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Characterisation of porcine cytomegalovirus and development as a vaccine vector for classical swine fever virus

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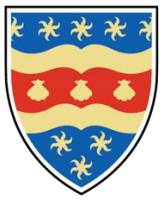

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Characterisation of porcine cytomegalovirus and development as a vaccine vector for classical swine fever virus

by

Thekla Mauch

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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ABSTRACT

Classical swine fever (CSF) is a highly infectious viral disease found in domestic pigs and wild boar populations. Outbreaks have a large economic cost for the swine industry and cause significant morbidity and mortality in pigs. Only one compatible vaccine that allows the differentiation between infected and vaccinated animals (DIVA), Suvaxyn® CSF Marker vaccine, is commercially available. However, due to e.g. trading issues caused by vaccination of animals this vaccine is not used prophylactically and is only used in emergency situations to prevent spread of CSFV to wider areas. An urgent need exists for development of a safe, effective, DIVA compatible vaccine. This study set out to address this need by taking the initial steps towards developing porcine cytomegalovirus (PCMV), as a vaccine platform. CMV-based vaccines have been shown to induce strong, durable immune responses against heterologous antigens, and also have the potential to be self-disseminating, which would facilitate vaccination of inaccessible animal populations such as wild boar. The aim of the present study was to further characterise the *in vitro* growth characteristics of a recent PCMV isolate. Our results show PCMV to have a slow growth, and low titre phenotype.

As a further step towards cloning of PCMV as an infectious BAC, recombinant BAC shuttle vectors were constructed and then characterised. Initial experiments using these shuttle vectors combined with homologous recombination and CRISPR/Cas9 technology to insert the BAC cassette within the PCMV genome are also described. In summary, the cloning of the shuttle vectors was successful but the results around PCMV replication kinetics provide preliminary evidence that there are difficulties to overcome before PCMV can be developed as a vaccine vector platform.

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Chapter 1

Introduction

1.1 Classical swine fever

Classical swine fever (CSF), also known as hog cholera, is a highly infectious viral disease found in domestic and wild pig populations (OIE. Classical Swine Fever Vol. 2015). It has a large impact on animal health and the swine industry worldwide and is classified as a disease that is reportable to the World Organisation for Animal Health (OIE). During an outbreak in the Netherlands in 1997, more than 10 million pigs were culled at a cost of more than 1 billion EUR (Food and Agriculture Organization of the United Nations, 2011).

CSF was identified about 200 years ago and work to combat its spread began in the nineteenth century (Edwards *et al*, 2000; Ji *et al*, 2015) but CSF is still widespread. It is endemic in Central and South America, parts of Eastern Europe and Asia (Blome *et al*, 2017b), but has been eradicated in Australia, Canada, New Zealand, and the USA. Most western and central European countries are considered CSF free (Figure 1), despite occasional outbreaks (EPIZONE, 2014; Risatti, G.R., Borca, M.V., 2016). Due to the global nature of pork production (with CSF being endemic in China, a leading country in pork production) and increasing international trade (Food and Agriculture Organization of the United Nations, 2011; McGlone, 2013), CSF is considered to be a biosafety and food security threat. Additionally, outbreaks in small production farms in low-income countries and rural areas threaten local food security and diminish local economies, with communities having less money available to spend in local economies, especially

in areas such as education and healthcare (Food and Agriculture Organization of the United Nations, 2011; 2014).

In CSF-free countries, a non-vaccination, 'stamping-out' policy has been adopted, whereby prophylactic vaccination is prohibited, other than in an emergency. Vaccination is only used when there is a threat of wider spread. In countries where CSF is endemic live attenuated CSFV vaccines are commonly used (Moennig, 2000; Chander et al, 2014; Madera et al, 2016). Different kinds of vaccines have been developed as discussed in more detail in section '1.3 Vaccination'.

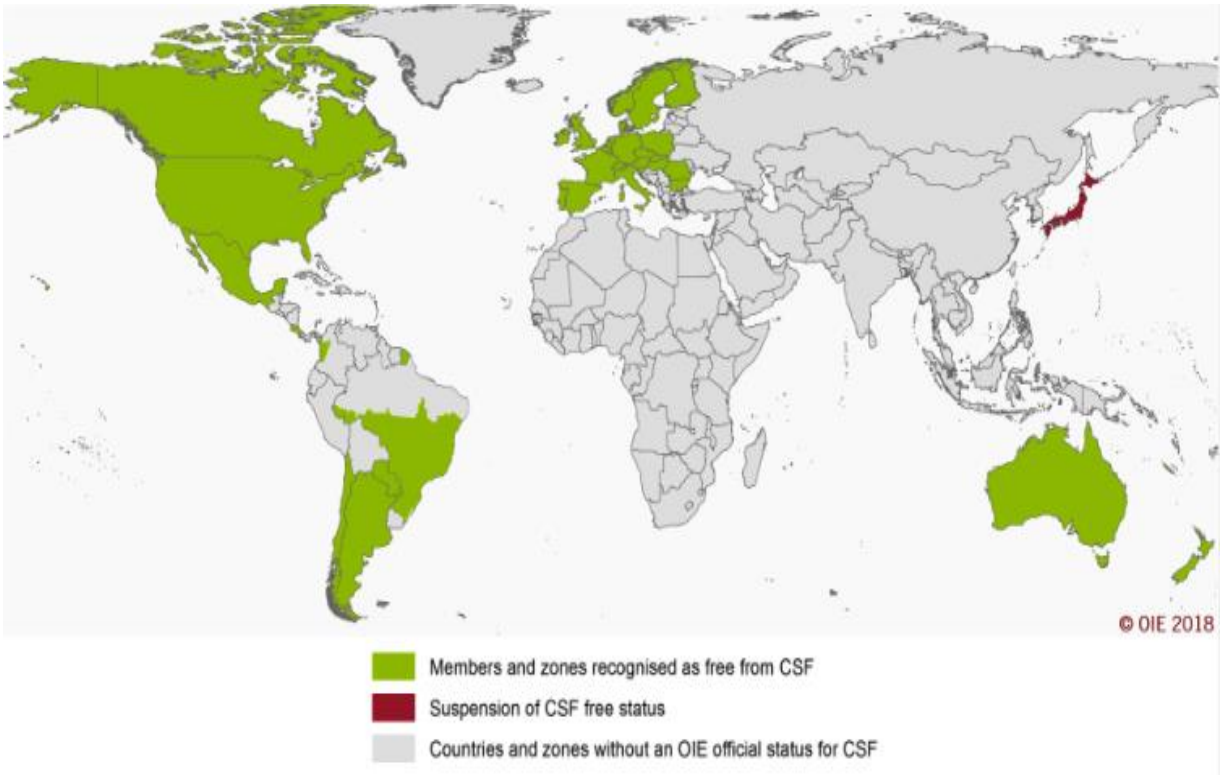


Figure 1. OIE Members' official CSF status map (Adapted from OIE, 2018)

1.2 Classical swine fever virus

1.2.1 Virus properties

CSF is caused by classical swine fever virus (CSFV), a small, 40 – 60 nm diameter, enveloped RNA virus. It has a genome of approximately 12.3 kilobases with one open reading frame (ORF) encoding one polyprotein of approximately 4000 amino acids. CSFV is classified as a positive sense single-stranded RNA virus. It belongs to the genus *Pestivirus* within the *Flaviviridae* family. It is closely related to other pestiviruses, such as bovine viral diarrhea virus species 1 and 2 (BVDV-1, BVDV-2) and border disease virus (BDV) of sheep but is only distantly related to more recently discovered pestiviruses, such as atypical porcine pestivirus (APPV), Bungowannah virus. CSFV has only 40% nucleotide identity with APPV (Yuan *et al*, 2017), compared to about 70% amino acid sequence identity with BDV (Vilcek & Belák, 1996; Strauss & Strauss, 2008) indicating wide genetic diversity among pestiviruses.

1.2.2 Protein structure

The infectious virus particle comprises four structural proteins and eight non-structural proteins (Figure 2). The structural proteins include the nucleocapsid core (C)-protein and three envelope glycoproteins called E(rns), E1 and E2 (Blome *et al*, 2017b). The core protein functions as an RNA chaperone (Riedel *et al*, 2012), and glycoproteins E(rns) and E2 play an essential role in attachment to the host cell surface. Glycoproteins E1 and E2 form a heterodimer which plays an essential role in virus entry into the cell. E2 also plays an important role as an immunological target, inducing neutralizing antibody production and cytotoxic T-lymphocyte responses (Franzoni *et al*, 2013; Wang *et al*, 2015). The non-structural proteins (N^{pro}, p7, NS2-3, NS2, NS3, NS4A, NS4B, NS5A, and NS5B)

have distinct functions in viral replication and virulence but, at this time have not been fully characterised (Blome *et al*, 2017b).

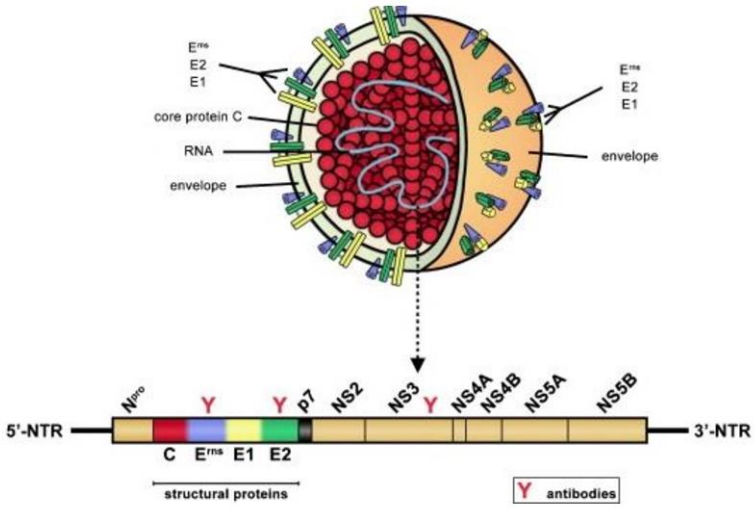


Figure 2. Structure of CSFV. The structure shows a virus particle consisting of an envelope with its four structural proteins and the core containing the genetic information (Adapted from Beer, 2007).

1.2.3 Transmission

Viral transmission can occur in two ways, horizontally and vertically. Horizontal transmission is the most effective way of spreading the virus (Ribbens *et al*, 2004). This happens primarily via oral and oronasal contact with infected pigs, and direct contact with secretions and excretions, such as saliva, urine, feces and semen. Indirect transmission can also occur, through contact with contaminated swill feed or equipment, such as transport vehicles/storage facilities. The virus can also be transmitted by contact with human handlers (OIE. Classical Swine Fever Vol. 2015; Floegel *et al*, 2000). Transmission to neighbouring pig farms via airborne transmission has also been reported (Weesendorp *et al*, 2008). Vertically, the virus can be transmitted transplacentally from pregnant sows to their offspring (de Smit *et al*, 2000).

1.2.4 Manifestations and clinical signs

Following infection, symptoms appear in various forms depending on the virus strain, age of pigs, inoculation dose and immunological host response. There are three different classified manifestations - acute, subacute / chronic and prenatal – which follow an incubation period of 4 to 10 days post-infection (Brown & Bevins, 2018; Blome *et al*, 2017c). Generally, the acute form is observed in piglets whereas the subacute/chronic form is observed in older animals (OIE. Classical Swine Fever Vol. 2015). However, clinical signs can vary from nearly inapparent to haemorrhagic fever-like illness.

Within the first three weeks of an acute manifestation, clinical signs such as high fever, anorexia, depression, conjunctivitis, coughing, wasting and constipation followed by diarrhoea can be detected. At around 14 days post-infection ataxia, haemorrhagic skin lesions, cyanosis especially at the extremities, paresis and convulsion are observed, leading to death within 5 to 30 days after onset of illness. The mortality rate can be as high as 100% (Blome *et al*, 2017c; OIE. Classical Swine Fever Vol. 2015; Petrov *et al*, 2014). The chronic manifestation of CSF elicits similar clinical symptoms, but due to lower virulence and/or higher immunity in the pig herd, the symptoms can appear less specific over time. However, recovery often seems to be feigned, and in these cases, death typically occurs within three months.

Prenatal infection, depending on the virulence of the virus strain and stage of gestation, can result either in stillbirth, mummification, embryonical death or abortion, or if born, in persistently infected piglets without initial clinical symptoms. These are often runts, which may or may not have a congenital tremor. Failure to thrive typically leads to death after a period of weeks to months. Although CSFV

infections are often lethal in younger animals, chronically infected older animals usually survive, with life-long immunity against CSF. However, they continue to shed the virus over their lifetime unless the virus is neutralized through specific antibodies (OIE. Classical Swine Fever Vol. 2015; Moennig *et al*, 2003).

1.2.5 Virus inactivation

Virus stability in offal was first reported in 1917 by Birch (Edwards, 2000). CSFV can survive more than 4 years in frozen pork, and up to 85 days in chilled pork. The virus can be inactivated rapidly by ultra-violet light, a pH of ≤ 3 or ≥ 10, pasteurisation or heat (Edwards, 2000).

1.3 Vaccination

Currently, there are several types of CSFV vaccines available. In CSF free countries a non-vaccination, 'stamping-out' policy (whereby prophylactic vaccination is prohibited other than in an emergency) is still in place although there is a DIVA compatible vaccine available. The reason for this policy may be two-fold. First, while vaccinated animals do not show symptoms of disease, they may not always be 100% protected against infection, leading to 'silent' circulation of the virus through herds. Second, commercial implications due to restrictions regarding trade imposed on vaccinated animals makes vaccination not attractive (European Commission, 2018).

1.3.1 Modified live virus (MLV) vaccine

In countries where CSF is endemic, conventional modified live virus (MLV) vaccines are commonly used to control the disease. The development of these vaccines began in the early 20th century (Blome *et al*, 2017a), but it took until the

middle of the 20th century (Kaden & Riebe, 2001) for promising candidates to be identified. Many different strains were used for vaccine development, including Lapinized Philippines Coronel (LPC) strain, Japanese guinea-pig exaltation-negative (GPE-) strain, Thiverval strain, Mexican PAV strain, low virulence strain of Miyagi (LOM) or China-strain (C-strain).

Individual MLVs differ in their efficacy. After vaccination with the LOM strain, CSF outbreaks were reported in South Korea in 2014 and 2016 (Je *et al*, 2018), demonstrating lack of protection against disease. Vaccines based on the China-strain (C-strain) of lapinized CSF virus are considered as safe and efficacious and induce both neutralizing antibody and T-cell responses. A single dose of this vaccine induces durable immunity for at least 6-18 months, and in some cases lifelong immunity, within a few days of vaccination (Moennig, 2000; van Oirschot, 2003; Huang *et al*, 2014). Although effective, MLVs are not suitable for CSF free countries because of DIVA issues as they don't allow differentiation between infected and vaccinated animals using standard immunological tests. The solution to this problem is to develop a vaccine which has the advantages of an MLV vaccine but also fulfils DIVA requirements.

1.3.2 Marker vaccine

The next generation of vaccines, known as marker vaccines, were developed in the early 1990s. A marker vaccine, also called DIVA vaccine, allows the differentiation between vaccinated and naturally infected animals. There were several important considerations for these vaccines. They needed to be safe, and also needed to be effective in preventing both clinical symptoms as well as transmission of CSFV. Additionally, the vaccine required its own matched companion immunological test for induction of CSFV-specific immune responses.

Marker vaccines were generated by genetically modifying CSFV, either by deleting antigenic proteins (negative marker), or substitution of antigenic protein(s) or epitope(s) on its own or with other markers (positive marker).

Most marker vaccines are negative marker vaccines. The matching companion immunological tests to detect antibodies against these deleted viral protein(s) and epitope(s) give negative results in vaccinated animals as the epitopes are no longer present. Deletion of antigens detected in DIVA assays from these vaccines made them DIVA compatible. Several marker vaccines were generated, including recombinant deletion vaccines, DNA vaccines, subunit vaccines, peptide vaccines and recombinant chimeric vaccines (Dong & Chen, 2007; Beer *et al*, 2007). However, there were several different problems identified with some of these different types of vaccines. DNA vaccines and subunit vaccines are associated with reduced immunogenicity with multiple application and high doses necessary (Beer *et al*, 2007; Li *et al*, 2012). Peptide vaccines are considered to not achieve complete protection (Dong & Chen, 2007) and viral vaccines harbour the risk of recombination with the live attenuated viral vaccines (Beer *et al*, 2007).

Glycoprotein E2 of CSFV emerged as an important target for the development of a subunit vaccine in which usually only a single antigenic protein or epitope was used to induce an immune response. E2 is the major immunogen and has shown neutralizing antibody induction (Dong & Chen, 2007; Huang *et al*, 2014; Wang *et al*, 2015). Baculovirus-expressed E2 subunit vaccines were well studied by several groups and found to be highly immunogenic, leading to commercialisation. However, it was shown that while a single vaccination could prevent clinical symptoms, it wasn't efficient enough to prevent transmission. Administration of an additional dose was unable to prevent horizontal and vertical

transmission. This may be due to antigen variety between different genotypes of CSFV as neutralizing antibody induction was more effective against homologous strains than heterologous strains (Dewulf *et al*, 2000; Huang *et al*, 2014). Even with use of multiple doses, full protection still was not achieved. The E2 subunit vaccines also only induced antibodies, without the induction of T-cell responses (Huang *et al*, 2014). Hence, while E2 subunit vaccines were a good step in CSFV vaccine development, further development using new approaches are required to develop a more effective vaccine.

1.3.2.1 Viral vector, chimeric pestivirus and porcine cytomegalovirus-based vaccines

Although the E2 subunit marker vaccines discussed above fulfilled DIVA requirements, they failed to provide adequate levels of protection. The next generation of vaccines built upon these findings to design more effective vaccines based on the use of viral vector vaccines including chimeric pestivirus vaccines (Beer *et al*, 2007).

1.3.2.1.1 Viral vector vaccines

Vaccinia virus and pseudorabies virus, genetically modified to express CSFV E2 protein were first developed in the 1990s (König *et al*, 1995; Peeters *et al*, 1997). These vaccines were followed by several other viral vector-based strategies such as porcine and human adenoviral vectors (Hammond & Johnson, 2005; Sun *et al*, 2011), swinepox viral vectors (Hahn *et al*, 2001), parapox, fowlpox and canarypox viral vectors (Dong & Chen, 2007). Although these vaccines achieved full protection (Beer *et al*, 2007) further investigation was necessary for example in regard to pre-existing immunity for some of the candidates, such as vaccinia viral vectors or adenoviral vectors (Ura *et al*, 2014).

1.3.2.1.2 Chimeric pestivirus vaccines

To date, the most successful CSFV vaccine strategy is based on a chimeric pestivirus vaccine expressing the E2 protein. A chimeric pestivirus is a recombinant CSFV, in which a region, or the entire E2 gene is replaced by the corresponding region of the E2 gene from a 'sister' pestivirus, such as BVDV or BDV. Chimeric viruses are generated in two different ways. A copy of CSFV serves as the backbone, with the E2 gene of a non-CSFV pestivirus inserted in place of the CSFV E2 gene. Alternatively, a copy of one of the 'sister' pestiviruses is used as the backbone, with the E2 gene from CSFV inserted to replace the native E2 gene. Wehrle and colleagues (Wehrle *et al*, 2007) developed a live-attenuated CSFV strain Riems expressing antigenic epitopes of the E2 gene from border disease virus strain Gifhorn. This resulted in the construction of three different mutants based on the exchange of the domains. The change of all three domains A, B and C resulted in the CSFV variant pRiems-ABC-Gif which, administered intramuscularly, showed full protection against wild-type CSFV. Additionally, pRiems-ABC-Gif vaccinated animals could be distinguished from wild-type CSFV infected animals by the absence of antibodies against CSFV E2 using commercially available CSFV E2-antibody ELISAs. The ability to differentiate between infected and vaccinated animals made this vaccine DIVA compatible. The other two candidates failed to show potential for development as a vaccine. Exchange of domain A only did not protect against the virus and exchange of domain B/C resulted in a vaccine that was not DIVA compatible.

Eblé and colleagues (Eblé *et al*, 2013) developed chimeric pestivirus vaccine candidates using BVDV as the backbone, carrying the E2 gene of classical swine fever virus, and vice versa. The live recombinant vaccine 'CP7_E2alf' therefore consists of a BVDV type 1 backbone ('CP7' strain) combined with the E2 gene of

CSFV strain ‘Alfort/187’ (Blome *et al*, 2017d). Two additional live recombinant vaccines were generated from a CSFV strain combined with the E2 genes of BVDV (‘Flc9’) and BVDV (‘Flc11’) respectively (de Smit *et al*, 2001). Under an EU-funded project to ‘Improve tools and strategies for the prevention and control of classical swine fever’, Grant agreement ID:227003 (European Commission, 2013), CP7_E2alf and Flc11 were chosen as the most promising candidates, based on data related to safety, efficacy, genetic stability, DIVA capability and comparative animal trials.

CP7_E2alf and Flc11 were compared (along with a control group of animals vaccinated with the C-strain “Riems’) using oral and intramuscular vaccination, in three independent domestic pig animal trials. At 14 and 21 days post-vaccination, the animals were challenged with the highly virulent CSFV strain ‘Koslov’ (European Commission, 2013). Animals were fully protected after challenge at 21 days post-vaccination with all three vaccines, up to at least 6 months post-vaccination. However, when challenge occurred 14 days post-vaccination, clinical protection was observed with CP7_E2alf, but only 50% protection was shown with Flc11, and 83% protection was observed with the C-strain (Blome *et al*, 2012; European Commission, 2013). This data led to the decision to license the CP7_E2alf vaccine, and a dossier for intramuscular vaccination was submitted to the European Medicines Agency (EMA).

In 2014 the BVDV type 1 backbone (‘CP7’ strain), expressing CSFV E2 from ‘Alfort/187’ strain, known as CP7_E2alf, became the first licensed live attenuated marker vaccine against CSF. It was named Suvaxyn® CSF Marker (Zoetis) (Blome *et al*, 2017d). However, there were still some remaining issues with CP7_E2alf that needed to be overcome. First, vaccination led to a reduction, but

not complete inhibition, of transplacental infection. Second, although the vaccine was licensed based on intramuscular vaccination performed in domestic pigs, wild boars, which act as a reservoir species, would still need to be vaccinated orally as it would not be feasible to trap and intramuscularly vaccinate wild populations of animals. Field studies from Italy monitoring virus neutralizing antibodies in wild boars found detectable antibodies in only 36.3% of wild boars after vaccination using oral bait. However, there was no evidence that the studied animals had eaten the baits. It was not known if all of the population came into contact with the baits due to environmental conditions including weather or other animal species having eaten the baits. Another study showed wild boar to be selective in their bait choice, with different bait types being preferred based on wild boar age (European Commission, 2013). There was no information in both studies about the density of the population though as this is one key factor for the transmission rate. Next to the density of the population the basic reproduction number (R_0) depends on the virulence of the CSF strain and the susceptibility of animals within the population. For domestic pigs the R_0 value was shown to be high in general but differ through the populations due to age and status (Weesendorp *et al*, 2009; Ribbens *et al*, 2004). One experimental study (Dewulf *et al*, 2001) estimated an R_0 of 13, another study during an outbreak in the Netherlands 1997 – 1998 (Stegeman *et al*, 1999) estimated R_0 as 2.9 in breeding pigs and 81 between weaned piglets. Therefore, the vaccine coverage calculated with the formular $V_c = (1-1/R_0)$ ranges between 92% based on the Dewulf study and 65% and 99% based on the Stegeman study.

1.3.2.1.3 Porcine cytomegalovirus based viral vector platform

To overcome issues mentioned above this study has been building on previous work directed towards developing a CSFV E2 based vaccine in a replication competent cytomegaloviral background, this time using a porcine cytomegalovirus based viral vector platform which is discussed in more detail in the following sections. As replication competent cytomegaloviruses spread easily within host populations, it would be ideal for vaccinating wild boar populations as it would only be necessary to trap and vaccinate a few 'founder' animals. This would then return to the herd and spread the vaccine naturally, eliminating the need for baiting.

1.4 Porcine cytomegalovirus (PCMV)

1.4.1 Lineage

PCMV (also known as suid herpesvirus-2), belongs to the subfamily *Betaherpesvirinae* of the family *Herpesviridae* and order *Herpesvirales*.

1.4.1.1 Herpesvirales

The order *Herpesvirales* is divided into three families: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*. The *Herpesviridae* family covers mammalian, avian and reptilian viruses. The *Alloherpesviridae* family includes fish and frog viruses, and the *Malacoherpesviridae* comprises molluscs viruses (Davison *et al*, 2009; Savin *et al*, 2010). Comprising over 100 viruses herpesviruses are among the most thoroughly studied group of large DNA viruses (McGeoch *et al*, 2006). Paleovirology indicates that the first *Herpesvirales* virus appeared around 200 million years ago, with further sublineages developing over

the past 80 million years. It is likely that these sublineages largely co-evolved with their host species (McGeoch *et al*, 1995).

