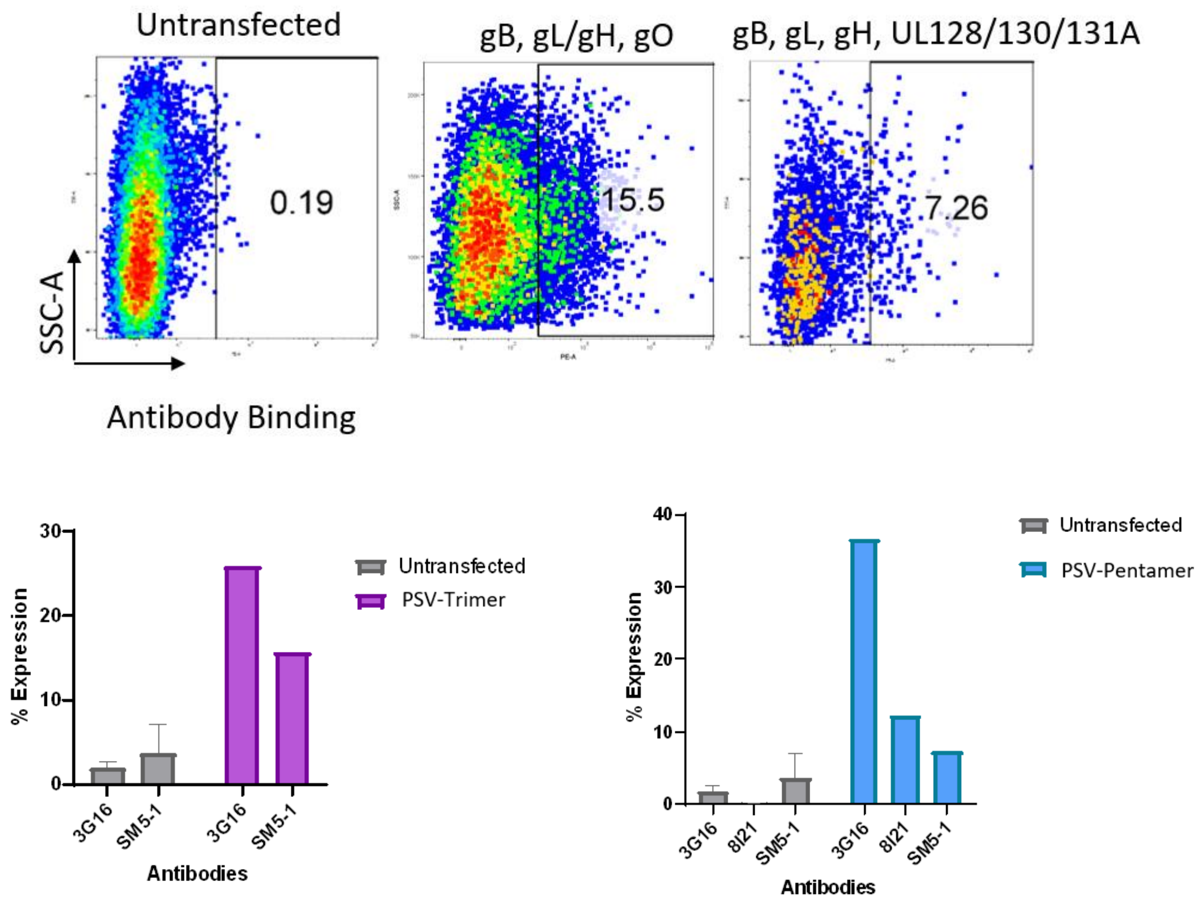

Figure 3-4: Flow Cytometry used to show that gB, gH, or gL and UL128/UL130 are expressed on the surface of 293T cells

Pseudovirus constructs were transfected into 293T cells and then harvested. Live/dead stain was performed with ZombieDye, then primary antibodies 3G16 (anti-gH), SM5-1 (anti-gB), or 8I21 (anti-gH/gL/UL128/130) were used to bind to antigens, then PE anti-Hu IgG used to bind to antibodies. (*Top*) Gating strategy for untransfected, PSV-Trimer (gB, gL/gH, gO), and PSV-Pentamer (gB, gL, gH, UL128/130/131A). (*Bottom Left*) PSV-Trimer (*Bottom Right*) PSV-Pentamer. Error bars on the untransfected represent experiment was done in triplicate, whereas the samples were done once.

3.4.2. HCMV pseudovirus infectivity in epithelial and fibroblast cells

Titration assays determined if the pseudovirus types could infect their respective cell types and the other cell type (Figure 3-5). Additionally, titration assays determined if the heterologous HCMV pseudovirus were infectious to target cells (Figure 3-6).

Homologous HCMV pseudovirus infectivity in epithelial and fibroblast cells

The PSV-Trimer and PSV-Pentamer can infect fibroblast and epithelial cells (Figure 3-5). The PSV-Trimer should infect fibroblast cells more efficiently than when infecting epithelial cells. Additionally, the PSV-Pentamer is meant to infect epithelial cells and efficiently infects these cells more than the fibroblast cells.

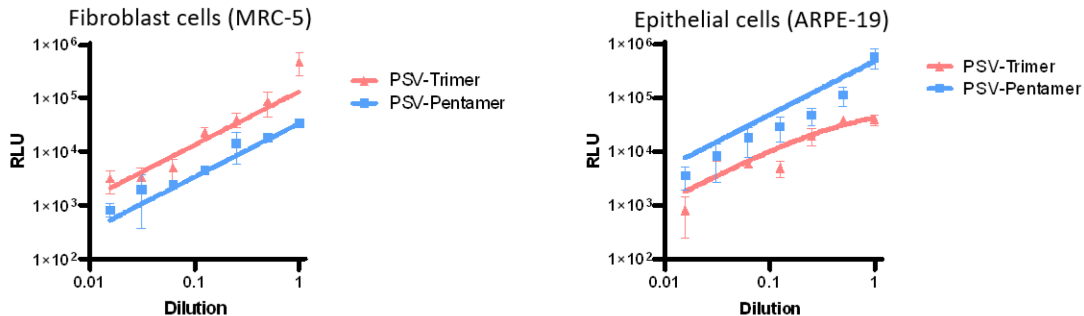

Figure 3-5: Homologous HCMV Pseudovirus is infectious in epithelial and fibroblast cells

Titration of VR1814 pseudovirus on MRC-5 fibroblast cells (*left)* or ARPE-19 epithelial cells (*right*) or showing how both are infectious. PSV-Trimer (pink triangles) infects MRC-5, and PSV-Pentamer (blue squares) infects ARPE-19, 10-fold better than the other cell line. RLU = Relative Luciferase Units. Error bars represent experiments done at least twice.

Heterologous strains of HCMV infectivity in epithelial and fibroblast cells

Researchers have shown and categorized the HCMV strains' sequences using bioinformatics tools[63,130–132]. The researchers also organized and grouped HCMV strains based on their gB sequence. According to Foglierini et al., HCMV might not be as diverse as previously thought, and the researchers show a lot of HCMV strain sequence similarity, especially in gB[63]. The HCMV Merlin strain is widely studied by researchers and can bind to 1G2, SM5-1, and ITC88[113]. The pseudovirus types were infectious when the Merlin-gB was expressed with the other VR1814 glycoproteins (Figure 3-6).

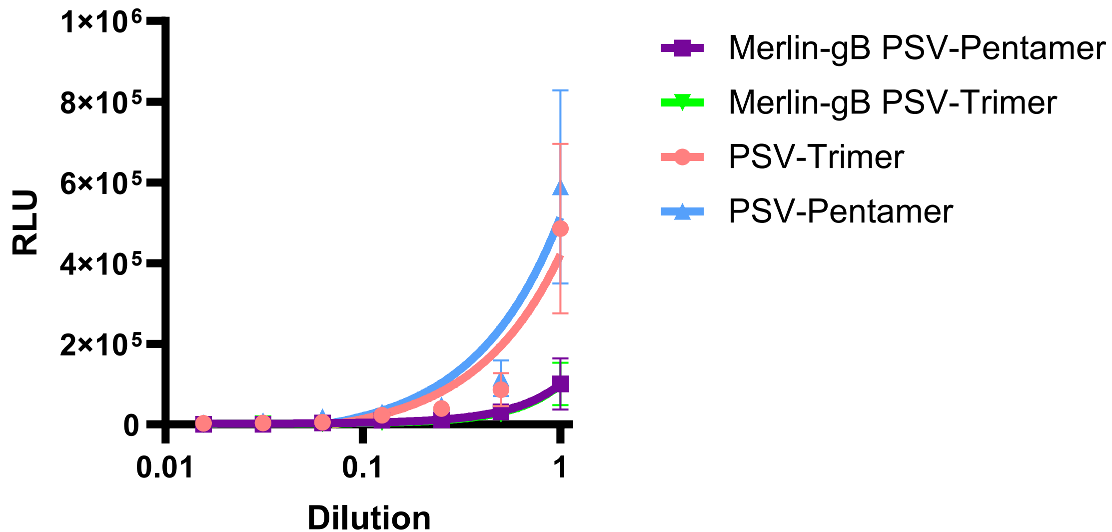

Figure 3-6: Heterologous strains of HCMV can be used to generate a pseudovirus that can still infect cells

Titration of HCMV Merlin-gB with VR1814 PSV-Pentamer or VR1814 PSV-Trimer pseudovirus infects both epithelial and fibroblast cells. Merlin PSV was made by switching the VR1814 gB with Merlin gB plasmid DNA during transfection. RLU = Relative Luciferase Units. Error bars represent experiments done at least twice.

3.4.3. Different conditions for the pseudovirus platform

Different conditions for the pseudovirus platform were explored. Different transfection agents in the lab were assessed to see if both could transfect the DNA plasmids efficiently to generate the pseudovirus (Figure 3-7). Additionally, different luciferases in the lab were evaluated to see if they could show the infection of the pseudovirus (Figure 3-8). Lastly, we also wanted to freeze the pseudovirus and see if we could use it for subsequent experiments (Figure 3-9).

Transporter-5 and FuGENE were used as transfection agents for infection of pseudovirus

Both transfection agents were used and were able to transfect the DNA plasmids to generate the pseudovirus. The Transporter-5 transfection agent was able to elicit a higher infection level.

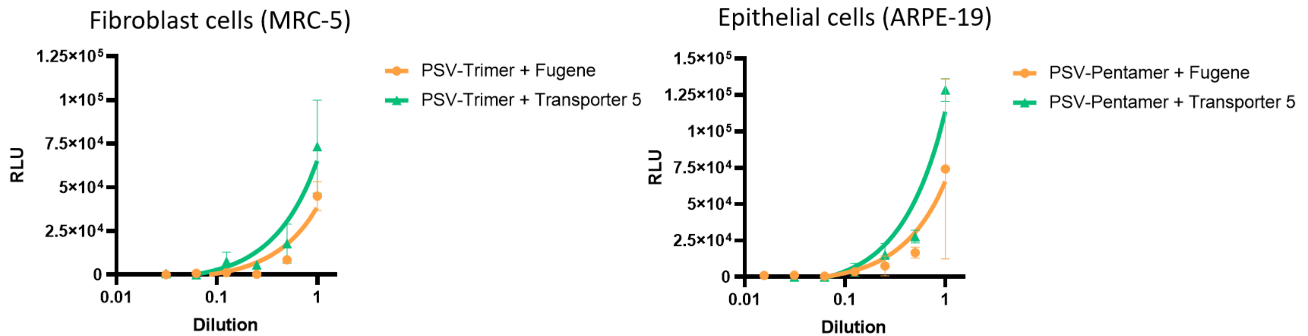

Figure 3-7: FuGENE and Transporter-5 are both transfection agents that successfully transfect the plasmids to generate the pseudovirus
Pseudovirus constructs were transfected with either FuGENE or Transporter-5. (*Left)* PSV-Trimer is used to infect fibroblast cells. RLU = Relative Luciferase Units. (*Right*) PSV-Pentamer is used to infect epithelial cells. Transporter-5 elicited a higher infectivity level for the pseudovirus constructs in both cell lines. Error bars represent experiments done at least twice.

Nanoluciferase and Brightglo (firefly) luciferase were used for infection of pseudovirus

Nanoluciferase is more sensitive than Brightglo (firefly) and, thus, can show a higher level of infectivity for the pseudovirus. However, either luciferase DNA plasmid can be transfected.

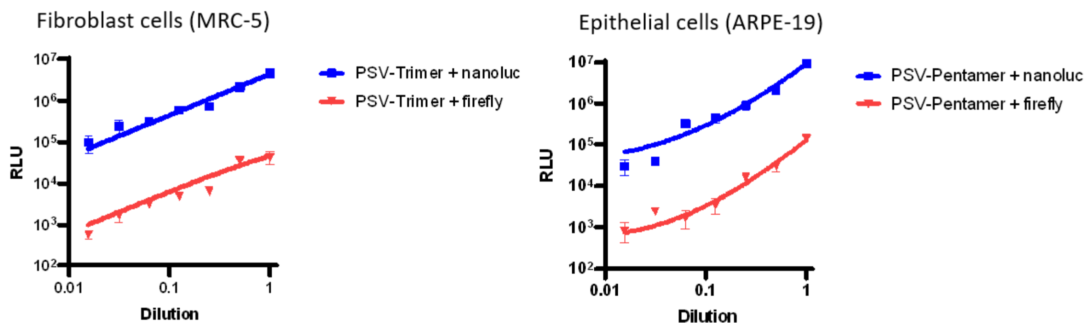

Figure 3-8: Brightglo (firefly) and nanoluciferase both show pseudovirus infection
Pseudovirus constructs were transfected into 293T cells with either nanoluciferase or firefly luciferase. Both show luminescence, but nanoluciferase is more sensitive than Brightglo. RLU = Relative Luciferase Units. (*Left*) PSV-Trimer used to infect fibroblast cells (*Right*) PSV-Pentamer used to infect epithelial cells. Error bars represent experiments done at least twice.

Pseudovirus was frozen and used for subsequent infection experiments

Frozen stocks were made by adding 20% FBS to harvested pseudovirus and mixed, then stored at -80 °C. Frozen stocks were used to infect target cells after one month of storage. Frozen pseudovirus made with nanoluciferase elicited diminished infectivity levels by 100-fold more compared to fresh pseudovirus.

