Development of a replication-defective baculovirus platform

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Baculovirus expression vector systems are used for the production of an increasing number of licensed commercial subunit vaccines. Though baculoviruses are incapable of replicating outside a narrow host range of lepidopteran insects, a replication-defective baculovirus system or platform provides an additional level of safety without the use of chemical inactivation methods such as binary ethylenimine. Circumventing the need for chemical inactivation protects sensitive proteins from degradation, and use of a live, yet replication-defective baculovirus has the potential to enhance antiviral immune response. These factors together make a replication-defective baculovirus platform highly desirable for subunit vaccine production. Choosing the essential baculovirus envelope protein gp64 as our target, we used recombination to delete gp64 from the baculovirus rendering it replication-defective. We used insect cell based plasmid expression vectors to generate insect cell lines stably expressing gp64 to allow passage of the replication-defective virus.

Porcine epidemic diarrhea virus (PEDV) remains an economically important disease in swine, and a highly effective vaccine remains elusive. To test the capacity of the replication-defective baculovirus system to express an antigenic target of interest, we used recombination to replace the gp64 in the baculovirus genome with a chimeric PEDV spike protein. We then infected Sf9 cells with the PEDV spike expressing replication-defective baculovirus. Analysis of insect cell supernatants indicated that yield of PEDV spike was low in non-complementing cells, but shows an association between the baculovirus capsid VP39 and PEDV spike suggesting that the pseudotyped spike chimera restores budding of replication-defective baculovirus particles in the absence of gp64 suggesting that the system also has potential as a vector for gene delivery.

CHAPTER 1. INTRODUCTION

Background

Baculovirus expression vector systems (BEVS) are important tools for the expression of subunit proteins and generation of virus like particles (VLPs) for veterinary vaccines. Chemical inactivation of baculovirus preparations by binary ethylenimine (BEI), a standard treatment for inactivation of live baculovirus present in antigens harvested from baculovirus-infected insect cells, bypasses the lengthy and costly regulatory processes required for the use of live, genetically modified viruses. While BEI modifies viral nucleic acids to render virions replication defective, increased temperatures employed during inactivation protocols can also alter or precipitate proteins during the inactivation process. A replication-defective baculovirus platform will allow for production of sensitive antigens without the need for BEI or other chemical viral inactivation methods. Thus, the utility of a replication-defective BEVS warrants further investigation as a potential platform for production of vaccine antigens.

Deletion of the gene encoding viral envelope glycoprotein gp64 from the viral genome renders the virus incapable of replication, and presents a feasible target for the generation of a replication-deficient BEVS.

Objectives

The research objectives were: 1. Generate an insect cell line stably expressing baculovirus envelope glycoprotein gp64 to complement a gp64 gene-deleted baculovirus. 2. Delete the envelope glycoprotein gp64 gene from the baculovirus genome 3. Express a protein of interest from an expression cassette inserted at the baculovirus gp64 locus.

Thesis Organization

This thesis consists of an introduction (Chapter 1), literature review (Chapter 2), original research section (Chapter 3) and conclusion (Chapter 4). The literature review includes a brief history of vaccines, describes the intersection of human and animal health, reviews the discoveries leading to the development of BEVS, summarizes notable improvements in the technology, and highlights the essential envelope protein gp64 and its role in viral replication and techniques for surface display of proteins in the BEVS. The literature review concludes with the selection of the PEDV spike envelope glycoprotein to replace gp64 in the final construct. Chapter 3 is comprised of original research detailing the design and construction of a replication-defective baculovirus expression vector platform carrying a foreign envelope glycoprotein at the gp64 locus of the baculovirus. The conclusion comprising Chapter 4 summarizes the material presented. The author's role in this research included the a) the stable expression of gp64 in a complementing insect cell line, b) purposeful genetic manipulation of the baculovirus bacmid and gene fragments, c) assays for gene deletions, insertions, viral competence and protein expression, , d) analysis and interpretation of results and e) generation of a manuscript for publication.

CHAPTER 2. LITERATURE REVIEW

A Brief History of Vaccines

The observation by Edward Jenner that infection with cowpox appeared to be protective against future exposure to smallpox illustrates connections between human and animal disease and formed the foundation of modern vaccinology. Building on Jenner's discovery, Louis Pasteur demonstrated that vaccination could be applied across species to many shared microbial diseases [1]. Pasteur developed a number of live attenuated vaccines with implications in the intersection of human and animal health including chicken cholera, anthrax and rabies. The first heat-inactivated killed vaccines [2] saw use shortly thereafter. A broad range of physical and chemical techniques for inactivation of bacterial and viral pathogens developed over the next century. Production of safe live attenuated viruses and the number of viruses that could be adapted to cell culture increased as techniques improved through the 20th century. Live attenuated vaccines for select pathogens present the advantage of strong immunogenicity, often providing life-long immunity. However, some live attenuated vaccines have proven difficult to produce, and potentially dangerous reversion to virulence presents a technical hurdle for others. While reversion to virulence is not possible with inactivated vaccines (thus providing an additional level of safety), and because the preparations are not infectious, they often require adjuvants to promote a protective immune response and may not offer life-long protection.

Improved laboratory techniques led to the development of new methods to produce even safer vaccines. The purification of soluble components from pertussis was the first such acellular vaccine developed, and was considered an improvement over the whole-cell preparations previously employed. The common occurrence of injection site reactions lead to the removal of the whole-cell based pertussis vaccine from the vaccine schedule in Sweden, which then saw a

resurgence in cases [3]. The acellular pertussis vaccine substantially reduced injection site reactions and resulted in improved vaccine compliance resulting in a reduction in pertussis cases [3]. The first recombinant subunit vaccine, generated for Hepatitis B virus using a yeast-based production system, came to market in 1986 and replaced a subunit vaccine comprised of Hepatitis B surface antigen (HBsAg) derived from human convalescent serum. Production of the HBsAg antigen by yeast alleviated the risks from exposure to human blood products including Hepatitis B virus infection [4]. Bacterial- and yeast-based expression systems were quickly adopted due to their low cost and relative ease of use, but these systems often generate misfolded proteins which fail to induce a protective immune response [5]. Baculovirus expression vector systems soon emerged as an attractive alternative to bacterial and yeast based systems, allowing high-level expression of target proteins with all of the post-translational machinery of complex eukaryotic cells.

Porcine Epidemic Diarrhea Virus as a Vaccine Target

Porcine epidemic diarrhea virus (PEDV) is an enveloped, single-stranded, positive sense RNA virus from the family *Coronaviridae,* genus *alphacoronavirus,* and causes acute diarrhea, vomiting, and dehydration resulting in a high mortality rate in naïve piglets [6]. The 28 kb genome of PEDV encodes the spike protein (S), which is the primary virulence factor distinguishing PEDV strains [6]. The spike protein serves a number of functions including receptor binding and cell fusion, and induces neutralizing antibodies in the host, making it an attractive target for a subunit vaccine [6].

The first reported cases of porcine epidemic diarrhea (PED) appeared in the United Kingdom in 1971, and the associated viral agent, PEDV, was identified as a new coronavirus designated CV777 in Belgium in 1978, and appeared in several other European countries over the following decades, but outbreaks remained isolated [7]. Both inactivated and modified live

vaccines based on the prototype CV777 strain eventually saw wide use in the Chinese swine industry where the disease is now considered endemic, and the prevalence of PEDV infection remained low with only sporadic outbreaks until late 2010 [6]. Starting in late 2010, a remarkable increase in PED outbreaks occurred in China [6]. Following the new Chinese outbreaks, the virus first appeared in the United States in April 2013 and spread rapidly, causing mortality rates in suckling piglets and substantial economic losses [7]. Conditionally approved inactivated vaccines are now available within the U.S., but to date, breeders have relied on deliberate exposure of sows and gilts (i.e. a process described as feedback) to control outbreaks along with strict disinfection and biosecurity controls. Development of safe and effective new PEDV vaccines with the capacity to be manufactured in bulk at low cost remains an attractive target for the vaccine industry. Several properties of the PEDV spike protein make a replication-defective baculovirus platform an ideal system for surface display of an envelope glycoprotein. First, the spike protein is expressed as a membrane anchored homotrimer, as is the baculovirus gp64 protein, increasing the likelihood of proper multimerization using the baculovirus display technique previously described. Second, the complete spike cDNA sequence is in excess of 4 kb allowing us to test the upper size limits of the system for insertion of a transgene at the gp64 locus of AcMNPV. Finally, reagents and cDNA sequences related to the U.S. outbreak were readily available, allowing direct analysis of protein products without the use of additional tags or markers, which have the potential to introduce confounding variables.

Baculovirus Expression Vector Systems

Baculoviruses are a family of rod shaped viruses (*Baculoviridae*) which are pathogens for a narrow range of insect hosts primarily from the order *Lepidoptera,* but also may affect Dipteran and Hymenopteran insects. Baculoviruses contain large, double stranded, circular dsDNA genomes ranging from 80-180 kb, and contain the genera *Alphabaculovirus*, *Betabaculovirus,*

Gammabaculovirus, and Deltabaculovirus. A member of group 1 of the genus *Alphabaculovirus*, the prototypical baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was recognized in the early 1980's for its ability to produce large amounts of recombinant protein within a eukaryotic system [8, 9]. The insect cells used in BEVS contain the required machinery for complex protein trafficking, oligomerization, and post-translational processing that are not available in bacterial or yeast-based expression systems. This advanced protein processing machinery is advantageous for vaccine target antigen production where proper folding of the target antigen is often critical for the induction of a neutralizing antibody response.

Crucial iterative discoveries led to the development of recombinant BEVS. First, the establishment of continuous insect cell lines allowed *in vitro* characterization of baculoviruses [10], including the isolation of AcMNPV (the model baculovirus and basis for BEVS). Incremental advances continued as researchers applied plaque assay techniques developed for mammalian cell culture to baculovirus. This allowed for viral quantification, purification and isolation of genetic variants [10].

The study of the baculovirus life cycle proved critical for its manipulation and development of BEVS. Interestingly, the virus replicates in two forms depending upon the stage of its life cycle. Initially, the baculovirus replication strategy relies on budding early in its life cycle to spread from the site of initial infection, and budded virus (BV) facilitates spread through the infected host. Later in its life cycle, the baculovirus replication strategy moves toward environmental spread by shifting from production of budded virus to the production of occlusion-derived virus (ODV) that facilitates transmission to new hosts [10]. In late life cycle, the baculovirus shifts its protein expression profile to the synthesis of large amounts of polyhedrin, which is essential to the formation of occlusion bodies (OB). The protein matrix of

the OBs protect ODV from environmental exposure conditions such as desiccation and UV light. As contemporaneous advances in genetic engineering allowed for cloning and sequencing of viral mRNA and DNA, further insight was provided into the location of key protein encoding regions within the viral genome and the expression of viral proteins during infection. These advances in sequencing technology allowed for the mapping and sequencing of the polyhedrin gene [11]. Transfection of purified baculovirus genomic DNA was shown to produce infectious virus [12] and allowed for avenues towards the *in vitro* manipulation of baculovirus DNA. The confluence of these discoveries allowed Smith et al. to determine that the polyhedrin gene was not essential for viral replication in cell culture [13]. Smith et al. then demonstrated robust expression of biologically active human interferon beta (IFN-β) protein in insect cells by the insertion of the IFN-β gene behind the polyhedrin promoter [8], marking the first use of baculovirus as a platform for foreign gene expression. The expression of proteins in BEVS for numerous applications followed, including investigational research, diagnostic applications, antibody development, X-ray crystallography and subunit or VLP-based vaccines.