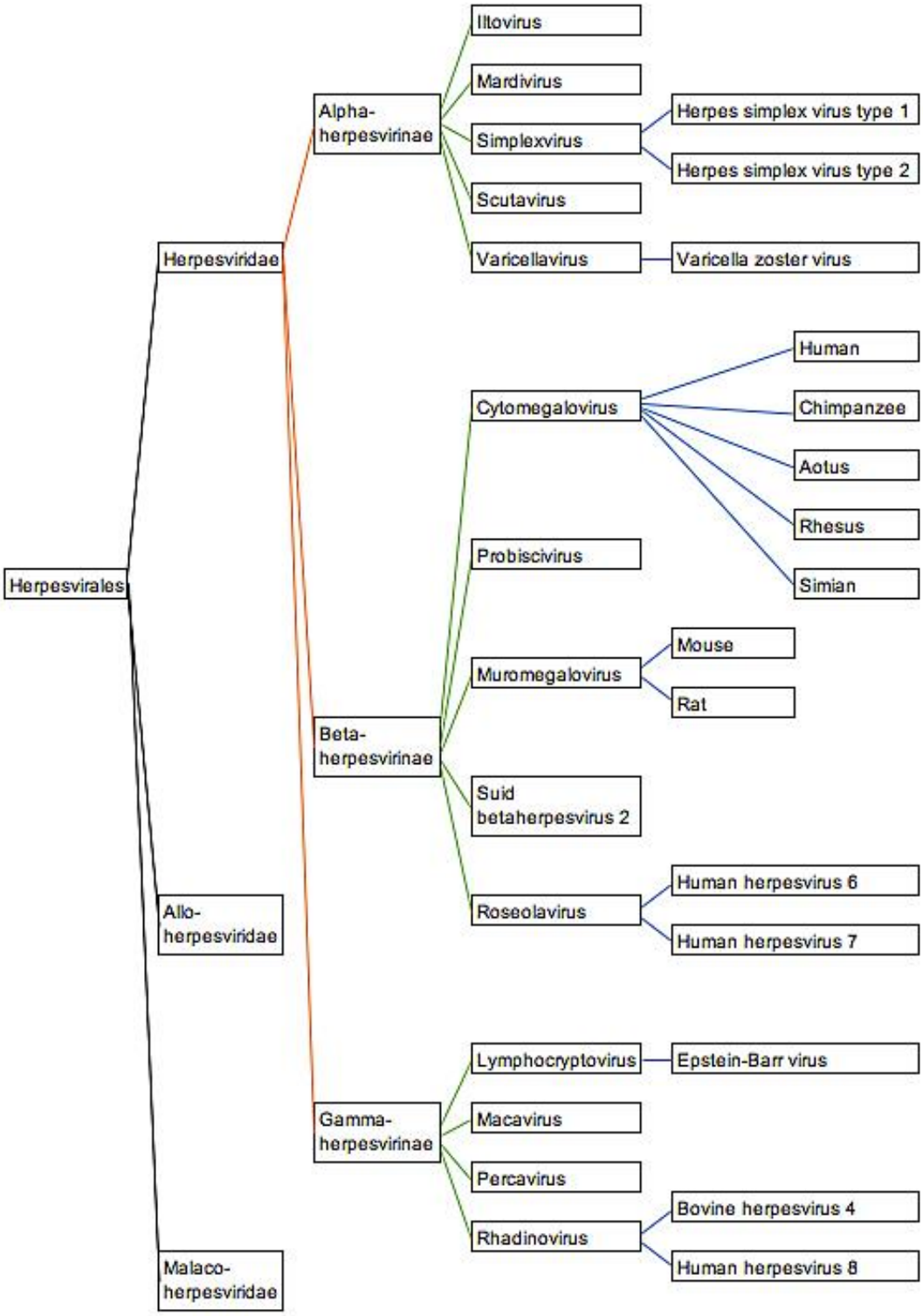


Figure 3. Herpesvirales classification (abstract of International Committee on Taxonomy of Viruses (ICTV) Master Species List 2018a v1)

1.4.1.2 Herpesviridae

Herpesviruses represent one of the three families in the order of Herpesvirales (Figure 3). Herpesviridae are further divided into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae*. These families contain several well-known human pathogens such as varicella zoster which is the cause of the common childhood disease chickenpox, Herpes simplex virus 1 which causes coldsores, cytomegalovirus (CMV) and Kaposi's sarcoma herpesvirus (KSHV; human herpesvirus-8). Infection with herpesviruses such as CMV and HSV1 is frequently asymptomatic being well-controlled by individuals with healthy immune systems. This is presumably due to extensive co-evolution leading to a balance between replication and immunological control within the host (Stuart-Harris, 1983; Grinde, 2013). However, co-existing with the host is a delicate balance, and these viruses can reactivate and cause disease when the host immune system is compromised. In the case of HSV-1, triggers such as stress, immune suppression or UV light can trigger reactivation and localized replication leading to the appearance of cold sores (Grinde, 2013; Hillaire *et al*, 2013). CMV can cause severe disease in immunocompromised patients, and in congenitally infected infants. KSHV is also known for being prominent in immune suppressed patients – commonly seen in patients with acquired immune deficiency syndrome (AIDS).

Common clinical manifestations of herpesvirus infection are shown in (Table 1) (WHO, 1985; Krug & Pellett, 2014; Hill & Zerr, 2014; The Pig Site; Center for Food Security and Public Health, Iowa State University, 2015; Rezk *et al*, 2018; ViPR, 2018; Donofrio *et al*, 2007).

Subfamily	Virus	Clinical manifestations
Alpha-herpesviruses	Herpes simplex virus type 1	Skin vesicles, labial mucosal lesions, keratoconjunctivitis, gingivostomatitis, encephalitis, meningitis
	Herpes simplex virus type 2	Genital lesions
	Varicella zoster virus	Chickenpox, Shingles
Beta-herpesviruses	Cytomegalovirus	Infectious mononucleosis, retinitis, anemia, thrombocytopenia, pneumonia, encephalitis, miscarriage, stillbirth, hearing loss, hepatitis
	Roseolavirus	roseola infantum, febrile rash in infants, Encephalitis and rejection in transplant patients, Hashimoto thyroiditis
	Suid betaherpesvirus 2	Rare except for young piglets: rhinitis, sneezing, respiratory distress, poor weight gain Pigs older than 3 weeks: subclinical to mild Infected pregnant sows: fetal death, mummified fetuses, stillbirth, weak piglets, nasal hemorrhage
Gamma-herpesviruses	Epstein-Barr virus	Infectious mononucleosis, associated with hematopoietic, epithelial and mesenchymal neoplasms
	Human herpesvirus 8	Kaposi's sarcoma with skin lesions, lymphoma, fever, weight loss
	Bovine herpesvirus 4	Uterine disease, metritis

Table 1. Clinical manifestations caused by Herpesvirinae

The herpesvirus life cycle can be divided into two phases – the lytic phase and the latent phase. During lytic replication, the viral genome is replicated, assembled into new virus particles which are then released from the host cell due to cell lysis. During the latent phase, the virus genome is maintained as an episome, largely concealed from the host's immune system, allowing herpesviruses to persist within the host (Guo *et al.*, 2010).

The ability to establish latency is a trait shared by all members of the *Herpesviridae* family (Whitley, 1996; Mettenleiter *et al.*, 2008); however, members of different subfamilies establish latency in distinct cell types. Alphaherpesvirus latency occurs in neuronal cells. When reactivation occurs, newly formed virions travel along neuronal cells where they can then cause a lytic infection in epithelial cells, facilitating spread of the virus (Stuart-Harris, 1983). Betaherpesviruses establish the latent phase within secretory glands and cells of the lymphoreticular system, while gammaherpesviruses latency involves B- and T- lymphocytes. The host range available also varies within the subfamilies from wide host range (alphaherpesviruses) to restricted host range (beta- and gammaherpesviruses).

Herpesviruses are linear double-stranded (ds) DNA viruses ranging from 120 to 260nm in diameter (Sadeghipour & Mathias, 2017). Genome size is variable, ranging between 108 to 300 kb. Depending on the virus, the number of proteins encoded by the genome can vary from 70 to 200 proteins (NCBI, 2013).

Herpesvirus virions consists of four layers: core, capsid, tegument and envelope. The core is the innermost area of the virion and contains the dsDNA viral genome encapsidated within an icosahedral capsid of 115 to 130 nm in diameter. This

nucleocapsid core is surrounded by the tegument. The tegument layer contains virus encoded proteins that are important in dampening initial host cell immune responses facilitating infection and replication. Proteins that play a role in assembly and egress of virus particles can also be found in the tegument. The envelope is a bilipid outer layer of the virion, and contains several different viral glycoproteins which often appear as spikes on the virion surface (Guo *et al.*, 2010; Sadeghipour & Mathias, 2017).

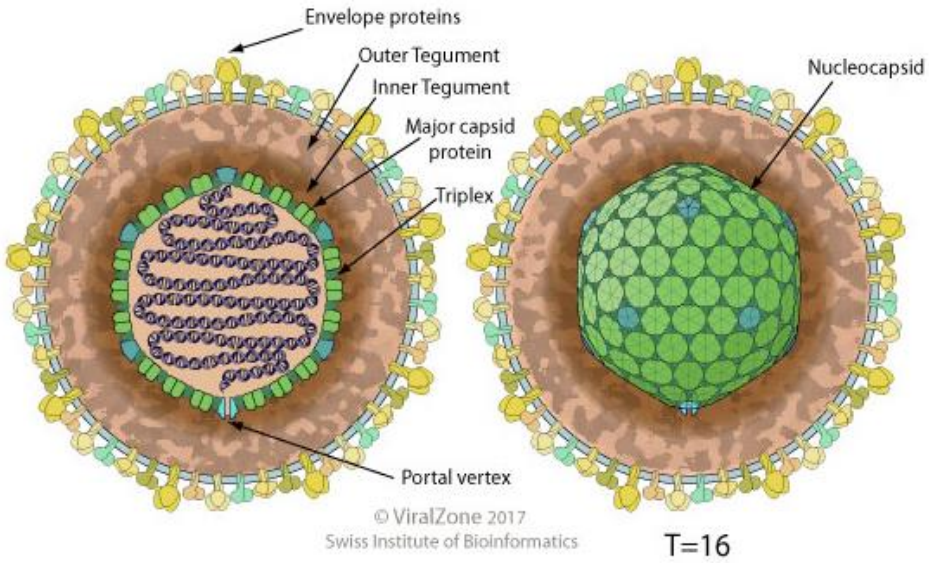


Figure 4. Structure of herpes virion. The double-stranded DNA is packaged within the icosahedral capsid. The nucleocapsid is surrounded by the tegument which contains virus encoded proteins. The outer envelope layer is a bilipid membrane spiked with viral glycoproteins. Adapted from (ViralZone 2017, Swiss Institute of Bioinformatics, with permission)

1.4.1.3 PCMV (suid herpesvirus-2, SuHV2)

1.4.1.3.1 History of the disease's name

Histological results from pig samples from several outbreaks in a Yorkshire pig herd in 1954 in England, and 1955 in Scotland indicated an outbreak of inclusion-body rhinitis (IBR) first reported by Done and colleagues (Mitchell & Corner, 1958a). While atrophic rhinitis was well known at the time, especially in Canada and the United States, this disease in the UK appeared to be different, warranting further investigation (Mitchell & Corner, 1958). In 1964 Corner et al. described the disease as cytomegalic inclusion disease (CID), caused by porcine cytomegalic inclusion disease virus, due to the similarity of inclusion bodies found in human, mouse and guinea pig cell-cultured cytomegalovirus (L'Ecuyer & Corner, 1966).

1.4.1.3.2 Taxonomy of PCMV

In the 1st report in 1971, the International Committee on Taxonomy of Viruses (ICTV) catalogued PCMV as a new species of herpesviruses, under the name porcine inclusion body rhinitis virus (International Committee on Taxonomy of Viruses). Following removal from the list in the 2nd report in 1976, the virus was reinstated in the 6th report in 1995 under an unassigned genus of the subfamily *Betaherpesvirinae*, species suid herpesvirus 2. In 2015 the virus was renamed as suid betaherpesvirus 2 [International Committee on Taxonomy of Viruses (ICTV)].

1.4.1.3.3 PCMV and Roseolavirus, a comparison

The only fully characterised genome of PCMV (BJ09 strain; GenBank no: KF017583) is comprised of 128,367 bp and a G/C content of 45.54% The PCMV

genome is shorter and has a higher G/C content than the Roseolavirus HHV-6A strain U1102 (159,322 bp; 42.44%), HHV-6B strain z29 (162,114 bp; 42.77%) and HHV-7 strain RK (153,080 bp; 36.22%) (Gu *et al*, 2014). The length of the direct repeats is also substantially shorter (363bp) (Gu *et al*, 2014) than those found in HHV-6A strain U1102 (8087 bp) (Megaw *et al*, 1998), HHV-6B strain z29 (8,793 bp) (Dominguez *et al*, 1999) and HHV-7 strain RK (5,814 bp) (Megaw *et al*, 1998). The unique region of PCMV contains 79 ORFs, flanked by direct repeats (Figure 5). Seventy-one ORFs of the PCMV genome have homologs in other herpesviruses, especially within the *Roseolavirus* genus. Only eight non-core genes (U13p, U24p, U26p, U83p, U85p, U89p, U90p and U100p) differ from HHV-6A, HHV-6B and HHV-7. These genes encode a transcriptional repressor (U13), membrane proteins (U24, U26), a CC chemokine (U83), glycoproteins (U85, U100) and IE-A proteins (U89, U90), while the functions in PCMV remain unknown. It has been suggested that PCMV be classified within the *Roseolavirus* genus rather than classifying it as a CMV - although there are some similarities between the unique regions of PCMV, HCMV, RCMV and MCMV. Similarities between PCMV genome and roseolavirus strains HHV-6A, HHV-6B and HHV-7 are shown in regard to genes encoding DNA polymerase, major capsid protein and glycoprotein B (gB) (Gu *et al*, 2014; Denner, 2015; Fiebig *et al*, 2017). PCMV gB showed high consensus with HHV-6 and HHV-7 (43.4% and 42.6%, respectively), much higher than with other herpesvirus gB genes (Widen *et al*, 2001).

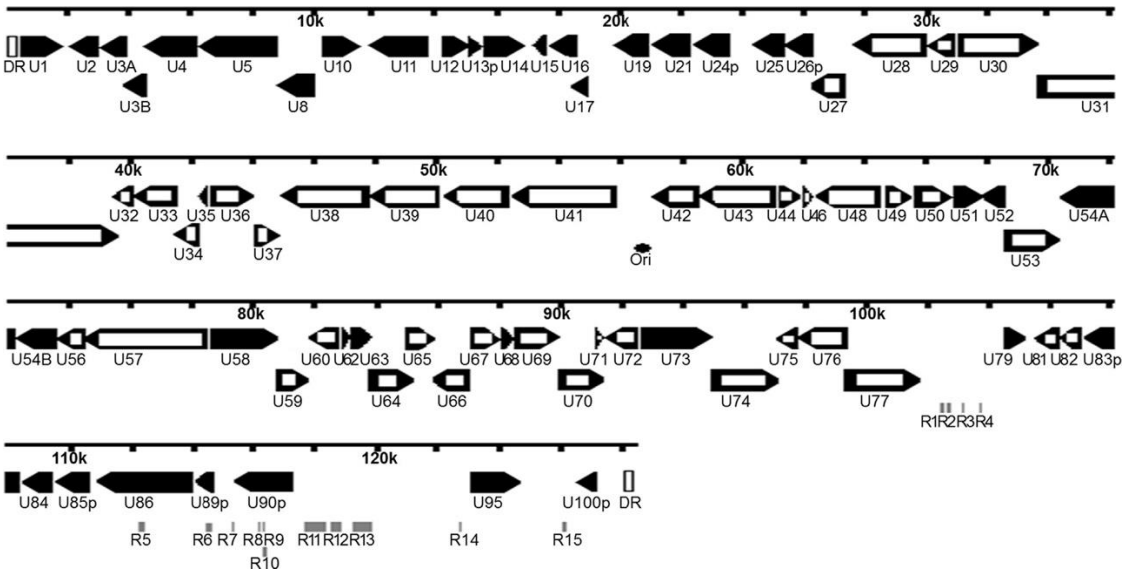


Figure 5. Characterisation of PCMV genome. 79 ORFs were identified, represented as arrows indicating location and orientation. Arrows with white rectangles illustrate so-called core genes which are present in all three subfamilies of the *Herpesviridae* family, due to a common ancestor. The unique regions are flanked by terminal direct repeats (DR), represented as white rectangles. Reprinted from (Gu *et al*, 2014).

1.4.1.3.4 Species specificity

Herpesviruses, especially CMV (a well-studied representative of the betaherpesvirus family), are known to be highly species specific with cross species infections inhibited, at least in part, due to prevention of viral replication in host cells by induction of the apoptotic pathway (Jurak & Brune, 2006).

When this project commenced PCMV was described as uncategorized within the betaherpesvirus subfamily, although studies had shown high similarities to the *Roseolavirus* genus. It is not known if PCMV is as highly species specific as other CMVs, or whether it is able to infect and replicate in other cell types. For example, the ability to replicate in human cells would be hugely problematic with regards to xenotransplantations from pigs into humans. Although under study, this question has not been fully answered (Denner, 2018). Studies to date have had

inconsistent results. Whittaker's group (Whitteker *et al*, 2008) confirmed PCMV infection of human fibroblasts using tissue culture, PCR and western blot, whereas Tucker and colleagues (Tucker *et al*, 1999) failed to demonstrate stable virus transmission of PCMV from infected primary porcine alveolar macrophages to a human detector cell line (RAJI), and a human epithelial cell line (293) by PCR. Fiebig and colleagues (Fiebig *et al*, 2017) were also unable to detect PCMV in five human cell lines (HeLa, 293T, TZM-bl, Jurkat and HepG2) by PCR, indicating that there was no virus transmission and replication following infection of these cell lines with serum from a PCMV-infected animal. They did however demonstrate antibody cross-activity between PCMV and the Roseolavirus HHV-6, which can likely be ascribed to the genomic similarity between PCMV and Roseolaviruses. Despite the likelihood of PCMV being able to infect humans being very low, the possibility has not been ruled out (Denner, 2017). In terms of xenotransplantation (e.g. heart valves are commonly used in human patients), PCMV has to be eradicated from pigs designated for organ donation and appropriate programs have been developed to ensure this goal (Clark *et al*, 2003; Tucker *et al*, 2003; Denner, 2015; Fiebig *et al*, 2018).

1.4.1.3.5 Transmission and clinical manifestation

Pigs are the natural host for PCMV. The virus is ubiquitous and is considered to be endemic in nearly all pig populations worldwide. It is thought that PCMV can be transmitted vertically, via transplacental transmission (Edington *et al*, 1988; Fryer *et al;* 2001 Clark *et al*, 2003), and also horizontally, via nasal and ocular secretions and urine (Morozov *et al*, 2017). Infection is usually latent, with the exception of infected piglets (Morozov *et al*, 2017). Symptoms can appear as mild sneezes, but infertility in older pigs has also been attributed to PCMV (Kavanová

et al, 2018). Clinical manifestations in piglets manifest as rhinitis with inclusion bodies observed in cells of the nasal mucosa (Basso *et al*, 2017). Infections in piglets less than 3-weeks old can lead to runting and death with a high mortality rate (Clark *et al*, 2003; Liu *et al*, 2014).

1.4.1.3.6 PCMV as a viral vector

While PCMV has been assigned to the *Roseolavirus* genus by ICTV since February 2019, it shares many similarities with the CMV genus. Infection of immunocompetent individuals with CMV results in an asymptomatic but persistent latency, and reactivations, which are actually consistent with low level chronic replication, occur frequently, evoking high levels of antibodies and T- cells directed against CMV (Sylwester *et al*, 2005). These strong immune responses, combined with the fact that CMV can also re-infect infected individuals, makes it ideal for use as a vector platform, as pre-existing immunity won't impede vaccination (Hansen *et al*, 2010; Tsuda *et al*, 2011; Tsuda *et al*, 2015). The use of CMV as a vaccine platform offers several advantages over conventional vaccines. It is ubiquitous, and its species-specific nature minimizes the risk of spread to other animals - as shown in a recent predator/prey study (Murthy *et al*, 2013). CMV spreads well through its host populations indicating a high transmission rate and provides long lasting durable immunity that can be achieved with a single vaccine dose (Redwood *et al*, 2005; Tsuda *et al*, 2015; Quinn *et al*, 2016) This is more cost effective than strategies which require multiple doses to achieve protection, or strategies where animals have to receive a vaccination individually, such as the chimeric vaccine described earlier. The ability of CMV to spread and essentially become 'self-disseminating' has great potential for controlling diseases in wild animal populations that can serve as a

reservoir of disease, and act as a source of infection for domestic animals and vice versa. A self-disseminating vaccine would be invaluable for targeting inaccessible wild-life populations such as wild boars and would eliminate the need for complex baiting strategies. Such a strategy would involve capturing and inoculating a small number of animals. When released back into the wild, the virus will then spread from animal to animal, through the whole population, spreading immunity as it goes (Murphy *et al*, 2016). In this study we hypothesised that PCMV will have similar properties, and in the future, it could be adapted for use as a self-disseminating viral vector platform.

1.4.2 PCMV mutagenesis

1.4.2.1 Cloning strategy using bacterial artificial chromosome (BAC-) based technology

Cloning strategies involving the insertion of large DNA fragments (12.5 kb) into a viral genome (baculovirus) that was propagated in Escherichia coli (*E. coli*) were first reported by Luckow and colleagues in the early nineties (Luckow *et al*, 1993). Generating an infectious BAC containing viral genomes allows genetic engineering of the viral genome in a circular plasmid form by using the *E. coli* recombination system. This is much faster than methods which rely on homologous recombination to insert / delete regions into / from viral genomic DNA. The genetically engineered BAC can then be transferred into eukaryotic cells to reconstitute the genetically modified virus (Adler *et al*, 2003). By the late 1990s, this strategy had been adapted to the mouse muromegalovirus model (murine cytomegalovirus, MCMV), to investigate the pathogenesis of CMV infection (Messerle *et al*, 1997). This technique is still widely used, and infectious

BACs have now been generated for many other DNA and RNA viruses (Hall *et al*, 2012).

BACs are supercoiled circular DNA plasmids based on the low-copy fertility factor (F-factor) (one to two copies per cell) of *E. coli*. Despite their often very large size (up to 300 kb pairs), they facilitate easy, quick editing of viral genomes. Other vector models such as plasmids or cosmids can maintain only small DNA constructs of 10 to 50 kb, respectively in comparison to BACs which can maintain the entire genome of a virus (Ramsay, 1994). BACs are also quite stable, as demonstrated through several serial passages. The low copy number of BACs minimizes the risk of rearrangements or deletions of sections within the cloned DNA (Shizuya *et al*, 1992), reducing the risk of mutations which would be present in the reconstituted virus (Osterrieder *et al*, 2003).

Other similar recombination systems also exist. Yeast artificial chromosomes (YACs) have also been used as a vector platform (Ketner *et al*, 1994), but have some disadvantages when compared to BACs. Although YACs can maintain 2000 kb DNA (Ramsay, 1994), they exist as linearized DNA constructs (as opposed to the circularized BACs), and therefore are much more susceptible to degradation (O'Connor *et al*, 1989), resulting in structural instability (Neil *et al*, 1990). It was decided to use a BAC based strategy for this project as several promising vaccine candidates have been generated using this technique with Human CMV (human cytomegalovirus), HSV-1 (herpes simplex virus 1), VZV (varicella zoster virus), MCMV (murine cytomegalovirus), GPCMV (guinea pig cytomegalovirus) and RhCMV (rhesus cytomegalovirus) BACs.

1.4.2.2 BAC vector

As described above BACs are large DNA plasmids that allow genetic manipulation of viral genomes in *E. coli*, rather than having to manipulate the viral DNA directly. BACs contain several genes that are required for maintenance and partitioning of the BAC in the bacterial cell. These regulatory genes are contained in an element known as a BAC cassette which is about 8 kb in size (Brune *et al*, 2000). This cassette contains an origin of replication (oriS) and repE, which are involved in reproduction of the plasmid within the bacteria. It also contains parA and parB genes which are involved in the partitioning of the BAC to daughter cells, forming part of the control mechanism for limitation of BAC copy numbers to one or two copies per bacterial cell. The BAC cassette also contains a selectable marker (frequently Chloramphenicol), which allows for selection of clones that carry the BAC. Additionally the cassette contains several restriction enzyme sites and some cleavage sites (e.g. loxP, locus of crossing over P1 phage) which facilitates the deletion of the BAC cassette after generation of the recombinant virus (Shizuya *et al*, 1992; Warden *et al*, 2011). Additional selectable markers which can be used to select eukaryotic cells infected with the resulting recombinant virus are also present. These include guanine phosphoribosyl transferase gene (GPT) and green fluorescent protein gene (GFP).