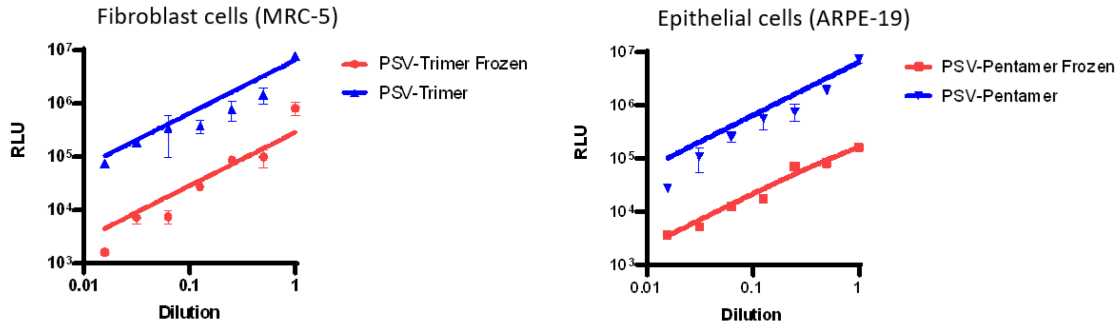

Figure 3-9: Fresh and frozen pseudovirus is both infectious
Pseudovirus constructs were transfected into 293T cells with nanoluciferase and then frozen for a month. Both show luminescence, meaning researchers can freeze and store pseudovirus for future use. RLU = Relative Luciferase Units. (*Left*) PSV-Trimer used to infect fibroblast cells (*Right*) PSV-Pentamer used to infect epithelial cells. Error bars represent experiments done at least twice.

3.4.4. gB and complete trimer and pentamer complexes are essential for pseudovirus infectivity

Experiments using virus samples expressing the trimer and pentamer complexes showed higher levels of infectivity than samples with viruses expressing only one complex (Figure 3-10). Furthermore, either one of the glycoprotein complexes, such as the trimer or the pentamer complex, needs to be expressed; otherwise, the pseudovirus shows no infectivity (Figure 3-11). Experiments where the disulfide bridges were disrupted, showing that the complexes cannot form properly, showed that the infectivity was mostly abolished (Figure 3-12). Both glycoprotein complexes with gB must be expressed simultaneously to have efficient pseudovirus infectivity.

Pseudovirus transfected with gB, trimer, and pentamer complexes elicit infectivity

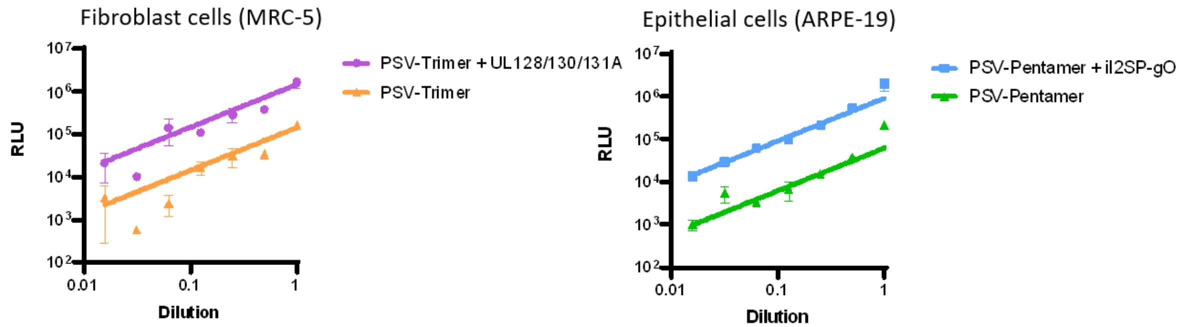

Figure 3-10: Pseudovirus generated where both trimer and pentamer complexes are expressed are considered more infectious

Pseudovirus generated with all glycoproteins is considered infectious. Pseudovirus, where both complexes are expressed, is more infectious than a pseudovirus with only a single complex. RLU = Relative Luciferase Units. Error bars represent experiments done at least twice.

Pseudovirus transfected with incomplete complexes expressed on the surface of the cell gB

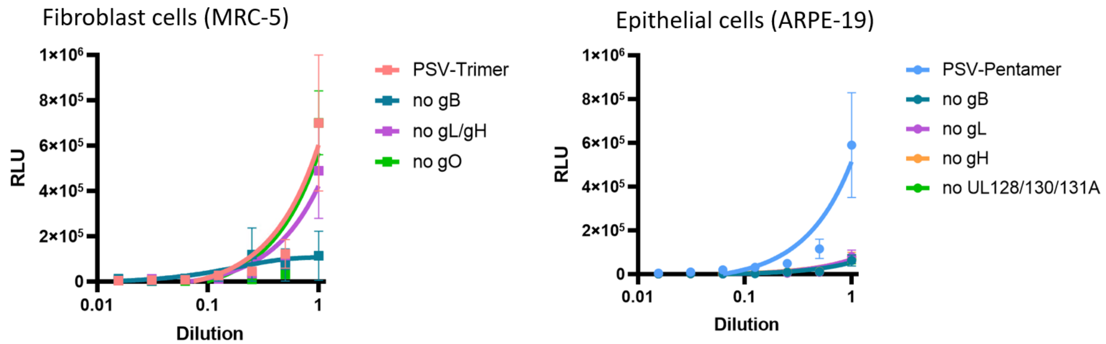

Figure 3-11: Omission of gB, gL, or gH significantly affects infectivity of pseudovirus

Titration of VR1814 pseudovirus on MRC-5 fibroblast cells (left) or ARPE-19 epithelial cells (right) with glycoproteins omitted one at a time. Each plasmid encoding the gene for the glycoprotein was omitted during transfection when creating the PSV. RLU = Relative Luciferase Units. Error bars represent experiments done at least twice. gB and complete trimer and pentamer complexes are known to be essential for pseudovirus infectivity

The proper structure of complexes is essential for pseudovirus infectivity

A couple of disulfide bonds bind glycoproteins to the other glycoprotein complexes (Figure 1-2). gL has a disulfide bond that attaches to gO or ULs. Additionally, gO has a disulfide bond that attaches it to gL. Finally, UL128 has a disulfide bond that attaches it to gL. Three different versions of glycoproteins were designed to replace cysteine with serine49: gL-144S, gO-351S, and UL128-162S.

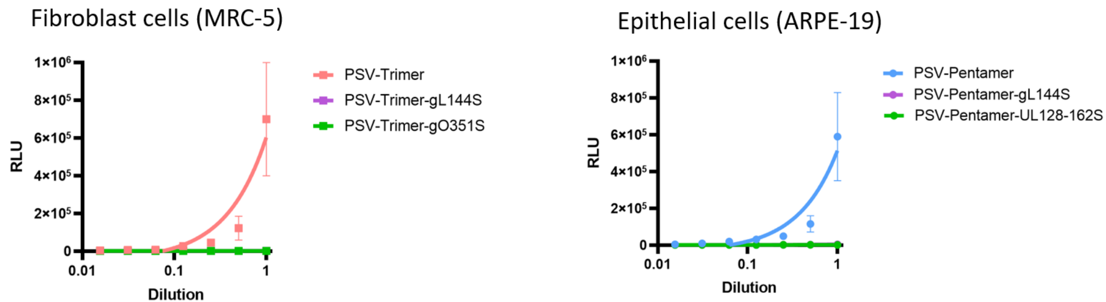

Figure 3-12: Mutations that Disrupt Disulfide Bridges affect infectivity
The titration of VR1814 pseudovirus with mutations gL-144S, UL128-162S, or gO-351S on MRC-5 fibroblast (*left*) or ARPE-19 epithelial (*right*) target cells affects infectivity. Point mutations were made from cysteine to serine to disrupt disulfide bridges. RLU = Relative Luciferase Units. Error bars represent experiments done at least twice. Prevention of disulfide bonds significantly decreases infectivity.

3.4.5. Pseudovirus entry neutralization assay

The neutralization assay measures the ability of antibodies to prevent or limit infection of target cells. A panel of antibodies was tested to see if the antibodies could neutralize the pseudovirus fusion to cells. Various antibodies against different glycoproteins of HCMV were: ITC88, SM5-1, and 1G2 are anti-gB; 3G16 is anti-gH, 8I21 is anti-UL128/130, and 10F7 is anti-UL130/131A (Figure 3-13). NAbs do not inhibit pseudovirus infection at the entry point of infection.

Pseudovirus entry neutralization assay

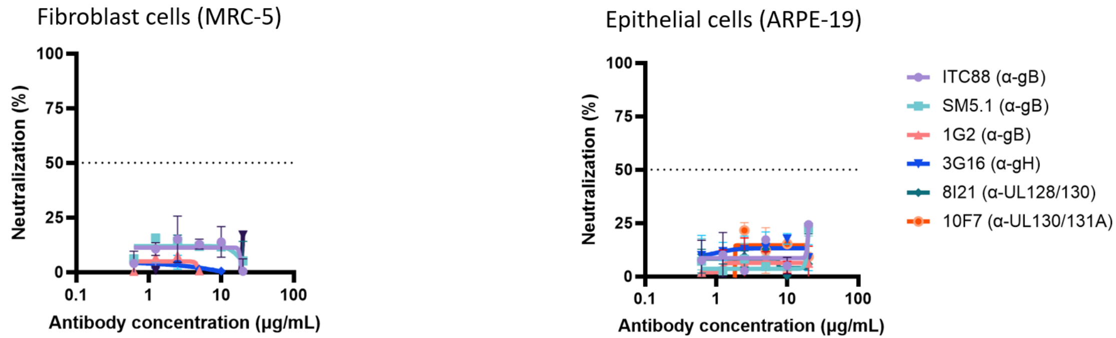

Figure 3-13: Antibodies against different HCMV glycoproteins do not inhibit entry of pseudovirus in cells

Neutralizing assay on PSV-Trimer (*left*) and PSV-Pentamer (*right*) on its respective target cells, fibroblast (MRC-5), or epithelial (ARPE-19) cells with a panel of known HCMV nAbs against various glycoproteins. ITC88, SM5-1, and 1G2 are anti-gB; 3G16 is anti-gH, 8I21 is anti-UL128/130, and 10F7 is anti-UL130/131A. The experiment was done once.

3.5. Discussion

Maternal immunity in the form of nAbs against glycoproteins in HCMV can protect against congenital infection. An effective vaccine given to women of childbearing age eliciting nAbs and a platform to categorize nAbs or screen sera from vaccinees reliably would be beneficial. A pseudovirus-based luciferase reporter assay was successfully developed and can be used in lower-level biosafety laboratories (Figure 3-1). Two main pseudovirus types were developed: gB, gL, gH, and gO, also known as the PSV-Trimer, and the other that contained gB, gL, gH, and UL128/130/131A (Figure 3-2). Researchers can comfortably use the pseudovirus platform in place of wild-type viruses and still be able to study conformational structures of the glycoproteins or even evaluate potential nAbs[124]. For example, many researchers use the pseudovirus platform for coronaviruses to evaluate nAbs rapidly[133].

To evaluate whether the pseudovirus platform can infect epithelial and fibroblast cells, both a homologous and heterologous HCMV pseudovirus was developed. Most glycoproteins used were from the VR1814 sequence, except for one gB, which contained Merlin-gB. As seen in Figure 3-5, the pseudovirus types were able to infect fibroblast and epithelial cells. The PSV-trimer could infect the fibroblast cells more efficiently than the epithelial cells. Likewise, the PSV-Pentamer could infect the epithelial

cells more efficiently than the fibroblast cells. For efficient infection of HCMV into the fibroblast cells, gB and the trimer complex would need to be transfected together[26]. For efficient infection of HCMV into epithelial cells, gB and the pentamer complex would need to be transfected together [26]. As seen in Figure 3-5, when gB and the pentamer complex infect fibroblast cells, has lower infectivity compared to gB and the trimer complex. Likewise, when gB and the trimer complex infect epithelial cells, it has lower infectivity than gB and the pentamer complex infecting fibroblast cells.

Furthermore, heterologous strains of HCMV can be used to generate a pseudovirus and can still infect cells (Figure 3-6). The pseudovirus was still infectious by replacing the VR1814 gB plasmid with a different strain, Merlin gB but keeping the other VR1814 strain sequenced glycoproteins. If researchers wanted to look at other strains, one would need only to clone the gB of the desired strain and keep the rest of the VR1814 glycoproteins the same as shown previously. Researchers could save time and money by cloning one glycoprotein of the desired strain.

Different conditions of the pseudovirus platform were experimented with to show its versatility. For example, FuGENE and Transporter-5 transfection reagents showed infectious pseudovirus (Figure 3-7). While Transporter-5 is more efficient and economical than FuGENE, either can be used as transfection reagents to avoid purchasing new materials. Platform users can also choose Brightglo (firefly) or nanoluciferase to generate fresh infectious pseudovirus (Figure 3-8). The pseudovirus can also be frozen at -80°C for future use if made with nanoluciferase (Figure 3-9). Therefore, the pseudovirus platform can be stored later or made with slightly different reagents and still be infectious.

All the glycoproteins must be present and functional for the pseudovirus to work correctly. Researchers can use the pseudovirus transfected with gB, trimer, and pentamer complexes expressed on the cell's surface and see infectivity for both epithelial and fibroblast cells (Figure 3-10). Researchers can also generate a pseudovirus that only has the trimer complex or the pentamer complex along with gB, as seen in Figure 3-5. Pseudovirus transfected with incomplete complexes expressed on the cell's surface is not infectious, as seen in Figure 3-11. To infect fibroblast cells, gB and the trimer complex must be functional. To infect epithelial cells, gB interacts with the pentamer complex (gL, gH, and UL128/130/131A) and needs to be functional. If one of

the glycoproteins is not expressed, then the infectivity diminishes. For both pseudovirus types, omitting gB has the most impact, and the pseudovirus is not considered infectious.

Pseudovirus transfected with disruption of disulfide bond formation in glycoproteins is not able to be infectious, as seen in Figure 3-12. Disulfide bonds are essential when forming the trimer complex or pentamer complex. As seen in Figure 1-2, gL has disulfide bonds that link to gO or UL128. Disulfide bonds were disrupted by designing glycoproteins with serine mutations: gL-144S, gO-351S, and UL128-162S. Thus, researchers can use this HCMV pseudovirus but must ensure that all the glycoproteins are expressed.