Researchers have made a number of important improvements to the BEVS since its inception. Initially, purified baculovirus DNA was co-transfected into cultured *Spodoptera frugiperda* cells with a plasmid transfer vector containing the desired insertion sequence flanked by DNA sequences homologous to the baculovirus DNA surrounding the polyhedrin gene [13]. The target sequence was inserted at the polyhedrin locus by homologous recombination, and recombinant viruses were isolated by plaque purification [13]. The early BEVS system developed by Smith et al. had very poor recombination efficiency with only 0.1-1% of plaques containing the desired transgene [10]. Kitts et al. inserted a unique restriction site into the baculovirus genome allowing for the linearization of baculovirus DNA prior to co-transfection

[14]. This linearized DNA was much less infectious than circular baculovirus DNA and was more likely to be restored and circularized by homologous recombination with the transfer plasmid subsequently increasing the frequency of transgene insertion to around 30%. Further optimization of this linearized DNA method was achieved by disruption of the essential gene *orf1629* adjacent to *polh* in the linearized DNA. The transfer vector would then provide the sequence restoring *orf1629* through homologous recombination reducing the number of rescued viruses lacking the desired transgene to approximately 2-3% [10]. This system remains the basis for vectors used in commercial antigen production since it requires no bacterial replication elements or antibiotic resistance markers, which could raise regulatory concerns. Bacmid-based systems containing bacterial origins of replication and antibiotic selection markers followed shortly after, and while not suitable for use in antigen production for the aforementioned reasons, provided many additional options for genome manipulation in bacterial systems desirable in research applications. BEVS have since been adapted to a variety of specialized applications including insertion of additional genes for the formation of complex multi-protein VLPs, or expression of proteins involved in alternate glycosylation pathways that are not normally present in insect cells.

Utility of the Baculovirus Platform and Potential Concerns

Production of recombinant subunit vaccines can occur in low biosafety level environments, which provides a crucial advantage over vaccine platforms dependent upon intact pathogens for production. AcMNPV is classified as a BSL-1 agent presenting minimal potential hazard to laboratory personnel and the environment [15]. In contrast, most influenza strains require BSL-2 containment, and highly pathogenic Avian H5N1 strains require BSL-3. Production at lower biosafety level allows much greater flexibility and more options in manufacturing than more limited and costly BSL-2 or BSL-3 facilities.

Baculoviruses can enhance humoral and cell-mediated immune responses against co-administered antigen. Ovalbumin (OVA) protein administered subcutaneously or intravenously with 10^3 to 10^6 PFU of live baculovirus elicited a potent and long lasting dose dependent increase in anti-OVA IgG titer in mice compared to the antigen administered alone [16]. This included an enhanced cytotoxic T lymphocyte (CTL) response and increased levels of interferon gamma (IFNγ) consistent with a type 1 CTL response to the antigen. Baculovirus inactivation abolished the adjuvant effects [16].

Thorough adventitious agent screening of insect cell lines used for baculovirus (replication-competent or -deficient) production is essential. A new adventitious agent, *Tricoplusia ni* cell line virus (TnCLV), was discovered in the commercial High Five™ cell line by a group at Japan's National Institute of Infectious diseases [17] after infection of High Five™ cells with recombinant baculovirus. Reverse transcription of viral RNA and sequencing of cDNA from viral particles led to its classification as an alphanodavirus with similarities to the well-characterized Flock House Virus [17]. Although TnCLV has shown no infectivity in mammalian cell lines and is not considered to be a mammalian pathogen, the potential biohazard of un-inactivated TnCLV particles remains unclear [17]. A number of TnCLV-free *T. ni* cell lines have since been identified [17].

Arifa Khan's group at the FDA's Center for Biologicals Evaluation and Research (CBER) identified a novel Rhabdovirus, Sf-rhabdovirus, in the *Spodoptera frugiperda* Sf9 cell line using 454 Roche massively parallel sequencing [18]. BLAST searches identified Sf-rhabdovirus elements in Sf9 cells and their parental line Sf21, though not in any other insect cell lines [18]. Although incapable of infecting mammalian cell lines to date, a Sf-rhabdovirus-negative cell line, Sf-RVN, is susceptible to reinfection [17]. Sf-RVN cells allow for testing of

inactivated baculovirus-based vaccines and therapies currently in production to assure inactivation of the Sf-rhabdovirus elements, and Sf-RVN or other Sf-rhabdovirus-free insect cell lines likely can be used safely for replication-defective baculovirus production after extensive adventitious agent testing. Overall, risks associated with adventitious agent contamination of insect cells are generally lower than those associated with the use of mammalian cells [18].

Plasmid-based Protein Expression in Insect Cells

The BEVS is not without limitations. Upon infection with baculovirus, a series of events are initiated that ultimately result in the death and lysis of the infected cell, highlighting that a fine balance of virus replication versus potential yield of the expressed antigen needs to be considered. The very strong polyhedrin promoter becomes active only in the very late phase of baculovirus infection requiring expression of other viral proteins in succession for its activation [19]. Additionally, the promoter is only active for a relatively short time before the cells are killed by the baculovirus infection, and some evidence suggests that secretory pathways responsible for post-translational processing of complex glycoproteins may also be compromised in the later stages of baculovirus infection [19]. To address these concerns, Jarvis et al. identified the AcMNPV promoter IE1 (AcIE1), an immediate early gene that is active very early in infection and requires only host proteins for transcription [19]. Jarvis et al. achieved stable expression of foreign gene products by insertion of the foreign gene under the control of the AcIE1 promoter on a plasmid containing a neomycin resistance cassette [19]. Transfection of Sf9 cells with this plasmid followed by neomycin selection of clones, generated stably transfected insect cell lines expressing the foreign gene [19]. Exploration of immediate early gene promoters OpIE1 and OpIE2 of *Orgyia pseudosugata* multicapsid nuclear polyhedrosis virus (OpMNPV) revealed that OpIE2 is 5- to 100-fold more active than AcIE1 in transiently transfected Sf9 cells [20]. The OpMNPV promoters are comprised of differing genetic sequences than those for

AcIE1, which should also reduce the probability of recombination with AcMNPV should the two expression systems be used in combination. Jarvis et al. demonstrated that expression levels of an *E. coli* β-galactosidase (β-gal) gene were 100- to 5000-fold greater in the BEVS than from stably transfected Sf9 cells, but expression of the complex, secreted glycoprotein human tissue plasminogen activator (tPA) was similar in quantity from either system [19]. They also demonstrated through radiolabeled pulse chase experiments that tPA was processed faster and more efficiently in stably transformed insect cells, and lacked an incompletely glycosylated precursor that was present in the BEVS-expressed protein. [19, 21]. Plasmid-based systems allow production of a foreign protein in insect cells without baculovirus infection. The ability to generate a cell line that stably expresses a foreign protein makes possible the selective complementation of a viral protein in insect cells facilitating deletion of the protein in the viral genome. The use of this system for complementation of an essential viral protein allows for the production of a replication defective virus that is only capable of reproducing in insect cells supplying the essential protein.

Lambda Red Recombineering

Manipulation of the baculovirus genome to establish the BEVS used homologous recombination in insect cells [13]. Addition of a mini-F bacterial origin of replication at the polyhedrin gene locus of AcMNPV allows for maintenance and manipulation of the resulting circular bacmid in *E. coli*. The labs of Kenan Murphy and Francis Stewart independently reported the technique termed Lambda Red recombination in 1998 [22, 23]. Lambda Red recombination is capable of replacing a chosen DNA sequence with a linear DNA sequence flanked by sequences of homology as small as 30 base pairs surrounding the sequence targeted for manipulation using recombination proteins derived from lambda bacteriophage. The Red system is comprised of three lambda phage proteins [24]. The lambda protein Gam binds to the

linear DNA preventing degradation by the *E. coli* RecBCD complex [24]. Beta anneals the single strand end of the linear DNA with the complementary sequences in the target bacmid, and protects the 3’ single strand extension of Exo, the homotrimeric 5’-3’ exonuclease that integrates the linear sequence into the replicating target DNA [24].

Subsequent iterations of the technique introduced arabinose-induced expression of the homing endonuclease I-SceI along with sequence duplication of the homology regions to remove introduced antibiotic resistance markers with a second round of recombination [25]. The lambda phage proteins required for recombination along with arabinose-induced I-sceI expression were integrated into *E. coli* chromosomal DNA in the GS1783 strain of *E. coli* allowing for highly efficient recombination without the maintenance and possible loss of extrachromosomal plasmids [24]. A two-step Red-mediated recombination method allows for modification of nearly any area of the baculovirus genome in engineered *E. coli* strains such as GS1783 without regard to the location of restriction sites, and leaving no residual sequences or markers [24].

Baculovirus Envelope Protein gp64

The envelope glycoprotein gp64 plays a central role in the formation of the viral envelope and viral budding [26] and is present in all members of group I within the genus *Alphabaculoviridae*, including AcMNPV. Monsma et al. demonstrated that gp64 is essential for production of infectious viral particles by disruption of gp64 within the AcMNPV genome. Infectivity of the gp64-deficient baculovirus was restored by expression of the gp64 homolog from OpMNPV *in trans* by stably transfected Sf9 cells [27]. The 64 kilodalton (kDa) membrane glycoprotein gp64 is expressed early in the viral life cycle, appearing from one to four hours after infection with peak expression at 20-24 hours [28]. Gp64 facilitates cell entry of BV produced within the BEVS primarily through clatherin-mediated endocytosis wherein gp64 binds to cellular receptors on the surface of clatherin-coated structures. Baculovirus binding triggers

the internalization of the bound virus into endosomal structures, and acidification of the endosome after internalization facilitates a conformational change in gp64 resulting in fusion of the viral envelope with the endosomal membrane, releasing the nucleocapsid into the cytoplasm [29]. Once the nucleocapsid has been released from the endosome, the viral capsid protein P78/83 facilitates actin-based transportation of the viral nucleocapsid into the cell nucleus where uncoating occurs and the viral genome can be transcribed [30].

Baculovirus Display of Envelope Glycoproteins

Phage display technology was developed in prokaryotic systems for the selection of high affinity binding ligands from large libraries of peptide epitopes fused to the capsid protein encoded by filamentous bacteriophages. The limitations of prokaryotic post-translational processing of eukaryotic proteins led to the utilization of BEVS to display complex eukaryotic proteins on the baculovirus surface by chimeric fusion of the foreign protein to baculovirus gp64. Both Glutathione S-transferase (GST) and HIV gp120 proteins were displayed on the baculovirus surface without disruption of proper folding or the infectivity of the baculovirus by insertion of the respective coding sequences into the gp64 encoding sequence immediately following the signal sequence [31].

Baculovirus display was adapted for use in experimental vaccines displaying influenza hemagglutinin (HA). Both influenza HA and baculovirus gp64 are homotrimeric envelope glycoproteins responsible for viral entry and budding and contain N-terminal signal peptides (SP) followed by their respective binding and membrane fusion domains and C-terminal transmembrane (TM) and cytoplasmic tail domains (CTD). Chimeric constructs where native HA sequences with gp64 SP TM, and CTD in all possible combinations were expressed in BEVS [32]. Constructs containing the gp64 TM domain produced soluble HA which was unbound to viral particles as it remained in the supernatant after ultracentrifugation, while HA with native

TM domain with gp64 SS and CTD was found only with the pelleted virions. Taken together, these findings demonstrate that gp64 SS and CTD are necessary for baculovirus surface display of the HA/gp64 chimeric proteins.