1.4.2.3 Red/ET homologous recombination

Homologous recombination (HR) was first introduced using YACs in the early 1990s (Baudin *et al*, 1993). Since then HR has become an important tool for gene editing to genetically modify specific genetic loci and is often referred to as recombinogenic engineering or recombineering. Although recombinogenic engineering in yeast pioneered HR technology, this approach had several

limitations. Structural instability of YACs was a primary problem. Yields of DNA were also quite low and working with YACs was labour-intensive. A prokaryotic based system, using bacterial artificial chromosomes (BACs) and an endogenous *E. coli* recombination system involved in DNA repair (RecA protein and RecBCD enzyme), was developed as an alternative strategy to overcome the disadvantages of YACs. Although more stable than YACs, a disadvantage of RecA-dependent recombinogenic engineering was the need for homologous regions ≥1000 bp in length, and the need for strong induction of RecBCD to degrade inserted linear DNA fragments.

An alternative bacterial recombination system based on the RecE and RecT proteins from the phage Rac, or Redα and Redβ from the phage λ (called E/T recombineering) overcame both of these limitations (Zhang *et al*, 1998; Muyrers *et al*, 1999; Rivero-Müller *et al*, 2007). Both E/T systems use similar pathways, and both require a Redγ protein which inhibits the activity of RecBCD, preventing degradation of the exogenously added DNA 'recombination fragment' containing the desired genetic alteration (Wagner & Koszinowski, 2004). RecE and Redα degrade the 5' end of the linear dsDNA recombination fragment to enable RecT and Redβ respectively, to anneal the resulting single-stranded homologous region (35-60 nucleotides) of the fragment to the targeted single-stranded circular DNA of the BAC genome being targeted, resulting in a strand exchange. Proteins from eukaryotes and viruses can also be used for E/T recombination (Kolodner *et al*, 1994; Muyrers *et al*, 2001), enabling E/T recombination to be performed in cell types other than bacteria.

E/T recombination has several advantages over other systems mentioned above, including the ability to insert large regions of DNA into the targeted BAC. The

relatively short size of the homologous region necessary to target the recombination fragment (35-60 nts) also means that the fragment can be amplified using polymerase chain reaction (PCR) (Zhang *et al*, 1998; Muyrers *et al*, 2001). Flanking marker genes by recognition sites such as FRT (FLP recognition target) or LoxP (locus of crossing over P1 phage) sites (Shizuya *et al*, 1992) can be used to enable marker gene removal from the targeted BAC genome following selection of recombinants. Due to these advantages, E/T recombination is regarded as a state-of-the-art, reliable technology for genetic modifications of virus genomes within BACs, with many studies having been performed in the years since its inception (Table 2).

Virus vector	Target	Studied by
Murine cytomegalovirus (MCMV)	Sperm binding protein in mice (Zona pellucida protein 3)	(Redwood *et al*, 2005)
	Ebolavirus (Zaire) nucleoprotein	(Tsuda *et al*, 2011) (Tsuda *et al*, 2015)
	Melanoma antigen (B16)	(Qiu *et al*, 2015)
Rhesus cytomegalovirus	Various proteins in simian immunodeficiency virus	(Hansen *et al*, 2011)
	Ebolavirus (Zaire) glycoprotein	(Marzi *et al*, 2016)
	Mycobacterium tuberculosis antigen	(Hansen *et al*, 2018)
Bovine herpesvirus 4 (BoHV-4)	Anti-complement protein I and II in Ixodes ricinus (tick) (IRAC I and IRAC II)	(Gillet *et al*, 2005)
	BVDV E2 protein	(Donofrio *et al*, 2007b)

Table 2. BAC-based vaccines

1.4.2.4 Genome editing by CRISPR/Cas9 technology

In addition to Red/ET HR, recently developed CRISPR/Cas9 technology can also be used to generate recombinant viruses.

1.4.2.4.1 Introduction

Although Red/ET based homologous recombination was a milestone in genome editing, the generation and subsequent screening of BAC clones is labour- and time intensive (Lin *et al*, 2016). A new powerful technology known as CRISPR/Cas9 has emerged in recent years. The first steps towards discovery of CRISPR began in the late 1980s (Ishino *et al*, 1987, Ishino *et al*, 2018). However, it has taken over 20 years to fully understand how CRISPR systems function in bacterial immunity (Hsu *et al*, 2014). CRISPR denotes clustered regularly interspaced short palindromic repeats and was discovered along with Cas (CRISPR-associated) proteins in prokaryotes where it functions to defend against bacteriophage infections (detailed below).

1.4.2.4.2 Classification

CRISPR-Cas systems are divided into two classes (Class 1 and Class 2) based on the presence of Cas1 and Cas2 genes, respectively. These two classes are then subdivided into different types based on the presence of additional accessory Cas subunit genes. Class 1 systems, which can contain several different Cas subunits, include type I, III and IV systems. Some of these are further sub-divided into different subtypes. Class 2 systems contain only one specific CRISPR ribonucleoprotein and are subdivided into types II, V and VI (Figure 6). As with Class 1, the Class 2 types are further sub-divided into subtypes (Koonin *et al*, 2017). The best characterised of all of these systems is the

CRISPR/Cas9 system, a type II system from *Streptococcus pyogenes*. This is the system that was utilized in this study and is described in more detail below.

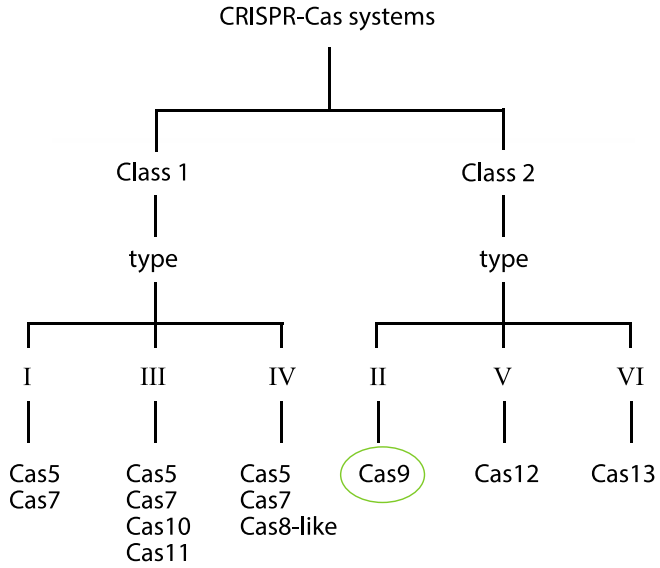


Figure 6. CRISPR/Cas classification showing two separate classes. Each class contains three 'types'. Cas 1 and Cas 2 proteins (not shown) are common to all 'types'. These 'types' can then be further divided into subtypes (not shown), on the basis of Cas proteins found in each subtype. The CRISPR/Cas9 system is the one used in this study.

1.4.2.4.3 CRISPR defence

CRISPR systems function as an adaptive immune system within prokaryotes. All of the different systems function in a very similar manner, proceeding through the same three steps of (spacer) acquisition, expression (also called crRNA biogenesis) and (target) interference (Yao *et al*, 2018) (Figure 7).

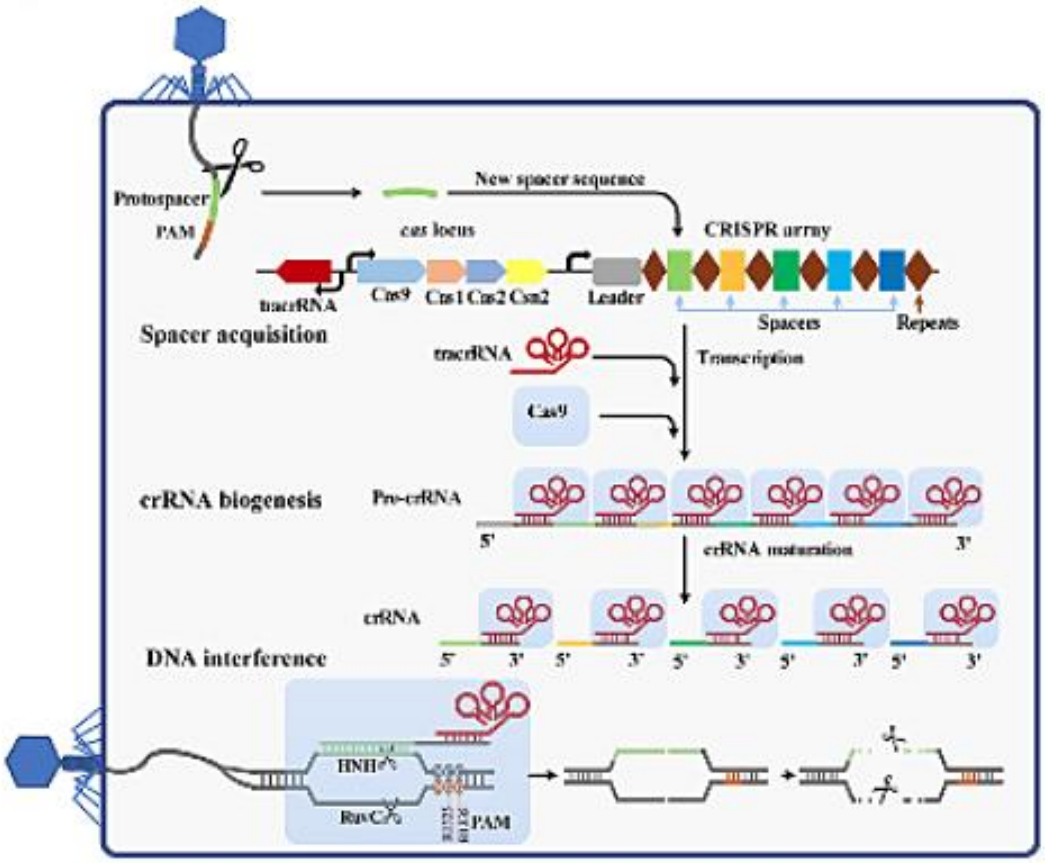


Figure 7. The natural bacterial adaptive immune defence system. This figure demonstrates the three stages of the CRISPR/Cas9 system (adapted from Yao *et al*, 2018)

1.4.2.4.3.1 Acquisition

When an external / invading genome is released into the bacterial cell for the first time, a copy of a short fragment (26 – 72 bp) of the invasive DNA (protospacer) is made and placed in a CRISPR array, downstream of a leader sequence. The leader sequence plays an important role in expression of pre-crRNA which is necessary for crRNA biogenesis (see below). The CRISPR array functions as a library record of prior infections, where all of these protospacer sequences are stored together, with each protospacer sequence being flanked by repeat sequences (21 – 48 bp) (Rath *et al*, 2015; Yao *et al*, 2018).

Protospacers are not randomly selected, but instead are captured using a sequence of 2 – 5 nucleotides adjacent to the protospacer, called the protospacer adjacent motif (PAM). The CRISPR array is surrounded by Cas genes which encode different Cas proteins depending on the specific CRISPR system (Hille & Charpentier, 2016; Yao *et al*, 2018). Cas1, Cas2 and Cas9 are all required for adaptation in type II systems but the process is not yet understood in full detail (Garneau *et al*, 2010; Wei *et al*, 2015). Together, the protospacer and Cas proteins form a ribonucleoprotein complex, forming a basis for the memory of viral invasion, leading to a defensive immune response during subsequent infections with the same pathogen.

1.4.2.4.3.2 crRNA biogenesis

After incorporation of the new protospacer the complete CRISPR array is transcribed into a precursor CRISPR RNA (pre-crRNA). During the maturation process, protospacers match with a complementary trans-activating RNA sequence (tracrRNA) at the repeat region, forming a unit called guide-RNA (gRNA), which is about 20 nucleotides long. The Cas9 protein recognizes the

CRISPR array, with its bound tracrRNAs, and clips the sequence to generate individual gRNAs. This event leads to the formation of a mature gRNA complex consisting of pre-crRNA and tracrRNA. For efficiency in genome editing technologies, pre-crRNA and tracrRNA can be synthesised to be used as a single guide RNA (sgRNA) (Hille & Charpentier, 2016; Yao *et al*, 2018). Together with the Cas9 protein, the gRNA forms the CRISPR/Cas9 complex which is widely used to efficiently edit genomes.

1.4.2.4.3.3 Interference

The CRISPR/Cas9 complex recognises a 20-nucleotide invasive DNA sequence (protospacer) that is complementary to the protospacer-derived gRNA sequence in the CRISPR array, when presented adjacent to a PAM (3-nucleotide sequence (NGG) downstream of the protospacer). The protospacer is cleaved 3 – 4 nucleotides upstream of the PAM by Cas9 nucleases. Cas9 HNH-like nuclease (endonuclease domain named for histidine and asparagine residues) cuts the strand complementary to the crRNA sequence and Cas9 RuvC-like nuclease (an endonuclease domain named for *E. coli* protein involved in DNA repair) cuts the non-complementary strand, resulting in a double-stranded break (DSB), leading to the destruction of the invasive DNA, preventing establishment of infection (Gasiunas *et al*, 2012; Jinek *et al*, 2012; Martinez-Lage *et al*, 2017).

1.4.2.4.4 Taking advantage of CRISPR/Cas9 mediated targeting

The development of CRISPR/Cas technologies has revolutionised the field of genetic engineering in eukaryotic systems, allowing easy and efficient targeted modifications to viral, bacterial and eukaryotic genomes. CRISPR/Cas is much more efficient than earlier technologies such as homologous recombination

(distinct from bacterial HR detailed above), the technology initially used in development of knock-in / knock-out animal models (Capecchi, 1989; Hsu *et al*, 2014). Several studies showed that by introducing targeted DNA DSB, CRISPR/Cas stimulated cellular repair events [either homologous direct repair (HR) or non-homologous end joining (NHEJ)], increasing recombination efficiency thereby becoming a powerful tool in genome editing technology (Rudin *et al*, 1989; Rouet *et al*, 1994; Bibikova *et al*, 2003). As Cas9 can be targeted to selected genomic regions based on the sequence of the gRNA, the DSBs can be targeted highly specifically to within the genome. Once a DSB occurs, one of the two distinct cellular repair mechanisms can be triggered: the more frequent but error-prone NHEJ pathway, and the high-fidelity HDR pathway. NHEJ repairs the DSB by processing the broken ends to fit together for ligation (Weterings & Chen, 2008). The repair is not always completely accurate and small deletions or insertions (indels) occur frequently (Gaj *et al*, 2013; Boel *et al*, 2018). These indels can lead to frameshifts in the genome, resulting in loss of functionality (Weterings & Chen, 2008). In contrast to the NHEJ pathway, repair by the HDR pathway is very precise as it requires a homologous stretch to the target sequence. HDR is the better tool for genome editing, as a gene of interest can be inserted into or deleted from the genome where desired, without the occurrence of indels - however, the efficiency is relatively low. To overcome this issue various approaches have been developed, including an NHEJ inhibitor SCR7 (used in this study), which tips the balance towards use of the HDR pathway [increases Cas9-mediated HDR by up to 19-fold (Aird *et al*, 2018)], over the NHEJ pathway by degrading DNA ligase IV, a ligase on which the NHEJ pathway is dependent (Maruyama *et al*, 2015; Aird *et al*, 2018); although a study by Zhang and colleagues (Zhang *et al*, 2017), reported that they did not find that SCR7

increased efficiency of HDR at their desired loci, but factors such as different cell lines and vectors may have contributed to this discrepancy. Hence, the role of SCR7 in increasing the efficiency of HDR still remains unclear.

1.5 Rationale for development of a PCMV vaccine vector

Cytomegalovirus (CMV), a member of the herpesvirus family, is a well-studied vaccine vector platform (McGeoch *et al*, 2006). Based on sequencing of one strain, the BJ09 strain, PCMV has been characterised within the *Roseolavirus* genus (Gu *et al*, 2014). However, PCMV is expected to share characteristics with other members of the beta-herpesvirus subfamily. In common with all members of the beta-herpesvirus subfamily, PCMV shares many open reading frames restricted to this subfamily such as UL83, which expresses the late tegument protein pp65. PCMV contains glycoprotein B (gB), an envelope protein which facilitates fusion of the virion with the host cell membrane and is essential for infectivity. PCMV also shares similarities in cytopathology with other beta-herpesviruses such as HCMV or MCMV: infection being associated with the development of cytomegalic intranuclear inclusions (Narita *et al*, 1987).

PCMV is ubiquitous and, where studied, is present in all pig populations worldwide (Edington *et al*, 1988; Fryer *et al*, 2001; Clark *et al*, 2003). Similar to other CMVs, PCMV infection is associated with asymptomatic, or mild disease. Only in the presence of immune suppression is PCMV associated with overt symptomology, for example causing disease or death in piglets, which is again comparable to other CMVs. Generally, this occurs through

congenital infection and only if the sow has a primary infection in late pregnancy (Morozov *et al*, 2017; The Pig Site).

CMVs have the capacity to induce high levels of antibody (Marzi *et al*, 2016) and T-cell responses against heterologous target antigens, even in CMV-infected individuals (Tsuda *et al*, 2011; Hansen *et al*, 2011; Tierney *et al*, 2012; Tsuda *et al*, 2015). Similarly, PCMV has been shown to induce high levels of antibodies, with one study reporting >99% pig sera from Japan testing positive for PCMV antibodies (Tajima *et al*, 1993; Plotzki *et al*, 2016). Previous studies by Edington and colleagues in 1976 and Staczek and colleagues in 1990, also reported high levels of antibodies in sera (Liu *et al*, 2012). There does not appear to be any published studies analysing T-cell responses during PCMV infection.

Based on the common characteristics PCMV shares with other members of the beta-herpesvirus subfamily, we hypothesized that PCMV has similar advantages for exploitation as a vaccine vector, with a capacity for inducing high levels of antibodies and T-cell responses against heterologous target antigens, even in PCMV-infected animals. Although betaherpesvirus-based vaccines have shown substantial promise in experimental studies (Tsuda *et al*, 2011; Tierney *et al*, 2012; Tsuda *et al*, 2015; Marzi *et al*, 2016), the utility of a mammalian herpesvirus platform is established commercially for a gE-deleted alphaherpesvirus-based vaccine (Bovilis, Rispoval, AKIPOR 6.3). The Bovilis-IBR marker live vaccine which targets bovine herpesvirus-1, the causative agent of infectious bovine rhinotracheitis (IBR) has shown to reduce clinical signs of IBR, and also reduce the shedding of virus from infected animals (Bosch *et al*, 1996). Zoetis also markets live and inactivated versions of this vaccine (Rispoval IBR Marker Vaccines). AKIPOR 6.3, marketed by

Merial, a live virus (Bartha-K-61 strain) vaccine in adjuvant targets pseudorabies virus (PRV), the causative agent of Aujeszky's disease. It has shown full protection against lethal challenge with the classical PRV SC strain and played an important role in eradication of PRV in e.g. Europe, North America and New Zealand. However, in 2011 it was shown in China that the Bartha-K61 strain showed only 50% protection against a challenge with HeN1 strain and therefore, does not protect effectively against this PRV strain (An *et al*, 2013).

As discussed earlier in this chapter, several types of vaccines against CSFV have been developed, but many of these vaccines do not provide adequate levels of protection or meet DIVA requirements. Live recombinant viruses showed the most efficacy, but to date, only one DIVA compatible live recombinant vaccine against CSFV called Suvaxyn® CSF Marker (Zoetis), is commercially available (Blome *et al*, 2017d). However, the Suvaxyn® CSF Marker (Zoetis) vaccine has some limitations (see above). The long-term goal of this project is to take advantage of the characteristics of beta-herpesviruses by generating a PCMV-based vaccine designed to overcome issues associated with existing CSF vaccines. The high antibody and T-cell responses seen with other beta-herpesvirus based vaccines, if recapitulated with a PCMV-based vaccine, might eliminate transplacental infection – something the Suvaxyn® CSF Marker (Zoetis) vaccine fails to do. Based on the capacity for CMVs to spread from animal to animal within defined species populations, a PCMV-based vaccine for CSF also has the potential to achieve substantial vaccine coverage in less accessible key animal populations, such as wild boar. As a licensed vaccine Suvaxyn® CSF Marker (Zoetis) requires direct intramuscular vaccination. Although it has shown potential for oral

vaccination (Blome *et al*, 2017d) further investigation might be necessary to achieve substantial coverage in wild animal reservoir species.

PCMV was initially identified over 60 years ago when Done and colleagues first reported about an outbreak in the UK that involved a rhinitis in pigs, with a histological discovery of 'inclusion bodies' (Mitchell & Corner, 1958a). This new condition was different than the already known disease of atrophic rhinitis, leading to the classification of a new disease in pigs called 'inclusion-body' rhinitis (Mitchell & Corner, 1958a). However, PCMV has remained largely unstudied both in terms of its growth characteristics *in vitro*, as well as immunology / virology *in vivo* (Liu *et al*, 2014). Combined with the generally benign nature of infection, the lack of exploration into PCMV probably also results from the viruses extremely slow *in vitro* growth characteristics, which amongst other things makes cloning of the virus as an infectious BAC difficult.

The aim of the present study was to further characterise the *in vitro* growth characteristics of a PCMV isolate provided by Dr. Jay Fishman (Section 2.2.1). As further steps towards cloning of PCMV as an infectious BAC, two recombinant BAC shuttle vectors were constructed to facilitate insertion of a BAC cassette into the PCMV genome at specific locations and were characterised. For development of a key reagent for these and subsequent studies, sera from a cohort of abattoir pigs were screened, and PCMV positive / negative sera were identified by ELISA. Initial experiments towards the use of homologous recombination and CRISPR/Cas9 technology to insert the BAC cassette within the PCMV genome are also described.

Chapter 2

Materials and Methods

All eukaryotic cell culture and virus work was performed in a Class II biological safety cabinet (Scanlaf Mars; LABOGENE, Leighton Buzzard, UK) using aseptic technique (ThermoFisher Scientific, Aseptic Technique).

2.1 Eukaryotic cell culture

2.1.1 Cell line

Porcine fallopian tube (PFT) cells, an adherent fibroblast-like cell line, were kindly provided by Dr. Jay Fishman, Harvard Mass General Hospital, Boston, USA forwarded by Dr. Helen Crooke, Animal and Plant Health Agency (APHA)-Weybridge, New Ham, Surrey, UK.

PFT cells were cultured and maintained in minimum essential media (Gibco™ MEM; Fisher Scientific, Loughborough, UK) supplemented with 10% (v/v) fetal bovine serum (Gibco™ FBS; US origin; heat inactivated at 56°C for 30 min; Fisher Scientific) and 1% (v/v) penicillin-streptomycin-glutamine (Gibco™ PSG; penicillin 100 U/ml; streptomycin 100 µg/ml; L-glutamine 0.29 mg/ml; Fisher Scientific) [complete MEM; MEM-10], at 37°C in a 5% CO_2 humidified atmosphere.

2.1.2 Maintenance of PFT cells

2.1.2.1 Thaw of cells

Cryovials containing 1 ml of PFT cells in 10% cryopreservation agent dimethyl sulfoxide (DMSO; Sigma-Aldrich Company Ltd., Gillingham, UK) diluted in FBS (see Section 2.1.2.3) were rapidly thawed in a 37°C water bath and transferred

into 5 ml prewarmed MEM-10. Cells were centrifuged at 296 x g (1200 rpm; Eppendorf Centrifuge 5810R, rotor S-4-104; Eppendorf Ltd, Stevenage, UK) for 5 min at room temperature (RT). After discarding the supernatant, the cell pellet was gently resuspended in 7 ml prewarmed MEM-10 and transferred to a 25 cm^2 tissue culture flask, which was incubated at 37°C in 5% CO_2 humidified atmosphere.

2.1.2.2 Growth of cells

Cell confluency was monitored under a light optical microscope (Motic AE 2000; VWR, Lutterworth, UK). Cells were passage into a new cell culture flask (generally every 3 – 4 days) after reaching a confluency of 90 - 100%. For passage, media was removed, and the monolayer was washed twice with 1X Dulbecco's phosphate buffered saline (Gibco™ DPBS; Fisher Scientific). The cell monolayer was detached by incubation with Trypsin-EDTA (Gibco™ Trypsin-EDTA (0.25%), Phenol red; Fisher Scientific) [1 ml for 25 cm^2, 2 ml for 75 cm^2, 3 ml for 175 cm^2] at 37°C for up to 5 min. Cells were resuspended in MEM-10 to inactivate the trypsin. For maintenance, cells were not counted but seeded at a 1:3 – 1:6 split ratio of the single cell suspension which was then transferred to a new flask. Cells used in experiments, with a defined number of cells required, were counted and seeded at a defined density (see Section 2.1.2.4).

2.1.2.3 Cryo-preservation of cells

Cells from one 175 cm^2 cell culture flasks were trypsinized as described above and centrifuged at 296 x g (1200 rpm; Eppendorf Centrifuge 5810R, rotor S-4-104; Eppendorf) for 5 min at RT. The cell pellet was resuspended to a concentration of 5 x 10^6 cells/ml in freezing media [FBS containing 10% (v/v)

DMSO] and 1 ml volumes were aliquoted into appropriately labelled 1.8 ml cryovials (Thermo Scientific™ Nunc™ Biobanking and Cell Culture Cryogenic Tubes; Fisher Scientific). Cells were then immediately transferred into a Mr. Frosty freezing container (Fisher Scientific) followed by storage at -80°C overnight (achieves controlled cooling a rate of -1°C/min). For short-term storage, vials were transferred to freezer boxes and maintained at -80°C. For storage longer-term, vials were transferred to a liquid nitrogen tank.