Unfortunately, the current entry neutralization assay shows no neutralization for the antibodies and needs further optimization. As seen in Figure 3-13, the entry neutralizing assay shows that the antibodies could not neutralize the pseudovirus when gB fuses to the trimer or pentamer complex but might neutralize at a different time. For example, ITC88 can bind at a different point in time and not at the entry. ITC88 is an AD-2-specific antibody, as previously discussed, and was shown to effectively neutralize HCMV infection by minimizing an effect mediated by gB so that it can stop HCMV from escaping natural apoptotic processes[134]. In the neutralizing assay, the pseudovirus was able to infect cells as gB could still escape the apoptotic processes. Additionally, 8I21 can bind to the pentamer complex in a region where NRP-2 does not bind[72], meaning that this antibody cannot stop the pentamer complex from binding NRP-2 and thus can still enter the cell. This antibody panel could not neutralize the pseudovirus at the point of entry.

Other neutralization points are possible, as seen in other viruses, but this could be the case for HCMV. For example, there are other ways antibodies can inhibit cells via budding inhibition, which is to inhibit the virus released from the infected cells[120]. Generally, antiviral antibodies are traditionally screened for their ability to inhibit virus entry into the target cells[120]. Stopping the interaction of the virus with the cells could stop the virus from spreading and infecting other cells. As this neutralizing assay only tests whether an antibody can neutralize the pseudovirus at the point of entry, another neutralizing assay is needed to evaluate neutralizing capacity at a different point, such as budding inhibition or neutralization at the point of egress.

HCMV entry into cells is complicated and can generate multiple alternative routes depending on the virus strain and the cell type[135]. For example, Patrone et al. found that UL128 could bind to the endothelial cell plasma membrane; thus, HCMV could obtain entry through receptor ligands to infect the cell[136]. Kschonsak et al. found that the HCMV pentamer complex can bind to multiple distinct receptors with high affinity and use different pathways as an entry for infection into the target cells[72]. Furthermore, Liu et al. found that the pentamer complex can promote egress from endosomes later in the entry pathway of HCMV[76]. Although not tested in epithelial cells, this could give insight into how the different glycoproteins on the surface of HCMV work together and could evade the nAbs. Other receptors are also present in the cell and could be another escape route for the pseudovirus so that it can still infect other cells. For example, 8I21 can bind to the pentamer complex but on a different area of glycoproteins gH/gL/UL128/130 where NRP-2 does not bind[72], meaning that HCMV can enter the cell still and can infect other cells. The other glycoproteins on the surface of HCMV are all working together to infect different cell types and infect them differently. How HCMV infects various cell types is still extensively studied as it is not widely understood.

An HCMV pseudovirus platform was designed successfully using DNA plasmids encoding virus surface glycoproteins. Additionally, examples of different reagents used or homologous or heterologous pseudovirus setups (different HCMV gB) were experimented with to show flexibility for the pseudovirus platform. With this pseudovirus platform, researchers could reliably study HCMV in a biosafety level 2 laboratory. gB with trimer (gL/gH/gO)- and pentamer (gL/gH/UL128/UL130/UL131A)-bearing pseudovirus types showed preferential infection of their corresponding target cells (i.e., fibroblast and epithelial cells). This infectivity depended on the presence of gB and the proper formation of the respective trimer and pentamer complexes. I showed that a pseudovirus type is infectious with the trimer and pentamer complexes. However, there was no meaningful neutralization with the HCMV nAbs, showing that these are not able to inhibit virus entry.

Chapter 4.

Future Directions

For these recombinant gB-based antigens, potentially doing a large-scale purification and then determining the genuine affinity (Kd) of nAbs (BLI) would be needed to characterize further and evaluate antigenicity. Potentially, looking at the crystal structure of the antibodies binding to see where these are binding to the recombinant gB-based antigen could give some insight into the epitopes presented. Similarities between the crystal structure of the recombinant gB-based antigen and HCMV gB with the same antibodies, such as SM5-1[112], would be interesting to analyze.

Immunizations can help to characterize and evaluate sgB-SS-His further. sgB-SS-His is a multimeric and oligomeric antigen that preserves nAb epitopes (AD-2, AD-4, and AD-5). Through immunization, potentially, a more significant nAb titer response can be induced. Mice immunizations of these recombinant gB-based antigens would be interesting as they could elicit several nAbs that could be further studied and characterized.

Furthermore, gB is widely studied, but newer research shows that the pentamer complex could elicit more potent nAbs. Potentially, making other recombinant versions of the pentamer complex to elicit more nAbs. For example, looking at gH and gL, both present on the trimer and pentamer complex, might be a good idea. Researchers have found that the gH/gL specific antibodies have lower neutralizing potency but can equally block infection in both fibroblast and epithelial cells[28]. By looking at gH/gL, it can be an advantage over gO or UL128/130/131A specific antibodies as anti-gH/gL antibodies an neutralize a wider breadth of target cell types. So far, researchers have found that the gH/gL specific antibodies can block the trimer complex by interacting with gB to stop the gB fusion in all cell types[28]. That way, the antibodies indirectly stop gB and thus stop HCMV infection. The pentamer complex can elicit more nAbs than gB[57]. Antibodies targeting the pentameric complex are found to be the most present in serum antibodies and are capable of neutralizing epithelial cells from HCMV infections[59]. Some antibodies to the pentameric complex exhibit a 1000-fold greater neutralizing potency than HCMV gB-specific antibodies[60]. Maybe targeting gH or gL in conjunction with gB would be the

better option. Interestingly, there has been data that pregnant mothers' sera contained trimer and pentamer-specific antibodies that worked together to neutralize the virus and successfully block cell-to-cell spread[107]. Since there are trimer and pentamer-specific antibodies, this could mean that looking into gO or UL128/130/131 specific nAbs might also be attractive targets.

Different strains of HCMV can be evaluated by changing the gB and measuring the levels of infectivity. Other strains have the same sequence as VR1814 either before or after the furin cleavage site on gB[130]. It might be beneficial to see how much this would change the infectivity levels and if certain nAbs are more effective against the different strains. For example, HAN28 has the same sequence for AD-2, AD-4, and AD-5 as VR1814. Researchers can use different strains of HCMV and analyze nAbs to see if there is any cross-reactivity between them.

Analyzing human sera to see which monoclonal antibodies can neutralize the different pseudovirus types might interest some researchers. Additionally, using the pseudovirus platform with other monoclonal antibodies of interest to analyze how potent they are. Other antibodies, such as TRL345, a high affinity neutralizing antibody on site I of AD-2 on gB, have also been shown to neutralize fibroblasts, epithelial, and endothelial cells[137]. Furthermore, 3-25 is another monoclonal antibody against a highly conserved region on AD-2 on gB. The crystal structure shows that 3-25 can bind and inhibit infection at a post-attachment step where it can interfere with the viral membrane fusion in ARPE-19 cells[138]. Therefore, the pseudovirus platform can quickly analyze different strains and potentially antibodies.

Cell lysate of the pseudovirus and live virus can be compared on a western blot. There are different amounts of the trimer complex compared to the pentamer complex on the HCMV virion envelope[28]. Another example is when Wu et al. found that the virions contain different relative amounts of the pentamer complex compared to the trimer complex[75]. The amount of glycoprotein found on HCMV is likely not a one-to-one ratio, and researchers might want to study different levels of glycoproteins found on the cell's surface. There might be more accurate infectivity by altering the ratios of the glycoproteins to make the pseudovirus mimic the same amount of glycoprotein expression in the live virus. Additionally, comparing the pseudovirus and the wild-type virus in terms of how the virus spreads in cultures would be beneficial.

By designing a recombinant gB-based antigen that preserves the conserved nAb epitopes and developing a pseudovirus platform that can measure infectivity levels, mice can be immunized, then sera-containing antibodies can be collected and analyzed to help identify potent nAbs for HCMV. Ideally, researchers would be able to immunize mice with a recombinant gB-based antigen, then harvest and use sera to discover nAbs then characterize them with the pseudovirus platform. Together, this could inform that the recombinant antigen can elicit potent nAbs at the entry point and be the first step in this lab toward creating an HCMV vaccine.

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Appendix A.

Sequences of plasmids used

Figure A.1 VR1814_gB_pcDNAZeo

```
NNNNNNNNNN TNNNCTTANC TTGGTACCGC CGCCACCATG GAGTCACGGA TTTGGTGCCT   60
CGTGGTCTGC GTGAACCTCT GCATCGTGTG CTTGGGCGCC GTGGTTTCGA GCTCCTCCAC  120
ATCCCATGCC ACCAGTAGTG CCCATAACGG CTCCCACACC TCCCGCACTA CCAGCGCCCA  180
GACTCGATCC GTGTCGTCGC AGCACGTGAC CTCGTCCGAA GCCGTGTCGC ATAGAGCCAA  240
CGAGACAATC TACAACACGA CCCTGAAATA CGGAGATGTG GTCGGTGTCA ACACTACCAA  300
GTACCCATAC AGAGTGTGCT CGATGGCCCA GGGAACCGAC CTGATCCGGT TCGAGAGAAA  360
TATCGTGTGT ACCCCCATGA AGCCGATTAA TGAGGACCTG GACGAGGGCA TCATGGTCGT  420
GTATAAGCGC AACATTGTGG CCCACACCTT CAAAGTCCGC GTGTACCAGA AGGTCCTTAC  480
CTTCCGGAGA AGCTACGCCT ACATCCACAC TACCTACTTG CTGGGGTCAA ACACTGAATA  540
CGTGGCCCCA CCTATGTGGG AGATCCATCA CATAAACCGC CACTCTCAGT GTTACTCATC  600
ATACAGCAGA GTGATCGCCG GCACCGTGTT CGTGGCCTAC CATCGGGACT CCTATGAGAA  660
CAAGACGATG CAGCTGATGC TGGATGACTA CTCGAACACT CACTCGACCC GCTACGTGAC  720
TGTGAAGGAT CAGTGGCACT CTAGGGGATC CACTTGGCTC TACCGGGAAA CCTGTAACCT  780
GAACTGCATG GTCACCATCA CCACCGCACG CTCCAAGTAC CCGTACCACT TCTTCGCCAC  840
TTCCACCGGC GACGTCGTGG ACATCTCCCC TTTCTACAAT GGAACCAACC GCAACACTAG  900
CTACTTTGGA GAAAACGCGG ACAAGTTCTT TATCTTCCCC AACTATACCA TTGTGTCCGA  960
CTTTGGGAGG GCGAACTCCG CACCCGAAAC CCNNAGATTG GTGGCGTTCC TGGANAGGGC 1020
CGANNGCGTC ATCAGCTGGG ANNTCCAGGA CNANAAAANN NNGACCTGTC NNCTGACCTT 1080
NGGNAAGCCN NNNGCGGANC ATCCGGTCCG AGGCTGAAGA NNNCNACCAT TNNNCCNNGG 1140
NAAANNNACN NNNACCTTNN NGAACAGAAA CCGNAANNAA NTNNNNGANC CNNNNNGGAN 1200
NGNNNGGGGA NNNGCCCTGA AANNNNNNNN NNNAANNTNN NNGGNCCNNA AANNNGNNNN 1260
NNNAA                                                             1265
```

Figure A.2 VR1814_sgB-HIS_pcDNAZeo

```
NNNNNNNNNN NNNANTTAGC TTGGTACCGC CGCCNCCATG GAGTCACGGA TTTGGTGCCT   60
CGTGGTCTGN NNGAACCTCT GCATCGTGTG CTTGGGCGCC GTGGTTTCGA GCTCCTCCAC  120
ATCCCATGCC ACCAGTAGTG CCCATAACGG CTCCCACACC TCCCGCACTA CCAGCGCCCA  180
GACTCGATCC GTGTCGTCGC AGCACGTGAC CTCGTCCGAA GCCGTGTCGC ATAGAGCCAA  240
CGAGACAATC TACAACACGA CCCTGAAATA CGGAGATGTG GTCGGTGTCA ACACTACCAA  300
GTACCCATAC AGAGTGTGCT CGATGGCCCA GGGAACCGAC CTGATCCGGT TCGAGAGAAA  360
TATCGTGTGT ACCCCCATGA AGCCGATTAA TGAGGACCTG GACGAGGGCA TCATGGTCGT  420
GTATAAGCGC AACATTGTGG CCCACACCTT CAAAGTCCGC GTGTACCANA ANGTCCTTAN  480
NNTCCGGAGA AGCTACGCCT ANNTGCACAC TATCTCTTTG TCTGGNGTCN AACACTGAAT  540
ACGNGGCTNC ANCTATGTGN GGAGATCCAT CCCNTANACN GCNACTNTCC TNGTNNNNCA  600
TCNTACNNCN NAGTGNGTCN GCNGGNANCN TTGTCTNTGN NNTNNNNTCGG GNACTCCCTN  660
TNNTCTCTAA NATCTACNCN NNCCGCACGN TGAAANAANN AANNNNNANA NNNTANNNCN  720
NCTACGCTAA NNNCATCCGT ANNCCNNCNN GT                                 752
```