CHAPTER 3. DEVELOPING A REPLICATION-DEFECTIVE BACULOVIRUS PLATFORM

Introduction

Use of binary ethylenimine (BEI) is a standard treatment for inactivation of live baculovirus present in antigens harvested from baculovirus-infected insect cells. In addition to the intended modification of viral nucleic acids, BEI in combination with increased temperatures employed during inactivation protocols can also alter proteins or result in precipitation of proteins during the inactivation process. Use of a replication-defective baculovirus platform would allow for production of sensitive antigens without the need for BEI inactivation. Baculoviruses are already highly specific to insect cells, and rendering baculovirus replication-defective adds an additional level of safety to a system that has an already excellent safety record with over a decade of use in humans and animals. USDA Veterinary Services Memorandum 800.213 allows for the licensure of non-replicating, non-viable platforms for vaccine production. The combination of these two factors makes the design of a replication-defective baculovirus vaccine platform desirable for production of vaccine antigens. The *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) envelope glycoprotein gp64 is essential for replication of infectious viral particles. Deletion of gp64 from the viral genome has been shown to leave the virus incapable of replication [27] in insect cells. Stably transfected insect cells expressing the deleted gp64 are required to complement the deleted gp64 and allow passage of the replication-defective virus.

Materials and Methods

Design of Expression vectors for gp64 Constitutive Expression in Insect Cells

The native gene sequence for the gp64 homolog from *Orgia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) preceded by a partial mammalian Kozak consensus sequence

(GCCACC) flanked by HindIII (5') and XbaI (3') restriction enzyme sites was synthesized by Integrated DNA Technologies, Inc. and inserted into the pIDT-Kan cloning plasmid. The OpMNPV gp64 insert was excised from the vector using HindIII and XbaI, gel purified, and ligated into the pIB/V5-His expression plasmid (Figure 1). The OpMNPV gp64 construct contained a stop codon prior to the V5/His tag sequence within the vector such that the tags were not expressed. Sf9 (*Spodoptera frugiperda*) cells were seeded at a density of 1 x 10^5 cells/cm^2 in Sf900III serum free insect cell media (Invitrogen). Cells were transfected with 1 µg or 5 µg of the OpMNPV gp64-pIB/V5-His plasmid DNA using 8 µL/mL Cellfectin II insect cell transfection reagent (Invitrogen). Each transfection was performed in duplicate. Transiently transfected cells were incubated for 72 hours at 27°C, and cells were assessed by immunofluorescence-based staining using anti-baculovirus gp64 AcV5 mAb. Transfected cells intended for selection were incubated for 48 hours post transfection and split at a 1:5 ratio. Sf9 cells with stably integrated plasmids were selected by addition of 80 µg/mL Blasticidin S HCl. Cells were grown to confluence and expanded. Two cell pools confirmed to express OpMNPV gp64 (designated Sf9 Op64 #1 and Sf9 Op64 #2) were expanded and cryopreserved for further use. Later, the process was repeated using the gp64 encoding nucleotide sequence from AcMNPV amplified from the BaculoG/mCherry-miniF-Bsu36I bacmid using primers containing the partial mammalian Kozak consensus sequence (GCCACC) at the 5' end flanked by HindIII (5') and XbaI (3') restriction enzyme sites. The PCR product was ligated into the PCR blunt II TOPO vector, and transformed into DH5α *E. coli*. TOPO clones were expanded, and the plasmid DNA was purified by QIAprep Spin Miniprep Kit (Qiagen). The nucleotide sequence of the cloned AcMNPV gp64 PCR product was confirmed by Sanger sequencing, and transferred to the pIB/V5-His expression plasmid as described above. Cell pools of Sf9 cells stably expressing

AcMNPV gp64 were generated as described for OpMNPV gp64, and selected cell clones designated as Sf9 Ac64 A4, Sf9 Ac64 C2, and Sf9 Ac64 E3, respectively.

Design and Construction of a Red Recombination Vector for the AcMNPV gp64 Locus

A vector was designed for Red recombination at the gp64 locus of AcMNPV using Lasergene Seqbuilder Pro. EcoRI and I-ceuI sites were followed by 203bp of sequence complementary to the 5' flank of gp64 containing a portion of the Ac-p24 gene along with the first 68bp of the gp64 promoter. Following the partial promoter sequence, an I-SceI restriction site and the kanamycin resistance gene (Kan) allow the use of kanamycin for selection of clones containing the inserted sequence. Subsequent recombination can result in deletion of the kanamycin resistance after induction of the I-SceI enzyme in GS1783 *E. coli* under an arabinose-induced promoter. The 5' flanking region was followed by a multiple cloning site for insertion of the desired foreign gene. The multiple cloning site region is followed by a 3' flanking region complementary to the 3' flank of AcMNPV gp64 containing 109 bp of intergenic region between AcMNPV gp64 ending in the last 26 bp of the Ac-v-cath gene. This is followed by another I-ceuI restriction site, and a PciI restriction site. This construct was synthesized as two gBlocks gene fragments from Integrated DNA Technologies (IDT) designated LH1 and LH2 to be joined by an XhoI restriction site at the 3' end of LH1 and 5' end of LH2. The two fragments were cloned into the pCR Blunt II TOPO vector. LH1 fragment was digested with EcoRI/XhoI. LH2 was digested with XhoI/PciI. The pUC19 vector was digested with EcoRI/PciI, dephosphorylated, and the components were ligated with T4 ligase and transformed into DH5α *E. coli*. Clones having the assembled LH1 and LH2 were expanded and DNA sequence was verified. The plasmid was designated pUC19-Δgp64-UTC.

Recombination to Remove gp64 from AcMNPV

The BaculoGold-mCherry-miniF bacmid (Figure 2) was transformed into GS1783 *E. coli* by electroporation in a 1mm cuvette (1.5 kV, 25μF, 200Ω), and plated on LB agar plates containing 30 μg/mL chloramphenicol. Chloramphenicol resistant clones containing the bacmid were expanded in LB broth and a stock of competent cells was prepared as described in Tischer et al. The recombination insert was digested from the pUC19-Δgp64-UTC overnight (18h) with Iceu-I and agarose gel purified. The purified recombination fragment was electroporated into the GS1783 cells containing the BaculoGold-mCherry-miniF bacmid. The cells were incubated in SOC media for 2 hours at 32˚C, and plated on LB agar with 30 μg/mL of chloramphenicol, and 50 μg/mL kanamycin (LB chloramphenicol/kanamycin). Kanamycin resistant colonies were then expanded and plasmid minipreps were performed for evaluation of target DNA by PCR screen. The second phase of Red recombination was performed using the PCR screened clones. The selected PCR screened clones were used to inoculate 2mL of LB broth with 30 μg/mL of chloramphenicol followed by incubation at 32˚C for 3.5 hours. After initial incubation 220 μL of 10% arabinose was added to a final arabinose concentration of 1% arabinose, and tubes were incubated an additional 1 hour at 32˚C, heat shocked at 42˚C for 30 minutes, and incubated at 32˚C for an additional 2 hours with shaking at 225 RPM. Serial tenfold dilutions of each tube were performed in LB and 100 μL of the 10^{-4} and 10^{-5} dilutions were plated on LB agar plates with 1% arabinose and 30 μg/mL of chloramphenicol and incubated overnight at 32˚C. The resulting colonies were screened on LB chloramphenicol (30 μg/mL) and LB chloramphenicol/kanamycin (30 μg/mL, 50 μg/mL) plates to test for kanamycin sensitivity. Kanamycin sensitive colonies were expanded, Bacmid DNA was purified, and PCR screened for the correct deletion. Deletion of gp64 from the Bacmid was confirmed by MiSeq analysis.

Alternate Recombination to Replace gp64 with PEDV Spike Protein

A synthetic gene was ordered from IDT as a gBlock. The sequence consisted of 221 bp of complementary sequence upstream of the gp64 gene including the gp64 promoter sequence followed immediately by an I-SceI restriction site, the coding sequence for EGFP, kanamycin resistance gene, MauBI restriction site, and 77 base pairs complementary to the 3' flank of gp64. The DNA was reconstituted in nuclease free water and 100ng was transformed by electroporation in a 1mm cuvette (1.5 kV, 25µF, 200Ω). Transformed GS1783 *E. coli* cells were incubated for two hours with SOC media at 32ºC with 250 RPM shaking, and then plated onto LB agar containing 30 µg/mL chloramphenicol and 50 µg/mL kanamycin. Resulting colonies were expanded in LB broth and Bacmid DNA was purified by Compact Prep Maxi DNA kit (Qiagen). Purified bacmid DNA was digested with I-SceI and MauBI to remove the EGFP and kanamycin resistance genes. The resulting linear bacmid DNA (100ng) was co-transfected using Escort IV transfection reagent into gp64-complementing insect cells with the digested and gel purified PEDV Spike BaculoDisplay sequence, which contained flanking recombination sequences from the pUC19-Δgp64 (300ng). Clones were then selected using two rounds of limiting dilution based on immunofluorescence-based staining with anti-PEDV rabbit serum. Viral DNA was extracted from the selected clones and transformed back into DH10B *E. coli* for maintenance.

Flow Cytometry Analysis of gp64 Expression by Transfected Insect Cells

Insect cells were fixed using BD Cytofix/Cytoperm™, washed with 1x BD Perm/Wash™ buffer, and 2 x 10^6 to 5 x 10^6 cells per sample were incubated with anti-gp64 monoclonal antibody AcV5 (100 µL diluted 1:100 in 1x perm/wash) for 30-60 min at 4ºC or with no primary antibody (1x Perm/Wash™ only). The fixed cells were then washed three times by re-suspending cells in 1 mL 1x Perm/Wash™, centrifugation at 1000 x g for 5 min at 4˚C and the supernatant

was removed from the pelleted cells. The pelleted cells were resuspended by mixing with FITC conjugated goat anti-mouse IgG (H+L) secondary antibody (100 µL diluted 1:100 in 1x Perm/Wash™) and then incubated 30-60 min at 4°C. Fixed cells were again washed three times as previously described, and the cells were then analyzed on a BD Accuri C6 cytometer with fluidics set to slow and a minimum of 10,000 events. Analysis was performed using BD Accuri C6 software.

Immunofluorescence-based Staining

Immunofluorescence-based staining was performed to test expression of target proteins. Cells were fixed to 96 well polystyrene cell culture plates using ice-cold 50% acetone, 50% methanol solution for 20 minutes, and dried. Wells were washed with 250 µL of PBS (pH 7.4), and 100uL of primary antibody diluted as indicated by antibody in PBS (pH 7.4). Plates were incubated for one hour at 37ºC. After incubation, each well was washed three times with 250 µL of PBS (pH 7.4). A species appropriate anti-IgG FITC conjugated secondary antibody diluted in PBS (pH 7.4) was added at 100 µL per well and plates were again incubated for one hour at 37ºC. Plates were again washed three times with 250 µL of PBS (pH 7.4), and observed by fluorescence microscopy with FITC filter. Primary and secondary antibodies used are listed in Table 1.

Sucrose Gradient Fractionation

Virus from 30mL of BaculoG/Δgp64 PEDVS BD supernatant was pelleted by ultracentrifugation at 100,000 x g for 2 hours at 4°C. The supernatant was removed and the resulting viral pellet was resuspended in 200 µL of TBS pH 7.6 (20mM Tris HCl pH 7.5, 150mM NaCl). The resuspended pellet was added to a 3.6 mL sucrose gradient consisting of 6 x 0.6 mL volumes of sucrose in TBS from 60% to 10%. The gradient was ultracentrifuged at

100,000 x g for 16 hours at 4°C. The gradient was then separated into 14 x 0.270 mL fractions for analysis by Western blot**.**

SDS-PAGE and Western Blotting

SDS-PAGE was performed using the NuPAGE electrophoresis system and 4-12% Bis-Tris MES mini gels (Invitrogen). Samples were separated under reducing conditions using 0.05M DTT at 150V for the appropriate time. Gels were transferred to nitrocellulose membranes using the Trans Blot Turbo system (Bio-Rad) for Western blots. Western blots were performed by the Snap ID 2.0 method (EMD Millipore) and developed using a one component TMB membrane peroxidase substrate (Seracare). Primary and secondary antibodies used are listed in Table 2.

qPCR of gp64 and Blasticidin Resistance Gene Integration Rates in Sf9 Cells

Genomic DNA was extracted from Sf9 Op64 #2 cells using a Qiagen DNeasy Blood & Tissue kit. Primers and probes were designed for the Blasticidin resistance gene in the pIB/V5-His vector, and for the inserted OpMNPV gp64 gene. Primers and probe for the OpMNPV gp64 gene were used at final concentrations of 500 nM for the primers, and 250 nM for the probe. The concentrations for the Blasticidin resistance gene were optimal at 125 nM for the primers, and 250 nM for the probe. Using 10ng of Sf9 Op64 #2 genomic DNA as a template, the primers and probes at the listed final concentrations were added to iTaq universal probes supermix (Bio-Rad), and cycled under the following conditions: 1 cycle at 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 15 seconds with fluorophore data collection. Primer and probe sequences are listed in Table 3.