2.1.2.4 Cell counting

Cell counts were performed under a light optical microscope (Motic AE 2000; VWR) using a haemocytometer (Hirschmann Instruments™ Counting Chamber; Fisher Scientific). Cells were counted in the four corner squares and the average of counted cells was used to calculate the concentration in cells / ml (multiplication factor 10^4, see Appendix IV) prior to expansion / maintenance or for use in experiments.

2.2 Porcine cytomegalovirus cultivation

2.2.1 Virus

Frozen PFT cells infected with porcine cytomegalovirus (PCMV) wild type (WT) were kindly provided by Dr. Jay Fishman, Harvard Mass General Hospital, Boston, USA forwarded by Dr. Helen Crooke, Animal and Plant Health Agency (APHA)-Weybridge, New Ham, Surrey, UK. Little information was given in regard to this WT strain hence this strain was called ‘JF strain’.

PCMV WT (JF strain) infected PFT cells were cultured and maintained in MEM-10 at 37°C in 5% CO_2 humidified atmosphere.

2.2.2 Culture maintenance

2.2.2.1 Thawing of cells

Cryovials containing 1 ml of PCMV WT (JF strain) infected PFT cells in 10% freezing media were rapidly thawed in a 37°C water bath and transferred into 10 ml prewarmed MEM-10. After centrifugation at 296 x g (1200 rpm; Eppendorf Centrifuge 5810R, rotor S-4-104; Eppendorf) for 5 min at RT, the supernatant was discarded, and the cell pellet was gently resuspended in 10 ml prewarmed MEM-10. Cells were counted and diluted to 2.1 x 10^4 cells / ml (Appendix IV) in MEM-10.

2.2.2.2 Growth of cells

For maintenance of PCMV WT (JF strain)-infected PFT cells, a 175 cm^2 cell culture flask of uninfected PFT cells was trypsinized (as described above) and resuspended to a concentration of 8.5 x 10^4 cells / ml MEM-10. A 1 ml volume of PCMV WT (JF strain) infected PFT cells (at a concentration of 2.1 x 10^4 cells / ml) was then mixed with an equivalent volume of uninfected PFT cells (at a concentration of 8.5 x 10^4 cells / ml), equivalent to a 1:4 ratio. The entire mixture was then transferred to a 75 cm^2 cell culture flask, the volume increased to 15 ml by addition of MEM-10 and cells were cultured at 37°C in a 5% CO_2 humidified atmosphere. Every 4 days, 5 ml of media was replaced, and cells were monitored via light optical microscopy until cells had reached confluency and cytopathic effect (CPE), characterised by swollen ‘cytomegalic’, rounded up cells.

2.2.2.3 Cell-free PCMV WT (JF strain) seed-stock production

Extracellular and cell-associated virus from a 175 cm^2 cell culture flask was harvested by scraping the cells into the supernatant, followed by centrifugation at

296 x g (1200 rpm; Eppendorf Centrifuge 5810R, rotor S-4-104; Eppendorf) for 5 min at RT. The clarified supernatant was then transferred to a fresh tube. Cell pellets were freeze-thawed three times, followed by resuspension in 5 – 10 ml of clarified supernatant. Resuspended cells were centrifuged again at 296 x g (1200 rpm; Eppendorf Centrifuge 5810R, rotor S-4-104; Eppendorf) for 5 min at RT. The residual pellet was discarded, and all clarified supernatants were combined, aliquoted into 1 ml aliquots in cryovials (Fisherbrand™ Polypropylene Microcentrifuge Tubes; Fisher Scientific) and stored in freezer boxes at -80°C.

2.2.2.4 Large-scale PCMV WT (JF strain) stock production

PFT cells were cultured as described (see Section 2.1.2.2) and expanded into ten 175 cm^2 cell culture flasks. After reaching a confluency of 70 – 80% media was removed and replaced by 20 ml fresh MEM-10 per flask. 10 ml seed stock were added to 40 ml MEM-10 and 5 ml of this suspension were added to each 175 cm^2 cell culture flask of PFT cells, followed by culturing until CPE was evident throughout the monolayer. The virus was harvested as previously described (Section 2.2.2.3) and stored as large 'bulk' stocks at -80°C. For final stock production, frozen 'bulk' stocks were rapidly thawed in a water bath at 37°C, pooled and then added to high speed polycarbonate centrifuge tubes (Thermo Scientific Nalgene™ Oak Ridge; Fisher Scientific) (31ml volume supernatant added per tube). Supernatants were underlaid with a 4 ml 20% (w/v) Sorbitol cushion and centrifuged at 69,600 x g (24,000 rpm; Beckmann Coulter Avanti® J-26XP, Rotor JA 25.50) for 80 min at RT. After centrifugation, supernatants were decanted, and virus pellet were resuspended in 2 ml of 2% FBS in DPBS. Virus stocks were then aliquoted and stored at -80°C.

2.2.2.5 Initiation of experimental infections

A T75 cm^2 cell culture flask of uninfected / PCMV WT (JF strain) infected PFT cells was set up in the same ratio as described (Section 2.2.2.2). 5 ml of supernatant were harvested after 5, 10, 16, 22, 30 and 37 days and replaced by MEM-10. The supernatant was aliquoted into 1 ml aliquots in cryovials (Fisherbrand™ Polypropylene Microcentrifuge Tubes; Fisher Scientific), stored in freezer boxes at -80°C and used in experiments.

2.2.3 Determination of infectious titre by $TCID_{50}$ (tissue culture infectious dose) assay

PFT cells were seeded at a density of 2 x 10^4 cells / well in 100 µl volumes of MEM-10 in flat-bottom 96-well plates and incubated overnight at 37°C in 5% CO_2 humidified atmosphere. Virus stocks to be titrated were ten-fold serially diluted and 100 µl of each dilution was added to each row. Ten replicate wells were used for each dilution, along with two uninfected control wells. Plates were incubated at 37°C in 5% CO_2 humidified atmosphere. Cells were visualised by light microscopy (Motic AE 2000; VWR) and scored as positive when CPE was detected. $TCID_{50}$ / ml was determined by the calculation of Reed and Muench (http://xenobiologista.com/wp-content/uploads/2014/05/Reed-Muench-Lindenbach-version-2008_$TCID_{50}$-calculator.xls). Titres are expressed as plaque forming units per ml (pfu / ml) by multiplying the $TCID_{50}$ / ml by a conversion factor of 0.7 (0.7 = natural logarithm (ln) of 0.5 where 0.5 states any titre expressed as a $TCID_{50}$; ATCC, converting $TCID_{50}$ to PFU).

2.2.4 Virus DNA extraction

Viral DNA was extracted using a QIAmp MinElute Virus Spin Kit (QIAGEN, Manchester, UK) according to the manufacturer's instructions. Briefly, high-titre crude viral stock aliquots were adjusted to 200 µl total volume with DPBS. 25 µl of QIAGEN Protease and 200 µl of Buffer AL (containing carrier RNA) were added and mixed thoroughly. Samples were then removed from the bio-safety cabinet and incubated at 56°C for 15 min. 250 µl ethanol (96 – 100%, Fisher Scientific) was added, mixed thoroughly and incubated for 5 min at RT. The lysate was transferred to a QIAmp MinElute column, and centrifugation and washing steps were carried out, as per manufacturer's instructions. Residual ethanol was removed by incubation at 56°C for 3 min. DNA was eluted from the column twice with 50 µl Buffer AVE and stored at -20°C.

2.2.5 Targeted degradation of non-encapsulated viral DNA

In order to distinguish between the total viral genome copy number and virion-protected genome copy number, viral supernatant was treated with DNase I to degrade viral DNA not protected within a viral particle. Twenty microliters of (10X) DNase I Reaction Buffer (NEB, Hitchin, UK) and 5 µl DNase I (RNase free; NEB) were mixed and added to 175 µl of viral supernatant. The reaction was pulse-vortexed to mix and incubated at 37°C for 10 min. After heat inactivation of the enzyme at 75°C for 10 min, DNA was extracted from the samples as previously described (Section 2.2.4).

2.2.6 Spinoculation of virus onto cell monolayer

PFT cells were seeded at a density of 5 x 10^5 cells / well in 2 ml volume of MEM-10 in two flat-bottom 6-well plates and incubated overnight at 37°C in 5% CO_2

humidified atmosphere. Plates were infected with 200 µl of day 22 PCMV WT (JF strain) supernatant (as detailed in Table 5). One plate was then centrifuged at 1000 x g for 30 minutes at RT, the control plate was not centrifuged. Both plates were incubated at 37°C in 5% CO_2 humidified atmosphere. Cells were fed with fresh media once per week. Virus titre (pfu/ml) was then determined by $TCID_{50}$ assay as described in Section 2.2.3.

2.3 Bacterial cell culture

2.3.1 Inoculation

Overnight bacterial cultures [3 – 5 ml Luria broth (LB); Fisher Scientific], with appropriate antibiotics (Appendix I) were inoculated either with a single colony from LB agar (Fisher Scientific) plates, or from frozen glycerol stocks (Section 2.3.2). Cultures were incubated at 30°C for 12 – 18 hr in a shaking incubator at 230 rpm (Incubating Orbital Shaker; VWR) with a vented cap. After incubation, culture growth was assessed by turbidity. Overnight cultures were then used for either preparing glycerol stocks for long term storage, or for extraction of DNA.

2.3.2 Glycerol stocks

Glycerol stocks were prepared by mixing equal amounts of overnight cultures (Section 2.3.1) with 80% (v/v) glycerol (molecular grade, Bp 229-1; Fisher Scientific) in 1.8 ml Nunc™ cryovials (Fisher Scientific). Glycerol stocks were then stored at -80°C.

2.4 Molecular biology methods

2.4.1 Plasmid DNA extraction (miniprep/midiprep)

Five millilitre cultures of *E. coli* containing plasmids of interest were cultured overnight with appropriate antibiotics (Appendix I); *E. coli* strain used for culture depended on the plasmid being amplified. Cultures were centrifuged at 2300 x g (3500 rpm; Heraeus™ Multifuge™ X1 Centrifuge, rotor TX-200; VWR) for 15 min at RT to pellet bacteria. Plasmid DNA was then extracted and purified using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) and a PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen; Fisher Scientific) respectively, in accordance with manufacturer's instructions. Briefly, bacterial pellets were resuspended in Resuspension Solution containing RNase A, lysed with Lysis Solution and pH neutralised and precipitated with Neutralization Solution and Precipitation Buffer, respectively. After centrifugation, clarified bacterial lysates were transferred to DNA Binding Columns, followed by several wash steps with centrifugation, as per manufacturer's instructions. Plasmid DNA was then eluted from columns with Elution Solution and stored at 4°C or -20°C for longer term storage. DNA concentration and purity were determined using a nanodrop-spectrophotometer (Section 2.4.4).

2.4.2 DNA restriction enzyme digestion

Plasmid DNA digestion was performed using restriction enzymes and buffers from New England Biolabs (NEB). High-Fidelity (HF®) enzymes were used when available. For sample reaction volumes, see Appendix II. Digestion was always performed for 2 h at 37°C. Prior to subsequent use in cloning, restriction enzymes were either heat inactivated when possible, or alternatively treated with SDS (0.1

– 0.5 %) and purified by PCR spin-column purification (see Section 2.4.3). Digestion was confirmed by using agarose gel electrophoresis (Section 2.4.6).

2.4.3 DNA purification

PCR products and digested plasmid DNA were purified to remove primers, dNTPs, enzymes and salt by using PureLink® PCR Purification Kit (Invitrogen; Fisher Scientific) according to the manufacturer's instructions. Briefly, 4 volumes of binding buffer (B2 or B3, depending on the DNA size) were mixed with 1 volume of DNA. The DNA mixture was then added to the spin-column and centrifuged at 12,000 x g (rpm 11807; Eppendorf Centrifuge 5418, rotor FA-45-18-11; Eppendorf) for 1 min at RT. After washing and centrifugation steps, DNA was eluted in Elution buffer and stored at -20°C. Purified DNA was visualized by agarose gel electrophoresis (Section 2.4.6).

2.4.4 DNA quantification (Nanodrop)

Purified DNA was quantified using a NanoDrop™ ND-2000 Spectrophotometer (ThermoFisher Scientific, Paisley, UK). One microliter of DNA was used for quantification. Measurements were taken at wavelengths of 230 nm, 260 nm and 280 nm. A ratio of 260 / 280 $\geq$ 1.8 and 260 / 230 between 2.0 and 2.2 indicate DNA suitably free from contaminants such as proteins or salt for down-stream application.

2.4.5 Polymerase chain reaction (PCR)

2.4.5.1 Standard PCR

All primers (Table 3) were purchased from Eurofins Genomics (Wolverhampton, UK). High quality HPLC- or PAGE-purified primers were used for all cloning and

E/T-based recombinations to minimize the risk of sequence errors being introduced at the primer level. Whilst preparing PCR reactions, all reagents were kept on ice to minimize primer dimer formation, and PCR enzymes were stored in a bench cooler to avoid denaturation. Non-template negative control reactions were included with every PCR, with molecular grade water substituted for the DNA template. PCR reactions were performed in duplicate. After preparing the reaction mix (Appendix III) PCR was carried out in duplicate in a T100™ Thermal Cycler (Bio-Rad Laboratories Ltd, Watford, UK). PCR programs and extension times were adjusted according to the nature of the template being amplified (Appendix III). PCR products were visualized by agarose gel electrophoresis (Section 2.4.6).

Primer	Sequence
BDV E2 Forward	GCGCGCTAGCACCATGGCATCGA GCAACAGCCTTC
BDV E2 Reverse	GCGCGCGGCCGCTCACGTAGAAT CGAGACCGAGGAGAGGGTTAGGG ATAGGCTTACCTGCTGATGCCATT GCTC
pMiniOri BGH pA FOR	GCGCCCTAGGAAGCCATAGAGCC CACCGCA
pMiniOri EF1a REV	GCGCCCTAGGTTCTGAATTTCGA TGCGC

Table 3. Primers for generating and sequencing E2 BDV Gifhorn recombinants

2.4.5.2 Droplet digital PCR (ddPCR)

PCMV primers and probes (Table 4), targeting the U38 gene (encoding a DNAse polymerase catalytic subunit), were kindly provided by Dr. Helen Crook. The ddPCR was performed using virus supernatant that had either been DNase I-treated or non-treated (Section 2.2.5) prior to DNA extraction (Section 2.2.4). This comparison allowed differences between total genome copy number and copy number within the viral particles. The Bio-Rad QX100™ Droplet Digital™ PCR System was used for ddPCR in accordance with the manufacturer's instructions. Briefly, the reaction mix (Appendix III) was thoroughly mixed and incubated for 3 min at RT. DG8^{TM} Cartridge wells were loaded with 20 µl of each reaction mix and 70 µl of Droplet Generator Oil for Probes. The cartridge was then placed in an Automated Droplet Generator. After droplets were generated the samples were transferred to a 96-well plate and sealed with a PX1 PCR Plate Sealer prior to PCR amplification in a C1000 Touch Thermal Cycler (Appendix III). After amplification, the plate was placed into the QX100 Droplet Reader. DNA concentration was measured using QuantaSoft^{TM} Software and reported as copies / µl. Viral supernatant from a harvest day 10 post-infection was used as a positive control and nuclease-free water was used as a negative control.

Primer	Sequence
Forward PCMV primer	GTTCTGGGATTCCGAGGTTG
Reverse PCMV primer	ACTTCATCGCAGCTCATCTGA
Probe	FAM CAGGGCGGCGGTCGAGCTC TAM

Table 4. Primers for quantifying viral DNA. The probe is dual labelled with a FAM as a 5' Fluorophore and a TAM as a 3' Quencher.

2.4.6 Agarose gel electrophoresis

Agarose gels (1%w/v) were prepared by dissolving molecular biology grade agarose powder (Fisher Scientific) in 1X Tris-acetate-EDTA buffer (50 x TAE Buffer; Fisher Scientific) in the microwave, allowing to cool and then pouring into a gel-forming tray. Ethidium bromide (EtBr, 0.5 µg/ml; Fisher Scientific) was added and mixed thoroughly. DNA samples were prepared by mixing with loading dye (gel loading dye purple (6X); NEB) to a final concentration of 1X and then loaded into the wells of the gel. A molecular weight marker (either Invitrogen™ 1 Kb Plus DNA Extension ladder or NEB Quick-Load® 1 Kb Extend DNA Ladder) was added to each gel. Gels were electrophoresed in a horizontal tank filled with 1X TAE buffer at 80 V for 1.5 hr. DNA was visualized under UV light and imaged using a gel imager (ChemiDoc-IH^2 with VisionWorks®LS Software, Ultra-Violet Products Ltd (UVP), Cambridge, UK).

2.4.7 DNA plasmid dephosphorylation

One microliter of Antarctic Phosphatase (NEB) together with 2 µl of Antarctic Phosphatase Reaction Buffer (10X concentration; NEB) were added to 20 µl of purified digested vector plasmid. After incubation at 37°C for 1 hour, the mixture was heat inactivated at 75°C for 5 min. In some cases, incubation times at 37°C were increased to 2 hr to further reduce parental plasmid contamination resulting from relegation of non-fully dephosphorylated vector. The dephosphorylated backbone vector was either used immediately for ligation reactions or stored at -20°C.

2.4.8 DNA ligation

Dephosphorylated vector backbone and purified E2 BDV Gifhorn PCR product (insert DNA) were ligated using DNA ligase (NEB). Dephosphorylated vector backbone (1ul), 3 µl insert DNA, 2 µl ligation buffer (10 x), 1 µl T4 DNA ligase and 13 µl molecular grade dH_2O were mixed together and incubated at RT for 30 min. The ligase was then heat inactivated at 65°C for 20 min. A negative control which lacked DNA insert was also included. Ligation reactions were either immediately transformed into bacteria or were stored at -20°C until use for bacterial transformation.

2.4.9 Transformation of bacteria

Chemically competent PIR1 *E. coli* (Invitrogen; Fisher Scientific) were thawed on ice and gently transferred into pre-chilled 12 ml polypropylene tubes. 0.5 - 1 µl plasmid, or 4 µl ligation product were added, gently mixed by shaking and incubated on ice for 30 min.

PIR1 bacteria contain a 'pir' gene which expresses the protein π. It is required for replication from an R6Kγ ori. This ori is called a 'suicide' ori as plasmids having this R6Kγ ori can only be replicated in PIR1 bacteria. The bacteria were heat shocked at 42°C for 30 sec without shaking and recovered on ice for 5 min. Prewarmed S.O.C medium (250 µl) was added and bacteria were allowed to recover at 30°C for 2 hours in a shaking incubator at 230 rpm (Incubating Orbital Shaker, VWR). Following recovery, bacteria were plated at different volumes (25 µl and 250 µl) on LB agar containing the relevant antibiotics. Plates were incubated at 30°C for 24 to 48 hours (INCU-Line, VWR). Colonies were picked, masterplated and DNA from overnight cultures was screened by restriction digest (Section 2.4.2). Digested DNA was visualised by agarose gel electrophoresis (Section 2.4.6) and band sizes were analysed to identify colonies carrying the correct recombinant plasmid. Once identified, the sequence of these clones was then confirmed by direct DNA Sanger sequencing (Eurofins Genomics). Glycerol stocks (Section 2.3.2) of selected clones were then prepared for long-term storage.

2.5 Biological methods

2.5.1 ELISA

Blood was collected from 20 abattoir pigs. Samples were allowed to clot by incubation at RT followed by centrifugation. Serum was removed and stored at -20°C. Prior to use, serum was heat inactivated at 56°C for 30 min to inactivate complement and unknown adventitious pathogens (Soltis *et al*, 1979). Serum samples were analysed by ELISA to detect PCMV-specific antibodies using a 'Porcine Anti-cytomegalovirus IgG antibody (Anti-CMV IgG) ELISA Kit' (Sincere;

2bscientific, Upper Heyford, UK), in accordance with the manufacturer's instructions. The starting standard used in this ELISA contained a Porcine Anti-CMV IgG concentration of 20 ng/ml which was then serially diluted to 0.312 ng/ml to generate a linear regression curve for analysis. Briefly, either 100 µl of standards, 1:2 diluted samples or standard diluent buffer (negative control) were added to pre-coated wells of a 96-well plate containing porcine anti-CMV IgG which, in this case, serves as the corresponding antigen for capturing porcine anti-CMV IgG antibodies in the sample. The plate was incubated and washed prior to addition of 100 µl of Detection Porcine Anti-CMV IgG solution, followed by 100 µl Avidin-Biotin-Peroxidase Complex and 100 µl tetramethylbenzidine (TMB). After development of blue colour in the 3 - 4 most concentrated standard solution wells, 100 µl acidic TMB stop solution was added to all wells, causing a colour change from blue to yellow. Within 10 min the OD absorbance was measured in a microplate reader (FLUOstar Omega, BMG LABTECH) at 450 nm. The concentration (ng / ml) was determined using linear regression based on blank corrected raw data, taking the dilution factor into account (Omega Control Software).

2.5.2 DNA transfection

Purified DNA was transfected into PFT cells and PCMV WT (JF strain) infected PFT cells, using Lipofectamine™ 3000 (Invitrogen, Fisher Scientific). Briefly, cells were seeded at a density expected to yield 80% confluent monolayers on the day of use. Prior to transfection the media in the wells was replaced with MEM containing 1% FCS, without antibiotics. Lipofectamine™ 3000 reagent was mixed with Opti-MEM™ Medium (Gibco™ Opti-MEM™ I Reduced Serum Medium, No Phenol Red; Fisher Scientific) and DNA was mixed with Opti-MEM™ Medium and

p3000™ Reagent, as per manufacturer's instructions. The Lipofectamine™ 3000 mix and the DNA were combined in a 1:1 ratio and incubated for 5 min at RT. The DNA-Lipofectamine mix was then added to wells in a dropwise manner and the plate was rocked to spread evenly. Plates were then centrifuged at 1000 x g (3000 rpm; Eppendorf Centrifuge 5810R, rotor S-4-104; Eppendorf) for 30 min at RT to 'spinoculate' the DNA-Lipofectamine mix onto the monolayers. After a few hours the media was replaced with MEM-10 and monitored under a fluorescence imaging system (ZOE Fluorescent Cell Imager; Bio-Rad Laboratories Ltd.).

Chapter 3

Results

3.1 Characterisation of PCMV *in vitro* in terms of distribution between cells and supernatant prior to growth of PCMV

3.1.1 Harvest of supernatant

CMVs are known to differ substantially in their characteristics of growth *in vitro* in regard to relative levels of cell-associated versus cell-free virus. Clinical isolates tend to be highly cell associated during initial passages, and this cell-associated phenotype is progressively lost following repeated passaging *in vitro* – usually correlating to changes within the viral genome (Yamane *et al*, 1983; Sinzger *et al*, 1999; Dargan *et al*, 2010). This characteristic can be influenced by the particular CMV, level of virus passage, as well as cell type being used for culture. The aim of these initial studies was to determine the replication kinetics of PCMV WT (JF strain) in porcine fallopian tube (PFT) cells and to assess the relative distribution of virus between cell-associated and cell-free compartments.

PFT cells were infected with PCMV WT (JF strain) as described in Section 2.2.2.2 and 2.2.2.5. Twice a week supernatant (5 ml) was harvested and replaced with fresh MEM-10. Harvested supernatant was stored at -80°C. Cytopathic effect (CPE) started to become visible around day 20, with

approximately 80% of PFT monolayers infected (determination based on experience) by day 34. PFT cells were harvested at day 37 post-infection.

3.1.2 Titration of infectious virus in harvested supernatant by $TCID_{50}$ assay

$TCID_{50}$ is a standard assay used in virus titration. In the case of PCMV, as we knew very little of its growth kinetics, we sought first to optimise this assay. Day 22 post-infection supernatant was used for the optimisation. The day 22 supernatant used was selected for the experiment as it appeared to have the highest titre as visual inspection indicated that approximately 80% of the monolayer was infected based on CPE. Following infection of the $TCID_{50}$ plate, $TCID_{50}$ readings were taken at day 22, 25, 27, 29 and 32 post-infection. As shown in Figure 8, titres did not significantly increase after reading at day 29, we therefore selected day 29 as suitable time for $TCID_{50}$ assays.

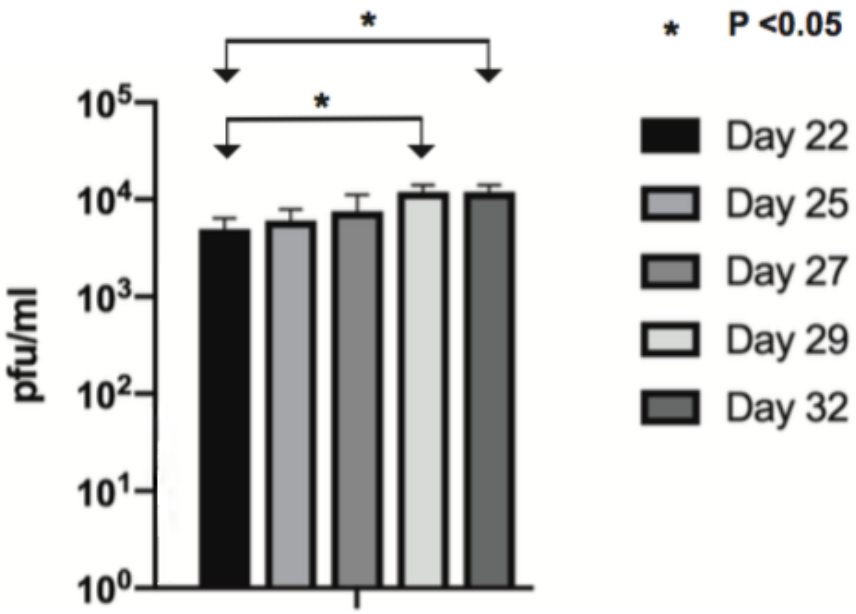


Figure 8. Titration of virus SN at increasing times on PFT cells**.** PFT cells were infected with day 22 post-infection supernatant and the virus was cultivated at 37°C. The virus titres were determined by $TCID_{50}$ assay at various days post-infection of $TCID_{50}$ plates. Data is presented as the average (+/- standard error of the mean) of three experiments, each carried out in 10 replicates.