Figure A.3 VR1814_dN_sgB-HIS_pcDNAZeo

```
GNNNNNNNNN NNNACTTANG CTTGGTACCG CCGCCACCAT GGAGTCACGG ATTTGGTGCC   60
TCGTGGTCTG NGTGNACCTC TGCATCGTGT GCTTGGGCGC CATGAAGCCG ATTAATGAGG  120
ACCTGGACGA GGGCATCATG GTCGTGTATA AGCGCAACAT TGTGGCCCAC ACCTTCAAAG  180
TCCGCGTGTA CCAGAGGGTC CTTACCTTCC GGAGAAGCTA CGCCTACATC CACACTACCT  240
ACTTGCTGGG GTCAAACACT GAATACGTGG CCCCACCTAT GTGGGAGATC CATCACATAA  300
ACCGCCACTC TCAGTGTTAC TCATCATACA GCAGAGTGAT CGCCGGCACC GTGTTCGTGG  360
CCTACCATCG GGACTCCTAT GAGAACAAGA CGATGCAGCT GATGCTGGAT GACTACTCGA  420
ACACTCACTC GACCCGCTAC GTGACTGTGA AGGATCAGTG GCACTCTAGG GGATCCACTT  480
GGCTCTACCG GGAAACCTGT AACCTGAACT GCATGGTCAC CATCACCACC GCACGCTCCA  540
AGTACCCGTA CCACTTCTTC GCCACTTCCA CCGGCGACGT CGTGGACATC TCCCCTTTCT  600
ACAATGGAAC CAACCGCAAC ACTAGCTACT TTGGAGAAAA CGCGGACAAG TCCTTTATCT  660
TCCCCAACTA TACCATTGTG TCCGACTTTG GGAGGGCGAA CTCCGCACCC GAAACCCACA  720
```

```
GATTGGTGGC GTTCCTGGAG AGGGCCGACA GCGTCATCAG CTGGGACATC CAGGACGAGA  780
AGAACGTGAC CTGTCAGCTG ACCTTCTGGG AAGCCAGCGA GCGGACCATC CGGTCCGAGG  840
CTGAGGACTC CTACCATTTC TCCTCGGCGA AGATGACCGC TACCTTTCTG AGCAAGAAGC  900
AGGAAGTGAA CATGAGCGAC CCAGTCCTGG ACTGCGTGCG GGATCAGGGC CTGAACAAGC  960
TGCAGCAGAT CTTNATNGCC TCCTACAACC AGACCTACGA AAATACGGAA ACGTGTCGTG 1020
TTCGAANCTA CGGGGGACTG GTGTGTTTGG GAGGTATTAA GCANNNTNCC TACTGGAGTG 1080
GACGGNTGGC ANNNNNCGGG NTAACGGNNN GCGGNTCGTC NTNTCTCACN CTTCCACTAT 1140
GANCGAGTNT NNGGGCGTTN AACCGTNNTN GCNNGTTNNG CTTTTTNGCN NCTTTNNNTN 1200
NCCNCCCNNN CTNNNNNNCN NNNANNGGCC                                  1230
```

Figure A.4 VR1814_sgB-Hpbf_pcDNAZeo

```
NNNNNNNNNN NNNTNNNCTT AAGCTTGGTA CCGCCGCCNC CATGGAGTCA CGGATTTGGT   60
GCCTCGTGGT CTGCGTGAAC CTCTGCATCG TGTGCTTGGG CGCCGTGGTT TCGAGCTCCT  120
CCACATCCCA TGCCACCAGT AGTGCCCATA ACGGCTCCCA CACCTCCCGC ACTACCAGCG  180
CCCAGACTCG ATCCGTGTCG TCGCAGCACG TGACCTCGTC CGAAGCCGTG TCGCATAGAG  240
CCAACGAGAC AATCTACAAC ACGACCCTGA AATACGGAGA TGTGGTCGGT GTCAACACTA  300
CCAAGTACCC ATACAGAGTG TGCTCGATGG CCCAGGGAAC CGACCTGATC CGGTTCGAGA  360
GAAATATCGT GTGTACCCCC ATGAAGCCGA TTAATGAGGA CCTGGACGAG GGCATCATGG  420
TCGTGTATAA GCGCAACATT GTGGCCCACA CCTTCAAAGT CCGCGTGTAC CAGAAGGTCC  480
TTACCTTCCG GAGAAGCTAC GCCTACATCC ACACTACCTA CTTGCTGGGG TCAAACACTG  540
AATACGTGGC CCCACCTATG TGGGAGATCC ATCACATAAA CCGCCACTCT CAGTGTTACT  600
CATCATACAG CAGAGTGATC GCCGGCACCG TGTTCGTGGC CTACCATCGG GACTCCTATG  660
AGAACAAGAC GATGCAGCTG ATGCTGGATG ACTACTCGAA CACTCACTCG ACCCGCTACG  720
TGACTGTGAA GGATCAGTGG CACTCTAGGG GATCCACTTG GCTCTACCGG GAAACCTGTA  780
ACCTGAACTG CATGGTCACC ATCACCACCG CACGCTCCAA GTACCCGTAC CACTTCTTCG  840
CCACTTCCAC CGGCGACGTC GTGGACATCT CCCCTTTCTA CAATGGAACC AACCGCAACA  900
CTAGCTACTT TGGAGAAAAC GCGGACAAGT TCTTTATCTT CCCCAACTAT ACCATTGTGT  960
CCGACTTTGG GAGGGCGAAC TCCGCACCCG AAACCCACAG ATTGGTGGCG TTCCTGNAAA 1020
```

```
GGGCCGANAG CGTCATCAGC TGGGANNTCC AGGACGANAA NAACGNGACC TGTCANCTGA 1080
ACTTNNGNGN AAGCCAGGNA GGGGANNATC CGGNNCCNAG GNNGNANNAN CNNCCATTTN 1140
CCCCNGNNAA ANNNACCNNN ACCTTNNNNN NNAAAANNNN NNNNNNNA              1188
```

Figure A.5 VR1814_sgB-SS-Hpbf_pcDNAZeo

```
NNNNNNNNNN NNNNNNNNCT TAAGCTTGGT ACCGCCGCCA CCATGGAGTC ACGGATTTGG   60
TGCCTCGTGG TCTGNNNGAA CCTCTGCATC GTGTGCTTGG GCGCCGTGGT TTCGAGCTCC  120
TCCACATCCC ATGCCACCAG TAGTGCCCAT AACGGCTCCC ACACCTCCCG CACTACCAGC  180
GCCCAGACTC GATCCGTGTC GTCGCAGCAC GTGACCTCGT CCGAAGCCGT GTCGCATAGA  240
GCCAACGAGA CAATCTACAA CACGACCCTG AAATACGGAG ATGTGGTCGG TGTCAACACT  300
ACCAAGTACC CATACAGAGT GTCCTCGATG GCCCAGGGAA CCGACCTGAT CCGGTTCGAG  360
AGAAATATCG TGTCTACCCC CATGAAGCCG ATTAATGAGG ACCTGGACGA GGGCATCATG  420
GTCGTGTATA AGCGCAACAT TGTGGCCCAC ACCTTCAAAG TCCGCGTGTA CCAGAAGGTC  480
CTTACCTTCC GGAGAAGCTA CGCCTACATC CACACTACCT ACTTGCTGGG GTCAAACACT  540
GAATACGTGG CCCCACCTAT GTGGGAGATC CATCACATAA ACCGCCACTC TCAGTGTTAC  600
TCATCATACA GCAGAGTGAT CGCCGGCACC GTGTTCGTGG CCTACCATCG GGACTCCTAT  660
GAGAACAAGA CGATGCAGCT GATGCTGGAT GACTACTCGA ACACTCACTC GACCCGCTAC  720
GTGACTGTGA AGGATCAGTG GCACTCTAGG GGATCCACTT GGCTCTACCG GGAAACCTGT  780
AACCTGAACT GCATGGTCAC CATCACCACC GCACGCTCCA AGTACCCGTA CCACTTCTTC  840
GCCACTTCCA NCGGCGACGT CGTGGACATC TCCCTTTCTA CAATGGAGCA ACNGNNNACT  900
AGCTANTTGG AGAANACGCG AACAAGTTNT TANNTACTCA ATTATACCAT TGNGTCCGAN  960
NTTGAGANGN NAAATCNNCN NANNNAACCC CAGAATGNNG CGTNNCNGGA ANGGT      1015
```

Figure A.6 VR1814_dN-sgB-Hpbf_pcDNAZeo

```
NNNNNNNNNN NNNACTNNNC TTGGTACCGC CGCCACCATG GAGTCACGGA TTTGGTGCCT   60
CGTGGTCTGC GTGANNNTCT GCATCGTGTG CTTGGGCGCC ATGAAGCCGA TTAATGAGGA  120
CCTGGACGAG GGCATCATGG TCGTGTATAA GCGCAACATT GTGGCCCACA CCTTCAAAGT  180
```

```
CCGCGTGTAC CAGAAGGTCC TTACCTTCCG GAGAAGCTAC GCCTACATCC ACACTACCTA  240
CTTGCTGGGG TCAAACACTG AATACGTGGC CCCACCTATG TGGGAGATCC ATCACATAAA  300
CCGCCACTCT CAGTGTTACT CATCATACAG CAGAGTGATC GCCGGCACCG TGTTCGTGGC  360
CTACCATCGG GACTCCTATG AGAACAAGAC GATGCAGCTG ATGCTGGATG ACTACTCGAA  420
CACTCACTCG ACCCGCTACG TGACTGTGAA GGATCAGTGG CACTCTAGGG GATCCACTTG  480
GCTCTACCGG GAAACCTGTA ACCTGAACTG CATGGTCACC ATCACCACCG CACGCTCCAA  540
GTACCCGTAC CACTTCTTCG CCACTTCCAC CGGCGACGTC GTGGACATCT CCCCTTTCTA  600
CAATGGAACC AACCGCAACA CTAGCTACTT TGGAGAAAAC GCGGACAAGT TCTTTATCTT  660
CCCCAACTAT ACCATTGTGT CCGACTTTGG GAGGGCGAAC TCCGCACCCG AAACCCACAG  720
ATTGGTGGCG TTCCTGGAGA GGGCCGACAG CGTCATCAGC TGGGACATCC AGGACGAGAA  780
GAACGTGACC TGTCAGCTGA CCTTCTGGGA AGCCAGCGAG CGGACCATCC GGTCCGAGGC  840
TGAGGACTCC TACCATTTCT CCTCGGCGAA GATGACCGCT ACCTTTCTGA GCAAGAAGCA  900
GGAAGTGAAC ATGAGCGACC CAGTCCTGGA CTGCGTGCGG GATCAGGCCC TGAACAAGCT  960
GCAGCAGATC TTCAATGCCT CCTACAACCA GACCTACGAA AAATACGGAA ACGTGTCCGG 1020
GTTCGAAACA CC                                                     1032
```

Figure A.7 VR1814_gB_ecto-HIS_pcDNAZeo

```
CCCAGAAAGA GGCCGTTTTA TACTTTAAAG CTTTGGTACC GCCGCCACCA TGGAGTCACG   60
GATTTGGTGC CTCGTGGTCT GCGTGAACCT CTGCATCGTG TGCTTGGGCG CCGTGGTTTC  120
GAGCTCCTCC ACATCCCATG CCACCAGTAG TGCCCATAAC GGCTCCCACA CCTCCCGCAC  180
TACCAGCGCC CAGACTCGAT CCGTGTCGTC GCAGCACGTG ACCTCGTCCG AAGCCGTGTC  240
GCATAGAGCC AACGAGACAA TCTACAACAC GACCCTGAAA TACGGAGATG TGGTCGGTGT  300
CAACACTACC AAGTACCCAT ACAGAGTGTG CTCGATGGCC CAGGGAACCG ACCTGATCCG  360
GTTCGAGAGA AATATCGTGT GTACCCCCAT GAAGCCGATT AATGAGGACC TGGACGAGGG  420
CATCATGGTC GTGTATAAGC GCAACATTGT GGCCCACACC TTCAAAGTCC GCGTGTACCA  480
GAAGGTCCTT ACCTTCCGGA GAAGCTACGC CTACATCCAC ACTACCTACT TGCTGGGGTC  540
AAACACTGAA TACGTGGCCC CACCTATGTG GGAGATCCAT CACATAAACC GCCACTCTCA  600
GTGTTACTCA TCATACAGCA GAGTGATCGC CGGGCACCGT GTTTCGTGGC CTACCATCTG  660
```

```
GACTCCTATG AGAACAAGAC TATGCAGCTG ATGCTGGATG ACTACTCAAA CACTCCACTC  720
GACCCGCTAC CTGACTGTGA AGGATCAGTG GCACTCTAAG GGCATCCTCT             770
```

Figure A.8 VR1814_gB-ecto-R6-P-HIS_pcDNAZeo

```
ANNNNCNNNA CNNNCGCTGG AGTCCGAAAG CGTCAGAAAC GTCCTGTACG CCCAACTGCA    60
GTTCACTTAC GATACTCTTC GGTCCTACAT CAACCGCGCT CTGGCCCAAA TTCCTGAGGC   120
TTGGTGCGTG GATCAGAGGC GGACCCTGGA GGTGTTCAAG GAACTGAGCA AGATTAACCC   180
CTCGGCGATT CTCTCCGCGA TCTATAACAA GCCGATTGCC GCCAGGTTCA TGGGCGATGT   240
GCTTGGGTTG GCCTCATGCG TGACTATCAA CCAAACCTCA GTGAAGGTCC TGCGGGACAT   300
GAACGTGAAG GAAAGCCCGG GACGGTGCTA CTCGCGGCCA GTCGTGATTT TTAACTTCGT   360
GAACTCCAGC TACGTGCAAT ACGGACAACT GGGAGAGGAC AACGAAATCC TCCTGGGAAA   420
CCACCGCACC GAAGAGTGCC AGTTCCCGTC GCTGAAGATT TTCATTGCCG GAAACAGCGC   480
TTACGAATAC GTGGACTACC TCTTCAAAAG AATGATCGAT CTGTCCTCAA TTTCAACTGT   540
GGACAGCATG ATCGCCCTGG ACATCGATCC TCTCGAAAAC ACCGATTTCC GGGTGCTGGA   600
GCTCTATTCC CAAAAGGAGC TGCGCTCGTC GAACGTGTTC GATCTGGAAG AAATCATGAG   660
AGAGTTCAAT TCATACAAGC AGCGCGTGAA ATATGTGGAG GACAAAGTGG TGGATCCGCT   720
CCCTCCGTAC GGCGGCAGCA CCGGATCCGC TCATCATCAT CACCACCATC ACCACTAATG   780
ACTCGAGTCT AGAGGGCCCG TTTAAACCCG CTGATCAGCC TCGACTGTGC CTTCTAGTTG   840
CCAGCCATCT GTTGTTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG GTGCCACTCC   900
CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA GGTGTCATNC   960
TATCNNGGGG GGGNGGGGTG GGGCNNGGAC NCCAGGGGGT AGGATGGGAN NNA         1013
```