DNA Purification

DNA was purified using a number of DNA extraction kits based on the application. The manufacturer recommended protocols were used unless otherwise specified. Baculovirus DNA

was extracted using Qiagen Viral RNA kit with the omission of the carrier RNA. Plasmid DNA was extracted by Qiagen QIAprep Mini Kit for high copy plasmids such as pIB/V5-His and its iterations. BaculoGold bacmid DNA was purified using Qiagen CompactPrep Maxi Kit using the vacuum manifold method using 0.75g of pelleted *E. coli* per 5mL of buffer P1. Sf9 cell DNA was extracted for qPCR analysis using Qiagen DNA Blood and Tissue Kit.

Immunoprecipitation

Antibodies were first covalently bound to Dynabeads M-270 Epoxy (Invitrogen). Ten µg of anti-PEDV S1 or anti-VP39 affinity purified polyclonal antibodies were each bound to 5 mg of Dynabeads M-270 according to the manufacturer protocol and resuspended in a final volume of 1mL PBS (pH 7.4). Fifty µL of each bead slurry was added to a 2mL microcentrifuge tube for each reaction. Beads were separated by placing the microcentrifuge tube in a magnetic separation tube tack for 1 minute. Beads were washed twice with 1 mL PBS (pH 7.4). Washed beads were then incubated with 1 mL each of BaculoG/Δgp64-mCherry-miniF (negative control) and BaculoG/Δgp64-PEDVS BD, and 300µL of BaculoG/PEDVS BD (positive control) at 37°C for 2 hours on a rotating mixer. Beads were separated using a magnetic bead separator, the supernatant was removed, and beads were resuspended in 1mL PBS (pH 7.4). Beads were washed two more times with PBS, and the bound antigen was then eluted with 50µL 0.1M Glycine buffer pH 3.0 at 50°C for 10 minutes, and neutralized with an equal volume of 0.5M Tris HCl pH 8.0. Eluates were then visualized by Western blotting using anti-PEDV S1 or anti-VP39 affinity purified polyclonal antibodies.

Next Generation Sequencing for Confirmation of Gene Deletion

Viral culture supernatant harvests were filtered via 0.45µM spin filter and were treated with a cocktail of RNase and DNase to digest free host DNA/RNA and enrich for viral particle protected nucleic acids. Following nuclease treatment, standard nucleic acid extraction was

performed using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) per the manufacturer's protocol. Libraries were generated using either the extracted viral nucleic acids or plasmid/bacmid constructs directly with the NexTera XT DNA Library Preparation Kit (Illumina, San Diego, CA) and purified using the MinElute® PCR Purification Kit (Qiagen, Hilden, Germany). Library concentrations were titrated, pooled and ran on a MiSeq using a 500-cycle MiSeq Reagent Kit v2 or v3 (Illumina, San Diego, CA). Data was analyzed using a combination of alignments to reference sequences and *de novo* assembly. Alignments and *de novo* assemblies were performed via NextGene software version 2.4.2.3 (Softgenetics, LLC, State College, PA) and subsequent analysis was performed using Sequencher software version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI). High quality sequences were selected as those containing a median Q-score of greater than 25 and trimmed to contain no more than three uncalled bases at 3'-end or three consecutive bases with Q-score measuring less than 16. Sequences were aligned to reference files using criteria of 75% or greater match over a 25bp stretch.

Results and Discussion

Preparation of gp64-Complementing Insect Cell Line

The first step required in generating a replication-defective baculovirus was to create a complementing cell line expressing the essential baculovirus envelope protein gp64. After deletion of gp64 from the viral genome, these cells complement the modified virus by providing the gp64 envelope protein necessary for production of viable budded virus. The pIB/V5-His vector (Figure 1) was utilized to express gp64 from OpMNPV (Op gp64) either transiently by transfection without selection or stably by selection with Blasticidin. Multiple methods were employed to evaluate expression of the complementing gp64 protein in the resulting Sf9 cells.

Firstly, immunofluorescence-based staining was performed on transfected Sf9 cells using available anti-gp64 antibodies (data not shown) confirming gp64 expression. Secondly, harvests of cell culture supernatants were evaluated by Snap ID Western blot using mouse anti-gp64 monoclonal antibody AcV5. Op gp64 transfections both showed strong bands at approximately 64 kDa indicating gp64 expression (Figure 5).

To determine the percentage of cells expressing gp64 as a result of transient transfection or stable transfection, gp64 expression was evaluated by flow cytometry using fluorescent antibody staining from the respective transfections (Figure 6). The samples were gated based on staining of the target cells with FITC-conjugated secondary antibody only. Baculovirus-infected Sf9 cells (positive control) at 3 days post-infection (dpi) clearly showed a shift in fluorescence with 90% of the cells expressing gp64. Cells transiently-transfected with the pIB Op gp64 plasmid displayed a broader range of fluorescence with fewer cells staining positive for gp64 than the baculovirus-infected control. After Blasticidin selection, the stably-transfected Sf9 line designated Sf9 Op64 #2 showed gp64 expression similar to that seen from baculovirus-infected Sf9 cells with 80% of the cells assayed expressing gp64. Given that the transiently-transfected Sf9 cells were found to have a lower percentage of gp64-positive cells, only the stably-transfected Sf9 cells were further investigated.

After establishing expression of gp64, flow cytometry was used to measure the impact of passage of stably transformed Sf9 Op64 #2 cells in the absence of Blasticidin (Figure 7). The cells were passed six times with and without Blasticidin, and compared by flow cytometry. After six passes without selection, the fluorescence measured from gp64 expression was similar to cells passed with Blasticidin maintenance. Following the initial assessment, Sf9 Op64 #2 cells were maintained for an additional 12 passes without Blasticidin and tested again. The percentage

of gp64 positive cells after a total of 18 passages with or without Blasticidin was similar (Figure 7). While the results do not rule out some loss of gp64 expression, it is likely that Blasticidin selection could be withheld for a number of passes so that selective antibiotics would not be present in the cell culture media in passages leading up to antigen production. More rigorous testing regarding the need for Blasticidin should be performed to confirm these preliminary data.

Deletion of gp64 from AcMNPV

With gp64-complementing Sf9 cells established, the next objective was to use Lambda Red recombination to remove the coding sequence for gp64 from the baculovirus bacmid. The linear recombination sequence DNA was maintained on a separate plasmid containing a high copy pUC19 origin of replication. Despite restriction enzyme digestion and gel-purification of the plasmid, carryover of small amounts of undigested pUC19 caused a large percentage of falsely positive colonies. This problem was addressed by using only 66 picograms of the gel-purified template for PCR amplification of the linear recombination sequence. Recombination was performed as described in Materials and Methods using the PCR-amplified fragment (Figure 3). After test transfection, it was discovered that the initial bacmid DNA prep contained a mixture of gp64-deleted and unmodified bacmid. The bacmid DNA prep containing the mixture of gp64-deleted and unmodified bacmid was transformed into DH10B *E. coli*, and the resulting clones were screened by PCR for the desired gp64 deletion. The rederived gp64-deleted bacmid, designated BaculoG/mCherry-miniF-Δgp64, was then transfected into insect cells. Deletion of gp64 was confirmed experimentally by transfection of BaculoG/mCherry-miniF-Δgp64 DNA. Transfection of the bacmid into insect cells was found to produce cells expressing mCherry from the polh promoter in both standard Sf9 cells and Sf9 Op64 #2 (gp64-complementing Sf9 cell line). It was also observed that foci of concentrated mCherry fluorescence, suggesting reinfection, occurred only in Sf9 Op64#2 cells (Figure 8). MiSeq next generation sequencing

analysis confirmed deletion of the entire coding sequence of AcMNPV gp64 from both the BaculoG/mCherry-miniF-Δgp64 bacmid, and the resulting baculovirus obtained by transfection of Sf9 Op64 #2 cells.

Recombination to Replace gp64 with PEDV Spike Protein

The spike envelope glycoprotein from PEDV was previously expressed in the BaculoDisplay system, which replaces the native signal peptide and cytoplasmic tail domain of PEDV spike with those of gp64. The PEDV spike antigen was shown to be very susceptible to degradation during inactivation with BEI, and this antigen would in turn serve as an excellent candidate to demonstrate the advantages of a replication-defective baculovirus system not requiring BEI inactivation. We replaced the deleted envelope protein, gp64, with an alternate envelope glycoprotein from PEDV creating a pseudotyped virus with the encoding sequence for the PEDV spike BaculoDisplay protein at the gp64 locus under the native gp64 promoter.

Use of PCR product to perform Lambda Red recombination to generate the initial gp64-deleted construct was problematic for insertion of the PEDV spike BaculoDisplay protein due to the large insert size. Low PCR yields resulted in several unsuccessful attempts to introduce the sequence using the lambda Red recombination protocol, so an alternate protocol was established (Figure 4). Rather than perform the recombination in two steps within GS1783 *E. coli*, an initial recombination step performed in GS1783 *E. coli* which inserted a Kanamycin resistance gene and GFP marker flanked by unique restriction sites and recombination sequences complementary to DNA sequences at the 5’ and 3’ regions of the gp64 locus of our BaculoGold bacmid. The bacmid DNA was then linearized using the unique restriction sites I-sceI and MauBI which removed the markers from the bacmid, and a second round of recombination was performed in insect cells to insert the PEDV spike BaculoDisplay sequence behind the gp64 promoter at the site of the deleted gp64 gene.

Expression of the PEDV spike, as well as the baculovirus envelope (gp64) and capsid proteins (VP39) were tested in immunofluorescence-based staining (Figure 9). The combined immunofluorescence-based staining results confirm deletion of gp64 from both the BaculoG/Δgp64-mCherry-miniF, and BaculoG/Δgp64 PEDVS BD constructs. Successful baculovirus entry was confirmed in all constructs by VP39 staining while foci of infection were only present in gp64-complementing cells. Expression of PEDV spike was confirmed in both the replication-competent control BaculoG/PEDVS BD and the gp64-deleted construct BaculoG/Δgp64 PEDVS BD. Expression of PEDV spike by BaculoG-Δgp64-PEDVS BD was compared to BaculoG-PEDVS BD by Western blot (Figure 10l). The Δgp64 virus showed reduced expression of the full-length PEDV spike BaculoDisplay protein under the gp64 promoter when compared to the replication-competent BaculoG/PEDVS BD expressing spike under the powerful polyhedrin promoter. Despite reduced expression level of PEDV spike under the gp64 promoter, the level of expression was surprisingly high given limitations of the replication-defective platform described in the subsequent sections.

Reduced Viral Titers in gp64-deleted Baculovirus Grown in gp64-Complementing Cells

Typical budded, replication-competent BaculoGold baculovirus virus titers from suspension culture infections of Sf9 cells range from 1×10^7 to 1×10^9 $TCID_{50}$/mL. Titrations of replication-competent BaculoGold baculovirus were assessed in both gp64-complementing Sf9 cells and in non-complementing Sf9 cells with comparable titers determined from each cell line. Titers of gp64-deleted virus were found to be 100- to 1000-fold lower than standard BaculoGold when evaluated on the gp64-complementing Sf9 cells.