Following optimisation, we sought to determine the kinetics of virus release from PCMV WT (JF strain) infected PFT cultures. PCMV WT (JF strain) titres released into supernatant at days 5, 10, 16, 22, 30 and 37 post-infection were measured by $TCID_{50}$ (Section 2.2.3). We had hypothesized that the highest titre would be determined after the monolayer was completely infected. However, as shown in Table 5, the highest titre was from day 22 at a time when visual inspection indicated that less than 80% of the monolayer was infected based on CPE. At later times, even though CPE was more extensive, reaching 100% of the monolayer, the titre did not increase.

SN harvested day	Titre (pfu/ml)
5	< 1.0E+02
10	< 1.0E+02
16	2.21E+03
22	4.77E+03
30	2.21E+03
37	3.68E+03

Table 5. Titration of supernatant by $TCID_{50}$ assay, harvested from PCMV WT (JF strain) infected PFT cells at different time points post-infection.

3.1.3 Quantification of infectious virus in harvested supernatant by droplet digital PCR (ddPCR)

Droplet digital PCR (ddPCR) (Section 2.4.5.2) was used for genome copy number quantitation. DNAse I treatment (Section 2.2.5) was used to enable distinction between total genome compared to virion-protected genome copy number released into the supernatant. Using this approach, DNA extracted from untreated supernatant is equivalent to the total genome number, whereas DNA extracted from DNAse I treated supernatant is equivalent to virion-protected genome numbers. The ratio of total genome copies to virion genome copies being greater than 1 (Table 6) indicated that a proportion of PCMV genomes were exposed to DNAse I and represented non-virion associated genome.

SN harvested day	Total genome copy (copies/ml)	Virion genome copy (copies/ml)	Ratio Total genome / Virion genome
5	2.60E+06	1.80E+06	1.4:1
10	3.32E+07	3.02E+07	1.1:1
16	8.58E+08	1.23E+08	7.0:1
22	4.20E+09	1.12E+09	3.8:1
30	2.34E+09	9.82E+08	2.4:1
37	>1.0E+10	>1.0E+10	**

Table 6. Quantitation and ratio of total genome and virion genome copy numbers in supernatant by ddPCR, harvested from PCMV WT (JF strain) infected PFT cells at different time points. ** Could not calculate ratio because the individual numbers were higher than the cut-off.

The particle to PFU ratio, represented below as the virion genome to infectious particles ratio, is a measure of virus particles that can complete an infection cycle (i.e. lead to formation of a plaque), relative to the total number of virus particles present. The lowest value possible is 1, and this means that every single virus present is capable of forming a plaque. This is quite unusual but has been observed with some viruses such as Semliki Forest virus (Klasse, 2015; Virology blog: Are all virus particles infectious?). A high particle to PFU ratio may indicate the presence of non-infectious defective particles, but often there is nothing 'wrong' with the particles other than they have been prevented from completing their infectious cycle for some reason e.g. unable to reach cells for attachment to occur, or perhaps underwent an abortive infection that did not result in plaque formation.

The ratio of virion-associated (DNAse I protected) genome copies to infectious particles on the basis of $TCID_{50}$ indicated that a proportion of virion-associated genome copies (DNAse I protected) were non-infectious (Table 7). Virion-associated genome copies and infectious particles were determined from the same sample. Of note, the sharp increase in the ratio seen at day 37 post-infection was also reflected in whole genome sequencing data, which reported large genome deletions in PCMV WT (JF strain) at this time-point, relative to genome samples from earlier time points. This indicates an accumulation of defective particles that have lost large segments of their genomes, at later time-points post-infection.

SN harvested day	**Virion genome copy (copies/ml)**	**Infectious particles (pfu/ml)**	**Ratio Virion genome / Infectious particles**
5	1.80E+06	< LOD	< LOD
10	3.02E+07	< LOD	< LOD
16	1.23E+08	2.21E+03	5.57E+04:1
22	1.12E+09	4.77E+03	2.35E+05:1
30	9.82E+08	2.21E+03	4.44E+05:1
37	>1.0E+10	3.68E+03	**

Table 7. Quantification and ratio of virion genome copy numbers and infectious particles in supernatant by ddPCR and $TCID_{50}$ assay, respectively**.** PCMV WT (JF strain) infected PFT cells were harvested at different days post-infection**.** Limit of detection (LOD) ** Could not calculate ratio because one individual number was higher than the cut-off.

3.1.4 Comparison of spinoculated virus versus non-spinoculated virus onto cell monolayer and time-dependent titre determination

Previously, low speed centrifugation has been shown to increase effective multiplicity of infection (MOI) for a virus inoculum (called spinoculation). Whilst the speeds used for centrifugation are not high enough to spin the virus down onto cells, one study using HIV-1 virus demonstrated that the effect seen was due to the effects of centrifugal stress on the cell monolayers, rather than directly on the virus. They found that spinoculation triggered dynamic actin and cofilin activity, enhancing HIV-1 binding and infection (Osborn & Walker, 1968; Cheng *et al*, 2007; Guo *et al*, 2011). In the next series of experiments, we determined whether PCMV WT (JF strain) infection of PFT cells was susceptible to enhancement by low speed centrifugation. Our hypothesis was that spinoculation of PCMV WT (JF strain) onto PFT cells would significantly enhance infection, which would be visualized by increased virus levels in the supernatant. The results are shown in Figure 9. Using a two-tailed t-test, we found there was a significant difference at day 29 ($P = 0.0154$) between spinoculated and non spinoculated plates.

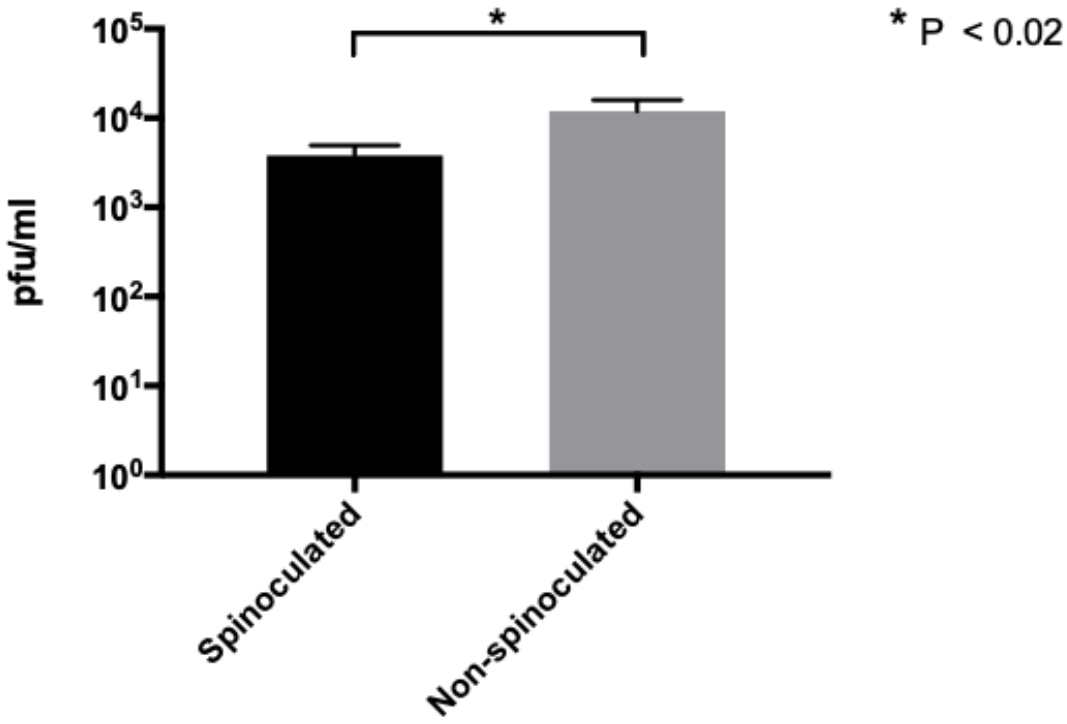


Figure 9. Titration of spinoculated and non-spinoculated virus onto PFT cells monitored at day 29. PFT cells were infected with PCMV WT (JF strain) SN harvested at day 22 post-infection and the virus was either spinoculated or not onto the monolayer and cultivated at 37°C. At day 29 post-infection, the virus titres were determined by $TCID_{50}$ assay. Data is presented as the average (+/- standard error of the mean) of three experiments, each carried out in 10 replicates.

3.1.5 Preparation and titration of virus stock

Concentrated virus stocks were prepared as described in (Section 2.2.2.4). After preparation of concentrated virus stock, the infectious titre was determined by $TCID_{50}$ assay (Section 2.2.3) as shown in Table 8. We hypothesized that the titre would be similar to other cytomegaloviruses that are in use in the lab such as rhesus macaque CMV (RhCMV) or murine CMV (MCMV) with pfu/ml at about 10^7 to 10^9. As shown in Table 8, although reproducible, PCMV WT (JF strain) has a significantly lower titre.

PCMV WT (JF strain) stock concentrated	Titre (pfu/ml)
21 June 2018	1.95E+04
10 July 2018	2.50E+04

Table 8. Titre of two different PCMV WT (JF strain) concentrated virus stocks were determined by $TCID_{50}$ assay.

3.1.6 Sequence analysis of concentrated virus stock

The PCMV WT (JF strain) virus stock was sent for full length sequencing to collaborators in the Davison Laboratory at the University of Glasgow. The PCMV WT (JF strain) genome matched fully characterised genome available in the GenBank database [GenBank no: KF017583] (mentioned in 1.4.1.3.3, (Gu *et al*, 2014)), however, PCMV WT (JF strain) had an additional 10 Kb terminal region, which is not present in the GenBank strain. This information was given by the collaborator as unpublished privileged information.

3.2 PCMV BAC cloning

For design of the shuttle vectors to be used in BAC cloning, an intergenic region (approximately 2800 bp) in the PCMV genome between U1 and U2 was selected as the site for BAC cassette insertion within the PCMV genome. The U1 and U2 genes run in opposite directions, meaning insertion into this location is less likely to cause issues with expression of either U1 or U2 as no

upstream regulatory sequences will be disrupted during the cloning. Additionally, U1 and U2 are non-essential genes within the PCMV genome as shown in Figure 5. The BAC cassette is approximately 8900 nt. An additional construct that would result in deletion of PCMV genes U2 through U4 (approximately 7000 bp) was also constructed to avoid over-size genome issues that can occur due to capsid packing size constraints. Homologous recombination is the means by which the BAC cassette will be inserted within the PCMV genome. PCMV recombinant plasmids comprised of a BAC cassette derived from pHA2, flanked by homologous regions to the site of insertion within the PCMV genome were available as pU1-BAC-U2 and pU1-BAC-U5 respectively at the beginning of this thesis work (generated by Dr. Michele Kiernan, University of Plymouth, UK), as was the g-RNA designed for insertion of pU1-BAC-U2 and pU1-BAC-U5 into the PCMV genome by CRISPR/Cas9 technology (generated by Dr. Michael Jarvis, University of Plymouth, UK).

3.2.1 Construction of a recombinant plasmid by cloning the BAC cassette between the U1 and U2 gene and the U1 and U5 gene, respectively in the PCMV genome

An infectious PCMV BAC would be extremely advantageous for manipulation of the virus genome. In order to achieve this aim, a BAC cassette containing genetic elements required for replication and maintenance of the genome in bacterial cells, must first be inserted within the virus genome. An aim of the current project was to construct plasmids that would enable insertion of the required BAC cassette within the virus genome. A subsequent modification of this approach inserted a eukaryotic expression cassette expressing a target

antigen from the simulant CSFV agent, border disease virus (BDV), at the same time as the BAC cassette. Two locations within the PCMV genome were chosen to insert the BAC cassette. The first location selected for insertion was between the U1 and U2 genes, and the second location was between the U1 and U5 genes. This second location was selected to result in removal of non-essential genes U2 through to U4, reducing the overall size of the genome. This alternative strategy was incorporated to address the possible concern of size related packaging problems when reconstituting the virus. Figure 10 outlines the cloning strategy, which is described in more detail below.

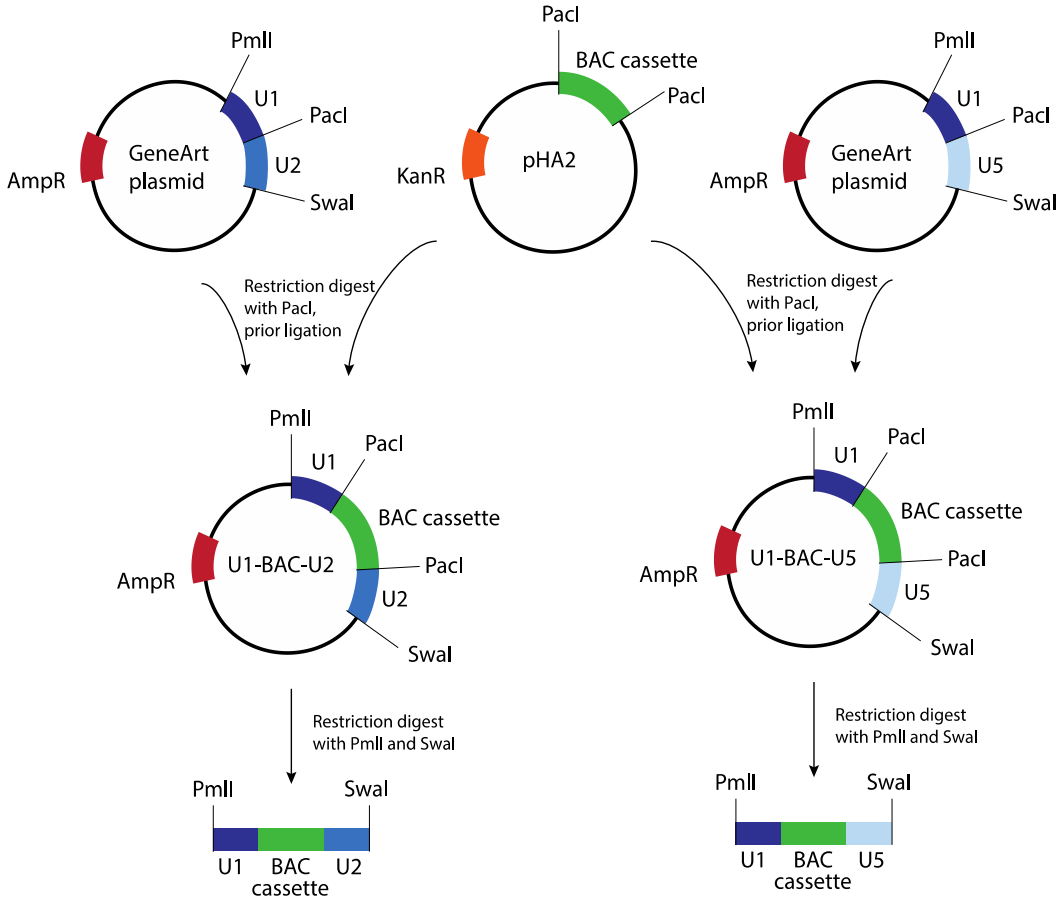


Figure 10. Schematic of construction of a PCMV recombinant U1-BAC-U2 and U1-BAC-U5 plasmid. The BAC cassette from plasmid pHA2 was cloned into a supplied GeneArt plasmid containing the desired flanking region U1-U2 and U1-U5 respectively, next to an Amp^R marker.

3.2.1.1 Excision of the BAC cassette from pHA2

The plasmid pHA2 (kindly provided by Professor Dr. Martin Messerle, Hannover Medical School, Hannover, Germany) which contains a BAC cassette flanked by two PacI restriction sites was transformed into DH10B *E. coli* cells. DNA was extracted (Section 2.4.1) and screened by restriction digest with PacI (Section 2.4.2) followed by gel electrophoresis (Section 2.4.6; Figure 11). The DNA gel confirmed the expected DNA band sizes of approximately 8.9 kb (BAC cassette) and 4.3 kb (pHA2 backbone).

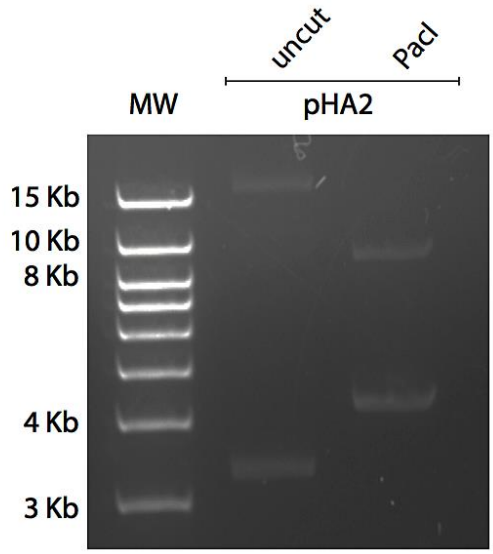


Figure 11. Gel electrophoresis showing PacI digestion of plasmid pHA2. The uncut plasmid was compared to PacI digested plasmid. The expected DNA bands of approximately 4.3 kb (pHA2 vector) and 8.9 kb (BAC cassette) were observed.

3.2.1.2 Insertion of the BAC cassette into U1-U2 and the U1-U5 shuttle plasmids

Insertion of the BAC cassette into the PCMV genome at the desired locations required flanking of the cassette by sequences homologous to PCMV U1 and U2 genes and U1 and U5 genes. U1-U2 and U1-U5 homologous regions were synthesized and supplied in plasmids by GeneArt. A PacI restriction site was placed between the U1 and U2, and U1 and U5 homologous regions in the plasmids, to enable cloning of the BAC cassette into the shuttle vector, as depicted in Figure 10. The GeneArt plasmid was transformed into DH10B *E. coli*. DNA was extracted from an overnight culture, miniprepped and digested with PacI. The digested plasmid was then dephosphorylated and ligated with the BAC cassette, liberated from pHA2 by PacI digestion. The ligation reaction was transformed into DH10B *E. coli*, followed by plating onto LB plates containing 100µg/ml Carbenicillin and 17.5 µg/ml Chloramphenicol and incubation at 30°C. Once colonies were visible, they were masterplated and overnight cultures were inoculated for screening. DNA was extracted (Section 2.4.1) from these overnight cultures and screened by restriction digestion with PacI and BamHI (Section 2.4.2), followed by gel electrophoresis (Section 2.4.6; Figure 12). PacI digests of U1-BAC-U2 and U1-BAC-U5 confirmed the presence of bands at the correct sizes (U1-BAC-U2 at 5.2 kb and 8.9 kb; U1-BAC-U5 at 4.9 kb and 8.9 kb). BamHI digests were used to determine the orientation of the BAC cassette within the shuttle vectors. DNA bands at a molecular weight of 4.6 kb and 9.4 kb confirmed insertion in the forward orientation for U1-BAC-U2, and DNA bands at 2.1kb and 11.6 kb confirmed insertion in the reverse orientation for U1-BAC-U5.

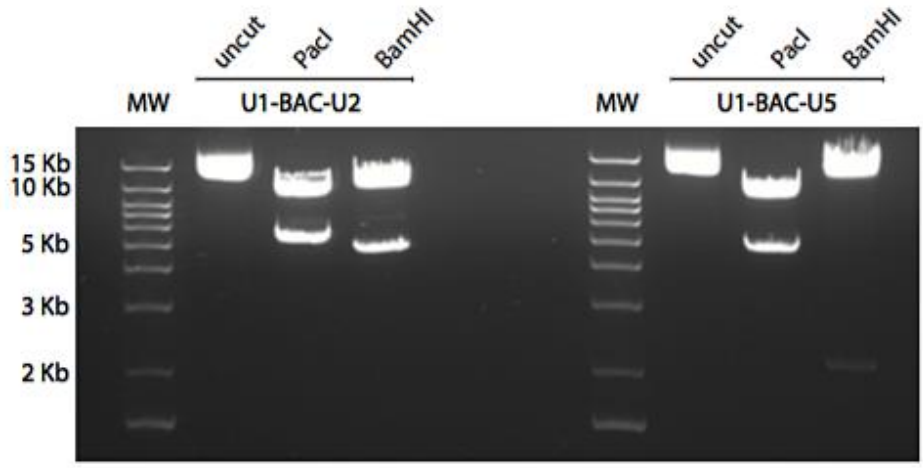


Figure 12. Gel electrophoresis showing PacI and BamHI digestion of U1-BAC-U2 and U1-BAC-U5. The uncut plasmid was compared to PacI and BamHI digested plasmids. The gel confirmed the correct DNA band sizes for PacI digested U1-BAC-U2 (5.2 kb and 8.9 kb) and U1-BAC-U5 (4.9 kb and 8.9 kb), and the BamHI digests confirmed orientation of BAC cassette insertion for U1-BAC-U2 (4.6 kb and 9.4 kb; forward orientation) and U1-BAC-U5 (2.1 kb and 11.6 kb; reverse orientation)

3.2.1.3 Excision of U1-BAC-U2 and U1-BAC-U5 from its backbone

Prior to use of the U1-BAC-U2 and U1-BAC-U5 fragments for transfection into eukaryotic PFT cells, the fragment needed to be excised from its vector (outlined in Figure 10). DNA was extracted (Section 2.4.1) from an overnight culture, miniprepped and quantified (Section 2.4.4). DNA was digested with PmlI and SwaI (Section 2.4.2), purified by using a PCR purification kit, followed by gel electrophoresis (Section 2.4.6; Figure 13). The PmlI / SwaI digests of U1-BAC-U2 and U1-BAC-U5 confirmed the presence of bands at the correct sizes (U1-BAC-U2 at 2.3 kb and 11.7 kb; U1-BAC-U5 at 2.3 kb and 11.4 kb). To summarize, a combination of state-of-the-art synthetic and more conventional molecular biology technologies resulted in production of two linear recombination fragments designed to target a BAC cassette to two distinct regions of the PCMV genome.

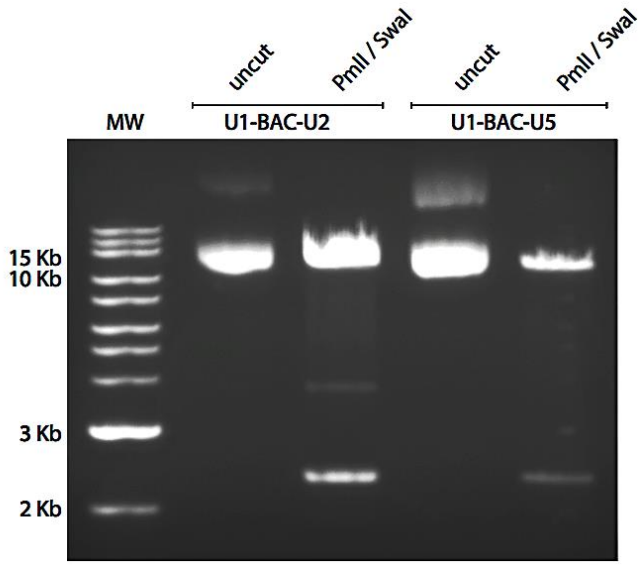


Figure 13. Gel electrophoresis showing PmlI / SwaI digestion of U1-BAC-U2 and U1-BAC-U5 after purification. The uncut plasmid was compared to restriction enzyme digestion with PmlI / SwaI. The gel confirmed the correct DNA band sizes in regard to U1-BAC-U2 of approximately 2.3 kb and 11.7 kb and U1-BAC-U5 of approximately 2.3 kb and 11.4 kb.

3.2.2 Cloning the gRNA into pX330 CRISPR/Cas9 plasmid

Two different approaches to generating a PCMV BAC were taken. One utilized CRISPR/Cas9, which we hypothesized would be more efficient, and the other was based on more conventional homologous recombination. The CRISPR/Cas9 approach required the design of gRNAs targeting the U1-BAC-U2 intergenic region of the PCMV genome for Cas9 cleavage, to induce a double-stranded break. Two independent gRNAs were designed. The gRNAs were inserted into a pX330 backbone vector carrying the Cas9 protein gene, an Amp^R resistance marker gene and several other elements not included in the schematic below. The vector plasmid, originally deposited by the Zhang group at MIT, was supplied by Addgene (Plasmid number: 42230). Once colonies were visible, overnight cultures were prepared. DNA was extracted from each of the two gRNA clones (Section 2.4.1), screened by restriction digest with EcoRI and BbsI to verify insertion, and then quantified (Table 9) to determine the amount of DNA to be used for transfection. gRNA sequence was verified by Sanger sequencing.