Figure A.9 IgSP-VR1814_gB.ecto-R6-P-HIS_pcDNAZeo

```
NNNNNNNNNN NNNNNGCAGA NCTCTCTGGC TACTAGAGAA CCCACTGCTT ACTGGCTTAT    60
CGAAATTAAT ACGACTCACT ATAGGGAGAC CCAAGCTGGC TAGCGTTTAA ACTTAAGCTT   120
GGTACCGCCG CCACCATGGG ATGGTCATGT ATCATCCTTT TTCTAGTAGC AACTGCAACC   180
GGTGTACACT CCGCCGTGGT TTCGAGCTCC TCCACATCCC ATGCCACCAG TAGTGCCCAT   240
AACGGCTCCC ACACCTCCCG CACTACCAGC GCCCAGACTC GATCCGTGTC GTCGCAGCAC   300
```

GTGACCTCGT CCGAAGCCGT GTCGCATAGA GCCAACGAGA CAATCTACAA CACGACCCTG 360

AAATACGGAG ATGTGGTCGG TGTCAACACT ACCAAGTACC CATACAGAGT GTGCTCGATG 420

GCCCAGGGAA CCGACCTGAT CCGGTTCGAG AGAAATATCG TGTGTACCCC CATGAAGCCG 480

ATTAATGAGG ACCTGGACGA GGGCATCATG GTCGTGTATA AGCGCAACAT TGTGGCCCAC 540

ACCTTCAAAG TCCGCGTGTA CCAGAAGGTC CTTACCTTCC GGAGAAGCTA CGCCTACATC 600

CACACTACCT ACTTGCTGGG GTCAAACACT GAATACGTGG CCCCACCTAT GTGGGAGATC 660

CATCACATAA ACCGCCACTC TCAGTGTTAC TCATCATACA GCAGAGTGAT CGCCGGCACC 720

GTGTTCGTGG CCTACCATCG GGACTCCTAT GAGAACAAGA CGATGCAGCT GATGCTGGAT 780

GACTACTCGA ACACTCACTC GACCCNCTAC GTGACTGTGA AGGATCAGTG GCACTCTATG 840

GGATCCGCTT GGCTCTACCG GGAAACCTGT AACCTGANAC TGCAGGGTCN CATCACCNCN 900

NACGCNNNAG TTACCCGTAN CANTANTAGC ACTTNTANNN GAGANNATNN NGAANNNNNN 960

NCTTTNNANN ANNGAANCGA CCANNNCATA ATNANNNNGN ANNNN 1005

Figure A.10 VR1814gBecto-T4.HIS_pcDNAZeo

NNNNNNNNNN GTTAACTTAA GCTTGGTACC GCCGCCACCA TGGAGTCACG GATTTGGTGC 60

CTCGTGGTCT GCGTGAACCT CTGCATCGTG TGCTTGGGCG CCGTGGTTTC GAGCTCCTCC 120

ACATCCCATG CCACCAGTAG TGCCCATAAC GGCTCCCACA CCTCCCGCAC TACCAGCGCC 180

CAGACTCGAT CCGTGTCGTC GCAGCACGTG ACCTCGTCCG AAGCCGTGTC GCATAGAGCC 240

AACGAGACAA TCTACAACAC GACCCTGAAA TACGGAGATG TGGTCGGTGT CAACACTACC 300

AAGTACCCAT ACAGAGTGTG CTCGATGGCC CAGGGAACCG ACCTGATCCG GTTCGAGAGA 360

AATATCGTGT GTACCCCCAT GAAGCCGATT AATGAGGACC TGGACGAGGG CATCATGGTC 420

GTGTATAAGC GCAACATTGT GGCCCACACC TTCAAAGTCC GCGTGTACCA GAAGGTCCTT 480

ACCTTCCGGA GAAGCTACGC CTACATCCAC ACTACCTACT TGCTGGGGTC AAACACTGAA 540

TACGTGGCCC CACCTATGTG GGAGATCCAT CACATAAACC GCCACTCTCA GTGTTACTCA 600

TCATACAGCA GAGTGATCGC CGGCACCGTG TTCGTGGCCT ACCATCGGGA CTCCTATGAG 660

AACAAGACGA TGCAGCTGAT GCTGGATGAC TACTCGAACA CTCACTCGAC CCGCTACGTG 720

ACTGTGAAGG ATCAGTGGCA CTCTAGGGGA TCCACTTGGC TCTACCGGGA AACCTGTAAC 780

CTGAACTGCA TGGTCACCAT CACCACCGCA CGCTCCAAGT ACCCGTACCA CTTCTTCGCC 840

```
ACTTCCACCG GCGACGTCGT GGACATCTCC CCTTTCTACA ATGGAACCAA CCGCAACACT  900
AGCTACTTTG GAGAAAACGC GGACAAGTTC TTTATCTTCC CCAACTATAC CATTGTGTCC  960
GACTTTGGGA GGGCGAACTC CGCACCCGAA ACCCACAGAT TGGTGGCGTT CCTGGAGAGG 1020
GCCGANNGCG TCATCAGCTG GGANNTCCAN GANNAGAAAA CGTGACTGTC NCNTGACTTN 1080
CGGGAAGCCA GNNAGNGGAN CATCCGGTCC AAGCTNA                         1117
```

Figure A.11 VR1814gBecto-R6-T4.HIS_pcDNAZeo

```
NNNNNNNNNN NNNNNNNNNT TAAGCTTGGT ACCGCCGCCA CCATGGAGTC ACGGATTTGG   60
TGCCTCGTGG TCTGCNNNNN CNTCTGCATC GTGTGCTTGG GCGCCGTGGT TTCGAGCTCC  120
TCCACATCCC ATGCCACCAG TAGTGCCCAT AACGGCTCCC ACACCTCCCG CACTACCAGC  180
GCCCAGACTC GATCCGTGTC GTCGCAGCAC GTGACCTCGT CCGAAGCCGT GTCGCATAGA  240
GCCAACGAGA CAATCTACAA CACGACCCTG AAATACGGAG ATGTGGTCGG TGTCAACACT  300
ACCAAGTACC CATACAGAGT GTGCTCGATG GCCCAGGGAA CCGACCTGAT CCGGTTCGAG  360
AGAAATATCG TGTGTACCCC CATGAAGCCG ATTAATGAGG ACCTGGACGA GGGCATCATG  420
GTCGTGTATA AGCGCAACAT TGTGGCCCAC ACCTTCAAAG TCCGCGTGTA CCAGAAGGTC  480
CTTACCTTCC GGAGAAGCTA CGCCTACATC CACACTACCT ACTTGCTGGG GTCAAACACT  540
GAATACGTGG CCCCACCTAT GTGGGAGATC CATCACATAA ACCGCCACTC TCAGTGTTAC  600
TCATCATACA GCAGAGTGAT CGCCGGCACC GTGTTCGTGG CCTACCATCG GGACTCCTAT  660
GAGAACAAGA CGATGCAGCT GATGCTGGAT GACTACTCGA ACACTCACTC GACCCGCTAC  720
GTGACTGTGA AGGATCAGTG GCACTCTAGG GGATCCACTT GGCTCTACCG GGAAACCTGT  780
AACCTGAACT GCATGGTCAC CATCACCACC GCACGCTCCA AGTACCCGTA CCACTTCTTC  840
GCCACTTCCA CCGGCGACGT CGTGGACATC TCCCCTTTCT ACAATGGAAC CAACCGCAAC  900
ACTAGCTACT TTGGAGAAAA CGCGGACAAG TTCTTTATCT TCCCAACTAT ACCATTGTGT  960
CCGACTTTGG GAGGGCGAAC TCCGCACCCG AAACCCACAG ATTGGTGGCG TTCCCGGAAA 1020
GGGCCGANNG CGTCATCAGC TGGGAAATCC AGGACAAGAA GAACTGACNT GTNNCTGACT 1080
TCTGGGAAGC NNNNN                                                 1095
```

Figure A.12 VR1814gBecto-R6-P-T4.HIS_pcDNAZeo

```
NNNNNNNNNN NNNNNNNNTT AAGCTTGGTA CCGCCGCCAC CATGGAGTCA CGGATTTGGT   60
GCCTCGTGGT CTGCGTGAAC CTCTGCATCG TGTGCTTGGG CGCCGTGGTT TCGAGCTCCT  120
CCACATCCCA TGCCACCAGT AGTGCCCATA ACGGCTCCCA CACCTCCCGC ACTACCAGCG  180
CCCAGACTCG ATCCGTGTCG TCGCAGCACG TGACCTCGTC CGAAGCCGTG TCGCATAGAG  240
CCAACGAGAC AATCTACAAC ACGACCCTGA AATACGGAGA TGTGGTCGGT GTCAACACTA  300
CCAAGTACCC ATACAGAGTG TGCTCGATGG CCCAGGGAAC CGACCTGATC CGGTTCGAGA  360
GAAATATCGT GTGTACCCCC ATGAAGCCGA TTAATGAGGA CCTGGACGAG GGCATCATGG  420
TCGTGTATAA GCGCAACATT GTGGCCCACA CCTTCAAAGT CCGCGTGTAC CAGAAGGTCC  480
TTACCTTCCG GAGAAGCTAC GCCTACATCC ACACTACCTA CTTGCTGGGG TCAAACACTG  540
AATACGTGGC CCCACCTATG TGGGAGATCC ATCACATAAA CCGCCACTCT CAGTGTTACT  600
CATCATACAG CAGAGTGATC GCCGGCACCG TGTTCGTGGC CTACCATCGG GACTCCTATG  660
AGAACAAGAC GATGCAGCTG ATGCTGGATG ACTACTCGAA CACTCACTCG ACCCGCTACG  720
TGACTGTGAA GGATCAGTGG CACTCTAGGG GATCCACTTG GCTCTACCGG GAAACCTGTA  780
ACCTGAACTG CATGGTCACC ATCACCACCG CACGCTCCAA GTACCCGTAC CACTTCTTCG  840
CCACTTCCAC CGGCGACGTC GTGGACATCT CCCCTTTCTA CAATGGAACC AACCGCAACA  900
CTAGCTACTT TGGAGAAAAC GCGGACAAGT TCTTTATCTT CCCCAACTAT ACCATTGTGT  960
CCGACTTTGG GAGGGCGAAC TCCGCACCCG AAACCCACAG ATTGGNGGCG TTCCTGGAGA 1020
GGGCNGACAG CGTCATCAGC TGGGACNTCC AGGACAAGAN AACGTGACTG TCAGCTGACT 1080
TTNGGGAAGC AGCNAGNGGA CATCCGGTCG NGGCTN                           1116
```

Figure A.13 IgSP-VR1814_gB.ecto-R6-P-T4.HIS_pcDNAZeo

```
NNNNNNNNNN NNNNTNNNTT AAGCTTGGTA CCGCCGCCAC CATGGGATGG TCATGTATCA   60
TCCTTTTTCT AGTAGCAACT GCAACCGGTG TACACTCCGC CGTGGTTTCG AGCTCCTCCA  120
CATCCCATGC CACCAGTAGT GCCCATAACG GCTCCCACAC CTCCCGCACT ACCAGCGCCC  180
AGACTCGATC CGTGTCGTCG CAGCACGTGA CCTCGTCCGA AGCCGTGTCG CATAGAGCCA  240
ACGAGACAAT CTACAACACG ACCCTGAAAT ACGGAGATGT GGTCGGTGTC AACACTACCA  300
AGTACCCATA CAGAGTGTGC TCGATGGCCC AGGGAACCGA CCTGATCCGG TTCGAGAGAA  360
```

```
ATATCGTGTG TACCCCCATG AAGCCGATTA ATGAGGACCT GGACGAGGGC ATCATGGTCG  420
TGTATAAGCG CAACATTGTG GCCCACACCT TCAAAGTCCG CGTGTACCAG AAGGTCCTTA  480
CCTTCCGGAG AAGCTACGCC TACATCCACA CTACCTACTT GCTGGGGTCA AACACTGAAT  540
ACGTGGCCCC ACCTATGTGG GAGATCCATC ACATAAACCG CCACTCTCAG TGTTACTCAT  600
CATACAGCAG AGTGATCGCC GGCACCGTGT TCGTGGCCTA CCATCGGGAC TCCTATGAGA  660
ACAAGACGAT GCAGCTGATG CTGGATGACT ACTCGAACAC TCACTCGACC CGCTACGTGA  720
CTGTGAAGGA TCAGTGGCAC TCTAGGGGAT CCACTTGGCT CTACCGGGAA ACCTGTAACC  780
TGAACTGCAT GGTCACCATC ACCACCGCAC GCTCCAAGTA CCCGTACCAC TTCTTCGCCA  840
CTTCCACCGG CGACGTCGTG GACATCTCCC CTTTCTACAA TGGAACCAAC CGCAACACTA  900
GCTACTTTGG AGAAAACGCG GACAAGTTCT TTATCTTCCC CAACTATACC ATTGTGTCCG  960
ACTTTGGGAG GGCGAACTCC GCACCCGAAA CCCACAGATT GGTGGCGTTC CTGGAGAGGG 1020
CCGANNGCGT CATCAGCTGG GANNTCCAGG ANNNNANAAC GTGACCTGTC NGCTGACCTC 1080
TGGGAAGCCG CNNGGNACAT CGGTCCN                                     1107
```