To determine the root cause of the reduction in viral titers, we investigated several possible factors. First, the pIB/V5-His expression plasmid randomly integrates into the host cell DNA, often in multiple locations, after transfection. Partial integration of the pIB/V5-His

expression plasmid could confer Blasticidin resistance and potentially provide reduced gp64 expression, which in turn, would result in lower viral titers obtained for gp64-deleted baculovirus grown in the gp64-complementing cells. A qPCR assay was designed to investigate whether the entire plasmid was integrated or whether pressure from Blasticidin selection could promote partial integration of the plasmid including only the Blasticidin resistance gene. Genomic DNA was extracted from the stably-transfected Sf9 Op64 cells, and qPCR was performed using primer sets specific for either the inserted Op gp64 gene or the Blasticidin resistance gene. The Cq values of 25.15 and 25.34 for the inserted Op gp64 gene and the Blasticidin resistance gene, respectively, indicate a 1:1 ratio of the two genes suggesting that the full plasmid is being integrated at the site of insertion.

Next, to evaluate whether the use of Op gp64 in the complementing cells contributes to the reduced viral titers, homologous recombination was used to generate a version of BaculoGold AcMNPV virus with the gp64 from AcMNPV replaced with the gp64 homolog from OpMNPV. Titers from the OpMNPV gp64-pseudotyped virus (BaculoG/Op gp64 mCherry miniF) showed a significant reduction in mean titer (Figure 11) when compared to that of the standard BaculoGold (Student t-test, $P = 0.0003$). The introduction of the gp64 homolog from OpMNPV appears to be responsible for some, but not all of the reduction in virus titers.

Given the titer reduction seen when AcMNPV gp64 was replaced with OpMNPV gp64, we generated stably-transfected Sf9 cells with a version of the pIB/V5 His plasmid carrying the AcMNPV gp64 coding sequence from BaculoGold. Sf9 cells stably-expressing gp64 from AcMNPV (Sf9 Ac64) showed similar titers for both replication defective viruses to Sf9 Op64 cells stably-expressing gp64 from OpMNPV by TCID50 assay (data not shown), but differences in infection kinetics were observed (Figure 12). These results suggest that the TCID50 titer of the

replication-defective, gp64-deleted virus does not accurately predict its ability to infect gp64-complementing Sf9 cells. Together these results implicate the use of the OpMNPV gp64 homolog as a factor, but not the only factor contributing to the observed reduction in infectivity of the gp64-deleted virus.

It has been previously demonstrated that plasmid-based expression of GFP under the OpIE2 promoter contained within the pIB/V5-His plasmid is reduced after baculovirus infection [33]. Three new plasmids replacing the OpIE2 promoter within the pIB/V5-His plasmid with alternate promoters expressing gp64 were constructed with the goal of improving replication-defective baculovirus titers (Table 4).

The first construct replaced the OpIE2 promoter with the native gp64 promoter from AcMNPV, and this plasmid was designated pBIIB1 (Table 4). Sf9 cells stably-transfected with pBIIB1 expressing Op gp64 (pBIIB1-Op64) failed to produce any viable virus. Additional constructs were produced including a hybrid promoter combining the hr5 enhancer region from AcMNPV followed by the OpIE2 promoter and then the AcMNPV p10 promoter (pBIIB2, Table 6), and the hr5-p10 promoter lacking the OpIE2 elements (pBIIB3, Table 6). The hybrid promoter plasmids were then used to generate stably transfected Sf9 cell lines. While both pBIIB2 and pBIIB3 transfected Sf9 cells produced viable virus, both constructs initiated frequent recombination events during replication-defective AcMNPV infection, which reintroduced functional gp64 into the genome of the gp64-deleted virus. It is likely that the homologous repeat region hr5 from AcMNPV is responsible for the aberrant recombination events. No recombination events restoring gp64 were observed when utilizing constructs lacking the hr5 sequence.

Given the opportunity to revisit insect cell expression of gp64, generating pIB-based plasmids with additional hybrid promoters lacking the hr5 element could improve complementing gp64 expression in insect cells. Combining the constitutive OpIE2 promoter with an essential AcMNPV gene promoter such as the capsid protein VP39 would be a logical next step. VP39 expression is temporally aligned with gp64 expression and use of this promoter could improve gp64 expression following baculovirus infection. Any recombination of Sf9 expressed gp64 with the VP39 promoter, which might restore gp64 to the replication-defective virus, should then disrupt VP39 providing negative selection against recombination. Other essential viral proteins requiring lower levels of transcription during the viral life cycle may also be targets for future work.

Expression of PEDV Spike in Standard Sf9 Cells without gp64

Production of a PEDV spike-expressing construct in a standard insect cell line would ideally produce a baculovirus capsid that has a viral envelope containing PEDV spike rather than gp64. To simulate final antigen production in an insect cell line lacking gp64 complementation, we infected Sf9 cells at a multiplicity of infection of 3, which was the highest possible when using BaculoG/Δgp64 PEDVS BD given its low titers from gp64-complementing cells. A viral particle enveloped with only the target protein should provide an improved immune response directed primarily at the target antigen displayed on the viral envelope. It was unknown whether the PEDV Spike BaculoDisplay protein would restore baculovirus budding in the absence of complementing gp64 with only the cytoplasmic tail domain of gp64. We performed a sucrose gradient purification of supernatant from BaculoG/Δgp64 PEDVS BD infection of Sf9 cells. Both PEDV spike and baculovirus capsid VP39 were present in the harvest supernatant, and sucrose gradient purification results in fractions containing both PEDV spike and VP39. The blots with anti-PEDV and anti-VP39 antibodies (Figure 13) show co-localization of both VP39

and PEDV Spike in gradient fractions 5-12 which suggests that the capsid and spike proteins are associated in at least some of these fractions.

After confirming that both the VP39 capsid protein and PEDV spike envelope proteins co-localize in gradient fractions 5-12, immunoprecipitation was performed with either anti-PEDV S1 or anti-VP39 conjugated Dynabeads M270 using BaculoG/Δgp64-PEDVS BD supernatant expressed in non-complementing Sf9 cells along with a matched BaculoG/Δgp64 with gp64-deleted as a negative control. A standard BaculoGold virus expressing PEDVS BD under the polh promoter was used as a positive control. Western blots were then performed using both antibodies (Figure 14). Immunoprecipitation utilizing anti-PEDV S1 revealed no PEDV spike or VP39 for the negative control, whereas the BaculoG/Δgp64-PEDVS BD revealed both PEDV spike and VP39 indicating an association of PEDV spike with the AcMNPV capsid VP39. The positive control, being replication-competent, contained both proteins at a higher concentration than the replication defective construct. Immunoprecipitation with anti-VP39 detected VP39 for all samples, but no PEDV spike for the negative control indicating that VP39 present in the supernatants likely comprised non-enveloped capsid which was released due to cell lysis. Small amounts of PEDV spike in both the BaculoG/Δgp64-PEDVS BD and BaculoG/PEDVS BD control supernatants that were immunoprecipitated with anti-VP39 suggest that some population of particles contained only a partial envelope allowing binding to the otherwise enveloped and inaccessible capsid proteins. This finding suggests that the replication-defective baculovirus pseudotyped with PEDV spike also has potential as a gene delivery vector. A cassette containing an additional copy of PEDV spike driven by a mammalian promoter inserted at the available polyhedrin site of the baculovirus could direct an immune response to the same swine intestinal cells targeted by PEDV.

CHAPTER 4. CONCLUSIONS

A strong need for an effective PEDV vaccine remains unmet after the 2013 U.S. outbreaks. Inactivated vaccines produced by Zoetis and VIDO-Intervac are now conditionally licensed along with the S-protein based iPED plus subunit vaccine produced by Harris Vaccines. The efficacy of these vaccines remains in question, and the practice of feedback, feeding back of minced intestines from infected piglets to sows, remains a common practice despite the associated risks. A replication-defective baculovirus platform provides a number of advantages that make it an excellent candidate for production of a subunit based PEDV vaccine. First, the baculovirus genome allows for large insertions making it possible to insert the entirety of the more than 4 kb coding sequence of the PEDV spike protein. Second, BEVS can rapidly be adapted to respond to relevant field strains of PEDV, requiring only the coding sequence without the need to adapt new PEDV strains to mammalian cell culture. The replication-defective nature of such a system provides a level of safety not possible with live attenuated vaccines, which have the potential to recombine with field strains and revert to virulence.

By bypassing chemical inactivation methods, a replication-defective baculovirus platform may provide additional advantages. Removing the requirement for chemical inactivation could protect sensitive antigen that might otherwise be damaged by the compounds or temperatures used during the inactivation process. In addition, stimulation of type I interferons, or activation of other innate antiviral pathways by baculovirus has been shown to be abrogated by inactivation. Thus, when viral inactivation is no longer required the potential immune stimulating properties of the baculovirus can be retained [16].

The research performed within this thesis met all of the stated objectives, but a number of obstacles remain for the system to perform as a practical vaccine production platform. The first

objective of producing an insect cell line stably expressing gp64 was ultimately the most problematic. Despite demonstrating expression of gp64 by stably transfected Sf9 cells, using these cells to complement gp64 in replication-defective viruses resulted in 2-3 log reductions in viral titers. Such a reduction in viral titers makes infection at high multiplicity of infection impractical, and places serious limitations on the use of the system as a large-scale vaccine production platform. Lambda Red recombination was successfully employed to produce a replication-defective baculovirus with the entire gp64 coding sequence deleted. A modification of this protocol allowed for the insertion of the large PEDV spike baculodisplay protein under the control of the gp64 promoter in its native location within the AcMNPV genome. The final objective of expressing the PEDV spike antigen in non-complementing cells was ultimately limited by the reduction in infectious viral titers for replication-defective baculovirus produced in complementing insect cells. Small amounts of antigen were successfully produced using replication-defective seed, but the system produces far less antigen than a replication-competent baculovirus expressing the same PEDVS baculodisplay protein under the polh promoter.

Despite the challenges remaining to produce a replication-defective baculovirus platform practical for commercial animal vaccine production, the techniques employed have provided valuable experience. Generating insect cell lines that stably express a complementary viral protein facilitate exploration of gene-deletion baculovirus mutants, which could improve understanding of viral protein functions. Adapting established recombination methods to manipulate the baculovirus genome allows for targeted gene deletion or replacement, as demonstrated for gp64, but may be applicable also for other potential gene targets.