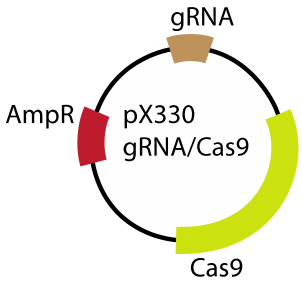


Figure 14. Schematic showing the CRISPR/Cas9 plasmid

3.2.3 Transfection of U1-BAC-U2 fragment and gRNA containing plasmids into PFT cells

For both strategies it was necessary to introduce PCMV and the U1-BAC-U2 fragment into PFT cells. For the CRISPR/Cas9 approach, it was also necessary to introduce the plasmid containing the gRNA, as this was required to introduce a double-stranded DNA break at the correct location on the PCMV genome (target sequence for the gRNA see Appendix VI). The U1-BAC-U2 fragment and the pX330 gRNA plasmids were quantified (Table 9) and transfected into PFT cells by using lipofectamine, a reagent known for high transfection efficiency and low toxicity in many cell types (Cardarelli *et al*, 2016). We anticipated that initially there would be a large number of green cells due to the presence of GFP in the BAC cassette. We then expected these cells to disappear over time as the unintegrated U1-BAC-U2 fragment was degraded, with the only remaining green cells representing cells in which the BAC cassette had integrated into the PCMV genome. As infection progressed we would expect to see expansion of green zones over time.

Multiple different conditions were evaluated in terms of experimental design. First, different amounts of DNA (1 µg and 0.5 µg) were compared for the U1-BAC-U2 fragment and both pX330 gRNA plasmids at a ratio of 1:1 (Appendix V), to assess the impact of DNA concentration on transfection efficiency. An EGFP-C1 plasmid (Clontech) was used as a transfection efficiency control. At day 3 post-transfection, the transfection efficiency rate was approximately 50% (Figure 15), determined by fluorescence read-out, when 0.5 µg of plasmid was used for transfection. As transfection efficiencies did not improve with increased (1 µg) amounts of plasmid, we decided to proceed with 0.5 µg for

further experiments. Transfection efficiencies were always lower with the U1-BAC-U2 fragments than the EGFP-C1 plasmid. This was not unexpected given the larger size of the fragments (approximately 14 kb) relative to the EGFP-C1 plasmid (4.7 kb). As shown in Figure 16, the transfection efficiency was lower again when the CRISPR/Cas9 plasmid was added into the mix. This may be due to additional toxicity caused by adding increased DNA to the cells.

BAC DNA fragments and plasmids were either i) transfected into uninfected PFT cells, which were then infected with PCMV the next day, or ii) transfected directly into infected PFT cells. Cells were fed twice a week with fresh media. In wells containing the pX330 CRISPR/Cas9 gRNA plasmid, non-homologous (NHEJ) end joining inhibitor reagent (SCR7) was added to the media.

Plasmid	ng / µl	260 / 280	260 / 230
PCMV U1-U2-BAC clone 1	1341.0	1.89	2.41
PCMV U1-U5-BAC clone 2	1476.4	1.91	2.44
gRNA pX330 #21 clone 2	246.0	1.91	3.74
gRNA pX330 #23 clone 2	152.7	1.89	5.64
EGFP-C1 (Clontech)	243.8	1.93	3.76

Table 9. Concentration and purity of relevant plasmids used for transfection into PFT cells based on absorbance.

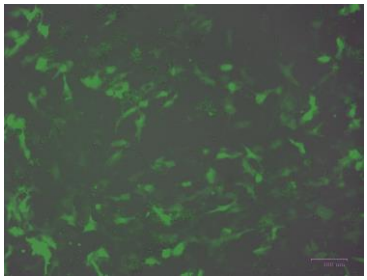


Figure 15. Evaluating transfection efficiency. 0.5 µg EGFP-C1 (Clontech) plasmid was transfected into PFT cells. At 3 days post-transfection the monolayers were imaged, revealing a transfection rate of approximately 50%.

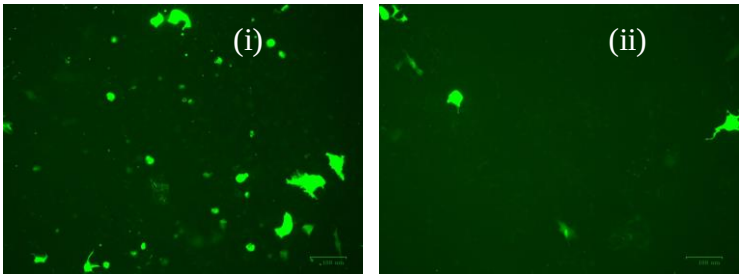


Figure 16. Decreased transfection efficiency when the pX330 CRISPR/Cas9 gRNA plasmid was included during transfection**. (i)** 0.5 µg of U1-BAC-U2 fragment was transfected into PFT cells. (ii) 0.5 µg of U1-BAC-U2 plasmid and 0.5 µg of pX330 CRISPR/Cas9 plasmid were transfected into PFT cells. Both monolayers were infected with PCMV the day after transfection, and monolayers were monitored daily and imaged at 11 days post-transfection. Transfection efficiency appeared reduced in the presence of the pX330 CRISPR/Cas9 plasmid.

Prior to our determination that spinoculation was not an effective means of increasing infection, we had hypothesized that spinoculation would increase the ongoing viral infection. Therefore after 11 days the transfected / infected PFT cells were centrifuged weekly at 1000 x g for 30 min and fed with fresh media. Once it was realized that titres were not enhanced by spinoculation (Section 3.1.4), this practice was stopped. In this initial experiment, cell monolayers partially detached after 22 days, without development of any GFP positive plaques. This was likely due to PCMV infection (i.e. non-recombinant wild-type PCMV), as it was not observed in uninfected control monolayers. To maintain the cultures, fresh PFT cells were seeded on top of the existing cell monolayers, and cells were fed twice a week with fresh media. The experiment was stopped after 20 days post-re-seeding as green fluorescent plaques were not observed.

Next, the U1-BAC-U2 fragment and pX330 CRISPR/Cas9 gRNA plasmid were transfected into PFT already infected with PCMV WT (JF strain) to determine whether this strategy would improve the outcome due to the anticipated higher probability of the transfected DNA entering an infected cell. Addition of the NHEJ inhibitor to the media prior to transfection was also evaluated (Appendix V). However, again no fluorescent green plaques were observed by 30 days.

The BAC cassette contains a gpt gene involved in guanine nucleotide synthesis using xanthine (Mulligan & Berg, 1981). Virus recombinants containing a BAC cassette with the gpt gene are able to form plaques in the presence of GPT, while wild-type (WT) virus cannot (Mulligan & Berg, 1981). Xanthine-guanine phosphoribosyltransferase (GPT) (Merck Millipore) selection reagent which contains mycophenolic acid, aminopterine, xanthine

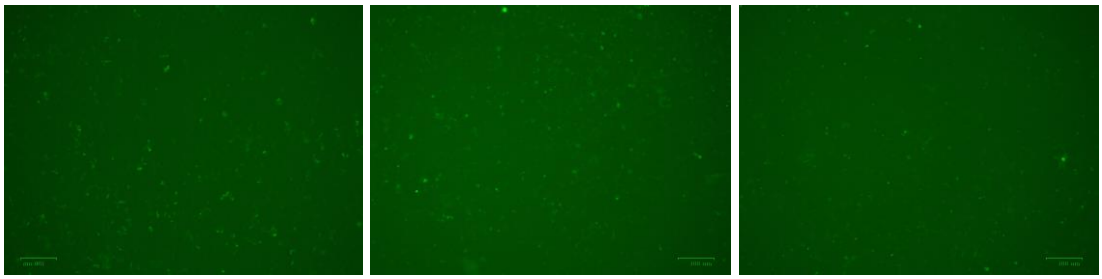
and hypoxanthine was therefore added to the medium post-infection in order to enrich for recombinant PCMV carrying the BAC cassette. Mycophenolic acid and aminopterine inhibit purine metabolism in mammalian cells. The GPT selection reagent was then added during feeding twice a week.

Initial experiments failed as the concentration of GPT was too high and cell monolayers died after 11 days. The experiment was repeated and the U1-BAC-U2 fragment was transfected into non-infected PFT cells, seeded in a 12-well plate at a density of $2x10^5$ cells / well. Day 1 post-transfection the cells were infected with 100 µl PCMV WT (JF strain). GPT selection reagent was added 2 days post-transfection (Appendix V). Fresh GPT selection reagent was added during feeding twice a week. At 15 days post-transfection, the experiment was stopped due to high auto-fluorescence background associated with GPT which made it impossible to distinguish between GFP positive virus spread and GPT background (Figure 17, I - VII).

I) - transfection
- infection
- GPT reagent

II) + transfection
- infection
- GPT reagent

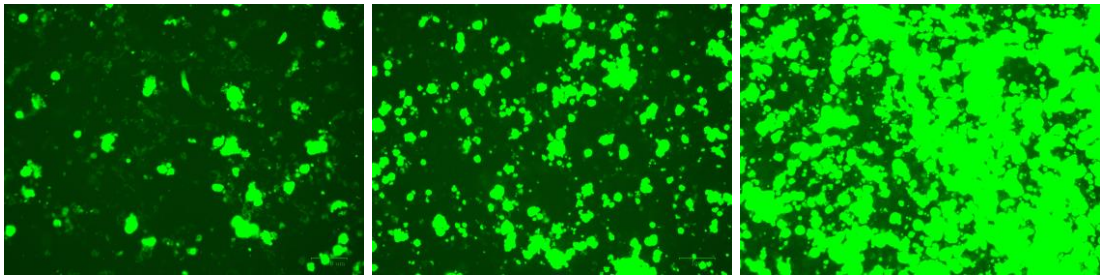
III) + transfection
+ infection
- GPT reagent



IV) + transfection
+ infection
+ GPT reagent

V) - transfection
+ infection
+ GPT reagent

VI) - transfection
- infection
+ GPT reagent



VII) + transfection
- infection
+ GPT reagent

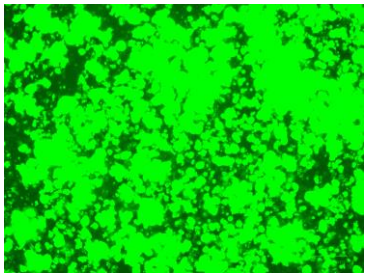


Figure 17. GPT induced fluorescence interfered with selection of GFP positive virus**.** I) to VII) showed PFT cells after 15 days grown under various conditions. GPT reagent alone, in untransfected, uninfected PFT cells (VI) caused high levels of green fluorescence that prevented screening for GFP positive virus.

3.2.4 Construction of PCMV recombinant plasmid encoding E2 BDV protein

As discussed above, when using the GPT selection agent to enhance the population of recombinant virus, the high fluorescence background induced by this reagent made it impossible to screen for GFP positive virus plaques. The overall aim of the study was to develop a prototype CSFV vaccine vector by inserting a simulant pathogen BDV E2 antigen into the PCMV virus. Originally the plan was to generate a PCMV BAC and then introduce the E2 gene at a later date. However, given the problems encountered above, it was decided to insert the E2 gene at the same time as the BAC cassette, so that we could screen for the V5 tag attached to the E2, rather than for GFP positive virus. The first step in this process was to clone the BDV E2 gene into a pMiniOri plasmid, to place it under the control of the constitutive EF1alpha promoter.

3.2.4.1 Cloning E2 BDV into pMiniOri plasmid

pMiniOri is a suicide plasmid, meaning it contains a suicide origin of replication (ori) which allows replication of the plasmid only in bacteria containing a 'pir' gene. In this instance, using the pMiniOri plasmid will allow the E2 BDV to be cloned under the control of the constitutively active EF1alpha promoter, and will provide a bovine growth hormone polyadenylation (BGH polyA) tail for the E2 BDV. Figure 18 outlines the cloning strategy used, which is described in more detail in Sections 3.2.4.1.1 to 3.2.4.1.3.

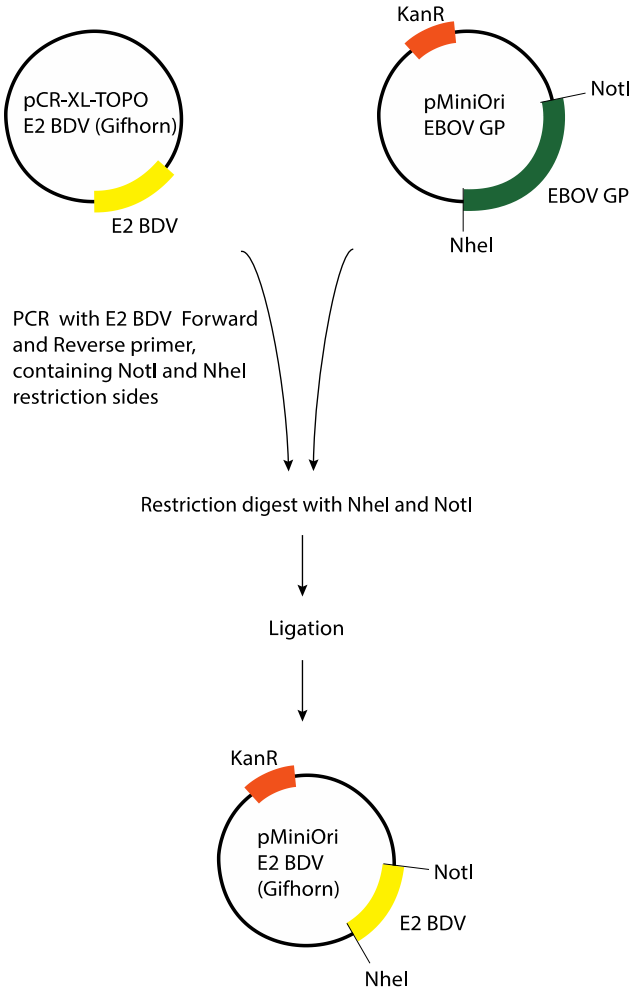


Figure 18. Schematic outlining the cloning strategy for insertion of E2 BDV Gifhorn into the pMiniOri vector.

3.2.4.1.1 Preparing E2 BDV (Gifhorn) fragment for cloning into the pMiniOri vector

The E2 BDV (Gifhorn) gene was kindly provided in pCR®-XL-TOPO (Invitrogen; Kanamycin resistance) by Dr. Thoman Bruun Rasmussen of the National Veterinary Institute DTU Denmark via Dr. Helen Crooke. The plasmid was transformed into One Shot® TOP10 *E. coli* (Section 2.4.9). DNA was extracted from an overnight culture (Section 2.4.1) and screened by restriction digest with EcoRI (Section 2.4.2) followed by gel electrophoresis (Section 2.4.6; Figure 19). EcoRI restriction digest confirmed the expected DNA band sizes of 3.5 kb (pCR-XL-TOPO vector) and 1.2 kb (E2 BDV (Gifhorn) insert). Glycerol stocks of successfully transformed bacteria were prepared and stored at -80°C (Section 2.3.2).

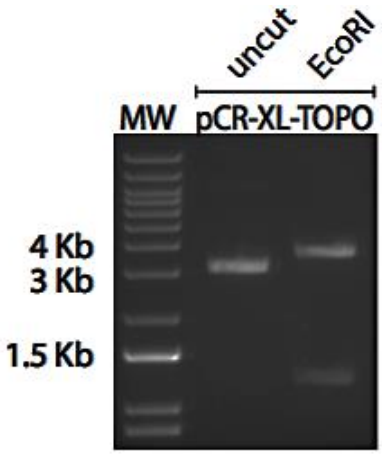


Figure 19. Gel electrophoresis showing EcoRI digestion of pCR-XL-TOPO E2 BDV plasmid after transformation into One Shot® TOP10**.** The gel confirmed the expected band sizes of 3.5 kb and 1.2 kb, for EcoRI digested pCR-XL-TOPO E2 BDV plasmid, representing the vector backbone and E2 BDV insert, respectively.

Using the pCR-XL-TOPO E2 BDV (Gifhorn) plasmid as a template, the E2 BDV (Gifhorn) sequence was amplified by PCR (Section 2.4.5.1; Appendix III). Oligonucleotide primers were synthesized (Table 3) that contained GCGC anchors and NheI and NotI restriction sites to enable cloning into the pMiniOri plasmid. A Kozak sequence was included on the 5' primer and a V5 tag sequence and stop codon were included on the 3' primer. The PCR reaction was performed in duplicate. PCR products were analysed on an agarose DNA gel (Section 2.4.6). A band of the expected size (approximately 1.3 kb) was observed (Figure 20). The E2 BDV PCR product was then digested with NheI and NotI, purified by spin-column purification (Section 2.4.3), and cloned into the NheI and NotI sites of the pMiniOri vector, as described below.

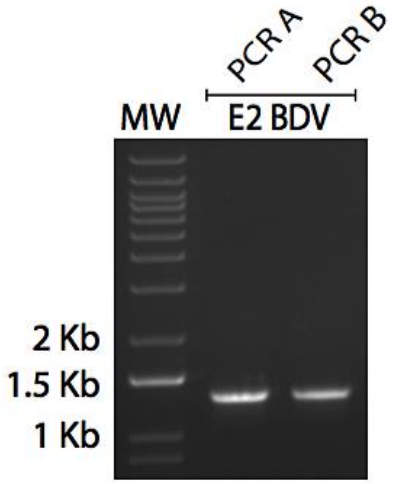


Figure 20. Analysis of E2 BDV PCR fragments used for cloning into pMiniOri vector**.** Gel electrophoresis confirmed the presence of DNA bands at the expected size of approximately 1.3 kb. Two independent PCR reactions were performed.

3.2.4.1.2 Restriction digest of pMiniOri vector plasmid

The NheI-E2 BDV-NotI PCR product (Section 3.2.4.1.1) was cloned into a pMiniOri Zaire EBOV GP plasmid, under the control of the EF1alpha promoter. The pMiniOri Zaire EBOV GP plasmid also contains a BGH-polyA tail, Kanamycin^R marker and R6Kγ origin of replication. The R6Kγ ori is known as a suicide ori, as it requires a 'pir' gene (which produces the π protein) to be present in bacteria for replication. This prevents the plasmid from replicating in bacteria that do not express the protein π. The pMiniOri Zaire EBOV GP vector plasmid was digested with NheI and NotI restriction enzymes. At the same time, the plasmid was also digested with BamHI (Section 2.4.2), which cuts within the Zaire EBOV GP gene that is being removed from the plasmid, to decrease the likelihood of re-insertion of the Zaire EBOV fragment during cloning. The digested plasmid was then run on a gel (Section 2.4.6) and spin-column purified (Section 2.4.3). Gel electrophoresis of the digested plasmid confirmed the presence of bands of the expected sizes of 3 kb, 1 kb, 0.6 kb and 0.5 kb (Figure 21). The digested vector was dephosphorylated as described in Section 2.4.7., prior to ligation with the NheI/NotI digested E2 BDV PCR product.

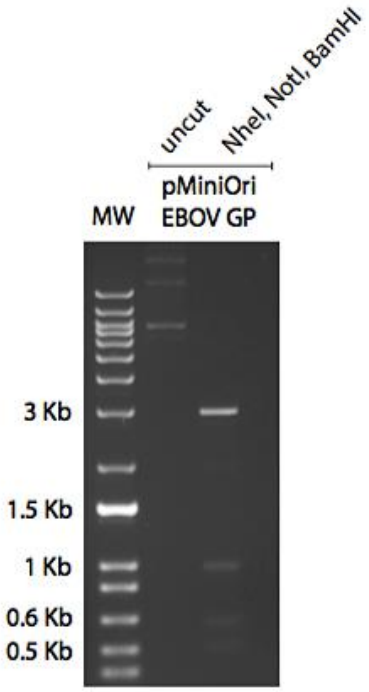


Figure 21. Gel electrophoresis showing NotI, NheI and BamHI digested pMiniOri Zaire EBOV GP vector plasmid**.** The gel confirmed the presence of the correct DNA band sizes of approximately 3 kb, 1 kb, 0.6 kb and 0.5 kb.

3.2.4.1.3 Construction of recombinant pMiniOri E2 BDV plasmid

The digested, purified E2 BDV PCR product (Section 3.2.4.1.1) was ligated (Section 2.4.8) into the linearized pMiniOri vector (Section 3.2.4.1.2; Figure 21), generating a pMiniOri E2 BDV plasmid. The ligated plasmid was transformed into PIR1 *E. coli* bacteria (Section 2.4.9), which express the required replication protein π (as described in Section 3.2.4.1.2). The bacteria were plated onto LB agar containing 50 µg/ml Kanamycin and incubated at 30°C for 24 to 48 hours. DNA was extracted from overnight cultures and analysed by restriction digest with NheI and NotI, followed by agarose gel electrophoresis. The DNA gel (Figure 22) confirmed the correct DNA band sizes for the parental pMiniOri Zaire EBOV GP plasmid (3.0 kb and 2.1 kb), and the recombinant pMiniOri E2 BDV plasmid clones (3.0 kb and 1.3 kb) confirming that the E2 BDV has been inserted into the correct position, placing it under the control of the EF1alpha promoter. Glycerol stocks of clones 1, 6 and 7 were prepared and stored at -80°C (Section 2.3.2). Sequence of the E2 BDV was confirmed by Sanger sequencing.

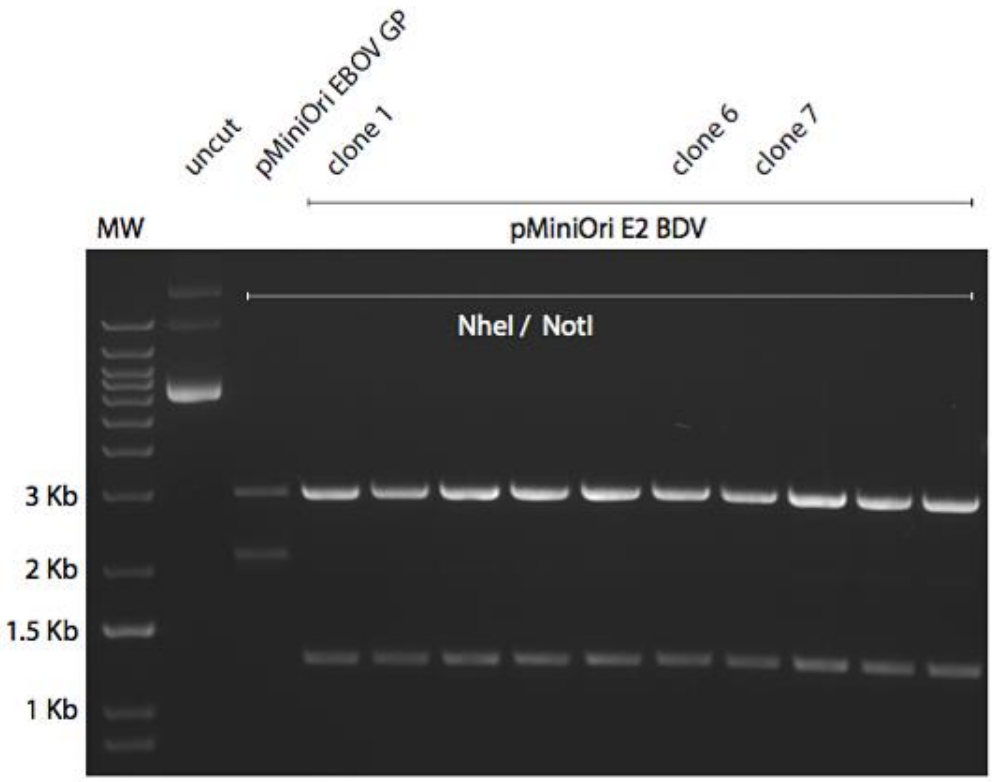


Figure 22. Gel electrophoresis showing NotI, NheI digestion of recombinant pMiniOri E2 BDV plasmid**.** The DNA gel confirmed the correct DNA band sizes for the parental pMiniOri Zaire EBOV GP plasmid (3.0 kb and 2.1 kb), and the recombinant pMiniOri E2 BDV plasmid clones 1-10 (3.0 kb and 1.3 kb). Clone 1, 6 and 7 were selected to move forward to the next step.

3.2.4.2 Cloning of E2 BDV-EF1alpha-BGH pA PCR fragment into U1-BAC-U2 plasmid

The next step in the cloning process was to clone the E2 BDV-EF1alpha-BGH poly A cassette into the shuttle vectors containing the U1-BAC-U2 and U1-BAC-U5. In order to do this the E2 BDV-EF1alpha-BGH pA- sequence was amplified off the pMiniOri plasmid by PCR, digested and then ligated into the shuttle vectors as detailed in this section. Figure 23 outlines the cloning strategy used in this step.