Figure A.14 VR1814_gH

```
GGTACCGCCG CCACCATGAG GCCAGGACTT CCCTTCTACC TCACCGTGTT CGCCGTGTAC   60
CTCCTCTCCC ACCTCCCAAG CCAGCGCTAC GGAGCCGACG CAGCTTCTGA AGCCCTAGAT  120
CCTCACGCCT TCCATCTGCT GCTGAATACT TATGGCCGGC CCATACGCTT CCTGCGCGAG  180
AACACCACCC AGTGCACTTA CAACAGCTCG CTTAGAAACA GCACCGTGGT CCGGGAGAAC  240
GCCATTTCCT TTAACTTCTT CCAATCATAC AACCAGTACT ACGTGTTCCA TATGCCCCGC  300
TGCCTGTTTG CCGGCCCATT AGCCGAGCAG TTCCTGAACC AAGTGGACCT GACTGAAACA  360
CTGGAAAGAT ACCAGCAGCG CCTGAACACC TACGCCTTGG TCAGCAAGGA CTTGGCGTCC  420
TACCGGAGCT TTCCCCAGCA ACTGAAGGCG CAGGATTCCC TCGGACAACA GCCCACCACT  480
GTGCCCCCTC CCATCGACCT GTCCATTCCC CACGTCTGGA TGCCGCCTCA AACCACTCCT  540
CACGACTGGA AGGGCTCGCA CACCACCTCC GGACTGCACA GGCCTCATTT CAATCAGACA  600
TGCATCCTGT TCGACGGTCA TGACCTCCTC TTCTCAACCG TGACCCCGTG TCTGCACCAA  660
GGATTCTACC TGATGGACGA ACTCAGATAT GTGAAGATTA CTCTGACCGA GGATTTCTTC  720
GTCGTCACCG TGTCGATTGA CGACGATACC CCCATGCTGC TGATTTTCGG TCATCTGCCG  780
```

```
CGGGTGCTGT TCAAGGCCCC TTACCAACGG GACAACTTTA TCCTGAGGCA GACCGAAAAG  840
CACGAACTGC TGGTGCTGGT CAAAAAGACC CAGCTGAATC GGCACAGCTA CCTGAAGGAC  900
TCAGACTTCC TGGATGCGGC CCTGGACTTT AACTACCTGG ACCTGTCGGC GCTGTTGAGA  960
AACTCGTTCC ATCGCTACGC CGTGGACGTG CTCAAGTCCG GCCGCTGTCA AATGCTGGAT 1020
AGGCGGACCG TGGAAATGGC CTTCGCCTAC GCCCTGGCAC TCTTCGCCGC CGCAAGACAG 1080
GAAGAGGCCG GCACCGAGAT CTCCATCCCG CGAGCCCTGG ATCGCCAAGC TGCCCTCCTG 1140
CAAATTCAGG AGTTCATGAT CACTTGCCTC TCCCAAACTC CTCCTCGCAC GACCCTCCTG 1200
CTATACCCAA CTGCCGTGGA TCTCGCCAAG AGAGCGCTGT GGACTCCGGA TCAGATCACC 1260
GACATCACCA GCCTTGTGCG GCTGGTGTAC ATTCTGAGCA AGCAGAACCA GCAGCACCTG 1320
ATCCCGCAGT GGGCACTGAG GCAGATCGCT GACTTTGCGC TCCAACTCCA CAAAACCCAC 1380
CTCGCTTCAT TCTTGTCCGC CTTCGCGAGA CAGGAACTGT ACCTGATGGG TTCGCTGGTG 1440
CACAGTATGC TCGTCCATAC CACCGAACGG CGGGAGATCT TCATTGTGGA AACCGGGCTG 1500
TGCTCCTTGG CGGAGCTGAG CCACTTCACC CAACTTCTGG CTCACCCGCA CCACGAGTAC 1560
CTGTCCGACC TGTACACGCC GTGCAGCAGC TCAGGACGCC GGGATCATTC GTTGGAACGG 1620
CTGACCCGGC TGTTCCCCGA CGCCACTGTG CCGGCAACCG TGCCAGCTGC CCTTAGCATC 1680
CTTAGCACCA TGCAGCCGTC CACGCTTGAA ACATTCCCGG ACCTGTTCTG CCTGCCGCTG 1740
GGAGAGTCGT TCTCGGCACT CACCGTGTCC GAGCACGTCA GCTACGTCGT GACTAACCAG 1800
TATCTGATTA AGGGGATTTC CTACCCCGTG TCGACCACTG TGGTCGGGCA GTCCCTGATC 1860
ATCACTCAGA CTGACTCCCA GTCTAAGTGC GAGCTGACCC GCAACATGCA TACCACCCAC 1920
TCCATCACCG CCGCCCTGAA CATCTCACTG GAGAACTGCG CATTCTGCCA ATCCGCTCTG 1980
CTTGAGTACG ACGACACCCA GGGAGTTATT AACATCATGT ACATGCACGA CTCCGATGAC 2040
GTGCTTTTTG CGCTGGACCC TTACAACGAA GTGGTCGTGT CCAGCCCTCG CACTCACTAC 2100
CTGATGCTCC TGAAGAACGG CACTGTGCTC GAAGTCACTG ACGTGGTGGT GGACGCCACT 2160
GACTCCCGGC TGCTGATGAT GTCCGTGTAC GCCCTGTCCG CCATCATCGG GATCTATTTG 2220
TTGTACCGGA TGCTCAAGAC CTGTTAATGA CTCGAGTCT                       2259
```

Figure A.15 VR1814_gH_pcDNAZeo

```
NNNNNNNNNN NNTTAAGCTT GGTACCGCCG CCACCATGAG GCCAGGACTT CCCTTCTACC   60
```

```
TCACCGTGTT CGCCGTGTAC CTCCTCTCCC ACCTCCCAAG CCAGCGCTAC GGAGCCGACG  120
CAGCTTCTGA AGCCCTAGAT CCTCACGCCT TCCATCTGCT GCTGAATACT TATGGCCGGC  180
CCATACGCTT CCTGCGCGAG AACACCACCC AGTGCACTTA CAACAGCTCG CTTAGAAACA  240
GCACCGTGGT CCGGGAGAAC GCCATTTCCT TTAACTTCTT CCAATCATAC AACCAGTACT  300
ACGTGTTCCA TATGCCCCGC TGCCTGTTTG CCGGCCCATT AGCCGAGCAG TTCCTGAACC  360
AAGTGGACCT GACTGAAACA CTGGAAAGAT ACCAGCAGCG CCTGAACACC TACGCCTTGG  420
TCAGCAAGGA CTTGGCGTCC TACCGGAGCT TTCCCCAGCA ACTGAAGGCG CAGGATTCCC  480
TCGGACAACA GCCCACCACT GTGCCCCCTC CCATCGACCT GTCCATTCCC CACGTCTGGA  540
TGCCGCCTCA AACCACTCCT CACGACTGGA AGGGCTCGCA CACCACCTCC GGACTGCACA  600
GGCCTCATTT CAATCAGACA TGCATCCTGT TCGACGGTCA TGACCTCCTC TTCTCAACCG  660
TGACCCCGTG TCTGCACCAA GGATTCTACC TGATGGACGA ACTCAGATAT GTGAAGATTA  720
CTCTGACCGA GGATTTCTTC GTCGTCACCG TGTCGATTGA CGACGATACC CCCATGCTGC  780
TGATTTTCGG TCATCTGCCG CGGGTGCTGT TCAAGGCCCC TTACCAACGG GACAACTTTA  840
TCCTGAGGCA GACCGAAAAG CACGAACTGC TGGTGCTGGT CAAAAAGACC CAGCTGAATC  900
GGCACAGCTA CCTGAAGGAC TCAGACTTCC TGGATGCGGC CCTGGACTTT AACTACCTGG  960
ACCTGTCGGC GCTGTTGAGA AACTCGTTCC ATCGCTACGC CGTGGACGTG CTCAAGTCCG 1020
GCCGCTGTCA ATGCTGGATA GGCGGACCGT GGAAATGGCC TTCGCCTACG CCCTGGCACT 1080
CTTCGCCGCN CAAGANNGGA AGAGGCCGNN CCGANATNNC ATCCCNNNNN CCTGGANNNC 1140
NAGCTGCCNN CTGCAATTCA GGNNTTNNNN NCCTTGCCNN NCCAAATCNN NNNNNNNCCN 1200
CNGGNNNACC NANTNNCNNG GANNNCCCNN AANGCCNNNG GANCCGGNNN AANCCGAANN 1260
NCNCCCTTGG GNGGGGNNNN TTTGNAAANN AANNC                           1295
```

Figure A.16 tpaSP-VR1814_gH_pcDNAZeo

```
NNNNNNNNNN NNNNNNNNNT AAGCTTGGTA CCGCCGCCAC CATGGATGCA ATGAAGAGAG   60
GGCTCTGCTG TGTGCTGCTG CTGTGTGGAG CAGTCTTCGT TTCGCCCGCG CGCGCTTCTG  120
AAGCCCTAGA TCCTCACGCC TTCCATCTGC TGCTGAATAC TTATGGCCGG CCCATACGCT  180
TCCTGCGCGA GAACACCACC CAGTGCACTT ACAACAGCTC GCTTAGAAAC AGCACCGTGG  240
TCCGGGAGAA CGCCATTTCC TTTAACTTCT TCCAATCATA CAACCAGTAC TACGTGTTCC  300
```

```
ATATGCCCCG CTGCCTGTTT GCCGGCCCAT TAGCCGAGCA GTTCCTGAAC CAAGTAGACC  360
TGACTGAAAC ACTGGAAAGA TACCAGCAGC GCCTGAACAC CTACGCCTTG GTCAGCAAGG  420
ACTTGGCGTC CTACCGGAGC TTTCCCCAGC AACTGAAGGC GCAGGATTCC CTCGGACAAC  480
AGCCCACCAC TGTGCCCCCT CCCATCGACC TGTCCATTCC CCACGTCTGG ATGCCGCCTC  540
AAACCACTCC TCACGACTGG AAGGGCTCGC ACACCACCTC CGGACTGCAC AGGCCTCATT  600
TCAATCAGAC ATGCATCCTG TTCGACGGTC ATGACCTCCT CTTCTCAACC GTGACCCCGT  660
GTCTGCACCA AGGATTCTAC CTGATGGACG AACTCAGATA TGTGAAGATT ACTCTGACCG  720
AGGATTTCTT CGTCGTCACC GTGTCGATTG ACGACGATAC CCCCATGCTG CTGATTTTCG  780
GTCATCTGCC GCGGGTGCTG TTCAAGGCCC CTTACCAACG GGACAACTTT ATCCTGAGGC  840
AGACCGAAAA GCACGAACTG CTGGTGCTGG TCAAAAAGAC CCAGCTGAAT CGGCACAGCT  900
ACCTGAAGGA CTCAGACTTC CTGGATGCGG CCCTGGACTT TAACTACCTG GACCTGTCGG  960
CGCTGTTGAG AAACTCGTTC NATCGCTACG CCGTGGACGT GCTCAAGTCC GGNGCTGTCA 1020
ATGCTGGATA GGCGGACCGN GGAAATGGCT TCGCTACGCC TGGCNNCTTC GCCNCGCAAA 1080
NAGGAAAAGG CGGCCCNANN TTNCNTCCNN NNGCCTGGAT CGCAANTGCC NCTGCAATTC 1140
AGANTCANNN NCTTGCNNTC CAANNCTCTN GNNACCNCGT NNCCAATGCG GGATNNCCAA 1200
AANGCTTGGG ATCCGNCAAT CCGAATCNNN CTTGGCGTGG NNATTNGAAN NNAACNNNNC 1260
CNNTNNN                                                           1267
```

Figure A.17 VR1814_gL

```
GGTACCGCCG CCACCATGTG TCGGAGGCCG GATTGCGGGT TCTCCTTCTC CCCCGGACCT   60
GTGGTGCTGC TCTGGTGCTG CCTGCTGCTC CCCATCGTGT CCTCCGTGGC CGTGTCAGTG  120
GCACCTACTG CCGCCGAGAA GGTCCCAGCC GAATGCCCCG AACTGACCCG GAGATGCCTG  180
CTCGGGGAGG TGTTCCAGGG CGACAAATAC GAATCCTGGC TGCGGCCCTT GGTGAACGTC  240
ACCGGACGCA ATGGCCCGCT GAGCCAACTG ATCAGATACC GGCCAGTGAC CCCTGAAGCC  300
GCCAACTCGG TGCTGCTCGA CGACGCCTTT CTCGATACCC TGGCCCTGCT GTACAACAAC  360
CCTGACCAGC TGCGGGCACT CCTGACCCTG CTGAGCTCCG ACACTGCGCC GAGATGGATG  420
ACTGTCATGC GCGGCTACTC AGAGTGCGGA GATGGATCGC CCGCCGTGTA TACATGCGTG  480
GACGATCTCT GTCGGGGCTA CGACCTGACC CGGCTGTCGT ACGGTCGCAG CATTTTCACC  540
```

```
GAGCACGTGC TGGGGTTCGA ACTGGTGCCC CCGTCTTTGT TCAACGTGGT GGTGGCGATT  600
CGCAACGAAG CCACGCGGAC CAACAGGGCT GTCAGGCTGC CAGTCAGCAC TGCTGCTGCG  660
CCTGAGGGTA TCACCCTGTT CTACGGCCTT TACAACGCCG TGAAGGAGTT TTGTCTGCGA  720
CACCAGCTGG ATCCGCCTCT TCTCCGGCAC CTTGACAAGT ACTATGCGGG ATTGCCTCCG  780
GAACTGAAGC AGACTCGCGT GAATCTCCCG GCACATTCCC GCTACGGACC GCAAGCCGTC  840
GACGCCAGAT AATGACTCGA GTCT                                         864
```

Figure A.18 VR1814_gL_pcDNAZeo

```
NNNNNNNNNN GNNNNNTTNA GCTTGGTACC GCCGCCACCA TGTGTCGGAG GCCGGATTGC   60
GGGTTCTCCT TCTCCCCCGG ACCTGTGGTG CTGCTCTGGT GCTGCCTGCT GCTCCCCATC  120
GTGTCCTCCG TGGCCGTGTC AGTGGCACCT ACTGCCGCCG AGAAGGTCCC AGCCGAATGC  180
CCCGAACTGA CCCGGAGATG CCTGCTCGGG GAGGTGTTCC AGGGCGACAA ATACGAATCC  240
TGGCTGCGGC CCTTGGTGAA CGTCACCGGA CGCAATGGCC CGCTGAGCCA ACTGATCAGA  300
TACCGGCCAG TGACCCCTGA AGCCGCCAAC TCGGTGCTGC TCGACGACGC CTTTCTCGAT  360
ACCCTGGCCC TGCTGTACAA CAACCCTGAC CAGCTGCGGG CACTCCTGAC CCTGCTGAGC  420
TCCGACACTG CGCCGAGATG GATGACTGTC ATGCGCGGCT ACTCAGAGTG CGGAGATGGA  480
TCGCCCGCCG TGTATACATG CGTGGACGAT CTCTGTCGGG GCTACGACCT GACCCGGCTG  540
TCGTACGGTC GCAGCATTTT CACCGAGCAC GTGCTGGGGT TCGAACTGGT GCCCCCGTCT  600
TTGTTCAACG TGGTGGTGGC GATTCGCAAC GAAGCCACGC GGACCAACAG GGCTGTCAGG  660
CTGCCAGTCA GCACTGCTGC TGCGCCTGAG GGTATCACCC TGTTCTACGG CCTTTACAAC  720
GCCGTGAAGG AGTTTTGTCT GCGACACCAG CTGGATCCGC CTCTTCTCCG GCACCTTGAC  780
AAGTACTATG CGGGATTGCC TCCGGAACTG AAGCAGACTC GCGTGAATCT CCCGGCACAT  840
TCCCGCTACG GACCGCAAGC CGTCGACGCC AGATAATGAC TCGAGTCTAG AGGGCCCGTT  900
TAAACCCGCT GATCAGCCTC GACTGTGCCT TCTAGTTGCC AGCCATCTGT TGTTTGCCCC  960
TCCCCCGTGC CTTCCTTGAC CCTGGAAGGT GCCACTCCCA CTGTCCTTTC CTAATAAAAT 1020
GAGGAAATTG CATCGCATTG TCTGANTAGG TGTCATTCNA TNNNGGGGGN NGGGNNGGGN 1080
CNNGGNNNCC NAAGGGNAGG AANGGNAAAA NNNNNNNNNN NNNNNNGGAA ANNNGNG    1137
```