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34. *pIB/V5-His.*

APPENDIX. TABLES AND FIGURES

Table 1. Antibodies for immunofluorescence-based staining

Primary Antibodies	**Dilution**
Mouse anti-baculovirus envelope protein gp64 MAb Clone AcV1 (eBioscience cat# 14-6991-82)	1:100
Mouse anti-baculovirus envelope protein gp64 Clone AcV5 (BIAH)	1:200
Rabbit anti-baculovirus capsid VP39 (BIAH)	1:200
Rabbit anti-PEDV pooled serum (BIAH)	1:200
Secondary Antibodies	
Goat anti-mouse IgG (H+L), FITC conjugated (Jackson Immuno Research cat# 115-095-003)	1:100
Goat anti-rabbit IgG (H+L), FITC conjugated (Jackson Immuno Research cat# 111-095-003)	1:100

Table 2. Antibodies for Western Blot

Primary Antibodies	**Dilution**
Mouse anti-baculovirus envelope protein gp64 Clone AcV5 (BIAH)	1:12000
Rabbit anti-baculovirus capsid VP39 protein A purified polyclonal IgG (BIAH)	1:2000, 1:5000
Rabbit anti-PEDV pooled serum (BIAH)	1:500
Rabbit anti-PEDV S1 protein A purified polyclonal IgG (BIAH)	1:5000
Secondary Antibodies	
Goat anti-mouse IgG (H+L), peroxidase conjugated (Jackson Immuno Research cat# 115-035-003)	1:500
Goat anti-rabbit IgG (H+L), peroxidase conjugated (Jackson Immuno Research cat# 111-035-003)	1:500

Table 3. qPCR Primer/Probe Sequences

Primer/Probe	**Sequence (5’ – 3’)**
*Op gp64 Fwd	CAA CAA ACT AAA CAA CAT GAT GCA CG
Op gp64 Rev	CGT CGG ACA GGA AAG TGG
Op gp64 Probe	56-FAM/TT GAC GAGC/ZEN/G GCT TAT CGG CAA CC/3IABkFQ
**BSD Fwd	CTT CTC GAT CTG CAT CCT GG
BSD Rev	ACA CAT AAC CAG AGG GCA GC
BSD Probe	56-FAM/CT GTC CAT C/ZEN/A CTG TCC TTC ACT ATG GC/3IABkFQ

*Op gp64 = gp64 gene from OpMNPV

**BSD = Blasticidin deaminase (antibiotic resistance)

Table 4. Insect Cell Expression Plasmids with Alternate Promoters

Plasmid	**Promoter Elements for Target Protein (gp64)**
pIB/V5-His	OpIE2
pBIIB1	gp64
pBIIB2	hr5-OpIE2-p10
pBIIB3	hr5-p10

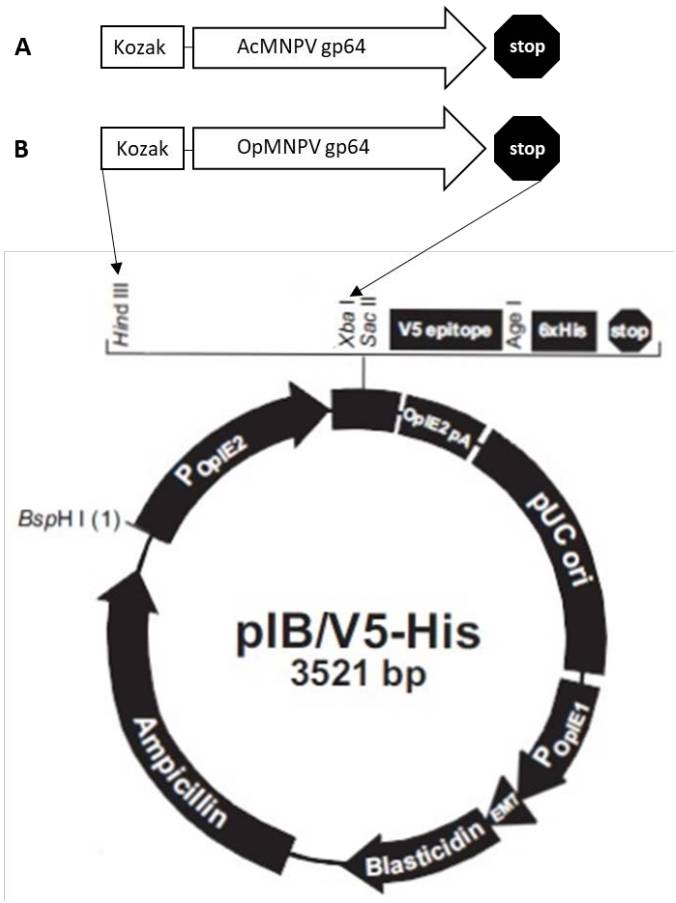


Figure 1. Map of the pIB V5/His plasmid. The pIB/V5/His plasmid was used to generate cells expressing the baculovirus envelope protein gp64. The two constructs each begin with a Kozak consensus sequence (gccacc) followed by the coding sequence for gp64 from either AcMNPV (A) or its homolog from OpMNPV (B), and ending with a stop codon prior to the V5 and 6xHis tags. These sequences were inserted at the vector's multiple cloning site using HindIII (5') and XbaI (3') restriction sites. Expression of gp64 is driven by the constitutive OpIE2 promoter while the Blasticidin resistance selection marker is driven by the OpIE1 promoter. Figure generated using the plasmid map retrieved from Thermo Fisher[34].

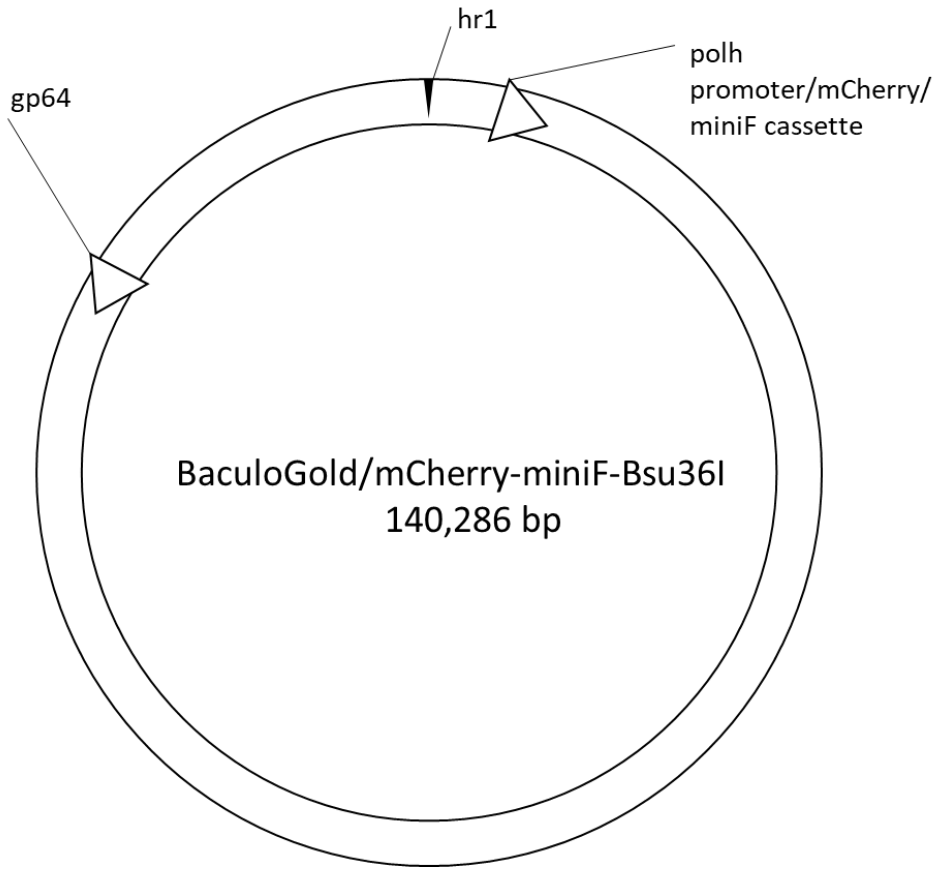


Figure 2. Relative location of polh and gp64 promoter regions of the AcMNPV bacmid

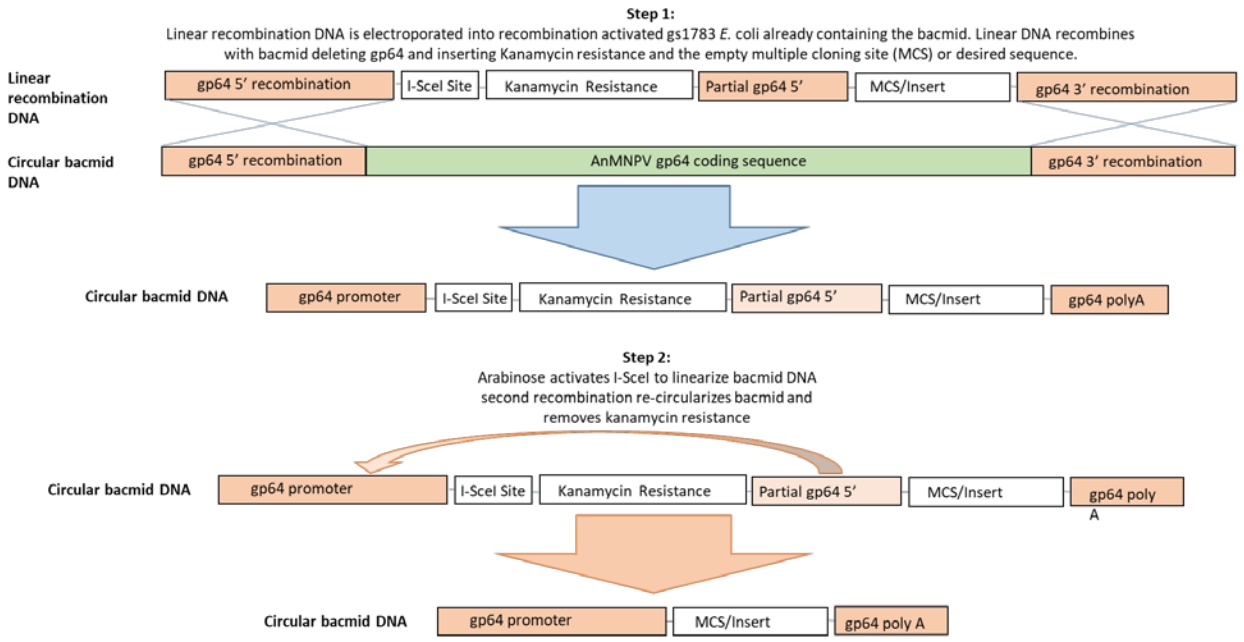


Figure 3. A visual representation of two-step Red recombination

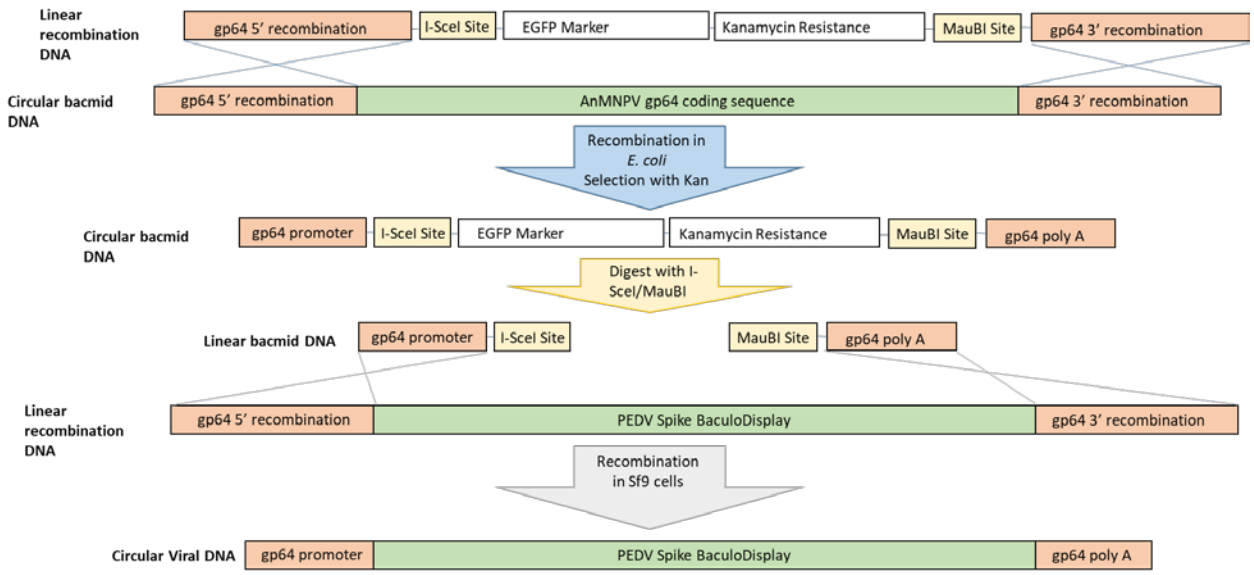


Figure 4. A visual representation of alternate recombination method

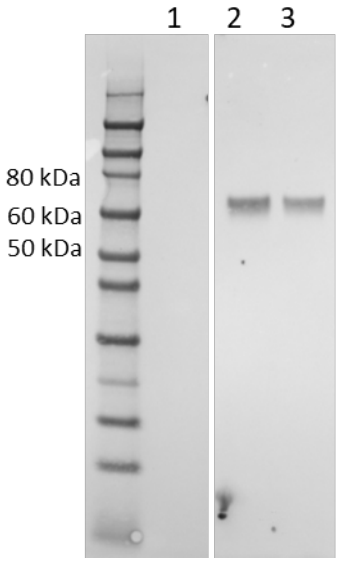


Figure 5. Western blot of transfection supernatants showing expression of gp64. Negative control supernatant collected from un-transfected Sf9 cells (lane 1) was compared to supernatants collected 72 hours after transient transfection with 1µg (lane 2) or 5µg (lane 3) by visible Snap-ID western blot with anti-gp64 mAb AcV5 primary Ab and peroxidase conjugated goat anti-mouse secondary Ab. The expected band for gp64 appears at approximately 64 kDa in both 1µg and 5µg transfections and is absent from the negative control.