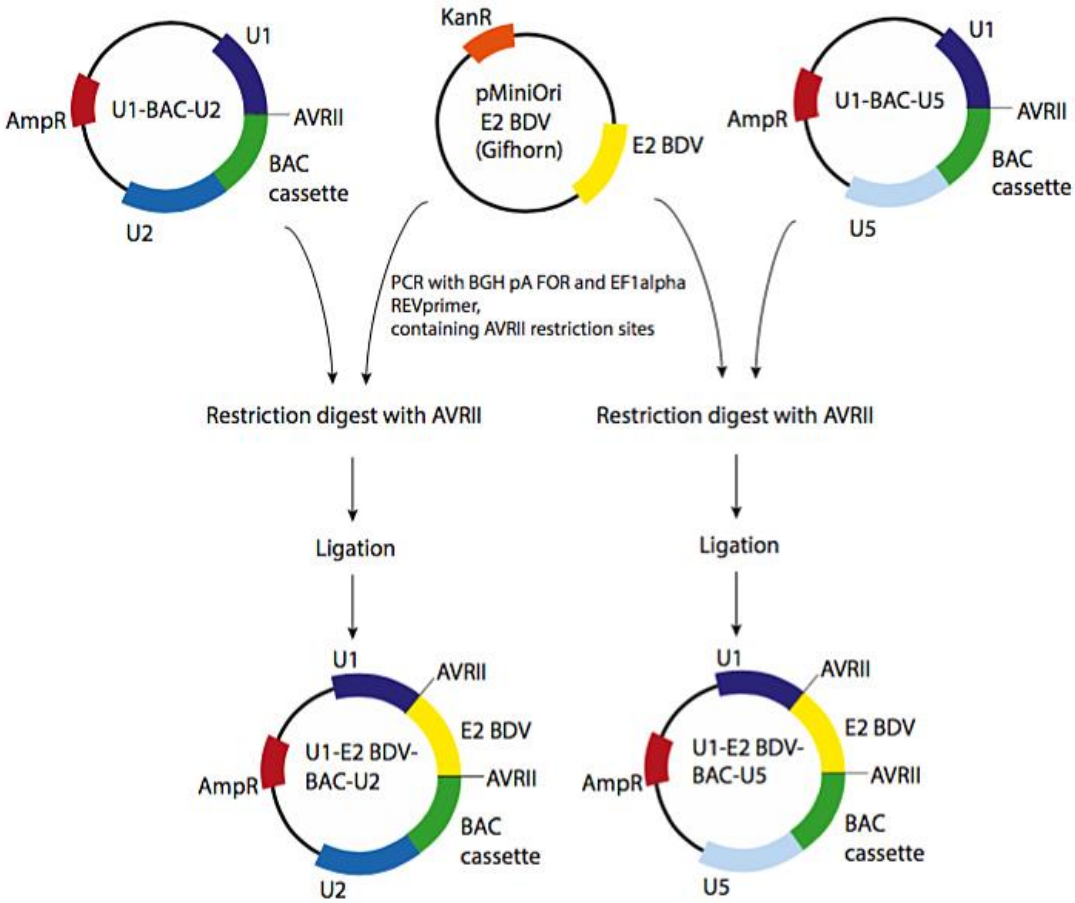


Figure 23. Schematic of cloning strategy for insertion of E2 BDV-EF1alpha- BGH pA into U1-BAC-U2 and U1-BAC-U5 shuttle vectors.

3.2.4.2.1 Preparing E2 BDV-EF1alpha-BGH pA cassette fragment for cloning into the U1-BAC-U2 and U1-BAC-U5 shuttle vectors

Using pMiniOri E2 BDV plasmid (Section 3.2.4.1) as a template, the E2 BDV-EF1alpha-BGH pA cassette was amplified using PCR (Section 2.4.5.1; Appendix III). Oligonucleotide primers that contained GCGC anchors and AvrII restriction sites to were designed to clone the cassette into the U1-BAC-U2 and U1-BAC-U5 shuttle vectors (Table 3). PCR products were analysed on an agarose gel (Section 2.4.6), with observation of the expected 2.7 kb band (Figure 24). The E2 BDV-EF1alpha-BGH pA PCR product was digested with AvrII, purified by spin-column purification, and cloned into the U1-BAC-U2 and U1-BAC-U5 shuttle vectors, as described below.

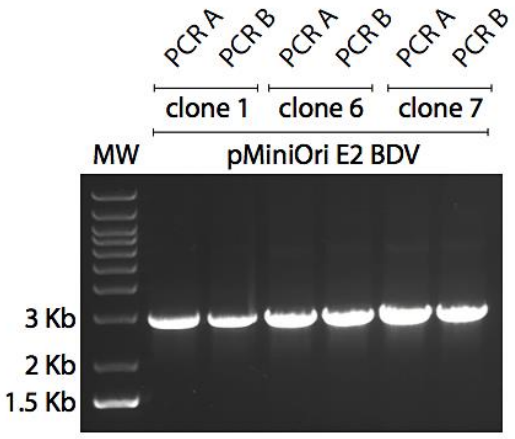


Figure 24. Analysis of E2 BDV- EF1alpha-BGH pA PCR fragments used for cloning into U1-BAC-U2 and U1-BAC-U5 vectors**.** Gel electrophoresis confirmed PCR products of E2 BDV-EF1alpha-BGH pA were of the expected band size of approximately 2.7 kb. Two independent PCR reactions of three different clones were performed.

3.2.4.2.2 Restriction digest of U1-BAC-U2 and U1-BAC-U5 vector plasmids

The U1-BAC-U2 and U1-BAC-U5 vector plasmids were digested with AvrII restriction enzyme (Section 2.4.2), to enable cloning of the AvrII digested E2 BDV-EF1alpha-BGH pA PCR product (Section 3.2.4.2.1). The digested plasmid was then run on a gel (Section 2.4.6) and spin-column purified (Section 2.4.3). Gel electrophoresis of the digested plasmid, alongside the digested PCR fragment, confirmed the presence of bands of the expected sizes of approximately 14 kb and 2.7 kb, representing the digested shuttle vectors and the digested BDV-EF1alpha-BGH pA PCR fragment respectively (Figure 25). The digested vector was dephosphorylated as described in Section 2.4.7., prior to ligation with the digested E2 BDV-EF1alpha-BGH pA PCR product.

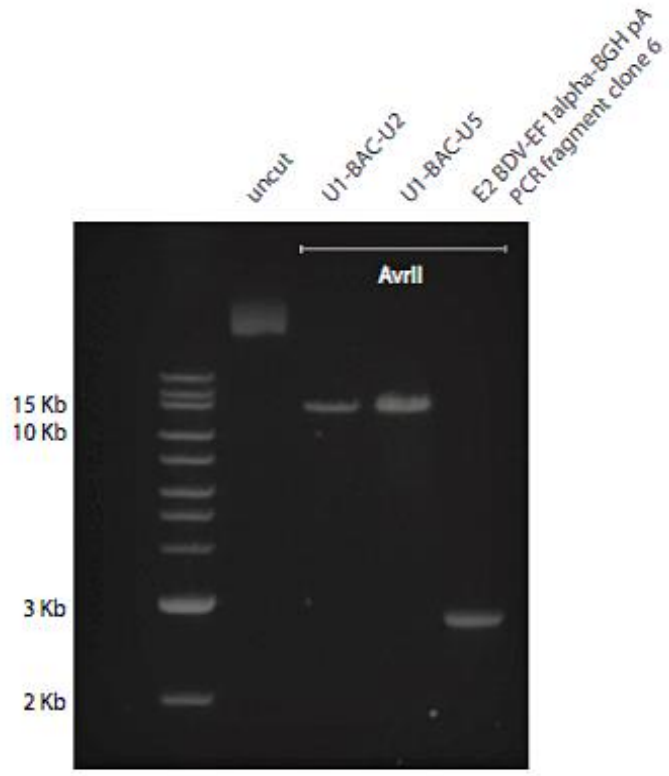


Figure 25. Gel electrophoresis showing AvrII digested U1-BAC-U2, U1-BAC-U5 and E2 BDV-EF1alpha-BGH pA PCR fragment. The gel confirmed the expected band sizes of approximately 14 kb for the linearized vectors, and 2.7 kb for the E2 BDV-EF1alpha-BGH pA PCR fragment.

3.2.4.2.3 Construction of U1-E2 BDV-BAC-U2 and U1-E2 BDV-BAC-U5 plasmids

The digested, purified E2 BDV-EF1alpha-BGH pA PCR product (Section 3.2.4.2.1) was ligated (Section 2.4.8) into the linearized U1-BAC-U2 and U1-BAC-U5 vectors (Section 3.2.4.2.2), generating U1-E2 BDV-BAC-U2 and U1-E2 BDV-BAC-U5 plasmids. The ligation product was transformed into DH10B *E. coli* bacteria. Bacteria were plated onto LB agar containing 100 µg/ml Carbenicillin and 17.5 µl/ml Chloramphenicol and the plates were incubated at 30°C for 24 to 48 hours. DNA was extracted from overnight cultures of colonies, and screened by restriction digest with AvrII and SacI, followed by agarose gel electrophoresis. AvrII digest DNA gels (Figure 26 & 27), revealed two possible U1-E2 BDV-BAC-U2 clones (clones 16 & 25) with the presence of bands at the expected sizes of 2.7 kb & 14.1 kb, and three possible U1-E2 BDV-BAC-U5 clones (clones 15, 29, & 34) with bands of 2.7 kb & 13.9 kb present. Parental plasmid linearized as expected, giving bands of 14 kb for U1-BAC-U2 and 13.8 kb for U1-BAC-U5.

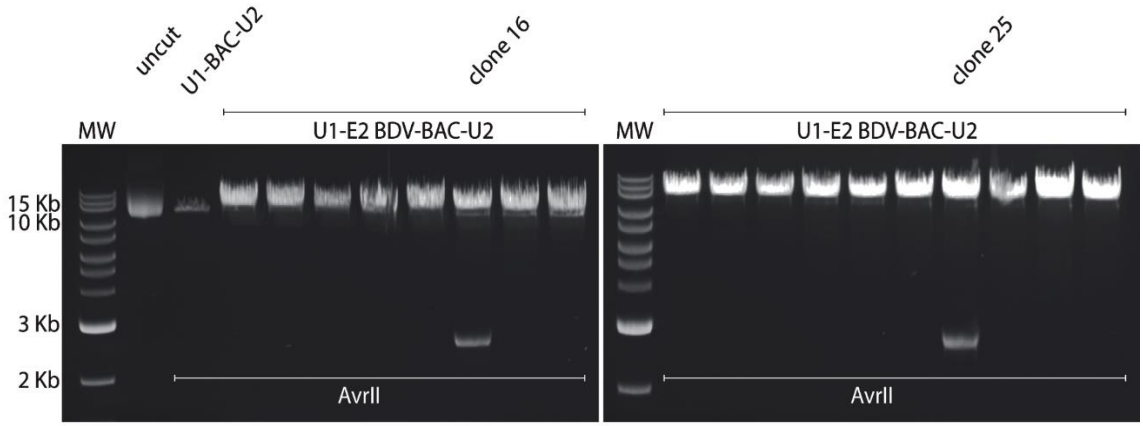


Figure 26. Gel electrophoresis showing AvrII digestion of U1-E2 BDV-BAC-U2 clones**.** This screen of AvrII digested U1-E2 BDV-BAC-U2 plasmids revealed two potential positive clones (16 & 25) with two bands present at 2.7 kb and 14.1 kb. The parental plasmid was linearized as expected, giving a band at the expected size of approximately 14 kb.

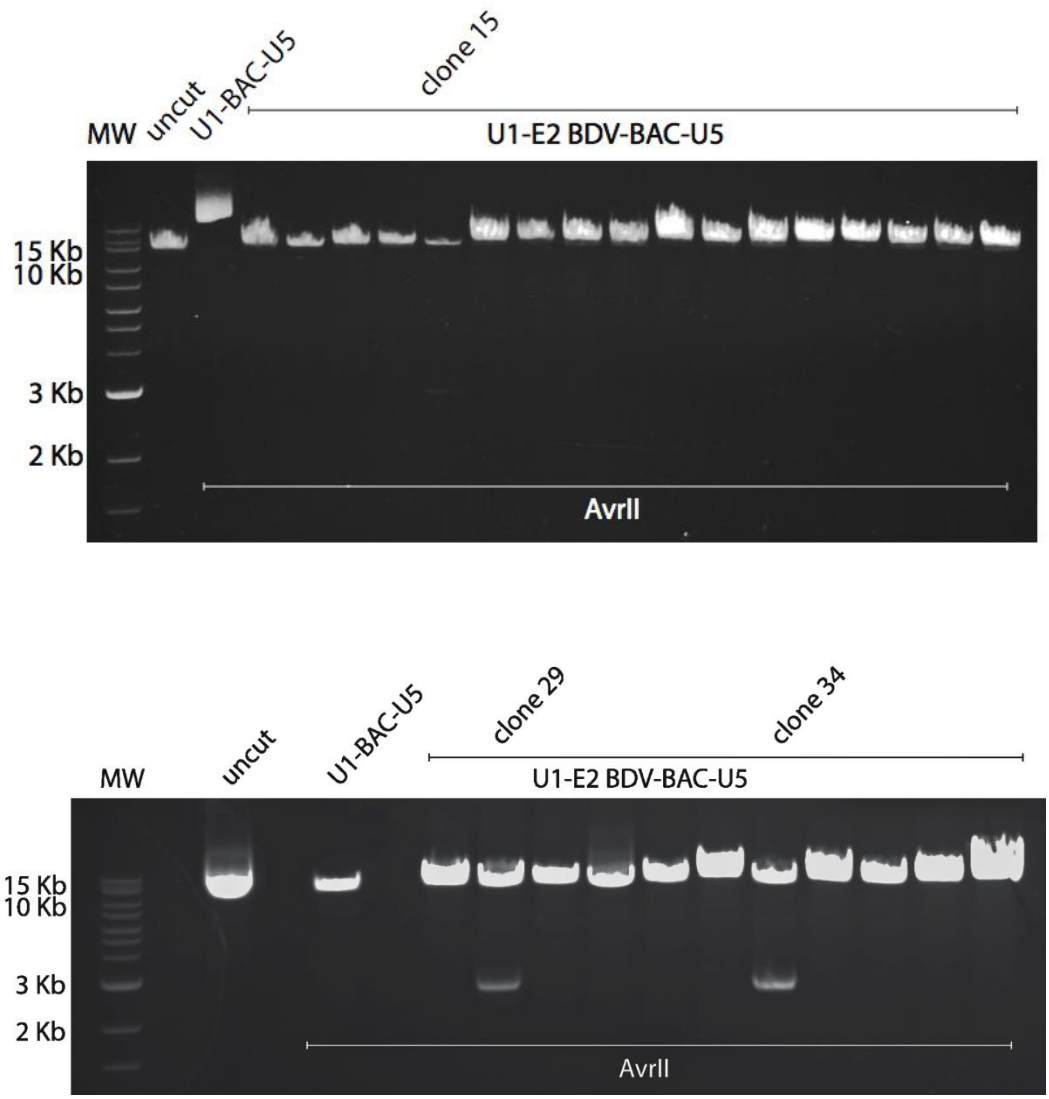


Figure 27. Gel electrophoresis showing AvrII digestion of U1-E2 BDV-BAC-U5 clones**.** This screen of AvrII digested U1-E2 BDV-BAC-U5 plasmids revealed three potential positive clones (15, 29 & 34) with two bands present at 2.7 kb and 13.9 kb. The parental plasmid was linearized as expected, giving a band at the expected size of approximately 13.9 kb.

A second digest with SacI (Figure 28 & 29) was used as an additional confirmation that the clones were correct. This second screen found only one of each of the U1-E2 BDV-BAC-U2 and U1-E2 BDV-BAC-U5 clones had the expected banding pattern, highlighting the importance of confirming construct through analysis with multiple restriction enzymes. U1-E2 BDV-BAC-U2 clone 25 had the expected banding pattern of 1.5 kb, 1.6 kb, 3.0 kb and 10.6 kb and U1-E2 BDV-BAC-U5 clone 29 had bands of 1.6 kb, 2.2 kb, 2.8 kb and 9.9 kb, as expected. The large size of the plasmids (kb) was a hurdle to the use of direct Sanger sequencing for final confirmation of the clones. The two clones were therefore sent to the Davison Laboratory for full length sequencing by using Illumina next-generation sequencing. Sequencing confirmed that the E2 BDV was inserted into U1-E2 BDV-BAC-U2 clone 25 in the correct position (Figure 30). The sequence data matched the in silico sequence prediction, although several indels were detected in regions of the BAC cassette, which were common to both clones and presumably reflect errors in the original pHA2 sequence. The U1-E2 BDV-BAC-U5 sequencing is still ongoing. In summary, a combination of two linear recombination fragments containing the BAC cassette and E2 BDV gene, flanked by regions homologous to either the U1 and U2, or U1 and U5 regions of PCMV were generated.

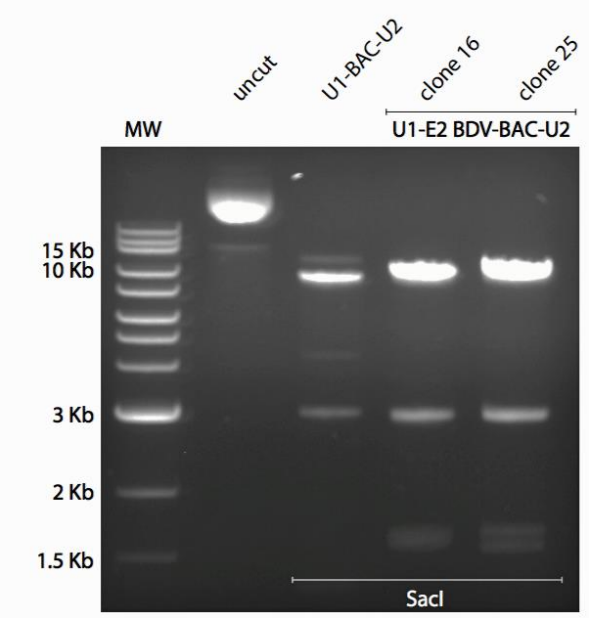


Figure 28. Gel electrophoresis showing SacI digestion of U1-E2 BDV-BAC-U2 clones**.** This screen of SacI digested U1-E2 BDV-BAC-U2 clones revealed that clone 25 had the correct banding pattern of 1.5 kb, 1.6 kb, 3.0 kb and 10.6 kb bands, while clone 16 did not. Parental band sizes were as expected.

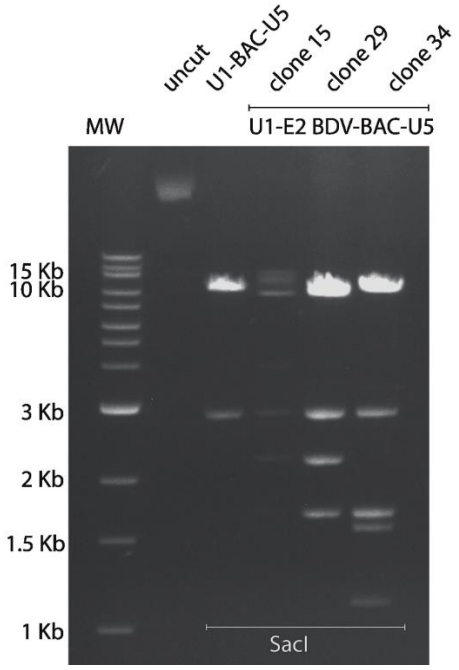


Figure 29. Gel electrophoresis showing SacI digestion of U1-E2 BDV-BAC-U5 clones**.** This screen of SacI digested U1-E2 BDV-BAC-U5 clones revealed that clone 29 had the correct banding pattern of 1.6 kb, 2.2 kb, 2.8 kb and 9.9 kb bands, while clone 15 and 34 did not. Parental band sizes were as expected.

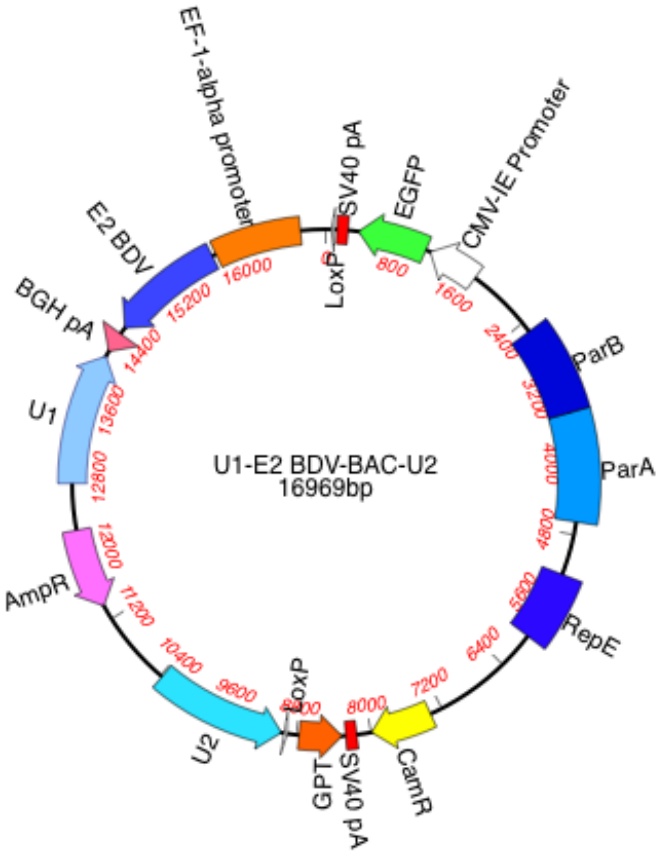


Figure 30. In silico map of U1-E2 BDV-BAC-U2 plasmid showing the insertion of E2 BDV-EF1alpha-BGHpA**.** Sequence was confirmed by whole genome sequencing.

3.3 Identification of PCMV-antibody positive pig sera

In these next studies, pig sera from a local abattoir was screened for PCMV-reactive antibodies. These studies were performed for two main reasons. Firstly, PCMV positive pig sera can be used for assays within the laboratory- it can be used as a primary antibody for western immunoblot analysis, and as control sera in ELISAs – both of which will prove very useful as this project progresses. Secondly, as the vaccine being constructed for use in swine, it is of interest to know the level of existing PCMV positivity within local farm populations, and it will also be of interest to compare the levels with other parts of the UK, if known, and other countries in Europe and beyond.

Sera from 20 pigs were screened using a commercial PCMV antibody-capture ELISA kit as described in Section 2.5.1. As a positive and negative control were not included in this ELISA Kit we had to set our own valuation by comparison of the individual values. From these, 3 PCMV-positive, 2 PCMV-borderline and 15 PCMV-negative sera were identified as shown in Table 13. These sera samples have been aliquoted and stored at -20°C for use later in the project.

Well Row	Well Col	Content	Dilutions	Raw Data (450)	Blank corrected based on Raw Data (450)	Linear regression fit based on Blank corrected in ng/ml (450)	
A	3	Sample X1	2	0,199	0,135	2,029	
B	3	Sample X1	2	0,221	0,156	2,436	
C	3	Sample X2	2	0,529	0,465	8,229	positive
D	3	Sample X2	2	0,533	0,469	8,299	
E	3	Sample X3	2	0,201	0,137	2,071	
F	3	Sample X3	2	0,21	0,146	2,241	
G	3	Sample X4	2	0,282	0,217	3,582	
H	3	Sample X4	2	0,256	0,192	3,107	
A	4	Sample X5	2	0,283	0,219	3,612	
B	4	Sample X5	2	0,242	0,178	2,841	
C	4	Sample X6	2	0,26	0,196	3,177	
D	4	Sample X6	2	0,273	0,209	3,419	
E	4	Sample X7	2	0,62	0,556	9,932	
F	4	Sample X7	2	0,253	0,189	3,047	
G	4	Sample X8	2	0,412	0,348	6,032	positive
H	4	Sample X8	2	0,38	0,316	5,428	
A	5	Sample X9	2	0,202	0,138	2,087	
B	5	Sample X9	2	0,153	0,089	1,169	
C	5	Sample X10	2	0,265	0,2	3,261	
D	5	Sample X10	2	0,236	0,172	2,723	
E	5	Sample X11	2	0,189	0,124	1,84	
F	5	Sample X11	2	0,202	0,138	2,086	
G	5	Sample X12	2	0,249	0,184	2,965	
H	5	Sample X12	2	0,272	0,208	3,402	
A	6	Sample X13	2	0,288	0,223	3,694	
B	6	Sample X13	2	0,275	0,21	3,45	
C	6	Sample X14	2	0,337	0,272	4,615	borderline
D	6	Sample X14	2	0,34	0,276	4,68	
E	6	Sample X15	2	0,474	0,409	7,183	positive
F	6	Sample X15	2	0,47	0,406	7,118	
G	6	Sample X16	2	0,274	0,21	3,435	
H	6	Sample X16	2	0,319	0,254	4,275	
A	7	Sample X17	2	0,266	0,202	3,291	
B	7	Sample X17	2	0,261	0,196	3,19	
C	7	Sample X18	2	0,309	0,245	4,103	borderline
D	7	Sample X18	2	0,311	0,246	4,125	
E	7	Sample X19	2	0,242	0,178	2,839	
F	7	Sample X19	2	0,242	0,178	2,841	
G	7	Sample X20	2	0,213	0,148	2,29	
H	7	Sample X20	2	0,249	0,185	2,967	

Table 10. ELISA results of pig sera**.** Three PCMV-positive and two PCMV-borderline sera were identified out of 20 pigs. The assay was performed in duplicate with a 2-fold dilution factor using Omega Control Software for data analysis.