Figure A.19 IgSP-VR1814-gL_pcDNAzeo

```
NNNNNNNNNN AACTTAAGCT TGGTACCGCC GCCACCATGG GATGGTCATG TATCATCCTT   60
TTTCTAGTAG CAACTGCAAC CGGTGTACAC TCCGTGGCCG TGTCAGTGGC ACCTACTGCC  120
GCCGAGAAGG TCCCAGCCGA ATGCCCCGAA CTGACCCGGA GATGCCTGCT CGGGGAGGTG  180
TTCCAGGGCG ACAAATACGA ATCCTGGCTG CGGCCCTTGG TGAACGTCAC CGGACGCAAT  240
GGCCCGCTGA GCCAACTGAT CAGATACCGG CCAGTGACCC CTGAAGCCGC CAACTCGGTG  300
CTGCTCGACG ACGCCTTTCT CGATACCCTG GCCCTGCTGT ACAACAACCC TGACCAGCTG  360
CGGGCACTCC TGACCCTGCT GAGCTCCGAC ACTGCGCCGA GATGGATGAC TGTCATGCGC  420
GGCTACTCAG AGTGCGGAGA TGGATCGCCC GCCGTGTATA CATGCGTGGA CGATCTCTGT  480
CGGGGCTACG ACCTGACCCG GCTGTCGTAC GGTCGCAGCA TTTTCACCGA GCACGTGCTG  540
GGGTTCGAGC TGGTGCCCCC GTCTTTGTTC AACGTGGTGG TGGCGATTCG CAACGAAGCC  600
ACGCGGACCA ACAGGGCTGT CAGGCTGCCA GTCAGCACTG CTGCTGCGCC TGAGGGTATC  660
ACCCTGTTCT ACAGCCTTTA CAANNNCGTG AAGGAGTTTT GTCTGCGACA CCAGCTGGAT  720
CCGCCTCTTC TCCGGCACCT TGACAAGTAC TATGCAGGAT TGCCTCCGGA ACTGAAGCAN  780
NCTCGCGTGA ATCTNNNNGC ACATTCNNGC TACAGACCGC AAGCCGNNNA NNNNNNATAA  840
NNNCNCTTNN NNN                                                     853
```

Figure A.20 IgSP-VR1814_gL-T2A-tpa-gH_pcDNAZeo

```
GNGNNNNNNN NNNTTANCTT GGTACCGCCG CCACCATGGG ATGGTCATGT ATCATCCTTT   60
TTCTAGTAGC AACTGCAACC GGTGTACACT CCGTGGCCGT GTCAGTGGCA CCTACTGCCG  120
CCGAGAAGGT CCCAGCCGAA TGCCCCGAAC TGACCCGGAG ATGCCTGCTC GGGGAGGTGT  180
TCCAGGGCGA CAAATACGAA TCCTGGCTGC GGCCCTTGGT GAACGTCACC GGACGCAATG  240
GCCCGCTGAG CCAACTGATC AGATACCGGC CAGTGACCCC TGAAGCCGCC AACTCGGTGC  300
TGCTCGACGA CGCCTTTCTC GATACCCTGG CCCTGCTGTA CTACTACCCT GACCAGCTGC  360
GGGCACTCCT GACCCTGCTG AGCTCCGACA CTGCGCCGAG ATGGATGACT GTCATGCGCG  420
GCTACTCAGA GTGCGGAGAT GGATCGCCCG CCGTGTATAC ATGCGTGGAC GATCTCTGTC  480
GGGGCTACGA CTTGACNTGG CTGTCGTACN GTCGCAGCAT TTTCNNCTAG NACGTGCTGG  540
CGTTCGAGCT GTTGTCGCAG TCTGTGCTCA CCATGGTGNN NNNNATTAAN NNATGAANNN  600
```

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ANNNANNACT ATNNATGGCT TTTTNTGNTC ACANNNNNNN NGNATAT              647
```

Figure A.21 VR1814_gO

```
GGTACCGCCG CCACCATGGG TCGCAAGGAG GACATGCGCT CCATCAGCAA GCTGTTCTTC   60
ATCATTTCAC TGACCGTCCT GCTGTTTTCC ATTATCAACT GCAAAGTCGT GCGCCCACCT  120
GGAAGATACT GGCTGGGAAC TGTGCTGTCC ACTATCGGAA AGCAGAAGCT CGACAAGTTC  180
AAGCTGGAGA TTCTGAAGCA GCTGGAGAGG GAACCCTACA CCAAGTACTT CAACATGACC  240
CGCCAGCACG TGAAGAATCT GACTATGAAC ATGACCCAGT TCCCCCAATA CTACATTCTT  300
GCGGGTCCCA TCAGAAATGA CTCCATCACC TACCTGTGGT TTGACTTCTA CTCGACCCAA  360
CTCCGGAAGC CCGCTAAATA CGTGTACAGC CAGTATAACC ACACAGCCAA GACTATCACC  420
TTCCGGCCTC CTTCCTGCGG AACCGTGCCG TCAATGACCT GTCTGAGCGA AATGCTGAAC  480
GTGTCGAAGC GGAACGATAC GGGAGAGCAG GGCTGCGGCA ACTTCACCAC TTTCAACCCG  540
ATGTTTTTCA ACGTCCCGCG GTGGAACACC AAGCTCTACG TGGGGCCTAC TAAGGTCAAC  600
GTGGACAGCC AGACTATCTA CTTCCTGGGC CTTACCGCAC TGCTGCTGAG ATACGCCCAG  660
CGCAACTGCA CCCACTCCTT CTACTTGGTG AACGCGATGT CCCGGAATCT CTTCCGGGTG  720
CCGAAGTACA TTAACGGCAC TAAGCTCAAG AACACTATGC GCAAGCTCAA AAGGAAGCAG  780
GCCCCCGTGA AGGAACAGCT CGAAAAGAAA ACCAAGAAGT CGCAGTCCAC CACCACCCCG  840
TACTTCTCCT ACACCACCTC AACTGCCCTG AACGTGACCA CCAACGCGAC GTACCGCGTG  900
ACCACTAGCG CCAAGCGGAT TCCGACCAGC ACCATCGCCT ATCGCCCTGA CTCTTCCTTC  960
ATGAAATCCA TCATGGCTAC CCAGTTGAGA GATCTGGCCA CCTGGGTGTA CACTACGCTG 1020
CGGTATAGGA ACGAACCATT CTGCAAGCCC GATAGAAACC GGACAGCCGT GTCCGAGTTC 1080
ATGAAGAACA CCCACGTGCT GATCCGAAAC GAAACCCCGT ACACCATCTA CGGGACCCTT 1140
GACATGAGCT CCCTGTACTA CAACGAAACT ATGTCCGTGG AGAATGAGAC TGCATCAGAC 1200
AACAACGAGA CTACTCCTAC CTCGCCAAGC ACCCGGTTTC AAAAGACCTT CATCGACCCC 1260
CTCTGGGATT ACCTGGATTC CCTGCTGTTC CTGGACAAGA TTCGCAACTT CTCCCTCCAA 1320
CTGCCTGCCT ACGGAAACCT CACTCCGCCC GAACATCGGA GGGCGGTCAA CTTGTCGACC 1380
CTTAATTCGT TGTGGTGGTG GCTCCAGTAA TGACTCGAGT CT                  1422
```

Figure A.22 VR1814_gO_pcDNAZeo

```
NNNNNNNNNN NCTTAAGCTT GGTACCGCCG CCACCATGGG TCGCAAGGAG GACATGCGCT   60
CCATCAGCAA GCTGTTCTTC ATCATTTCAC TGACCGTCCT GCTGTTTTCC ATTATCAACT  120
GCAAAGTCGT GCGCCCACCT GGAAGATACT GGCTGGGAAC TGTGCTGTCC ACTATCGGAA  180
AGCAGAAGCT CGACAAGTTC AAGCTGGAGA TTCTGAAGCA GCTGGAGAGG GAACCCTACA  240
CCAAGTACTT CAACATGACC CGCCAGCACG TGAAGAATCT GACTATGAAC ATGACCCAGT  300
TCCCCCAATA CTACATTCTT GCGGGTCCCA TCAGAAATGA CTCCATCACC TACCTGTGGT  360
TTGACTTCTA CTCGACCCAA CTCCGGAAGC CCGCTAAATA CGTGTACAGC CAGTATAACC  420
ACACAGCCAA GACTATCACC TTCCGGCCTC CTTCCTGCGG AACCGTGCCG TCAATGACCT  480
GTCTGAGCGA AATGCTGAAC GTGTCGAAGC GGAACGATAC GGGAGAGCAG GGCTGCGGCA  540
ACTTCACCAC TTTCAACCCG ATGTTTTTCA ACGTCCCGCG GTGGAACACC AAGCTCTACG  600
TGGGGCCTAC TAAGGTCAAC GTGGACAGCC AGACTATCTA CTTCCTGGGC CTTACCGCAC  660
TGCTGCTGAG ATACGCCCAG CGCAACTGCA CCCACTCCTT CTACTTGGTG AACGCGATGT  720
CCCGGAATCT CTTCCGGGTG CCGAAGTACA TTAACGGCAC TAAGCTCAAG AACACTATGC  780
GCAAGCTCAA AAGGAAGCAG GCCCCCGTGA AGGAACAGCT CGAAAAGAAA ACCAAGAAGT  840
CGCAGTCCAC CACCACCCCG TACTTCTCCT ACACCACCTC AACTGCCCTG AACGTGACCA  900
CCAACGCGAC GTACCGCGTG ACCACTAGCG CCAAGCGGAT TCCGACCAGC ACCATCGCCT  960
ATCGCCCTGA CTCTTCCTTC ATGAAATCCA TCATGGCTAC CCAGTTGANA GATCTGGCCA 1020
CCTGGGTGTA CACTACGCTG CGGTATAGGA ACGAACCATT CTGCAAGCCC GATAGAAACC 1080
GGANNGCCNN GTCCGANTTC NTGAAGNANN NCCCACGTGC TGATCCGAAA CGAAACCCCG 1140
TANNCCATNN ACGGGANCCT TGNNNTGNNN NCCNTGTACT NNANGAANNN NNNNNTCCGG 1200
GNAAANNNAN NNNNNNCAAN NNNANNGAAN NNNNNCNNCN NNNCAANNNC CGGTTNNNAN 1260
ACTNNNCNNA CCCNNNGGNA NTACCGGNNT CCNGGNNTTC NNGGAAAANT CCNAANTTC  1319
```

Figure A.23 IL2sp-VR1814_gO_pcDNAZeo

```
NNNNNNNNNN NNNNTTNNGC TTGGTACCGC CGCCACCATG TACAGGATGC AACTCCTGTC   60
TTGCATTGCA CTAAGTCTTG CACTTGTCAC GAATTCGAAA GTCGTGCGCC CACCTGGAAG  120
ATACTGGCTG GGAACTGTGC TGTCCACTAT CGGAAAGCAG AAGCTCGACA AGTTCAAGCT  180
```

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GGAGATTCTG AAGCAGCTGG AGAGGGAACC CTACACCAAG TACTTCAACA TGACCCGCCA  240
GCACGTGAAG AATCTGACTA TGAACATGAC CCAGTTCCCC CAATACTACA TTCTTGCGGG  300
TCCCATCAGA AATGACTCCA TCACCTACCT GTGGTTTGAC TTCTACTCGA CCCAACTCCG  360
GAAGCCCGCT AAATACGTGT ACAGCCAGTA TAACCACACA GCCAAGACTA TCACCTTCCG  420
GCCTCCTTCC TGCGGAACCG TGCCGTCAAT GACCTGTCTG AGCGAAATGC TGAACGTGTC  480
GAAGCGGAAC GATACGGGAG AGCAGGGCTG CGGCAACTTC ACCACTTTCA ACCCGATGTT  540
TTTCAACGTC CCGCGGTGGA ACACCAAGCT CTACGTGGGG CCTACTAAGG TCAACGTGGA  600
CAGCCAGACT ATCTACTTCC TGGGCCTTAC CGCACTGCTG CTGAGATACG CCCAGCGCAA  660
CTGCACCCAC TCCTTCTACT TGGTGAACGC GATGTCCCGG AATCTCTTCC GGGTGCCGAA  720
GTACATTAAC GGCACTAAGC TCAAGAACAC TATGCGCAAG CTCAAAAGGA AGCAGGCCCC  780
CGTGAAGGAA CAGCTCGAAA AGAAAACCAA GAAGTCGCAG TCCACCACCA CCCCGTACTT  840
CTCCTACACC ACCTCAACTG CCCTGAACGT GACCACCAAC GCGACGTACC GCGTGACCAC  900
TAGCGCCAAG CGGATTCCGA CAGCACATCG CCTATCGCCT GACTCTCCTT CNGAAATCAT  960
CAGGCTACCC NTTGAGANNN TGGCCCCNGG GGGACACTAN CTGCGGTAAG GAACGAACAT 1020
TCTGCAGCCC AAAANAAACG GAAAGCCGGT CCAATTCAGA AAANCCCCNG GCTGATCGAA 1080
ACAANCCCGG AACCATC                                                1097
```