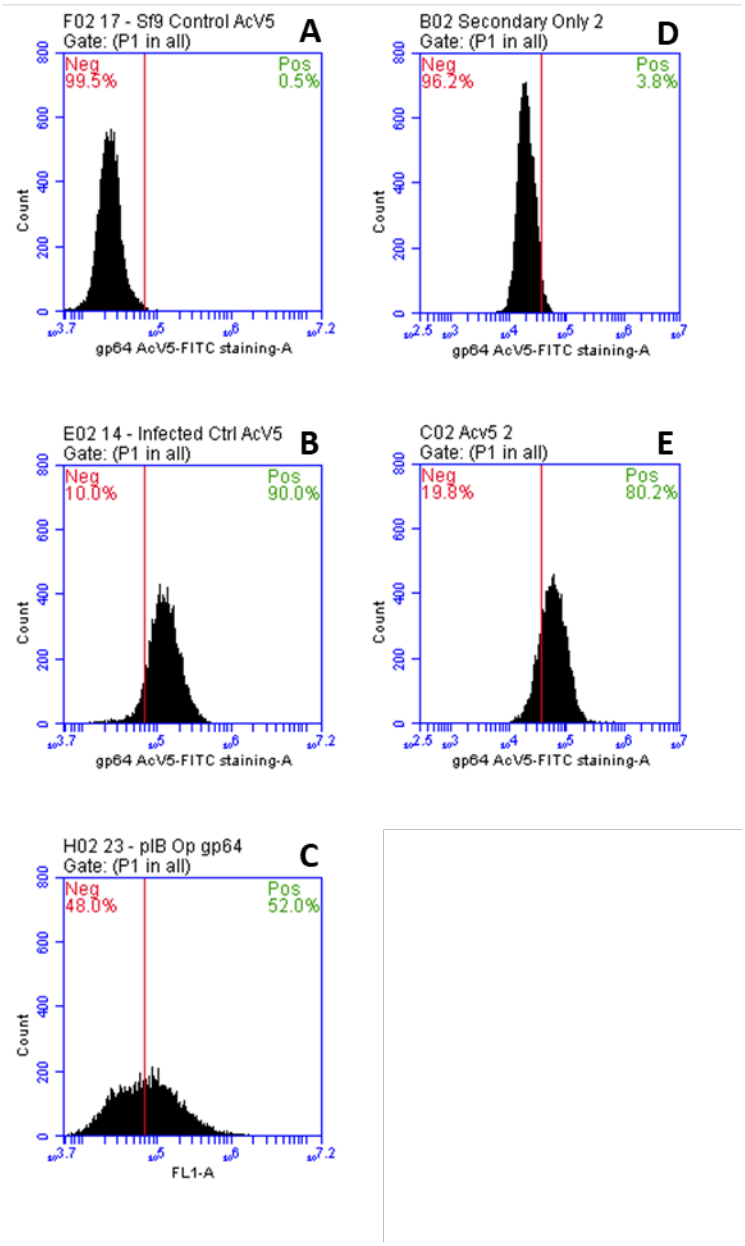


Figure 6. Flow cytometric evaluation of gp64 expression with anti-gp64 mAb Acv5. Cells were gated based on staining of gp64 negative Sf9 cells (A). Baculovirus infected Sf9 cells at 3dpi (B) show an apparent shift in fluorescence with 90% above the gated threshold. Sf9 cells transiently transfected with pIB/V5-His Op gp64 (C) have a broader range of staining with only 52% of cells positive. After selection of stably transfected cells, staining was performed again, and gated based on staining with secondary Ab only (D). The stably transfected cells (E) generate a peak similar to baculovirus infected cells.

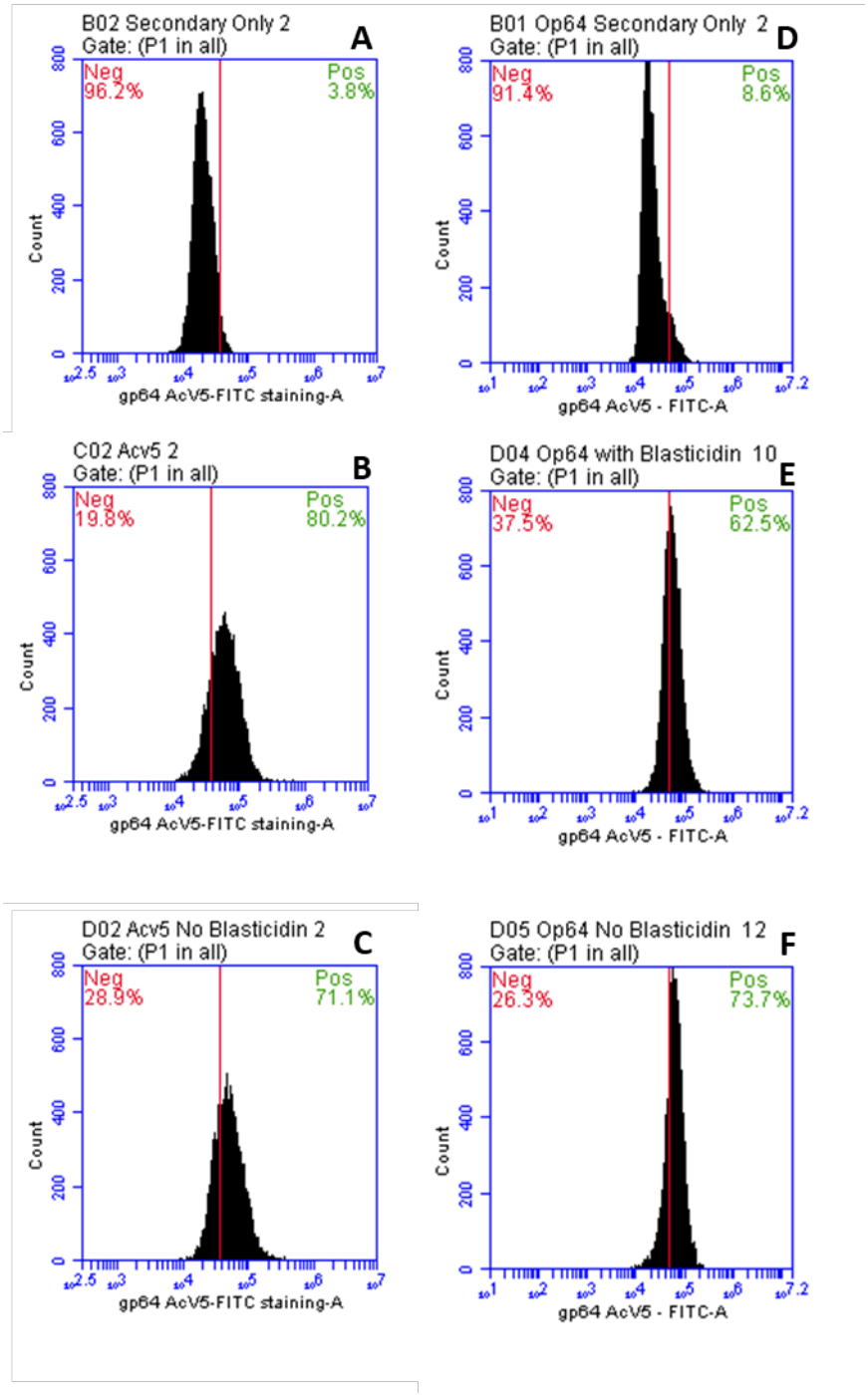
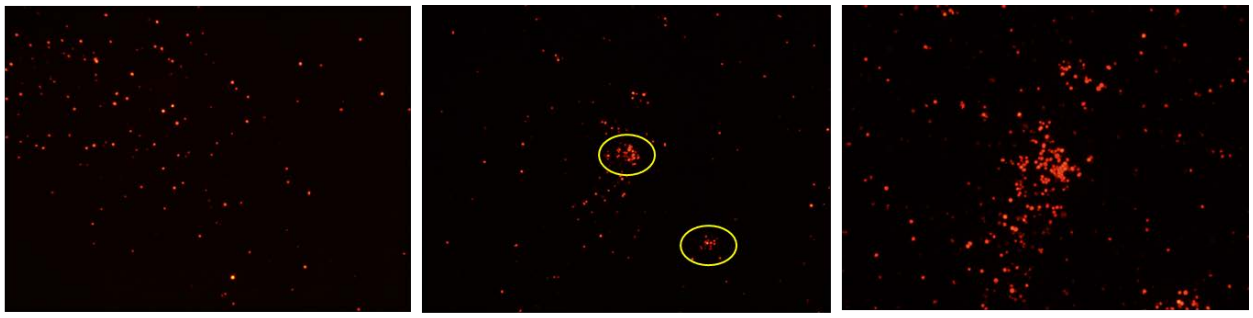


Figure 7. gp64 expression in stably transfected Sf9 cells without Blasticidin maintenance. In two separate experiments, cells were passed six times (A-C) or eighteen times (D-F) both with and without Blasticidin S HCl selection. Gates were placed based on secondary Ab only staining (A, D). Staining was maintained at similar levels with Blasticidin maintenance (B, E) or without Blasticidin in the cell culture medium (C, F)



A. Sf9 Cells Day 4 B. Sf9 Op64 #2 Cells Day 4 C. Sf9 Op64 #2 Cells Day 5

Figure 8. Transfection of standard Sf9 cells and gp64-complementing Sf9 Op64 #2 cells with gp64-deleted bacmid. (A) Standard Sf9 cells show mCherry expression from the transfected BaculoG/mCherry-miniF-Δgp64 bacmid, but no spread to surrounding cells was observed. At Day 5, mCherry expression in standard Sf9 cells remained similar to that observed for day 4 (image not shown). (B) Transfection of BaculoG/mCherry-miniF-Δgp64 bacmid into Sf9 Op64 #2 cells at four days post-transfection show clustering of mCherry (circled in yellow) indicating that the infection is spreading from new foci in gp64-complementing cells. (C) Expression of mCherry continues to expand at five days post transfection Sf9 Op64 #2 cells.