3.4 Discussion

The long-term goal of this project is to develop PCMV as a vaccine vector platform, using it to vaccinate against CSFV, as well as potentially other swine diseases. To date, PCMV has not been well characterised, so the initial focus was on characterising *in vitro* growth characteristics.

Given the lack of information available with regards to culturing and titration of PCMV, initial experiments were focused on optimisation of the $TCID_{50}$ assay, a standard assay used in virus titration. After reading the $TCID_{50}$ plates at several timepoints post-infection, we observed that the readings reached their maximum at 28/29 days post-infection. This time-point was therefore selected to be the reading point for all PCMV based $TCID_{50}$ assays moving forward. Other CMVs within the lab have a readout time of 7 – 10 days (MCMV & RhCMV), so these initial data suggested a slow growing phenotype for PCMV relative to other CMVs.

Having optimised the $TCID_{50}$ assay, we then looked at the kinetics of virus release from infected cells into the supernatant. CPE was only visible after 20 days post-infection, further supporting a slow growing phenotype. Additionally, titration of supernatants from several different timepoints post-infection revealed relatively low titres, with a maximum titre of 4.77E+03 pfu/ml observed at 22 days post-infection. This compares with maximum titres for MCMV and RhCMV on the order of 1.00E+06 to 1.00E+07 pfu/ml. The PCMV titre did not increase further with additional incubation time, despite progression of visible CPE. Using ddPCR we demonstrated that the ratio of virion genome copy number to infectious virion particles increased over time, indicating an accumulation of defective, non-infectious virus particles in the supernatant. Whole genome sequencing of the

supernatants supports this scenario, with analysis of day 37 supernatant revealing large genome deletions in the PCMV genome, relative to earlier timepoints. The total/infectious particle ratio has been measured for many viruses (Klasse, 2015), including the alphaherpesvirus HSV-1 which has a ratio of ~ 50 – 200. Interestingly, another alphaherpesvirus, varicella-zoster virus, which is reported to be difficult to work with due to difficulties obtaining high titre cell free virus stocks (Harper *et al*, 1998), also has a much higher ratio of ~ 4E+04 (Carpenter *et al*, 2009). Harper and colleagues devised a method to obtain slightly higher titre virus stocks by harvesting and freezing virus in a sucrose-based buffer, so it is possible that the low titres observed with PCMV could be improved with further optimisation in future studies.

Given the low titres of PCMV observed in supernatants, we tried low speed centrifugation/spinoculation to determine whether this technique would increase the effective MOI, as has been previously reported for several other viruses (Osborn & Walker, 1968; Cheng *et al*, 2007; Guo *et al*, 2011). As can be seen in Figure 8, spinoculation did not result in increased supernatant virus titres, [caused a small, but significant decrease in supernatant virus titres]. Attempts to prepare a concentrated virus stock with a higher titre were also unsuccessful, yielding maximum titres of ~2.5E+04 pfu/ml, indicating that the concentration process most likely increased the amount of non-infectious (or possibly non-infecting/un-available to infect/aggregated) particles. Overall, the data obtained in this study indicate that the WT (JF strain) has a slow growing, low titre phenotype. Our data concurs with two previous studies which reported PCMV to be a slow growing virus in PFT cells (Fryer *et al*, 2004). There are, however two published studies that report significant visible CPE within 7 days (Whitteker *et al*, 2008) and 7-10 days (Rupasinghe *et al*, 2001), which is significantly earlier

than the 20 days at which we first observed CPE, but it is possible that this may be due to differences in the cell lines and virus strains used. Further optimisation will need to be carried out in the future to try and improve virus yields.

The overall aim of this study is to develop PCMV as a vaccine platform (Section 3.2). To accomplish this, heterologous antigens needed to be inserted into the genome such that they can be expressed during virus infection and elicit protective immune responses. In order to facilitate insertion of antigens into this slow growing PCMV genome, it was decided to clone it as a BAC (Section 3.2), thereby allowing the genome to be manipulated in bacterial cells. As outlined in Figure 9, the BAC cassette, which contains all of the elements necessary to allow replication within bacterial cells (Shizuya *et al*, 1992; Warden *et al*, 2011), was cloned into shuttle vectors to position it between flanking homologous targeting sequences, to direct the cassette to the correct genomic locations for insertion. Two different locations within the genome were chosen for insertion of a BAC cassette – between U1 and U2, and U1 and U5 (Section 3.2). Insertion of the BAC cassette into the shuttle vectors was confirmed by restriction digest analysis (Figure 11), and then the BAC cassettes, flanked by the homologous regions was released from the vectors as linear fragments by restriction digests with PmlI and SwaI (Figure 12). Two different approaches were selected to introduce this linearized fragment into the PCMV genome – one based on homologous recombination (Section 1.4.2.3) (Zhang *et al*, 1998; Muyrers *et al*, 1999; Rivero-Müller *et al*, 2007) and one based on CRISPR/Cas9 technology (Section 1.4.2.4) (Hsu *et al*, 2014; Ishino *et al*, 2018). The CRISR/Cas9 approach required an extra plasmid to supply the gRNA and CRISPR proteins (Section 1.4.2.4.3.3) (Yao *et al*, 2018). Cloning of the shuttle vectors and gRNA CRISPR plasmid was straightforward and no issues were encountered.

After preparation by restriction enzyme digestion and purification, the linearized fragments U1-BAC-U2 and U1-BAC-U5 were transfected into either uninfected or PCMV infected PFT cells. As the BAC cassette contains GFP we were able to monitor transfection efficiency. The expectation was that initially there would be a lot of GFP positive cells. Over time, we expected that this number would decrease as the linearized fragment was degraded, and the only remaining GFP positive cells would be those where the BAC cassette had recombined into the PCMV genome. After transfection we observed that the transfection efficiency of the U1-BAC-U2 and U1-BAC-U5 fragments (Figure 15) was very low in comparison to transfection efficiency of an EGFP-C1 plasmid which was used as a control (Figure 14). According to a study from Kreiss and colleagues (Kreiss *et al*, 1999), this is likely due to the larger size of the linearized fragments (~14kb), relative to the EGFP-C1 plasmid (~4.7kb). Transfecting a higher quantity of DNA did not improve the transfection efficiency. When the CRISPR/Cas9 plasmid was co-transfected into cells, the transfection efficiency was even lower again (Figures 16), possibly due to increased toxicity caused by introducing increased levels of DNA.

In order for recombination of the BAC cassette into the PCMV genome to take place by HR, the PCMV genome and the linearized fragment must be present in the same cell. For the CRISPR/Cas9 approach, the CRISPR/Cas9 plasmid must also be present. Low transfection efficiencies, combined with slow growing, low titre PCMV did not generate ideal conditions for this event. To try to maximize the chances of the linearized fragments entering a cell that contained virus, we also tried transfecting PCMV-infected cells. Regardless of the timing of infection with PCMV, we did not observe the development of any GFP positive virus plaques.

It was assumed that the replication of PCMV WT (JF strain) virus might be outgrowing any recombinant clones, causing CPE and monolayer detachment before any GFP positive plaques could be observed. To address this, GPT selection agent was added to the media (Section 3.2.3). The BAC cassette contains a gpt gene, so that only recombinant PCMV containing a BAC cassette should be able to grow in the presence of GPT reagent, while WT (JF strain) virus should not be able to replicate (Mulligan & Berg, 1981; Falkner & Moss, 1988). Unfortunately, as shown in Figure 17, the GPT reagent was associated with high auto-fluorescence background, precluding screening for GFP positive cells / plaques containing recombinant virus. While the increased levels of auto-fluorescence prevents screening for GFP positive plaques, we could potentially monitor for the presence of recombinant virus using PCR with primers specific for the BAC cassette region. An increase in levels of the BAC cassette over time would indicate it had been recombined into the viral genome.

Initially we had hoped to insert the BAC cassette into the PCMV genome first, and then perform subsequent manipulations such as insertion of the E2 BDV antigen in bacteria. However, the problems encountered while trying to insert the BAC cassette into the genome necessitated a change in approach. We still wanted to use GPT selection to prevent overgrowth of cultures with WT (JF strain) virus, however, this meant we could no longer use GFP positivity as a means of detecting recombinant virus. We decided to insert the E2 BDV protein along with the BAC cassette. This meant we would still be able to use GPT selection and could screen for E2 BDV positive virus plaques rather than GFP positive plaques. The E2 BDV gene was first cloned into a pMiniOri vector to place it under the control of an EF1alpha promoter (Figure 17), followed by cloning of the E2 BDV expression cassette into shuttle vectors flanked by regions homologous to U1

and U2, and U1 and U5, as outlined in Figure 22. Clones were confirmed by restriction digest with two different enzymes – AvrII and SacI. The large size of the plasmids (approx. 15kb) precluded direct DNA Sanger sequencing. Instead, NGS sequencing using the Illumina platform was used to confirm plasmid integrity. Due to time constraints it was not possible to transfect these into cells, but this work will be continued in the future.

Similar to other CMV, PCMV may have substantial utility for development as a vaccine platform. However, the slow growth characteristics, combined with low-titre phenotype and transfection efficiencies of PCMV permissive cells presents unique issues to cloning of PCMV as an infectious BAC, which is required for subsequent genetic manipulation of the virus. After characterisation of PCMV *in vitro* growth, all the shuttle vectors required for the project were constructed without any issues and all were verified by restriction digest and sequence analysis. Initial studies towards use of these constructs for the BAC cloning of PCMV encountered a number of hurdles. Future studies towards the cloning of the PCMV BAC will focus on optimisation of these factors, including virus growth, transfection efficiency and selection conditions.

Chapter 4

Conclusions and future directions

Classical swine fever is a notifiable disease which causes significant morbidity and mortality in pigs, and also, causes large economic losses in the swine industry. One reported outbreak in the Netherlands resulted in the culling of over 10 million pigs, costing over 1 billion Euro (Food and Agriculture Organization of the United Nations, 2011). Although most western and central European countries are classified as being CSF free, CSF is endemic in many countries, including China which is a leading producer of pork. High levels of international trade mean the risk of CSFV introduction into CSF free territories is ever present. Currently CSF free countries use a non-vaccination, stamping out policy rather than prophylactic vaccination. This is due to concerns surrounding existing vaccines that include incomplete protection against infection, and DIVA incompatibility issues which would affect ability to trade post-vaccination, as discussed in Section 1.3.

To date, several different vaccines have been developed (Section 1.3), but only one has made it onto the European market - Suvaxyn® CSF Marker vaccine, a chimeric pestivirus vaccine expressing the E2 protein of CSFV. Transplacental CSFV infection is not completely inhibited by Suvaxyn® CSF Marker vaccine, so the virus may still spread through unvaccinated piglets within the herd, preventing use of this vaccine prophylactically. This vaccine is also not suitable for use in vaccination of wild boar populations which serve as a reservoir for CSFV, as it requires direct intramuscular inoculation of each animal, and that is not feasible when dealing with inaccessible wild animal

populations. This study set out to address the urgent need for development of a safe, effective, DIVA compatible vaccine using a herpesvirus-based platform.

Herpesvirus based vaccines, and in particular, CMV-based vaccines, have been shown to introduce strong, durable antibody (Marzi *et al*, 2016) and T-cell responses (Hansen *et al*, 2010) against several different heterologous antigens (Table 2) (Hansen *et al*, 2011; (Tsuda *et al*, 2015) Hansen *et al*, 2018), and also have the potential to self-disseminate (Murphy *et al*, 2016), which would facilitate vaccination of inaccessible wild-boar populations. PCMV, classified as a Roseolavirus, shares biological similarities with other betaherpesviruses, and also in terms of cytopathology, with development of inclusion bodies and induction of cytomegaly in infected tissues. The overall long-term aim of this project is to develop PCMV as a vaccine vector platform, using it for vaccination against CSFV. We set out with the intention of inserting a BAC cassette into the PCMV genome, either by homologous recombination or through the use of CRISPR/Cas9 technology, to allow easier and more efficient manipulation of the PCMV genome in bacterial cells.

Information regarding PCMV growth *in vitro* was lacking in the literature. This study took some initial steps towards characterisation, revealing that the PCMV WT (JF strain) virus is quite slow growing, with CPE only being visible after about 20 days post-infection. We also found that the virus titres released into the supernatant were very low, reaching a maximum level of 4.77E+04 pfu/ml. Using ddPCR to measure the total virion genome copy number, we observed quite a high genome to pfu ratio for PCMV (Table 7), which increased towards later time-points post- infection. This data, combined with whole genome sequencing data

of viral supernatants, which showed large genome deletions at later times post-infection (Section 3.1.3), indicated an accumulation of defective non-infectious particles over time. A greater understanding of PCMV replication and growth kinetics will be required moving forward, and future studies should focus on improving virus yields as this was one limiting factor in our attempts to generate a recombinant PCMV BAC (Section 3.2.3). Our current understanding of genome to pfu ratios indicates that not all non-infecting particles are defective (Klasse, 2015; Virology blog: Are all virus particles infectious?), and that under the right conditions, many of them could complete an infectious cycle. Experimentation with different cell lines, along with techniques such as sonication of viral stocks may help to improve PCMV titres.

We were able to successfully clone two shuttle vectors – one targeting the BAC cassette into the U1 and U2 region of the PCMV genome, and the other targeting the BAC cassette between the U1 and U5 regions of the genome, deleting the intervening non-essential genes (Section 3.2.1). However, transfection efficiency was quite low when the linearized recombinant fragments were transfected into either uninfected or PCMV infected PFT cells. As discussed previously, in order for a recombination event to occur, the PCMV genome and the recombination fragment must be present in the same cell. When CRISPR/Cas9 approach is used, the CRISPR/Cas9 plasmid must also be present in the cell. Low transfection efficiencies, combined with slow growing, low titre PCMV infections made it quite unlikely that all the required DNA elements would end up in the same cell at the same time. There is evidence in the literature that transfection of linearized DNA is less efficient than transfection of supercoiled DNA (von Groll *et al*, 2006). When complexed with lipofectamine, circular DNA forms a compact spherical shape, whereas linearized DNA forms ‘worm-like’ strands, which may

affect uptake of the complex into the cell (von Groll *et al*, 2006; Lehner *et al*, 2013). In the present study, the linear recombinant fragments were released from a circular shuttle vector plasmid by restriction digest, as outline in Figure 10, prior to transfection. Future studies should also evaluate using the recombinant fragment within the shuttle vector, rather than excising it. Taking low transfection efficiencies into account, electroporation should also be evaluated as a means of transferring the recombinant fragments into cells, to determine whether this technique would be more efficient than transfection.

Following transfection, our expectation was that there would be a large number of green cells due to the BAC cassette containing a GFP gene. The GFP expression was expected to fade away over time as the linearized recombination fragment was degraded. Any green that emerged after this time and spread from cell to cell forming a GFP positive plaque would be a recombinant virus that contained the BAC cassette. We did not see any GFP positive plaques, and the monolayers were usually destroyed by replication of PCMV WT (JF strain) virus. In an effort to restrict WT (JF strain) virus growth, we added GPT selection agent to the media (Section 3.2.3). In the presence of GPT reagent, only recombinant virus containing a gpt gene from the BAC cassette would be able to grow, preventing the cells from being overgrown by WT (JF strain) virus. Unfortunately, GPT reagent had the unexpected property of causing bright auto-fluorescence in the GFP fluorescence channel (Figure 17), making it impossible to screen for GFP positive virus / plaques. To overcome this, we needed a means to screen for recombinant virus that was not GFP dependent. Another set of shuttle vectors targeting the same PCMV genomic regions for insertion was constructed, but this time the BDV E2 gene was also cloned into the shuttle vectors. The idea behind this was that recombinant plaques could be selected by screening for expression

of the E2 BDV protein rather than GFP. There was no time to test this strategy, but the vectors will be used in future studies.

In conclusion, we have taken the initial steps towards developing PCMV as a vaccine vector platform. Difficulties with low virus titres and low transfection efficiencies hindered experiments, and further optimisation is required to overcome these issues and improve the likelihood of a recombination event occurring. All of the shuttle vectors required for the project were successfully cloned and are now available, and the data gathered about PCMV replication kinetics is a good resource moving forward with the development of PCMV as a vaccine vector platform.

Appendix I

Plasmid	Antibiotic selection	Concentration (µg/ml)	Bacteria
pHA2	Kanamycin Chloramphenicol	50 17.5	DH10B
U1-U2 (GeneArt)	Carbenicillin	100	DH10B
U1-U5 (GeneArt)	Carbenicillin	100	DH10B
U1-BAC-U2	Carbenicillin Chloramphenicol	100 17.5	DH10B
U1-BAC-U5	Carbenicillin Chloramphenicol	100 17.5	DH10B
EGFP-C1 (Clontech)	Kanamycin	50	n/a
pX330 + gRNA/Cas9	Carbenicillin	100	DH10B
pCR-XL-TOPO E2 BDV (Gifhorn)	Kanamycin	50	TOP10
pMiniOri Zaire EBOV GP	Kanamycin	50	PIR1
pMiniOri BDV E2 (Gifhorn)	Kanamycin	50	PIR1
U1-E2 BDV-BAC-U2	Carbenicillin Chloramphenicol	100 17.5	DH10B
U1-E2 BDV-BAC-U5	Carbenicillin Chloramphenicol	100 17.5	DH10B

Antibiotic	Stock solution	Supplier
Carbenicillin	50 mg/ml	Melford
Chloramphenicol	34 mg/ml	Sigma-Aldrich
Kanamycin	50 mg/ml	Sigma-Aldrich

Appendix II

Restriction digest reaction setup

	volume (µl)	
	Screening	Cloning
DNA	5	40
Buffer (10X)	1	5
dH_2O, molecular grade	3	3
High-Fidelity enzyme	1	2

Dephosphorylation and ligation reaction setup

	volume (µl)	
	1:3 ratio	no insert control
Dephosphorylated vector	1	1
Insert	3	X
10x ligation buffer	2	2
T4 DNA ligase	1	1
dH_2O	16	13

Appendix III

Standard PCR reaction setup and thermal cycling conditions

Component	Volume per reaction (µl)
5x Q5™ Reaction Buffer	10
dNTP (10 mM)	1
Forward primer (50 µM)	1
Reverse primer (50 µM)	1
Template DNA	1
Q5™ Hot Start HF DNA Polymerase	0.5
5x Q5™ High GC Enhancer	10
Molecular water	25.5

Cycling Step	Temp, °C	Time	cycles
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	34
Annealing	72	30 sec	34
Extension	72	90 sec	34
Final extension	72	2 min	1
Hold	12	Infinite	

Using Q5™ High-Fidelity DNA Polymerase annealing temperatures and extension times can vary depending on melting temperature of the primers and complexity of genomic templates respectively.

ddPCR reaction setup and thermal cycling conditions

Component	Volume per reaction (µl)
2x ddPCR Supermix for Probes (no dUTP)	10
Forward primer (2 uM)	1
Reverse primer (2 uM)	1
Probe (2 µM)	2
Molecular water	5
DNA (0.2 ng/µl)	1

Cycling Step	Temp, °C	Time	Ramp Rate	cycles
Enzyme activation	95	10 min	2°C/sec	1
Denaturation	94	30 sec	2°C/sec	40
Annealing/extension	60	1 min	2°C/sec	40
Enzyme deactivation	98	10 min	2°C/sec	1
Hold	12	Infinite	2°C/sec	1

Appendix IV

Density

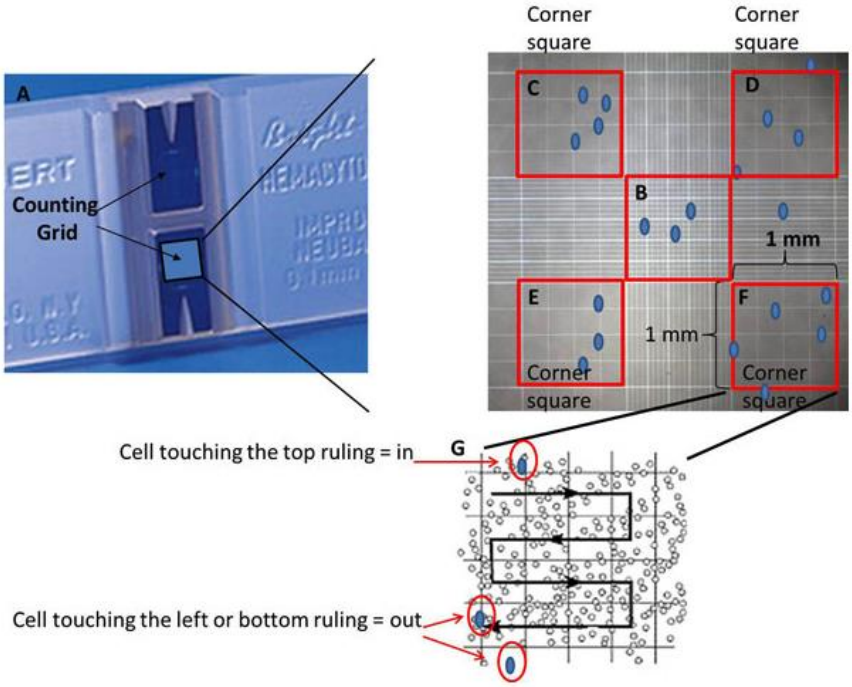
Plates	Seeding density per well	Growth medium (ml)	Surface area (cm^2)
6-well	0.25 - 1 x 10^6	2	9
12-well	1 x 10^5	1 - 2	4
24-well	0.5 - 2 x 10^5	0.5 - 1	2
96-well	1 - 4 x 10^4	0.1 - 0.3	n.a.

From 'Useful numbers for Cell Culture' (ThermoFisher) and Lipofectamine® 3000 Reagent Protocol (Invitrogen, Fisher Scientific)

Cell count

A Neubauer chamber consists of 9 big squares, each of 1 mm^2. The depth of the chamber is 0.1 mm. The total volume amounts to 0.1 mm^3 = 0.0001 ml.

$$\text{Concentration of cells / ml} = \frac{\text{Number of cells}}{\text{Number of squares}} \times 10^4$$



Neubauer chamber and the method how to count cells.

(Reprinted form BiteSizeBio).

Appendix V

Transfection of DNA into PFT cells

1.1 Red/ET recombination technology

24 well plate

Lipofectamine mix / RXN :

Opti-MEM™	24 µl
Lipofectamine™ 3000	1 µl
	25 µl

DNA mix / RXN :

Opti-MEM™	12 µl	17.5 µl	18.9 µl	20.95 µl
p3000	2 µl	2 µl	2 µl	2 µl
DNA	1 µg	0.5 µg		
EGFP-C1 plasmid			1 µg	0.5 µg
	25 µl	25 µl	25 µl	25 µl

1.2 CRISPR/Cas9 technology

24 well plate

Lipofectamine mix / RXN :

Opti-MEM™	24 µl
Lipofectamine™ 3000	1 µl
	25 µl

DNA mix / RXN :

Opti-MEM™	6.7 µl	14.85 µl
p3000	2 µl	2 µl
DNA	1 µg	0.5 µg
gRNA (# 21)	0.5 µg	0.25 µg
gRNA (# 23)	0.5 µg	0.25 µg
	25 µl	25 µl
NHEJ Reagent (SCR7)	1 µM	1 µM

Transfection of DNA in PCMV infected PFT cells

2.1 Red/ET recombination technology

24 well plate

Lipofectamine mix / RXN :

Opti-MEM™	24 µl
Lipofectamine™ 3000	1 µl
	25 µl

DNA mix / RXN :

Opti-MEM™	18 µl	22 µl
p3000	2 µl	2 µl
DNA / EGFP-C1 plasmid	0.5 µg	0.1 µg
	25 µl	25 µl

2.2 CRISPR/Cas9 technology

24 well plate

Lipofectamine mix / RXN :

Opti-MEM™	24 µl
Lipofectamine™ 3000	1 µl
	25 µl

DNA mix / RXN :

Opti-MEM™	15.35 µl	21.47 µl
p3000	2 µl	2 µl
DNA	0.5 µg	0.1 µg
gRNA (# 21)	0.25 µg	0.05 µg
gRNA (# 23)	0.25 µg	0.05 µg
	25 µl	25 µl
NHEJ Reagent (SCR7)	1 µM	1 µM

Transfection of DNA in PFT cells, selecting for GPT

Red/ET recombination technology

12 well plate

Lipofectamine mix / RXN :

Opti-MEM™	48 µl
Lipofectamine™ 3000	2 µl
	50 µl

DNA mix / RXN :

Opti-MEM™	24 µl	37.8 µl
p3000	4 µl	4 µl
DNA	2 µg	
EGFP-C1 plasmid		2 µg
	50 µl	50 µl

GPT Reagent:

Mycophenolic acid in EtOH 500x	12.5 mg / ml
1x	25 µg / ml
Aminopterine 100x containing Xanthine	25 mg / ml
Hypoxanthine	1.5 mg / ml
1x containing Xanthine	250 µg / ml
Hypoxanthine	15 µg / ml

Appendix VI

Target sequence for gRNA

gRNA	Target sequence
pX330 #21 clone 2	CCTGCGCCTTCTTCTACCGA
pX330 #23 clone 2	CAAAAATAACTCTAATTCTAC

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