Figure A.24 VR1814_UL128

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GGTACCGCCG CCACCATGTC GCCCAAGAAC CTTACGCCCT TTCTCACTGC CCTGTGGCTT   60
CTGCTGGGCC ATTCCCGCGT GCCAAGAGTG CGCGCGGAAG AGTGCTGCGA GTTCATCAAC  120
GTGAACCACC CGCCTGAACG GTGTTACGAC TTCAAGATGT GCAACCGGTT CACTGTGGCC  180
CTGAGATGTC CTGATGGCGA AGTCTGCTAC TCCCCGGAGA AAACCGCTGA AATCCGGGGG  240
ATTGTGACCA CCATGACTCA CTCCCTCACC CGCCAAGTCG TGCACAACAA GCTCACCAGC  300
TGCAATTACA ACCCCCTGTA CCTGGAGGCC GATGGAAGAA TCCGCTGCGG AAAGGTCAAC  360
GACAAGGCCC AGTACCTGTT GGGTGCAGCC GGATCAGTGC CGTATCGGTG GATCAACCTC  420
GAATACGACA AGATTACCCG GATCGTGGGC CTGGACCAGT ACCTGGAGAG CGTGAAGAAG  480
CACAAGAGGC TGGACGTGTG CAGGGCGAAG ATGGGGTACA TGCTGCAGTA ATGACTCGAG  540
TCT                                                                543
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Figure A.25 VR1814_UL128_pcDNAZeo

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NNNNNNNNNN NNNTTAAGCT TGGTACCGCC GCCACCATGT CGCCCAAGAA CCTTACGCCC   60
TTTCTCACTG CCCTGTGGCT TCTGCTGGGC CATTCCCGCG TGCCAAGAGT GCGCGCGGAA  120
GAGTGCTGCG AGTTCATCAA CGTGAACCAC CCGCCTGAAC GGTGTTACGA CTTCAAGATG  180
TGCAACCGGT TCACTGTGGC CCTGAGATGT CCTGATGGCG AAGTCTGCTA CTCCCCGGAG  240
AAAACCGCTG AAATCCGGGG GATTGTGACC ACCATGACTC ACTCCCTCAC CCGCCAAGTC  300
GTGCACAACA AGCTCACCAG CTGCAATTAC AACCCCCTGT ACCTGGAGGC CGATGGAAGA  360
ATCCGCTGCG GAAAGGTCAA CGACAAGGCC CAGTACCTGT TGGGTGCAGC CGGATCAGTG  420
CCGTATCGGT GGATCAACCT CGAATACGAC AAGATTACCC GGATCGTGGG CCTGGACCAG  480
TACCTGGAGA GCGTGAAGAA GCACAAGAGG CTGGACGTGT GCAGGGCGAA GATGGGGTAC  540
ATGCTGCAGT AATGACTCGA GTCTAGAGGG CCCGTTTAAA CCCGCTGATC AGCCTCGACT  600
GTGCCTTCTA GTTGCCAGCC ATCTGTTGTT TGCCCCTCCC CCGTGCCTTC CTTGACCCTG  660
GAAGGTGCCA CTCCCACTGT CCTTTCCTAA TAAAATGAGG AAATTGCATC GCATTGTCTG  720
AGTAGGTGTC ATTCTATTCT GGGGGGGTGG GGTGGGGCAG GACAGCAAGG GGGAGGANNG  780
NGAAGANNNN ANNNGGNNNG NNGGGGANGN GGNGGGGNNN NANGGNNNNN GAGGNGGAAA  840
GAANNAGNNG GGGNNNNNNA                                              860
```

Figure A.26 VR1814_UL128-130-131A_pcDNAZeo

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NNNNNNNNNN NNNNNNNNNN NNNNTTANCT TGGTACCGCC GCCNCCATGT CGCCCAAGAA   60
CCTTACGCCC TTTCTCACTG CNNTGTGGCT TCTGCTGGGC CATTCCCGCG TGCCAAGAGT  120
GCGCGCGGAA GAGTGCTGCG AGTTCATCAA CGTGAACCAC CCGCCTGAAC GGTGTTACGA  180
CTTCAAGATG TGCAACCGGT TCACTGTGGC CCTGAGATGT CCTGATGGCG AAGTCTGCTA  240
CTCCCCGGAG AAAACCGCTG AAATCCGGGG GATTGTGACC ACCATGACTC ACTCCCTCAC  300
CCGCCAAGTC GTGCACAACA AGCTCACCAG CTGCAATTAC AACCCCCTGT ACCTGGAGGC  360
CGATGGAAGA ATCCGCTGCG GAAAGGTCAA CGACAAGGCC CAGTACCTGT TGGGTGCAGC  420
CGGATCAGTG CCGTATCGGT GGATCAACCT CGAATACGAC AAGATTACCC GGATCGTGGG  480
```

CCTGGACCAG TACCTGGAGA GCGTGAAGAA GCACAAGAGG CTGGACGTGT GCAGGGCGAA 540

GATGGGGTAC ATGCTGCAGG AGGGCAGAGG AAGTCTGCTA ACATGCGGTG ACGTCGAGGA 600

GAATCCTGGC CCAATGCTGA GACTTCTGCT GCGGCATCAC TTCCACTGCC TCCTGCTTTG 660

CGCTGTGTGG GCCACCCCTT GTCTGGCCTC CCCCTGTTTC ACCCTGACTG CGAANNANAA 720

TCCAAGCCNN CC 732

Figure A.27 VR1814_UL128-162S-130-131A_pcDNAZeo

CCACTNGNAC GTTTACTTAA GCTTGGTACC GCCGCCACCA TGTCGCCCAA GAACCTTACG 60

CCCTTTCTCA CTGCCCTGTG GCTTCTGCTG GGCCATTCCC GCGTGCCAAG AGTGCGCGCG 120

GAAGAGTGCT GCGAGTTCAT CAACGTGAAC CACCCGCCTG AACGGTGTTA CGACTTCAAG 180

ATGTGCAACC GGTTCACTGT GGCCCTGAGA TGTCCTGATG GCGAAGTCTG CTACTCCCCG 240

GAGAAAACCG CTGAAATCCG GGGGATTGTG ACCACCATGA CTCACTCCCT CACCCGCCAA 300

GTCGTGCACA ACAAGCTCAC CAGCTGCAAT TACAACCCCC TGTACCTGGA GGCCGATGGA 360

AGAATCCGCT GCGGAAAGGT CAACGACAAG GCCCAGTACC TGTTGGGTGC AGCCGGATCA 420

GTGCCGTATC GGTGGATCAA CCTCGAATAC GACAAGATTA CCCGGATCGT GGGCCTGGAC 480

CAGTACCTGG AGAGCGTGAA GAAGCACAAG AGGCTGGACG TGTCCAGGGC GAAGATGGGG 540

TACATGCTGC AGGAGGGCAG AGGAAGTCTG CTAACATGCG GTGACGTCGA GGAGAATCCT 600

GGCCCAATGC TGAGACTTCT GCTGCGGCAT CACTTCCACT GCCTCCTGCT TTGCGCTGTG 660

TGGGCCACCC CTTGACTGGC CTCCCCCTGG TTCACCCTGA CTGCGAATCA GAATCCAAGT 720

CCGTCTTGNN CT 732

Figure A.28 VR1814_UL130

GGTACCGCCG CCACCATGCT GAGACTTCTG CTGCGGCATC ACTTCCACTG CCTCCTGCTT 60

TGCGCTGTGT GGGCCACCCC TTGTCTGGCC TCCCCCTGGT TCACCCTGAC TGCGAATCAG 120

AATCCAAGCC CGCCTTGGTC TAAGCTGACC TACCCGAAGC CTCACGATGC CGCCACCTTT 180

TACTGCCCCT TCCTGTACCC GTCACCGCCA AGGTCGCCCA GCCAATTCAG CGGATTTCAG 240

AGGGTGTCCA CTGGCCCTGA GTGTCGCAAC GAAACACTGT ACCTCCTCTA TAACCGGGAA 300

GGGCAGACCC TGGTGGAACG GTCCTCCACC TGGGTCAAGA AAGTCATTTG GTACTTGAGC 360

GGAAGAAACC AGACCATCCT CCAACGCATG CCCAGAACTG CATCGAAGCC GTCCGACGGC 420

AACGTGCAGA TTTCCGTGGA GGACGCCAAG ATCTTCGGTG CCCATATGGT GCCGAAGCAG 480

ACCAAGCTCC TGCGGTTCGT GGTCAACGAC GGAACGCGCT ACCAGATGTG CGTGATGAAG 540

TTGGAGTCAT GGGCGCACGT GTTCCGCGAT TACTCCGTGT CGTTCCAAGT CCGGCTGACT 600

TTCACCGAGG CCAACAACCA GACTTACACC TTCTGCACCC ACCCCAACCT GATCGTGTAA 660

TGACTCGAGT CT 672

Figure A.29 VR1814_UL130_pcDNAZeo

NNNNNNNNNN NNNNTNNNTT AGCTTGGTAC CGCCGCCACC ATGCTGAGAC TTCTGCTGCG 60

GCATCACTTC CACTGCCTCC TGCTTTGCGC TGTGTGGGCC ACCCCTTGTC TGGCCTCCCC 120

CTGGTTCACC CTGACTGCGA ATCAGAATCC AAGCCCGCCT TGGTCTAAGC TGACCTACCC 180

GAAGCCTCAC GATGCCGCCA CCTTTTACTG CCCCTTCCTG TACCCGTCAC CGCCAAGGTC 240

GCCCAGCCAA TTCAGCGGAT TTCAGAGGGT GTCCACTGGC CCTGAGTGTC GCAACGAAAC 300

ACTGTACCTC CTCTATAACC GGGAAGGGCA GACCCTGGTG GAACGGTCCT CCACCTGGGT 360

CAAGAAAGTC ATTTGGTACT TGAGCGGAAG AAACCAGACC ATCCTCCAAC GCATGCCCAG 420

AACTGCATCG AAGCCGTCCG ACGGCAACGT GCAGATTTCC GTGGAGGACG CCAAGATCTT 480

CGGTGCCCAT ATGGTGCCGA AGCAGACCAA GCTCCTGCGG TTCGTGGTCA ACGACGGAAC 540

GCGCTACCAG ATGTGCGTGA TGAAGTTGGA GTCATGGGCG CACGTGTTCC GCGATTACTC 600

CGTGTCGTTC CAAGTCCGGC TGACTTTCAC CGAGGCCAAC AACCAGACTT ACACCTTCTG 660

CACCCACCCC AACCTGATCG TGTAATGACT CGAGTCTAGA GGGCCCGTTT AAACCCGCTG 720

ATCAGCCTCG ACTGTGCCTT CTAGTTGCCA GCCATCTGTT GTTTGCCCCT CCCCCGTGCC 780

TTCCTTGACC CTGGNAAGGT GCCACTCCCA CTGTCCNTTT CCTAATAAAA TGAAGGAAAT 840

TGCATCGCCA TGGNNGGAGT ANGNGT 866

Figure A.30 VR1814_UL131A

GGTACCGCCG CCACCATGAG ACTTTGCCGC GTGTGGCTCA GCGTGTGCTT GTGTGCCGTC 60

GTCCTGGGAC AGTGCCAGAG AGAAACCGCC GAGAAGAACG ATTACTACCG GGTGCCGCAC 120

```
TACTGGGACG CCTGCTCAAG GGCGCTGCCC GATCAGACCC GGTACAAATA CGTGGAGCAG  180
CTCGTCGACC TCACCCTGAA TTACCACTAT GACGCGTCCC ACGGACTGGA TAACTTCGAC  240
GTGCTGAAGA GGATCAACGT GACCGAAGTG TCCCTGCTGA TTTCGGACTT CCGCCGCCAA  300
AACAGACGGG GCGGTACTAA CAAGCGGACT ACCTTCAACG CTGCTGGGAG CCTCGCACCT  360
CATGCCCGGT CCCTGGAATT TTCCGTGCGC CTGTTCGCCA ACTAATGACT CGAGTCT     417
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Figure A.31 VR1814_UL131_pcDNAZeo

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NNNNNNNNGNG NNNNNTTAAG CTTGGTACCG CCGCCACCAT GAGACTTTGC CGCGTGTGGC   60
TCAGCGTGTG CTTGTGTGCC GTCGTCCTGG GACAGTGCCA GAGAGAAACC GCCGAGAAGA  120
ACGATTACTA CCGGGTGCCG CACTACTGGG ACGCCTGCTC AAGGGCGCTG CCCGATCAGA  180
CCCGGTACAA ATACGTGGAG CAGCTCGTCG ACCTCACCCT GAATTACCAC TATGACGCGT  240
CCCACGGACT GGATAACTTC GACGTGCTGA AGAGGATCAA CGTGACCGAA GTGTCCCTGC  300
TGATTTCGGA CTTCCGCCGC CAAAACAGAC GGGGCGGTAC TAACAAGCGG ACTACCTTCA  360
ACGCTGCTGG GAGCCTCGCA CCTCATGCCC GGTCCCTGGA ATTTTCCGTG CGCCTGTTCG  420
CCAACTAATG ACTCGAGTCT AGAGGGCCCG TTTAAACCCG CTGATCAGCC TCGACTGTGC  480
CTTCTAGTTG CCAGCCATCT GTTGTTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG  540
GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA  600
GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG GATTGGGAAG  660
ACAATAGCAG GCATGCTGGG GATGCGGTGG GCTCTATGGC TTCTGAGGCG GAAAGAACCA  720
GCTGGGGCTC TAGGGGGTAT CCCCACGCGC CCTGTAGCGG CGCATTAAGC GCGGCGGGTG  780
TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC GCTCCTTTCG  840
CTTTCTTCCC TTCCTTTCTC GCCACGTTCG CCGGCTTTCC CCGTCAAGCT CTAAATCGGG  900
GGCTCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT CGACCCCAAA AACTTGATTA  960
GGGTGATGGT TCACGTAGTG GGCCATCGCC CTGATAGACG GTTTTTCGCC CTTTGACGTT 1020
GGAGTCCACG TTCTTTAANA GTGGANNCTT GTTCCNNACT GGAACAANNN TCAACCCTNN 1080
NNCGGNNNNN TNNTTNGAAN T                                           1101
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