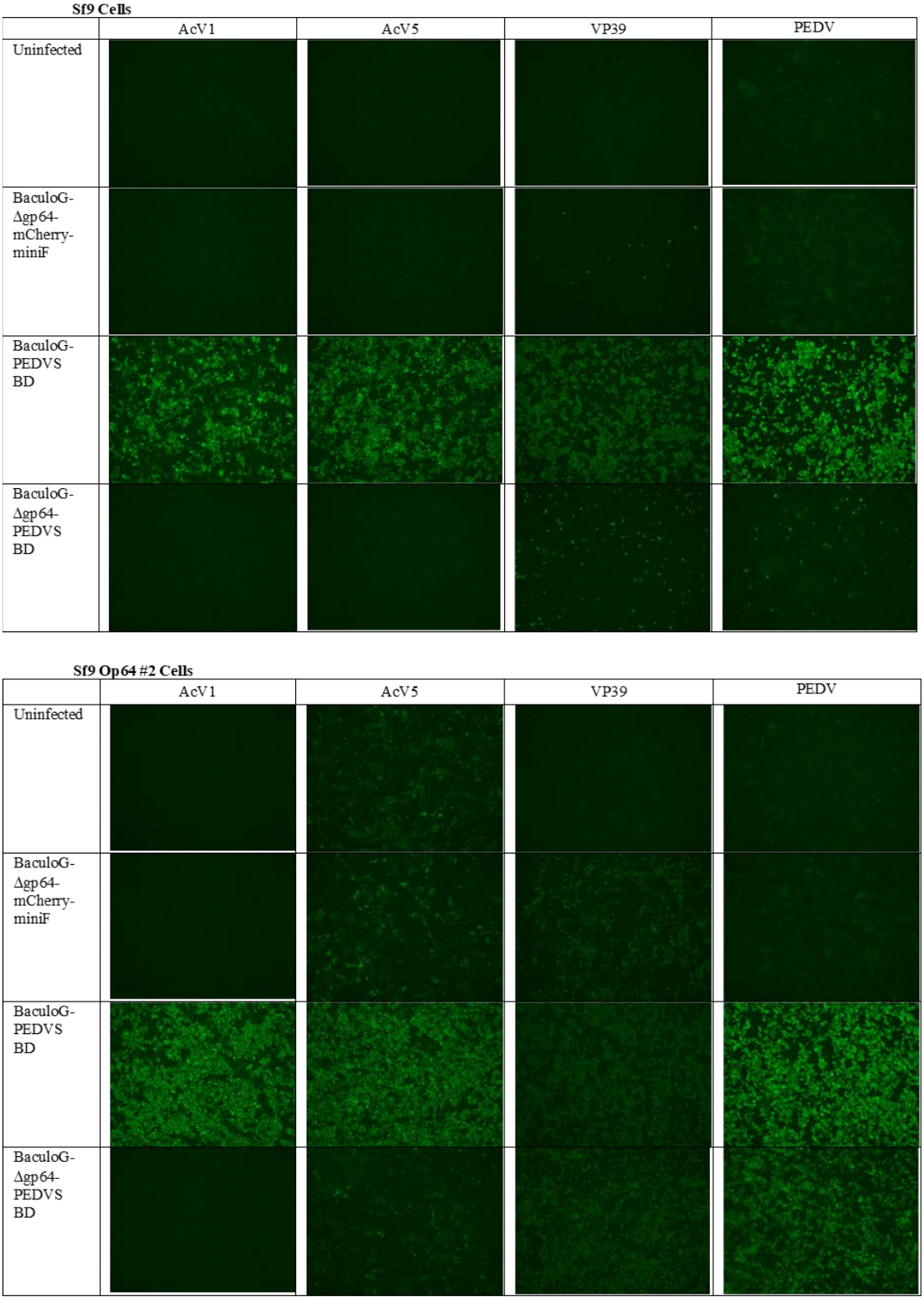


Figure 9. Immunofluorescence-based staining of Replication-defective Baculovirus Constructs in Sf9 and Sf9 Op64 #2 Cells.

Both the gp64-deleted (BaculoG-Δgp64-mCherry-miniF) and PEDV spike-pseudotyped (BaculoG-Δgp64-PEDVS BD) viruses along with a replication-competent positive control (BaculoG-PEDVS BD) were used to infect both Standard, non-complementing, Sf9 (A) cells along with OpMNPV gp64-expressing Sf9 Op64 #2 cells (B) for 72 hours. Respective cell cultures were then fixed and stained with one of four antibodies, 1) AcV1 which recognizes a conformational epitope on AcMNPV gp64 that is not present on OpMNPV gp64. 2) AcV5, which recognizes the linear V5 epitope conserved in both versions of gp64, 3) anti-VP39, which recognizes the AcMNPV capsid protein, and 4) anti-PEDV serum generated against inactivated PEDV along with an appropriate FITC conjugated secondary Ab. In Figure 9. A, Sf9 cells infected with BaculoGold expressing the PEDV spike baculodisplay show strong staining with all of the antibodies as expected. A small number of cells are positive for VP39 for the BaculoG-Δgp64-mCherry-miniF virus, indicating cell entry, but no foci of infection. This is also seen with the BaculoG-Δgp64-PEDVS BD virus including some expression of PEDV spike. In Figure 9.B, Sf9 Op64 #2 cells express OpMNPV gp64 at a lower level than seen with the replication-competent BaculoG-PEDVS BD control. Expression of VP39 appears similar though slightly reduced for both replication-defective constructs when compared to standard BaculoGold. Spike expression from BaculoG-Δgp64-PEDVS BD also appears slightly reduced, but similar to that of BaculoG-PEDVS BD.

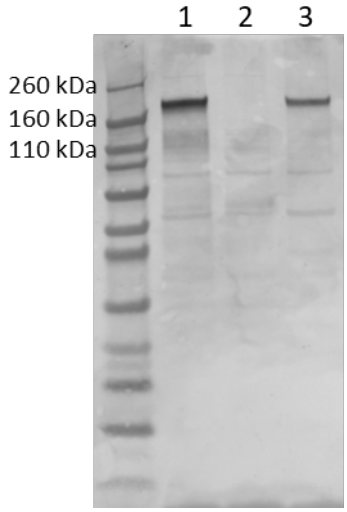


Figure 10. Western blot of PEDV Spike Expression. Harvest supernatants of BaculoG/PEDVS BD (lane 1) and a BaculoGold control containing no transgene insert (lane 2) were compared to expression of BaculoG/Δgp64-PEDVS BD expressed in Sf9 Ac64 cells (lane 3) by visible Snap-ID western blot with rabbit anti-PEDV primary Ab and peroxidase conjugated goat anti-rabbit secondary Ab. The expected band for PEDV Spike appears ca 180 kDa for both PEDV constructs and is absent from the negative control.

Baculovirus Construct	Repeats	Log10 Mean TCID50/mL	Standard Deviation
Standard BaculoGold	12	7.97	0.214
BaculoGold Op gp64 mCherry miniF	4	7.08	0.553
BaculoGold Δgp64 mCherry miniF	10	6.16	0.536

Figure 11. gp64 Modified Baculovirus Titers. Titers of standard BaculoGold and a modified BaculoGold replacing the native gp64 with its homolog from OpMNPV (BaculoGold Op gp64 mCherry miniF) and gp64-deleted BaculoGold Δgp64 mCherry miniF were compared. The gp64 swap appears to be responsible for some, but not all of the reduction in virus titers

Viable Cells/mL x 10^6 During Peak Infection			
Days Post Infection (DPI)	D3	D4	D5
Sf9 Ac64	1.24	0.61	0.29
Sf9 Op64	4.65	5.94	7.70

Figure 12. Differing Infectivity of gp64 Deleted Baculovirus in Sf9 Op64 Cells vs Sf9 Ac64 Cells. Fifty (50) mL suspension cultures of each gp64-complementing cell line infected with 0.5mL of the same BaculoG/Δgp64 PEDVS BD virus stock show very different infection kinetics. The infection in Sf9 Ac64 cells appeared to be resolved by day 5 post-infection while viable cells continued to rise from day 3 post-infection to day 5 post-infection indicating that the infection was unsuccessful.

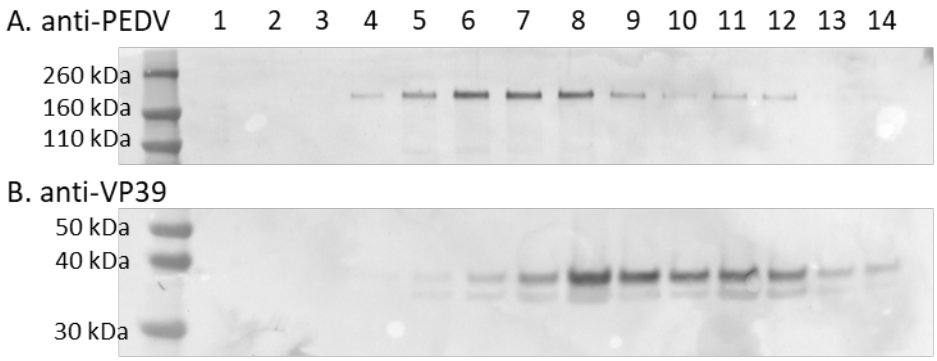


Figure 13. Sucrose gradient fractions of BaculoG/Δgp64 PEDVS BD. Western blots of the sucrose gradient fractions were performed using both Rabbit anti-PEDV (A) and Rabbit anti-VP39 (B) antibodies. The intensity of the banding at different fractions suggests that at least some of the spike is not co-located with VP39, but co-migration in fractions 6-12 does not rule out the possibility of budding being restored by PEDV spike containing only the gp64 CTD.

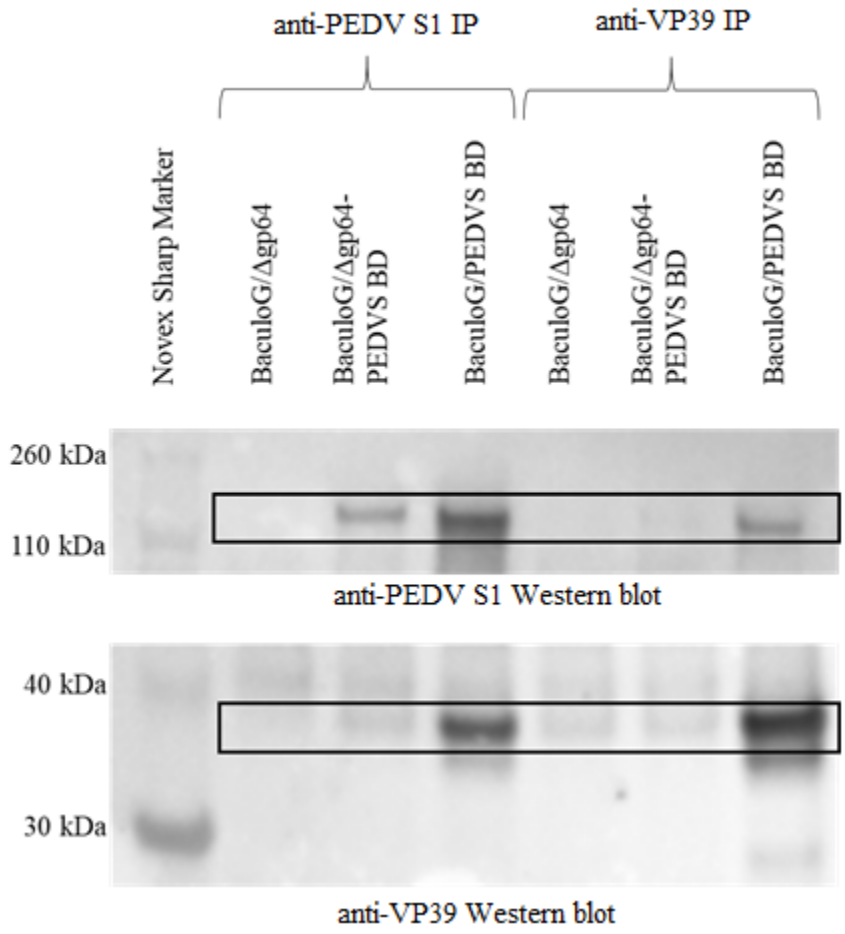


Figure 14. Western Blots of BaculoG/Δgp64 PEDVS BD Immunoprecipitation. Each of the three samples from both the anti-PEDV S1 and anti-VP39 immunoprecipitation were analyzed by Western blot with either anti-PEDV S1 and anti-VP39 antibodies as indicated. PEDV spike appears ca. 180 kDa andVP39 appears ca. 39kDa. IP = immunoprecipitation.